Vascular endothelial growth factor A isoform-specific regulation of endothelial cell function

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Submitted in accordance with the requirement's for the degree of
Doctor of Philosophy

The University of Leeds
Faculty of Biological Sciences

November 2015

The candidate confirms that the work submitted is his own, except where work which has formed part of jointly-authored publications has been included. The contribution of the candidate and the other authors to this work has been explicitly indicated below. The candidate confirms that the appropriate credit has been given within the thesis where reference has been made to the work of others.

Jointly authored publications

Chapter 1

Fearnley GW, Smith GA, Harrison MA, Wheatcroft SB, Tomlinson DC, Ponnambalam S (2013) The biochemistry of new blood vessel formation in health and disease. *OA Biochemistry* **1**:5.

G.W.F, G.A.S and S.V.P wrote the manuscript. **G.W.F**, G.A.S, M.A.H, S.B.W, D.C.T and S.V.P revised the manuscript.

Smith GA, **Fearnley GW**, Harrison MA, Tomlinson DC, Wheatcroft SB, Ponnambalam S (2014) Vascular endothelial growth factors: multitasking functionality in metabolism, health and disease. *J Inherited Metab. Dis.* **38**:753-763.

G.A.S, **G.W.F,** and S.V.P wrote the manuscript. G.A.S, **G.W.F** M.A.H, D.C.T, S.B.W and S.V.P revised the manuscript.

Chapter 2

Fearnley GW, Wheatcroft SB, Ponnambalam S (2014) Detection and quantification of VEGF receptor tyrosine kinases in human endothelial cells. *Meth. Mol. Biol.* **1332**:49-65.

G.W.F, S.B.W and S.V.P conceived and designed the experiments. **G.W.F** performed the experiments. **G.W.F** and S.V.P wrote the manuscript. **G.W.F**, S.B.W and S.V.P revised the manuscript.

Fearnley GW, Smith GA, Odell AF, Latham AM, Wheatcroft SB, Harrison MA, Tomlinson DC, Ponnambalam S (2014) Vascular endothelial growth factor A-stimulated signaling from endosomes in primary endothelial cells. *Meth. Enzymol.* **535**:265-292.

G.W.F, A.F.O, A.M.L, S.B.W and S.V.P conceived and designed the experiments. **G.W.F**, A.F.O, A.M.L performed the experiments. **G.W.F**, G.A.S and S.V.P wrote the manuscript. **G.W.F**, G.A.S, A.F.O, A.M.L, S.B.W, M.A.H, D.C.T and S.V.P revised the manuscript.

Latham AM, Kankanala J, **Fearnley GW**, Gage MC, Kearney MT, Homer-Vanniasinkam S, Wheatcroft SB, Fishwick CWG, Ponnambalam S *(2014) In Silico* Design and Biological Evaluation of a Dual Specificity Kinase Inhibitor Targeting Cell Cycle Progression and Angiogenesis**.** *PLoS ONE* **9:**e110997**.**

A.M.L, J.K, **G.W.F**, M.C.G, C.W.G.F and S.V.P conceived and designed the experiments. A.M.L, J.K, **G.W.F**, M.C.G performed the experiments. A.M.L, J.K, **G.W.F** analysed the data. A.M.L, J.K, **G.W.F** and S.V.P wrote the manuscript. A.M.L, J.K, **G.W.F**, M.C.G, M.K, S.H, S.B.W, C.W.G.F and S.V.P revised the manuscript.

Chapter 3

Fearnley GW, Yuldasheva N, Abdul Zani I, Smith GA, Mughal NA, Kearney MT, Homer Vanniasinkam S, Tomlinson DC, Harrison MA, Zachary IC, Wheatcroft SB, Ponnambalam S (2015) VEGF-A isoform-specific VEGFR2 trafficking regulates signal transduction, cellular responses and arterial regeneration. **Submitted.**

G.W.F, N.Y, S.B.W and S.V.P conceived and designed the experiments. **G.W.F**, N.Y, I.A.Z., G.A.S and N.A.M performed the experiments. **G.W.F** and S.V.P wrote the manuscript. **G.W.F**, N.Y, I.A, G.A.S, N.A.M, M.T.K, S.H, D.C.T, S.H.V., M.A.H, I.C.Z, S.B.W and S.V.P revised the manuscript.

Chapter 4

***Fearnley GW**, Odell AF, Latham AM, Mughal NA, Bruns AF, Burgoyne NJ, Homer-Vanniasinkam S, Zachary IC, Hollstein M, Wheatcroft SB, Ponnambalam S (2014) VEGF-A isoforms differentially regulate ATF-2-dependent VCAM-1 gene expression and endothelial-leukocyte interactions. *Mol. Biol. Cell.* **25**:2509-2521. ***Cover article.**

G.W.F, A.F.O, A.M.L S.B.W and S.V.P conceived and designed the experiments. **G.W.F**, A.F.O, A.M.L., N.A.M performed the experiments. N.J.B preformed the bioinformatics **G.W.F** and S.V.P wrote the manuscript. **G.W.F**, A.F.O, A.M.L, N.A.M, A.F.B, S.H, I.C.Z, M.H, S.B.W and S.V.P revised the manuscript.

Chapter 5

Fearnley GW, Odell AF, Abdul Zani I, Latham AM, Hollstein M, Wheatcroft SB, Ponnambalam S (2015) Tpl2 is a signaling nexus in endothelial gene expression and cell cycle progression. **Submitted.**

G.W.F, A.F.O, S.B.W and S.V.P conceived and designed the experiments. **G.W.F**, A.F.O, I.A.Z, A.M.L performed the experiments. **G.W.F** and S.V.P wrote the manuscript. **G.W.F**, A.F.O, I.A.Z, A.M.L, M.H, S.B.W and S.V.P revised the manuscript.

Chapter 6

Fearnley GW, Bruns AF, Wheatcroft SB, Ponnambalam S (2014) VEGF-A isoform-specific regulation of endothelial cell migration via differential Ca^{2+}-dependent NFATc2 activation. *BiO.* **4**:731-742.

G.W.F, S.B.W and S.V.P conceived and designed the experiments. **G.W.F** and A.F.B performed the experiments. **G.W.F** and S.V.P wrote the manuscript. **G.W.F**, A.F.B, S.B.W and S.V.P revised the manuscript.

ACKNOWLEDGEMENTS

ABSTRACT

Vascular endothelial growth factor A (VEGF-A) binding to the receptor tyrosine kinase (RTK) vascular endothelial growth factor 2 (VEGFR2) triggers an array of downstream signal transduction pathways which modulate a multitude of endothelial cell responses, such as cell migration, proliferation, tubulogenesis and cell-cell interactions. Multiple splice isoforms of VEGF-A exist, yet it is unclear how different VEGF-A isoforms bind to the same RTK to program distinct cellular responses. The work presented in this PhD thesis evaluated VEGF-A isoforms for their ability to program VEGFR2 endocytosis, post-translational modification, proteolysis and terminal degradation. Such changes in VEGFR2 status were linked to downstream signal transduction and gene expression, with relevance to cell function and vascular physiology. VEGF-A isoforms differentially promoted VEGFR2 tyrosine transautophosphorylation and endocytosis. Different VEGFR2-VEGF-A complexes exhibit altered ubiquitination, a hallmark of trafficking through the endosome-lysosome system for subsequent terminal degradation and proteolysis. VEGF-A isoform-specific VEGFR2 phosphorylation coupled with endocytosis and delivery to early endosomes is required for isoform-specific activation of the MEK1-ERK1/2 signal transduction pathway and endothelial cell proliferation. VEGF-A isoforms also exhibited differences in their ability to stimulate arterial regeneration in a mouse hind limb ischaemia model. VEGF-A isoform-specific ERK1/2 activation was essential for the phosphorylation of activating transcription factor 2 (ATF-2) at residue T71. Differential activation of ATF-2 regulated VEGF-A isoform-specific gene transcription (e.g. VCAM-1) and endothelial cell responses, such as leukocyte recruitment. Additionally, basal ATF-2-pT71 levels are required to maintain endothelial cell cycle commitment, via repressing p53-dependent gene transcription. Furthermore, VEGF-A isoforms promoted differential PLCγ1 phosphorylation and a subsequent isoform-specific increase in cytosolic calcium ions. A functional consequence of this VEGF-A isoform-specific calcium ion flux, was differential dephosphorylation and subsequent nuclear translocation of the transcription factor NFATc2 (NFAT1) in order to regulate endothelial cell migration. Thus, this study provides a mechanistic framework for understanding how different ligand isoforms differentially program RTK functionality in health and disease.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AJ	adherens junctions
Akt	protein kinase B
ANG	angiopoietin
AP-1	activator protein 1
ATF-2	activating transcription factor 2
ATP	adenosine triphosphate
BAD	Bcl2-associated death promoter
bZIP	basic region subdomain/leucine zipper
CDC42	cell division cycle 42
CDE	clathrin-dependent endocytosis
CID	clathrin-independent endocytosis
CLI	critical limb ischaemia
$cPLA_2$	cytosolic phospholipase A_2
CREB	cyclic AMP response element binding protein
CXCL	chemokine (C-X-C motif) ligand
DAG	diacylglycerol
Dll4	delta-like 4
DMEM	Dulbecco's modified Eagle's medium
ECD	extracellular domain
ECGM	endothelial cell growth medium
ECM	endothelial cell matrix
EGF	epidermal growth factor
EGFL7	EGF-like domain-containing protein 7
Egr	early growth response
eNOS	endothelial nitric oxide synthase
ER	endoplasmic reticulum
ERK1/2	extracellular signal-regulated kinase 1/2 (p42/44)
ESAM	endothelial-cell-selective adhesion molecule
FABP4	fatty-acid-binding protein 4
FAK	focal adhesion kinase
FAS	fatty acid synthase
FATP	fatty-acid-transport protein
FGF	fibroblast growth factor

FGFR	fibroblast growth factor receptor
FIH-1	hydroxylase factor inhibiting HIF-1
Flk-1	fetal liver kinase-1/VEGFR2
Flt-1	FMS-like tyrosine kinase-1/VEGFR1
Flt-4	FMS-like tyrosine kinase-4/VEGFR3
FOXO	forkhead box O
G6PD	glucose-6-phosphate dehydrogenase
GAB1	Grb2-associated binding protein 1
GLUT-1	glucose transporter 1
GRB2	growth factor receptor-bound protein 2
GSH	reduced glutathione
HDAC	histone deacetylase
HIF-1	hypoxia-inducible factor-1
HRE	hypoxia-responsive element
HSP27	heat-shock protein of 27 kDa
JAK	Janus kinase
JAMs	junction adhesion molecules
IAP	inhibitor of apoptosis
ICAM-1	intracellular adhesion molecule 1
Ig	immunoglobulin
IL-1β	interleukin 1beta
IP$_3$	inositol trisphosphate
IP3R	inositol trisphosphate receptor
KDR	kinase-insert domain containing receptor/VEGFR2
LDH-A	lactate dehydrogenase-A
MAPK	mitogen-activated protein kinase
MAPKAPK2	mitogen-activated protein kinase-activated protein kinase 2
MEK1	mitogen-activated protein/ERK kinase 1
MMPs	matrix metalloproteinases
MT1-MMP	membrane type I matrix metalloproteinase
NADPH	nicotinamide adenine dinucleotide phosphate
NFAT	nuclear factor of activated T-cells
NFκB	nuclear factor kappa B
NICD	notch intracellular domain
NO	nitric oxide
NRARP	notch-regulated ankyrin repeat protein

NRP	neuropilin
oxPPP	oxidative pentose phosphate pathway
p38 MAPK	mitogen-activate protein kinase of 38 kDa
PAD	peripheral arterial disease
PAEC	porcine aortic endothelial cells
PAI-1	plasminogen activator inhibitor 1
PAK2	p21-activated protein kinase 2
PCG-1α	peroxisomes proliferator-activated receptor gamma coactivator-1 alpha
PDGF	platelet-derived growth factor
PDGFR	platelet-derived growth factor receptor
PDK	phosphoinositide-dependent kinase
PDZ	PSD95, DLGA and ZO1 homology
PECAM-1	platelet endothelial cell adhesion molecule 1
PFKFB3	phosphofructokinase-2/fructose-2,6-bisphosphate
PHD	prolyl hydroxylase domain enzyme
PIP$_2$	phosphatidylinositol 4,5-bisphosphate
PI3K	phosphoinositide 3-kinase
PKB	protein kinase B
PKC	protein kinase C
PLCβ3	phospholipase C beta 3
PLCγ1	phospholipase C gamma 1
PlGF	placental growth factor
PPP	pentose phosphate pathway
PSG	pig skin gelatin
PTP	protein tyrosine phosphatase
qRT-PCR	quantitative real time polymerase chain reaction
RACK1	receptor for activated protein kinase C 1
RNAi	RNA interference
ROS	reactive oxygen species
RTK	receptor tyrosine kinase
SEA motif	Ser-Glu-Ala motif
sFlt-1	soluble FMS-like tyrosine kinase 1
SCK	SHC-related adaptor protein
SDF-1α	stromal cell-derived factor-1 alpha
SH2	Src homology 2

SHC	SH2-domain containing transforming protein
SHP-2	SH2-domain containing protein tyrosine phosphatase 2
SOS	son of sevenless
SP1	specificity protein 1
SPK	sphingosine kinase
STAT	signal transducer and activator of transcription
sVEGFR1	soluble VEGFR1
sVEGFR2	soluble VEGFR2
TAD	transactivation domain
TEM	transendothelial migration
TfR	transferrin receptor
TGF-β	transforming growth factor beta
TIMPs	tissue inhibitor of metalloproteinases
TJ	tight junctions
TK	tyrosine kinase
TNFα	tumour necrosis factor alpha
Tpl2	tumour progression locus 2
TSAd	T-cell-specific adaptor
uPA	urokinase plasminogen activator
UPR	unfolded protein response
VCAM1	vascular cell adhesion molecule 1
VE-cadherin	vascular endothelial cadherin
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
VEPTP	vascular endothelial PTP
VHL	von Hippel-Lindeau factor
vWF	von Williebrand factor
WNT	wingless-related integration site
XBP-1	X-box binding protein

CHAPTER 1

INTRODUCTION

1.1.   A brief history of angiogenesis

The term angiogenesis was first employed by British surgeon Dr John Hunter in 1787 to describe new blood vessel growth to supply the growing antlers from the Fallow Deer's skull. He stated that blood vessels have a unique capacity to repair themselves upon injury; in comparison to other parts of the body (Stephenson et al., 2013). Nearly 2 centuries later in the 1970s, Dr Judah Folkman, deemed the 'father of angiogenesis', kick-started the field with his landmark study published in the *New England Journal of Medicine* (Folkman, 1971). This study identified that growing solid tumours stimulated the growth and recruitment of new blood vessels from the pre-existing vasculature. Folkman concluded that simple diffusion was not sufficient for a tumour to receive enough nutrients and O_2 to grow beyond 2 mm in size. Therefore, tumourigenesis requires a healthy blood supply and is thus angiogenesis-dependent (Folkman, 1971). In addition, Folkman coined the term anti-angiogenesis by proposing that the removal of an angiogenesis promoting factor could prove an effective treatment for cancer, as preventing blood vessel growth could halt the tumour in a dormant state (<2mm) (Folkman, 1971). Folkman's conclusions at the time of publication were highly controversial, with the accepted belief being that tumour vascularity was non-specific inflammation and that tumours grew around pre-existing capillary blood vessels (Stephenson et al., 2013). Folkman followed up his 1971 hypothesis by providing evidence that anti-angiogenic factors existed, and that inhibition of angiogenesis prevented tumour growth and that the removal of an angiogenic stimulus promoted neovascular regression (Cao and Langer, 2008). Finally, almost a decade after Folkman's seminal publication, his premise on tumour angiogenesis started to become widely accepted; by the mid-1980s, Folkman began to convert his critics into competitors, resulting in a surge in angiogenesis research (Cao and Langer, 2008; Stephenson et al., 2013; Epps, 2005).

In 1983, Harold Dvorak and colleagues discovered a potent angiogenic factor from a guinea pig tumour cell line which they called vascular permeability factor (VPF) (Senger et al., 1983). VPF was categorised as having the ability to induce vascular

leakage; it was suggested that VPF was accountable for the leakiness of blood vessels associated with tumours (Senger et al., 1983). However, this study did not go as far as fully purifying the VPF protein, thus a lack of primary sequence prevented the establishment of its identity (Ferrara, 2009). However in 1989, Napoleone Ferrara and colleagues isolated and cloned a novel heparin-binding endothelial cell mitogen from media conditioned by bovine pituitary follicular cells. Ferrara and colleagues proposed the name vascular endothelial growth factor (VEGF), due to its ability to stimulate growth-promoting activity only towards vascular endothelial cells (Ferrara and Henzel, 1989; Ferrara, 2009). Interestingly, later that year Ferrara and colleagues proved the existence of several VEGF isoforms by screening human cDNA libraries and isolating several VEGF clones (VEGF$_{121}$, VEGF$_{165}$ and VEGF$_{189}$), they suggested that these isoforms arose from alternative mRNA splicing (Leung et al., 1989; Ferrara, 2009). Surprisingly almost simultaneously, a group led by Daniel Connolly had submitted a manuscript reporting on the cloning of the VPF protein, this protein turned out to be identical to VEGF$_{189}$ (Keck et al., 1989; Ferrara, 2009). Therefore VPF and VEGF were the equivalent protein (now termed VEGF-A as the founding member of this family) which exhibits both mitogenic and permeability-enhancing properties.

2008 saw the passing of Judah Folkman whilst on his way to deliver the keynote speech at a symposium on molecular mechanisms of angiogenesis in development and disease. However, Folkman's legacy still remains strong, with research into angiogenesis still yielding new insights into the molecular mechanisms involved. Folkman's prediction of anti-angiogenic therapies has led to the currently available drugs for the treatment of a wide range of cancers and age-related macular degeneration (AMD) (Welti et al., 2013).

1.2.   The vascular endothelium

The vascular endothelium is an autocrine and paracrine organ which lines the internal surfaces of all blood vessels and plays a vital role in regulating many vascular responses such as vessel tone, thrombosis, immune function and blood pressure. It achieves this by expressing an array of membrane-bound receptors which respond to many changing stimuli, as well as secreting a varying cocktail of growth factors, receptors and other small molecules (Onat et al., 2011). Vascular endothelial cell monolayers exhibit a characteristic 'cobblestone' morphology: this monolayer or phalanx layer in mature stable blood vessels is ordinarily quiescent and arrested in cell cycle phase G$_0$ (Lampugnani and Dejana, 2007). Vascular

endothelial cells are thought to constitute an estimated 1 kg of tissue (Cines et al., 1998; Roskoski, 2007). A key hallmark of the vascular endothelium is the presence of cell-cell junctions; these endothelial junctions exist as one of two types, namely tight (TJs) and adherens (AJs) junctions. Other adhesion molecules such as platelet-endothelial cell adhesion molecules (PECAM-1) are concentrated in regions outside of TJs and AJs (Dejana and Orsenigo, 2013). Endothelial cell-cell junctions are vital for maintaining vascular integrity such as inhibiting proliferation and apoptosis, help control apical-basal polarity, allow for selective permeability and aid leukocyte trafficking (Dejana and Orsenigo, 2013; Giannotta et al., 2013; Lampugnani and Dejana, 2007).

1.2.1.   Adherens junctions (AJs)

Adhesion at AJs is mediated by calcium-dependent adhesion molecules called cadherins (Giannotta et al., 2013), Cadherins interact in *trans* with neighbouring cadherins, and form lateral interactions in *cis* with other cadherin-cadherin complexes. This creates zipper-like structures which allows for stable adhesion between neighbouring cells, with the exception of cells within lymphatic vessels (Dejana and Orsenigo, 2013; Shapiro et al., 1995). Endothelial cells express 2 forms of cadherin, namely vascular endothelial (VE)-cadherin and N-cadherin. VE-cadherin is clustered at cell-cell contacts, unlike N-cadherin which is usually excluded from these structures (Giannotta et al., 2013). VE-cadherin and the non-vascular and more ubiquitous N-cadherin have been implemented in regulating endothelial cell function. The presence of both these cadherins inhibits endothelial cell proliferation and apoptosis; however, they have inverse effects on cell mobility with VE-cadherin inhibiting cell mobility but N-cadherin promoting it (Giampietro et al., 2012). This difference in ability to promote cell mobility was linked to their differential capacities to regulate fibroblast-growth factor receptor (FGFR) activity. VE-cadherin was shown to interact with FGFR and reduce its level of phosphorylation and downstream signal transduction, conversely, N-cadherin maintained FGFR phosphorylation and downstream gene expression required for cell mobility (Giampietro et al., 2012). Additionally, endothelial-specific deletion of N-cadherin and VE-cadherin are embryonically lethal due to vascular defects (Luo and Radice, 2005; Gory-Faure et al., 1999; Crosby et al., 2005). However, studies have shown that VE-cadherin is not vital for *de novo* blood vessel formation up to the point of vascular epithelium formation (i.e., nascent vessels with lumens). Nonetheless, it is required for nascent vessel stabilisation and to prevent nascent vessels from undergoing disassembly (Crosby et al., 2005).

1.2.2. Tight Junctions (TJs)

Endothelial tight junctions are made up of several protein families. These include claudins, a family of tetraspanin trans-membrane proteins which promote tight adhesion between neighbouring cells, junction adhesion molecules (JAMs) endothelial-cell-selective adhesion molecule (ESAM), nectins, and occludins (Dejana and Orsenigo, 2013). Currently there is only a limited amount of information about the cell-specific characteristics of TJs in endothelial cells available (Dejana and Orsenigo, 2013).

1.3. Vasculogenesis

The human circulatory network of vascular endothelial and supporting cells is crucial for transporting oxygen, nutrients and signalling molecules; as well as the removal of carbon dioxide and metabolic end products from, cells, tissues and organs (Roskoski, 2007). The growth of new blood vessels can be split into two physiological processes, vasculogenesis and angiogenesis. Vasculogenesis is the process of new blood vessel formation mediated by hematopoietic stem cells called hemangioblasts which differentiate into blood cells and mature endothelial cells (Dvorak, 2005).

1.4. Angiogenesis

Angiogenesis is the complex process whereby new blood vessels sprout from the pre-existing vasculature and is essential for normal physiology. Angiogenesis involves the role of many different cell types and various angiogenic regulators working in sync to build and direct the developing blood vessel. This process is tightly regulated via a so call angiogenic 'switch' which is turned 'on' or 'off' depending on the surrounding levels of pro- vs. anti- angiogenic factors. Physiological angiogenesis occurs during embryo development, organ growth and wound repair (Carmeliet and Jain, 2011). However, uncontrollable or excessive angiogenesis occurs in pathological disease states such as tumour growth and metastasis, atherosclerosis and age-related macular degeneration (AMD). In contrast, inadequate angiogenesis results in a deficient vascular network which can result in compromised wound healing, ulcers and ischaemic heart disease (Carmeliet and Jain, 2011; Hanahan and Weinberg, 2011).

1.4.1. Model of vessel sprouting

Angiogenesis is a multi-stage process, and occurs when quiescent endothelial cells sense pro-angiogenic signals. Firstly, in response to AngII binding to the membrane

receptor Tie2, pericytes detach themselves from the basement membrane with the aid of matrix matalloproteinases (MMPs; Figure 1.1) (Welti et al., 2013; Eelen et al., 2013). Secondly, VEGF-A which is synthesised and secreted by injured or hypoxic cells binds to its receptor present on endothelial cells. VEGF-A binding to membrane receptors results in increased vascular permeability due to a breakdown of endothelial cell-cell cadherin complexes (e.g. VE-cadherin) and adhesion molecules (e.g., Z0-1) (Schulte et al., 2011; Welti et al., 2013). Increased vascular permeability allows for the extravasation of plasma proteins from within the blood vessel allowing them to construct a temporary ECM scaffold, on to which endothelial cells begin to migrate (Figure 1.1). Proteases such as MT1-MMP cleave further growth factors from the ECM (e.g. VEGF-A) (Sounni et al., 2011), this results in the formation of an environment primed for angiogenesis (Carmeliet and Jain, 2011). To prevent uncontrollable chemotaxis in the direction of the chemo-attractant, one endothelial cell known as the 'tip cell' is promoted to lead the migrating endothelial cells (Figure 1.1), whereas the remaining endothelial cells take up secondary roles as stalk cells (Carmeliet and Jain, 2011; Adams and Eichmann, 2010). Endothelial 'tip cells' are motile, invasive and have numerous dynamic filopodia which sense and respond to guidance cues such as ephrin-B2, semaphorins, netrins and slit proteins present within their immediate microenvironment (Adams and Eichmann, 2010; Carmeliet and Jain, 2011). Thus, 'tip cells' have many morphological and functional similarities with neuronal growth cones which regulate axon guidance (Adams and Eichmann, 2010; Herbert and Stainier, 2011).

Tip cell specification is regulated by VEGF-A-stimulated upregulation of Notch ligand delta-like 4 (Dll4), which binds to the membrane receptor Notch 4 on neighbouring endothelial cells. Upon receptor-ligand interaction, the Notch intracellular domain (NICD) is released by controlled proteolysis. NICD acts as a transcription factor repressing the levels of VEGFR2 and NRP1 and promoting transcription of VEGFR1; thus angiogenic signal transduction is dramatically reduced (Welti et al., 2013; Siekmann et al., 2013; Herbert and Stainier, 2011). In contrast, another Notch ligand called Jagged 1 acts in a pro-angiogenic manner by acting as an antagonist for Dll4-Notch signalling (Benedito et al., 2009; Adams and Eichmann, 2010; Herbert and Stainier, 2011). This has particular importance in endothelial stalk cells where the levels of Jagged 1 are high, thus effectively antagonising the more potent Dll4 ligand and thereby preventing Dll4-Notch signalling in neighbouring 'tip cells' (Benedito et al., 2009).

Lagging stalk cells undergo rapid proliferation to elongate the sprout and promote formation of the vessel lumen, this process is modulated by various factors including VE-cadherin and VEGF (Welti et al., 2013). In response to the interaction between the filopodia of two independent 'tip cells', these meeting tip cells undergo anastomosis to initiate blood flow (Figure 1.1). 'Tip cell' anastomosis is regulated by macrophages which act as 'bridge cells' by secreting angiogenic factors (Siekmann et al., 2013; Welti et al., 2013; Carmeliet and Jain, 2011). This is followed by vessel maturation and a subsequent return to its quiescent phalanx state. Vessel maturation occurs when oxygen is readily available for the hypoxic tissue, promoting a fall in levels of pro-angiogenic factors. Autocrine molecules including VEGF-A, Notch, AngI, FGF and ephrin-B2 maintain the endothelial cells in a quiescent state and promote pericyte re-coverage (Carmeliet and Jain, 2011; Welti et al., 2013). Tissue inhibitors of metalloprotease (TIMPs) and plasminogen activator inhibitor-1 (PAI-1) repress MMP activity, resulting in re-formation of the ECM, allowing endothelial cells to re-adhere and form tight cell-cell contacts (Carmeliet and Jain, 2011; Welti et al., 2013).

1.4.2. Angiogenesis in disease states

The blood vessels of a developed adult are mostly quiescent in nature, yet retain their ability to respond to an array of physiological stimuli to permit wound healing and repair (Carmeliet, 2005). However, if levels of such stimuli become dysregulated, this results in excessive or inferior angiogenesis which contributes to the formation of multiple pathological conditions (Carmeliet, 2005). Excessive angiogenesis is characteristic of atherosclerosis, rheumatoid arthritis, age-related macular degeneration (AMD), diabetic retinopathy, tumour development and metastasis (Carmeliet and Jain, 2011; Chung and Ferrara, 2011). However, deficiencies in angiogenesis can contribute to limb ischaemia, impaired wound healing and ulcers (Carmeliet and Jain, 2011; Chung and Ferrara, 2011). There is also increasing evidence for pathological angiogenesis in the development of neurodegenerative diseases, such as Alzheimer's disease (Qin et al., 2015; Ponnambalam and Alberghina, 2011; Ruiz de Almodovar et al., 2009; Grammas et al., 2014; Provias and Jeynes, 2014; Jefferies et al., 2013).

1.5. Vascular endothelial growth factor family

The mammalian human vascular endothelial growth factor (VEGF) family consists of five structurally related members (VEGF-A, VEGF-B, VEGF-C, VEGF-D and placental growth factor (PlGF); Figure 1.2) which between them regulate
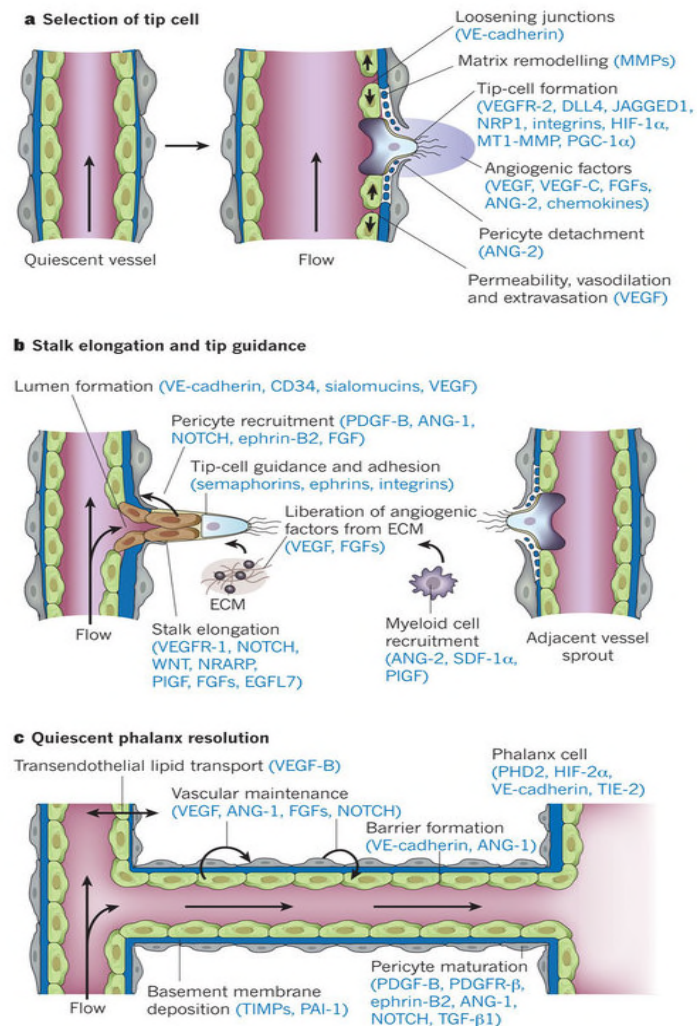
Figure 1.1. Model of angiogenesis. Schematics depicting the consecutive steps of blood vessel branching and the key molecular players involved. (A) Upon stimulation with angiogenic factors an endothelial cell tip cell becomes selected. Tip-cell formation requires degradation of the basement membrane, pericyte detachment and loosening of endothelial cell junctions. (B) Tip cells then navigate in response to guidance cues and adhere to the extracellular matrix to migrate. Stalk cells behind the tip cell proliferate and elongate to form a lumen. (C) Neighbouring tip cells may fuse to form collateral blood vessels, with lumen formation allowing the perfusion of the newly formed vessel. Quiescence is achieved upon re-establishment of junctions, deposition of basement membrane, maturation of pericytes and production of vascular maintenance signals. ANG, angiopoietin ; DLL4, delta-like 4; EGFL7, EGF-like domain-containing protein 7; FGF, fibroblast growth factor; HIF, hypoxia-inducible factor; MT1-MMP, membrane type I matrix metalloproteinase; NRARP, notch-regulated ankyrin repeat protein; NRP, Neuropilin; PCG-1a, peroxisomes proliferator-activated receptor gamma coactivator-1 alpha; PDGF, platelet-derived growth factor; PDGFR platelet-derived growth factor receptor; PIGF, placental growth factor; PHD, prolyl hydroxylase domain enzyme; SDF-1a, stromal cell-derived factor-1 alpha; TGF-b, transforming growth factor beta; TIMP, tissue inhibitor of metalloproteinases; VE-cadherin, vascular endothelial cadherin; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; WNT, wingless-related integration site. Figure taken from Carmeliet and Jain (2011).

angiogenesis, vasculogenesis and lymphanogenesis. These ligands differentially bind to class V receptor tyrosine kinases, called vascular endothelial growth factor receptors (VEGFR 1-3) and co-receptors such as Neuropilins i.e. NRP1 and NRP2 (Figure. 1.3) (Koch et al., 2011). Interestingly, some non-vertebrate polypeptides are structurally and functionally related to mammalian VEGFs. These include the parapox virus open reading frame, VEGF-E (Ogawa et al., 1998) and VEGF-F, a VEGF-related gene product present in some snake venoms (Yamazaki et al., 2005). VEGF polypeptides generally function as homodimers. However, heterodimers of VEGF-A and PlGF have been found to occur naturally (DiSalvo et al., 1995). The functional complexity of the VEGF family is emphasised by the large number of existing splice variants, created through either alternative RNA splicing (VEGF-A, VEGF-B, VEGF-C and PlGF) or via proteolytic processing (VEGF-A, VEGF-C and VEGF-D). Thus, multiple VEGF isoforms with distinct receptor and extracellular matrix-binding properties are encoded by each gene (Ferrara and Kerbel, 2005).

1.5.1.   VEGF-A

The founding member of this gene family, *VEGF-A*, is a crucial regulator of angiogenesis, mediating an array of endothelial functions such as cell proliferation, migration, tubulogenesis, leukocyte recruitment and vascular permeability. VEGF-A gene dosage is essential for normal mammalian development and homeostasis, as heterozygous (+/-) *VEGFA* knockout mice die between embryonic day E11 and E12 due to a deformed vascular network (Carmeliet et al., 1996; Ferrara et al., 1996). However, dysfunction in the cellular response to VEGF-A can cause pathological angiogenesis which contributes towards chronic inflammatory diseases, ischaemic heart disease, cancer and retinopathy (Carmeliet, 2005; Coultas et al., 2005; Ferrara and Kerbel, 2005). Almost all parenchymal cells express and secrete various VEGF ligands, particularly VEGF-A. VEGF-A can act either in a paracrine manner on neighbouring endothelial cells to regulate VEGFR-mediated signalling and angiogenesis, or in an autocrine fashion, which is considered essential for maintaining endothelial cell survival (Lee et al., 2007).

The *VEGF-A* gene is on chromosome 6p21.3 (Vincenti et al., 1996); transcription of this gene leads to the formation of pre-mRNA with a coding region that spans approximately 14 kb and contains 8 exons and 7 introns (Robinson and Stringer, 2001). Alternative splicing of this pre-mRNA transcript leads to the production of multiple VEGF-A isoforms (Figure 1.4). There are at least 7 pro-angiogenic isoforms of human VEGF-A which encode polypeptides of 121, 145, 148, 165, 183, 189
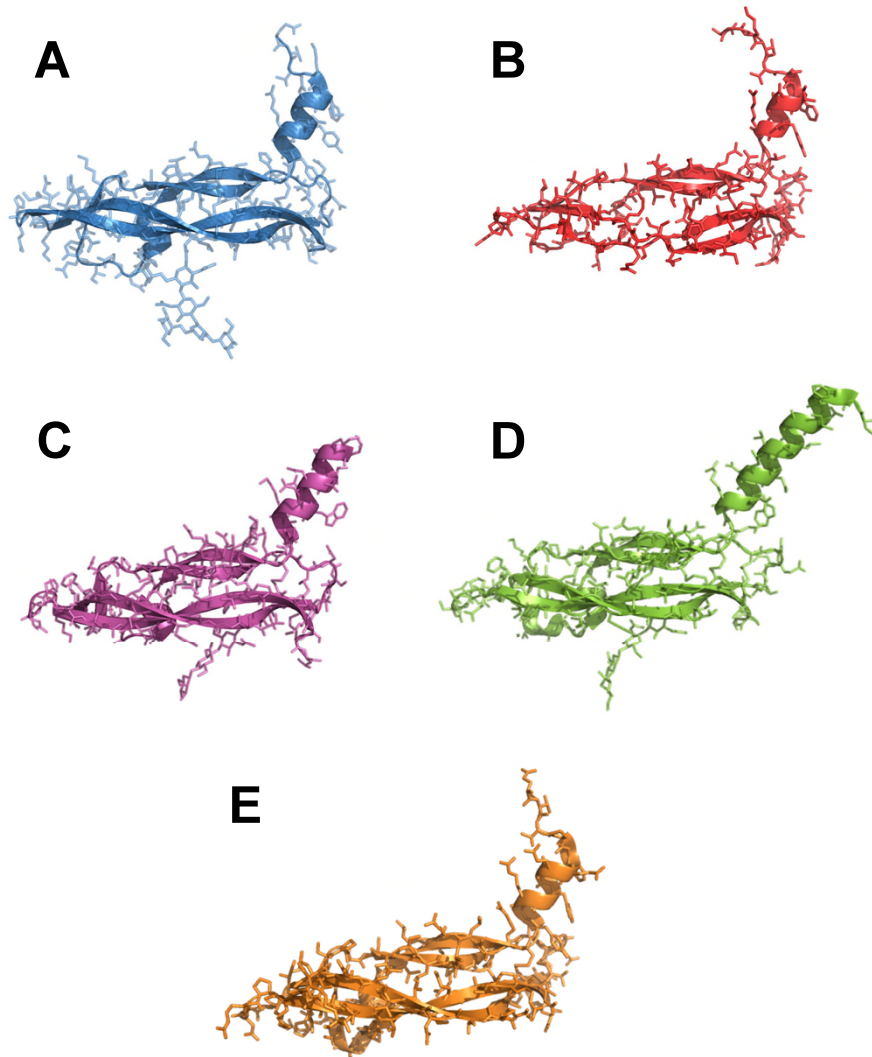
Figure 1.2. VEGF in situ protein structures. Ribbon and stick diagrams depicting the structure of receptor bound (A) VEGF-A (PDB id: 4KZN), (B) VEGF-B (PDB id: 2XAC), (C) VEGF-C (PDB id: 2X1W), (D) VEGF-D (PDB id: 2XV7) and (E) PlGF (PBD id: 1RV6). Figure and legend adapted from Smith et al. (2015).
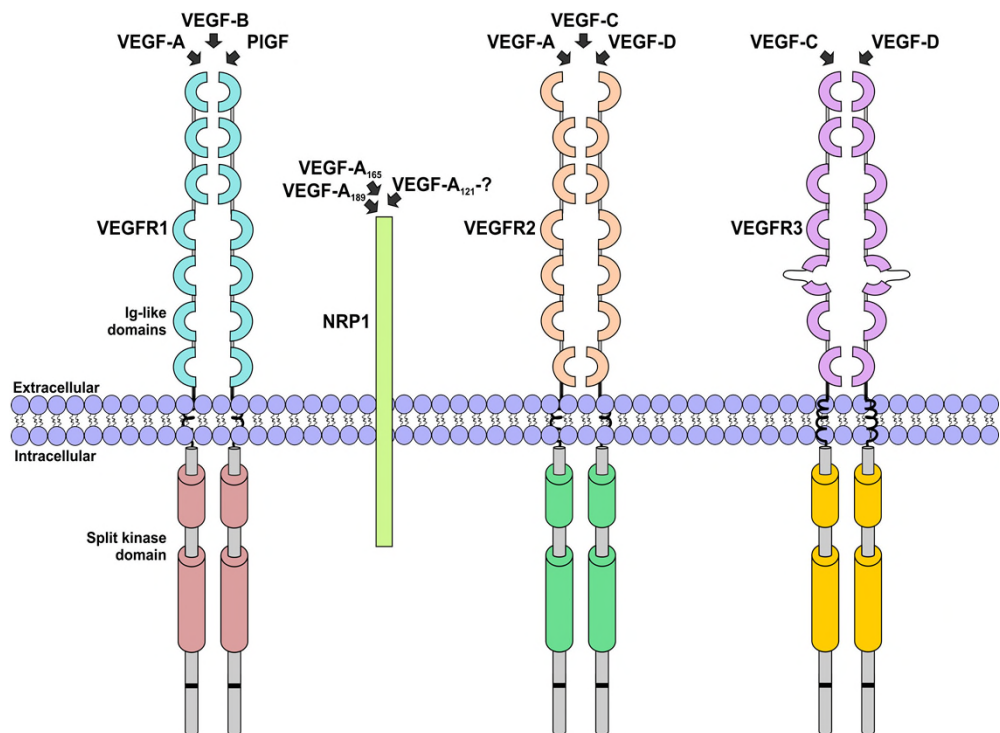
Figure 1.3. VEGFR receptor tyrosine kinase subfamily. Schematic illustrating interacting VEGF ligands associated with each VEGFR. Upon VEGF ligand binding, Ig-like domains can mediate further interaction between VEGFR monomers to promote complex assembly. Question mark (?) depicts debated interaction. Figure and legend adapted from Smith et al. (2015).

or 206 residues (a isoforms; Figure 1.4). Each VEGF-A isoform contains exons 1-5 which encode the signal sequence (exon 1), an N-terminus dimerisation domain (exon 2), VEGFR1-binding region, N-glycosylation sites (exon 3), a VEGFR2-binding site (exon 4) and a plasmin cleavage site (exon 5). Variable inclusion of exons 6a, 6b, 7a and 7b which encode the heparin-binding domain (HBD) largely resolves the identity of each VEGF-A isoform (Figure 1.4). Exon 6a is dominated by basic amino acids and acts as a direct inhibitor of VEGF-A activity by interfering with VEGFR2-VEGF-A binding (Jia et al., 2001), isoforms which contain exon 6a (i.e. VEGF-A$_{145}$ and VEGF-A$_{189}$) are weaker chemotactic cytokines and mitogens (Plouet et al., 1997; Lee et al., 2010). In addition to generating isoforms that differ in length and domain composition, the pre-mRNA splicing machinery can also give rise to at least 5 anti-angiogenic isoforms of 121, 145, 165, 183 and 189 residues in length (Figure 1.4).

However, the physiological existence of these anti-angiogenic isoforms has been highly debated (Harris et al., 2012). This generation of these anti-angiogenic isoforms occurs due to two splice site selection events, termed proximal splice site selection (PSS) and distal splice site selection (DSS). These selection events determine the terminal sequence of 6 amino acids (exon 8), namely either the pro-angiogenic sequence CDKPRR (exon 8a) or the anti-angiogenic sequence SLTRKD (exon 8b; Figure 1.4) (Harper and Bates, 2008). The change in carboxyl–terminal amino acid sequence between VEGF-A$_{165a}$ and VEGF-A$_{165b}$ has a tremendous effect on the structure and function of VEGF-A. VEGF-A$_{165a}$ can bind to VEGFR2 and Neuropilin 1, which results in a conformational change of VEGFR2 leading to internal rotation and autophosphorylation of its intracellular domain, thus inducing several signalling outputs. However, although VEGF-A$_{165b}$ binds to VEGFR2 with the same affinity as VEGF-A$_{165a}$, it elicits insufficient torsional rotation, which results in rapid closing of the VEGFR2-ATP binding site and receptor inactivation; hence this produces weak or transient downstream intracellular signalling outputs. Additionally, VEGF-A$_{165b}$ cannot bind Neuropilin 1 as the basic C-terminal amino acids (believed to be responsible in VEGF-A-NRP1 interactions) are substituted for a different sequence. Therefore a combination of weak receptor activation, lack of Neuropilin 1 co-signalling and competing for binding sites with its pro-angiogenic partner may contribute to its anti-angiogenic properties (Harper and Bates, 2008; Kawamura et al., 2008). Furthermore, as VEGF-A usually functions as a homodimer, there is a theoretical possibility of heterodimers, this phenomenon would allow for increased
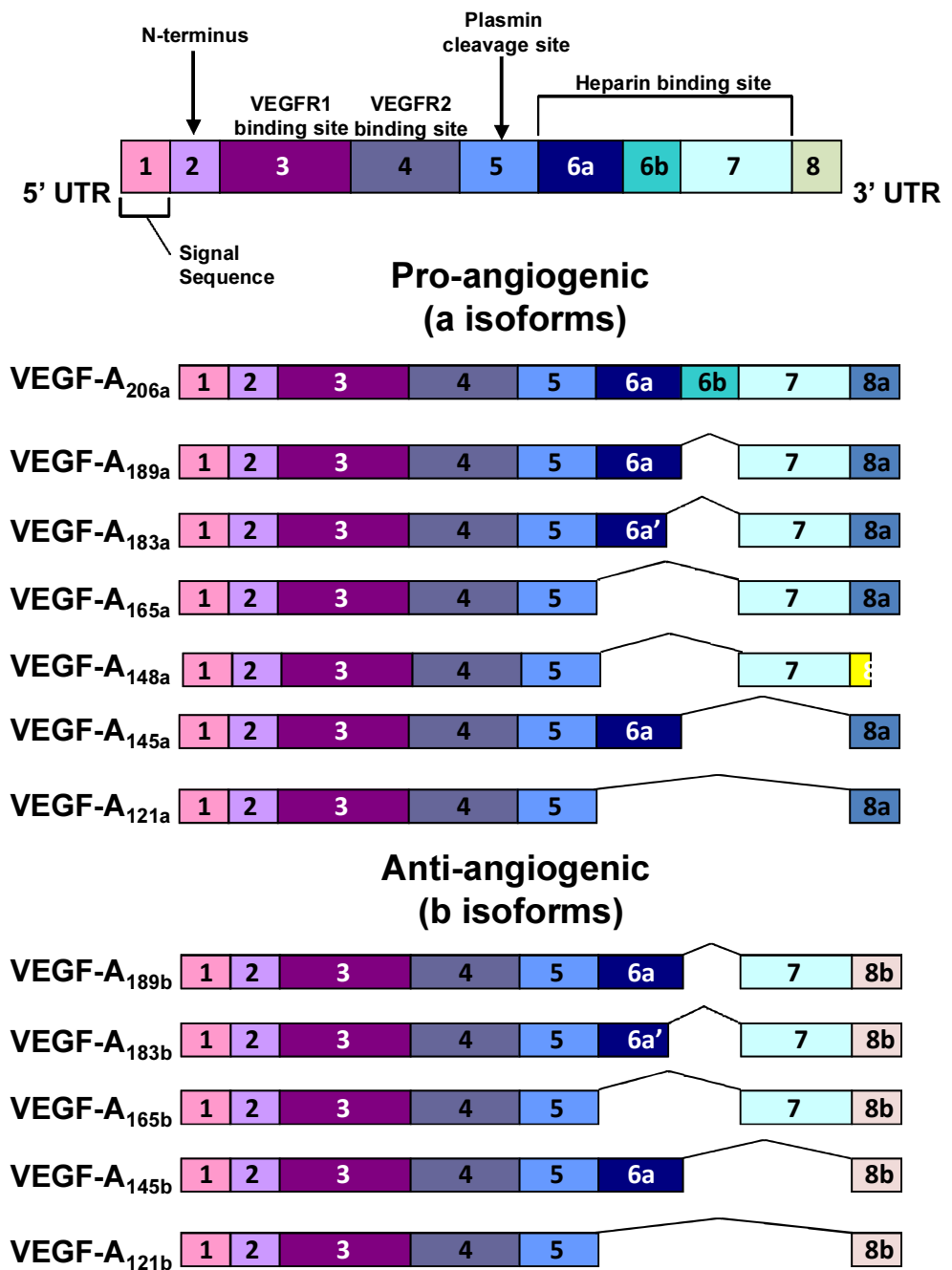
Figure 1.4. VEGF-A splice isoforms and exon arrangement. VEGF-A isoforms are made up from 8 exons and alternative splicing of these exons give rise to at least 7 pro-angiogenic (a isoforms) and 5 anti-angiogenic (b isoforms). 6a' indicates truncated form of exon 6a. VEGF-A_{148} is generated by truncation due to premature stop codon (yellow). VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor tyrosine kinase. Figure and legend adapted from Fearnley *et al.* (2013).

levels of complexity in regulating angiogenesis (Harper and Bates, 2008). Downregulation of VEGF-A$_{165b}$ expression promotes the switching of endothelial cells towards a pro-angiogenic phenotype. This 'switch' is associated with multiple diseases including diabetic retinopathy (Perrin et al., 2005) and several adult epithelial cancers (Varey et al., 2008). Conversely, VEGF-A$_{165b}$ upregulation in patients with systemic sclerosis results in impaired angiogenesis and reduced vascular repair (Manetti et al., 2010).

1.5.2. Transcriptional regulation of VEGF-A

The oxygen concentration within cells plays a crucial role in regulating the expression of hundreds of genes. During periods of hypoxia (1% or less O$_2$ levels), the expression of many proteins such as glucose transporters, glycolytic enzymes, erythropoietin and VEGFs increases. Together these proteins produce an adaptive response to hypoxia. This occurs by augmenting cellular ATP/O$_2$ levels (via activating ATP-synthesising pathways) and red blood cell count (via increasing blood supply via VEGF-mediated angiogenesis) (Roskoski, 2007). The main regulatory element in this process is the transcription factor, hypoxia-inducible factor-1 (HIF-1). HIF-1 is a heterodimeric nuclear transcription factor which makes a vital contribution to neovascularisation in wound healing and diseases, via modulating the expression of several target genes (including VEGF-A). These gene products regulate endothelial cell proliferation and migration, microvessel tone, vascular remodelling and proangiogenic myeloid cell recruitment (Evans et al., 2011; Oladipupo et al., 2011).

HIFs are master regulatory transcription factors, with more than 100 known target genes (Oladipupo et al., 2011). The HIF-1 heterodimer compromises of the HIF-1α and HIF-1β subunits. HIF-1β is constitutively expressed in cells; however HIF-1α levels are regulated in an oxygen-dependent manner. Under normoxia (physiological levels of 3-5% O$_2$) a prolyl hydroxylase domain enzyme (PHD) hydroxylates HIF-1α at two critical proline residues, forming a binding site for the von Hippel-Lindau factor (VHL). VHL is a component of the E3 ubiquitin ligase complex, thus binding of HIF-1α by VHL targets it for proteasomal degradation (Ivan et al., 2001). HIF-1α degradation inhibits HIF-1 function and subsequent gene transcription. Conversely, during periods of hypoxia PHD activity becomes compromised which prevents it from hydroxylating HIF-1α. HIF-1α is then free to translocate to the nucleus and form a heterodimer with HIF-1β. This HIF-1α-HIF-1β heterodimer then binds to the hypoxia-responsive element (HRE) promoting

transcriptional upregulation of its target genes, such as VEGF-A (Evans et al., 2011). In addition to prolyl residues, asparaginyl residues have also been implicated as a means of inactivating HIF-1α. Under normoxia, an asparagine residue (N803) located in the transactivation domain (TAD-C) of HIF-1α is hydroxylated by the hydroxylase factor inhibiting HIF-1 (FIH-1) (Mahon et al., 2001). This hydroxylation prevents the binding of co-activators CBP and P300, which under hypoxic conditions promote gene transcription via acetylating lysine residues within histones. Histone lysine acetylation weakens the association with DNA, giving HIF-1 better access to bind HREs (Lando et al., 2002). Thus, the oxygen-sensing ability of this system is tightly linked to the PHD enzyme activity (Berra et al., 2003).

The unfolded protein response (UPR) pathway is activated when normal homeostasis of the endoplasmic reticulum (ER) is disrupted due to changes in the extracellular environment (e.g. hypoxia). The UPR is a cytoprotective multi-component signal transduction pathway, which serves to reduce the adverse effects of accumulated unfolded proteins. This is mediated by regulating the levels of molecular chaperones, downregulating new protein synthesis and increasing the degradative capacity of the cell (Pereira et al., 2010). In mammalian cells, the UPR pathway is regulated by three transmembrane proteins that "sense" ER stress: namely Ire-1, PERK and ATF6 (Pereira et al., 2010). Ire-1 is an ER localised transmembrane protein, which upon sensing ER stress is trans-phosphorylated and its endonuclease domain becomes activated. Ire-1 activation results in the exclusion of a specific sequence (26 bases) from the X-box binding protein pre-mRNA transcript (Lee et al., 2003). This mRNA frameshift now encodes a functional XBP-1 transcription factor which stimulates transcription of various resident ER proteins which assist in the degradation or folding of defective proteins (Lee et al., 2003). XBP-1 has been shown to bind to two regions of the VEGF-A promoter (Pereira et al., 2010) leading to elevated levels of VEGF-A.

1.5.3.   VEGF-A isoforms

Multiple studies have shown that VEGF-A isoforms differentially regulate endothelial cell responses *in vitro*, with most studies in agreement that VEGF-A$_{165}$ produces the greatest effect in most instances (Kawamura et al., 2008; Pan et al., 2007). Additionally their individual properties *in vivo* have been investigated using Cre-LoxP technology. The phenotypic vascular defects witnessed are dependent on which VEGF-A isoforms are depleted. For instance blood vessels have an unnaturally large diameter and suffer from hypobranching in *VEGFA*$^{120/120}$ mice

(mice only expressing VEGF-A$_{120}$; VEGF-A$_{121}$ in humans), whereas in *VEGFA*$^{188/188}$ (mice only expressing VEGF-A$_{188}$; VEGF-A$_{189}$ in humans) blood vessels appear spindle-like and suffer from irregular branching (Ruhrberg et al., 2002). Contrastingly, *VEGFA*$^{164/164}$ mice which only express VEGF-A$_{164}$ (VEGF-A$_{165}$ in human) exhibit a relatively normal vascular phenotype (Ruhrberg et al., 2002). Intriguingly, both the morphological and branching defects associated with VEGFA$^{120/120}$ and VEGFA$^{188/188}$ mice are recovered in mice expressing both VEGF-A$_{120}$ and VEGF-A$_{188}$ (*VEGFA*$^{120/188}$ mice) (Ruhrberg et al., 2002). This data shows that both diffusible (VEGF-A$_{120}$) and matrix binding (VEGF-A$_{188}$) isoforms act as spatial cues to guide the developing blood vessel; both of which are required for normal healthy vasculogenesis (Ruhrberg et al., 2002; Haigh, 2008).

Aside from vascular defects, these mice also suffer from a multitude of other problems, including irregular organ development. *VEGFA*$^{120/120}$ mice overexpress VEGF-A$_{120}$ in comparison to wild type mice and stimulate enough vasculogenesis to survive until birth. However, these mice have multiple organ system defects affecting the heart (Carmeliet et al., 1999), bone (Zelzer et al., 2002; Maes et al., 2002), retina (Stalmans et al., 2002) and lung (Ng et al., 2001). Due to this array of defects ~99.5% of *VEGFA*$^{120/120}$ mice die ~2 weeks after birth, due to inferior cardiac function (Carmeliet et al., 1999). *VEGFA*$^{188/188}$ mice have serve defects in aortic arch remodelling leading to ~50% prenatal mortality. Those which survive until birth have defective artery development and suffer from dwarfism (Maes et al., 2004; Stalmans et al., 2002). Contrastingly, *VEGFA*$^{164/164}$ mice have relatively normal phenotypes (Stalmans et al., 2002). Again, mice which express both VEGF-A$_{120}$ and VEGF-A$_{188}$ (*VEGFA*$^{120/188}$ mice) alone, as opposed to *VEGFA*$^{120/120}$ or *VEGFA*$^{188/188}$ mice alone, also show no discernible phenotypic differences (Ruhrberg et al., 2002).

1.6.   Vascular endothelial growth factor receptors (VEGFRs)

Vascular endothelial growth factor receptors (VEGFRs) are a family of class V membrane bound receptor tyrosine kinases (RTKs) comprising VEGFR1, VEGFR2 and VEGFR3. These VEGFRs are structurally similar and comprise of an extracellular ligand-binding domain, a transmembrane domain, a juxtamembrane domain, a split tyrosine kinase domain and a C-terminal tail (Figure 1.3). VEGFs bind to the extracellular domain of VEGFRs and can also recruit additional cell surface-expressed co-receptors i.e. Neuropilins (NRP1 and NRP2), heparin sulphate glycoproteins (HSPGs), integrins and ephrin B2 (Grunewald et al., 2010; Sawamiphak et al., 2010; Zachary et al., 2009; Koch and Claesson-Welsh, 2012).

Not all members of the VEGF family bind each of the VEGFRs, VEGF-B and PlGF specifically bind VEGFR1 whilst VEGF-A binds both VEGFR1 and VEGFR2 (Errico et al., 2004; Olofsson et al., 1998), whereas, the non-human structurally related VEGF-E and VEGF-F bind exclusively to VEGFR2 (Wise et al., 2003). Finally, VEGF-C and VEGF-D bind VEGFR2, VEGFR3 and co-receptor NRP2 (Takahashi and Shibuya, 2005). Although all VEGF-A isoforms bind VEGFR2, distinct splice variants of VEGF-A can form specific receptor/co-receptor complexes. VEGF-A$_{165}$ and VEGF-A$_{189}$ bind to HSPGs and NRP1, however, VEGF-A$_{121}$ does not bind either of these co-receptors. The affinity of VEGF-A for HSPGs influences their extracellular matrix binding propertiesm, which regulates VEGFR signalling (Koch et al., 2011). VEGF-A binding to NRP1 is not thought to directly stimulated signal transduction but rather NRP1 presents bound-VEGF-A to VEGFR2 (either in *cis* or *trans*), thus selectively enhancing VEGFR2-VEGF-A complex signal transduction (Koch et al., 2014).

VEGFR-VEGF-A binding regulates a whole array of endothelial cell responses and occurs via either ligand presentation from co-receptors or from freely diffusible VEGF. Upon VEGF binding to the monomeric VEGFR N-terminal extracellular domain, VEGFR monomers undergo dimerisation which leads to a conformational change within the receptor's kinase domain. This conformational change leads to the exposure of an ATP binding site, resulting in subsequent trans-autophosphorylation of various key tyrosine residues within their carboxy-proximal kinase domains (Nilsson et al., 2010; Stuttfeld and Ballmer-Hofer, 2009). VEGF receptor activation is tightly regulated through receptor internalisation, degradation and via dephosphorylation by protein tyrosine phosphatases (PTPs); such as VEPTP (vascular endothelial PTP) and PTP1B (Bruns et al., 2009; Kappert et al., 2005; Lanahan et al., 2014).

1.6.1.   VEGFR1

The *VEGFR1* (*FLT1*) gene, located on human chromosome 13q12, contains 30 exons encoding an estimated 151 kDa membrane-bound receptor which undergoes post-translational modifications to produce a ~180 kDa mature glycoprotein (Kendall and Thomas, 1993; Devries et al., 1992). VEGFR1 binds VEGF-A, VEGF-B and PlGF (Figure 1.3) and is expressed in a variety of cell types including both quiescent and actively proliferating endothelial cells, hematopoietic stem cells, monocytes, macrophages, epithelial, tumour and neuronal cells (Roskoski, 2007; Koch and Claesson-Welsh, 2012; Robinson and Stringer, 2001). During embryonic

development, *VEGFR1* expression and functional requirement is essential: homozygous *VEGFR1*^{-/-} knock out mice die between embryonic days E8.5 and E9.5 due to endothelial hyperproliferation which causes blood vessel obstruction (Fong et al., 1995). VEGFR1 gene expression is hypoxia-sensitive, due to a hypoxia-inducible enhancer element located within the *VEGFR1* promoter region (Ulyatt et al., 2011). In addition to the mature full length ~180 kDa protein, the VEGFR1 pre-mRNA transcript undergoes alternative splicing to generate a soluble VEGFR1 isoform (sFlt-1; sVEGFR1) of ~110 kDa (He et al., 1999). Soluble VEGFR1 comprises of the immunoglobulin-like domains 1-6 present within mature VEGFR1, but also includes a unique 31-residue stretch encoded by intron 13 (Shibuya, 2001).

In endothelial cells, a significant proportion (~80%) of VEGFR1 is postulated to be stably localised to a Golgi-like compartment away from circulating ligands (Mittar et al., 2009). Upon VEGF-A stimulation, VEGFR1 undergoes trafficking from the *trans*-Golgi-network (TGN) to the plasma membrane (PM) via a calcium-dependent mechanism and pathway (Mittar et al., 2009). Despite lower VEGFR1 levels in endothelial cells, this receptor nonetheless binds VEGF-A with a higher affinity, compared to VEGFR2 (~10-30 pM vs ~75-125 pM) (Waltenberger et al., 1994; Devries et al., 1992). However, ligand-stimulated VEGFR1 kinase activity is weaker and thus forms a relatively non-productive signalling complex (Robinson and Stringer, 2001). Reduced VEGFR1 kinase activity is thought to arise through a combination of a repressor sequence in its juxtamembrane domain and a lack of positive regulatory tyrosine residues (Ito et al., 1998; Gille et al., 2000). Due to its weak kinase activity and strong affinity for VEGF-A, it is thought that both mature and soluble VEGFR1 act as 'decoy receptors', sequestering circulating VEGF-A and thus reducing its bioavailability for VEGFR2 (Rahimi, 2006). Furthermore, sVEGFR1 is also a potent inhibitor of both VEGF-B and PlGF-mediated signal transduction (Olsson et al., 2006). VEGFR1 plays an important role in a range of inflammatory pathological conditions; such as rheumatoid arthritis (Murakami et al., 2006). Soluble VEGFR1 has been implicated in the pathogenesis of pre-eclampsia (Maynard et al., 2003). Pre-eclampsia is characterised by hypertension, proteinuria and accumulation of a sVEGFR1 variant containing exon 14 in the circulation of women with the disease (Sela et al., 2008). Besides its well-known anti-angiogenic role as a 'VEGF trap', VEGFR1 can also be weakly phosphorylated on multiple specific tyrosine residues (Y794, Y1169, Y1213, Y1242, Y1327, and Y1333; Figure 1.5) located within its cytoplasmic domain (Wiesmann et al., 1997; Cunningham et al., 1997; Yu et al., 2001). Interestingly, the pattern of VEGFR1 tyrosine

Figure 1.5. VEGFR1-mediated signal transduction. Schematic depiciting vascular endothelial growth factor 1 (VEGFR1)-mediated signal transduction in response to VEGF. Y1309 (boxed italics) is phosphorylated only upon PlGF stimulation. Biological functions of VEGFR1 are summarized in the bottom box. 3D, three-dimensional; GRB2, growth factor receptor-bound protein 2; JAK, Janus kinase; NFAT, nuclear factor of activated T-cells; PKB, protein kinase B; PI3K, phosphoinositide 3-kinase; PLCγ1, phospholipase c gamma 1; PlGF, placental growth factor; NO, nitric oxide; RACK1, receptor for activated C-kinase 1; SHP2, Src homology 2-containing phosphotyrosine phosphatase 2; STAT, signal transducer and activator of transcription; VEGF, vascular endothelial cell growth factor. Figure and legend adapted from Koch *et al.* (2011).

phosphorylation is ligand-dependent i.e. Y1309 phosphorylation is specific to PlGF-stimulation and results in subsequent activation of downstream Akt (Autiero et al., 2003). VEGFR1-mediated signal transduction (Figure 1.5) is implicated in endothelial cell migration and actin re-organisation via regulating RACK1 (receptor for activated C-kinase 1) activation (Wang et al., 2011). VEGFR1-mediated PI3K/Akt activation is linked to endothelial cell differentiation and sustaining angiogenesis (Cai et al., 2003). VEGFR1 also mediates other signalling transduction pathways including, PLCγ1 via recruiting it onto pY1169 and pY794 epitopes within the cytoplasmic domain of activated VEGFR1. Additionally, activated VEGFR1 can also recruit growth-factor-receptor bound protein 2 (Grb2) and SH2-domain-containing protein tyrosine phosphatase 2 (SHP-2) factors (Roskoski, 2007; Cunningham et al., 1997; Ito et al., 1998). Thus, due to its contrasting functions, VEGFR1 has the potential to positively or negatively influence *de novo* blood vessel growth, under varying biological conditions. Surprisingly, mice expressing tyrosine kinase-dead VEGFR1 (TK$^{-/-}$) mutants are viable and exhibit normal developmental blood vessel formation; yet these mice have defective VEGF-A-mediated macrophage migration (Shibuya, 2014). Consequently, its precise signalling role in regulating endothelial cell function is disputed. However, a multitude of studies have demonstrated that VEGFR1 kinase activity is required for angiogenesis during, tumour metastasis, inflammatory diseases, stroke, liver repair and ulcer healing (Shibuya, 2014). Therefore, despite its lowered tyrosine kinase activity, VEGFR1-targeted therapeutics may be beneficial during certain disease conditions (Shibuya, 2006).

The VEGFR1-specific ligands VEGF-B and PlGF have widely different properties: it is possible that VEGFR1-mediated effects on cell function are regulated via co-receptor binding or through cell-specific signal transduction (Koch and Claesson-Welsh, 2012). VEGF-B-stimulated endothelial cell fatty acid uptake is crucial in organs which experience high metabolic stress (such as the heart) and requires both VEGFR1 and NRP1 activation (Hagberg et al., 2010). Additionally, VEGF-B stimulation of cardiac endothelial cells mediates revascularisation of ischaemic myocardium (Li et al., 2008). In contrast, PlGF has no role in fatty acid uptake nor physiological angiogenesis; however, PlGF promotes inflammation-associated pathological angiogenesis via VEGFR1-mediated recruitment of monocytes which secrete angiogenic growth factors (Carmeliet et al., 2001).

1.6.2.　　　　VEGFR2

First isolated in 1991, vascular endothelial growth factor receptor 2 (VEGFR2; Kinase-insert domain receptor (KDR); foetal liver kinase (Flk)-1) is a class V receptor tyrosine kinase (RTK) which binds to a family of vascular endothelial growth factors (VEGFs) (Terman et al., 1991). Human VEGFR2 is ~1356 residues in length and consists of an extracellular domain composing of seven immunoglobulin (Ig)-like domains, a short transmembrane domain and a split tyrosine kinase domain (Holmes et al., 2007). Prior to ligand activation, immature VEGFR2 (~150 kDa) must undergo N-linked glycosylation along the secretory pathway to produce a mature glycoprotein with an approximate mass of 200-230 kDa (Waltenberger et al., 1994; Koch and Claesson-Welsh, 2012). This process is essential to ensure efficient ligand-stimulated VEGFR2 trans-autophosphorylation (Takahashi and Shibuya, 1997). Contrastingly and unlike VEGFR1, the expression of VEGFR2 is mostly restricted to vascular endothelial cells and their embryonic precursors (Millauer et al., 1993). However, VEGFR2 is also expressed in other cell types such as neurons and immune cells (Ponnambalam and Alberghina, 2011; Adham and Coomber, 2009). Similarly to VEGFR1, alternative splicing can generate a soluble form of VEGFR2 (sVEGFR2) which is present in multiple tissues including the heart, spleen, skin, ovary and kidney, as well as in the circulating plasma (Albuquerque et al., 2009). The *VEGFR2* gene is essential for healthy mammalian development as homozygous *VEGFR2$^{-/-}$* knock out mice die at embryonic day E8.5 due to impaired development of hematopoietic and endothelial cells (Shalaby et al., 1995). This leads to the formation of an insufficient vascular network; a phenotype which mimics that of the *VEGFA$^{-/-}$* knock out mice (Ferrara et al., 1996; Carmeliet et al., 1996; Koch and Claesson-Welsh, 2012; Shalaby et al., 1995).

The binding affinity of VEGF-A for VEGFR2 is ~5-10-fold lower than for VEGFR1. However, despite this lower affinity, most VEGF-A-stimulated angiogenesis is programed through VEGFR2/VEGF-A mediated-signal transduction. This is primarily due to the increased kinase activity displayed by VEGFR2 upon ligand binding and activation (Roskoski, 2007; Quinn et al., 1993), thus VEGFR2 is considered the major pro-angiogenic receptor through which VEGFs regulate blood vessel development and homeostasis (Roskoski, 2007; Quinn et al., 1993). Intriguingly, and in contrast to VEGFR1, VEGFR2 expression is downregulated in quiescent adult vasculature as a means of attenuating VEGFR2-regulated pro-angiogenic gene expression (Eichmann et al., 1997; Kanno et al., 2000). VEGFR2 is localised to the Golgi, plasma membrane, early endosomes and perinuclear

caveolae in non-stimulated endothelial cells (Bruns et al., 2009; Jopling et al., 2011; Manickam et al., 2011; Bhattacharya et al., 2005). Approximately 60% of resting VEGFR2 is situated at the plasma membrane, with the remainder located in an internal early endosomal pool (Gampel et al., 2006; Jopling et al., 2011; Jopling et al., 2014). Unstimulated VEGFR2 undergoes a relatively fast rate of ligand-independent, constitutive internalisation (Gampel et al., 2006; Lampugnani et al., 2006); which does not require tyrosine kinase activity (Jopling et al., 2011).

VEGFR2-mediated signalling regulates a wide array of endothelial cell responses ranging from proliferation, migration, survival, vascular tube formation to cell-cell interactions (Kawamura et al., 2008; Koch and Claesson-Welsh, 2012). VEGF-A interacts with VEGFR2 via binding to Ig-like domains 2 and 3 on its extracellular domain (ECD; Figure 1.3) (Shinkai et al., 1998). Ligand binding promotes monomeric VEGFR2 dimerisation which is stabilised via low-affinity homotypic interactions between the 7th Ig-like domains within the VEGFR2 extracellular domain (Figure 1.3) (Ruch et al., 2007). Interestingly, deletion of the last four Ig-like domains decreases the VEGFR2 ligand-binding affinity by ~1000-fold (Shinkai et al., 1998). VEGF-induced interactions between monomeric VEGFR2 ECDs bring adjacent RTK domains into contact alignment allowing torsional rotation and exposure of an ATP binding site. Exposure of this site allows ATP binding and subsequent transfer of the ATP γ-phosphate onto the hydroxyl-group of specific tyrosine residues within its cytoplasmic kinase domain e.g. pY951, pY1054, pY1059, pY1175 and pY1214 (Figure 1.6) (Koch et al., 2011; Roskoski, 2007). Phosphorylation of these tyrosine residues leads to the recruitment of numerous SH2 domain-containing adaptor proteins and the subsequent activation of multiple downstream signal transduction pathways.

Ligand-induced phosphorylation of VEGFR2-Y951 provides a binding site for the SH2-domain-containing T-cell-specific adaptor molecule (TSAd) and is associated with endothelial cell migration and vascular permeability (Matsumoto et al., 2005). Whereas the phosphorylation of VEGFR2-Y1054 and VEGFR2-Y1059 is crucial for kinase activity (Kendall et al., 1999). Phosphorylation of VEGFR2-Y1059 promotes recruitment of Src which phosphorylates other specific-residues within the VEGFR2 kinase domain, such as Y1175. Furthermore, activated Src phosphorylates other downstream signal transducers (Figure 1.6).
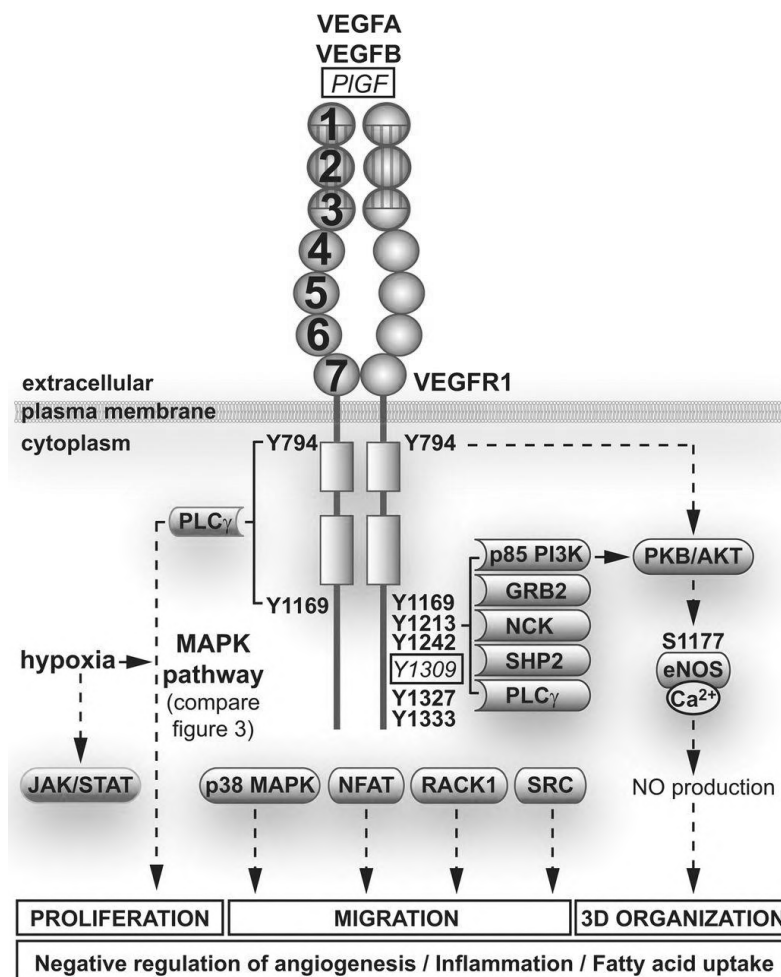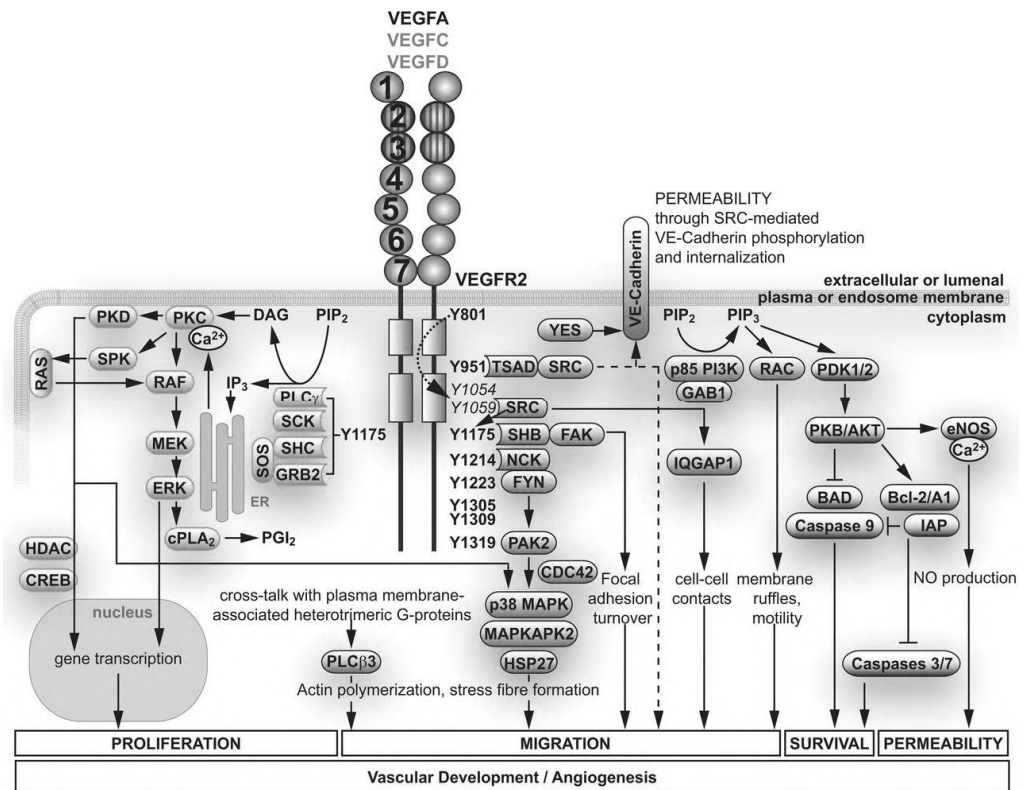
Figure 1.6. VEGFR2-mediated signal transduction. Schematic depicting vascular endothelial growth factor 2 (VEGFR2)-mediated signal transduction in response to VEGF. Y1054 and Y1059 are crucial for VEGFR2 kinase activity (italics). The complex network of intracellular signal transduction modulates and array of endothelial cell responses such as proliferation, migration, survival and permeability. BAD, Bcl2-associated death promoter; CDC42, cell division cycle 42; $cPLA_2$, cytosolic phospholipase A_2; CREB, cyclic AMP response element binding protein; DAG, diacylglycerol; eNOS, endothelial nitric oxide synthase; ER, endoplasmic reticulum; ERK, extracellular signal-regulated kinase; FAK, focal adhesion kinase; GAB1, GRB2-associated binding protein 1; GRB2, growth factor receptor-bound protein 2; HDAC, histone deacetylase; HSP27, heat-shock protein of 27 kDa; IAP, inhibitor of apoptosis; IP_3, inositol 1,4,5-trisphosphate; MAPK, mitogen-activated protein kinase; MAPKAPK2, MAPK-activated protein kinase 2; MEK, mitogen-activated protein kinase kinase; NO, nitric oxide; PAK2, p21-activated protein kinase 2; PDK, phosphoinositide-dependent kinase; PGI_2, prostaglandin I2; PIP_2, phosphatidylinositol 4,5-bisphosphate; PLCβ3, phospholipase c beta 3; PLCγ1, phospholipase c gamma 1; SCK, SHC-related adaptor protein; SHC, Src homology 2 domain containing transforming protein; SOS, son of sevenless; SPK, sphingosine kinase; TSAD, T-cell-specific adaptor; VEGF, vascular endothelial growth factor. Figure and legend adapted from Koch *et al*. (2011).

VEGFR2-Y1175 phosphorylation (Figure 1.6) recruits PLCγ1 and adaptor protein SH2-domain-containing adaptor protein B (Shb) which in turn binds to FAK (focal adhesion kinase) and contributes to endothelial cell migration and attachment (Holmqvist et al., 2003). PLCγ1 activation induces hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2) to membrane-bound diacylglycerol (DAG) and soluble IP_3. Subsequent binding of IP_3 to the IP_3 receptor leads to the translocation of intracellular Ca^{2+} from the endoplasmic reticulum to the cytosol, resulting in the dephosphorylation and activation of the transcription factor nuclear factor of activated T-cells (NFAT) via a calmodulin-calcineurin-mediated pathway (Armesilla et al., 1999). This proangiogenic pathway promotes an inflammatory response that is similar to NFκB activation downstream of Akt (Jiang and Liu, 2009). In parallel, increased DAG results in elevated protein kinase C (PKC) activity and subsequent activation of the MAPK pathway. PLCγ1 activation of PKC also regulates cell proliferation and gene expression, via the MEK1-ERK pathway (Takahashi et al., 2001). Furthermore, Shb-mediated activation of PI3K results in sequential activation of Akt and endothelial nitric oxide synthase (eNOS), resulting in increased cell survival and nitric oxide (NO) production, respectively (Olsson et al., 2006). Grb2 binding to Y1175 recruits nucleotide-exchange factor SOS (Son of Sevenless) which controls Ras activation and mitogenicity in response to VEGF-A (Warner et al., 2000; Meadows et al., 2001). The VEGFR2-pY1175 phospho-epitope has been widely studied and is crucial for VEGFR2-activity in mammalian development. Mice expressing Y1173F (corresponding to Y1175F in humans) VEGFR2 mutants die at embryonic day E8.5-9, due to phenotypic defects mimicking that of full $VEGFR2^{-/-}$ knockout mice (Sakurai et al., 2005; Shalaby et al., 1995; Koch and Claesson-Welsh, 2012).

VEGFR2-Y1214 phosphorylation (Figure 1.6) leads to the recruitment of Nck and the cytoplasmic tyrosine kinase, Fyn. Nck-Fyn complex formation regulates PAK2 (p21-activated protein kinase 2) phosphorylation which in turn activates cell division cycle 42 GTPase (Cdc42) and p38 MAPK signal transduction (Lamalice et al., 2006). Cdc42 and p38 kinase activation impacts on cell migration, by increasing actin remodelling. Contrastingly, unlike the VEGFR2-pY1175 epitope, the phosphorylation of Y1214 does not appear to be essential for mammalian development, as mice expressing the VEGFR2-Y1212F knock-in mutation (corresponding to VEGFR2-Y1214F in humans) are viable (Sakurai et al., 2005).

This raises the question of the exact role of Y1214 in VEGFR2-mediated angiognesis.

Following VEGF-A stimulation, VEGFR2 undergoes ubiquitination and internalisation into early endosomes (Figure 1.7). Activated VEGFR2 is then either targeted for lysosomal degradation or recycled back to the plasma membrane (Figure 1.7) (Miaczynska et al., 2004). Early endosomal localisation of VEGFR2 is essential for optimal tyrosine phosphorylation and subsequent activation of the MEK1-ERK1/2 signalling pathways (Gourlaouen et al., 2013). In contrast, activation of the p38 MAPK signal transduction pathway is believed to be initiated by cell surface VEGFR2 only (Lampugnani et al., 2006; Chen et al., 2010; Sawamiphak et al., 2010). Upon VEGF-A stimulation, the relative distribution of intracellular VEGFR2 shifts from early to late endosomes depending upon the intensity and duration of VEGF-A stimulation (Gampel et al., 2006). The remodeling of early or late endosomal membranes is closely associated with the Rab5 and Rab7 GTPases, respectively (Arlt et al., 2015). It has been proposed that the Rab5a and Rab7a GTPases regulate VEGFR2 early endosomal trafficking and signal transduction and early to late endosomal trafficking (Figure 1.7), respectively (Jopling et al., 2009; Rodman and Wandinger-Ness, 2000). Inactive Rabs are located within the cytosol and are kept soluble via binding to the GDP dissociation inhibitor (GDI) (Arlt et al., 2015; Barr, 2013). However, membrane-associated guanine exchange factors (GEFs) recruit Rabs onto the endosomes, triggering the exchange of Rab bound GDP for GTP and promoting its subsequent activation (Arlt et al., 2015; Barr, 2013). Activation of Rabs, results in a conformational change, which allows them to bind Rab effector protein complexes present on target membranes. The binding of Rabs to specific effector proteins allows the docking of transport vesicles with the appropriate target membrane; for instance the binding of Rab5 with the early endosome marker EEA1 (Zerial and McBride, 2001). Once docked, v-SNARE proteins located within the transport vesicles plasma membrane bind to specific t-SNARE proteins present on the target membrane, resulting in the formation of a SNARE complex (Schekman, 1998; Chen and Scheller, 2001). Upon formation, this SNARE complex drives the fusion of adjacent membranes, yet the precise mechanism by which this occurs is unclear (Chen and Scheller, 2001). Additionally, recycling of activated VEGFR2 has been said to occur either via Rab4a- or Rab11a-positive endosomes and follows a short loop (Rab4a) or long loop (Rab11a) pathway (Figure 1.7) (Ballmer-Hofer et al., 2011; Jopling et al., 2014). Long loop recycling is believed to occur in coordination with NRP1 trafficking
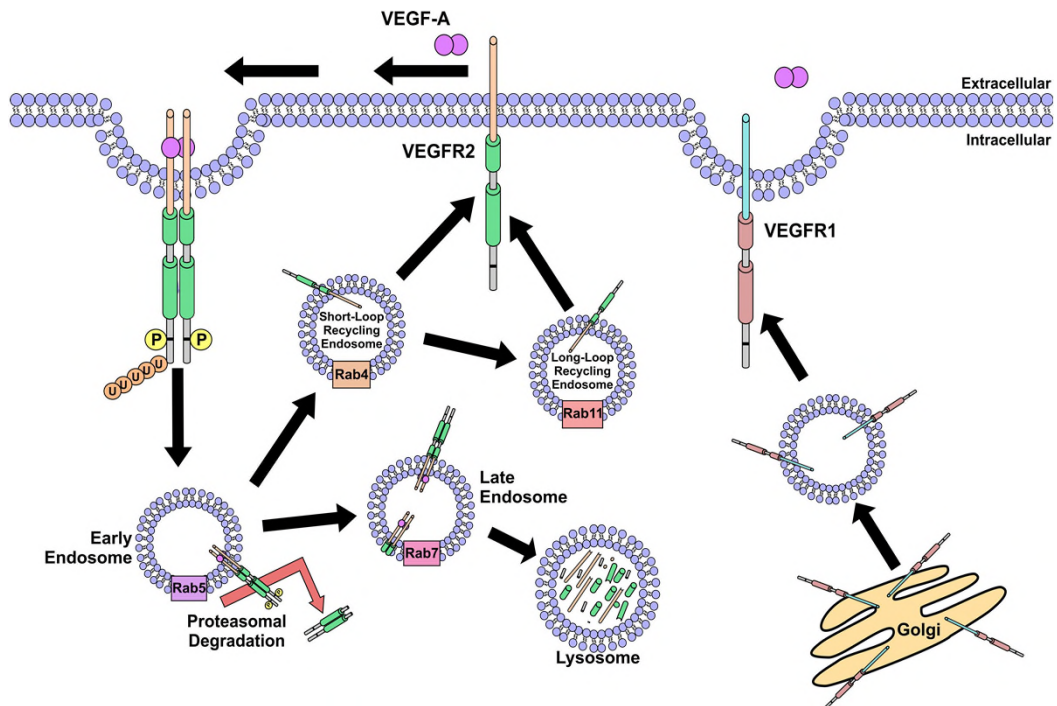
Figure 1.7. VEGFR activation, trafficking and proteolysis. Schematic depicting constitutive and ligand-dependent VEGFR trafficking and proteolysis. Upon VEGF-A binding, VEGFR2 undergoes dimerisation, transautophosphorylation, ubiquitination and internilisation. Following internalisation into early endosomes, both activated and quiescent VEGFR2 can undergo recycling back to the plasma membrane via short- or long-loop recycling pathways. Alternatively, ubiquitinated VEGFR2 undergoes 26S proteasome-regulated cleavage of its C-terminus, followed by trafficking towards late endosomes and lysosomes for terminal degradation. Furthermore, VEGF-A-stimulated VEGFR2 signal transduction promotes increased calcium-dependent plasma membrane translocation of VEGFR1 thus generating a negative feedback loop to attenuate VEGFR2 activity. Figure and legend adapted from Smith *et al.* (2015).

following transition from Rab4a-postive vesicles. Here, Rab4a-Rab11a transition is coordinated by the interaction between the C-terminal PDZ-binding motif of synectin, myosin VI and the NRP1 C-terminal motif, SEA (Ser-Glu-Ala) (Ballmer-Hofer et al., 2011; Cai and Reed, 1999; Wang et al., 2003; Chittenden et al., 2006). Furthermore, it is also believed that VEGF-A isoforms unable to bind NRP1, such as VEGF-A$_{165b}$, fail to promote Rab11a-dependent recycling (Ballmer-Hofer et al., 2011).

Internalised VEGFR2 continues to signal from intracellular compartments until it is committed for recycling or degradation (Murdaca et al., 2004). Internalised VEGFR2 undergoes 26S proteasome-mediated proteolysis in early endosomes which results in the cleavage of its C-terminal domain prior to lysosomal processing (Ewan et al., 2006; Bruns et al., 2010). Proteasome-mediated VEGFR2 proteolysis regulates VEGFR2 signal transduction through the Akt, eNOS and MAPK pathways.

1.6.3.   VEGFR2-VEGF-A signalling in disease states

Dysfunctional VEGFR2-VEGF-A signalling has been linked to multiple disease pathologies such as tumour development and metatasis, atherosclerosis, ischaemia, diabetes and age-related macular degeneration (AMD). Pathological angiogenesis is a hallmark of cancer, as like all tissues, tumours require a healthy blood supply to provided nutrients and oxygen as well as to allow for the removal of metabolic wastes and carbon dioxide (Hanahan and Weinberg, 2011). In order for a solid tumour to grow beyond 1-2 mm in diamter, it must recruit the surrounding vasculaculture via stimulating angiogenesis (Folkman, 1971). One way in which tumours promote angiogenesis is by stimulating VEGF-A expression through hypoxia-induced HIF-1-dependent *VEGFA* gene transcription (see previous section 1.5.2). Increased VEGFR2 and VEGF-A expression has been implicated in a wide range of cancers including: bladder, brain, breast, colon, gastric, lung, and prostate (see (Goel and Mercurio, 2013) and refrences therin). However, in contrast to acting in a paracrine fashion on neigbouring endothelial cells to promote increased blood vessel growth, autocrine VEGF-A-stimulated signal trasduction has also been attributed to tumour progression (Lichtenberger et al., 2010; Schoeffner et al., 2005). Autocrine VEGF-A signalling in tumour cells occurs via VEGFR2 and NRPs, and promotes cell growth, migration, survival and invasion, via activating PI3K-Akt and MEK1-ERK1/2 signalling pathways (Goel and Mercurio, 2013).

Hypoxic tumour cells primarliy rely on glucose as a means of generating ATP (Ruan and Kazlauskas, 2013). Additionally, oxygenated tumour cells also use glucose (aerobic glycolysis), a phenomenon known as the Warburg effect (Warburg et al., 1927). Both of these metabolic process generate lactate as a byproduct. High levels of lactate have the ability to stimulate angiogenesis via promoting ligand-independent activation of VEGFR2 and subsequent activation of the PI3K/Akt pathway (Ruan and Kazlauskas, 2013). This occurs via a complex signalling process comprising of three RTKs, namley VEGFR2, Axl and Tie2 (Ruan and Kazlauskas, 2013). Additionally, lactate enters endothelial cells via the MCT1 transporter and inhibits the oxygen-sensing prolyl hydoxylase (PHD2), thus stabilising hypoxia-inducible factor 1α (HIF-1α) resulting in the elevated transcription of multiple angiogenic genes including, VEGF-A (Sonveaux et al., 2012; De Saedeleer et al., 2012).

Futhermore, under conditions of wound healing and tissue ischaemia, a combination of reduced vascular perfusion and increased inflamatory cell oxygen consuption leads to hypoxia (Trabold et al., 2003; Ruan and Kazlauskas, 2013; Porporato et al., 2012). This triggers a metabolic switch towards glycolysis resulting in increased lactate production (Porporato et al., 2012; Ruan and Kazlauskas, 2013). Elevated lactate levels stimulate angigoenesis possibly via the mechanisms decribed above, as a means of promoting blood vessel growth to combat tissue ischaemia and injury. Hypercholesterolemia is associated with atherosclerosis, an underlying condition of ischaemic disease (Jin et al., 2013). During periods of hypercholesterolemia, both vascular function and *de novo* blood vessel formation are impaired (Jin et al., 2013; Tirziu et al., 2005; Van Belle et al., 1997). One reason behind this is that increased circulating low density lipoprotein (LDL) levels attenuate VEGF-A-stimulated signal transduction (Jin et al., 2013). Endothelial cell exposure to LDL depletes VEGFR2 from the cell surface, by promoting VEGFR2 turnover via a syntaxin-16-dependent mechanism (Jin et al., 2013). Reduction in VEGFR2 levels results in diminished VEGF-A-stimulated Akt and ERK1/2 signal transduction and subsequently imparied endothelial cell proliferation, migration and tubulogenesis (Jin et al., 2013).

Diabetics suffer from multiply vascular problems and poor wound healing due to endothelial dysfunction (Rask-Madsen and King, 2013). Chronic diabetes illustrates an angiogenic paradox, whereby both exacerbated and inferior angiogenesis occurs (Simons, 2005; Werner et al., 2003). Hyperglycaemia as a result of diabetes leads

to the generation of reactive oxygen species (ROS) through the activation of protein kinase C, as well as among other mechanisms (Rask-Madsen and King, 2013). Elevated ROS levels lead to ligand-dependent VEGFR2 activation within the Golgi network (Warren et al., 2014). VEGFR2 activation promotes its turnover, resulting in attenuated Golgi-associated VEGFR2 stores and VEGFR2 cell surface levels (Warren et al., 2014). Depleted levels of cell surface-associated VEGFR2 results in diminished VEGF-A-stimulated signal transduction and subsequent endothelial responses (Warren et al., 2014). A recent study has suggested that the anti-angiogenic VEGF-A isoform VEGF-A$_{165b}$ can influence prostate cancer progression (Mavrou et al., 2014) via repressing blood vessel development. Furthermore, VEGF-A$_{165b}$ over expression in ischaemic tissues was shown to impair revascularisation of ischaemic limbs in a mouse model of peripheral arterial disease (Kikuchi et al., 2014).

1.6.4. Complexities within VEGFR1-VEGFR2 signal transduction

Many studies show that VEGFR1 and VEGFR2 mediate cross-talk between their signalling pathways. VEGFR1-mediated PI3K pathway activation inhibits VEGFR2-associated endothelial cell proliferation (Zeng et al., 2001). Furthermore, embryonic stem cells lacking VEGFR1 elicit a higher level of VEGFR2 signalling (Roberts et al., 2004), probably due to the higher bioavailability of VEGF-A for VEGFR2. However, upon combined PlGF and VEGF-A treatment, the activation of VEGFR1 by PlGF increased VEGFR2 phosphorylation (Autiero et al., 2003), again this is probably due to the increased bioavailability of VEGF-A for VEGFR2 (Olsson et al., 2006). Additionally, differential apico-basal positioning of VEGFR1/VEGFR2 at the vascular blood-neural barriers facilitates specific-signal transduction and cellular responses (Hudson et al., 2014). Thus, regulating endothelial cell polarisation and VEGFR clustering allows for tighter control of VEGFR-mediated endothelial cell functions.

Concerns over using VEGF-A to promote angiogenesis in patients arise from its ability to stimulate fluid retention, inflammation and tumour growth. However, the virally-encoded VEGF-E (which only binds VEGFR2) has similar properties to VEGF-A (Meyer et al., 1999) but has reduced effects on permeability and inflammation (mediated via VEGFR1-activation) (Shibuya, 2009). A chimeric VEGF-E$_{NZ7}$/PlGF heterodimer has been shown to elicit a strong angiogenic response in transgenic mice, while reducing the high levels of inflammation and blood vessel permeability associated with VEGF-A. Thus leading to the possibility of a safer

therapeutic pro-angiogenic agent (Zheng et al., 2006). Therefore, understanding how co-operative VEGFR1 and VEGFR2 signal transduction regulates vascular homeostasis is essential for the development of effective and safer therapeutics for many diseases.

1.6.5.   VEGFR3

VEGFR3 (Flt-4) is an essential regulator of lymphendothelial function and ultimately lymphanogenesis (Figure 1.8). Mutations within the VEGFR3 tyrosine kinase domain are associated with variants of hereditary lymphoedema emphasising its pivotal role in lymphatic endothelial cell function (Irrthum et al., 2000). The VEGFR3 precursor protein is ~195 kDa and undergoes proteolytic cleavage within the 5th Ig-like domain to generate an N-terminal polypeptide which remains bound to the carboxy-terminal polypeptide via a disulphide linkage (Pajusola et al., 1993) (Figure 1.3). Alternative splicing of the *VEGFR3* primary transcript produces both long and short isoforms (Pajusola et al., 1993; Galland et al., 1993). This shorter VEGFR3 isoform lacks 65 C-terminal amino acids which include two specific C-terminal phosphorylation sites that are normally phosphorylated in VEGFR3 homodimers but not in VEGFR2/VEGFR3 heterodimers (Dixelius et al., 2003). VEGFR3$^{-/-}$ knockout mice die at embryonic day E10-11; this is however not because of disruption to the hosts lymphatic system but due to impaired hierarchical formation of the peripheral blood vasculature and cardiac remodelling defects (Dumont et al., 1998). Although VEGFR3 plays a well-characterised role in lymphatic endothelial cells, it is first expressed on venous endothelial cells during development before becoming restricted to the lymphatic endothelium. However, its expression is also upregulated in vascular endothelial cells involved in active angiogenesis (Figure 1.8), such as those associated with angiogenic sprout tip cells, retina development and with the tumour vasculature (Koch and Claesson-Welsh, 2012; Carmeliet et al., 2009). In addition to endothelial cells, VEGFR3 is also expressed in an assortment of other non-endothelial cell types such as osteoblasts, neuronal progenitors and macrophages, however its expression in tumour cells is widely disputed (Koch and Claesson-Welsh, 2012). Interestingly, mice expressing a kinase-deficient VEGFR3 receptor maintain normal physiological blood vessel development, but suffer from impaired lymphatic development (Zhang et al., 2010). This stresses the importance of VEGFR2/VEGFR3 homodimers in regulating blood vessel formation during development (Koch et al., 2011).
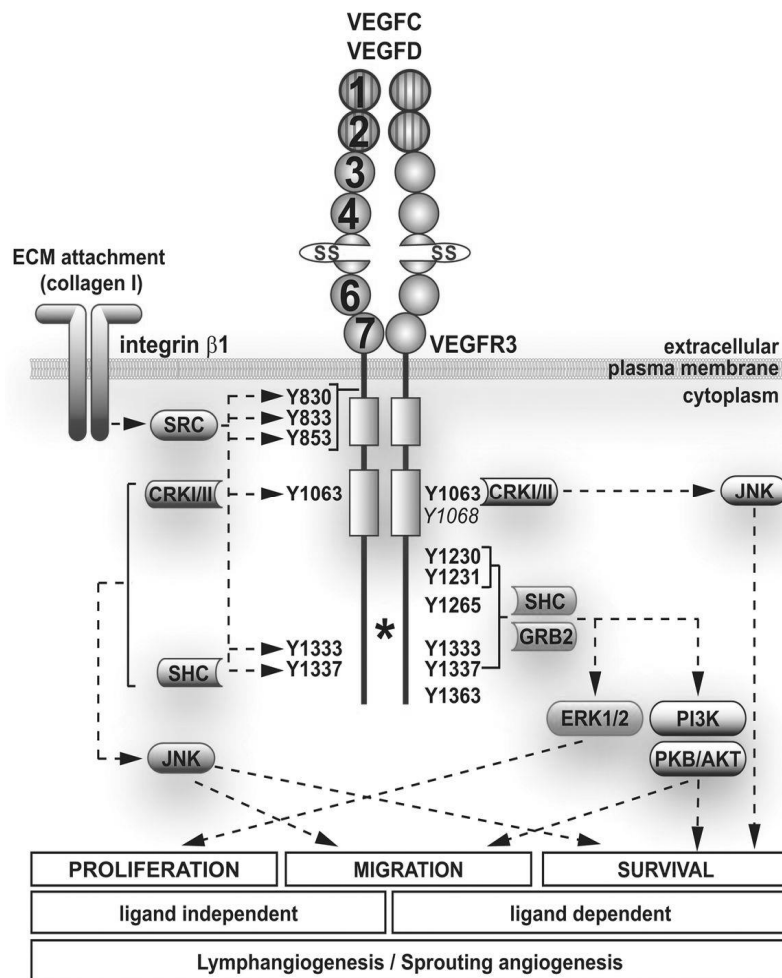
Figure 1.8. VEGFR3-mediated signal transduction. Schematic depiciting vascular endothelial growth factor 3 (VEGFR3)-mediated signal transduction in response to VEGF. Y1068 (italics) is crucial for VEGFR3 kinase activity. VEGFR3 occurs as a short and a long isoform (the length varying in the region indicated by *). Y1337 and Y1363 are transautophosphorylated only in the homodimeric VEGFR3 long isoforms. VEGFR3 may contribute to proliferation, migration and survival of lymphendothelial cells through ligand-dependent or ligand-independent mechanisms. ECM, extracellular matrix; ERK, extracellular signal-regulated kinase 1/2; GRB2, growth factor receptor-bound protein 2; JNK, janus kinase; PI3K, phosphoinositide 3-kinase; PKB, protein kinase B; SHC, Src homology 2 domain containing transforming protein; VEGF, vascular endothelial growth factor. Figure and legend adapted from Koch *et al.* (2011).

1.6.6. Neuropilins

Neuropilin 1 and its homologue Neuropilin 2 (NRP1 and NRP2) are single pass transmembrane glycoproteins, consisting of a large extracellular domain, short transmembrane domain and a small cytoplasmic tail (Figure 1.9) (Lampropoulou and Ruhrberg, 2014; Roskoski, 2007). Both NRP1 and NRP2 were first identified as receptors for class III semaphorins (e.g. SEMA3A and SEMA3F respectively) in the regulation of axon guidance (He and Tessier-Lavigne, 1997; Chen et al., 1997). However, NRP1 and NRP2 also bind members of the VEGF family to regulate both embryonic and pathological angiogenesis (Zachary, 2011). NRP1 and NRP2 share ~44% sequence homology, yet interact with different ligands (Schwarz and Ruhrberg, 2010). The NRP extracellular domain contains two complement-binding homology domains termed a1 and a2, two coagulation factor V/VIII homology domains, termed b1 and b2 and a meprin domain, termed c (Figure 1.9). Domains a- and b- are essential for mediating semaphorin and semaphorin/VEGF binding respectively, whereas the c domain helps promote receptor dimerisation (Figure 1.9) (Schwarz and Ruhrberg, 2010; Zachary, 2011). Due to the short nature of the NRP cytoplasmic domain, it is believed to lack enzymatic activity but does contain a SEA (Ser-Glu-Ala) motif implicated in binding PDZ domains on interacting proteins (Figure 1.9) (Ballmer-Hofer et al., 2011; Lanahan et al., 2013; Zachary, 2011).

1.6.6.1. NRP1's role in angiogenesis

NRP1 is 923 residues in length (~120-140 kDa) and is encoded by the *NRP1* gene located on human chromosome 10p12 (Pellet-Many et al., 2008; Plein et al., 2014; Zachary, 2014; Rossignol et al., 2000). The *NRP1* gene spans over 120 kb and is composed of 17 exons; alternative splicing of this gene gives rise to multiple NRP1 isoforms (Zachary, 2014). One of these NRP1 isoforms is a membrane-associated variant lacking 51 nucleotides corresponding to exon 16 (NRP1(Δexon16)) (Tao et al., 2003). In addition, the mRNA of 4 additional soluble versions of NRP1 has also been reported, although only two of these (NRP1 isoform b (s_{12}NRP1) and NRP1 isoform c (s_{IV}NRP1)) have been conclusively detected at the protein level (Zachary, 2014). NRP1 interacts with a wide variety of ligands including VEGF-A_{165}, VEGF-A_{121}, VEGF-B_{167}, VEGF-C, VEGF-D, VEGF-E (NZ2) and PlGF-2, to regulate an array of physiological responses (Zachary, 2014; Schwarz and Ruhrberg, 2010; Plein et al., 2014; Roskoski, 2007). One possible role of these sNRP isoforms is to bind to and sequester free VEGF-A to negatively regulate angiogenesis. Such a role is supported by the fact that sNRP administration inhibits tumour growth in a fashion which mimics VEGF-A withdrawal (Gagnon et al., 2000).
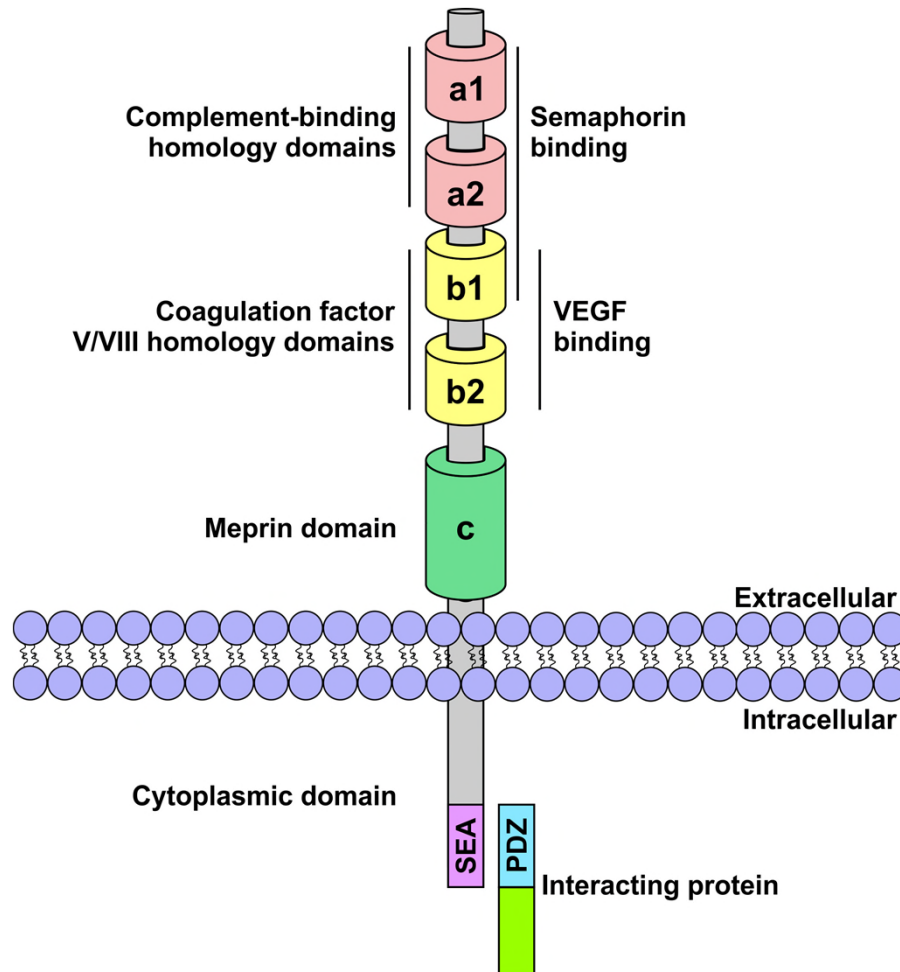
Figure 1.9. Neuropilin co-receptors. Schematic depecting the structure and binding sites of Neuropilin co-receptors. Neuropilins (NRPs) consist of two complement-binding homology domains (a1 and a2), two coagulation factor V and VII homology domains (b1 and b2) and a meprin domain (c) in their extracellular domain. Domains a1, a2 and b1 enable semaphorin binding (e.g. SEMA3A). Whereas domains b1 and b2 facilitate VEGF binding (e.g. VEGF-A$_{165}$). SEA represent the last amino acid residues (Ser, Glu and Ala) of the cytoplasmic domain, which enable the interaction with PDZ (PSD95, DLGA and ZO1 homology) domain-containing proteins, such as synectin.

NRP1 has a defined role in embryonic development, with NRP1 knockout mice dying between E10.5-E14.5 due to a spectrum of both cardiovascular and neuronal defects (Kawasaki et al., 1999). However, the embryonic lethality of endothelial cell-specific NRP1 knockout mice only occurs due to cardiovascular deformities (Gu et al., 2003). Furthermore, overexpression of NRP1 also results in in embryonic lethality due to dysregulated capillary growth and heart formation (Kitsukawa et al., 1995). Within the endothelium, the current consensus is that NRP1 functions primarily as a co-receptor for VEGFR2. Co-expression of NRP1 enhances VEGF-A-stimulated VEGFR2 phosphorylation, downstream signal transduction and cellular response (Whitaker et al., 2001; Soker et al., 2002; Pan et al., 2007; Herzog et al., 2011). However, NRP1 is not fundamental for VEGFR2 activation, although siRNA mediated knockdown of NRP1 results in 50% reduction in VEGFR2-Y1175 phosphorylation (Evans et al., 2011; Herzog et al., 2011; Shraga-Heled et al., 2007). NRP1 forms a constitutive ligand-independent complex with VEGFR2; however, levels of this complex are enhanced upon VEGF-A binding (Whitaker et al., 2001; Soker et al., 2002; Shraga-Heled et al., 2007). The formation of this VEGFR2/VEGF-A/NRP1 trimeric complex is likely to result from the dual binding of the cysteine knot motif of VEGF-A in the core VEGF homology region to VEGFR2, and of the exon 7/8 encoded carboxy-terminal moiety of VEGF-A to the b1 domain of NRP1 (Zachary, 2014). Deletion of the NRP1 carboxy-terminal PDZ domain binding motif decreased NRP1/VEGFR2 co-immunoprepcipitation (Zachary, 2014). Multiple studies have identified a role for NRP1 in modulating VEGFR2-mediated signal-transduction pathways. One such study looked at the adaptor protein p130Cas which becomes activated upon VEGFR2 phosphorylation and interacts with several other signal transduction enzymes to regulate endothelial cell migration (Evans et al., 2011). Here, depletion of NRP1 or expression of a NRP1 mutant lacking the intracellular domain (NRP1ΔC) resulted in impaired Pyk2-mediated activation of p130Cas and subsequently diminished cellular migration. Furthermore, NRP1 is required for p130Cas activation and cellular migration in human coronary artery smooth muscle cells (HCASMCs) (Pellet-Many et al., 2011). In addition to p130Cas, depletion of NRP1 also impaired VEGF-A-stimulated FAK-Y407 phosphorylation (Herzog et al., 2011). Conversely, the major autophosphorylation site FAK-Y397 was not affected (Herzog et al., 2011). Likewise, depletion of NRP1 was shown to regulate VEGF-A-stimulated phosphorylation of ERK1/2 (Lanahan et al., 2013). However, other studies have argued against the role of NRP1 in modulating VEGF-A-stimulated ERK1/2 activation (Herzog et al., 2011; Evans et al., 2011). Furthermore, NRP1 has been implicated in regulating downstream VEGF-A-

stimulated p38 MAPK activation and subsequent endothelial cell migration (Kawamura et al., 2008).

Whilst the current consensus is that NRP1 lacks independent signalling roles in endothelial cells, the NRP1 cytoplasmic domain has been implicated in the regulation of VEGFR2 trafficking (Ballmer-Hofer et al., 2011; Lanahan et al., 2013; Salikhova et al., 2008). One study showed that recruitment of the PDZ domain-containing protein synectin (GIPC1) onto the NRP1 C-terminal SEA motif, facilitated VEGF-A-stimulated VEGFR2 recycling via long loop Rab11a-positive endosomes (Ballmer-Hofer et al., 2011). Here, prevention of synectin binding to NRP1 resulted in attenuated VEGF-A-stimulated signal transduction and endothelial function (Ballmer-Hofer et al., 2011). Furthermore, depletion of the NRP1 cytoplasmic domain impaired developmental and adult arteriogenesis but this did not affect angiogenesis (Lanahan et al., 2013). This effect was linked to attenuated ERK1/2 signal transduction as a result of reduced VEGFR2 synectin-mediated internalisation (Lanahan et al., 2013). Further support for the role of synectins in NRP1-mediated regulation of angiogenesis, came from studies on zebrafish expressing NRP1, which lacked the c-terminal SEA motif or those genetically manipulated to impair either synectin or NRP1 function which produced similar impaired vascular phenotypes (Wang et al., 2006).

1.6.6.2. VEGF-A-independent roles for NRP1

In contrast to regulating VEGF-A signal transduction, many studies have revealed VEGF-A-independent roles of NRP1 in the endothelium. Such a role is exemplified by the fact that VEGF-A-binding to NRP1 is not required for developmental angiogenesis, despite genetic ablation of NRP1 being embryonically lethal (Gelfand et al., 2014; Fantin et al., 2014). Furthermore, genetic ablation of NRP1, but not VEGF-A binding to NRP1, reduces cell surface VEGFR2 levels (Gelfand et al., 2014). Additionally, interactions with integrins can also regulate endothelial cell function. Association between NRP1 and the cytoplasmic domain of the $\beta3$ integrin subunit negatively regulates angiogenesis by limiting the availability of NRP1 which can form complexes with VEGFR2 (Robinson et al., 2009). In addition, the interaction between NRP1 and integrin $\alpha5\beta1$ promotes $\alpha5\beta1$ internalisation and endothelial cell adhesion in response to fibronectin (Valdembri et al., 2009). Furthermore, the integrin ligand fibronectin stimulates actin remodelling due to the phosphorylation of paxillin in a VEGF-A/VEGFR2-independent but NRP1-dependent

manner (Raimondi et al., 2014). NRP1 forms a complex with ABL1 which is required for fibronectin-stimulated paxillin phosphorylation, subsequent actin remodelling and angiogenesis (Raimondi et al., 2014). Additionally, a recent study has identified further roles for NRP1 in mediating fibronectin-stimulated angiogenesis (Fantin et al., 2015). Here NRP1 was essential for the activation of CDC42 in response to fibronectin (Fantin et al., 2015). NRP1 gene silencing resulted in impaired fibronectin-stimulated CDC42 activation, subsequent actin remodelling and endothelial tip cell function (Fantin et al., 2015). Together, these studies highlight the importance of NRP1 in regulating both VEGF-A- and ECM-mediated signal transduction in order to modulate endothelial cell function and regulate angiogenesis.

1.7.   Metabolic determinants of angiogenesis

Endothelial cells, in contrast to other healthy cells, rely predominantly on glycolysis as a means of generating ATP, with very little dependence on glucose oxidation or mitochondrial respiration. This glycolytic flux is >200-fold higher in comparison to glucose, fatty acid or glutamine oxidation (De Bock et al., 2013). However, mitochondrial respiration can serve as a reserve mechanism of ATP-production during periods of cell stress (Eelen et al., 2013). VEGFs have been implicated in regulating endothelial cell metabolism as a means of promoting angiogenesis. Endothelial cells passively acquire glucose through glucose transporter 1 (GLUT-1)-mediated diffusion (De Bock et al., 2013). VEGF-A-stimulation promotes a surge in endothelial cell glycolysis via increasing PI3K-Akt-mediated GLUT-1 expression, in addition to lactate dehydrogenase-A (LDH-A) and phosphofructokinase-2/fructose-2,6-bisphosphate (PFKFB3) (De Bock et al., 2013; Eelen et al., 2013; Yeh et al., 2008). VEGF-A particularly increases PFKFB3-driven glycolysis in endothelial tip cells, indicating a dependence on PFKFB3-driven glycolysis within these cells (De Bock et al., 2013). Inhibition of glycolysis via silencing PFKFB3 gene expression impairs endothelial cell migration, proliferation and vascular sprouting (De Bock et al., 2013). Overexpression of PFKFB3 in stalk cells promoted tip cell-like behaviour, remarkably superseding other pro-stalk signals (Eelen et al., 2013; De Bock et al., 2013). These findings imply that glucose metabolism has the ability to determine angiogenic fate, rather than just drive it (Jang and Arany, 2013).

Oxidative phosphorylation produces ~20-fold more ATP, compared to glycolysis (Locasale and Cantley, 2011). Therefore, due to the exposure of endothelial cells to relatively high circulating blood oxygen levels, the significance of glycolysis in

endothelial cell metabolism seems somewhat paradoxical (Eelen et al., 2013). One explanation for this phenomenon is that the endothelium is required to be highly active within hypoxic environments in order to facilitate angiogenesis. Thus having low oxygen-dependency on ATP synthesis could prepare them for such a case (Eelen et al., 2013).

Endothelial cell reliance on glycolysis allows for the diversion of glycolytic intermediates into several side branches of this pathway, including the pentose phosphate pathway (PPP). Glucose-6-phosphate dehydrogenase (G6PD)-mediated oxidation of glucose-6-phosphate into pentose sugars, facilitates nucleotide synthesis, NO production, reductive biosynthesis of lipids and production of reduced glutathione (GSH) (Cairns et al., 2011). Inhibition of G6PD decreases VEGF-mediated VEGFR2 tyrosine phosphorylation, eNOS activation and NO production, as well as attenuating endothelial cell migration proliferation and tubulogenesis (Leopold et al., 2003; Pan et al., 2009). The oxidative branch of the PPP (oxPPP) generates NADPH and ribose-5-phosphate. VEGF-A stimulation increases the rate of oxPPP (Vizan et al., 2009) via increasing both G6PD plasma membrane localisation and activity (Pan et al., 2009).

Hyperglycaemia (elevated glucose levels) is characteristic of diabetes and promotes vascular dysfunction. The polyol pathway converts excess glucose to sorbitol (which is then converted to fructose) via an aldose reductase-catalysed reduction (Tang et al., 2012). Inactivation of aldolase reductase inhibits VEGFR2-stimulated PI3K/Akt and NFκB signalling via suppressing VEGFR2 protein levels (Tammali et al., 2011). Furthermore, inactivation of aldolase reductase also attenuated downstream VEGF-A-stimulated ICAM, VCAM, IL-6, MMP2 and MMP9 gene expression and secretion (Tammali et al., 2011).

In addition to glycolysis, endothelial cells can also utilise fatty acid oxidation (FAO) in the absence of glucose to compensate for a lack of glycolytic ATP production (Dagher et al., 2001). VEGF-A-stimulated VEGFR2 activation promotes increased expression of fatty-acid-binding protein 4 (FABP4), which is required for effective endothelial cell proliferation (Elmasri et al., 2009). Furthermore, VEGF-B stimulation promotes increased expression of fatty acid transporter protein-3 and -4 (FATP3 and FATP4), thus increasing endothelial lipid uptake and transport into peripheral tissues (heart and skeletal muscle) (Hagberg et al., 2010). Fatty acid synthase (FAS) regulates *de novo* lipid synthesis and its expression is highly upregulated in

cancer cells (Santos and Schulze, 2012). Inhibition of endothelial cell FAS attenuates eNOS activation, thus promoting vascular dysfunction (Wei et al., 2011).

1.8. VEGF-A regulated gene transcription

Gene expression is essential to convert short lived signal transduction into longer term cellular responses. VEGF-A stimulation induces the expression of specific gene products which act to further modulate endothelial cell outcomes. Amongst these are target genes encoding transcription factors (Egr-3), cytokines and growth factors, adhesion molecules (VCAM-1) and phosphatases (DUSP-1 and DUSP-5) (Rivera et al., 2011; Schweighofer et al., 2009). Although a lot is known about VEGF-A target genes, less is known regarding the transcription factors involved.

One transcription factor activated upon VEGF-A stimulation is activating transcription factor 2 (ATF-2) (Seko et al., 1998; Salameh et al., 2010). Furthermore, ATF2 was shown to regulate endothelial cell survival by promoting upregulation of Bcl-X_L in response to VEGF (Salameh et al., 2010). Additionally, VEGF-A activation of NF-κB has also been shown to upregulate cell adhesion molecules, including VCAM-1 (Kim et al., 2001). Another transcription factor linked to regulating VEGF-A isoform-specific endothelial responses is NFATc2 (NFAT1) (Armesilla et al., 1999). Increased NFATc2 activity has also been linked to elevated endothelial cell tubulogenesis (Bala et al., 2012). In addition, VEGF-A upregulation of Down's syndrome critical region 1 (DSCR1; RCAN1) via PKCδ-mediated $Ca^{2+/}$calcineurin-dependent activation of NFATc2, acts to regulate endothelial cell migration and tube formation (Holmes et al., 2010).

The VEGF-regulated transcription factor HLX controls the expression of guidance cues and negatively regulates sprouting of endothelial cells (Testori et al., 2011). Furthermore, autocrine VEGF-A signal transduction represses the levels of the transcription factor FOXO1 in the endothelial cell, thus maintaining endothelial cell metabolism and survival (Domigan et al., 2015). Additionally FOXO1 is critical for early vascular development with FOXO1 knockout mice dying at embryonic day 9.5 due to abnormal angiogenesis within the yolk sac (Furuyama et al., 2004). This was attributed due to a defective endothelial cell response to VEGF-A (Furuyama et al., 2004). The transcription factor early growth response 3 (Egr-3) has also been linked to VEGF-A-stimulated endothelial cell response (Suehiro et al., 2010). Gene silencing of Egr-3 impaired VEGF-A stimulated endothelial cell proliferation,

migration, tubulogenesis and monocyte adhesion (Suehiro et al., 2010). Additionally, VEGF-A activation of the MEK1-ERK1/2 signal transduction pathway was shown to upregulate Egr-3, however the transcriptional mechanism was not defined (Liu et al., 2008).

1.10.   Angiogenesis and Inflammation

Aulus Celsus documented the 4 hallmarks of inflammation over 2 millennia ago as redness and swelling associated with heat and pain (Scott et al., 2004). Inflammation involves the recruitment of immune cells such as monocytes, macrophages and mast cells to the area of infection or injury. Immune cell recruitment has the ability to regulate angiogenesis due to their capacities to secrete pro- and anti-angiogenic cytokines and growth factors (e.g. VEGF-A)

(Lingen, 2001; Naldini and Carraro, 2005). Additionally, during an injury or immune response the endothelium plays a vital role in regulating the transendothelial migration (TEM) of circulating leukocytes from the blood stream into the extravascular tissue (Nourshargh et al., 2010). TEM is the process whereby, circulating leukocytes are recruited onto the endothelial cell boarder before proceeding to migrate into the extravascular tissue via the interstitial tissue. This occurs via a series of steps, namely, endothelial cell capture, rolling, arrest, adhesion, crawling and finally migration through the endothelial monolayer (Figure 1.10). TEM is regulated by an array of adhesion molecules (e.g., ICAM-1, VCAM-1 and PECAM-1) and soluble cytokines (Nourshargh et al., 2010). *In vivo* TEM primarily occurs in venules; small blood vessels which drain capillary beds (Nourshargh et al., 2010). Over the years the link between angiogenesis and inflammation has been intensively studied multiple 'classical' inflammatory cytokines have been shown to promote angiogenesis including TNFα, CXCL ligands and interleukins (ILs) (Lee et al., 2013; Sainson et al., 2008; Salven et al., 2002; Angiolillo et al., 1997). These cytokines function by either directly stimulating endothelial cells or via promoting immune cell secretion of pro-angiogenic ligands such as VEGF-A. In addition to regulating endothelial cell responses, inflammatory cytokines also play a role in blood vessel maturation (Ivins et al., 2015; Cavallero et al., 2015; Harrison et al., 2015). However, not all inflammatory cytokines stimulate angiogenesis, some such as IL-32 act in an inhibitory manner (Meyer et al., 2012). Furthermore, there is increasing evidence that 'classical' pro-angiogenic ligands (e.g. VEGF-A) help to modulate pro-inflammatory gene expression and inflammatory responses and leukocyte recruitment (Schweighofer et al., 2009).
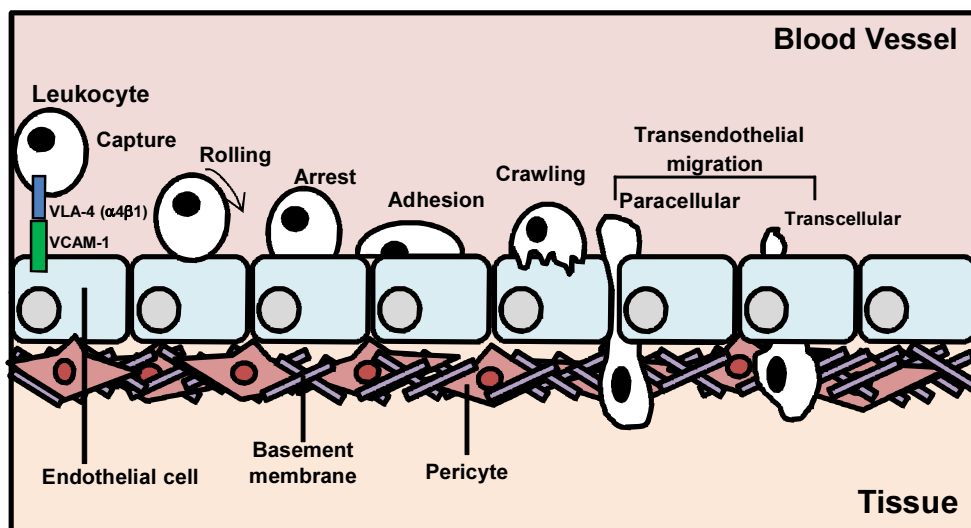
Figure 1.10. Transendothelial migration. Schematic depiciting key cellular events in the leukocyte adhesion cascade. Leukocyte capture, rolling, adesision and intravascular crawling are considered as prerequisites to transendothelial migration. Figure and legend adapted from Nourshargh *et al.* (2010).

1.11.   Aims

Vascular endothelial growth factor A (VEGF-A) binding to the receptor tyrosine kinase (RTK) vascular endothelial growth factor 2 (VEGFR2) triggers an array of downstream signal transduction pathways which modulate a plethora of endothelial cell responses, such as migration, proliferation, tubulogenesis and cell-cell interactions. Various splice isoforms of VEGF-A exist, yet it is unclear how multiple VEGF-A isoforms bind to the same RTK to program distinct cellular responses. Recent studies have shown that VEGFR2/VEGF-A-mediated signal transduction is dependent on the residence time of the activated VEGFR2-signalling complex, either at the plasma membrane or within the endosome lysosome system (Zhang et al., 2013, Gourlaouen et al., 2013, Lanahan et al., 2014, Jopling et al., 2009, Lanahan et al., 2010, Manickam et al., 2011, Yamada et al., 2014). Thus our first aim is to assess the effect of multiple VEGF-A isoforms on promoting VEGFR2 internalisation and degradation, linked to receptor activation, downstream signal transduction and post-translational modification (e.g. ubiquitination).

In order to regulate longer term (≥24 h) endothelial cell responses (e.g. migration, proliferation and tubulogenesis) short-lived (≤30 min) signal transduction needs to be converted into gene expression, via transcription factor activation. However, the transcription factors (and their associated target genes) involved in regulating isoform-specific endothelial cell responses are ill defined. Activating transcription factor (ATF-2) belongs to the bZIP (basic region subdomain/leucine zipper) family of DNA-binding transcription factors and undergoes activation upon cellular stress or plasma membrane receptor activation (Lau and Ronai, 2012). ATF-2 undergoes VEGF-A-stimulated phosphorylation in cardiac myocytes and endothelial cells (Seko et al., 1998, Salameh et al., 2010), with multiple signal transduction pathways linked to regulating its phosphorylation (Lau and Ronai, 2012). Thus, our second aim is to investigate the capacity of different VEGF-A isoforms to promote ligand-stimulated ATF-2 activation and to identify the signal transduction pathway responsible using small molecule inhibitors. Additionally, building on previously acquired gene array data (carried out by Dr A. Latham, University of Leeds, 2012) we will use reverse genetics and an array of cellular assays to identify the role of ATF-2 and its target genes in regulating differential VEGF-A isoform-specific endothelial cell responses.

VEGF-A-stimulated VEGFR2 dimerisation and transautophosphorylation of Y1175, enables the recruitment of PLCγ1 to VEGFR2 at the plasma membrane (Takahashi et al., 2001). Subsequent, VEGFR2-mediated PLCγ1 phosphorylation (PLCγ1-

pY783) leads to enzymatic activation and hydrolysis of plasma membrane phosphatidylinositol 4,5-bisphosphate (PIP_2) to diacylglycerol (DAG) and inositol triphosphate (IP_3), thus triggering a rise in cytosolic calcium ions and altered endothelial responses. Cytosolic calcium ion fluxes can regulate endothelial cell gene transcription via the protein phosphatase calcineurin (Zarain-Herzberg et al., 2011, Dominguez-Rodriguez et al., 2012), which promotes dephosphorylation and subsequent nuclear translocation of the nuclear factor of activated T-cells (NFAT) family of transcription factors (Rao et al., 1997, Wu et al., 2007). VEGF-A$_{165}$ has been shown to promote nuclear translocation of NFAT family members such as NFATc2 (Goyal et al., 2012, Zaichuk et al., 2004). Therefore, our final aim is to determine the capacity of different VEGF-A isoforms to promote PLCγ1-mediated cytosolic calcium ion release, NFATc2 activation and nuclear translocation. Additionally using RNA interference (RNAi) technology to identify the role of NFATc2 in regulating VEGF-A isoform-specific endothelial responses.

Thus, we aim to carry out an in depth analysis of how different VEGF-A isoforms regulate specific endothelial responses via promoting VEGFR2 activation, post-translational modification and turnover; linked to its impact on downstream signal transduction, transcription factor activation and ultimately gene expression. Our experimental study will further our understanding of how multiple growth factor isoforms bind to the same receptor tyrosine kinase to mediate distinct cellular outcomes and hopefully identify novel targets which will contribute to the development of more effective therapeutics.

CHAPTER 2

MATERIALS AND METHODS

2.1.   Materials

2.1.1.   General reagents

Recombinant VEGF-A$_{165}$ was a gift from Genentech Inc. (San Francisco, CA, USA). VEGF-A$_{121}$ and VEGF-A$_{145}$ was purchased from Promocell (Heidelberg, Germany). All chemicals were of analytical grade and obtained from Sigma-Aldrich (Poole, UK) unless otherwise stated. VEGF-A activity was verified by immunoblot assessment of active dimeric or inactive monomeric species (Appendix B).

2.1.2.   Pharmacological reagents

Pharmacological reagents were used as described in Table 2.1.

Compound	Target	Working concentration	Pre-treatment incubation time	Source
SB203580	p38 MAPK	10 μM	30 min	LC Labs (Boston, MA, USA)
U1026	MEK1	10 μM	30 min	LC Labs (Boston, MA, USA)
CAS 871307-18-5	Tpl2	2 μM	30 min	Calbiochem (San Diego, CA USA)

Table 2.1: Details of pharmacological reagents. The commercial supplier, enzyme target, and recommended working concentration and pre-incubation period are shown.

2.1.3.   Immunological reagents

Primary and secondary antibodies were used as described in Table 2.2.

2.1.4.   Primary cells and immortalised cell lines

Primary human foreskin fibroblasts (pHFF) were purchased from Promocell (Heidelberg, Germany). The human HL-60 leukocyte cell line was provided by Dr Adam Odell (Faculty of Medicine & Health, University of Leeds). Human umbilical vein endothelial cells (HUVECs) were isolated from umbilical cords obtained with informed patient consent from patients undergoing elective Caesarean section at Leeds General Infirmary. This is described in detail in section 2.2.1.1.

Antigen	Species	Concentration mg ml^{-1}	Dilution Factor	Source
Akt	Rabbit	0.5	IB: 1:1000	Cell Signalling Technology (Danvers, MA, USA)
Akt, Phospho-S473	Rabbit	0.5	IB: 1:1000	Cell Signalling Technology (Danvers, MA, USA)
ATF-2	Rabbit	0.5	IB: 1:1000	Cell Signalling Technology (Danvers, MA, USA)
ATF-2, Phospho-T71	Rabbit	0.5	IB: 1:1000 IF: 1:100	Cell Signalling Technology (Danvers, MA, USA)
Cyclin A2	Mouse	0.2	IB: 1:1000	BD transduction labs (Oxford, UK)
Cyclin B	Mouse	0.2	IB: 1:1000	BD transduction labs (Oxford, UK)
Cyclin D1 (DCS6)	Mouse	0.5	IB: 1:1000	Cell Signalling Technology (Danvers, MA, USA)
eNOS	Rabbit	0.5	IB: 1:1000	Cell Signalling Technology (Danvers, MA, USA)
eNOS, Phospho-S1177	Rabbit	0.5	IB: 1:1000	Cell Signalling Technology (Danvers, MA, USA)
EEA1	Rabbit	0.25	IF: 1:200	BD transduction labs (Oxford, UK)
ERK1/2	Rabbit	0.5	IB: 1:1000	Cell Signalling Technology (Danvers, MA, USA)
ERK1/2, Phospho-T202/Y204	Mouse	0.5	IB: 1:1000 IF: 1:100	Cell Signalling Technology (Danvers, MA, USA)
FK2	Mouse	0.5	IB: 1:1000	R&D Systems (Minneapolis, MN, USA)
Goat IgG	Donkey, HRP	0.4	IB: 1:5000	Stratech Scientific (Newmarket, UK)
Goat IgG	Donkey, Alexa Fluor 488	2.0	IF: 1:250	Life Technologies (Grand Island, NY.USA)
Mouse IgG	Donkey, HRP	0.4	IB: 1:5000	Stratech Scientific (Newmarket, UK)
Mouse IgG	Donkey, Alexa Fluor 594	2.0	IF: 1:250	Life Technologies (Grand Island, NY.USA)
NRP1	Rabbit	0.5	IB: 1:1000	Cell Signalling Technology (Danvers, MA, USA)
NFATc2	Rabbit	0.5	IB: 1:1000	Cell Signalling Technology (Danvers, MA, USA)
p21 (DCS60)	Mouse	0.5	IB: 1:1000	Cell Signalling Technology (Danvers, MA, USA)
p38 MAPK	Rabbit	0.5	IB: 1:1000	Cell Signalling Technology (Danvers, MA, USA)
p38 MAPK, Phospho-T180/Y182	Rabbit	0.5	IB: 1:1000	Cell Signalling Technology (Danvers, MA, USA)
p53	Mouse	0.2	IB: 1:1000	BD transduction labs (Oxford, UK)
PECAM-1 (CD31)	Mouse	0.2	IF: 1:1000	Santa Cruz antibodies (CA, USA)
PLCγ1	Rabbit	0.5	IB: 1:1000	Cell Signalling Technology (Danvers, MA, USA)
PLCγ1, Phospho-Y783	Rabbit	0.5	IB: 1:1000	Cell Signalling Technology (Danvers, MA, USA)
Rabbit IgG	Donkey, HRP	0.4	IB: 1:5000	Stratech Scientific (Newmarket, UK)
Tpl2 (H-7)	Mouse	0.2	IB: 1:1000	Santa Cruz antibodies (CA, USA)
Transferrin receptor (TfR; CD71)	Mouse	0.2	IB: 1:1000	Santa Cruz antibodies (CA, USA)
VCAM-1	Mouse	0.5	IB: 1:1000	Sigma-Aldrich (Poole, UK)
VEGFR1 extracellular domain	Goat	0.1	IB: 1:1000	R&D Systems (Minneapolis, MN, USA)
VEGFR2 extracellular domain	Goat	0.1	IB: 1:1000 IF: 1:100	R&D Systems (Minneapolis, MN, USA)
VEGFR2, Phospho-Y1175	Rabbit	0.5	IB: 1:1000	Cell Signalling Technology (Danvers, MA, USA)
VEGFR2, Phospho-Y1214	Rabbit	0.5	IB: 1:1000	R&D Systems (Minneapolis, MN, USA)
α-Tubulin	Mouse	2.0	IB: 1:8000	Sigma-Aldrich (Poole, UK)

Table 2.2: Primary and secondary antibody information. Details of antibody species, concentration, dilution factor and commercial source for reagents used within this study. HRP, horseradish peroxidase; IB, immunoblot; IF, immunofluorescence.

2.2.   Methods

2.2.1.   Mammalian cell culture techniques

2.2.1.1.   Isolation of primary human umbilical vein endothelial cells (HUVECs)

Primary endothelial cells were obtained from fresh (~3 h old) human umbilical cords (~20 cm in length) from donors through an ethically approved program linked to Leeds General Infirmary and Leeds Hospital NHS Trust (Reference #CA03/020). The umbilical vein was cannulated with an 18 gauge blunt syringe needle and flushed twice with 50 ml PBS. Cells were detached via digestion with 0.1% (w/v) type II-S collagenase (Sigma-Aldrich, Poole, UK). A haemostat was use to clamp the cord at one end and the umbilical vein was filled with 10 ml 0.1% (w/v) type II-S collagenase in MCDB131, before clamping the other end. The cord was the incubated at 37°C for 20 min and the contents were drained and collected. The vein was then flushed with PBS to remove all contents and to acquire a total collected volume of 50 ml. Cells were pelleted via centrifugation at 140 g for 5 min at 37°C. Supernatant was then aspirated and the cell pellet resuspended in endothelial cell growth medium (ECGM) containing 10 U/ml penicillin, 100 μg/ml streptomycin and 50 ng/ml amphotericin B. Cells where then seeded into one 75 cm^2 vented tissue culture flask (Nunc, Copenhagen, Denmark) which was pre-coated with 0.1% (w/v) pig skin gelatin (PSG) for 30 min. After 24 h, the media was aspirated and cells washed five times in PBS before applying fresh ECGM. Isolated cells were routinely characterised via immunofluorescence analysis of common endothelial cell markers (Figure 2.1), as previously described (Fearnley et al., 2014).

2.2.1.2.   Cell passage

HUVECs were cultured in ECGM in a 75 cm^2 vented tissue culture flask pre-coated with 0.1% (w/v) PSG and incubated at 37°C in a hydrated 5% CO_2 atmosphere until ~80% confluent. ECGM was then aspirated and the cells washed twice with 6 ml PBS. 1 ml of TrypLE^{TM} Express (Invitrogen, Amsterdam, Netherlands) was added, followed by incubation at 37°C for 3 min. The flask was then tapped gently to remove any adherent cells and the trypsinisation was quenched with 5 ml DMEM+10% (v/v) foetal calf serum (FCS; Life Technologies, Paisley, UK). Cells were pelleted via centrifugation at 140 g for 5 min at 37°C, the supernatant was aspirated and cell pellet resuspended in ECGM. Cells were seeded into 75 cm^2 vented culture flasks or experimental 10 cm diameter round dishes pre-coated with 0.1% (w/v) PSG. 75 cm^2 vented culture flasks were not split more than 1:3 as this
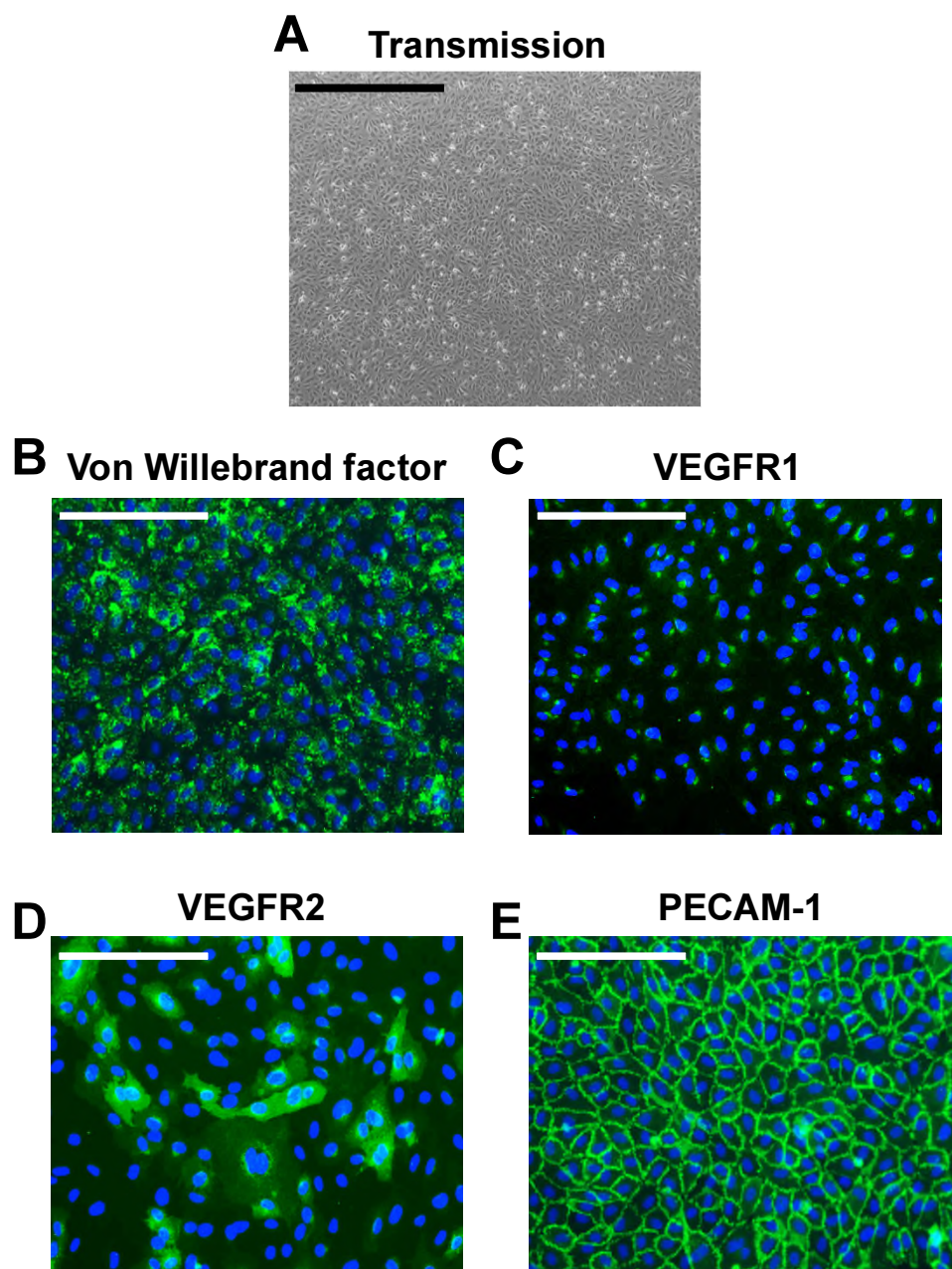
Figure 2.1. Primary endothelial cell morphology. (A) Confluent HUVEC monolayer grown on gelatin-coated surfaces and visualised by phase-contrast microscopy. Bar, 1000 μm. Using immunofluorescence microscopy, confluent HUVECs were labelled with (B) anti-Von Willebrand Factor (VWF), (C) anti-VEGFR1, (D) anti-VEGFR2 and (E) anti-PECAM-1 (CD31) primary antibodies followed by Alexa Fluor 488-conjugated species-specific secondary antibodies (green). Nuclei labeled with DAPI (blue). Bar, 200 μm.

was detrimental to HUVEC growth. For all experiments, HUVECs were used between passages 0 and 5.

2.2.1.3.   Pharmacological inhibition of signal transduction pathways

Cells were seeded and starved as stated below (2.2.1.5.), before pre-treatment with desired small molecule kinase inhibitor (as described in Table 2.1) prior to stimulation. Cells were then processed via SDS-PAGE before immunoblot analysis.

2.2.1.4.   Preparation of total cell lysates

Media was aspirated and the cells were washed twice with ice-cold PBS. Cells where then lysed in 2% (w/v) SDS (in PBS) containing 1 mM PMSF and protease inhibitor cocktail (Roche) (100 µl per well of 6-well plate); remaining cells were detached using a sterile cell scraper. Lysates where then transferred into 1.5 ml centrifuge tubes. Lysates were stored at -20°C until needed. When required lysates were incubated at $97^{\circ}C$ for 5 min before being sonicated for 3 seconds. Protein concentration was then determined using the biocinchoninic acid (BCA) assay.

2.2.1.5.   Analysis of intracellular signalling pathways

Cells were seeded into 6-well plates and cultured for at least 24 h in ECGM until ~80% confluent. ECGM was then aspirated and cells washed 3 times with 1 ml PBS. Cells were then starved in MCDB131 + 0.2% (w/v) BSA for 2-3 h prior to stimulation with 0, 0.025, 0.250 or 1.25 nM VEGF-A isoform as specified in each experiment. After the time course, plates were put on ice and media aspirated; cells were then washed twice with 1 ml ice cold PBS. Plates were then removed from ice and cells lysed using an appropriate buffer.

2.2.1.6.   Immunoblot analysis of p53, p21 and Cyclin D1 protein levels

Cells were seeded into 6-well plates and cultured in ECGM (for at least 24 h) until ~80% confluent, cells were washed twice with PBS and starved overnight in MCDB131 + 0.2% (w/v) BSA and 2 mM thymidine, to stimulate cell cycle arrest. Starvation media was aspirated and cells stimulated in ECGM + 25 µM 2-deoxycytidine, containing (0.25 nM) VEGF-A isoform or Tpl2 kinase inhibitor for desired time period. Cells were then lysed and processed for immunoblot analysis.

2.2.1.7.   Lipid-based transfection of siRNA duplexes

Cells were transfected with siRNA duplexes using lipofectamine RNAiMAX (Invitrogen). Per well of a 6-well plate, 15 µl of 2 µM siRNA duplexes was added to

481 μl of serum/antibiotic-free OptiMEM (Invitrogen) and allowed to settle at room temperature for 5 min. 4 μl of lipofectamine was then added and the mixture was inverted briefly and incubated at room temperature for 20 min. HUVECs were seeded at 2.5×10^5 cells/ml in a 1 ml volume of OptiMEM, followed by immediate dropwise addition of the siRNA/lipofectamine mixture. Cells were left at room temperature for 30 min before being returned to the incubator. After 6 h total incubation, media was replaced for ECGM. Cells were allowed to recover for 72 h prior to treatment or processing for analysis. All siRNA duplexes where purchased as siGENOME SMARTpools from Dharmacon (GE Healthcare, Buckinghamshire, UK).

Scrambled siRNA

Target sequence	5'- UAGCGACUAAACACAUCAA -3'
	5'- UAAGGCUAUGAAGAGAUAC -3'
	5'- AUGUAUUGGCCUGUAUUAG -3'
	5'- AUGAACGUGAAUUGCUCAA -3'

Tpl2-specific siRNA

Target sequence	5'- AAGCUGACUUACAGGAAUA -3'
	5'- GCCAAGAGGUACCAUGGUU -3'
	5'- GUAGAUCAAUUUAAGCCAU -3'
	5'- CCAAAUAGAUUCCGAUGUU -3'

ATF-2-specific siRNA

Target sequence	5'- GAAGAAAUCUGGCUAUCAU -3'
	5'- GACAAACCCUUUCUAUGUA -3'
	5'- GAAGUGGGUUUGUUUAAUG -3'
	5'- GGACAAACCAUGCCUGUUG -3'

NRP1-specific siRNA

Target sequence	5'- GGACAGAGACUGCAAGUAU -3'
	5'- CAAGAGAGGUCCUGAAUGU -3'
	5'- CAACAACUAUGAUACACCU -3'
	5'- GUAUACGGUUGCAAGAUAA -3'

p53-specific siRNA

Target sequence	5'- GAGGUUGGCUCUGACUGUA -3'

5'- GCACAGAGGAAGAGAAUCU -3'

5'- GAAGAAACCACUGGAUGGA -3'

5'- GCUUCGAGAUGUUCCGAGA -3'

NFATc2-specific siRNA

Target sequence   5'- GCUUAGAAACGCCGACAUU -3'

5'- AGACGGAGCCCACGGAUGA -3'

5'- GCAGAAUCGUCUCUUUACA -3'

5'- GAACCUCGCCAAUAAUGUC -3'

2.2.1.8.   SDS-PAGE

15-25 μg of total cell lysate was resuspended in an equal volume of 2X SDS-PAGE sample buffer (1 M Tris-HCl pH 6.8, 4% (w/v) SDS, 20% (v/v) glycerol, 0.1% (w/v) bromophenol blue, 4% (v/v) β-mercaptoethanol) and incubated at 97°C for 5 min. Samples were loaded onto a 8-12% (v/v) SDS-polyacrylamide resolving gel containing 5% (v/v) SDS-PAGE stacking gel. Samples were then subjected to electrophoresis at 120-130 V for ~1-2 h in 1X SDS-PAGE running buffer (25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS).

2.2.1.9.   Immunoblotting

Proteins subject to SDS-PAGE were transferred onto 0.2 μm pore size reinforced nitrocellulose membrane (Schleicher & Schuell, Bath, UK) in transfer buffer (25 mM Tris, 106 mM glycine, 20% (v/v) methanol, 0.1% (w/v) SDS) at 4°C for either 3 h at 300 mA or overnight at 30 mA. Membranes were briefly stained in Ponceau S solution (1 g/l Ponceau S in 5% (v/v) glacial acetic acid) to check for successful transfer. Membranes were then rinsed in TBS-T (20 mM Tris pH 7.6, 137 mM NaCl, 0.1% (v/v) Tween-20) to remove all traces of Ponceau S. Membranes were then incubated for 20-60 min in 5% (w/v) skimmed milk in TBS-T to block non-specific antibody binding. The blocking solution was then removed and the membrane rinsed in TBS-T. Membranes were then incubated with primary antibody (Table 2.2) overnight at 4°C or room temperature (As per manufacturer's instructions). Primary antibody was discarded and membranes washed three times for 10 min in TBS-T before being incubated with secondary HRP-conjugate antibodies (Table 2.2; Jackson ImmunoResearch, Stratech, Suffolk, UK) in TBS-T for 1-2 h at room temperature. Secondary antibodies were discarded and membranes were washed three times for 10 min in TBS-T. Membranes were then incubated briefly with combined enhanced chemiluminescence solution (EZ-ECL chemiluminescence

detection kit, Geneflow, Nottingham, UK). Blots were imaged using a G:BOX XT4 Chemi imaging system (Syngene, Cambridge, UK). Band intensity was determined using 2-D densitometry running on dedicated image analysis software (Syngene).

2.2.1.10.   Immunoprecipitation and detection of VEGFR2 ubiquitination

Cells were stimulated (1.25 nM VEGF-A isoform in MCDB131 + 0.2% (w/v) BSA; 2 wells per condition) before washing twice with ice-cold PBS and lysed in RIPA buffer (150 mM NaCl, 50 mM Tris-HCl pH7.4, 0.1% (w/v) SDS, 0.5% (w/v) sodium deoxycholate, 2 mM EDTA, 1% (v/v) NP-40, 50 mM NaF) with freshly added 1 mM phenylmethylsulfonyl fluoride (PMSF) and 10 mM iodoacetamide, and incubated for 5 min on ice at 4°C. Lysates were cleared by centrifugation at 16000 g for 30 min at 4°C. Equal concentrations of supernatant were incubated with VEGFR2 antibody for 2 h at 4°C. 35 μl of 50:50 Protein G-Sepharose slurry (Millipore, Watford, UK) was added and incubated overnight at 4°C. Beads were pelleted by brief centrifugation, supernatant removed and beads washed 4 times with 500 μl ice-cold RIPA buffer. 50 μl of 2X SDS-PAGE sample buffer was added and proteins eluted by heating at 92°C for 10 min. Eluted protein was then subjected to analysis via SDS-PAGE and immunoblotting. VEGFR2 ubiquitination was monitored using mouse anti-FK2 antibody which detects both poly- and mono-ubiquitination.

2.2.1.11.   Cell surface biotinylation

Cells were stimulated (1.25 nM VEGF-A isoform in MCDB131 + 0.2% (w/v) BSA) before washing twice with ice-cold PBS and incubated with 0.5 mg/ml EZ-Link Sulfo-NHS-LC-Biotin (ThermoFisher) in PBS containing 2 mM MgCl$_2$ and 2 mM CaCl$_2$ for 30 min at 4°C. Biotinylation was quenched by washing twice with ice-cold TBS followed by washing twice with ice-cold PBS. Cells were lysed in 500 μl RIPA buffer for 1 h at 4°C. Lysates were cleared by centrifugation at 16000 g for 30 min at 4°C. Equivalent protein amounts were incubated with 35 μl neutravidin-agarose beads (ThermoFisher) overnight at 4°C. Beads were pelleted by brief centrifugation, supernatant removed and beads washed 4 times with 500 μl ice-cold RIPA buffer. 50 μl of 2X SDS-PAGE sample buffer was added and proteins eluted by heating at 92°C for 10 min before analysis via SDS-PAGE and immunoblotting.

2.2.1.12.   Immunofluorescence analysis

Medium was aspirated from cells seeded on PSG (0.1% (w/v)) coated coverslips in 24-well plates and cells were rinsed twice in 500 μl of PBS. Cells were fixed in 400

μl 10% (v/v) formalin (Sigma-Aldrich, Poole, UK) for 5 min at 37°C. Fixative was aspirated and coverslips rinsed twice in 500 μl of PBS. Cells were then permabilised for 4 min in 1 ml of 0.2% (v/v) Triton X-100 in PBS. Coverslips were rinsed twice in 500 μl of PBS and incubated for 30 min in 5% (w/v) BSA in PBS to block non-specific antibody binding to cells. Coverslips were inverted onto a 20 μl drop of primary antibody solution diluted in 1% (w/v) BSA in PBS (Table 2.2) in a moist staining chamber and incubated overnight at room temperature. Coverslips were washed three times with 500 μl of PBS and inverted onto a 20 μl secondary antibody solution containing 4 μg/ml donkey Alexa Fluor-conjugated secondary antibody (Invitrogen, Amsterdam, Netherlands), 2 μg/ml 4,6-diamidino-2-phenylidole (DAPI) in 1% (w/v) BSA in PBS and incubated for 2 h at room temperature. Coverslips were washed three times with 500 μl of PBS and mounted onto slides using Fluoromount G (Southern Biotech, Birmingham, Alabama, USA). Images were acquired either using a DeltaVision wide-field deconvolution microscope (Applied Precision Inc., Issaquah, USA) or an EVOS-fl inverted digital microscope (Life Technologies, Paisley, UK). Relative VEGFR2 co-distribution or protein levels were quantified using Image J (NIH, Bethesda, USA).

2.2.1.13.   Monitoring cytosolic calcium ion flux.

2.5×10^4 cells were seeded per well of a 96-well plate and cultured for 48 h until 100% confluent. Cells were washed twice with 100 μl SBS buffer (130 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, 8 mM glucose, 10 mM HEPES, 1.5 mM $CaCl_2$, pH 7.4) and loaded with 50 μl Fura-2 AM/ SBS (2 μM Fura-2 AM, 0.01% Pluronic F-127) for 60 min at 37°C. Cells were then washed twice with 100 μl SBS and left at room temperature for 30 min to allow complete de-esterification of Fura-2 AM. Desired concentration of VEGF-A isoform was made up as a 5X stock in a compound plate. Ca^{2+} release was monitored by measuring the ratio of 510 nm emission achieved from excitation at 340 nm vs. 380 nm using a FlexStation Benchtop Microplate Reader (Molecular Devices, Sunnyvale, USA). VEGF-A was added automatically after 32 sec and Ca^{2+} levels were measured every 5 sec for a total of 900 sec. Change in cytosolic Ca^{2+} fluctuations as a function of time were plotted and quantified by calculating the area under the curve using OriginPro 8.6 (OriginLab, USA) .

2.2.1.14.   BrdU incorporation cell proliferation assay

2,500 cells were seeded/well of a 96-well plate and left to acclimatise in ECGM overnight. ECGM was then aspirated and cells starved in MCDB131 + 0.2% (w/v) for 2-3 h. Cells were then stimulated with desired concentration of VEGF-A isoforms in 90 μl total volumes for 24 h. Cells were incubated with 10 μM BrdU at t=20 h. A cell proliferation ELISA (Roche Diagnostics, Mannhein, Germany) was performed according to manufacturer's instructions. The colour change was developed using 3,3'5,5'-tetramethylbenzidine solution and the reaction quenched with 1 M H_2SO_4. The absorbance was measured at 450 nm using the 96-well plate reader.

2.2.1.15.   Cell migration assay

Cells were seeded at 3 x 10^4 cells/well into a 8 μm pore size Transwell filter inserted into a 24-well companion plate (BD Biosciences, Oxford, UK) in MCDB131 + 0.2% BSA. ECGM or MCDB131 + 0.2% BSA containing the desired concentration of VEGF-A was added to the lower chambers to stimulate cell migration. Cells were allowed to migrate for 24 h before being fixed and stained with 0.2% (w/v) crystal violet in 20% (v/v) methanol. Non-migrated cells were then removed from the upper chamber using a moist cotton bud, chambers were rinsed using double-distilled water. 3-5 random fields were imaged per Transwell filter and the average number of migratory cells calculated.

2.2.1.16.   Endothelial cell tubulogenesis assay

Primary human foreskin fibroblasts (pHFFs) (Promocell, Heidelberg, Germany) were cultured to confluency in 48-well plates in Q333 fibroblasts growth media (PAA Laboratories, Pasching, Austria) or   DMEM contain 10% (v/v) FCS, 1% (v/v) non-essential amino acids and 1% (v/v) sodium pyruvate. 6500 HUVECs were then seeded onto the fibroblast monolayer in a 1:1 mixture of Q333 and ECGM and left for 24 h. Media was then removed and replaced with fresh ECGM ±VEGF-A as desired; media was replaced every 2-3 days for 7 days. Co-cultures were then fixed in 200 μl 10% (v/v) formalin for 20 min and blocked in 1% (w/v) BSA for 30 min at room temperature. Co-cultures were then incubated with 1 μg/ml mouse anti-human PECAM-1 (CD31, Santa Cruz, CA, USA) overnight at room temperature. Cells were washed three times with PBS before incubation with donkey secondary anti-mouse Alexa Fluor 594 conjugate (Invitrogen) for 2-3 h at room temperature. Wells were then washed three times with PBS. Endothelial tubules were then visualised by immunofluorescence microscopy using an EVOS-fl inverted digital microscope (Life

Technologies, Paisley, UK). 5 random fields were imaged per well. Both the number of branch points and the total tubule length was then quantified from each photographic field using the open source software AngioQuant (www.cs.tut.fi/sgn/csb/angioquant) and values averaged.

2.2.1.17. *Ex vivo* aortic sprouting assay

This assay is based on a protocol adapted from a published study (Baker et al., 2012). All procedures involving animal and their tissues were carried out in accordance to local and national regulations under a UK Home Office project license held by Dr. S. Wheatcroft (Faculty of Medicine & Health, University of Leeds) and a UK Home Office personal License (GWF, NAM). All work was carried out in the Central Biological Services animal facility at room temperature unless otherwise stated. Briefly, male wild-type C57Bl/6 mice were sacrificed by Schedule 1 in accordance with UK Home Office regulations. The thoracic aorta was harvested from aortic arch to diaphragm, fat and fascia were removed from the aorta by sharp dissection and the vessel sliced into 0.5 mm rings. Aortic rings were serum-starved overnight at 37°C in 5 ml OptiMEM supplemented with penicillin-streptomycin. On ice, purified type 1 collagen from rat tail (Millipore, Watford, UK) was diluted to 1 mg/ml with DMEM before adding 2 μl per ml of 5 M NaOH. 55 μl of this embedding matrix was pipetted per well into a 96-well plate and aortic ring submerged within. Plates were incubated at room temperature for 15 min before incubation at 37°C for 60 min. 150 μl OptiMEM containing 2.5% (v/v) FCS and penicillin-streptomycin was added per well containing the appropriate VEGF-A isoform. Aortic rings were incubated at 37°C for 5 days with a media change on day 3. Wells were washed with 150 μl PBS containing 2 mM $CaCl_2$ and 2 mM $MgCl_2$ and fixed in 10% (v/v) formalin for 30 min. The collagen was permeabilised with three 15 min washes with PBS buffer containing 2 mM $MgCl_2$, 2 mM $CaCl_2$, 0.25% (v/v) Triton X-100. Rings were blocked in 30 μl 1% (w/v) BSA in PBLEC (PBS containing 100 μM $MnCl_2$, 1% (v/v) Tween-20, 2 mM $CaCl_2$, 2 mM $MgCl_2$) for 30 min at 37°C. 2.5 μg BS1 lectin-FITC (Sigma-Aldrich, Poole, UK) in PBLEC was added per well and incubated overnight at 4°C. Wells were washed three times with 100 μl PBS containing 2 mM $MgCl_2$, 2 mM $CaCl_2$ and 0.25% (v/v) Triton X-100 before incubation for 2 h with 1 μg/ml DAPI (in PBLEC). Wells were washed 3 times with 100 μl PBS containing 0.1% (v/v) Triton X-100 and then with 100 μl sterile water. Aortic sprouts were imaged using an EVOS-fl inverted digital microscope. Number of initial sprouts

(vascular sprouts emanating directly from the aortic ring) were counted and average.

2.2.1.18. Leukocyte binding assay

2×10^5 HL-60 leukocytes per well were labelled with 0.5 µg/ml calcein-AM (Invitrogen) for 30 min at 37°C. Cells were then pelleted and washed twice in 5 ml RPMI + 10% (v/v) FCS (Invitrogen). Cells were left for 30 min at 37°C to allow de-esterification of calcein-AM agent. 100 nM phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) was then added and the cells left to incubate for 30 min at 37°C. Cells were again pelleted and washed twice in 5 ml RPMI. 2×10^5 HL-60 leukocytes per well were then added onto a confluent layer of HUVECs which had been previously stimulated with full growth media (± VEGF-A$_{165}$ or VEGF-A$_{121}$ for 7h) and left to adhere for 1 h at 37°C. Non-adhered leukocytes were removed by gentle rinsing with PBS. Cells were then lysed in 200 µl RIPA buffer. 50 µl was then analysed by fluorescence excitation at 488 nm and emission at 520 nm in a multiwell plate format using a 96-well FLUOstar OPTIMA fluorescence plate reader (BMG LABTECH, Buckinghamshire, UK). Values were compared to controls where VEGF-A was absent.

2.2.1.19. Cell cycle analysis
2.2.1.19.1. Flow cytometry analysis of cell cycle progression

Control cells or cells subjected to siRNA treatment were cultured and reseeded at 60–70% confluency in ECGM. Cells were left to attach for 6-8 h, media was aspirated and cells serum starved overnight in MCBD131 containing 0.2% (w/v) BSA and 2 mM thymidine, to promote cell cycle arrest. Starvation media was aspirated and cells incubated with ECGM containing 25 µM 2-deoxycytidine, with or without Tpl2-specific kinase inhibitor, for 24 h at 37°C. Endothelial cells were detached using TrypLE Express (Invitrogen), quenched with DMEM containing 10% (v/v) FCS and cells transferred into 1.5 or 15 ml centrifuge tubes as appropriate. Cells were then pelleted at 140 g at 4°C for 5 min and supernatant discarded. Cells were then fixed with dropwise addition of ice-cold 70% (v/v) ethanol and stored at -20°C prior to analysis. Immediately prior to flow cytometry, cells were pelleted (140 g at 4°C for 5 min), supernatant removed and cells washed twice in 500 µl PBS. 100 mg/ml ribonuclease and 50 mg/ml propidium iodide (Sigma-Aldrich) was added to each sample and incubated for 2–3 h at 37°C. Cells were pelleted (140 g at 4°C for 5 min), supernatant removed and cells washed in 500 µl PBS before being

resuspended in 500 μl PBS buffer containing 2.5 mM EDTA. Samples were gated and run at a low flow rate (1000 events s^{-1}) on a Fortessa flow cytometer (Becton Dickinson, Oxford, UK) with multi-laser and detection capabilities. The information from 10000 events was collect and data was analysed using ModFit software (Becton Dickinson).

2.2.1.19.2. Analysis of cell cycle progression using the FUCCI expression system

cDNA encoding the FUCCI constructs cloned into the CSII-EF-MCS vector were obtained from Dr Atsushi Miyawaki (Riken Institute of Brain Science, Saitama, Japan). Endothelial cells were transduced with replication-defective, self-inactivating lentiviral vectors encoding mAG-hGeminin (1/110) and mKO2-hCdt (30/120) as outlined in Sakaue-Sawano et al. (Sakaue-Sawano et al., 2008). Following transduction with high-titre viral solutions generated in HEK293T cells, endothelial cells were transfected with specific siRNA duplexes for 48 h prior to serum-starvation-induced cell cycle arrest. Cells were subsequently trypsinised and reseeded into 96-well, black-walled plates at 2.5×10^{3} cells per well and imaged at 4, 8, 16 and 30 h post release following Hoechst 33342 addition. Image acquisition was carried out using either a BD Pathway 435 imager or Olympus X81 immunofluorescence microscope equipped with 405 nm, 488 nm, and 543 nm light-source lines. Image analysis was performed using ImageJ and Metamorph 6 software (Universal Imaging, Media, PA) on between 400-2000 cells at each time-point per siRNA treatment. Quantifications are from three independent experiments performed in duplicate.

2.2.1.20. Evaluation of angiogenesis using the mouse hind limb ischaemia model

Animal experiments were carried out in accordance to UK Home Office regulations and guidance. Murine surgery was performed by Dr Nadira Yuldasheva (Faculty of Medicine & Health, University of Leeds). Prior to surgery the lower abdomen and both groins of each mouse were depilated with Veet® hair removal cream. In brief, 8-week old C57Bl/6 male mice (Charles River, Kent, UK) were anaesthetised via inhalation agent isofluorane 1.5-2.0 L (Merial Animal Health Ltd, Essex, UK) saturated with O_2 in an induction chamber. The mouse was then place in the supine position on a heating plate (Vet-tech, Cheshire, UK) with its upper paws fixed onto an anaesthetic nose mask and its lower extremities abducted and extended. Analgesia of 0.25 mg/kg buprenorphine in 0.9% NaCl was administered via

intraperitoneal injection and the surgical site cleaned using povidone-Iodine 0.75% (w/w) available iodine (Animalcare, UK). All surgical procedures were performed with the assistance of a Zeiss OPMI 1 FC surgical microscope (Carl Zeiss, Jena, Germany) under appropriate magnification. An initial 1.2 cm longitudinal groin incision was made on the lower-left extremity, crossing the inguinal ligament. Femoral vessels were then exposed from the region distally to inguinal ligament to saphenous artery. Femoral artery (just under the inguinal ligament) was dissected and ligated with 8.0 vicryl suture (Ethicon, Belgium), two ligatures were made in space of 1 mm. Proximally to bifurcation of femoral artery, the vessel was dissected and two ligatures were made in space of 1 mm. Femoral artery was separated from vein and excised. The skin incision was closed with a continuous 8.0 vicryl suture and povidone-Iodine was applied onto the wound. A sham operation was then preformed on the contralateral limb.

The mouse was then placed in the prone position and a longitudinal skin incision was made between the shoulder blades, subcutaneous pocket was extended and an ALZET model 1004 osmotic pump (Charles River) pre filled with either sterile PBS or the desired VEGF-A isoform (to give a calculated dose of 20 ng VEGF-A/day) was inserted. The subcutaneous incision was then closed using a continuous 8.0 vicryl suture and povidone-Iodine was applied onto the wound. Finally, the mouse was injected intraperitoneally with 300 μl 0.9% NaCl solution before being transferred to a pre-warmed 38°C chamber where it awoke within 1-2 min. Mice were closely monitored at regular intervals after surgery to ensure no post-operative complications arose.

2.2.1.21.   Laser Doppler assessment of hind limb reperfusion after injury

60-90 min after surgery, mice were place in a temperature controlled 37°C environment and allowed to warm prior to assessment of hind limb blood flow. Mice were then anaesthetised via inhalation agent isofluorane saturated with O_2 in an induction chamber, prior to being placed in the supine position with its upper paws fixed onto an anaesthetic nose mask and its lower extremities abducted and extended. Laser Doppler analysis using the high resolution Moor LDI2-HR imager with spatial resolution of 100 μm (Moor Instruments, Axminster, UK) on ischaemic and non-ischaemic limbs was performed to confirm surgical induction of ischaemia; this was then performed once/week for three weeks to assess hind limb reperfusion. Quantification of hind limb blood flow was carried out using moorLDITM analysis

software (Moor Instruments); the area from just above the mouse knee was quantified. A reperfusion index was calculated by comparing ischaemic to non-ischaemic (control) hind limb blood flow. Data was then compared to each group's individual reperfusion index at week=0 to give a relative increase in blood flow regeneration.

2.2.1.22. Statistical analysis

This was performed using a one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test or two-way ANOVA followed by Bonferroni multiple comparison test using GraphPad Prism software (La Jolla, CA, USA). Significant differences between control and test groups were evaluated with p values less than 0.05 (*), 0.01 (**), 0.001 (***) and 0.0001 (****) indicated on the graphs. Error bars in graphs and histograms denote ±SEM (Standard error of mean).

2.2.2. Molecular biology techniques

2.2.2.1. Quantitative reverse transcription PCR (qRT-PCR)

Cells were serum-starved and stimulated with 0.25 nM VEGF-A$_{165}$ or VEGF-A$_{121}$ prior to extraction of total RNA with RNeasy Plus Mini Kit (Qiagen, UK). 1 μg total RNA was reverse transcribed using GoScript Reverse Transcription System (Promega, UK). Real time quantitative reverse transcription PCR was performed using Power SYBR Green master mix (Applied Biosystems, Warrington, UK) with desired forward and reverse primer sets (see Table 2.3). qRT-PCR was carried out in multiwell plates run on an ABI 7900HT Fast Real-Time PCR System (Applied Biosystems, UK) and gene expression analysed using the delta/delta CT method standardised against an endogenous control, GAPDH.

Target	Forward sequence	Reverse sequence
GAPDH	5'– GTC TCC TCT GAC TTC AAC AGC G -3',	5'– ACC ACC CTG TTG CTG TAG CCA A -3';
ATF-2	5'– GGT AGC GGA TTG GTT AGG ACT C -3',	5'– TGC TCT TCT CCG ACG ACC ACT T -3';
VCAM-1	5'– GAT TCT GTG CCC ACA GTA AGG C -3',	5'– TGG TCA CAG AGC CAC CTT CTT G -3'.
Tpl-2	5'– CGC AAG AGG CTG CTG AGT A -3'	5'– TTC CTG TGC ACG AAG AAT CA -3'.

Table 2.3: Forward and reverse primer sequences for qRT-PCR. Nucleotide sequences of forward or reverse DNA primers used in qRT-PCR analysis.

CHAPTER 3

VEGF-A isoform-specific VEGFR2 trafficking regulates signal transduction and endothelial responses

3.1.   INTRODUCTION

The 58 human receptor tyrosine kinases (RTKs) can be divided into 20 subfamilies which regulate health and disease outcomes (Lemmon and Schlessinger, 2010). Many of these RTKs exist as monomers which upon ligand binding undergo dimerisation, tyrosine transautophosphorylation and increased enzymatic activity. This mode of enzymatic activation enables such membrane-bound enzymes to recruit an array of signal transduction and adaptor proteins (Koch et al., 2011). Although RTKs are key therapeutic targets, successful drug design is complicated by our lack of understanding of how RTK function is controlled by ligand diversity. The human vascular endothelial growth factor (VEGF) family comprises 5 family members: VEGF-A, VEGF-B, VEGF-C, VEGF-D and placental growth factor (PlGF). These soluble growth factors regulate angiogenesis and lymphangiogenesis by complex interactions with Class V RTKs (VEGFR1-3) and the co-receptors Neuropilin 1 (NRP1), NRP2 and heparan sulphate proteoglycans (HSPGs) (Koch et al., 2011). The *VEGFA* gene (located on chromosome 6p21.3) encodes a pre-mRNA transcript that contains at least 8 exons and 7 introns. Alternative pre-mRNA splicing gives rise to at least 7 pro-angiogenic and 1 anti-angiogenic isoforms (Vincenti et al., 1996; Robinson and Stringer, 2001; Kikuchi et al., 2014; Mavrou et al., 2014). VEGF-A is crucial for normal mammalian development as the targeted deletion of a single *VEGFA* allele (contained within exon 3) is sufficient to promote embryonic lethality between days E11 to E12 due to abnormal blood vessel and heart development (Carmeliet et al., 1996; Ferrara et al., 1996). VEGF-A isoforms have specific roles within vascular development, for instance *VEGFA*$^{120/120}$ mice (mice only expressing VEGF-A$_{120}$; VEGF-A$_{121}$ in humans) have blood vessel, which have an unnaturally large diameter and suffer from hypobranching, whereas the blood vessels of *VEGFA*$^{188/188}$ (mice only expressing VEGF-A$_{188}$; VEGF-A$_{189}$ in humans) appear spindle-like and suffer from irregular branching (Ruhrberg et al., 2002). Additionally, *VEGFA*$^{188/188}$ mice also suffer from inferior aortic arch remodelling and abnormal artery formation (Stalmans et al., 2002). Contrastingly,

VEGFA$^{164/164}$ mice which only express VEGF-A$_{164}$ (VEGF-A$_{165}$ in humans) exhibit a relatively normal vascular phenotype (Ruhrberg et al., 2002). Intriguingly, both the morphological and branching defects associated with VEGFA$^{120/120}$ and VEGFA$^{188/188}$ mice are recovered in mice expressing both VEGF-A$_{120}$ and VEGF-A$_{188}$ (Ruhrberg et al., 2002). Such studies suggest that a combination of rapidly diffusible (VEGF-A$_{120}$) and matrix-binding (VEGF-A$_{188}$) isoforms act as spatial cues to guide the developing blood vessel; both of which are required for normal healthy vasculogenesis (Ruhrberg et al., 2002; Haigh, 2008). All VEGF-A isoforms bind both VEGFR1 (Flt-1) and VEGFR2 (KDR), but selectively recruit neuropilin co-receptors (Koch et al., 2011). Most studies have focused on the relatively abundant and major VEGF-A$_{165}$ isoform which is secreted by most cells and tissues.

VEGF-A$_{165}$ binding to the VEGFR2 extracellular domain triggers a pro-angiogenic response by endothelial cells, involving cell proliferation, migration, tubulogenesis, and vascular permeability (Takahashi et al., 1999; Nakatsu et al., 2003; Yilmaz et al., 2003; Xu et al., 2011). Such RTK-ligand interactions sequentially cause receptor phosphorylation, ubiquitination, trafficking and proteolysis (Bruns et al., 2009; Horowitz and Seerapu, 2012; Koch and Claesson-Welsh, 2012; Nakayama and Berger, 2013). Studies have shown that VEGF-A isoforms differentially promote VEGFR2-dependent signal transduction and cellular responses (Kawamura et al., 2008; Kawamura et al., 2008; Zhang et al., 2000). Furthermore, recent studies have suggested that the human anti-angiogenic (VEGF-A$_{165b}$) isoform influences prostate cancer progression (Mavrou et al., 2014) and peripheral arterial disease (Kikuchi et al., 2014). However, the underlying mechanism(s) are still unclear. More recently, it has been shown that VEGF-A$_{165}$-stimulated signal transduction is not only dependent on VEGFR2 tyrosine kinase activation, but is also dependent on VEGFR2 trafficking and turnover within the biosynthetic secretory pathway and delivery from the plasma membrane to the endosome-lysosome system (Zhang et al., 2013; Gourlaouen et al., 2013; Lanahan et al., 2014; Jopling et al., 2009; Lanahan et al., 2010; Manickam et al., 2011; Yamada et al., 2014). It is therefore important to address whether VEGF-A isoforms can thus 'program' differential VEGFR2 trafficking and turnover which would have consequences for endothelial cell function in vascular physiology.

To address this question, the work carried out in this chapter evaluated the VEGF-A isoforms, VEGF-A$_{121}$, VEGF-A$_{145}$ and VEGF-A$_{165}$ for their ability to regulate VEGFR2 activation, downstream signal transduction, post-translational modification,

trafficking and proteolysis linked to physiological responses. Here, we show that these three VEGF-A isoforms exhibit differential VEGFR2 phosphorylation and delivery into early endosomes. This co-operatively impacts on isoform-specific signal transduction through the MAPK pathway. A key feature is that different VEGFR2-VEGF-A isoform complexes exhibit distinct patterns of plasma membrane trafficking, ubiquitination and proteolysis. The function of VEGF-A isoforms is also different in arterial regeneration in an animal model. These findings show that VEGF-A-isoform-specific RTK function is programmed by linking post-translation modifications to residence within different intracellular locations.

3.2.   RESULTS

3.2.1. VEGF-A isoforms trigger differential VEGFR2 plasma membrane-to-endosome trafficking

Activated VEGFR2 undergoes dimerisation and transautophosphorylation at specific cytoplasmic tyrosine residues linked to downstream signal transduction pathways (Bruns et al., 2010; Koch et al., 2011). VEGFR2 tyrosine phosphorylation precedes ubiquitination and delivery to early endosomes (Jopling et al., 2009; Scott and Mellor, 2009; Bruns et al., 2010). Firstly, activated VEGFR2 is trafficked into endosomes prior to proteasome-dependent, cleavage before trafficking into lysosomes for terminal degradation (Bruns et al., 2010; Jopling et al., 2009). Secondly, internalised VEGFR2 can be redirected into short-loop/Rab4-positive or long-loop/Rab11-positive recycling endosomes for trafficking back to the plasma membrane in order to mediate a subsequent round of receptor-ligand engagement (Ballmer-Hofer et al., 2011; Jopling et al., 2014). We hypothesised that VEGF-A isoforms could differentially regulate activated-VEGFR2 internalisation in order to modulate isoform-specific downstream signal transduction and endothelial cell responses.

To test this idea, primary human umbilical vein endothelial cells (HUVECs) were stimulated for 0, 5, 15, 30 or 60 min with either VEGF-A$_{165}$, VEGF-A$_{121}$ or VEGF-A$_{145}$ (1.25 nM) prior to monitoring VEGFR2 dynamics using cell surface biotinylation. In order to measure plasma membrane-associated VEGFR2 levels and VEGFR2 activity, both biotinylated cell surface (affinity isolation) and total cellular protein (total lysates) pools were subjected to immunoblotting for either total VEGFR2 or VEGFR2 phosphorylated at Y1175 (Figure 3.1A). This VEGFR2-pY1175 phospho-epitope is essential for VEGFR2 functional output(s). Quantification revealed that all

three VEGF-A isoforms promoted a significant rise in cell surface VEGFR2-pY1175 levels, peaking at either 5 (VEGF-A$_{165}$ and VEGF-A$_{121}$) or 15 min (VEGF-A$_{145}$) respectively (Figure 3.1B). Interestingly, cell surface VEGFR2-pY1175 levels were significantly decreased after 30 min of VEGF-A$_{165}$ or VEGF-A$_{145}$ stimulation (Figure 3.1B). In comparison, 30 min stimulation with VEGF-A$_{121}$ revealed greater stability and longevity in cell surface VEGFR2-pY1175 levels (Figure 3.1B). This latter phenomenon coincided with reduced total VEGFR2 internalisation, which contrasted with effects observed upon VEGF-A$_{165}$ or VEGF-A$_{145}$ stimulation (Figure 3.1C).

To further assess the ability of each VEGF-A isoform to promote VEGFR2 internalisation, endothelial cells were first treated (1.5 h) with cycloheximide to block new VEGFR2 biosynthesis and then subjected to a VEGF-A isoform stimulation time course followed by analysis using fluorescence-based microscopy (Figure 3.2A). Quantification of VEGFR2 plasma membrane-to-endosome trafficking after VEGF-A$_{165}$ or VEGF-A$_{145}$ stimulation revealed a 40-50% increase in co-distribution with the early endosome marker, EEA1 (Figure 3.2B). Conversely, VEGF-A$_{121}$ stimulation failed to reveal a significant increase in VEGFR2 co-distribution with EEA1 (Figure 3.2B). Taken together, these findings suggest that VEGF-A isoforms can cause unique VEGF-A signal transduction events which are linked to differential localisation to endosomes.

3.2.2. VEGF-A isoforms program differential VEGFR2 ubiquitination, proteolysis and terminal degradation

VEGF-A binding to VEGFR2 promotes proteolysis and terminal degradation in the endosome-lysosome system. As VEGF-A isoforms exhibit different abilities in stimulating VEGFR2 endocytosis, we hypothesised that such trafficking is linked to VEGFR2 terminal degradation. To investigate this, endothelial cells were subjected to a VEGF-A isoform stimulated time course before immunoblot analyses (Figure 3.3A). Stimulation with VEGF-A$_{165}$ or VEGF-A$_{145}$ promoted a steady turnover of total VEGFR2 levels over 120 min, resulting in ~30% reduction in basal VEGFR2 levels (Figure 3.3B). However, stimulation with VEGF-A$_{121}$ promoted a modest, statistically insignificant ~10% reduction in total VEGFR2 levels over the same period (Figure 3.3B). Thus, exogenously added VEGF-A isoforms have differential effects on the VEGFR2 pool associated with the endocytic pathway.

VEGF-A$_{165}$ binds to the co-receptor NRP1 to form a VEGFR2/NRP1/VEGF-A$_{165}$ complex (Fantin et al., 2013; Herzog et al., 2011; Zachary et al., 2009; Raimondi
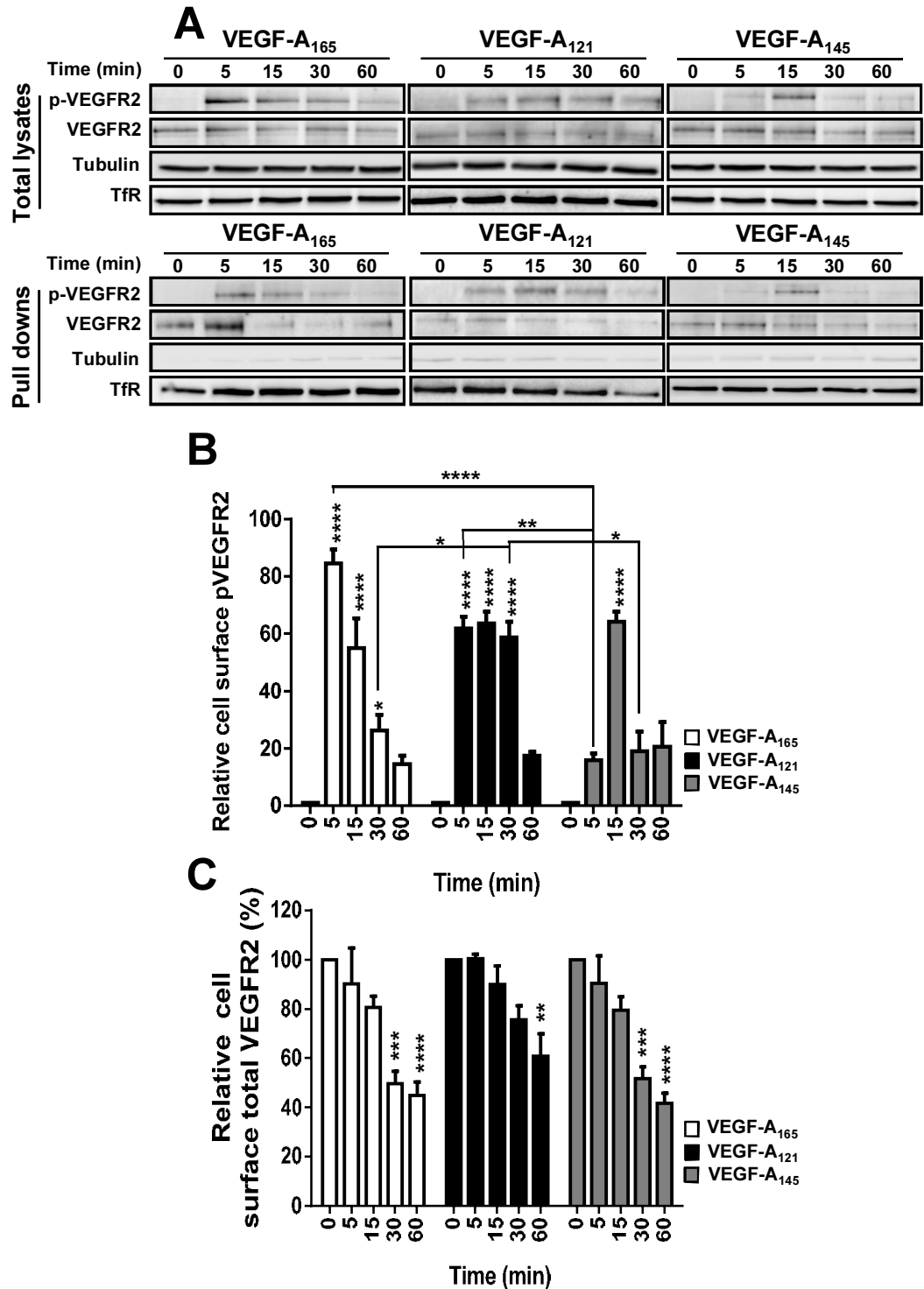
Figure 3.1. VEGF-A isoform-specific VEGFR2 internalisation. (A) Human endothelial cells were stimulated with either VEGF-A$_{165}$, VEGF-A$_{121}$ or VEGF-A$_{145}$ (1.25 nM) for 5, 15, 30 or 60 min before cell surface biotinylation, affinity isolation and immunoblot analysis of total cell lysates or biotinylated cell surface proteins. Negative control (tubulin) and positive control (transferrin receptor, TfR). (B and C) Quantification of cell surface (B) activated VEGFR2-pY1175 or (C) Mature total VEGFR2 levels upon VEGF-A$_{165}$, VEGF-A$_{121}$ or VEGF-A$_{145}$ stimulation. Error bars indicate ±SEM (n≥3). $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$ (****).
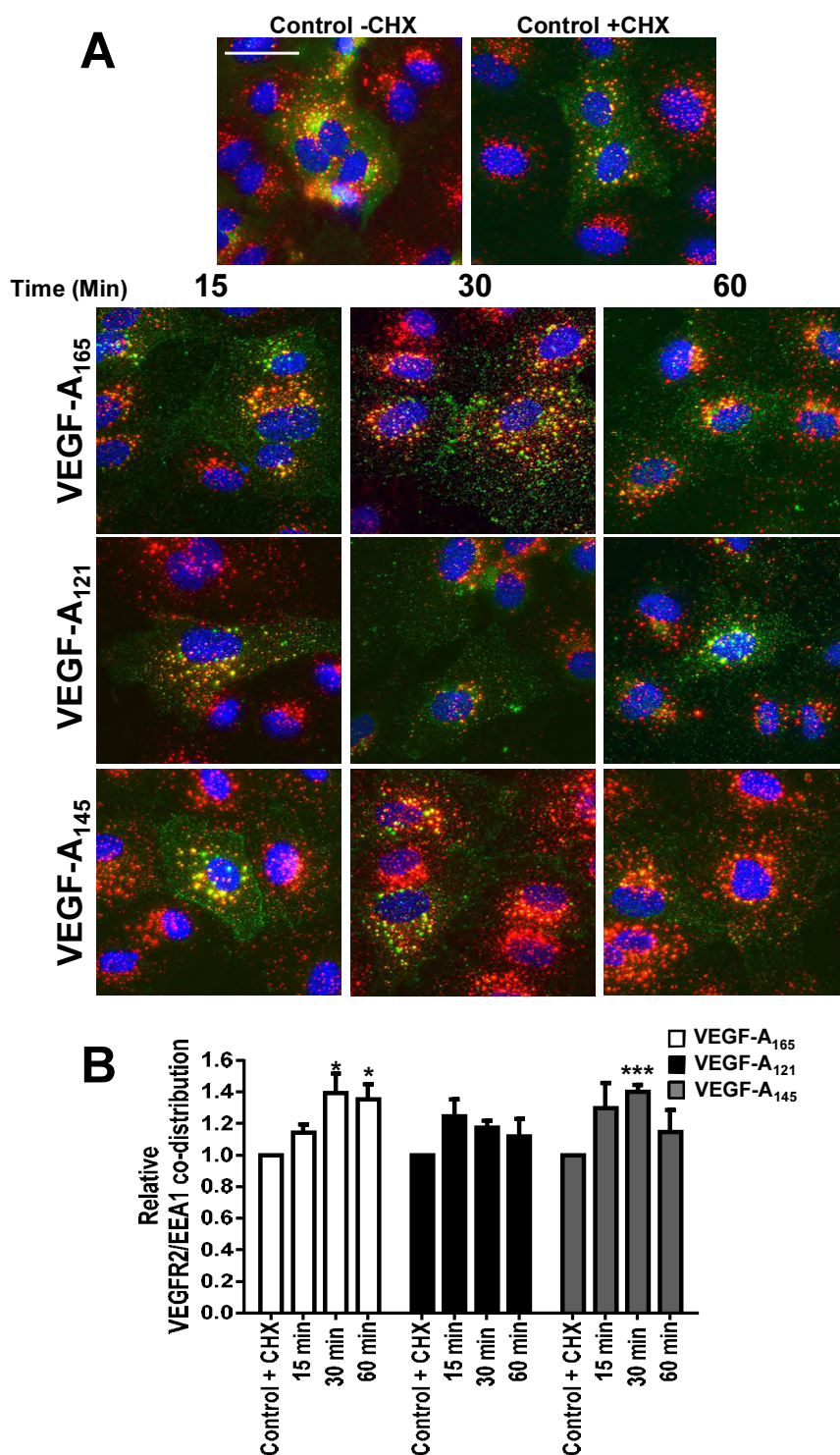
Figure 3.2. Activated VEGFR2 localisation to early endosomes. (A) Human endothelial cells were pre-treated with cycloheximide (CHX; 2 μg/ml) for 2 h prior to treatment with either VEGF-A$_{165}$, VEGF-A$_{121}$ or VEGF-A$_{145}$ (1.25 nM) for 15, 30 or 60 min. Endothelial cells were fixed and processed for immunofluorescence microscopy using goat anti-VEGFR2 (green) and rabbit anti-EEA1 (red) antibodies; nuclei stained using DAPI (blue). Scale bar, 20 μm. (B) Quantification of VEGFR2/EEA1 co-distribution upon treatment with VEGF-A$_{165}$, VEGF-A$_{121}$ or VEGF-A$_{145}$. Error bars indicate ±SEM (n=3). $p < 0.05$ (*), $p < 0.001$ (***).
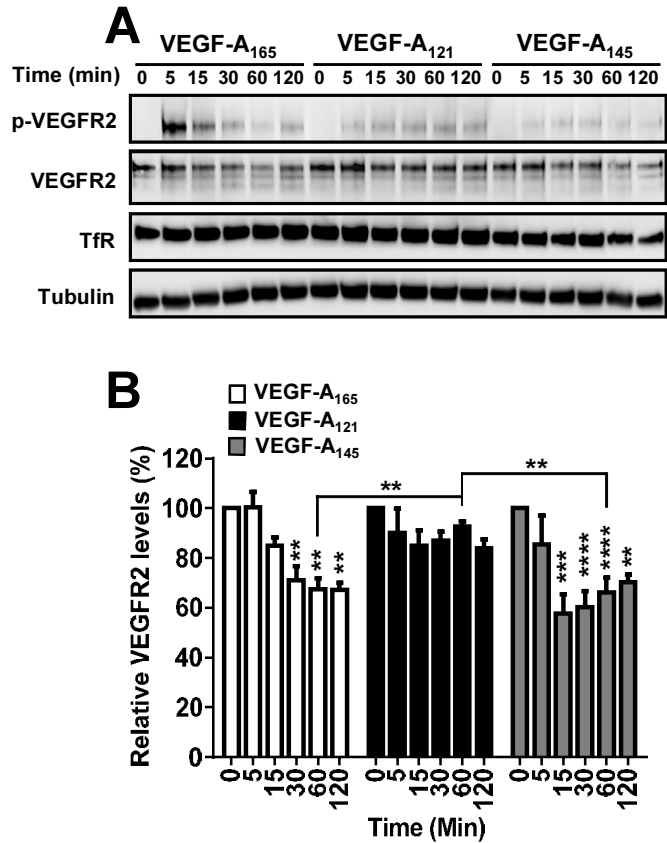
Figure 3.3. VEGF-A isoform-specific stimulation of VEGFR2 degradation. (A) Human endothelial cells were stimulated with either VEGF-A$_{165}$, VEGF-A$_{121}$ or VEGF-A$_{145}$ (1.25 nM) for 5, 15, 30, 60 or 120 min before lysis and processing for immunoblot analysis to asses total VEGFR2 levels. (B) Quantification of total VEGFR2 levels upon treatment with VEGF-A$_{165}$, VEGF-A$_{121}$ or VEGF-A$_{145}$. Error bars indicate ±SEM (n≥3). $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$ (****).

and Ruhrberg, 2013). NRP1 recruitment modulates the VEGF-A$_{165}$-mediated response (Allain et al., 2012; Kawamura et al., 2008; Koch, 2012) but exactly how this occurs is unclear. Furthermore, VEGF-A$_{121}$ binds more weakly to NRP1 and does not promote NRP1 complex formation with VEGFR2 (Pan et al., 2007). Additionally, NRP1 regulates VEGFR2 trafficking in response to VEGF-A$_{165}$ (Ballmer-Hofer et al., 2011; Koch et al., 2014). To test the significance of NRP1 in the VEGF-A isoform-specific endothelial response, endothelial cells were pre-treated with EG00229, a specific antagonist of VEGF-A$_{165}$ binding to NRP1 (Jarvis et al., 2010), prior to either VEGF-A$_{165}$, VEGF-A$_{121}$ or VEGF-A$_{145}$ treatment before microscopy-based analysis of VEGFR2 trafficking (Figure 3.4A). Quantification of total VEGFR2 levels in control and EG00229-treated cells revealed that inhibition of VEGF-A$_{165}$ binding to NRP1 significantly increased ligand-dependent VEGFR2 degradation (~1.6-fold) (Figure 3.4B). However, this molecule had no effect on VEGF-A$_{121}$ or VEGF-A$_{145}$-dependent VEGFR2 degradation (Figure 3.4B).

Prior to terminal degradation, VEGFR2 undergoes 26S proteasome-dependent proteolysis, within late endosomes (~30 min post-stimulation) to generate a 160 kDa C-terminal fragment (Bruns et al., 2010). Thus to assess each VEGF-A isoform for its ability to promote VEGFR2 proteolysis endothelial cells were stimulated with either VEGF-A$_{165}$, VEGF-A$_{121}$ or VEGF-A$_{145}$ followed by immunoblotting to assess the levels of the ~160 kDa proteolytic VEGFR2 fragment generated in endosomes (marked by black arrow) (Figure 3.5A). Quantification of the 160 kDa VEGFR2 fragment showed that all three VEGF-A isoforms significantly promoted VEGFR2 proteolysis, which peaked at ~30 min post stimulation (Figure 3.5B). Interestingly, VEGF-A$_{165}$-stimulated VEGFR2 proteolysis was higher (~1.5-fold) than that caused by either VEGF-A$_{121}$ or VEGF-A$_{145}$ (Figure 3.5B). Notably, VEGF-A$_{121}$ stimulation promoted similar levels of VEGFR2 proteolysis compared to VEGF-A$_{145}$ treatment (Figure 3.5B), despite reduced ligand-stimulated VEGFR2 internalisation (see earlier in Figure 3.1C and Figure 3.2B).

VEGF-A-stimulated VEGFR2 activation causes tyrosine transautophosphorylation which precedes ubiquitination, which has been implicated in targeting VEGFR2 for proteolysis and terminal degradation (Bruns et al., 2010; Ewan et al., 2006). One possible explanation for the differential ability of VEGF-A isoforms to mediate ligand-dependent VEGFR2 terminal degradation and proteolysis, could be their capacity to promote VEGFR2 ubiquitination. To evaluate this possibility, a time course of VEGF-A stimulation was followed by an analysis of immunoprecipitated VEGFR2 complexes for ubiquitination (Figure 3.6A). Quantification of these

A

B
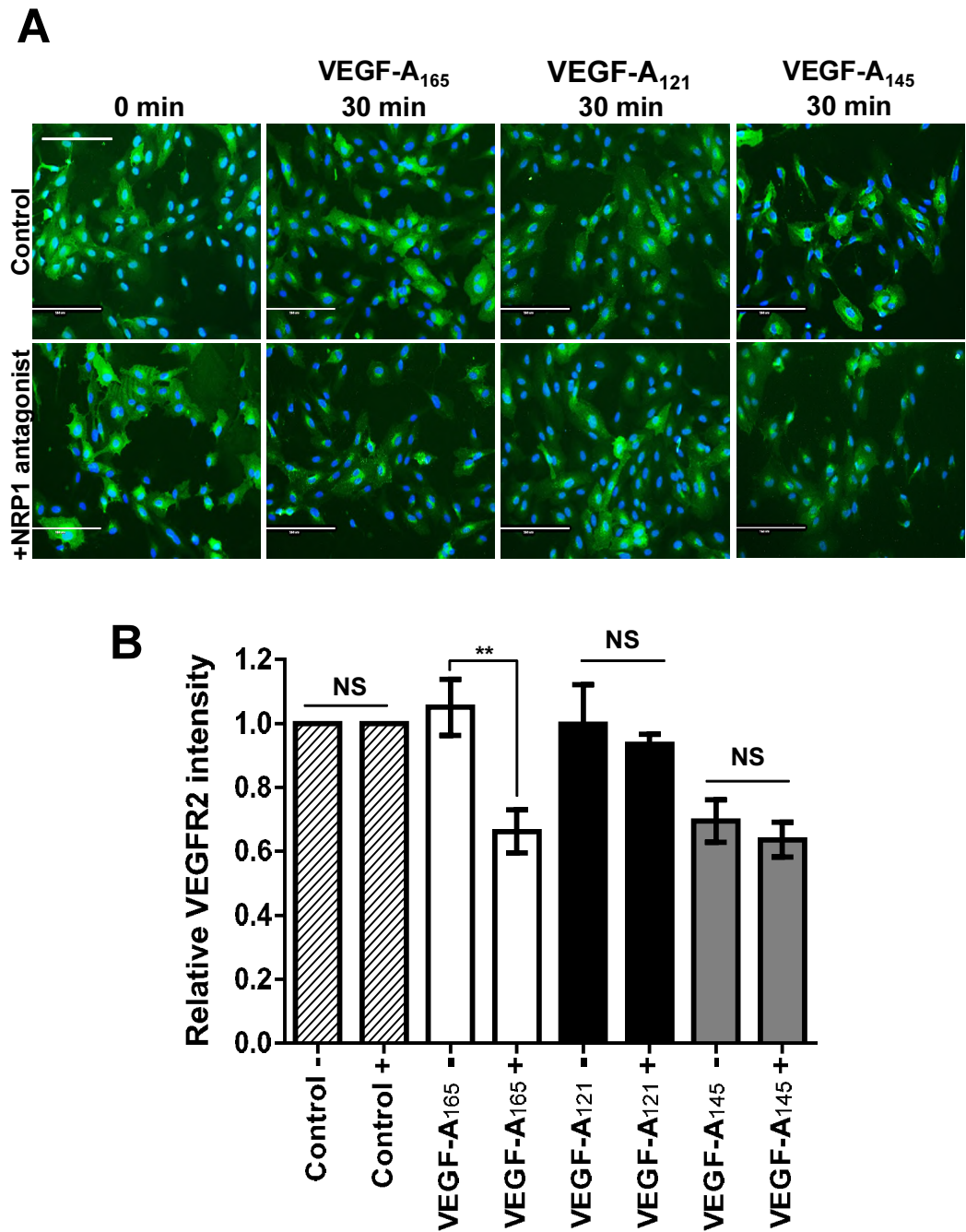
Figure 3.4. An NRP1 antagonist promotes activated VEGFR2 degradation. (A) Human endothelial cells were pre-treated with 1 mM EG00229, a membrane-impermeable antagonist for NRP1 (30 min) prior to treatment with either VEGF-A$_{165}$, VEGF-A$_{121}$ or VEGF-A$_{145}$ (1.25 nM) for 30 min. Endothelial cells were fixed and processed for immunofluorescence microscopy using goat anti-VEGFR2 (green); nuclei stained using DAPI (blue). Scale bar, 200 μm. (B) Quantification of VEGR2 levels upon treatment with VEGF-A$_{165}$, VEGF-A$_{121}$ or VEGF-A$_{145}$. Error bars indicate ±SEM (n=3). $p<0.01$ (**), NS = non-significant.
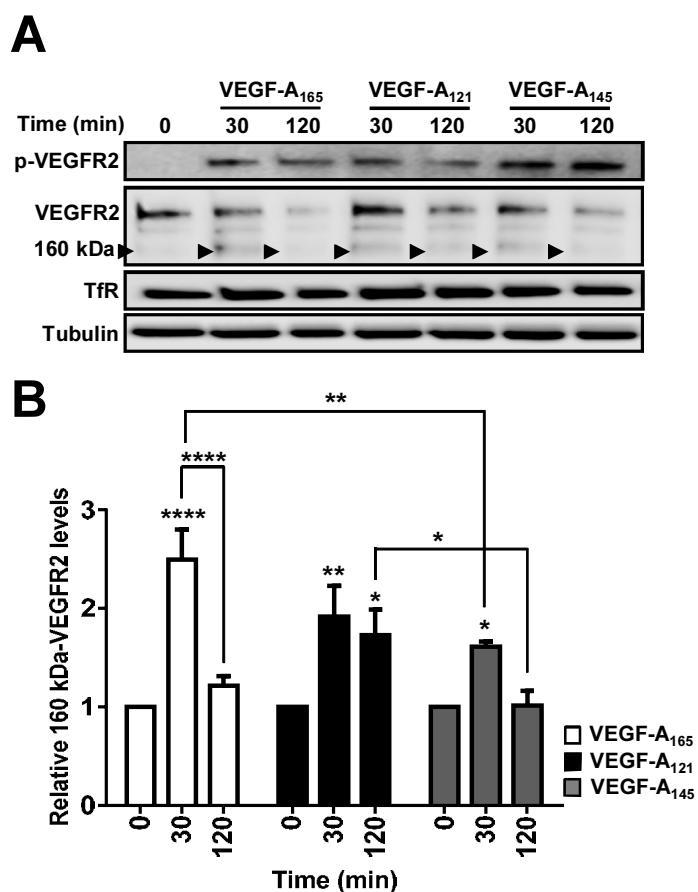
Figure 3.5. VEGF-A isoform-specific regulation of ligand-stimulated VEGFR2 proteolysis. (A) Human endothelial cells were stimulated with either VEGF-A$_{165}$, VEGF-A$_{121}$ or VEGF-A$_{145}$ (1.25 nM) for 30 or 120 min before lysis and processing for immunoblot analysis to asses VEGFR2 proteolysis. (B) Quantification of VEGFR2-160 kDa proteolytic fragment (Arrow) levels upon treatment with VEGF-A$_{165}$, VEGF-A$_{121}$ or VEGF-A$_{145}$.. Error bars indicate ±SEM (n≥3). p<0.05 (*), p<0.0001 (****).
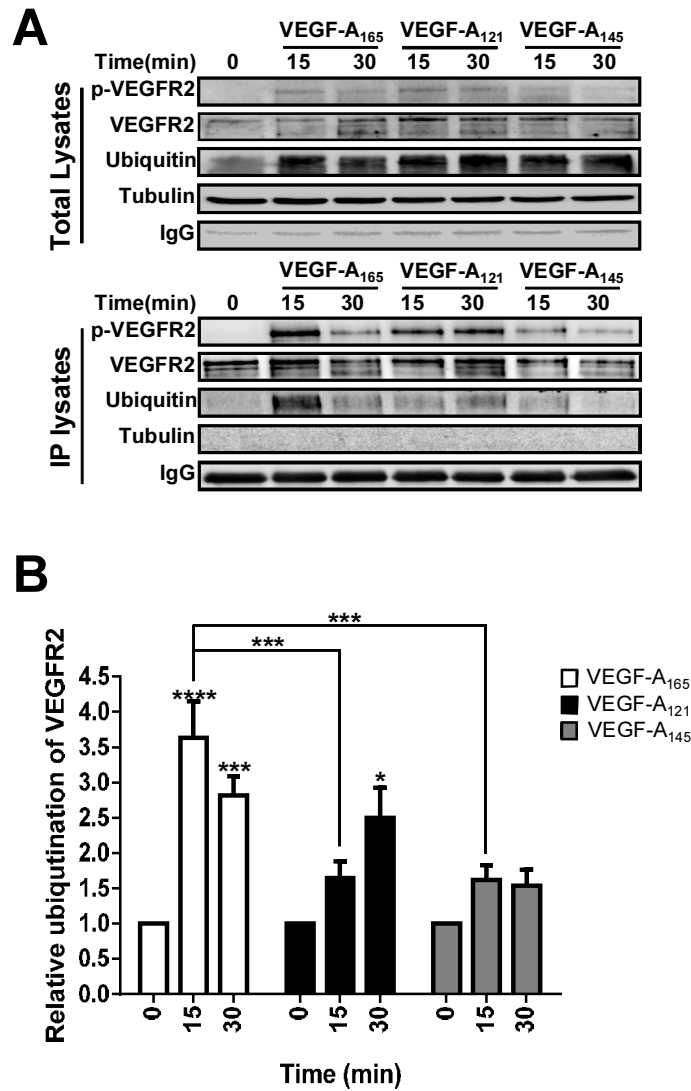
Figure 3.6. VEGF-A isoform-specific effects on VEGFR2 ubiquitination. (A) Human endothelial cells were stimulated with either VEGF-A$_{165}$, VEGF-A$_{121}$ or VEGF-A$_{145}$ (1.25 nM) for 15 or 30 min before being subjected to immunoprecipitation (IP) using an antibody against total VEGFR2. Total cell or IP lysates were processed for immunoblot analysis to asses VEGFR2 ubiquitination status using an antibody against poly-ubiquitin (FK2). Negative control (tubulin) and positive control (IgG). (B) Quantification of VEGFR2 ubiquitination status upon treatment with VEGF-A$_{165}$, VEGF-A$_{121}$ or VEGF-A$_{145}$. Error bars indicate ±SEM (n≥3). $p<0.05$ (*), $p<0.001$ (***), $p<0.0001$ (****). Panel A and selected data from panel B in this figure was kindly provided by Miss G. A. Smith (Ponnambalam laboratory, University of Leeds, UK).

combined immunoisolation and immunoblotting experiments revealed VEGF-A$_{165}$ stimulation caused maximal VEGFR2 ubiquitination (~3.5-fold relative to non-stimulated control) in comparison to either VEGF-A$_{121}$ or VEGF-A$_{145}$ stimulation (Figure 3.6B). Interestingly, VEGF-A$_{145}$ caused significant VEGFR2 terminal degradation (Figure 3.3A), yet this ligand did not cause a corresponding increase in VEGFR2 ubiquitination over a similar time period (Figure 3.6B). In contrast, although VEGF-A$_{121}$ stimulation caused significant yet delayed ubiquitination in VEGFR2 (Figure 3.6B), terminal degradation was greatly reduced (Figure 3.3B). These findings suggest that ubiquitination of VEGFR2 is not a prerequisite for terminal degradation but may be required for proteasome-regulated cleavage associated with endosomes.

3.2.3. VEGF-A isoform-specific VEGFR2 endocytic trafficking regulates ERK1/2 activation and endothelial cell proliferation.

VEGF-A binding to VEGFR2 activates multiple downstream signalling pathways (e.g. ERK1/2, p38 MAPK and Akt) which display VEGF-A isoform-specific differences (Kawamura et al., 2008; Pan et al., 2007). Such signal transduction is dependent on the phosphorylation of key VEGFR2 tyrosine residues such as Y951, Y1054, Y1059, Y1175 and Y1214 (Koch et al., 2011). We further investigated the link between isoform-specific VEGFR2 activation and downstream signal transduction, by monitoring two phosphotyrosine epitopes, namely pY1175 and pY1214 (Figure 3.7A). Quantification revealed that these three VEGF-A isoforms promoted differential activation of VEGFR2-pY1175 (Figure 3.7B). However, under the same conditions, the levels of VEGFR2-pY1214 were relatively similar (Figure 3.7C). In contrast to the other phospho-epitope, VEGFR2-pY1214 was already evident in non-stimulated cells and only displayed a modest ~2-fold rise upon stimulation with the various VEGF-A isoforms (Figure 3.7, A to C). Notably, although the intensity of the VEGFR2-pY1214 was lower, the duration was clearly more sustained (Figure 3.7C). This raises the likelihood that these two phospho-epitopes function differently within the lifetime of the VEGFR2 complex and integrate different aspects of signal transduction, post-translational modifications and trafficking.

VEGF-A isoform-specific VEGFR2 activation triggers differential signalling through the MEK1-ERK1/2 pathway (Zhang et al., 2008). VEGF-A-stimulated VEGFR2-pY1175 activation is linked to the generation of phospho-ERK1/2 (McLaughlin and De Vries, 2001; Takahashi et al., 2001; Koch et al., 2011). Additionally, activation of
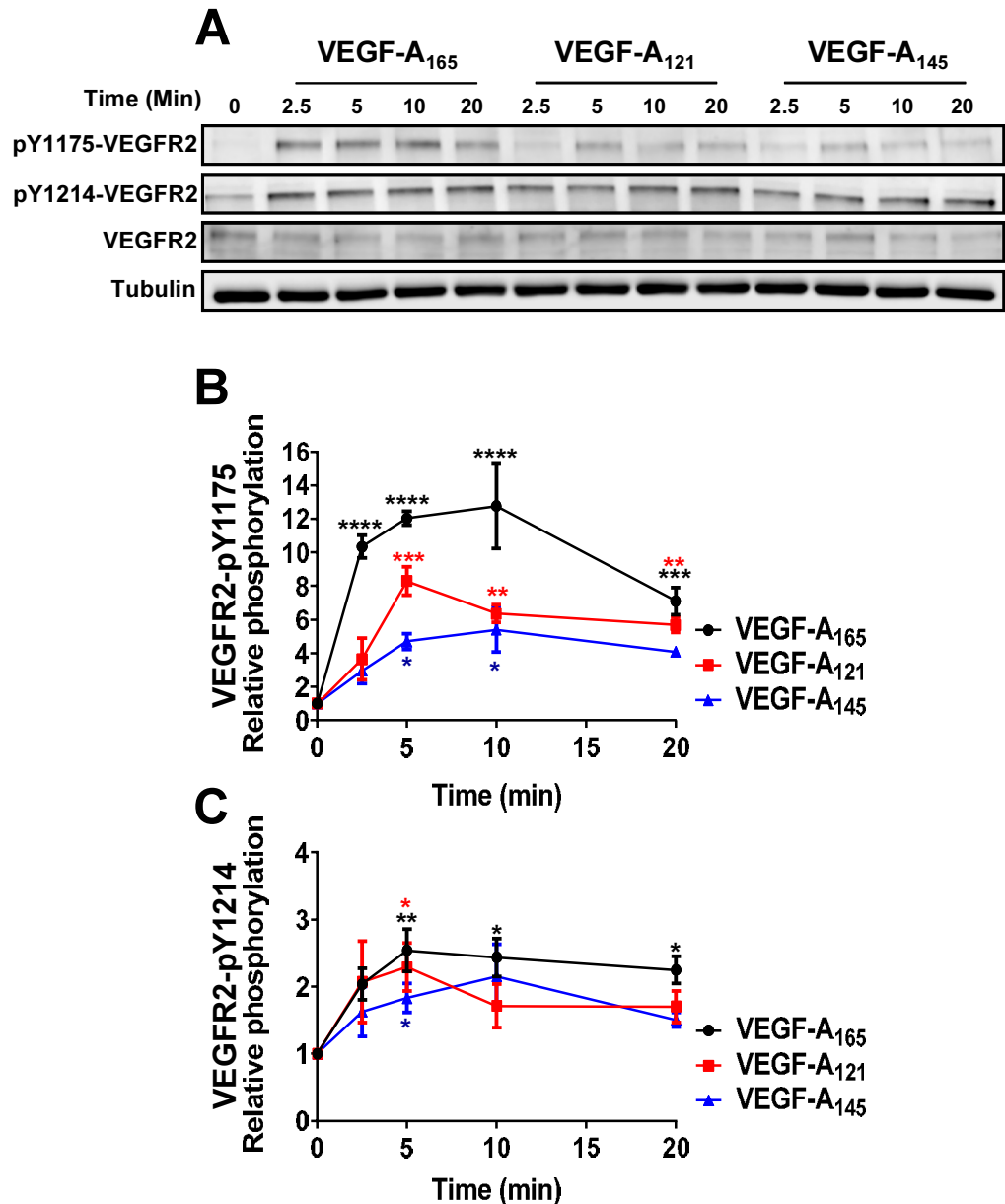
Figure 3.7. VEGF-A isoforms promote differential VEGFR2 tyrosine phosphorylation. (A) Human endothelial cells were stimulated with either VEGF-A$_{165}$, VEGF-A$_{121}$ or VEGF-A$_{145}$ (1.25 nM) for 2.5, 5, 10 or 20 min before lysis and processing for immunoblot analysis using site-specific phospho- antibodies against p-VEGFR2. (B and C) Quantification of VEGF-A isoform-specific phosphorylation of specific VEGFR2 tyrosine residues upon treatment with VEGF-A$_{165}$, VEGF-A$_{121}$ or VEGF-A$_{145}$. (B) VEGFR2-pY1175 and (C) VEGFR2-pY1214. Error bars indicate ±SEM (n≥4). $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$ (****).

the MEK1-ERK1/2 pathway is associated with VEGFR2 residence in endosomes (Lanahan et al., 2010; Yilmaz et al., 2003). Our findings suggested that differential VEGF-A isoform-specific 'programming' of VEGFR2 functionality could be associated with its residence time within endosomes. To test this idea, VEGF-A isoform-specific stimulation of endothelial cells was followed by immunoblot analysis of phospho-ERK1/2 and VEGFR2-pY1175 levels (Figure 3.8A). Quantification of phospho-ERK1/2 levels revealed that whereas VEGF-A$_{165}$ stimulation produced the highest increase in ERK1/2 activation (~2-fold higher); there was no difference between the reduced levels of activation in VEGF-A$_{121}$ or VEGF-A$_{145}$-stimulated cells (Figure 3.8B). Although, there were consistent increases in VEGFR2-pY1175 levels (Figure 3.8C), thus this did not appear to be directly coupled to phospho-ERK1/2 levels. To investigate if this phenomenon was functionally linked, endothelial cells were stimulated with each VEGF-A ligand prior to assessment of endothelial cell proliferation (Figure 3.8D). Quantification revealed that the VEGF-A$_{165}$ stimulation promoted the largest increase in cell proliferation which was significantly higher (~25%) than either VEGF-A$_{121}$ or VEGF-A$_{145}$ (Figure 3.8D). Conversely, endothelial cells stimulated with VEGF-A$_{121}$ or VEGF-A$_{145}$ did not show any significant differences (Figure 3.8D). These findings indicate that VEGF-A isoform-specific programming of VEGFR2 function is dependent on both tyrosine phosphorylation and trafficking to endosomes; thus reduced capacity in either of these events substantially decreases cellular responses such as proliferation.

3.2.4. VEGF-A isoforms differentially regulate tubulogenesis and arterial regeneration

Endothelial cell tube formation is one of several key endothelial cell responses required for VEGF-A-stimulated angiogenesis. To assess the ability of each VEGF-A to promote the formation of hollow endothelial cell tubes from isolated cells, we used an endothelial cell/fibroblast co-culture assay (Figure 3.9A). Quantification of total endothelial tubule length (Figure 3.9B) or branch point number (Figure 3.9C) revealed that VEGF-A$_{165}$ treatment promoted significantly longer and more extensively branched tubule networks, as opposed to either VEGF-A$_{121}$ or VEGF-A$_{145}$ treatment (Figure 3.9, B and C). However, there was little difference in the size and complexity of the tubule networks formed in comparison of VEGF-A$_{121}$ vs. VEGF-A$_{145}$ treatment (Figure 3.9, B and C). Interestingly, VEGF-A$_{121}$-stimulated tubulogenesis peaked after 5 days, with a decline in both total tubule length and branch point number upon continued growth factor stimulation, whereas the effects of VEGF-A$_{165}$ and VEGF-A$_{145}$ were more sustained (Figure 3.9, B and C).
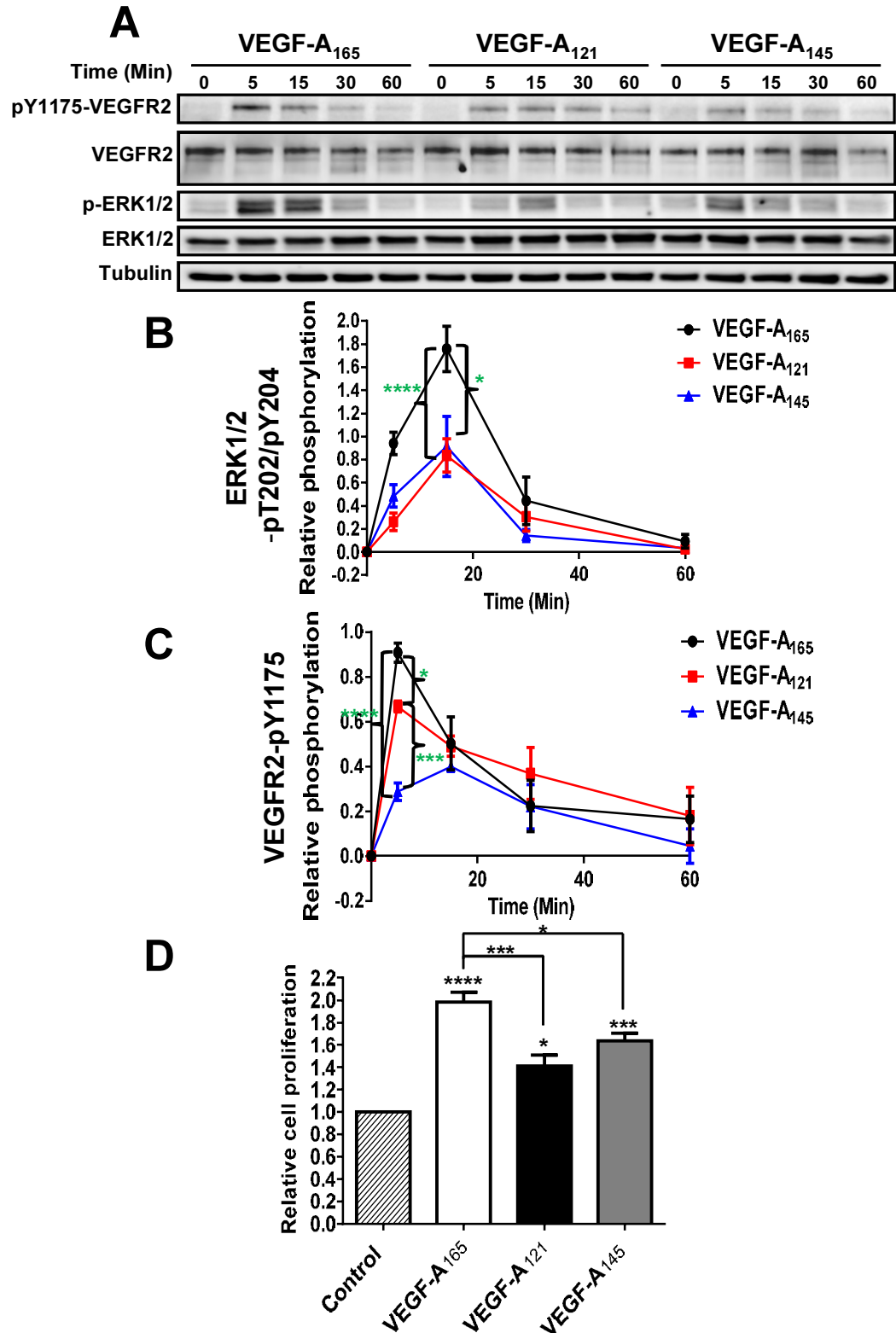
Figure 3.8. VEGF-A isoform-specific ERK1/2 activation and endothelial cell proliferation. (A) Human endothelial cells stimulated with either VEGF-A$_{165}$, VEGF-A$_{121}$ or VEGF-A$_{145}$ (1.25 nM) for 5, 15, 30 or 60 min before lysis and processing for immunoblot analysis using phospho-specific antibodies against VEGFR2-pY1175 and p-ERK1/2. (B and C) Quantification of (B) ERK1/2-pT202/pY204 and (C) VEGFR2-pY1175 activation upon VEGF treatment. (D) Endothelial cell proliferation upon VEGF-A$_{165}$, VEGF-A$_{121}$ or VEGF-A$_{145}$ stimulation (1.25 nM; 24 h). Error bars indicate ±SEM (n≥4). $p<0.05$ (*), $p<0.001$ (***), $p<0.0001$ (****).
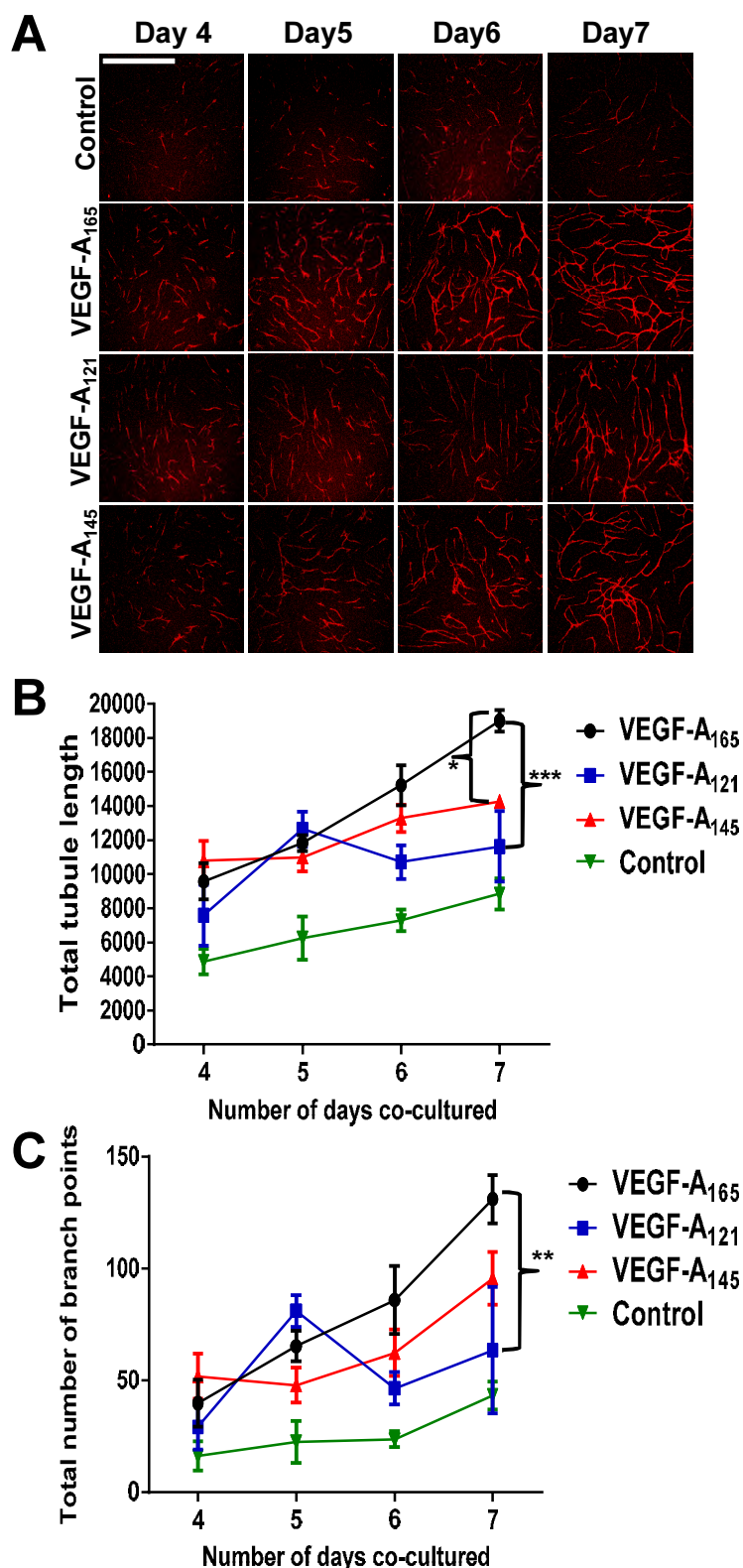
Figure 3.9. VEGF-A isoforms differentially promote endothelial tubulogenesis. (A) Human endothelial cells were co-cultured on a bed of primary human fibroblasts for 4, 5, 6 or 7 days and stimulated with either VEGF-A$_{165}$, VEGF-A$_{121}$ or VEGF-A$_{145}$ (1.25 nM). Co-cultures were fixed and endothelial tubules visualised by staining using a mouse anti-PECAM1 antibody followed by fluorescent secondary antibody labelling. Scale bar, 1000 μm. (B and C) Quantification of endothelial cell tubulogenesis including total (B) tubule length (AU) and (C) number of branch points. Error bars indicate ±SEM (n=3). $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***).

To investigate the individual capacity of each VEGF-A isoform to promote blood vessel sprouting we used an *ex vivo* sprouting assay using slices of mouse aorta embedded within a collagen matrix (Figure 3.10A). Quantification revealed that all three VEGF-A isoforms have the potential to induce blood vessel sprouting (Figure 3.10B). Stimulation with VEGF-A$_{165}$ promoted the largest relative increase in sprout number (~4-fold) followed by VEGF-A$_{145}$ (~3-fold) and finally VEGF-A$_{121}$ (~2.5-fold; Figure 3.10B).

We then asked whether such phenomena could be recapitulated *in vivo* using a mouse hind limb ischaemia model. After the surgical induction of ischaemia within the hind limbs, mice were given either vehicle (PBS) (Figure 3.11A), VEGF-A$_{165}$ (Figure 3.11B) or VEGF-A$_{121}$ (Figure 3.11C) as described over a period of 21 days and arterial blood flow analysed using laser Doppler imaging (Figure 3.11, A to C). Quantification of relative hind limb reperfusion (see Materials and Methods) showed that due to their natural healing ability alone (treatment with PBS), mice promoted a maximal peak recovery in blood flow (~2-fold) after a 14 day period and this was still evident after 21 days post injury (Figure 3.11D). This was similar to that observed upon administration of VEGF-A$_{121}$ (Figure 3.11D). Conversely, VEGF-A$_{165}$ treatment promoted a significant ~4-fold peak increase in hind limb reperfusion after just 7 days compared to administration of vehicle or VEGF-A$_{121}$ (Figure 3.11D). Thus VEGF-A isoforms have differential capacities to promote blood vessel formation and regeneration in both *in vitro* and *in vivo* models.
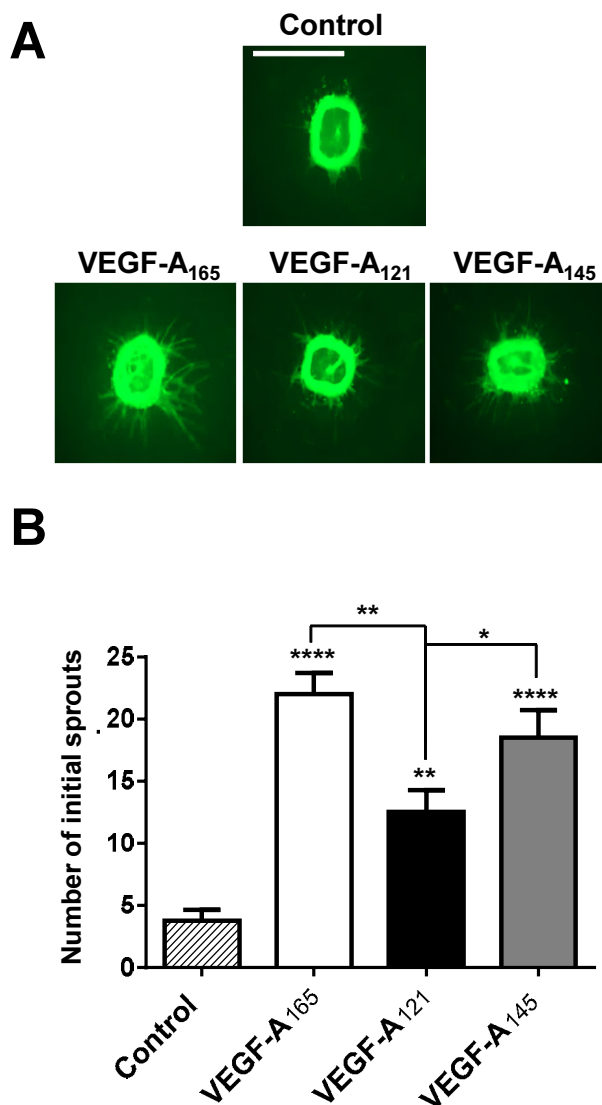
Figure 3.10. VEGF-A isoforms differentially promote angiogenesis in an *ex vivo* mouse model. (A) Mouse aortic ring slices were seeded into a collagen gel (see Materials and methods) and stimulated with either VEGF-A$_{165}$, VEGF-A$_{121}$ or VEGF-A$_{145}$ (1.25 nM) prior to fixation and labelling using FITC-labelled lectin conjugate. (B) Quantification of *ex vivo* aortic endothelial sprouting. Error bars indicate ±SEM (n=3). $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$ (****). The data in this figure was provided in part by N. A. Mughal, (Ponnambalam laboratory, University of Leeds, UK).
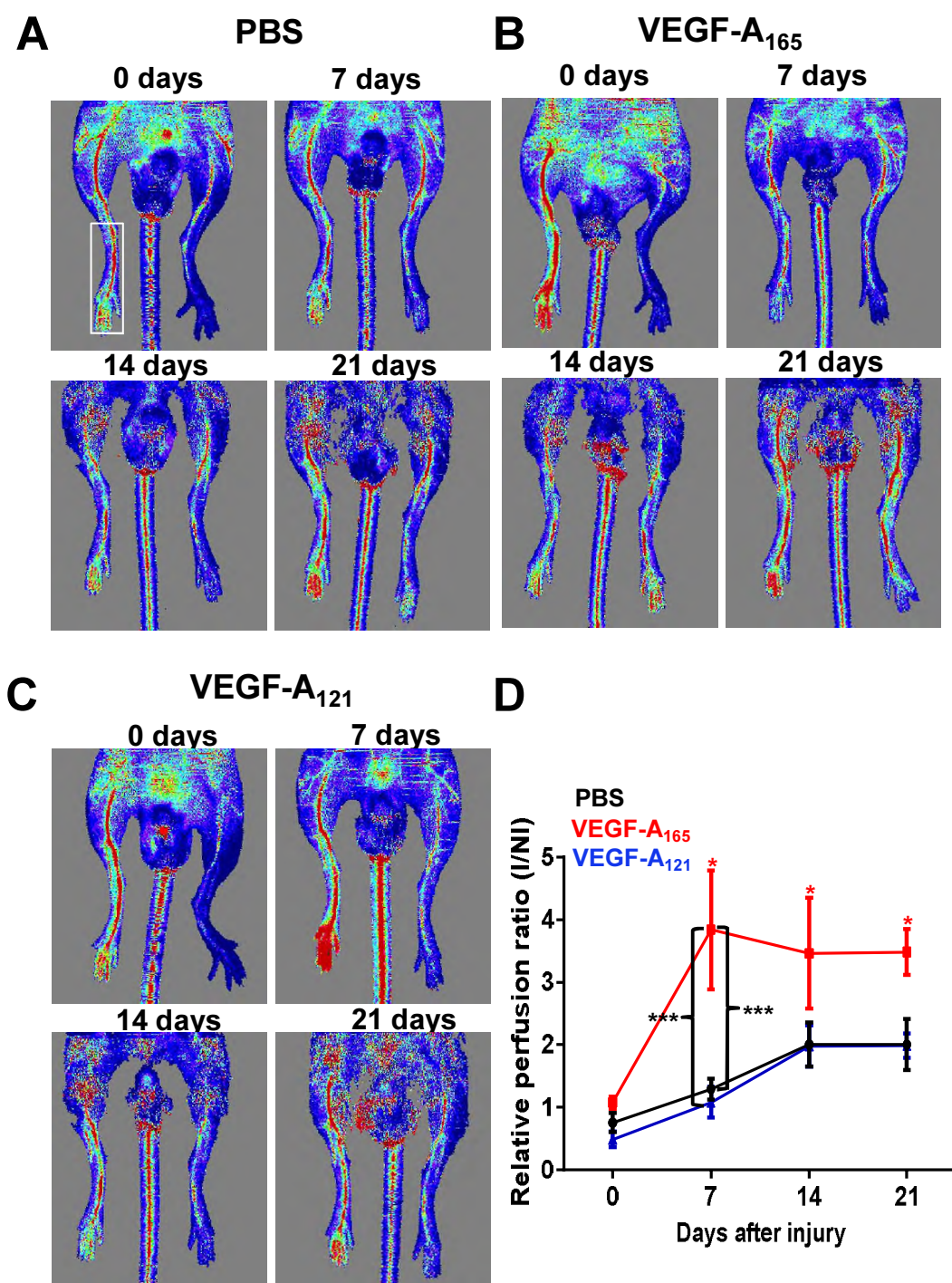
Figure 3.11. VEGF-A isoforms differentially promote *in vivo* angiogenesis in a mouse hind limb ischaemia model. (A to C) 8 week old C57Bl/6 male mice were anesthetised and subjected to ligation of the femoral artery (Right) and a sham operation on their contralateral limb (Left). Mice were then given either (A) PBS, (B) VEGF-A$_{165}$ (20 ng/day) or (C) VEGF-A$_{121}$ (20 ng/day) via implanted osmotic pump. Hind limb blood flow was analysed via laser Doppler imaging at 0, 7, 14 and 21 days post operation. Box depicts area quantified (D). Quantification of hind limb reperfusion after treatment with either PBS, VEGF-A$_{165}$ or VEGF-A$_{121}$. Error bars indicate ±SEM (n≥6). $p<0.001$ (***). The data in this figure was provided in part by Dr N. Yuldasheva (Faculty of Medicine & Health, University of Leeds, UK) and Miss I. Abdul Zani (Ponnambalam laboratory, University of Leeds, UK).

3.3. DISCUSSION

In this study, we show that different VEGF-A isoforms differential promote ligand-dependent VEGFR2 internalisation, which impacts on VEGF-A isoform-specific downstream signal transduction and this influences endothelial cell proliferation, tube formation and arterial regeneration (Figure 3.12). In this model, three VEGF-A isoforms with similar affinities for VEGFR2, differentially program ligand-dependent receptor internalisation, proteolysis and turnover linked to tyrosine phosphorylation and ubiquitination (Figure 3.12). Our findings reveal that VEGF-A isoform-specific VEGFR2 internalisation into EEA1-positive early endosomes, in conjunction with VEGFR2-pY1175 levels, programs isoform-specific ERK1/2 signal transduction (Figure 3.12). This integrated mechanism helps determine VEGF-A isoform-specific endothelial cell outcomes (Figure 3.12).

A key feature of VEGF-A binding to VEGFR2 is receptor internalisation, linked to its ubiquitination and terminal degradation. Ligand-dependent VEGFR2 internalisation was increased in endothelial cells stimulated with either VEGF-A$_{165}$ or VEGF-A$_{145}$, in comparison to those stimulated with VEGF-A$_{121}$. This correlated with increased co-distribution with EEA1-positive early endosomes and rate of VEGFR2 degradation in those cells. The mechanisms behind how different VEGF-A isoforms promote specific VEGFR2 internalisation are still unclear. However, the VEGF-A isoform-specific co-receptor neuropilin 1 (NRP1) appears to play a role in regulating VEGFR2 turnover and trafficking. Blocking VEGF-A/NRP1 interactions increased VEGF-A$_{165}$-dependent VEGFR2 degradation. Conversely, NRP1 perturbation did not affect VEGFR2 turnover upon stimulation with VEGF-A$_{121}$ or VEGF-A$_{145}$, where NRP1 is not recruited to such VEGFR2-VEGF-A complexes (Pan et al., 2007; Neufeld et al., 2002; Koch and Claesson-Welsh, 2012; Koch, 2012). Additionally, NRP1 is implicated in Rab11-dependent long loop VEGFR2 recycling upon VEGF-A$_{165}$ stimulation (Ballmer-Hofer et al., 2011). Therefore, attenuation of VEGFR2 recycling could account for increased VEGF-A$_{165}$-stimulated VEGFR2 turnover in NRP1 antagonist-treated endothelial cells.

One explanation for how different VEGF-A isoforms promote differential VEGFR2 internalisation and trafficking, is through the utilisation of different internalisation pathways at the plasma membrane. VEGFR2 internalisation is regulated by both classical clathrin-dependent endocytosis (CDE) and clathrin-independent endocytosis (CIE) (Ewan et al., 2006; Bruns et al., 2010; Labrecque et al., 2003; Lampugnani et al., 2006). Activated VEGFR2 endocytosis via different pathways
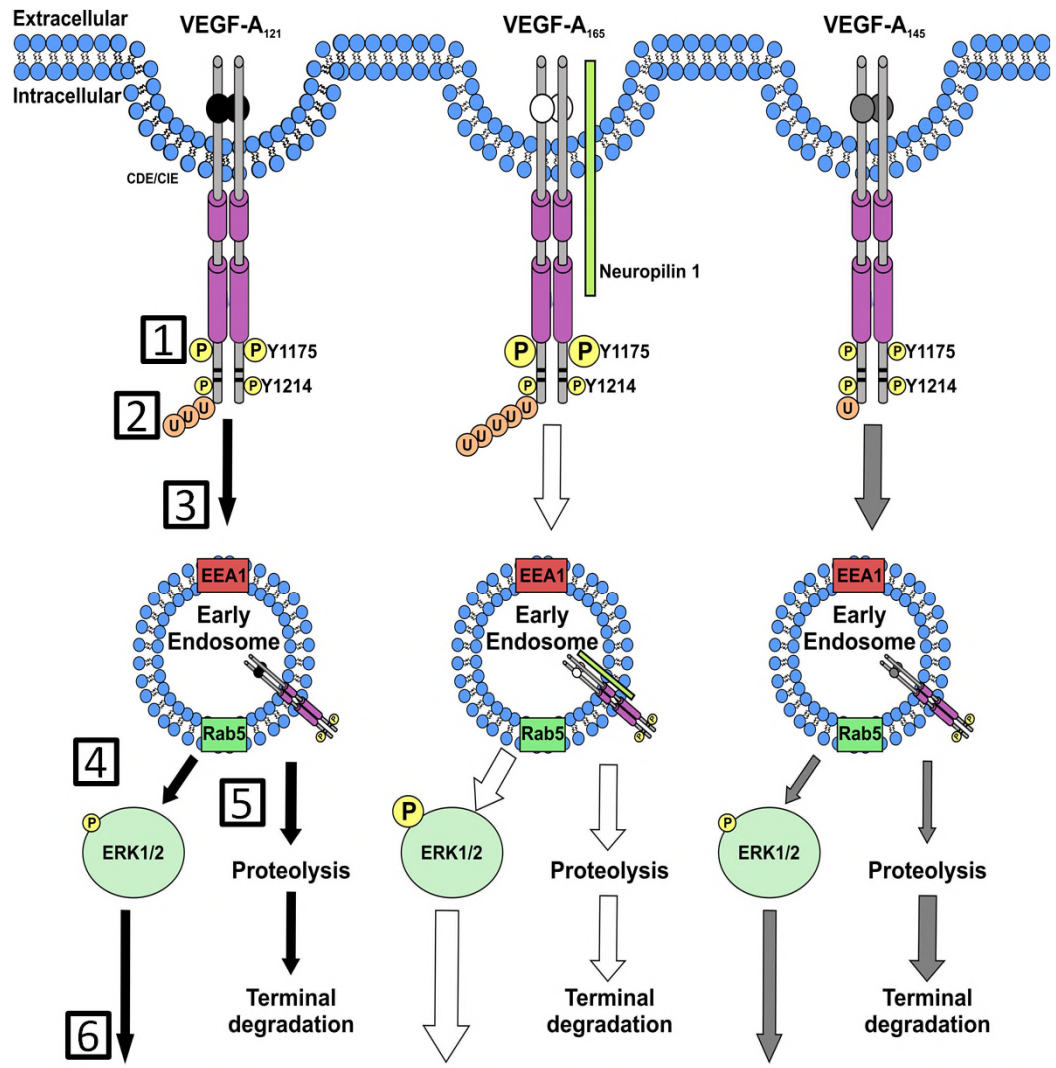
Regulation of endothelial cell proliferation and blood vessel formation

Figure 3.12. Mechanism for VEGF-A isoform-specific VEGFR2 signal transduction and membrane trafficking. (1) Ligand binding causes VEGFR2 dimerisation and either (Y1175) or (Y1214) transautophosphorylation of specific tyrosine residues, depending on the VEGF-A isoform used. (2) Differential receptor ubiquitination, and (3) VEGFR2 internalisation into EEA1-positive early endosomes. (4) Differential levels of VEGF-A isoform-stimulated VEGFR2 internalisation impacts on downstream ERK1/2 activation in combination with VEGFR2-pY1175 levels i.e. a high level of Y1175 activation and high level of VEGFR2 internalisation equals higher ERK1/2 signalling; a low level of Y1175 activation and high level of VEGFR2 internalisation (or vice versa) equals lower ERK1/2 signalling; as with VEGF-A$_{165}$ or VEGF-A$_{145}$ and VEGF-A$_{121}$ respectively. (5) VEGFR2 is trafficked into late endosomes where it is programmed for VEGF-A isoform-specific proteolysis prior to lysosomal degradation. (6) VEGF-A isoform-specific VEGFR2 activation and receptor trafficking programs endothelial cell proliferation and blood vessel formation. VEGF-A$_{121}$ pathway denoted by black arrow; VEGF-A$_{165}$ pathway denoted by white arrow; VEGF-A$_{145}$ pathway denoted by grey arrow. Size of arrow denotes magnitude of response.

could explain how different VEGF-A isoforms promote differential trafficking and turnover, thus modulating downstream pathways and cellular responses.

VEGFR2 ubiquitination has been strongly linked to terminal degradation (Bruns et al., 2010). Surprisingly, our work now argues that ligand-stimulated VEGFR2 ubiquitination is not directly coupled to endosome-lysosome trafficking for terminal degradation. Although VEGF-A$_{165}$ clearly triggers maximal VEGFR2 ubiquitination and terminal degradation, VEGF-A$_{145}$ causes equivalent VEGFR2 terminal degradation without corresponding ubiquitination (Figure 3.12). One possibility is that VEGFR2 ubiquitination reflects membrane protein recycling or its level of proteolysis rather than marking the receptor for terminal degradation.

VEGF-A-stimulated VEGFR2 dimerisation promotes transautophosphorylation of multiple tyrosine e.g. residues Y1175 and Y1214. Whereas VEGFR2-pY1175 levels were variable depending on the VEGF-A isoform involved; VEGFR2-pY1214 levels were relatively similar between all the VEGF-A isoforms (Figure 3.12). In addition, the VEGFR2-pY1214 epitope levels are already evident in quiescent VEGFR2 and increase slowly over time; these kinetics are substantially different to that of VEGFR2-pY1175. Transgenic mouse studies reveal that VEGFR2-Y1175F mutation causes embryonic lethality whereas VEGFR2-Y1214F mice are viable and fertile (Sakurai et al., 2005). This suggests that these two VEGFR2 phospho-epitopes have different functional roles in the endothelial cell response to VEGF-A.

Lanahan *et al.* have shown that upon VEGF-A$_{165}$ activation, internalised VEGFR2 is trafficked away from the plasma membrane into early endosomes, via a NRP1-dependent mechanism involving synectin and myosin VI (Lanahan et al., 2010). Trafficking into early endosomes prevents VEGFR2-pY1175 dephosphorylation via the protein phosphatase PTP1B, and this is required for signal transduction and ERK1/2 activation (Lanahan et al., 2014). Our results show that VEGF-A$_{165}$ stimulation promoted the highest activation of ERK1/2 activation which correlated with increased VEGFR2-pY1175 levels. Yet, stimulation with VEGF-A$_{121}$ and VEGF-A$_{145}$ resulted in comparable levels of ERK1/2 activation and cellular proliferation, despite significantly different levels of VEGFR2-Y1175 activation (Figure 3.12). Although VEGF-A$_{121}$ promotes increased VEGFR2 activation and phosphorylation, the rate of internalisation and co-distribution with EEA1-positive endosomes is diminished in comparison to VEGF-A$_{145}$ (Figure 3.12). Thus the differences in VEGF-A isoform specific VEGFR2 endocytosis provides an explanation for the

comparable levels in MEK1-ERK1/2 pathway activation and cell proliferation, in VEGF-A$_{121}$ and VEGF-A$_{145}$ stimulated endothelial cells.

Other signalling pathways such as the p38 MAPK pathway are implicated in VEGFR2 activation (Chen et al., 2010). Thus, It is feasible that differences in VEGF-A isoform-specific VEGFR2 trafficking could also differentially regulate other such signalling pathways. VEGF-A-stimulated VEGFR2 cleavage and proteolysis of the cytoplasmic domain by a 26S proteasome-dependent pathway has been shown to perturb signal transduction through the ERK1/2 and Akt pathways (Bruns et al., 2010). Therefore, differential levels of VEGFR2 proteolysis, could also contribute towards modulating VEGF-A isoform-specific signal transduction.

VEGF-A isoforms thus have unique abilities to program VEGFR2 internalisation, ubiquitination, cytoplasmic domain proteolysis and terminal degradation in lysosomes (Figure 3.12). Differential ligand-dependent VEGFR2 trafficking regulates VEGF-A isoform-specific ERK1/2 signal transduction, subsequent to generation of the key VEGFR2-pY1175 phospho-epitope (Figure 3.12). This complex and integrated mechanism has a direct effect on endothelial cell proliferation, blood vessel formation and arterial regeneration. This study explains how multiple VEGF-A isoforms bind to the same pro-angiogenic receptor, yet orchestrate diverse cellular outcomes. A future challenge will be to decipher the mechanism(s) regulating VEGF-A isoform-specific VEGFR2 trafficking and turnover, to specifically manipulate endothelial cell signal transduction to regulate angiogenesis in healthy and diseased states.

CHAPTER 4

VEGF-A isoforms differentially regulate ATF-2-dependent VCAM-1 gene expression and endothelial-leukocyte interactions

4.1. INTRODUCTION

The vascular endothelial growth factor (VEGF) family bind Class V RTKs (VEGFR1-3) and co-receptors such as Neuropilins i.e. NRP1 and NRP2 (Koch et al., 2011). The *VEGFA* gene alone encodes 7 or more different isoforms that bind VEGFR1 (Flt-1), VEGFR2 (KDR) and Neuropilins (Harper and Bates, 2008). *VEGFA* gene dosage is critical as heterozygous *VEGFA* (+/-) knockout mice embryos die during embryogenesis (Keyt et al., 1996, Carmeliet et al., 1996); *VEGFR2* (-/-) knockout mice also result in embryonic lethality (Shalaby et al., 1995). The most studied VEGF-A ligand is a mature 165 residue processed polypeptide (i.e. VEGF-A$_{165}$) which promotes endothelial cell survival, proliferation, migration and angiogenesis. VEGF-A-regulated endothelial responses are especially associated with pathological conditions such as tumour progression (Chung and Ferrara, 2011, Meadows and Hurwitz, 2012).

The binding of VEGF-A to VEGFR2 triggers sustained signal transduction, increased trafficking and proteolysis (Bruns et al., 2009; Koch and Claesson-Welsh, 2012; Horowitz and Seerapu, 2012; Nakayama and Berger, 2013). A key aspect of VEGF-A-stimulated re-programming of endothelial cell function is elevated expression of 100-200 genes (Schweighofer et al., 2009; Rivera et al., 2011). VEGF-A-regulated target genes are implicated in a multitude of cellular functions ranging from cell adhesion, signal transduction and transcriptional control. A major question is the nature of the mechanism(s) that control VEGF-A-stimulated gene expression. Although VEGF-A-stimulated signal transduction via MEK1-ERK1/2, p38 MAPK and JNK pathways could potentially provide multiple means of elevating gene expression, the exact mechanism by which such signal transduction is integrated with nuclear transcriptional control is unclear. One well-known target is the membrane receptor VCAM-1, whose expression on endothelial cells promotes

binding to leukocyte integrin $\alpha4\beta1$ (VLA-4), thus promoting endothelial-leukocyte interactions (Jain et al., 1996; Melder et al., 1996). The mechanism underlying this VEGF-A-stimulated gene expression is unclear, with studies suggesting roles for NFκB (Kim et al., 2001) and forkhead (Abid et al., 2008) transcription factors in regulating *VCAM-1* gene transcription.

Discovery of increasing numbers of VEGF splice isoforms raises questions as to their role(s) in vascular and animal function. The human VEGF-A gene alone expresses 8 isoforms ranging from 121 to 206 residues in length. One idea is that the *VEGFA* gene encodes both pro- and anti-angiogenic isoforms that are expressed in different tissues to modulate the vascular response during health and disease (Harper and Bates, 2008). To evaluate the link between VEGF-A-stimulated gene expression and isoform functionality, we investigated the mechanism underlying VEGF-A-stimulated VCAM-1 expression. By comparing VEGF-A$_{165}$ and VEGF-A$_{121}$ isoform-mediated effects, we showed that these growth factor isoforms differentially activated signal transduction pathways that control nuclear *VCAM-1* gene transcription.

4.2. RESULTS

4.2.1. VEGF-A isoforms differentially regulate VCAM-1 and VEGFR2 turnover and synthesis

The VEGF-A$_{165}$ isoform promotes increased endothelial VCAM-1 expression (Melder et al., 1996; Kim et al., 2001; Schweighofer et al., 2009). Although the pathway of VEGF-A-VEGFR2 signal transduction is well-studied, it is unclear how such events are communicated to the nucleus to control nuclear gene transcription, such as increased VCAM-1 expression. To investigate this phenomenon, we first asked whether two different VEGF-A isoforms that differ in their carboxy-proximal regions caused similar or different effects on VCAM-1 expression levels in primary human endothelial cells (Figure 4.1A). We first compared VCAM-1 and VEGFR2 levels in endothelial cells stimulated with a maximal stimulatory dose (0.25 nM) of either VEGF-A$_{165}$ or VEGF-A$_{121}$ for 2, 4, 6, 8 and 24 h (Figure 4.1A). We used endothelial tubulin levels as a control and such comparisons were used to evaluate varying protein expression in response to VEGF-A stimulation (Figure 4.1A). Quantification of endothelial VCAM-1 levels revealed a peak of VCAM-1 expression after VEGF-A$_{165}$ stimulation for 8 h corresponding to ~6.5-fold increase compared to the 0 h time point (Figure 4.1B). This peak in VCAM-1 levels was transient and

dropped to ~2.5-fold rise after VEGF-A$_{165}$ stimulation for 24 h (Figure 4.1B). In comparison, VEGF-A$_{121}$ stimulation failed to significantly elevate VCAM-1 levels (Figure 4.1B).

VEGFR2 activation leads to transautophosphorylation of multiple tyrosine residues: Y1175 is a key site that undergoes such phosphorylation (Holmqvist et al., 2004; Takahashi et al., 2001; Koch et al., 2011). Monitoring VEGFR2-pY1175 appearance showed that VEGF-A$_{165}$ stimulates rapid and transient phosphorylation of this site whereas VEGF-A$_{121}$ treatment did not produce significant Y1175 phosphorylation (Figure 4.1A). VEGF-A-stimulation promotes VEGFR2 ubiquitination, endocytosis and proteolysis (Ewan et al., 2006). We then asked whether VEGFR2 turnover and synthesis was different upon treatment with either VEGF-A$_{165}$ or VEGF-A$_{121}$ isoform (Figure 4.1C). VEGF-A$_{165}$ stimulation promoted VEGFR2 degradation over a short time period (2-4 h), with VEGFR2 levels reduced by ~60% after 2 h (Figure 4.1C). However, VEGFR2 levels returned to baseline after VEGF-A$_{165}$ stimulation for 8 h (Figure 4.1C). VEGFR2 levels continued on an upward trajectory with ~50% increase after VEGF-A$_{165}$ stimulation for 24 h (Figure 4.1C). In contrast, VEGF-A$_{121}$ stimulation appeared to have little effect on VEGFR2 protein levels (Figure 4.1C). These findings show that two different VEGF-A isoforms have different capabilities in stimulating the turnover and synthesis of not only VEGFR2, but another membrane receptor, VCAM-1.

4.2.2. VEGF-A isoforms differentially regulate multiple signal transduction pathways

VEGF-A stimulates multiple MAPK signal transduction pathways in endothelial cells (Horowitz and Seerapu, 2012; Koch and Claesson-Welsh, 2012), that regulate multiple cellular outcomes (Karihaloo et al., 2005; Nakatsu et al., 2003; Zhang et al., 2008; Xu et al., 2011). In this context, we asked whether the increase in endothelial VCAM-1 levels (Figure 4.1B) was linked to altered signal transduction pathways activated by the two VEGF-A isoforms using ligand titration followed by signal transduction pathway analysis (Figure 4.2, A and B). Activation of VEGFR2 and downstream signalling events were first assessed by monitoring transautophosphorylation at cytoplasmic residue Y1175 (Figure 4.2, A and B). Phosphorylation of Y1175 could be detected within 5 min of stimulation with either VEGF-A$_{165}$ or VEGF-A$_{121}$ but there were concentration-dependent effects (Figure 4.3A). Quantification showed that VEGF-A$_{121}$-stimulated VEGFR2-Y1175 phosphorylation at 0.025 and 0.25 nM was significantly reduced (Figure 4.3B) in
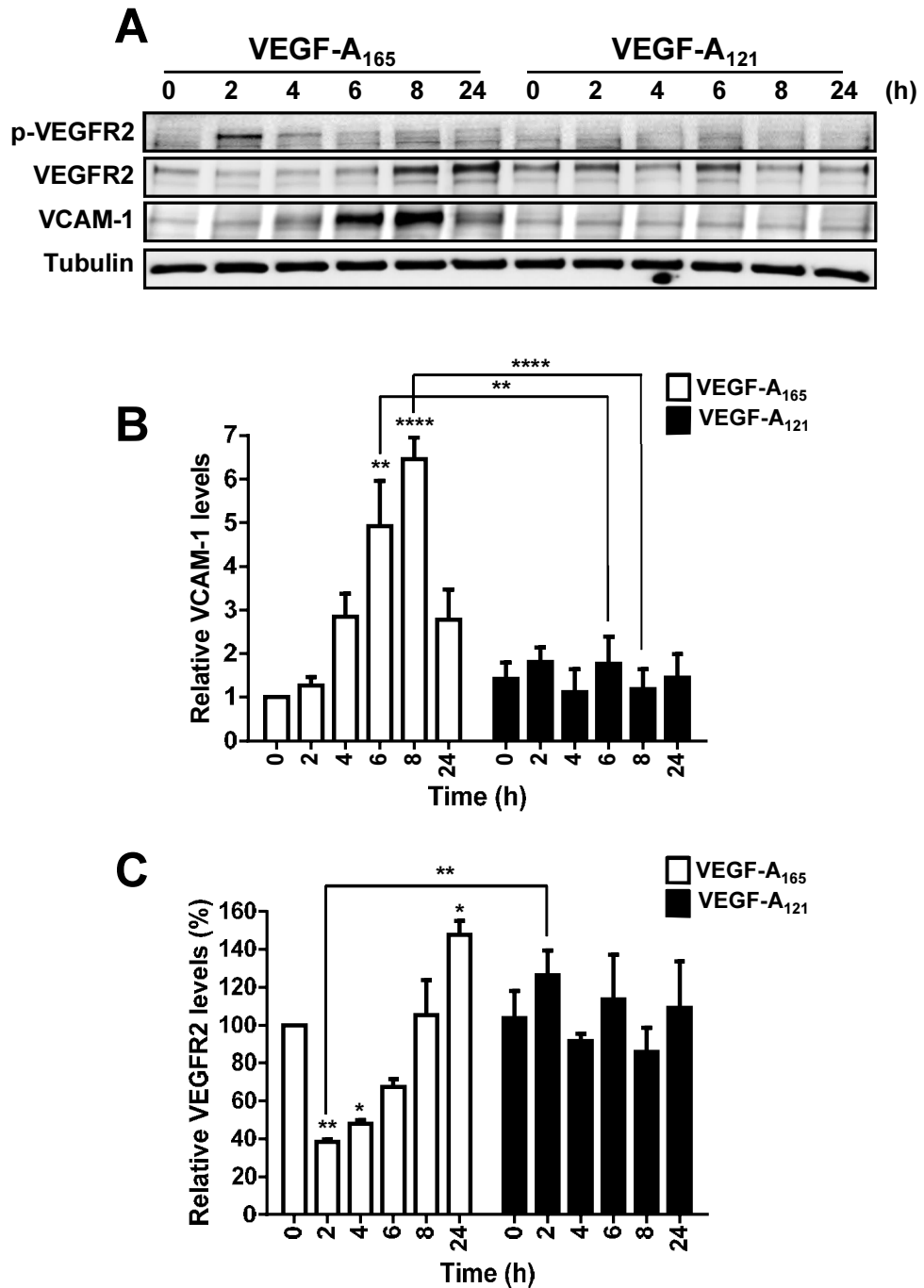
Figure 4.1. VEGF-A isoform-specific regulation of VCAM-1 gene expression. (A) Endothelial cells subjected to 0.25 nM VEGF-A$_{165}$ or VEGF-A$_{121}$ for the specified times indicated (h), lysed and probed by immunoblotting to assess phospho-VEGFR2 (VEGFR2-pY1175), VEGFR2, VCAM-1 or tubulin protein levels. (B and C) Quantification of (B) VCAM-1 or (C) VEGFR2 protein levels from immunoblotting data of VEGF-A$_{165}$ and VEGF-A$_{121}$ stimulated endothelial cells. Error bars indicate ±SEM (n=3). $p<0.05$ (*), $p<0.005$ (***), $p<0.0001$ (****).

comparison to VEGF-A$_{165}$ (Figure 4.3A). However, under saturating conditions of VEGF-A (1.25 nM), the peak level of VEGFR2 activation in response to VEGF-A$_{165}$ (Figure 4.3A) was similar to that induced by VEGF-A$_{121}$ (Figure 4.3B). VEGF-A activates ERK1/2, p38 MAPK and Akt pathways in endothelial cells (Koch et al., 2011; Koch and Claesson-Welsh, 2012).   Both VEGF-A$_{165}$ and VEGF-A$_{121}$ stimulation promoted a rapid and transient peak in ERK1/2 activation within 15 min, with differing magnitudes (Figure 4.2). Quantification showed that VEGF-A$_{121}$ stimulation resulted in a generally lower level of peak activation (Figure 4.3D) compared to VEGF-A$_{165}$ (Figure 4.3C). Interestingly, saturating conditions of VEGF-A, which resulted in similar levels of VEGFR2 peak activation (Figure 4.3, A and B), exhibited an ~2-fold difference in ERK1/2 peak activation between the two isoforms (Figure 4.3, C and D). VEGF-A$_{165}$ and VEGF-A$_{121}$ also triggered sustained and pronounced p38 MAPK activation (Figure 4.2, A and B). Quantification showed that VEGF-A$_{121}$-stimulated p38 MAPK activation was more pronounced (Figure 4.3F) than for VEGF-A$_{165}$ (Figure 4.3E) under saturating ligand conditions. VEGF-A$_{165}$ and VEGF-A$_{121}$ also caused differential Akt activation (Figure 4.2, A and B). Quantification showed that both VEGF-A$_{165}$ (Figure 4.3G) and VEGF-A$_{121}$ (Figure 4.3H) promoted a rapid peak in Akt activation within 15 min. However VEGF-A$_{165}$ (Figure 4.3G), had a greater efficacy compared to VEGF-A$_{121}$ (Figure 4.3H) as a much lower concentration of ligand was required to achieve a significant response. Interestingly, at saturating ligand conditions (1.25 nM), the peak in Akt activation was comparable between the two VEGF-A isoforms (Figure 4.3, G and H). This data suggest that these two different VEGF-A isoforms have differential capabilities in not only stimulating VEGFR2 activation but other downstream signal transduction pathways.

4.2.3. VEGF-A isoform-specific stimulation of activating transcription factor 2

Exactly how short-lived RTK signal transduction integrates with long-term cellular responses is not well understood (Lemmon and Schlessinger, 2010). The VEGFR-VEGF-A axis stimulates intracellular signalling over a short time frame (0-1 h) and regulates long-term endothelial responses such as leukocyte recruitment, cell migration (>24 h) and tubulogenesis (5-7 days) (Koch et al., 2011; Chung and Ferrara, 2011). To identify a nuclear switch that was responsive to VEGF-A isoform-specific MAPK signal transduction that could influence VCAM-1 expression, we focused on activating transcription factor 2 (ATF-2) which is known to undergo VEGF-A-stimulated phosphorylation in cardiac myocytes and endothelial cells (Seko et al., 1998; Salameh et al., 2010). ATF-2 belongs to the bZIP (basic region

subdomain/leucine zipper) family of DNA-binding transcription factors which undergoes activation upon cellular stress or plasma membrane receptor activation (Lau and Ronai, 2012). To determine whether VEGF-A isoforms differentially regulate ATF-2 activation, we monitored ATF-2-pT71 levels (Figure 4.4A). Endothelial cells contain basal phospho-ATF-2 which is further elevated upon VEGF-A stimulation (Figure 4.4A). Notably, maximal ATF-2-pT71 levels detected upon VEGF-A$_{165}$ addition were ~2-5-fold higher (Figure 4.4B) to comparable VEGF-A$_{121}$ (Figure 4.4C) ligand concentrations.

Multiple signal transduction pathways including JNK, p38 MAPK, ERK1/2 and ATM stimulate phosphorylation at different sites on ATF-2 (Lau and Ronai, 2012). To assess whether the increase in phospho-ATF-2 was dependent on MEK1-ERK1/2 or p38 MAPK pathways, we used cell-permeable small molecule inhibitors specific for either pathway (Figure 4.5A). VEGF-A-stimulated activation of ERK1/2 is significantly reduced by addition of U0126, a MEK1 inhibitor (Figure 4.5A). In contrast, VEGF-A-stimulated activation of p38 MAPK is significantly reduced by addition of SB203580, which inhibits both the α and β forms of p38 MAPK (Figure 4.5A). Quantification of VEGF-A-stimulated phospho-ATF-2 levels showed that that U0126 (MEK1 inhibitor) but not SB203580 (p38 MAPK inhibitor) completely blocked ligand-stimulated phosphorylation of ATF-2 (Figure 4.5B).

A key aspect of growth factor-stimulated MAPK activation is nuclear translocation of activated protein kinases and phosphorylation of key substrates which in turn regulate gene transcription (Plotnikov et al., 2011). We have previously shown that VEGF-A-stimulation causes translocation of activated ERK1/2 into the nucleus of endothelial cells (Jopling et al., 2009). To correlate ERK1/2 translocation with ATF-2 activation, we monitored the intracellular distribution of phospho-ERK1/2 and ATF-2-pT71 using microscopy (Figure 4.6A). Activated phospho-ERK1/2 was detected in both the cytoplasm and nucleus within 15 min; this also correlated with a peak of phospho-ATF-2 in the nucleus (Figure 4.6A). Microscopy analysis of phospho-ERK1/2 and phospho-ATF-2 in the nucleus revealed substantial co-distribution of both proteins at ~15 min post-VEGF-A$_{165}$ stimulation (Figure 4.6B). Quantification of nuclear phospho-ERK1/2 and phospho-ATF-2 showed a >10-fold rise in co-distribution after VEGF-A$_{165}$ stimulation (Figure 4.6C). Such findings show a close link between VEGF-A$_{165}$-stimulated MAPK signal transduction leading to ERK1/2 activation, nuclear translocation and downstream activation of ATF-2. The VEGF-A
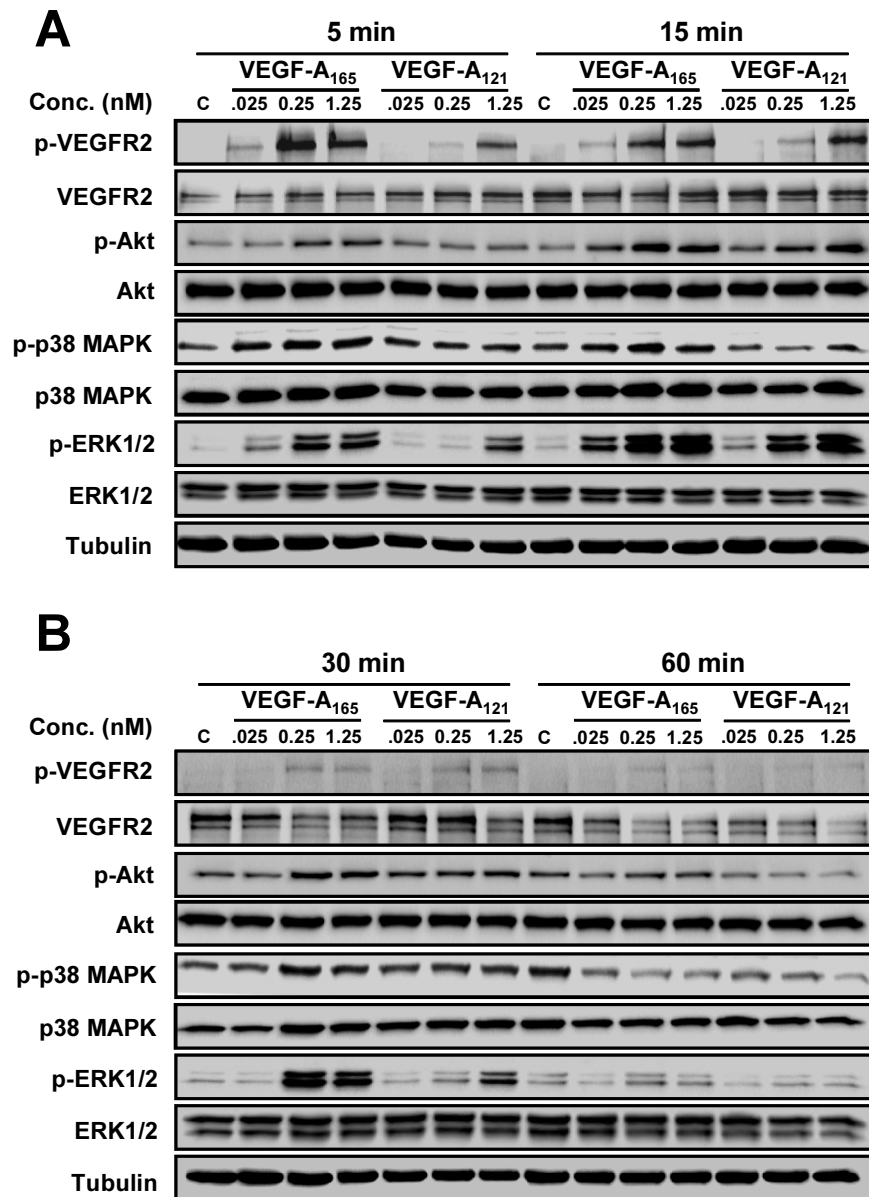
Figure 4.2. VEGF-A isoform-specific activation of signal transduction. (A and B) Endothelial cells subjected to different VEGF-A$_{165}$ or VEGF-A$_{121}$ concentrations (0, 0.025, 0.25 or 1.25 nM) for (A) 5 and 15 or (B) 30 and 60 min were lysed and probed for phospho-VEGFR2, phospho-ERK1/2, phospho-p38 MAPK and phospho-Akt levels using immunoblotting.
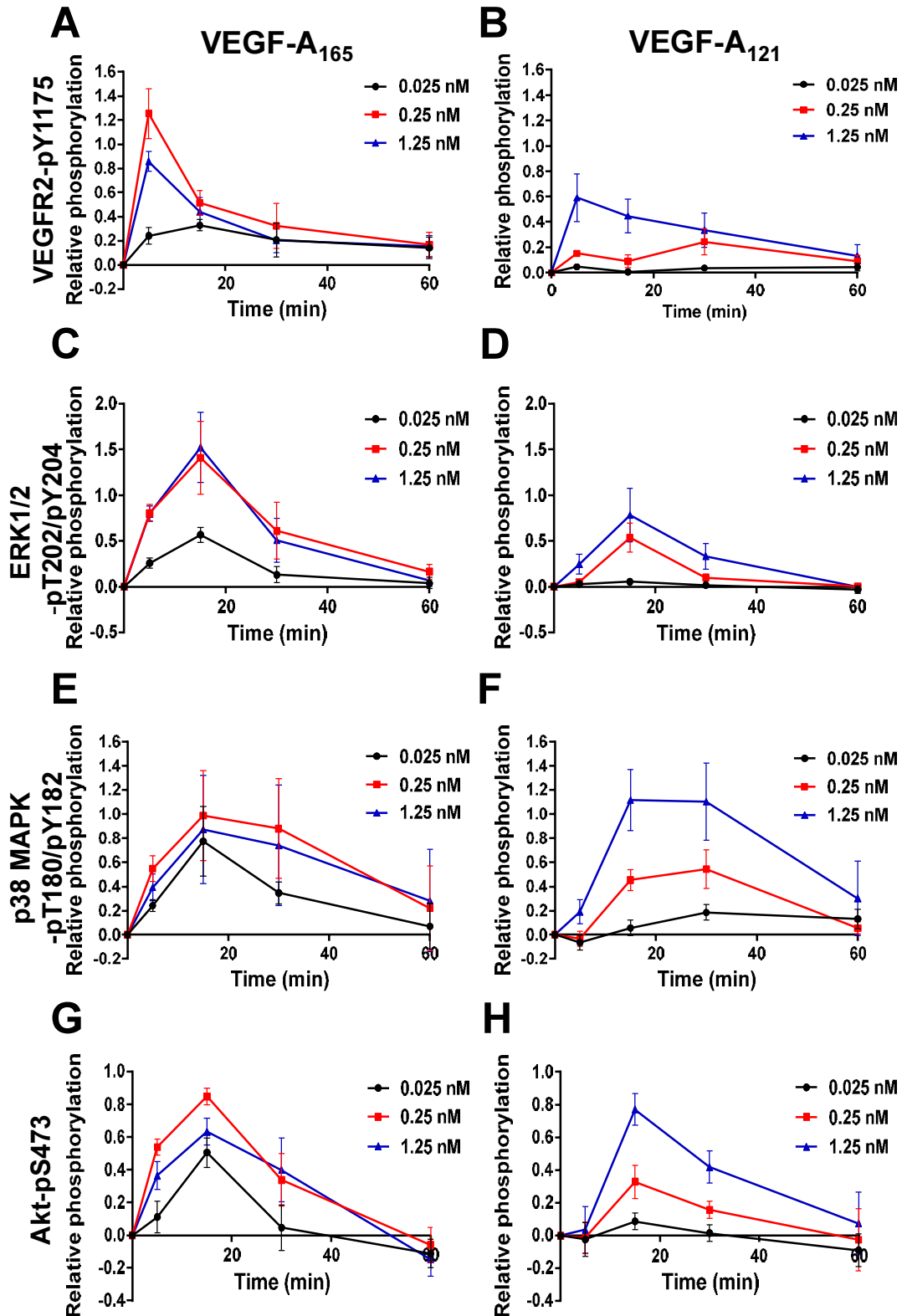
Figure 4.3. Quantification of VEGF-A isoform-specific signal transduction. Quantification of (A and B) VEGFR2-pY1175, (C and D) ERK1/2-pT202/pY204, (E and F) p38-pT180/pY182 or (G, H) Akt-pS473 levels upon (A, C and E) VEGF-A$_{165}$ or (B, D and F) VEGF-A$_{121}$ titration. Error bars indicate ±SEM (n≥4).

co-receptor Neuropilin 1 (NRP1) has been shown to attenuate VEGF-A-stimulated signal transduction (Pan et al., 2007; Herzog et al., 2011). Therefore, we evaluated whether NRP1 was essential for optimal VEGF-A-stimulated ATF-2 activation. Using immunoblotting, we monitored ATF-2-pT71 levels after 15 min stimulation with either VEGF-A$_{165}$ or VEGF-A$_{121}$ in NRP1-depleted and control endothelial cells (Figure 4.7A). Quantification revealed that both VEGF-A$_{165}$ and VEGF-A$_{121}$ stimulated ATF-2 activation was reduced in NRP1-depleted endothelial cells (Figure 4.7B). One consequence of a reduction in NRP1 levels was a concomitant reduction in VEGF-A-stimulated ERK1/2 activation (Figure 4.7C). These data suggest that NRP1 influences the ability of the VEGFR2-VEGF-A$_{165}$ complex to effectively activate downstream ERK1/2 and thus ATF-2.

4.2.4. VEGF-A and ATF-2 are required for VCAM-1 expression and endothelial-leukocyte interactions

VEGF-A-stimulated *VCAM-1* gene expression has implicated both the NFκB pathway and forkhead transcription factors (Kim et al., 2001; Kim et al., 2001; Abid et al., 2006; Dejana et al., 2007). Based on our data, we hypothesised that ATF-2 acts as a nuclear 'switch' for converting VEGF-A isoform-specific short-term cytosol-to-nucleus signal transduction (via the MEK1-ERK1/2 pathway) into *VCAM-1* gene transcription, thus modulating VEGF-A isoform-specific long term endothelial responses (including leukocyte recruitment). To evaluate ATF-2 requirement in VEGF-A-stimulated gene transcription, we first used specific siRNA duplexes to deplete endothelial ATF-2. As expected, ATF-2 mRNA levels were depleted only in endothelial cells treated with ATF-2-specific siRNA duplexes (ATF-2 knockdown) in comparison to scrambled siRNA duplex treatment (control), under both non-stimulated and VEGF-A-stimulated conditions (Figure 4.8A). We then analysed whether ATF-2 depletion affected VCAM-1 mRNA levels (Figure 4.8B). Upon VEGF-A$_{165}$ stimulation, we detected ~1.4-fold increase in VCAM-1 mRNA levels compared to controls (Figure 4.8B). VEGF-A$_{121}$-stimulated endothelial cells produced ~1.2-fold rise in VCAM-1 mRNA levels but this was substantially less than that observed for VEGF-A$_{165}$ (Figure 4.8B). ATF-2 knockdown substantially reduced the VEGF-A-stimulated rise in VCAM-1 mRNA levels by ~25% (Figure 4.8B). Thus there is a functional requirement for the presence of ATF-2 in VEGF-A-stimulated VCAM-1 expression in endothelial cells.
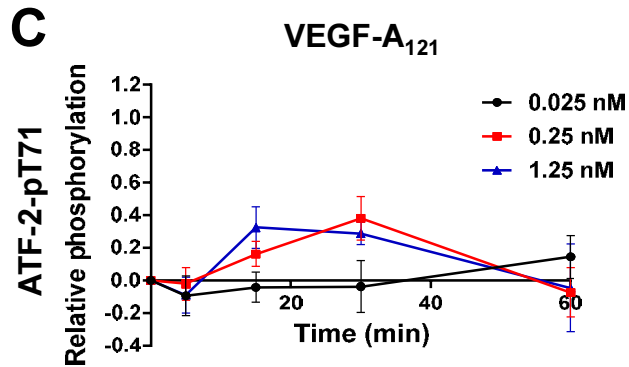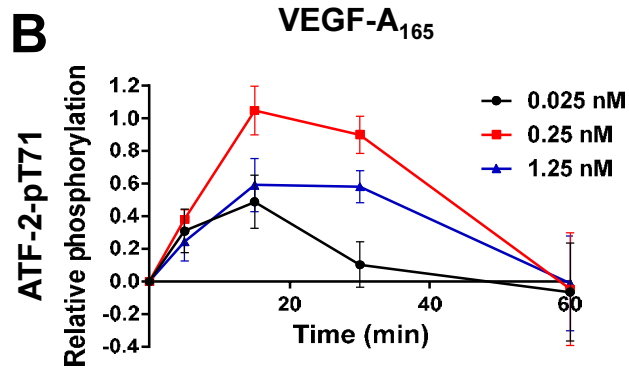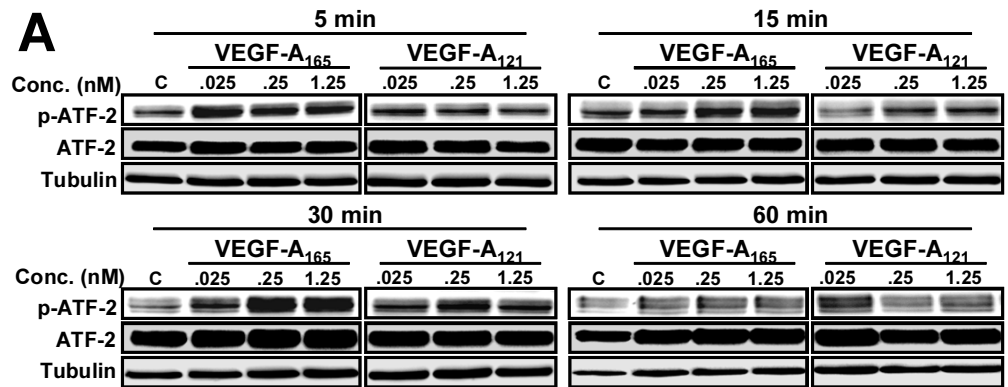
Figure 4.4. VEGF-A isoform-specific ATF-2 phosphorylation. (A) Immunoblotting of VEGF-A-stimulated endothelial cells for ATF-2-pT71, total ATF-2 and tubulin upon ligand titration. (B and C) Quantification of ATF-2-pT71 levels upon (B) VEGF-A$_{165}$ or (C) VEGF-A$_{121}$ titration. Error bars indicate ±SEM (n=4).
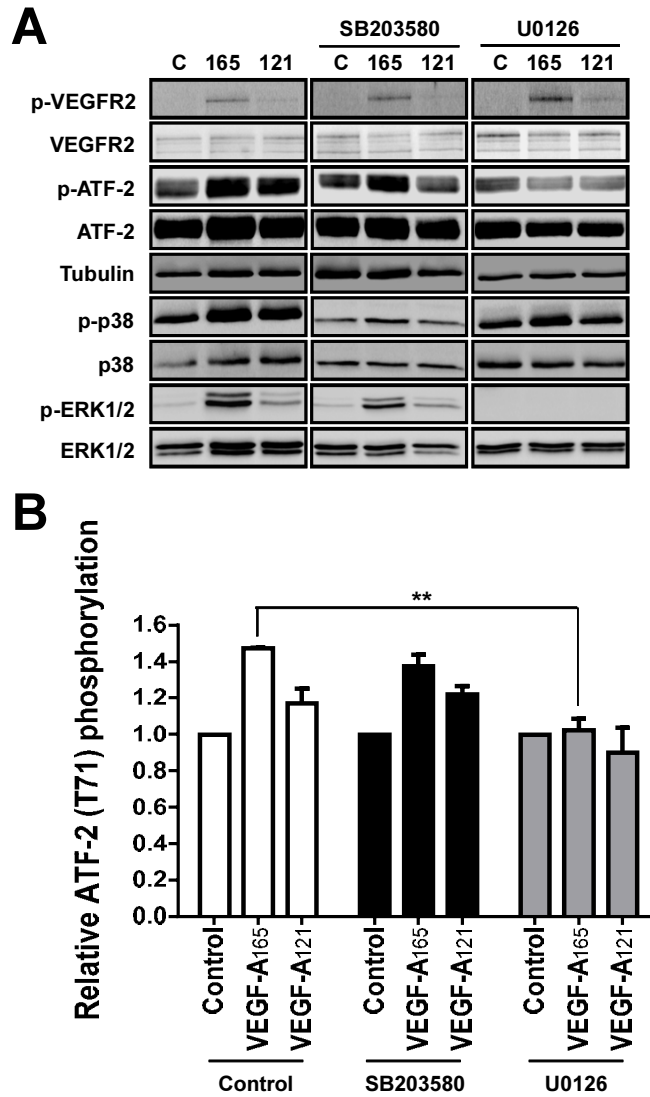
Figure 4.5. MEK1-ERK1/2 pathway is required for VEGF-A isoform-specific ATF-2 phosphorylation. (A) Immunoblotting of total and phosphorylated VEGFR2, ATF-2, ERK1/2 and p38 MAPK levels following pre-incubation with MEK1 inhibitor (U0126) or p38 MAPK inhibitor (SB203580) prior to VEGF-A isoform (1.25 nM) stimulation for 15 min. (B) Quantification of ATF-2-pT71 levels upon activation by VEGF-A$_{165}$ or VEGF-A$_{121}$ in the presence of MEK1 inhibitor (U0126) or p38 MAPK inhibitor (SB203580). Error bars indicate ±SEM (n=3). $p<0.01$ (**).
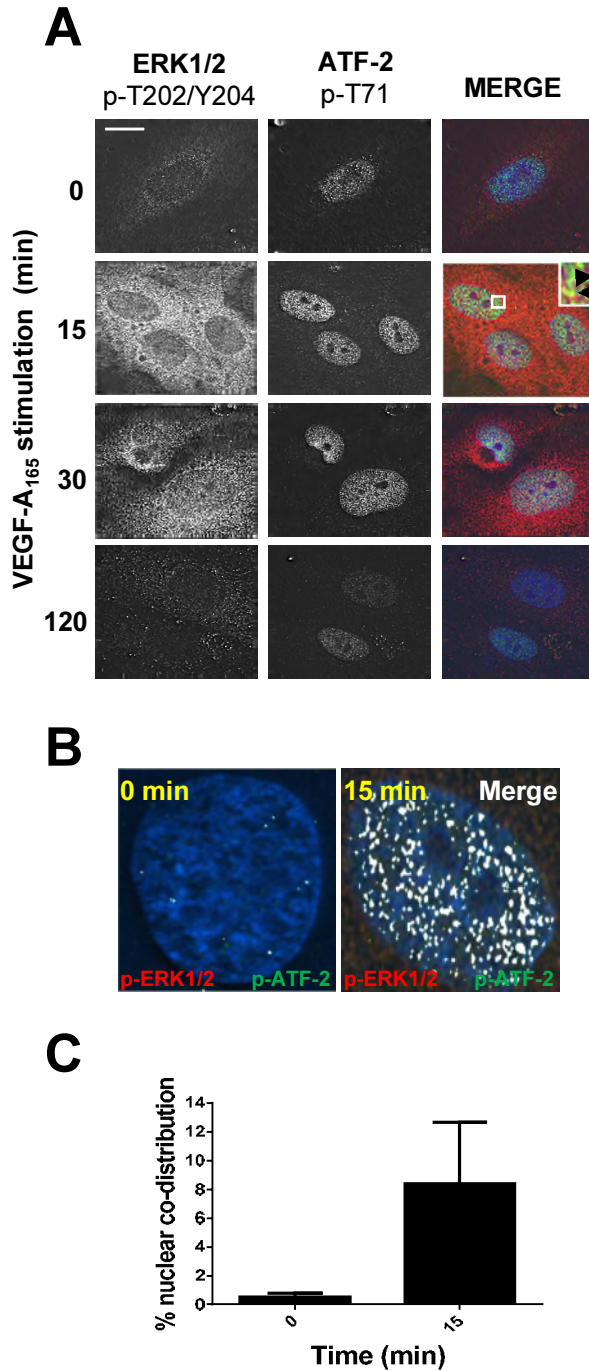
Figure 4.6. VEGF-A stimulation increases ERK1/2 and ATF-2 co-distribution. (A) Endothelial cells stimulated with VEGF-A$_{165}$ for 0, 15 or 30 min were processed for immunofluorescence and confocal laser microscopy using mouse anti-ERK1/2-pT202/pY204 or rabbit anti-ATF-2-pT71 followed by fluorescent labelled secondary antibodies. Overlay of phospho-ERK1/2 (red) and phospho-ATF-2 (green) staining patterns shown with inset box indicating regions of co-distribution within the nucleus (yellow). Bar, 10 μm. (B) Nuclear co-distribution of phospho-ERK1/2 and phospho-ATF-2 (white) 15 min post-stimulation. (C) Quantification of nuclear phospho-ERK1/2 and phospho-ATF-2 co-distribution at 0 and 15 min after VEGF-A$_{165}$ treatment. The data presented was provided by Dr A. Latham (Ponnambalam laboratory, University of Leeds, UK).
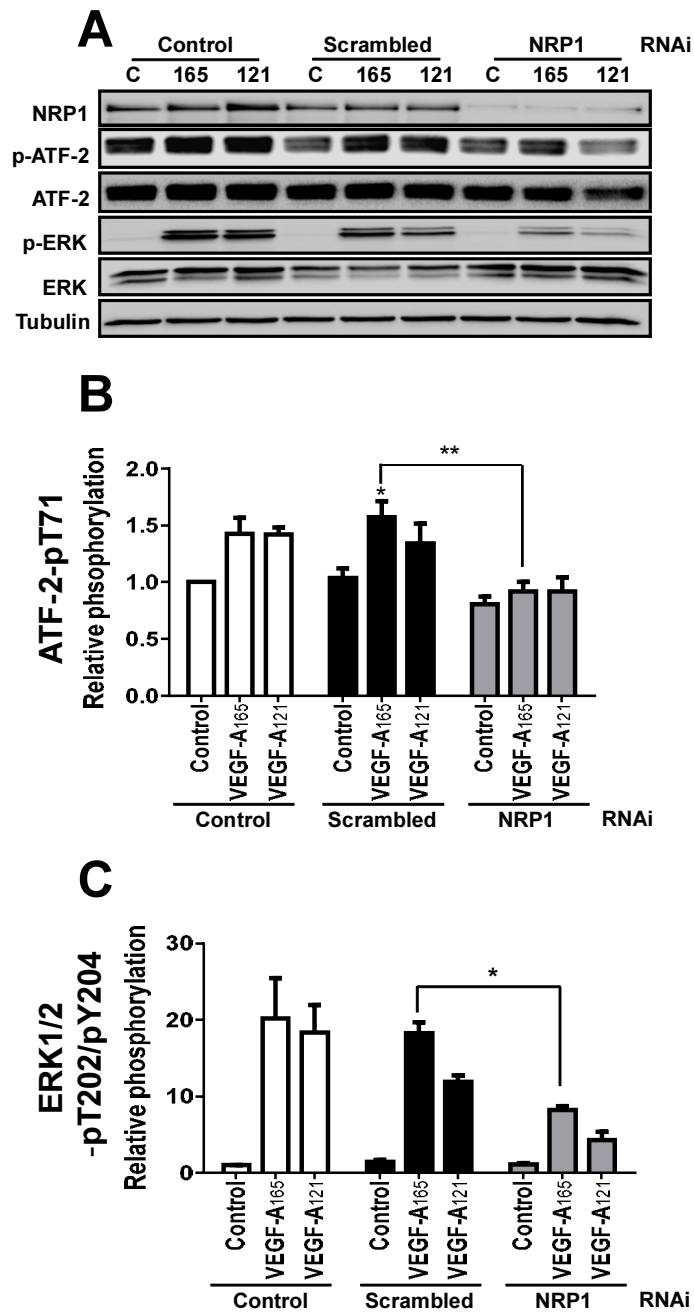
Figure 4.7. NRP1 is required for ERK1/2-dependent ATF-2 phosphorylation. (A) NRP1 depletion in endothelial cells followed by 1.25 nM VEGF-A$_{165}$ (165) or VEGF-A$_{121}$ (121) stimulation as indicated. (B and C) Effects of NRP1 depletion on 1.25 nM VEGF-A$_{165}$ or VEGF-A$_{121}$-stimulated intracellular signalling monitored by immunoblotting for (B) ATF-2-pT71 or (C) ERK1/2-pT202/pY204. Error bars indicate ±SEM (n=3). $p<0.05$ (*), $p<0.01$ (**), $p<0.005$ (***).

One question is the link between ATF-2 in endothelial signal transduction and protein expression. To address this we used immunoblotting to monitor protein levels and phosphorylation events 8 h after VEGF-A stimulation in control or ATF-2-depleted endothelial cells (Figure 4.8C). ATF-2 knockdown caused ~75% reduction in ATF-2 protein levels (Figure 4.8D). Activated VEGFR2-pY1175 levels were significantly elevated at this time point but were mirrored by a large decrease in VEGFR2 levels (Figure 4.8C). Differential phosphorylation in ERK1/2 and PLCγ1 was also evident (Figure 4.8C). These findings showed that ATF-2 knockdown did not significantly affect VEGFR2 turnover and downstream signal transduction in response to VEGF-A stimulation. We probed for VCAM-1 in control or ATF-2 depleted cells 8 h after VEGF-A isoform stimulation (Figure 4.8C). Quantification of VCAM-1 levels highlighted a relatively large (>3-fold) rise in VCAM-1 levels observed upon VEGF-A$_{165}$ stimulation compared to cells incubated in complete medium (Figure 4.8E). Depletion of ATF-2 reduced VCAM-1 levels ~2-fold in VEGF-A$_{165}$-stimulated cells (Figure 4.8E).

Endothelial VCAM-1 binds leukocyte α4β1 integrin (VLA-4) and promotes leukocyte recruitment onto the endothelium prior to transendothelial migration (Sixt et al., 2006; Reglero-Real et al., 2012; Nourshargh et al., 2010). A major question is whether VEGF-A-stimulated and ATF-2-dependent *VCAM-1* gene expression can influence leukocyte binding to endothelial cells. To test this idea, we used a binding assay that monitored the binding of fluorescent labelled human leukocyte HL-60 cells to an endothelial cell monolayer (Figure 4.8F). VEGF-A$_{165}$ stimulated ~75% increase in leukocyte binding to the endothelial monolayer (Figure 4.8F). However, VEGF-A$_{121}$ only caused a small ~15% increase in leukocyte binding to endothelial cells (Figure 4.8F). ATF-2 knockdown completely ablated VEGF-A-stimulated binding of leukocytes to endothelial cells (Figure 4.8F). This phenomenon was VCAM-1-dependent, as VCAM-1 knockdown also completely inhibited endothelial-leukocyte interactions (Figure 4.8F). This data confirms that the VEGF-A-stimulated expression of endothelial VCAM-1 is not only ATF-2 dependent but also sufficient to enable recruitment of leukocytes and enhance cell-cell interactions.

4.2.5. ATF-2 is required for VEGF-A-stimulated endothelial cell responses

VEGF-A-stimulated signal transduction regulates diverse long-term responses in endothelial cells such as cell migration and tubulogenesis (Koch et al., 2011; Chung and Ferrara, 2011). This raises the question as to the importance of ATF-2 in

endothelial cell function and responses such as cell migration and tubule formation (tubulogenesis). To address this, we first compared the roles of these two VEGF-A isoforms in promoting endothelial cell migration, tubulogenesis and *ex vivo* angiogenesis. VEGF-A$_{165}$ produced a marked dose-dependent stimulation in endothelial cell migration (Figure 4.9, A and B) and tubulogenesis (Figure 4.9, C and D). However, VEGF-A$_{121}$ showed a much reduced or modest stimulation in such endothelial cell responses and such effects were especially evident at intermediate or sub-stoichiometric concentrations of VEGF-A (Figure 4.9, B and D). *Ex vivo* angiogenesis assays using mouse aortic slices (Figure 4.9E) showed VEGF-A$_{165}$ had ~3-fold higher capacity to stimulate vascular sprouting (Figure 4.9F). Thus each VEGF-A isoform had a distinct capacity to promote differential endothelial cell outputs; VEGF-A$_{165}$ was generally more biologically active at low or sub-stoichiometric concentrations. This raised the question as to whether ATF-2 was equally important for such differential programming of these endothelial cell responses. ATF-2 knockdown completely abolished either VEGF-A$_{165}$ or VEGF-A$_{121}$-stimulated cell migration (Figure 4.10, A and B). ATF-2 knockdown also inhibited (~ 50%) both VEGF-A$_{165}$ and VEGF-A$_{121}$-stimulated tubulogenesis (Figure 4.10, C and D). ATF-2 was also required for endothelial cell proliferation (Figure 4.11). Depletion of ATF-2 resulted in a ~2-fold decrease in VEGF-A$_{165}$-stimulated cell proliferation (Figure 4.11). Interestingly, depletion of ATF-2 also significantly reduced endothelial cell proliferation in complete media (Figure 4.11). Since VCAM-1 was required for endothelial-leukocyte adhesion (Figure 4.8F), it raised the possibility that it may also be required for other endothelial responses. To test this idea, we treated endothelial cells with scrambled or VCAM-1-specific siRNA duplexes before assessing VEGF-A isoform-specific endothelial tubulogenesis (Figure 4.12, A and B). However, there was no significant difference in tubulogenesis between control and VCAM-1-depleted endothelial cells (Figure 4.12B).
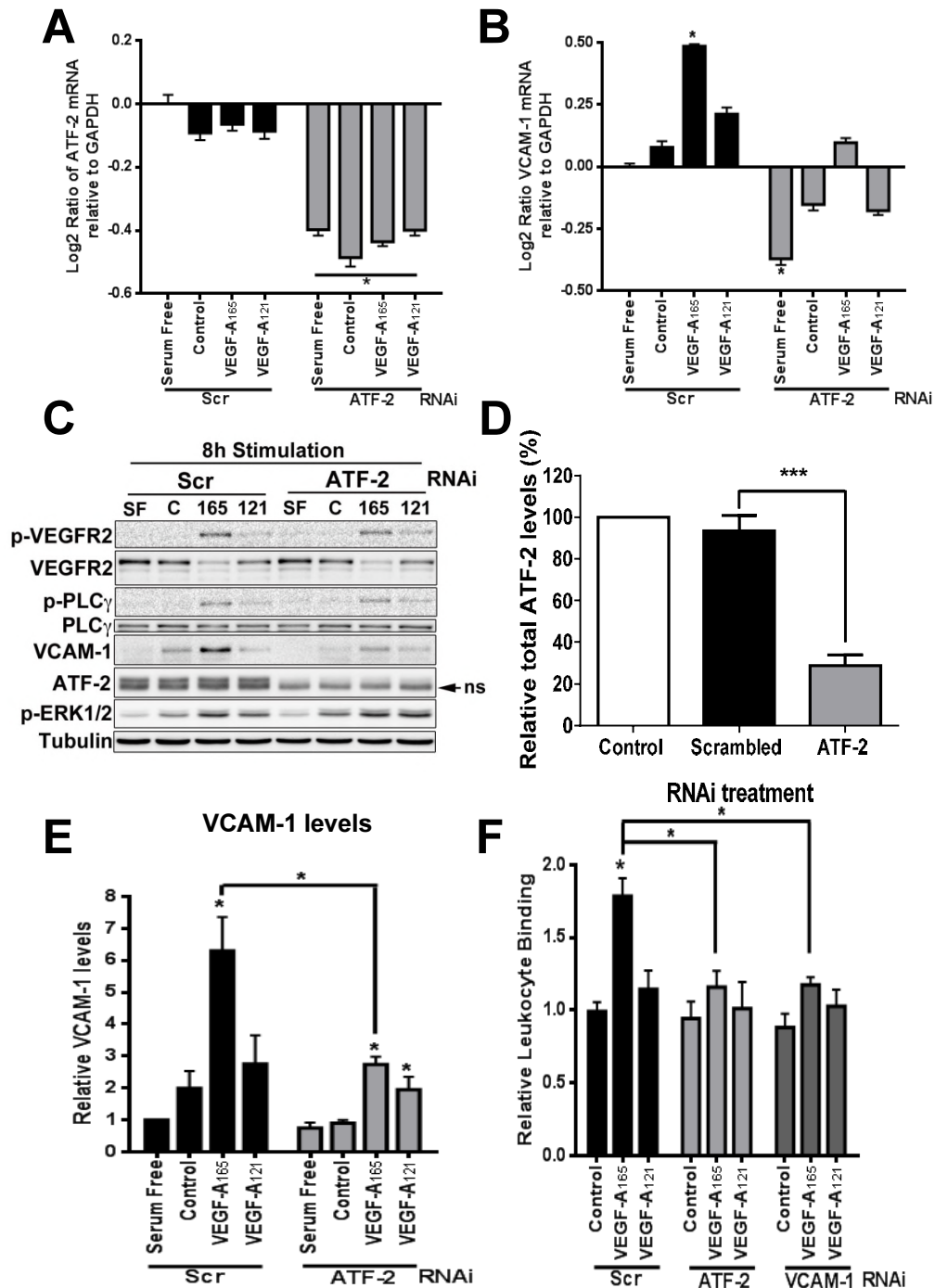
Figure 4.8. ATF-2 requirement for VEGF-A isoform-specific *VCAM-1* gene expression. (A and B) Endothelial cells stimulated with 0.25 nM VEGF-A$_{165}$ or VEGF-A$_{121}$ for 4 h were analysed by qRT-PCR for (A) ATF-2 or (B) VCAM-1 mRNA levels. GAPDH mRNA was used as an internal control. (C) Endothelial cells subjected to either scrambled (Scr) or ATF-2-specific (ATF-2) siRNA duplexes were then stimulated with 0.25 nM VEGF-A$_{165}$ or VEGF-A$_{121}$ for 8 h, prior to immunoblot analysis for a variety of proteins including ATF-2, VEGFR2 and VCAM-1. (D) Quantification of ATF-2 knockdown. (E) Quantification of VCAM-1 levels after 8 h VEGF-A stimulation. (F) Endothelial cells treated with scrambled (Scr), ATF-2 or VCAM-1 siRNA duplexes were stimulated with 0.25 nM VEGF-A$_{165}$ or VEGF-A$_{121}$ (7 h) prior to binding of calcein-labeled activated HL-60 leukocytes before lysis and measurement. Error bars denote ±SEM (n≥3). $p<0.05$ (*), $p<0.001$ (***). Selected data in this figure was provided by Dr A. F. Odell (Faculty of Medicine & Health, University of Leeds, UK).
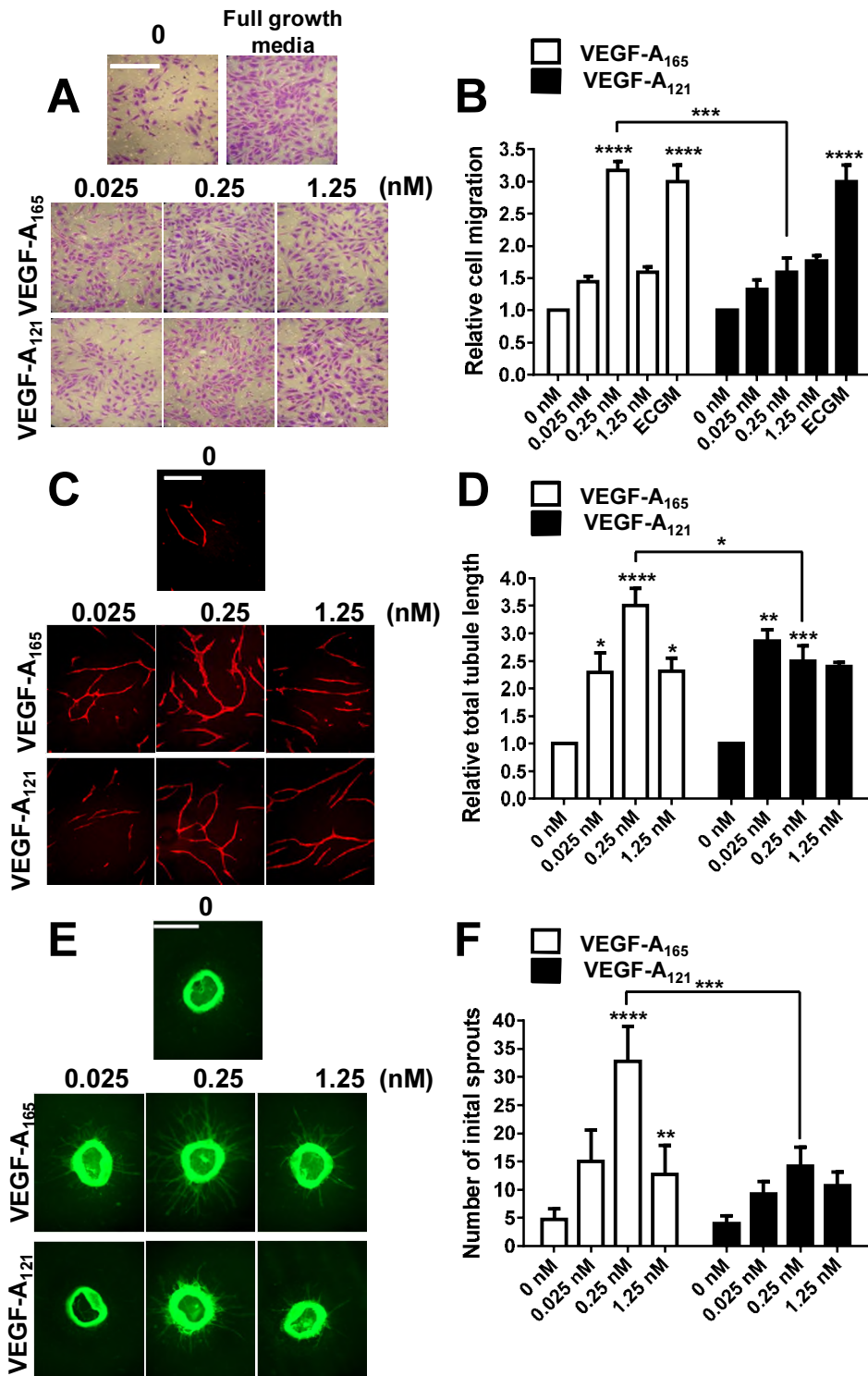
Figure 4.9. VEGF-A isoform-specific regulation of endothelial cell outcomes. (A-D) Endothelial cells were seeded into different cellular assays and subjected to 0, 0.025, 0.25 or 1.25 nM VEGF-A$_{165}$ or VEGF-A$_{121}$ prior to assessment of endothelial cell (A and B) migration or (C and D) tubulogenesis. Error bars indicate ±SEM (n=4). (E) *Ex vivo* angiogenesis assay using mouse aortic slices seeded into a collagen gel prior to stimulation with 0, 0.025, 0.25 or 1.25 nM VEGF-A$_{165}$ or VEGF-A$_{121}$. (F) Quantification of *ex vivo* aortic endothelial sprouting. Scale bars indicate, 200 μm, 400 μm or 1000 μm respectively. Error bars indicate ±SEM (n=3). $p<0.05$ (*), $p<0.01$ (**), $p<0.005$ (***), $p<0.0001$ (****). The data shown in panels E and F was provided by N. A. Mughal (Ponnambalam laboratory, University of Leeds, UK).
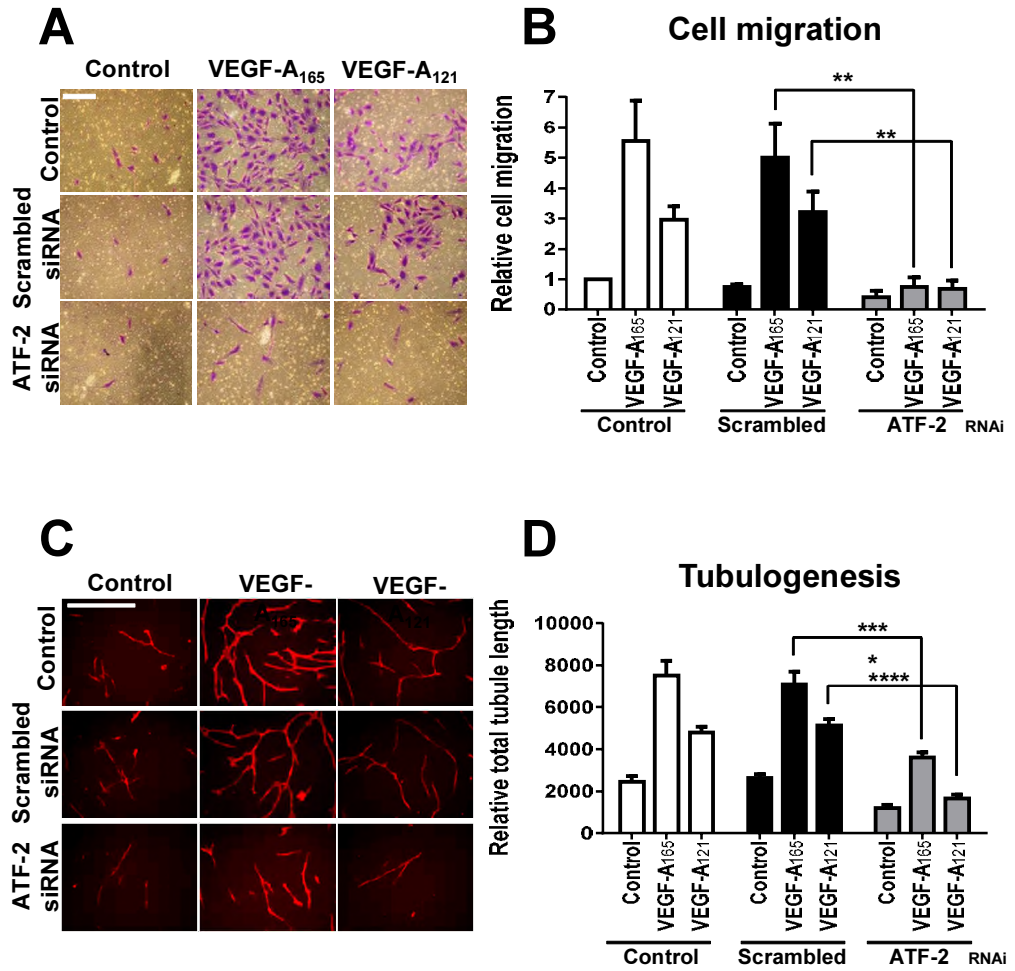
Figure 4.10. VEGF-A and ATF-2 regulation of endothelial cell responses. (A-D) Control, scrambled or ATF-2-specific siRNA duplex-treated endothelial cells were seeded into different assays and stimulated with 0.25 nM VEGF-A$_{165}$ or VEGF-A$_{121}$ and assessed for endothelial cell (A, B) migration, or (C-D) tubulogenesis. Scale bars indicate, 200 μm or 400 μm respectively. Error bars indicate ±SEM (n=3). $p<0.01$ (**), $p<0.0001$ (****).

Cell proliferation
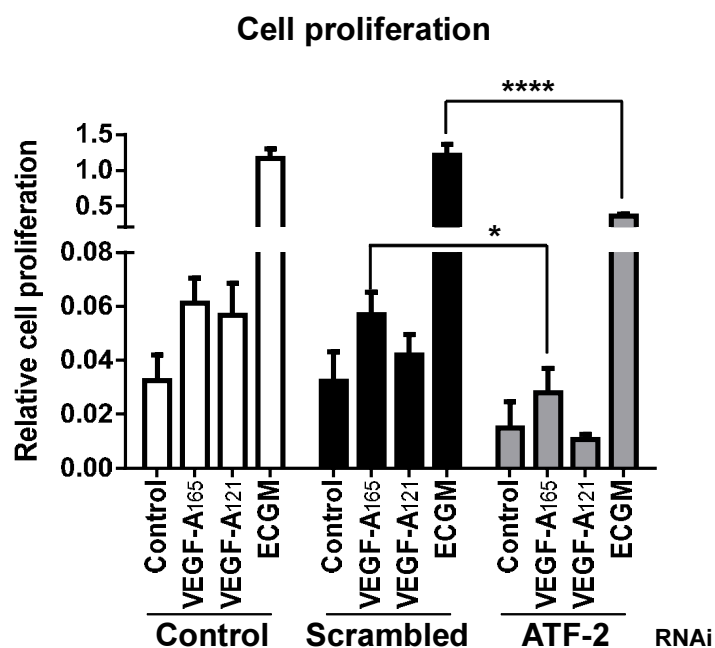
Figure 4.11. ATF-2 activity is required for endothelial cell proliferation. Control, scrambled or ATF-2-specific siRNA duplex-treated endothelial cells were seeded into 96-well plates and stimulated with 0.25 nM VEGF-A$_{165}$, VEGF-A$_{121}$ or cultured in full endothelial cell growth media (ECGM). Endothelial cell proliferation was assed using a BrdU incorporation cell proliferation ELISA (see Materials and Methods). Error bars indicate ±SEM (n=3). $p<0.05$ (*), $p<0.0001$ (****).
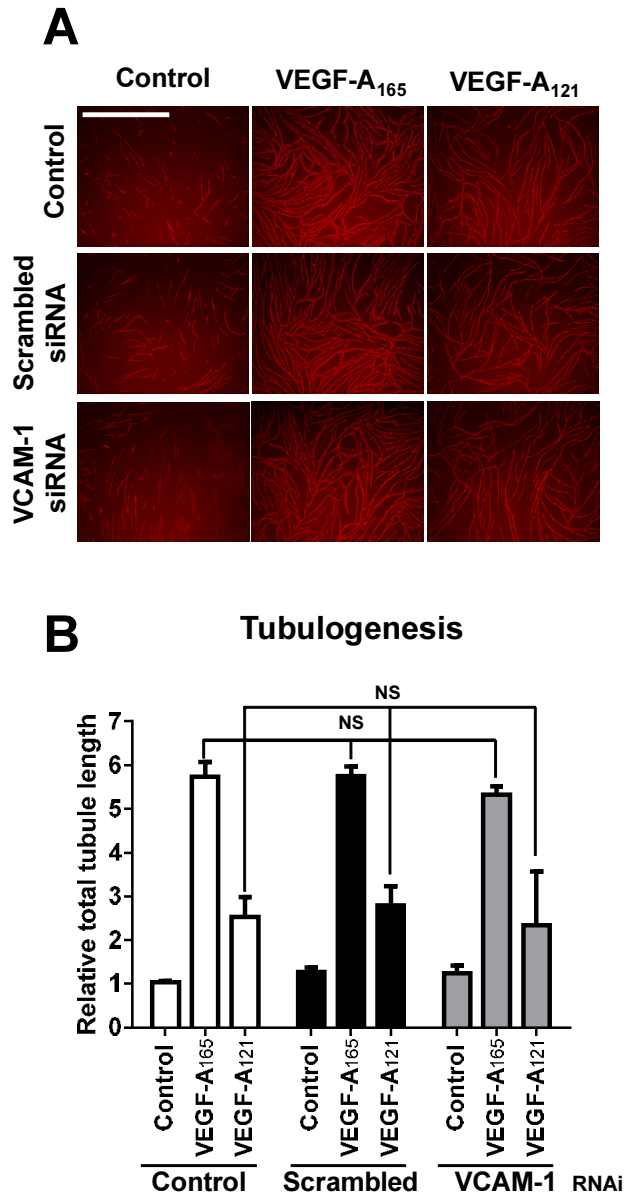
Figure 4.12. VEGF-A isoform-specific *VCAM-1* gene expression is not required for endothelial tubulogenesis. (A and B) Control, scrambled or VCAM-1-specific siRNA duplex-treated endothelial cells were seeded onto a bed of confluent human fibroblasts and stimulated with 0.25 nM VEGF-A$_{165}$ or VEGF-A$_{121}$ every 2-3 days for 7 days. (A) Endothelial tubules were visualised and captured via immunofluorescence microscopy (see Materials and Methods). (B) Quantification of total tubule length. Scale bars indicates 1000 μm. Error bars indicate ±SEM (n=3). NS; non-significant.

4.3.   DISCUSSION

In this study, we show that different VEGF-A isoforms have differential capacities to regulate *VCAM-1* gene expression and modulate endothelial-leukocyte binding via a novel mechanism (Figure 4.13). In this model, two VEGF-A isoforms with similar binding affinities differentially program VEGFR2 activation and downstream signal transduction to act on a common nuclear 'switch' that regulates *VCAM-1* gene expression. This 'switch' comprised of nuclear ATF-2, a transcription factor that is regulated by increased signal transduction from the MEK1-ERK1/2 pathway. Our findings show that ATF-2 is an important factor that regulates both VEGF-A regulated responses as well as other essential pathways.

A key feature in VEGF-A-stimulated *VCAM-1* gene expression is the requirement for ERK1/2 activation and ATF-2 expression. Maximal *VCAM-1* gene expression is dependent on endothelial cell stimulation by a specific VEGF-A$_{165}$ isoform. This isoform greatly increased VEGFR2 phosphorylation at residue Y1175 in comparison to the VEGF-A$_{121}$ isoform. This correlated with an increased ability to promote ERK1/2 activation and nuclear translocation (Figure 4.13). Translocation of activated ERK1/2 into the nucleus revealed close proximity to activated phospho-ATF-2. It is feasible that the T71 residue on ATF-2 is directly phosphorylated by ERK1/2. Alternatively, another target of ERK1/2 such as the p90 ribosomal S6 kinase (p90rsk or MAPKAP-K1) which can also translocate into the nucleus and phosphorylate key transcription factors (Gerits et al., 2008; Arthur, 2008). VEGF-A-stimulated intracellular signalling over a short-time frame (0-2 h) caused early *VCAM-1* gene transcription and increased mRNA levels (2-8 h) with concomitant peak in VCAM-1 protein levels at the cell surface after 8 h. In this way, short range signal transduction is translated into intermediate and long range effects such as membrane protein expression and subsequent interactions which modulate endothelial interactions with the environment.

Another key feature is that two different VEGF-A isoforms show significantly altered ability to promote *VCAM-1* gene expression. This is largely due to decreased ERK1/2 activation by the shorter VEGF-A$_{121}$ isoform. The different VEGF-A isoforms have a conserved N-proximal region (residues 1-111) and variable C-terminus (112-206). Notably all VEGF-A isoforms display similar binding affinity to VEGFR2 (Keyt et al., 1996; Delcombel et al., 2013) but unique receptor-ligand complexes can produce different functional outputs. Interestingly, it has been noted that the murine
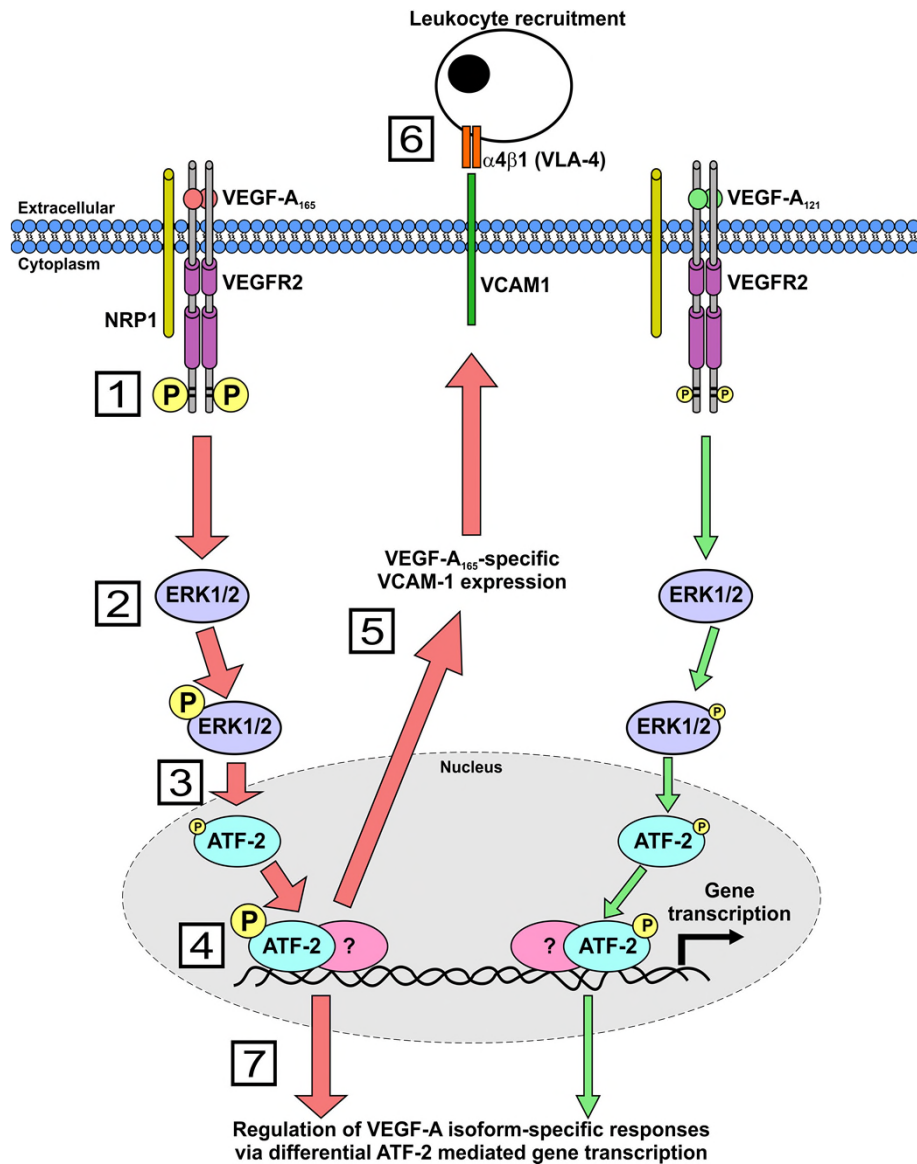
Figure 4.13. A mechanism for VEGF-A isoform-specific-regulation of *VCAM-1* gene expression and endothelial-leukocyte interactions. Schematic describing VEGF-A isoform-specific stimulation of intracellular signalling and ATF-2-regulated *VCAM-1* gene expression. (1) VEGFR2 activation by either VEGF-A$_{165}$ or VEGF-A$_{121}$ programs differential phosphorylation of residue Y1175. (2) VEGF-A$_{165}$ stimulation results in increased ERK1/2 phosphorylation and activation, compared to VEGF-A$_{121}$. (3) Phosphorylated activated ERK1/2 translocates to the nucleus and regulates ATF-2 phosphorylation. (4) VEGF-A$_{165}$ is more potent than VEGF-A$_{121}$ in promoting ERK1/2-dependent ATF-2 phosphorylation. (5) VEGF-A$_{165}$-stimulated ATF-2 activity regulates *VCAM-1* gene transcription. (6) *VCAM-1* gene expression promotes endothelial-leukocyte interactions. (7) VEGF-A stimulated ATF-2 activity also modulates other VEGF-A-isoform-specific endothelial responses.

orthologs of VEGF-A$_{121}$ and VEGF-A$_{165}$ show capacity to differentially elevate expression of another cell adhesion molecule, ICAM-1 in the mouse ocular endothelium (Usui et al., 2004). The underlying mechanism regulating such differential VEGF-A-regulated ICAM-1 expression is unknown but raises speculations that ATF-2 may be involved in this phenomenon as well. In this context, it is well-known that different VEGF-A isoforms have the capacity to trigger differential VEGFR2 activation and downstream signal transduction (Zhang et al., 2000; Bates et al., 2002; Herve et al., 2005; Nakatsu et al., 2003; Zhang et al., 2008; Chen et al., 2010). An important question is the mechanism underlying VEGF-A-stimulated gene transcription (Goddard and Iruela-Arispe, 2013). STAT3 (Bartoli et al., 2003), Egr3 (Liu et al., 2003), forkhead-like transcription factors (Abid et al., 2006), FoxO- and Ets-related factors (Dejana et al., 2007) and HLX (Testori et al., 2011) have all been implicated in regulating VEGF-A-dependent gene transcription.

How ATF-2 regulates *VCAM-1* gene transcription is intriguing. ATF-2 was originally identified as a nuclear transcriptional switch that was activated upon DNA damage or stress thus enabling gene expression linked to an anti-apoptotic response or cell proliferation (Lau and Ronai, 2012). The ATF-2 polypeptide can undergo phosphorylation at different Ser/Thr residues at the N- (T52, S62, T69, T71, S73, S121) or C-termini (S490, S498) (Lau and Ronai, 2012). One possibility is that phospho-ATF-2 directly binds to the VCAM-1 promoter to stimulate early gene transcription: this may occur via the formation of ATF-2 homodimers or heterodimers with factors such as c-jun. In addition, ATF-2 may recruit other factors such as p300 (Kawasaki et al., 1998) or IRF3 (Panne et al., 2004) to promoter loci which further modulate target gene expression. It has also been proposed that ATF-2 has intrinsic histone acetyltransferase activity (HAT) such that it acts to de-repress gene transcription upon recruitment (Kawasaki et al., 2000) and other transcription factors such as NFκB or forkhead could directly stimulate *VCAM-1* gene transcription.

Our study shows evidence that different VEGF-A isoforms regulate both angiogenesis and inflammation via an ATF-2-dpendent mechanism. The increased recruitment of activated leukocytes to VEGF-A$_{165}$-stimulated cells in contrast to VEGF-A$_{121}$-stimulation, argues that this has a functional role *in vivo*. This could be useful in the VEGF-A-isoform-specific recruitment of leukocytes into a developing blood vessel during angiogenesis. This would be advantageous in controlling

vascular development and endothelial-leukocyte balance within a vascular niche. Alternatively, such a phenomenon could be extremely useful during pathogenic infection or injury: the release of specific VEGF-A isoforms into the damaged vasculature could attune leukocyte recruitment to the extent of infection or injury. VEGF-A$_{165}$ has been shown to stimulate *VCAM-1* gene expression (Kim et al., 2001; Abid et al., 2006) but this has also been contradicted (Stannard et al., 2007). In this context, it has been shown that pro-inflammatory cytokines such as IL-1β or TNFα promote NFκB, SP1, AP-1 and IRF recruitment to the *VCAM-1* locus to stimulate VCAM-1 expression (Collins et al., 1995; Sixt et al., 2006; Hordijk, 2006; Weber, 1996). A link between ATF-2 and NFκB has also been proposed in regulating VCAM-1 during shear stress (Cuhlmann et al., 2011). Expression of a mutant ATF-2 in a mouse model has been shown to inhibit *VCAM-1* gene expression (Reimold et al., 2001).

The interactions between endothelial cells and leukocytes can be subverted in major disease states ranging from atherosclerosis, rheumatoid arthritis, pathogenic infection to cancer. This study now provides a mechanism to explain how different VEGF-A isoforms regulate not only angiogenesis but also inflammation in such disease states. Immune cells are well-known to secrete pro-angiogenic cytokines such as TNFα and VEGF-A (Griffioen and Molema, 2000; Naldini and Carraro, 2005). The angiocrine model postulated by Rafii and colleagues suggests that the endothelium secretes soluble and membrane-bound factors that act in a paracrine manner on neighbouring cells to influence vascular development in tissues such as liver (Butler et al., 2010; Ding et al., 2010). Our work shows that VEGF-A isoforms have unique abilities to instruct the endothelium and influence leukocyte recruitment at local sites through cell-cell interactions. A challenge will be to decipher the myriad biological properties of the VEGF family with functional roles in both angiogenesis and inflammation in healthy and diseased states.

CHAPTER 5

Tpl2 is a signalling nexus in endothelial gene expression and cell cycle progression

5.1.   INTRODUCTION

Eukaryotes use complex signal transduction pathways to communicate information from the plasma membrane to the cell interior. The interaction between membrane receptors and substances in the extracellular medium (ranging from growth factors, lipid particles to relatively simple molecules), can trigger a vast array of signals which rapidly reach different intracellular compartments. A critical step in eukaryote cell function is the decision between cellular quiescence, proliferation or death. Such biological processes are deregulated in disease states such as cancer. Here, the switch from a quiescent or non-proliferative state into a rapidly proliferating one drives tumour initiation and disease progression. Genes which promote or suppress such proliferative states are respectively termed as proto-oncogenes or tumour suppressors (e.g. p53) (Hanahan and Weinberg, 2011).

The mammalian endothelial cell is a useful system for studying such decision making events and their effect on complex cellular outcomes including cell migration, proliferation and vascular tube formation. Endothelial cells express a complex array of membrane receptors, which interact with soluble factors, membrane proteins (on other cells) and the substratum, thus allowing a rapid response to changes within the cellular milieu (Koch et al., 2011; Chen et al., 2010; Herbert and Stainier, 2011). One such receptor-ligand system comprises the vascular endothelial growth factor (VEGF) family, which binds a receptor tyrosine kinase (RTK) subfamily comprising vascular endothelial growth factor receptor VEGFR1, 2 and 3; in addition, to co-receptors such as Neuropilins which can further modulate signal transduction and cell outcomes (Koch et al., 2011). Binding of VEGF-A to endothelial VEGFR2 mediates new blood vessel sprouting (i.e. angiogenesis) and other cellular outcomes such as vasodilation (Koch et al., 2011). *VEGFA* and *VEGFR* genes are essential for mammalian development and vascular function (Keyt et al., 1996; Carmeliet et al., 1996; Shalaby et al., 1995). A dysfunctional response to VEGF-A by the endothelium is implicated in many

diseases, including cancer, pre-eclampsia and cardiovascular disease (Carmeliet and Jain, 2011; Hanahan and Weinberg, 2011; Chung and Ferrara, 2011; Meadows and Hurwitz, 2012).

It is poorly understood how short lived signal transduction (0-2 h) is integrated with long term cellular outcomes to impact on states such as cellular quiescence, proliferation, migration and complex organ formation e.g. vascular tubes (days to weeks). On the shorter time scale, VEGF-A binding to VEGFR2 triggers dimerisation and transautophosphorylation of multiple tyrosine residues within its cytoplasmic kinase domain, enabling the recruitment of Src homology 2 (SH2) domain containing adaptor proteins and signal transduction enzymes (Koch et al., 2011; Bruns et al., 2010). This process is further complicated by the fact that the *VEGFA* gene alone encodes at least 7 different isoforms with both pro- and anti-angiogenic activity (Harper and Bates, 2008). A general consensus is that VEGF-A binding to endothelial VEGFR2, activates multiple signal transduction pathways which impact on different cellular responses (Lee et al., 2007; Gerber et al., 1998; Matsumoto et al., 2005; Olsson et al., 2006; Lampugnani et al., 2006; Koch and Claesson-Welsh, 2012). Nonetheless, we still lack an understanding of how a single VEGF-A isoform can simultaneously influence multiple long term cellular decision events.

It is well-known that the mitogen-activated protein kinase (MAPK) pathways are activated in response to VEGF-A binding to endothelial VEGFRs (Koch et al., 2011). Both the MEK1-ERK1/2 and p38 MAPK pathways are activated by VEGF-A; however, this activation occurs within a relatively rapid time period (15-30 min) and thus, does not fully explain the subsequent effects on longer term cellular responses. One possible candidate enzyme, which could play a role in modulating VEGF-A-stimulated signal transduction and endothelial cell outcomes, is the serine/threonine protein kinase, tumour progression locus 2 or Tpl2 (MAP3K8, Cot). Tpl2 has recently been implicated in regulating angiogenesis (Lee et al., 2013; Gantke et al., 2011; Lee et al., 2015). Furthermore, a Tpl2-specific small molecule inhibitor impairs *in vivo* angiogenesis by attenuating endothelial cell proliferation (Lee et al., 2013). However, despite its role in VEGF-A-stimulated endothelial responses being unclear, Tpl2 has a well-established role as a regulator of NFκB status (Gantke et al., 2011) and a more recent link to p53 tumour suppressor function in lung carcinogenesis (Gkirtzimanaki et al., 2013).

A major question is the role of Tpl2 in VEGF-A-stimulated endothelial cell outcomes and angiogenesis. To address this, we tested the hypothesis that Tpl2 is part of a regulatory axis which modulates short and long term cellular decisions. Here, we show that stimulation with VEGF-A$_{165}$ but not VEGF-A$_{121}$ increased Tpl2 mRNA expression and protein levels. Tpl2 was also required for VEGF-A stimulated tubulogenesis and endothelial-leukocyte interactions. Furthermore, basal Tpl2 activity is essential for maintaining constitutive phosphorylation and stabilisation of a key VEGF-A-stimulated transcription factor, activating transcription factor 2 (ATF-2). Loss of Tpl2 kinase activity resulted in decreased ATF-2 phosphorylation and impaired VEGF-A-stimulated *VCAM-1* gene expression. Furthermore, reduced ATF-2 activity resulted in cell cycle arrest and subsequent attenuation of endothelial cell responses. This phenomenon was dependent on both Tpl2 and the tumour suppressor p53. Our findings thus present a novel mechanism, whereby Tpl2 kinase activity is crucial for endothelial cell function, via regulating ligand-stimulated gene expression and cell cycle progression.

5.2.   RESULTS

5.2.1. VEGF-A-stimulated Tpl2 expression modulates endothelial cell responses

VEGF-A binding to VEGFR2 stimulates receptor dimerisation, signal transduction and endothelial cell responses (Koch and Claesson-Welsh, 2012). VEGF-A isoforms elicit different VEGFR2-mediated signal transduction and endothelial outcomes (Pan et al., 2007; Kawamura et al., 2008). Recent work revealed a role for the MAPK family member, Tpl2 in regulating angiogenesis in both cell and animal models (Lee et al., 2013; Gantke et al., 2011). We hypothesised that this Tpl2 serine/threonine protein kinase is part of a regulatory circuit which controls VEGF-A-stimulated signal transduction in endothelial cells. To test this idea, we stimulated endothelial cells with either the VEGF-A$_{165}$ or VEGF-A$_{121}$ isoform for 2, 4, 6, 8 or 24 h prior to monitoring VEGFR2 and Tpl2 protein levels (Figure 5.1A). Quantification revealed that VEGF-A$_{165}$ caused a relatively rapid ~2.5-fold increase in Tpl2 protein levels (doublet of 52 and 58 kDa) after 2 h which remained elevated over a 24 h period (Figure 5.1B). Conversely, VEGF-A$_{121}$ treatment caused no significant change in Tpl2 protein levels over the same time period (Figure 5.1B). To address whether this rise in Tpl2 protein levels was due to altered mRNA levels, we used qRT-PCR to evaluate Tpl2 mRNA after VEGF-A stimulation (Figure 5.1C). Quantification revealed that VEGF-A$_{165}$ stimulation caused an ~3.5-fold and ~2.5-
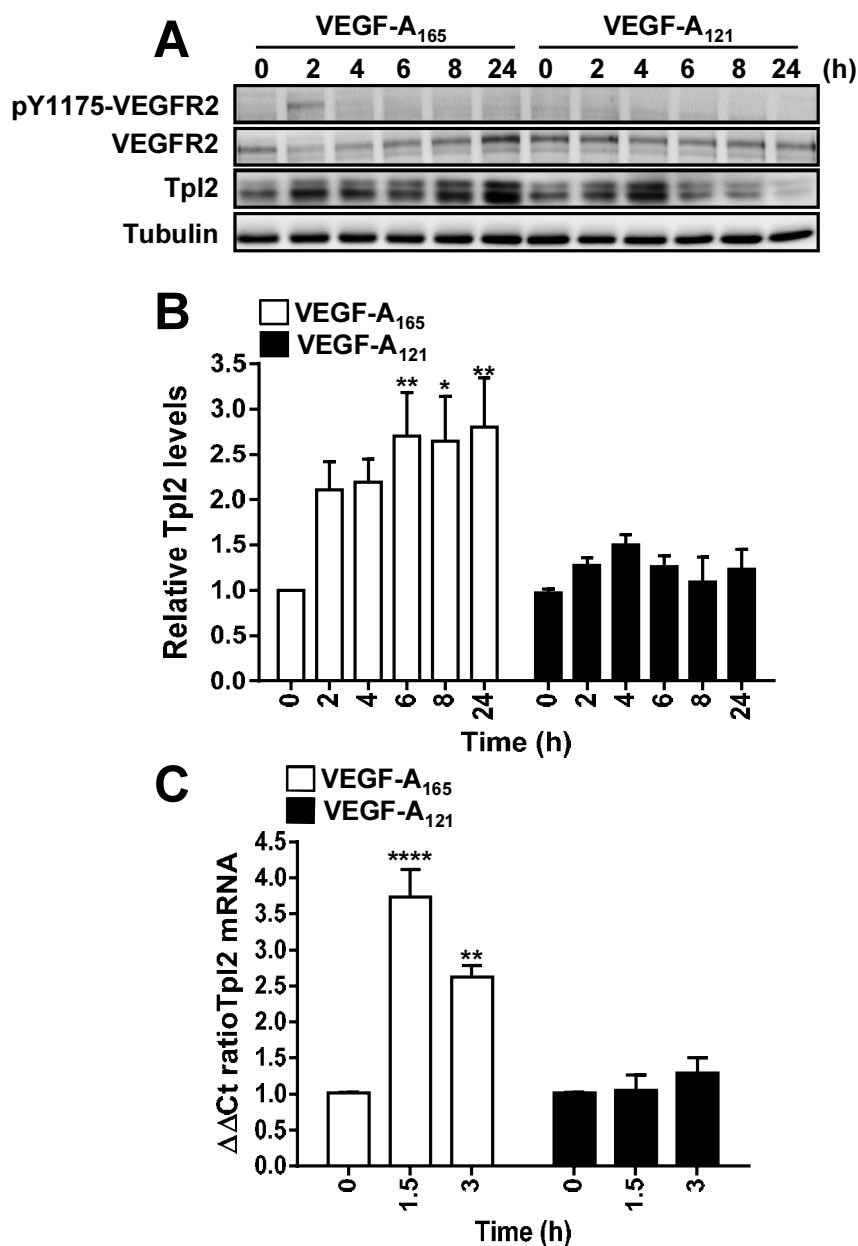
Figure 5.1. VEGF-A isoform-specific effects on Tpl2 expression. (A) Human endothelial cells were stimulated with either VEGF-A$_{165}$ or VEGF-A$_{121}$ (0.25 nM) for 2, 4, 6 8 or 24 h before lysis and processing for immunoblot analysis using antibodies against VEGFR2 and Tpl2. (B) Quantification of Tpl2 protein levels upon VEGF-A$_{165}$ or VEGF-A$_{121}$ treatment. (C) Quantification of relative Tpl2 mRNA levels via qRT-PCR (see Materials and Methods). Error bars indicate ±SEM (n=3). $p<0.05$ (*), $p<0.01$ (**), $p<0.0001$ (****). The data provided in panel C was provided by I. Abdul Zani, (Ponnambalam laboratory, University of Leeds, UK).

fold increase in Tpl2 mRNA after 1.5 and 3 h respectively (Figure 5.1C). In comparison, VEGF-A$_{121}$ stimulation had a relatively small or negligible effect on Tpl2 mRNA levels (Figure 5.1C). These data show that VEGF-A$_{165}$-stimulated signal transduction promotes *Tpl2* gene transcription and mRNA synthesis.

VEGF-A isoforms differentially regulate pro-angiogenic (Pan et al., 2007; Kawamura et al., 2008) and pro-inflammatory (see earlier in chapter 4) endothelial cell responses. To determine whether VEGF-A-stimulated Tpl2 expression modulates cellular responses in a VEGF-A isoform-specific manner, we performed RNA interference (RNAi) using synthetic Tpl2-specific siRNA-duplexes prior to evaluating endothelial tubulogenesis (Figure 5.2, A and B) and endothelial-leukocyte interactions (Figure 5.3, A and B). Under such conditions, Tpl2 is depleted by ~75% (see later in Figure 5.4C). Quantification revealed that Tpl2 depletion caused ~25% reduction in endothelial tubulogenesis (Figure 5.2B) and ~35% reduction in endothelial-leukocyte binding (Figure 5.3B). In contrast, VEGF-A$_{121}$-stimulated endothelial cell responses were not significantly affected by Tpl2 depletion (Figure 5.2A and 5.3B). VEGF-A$_{165}$-stimulated Tpl2 expression is thus required for long term endothelial cell responses, such as tubulogenesis and endothelial-leukocyte interactions.

5.2.2. Tpl2 regulates ATF-2 phosphorylation and *VCAM-1* gene expression

VCAM-1 expression on the surface of the endothelium is a critical regulator of leukocyte binding (Nourshargh et al., 2010). Previous work shown in this PhD thesis found that VEGF-A$_{165}$-stimulated expression of endothelial VCAM-1 is dependent on a signal transduction pathway involving ERK1/2 and activating transcription factor 2, ATF-2 (see earlier in chapter 4). One prediction is that Tpl2 modulates ATF-2 status or activity which subsequently impacts on *VCAM-1* gene expression. To test this idea, we compared protein levels in control, ATF-2 or Tpl2-depleted endothelial cells subjected to VEGF-A$_{165}$ or VEGF-A$_{121}$ (negative control) stimulation for 4 or 8 h (Figure 5.4A). ATF-2 depletion completely ablated VEGF-A$_{165}$-stimulated VCAM-1 expression, whereas Tpl2 depletion caused an ~2.8-fold reduction (Figure 5.4B). This data thus confirmed Tpl2 requirement for VEGF-A$_{165}$-stimulated *VCAM-1* gene expression via ATF-2.
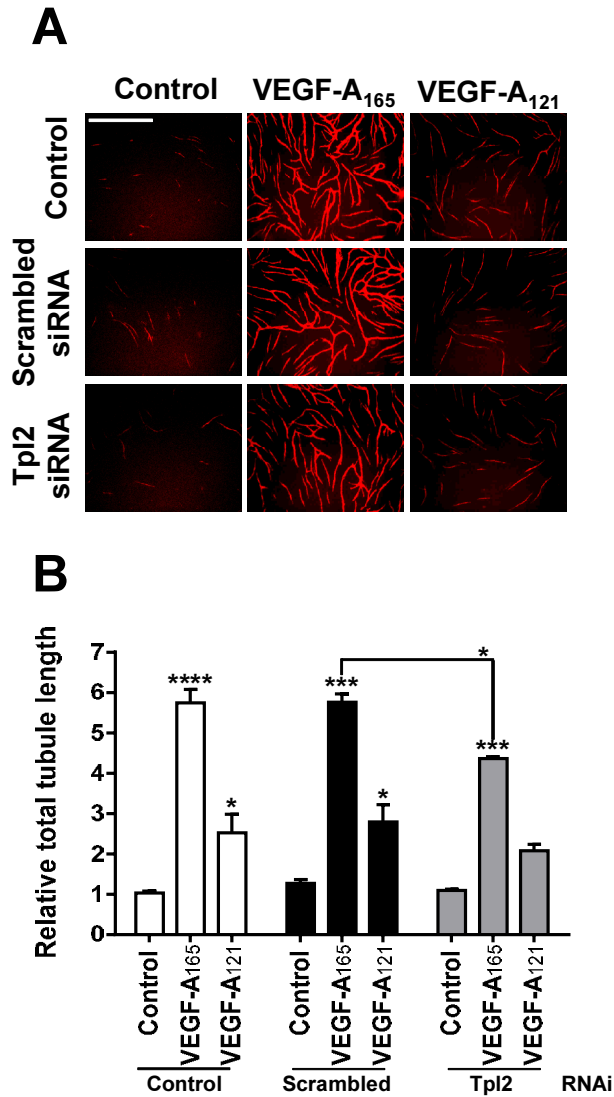
Figure 5.2. Endothelial cell Tpl2 is required for VEGF-A-stimulated endothelial cell tubulogenesis. (A and B) Control, scrambled or Tpl2-depleted human endothelial cells were seeded into cellular assays to assess endothelial tubulogenesis, upon VEGF-A$_{165}$ or VEGF-A$_{121}$ (0.25 nM) stimulation. Scale bar, 1000 μm. (B) Quantification of VEGF-A-stimulated endothelial cell tubule length in control or Tpl2-depleted endothelial cells. Error bars indicate ±SEM (n≥3). $p<0.05$ (*), $p<0.001$ (***), $p<0.0001$ (****).
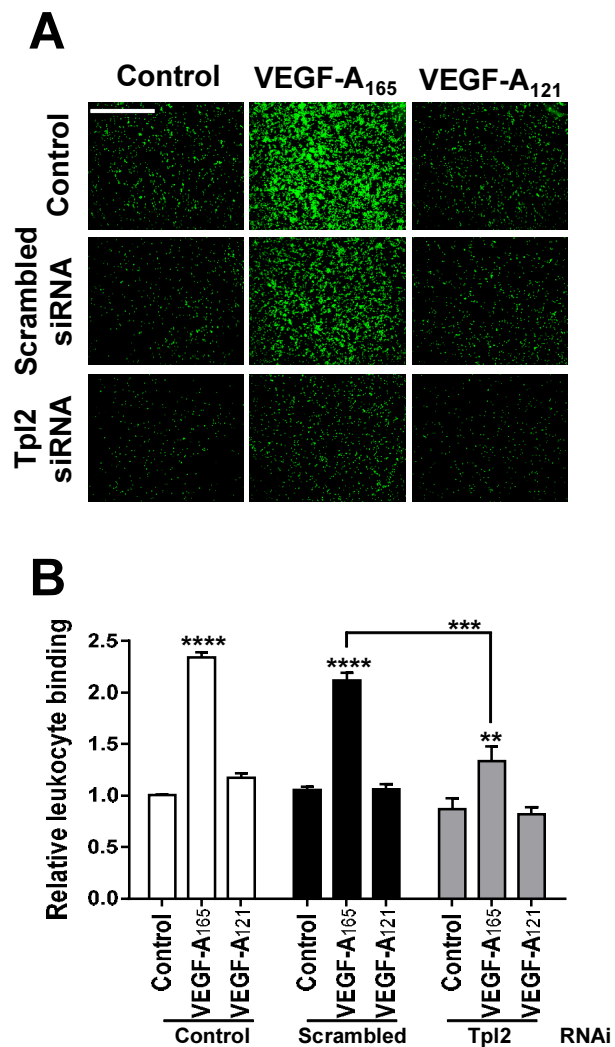
Figure 5.3. Endothelial cell Tpl2 is required for VEGF-A-stimulated endothelial-leukocyte interactions. (A-B) Control, scrambled or Tpl2-depleted human endothelial cells were seeded into cellular assays to assess endothelial-leukocyte interactions, upon VEGF-A$_{165}$ or VEGF-A$_{121}$ (0.25 nM) stimulation. Scale bar, 1000 μm. (B) Quantification of endothelial-leukocyte binding in control or Tpl2-depleted endothelial cells. Error bars indicate ±SEM (n≥3). $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$ (****).

VEGF-A$_{165}$ stimulates an ~2-fold rise in Tpl2 levels (Figure 5.4C); interestingly, depletion of ATF-2 caused an ~2-fold increase in basal Tpl2 levels which were not further elevated upon VEGF-A$_{165}$-stimulation (Figure 5.4C). Therefore, ATF-2 activity is required for VEGF-A$_{165}$-stimulated Tpl2 expression. VEGF-A stimulation produced ~2-fold increase in ATF-2 phosphorylation at residue T71 (ATF-2-pT71) (Figure 5.4D), corresponding to increased transcription factor activity. However, this had no effect on total ATF-2 levels (Figure 5.4E). As expected, both phospho-ATF-2 levels (Figure 5.4D) and total ATF-2 (Figure 5.4E) levels were reduced (~50% or ~75% respectively) in ATF-2-depleted endothelial cells under both resting and stimulatory conditions. Consistent with our prediction, both phospho- and native ATF-2 levels were similarly reduced in Tpl2-depleted endothelial cells (Figure 5.4, D and E). Taken together, this data suggest that Tpl2 is important for regulating both ATF-2 levels and activation by phosphorylation.

The Tpl2-specific kinase inhibitor CAS 871307-18-5 targets Tpl2 activity with an IC$_{50}$ ~50 nM. Endothelial cells were subjected to a time course of inhibitor treatment to block Tpl2 kinase activity prior to evaluating phospho-ATF-2, ATF-2 and Tpl2 levels (Figure 5.5A). Quantification showed Tpl2 kinase inhibition reduced basal ATF-2-pT71 levels by ~60% after just 1 h and was sustained for up to 8 h (Figure 5.5B). In contrast to Tpl2 depletion by RNAi, pharmacological inhibition of Tpl2 activity did not significantly affect ATF-2 levels (Figure 5.5C). These findings reveal a dichotomy between Tpl2 depletion and Tpl2 kinase inhibition on ATF-2 phosphorylation and transcriptional status.

Our findings showed that ATF-2 depletion resulted in elevated Tpl2 levels (Figure 5.4C). Therefore, as ATF-2 dephosphorylation is linked to its stability and proteolysis (Fuchs et al., 2000), one possibility is that pharmacological inhibition of Tpl2 kinase activity stimulates elevation of Tpl2 levels as a consequence of reduced ATF-2 phosphorylation, which may in turn positively feedback to maintain phospho-ATF-2 levels. To test this idea, we monitored Tpl2 levels over an inhibitor time course. Quantification revealed a steady-rise in Tpl2 levels upon inhibitor treatment, with ~70% increase after 8 h (Figure 5.5D). This data implied a close functional link between Tpl2 activity and phospho-ATF-2 status which impacts on total ATF-2 levels. A prominent functional consequence of elevated phospho-ATF-2 levels is increased *VCAM-1* gene expression (Figure 5.4B), thus WE next evaluated the effects of Tpl2 kinase activity on VEGF-A-stimulated *VCAM-1* gene expression.
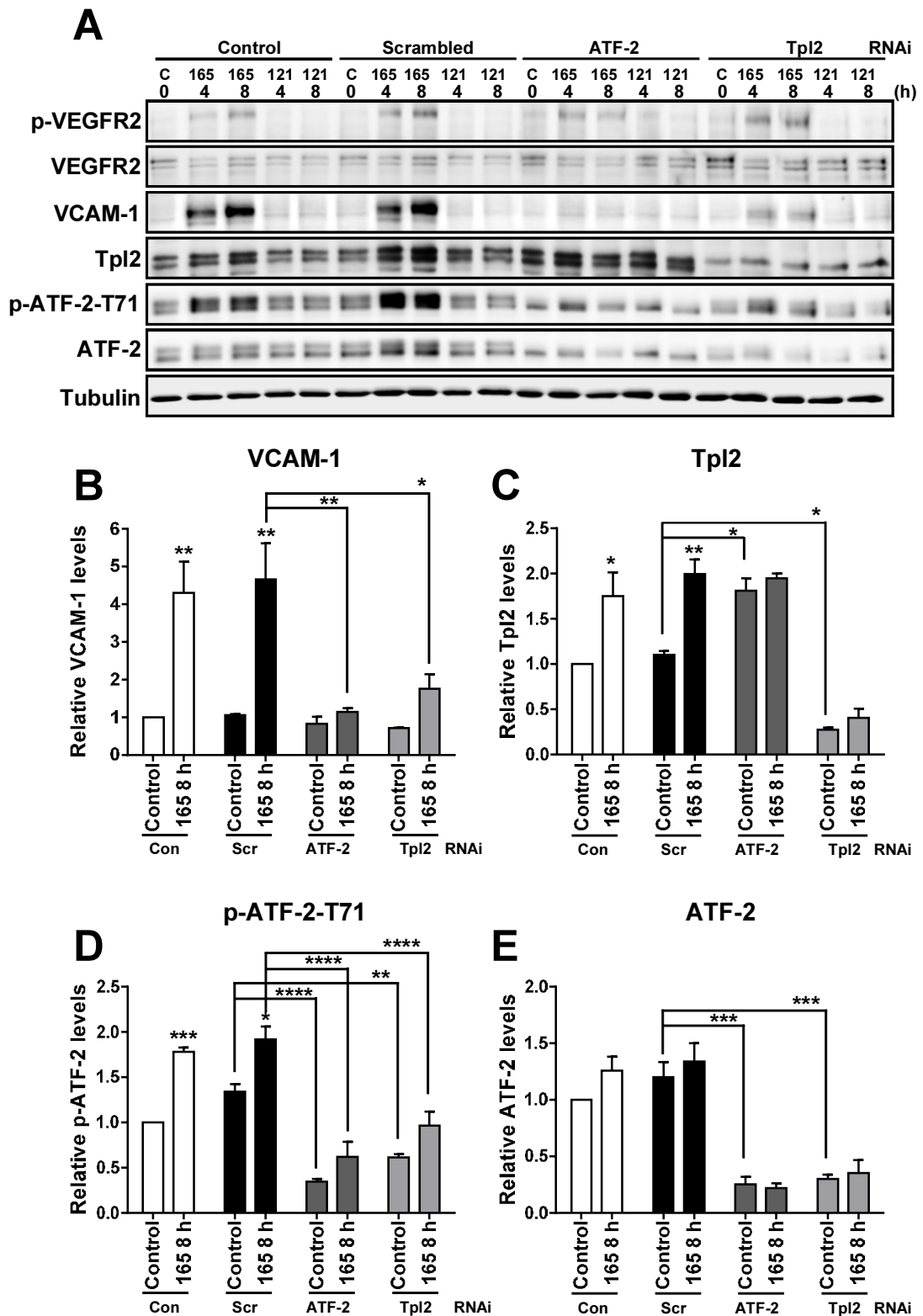
Figure 5.4. Tpl2 is essential for VEGF-A$_{165}$-stimulated ATF-2-dependent VCAM-1 expression. Control, scrambled or human endothelial cells depleted of ATF-2 or Tpl2 were stimulated (0.25 nM) with either VEGF-A$_{165}$ or VEGF-A$_{121}$ (negative-control) for 4 or 8 h, were lysed prior to processing for immunoblot analysis using antibodies against VCAM-1, Tpl2 and ATF-2. Quantification of (B) VCAM-1, (C) Tpl2, (D) ATF-2-pT71 and (E) ATF-2 levels after 8 h VEGF-A$_{165}$ stimulation. Error bars indicate ±SEM (n=3). $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$ (****).
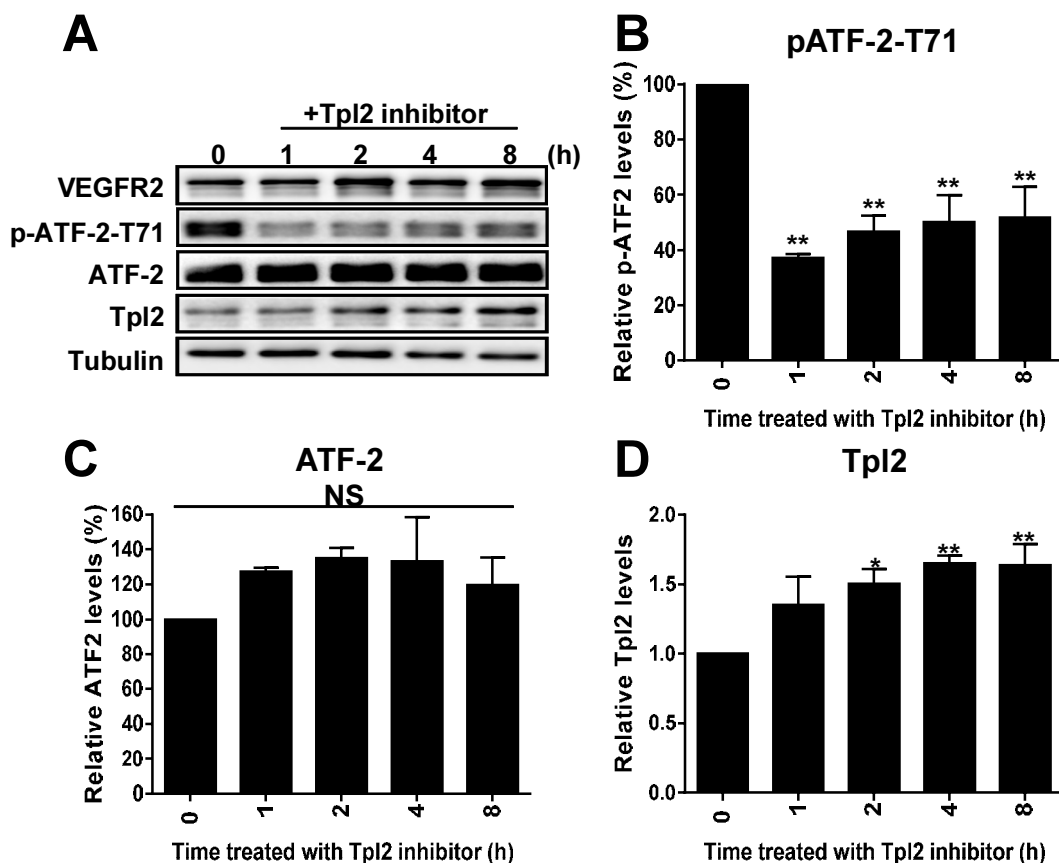
Figure 5.5. Tpl2 kinase activity regulates ATF-2 phosphorylation. (A) Human endothelial cells treated with a Tpl2-specific kinase inhibitor (2 μM; 30 min) for 0, 1, 2, 4 or 8 h were lysed prior to immunoblot analysis and quantification of (B) ATF-2-pT71, (C) ATF-2 and (D) Tpl2 protein levels. Error bars indicate ±SEM (n≥3). NS = non-significant, $p<0.05$ (*), $p<0.01$ (**), $p<0.0001$ (****).
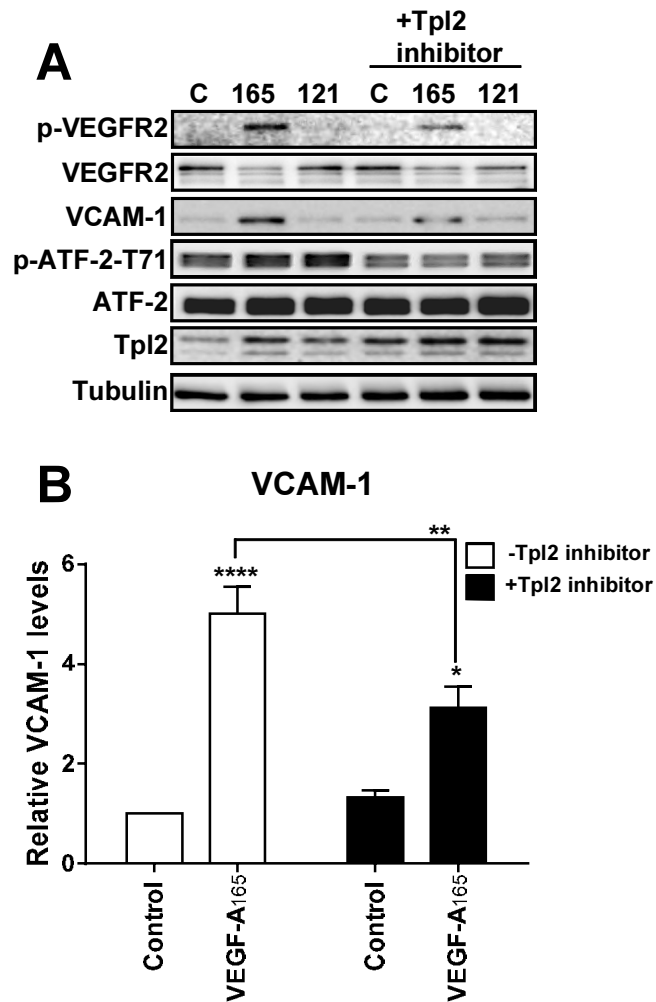
Figure 5.6. Tpl2 kinase activity regulates VEGF-A$_{165}$-stimulated VCAM-1 expression. (A) Human endothelial cells were pre-treated with a Tpl2 kinase inhibitor (2 μM; 30 min) prior to stimulation with either (0.25 nM) VEGF-A$_{165}$ (165) or VEGF-A$_{121}$ (121, negative-control) for 0 or 8 h before lysis and processing for immunoblot analysis. (B) Quantification of VCAM-1 levels upon VEGF-A$_{165}$ treatment. Error bars indicate ±SEM (n≥3). $p<0.05$ (*), $p<0.01$ (**), $p<0.0001$ (****).

Endothelial cells were pre-treated with Tpl2 kinase inhibitor prior to VEGF-A$_{165}$ or VEGF-A$_{121}$ stimulation, followed by lysis and immunoblotting (Figure 5.6A). Quantification showed that inhibition of Tpl2 kinase activity caused ~38% reduction in VEGF-A$_{165}$-stimulated *VCAM-1* gene expression over this 8 h period (Figure 5.6B). Therefore, these findings suggest that Tpl2 kinase activity is required for maintaining ATF-2 phosphorylation and stability, thus permitting VEGF-A$_{165}$-stimulated *VCAM-1* gene expression.

5.2.3. ATF-2 status modulates p53 tumour suppressor levels and function

ATF-2 is essential for endothelial cell proliferation and VEGF-A-stimulated tubulogenesis (see earlier in chapter 4). One possibility is that ATF-2 is required for endothelial cell cycle progression. To test this idea, ATF-2-depleted endothelial cells were analysed for key proteins that regulate cell cycle progression (Figure 5.7). Control or ATF-2-depleted cells were synchronised using a thymidine block, prior to stimulation for 8 h with either VEGF-A$_{165}$ or VEGF-A$_{121}$ followed by lysis and immunoblotting (Figure 5.7). Compared to controls, ATF-2-depleted endothelial cells displayed increased expression of both Cyclin D1 and p21 (Figure 5.7), whereas the levels of Cyclin A2 or Cyclin B remained relatively unchanged (Figure 5.7). These findings suggested that ATF-2 depletion promotes increased Cyclin D1 and p21, which could in turn perturb endothelial cell cycle progression.

The p53 tumour suppressor is a key cell cycle regulator whose levels are elevated during cellular stress to promote the expression of the cyclin-dependent kinase inhibitor, p21 (Klein and Assoian, 2008; Bieging et al., 2014; Galbiati et al., 2001; Gkirtzimanaki et al., 2013). The discovery of elevated p21 levels in ATF-2-depleted cells (Figure 5.7) suggested this phenomenon may be p53-dependent. To test this idea, endothelial cells subjected to ATF-2, p53 or combined ATF-2/p53 depletion followed by either VEGF-A$_{165}$ or VEGF-A$_{121}$ stimulation were analysed for p53, p21 and Cyclin D1 levels (Figure 5.8A). All these proteins were elevated upon ATF-2 depletion but neither VEGF-A isoform addition showed any effect (Figure 5.8A). Quantification showed that ATF-2-depleted cells exhibited elevated levels of p53 (~2.5-fold), p21 (~2.5-fold) and Cyclin D1 (~3.5-fold) compared to controls (Figure 5.8B). However, simultaneous depletion of ATF-2 and p53 (ATF-2/p53) reduced protein expression back to baseline levels (Figure 5.8B). These findings suggest that endothelial ATF-2 activity represses p53-dependent p21 and Cyclin D1 expression. To determine the requirement for p53 in VEGF-A-stimulated ATF-2-
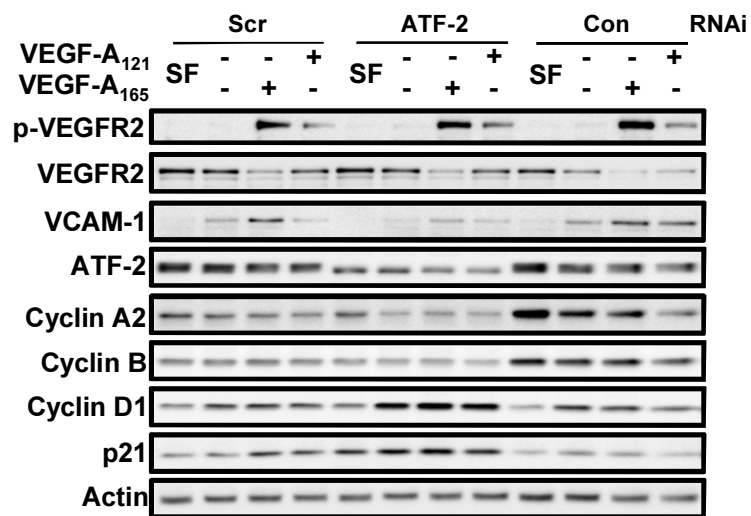
Figure 5.7. Cyclin D1 and p21 are elevated in ATF-2-depleted endothelial cells. Control, scrambled (Scr) or human endothelial cells depleted of ATF-2 were synchronised, prior to cell cycle release in either serum-free (SF), or full growth media supplemented with either (0.25 nM) VEGF-A$_{165}$ or VEGF-A$_{121}$ for 0 or 8 h. Endothelial cells were then lysed and processed for immunoblot analysis using antibodies against Cyclin A2, Cyclin B, Cyclin D1, p21, VCAM-1 and ATF-2. The data in this figure was provided by Dr A. F. Odell (Faculty of Medicine & Health, University of Leeds, UK).
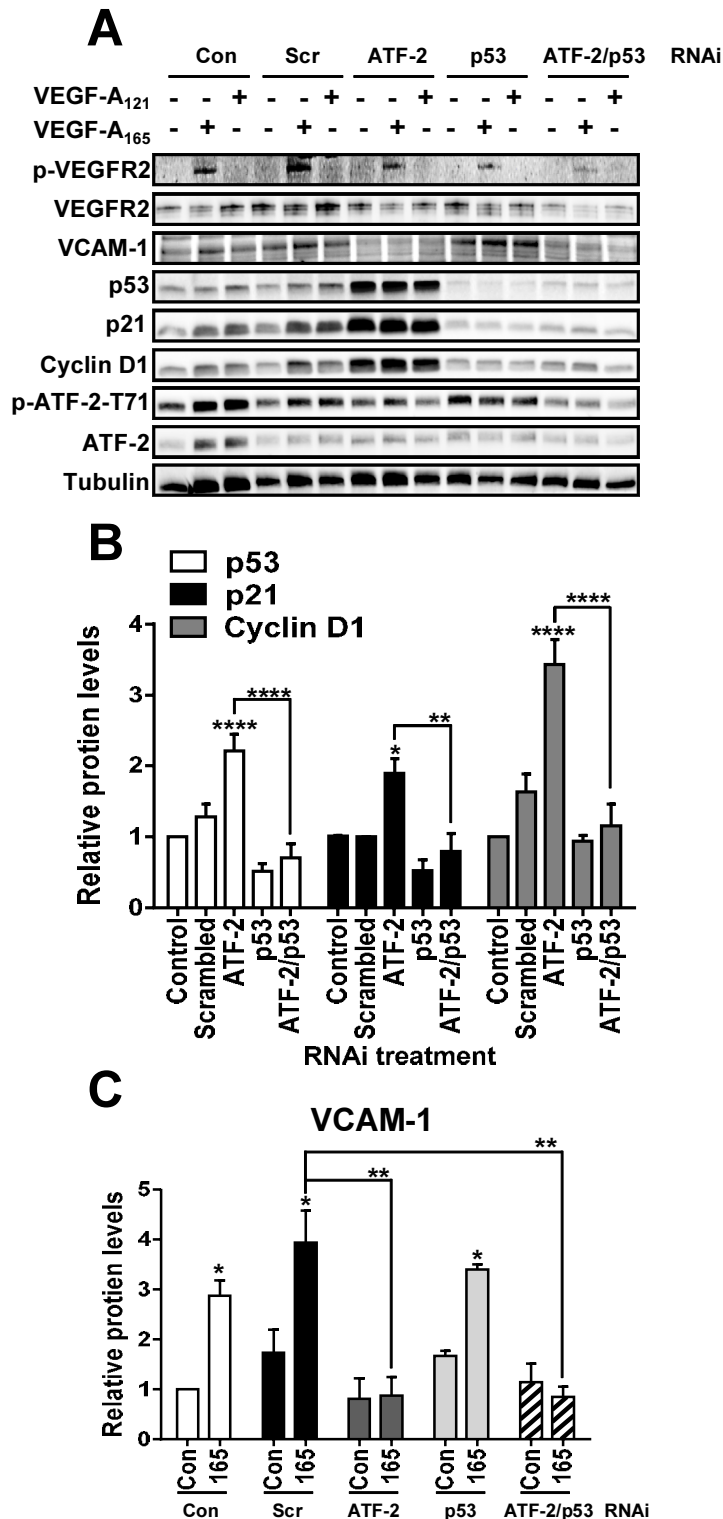
Figure 5.8. ATF-2 activity represses p53-dependent gene expression.
(A) Control, Scrambled (Scr) or human endothelial cells depleted of ATF-2, p53 or ATF-2/p53 were synchronised, prior to cell cycle release and stimulation with either (0.25 nM) VEGF-A$_{165}$ or VEGF-A$_{121}$ for 0 or 8 h. Endothelial cells were then lysed prior to immunoblot analysis and quantification of basal (B) p53, p21, Cyclin D1, and (C) VEGF-A$_{165}$-stimulated VCAM-1 protein levels. Error bars indicate ±SEM (n≥3). p<0.05 (*), *p*<0.01 (**), *p*<0.0001 (****). The data in panels B and C was provided in part by Dr A. F. Odell (Faculty of Medicine & Health, University of Leeds, UK).

regulated gene expression, we also analysed VCAM-1 levels in these experiments (Figure 5.8A). Quantification showed that VEGF-A$_{165}$-stimulated increase in VCAM-1 expression was completely blocked when ATF-2 was depleted using RNAi (Figure. 5.8C). However, p53 depletion alone did not significantly affect VCAM-1 expression (Figure 5.8C). Furthermore, combined ATF-2/p53-depletion did not rescue VEGF-A$_{165}$-stimulated VCAM-1 expression (Figure 5.8C). These findings argue that ATF-2-regulated *VCAM-1* gene expression operates independently of the p53 tumour suppressor.

5.2.4. Tpl2 modulates elevated p53 tumour suppressor levels

Our findings showed that the depletion of ATF-2 levels caused a subsequent increase in Tpl2 levels (Figure 5.4D). Therefore, one possibility is that increased Tpl2 activity elevates p53 levels. To test this idea, we compared controls with ATF-2, Tpl2 or ATF-2/Tpl2-depleted endothelial cells which had been synchronised using a thymidine block (Figure 5.9A). As predicted, endothelial cells depleted of ATF-2 displayed increased p53 (~2-fold), p21 (~2-fold) and Cyclin D1 (~3.5-fold; Figure 5.9B). However, Tpl2 depletion had no effect (Figure 5.9, A and B). Interestingly, combined ATF-2/Tpl2 depletion caused significant reduction in all three proteins (p53, p21, Cyclin D1), compared to ATF-2 depletion (Figure 5.9B). This data suggest that in response to ATF-2 depletion, elevated Tpl2 levels are responsible for the p53-dependent elevation of *p21* and *Cyclin D1* gene expression.

5.2.5. ATF-2 is required for endothelial cell cycle progression

Elevated p53 expression and increased transcription of its target genes can promote cell cycle arrest at both G1 and G2/M in different cell types (Agarwal et al., 1995; Taylor and Stark, 2001). One prediction is that Tpl2 regulation of p53 could alter endothelial cell cycle progression. To test this, endothelial cells expressing transgene fluorescent ubiquitination cell cycle indicator (FUCCI) proteins (see Materials and Methods) were used to assess cell cycle progression (Figure 5.10A and 5.11, A-L). Lentiviral-transduced endothelial cells stably expressing the mAG-hGeminin reporter show staining during G2/M (Figure 5.10A, green) whereas expression of the mKO2-hCdt reporter is only evident during G1 (Figure 5.10A, red). Endothelial cells expressing these reporter constructs were subjected to control, ATF-2, p53, Tpl2, ATF-2/p53 or ATF-2/Tpl2 depletion and synchronised in G1 using thymidine block and serum starvation. Following cell cycle release in complete growth media, cells were examined by microscopy at 8 h or 30 h for hGeminin and hCdt expression (Figure 5.10A and 5.11, A-L). Quantification of the number of cells
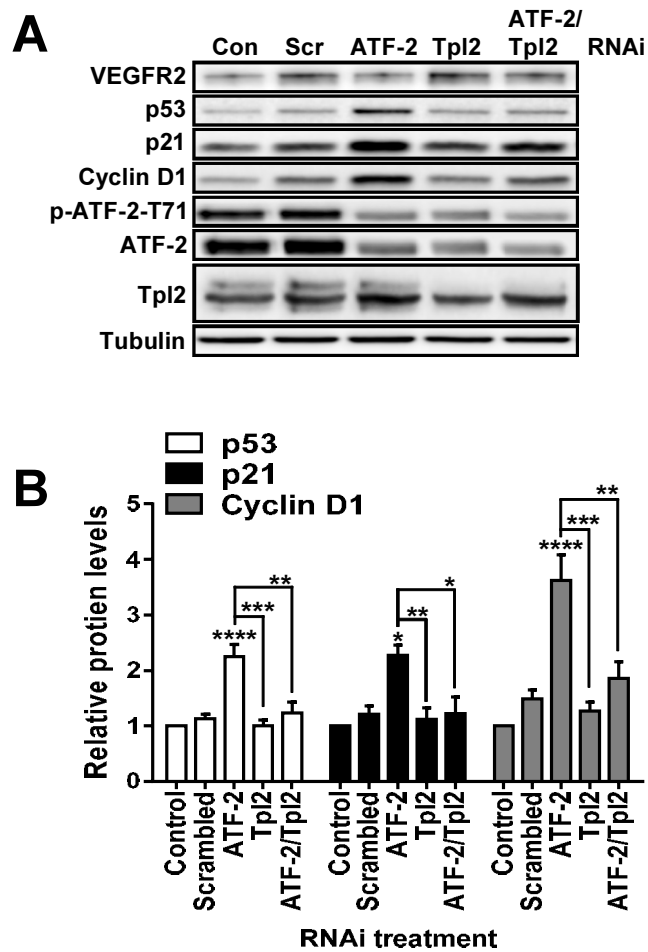
Figure 5.9. Tpl2 modulates p53 levels in ATF-2-depleted endothelial cells. Control, scrambled (Scr) or human endothelial cells depleted of ATF-2, Tpl2 or ATF-2/Tpl2 were synchronised, prior to cell cycle release (8 h). Endothelial cells were then lysed prior to (A) immunoblot analysis and quantification of (B) p53, p21 and Cyclin D1 protein levels. Error bars indicate ±SEM (n≥3). $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$ (****).
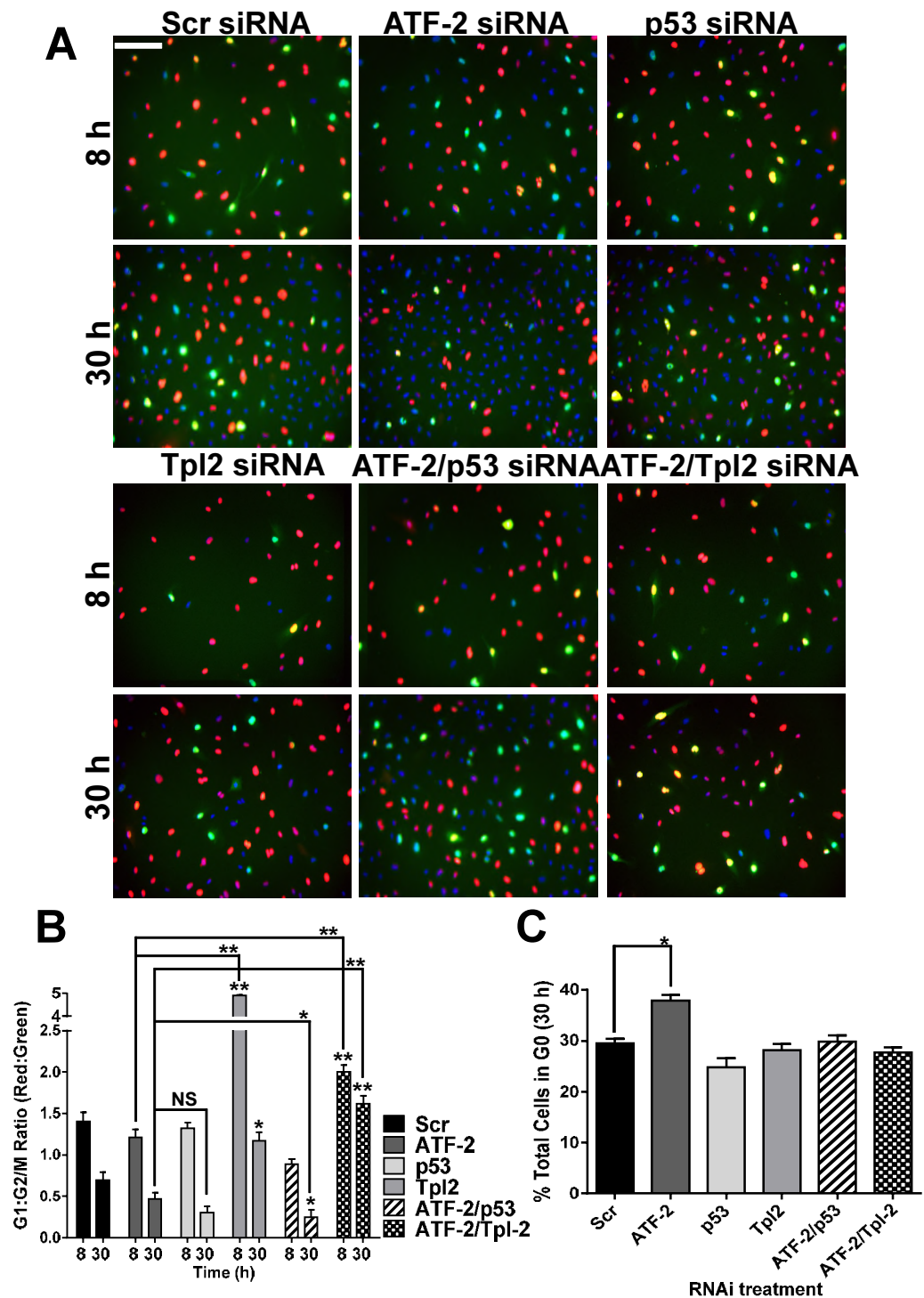
Figure 5.10. Loss of ATF-2 causes endothelial cell quiescence. (A) Endothelial cells transduced with FUCCI-encoding lentiviral vectors (see Material and Methods) were treated with scrambled (Scr), ATF-2, p53, Tpl2 ATF-2/p53 or ATF-2/Tpl2-specific siRNA duplexes prior to synchronisation using thymidine block. Cells were imaged at 8 and 30 h following cell cycle release. Scale bar, 50 μm. (B) The intensity of mAG-hGeminin (green) and mKO2-hCdt (red) expression was determined together with Hoechst 33342 counterstaining (blue) and background corrected using Metamorph 6 software. Expression was stratified and the ratio of G1:G2/M cells determined. (C) The total number of cells across all time points displaying neither hGeminin nor hCdt expression (i.e. G0 population of cells; shown in blue) was quantified and expressed as a percentage of total cells imaged. Error bars indicate ±SEM (n≥3).NS=non-significant, $p<0.05$ (*), $p<0.01$ (**). Data present in this figure was provided by Dr. A. F. Odell (Faculty of Medicine & Health, University of Leeds, UK).
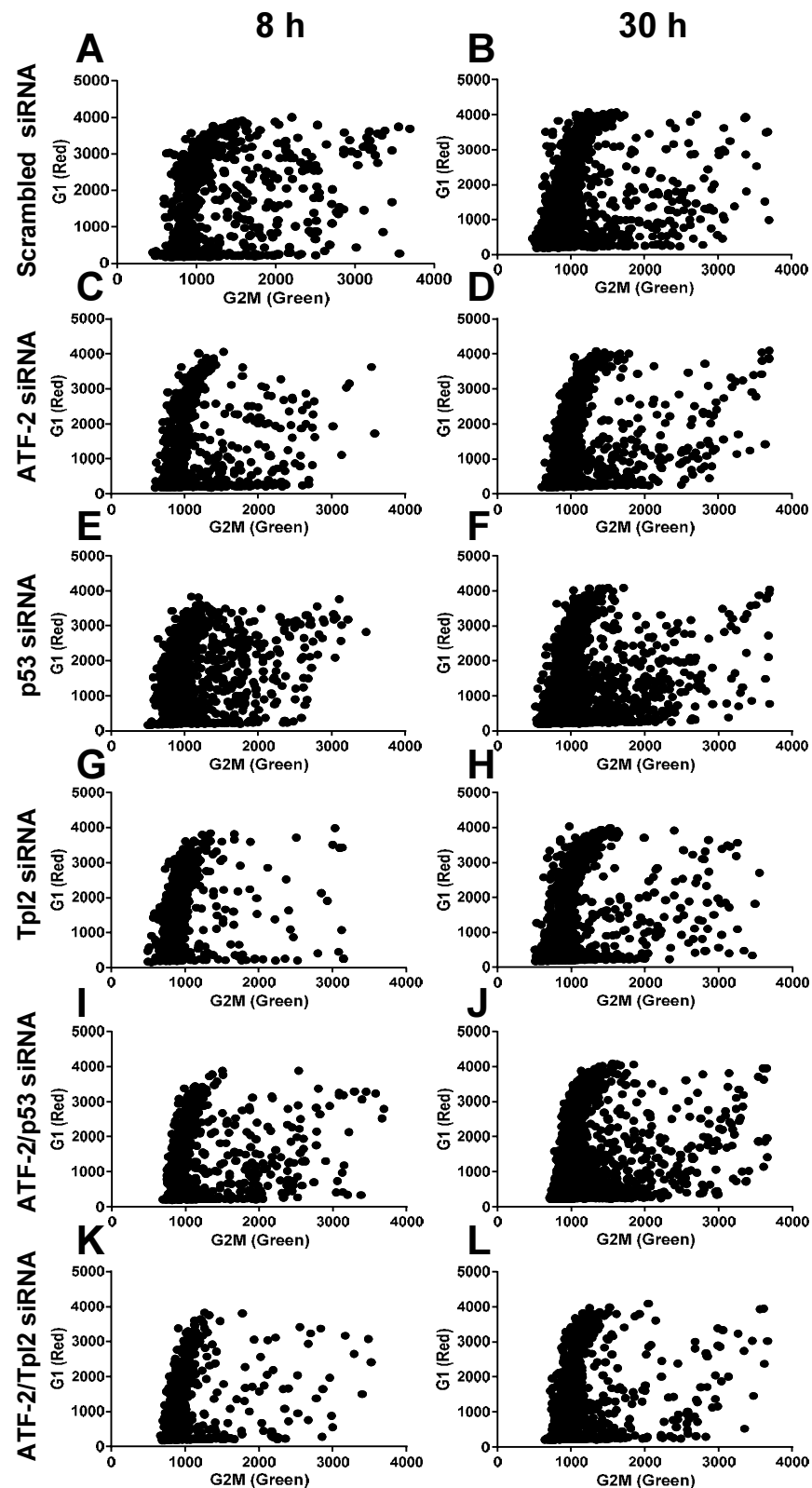
Figure 5.11. Analysis of ATF-2 depletion on cell cycle progression using FUCCI technology. Representative scatter plots of images quantified in Figure 5.10 displaying the relative expression of hGeminin (G2/M, Green) in comparison to hCdt (G1, Red) at 8 and 30 h post-cell cycle release in endothelial cells treated with (A-B) scrambled, (C-D) ATF-2, (E-F) p53, (G-H) Tpl2, (I-J) ATF-2/p53 or (K-L) ATF-2/Tpl2-specific siRNA duplexes. The data presented in this figure was provided by Dr A. F. Odell, (Faculty of Medicine & Health, University of Leeds, UK).

within the G1:G2/M ratio within each experiment showed that depletion of ATF-2 had little or no effect on the population of cells within G1 or G2/M compared to scrambled cells (Figure. 5.10B). In contrast, Tpl2 depletion dramatically increased the number of cells in G1 after 8 h, although this effect was substantially decreased after 30 h (Figure 5.10B), indicating that Tpl2 depletion delays cell cycle progression. Interestingly, combined depletion of ATF-2 and Tpl2 increased the proportion of cells in G1 at 8 and 30 h in comparison to control or ATF-2-depleted endothelial cells (Figure 5.10B).

Although this analysis did not show a definitive block in endothelial cell cycle progression in ATF-2-depleted endothelial cells, another possibility was that depletion of Tpl2, ATF2 or p53 promotes cellular quiescence (i.e. increased residence in G_0). This can be quantified by comparing the total number of cells exhibiting DAPI staining of nuclear DNA (Figure. 5.10A, blue), to the proportion of cells exhibiting either mKO2 or mAG expression (endothelial cells in G_0 should express neither hGeminin (green) nor hCdt (red)). Quantification revealed an elevated G_0 population only in ATF-2-depleted cells (Figure. 5.10C); an effect that was lost upon combined depletion of ATF-2 with either p53 or Tpl2 (Figure. 5.10C). Constitutive ATF-2 expression is thus required to maintain effective endothelial cell cycle progression by preventing Tpl2-mediated p53-dependent cellular quiescence.

Previous work carried out for this thesis showed that ATF-2 depletion caused reduced endothelial cell proliferation (see earlier in chapter 4). To further investigate the roles played by Tpl2, ATF-2 and p53 in this pathway, we used RNAi to deplete these proteins either alone or in combination followed by cell proliferation analyses (Figure 5.12). Depletion of ATF-2 caused ~40% reduction in basal endothelial cell proliferation compared to controls (Figure 5.12). However, either combined ATF-2/p53 or ATF-2/Tpl2 depletion restored endothelial cell proliferation to baseline levels (Figure 5.12). To determine whether such regulation of endothelial proliferation was important in VEGF-A-stimulated tubulogenesis we performed an endothelial-fibroblast co-culture assay (Figure 5.13A). Quantification revealed that Tpl2 depletion resulted in an ~25% reduction in endothelial tubule length in comparison to controls (Figure 5.13B). However, p53 depletion had little or no effect (Figure 5.13B). As expected, ATF-2 depletion reduced endothelial tubulogenesis by ~50% (Figure 5.13B); however such effects were absent under conditions of ATF-2/p53 or ATF-2/Tpl2 depletion (Figure 5.13B). The loss of ATF-2 expression thus correlates with increased Tpl2-mediated p53-dependent cellular quiescence (G0)
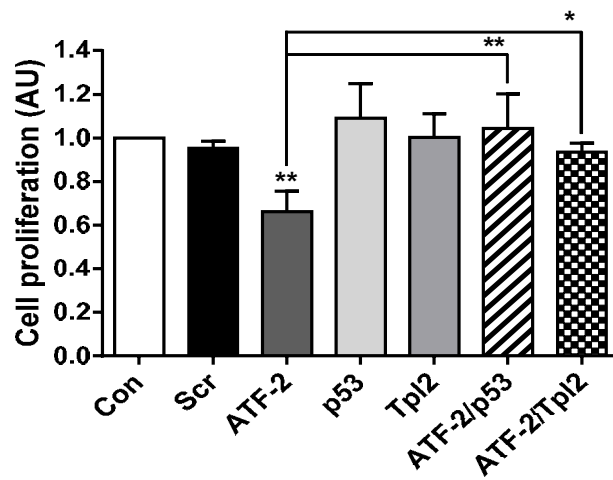
Figure 5.12. Re-engagement of cell cycle progression rescues endothelial cell proliferation. (A) Control, scrambled (Scr) or human endothelial cells depleted of ATF-2, p53, Tpl2, ATF-2/p53 or ATF-2/Tpl2 were seeded into a cellular assay to assess basal endothelial cell proliferation. Error bars indicate ±SEM (n=3). $p<0.05$ (*), $p<0.01$ (**).
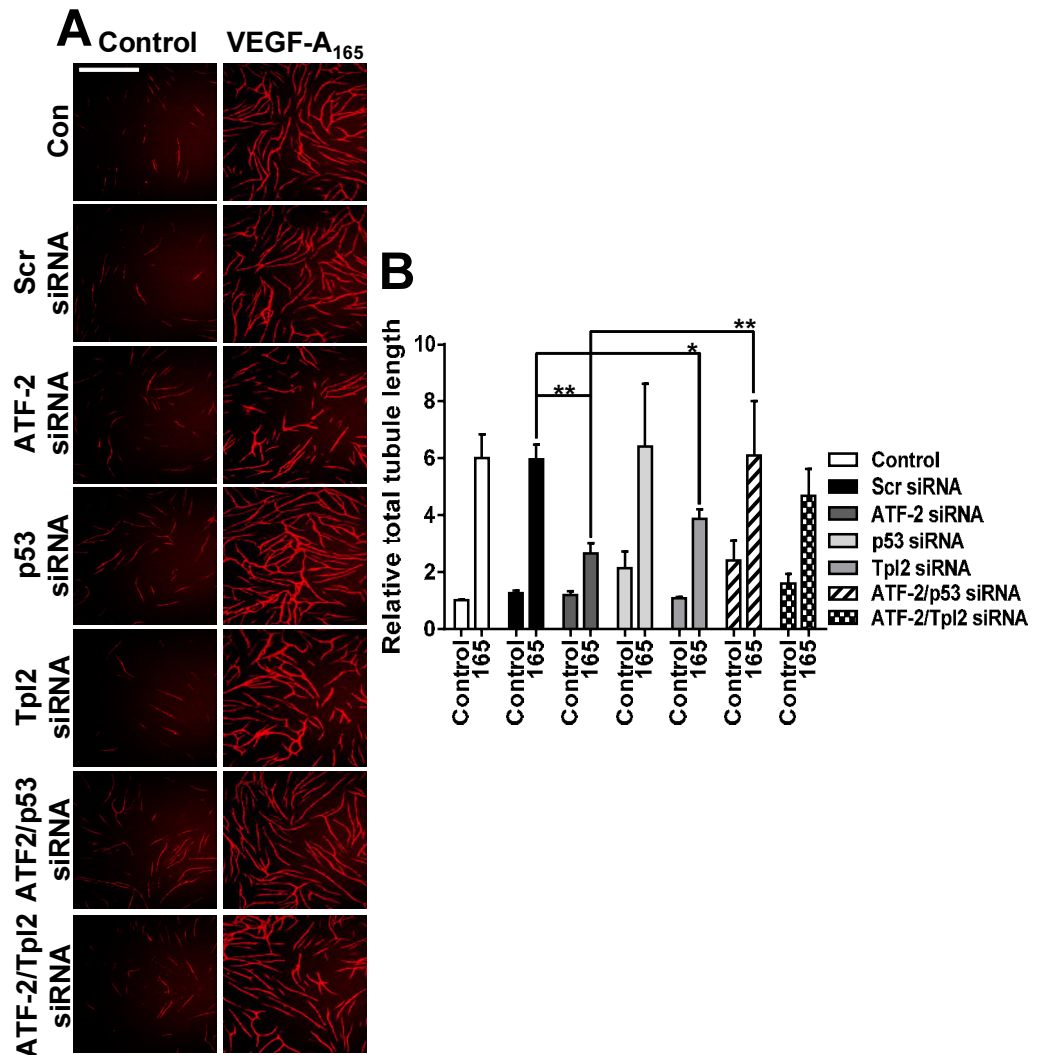
Figure 5.13. Re-engagement of cell cycle progression rescues endothelial tubulogenesis. (A-B) Control, scrambled (Scr) or human endothelial cells depleted of ATF-2, p53, Tpl2, ATF-2/p53 or ATF-2/Tpl2 were seeded into a cellular assay to assess and quantify VEGF-A$_{165}$ (0.25 nM) stimulated tubulogenesis. Scale bar, 1000 μm. Error bars indicate ±SEM (n=3). $p<0.05$ (*), $p<0.01$ (**).

and a reduced capacity for endothelial cell responses such as tubulogenesis and proliferation.

5.2.6. Tpl2 kinase inhibition regulates p53 status and endothelial cell function

Pharmacological inhibitors that target Tpl2 kinase activity also attenuate angiogenesis and endothelial cell proliferation *in vivo,* but the mechanism underlying such regulation is unclear (Lee et al., 2013b). One such small molecule Tpl2 inhibitor reduced ATF-2 phosphorylation and increased Tpl2 expression (Figure 5.4). One possibility is that Tpl2 kinase inhibition modulates p53 status and thus endothelial cell function via elevation of Tpl2 expression. To test this idea, endothelial cells were treated with the same Tpl2-specific kinase inhibitor over a 0-8 h time course, followed by lysis and analysis of p53, p21 and Cyclin D1 protein levels (Figure 5.14A). Quantification showed that Tpl2 kinase inhibition promoted a gradual but significant rise in p53 levels over a 8 h period (Figure 5.14B) which was similar to that observed for Cyclin D1 (Figure 5.14D). However, p21 levels were relatively static but suddenly increased at the 8 h time point (Figure 5.14C). To further analyse the link between Tpl2 kinase inhibition and cell cycle progression, we used flow cytometry to monitor DNA content (Figure 5.15, A and B). Quantification showed an ~2.3-fold increase in the G1:G2/M ratio in comparison to control cells, which was indicative of proportionally increased G1 and reduced G2/M cell populations (Figure 5.15C).

Further analysis was performed to assess whether such a block in Tpl2 kinase activity could inhibit outputs such as endothelial tubulogenesis (Figure 5.16, A and B) and aortic sprouting (Figure 5.17, A and B). Treatment with a Tpl2-specific kinase inhibitor caused ~50% decrease in basal and VEGF-A-stimulated tubulogenesis compared to controls (Figure 5.16B). Furthermore, VEGF-A$_{165}$-stimulated sprouting from mouse aortic rings was reduced ~5-fold upon Tpl2 kinase inhibition (Figure 5.17B). These findings support the concept of a Tpl2/ATF-2 regulatory axis which modulates p53 status, cell cycle progression and thus, the endothelial cell response.
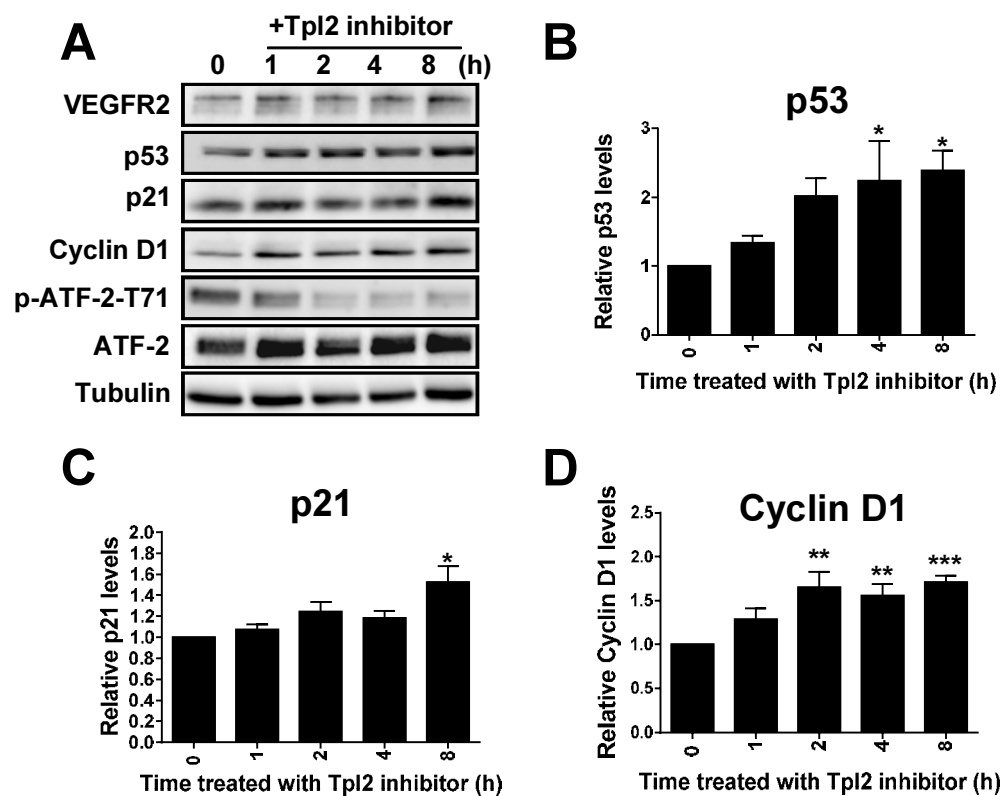
Figure 5.14. Pharamcological inhibition of Tpl2 kinase activity promotes p53-dependent gene expression. (A) Human endothelial cells treated with a Tpl2-specific kinase inhibitor for 0, 1, 2, 4 or 8 h, were lysed prior to immunoblot analysis and quantification of (B) p53, (C) p21 and (D) Cyclin D1 protein levels. Error bars indicate ±SEM (n≥3). $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***).
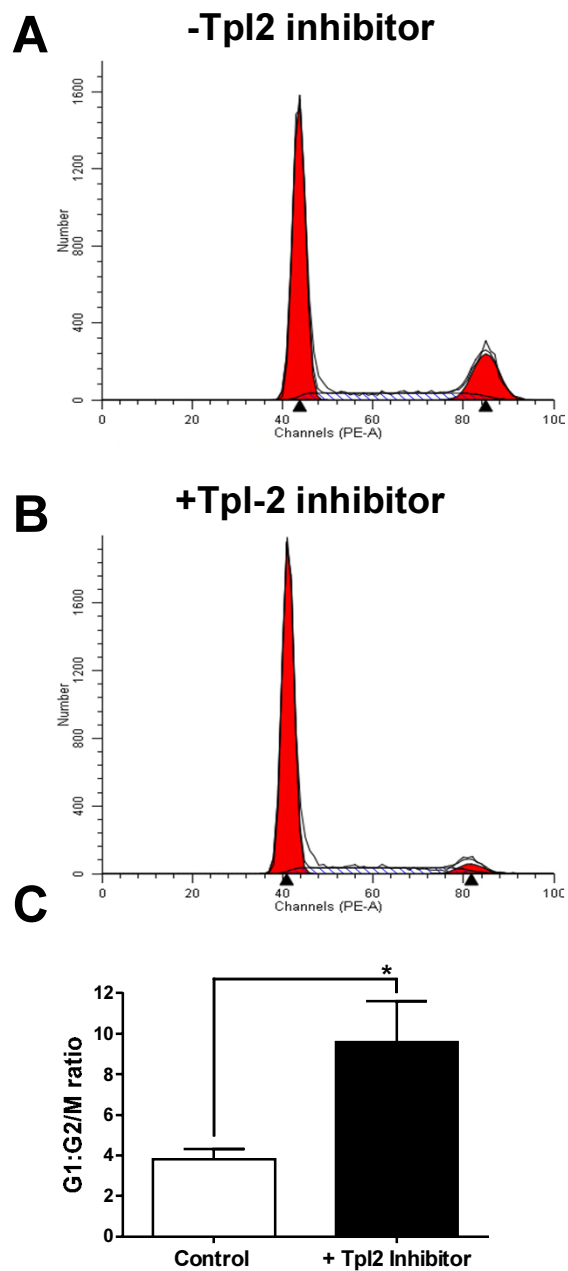
Figure 5.15. Inhibition of Tpl2 kinase activity promotes endothelial cell cycle arrest. (A-B) Human endothelial cells were synchronised using thymidine block prior to cell cycle release (24 h) in the (A) absence or (B) presence of 2 μM Tpl2 kinase inhibitor. Endothelial cells were then fixed and stained using propidium iodide prior to assessment of DNA content via flow cytometry. (C) Quantification of the G1:G2/M ratio. Error bars indicate ±SEM (n≥3). $p<0.05$ (*).
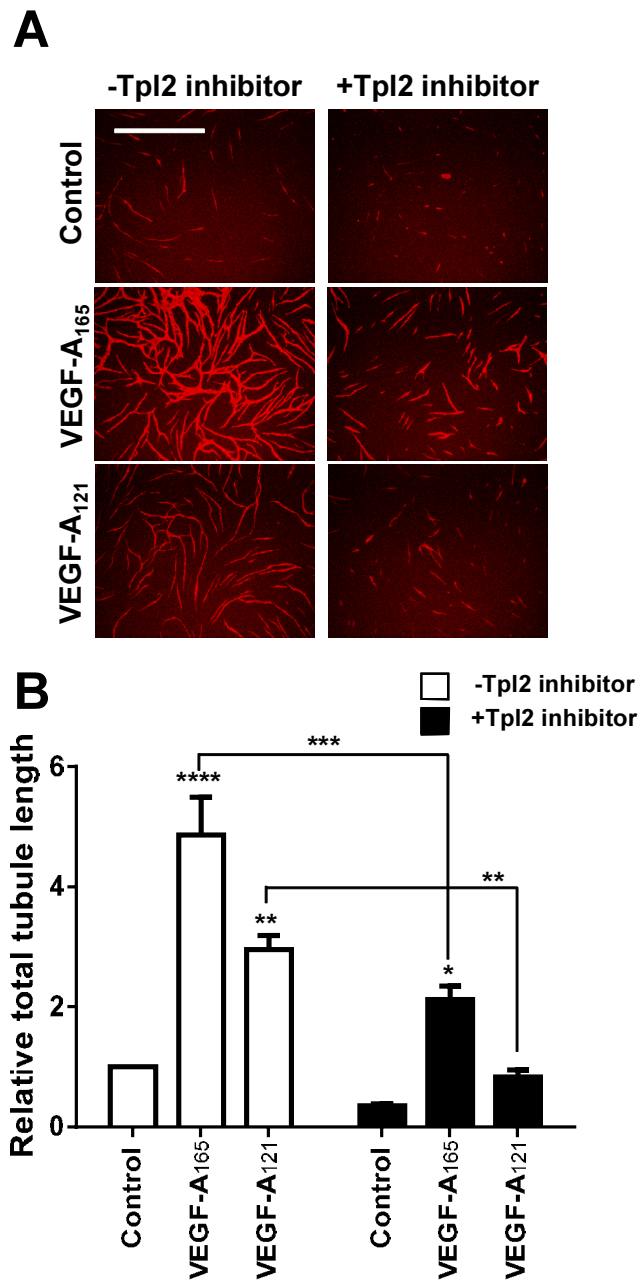
Figure 5.16. Inhibition of Tpl2 kinase activity perturbs VEGF-A-stimulated endothelial cell tubulogenesis. (A) Human endothelial cells were co-cultured on a bed of primary human fibroblasts for 7 days and stimulated with either VEGF-A$_{165}$ or VEGF-A$_{121}$ (0.25 nM) ±2 µM Tpl2 kinase inhibitor. Co-cultures were fixed and stained, prior to visualisation using immunoflurescence microscopy. (B) Quantification of endothelial cell total tubule length. Error bars indicate ±SEM (n≥3). $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$ (****).
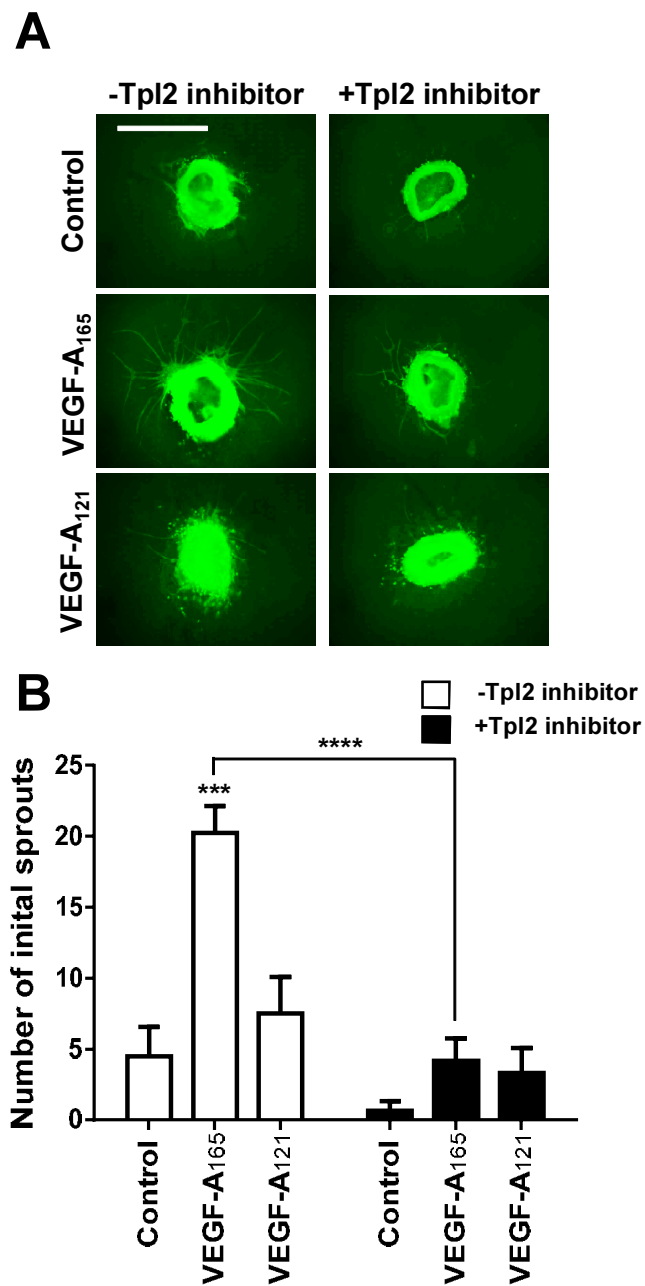
Figure 5.17. Inhibition of Tpl2 kinase activity perturbs VEGF-A-stimulated aortic sprouting. (A) Mouse aortic rings were stimulated with either VEGF-A$_{165}$ or VEGF-A$_{121}$ (0.25 nM) ±2 μM Tpl2 kinase inhibitor. Scale bar, 1000 μm. Aortic rings were fixed and stained, prior to visualisation using lectin-based staining and fluorescence microscopy. (B) Quantification of endothelial sprouting. Error bars indicate ±SEM (n≥3). $p < 0.001$ (***), $p < 0.0001$ (****).

5.3. DISCUSSION

The findings presented here support a role for Tpl2 (MAP3K8) as a signalling nexus which co-ordinates cellular decision-making pathways that impacts on essential endothelial cell outcomes. Here, we show that VEGF-A$_{165}$ stimulates increased Tpl2 expression and this is required for endothelial cell responses. Furthermore, they show that basal endothelial Tpl2 kinase activity stabilises ATF-2 turnover via promoting its constitutive phosphorylation on residue T71 (ATF-2-pT71; Figure 5.18A). This novel function modulates VEGF-A-stimulated ATF-2-dependent gene transcription and cellular responses (Figure 5.18A). Furthermore, ATF-2 is essential for endothelial cell cycle progression with loss of ATF-2 triggering cellular quiescence (Figure 5.18B). One explanation for this is elevated *p21* and *Cyclin D1* gene expression in response to ATF-2 depletion; which is reliant on both Tpl2 and p53 (Figure 5.18B). Therefore, Tpl2 and ATF-2 form a regulatory axis, which modulates endothelial gene expression and cell cycle progression (Figure 5.18, A and B).

These findings suggest a model whereby Tpl2 integrates information conveyed through VEGFR2 and stress-activated signal transduction pathways to modulate endothelial cell function (Figure 5.18, A and B). Several lines of evidence support this. Firstly, endothelial cell *Tpl2* gene transcription is regulated by VEGF-A with ~3.7-fold rise in Tpl2 mRNA levels evident upon stimulation. This is important for the endothelial response as Tpl2 is required for the expression of other VEGF-A regulated genes (e.g. *VCAM-1*) by modulating the phosphorylation status of ATF-2. Notably, Tpl2 depletion or pharmacological inhibition of kinase activity drastically reduces ATF-2-T71 phosphorylation and attenuates ATF-2-dependent *VCAM-1* gene expression. Furthermore, loss of Tpl2 attenuates endothelial cell responses such as endothelial-leukocyte interactions and tubulogenesis. Secondly, depletion of ATF-2 causes a striking increase in Tpl2 levels, likely triggered by an endothelial stress response. Additionally, this stress response triggered upon loss of ATF-2 results in increased p53 expression and cellular quiescence (i.e. G$_0$). Furthermore, this phenomenon is reliant on both Tpl2 and p53 as combined depletion of ATF-2/Tpl2 or ATF-2/p53 alleviates cell cycle arrest, allowing endothelial cell cycle progression, proliferation and tubulogenesis. Finally, pharmacological inhibition of Tpl2 kinase activity attenuates ATF-2 phosphorylation which triggers a stress response resulting in elevated Tpl2 levels, p53 expression and subsequent cell cycle arrest. Therefore, a Tpl2-centred regulatory mechanism coordinates signal
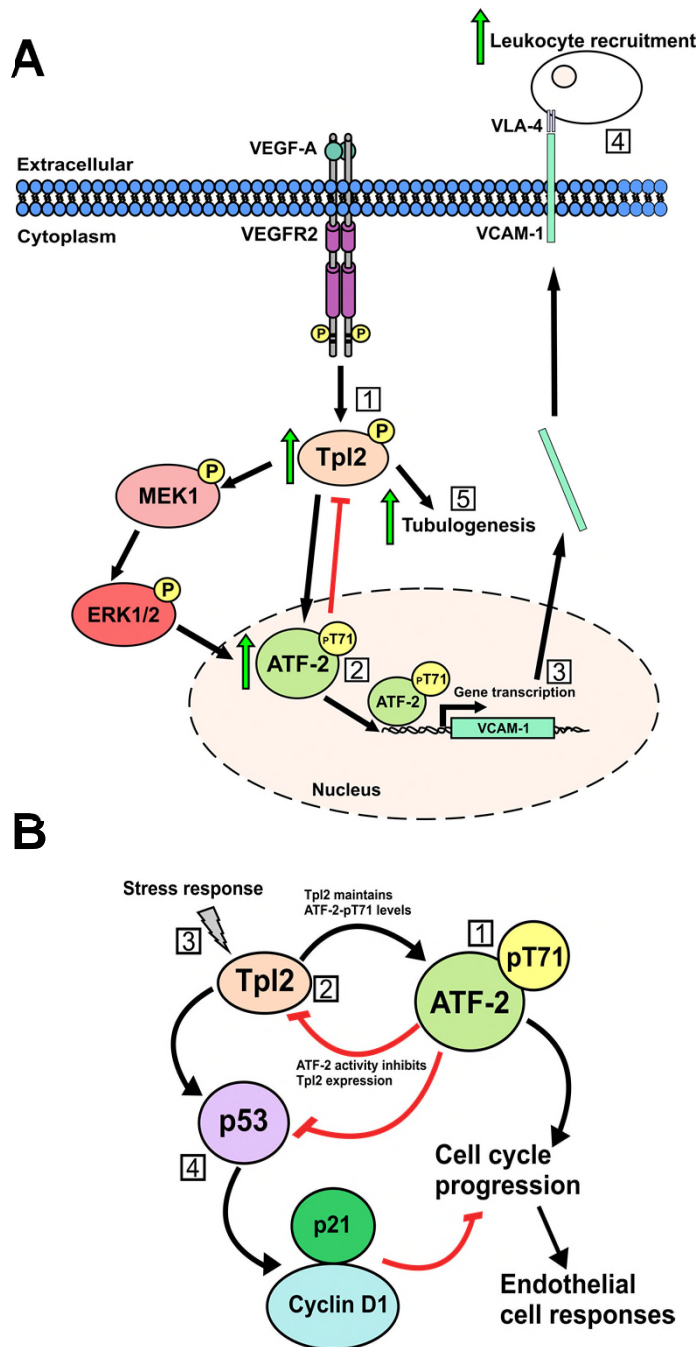
Figure 5.18. Tpl2 is a signalling nexus which regulates endothelial gene expression and cell cycle progression. (A) Step 1 - VEGF -A$_{165}$ stimulation promotes increased Tpl2 expression and activation. Step 2 - Elevated Tpl2 kinase activity stimulates increased phosphorylation of ATF-2-pT71. Step 3 - Hyperphosphorylation of ATF-2-pT71 triggers *VCAM-1* gene expression. Step 4 - Elevated *VCAM-1* gene expression facilitates increased VEGF-A-stimulated leukocyte recruitment. Step 5 - Furthermore, Tpl2 kinase activity is required for VEGF-A$_{165}$ stimulated tubulogenesis. (B) Step 1 - ATF-2 activity is integral for endothelial cell cycle progression and cellular responses. Step 2 - Tpl2 modulates ATF-2 activity by maintaining constitutive levels of ATF-2-pT71. This constitutive phosphorylation feeds back to control Tpl2 and p53 protein levels. Step 3 - Reduced ATF-2 activity triggers a stress response and elevation of Tpl2 protein levels. Step 4 - Elevated Tpl2 protein levels stimulate p53-dependent p21 and Cyclin D1 gene expression and subsequent cell cycle arrest.

transduction via separate but parallel pathways to balance cellular commitments to gene expression and cell division.

This data now shows that Tpl2 is essential for the pro-angiogenic response by regulating the status of ATF-2 and p53, two key factors which control nuclear gene expression and cell cycle progression. A recent study showed that inhibition of Tpl2 kinase activity with the same small molecule kinase inhibitor used here (CAS 871307-18-5), impaired tumor angiogenesis and the pro-angiogenic response to VEGF-A$_{165}$ in mouse models (Lee et al., 2013), however a mechanistic explanation was lacking. Our work now shows that pharmacological inhibition of Tpl2 triggers increased p53 expression and subsequent cell cycle arrest, resulting in perturbed endothelial cell tubulogenesis and aortic sprouting. Interestingly, Tpl2 is also essential for pro-inflammatory endothelial cell responses. VEGF-A$_{165}$-stimulated MEK1-ERK1/2-dependent hyperphosphorylation of ATF-2-T71 is essential for isoform-specific programing of *VCAM-1* gene expression and endothelial responses (see earlier in chapter 4). This study now identifies Tpl2 as an additional regulator of this mechanism (Figure 5.18A). Furthermore, Tpl2 becomes phosphorylated in response to VEGF-A (Lee et al., 2013) and is a known activator of the MEK1-ERK1/2 pathway (Beinke et al., 2003); thus it is likely that Tpl2 acts upstream of MEK1 in response to VEGF-A-stimulation to modulate ATF-2 phosphorylation (Figure 5.18A). In addition to its role in angiogenesis, work elsewhere has described the requirement of Tpl2 in immune cell functions. In the context of inflammation and disease, inhibition of Tpl2 kinase activity has been shown to alleviate symptoms of rheumatoid arthritis and inflammatory bowel disease (Gantke et al., 2011; Dumitru et al., 2000; Kontoyiannis et al., 2002; Vougioukalaki et al., 2011; Hall et al., 2007). Aberrant and elevated angiogenesis is also implicated in rheumatoid arthritis (Carmeliet, 2005). Therefore, Tpl2 kinase inhibitors acting at the level of endothelial cells may help alleviate symptoms by contributing to both diminished inflammation and angiogenesis by our proposed model (Figure 5.18A).

We now show that ATF-2 is essential for endothelial cell cycle progression, a phenomenon previously documented in other cell types (An et al., 2013; Walluscheck et al., 2013). Depletion of ATF-2 likely triggers a cellular stress response which elevates Tpl2 levels; through which it further acts to stimulate p53 expression. In support of this finding elevated Tpl2 expression is also linked to increased p53 and p21 expression in lung cancer (Gkirtzimanaki et al., 2013). However, other studies suggest elevated p53 levels repress Cyclin D1 expression

(Rocha et al., 2003). In our model, elevated endothelial p53 expression is linked to increased p21 and Cyclin D1 expression and subsequent cell cycle arrest (Figure 5.18B). It has previously been shown that Tpl2 kinase stability and activity can be regulated through binding to the NFκB precursor protein, p105 (Beinke et al., 2003; Cho and Tsichlis, 2005; Waterfield et al., 2003). A possible model for Tpl2 control in the endothelium is that Tpl2 phosphorylation by upstream kinases causes activation and dissociation from p105. Furthermore, dissociated p105 then undergoes proteolytic cleavage to release the p50 subunit, which forms a heterodimer with p65 RelA. This p50/p65 complex can translocate into the nucleus and modulate both pro-angiogenic and pro-inflammatory gene expression. Interestingly, one such target gene is p53 (Yu et al., 2014; Lin et al., 2012). Additionally, NFκB is known to regulate both *Cyclin D1* and *p21* gene expression (Basile et al., 2003; Guttridge et al., 1999; Wu and Lozano, 1994; Mauro et al., 2011; Johnson and Perkins, 2012). Stress-induced increases in Tpl2 levels and kinase activity are linked to elevated NFκB-regulated pathways (Lee et al., 2009; Lee et al., 2015). Therefore, increased NFκB activity may link Tpl2 status to p53 expression and cell cycle arrest. Alternatively, increased Tpl2 levels and activity could stimulate JNK signal transduction to regulate p53-dependent gene expression (Gkirtzimanaki et al., 2013).

There is much debate as to whether Tpl2 is a proto-oncogene or a tumour suppressor (Lee et al., 2015). Increased Tpl2 expression is linked to poor patient prognosis and increased tumorigenesis, whereas inhibition of Tpl2 kinase activity can cause reduced tumour angiogenesis and metastasis (Lee et al., 2013; Pan et al., 2013; Lee et al., 2013). Conversely, decreased Tpl2 expression was found to confer an increased incidence of lung tumours and reduced patient survival (Gkirtzimanaki et al., 2013; Serebrennikova et al., 2012). One argument is that reduced Tpl2 levels prevent elevated p53 levels in response to oncogenic stress, thus allowing cells to better progress into a proliferative, cancer-like state (Gkirtzimanaki et al., 2013). This study now provides new insights to support these dual properties of Tpl2. Under certain conditions Tpl2 can help promote cellular growth, with loss of its activity attributing to diminished cellular responses (Figure 5.18A). However, during cellular stress, Tpl2 could promote cell cycle arrest; in this context, loss of Tpl2 activity could be viewed as pathological as progression through the cell cycle could contribute to increased incidence of cellular transformation (Figure 5.18B).

The mechanisms by which endothelial cells respond to VEGF-A by integrating short lived signal transduction (0-2 h) into long term (days to weeks) cellular responses are ill-defined. Our study now provides a framework that integrates Tpl2-mediated signal transduction pathways into the control of gene expression and cell cycle progression to impact on various cellular outcomes. For example, VEGF-A$_{165}$ but not VEGF-A$_{121}$ promotes short term Tpl2 gene transcription; which is a critical feature in long term endothelial decisions such as whether to undergo tubulogenesis or interact with leukocytes. Furthermore as Tpl2 is activated upon VEGF-A$_{165}$ stimulation (Lee et al., 2013), the initial elevation of Tpl2 levels by VEGF-A$_{165}$ may prime endothelial cells for further stimulation via these signal transduction pathway (Figure 5.18A).

In summary, this study supports the role of Tpl2 as a signalling nexus which integrates the flow of information conveyed from different signal transduction pathways. VEGF-A/VEGFR2-mediated signal transduction requires Tpl2 activity to trigger activation of the phosphorylation cascade, impacting on key nuclear regulators such as ATF-2. Furthermore, cellular stress-responsive signal transduction pathways impact on Tpl2, drastically promoting p53 protein stabilisation, *p21* and *Cyclin D1* gene expression and cell cycle arrest. Such events can be further integrated into a temporal framework which allows the endothelial cell to respond to different environmental cues that not only regulate short lived signal transduction but also lay the foundations for sustained changes in long term responses (e.g. cell proliferation and tubulogenesis). Finally, this work now provides a model for understanding and targeting such signal transduction pathways in a cell-specific manner to normalise altered endothelial cell function in pathologies such as cancer and cardiovascular disease.

CHAPTER 6

VEGF-A isoform-specific regulation of calcium ion flux, transcriptional activation and endothelial cell migration

6.1.   INTRODUCTION

VEGF-A binding to VEGFR2 triggers receptor dimerisation, linked to the activation of its tyrosine kinase domain, which elicits sustained downstream signal transduction integrated with receptor ubiquitination, trafficking and proteolysis (Bruns et al., 2009; Horowitz and Seerapu, 2012; Koch and Claesson-Welsh, 2012; Nakayama and Berger, 2013). A key aspect of VEGF-A-stimulated endothelial cell signal transduction is the elevated transcription of 100-200 target genes, which regulate a variety of cellular responses (Schweighofer et al., 2009; Rivera et al., 2011). Various studies have shown that VEGF-A isoforms differentially promote VEGFR2-dependent signal transduction and cellular outcomes (Kawamura et al., 2008; Kawamura et al., 2008; Zhang et al., 2000). However, the mechanism(s) which link VEGF-A isoform-specific signal transduction to nuclear gene transcription and specific endothelial responses are ill-defined.

To address the individual role of each VEGF-A splice isoform in regulating vascular function, we evaluated VEGF-A$_{121}$ and VEGF-A$_{165}$ for their ability to regulate signal transduction events linked to physiological responses. Here, we show that these two VEGF-A isoforms produce different intracellular signalling outcomes which impact on a calcium ion-dependent transcriptional 'switch' allowing for isoform-specific regulation of endothelial cell migration. Thus, VEGF-A isoforms could act as temporal and spatial cues that program endothelial responses that are calcium-dependent and essential for building unique vascular networks.

6.2.   RESULTS

6.2.1. VEGF-A isoforms cause differential VEGFR2 activation and signal transduction

VEGF-A-stimulation promotes VEGFR2 dimerisation and transautophosphorylation of several key tyrosine residues within its cytoplasmic domain (Koch and Claesson-Welsh, 2012), which stimulates downstream signal transduction pathways (Figure 6.1A). Recruitment of factors and enzymes that bind activated VEGFR2 stimulates

intracellular signalling events which modulate an array of endothelial cell responses in order to promote angiogenesis and regulate vascular development (Figure 6.1A). Various studies have shown that VEGF-A isoforms promote differential VEGFR2 activation and downstream signal transduction (Kawamura et al., 2008; Pan et al., 2007). Although, VEGF-A-stimulated VEGFR2-dependent signalling is well understood, it is still unclear how VEGF-A isoform-specific signal transduction is converted into nuclear gene transcription to differentially regulate endothelial cell responses. In order to further investigate this phenomenon, we first compared the ability of two VEGF-A isoforms (VEGF-A$_{165}$ and VEGF-A$_{121}$) to regulate signal transduction events via the VEGF-A/VEGFR2 signalling axis. Primary human umbilical vein endothelial cells (HUVECs) were titrated with 0.025, 0.25 and 1.25 nM of either VEGF-A$_{165}$ or VEGF-A$_{121}$ for 5 or 15 min prior to processing and immunoblot analysis of VEGFR2 activation and downstream signalling pathways (Figure 6.1B). Quantification of the relative changes in phosphorylation status of VEGFR2-pY1175 in response to a dose-dependent titration of VEGF-A$_{165}$ (Figure 6.2A) or VEGF-A$_{121}$ (Figure 6.2B) revealed that peak activation occurred within 5 min of ligand treatment. However, VEGF-A$_{121}$-stimulated VEGFR2-Y1175 phosphorylation (Figure 6.2B) was significantly reduced versus VEGF-A$_{165}$ treatment (Figure 6.2A) at 0.025 and 0.25 nM. However, upon stimulation with saturating levels of VEGF-A (1.25 nM), the peak level of VEGFR2 activation achieved in response to VEGF-A$_{165}$ (Figure 6.2A) was comparable to that induced by VEGF-A$_{121}$ (Figure 6.2B). Interestingly, at this VEGF-A concentration, activated VEGFR2 appeared to be dephosphorylated at an increased rate upon treatment with VEGF-A$_{121}$ (Figure 6.2B) compared to cells treated with VEGF-A$_{165}$ (Figure 6.2A).

Angiogenesis is a tightly regulated process and many endothelial cell responses constantly need fine-tuning for homeostasis and maintenance of vascular physiology (Carmeliet, 2005). VEGF-A stimulation regulates several endothelial cell responses through the activation of downstream signaling events including those that affect endothelial cell permeability (Akt-eNOS pathway), cell migration (p38 MAPK pathway) and cell proliferation (ERK1/2 pathway). Stimulation with either VEGF-A$_{165}$ or VEGF-A$_{121}$ promoted phosphorylation of endothelial nitric oxide synthase (eNOS), p38 MAPK and ERK1/2 (Figure 6.1A) enzymes. Quantification of eNOS-pS1177 levels revealed that stimulation with VEGF-A$_{121}$ (Figure 6.2D) resulted in a generally lower level of activation compared to VEGF-A$_{165}$ stimulation (Figure 6.2C). However, peak levels of eNOS activation were comparable between the two isoforms (Figure 6.2, C and D). Additionally both VEGF-A isoforms caused a
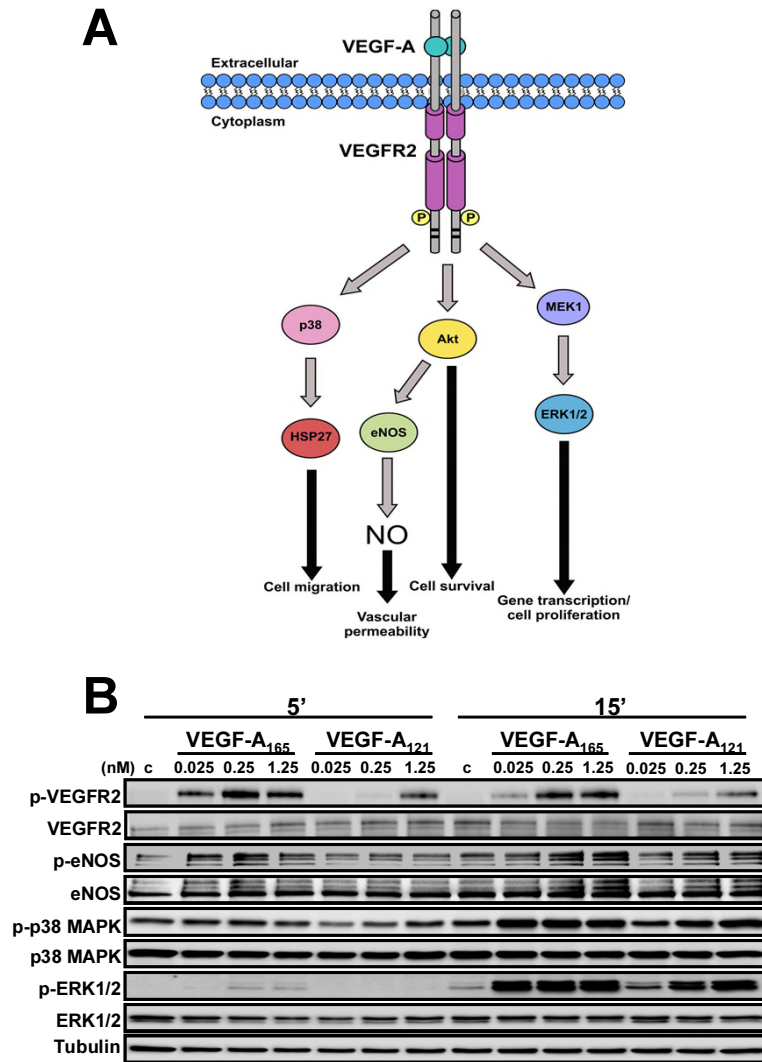
Figure 6.1. VEGF-A isoform-specific stimulated endothelial cell signalling. (A) Schematic depicting VEGF-A stimulated VEGFR2 phosphorylation, activation of downstream signalling pathways linking to cellular responses. Abbreviations: p38, p38 mitogen-activated protein kinase; HSP27, heat-shock protein of 27 kDa; Akt, Protein kinase B; eNOS, endothelial nitric oxide synthase; NO, nitric oxide; MEK1, mitogen-activated protein kinase 1; ERK1/2, p42/44 mitogen-activated protein kinase. (B) Endothelial cells subjected to different VEGF-A$_{165}$ or VEGF-A$_{121}$ concentrations (0, 0.025, 0.25 or 1.25 nM) for 5 or 15 min were lysed and processed for immunoblot analysis using phospho-specific antibodies against p-VEGFR2, p-eNOS, p-p38 and p-ERK1/2.

similar increase in p38 MAPK-pT180/pY182 levels at 1.25 nM ligand stimulation, with peak levels detected after 15 min (Figure 6.2, E and F). In contrast, VEGF-A$_{165}$ promoted ~2-fold increase in ERK1/2-T202/Y204 phosphorylation compared to VEGF-A$_{121}$ at the same concentration (1.25 nM); peak levels were still detected 15 min post stimulation (Figure 6.2, G and H). These findings suggest that VEGF-A isoforms have the capability to differentially stimulate multiple signal transduction pathways in endothelial cells.

6.2.2. VEGF-A isoforms promote differential PLCγ1 activation and corresponding cytosolic calcium ion flux

VEGF-A-stimulated generation of VEGFR2-pY1175 creates a phosphotyrosine-based epitope that recruits PLCγ1 to activated VEGFR2 at the plasma membrane (Takahashi et al., 2001). Subsequently, VEGFR2-mediated PLCγ1 phosphorylation on residue Y783 (PLCγ1-pY783) leads to enzymatic activation and hydrolysis of plasma membrane PIP$_2$ to DAG and IP$_3$, thus triggering a rise in cytosolic calcium ion flux and altered endothelial responses. To investigate VEGF-A isoform-specific PLCγ1-mediated responses, we monitored PLCγ1-pY783 levels upon titration with VEGF-A$_{165}$ or VEGF-A$_{121}$ for 5, 15, 30 or 60 min (Figure 6.3A). PLCγ1-pY783 levels were clearly elevated upon addition of VEGF-A$_{165}$ compared to VEGF-A$_{121}$ (Figure 6.3A) addition to endothelial cells. Quantification revealed VEGF-A$_{165}$ stimulated a rapid and transient rise in endothelial PLCγ1-pY783 levels within 5 min (Figure 6.3B) but VEGF-A$_{121}$-stimulated PLCγ1-pY783 peak levels were ~2-3-fold lower (Figure 6.3C).

PLCγ1-mediated IP$_3$ stimulates the release of intracellular Ca^{2+} stores (Figure 6.4A). Thus, we then carried out an analysis of cytosolic calcium ion levels in VEGF-A isoform-stimulated endothelial cells using a cell-permeable Ca^{2+}-sensitive fluorescent probe (Fura-2 AM). Upon titration of either VEGF-A$_{165}$ (Figure 6.4B) or VEGF-A$_{121}$ (Figure 6.4C) we observed different patterns of cytosolic calcium ion flux. The lowest concentration (0.025 nM) of VEGF-A$_{165}$ ligand caused a slow and sustained rise in cytosolic calcium ions (Figure 6.4B); this was not observed upon treatment with 0.025 nM VEGF-A$_{121.}$ A similar effect was observed with 0.25 nM VEGF-A isoform (Figure 6.4, B and C). Interestingly, the highest concentration ligand elicited similar rises in cytosolic Ca^{2+} levels with both VEGF-A$_{165}$ (Figure 6.4B) and VEGF-$_{A121}$ (Figure 6.4C). Quantification of the peak magnitude in ligand-
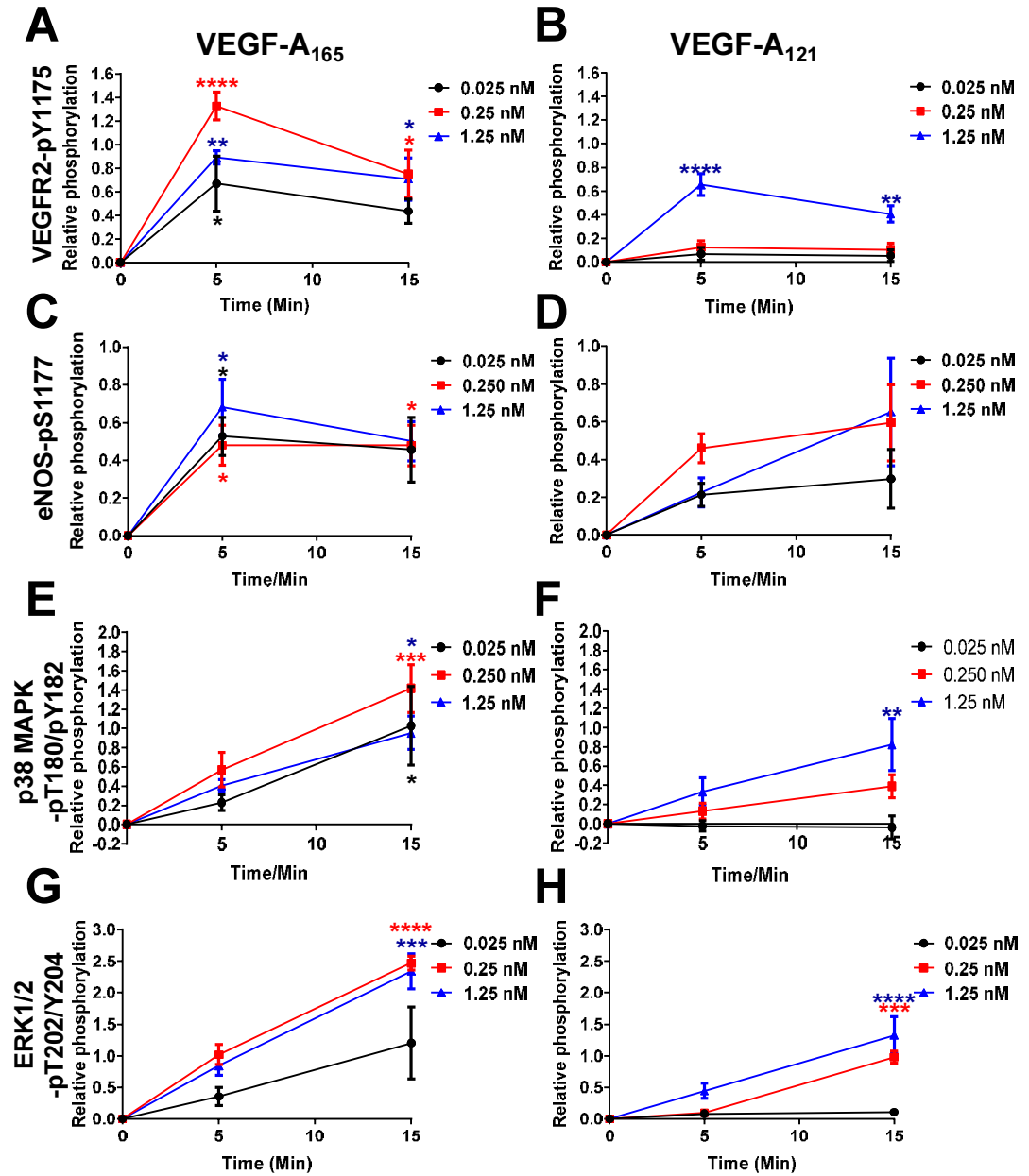
Figure 6.2. Quantification of VEGF-A isoform-specific stimulated endothelial cell signal transduction. (A-H) Quantification of VEGF-A isoform-specific signal transduction events. Quantification of (A and B) VEGFR2-pY1175, (C and D) eNOS-pS1177, (E and F) p38 MAPK-pT180/pY182 or (G and H) ERK1/2-pT202/Y204 upon (A, C, E and G) VEGF-A$_{165}$ or (B, D, F and H) VEGF-A$_{121}$ stimulation. Error bars indicate ±SEM (n≥3). $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****).
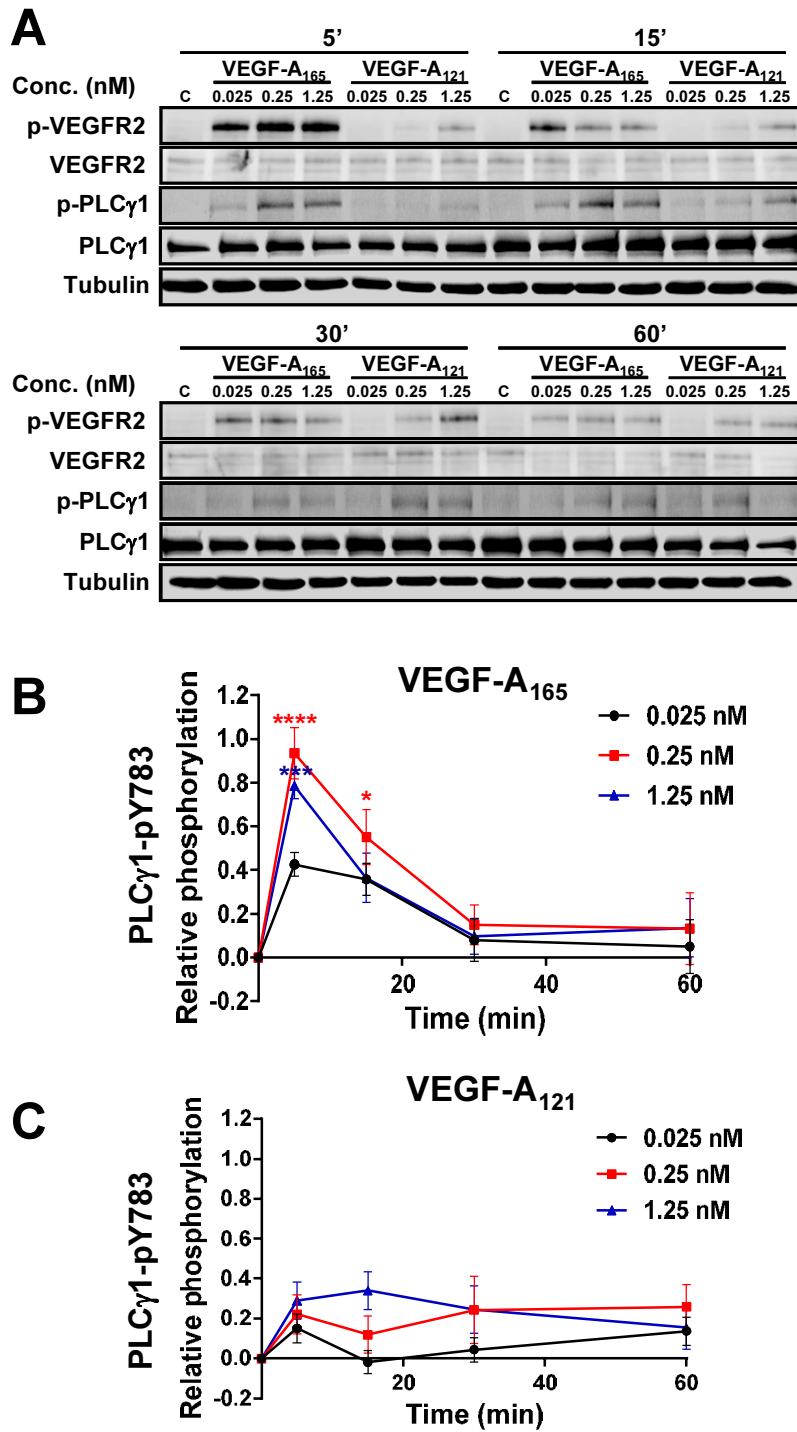
Figure 6.3. VEGF-A isoform-specific PLCγ1 activation. (A) Endothelial cells subjected to different VEGF-A$_{165}$ or VEGF-A$_{121}$ concentrations (0, 0.025, 0.25 or 1.25 nM) for 5, 15, 30 or 60 min were lysed and probed for phospho-PLCγ1. (B and C) Quantification of PLCγ1-pY783 upon (B) VEGF-A$_{165}$ or (C) VEGF-A$_{121}$ stimulation. Error bars indicate ±SEM (n≥3). $p<0.001$ (***), $p<0.0001$ (****).
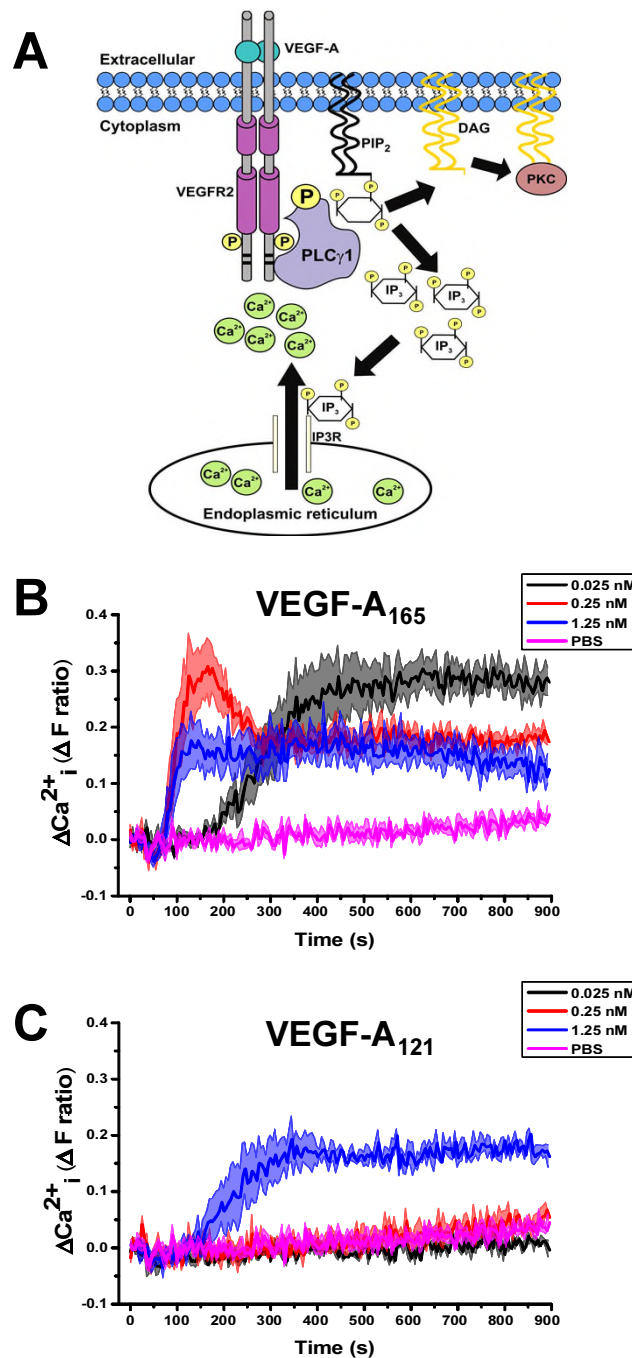
Figure 6.4. VEGF-A isoform-specific rise in cytosolic calcium ions. (A) Schematic depicting VEGF-A-stimulated second messenger targeting of $InsP_3$ receptors ($InsP_3R$) and subsequent cytosolic calcium ion rise. $PtdIns(4,5)P_2$ hydrolysis to generate diacylglycerol (DAG) and $Ins(1,4,5)P_3$ is depicted. (B, C) Real-time monitoring of cytosolic calcium ion flux with (B) VEGF-A$_{165}$ or (C) VEGF-A$_{121}$ titration; graphs show a representative plot of multiple experiments. Error bars indicate ±SEM (n=1, N=4).

stimulated rise in cytosolic calcium ion levels revealed that both VEGF-A$_{165}$ and VEGF-A$_{121}$ acted in a concentration-dependent manner (Figure 6.5A). VEGF-A$_{165}$ stimulation generally promoted an increased level in intracellular Ca^{2+} versus treatment with VEGF-A$_{121}$ (Figure 6.5A). However, at saturating levels of VEGF-A (1.25 nM) the peak magnitude of cytosolic calcium ion rise was comparable upon stimulation with either VEGF-A$_{165}$ or VEGF-A$_{121}$ (Figure 6.5A). In contrast, the time taken to reach this peak magnitude was ~2-fold longer in VEGF-A$_{121}$-stimulated cells in comparison to VEGF-A$_{165}$ treatment (Figure 6.5B). Quantification of the relative curve area at different VEGF-A concentrations revealed a similar magnitude of response for VEGF-A$_{165}$ regardless of concentration; however, VEGF-A$_{121}$ only induced significant changes in intracellular calcium ion levels at the maximum concentration of 1.25 nM (Figure 6.5C). These findings suggest that the cytosolic calcium ion flux caused by the VEGFR2-PLCγ1 axis is VEGF-A isoform-dependent.

6.2.3. VEGF-A isoform-dependent NFATc2 and VEGFR1 translocation

Cytosolic calcium ion fluxes can regulate endothelial cell gene transcription via the calcium ion-regulated and calmodulin-dependent activation of the protein phosphatase calcineurin (Zarain-Herzberg et al., 2011; Dominguez-Rodriguez et al., 2012). This enzyme regulates dephosphorylation and subsequent nuclear translocation of the nuclear factor of activated T-cells (NFAT) family of transcription factors (Rao et al., 1997; Wu et al., 2007) (Figure 6.6). VEGF-A can promote nuclear translocation of NFAT family members such as NFATc2 (NFAT1) (Goyal et al., 2012; Zaichuk et al., 2004). We hypothesised that the differential VEGF-A isoform-specific regulation of cytosolic calcium ion flux could modulate activation of endothelial NFATc2. To test this idea, endothelial cells were stimulated with 0.25 nM of either VEGF-A$_{165}$ or VEGF-A$_{121}$ which elicited the major differences in cytosolic calcium ion flux, followed by immunoblot analysis of NFATc2 phosphorylation status (Figure 6.7A). This revealed that at the peak of VEGF-A$_{165}$-stimulated PLCγ1 phosphorylation (PLCγ1-pY783), there was a corresponding increase in NFATc2 activation, reflecting dephosphorylation of this transcription factor (Figure 6.7A). However, at a comparable level of VEGF-A$_{121}$, we found a relatively small rise in NFATc2 activation (Figure 6.7A). Quantification of the relative levels of dephosphorylated (active) vs. phosphorylated (inactive) NFATc2 revealed that both VEGF-A isoforms stimulated rapid and transient dephosphorylation, with peak dephospho-NFATc2 levels occurring after ~5 min (Figure 6.7B). However, VEGF-A$_{165}$ stimulation promoted ~22-fold increase in dephospho-NFATc2, whereas
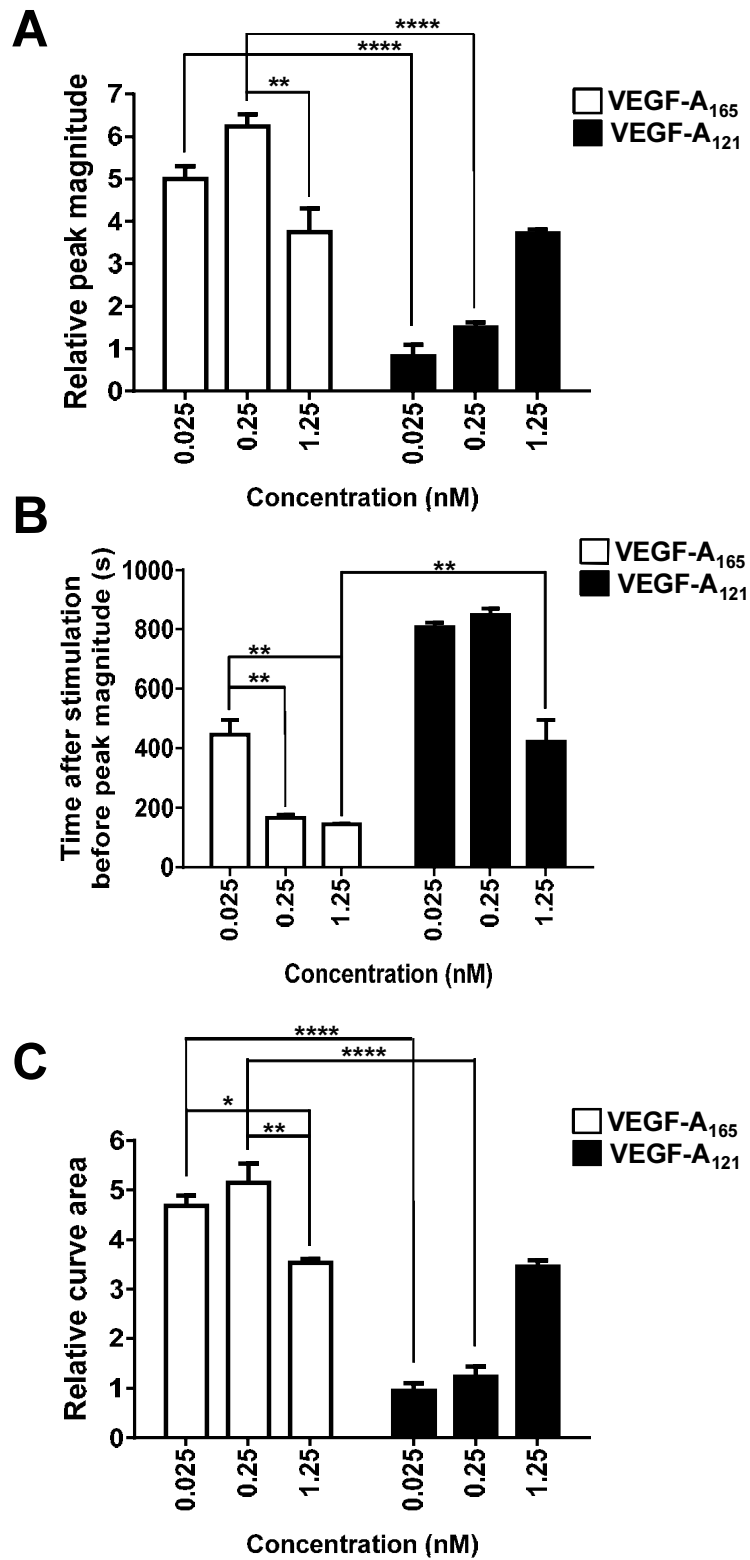
Figure 6.5. Quantification of VEGF-A isoform-specific rise in cytosolic calcium ions. (A-C) Quantification of (A) peak magnitude, (B) time taken to reach peak magnitude or (C) the total curve area for each Ca^{2+} flux upon stimulation with VEGF-A$_{165}$ and VEGF-A$_{121}$. Error bars indicate ±SEM (n=3). $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$ (****).
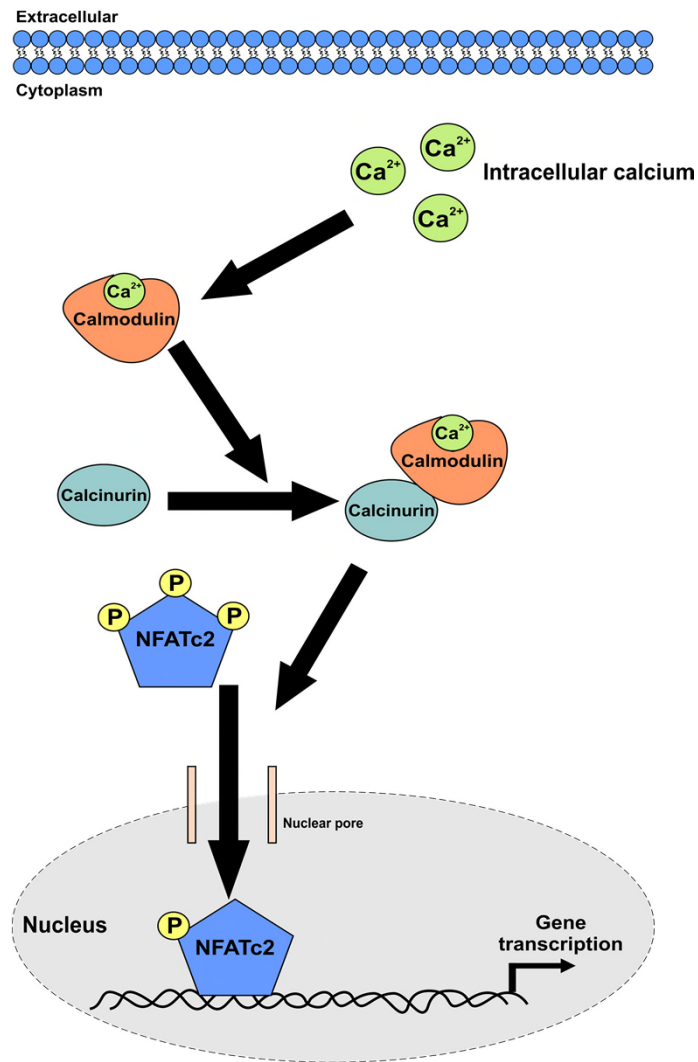
Figure 6.6. Schematic depicting Ca^{2+}-dependent regulation of NFATc2 dephosphorylation and nuclear translocation. Increase in cytosolic calcium ion levels promotes calmodulin-dependent activation of the protein phosphatase, calcineurin. Activated calcineurin promotes NFATc2 dephosphorylation, subsequent nuclear translocation and NFATc2-dependent gene transcription.

VEGF-A$_{121}$ stimulation caused a significantly lower rise (~2-fold) in dephospho-NFATc2 levels (Figure 6.7B). Nuclear translocation of dephosphorylated NFATc2 is required for the modulation of endothelial gene expression (Wu et al., 2007). To test the idea that VEGF-A isoform-specific NFATc2 dephosphorylation regulates its nuclear translocation we evaluated the intracellular distribution of NFATc2 in response to VEGF-A isoform stimulation (Figure 6.6). Endothelial cells were stimulated with either VEGF-A$_{165}$ or VEGF-A$_{121}$ (0.25 nM) for 0, 5, 15 or 30 min before fixation and processing for immunofluorescence analysis (Figure 6.8A). Addition of VEGF-A$_{165}$ caused rapid and sustained NFATc2 accumulation within the nucleus within a 30 min period (Figure 6.8A). However, VEGF-A$_{121}$ stimulation resulted in relatively low nuclear accumulation within a 30 min period (Figure 6.8A). Quantification of NFATc2 nuclear co-distribution in a ligand- and time-dependent manner revealed an ~18-fold (VEGF-A$_{165}$) or ~3-fold (VEGF-A$_{121}$) increase in peak nuclear NFATc2 levels (Figure 6.8B). Interestingly, biochemical analysis of NFATc2 phosphorylation status after VEGF-A$_{165}$ stimulation for 30 min showed that phosphorylated activated NFATc2 levels returned to baseline levels (Figure 6.8B) but the morphological analysis revealed a major pool of NFATc2 still present within the nucleus at the 30 min time point (Figure 6.8, A and B). Thus initial dephosphorylation of NFATc2 is sufficient to promote rapid cytosol-to-nuclear translocation but nuclear retention occurs via a different mechanism.

Cytosolic calcium ion fluxes have also been linked to promoting trafficking of another endothelial receptor tyrosine kinase (VEGFR1) from the Golgi apparatus to the plasma membrane (Mittar et al., 2009; Bruns et al., 2009). Due to the different abilities of the two VEGF-A isoforms in promoting the release of intracellular calcium ion stores, we hypothesised about the effects on VEGFR1 translocation to the plasma membrane. To test this idea, we stimulated endothelial cells with either VEGF-A$_{165}$ or VEGF-A$_{121}$ for 0, 15, 30 or 60 min prior to assessing plasma membrane VEGFR1 using cell surface biotinylation (Figure 6.9A). Immunoblot analysis of mature and soluble VEGFR1 expression revealed that in non-stimulated endothelial cells, both VEGFR1 and VEGFR2 are predominately located within internal, biotin probe-inaccessible compartments and not at the plasma membrane (Figure 6.9A). However, upon stimulation with VEGF-A$_{165}$ there was a significant increase (~2.5-fold) in both mature (Figure 6.9B) and soluble (Figure 6.9C) VEGFR1 (sVEGFR1/sFlt1) at the cell surface. Contrastingly, VEGF-A$_{121}$ treatment failed to promote a significant increase in cell surface levels of either mature or sVEGFR1 (Figure 6.9, B and C).

A

B
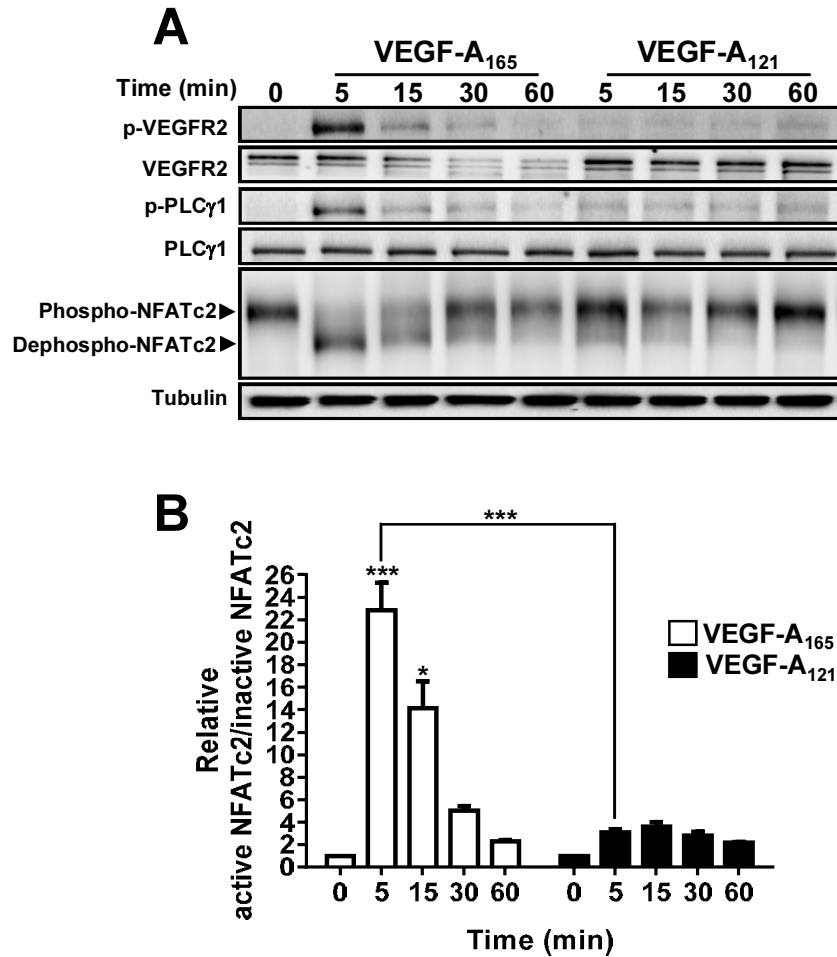
Figure 6.7. VEGF-A isoform-specific cytosolic calcium ion flux promotes differential NFATc2 activation. (A) Endothelial cells subjected to stimulation with 0.25 nM VEGF-A$_{165}$ or VEGF-A$_{121}$ for 5, 15, 30 and 60 min were lysed and process for immunoblot analysis to detect phospho- (inactive) and dephospho- (active) NFATc2 species. (B) Quantification of relative active versus inactive NFATc2 levels upon stimulation with VEGF-A$_{165}$ and VEGF-A$_{121}$. Error bars indicate ±SEM (n=3). $p<0.05$ (*), $p<0.001$ (***).
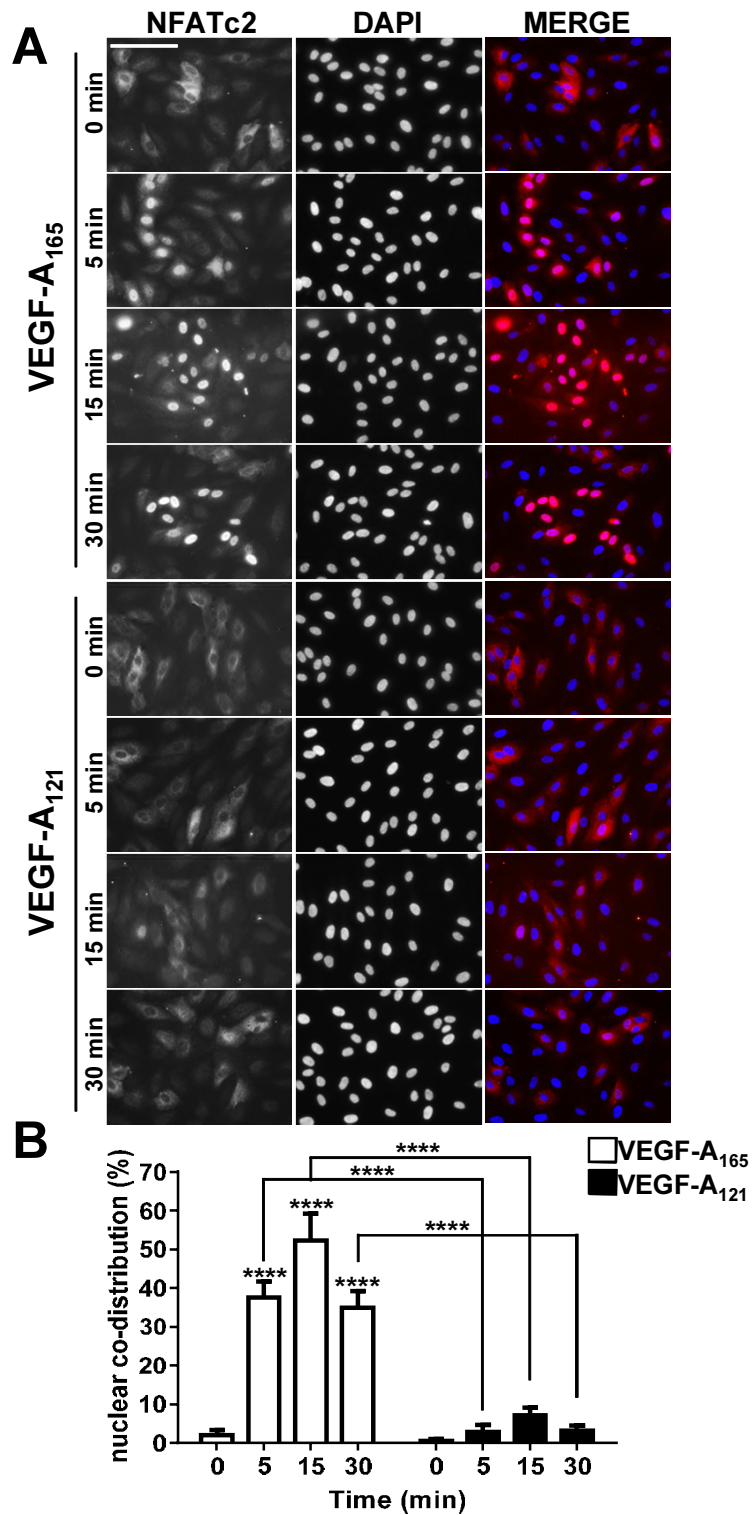
Figure 6.8. VEGF-A isoforms promote differential NFATc2 nuclear translocation. (A) Endothelial cells stimulated with 0.25 nM VEGF-A$_{165}$ or VEGF-A$_{121}$ for 5, 15 and 30 min were fixed and processed for immunofluorescence microscopy using anti-NFATc2 (green); nuclei stained using DAPI (blue). Scale bar, 1000 μm. (B) Quantification of NFATc2 nuclear co-distribution at 0, 5, 15 and 30 min after stimulation with VEGF-A$_{165}$ or VEGF-A$_{121}$. Error bars indicate ±SEM (n=3). $p < 0.0001$ (****).

6.2.4. VEGF-A-stimulated endothelial cell migration is NFATc2-dependent

VEGF-A-stimulated signal transduction regulates a diverse number of long-term endothelial cell responses, such as cell migration and tubulogenesis (Koch et al., 2011; Chung and Ferrara, 2011). VEGF-A isoforms have been shown to differentially regulate endothelial cell responses (Kawamura et al., 2008). However, the transcription factors involved in regulating these isoform-specific cellular responses are not well defined. To determine whether NFATc2 is required for the diverse array of VEGF-A-stimulated endothelial cell responses, we used reverse genetics combined with ligand-stimulated cellular assays (Figure 6.10 and 6.11). siRNA duplexes were used to knockdown NFATc2 levels in endothelial cells and we compared these cells to scrambled siRNA duplex-treated or non-transfected controls. Treatment with NFATc2-specific siRNA duplexes caused ~90% knockdown in endothelial NFATc2 levels (Figure 6.12), but did not affect expression of other endothelial proteins such as VEGFR2, VEGFR1 and ERK1/2 (Figure 6.12). To determine the effect of NFATc2 knockdown on endothelial cell migration NFATc2-depleted or control endothelial cells were seeded into 8.0 μm transwell filters prior to stimulation with 0.25 nM VEGF-A$_{165}$ or VEGF-A$_{121}$, before fixation, staining and processing for light microscopy (Figure 6.10A). As previously reported, VEGF-A$_{165}$ has an ~2-3-fold higher efficacy for promoting endothelial cell migration (Figure 6.10, B and C). Interestingly, quantification revealed that depletion of NFATc2 caused an ~2-3-fold increase in basal cell migration in non-stimulated endothelial cells (Figure 6.10), during comparison of all values to non-transfected non-stimulated endothelial cells. In such an analysis, there appears to be relatively little change in VEGF-A isoform-stimulated cell migration upon NFATc2 knockdown (Figure 6.10B). However, when each experiment was compared to their own non-stimulated control which had also been subjected to scrambled or NFATc2 siRNA, a different pattern emerged (Figure 6.10C). There was now a substantial 3-4-fold decrease in both VEGF-A$_{165}$ and VEGF-A$_{121}$-stimulated endothelial cell migration in NFATc2 depleted endothelial cells (Figure 6.10C).

An important aspect of the VEGF-A-stimulated endothelial cell response is the capacity to build hollow tubes (tubulogenesis), which can be monitored using an organotypic assay. Using this technique, we asked whether NFATc2 was required for VEGF-A-stimulated tubulogenesis (Figure 6.11A). As previously reported, VEGF-A$_{165}$ has a ~2-3-fold higher efficacy for promoting endothelial cell tubulogenesis, in comparison to VEGF-A$_{121}$ (Figure 6.11, A and B). Intriguingly, NFATc2 depletion did not significantly affect VEGF-A stimulated endothelial cell
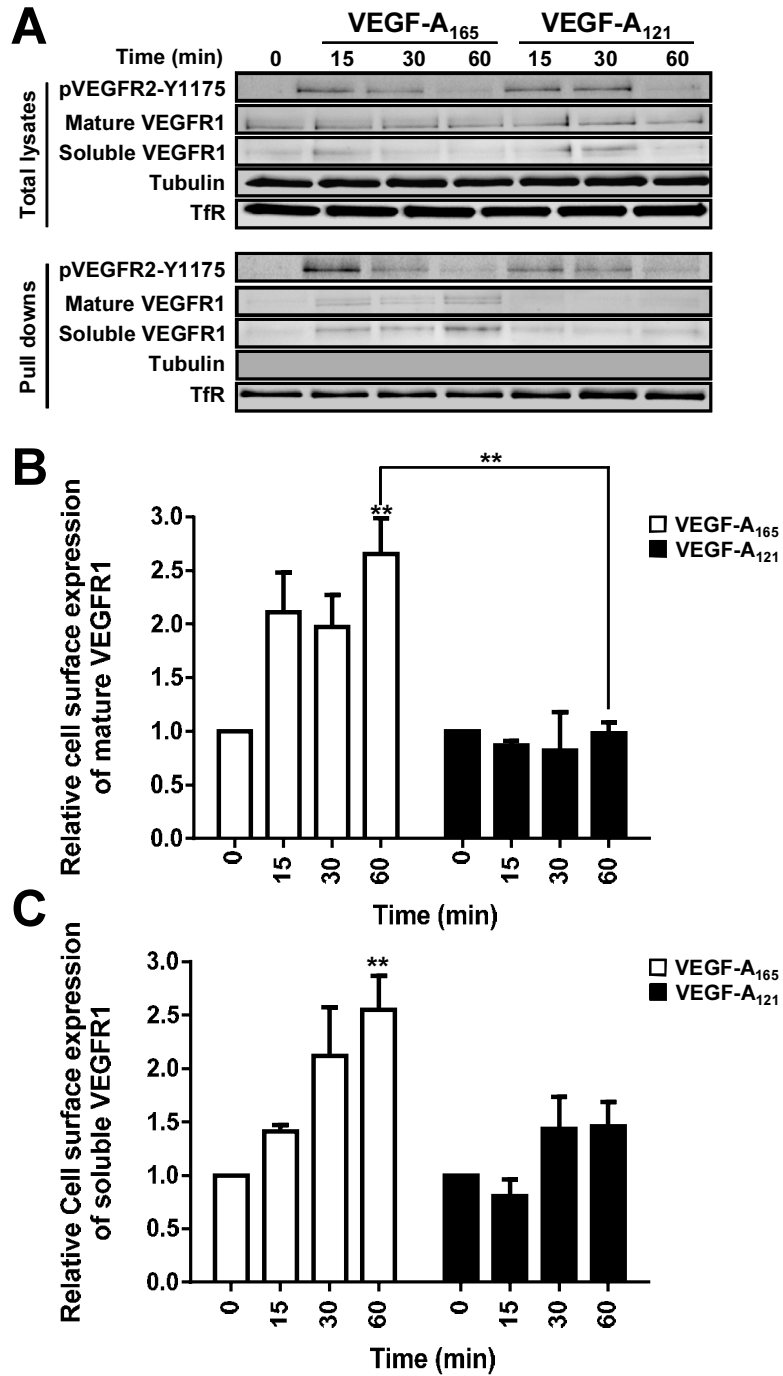
Figure 6.9. VEGF-A isoforms promote differential trafficking of VEGFR1. (A) Cell surface biotinylation, affinity isolation and immunoblot analysis of total cell lysates or biotinylated cell surface protein pools. Negative control (tubulin) and positive control (transferrin receptor, TfR). (B) Quantification of cell surface mature VEGFR1 or (C) soluble VEGFR1 levels upon VEGF-A$_{165}$ or VEGF-A$_{121}$ stimulation. Error bars indicate ±SEM (n=3). $p<0.01$ (**).
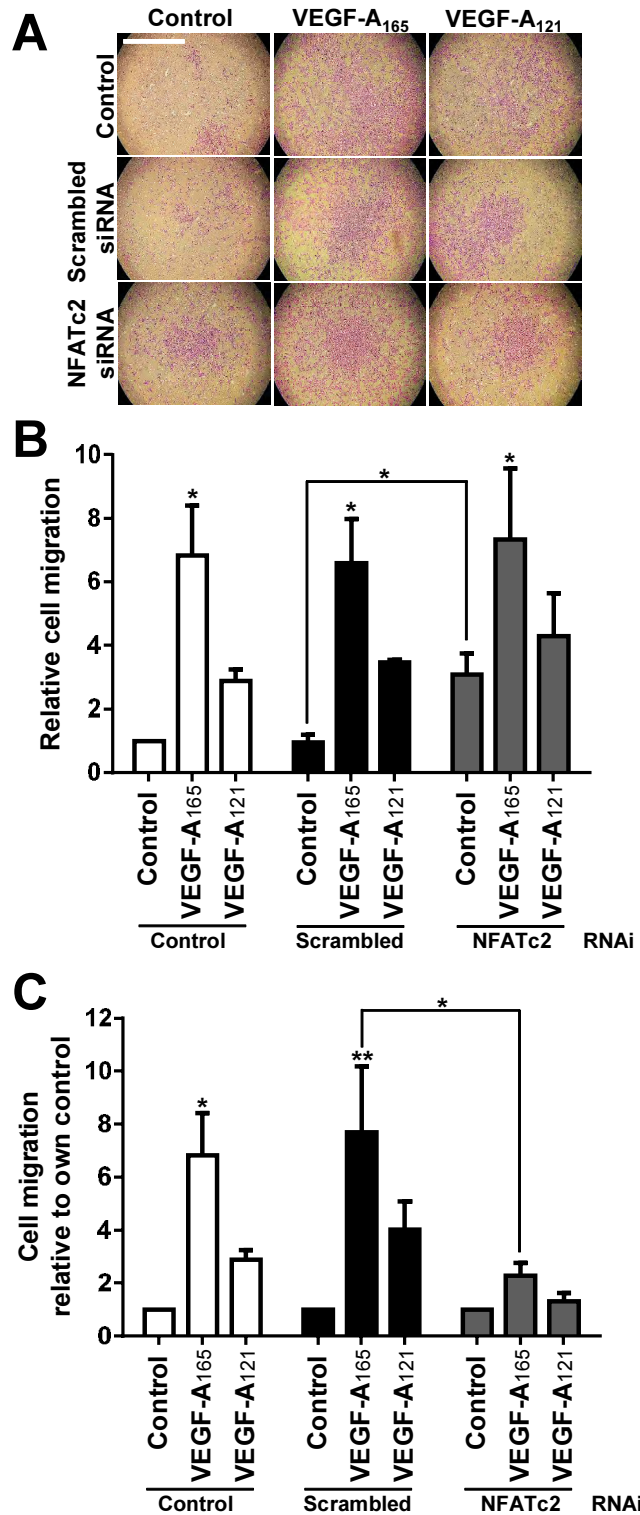
Figure 6.10. VEGF-A isoform-specific endothelial cell migration requires NFATc2. (A) Control, scrambled or NFATc2-specific siRNA duplex-treated endothelial cells were seeded into Transwell filters and stimulated with 0.25 nM VEGF-A$_{165}$ or VEGF-A$_{121}$ for 24 h before being fixed and stained with 20% (v/v) crystal violet. Scale bar, 1000 µm. (B, C) Quantification of endothelial cell migration compared to (B) non-transfected or (C) individual controls. Error bars indicate ±SEM (n≥3). $p<0.05$ (*), $p<0.01$ (**).

tubulogenesis (Figure 6.11A). Quantification revealed that NFATc2 knockdown did not affect endothelial tubule length (Figure 6.11B) nor branch point complexity (Figure 6.11C).
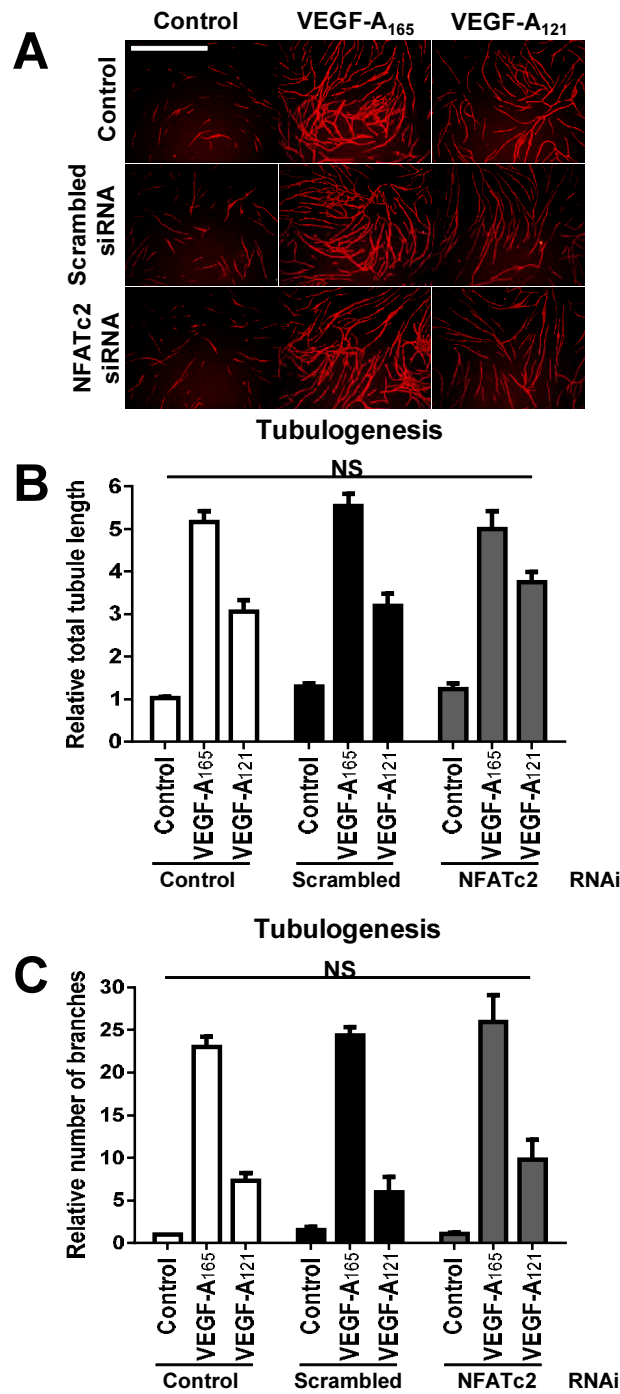
Figure 6.11. NFATc2 does not regulate VEGF-A-stimulated endothelial tubulogenesis. (A) Control, scrambled or NFATc2-specific siRNA duplex-treated endothelial cells were co-cultured on a bed of primary human fibroblast for 7 days and stimulated with 0.25 nM VEGF-A$_{165}$ or VEGF-A$_{121}$. Co-cultures were fixed and endothelial tubules were stained and visualized using an anti-PECAM1 antibody followed by fluorescent secondary antibody. Scale bar, 1000 μm. (B, C) Quantification of endothelial cell tubulogenesis including total (B) tubule length or (C) number of branch points. Error bars indicate ±SEM (n≥3). NS=non-specific.
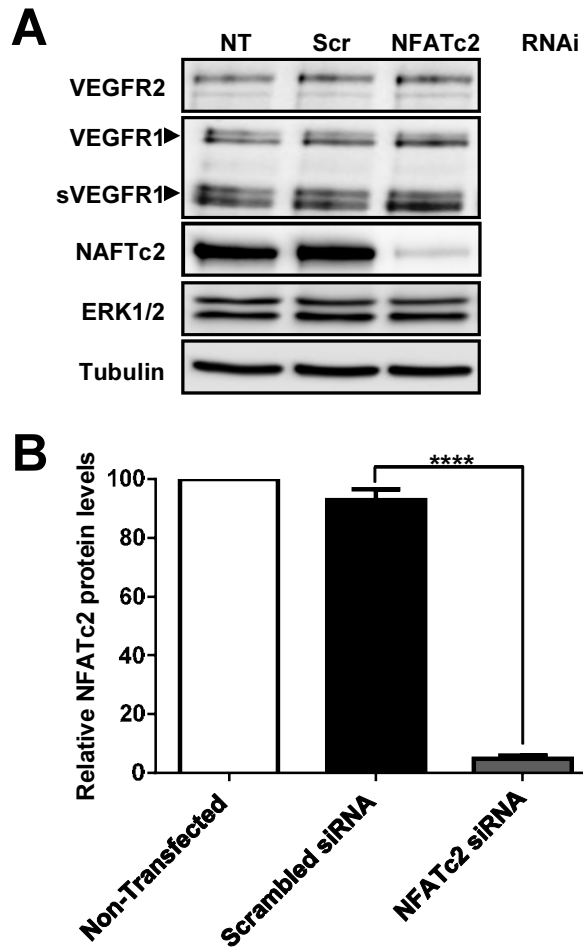
Figure 6.12. Quantification of endothelial NFATc2 depletion. (A) Control, scrambled or NFATc2 specific siRNA duplex-treated endothelial cells were lysed and process for immunoblot analysis to determine NFATc2 protein expression levels. (B) Quantification of NFATc2 protein expression levels in control, scrambled or NFATc2-specific siRNA duplex-treated endothelial cells. Error bars indicate ±SEM (n=3). *p*<0.0001 (****).

6.3.   DISCUSSION

The work carried out in this chapter showed that different VEGF-A isoforms have differing capabilities to modulate endothelial cell migration by regulating the activation and nuclear localisation of a key calcium ion-regulated transcription factor, NFATc2 (Figure 6.13). In our proposed model, two VEGF-A isoforms with similar binding affinities differentially program VEGFR2 activation and downstream signal transduction which controls a transcriptional 'switch' that regulates endothelial cell migration. This 'switch' comprises the calcium-dependent activation of the calmodulin-calcineurin pathway which targets endothelial NFATc2 (Figure 6.13).

A key feature of endothelial cell migration is the VEGF-A isoform-specific programming of signal transduction and this cellular response. VEGF-A$_{165}$ significantly promoted increased VEGFR2 phosphorylation at residue Y1175 in comparison to the VEGF-A$_{121}$. The generation of this unique pY1175 binding site enables recruitment of PLCγ1 via its SH2 domains and subsequent phosphorylation on residue Y783. VEGF-A isoform-specific stimulation of PLCγ1-pY783 levels correlated with increased cytosolic calcium ion flux (Figure 6.13). Calmodulin is a key target of elevated cytosolic calcium ion levels and upon calcium ion binding to one of its 4 EF hands, undergoes a conformational change that promotes interaction with new cellular targets, including the protein phosphatase calcineurin (Berchtold and Villalobo, 2014). Calmodulin binding to calcineurin promotes enzymatic activation; one such target is NFATc2, resulting in its subsequent rapid dephosphorylation and nuclear targeting (Luo et al., 1996; Okamura et al., 2000). Both VEGF-A$_{165}$ and VEGF-A$_{121}$ stimulation promote cytosolic calcium ion flux, NFATc2 dephosphorylation and nuclear translocation, but to widely differing extents (Figure 6.13). Depletion of endothelial NFATc2 levels significantly reduced VEGF-A-stimulated endothelial cell migration suggesting that this factor plays a role in controlling gene expression linked to cell migration processes e.g. focal adhesion, stress fibre formation and actin polymerisation (Figure 6.13). Although, our results show that VEGF-A isoforms promote NFATc2 translocation into the nucleus, a lacking area of investigation is to determine if VEGF-A-stimulated NFATc2 dephosphorylation and nuclear translocation leads to the transcription of well-known NFATc2 regulated genes. However, it is highly likely that this is the case, due to the effect that depletion of NFATc2 has on VEGF-A-stimulated endothelial cell migration. Various transcription factors such as ATF-2, NFAT, STAT3, forkhead-like transcription factors, FoxO- and ETS- related transcription factors are implicated in
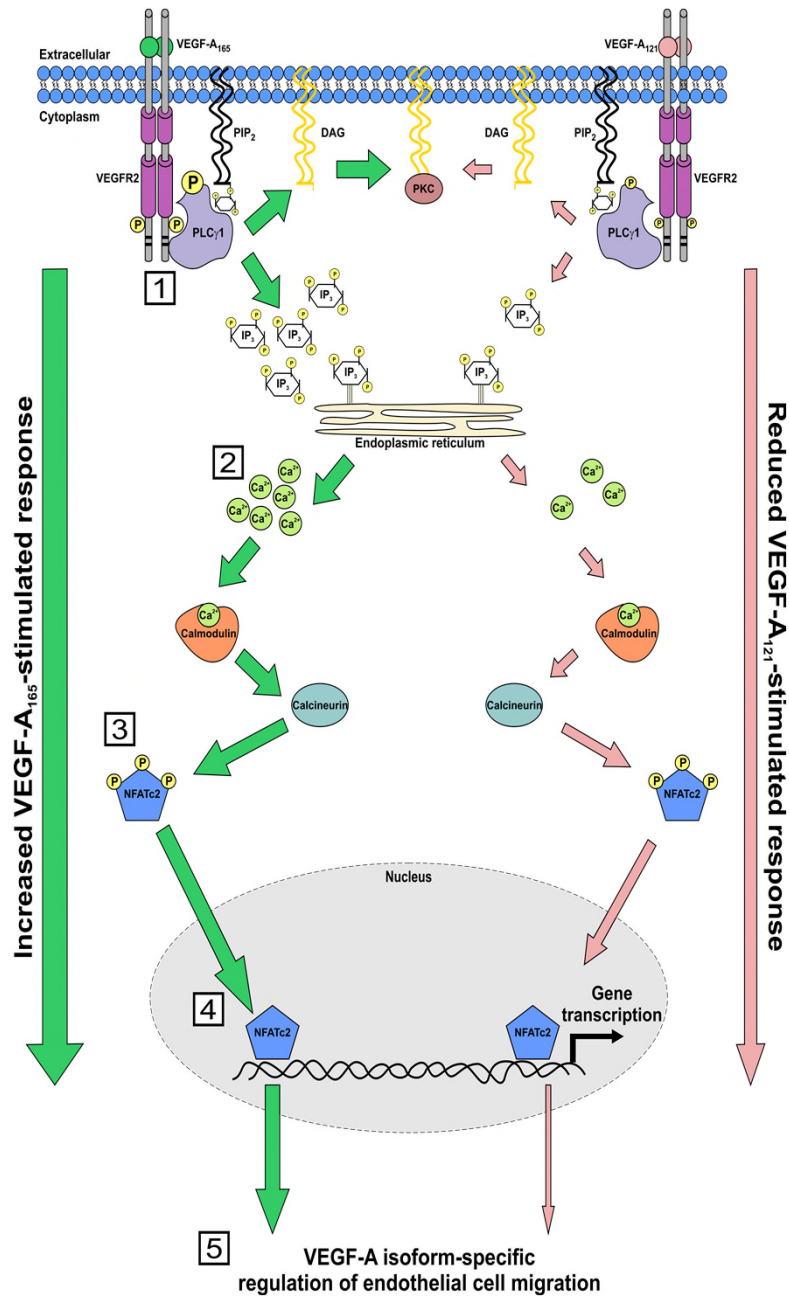
Figure 6.13. Mechanism for VEGF-A isoform-specific regulation of endothelial cell migration. Schematic depicting VEGF-A isoform-specific regulation of NFATc2 activation and regulation of endothelial cell migration. Numbered steps denote: (1) VEGF-A isoforms promote differential recruitment and activation of PLCγ1 through interaction with VEGFR2, (2) due to increased PLCγ1, VEGF-A$_{165}$ promotes an increased rise in intracellular Ca^{2+}, versus VEGF-A$_{121}$. (3) Increased VEGF-A$_{165}$ stimulated intracellular Ca^{2+} flux results in increased NFATc2 dephosphorylation, versus VEGF-A121, (4) dephosphorylated NFATc2 translocates into the nucleus where it regulates endothelial gene transcription. (5) VEGF-A-stimulated NFATc2 regulated gene expression promotes endothelial cell migration.

VEGF-A-dependent gene expression and physiological responses (Armesilla et al., 1999; Potente et al., 2005; Liu et al., 2003; Dejana et al., 2007; Bartoli et al., 2003; Abid et al., 2008). However, the role of NFATc2 in VEGF-A stimulated angiogenesis is poorly defined. Previous studies have suggested a role for the transcriptional activity of endothelial NFATc2 in mediating VEGF-A-stimulated retinal angiogenesis (Zaichuk et al., 2004) and endothelial cell tubulogenesis (Goyal et al., 2012). Thus, our study now identifies a novel role for NFATc2 in the regulation of VEGF-A isoform-specific endothelial cell migration (Figure 7). However, in contrast to work elsewhere (Bala et al., 2012), we did not find that NFATc2 was essential for VEGF-A-stimulated endothelial cell tubulogenesis. One possible explanation lies within the choice of assay used to monitor endothelial cell tubulogenesis. In our study we used a 7 day human fibroblast-endothelial cell co-culture assay to monitor the formation of PECAM-1 positive endothelial tubules, whereas Bala and colleagues performed a Matrigel co-culture assay (Bala et al., 2012); one drawback of this assay is that does not give an accurate index of endothelial cell tube formation (Donovan et al., 2001). Another reason could be the method used to look at NFATc2 involvement in endothelial cell function. In our study we used RNAi to directly deplete 90% of endothelial NFATc2. Bala *et al* were studying the effects of a Kaposi's Sarcoma Herpesvirus protein (K15) on stimulating NFATc2-dependent RCAN1 expression (Bala et al., 2012).

Therefore, it is possible that VEGF-A and K15-stimulated NFATc2 activation play different roles in determining endothelial cell fate. The fact that NFATc2 does not appear to be required for endothelial tube formation but is required for endothelial cell motility is surprising, as the latter is required for the former. This is a serious limitation to our current study and further work is required; the use of more robust 3-D angiogenesis assays such as the fibrin bead and '*ex vivo*' mouse aortic ring assay would help strengthen the conclusions made here. Additionally, the monitoring of endothelial cell motility in real-time would further strengthen the role of NFATc2 in regulating VEGF-A isoform-specific cell migration. Likewise, analysis of other VEGF-A-stimulated endothelial responses (e.g. cell proliferation and permeability) for NFATc2 dependency would prove informative.

Interestingly, we found that basal NFATc2 activity inhibits the rate of cell migration in non-stimulated endothelial cells. One possible explanation could lie within the fact that NFATc2 naturally functions in an auto-inhibitory manner by promoting the expression of regulator of calcineurin 1 (RCAN1). RCAN1 forms a complex with

calcineurin to directly inhibit its phosphatase activity, thus preventing calcineurin-dependent NFATc2 dephosphorylation and nuclear translocation (Li et al., 2011; Holmes et al., 2010). However, calcineurin also activates receptor for activated protein kinase C 1 (RACK1), which has been implicated in regulating cell migration through binding to an array of signalling proteins. Endothelial cells constitutively express RCAN1 at a basal level (Mendez-Barbero et al., 2013; Holmes et al., 2010). Thus, depleting NFATc2 could reduce basal levels of RCAN1, subsequently leading to increased endothelial cell migration through attenuated calcineurin-stimulated RACK1 signalling. Calcineurin inhibitors have also been shown to inhibit cell migration (Siamakpour-Reihani et al., 2011; Espinosa et al., 2009), thus strengthening this proposed mechanism. RCAN1 is one of the most highly expressed gene products in response to VEGF-A-stimulation (Schweighofer et al., 2009; Rivera et al., 2011). Therefore, it is likely to have an essential role in regulating VEGF-A mediate endothelial responses. Various studies have shown that RCAN1 is essential for cellular migration (Ryeom et al., 2008; Iizuka et al., 2004; Holmes et al., 2010; Espinosa et al., 2009). Hence, VEGF-A isoform-specific elevation in RCAN1 expression could account for the reduction in VEGF-A-stimulated endothelial cell migration in NFATc2-depleted endothelial cells. Additionally, VEGF-A isoform-specific NFATc2-dependent upregulation of RCAN1 could also account for the differences in their abilities to stimulate endothelial cell migration.

One question left unanswered is what is the mechanism(s) behind how these two VEGF-A isoforms with similar binding affinities, promote such diverse activation of VEGFR2 and downstream signalling enzymes. One possible answer is through the differential binding and recruitment of co-receptors (e.g. NRP1, NRP2 and HSPG). Neuropilin 1 (NRP1) is a VEGF-A isoform-specific co-receptor (Zachary et al., 2009). VEGF-A$_{165}$ binds to the co-receptor Neuropilin 1 (NRP1) to form a VEGFR2/NRP1/VEGF-A$_{165}$ signalling complex (Fantin et al., 2013; Herzog et al., 2011; Zachary et al., 2009; Raimondi and Ruhrberg, 2013). Formation of this trimeric complex has been shown to increase VEGF-A$_{165}$-stimulated VEGFR2 activation, downstream signalling and endothelial responses (Allain et al., 2012; Kawamura et al., 2008; Koch, 2012). However, VEGF-A$_{121}$ binding simultaneously to VEGFR2 and NRP1 has been contradicted (Pan et al., 2007). Therefore, VEGF-A isoform-specific recruitment of NRP1 could account for the differences in signalling between the 2 isoforms.

VEGF-A-stimulated NFATc2-dependent gene expression is said to occur in co-operation with the transcription factor early growth response 1 (EGR-1) (Schweighofer et al., 2007). VEGF-A stimulates *EGR-1* gene expression through the MEK1-ERK1/2 pathway (Schweighofer et al., 2007). As we show that VEGF-A$_{165}$ and VEGF-A$_{121}$ have differential effects on ERK1/2 activation, one future study would be to investigate if these 2 isoforms differentially activated EGR-1 as a means of programing isoform-specific endothelial cell responses.

VEGFR1 binds VEGF-A with a much higher affinity than VEGFR2 (de Vries et al., 1992), yet its involvement in VEGF-A stimulated angiogenesis is not well understood. One view is that both membrane-bound and sVEGFR1 proteins act primarily as VEGF-A 'traps', thus limiting ligand availability for the major pro-angiogenic receptor, VEGFR2 (Rahimi, 2006). However, VEGF-A-regulated VEGFR1-linked signal transduction has been implicated in certain aspects of endothelial cell physiology (Koch and Claesson-Welsh, 2012). In non-stimulated endothelial cells, VEGFR1 is primarily inaccessible to VEGF-A, as it is located within an internal compartment resembling the Golgi apparatus (Mittar et al., 2009). VEGF-A$_{165}$ stimulation promotes VEGFR1 translocation to the cell surface via a cytosolic calcium ion-dependent mechanism where it can bind exogenous VEGF-A. In this study, VEGF-A$_{165}$ promoted significant trafficking of VEGFR1 to the plasma membrane whereas VEGF-A$_{121}$ was largely ineffective in this context; again this could be explained by signal transduction effects on cytosolic calcium ion levels. The role of VEGF-A isoforms in differential VEGFR1 trafficking could further modulate the endothelial cell response to this ligand, as not only would this affect VEGF-A bioavailability for VEGFR2, but regulate VEGFR1-specific signal transduction in response to VEGF-A, VEGF-B and PlGF isoforms and thus regulate vascular physiology.

The physiological process of angiogenesis can be destabilised in a wide variety of major disease states ranging from atherosclerosis, rheumatoid arthritis, pathogenic infection to cancer. Current anti-angiogenic therapies which try to restrict pathological angiogenesis, by sequester endogenous VEGF-A or via inhibiting RTK activity, are not as successful as first hoped. This is partially due to tumour cell-acquired resistance through the activation of alternative angiogenic pathways, in addition to other assorted mechanisms (Vasudev and Reynolds, 2014). Therefore, further investigation into the mechanisms which regulate angiogenesis is required. This will hopefully lead to improved patient outcomes in various disease states,

through the design of better therapeutics. Our study now provides a novel mechanism to explain how different VEGF-A isoforms act on a common receptor tyrosine kinase (VEGFR2) to differentially determine a specific endothelial cell outcome (i.e. cell migration; Figure 6.13). Additionally, this work shines new light on the physiological importance of NFATc2 in regulating VEGF-A-stimulated endothelial cell migration. A substantial future challenge will be to determine the biological significance of each VEGF-A isoform in healthy and diseased states.

CHAPTER 7

GENERAL DISCUSSION

The studies presented in this thesis provided novel insights into how VEGF-A isoforms regulate VEGFR2 trafficking, downstream signal transduction and gene expression to modulate endothelial cell function. This chapter will now provide an overview on the subject matter in the context of the current understanding within the field. Furthermore, any potential therapeutic implications of these studies in area of vascular disease or cancer will also be highlighted.

7.1. VEGF-A isoform-specific modulation of VEGFR2 trafficking, downstream signal transduction and gene expression

Upon VEGF-A binding, VEGFR2 undergoes rapid and transient phosphorylation at multiple tyrosine residues within its cytoplasmic domain. This transautophosphorylation stimulates kinase activation and also enables the recruitment and subsequent activation of an array of adaptor proteins and downstream signal transduction enzymes (e.g. Src, p38 MAPK and ERK1/2) (Koch and Claesson-Welsh, 2012). In order to regulate long-term endothelial cell responses (proliferation, migration, tubulogenesis and cell-cell interactions), such short-lived signal transduction needs to be converted into gene expression via the activation of downstream signal transduction enzymes. VEGF-A isoforms stimulate differential VEGFR2-mediated downstream signal transduction and endothelial cell responses (Pan et al., 2007; Kawamura et al., 2008). However, the mechanisms which regulate such process are ill-defined.

Following VEGF-A binding, VEGFR2 undergoes ubiquitination and internalisation into early endosomes (Jopling et al., 2009; Bruns et al., 2010). From here VEGFR2 can either be targeted for degradation or recycled back to the plasma membrane (Miaczynska et al., 2004). Recent studies have highlighted the importance of activated VEGFR2 internalisation with the regulation of downstream signal transduction (Jopling et al., 2009; Gourlaouen et al., 2013; Lanahan et al., 2010; Lanahan et al., 2013). One such signal transduction enzyme which is robustly dependent on VEGFR2 trafficking into early endosomes is ERK1/2 (Lanahan et al., 2013; Gourlaouen et al., 2013). The studies presented in this thesis reveal for the

first time that VEGF-A isoforms differential program VEGFR2 trafficking, turnover and post-translational modifications in order to impact on downstream signal transduction via the MEK1-ERK1/2 signal transduction pathway (Figure 7.1). However, a major drawback to this study was the absence of a specific mechanism by which VEGF-A isoforms promote differential VEGFR2 trafficking and turnover. NRP1 is required for VEGF-A-stimulated ERK1/2 activation (Lanahan et al., 2013). NRP co-receptors have been linked to regulating VEGFR2 trafficking in response to VEGF-A (Lanahan et al., 2013; Ballmer-Hofer et al., 2011). Therefore, one possibility is that selective recruitment of the NRP co-receptors could confer isoform-specific VEGFR2 trafficking, turnover and post-translational modifications, as a means of modulating isoform-specific signal transduction via the MEK-ERK1/2 pathway. In order to test this hypothesis, NRP antagonists should be used rather than gene silencing, as NRP1 has functional roles in regulating steady state cell surface VEGFR2 levels (Gelfand et al., 2014).

ERK1/2 has a well-defined role in angiogenesis (Lanahan et al., 2013; Koch et al., 2011). Upon activation, ERK1/2 translocates into the nucleus where it phosphorylates an array of transcription factors, one of which is the transcription factor ATF-2 (Ouwens et al., 2002; Glauser and Schlegel, 2007; Roskoski, 2012). Similarly, VEGF-A$_{165}$ stimulation has been shown to promote ATF-2 activation, yet prior to the studies presented in this thesis, a detailed intracellular signalling cascade was not defined (Seko et al., 1998; Salameh et al., 2010). The work presented here now shows that VEGF-A$_{165}$ but not VEGF-A$_{121}$ stimulation promotes ATF-2 phosphorylation via the MEK1-ERK1/2 pathway (Figure 7.1). Expression of a mutant ATF-2 in a mouse model has been shown to inhibit VCAM-1 expression (Reimold et al., 2001). Therefore, one hypothesis was that ATF-2 regulated VCAM-1 levels in response to VEGF-A. Our studies showed that elevated ATF-2 activity resulted in increased VEGF-A$_{165}$-specific VCAM-1. VEGF-A$_{165}$-stimulated elevation of VCAM-1 resulted in increased endothelial-leukocyte binding. Contrastingly, the mechanism underlying VEGF-A-stimulated *VCAM-1* gene expression is contradicted by other studies, which suggest roles for NF-κB (Kim et al., 2001) and forkhead (Abid et al., 2008) transcription factors.

Additionally, studies carried out for this thesis also revealed that ATF2 activity was required for VEGF-A$_{165}$ isoform-specific upregulation of the serine/threonine protein kinase Tpl2 (Figure 7.1). Tpl2 has recently been implicated in tumour angiogenesis (Lee et al., 2013).

Figure 7.1. Mechanisms involved in regulating VEGF-A isoform-specific endothelial cell function. VEGF-A isoforms differentially stimulate VEGFR2 activation, internalisation, downstream signal transduction and gene expression to control endothelial cell function. Differential VEGF-A isoform-specific programing of VEGFR2 internalisation impacts on downstream ERK1/2 phosphorylation and subsequent ATF-2 activation. Increased ATF-2 activity is associated with elevated *VCAM-1* and *Tpl2* gene expression. Elevated VCAM-1 levels promote endothelial-leukocyte interactions, whereas increased Tpl2 levels triggers subsequent waves of intracellular signal transduction required for VEGF-A-stimulated tubulogenesis. Elevated Tpl2 activity in response to reduced ATF-2 activity triggers p53-dependent p21 and Cyclin D1 expression and cell cycle arrest. VEGF-A isoform-specific PLCγ1 phosphorylation stimulates differential cytosolic calcium ion flux and calcineurin-dependent dephosphorylation of NFATc2. Dephosphorylated NFATc2 translocates into the nucleus where it regulates the transcription of target genes to promote endothelial cell migration. Dashed arrows denote undefined mechanisms. Grey arrows denote VEGF-A stimulated pathways. Blue arrows indicate pathways activated by cellular stress. Green arrows denote constitutive functions. Red arrows denote inhibitory functions. Black arrows denote canonical pathways.
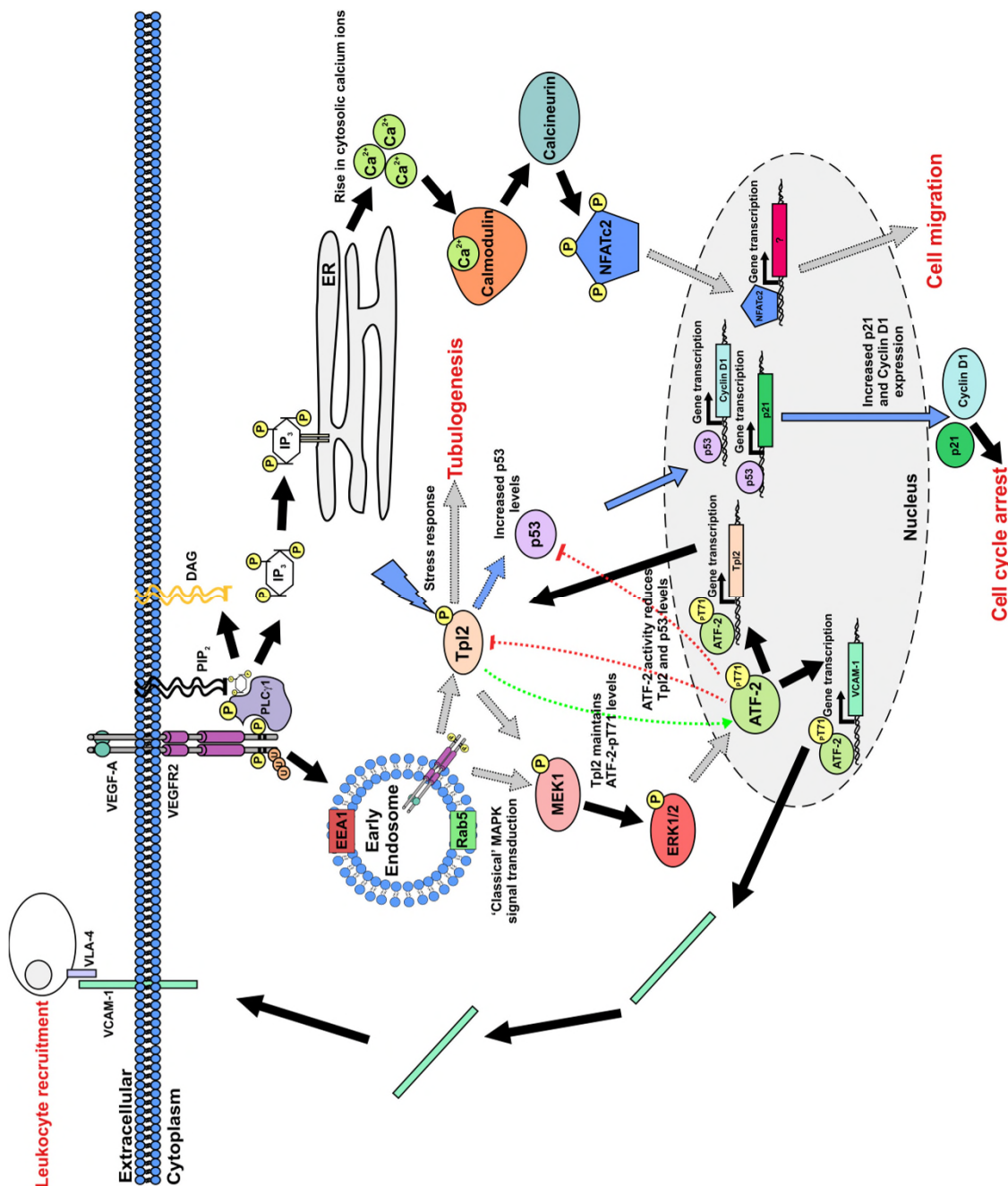
Furthermore, a Tpl2-specific small molecule inhibitor impairs *in vivo* angiogenesis by attenuating endothelial cell proliferation (Lee et al., 2013). However, prior to the work carried out in this thesis, the mechanisms by which Tpl2 modulates endothelial function were ill defined. One hypothesis was that Tpl2 acted as a central signalling nexus that integrates VEGF-A-stimulated short-term signal transduction with long-term cellular decisions. This study supported this role as Tpl2 was essential to regulate both pro- and anti-angiogenic cellular decision-making pathways, which impact on essential endothelial cell outcomes. The studies carried out here revealed a novel role for endothelial Tpl2 kinase activity in the stabilisation of ATF-2 turnover via promoting its constitutive phosphorylation on residue T71 (ATF-2-pT71; Figure 7.1). This novel function modulates VEGF-A isoform-specific ATF-2-dependent gene transcription (VCAM-1) and cellular responses (endothelial-leukocyte interactions) (Figure 7.1). However, the mechanism by which Tpl2 regulates ATF-2 phosphorylation is still unknown. Tpl2 has been shown to activate ERK1/2 (Beinke et al., 2003), thus one possibility is that Tpl2 acts through the MEK1-ERK1/2 pathway in order to stimulate ATF-2 phosphorylation. Furthermore, our study highlighted ATF-2 as being essential for endothelial cell cycle progression with loss of ATF-2 triggering *p21* and *Cyclin D1* gene expression and cellular quiescence. This phenomenon is both Tpl2 and p53-depedenent, as simultaneous depletion of either ATF-2/Tpl2 or ATF-2/p53 was sufficient to rescue endothelial responses. Previous work has already implicated increased Tpl2 activity with mediating p53-dependent cell cycle arrest (Gkirtzimanaki et al., 2013) and ATF-2 in modulating cell cycle progression (Walluscheck et al., 2013; An et al., 2013). Therefore, the work here in this thesis defines a novel mechanism whereby a Tpl2/ATF-2 regulatory axis, modulates endothelial gene expression and cell cycle progression (Figure 7.1).

VEGF-A binding promotes VEGFR2 phosphorylation on cytoplasmic residue Y1175, which promotes the recruitment and activation of PLCγ1 (Koch and Claesson-Welsh, 2012). PLCγ1 activation causes production of IP$_3$ which stimulates a rise in cytosolic calcium ions. This led us to ask whether VEGF-A isoforms could promote differential PLCγ1 activation and elevation of cytosolic calcium ions. Indeed our results revealed that VEGF-A$_{165}$ was more effective than VEGF-A$_{121}$ in stimulating phosphorylation and activation of PLCγ1, and subsequent elevation of cytosolic calcium ions (Figure 7.1).

Cytosolic calcium ions are known to regulate a vast number of cellular responses (Hogan et al., 2003; Crabtree, 2001). One way it can do this, is by binding and subsequently activating the protein calmodulin (Hogan et al., 2003; Crabtree, 2001). Activated calmodulin can then act to activate the protein phosphatase calcineurin, which then promotes the dephosphorylation of calcineurin target proteins. One such target is NFATc2 (NFAT1; Figure 7.1), resulting in its subsequent rapid dephosphorylation and nuclear translocation (Luo et al., 1996; Okamura et al., 2000). Our results, reveal that VEGF-A$_{165}$ and VEGF-A$_{121}$ stimulation promotes cytosolic calcium ion flux, NFATc2 dephosphorylation and nuclear translocation but to widely differing extents. Furthermore, we showed for the first time that depletion of endothelial NFATc2 levels significantly reduced VEGF-A stimulated endothelial cell migration (Figure 7.1) suggesting that this factor plays a role in controlling gene expression linked to cell migration processes e.g. focal adhesion, stress fibre formation. Additionally, NFATc2 was also required for the regulation of basal endothelial cell migration. However, one major drawback of the work present in this thesis is the lack of the identification of the NFATc2 target gene responsible. Interestingly, NFATc2 has also been linked to regulating VEGF-A-stimulated tubulogenesis (Bala et al., 2012), a phenomenon which was not detected in the studies carried out for this thesis. NFATc2 has a well-established role in regulating VEGF-A stimulated endothelial responses and angiogenesis (Zaichuk et al., 2004; Goyal et al., 2012), thus the study presented here strengthens the importance of NFATc2 within the endothelium.

Although the work presented in this thesis advances our knowledge of how VEGF-A isoforms differentially regulate VEGFR2 trafficking and turnover to impact on downstream signal transduction, gene expression and endothelial cell function, there are some drawbacks with our current study. Firstly, there is a lack of animal model data to confirm the *in vivo* functionality of our mechanisms derived from cellular models. Furthermore, a lot of our conclusions were based on siRNA-mediated gene silencing using a pool of siRNA duplexes. Therefore, further experiments using multiple single siRNA duplexes followed by lentiviral expression of wild type of mutant transcription factors will enable us to detect any off target effects, associated with RNAi-mediated gene silencing. Finally, most of our study focused on just 2 VEGF-A isoforms (VEGF-A$_{165}$ and VEGF-A$_{121}$). Repeating such experiments using a wider range of pro- or anti-angiogenic VEGF-A isoforms may help us better understand their individual roles in modulating endothelial cell function and ultimately angiogenesis.

7.2.   VEGF-A isoforms as therapeutic agents.

Peripheral artery disease (PAD), ischaemic stroke and coronary artery diseases generate tissue ischaemia through arterial occlusions (caused by atherosclerosis and thrombosis) and insufficient collateral vessel formation (Shimamura et al., 2013; Mughal et al., 2012). Critical limb ischaemia (CLI) is a complication associated with PAD and causes pain on walking (claudication), pain at rest, and non-healing ulcers (Shimamura et al., 2013; Mughal et al., 2012). Patients suffering from CLI are commonly treated with statins, anti-platelet drugs, and angioplasty, however these treatments occasionally fail to recover sufficient blood flow in order to maintain normal tissue function, resulting in amputation (Shimamura et al., 2013; Mughal et al., 2012). Therefore, there is a desire to therapeutically stimulate new blood vessel growth (angiogenesis) in such disease settings to try and overcome clinical complication and ultimately improve patient outcomes.

Current, pro-angiogenic therapies have focused on using gene therapy to express an array of pro-angiogenic factors (e.g. VEGF-A, fibroblast growth factor (FGF), and hepatocyte growth factor (HGF)) in order to achieve therapeutic angiogenesis (Shimamura et al., 2013). As the most extensively studied pro-angiogenic growth factor, VEGF-A has been the subject of multiple clinical trials aimed at stimulating new blood vessel formation in such disease states (Rajagopalan et al., 2001; Rajagopalan et al., 2003; Muona et al., 2012; Kusumanto et al., 2006). Many of these clinical trials have reported that VEGF-A therapy promotes increase blood vessel formation in ischaemic tissue (Makinen et al., 2002; Isner, 1998), however two Phase II clinical trials failed to meet their primary endpoints (Rajagopalan et al., 2003). Therefore, even though VEGF-A gene therapy appears promising, its efficacy remains controversial; thus further research is required in order to improve its therapeutic benefit (Shimamura et al., 2013). One hypothesis was that different VEGF-A isoforms could stimulate varying levels of angiogenesis and thus prove viable alternatives to the more physiological abundant VEGF-A$_{165}$. The data presented here suggest that VEGF-A isoforms do indeed have differential capacities for stimulating collateral blood vessel formation in ischaemic tissue. Here VEGF-A$_{165}$ (20 ng daily via subcutaneous administration) treatment promoted increased blood vessel formation and limb reperfusion in comparison to both VEGF-A$_{121}$ and vehicle alone. Furthermore, the maximal therapeutic benefit of VEGF-A$_{165}$ treatment was detected up to 1 week sooner than treatment with VEGF-A$_{121}$ or vehicle alone. Therefore, this *in vivo* study supports various human clinical trials which suggested

that VEGF-A$_{165}$ pro-angiogenic therapy stimulated new blood vessel growth in ischaemic tissue (Tsurumi et al., 1997; Kusumanto et al., 2006). Such conclusions were similar to those reached in a Phase II clinical trial (Rajagopalan et al., 2003) despite promising Phase I results (Rajagopalan et al., 2001), as our studies failed to detect any added benefit as a result of VEGF-A$_{121}$ treatment in comparison to mice treated with vehicle alone. One aspect of this clinical Phase II study was that the circulating levels of VEGF-A$_{121}$ produced as a result of adenoviral gene-transfer might have been insufficient to stimulate adequate blood vessel growth. Therefore, it is possible that the concentration of VEGF-A$_{121}$ used in our study was also insufficient to promote therapeutic angiogenesis, thus further experiments to analyses the effect of increasing the concentration of VEGF-A$_{121}$ are needed. Interestingly, in contrast to other studies (Rajagopalan et al., 2001; Rajagopalan et al., 2003; Kusumanto et al., 2006) our animal study used the direct administration of VEGF-A protein rather than adenovirus-mediated or direct injection of VEGF-A plasmid DNA.

Interestingly, patients suffering from PAD have a relatively high level of circulating VEGF-A in their ischaemic tissues, which is partially due to increased hypoxia-driven *VEGF-A* gene expression (Kikuchi et al., 2014; Findley et al., 2008). However, despite this, patients suffering from PAD have paradoxically insufficient levels of angiogenesis and collateral vessel formation (Kikuchi et al., 2014). In addition to pro-angiogenic VEGF-A isoforms, anti-angiogenic forms have also been documented (Varey et al., 2008; Mavrou et al., 2014; Kikuchi et al., 2014; Kawamura et al., 2008; Harper and Bates, 2008; Cui et al., 2002). VEGF-A$_{165b}$ is the anti-angiogenic counterpart of the physiological abundant VEGF-A$_{165a}$ (commonly referred to as VEGF-A$_{165}$) and has been reported to be upregulated in patients suffering from PAD. Here, immune cell secretion of VEGF-A$_{165b}$ contributes to impaired collateral blood vessel formation (Kikuchi et al., 2014).

One clinical complication of VEGF-A therapy is increased peripheral oedema, which is thought to be as a result of increased vascular permeability and inflammation (Rajagopalan et al., 2003; Mughal et al., 2012; Kusumanto et al., 2006). In addition to VEGF-A, leukocytes can also promote increased vascular permeability in the process of transendothelial cell migration. Here, leukocytes attach themselves on the endothelium and trigger the dismantling of integral cell-cell junctions between neighbouring endothelial cells, thus increasing vascular permeability (Nourshargh et al., 2010; Vestweber et al., 2014; Wessel et al., 2014). Furthermore, circulating

immune cells can secrete a variety of factors which can stimulate increased vascular permeability (Newton and Dixit, 2012). Therefore, by selecting for VEGF-A isoforms which minimise endothelial-leukocyte interactions it may be possible to reduce the negative side effects associated with VEGF-A therapy. Work carried out in this thesis revealed that VEGF-A$_{165}$ and VEGF-A$_{121}$ have distinct capacities to stimulated ATF-2-dependent *VCAM-1* gene expression and endothelial cell-leukocyte interactions. Here, VEGF-A$_{165}$ significantly increased *VCAM-1* gene expression and leukocyte binding, whereas VEGF-A$_{121}$ failed to do so. Furthermore, VEGF-A$_{165}$ but not VEGF-A$_{121}$ also increased ATF-2-dependent *Tpl2* gene expression, which is essential for ATF-2 activity. Hence, VEGF-A$_{121}$ may be a better therapeutic choice in order to stimulate collateral vessel formation as it may elicit reduced immune cell-mediated vascular permeability and peripheral oedema. However, adenoviral VEGF-A$_{121}$ gene therapy has been documented to stimulate leg oedema, but was unclear if this was due to an inflammatory response to the adenovirus (Rajagopalan et al., 2003). Intriguingly, a non-mammalian VEGF family member, VEGF-E has been reported to stimulate angiogenesis in mice without promoting vascular permeability or inflammation and thus could prove a promising and alternative therapeutic option (Zheng et al., 2006).

7.3.   Conclusion

In conclusion this work has provided new insights into how VEGF-A isoforms differentially regulate the endothelial cell response. VEGF-A isoforms promote specific phosphorylation, ubiquitination, trafficking, proteolysis and turnover of VEGFR2 which helps confer isoform-specific downstream signalling outputs e.g. ERK1/2 (Figure 7.1). This differential downstream signal transduction results in altered transcription factor activation (e.g. ATF-2 and NFATc2), gene expression (e.g. VCAM-1 and Tpl2) and cellular responses (e.g. migration, tubulogenesis and endothelial-leukocyte interactions) (Figure 7.1). This data helps us to further understand how short-lived signal transduction is converted into gene expression to modulate long-term endothelial response. Furthermore, this work presents a novel role for Tpl2 in the dual regulation of endothelial gene expression and cell cycle progression (Figure 7.1). Here endothelial Tpl2 is essential to maintain constitutive phosphorylation of ATF-2 and is required for VEGF-A$_{165}$ stimulated endothelial cell responses. Loss of ATF-2 activity impaired endothelial cell cycle progression, a phenomenon reliant on p53 and Tpl2. Therefore, Tpl2 acts as a signalling nexus which relays multiple cellular signals to promote or impair endothelial cell function and angiogenesis (Figure 7.1). This model now provides a framework for targeting

Tpl2 to modulate altered endothelial cell function in wide number of disease states. Finally, this study confirms that VEGF-A isoforms have different capacities to stimulating collateral blood vessel formation and reperfusion of ischaemic tissues. Therefore, it is possible that the use of an alternate VEGF-A isoform may prove a promising therapeutic option for the treatment of PAD, by reducing associated side effects and improve patient outcomes.

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Appendix A

Publications, Conference proceedings and Awards

Publications (10):

Smith GA, **Fearnley GW**, Abdul Zani I, Wheatcroft SB, Tomlinson DC, Harrison MA, Ponnambalam S (2015) VEGFR2 signaling, trafficking and proteolysis is regulated by the ubiquitin isopeptidase USP8. *Traffic*. **ePub ahead of print**.

Smith GA, **Fearnley GW**, Tomlinson DC, Harrison MA, Ponnambalam S (2015) The cellular response to vascular endothelial growth factors requires co-ordinated signal transduction, trafficking and proteolysis. *Bioscience. Rep*. **35**:e00253.

Karpov OA, **Fearnley GW**, Smith GA, Kankanala J, McPherson MJ, Tomlinson DC, Harrison MA, Ponnambalam S (2015) Receptor tyrosine kinase structure and function in health and disease. *AIMS Biophysics*. **2**:476-502.

Fearnley GW, Wheatcroft SB, Ponnambalam S (2015) Detection and quantification of VEGF receptor tyrosine kinases in human endothelial cells. *Meth Mol Biol*. **1332**:49-65.

Smith GA, **Fearnley GW**, Harrison MA, Tomlinson DC, Wheatcroft SB, Ponnambalam S (2015) Vascular endothelial growth factors: multitasking functionality in metabolism, health and disease. *J Inherited Metab. Dis.* **38**:753-763.

Fearnley GW, Bruns AF, Wheatcroft SB, Ponnambalam S (2015) VEGF-A isoform-specific regulation of endothelial cell migration via differential Ca^{2+}-dependent NFATc2 activation**.** *BiO*. **4**:731-742*.*

Latham AM, Kankanala J, **Fearnley GW**, Gage MC, Kearney MT, Homer-Vanniasinkam S, Wheatcroft SB, Fishwick CWG, Ponnambalam S (2014) *In Silico* Design and Biological Evaluation of a Dual Specificity Kinase Inhibitor Targeting Cell Cycle Progression and Angiogenesis**.** *PLoS One* **9**:e110997.

*Fearnley GW, Odell AF, Latham AM, Mughal NA, Bruns AF, Burgoyne NJ, Homer-Vanniasinkam S, Zachary IC, Hollstein M, Wheatcroft SB, Ponnambalam S (2014) VEGF-A isoforms differentially regulate ATF-2-dependent VCAM-1 gene expression and endothelial-leukocyte interactions. *Mol Biol Cell*. **25**:2509-2521. ***Cover article**.

Fearnley GW, Smith GA, Odell AF, Latham AM, Wheatcroft SB, Harrison MA, Tomlinson DC, Ponnambalam S (2014) Vascular endothelial growth factor A-stimulated signaling from endosomes in primary endothelial cells. *Meth Enzymol.* **535**:265-292.

Fearnley GW, Smith GA, Harrison MA, Wheatcroft SB, Tomlinson DC, Ponnambalam S (2013) The biochemistry of new blood vessel formation in health and disease. *OA Biochemistry.* **1**:5.

Manuscripts submitted/under review (4):

Fearnley GW, Odell AF, Abdul Zani I, Latham AM, Hollstein M, Wheatcroft SB, Ponnambalam S (2015) Tpl2 is a signaling nexus in endothelial gene expression and cell cycle progression. **Submitted**.

López-García M, Nowicka M, **Fearnley GW**, Ponnambalam S. Lythe GD, Molina-París C (2015) Matrix-analytic solution schemes. *Quant. Biol.* **Submitted**.

Fearnley GW, Smith GA, Abdul Zani I, Yuldasheva N, Mughal NA, Homer-Vanniasinkam S, Tomlinson DC, Harrison, MA, Zachary IC, Kearney MT, Wheatcroft SB, Ponnambalam S (2015) VEGF-A isoform-specific signal transduction is modulated by VEGFR2 endocytosis. **Submitted**.

Smith GA, **Fearnley GW**, Wheatcroft SB, Tomlinson DC, Harrison MA, Ponnambalam S (2015) Basal VEGFR2 ubiquitination modulates signal transduction and endothelial function. **Submitted**.

Manuscripts in preparation (2):

Meeting Presentations & Participation:
Oral presentations:

Fearnley GW, Wheatcroft SB, Ponnambalam S. VEGF-A isoform-specific regulation of gene expression and leukocyte recruitment. *University of Leeds Postgraduate Symposium*, *University of Leeds, UK:* **9th-10th Apr 2014. (20 min talk)**

Fearnley GW, Wheatcroft SB, Ponnambalam S. VEGF-A isoform-specific regulation of gene expression and leukocyte recruitment. *MCRC Retreat 'Cardiovascular and Diabetes Research at Leeds: Bench to Bedside', Weetwood Hall, Leeds, UK*: **20th-21st Mar. 2014. (20 min talk)**

Fearnley GW, Wheatcroft SB, Ponnambalam S. VEGF isoforms differentially program VEGFR2 receptor tyrosine kinase activation, signalling, trafficking and turnover in endothelial cells. *North of England Cell Biology (NECB)*

meeting, Foresight centre, University of Liverpool, UK: **11**th **Sep. 2013. (20 min talk)**

<u>Fearnley GW</u>, Wheatcroft SB, Ponnambalam S. Differential regulation of the endothelial response by VEGF-A isoforms. *University of Leeds Endothelial retreat, the Inn on the Lake, Ullswater, UK*: **24**th**-25**th **Jan. 2013. (20 min talk)**

Poster Presentations:

<u>Fearnley GW</u>, Odell AF, Wheatcroft SB, Ponnambalam S. Integration of signal transduction and gene expression in controlling endothelial cell function. *78*th *Harden Conference on* 'Protein Kinases in Health and Disease', Norton Park Hotel, Winchester, UK. **15**th**-18**th **Sep. 2015**.

<u>Fearnley GW</u>, Odell AF, Wheatcroft SB, Ponnambalam S. Integration of signal transduction and gene expression in controlling endothelial cell function*. North of England Cell Biology (NECB) meeting, University of York, UK.* **7**th **Sep. 2015**.

<u>Fearnley GW</u>, Odell AF, Wheatcroft SB, Ponnambalam S. VEGF-A isoforms differentially regulate ATF-2-dependent VCAM-1 gene expression and endothelial-leukocyte interactions. *Northern Vascular Biology Forum, Manchester Metropolitan University, UK:* **8**th **Dec. 2014**.

*****<u>Fearnley GW</u>**, Odell AF, Wheatcroft SB, Ponnambalam S. VEGF-A isoforms differentially regulate ATF-2-dependent VCAM-1 gene expression and endothelial-leukocyte interactions. *North of England Cell Biology (NECB) meeting, University of Leeds, UK:* **12**th **Sep. 2014**. *<u>Poster Prize</u>

<u>Fearnley GW</u>, Odell AF, Wheatcroft SB, Ponnambalam S. VEGF-A isoforms differentially regulate ATF-2-dependent VCAM-1 gene expression and endothelial-leukocyte interactions. 'Angiogenesis and vascular remodelling: New perspectives' *University of Chester, UK.* **14**th**-15**th **Jul. 2014**.

<u>Fearnley GW</u>, Odell AF, Wheatcroft SB, Ponnambalam S. VEGF-A isoforms differentially regulate ATF-2-dependent VCAM-1 gene expression and endothelial-leukocyte interactions. Gordon Research Conference on 'Endothelial cell phenotypes in health and disease'*. Melia Golf Vinchy, Catalan business and conference centre, Girona, Spain*. **6**th**-11**th **Jul. 2014**.

<u>Fearnley GW</u>, Smith GA, Wheatcroft SB, Ponnambalam S. Vascular endothelial growth factor A (VEGF-A) isoforms show differential regulation of the endothelial response. *North of England Cell Biology (NECB) meeting, Foresight centre, University of Liverpool, UK.* **11**th **Sep. 2013**.

Fearnley GW, Smith GA, Wheatcroft SB, Ponnambalam S. Vascular endothelial growth factor A (VEGF-A) isoforms show differential regulation of the endothelial response. *75th Harden Conference on 'Receptor tyrosine kinase structure and function in health and disease'. The Endcliffe Village, University of Sheffield, UK.* **1st-4th Sep. 2013**.

Fearnley GW, Wheatcroft SB, Ponnambalam S. Vascular endothelial growth factor A isoforms differentially regulate the endothelial response. *University of Leeds Postgraduate Symposium, University of Leeds, UK.* **10-11 Apr. 2013**.

Session Chairs:

North of England Cell Biology meeting, chair of morning session, University of Leeds, UK. **12th Sep 2014.**

Seminars:

Fearnley GW, Wheatcroft SB, Ponnambalam S. VEGF-A isoform-specific regulation of endothelial cell function. *Centre for Cardiovascular Biology and Medicine, University College London, UK:* **16th Mar. 2015. (40 min talk)**

Awards & Prizes;

Poster 1st Prize. VEGF-A isoforms differentially regulate ATF-2-dependent VCAM-1 gene expression and endothelial-leukocyte interactions. *North of England Cell Biology (NECB) meeting, University of Leeds, UK:* **12th Sep 2014.**

Appendix B

Characterisation of recombinant VEGF-A proteins
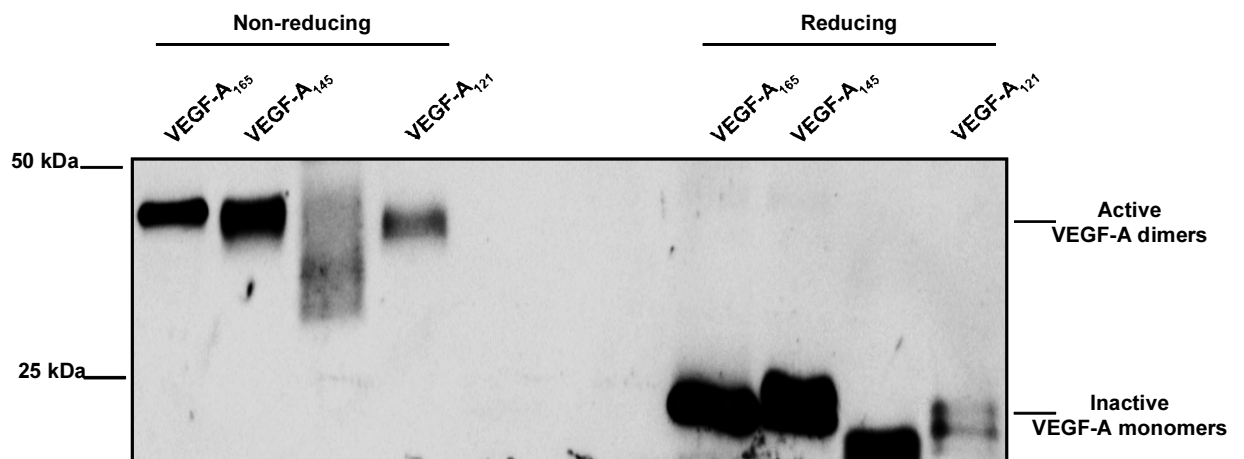
Appendix B. Immunoblot assessment of recombinant VEGF-A activity. 10 μg of recombinant VEGF-A$_{165}$, VEGF-A$_{145}$ or VEGF-A$_{121}$ was loaded onto a SDS-PAGE gel under non-reducing (-β-mercaptoethanol) or reducing (+β-mercaptoethanol) conditions prior to immunoblot assessment of active dimers using a pan anti-human VEGF-A antibody.