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The role of the extracellular signal-regulated kinase 5 (ERK5) signalling axis in angiogenesis

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THE ROLE OF THE EXTRACELLULAR SIGNAL-REGULATED KINASE 5 (ERK5) SIGNALLING AXIS IN ANGIOGENESIS

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Swyddogaeth echelin signalu y cinas allgellog-reoledig 5 (ERK5) mewn angiogenesis

Mae angiogenesis – ffurfiant gwaedlestri newydd o waedlestri sydd eisioes mewn bodolaeth – yn hanfodol ar gyfer datblygiad embryonaidd ac ar gyfer atgyweiriad meinweol mewn oedolion. Ers y darganfyddiad fod angiogenesis yn allweddol ar gyfer datblygiad sawl anhwylder, gan gynnwys tŵf ac ymlediad cancr, bu ymchwil frwd mewn ymdrech i geisio esbonio dulliau angiogenesis. Caiff y protein cinas mitogen-egnïoledig, ERK5 ei egnïoli mewn ymateb i amrywiaeth o stimwli allgellog, gan gynnwys ffactorau tŵf pro-angiogenaidd mewn celloedd endothelaidd. Mae diddymiant y gennyn *Erk5* mewn llygod yn arwain at angeuoldeb embryonaidd o ganlyniad i amhariad mewn datblygiad cardiofasgwlaidd a cholled cyfundeb gwaedlestrol. Yn yr astudiaeth hon, ymchwilwyd swyddogaeth ddichonol ERK5 fel cyfryngydd o gamau amrywiol angiogenesis yn dilyn ysgogiad y factor tŵf VEGF mewn celloedd endotheliaidd microfascwlaidd dynol cynradd (HDMEC). Darganfyddwyd fod ERK5 yn cael ei egnïoli gan VEGF mewn HDMEC drwy gyfrwng y derbynnydd VEGFR-2 a'r protein cinas PKC-delta (PKCδ). Trwy dawelu mynegiant ERK5 mewn HDMEC gyda RNA bychan ymyrgar (siRNA), datgelwyd fod ERK5 yn angenrheidiol ar gyfer goroesiad celloedd endothelaidd yn ystod eu gwahanrediad i ffurfio strwythurau capilariadd yn dilyn ysgogiad VEGF ar factics colagen tri dimensiwn, ond nid ar gyfer amlhad nac ymfudiad VEGF-ysgodedig y celloedd hyn. Ymhellach, darganfyddwyd fod actifiant cyfansoddol ERK5 yn ddigonol i ysgogi morffogenesis tiwbwlaidd yn absenoldeb VEGF, a chaniatau goroesiad estynedig ffurfiannau capilariaidd ym mhresenoldeb VEGF. Dangosodd dadansoddiad o lwybrau signalu mewngellol fod ERK5 yn cyfryngu goroesiad celloedd endothelaidd yn dilyn ysgogiad VEGF drwy reoli egnioliad y protein cinas AKT, gan arwain at lonyddiad y protein pro-apoptotig BAD, tra ar yr un pryd yn caniatau mynegiant VEGF-ysgogedig y protein gwrth-apoptotig BCL-2, gan arwain at rwystro adweithedd caspase-3 ac ataliad apoptosis o ganlyniad i hyn. Er mwyn astudio swyddogaeth ERK5 mewn angiogenesis ymhellach, datblygwyd model mewn llestr o angiogenesis ble cyd-feithrinwyd ffibroblastau a chelloedd endothelaidd. Dangosodd y model hwn fod trinaiaeth hirfaith gyda siRNA ERK5-neilltuol yn ddigonol i rwystro angiogenesis a ysgogwyd gan y ffactorau tŵf VEGF ac FGF-2 drwy beri apoptosis yn benodol mewn celloedd endothelaidd, ond nid mewn celloedd ffibroblast. Fel cyfangorff, mae'r astudiaeth hon yn dynodi ERK5 fel cyfryngydd hanfodol ar gyfer goroesiad VEGF-ysgogedig celloedd endothelaidd dynol yn ystod camau penodol o angiogenesis, gan hefyd awgrymu y gallai atal actifedd ERK5 gynnig modd posib o rwystro angiogenesis patholegol yn y corff byw.

The role of the extracellular signal-regulated kinase 5 (ERK5) signalling axis in angiogenesis

Angiogenesis - the formation of new blood vessels from pre-existing blood vessels - is vital for normal development in embryos and for tissue repair in adults. Elucidating the mechanisms of angiogenesis has been subject to intense research following the discovery that angiogenesis is critical for the progression of several pathologies, including cancer. The mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase 5 (ERK5) is activated in response to a variety of extracellular stimuli, including pro-angiogenic stimuli in endothelial cells. Ablation of the *Erk5* gene in mice is embryonically lethal due to disruption of cardiovascular development and loss of vascular integrity. In this study, the potential role of ERK5 in mediating various stages of vascular endothelial growth factor (VEGF)-stimulated angiogenesis in primary human dermal microvascular endothelial cells (HDMECs) was investigated. It was found that VEGF stimulated ERK5 activation in HDMECs via VEGF receptor-2 (VEGFR-2) and protein kinase C-delta (PKCδ). Small interfering RNA (siRNA)-mediated silencing of ERK5 expression revealed that ERK5 was required for HDMEC survival during VEGF-stimulated differentiation of HDMECs to form capillary structures within a 3-D collagen gel matrix, but not for VEGF-stimulated proliferation or migration. Furthermore, constitutive activation of ERK5 induced tubular morphogenesis in the absence of VEGF, and facilitated sustained survival of HDMEC capillary structures in the presence of VEGF. Analysis of intracellular signalling pathways showed that ERK5 mediated VEGF-induced endothelial cell survival via regulation of AKT activation and subsequent inactivation of the pro-apoptotic protein BAD, whilst concomitantly facilitating VEGF-stimulated expression of the anti-apoptotic protein *BCL2*, resulting in decreased caspase-3 activity and suppression of apoptosis. To further investigate the role of ERK5 in angiogenesis, a HDMEC/NHDF (normal human dermal fibroblast) co-culture *in vitro* angiogenesis assay was developed, revealing that chronic treatment with ERK5-specific siRNA prevented VEGF and fibroblast growth factor-2 (FGF-2)-stimulated angiogenesis by specifically inducing apoptosis of HDMECs, but not NHDFs. Together, this study identifies ERK5 as a critical mediator of VEGF-induced human endothelial cell survival during a specific stage of angiogenesis, and suggests that inhibition of ERK5 activity may prevent aberrant angiogenesis *in vivo.*

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This thesis is dedicated to the memory of my grandfathers: Trefor Roberts and Merville Stephen Roberts; two men who profoundly influenced my life, and whom are sadly missed.

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Abbreviations

3-D	Three-dimensional
AKT	v-akt murine thymoma viral oncogene homolog (PKB)
Ang-1	Angiopoietin-1
aPKC	Atypical PKC
BAD	BCL2-antagonist of cell death
BAEC	Bovine aortic endothelial cell
BAPTA-AM	1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis-acetoxymethyl ester
BCIP/NBT	5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium
BCL2	B-cell lymphoma 2
BDNF	Brain-derived neurotrophic factor
BH3	BCL2 homology 3
BIM	BCL2-interacting mediator of cell death
BLMEC	Bovine lung microvascular endothelial cell
BMK1	Big MAPK-1
BSA	Bovine serum albumin
CA	Constitutively active
CaMK	Ca^{2+}/Calmodulin-dependent kinase
cAMP	Cyclic adenosine monophosphate
CD	Common docking
CKR	CSFR-1-Flk-1 receptor
CMFDA	5-chloromethylfluorescein diacetate
COT	Cancer Osaka thyroid
cPKC	Classical PKC
CREB	cAMP-responsive element binding-protein
CSF-1	Colony stimulating factor-1
CSFR-1	CSF-1 receptor-1
C_T	Cycle threshold
DAG	Diacylglycerol
DMSO	Dimethyl sulfoxide
DN	Dominant-negative
dNTP	Deoxynucleotide triphosphate
DOG	1,2-dioctanoyl-*sn*-glycerol
DOPA	1,2-dioctanoyl-*sn*-glycero-3-phosphate
DOPC	1,2-dioctanoyl-*sn*-glycero-3-phosphocholine
DPBS	Dulbecco's phosphate-buffered saline
DRG	Dorsal root ganglion
dsRNA	Double-stranded RNA
DTT	Dithiothreitol
DUSP	Dual-specificity protein phosphatase
E	Embryonic day
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor

EGTA	Ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid
eNOS	Endothelial nitric oxide synthase
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
F.G.M.	Full growth medium
FAK	Focal adhesion kinase
FasL	Fas ligand
FCS	Foetal calf serum
FDA	Food and drug administration
FGF	Fibroblast growth factor
FKBP12	FK506-binding protein 12
FLIP	FLICE-like inhibitory protein
Flk1	Foetal liver kinase 1
Flt1	Fms-related tyrosine kinase 1
FOXO3a	Forkhead box O3a
FPP	Farnesyl pyrophosphate
FTS	Farnesylthiosalicylic acid
g	Gravitational force
Gab1	Grb2-associated binder-1
GFP	Green fluorescent protein
Grb2	Growth factor-receptor-bound 2
HA	Haemagluttinin
HAEC	Human aortic endothelial cell
HDMEC	Human dermal microvascular endothelial cell
HEK	Human embryonic kidney
HGF	Hepatocyte growth factor
HIF-1α	Hypoxia-inducible factor-1α
HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme A
HREC	Human retinal endothelial cell
HRP	Horseradish peroxidase
HSP27	Heat-shock protein 27
HSPG	Heparan sulfate proteoglycan
HUVEC	Human umbilical vein endothelial cell
IAP	Inhibitor of apoptosis protein
IGF-1	Insulin-like growth factor 1
IL-8	Interleukin-8
ILK	Integrin-linked kinase
IP_3	Inositol (1,4,5)-trisphosphate
IP_3R	IP_3 receptor
ISG20	Interferon-stimulated gene (20kD)
JNK	c-Jun NH_2-terminal kinase
KDR	Kinase-insert domain-containing receptor
KLF2	Krüppel-like factor 2
LAD	Lck-associated adapter
LB	Luria-Bertani
LDS	Lithium dodecyl sulfate
LPA	Lysophosphatidic acid

MADS	MCM1, agamous, deficiens, serum response factor
MAPK	Mitogen-activated protein kinase
MAPKAPK	MAPK-activated protein kinase
MAPKK	MAPK kinase
MAPKKK	MAPKK kinase
MEF2	Myocyte enhancer factor 2
MEK	MAPK/ERK kinase
MEKK	MEK kinase
MKP	MAP kinase phosphatase
MLCEC	Mouse lung capillary endothelial cell
mLST8	Mammalian lethal with SEC13 protein 8
M-MLV	Moloney murine leukemia virus
MMP	Matrix metalloproteinases
mRNA	Messenger RNA
mTOR	Mammalian target of rapamycin
mTORC	mTOR complex
Mx1	Myxovirus resistance 1
N.S.	Non-silencing
NFAT	Nuclear factor of activated T-cells
NF-κB	Nuclear factor-κB
NGF	Nerve growth factor
NHDF	Normal human dermal fibroblast
NLS	Nuclear localisation signal
NO	Nitric oxide
nPKC	Novel PKC
NRP	Neuropillin
NT	Neurotrophin
OAS1	2′, 5′-oligoadenylate synthetase
$p90^{RSK}$	90 kDa ribosomal S6 kinase
PAE	Porcine aortic endothelial
PAGE	Polyacrylamide gel electrophoresis
PB1	Phox and Bem1p
PC12	Pheochromocytoma 12
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PDK1	3'-phosphoinositide-dependent kinase-1
PECAM-1	Platelet endothelial cell adhesion molecule-1
PH	Pleckstrin-homology
PI3K	Phosphoinositide 3'-kinase
PIKK	PI3K-related protein kinase
PIP_2	Phosphatidylinositol (4,5)-bisphosphate
PIP_3	Phosphatidylinositol (3,4,5)-trisphosphate
pIpC	Polyinosinicpolycytidylic acid
PKA	Protein kinase A
PKB	Protein kinase B
PKC	Protein kinase C
PKD	Protein kinase D

PLC-γ	Phospholipase C-γ
PlGF	Placental growth factor
PMA	Phorbol 12-myristate 13-acetate
PMSF	Phenylmethanesulfonyl fluoride
PP2A	Protein phosphatase 2A
PP2B	Protein phosphatase 2B
PPAR-γ1	Peroxisome proliferator-activated receptor-γ1
PR	Proline rich
PS	Phosphatidylserine
PTEN	Phosphatase and tensin homologue
qRT-PCR	Quantitative real-time PCR
r.p.m.	Revolutions per minute
r.t.	Room temperature
RAPTOR	Regulatory associated protein of mTOR
RCAN1	Regulator of calcineurin 1
RELA	v-rel reticuloendotheliosis viral oncogene homolog A (p65 NF-κB)
RICTOR	Rapamycin-insensitive companion of mTOR
RIPA	Radio-immunoprecipitation assay
RISC	RNA-induced silencing complex
RNAi	RNA interference
rpS6	Ribosomal protein S6
RTK	Receptor tyrosine kinase
SAPK	Stress-activated protein kinase
Sck	Shc-like protein
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SGK	Serum and glucocorticoid-inducible kinase
SH2	Src homology 2
SH3	Src homology 3
Shb	Src homology 2 protein B
SIN1	Stress-activated-protein-kinase-interacting protein 1
siRNA	Small interfering RNA
SOS	Son of sevenless
SUMO	Small ubiquitin-related modifier
sVEGFR-1	Soluble VEGFR-1
TBE	Tris-borate EDTA
TBS	Tris-buffered saline
TBST	TBS Tween
TE	Tris EDTA
TEMED	N,N,N′,N′-tetramethylethylenediamine
TGF-β	Tumour growth factor-β
TIME cells	Telomerase-immortalised microvascular endothelial cells
TNF-α	Tumour necrosis factor-α
TPL2	Tumour progression locus 2
TSAd	T-cell specific adapter
TSP-1	Thrombospondin-1
VCAM	Vascular cell adhesion molecule

VE-cadherin	Vascular endothelial cadherin
VEGF	Vascular endothelial growth factor
VEGFR	VEGF receptor
VPF	Vascular permeability factor
VRAP	VEGF receptor-associated protein
WB	Western blot
wt	Wild-type
XIAP	X-chromosome-linked IAP

CHAPTER ONE

Introduction

1.1 Vascular development

A complex vascular system is of vital importance in all vertebrates to allow an adequate supply of oxygen and nutrients to their organs and the concomitant removal of the waste products of metabolism. Furthermore, a vascular system allows the rapid transport of endocrine signals to their effector organs, and the movement of immune cells to sites of infection and inflammation. As such, the cardiovascular system is the first functional organ to develop during embryogenesis (Rossant & Howard, 2002).

The vascular system is composed of a continuous structure of highly-branched tubular networks consisting of blood vessels and lymphatic vessels, the lumen of which is lined by a monolayer of endothelial cells. The structure of large vessels such as arteries and veins is composed of an inner layer (*tunica intima*), comprising a monolayer of endothelial cells, which are surrounded by a sheet-like basement membrane consisting of the extracellular matrix (ECM) proteins. The basement membrane is surrounded by the *tunica media* layer consisting of vascular smooth muscle cells, which allow vessel contraction, as well as the outermost *tunica adventia* layer which consists of the supporting connective tissue (**Fig. 1.1 A**). By contrast, the endothelial cells of the small blood vessels such as capillaries and arterioles are surrounded by basement membrane, and are stabilised by specialised smooth muscle cells known as pericytes (**Fig. 1.1 B**; Gallagher & van der Wal, 2006).

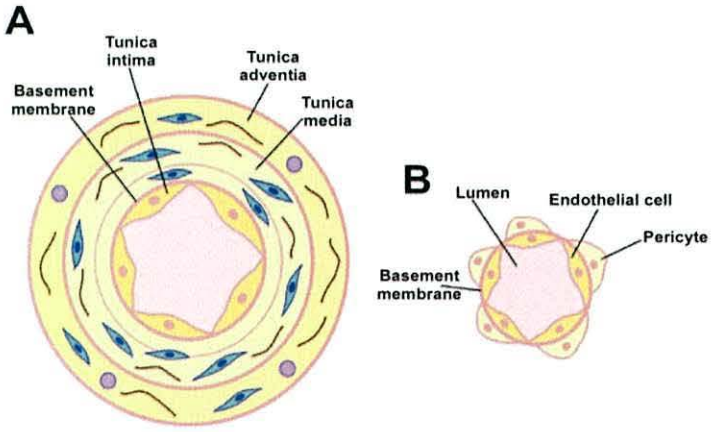


Figure 1.1 Blood vessel structures. (**A**) Schematic illustration representing a cross-section of large vessel such as an artery or vein. (**B**) Schematic illustration representing a cross-section of a small vessel such as a capillary, post-capillary venule or arteriole. Diagram modified from (Lafleur *et al.*, 2003).

It should be noted that endothelial cells originating from different organs display considerable heterogeneity, most prominently in their size and morphology (Aird, 2003), but also in their immunological and metabolic properties, as well as their responses to extracellular stimuli (Kumar *et al.*, 1987; Detmar *et al.*, 1992; Jackson & Nguyen, 1997; Lang et al., 2001; Conway & Carmeliet, 2004). The vast majority (95%) of the endothelial cells in the human vascular system are microvascular endothelial cells (Hewett, 2009).

1.1.1 Vasculogenesis

The initial establishment of a vascular network during early embryogenesis, termed vasculogenesis, occurs extra-embryonically, and involves the differentiation of mesodermal cells into hemangioblasts (Risau, 1995). Hemangioblasts subsequently form aggregates known as primitive blood islands, which later differentiate to form mature blood islands, consisting of an outer layer of endothelial cell precursors known as angioblasts, and an inner mass of hematopoietic stem cells. Fusion of blood islands and the subsequent differentiation of the angioblasts and hematopoietic precursor cells, to endothelial cells and mature hematopoietic cells respectively, give rise to a primary capillary plexus in the yolk sac that surrounds the embryo (Choi *et al.*, 1998). A similar, but distinct process of *de novo* blood vessel formation occurs in the embryo proper, whereby mesoderm-derived cells differentiate directly into angioblasts that later differentiate into endothelial cells (Risau & Flamme, 1995). In mice, angioblast aggregation occurs at embryonic day 6.5 (E6.5), and a primary vascular plexus is established at E8.0-E8.5 (Haar & Ackerman, 1971). The establishment of a functional primary vascular plexus is critical for the survival of murine embryos beyond E9.5 (Srivastava & Olson, 2000).

1.1.2 Angiogenesis

Angiogenesis is defined as the formation of new blood vessels from pre-existing blood vessels (Risau, 1997). During normal embryonic development, following vasculogenesis, angiogenesis occurs, whereby the newly formed primary capillary plexus is remodelled to allow blood vessels to reach the avascular extremities of the developing embryo (Carmeliet, 2005). Angiogenesis is critical for normal embryonic

development and is a rare event in postnatal mammals; however, angiogenesis does occur in specialised physiological processes in adults, including the female reproductive cycle (Groothuis *et al.*, 2005), and during wound healing (Li *et al.*, 2003). Furthermore, aberrant angiogenesis occurs in a number of pathologies, and is a key feature of rheumatoid arthritis, macular degeneration, psoriasis, trachoma, diabetic retinopathy as well as tumour development and metastasis (Carmeliet, 2005). By contrast, pathologies such as ischemia of limbs and the myocardium are known to be caused by insufficient angiogenesis (Folkman, 1995).

Sprouting angiogenesis is a dynamic, multi-step process, which is tightly-regulated by the balance of endogenous pro-angiogenic and anti-angiogenic factors ((Carmeliet, 2005). Endothelial cells are normally quiescent (Pearson, 2000). However, an increase in the concentration of pro-angiogenic factors, including growth factors such as vascular endothelial growth factor (VEGF) and fibroblast growth factor-2 (FGF-2); and cytokines such as interleukin 8 (IL-8), or a net decrease in anti-angiogenic factors such as angiostatin, endostatin and thrombospondin-1 (TSP-1) in the immediate microenvironment surrounding endothelial cells, can induce a phenotypic change whereby the so-called 'angiogenic switch' is switched on, and endothelial cells become activated, adopting an angiogenic phenotype (Hanahan & Folkman, 1996; Carmeliet, 2000).

Pro-angiogenic growth factors bind to their cognate receptors on endothelial cells, inducing an increase in vascular permeability and loss of pericyte coverage (Dvorak, 2005). Subsequently, endothelial cells release proteases such as matrix metalloproteinases (MMPs), which degrade the basement membrane surrounding endothelial cells, providing space and cryptic sites to facilitate endothelial cell migration (van Hinsbergh & Koolwijk, 2008). Invasion of the degraded ECM is led by 'tip' endothelial cells, along a concentration gradient of pro-angiogenic growth factors to form a capillary sprout (Lamalice *et al.*, 2007). Proliferation of so-called 'stalk' endothelial cells provides support for elongating capillary sprouts, prior to the anastomosis and differentiation of endothelial cells to form lumen-containing capillary

tubes. Newly-formed tubes are subsequently stabilised by the recruitment of pericytes and the secretion of ECM proteins to form a basement membrane, thus forming a mature vessel (Carmeliet, 2000; Jain, 2003).

1.1.3 Tumour angiogenesis

The process of angiogenesis has been subject to intense research for several years, mainly due to the identification of numerous 'angiogenesis-dependent diseases' (Folkman, 2007). Of these pathologies, the role of angiogenesis in tumour progression has received particular attention. In 1971, it was first suggested by Folkman that tumour growth was fundamentally dependent upon an adequate blood supply, and that tumour metastasis was also angiogenesis-dependent (Folkman, 1971). Today, it is well established that the growth of solid tumours beyond 1-2 mm^3 is angiogenesis-dependent (Hanahan & Weinberg, 2000; Folkman, 2002). Hypoxic conditions caused by the limited capacity of oxygen to diffuse into the tumour mass (Dor *et al.*, 2001), initiate the transcriptional up-regulation of the expression of various pro-angiogenic growth factors, such as VEGF and FGF-2, which are released into the tumour stroma, and which subsequently bind to their associated growth factor receptors on endothelial cells within neighbouring blood vessels. Growth factor-induced activation of endothelial cells initiates an angiogenic response, which culminates in the vascularisation of the tumour (**Fig. 1.2**). The vessels that are formed during tumour angiogenesis are structurally distinct from those of the normal vasculature (Carmeliet & Jain, 2000; Ruoslahti, 2002a). Unlike normal endothelium, tumour endothelium fails to become quiescent; as a result, the tumour vasculature is characterised by leaky, irregularly-shaped vessels that contain several loops and *cul de sacs* (Bergers & Benjamin, 2003). The tumour vasculature also fails to mature, and is not stabilised by pericytes (Morikawa *et al.*, 2002). In addition, it has been shown that endothelial cells derived from normal and tumour vasculature display differential gene expression (St Croix *et al.*, 2000; Seaman *et al.*, 2007). Notably, capillaries within tumours are characterised by high level of expression of the VEGF receptor (VEGFR)-1 and VEGFR-2 (Plate *et al.*, 1993; Ferrara & Davis-Smyth, 1997). Tumour angiogenesis is recognised as being a rate-limiting step in carcinogenesis (Hanahan & Weinberg, 2000), as

vascularisation not only promotes tumour growth, but also facilitates tumour metastasis, events that are associated with poor prognosis in the majority of cancers (Hillen & Griffioen, 2007; Longo & Gasparini, 2007).

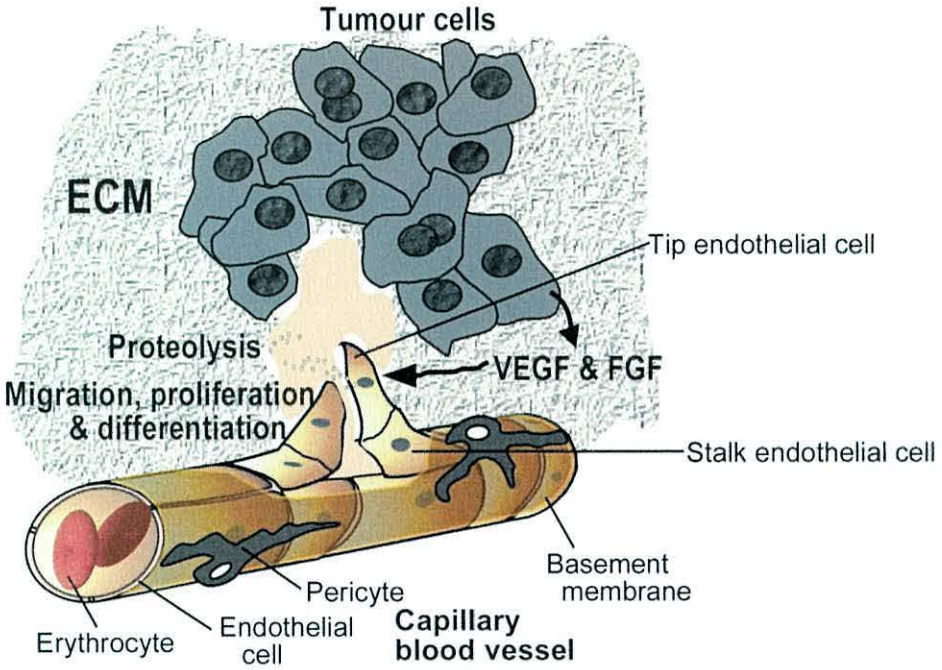


Figure 1.2 Principal steps in tumour angiogenesis. Pro-angiogenic growth factors such as VEGF and FGF-2 are secreted from tumour cells and bind to their respective associated receptors, VEGFR-2 and FGFR-1, on endothelial cells of neighbouring capillary blood vessels. Binding of growth factors to their cognate receptors activates several intracellular signalling pathways that direct the angiogenic response. Activated endothelial cells release MMPs, which induce proteolysis of the surrounding ECM, releasing further matrix-bound growth factors from the stroma. Proteolysis of the ECM facilitates VEGF-induced sprouting of tip endothelial cells. Capillary sprouting involves integrin-dependent attachment of endothelial cells to ECM proteins such as collagens and fibronectin. Sporadic detachment of endothelial cells allows migration of tip endothelial cells along a VEGF concentration gradient towards the tumour mass. Concomitantly, stalk endothelial cells undergo proliferation in response to VEGF and FGF-2 stimulation allowing cord formation to occur. Finally, endothelial cells differentiate to form a lumen-containing vessel, which is subsequently stabilised by the recruitment of pericytes. *ECM, extracellular matrix; FGF-2, fibroblast growth factor 2; FGFR-1, FGF receptor-1; MMP, matrix metalloproteinase; VEGF, vascular endothelial growth factor; VEGFR-2, VEGF receptor 2.* Diagram modified from Dr. M.J. Cross.

1.1.4 The role of the extracellular matrix in angiogenesis

Angiogenesis is not only dependent upon the balance of pro- and anti-angiogenic factors, but is also directed by the interactions of endothelial cells with the ECM (Rongish *et al.*, 1996; Davis & Senger, 2005). The ECM is composed of two layers, the first being the interstitial matrix, which is comprised of a mass of long, filamentous proteins including fibrillar collagens and glycoproteins such as fibronectin that is present between clusters of cells in all tissues (Kalluri, 2003). The second layer of the ECM is in direct contact with the abluminal surface of endothelial cells and is termed

the basement membrane (**Fig. 1.1**). The basement membrane is denser than the interstitial matrix, and is largely comprised of type IV collagen, laminins and heparan sulfate proteoglycans (HSPGs) such as nidogen and perlecan (Kalluri, 2003; Arnaoutova *et al.*, 2009).

Endothelial cell adhesion to the protein components of the ECM is vital for endothelial cell survival during sprouting angiogenesis (Re *et al.*, 1994; Stupack & Cheresh, 2003). In addition, endothelial cell-ECM interaction directs endothelial cell migration (Lamalice *et al.,* 2007), and proliferation (Senger *et al.*, 2002). For example, following the initial degradation of the basement membrane and the subsequent invasion of the interstitial matrix, endothelial cells bind to fibrillar collagens such as collagen I, which is a well known stimulator of angiogenesis (Delvos *et al.*, 1982; Davis & Senger, 2005).

The ECM is also important for the maintenance of vessel integrity by providing a scaffold onto which pericytes can adhere to provide structural support to newly formed capillaries (Hirschi & D'Amore, 1996; Gerhardt & Betsholtz, 2003). Furthermore, components of the basement membrane can themselves act as potent pro- or anti-angiogenic molecules (Kalluri, 2003; Sund *et al.*, 2004; Sottile, 2004). HSPGs can bind, and store certain pro-angiogenic growth factors including some VEGFs and FGF-2, making them inaccessible to endothelial cells until the induction of angiogenesis, where the action of proteolytic enzymes such as MMPs induces the release of these growth factors, allowing their binding to cognate receptors on endothelial cells (Folkman *et al.*, 1988; Leung *et al.*, 1989).

Endothelial cell behaviour is highly dependent upon the ECM components with which they are in intimate contact (Folkman & Haudenschild, 1980; Kalluri, 2003; Soucy & Romer, 2009). This characteristic has been exploited in angiogenesis assays that allow distinct stages of the *in vivo* angiogenic response to be recapitulated *in vitro*. For example, endothelial cells stimulated with pro-angiogenic factors undergo proliferation on a fibronectin or gelatin matrix (Form *et al.*, 1986; Schelling *et al.*, 1988). By contrast, endothelial cells plated within three-dimensional (3-D) collagen

matrices differentiate in response to growth factor stimulation to form lumen-containing vessels (Montesano *et al.*, 1983; Ingber & Folkman, 1989; Davis *et al.*, 2002; Egginton & Gerritsen, 2003; Davis & Senger, 2008). *In vitro* investigations have revealed that endothelial cell responses to anti-angiogenic molecules are also dependent upon the identity of the ECM proteins to which endothelial cells are attached (Addison *et al.*, 2005; Delaney *et al.*, 2006). Furthermore, distinct gene expression profiles have been observed under conditions of growth factor-induced endothelial cell proliferation and tubular morphogenesis within collagen gels *in vitro* (Bell *et al.*, 2001; Mellberg *et al.*, 2009), highlighting the importance of ECM in the regulation of angiogenesis.

1.1.5 Cell-matrix and cell-cell interactions in angiogenesis

Interaction between endothelial cells and the ECM is mediated by integrins, a specialised group of heterodimeric cell adhesion receptors. Integrins are comprised of at least 16 α-subunits and 8 β-subunits that are currently known to give rise to 24 different heterodimers to mediate endothelial cell adhesion to the ECM (Takada *et al.*, 2007). Integrins recognise and bind to Arg-Gly-Asp (RGD) motifs exposed on ECM protein ligands to facilitate cell-matrix adhesion (Hynes, 2007; Harburger & Calderwood, 2009). Integrin-mediated adhesion of endothelial cells to the ECM is critical for endothelial cell survival (Re *et al.*, 1994; Chavakis & Dimmeler, 2002; Davis & Senger, 2005; Cheresh & Stupack, 2008), and is vital for the mediation of angiogenesis and vascular development (Silva *et al.*, 2008; Ramjaun & Hodivala-Dilke, 2009). Importantly, intracellular signalling proteins can increase avidity of the integrin for the ECM, whilst integrin binding to the ECM can also influence intracellular signalling. This two-way 'cross-talk' between intracellular signalling molecules and integrins remains poorly understood, but is known to be critical during angiogenesis (Streuli & Akhtar, 2009; Ramjaun & Hodivala-Dilke, 2009). Endothelial cells are reported to express 11 integrins: α_1, α_2, α_3, α_4, α_5, α_6, α_V, β_1, β_3, β_4 and β_5, which form heterodimers that bind with different affinities to various ECM components (Hynes, 2007). Amongst others, the collagen-binding integrins $\alpha_1\beta_1$ and $\alpha_2\beta_1$ and the fibronectin-binding integrins $\alpha_4\beta_1$ and $\alpha_5\beta_1$ have been implicated in angiogenesis (Davis

& Camarillo, 1996; Senger *et al.*, 1997; Senger *et al.*, 2002; Ramjaun & Hodivala-Dilke, 2009; Hynes, 2007). However, whilst the $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins have been intensively studied, much less is known about the relative contribution of other integrins in the process of angiogenesis (Hynes, 2007). Importantly, integrin-mediated adhesion of endothelial cells to basement membrane proteins has been shown to inhibit endothelial cell proliferation to facilitate cell-cell adhesion (Form *et al.*, 1986).

In addition to integrin-mediated endothelial cell-ECM interactions, homophilic interactions between endothelial cells and other endothelial cells as well as heterophilic interactions between endothelial cells and mural cells, such as pericytes and fibroblasts, are essential for angiogenesis to occur *in vivo* (Jain, 2003; Dejana, 2004; Gerhardt & Betsholtz, 2003; Armulik *et al.*, 2005; Hughes, 2008). The transmembrane adhesive protein, vascular endothelial cadherin (VE-cadherin), is a central constituent of endothelial cell adherens junctions and a key mediator of homophilic endothelial cell interactions, which is essential for normal vascular development and the maintenance of vascular integrity in adult mice (Vittet *et al.*, 1997). VE-cadherin is also critically involved in the transmission of VEGF-induced survival signalling (Carmeliet *et al.*, 1999; Wallez *et al.*, 2006; Vestweber *et al.*, 2009). Another transmembrane adhesive protein involved in forming homophilic intracellular contacts is the immunoglobulin superfamily member: vascular cell adhesion molecule-1 (VCAM-1), which is critical for vasculogenesis and has been shown to have a role in angiogenesis, as well as for facilitating adhesion of mural cells to endothelial cells to stabilise newly-formed vessels (Kwee *et al.*, 1995; Brooks, 1996; Koch *et al.*, 1995; Garmy-Susini *et al.*, 2005).

1.1.6 Therapeutic targeting of angiogenesis

Modulation of angiogenesis to treat various pathologies is a goal that has attracted much research attention (Ferrara & Kerbel, 2005). Therapeutic angiogenesis, the promotion of vessel growth in ischemic tissues, has met with some success in animal models, but has thus far not proven to be clinically relevant in humans (Taniyama *et al.*, 2001; Khan *et al.*, 2003; Zhou *et al.*, 2007; Yla-Herttuala *et al.*, 2007). However, the

major focus of investigation has been in the development of anti-angiogenic strategies for the treatment of cancer and inflammatory diseases (Ferrara & Kerbel, 2005; Quesada *et al.*, 2006; Shojaei & Ferrara, 2007).

As the endothelium is in direct contact with the blood, it presents an easily accessible target for intravenous administration of anti-vascular therapies. In addition, the genetic stability of endothelial cells makes them less prone to the development of resistance to chemotherapeutic agents (Ruoslahti, 2002b). Furthermore, it has been suggested that differential expression of cell markers in the tumour endothelium may also allow the tumour vasculature to be specifically targeted in future therapies (Li & Harris, 2007).

Several approaches aimed at inhibition of angiogenesis *in vivo* have been taken (Folkman, 2007; Hirte, 2009). However, the most successful strategies thus far have been those targeting VEGF, or the activity of VEGF receptors (Folkman, 2007; Holmes *et al.*, 2007; Ellis & Hicklin, 2008; Soltau & Drevs, 2009). In 2004, the humanised variant of a murine anti-VEGF neutralising antibody, bevacizumab (Avastin®), became the first anti-angiogenic agent to be approved by the U.S. Food and Drug Administration (FDA) for use in the treatment of cancer (Hurwitz *et al.*, 2004; Ferrara *et al.*, 2004). Importantly, clinical efficacy of anti-angiogenic treatment with bevacizumab is only reached by combination treatment with chemotherapeutic agents (Hurwitz *et al.*, 2004). Indeed, it has since been shown that bevacizumab acts, in part, by normalising the tumour vasculature to facilitate improved delivery of chemotherapeutic agents into the tumour mass (Jain, 2005; Fukumura & Jain, 2007). The small-molecule VEGF receptor tyrosine kinase (RTK) inhibitors sorafenib (Nevaxar®) and sunitinib (Sutent®), which act by blocking VEGFR-2 tyrosine kinase activity and thus preventing the subsequent activation of signalling pathways downstream of VEGFR-2, have been approved by the FDA for use as monotherapies to treat renal cell carcinoma and hepatocellular carcinoma (Mendel *et al.*, 2003; Motzer *et al.*, 2007; Escudier *et al.*, 2007; Llovet *et al.*, 2008). In addition, the VEGFR-2 tyrosine kinase inhibitor AZD2171/cediranib (Recentin®), has been shown to inhibit

angiogenesis *in vivo* and is currently in phase III clinical trials for the treatment of colorectal cancer (Wedge *et al.*, 2005; Lindsay *et al.*, 2009).

However, despite the relative success of anti-VEGF/VEGFR therapies, the benefits of treating cancer patients with these agents are short-lived, due to either the rapid development of adaptive resistance, or to the inherent resistance of tumours to these treatments (Bergers & Hanahan, 2008). Acquired resistance is caused, in part, by redundancy in angiogenic signalling pathways, whereby when a particular signalling pathway is blocked; another pathway is utilised to compensate for this loss (Stommel *et al.*, 2007; Eikesdal & Kalluri, 2009). Together, these issues highlight a need to identify new targets for the development of novel anti-angiogenic therapies. As pro-angiogenic factors acting on distinct receptors often share common intracellular signal transduction pathways downstream of their cognate receptors (Rossant & Howard, 2002; Zachary, 2005), inhibition of these intracellular signalling pathways, rather than targeting the ligand or upstream receptor directly, may therefore represent a more promising strategy for the therapeutic inhibition of angiogenesis.

1.2 The role of VEGF and VEGFR-2 in angiogenesis

1.2.1 VEGFs

VEGF is an endothelial cell-specific mitogen and a critical regulator of vasculogenesis and angiogenesis *in vivo* (Ferrara & Davis-Smyth, 1997; Olsson *et al.*, 2006). VEGFs are a family of homodimeric glycoproteins that are secreted from various cell types, including fibroblasts, tumour cells and endothelial cells themselves (Olsson *et al.*, 2006). The VEGF family is comprised of VEGF-A, VEGF-B, VEGF-C, VEGF-D and placenta growth factor (PlGF) (Maglione *et al.*, 1991; Olofsson *et al.*, 1996; Joukov *et al.*, 1996; Lee *et al.*, 1996; Orlandini *et al.*, 1996). VEGF homologues have also been described, including the papoxyvirus *Orf* virus-encoded VEGF, termed VEGF-E (Lyttle *et al.*, 1994) and snake venom VEGF, VEGF-F (Yamazaki & Morita, 2006). However, it is the prototypic VEGF, VEGF-A (also known simply as VEGF, and referred to hereinafter as such), which plays the predominant role in vasculogenesis and angiogenesis (Coultas *et al.*, 2005).

Alternative splicing of the *VEGF* gene in humans gives rise to six variants of 121, 145, 165, 183, 189 and 206 amino acids in length, denoted: $VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{183}$, $VEGF_{189}$ and $VEGF_{206}$ respectively (Tischer *et al.*, 1991; Poltorak *et al.*, 1997; Jingjing *et al.*, 1999; Robinson & Stringer, 2001). $VEGF_{165}$ is the most widely-expressed and biologically active VEGF isoform (Neufeld *et al.*, 1996). Most of the VEGF isoforms, with the exception of $VEGF_{121}$, contain a heparin binding domain. These proteins bind to HSPGs in the ECM, which aid in facilitating the activation and subsequent migration of tip endothelial cells along a concentration gradient during sprouting angiogenesis. Expression of *VEGF* is low under normal physiological conditions, but can be up-regulated by various growth factors including the pro-angiogenic molecules FGF-2 and transforming growth factor-β (TGF-β) (Stavri *et al.*, 1995; Tuder *et al.*, 1995). Furthermore, environmental conditions such as hypoxia and low pH in developing tumours can also trigger up-regulation of *VEGF* expression (Carmeliet & Jain, 2000).

1.2.2 The role of VEGF *in vivo*

The importance of VEGF during embryonic vascular development was highlighted in gene deletion studies in mice. Interestingly, knockout of a single murine *Vegf* allele was found to be sufficient to cause embryonic lethality between E11.0 and E12.0 due to abnormal blood-island formation and defective angiogenesis (Ferrara *et al.*, 1996). *Vegf*-null mice died at an earlier time-point (E9.5-10.5) with more profound vascular defects (Carmeliet *et al.*, 1996), thus suggesting that both vasculogenesis and angiogenesis are regulated by VEGF in a concentration-dependent manner. Gerber and co-workers subsequently showed that conditional knockout of the *Vegf* gene in neonatal mice also resulted in death, caused by reduced blood vessel formation (Gerber *et al.*, 1999). However, in the same study, it was shown that knockout of *Vegf* did not result in the death of adult mice (Gerber *et al.*, 1999), possibly due to the fact that adult vessels are pericyte-covered, and are thus more stable (Benjamin & Keshet, 1997; Gerber *et al.*, 1999). The critical dependence of tightly-regulated VEGF expression in the developing embryo was further exemplified in experiments where mouse embryos carrying a mutation inducing a 3-fold increase in VEGF levels

underwent embryonic death at E12.5-E14.0 due to severe cardiovascular defects (Miquerol *et al.*, 2000).

1.2.3 VEGF receptors

VEGFs exert their biological effects by binding, in an overlapping pattern, to three distinct, but structurally related, class III RTKs, denoted VEGF receptor-1, -2 and -3 (VEGFR-1-3), as well as to two non-tyrosine kinase receptors, termed neuropillin-1 (NRP1) and neuropillin-2 (NRP2). VEGFRs consist of an extracellular domain containing seven immunoglobulin-like domains, a hydrophobic transmembrane domain, and an intracellular region containing two tyrosine kinase domains separated by a 70 amino acid kinase-insert domain (Olsson *et al.*, 2006; Roskoski, 2007).

1.2.3.1 VEGFR-1

VEGFR-1, also known as Fms-like tyrosine kinase 1 (FLT1), is expressed on monocytes, haematopoietic cells and endothelial cells and can bind to VEGF, VEGF-B and PlGF. A splice variant of VEGFR-1, gives rise to a receptor which lacks the transmembrane and internal signalling domains of VEGFR-1 and is denoted sVEGFR-1 (soluble VEGFR-1). It is believed that sVEGFR-1 acts mainly as a decoy receptor, sequestering VEGF, thus negatively-regulating VEGFR-2 signalling (Shibuya *et al.*, 1990; Kendall & Thomas, 1993; Roberts *et al.*, 2004b). *Vegfr-1*$^{-/-}$ mice die at E8.5-E9.0 due to disorganised vasculature caused by over-production of haemangioblasts (Fong *et al.*, 1995; Fong *et al.*, 1999). Interestingly, mice expressing the VEGFR-1 ligand-binding domain, but lacking the kinase domain of VEGFR-1 developed normally (Hiratsuka *et al.*, 1998). It has since been shown that fixation of the extracellular domain of VEGFR-1 via the transmembrane domain in endothelial cells is also essential for normal development (Hiratsuka *et al.*, 2005), showing that binding of VEGFR-1 to the membrane is essential, but that potential signalling occurring downstream of VEGFR-1 is disposable for normal vascular development.

1.2.3.2 VEGFR-2

VEGFR-2 (kinase-insert domain-containing receptor (KDR) in humans) is expressed on vascular and lymphatic endothelial cells, and is the most physiologically significant VEGFR (Waltenberger *et al.*, 1994; Seetharam *et al.*, 1995; Wheeler-Jones *et al.*, 1997; Olsson *et al.*, 2006; Holmes *et al.*, 2007; Lohela *et al.*, 2009). Foetal liver kinase-1 (Flk-1) is the murine homologue of VEGFR-2 and shares 85% sequence homology with KDR, but is 2 amino acids shorter than KDR (Holmes *et al.*, 2007). Genetic ablation of *Flk-1* in mice is embryonically lethal between E8.5 and E9.5 due to impaired blood-island formation and vasculogenesis (Shalaby *et al.*, 1995), a phenotype similar to that found in *Vegf*$^{-/-}$ mice (Carmeliet *et al.*, 1996). VEGF-A binds to VEGFR-1 and VEGFR-2, but binds to VEGFR-1 with a greater affinity (K_d 2-10 pM) (de Vries *et al.*, 1992) than VEGFR-2 (K_d 75-125 pM) (Shinkai *et al.*, 1998; Fuh *et al.*, 1998). Interestingly however, the tyrosine kinase activity of VEGFR-1 is significantly less than that of VEGFR-2, and it remains unclear whether or not VEGFR-1 mediates a biologically relevant signal (Waltenberger *et al.*, 1994; Seetharam *et al.*, 1995; Olsson *et al.*, 2006).

1.2.3.3 VEGFR-3

During development, VEGFR-3 (Flt-4) is expressed on all endothelial cells, but its expression in adults is restricted to lymphatic endothelial cells (Olsson *et al.*, 2006; Lohela *et al.*, 2009). VEGF-C and VEGF-D are the only ligands that can bind to VEGFR-3 (Karkkainen *et al.*, 2002). Gene targeting studies in which *Vegfc* was deleted has shown that *Vegfc* is critical for lymphatic sprouting angiogenesis (Karkkainen *et al.*, 2004). Ablation of *Flt-4* in mice led to fluid accumulation in the pericardium of *Flt4*$^{-/-}$ embryos caused by leaky vasculature which led to embryonic lethality by E9.5, prior to the onset of lymphangiogenesis (Dumont *et al.*, 1998).

1.2.3.4 NRP1 and NRP2

Further receptors for VEGF are the cell-surface glycoproteins NRP1 and NRP2. NRPs are members of class 3 semaphorins, which lack kinase activity, and bind to $VEGF_{165}$, but not $VEGF_{121}$ (Neufeld *et al.*, 2002). NRP1 expression is largely restricted to the heart vasculature where its main function is to act as a co-receptor for VEGFR-2

(Partanen *et al.*, 1999; Whitaker *et al.*, 2001; Soker *et al.*, 2002). Interestingly, $VEGF_{165}$ binds to VEGFR-2/NRP1 heterodimers with a four-fold greater affinity than to VEGFR-2 homodimers, an effect that has been shown to enhance VEGF-induced endothelial cell proliferation and migration (Soker *et al.*, 1998; Soker *et al.*, 2002). NRP1 is critical for vascular development in the embryo as *Nrp1*$^{-/-}$ die at E14.0 with cardiovascular defects (Kawasaki *et al.*, 1999), and endothelial cell-specific deletion of *Nrp1* results in vascular abnormalities (Gu *et al.*, 2003). NRP2 appears to have an important role in lymphatic vessel development, as *Nrp2*$^{-/-}$ mice are viable, but display reduced lymphatic growth (Yuan *et al.*, 2002). More recently it was shown that NRP2 acts as a co-receptor for VEGFR-2 and VEGFR-3 in endothelial cells, and plays an important role in the enhancement of VEGF-stimulated proliferation and migration (Favier *et al.*, 2006).

1.2.4 VEGFR-2-mediated intracellular signalling

Intensive research has revealed that VEGF-stimulated angiogenic responses are principally mediated by VEGFR-2 (Holmes *et al.*, 2007). Specifically, VEGF-stimulated activation of VEGFR-2 can induce endothelial cell proliferation, migration, survival, differentiation and permeability (Zachary, 2003; Holmes *et al.*, 2007). These diverse responses to VEGF stimulation are arbitrated by a range of intracellular signalling pathways acting downstream of VEGFR-2, as illustrated in **Figure 1.3**. Key events in VEGFR-2 activation and the activation of selected pathways downstream of VEGFR-2 are presented below.

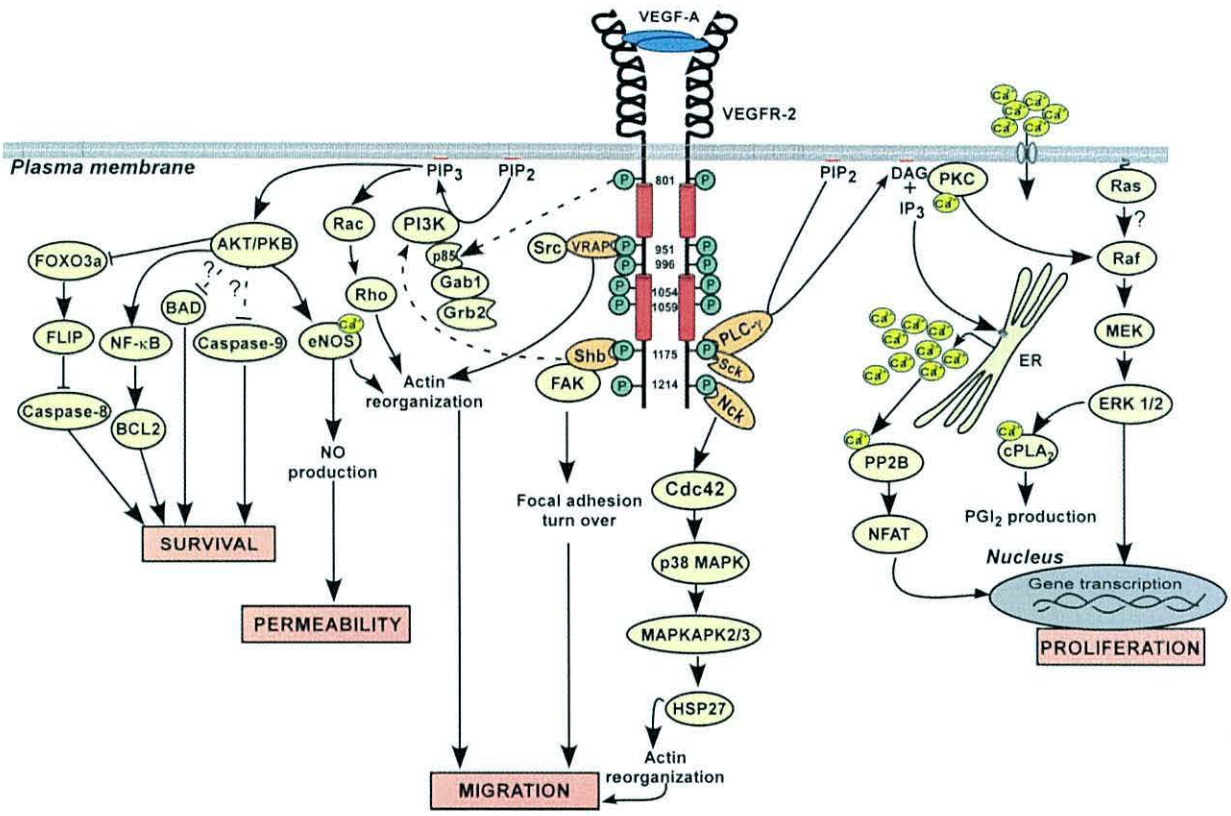


Figure 1.3 VEGFR-2-mediated intracellular signalling in endothelial cells. Binding of VEGF to VEGFR-2 induces dimerisation and trans-autophosphorylation of intracellular tyrosine residues in the cytoplasmic domain of the receptor. Adapter molecules bind to specific phosphorylated tyrosine (pY) residues, leading to the phosphorylation of downstream proteins and the generation of second messengers. The binding of PLC-γ to pY^{1175} results in the hydrolysis of PIP_2, generating DAG and IP_3. IP_3 binds to IP_3Rs on the ER, inducing the release of Ca^{2+} into the cytoplasm, which is required for the activation of several proteins including PP2B/calcineurin, and classical PKC isoforms. DAG is a physiological activator of novel and classical PKCs. VRAP (also known as TSAd) binds to pY^{951}, and forms a complex with Src, leading to actin reorganisation. VEGF also induces the influx of extracellular Ca^{2+} via specific membrane channels. Activation of PI3K induces phosphorylation of PIP_2 into PIP_3, leading to the activation of AKT. Activated AKT phosphorylates IKKα, releasing it from NF-κB, allowing transcription of the pro-survival protein BCL2. AKT also phosphorylates FOXO3a, sequestering it in the cytoplasm and preventing the transcription of death-promoting proteins. VEGF may also stimulate activation of other AKT target proteins, such as the pro-apoptotic protein BAD. *AKT/PKB, protein kinase B; BAD, BCL2-antagonist of cell death; BCL2, B-cell lymphoma 2; cPLA2, cytosolic phospholipase A2; DAG, diacylglycerol; eNOS, endothelial nitric oxide synthase; ER, endoplasmic reticulum; ERK1/2, extracellular signal-regulated kinases 1 and 2; FAK, focal adhesion kinase; FLIP, FLICE-like inhibitory protein; FOXO3a, Forkhead box O3a; Gab1, Grb2-associated binder-1; Grb2, growth-factor receptor-bound 2; HSP27, heat-shock protein 27; IKKα, IκB kinase alpha; IP_3, inositol (1,4,5)-trisphosphate; IP_3R, IP_3 receptor; MAPKAPK2/3, MAPK-activated protein kinase-2 and -3; MEK, MAPK/ERK kinase; NFAT, nuclear factor of activated T cells; NF-κB, nuclear factor κB; NO, nitric oxide; p38 MAPK, p38 mitogen-activated protein kinase; PI3K, phosphoinositide 3-kinase; PGI_2, prostacyclin; PIP_2, phosphatidylinositol (4,5)-bisphosphate; PIP_3, phosphatidylinositol (3,4,5)-trisphosphate; PKC, protein kinase C; PLC-γ, phospholipase C-γ; PP2B, protein phosphatase 2B; Sck, Shc-like protein; VEGF, Shb, Src homology 2 protein B; vascular endothelial growth factor; TSAd, T-cell-specific adaptor molecule; VEGFR-2, VEGF receptor-2; VRAP, VEGFR-associated protein. Diagram modified from Holmes et al., 2007.*

1.2.4.1 Activation of VEGFR-2 and initiation of downstream signalling

Binding of VEGF to the extracellular domain of VEGFR-2 induces a change in the conformation of VEGFR-2, leading to receptor dimerisation and resulting in the trans-autophosphorylation of at least seven tyrosine residues located in intracellular domain

of the receptor, namely: Tyr^{801} in the juxtamembrane domain; Tyr^{951} and Tyr^{996} in the kinase-insert domain; Tyr^{1054} and Tyr^{1059} in the second tyrosine kinase domain; and Tyr^{1175} and Tyr^{1214} in the C-terminal tail (Dougher-Vermazen *et al.*, 1994; Takahashi *et al.*, 2001; Blanes *et al.*, 2007; **Fig. 1.3**). Specific Src homology 2 (SH2)-domain-containing adapter molecules have been shown to interact with some of these autophosphorylated tyrosines (Petrova *et al.*, 1999; **Fig. 1.3**). Phosphorylated VEGFR-2 Tyr^{1175} provides a docking site for phospholipase C-γ (PLC-γ) (Takahashi *et al.*, 2001), Shb (Src homology 2 protein B) (Holmqvist *et al.*, 2004) and Sck (Shc-like protein) (Warner *et al.*, 2000). The adapter molecule VRAP/TSAd (VEGF receptor-associated protein/T-cell specific adapter) binds to phospho-Tyr^{951} of VEGFR-2 (Wu *et al.*, 2000b), whilst the adapter protein Nck binds to phospho-Tyr^{1214} (Lamalice *et al.*, 2006; **Fig. 1.3**). The importance of Tyr^{1175} in angiogenesis has been established from knock-in studies in mice. Mutation of Tyr^{1173} of Flk-1 (Tyr^{1175} in KDR) to give Phe^{1173}, which cannot undergo autophosphorylation, resulted in embryonic lethality at E8.5-E9.5 due to severe defects in vasculogenesis, with an absence of blood vessels and blood islands and a reduced amount of haematopoietic progenitors (Sakurai *et al.*, 2005), a phenotype almost identical to that of *Flk-1*$^{-/-}$ mice (Shalaby *et al.*, 1995). Similarly, homozygous deletion of the *Plc-γ1* gene in mice also resulted in embryonic cell death at around E9.0 due to defects in vasculogenesis and erythropoiesis (Ji *et al.*, 1997; Liao *et al.*, 2002). By contrast, mutation of VEGFR-2 Tyr^{1214} to Phe^{1214} has no adverse phenotypic effects upon mouse development (Sakurai *et al.*, 2005). These results strongly suggest that Tyr^{1175} is essential for mediating the downstream biological effects of VEGFR-2 in endothelial cells during development, possibly due to a critical role played by PLC-γ.

1.2.4.2 VEGFR-2-mediated activation of PLC-γ and protein kinase C

Binding of PLC-γ to phosphorylated Tyr^{1175} induces PLC-γ activation by phosphorylation (Wu *et al.,* 2000). Phosphorylated PLC-γ then hydrolyses the membrane phospholipid phosphatidylinositol (4,5)-bisphosphate (PIP_2). Cleavage of PIP_2 generates the second messengers inositol (1,4,5)-trisphosphate (IP_3) and diacylglycerol (DAG). DAG is a physiological activator of classical and novel protein kinase C (PKC) isoforms, whilst IP_3

binds to IP_3 receptors (IP_3Rs) on the endoplasmic reticulum (ER) to mobilise the release of Ca^{2+} into the cytoplasm (Nishizuka, 1992; **Fig. 1.3**).

The PKC isoforms are a family of phospholipid-dependent serine/threonine kinases, which are critical for arbitrating intracellular signals that regulate various cellular processes including proliferation and survival in several cell types (Dempsey *et al.*, 2000; Rosse *et al.*, 2010). The PKC family consists of at least 10 isoforms, which have been separated into three classes based on their structure and mode of activation (Newton, 2001). The classical PKCs (cPKCs), comprising the isoforms PKCα, PKCγ and the splice variants $PKC\beta_I$ and $PKC\beta_{II}$, are dependent upon both DAG and Ca^{2+} for their activation. Activation of the novel PKC (nPKC) isoforms, PKCδ, PKCε, PKCη and PKCθ is DAG-dependent, but Ca^{2+}-independent, whilst the atypical PKCs (aPKCs), PKCι and PKCζ do not require either Ca^{2+} or DAG for their activation (Newton, 1997; Mellor & Parker, 1998). A more recently discovered isoform termed PKCμ (also known as protein kinase D, PKD), is regulated in a similar way to the nPKCs, requiring DAG for its activation, but forms a distinct group (Newton, 2001), and has recently been classified as a member of the Ca^{2+}/calmodulin-dependent kinase (CaMK) superfamily (Rozengurt *et al.*, 2005; Wang, 2006).

VEGF stimulation of endothelial cells has been reported to induce the activation of PKCα (Xia *et al.*, 1996; Wellner *et al.*, 1999; Gliki *et al.*, 2001), $PKC\beta_{I/II}$ (Xia *et al.*, 1996; Takahashi *et al.*, 1999), PKCδ (Gliki *et al.*, 2001; Gliki *et al.*, 2002), PKCε (Wu *et al.*, 2000a; Gliki *et al.*, 2001), PKCζ (Wellner *et al.*, 1999) and PKD/PKCμ (Wong & Jin, 2005; Evans *et al.*, 2008). Inhibition of PKC activation in endothelial cells *in vitro* has revealed PKCs as important arbitrators of VEGF-stimulated endothelial cell proliferation, migration, permeability, prostacyclin production, survival and tubular morphogenesis (Montesano *et al.*, 1992; Wellner *et al.*, 1999; Wu *et al.*, 2000a; Gliki *et al.*, 2001; Gliki *et al.*, 2002; Wang *et al.*, 2002; Harhaj *et al.*, 2006; Steinberg *et al.*, 2007; Xu *et al.*, 2008). Whilst a role for individual PKC isoforms in some VEGF-stimulated physiological responses has been suggested, this appears to be endothelial cell type-dependent,

possibly indicating functional redundancy between distinct PKCs (Wellner *et al.*, 1999; Parker & Murray-Rust, 2004; Xu *et al.*, 2008).

1.2.4.3 VEGFR-2-mediated activation of the PI3K-AKT pathway

The phosphoinositide 3'-kinases (PI3Ks) are a family of lipid kinases that catalyse the phosphorylation of the inositol ring of plasma membrane-associated phosphoinositides. Upon growth factor stimulation, PI3K phosphorylates PIP_2 to generate phosphatidylinositol (3,4,5)-trisphosphate (PIP_3) (Wymann & Pirola, 1998; Cantley, 2002). The serine/threonine kinase AKT/PKB (protein kinase B) and 3'-phosphoinositide-dependent kinase-1 (PDK1) bind to membrane-bound PIP_3 via their respective pleckstrin-homology (PH)-domains, anchoring these proteins to the plasma membrane to facilitate PDK1-mediated phosphorylation of AKT on Thr^{308} within its catalytic domain (Stokoe *et al.*, 1997; Alessi *et al.*, 1997; Lawlor & Alessi, 2001). Further phosphorylation of AKT on the Ser^{473} residue, located within a hydrophobic region in the C-terminus of AKT, is required for AKT to become fully activated (Alessi *et al.*, 1996; Coffer *et al.*, 1998). At least 10 candidate kinases have been reported to act as a so-called 'PDK2' capable of phosphorylating AKT on Ser^{473} *in vitro* (Dong & Liu, 2005). However, it was recently shown that the most physiologically relevant kinase acting as a 'PDK2' in regulation of AKT phosphorylation on Ser^{473} is the mammalian target of rapamycin (mTOR)-RICTOR (rapamycin-insensitive companion of mTOR)-SIN1 (stress-activated-protein-kinase-interacting protein 1)-mLST8 (mammalian lethal with SEC13 protein 8) complex, termed mTOR complex 2 (mTORC2) (Sarbassov *et al.*, 2005; Jacinto *et al.*, 2006). The lipid phosphatse, PTEN (phosphatase and tensin homologue), negatively-regulates PI3K/AKT activity by de-phosphorylating PIP_3 thus preventing the binding and subsequent activation of AKT (Maehama & Dixon, 1998).

The PI3K-AKT pathway is critical for mediating anti-apoptotic signals in several cell types (Nunez & del Peso, 1998). In endothelial cells, VEGF stimulation has been shown to activate PI3K (Guo *et al.*, 1995; Gerber *et al.*, 1998b; Thakker *et al.*, 1999; Yu & Sato, 1999). The mechanism of VEGF-mediated PI3K activation is not currently clear, but some evidence suggests that it is dependent upon phosphorylated Tyr^{1175} and the

adapter protein Shb (Dayanir *et al.*, 2001; Holmqvist *et al.*, 2004; **Fig. 1.3**). It has also been shown that the scaffold adapter protein Gab1 (Grb2-associated binder-1) is phosphorylated following VEGF stimulation, and interacts with the p85 regulatory subunit of PI3K to facilitate PI3K activation (Dance *et al.*, 2006; Laramee *et al.*, 2007). More recently, Blanes and co-workers provided evidence to suggest that autophosphorylated Tyr^{801} of VEGFR-2 can bind, and activate the p85 regulatory subunit of PI3K, to directly induce PI3K activation (Blanes *et al.*, 2007; **Fig. 1.3**). VEGF-stimulated activation of PI3K induces AKT activation (Gerber *et al.*, 1998b), resulting in a wide range of physiological responses including endothelial cell survival, permeability and migration (Gerber *et al.*, 1998b; Fujio & Walsh, 1999; Radisavljevic *et al.*, 2000; Shiojima & Walsh, 2002; Abid *et al.*, 2004; Holmes *et al.*, 2007). The importance of PI3K-AKT signalling in angiogenesis has been underlined in studies of transgenic mice, where ablation of the p110 catalytic subunit of PI3K resulted in embryonic death at E9.5-E10.5 with defective angiogenesis caused by reduced cell proliferation (Bi *et al.*, 1999a). Similarly, $Pten^{-/-}$ mice die before E11.5 due to haemorrhaging caused by impaired recruitment of mural cells to developing blood vessels (Hamada *et al.*, 2005). In addition, endothelial cell-specific expression of a constitutively active form of Akt also induced embryonic lethality due to vascular malformations (Sun *et al.*, 2005).

1.2.5 Physiological consequences of VEGFR-2 activation

1.2.5.1 Permeability

VEGF was originally identified as a permeability-inducing factor in endothelial cells and is alternatively termed vascular permeability factor (VPF) (Senger *et al.*, 1983). It was later discovered that VPF was a potent inducer of endothelial cell proliferation *in vitro* (Connolly *et al.*, 1989) and that VPF was in fact the same molecule as VEGF (Leung *et al.*, 1989). VEGF-induces endothelial cell permeability via AKT-mediated phosphorylation of endothelial nitric oxide synthase (eNOS) on Ser^{1179} leading to increased eNOS activity and nitric oxide (NO) release (Dimmeler *et al.*, 1999; Fulton *et al.*, 1999; **Fig. 1.3**). PLC-γ-induced increase in intracellular Ca^{2+} has also been implicated in eNOS activation, leading to the generation of NO (Fukumura *et al.*, 2001; Dimmeler *et al.*, 1999; Fulton *et al.*, 1999). More recently, auto-phosphorylation of

VEGFR-2 on Tyr^{801} was shown to be essential for NO production via a pathway involving p85 binding to VEGFR-2 and subsequent activation of the PI3K/AKT pathway (Blanes *et al.*, 2007; **Fig. 1.3**).

1.2.5.2 Proliferation

VEGF is a potent endothelial cell mitogen, which stimulates endothelial cell mitogenesis via activation of extracellular signal-regulated kinase-1 and -2 (ERK1/2) (Kroll & Waltenberger, 1997; Vinals & Pouyssegur, 1999; Wu *et al.*, 2000a; **Fig. 1.3**). In addition to ERK1/2-mediated proliferation, some evidence suggests that VEGF-stimulated endothelial cell proliferation can occur via a pathway requiring PI3K and p70 S6 kinase (Yu & Sato, 1999; Dayanir *et al.*, 2001).

The canonical pathway mediating ERK1/2 activation following RTK activation involves phosphorylation of the adapter molecule Grb2 (growth-factor-receptor-bound 2), leading to activation of the guanine nucleotide-exchange factor SOS (son of sevenless), which mediates activation of the monomeric GTPase Ras and the subsequent activation of the classical Raf-1→MEK1/2→ERK1/2 cascade (Buday & Downward, 1993). However, the potential role of Ras in mediation of VEGF-stimulated ERK1/2 activation remains unclear. VEGF stimulation has been shown to potently activate Ras in human umbilical vein endothelial cells (HUVECs), and inhibition of Ras activity in these cells has been reported to block VEGF-stimulated ERK1/2-mediated proliferation (Meadows *et al.*, 2001). On the other hand, evidence gained from primary liver sinusoidal endothelial cells suggests that VEGF/VEGFR-2-mediated activation of ERK1/2 can occur independently of the canonical Grb2→SOS→Ras pathway (Takahashi *et al.*, 1999). Instead, it has been shown that VEGF/VEGFR-2-stimulated ERK1/2 activation occurs via a PLC-γ/PKC-dependent pathway (Doanes *et al.*, 1999; Takahashi *et al.*, 2001; **Fig. 1.3**). Interestingly, VEGF-stimulated, Ras-mediated ERK1/2 activation has also been shown to be dependent upon PKC and sphingosine kinase activity (Shu *et al.*, 2002; **Fig. 1.3**).

1.2.5.3 Survival

Alon and co-workers were the first to report that VEGF can act as a survival factor during angiogenesis (Alon *et al.*, 1995). Importantly, VEGF is also a key regulator of endothelial cell survival in the tumour vasculature (Benjamin & Keshet, 1997; Liu *et al.*, 2000). It has since been shown that VEGF induces the expression of the pro-survival proteins B-cell lymphoma-2 (BCL2) and A1 in HUVECs (Gerber *et al.*, 1998a); whilst inhibitor of apoptosis protein (IAP) family members X-chromosome-linked IAP (XIAP) and survivin have been shown to become up-regulated following VEGF stimulation to mediate endothelial cell survival during angiogenesis (Tran *et al.*, 1999; O'Connor *et al.*, 2000). VEGF exerts its pro-survival effects upon endothelial cells by activating the PI3K-AKT pathway (Gerber *et al.*, 1998b; Carmeliet *et al.*, 1999; Fujio and Walsh, 1999; **Fig. 1.3**). The forkhead transcription factor FOXO3a (Forkhead box O3a) has also been shown to be a downstream target of VEGF-stimulated PI3K-AKT activation in endothelial cells (Abid *et al.*, 2004). Blockade of FOXO3a activity in endothelial cells leads to down-regulation of expression of the caspase-8 inhibitor FLIP (FLICE-like inhibitory protein) leading to increased caspase-8 activation (Skurk *et al.*, 2004). The transcription factor NF-κB (nuclear factor kappa B) has also been shown to become activated following VEGF-stimulated endothelial cell survival via an AKT-dependent pathway (Karl *et al.*, 2005; Grosjean *et al.*, 2006). VEGF-stimulated NF-κB activation is required for VEGF-dependent up-regulation of the expression of the pro-survival protein BCL2 (Grosjean *et al.*, 2006). Other candidate AKT targets that may facilitate VEGF-stimulated endothelial cell survival include caspase-9 (Cardone *et al.*, 1998) and BAD (Datta *et al.*, 1997; **Fig. 1.3**). Importantly, VEGFR-2 can also associate with $\alpha_v\beta_3$ integrins, resulting in enhanced activation of VEGFR-2 and of AKT (Soldi *et al.*, 1999; Masson-Gadais *et al.*, 2003; Streuli & Akhtar, 2009; Somanath *et al.*, 2009). Reciprocally, VEGF has been reported to activate $\alpha_v\beta_3$ integrins via a PI3K/AKT-dependent pathway (Byzova *et al.*, 2000).

1.2.5.4 Migration

Directed endothelial cell migration in response to VEGF stimulation represents one of the earliest steps in sprouting angiogenesis, and is essential for the endothelial cell

invasion of the ECM (Lamalice *et al.*, 2007). Rousseau and co-workers first demonstrated that VEGF-stimulated activation of p38 mitogen-activated protein kinase (p38 MAPK) was required to induce actin remodelling to facilitate endothelial cell motility (Rousseau *et al.*, 1997). It has since been shown that VEGF-induced activation of p38 MAPK is mediated by the small GTPase Cdc42 via a pathway requiring binding of the adapter protein Nck to autophosphorylated VEGFR-2 Tyr^{1214} and the subsequent activation of the Src family member, Fyn (Lamalice *et al.*, 2006). Fyn binds to Nck in response to VEGF stimulation, resulting in the phosphorylation of PAK-2 (p21-activated protein kinase-2), which induces activation of Cdc42 (cell division cycle 42) and p38 MAPK (Lamalice *et al.,* 2006; **Fig. 1.3**). Downstream of p38 MAPK, MAPKAPK2/3 (MAPK-activated protein kinase-2 and -3) are activated following VEGF stimulation, leading to the phosphorylation of heat-shock protein 27 (HSP27), and subsequent actin polymerisation (Lamalice *et al.,* 2004; **Fig. 1.3**). It has been shown that VEGF-stimulated phosphorylation of HSP27 may also occur via a mechanism involving PKD, independently of p38 MAPK (Evans *et al.*, 2008). Furthermore, a role for phospholipase C-β3 (PLC-β3) acting via Cdc42, as a mediator of VEGF-induced endothelial chemotaxis has also recently emerged (Bhattacharya *et al.*, 2009).

In a subset of endothelial cells undergoing pathological angiogenesis, VRAP/TSAd binds to phosphorylated Tyr^{951}, and forms a complex with the cytoplasmic tyrosine kinase Src to regulate actin polymerisation and endothelial cell migration via a p38 MAPK-independent pathway (Matsumoto *et al.,* 2005; **Fig. 1.3**). VEGF-stimulated activation of the PI3K/AKT pathway and the subsequent phosphorylation of eNOS on Ser^{1177} (Ser^{1179} in humans) has been shown to mediate endothelial cell migration (Dimmeler *et al.,* 2000; **Fig. 1.3**). More recently, it was shown that the association of the scaffold protein Gab1 with VEGFR-2 is essential for mediating VEGF-stimulated endothelial cell migration due to its role in regulating PI3K/AKT activation (Dance *et al.*, 2006; Laramee *et al.*, 2007). An important role for small GTPase, RhoA in mediating VEGF-stimulated endothelial cell chemotaxis has also been demonstrated (van Nieuw Amerongen *et al.*, 2003). Following VEGF stimulation, GTP-bound, active RhoA induces the activation of ROCK (Rho-associated kinase), leading to phosphorylation, and activation of the

protein tyrosine kinase focal adhesion kinase (FAK), which acts by regulating the assembly and disassembly of focal adhesions (focal adhesion turnover), a process that also requires FAK-mediated cross-talk between VEGFR-2 and integrin $\alpha_v\beta_3$ (Le Boeuf *et al.*, 2004; Le Boeuf *et al.*, 2006; Lamalice *et al.*, 2007). FAK-mediated focal adhesion turnover may also occur in a Src-dependent manner via association of FAK with the adapter protein Shb (Holmqvist *et al.*, 2003). Furthermore, the GTP-binding protein Rac has also been implicated in the formation of membrane ruffles to mediate endothelial cell migration following VEGF stimulation, in a p38 MAPK-independent manner (Lamalice *et al.,* 2006). Together, these studies indicate that VEGF, acting via VEGFR-2, can induce endothelial cell motility via several distinct and complementary pathways.

1.3 Mitogen-activated protein kinase (MAPK) signalling

1.3.1 Role of MAPKs

The transmission of an extracellular signal from the cell surface into the cell nucleus to elicit an intracellular response requires the involvement of several intermediary signalling molecules. Mitogen-activated protein kinases (MAPKs) are proline-directed protein-serine/threonine kinases, which phosphorylate substrates on Ser or Thr residues that are immediately followed by a Pro residue. MAPKs are expressed in all eukaryotic cells and are highly conserved signalling proteins that relay extracellular-derived signals to evoke intracellular responses, which enable cells to adapt to changes in their surrounding environment (Widmann *et al.*, 1999; Pearson *et al.*, 2001b; Turjanski *et al.*, 2007). Activation of MAPKs occurs by phosphorylation, and can be induced by a diverse range of stimuli, including: growth factors, hormones, inflammatory cytokines and physical or chemical stresses (Derijard *et al.*, 1994; Han *et al.*, 1994; Cano & Mahadevan, 1995). The substrates of activated MAPKs are often transcription factors that regulate expression of genes involved in a wide-range of processes during normal cell function (Yang *et al.*, 2003). Alternative substrates phosphorylated by MAPKs include: other kinases, structural proteins and phospholipids (Chang & Karin, 2001). Together, MAPKs engage in cross-talk with other

MAPKs and are critical regulators of cell differentiation, cell migration, homeostasis, cell proliferation, cell survival and cell death (Qi & Elion, 2005).

1.3.2 MAPK cascades: organisation, activation, signal transfer and inactivation

MAPKs are terminal members of a three-tiered hierarchical cascade of sequentially-activated protein kinases (**Fig. 1.4**). The apical kinase of MAPK modules is termed MAPK kinase kinase (MAPKKK) (also known as MAPK/ERK kinase kinase or MEKK). The MAPKKK activates its downstream target, termed MAPK kinase (MAPKK) (alternatively known as MAPK/ERK kinase or MEK) by phosphorylation of specific serine and threonine residues within MAPKK. In turn, the activated MAPKK can catalyse the activation of the MAPK by dual-phosphorylation of specific threonine and tyrosine residues in a conserved T-X-Y motif present within the activation loop of the terminal MAPKs (Zhang & Dong, 2007). Consecutive activation of each component in the MAPK cascade allows the activated MAPK to phosphorylate, and thus modify the activity of one of potentially many downstream effector molecules, which may include transcription factors, other protein kinases, phospholipases, or other intracellular proteins, to generate a specific intracellular response (Lewis et al., 1998; Yoon & Seger, 2006; **Fig. 1.4**). Specificity of signal transfer to MAPKs is maintained by scaffold proteins, which organise components of MAPK modules into signalling complexes (Morrison & Davis, 2003; Dhanasekaran *et al.*, 2007), as well as by specific docking interactions between the individual components of each MAPK cascade, thus ensuring efficient signal transfer to give the required cellular response (Tanoue & Nishida, 2003; Raman *et al.*, 2007). The strength and duration of MAPK activation is an important determinant of the biological outcome of MAPK signalling (Marshall, 1995; Cook *et al.*, 1999; Murphy & Blenis, 2006; Chung *et al.*, 2010). MAPK phosphorylation is reversed by a subfamily of dual-specificity protein phosphatases (DUSPs) known as MAP kinase phosphatases (MKPs), which catalyse de-phosphorylation of the threonine and tyrosine residues in the activation loop of MAPKs to inactivate them (Dickinson & Keyse, 2006; Keyse, 2008).

1.3.3 Classification of mammalian MAPKs

In mammalian cells, fourteen MAPKs have been identified; these have been subdivided into MAPK subfamilies, according to the identity of the central amino acid residue in their respective T-X-Y dual phosphorylation motifs, their sequence homology and their mode of activation by different agonists (Raman *et al.,* 2007; **Fig. 1.4**). Four major MAPK cascades exist, namely: extracellular signal-regulated kinase-1 and -2 (ERK1/2), which contain the activation motif T-E-Y (Payne *et al.*, 1991); c-Jun NH_2-terminal kinase-1, -2 and -3 (JNK1-3) (Kyriakis *et al.*, 1994; Johnson & Nakamura, 2007), which contain a T-P-Y dual-phosphorylation motif; and the p38 MAPKs (p38α, -β, -γ and -δ) (Han *et al.*, 1994; Cuenda & Rousseau, 2007), which contain a T-G-Y activation motif. The fourth, and most recently discovered MAPK cascade, termed ERK5, also contains a T-E-Y activation motif and is hence classified as an ERK, but it is distinct from ERK1/2 as it has a unique structure and is much larger than ERK1/2 (Zhou et al., 1995; Lee et al., 1995; Nishimoto & Nishida, 2006; *Section 1.4*).

It is noteworthy that a further class of MAPKs, termed 'atypical MAPKs,' has also been described. The atypical MAPKs comprise ERK3, ERK4, ERK7, ERK8 and Nemo-like kinase (NLK), and are distinct from conventional MAPKs as they are not activated by an upstream MAPKKK or MAPKK and generally do not contain a T-X-Y activation motif present in conventional MAPKs (Coulombe & Meloche, 2007). This class of MAPKs remain poorly characterised and their function *in vivo* remains largely unknown (Coulombe & Meloche, 2007).

The classical MAPKs, ERK1/2, are preferentially activated by growth factors (Rubinfeld & Seger, 2005). By contrast, activation of the JNKs and p38 MAPKs is generally induced by exposure of cells to environmental stresses (Kyriakis & Avruch, 2001), and as such they are sometimes referred to collectively as stress-activated protein kinases (SAPKs). These differences in activating stimuli combined with the different substrate specificities of the classical and stress-activated MAPKs is thought to account for the diverse biological functions played by these respective groups (Roux & Blenis, 2004). MAPKs are ubiquitously expressed in all mammalian cells; however, the function of a

given MAPK can vary significantly between organisms, as well as between cell types within the same organism. It has been suggested that the reason for this variation in MAPK function between cell types is partly due to differential expression of transcription factors in distinct cell types (Chang & Karin, 2001).

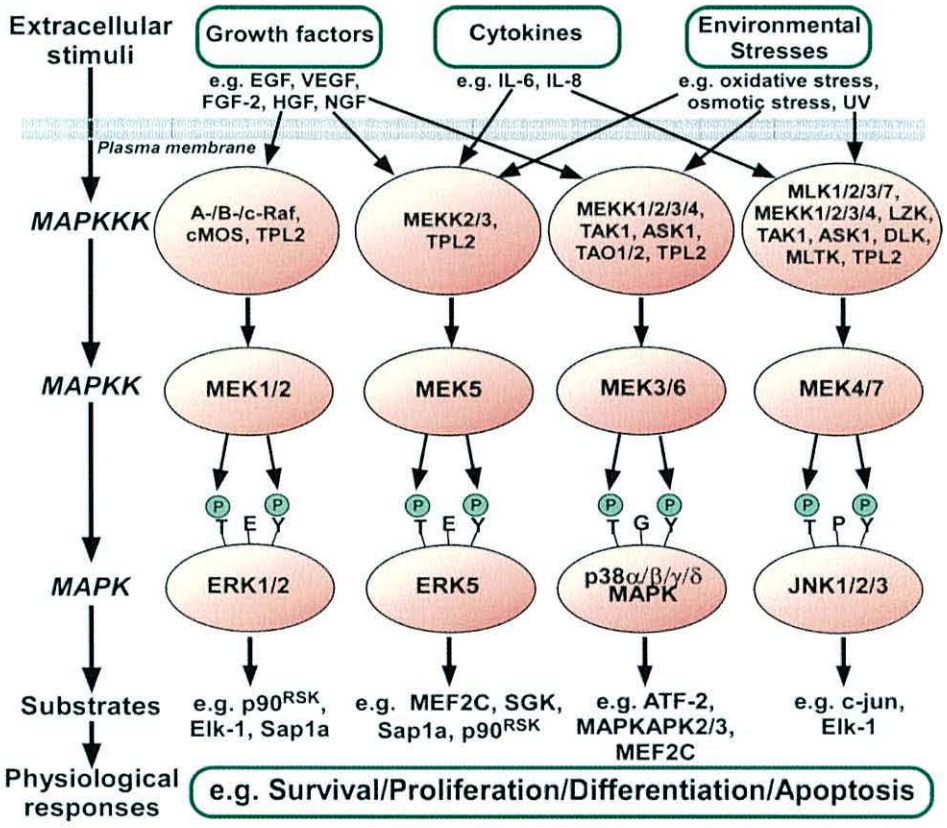


Figure 1.4 Mitogen-activated protein kinase (MAPK) signalling cascades in mammalian cells. Mammalian cells have four distinct conventional MAPK cascades termed: ERK1/2, ERK5, p38 MAPKs (p38α, -β, -γ and -δ), and JNK1/2/3, which are each arranged into a hierarchal, three-tier system. Mitogens (such as cytokines or growth factors), or environmental stresses, (such as hyperosmolarity), as well as other extracellular stimuli, act via specific receptors to stimulate MAPK cascade activation. MAPK activation is initiated by phosphorylation of an apical MAPKKK (or MEKK). Activation of MAPKKK leads to the sequential phosphorylation, and concomitant activation of the MAPKK (or MEK), which in turn, phosphorylates (denoted **P**) specific threonine (T) and tyrosine (Y) residues present within a conserved T-X-Y activation motif. The central amino acid present in the T-X-Y motif is glutamate (E) in the case of ERK1/2 and ERK5, glycine (G) in the case of p38 MAPKs and proline (P) in the case of JNKs. Dual-phosphorylation of Thr and Tyr within the T-X-Y motif activates each MAPK, facilitating phosphorylation of a variety of downstream cytosolic or nuclear substrates, which may include other protein kinases (e.g. $p90^{RSK}$ and SGK) or transcription factors (e.g. MEF2C, Elk-1, c-Myc). MAPK-mediated phosphorylation of downstream substrates can elicit a range of transcriptional or non-transcriptional changes, leading to specific cellular responses which may include the induction of cellular proliferation, migration, differentiation, survival or the initiation of cell death by apoptosis. *ASK, apoptosis signal-regulating kinase/MEKK5; ATF-2, activating transcription factor-2 ; cMOS, moloney sarcoma oncogene; DLK, mitogen-activated protein kinase kinase kinase 12; Elk-1, similar to ETS domain protein Elk-1; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; FGF-2, fibroblast growth factor-2; HGF, hepatocyte growth factor; JNK, c-Jun NH_2-terminal kinase; IL-6, interleukin-6; IL-8, interleukin-8; LZK, mitogen-activated protein kinase kinase kinase 13; MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; MAPKKK, MAPKK kinase; MAPKAPK2/3 MAPK-activated protein kinase 2 and 3; MEF2C, myocyte enhancer factor 2C; MEK, MAPK/ERK kinase; MEKK, MAPK/ERK kinase kinase; MLK1/2/3, mixed-lineage kinase 1/2/3; MLTK, sterile alpha motif and leucine zipper containing kinase; NGF, nerve growth factor; $p90^{RSK}$, p90 ribosomal S6 kinase; Sap1a, serum response factor accessory protein 1a; SGK, serum and glucocorticoid-inducible kinase; TAK1, TGFβ-activated kinase/mitogen-activated protein kinase kinase kinase 7; TAO1/2, thousand and one amino acids/TAO kinase 1/2; TPL2, tumour progression locus 2; UV, ultra-violet; VEGF, vascular endothelial growth factor.*

1.3.4 The role of MAPKs in angiogenesis

Considerable insight into the role of MAPKs *in vivo* has been attained from the analysis of embryonic stem-cell-derived mice in which genes encoding specific MAPKs have been deleted (Kuida & Boucher, 2004; Krens *et al.*, 2006; Gerits *et al.*, 2007). These studies have revealed that certain MAPKs are critical for normal development whilst others are not (Gerits *et al.*, 2007). Analysis of the cause of death of embryos following ablation of certain components of MAPK cascades has revealed a range of abnormal phenotypes including defective neural and cardiovascular development (Krens *et al.*, 2006; Gerits *et al.*, 2007). Defective cardiovascular development following ablation of certain genes in mice has provided an important clue to suggest a possible role of particular MAPKs in neovascularisation. However, as angiogenesis is a multi-faceted process it can often be difficult to pinpoint the specific role of a given signalling molecule in this process based solely upon the phenotype of knockout mice, as signalling proteins and MAPKs may be involved in several distinct steps of the angiogenic response (Hanahan, 1997; Rossant & Howard, 2002). This has meant that manipulation of MAPK expression and activity in endothelial cells *in vitro* is often necessary to complement *in vivo* studies. The phenotypes of mice in which individual conventional MAPK isoforms have been ablated are presented below. In addition, the findings of selected *in vitro* studies in which the role of MAPKs in growth factor-stimulated angiogenesis has been investigated are summarised. The role of the ERK5 signalling axis *in vivo* is discussed elsewhere (*section 1.4.8*).

1.3.4.1 ERK1/2

The prototypic MAPKs, ERK1 ($p42^{MAPK}$, MAPK3) and ERK2 ($p44^{MAPK}$, MAPK1) have been intensively studied and represent the best characterised MAPKs (Rubinfeld & Seger, 2005). It has emerged that a central role played by ERK1/2 is in relaying growth factor-induced mitogenic signals, to facilitate cell-cycle progression and cellular proliferation (Brunet *et al.*, 1999b). Redundancy in function exists between ERK1 and ERK2 as they share 84% sequence identity (Boulton *et al.*, 1990; Boulton *et al.*, 1991). Interestingly however, genetic studies in mice in which individual components of the classical MEK/ERK cascade have been deleted have revealed that ERK1 and ERK2 perform

distinct functions during development. *Mek1*$^{-/-}$ and *Erk2*$^{-/-}$ mice die *in utero* at between E10.5 and E11.5 with defective placental development (Giroux *et al.*, 1999; Hatano *et al.*, 2003). By contrast, *Mek2*$^{-/-}$ and *Erk1*$^{-/-}$ mice develop normally and are viable, with no vascular defects (Pages *et al.*, 1999; Belanger *et al.*, 2003). Most recently, it was reported that the combined deletion of *Erk1* and *Erk2* specifically in the endothelial cells of developing mice, induced embryonic lethality at E10.5 due to defective angiogenesis in the yolk sac and the embryo proper (Srinivasan *et al.*, 2009).

Pouysségur and co-workers provided evidence that ERK1/2 is activated in endothelial cells following hypoxia, leading to the promotion of VEGF expression by recruitment of the activator protein-2 (AP-2) complex to the *VEGF* promoter and by direct phosphorylation and stabilisation of hypoxia-inducible factor-1α (HIF-1α), leading to increased HIF-1 transcriptional activity (Berra *et al.*, 2000). In addition, ERK1/2 regulates growth factor-induced endothelial cell proliferation as well as cell-cycle arrest at confluency (Berra *et al.*, 2000; Pages *et al.*, 2000; Wu *et al.*, 2000a).

ERK1/2 are activated by a diverse array of growth factors in endothelial cells, including VEGF and FGF-2 as well as by ECM-integrin interactions, and are known to positively-regulate angiogenesis, mainly by facilitating endothelial cell proliferation (Eliceiri *et al.*, 1998; Zachary, 2003; Bullard *et al.*, 2003). Cell-cycle progression and endothelial cell proliferation following stimulation with growth factors such as VEGF, is a critical process in angiogenesis and has been shown to be chiefly regulated by ERK1/2 (Rousseau *et al.*, 1997; Pedram *et al.*, 1998; Berra *et al.*, 2000; Wu *et al.*, 2000a). More recently, FGF-2-stimulated ERK1/2 activation was found to be important for endothelial cell migration (Pintucci *et al.*, 2002). FGF-2-induced ERK1/2 activation occurs via the classical Ras→Raf-1→MEK1/2→ERK1/2 pathway (Klint *et al.*, 1999), whereas VEGF-stimulated ERK1/2 activation has been reported to occur via either Ras-dependent or PKC-dependent pathways (Takahashi *et al.*, 1999; Meadows *et al.*, 2001). In addition to its role in proliferation, VEGF-stimulated ERK1/2 activity has also been shown to be important for prostacyclin production in endothelial cells (Gliki *et al.*, 2001).

1.3.4.2 p38 MAPK

Four isoforms of the p38 MAPKs have been identified in mammalian cells, namely p38α (SAPK2a), p38β (SAPK2b), p38γ (SAPK3), p38δ (SAPK4). Inducers of p38 MAPK cascade activation include cellular stresses such as osmotic and chemical shock, as well as pro-inflammatory cytokines and certain growth factors (Zarubin & Han, 2005). The p38 MAPKs have been implicated in the regulation of cell migration, differentiation and apoptosis (Cuenda & Rousseau, 2007). Deletion of p38α MAPK in mice results in embryonic cell death at E11.5 due to defective angiogenesis in the placenta and in the embryo proper (Mudgett *et al.*, 2000; Adams *et al.*, 2000). By contrast, targeted ablation of p38β, p38γ or p38δ MAPK isoforms in mice did not show any abnormal phenotypes (Beardmore *et al.*, 2005; Sabio *et al.*, 2005), thus suggesting that p38α has a unique role in vascular development.

The role of p38α in angiogenesis may be restricted to embryonic development, as inhibition of p38α activity in endothelial cells *in vitro* has been reported to enhance VEGF- and FGF-2-induced tubular morphogenesis and endothelial cell survival, suggesting that p38 MAPK negatively-regulates these responses (Gratton *et al.*, 2001; Matsumoto *et al.*, 2002; Issbrucker *et al.*, 2003; Yang *et al.*, 2004a). As described previously (*section 1.2.5.4*), VEGF-stimulated p38 MAPK activation is important for actin reorganisation in endothelial cells and subsequent endothelial cell migration via a HSP27-dependent pathway (Rousseau *et al.*, 1997; Rousseau *et al.*, 2000; Lamalice *et al.*, 2004; Lamalice *et al.*, 2006). A role for p38 MAPK as an enhancer of VEGF-induced endothelial cell permeability has also been suggested (Issbrucker *et al.*, 2003). In addition, p38 MAPK may have a role in the stabilisation of newly formed angiogenic sprouts, as angiopoietin-1 (Ang-1)-directed mural cell recruitment was found to be p38 MAPK-dependent in a rat aorta model (Zhu *et al.*, 2003).

1.3.4.3 JNK

There are three members of the JNK family, termed: JNK1 (SAPKβ) (Derijard *et al.*, 1994), JNK2 (SAPKα) (Sanchez *et al.*, 1994) and JNK3 (SAPKγ) (Lin *et al.*, 1995). The JNKs have been implicated in the regulation of various cellular functions, including mediation of cell survival, cell proliferation and apoptosis (Weston & Davis, 2007;

Johnson & Nakamura, 2007). In mice, Jnk1 and Jnk2 are ubiquitously expressed, whereas Jnk3 is expressed mainly in the brain and in the heart (Martin *et al.*, 1996; Kuan *et al.*, 1999; Sabapathy *et al.*, 1999a). Mice lacking *Jnk1*, *Jnk2* or *Jnk3* individually are viable and develop normally (Dong *et al.*, 1998a; Yang *et al.*, 1997; Yang *et al.*, 1998b). However, compound murine mutants lacking both *Jnk1* and *Jnk2* die during embryogenesis at between E11.0 and E12.5 due to dysregulated region-specific apoptosis leading to defective neural tube closure during early brain development (Kuan *et al.*, 1999; Sabapathy *et al.*, 1999b). The absence of cardiovascular defects in *Jnk1-3*-null mice suggests that these isoforms are not required for vasculogenesis or angiogenesis.

Studies of the potential role of JNKs in endothelial cell function *in vitro* has revealed that VEGF attenuates JNK activation in endothelial cells under conditions of apoptosis in a dose-dependent manner, whereas inhibition of the JNK pathway sustained VEGF-mediated HDMEC survival, together suggesting that JNK acts as a negative-regulator of VEGF-induced cell survival (Gupta *et al.*, 1999). It is possible that JNKs may play a role in angiogenesis by regulating production of matrix-degrading proteases necessary to allow endothelial cell invasion of the ECM, as inhibition of JNK in endothelial cells plated in a type I collagen matrix exhibited diminished tubular morphogenesis due to decreased migration caused by reduced expression of MMP2 and MT1 (membrane-tethered protease 1) (Boyd *et al.*, 2005). More recently, JNK has been shown to be required for up-regulation of expression of the immediate-early gene *Egr-1* (early growth response-1) in endothelial cells undergoing capillary morphogenesis (Uchida *et al.*, 2008).

1.4 Extracellular signal-regulated kinase 5 (ERK5)

1.4.1 Discovery of ERK5

Extracellular signal-regulated kinase 5 (ERK5) was cloned by two independent research groups in 1995, following a degenerate PCR-based search for novel MAPKs with similar sequences to MEK1, which led to the discovery of a previously unknown MAPKK, termed MAP/ERK kinase 5 (MEK5) (Zhou *et al.*, 1995; English *et al.*, 1995). Subsequent yeast two-hybrid analysis of MEK5 binding partners led to the discovery of ERK5 (Zhou

et al., 1995). In a separate study, Lee *et al.* used degenerate PCR to screen a human placenta cDNA library, and isolated a novel MAPK, which, owing to the fact that it is nearly twice the size of ERK1 and ERK2, was termed big MAPK 1 (BMK1) (Lee *et al.*, 1995). It later became apparent that BMK1 and ERK5 were in fact the same protein. In vertebrates, ERK5 is expressed in a variety of tissues, but is most abundant in the heart, skeletal muscle, placenta and kidneys (Lee *et al.*, 1995; Zhou *et al.*, 1995).

1.4.2 ERK5 structure and functional domains

The human *ERK5* gene (also termed *MAPK7*), is present on chromosome 17p11.2 and spans 5.79 kb (Purandare *et al.*, 1998). It has an open reading frame of 2445 base pairs encoding a protein of 816 amino acids with a predicted molecular mass of 98 kDa (Lee *et al.,* 1995; **Fig. 1.5**). ERK5 shares 66% sequence identity with ERK2 within the kinase domain (Zhou *et al.,* 1995). The N-terminal domain of ERK5 contains the kinase domain (amino acids 78-406), which contains a T-E-Y dual-phosphorylation motif present in the activation loop (Zhou *et al.,* 1995; **Fig. 1.5**). In addition, the N-terminus is important for cytoplasmic targeting (amino acids 1-77), interaction with MEK5 (amino acids 78-139) and oligomerisation (amino acids 140-406) (Kasler et al., 2000; Yan et al., 2001; **Fig. 1.5**). Similar to other MAPKs, ERK5 also contains a common docking (CD) domain, consisting of a short sequence of negatively-charged amino acids residues (amino acids 350-358; **Fig. 1.5**), which allows ERK5 to form associations with certain docking (D)-domain-containing substrates (Tanoue & Nishida, 2002; Ranganathan et al., 2006).

The large size of ERK5 is attributable to its long carboxyl-terminal tail of approximately 400 amino acids, which is unique amongst the conventional MAPKs (**Fig. 1.5**). Several of the unusual characteristics of ERK5 have been attributed to this C-terminal domain, including the transactivational activity of ERK5 (Kasler *et al.*, 2000; Akaike *et al.*, 2004), and the shuttling of ERK5 to the nucleus following its activation (Buschbeck & Ullrich, 2005; McCaw *et al.*, 2005). The C-terminal domain also contains a nuclear localisation signal (NLS) (amino acids 505-539), which is important for ERK5 nuclear targeting (Yan *et al.,* 2001; Buschbeck & Ullrich, 2005; **Fig. 1.5**), and two proline-rich (PR) domains,

termed PR1 and PR2 (amino acids 434-465 and amino acids 578-701 respectively) that are proposed to serve as binding sites for SH3-domain-containing proteins (Zhou *et al.,* 1995; Yan *et al.,* 2001; **Fig. 1.5**). The C-terminal region also contains a myocyte enhancer factor 2 (MEF2)-interacting region (amino acids 440-501) and a peroxisome proliferator-activated receptor-γ1 (PPAR-γ1)-interacting region (amino acids 412-577), which regulate the activity of MEF2 and PPAR-γ1 transcription factors, respectively (Kasler *et al.,* 2000; Akaike *et al.,* 2004; **Fig. 1.5**). In addition, ERK5 contains a potent transcriptional activation domain (amino acids 664-789) that is activated by autophosphorylation, which affords ERK5 the unique ability amongst conventional MAPKs to directly regulate gene transcription (Kasler *et al.,* 2000; Morimoto *et al.,* 2007; **Fig. 1.5**). Truncation of the C-terminal tail results in increased ERK5 kinase activity, revealing that the ERK5 C-terminus also has an auto-inhibitory function (Buschbeck and Ullrich, 2005).

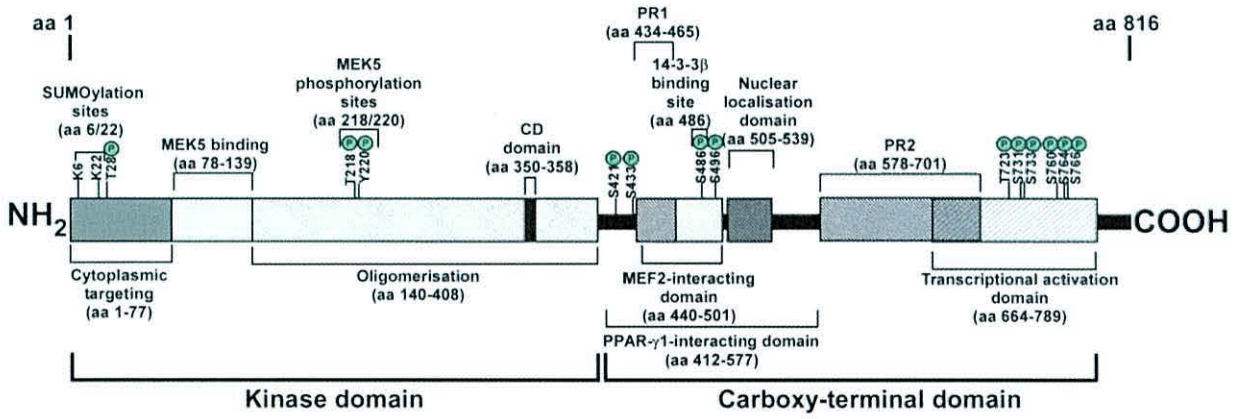


Figure 1.5 ERK5 structure and functional domains. ERK5 consists of 816 amino acid (aa) residues, and contains an N-terminal kinase domain, and a large C-terminal tail of approximately 400 aa. The N-terminus contains a domain that maintains ERK5 in a cytoplasmic location (aa 1-77), binding of MEK5 – the upstream kinase of ERK5 – occurs between (aa 78-139). Oligomerisation of ERK5 monomers is dependent upon aa 140-408. A CD domain (aa 350-358) is involved in allowing ERK5 to form associations with D-domain-containing proteins. The large C-terminal tail of ERK5 contains two Pro-rich (PR) regions - PR1 (aa 434-485) and PR2 (aa 578-701), believed to act as binding sites for SH3-domain containing proteins. Domains that allow interaction with the MEF2 transcription factors (aa 440-501), and PPAR-γ1 (aa 412-570) to regulate gene-expression are also located in the C-terminal tail of ERK5. ERK5 also contains a transcriptional activation domain (aa 664-789), which allows ERK5 to directly regulate gene expression. Phosphorylation (**P**) of Thr^{218} and Tyr^{220} within the activation loop of ERK5, by MEK5, is critical for ERK5 activation. ERK5 also undergoes activation-dependent autophosphorylation of several residues, mainly within the transcriptional activation domain, that are important for ERK5-regulated gene transcription. Activation-independent SUMOylation of ERK5 on Lys^{6} and Lys^{22} negatively-regulates ERK5-regulated gene transcription, whilst binding of the regulatory protein 14-3-3β to phosphorylated Ser^{486} can negatively-regulate ERK5 kinase activity. *CD, common docking; D, docking; ERK5, extracellular signal-regulated kinase 5; MAPK, mitogen-activated protein kinase; MEF2, myocyte enhancer factor 2; MEK5, MAPK/ERK kinase 5; PPAR-γ1, peroxisome proliferator-activated receptor-γ1; PR, proline rich; SH3, Src-homology 3; SUMO, small ubiquitin-like modifier.*

1.4.3 Regulation of ERK5 activity

1.4.3.1 Activation of the ERK5 signalling axis

ERK5 was originally identified as a stress-activated MAPK, activated by both osmotic and oxidative stresses (Abe *et al.*, 1996). Subsequent studies have revealed that ERK5 is also activated by serum (Kato *et al.*, 1997; Squires *et al.*, 2002) and a range of growth factors including: epidermal growth factor (EGF) (Kato *et al.*, 1998; Kamakura *et al.*, 1999), VEGF (Hayashi *et al.,* 2005), FGF-2 (Kesavan *et al.*, 2004; Kondoh *et al.*, 2006), TGF-β (Browne *et al.*, 2008); platelet-derived growth factor (PDGF) (Izawa *et al.*, 2007) and by certain inflammatory cytokines such as interleukin 6 (IL-6) (Carvajal-Vergara *et al.*, 2005). In neurones, ERK5 is known to be activated by trophic factors such as brain-derived neurotrophic factor (BDNF) (Cavanaugh *et al.*, 2001) and nerve growth factor (NGF) (Watson *et al.*, 2001; Finegan *et al.*, 2009). ERK5 is also activated by a range of physiological and pathological conditions including laminar shear-stress (Yan *et al.*, 1999), hypoxia (Sohn *et al.,* 2002) and ischemia (Takeishi *et al.*, 1999).

Activation of ERK5 involves phosphorylation of residues Thr^{218}/Tyr^{220} within the T-E-Y dual phosphorylation motif by MEK5 (Mody *et al.,* 2003; **Fig. 1.5**). MEK5 preferentially phosphorylates ERK5 on Thr^{218}, but phosphorylation of both Thr^{218} and Tyr^{220} is required for full catalytic activity of ERK5 (Mody *et al.*, 2003). MEK5 is the only upstream MAPKK that directly phosphorylates ERK5 (English *et al.,* 1995; Mody *et al.,* 2003; **Fig. 1.4**). Whilst MEK1 shares 48% sequence identity with MEK5, MEK1 does not interact with, or phosphorylate ERK5, and in the same way, MEK5 does not interact with, or activate ERK1/2 (English *et al.*, 1995; Zhou *et al.*, 1995; Kato *et al.*, 1997; Wang *et al.*, 2005a). MEK5 contains a Raf-1 activation motif ($S^{313}XXXT^{317}$) analogous to the one found in MEK1 (Zhou *et al.,* 1995), and growth factor stimulation induces MEK5 phosphorylation on Ser^{313}/Thr^{317} to induce MEK5 activity (Mody *et al.*, 2001).

1.4.3.2 Inactivation of the ERK5 signalling axis

Currently, no DUSP has been identified that can de-phosphorylate the Thr^{218}/Tyr^{220} residues within the T-E-Y activation motif of ERK5. It has been reported that ERK5 can be de-phosphorylated by the phosphotyrosine-specific phosphatase PTP-SL (protein

tyrosine phosphatase STEP-like), which interacts with ERK5 and impedes its translocation to the nucleus (Buschbeck *et al.*, 2002). Whilst both MKP-1 (DUSP1) and MKP-3 (DUSP6) had previously been reported as being capable of inactivating EGF-stimulated ERK5 phosphorylation in COS7 cells (Kamakura *et al.*, 1999), it was recently shown that although DUSP6 inactivates ERK1/2, it does not bind to, or inactivate ERK5 (Arkell *et al.*, 2008).

1.4.3.3 Other regulators of ERK5 activity

Small ubiquitin-related modifier (SUMO) polypeptides can alter protein function by covalently attaching to specific residues of certain target proteins (Wang & Dasso, 2009). ERK5 undergoes SUMOylation by SUMO3 on Lys^6 and Lys^{22} following exposure to either H_2O_2 or advanced glycation end-stage products (AGE) in HUVECs (Woo *et al.,* 2008; **Fig. 1.5**). Interestingly, SUMOylation of ERK5 was found to inhibit shear-stress-induced ERK5 transcriptional activity without affecting ERK5 phosphorylation in HUVECs (Woo *et al.,* 2008). In addition, Zheng and co-workers performed yeast two-hybrid analysis to reveal that the scaffold protein 14-3-3β can interact with ERK5 to regulate its activity (Zheng *et al.*, 2004). Specifically, it was shown that under basal conditions, 14-3-3β binds to phosphorylated Ser^{486} of ERK5, and acts as a negative-regulator of MEK5-mediated ERK5 activation (Zheng *et al.,* 2004; **Fig. 1.5**). Together, these findings show that ERK5 activity may be regulated by various mechanisms in addition to phosphorylation on the T-E-Y activation motif.

1.4.4 Kinases upstream of MEK5/ERK5

1.4.4.1 MEKK2 and MEKK3

The upstream MAPKKKs that specifically activate MEK5-ERK5 signalling axis by phosphorylating Ser^{313}/Thr^{317} of MEK5 are MEKK2 and MEKK3 (Sun et al., 2001; Chao et al., 1999; **Fig. 1.4**). Specificity of signal transduction between MEKK2/MEKK3 and MEK5 itself is ensured in part, by heterodimerisation arbitrated by phox and Bem1p (PB1) domains present in the respective N-termini of MEK5 and MEKK2/MEKK3 (Ponting *et al.*, 2002; Nakamura & Johnson, 2003).

MEKK2 and MEKK3 share 94% sequence identity (Blank *et al.*, 1996) and these proteins contain PB1 domains in their N-terminal regions (Wilson *et al.*, 2003; Lamark *et al.*, 2003). However, they are sufficiently divergent in their N-termini to allow differential regulation of the ERK5 signalling axis. In accordance with this, MEKK2, but not MEKK3, binds to the scaffold protein Lad (Lck-associated adapter). MEKK2 binds to MEK5 more strongly than MEKK3, and enhances ERK5 activation to a greater extent than MEKK3 (Sun *et al.,* 2001). It is believed that the MEKK2-Lad-MEK5 complex acts as a scaffold protein to allow enhanced ERK5 activation (Sun *et al.,* 2001).

1.4.4.2 Other kinases implicated in the ERK5 signalling axis

Interaction between the protein kinase WNK1 (with no lysine (K)-1) and MEKK2/MEKK3 has been shown to be important for EGF-stimulated ERK5 activation (Xu *et al.*, 2004). Over-expression of the serine/threonine protein kinase COT (cancer Osaka thyroid), which is also known as TPL2 (tumour progression locus 2) or MAPKKK8 has also been shown to be sufficient to activate ERK5 (Chiariello *et al.*, 2000). In addition, the mixed-lineage kinase (MLK)-like mitogen-activated protein triple kinase (MLTK) has been reported to act as a MAPKKK capable of activating MEK5 (Gotoh *et al.*, 2001).

1.4.5 Upstream activators of the ERK5 signalling axis

1.4.5.1 Ras/Raf-1

As MEK5 contains a Raf-1 phosphorylation motif - $S^{311}XXXT^{315}$ - similar to the $S^{218}XXXS^{222}$ motif found in MEK1, it was initially proposed that Ras/Raf-1 may act as upstream activators of MEK5, akin to the canonical Ras→Raf-1→MEK1/2→ERK1/2 cascade (English *et al.*, 1995; English *et al.*, 1998). Interestingly, co-transfection of constitutively active Ras with ERK5 in human embryonic kidney (HEK) 293 cells was found to induce ERK5 activation independently of Raf-1, thus suggesting that ERK5 may be activated by a novel Ras-dependent, Raf-independent pathway (English *et al.,* 1998). However, Ras-mediated activation of ERK5 appears to be cell type-dependent. For example, whilst both ERK5 and ERK1/2 were strongly activated by expression of constitutively active Ras in pheochromocytoma 12 (PC12) cells and in C2C12 myoblasts

(Kamakura *et al.,* 1999). By contrast, in HeLa or COS7 cells, expression of constitutively active Ras activated ERK1/2, but not ERK5 (Kato *et al.,* 1998; English *et al.,* 1998; Kamakura *et al.,* 1999). In NIH 3T3 fibroblasts, whilst Raf-1 was required to activate the MEK5/ERK5 pathway, this was independent of the Raf-1 kinase activity (English *et al.,* 1999). More recently, insulin-stimulated ERK5 activation in 3T3-L1 pre-adipocytes was reported to be inhibited by a dominant-negative farnesyltransferase, suggesting that ERK5 may be downstream of Ras, and is affected by Ras prenylation in these cells (Sharma & Goalstone, 2005). In total therefore, the current evidence of the requirement of Ras/Raf-1 to ERK5 activation remains conflicting and appears to be highly dependent upon cell type.

1.4.5.2 Protein kinase C (PKC)

The PKC activator phorbol 12-myristate 13-acetate (PMA) has been reported to stimulate ERK5 activation in various cell types (Kamakura *et al.*, 1999; Reddy *et al.*, 2002; Villa-Moruzzi, 2007). Growth factor-stimulated MEK5 activation in HeLa and HEK 293 cells was found to be mediated by PB1 domain-mediated physical interaction between the aPKC isoforms PKCζ or PKCι and MEK5 to form a ternary complex (Diaz-Meco & Moscat, 2001). Interestingly, PKCζ/ PKCι kinase activity was not required for MEK5 activation (Diaz-Meco *et al.,* 2001). In addition, Li *et al.* (2005) demonstrated that over-expression of PKCα or PKCβ_I/β_{II} in bone marrow-derived mast cells stimulated ERK5 activation, and cytokine production, thus suggesting that these cPKCs are upstream regulators of ERK5 activation in these cells. Most recently, Zhao *et al.* provided evidence to show that angiotensin II (Ang II)-stimulated activation of ERK5 in cardiomyocytes is mediated by PKCε, a nPKC (Zhao *et al.*, 2010). These studies collectively reveal that ERK5 activity can be regulated by various PKC isoforms. However, it should also be noted that some studies have also shown that ERK5 activation does not occur via PKC (Abe *et al.,* 1996; Kato *et al.,* 1998; Yan *et al.,* 1999). Furthermore, pharmacologic inhibition of PKC activity in BAF3 cells was found to augment granulocyte colony-stimulating factor (G-CSF)-stimulated ERK5 activity, suggesting that PKC may even negatively-regulate ERK5 activity in certain cell types (Dong *et al.*, 2001).

1.4.6 Downstream substrates of ERK5

Compared to ERK1/2, which has over 150 reported substrates (Yoon & Seger, 2006), relatively few substrates of ERK5 have been identified. The prototypic and best-characterised ERK5 substrate is the transcription factor MEF2C (Kato *et al.*, 1997; Yang *et al.*, 1998a). Other targets of ERK5 include MEF2A and MEF2D (Kato *et al.*, 2000), the Ets domain transcription factor serum response factor accessory protein 1a (Sap1a; Kamakura *et al.,* 1999), serum and glucocorticoid-inducible kinase (SGK) (Hayashi *et al.*, 2001) and 90 kDa ribosomal S6 kinase ($p90^{RSK}$) (Ranganathan *et al.,* 2006). Several other downstream effectors of ERK5 have been identified, whose activity is regulated by ERK5, but which may not be *bona fide* targets of ERK5. These include the pro-apoptotic protein Bcl2 antagonist of cell death (Bad) (Pi *et al.*, 2004), NF-κB (Garaude *et al.,* 2006), the forkhead box transcription factor FoxO3a (Wang *et al.*, 2006), FAK (Villa-Moruzzi, 2007) and glycogen synthase kinase-3β (GSK-3β) (Marchetti *et al.*, 2008).

Like other MAPKs, ERK5 can phosphorylate substrates on Ser/Thr residues immediately preceding a Pro residue. Intriguingly however, residues Thr^{28} in the ERK5 N-terminal domain, and residues Ser^{421}, Ser^{433}, Ser^{496}, Ser^{731} and Thr^{733} in the C-terminus of ERK5 are not followed by Pro, but undergo autophosphorylation by ERK5 (Mody *et al.,* 2003; **Fig. 1.5**). In addition, ERK5 can also phosphorylate MEK5 on the non-proline directed residues Ser^{129}, Ser^{137}, Ser^{142} and Ser^{149} (Mody *et al.*, 2003). Together, these findings suggest that the substrate specificity of ERK5 may differ from that of other MAPK family members.

1.4.7 The role of ERK5 in cell physiology

ERK5 is widely expressed in several tissues and cell lines (Regan *et al.*, 2002; Yan *et al.*, 2003; Buschbeck & Ullrich, 2005), but studies suggest that ERK5 may have specific roles in particular cell types (Hayashi & Lee, 2004; Sohn *et al.,* 2005; Wang *et al.,* 2005a; Wang & Tournier, 2006; Spiering *et al.,* 2009). Several studies have been conducted aimed at determining the relative contribution of ERK5 in arbitrating distinct physiological responses. In various cellular contexts, ERK5 has been implicated

in the regulation of cell differentiation, survival, proliferation, migration and adhesion (Kato *et al.*, 1998; Dinev *et al.*, 2001; Wang & Tournier, 2006; Sawhney *et al.*, 2009; Carter *et al.*, 2009). Furthermore, ERK5 appears to have an important role in the progression of certain pathologies including cancer, ischemia and cardiac hypertrophy (Takeishi *et al.*, 1999; Wang & Tournier, 2006; Montero *et al.*, 2009). Presented below are the principal findings of a series of studies, which implicate ERK5 in cellular survival, proliferation, migration and adhesion. Much evidence has emerged to suggest that ERK5 has a unique and important role in the regulation of normal endothelial cell function (Hayashi & Lee, 2004; Roberts *et al.*, 2009), hence, the role of ERK5 in endothelial cell physiology is discussed separately (*section 1.4.9*).

1.4.7.1 ERK5 and cell survival

1.4.7.1.1 ERK5 regulation of neuronal cell survival

Numerous studies have shown that ERK5 is an important facilitator of neuronal cell survival in response to diverse neurotrophic pro-survival stimuli (Cavanaugh, 2004; Wang & Tournier, 2006; **Fig. 1.6**). In response to stimulation with the neurotrophins BDNF and NGF in sensory neurones of rat dorsal root ganglia (DRG), Erk5 mediates phosphorylation of the transcription factor cAMP-responsive element binding protein (CREB) to facilitate neuronal cell survival (Watson *et al.*, 2001). Recent data has revealed that in murine superior cervical ganglion sympathetic neurons, Erk5 is an important regulator of NGF-induced transcription of the pro-apoptotic proteins *Bad*, Bim_L (BCL2-interacting mediator of cell death long) and Bim_{EL} (Bim extra-long) (Finegan *et al.*, 2009). Specifically, Erk5 was found to be required for down-regulating transcription of *Bad* via phosphorylation of $p90^{RSK}$ and subsequent activation of CREB. CREB exerts its anti-apoptotic activity by binding to CRE sites in the promoter region of genes such as *Bad* to prevent their transcription (Finegan *et al.,* 2009; **Fig. 1.6**). On the other hand, Erk5-mediated down-regulation of *Bim* expression was due to the ability of Erk5 to activate Akt, leading to phosphorylation and cytosolic sequesterisation of the forkhead transcription factor FoxO3a, and hence prevention FoxO3a-mediated transcription of *Bim* (Finegan *et al.,* 2009; **Fig. 1.6**).

The MADS (MCM1, Agamous, Deficiens, serum response factor)-box-domain MEF2-family of transcription factors were originally identified as regulators of myoblast differentiation, but have since been shown to have diverse roles, including acting as regulators of apoptosis in various cell types (McKinsey *et al.*, 2002). MEF2 family members have also emerged as common effectors of ERK5-mediated survival in neuronal cells. Shalizi and co-workers demonstrated that the protective effect of BDNF in newly-generated cerebellar granule neurones was mediated by ERK5, leading to MEF2-dependent transcription of the pro-survival factor neurotrophin-3 (NT3) to facilitate cell survival (Shalizi *et al.,* 2003; **Fig. 1.6**). In a separate study, BDNF-mediated survival of cortical neurons in developing rat embryos was also shown to be dependent upon ERK5-MEF2C activity (Liu *et al.,* 2003; **Fig. 1.6**). Interestingly however, ERK5 was not required for BDNF-induced neuronal survival in postnatal rats (Liu *et al.*, 2003b), suggesting that the requirement for ERK5 in cell survival is dependent upon the stage of development. Most recently, it was reported that Trk-dependent activation of ERK5 in DRG sensory neurones was required for *MEF2D* transcription and the subsequent up-regulation of *Bcl-w* (*BCL-2L2*), an anti-apoptotic member of the BCL2 family (Pazyra-Murphy *et al.,* 2009; **Fig. 1.6**).

1.4.7.1.2 ERK5 regulation of non-neuronal cell survival

In murine fibroblasts, ERK5 protects against the apoptotic effects of hyperosmolar stress by regulating the activation of Akt, to facilitate Akt-mediated activation of FoxO3a (Wang *et al.,* 2006a). Phosphorylation of FoxO3a facilitates the binding of the scaffold protein 14-3-3 (Brunet *et al.,* 1999a), resulting in the sequestration of FoxO3a in the cytoplasm, and the prevention of the transcription of the death-inducing ligand FasL (Fas ligand) (Wang *et al.,* 2006a; **Fig. 1.6**).

ERK5 has also been implicated in the regulation of the activity of the pro-apoptotic BCL2 homology 3 (BH3)-only protein Bim (BCL2 interacting mediator of cell death; Gírio *et al.,* 2007). When phosphorylated, Bim is inactivated and is sequestered in the mitochondria. De-phosphorylation of Bim allows its interaction with Bax (BCL2-associated X protein) to induce apoptosis via the mitochondrial pathway leading to

caspase activation and cell death (Le Bras *et al.*, 2006; Czabotar *et al.*, 2009). In HeLa cells, EGF-stimulated activation of ERK5 was recently shown to be required for phosphorylation and inactivation of Bim to confer cell survival during mitosis (Gírio *et al.,* 2007; **Fig. 1.6**), thus raising the possibility that ERK5 may regulate the activity of other pro-apoptotic BCL2 family proteins to facilitate cell survival.

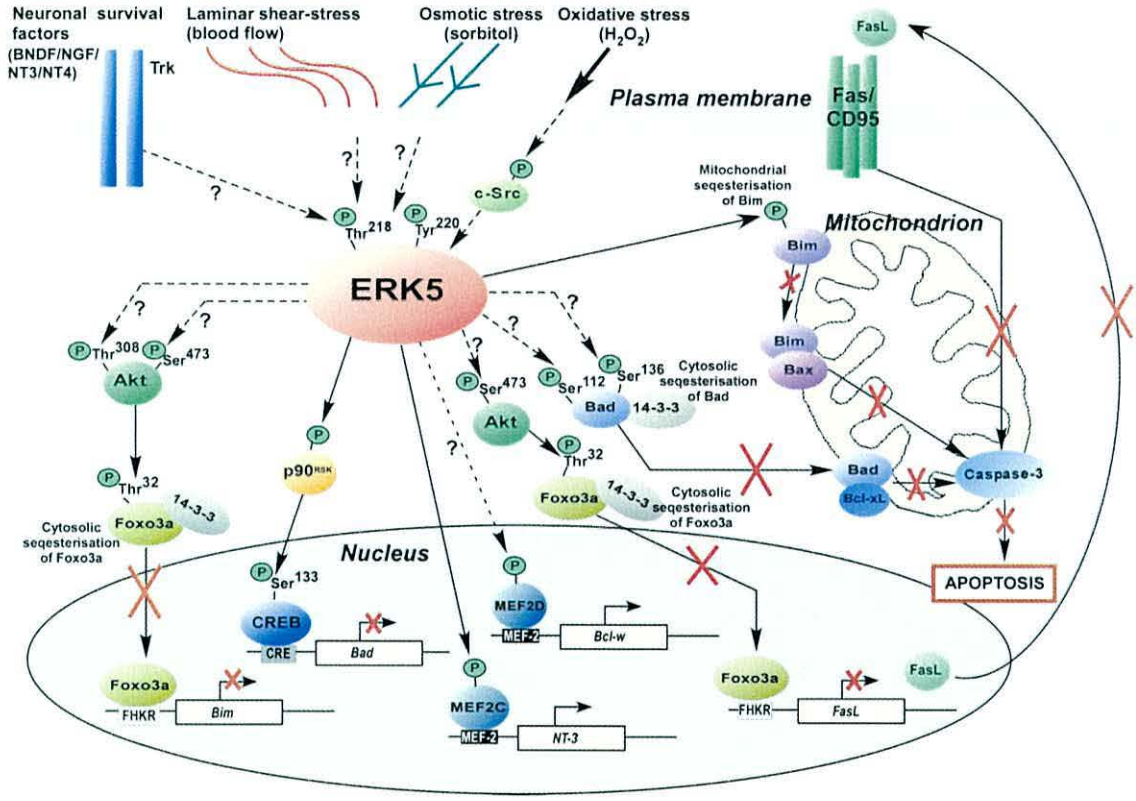


Figure 1.6 Mechanisms of ERK5-mediated cell survival. ERK5 mediates mammalian cell survival in response to diverse pro-apoptotic and anti-apoptotic stimuli by regulating several downstream pathways. ERK5 was first implicated as a mediator of cell survival from the observation that oxidative stress induced ERK5 activity in a c-Src dependent manner leading to MEF2C activation and protection against cell death (Suzaki *et al.*, 2002). In response to osmotic stress in murine fibroblasts ERK5 induces Akt activation via an unknown mechanism leading to phosphorylation of FoxO3a on Thr^{32}. ERK5-mediated phosphorylation of FoxO3a sequesters FoxO3a in the cytoplasm, preventing the transcription of the death-inducing ligand FasL, and subsequent apoptotic cell death via an extrinsic Fas (CD95)-mediated pathway (Wang *et al.*, 2006). Similarly, in response to NGF stimulation in mouse superior cervical ganglion (SCG) neuronal cells, ERK5 regulates Akt-dependent phosphorylation of FoxO3a to inhibit its entry into the nucleus and transcription of the pro-apoptotic protein *Bim* (Finegan *et al.*, 2009). ERK5-mediated phosphorylation of $p90^{RSK}$ in SCG neurones results in CREB phosphorylation on Ser^{133}, and the subsequent inhibition of the transcription of the pro-apoptotic protein *Bad* (Finegan *et al.*, 2009). Neurotrophic factors stimulate neuronal cell survival by binding to transmembrane tyrosine kinase (Trk) receptors termed collectively Trks. Stimulation of rat neuronal cells with the survival factor BDNF activates ERK5, inducing MEF2-dependent up-regulation of the survival factor NT3 (Shalizi *et al.*, 2003). Furthermore, ERK5 has been shown to regulate MEF2D-dependent expression of the pro-survival factor Bcl-w (Pazyra-Murphy *et al.*, 2009). In bovine endothelial cells, laminar flow-induced ERK5 activation leads to phosphorylation, and cytosolic sequesterisation of the pro-apoptotic protein Bad, via an unknown mechanism. This prevents Bad from translocating to the mitochondria and initiating an intrinsic apoptosis pathway involving Bad heterodimerisation with the pro-survival proteins Bcl2 and $Bcl2_{XL}$, terminating in the activation of capsase-3, and apoptosis (Pi *et al.*, 2004). In mitotic cells, ERK5-dependent phosphorylation of the pro-apoptotic protein Bim, leads to sequesterisation of Bim within the mitochondria, a process that prevents Bim-Bax association and subsequent caspase-3 activation and apoptosis (Gírio *et al.*, 2007). Dashed arrows represent instances in which connections between inducers of ERK5 activation and ERK5, or between ERK5 and downstream effectors have been shown, but in which the exact mechanisms remains unclear. *Akt, protein kinase B; Bad, BCL2-antagonist of cell death; Bax, BCL2–associated X protein; BCL2, B-cell lymphoma 2; Bcl-w, BCL2-like 2; Bcl-xL, BCL2 like 1; BDNF, brain-derived neurotrophic factor; Bim, BCL2-interacting mediator of cell death; CRE, cAMP-responsive element; CREB, cAMP-responsive element binding-protein; DRG, dorsal root ganglion; ERK5, extracellular signal-regulated kinase 5; FasL, fas ligand; FoxO3a, forkhead transcription factor box O3a; MEF2, myocyte enhancer factor-2; NGF, nerve growth factor; NT-3, neurotrophin-3; NT-4, neurotrophin-4;* $p90^{RSK}$*, p90 ribosomal S6 kinase; SCG, superior cervical ganglion.*

1.4.7.2 ERK5 and cell migration and adhesion

Growth factor-stimulated migration of renal epithelial cells and rat aortic smooth muscle cells has been shown to be mediated by ERK5 (Karihaloo *et al.*, 2001; Izawa *et al.*, 2007). By contrast, in hepatic stellate cells, ERK5 was reported to mediate FAK phosphorylation to enhance focal adhesion, resulting in diminished cell migration (Rovida *et al.*, 2008a). Interestingly, whilst a functional relationship between FAK and ERK5 has been demonstrated in several studies, the precise nature of this relationship seems to vary between cell types, with ERK5 appearing to act upstream of FAK in some cells (Villa-Moruzzi, 2007; Rovida *et al.*, 2008a), and downstream of FAK in others (Sawhney *et al.*, 2009). It has also been shown that ERK5 forms a complex with $\alpha_v\beta_3$ integrins and FAK in prostate and breast cancer cells to regulate adhesion and migration (Sawhney *et al.*, 2009), which raises the prospect that ERK5 may play a similar role in non-cancerous cells. Over-expression of constitutively active MEK5 led to hyper-phosphorylation of FAK (Sawhney *et al.*, 2009). Taken together, these data suggest that ERK5 may have an important role in cell attachment to the ECM and in cell migration.

1.4.7.3 ERK5 and cell proliferation

Kato and co-workers demonstrated that EGF-induced proliferation of MCF10A cells was dependent upon ERK5 activity (Kato *et al.*, 1998). The mechanism of ERK5-mediated cell-cycle progression in these cells was subsequently revealed from the finding that ERK5 was required for the phosphorylation of the serine/threonine kinase SGK on Ser^{78} to allow entry into S-phase, and subsequent cell proliferation following EGF stimulation (Hayashi *et al.*, 2001). Mulloy *et al.* (2003) since demonstrated that ERK5 can regulate cyclin D1 expression to allow cell-cycle progression from G_1-phase to S-phase in a variety of cells (Mulloy *et al.*, 2003). More recently however, Cude and co-workers provided evidence that ERK5 activity peaks at G_2-M phase and is required for the regulation of NF-κB activity and subsequent expression of mitosis-promoting genes to allow cell proliferation (Cude *et al.*, 2007).

In contrast to these results, Squires and co-workers demonstrated that by using a low dose of the MEK1/2 inhibitor PD184252 to specifically inhibit the activity of ERK1/2,

but not the activity of ERK5, EGF-stimulated cell-cycle progression and proliferation of CCl39 fibroblasts was blocked (Squires *et al.,* 2002). Similarly, aldosterone-stimulated proliferation of vascular smooth muscle cells was found to be independent of the ERK5 pathway (Ishizawa *et al.,* 2005). Furthermore, Garaude *et al.* showed that knockdown of ERK5 with small hairpin RNA had no effect upon the cell-cycle progression and proliferation of leukaemic T-cells (Garaude *et al.*, 2006). Together, these results show that whilst the ERK5 signalling axis may be required for the proliferation of certain cell types, it appears to be dispensable for growth factor-induced proliferation in others.

1.4.8 The role of the ERK5 signalling axis *in vivo*

To address the physiological role of the ERK5 signalling axis *in vivo* researchers utilised gene-targeting in mice to ablate specific genes (Hayashi & Lee, 2004; **Table 1.1**). *Erk5*-deficient mice died around E10.5 due to cardiovascular defects and angiogenic failure in embryonic and extra-embryonic tissues. In these mice, the developing vasculature fails to mature, with endothelial cells becoming disorganised and rounded, leading to a loss of vascular integrity and increased vessel leakiness, which culminates in embryonic death by haemorrhaging (Regan *et al.,* 2002; Sohn *et al.,* 2002; Yan *et al.,* 2003). Similar phenotypic abnormalities were seen in mice lacking *Mek5* (Wang *et al.,* 2005a) and *Mekk3* (Yang *et al.*, 2000), suggesting that the ERK5 signalling axis is critical for angiogenesis (**Table 1.1**). In an attempt to determine the primary defect upon Erk5 gene ablation, researchers generated conditional tissue-specific Erk5 knockout mice. Endothelial-specific *Erk5* knockout mice showed cardiovascular defects and died around E10.0, similar to the conventional *Erk5* knockout mice (Hayashi *et al.,* 2004). However, specific knockout of *Erk5* in cardiomyocytes, hepatocytes or neuronal cells does not affect development (Hayashi *et al.,* 2004; Hayashi & Lee, 2004). These important data suggest that whilst global *Erk5* knockout affects cardiovascular development, the initial defect occurs in the endothelium and that ERK5 is critical for endothelial cell function. The requirement of ERK5 in the maintenance of vascular integrity is highlighted by the fact that induced ablation of *Erk5* in adult mice is lethal within 2-3 weeks as blood vessels become leaky due to endothelial cell apoptosis (Hayashi *et al.,* 2004).

Table 1.1 Phenotypes of mice following global ablation of specific components of the Erk5 signalling axis, inducible ablation of *Erk5* in adult mice and following conditional ablation of *Erk5* in specific murine tissues.

Genotype	Phenotype	Reference
Mekk2$^{-/-}$	Mice are viable, develop normally and are fertile. Mice are viable and develop normally, but exhibit altered cytokine expression in thymocytes. Mice are viable and develop normally, but exhibit reduced cytokine expression in embryonic stem cell-derived mast cells.	Kesavan et al., 2004 Guo et al., 2002 Garrington et al., 2000
Mekk3$^{-/-}$	Embryonically lethal at E11.0 with severe defects in early angiogenesis. Did not prevent early formation of blood islands, suggesting that vasculogenesis was not affected.	Yang *et al.*, 2000
Mek5$^{-/-}$	Embryonically lethal at E10.5 due to defective cardiac development, increased apoptosis and decreased proliferation in the heart, head and dorsal regions.	Wang *et al.*, 2005
Erk5$^{-/-}$	Embryonically lethal at E9.5-E10.5 due to defects in normal heart looping, cardiac development, vascular maturation and angiogenesis. Embryonically lethal at E10.5-E11.5. Embryos displayed stunted growth, especially in the head and lower trunk with dilated pericardial sacs. Impaired angiogenesis in the embryo and placenta. Embryonically lethal at E10.5-E11.0 due to impaired angiogenesis in the embryo and the placenta. Embryonic endothelial cell apoptosis evident. Embryonically lethal at E9.5-E10.5 with growth retardation and underdeveloped yolk sac vasculature.	Regan *et al.*, 2002 Sohn *et al.*, 2002 Yan *et al.*, 2003 Hayashi *et al.*, 2004
Erk5$^{-/-}$ *Endothelial cell*	Embryonically lethal at E9.5-10.5 due to cardiovascular defects. Identical phenotype to that of global *Erk5*$^{-/-}$ mice.	Hayashi *et al.*, 2004
Erk5$^{-/-}$ *Cardiomyocyte*	Mice are viable and develop normally.	Hayashi *et al.*, 2004
Erk5$^{-/-}$ *Hepatocyte*	Mice are viable and develop normally.	Hayashi & Lee, 2004
Erk5$^{-/-}$ *Inducible knockout*	Lethality of adult mice within 2 weeks following induced ablation of *Erk5*. Mice display degeneration of the cardiovascular system with endothelial cell apoptosis.	Hayashi *et al.*, 2004
Mef2c$^{-/-}$	Embryonically lethal at E9.5 due to failure of the heart to undergo rightward loop morphogenesis, leading to right ventricle malformation. Endothelial cells fail to organise normally into a vascular plexus. Embryonically lethal at E9.5 due to cardiac and vascular malformations.	Lin *et al.*, 1997 Lin *et al.*, 1998 Bi *et al.*, 1999

1.4.9 ERK5 and endothelial cell physiology

1.4.9.1 ERK5 activation in endothelial cells

Initial studies using HUVECs stimulated with H_2O_2 showed that ERK5 was activated by oxidative stress in these cells (Abe *et al.,* 1996). Further studies demonstrated that flow-induced shear-stress could stimulate ERK5 activity in bovine aortic endothelial cells (BAECs) via a pathway that required intracellular Ca^{2+} (Yan *et al.,* 1999). Interestingly, VEGF and FGF-2 did not stimulate ERK5 activity in BAECs (Yan *et al.,* 1999); however, these pro-angiogenic molecules have since been reported to induce ERK5 activation in HUVECs (Hayashi *et al.,* 2004).

1.4.9.2 Inhibition of endothelial apoptosis

Targeted deletion of specific components of the Erk5 signalling axis in mice suggests that Erk5 plays an essential role in murine endothelial cell survival (Hayashi *et al.,* 2004; Hayashi & Lee, 2004; **Table 1.1**). Given that the phenotype of *Mef2c*$^{-/-}$ mice is similar to that of *Erk5*$^{-/-}$ mice (Lin *et al.,* 1997; Lin *et al.,* 1998; Bi *et al.,* 1999; **Table 1.1**), it has been suggested that ERK5 may mediate endothelial cell survival by signalling to MEF2C (Hayashi *et al.,* 2004; Olson, 2004). This premise was further supported by the finding that over-expression of a constitutively active form of Mef2c in *Erk5*$^{-/-}$ murine endothelial cells could partially rescue these cells from apoptosis (Hayashi *et al.,* 2004; Olson, 2004). However, MEF2C is also a substrate for p38α MAPK (Han *et al.,* 1997), and given that *p38α*$^{-/-}$ mice are also embryonically lethal due to cardiovascular abnormalities (Mudgett *et al.*, 2000; Adams *et al.*, 2000), it remains unclear whether the role of MEF2C in vascular development is due to its function as an arbitrator of ERK5- or p38 MAPK-derived signals (Regan *et al.,* 2002; Sohn *et al.,* 2002; Yan *et al.,* 2003; Hayashi & Lee, 2004). Nevertheless, it is generally accepted that other effectors in addition to Mef2C, acting downstream of Erk5, are also important in mediating endothelial cell survival (Sohn *et al.,* 2002; Hayashi *et al.,* 2004; Olson, 2004).

The luminal surface of endothelial cells *in vivo* is exposed to continuous laminar shear-stress caused by blood flow, which is known to confer atheroprotective effects (Traub & Berk, 1998). Pi and co-workers provided evidence to demonstrate that ERK5 is required for mediating flow-stimulated survival in bovine lung microvascular endothelial cells (BLMECs) (Pi *et al.,* 2004). Specifically, it was shown that ERK5 induced the phosphorylation and inactivation of the pro-apoptotic protein Bad on Ser^{112} and on Ser^{136} (Pi *et al.,* 2004). Phosphorylation of Bad on these residues prevents the interaction of Bad with the pro-survival proteins Bcl2 and $Bcl2_{XL}$ in the mitochondria (Zha *et al.*, 1996), thus preventing activation of the intrinsic apoptosis pathway, cleavage of caspase-3 and subsequent cell death (Pi *et al.,* 2004; **Fig. 1.6**). Surprisingly, candidate kinases such as Akt, protein kinase A (PKA) and $p90^{RSK}$, which are known to phosphorylate Bad (Datta *et al.*, 1997; Harada *et al.*, 1999; Bonni *et al.*, 1999; Tan *et al.*, 1999), were not responsible for mediating ERK5-induced

phosphorylation of Bad in BLMECs (Pi *et al.,* 2004). However, it was recently shown that siRNA-mediated down-regulation of Erk5 expression prevented sustained Akt phosphorylation on Ser^{473} following PDGF stimulation in porcine aortic endothelial (PAE) cells (Lennartsson *et al.*, 2010), which suggests that ERK5 can regulate Akt activation in certain endothelial cells.

1.4.9.3 Mediation of shear-stress signalling

Fluid shear-stress-mediated ERK5 activation has been shown to confer an atheroprotective effect by negatively-regulating tumour necrosis factor-α (TNF-α)-stimulated expression of the adhesion molecules *VCAM-1* and *E-selectin* in endothelial cells (Akaike *et al.,* 2004). A more recent study utilising the novel MEK5 inhibitor BIX02188 revealed that the MEK5-ERK5 pathway mediates flow-dependent inhibition of TNF-α signalling in BLMECs (Li *et al.,* 2008). Analysis of laminar shear-stress induced transcriptional responses in endothelial cells has identified Krüppel-like factor 2 (KLF2) as a mechano-stress induced gene (Dekker *et al.*, 2002; SenBanerjee *et al.*, 2004) SenBanerjee *et al.,* 2004). KLF2 is responsible for negatively-regulating inflammation and angiogenesis, promoting vascular stabilisation and maintaining vascular quiescence (Dekker *et al.,* 2002; SenBanerjee *et al.,* 2004; Boon & Horrevoets, 2009). It has also been shown that ERK5 regulates KLF2 expression in mouse embryonic fibroblasts via a pathway requiring the MEF2 transcription factor (Sohn *et al.,* 2005). In addition, recent studies have revealed that ERK5 is required for flow-induced expression of KLF2 in HUVECs (Parmar *et al.*, 2006), and the subsequent increased cell-surface expression of CD59 (Kinderlerer *et al.*, 2008).

1.4.9.4 Regulation of hypoxic responses

ERK5 is activated under hypoxic conditions, and has been reported to negatively-regulate *Vegf* expression in murine fibroblasts (Sohn *et al.,* 2002). Furthermore, increased *Vegf* expression was observed in $Erk5^{-/-}$ embryos compared with wild-type and $Erk5^{+/-}$ mice (Sohn *et al.,* 2002). However, it is unlikely that increased Vegf expression in endothelial cells is the primary causative factor leading to death in $Erk5^{-/-}$ mice at E11.0 (Sohn *et al.,* 2002), as over-expression of *Vegf* in murine embryos results

in normal development up to E12.5, with lethality due to cardiovascular abnormalities only evident at E12.5-14.5 (Miquerol *et al.,* 2000). ERK5 has been shown to regulate levels of the hypoxia-inducible transcription factor HIF-1α, by promoting HIF-1α ubiquitination and proteolysis in BLMECs, leading to a decrease in hypoxia-induced *Vegf* mRNA levels (Pi *et al.*, 2005).

1.4.9.5 Regulation of endothelial cell migration

Pi *et al.* observed that enhanced migration of BLMECs following transfection with a stable mutant of HIF-1α was abolished following co-expression of CA-MEK5 and HIF-1α (Pi *et al.,* 2005). It was suggested that this effect was attributable to a reduction in *Vegf* expression following ERK5-regulated HIF1-α degradation (Pi *et al.,* 2005); however, the potential role of ERK5 in regulating VEGF-stimulated endothelial cell migration is currently unknown.

More recently, a role for the MEK5-ERK5 pathway in regulating endothelial cell migration and focal contact turnover was demonstrated (Spiering *et al.*, 2009). Over-expression of a constitutively active form of MEK5 led to reduced focal adhesion turnover in HUVECs, resulting in reduced HUVEC migration on a fibronectin matrix (Spiering *et al.,* 2009). Seemingly paradoxically, constitutive activation of MEK5 in HUVECs increased phosphorylation of FAK on residues Tyr^{861}, Ser^{910} and Tyr^{925}, but this did not result in enhanced cell migration. However, it was found that ERK5 activation resulted in decreased expression of p130Cas, a downstream target of FAK, by an as-yet-unidentified mechanism (Spiering *et al.,* 2009).

1.4.9.6 Tumour angiogenesis

Hayashi and co-workers provided evidence to show that ERK5 regulates tumour angiogenesis (Hayashi *et al.*, 2005). Following the establishment of melanoma and Lewis lung carcinoma tumour xenografts in mice, polyinosinicpolycytidylic acid (pIpC)-induced ablation of *Erk5* in $Erk5^{flox/flox}$ mice carrying an inducible (myxovirus resistance-1) Mx1-Cre transgene, resulted in a regression of the tumour vasculature and a concomitant reduction in tumour volume by 63% and 72% respectively. Furthermore,

screening of potential ERK5 targets using a Pepchip array revealed that ablation of *Erk5* in mouse lung endothelial cells prevented phosphorylation of ribosomal protein S6 (rpS6) on $Ser^{235/236}$ by $p90^{RSK}$ (Hayashi *et al.,* 2005). Interestingly, ablation of *Erk5* in mouse fibroblasts did not affect phosphorylation of rpS6 (Hayashi *et al.,* 2005), suggesting that activation of this signalling pathway may be cell-type specific.

It is known that the pro-angiogenic factors VEGF, FGF-2 and EGF stimulate ERK5 activity in mouse lung capillary endothelial cells (MLCECs) and HUVECs (Hayashi *et al.,* 2004). Furthermore, *Erk5*$^{-/-}$ mice showed impaired formation of functional blood vessels in Matrigel plug implants soaked in either VEGF or FGF-2 (Hayashi *et al.,* 2005), thus suggesting that Erk5 may be required for sprouting angiogenesis in response to either VEGF or FGF-2 stimulation in murine endothelial cells. However, the precise role played by Erk5 in this process is far from clear and the potential physiological importance of VEGF-stimulated ERK5 activation in endothelial cells remains unknown. It therefore remains a distinct possibility that ERK5 is an important component of the VEGF/VEGFR-2 signalling cascade in endothelial cells, with potential roles in the regulation of VEGF-stimulated endothelial cell survival, migration, proliferation and differentiation (Holmes *et al.,* 2007).

1.5 Project aims

The broad objective of this study was to define the role of ERK5 in growth factor-induced angiogenesis *in vitro* in primary human dermal microvascular endothelial cells (HDMECs).

In order to achieve this objective, this project aimed to:

1. Characterise the activation of ERK5 in response to VEGF and other pro-angiogenic stimuli in HDMECs and delineate the upstream regulatory pathways mediating ERK5 activation in response to these stimuli in HDMECs.
2. Define the functional role of ERK5 in the angiogenic response to VEGF stimulation by manipulation of ERK5 expression and activity in HDMECs and the quantification of the effects of these measures upon distinct stages of the angiogenic response – proliferation, adhesion, migration, survival and differentiation - using a range of *in vitro* angiogenesis assays.
3. Identify downstream growth factor-activated signalling pathways that may be regulated by ERK5 in HDMECs and establish whether or not the MEK5/ERK5 pathway is involved in regulating the transcription of genes involved in angiogenesis.
4. Determine the effects of manipulation of ERK5 expression levels upon both established capillary structures, and newly-formed vessels, in a HDMEC/NHDF (normal human dermal fibroblast) co-culture *in vitro* angiogenesis assay, to assess the feasibility of targeting ERK5 for the therapeutic inhibition of angiogenesis.

CHAPTER TWO

Materials and Methods

2.1 Materials

2.1.1 Reagents

Recombinant human VEGF-A_{165}; VEGF-A_{121}; EGF; and HGF were purchased from Peprotech EC (Rocky Hill, NJ, U.S.A.). Recombinant human macrophage colony stimulating factor (CSF-1) and FGF-2 were purchased from R&D Systems Inc. (Minneapolis, MN, U.S.A.). Phorbol-12-myristate-13-acetate (PMA); A23187, free acid, *Streptomyces chartreusensis*; 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid tetrakis (acetoxymethyl ester) (BAPTA-AM); hydrogen peroxide; Gö6976; GF109203X; rottlerin; calphostin C, *Cladosporium cladosporioides*; U0126; rapamycin; wortmannin; SB202474 and SB202190 were purchased from Calbiochem (Merck Chemicals Ltd., Nottingham, U.K.). ZM323881 hydrochloride was purchased from Tocris Bioscience (Bristol, U.K.). Farnesylthiosalicylic acid was purchased from Biomol® International (Exeter, U.K.). RNeasy mini RNA extraction kit; QIAshredder kit; and RNase-free DNase set were bought from Qiagen (Crawley, U.K.). 2 x Power SYBR® Green mastermix was purchased from Applied Biosystems (Warrington, U.K.). Ethylenediaminetetraacetic acid (EDTA); sodium hydroxide; sodium chloride; N,N,N',N'-tetramethylethylenediamine (TEMED); ammonium persulfate (APS); aprotinin; leupeptin; phenylmethylsulfonyl fluoride (PMSF); dimethyl sulfoxide (DMSO); sterile DNase- and RNase-free dH_2O; Tris-EDTA (TE) buffer solution (pH 8.0); Tris-Base; boric acid; sodium ethylenediaminetetraacetic acid (Na_4EDTA); sodium dodecyl sulfate (SDS); oleoyl-L-α-lysophosphatidic acid (LPA); sodium orthovanadate (Na_3VO_4); paraformaldehyde; Triton X-100; mitomycin C from *Streptomyces caespitosus;* 2-mercaptoethanol for electrophoresis, ≥98% (GC/titration); SIGMA *FAST*™ BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium) tablets; N-acetyl-D-sphingosine; ponceau S solution 0.1% (w/v) in 5% (v/v) acetic acid; staurosporine solution from *Streptomyces* sp. and ethidium bromide were purchased from Sigma-Aldrich (Poole, U.K.). SuperSignal® West pico chemiluminescent substrate was provided by Fisher Scientific (Loughborough, U.K.). Full-range (10-250 kDa) rainbow molecular weight markers; Hybond™ ECL™ nitrocellulose membrane; and enhanced chemiluminescence (ECL) Western blotting detection reagents were purchased from GE Healthcare (Amersham, U.K.). TransPass™ HUVEC transfection

reagent; restriction endonucleases; T4 DNA ligase; acetylated bovine serum albumin (BSA) (10 µg/µl); 10x restriction endonuclease buffer; 2x rapid ligation buffer; and 1 kb and 100 b.p. DNA ladders were purchased from New England Biolabs (Hitchin, U.K.). Moloney murine leukemia virus (M-MLV) reverse transcriptase; 0.1 M dithiothreitol (DTT); 5 x first strand buffer; 10 mM PCR grade dNTP mix; RNaseOUT™ recombinant ribonuclease inhibitor; Lipofecatamine™ RNAiMAX transfection reagent; Lipofectamine™ 2000; NuPAGE® 3-(N-Morpholino)propanesulfonic acid (MOPS) SDS running buffer; NuPAGE® 4X lithium dodecyl sulfate (LDS) sample buffer and NuPAGE® 4-12% pre-cast gradient gels were provided by Invitrogen (Paisley, U.K.). Tween-20; tryptone; glycerol; and agarose (electrophoresis grade) were purchased from Melford (Ipswich, U.K.). UltraPure ProtoGel® 30% (w/v) acrylamide was purchased from National Diagnostics (Hessle, U.K.). BSA fraction V was purchased from Roche (Welwyn Garden City, U.K.). GoTaq® Flexi DNA polymerase; $MgCl_2$ (25 mM); 5X colourless GoTaq® Flexi Buffer; ultra-pure deoxynucleotide triphosphates (dNTPs) (100 mM of each dNTP in ddH_2O), pH 7.0; restriction endonucleases; CellTiter-Glo™ luminescent cell viability assay; and Caspase-Glo® 3/7 assay were from Promega (Southampton, U.K.). CellTracker™ Green CMFDA (5-chloromethylfluorescein diacetate); Alexa Fluor® 546 phalloidin; Fluo-4; Fura Red™; annexin V Alexa Fluor® 488 conjugate; and Hoechst 33342 were purchased from Molecular Probes Europe BV (Leiden, The Netherlands). Optical-grade sealing film for 96-well qRT-PCR plates was purchased from Bioline (London, U.K.). Normal goat serum was bought from Vector Laboratories (Burlingame, CA, U.S.A.). Whatman 3 MM chromatography paper was purchased from VWR (Lutterworth, U.K.). The Essen Woundmaker™ and 24-well ImageLock™ microplates were from Essen Instruments (West Wickham, U.K.). Eppendorf 0.1-10 µl epT.I.P.S were provided by Eppendorf (Cambridge, U.K.). Bottle-top filters (0.22 µm) were obtained from Millipore Ltd. (Watford, U.K.).

Water

Distilled, deionised water (ddH_2O) was passed through a 0.22 µm filter and treated with UV light in a Millipore Synergy 185 unit (Millipore Ltd., Watford, U.K.) prior to use in the preparation of buffers and solutions.

2.1.2 Antibodies

Table 2.1 Primary antibodies used in this study.

Antibody name	Source	Host species	Dilution factor	Catalogue number	Appl.
Actin (C-11)	Santa Cruz Biotechnology (CA, U.S.A.)	Goat	1000	sc-1615-R	WB
Akt Antibody	New England Biolabs (Hitchin, U.K.)	Rabbit	1000	#9271	WB
Anti-BMK-1	Prof. J.-D. Lee, The Scripps Research Institute, La Jolla, CA, U.S.A.	Rabbit	5000	N/A	WB
Anti-FAK [pY^{576}] phosphospecific Antibody	Invitrogen (Paisley, U.K.)	Rabbit	1000	44652G	WB
Anti-NFκB p65 (RelA) Subunit Polyclonal Antibody	Millipore (U.K.) Ltd. (Watford, U.K.)	Rabbit	1000	AB1604	WB
Anti-phospho-FKHRL1/FOXO3A (Thr32)	Upstate® Cell Signaling Solutions (Lake Placid, NY, U.S.A.)	Rabbit	1000	#07-695	WB
Anti-PKCε	Upstate® Cell Signaling Solutions (Lake Placid, NY, U.S.A.)	Mouse	1000	#06-991	WB
Bcl-2 Antibody (Human Specific)	New England Biolabs (Hitchin, U.K.)	Rabbit	1000	#2872	WB
Cleaved Caspase-3 (Asp^{175}) Antibody	New England Biolabs (Hitchin, U.K.)	Rabbit	1000	#9661	WB
Cleaved Caspase-3 (Asp^{175}) Antibody (Alexa Fluor® 488 Conjugate)	New England Biolabs (Hitchin, U.K.)	Rabbit	25	#9669	IF
ERK5 Antibody	New England Biolabs (Hitchin, U.K.)	Rabbit	1000	#3372	WB
FAK Antibody	Santa Cruz Biotechnology (CA, U.S.A.)	Rabbit	1000	A-17	WB
Flag-Tag (DYKDDDDK Tag) Antibody	New England Biolabs (Hitchin, U.K.)	Rabbit	1000	#2368	WB
HA-Tag (6E2) Antibody	New England Biolabs (Hitchin, U.K.)	Mouse	1000	#2367	WB
Lamin B1 (H-90)	Santa Cruz Biotechnology (CA, U.S.A.)	Rabbit	2000	sc-20682	WB
M-CSF Receptor Antibody	New England Biolabs (Hitchin, U.K.)	Rabbit	1000	#3152	WB
MEF2C Antibody	New England Biolabs (Hitchin, U.K.)	Rabbit	1000	#9792	WB
MEK-5 (H-94)	Santa Cruz Biotechnology (CA, U.S.A.)	Rabbit	500	sc-10795	WB
Monoclonal Mouse Anti-Human CD31, Endothelial Cell Clone JC70A	Dako Cytomation (Glostrup, Denmark)	Mouse	750	M 0823	CCA
p44/42 MAPK (Erk1/2) Antibody	New England Biolabs (Hitchin, U.K.)	Rabbit	1000	#9102	WB
Phospho-p44/42 MAP Kinase (Thr^{202}/Tyr^{204}) Antibody	New England Biolabs (Hitchin, U.K.)	Rabbit	1000	#9101	WB
PKCα Antibody	New England Biolabs (Hitchin, U.K.)	Rabbit	1000	#2056	WB
Phospho Akt (Thr^{308}) (244F9)	New England Biolabs (Hitchin, U.K.)	Rabbit	1000	#4056	WB
Phospho Akt (Ser^{473}) (193H12)	New England Biolabs (Hitchin, U.K.)	Rabbit	1000	#4058	WB
Phospho-BAD (Ser^{112}) Antibody	New England Biolabs (Hitchin, U.K.)	Rabbit	2000	#9291	WB
Phospho-BAD (Ser^{136}) Antibody	New England Biolabs (Hitchin, U.K.)	Rabbit	1000	#9295	WB
PhosphoDetect™ Anti-VEGF Receptor 2 (pTyr$^{1054/1059}$) Antibody	Calbiochem (Nottingham, U.K.)	Rabbit	1000	PS1013	WB
Phospho-Erk5 (Thr^{218}/Tyr^{220}) Antibody	New England Biolabs (Hitchin, U.K.)	Rabbit	2500	#3371	WB
Phospho-NF-κB p65 (Ser^{536}) (93H1)	New England Biolabs (Hitchin, U.K.)	Rabbit	1000	#3033	WB
Phospho-p38 MAP Kinase (Thr^{180}/Tyr^{182}) Antibody	New England Biolabs (Hitchin, U.K.)	Rabbit	1000	#9211	WB
Phospho-p70 S6 Kinase (Thr^{389}) (108D2) Antibody	New England Biolabs (Hitchin, U.K.)	Rabbit	1000	#9234	WB
Phospho-p90^{RSK} (Ser^{380}) Antibody	New England Biolabs (Hitchin, U.K.)	Rabbit	1000	#9341	WB
Phospho-PKC (pan) (βII Ser^{660}) Antibody	New England Biolabs (Hitchin, U.K.)	Rabbit	2000	#9371	WB

Phospho-S6 Ribosomal Protein ($Ser^{235/236}$) Antibody	New England Biolabs (Hitchin, U.K.)	Rabbit	5000	#2211	WB
Phospho-SAPK/JNK (Thr^{183}/Tyr^{185}) Antibody	New England Biolabs (Hitchin, U.K.)	Rabbit	1000	#9251	WB
Phospho-VEGFR2 Tyr^{1175} Antibody	New England Biolabs (Hitchin, U.K.)	Rabbit	1000	#2478	WB
PKC Sampler Kit	BD Transduction Laboratories (Oxford, U.K.)	Various	Various	6E+05	WB
PKCδ Antibody	New England Biolabs (Hitchin, U.K.)	Rabbit	1000	#2058	WB
p-MEF2C (Ser^{387})	Santa Cruz Biotechnology (CA, U.S.A.)	Rabbit	2000	sc-13920-R	WB
p-MEF-2 (Ser^{59})	Santa Cruz Biotechnology (CA, U.S.A.)	Rabbit	2000	sc-13919-R	WB
VEGF Receptor 2 (55B11)	New England Biolabs (Hitchin, U.K.)	Rabbit	1000	#2479	WB

WB, Western blotting; IF, immuofluorescence; CCA, co-culture assay

Table 2.2 Secondary antibodies used in this study.

Cell type	Source	Dilution factor	Catalogue number
Anti-mouse IgG (whole molecule) alkaline phosphatase conjugate antibody	Sigma-Aldrich (Poole, U.K.)	250	A3688
Anti-goat IgG (whole molecule)-peroxidase antibody (from rabbit)	Sigma-Aldrich (Poole, U.K.)	10000	A5420
Anti-rabbit IgG, horseradish peroxidise (HRP)-linked whole antibody (from donkey)	GE Healthcare (Amersham, U.K.)	5000	NA934
Anti-mouse IgG, HRP-linked whole antibody (from sheep)	GE Healthcare (Amersham, U.K.)	5000	NA931
Alexa Fluor® 568 goat anti-mouse IgG	Molecular Probes Europe BV (Leiden, The Netherlands)	200	A11031

WB, Western blotting; IF, immuofluorescence; CCA, co-culture assay

2.1.3 Bacterial strains and bacterial media

The TOP10 strain of *Escherichia coli* (*E. coli*), purchased from Invitrogen (Paisley, U.K.), was used for plasmid propagation. Luria-Bertani (LB) medium was prepared by adding 5 g yeast extract, 5 g NaCl, 10 g of tryptone and 1 ml of 1 M NaOH to 1 l of ddH_2O, and autoclaving for 15 min at 120 °C in a Boxer 200/200L autoclave (Boxer Laboratory Equipment Ltd., Ware, U.K.). LB agar was prepared by adding 15 g of agar, 5 g yeast extract, 5 g NaCl, 10 g of tryptone and 1 ml of 1 M NaOH to 1 l of ddH_2O. For ampicillin resistance, ampicillin solution (Sigma-Aldrich) was added to a final concentration of 50 µg/ml when the temperature of the LB agar was below 60 °C.

2.1.4 Cell lines

Table 2.3 Cell lines used in this study.

Name	Cell type	Source
HDMEC	Human dermal microvascular endothelial cells (Lot-No. 5090501.1).	PromoCell (Heidelberg, Germany).
NHDF	Normal human dermal fibroblasts.	PromoCell (Heidelberg, Germany).
HEK 293 cells	Human embryonic kidney cells	Dr. Jürgen Müller, NWCRF Institute, Bangor University, Bangor, Gwynedd
PAE cells	Porcine aortic endothelial cells.	Dr. Nader Rahimi, Boston University, School of Medicine, Departments of Opthalmology & Biochemistry, Boston, Massachusetts 02118, U.S.A.
PAE/CKR/Flk-1 wt cells	PAE cells stably expressing a chimeric receptor containing the extracellular domain of human CSF-1R/c-fms, fused with the transmembrane and cytoplasmic domains of murine VEGFR-2 (Flk-1).	Dr. Nader Rahimi, Boston University, School of Medicine, Departments of Opthalmology & Biochemistry, Boston, Massachusetts 02118, U.S.A.
PAE/CKR/Flk-1 Y1173F cells	PAE cells stably expressing a chimeric receptor containing the extracellular domain of human CSF-1R/c-fms, fused with the transmembrane and cytoplasmic domains of murine VEGFR-2 (Flk-1), with a point mutation at Tyr^{1173} to give Phe^{1173} in the cytoplasmic domain of Flk-1.	Dr. Nader Rahimi, Boston University, School of Medicine, Departments of Opthalmology & Biochemistry, Boston, Massachusetts 02118, U.S.A.

2.1.5 Cell culture media and cell culture solutions

Table 2.4 Composition of cell culture media.

Name	Composition	Source
EBM MV2 basal medium containing 1% (v/v) FCS	EBM MV2 basal medium supplemented with 1% (v/v) foetal calf serum (FCS), (Cat. No.: C-22221).	PromoCell (Heidelberg, Germany).
Fibroblast growth medium	Fibroblast growth medium supplemented with 1.0 ng/ml bFGF and 5.0 µg/ml insulin, (Cat. No.: C-23110).	PromoCell (Heidelberg, Germany).
EBM MV2 growth medium	EBM MV2 basal medium supplemented with 5% (v/v) foetal calf serum (FCS), EGF (5.0 ng/ml), hydrocortisone (0.2 µg/ml), VEGF (0.5 ng/ml), FGF-2 (10.0 ng/ml), insulin-like growth factor-1 (20.0 ng/ml) and ascorbic acid (1.0 µg/ml), (Cat. No.: C-22121).	PromoCell (Heidelberg, Germany).
F-12 nutrient mixture (Ham) (1X), liquid	Containing GlutaMAX™ I, (Cat. No.: 31765-027).	GIBCO (Paisley, U.K.).
D-MEM	Dulbecco's modified Eagle medium (D-MEM) containing 4500 mg/l D-glucose, L-glutamine, and 25 mM HEPES buffer, without sodium pyruvate, (Cat. No.: 42430-025).	GIBCO (Paisley, UK).
OptiMEM® I reduced serum medium (1X), liquid - with GlutaMAX™	Containing GlutaMAX™ I, 2400 mg/l sodium bicarbonate, HEPES, sodium pyruvate, hypoxanthine, thymidine, trace elements, growth factors, 1.1 mg/l phenol red, (Cat. No.: 51985-026).	GIBCO (Paisley, U.K.).
Serum-free medium	EBM MV2 basal medium with no added growth factors or serum, (Cat. No.: C-22221).	PromoCell (Heidelberg, Germany).

Table 2.5 Composition of cell culture solutions.

Solution	Composition	Source
0.05% Trypsin-EDTA solution	Trypsin (0.05%), EDTA•4Na (0.53 mM) in PBS, pH 7.4.	GIBCO (Paisley, U.K.).
1% (w/v) gelatin solution	1% (w/v) gelatin from porcine skin, (type A, cell culture tested) in ddH_2O, autoclaved and sterile-filtered.	Sigma-Aldrich (Poole, U.K.).
Dulbecco's phosphate-buffered saline (with Ca^{2+}/Mg^{2+})	$CaCl_2 \bullet 2H_2O$ (130 mg/l), KCl (200 mg/l), KH_2PO_4 (200 mg/l), $MgCl_2 \bullet 6H_2O$ (100 mg/l), NaCl (8,000 mg/l), $Na_2HPO_4 \bullet 7H_2O$ (2,160 mg/l).	Lonza (Basel, Switzerland).
Dulbecco's phosphate-buffered saline (without Ca^{2+}/Mg^{2+})	KCl (200 mg/l), KH_2PO_4 (200 mg/l), NaCl (8,000 mg/l), $Na_2HPO_4 \bullet 7H_2O$ (2,160 mg/l).	Lonza (Basel, Switzerland).
Foetal bovine serum (FBS)	-	GIBCO (Paisley, U.K.).
GlutaMAX™-I supplement	L-alanyl-L-glutamine (200 mM) in 0.85% (w/v) NaCl solution.	GIBCO (Paisley, U.K.).
Heat-denatured 1% (w/v) BSA solution	1% (w/v) BSA (fraction V) in DPBS (without Ca^{2+}/Mg^{2+}) heated to 80 °C for 30 min.	Roche (Welwyn Garden City, U.K.).
HEPES buffer solution (1M)	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (238.3 g/l) in distilled water (pH 7.2 to 7.5).	GIBCO (Paisley, U.K.).
Human fibronectin	Purified human fibronectin (0.5 mg/ml) in 20 mM Tris/HCl, pH 7.0 containing NaCl (0.45 M) and 12% glycerol.	Invitrogen (Paisley, U.K.).
PureCol collagen	97% type I collagen (3 mg/ml), 3% type III collagen from bovine hide in 0.01 M HCl, pH 2.0.	Inamed Biomaterials (Leimuiden, The Netherlands).
Purified human vitronectin	Purified human vitronectin (0.5 mg/ml) in NaCl (0.15 M), HEPES (0.005 M), pH 7.4.	Invitrogen (Paisley, U.K.).
Sodium bicarbonate solution 7.5% (w/v)	Sodium bicarbonate in ddH_2O, 7.5% (w/v).	GIBCO (Paisley, U.K.).
UltraPure™ 0.5 M EDTA, pH 8.0	$Na_2EDTA \bullet 2H_2O$ in ddH_2O, pH 8.0.	GIBCO (Paisley, U.K.).
X10 Conc. Ham's F-12 w/o L-Gln, w/o sodium bicarbonate	-	PromoCell (Heidelberg, Germany).

2.1.6 Cell culture materials

Ten centimetre diameter polystyrene tissue-culture dishes, 6-, 12-, 24- and 96-well cell-culture plates, black-walled 96-well cell culture plates, 96-well polypropylene PCR microplates, centrifuge tubes, cell scrapers, cryovials, microfuge tubes, filter pipette tips and serological pipettes were bought from Greiner Bio-One (Stonehouse, U.K.).

2.1.7 Deoxyribonucleic acid (DNA)

2.1.7.1 Plasmid constructs

Table 2.6 Plasmid constructs used in this study.

Plasmid	Description	Source
pcDNA3.1-ERK5	pcDNA3.1 mammalian expression vector containing human wild-type *ERK5* cDNA, and a FLAG epitope tag at the N-terminus (Kato *et al.*, 1997).	Prof. J.-D. Lee, The Scripps Research Institute, La Jolla, CA, U.S.A.
pcDNA3.1-ERK5(AEF)	pcDNA3.1 mammalian expression vector containing a dominant-negative human *ERK5* cDNA in which the TEY dual phosphorylation site was mutated to AEF, with a FLAG epitope tag added at the N-terminal (Kato *et al.*, 1997).	Prof. J.-D. Lee, The Scripps Research Institute, La Jolla, CA, U.S.A.
pCMV5-MEK5(D)	pCMV5 mammalian expression vector containing a constitutively active rat *Mek5α-1* cDNA in which the phosphorylation sites Ser^{313} and Thr^{317} have been replaced with Asp, and 3 copies of the HA epitope added at the C-terminus (Kato *et al.*, 1997).	Prof. J.-D. Lee, The Scripps Research Institute, La Jolla, CA, U.S.A.
pcDNA3.1-MEK5(D)	pcDNA3.1 mammalian expression vector containing constitutively active rat *Mek5α-1* cDNA in which the phosphorylation sites Ser^{313} and Thr^{317} have been replaced with Asp, and 3 copies of the HA-epitope at the C-terminus, restriction digested from pCMV5-MEK5(D) with *Xba* I and *Kpn* I subcloned into pcDNA3.1 (Invitrogen).	This study.

2.1.7.2 Oligonucleotide primers

Sequences of each oligonucleotide primer pair used in this study are provided in **Appendix 1**. Unless stated otherwise, primers were purchased from Invitrogen (Paisley, U.K.), and were designed using the Invitrogen OligoPerfect™ designer facility: (https://www.invitrogen.com/content.cfm?pageID=9716), using the following parameters: Primer size (bases): min 18, opt 20, max 27. Primer melting temperature (T_m(°C)): min 57, opt 60, max 63. Primer GC content (%GC): min 40, opt 50, max 60. Product size (bp): min 100, max 150. Salt conc.: 50 mM. Primer conc.: 50 nM. The published sequence of each gene of interest was used for primer design in each case (http://www.ncbi.nlm.nih.gov/sites/entrez?db=Nucleotide). Following primer design, the specificity of each primer was tested by performing a basic local alignment search tool (BLAST) search (http://www.ncbi.nlm.nih.gov/BLAST) using the Blastn facility with the following parameters: Word size: 7, Expect Value: 1000, and with the low complexity filter switched off.

Handling of oligonucleotide primers

Oligonucleotide primers were re-constituted to 100 µM using Tris-EDTA (TE) buffer solution (pH 8.0). Qiagen primers were re-constituted to 100 µM by adding 55 µl of TE buffer solution. Primers were incubated at room temperature (r.t.) for 2 min to allow re-hydration to occur, vortexed for 15 s and stored at -80 °C. Aliquots of the re-constituted primers were diluted 40-fold in sterile, RNase-free ddH_2O (Sigma-Aldrich), to a final concentration of 2.5 µM prior to use in qRT-PCR reactions.

2.2 Methods

2.2.1 Manipulation of bacterial cells

2.2.1.1 Bacterial cultures

TOP10 *E. coli* were grown, as colonies at 37 °C on LB agar plates, or as cultures in LB medium at 37 °C in an orbital shaker incubator (SANYO E&E Europe BV, Loughborough, U.K.).

2.2.1.2 Preparation of competent cells

A single colony of TOP10 *E. coli* was picked from a LB agar plate without antibiotics, and used to inoculate 5 ml LB medium prior to incubation with moderate shaking (250 r.p.m.) in an orbital shaker at 37 °C. Following 20 h incubation, the optical density (OD) at 590 nm (OD_{590}) of the culture was measured using a SP *BIO* spectrophotometer (Sanyo-Gallenkamp, Leicester, U.K.). Having reached an OD_{590} of 0.02, 2 ml of the culture was used to inoculate 400 ml of LB medium in a sterile 2 l conical flask and incubated at 37 °C with moderate shaking (250 r.p.m.) in an orbital shaker for 2 h until cells had grown to mid log-phase (OD_{590} = 0.375). The culture was then chilled on ice, aliquotted into sterile 50 ml centrifuge tubes and left on ice for a further 10 min. Cells were centrifuged at 3,000 r.p.m. for 7 min at 4 °C in a Sorvall® Legend *T* centrifuge (Kendro Laboratory Products, Stortford, U.K.). The supernatant was discarded and each pellet was re-suspended in 10 ml ice-cold $CaCl_2$ solution (60 mM $CaCl_2$, 15% (v/v) glycerol, 10 mM PIPES, pH 7.0). Resuspended cells were centrifuged at 2500 r.p.m. for 5 min at 4 °C, and the supernatant was discarded, and the pellet re-suspended in 10 ml of ice-cold $CaCl_2$ solution, and incubated on ice for 30 min. Re-suspended cells were

then centrifuged again at 2500 r.p.m. for 5 min at 4 °C. The supernatant was discarded and each pellet was re-suspended in 2 ml of ice-cold $CaCl_2$ solution. Cell suspensions were aliquotted into 500 µl aliquots in sterile cryovials and used for transformation, or transferred for long-term storage in the -80 °C freezer.

2.2.1.3 Transformation of competent bacteria

Chemically-competent TOP10 *E. coli* were transformed with plasmid DNA or ligation reaction products using the heat-shock method. After incubation on ice for 30 min, cells were heat-shocked at 42 °C for 30 s and then rapidly cooled on ice for 2 min. Two hundred and fifty microlitres of LB media was added, to the cells, and the suspension was incubated in an orbital shaker for 1 h at 37 °C. For selection purposes, cell suspensions were plated on LB agar plates containing ampicillin, and incubated for 20 h at 37 °C.

2.2.2 Manipulation of mammalian cells

2.2.2.1 Cell culture techniques

Sterile conditions were maintained at all times during culturing and manipulation of mammalian cells by wearing nitrile examination gloves, using sterile cell culture equipment and working in a Kojair KR BioWizard 130 Class II laminar flow cabinet (Kojair Tech Oy, Vilppula, Finland). Culture medium was warmed in a water bath to 37 °C prior to use. All work surfaces were disinfected by wiping with 70% (v/v) ethanol before and after cell culture work.

2.2.2.2 Gelatin-coating of cell culture plates and dishes

All cell lines were routinely cultured on sterile, 10 cm diameter polystyrene cell culture dishes. Dishes were coated with 5 ml of autoclaved and sterile-filtered gelatin solution (1% (w/v) in ddH_2O), and incubated at 37 °C in a humidified, 5% (v/v) CO_2, atmosphere in a Sanyo MCO-17AC incubator (SANYO E&E Europe BV, Loughborough, U.K.) for 30 min prior to use. Gelatin was aspirated from the dishes prior to the plating of cells.

2.2.2.3 Thawing of cryopreserved cell stocks

Cryopreserved cells stored at -135 °C were thawed in a 37 °C water bath for 2-3 min with gentle agitation. The exterior of each cryovial was carefully disinfected with 70% (v/v) ethanol prior to pipetting the contents of the vial into to a gelatinized cell culture dish containing 9 ml of cell culture medium which had been pre-warmed at 37 °C. Dishes were then incubated for 24 h at 37 °C, after which time the cell culture medium was removed and replaced with 10 ml of fresh cell culture medium.

2.2.2.4 Passaging of cells and routine cell culture

Following incubation at 37 °C for 2 days in the appropriate cell growth culture medium, to expand cell numbers, cells were briefly washed with 10 ml of DPBS (without Ca^{2+}/Mg^{2+}) per 10 cm dish. DPBS was then aspirated, and 1 ml of 0.05% Trypsin-EDTA was added. Dishes were returned to the incubator for 2 min and tapped gently to aid cell detachment. When 95-100% of the cells had detached, as judged by viewing under an inverted light microscope, cells were resuspended in the appropriate cell culture medium and split as described in **Table 2.7**. All cells were routinely grown on gelatin-coated 10 cm diameter dishes in 10 ml of the appropriate cell culture medium (**Table 2.4**).

Table 2.7 Routine culture conditions for cells used in this study.

Cell type	Cell culture medium used for routine culture	Ratio and frequency of cell splitting	*Passages* used for experiments (from-to)
HDMECs	EBM MV2 growth medium.	1:4, every 2 days	*p*4-*p*11
NHDFs	Fibroblast growth medium.	1:5, every 2 days	*p*4-*p*22
PAE cells	F-12 Nutrient Mixture (Ham) (1X), liquid containing 10% (v/v) FCS.	1:8, every 3 days	*p*1-*p*15
HEK 293 cells	D-MEM containing 10% (v/v) FBS.	1:8, every 2 days	*p*1-*p*20

2.2.2.5 Cell counting for experiments

Prior to use in experiments, cells growing on 10 cm dishes were trypsinised in 1 ml of 0.05% Trypsin-EDTA for 2 min. The trypsin was quenched by the addition of 3 ml of EBM MV2 growth medium. One hundred microlitres of the cell suspension was then pipetted onto a Neubauer Improved (0.0025 mm^2 and 0.100 mm depth) haemocytometer (Hecht-Assistent, Sondheim/Rhön, Germany) for counting.

2.2.2.6 Freezing of cell stocks

Cells were purchased cryopreserved at *p*2. Following their initial thawing, cells were passaged twice and prepared for freezing at *p*4, by trypsinisation and re-suspension at a density of 5.0 x 10^5 cells/ml in 1 ml of cell freezing medium (90% (v/v) FCS and 10% (v/v) DMSO). Resultant cell suspensions were aliquotted into sterile cryovials, and transferred to the -135 °C freezer for long-term storage.

2.2.2.7 Cell transfection

2.2.2.7.1 Transient transfection with plasmid DNA

TransPass™ HUVEC transfection reagent, consisting of a non-lipid cationic transfection reagent (HUVEC reagent component), and a replication-deficient adenovirus component (TransPass™ V) (New England Biolabs, Hitchin, U.K.), was used to transfect HDMECs (*p*5-*p*7) with plasmid DNA. Prior to transfection, cells were seeded on 12-well or 6-well gelatinised cell culture plates at the densities shown in **Table 2.8** and incubated at 37 °C. Following 24 h incubation, cells were placed in fresh EBM MV2 growth medium. Plasmid DNA was mixed with the appropriate volume of serum-free OptiMEM® medium as indicated in **Table 2.8**, in a sterile cryovial. HUVEC reagent component was equilibrated to r.t., and was mixed by gently flicking the tube, prior to addition of the appropriate volume to the OptiMEM®/plasmid DNA mixture (**Table 2.8**). TransPass V component was vortexed for 30 s, and the appropriate volume was added to the OptiMEM®/plasmid DNA/HUVEC reagent component mixture (**Table 2.8**). The transfection mixture was then mixed gently by flicking, and was incubated at r.t. Following 30 min incubation, the transfection complex mixture was added drop-wise to the cells, and the plate was swirled gently five times to ensure even distribution of the transfection complex components. Following 4 h incubation at 37 °C, cells were washed twice with 2 ml of DPBS (containing Ca^{2+}/Mg^{2+}), media was then replaced with the appropriate volume of EBM MV2 growth medium, and plates were returned to the incubator at 37 °C. Following 24 h incubation, cells were washed in 2 ml DPBS and the medium was replaced with the appropriate volume of EBM MV2 basal medium containing 1% (v/v) FCS, and incubated at 37 °C. Following 20 h incubation, cells were stimulated, or not, with VEGF (*section 2.2.2.8.1*) followed by cell lysis (*section 2.2.6.1*) or used in cell-based assays (*sections 2.2.7-2.2.12*).

Table 2.8 Experimental parameters for DNA transfection of HDMECs using TransPass™ HUVEC Transfection Reagent.

Culture plate type	Number of HDMECs seeded/well	Volume of EBM MV2 growth medium used/well (ml)	Amount of DNA in OptiMEM®	Volume of HUVEC reagent component in transfection complex mixture (µl)	Volume of TransPass™V reagent component in transfection complex mixture (µl)
12-well	4.0×10^4	1	1.25 µg in 100 µl	2	2
6-well	9.3×10^4	2	2.50 µg in 200 µl	4	4

2.2.2.7.2 Transfection with small interfering RNA (siRNA)

A list of all the siRNA duplexes used in this study is provided in **Appendix 2**. The conditions for transfection of HDMECs in various cell culture dishes are provided in **Table 2.9**. Routine transfection of HDMECs was performed in 6-well cell culture dishes. HDMECs were seeded at 1.0×10^5 cells per well in gelatin-coated 6-well dishes in 2 ml of EBM MV2 growth medium and incubated at 37 °C. Following 24 h incubation, HDMECs were typically ~70% confluent and were transfected with siRNA and Lipofectamine™ RNAiMAX transfection reagent in a total volume of 2.5 ml OptiMEM® media. SiRNA stock solution was diluted to the required final concentration in 250 µl of OptiMEM®, and in a separate tube, Lipofectamine™ RNAiMAX transfection reagent was diluted to give a 0.1% (v/v) final concentration in 250 µl of OptiMEM®. The diluted Lipofectamine™ RNAiMAX and siRNA components were combined and mixed well by gentle pipetting, to give a 500 µl transfection mix, and incubated for 25 min at r.t. Media was aspirated from the 6-well dishes, and replaced with 2 ml of OptiMEM® medium. The transfection mix was added drop-wise to each well, and the plates were swirled gently to ensure even distribution of the siRNA. Cells were incubated with the transfection mix at 37 °C. Following 4 h incubation, cells were washed twice with 2 ml of DPBS (containing Ca^{2+}/Mg^{2+}), and the medium was replaced with 2 ml EBM MV2 growth medium. Following 24 h incubation at 37 °C, cells were washed in DPBS and serum starved in EBM MV2 basal medium containing 1% (v/v) FCS for 20 h prior to cell lysis (*section 2.2.6.1*) and Western blot analysis (*section 2.2.6.3*). Alternatively, following serum starvation, HDMECs were seeded for use in cell-based assays (*sections 2.2.7-2.2.12*).

Table 2.9 Summary of conditions used for siRNA transfection of HDMECs.

Culture vessel	Well diameter (mm)	Well area (mm^2)	Cell number for seeding	Total transfection volume (µl)
24-well plate	16	201.06	2.1×10^4	625
12-well plate	23	415.48	4.3×10^4	1250
6-well plate	35	962.11	1.0×10^5	2500
10 cm dish	100	7853.98	8.25×10^5	20400

2.2.2.8 Cell treatments and use of signal transduction inhibitors

2.2.2.8.1 Cell stimulation

Prior to the stimulation of cells with growth factors, chemicals or polar lipids, HDMECs were serum-starved in EBM MV2 basal medium containing 1% (v/v) FCS and no other supplements for 20 h at 37 °C. DPBS (without Ca^{2+}/Mg^{2+}) containing 0.1% (w/v) BSA was sterile filtered through a 0.22 µm bottle-top filter prior to use as a diluent to prepare a dilution of the growth factor, or chemical to be used for cell stimulation, X100-fold greater than that of the final concentration to be added to the cells. For cells growing in 6-well cell culture dishes that had been serum-starved in 2 ml of EBM MV2 basal medium containing 1% (v/v) FCS, 20 µl of this diluted cell agonist was added to give the required X1 final concentration of cell agonist. The final concentrations and the respective incubation times, for each growth factor and cell agonist used in this study are provided in the text.

2.2.2.8.2 Treatment with polar lipids

The cell-permeable polar lipids: 1,2-dioctanoyl-*sn*-glycerol (DOG), 1 ,2- dioctanoyl-*sn*-glycero-3-phosphate (DOPA) and 1,2-dioctanoyl-*sn*-glycero-3-phosphocholine (DOPC) (Avanti Polar Lipids Inc., Alabaster, AL, U.S.A.), were re-suspended in a 19:1 chloroform:methanol solution to 5 mg/ml. For use in experiments, the desired volume of polar lipid was removed with a microman positive-displacement pipette, and transferred to a sterile glass test tube under the fume hood. The chloroform solvent was removed from the polar lipid by blowing a slow stream of nitrogen gas over the chloroform solution using a Pasteur pipette until the chloroform had evaporated to dryness. Once the polar lipid was dry, EBM MV2 basal medium containing 1% (v/v) FCS and 0.1% (v/v) BSA was added to the test tube to give the desired concentration of lipid, and sonicated in a water bath sonicator (Lucas Dawe Ultrasonics Sonicleaner,

Dawe Instruments Ltd., London, U.K.) until no lipid droplets were visible. Finally, the solution was warmed to 37 °C in a water bath. Cells were treated for the indicated time with the polar lipid solution, or with EBM MV2 basal medium containing 1% (v/v) FCS and containing 0.1% BSA (v/v) in control experiments.

2.2.2.8.3 Use of signal transduction inhibitors

All signal transduction inhibitors were dissolved in sterile, hygroscopic DMSO to give a stock solution. Stock solutions were stored as single-use aliquots at -80 °C. Inhibitors were thawed immediately prior to use, and were diluted in cell culture medium. In control experiments where no inhibitor was present, the equivalent volume of DMSO was added to the cell culture medium as a vehicle control. The final concentrations of inhibitors used and their respective incubation times with the cells are indicated in the text. The final concentration of DMSO was less than 0.1% (v/v) in each case, a concentration at which no effects of DMSO were observed.

2.2.2.8.4 Short-wave ultraviolet treatment of cells

For treatment of cells with short-wave ultraviolet (UV) radiation, endothelial cell medium was aspirated and briefly replaced with 2 ml of DPBS (containing Ca^{2+}/Mg^{2+}). Cell culture plates were then placed immediately in a CL-1000 Ultraviolet Crosslinker (Ultra-Violet Products Ltd., Cambridge, U.K.) and subjected to a 60 J/m^2 dose of UV-C. Following UV-C treatment, the DPBS was immediately aspirated, and replaced with 2 ml of EBM MV2 basal medium containing 1% (v/v) FCS; cells were then incubated at 37 °C for 30 min.

2.2.3 Recombinant DNA methodology

2.2.3.1 Restriction endonuclease digestion of DNA

Restriction endonuclease digestions were performed with NEB restriction endonucleases (10U/µl) together with the compatible buffer according to NEB's guidelines. One microgram of vector DNA was incubated for 1 h at 37 °C with a total of 10 units of restriction enzyme(s), in the presence of 2 µl of 10x NEB buffer, and 2 µg of acetylated BSA, and sterile, nuclease-free ddH_2O to a final volume of 20 µl. When performing restriction digests on insert DNA, 10 µl of purified DNA was incubated for

16 h at 37 °C with 10 units of restriction enzyme, 2 µl of 10x NEB buffer, 20 µg of acetylated BSA, and sterile, nuclease-free ddH_2O to a final volume of 20 µl.

2.2.3.2 Agarose gel electrophoresis of DNA

For resolution of DNA restriction digests, 1% (w/v) agarose gels were used, whilst PCR products of primer testing for qRT-PCR were resolved on 3% (w/v) agarose gels. One percent and 3% (w/v) agarose gels were prepared by adding 1.2 g, or 3.6 g of agarose respectively, to 120 ml Tris-Borate EDTA (TBE) electrophoresis buffer (89 mM Tris-base, 89 mM boric acid, 2 µM Na_4EDTA) and heating the mixture in a microwave for approximately 2 min, until all the agarose had dissolved. The solution was allowed to cool for a few min, until hand-hot, prior to adding ethidium bromide to a final concentration of 1 µg/ml and careful mixing of the solution by swirling. The solution was then poured into a gel tank, and a 10- or 16-well comb was added, and was allowed to set.

PCR products or restriction digests were mixed with type I gel loading solution (bromphenol blue, 0.25% (w/v); xylene cyanole FF, 0.25% (w/v); sucrose, 40% (w/v) (Sigma-Aldrich), and 20 µl of each sample was loaded per well. In order to estimate the length of the DNA products resolved, 5 µl of either 1 kb (1% (w/v) gels) or 100 bp (3% (w/v) gels) DNA ladders were loaded, and run alongside the samples. DNA electrophoresis was routinely carried out at 120 V for 1 h, or until the bromophenol blue dye front had migrated to the end of the gel. Bands were viewed and photographed using a Bio-Rad Gel Doc 2000 UV transilluminator (Bio-Rad, Hemel Hempstead, U.K.).

Extraction of DNA from gels

For improved band resolution, electrophoresis was carried out at 80 V when extracting DNA. Bands were viewed on an UV light box on a Bio-Rad Gel Doc 2000 transilluminator. Restriction digest products were excised from the gel using a sterile razor blade, and excised products were purified using a Wizard® SV Gel and PCR clean-up system (Promega, Southampton, U.K.) according to the manufacturer's instructions,

and eluted in 50 µl of H_2O. The quality of excised samples was analysed by running 5 µl of each extracted sample on a 1% (w/v) agarose gel.

2.2.3.3 DNA ligation

DNA ligation reactions were prepared using 1 µl of T4 DNA ligase (1 U/µl), 5 µl of 2x rapid ligation buffer (NEB, Hitchin, U.K.), 1 µl vector and 3 µl insert. The reaction mixture was mixed gently, and incubated at r.t. for 15 min, after which 5 µl of the mixture was used to transform chemically competent TOP10 *E. coli*.

2.2.3.4 Mini-preparation of plasmid DNA

Small-scale preparations of plasmid DNA was isolated from *E. coli* cultures using a Sigma® GenElute™ plasmid miniprep kit (Sigma-Aldrich), according to the manufacturer's instructions. A single, isolated colony of *E. coli* grown on selective LB agar plates containing ampicillin (50 µg/ml) was used to inoculate 5 ml of LB medium containing ampicillin (50 µg/ml), and was grown for 20 h, at 37 °C in an orbital shaker incubator at 250 r.p.m. A 1 ml suspension of the culture was transferred to a microfuge tube, and cells were pelleted by centrifugation at 15,000 r.p.m. for 1 min. The supernatant was discarded, and the bacterial pellet was fully resuspended by vortexing in 200 µl of resuspension solution containing RNase A. Resuspended cells were lysed by the addition of 200 µl of lysis buffer, and inverting the tube gently six times. Cell debris was precipitated by adding 350 µl of neutralization/binding buffer, and inverting the tube gently six times. The precipitate was pelleted by centrifugation at 15,000 r.p.m. for 1 min. A GenElute™ miniprep binding column was placed in a collection tube, and equilibrated by the addition of 500 µl of column preparation solution followed by centrifugation at 15,000 r.p.m. for 1 min. The cleared bacterial cell lysate was then transferred to the binding column and centrifuged at 15,000 r.p.m. for 1 min. Seven hundred and fifty microlitres of wash solution containing ethanol was added to the binding column, and centrifuged at 15,000 r.p.m. for 1 min to remove residual salt from the column. After discarding the flow-through, the binding column was centrifuged again, at 15,000 r.p.m. for 2 min to remove excess ethanol. DNA was eluted by the addition of 100 µl of sterile, nuclease-free water, and centrifuging at 15,000 r.p.m. for 1 min.

2.2.3.5 Maxi-preparation of plasmid DNA

Endotoxin-free large-scale preparations of plasmid DNA were prepared by a modified alkaline lysis method using an EndoFree® plasmid purification kit (Qiagen, Crawley, U.K.) according to the manufacturer's instructions. Briefly, a single bacterial colony picked from a selective LB agar plate containing ampicillin (50 µg/ml) was used to inoculate a starter culture of 5 ml of LB medium containing ampicillin (50 µg/ml) and was grown for 8 h at 37 °C in an orbital shaker incubator at 250 r.p.m. The starter culture was then diluted 1:500 in 250 ml of LB medium containing ampicillin (50 µg/ml), and grown at 37 °C for 16 h in an orbital shaker incubator at 250 r.p.m. Bacterial cells were then harvested by centrifugation in a polypropylene wide-mouth bottle with screw-top cap assembly in a JLA-16.250 rotor in a Beckman Coulter Avanti® J-E centrifuge at 6,000 x *g* for 15 min at 4 °C (Beckman Coulter Ltd., High Wycombe, U.K.). The bacterial pellet was resuspended completely in 10 ml of Buffer P1 (50 mM Tris/HCl, pH 8.0; 10 mM EDTA) containing RNase A and LyseBlue by vortexing using a Vortex-Genie® (VWR, Lutterworth, U.K.) until no cell-clumps were visible. Cells were lysed by adding 10 ml of Buffer P2 (200 mM NaOH, 1% (w/v) SDS) to the re-suspended bacterial cells, mixing well by inversion until a homogenous blue-coloured suspension was achieved, and incubating at r.t. for 5 min. Ten ml of chilled buffer P3 (3.0 M potassium acetate, pH 5.5) was added to the lysate, and mixed well by vigorous inversion. The mixture was then transferred to a QIAfilter maxi cartridge and was incubated at r.t. for 10 min, to allow precipitation to occur, after which the precipitate was removed by allowing the lysate to pass through the QIAfilter maxi cartridge. A QIAGEN-tip 500 was equilibrated by the addition of 10 ml of Buffer QBT (750 mM NaCl; 50 mM MOPS, pH 7.0; 15% (v/v) isopropanol; 0.15% (v/v) Triton® X-100). The cleared lysate was added to the QIAGEN-tip 500 and was allowed to enter the resin by gravity flow. The QIAGEN-tip 500 was then washed twice with 30 ml of Buffer QC (1.0 M NaCl; 50 mM MOPS, pH 7.0; 15% (v/v) isopropanol). Plasmid DNA was eluted into an endotoxin-free 50 ml Beckman polyallomer screw-top bottle (Beckman Coulter Ltd., High Wycombe, U.K.) by the addition of 15 ml of Buffer QN (1.6 M NaCl; 50 mM MOPS, pH 7.0; 15% (v/v) isopropanol). DNA was then precipitated by the addition of 10.5 ml of isopropanol, mixing briefly and then centrifuging at 15,000 x *g* for 30 min at 4 °C in a JA-20 rotor in a Beckman Coulter Avanti® J-E centrifuge (Beckman Coulter Ltd., High

Wycombe, U.K.). The supernatant was carefully removed, and the DNA pellet was washed with 5 ml of endotoxin-free 70% (v/v) ethanol, followed by centrifuging at 15,000 x *g* for 30 min at 4 °C in a JA-20 rotor in a Beckman Coulter Avanti® J-E centrifuge. The pellet was air-dried for 10 min, and resuspended in nuclease-free ddH_2O.

2.2.3.6 Quantification of nucleic acid concentration and purity

The concentration of RNA and DNA samples was determined by spectrophotometric measurement of the absorbance at 230 nm (A_{230}) and 260 nm (A_{260}) respectively, using a NanoDrop ND-1000 spectrophotometer (Labtech International, Lewes, U.K.) and the associated ND-1000 software, version 3.3.0. DNA with an A_{260} /A_{280} ratio of between 1.6-1.8, and RNA with an A_{260} /A_{280} ratio of 1.8-2.0 was considered to be pure.

2.2.3.7 DNA sequencing

DNA sequencing primers were designed using the sequencing application on the OligoPerfect™ Designer (https://tools.invitrogen.com/content.cfm?pageid=9716). Primers were designed from accurate sequence data, as judged from well-formed peaks on sequence data chromatograms. Primers were designed to be 20-25 nucleotides in length, with a G-C content of 40-60%, and a T_m of 55-75 °C. Prior to ordering, the specificity of each primer was verified by performing a nucleotide BLAST search http://blast.ncbi.nlm.nih.gov/Blast.cgi. DNA sequencing primers were synthesized by Eurofins MWG Operon (London, U.K.). Two micrograms of each purified DNA sample was air-dried in a sterile, 1.5 ml microfuge tube, using a Jouan RC 10-22 speed vacuum centrifuge linked to a Jouan RCT 90 refrigerated cold trap (Jouan, Saint Herblain, France). Sealed tubes were then sent for sequencing using the "Value Read Tube" service provided by Eurofins MWG Operon (London, U.K.).

2.2.3.8 Polymerase chain reaction (PCR)

PCR reactions were carried out using GoTaq® Flexi DNA polymerase (Promega, Southampton, U.K.). A PCR master mix consisting of 4 µl 5X Colorless GoTaq® Flexi Buffer (Magnesium-free, proprietary formulation, pH 8.5), 2.4 µl of $MgCl_2$ (25 mM), 2 µl of dNTP mix (100 µM of each dNTP) (Promega, Southampton, U.K.), 5.8 µl sterile,

nuclease-free water (Sigma-Aldrich), and 0.2 µl GoTaq® Flexi DNA polymerase was prepared and was added to 250 nM of forward and 250 nM of reverse primers (*Appendix 1*), and 1.6 µl of cDNA (*section 2.2.4.2*) in a total volume of 20 µl in a sterile 200 µl PCR tube. Thermal cycling was carried out in a Techne TC-512 thermal cycler (Bibby Scientific, Stone, U.K.) with the following parameters: 95 °C for 10 min followed by 30 cycles of amplification at 95 °C for 15 s and 60 °C for 1 min.

2.2.4 Working with RNA

2.2.4.1 RNA extraction and DNase I treatment

All stages of the RNA extraction procedures were carried out using sterile, RNase-free plastic ware, filter-tip pipette tips, and RNase-free water. RNA samples were kept on ice at all times, to minimise the possibility of RNA degradation by RNases, and all work surfaces and nitrile examination gloves were treated with RNase*Zap*® RNase decontamination solution (Ambion Inc., Huntingdon, U.K.). Total RNA was extracted from cells using the RNeasy mini kit (Qiagen, Crawley, U.K.). Following treatment with siRNA or signal transduction inhibitors, and/or stimulation with agonists for the indicated times, cells in 6-well cell culture dishes were washed twice in DPBS (without Ca^{2+}/Mg^{2+}). DPBS was aspirated and 350 µl of Buffer RLT containing 1% (v/v) 2-mercaptoethanol was added to each well. Cells were harvested by scraping in Buffer RLT, and the resultant lysates were transferred into a QIAshredder spin column placed in a 2 ml centrifuge tube, and spun for 2 min at 15,000 r.p.m. in a Sanyo MSE HAWK 15/05 centrifuge (MSE, Lower Sydenham, U.K.). Two hundred and forty five microlitres, 70% (v/v), of ethanol USP/NF (200 proof) (Sigma-Aldrich) was added to each homogenised lysate, and mixed well by pipetting.

When lysing HDMECs sandwiched between two layers of collagen I gel in a 3-D collagen tube formation assay (*section 2.2.7.1*), 2.5 ml of Buffer RLT containing 1% (v/v) 2-mercaptoethanol was added to each well, and the collagen gel was allowed to dissolve at r.t. for 20 min. The lystate was then passed through a sterile, blunt, 20-gauge needle (0.9 mm diameter) fitted to a 20 ml RNase-free Luer-Lok™ syringe (BD Biosciences, Oxford, U.K.) five times, to ensure that no cell clumps remained. After

mixing, the homogenised lysate was collected in the syringe, the needle was removed, and the syringe was attached to a QIAshredder column. The lysate was then forced through the QIAshredder column to shear high-molecular-weight cellular components and genomic DNA, and the homogenate was collected in a sterile, RNase-free 50 ml centrifuge tube. The volume of the homogenised lysate was measured using a sterile 5 ml serological pipette, and 0.56X the measured volume, of ethanol USP/NF (200 proof), was added, and mixed well by gentle pipetting.

Samples, from both collagen and gelatin lysates, were then transferred into individual RNeasy spin columns, placed in 2 ml collection tubes and spun for 15 s at 15,000 r.p.m., the flow-through was discarded. As each RNeasy spin column could only hold approximately 700 µl of lysate, this procedure was repeated as many times as necessary to allow all of the homogenised lysate from the collagen-prepared samples to pass through the column. Each RNeasy spin column was then washed by adding 350 µl of Buffer RW1 to the column, and centrifuging for 15 s at 15,000 r.p.m., genomic DNA contamination was eliminated by performing an on-column DNase digestion step. DNase I stock solution was prepared by injecting 550 µl RNasefree water into the glass vial containing lyophilized DNase I (1500 Kunitz units) using a sterile, RNase free, syringe and needle. DNase I was allowed to dissolve for 2 min whilst gently inverting the sealed vial, the DNase I stock solution was then either used immediately, or stored for up to 9 months at -20 °C. Ten microlitres of DNase I stock solution was added to 70 µl Buffer RDD in a sterile, RNase-free microfuge tube, and the solution was mixed by gentle inversion. Seventy-seven microlitres of the DNase I incubation mix was added directly to the membrane of each RNeasy spin column, and the tube was incubated at r.t. for 15 min to allow complete digestion of any possible contaminating genomic DNA. Each column was then washed once more with 350 µl of Buffer RW1 for and centrifuged for 15 s at 15,000 r.p.m. RNA quality was assessed spectrophotometrically, as described (*section 2.2.3.6*).

2.2.4.2 Reverse transcription of mRNA

Total RNA (1 µg) was used for cDNA synthesis. Reverse transcription reactions were carried out in sterile, RNase- and DNase-free 0.2 ml PCR tubes. To each tube, 1µl of

Oligo $d(T)_{18}$ (Invitrogen Custom Primer, 500 µg/ml), 1 µg total RNA, 1 µl of dNTP mix (10 mM) and sterile, nuclease-free water (Sigma-Aldrich) to a final reaction volume of 12 µl, was added. The contents of each tube was mixed well by gentle pipetting, and tubes were heated to 65 °C for 5 min in a Techne TC-512 thermal cycler, and then placed on ice for 2 min to cool. A master mix containing 4 µl of 5x first strand buffer, 2 µl 0.1 M DTT and 1 µl RNaseOUT recombinant RNase Inhibitor per tube was prepared, and 7 µl of this master mix was added to each tube, mixed gently by pipetting and then incubated at 37 °C for 2 min in a Techne TC-512 thermal cycler. Reverse transcription was carried out by adding 1 µl of M-MLV reverse transcriptase to each tube, and incubating at 37 °C for 50 min, followed by a 15 min incubation at 70 °C to inactivate the reaction. As a negative control, to confirm that there was no genomic DNA contamination in the RNA samples, two reactions were set up, one containing M-MLV RT and the other containing 1 µl of sterile ddH_2O. Finally, each sample was diluted with 130 µl of ddH_2O and cDNA was stored at -80 °C, or used in quantitative real-time PCR (qRT-PCR) reactions immediately. Based on the assumption that reverse transcription reactions occurred with 100% efficiency, the cDNA concentration was assumed, from the quantification of the original RNA to be 6.67 ng/µl.

2.2.5 Quantitative real-time PCR (qRT-PCR)

Power SYBR® Green chemistry was used for analysis of gene expression in two-step qRT-PCR reactions. Power SYBR® Green contains a highly specific dye - SYBR® Green I - which fluoresces when bound to dsDNA. Reaction mixtures for qRT-PCR contained 1.5 µl (10 ng) cDNA template, 4.5 µl sterile, nuclease-free ddH_2O, 10 µl 2X Power SYBR® Green Mastermix (Applied Biosystems) and 250 nM of each of the forward and reverse primers in a final reaction volume of 20 µl. QRT-PCR reactions were performed in a 7900 HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, U.S.A.) with the following parameters: 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. All reagents were thoroughly mixed by vortexing prior to use. Following the loading of each 96-well PCR plate, plates were sealed with optically clear sealing film, and the plate was vortexed gently. Sealed plates were centrifuged for 1 min at 3,000 r.p.m. at 4 °C in a Beckman Coulter Allegra™ 25R

centrifuge (Beckman Coulter (U.K.) Ltd, High Wycombe, U.K.) prior to loading the plate into the RT-PCR machine.

Analysis of qRT-PCR data

Absolute quantification and relative quantification are the most commonly used methods to analyze data from qRT-PCR experiments (Livak & Schmittgen, 2001). Both methods of qRT-PCR data analysis involve the use of cycle threshold (C_T) values. C_T values can be defined as the number of cycles at which the amplification curve of a gene crosses a certain threshold of fluorescence. C_T values were recorded by SDS2.3 software linked to the 7900 HT Fast Real-Time PCR machine. When analysing C_T values, a threshold value of 0.2 was manually selected in each case.

The expression of an invariant endogenous control (housekeeping gene) can be used as an active reference to correct minor intra- and inter-assay variations (sample-to-sample and run-to-run variations respectively) (Pfaffl & Hageleit, 2001). In each case, the level of expression of the mRNA of interest was normalized to the expression level of *β-actin* mRNA, which was quantified in a separate set of reactions run in parallel with those used to determine the expression level of the gene of interest. Three identical replicates of each sample were included on each plate. C_T values for the mRNA of the gene of interest and for the *β-actin* control were recorded, and changes in gene expression were determined by either the comparative C_T ($2^{-\Delta\Delta C_T}$) method (Livak & Schmittgen, 2001) or the standard curve method (Morrison *et al.*, 1998).

2.2.5.1 Absolute quantification of samples using qRT-PCR

To quantify the effects of siRNA-induced gene silencing on gene expression, absolute quantification of samples was carried out using the standard curve method (Morrison *et al.*, 1998). A standard curve was prepared for the gene of interest and for the housekeeping gene (*β-actin*) in each case, by accurately preparing two-fold serial dilutions of the untransfected basal cDNA sample in sterile, nuclease-free ddH_2O (Sigma-Aldrich) to obtain six samples containing between 10 ng (undiluted) and 0.3125 ng of cDNA. Samples were loaded in triplicate, and run on the same 96-well qRT-PCR plate as the unknown samples. The mean C_T values of the gene of interest and the

reference gene from each sample were calculated, and were plotted on the Y-axes of two separate graphs, with the $\log_{10}$ amount of RNA in nanograms plotted on the X axis of each graph. The straight line equation y = mx + c (where y = C_T value, and x = $\log_{10}$ RNA) was then used to interpolate the amount mRNA in each sample. Gene expression was normalised to the untreated control sample in each case, by dividing the mean C_T value of the gene of interest with the mean C_T value of the housekeeping gene. The expression of the untreated control sample was arbitrarily set at 1, and the expression of the treated samples was calculated relative to the untreated calibrator by dividing the expression value with that of the calibrator sample.

2.2.5.2 Relative quantification of samples using qRT-PCR

In gene regulation experiments involving large numbers of samples, the relative expression of the gene of interest was determined by using the comparative C_T ($2^{-\Delta\Delta C_T}$) method (Livak & Schmittgen, 2001). Firstly, the mean, and standard deviation (SD), of three replicate samples was calculated. Secondly, the ΔC_T value of each sample was calculated by subtracting the C_T value of the target gene (gene of interest) from the C_T value for *β-actin*. Thirdly, the SD of the ΔC_T value ($SD\Delta C_T$) was calculated from the SD of the target and that of *β-actin* as shown below:

$$SD(\Delta C_T) = (SD_{target}^2 + SD_{\beta\text{-}actin}^2)^{1/2}$$

Fourthly, the $\Delta\Delta C_T$ value was then calculated by subtracting the ΔC_T of the test sample from the ΔC_T of the calibrator sample. Finally, the fold-change in gene expression was calculated by subtracting the $\Delta\Delta C_T$ value from 2. As the SD of the ΔC_T values is the same as the SD $\Delta\Delta C_T$ value in each case, this value was used to calculate the range of the fold-change in expression of the target relative to that of the *β-actin,* which was calculated as follows:

$$\text{From: } 2^{-(\Delta\Delta C_T) + (SD(\Delta\Delta C_T))} \text{ To: } 2^{-(\Delta\Delta C_T) - (SD(\Delta\Delta C_T))}$$

2.2.6 Working with proteins

2.2.6.1 Preparation of cell lysates

Unless stated otherwise (*section 2.2.6.1.2*), cell lysates were routinely prepared by placing cell culture plates on ice, prior to washing cells twice in ice-cold DPBS (without

Ca^{2+}/Mg^{2+}) and the addition of modified radio-immunoprecipitation assay (RIPA) lysis buffer (20 mM Tris/HCl, pH 7.5; 150 mM NaCl; 2.5 mM EDTA; 10% (v/v) glycerol; 1% (v/v) Triton-X-100; 1 mM Na_3VO_4; 10 µg/ml aprotinin; 10 µg/ml leupeptin and 1 mM PMSF) supplemented with SDS 0.1% (w/v), and sodium deoxycholate 0.5% (w/v). Cells were incubated on ice for 5 min, with gentle rocking to allow complete cell lysis. Lysed cells were then scraped and transferred into microfuge tubes and cleared of cell debris by centrifugation at 15,000 r.p.m. for 20 min at 4 °C in a Sanyo MSE HAWK 15/05 refrigerated centrifuge.

2.2.6.1.2 Preparation of cell lysates for detection of cleaved caspase-3

When lysing cells within 3-D collagen gels plated in 12-well plates, with the ultimate aim of detecting cleaved caspase-3 levels by SDS-PAGE, plates were placed on ice, prior to the removal of cell media and the apical layer of collagen by direct aspiration. Cells were then washed briefly in 500 µl ice-cold DPBS (without Ca^{2+}/Mg^{2+}), and 150 µl of 1X LDS buffer, containing 5% (v/v) 2-mercaptoethanol, was added per well, incubated at r.t. for 10 s, then swirled around the well three times to ensure complete lysis of the cells, before being pipetted into a clean microfuge tube placed on ice. Lysates were then sonicated on ice for 10 s using a Soniprep 150 (MSE) sonicator (Sanyo-Gallenkamp, Leicester, U.K.) at a wavelength of 7 µm to reduce sample viscosity and then heated for 5 min at 90 °C to heat-denature the proteins.

2.2.6.2 Cell fractionation

An assay adapted from Gliki *et al.* (2001) was used to assess PKC translocation from the cytosol to the membrane. Following treatment with growth factors in EBM MV2 basal medium containing 1% (v/v) FCS, cells were washed once in ice-cold DPBS, and then scraped in ice-cold homogenization buffer (20 mM Tris/HCl, pH 7.5; 5 mM EDTA; 0.1 mM PMSF; 10 mM aprotinin; 10 mM leupeptin; 0.3% (v/v) 2-mercaptoethanol). Cells were then sonicated on ice three times, for 5 s each time at 7 µm using a Soniprep 150 (MSE) sonicator. The homogenate was then centrifuged at 21,000 r.p.m. (55,000 x *g*) for 2 h at 4 °C. The supernatant was retained, and used as the cytosolic fraction. The pellet was re-suspended in homogenization buffer containing 0.5% (v/v) Triton-X-100, and mixed with end-over-end mixing for 30 min at 4 °C prior to

centrifuging again at 21,000 r.p.m. for 2 h at 4 °C. The supernatant was retained and used as the membranous fraction. Fractions were separated on an 8% SDS-PAGE gel (*section 2.2.6.3*), and analysed by immunoblotting.

2.2.6.3 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was routinely carried out using a Hoefer™ SE 400 vertical slab gel electrophoresis unit (GE Healthcare, Amersham, U.K.). The gel casting assembly consisted of two 16 cm x 18 cm glass plates separated by 1.5 mm plastic spacers. When two gels were to be run simultaneously, a glass divider plate was added between the two glass plates. Running gels were prepared as shown in **Table 2.10** below, and slowly pipetted into the gel casting assembly, until approximately 4.5 cm from the top of the glass plates. To eliminate air bubbles and ensure that an even edge to the top of the gel was achieved, 1 ml of isopropanol was added to the top of the unpolymerised gel, and was allowed to set. After 45 min, the isopropanol layer was drained away, and the stacking gel (**Table 2.10**) was carefully pipetted to the top of the polymerised running gel. At this point, a 15-well, 1.5 mm comb was inserted between the glass plates at the top of each gel un-polymerised stacking gel, and the gel was allowed to set for 45 min.

Table 2.10 Composition of SDS-PAGE gels.

	6% running gel	**8% running gel**	**10% running gel**	**Stacking gel**
Acrylamide solution (30% w/v)	7 ml	9.5 ml	11.5 ml	1.4 ml
Running gel buffer (2.0 M Tris/HCl pH 8.8, 0.4% (w/v) SDS)	7 ml	7 ml	7 ml	—
Stacking gel buffer (0.5 M Tris/HCl pH 6.8, 0.4% (w/v) SDS)	—	—	—	1.4 ml
Glycerol (87% v/v)	2.5 ml	2.5 ml	2.5 ml	—
ddH_2O (Milli-Q)	18.5 ml	16 ml	14 ml	7.2 ml
TEMED	17.5 µl	17.5 µl	17.5 µl	10 µl
APS (10% w/v in ddH_2O)	80 µl	80 µl	80 µl	50 µl

2.2.6.3.1 Electrophoresis

SDS-PAGE Tris-glycine tank buffer (25 mM Tris base, 0.192 M glycine, 0.1% (w/v) SDS, pH 8.3) was prepared as a 10X stock solution and diluted 1:10 in ddH_2O prior to use. The gel assembly was placed into the Hoefer™ SE 400 vertical slab gel electrophoresis

unit trough and the trough was filled with 500 ml of 1X SDS-PAGE Tris-glycine tank buffer. Combs were removed from each gel, and the wells were flushed with 1X Tank Buffer to remove un-polymerised acrylamide. Three microlitres of full-range (12-225 kDa) rainbow molecular weight markers was added to 67 µl of 1xLDS sample buffer, and loaded into the first well of each SDS-PAGE gel. Seventy microlitres of reduced total cell lysate in LDS sample buffer was loaded per well, any empty wells were loaded with 70 µl of 1X LDS sample buffer. Following the loading of all samples, the plastic trough was locked on top of the gel assembly and 500 ml of 1X SDS-PAGE Tris-glycine tank buffer was poured in. Finally, the apparatus was connected to a Consort E863 electrophoresis power supply (VWR, Lutterworth, U.K.) and run at 250 V, 9 mA, 10 W for 16 h. When running two gels simultaneously, the running parameters were changed accordingly to 250 V, 18 mA, 20 W for 16 h, or until the bromophenol blue dye front had migrated out of the bottom of the gel.

2.2.6.3.2 NuPAGE® Gels

Pre-cast, NuPAGE® 4-12% Bis-Tris 1.5 mm x 15-well or 12-well polyacrylamide gels (Invitrogen) were run in XCell *SureLock*™ Mini-Cell tanks in 1 l of 1X NuPAGE® MOPS SDS running buffer. The tank was connected to a PowerEase® 500 Power Supply and samples were resolved at a constant voltage of 180 V for 2 h.

2.2.6.4 Western blot analysis

Proteins resolved by SDS-PAGE were electrophoretically transferred onto Hybond ECL nitrocellulose membranes in 5 l of Towbin transfer buffer (120 mM Tris-base, 192 mM glycine, 20% (v/v) methanol, pH 8.6) in a Hoefer™ TE 62 Tank Transfer Unit (GE Healthcare, Amersham, U.K.). The unit was connected to a Grant Optima™ GD120-R2 pump refrigeration unit (Grant Instruments, Shepreth, U.K.) set at 7 °C. Gels to be transferred, together with nitrocellulose membrane, Whatman® 3MM CHR chromatography paper (VWR, Lutterworth, U.K.) measuring 11 x 15 cm and 3 mm transfer unit blotting sponges were pre-soaked in Towbin buffer for 10 min prior to assembling the gel-membrane sandwich as described in the manufacturer's instructions (http://www.hoeferinc.com/downloads/TE42-IMF0.pdf). The tank transfer unit was connected to an Apelex™ PS 202 Electrophoresis power supply, and

proteins were transferred at 200 V, 400 mA for 4 h. Transferred membranes were washed once with 25 ml of Tris-buffered saline (TBS) wash buffer (137 mM NaCl, 20 mM Tris-base/HCl, pH 7.6). To assess transfer efficiency, transferred proteins on nitrocellulose membranes were visualised by staining with Ponceau S as described previously (Salinovich & Montelaro, 1986). When necessary, individual lanes were cut from nitrocellulose membranes with a scalpel. Ponceau S stain was removed by washing twice in 10 ml of ddH_2O. Washed membranes were incubated in 25 ml of blocking buffer (5% (w/v) BSA in TBS containing 0.1% (v/v) Tween-20 (TBST)) for 1 h at r.t. to block non-specific antibody binding sites. Membranes were placed in polythene tubing (Polywell Industrial Co., Hong Kong), sealed using a Hulme-Martin heat sealer (Woking, U.K.), and 10 ml of the appropriate dilution of primary antibody (**Table 2.1**) in TBST containing 2% (w/v) BSA was poured into the bag. Membranes were incubated for 20 h with primary antibody in sealed bags at 4 °C with gentle agitation on a Stuart gyro-rocker SSL3 (Wolf Laboratories Ltd., York, U.K.). Following incubation with primary antibody, membranes were washed three times in TBST, with gentle rocking, for 10 min each. A horseradish peroxidise (HRP)-linked secondary antibody of the appropriate dilution (**Table 2.2**) in 10 ml of TBST containing 2% (w/v) BSA was added to each blot and sealed in polythene tubing, followed by incubation at 4 °C for 1.5 h with gentle agitation. Membranes were then washed twice, for 10 min each in TBST, followed by a single 10 min wash in TBS. Three millilitres of enhanced chemiluminescence (ECL) Western blotting detection reagent 1 was added to 3 ml of ECL Western blotting detection reagent 2, and was mixed well by vortexing. Membranes were immersed in 6 ml of the ECL reagent mix for 2 min, and then sealed in plastic, before exposure to Fuji Super RX film (Jet X-Ray, London, U.K.) in a sealed X-ray film cassette. X-ray films were developed in photosol RG developer and fixed in photosol RG fixer (Jet X-Ray) at 28 °C in a MI-5 X-ray film processor (Medical Index GmbH, Bad Rappenau, Germany).

2.2.6.4.1 Immunoblot analysis using NuPAGE® gels

Proteins resolved on NuPAGE® Novex gels were transferred onto Hybond ECL membranes in a XCell II™ Blot Module in 1X Novex® Tris-Glycine Transfer Buffer (12 mM Tris Base, 96 mM Glycine, 20% (v/v) methanol) for 2 h at a constant voltage of 25

V. Transferred blots were incubated for 1 h in 5% (w/v) BSA in 1X TBST, or alternatively (when blotting for cleaved caspase-3) membranes were incubated in 5% (w/v) non-fat dried milk in 1X TBST for 1 h with gentle rocking. Detection of cleaved caspase-3 was achieved using SuperSignal® West pico chemiluminescent substrate according to the manufacturer's instructions.

2.2.6.4.2 Densitometric quantification of protein expression/phosphorylation

Following immunoblotting with phospho-specific antibodies, and detection of the immunoreactive band on X-ray film, in cases where it was necessary to quantify the relative degree of activation of certain phosphorylated proteins, densitometric analysis was performed. Developed X-ray films, within the linear range of the film, were scanned using an Epson® Perfection 4490 photo scanner (Epson (U.K.) Ltd., Hemel Hempstead, U.K.), and the densitometric value of each band in the resulting image was quantified using National Institutes of Health (NIH) ImageJ, version 1.39 (http://rsbweb.nih.gov/ij/). To correct for protein loading, the corresponding actin band was also quantified. The untreated control sample in each case was arbitrarily assigned a value of 1.0, and the remaining bands were expressed relative to this value.

2.2.7 *In vitro* angiogenesis assays

2.2.7.1 3-D-collagen matrix tube formation assay

The 3-D collagen I matrix tubular morphogenesis assay was routinely set-up in 12-well cell-culture plates. Lower collagen layers were prepared 16-20 h prior to commencing the assay.

2.2.7.1.1 Preparation of the collagen mix

To prepare the collagen mix, 8 parts PureCol™ type I collagen (final concentration 2.4 mg/ml), 1 part sterile-filtered 0.1 M NaOH (final concentration 10 mM), 1 part X10 conc. Ham's F-12 medium without L-glutamine or sodium bicarbonate, 1:50 dilution of HEPES buffer solution (1 M) liquid (final concentration 20 mM), 1:64 dilution of sodium bicarbonate (7.5%) liquid (final concentration 0.117%), 1:100 dilution of GlutaMAX™-I supplement (200 mM) (final concentration 2 mM) were added under sterile conditions,

in that particular order, to a sterile 50 ml centrifuge tube, on ice. The collagen mix was then mixed by gentle pipetting, taking care to avoid the formation of air bubbles, to give a homogenous light-straw-coloured solution. Three hundred microlitres of the collagen mix was then added per well of 24-well plates by slowly pipetting the solution against the wall of each well. Plates were tapped gently, and the collagen solution was swirled to ensure an even coating of the bottom of each well, prior to allowing the collagen to set at 37 °C for 20 h.

2.2.7.1.2 Seeding of HDMECs

HDMECs were placed in EBM MV2 basal medium containing 1% (v/v) FCS for 20 h prior to seeding. In order to aid adhesion of the HDMECs to the collagen layer, 0.5 ml of serum-free EBM MV2 basal medium was added to each well for 10 min, which was then aspirated immediately prior to seeding the cells. Cells were seeded at the appropriate density in EBM MV2 basal medium containing 1% (v/v) FCS (**Table 2.11**), and placed in the incubator at 37 °C for 2 h.

Table 2.11 Collagen gel volumes and cell seeding conditions in the 3-D collagen gel tube formation assay.

Culture vessel	Well diameter (mm)	Number of HDMECs seeded/well	Volume of EBM MV2 basal medium containing 1% (v/v) FCS used for cell seeding (ml)	Volume of collagen in lower layer (µl)	Volume of collagen in upper layer (µl)
24-well	16	1.25×10^5	1.0	300	200
12-well	23	2.6×10^5	1.5	600	400
6-well	35	6.0×10^5	2.5	1500	1000

2.2.7.1.3 Preparation of the upper collagen layer, and stimulation of cells

At 2 h post-seeding, cells were viewed under an inverted light microscope to confirm that all cells had adhered to the lower collagen matrix. If the cells had adhered, the upper layer of collagen was prepared as described above (*section 2.2.7.1.1*). Prior to the addition of the upper collagen layer, all media was carefully removed from each well by aspirating to dryness by tipping the plate at 45° and aspirating the media from each well twice, without disturbing the lower collagen layer. The appropriate volume of collagen mix (**Table 2.11**) was then carefully pipetted against the side of the well, and the plate was swirled gently to ensure an even coating the cell monolayer with

collagen gel. Plates were incubated at 37 °C, and the upper layer of collagen was allowed to solidify for 1 h. After 1 h, 2X concentration of growth factors (and/or inhibitors) were prepared in EBM MV2 basal medium containing 1% (v/v) FCS, and 0.5 ml was pipetted gently against the side of each well so as not to disturb the upper collagen layer. Plates were returned to the incubator for 20-24 h, after which time tubes were visible. Tube-forming cells were then either lysed (*section 2.2.6.1.2*) or fixed (*section 2.2.7.1.5*).

2.2.7.1.4 Preparation of paraformaldehyde

Four grams of paraformaldehyde was dissolved in 90 ml of DPBS. The solution was heated to 60 °C with stirring on a heated hot plate in a fume hood. A few drops of 1 M NaOH were added to clear the solution (approximately pH 10.0). The solution was adjusted to pH 7.4 with HCl, and the volume was adjusted to 100 ml with DPBS. Finally, the solution was filter-sterilised through a 0.22 µm filter and stored at -20 °C until required.

2.2.7.1.5 Fixing and permeablising cells in 3-D collagen gels

HDMECs having undergone tubular morphogenesis in collagen gels were fixed in 0.5 ml of 4% (w/v) paraformaldehyde (*section 2.2.7.1.4*) in DPBS at r.t. for 20 min, washed twice in DPBS and permeablised with DPBS containing 0.2% (v/v) Triton-X-100 for 10 min at r.t. Plates containing fixed and permeablised cells in 1 ml of DPBS were then immunostained as described (*section 2.2.7.1.6*).

2.2.7.1.6 Immunostaining of F-actin fibres and nuclei in HDMECs within 3-D collagen gels

After fixing and permeablising the cells, collagen gel structures were washed once with 1 ml of DPBS and blocked with 500 µl blocking solution (2% (v/v) normal goat serum in DPBS) for 30 min. To visualise the F-actin fibres within the tube-forming cells, collagen gels were incubated with Alexa Fluor® 546 phalloidin diluted 1:50 in blocking solution for 45 min at r.t. in the dark with gentle rocking. Collagen gel structures were then washed 3 times with 1 ml of DPBS for five min each, in the dark with gentle rocking. To stain cell nuclei, Hoechst 33342 (2 µg/ml) was added during the first washing step.

2.2.7.1.7 Visualisation and quantification of tubes within 3-D collagen gels

HDMECs that had undergone tubular morphogenesis in collagen gels were examined and photographed using a Nikon Eclipse TE2000U inverted fluorescence microscope (Nikon (U.K.) Ltd., Kingston upon Thames, U.K.), attached to a Hamamatsu 1394 ORCA-285 camera (Hamamatsu Photonics U.K. Ltd., Welwyn Garden City, U.K.), operated using IPLab software (Becton-Dickinson Biosciences, Oxford, U.K.). Tubes were defined as structures containing four or more fused cells counted in five fields per well and in three wells per condition. Images were analysed using the NIH ImageJ software (version 1.39, http://rsb.info.nih.gov/ij/), as described previously (Matsumoto *et al.,* 2002).

2.2.7.2 HDMEC/NHDF co-culture *in vitro* angiogenesis assay

2.2.7.2.1 Normal NHDF/HDMEC co-culture

On the first day of the assay (day 1), NHDFs were seeded at 1.0 x 10^4 cells per well of a gelatin-coated 24-well plate in 1 ml fibroblast growth medium, and incubated for 3 days at 37 °C. After 3 days (day 4), the NHDF layer had grown to confluence. Media was aspirated, the NHDF layer was washed once in 1 ml of DPBS, and HDMECs were plated at 4.5 x 10^4 cells per well in 0.5 ml of EBM MV2 growth medium, and plates were returned to the incubator for a further two days. On day 6, the media was aspirated from each well, cells were washed once in DPBS, and 0.5 ml of EBM MV2 basal medium containing 1% (v/v) FCS, and containing growth factors and/or inhibitors was added to each well, and the plates were returned to the incubator for a further two days. On day 8, media was aspirated from each well, then co-cultures were washed gently with 1 ml of DPBS per well, and the media was replaced with fresh EBM MV2 basal medium containing 1% (v/v) FCS medium and containing growth factors and/or inhibitors. On day 10 of the assay, media was aspirated from the wells and the co-cultures were washed twice by gently pipetting 1 ml of DPBS against the side of each well. DPBS was aspirated and the cells were fixed in 1 ml of ice-cold 70% (v/v) ethanol per well and incubated at r.t. for 30 min.

2.2.7.2.2 Transfection of siRNAs in the HDMEC/NHDF co-culture assay

In certain experiments, the HDMEC/NHDF angiogenesis assay described above (*section 2.2.7.2.1*), was modified to incorporate the use of siRNA to silence gene-expression and assess the functional effects of this treatment on angiogenesis. Where indicated, HDMECs growing on 10 cm diameter gelatin-coated cell culture dishes were transfected with siRNA (*section 2.2.2.7.2*) on day 3, one day prior to seeding HDMECs on the NHDF monolayer. On day 5 of the assay, 24 h post-seeding of HDMECs onto the NHDF monolayer, the HDMEC/NHDF co-cultures were transfected, where indicated, with siRNA as described (*section 2.2.2.7.2*) in a total volume 1.25 ml of the transfection mixture (OptiMEM® medium containing 0.1% (v/v) Lipofectamine™ RNAiMAX, and the indicated concentration of siRNA). Cells were incubated with the transfection mixture at 37 °C for 4 h in OptiMEM® then washed twice with 1 ml of DPBS containing Ca^{2+}/Mg^{2+}. Finally, 0.5 ml of EBM MV2 growth medium was added to each well, and the plates were returned to the incubator at 37 °C for 24 h. In certain experiments, cells were also transfected with siRNA on days 7 and 9 of the assay, following the procedure described above, with the exception that following incubation with the transfection mixture for 4 h, cells were washed twice with 1 ml of DPBS containing Ca^{2+}/Mg^{2+}, and finally 0.5 ml of EBM MV2 basal medium containing 1% (v/v) FCS and containing growth factors and/or inhibitors was added to each well. Cells were fixed on day 10 of the assay, as described above (*section 2.2.7.2.1*).

2.2.7.2.3 Staining of HDMECs in HDMEC/NHDF co-cultures

After fixing the co-cultures, ethanol was poured from the plates, and plates were left face-down on blue laboratory roll (Wypall*, Kimberly-Clark, West Malling, U.K.) for 1 min, and tapped gently to remove residual ethanol. One millilitre of block buffer (1% (w/v) BSA in DPBS) was added to each well, and plates were incubated at 37°C for 30 min, after which time the block buffer was poured from the plates, and the plates were blotted gently on blue roll. Monoclonal mouse anti-human CD31, endothelial cell clone JC70A antibody was diluted 1:750 in block buffer, and 200 µl was added to each well and incubated at 37°C for 1 h. The primary antibody solution was poured from the plates, and plates were washed three times in 1 ml of block buffer, for five min each time. Two hundred and fifty microlitres of anti-mouse IgG (whole molecule)

alkaline phosphatase conjugate antibody in block buffer (1:200 dilution) was added to each well, and the plates were incubated at 37°C for 1 h. Secondary antibody dilution was poured from the plates prior to washing each well three times with ddH_2O. One SIGMA*FAST*™ BCIP®/NBT tablet was added to 10 ml ddH_2O, and was allowed to dissolve for 30 min prior to vortexing to give a homogenous yellow-coloured solution that acts as a substrate of alkaline phosphatase to form a purple-coloured precipitate. Two hundred and fifty microlitres of the BCIP®/NBT solution was added to each well of the co-culture plates, and plates were incubated at 37°C for 10 min, after which time a purple-coloured precipitate had formed in tubule-containing wells. The substrate solution was then poured from the plates, and plates were washed three times, for five min each, with 1 ml of ddH_2O per well. Plates were left to air-dry, and tubes were visualised, photographed and quantified as described below (*section 2.2.7.2.4*).

2.2.7.2.4 Image analysis and quantification of tube formation in HDMEC/NHDF co-cultures

Co-culture plates were analysed by photographing three, randomly selected fields of view, from triplicate wells per condition at low magnification (X 4 objective) using a Nikon Eclipse TS 100 inverted light microscope attached to a Nikon DS-Fi1-L2 digital camera (Nikon (U.K.) Ltd.). Images were saved as TIFF files, and total tube length (in pixels) was quantified using the AngioQuant programme (Niemisto *et al.*, 2005): (http://www.cs.tut.fi/sgn/csb/angioquant/) using the following parameters: kernel size = 1; and prune size = 10.

2.2.8 Cell proliferation assay

A commercially available CellTiter-Glo® luminescent cell viability kit (Promega, Southampton, U.K.), was used to determine the number of viable cells in culture. This assay utilises the luciferin-luciferase reaction to detect the amount of ATP present in cultured cells, which is directly correlated with the number of viable cells, and has been shown to be an accurate measure of cytokine-dependent proliferation in cultured cells (Crouch *et al.*, 1993). CellTiter-Glo® reagent lyses cell membranes to release ATP and inhibits endogenous ATPases, whilst also providing beetle luciferin and recombinant firefly luciferase to the reaction mixture. HDMECs were seeded at

1.5×10^4 cells per well in gelatin-coated 24-well plates in 0.5 ml EBM MV2 growth medium per well and incubated at 37 °C. After 24 h incubation, cells were washed in 1 ml of DPBS per well, and the medium was replaced with 0.5 ml EBM MV2 basal medium containing 1% (v/v) FCS. The following day, cells were stimulated, or not, with agonists, and cells were incubated for a further 72 h. Each condition was tested in triplicate. On the sixth day of the assay, a standard curve was prepared by seeding HDMECs, in triplicate wells, at densities of: 0; 1.0×10^4; 2.0×10^4; 4.0×10^4; 6.0×10^4; 8.0×10^4 and 1.0×10^5 cells per well in 0.5 ml of EBM MV2 growth medium. The cells were incubated at 37 °C for 4 h to allow cells to adhere. After 4 h incubation, CellTiter-Glo® buffer and CellTiter-Glo® substrate were equilibrated to r.t. prior to mixing the two components by vortexing for 1 min to provide CellTiter-Glo® reagent. Media was aspirated from the 24-well cell culture plates, and 200 µl of serum-free EBM MV2 basal medium was added to each well, followed by the addition of 200 µl of CellTiter-Glo® reagent to each well. Plates were shaken at 600 r.p.m. for 2 min on a Heidolph® Vibramax 100 (Heidolph U.K. – Radleys, Saffron Walden, U.K.) platform shaker to ensure complete mixing of the cells with the CellTiter-Glo® reagent. One hundred microlitre aliquots of the mixture from each well of a 24-well cell culture plate were then transferred, in triplicate, to wells of a black-walled 96-well plate. Luminescence was measured between 230-410 nm using the luminescence protocol on a Wallac Victor^2 1420 multilabel counter equipped with a D320 filter (Perkin Elmer Life Sciences, Fremont, CA, U.S.A.). The standard-curve of cell number was used to calculate the number of cells present in test samples.

2.2.9 Calcium measurements

Measurement of intracellular calcium $[Ca^{2+}]_i$ within HDMECs was conducted by Dr. Katherine Holmes (University of Liverpool). Briefly, HDMECs in 35 mm glass coverslip Iwaki culture dishes (Lennox Laboratory Supplies Ltd., Dublin, Ireland) were incubated at 37°C for 20 min with the cell-permeant acetoxymethyl esters of Fluo-4 (1.25 µM Fluo-4-AM) and Fura Red™ (1.25 µM Fura Red™-AM). Cells were then washed briefly with DPBS (without Ca^{2+}/Mg^{2+}), and changes in fluorescence intensity at 515 nm/660 nm was measured by time-lapse fluorescence confocal microscopy using a LSM510

laser scanning microscope (Carl Zeiss MicroImaging, Inc., Welwyn Garden City, U.K.) equipped with a F-FLUAR 40×/1.3 NA oil-immersion objective.

2.2.10 Scratch-wound healing cell migration assay

HDMECs were seeded at 2.0 x 10^5 cells per well on gelatinized 24-well ImageLock™ microplates (Essen Instruments, West Wickham, U.K.) in 1 ml of EBM MV2 growth medium, and incubated at 37 °C. After 24 h incubation, the medium was aspirated from the plates, and cells were washed once with 1 ml of DPBS. The medium was replaced with 1 ml of EBM MV2 basal medium containing 1 % (v/v) FCS, and the plates were incubated at 37 °C. After 24 h incubation, 10 µl Eppendorf epT.I.P.S. pipette tips, attached to an Essen Woundmaker™ (Essen Instruments, West Wickham, U.K.) were used to mechanically form a single scratch of uniform width, denuding the HDMEC monolayer across the centre of each well. The cell medium was then aspirated, and cells were washed twice with 1 ml of DPBS per well, to remove cell debris. One millilitre of EBM MV2 basal medium containing 1% (v/v) FCS, supplemented with mitomycin C (2.5 µg/ml) was added to each well, and the cells were stimulated, or not, with growth factors (*section 2.2.2.8.1*). Plates were then placed into the IncuCyte™ incubator (Essen Instruments, West Wickham, U.K.) at 37 °C, and incubated for 24 h. Three images of the centre of each well were captured at 2 h intervals for 24 h by time-lapse microphotography using a camera within the IncuCyte™ incubator to document the rate of migration. Images were analysed, and the wound confluence was calculated by IncuCyte™ software (GUI Version 20081.2.306.26250) (Essen Instruments, West Wickham, U.K.).

2.2.11 Cell adhesion assay

Stock solutions of vitronectin, fibronectin and collagen were diluted in DPBS (without Ca^{2+}/Mg^{2+}) to give 5 µg/ml fibronectin; 5 µg/ml vitronectin and 5 µg/ml type I collagen. Wells of black-walled 96-well plates were coated with 50 µl of each matrix, in triplicate for each condition to be tested, and plates were incubated at 37 °C for 20 h. HDMECs growing on 10 cm cell culture dishes, having been treated, or not, with siRNA, were serum-starved for 20 h in EBM MV2 basal medium containing 1% (v/v) FCS, and

stimulated with, or without, VEGF (50 ng/ml) for 4 h. After 20 h incubation, the various matrices were carefully aspirated from 96-well plates, and each well was coated with 50 µl of heat-denatured 1% (w/v) BSA solution in DPBS (without Ca^{2+}/Mg^{2+}) and incubated for 1 h at 37 °C, to block non-specific binding sites. Following incubation for 4 h with, or without VEGF, media was aspirated from the plates, cells were washed once in DPBS (without Ca^{2+}/Mg^{2+}), and 10 ml of warm OptiMEM® containing 5 µM of CellTracker™ Green CMFDA was added to each 10 cm plate, and incubated for 1 h at 37 °C. CellTracker™ Green CMFDA freely diffuses through the membranes of live cells, once inside the cell, the action of esterases hydrolyse the acetate groups on the non-fluorescent 5-chloromethylfluorescein diacetate molecule, to yield the fluorescent molecule 5-chloromethylfluorescein (excitation wavelength 492 nm/emission wavelength 535 nm). After 1 h incubation, heat-denatured 1% (w/v) BSA was aspirated from the wells. Media containing CellTracker™ Green CMFDA was aspirated from the cells, and cells were then washed once with 10 ml of DPBS (without Ca^{2+}/Mg^{2+}). Two millilitres of 0.5 mM EDTA in DPBS (without Ca^{2+}/Mg^{2+}) was added to each 10 cm dish and was then incubated at 37 °C for 5 min, followed by gentle tapping of the plates to aid cell detachment. HDMECs were re-suspended in EBM MV2 basal medium containing 1% (v/v) FCS, and seeded at 1.0 x 10^4 cells in 100 µl of medium per well. After 1 h incubation, plates were tapped gently to aid cell detachment. Media was aspirated from each test well, and three wells from each condition were left without aspirating media (control), to obtain a relative value of the total number of cells seeded in each case. Test wells were washed twice with 100 µl of DPBS, prior to the addition of 100 µl of EBM MV2 basal medium containing 1% (v/v) FCS to the washed wells. By recording the fluorescence of each sample using the 1 s fluorescein protocol on a $Victor^2$ 1420 multilabel counter equipped with F485 and F535 filters with a centre wavelength of 485 nm and 535 nm respectively (Perkin Elmer Life Sciences, Fremont, CA, U.S.A.). The relative number of attached cells was calculated by comparing the mean fluorescence value of each test well with the mean fluorescence value of the control samples in each case.

2.2.12 Apoptosis Assays

2.2.12.1 Annexin V assay

Early apoptosis in HDMECs undergoing tubular morphogenesis within collagen gels was detected using a method adapted from Dimberg *et al.* (2008). Cells treated with ERK5-specific siRNA or with non-silencing (N.S.) siRNA as described (*section 2.2.2.7.2*) and serum-starved for 20 h in EBM MV2 basal medium containing 1% (v/v) FCS were seeded on gelatin-coated 24-well plates or within 3-D collagen gels in 24-well plates, as described (*section 2.2.7.1*). Following stimulation with VEGF for 1 h, media was aspirated, and collagen gel structures or cells on a gelatin matrix were washed by adding 250 µl of DPBS per well, and incubating on ice for 20 min with gentle rocking. Thirty eight microlitres of annexin V, Alexa Fluor® 488 conjugate was diluted 33-fold in 1250 µl of annexin V binding buffer (10 mM HEPES; 140 mM NaCl; 2.5 mM $CaCl_2$, pH 7.4). One hundred and forty five microlitres of the solution was then added per well of a 24-well dish, and incubated for 2.5 h at 4 °C, in the dark with gentle rocking. Following incubation, cells were washed in 1 ml of DPBS (without Ca^{2+}/Mg^{2+}), containing 2 µg/ml of Hoechst 33342, at 4 °C, in the dark with gentle rocking for 10 min, to counterstain nuclei. Cells were then washed twice in 1 ml of DPBS (without Ca^{2+}/Mg^{2+}) for 10 min each at 4 °C, in the dark, with gentle rocking. Cells were then examined using a Nikon Eclipse TE2000U inverted fluorescence microscope (Nikon (U.K.) Ltd., Kingston upon Thames, U.K.), and six fields were randomly photographed using a Hamamatsu 1394 ORCA-285 camera (Hamamatsu Photonics U.K. Ltd., Welwyn Garden City, U.K.). Numbers of annexin V-positive cells, and total (Hoechst-positive) cells were determined using IPLabs quantification. The proportion of annexin V-positive cells was normalised to the N.S. siRNA-treated cells in each case.

2.2.12.2 Caspase-Glo® 3/7 assay

A luminescent assay was used to rapidly assess the relative activity of the executioner caspases, caspases-3/7, in HDMECs seeded on gelatin, or within 3-D collagen gels (*section 2.2.7.1*) in 24-well plates. Each condition to be tested was prepared in triplicate wells. Following treatment with siRNA and/or VEGF, media and the top layer of collagen was removed from the plates by direct aspiration. The cells were then

washed briefly in 1 ml of DPBS (containing Ca^{2+}/Mg^{2+}), prior to the addition of 200 µl of Caspase-Glo® 3/7 reagent (Promega) to each well, and incubation at r.t. with orbital shaking on a Heidolph® Vibramax 100 platform shaker (Heidolph U.K. – Radleys, Saffron Walden, U.K.) at 600 r.p.m. for 1 h to ensure complete lysis of the cells, and adequate mixing of the substrate with the cellular components. The Caspase-Glo® 3/7 reagent lyses the cells, and contains a luminogenic caspase-3/7 substrate containing the tetrapeptide sequence DEVD and a proprietary, thermostable, Ultra-Glo™ recombinant luciferase. Upon lysis of the cells, the DEVD sequence of the substrate - DEVD-6′-aminoluciferin - is cleaved by caspase-3/7 activity, thus releasing aminoluciferin, which results in the luciferase reaction, and the production of light. Following mixing of the lysates, 60 µl aliquots were transferred, in triplicate, from each well of the 24-well plates into separate wells of a black-walled 96-well plate. The 96-well plates were then placed in Wallac Victor^2 1420 multilabel counter (Perkin Elmer Life Sciences, Fremont, CA, U.S.A.) and the absorbance was determined using the luminescence protocol and a D320 filter (230-410 nm).

2.2.12.3 Immunofluorescence double-labelling of HDMEC/NHDF co-culture plates to detect cleaved caspase-3 positive cells

Co-cultures were fixed in ice-cold 70% (v/v) ethanol on day 10 of the assay, as described above (*section 2.2.7.2.1*). Five hundred microlitres of block buffer (5% (v/v) normal goat serum in DPBS containing 0.3% (v/v) Triton-X-100) was added per well and the plates were incubated at 37°C for 30 min. A 1:750 dilution of monoclonal mouse anti-human CD31, endothelial cell clone JC70A antibody, together with a 1:10 dilution of cleaved caspase-3 (Asp^{175}) antibody Alexa Fluor® 488 conjugate, was prepared in antibody dilution buffer (1% (w/v) BSA in DPBS containing 0.3% (v/v) Triton-X-100). One hundred and fifty microlitres of the antibody dilution was added to each well, and plates were incubated at 4 °C, in the dark for 16 h, with gentle rocking. Plates were washed three times with 1 ml DPBS per well for 5 min each at r.t. Alexa Fluor® 568 goat anti-mouse IgG was diluted 1:200 in antibody dilution buffer. One hundred and fifty microlitres of the antibody dilution was added to each well, and plates were left at r.t. for 2 h. After 2 h, each well was washed twice, for 5 min each, with ddH_2O. A final

1 ml of ddH_2O was then added per well, and plates were analysed using a Nikon TE2000 inverted fluorescence microscope.

2.2.13 Statistical analysis

Data are reported as mean ± SD. Statistical analysis was performed using SPSS software, version 16.0 (SPSS Inc., Chicago, U.S.A.). Two-tailed unpaired Student's *t*-test was used to compare the means of two independent groups. The non-parametric, two-tailed Mann-Whitney *U*-test was used to compare two groups where it could not be assumed that the data was normally distributed. Differences with *p* values less than or equal to 0.05 were considered statistically significant.

CHAPTER THREE

Characterisation of signalling pathways mediating ERK5 activation in HDMECs

3.1 Introduction

Targeted ablation of *Erk5* in murine endothelial cells results in death at E10.5, due to defects in vascular development and endothelial cell integrity (Hayashi *et al.*, 2004), suggesting that ERK5 plays a critical role in endothelial cell function (Roberts *et al.*, 2009). Studies aimed at defining the function of ERK5 in endothelial cells have hitherto been largely confined to the characterisation of the role of ERK5 in fluid shear-stress signalling (Yan *et al.*, 1999; Pi *et al.*, 2004; Akaike *et al.*, 2004; Parmar *et al.*, 2006). Whilst these studies have revealed that ERK5 plays a key role in maintaining vascular homeostasis in response to laminar blood flow, and have identified ERK5 as a key atheroprotective molecule, they have provided little insight into the potential role of ERK5 in angiogenesis.

It should be noted that the majority of studies aimed at investigating the role of ERK5 in endothelial cells have utilised endothelial cells of non-human origin including BAECs, BLMECs and MLCECs (Yan *et al.*, 1999; Pi *et al.*, 2004; Akaike *et al.*, 2004; Parmar *et al.*, 2006; Hayashi *et al.*, 2005; Li *et al.*, 2008). Published data documenting the role of ERK5 in human endothelial cells has been confined to the use of HUVECs (Abe *et al.*, 1996; Hayashi *et al.*, 2004; Hayashi *et al.*, 2005; Woo *et al.*, 2008). However, given the heterogeneity of endothelial cells originating from different vascular beds, the use of large-vessel endothelial cells such as HUVECs provide relatively little useful information regarding the importance of a given protein of interest in angiogenesis *per se* (Cines *et al.*, 1998; Conway & Carmeliet, 2004).

Angiogenesis is a process largely confined to the microvasculature; therefore the *in vitro* study of human endothelial cells of microvascular origin, as opposed to large vessel endothelial cells offers the most representative view of physiological events occurring during angiogenesis in humans *in vivo* (Cines *et al.*, 1998; Bouis *et al.*, 2001; Hewett, 2009). Hayashi *et al.* provided an intriguing insight into the potential role of ERK5 in mediating growth factor-stimulated angiogenesis with the finding that ERK5 is activated by the pro-angiogenic growth factors EGF, FGF-2 and VEGF in MLCECs and HUVECs (Hayashi *et al.*, 2004). Surprisingly however, there has been no attempt to

delineate the upstream pathways leading to ERK5 activation by these growth factors in endothelial cells.

This chapter describes experiments performed in an effort to characterise ERK5 activation in HDMECs, with the aim of defining the signalling pathways mediating pro-angiogenic growth factor-stimulated activation of ERK5 in these cells. Delineating the role of ERK5 in angiogenesis firstly required the identification of pro-angiogenic activators of the ERK5 pathway in HDMECs. Secondly, by utilising a range of specific pharmacologic inhibitors of key intracellular signalling molecules, the regulatory pathways upstream of ERK5 were broadly defined. Finally, siRNA technology was employed to further define the upstream regulators of ERK5 activation in these cells.

3.2 Characterisation of MAPK activity in HDMECs

3.2.1 Detection of ERK5 expression and activation in HDMECs

Early studies detailing ERK5 activation involved the use of a polyclonal anti-BMK1/ERK5 antibody, referred to hereinafter as anti-BMK1 (Abe *et al.*, 1996). During SDS-PAGE on a 6% gel, phosphorylated ERK5 exhibits reduced electrophoretic mobility, migrating slightly more slowly than non-phosphorylated ERK5. This phenomenon leads to a 'bandshift', with the appearance of a discrete band, located above the main ERK5 protein band when cells are resolved on a 6% SDS-PAGE gel, followed by immunoblotting with the anti-BMK-1 antibody (Abe *et al.*, 1997; Kato *et al.*, 1997; Kato *et al.*, 1998; Cavanaugh *et al.*, 2001; Hayashi *et al.*, 2004; Cavanaugh *et al.*, 2006). Using the same anti-BMK1 antibody (kindly donated by Prof. Jing-Dwan Lee, The Scripps Research Institute, La Jolla, CA, U.S.A.), this 'bandshift' assay was used as a positive control to confirm ERK5 activation in HDMECs.

Since VEGF and FGF-2 had been reported to induce ERK5 activation in HUVECs and MLCECs using the anti-BMK1 antibody (Hayashi *et al.*, 2004), this antibody was used as a positive control to determine whether ERK5 activation was induced by VEGF- or FGF-2-stimulation in HDMECs. ERK1 and ERK2 are also known to be activated by VEGF and FGF-2 in various endothelial cells (D'Angelo *et al.*, 1995; Kroll & Waltenberger, 1997; Klint *et al.*, 1999); therefore ERK1/2 phosphorylation was also assessed as a positive

control to determine whether these growth factors were biologically active. HDMECs were stimulated, or not, with the pro-angiogenic growth factors $VEGF_{165}$, $VEGF_{121}$ or FGF-2 for 10 min prior to cell lysis, and total cell lysates were separated on a 6% acrylamide SDS-PAGE gel. Western blot analysis using anti-BMK1 revealed a single major band resolving at approximately 110 kDa, which was apparent in all samples (**Fig. 3.1 A, *ERK5***). In the samples that had been stimulated with $VEGF_{165}$ or $VEGF_{121}$, but not in FGF-2-stimulated samples, a second, slightly more slowly-migrating band was apparent at approximately 115 kDa, suggesting that ERK5 is activated by VEGF, but not by FGF-2 in these cells (**Fig. 3.1 A, *p-ERK5***).

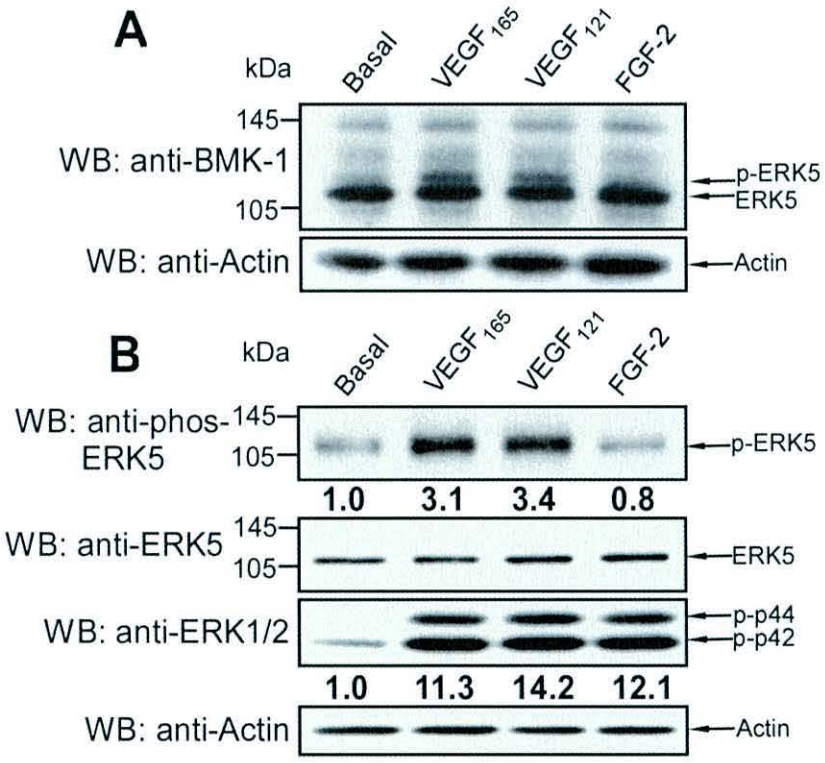


Figure 3.1 $VEGF_{121}$ and $VEGF_{165}$, but not FGF-2, induce ERK5 activation in HDMECs. (**A**) HDMECs seeded at 2.0×10^5 cells per well on gelatin-coated 6-well dishes were grown in EBM MV2 growth medium for 24 h prior to serum starvation in EBM MV2 basal medium containing 1% (v/v) FCS for 20 h. Cells were stimulated with $VEGF_{121}$ (50 ng/ml), $VEGF_{165}$ (50 ng/ml) or FGF-2 (50 ng/ml) for 10 min, or left unstimulated (Basal). Total cell lysates were separated on a 6% SDS-PAGE gel and subjected to Western blot (*WB*) analysis with anti-BMK-1 antibody or an antibody directed towards actin as a loading control, as indicated. (**B**) Identical cell lysates to those described in (*A*) above, were resolved on a 10% SDS-PAGE gel and were subjected to Western blot (*WB*) analysis with antibodies directed against phos-ERK5, ERK5 antibody and actin as a loading control, as indicated. Densitometric analysis of ERK5 and ERK1/2 relative to actin displayed beneath the respective lanes of each blot was performed using NIH ImageJ software, with the basal control set arbitrarily as 1.0. The result shown is representative of at least three independent experiments. *ERK5, extracellular signal-regulated kinase; FCS, foetal calf serum; FGF-2, fibroblast growth factor-2; HDMEC, human dermal microvascular endothelial cell; VEGF, vascular endothelial growth factor; WB, Western blot.*

Detection of ERK5 activation via dual-phosphorylation of the Thr^{218} /Tyr^{220} residues present in the T-E-Y motif of the ERK5 activation loop (Zhou *et al.*, 1995) has been made possible with the advent of phospho-specific ERK5 (p-ERK5) antibodies. A Thr^{218}/Tyr^{220}-specific p-ERK5 antibody produced by New England Biolabs (Hitchin, U.K.), has been used to detect ERK5 activation in response to a range of stimuli in a variety of cell lines (Mulloy *et al.*, 2003; Ishizawa *et al.*, 2005; Sharma & Goalstone, 2005; Morimoto *et al.*, 2007; Arkell *et al.*, 2008; Browne *et al.*, 2008; Gilley *et al.*, 2009; Tan *et al.*, 2009; Pazyra-Murphy *et al.*, 2009; Lennartsson *et al.*, 2010). This anti-phos-ERK5 Thr^{218}/Tyr^{220} antibody was used during Western blotting following 10% SDS-PAGE of identical lysates to those used in **Fig. 3.1 A**, to confirm whether the bandshift apparent on 6% SDS-PAGE gels was indicative of ERK5 activation. Indeed, as shown in **Fig 3.1 B**, both $VEGF_{165}$ and the non-heparin-binding VEGF isoform $VEGF_{121}$, induced a 3-fold increase in ERK5 phosphorylation compared to the unstimulated basal control. Stimulation with FGF-2 did not induce ERK5 phosphorylation on Thr^{218} /Tyr^{220} (**Fig. 3.1 B**), in agreement with the lack of ERK5 'bandshift' in the same lysates using the anti-BMK1 antibody (**Fig. 3.1 A**). Maximal doses of $VEGF_{121}$, $VEGF_{165}$ and FGF-2 elicited similar levels of ERK1/2 phosphorylation in HDMECs (**Fig. 3.1 B**), suggesting that all of these growth factors are biologically active in these cells. Western blot analysis of the same lysates following 10% SDS-PAGE, with a commercially-available anti-ERK5 antibody, revealed no change in the total amount of ERK5 present following treatment with these growth factors (**Fig 3.1 B**). Together, these results confirm that ERK5 activation can be detected using a commercially available anti-p-ERK5 antibody in HDMECs, and suggest that both $VEGF_{165}$ and $VEGF_{121}$ are activators of ERK5 in these cells, whereas FGF-2 is not.

3.2.2 Characterisation of ERK5 activity following cellular stress insults in HDMECs

ERK5 was originally identified as a stress-activated MAPK in several cell lines, including HUVECs (Abe *et al.*, 1996). To further characterise MAPK activity in HDMECs, and to test whether ERK5 is activated by cellular stresses in these cells, a stress agent screen was performed. HDMECs were exposed to 200 μM hydrogen peroxide (H_2O_2), for 5 min, as this treatment had been shown to induce ERK5 activation in HUVECs (Abe *et*

al., 1996). Hyperosmolar conditions had also been shown to activate ERK5, and other MAPKs in several cell lines (Kato *et al.*, 1997; Duzgun *et al.*, 2000; Wang *et al.*, 2006). In order to assess the impact of osmotic shock upon ERK5 activation in HDMECs, cells were incubated with NaCl (300 mM) for 30 min, conditions that had previously been shown to induce MAPK activation in endothelial cells (Duzgun *et al.*, 2000).

A cell-permeable, biologically active ceramide, N-Acetyl-D-sphingosine (C2 ceramide), was added to HDMECs for 60 min (100 µM), conditions that are known to mediate stress-induced apoptosis signals in HDMECs (Gupta *et al.*, 1999; Kolesnick & Fuks, 2003). As a positive control for ERK5 activation in HDMECs, cells were also treated with VEGF (50 ng/ml) for 10 min. Activation of MAPKs was assessed by 10% SDS-PAGE and Western blot analysis with phospho-specific antibodies directed against p-ERK5, p-ERK1/2, p-p38 MAPK and p-SAPK/JNK (**Fig. 3.2**).

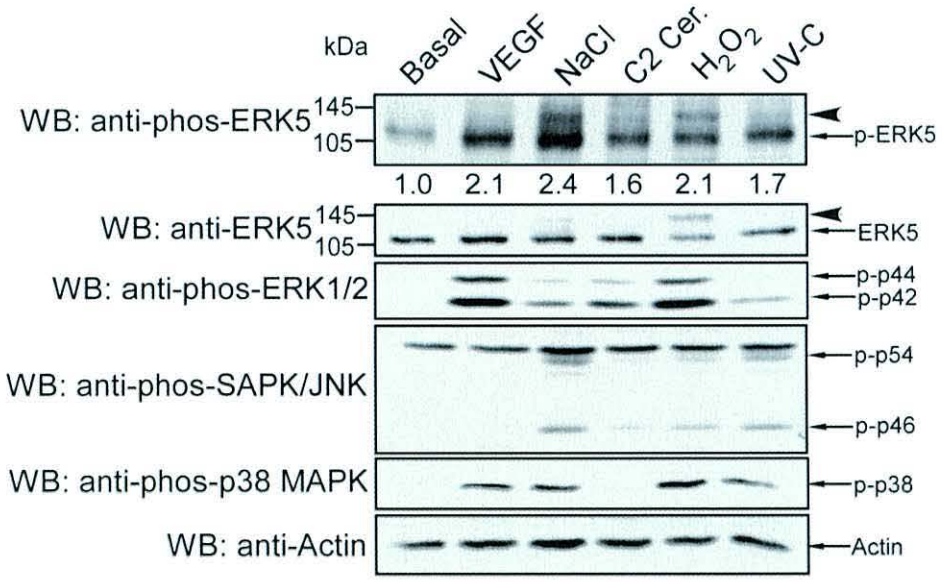


Figure 3.2 ERK5 is activated by stress stimuli in HDMECs. HDMECs were seeded at 2.0 x 10^5 cells per well on gelatin-coated 6-well dishes in EBM MV2 growth medium, and incubated for 24 h at 37 °C, then serum-starved in EBM MV2 basal medium containing 1% (v/v) FCS for 20 h prior to stimulation, or not (Basal), with VEGF (50 ng/ml) for 10 min; 300 mM NaCl for 30 min; 100 µM C2 Ceramide (C2 Cer.) for 60 min; 200 µM H_2O_2 for 5 min or subjected to a 60 J/m^2 dose of UV-C irradiation, followed by incubation at 37 °C for 30 min. Cells were lysed and total cell lysates were separated on a 10% SDS-PAGE gel and subjected to Western blot (*WB*) analysis with antibodies directed against ERK5, phos-ERK5, phos-ERK1/2, phos-p38 MAPK, phos-SAPK/JNK, and actin as a loading control, as indicated. Arrowheads indicate a slower-migrating phos-ERK5 band. The result shown is representative of two independent experiments. Densitometric analysis of ERK5 phosphorylation on Thr^{218}/Tyr^{220} relative to actin displayed beneath the p-ERK5 blot was performed using NIH ImageJ software, with the basal control set arbitrarily as 1.0. *C2 Cer., C2 Ceramide/N-acetyl-D-sphingosine; FCS, foetal calf serum; HDMEC: human dermal microvascular endothelial cell; ERK, extracellular signal-regulated kinase; SAPK/JNK, stress-activated protein kinase/c-Jun NH_2-terminal kinase; VEGF, vascular endothelial growth factor; UV-C, ultra violet C; WB, Western blot.*

Densitometric analysis showed that ERK5 was robustly activated by VEGF, NaCl and H_2O_2 (**Fig. 3.2, *p-ERK5***). Interestingly, Western blot analysis with an anti-ERK5 antibody revealed that treatment with H_2O_2, and to a lesser degree, NaCl, but not VEGF, induced a discrete anti-ERK5-immunoreactive band at approximately 140 kDa (**Fig. 3.2, *ERK5, arrowhead***) corresponding to the 'distinct band of higher molecular weight' first described by Abe and co-workers (Abe *et al.*, 1996). This band also cross-reacted with the anti-phos-ERK5 antibody (**Fig. 3.2, *p-ERK5, arrowhead***), thus showing that the higher molecular weight ERK5-immunoreactive band was also phosphorylated on the Thr^{218}/Tyr^{220} residues recognised by this antibody. It is suggested that the appearance of the ERK5-immunoreactive 140 kDa band, following exposure of HDMECs to oxidative and osmotic stresses, represents a hyperphosphorylated form of ERK5, arising from phosphorylation of other residues in addition to the Thr^{218}/Tyr^{220}. Indeed, it was recently demonstrated that phosphorylation of ERK5 on Thr^{218}/Tyr^{220} is required for autophosphorylation of residues Thr^{723}, Ser^{760}, Ser^{764} and Ser^{766} located in the transcriptional activation domain of the C-terminal tail, and that phosphorylation of these sites is necessary for the electrophoretic mobility shift of ERK5 to take place (Morimoto *et al.*, 2007). It would appear from these results that hyperphosphorylation of ERK5 only occurs in response to certain stimuli, and that phosphorylation of Thr^{218}/Tyr^{220} can occur without necessarily inducing ERK5 hyperphosphorylation.

Consonant with its role as a stress-activated protein kinase, SAPK/JNK was activated by each stress agent used in the screen, with osmotic-, and UV-induced stresses being the strongest activators of SAPK/JNK, in agreement with previous findings (Hibi *et al.*, 1993; Gupta *et al.*, 1995; Duzgun *et al.*, 2000). Both ERK1/2 (**Fig. 3.2, *ERK1/2***), and p38 MAPK (**Fig. 3.2, *p38 MAPK***) were activated by VEGF, as well as by H_2O_2 in accordance with previous reports (Rousseau *et al.*, 1997; Nguyen *et al.*, 2004). Together, these data serve to document the activation profile of various MAPKs in HDMECs, and confirm that ERK5 displays typical activity, becoming activated in response to stimulation with both mitogenic and stress-induced stimuli in these cells.

3.2.3 ERK5 is activated by a variety of growth factors and angiogenesis inducers in HDMECs

In order to assess the potential effects of other pro-angiogenic growth factors upon ERK5 activation in HDMECs, and to determine the profile of MAPK activation in response to stimulation with pro-angiogenic factors, an agonist screen was conducted. HDMECs were treated with pro-angiogenic growth factors (VEGF, FGF-2, EGF, HGF) and angiogenesis inducers (LPA, PMA), followed by cell lysis and analysis of MAPK activation by Western blotting with phospho-specific antibodies (**Fig. 3.3**). It was found that EGF, a potent activator of ERK5 activation in several cell types including HUVECs (Kato *et al.*, 1998; Kamakura *et al.*, 1999; Hayashi *et al.*, 2004; Kondoh *et al.*, 2006), did not induce ERK5 activation in HDMECs, despite its ability to activate ERK1/2 (**Fig. 3.3**). In addition to inducing the activation of ERK5 and ERK1/2, VEGF also stimulated p38 MAPK activity, but did not activate either the p54 or the p46 isoforms of SAPK/JNK (**Fig. 3.3**), in agreement with the reported response of SAPK/JNK to VEGF stimulation in HUVECs (Yu & Sato, 1999). FGF-2 induced ERK1/2 activation and weakly stimulated p38 MAPK activation, but did not induce activation of either ERK5 or SAPK/JNK (**Fig. 3.3**). Hepatocyte growth factor (HGF) was recently described as an activator of ERK5 in malignant mesothelioma cells (Ramos-Nino *et al.*, 2008). In addition, HGF is known to induce angiogenesis both *in vivo* and *in vitro* (Morishita *et al.*, 1999; Nakagami *et al.*, 2001). HGF has also been shown to stimulate ERK1/2 activation in human aortic endothelial cells (HAECs) *in vitro* (Endo *et al.*, 2003). In HDMECs, HGF stimulation induced ERK5 activation, as well as activating ERK1/2 and p38 MAPK (**Fig. 3.3**).

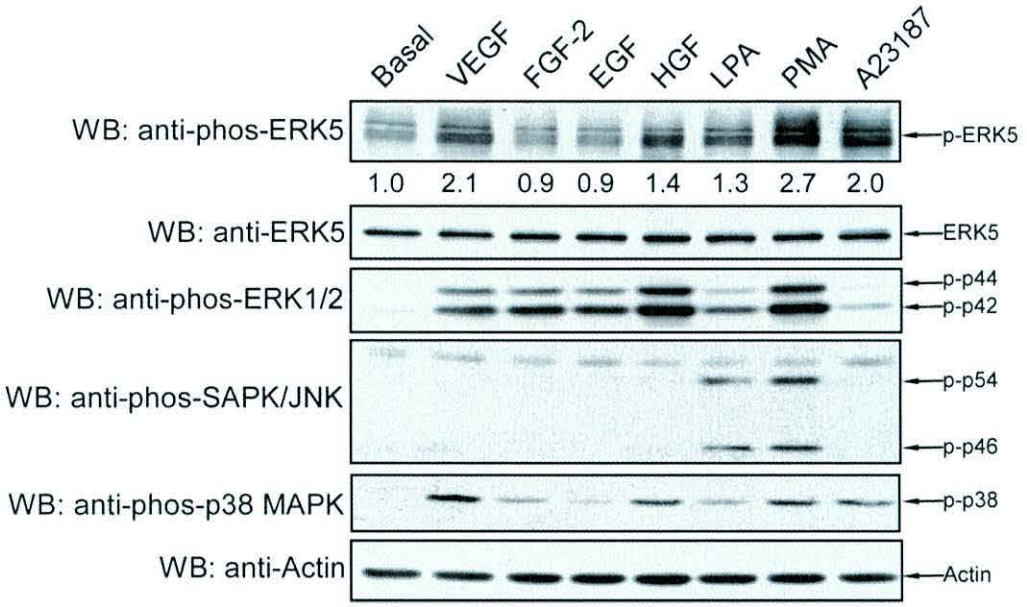


Figure 3.3 ERK5 activation in HDMECs is induced by pro-angiogenic growth factors and signalling modulators. HDMECs were seeded at 2.0 x 10^5 cells per well on gelatin-coated 6-well dishes in EBM MV2 growth medium and incubated for 24 h, then serum-starved in EBM MV2 basal medium containing 1% (v/v) FCS for 20 h prior to stimulation, or not (Basal), with 50 ng/ml VEGF; 50 ng/ml FGF-2; 50 ng/ml EGF; 50 ng/ml HGF or 10 μM LPA for 10 min each, or with 5 μM A23187 or 100 nM PMA for 20 min, as indicated. Cells were lysed and total cell lysates were separated on a 10% SDS-PAGE gel and subjected to Western blot (*WB*) analysis as described (*section 2.2.6.4*) with antibodies directed against ERK5, phos-ERK5, phos-ERK1/2, phos-p38 MAPK, phos-SAPK/JNK, and actin as a loading control, as indicated. Densitometric analysis of ERK5 phosphorylation on Thr^{218}/Tyr^{220} relative to actin displayed beneath the p-ERK5 blot was performed using NIH ImageJ software, with the basal control set arbitrarily as 1.0. The results shown are representative of five independent experiments. *EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; FCS, foetal calf serum; FGF-2, fibroblast growth factor-2; HDMEC, human dermal microvascular endothelial cell; HGF, hepatocyte growth factor; SAPK/JNK, stress-activated protein kinase/c-Jun NH_2-terminal kinase; LPA, lysophosphatidic acid; PMA, phorbol 12-myristate 13-acetate; VEGF, vascular endothelial growth factor; WB, Western blot.*

The lysophospholipid, 1-acyl-2-hydroxy-*sn*-glycero-3-phosphate, commonly referred to as lysophosphatidic acid (LPA), is an activator of G protein-coupled receptors (GPCRs) (Ishii *et al.*, 2004), and has been shown to induce angiogenesis *in vitro* (Rivera & Chun, 2008; Teo *et al.*, 2009). LPA has been reported to stimulate GPCR-mediated ERK5 activation in certain cell types, but has also been shown to antagonise growth factor-stimulated ERK5 activation in other cells (Obara & Nakahata, 2010). Treatment of HDMECs with LPA was sufficient to moderately stimulate activation of ERK5, SAPK/JNK, p38 MAPK and ERK1/2 (**Fig. 3.3**).

To test whether ERK5 activity was regulated by Ca^{2+}, HDMECs were treated with the calcium ionophore A23187 (Reed & Lardy, 1972). Interestingly, A23187 induced a 2-fold increase in ERK5 phosphorylation compared to the basal, unstimulated control (**Fig. 3.3**). By contrast, A23187 treatment had a negligible effect on p38 MAPK

activation and did not induce activation of either ERK1/2 or SAPK/JNK (**Fig. 3.3**). This finding suggested that ERK5 activation can be induced by elevated Ca^{2+} levels in HDMECs.

The phorbol ester, PMA (also known as 12-O-tetradecanoylphorbol-13-acetate, TPA), is an activator of DAG-dependent PKC isoforms (cPKCs and nPKCs) in a variety of cells, including endothelial cells (Liu & Heckman, 1998), and is a potent inducer of angiogenesis *in vitro* (Montesano & Orci, 1985; Taylor *et al.*, 2006; Xu *et al.*, 2008). Treatment with 100 nM PMA potently induced ERK5 phosphorylation by 2.7-fold compared to the unstimulated basal control (**Fig. 3.3**). PMA treatment also induced activation of ERK1/2, SAPK/JNK and p38 MAPK (**Fig. 3.3**). Together, these results revealed that ERK5 is activated by distinct pro-angiogenic growth factors and angiogenesis inducers in HDMECs, and suggest that ERK5 activation in these cells may be mediated by Ca^{2+}- and PKC-dependent pathways.

3.3 VEGF-induced activation of ERK5 is mediated by VEGFR-2

3.3.1 VEGF-induced ERK5 activation is dependent upon VEGFR-2 tyrosine kinase activity

Given the importance of VEGF as a pro-angiogenic molecule, efforts were focused upon attempting to delineate the upstream mechanisms mediating VEGF-induced ERK5 activation in HDMECs. VEGF exerts most of its biological effects via activation of VEGFR-2 (Waltenberger *et al.*, 1994; Kroll & Waltenberger, 1997; Gille *et al.*, 2001; Holmes *et al.*, 2007). Whilst VEGFR-1 is widely acknowledged to act as a decoy receptor for VEGF, to negatively-regulate VEGFR-2 activation, certain VEGF-stimulated signalling events and biological responses have been reported to be mediated by VEGFR-1 (Clauss *et al.*, 1996; Maru *et al.*, 2000; Kanno *et al.*, 2000; Bussolati *et al.*, 2001). Agarose gel analysis of PCR products using gene-specific primers to assess the expression of VEGF receptors in HDMECs revealed that these cells expressed VEGFR-1 and VEGFR-2 as well as VEGFR-3 (data not shown). It was thus possible that VEGF-induced ERK5 activation may be mediated by VEGFR-1, VEGFR-2 or VEGFR-3. It was hypothesised that VEGF-mediated activation of ERK5 may occur via VEGFR-2. To test this, VEGFR-2 activation was blocked using ZM323881, a potent and selective

anilinoquinazoline inhibitor of VEGFR-2 kinase activity (Whittles *et al.*, 2002; Endo *et al.*, 2003).

HDMECs were pre-incubated with 3 μM ZM323881 for 30 min prior to stimulation with 50 ng/ml VEGF for 10 min, conditions that had been previously shown to block VEGF-induced ERK1/2 activation in HAECs (Endo *et al.*, 2003). VEGF induced the phosphorylation of VEGFR-2 Tyr^{1175}, as well as the activation of both ERK1/2 and ERK5 (**Fig. 3.4**), an effect that was abolished following treatment with ZM323881 (**Fig. 3.4**). As a negative control, HDMECs were stimulated with PMA. ZM323881 had no effect upon PMA-stimulated activation of either ERK1/2, or ERK5 (**Fig. 3.4**), thus indicating that the effect of ZM323881 was not due to non-specific inhibition of kinase activity. Western blot analysis of p-PLC-γ, a key intermediary of VEGF/VEGFR-2-mediated signalling, revealed that ZM323881 also blocked VEGF-induced activation of PLC-γ (**Fig. 3.4, *p-PLC-γ***). Together, these results showed that intrinsic VEGFR-2 tyrosine kinase activity is required for VEGF-stimulated ERK5 activation in HDMECs.

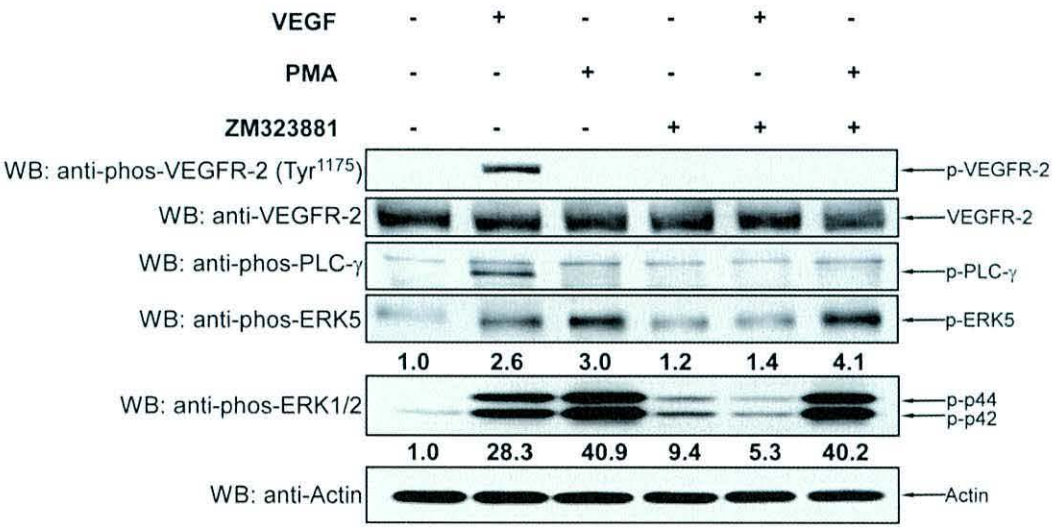


Figure 3.4 ZM323881 treatment inhibits VEGF-induced ERK5 activation in HDMECs. HDMECs were seeded at 2.0 x 10^5 cells per well on gelatin-coated 6-well dishes in EBM MV2 growth medium, and incubated for 24 h. Cells were serum-starved in EBM MV2 basal medium containing 1% (v/v) FCS for 20 h, and then were pre-incubated with 3 μM ZM323881 or 0.1% (v/v) DMSO (vehicle control) for 30 min prior to stimulation, or not (control), with 50 ng/ml VEGF for 10 min or 100 nM PMA for 20 min. Cells were lysed, and total cell lysates were separated on a 10 % SDS-PAGE gel and subjected to Western blot (*WB*) analysis with antibodies directed against phos-VEGFR-2 (Tyr^{1175}), VEGFR-2, phos-PLC-γ, phos-ERK5, phos-ERK1/2 or actin as a loading control, as indicated. Densitometric analysis of ERK5 or ERK1/2 phosphorylation relative to actin is displayed beneath the respective lanes of each blot, and was performed using NIH ImageJ software, with the basal, unstimulated control set arbitrarily as 1.0. This experiment was repeated three times with similar results. *DMSO, dimethyl sulfoxide; ERK5, extracellular signal-regulated kinase 5; FCS, foetal calf serum; HDMEC, human dermal microvascular endothelial cell; PLC-γ, phospholipase C-γ; PMA, phorbol 12-myristate 13-acetate, VEGF, vascular endothelial growth factor; VEGFR-2, VEGF receptor-2; WB, Western blot.*

3.3.2 VEGF-induced ERK5 activation is dependent upon VEGFR-2 Tyr^{1175}

Trans-autophosphorylation of the Tyr^{1175} residue located within the C-terminal tail of VEGFR-2, following the binding of VEGF to the extracellular domain of VEGFR-2, represents a critical step in VEGF/VEGFR-2 signalling during angiogenesis. Phospho-Tyr^{1175} provides a docking site for PLC-γ (Takahashi *et al.*, 2001), as well as for the SH2-domain-containing adaptor proteins Sck (Warner *et al.*, 2000) and Shb (Holmqvist *et al.*, 2004). PLC-γ undergoes phosphorylation and activation in response to VEGFR-2 activation (Cunningham *et al.*, 1997), and has been shown to be an important regulator of ERK1/2 activation in response to VEGF stimulation, by stimulating PKC-regulated Raf→MEK→ERK activation (Takahashi *et al.*, 2001). More recently, VEGF-stimulated PLC-γ activity has also been shown to be required for VEGF-stimulated tubular morphogenesis of PAE cells (Meyer *et al.*, 2003). To investigate the potential role of VEGFR-2 Tyr^{1175} in VEGF-stimulated ERK5 activation, wild-type PAE cells, and PAE cells stably expressing chimeric receptors were used. The first chimeric receptor consisted of the extracellular ligand-binding domain of human CSF-1R fused with the transmembrane and cytoplasmic domains of murine VEGFR-2 (Flk-1), termed CKR (CSFR-1-Flk-1 receptor) (Rahimi *et al.*, 2000). The second receptor chimera stably expressed in PAE cells consisted of CKR in which a point mutation of Tyr^{1173} of *Flk-1* (corresponding to the conserved Tyr^{1175} in KDR) to give Phe^{1173}, termed CKR/Flk-1 Y1173F, had been generated (Rahimi *et al.*, 2000; Dayanir *et al.*, 2001). The Phe^{1173} residue within CKR/Flk-1 Y1173F is unable to undergo phosphorylation following receptor activation, thus preventing the recruitment and phosphorylation of PLC-γ (Dayanir *et al.*, 2001) or the adaptor protein Shb (Holmqvist *et al.*, 2004).

Western blot analysis with an antibody directed against human CSF-R, confirmed that the extracellular domain of CSF1-R was expressed equally well in both PAE CKR/Flk-1 wt and PAE CKR/Flk-1 Y1173F cells, but was absent from wild type PAE cells (**Fig. 3.5, *CSF1-R***). Treatment of all three PAE cell types with CSF-1 thus allowed selective activation of the receptor chimeras. Stimulation with CSF-1 (50 ng/ml) resulted in the activation of Tyr^{1052} and Tyr^{1057} ($Tyr^{1054/1059}$ in human KDR) within the kinase domain of the murine VEGFR-2 (Flk-1) of both PAE CKR/Flk-1 wt and PAE CKR/Flk-1 Y1173F cells,

but not in wild-type PAE cells, as determined by Western blot analysis (**Fig. 3.5, *VEGFR-2 Tyr$^{1054/1059}$***).

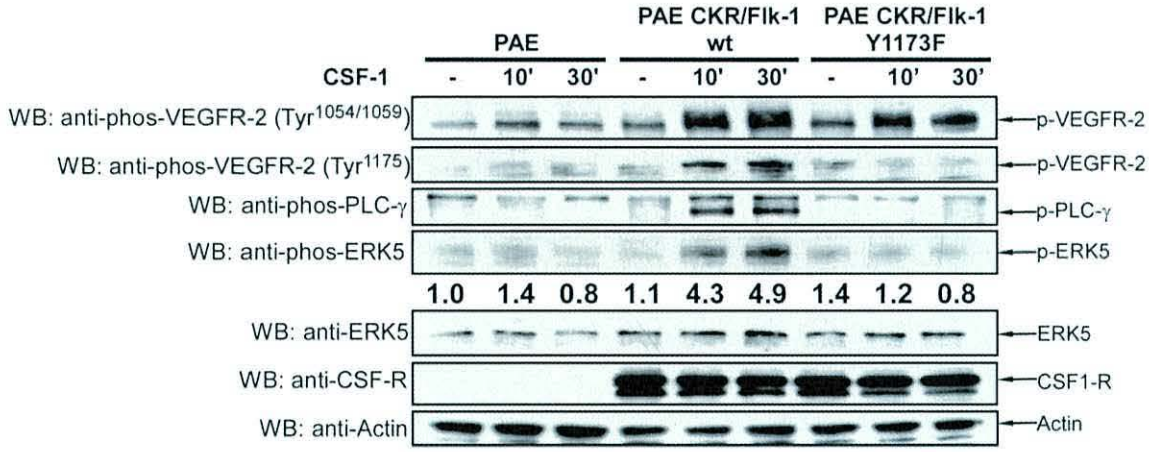


Figure 3.5 Phosphorylation of VEGFR-2 Tyr^{1175} is required for VEGFR-2-mediated ERK5 activation. PAE cells, PAE CKR/Flk-1 wt cells, or PAE CKR/Flk-1 Y1173F cells were seeded at 5.0 x 10^5 cells per well on gelatin-coated 6-well dishes in Hams' F-12 medium containing 10% (v/v) FCS, and incubated for 48 h. Cells were serum-starved in Hams' F-12 medium containing 1% (v/v) FCS for 20 h, prior to stimulation, or not (control), with CSF-1 (50 ng/ml) for 10 min or 30 min, as indicated. Cells were lysed, and total cell lysates were separated on a 10% SDS-PAGE gel as described (*section 2.2.6.3*) and subjected to Western blot (*WB*) analysis with antibodies directed against phos-VEGFR-2 (Tyr$^{1054/1059}$). phos-VEGFR-2 (Tyr^{1175}), phos-PLC-γ, phos-ERK5, ERK5, CSF-R or actin as a loading control, as indicated. Densitometric analysis of ERK5 phosphorylation relative to actin displayed beneath the p-ERK5 blot was performed using NIH ImageJ software, with the basal control set arbitrarily as 1.0. This experiment was repeated three times with similar results. *CKR, CSFR-1-Flk-1 receptor; CSF-1, colony-stimulating factor-1; CSF-R, CSF-1 receptor; ERK5, extracellular signal-regulated kinase 5; FCS, foetal calf serum; Flk-1, foetal liver kinase-1; PAE, porcine aortic endothelial, PLC-γ, phospholipase C-γ; VEGFR-2, VEGF receptor-2; WB, Western blot; wt, wild-type.*

As expected, phosphorylation of Tyr^{1175} following CSF-1 stimulation only occurred in PAE CKR/Flk-1 cells (**Fig. 3.5, *VEGFR-2 Tyr^{1175}***). Similarly, phosphorylation of PLC-γ was blocked in PAE CKR/Flk-1 Y1173F cells, but not in PAE CKR/Flk-1 wt cells, thus confirming that the Y1173F mutation prevented activation of PLC-γ (**Fig. 3.5, *PLC-γ***). Western blot analysis revealed that ERK5 was expressed at similar levels in all cells (**Fig. 3.5, *ERK5***). However, CSF-1-stimulated activation of ERK5 was abolished in PAE CKR/Flk-1 Y1173F cells, but not in PAE CKR/Flk-1 wt (**Fig. 3.5, *p-ERK5***). Together, these data suggest that VEGFR-2-mediated activation of ERK5 in endothelial cells is critically dependent upon VEGFR-2 Tyr^{1175} phosphorylation.

3.4 VEGF-induced ERK5 activation is dependent upon intracellular Ca^{2+}

Ca^{2+} is a critical second messenger in numerous signalling pathways within all eukaryotic cells (Clapham, 2007). Furthermore, Ca^{2+} signalling is important for

mediating both FGF-2- and VEGF-stimulated cell adhesion and spreading of endothelial cells during angiogenesis (Alessandro *et al.*, 1996; Munaron, 2006). VEGF stimulation of endothelial cells induces an increase in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$), by stimulating release of Ca^{2+} from intracellular stores such as the ER, as well as inducing an influx of extracellular Ca^{2+} ($[Ca^{2+}]_e$) via activation of receptor-operated cation channels (Brock *et al.*, 1991; Faehling *et al.*, 2002; Cheng *et al.*, 2006). VEGF/VEGFR-2-mediated phosphorylation of PLC-γ leads to PLC-γ-induced hydrolysis of the membrane phospholipid PIP_2, resulting in the generation of the second messengers DAG and IP_3 (Faehling *et al.*, 2002). The action of IP_3 on cognate IP_3Rs on the ER induces release of Ca^{2+} from within the ER into the cytoplasm (Patel *et al.*, 1999).

Given that the calcium ionophore A23187 was found to induce ERK5 activation in HDMECs (**Fig. 3.2**), it was hypothesised that Ca^{2+} was likely to play a role in ERK5 activation in these cells. To determine whether VEGF-induced ERK5 activation in HDMECs was dependent upon Ca^{2+}, various modulators of Ca^{2+} levels were used. Treatment with the cell-permeable $[Ca^{2+}]_i$ chelator, BAPTA-AM had been previously shown to inhibit VEGF-induced $[Ca^{2+}]_i$ mobilisation in HUVECs (Gliki *et al.*, 2001). The capacity of BAPTA-AM to chelate $[Ca^{2+}]_i$ in HDMECs was assessed by microspectrofluorometry Ca^{2+}-imaging carried out by Dr. Katherine Holmes at the University of Liverpool. Quantification of $[Ca^{2+}]_i$ in HDMECs following VEGF stimulation, revealed that VEGF induces a sharp increase in $[Ca^{2+}]_i$, an effect that was abolished by pre-incubation of HDMECs with 20 μM BAPTA-AM for 45 min (**Fig. 3.6 A**), thus confirming the effectiveness of BAPTA-AM in prevention of VEGF-stimulated $[Ca^{2+}]_i$ release in these cells.

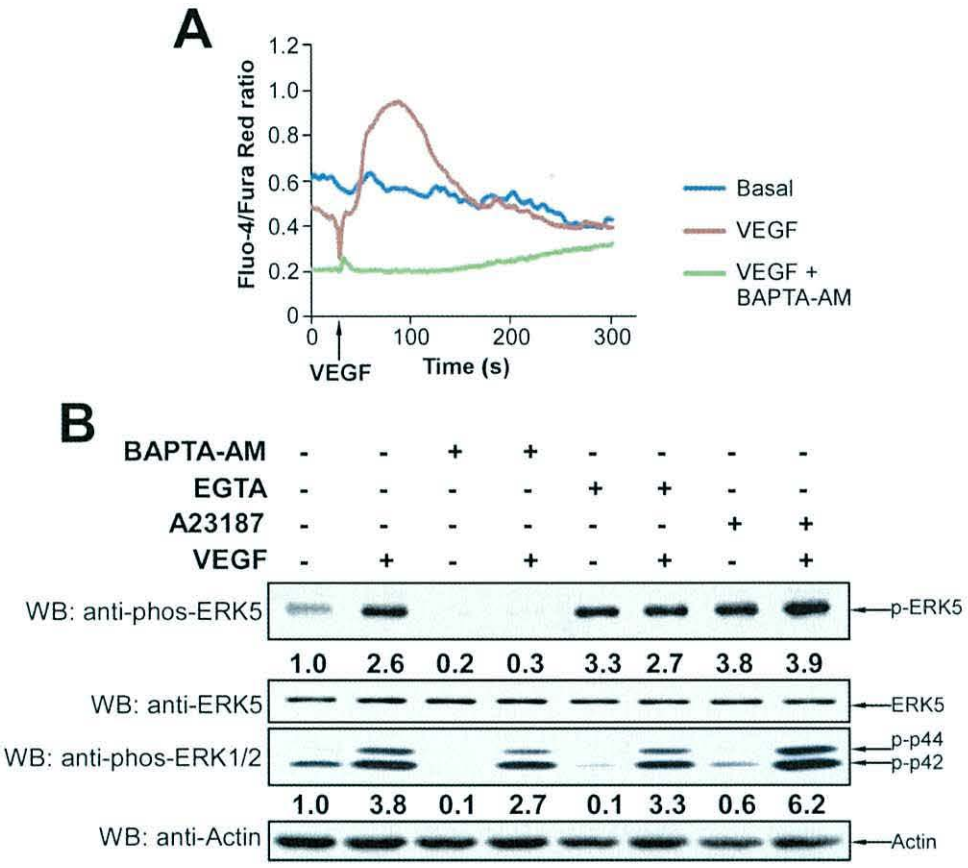


Figure 3.6 VEGF-stimulated ERK5 activation is dependent upon intracellular Ca^{2+}. HDMECs were seeded at 2.0 x 10^5 cells per well on gelatin-coated 6-well dishes in EBM MV2 growth medium, and incubated for 24 h, prior to serum-starvation in EBM MV2 basal medium containing 1% (v/v) FCS for 20 h. (**A**) Measurement of $[Ca^{2+}]_i$ in HDMECs following VEGF stimulation in the presence or absence of BAPTA-AM. Cells were pre-treated with the cell-permeant calcium-responsive dyes Fluo-4-AM (1.25 µM) and Fura Red™-AM (1.25 µM) for 20 min, and incubated with 0.1% (v/v) DMSO alone (vehicle control) or with 20 µM BAPTA-AM for 45 min, prior to stimulation, or not (control), with 50 ng/ml VEGF (arrow). Changes in fluorescence intensity were measured by microspectrofluorometry as described (*section 2.2.9*). (**B**) Cells were pre-incubated with 0.1% (v/v) DMSO (vehicle control) for 45 min; 20 µM BAPTA-AM for 45 min; 2.5 mM EGTA for 15 min or with 5 µM A23187 for 20 min, prior to stimulation, or not (control), with VEGF (50 ng/ml) for 10 min. Cells were lysed, and total cell lysates were separated on a 10% SDS-PAGE gel and subjected to Western blot (*WB*) analysis with antibodies directed against ERK5, phos-ERK5, phos-ERK1/2 or actin as a loading control, as indicated. Densitometric analysis of ERK5 or ERK1/2 phosphorylation relative to actin displayed beneath the respective blots was performed using NIH ImageJ software, with the basal vehicle control set arbitrarily as 1.0. This experiment was repeated three times with similar results. *BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis-acetoxymethyl ester; DMSO, dimethyl sulfoxide; EGTA, ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid; ERK, extracellular signal-regulated kinase; FCS, foetal calf serum; HDMEC, human dermal microvascular endothelial cell; VEGF, vascular endothelial growth factor; WB, Western blot.*

Treatment with BAPTA-AM abolished both basal and VEGF-induced ERK5 activation in HDMECs, but had no effect on total ERK5 expression (**Fig. 3.6 B**). By contrast, treatment with EGTA, a chelator of $[Ca^{2+}]_e$ that does not affect $[Ca^{2+}]_i$ levels, had no effect upon VEGF-stimulated ERK5 activation, but induced ERK5 activation in the absence of VEGF (**Fig. 3.6 B**). Treatment with A23187 alone strongly induced ERK5 activation by 3.8-fold (**Fig. 3.6 B**), but did not affect ERK1/2 activation levels. The effect of A23187 upon ERK5 activation appeared to be maximal, as stimulation with

VEGF and A23187 together did not induce a further increase in ERK5 phosphorylation (**Fig. 3.6 B**).

In agreement with the findings of Gliki *et al.* (2001), VEGF-induced ERK1/2 activation was only marginally reduced, by approximately 29%, following BAPTA-AM treatment. This result contrasts with the effect of BAPTA-AM upon VEGF-stimulated ERK5 activation, and suggests that ERK1/2 and ERK5 are regulated by distinct upstream pathways in HDMECs. In combination, these results suggest that VEGF-induced ERK1/2 activation in HDMECs is partly dependent upon $[Ca^{2+}]_i$ levels, whereas ERK5 activation is fully dependent upon $[Ca^{2+}]_i$ in these cells.

3.5 ERK5 activation in HDMECs is mediated by PKC

The observations that ERK5 activity was induced by the PKC activator PMA (**Fig. 3.3**), and was dependent upon $[Ca^{2+}]_i$, a known regulator of cPKC activation (**Fig. 3.6**), raised the possibility that ERK5 activation in HDMECs may be mediated by PKC isoforms. Furthermore, the finding that VEGFR-2-mediated ERK5 activation was prevented by mutation of the PLC-γ binding site within VEGFR-2 (**Fig. 3.5**) provided further evidence to imply that VEGF/VEGFR-2-stimulated ERK5 activation was downstream of PKC. Given that the PKC family are important downstream mediators of VEGF/VEGFR-2-induced signalling (Xia *et al.*, 1996; Takahashi *et al.*, 1999; Shen *et al.*, 1999; Wellner *et al.*, 1999; Gliki *et al.*, 2001; Rask-Madsen & King, 2008), it was hypothesised that PKC may also regulate ERK5 activation in HDMECs.

3.5.1 ERK5 is activated by a DAG mimetic in HDMECs

Activation of cPKC and nPKC isoforms is dependent on the lipophillic second messenger DAG (Nishizuka, 1992). DAG binds to one of two cysteine-rich C1 domains, termed C1a and C1b, present in the N-termini of the cPKC isoforms (PKCα, PKCβ, PKCγ), nPKC isoforms (PKCδ, PKCε, PKCη, PKCθ) and in PKCμ/PKD (Liu & Heckman, 1998). In addition, cPKC isoforms contain a Ca^{2+}-binding domain termed C2, which is required for the activation of these isoforms. PMA stimulates the activation of DAG-dependent cPKC and nPKC isoforms by binding to their C1 domain and increasing the membrane affinity of these isoforms (Liu & Heckman, 1998; Newton, 2001). Given

that PMA is a robust activator of ERK5 in HDMECs, it was hypothesised that ERK5 activation may be mediated by DAG-regulated PKC isoforms.

To gain further insight into the potential role of DAG in activating ERK5 in HDMECs, cells were stimulated with a synthetic, cell-permeable, short-chain fatty acid analogue of DAG termed 1,2-dioctanoyl-*sn*-glycerol (DOG). DOG is structurally more similar to DAG than PMA and selectively activates the DAG-regulated cPKC and nPKC isoforms both *in vitro* and *in vivo* (Ebeling *et al.*, 1985; Lapetina *et al.*, 1985). The physiological importance of DOG-induced PKC activity in angiogenesis has also been underlined by the finding that DOG induces neovascularisation in a chick chorioallantoic membrane *in vivo* model of angiogenesis (Tsopanoglou *et al.*, 1993).

HDMECs were treated with 100 μM DOG for 30 min, conditions that had been previously shown to activate ERK1/2 in endothelial cells (Tsopanoglou *et al.*, 1993). As positive controls for ERK5 activation, HDMECs were stimulated with VEGF (50 ng/ml) for 10 min, or with PMA (100 nM) for 20 min (**Fig. 3.7**). To act as an effective control for the potential non-specific activation of ERK5 by DOG, which may be induced by lipid treatment, HDMECs were treated with the cell-permeable phosphatidylcholine mimetic 1,2-dioctanoyl-*sn*-glycero-3-phosphocholine (DOPC), a phospholipid that is structurally similar to DOG, but that contains a phosphocholine head-group and so cannot act as a direct physiological activator of PKC. In addition, cells were treated with the short-chain fatty acid, cell-permeable analogue of phosphatidic acid, 1,2-dioctanoyl-*sn*-glycero-3-phosphate (DOPA) to determine whether phosphatidic acid was capable of activating ERK5. Western blot analysis of whole cell lysates revealed that ERK5 was activated by DOG to a similar degree as VEGF and PMA thus suggesting that ERK5 is activated by DAG (**Fig. 3.7**). By contrast, treatment with either DOPC or DOPA did not result in ERK5 activation, although some activation of ERK1/2 was apparent. Together, these results suggested that ERK5 activation in HDMECs is induced by DAG derived from PIP_2 hydrolysis, raising the possibility that ERK5 activation may be mediated by DAG-regulated PKC isoforms in these cells (**Fig. 3.7**).

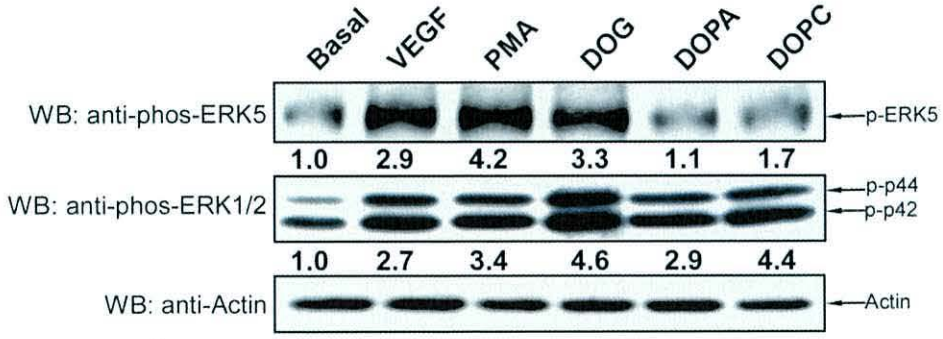


Figure 3.7 ERK5 is activated by a cell-permeable DAG mimetic. HDMECs were seeded at 2.0 x 10^5 cells per well on gelatin-coated 6-well dishes in EBM MV2 growth medium, and incubated for 24 h. Cells were serum-starved in EBM MV2 basal medium containing 1% (v/v) FCS for 20 h, prior to stimulation, with 50 ng/ml VEGF for 10 min, 100 nM PMA for 20 min or with 100 μM DOG or 100 μM DOPA or 100 μM DOPC (control) for 15 min as described (*section 2.2.2.8.2*). Cells were lysed, and total cell lysates were separated on a 10% SDS-PAGE gel as described (*section 2.2.6.3*) and subjected to Western blot (*WB*) analysis with antibodies directed against phos-ERK5, phos-ERK1/2, or actin as a loading control, as indicated. Densitometric analysis of ERK5 or ERK1/2 phosphorylation relative to actin displayed beneath the respective blots was performed using NIH ImageJ software, with the basal control set arbitrarily as 1.0. This experiment was repeated three times with similar results. *DOG, 1,2-dioctanoyl-sn-glycerol; DOPA, 1,2-dioctanoyl-sn-glycero-3-phosphate; DOPC, 1,2-dioctanoyl-sn-glycero-3-phosphocholine; ERK, extracellular signal-regulated kinase; FCS, foetal calf serum; HDMEC, human dermal microvascular endothelial cell; PMA, phorbol 12-myristate 13-acetate; VEGF, vascular endothelial growth factor; WB, Western blot.*

3.5.2 ERK5 activation is blocked by GF109203X but not by Gö6976

The potential role of PKC isoforms as upstream mediators of ERK5 activation in HDMECs was assessed by selective pharmacological inhibition of specific PKCs using the compounds GF109203X and Gö6976. GF109203X is a potent broad-spectrum inhibitor of cPKCs and nPKCs, which is reported to inhibit PKCα, PKCβ, PKCδ and PKCε with an IC_{50} < 1 μM (Toullec *et al.*, 1991; Wilkinson *et al.*, 1993). The nonglycosidic indolocarbazole Gö6976 is a selective inhibitor of the cPKC isoforms PKCα and PKCβ (Qatsha *et al.*, 1993; Martiny-Baron *et al.*, 1993).

Phosphorylation of a highly-conserved serine residue corresponding to Ser^{660} of $PKC\beta_{II}$, present within the C-terminal hydrophobic FXXFS/TF/Y motif in cPKCs and nPKCs, represents a critical phase in PKC activation (Keranen *et al.*, 1995). To determine the activation state of PKC isoforms in response to VEGF and PMA stimulation in HDMECs, Western blot analysis using an anti-(pan)phos-PKC antibody, directed against the conserved Ser^{660} residue of $PKC\beta_{II}$ was carried out.

PKCα was found to be strongly phosphorylated on Ser^{657} in unstimulated cells (**Fig. 3.8 A, *p-PKCα***), thus suggesting that this isoform is constitutively activated in HDMECs. Stimulation of HDMECs with either VEGF or PMA only marginally increased detectable

PKCα phosphorylation on Ser[657], from 1.0 to 1.45 and from 1.0 to 1.59 for VEGF and PMA respectively (**Fig. 3.8 B**).

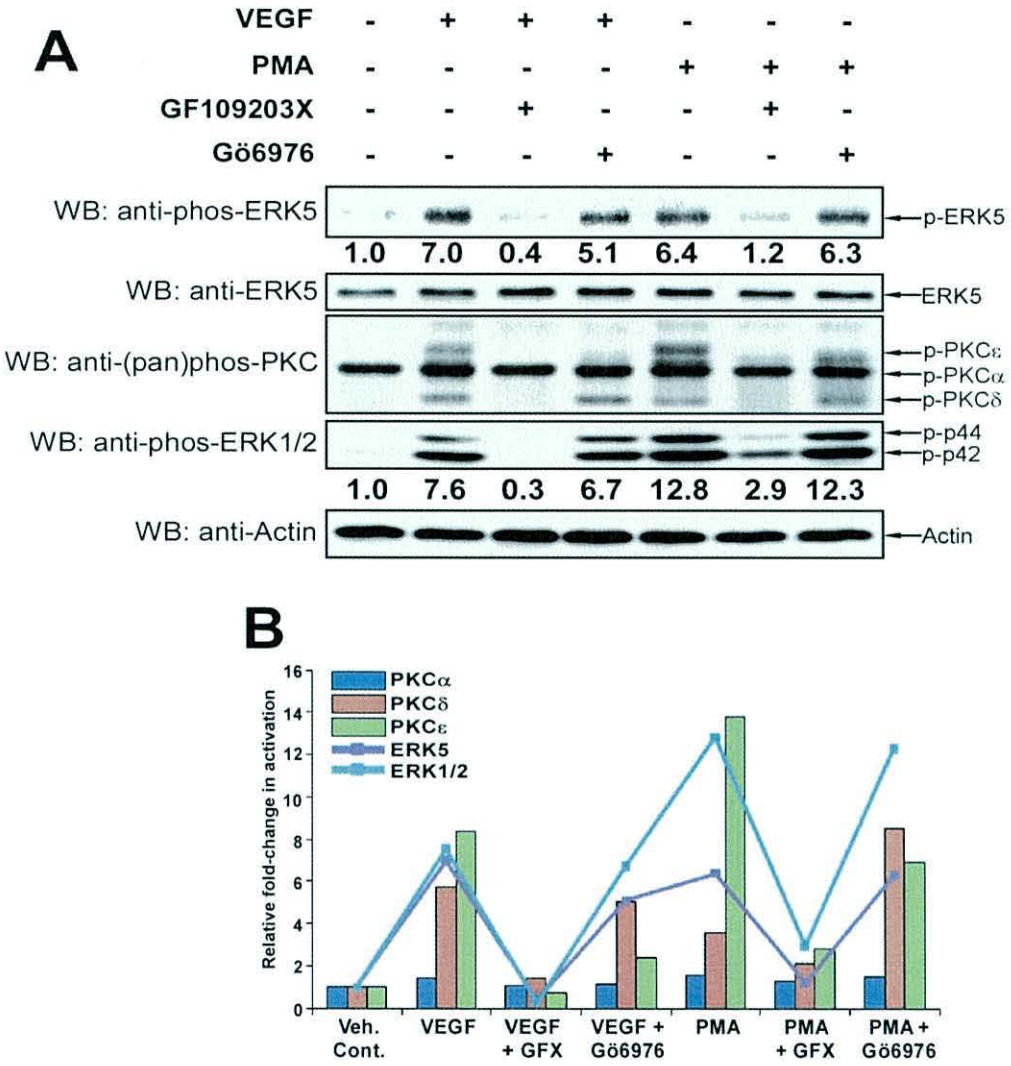


Figure 3.8 Treatment with GF109203X, but not Gö6976, inhibits ERK5 activation in HDMECs. (**A**) HDMECs were seeded at 2.0 x 10^5 cells per well on gelatin-coated 6-well dishes in EBM MV2 growth medium and incubated for 24 h. Cells were serum-starved in EBM MV2 basal medium containing 1% (v/v) FCS for 20 h, then pre-incubated with 3 μM GF109203X (GFX), or with 3 μM Gö6976, or with 0.1% (v/v) DMSO (vehicle control) for 30 min (Veh. Cont). Cells were then stimulated with 50 ng/ml VEGF for 10 min or with 100 nM PMA for 20 min. Cells were lysed, and total cellular proteins were separated on a 10% SDS-PAGE gel and subjected to Western blot (*WB*) analysis with antibodies directed against phos-ERK5, ERK5, (pan)phos-PKC, phos-ERK1/2 or actin as a loading control, as indicated. (**B**) Densitometric analysis of the phosphorylation of ERK5, ERK1/2, PKCα, PKCδ and PKCε relative to actin, displayed beneath each respective blot, and/or displayed graphically was performed using NIH ImageJ software, with the basal vehicle control set arbitrarily as 1.0. The result shown is representative of three independent experiments. *DMSO, dimethyl sulfoxide; ERK, extracellular signal-regulated kinase; FCS, foetal calf serum; HDMEC, human dermal microvascular endothelial cell; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; VEGF, vascular endothelial growth factor; WB, Western blot.*

In contrast to PKCα activation, stimulation of HDMECs with VEGF or PMA led to a marked increase in the phosphorylation of both PKCδ and PKCε (**Fig. 3.8, A and B**). Treatment with the broad-spectrum PKC inhibitor GF109203X abolished activation of both PKCδ and PKCε (**Fig. 3.8**). Interestingly however, whilst treatment with the PKCα-

specific inhibitor Gö6976 did not affect VEGF-stimulated PKCδ activation, this treatment inhibited VEGF-stimulated PKCε activation (**Fig. 3.8**). This may be indicative of a VEGF-induced PKCα-dependent activation of PKCε, as previously reported in cyclic-strain-induced PKC activation in endothelial cells (Cheng *et al.*, 2001).

Pre-treatment of HDMECs with GF109203X was sufficient to lower VEGF-stimulated ERK5 activation by 94% and PMA-stimulated ERK5 activation by 80% (**Fig. 3.8, *p-ERK5***). However, treatment with the PKCα inhibitor Gö6976 had no effect upon PMA-stimulated ERK5 activation, and only marginally affected VEGF-stimulated ERK5 activation. In agreement with the findings of Gliki *et al.* (2002), pre-treatment with 3 μM GF109203X abolished VEGF-induced ERK1/2 activation, whilst treatment with Gö6976 did not affect VEGF-stimulated ERK1/2 activation (**Fig. 3.8, *p-ERK1/2***). In addition, whilst PMA-stimulated ERK1/2 activation was severely inhibited by GF109203X treatment, Gö6976 did not inhibit PMA-induced ERK1/2 activation. Together, these results suggest that activation of both ERK1/2 and ERK5 in response to VEGF is mediated by nPKC isoforms.

3.5.3 Rottlerin inhibits VEGF-stimulated ERK5 activation in HDMECs

PKCδ has been previously shown to be an important mediator of VEGF-induced intracellular signalling and survival in endothelial cells (Takahashi *et al.*, 1999; Gliki *et al.*, 2001; Gliki *et al.*, 2002). Rottlerin has been described as a PKCδ-specific inhibitor with a reported IC_{50} of 3-6 μM (Gschwendt *et al.*, 1994) and has been previously used to specifically inhibit PKCδ in HUVECs, leading to the inhibition of VEGF-induced ERK1/2 activation (Gliki *et al.*, 2001).

To investigate the effects of rottlerin upon ERK5 activation in HDMECs, cells were pre-treated with various concentrations of rottlerin, prior to stimulation with VEGF. It was found that 1 μM rottlerin was sufficient to lower VEGF-induced ERK5 activation by 70% (**Fig. 3.9**). Interestingly, and in contrast to the reported effect in HUVECs (Gliki *et al.*, 2001), treatment with 1 μM rottlerin increased ERK1/2 phosphorylation compared to cells treated with VEGF and vehicle control alone (**Fig. 3.9**). Increasing the dose of rottlerin to 5 μM led to a further inhibition of VEGF-stimulated ERK5 activation, and

lowered ERK1/2 activation in a dose-dependent manner (**Fig. 3.9**). These data show that VEGF-stimulated ERK5 activation is sensitive to rottlerin treatment, whereas VEGF-induced ERK1/2 activity is less sensitive to treatment with this inhibitor, collectively suggesting that PKCδ may be an important mediator of VEGF-induced activation of ERK5 but not ERK1/2.

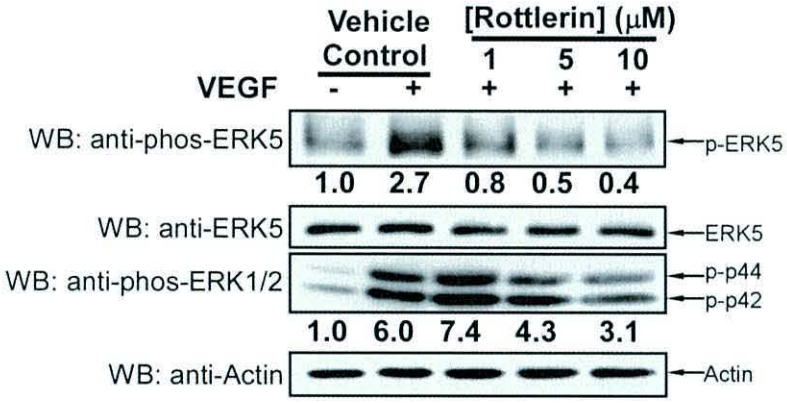


Figure 3.9 Treatment with rottlerin inhibits VEGF-induced ERK5 activation in HDMECs. HDMECs were seeded at 2.0 x 10^5 cells per well on gelatin-coated 6-well dishes in EBM MV2 growth medium and incubated for 24 h. Cells were serum-starved in EBM MV2 basal medium containing 1% (v/v) FCS for 20 h, then pre-incubated with the indicated concentrations of rottlerin or with 0.1% (v/v) DMSO (vehicle control) for 30 min, followed by stimulation with 50 ng/ml VEGF for 10 min. Cells were lysed, and total cellular proteins were separated on a 10% SDS-PAGE gel and subjected to Western blot (*WB*) analysis with antibodies directed against phos-ERK5, ERK5, phos-ERK1/2 or actin as a loading control, as indicated. Densitometric analysis of ERK5 or ERK1/2 phosphorylation relative to actin displayed beneath the respective lanes of each blot was performed using NIH ImageJ software, with the basal vehicle control set arbitrarily as 1.0. This result is representative of three independent experiments. *DMSO, dimethyl sulfoxide; ERK, extracellular signal-regulated kinase; FCS, foetal calf serum; HDMEC, human dermal microvascular endothelial cell; VEGF, vascular endothelial growth factor; WB, Western blot.*

3.5.4 Profiling of PKC isoform expression and activity in HDMECs

Stimulation of HDMECs with PMA or the DAG-mimetic, DOG, suggested that ERK5 activation in HDMECs was dependent upon a DAG-regulated PKC isoform (**Fig. 3.7**). Furthermore, selective inhibition of PKC isoforms with small-molecule pharmacological inhibitors suggested that ERK5 activation in HDMECs was dependent upon a nPKC isoform (**Fig. 3.8 and Fig. 3.9**). In order to further investigate the relative contribution of distinct PKC isoforms in the mediation of VEGF-induced activation of ERK5, it was firstly necessary to determine which PKC isoforms were expressed in HDMECs.

PKCα, PKCδ, PKCε, and PKCζ had been previously shown to be expressed in HUVECs (Haller *et al.*, 1996; Wellner *et al.*, 1999; Gliki *et al.*, 2001); however, the PKC complement of HDMECs had not been previously reported. Profiling PKC expression in HDMECs was performed by two different approaches. Firstly, gene-specific primers to

nine PKC isoforms: PKCα, PKCγ, PKCδ, PKCε, PKCη, PKCι, PKCθ, PKCζ as well as a primer targeting a common sequence shared by $PKC\beta_I$ and $PKC\beta_{II}$, were designed (*Appendix 1*), and used in conventional PCR reactions to probe for the expression of these genes in HDMEC cDNA. As a positive control, cDNA, prepared from RNA extracted from HEK 293 cells was probed in parallel, to determine the effectiveness of these gene-specific primers (**Fig. 3.10 A**). A second approach involved analysis of PKC expression at the protein level. HDMECs were lysed in RIPA buffer, and total cell proteins were separated on an 8% SDS-PAGE gel. Following protein transfer onto nitrocellulose membranes, individual lanes were cut from the membrane, and were probed with antibodies directed against the isoforms PKCα, PKCβ, PKCγ, PKCδ, PKCε, PKCη, PKCθ, PKCι and PKCμ/PKD) (**Fig. 3.10 B**). As a positive control, HEK 293 cells were also lysed, and analysed in the same way (**Fig. 3.10 B**).

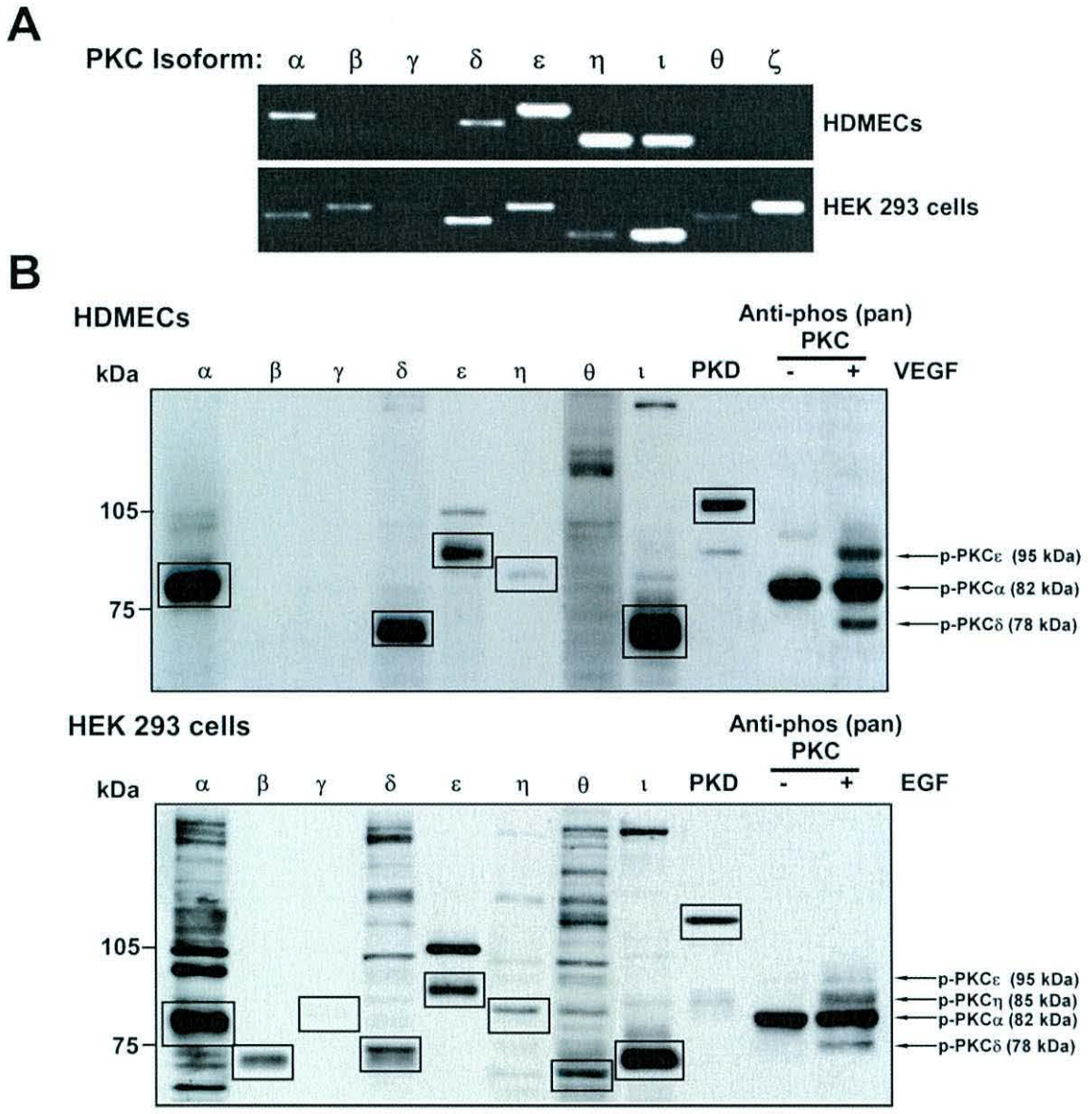


Figure 3.10 HDMECs express the isoforms PKCα, PKCδ, PKCε, PKCη, PKCι and PKD. (**A**) HDMECs were seeded in gelatin-coated 10 cm cell culture dishes at 1.0 x 10^6 cells per dish in EBM MV2 growth medium whilst HEK293 cells were seeded at 1.0 x 10^6 cells per dish, and were grown in D-MEM medium containing 10% (v/v) FCS. At 48 h post-seeding, total RNA was extracted from cells as described (*section 2.2.4.1*) followed by first-strand cDNA synthesis by reverse-transcription as described (*section 2.2.4.2*). Standard PCR (*section 2.2.3.8*) was carried out on equal amounts of HDMEC and HEK293 cDNA using PCR primers directed against PKC-α, -β, -γ, -δ, -ε, -η, -θ, -ι, and -ζ isoforms (*Appendix 1*). Equal amounts of the PCR products were resolved by electrophoresis on a 3% (w/v) agarose gel and visualised as described (*section 2.2.3.2*). (**B**) HDMECs were seeded in gelatin-coated 10 cm cell culture dishes at 1.0 x 10^6 cells per dish in EBM MV2 growth medium whilst HEK293 cells were seeded at 1.0 x 10^6 cells per dish, and were grown in D-MEM medium containing 10% (v/v) FCS. At 24 h post-seeding, HDMECs were serum-starved in EBM MV2 basal medium containing 1% (v/v) FCS, whilst HEK293 cells were serum-starved in D-MEM containing 1% (v/v) FCS. Following 20 h of serum-starvation, cells were stimulated (or not) with 50 ng/ml VEGF (HDMECs) or 50 ng/ml EGF (HEK 293 cells) for 10 min. Total cell lysates were prepared, and lysates were separated on an 8% SDS-PAGE gel, prior to transfer, followed by Ponceau S staining as described (*section 2.2.6.4*). Individual lanes were cut from the membrane prior to detection of the PKC isoforms indicated using the PKC sampler kit (BD Biosciences). VEGF- or EGF-induced phosphorylation of PKC isoforms following treatment were detected by immunoblot analysis with an antibody directed against phos(pan)-PKC, as indicated. Individual PKC isoforms are indicated as boxed sections. *D-MEM, Dulbecco's modified Eagle medium; EGF, epidermal growth factor; FCS, foetal calf serum; HDMEC, human dermal microvascular endothelial cell; HEK293, human embryonic kidney 293; PKC, protein kinase C; VEGF, vascular endothelial growth factor.*

Agarose gel analysis of the products of conventional PCR using PKC-specific gene-specific primers revealed that HEK 293 cells expressed PKCα, PKCβ, PKCδ, PKCε, PKCη, PKCι, PKCθ and PKCζ, whilst HDMECs were found to express five PKC isoforms, namely: the cPKC, PKCα; the nPKCs, PKCδ, PKCε and PKCη; and the aPKC, PKCι (**Fig. 3.10 A**). Immunoblot analysis with PKC isoform-specific antibodies confirmed that PKCα, PKCδ, PKCε, PKCη and PKCι were expressed in HDMECs. In addition, PKD was detected by this method in both HDMECs and HEK 293 cells (**Fig. 3.10 B**). PKCβ, PKCγ and PKCθ were not detectable in HDMECs by either conventional PCR analysis, or Western blotting (**Fig. 3.10, A and B**). Together, these results collectively indicate that HDMECs express PKCα, PKCδ, PKCε, PKCη, PKCι and PKD.

3.5.5 VEGF stimulates translocation of PKCα, PKCδ and PKCε, but not PKCη or PKCι, to the cell membrane in HDMECs

Translocation of PKCs from the cytoplasm to cellular membranes represents a critical step required for PKC activation (Newton, 2001). It has been previously reported that PKCα, PKCδ and PKCε translocate to the membrane in response to VEGF stimulation in HUVECs (Gliki *et al.*, 2001). In a separate study, PKCα and PKCζ underwent translocation following VEGF-treatment, whilst the cellular distribution of PKCδ and PKCε did not change (Wellner *et al.*, 1999). Furthermore, Shen *et al.* demonstrated that VEGF induced translocation of PKCα, PKCγ and PKCε in bovine adrenal cortex capillary endothelial cells (Shen *et al.*, 1999). To investigate which PKC isoforms translocate to the particulate fraction in response to VEGF stimulation in HDMECs, cells were stimulated with VEGF for various times, prior to cell lysis and fractionation. Membrane and cytosolic levels of PKCα, PKCδ, PKCε, PKCη and PKCι were analysed by Western blotting (**Fig. 3.11**).

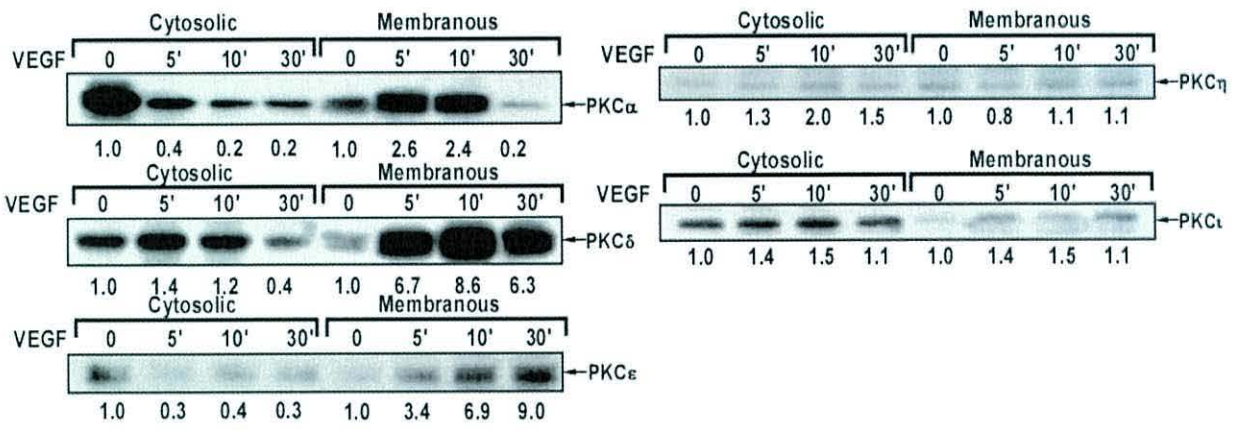


Figure 3.11 Stimulation of HDMECs with VEGF induces translocation of PKCα, PKCδ and PKCε to the cell membrane. HDMECs were seeded on gelatin-coated 10 cm cell culture dishes at 1.0 x 10^6 cells per dish in EBM MV2 growth medium. After 24 h incubation, cells were serum-starved in EBM MV2 basal medium containing 1% (v/v) FCS for 20 h. HDMECs were stimulated with VEGF (50 ng/ml) for the indicated times, and membranous and cytosolic fractions were prepared as described in (*section 2.2.6.2*). Equal amounts of protein were resolved on an 8% SDS-PAGE gel followed by Western blotting with antibodies directed against PKCα, PKCδ, PKCε, PKCη or PKCι, as indicated. The results shown are representative of two independent experiments. Quantification of PKC translocation was conducted by densitometric analysis of scanned X-ray films using NIH ImageJ software, with the unstimulated control set arbitrarily at 1.0 in each case. Data is presented as fold-change relative to the untreated control in each case and is presented at below each respective lane. *FCS, foetal calf serum; HDMEC, human dermal microvascular endothelial cell; PKC, protein kinase C; VEGF, vascular endothelial growth factor.*

VEGF stimulation induced translocation of PKCα, PKCδ and PKCε to the membranous fraction in HDMECs (**Fig. 3.11**). Notably, VEGF stimulation induced rapid, robust and sustained translocation of PKCδ to the cell membrane (**Fig 3.11, *PKCδ***). By contrast, VEGF-induced translocation of PKCα was transient (**Fig 3.11, *PKCε***), whereas VEGF-induced translocation of PKCε increased steadily with prolonged exposure to VEGF (**Fig 3.11, *PKCα***). The cellular distribution of PKCη and PKCι did not change in response to VEGF stimulation (**Fig 3.11, *PKCη*; Fig 3.11, *PKCι***).

3.5.6 SiRNA-mediated down-regulation of PKCδ attenuates ERK5 activation in HDMECs

The findings that PKCα, PKCδ and PKCε were activated by VEGF (**Fig. 3.8**) and that VEGF stimulation induced translocation of these isoforms to the membranous fraction of the cell (**Fig. 3.11**), suggested these isoforms as candidate arbitrators of VEGF-induced ERK5 activation. Treatment with the PKCα inhibitor Gö6976 did not affect ERK5 activation, suggesting that PKCα was not involved in ERK5 activation (**Fig. 3.8**). However, the efficacy of this inhibitor at inhibiting PKCα activation in these cells was not fully quantifiable with the methods used, and thus would require further validation. Furthermore, pre-treatment with the PKCδ inhibitor rottlerin prevented

VEGF-induced activation of ERK5 (**Fig. 3.9**); however, rottlerin has been shown to inhibit PKCδ activity in a non-specific manner by uncoupling mitochondria (Soltoff, 2001). Furthermore, rottlerin has also been reported to non-specifically inhibit targets other than PKCδ (Davies *et al.*, 2000; Soltoff, 2007). Therefore, the observation that rottlerin prevents VEGF-stimulated ERK5 activation may not be considered fully conclusive evidence of the involvement of PKCδ in this process. To circumvent potential non-specific inhibition of other kinases, or other PKC isoforms, siRNA-mediated gene silencing (see *section 4.1*) was utilised to selectively down-regulate the expression of individual PKC isoforms, and the effect of siRNA-mediated down-regulation of PKC isoforms upon VEGF- and PMA-stimulated ERK5 and ERK1/2 activity was subsequently assessed.

HDMECs were treated with siRNAs directed against PKCα, PKCδ or PKCε, or were treated with N.S. siRNA for 24 h, prior to serum-starvation of cells for 20 h and stimulation with VEGF, or PMA as described in the legend to **Fig. 3.12**. Western blot analysis revealed that the expression of each individual PKC isoform was silenced by 70-90% compared to N.S. siRNA-treated cells (**Fig. 3.12**). Importantly, the expression of other PKC isoforms was not affected by transfection of siRNAs directed against individual PKC isoforms (**Fig. 3.12**), thus showing the specificity of siRNA treatment. Down-regulation of PKCδ expression severely attenuated VEGF- and PMA-induced ERK5 activation by 85% and 68% respectively (**Fig. 3.12**). By contrast, siRNA-mediated silencing of PKCα had limited effects upon ERK5 activity, lowering VEGF-stimulated ERK5 activity by 28%, and had no effect upon PMA-stimulated ERK5 activity (**Fig. 3.12, *p-ERK5***). Similarly, PKCε siRNA treatment had negligible effects upon VEGF- or PMA-induced ERK5 activation (**Fig. 3.12, *p-ERK5***). VEGF-stimulated activation of ERK1/2 was lowered by treatment with PKCα-, PKCδ- and PKCε-siRNA, but was most severely attenuated following down-regulation of PKCε (**Fig. 3.12, *p-ERK1/2***). Densitometric analysis revealed that siRNA-mediated down-regulation of PKCε expression lowered VEGF- and PMA-induced ERK1/2 activation by 73% and 63% respectively (**Fig. 3.12, *p-ERK1/2***). Together, these data suggest that both VEGF- and PMA-induced activation of ERK5 is PKCδ-dependent, whereas ERK1/2 activation is primarily mediated by PKCε.

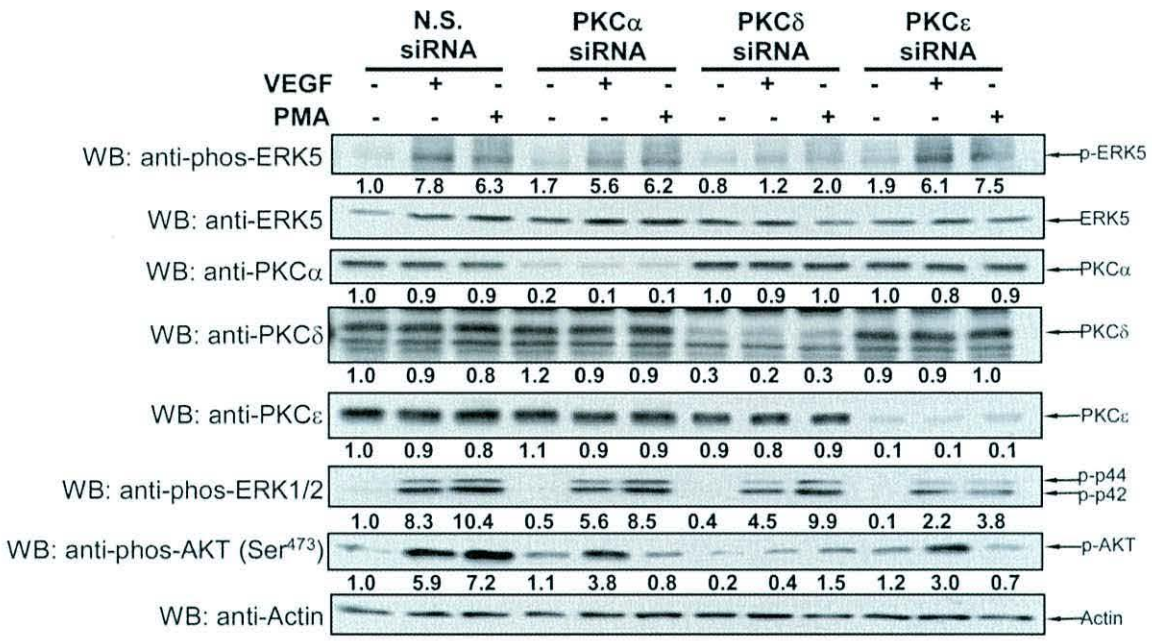


Figure 3.12 SiRNA-mediated down-regulation of PKCδ expression attenuates VEGF- and PMA-induced ERK5 activation in HDMECs. HDMECs were seeded at 1.0 x 10^5 cells per well on gelatin-coated 6-well dishes in EBM MV2 growth medium and incubated for 24 h. Cells were transfected as described (*section 2.2.2.7.2*) with siRNA directed against the PKC isoforms PKCα (5 nM Hs_PRKA_5_HP + 5 nM Hs_PRKA_6_HP), PKCδ (5 nM Hs_PRKD_8_HP + 5 nM Hs_PRKD_11_HP), PKCε (5 nM Hs_PRKE_5_HP + 5 nM Hs_PRKE_6_HP) or with 10 nM of non-silencing (N.S.) control siRNA, as indicated. At 24 h post-transfection, cells were serum-starved in EBM MV2 basal medium containing 1% (v/v) FCS for 20 h prior to cell stimulation, or not, with 50 ng/ml VEGF for 10 min or with 100 nM PMA for 20 min. Cells were lysed, and total cell lysates were separated on an 8% SDS-PAGE gel and subjected to Western blot (*WB*) analysis with antibodies directed against phos-ERK5, ERK5, PKCα, PKCδ, PKCε, phos-ERK1/2, phos-AKT (Ser^{473}) or actin as a loading control, as indicated. Densitometric analysis of protein phosphorylation or protein expression relative to actin is displayed beneath each blot, and was performed using NIH ImageJ software, with the basal control set arbitrarily as 1.0. This result is representative of three independent experiments. *ERK, extracellular signal-regulated kinase; FCS, foetal calf serum; HDMEC, human dermal microvascular endothelial cell; N.S., non-silencing; PKC, protein kinase C; siRNA, small interfering RNA; VEGF, vascular endothelial growth factor; WB, Western blot.*

PKCδ had been previously shown to regulate VEGF-induced AKT activation in HUVECs (Gliki *et al.*, 2002). In order to assess the potential effects of PKCδ down-regulation upon AKT activation in HDMECs, Western blot analysis with an antibody directed against p-AKT Ser^{473} was carried out (**Fig. 3.12, *p-AKT***). Treatment with either PKCα- or PKCε-specific siRNA lowered VEGF-induced phosphorylation of AKT on Ser^{473} by approximately 50% in each case, whereas down-regulation of PKCδ resulted in a 93% reduction in VEGF-induced levels of p-AKT-Ser^{473} (**Fig. 3.12, *p-AKT***). By contrast, siRNA-mediated down-regulation of PKCα, PKCδ, and PKCε inhibited PMA-induced AKT-Ser^{473} phosphorylation (**Fig. 3.12, *p-AKT***).

3.6 ERK5 activation in HDMECs is not dependent upon Ras

It had been suggested that VEGF-induced activation of the small, membrane-anchored GTPase, Ras, represents a pathway leading to ERK1/2 activation (Kroll & Waltenberger, 1997; Meadows *et al.*, 2001). Furthermore, VEGF-stimulated Ras activation is sufficient to induce angiogenesis *in vitro* in some endothelial cell types (Meadows *et al.*, 2004). However, conflicting evidence exists regarding the importance of Ras in mediating ERK1/2-activating signals originating from VEGFR-2 (Doanes *et al.*, 1999; Takahashi *et al.*, 2001). Similarly, the involvement of Ras in ERK5 activation is unclear (English *et al.*, 1999; Sharma & Goalstone, 2005; Carvajal-Vergara *et al.*, 2005).

To determine whether or not Ras was required for ERK1/2 and ERK5 activation in HDMECs, cells were treated separately with farnesylthiosalicylic acid (FTS) or simvastatin, two inhibitors of Ras activity with independent mechanisms of action. Attachment of Ras to the plasma membrane is essential for Ras activity, and the subsequent activation of Raf-1 (Casey *et al.*, 1989; Kato *et al.*, 1992; Stokoe *et al.*, 1994). Farnesylation of Ras, by transfer of the farnesyl moiety from farnesyl pyrophosphate (FPP), to a cysteine residue in Ras, mediated by farnesyl transferase, is critical for the attachment of Ras to the plasma membrane (Hancock *et al.*, 1990; Reiss *et al.*, 1990). FTS mimics the farnesyl moiety of FPP, and inhibits Ras activity by inducing dislodgment of Ras from the cell membrane (Marciano *et al.*, 1995; Haklai *et al.*, 1998). FTS-mediated inhibition of Ras has been shown to inhibit serum-induced activation of Raf-1, and subsequent activation of the classical ERK1/2 pathway (Gana-Weisz *et al.*, 1997). Simvastatin (Synvinolin, MK-733) is a 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitor (Olsson *et al.*, 1986). HMG-CoA reductase is required for FPP synthesis; thus, inhibition of HMG-CoA reductase indirectly inhibits post-translational farnesylation of Ras, and hence inhibits Ras activation.

HDMECs were pre-incubated with FTS (50 μM) or simvastatin (500 nM) or with 0.1% (v/v) DMSO as a vehicle control for 24 h, and were subsequently stimulated with VEGF (50 ng/ml), FGF-2 (50 ng/ml), PMA (100 nM) or were left unstimulated. VEGF-induced activation of ERK5 and ERK1/2 was not affected by treatment with FTS or simvastatin (**Fig. 3.13**). However, similar to the reported findings of Klint et al. (1999), pre-

incubation of HDMECs with FTS lowered FGF-2-stimulated ERK1/2 activity by 52% (**Fig. 3.13**). A similar reduction in FGF-2-induced ERK1/2 activation was also apparent following simvastatin treatment (**Fig. 3.13**). Together, these results suggest that FGF-2-stimulated ERK1/2 activation is, at least in part, mediated by Ras in HDMECs, whereas VEGF-stimulated activation of both ERK1/2 and ERK5 appears to be Ras-independent in these cells.

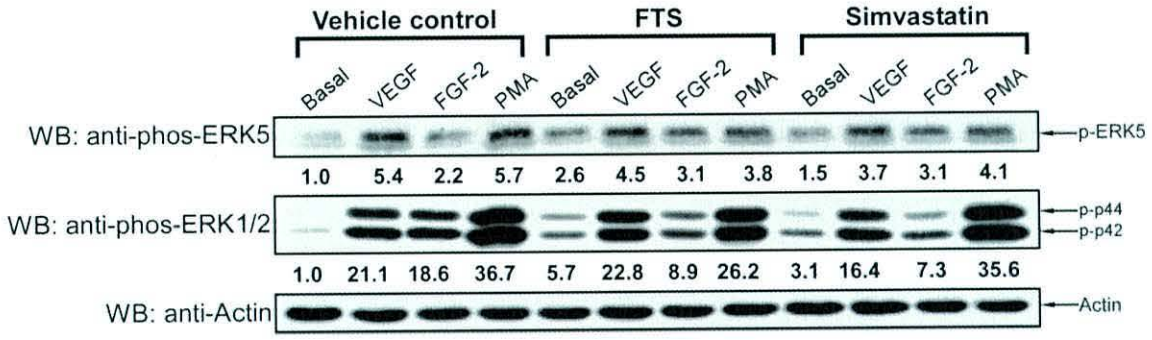


Figure 3.13 VEGF-stimulated ERK5 activation is not dependent upon Ras. HDMECs were seeded at 2.0 x 10^5 cells per well on gelatin-coated 6-well dishes in EBM MV2 growth medium, and incubated for 24 h. Cells were pre-incubated with 50 µM farnesylthiosalicylic acid (FTS); 500 nM simvastatin or with 0.1% (v/v) DMSO (vehicle control) in EBM MV2 growth medium for 12 h prior to serum-starvation in EBM MV2 basal medium containing 1% (v/v) FCS and containing 50 µM FTS, 500 nM simvastatin, as indicated, or 0.1% (v/v) DMSO as a vehicle control (vehicle control) for a further 12 h, prior to stimulation, or not (control), with 50 ng/ml VEGF or 50 ng/ml FGF-2 for 10 min or 100 nM PMA for 20 min. Cells were lysed, and total cell lysates were separated on a 10% SDS-PAGE gel and subjected to Western blot (*WB*) analysis with antibodies directed against ERK5, phos-ERK5, phos-ERK1/2 or actin as a loading control, as indicated. Densitometric analysis of ERK5 or ERK1/2 phosphorylation relative to actin displayed beneath the respective blots was performed using NIH ImageJ software, with the basal vehicle control set arbitrarily as 1.0. This experiment was repeated twice with similar results. *DMSO, dimethyl sulfoxide; ERK5, extracellular signal-regulated kinase 5; FCS, foetal calf serum; FTS, farnesylthiosalicylic acid; HDMEC, human dermal microvascular endothelial cell; PMA, phorbol 12-myristate 13-acetate; VEGF, vascular endothelial growth factor; WB, Western blot.*

3.7 VEGF-induced ERK5 activation is not mediated by PI3K or mTOR

3.7.1 Inhibition of PI3K or mTORC1 activity does not prevent VEGF-stimulated ERK5 activation

VEGF-stimulated activation of the PI3K-AKT signalling pathway has been shown to be important for the mediation of diverse physiological responses in endothelial cells, including: survival, migration and permeability (Gerber *et al.*, 1998b; Shiojima & Walsh, 2002). The catalytic activity of PI3K can be inhibited by the use of wortmannin, a potent and specific, non-competitive, irreversible inhibitor of PI3K activity (Arcaro & Wymann, 1993; Powis *et al.*, 1994). Wortmannin has been previously used at a concentration of 100 nM to inhibit VEGF-induced PI3K activation and to suppress

VEGF-induced activation of AKT in bovine capillary endothelial cells and HUVECs (Qi *et al.*, 1999; Gliki *et al.*, 2001).

To determine whether VEGF-stimulated ERK5 activation occurs via a PI3K-dependent pathway in HDMECs, cells were pre-incubated with various concentrations of wortmannin, prior to stimulation with VEGF (50 ng/ml) for 10 min. As a positive control to confirm that wortmannin was effective at inhibiting VEGF-induced activation of PI3K, Western blot analysis of cell lysates using an antibody directed against phos-AKT Ser^{473} was carried out (Cuenda & Alessi, 2000). It was found that pre-treatment with 100 nM wortmannin was sufficient to abolish VEGF-induced AKT Ser^{473} phosphorylation (**Fig. 3.14**), thus showing that VEGF-stimulates activation of AKT via a PI3K-dependent pathway in HDMECs.

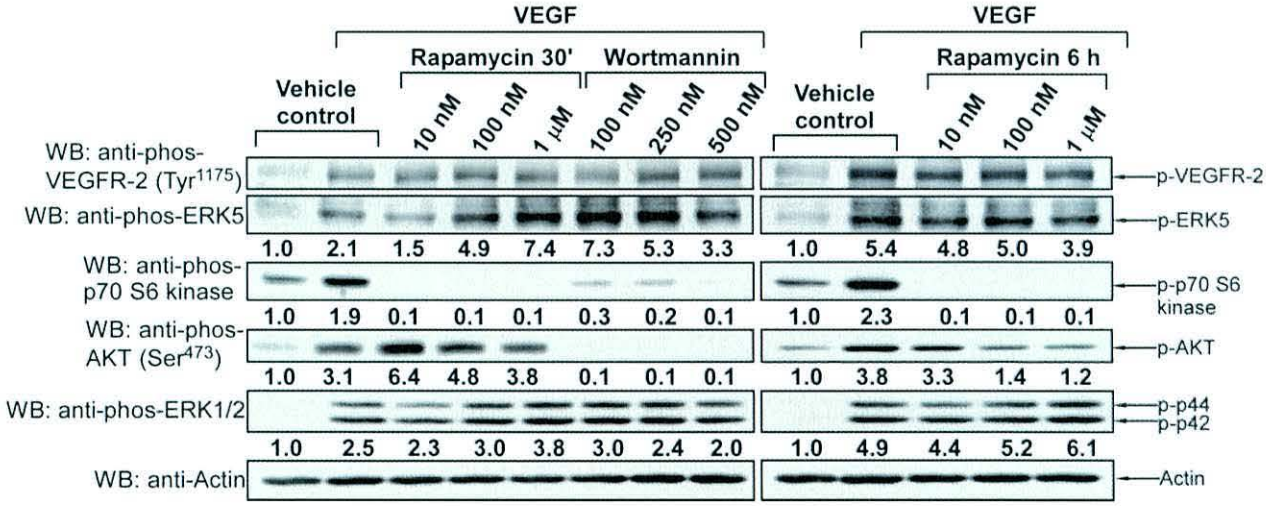


Figure 3.14 Effects of rapamycin and wortmannin treatment upon VEGF-stimulated ERK5 activation in HDMECs. HDMECs were seeded at 2.0 x 10^5 cells per well on gelatin-coated 6-well dishes in EBM MV2 growth medium and incubated for 24 h. Cells were serum-starved in EBM MV2 basal medium containing 1% (v/v) FCS for 20 h, then incubated with 0.1% (v/v) DMSO (vehicle control), or with the indicated concentrations of rapamycin for 30 min, or 6 h, as described or with the indicated concentrations of wortmannin for 30 min. Cells were then stimulated with VEGF (50 ng/ml) for 10 min. Cells were lysed, and total cell lysates were separated on a 10% SDS-PAGE gel and subjected to Western blot (*WB*) analysis with antibodies directed against phos-VEGFR-2^{1175}, phos-ERK5, phos-p70 S6 kinase, phos-AKT Ser^{473}, phos-ERK1/2 or actin as a loading control, as indicated. Densitometric analysis of protein phosphorylation relative to actin displayed beneath each blot was performed using NIH ImageJ software, with the basal vehicle control set arbitrarily as 1.0. This experiment was repeated three times with similar results. *DMSO, dimethyl sulfoxide; ERK5, extracellular signal-regulated kinase 5; FCS, foetal calf serum; HDMEC, human dermal microvascular endothelial cell; VEGF, vascular endothelial growth factor; VEGFR-2, VEGF receptor-2; WB, Western blot.*

Interestingly, treatment with 100 nM wortmannin potentiated VEGF-induced ERK5 activation, inducing a 3.5-fold increase in ERK5 phosphorylation by comparison to VEGF-treatment alone (**Fig. 3.14**). Increasing the dose of wortmannin above 100 nM had no inhibitory effect upon VEGF-induced ERK5 activation (**Fig. 3.14**). Wortmannin

did not affect VEGF-stimulated ERK1/2 activity (**Fig. 3.14**), in accordance with the reported findings of Gliki *et al.* (2001) in HUVECs. VEGF-stimulated VEGFR-2 Tyr^{1175} autophosphorylation was also unaffected by pre-incubation with wortmannin, as determined by Western blot analysis (**Fig. 3.14**), thus illustrating that wortmannin-induced augmentation of ERK5 activation was unrelated to any possible effects upon VEGFR-2.

Mammalian target of rapamycin (mTOR) protein is a serine/theonine kinase that is structurally similar to PI3K in its catalytic domain, and is a member of the PI3K-related protein kinases (Lovejoy & Cortez, 2009). The function of mTOR is dependent upon which proteins it forms complexes with. The mTOR complex 1 (mTORC1), consisting of mTOR, regulatory associated protein of mTOR (RAPTOR) and mLST8, has been implicated in the activation of p70 S6 kinase and regulation of cellular proliferation (Burnett *et al.*, 1998). By comparison, mTOR complex 2 (mTORC2), comprising mTOR, RICTOR, mLST8 and SIN1, has recently been shown to function as a so-called 'PDK2', and regulates AKT activity by phosphorylation of AKT on Ser^{473} (Sarbassov *et al.*, 2005; Jacinto *et al.*, 2006).

The immunosuppressive macrolide rapamycin, represents the prototypic mTOR inhibitor (Ballou & Lin, 2008). Rapamycin blocks mTOR activity indirectly by binding to FKBP12 (FK506-binding protein 12), forming an inhibitory complex, which then binds with high specificity to mTOR, and inhibits its association with RAPTOR, thus blocking mTORC1 activity (Abraham & Wiederrecht, 1996; Inoki *et al.*, 2005). Rapamycin-mediated inhibition of mTOR has been shown to inhibit endothelial cell proliferation and VEGF-driven angiogenesis *in vitro* and *in vivo* (Yu & Sato, 1999; Guba *et al.*, 2002; Phung *et al.*, 2006; Xue *et al.*, 2009). To investigate the potential effects of inhibition of the mTORC1→p70 S6 kinase pathway upon VEGF-induced activation of ERK5, HDMECs were subjected to acute (30 min) exposure to various concentrations of rapamycin, prior to stimulation with VEGF for 10 min, followed by cell lysis. Western blot analysis of the phosphorylation status of p70 S6 kinase on Thr^{389} was carried out as a positive control to test the effectiveness of rapamycin at inhibiting mTORC1 activity in HDMECs (Cuenda & Alessi, 2000). It was found that acute exposure to 10

nM rapamycin was sufficient to abolish VEGF-induced p70 S6 kinase phosphorylation in these cells (**Fig. 3.14, *p70 S6 kinase***). Analysis of phos-AKT Ser^{473} levels revealed that acute treatment with 10 nM or 100 nM of rapamycin induced a significant increase in phosphorylation of AKT on Ser^{473} by 52% and 35% respectively (**Fig. 3.14, *p-AKT***). Interestingly, this was accompanied by a concomitant increase in VEGF-stimulated ERK5 activation (**Fig. 3.14, *p-ERK5***). Together, these results suggest that VEGF-stimulated mTORC1 activity can be blocked by acute exposure to rapamycin (0.01-1 μM) in HDMECs, but that this is insufficient to inhibit VEGF-stimulated mTORC2 activity, in agreement with recent findings in HUVECs (Dormond *et al.*, 2007). Furthermore, the simultaneous increase in p-ERK5 levels in response to treatment with 0.1-1 μM may also indicate that mTORC1 negatively-regulates VEGF-induced ERK5 activity in HDMECs (**Fig. 3.14, *p-ERK5***).

3.7.2 Inhibition of mTORC2 activity does not prevent VEGF-stimulated ERK5 activation

By contrast to acute treatment of HDMECs with rapamycin, chronic exposure of cells to this compound has been shown to be sufficient to block the activity of the 'rapamycin-resistant' mTORC2 complex, resulting in the inhibition of AKT Ser^{473} phosphorylation (Sarbassov *et al.*, 2006). In agreement with this premise, chronic exposure to rapamycin has also been shown to inhibit VEGF-stimulated, mTORC2-mediated, phosphorylation of AKT on Ser^{473} in HUVECs (Dormond *et al.*, 2007). In order to assess whether prolonged exposure with rapamycin had an effect upon VEGF-stimulated ERK5 activation, HDMECs were pre-incubated with various concentrations of rapamycin for 6 h, prior to stimulation with VEGF (50 ng/ml) for 10 min. Western blot analysis of cell lysates revealed that chronic exposure of HDMECs to 100 nM rapamycin for 6 h lowered VEGF-stimulated AKT Ser^{473} phosphorylation by 63% (**Fig. 3.14**, ***rapamycin 6 h***). However, VEGF-stimulated ERK5 activation was not affected by these conditions (**Fig. 3.14**, ***rapamycin 6 h***). Together, these results suggest that mTORC2 activity is inhibited by chronic exposure to rapamycin in HDMECs, which prevents mTORC2-mediated phosphorylation of AKT on Ser^{473}. As ERK5 activation was not affected by these conditions, it can be concluded that it is unlikely that PI3K/AKT mediates VEGF-stimulated activation of ERK5 in these cells.

3.8 The MEK inhibitor U0126 does not inhibit VEGF-induced ERK5 activation in HDMECs

U0126 blocks ERK1/2 activity indirectly, by binding to, and inhibiting the activation of MEK1, in a non-ATP-competitive manner (Favata *et al.*, 1998). However, U0126 has also been reported to inhibit EGF-induced activation of transfected ERK5 in COS7 cells (Kamakura *et al.*, 1999) and endogenous ERK5 in HeLa cells (Mody *et al.*, 2001). It was therefore a possibility that U0126 may block VEGF-stimulated ERK5 activity in HDMECs. To test this, cells were incubated for 30 min with a range of concentrations of U0126 (1-100 μM), prior to stimulation, or not, with VEGF for 10 min (**Fig. 3.15**).

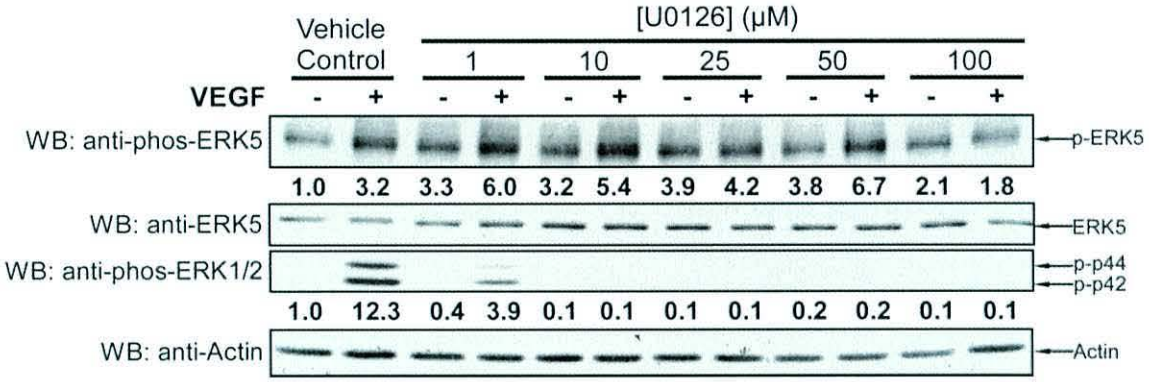


Figure 3.15 Effects of U0126 treatment upon VEGF-induced ERK5 activation in HDMECs. HDMECs were seeded at 2.0 x 10^5 cells per well on gelatin-coated 6-well dishes in EBM MV2 growth medium, and incubated for 24 h. Cells were serum-starved in EBM MV2 medium containing 1% (v/v) FCS for 20 h, then pre-incubated with the indicated concentrations of U0126 or 0.1% (v/v) DMSO (vehicle control) for 30 min prior to stimulation, or not (control), with 50 ng/ml VEGF for 10 min. Cells were lysed, and total cell lysates were separated on a 10% SDS-PAGE gel as described (*section 2.2.6.3*) and subjected to Western blot (*WB*) analysis with antibodies directed against phos-ERK5, ERK5, phos-ERK1/2 or actin as a loading control, as indicated. Densitometric analysis of ERK5 or ERK1/2 phosphorylation relative to actin displayed beneath the respective blots was performed using NIH ImageJ software, with the basal vehicle control set arbitrarily as 1.0. This experiment was repeated five times with similar results. *DMSO, dimethyl sulfoxide ; ERK, extracellular signal-regulated kinase; FCS, foetal calf serum; HDMEC, human dermal microvascular endothelial cell; VEGF, vascular endothelial growth factor; WB, Western blot.*

It was found that VEGF-stimulated ERK1/2 activation was lowered by 68% following treatment with 1 μM U0126 (**Fig. 3.15, *p-ERK1/2***), and was abolished at concentrations ≥10 μM (**Fig. 3.15, *p-ERK1/2***), thus showing, as expected, that U0126 is an effective inhibitor of VEGF-induced ERK1/2 activity in these cells. By contrast, both basal and VEGF-stimulated phosphorylation of ERK5 on Thr^{218}/Tyr^{220} was increased by U0126 treatment when used at 1-50 μM (**Fig. 3.15, *p-ERK5***). Although 100 μM U0126 decreased VEGF-stimulated ERK5 activation by 44%, this effect is unlikely to have been caused by specific inhibition of MEK5/ERK5 signalling (**Fig. 3.15, *p-ERK5***). In

conclusion, these data show that U0126 does not inhibit VEGF-induced ERK5 activation in HDMECs, and suggests that U0126-mediated inhibition of ERK1/2 activation leads to the potentiation of VEGF-stimulated ERK5 activation in these cells. This effect is similar to the reported effects of U0126 treatment upon oncostatin M- and EGF-stimulated ERK5 activation in human kidney-2 cells (Sarkozi *et al.*, 2007; Pollack *et al.*, 2007), and the effect of U0126 upon EGF- and H_2O_2-stimulated ERK5 activity in HeLa cells (Mody *et al.*, 2001). Thus providing further evidence of cross-talk between the ERK5 and ERK1/2 signalling pathways, in which ERK1/2 may exert a negative-regulatory effect upon ERK5 activation, as first suggested by Mody *et al.* (2001).

3.9 Discussion

The aim of this chapter was to characterise ERK5 activation in HDMECs, in an effort to define the upstream signalling pathways mediating ERK5 activation by pro-angiogenic molecules. Preliminary experiments showed that ERK5 underwent activation in response to certain stresses in HDMECs (**Fig. 1.2**), in agreement with the premise that ERK5 is a stress-activated kinase (Abe *et al.*, 1996; Wang *et al.*, 2006). ERK5 had also been shown to undergo activation in response to growth factor stimulation in a variety of cell types (Kamakura *et al.*, 1999). Accordingly, ERK5 activation in HDMECs was induced by the pro-angiogenic growth factors HGF and VEGF (**Fig. 1.3**). However, FGF-2 and EGF, both of which strongly induced ERK5 activation in HUVECs (Hayashi *et al.*, 2004), failed to stimulate ERK5 activity in HDMECs, thus likely further highlighting the existence of phenotypic differences between these endothelial cell types. All growth factors tested induced ERK1/2 activation in HDMECs (**Fig. 1.3**), suggesting that ERK1/2 can be activated by upstream pathways independent of those regulating ERK5 in these cells.

3.9.1 VEGF stimulates ERK5 activation via a VEGFR-2 Tyr^{1175}-dependent pathway

VEGF is critical for angiogenesis during embryonic development (Carmeliet *et al.*, 1996; Ferrara *et al.*, 1996), as well as for mediating endothelial cell survival during tumour angiogenesis (Benjamin & Keshet, 1997; Liu *et al.*, 2000). Research aimed at identifying intracellular arbitrators of VEGF-induced signalling events in endothelial cells has shown that VEGF, acting mainly via VEGFR-2, induces the activation of several

intracellular pathways (Olsson *et al.*, 2006; Holmes *et al.*, 2007). However, whilst VEGF has been previously reported to induce ERK5 activation in MLCECs and HUVECs (Hayashi *et al.*, 2004), the mechanisms by which VEGF induces ERK5 activation in human microvascular endothelial cells had not been previously demonstrated.

This study has shown that selective inhibition of VEGFR-2 activity using ZM323881 (Whittles *et al.*, 2002; Endo *et al.*, 2003) prevented VEGF-stimulated ERK5 activation in HDMECs, revealing that ERK5 activation is mediated by VEGFR-2 (**Fig. 3.4**). Furthermore, a particular requirement for phosphorylation of VEGFR-2 on Tyr^{1175} for VEGF-stimulated ERK5 activation was demonstrated (**Fig. 3.5**). Auto-phosphorylation of VEGFR-2 on Tyr^{1175} provides a binding site for a number of adaptor proteins including PLC-γ (Takahashi *et al.*, 2001) and is critical for normal VEGF-stimulated endothelial cell signalling, leading to certain VEGF-induced physiological responses in endothelial cells (Takahashi *et al.*, 2001; Meyer *et al.*, 2003; Dayanir *et al.*, 2001). Whilst further work would be required to confirm the exact mechanism linking VEGFR-2 Tyr^{1175} phosphorylation with ERK5 activation, evidence presented herein implies that VEGF-stimulated ERK5 activation may proceed via a PLC-γ-dependent pathway.

Knock-in mice in which Tyr^{1173} of *Flk-1* was substituted for Phe^{1173} were found to die during embryogenesis at E8.5-E9.5 due to defective vasculogenesis, characterised by a lack of organised blood vessels and yolk sac blood islands (Sakurai *et al.*, 2005), akin to the phenotype of *Flk1*-null mice (Shalaby *et al.*, 1995). Similarly, *Plcγ1*$^{-/-}$ mice die at approximately E9.0, with impaired erythrogenesis and vasculogenesis (Ji *et al.*, 1997; Liao *et al.*, 2002). In contrast, *Erk5*-null mice die at a later time point during development (E9.5-E11.5) with distinct vascular defects (Regan *et al.*, 2002; Sohn *et al.*, 2002; Yan *et al.*, 2003), suggesting that the requirement for *Plc-γ* for normal vascular development during embryogenesis is unlikely to be related to a conceivable involvement of ERK5 as a downstream effector of PLC-γ signalling. However, it remains feasible that VEGF-stimulated activation of ERK5, mediated via VEGFR-2 Tyr^{1175} and possibly PLC-γ, may have a functional role in endothelial cells *in vivo,* possibly at a later stage in development. Furthermore, the involvement of other phospho-VEGFR-2 Tyr^{1175}-binding adapter proteins such as Sck and Shb as possible

mediators of VEGF-stimulated ERK5 activation cannot be fully ruled out from the data presented in this chapter. It is suggested that further studies should aim to address these possibilities.

3.9.2 VEGF-stimulated ERK5 activation is dependent upon intracellular Ca^{2+}

Treatment with the cell-permeable Ca^{2+} chelator, BAPTA-AM (**Fig. 3.6**), revealed that VEGF-stimulated ERK5 activity in HDMECs is dependent upon $[Ca^{2+}]_i$. This finding is in accordance with previous reports that BAPTA-AM treatment abolished laminar flow-induced ERK5 activation in BAECs (Yan *et al.*, 1999), and EGF-stimulated ERK5 activation in mouse embryonic fibroblasts (Ji and Carpenter, 2000). Yan *et al.* also showed that treatment with the Ca^{2+} ionophore A23187 did not stimulate ERK5 activation in BAECs (Yan *et al.*, 1999). Similarly, ERK5 activity was not stimulated by A23187 treatment in COS7 cells (Kamakura *et al.,* 1999), which has led to the suggestion that ERK5 activation is Ca^{2+}-dependent, but that increased $[Ca^{2+}]_i$ alone is insufficient to induce ERK5 activation (Yan *et al.*, 1999). In contrast to these reports, this study has shown that treatment with A23187 strongly induced ERK5 activation in HDMECs (**Fig. 3.3; Fig. 3.6**), suggesting that Ca^{2+} is both necessary and sufficient to induce ERK5 activation in these cells.

Ca^{2+} is an ubiquitous second messenger critical for cell signalling and almost every aspect of normal cellular function (Clapham, 2007; Roderick & Cook, 2008). The finding that $[Ca^{2+}]_i$ was required for VEGF-stimulated activation of ERK5, but not that of ERK1/2, is intriguing and suggests that ERK5 is regulated by Ca^{2+}-dependent mechanisms independent to those regulating ERK1/2 activation in these cells. Yan *et al.* reported that Ca^{2+}-dependent laminar shear-stress-stimulated ERK5 activity was not affected following treatment with the Ca^{2+}/calmodulin-dependent kinase II (CaMK II) inhibitor KN-62 in BAECs (Yan *et al.*, 1999), suggesting that CaMK II may not be required for regulation of ERK5 activity in these cells. Two recent reports have implicated ERK5 as both a positive and negative regulator of the activity of the CaMK-dependent phosphatase, calcineurin (Abbasi *et al.*, 2006; Yang *et al.*, 2008). However, the identity of Ca^{2+}-dependent pathway(s) regulating VEGF-stimulated ERK5 activation in HDMECs were not investigated here. Given the profound effect of modulating

$[Ca^{2+}]_i$ levels upon ERK5 activation in endothelial cells, it is suggested that delineating the mechanisms regulating Ca^{2+}-dependent ERK5 activation in these cells would represent a key advancement in our comprehension of how ERK5 is regulated, and may prove to be of vital importance in fully understanding the role of ERK5 in endothelial cell function.

3.9.3 VEGF-stimulated ERK5 activity is not mediated by PI3K/mTOR in HDMECs

Whilst the majority of studies have shown that ERK5 is not activated downstream of PI3K (Sharma & Goalstone, 2005; Rovida *et al.*, 2008b; Finegan *et al.*, 2009; Lennartsson *et al.*, 2010), a recent study demonstrated that HGF-stimulated ERK5 activation in malignant mesothelioma cells was mediated by PI3K (Ramos-Nino *et al.*, 2008). VEGF stimulation has been previously shown to stimulate PI3K activation, and subsequent activation of AKT and p70 S6 kinase (Gerber *et al.*, 1998b; Yu & Sato, 1999). Results presented in this chapter confirm that PI3K mediates VEGF-induced activation of AKT in HDMECs. However, treatment with the PI3K inhibitor wortmannin did not inhibit VEGF-induced ERK5 activation (**Fig. 3.14**), indicating that ERK5 is not downstream of either PI3K or AKT in these cells (**Fig. 3.14**). Interestingly, treatment with wortmannin was found to potentiate VEGF-stimulated ERK5 activation, thus suggesting that PI3K may act as a negative-regulator of ERK5 activity in HDMECs. Similarly, inhibition of the mTORC1→p70 S6 kinase pathway by acute exposure to rapamycin, or inhibition of the mTORC2→AKT pathway by chronic exposure of HDMECs to rapamycin (Sarbassov *et al.,* 2006; Dormond *et al.,* 2007), did not affect VEGF-induced ERK5 activation (**Fig. 3.14**). Together these data suggest that ERK5 is not downstream of either p70 S6 kinase or AKT.

3.9.4 ERK5 activity is mediated by PKCδ in HDMECs

A number of studies have identified PKC as an important mediator of VEGF-induced signalling events in endothelial cells, including: endothelial cell proliferation, permeability, differentiation and survival (Xia *et al.*, 1996; Shen *et al.*, 1999; Taylor *et al.*, 2006). Current evidence suggests that a high degree of redundancy in function exists between individual PKC isoforms, despite the fact that PKC isoforms are widely expressed, and are highly conserved amongst eukaryotes, which would also imply that

distinct PKCs have unique roles within the cell (Parker & Murray-Rust, 2004). In endothelial cells, individual PKC isoforms have been shown to mediate diverse processes. For example, PKCα has been reported to regulate VEGF-induced endothelial cell proliferation, migration and tubular morphogenesis *in vitro* (Wellner *et al.*, 1999; Wang *et al.*, 2002). In addition, PKCε has been implicated in mediating several processes, including: VEGF-induced ERK1/2 activation, cell-cycle progression and endothelial cell survival (Rask-Madsen & King, 2008; Wu *et al.*, 2000a; Steinberg *et al.*, 2007).

A key finding presented herein was that VEGF-stimulated ERK5 activation is mediated by PKC, and is specifically dependent upon PKCδ in HDMECs. The requirement of PKC for ERK5 activation seems to be cell-type-dependent. For example, PMA has been reported to induce ERK5 activation in human Clara-like H441 cells (Reddy *et al.*, 2002) and in breast cancer cells (Villa-Moruzzi, 2007), suggesting that ERK5 may be regulated via DAG-dependent PKC isoforms in these cells. In contrast, ERK5 activity in rat vascular smooth muscle cells (Abe *et al.*, 1996) and in HeLa cells (Kato *et al.*, 1998), appears to be PKC-independent. In HDMECs, ERK5 activation was potently induced by treatment with PMA or the DAG-mimetic, DOG (**Fig. 3.7**), suggesting that ERK5 is activated via a PKC-dependent pathway in these cells. This hypothesis was confirmed by pharmacological inhibition of ERK5 activation using pharmalogical inhibitors of PKC activity: GFX109203X (**Fig. 3.8**) and calphostin C (data not shown), which both inhibit a broad-range of PKC isoforms via distinct mechanisms of action. Finally, the specific PKC isoform mediating VEGF-stimulated ERK5 activatory signals in HDMECs was identified as PKCδ (**Fig. 3.12**). When considered together, data presented in this chapter suggest that VEGF can stimulate ERK5 activation in HDMECs via a VEGFR-2→PLC-γ→DAG→PKCδ-dependent pathway as outlined in **Fig. 3.16**.

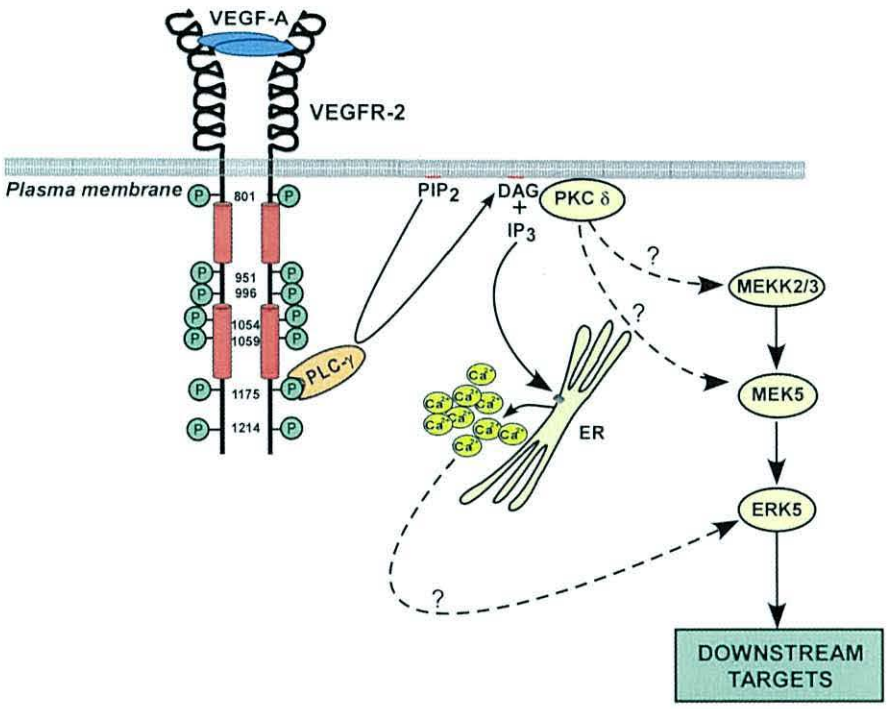


Figure 3.16 Hypothesised mechanism of VEGF-induced activation of ERK5 in HDMECs. VEGF stimulates ERK5 activation via activation of VEGFR-2. PLC-γ binding to phosphorylated Tyr^{1175} of VEGFR-2, results in the hydrolysis of PIP_2, generating DAG and IP_3. IP_3 acts on its cognate receptors (IP_3Rs) in the ER, inducing the release of Ca^{2+} from this intracellular store. Intracellular Ca^{2+} regulates ERK5 activation in HDMECs via an unknown mechanism. DAG is a physiological activator of classical and novel PKC isoforms, and is essential for ERK5 activation in HDMECs. The DAG-regulated, novel PKC isoform, PKCδ, is a mediator of VEGF-stimulated ERK5 activation in HDMECs. VEGF-induced activation of ERK5 via this pathway is hypothesised to phosphorylate downstream targets to modulate the angiogenic response of endothelial cells to VEGF-stimulation. *DAG, diacylglycerol; ER, endoplasmic reticulum; ERK5, extracellular signal-regulated kinase 5; HDMEC, human dermal microvascular endothelial cell; IP_3, inositol (1,4,5)-trisphosphate; IP_3R, IP_3 receptor; MEK5, mitogen-activated protein kinase(MAPK)/ERK kinase 5; MEKK2/3, MAPK/ERK kinase kinase 2/3; PIP_2, phosphatidylinositol (4,5)-bisphosphate; PKC, protein kinase C; PLC-γ, phospholipase C-γ; VEGF, vascular endothelial growth factor; VEGFR-2, VEGF receptor-2.*

Additional work would be required to confirm the functional significance of PKCδ-mediated regulation of VEGF-induced ERK5 activation in HDMECs. Phosphorylation of PKCδ on distinct tyrosine residues has been shown to be a critical determinant of its cellular function including protection against apoptosis (Kronfeld *et al.*, 2000; Okhrimenko *et al.*, 2005). In endothelial cells, phosphorylation of PKCδ on Thr^{505} was necessary to mediate VEGF-induced AKT phosphorylation (Gliki *et al.*, 2002). Whilst the observation that PKCδ regulates ERK5 activation in response to VEGF is interesting, it is most probable that other pathways are also involved in regulating ERK5 activity in endothelial cells. Deletion of *Erk5* in mice leads to death at around E10.5 due to cardiovascular defects and loss of endothelial cell integrity (Regan *et al.*, 2002; Sohn *et al.*, 2002; Yan *et al.*, 2003). However, *Pkcδ*-null mice are reported to develop normally and are fertile (Leitges *et al.*, 2001; Miyamoto *et al.*, 2002), with no reported defects in endothelial cells. Even so, Leitges and co-workers have demonstrated that $Pkc\delta^{-/-}$ mice

exhibited increased arteriosclerosis due to decreased smooth muscle cell apoptosis (Leitges *et al.*, 2001). Considerable redundancy in function of individual PKC isoforms is believed to occur (Parker & Murray-Rust, 2004); it is therefore possible that other PKC isoforms are capable of arbitrating ERK5 activation. Furthermore, whilst PKCδ may be dispensable during embryonic development, it may be required for endothelial cell signalling in adult cells. Alternatively, PKCδ may be required exclusively during VEGF-stimulated ERK5 activation. It would be of interest to determine the physiological effects of siRNA-mediated down-regulation of PKCδ expression in HDMECs by means of various functional assays. Further studies should also aim to over-express constitutively active PKCδ to ascertain whether activation of ERK5 and AKT may be induced in HDMECs by such treatment.

3.9.5 Concluding remarks

Defining the regulatory pathways that arbitrate activation of a given MAPK, in response to extracellular stimuli, can provide important clues regarding the likely function of that protein within the cell. In summary, this chapter represents the first study aimed at delineating the pathways that mediate pro-angiogenic growth factor-induced activation of ERK5 in endothelial cells. The data shows, for the first time in human microvascular endothelial cells, that VEGF is a potent inducer of ERK5 activity; furthermore, the upstream pathways by which VEGF mediates ERK5 activity in HDMECs have begun to be uncovered. This work establishes ERK5 as a signalling molecule operating downstream of VEGFR-2, which is dependent upon VEGFR-2 Tyr^{1175} phosphorylation, $[Ca^{2+}]_i$, DAG and PKCδ. In addition, VEGF-stimulated ERK5 activity was found to occur independently of PI3K, mTORC2, and Ras activity in HDMECs. Importantly, this chapter also shows that ERK5 is activated by pathways different from those that mediate VEGF-stimulated activation of ERK1/2 - the MAPKs that share most similarity with ERK5 – suggesting that these MAPKs may perform distinct functions in response to VEGF-stimulation in endothelial cells.

CHAPTER FOUR

Physiological role of VEGF-stimulated ERK5 activity in HDMECs: Studies using small interfering RNA and over-expression

4.1 Introduction

VEGF-induced signalling via its cognate receptor VEGFR-2, is essential for vasculogenesis and angiogenesis during embryonic development, and in early postnatal life (Carmeliet *et al.*, 1996; Ferrara *et al.*, 1996; Gerber *et al.*, 1999). Furthermore, VEGF-VEGFR-2 signalling is critical for endothelial cell survival during tumour angiogenesis, which ultimately facilitates tumour progression and metastasis (Benjamin & Keshet, 1997; Liu *et al.*, 2000). It is known that VEGF-induced, VEGFR-2-mediated signals bring about a diverse array of physiological responses in endothelial cells including: proliferation, migration, differentiation and survival (Holmes *et al.*, 2007). These responses are regulated within the cell by various intracellular signalling pathways downstream of VEGFR-2 (Olsson *et al.*, 2006; Holmes *et al.*, 2007).

ERK5 had been reported to become activated following VEGF stimulation in HUVECs and MLCECs (Hayashi *et al.*, 2004). Furthermore, data presented in chapter 3 of this study revealed that ERK5 is strongly activated in response to VEGF stimulation via a VEGFR-2-dependent pathway in HDMECs (*chapter 3*). Importantly however, the physiological significance of VEGF-stimulated ERK5 activity in response to VEGF stimulation in endothelial cells had not been previously reported. It therefore remained a distinct possibility that ERK5 may mediate VEGF/VEGFR-2-stimulated signals to regulate endothelial cell migration, proliferation, differentiation, and survival to facilitate angiogenesis. To address this possibility, it was firstly necessary to specifically manipulate ERK5 expression levels and ERK5 activity in HDMECs prior to quantifying the resultant effects upon VEGF-induced physiological responses in these cells.

Available approaches to assess the functional role of a protein kinase *in vitro* include the inhibition of kinase activity by means of specific small-molecule pharmacological inhibitors. However, at the commencement of this work, no MEK5/ERK5-specific inhibitor was available. Furthermore, studies aimed at characterising commercially available MEK1/2 inhibitors that had been reported to also inhibit ERK5 activity in some cells (Kamakura *et al.*, 1999; Mody *et al.*, 2001), had proven that these agents did

not inhibit VEGF-induced ERK5 activation in HDMECs (**Fig. 3.15** and data not shown). Use of a pharmacological inhibitor to directly inhibit ERK5 activity was therefore not an option. In the absence of a specific ERK5 inhibitor, alternative *in vitro* methods of manipulating ERK5 activity included silencing of ERK5 expression by RNA interference (RNAi) technology or over-expression of constitutively active (CA) or dominant-negative (DN) versions of ERK5, to enhance, or abolish ERK5 activity, respectively.

The use of RNAi as a tool to assess gene function has developed rapidly from the initial discovery in *Caenorhabditis elegans* that introduction of small double-stranded RNA (dsRNA), complementary to the sequence of a gene of interest would allow robust, specific degradation of a target mRNA, thereby inhibiting the mRNA function, thus down-regulating the expression of the protein (Fire *et al.*, 1998). RNAi technology has been shown to be an effective method of post-transcriptional gene silencing in a wide range of eukaryotic organisms (Sharp, 2001). The mechanism of RNAi is triggered by the initial recognition and cleavage of foreign dsRNA by an RNA III nuclease termed Dicer, into short dsRNA measuring 21-28 nucleotides in length termed small interfering RNA (siRNA) (Bernstein *et al.*, 2001; Zhang *et al.*, 2002). SiRNAs are then incorporated into a silencing complex termed RISC (RNA-induced silencing complex), allowing the complex to be guided to the target mRNA sequence, leading to consequent degradation of the target mRNA, thus preventing its translation into a functional protein (Hammond *et al.*, 2000).

Transfection of dsRNA is an effective method of gene silencing in most eukaryotic cell types, however in mammalian cells, transfection of dsRNAs greater than 30 nucleotides in length can activate the interferon response, resulting in non-specific inhibition of mRNA translation and degradation of global mRNA (McManus & Sharp, 2002; Stark *et al.*, 1998). It is now generally accepted that by transfecting synthetic siRNAs of 21-22 nucleotides in length, induction of the interferon response can be circumvented, whilst still allowing knockdown of gene expression (Elbashir *et al.*, 2002). However, some cases of sequence- and concentration-dependent siRNA-stimulated transcriptional up-regulation of interferon-response genes in mammalian cells have been reported, illustrating that the interferon response should be assessed

when using siRNA in functional studies (Persengiev *et al.*, 2004; Sledz & Williams, 2004). SiRNA-mediated silencing of gene expression in endothelial cells has been successfully applied in several recent studies to allow the study of the functional role of proteins of interest in regulating endothelial cell function (Holmqvist *et al.*, 2004; Tomasi *et al.*, 2006; Dimberg *et al.*, 2008; Mellberg *et al.*, 2009).

Plasmid constructs containing CA rat *Mek5* and DN human *ERK5* have been generated (Kato *et al.*, 1997). Transfection of these constructs has been shown to offer an effective means to induce, and abolish, ERK5 activity respectively, in various cells (Kato *et al.*, 1997; Barros & Marshall, 2005; Schweppe *et al.*, 2006). Therefore, in this study, to further confirm the effects of siRNA-mediated silencing of ERK5 expression in HDMECs, cells were transiently transfected with wild-type (wt) ERK5, ERK5(wt); DN ERK5, DN-ERK5(AEF); and CA MEK5, CA-MEK5(D).

The process of angiogenesis *in vivo* involves several co-ordinated steps whereby endothelial cells adhere to the ECM, migrate, proliferate, and ultimately differentiate to form a functional lumen-containing vessel. Each of these distinct steps can be effectively modelled by the use of various specialised assays that have facilitated the study of angiogenesis *in vitro* (Donovan *et al.*, 2001; Tomasi *et al.*, 2006; Goodwin, 2007; Staton *et al.*, 2009). Importantly, this has offered the added benefit of the study of the relative contribution of a given protein in discrete phases of angiogenesis. Experiments presented within this chapter detail the optimisation, and validation of siRNA-mediated knockdown of various components of the ERK5 signalling cascade and the subsequent use of siRNAs in *in vitro* functional angiogenesis assays, to assess the role of the ERK5 signalling pathway in distinct phases of angiogenesis, namely: endothelial cell proliferation, adhesion, migration, tubular morphogenesis and survival. Furthermore, the functional effects of transient transfection of ERK5, CA-MEK5(D) and DN-ERK5(AEF) on HDMEC proliferation and tubular morphogenesis were also assessed, and are described herein.

4.2 Optimisation of siRNA transfections in HDMECs

4.2.1 Selection of siRNA

Three commercially-available siRNA duplexes targeting the human *ERK5* gene (*MAPK7*) namely: Hs_MAPK7_9; Hs_MAPK7_5 and Hs_MAPK7_10 – termed ERK5 siRNA 1, ERK5 siRNA 2 and ERK5 siRNA 3 respectively, for the purpose of this study – (*Appendix 2*), were purchased from Qiagen (Crawley, U.K.). In addition, as a negative control in all experiments, cells were transfected with a non-silencing (N.S.) control siRNA (Qiagen).

4.2.2 Optimisation of ERK5 siRNA

Initial transfections were performed using the transfection reagent Lipofectamine™ 2000 (Invitrogen, Paisley, U.K.) following a protocol described by (Nishiwaki *et al.*, 2003). However, the use of a novel transfection reagent termed Lipofectamine™ RNAi MAX (Invitrogen) was found to provide efficient knockdown but with considerably less cell death, as assessed by the number of detached cells 24 h post-transfection (data not shown). It was also observed that the amount of Lipofectamine™ RNAi MAX used could be reduced to 0.1% (v/v) of the total transfection volume, whilst maintaining a similar transfection efficiency of 95%, as determined by fluorescent analysis of cells transfected with Alexa Fluor® 488-labelled N.S. siRNA at 24 h post-transfection (data not shown).

4.2.3 Validation and optimisation of ERK5 siRNA

In order to verify whether all three ERK5-specific siRNA duplexes were able to down-regulate ERK5 expression in HDMECs, and to optimise the concentration of siRNA required to achieve ERK5 knockdown, ERK5 siRNAs -1, -2 and -3, as well as the N.S. siRNA control siRNA were titrated, and ERK5 expression was assessed by lysing cells at 48 h post-transfection, and performing Western blot analysis using an anti-ERK5 antibody (**Fig. 4.1 A**). The validated siRNA, ERK5 siRNA 1, and the non-validated siRNA, ERK5 siRNA 2, both down-regulated ERK5 expression by 80-90% when used at a concentration of 10 nM (**Fig. 4.1 A**). By contrast, transfection of HDMECs with ERK5 siRNA 3 did not efficiently down-regulate ERK5 expression, only inducing a 40% decrease in ERK5 expression when used at 10 nM (**Fig. 4.1 A**). Whilst N.S. siRNA had

no effect upon ERK5 expression at concentrations <25 nM, N.S. siRNA treatment non-specifically lowered ERK5 expression by 50% when used at 50 nM (**Fig. 4.1 A**). Based on these observations, ERK5 siRNA was used at a final concentration of 10 nM, for silencing of ERK5 expression in future experiments, and N.S. siRNA was used at a final concentration of 10 nM as a negative control.

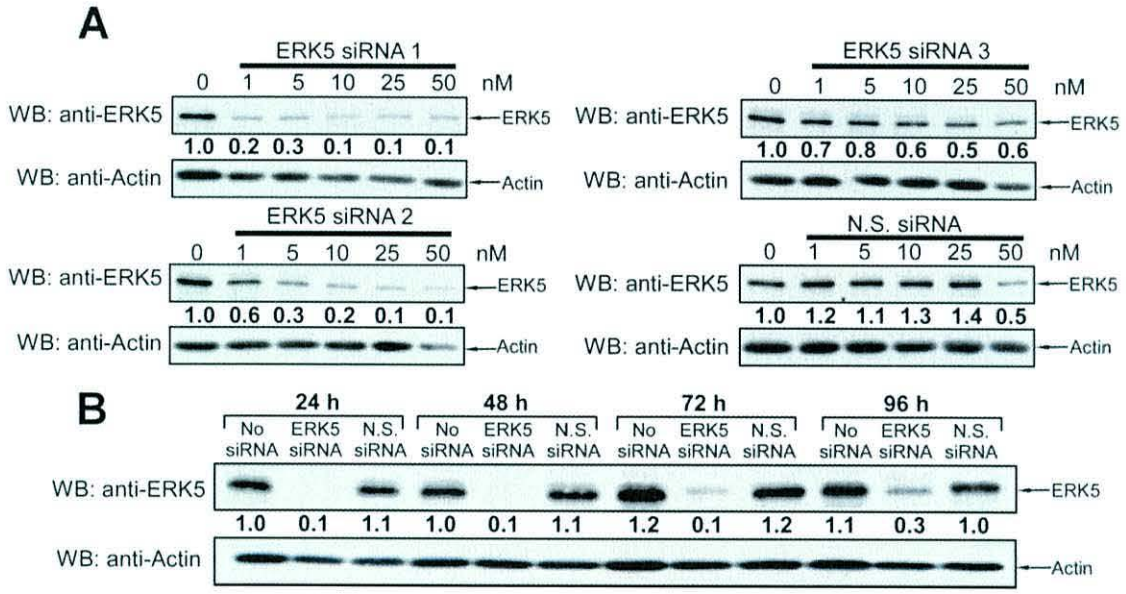


Figure 4.1 Validation of ERK5 down-regulation with siRNA. (**A**) HDMECs were seeded at 1.0 x 10^5 cells per well on gelatin-coated 6-well plates for 24 h. Cells were transfected with the indicated amounts of siRNA duplexes directed against *ERK5* - Hs_MAPK7_9 siRNA; Hs_MAPK7_5 siRNA; Hs_MAPK7_10 siRNA – (ERK5 siRNA 1, 2 and 3 respectively) or with a non-silencing (N.S.) control siRNA as described (*section 2.2.2.7.2*). Alternatively, cells were treated with the transfection reagent Lipofectamine™ RNAi MAX as a mock transfection control. At 48 h post-transfection, cells were lysed and proteins separated on a 10% SDS-PAGE gel, prior to Western blotting (*WB*) with antibodies directed against ERK5 and actin as a loading control. (**B**) ERK5 siRNA induced sustained down-regulation of ERK5 expression. HDMECs were seeded at 1.0 x 10^5 cells per well on gelatin-coated 6-well plates for 24 h. Cells were transfected with ERK5 siRNA 1 (5 nM) and ERK5 siRNA 2 (5 nM) (ERK5 siRNA), or with N.S. siRNA (10 nM) as described (*section 2.2.2.7.2*), or without siRNA, but with the transfection reagent Lipofectamine™ RNAi MAX as a mock transfection control (No siRNA). Cells were lysed at 24 h, 48 h, 72 h and 96 h post-transfection, as indicated, and resolved on a 10% SDS-PAGE gel prior to Western blotting (*WB*) with antibodies directed against ERK5, or actin as a loading control. Expression of ERK5 relative to actin was quantified by densitometric analysis with NIH ImageJ software, and is displayed at the bottom of each blot. *ERK5, extracellular signal-regulated kinase 5; HDMEC, human dermal microvascular endothelial cell; N.S., non-silencing; siRNA, small interfering RNA; WB, Western blot.*

The intended end use of siRNA-mediated silencing of ERK5 expression in HDMECs was as a tool to determine the effects of silencing ERK5 expression in functional cell-based assays, to assess the relative importance of ERK5 in distinct stages of angiogenesis. As these assays often involved long incubation periods of >48 h, the relative efficiency of siRNA-induced down-regulation of ERK5 over four days was quantified to determine the level of siRNA-mediated ERK5 down-regulation over this time. HDMECs were mock-transfected (treated with Lipofectamine™ RNAi MAX only), or were transfected

with 10 nM ERK5 siRNA (5 nM ERK5 siRNA 1 + 5 nM ERK5 siRNA 2) or with 10 nM N.S. siRNA as a negative control. Cells were lysed at 24-, 48-, 72- and 96-h post-transfection, and ERK5 expression was analysed by Western blotting. It was found that transfection of ERK5 siRNA down-regulated ERK5 expression by 90% compared to mock-transfected cells by 24 h post-transfection, and that this level of knockdown was maintained for at least 72 h (**Fig. 4.1 B**). By 96 h post-transfection, ERK5 expression in HDMECs had partially recovered, but remained 70% lower than the N.S. siRNA control transfected cells (**Fig. 4.1 B**). These results revealed that ERK5 expression could be efficiently down-regulated over several days using ERK5-specific siRNAs.

4.2.4 Validation and optimisation of MEK5 siRNA

Four commercially-available siRNA duplexes directed against MEK5, the kinase directly upstream of ERK5, were purchased from Qiagen (*Appendix 2*). Optimisation experiments designed to assess the knockdown efficiency of these siRNAs, termed herein MEK5 siRNA -1, -2, -3 and -4, involved titration of the siRNAs to be transfected and assessment of knockdown efficiency, by qRT-PCR (**Fig. 4.2 A**) and by Western blot-analysis (**Fig. 4.2 B**), at 24 h- and 48 h-post-transfection, respectively. MEK5 siRNA 1 and MEK5 siRNA 2 induced a 90% down-regulation of both *MEK5* mRNA and MEK5 protein levels compared to that of N.S. siRNA when used at 5 nM (**Fig. 4.2, A and B**). By contrast, MEK5 siRNA 3 treatment only induced a 50% reduction in MEK5 expression when used at 10 nM. Furthermore, analysis of actin expression at 48 h post-transfection in MEK5 siRNA 3- and MEK5 siRNA 4-transfected cells suggested that these duplexes were also non-specifically lowering actin expression (**Fig. 4.2 B**). From these findings, it was decided to use MEK5 siRNA 1 and MEK5 siRNA 2 at 10 nM for future experiments. Analysis of ERK5 Thr^{218}/Tyr^{220} phosphorylation by Western blotting confirmed that siRNA-mediated down-regulation of MEK5 expression in HDMECs was sufficient to block VEGF-stimulated activation of ERK5 (**Fig. 4.2 C**).

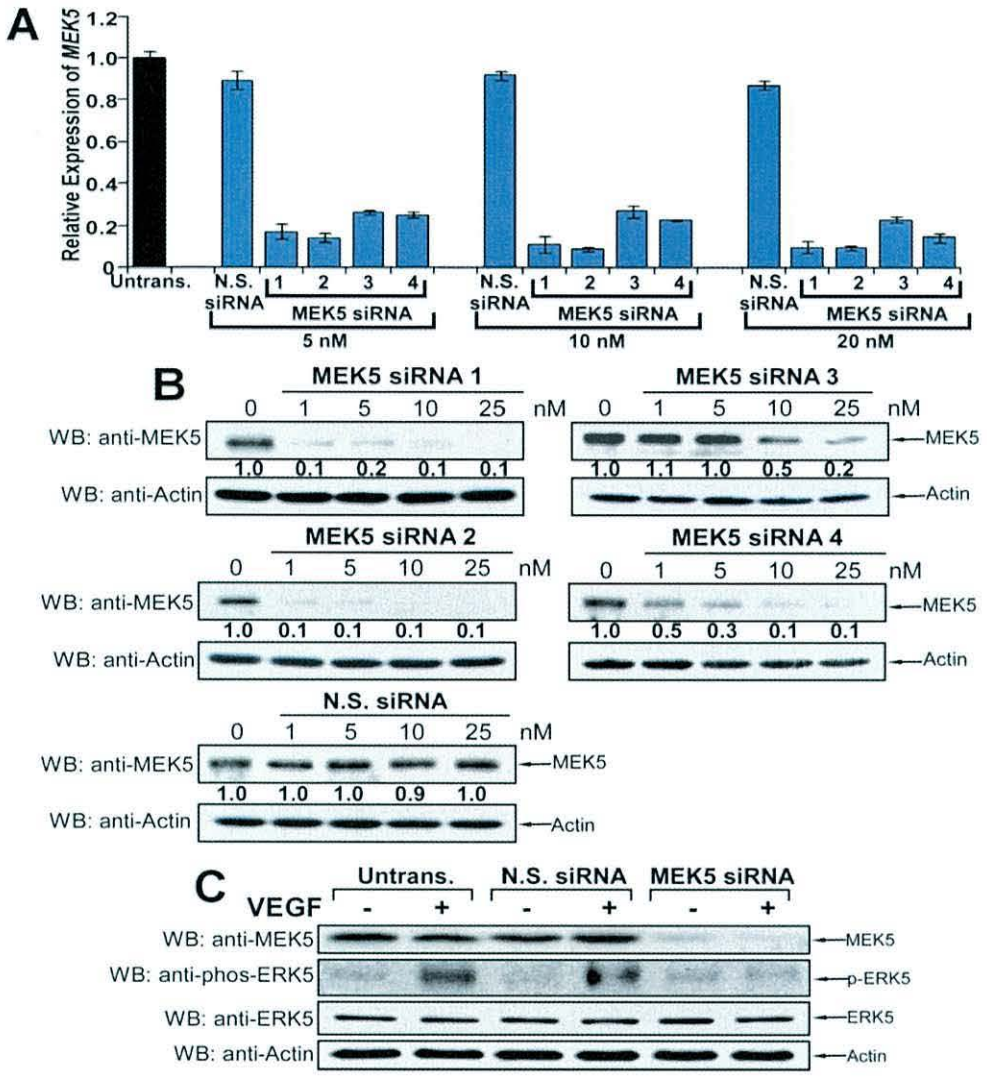


Figure 4.2 Validation of MEK5 down-regulation with siRNA. (**A**) HDMECs were seeded at 1.0 x 10^5 cells per well on gelatin-coated 6-well plates for 24 h. Cells were transfected with the indicated amounts of siRNA duplexes directed against *MEK5*, termed: MEK5 siRNA -1, -2, -3 and -4 (Hs_MAP2K5_11 siRNA; Hs_MAP2K5_12 siRNA; Hs_MAP2K5_9 siRNA and Hs_MAP2K5_10 siRNA respectively) or with a non-silencing (N.S.) control siRNA as described (*section 2.2.2.7.2*). Alternatively, cells were treated with the transfection reagent Lipofectamine™ RNAi MAX as a mock transfection control. At 24 h post-transfection, total RNA was harvested, followed by reverse-transcription cDNA synthesis. cDNA from each experimental condition was analysed by quantitative real-time PCR (qRT-PCR), for expression of *MEK5* and the control housekeeping gene *β-actin*. Absolute quantification of *MEK5* expression was performed by standard-curve analysis (*section 2.2.5.1*). Normalised *MEK5* expression is presented relative to the mock-transfected control, which was set arbitrarily as 1.0. (n = 3; mean ± SD). (**B**) Effects of MEK5 siRNA on MEK5 protein expression. HDMECs were seeded at 1.0 x 10^5 cells per well on gelatin-coated 6-well plates for 24 h. Cells were transfected with the indicated amounts of siRNA duplexes directed against MEK5: Hs_MAP2K5_11 siRNA; Hs_MAP2K5_12 siRNA; Hs_MAP2K5_9 siRNA and Hs_MAP2K5_10 siRNA (MEK5 siRNA 1, 2, 3 and 4 respectively) or against a non-silencing (N.S.) control siRNA as described (*section 2.2.2.7.2*). Alternatively, cells were treated with the transfection reagent Lipofectamine™ RNAi MAX as a mock transfection control. At 48 h post-transfection, cells were lysed and total cellular proteins were separated on a 10% SDS-PAGE gel, prior to Western blot (*WB*) analysis with antibodies directed against MEK5 and actin as a loading control. Expression of MEK5 relative to actin was quantified by densitometric analysis with NIH ImageJ software, and is displayed at the bottom of each blot. (**C**) Effects of MEK5 siRNA on VEGF-stimulated ERK5 activation. HDMECs were seeded at 1.0 x 10^5 cells per well on gelatin-coated 6-well plates for 24 h. Cells were transfected with 10 nM of MEK5 siRNA 1 or with 10 nM N.S. siRNA as described (*section 2.2.2.7.2*), or were left untransfected (Untrans.). At 24 h post-transfection, cells were lysed serum starved in EBM MV2 basal medium containing 1% (v/v) FCS for 20 h prior to stimulation with VEGF (50 ng/ml) for 10 min. Cells were lysed, and total cellular proteins were separated on a 10% SDS-PAGE gel, prior to Western blot (*WB*) analysis with antibodies directed against MEK5, p-ERK5, ERK5 and actin as a loading control. *MEK5, MAPK/ERK kinase 5; HDMEC, human dermal microvascular endothelial cell; N.S., non-silencing; siRNA, small interfering RNA; WB, Western blot.*

4.2.5 Validation and optimisation of MEF2C siRNA

MEF2C represents the best characterised downstream target of ERK5 (Kato *et al.*, 1997; Yang *et al.*, 1998a; Kato *et al.*, 2000). In addition, it has been suggested that MEF2C acts as a critical effector of ERK5-mediated endothelial cell survival (Hayashi *et al.*, 2004; Olson, 2004). Due to these factors, it was of interest to attempt to manipulate MEF2C expression in HDMECs, with the ultimate goal of assessing the relative contribution of MEF2C in distinct phases of angiogenesis in HDMECs.

Preliminary validation experiments, in which HDMECs were transfected with commercially-available MEF2C siRNAs purchased from Qiagen (Crawley, U.K.), did not result in the efficient down-regulation of MEF2C expression (Dr. Katherine Holmes, University of Liverpool, personal communication, data not shown). A search for alternative commercially-available siRNAs led to the purchase from Dharmacon RNAi technologies (Lafayette, CO, U.S.A.), four MEF2C-specific siRNA duplexes termed: J-009455-07, J-009455-08, J-009455-05 and J-009455-06, referred to hereinafter as MEF2C siRNA 1, MEF2C siRNA 2, MEF2C siRNA 3 and MEF2C siRNA 4 respectively (*Appendix 2*). Validation of MEF2C siRNA was conducted by Dr. Katherine Holmes (University of Liverpool). Quantitative RT-PCR analysis of *MEF2C* mRNA expression in HDMECs at 24 h post-transfection with 10 nM of each MEF2C-specific siRNA or with 10 nM of N.S. siRNA revealed that MEF2C siRNA 1 and MEF2C siRNA 2 down-regulated *MEF2C* expression by more than 80% in HDMECs, whereas transfection of MEF2C siRNA 3 and MEF2C siRNA 4 had a less substantial effect upon *MEF2C* expression (**Fig. 4.3 A**). To confirm that MEF2C siRNA 1 and MEF2C siRNA 2 were effective at down-regulating MEF2C protein expression, these siRNAs were used at 10 nM to transfect HDMECs and cell lysates were prepared 48 h post-transfection. Western blot analysis confirmed that treatment with MEF2C siRNA 1 or MEF2C siRNA 2 was sufficient to lower MEF2C expression by 80% and 90% respectively, by comparison to the untransfected control (**Fig. 4.3 B**).

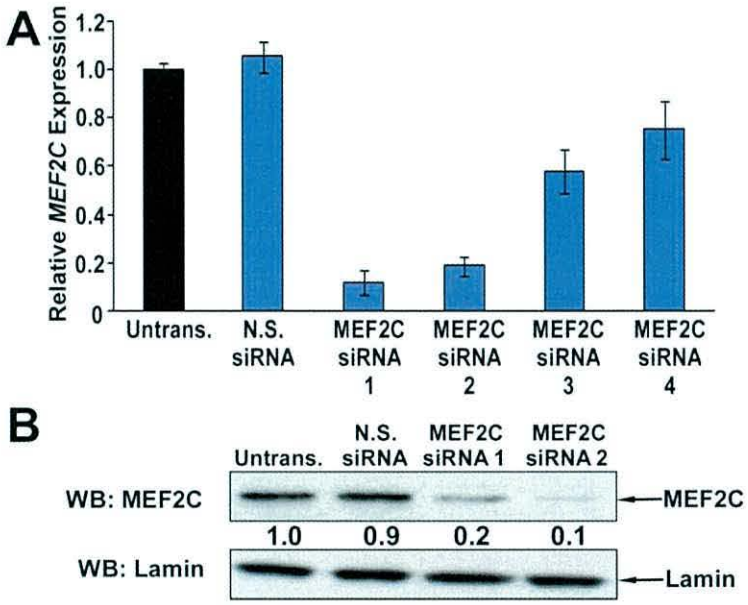


Figure 4.3 Validation of MEF2C down-regulation with siRNA. HDMECs were seeded at 1.0 x 10^5 cells per well on gelatin-coated 6-well plates for 24 h. Cells were transfected with the 10 nM of siRNA directed against *MEF2C* (MEF2C siRNA 1, MEF2C siRNA 2, MEF2C siRNA 3 or MEF2C siRNA 4), or with siRNA directed against a non-silencing (N.S.) control siRNA as described (*section 2.2.2.7.2*). Alternatively, cells were left untransfected (Untrans.). (**A**) Effects of MEF2C siRNA on MEF2C mRNA expression. At 24 h post-transfection with siRNA, total RNA was extracted from the cells as described (*section 2.2.4.1*), and expression of MEF2C and the housekeeping gene *β-actin* was analysed by qRT-PCR. Absolute quantification of *MEF2C* expression was performed by standard-curve analysis (*section 2.2.5.1*). Normalised *MEF2C* expression is presented relative to the mock-transfected control, which was set arbitrarily as 1.0. (n = 3; mean ± SD). (**B**) At 48 h post-transfection, cells were lysed and total cellular proteins were separated on a 10% SDS-PAGE gel, prior to Western blot (*WB*) analysis with antibodies directed against ERK1/2, ERK5 or Lamin B1 as a loading control. Expression of MEF2C relative to actin was quantified by densitometric analysis with NIH ImageJ software, and is displayed at the bottom of each respective lane. *ERK1/2, extracellular signal-regulated kinase 1 and 2; ERK5, extracellular signal-regulated kinase 5; HDMEC, human dermal microvascular endothelial cell; MEF2C, myocyte enhancer factor 2C; N.S., non-silencing; siRNA, small interfering RNA; WB, Western blot.*

4.2.6 Validation and optimisation of ERK1 and ERK2 siRNA

ERK5 shares greatest sequence identity with the classical MAPK members ERK1 and ERK2 (Lee *et al.*, 1995; Zhou *et al.*, 1995). It was therefore decided to utilise siRNA-mediated gene silencing of these MAPKs as controls in certain functional studies when analysing the effects of ERK5 siRNA transfection on HDMEC physiology. The knockdown efficiency of two siRNAs directed against human *ERK1* (Hs_MAPK3_6 HP and Hs_MAPK3_7 HP) and two siRNAs directed against ERK2 (Hs_MAPK1_10_HP and Hs_MAPK1_11 HP) or four siRNAs in combination to target both ERK1 and ERK2 (*Appendix 2*), was assessed by Western blotting (**Fig. 4.4**). Treatment with 2.5 nM of Hs_MAPK3_6 HP and 2.5 nM of Hs_MAPK3_7 HP siRNA, to give a total concentration of 5 nM ERK1 siRNA, was sufficient to inhibit ERK1 expression without affecting ERK2 expression (**Fig. 4.4**). Similarly, a total concentration of 5 nM ERK2 siRNA was sufficient

to abolish the expression of ERK2 without affecting ERK1 expression (**Fig. 4.4**). Western blot analysis revealed that ERK5 expression was unaffected by treatment with ERK1 siRNA, ERK2 siRNA or ERK1/2 siRNAs in combination, even at concentrations of 25 nM, thus confirming the specificity of the knockdown. From these experiments ERK1 siRNA, ERK2 siRNA and ERK1/2 siRNA were used at a final concentration of 10 nM in functional studies.

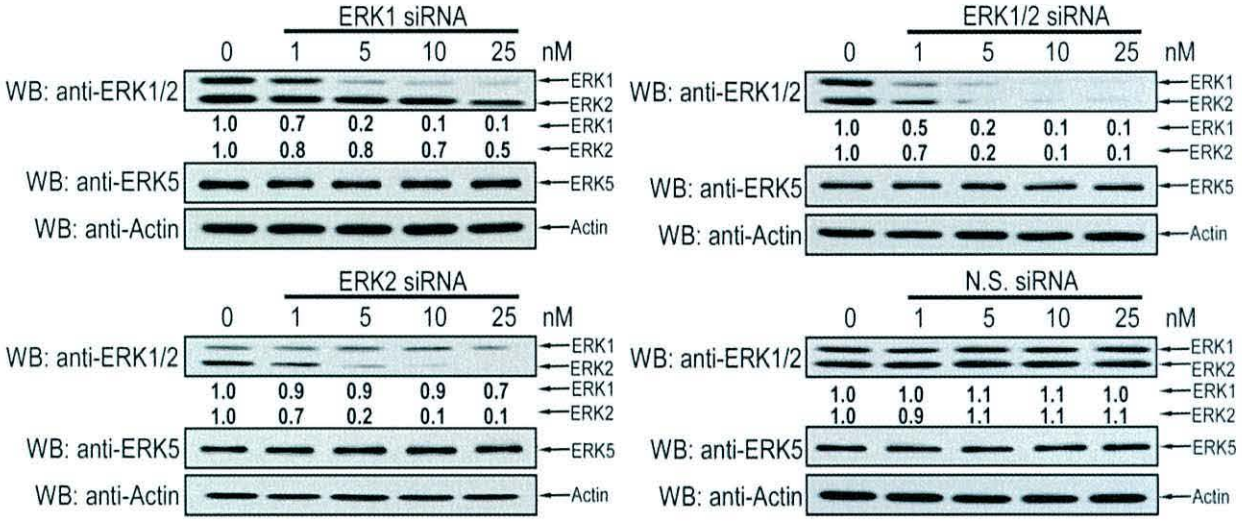


Figure 4.4 Validation of ERK1 and ERK2 down-regulation with siRNA. Effects of ERK1- and ERK2-specific siRNA treatment on ERK1 and ERK2 protein expression. HDMECs were seeded at 1.0 x 10^5 cells per well on gelatin-coated 6-well plates for 24 h. Cells were transfected with the indicated amounts of siRNA duplexes directed against ERK1 (equal amounts of Hs_MAPK3_6 HP and Hs_MAPK3_7 HP), ERK2 (equal amounts of Hs_MAPK1_10_HP and Hs_MAPK1_11 HP) or ERK1/2 (equal amounts of siRNAs directed against ERK1 and ERK2 together), or against a non-silencing (N.S.) control siRNA as described (*section 2.2.2.7.2*). Alternatively, cells were treated with the transfection reagent Lipofectamine™ RNAi MAX as a mock transfection control. At 48 h post-transfection, cells were lysed and separated on a 10% SDS-PAGE gel, prior to Western blotting (*WB*) with antibodies directed against total ERK1/2, total ERK5 and actin as a loading control. Expression of ERK1 and ERK2 relative to actin was quantified by densitometric analysis with NIH Image J software, and is displayed at the bottom of each blot. *ERK1/2, extracellular signal-regulated kinase 1 and 2; ERK5, extracellular signal-regulated kinase 5; HDMEC, human dermal microvascular endothelial cell; N.S., non-silencing; siRNA, small interfering RNA; WB, Western blot.*

4.3 Effects of ERK5 down-regulation on interferon gene expression

4.3.1 ERK5 siRNA does not induce an interferon response

Transfection of certain siRNA duplexes into mammalian cells can induce activation of the interferon response, leading to up-regulation of interferon gene expression, non-specific degradation of mRNAs and eventual cell death (Sledz & Williams, 2004; Persengiev *et al.*, 2004). To ascertain whether introduction of ERK5 siRNA induced a similar interferon response in HDMECs, cells were transfected, or not, with ERK5 siRNA 1, ERK5 siRNA 2 or with N.S. siRNA. It was decided to measure the potential change in

gene expression of two interferon response genes, namely *OAS1* (2′, 5′-oligoadenylate synthetase), and *ISG20* (interferon-stimulated gene 20kD). *OAS1* had been previously used as a marker of the interferon response following treatment with siRNA (Pebernard & Iggo, 2004). Similarly, expression of *ISG20* had been shown to be up-regulated in response to transfection of synthetic dsRNA in mammalian cells (Espert *et al.*, 2003).

The relative expression of *ISG20* (**Fig. 4.5 A**) and *OAS1* (**Fig. 4.5 B**) mRNA was quantified by qRT-PCR, normalised to the housekeeping gene *β-actin*, in RNA extracted from HDMECs 24 h post-transfection with siRNAs. No significant change in expression of either *OAS1* or *ISG20* was observed following transfection with N.S. siRNA or ERK5 siRNA compared to the untransfected control (**Fig. 4.5**). Together, these results suggested that transfection of either N.S. siRNA or ERK5 siRNA did not induce expression of interferon response genes.

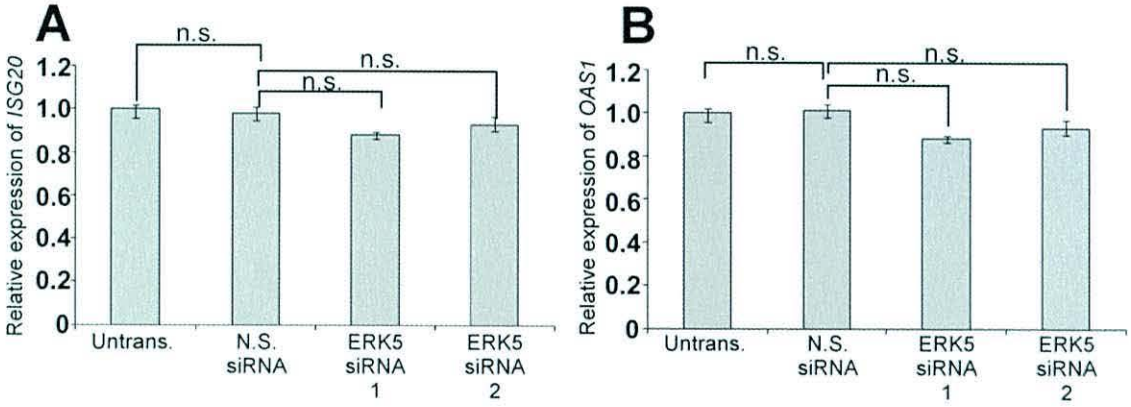


Figure 4.5 Expression of interferon response genes following transfection with ERK5 siRNA. HDMECs were plated at 1.0 x 10^5 cells per well on gelatin-coated 6-well plates for 24 h prior to transfection, or not (untransfected control; Untrans.) with 10 nM ERK5 siRNA 1 (Hs_MAPK7_9), ERK5 siRNA 2 (Hs_MAPK7_5) or 10 nM of non-silencing control siRNA (N.S. siRNA) as described (*section 2.2.2.7.2*). At 24 h post-transfection, RNA was harvested from the cells (*section 2.2.4.1*), followed by reverse-transcription cDNA synthesis. cDNA from each experimental condition was analysed by quantitative real-time PCR (qRT-PCR) in triplicate, for expression of MEK5 and the control housekeeping gene *β-actin*. Relative quantification was performed by Comparative C_T ($2^{-\Delta\Delta C_T}$) method (*section 2.2.5.2*). Normalised (**A**) *ISG20* and (**B**) *OAS1* mRNA expression is presented relative to that of the untransfected sample, which was set arbitrarily as 1.0. Data is presented is representative of three independent transfections, (n = 3; mean ± SD; n.s. = not significant; unpaired Student's *t* test). *HDMEC, human dermal microvascular endothelial cell; ERK5, extracellular signal-regulated kinase 5; ISG20, interferon-stimulated gene (20kD); N.S., non-silencing; OAS1, 2′, 5′-oligoadenylate synthetase; siRNA, small interfering RNA.*

4.4 Effects of ERK5 down-regulation on HDMEC proliferation

4.4.1 HDMEC proliferation is induced by various pro-angiogenic growth factors

Primary endothelial cells undergo proliferation when plated on a gelatin or fibronectin matrix and stimulated with pro-angiogenic mitogens such as FGF-2 or VEGF (Guo *et al.*, 1995; Bohman *et al.*, 2005; Mellberg *et al.*, 2009). Endothelial cells from different vascular beds often respond differently to growth factor stimulation (Cines *et al.*, 1998; Wu *et al.*, 2000a; Zubilewicz *et al.*, 2001). To characterise the proliferative response of HDMECs to growth factor stimulation, the number of viable cells was measured using a commercially-available luminescent cell viability assay, as previously described (Crouch *et al.,* 1993; *section 2.2.8.1*).

In an initial screen, the mitogenic effect of a range of pro-angiogenic inducers was assessed. VEGF and FGF-2 were used due to their reported ability to stimulate endothelial cell proliferation *in vitro* (Ferrara & Henzel, 1989; Pintucci *et al.*, 2002). In addition, cells were stimulated with HGF, PMA and A23187, as these factors had been shown to activate ERK5 in HDMECs (**Fig. 3.3**). Serum-starved HDMECs were treated with VEGF, FGF-2, EGF, HGF, PMA or A23187, and then allowed to proliferate at 37 ˚C for three days. Stimulation with VEGF, FGF-2, EGF, HGF or PMA was sufficient to induce HDMEC proliferation on a gelatin matrix compared to the unstimulated basal control (**Fig. 4.6**), whereas A23187 caused a dramatic drop in detectable cell numbers due to total cell death induced by this treatment (**Fig. 4.6**). By contrast, under the same conditions, treatment with VEGF induced a 2.3-fold increase in HDMEC cell numbers over 3 days (**Fig. 4.6**).

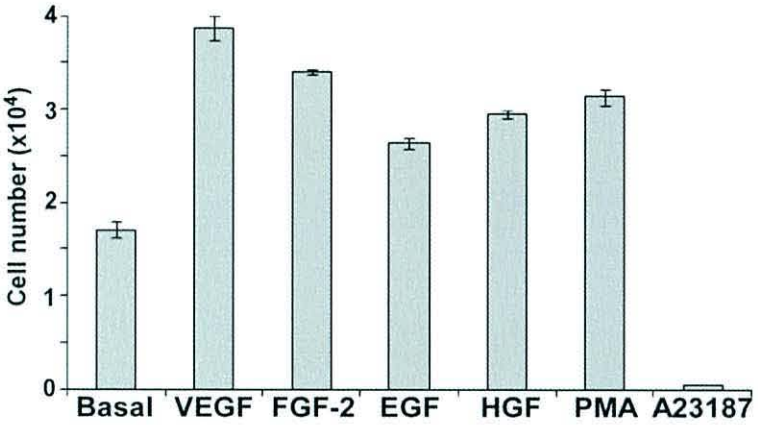


Figure 4.6 VEGF induces proliferation of HDMECs when plated on a gelatin matrix. HDMECs were seeded at 1.5 x 10^4 cells per well in EBM MV2 growth medium on gelatin-coated 24-well plates and incubated for 24 h, followed by serum-starvation in EBM MV2 basal medium containing 1% (v/v) FCS for 20 h. Cells were then treated with fresh EBM MV2 medium containing 1% (v/v) FCS alone (Basal), or containing VEGF (50 ng/ml), FGF-2 (50 ng/ml), EGF (50 ng/ml), HGF (50 ng/ml), PMA (100 nM) or A23187 (5 µM), and incubated at 37 °C for 72 h. On the fifth day of the assay, a standard curve was prepared, and cell numbers were calculated using the CellTiter-Glo® luminescent cell viability assay (*section 2.2.8.1*). *EGF, epidermal growth factor; FCS, foetal calf serum; FGF-2, fibroblast growth factor-2; HDMEC, human dermal microvascular endothelial cell; HGF, hepatocyte growth factor; PMA, phorbol-12-myristate-13-acetate; VEGF, vascular endothelial growth factor.*

4.4.2 ERK5 is not required for VEGF-induced HDMEC proliferation

ERK5 has been implicated in growth factor-induced cellular proliferation of several cell types including: MCF10A epithelial cells (Kato *et al.*, 1998; Hayashi *et al.*, 2001), hepatic stellate cells (Rovida *et al.*, 2008b) and glomerular mesangial cells (Dorado *et al.*, 2008). However, the potential role of ERK5 in regulating VEGF-stimulated endothelial cell proliferation had not been previously investigated.

To investigate the role of ERK5 in VEGF-stimulated proliferation of HDMECs, ERK5 expression was down-regulated with ERK5 siRNA 1 or ERK5 siRNA 2, prior to serum starvation, followed by stimulation with VEGF, and quantification of cell numbers 3 days post-stimulation (*section 2.2.8.1*). Given that the proliferative response of various endothelial cell types to VEGF stimulation had been reported to be dependent upon ERK1/2 (Rousseau *et al.*, 1997; Kroll & Waltenberger, 1997; Vinals & Pouyssegur, 1999; Wu *et al.*, 2000a), the effects of ERK1/2 siRNA upon VEGF-stimulated HDMEC proliferation was also quantified.

Total cell lysates from identically-treated cells were prepared, and proteins were analysed by Western blotting to assess the relative expression of ERK1/2 and ERK5.

SiRNA-mediated down-regulation of ERK1, ERK2 or ERK5 lowered the expression of these proteins by 70-90% compared to N.S. siRNA control-treated cells (**Fig. 4.7 A**). Quantification of cell numbers showed that N.S. siRNA did not affect VEGF-induced doubling in cell numbers observed over 3 days (**Fig. 4.7 B**). Similarly, ERK5 silencing with either ERK5 siRNA 1 or with ERK5 siRNA 2 had no significant effect on VEGF-induced HDMEC proliferation (**Fig. 4.7 B**). By contrast, treatment with ERK1- or ERK2-specific siRNA significantly impeded VEGF-stimulated HDMEC proliferation by approximately 33% compared to the N.S. control siRNA-treated cells (**Fig. 4.7 B**). SiRNA-mediated down-regulation of ERK1 and ERK2 expression together, resulted in a further lowering in VEGF-induced HDMEC proliferation, compared to the effects of siRNA-mediated down-regulation of the expression of either ERK1 or ERK2 alone (**Fig. 4.7 B**).

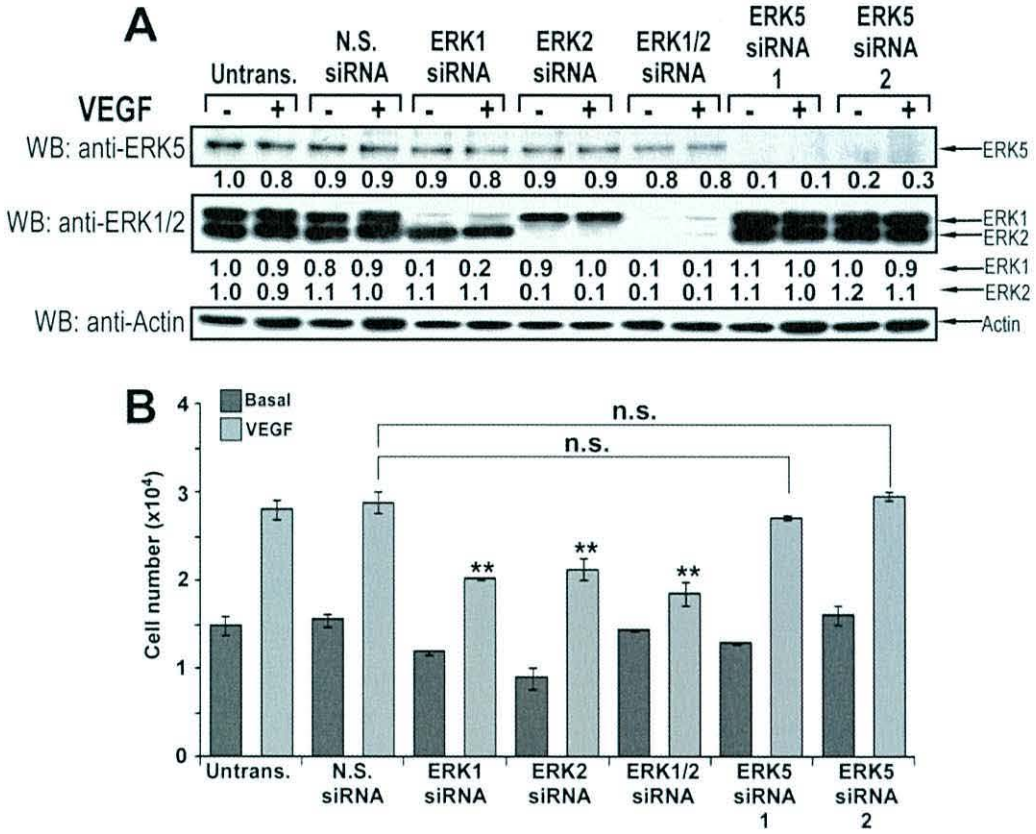


Figure 4.7 Effects of siRNA-mediated down-regulation of ERK1, ERK2, ERK1/2 or ERK5 upon VEGF-stimulated HDMEC proliferation. HDMECs were transfected with 10 nM of N.S. siRNA, ERK5 siRNA 1, ERK5 siRNA 2, ERK1 siRNA, ERK2 siRNA or ERK1/2 siRNA 24 h prior to seeding at 1.5 x 10^4 cells per well on gelatin-coated 24-well plates and incubation for 24 h. Cells were serum-starved in EBM MV2 basal medium containing 1% (v/v) FCS for 20 h prior to the addition of fresh EBM MV2 basal medium containing 1% (v/v) FCS alone (Basal) or supplemented with 50 ng/ml VEGF (VEGF) in each case. Cells were incubated at 37 °C for 72 h. On the fifth day of the assay, (***A***) cells were lysed and proteins separated on a 10% SDS-PAGE gel prior to Western blotting (*WB*) with antibodies directed against ERK5, ERK1/2 and actin, as a loading control. Expression of ERK5, ERK1 and ERK2 relative to actin was quantified by densitometric analysis with NIH ImageJ software, and is displayed at the bottom of each respective blot. (***B***) HDMEC numbers were quantified using the CellTiter-Glo® luminescent cell viability assay using a standard curve method as previously described (*section 2.2.8.1*). (n = 3; mean ± SD; n.s., not significant; $^{**}p < 0.01$; unpaired Student's *t* test, compared to N.S. siRNA control-treated cells). *ERK1/2, extracellular signal-regulated kinase 1/2; ERK5, extracellular signal-regulated kinase 5; FCS, foetal calf serum; HDMEC, human dermal microvascular endothelial cell; N.S., non-silencing; siRNA, small interfering RNA; VEGF, vascular endothelial growth factor, WB, Western blot.*

Together, these results suggest that both ERK1 and ERK2 are involved in mediating VEGF-induced HDMEC proliferation, and are equally important for this process. By contrast, the lack of effect of siRNA-mediated silencing of ERK5 upon VEGF-induced HDMEC proliferation suggests that ERK5 is not required to mediate this response in these cells.

4.5 Effects of ERK5 down-regulation on HDMEC adhesion

4.5.1 SiRNA-mediated down-regulation of ERK5 expression prevents adhesion of HDMECs to vitronectin and fibronectin matrices, but not to a collagen I matrix

Adhesion of endothelial cells to the ECM is a key event in angiogenesis (Eliceiri & Cheresh, 2001; Chavakis & Dimmeler, 2002; Cheresh & Stupack, 2008; Ramjaun & Hodivala-Dilke, 2009), required for both endothelial cell migration and the prevention of cell death by anoikis. During routine observations of HDMECs using an inverted light microscope, it was observed that an estimated 95% of N.S. siRNA-treated cells adhered to gelatin-coated cell culture dishes within 1 h of plating; however, only ~50% of ERK5-siRNA-treated HDMECs plated on a gelatin matrix had adhered by 1 h (data not shown). Interestingly, both N.S. siRNA-treated cells and ERK5 siRNA-treated cells adhered equally well to collagen matrices within 1 h post-plating (data not shown). It was thus hypothesised that ERK5 may differentially regulate HDMEC adhesion to various matrices. To confirm or refute these initial observations, an adhesion assay was designed, optimised and conducted.

A fluorescence-based adhesion assay was developed, which involved labelling HDMECs with CellTracker™ Green CMFDA dye 28 h post-transfection with ERK5 siRNA, and 4 h post-stimulation with VEGF (*section 2.2.11*). Optimisation of assay conditions involved the quantification of the minimum cell number detectable following CellTracker™ Green CMFDA treatment, measurable by a fluorescence plate reader. Tests revealed that it was possible to detect cell numbers as low as 2.5×10^3 using this system following incubation of the cells for 1 h (data not shown). Subsequent experiments were carried out using 1.0×10^4 cells per well. As initial observations had revealed that the apparent effect of ERK5 siRNA upon the adhesion of HDMECs to a gelatin matrix was only noticeable for a few hours, it was deemed important to quantify the effects of siRNA-mediated knockdown of ERK5 upon HDMEC adhesion at various time points post-seeding. HDMEC adhesion to the ECM component proteins vitronectin and fibronectin, as well as to collagen I and gelatin, was assessed. VEGF had been shown to enhance endothelial cell adhesion to the ECM by inducing integrin expression

(Senger *et al.*, 1997; Byzova *et al.*, 2000), therefore the potential effects of VEGF in modulating HDMEC adhesion was also assessed in this assay.

It was found that, in agreement with preliminary observations, both N.S. siRNA-treated and ERK5 siRNA-treated HDMECs adhered equally well to a collagen matrix. Specifically, approximately 40% of cells had adhered to collagen I within 10 min of plating, with >80% of cells having adhered by 1 h post-plating on this matrix, regardless of treatment with ERK5 siRNA or with N.S. siRNA (**Fig. 4.8 A**). By contrast, N.S. siRNA-treated cells adhered more slowly to a gelatin matrix, with only ~70% cells having adhered to this matrix by 1 h post-plating (**Fig. 4.8 B**). Strikingly, less than 30% of ERK5 siRNA-treated cells were found to adhere to a gelatin matrix by 1 h post-plating. By 2 h post-plating, the numbers of N.S. siRNA-treated adherent cells on a gelatin matrix had reached 90% of that of the cell seeding control, whereas only 60% of ERK5 siRNA-treated cells had adhered to gelatin at 1 h post-plating (**Fig. 4.8 B**). Importantly however, by 4 h post-plating, both N.S. siRNA- and ERK5 siRNA-treated cells had adhered equally well to gelatin matrices.

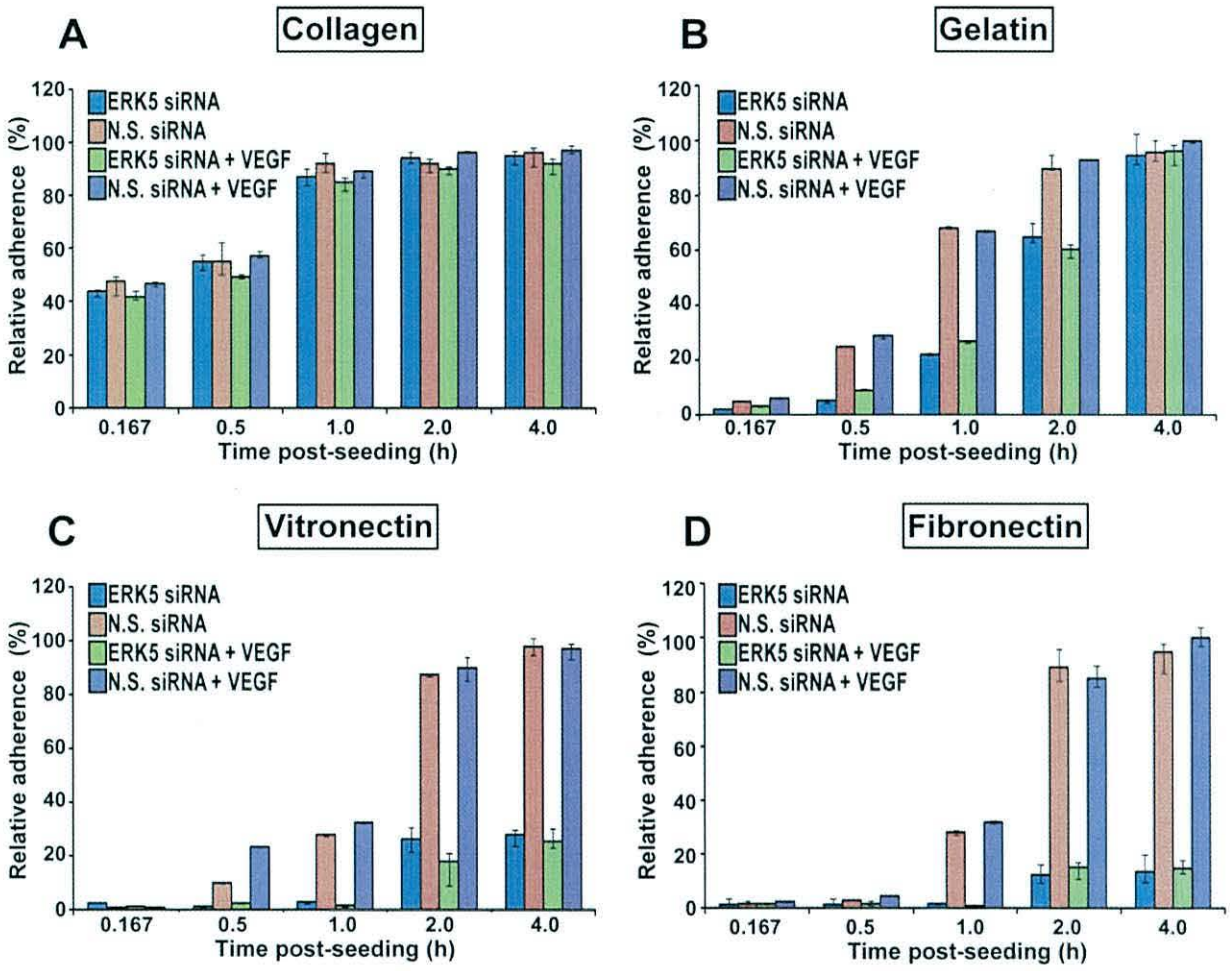


Figure 4.8 Effects of siRNA-mediated down-regulation of ERK5 on HDMEC adhesion to various matrices. HDMECs were transfected with 5 nM of ERK5 siRNA 1 and 5 nM of ERK5 siRNA 2 (ERK5 siRNA), or with 10 nM non-silencing (N.S.) siRNA as described (*section 2.2.2.7.2*). At 24 h post-transfection, cells were stimulated, or not, with 50 ng/ml VEGF for 4 h. Following stimulation, cells were labelled with 5 µM CellTracker™ Green CMFDA for 1 h prior to harvesting (using 0.5 mM EDTA in DPBS), counting, and seeding cells in triplicate wells at 1.0 x 10^4 cells per well in 96-well plates coated with (**A**) 5 µg/ml collagen I; (**B**) 1% (w/v) gelatin (in ddH_2O) (**C**) 5 µg/ml vitronectin; (**D**) 5 µg/ml fibronectin. At 10 min, 30 min, 1 h, 2 h and 4 h post-seeding, wells were washed or not, (control) and fluorescence was measured at 492/535 nm as described (*section 2.2.11*). Additional unwashed wells containing CellTracker™ Green CMFDA-labelled medium alone on each matrix was also quantified in order to normalise for variations in background fluorescence. Data is presented as mean ± SD of triplicate wells. This experiment was repeated twice with similar results. *CMFDA, 5-chloromethylfluorescein diacetate; ERK5, extracellular signal-regulated kinase 5; FCS, foetal calf serum; HDMEC, human dermal microvascular endothelial cell; N.S., non-silencing; siRNA, small interfering RNA; VEGF, vascular endothelial growth factor.*

Profiling of HDMEC adhesion to vitronectin and fibronectin revealed that cells adhered relatively less rapidly to these matrices compared to the profile of cell adhesion to gelatin-coated wells (**Fig. 4.8, C and D**). Less than 30% of N.S. siRNA-treated cells had adhered to either vitronectin (**Fig. 4.8 C**), or fibronectin (**Fig. 4.8 D**) by 1 h post-plating; however, this rose to >80% adhesion of N.S. siRNA-treated cells 2 h after seeding on both matrices (**Fig. 4.8, C and D**). Notably however, on both vitronectin and fibronectin matrices, down-regulation of ERK5 expression by siRNA treatment severely

abrogated HDMEC adhesion, with <30% adherent ERK5 siRNA-treated cells on a vitronectin matrix by 4 h post-plating (**Fig. 4.8, C and D**). This effect was most apparent in cells plated on fibronectin, where <20% of ERK5-siRNA-treated cells had adhered to this matrix by 4 h post-plating (**Fig. 4.8 D**). Treatment with VEGF had no discernable effect upon adhesion of HDMECs to any of the matrices tested (**Fig. 4.8, A-D**). Together, these results confirmed the preliminary observations, showing that siRNA-mediated down-regulation of ERK5 in HDMECs had no effect upon adhesion of these cells to collagen I, whereas transfection with ERK5 siRNA delayed, but did not prevent, full adhesion of HDMECs to a gelatin matrix. Furthermore, HDMEC adhesion to vitronectin and fibronectin was prevented by ERK5 siRNA treatment.

4.6 Effects of ERK5 down-regulation on HDMEC migration

4.6.1 ERK5 is not required for VEGF-stimulated HDMEC migration

Endothelial cell migration represents a key step in the angiogenic process (Lamalice *et al.*, 2007). VEGF induces endothelial cell migration *in vivo* and *in vitro* (Rousseau *et al.*, 1997; Rousseau *et al.*, 2000; Lamalice *et al.*, 2006). As the potential role of ERK5 in regulating growth-factor stimulated endothelial cell migration had not been previously reported, it was decided to assess this by an *in vitro* scratch-wound assay (*section 2.2.10*). This method of assessing endothelial cell migration is highly representative of endothelial cell migration *in vivo,* and involves the quantification of the migration of cells from the leading edge of newly-formed 'scratch' in a confluent monolayer of cells into the denuded area (Liang *et al.*, 2007). Preliminary experiments involved assessing the relative degree of HDMEC cell migration induced by various pro-angiogenic growth factors such as VEGF, FGF-2, EGF and HGF. Of the growth factors tested, VEGF stimulation induced the greatest degree of HDMEC migration (data not shown), in agreement with the published reports on growth factor-stimulated migration of HUVECs (Rousseau *et al.*, 1997; Rousseau *et al.*, 2000).

The degree of cell migration into the denuded area was assessed by time-lapse microphotography using an IncuCyte™ incubator. To ensure that wound closure was due to HDMEC migration, and not due to VEGF-induced proliferation, the anti-proliferative agent mitomycin C was added to the cell culture medium (Erzurum *et al.*,

2003). Mitomycin C inhibited HDMEC proliferation as determined by proliferation assay, without increasing cell death over 24 h (data not shown). Using this system, HDMECs began to migrate into the denuded area within 6 h post-VEGF stimulation.

HDMECs were seeded for use in the assay 24 h post-transfection with 10 nM of ERK5 siRNA 1, 10 nM of ERK5 siRNA 2, or with 10 nM N.S. siRNA. Cells were then serum-starved, and incubated for a further 24 h prior to forming a scratch in the HDMEC monolayer, followed by the addition, or not, of VEGF. Identically-treated wells were prepared for cell lysis, and Western blot analysis, which confirmed that ERK5 expression was down-regulated by at least 80% throughout the duration of this assay (data not shown).

It was found that N.S. siRNA-transfected HDMECs underwent minimal migration in the absence of VEGF (**Fig. 4.9, A**), reaching 17.3% (±1.5% SD) confluence by 24 h. By comparison, VEGF induced 93% (±1.5% SD) wound confluence by 24 post-stimulation (**Fig. 4.9, B**). Interestingly, down-regulation of siRNA-mediated down-regulation of ERK5 expression had no effect upon migration at early time points (6-16 h), but induced a doubling in HDMEC migration at later time points (16-24 h) compared to the N.S. control siRNA-treated cells in the absence of VEGF ($p<0.05$) (**Fig. 4.9**). However, in the presence of VEGF, siRNA-mediated ERK5 silencing had no effect upon HDMEC migration (**Fig. 4.9, A and B)**. Together, these data suggest that ERK5 negatively-regulates HDMEC migration, an effect that was not apparent in the presence of VEGF.

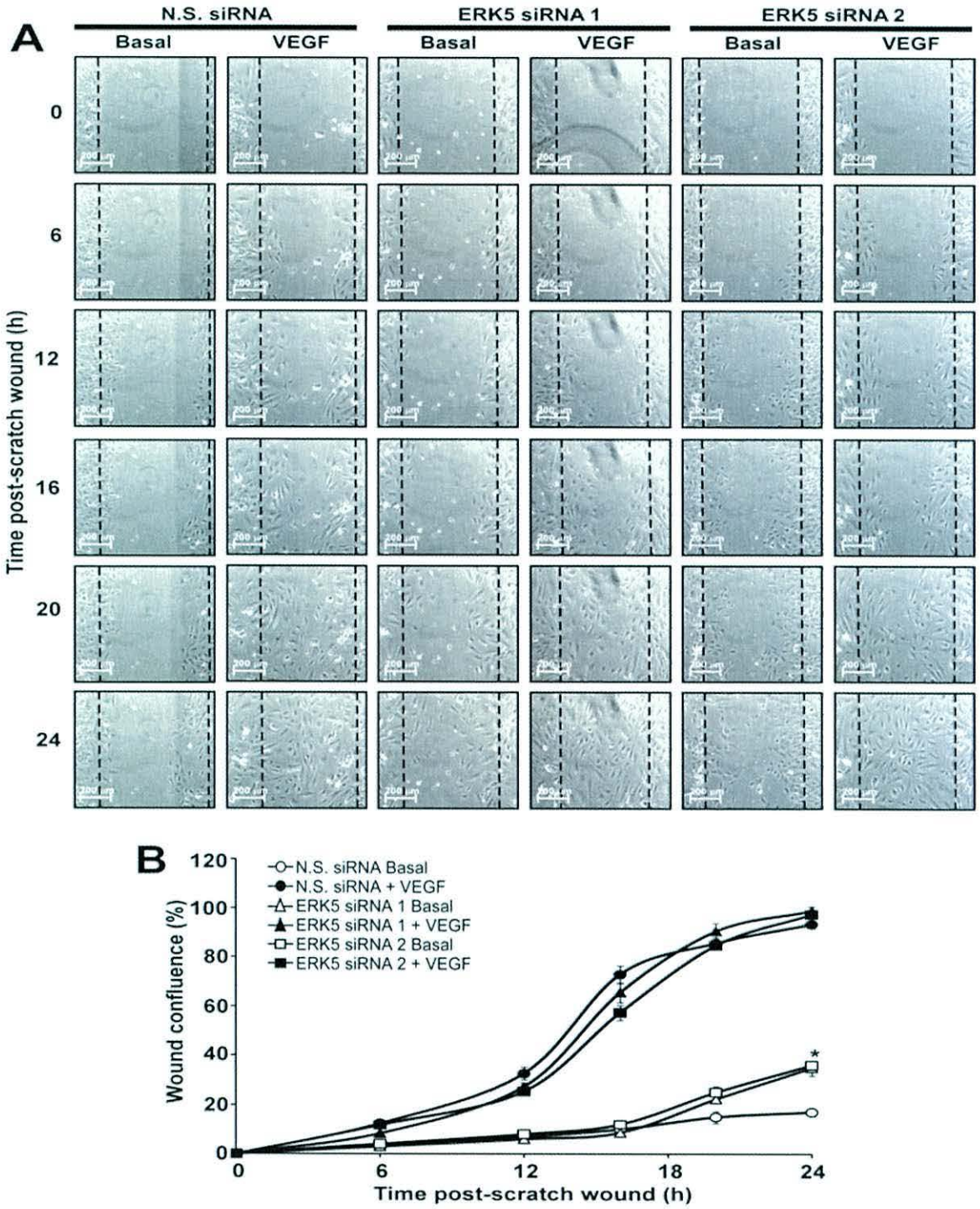


Figure 4.9 Effects of siRNA-mediated down-regulation of ERK5 expression upon VEGF-induced HDMEC migration in a scratch-wound healing assay. (**A**) HDMECs were treated with 10 nM of non-silencing (N.S.) control siRNA or with 10 nM ERK5-specific siRNAs ERK5 siRNA 1 or ERK5 siRNA 2 for 24 h prior to seeding in triplicate at 2.0 x 10^5 cells per well in 24-well ImageLok™ plates for 24 h, prior to serum starvation for a further 20 h. The cell monolayer was then scratched and HDMECs were incubated with EBM MV2 basal medium containing 1% (v/v) FCS and 2.5 µg/ml Mitomycin C alone (Basal) or supplemented with 50 ng/ml VEGF (VEGF). Photomicrographs were taken in the same position at 0, 6, 12, 16, 20 and 24 h post-scratch-wound with IncuCyte™ apparatus (*section 2.2.10*). The original denuded area is represented by two dashed lines in each case. The images shown are representative of results obtained from three independent experiments. (**B**) Percentage wound confluence was determined by IncuCyte™ software. (n = 3; mean ± SD; *p<0.05; Mann-Whitney U test, compared to N.S. siRNA control-treated cells). Scale bars = 200 µm. *ERK5, extracellular signal-regulated kinase 5; FCS, foetal calf serum; HDMEC, human dermal microvascular endothelial cell; N.S., non-silencing; siRNA, small interfering RNA; VEGF, vascular endothelial growth factor.*

4.7 Determination of the effects of siRNA-mediated down-regulation of MEK5/ERK5 expression upon VEGF-stimulated HDMEC morphogenesis within 3-D collagen gels

4.7.1 VEGF stimulates tubular morphogenesis of HDMECs in 3-D collagen gel structures

It is well established that in response to stimulation with certain pro-angiogenic growth factors such as VEGF and FGF-2, endothelial cells undergo tubular morphogenesis to form capillary-like structures in 3-D collagen I gels (Montesano et al., 1983; Qi et al., 1999; Yang et al., 2004; Matsumoto et al., 2002; Bohman et al., 2005). However, the response of HDMECs to growth factor stimulation when plated within a 3-D collagen I gel had not been previously reported. To assess whether growth factor stimulation could initiate tubular morphogenesis in HDMECs, cells were seeded between two layers of collagen (*section 2.2.7.1*), and stimulated with various growth factors (**Fig. 4.10**). At 24 h post-growth factor-stimulation, cells within collagen gels were fixed. Fluorescent staining of the actin cytoskeleton and nuclei, with Alexa Fluor® 546-conjugated phalloidin and Hoechst 33342 respectively, allowed visualisation of anastomosed tubules by inverted fluorescent microscopy (*section 2.2.7.1.7*). In the absence of growth factors, HDMECs did not undergo any tubule formation (**Fig. 4.10, *Basal***). By contrast, stimulation with VEGF induced tubular morphogenesis of HDMECs, with the formation of distinctive interconnecting capillary-like structures, consisting of several anastomosed cells (**Fig. 4.10, *VEGF***). Although stimulation of HDMECs with FGF-2, EGF or HGF induced some HDMEC capillary sprouting (**Fig. 4.10, *FGF-2, EGF, HGF***), these capillary sprouts failed to interconnect to form *de novo* vessels. PMA had been previously reported to induce HUVEC tube formation of PMA-stimulation in a collagen gel angiogenesis assay (Ilan *et al.*, 1998). However, PMA stimulation of HDMECs in the present 3-D collagen assay appeared to induce HDMEC cell proliferation rather than differentiation, an effect characterised by large sheet-like masses of cells within the collagen I matrix (**Fig. 4.10, *PMA***). By contrast, chronic treatment with the calcium ionophore A23187 induced the rounding of cells, indicative of cell death (**Fig. 4.10, *A23187***).

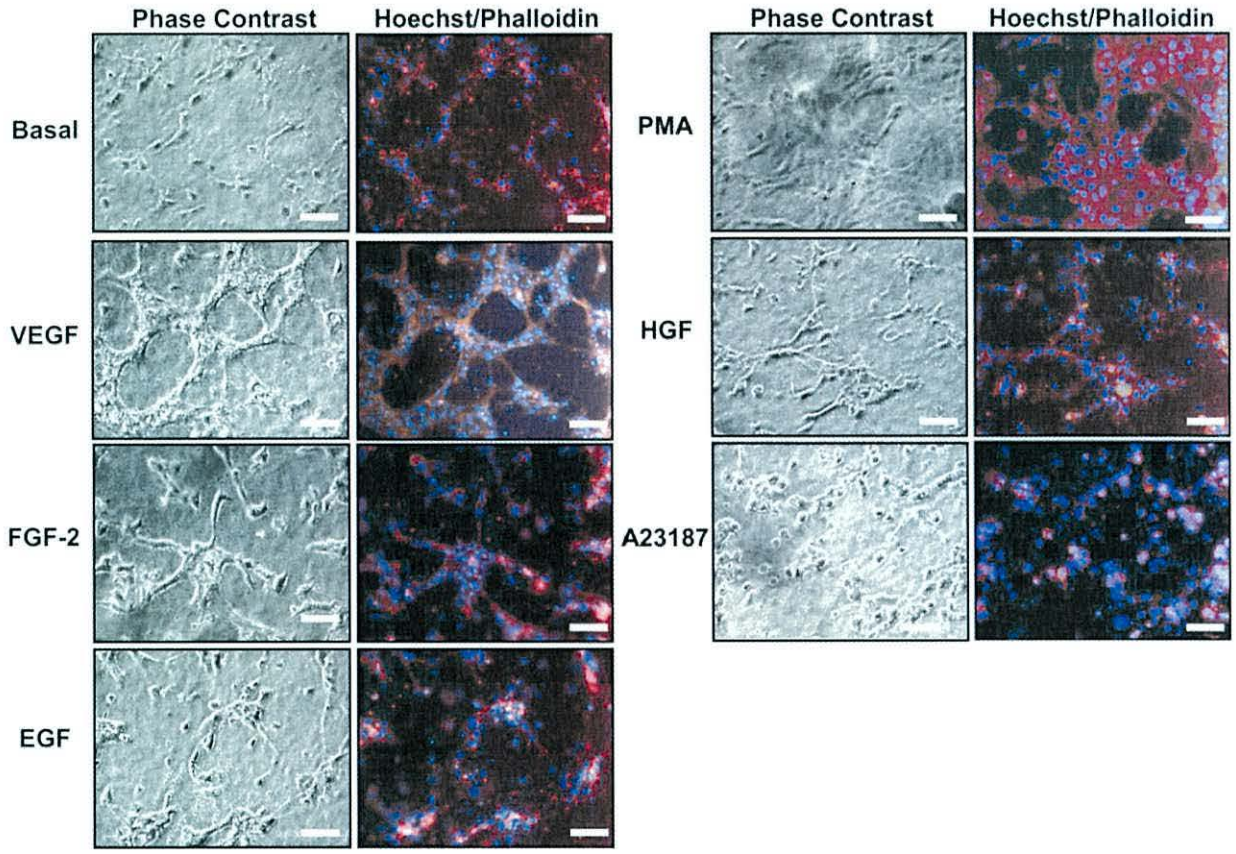
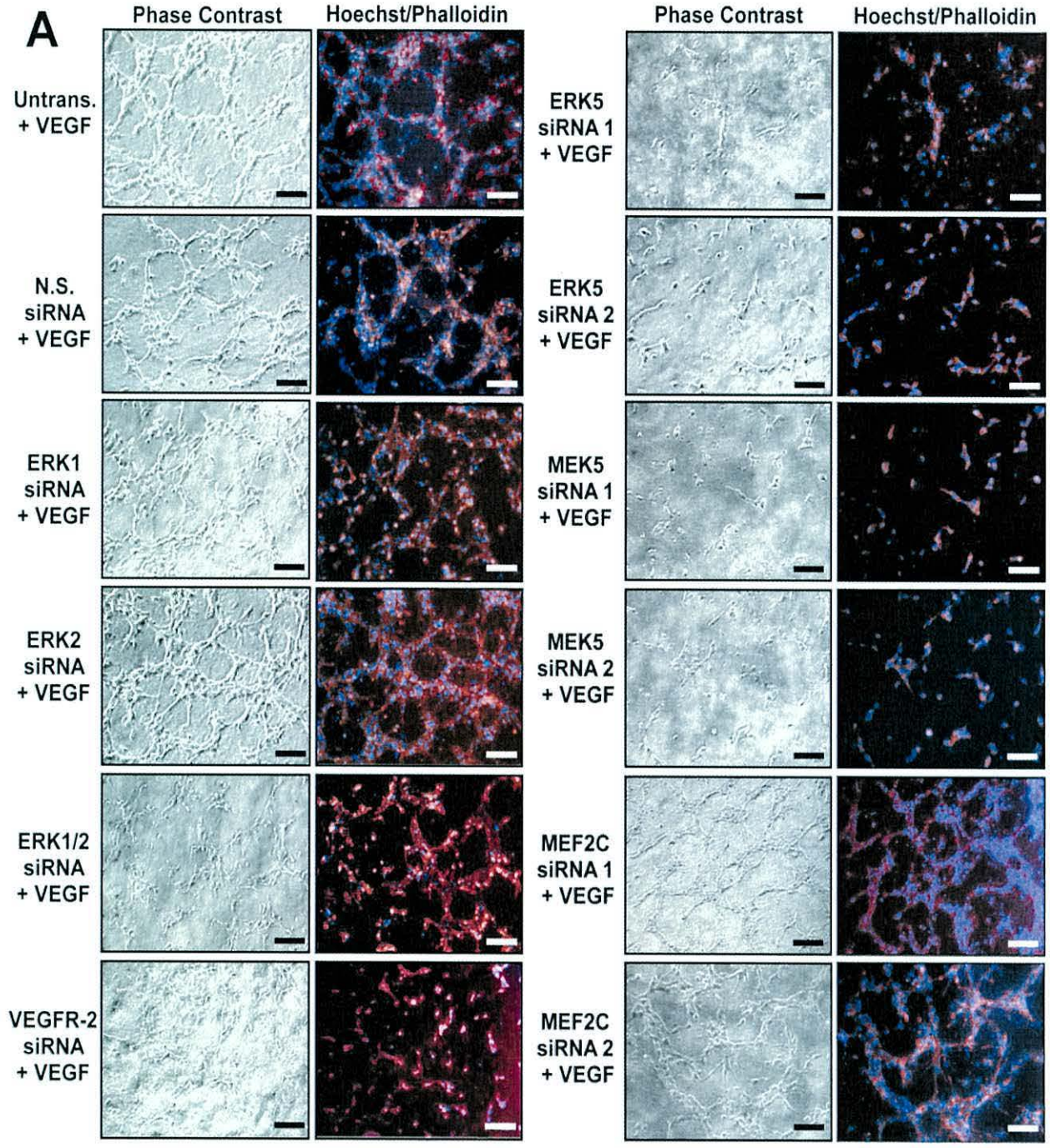


Figure 4.10 VEGF-stimulates tubular morphogenesis of HDMECs within 3-D collagen I gel matrices. HDMECs were serum-starved in EBM MV2 basal medium containing 1% (v/v) FCS for 20 h prior to seeding at 2.6 x 10^5 cells between two layers of collagen I gels in 12-well cell-culture dishes (*section 2.2.7.1*). Following incubation for 1 h at 37 °C, cells were treated with EBM MV2 basal medium containing 1% (v/v) FCS alone (Basal), or containing VEGF (50 ng/ml); FGF-2 (50 ng/ml); EGF (50 ng/ml); HGF (50 ng/ml); PMA (100 nM) or the calcium ionophore A23187 (5 µM). Cells within the collagen I gel structures were incubated for 24 h at 37 °C prior to fixing in 4% (w/v) paraformaldehyde. Cell nuclei were stained with Hoechst 33342 (blue) and F-actin fibres within the cells were stained with Alexa Fluor® 546 phalloidin (red), and visualised by inverted fluorescence microscopy (*section 2.2.7.1.7*). The data is representative of three independent experiments. Scale bars = 100 µm. *EGF, epidermal growth factor; ERK5, extracellular signal-regulated kinase 5; FCS, foetal calf serum; FGF-2, fibroblast growth factor-2; HDMEC, human dermal microvascular endothelial cell; HGF, hepatocyte growth factor; N.S., non-silencing; PMA, phorbol-12-myristate-13-acetate; siRNA, small interfering RNA; VEGF, vascular endothelial growth factor.*

The effect of VEGF in inducing tubular morphogenesis of HDMECs in a 3-D collagen I gel was unique amongst the growth factors tested. VEGF stimulation of HDMECs plated on a gelatin matrix was found to stimulate cell-cycle entry (data not shown) to induce cellular proliferation of HDMECs (**Fig. 4.6**). By contrast HDMECs plated within a 3-D collagen I matrix and stimulated with VEGF exit the cell cycle (data not shown) and undergo differentiation to form tube-like structures. In agreement with the reported behaviour of other endothelial cell types in this assay (Matsumoto *et al.*, 2002; Bohman *et al.*, 2005), VEGF-induced tubular morphogenesis of HDMECs reached a peak of tube-like structure formation at 24 h post-VEGF stimulation, after which time, tubes regressed slowly and had disappeared by 72 h (data not shown).

4.7.2 The MEK5/ERK5 pathway is required to mediate VEGF-induced tubular morphogenesis of HDMECs in 3-D collagen gel structures

The relative importance of the MEK5/ERK5 signalling axis in VEGF-stimulated HDMEC tubular morphogenesis was assessed by siRNA-mediated down-regulation of ERK5 expression, prior to serum-starvation of cells, followed by plating these cells between two layers of collagen I gel, and stimulation with VEGF. Transfection of HDMECs with N.S. siRNA had no significant effect on VEGF-induced tube morphology or length in a 3-D collagen gel (**Fig. 4.11, A and B**). By contrast, treatment with ERK5 siRNA had a profound effect upon VEGF-induced HDMEC tubular morphogenesis. Specifically, HDMECs were unable to form a network of vessels in 3-D collagen I gel in response to VEGF stimulation following pre-treatment with ERK5-specific siRNA (**Fig. 4.11 A**). Analysis of cellular morphology revealed that cells were rounded, with pyknotic, hypercondensed nuclei (**Fig. 4.11 A**), indicating that HDMECs underwent widespread cell death in the absence of ERK5.



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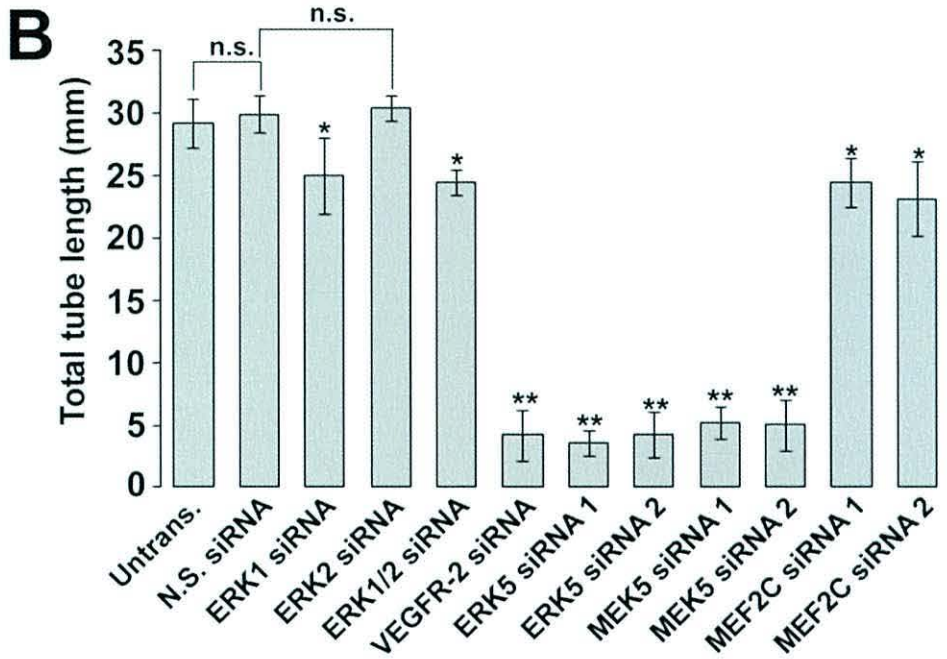


Figure 4.11 SiRNA-mediated down-regulation of MEK5 or ERK5 expression prevents VEGF-stimulated HDMEC tubular morphogenesis within 3-D collagen I matrices. (**A**) HDMECs were seeded at 8.25 x 10^5 cells per dish in 10 cm dishes in 10 ml of EBM MV2 growth medium, and grown for 24 h, prior to transfection with 10 nM siRNA directed against *ERK5, MEK5, ERK1, ERK2, ERK1/2, MEF2C* or 10 nM of non-silencing control siRNA (N.S. siRNA) as described (*section 2.2.2.7.2*). At 24 h post-transfection, cells were serum-starved in EBM MV2 basal medium containing 1% (v/v) FCS for 20 h prior to seeding between two layers of collagen I gel at 2.6 x 10^5 cells per well in 12-well cell-culture dishes (*section 2.2.7.1*). Cells seeded within the collagen I gels were stimulated with EBM MV2 basal medium containing 1% (v/v) FCS supplemented with 50 ng/ml VEGF and incubated for 24 h at 37 °C, after which time cells were fixed in 4% paraformaldehyde. Cell nuclei were stained with Hoechst 33342 (blue) and the F-actin fibres within the cells were stained with Alexa Fluor® 546 phalloidin (red) as described (*section 2.2.7.1.6*), and visualised by inverted fluorescence microscopy (*section 2.2.7.1.7*). (**B**) The total length of tubular structures were quantified from three fields (X 10 objective) per well using NIH ImageJ software, as described (*section 2.2.7.1.7*). (n = 3; mean ± SD; n.s., not significant; *p<0.05; **p<0.01; unpaired Student's t test, compared to N.S. siRNA control-treated cells). Scale bars = 100 µm. *ERK5, extracellular signal-regulated kinase 5; FCS, foetal calf serum; HDMEC, human dermal microvascular endothelial cell; MEF2C, myocyte enhancer factor 2C; MEK5, MAPK/ERK kinase 5; N.S., non-silencing; siRNA, small interfering RNA; VEGF, vascular endothelial growth factor, VEGFR-2, VEGF receptor-2.*

Similar to ERK5 siRNA-treated cells, treatment with MEK5-specific siRNA also abolished VEGF-induced HDMEC tube formation over 24 h, with cells having an almost identical appearance to those of ERK5-siRNA treated cells (**Fig. 4.11 A**). Transfection of HDMECs with VEGFR-2-specific siRNA induced at least a 95% down-regulation of *VEGFR-2* expression, as assessed by qRT-PCR analysis (data not shown). Consonant with the accepted role of VEGFR-2 in mediating VEGF-induced tubular morphogenesis of endothelial cells *in vitro* (Yang *et al.,* 2001), VEGFR-2-specific siRNA-treatment prevented VEGF-induced tubular morphogenesis of HDMECs (**Fig. 4.11 A**). Quantification of tubule length revealed that transfection with MEK5-, ERK5- or VEGFR-2-specific siRNA induced a 10-fold lowering in tubule length compared to the

N.S. siRNA control (**Fig. 4.11 B**). Together, these results show that MEK5 and ERK5 are critical for VEGF-induced tubular morphogenesis of HDMECs, and that this response also requires VEGFR-2.

The effects of treating HDMECs with siRNA directed against ERK1 and ERK2 upon VEGF-stimulated HDMEC tube formation was also assessed. It was found that down-regulation of ERK1 had a slight effect upon VEGF-induced morphogenesis. Specifically, VEGF-induced tube formation occurred following siRNA-mediated down-regulation of *ERK1* expression, but these tubes exhibited a reduced degree of branching and anastomosis, compared to N.S. siRNA-treated cells. Knockdown of both ERK1 and ERK2 together had a similar effect upon VEGF-induced tube formation with overall tubule length reduced by approximately 20% in these cells (**Fig. 4.11, A and B**). By contrast, treatment with ERK2-specific siRNA had no discernable effect upon VEGF-induced tube formation (**Fig. 4.11 A**).

It has been suggested that the transcription factor MEF2C mediates ERK5-regulated endothelial cell survival (Hayashi *et al.*, 2004; Olson, 2004). To assess the effects of MEF2C knockdown upon VEGF-induced tube formation in this assay, MEF2C expression was down-regulated by treatment with MEF2C siRNA 1 and MEF2C siRNA 2. SiRNA-mediated down-regulation of MEF2C impaired VEGF-induced tubular morphogenesis, reducing overall tubule length by ~20% (**Fig. 4.11, A and B**). However, whilst some cells displayed signs of impaired migration, with thread-like protrusions, the majority of the cells underwent normal anastomosis to form branching vessels (**Fig. 4.11 A**). Importantly, the appearance of MEF2C siRNA-treated cells was unlike that of MEK5- or ERK5-siRNA-treated cells, suggesting that MEF2C is not involved in VEGF-induced MEK5/ERK5-mediated tube formation of HDMECs (**Fig. 4.11 A**).

4.7.3 SiRNA-mediated down-regulation of ERK5 expression prevents tubular morphogenesis at an early stage during VEGF-induced tubule formation

To establish whether siRNA-mediated knockdown of ERK5 expression was affecting the kinetics of ERK5 VEGF-induced tubular morphogenesis of HDMECs, a time-course study was conducted. HDMECs transfected with N.S. siRNA or ERK5 siRNA, were seeded

between two layers of collagen I, and stimulated with VEGF. Cells within the 3-D collagen matrix were fixed at 3 h post-seeding (0 h time point) and thereafter at 1, 3, 6, 9, 18 and 24 h post-VEGF-stimulation. Assessment of cellular morphology by light microscopy revealed that HDMECs transfected with N.S. siRNA underwent cell spreading at 1 h post-VEGF stimulation (**Fig. 4.12 A, *ii***). Cells then migrated and formed interconnections by 3 h (**Fig. 4.12 A, *iii***); this was followed by cell anastomosis at 6-9 h (**Fig. 4.12 A, *iv-v***). Distinctive tubular structures had formed by 18 h, which later developed into a mature capillary-like network by 24 h (**Fig. 4.12 A, *vi-vii***).

Although ERK5 siRNA-treated cells showed some signs of cell spreading at 1 h post VEGF-stimulation, a notable increase in single, rounded cells was apparent compared to N.S. siRNA-treated cells at the same time point (**Fig. 4.12 B, *ii***). By 3 h post-VEGF-stimulation, the majority of ERK5-siRNA-treated cells appeared rounded, with few interconnecting cells (**Fig. 4.12 B, *iii***). Some fused cells were apparent at 6 h post-induction of tubulogenesis; however, the majority of cells seemed to have undergone apoptosis (**Fig. 4.12 B, *iv***). Nevertheless, the most striking difference in cellular morphology between ERK5-siRNA- and N.S.-siRNA-treated cells was evident at 9 h post-VEGF-stimulation (**Fig. 4.12 B, *iv***). At this time point, few of the cells appeared viable; and those cells that had survived were characterised as fused cell clusters, forming isolated islands within the collagen matrix. Cytoskeletal and nuclear staining of cells fixed at 24 h post-VEGF-stimulation, revealed that whereas N.S. siRNA-treated HDMECs formed capillary networks consisting of several anastomosed cells (**Fig. 4.12 A, *viii***), ERK5-siRNA-treated cells displayed no tubular morphogenesis, and possessed both hypercondensed and fragmented nuclei, two characteristic signs of apoptosis (**Fig. 4.12 A, *viii***) (Wyllie *et al.*, 1980).

In conclusion, down-regulation of ERK5 expression by ERK5-specific siRNA prevented VEGF-induced morphogenesis of HDMECs over 24 h at an early stage, impeding tubule network formation prior to 9 h post-VEGF stimulation. Based on the morphology of ERK5 siRNA-treated cells at the 24 h time point (**Fig. 4.12 B, *vii-viii***); it was deemed likely that ERK5 is necessary for mediating HDMEC survival following VEGF-stimulation on a collagen matrix. By contrast, HDMECs treated with either ERK5-specific siRNA or

with N.S. siRNA, and stimulated with VEGF for 24 h, but plated on a gelatin matrix rather than within a collagen I matrix, displayed a typical 'cobblestone' appearance, with no detectable difference in cellular morphology in the ERK5 siRNA-treated cells compared to the N.S. siRNA-treated cells (**Fig. 4.12 A, *ix* and Fig. 4.12 B, *ix***).

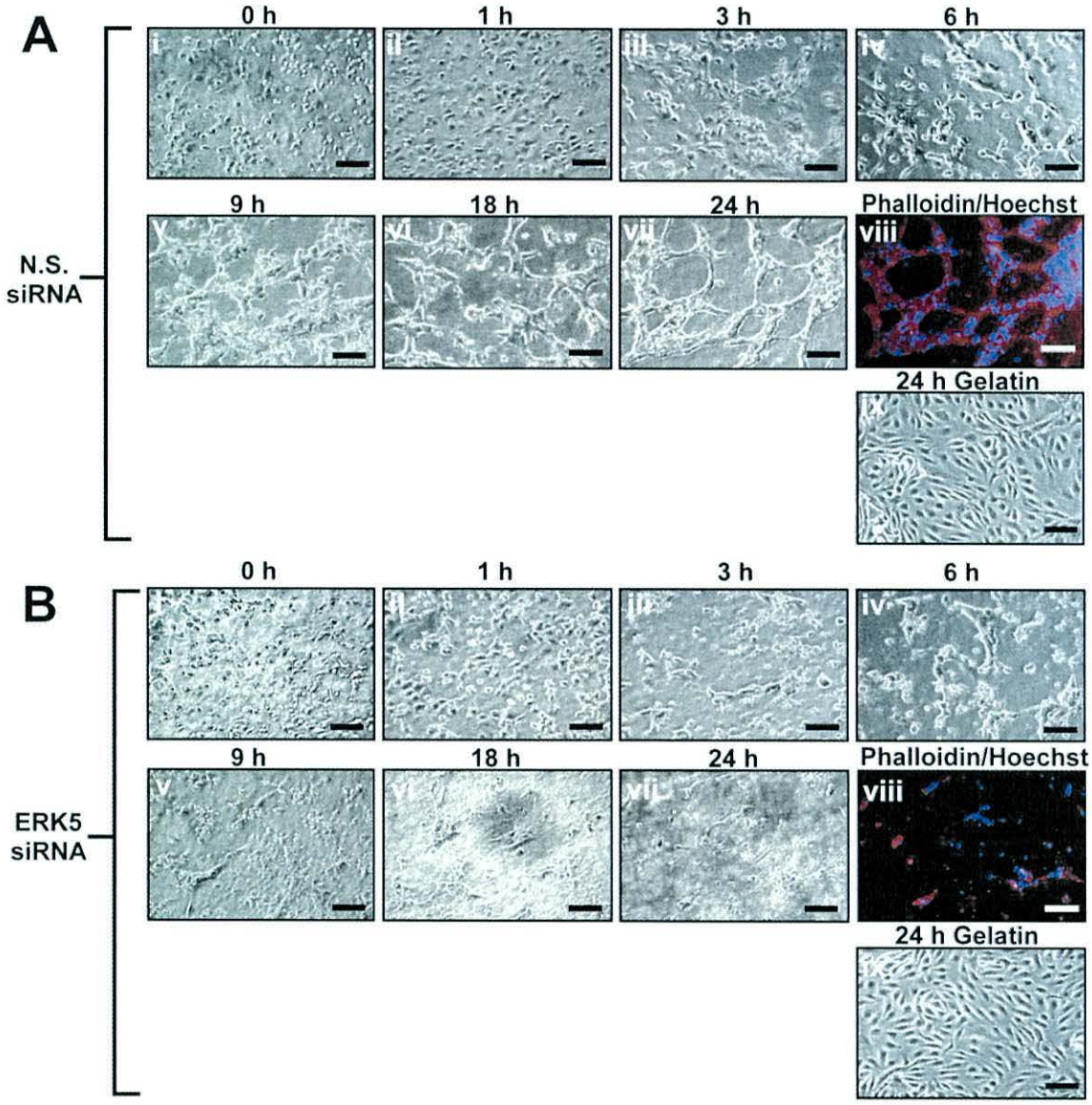


Figure 4.12 SiRNA-mediated down-regulation of ERK5 expression prevents VEGF-stimulated tubular morphogenesis at an early stage in 3-D collagen I structures. HDMECs seeded at 8.25 x 10^5 cells per dish in EBM MV2 growth medium on 10 cm dishes were grown for 24 h, prior to transfection with 5 nM ERK5 siRNA 1 and 5 nM ERK5 siRNA 2 (ERK5 siRNA), or 10 nM of non-silencing control siRNA (N.S. siRNA) as described (*section 2.2.2.7.2*). At 24 h post-transfection, cells were serum starved in EBM MV2 basal medium containing 1% (v/v) FCS for 20 h prior to seeding between two layers of collagen I gels at 2.6 x 10^5 cells per well in 12-well cell-culture dishes (*section 2.2.7.1*). Cells seeded between the collagen I gels were stimulated with EBM MV2 basal medium containing 1% (v/v) FCS supplemented with 50 ng/ml VEGF. Cells were seeded in separate plates, and were either fixed immediately (0 h time point) in 4% (w/v) paraformaldehyde (*section 2.2.7.1.5*), or were fixed at 1 h, 3 h, 6 h, 9 h, 18 h or 24 h thereafter. The nuclei and F-actin fibres of VEGF-stimulated HDMECs were stained with Hoechst 33342 (blue) and Alexa Fluor® 546 phalloidin (red) as described (*section 2.2.7.1.6*), and visualised by inverted fluorescence microscopy (*section 2.2.7.1.7*). This experiment was repeated twice with similar results. Scale bars = 100 µm. *ERK5, extracellular signal-regulated kinase 5; FCS, foetal calf serum; HDMEC, human dermal microvascular endothelial cell; N.S., non-silencing; siRNA, small interfering RNA; VEGF, vascular endothelial growth factor.*

4.8 Effects of siRNA-mediated down-regulation of ERK5 expression on VEGF-induced survival of HDMECs plated on a gelatin matrix or within 3-D collagen gels

4.8.1 SiRNA-mediated down-regulation of ERK5 expression induces early apoptosis in HDMECs during VEGF-induced tubular morphogenesis on a collagen matrix, but not during VEGF-induced proliferation on a gelatin matrix

It was hypothesised that the lack of tubes formed following VEGF stimulation of HDMECs in a 3-D collagen matrix (**Fig. 4.11 – Fig. 4.12 B**) was due to the onset of early apoptosis. To test this, the expression of phosphatidylserine (PS) was quantified by an annexin V assay. Annexin V binds to the cell membrane molecule PS only when PS is exposed on the outer surface of the cell, a process that is a hallmark of early apoptosis (Martin *et al.*, 1995; van Engeland *et al.*, 1996). HDMECs were treated with ERK5-specific siRNA (5 nM ERK5 siRNA 1 and 5 nM ERK5 siRNA 2) prior to serum-starvation for 20 h, and the plating of cells on a gelatin matrix or within a collagen matrix. After 1 h incubation on each matrix, cells were stained with annexin V Alexa Fluor® 488 conjugate and counterstained with Hoechst 33342 to detect cell nuclei as described (*section 2.2.12.1*). Cells were viewed under an inverted fluorescence microscope (**Fig. 4.13 A**), and the abundance of annexin V-positive cells following ERK5-siRNA treatment, relative to the those apparent following N.S.-siRNA treatment was quantified (**Fig. 4.13 B**).

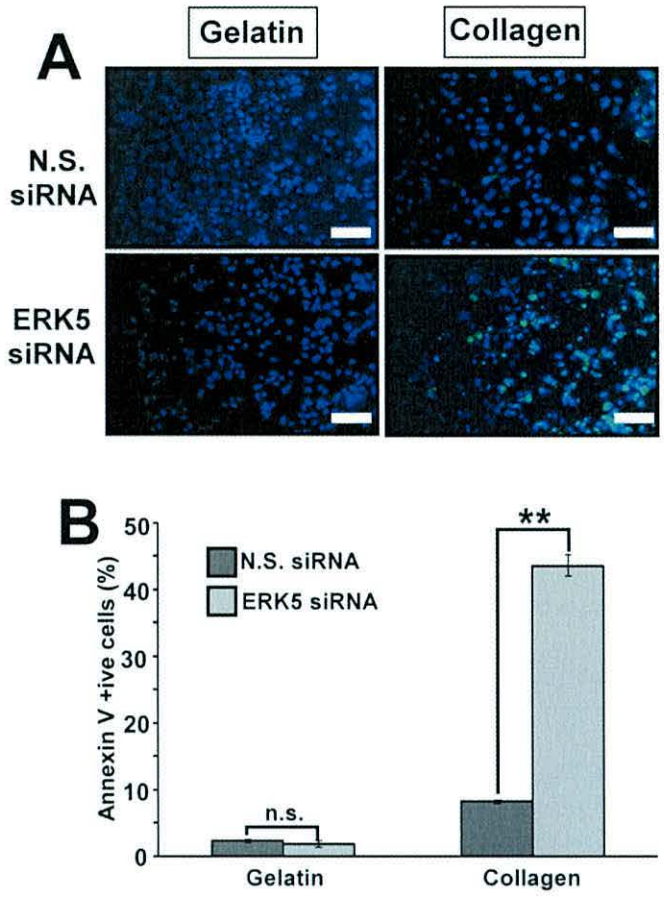


Figure 4.13 ERK5 siRNA treatment induces early apoptosis of HDMECs plated within a collagen matrix, but not in cells plated on a gelatin matrix. (**A**) HDMECs were plated at 8.25 x 10^5 cells per plate on gelatin-coated 10 cm dishes in 10 ml of EBM MV2 growth medium. At 24 h post-seeding, HDMECs were transfected with ERK5 siRNA (5 nM ERK5 siRNA 1 + 5 nM ERK5 2 siRNA) or N.S. siRNA (10 nM) as described (*section 2.2.2.7.2*). At 24 h post-transfection, cells were serum-starved in EBM MV2 basal medium containing 1% (v/v) FCS. Following 20 h serum starvation, cells were plated on gelatin-coated 24-well plates, or between two layers of collagen I, and stimulated with 50 ng/ml VEGF (*section 2.2.7.1*). At 1 h post-VEGF-stimulation, cells were stained with annexin V-Alexa Fluor® 488 conjugate (green) and Hoechst 33342 (blue) as described (*section 2.2.12.1*). (**B**) Quantification of early apoptosis in siRNA-transfected HDMECs after 1 h of tube formation (collagen) or proliferation (gelatin). Data is presented as percentage of annexin V-positive cells (n = 3; mean ± SD; n.s., not significant; $^{**}p<0.01$; unpaired Student's *t* test). Scale bars = 100 µm. *ERK5, extracellular signal-regulated kinase 5; FCS, foetal calf serum; HDMEC, human dermal microvascular endothelial cell; N.S., non-silencing; siRNA, small interfering RNA; VEGF, vascular endothelial growth factor.*

Within collagen gels, ERK5 siRNA-treated cells displayed a 4-fold increase in apoptosis compared to cells transfected with N.S. siRNA (**Fig. 4.13, A and B**). By contrast, ERK5-siRNA-treated cells plated on a gelatin matrix showed no significant difference in the number of annexin V-positive cells, compared to that of the N.S. siRNA-treated cells (**Fig. 4.13, A and B**). When considered together with **Fig. 4.12**, these data indicate that expression of ERK5 is necessary for early tubular morphogenesis of HDMECs within a 3-D collagen gel in response to VEGF by preventing apoptosis of these cells within this matrix.

4.8.2 ERK5 is required to prevent caspase-3/7 activation during conditions of VEGF-stimulated tubular morphogenesis of HDMECs on a collagen matrix, but not during HDMEC proliferation on a gelatin matrix

Activation of the cysteine-dependent aspartyl-specific proteases (caspases) represents one of the terminal stages of pathways leading from death receptor-, or mitochondrial-induced signals to mediate apoptosis in endothelial cells (Riedl & Shi, 2004; Pober *et al.*, 2009). The observed effects of ERK5 down-regulation upon VEGF-induced tubular morphogenesis in a collagen gel (**Fig. 4.12 B**), in combination with the observation that ERK5 siRNA-treated cells were annexin V-positive on a collagen matrix (**Fig, 4.13**) strongly suggested that loss of ERK5 induced apoptosis of HDMECs plated within a 3-D collagen gel. To investigate this premise further, the activity of the executioner caspases, caspase-3 and -7, was compared in HDMECs plated on a gelatin matrix, and in HDMECs plated within a collagen matrix at 1 h, 6 h and 24 h post-stimulation with VEGF, using a fluorescence-based assay.

The microbial alkaloid, staurosporine (STS), had been previously shown to induce apoptosis in endothelial cells (Kabir *et al.*, 2002), and so was used as a positive control to induce caspase-3/7 activation in this assay. Treatment with STS induced maximal activation of caspases-3/7 after 1 h on a gelatin or collagen matrix (**Fig. 4.14, A and B**). At this time point, ERK5 siRNA-treated cells induced 40% of the caspase-3/7 activity induced by STS treatment, an effect that was not suppressed by VEGF treatment (**Fig. 4.14 B**). By comparison, the caspase-3/7 activity of ERK5 siRNA-treated cells plated on a gelatin matrix was approximately 20%, a level similar to that observed in N.S. siRNA-treated cells plated on this matrix (**Fig, 4.14 A**).

Following 6 h incubation on a gelatin matrix, ERK5 siRNA-treated and N.S. siRNA-treated cells displayed approximately 20% of the of the maximal caspase-3/7 activity induced by STS (**Fig. 4.14 C**). By comparison, on a collagen gel, maximal apoptosis, equivalent to the level observed in STS-treated cells was induced by siRNA-mediated down-regulation of ERK5 (**Fig. 4.14 D**). Maximal apoptosis was also evident after 24 h incubation of ERK5 siRNA-treated cells on a collagen matrix (**Fig. 4.14 F**), but this effect was not seen in ERK5-siRNA-treated cells incubated on a gelatin matrix (**Fig. 4.14 E**).

After 24 h incubation, under basal conditions, caspase-3/7 activity was induced in HDMECs plated within a collagen gel, an effect that was also apparent in N.S. siRNA-treated wells, but that was partly suppressed by the addition of VEGF (**Fig. 4.14 F**).

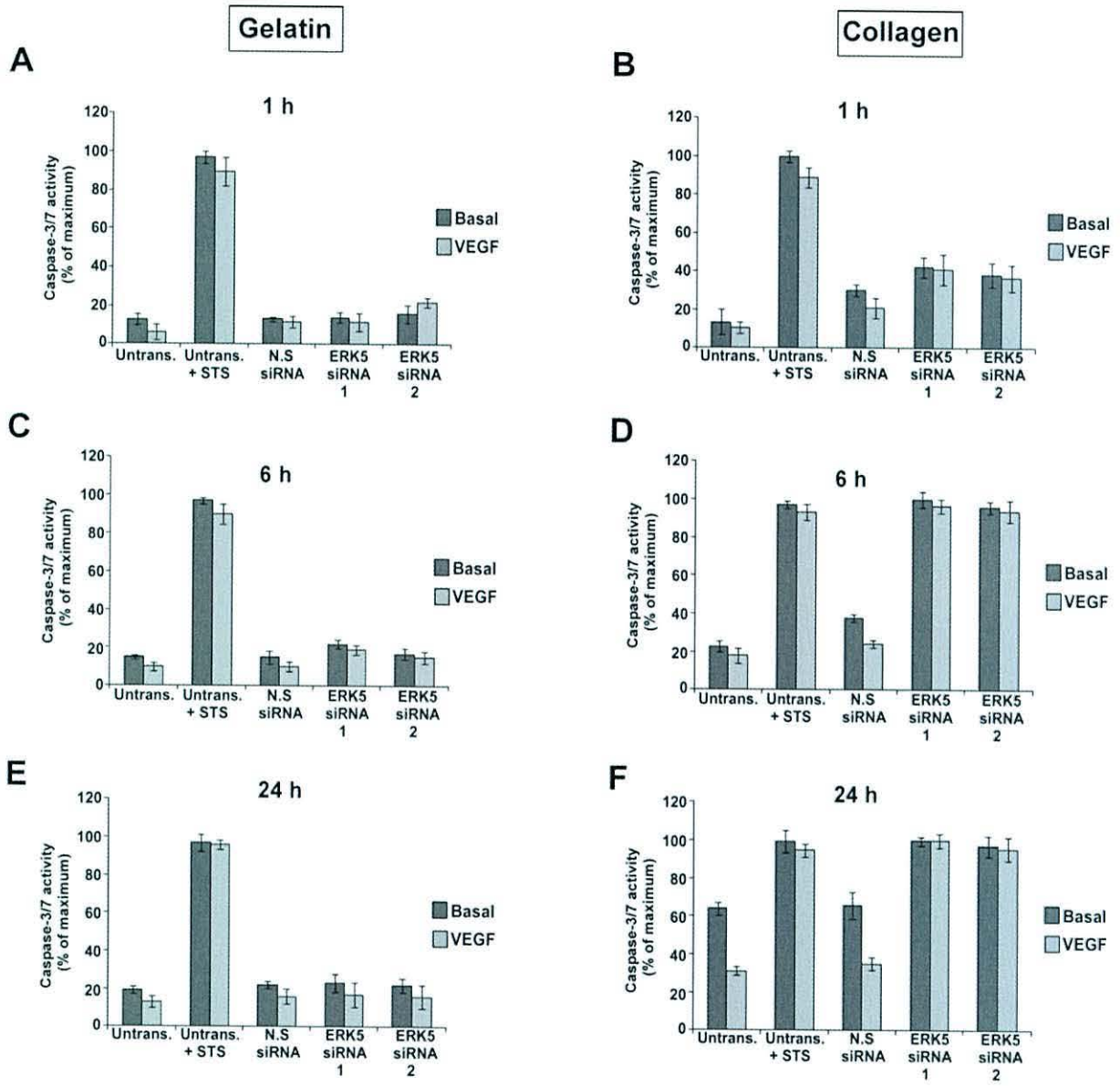


Figure 4.14 SiRNA-mediated down-regulation of ERK5 expression induces activation of caspases-3/7 during HDMEC tubular morphogenesis, but not during proliferation. HDMECs were transfected with 10 nM of ERK5 siRNA 1, or 10 nM of ERK5 siRNA 2, or with 10 nM of non-silencing (N.S.) siRNA as described (*section 2.2.2.7.2*), or left untransfected (Untrans.). At 24 h post-transfection, cells were serum-starved in EBM MV2 basal medium containing 1% (v/v) FCS for 20 h, prior to plating on a gelatin matrix, or between two layers of a collagen I gel as described (*section 2.2.7.1*). Cells were then stimulated with VEGF (50 ng/ml) and/or 50 mM staurosporine (STS) as indicated, and lysed in Caspase-Glo® 3/7 reagent at 1 h (**A** and **B**), 6 h (**C** and **D**) or 24 h (**E** and **F**), post-treatment with VEGF or STS as described (*section 2.2.12.2*). Data is presented as percentage of maximal caspase-3/7 activity (n = 3; mean ± SD). This experiment was repeated twice with similar results. *ERK5, extracellular signal-regulated kinase 5; FCS, foetal calf serum; HDMEC, human dermal microvascular endothelial cell; N.S., non-silencing; siRNA, small interfering RNA; STS, staurosporine; VEGF, vascular endothelial growth factor.*

To further confirm the role of caspase-3 in HDMEC apoptosis caused by loss of ERK5, the levels of the active 17 kDa and 19 kDa caspase-3 proteolytic fragments were analysed by Western blotting. SiRNA-mediated down-regulation of ERK5 occurred to similar levels on both collagen and gelatin matrices (**Fig. 4.15, A and B**). In agreement with the findings shown in **Fig. 4.14**, whilst STS treatment induced cleavage of pro-caspase-3 into the active cleaved-caspase-3 fragments, loss of ERK5 was not sufficient to induce cleavage of caspase-3 in HDMECs plated on a gelatin matrix (**Fig. 4.15, A and C**). Within a 3-D collagen gel, treatment with N.S. siRNA induced a low level expression of cleaved caspase-3, an effect that was prevented in the presence of VEGF (**Fig. 4.15, B and D**). By contrast, in HDMECs treated with ERK5-specific siRNA and plated within a 3-D collagen matrix cleaved caspases-3 was detectable in both the presence and the absence of VEGF (**Fig. 4.15, B and D**). Addition of STS was sufficient to give rise to the 17 kDa and 19 kDa cleaved caspase-3 fragments on both gelatin and collagen matrices (**Fig. 4.15, A and B**). Taken together, these data show that under conditions of tubular morphogenesis, ERK5 is critical for mediating VEGF-induced suppression of caspase-3 cleavage into the active 17 kDa and 19 kDa fragments, to facilitate endothelial cell survival. Conversely, on a gelatin matrix, where proliferation occurs, ERK5 is not required for suppression of caspase-3 activity, in either the presence or the absence of VEGF.

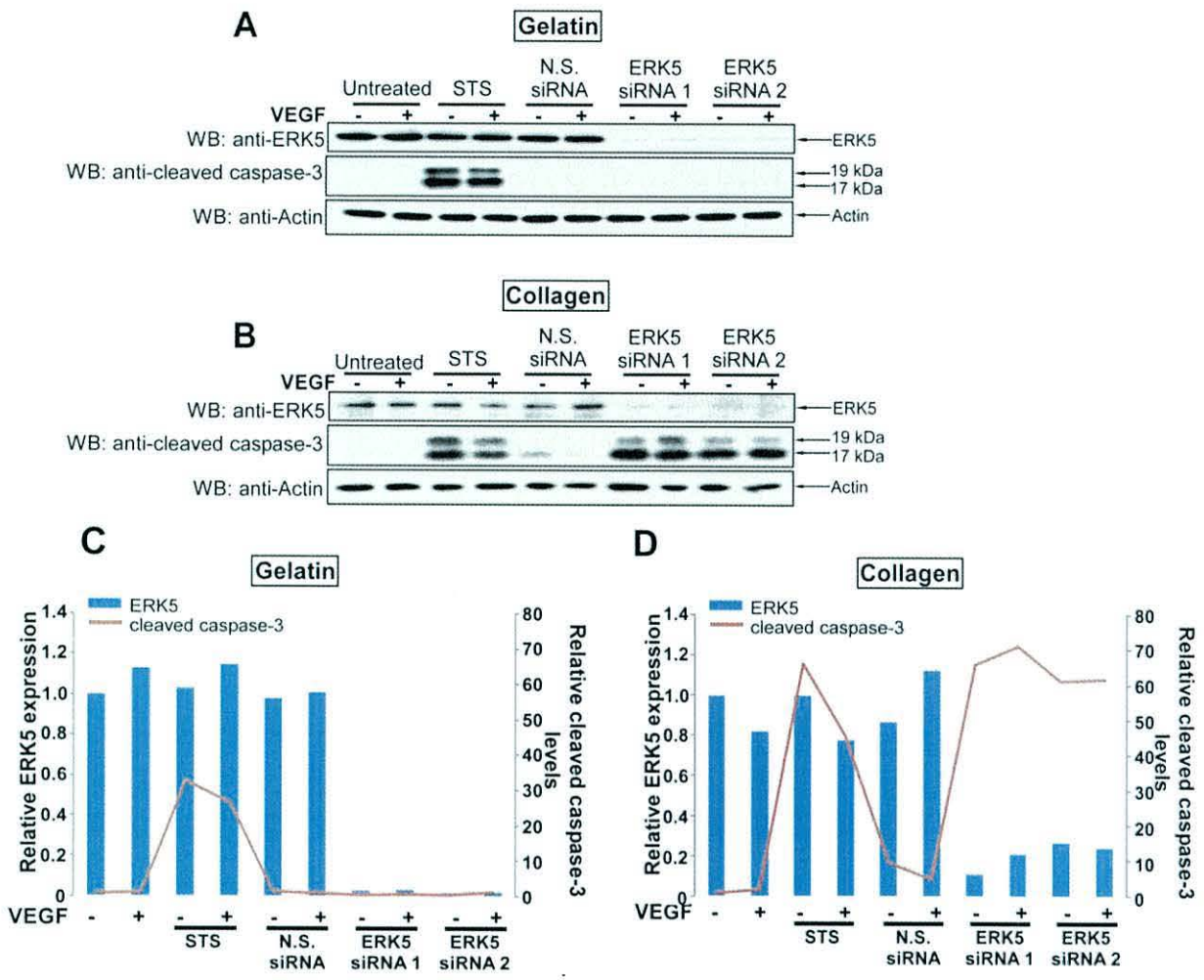


Figure 4.15 SiRNA-mediated down-regulation of ERK5 expression induces caspase-3 cleavage in HDMECs plated within collagen matrices, but not in cells plated on gelatin matrices, in the presence or in the absence of VEGF. HDMECs were transfected with ERK5 siRNA 1 (10 nM) or ERK5 siRNA 2 (10 nM) or with 10 nM non-silencing (N.S.) control siRNA as described (*section 2.2.2.7.2*) or were left untransfected. At 24 h post-transfection all cells were serum-starved in EBM MV2 basal medium containing 1% (v/v) FCS for 20 h, prior to plating on (**A**) a gelatin-coated 12-well dishes or (**B**) between two layers of collagen I gels in 12-well dishes as described (*section 2.2.7.1*), and treated (or not) with 50 ng/ml VEGF as indicated. As a positive control, certain cells were treated with 50 nM staurosporine (STS) to induce apoptosis. Cells were lysed (*section 2.2.6.1.2*) at 6 h post-VEGF treatment on both matrices. Total cell lysates were analysed by SDS-PAGE on NuPAGE® 4-12% gradient Bis-Tris gels followed by immunoblotting with antibodies directed against ERK5 and cleaved caspase-3 (17 kDa and 19 kDa fragments). Actin expression was analyzed as a loading control. Quantification of the relative expression of ERK5 and relative cleaved caspase-3 expression of cells on (**C**) gelatin and (**D**) collagen was normalized to actin expression on the respective matrices using NIH ImageJ software (*section 2.2.6.4.2*). This experiment was repeated twice with similar results. *ERK5, extracellular signal-regulated kinase 5; FCS, foetal calf serum; HDMEC, human dermal microvascular endothelial cell; N.S., non-silencing; siRNA, small interfering RNA; STS, staurosporine; VEGF, vascular endothelial growth factor*

4.9 Effects of transient expression of a dominant-negative mutant of ERK5, or a constitutively active mutant of MEK5, on HDMEC physiology

4.9.1 Optimisation of conditions for transient expression of plasmid constructs in HDMECs

Transient transfection of primary endothelial cells is difficult to achieve (Kovala *et al.,* 2000; Ear *et al.,* 2001). Early experiments aimed at over-expressing plasmid constructs in HDMECs performed within our laboratory were carried out using Amaxa® HMVEC-L Nucleofector® technology (Lonza Cologne AG, Cologne, Germany), a method that had been successfully utilised in other studies (Ear *et al.*, 2001; Kang *et al.*, 2009). However, assessment of transfection efficiency by transfection of green fluorescent protein (GFP)-containing plasmids in HDMECs revealed that, in general, only approximately 20% of HDMECs were successfully transfected using this method (data not shown). Furthermore, the numerous cell-manipulation stages involved in this procedure resulted in the death of at least 50% of cells, as determined by inverted light microscopy, which revealed the cells as being rounded and detached from the gelatin matrix at 24 h post-transfection (data not shown), thus suggesting that cells were unable to recover from the effects of transfection. Overall, this technique was deemed inappropriate for the purpose of investigating the effects of transient transfection of ERK5 cascade mutant proteins upon intracellular signalling in HDMECs, and the resultant effects of these treatments upon HDMEC physiology.

In a search for alternative transfection methods to the Amaxa® HMVEC-L Nucleofector® method, several commercially-available transfection reagents including: Lipofectamine™ 2000 (Invitrogen, Paisley, U.K.), Lipofectamine™ LTX (Invitrogen, Paisley, U.K.) and NanoJuice™ (Merck Chemicals Ltd., Nottingham, U.K.) were tested for transfection efficiency and the resultant effects of transfection upon cell morphology (data not shown). Of the transfection reagents tested, a recently developed transfection reagent termed TransPass™ V HUVEC transfection reagent (New England Biolabs, Hitchin, U.K.), which had been specifically optimised for the transfection of endothelial cells, was found to provide the highest transfection

efficiency (70-80%), with minimal effects on HDMEC morphology post-transfection (data not shown).

4.9.2 Optimisation of conditions for the transient transfection of components of the ERK5 signalling axis in HDMECs using TransPass™ HUVEC transfection reagent

Two plasmid constructs, one containing the wild-type (wt) human *ERK5* gene, termed ERK5(wt), and another containing a dominant-negative (DN) version of human *ERK5*, termed DN-ERK5(AEF), both of which containing an N-terminus Flag (DYKDDDDK) tag, have been described previously (Kato *et al.*, 1997). These plasmids were kindly donated by Prof. J.-D. Lee (The Scripps Research Institute, La Jolla, CA, U.S.A.) for use in this study. DN-ERK5(AEF) cannot be phosphorylated by MEK5, as the Thr^{218} and Tyr^{220} residues of the T-E-Y dual-phosphorylation motif required for ERK5 activation have been mutated by site-directed mutagenesis to give to Ala^{218} and Phe^{220} (A-E-F) respectively (Kato *et al.*, 1997). These constructs were verified by nucleotide sequencing (*section 2.2.3.7*).

Phosphorylation of the residues Ser^{313} and Ser^{317}, present in the kinase domain of MEK5, is required for activation of MEK5 and the subsequent activation of ERK5 (Zhou *et al.*, 1995; Kato *et al.*, 1997; Mody *et al.*, 2003). By using site-directed mutagenesis to replace the Ser^{313} and Ser^{317} with Asp^{313} and Asp^{317} residues, thus giving permanent negative charge at these sites, Kato *et al.* constructed a CA, phosphomimetic form of rat *Mek5α-1*, termed CA-MEK5(D) (Kato *et al.*, 1997). CA-MEK5(D) was obtained from Prof. J.-D. Lee in the expression vector pCMV5 and contained 3 copies of the haemagluttinin (HA) epitope at the C-terminus (Kato *et al.*, 1997).

With the exception of CA-MEK5(D), all of the cDNAs of interest for this study had been cloned into the mammalian expression vector pcDNA3.1. In order to maintain consistency, and to reduce the possibility of experimental variation that may be caused by differential expression due to the use of different vectors, it was decided to excise MEK5(D)$(HA)_3$ from pCMV5-MEK5(D)$(HA)_3$ using the restriction endonucleases *Xba* I and *Kpn* I, prior to subcloning MEK5(D)$(HA)_3$ into the expression vector pcDNA3.1 which had been cut with the same enzymes, to give the plasmid pcDNA3.1-

MEK5(D)$(HA)_3$. This plasmid was sequenced, confirming that the full protein, together with the three copies of the HA tag at the C-terminus was present, and that only mutations giving rise to Asp^{313} and Asp^{317} were present.

To optimise conditions for over-expression of ERK5, it was firstly necessary to establish the optimal amount of TransPass™ V component relative to the TransPass™ HUVEC transfection reagent component to use. Preliminary experiments revealed that when used at the volumes and ratios recommended by the manufacturers for transfection of HUVECs, exposure to the transfection mixture resulted in widespread death of HDMECs (data not shown). The amount of transfection reagent was thus titrated from 8 µl to 4 µl. Interestingly, the recommended 1:2 ratio of HUVEC transfection reagent to TransPass™ V component led to poor transfection efficiency and increased cell death, suggesting that large quantities of the TransPass™ V adenoviral component is toxic to HDMECs (**Fig. 4.16**). However, when used at a volume of 4 µl and at a 1:1 ratio of HUVEC transfection reagent to TransPass™ V component, no adverse effects of this transfection component could be observed upon the HDMECs (data not shown). Over-expression of both ERK5(wt) and DN-ERK5(AEF) to similar levels under these experimental conditions was confirmed by Western blot analysis with anti-ERK5 antibody (**Fig. 4.16**). Increasing the amount of each plasmid transfected from 2.5 µg to 5 µg had no effect upon the expression levels of ERK5 (**Fig. 4.16**). Based on these results, future experiments were performed using 2.5 µg of plasmid DNA and a 4 µl: 4 µl ratio of HUVEC transfection reagent to TransPass™ V component.

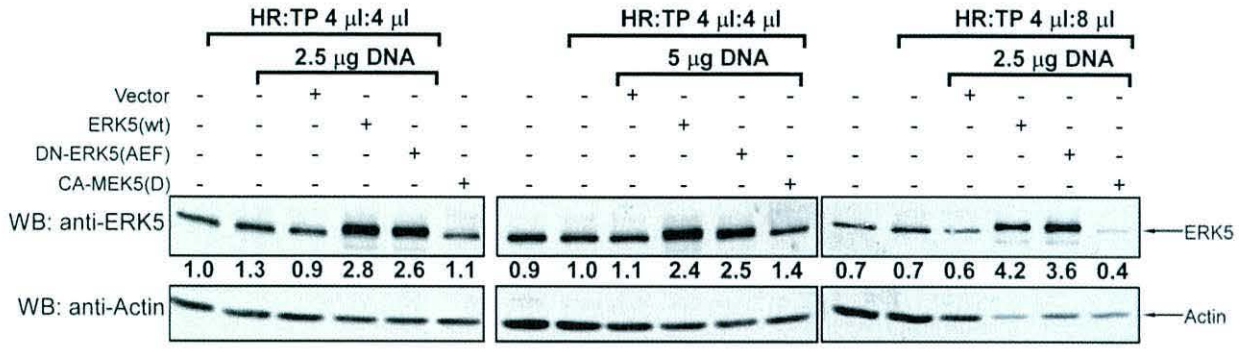


Figure 4.16 Optimisation of experimental conditions for transfection of HDMECs with plasmid constructs using TransPass™ V HUVEC transfection reagent. Cells were seeded at 9.3 x 10^4 cells per well in 6-well plates in EBM MV2 growth medium and allowed to grow for 24 h, prior to the addition of fresh EBM MV2 growth medium. Cells were transfected as described (*section 2.2.2.7.1*) for 4 h with the indicated amounts of specified plasmids using TransPass™ V HUVEC transfection reagent, prior to the addition of EBM MV2 growth medium. The ratio, and volumes, of HUVEC Reagent (HR) component to the adenoviral TransPass™ V (TP) component used is indicated in each case. Total cell lysates were prepared 48 h post-transfection, and total cellular proteins were separated on a 10% SDS-PAGE gel followed by Western blotting (*WB*) with antibodies directed against ERK5, and actin as a loading control. *ERK5, extracellular signal-regulated kinase 5; HDMEC, human dermal microvascular endothelial cell; HR, HUVEC reagent component; MEK5, MAPK/ERK kinase 5; TP, TransPass™ V component; WB, Western blot.*

4.9.3 Co-transfection of CA-MEK5(D) and ERK5(wt) activates ERK5, and potentiates VEGF-stimulated ERK5 activation

To verify the effects of ERK5, CA-MEK5(D) and DN-ERK5(AEF) on ERK5 activation in HDMECs, these constructs were transiently expressed in HDMECs either alone, or together, and cells were stimulated, or not, with VEGF. Transpass™ V-mediated transfection of ERK5(wt) alone did not affect VEGF-induced ERK5 phosphorylation (**Fig. 4.17**). However, in agreement with previous studies (Barros & Marshall, 2005; Schweppe *et al.*, 2006), co-transfection of HDMECs with ERK5(wt) and CA-MEK5(D) induced a 5.7-fold increase in ERK5 phosphorylation at Thr^{218}/Tyr^{220} in comparison to the unstimulated basal control (**Fig. 4.17**). This increase in ERK5 activation was augmented by stimulation with VEGF (**Fig. 4.17**), and blocked by co-transfection of ERK5(AEF) and MEK5(D) (**Fig. 4.17**). These data served to confirm biochemically that transient co-expression of CA-MEK5(D) and ERK5(wt), or CA-MEK5(D) and DN-ERK5(AEF) in HDMECs, offer an effective means to respectively induce, or abolish, ERK5 activity in these cells.

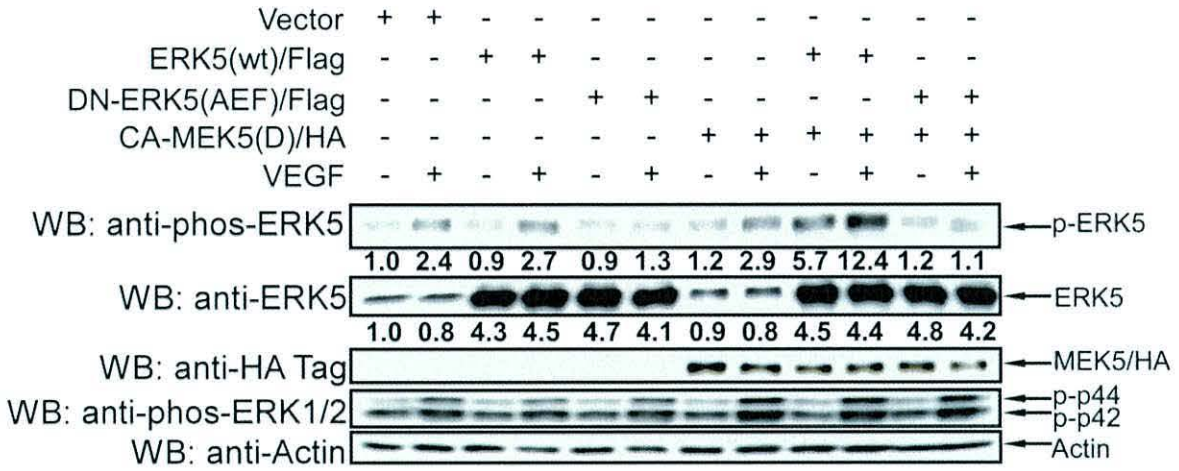


Figure 4.17 Co-expression of ERK5(wt) and CA-MEK5(D) induces ERK5 activation and augments VEGF-stimulated ERK5 activation. HDMECs were seeded in 12-well plates, and transiently transfected as described (*section 2.2.2.7.1*) with 2.5 µg wild-type (wt) ERK5; or 2.5 µg dominant-negative (DN)-ERK5(AEF); or 2.5 µg constitutively-active (CA)-MEK5(D); or 2.5 µg ERK5(wt) and 2.5 µg CA-MEK5(D); or 2.5 µg DN-ERK5(AEF) + 2.5 µg CA-MEK5(D); or alternatively, cells were mock-transfected with 2.5 µg empty pcDNA3.1 (Vector). At 24 h post-transfection, cells were serum-starved in EBM MV2 basal medium containing 1% (v/v) FCS for 20 h, prior to stimulation, or not with VEGF (50 ng/ml) for 10 min. The cells were then lysed, and total cellular proteins were separated on a 10% SDS-PAGE gel and Western blotted (*WB*) with antibodies directed against ERK5, HA-Tag, phos-ERK5, phos-ERK1/2 and actin as a loading control. *CA, constitutively active; DN, dominant-negative; ERK5, extracellular signal-regulated kinase 5; FCS, foetal calf serum; HDMEC, human dermal microvascular endothelial cell; MEK5, MAPK/ERK kinase 5; VEGF, vascular endothelial growth factor; WB, Western blot; wt, wild-type.*

4.9.4 Induction or abolition of ERK5 activity in HDMECs does not affect cell proliferation

Having established that transient transfection of CA-MEK5(D) and DN-ERK5(AEF) constructs in HDEMCs could allow the respective enhancement and blockade of ERK5 activation in HDMECs (**Fig. 4.17**), the physiological effects of transfection of these plasmid constructs upon VEGF-induced HDMEC proliferation was assessed. A comparison of cell numbers in untransfected cells and cells that had been transfected with empty pcDNA3.1 vector alone revealed that this treatment had no stimulatory or inhibitory effects upon VEGF-stimulated proliferation of HDMECs (**Fig. 4.18**). Inhibition of ERK5 activity by co-expression of DN-ERK5(AEF) and CA-MEK5(D) had no significant effect upon VEGF-stimulated HDMEC proliferation (**Fig. 4.18**). Similarly, co-transfection of HDMECs with ERK5(wt) and CA-MEK5(D) did not induce, or inhibit HDMEC proliferation (**Fig. 4.18**).

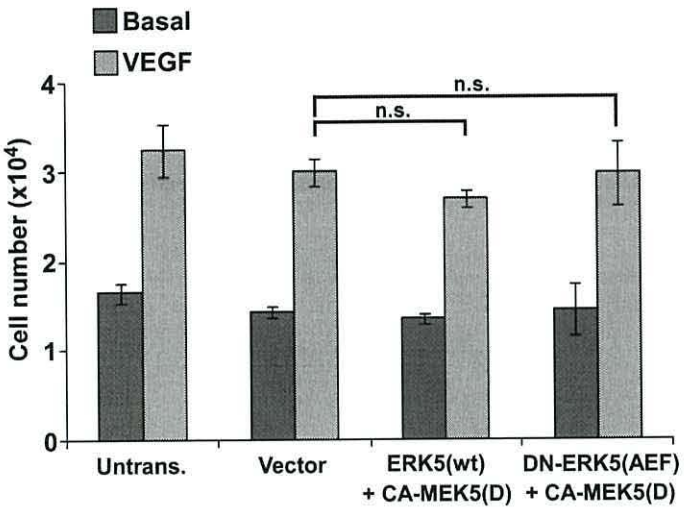


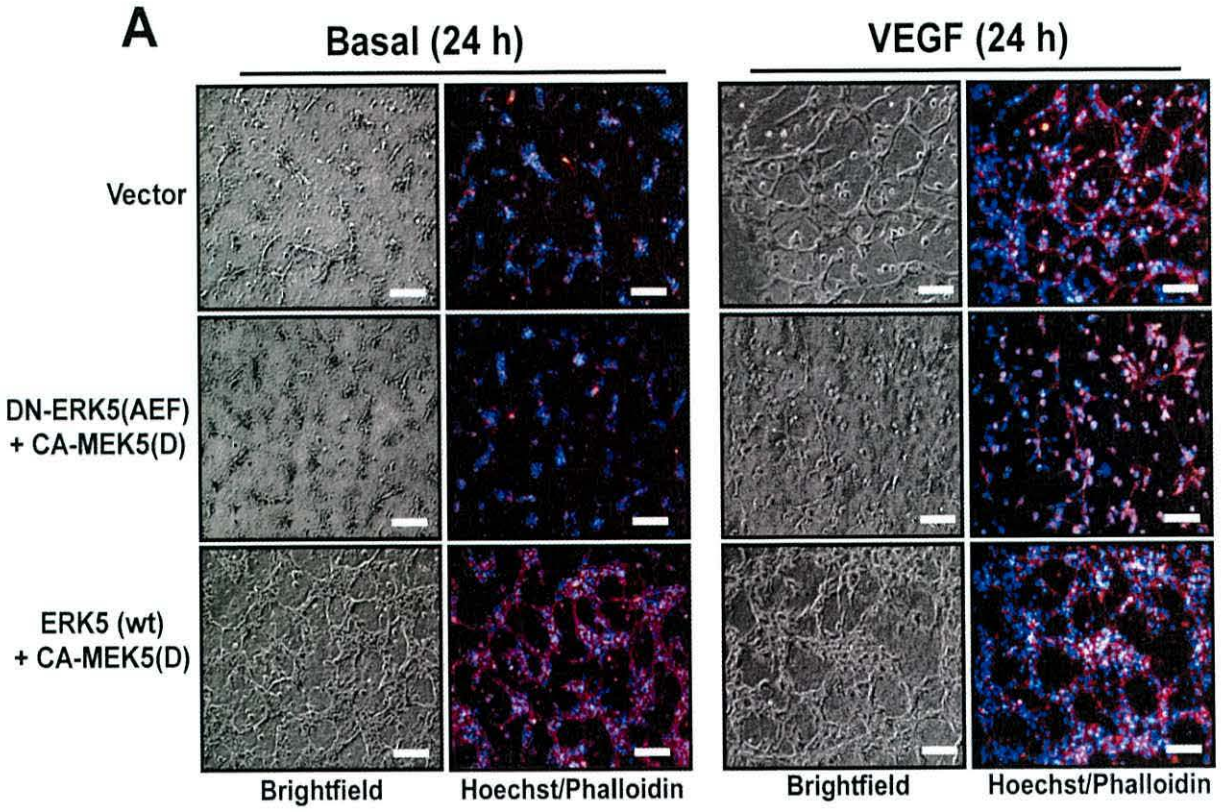
Figure 4.18 Effects of transient expression of ERK5 cascade proteins upon HDMEC proliferation. HDMECs were seeded in 6-well plates, and transiently transfected as described (*section 2.2.2.7.1*) with 2.5 µg ERK5(wt) and 2.5 µg CA-MEK5(D); or 2.5 µg DN-ERK5(AEF) + 2.5 µg CA-MEK5(D); or mock-transfected with 5 µg empty pcDNA3.1 (Vector), or alternatively left untansfected (Untrans.). Cells were incubated at 37 °C for 24 h post-transfection. At 24 h post-transfection, HDMECs were seeded at 15,000 cells per well on gelatin-coated 24-well plates and incubated at 37 °C for 24 h. Cells were serum-starved in EBM MV2 medium containing 1% (v/v) FCS for 20 h, prior to the addition of fresh EBM MV2 basal medium containing 1% (v/v) FCS alone (Basal), or supplemented with VEGF (50 ng/ml). Cells were allowed to proliferate by incubation at 37 °C for 48 h. On the fifth day of the assay cell numbers were quantified using the CellTiter-Glo® luminescent cell viability assay using a standard curve method as previously described (*section 2.2.8.1*). The data shown is representative of one of three independent experiments. (n = 3; mean ± S.D; n.s., not significant; unpaired Student's *t* test). *CA, constitutively active; DN, dominant-negative; ERK5, extracellular signal-regulated kinase 5; FCS, foetal calf serum; HDMEC, human dermal microvascular endothelial cell; MEK5, MAPK/ERK kinase 5; VEGF, vascular endothelial growth factor; wt, wild-type.*

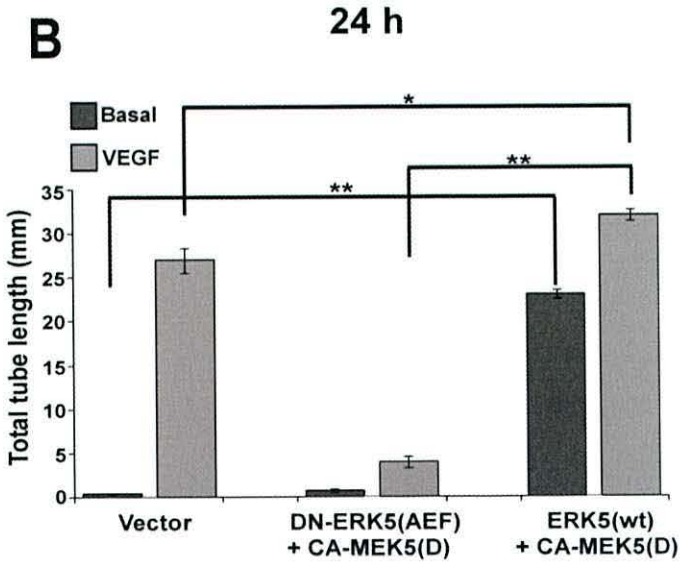
These results are in accordance with the lack of effect of siRNA-mediated ERK5 down-regulation upon HDMEC proliferation (**Fig. 4.7**). Together these data suggest that neither ERK5 itself, nor ERK5 activity is required to mediate HDMEC proliferation.

4.9.5 Constitutive activation of ERK5 stimulates angiogenesis *in vitro* and sustains VEGF-induced tubular morphogenesis

Given the profound effect of siRNA-mediated down-regulation of ERK5 expression upon VEGF-induced tubular morphogenesis of HDMECs in a 3-D collagen gel, it was of interest to investigate the effects of constitutive activation of ERK5 in the same assay. It was found that constitutive activation of ERK5 by transient co-expression of ERK5(wt) and CA-MEK5(D) stimulated HDMEC tubular morphogenesis in the absence of VEGF over 24 h (**Fig. 4.19 A**). Compared to VEGF-stimulated cells that had been transfected with the empty vector, cells transfected with ERK5(wt) and CA-MEK5(D) formed thicker tubes (**Fig. 4.19, A and B**). However, these capillary structures displayed less branching (**Fig. 4.19 A**) than cells that had been transfected with empty

vector and stimulated with VEGF. In addition, quantification of total tubule length revealed that these tubular structures were shorter than those found in vector-transfected cells stimulated with VEGF (**Fig. 4.19 B**). Following VEGF stimulation, ERK5(wt) and CA-MEK5(D)-co-transfected cells plated within a 3-D collagen I matrix displayed enhanced tubular morphogenesis, with thicker vessels, increased branching and enhanced tubule length compared to pcDNA3.1 transfected, VEGF-stimulated cells ($p<0.05$) (**Fig. 4.19, A and B**). By contrast, to the effect of constitutive activation of ERK5, blockade of ERK5 activity by co-transfection of HDMECs with DN-ERK5(AEF) and CA-MEK5(D), did not stimulate tubular morphogenesis under basal conditions, and blocked VEGF-stimulated morphogenesis over 24 h (**Fig. 4.19, A and B**).





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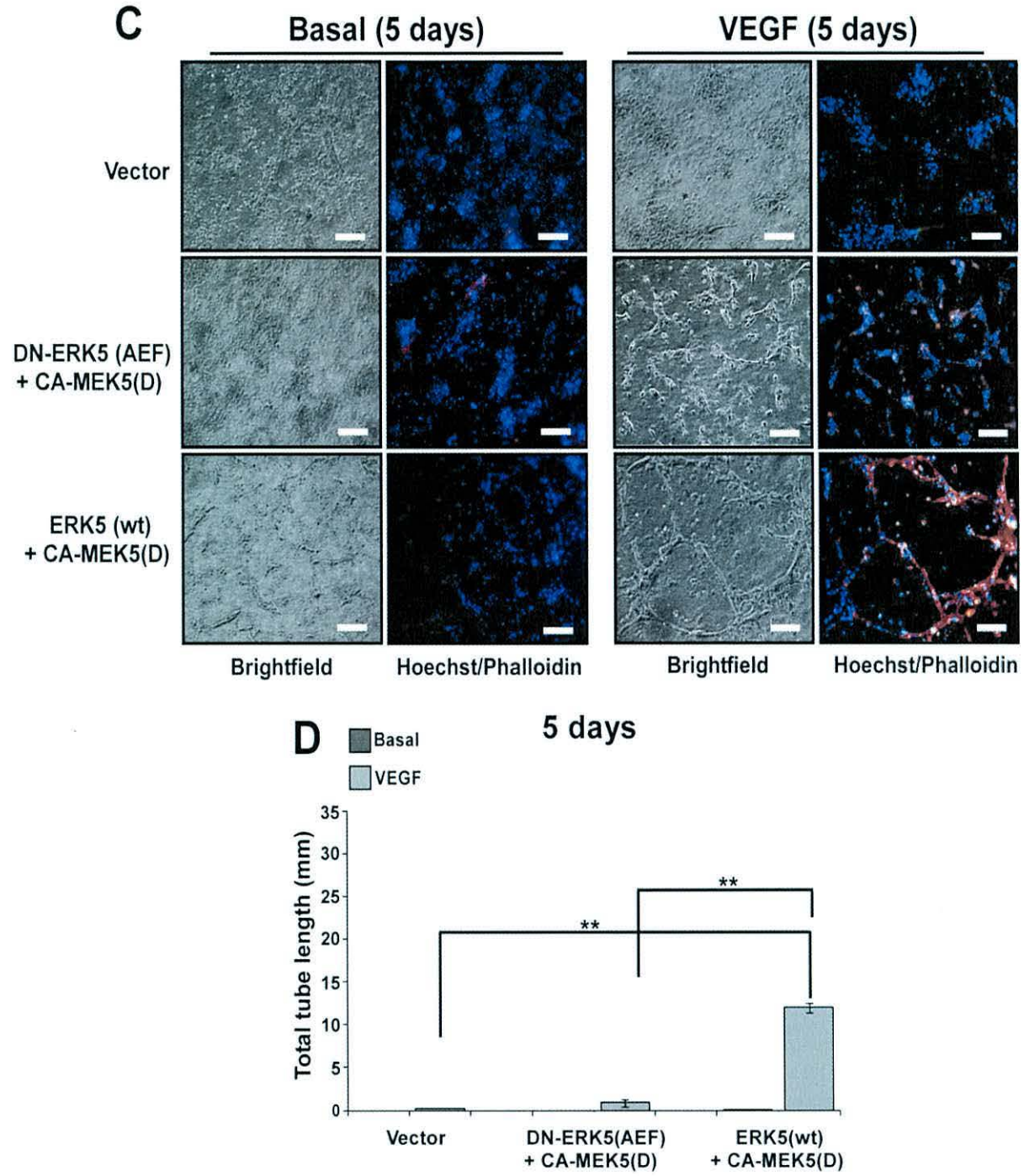

Figure 4.19 Co-expression of ERK5(wt) and CA-MEK5(D) stimulates HDMEC tubular morphogenesis in the absence of VEGF, and sustains HDMEC tube formation in the presence of VEGF. HDMECs were transiently transfected with 2.5 µg ERK5(wt) and 2.5 µg CA-MEK5(D); or with 2.5 µg DN-ERK5(AEF) + 2.5 µg CA-MEK5(D); alternatively, cells were transfected with 5.0 µg empty pcDNA3.1 vector alone (Vector). At 24 h post-transfection, HDMECs were serum-starved in EBM MV2 basal medium containing 1% (v/v) FCS for 20 h, and seeded at 1.25 x 10^5 cells per well between two layers of collagen I, prior to stimulation with VEGF (50 ng/ml). At 24 h (**A**), and 5 days (**C**) post-VEGF stimulation, cells were fixed, and the actin stress fibres, and nuclei were stained with Alexa Fluor™ 546-coupled phalloidin (red), and Hoechst 33342 (blue) respectively. (**B** and **D**) The total length of tubular structures were quantified from three fields (x10 objective) per well using NIH ImageJ software as described (*section 2.2.7.1.7*). The result shown is representative of three independent experiments. (n = 3; mean ± SD; *p<0.05; **p<0.01; unpaired Student's t test). Scale bars = 100 µm. *CA, constitutively active; DN, dominant-negative; ERK5, extracellular signal-regulated kinase 5; HDMEC, human dermal microvascular endothelial cell; MEK5, MAPK/ERK kinase 5; VEGF, vascular endothelial growth factor; wt, wild-type.*

Normal VEGF-stimulated tubular morphogenesis in this assay is maximal at 24 h, after which time tubes rapidly regress due to apoptosis, and disappear completely by 72 h (Bohman *et al.*, 2005; Dimberg *et al.*, 2008). In the absence of VEGF, co-transfection of HDMECs with ERK5(wt) and CA-MEK5(D) was not sufficient to sustain tubular morphogenesis beyond 24 h (**Fig. 4.19 C**). Strikingly however, co-transfection of ERK5(wt) with CA-MEK5(D) in the presence of VEGF sustained tubular morphogenesis for 5 days (**Fig. 4.19 C**). Together, these data demonstrate that ERK5 activity is sufficient to stimulate angiogenesis in a 3-D collagen I matrix in the absence of VEGF over 24 h, and is essential for VEGF-stimulated tube formation. Furthermore, VEGF-induced augmentation of ERK5 activity (**Fig. 4.17**) was sufficient to both enhance, and sustain tubular morphogenesis for prolonged periods.

4.10 Discussion

In this chapter, the potential role of ERK5 in mediating various aspects of growth factor-induced angiogenesis was investigated. The pro-angiogenic growth factor VEGF was found to induce tubular morphogenesis, proliferation, migration and survival of HDMECs in various angiogenic assays. VEGF had been previously shown to activate ERK5 in HUVECs (Hayashi *et al.*, 2004) and in HDMECs (*chapter 3*). However, the potential role of ERK5 in regulating individual phases of VEGF-stimulated angiogenesis in primary human endothelial cells had not been previously reported. A novel role for ERK5 as a facilitator of VEGF-induced HDMEC survival and tubular morphogenesis on a collagen I gel was described. In contrast, on a gelatin matrix ERK5 was not required for VEGF-induced HDMEC proliferation, migration or survival. These findings are discussed in further detail below.

4.10.1 ERK5 is required for VEGF-mediated survival of HDMECs during tubular morphogenesis

Hayashi *et al.* showed that pIpC-induced ablation of the *Erk5* gene in *Erk5*$^{flox/flox}$ adult mice prevented endothelial capillary network formation in VEGF- or FGF-2-treated Matrigel plugs implants in these mice (Hayashi *et al.*, 2005). However, the authors did not investigate the physiological causes of the failure of endothelial cells to form

capillary networks within this matrix. Furthermore, 'tube formation' on Matrigel (a gel-like matrix prepared from murine Engelbreth–Holm–Swarm tumour cells (Kubota *et al.*, 1988), is not representative of angiogenesis *per se* as this matrix induces migration, and 'tube formation' of non-endothelial cells such as fibroblasts, even in the absence of growth factor stimulation (Obeso *et al.*, 1990; Vernon *et al.*, 1992; Donovan *et al.*, 2001; Auerbach *et al.*, 2003).

In this chapter, a 3-D collagen I angiogenesis assay, which is highly representative of sprouting angiogenesis *in vivo* (Kamei *et al.*, 2006; Dimberg *et al.*, 2008), was used to assess the effects of siRNA-mediated ERK5 down-regulation upon VEGF-stimulated tube formation in human microvascular endothelial cells. Importantly, in contrast to Matrigel, formation of tubular structures within collagen gels is endothelial cell-specific (Delvos *et al.*, 1982), and is physiologically relevant, as endothelial cells form lumen-containing vessels that are capable of allowing blood flow when implanted into mice (Koike *et al.*, 2004). The significance of the finding that ERK5 is required for VEGF-induced tubular morphogenesis is two-fold. Firstly, it shows, for the first time in human cells, that ERK5 is required for endothelial cell survival. Secondly, it provides the first evidence that ERK5 is required to mediate VEGF-induced endothelial cell survival.

VEGF is critical for endothelial cell survival both *in vivo* and *in vitro* (Alon *et al.*, 1995; Gerber *et al.*, 1998b). The composition of the ECM in immediate contact with endothelial cells is also an important determinant of endothelial cell survival and induction of the angiogenic response following exposure to growth factors (Cheresh & Stupack, 2008). It is well established that endothelial cells plated in an *in vitro* 3-D collagen I assay rapidly undergo programmed cell death in the absence of survival factors (Ilan *et al.*, 1998; Yang *et al.*, 1999; Matsumoto *et al.*, 2002; Bohman *et al.*, 2005). A key finding presented in this chapter was that VEGF-induced tubular morphogenesis of HDMECs in a 3-D collagen I assay was inhibited by siRNA-mediated down-regulation of ERK5, MEK5 or VEGFR-2 with an almost identical effect on tubes (**Fig. 4.11**), thus confirming the observations of Hayashi *et al.* (2005). Importantly, this

study is the first to show that MEK5/ERK5 is required for endothelial tubular network formation in human endothelial cells.

A time-course observation of VEGF-stimulated HDMEC tube formation within collagen I gels revealed that ERK5-siRNA-treated HDMECs failed to form stable interconnecting vessels in response to VEGF, but rather appear to undergo apoptosis at an early time point, with cells appearing rounded and with hypercondensed nuclei (**Fig. 4.12**). Further analysis confirmed that the loss of ERK5 induced early apoptosis in HDMECs (**Fig. 4.13**), culminating in caspase-3 activation only in HDMECs plated within a collagen I matrix (**Fig. 4.14; Fig. 4.15**), an effect that was not suppressed by VEGF (**Fig. 4.14; Fig. 4.15**). Collectively, these results indicate that VEGF-induced tubular morphogenesis is facilitated by ERK5-mediated survival of HDMECs on a collagen I matrix. By analogy with the pro-survival effects of trophic factors such as BDNF and NGF, which activate ERK5 to facilitate the survival of neuronal cells (Watson *et al.*, 2001; Liu *et al.*, 2003b; Shalizi *et al.*, 2003; Finegan *et al.*, 2009), it was first hypothesised by Hayashi and co-workers that VEGF-stimulated activation of ERK5 may mediate a similar survival response in endothelial cells (Hayashi *et al.*, 2004; Hayashi & Lee, 2004); however, prior to this study, no experimental evidence had been provided to prove this theory. ERK5 has been implicated in the mediation of shear-stress-induced survival of BAECs (Pi *et al.*, 2004), this chapter now presents the first evidence to show that ERK5 is required for VEGF-induced survival of endothelial cells.

MEF2C is a well-characterised substrate of ERK5 (Kato *et al.*, 1997; Yang *et al.*, 1998a; Kato *et al.*, 2000), which has been proposed to mediate Erk5-dependent angiogenesis and endothelial cell survival in mice (Hayashi *et al.*, 2004). However, whilst siRNA-mediated MEF2C down-regulation in HDMECs did lower VEGF-stimulated tube formation *in vitro,* reducing overall tube length by ~20% (**Fig. 4.11**), this effect was dissimilar to the phenotype of ERK5 siRNA-treated cells, in which VEGF-induced tube formation was abolished (**Fig. 4.11**). This suggests that the primary effector mediating VEGF-induced survival and tubular morphogenesis downstream of ERK5 in HDMECs is not likely to be MEF2C. Although it remains possible that this effect may be the result of incomplete down-regulation of MEF2C, with sufficient MEF2C expressed in HDMECs

to mediate ERK5-regulated endothelial cell survival in response to VEGF, this seems unlikely, as siRNA-treatment induced a 90% lowering of MEF2C expression in these cells (**Fig. 4.3**). It should be noted that infection of *Erk5*$^{-/-}$ embryos with an adenovirus encoding a constitutively active Mef2c was only able to partially protect endothelial cells from apoptosis (Hayashi *et al.,* 2004), suggesting the existence of additional effectors downstream of ERK5 to mediate endothelial cell survival (Olson, 2004; Hayashi *et al.*, 2004). Based on the results presented in this chapter, it is hypothesised that ERK5 is likely to mediate VEGF-stimulated endothelial cell survival on a collagen matrix via effectors distinct from MEF2C.

Further confirmation of the role of ERK5 as a critical mediator of VEGF-induced tubular morphogenesis was gained from the observation that VEGF-induced tubular morphogenesis was abrogated by co-expression of CA-MEK5(D) with DN-ERK5(AEF), which suggested that ERK5 activation is critical for this process (**Fig. 4.19 A**). Strikingly, constitutive activation of ERK5 by co-transfection of ERK5(wt) and CA-MEK5(D) was sufficient to induce tubular morphogenesis of HDMECs on a 3-D collagen matrix in the absence of VEGF (**Fig. 4.19 A**). This finding provides the first evidence of VEGF-independent survival and tubular morphogenesis in this assay. Interestingly, co-transfection of ERK5(wt) and CA-MEK5(D) in the presence of VEGF enhanced tubular morphogenesis by increasing total tubule length and HDMEC branching over 24 h (**Fig. 4.19, A and C**). It should be noted that ERK5 activation, which was induced by co-transfection with ERK5(wt) and CA-MEK5(D), was augmented by stimulation with VEGF (**Fig. 4.17**). Under these conditions, enhanced capillary branching and anastomosis was observed in this assay over 24 h, and sustained tube formation was seen over 5 days (**Fig. 4.19, B and D**). These observations suggest that whilst ERK5 activity alone is sufficient to induce tubular morphogenesis, VEGF-potentiated ERK5 activation enhances this effect, possibly by sustaining ERK5 activation for prolonged periods or otherwise by allowing the activation of other signalling cascades, which may work together with ERK5 to sustain endothelial cell survival.

Blockade of the ERK1/2 signalling pathway had previously been shown to prevent PMA-induced endothelial cell tubulogenesis *in vitro* (Ilan *et al.*, 1998; Zachary, 2003).

However, in response to VEGF stimulation on a 3-D collagen I gel matrix, siRNA-mediated silencing of either ERK1 or ERK1/2, had a small, but significant inhibitory effect upon VEGF-induced tube formation in a 3-D collagen I gel (**Fig. 4.11**), whereas ERK2-specific siRNA treatment of HDMECs had no apparent effects upon VEGF-stimulated tubular morphogenesis. Interestingly, in developing mice, deletion of *Erk2* results in death *in utero* at E11.5 with impaired placental angiogenesis (Hatano *et al.*, 2003), whilst *Erk1*-null mice develop normally (Pages & Pouyssegur, 2004). Most recently, by generating endothelial cell-specific *Erk1*- and *Erk2*-null mice, a critical role for both Erk1 and Erk2 in regulating endothelial cell migration and proliferation has been revealed, with compound mutants lacking both *Erk1* and *Erk2* dying at E10.5 due to impaired angiogenesis (Srinivasan *et al.*, 2009). The apparent discrepancy between these findings and the effects of siRNA-mediated silencing of ERK1/2 expression in HDMECs may reflect a species-specific requirement for ERK1/2 expression. Alternatively, the necessity for ERK1/2 expression in endothelial cells may be confined to embryogenesis, but not in adult endothelial cells, where the requirement for endothelial cell proliferation is limited. It is suggested that the generation of inducible *Erk1*- and *Erk2*-knockout mice may provide further insight into the role of ERK1/2 in angiogenesis *in vivo*.

4.10.2 ERK5 is not required for VEGF-stimulated HDMEC proliferation

SiRNA-mediated down-regulation of ERK5 expression in HDMECs had no significant effect on the proliferative response of these cells following VEGF stimulation when plated on a gelatin matrix (**Fig. 4.7**). Accordingly, abolishment of ERK5 activity by over-expression of DN-ERK5(AEF), had no effect upon VEGF-stimulated HDMEC proliferation (**Fig. 4.18**). These data collectively showed that neither ERK5 expression, nor ERK5 activity, is required for VEGF-induced HDMEC proliferation. ERK5 has been reported to be required for growth factor-induced proliferation of HeLa cells (Kato *et al.*, 1998; Hayashi *et al.*, 2001), hematopoietic cells (Dong *et al.*, 2001) and hepatic stellate cells (Rovida *et al.*, 2008b). However, serum-stimulated cell-cycle progression and proliferation of CCl39 cells was found to be dependent upon ERK1/2, but not ERK5 (Squires *et al.*, 2002). In addition, cell-cycle progression was not altered in murine endothelial cells lacking *Erk5* (Hayashi *et al.*, 2004), or in fibroblasts taken from *Mek5*$^{-/-}$

mice (Wang *et al.*, 2005a). Together, these studies suggest that the role of ERK5 in regulating cell-cycle progression and mitosis may be cell type- or stimulus-dependent. Further analysis of the mechanisms regulating VEGF-induced proliferation in HDMECs, revealed that siRNA-mediated silencing of ERK1/2 expression abrogated this response in HDMECs (**Fig. 4.7**). This observation is in agreement with the established role of ERK1/2 in regulating cell-cycle entry (Brunet *et al.*, 1999b; Lefloch *et al.*, 2009), and endothelial cell proliferation (Vinals & Pouyssegur, 1999). In studies of other endothelial cell types, VEGF-stimulated ERK1/2 activation has been shown to induce cell-cycle progression and endothelial cell proliferation *in vitro* (Kroll & Waltenberger, 1997; Pedram *et al.*, 1998) and has recently been shown to regulate endothelial cell proliferation during embryonic angiogenesis *in vivo* (Srinivasan *et al.*, 2009).

4.10.3 ERK5 is not required for VEGF-stimulated HDMEC migration

In HUVECs, constitutive activation of ERK5 by over-expression of CA-MEK5(D), has been reported to prevent hypoxia-induced BLMEC migration; an effect that was reversed by over-expression of DN-ERK5(AEF) (Pi *et al.*, 2005). In addition, in a recent report, Spiering *et al.* demonstrated that constitutive activation of ERK5 inhibited HUVEC migration by reducing focal turnover (Spiering *et al.*, 2009). Collectively, these reports suggest that ERK5 acts to negatively-regulate endothelial cell migration. In agreement with these findings, siRNA-mediated silencing of ERK5 expression induced a small, but significant increase in basal HDMEC migration (**Fig. 4.9**). Importantly however, treatment with ERK5-specific siRNA did not affect VEGF-stimulated migration of HDMECs (**Fig. 4.9**). VEGF-induced endothelial cell migration has been shown to be regulated by diverse and complementary pathways (*section 1.2.5.4*). Data presented herein suggest that ERK5 is not an important regulator of VEGF-stimulated chemotaxis. However, endothelial cell migration is not only a function of chemotaxis, but is also regulated by the ECM component in direct contact with the endothelial cells; a process termed haptotaxis (Stupack & Cheresh, 2003; Lamalice *et al.*, 2007). A possible limitation of this study, therefore, is that the migratory response of HDMECs to VEGF was only quantified on a gelatin matrix. Thus, it may be of interest to further investigate the relative contribution of various ECM proteins to HDMEC migration following ERK5 siRNA treatment in future studies.

4.10.4 ERK5 is differentially required for HDMEC adhesion to ECM components

ERK5 siRNA treatment delayed HDMEC adhesion on a gelatin matrix (**Fig. 4.8 B**); however, this did not lead to apoptosis (**Fig. 4.13**). Adhesion of endothelial cells to the ECM is critical to prevent cell death by anoikis (Fujio & Walsh, 1999; Reddig & Juliano, 2005); furthermore, endothelial cell interactions with the ECM governs each aspect of the angiogenic response (Davis & Senger, 2005; Cheresh & Stupack, 2008). Previous studies have shown that prolonged detachment from the extracellular matrix is necessary to induce anoikis (Re *et al.*, 1994; Aoudjit & Vuori, 2001); as ERK5 siRNA-treated cells eventually adhered to the gelatin matrix by 4 h post-plating (**Fig. 4.8 B**), it is therefore likely that impairment of HDMEC adhesion prior to this point is not sufficient to induce apoptosis. Endothelial cell attachment to the ECM is mediated by the cell surface receptors, integrins (Hynes, 2007). Integrins are critical for each phase of the angiogenic response, however the distinct roles of these molecules are difficult to characterise due to overlapping substrate specificities (Perruzzi *et al.*, 2003), combined with the fact that the relative contribution of several integrins in angiogenesis has not been characterised (Hynes, 2007).

Endothelial cells bind to collagen matrices via the integrins $\alpha_1\beta_1$ and $\alpha_2\beta_1$ (Avraamides *et al.*, 2008). Given that HDMEC adhesion to collagen I was not impaired by ERK5 down-regulation, it seems unlikely that ERK5 regulates activity or expression of these integrins. By contrast, ERK5 siRNA treatment had a profound effect upon adhesion of HDMECs to either fibronectin or vitronectin matrices compared to N.S. siRNA-treated cells (**Fig. 4.8, C and D**). The effects of ERK5 siRNA upon HDMEC survival on these matrices was not assessed in this study, but attachment of endothelial cells to either of these matrices is essential for endothelial cell survival (Isik *et al.*, 1998; Francis *et al.*, 2002) via a mechanisms involving the well-studied $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrins. Attachment of HUVECs to fibronectin is mediated by $\alpha_V\beta_1$ and $\alpha_5\beta_1$ integrins (Ruegg *et al.*, 1998). Future studies should aim to further characterise the potential role of ERK5 in modulating integrin activity or integrin expression in endothelial cells, and the implications of this upon various stages of angiogenesis.

It is conceivable that ERK5 may engage in "inside out" signalling crosstalk with certain integrins (Streuli & Akhtar, 2009; Ramjaun & Hodivala-Dilke, 2009), to activate, and thus increase the affinity of these integrins to ECM protein ligands. Alternatively, ERK5 may also regulate integrin expression. The recent finding that ERK5 immunoprecipitates with integrins β_1, $\alpha_v\beta_3$ and $\alpha_v\beta_5$ in breast cancer cells (Sawhney et al., 2009), raises the possibility that ERK5 may bind to specific integrins in HDMECs to integrate signals from both the ECM and VEGFR-2 to facilitate tubular morphogenesis and survival in endothelial cells.

4.10.5 Conclusions

The precise role of ERK5 as a potential mediator of pro-angiogenic signals in response to VEGF stimulation in endothelial cells was previously unknown. This chapter has addressed this issue by identifying ERK5 as a critical regulator of VEGF-stimulated endothelial cell survival HDMECs undergoing tubular morphogenesis within a 3-D collagen matrix. Interestingly, ERK5 was not required for mediating the survival of HDMECs plated on a gelatin matrix; furthermore, neither VEGF-directed proliferation nor VEGF-induced migration of these cells on this matrix was affected by siRNA-mediated silencing of ERK5 expression. It was also shown that ERK5 expression is differentially required for HDMEC adhesion to various ECM proteins, which suggests that ERK5 may be an important regulator of integrin avidity in endothelial cells. A salient finding was that constitutive activation of ERK5 was sufficient to induce HDMEC tube formation in a collagen matrix in the absence of VEGF, and to sustain VEGF-stimulated tubular morphogenesis in the presence of VEGF. Together, these results suggest that inhibitors targeting the ERK5 pathway *in vivo* may allow a unique opportunity to selectively interfere with a specific component of angiogenesis. Conversely, constitutive activation of ERK5 may feasibly be used in gene therapy to stimulate neo-angiogenesis and revascularisation in conditions characterised by a deficiency in functioning vessels, such as ischemia.

CHAPTER FIVE

ERK5 and the regulation of VEGF-stimulated intracellular signalling and gene expression in HDMECs

5.1 Introduction

SiRNA-mediated gene silencing has been used increasingly over the past few years as a tool to define the role of given proteins of interest in the regulation of intracellular signalling and gene expression (Scherr *et al.*, 2003; Czauderna *et al.*, 2003). In endothelial cells, siRNA-mediated down-regulation of specific proteins of interest has allowed their functional importance in distinct phases of angiogenesis, as well as in the regulation of downstream intracellular signalling events to be evaluated (Lu *et al.*, 2005; Tomasi *et al.*, 2006). Silencing of ERK5 expression in HDMECs by the use of siRNA in functional angiogenesis assays (*Chapter 4*), revealed a specific requirement for ERK5 during VEGF-mediated HDMEC tubular morphogenesis, by facilitating endothelial cell survival in cells plated within a 3-D collagen matrix. In contrast, ERK5 was found to be dispensable for HDMEC proliferation and migration in response to VEGF-stimulation on a 2-D gelatin matrix (*Chapter 4*). It was deemed possible that VEGF stimulation may result in differential expression and/or activation of ERK5 in HDMECs on these matrices to account for these distinct phenotypes. Importantly, as the effects of ERK5 siRNA treatment upon potential VEGF-stimulated intracellular signalling pathways operating downstream of ERK5 in endothelial cells had not been previously explored; it was therefore also a possibility that ERK5 may act as a regulator of signalling pathways which mediate VEGF-stimulated endothelial cell survival.

This chapter details experiments conducted to characterise ERK5-regulated intracellular signalling in response to VEGF-stimulation, in a bid to understand the molecular mechanisms giving rise to the observed effects of siRNA-induced down-regulation of ERK5 in HDMECs on collagen and gelatin matrices. VEGF is also known to induce transcriptional up-regulation of several genes in endothelial cells, some of which have been implicated in endothelial cell survival and angiogenesis (Gerber *et al.*, 1998a; Abe & Sato, 2001; Grosjean *et al.*, 2006; Mellberg *et al.*, 2009). To gain an insight into whether ERK5 differentially regulates VEGF-induced gene expression in HDMECs within 3-D collagen matrices and on gelatin matrices, the effects of ERK5 siRNA treatment upon the expression of a selected panel of genes that had been previously implicated in angiogenesis was quantified by qRT-PCR. Furthermore,

constitutive activation of ERK5 by co-transfection of CA-MEK5(D) and ERK5(wt) had been found to induce tubular morphogenesis, an effect that was augmented by VEGF stimulation (**Fig. 4.19**). In a bid to elucidate the molecular mechanisms of this process, the effects of constitutive activation of ERK5, as well as blockade of ERK5 activity, induced by transient expression of these constructs upon VEGF-stimulated activation of intracellular signalling pathways in HDMECs within 3-D collagen gels were also determined, and are described herein.

5.2 Characterisation of ERK5 expression and activity in response to VEGF-stimulation of HDMECs plated on a gelatin matrix or within a 3-D collagen gel matrix

5.2.1 VEGF stimulates sustained ERK5 activation in HDMECs on both gelatin and collagen matrices

ERK5 was found to be required for mediating VEGF-induced survival of HDMECs to facilitate differentiation of these cells into tubular structures on a 3-D collagen matrix (**Fig. 4.11**), but was dispensable for VEGF-induced proliferation on a gelatin matrix (**Fig. 4.7**). Given that cellular responses to extracellular stimuli are often determined by the strength and duration of MAPK activation (Marshall, 1995; Cook *et al.*, 1999; Murphy & Blenis, 2006; Chung *et al.*, 2010), it was hypothesised that possible differences in the kinetics of ERK5 activation in response to VEGF stimulation on collagen and gelatin matrices may exist, to account for the distinct phenotypes of ERK5 loss on these matrices. A further possibility was that *ERK5* expression may be differentially regulated by VEGF stimulation on collagen or gelatin matrices. To determine whether expression or activation of ERK5 was differentially induced in cells plated on these matrices, serum-starved HDMECs were plated on gelatin matrices, or within 3-D collagen gels prior to stimulation with VEGF (50 ng/ml), followed by cell lysis at various time points between 10 min and 24 h (**Fig. 5.1 A**). Western blot analysis of whole cell lysates using a phospho-ERK5 antibody, directed against phosphorylated Thr^{218}/Tyr^{220} residues within the T-E-Y motif in the ERK5 activation loop, revealed that VEGF stimulation induced activation of ERK5 in HDMECs on both gelatin and collagen matrices within 10 min (**Fig. 5.1, A and B**).

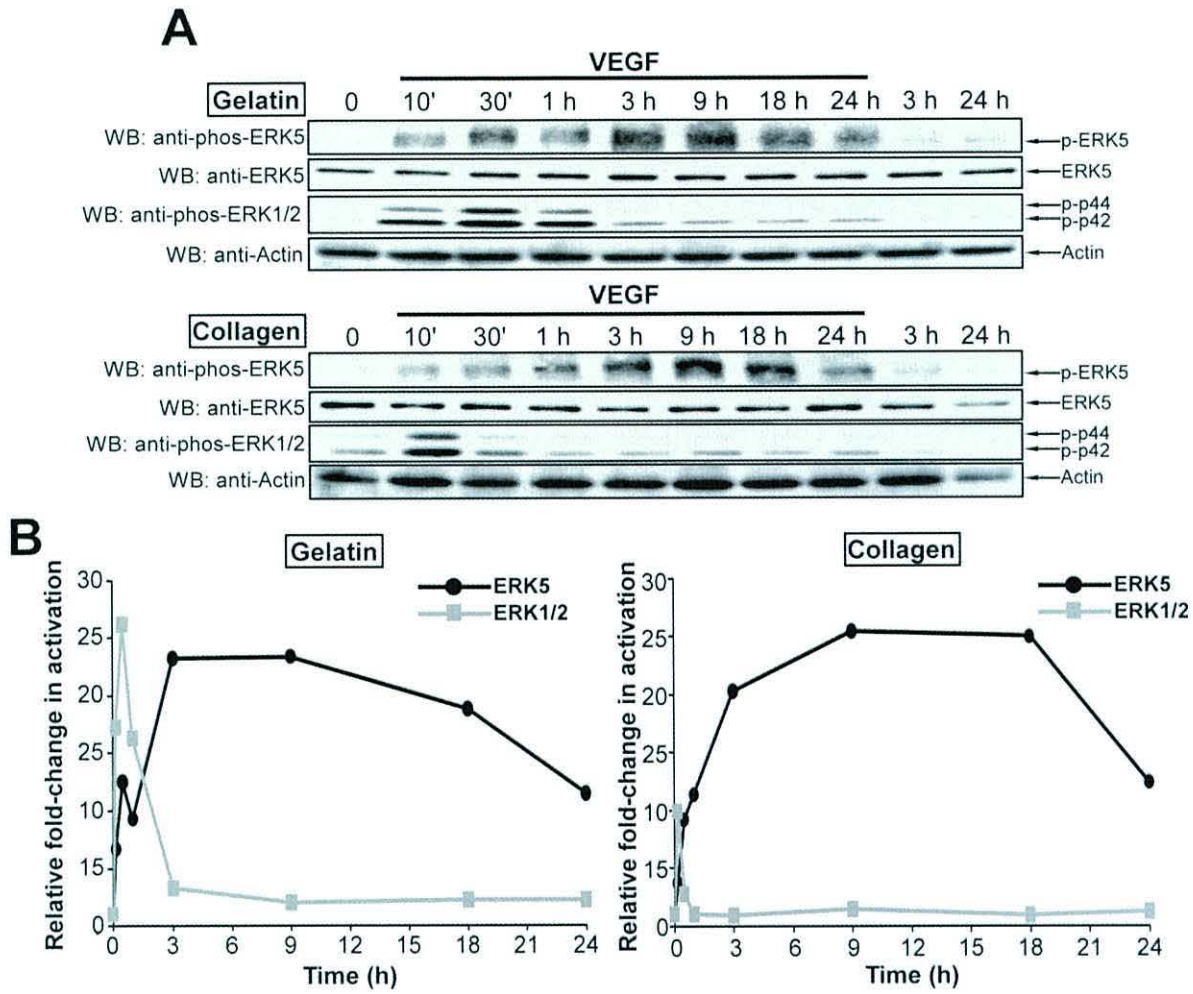

Figure 5.1 VEGF stimulates activation of ERK5 under conditions of HDMEC proliferation and tubular morphogenesis. (**A**) HDMECs were serum-starved in EBM MV2 basal medium containing 1% (v/v) FCS for 20 h prior to plating at 5.0 x 10^5 cells per well of 6-well gelatin-coated plates in 2 ml of EBM MV2 basal medium containing 1% (v/v) FCS on a gelatin matrix, or at 6.25 x 10^5 cells per well of a 6-well plate between two layers of collagen I gel. After 1 h (0 h time point) cells were lysed, or stimulated with 50 ng/ml VEGF. The remaining VEGF-stimulated cells on each matrix were lysed at the indicated times thereafter, and proteins were separated on a 10% SDS-PAGE gel, and analysed by Western blotting (*WB*) with antibodies directed against phos-ERK5, ERK5, phos-ERK1/2 and actin as a loading control, as indicated. (**B**) The degree of ERK5 and ERK1/2 phosphorylation, relative to actin expression, was quantified by densitometric analysis using NIH ImageJ software. *ERK, extracellular signal-regulated kinase; FCS, foetal calf serum; VEGF, vascular endothelial growth factor; WB, Western blot.*

The kinetics of VEGF-induced ERK5 activation in HDMECs plated on these matrices was similar. Specifically, ERK5 phosphorylation increased with time, reaching a maximal level at 3 h in cells plated on either matrix, and was sustained for 18 h on a gelatin matrix, and for 24 h on a collagen matrix (**Fig. 5.1, A and B**). Western blot analysis of total ERK5 expression levels revealed that expression of ERK5 in HDMECs did not alter on either matrix (**Fig. 5.1 A**). In contrast to ERK5 activation, VEGF-induced activation of ERK1/2 was relatively transient, with phospho-ERK1/2 returning to basal levels by 3 h on a gelatin matrix and by 1 h in a collagen gel (**Fig. 5.1, A and B**).

As plating endothelial cells within a 3-D collagen gel matrix had been previously shown to modulate MAPK activation (Boyd *et al.*, 2005), it was important to determine the effects of seeding HDMECs within a 3-D collagen I gel or on a gelatin matrix upon ERK5 and ERK1/2 activation in the absence of VEGF stimulation, as a negative control. It was found that the seeding of HDMECs between two layers of collagen I gel or on a gelatin matrix alone for 3 h or 24 h did not induce activation of either ERK5 or ERK1/2 (**Fig. 5.1 A**). Together, these data showed that VEGF-induced ERK5 activation is not matrix-dependent, suggesting that the selective role of ERK5 in facilitating VEGF-stimulated survival of HDMECs plated within a 3-D collagen gel was not due a potential disparity in the strength or duration of VEGF-induced ERK5 activation in cells plated within this matrix.

5.2.2 VEGF stimulation does not affect expression of *ERK5*, but induces up-regulation of *MEF2C* expression in HDMECs

Microarray analysis of gene expression in response to VEGF stimulation in telomerase-immortalised human microvascular endothelial (TIME) cells had revealed that transcription of *MEF2C*, a well-characterised downstream target of ERK5 (Kato *et al.*, 1997; Yang *et al.*, 1998a; Kato *et al.*, 2000), was up-regulated in response to VEGF stimulation (Dr. Michael Cross, University of Liverpool, unpublished data). Interestingly, the expression of *MEF2C* was most strongly up-regulated in response to VEGF stimulation in TIME cells plated on a collagen matrix (Dr. Michael Cross, University of Liverpool, unpublished data). It was initially hypothesised that *MEF2C* up-regulation in response to VEGF stimulation may occur to provide additional substrate to ERK5 to confer VEGF-stimulated survival of endothelial cells. Furthermore, as the potential effects of VEGF stimulation upon *ERK5* expression had not been previously quantified, it was of interest to determine whether or not *ERK5* may be transcriptionally regulated in response to VEGF stimulation. To measure the expression of *ERK5* and *MEF2C* in HDMECs in response to VEGF stimulation, a time-course study was conducted by Dr. Katherine Holmes (University of Liverpool) to analyse gene expression in cells plated on a gelatin matrix, or within a 3-D collagen

matrix over 24 h. Total RNA was extracted from HDMECs at 3 h, 6 h, 9 h, 12 h and 24 h post-stimulation with VEGF (**Fig. 5.2**).

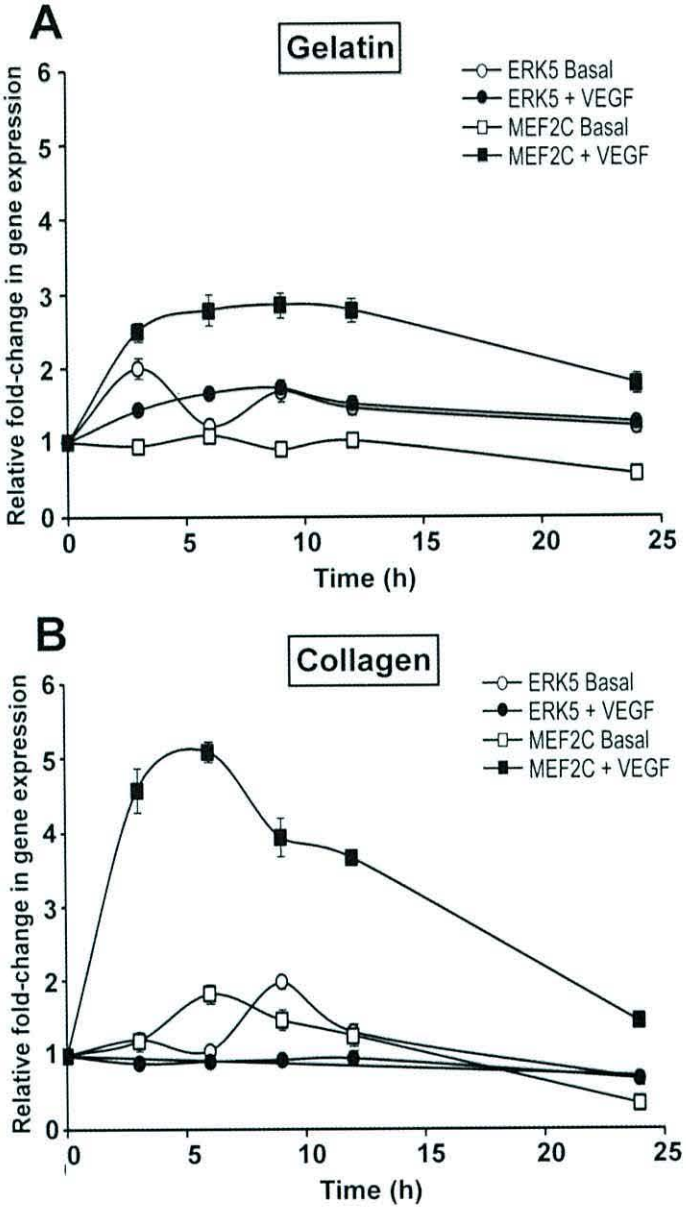

Figure 5.2 Time-course of *ERK5* and *MEF2C* mRNA expression on collagen and gelatin matrices following VEGF stimulation. HDMECs were serum-starved for 20 h prior to seeding at 5.0 x 10^5 cells per well of a 6-well gelatin-coated plate in 2 ml of EBM MV2 basal medium containing 1% (v/v) FCS (**A**), or at 6.25 x 10^5 cells per well of a 6-well plate between two layers of collagen I gel (**B**). Total RNA was extracted immediately (0 h), or at 3 h, 6 h, 9 h, 12 h and 24 h post-stimulation or not (basal), with VEGF (50 ng/ml) each matrix as described (*section 2.2.4.1*). Following cDNA synthesis (*section 2.2.4.2*), *ERK5* and *MEF2C* mRNA expression was quantified by qRT-PCR analysis by standard curve analysis (*section 2.2.5.1*). Data is presented as relative fold-change in *ERK5* and *MEF2C* mRNA expression levels relative to the 0 h basal sample on each matrix (n = 3; mean ± SD). *ERK5, extracellular signal-regulated kinase 5; HDMEC, human dermal microvascular endothelial cell; MEF2C, myocyte enhancer factor 2C; VEGF, vascular endothelial growth factor.*

VEGF induced a 2.5-fold increase in *MEF2C* expression by 3 h in HDMECs plated on a gelatin matrix, an effect that was sustained for 12 h (**Fig. 5.2 A**). However, on a

collagen matrix, *MEF2C* was more robustly up-regulated, with a 5-fold increase in *MEF2C* expression observed by 6 h. *MEF2C* expression returned to near basal levels by 24 h (**Fig. 5.2 B**). In contrast, *ERK5* expression did not change in response to VEGF stimulation on either matrix (**Fig. 5.2, A and B**). These data show that *ERK5* expression is not induced by VEGF stimulation, and is not altered by seeding on collagen I or gelatin matrices, in agreement with previous analyses of ERK5 protein expression levels following VEGF stimulation in HDMECs plated on these matrices (**Fig. 5.1**). In contrast, *MEF2C* expression was up-regulated following VEGF stimulation in HDMECs, consonant with the recent observations of Maiti et al. (2008) in retinal endothelial cells. Expression of *MEF2C* was most robustly induced in HDMECs plated within a 3-D collagen I gel (**Fig. 5.2 B**) in agreement with previous microarray data in TIME cells (data shown), further supporting the hypothesis that MEF2C may be specifically required during VEGF-stimulated HDMEC tubular morphogenesis on this matrix.

5.3 Analysis of the effects of siRNA-mediated down-regulation of ERK5 expression on VEGF-stimulated intracellular signalling in HDMECs plated on a gelatin matrix or within a 3-D collagen matrix

5.3.1 ERK5 differentially regulates VEGF-stimulated AKT activity in HDMECs on gelatin and collagen matrices

The observation that down-regulation of ERK5 expression abrogated VEGF-induced tubular morphogenesis of HDMECs within a 3-D collagen gel with a concomitant increase in annexin V-positive cells by 1 h (**Fig. 4.13**), an effect not observed in cells plated on a gelatin matrix, suggested that ERK5 may differentially regulate VEGF-stimulated activation of intracellular pathways that mediate endothelial cell survival on these matrices. To investigate this possibility, the effect of siRNA-induced silencing of ERK5 expression upon VEGF-stimulated activation of selected intracellular signalling pathways in HDMECs plated within a 3-D collagen I gels or on 2-D gelatin matrices was determined.

Cells were treated with ERK5-specific siRNA or with N.S. siRNA, as described in the legend to **Fig. 5.3**. Western blot analysis confirmed that both ERK5 siRNA 1 and ERK5

siRNA 2 down-regulated ERK5 expression by 90% in cells on both gelatin and collagen matrices (**Fig. 5.3, *ERK5***). Similarly, treatment with either ERK5 siRNA 1 or ERK5 siRNA 2 prevented VEGF-stimulated phosphorylation of ERK5 on Thr^{218}/Tyr^{220} equally well in HDMECs plated on either matrix (**Fig. 5.3, *p-ERK5***). Together, these results serve to show that the specific requirement for ERK5 to mediate HDMEC survival in response to VEGF stimulation on a 3-D collagen matrix is not due to differential activation of ERK5 on in cells plated within a collagen matrix or on a gelatin matrix, nor due to differential siRNA-mediated down-regulation of ERK5 expression on these matrices. VEGF-induced VEGFR-2 phosphorylation on Tyr^{1175} was detectable in HDMECs plated on gelatin or within a collagen gel, an effect that was not altered by siRNA-mediated silencing of ERK5 expression (**Fig. 5.3, *p-VEGFR-2***). Similarly, VEGF-stimulated phosphorylation of ERK1/2 was not blocked by treatment with ERK5 siRNA on either matrix (**Fig. 5.3, *p-ERK1/2***).

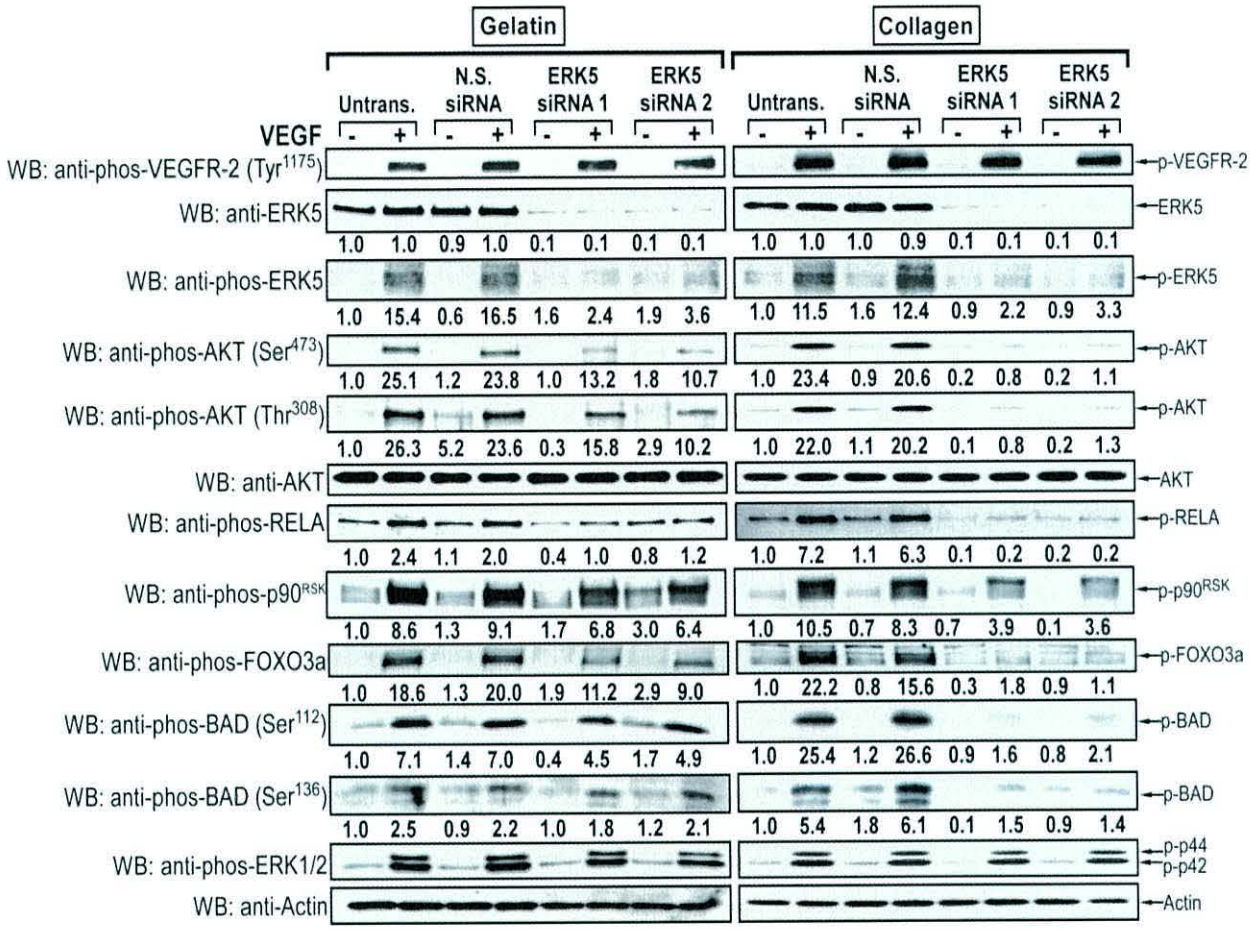

Figure 5.3 ERK5 activation is required for VEGF-stimulated phosphorylation of AKT, p90^{RSK}, RELA, FOXO3a and BAD. HDMECs were plated at 8.2 x 10^5 cells per dish on gelatin-coated 10 cm cell culture dishes in 10 ml of EBM MV2 growth medium. At 1 day post-seeding, cells were transfected as described (*section 2.2.2.7.2*) with 10 nM of ERK5 siRNA 1, or 10 nM of ERK5 siRNA 2, or with 10 nM of non-silencing (N.S.) siRNA, or were left untransfected (Untrans.). At 24 h post-transfection, cells were serum-starved for 20 h, prior to plating at 5.0 x 10^5 cells per well of a 6-well gelatin-coated plate, or at 6.25 x 10^5 cells per well of a 6-well plate between two layers of collagen I gel, and stimulated, or not, with VEGF (50 ng/ml) for 10 min. Cells were lysed in RIPA buffer and proteins were separated on a 10% SDS-PAGE gel and analysed by Western blotting (*WB*) with antibodies directed against ERK5, phos-ERK5, phos-VEGFR-2 Tyr^{1175}, phos-AKT Ser^{473}, phos-AKT Thr^{308}, total AKT, phos-RELA Ser^{536}, phos-FOXO3a Thr^{32}, phos-p90^{RSK} Ser^{380}, phos-BAD Ser^{136}, phos-BAD Ser^{112} and phos-ERK1/2, and actin, as indicated. The degree of phosphorylation of ERK5, AKT Ser^{473}, AKT Thr^{308}, RELA Ser^{536}, p90^{RSK} Ser^{380}, FOXO3a Thr^{32}, BAD Ser^{136}, BAD Ser^{112} and the expression of ERK5 relative to actin was quantified by densitometric analysis using NIH ImageJ software, and is indicated below each respective lane. The results shown are representative of three independent experiments. *AKT/PKB, protein kinase B; BAD, BCL2-antagonist of cell death; ERK, extracellular signal-regulated kinase; FOXO3a; forkhead box transcription factor O3a; HDMEC, human dermal microvascular endothelial cell; N.S., non-silencing; RELA, p65 nuclear factor-κB (NF-κB); p90^{RSK}, p90 ribosomal S6 kinase; rps6, ribosomal protein S6; siRNA, small interfering RNA; VEGF, vascular endothelial growth factor; VEGFR-2; VEGF receptor-2; WB, Western blot.*

The serine/threonine protein kinase AKT is a critical mediator of VEGF-induced pro-survival signals in endothelial cells (Gerber *et al.*, 1998b; Fujio & Walsh, 1999). It was hypothesised that ERK5 may mediate VEGF-stimulated activation of AKT in HDMECs. To explore this possibility, Western blot analysis of the phosphorylation status of AKT on residues Ser^{473} and Thr^{308} in response to acute VEGF stimulation in ERK5 siRNA-treated HDMECs was carried out (**Fig. 5.3**). It was found that siRNA-mediated down-

regulation of ERK5 expression decreased VEGF-stimulated AKT phosphorylation on both Ser^{473} and Thr^{308} by approximately 50% in cells plated on a gelatin matrix compared to cells transfected with N.S. siRNA (**Fig. 5.3, *p-AKT***). By contrast, in HDMECs plated within a collagen matrix, this inhibitory effect was much more profound, with VEGF-induced AKT phosphorylation reduced by at least 94% (**Fig. 5.3, *p-AKT***). Importantly, the total level of AKT protein was not affected on either matrix, thus indicating that the siRNA-mediated inhibition of ERK5 activity was specifically affecting AKT phosphorylation, and not expression of AKT (**Fig. 5.3, *AKT***).

5.3.2 ERK5 differentially regulates VEGF-stimulated phosphorylation of BAD on Ser^{112} and Ser^{136} in HDMECs on gelatin and collagen matrices

AKT confers its anti-apoptotic effects by phosphorylating a number of downstream effector proteins, including the pro-apoptotic protein BAD (Datta *et al.*, 1997). Phosphorylation of BAD allows the binding of the scaffold protein 14-3-3 to BAD, which sequesters BAD in the cytoplasm, thus inactivating BAD by preventing the death-promoting interaction of BAD with the anti-apoptotic proteins BCL2 and BCL-xL in the mitochondria (Zha *et al.*, 1996). It had been previously shown that ERK5 was required for phosphorylation of BAD on residues Ser^{112} and Ser^{136}, via a mechanism reportedly independent of AKT, PKA and $p90^{RSK}$, to confer survival of bovine endothelial cells in response to laminar-shear-stress (Pi *et al.*, 2004). It was therefore of interest to determine whether siRNA-mediated down-regulation of ERK5 expression would affect phosphorylation of BAD on these residues in primary human endothelial cells in response to VEGF stimulation. It was found that siRNA-mediated down-regulation of ERK5 expression resulted in a marked reduction in VEGF-induced phosphorylation of BAD on both Ser^{112} and Ser^{136} in cells plated within a collagen matrix (**Fig. 5.3, *p-BAD***). Densitometric quantification revealed that ERK5 siRNA lowered VEGF-induced phosphorylation of BAD on Ser^{136} in cells plated within a collagen matrix by at least 75% (**Fig. 5.3, *p-BAD***). By contrast, in HDMECs plated on a gelatin matrix, the effect of siRNA-mediated silencing of ERK5 expression upon VEGF-induced phosphorylation of BAD on Ser^{136} was more subtle, with phospho-BAD Ser^{136} levels lowered by 11% in comparison to N.S. siRNA-treated cells (**Fig. 5.3, *p-BAD***).

VEGF-stimulated phosphorylation of BAD on Ser^{112} was also affected by ERK5 siRNA treatment in HDMECs plated on a collagen gel (**Fig. 5.3, *p-BAD***). Densitometric quantification revealed that ERK5 siRNA treatment attenuated phosphorylation of BAD on Ser^{112} by 90% on a collagen gel, but only by 35% on a gelatin matrix (**Fig. 5.3, *p-BAD***). Phosphorylation of BAD on Ser^{112} has been shown to be mediated by $p90^{RSK}$ (Bonni *et al.*, 1999; Tan *et al.*, 1999). Analysis of $p90^{RSK}$ phosphorylation on Ser^{380}, a residue that is required for full activation of $p90^{RSK}$ (Dalby *et al.*, 1998), revealed that VEGF-induced activation of $p90^{RSK}$ was lowered in HDMECs treated with ERK5 siRNA, an effect that was most evident in cells plated within a collagen matrix (**Fig. 5.3, *p-p90^{RSK}***). Together, these results showed that ERK5 is critical for VEGF-induced phosphorylation of BAD on both Ser^{136} and Ser^{112} in HDMECs plated on a collagen matrix, but is only partly required for phosphorylation of BAD in cells plated on a gelatin matrix.

5.3.3 ERK5 differentially regulates VEGF-stimulated phosphorylation of FOXO3a in HDMECs on gelatin and collagen matrices

The forkhead/winged helix transcription factor family member, FOXO3a is a key regulator of apoptosis (Birkenkamp & Coffer, 2003). FOXO3a is phosphorylated in an AKT-dependent manner in response to VEGF stimulation in endothelial cells (Brunet *et al.*, 1999a; Abid *et al.*, 2004). Phosphorylation of FOXO3a allows its binding to the scaffold protein 14-3-3, an action that sequesters FOXO3a in the cytoplasm, preventing its nuclear entry and target gene activation (Fu & Tindall, 2008). Western blot analysis revealed that FOXO3a was strongly phosphorylated in response to VEGF stimulation in HDMECs plated on both collagen and gelatin matrices (**Fig. 5.3, *p-FOXO3a***). ERK5 siRNA treatment attenuated VEGF-induced FOXO3a phosphorylation by approximately 50% on a gelatin matrix, whereas in cells plated within a 3-D collagen gel a more profound effect was observed, with VEGF-induced FOXO3a phosphorylation being lowered by approximately 90% following ERK5 siRNA treatment, compared to N.S. siRNA treated cells (**Fig. 5.3, *p-FOXO3a***). This result suggests that FOXO3a is a further downstream effector of ERK5-regulated VEGF-mediated signalling in HDMECs, and supports the premise that ERK5 differentially regulates AKT activation in HDMECs on collagen and gelatin matrices.

5.3.4 ERK5 differentially regulates VEGF-stimulated phosphorylation of RELA in HDMECs plated on gelatin and collagen matrices

The nuclear factor kappa B (NF-κB) family of transcription factors consist of five members: p50, p65/RELA, RELB, p52, and c-REL (Karin & Lin, 2002). Of these, the p65 subunit of NF-κB – RELA - is well known to undergo activation in response to VEGF stimulation (Marumo *et al.*, 1999; Kim *et al.*, 2001). Recently, RELA has also been demonstrated to be critical for mediating VEGF-stimulated endothelial cell survival via an AKT-dependent mechanism (Grosjean *et al.*, 2006). ERK5 has been shown to act as an upstream regulator of NF-κB activity (Pearson *et al.*, 2001a). Furthermore, ERK5-mediated growth factor-induced phosphorylation of RELA was found to be essential for the mediation Jurkat T-cell survival (Garaude *et al.*, 2006). RELA is known to become activated via an AKT-dependent mechanism to facilitate cell survival (Ozes *et al.*, 1999; Madrid *et al.*, 2000; Hayden & Ghosh, 2008). Given that siRNA-mediated down-regulation of ERK5 expression was found to result in diminished AKT activation (**Fig. 5.3, *p-AKT***), it was hypothesised that silencing of ERK5 expression would also lead to diminished VEGF-induced phosphorylation of RELA in HDMECs. Indeed, Western blot analysis using a phospho-specific antibody directed against Ser^{536}, located in the transactivation domain of RELA, revealed that siRNA-mediated down-regulation of ERK5 in HDMECs plated within a 3-D collagen matrix reduced VEGF-stimulated phosphorylation of RELA on Ser^{536} by 97% compared to the non-silencing control (**Fig. 5.3, *p-RELA***). VEGF-induced activation of RELA was less affected by siRNA-mediated silencing of ERK5 expression in cells plated on a gelatin matrix, in which a 45% decrease in VEGF-stimulated RELA phospho-Ser^{536} was observed (**Fig. 5.3, *p-RELA***). Expression of total RELA was not altered by ERK5 siRNA treatment on either matrix (data not shown). These results show that ERK5 regulates VEGF-induced activation of RELA in HDMECs in a matrix-dependent manner.

5.3.5 Effects of siRNA-mediated down-regulation of ERK5 expression upon VEGF-stimulated phosphorylation of p70 S6 kinase and ribosomal protein S6 activation in HDMECs on collagen or gelatin matrices

Hayashi and co-workers provided evidence to show that FGF-2-stimulated phosphorylation of rpS6 on residues Ser^{235} and Ser^{236} in MLCECs occurs via an ERK5-regulated, $p90^{RSK}$-mediated mechanism (Hayashi *et al.*, 2005). Given that rpS6 has roles in regulating proliferation and survival (Anjum & Blenis, 2008), Hayashi *et al.* proposed that FGF-2-stimulated Erk5-mediated activation of rpS6 via $p90^{RSK}$ in murine endothelial cells was partly accountable for the requirement of Erk5 for tumour associated angiogenesis (Hayashi *et al.*, 2005). In the current study, siRNA-mediated silencing of ERK5 expression diminished VEGF-induced activation of $p90^{RSK}$ (**Fig. 5.3, *p-p90^{RSK}***), but had no effect upon VEGF-induced proliferation (**Fig. 4.7**).

To determine the potential effects of siRNA-mediated ERK5 knockdown upon VEGF-induced rpS6 phosphorylation on Ser^{235}/Ser^{236} in HDMECs, cells were treated with ERK5 siRNA for 24 h prior to serum starvation for 20 h, followed by plating on a gelatin matrix or between two layers of collagen I gel and acute VEGF stimulation for 10 min. Western blot analysis with phospho-specific antibodies revealed that rpS6 was constitutively phosphorylated on Ser^{235}/Ser^{236} in HDMECs under basal, unstimulated conditions (**Fig. 5.4, *p-rpS6***); however, stimulation with VEGF induced a noticeable increase in rpS6 phosphorylation on Ser^{235}/Ser^{236}, an effect that was most apparent in cells plated within a collagen gel (**Fig. 5.4, *p-rpS6***). ERK5 siRNA treatment had no effect upon either basal or VEGF-stimulated rpS6 phosphorylation on Ser^{235}/Ser^{236} (**Fig. 5.4, *p-rpS6***), which suggested that ERK5 is not required for VEGF-induced phosphorylation of rpS6 on these residues in HDMECs.

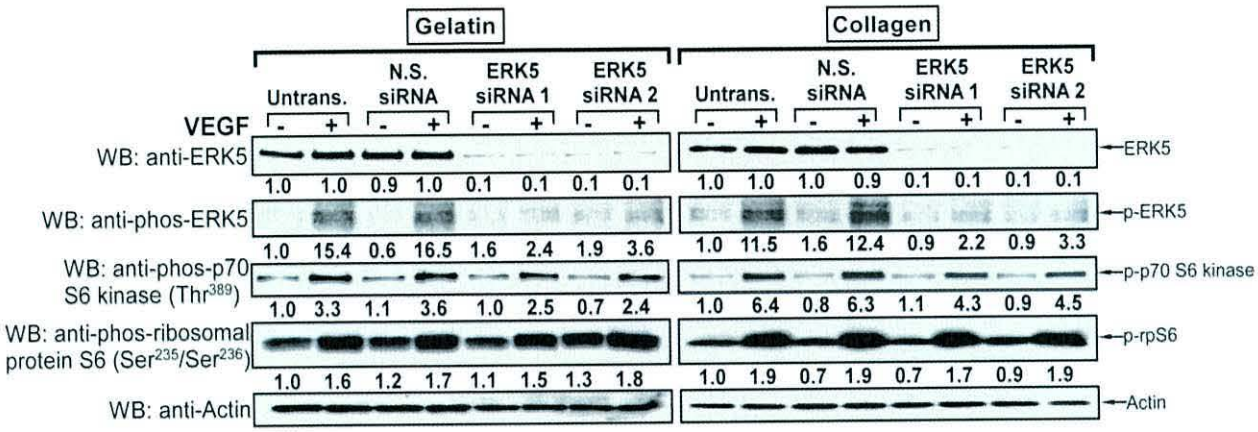

Figure 5.4 Effects of siRNA-mediated silencing of ERK5 expression upon VEGF-induced activation of p70 S6 kinase and ribosomal protein S6. HDMECs were plated at 8.2 x 10^5 cells per dish on gelatin-coated 10 cm cell culture dishes in 10 ml of EBM MV2 growth medium. At 1 day post-seeding, cells were transfected as described (*section 2.2.2.7.2*) with 10 nM of ERK5 siRNA 1, or 10 nM of ERK5 siRNA 2, or with 10 nM of non-silencing (N.S.) siRNA, or were left untransfected (Untrans.). At 24 h post-transfection, cells were serum-starved for 20 h, prior to plating at 5.0 x 10^5 cells per well of a 6-well gelatin-coated plate, or at 6.25 x 10^5 cells per well of a 6-well plate between two layers of collagen I gel, and stimulated, or not, with VEGF (50 ng/ml) for 10 min. Cells were lysed in RIPA buffer and proteins were separated on a 10% SDS-PAGE gel and analysed by Western blotting (*WB*) with antibodies directed against ERK5, phos-ERK5, phos-p70 S6 kinase Thr^{389}, phos-ribosomal protein S6 $Ser^{235/236}$ and actin, as indicated. The degree of phosphorylation of ERK5, p70 S6 kinase, ribosomal protein S6 and the expression of ERK5 relative to actin was quantified by densitometric analysis using NIH ImageJ software, and is indicated below each respective lane. The results shown are representative of three independent experiments. *ERK5, extracellular signal-regulated kinase 5; HDMEC, human dermal microvascular endothelial cell; MEF2C, myocyte enhancer factor 2C; N.S., non-silencing; p70 S6 kinase, p70 ribosomal protein S6 kinase; rpS6, ribosomal protein S6; siRNA, small interfering RNA; VEGF, vascular endothelial growth factor; WB, Western blot.*

The p70 S6 kinase is involved in regulating several growth factor-induced cellular responses including cell proliferation (Cho *et al.*, 2006) and cell survival (Moon *et al.*, 2005) and was recently shown to be an important regulator of tumour angiogenesis (Liu *et al.*, 2008). Phosphorylation of p70 S6 kinase on Thr^{389} is highly representative of p70 S6 kinase activity *in vivo* (Weng *et al.*, 1998). Hayashi *et al.* demonstrated that deletion of *Erk5* in murine endothelial cells had no effect upon FGF-2-stimulated phosphorylation of p70 S6 kinase on Thr^{389}, but that FGF-2-induced phosphorylation of $p90^{RSK}$ on Thr^{359}/Ser^{363} was blocked by the loss of *Erk5* in MLCECs (Hayashi *et al.*, 2005). In contrast, siRNA-mediated down-regulation of ERK5 expression in HDMECs, attenuated VEGF-induced p70 S6 kinase phosphorylation on Thr^{389} by approximately 30% compared to N.S. siRNA-treated cells, an effect that was apparent in cells plated on either gelatin or collagen matrices (**Fig. 5.4, *p-70 S6 kinase***). Both p70 S6 kinase and $p90^{RSK}$ have been shown to phosphorylate rpS6 *in vitro* (Pullen & Thomas, 1997; Frodin & Gammeltoft, 1999). However, the effect of siRNA-mediated silencing of ERK5 expression upon VEGF-induced activity of these kinases was not reflected in a similar

lowering in p-rpS6 levels (**Fig. 5.4**). These results suggest that ERK5 is an upstream regulator of both p70 S6 kinase and $p90^{RSK}$ in HDMECs; however, it is possible that in these cells, ERK5 is only partly responsible for mediating activation of these kinases, and that the effects of the ERK5 loss may be circumvented, with no direct impact upon rpS6 activation in these cells, by phosphorylation of p70 S6 kinase by other kinases such as ERK1/2 (Wang *et al.*, 2001).

5.3.6 ERK5 siRNA treatment does not affect VEGF-stimulated MEF2C activation in HDMECs

MEF2C has been shown to act as a downstream effector of ERK5-mediated survival of both PC12 cells and cortical neurones (Suzaki *et al.*, 2002; Liu *et al.*, 2003b). In addition, Hayashi *et al.* reported that apoptosis of *Erk5*$^{-/-}$ murine endothelial cells could be partly rescued by over-expression of a constitutively active form of *Mef2c* (Hayashi *et al.,* 2004). These reports raised the question: does ERK5 regulate VEGF-stimulated MEF2C phosphorylation in HDMECs? MEF2C undergoes phosphorylation on residues Thr^{293}, Thr^{300}, and Ser^{387} within its transactivation domain in response to agonist stimulation, to confer transactivational activity (Han *et al.*, 1997). Importantly, ERK5 has only been shown to phosphorylate MEF2C on Ser^{387} (Kato *et al.*, 1997), a residue that is also phosphorylated by p38 MAPK (Han *et al.*, 1997). Western blot analysis with an antibody directed against phos-MEF2C Ser^{387} revealed that VEGF strongly induced MEF2C phosphorylation of this site in HDMECs plated on either a gelatin matrix or within a collagen matrix (**Fig. 5.5, *p-MEF2C Ser*^{387}**); however, silencing of ERK5 expression had no effect upon VEGF-induced MEF2C activation on Ser^{387} (**Fig. 5.5, *p-MEF2C Ser*^{387}**). As a negative control, phosphorylation of MEF2C on Ser^{59}, a residue that is phosphorylated by casein kinase II (Molkentin et al., 1996), but not by p38 MAPK or ERK5, was also assessed. It was found that siRNA-mediated silencing of ERK5 expression had a minimal effect upon VEGF-induced phosphorylation of MEF2C on Ser^{59} (**Fig. 5.5, *p-MEF2C Ser*^{59}**). These data suggest that ERK5 is not required for VEGF-stimulated MEF2C phosphorylation on Ser^{387} or Ser^{59} in HDMECs.

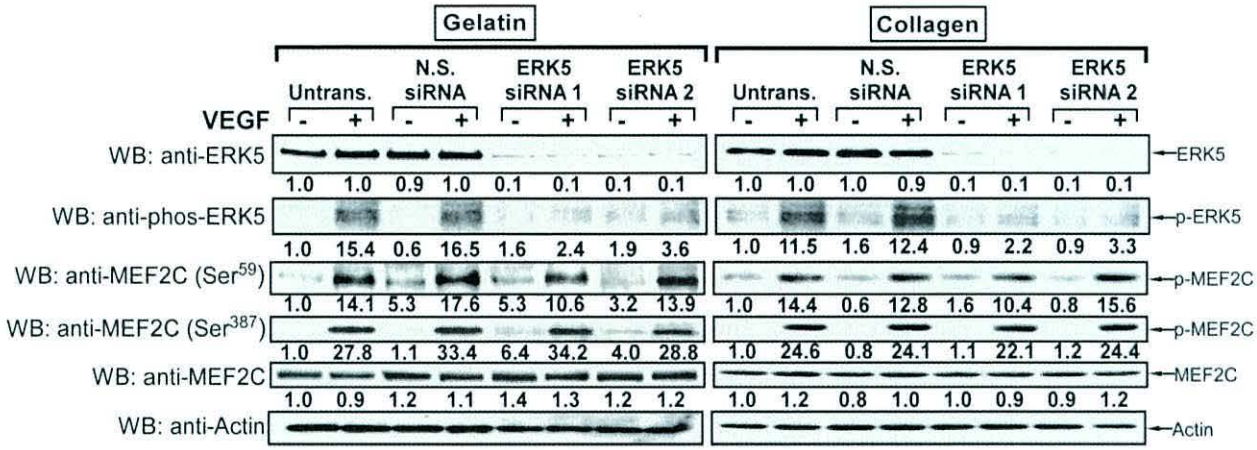

Figure 5.5 ERK5 does not mediate VEGF-stimulated phosphorylation of MEF2C on Ser^{59} or Ser^{397}. HDMECs were plated at 8.2 x 10^5 cells per dish on gelatin-coated 10 cm cell culture dishes in 10 ml of EBM MV2 growth medium. At 1 day post-seeding, cells were transfected as described (*section 2.2.2.7.2*) with 10 nM of ERK5 siRNA 1, or 10 nM of ERK5 siRNA 2, or with 10 nM of non-silencing (N.S.) siRNA, or were left untransfected (Untrans.). At 24 h post-transfection, cells were serum-starved for 20 h, prior to plating at 5.0 x 10^5 cells per well of a 6-well gelatin-coated plate, or at 6.25 x 10^5 cells per well of a 6-well plate between two layers of collagen I gel, and stimulated, or not, with VEGF (50 ng/ml) for 10 min. Cells were lysed in RIPA buffer and proteins were separated on a 10% SDS-PAGE gel and analysed by Western blotting (*WB*) with antibodies directed against ERK5, phos-ERK5, phos-MEF2C Ser^{59}, phos-MEF2C Ser^{387}, MEF2C and actin, as indicated. The degree of phosphorylation of ERK5, MEF2C Ser^{59}, MEF2C Ser^{387} and the expression of ERK5 and MEF2C relative to actin was quantified by densitometric analysis using NIH ImageJ software, and is indicated below each respective lane. The results shown are representative of three independent experiments. *ERK5, extracellular signal-regulated kinase 5; HDMEC, human dermal microvascular endothelial cell; MEF2C, myocyte enhancer factor 2C; N.S., non-silencing; siRNA, small interfering RNA; VEGF, vascular endothelial growth factor; WB, Western blot.*

5.3.7 VEGF-stimulated phosphorylation of MEF2C Ser^{387} in HDMECs is dependent upon p38 MAPK activity

Given that siRNA-induced down-regulation of ERK5 expression had no effect upon VEGF-induced phosphorylation of MEF2C on Ser^{387} (**Fig. 5.5, *p-MEF2C Ser^{387}***), it was hypothesised that VEGF-stimulated p38 MAPK activity (**Fig. 3.3, *p-p38 MAPK***) was likely to mediate MEF2C phosphorylation on Ser^{387} in HDMECs. To further characterise VEGF-induced MEF2C activation in HDMECs, and to test this hypothesis, p38 activation was inhibited by the use of SB202190, an ATP-competitive inhibitor of p38 MAPK activation, which is reported to be selective for p38α, -β and -γ MAPK (Lee *et al.*, 1994; Li *et al.*, 1996; Jiang *et al.*, 1997). As a negative control, cells were also treated with a structural analogue of SB202190, termed SB202474, which is reported to have no inhibitory effects upon p38 MAPK activation (Lee *et al.*, 1994).

HDMECs were serum-starved for 20 h prior to pre-incubation with various concentrations (1-100 μM) of SB202190 or SB202474, followed by stimulation, or not,

with VEGF. Western blot analysis revealed that SB202474 had no substantial effects upon VEGF-induced phosphorylation of ERK5 or p38 MAPK, even when used at a concentration of 50 µM (**Fig. 5.6 A**). Similarly, treatment with SB202474 did not block VEGF-induced MEF2C phosphorylation on Ser^{387} (**Fig. 5.6 A, *p-MEF2C Ser^{387}***). In contrast, incubation of HDMECs with the p38 MAPK inhibitor SB202190 lowered VEGF-induced phosphorylation of p38 MAPK by 90% when used at 10 nM compared to vehicle control-treated cells (**Fig. 5.6 B, *p-p38 MAPK***). When used at concentrations >10 µM, SB202190 treatment abolished VEGF-induced p38 MAPK activation (**Fig. 5.6 B, *p-p38 MAPK***). Concomitantly, 10 µM SB202190 abrogated VEGF-induced phosphorylation of MEF2C on Ser^{387} (**Fig. 5.6 B, *p-MEF2C Ser^{387}***). Treatment of HDMECs with SB202190 had no effect upon VEGF-induced ERK5 phosphorylation on Thr^{218}/Tyr^{220} when used at concentrations up to 25 µM (**Fig. 5.6 B, *p-ERK5***); however, non-specific inhibition of ERK5 activity became apparent when SB202190 was used at concentrations >50 µM (**Fig. 5.6 B, *p-ERK5***). Interestingly, treatment with 10 µM of SB202190 induced the appearance of a distinct, slower-migrating band above the main ERK5 band in both the presence, and absence of VEGF (**Fig. 5.6 B, *ERK5, arrowhead***), which suggests that this treatment induces hyper-phosphorylation of ERK5, an effect that was not apparent when SB202190 was used at a concentration of 25 µM or greater. Considered together, these results suggest that p38 MAPK mediates VEGF-stimulated MEF2C phosphorylation on Ser^{387} in HDMECs, and that ERK5 is not involved in this process.

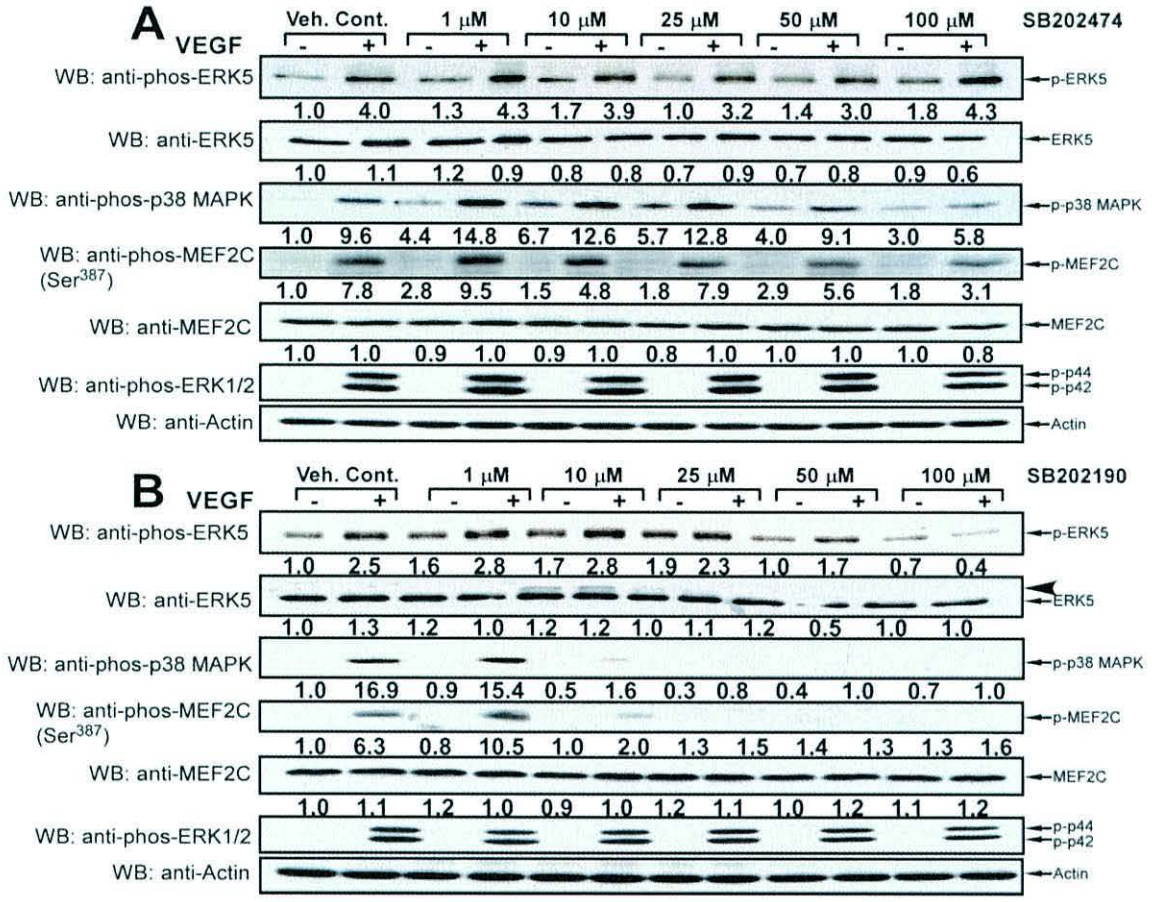

Figure 5.6 VEGF-induced phosphorylation of MEF2C on Ser^{387} is mediated by p38 MAPK. HDMECs were seeded at 2.0 x 10^5 cells per well on gelatin-coated 6-well dishes in EBM MV2 growth medium and incubated for 24 h. Cells were serum-starved in EBM MV2 basal medium containing 1% (v/v) FCS for 20 h, then incubated with 0.1% (v/v) DMSO as a vehicle control (Veh. Cont.), or with the indicated concentrations of (**A**) SB202474 or (**B**) SB202190 for 30 min. Cells were then stimulated, or not (control), with VEGF (50 ng/ml) for 10 min. Cells were lysed, and total cellular proteins were separated on a 10% SDS-PAGE gel and subjected to Western blot (*WB*) analysis with antibodies directed against phos-ERK5 (Thr^{218}/Tyr^{220}), ERK5, phos-p38 MAPK (Thr^{180}/Tyr^{182}), phos-MEF2C (Ser^{387}), MEF2C, phos-ERK1/2 (Thr^{202}/Tyr^{204}) and actin as a loading control, as indicated. The degree of phosphorylation of ERK5, p38 MAPK, MEF2C Ser^{387} and the expression of ERK5 and MEF2C relative to actin was quantified by densitometric analysis using NIH ImageJ software, and is indicated below each respective lane. The result shown is representative of two independent experiments. *DMSO, dimethyl sulfoxide; ERK, extracellular signal-regulated kinase; FCS, foetal calf serum; HDMEC, human dermal microvascular endothelial cell; MEF2C, myocyte enhancer factor 2C; p38 MAPK, p38 mitogen-activated protein kinase; VEGF, vascular endothelial growth factor; WB, Western blot.*

5.4 Profiling the effects of siRNA-mediated down-regulation of ERK5 expression upon VEGF-stimulated gene expression in HDMECs plated on a gelatin matrix or within a 3-D collagen matrix

Endothelial cell stimulation with pro-angiogenic growth factors such as VEGF is known to up-regulate the expression of several genes, some of which have been implicated in angiogenesis (Jih *et al.*, 2001; Abe & Sato, 2001; Wary *et al.*, 2003; Liu *et al.*, 2003a; Gerritsen *et al.*, 2003; Glesne *et al.*, 2006). More recently, microarray and qRT-PCR analyses of VEGF-stimulated gene expression in human microvascular endothelial cells

plated within collagen gels or on a gelatin matrix have shown that genes involved in differentiation, survival and proliferation are specifically up-regulated in endothelial cells plated on these respective matrices in response to VEGF stimulation (Rennel *et al.*, 2007; Mellberg *et al.*, 2009). In an effort to further define the mechanisms whereby ERK5 facilitates VEGF-induced tubular morphogenesis and survival of HDMECs on a collagen gel, the expression of a selected panel of genes that had been previously shown to be involved in VEGF-induced angiogenesis was profiled by qRT-PCR following siRNA-mediated silencing of ERK5 expression, or treatment with N.S. siRNA.

5.4.1 ERK5 regulates VEGF-induced transcription of *BCL2*

The cytoprotective effect of VEGF, which is critical for long-term survival of endothelial cells is arbitrated in part via VEGF-induced transcriptional up-regulation of anti-apoptotic genes (Zachary, 2003; Holmes *et al.*, 2007), which promote endothelial cell survival, and prevent programmed cell death. To date, VEGF has been shown to induce the expression of four anti-apoptotic genes, namely: the IAP family members *Survivin* (Tran *et al.*, 1999; O'Connor *et al.*, 2000) and *XIAP* (X-chromosome linked IAP) (Tran *et al.*, 1999), the anti-apoptotic protein *BCL2* (Gerber *et al.*, 1998a; Tran *et al.*, 1999; Nor *et al.*, 2001; Cai *et al.*, 2003; Grosjean *et al.*, 2006) and the anti-apoptotic BCL2 family member protein *A1* (Gerber *et al.*, 1998a).

It was revealed in chapter 4 of this study that siRNA-mediated down-regulation of ERK5 expression induced apoptosis of HDMECs at an early time point when plated within a 3-D collagen gel, an effect not observed in cells plated on a gelatin matrix (**Fig. 4.12 – Fig. 4.15**). To investigate the potential involvement of ERK5 in regulating VEGF-stimulated expression of anti-apoptotic genes in HDMECs within a collagen I gel, or on a gelatin matrix, a nine hour time-course study was carried out. Cells were treated with ERK5 siRNA, or with N.S. siRNA as a negative control, and plated on a gelatin matrix or between two layers of collagen I, prior to stimulation with 50 ng/ml VEGF. Total RNA was extracted from cells at 3 h post plating (0 h time point) and at 3 h and 9 h post-VEGF stimulation thereafter. Following reverse transcription of RNA, cDNA was

used as a template in qRT-PCR reactions with gene-specific primers to detect expression of *BCL2, Survivin, XIAP* and *A1*. Analysis of *ERK5* mRNA expression levels confirmed that transfection of ERK5 siRNA attenuated *ERK5* expression by at least 80% on both collagen and gelatin matrices in the presence and the absence of VEGF, at all time points (**Fig. 5.7 A**).

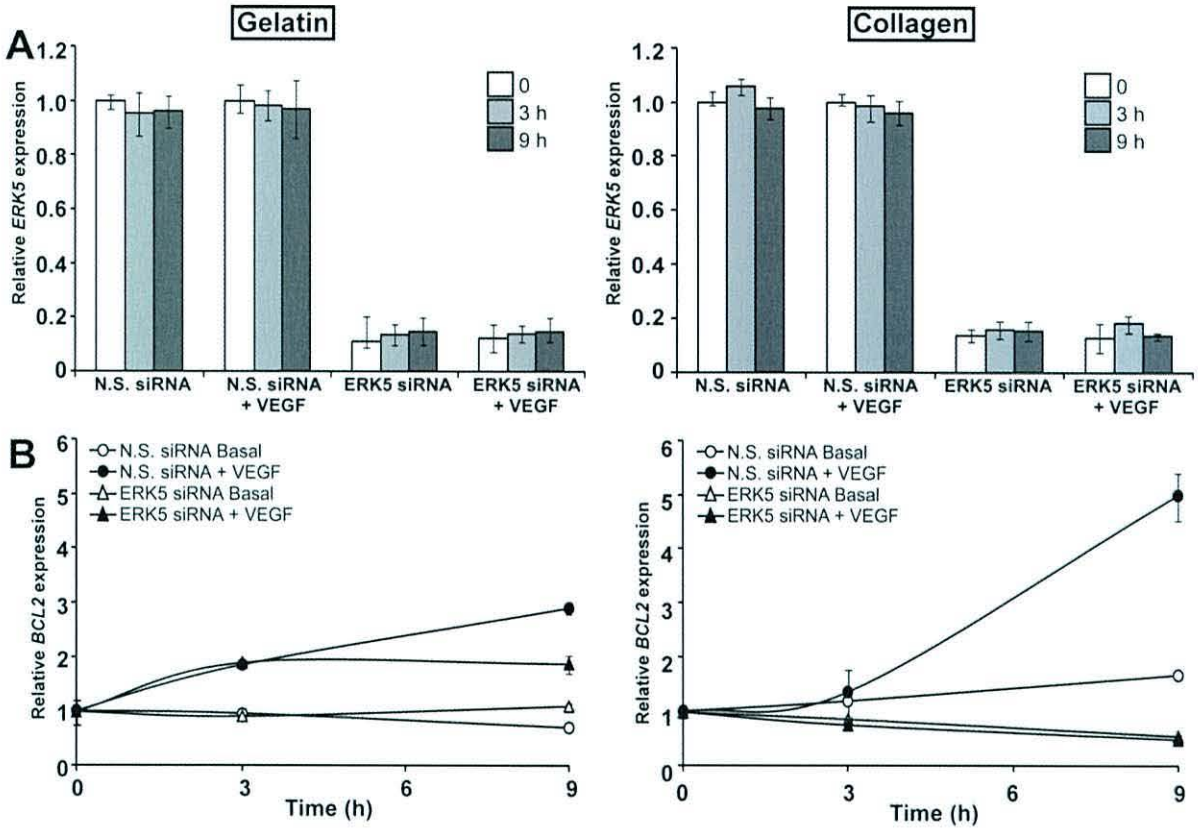

Figure 5.7 ERK5 differentially regulates VEGF-induced expression of *BCL2* in HDMECs on collagen and gelatin matrices. HDMECs were plated at 8.2 x 10^5 cells per dish on gelatin-coated 10 cm cell culture dishes in 10 ml of EBM MV2 growth medium. At 1 day post-seeding, cells were transfected with ERK5 siRNA (5 nM ERK5 siRNA 1 + 5 nM ERK5 siRNA 2) or with 10 nM non-silencing (N.S.) control siRNA as described (*section 2.2.2.7.2*). At 24 h post-transfection, cells were serum-starved for 20 h in 10 ml of EBM MV2 basal medium containing 1% (v/v) FCS, prior to plating at 5.0 x 10^5 cells per well in a 6-well gelatin-coated plate in 2 ml of EBM MV2 basal medium containing 1% (v/v) FCS on a gelatin matrix, or at 6.25 x 10^5 cells per well in a 6-well plate between two layers of a type I collagen gel. After 1 h, cells were stimulated with VEGF (50 ng/ml). Total RNA was extracted immediately (0 h time point) and at 3 h and 9 h post-stimulation with VEGF on both matrices thereafter (*section 2.2.4.1*), followed by reverse-transcription cDNA synthesis. cDNA from each experimental condition was analysed by quantitative real-time PCR (qRT-PCR) in triplicate, for expression of (**A**) *ERK5* (**B**) *BCL2* and the control housekeeping gene *β-actin*. Relative quantification was performed by the comparative C_T ($2^{-\Delta\Delta C}{}_T$) method (*section 2.2.5.2*). Gene expression is presented relative to the 0 h basal sample on each matrix (n = 3; mean ± SD of triplicate wells). The result shown is representative of three independent experiments. *BCL2, B-cell lymphoma 2; ERK5, extracellular signal-regulated kinase 5; HDMEC, human dermal microvascular endothelial cell; N.S., non-silencing; siRNA, small interfering RNA; VEGF, vascular endothelial growth factor.*

Under the conditions tested, VEGF-stimulation did not induce transcriptional up-regulation of *XIAP, A1* or *Survivin* in HDMECs plated either on a gelatin matrix or within

a 3-D collagen gel. Furthermore, treatment with ERK5 siRNA did not affect basal expression levels of any of these genes on either matrix (data not shown). However, qRT-PCR analysis of *BCL2* expression revealed that at 9 h post-VEGF stimulation, *BCL2* expression was significantly up-regulated (2.9-fold, ± 0.1-fold SD) on a gelatin matrix and (4.8-fold, ± 0.4-fold SD) on a collagen matrix compared to the respective unstimulated basal controls on each matrix (**Fig. 5.7 B**). VEGF-induced expression of *BCL2* was lowered by 35% on a gelatin matrix following ERK5 siRNA treatment compared to N.S. siRNA-treated cells, whereas siRNA-induced down-regulation of ERK5 expression in cells plated within a collagen matrix resulted in a 90% reduction in *BCL2* expression (**Fig. 5.7 B**). Furthermore, in the absence of VEGF, basal expression levels of *BCL2* were lowered by 68% following ERK5 siRNA treatment compared to the N.S. siRNA control on a collagen matrix (**Fig. 5.7 B**). These results suggest that ERK5 is essential for both VEGF-stimulated and basal expression of *BCL2* in HDMECs on a collagen gel, whereas under conditions of proliferation on a gelatin matrix, the effect of ERK5 loss upon *BCL2* expression was less profound, suggesting that the role of ERK5 in regulating *BCL2* expression is also substrate dependent.

5.4.2 SiRNA-mediated silencing of ERK5 expression induces up-regulation of *MEF2C* expression

Given that VEGF stimulation induced up-regulation of *MEF2C* expression in HDMECs (**Fig. 5.2**), it was of interest to determine whether siRNA-induced down-regulation of ERK5 expression would affect VEGF-stimulated expression of *MEF2C* in HDMECs. To test this, cells were treated with ERK5 siRNA or with N.S. control siRNA prior to serum-starvation for 20 h, followed by plating on a gelatin matrix, or between two layers of collagen I gel and stimulation with VEGF. Total RNA was extracted at 1 h post-plating (0 h), and at 3 h and 9 h post-stimulation with VEGF, and was reverse-transcribed for use in qRT-PCR analysis. It was found that N.S. siRNA-treated cells exhibited a 1.7-fold and a 4.2-fold increase in *MEF2C* expression following 9 h VEGF stimulation on gelatin and collagen matrices respectively (**Fig. 5.8**), similar to that described previously (**Fig. 5.2**). In addition, *MEF2C* expression levels did not change in the absence of VEGF (**Fig. 5.8**). Strikingly however, in cells plated within a 3-D collagen matrix, silencing of ERK5

expression induced an 8-fold up-regulation in *MEF2C* expression, an effect that was further enhanced by stimulation with VEGF compared to the N.S. siRNA-treated cells (**Fig. 5.8**). Interestingly, this effect was confined to cells plated within a 3-D collagen matrix (**Fig. 5.8**).

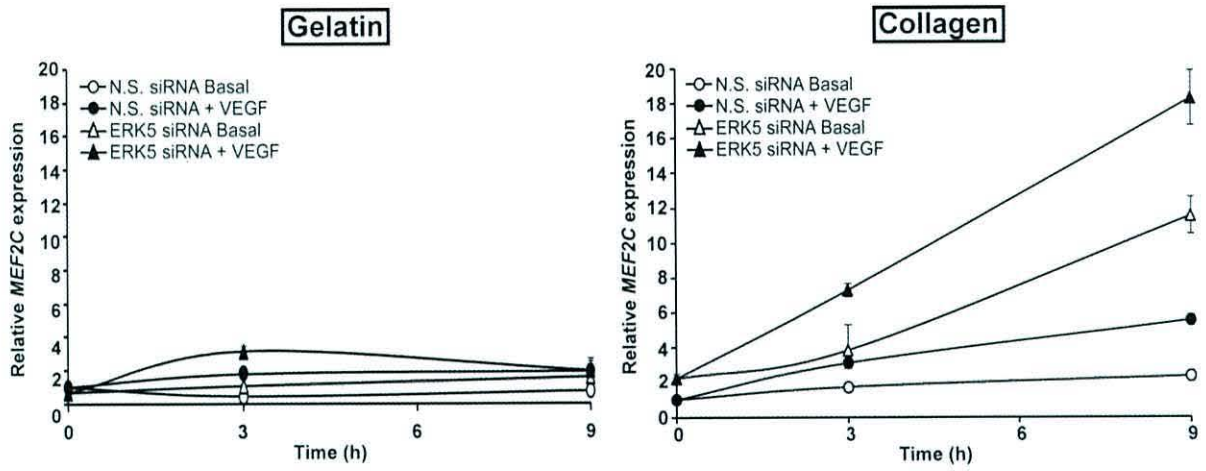

Figure 5.8 SiRNA-mediated silencing of ERK5 expression induces up-regulation of *MEF2C* expression in HDMECs on a collagen matrix. HDMECs were plated at 8.2 x 10^5 cells per dish on gelatin-coated 10 cm cell culture dishes in 10 ml of EBM MV2 growth medium. At 1 day post-seeding, cells were transfected with ERK5 siRNA (5 nM ERK5 siRNA 1 + 5 nM ERK5 siRNA 2) or with 10 nM non-silencing (N.S.) control siRNA as described (*section 2.2.2.7.2*). At 24 h post-transfection, cells were serum-starved for 20 h in 10 ml of EBM MV2 basal medium containing 1% (v/v) FCS, prior to plating at 5.0 x 10^5 cells per well in a 6-well gelatin-coated plate in 2 ml of EBM MV2 basal medium containing 1% (v/v) FCS on a gelatin matrix, or at 6.25 x 10^5 cells per well in a 6-well plate between two layers of a type I collagen gel. After 1 h, cells were stimulated with VEGF (50 ng/ml). Total RNA was extracted immediately (0 h time point) and at 3 h and 9 h post-stimulation with VEGF on both matrices thereafter (*section 2.2.4.1*), followed by reverse-transcription cDNA synthesis. cDNA from each experimental condition was analysed by quantitative real-time PCR (qRT-PCR) in triplicate, for expression of *MEF2C* and the control housekeeping gene *β-actin*. Relative quantification was performed by Comparative C_T ($2^{-\Delta\Delta C_T}$) method (*section 2.2.5.2*). Gene expression is presented relative to the 0 h basal sample on each matrix (n = 3; mean ± SD of triplicate wells). The result shown is representative of three independent experiments. *ERK5, extracellular signal-regulated kinase 5; FCS, foetal calf serum; HDMEC, human dermal microvascular endothelial cell; MEF2C, myocyte enhancer factor 2C; N.S., non-silencing; siRNA, small interfering RNA; VEGF, vascular endothelial growth factor.*

5.4.3 SiRNA-mediated down-regulation of ERK5 expression does not affect expression of the VEGF-responsive genes *Nur77* and *RCAN1*

The expression of the calcineurin inhibitor protein *RCAN1* (regulator of calcineurin 1) is well documented to be strongly up-regulated in response to VEGF stimulation in endothelial cells (Abe & Sato, 2001; Liu *et al.*, 2003a). More recent work has revealed that RCAN1 plays an important role in the regulation of endothelial cell migration and tubular morphogenesis via a mechanism that involves regulation of the calcineurin-NFAT pathway, but which is not yet fully understood (Yao & Duh, 2004; Qin *et al.*, 2006; Schabbauer *et al.*, 2007). In addition, expression of the NR4A orphan nuclear receptor family transcription factor *Nur77* (also known as TR3), has been shown to be

robustly up-regulated following VEGF stimulation in endothelial cells (Liu *et al.*, 2003a), and has been implicated in the regulation of endothelial cell proliferation (Arkenbout *et al.*, 2003). Interestingly, over-expression of *Nur77* in HUVECs has been shown to be sufficient to induce endothelial cell survival and angiogenesis in the absence of VEGF (Zeng *et al.*, 2006). It was of interest to investigate the potential role of ERK5 as a potential transcriptional regulator of VEGF-stimulated expression of *RCAN1* and *Nur77.*

VEGF-stimulated expression of *RCAN1* was most robustly induced on a collagen matrix (**Fig. 5.9 A**). It was found that siRNA-mediated silencing of ERK5 had no significant effect upon VEGF-induced expression of either *RCAN1*. Consistent with its reported role as a regulator of endothelial cell proliferation, VEGF induced a 4-fold increase in *Nur77* expression on a gelatin matrix (Arkenbout *et al.*, 2003), but its expression was more weakly induced following VEGF stimulation on a collagen matrix (**Fig. 5.9 B**). SiRNA-mediated silencing of ERK5 expression had no effect on VEGF-stimulated expression of *Nur77* (**Fig. 5.9**).

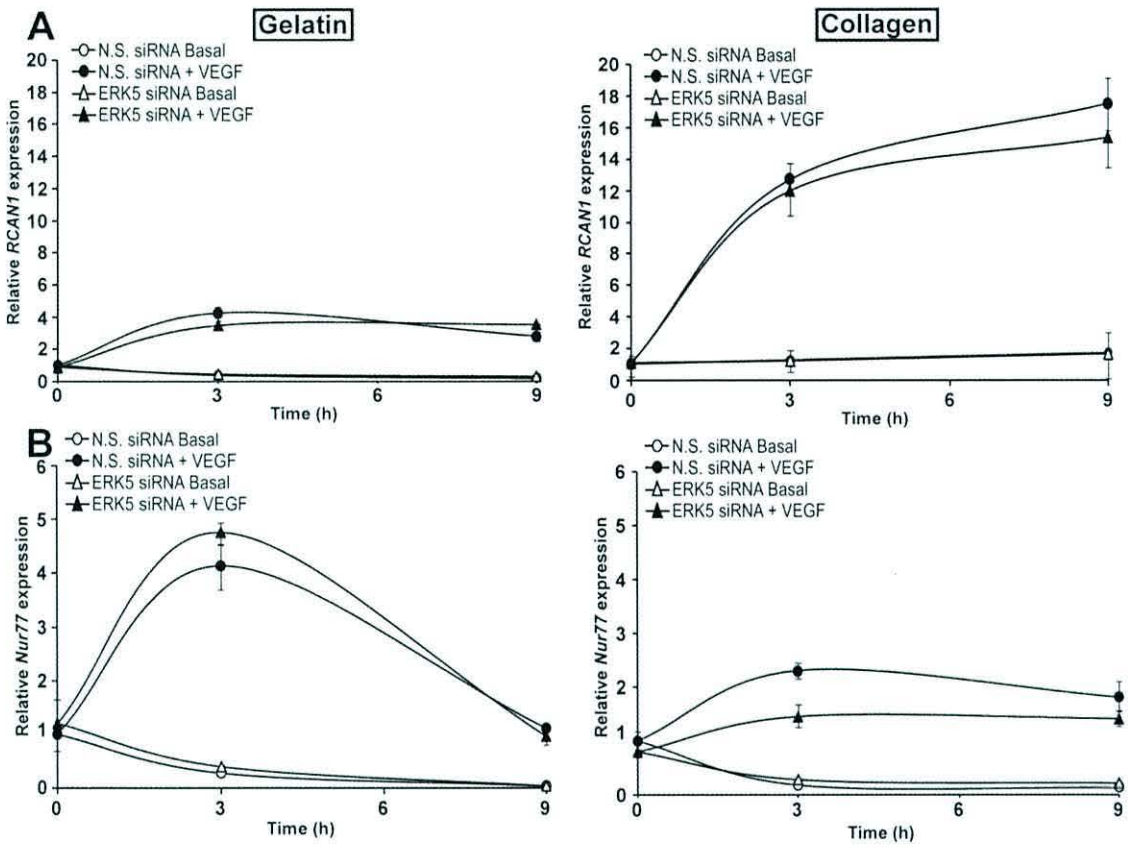

Figure 5.9 Effects of ERK5 siRNA on expression of *RCAN1* and *Nur77* in HDMECs on collagen and gelatin matrices following VEGF stimulation. HDMECs were plated at 8.2 x 10^5 cells per dish on gelatin-coated 10 cm cell culture dishes in 10 ml of EBM MV2 growth medium. At 1 day post-seeding, cells were transfected with ERK5 siRNA (5 nM ERK5 siRNA 1 + 5 nM ERK5 siRNA 2) or with 10 nM non-silencing (N.S.) control siRNA as described (*section 2.2.2.7.2*). At 24 h post-transfection, cells were serum-starved for 20 h in 10 ml of EBM MV2 basal medium containing 1% (v/v) FCS, prior to plating at 5.0 x 10^5 cells per well in a 6-well gelatin-coated plate in 2 ml of EBM MV2 basal medium containing 1% (v/v) FCS on a gelatin matrix, or at 6.25 x 10^5 cells per well in a 6-well plate between two layers of a type I collagen gel. After 1 h, cells were stimulated with VEGF (50 ng/ml). Total RNA was extracted immediately (0 h time point) and at 3 h and 9 h post-stimulation with VEGF on both matrices thereafter (*section 2.2.4.1*), followed by reverse-transcription cDNA synthesis. cDNA from each experimental condition was analysed by quantitative real-time PCR (qRT-PCR) in triplicate, for expression of (**A**) *RCAN1*, (**B**) *Nur77* and the control housekeeping gene *β-actin*. Relative quantification was performed by Comparative C_T ($2^{-\Delta\Delta C_T}$) method (*section 2.2.5.2*). Gene expression is presented relative to the 0 h basal sample on each matrix (n = 3; mean ± SD of triplicate wells). The result shown is representative of three independent experiments. *ERK, extracellular signal-regulated kinase; HDMEC, human dermal microvascular endothelial cell; N.S., non-silencing; RCAN1, regulator of calcineurin 1; siRNA, small interfering RNA; VEGF, vascular endothelial growth factor.*

5.4.4 ERK5 regulates VEGF-stimulated expression of *VCAM-1* and *E-selectin*

The transmembrane adhesion molecules E-selectin and VCAM-1 have important roles during vascular development (Kwee *et al.*, 1995), as well as during angiogenesis *in vitro* and *in vivo* (Koch *et al.*, 1995; Radisavljevic *et al.*, 2000; Aoki *et al.*, 2001). It had been previously shown that VEGF stimulation induces up-regulation of *VCAM-1* and *E-selectin* expression in HUVECs via a NF-κB-dependent mechanism (Kim *et al.*, 2001). Given the effects of ERK5 knockdown on VEGF-induced RELA activation in HDMECs (**Fig. 5.3**), it was of interest to determine the effects of ERK5 siRNA treatment upon

VEGF-induced expression of these genes in HDMECs under conditions of endothelial cell proliferation and tubular morphogenesis *in vitro*. It was found that VEGF stimulation of HDMECs strongly induced up-regulation of *VCAM-1* and *E-selectin* expression (**Fig. 5.10, A and B**), an effect that was most pronounced in cells plated on a collagen matrix. SiRNA-mediated silencing of ERK5 expression lowered VEGF-stimulated expression of *VCAM-1* and *E-selectin* by ~50% in cells plated on a gelatin matrix, whereas siRNA-mediated down-regulation of ERK5 expression abolished VEGF-stimulated expression of these genes in HDMECs plated within a collagen matrix (**Fig. 5.10 B**). These data showed that ERK5 regulates the expression of these adhesion molecules in response to VEGF stimulation in a matrix-dependent manner.

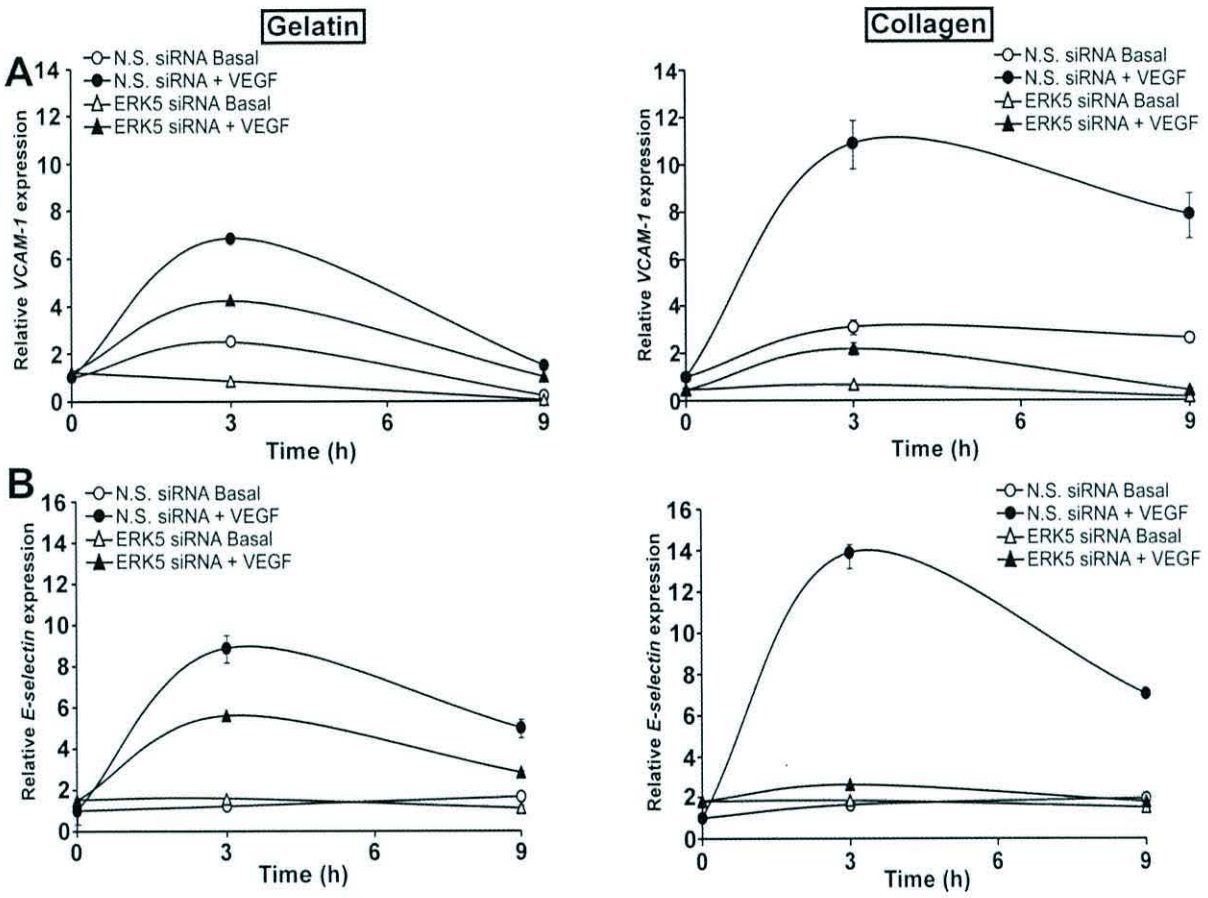

Figure 5.10 Effects of ERK5 siRNA on expression of *VCAM-1* and *E-Selectin* in HDMECs on collagen and gelatin matrices following VEGF stimulation. HDMECs were plated at 8.2 x 10^5 cells per dish on gelatin-coated 10 cm cell culture dishes in 10 ml of EBM MV2 growth medium. At 1 day post-seeding, cells were transfected with ERK5 siRNA (5 nM ERK5 siRNA 1 + 5 nM ERK5 siRNA 2) or with 10 nM non-silencing (N.S.) control siRNA as described (*section 2.2.2.7.2*). At 24 h post-transfection, cells were serum-starved for 20 h in 10 ml of EBM MV2 basal medium containing 1% (v/v) FCS, prior to plating at 5.0 x 10^5 cells per well in a 6-well gelatin-coated plate in 2 ml of EBM MV2 basal medium containing 1% (v/v) FCS on a gelatin matrix, or at 6.25 x 10^5 cells per well in a 6-well plate between two layers of a type I collagen gel. After 1 h, cells were stimulated with VEGF (50 ng/ml). Total RNA was extracted immediately (0 h time point) and at 3 h and 9 h post-stimulation with VEGF on both matrices thereafter (*section 2.2.4.1*), followed by reverse-transcription cDNA synthesis. cDNA from each experimental condition was analysed by quantitative real-time PCR (qRT-PCR) in triplicate, for expression of (**A**) *VCAM-1*, (**B**) *E-Selectin* and the control housekeeping gene *β-actin*. Relative quantification was performed by Comparative C_T ($2^{-\Delta\Delta C_T}$) method (*section 2.2.5.2*). Gene expression is presented relative to the 0 h basal sample on each matrix (n = 3; mean ± SD of triplicate wells). The result shown is representative of three independent experiments. *ERK, extracellular signal-regulated kinase; FCS, foetal calf serum; HDMEC, human dermal microvascular endothelial cell; N.S., non-silencing; siRNA, small interfering RNA; VCAM-1, vascular endothelial adhesion molecule-1; VEGF, vascular endothelial growth factor.*

5.4.5 ERK5 is required for VEGF-induced *IL-8* expression in HDMECs

VEGF induces expression of the pro-angiogenic chemokine *IL-8* in endothelial cells via a NF-κB-dependent mechanism (Lee *et al.*, 2002; Liu *et al.*, 2003a; Karl *et al.*, 2005). Analysis of *IL-8* expression in HDMECs revealed that VEGF stimulation induced a 4.5-fold and a 7-fold increase in *IL-8* expression in HDMECs plated on a gelatin matrix and within a collagen gel respectively (**Fig. 5.11**). SiRNA-mediated down-regulation of ERK5

expression abolished VEGF-stimulated expression of *IL-8* in HDMECs on a collagen matrix, but had a less pronounced effect upon VEGF-stimulated *IL-8* expression in HDMECs plated on a gelatin matrix (**Fig. 5.11**), thus showing that ERK5 is differentially required for mediation of *IL-8* gene expression in these cells in response to VEGF stimulation.

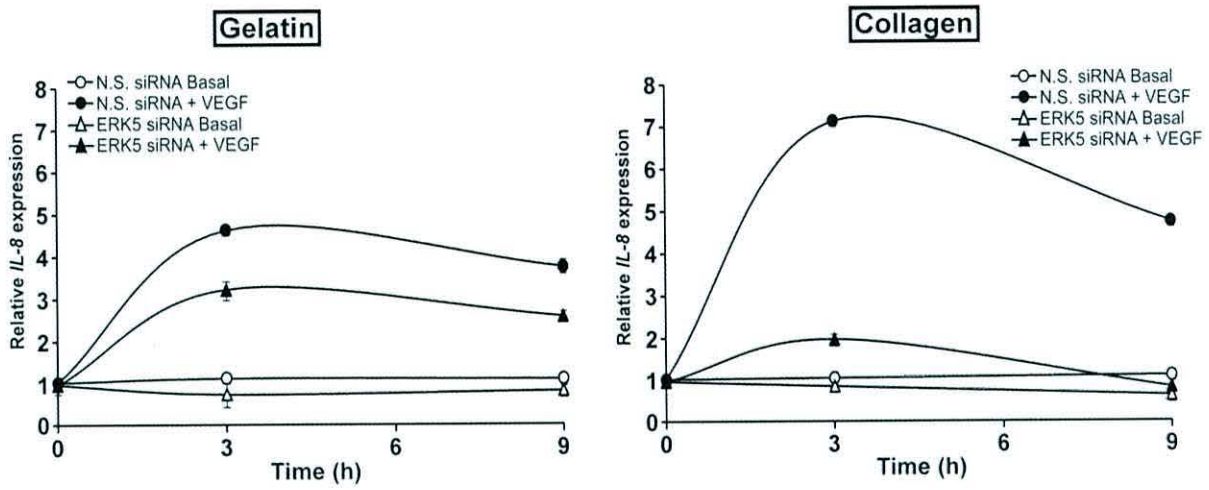

Figure 5.11 Effects of ERK5 siRNA upon VEGF-induced expression of *IL-8* in HDMECs on collagen and gelatin matrices. HDMECs were plated at 8.2 x 10^5 cells per dish on gelatin-coated 10 cm cell culture dishes in 10 ml of EBM MV2 growth medium. At 1 day post-seeding, cells were transfected with ERK5 siRNA (5 nM ERK5 siRNA 1 + 5 nM ERK5 siRNA 2) or with 10 nM non-silencing (N.S.) control siRNA as described (*section 2.2.2.7.2*). At 24 h post-transfection, cells were serum-starved for 20 h in 10 ml of EBM MV2 basal medium containing 1% (v/v) FCS, prior to plating at 5.0 x 10^5 cells per well in a 6-well gelatin-coated plate in 2 ml of EBM MV2 basal medium containing 1% (v/v) FCS on a gelatin matrix, or at 6.25 x 10^5 cells per well in a 6-well plate between two layers of a type I collagen gel. After 1 h, cells were stimulated with VEGF (50 ng/ml). Total RNA was extracted immediately (0 h time point) and at 3 h and 9 h post-stimulation with VEGF on both matrices thereafter (*section 2.2.4.1*), followed by reverse-transcription cDNA synthesis. cDNA from each experimental condition was analysed by quantitative real-time PCR (qRT-PCR) in triplicate, for expression of *IL-8* and the control housekeeping gene *β-actin*. Relative quantification was performed by the comparative C_T ($2^{-\Delta\Delta C_T}$) method (*section 2.2.5.2*). Gene expression is presented relative to the 0 h basal sample on each matrix (n = 3; mean ± SD of triplicate wells). The result shown is representative of three independent experiments. *ERK5, extracellular signal-regulated kinase 5; HDMEC, human dermal microvascular endothelial cell; IL-8, interleukin 8; N.S., non-silencing; siRNA, small interfering RNA; VEGF, vascular endothelial growth factor.*

5.5 Effects of transient expression of a dominant-negative mutant of ERK5, or a constitutively active mutant of MEK5, upon intracellular signalling in HDMECs

5.5.1 Co-expression of ERK5(wt) and CA-MEK5(D) leads to phosphorylation of AKT and BAD and induces expression of BCL2 whilst suppressing caspase-3 activity in HDMECs within a 3-D collagen gel

Constitutive activation of ERK5 by co-expression of CA-MEK5(D) and ERK5(wt) induced tubular morphogenesis of HDMECs within a collagen I gel in the absence of VEGF (**Fig. 4.19**). In an effort to define the molecular mechanisms regulating this process, it was

of interest to assess the intracellular signalling events occurring in HDMECs under these conditions. HDMECs were plated within a 3-D collagen matrix in the presence, or absence of VEGF following constitutive activation of ERK5 or abolition of ERK5 activity by transient transfection with HA-tagged CA-MEK5(D) and Flag-tagged ERK5(wt), or with HA-tagged CA-MEK5(D) and Flag-tagged DN-ERK5(AEF) respectively, followed by cell lysis and Western blot analysis.

As described previously (**Fig. 4.17**), constitutive activation of ERK5 by co-transfection with ERK5(wt) and CA-MEK5(D) induced ERK5 phosphorylation on Thr^{218}/Tyr^{220} to a similar degree to that observed in cells transfected with empty pcDNA3.1 vector alone and stimulated with VEGF (**Fig. 5.12, *p-ERK5***). ERK5(wt) and CA-MEK5(D)-induced ERK5 activation was augmented by stimulation with VEGF (**Fig. 5.12**). Co-expression of ERK5(wt) and CA-MEK5(D) was sufficient to induce phosphorylation of AKT on both Ser^{473} and Thr^{308} in the absence of VEGF (**Fig. 5.12, *p-AKT***), an effect that was enhanced by VEGF stimulation. Interestingly, maximal phosphorylation of BAD on Ser^{136} and elevated expression of BCL2 was observed in HDMECs co-transfected with CA-MEK5 and ERK5(wt) (**Fig. 5.12**), an effect that was not enhanced by VEGF stimulation, suggesting that constitutive activation of ERK5 alone was sufficient to exert these effects. Analysis of cleaved caspase-3 levels revealed that co-expression of ERK5(wt) and CA-MEK5(D) also resulted in suppression of caspase-3 cleavage, even in the absence of VEGF (**Fig. 5.12, *cleaved caspase-3***).

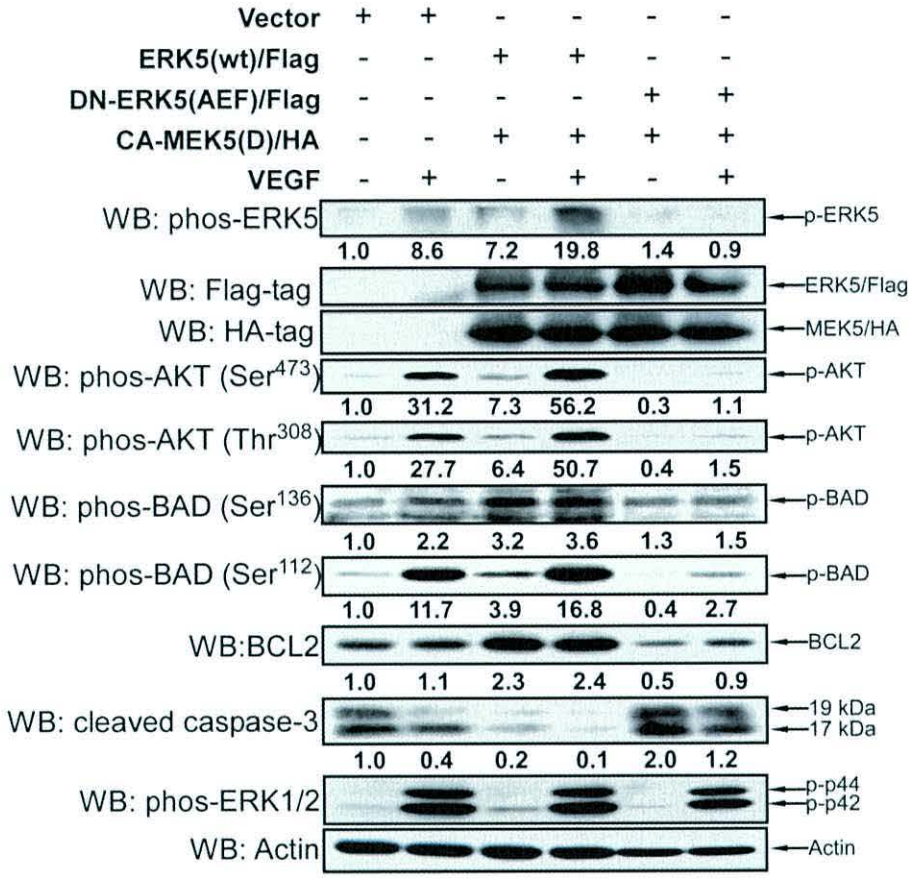

Figure 5.12 Constitutive activation of ERK5 activity by co-expression of ERK5(wt) and CA-MEK5(D) stimulates phosphorylation of ERK5 and AKT and induces expression of BCL2 whilst suppressing caspase-3 activity in HDMECs within a 3-D collagen gel. HDMECs were transiently transfected with 2.5 µg ERK5(wt) and 2.5 µg CA-MEK5(D); or with 2.5 µg DN-ERK5(AEF) and 2.5 µg CA-MEK5(D); alternatively, cells were mock-transfected with 5.0 µg empty pcDNA3.1 (Vector) as described (*section 2.2.2.7.1*). At 24 h post-transfection, HDMECs were serum-starved for 20 h, prior to plating between two layers of collagen I, and stimulation with VEGF (50 ng/ml) for 24 h. Cells were stimulated again with VEGF (50 ng/ml) for 10 min prior to lysis in 1x LDS sample buffer. Lysates were separated on a 4-12% NuPAGE® gel. Western blot (*WB*) analysis was carried out with antibodies directed against p-ERK5, ERK5, HA-Tag, Flag-Tag, p-AKT (Ser^{473}), p-AKT (Thr^{308}), p-BAD (Ser^{136}), BCL2, cleaved caspase-3, p-ERK1/2 and actin, as indicated. The degree of phosphorylation of ERK5, AKT (Ser^{473}), AKT (Thr^{308}), BAD (Ser^{136}), and the expression levels of ERK5 and BCL2 relative to actin was quantified by densitometric analysis using NIH ImageJ software, and is indicated below each respective lane. The result shown is representative of three independent experiments. *AKT/PKB, protein kinase B; BAD, BCL2-antagonist of cell death; BCL2, B-cell lymphoma 2; CA, constitutively active; DN, dominant-negative; ERK, extracellular signal-regulated kinase; HA, haemagluttinin; HDMEC, human dermal microvascular endothelial cell; MEK, MAPK/ERK kinase; N.S., non-silencing; siRNA, small interfering RNA; VEGF, vascular endothelial growth factor; WB, Western blot; wt; wild type.*

In contrast, co-expression of DN-ERK5(AEF) and CA-MEK5(D) did not stimulate ERK5 phosphorylation, AKT phosphorylation, BAD phosphorylation or BCL2 expression under basal conditions. Furthermore, co-transfection of DN-ERK5(AEF) and CA-MEK5(D) blocked VEGF-stimulated ERK5 phosphorylation, AKT phosphorylation and BAD phosphorylation (**Fig. 5.12**). VEGF-induced *BCL2* expression and suppression of cleaved caspase-3 was also blocked this treatment (**Fig. 5.12**). Collectively, these results confirm the previous biochemical analyses with siRNA-mediated silencing of

ERK5 (**Fig. 5.3**), whilst also showing that ERK5 phosphorylation on Thr^{218}/Tyr^{220} is necessary for the activation of AKT, and the downstream phosphorylation and inactivation of BAD, together with the concomitant increase in BCL2 levels, resulting in suppression of caspase-3 cleavage. In conclusion, these data suggest that constitutive activation of ERK5 is sufficient to inhibit apoptosis of HDMECs in the absence of VEGF thus providing a likely mechanism to account for the sustained survival of HDMECs under these conditions (**Fig. 4.19**).

5.6 Discussion

Whilst VEGF had previously been reported to induce activation of ERK5 in HUVECs and HDMECs (Hayashi *et al.*, 2004), the potential role of ERK5 as a mediator of VEGF-stimulated signalling and gene expression had not been previously investigated, and so had remained hitherto unknown. A selective requirement for ERK5 in mediating VEGF-induced survival and tubular morphogenesis of HDMECs within a 3-D collagen gel, but not under conditions of VEGF-induced proliferation on a gelatin matrix, was described earlier in this study (*chapter 4*). The experiments detailed in this chapter were conducted with the overall aim of characterising VEGF-induced activation of ERK5 and ERK5-regulated downstream signalling and gene expression under these conditions, which recapitulate distinct phases of the angiogenic response, in order to gain an understanding of the mechanisms by which ERK5 regulates VEGF-induced angiogenesis.

5.6.1 Characterisation of VEGF-stimulated activation of ERK5 in HDMECs under conditions of proliferation and tubular morphogenesis

It is well established that the duration of ERK1/2 activation is a major determinant of whether cells undergo differentiation or proliferation. Classical studies of NGF-stimulated ERK1/2 activation in PC12 cells have established that transient ERK1/2 activation correlates with PC12 proliferation, whereas sustained activation of ERK1/2 induces the differentiation of PC12 cells into sympathetic neurons (Marshall, 1995). The importance of ERK5 signal duration or intensity in conferring various cellular responses is less well understood. This study reveals that in contrast to ERK1/2, VEGF-

mediated activation of ERK5 was sustained under conditions of both proliferation and differentiation (**Fig. 5.1**), suggesting that the requirement for ERK5 in HDMEC differentiation, but not in proliferation was not attributable to possible differential activation of ERK5 on collagen and gelatin matrices.

Numerous reports in which the profile of ERK5 and ERK1/2 activation in various cell types, and in response to diverse stimuli, including: hypoxia-, and shear-stress-induced activation of ERK1/2 and ERK5 in endothelial cells, have shown that ERK5 activation is generally sustained, whilst that of ERK1/2 is generally more transient (Yan *et al.*, 1999; Cavanaugh *et al.*, 2001; Mody *et al.*, 2001; Pi *et al.*, 2005; Fujii *et al.*, 2008). These findings suggest that the mechanisms regulating de-activation of ERK5 are distinct from those regulating ERK1/2 de-phosphorylation. Indeed, whilst it had been suggested that de-phosphorylation, and de-activation of ERK5 may be mediated by DUSP6 (dual-specificity phosphatase 6)/MKP-3 (MAP kinase phosphatase-3) (Kamakura *et al.*, 1999; Zou *et al.*, 2006; Sarkozi *et al.*, 2007), it was recently shown that this was not the case (Arkell *et al.*, 2008). The specific MKP responsible for de-phosphorylating ERK5 has yet to be identified. Further studies comparing ERK1/2 and ERK5 activation are therefore merited to further understand the mechanisms regulating ERK5 signal duration, and the relative importance of ERK5 signal length in dictating cellular responses.

5.6.1.1 MEF2C is not required for ERK5-mediated HDMEC survival

MEF2C represents the best-characterised substrate of ERK5 (Kato *et al.*, 1997; Yang *et al.*, 1998a), and has been implicated as a downstream effector of BDNF-induced ERK5-regulated survival of developing neurones (Liu *et al.*, 2003b) and for ERK5-mediated survival of PC12 cells following oxidative insults (Suzaki *et al.*, 2002). In addition, it has been suggested that serum-induced survival of mouse endothelial cells is arbitrated by an Erk5-Mef2c-dependent pathway (Hayashi *et al.*, 2004), it was therefore of particular interest to determine whether MEF2C acted downstream of ERK5 to facilitate VEGF-stimulated survival. VEGF stimulation was recently reported to induce expression of *MEF2C* in human retinal endothelial cells (HRECs) and to enhance MEF2C

transcriptional activity (Maiti *et al.*, 2008). Results presented in this chapter reveal that VEGF-mediated up-regulation of *MEF2C* expression was most pronounced under conditions of tubular morphogenesis (**Fig. 5.2 B**), possibly indicating a specific requirement for MEF2C in this response. However, whilst siRNA-mediated down-regulation of MEF2C expression attenuated VEGF-induced tubular morphogenesis (**Fig. 4.11**), this effect was clearly different from the observed effects of siRNA-induced silencing of ERK5 expression upon VEGF-tube formation (**Fig. 4.11**), thus suggesting that MEF2C is not the primary effector mediating VEGF-stimulated pro-survival effects of ERK5 in HDMECs.

Ablation of either *Mef2c* (Lin *et al.*, 1997; Lin *et al.*, 1998; Bi *et al.*, 1999b), *Erk5* (Regan *et al.*, 2002; Sohn *et al.*, 2002; Yan *et al.*, 2003) or *p38α MAPK* (Mudgett *et al.*, 2000; Adams *et al.*, 2000) in mice, results in embryonic lethality at around the same point during in embryogenesis (E9.5-E11.5). It has been the subject of some debate whether *Mef2c*-null mice most closely resemble the phenotype of *Erk5*-null mice or that of *p38α MAPK*-null mice (Regan *et al.*, 2002; Sohn *et al.*, 2002; Yan *et al.*, 2003; Hayashi & Lee, 2004). Phosphorylation of MEF2C on Ser^{387} either by ERK5, or by p38 MAPK, is required for full MEF2C transactivational activity (Kato *et al.*, 1997; Han *et al.*, 1997). However, VEGF-induced MEF2C transcriptional activity in HRECs was recently shown to be independent of ERK5, but dependent upon p38 MAPK (Maiti *et al.*, 2008). In accordance with the findings of Maiti *et al.*, it was found that siRNA-mediated silencing of ERK5 expression did not prevent VEGF-induced phosphorylation of MEF2C on Ser^{387} (**Fig. 5.5**), whereas pharmacologic inhibition of p38 MAPK activity abolished VEGF-induced phosphorylation of MEF2C at this residue (**Fig. 5.6**). In addition, in agreement with the findings of Yang *et al.*, pharmacological inhibition of VEGF-induced p38 MAPK activation did not inhibit VEGF-induced tubular morphogenesis of HDMECs (Yang et al., 2004; *Appendix 3*). It can therefore be concluded that VEGF-induced phosphorylation of MEF2C on Ser^{387} is not likely to be required for VEGF-induced tubular morphogenesis. It is noteworthy that endothelial cells lacking *Erk5* could only be partially rescued from apoptosis following expression of a constitutively active form of *Mef2c* (Hayashi *et al.*, 2004; Olson, 2004), implying that Mef2c is only partly

responsible for conferring endothelial cell survival, and that additional downstream effectors are required for this response (Hayashi *et al.*, 2004; Olson, 2004). It should also be noted that the reported pro-survival effects of over-expressing constitutive active Mef2c in endothelial cells (Hayashi *et al.*, 2004), does not necessarily reflect a requirement for Erk5 in this survival pathway.

Ablation of the *Erk5* gene in mice has been reported to have no effect upon expression of *Mef2c* (Regan *et al.*, 2002). Intriguingly however, siRNA-mediated down-regulation of ERK5 expression led to a robust up-regulation of *MEF2C* expression on a collagen matrix (**Fig. 5.9**). A possible interpretation of this observation is that MEF2C is partly required for endothelial cell survival (**Fig. 4.11;** Hayashi *et al.,* 2004), and that *MEF2C* may be up-regulated in an attempt by the cell to protect HDMECs undergoing apoptosis to compensate for loss of the protective effects of ERK5 in these cells. The identity of the kinase regulating MEF2C phosphorylation in this hypothesised process remains to be determined. In murine P19 cells, under conditions differentiation, *Mef2c* has been shown to be activated to protect these cells from apoptosis via a p38 MAPK-dependent mechanism (Okamoto *et al.*, 2000). However, based on the fact that inhibition of p38 MAPK did not prevent VEGF-induced tube formation in HDMECs (*Appendix 3*), it seems unlikely that p38 MAPK mediates a similar process in these cells. MEF2C may be phosphorylated by other kinases such as PKA (Wang *et al.*, 2005b) or $p90^{RSK}$ (Wang *et al.*, 2007). Overall, it may be concluded that ERK5 does not regulate VEGF-induced phosphorylation of MEF2C on Ser^{387} in HDMECs and whatever role MEF2C may have in mediating the survival of HDMECs in response to VEGF stimulation, this is not likely to be regulated by ERK5.

5.6.1.2 ERK5 differentially regulates VEGF-induced activation of AKT on collagen and gelatin matrices in HDMECs

Hayashi *et al.* speculated that the ERK5 signalling axis may represent a signalling pathway required for endothelial cell survival, which was distinct from, and additional to, the canonical PI3K/AKT survival pathway (Hayashi *et al.*, 2004); however, these researchers failed to examine the effects of *Erk5* deletion upon activation of the

PI3K/AKT pathway in murine endothelial cells. It has been subsequently reported that deletion of *Erk5* in mice prevents sorbitol-induced activation of Akt in mouse embryonic fibroblasts (Wang *et al.*, 2006) and NGF-induced Akt activity in murine sympathetic neurons (Finegan *et al.*, 2009), to confer survival of these two cell types. Results presented in this chapter now reveal for the first time that ERK5 also regulates AKT activation in response to VEGF stimulation in HDMECs (**Fig. 5.3**), thus showing that ERK5 forms part of the AKT signalling pathway in these cells. In accordance with this finding, Lennartsson *et al.* recently reported that ERK5 regulates PDGF-induced activation of AKT and subsequent suppression of caspase-3 cleavage in PAE cells (Lennartsson *et al.*, 2010), which suggests that ERK5 may mediate endothelial cell survival in response to diverse pro-survival stimuli by regulating AKT activation.

The canonical pathway leading to AKT activation by the prototypic AKT activator IGF-1 (insulin-like growth factor 1), requires the binding of AKT, via its PH domain, to PIP_3 allowing phosphorylation of AKT on Thr^{308} in the activation loop by PDK1 as well as phosphorylation on Ser^{473} in the carboxy-terminus (Alessi et al., 1996; Stokoe et al., 1997). The mTORC2 protein complex, is able to phosphorylate AKT on Ser^{473} (Sarbassov *et al.*, 2005; Jacinto *et al.*, 2006), whereas PDK1 phosphorylates AKT on Thr^{308} (Alessi et al., 1996). VEGF-stimulated activation of AKT has been shown to be regulated by PI3K, to confer endothelial cell survival (Gerber *et al.*, 1998b; Thakker *et al.*, 1999). The ability of both wortmannin and chronic exposure to rapamycin (Sarbassov et al., 2006) to inhibit VEGF-stimulated phosphorylation of AKT, confirmed that the classical PI3K→PDK1 and mTORC2 pathways are able to regulate AKT phosphorylation in these cells (**Fig. 3.14**). The importance of AKT in mediating VEGF-stimulated survival during HDMEC differentiation in a 3-D collagen gel was confirmed by the observation that treatment with wortmannin prevented VEGF-stimulated HDMEC tubular morphogenesis (*Appendix 3*). Interestingly however, VEGF-induced activation of ERK5 in HDMECs was not inhibited by either the PI3K inhibitor wortmannin, or the mTOR inhibitor rapamycin (**Fig. 3.14**), suggesting that ERK5 is not downstream of PI3K, PDK1 or mTORC2 in these cells.

Characterisation of the precise mechanism to account for substrate-dependent ERK5-regulated AKT activation was beyond the scope of this study. However, it is possible that ERK5 regulates matrix-dependent VEGF-induced AKT activation via a distinct kinase upstream of PI3K and AKT. Several proteins including FAK (Mitra *et al.*, 2005), integrin-linked kinase (ILK) (Persad *et al.*, 2001) and Shc (Berra *et al.*, 2000) have been shown to associate with integrin cytoplasmic tails to mediate "outside in" signals from integrins to activate AKT and mediate cell survival (Reddig & Juliano, 2005).

Xia *et al.* provided evidence of direct relevance to this study, showing that survival of fibroblasts plated on collagen I gels was dependent on a β_1 integrin→FAK→PI3K→AKT signalling pathway (Xia *et al.*, 2004), which raises the question: could ERK5 form part of such a pathway? ERK5 has been reported to mediate phosphorylation of FAK on Tyr^{576}/Tyr^{577} in hepatic stellate cells (Rovida *et al.*, 2008a), Ser^{910} in breast cancer cells (Villa-Moruzzi, 2007) and on Tyr^{397}, Tyr^{861}, Ser^{910} and Tyr^{925} in HUVECs (Spiering *et al.*, 2009), revealing that ERK5 can act as an upstream regulator of FAK phosphorylation on these residues in certain cells. Conversely, expression of a dominant-negative version of FAK, incapable of undergoing phosphorylation on Tyr^{397}, was found to block ERK5 activation in MDA-MB-231 breast cancer cells and PC3 prostate cancer cells, suggesting that ERK5 may also contribute to intracellular signalling downstream of FAK (Sawhney *et al.*, 2009). Thus, the relationship between FAK and ERK5 is currently unclear, and may be cell type-dependent.

Interestingly, ablation of *Fak* in mouse embryos has been shown to result in embryonic lethality at E10.5-E11.5 due to endothelial cell apoptosis and loss of vascular integrity (Braren *et al.*, 2006). The similarity in the phenotype of $Fak^{-/-}$ mice and that of $Erk5^{-/-}$ mice, raises the question: do ERK5 and FAK form part of a functional pathway required for vascular development? SiRNA-induced down-regulation of ERK5 expression did not have an effect upon VEGF-stimulated FAK phosphorylation on Tyr^{576} in HDMECs plated on either collagen or gelatin matrices (data not shown). However, FAK activity is regulated by phosphorylation on multiple residues (Parsons, 2003). Additional studies are therefore warranted to evaluate the potential contribution of ERK5 as an upstream regulator or downstream target of FAK, in the mediation of potential matrix-dependent VEGF-stimulated FAK-AKT signalling in endothelial cells.

A further candidate protein kinase that may mediate VEGF-stimulated signals to AKT from ERK5 is integrin-linked kinase (ILK) (Persad et al., 2001; Troussard et al., 2003), which has been reported to regulate AKT Ser^{473} phosphorylation in fibroblasts on collagen matrices via a pathway requiring β_1 integrin (Nho et al., 2005). Furthermore, Kanenko and associates demonstrated that inhibition of ILK activity in HUVECs plated on a collagen I gel prevented VEGF-induced tubular morphogenesis by inhibition of AKT survival and subsequent activation of the executioner caspases-3/7 (Kaneko *et al.*, 2004). In addition, ILK has been shown to be required for endothelial cell survival and vascular development *in vivo* (Friedrich *et al.*, 2004). It was recently shown that ERK5 interacts with β_1 integrin and other integrins in breast cancer cells (Sawhney et al., 2009); therefore, it may be possible that ERK5 may bind to β_1 integrin to integrate signals derived from both the cell matrix and from VEGFR-2 to regulate AKT phosphorylation to facilitate endothelial cell survival under conditions of tubular morphogenesis (**Fig. 5.13**). However, whilst cross-talk between VEGFR-2 and various integrins has been reported (Senger *et al.*, 1997; Soldi *et al.*, 1999; Somanath *et al.*, 2009), little is known of the molecular mechanisms mediating this relationship (Ramjaun & Hodivala-Dilke, 2009); it therefore remains an intriguing possibility, meriting further study, that ERK5 has a role in mediating these signals.

5.6.1.3 ERK5 regulates VEGF-stimulated BAD phosphorylation

Phosphorylation of the pro-apoptotic protein BAD in response to various pro-survival factors has been shown mediate endothelial cell survival (Hermann *et al.*, 2000; Nofer *et al.*, 2001; Ohi *et al.*, 2006; Grethe *et al.*, 2006). When phosphorylated, BAD is sequestered in the cytosol by binding to 14-3-3, thus preventing BAD from translocating to the mitochondria where it heterodimerises with BCL2 and BCL-xL to induce apoptosis (Yang et al., 1995; Zha et al., 1996; Downward, 1999). PKA and $p90^{RSK}$ have been shown to mediate phosphorylation of BAD on Ser^{112} (Bonni *et al.*, 1999; Tan *et al.*, 1999; Harada *et al.*, 1999), whereas BAD phosphorylation on Ser^{136} is well established to be mediated by AKT (Datta *et al.*, 1997).

ERK5 had been previously implicated as an upstream regulator of BAD phosphorylation to facilitate the survival of bovine endothelial cells in response to laminar shear-stress (Pi *et al.,* 2004); surprisingly however, these authors suggested that ERK5 regulated phosphorylation of BAD independently of PKA, $p90^{RSK}$ or Akt (Pi *et al.,* 2004). Data presented in this chapter reveals that siRNA-mediated down-regulation of ERK5 expression prevents VEGF-induced phosphorylation of BAD on Ser^{112} and Ser^{136} in HDMECs plated within a 3-D collagen gel matrix (**Fig. 5.3**), an effect that also coincided with a decrease in VEGF-induced phosphorylation of AKT and $p90^{RSK}$ on this matrix (**Fig. 5.3**). In rat neuronal cells, Erk5 has been shown to mediate NGF stimulated Akt phosphorylation (Finegan *et al.*, 2009). Furthermore, ERK5 is known to directly interact with and phosphorylate $p90^{RSK}$ (Ranganathan *et al.*, 2006). The susceptibility of HDMECs to undergo apoptosis in the absence of ERK5 when plated on a collagen matrix (**Fig. 4.12 - Fig. 4.15**) is reflected in the more profound effect of the loss of ERK5 upon VEGF-stimulated activation of $p90^{RSK}$ and AKT on this matrix. Together, these observations suggest a model whereby ERK5 regulates VEGF-induced endothelial cell survival under conditions of tubular morphogenesis within a collagen gel, in part by acting as an upstream regulator of VEGF-stimulated activation of $p90^{RSK}$ and AKT. Based upon the results presented in this chapter, the hypothesised signal transduction pathways operating downstream of ERK5 activity in HDMECs to mediate VEGF/VEGFR-2-stimulated HDMEC survival within a collagen matrix are summarised in **Fig. 5.13**.

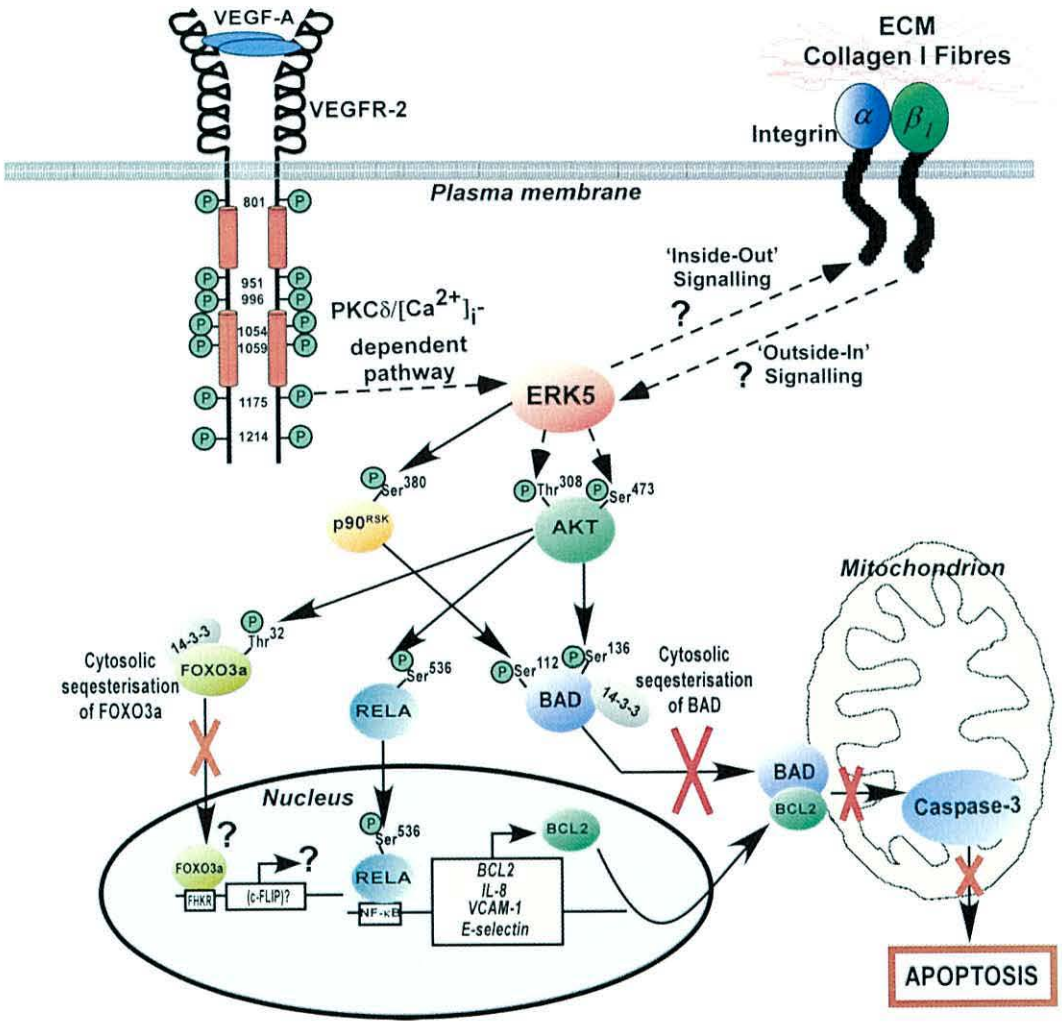

Figure 5.13 Schematic illustration of the hypothesised intracellular signalling pathway mediating VEGF/VEGFR-2-stimulated ERK5-regulated survival of endothelial cells within a 3-D collagen matrix. VEGF stimulates VEGFR-2 trans-autophosphorylation leading to activation of the ERK5 signalling axis by a pathway requiring VEGFR-2 Tyr^{1175} phosphorylation, PKCδ and $[Ca^{2+}]_i$ (*Chapter 3*). VEGF-stimulated AKT activation is mediated by ERK5 by an unknown mechanism that does not involve PI3K, PDK1 or mTORC2 (*Chapter 3*), but which is likely to involve cross-talk with the collagen-binding β_1 integrins. Activation of AKT leads to phosphorylation of the pro-apoptotic protein BAD on Ser^{136} (Datta *et al.*, 1997), allowing binding of 14-3-3, and cytosolic sequesterisation of BAD, preventing BAD migration to the mitochondria where it interacts with the anti-apoptotic protein BCL2, leading to mitochondrial permeabilisation and activation of the executioner caspase-3 (Zha *et al.*, 1996). Phosphorylation of the transcription factor FOXO3a by AKT (Brunet *et al.*, 1999a) allows binding of 14-3-3 and sequesterisation of FOXO3a in the cytosol, preventing its migration to the nucleus where it regulates transcription of various genes, including *cFLIP* (Skurk *et al.*, 2004), it remains to be seen whether ERK5 plays a functional role in regulating FOXO3a-mediated gene transcription. ERK5 regulates VEGF-stimulated phosphorylation of $p90^{RSK}$ in HDMECs, possibly by direct interaction (Ranganathan *et al.*, 2006). Phosphorylation of BAD on Ser^{112} is regulated by $p90^{RSK}$ (Bonni *et al.*, 1999; Tan *et al.*, 1999). ERK5 is also required for VEGF-mediated expression of the anti-apoptotic protein *BCL2*, likely via regulation of RELA (Grosjean *et al.*, 2006). RELA induces expression of several other genes in response to VEGF stimulation including *IL-8* (Lee *et al.*, 2002), *VCAM-1* and *E-selectin* (Kim *et al.*, 2001). Arrows with solid lines represent probable signal transduction pathways based on experimental evidence and pathways reported in the literature. Arrows with dashed lines represent possible alternate signal transduction pathways based on literature searches. *AKT/PKB, protein kinase B; BAD, BCL2-associated antagonist of cell death; BCL2, B cell lymphoma 2; c-FLIP, cellular Fas-associated death domain-like interleukin 1β-converting enzyme (FLICE)-like inhibitory protein; CRE, Ca^{2+}/cAMP response element; CXCR2, cysteine-X-cysteine motif receptor-2; ERK, extracellular signal-regulated kinase; ECM, extracellular matrix; FOXO3a, forkhead box O3a; IL-8, interleukin 8; MEK, MAPK/ERK kinase; mTORC2, mammalian target of rapamycin complex 2; NFκB, nuclear factor κB; $p90^{RSK}$, p90 ribosomal S6 kinase; PDK1, 3'-phosphoinositide-dependent kinase-1; PI3K, phosphatidylinositol 3-kinase; RELA, p65 NFκB; VCAM-1, vascular cell adhesion molecule-1; VEGF, vascular endothelial growth factor; VEGFR-2, VEGF receptor-2.*

5.6.1.4 ERK5 regulates VEGF-induced activation of FOXO3a

It is well established that phosphorylation of the FOXO3a transcription factor is mediated by AKT, to confer cell survival (Brunet *et al.*, 1999a). Given the role of FOXO3a in the regulation of cell survival (Birkenkamp & Coffer, 2003), combined with the fact that FOXO3a activity has been shown be regulated by ERK5 to confer the survival of murine fibroblasts in response to osmotic stress (Wang *et al.*, 2006), it was of direct relevance to the current study to establish whether down-regulation of ERK5 expression would affect VEGF-induced FOXO3a activity. Indeed, it was found that siRNA-mediated silencing of ERK5 expression prevented VEGF-induced activation of FOXO3a on a collagen matrix, and attenuated FOXO3a phosphorylation on a gelatin matrix. These findings provide further support to the notion that ERK5 is an upstream regulator of AKT activity in these cells.

ERK5 protects mouse embryonic fibroblasts against apoptosis caused by hyperosmolar stress by preventing FOXO3a-mediated expression of the death-promoting ligand FasL, leading to the induction of cell death via the extrinsic apoptosis pathway (Wang *et al.*, 2006). However, whilst FasL expression has been reported in HUVECs (Chen *et al.*, 2003), its expression was not detectable in HDMECs under any conditions tested in HDMECs (data not shown). However, FOXO3a is known to regulate expression of other genes encoding proteins that are involved in the regulation of apoptosis, including *BIM* (Stahl *et al.*, 2002) and *FLIP* (FLICE-like inhibitory protein) (Panka *et al.*, 2001). FLIP inhibits caspase-8 activity in endothelial cells to promote endothelial cell survival (Aoudjit & Vuori, 2001; Skurk *et al.*, 2004). Furthermore, over-expression of a non-phosphorylatable, constitutively active form of FOXO3a has been recently reported to induce the expression of MMPs and suppress cell-cell and cell-matrix interactions in HUVECs, to induce endothelial cell apoptosis (Lee *et al.*, 2008). By regulating FOXO3a activity in HDMECs, it remains possible that ERK5 may be involved in one, or more, of these pathways. Future studies may therefore aim to address this possibility.

5.6.1.5 ERK5 regulates VEGF-stimulated RELA activation

Activation of RELA occurs in an AKT-dependent manner to confer the survival of several cell types (Romashkova & Makarov, 1999; Ozes *et al.*, 1999; Hayden & Ghosh, 2008). Importantly, RELA has been shown to be a critical mediator of VEGF-stimulated pro-survival signals in endothelial cells. Results presented in this chapter, revealing that VEGF-stimulated phos-RELA Ser^{536} levels are diminished following siRNA-mediated down-regulation of ERK5 expression in HDMECs indicates that VEGF-stimulated activation of RELA is ERK5-dependent (**Fig. 5.3, *p-RELA***). This finding is analogous to the reported role of ERK5 as an upstream arbitrator of IGF-1-induced activation of RELA to facilitate survival of Jurkat T-cells (Garaude *et al.*, 2006). Importantly, VEGF-induced RELA activation was most severely diminished on a collagen matrix (**Fig. 5.3, *p-RELA***), consistent with the observed effects of siRNA-mediated down-regulation of ERK5 expression upon AKT activity on this matrix (**Fig. 5.3, *p-AKT***). Treatment with the PI3K inhibitor wortmannin, abolished VEGF-stimulated RELA activity (data not shown), thus re-affirming the importance of AKT as an upstream mediator of VEGF-stimulated RELA activity. Taken together, these data suggest the existence of a VEGF-stimulated ERK5→AKT→RELA signalling pathway in HDMECs (**Fig. 5.13**).

5.6.2 ERK5-mediated regulation of VEGF-induced gene expression

5.6.2.1 ERK5-regulates VEGF-induced expression of *BCL2*

VEGF failed to induce a significant (2-fold increase or 0.5-fold decrease) change in expression of the anti-apoptotic genes *Survivin, A1* or *XIAP* in HDMECs plated on either a gelatin matrix, or within a 3-D collagen matrix, at times up to 24 h (data not shown). This observation was not entirely unexpected, as some studies have shown that these genes require prolonged exposure to VEGF to induce their expression (Gerber *et al.*, 1998a; Tran *et al.*, 1999; Grosjean *et al.*, 2006), and that even then, only subtle changes in gene expression were observed (Tran *et al.*, 1999; Grosjean *et al.*, 2006). By contrast, analysis of *BCL2* mRNA expression levels revealed that *BCL2* is transcriptionally up-regulated in response to VEGF stimulation in HDMECs plated on either collagen or gelatin matrices (**Fig. 5.7 B**). Strikingly, treatment with ERK5-specific siRNA abrogated VEGF-induced expression of *BCL2* in HDMECs plated within a collagen matrix, and also lowered the basal level of *BCL2* expression (**Fig. 5.7 B, collagen**),

whereas VEGF-stimulated expression of *BCL2* on a gelatin matrix was not as severely affected (**Fig. 5.7 B, gelatin**), thus revealing for the first time that ERK5 is an important regulator of VEGF-stimulated *BCL2* expression in HDMECs under conditions of tubular morphogenesis.

BCL2 protein family members are well-documented regulators of cell survival (Cory *et al.*, 2003; Adams & Cory, 2007). In HDMECs, BCL2 is constitutively expressed (Pammer *et al.*, 1999) but its expression is up-regulated following VEGF-stimulation (Gerber *et al.*, 1998a; Nor *et al.*, 1999; Cai *et al.*, 2003). VEGF has been shown to mediate endothelial cell survival both *in vitro* and *in vivo*, partly via AKT-dependent up-regulation of BCL2 expression (Gerber *et al.*, 1998a; Cai *et al.*, 2003; Kumar *et al.*, 2004; Karl *et al.*, 2005; Grosjean *et al.*, 2006). Grosjean and co-workers demonstrated that VEGF-induced *BCL2* up-regulation occurs via a RELA-dependent mechanism to facilitate endothelial cell survival (Grosjean *et al.*, 2006). Combined with the findings discussed above (*section 5.6.1.5*), it is inferred that ERK5 mediates VEGF-stimulated *BCL2* expression via an ERK5→AKT→RELA pathway in HDMECs (**Fig. 5.13**).

Pi *et al.* reported that expression of CA-MEK5(D) alone did not induce expression of *BCL2* in BLMECs (Pi *et al.*, 2004). However, in the current study, it was found that constitutive activation of ERK5 by co-expression of CA-MEK5(D) and ERK5(wt) was sufficient to up-regulate *BCL2* mRNA expression in HDMECs (**Fig. 5.12**), suggesting that co-transfection of these constructs may be critical for this response. Interestingly, Nor and co-workers had previously reported that stable over-expression of BCL2 in HDMECs did not affect HDMEC proliferation, but enhanced HDMEC survival and induced sustained tubular morphogenesis in the absence of exogenous VEGF both *in vitro* and *in vivo* (Nor *et al.*, 1999; Karl *et al.*, 2005). In light of these findings, given that co-transfection of HDMECs with CA-MEK5(D) and ERK5(wt) also induced tubular morphogenesis of HDMECs in the absence of VEGF (**Fig. 4.19**), it is interesting to speculate that the ability of HDMECs, in which ERK5 is constitutively activated, to form tubes in the absence of VEGF may be attributable, at least in part, to the increased

expression of *BCL2* observed under these conditions, conferring HDMEC survival (**Fig. 5.12**).

5.6.2.2 ERK5-mediates VEGF-induced expression of NF-κB-dependent genes *VCAM-1, E-selectin* and *IL-8*

It had been previously reported that TNFα-induced expression of *VCAM-1* and *E-selectin* in endothelial cells is negatively-regulated by ERK5 activity under conditions of laminar shear-stress (Akaike *et al.*, 2004; Li *et al.*, 2008). Interestingly however, data presented in this chapter show that under conditions of VEGF-stimulation, ERK5 can positively-regulate expression of these adhesion molecules in HDMECs (**Fig. 5.10**). A possible explanation for the apparent discrepancies between these results could be that ERK5 activity in endothelial cells may be differentially-regulated under conditions of laminar shear-stress and under static conditions. For example, under normal physiological conditions, ERK5 may negatively-regulate TNF-α and VEGF-stimulated expression of adhesion molecules such as *VCAM-1* and *E-selectin* in the endothelium, whereas under conditions of reduced flow, this effect may be lost. As impaired blood flow is a characteristic feature of the tumour vasculature (Raghunand *et al.*, 2003), it is proposed that under these conditions, ERK5 is likely to be critical in positively-regulating VEGF-induced expression of *VCAM-1* and *E-selectin*, molecules that are known play important roles in angiogenesis (Bischoff *et al.*, 1997; Yasuda *et al.*, 2002).

SiRNA-mediated silencing of ERK5 expression also prevented VEGF-stimulated up-regulation of *IL-8* expression in HDMECs (**Fig. 5.11**), an effect that was most evident in cells plated within a 3-D collagen matrix. IL-8 is a pro-angiogenic chemokine that has been shown to be sufficient to induce endothelial cell sprouting *in vitro* and angiogenesis *in vivo* (Strieter *et al.*, 1992; Strieter *et al.*, 2004). Emerging evidence has shown that IL-8 released from endothelial cells can participate in a paracrine signalling loop with tumour cells, whereby VEGF released from tumour cells induces expression of *IL-8* in endothelial cells, which in turn induces tumour cell invasiveness and survival (Warner *et al.*, 2008; Neiva *et al.*, 2009). It is an intriguing possibility that inhibition of ERK5 activity either by siRNA-mediated therapy or by use of a specific pharmacological inhibitor in endothelial cells *in vivo* may attenuate IL-8 expression and resultant

tumour cell migration and survival. In addition, other recent work has revealed that IL-8 released from endothelial cells can partake in autocrine signalling by binding to its cognate receptor CXC2R (CXC2 receptor) to induce VEGF expression in endothelial cells, resulting in VEGFR-2 activation (Martin *et al.*, 2009). Based on these recent reports, and data presented in this chapter, it is hypothesised that lowering IL-8 expression by inhibition of ERK5 activation *in vivo* may also prevent autocrine activation of VEGFR-2 in this manner. Future studies should firstly aim to establish whether ERK5 regulates IL-8 expression at the protein level in endothelial cells, prior to further investigating this premise.

VEGF-induced expression of *VCAM-1* and *E-selectin* and *IL-8* in endothelial cells has been shown to be regulated via a RELA-dependent mechanism (Kim *et al.*, 2001; Lee *et al.*, 2002). Whilst the data presented in this chapter is not sufficient to delineate the exact means by which ERK5 regulates expression of these genes in response to VEGF-stimulation, it is suggested that ERK5 may regulate this process via the aforementioned VEGF-stimulated ERK5→AKT→RELA signalling pathway (**Fig. 5.13**).

5.6.3 Concluding remarks

VEGF stimulates the activation of a number of intracellular signalling pathways, which regulate cellular proliferation, migration and survival (Holmes *et al.*, 2007); however, the potential role of ERK5 as a mediator of VEGF-stimulated signalling pathways had not been previously explored. Data presented in this chapter have provided an insight into the mechanisms by which ERK5 regulates VEGF-stimulated survival of HDMECs during tubular morphogenesis within a 3-D collagen matrix, namely by regulating AKT activation and the resultant phosphorylation, and functional inactivation of the pro-apoptotic protein BAD. Furthermore, siRNA-mediated down-regulation of ERK5 expression and constitutive activation of ERK5 revealed that ERK5 regulates the expression of *BCL2* in these cells, likely via regulation of AKT-mediated RELA activation. In conclusion, these results suggest that ERK5 can mediate VEGF-induced HDMEC survival during tubular morphogenesis by regulating several downstream effectors, revealing a possible mechanism to account for the adverse effects on angiogenesis seen in *Erk5*-deficient mice.

CHAPTER SIX

Development and application of an *in vitro* endothelial cell/fibroblast co-culture assay to assess the effects of siRNA-induced down-regulation of ERK5 expression on angiogenesis

6.1 Introduction

Endothelial cells represent the principal cells involved in angiogenesis; however, these cells are critically dependent upon heterotypic interaction with mural cells (fibroblasts, smooth muscle cells and pericytes) for their continued survival, and for angiogenesis to occur *in vivo* (Ingber & Folkman, 1989; Hellstrom *et al.*, 2001; Jain, 2003; Armulik *et al.*, 2005; Hughes, 2008). Montesano and associates provided the first evidence demonstrating that factors released from Swiss 3T3 fibroblasts could induce angiogenesis in endothelial cells in a paracrine fashion (Montesano *et al.*, 1993). Since then, several reports have shown that endothelial cells, when co-cultured with fibroblasts, undergo migration, proliferation and remodelling to form lumen-containing capillary-like structures in response to pro-angiogenic stimuli (Nehls *et al.*, 1994; Bishop *et al.*, 1999; Donovan *et al.*, 2001; Saito *et al.*, 2003; Kunz-Schughart *et al.*, 2006; Sorrell *et al.*, 2007; Sorrell *et al.*, 2008; Chen *et al.*, 2008; Lilly & Kennard, 2009). Fibroblasts secrete ECM proteins such as collagens and laminins, which provide a substratum to facilitate endothelial migration and act as a scaffold to allow vessel remodelling (Bishop *et al.*, 1999; Berthod *et al.*, 2006; Staton *et al.*, 2009). Furthermore, fibroblasts produce growth factors such as VEGF, to induce endothelial cell proliferation and migration both *in vitro* and *in vivo*. These pro-angiogenic growth factors also bind to a fibroblast-derived ECM to facilitate 3-D organisation, tubulogenesis and long-term survival of endothelial cells (Gabbiani, 2003; Hartlapp *et al.*, 2001; Lerman *et al.*, 2003; Sorrell *et al.*, 2007). Thus, endothelial cell/fibroblast co-cultures provide an accurate *in vitro* representation of *in vivo* angiogenesis (Donovan *et al.*, 2001).

Whilst the 3-D collagen gel *in vitro* angiogenesis assay described in chapter 4 of this study accurately represents the early stages of *in vivo* sprouting angiogenesis (Montesano *et al.*, 1983; Koike *et al.*, 2004), a limitation of using this model is the short–term nature of tubule formation, with tubular structures typically regressing within 48 h of formation (Ilan *et al.*, 1998; Matsumoto *et al.*, 2002; Bohman *et al.*, 2005). This caveat confines the usefulness of the 3-D collagen gel assay as a tool in cases where it is desirable to monitor the process of blood vessel regression, as

opposed to the inhibition of vessel formation. In contrast, endothelial cells in co-culture with fibroblasts undergo differentiation over a period of several days to form well-established capillary structures that are highly representative of capillary formation *in vivo*, and that have been reported to remain stable for up to one month after formation (Montesano *et al.,* 1993; Black *et al.,* 1998; Bishop *et al.,* 1999; Donovan *et al.,* 2001; Saito *et al.,* 2003; Berthod *et al.,* 2006). The benefits of endothelial cell/fibroblast co-culture assays has prompted their use to model the effects of potential angiogenesis inhibitors on established vessels *in vitro* (Donovan *et al.*, 2001; Beilmann *et al.*, 2004; Friis *et al.*, 2003; Friis *et al.*, 2006). A further asset of endothelial cell/fibroblast co-culture assays is the fact that they are more easily quantified by automated computer analysis than 3-D gel-based assays (Donovan *et al.,* 2001; Staton, 2009).

Endothelial cells are heterogeneous, and as such, it has been stated that the appropriateness of a given endothelial cell type for use in *in vitro* angiogenesis assays should be carefully considered (Goodwin, 2007). Most studies in which endothelial cells and fibroblasts have been co-cultured to study angiogenesis have been performed using HUVECs (Bishop *et al.,* 1999; Friis *et al.,* 2003; Beilmann *et al.,* 2004; Friis *et al.,* 2006; Sorell *et al.,* 2008). However, despite their popularity as a model cell line, HUVECs rarely, if ever, undergo angiogenesis *in vivo* (Cines et al., 1998; Conway and Carmeliet, 2004); therefore, their use to represent angiogenesis *in vitro* is inherently inappropriate. Co-culture of HDMEC/NHDF in a 3-D collagen assay has recently been described (Sorrell *et al.*, 2007; Neeley *et al.*, 2010). However, direct co-culture of HDMECs with NHDFs in a 2-D assay to form stable capillary networks without the requirement for the addition of exogenous factors has hitherto been unsuccessful as HDMECs were found to undergo apoptosis, resulting in short tubes that fail to interconnect to form a stable network (Donovan *et al.*, 2001; Sorrell *et al.*, 2007).

It had been previously shown that ERK5 siRNA treatment prevented VEGF-induced HDMEC capillary sprouting in a 3-D collagen assay (*chapter 4*), raising the possibility

that ERK5 may also be required for growth factor-mediated angiogenesis in endothelial cell/fibroblast co-cultures. This chapter details experiments conducted in an effort to further investigate the role of ERK5 in growth factor-induced capillary network formation of human endothelial cells, by use of an *in vitro* model that is more representative of *in vivo* angiogenesis (Donavan *et al.,* 2001; Staton *et al.,* 2009). Specifically, the effect of ERK5 siRNA treatment upon nascent vessel formation and on established capillary networks was determined in a HDMEC/NHDF co-culture assay. In addition, the effects of the VEGFR-2 inhibitor, ZM323881 alone, and in combination with ERK5 siRNA on VEGF- and FGF-2 induced angiogenesis in a HDMEC/NHDF co-culture was assessed, to determine if ERK5 may represent a feasible target for siRNA-based therapeutic inhibition of neo-vascularisation.

6.2 Development of a HDMEC/NHDF co-culture *in vitro* angiogenesis assay

6.2.1 Characterisation of HDMEC tubular morphogenesis and optimisation of assay length

Previous experiments in this study aimed at characterising the role of ERK5 in angiogenesis had been done using HDMECs, a model cell line that is representative of angiogenesis *in vivo* (Cines *et al.*, 1998; Conway & Carmeliet, 2004). For the sake of consistency it was pertinent to develop a HDMEC/NHDF co-culture assay, and to establish a means of quantifying tube formation in this assay.

A protocol for an 18 day HUVEC/NHDF co-culture assay, based upon that described by Bishop *et al.* (1999) had been developed by Clare Barnes (AstraZeneca Ltd.). This assay involved plating NHDFs on day 1, to obtain a confluent monolayer upon which endothelial cells were plated (day 4), followed by feeding of the co-cultures on days 8, 10, 12, 14, 15 and 16 of the assay (**Fig. 6.1 A**). Co-cultures were later fixed, and stained for expression of the endothelial specific marker PECAM-1 (platelet endothelial cell adhesion molecule-1)/CD31 (Baldwin *et al.,* 1994), and visualised by immunohistochemistry as described by Bishop *et al.* (1999) (*section 2.2.7.2.3*).

The HUVEC/NHDF co-culture protocol was adapted for the co-culture of HDMECs and NHDFs. Preliminary experiments revealed that seeding HDMECs at the same density as HUVECs (3.0×10^4 cells per well) gave shortened tubes following VEGF or FGF-2 stimulation in comparison to tubes formed in a HUVEC/NHDF co-culture (data not shown), as previously reported (Donovan *et al.*, 2001). Optimisation of the number of HDMECs seeded on confluent NHDF layers led to the discovery that by increasing the initial number of HDMECs plated on the NHDF monolayer to 4.5×10^4 per well, longer tubes were obtained, which anastomosed to form a highly interconnected network in response to VEGF or FGF-2 stimulation over 18 days (**Fig. 6.1 B, *Day 18, VEGF/FGF-2***).

A comparison of VEGF- and FGF-2-stimulated capillary-like structures in HDMEC/NHDF co-cultures revealed that VEGF-stimulated capillary networks were characterised by thinner, shorter tubes, typically measuring <100 µm, with enhanced branching and shorter inter-nodal lengths than those capillary networks observed following FGF-2 stimulation (**Fig. 6.1 B, *Day 18, VEGF/FGF-2***). Given that fully supplemented EBM MV2 endothelial growth medium (full growth medium; F.G.M.) had been described to induce tube formation in a HUVEC/NHDF co-culture assay (Bishop *et al.*, 1999), the ability of this medium to stimulate capillary formation in the HDMEC/NHDF co-culture model was also assessed. It was found that at early time points, EBM MV2 growth medium induced proliferation of HDMECs, characterised by the formation of CD31-positive aggregates (**Fig. 6.1 B, *F.G.M., Days 10-12***). Although some HDMECs showed evidence of tubule formation at later time points, these cells did not fully differentiate to form a substantial network of interconnecting, branching vessels (**Fig. 6.1 B, *F.G.M., Days 14-18***). From these findings, future experiments were conducted using VEGF or FGF-2 to stimulate tube formation.

Despite the benefits of the use of endothelial cell/fibroblast co-culture assay in the assessment of angiogenesis (Donovan *et al.*, 2001; Staton *et al.*, 2009), a major drawback of using this system was that it involved several steps and took 18 days to complete, making it both labour-intensive and time consuming. To investigate the feasibility of shortening the length of the HDMEC/NHDF assay, a time course study was

conducted to analyse VEGF-, FGF-2- or F.G.M.-stimulated angiogenesis over several days. Earlier work had shown that it was necessary to allow NHDFs to proliferate for four days post-plating to reach a 95-100% confluent monolayer prior to seeding HDMECs. In cases where HDMECs were plated on a sub-confluent NHDF monolayer, HDMECs preferentially occupied fibroblast-free areas on the cell culture dish, and did not differentiate to form tubes, but rather underwent proliferation to form islands of cells (data not shown). HDMECs plated on day 4 of the assay were 'fed' on day 8 of the assay with EBM MV2 basal medium containing 1% (v/v) FCS, and supplemented with growth factors as indicated in **Figure 6.1 A**, and were either treated with fresh growth factors on days 10, 12, 14, 15, 16 and 18 or were fixed in ice-cold 70% (v/v) ethanol at these points.

Specific staining of HDMECs with anti-CD31 antibody followed by immunohistochemical analysis (*section 2.2.7.2.3*) revealed that HDMECs differentiated to form capillary-like structures by day 10 (**Fig. 6.1 B, *Day 10***). No substantial change in VEGF-induced HDMEC network capillary formation was apparent following the addition of further VEGF (**Fig. 6.1 B, *VEGF, Days 12-18***). By contrast, FGF-2-induced HDMEC capillary networks became noticeably thicker at day 14, with an increased number of interconnecting vessels becoming apparent thereafter (**Fig. 6.1 B, *FGF-2, Days 14-18***). Co-cultures treated with EBM MV2 basal medium containing 1% (v/v) FCS alone (Basal), displayed little tubule formation, with few interconnecting tubes (**Fig. 6.1 B, *Basal, Day 10-18***). Together, these data showed that VEGF- and FGF-2-stimulation was necessary for HDMEC network formation in the HDMEC/NHDF co-culture model, whereas F.G.M. appeared to induce proliferation of HDMECs. Furthermore, the finding that these factors could stimulate the formation of a stable capillary network by day 10 of the assay suggested that this assay could potentially be shortened to 10 days, compared to the classical 18 day HUVEC/NHDF co-culture assay described by Bishop and colleagues (Bishop *et al.*, 1999).

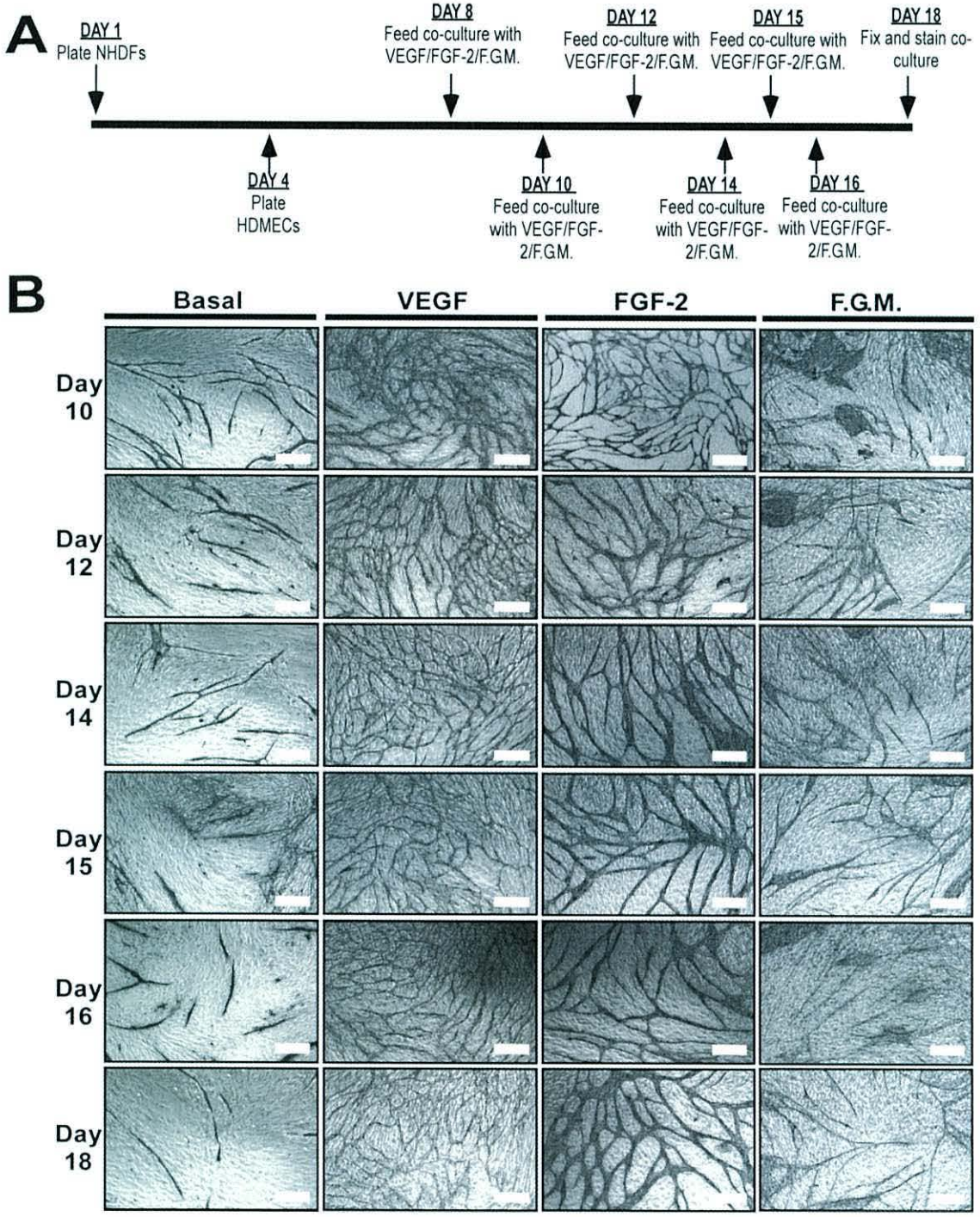

Figure 6.1 Profiling of the effects of VEGF, FGF-2 and F.G.M. upon HDMEC morphology in a HDMEC/NHDF co-culture over time. (**A**) Treatment schedule. NHDFs were seeded in fibroblast growth medium at 1.5 x 10^4 cells per well on gelatin-coated 24-well plates and incubated for 3 days. On day 4, HDMECs were seeded at 4.5 x 10^4 cells per well, on top of the NHDF monolayer in EBM MV2 growth medium. On days 8, 10, 12, 14, 15 and 16 cells were placed in EBM MV2 basal medium containing 1% (v/v) FCS and supplemented, or not with VEGF (50 ng/ml), FGF-2 (50 ng/ml) or were placed in EBM MV2 full growth medium (F.G.M.). On day 18 of the assay, cells were fixed and stained as described (*section 2.2.7.2.3*). (**B**) Representative morphology of HDMECs at different time points in the assay. Following the treatment schedule (A), co-cultures were fixed and stained as described (*section 2.2.7.2.3*), at days 10, 12, 14, 15, 16 and 18 of the assay, to assess capillary network formation in response to VEGF, FGF-2 or F.G.M. over time. X100 magnification. Bars = 100 µm. *FCS, foetal calf serum; FGF-2, fibroblast growth factor-2; F.G.M., full growth medium; HDMEC, human dermal microvascular endothelial cell; NHDF, normal human dermal fibroblast; VEGF, vascular endothelial growth factor.*

6.2.2 Quantification of VEGF- and FGF-2-induced HDMEC tubular morphogenesis in a HDMEC/NHDF co-culture assay

HDMEC differentiation into capillary-like structures could be detected as early as day 10 of the co-culture assay (**Fig. 6.1 B**). However, as FGF-2-stimulated capillary network formation appeared to become more robust following chronic stimulation with FGF-2, compared to those tubes formed following FGF-2 stimulation on day 8 alone (**Fig. 6.1 B, *FGF-2, Days 10-14***), the shortened 10 day assay was modified to incorporate a further feed point at day 6 (**Fig. 6.2 B**). It was found that this modification induced robust FGF-2-stimulated HDMEC capillary formation (**Fig. 6.2 B, *FGF-2, Brightfield***).

It was next asked whether VEGF- and FGF-2-stimulated HDMEC tube formation in the 10 day assay could be accurately quantified. Traditionally, the extent of capillary network formation following endothelial tubular morphogenesis in co-culture assays had been quantified by image analysis programs such as NIH ImageJ, Scion image and Image Pro Plus (Bishop *et al.*, 1999; Donovan *et al.*, 2001; Sorrell *et al.*, 2008). However, these methods require user intervention and are time-consuming to perform. In this study, the usefulness of image analysis software termed 'AngioQuant', which was recently developed for quantification of tubule formation in the commercially available TCS CellWorks AngioKit™ (Buckingham, U.K.) HUVEC/NHDF co-culture assays (Niemisto *et al.*, 2005), was assessed.

HDMEC/NHDF co-cultures treated with VEGF or FGF-2 or left untreated (basal) (**Fig. 6.2 B**), were fixed and stained on day 10 of the assay (*section 2.2.7.2.3*). Segmentation analysis of representative X40 magnification brightfield photomicrographs produced an accurate binary representation of the darker tubular networks, making a clear distinction between the CD31-postitive HDMEC tubules and the unstained NHDF layer (**Fig. 6.2 A, *Segmented***). Thinning of the tubules produced a skeletonised, one-pixel-wide depiction of the tubes as described in HUVEC/NHDF co-cultures (Niemisto *et al.*, 2005; **Fig. 6.2 A, *Skeletonised***). Quantification of the skeletonised image revealed that both VEGF and FGF-2 induced a similar level of tubule formation, which was approximately 3-fold greater than that observed in basal, unstimulated co-cultures

(**Fig. 6.2 C**). Together, these results confirmed that VEGF or FGF-2 stimulated *in vitro* angiogenesis in HDMEC/NHDF co-cultures could be accurately quantified in an automated manner using AngioQuant software.

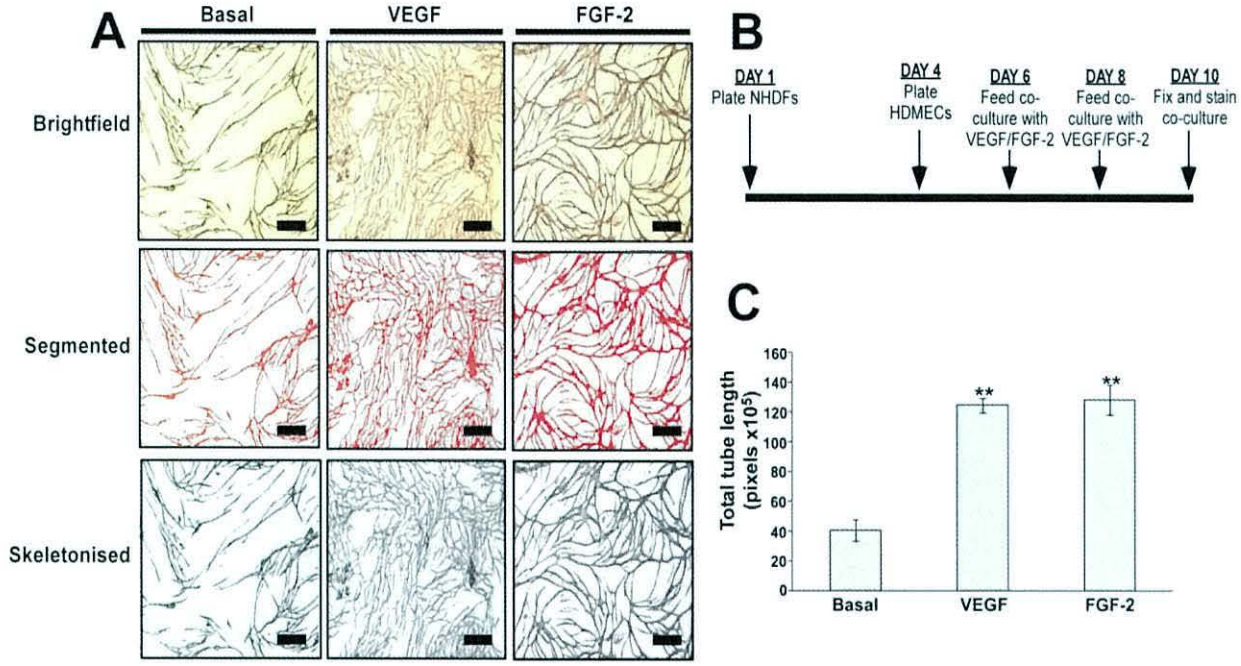

Figure 6.2 HDMEC capillary structure formation in a HDMEC/NHDF co-culture assay can be quantified by AngioQuant software. (**A**) NHDFs were seeded in fibroblast growth medium at 1.5 x 10^4 cells per well on gelatin-coated 24-well plates and incubated for 3 days. On day 4, HDMECs were seeded at 4.5 x 10^4 cells per well, on top of the NHDF monolayer in EBM MV2 growth medium. On days 6 and 8, co-cultures were placed in EBM MV2 basal medium containing 1% (v/v) FCS and supplemented, or not, with VEGF (50 ng/ml) or FGF-2 (50 ng/ml). On day 10 of the assay, co-cultures were fixed and stained as described (*section 2.2.7.2.3*), and total tubule length was quantified by AngioQuant image analysis software as described (*section 2.2.7.2.4*). (**B**) Treatment schedule. (**C**) Data is presented as total tubule length from triplicate wells (n = 3, mean ± SD, $^{**}p<0.01$ compared to basal, Student *t* test). The result shown is representative of three independent experiments. X40 magnification. Bars = 400 µm. *FCS, foetal calf serum; FGF-2, fibroblast growth factor-2; HDMEC, human dermal microvascular endothelial cell; NHDF, normal human dermal fibroblast; VEGF, vascular endothelial growth factor.*

6.3 Assessment of the effects of ERK5 siRNA upon VEGF- and FGF-2-induced angiogenesis in a HDMEC/NHDF co-culture assay

6.3.1 ERK5 siRNA treatment on HDMECs prior to VEGF- or FGF-2-stimulation and following VEGF- and FGF-2-stimulated angiogenesis in co-culture

Data from the 3-D collagen gel assay had suggested that ERK5 was required for tubular morphogenesis at an early time point (**Fig. 4.12 - Fig. 4.15**). It was hypothesised that ERK5 may be required for early neovascularisation in response to VEGF or FGF-2 stimulation in the HDMEC/NHDF co-culture assay. However, it had also been reported that conditional deletion of *Erk5* in adult mice induced lethality due to endothelial

apoptosis leading to cardiovascular degeneration and haemorrhaging (Hayashi *et al.*, 2004), suggesting that Erk5 is also critical for endothelial cell survival in established vessels. To determine the effects of ERK5 siRNA treatment upon VEGF- or FGF-2-stimulated angiogenesis of human microvascular endothelial cells in a co-culture assay, two approaches were taken. Firstly, HDMECs were transfected with ERK5 or N.S. control siRNA on day 3 of the assay prior to plating onto a confluent NHDF monolayer on day 4 as illustrated in **Fig. 6.3 B**. A second approach involved combined transfection of HDMECs and NHDFs on the ninth day of the assay in co-cultures that had already undergone growth factor-stimulated angiogenesis in response to VEGF or FGF-2 stimulation on days 6 and 8 (**Fig. 6.3 B**). Treatment of HDMECs with N.S. siRNA prior to plating on NHDFs had minimal effect upon capillary network formation compared to untransfected cells (**Fig. 6.3 A, *N.S. siRNA, Day 3***). By contrast, ERK5 siRNA treatment of HDMECs on day 3 of the assay had a noticeable effect upon VEGF-stimulated tube formation, with a reduction in interconnections between tubules and less capillary branching apparent (**Fig. 6.3 A, *ERK5 siRNA, Day 3***). Similarly, FGF-2 stimulated angiogenesis was impaired following ERK5 siRNA treatment on day 3 of the assay (**Fig. 6.3 A, *ERK5 siRNA, Day 3***). Quantification of total tube length revealed that ERK5 siRNA treatment reduced both VEGF- and FGF-2-induced tubule formation by 18% (±5.1% SD) and 14% (±2.3% SD) respectively compared to N.S. siRNA-treated cells ($p<0.05$, Student *t* test) (**Fig. 6.3 C**). By contrast, treatment with ERK5 siRNA on day 9 of the co-culture assay had no significant effect upon formation of either VEGF- or FGF-2-stimulated tube formation (**Fig. 6.3, A and C**). Assessment of siRNA transfection efficiency using Alexa Fluor 488®-coupled N.S. siRNA on days 3 and 9 revealed that approximately 95% of HDMECs had been transfected on day 3 (data not shown). Surprisingly, transfection of HDMECs and NHDFs in co-culture on day 9 of the assay was also found to be at least 95% efficient (data not shown). These observations suggested that the differential effects of ERK5 siRNA on VEGF- and FGF-2-stimulated tube formation were not due to differences in transfection efficiency. Together, these data suggested a requirement for ERK5 to enable maximal growth factor-induced neo-vascularisation at an early stage in tubule development, but that ERK5 was not critical for the maintenance of established tubes.

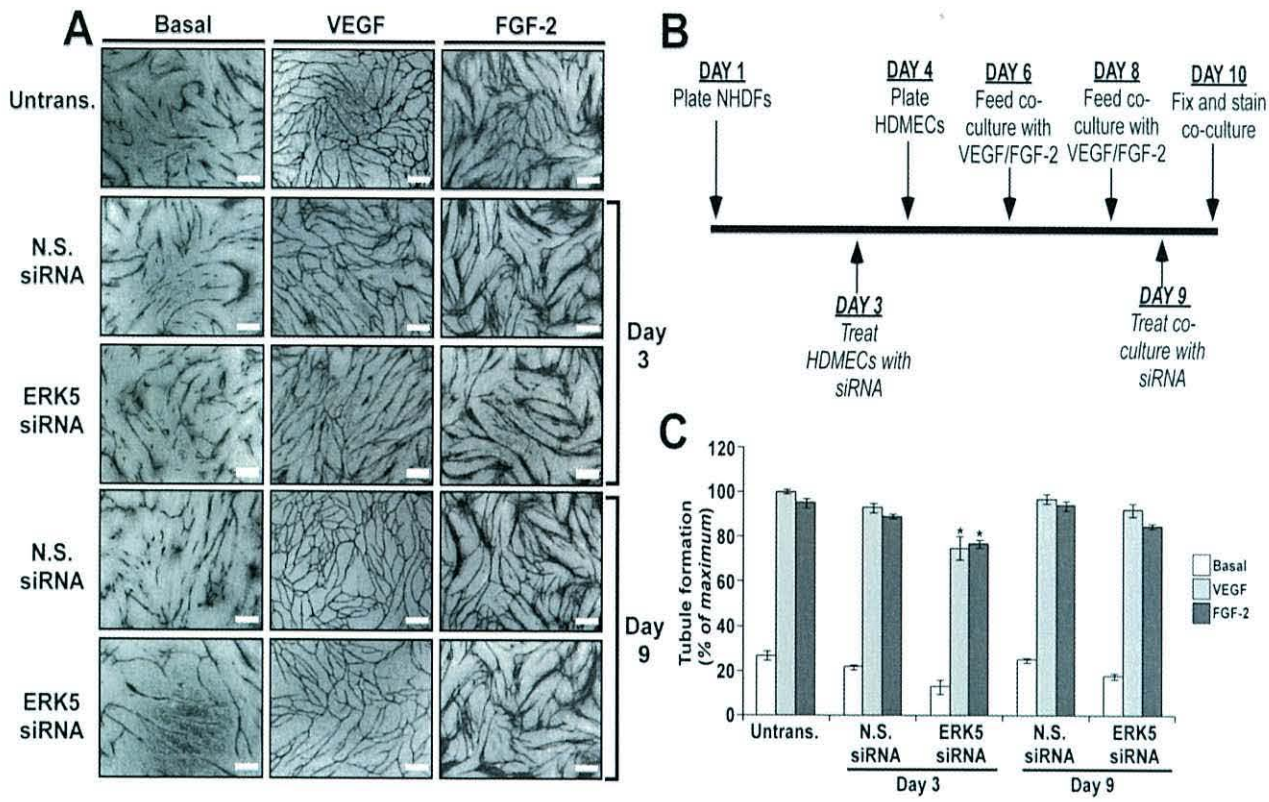

Figure 6.3 Effects of ERK5 siRNA treatment in HDMECs prior to plating on NHDF cells or following 5 days in co-culture upon VEGF and FGF-2 -stimulated angiogenesis in a HDMEC/NHDF co-culture assay. (**A**) NHDFs were seeded in fibroblast growth medium at 1.5 x 10^4 cells per well on gelatin-coated 24-well plates and incubated for 3 days. On day 3, HDMECs were transfected with 5 nM ERK5 siRNA 1 and 5 nM ERK5 siRNA 2, 10 nM non-silencing (N.S.) siRNA, or were left untransfected (Untrans.). On day 4 of the assay, 24 h post-transfection of HDMECs with siRNA, HDMECs were plated at 4.5 x 10^4 cells per well onto a confluent NHDF monolayer, and treated with EBM MV2 basal medium containing 1% (v/v) FCS alone (Basal) or supplemented with VEGF (50 ng/ml), or FGF-2 (50 ng/ml). Previously untransfected co-cultures were transfected with siRNAs on day 9 of the assay (Day 9) as indicated in the treatment schedule (**B**). (**C**) On day 10 of the assay, cells were fixed and stained as described (*section 2.2.7.2.3*), and total tubule length was quantified by AngioQuant image analysis software as described (*section 2.2.7.2.4*). Data is presented as percentage of maximum tube formation from triplicate wells (n = 3, mean ± SD, *p<0.05, Student t test, compared to N.S. control siRNA treated cells). The result shown is representative of three independent experiments. X40 magnification. Bars = 400 µm. *ERK5, extracellular signal-regulated kinase 5; FCS, foetal calf serum; FGF-2, fibroblast growth factor-2; HDMEC, human dermal microvascular endothelial cell; NHDF, normal human dermal fibroblast; N.S., non-silencing; siRNA, short interfering RNA; VEGF, vascular endothelial growth factor.*

6.3.2 Acute ERK5 siRNA treatment attenuates nascent HDMEC capillary morphogenesis, but is insufficient to induce regression of established HDMEC capillary networks

As treatment with ERK5 on day 3 of the HDMEC/NHDF assay was found to impair the formation of VEGF and FGF-2-induced formation of capillary-like structures (**Fig. 6.3**), it was decided to further explore the effects of ERK5 siRNA treatment upon VEGF- and FGF-2-stimulated angiogenesis by transfecting co-cultures with siRNA twice rather than singly. HDMECs were transfected with ERK5 or N.S. siRNA on day 3, prior to plating cells onto a NHDF monolayer on day 4, and transfection of the co-culture with

siRNA on day 5, prior to stimulation of co-cultures with VEGF or FGF-2 on days 6 and 8 (**Fig. 6.4 B**). It was found that ERK5 siRNA treatment on days 3 and 5 significantly attenuated VEGF and FGF-2-stimulated tubule formation compared to the N.S. control siRNA treated cells by 54.4% (±3.5% SD) and 25% (±2.6% SD) respectively ($p<0.01$, Student *t* test). By contrast, the effects of ERK5 siRNA on established VEGF- and FGF-2-stimulated capillary networks were less profound, transfection of ERK5 siRNA on days 7 and 9 of the assay only induced a 13.3% (±4.3% SD) lowering in VEGF-stimulated tube formation and 4.2% (±3.1% SD) lowering in FGF-2 stimulated tubes in comparison to the N.S. siRNA-treated co-cultures. Together, these data suggest that capillary-like structures in HDMEC/NHDF co-cultures are most sensitive to ERK5 siRNA treatment during early vessel development, and that transfecting HDMECs on day 3 and HDMEC/NHDF co-cultures on day 5 enhances this effect. These data also imply that VEGF-stimulated tubules are more susceptible to ERK5 siRNA treatment than are FGF-2-stimulated capillary structures.

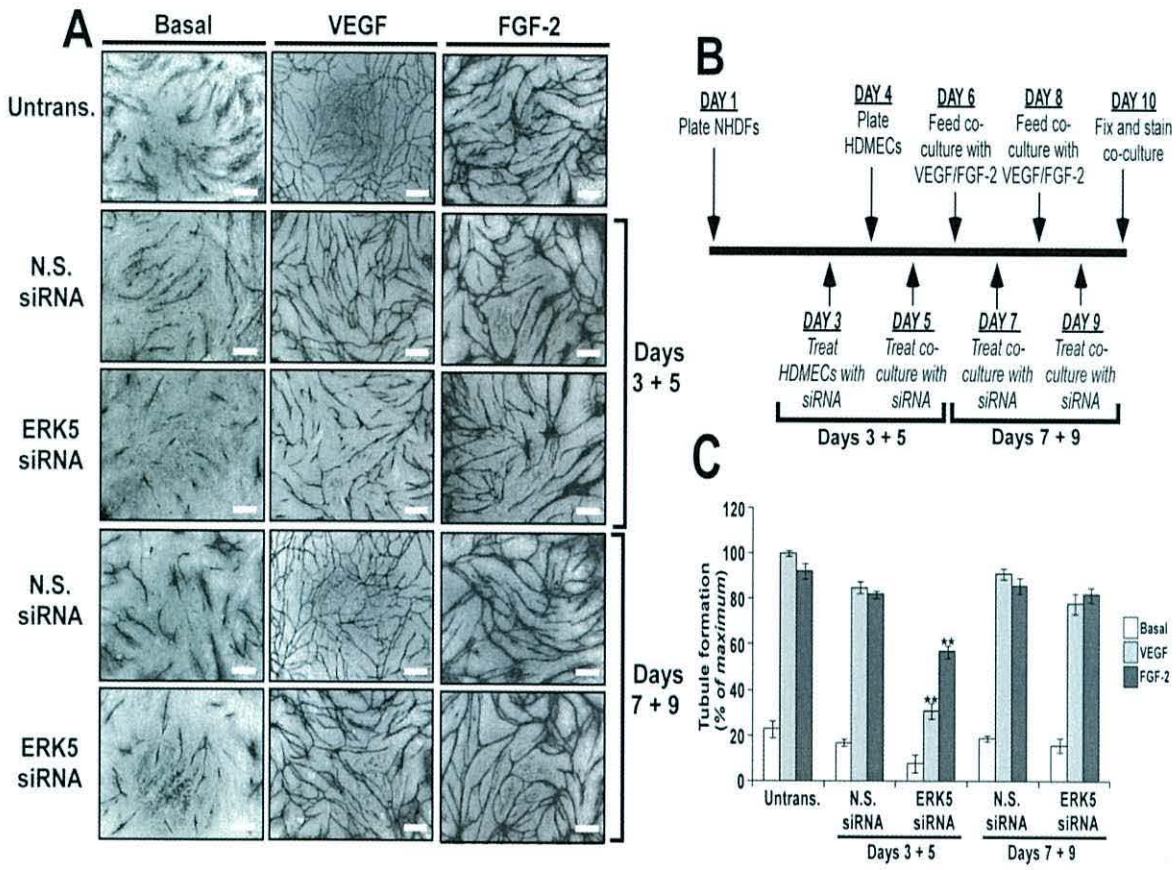

Figure 6.4 ERK5 siRNA attenuates VEGF- and FGF-2 stimulated angiogenesis in a HDMEC/NHDF co-culture assay. (**A**) NHDFs were seeded in fibroblast growth medium at 1.5 x 10^4 cells per well on gelatin-coated 24-well plates and incubated for 3 days. On day 3, HDMECs were transfected with 5 nM ERK5 siRNA 1 and 5 nM ERK5 siRNA 2, 10 nM non-silencing (N.S.) siRNA, or were left untransfected (Untrans.). On day 4 of the assay, 24 h post-transfection of HDMECs with siRNA, HDMECs were plated at 4.5 x 10^4 cells per well onto a confluent NHDF monolayer, and treated with EBM MV2 basal medium containing 1% (v/v) FCS alone (Basal) or supplemented with VEGF (50 ng/ml), or FGF-2 (50 ng/ml). Co-cultures were treated with further siRNA at day 5 (Days 3 + 5) as described (*section 2.2.7.2.2*). Alternatively, previously untransfected co-cultures were transfected with siRNAs at days 7 and 9 (Days 7 + 9) of the assay as indicated in the treatment schedule (**B**). (**C**) On day 10 of the assay, cells were fixed and stained as described (*section 2.2.7.2.3*), and total tubule length was quantified by AngioQuant image analysis software as described (*section 2.2.7.2.4*). Data is presented as percentage of maximum tube formation from triplicate wells (n = 3, mean ± SD, $^{**}p<0.01$, Student *t* test, compared to N.S. control siRNA treated cells). The result shown is representative of three independent experiments. X40 magnification. Bars = 400 µm. *ERK5, extracellular signal-regulated kinase 5; FCS, foetal calf serum; FGF-2, fibroblast growth factor-2; HDMEC, human dermal microvascular endothelial cell; NHDF, normal human dermal fibroblast; N.S., non-silencing; siRNA, short interfering RNA; VEGF, vascular endothelial growth factor.*

6.3.3 Chronic siRNA-mediated down-regulation of the MEK5/ERK5 abolishes VEGF- and FGF-2-stimulated HDMEC capillary morphogenesis

Given that treatment with ERK5 siRNA on days 3 and 5 of the co-culture assay had been found to significantly diminish VEGF- and FGF-2-induced tube formation (**Fig. 6.4**), it was of interest to determine the effects of chronic treatment with ERK5 siRNA upon VEGF- and FGF-2-induced angiogenesis in this assay. In addition, to further confirm the effects of the ERK5 signalling axis in this process, the effects of repeated

transfection of MEK5 siRNA in co-cultures was also assessed. HDMECs were left untransfected, or were transfected with ERK5 siRNA, MEK5 siRNA or with N.S. control siRNA on day 3, prior to plating on a confluent NHDF monolayer. Co-cultures were transfected with siRNAs on days 5, 7 and 9 thereafter as shown in **Fig. 6.5 B**.

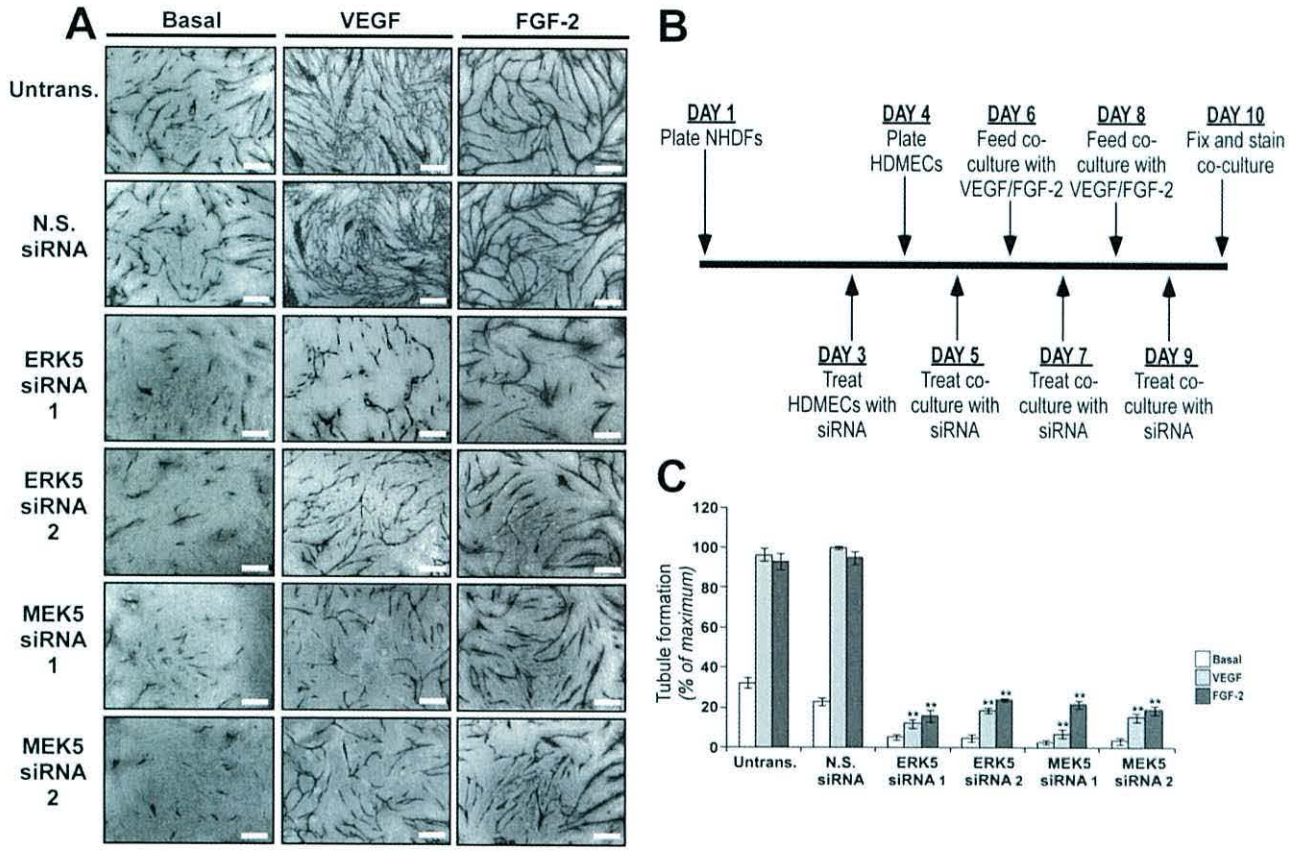

Figure 6.5 Chronic treatment with ERK5-specific siRNA or with MEK5-specific siRNA prevents VEGF- and FGF-2-stimulated angiogenesis in a HDMEC/NHDF co-culture assay. NHDFs were seeded in fibroblast growth medium at 1.5 x 10^4 cells per well on gelatin-coated 24-well plates and incubated for 3 days. On day 3, HDMECs were transfected with 10 nM ERK5 siRNA 1, 10 nM ERK5 siRNA 2, 10 nM MEK5 siRNA 1, 10 nM MEK5 siRNA 2, 10 nM non-silencing (N.S.) siRNA, or left untransfected (Untrans.). (**A**) At 24 h post-transfection, or not, HDMECs were plated at 4.5 x 10^4 cells per well onto a confluent NHDF monolayer, and treated with EBM MV2 basal medium containing 1% (v/v) FCS alone (Basal) or supplemented with VEGF (50 ng/ml), or FGF-2 (50 ng/ml). Co-cultures were transfected, or not, with ERK5 siRNA, MEK5 siRNA, or with N.S. siRNA on days 5, 7 and 9 of the assay (*section 2.2.7.2.2*) as indicated in the treatment schedule (**B**). (**C**) On day 10 of the assay, cells were fixed and stained as described (*section 2.2.7.2.3*), and total tubule length was quantified by AngioQuant image analysis software as described (*section 2.2.7.2.4*). Data is presented as percentage of maximum tube formation from triplicate wells (n = 3, mean ± SD, $^{**}p<0.01$, Student *t* test, compared to N.S. control siRNA treated cells). The result shown is representative of three independent experiments. X40 magnification. Bars = 400 µm. *ERK5, extracellular signal-regulated kinase 5; FCS, foetal calf serum; FGF-2, fibroblast growth factor-2; HDMEC, human dermal microvascular endothelial cell; NHDF, normal human dermal fibroblast; MEK5, MAPK/ERK kinase 5; N.S., non-silencing; siRNA, short interfering RNA; VEGF, vascular endothelial growth factor.*

Chronic transfection of co-cultures with ERK5- or MEK5-specific siRNAs had a profound effect upon both VEGF- and FGF-2-stimulated tube development (**Fig. 6.5 A**). CD31-positive HDMEC chord-like structures failed to interconnect, resulting in total abrogation of capillary network formation (**Fig. 6.5 A**). Quantification of tubule formation revealed that transfection with ERK5 siRNA attenuated VEGF-stimulated tubule formation by at least 80% and lowered FGF-2-stimulated tubule formation by at least 75% in each case (**Fig. 6.5 C**). Whilst previous data suggested that repeated treatment with ERK5 siRNA lowered early tubulogenesis, but was less effective at inducing the regression of more established vessels (**Fig. 6.4 A**), these results showed that continuous blockade of the MEK5-ERK5 signalling axis was sufficient to abolish both VEGF- and FGF-2-induced capillary network formation in HDMEC/NHDF co-cultures.

6.3.4 Chronic ERK5 siRNA treatment of HDMEC/NHDF co-cultures induces caspase-3 activation in HDMECs but not in NHDFs

Given the profound effects of chronic ERK5 siRNA treatment upon HDMEC capillary network formation (**Fig. 6.5**), it was hypothesised that ERK5 may be required for HDMEC survival in this context. To test this hypothesis, HDMEC/NHDF co-cultures were persistently treated with ERK5 siRNA or with N.S. control siRNA on days 3, 5, 7 and 9 of the assay and were treated with VEGF on days 6 and 8 of the assay prior to specific immunofluorescent staining of HDMECs with a mouse anti-CD31 primary antibody and an anti-mouse Alexa Fluor® 568 secondary antibody. Co-cultures were also probed with an anti-cleaved-caspase-3 antibody conjugated to an Alexa Fluor® 488 fluorophore. Fluorescence microscopy revealed that N.S. siRNA-treated cells exhibited an extensive capillary network with occasional punctuate cleaved caspase-3-positive cells (**Fig. 6.6 A**). By contrast, CD31 staining of ERK5 siRNA-treated co-cultures revealed very few tubule-like structures, and no capillary network formation. Concomitantly, siRNA-mediated silencing of ERK5 expression resulted in an increase in cleaved caspase-3, which co-localised to the CD31-positive HDMECs within the co-culture (**Fig. 6.6 A**). By contrast, no cleaved caspase-3 expression was detectable in the CD31-negative fibroblast layer (**Fig. 6.6 A**). As it was a possibility that these effects may be due to unequal silencing of ERK5 expression in NHDFs and HDMECs and/or

differential expression of ERK5, the efficiency of siRNA-mediated ERK5 silencing in NHDFs and HDMECs was compared by Western blot analysis. It was found that ERK5 was down-regulated by ERK5 siRNA treatment by 90% in both NHDFs and HDMECs, whereas ERK5 expression was not silenced by N.S. siRNA treatment in either cell type (**Fig. 6.6 B**). Together, these data suggested that human endothelial cells have a specific requirement for ERK5 to suppress caspase-3 activation and ultimately facilitate VEGF-mediated survival and tubular morphogenesis.

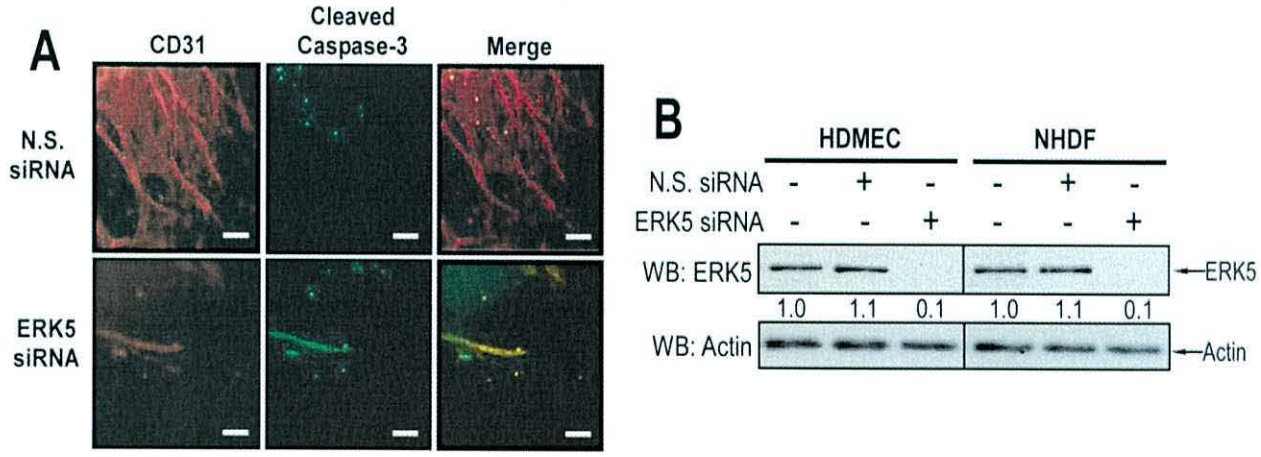

Figure 6.6 Treatment with ERK5 siRNA specifically induces caspase-3 activation in HDMECs, but not in NHDFs in a HDMEC/NHDF co-culture assay. (**A**) NHDFs were seeded in fibroblast growth medium at 1.5 x 10^4 cells per well on gelatin-coated 24-well plates and incubated for 3 days. On day 3, HDMECs were transfected with 5 nM ERK5 siRNA 1 and 5 nM ERK5 siRNA 2 or with 10 nM non-silencing (N.S.) siRNA. At 24 h post-transfection, HDMECs were plated at 4.5 x 10^4 cells per well onto a confluent NHDF monolayer. Co-cultures were treated with EBM MV2 basal medium containing 1% (v/v) FCS alone (Basal) or supplemented with VEGF (50 ng/ml), or FGF-2 (50 ng/ml) on days 6 and 8, as described (*section 2.2.7.2.1*). Co-cultures were treated with siRNA on days 5, 7 and 9 of the assay as described (*section 2.2.7.2.2*). On day 10 of the assay, cells were fixed, and stained with anti-CD31 antibody (red) and Alexa Fluor® 568-conjugated mouse secondary antibody as described (*section 2.2.12.3*) or with Alexa Fluor® 488 conjugated anti-cleaved caspase-3 (Asp^{175}) antibody (green), as described (*section 2.2.12.3*). X100 magnification. Bars = 100 µm. (**B**) HDMECs and NHDFs were seeded separately at 1.0 x 10^5 cells per well in a 6-well plate, and left untransfected, or were transfected with 5 nM ERK5 siRNA 1 + 5 nM ERK5 siRNA 2, or with 10 nM non-silencing (N.S.) siRNA as described (*section 2.2.7.2.2*). At 48 h post-transfection, cells were lysed and separated on a 10% SDS-PAGE gel followed by Western blotting (*WB*) with antibodies directed against ERK5, and actin, as indicated. Expression of ERK5 relative to actin was quantified by densitometric analysis using NIH ImageJ software as described (*section 2.2.6.4.2*).*ERK5, extracellular signal-regulated kinase 5; FCS, foetal calf serum; HDMEC, human dermal microvascular endothelial cell; NHDF, normal human dermal fibroblast; N.S., non-silencing; siRNA, short interfering RNA; VEGF, vascular endothelial growth factor; WB, Western blot.*

6.4 Assessment of the effects ZM323881 treatment on VEGF and FGF-2-driven angiogenesis in a HDMEC/NHDF co-culture assay

6.4.1 Chronic treatment with a low dose of the VEGFR-2 inhibitor ZM323881 inhibits VEGF-and FGF-2 stimulated tube formation in a HDMEC/NHDF co-culture assay

The polysulfonated naphthylurea compound suramin, which acts as an inhibitor of several RTKs, including VEGFR-2 (Waltenberger *et al.*, 1996), has been shown to prevent capillary network formation in HUVEC/NHDF co-culture angiogenesis assays by several independent groups (Bishop *et al.*, 1999; Donovan *et al.*, 2001; Beilmann *et al.*, 2004; Friis *et al.*, 2006). These studies have revealed the usefulness of endothelial cell/fibroblast co-culture assays as a means to assess vessel regression *in vitro.* In addition, the selective VEGFR-2 inhibitor AZD2171/cediranib (Recentin®) has also been shown to prevent VEGF-stimulated angiogenesis in the TCS CellWorks AngioKit™ assay (Wedge *et al.*, 2005).

To determine the relative importance of VEGFR-2-mediated signalling in the HDMEC/NHDF co-culture assay, co-cultures were treated with the selective VEGFR-2 inhibitor ZM323881 (Whittles *et al.*, 2002; Endo *et al.*, 2003). Single-dose treatments with 0.3 μM - 3.0 μM of ZM323881 were performed on either day 6 or day 8 of the co-culture assay; alternatively, co-cultures were treated twice with ZM323881 on both day 6 and day 8 of the assay (**Fig. 6.7 B**). As a vehicle control, co-cultures were treated with 0.1% (v/v) DMSO, which did not prevent VEGF- or FGF-2-stimulated tube formation at any time point tested (**Fig. 6.7 A**). In addition, HDMECs and NHDFs plated separately and treated with 3.0 μM ZM323881 over five days showed no detectable changes in morphology, suggesting that prolonged exposure to this inhibitor was not toxic to either HDMECs or NHDFs (data not shown).

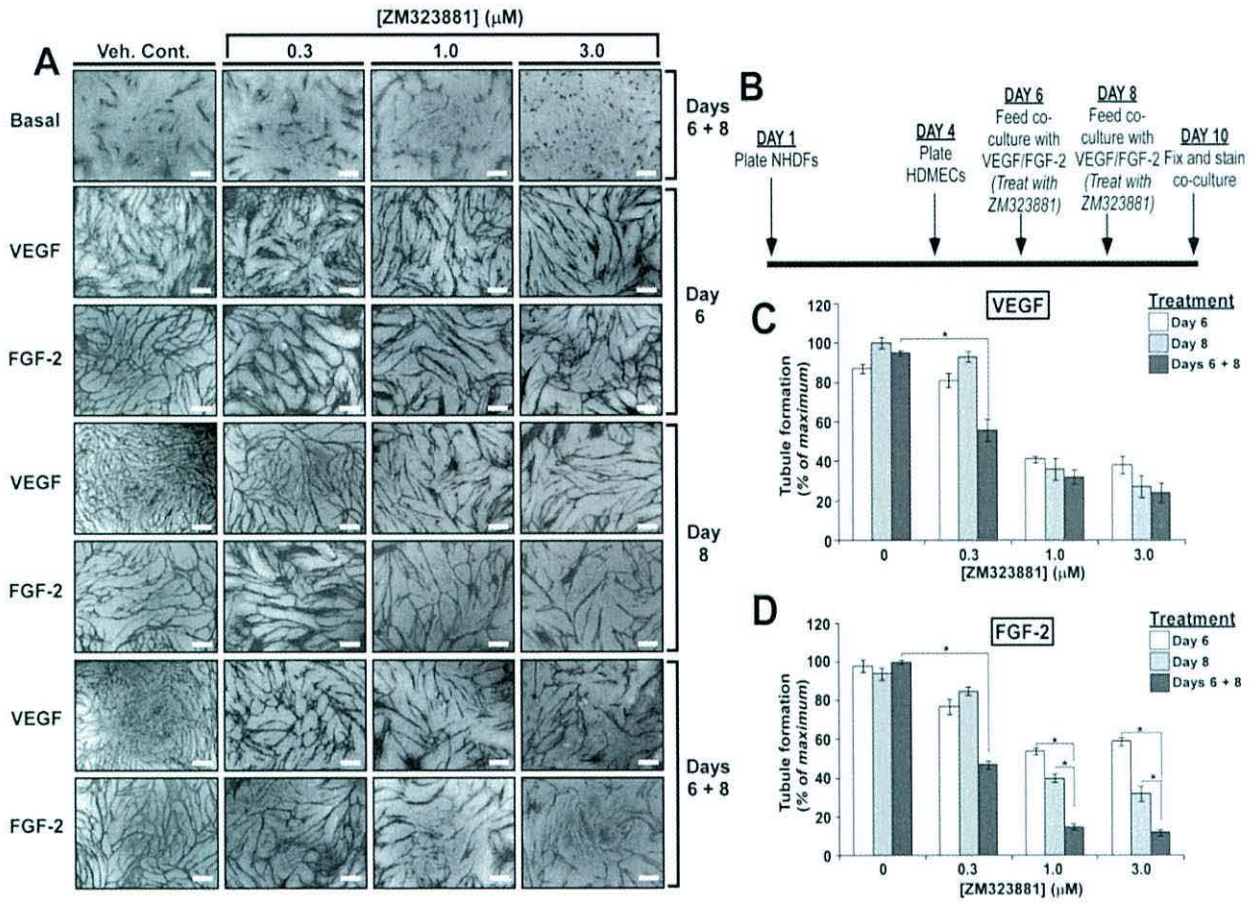

Figure 6.7 Effects of the VEGFR-2 inhibitor ZM323881 upon VEGF- and FGF-2-stimulated capillary morphogenesis of HDMECs in HDMEC/NHDF co-cultures. NHDFs were seeded in fibroblast growth medium at 1.5 x 10^4 cells per well on gelatin-coated 24-well plates and incubated for 3 days. On day 4 of the assay, HDMECs were seeded at 4.5 x 10^4 cells per well on top of the confluent NHDF monolayer. On days 6 and 8 of the assay, cells were treated with EBM MV2 basal medium containing 1% (v/v) FCS alone (Basal), or supplemented with VEGF (50 ng/ml), or FGF-2 (50 ng/ml). At the same time, co-cultures were treated with 0.1% (v/v) DMSO as a vehicle control (Veh. Cont.), or with the indicated amounts of ZM323881, as shown in the treatment schedule (**B**). On day 10 of the assay, cells were fixed and stained as described (*section 2.2.7.2.3*). Total tubule length of VEGF-stimulated (**C**) and FGF-2-stimulated (**D**) tubes was quantified using AngioQuant image analysis software as described (*section 2.2.7.2.4*). Data is presented as percentage of maximum tube formation from triplicate wells (n = 3, mean ± SD, *p<0.05, Student t test). The result shown is representative of three independent experiments. X40 magnification. Bars = 400 μm. *DMSO, dimethyl sulfoxide; ERK5, extracellular signal-regulated kinase 5; FCS, foetal calf serum; FGF-2, fibroblast growth factor-2; HDMEC, human dermal microvascular endothelial cell; NHDF, normal human dermal fibroblast; VEGF, vascular endothelial growth factor; VEGFR-2, VEGF receptor 2.*

Single-dose treatment with 0.3 μM ZM323881 on either day 6 or day 8 marginally lowered VEGF-stimulated capillary morphogenesis by 6% (±3.6% SD) and 7% (±2.5% SD) respectively, compared to vehicle control-treated co-cultures (**Fig. 6.7, A and C**), an effect that did not reach statistical significance (p>0.05, Student t test). By comparison, repeated exposure of co-cultures to 0.3 μM ZM323881 on days 6 and 8 significantly inhibited VEGF-induced tube formation by 39% (±5.4% SD) compared to vehicle control-treated cells (p<0.05, Student t test) (**Fig. 6.7, A and C**), thus showing

that repeated treatment with a low dose of ZM323881 was sufficient to partially attenuate VEGF-induced tube formation.

Treatment with 1 μM ZM323881 on day 6 or day 8 alone decreased VEGF-driven angiogenesis by more than 60% compared to VEGF-induced tube formation in vehicle control-treated co-cultures (**Fig. 6.7, A and C**). This effect appeared to be maximal, as treatment with 3 μM exerted a similar inhibitory effect upon VEGF-induced tube formation (**Fig. 6.7, A and C**). The inhibitory effect of ZM323881 was only marginally increased by repeated exposure of co-cultures to 1 - 3 μM ZM323881 on days 6 and 8 (**Fig. 6.7, A and C**). This observation suggested that 1 μM ZM323881 was sufficient to maximally attenuate VEGF-induced tube formation in this assay, and that this effect was independent of the day of treatment.

Whilst treatment with 1 μM ZM323881 also maximally inhibited FGF-2-induced angiogenesis in the co-culture assay (**Fig. 6.7, A and D**), the inhibitory effect of ZM323881 upon FGF-2-stimulated capillary network formation was more dependent upon the treatment regimen than the effects upon VEGF-induced tube formation. By contrast to the effect of ZM323881 on VEGF-induced tube formation, FGF-2-induced capillary network formation was more profoundly inhibited by treatment with ZM323881 on day 8 than day 6, whereas treatment on both days significantly increased this inhibitory effect, lowering FGF-2-induced tube formation by at least 85% of maximal tube formation (**Fig. 6.7, A and D**). These results suggest that whilst the profile of ZM323881-mediated inhibition was dependent on the growth factors used to stimulate tube formation, both VEGF- and FGF-2-induced angiogenesis in this assay appears dependent upon VEGFR-2 activity. However, the specificity of ZM323881 as an inhibitor of only VEGFR-2 and not FGFR-1 should be tested to confirm this premise.

6.4.2 Combination treatment with low-dose ZM323881 and low-dose ERK5 siRNA synergistically inhibits VEGF- and FGF-2-stimulated tube formation in a HDMEC/NHDF co-culture assay

Chronic treatment of co-cultures with ERK5 siRNA, by transfecting HDMECs and co-cultures at days 3, 5, 7 and 9 was necessary to prevent growth factor-induced neo-vascularisation (**Fig. 6.5, A and C**). Furthermore, repeated treatment with ZM323881, when used at 0.3 µM, was necessary to significantly attenuate VEGF- or FGF-2-induced angiogenesis in the HDMEC/NHDF co-culture assay (**Fig. 6.7, A and C**). Clinical efficacy of currently approved anti-angiogenic therapies is often only attained by combination treatment with other chemotherapeutic agents (Gasparini *et al.*, 2005; Sartore-Bianchi *et al.*, 2007; Ellis & Hicklin, 2008).

It had been previously shown that transfection with 1 nM ERK5 siRNA was sufficient to lower ERK5 expression by up to 80% (**Fig. 4.1 A**). Given that non-specific toxic effects of siRNAs in cells have been shown to occur in a dose-dependent manner, which can be avoided by transfecting siRNAs at lower concentrations (Fedorov *et al.*, 2006; Bumcrot *et al.*, 2006), it was of interest to determine whether combined treatment with a low dose of ERK5 siRNA and a low-dose treatment with ZM323881 could work synergistically to attenuate VEGF- and FGF-2-stimulated neo-vascularisation. To test this in HDMEC/NHDF co-cultures, HDMECs were transfected with 1 nM ERK5 siRNA or 1 nM N.S. siRNA or were left untransfected, prior to plating on a confluent NHDF lawn, and the subsequent transfection of co-cultures with 1 nM siRNAs on day 5. Co-cultures, transfected with siRNA or not, were then treated with 0.3 µM ZM323881 on either day 6 or day 8 of the assay, as shown in **Fig. 6.8 B**.

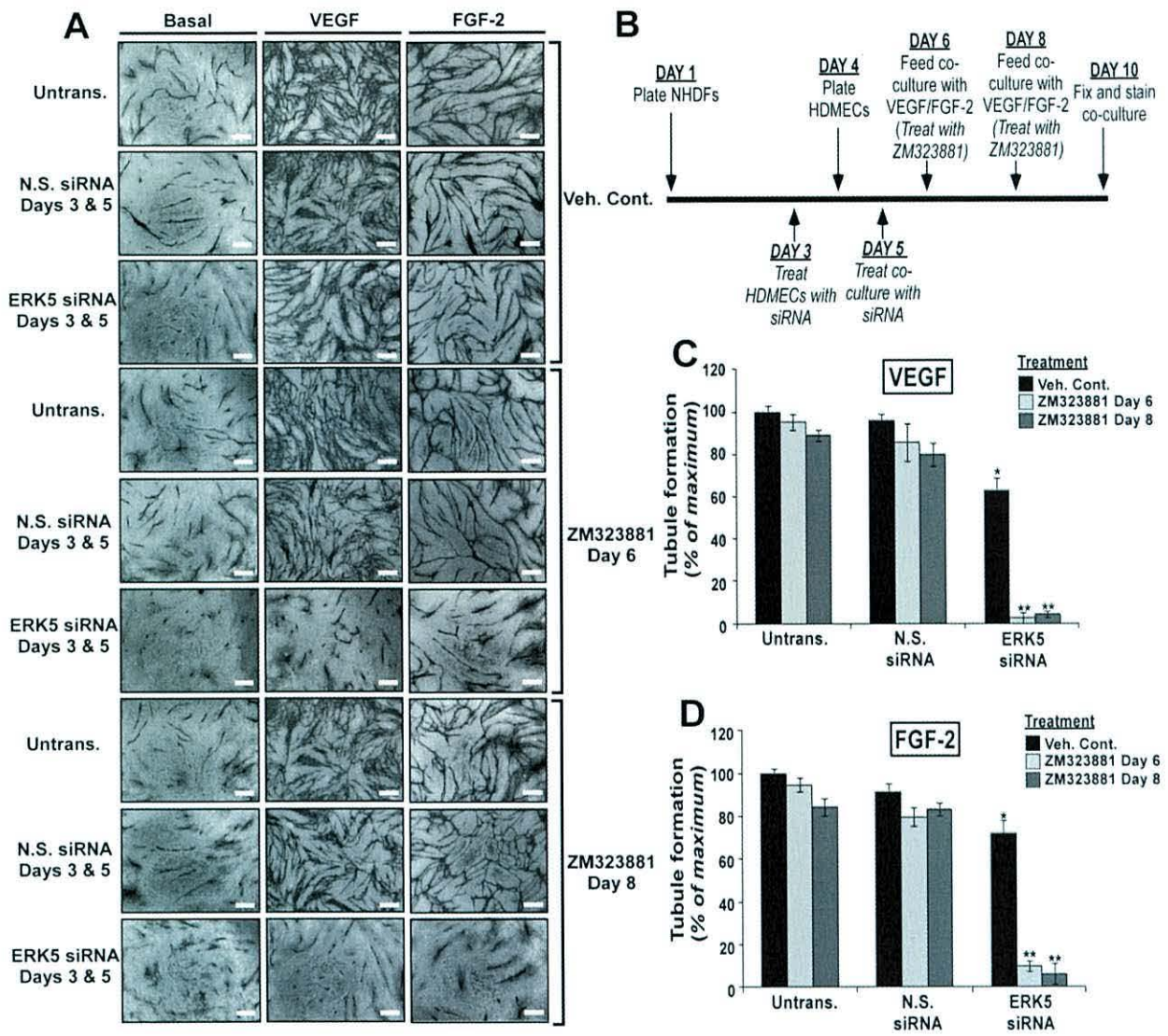

Figure 6.8 Effects of combination treatment with a low dose of the VEGFR-2 inhibitor ZM323881, and a low dose ERK5 siRNA upon VEGF- and FGF-2-stimulated capillary morphogenesis of HDMECs in HDMEC/NHDF co-cultures. NHDFs were seeded in fibroblast growth medium at 1.5 x 10^4 cells per well on gelatin-coated 24-well plates and incubated for 3 days. On day 3 of the assay, HDMECs were transfected with 1 nM ERK5 siRNA (0.5 nM ERK5 siRNA 1 + 0.5 nM ERK5 siRNA 2) or with 1 nM N.S. siRNA as described (*section 2.2.2.7.2*), or were left untransfected (Untrans.). On day 4 of the assay, HDMECs treated with siRNA, or not, were seeded at 4.5 x 10^4 cells per well on top of a confluent NHDF monolayer. On day 5, co-cultures were transfected with 1 nM ERK5 siRNA (0.5 nM ERK5 siRNA 1 + 0.5 nM ERK5 siRNA 2) or with 1 nM N.S. siRNA as described (*section 2.2.7.2.2*). On days 6 and 8 of the assay, cells were treated with EBM MV2 basal medium containing 1% (v/v) FCS alone (Basal), or supplemented with VEGF (50 ng/ml), or FGF-2 (50 ng/ml). At the same time, co-cultures were treated with 0.1% (v/v) DMSO as a vehicle control (Veh. Cont.), or with 0.3 µM ZM323881, as indicated in the treatment schedule (**B**). (**C**) On day 10 of the assay, cells were fixed and stained as described (*section 2.2.7.2.3*). Total tubule length of VEGF-stimulated (**C**) and FGF-2-stimulated (**D**) tubes was quantified using AngioQuant image analysis software as described (*section 2.2.7.2.4*). Data is presented as percentage of maximum tube formation from triplicate wells (n = 3, mean ± SD, *p<0.05, **p<0.01, Student t test, compared to N.S. control siRNA treated cells). The result shown is representative of three independent experiments. X40 magnification. Bars = 400 µm. *DMSO, dimethyl sulfoxide; ERK5, extracellular signal-regulated kinase 5; FCS, foetal calf serum; FGF-2, fibroblast growth factor-2; HDMEC, human dermal microvascular endothelial cell; NHDF, normal human dermal fibroblast; N.S., non-silencing; siRNA, short interfering RNA; VEGF, vascular endothelial growth factor; VEGFR-2, VEGF receptor 2.*

It was found that single-dose treatments on day 6 or day 8 with 0.3 μM ZM323881 alone had negligible effect on VEGF-induced tube formation in untransfected co-cultures (**Fig. 6.8, A and C**). Treatment with 1 nM ERK5 siRNA on days 3 and 5 followed by treatment with 0.1% (v/v) DMSO vehicle control on day 6 or day 8 was sufficient to lower FGF-2-stimulated tube formation by 18.9% (±6.1% SD), and VEGF-induced angiogenesis by 33.2% (±5.9% SD), showing that a low dose of ERK5 siRNA was able to lower capillary vessel formation. In contrast, treatment of co-cultures with 1 nM ERK5 siRNA in combination with 0.3 μM ZM323881 resulted in a 83% (±2.5% SD) and 75.6% (±1.6% SD) decrease in VEGF-induced tube formation following treatment on day 6 and day 8 respectively, compared to N.S. siRNA-treated controls ($p<0.01$, Student *t* test) (**Fig. 6.8, A and C**). Similarly, combination treatment with low dose ERK5 siRNA and 0.3 μM ZM323881 on days 6 and 8 reduced FGF-2-driven angiogenesis by 69.7% (±2.6% SD) and 76.9% (±4.8% SD) ($p<0.01$, Student *t* test) (**Fig. 6.8, A and C**). Collectively, these data showed that early treatment with 1 nM ERK5 siRNA followed by 0.3 μM ZM323881 at a later time point was sufficient to inhibit neo-vascularisation in a HDMEC/NHDF co-culture assay.

6.5 Discussion

6.5.1 Development of a HDMEC/NHDF *in vitro* angiogenesis assay

The purpose of conducting the experiments detailed in this chapter was to assess the effects of ERK5 siRNA treatment on tubule development as well as long-term capillary structure formation in a HDMEC/NHDF co-culture *in vitro* angiogenesis assay, in a feasibility study to indicate whether targeted down-regulation of ERK5 by siRNA may represent a possible therapeutic strategy to inhibit aberrant angiogenesis *in vivo*. HDMECs had been used as a model cell line throughout this study to characterise the role of ERK5 in angiogenesis; however, given that previous reported attempts to establish a 2-D HDMEC/NHDF co-culture angiogenesis assay had not been successful (Donovan *et al.*, 2001; Sorrell *et al.*, 2007), it was firstly necessary to develop and validate a HDMEC/NHDF *in vitro* angiogenesis assay.

A HDMEC/NHDF co-culture assay was developed based upon the outline method of the quasi 2-D HUVEC/NHDF co-culture assay first described by Bishop and colleagues (Bishop *et al.*, 1999). The assay described herein represents the first successful co-culture of HDMECs and NHDFs to give interconnecting tubules without the need to add supplementary collagen or ascorbate to promote tube formation (Sorrell *et al.*, 2007; Neeley *et al.*, 2010). It is suggested that the success of the current assay is attributable to seeding HDMECs at a higher density, and the use of EBM MV2 growth medium for culturing these cells as opposed to the co-culture conditions used in previous, less successful studies (Donovan *et al.*, 2001; Sorrell *et al.*, 2007).

In a recent review of *in vitro* angiogenesis assays, Staton and co-workers identified the characteristics of a hypothetical 'ideal angiogenesis assay', including the need for a rapid, reproducible, robust assay with automated computational analysis (Staton *et al.*, 2009). The current assay has proven to give reproducible results with growth factor-dependent HDMEC capillary network formation observed within 10 days (**Fig. 6.1 B**), which was fully quantifiable using AngioQuant image analysis software (**Fig. 6.2**). An additional important asset of this assay is that it took 10 days to complete, rather than the classical 18 day HUVEC/NHDF assay described by Bishop *et al.* (1999), representing a further reason to advocate its future use as an angiogenesis assay.

6.5.2 Effects of ERK5 siRNA transfection in HDMEC/NHDF co-cultures

Whilst siRNA-mediated gene silencing has been used extensively as a tool to study the roles of individual proteins in various stages of angiogenesis (Tomasi *et al.*, 2006), the current study is the first to describe a method for evaluating the potential inhibitory effects of siRNA-mediated down-regulation of protein expression upon angiogenesis in an endothelial cell/fibroblast co-culture assay. It is thus suggested that this represents an important addition to the tools currently available for the *in vitro* study of angiogenesis, which allows the relative contribution of specific proteins in the process of angiogenesis to be measured *in vitro,* in an assay that is highly representative of angiogenesis *in vivo* (Donovan *et al.*, 2001; Staton *et al.*, 2009).

Transfection of HDMECs with ERK5 siRNA at the start of the assay (days 3 and 5), prior to growth factor stimulation, and at the end of the assay, following the establishment of capillary-like structures (days 7 and 9), allowed the role of ERK5 in nascent tubule formation and the maintenance of established tubes to be evaluated respectively. In accordance with the previously reported effects of ERK5 siRNA on VEGF-induced tubular morphogenesis within 3-D collagen gels (**Fig. 4.11 - Fig. 4.12**), a particular requirement for ERK5 during the early stages of both VEGF- and FGF-2-driven angiogenesis was revealed (**Fig. 6.3**). Transfection of HDMECs alone and HDMEC/NHDF co-cultures together, with ERK5 siRNA on days 3, and 5 respectively led to a more profound inhibition of HDMEC capillary formation (**Fig. 6.4**). Previous work had shown that ERK5 siRNA-mediated knockdown was sustained for 96 h post-transfection (**Fig. 4.1**). However, in the context of the co-culture, it is possible that the down-regulation of ERK5 in HDMECs is not as sustained, bringing about a need for repeated transfection with ERK5 siRNA to exert an inhibitory effect on tubule formation.

It was found that chronic treatment with ERK5 siRNA or MEK5 siRNA in HDMEC/NHDF co-cultures abolished VEGF- and FGF-2-induced angiogenesis (**Fig. 6.5**). Interestingly, whilst ERK5 expression was down-regulated equally well in both NHDFs and HDMECs in response to ERK5 siRNA treatment (**Fig. 6.6 B**), HDMECs underwent apoptosis in response to ERK5 siRNA-treatment, whereas NHDFs did not (**Fig. 6.6 A**). These findings are in agreement with reports that deletion of *Erk5* or *Mek5* in mice induce endothelial cell apoptosis leading to cardiovascular defects that result in embryonic cell death (Regan *et al.*, 2002; Sohn *et al.*, 2002; Yan *et al.*, 2003; Wang *et al.*, 2005a). Wang and co-workers also reported that mouse embryonic fibroblasts from *Mek5*$^{-/-}$ mice did not show signs of apoptosis (Wang *et al.*, 2005a). Furthermore, targeted deletion of *Erk5* in murine endothelial cells induced endothelial cell death, whereas no adverse phenotype was reported following cell-specific ablation of *Erk5* in hepatocytes or neuronal cells (Hayashi *et al.*, 2004; Hayashi & Lee, 2004). Data presented in the current study are the first to show that siRNA-mediated down-regulation of ERK5 expression induces apoptosis in human endothelial cells, but not in human fibroblasts,

suggesting that ERK5 may have a similar cell-type-specific anti-apoptotic role in human endothelium, to that described in mice (Hayashi & Lee, 2004; Wang *et al.*, 2005a).

The physiological significance of endothelial cell/fibroblast co-culture assays has been demonstrated by the observation of inosculation and blood perfusion of capillary-like structures formed in an endothelial-keratinocyte-fibroblast co-culture, with the host vasculature following transplantation of co-cultures onto nude mice skin (Tremblay *et al.*, 2005). However, an important question rising from the findings presented in this chapter is the phenotypic difference observed following ablation of *Erk5* in adult mice (Hayashi *et al.*, 2004), and the lack of apparent effects on established vessels following transfection with ERK5 siRNA on day 9 alone (**Fig. 6.3**) or on days 7 and 9 together (**Fig. 6.4**). Whilst there may be several reasons for this apparent discrepancy, it is suggested that the most likely explanation is that the observed effects of loss of *Erk5* in adult mice is a reflection of the effects of haemodynamic flow *in vivo*, which is likely to exacerbate the effects of a weakened vasculature caused by the rounding of endothelial cells undergoing apoptosis following the loss of *Erk5* (Regan *et al.*, 2002; Hayashi *et al.*, 2004). This scenario is feasible, as conditional ablation of *Erk5* in adult mice does not induce lethality immediately, but rather, two weeks after induction of the deletion (Hayashi *et al.*, 2004), which suggests that continual haemodynamic flow over several days on apoptotic *Erk5*$^{-/-}$ mouse endothelial cells weakens the vasculature, culminating in haemorrhage. Indeed, whilst it had been previously reported that targeted deletion of *Erk5* in murine cardiomyocytes resulted in no adverse phenotype (Hayashi & Lee, 2004), recent work has revealed that under hypertrophic conditions, *Erk5*$^{-/-}$ cardiomyocytes undergo enhanced apoptosis, diminishing the hypertrophic response (Kimura *et al.*, 2010). These data suggest that the physiological conditions to which cells lacking ERK5 are exposed may be critical in determining the role of ERK5 in any given biological process. In total, these observations serve to highlight the need to thoroughly assess the role of ERK5 in *in vivo* models to further validate these findings.

6.5.3 Effects of combination treatment of HDMEC/NHDF co-cultures with ERK5 siRNA and ZM323881

Comparison of the inhibitory effects of the VEGFR-2 inhibitor ZM323881 (**Fig. 6.7**) or the effects of ERK5 siRNA when used alone (**Fig. 6.4**) with the effects of combinatory treatment of low-dose (1 nM) ERK5 siRNA and low-dose (0.3 μM) ZM323881 upon VEGF- and FGF-2-induced angiogenesis (**Fig. 6.8**), suggest that the effects of these agents are both additive and synergistic. The implications of this finding are potentially far-reaching; as, despite the relative success of anti-VEGF/VEGFR-2 treatments as anti-angiogenic agents, their long-term success is often limited due to the development of resistance (Bergers & Hanahan, 2008).

ZM323881 was sufficient to inhibit VEGF-stimulated ERK5 activation in HDMECs when used at 1-3 μM (**Fig. 3.4**). However, it is likely that prolonged stimulation of HDMEC/NHDF co-cultures with VEGF or FGF-2 evokes the release of several other growth factors from both NHDFs and HDMECs, that are likely to act in both a paracrine and autocrine fashion to stimulate HDMEC differentiation, as has been demonstrated in HUVEC/NHDF co-cultures (Saito *et al.*, 2003; Berthod *et al.*, 2006). It was shown elsewhere in this study that VEGF-induced HDMEC survival during conditions of tubular morphogenesis was dependent upon both the expression and activity of ERK5 (*Chapters 4 and 5*). Recent work has shown that PDGF-stimulated survival of PAE cells is also regulated by ERK5 (Lennartsson *et al.*, 2010), thus suggesting that blockade of VEGFR-2 activity alone may only inhibit the action of one of potentially many pro-angiogenic inducers of ERK5 activation, and subsequent endothelial cell survival. Data presented herein, showing that combination treatment with a low dose of ZM323881 and a low dose of ERK5 siRNA, suggests a model whereby down-regulation of ERK5 expression prevents HDMEC survival by blocking activation of AKT and other downstream proteins (*Chapter 5*) in response to VEGF, and possibly other growth factors. At the same time, ZM323881-mediated inhibition of VEGFR-2 is likely to contribute to inhibition of VEGF-induced survival signalling, whilst also blocking other VEGF-induced endothelial cell responses, such as proliferation and migration (Holmes

et al., 2007), that are known to occur in this assay (Donovan *et al.*, 2001), as is the case *in vivo*.

6.5.4 Conclusions and future perspectives

This study is the first to demonstrate the successful co-culture of HDMECs and NHDFs in a 2-D angiogenesis assay. Furthermore, this chapter documents the first use of siRNA to evaluate the relative importance of a given protein in a co-culture assay. In summary, ERK5 siRNA-mediated inhibition of angiogenesis was most effective when used on nascent vessels, whilst chronic exposure to ERK5 siRNA was sufficient to abolish VEGF- and FGF-2-stimulated tube development. Further experiments revealed that a combination treatment with low dose (1 nM) ERK5 siRNA at an early stage in angiogenesis, followed by low dose (0.3 μM) ZM323881 treatment at a later point was also an effective method of inhibiting capillary vessel growth. The finding that chronic treatment with ERK5-specific siRNA induced apoptosis of HDMECs, but not of NHDFs, represents the first evidence in human cells that endothelial cells may be more sensitive to the loss of ERK5 than other cells. This finding, in combination with the finding that ERK5 siRNA treatment preferentially targets new vessels, with less profound effects on established vessels, points towards the possible emergence of ERK5 as a promising target for the inhibition of aberrant angiogenesis in humans.

In conclusion, these results serve as the first proof-of-concept that siRNA-mediated silencing of ERK5 expression may provide a possible means for the therapeutic inhibition of angiogenesis *in vivo.* It is suggested that future studies should aim to optimise transfection methods for transient expression of CA-MEK5(D) and ERK5(wt) in order to establish whether or not this treatment may induce neovascularisation in this assay. In addition, it would be of interest to conduct studies to investigate the effects the recently described MEK5 inhibitors BIX02188 and BIX02189 (Tatake *et al.*, 2008; Li *et al.*, 2008), in HDMEC/NHDF co-cultures to further validate this model.

CHAPTER SEVEN

General Discussion

7.1 The role of the ERK5 signalling axis in angiogenesis

At the commencement of this project, gene targeting studies in mice had shown that Erk5 is critical for cardiovascular development (Regan *et al.*, 2002; Sohn *et al.*, 2002; Yan *et al.*, 2003; Hayashi *et al.*, 2004; Hayashi & Lee, 2004); however, the role played by ERK5 in distinct stages of angiogenesis had not been characterised. Furthermore, the potential function of ERK5 in regulating intracellular signalling and gene expression in response to VEGF stimulation in human endothelial cells was hitherto unknown. This thesis has addressed these important issues by evaluating the relative contribution of ERK5 in discrete stages of VEGF-induced angiogenesis in primary HDMECs as well as assessing the potential for targeting ERK5 for therapeutic inhibition of angiogenesis. The main findings of this study, their significance, and their wider implications are discussed below.

7.1.1 VEGF induces ERK5 activation via VEGFR-2 and PKCδ

Experiments aimed at characterising ERK5 activation in HDMECs revealed that VEGF-stimulated activation of ERK5 is mediated via VEGFR-2 and requires autophosphorylation of VEGFR-2 on Tyr^{1175} (**Fig. 3.4; Fig. 3.5**), raising the possibility that ERK5 may be an important downstream effector of the VEGFR-2-phospho-Tyr^{1175}-binding adaptor protein PLC-γ (Takahashi *et al.*, 2001). Based on studies involving down-regulation of specific PKC isoforms using siRNA (**Fig. 3.12**), it is inferred that PLC-γ may mediate VEGF-induced ERK5 activation via a p-VEGFR-2 Tyr^{1175}→p-PLC-γ→DAG→PKCδ signalling pathway (Takahashi *et al.*, 1999). However, these observations do not rule out the possibility that activation of ERK5 may be mediated by other VEGFR-2-phospho-Tyr^{1175}-binding adaptor molecules such as Sck (Warner *et al.*, 2000) or Shb (Holmqvist *et al.*, 2004). The upstream signalling pathways mediating activation of MEK5/ERK5 remain incompletely characterised; therefore, further studies will be required to confirm the exact mechanisms relaying activatory signals from VEGFR-2 to ERK5. Based upon data presented in this thesis, the hypothesised signal transduction pathway leading to ERK5 activation in response to VEGF stimulation is summarised in **Fig. 7.1**.

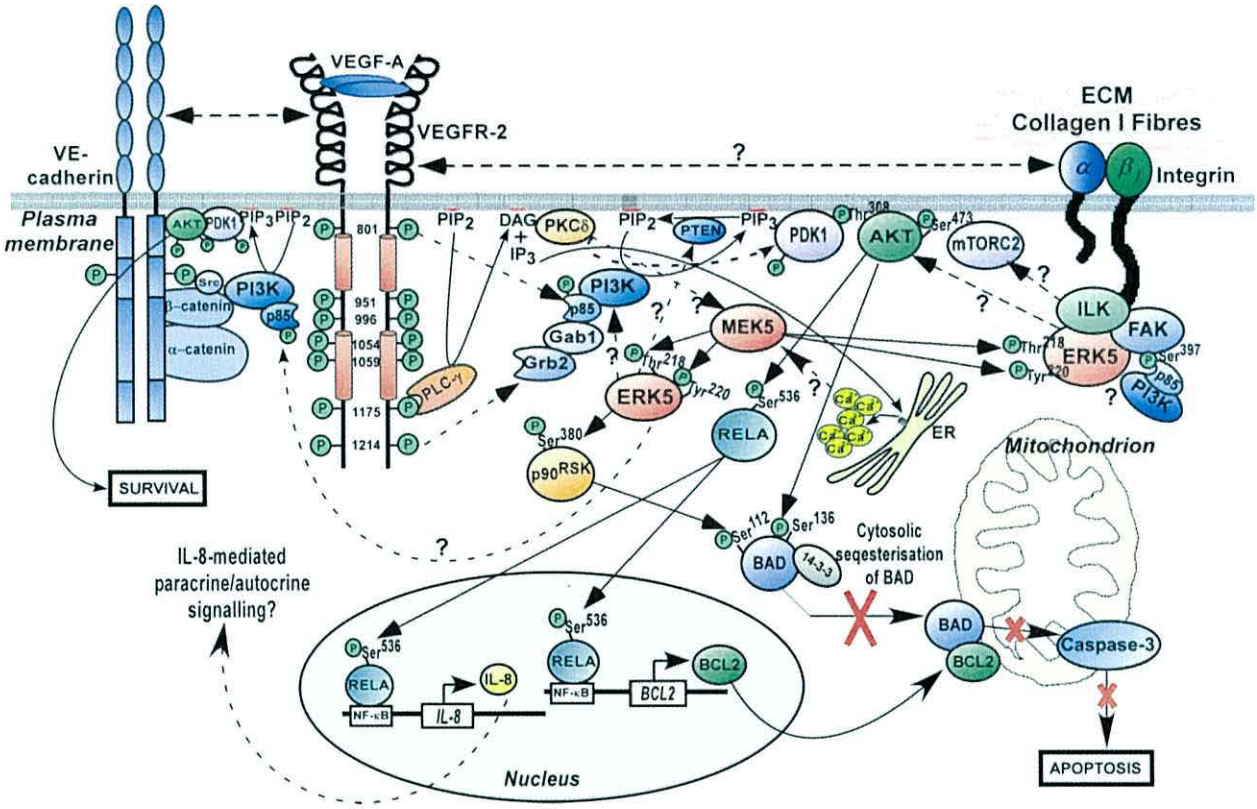

Figure 7.1 Model showing the hypothesised involvement of the ERK5 signalling axis as a mediator of VEGF-stimulated intracellular signalling conferring endothelial cell survival during angiogenesis. Binding of VEGF to VEGFR-2 induces trans-autophosphorylation of several tyrosine residues in the cytoplasmic domain of VEGFR-2, and allows direct interaction (indicated by double-headed dashed arrows) between VEGFR-2 and VE-cadherin as well as between VEGFR-2 and collagen-binding β_1 integrins. VEGF-induced activation of ERK5 is dependent upon phosphorylation of VEGFR-2 Tyr^{1175}. PLC-γ binds to VEGFR-2 Tyr^{1175} leading to hydrolysis of PIP_2 to give DAG and IP_3. IP_3 induces the release of Ca^{2+} from the ER. Intracellular Ca^{2+} is critical for VEGF-induced activation of ERK5, via an unknown mechanism. DAG induces the activation of PKCδ, which mediates the activation of the ERK5 signalling axis via an unknown mechanism. PKCδ can also mediate activation of PDK1 independently of PI3K. ERK5 may regulate PI3K activation, or inactivation, by as-yet-unidentified mechanisms to regulate AKT activation. Binding of the β_1 integrins to collagen I matrices induces the association of ILK with the cytoplasmic tails of the integrins, as well as autophosphorylation of FAK. ERK5 may be involved in FAK- or ILK-mediated activation of AKT. ERK5 may also phosphorylate AKT on Ser^{473} and Thr^{308} directly. ERK5 regulates VEGF-stimulated activation of AKT, RELA and the expression of the anti-apoptotic protein BCL2 and the pro-angiogenic chemokine IL-8. Phosphorylation of the pro-apoptotic protein BAD on Ser^{112} and Ser^{136} by $p90^{RSK}$ and AKT prevents BAD-BCL2 heterodimerisation, caspase-3 activation and subsequent cell death. IL-8 may be involved in autocrine and paracrine signalling to induce VEGF expression in endothelial cells and tumour cells. The transmembrane adherens junction molecule VE-cadherin associates with VEGFR-2, PI3K, β-catenin and Src, and is critical for VEGF-induced activation of AKT, and subsequent endothelial cell survival. ERK5 may regulate VE-cadherin-mediated activation of AKT via unknown mechanisms. Arrows with solid lines represent probable signal transduction pathways based on experimental evidence and pathways reported in the literature. Arrows with dashed lines represent alternate or hypothesised signal transduction pathways based on literature searches. *AKT/PKB, protein kinase B; BAD, BCL2-antagonist of cell death; BCL2, B-cell lymphoma 2; ER, endoplasmic reticulum; ERK5, extracellular signal-regulated kinase 5; ECM, extracellular matrix; FAK, focal adhesion kinase; Gab1, Grb2-associated binder-1; Grb2, growth-factor-receptor-bound 2; IL-8, interleukin 8; ILK, integrin-linked kinase; IP_3, inositol (1,4,5)-trisphosphte; MEK5, MAPK/ERK kinase 5; mTORC2, mammalian target of rapamycin complex 2; NFκB, nuclear factor κB; $p90^{RSK}$, p90 ribosomal S6 kinase; PDK1, 3'-phosphoinositide-dependent kinase-1; PI3K, phosphoinositide 3-kinase; PIP_2, phosphatidylinositol (4,5)-bisphoisphate; PIP_3, phosphatidylinositol (3,4,5)-trisphosphate; PTEN, phosphatase and tensin homologue; RELA, p65 NFκB; VE-cadherin, vascular endothelial cadherin; VEGF, vascular endothelial growth factor; VEGFR-2, VEGF receptor-2.*

PKCδ is a well-characterised nPKC isoform (Reyland, 2009), which has a notable, well established role as a regulator of cell fate, mediating cell survival in particular cell

types, whilst initiating and amplifying apoptosis in certain other cells (Emoto *et al.*, 1995; Heidkamp *et al.*, 2001; Xia *et al.*, 2009; Ringshausen *et al.*, 2002). In endothelial cells, PKCδ has been shown to be important for the control of several cellular functions, including regulation of endothelial cell survival (Takahashi & Shibuya, 2001), and the regulation of VEGF-induced activation of AKT (Gliki *et al.*, 2002). This study now reveals that PKCδ can also mediate VEGF-induced activation of ERK5 in endothelial cells, raising the possibility that at least some of the physiological roles played by PKCδ may also be mediated by ERK5 in these cells.

Unlike *Erk5*$^{-/-}$ mice, *Pkcδ*$^{-/-}$ mice are viable with no obvious vascular defects (Leitges *et al.*, 2001; Miyamoto *et al.*, 2002), suggesting that ERK5 is regulated by other pathways distinct from PKCδ during development. Due to inherent redundancy between PKC isoforms (Parker & Murray-Rust, 2004), it is possible that other PKC isoforms may be involved in this process, or that distinct signal transduction pathways, acting via ERK5, are utilised specifically during embryonic development to facilitate endothelial cell survival. A further possibility is that PKCδ-mediated ERK5 activation may be species-specific, occurring in human endothelial cells, but not in murine endothelial cells.

It is interesting to note that siRNA-mediated down-regulation of the expression of either ERK5 or PKCδ in HDMECs resulted in the attenuation of VEGF-induced AKT activation (**Fig. 3.12**). However, it is not clear whether or not ERK5 represents an intermediary molecule regulating AKT activation downstream of PKCδ in these cells. Brand *et al.* provided evidence to show that insulin-mediated AKT activation in muscle cells is regulated by the direct association of PKCδ with PDK1 independently of PI3K (Brand *et al.*, 2006). It is therefore possible that ERK5 and PKCδ regulate AKT activation independently of each other. Future studies should aim to further define the relationship between PKCδ and ERK5 by establishing the mode by which PKCδ activates ERK5, as well as determining the physiological significance of VEGF-stimulated PKCδ-mediated ERK5 activation.

7.1.2 The physiological role of ERK5 in HDMECs

Angiogenesis is a multi-faceted process involving endothelial cell activation, proteosomal degradation of the ECM, migration of 'tip' endothelial cells into the degraded ECM, proliferation of 'stalk' endothelial cells and subsequent endothelial cell differentiation to form lumen-containing vessels that are finally stabilised by mural cells. This study is the first to define a specific requirement for ERK5 as a mediator of VEGF-stimulated endothelial cell survival during tubular morphogenesis (**Fig. 4.12 - Fig. 4.15**). Interestingly, ERK5 was not required for VEGF-stimulated HDMEC survival on a gelatin matrix (**Fig. 4.12 - Fig. 4.15**). In addition, the proliferative and migratory responses of these cells to VEGF stimulation were not affected by siRNA-mediated down-regulation of ERK5 expression (**Fig. 4.7 and Fig. 4.9**). An attempt to define the molecular mechanisms by which ERK5 mediates endothelial cell survival revealed that VEGF-induced activation of AKT and $p90^{RSK}$, as well as phosphorylation of BAD on Ser^{112} and Ser^{136}, was severely affected following siRNA-mediated down-regulation of ERK5 expression in HDMECs plated on a collagen matrix, whereas phosphorylation of these key regulators of cell survival remained relatively unaffected in cells plated on a gelatin matrix (**Fig. 5.3**). This raises a key question: why do these phenotypic differences exist? In search for an answer to this question, the importance of integrins, not only in mediating cellular attachment to specific ECM components, but also in the interpretation of cell polarity in 2-D and 3-D environments and their influence upon cellular signalling events and subsequent physiological responses in various cell types must be considered (Cukierman *et al.*, 2001; Yamada & Clark, 2002; Larsen *et al.*, 2006; Green & Yamada, 2007; Ramjaun & Hodivala-Dilke, 2009). The collagen-binding integrins $\alpha_1\beta_1$ and $\alpha_2\beta_1$ have been assigned important roles for allowing endothelial cell tube formation *in vitro* (Senger *et al.*, 1997; Senger *et al.*, 2002; Whelan & Senger, 2003; Davis & Senger, 2008). It is proposed that ERK5 may be involved in incorporating signals from such collagen-binding integrins to facilitate activation of downstream effectors such as AKT. Indeed, recent work by Sawhney *et al.* has shown that ERK5 can associate with certain integrins, including β_1 integrins, in cancer cells (Sawhney *et al.*, 2009), making this concept a real possibility. ERK5 may therefore bind directly to integrins themselves or to integrin-associated adapter

proteins, to regulate AKT activation. The hypothesised mechanisms by which ERK5 mediates AKT activation are summarised in **Fig. 7.1**, and are discussed below.

7.1.3 Possible mechanisms of ERK5-mediated regulation of VEGF-induced AKT activity

During the course of this study, an increasing number of reports have revealed that ERK5 may be an important regulator of AKT activation in different cell types in response to various stimuli (Wang *et al.*, 2006; Finegan *et al.*, 2009; Lennartsson *et al.*, 2010). However, the exact mechanism by which ERK5 regulates AKT activation remains unknown. In HDMECs, wortmannin-mediated inhibition of PI3K, or inhibition of mTORC2 by chronic exposure to rapamycin blocked VEGF-induced AKT activation, but not VEGF-stimulated ERK5 activation in HDMECs, suggesting that ERK5 regulates AKT activity either upstream of PI3K/mTORC2, or independently of these entities. Lennartson *et al.* recently proposed that ERK5 may regulate PDGF-induced AKT activation in PAE cells indirectly, by regulating PTEN activity resulting in de-phosphorylation of the phospholipid PIP_3 (Lennartsson *et al.*, 2010). Nevertheless, this was not demonstrated experimentally, and so it would be of interest to conduct further work to confirm or refute this hypothesis.

The mechanisms by which ERK5 regulates AKT activation may differ between cells. Indeed, within mouse embryonic fibroblasts, stress- and growth factor-stimuli appear to induce AKT activation via ERK5-dependent and ERK5-independent pathways respectively (Wang *et al.*, 2006). The present study now shows that the environmental context of the cell is also important in determining the relative contribution of ERK5 to the mediation of AKT activation. It is suggested that two possible candidate proteins with which ERK5 may interact to differentially regulate AKT activation in response to VEGF stimulation on collagen and gelatin matrices may be the β_1 integrin-associated proteins ILK and FAK (**Fig. 7.1**).

ILK binds to the cytoplasmic tails of β_1 integrins, and forms complexes with specific proteins to regulate numerous cellular signalling events (Wickstrom *et al.*, 2010). ILK has also been shown to mediate AKT phosphorylation and subsequent survival of

various cell types including cancer cells, endothelial cells and fibroblasts plated within a 3-D collagen matrix (Persad *et al.*, 2001; Kaneko *et al.*, 2004; Nho *et al.*, 2005; McDonald *et al.*, 2008). However, it was recently revealed that ILK lacks kinase activity, but rather acts as an adapter protein to facilitate phosphorylation of various proteins (Wickstrom *et al.*, 2010). Indeed, ILK has been shown to mediate AKT phosphorylation on Ser^{473} via direct interaction with RICTOR (McDonald *et al.*, 2008). It may then be possible, that ERK5 associates with ILK/RICTOR to regulate AKT phosphorylation on Ser^{473}. A caveat of this hypothesis is the requirement of ERK5 for VEGF-stimulated AKT activation on both Thr^{308} and Ser^{473} in cells plated on within a 3-D collagen gel (**Fig. 5.3**), whereas ILK has been shown to specifically mediate AKT activation on Ser^{473}, but not Thr^{308} (Persad *et al.*, 2000; Persad *et al.*, 2001; Troussard *et al.*, 2003); thus suggesting that if ILK is indeed a mediator of ERK5-regulated AKT activation in these cells, then ERK5 must mediate Thr^{308} activation via a distinct pathway.

The protein tyrosine kinase FAK may be a further possible candidate mediator of VEGF-stimulated, ERK5-regulated regulation of AKT activation in HDMECs plated within a collagen matrix. Attachment of β_1 integrins to the ECM leads to the recruitment of FAK to the cytoplasmic tails of β_1 integrins inducing the autophosphorylation of FAK on Tyr^{397}, which serves as a binding site for the p85 regulatory subunit of PI3K, facilitating PI3K activation and subsequent activation of AKT (Chen *et al.*, 1996). In a separate study, Xia *et al.* provided evidence to show that survival of fibroblasts within 3-D collagen I gels involved the binding of β_1 integrins to the ECM to stimulate FAK phosphorylation on Tyr^{397} and Tyr^{407} and the subsequent activation of PI3K (Xia *et al.*, 2004). More recently, it has been shown that β_1 integrins can mediate AKT phosphorylation on Thr^{308} and Ser^{473} independently of FAK or Src family kinases via a mechanism involving PI3K (Velling *et al.*, 2004; Velling *et al.*, 2008). It is therefore interesting to speculate that ERK5 may be involved in one or more of these processes to facilitate β_1-mediated activation of PI3K and subsequent phosphorylation of AKT on Thr^{308} and Ser^{473} in HDMECs in response to VEGF stimulation within a 3-D collagen matrix.

The possibility that ERK5 may directly phosphorylate AKT on Thr^{308} and Ser^{473} without the involvement of an intervening kinase should also be considered. Whilst this may at first seem unlikely, it is noteworthy that ERK5 has been reported to phosphorylate non-proline directed serine and threonine residues within itself and within MEK5 (Mody *et al.*, 2003), raising the prospect that ERK5 may also be able to phosphorylate the non-proline-directed Thr^{308} and Ser^{473} residues required for AKT activation.

Finally, it is hypothesised that certain protein phosphatases should also be considered as possible targets of ERK5 that may be involved in regulating AKT activity. The outcomes of this study, showing that ERK5 is regulated in a matrix-dependent manner, suggests that a possible candidate phosphatase for this role may be protein phosphatase 2A (PP2A), which has been shown to become activated following interaction with the collagen-binding β_1 integrins to directly de-phosphorylate AKT on both Ser^{473} and Thr^{308} (Ivaska *et al.*, 2002; Pankov *et al.*, 2003).

7.1.4 Downstream effectors of the ERK5/AKT survival signalling in endothelial cells

Evidence gained from this study strongly implies that ERK5 mediates VEGF-induced HDMEC survival during tubular morphogenesis independently of MEF2C (**Fig. 4.11**). Furthermore, VEGF-induced phosphorylation of MEF2C on Ser^{387} was not affected by siRNA-mediated down-regulation of ERK5 expression (**Fig. 5.5**), suggesting that ERK5 does not regulate MEF2C transactivational activity in response to VEGF in endothelial cells, a premise supported by the recent findings of Maiti *et al.* (2008). These results also collectively suggest that MEF2C is not the primary effector that mediates VEGF-induced cell survival signals downstream of ERK5 in HDMECs. In contrast, VEGF-stimulated phosphorylation, and inactivation, of the pro-apoptotic protein BAD was blocked following siRNA-mediated down-regulation of ERK5 expression in HDMECs. Phosphorylation of BAD had been previously shown to be mediated by ERK5, to confer survival of BAECs in response to laminar shear-stress; importantly however, the kinases responsible for mediating ERK5-regulated phosphorylation of BAD in BAECs had not been identified (Pi *et al.*, 2004). Analysis of the effects of siRNA-mediated silencing of ERK5 expression upon VEGF-stimulated activation of candidate kinases acting upstream of BAD performed in this study, suggests a model whereby ERK5

regulates BAD phosphorylation on Ser^{112} and Ser^{136} via regulation of $p90^{RSK}$ and AKT activity respectively (**Fig. 7.1**).

Data presented herein supports the view that ERK5 mediates endothelial cell survival in response to VEGF stimulation by regulating the apoptotic balance of BAD phosphorylation and BCL2 expression (*chapter 5*). SiRNA-mediated down-regulation of ERK5 expression prevented VEGF-induced up-regulation of *BCL2* mRNA whilst also lowering basal expression levels of *BCL2,* specifically in cells on a collagen matrix. VEGF-stimulated, AKT-regulated, activation of RELA has been shown to directly regulate BCL2 expression (Grosjean *et al.*, 2006), suggesting a mechanism whereby ERK5 may regulate the transcription of *BCL2*, via a RELA-dependent pathway.

7.1.5 Constitutive activation of ERK5 stimulates tubular morphogenesis of HDMECs

Constitutive activation of ERK5 in HDMECs was sufficient to induce tubular morphogenesis in the absence of VEGF and was accompanied by an increase in phosphorylation of AKT and BAD as well as increased BCL2 expression (**Fig. 5.3**; **Fig. 5.7**). Nör and co-workers demonstrated that over-expression of BCL2 in HDMECs was sufficient to induce tubular morphogenesis of HDMECs within 3-D collagen gels in the absence of VEGF, an effect partly attributable to the enhanced cell survival of HDMECs, but which also critically involved the production of IL-8 to induce endothelial cell migration and capillary sprouting (Nor *et al.*, 2001). Intriguingly, siRNA-mediated down-regulation of ERK5 expression also prevented VEGF-stimulated expression of *IL-8* in HDMECs (**Fig. 5.11**). In addition, Schweppe *et al.* revealed that expression of *IL-8,* amongst other genes, was up-regulated following over-expression of CA-MEK5(D) in immortalised human retinal pigment epithelial cells (Schweppe *et al.*, 2006). Whilst the effects of co-expressing CA-MEK5(D) and ERK5(wt) on *IL-8* gene expression were not measured in this study, it is tempting to speculate that constitutive activation of ERK5 may result in the possible up-regulation of *IL-8* expression, together with *BCL2* expression, to facilitate endothelial cell sprouting, and survival in the absence of VEGF. Future studies should aim to firstly determine whether co-transfection of CA-MEK5(D) and ERK5(wt) induces up-regulation of *IL-8* mRNA expression in HDMECs and whether or not IL-8 protein is secreted from HDMECs following constitutive activation of ERK5.

It is noteworthy that both IL-6 and IL-7 have been shown to activate ERK5 (Carvajal-Vergara *et al.*, 2005). Whilst it is not currently known whether ERK5 can be activated by IL-8, it remains possible that IL-8 may stimulate activation of ERK5 to mediate certain pro-angiogenic effects.

The importance of BCL2 expression in endothelial cells *in vivo* has been demonstrated from the observation that over-expressing BCL2 in HDMECs induces endothelial cell survival, leading to enhanced tumour growth in a tumour xenograft model (Nor *et al.*, 2001). Interestingly, BCL2 expression has also been shown to be 4-fold greater in endothelial cells within the tumour microvasculature of head and neck squamous cell carcinomas (Kumar *et al.*, 2007). Together, these studies show that BCL2 expression is important for tumour angiogenesis and appears to be differentially expressed in endothelial cells lining the tumour vasculature. Could this pattern of *BCL2* expression be a reflection of disparate ERK5 expression or ERK5 activation between normal endothelium and that of the tumour vasculature endothelium? Further work to assess the expression and the activation status of ERK5 in endothelial cells derived from the tumour vasculature should aim to clarify this notion.

7.1.6 SiRNA-mediated down-regulation of ERK5 prevents growth factor-induced neovascularisation in a HDMEC/NHDF co-culture *in vitro* angiogenesis model

One of the principal reasons for characterising the role of ERK5 in angiogenesis was to assess the feasibility of targeting ERK5 for therapeutic inhibition of angiogenesis. Commercially available HUVEC/NHDF co-culture *in vitro* angiogenesis assays have been widely used for testing the anti-angiogenic potential of various compounds (Wedge *et al.*, 2005; Secchiero *et al.*, 2007; Wang *et al.*, 2010). Data presented in chapter 6 of this study serves as the first proof-of-concept study to show that siRNA-mediated down-regulation of ERK5 expression prevents VEGF- and FGF-2-induced neovascularisation in a HDMEC/NHDF co-culture *in vitro* angiogenesis model. The use of siRNA to specifically down-regulate molecules involved in angiogenesis has been considered as a potentially promising anti-angiogenic therapeutic strategy (Lu *et al.*, 2005; Hadj-Slimane *et al.*, 2007). Until recently however, the lack of a safe and effective method of delivering siRNA *in vivo* has hampered the development of this

method (Aigner, 2006; Aigner, 2007). Nevertheless, it was recently reported that polyethylenimine-mediated delivery of siRNA targeting VEGF *in vivo* can work synergistically with the humanised anti-VEGF-A antibody bevacizumab, to inhibit tumour growth in mouse tumour xenograft models (Hobel *et al.*, 2010).

ERK5 siRNA treatment of co-cultures proved more effective at impeding the growth of vessels undergoing angiogenesis, rather than promoting the regression of established tubes in a HDMEC/NHDF co-culture model (**Fig. 6.4**), suggesting that nascent vessels are more sensitive to the loss of ERK5 than are fully-formed capillary networks. This observation is in agreement with the findings of Hayashi *et al.,* who reported that repeated administration of pIpC in adult *Erk5*$^{flox/flox}$ mice expressing the Mx1-Cre transgene resulted in a more rapid onset of death due to loss of blood vessel integrity, than in those mice injected once (Hayashi *et al.*, 2005). It is therefore possible that low-level expression of ERK5 in endothelial cells may be sufficient to permit normal endothelial cell functioning. Data presented herein suggests that ERK5 represents an attractive target for inhibition of aberrant angiogenesis. It remains to be seen what the effects of a low dose ERK5 inhibitor or siRNA treatment will be in mouse tumour models. However, based on the outcomes of this study, it is anticipated that targeting ERK5 may induce pruning of angiogenic vessels, which may be effective as a monotherapy. Alternatively, the anticipated vessel 'pruning' effect of targeting ERK5 may aid delivery of chemotherapeutic agents, as has been recently shown to be the case with bevacizumab (Jain, 2005; Fukumura & Jain, 2007).

Resistance to anti-angiogenic treatments that target VEGF/VEGFR-2 often arise from the utilisation of alternative pro-angiogenic pathways during tumour progression, which is a major contributory factor in the limited long-term success of this strategy in cancer treatment (Shojaei & Ferrara, 2007; Bergers & Hanahan, 2008). Based on the outcomes of this study, showing that ERK5 is critical for VEGF-induced endothelial cell survival by mediating activation of AKT, and the recently reported finding that ERK5 is required for sustained PDGF-induced activation of AKT in PAE cells (Lennartsson *et al.*, 2010), it is suggested that ERK5 may represent a critical common integrator of several growth factor-induced signals to confer survival of various endothelial cell types. If this proves to be the case, then specific inhibition of the ERK5 pathway *in vivo* either by

siRNA, or by the use of a MEK5/ERK5 inhibitor such as the recently developed MEK5-specific inhibitors, BIX02188 and BIX02189 (Tatake *et al.*, 2008), would be expected to prevent angiogenesis by preventing endothelial cell survival in response to stimulation with various growth factors by impeding AKT activation. AKT is a point of convergence for several growth-factor induced signals mediating cell survival including: VEGF, PDGF, FGF-2 and Ang-1 (Zachary, 2005). Thus, it is possible that targeted inhibition of ERK5 in endothelial cells would also make these cells less prone to the development of resistance to such an anti-angiogenic therapy than is normally the case when VEGF/VEGFR-2 alone is targeted.

Induction of endothelial apoptosis to inhibit neovascularisation and promote vessel regression in the tumour microenvironment has long been considered as a possible means of limiting angiogenesis (Folkman, 1971; Dimmeler & Zeiher, 2000b); however, the lack of specific regulators of endothelial cell apoptosis has thus far hampered progress of potential anti-angiogenic strategies. Gene-targeting studies in mice have suggested that Mek5/Erk5 have a unique role in regulating the viability of endothelial cells (Hayashi *et al.*, 2004; Wang *et al.*, 2005a). Interestingly, siRNA-induced down-regulation of ERK5 expression in NHDF/HDMEC co-cultures induced apoptosis in HDMECs but not in NHDFs, suggesting that human endothelial cells are also more sensitive to the loss of ERK5 than are fibroblasts. This raises the possibility that systemic administration of MEK5/ERK5-specific siRNA or a small molecule inhibitor directed against MEK5/ERK5 may preferentially target the endothelium, with limited effects in other cell types.

The orally-active VEGFR-2 inhibitor ZM323881 has been recently shown to attenuate neovascularisation *in vivo* in a Zebrafish model (Cao *et al.,* 2008). The current study now reveals that administration of low dose ZM323881 together with low dose ERK5 siRNA, can work synergistically to prevent VEGF- and FGF-2-driven angiogenesis in HDMEC/NHDF co-cultures (**Fig. 6.8**). The importance of this finding lies in the fact that a lower dose of therapeutics targeting VEGF/VEGFR-2 is known to reduce the toxic side effects of such treatment (Chen & Cleck, 2009). These findings advocate the need for further *in vitro* studies to determine whether or not combination therapy with either

low-dose ERK5 siRNA or MEK5 inhibitor (BIX02188), together with low-dose ZM323881 will prove to be an effective method for inhibiting angiogenesis *in vivo.*

7.2 Study limitations and future directions

Given the importance of VEGF as a pro-angiogenic molecule, this study has focused upon determining the physiological relevance of VEGF-induced ERK5 activity in HDMECs during distinct phases of angiogenesis, as well as aiming to elucidate the importance of ERK5 as a mediator of VEGF-stimulated intracellular signalling and gene expression. However, this work has also revealed that other angiogenesis inducers such as HGF can also stimulate ERK5 activation in HDMECs. In addition, other pro-angiogenic growth factors such as Ang-1, which exerts its physiological effects via its cognate receptor tyrosine kinase Tie-2, have been previously hypothesised to mediate pro-angiogenic signals via ERK5 (Regan *et al.*, 2002; Yan *et al.*, 2003). Interestingly, Ang-1 induces endothelial cell sprouting and tubular morphogenesis *in vitro* (Koblizek *et al.*, 1998), but does not stimulate endothelial cell proliferation (Kim *et al.*, 1999). Similar to VEGF, Ang-1 has also been shown to inhibit endothelial cell apoptosis by activation of AKT pathway (Kim *et al.*, 2000; Papapetropoulos *et al.*, 2000). It was recently shown that Ang-1 can induce ERK5 activation in endothelial cells (Deng *et al.*, 2007); however, the functional significance of Ang-1-induced ERK5 activation, and the potential downstream effectors were not reported. It is therefore suggested that future studies should aim to determine whether Ang-1 and HGF can induce physiologically meaningful signals via ERK5 in endothelial cells.

It should be noted that in light of the outcomes of this study, combined with the knowledge of the phenotypic effects of ablation of *Erk5* in mice, it is hypothesised that a potential role for ERK5 in the regulation of the function of the adherens junction protein, VE-cadherin may exist (**Fig. 7.1**). Expression of VE-cadherin is critical for the maintenance of homophilic endothelial cell-cell contacts to maintain blood vessel integrity and the formation of a functional vascular system in mice (Vittet *et al.*, 1997). Truncation of the cytoplasmic domain of VE-cadherin in developing mice induces embryonic lethality at E9.5, due to loss of vascular integrity caused by endothelial cell

apoptosis (Carmeliet *et al.*, 1999), a phenotype similar to that observed in *Erk5*$^{-/-}$ mice. Interestingly, the mechanism leading to endothelial cell apoptosis following the deletion of *VE-cadherin* or truncation of the cytoplasmic portion of *VE-cadherin* in mice is due to the requirement for interaction between VE-cadherin, β-catenin, VEGFR-2 and PI3K for both VEGF-induced AKT activation and up-regulation of *BCL2* expression (Carmeliet *et al.*, 1999). VE-cadherin is also known to be differentially activated in endothelial cells undergoing differentiation and proliferation (Wallez *et al.*, 2006). Clear parallels seem to exist between the roles of ERK5 and VE-cadherin in mediating VEGF-stimulated endothelial cell survival, it is therefore interesting to consider the prospect that ERK5 and VE-cadherin may regulate each others' activity in some way. Future studies should aim to investigate this possibility.

Understanding the mechanism by which ERK5 mediates activation of AKT in response to VEGF stimulation in HDMECs would be of great interest. Future studies should aim to determine the expression profile of integrins in HDMECs prior to establishing whether or not ERK5 co-immunoprecipitates with certain integrins or integrin-binding proteins such as FAK or ILK in these cells. In addition, the use of blocking antibodies, to specifically block integrin binding to ECM substrates, may be useful to establish which integrins function in mediating VEGF-induced ERK5 activation and subsequent activation of AKT in HDMECs. A further approach to investigate the putative relationship between FAK and ILK with ERK5 may be to use siRNA-to down-regulate FAK or ILK expression and to subsequently assess the effects of VEGF-stimulation on ERK5 and AKT activation.

This study has revealed that ERK5 is an important regulator of VEGF-induced signalling, and gene expression. Even so, the scope of the study prevented the mechanisms by which ERK5 regulates gene expression to be fully investigated. SiRNA-mediated silencing of ERK5 expression did not affect VEGF-stimulated MEF2C phosphorylation on Ser^{387}, suggesting that ERK5 is not required for transcriptional activation of MEF2C in response to VEGF, in agreement with the recently reported findings of (Maiti *et al.*, 2008). However, VEGF-stimulated transcriptional activation of MEF2C was not measured directly in the current study. In addition, the observations that siRNA-

mediated down-regulation of ERK5 expression attenuated VEGF-stimulated activation of RELA, and lowered expression of *VCAM-1, E-selectin, IL-8* and *BCL2* suggested that ERK5 regulates expression of these genes via regulation of RELA activation. However, the transcriptional activity of RELA in response to VEGF and following ERK5 down-regulation was not directly measured in this study. It is suggested that MEF2- and RELA-dependent *cis*-reporter assays should be conducted to clarify the role of ERK5 as a regulator of RELA-dependent transcription.

Further insight into the potential role of ERK5 in regulation of VEGF-stimulated gene expression would have been gained from analysing the effects of siRNA-mediated down-regulation of ERK5 in HDMECs over a greater number of time points, and possibly extending the length of these experiments to measure gene expression at 24 h post-VEGF stimulation. However, the time-consuming nature of RNA extraction from HDMECs plated within 3-D collagen matrices would have made data collection at more acute time points technically difficult to achieve. Affymetrix oligonucleotide GeneChip™ arrays (Affymetrix UK Ltd., High Wycombe, U.K.) have been used as a means to simultaneously profile the expression of several genes in endothelial cells undergoing angiogenesis (Gerritsen *et al.*, 2003; Mellberg *et al.*, 2009). The use of such microarrays would have offered a more unbiased view of the role of ERK5 in the regulation of gene expression in HDMECs during distinct phases of angiogenesis. However, the cost of such technology prohibited their use in this project. An alternative, less costly strategy to determine the potential role of ERK5 in regulating gene expression would have been to use the commercially-available PCR arrays 'Human Angiogenesis RT^{2}' (SABiosciences™, Frederick, U.S.A.), to simultaneously profile the expression of a range of genes implicated in angiogenesis, as demonstrated in recent studies (Basile *et al.*, 2008; Bhuvaneswari *et al.*, 2008). It is suggested that future studies may benefit from the use of this technology to potentially identify additional genes of importance in angiogenesis that may be transcriptionally regulated directly, or indirectly, by ERK5. This study has shown that ERK5 regulates the phosphorylation of several proteins in response to VEGF stimulation. However, a more exhaustive study is warranted to identify additional proteins that may act as downstream effectors of ERK5 following VEGF stimulation. Hayashi *et al.* reported the

use of PepChip™ kinase arrays (Pepscan Presto BV, Lelystad, The Netherlands), which allow the simultaneous screening of several peptide kinase substrates, to determine the effects of *Erk5* ablation upon FGF-2-stimulated phosphorylation of various substrates in MLCECs (Hayashi *et al.*, 2005).

The emergence of ERK5 as a regulator of VEGF-stimulated activation of AKT raises the possibility that ERK5 may also regulate other VEGF-stimulated, AKT-regulated processes in endothelial cells, including NO production and endothelial cell permeability (Shiojima & Walsh, 2002). It therefore remains possible that ERK5 may also play an important function in regulating other physiological responses in endothelial cells that are mediated by AKT.

The production of ECM-degrading proteolytic enzymes such as MMPs is critical for the initial degradation of the ECM to allow endothelial cell migration (van Hinsbergh & Koolwijk, 2008). VEGF up-regulates expression of MMPs in endothelial cells (Zucker *et al.*, 1998). Furthermore, over-expression of MEK5 has been shown to induce MMP-9 expression in prostate cells (Mehta *et al.*, 2003). This study did not address the effects of siRNA-mediated down-regulation of ERK5 upon MMP expression in HDMECs; therefore it remains a possibility that ERK5 may regulate the expression of MMP-9 or other MMPs in endothelial cells.

7.3 Conclusions and perspectives

As the most recently discovered conventional MAPK cascade, the ERK5 signalling axis has been subject to an increasing number of studies aimed at identifying its upstream regulators and downstream effectors, as well as, crucially, aiming to determine the function of this pathway in various cellular contexts and in response to diverse extracellular stimuli. Whilst the ERK1/2 and p38 MAPK pathways are well-established as respective regulators of VEGF-stimulated endothelial cell proliferation (Wu *et al.*, 2000a), and migration (Rousseau *et al.*, 1997), the potential role of the ERK5 pathway in mediating VEGF-induced physiological responses had not been previously explored. In summary, this study has established ERK5 as a signalling protein activated

downstream of p-VEGFR-2 Tyr^{1175} and PKCδ in endothelial cells. It was found that ERK5 was not required for VEGF-stimulated proliferation or migration of HDMECs, but that ERK5 served a specific role in growth factor-induced angiogenesis by mediating endothelial cell survival in response to VEGF stimulation during tubular morphogenesis. A hitherto unknown role of ERK5 as an upstream regulator of VEGF-stimulated AKT activation leading to the regulation of BAD phosphorylation and the expression of *BCL2* has also been revealed. In addition, constitutive activation of ERK5 was found to be sufficient to facilitate tubular morphogenesis in the absence of VEGF, and to sustain capillary structure survival in the presence of VEGF. ERK5 was also shown to have an important role in regulating the expression of the pro-angiogenic chemokine *IL-8* in response to VEGF stimulation. Finally, it was demonstrated that chronic treatment with ERK5 siRNA specifically induced apoptosis of endothelial cells, but not fibroblasts, to prevent capillary network formation in a HDMEC/NHDF co-culture *in vitro* angiogenesis model.

In conclusion, this thesis demonstrates that ERK5 is necessary for mediating the survival of human microvascular endothelial cells during conditions of tubular morphogenesis, and has begun to unravel the mechanisms by which the ERK5 signalling axis regulates this process. Several new questions have arisen from this research, which hold much promise as interesting avenues of future investigation. Of particular interest is the finding that inhibition, or constitutive activation of ERK5 in HDMECs, can respectively prevent, or promote, capillary formation *in vitro,* leading to the concept that ERK5 may prove to be a feasible target for modulating angiogenesis *in vivo.* Currently approved anti-angiogenic therapies are centred around the use of inhibitors or antibodies that target VEGF or VEGFR-2 (Ferrara & Kerbel, 2005; Ellis & Hicklin, 2008), which indiscriminately inhibit all of the actions of VEGF on endothelial cells. These treatments commonly lead to complications such as the development of resistance to therapy as well as toxic side effects (Ellis & Hicklin, 2008; Bergers & Hanahan, 2008; Chen & Cleck, 2009). Data presented in this thesis suggests that targeting the ERK5 pathway *in vivo* may allow the unique opportunity to selectively induce apoptosis of endothelial cells to specifically impede a critical component of angiogenic response. Conversely, stimulating ERK5 activity by means of gene therapy

may conceivably offer a means to promote endothelial cell survival, to allow therapeutic revascularisation in conditions such as ischemia.

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APPENDICES

APPENDIX 1
Primer sequences used in this study

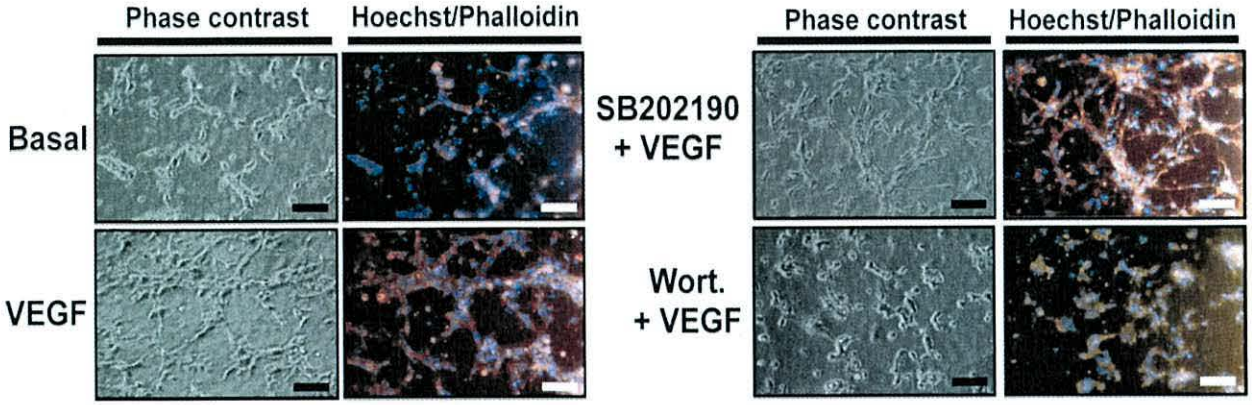
Target gene	Accession number	Forward primer	Reverse primer
A1	NM_004049	5′-TCT CAG CAC ATT GCC TCA AC-3′	5′-AGT CCT GAG CCA GCC TGT AA-3′
β-Actin	NM_001101	5′-ATG GAT GAT GAT ATC GCC GC-3′	5′-AAG CCG GCC TTG CAC AT-3′
BCL2	NM_000633	5′-GTC TGG GAA TCG ATC TGG AA-3′	5′-GCA ACG ATC CCA TCA ATC TT-3′
ERK5	NM_002749	Hs_MAPK7_1_SG QuantiTect primer assay (Qiagen, Crawley, U.K.).	Hs_MAPK7_1_SG QuantiTect primer assay (Qiagen, Crawley, U.K.).
E-Selectin	NM_000450	5′-ATC CAA AAG GCT CCA ATG TG-3′	5′-CTC CAA TAG GGG AAT GAG CA-3′
IL-8	NM_000584	5′-CAG GAA TTG AAT GGG TTT GC-3′	5′-AGC AGA CTA GGG TTG CCA GA-3′
ISG20	NM_002201	5′-AAA GAG GAC ATG AGC GGC TA-3′	5′-TTC TGG ATG CTC TTG TGC AG-3′
MEF2C	NM_002397	5′-TCT TCA ACA GCA CCA ACA AGC T-3′	5′-TCA TGC GGC TCG TTG TAC TC-3′
MEK5	NM_002757	5′-AAC CAG GTG CTG GTA ATT CG-3′	5′-ACC TGG CCT ATC ACA TCC AG-3′
Nur77	NM_002135	5′-AGT GCA GAA AAA CGC CAA GT-3′	5′-CGG ACA ACT TCC TTC ACC AT-3′
OAS1	NM_001032409	5′-ACA GGC AGA AGA GGA CTG GA-3′	5′-GGA TCA AGA GTC CCA CCT GA-3′
PKCα	NM_002737	5′-GTG TAC CCC AGA GGC CAG TA-3′	5′-GTG GGA ACT GCG GTG TTA GT-3′
PKCβ	NM_002738	5′-CTC CAC TCC TGC TTC CAG AC-3′	5′-GAA CAG ACC GAT GGC AAT TT-3′
PKCγ	NM_002739	5′-GTC TGC AGC TTT GTG GTT CA-3′	5′-TAG CTA TGC AGG CGG AAC TT-3′
PKCδ	NM_006254	5′-TTC GAT GCC CAC ATC TAT GA-3′	5′-TGC CAT TGT TCT TCT TGC AG-3′
PKCε	NM_005400	5′-AAG GCA GCC AGT CAG TCT GT-3′	5′-TAC TCA ACG AAC ACG CAA GC-3′
PKCζ	NM_001033581	5′-CCA AGA GCC TCC AGT AGA CG-3′	5′-CCA TCC ATC CCA TCG ATA AC-3′
PKCη	NM_006255	5′-CCC AGC ATC AGC CTT AGA AC-3′	5′-TGG CTC CCT CTG GAC TCT AA-3′
PKCϑ	NM_006257	5′-TGA ACG GAA GGA GAT TGA CC-3′	5′-CCA TGC TGT TGA TCA GTG CT-3′
PKCι	NM_002740	5′-TAC GGC CAG GAG ATA CAA CC-3′	5′-AAG AGC CCA CCA GTC AAC AC-3′
RCAN1	NM_004414	5′-AAA AGT ATG AAT TGC ACG CAG C-3′	5′-TCA CAT ACA TGG ACC ACC ACG-3′
Survivin	NM_001012270	5′-CAC CTG AAA GCT TCC TCG AC-3′	5′-AAC CCT TCC CAG ACT CCA CT-3′
VCAM-1	NM_001078	5′-CCT GGC TCA AGC AAC CCA AAC A-3′	5′-CCT GGC TCA AGC ATG TCA TA-3′
VEGFR-1	NM_002019	5′-TGT GCC AAA TGG GTT TCA TG-3′	5′-TGC AAG ACA GTT TCA GGT CCT C-3′
VEGFR-2	NM_002253	5′-CAA ACG CTG ACA TGT ACG GTC T-3′	5′-CCA ACT GCC AAT ACC AGT GGA-3′
VEGFR-3	NM_002020	5′-GCT CCT ACG TGT TCG TGA GAG A-3′	5′-TCC TGT TGA CCA AGA GCG TG-3′
XIAP	NM_001167	5′-CTC CCA AAG TGC TGG GAT TA-3′	5′-GCA GAC AGT GCA AAG GTG AA-3′

APPENDIX 2
siRNAs used in this study

SiRNA name	Duplex name	Target gene	Accession number	Sense sequence	Source
N.S. siRNA	Control (non-sil.) siRNA	-	-	5′-UUC UCC GAA CGU GUC ACG Utt-3′	Qiagen (Crawley, U.K.)
ERK1 siRNA 1	Hs_MAPK3_6 HP	*ERK1*	NM_001040056	5′-CGU CUA AUA UAU AAA UAU Att-3′	Qiagen (Crawley, U.K.)
ERK1 siRNA 2	Hs_MAPK3_7 HP	*ERK1*	NM_001040056	5′-CCC UGA CCC GUC UAA UAU Att-3′	Qiagen (Crawley, U.K.)
ERK2 siRNA 1	Hs_MAPK1_9 HP	*ERK2*	NM_002745	5′-CAA AGU UCG AGU AGC UAU Ctt-3′	Qiagen (Crawley, U.K.)
ERK2 siRNA 2	Hs_MAPK1_10 HP	*ERK2*	NM_002745	5′- GUU CGA GUA GCU AUC AAG Att -3′	Qiagen (Crawley, U.K.)
ERK5 siRNA 1	Hs_MAPK7_9_HP	*ERK5*	NM_002749	5′- GAC CCA CCU UUC AGC CUU Att -3′	Qiagen (Crawley, U.K.)
ERK5 siRNA 2	Hs_MAPK7_5_HP	*ERK5*	NM_002749	5′- GGA UGG CCA GGC AGA UUC Att -3′	Qiagen (Crawley, U.K.)
ERK5 siRNA 3	Hs_MAPK7_10_HP	*ERK5*	NM_002749	5′-CGA GAU CAU CGA GAC CAU Att-3′	Qiagen (Crawley, U.K.)
MEF2C siRNA 1	J-009455-05	*MEF2C*	NM_002397	5′-GAC AAG GAA UGG GAG GAU AUU-3′	Dharmacon Inc. (Lafayette, U.S.A.)
MEF2C siRNA 2	J-009455-06	*MEF2C*	NM_002397	5′-UAA CAC AGG UGG UCU GAU GUU-3′	Dharmacon Inc. (Lafayette, U.S.A.)
MEF2C siRNA 3	J-009455-07	*MEF2C*	NM_002397	5′-GAA UAA CCG UAA ACC AGA UUU-3′	Dharmacon Inc. (Lafayette, U.S.A.)
MEF2C siRNA 4	J-009455-08	*MEF2C*	NM_002397	5′-GAU CAG CAG GCA AAG AUU GUU-3′	Dharmacon Inc. (Lafayette, U.S.A.)
MEK5 siRNA 1	Hs_MAP2K5_11_HP	*MEK5*	NM_002757	5′-GAC GUA UGU UGG AAC AAA Utt-3′	Qiagen (Crawley, U.K.)
MEK5 siRNA 2	Hs_MAP2K5_12_HP	*MEK5*	NM_002757	5′-AGA CGU AUG UUG GAA CAA Att-3′	Qiagen (Crawley, U.K.)
MEK5 siRNA 3	Hs_MAP2K5_9_HP	*MEK5*	NM_002757	5′-GGC AAU GCU GUC AUA UUA Utt-3′	Qiagen (Crawley, U.K.)
MEK5 siRNA 4	Hs_MAP2K5_10_HP	*MEK5*	NM_002757	5′- AGG CCA GCA CCU GAA GAA Utt -3′	Qiagen (Crawley, U.K.)
PKCα siRNA 1	Hs_PRKCA_5_HP	*PKC alpha*	NM_002737	5′-CCA UCC GCU CCA CAC UAA Att-3′	Qiagen (Crawley, U.K.)
PKCα siRNA 2	Hs_PRKCA_6_HP	*PKC alpha*	NM_002737	5′-CAG UGG AAU GAG UCC UUU Att-3′	Qiagen (Crawley, U.K.)
PKCδ siRNA 1	Hs_PRKCD_8_HP	*PKC delta*	NM_006254	5′-GCU UCA AGG UUC ACA ACU Att-3′	Qiagen (Crawley, U.K.)
PKCδ siRNA 2	Hs_PRKCD_11_HP	*PKC delta*	NM_006254	5′-GCA GCA AUG UCA ACA UCA Att-3′	Qiagen (Crawley, U.K.)
PKCε siRNA 1	Hs_PRKCE_5_HP	*PKC epsilon*	NM_005400	5′-CGA CCA UGG UAG UGU UCA Att-3′	Qiagen (Crawley, U.K.)
PKCε siRNA 2	Hs_PRKCE_6_HP	*PKC epsilon*	NM_005400	5′-CGG AAA CAC CCG UAC CUU Att-3′	Qiagen (Crawley, U.K.)
VEGFR-2 siRNA	Hs_KDR_5 HP	*KDR*	NM_002253	5′-CGC UGA CAU GUA CGG UCU Att-3′	Qiagen (Crawley, U.K.)

APPENDIX 3

Effects of the p38 MAPK inhibitor SB202190 and the PI3K inhibitor wortmannin on VEGF-stimulated HDMEC tubular morphogenesis within 3-D collagen I gel structures



Appendix 3 Effects of the p38 MAPK inhibitor SB202190 and the PI3K inhibitor wortmannin on VEGF-stimulated HDMEC tubular morphogenesis within 3-D collagen I gel structures. HDMECs were serum-starved in EBM MV2 basal medium containing 1% (v/v) FCS for 20 h prior to seeding at 2.6 x 10^5 cells between two layers of collagen I gels in 12-well cell-culture dishes (*section 2.2.7.1*). Following incubation for 1 h at 37 °C, cells were treated with EBM MV2 basal medium containing 1% (v/v) FCS and 0.1% (v/v) DMSO (Basal), or containing VEGF (50 ng/ml) and 0.1% (v/v) DMSO (VEGF), or with VEGF (50 ng/ml) and SB202190 (10 μM) (SB202190 + VEGF), or with VEGF (50 ng/ml) and wortmannin (100 nM) (Wort. + VEGF). Cells within the collagen I gel structures were incubated for 24 h at 37 °C prior to fixing in 4% paraformaldehyde. Cell nuclei were stained with Hoechst 33342 (blue) and the F-actin fibres within the cells were stained with Alexa Fluor® 546 phalloidin (red), and visualised by inverted fluorescence microscopy (*section 2.2.7.1.7*). Scale bars = 100 μm. *DMSO, dimethyl sulfoxide; FCS, foetal calf serum; HDMEC, human dermal microvascular endothelial cell; VEGF, vascular endothelial growth factor.*