The role of αvβ3 and VEGFR2 endocytic recycling in angiogenesis

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Matthew Callum Jones (BSc Hons) MSc

Department of Biochemistry

University of Leicester

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ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

STATEMENT

The work recorded in this thesis is original and was carried out by the author, unless otherwise acknowledged in the text or by reference, in the Department of Biochemistry at the University of Leicester and at the Beatson Institute for Cancer Research between October 2002 and August 2007. No part of this work has been submitted for any degree at the University of Leicester or at any other institution.

Signed: Date:

Department of Biochemistry
Henry Wellcome Building
University of Leicester
Lancaster Road
Leicester
LE1 9HN
United Kingdom

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Matthew Jones

Abstract

Placental growth factor (PlGF) binds to VEGFR1 and is known to play a role in pathological angiogenesis, but its mechanism of action remains unclear. Endothelial cell migration in response to angiogenic stimuli requires coordination of adhesive function with VEGFR signalling, and I have studied the intracellular trafficking of integrins and VEGFRs in primary cultured human umbilical vein endothelial cells (HUVECs). VEGFR2 and αvβ3 integrin cycled rapidly between the plasma membrane and an internal pool, and treatment of HUVECs with PlGF promoted the rapid mobilisation of both VEGFR2 and αvβ3 integrin from this internal pool to the plasma membrane. This mobilisation occurred via a mechanism that was dependent on the presence of VEGFR1 and Rab4a, and required the inactivation of GSK3β, but did not require the activity of PKD1 nor the tyrosine kinase activity of VEGFRs. Furthermore, RNAi of Rab4a, αvβ3 and VEGFR1 opposed PlGF-promoted endothelial cell tubule-like structure branching and cross-bridge formation in an organotypic tube formation assay.

Taken together, these data show that PlGF can influence endothelial cell function by controlling the endocytic function of αvβ3 integrin. This recycling mechanism is required to induce endothelial cell structure branching and formation of a complex endothelial cell vessel network. In addition, regulation of VEGFR2 recycling by VEGFR1 represents a novel mechanism for mediating receptor cross talk during angiogenesis. Therefore, I have identified a novel pathway by which PlGF/VEGFR1 is able to positively influence the angiogenic response.

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ABBREVIATIONS

AEBSF	4-(2-Aminoethyl) benzynesulphonyl fluoride
AP2	Adaptor Protein-2
APC	Adenomatous polyposis coli protein
ARF	ADF-ribosylation factor
ATP	Adenosine triphosphate
BAE	Bovine aortic endothelial
BSA	Bovine serum albumin
CAM	Chick chorioallantoic membrane
CCP	Clathrin-coated pit
CEMM	Cholesterol-enriched membrane microdomain
CHO	Chinese hamster ovary
CNF	Cytotoxic necrotising factor
DAG	Diacylglycerol
DMEM	Dulbeco modified eagle medium
DN	Dominant negative
DNA	Deoxyribonucleic acid
DRG	Dorsal root ganglion
EC	Endothelial cell
ECL	Enhanced chemi-luminescence
EE	Early endosome
EEA	Early endosomal antigen
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbant assay
EPC	Endothelial progenitor cell
ERK	Extracellular related kinase
FAK	Focal adhesion kinase
FCS	Foetal calf serum
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor

FIP	Rab11 family of interacting protein
FN	Fibronectin
GAP	GTPase activating protein
GDF	GDI-displacement factor
GDI	GDP dissociation inhibitor
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GLUT4	Glucose transporter
GRB	Growth factor receptor-binding protein 2
GSK	Glycogen synthase kinase
GTP	Guanosine triphosphate
HDF	Human dermal fibroblast
HGFR	Hepatocyte growth factor receptor
HUVEC	Human umbilical vein endothelial cell
IAA	Iodoacetamide
IGF	Insulin growth factor
IL	Interleukin
ILK	Integrin-linked kinase
IP	Immunoprecipitation
IP3	Inositol Phosphate
IR	Insulin receptor
JNK	c-jun N-terminal kinase
kDa	Kilo Daltons
KGFR	Keratinocyte growth factor receptor
LDL	Low density lipoprotein
LPA	Lysophosphatidic acid
MAPK	Mitogen activated protein kinase
MDC	Monodansyl cadaverine
MEK	MAPK/ERK kinase
MLC	Myosin light chain
MMP	Matrix metalloproteinase
MPR	Mannose-6-phosphate receptor
NGF	Neuronal growth factor
NOS	Nitric oxide synthase

PAE	Porcine aortic endothelial
PAGE	Polyacrylamide gel electrophoresis
PAI-1	Plasminogen activator inhibitor type-1
PAK	Rac-activated serine/threonine kinase
	p21 activated kinase
PBS	Phosphate buffered saline
PDGF	Platelet derived growth factor
PDGFR	Platelet derived growth factor receptor
PDK	3-phosphoinositide dependent kinase
PECAM	Platelet endothelial cell adhesion molecule
PI(3)K	Phosphoinositide 3-kinase
PIP2	Phosphatidylinositol bisphosphate
PKB	Protein kinase B
PKC	Protein kinase C
PKD	Protein kinase D
PlGF	Placental growth factor
PLC	Phospholipase C
PNRC	Perinuclear recycling compartment
PQ	Primaquine
PVR	PDGF- and VEGF-related receptor
RAB	Ras related proteins in the brain
RABF	Rab family domain
RABSF	Rab subfamily domain
REP	Rab escort protein
RGGT	Rab geranyl geranyl transferase
RILP	Rab7 interacting lysosomal protein
ROCK	Rho-kinase
RTK	Receptor tyrosine kinase
SDS	Sodium dodecyl sulphate
SH2 and SH3 domain	Src homology region
TGF	Transforming growth factor
TGN	Trans golgi network
TM	Transmembrane
TNFα	Tumour necrosis factor alpha

UB	Ureteric bud
uPA	Urokinase plaminogen activator
uPAR	Urokinase plasminogen activator receptor
VEGF	Vascular endothelial growth factor
VEGFR1	Vascular endothelial growth factor receptor-1
VEGFR2	Vascular endothelial growth factor receptor-2
VSMC	Vascular smooth muscle cell

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Chapter One: Introduction

1.1 ANGIOGENESIS

1.1.1 The vasculature

In higher organisms the blood vasculature represents one system through which gases, liquids, nutrients, signalling molecules and cells are transported between tissues and organs. The vascular system consists of two largely distinct networks of arterial and venous vessels that have clear structural similarities. Both are composed of an inner endothelium surrounded by internal elastic tissue, a smooth muscle layer, external elastic tissue and then fibrous connective tissue (Figure 1.1). Veins however have a markedly thinner smooth muscle cell layer and contain specialist structures such as valves to prevent backflow [1]. These structural differences reflect the differences in physiological parameters such as blood flow pressure and shear stress that the types of vessel have to endure, and for a while it was believed that these structural changes were the only differences between veins and arteries.

It is now becoming clear that molecular differences between artereous and venous endothelial cells exist even prior to the formation of blood vessels. For example, proteins in the Notch pathway may be involved in the differentiation of the arterial branch. Notch receptors are transmembrane proteins that mediate signalling between neighbouring cells via interactions with transmembrane ligands known collectively as DSL ligands (Delta, Serrate and Lag2). Notch-1 and Notch-4 are expressed specifically in arterial endothelial cells whereas Notch-3 is expressed in arterial smooth muscle cells [2]. In addition the Notch ligands Delta-4, Jagged-1 and Jagged-2 are expressed in arterial endothelial cells [2]. Therefore Notch signalling

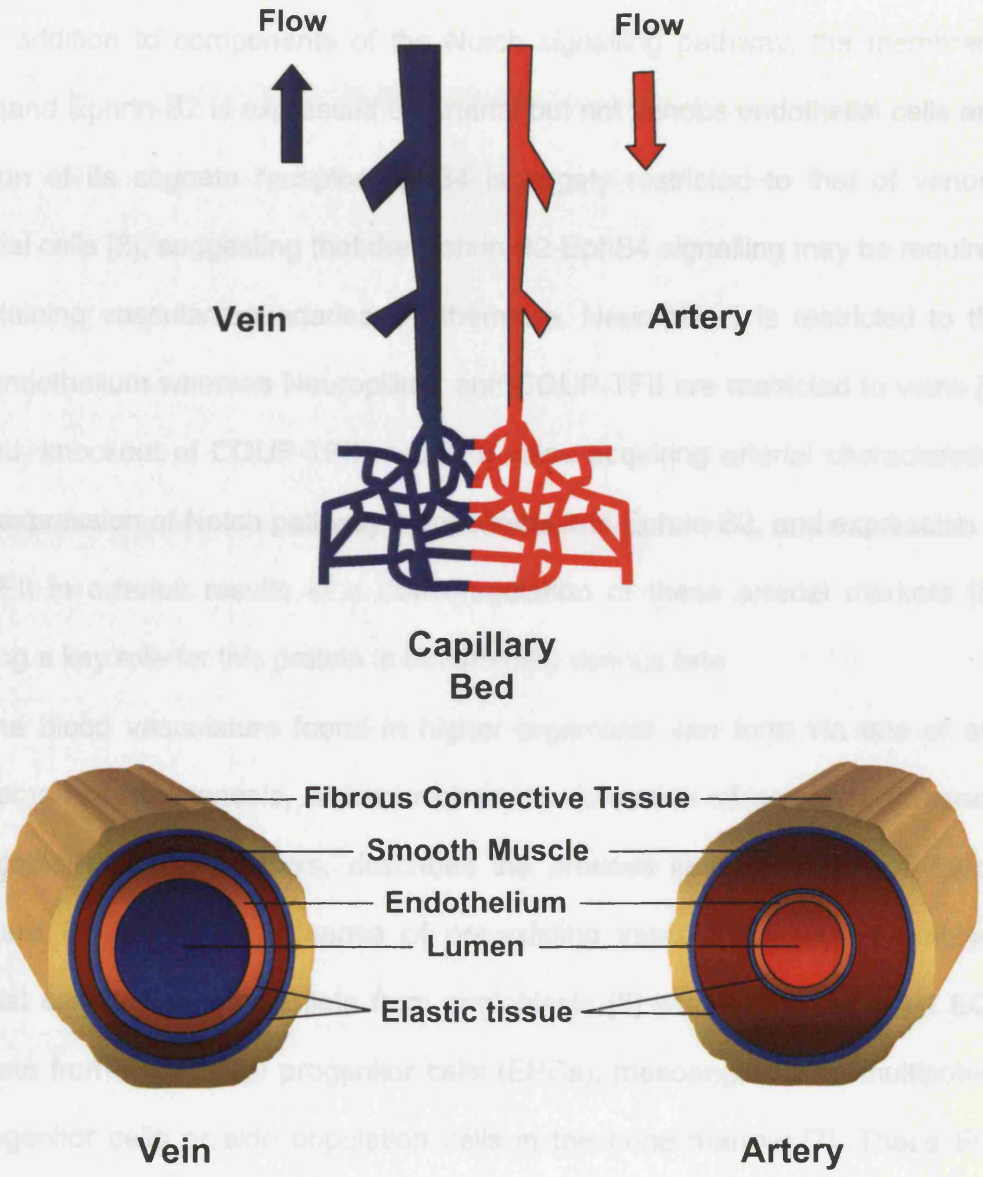

Figure 1.1: Arteries and Veins

Arteries and veins are both composed of an inner endothelium surrounded by inner elastic tissue, smooth muscle cells, external elastic tissue and fibrous connective tissue. Larger arteries have thicker smooth muscle cell layers while larger veins contain specialised structures such as valves. The two networks are largely distinct but are linked together distally via a system of capillaries found throughout all tissues.

appears to occur specifically in arteries and, as such, Notch-regulated expression of genes may be important in arterial differentiation.

In addition to components of the Notch signalling pathway, the membrane bound ligand Ephrin-B2 is expressed by arterial but not venous endothelial cells and expression of its cognate receptor EphB4 is largely restricted to that of venous endothelial cells [3], suggesting that the Ephrin-B2-EphB4 signalling may be required for maintaining vascular boundaries. Furthermore, Neuropilin-1 is restricted to the arterial endothelium whereas Neuropilin-2 and COUP-TFII are restricted to veins [4, 5]. Indeed, knockout of COUP-TFII results in veins acquiring arterial characterstics such as expression of Notch pathway components and Ephrin-B2, and expression of COUP-TFII in arteries results in a down-regulation of these arterial markers [5], suggesting a key role for this protein in determining venous fate.

The blood vasculature found in higher organisms can form via one of two mechanisms. Vasculogenesis, which involves the formation of new blood vessels from endothelial cell precursors, describes the process in which *de novo* blood vessels are formed in the absence of pre-existing vasculature. In the embryo, endothelial cells (ECs) differentiate from angioblasts [6] whereas in the adult ECs differentiate from endothelial progenitor cells (EPCs), mesoangioblasts, multipotent adult progenitor cells or side population cells in the bone marrow [7]. These EC-precursors migrate into defined locations and then differentiate forming solid endothelial cords that subsequently develop a lumen. The subsequent formation of new vessels from a pre-existing vasculature is termed angiogenesis, and this process is responsible for creating the complex branched morphology of the vascular network. Angiogenesis is thought to occur via three mechanisms: The formation of transendothelial bridges and intussusception that act to separate large parent

vessels into smaller daughter vessels, and sprouting of new vessels from the exterior of existing one. It is likely that all three mechanisms combine during development, whereas sprouting angiogenesis is the key mechanism by which new vessels are formed in response to angiogenic stimuli in the adult. In view of this, it is this mechanism which will be referred to whenever angiogenesis is mentioned.

1.1.2 Sprouting angiogenesis

Sprouting angiogenesis can be thought of as occurring in a step-wise fashion (Summarised in Figure 1.2). Following an angiogenic stimulus, vessels first dilate and an increase in vascular permeability is observed following a redistribuion of adhesion molecules such as Platelet Endothelial Cell Adhesion Molecule (PECAM)-1 and Vascular Endothelial (VE)-Cadherin. Extravasation of plasma proteins such as fibrinogen follows which is proteolytically cleaved to form fibrin, which constitutes a major component of the provisional matrix through which endothelial cells migrate [8]. Vascular permeability must be tightly regulated and Angiopoietin-1 (Ang1) is an anti-permeability factor that provides protection against excessive permeability via its interactions with the Tie2 receptor [9]. In contrast, Angiopoietin-2 (Ang2) is an antagonist of Tie2 signalling and is involved in detaching vascular smooth muscle cells and modifying the surrounding matrix to allow endothelial cell migration [10]. Therefore the balance between Ang1 and Ang2 is key to the initial stages of angiogenesis.

Degradation of the extracellular matrix is also critical to the initiation of angiogenesis, and a number of matrix metalloproteinases (MMPs) have been linked to this process. MMPs 2,3,7 and 9 have been shown to be required for induction of angiogenesis [11, 12] and Thrombospondin-1 (TSP-1) is believed to exert its anti-

angiogenic function via inhibition of MMP-2 and MMP-9 activity [13]. It is clear that a correct balance between proteinase activity and inhibition is required for efficient angiogenesis to occur. Endothelial cells produce TIMP-1, -2 and -3 which are inhibitors of MMP activity [14] and TIMP-1 acts to promote vascularisation of mouse retina [15].

As matrix barriers are degraded this allows endothelial cells to migrate into distant sites. The growing sprout is lead by 'tip' cells selected from the vessel walls whose polarity is reversed prior to leading new sprouts from the outer side of the endothelium. Sprout elongation must then continue in a polarised and directional manner, and this is achieved via a concentration gradient of matrix bound Vascular Endothelial Growth Factor (VEGF) binding to its cognate receptor VEGFR2 expressed at sprout tips resulting in tip cell extension of filopodia [16]. This extension of filopodia allows tip cells to sense positive or negative guidance cues in the surrounding tissue environment. For example members of the Class-3 Semaphorin family have been linked to both axonal and vascular patterning with SEMA3E and its receptor PlexinD1 in particular having been identified as inducing a repulsive cue in endothelial cells [17, 18]. Furthermore the Netrin receptor UNC5B is expressed in capillaries and endothelial tip cells, and stimulation of this receptor with Netrin-1 results in the retraction of tip cell filopodia [19] suggesting a negative role for this receptor-ligand pair in regulating vascular sprouting. However, other Netrins have been implicated in the promotion of angiogenesis during development and in pathological settings [20].

Once tip cells reach their destination they need to suppress their motile behaviour upon interaction with their targets, such as other sprouts or existing stable vessels, and this presumably involves the formation of EC-EC contacts, but how this

process occurs and is regulated is unknown. Once this has occurred endothelial cords acquire lumen, although little is known as to how lumen are formed in the vasculature. Currently it is thought that lumen formation involves the process of pinocytosis and vacuole formation involving the small GTPases cdc42 and Rac1 [21] followed by intra- and intercellular fusion of large vacuoles [22]. These newly formed tubules are perfused and then stabilised by mural cells – pericytes and vascular smooth muscle cells – which inhibit endothelial cell proliferation and migration [23] in conjunction with deposition of subendothelial basement membranes that provide endothelial growth arrest cues [8], resulting in mature functioning blood vessels.

Angiogenesis is critical in a number of physiological and pathophysiological processes. In the normal adult, angiogenesis occurs during the ovarian cycle and in processes such as wound healing. Overall though, little turnover of endothelial cells is observed [24]. When blood vessel growth is deregulated, however, it can contribute to a wide range of disorders. In the case of tumour growth, angiogenesis is essential for establishing a nutrient supply to allow continued growth. In addition, the formation of the unstable blood vessels associated with tumours provides a route for invasive malignant cells to enter the circulation and seed metastases [25]. Hence, it is apparent that the induction of angiogenesis by a tumour is a critical step in malignant progression.

In addition to cancer, excessive angiogenesis has also been linked with a variety of infectious diseases [26, 27], psoriasis and arthritis [28], among other disorders. In addition, insufficient angiogenesis or vessel regression is a characteristic of many other disorders, for example: alzheimers disease [29], pulmonary fibrosis and emphysema [30], diabetes [31, 32] and strokes [33].

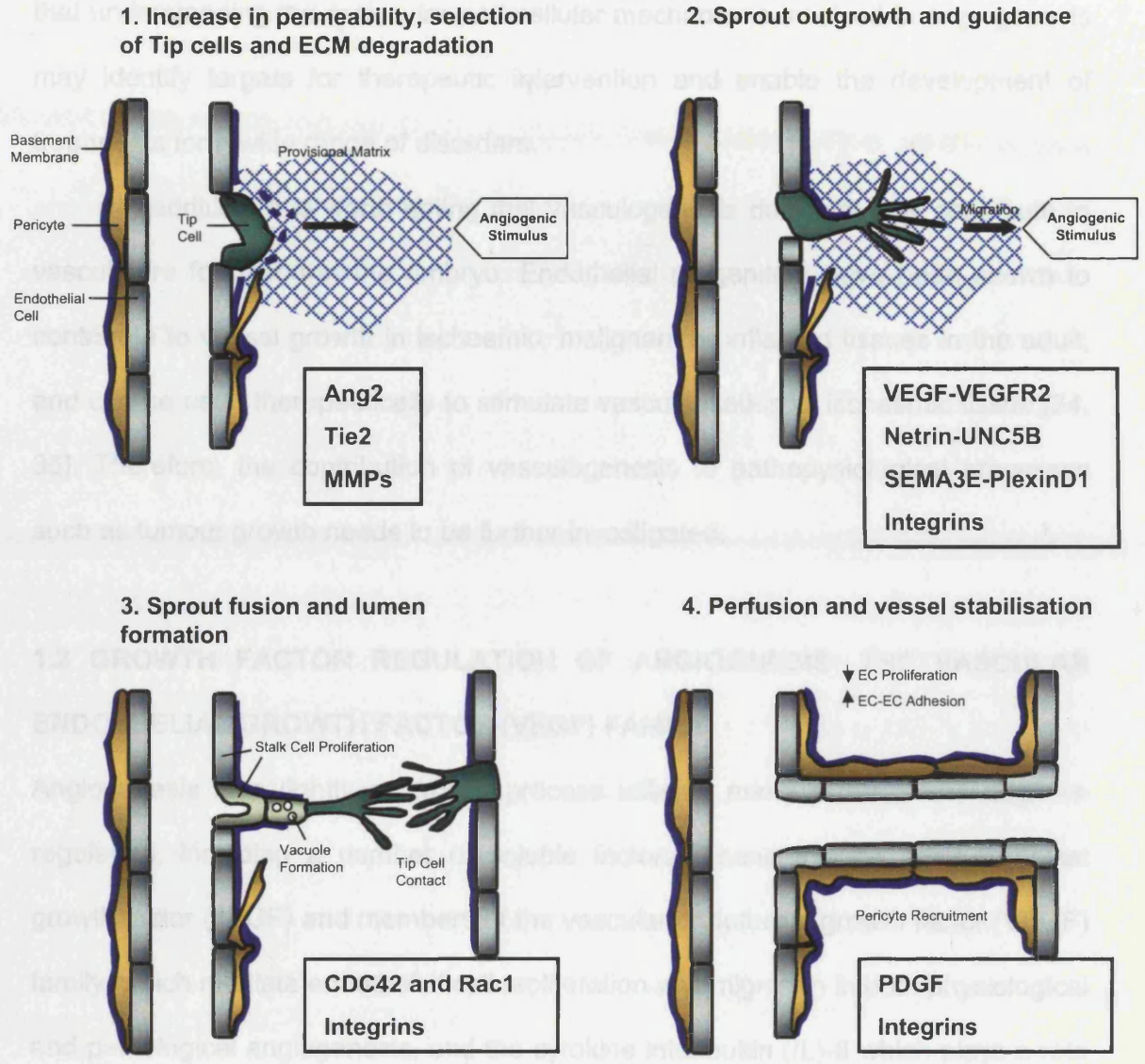

Figure 1.2: Sprouting Angiogenesis

1. In response to angiogenic stimuli such as the presence of Vascular Endothelial Growth Factor (VEGF), vascular permeability is increased following vessel destabilisation resulting in extravasation of plasma proteins that form the provisional matrix. A Tip endothelial cell (EC) is selected which flips its apical-basal polarity and migrates through the matrix, a process which is believed to require MMP mediated matrix degradation. **2.** The growing EC sprout is guided by VEGF gradients along with potentially other environmental cues. **3.** Tip cells reach their destination and regulate the fusion of sprouts with adjacent vessels. Stalk cells proliferate and form lumen via the fusion of vacuoles, although other mechanisms may be involved. **4.** Release of PDGF by endothelial cells, in conjunction with the initiation of blood flow, promotes vessel stabilisation and maturation via tightening of EC-EC junctions and recruitment of pericytes.

Given the importance of the vasculature in a multitude of disorders, it is clear that understanding the molecular and cellular mechanisms involved in angiogenesis may identify targets for therapeutic intervention and enable the development of treatments for a wide range of disorders.

In addition, it is worth noting that vasculogenesis does not only contribute to vasculature formation in the embryo. Endothelial progenitors have been shown to contribute to vessel growth in ischaemic, malignant or inflamed tissues in the adult, and can be used therapeutically to stimulate vascularisation of ischaemic tissue [34, 35]. Therefore, the contribution of vasculogenesis to pathopysiological processes such as tumour growth needs to be further investigated.

1.2 GROWTH FACTOR REGULATION OF ANGIOGNESIS: THE VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF) FAMILY

Angiogenesis is a tightly controlled process utilising many positive and negative regulators, including a number of soluble factors. These include basic-fibroblast growth factor (bFGF) and members of the vascular endothelial growth factor (VEGF) family, which mediate endothelial cell proliferation and migration in both physiological and pathological angiogenesis, and the cytokine interleukin (IL)-8 which plays a role in tumour neovascularisation [36].

Both physiological and pathophysiological angiogenesis appear to rely heavily on VEGFs. VEGFs are members of the platelet-derived growth factor (PDGF) family of growth factors and are encoded by a family of genes consisting of VEGF-A, -B, -C, -D [37] and placental growth factor (PlGF) [38]. In terms of effect, VEGF-A, -B and PlGF are linked to blood vessel formation whilst VEGF-C and –D are linked to the formation of lymphatic vessels [39]. In addition to these mammalian genes, viral

VEGFs expressed by pox viruses of the Orf family are referred to as VEGF-E [27] and a family of snake venom VEGF variants are called VEGF-F collectively [40, 41].

VEGF variants are expressed as a number of different isoforms demonstrating different signalling properties. For example, the VEGF-A gene encodes peptides of 206, 189, 165 and 121 amino acids. In addition, alternative splicing of each splice variant can introduce an extra level of variability. For example, VEGF-A165b carries sequences encoded by exon 9 at the carboxy terminus as opposed to sequences encoded by exon 8 seen in VEGF-A165. This alternative form of VEGF-A165 inhibits VEGF-A driven signalling [42], demonstrating that multiple isoforms of VEGF-A are able to influence and regulate the complexity of VEGF-signalling in both negative and positive fashions.

All the VEGFs outlined are able to influence either blood or lymph vessel formation, but VEGF-A is by far the best characterised and possibly the most biologically significant variant in terms of blood vasculature formation. The presence of both VEGF-A and its receptor VEGFR2 is essential for both embryonic development [43] and tumour vascularisation [44, 45], with VEGF-A165 being the main isoform studied for the ability to induce angiogenesis [46]. For this reason, the biological effects of VEGF-A will be further considered along with that of PlGF. The precise role for PlGF in angiogenesis remains unclear, although it has a particular significance for pathological angiogenesis.

1.2.1 VEGF-A

VEGF-A is a cytokine necessary for vascular development and was first identified as a vascular permeability factor released by tumours [47], a process mediated by calcium influx [48] and the redistribution of intercellular adhesion molecules such as

platelet endothelial cell adhesion molecule (PECAM)-1 and vascular endothelial (VE)-Cadherin. Indeed, phosphorylation of VE-cadherin [49] β-catenin [50] and connexin 43 [51], all major components of tight, gap and adherens junctions have been reported in response to VEGF-A.

In vitro, VEGF-A is able to act as a survival factor for ECs, as shown by the ability of VEGF-A to prevent apoptosis in ECs grown in the absence of serum. This occurs via activation of the PI3-kinase/Akt pathway [52] and the induction of expression of anti-apoptotic caspase inhibitor molecules such as Bcl-2 and A1 [53]. This ability of VEGF-A to act as a survival factor and influence EC apoptosis has also been shown *in vivo*, where the endothelium of newly formed vessels in tumours displays VEGF-A dependence. This dependence on VEGF-A is subsequently lost when vessels are stabilised by pericyte recruitment and become established [54]. In addition to acting as an EC survival factor and mediating the dissolution of cell-cell contacts, VEGF-A is able to contribute to many of the subsequent steps involved in angiogenesis. VEGF-A is able to stimulate EC proliferation [55] and migration [56], and has been shown to increase lumen formation following assembly of EC cords [57]. Therefore, it is clear that VEGF-A is able to stimulate and mediate all the major stages of vascular sprouting.

1.2.2 PlGF

While PlGF is primarily expressed in the placenta, transcripts have also been identified in tissues of the lung, heart, thyroid gland and skeletal muscle [58]. PlGF is expressed at low levels by quiescent ECs as well as vascular smooth muscle cells, bone marrow cells, neurons and inflammatory cells [28, 59]. Upon activation, angiogenic ECs upregulate expression of PlGF [60], suggesting that PlGF plays a

role in initiating angiogenesis. However, whilst PlGF is able to affect vascular development it is not required for angiogenesis during development [60].

PlGF contributes to revascularisation of ischaemic tissues [28] and may even be more potent than VEGF-A in this regard. Genetic deletion of PlGF significantly impairs revascularisation of ischaemic tissues [28] as well as angiogenesis associated with tumour growth [61]. Therefore, PlGF is a potent regulator of pathological angiogenesis. PlGF has been reported to stimulate EC proliferation [62, 63] and migration [63] and hence can influence EC behaviour and the angiogenic response. This role, however, may not be due to the direct effects of PlGF on ECs, as is the case for VEGF-A, as PlGF has also been reported to enhance the EC response to VEGF-A [60]. Therefore, the direct effect of PlGF on ECs is open to debate, as any observed response to PlGF could be due to the influence that PlGF has on VEGF-A signalling.

PlGF may not, however, only act through ECs to regulate angiogenesis as it is also able to affect multiple other cell types involved in vascular development, such as smooth muscle cells and haematopoietic progenitor cells [60]. In addition, PlGF is chemoattractant for monocytes and macrophages [28, 64], cell types required to induce remodelling of the extracellular matrix and allow smooth muscle cell migration. Monocytes and macrophages in turn produce PlGF when activated [60], thus establishing a positive feedback loop.

PlGF clearly influences angiogenesis, although via differing mechanisms to that of VEGF-A. The ability of both VEGF-A and PlGF to effect EC behaviour, and how they differ, can be further understood when signalling via their cognate receptors is considered.

1.2.3 VEGF-Receptors

VEGF receptors (VEGFRs) are type-III receptor tyrosine kinases (RTKs), closely related to Fms and PDGF receptors, consisting of seven extracellular immunoglobulin (Ig)- like domains, a transmembrane (TM) domain, a regulatory juxtamembrane domain, an intracellular kinase domain interrupted by a kinase insert domain followed by a series of tyrosine residues involved in recruitment of adaptor molecules. VEGFRs are found almost exclusively on endothelial cells [56], but members of the VEGFR family are also expressed in haematopoietic cells [65], macrophages [56], vascular smooth muscle cells [66] as well as some malignant cells [67]. These VEGFRs relay signals for processes essential in the stimulation of vessel growth, such as: vasorelaxation, induction of vascular permeability and endothelial cell proliferation, migration and survival [68].

The VEGFR family consists of 3 members: VEGFR1 (Flt-1), VEGFR2 (KDR) and VEGFR3 (Flt-4), which are activated by specific ligands of the VEGF family. VEGF-A binds to VEGFR1 and VEGFR2 and VEGF-C and -D bind VEGFR2 and VEGFR3. PlGF and VEGF-B exhibit individual receptor specificity towards VEGFR1 [69, 70] and VEGF-E binds VEGFR2 only [27] (summarised in Figure 1.3). VEGF-F variants can bind to either VEGFR1 [71] or VEGFR2 [40] specifically.

Like other RTKs, VEGFRs are activated upon ligand binding induced receptor dimerisation [72]. This induces a conformational change in the kinase domain, via a mechanism involving rearrangement of the TM and juxtamembrane domains, and activation of kinase activity leading to autophosphorylation of VEGFR tyrosine residues which have either regulatory or signalling properties.

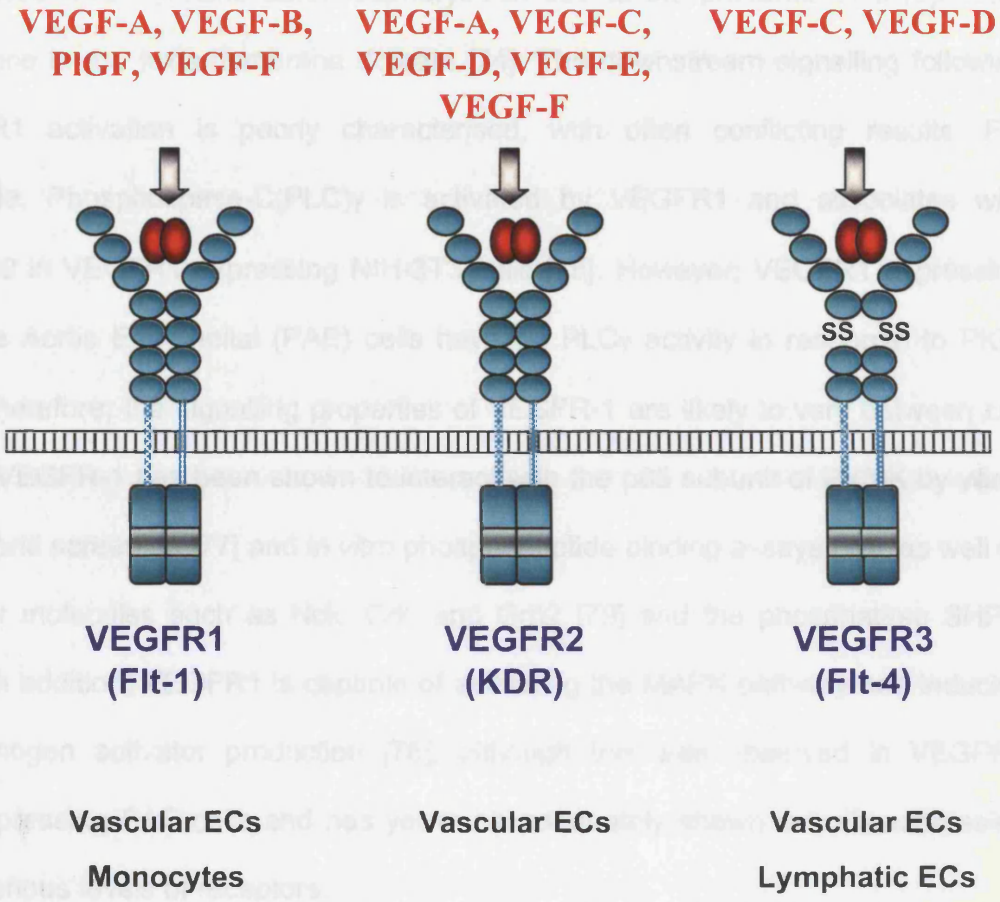

Figure 1.3: VEGF receptors and their ligands

VEGF receptors (VEGFRs) are type-III receptor tyrosine kinases (RTKs) consisting of seven extracellular immunoglobulin (Ig)- like domains, a transmembrane (TM) domain, a regulatory juxtamembrane domain, an intracellular kinase domain interrupted by a kinase insert domain followed by a series of tyrosine residues involved in recruitment of adaptor molecules. Members of the VEGFR family are expressed in vascular endothelial cells with VEGFR1 also being exclusively expressed on monocytes and VEGFR3 being expressed in lymphatic endothelial cells. VEGF-A binds to VEGFR1 and VEGFR2 and VEGF-C and -D bind VEGFR2 and VEGFR3. PlGF and VEGF-B exhibit individual receptor specificity towards VEGFR1 and VEGF-E binds VEGFR2. VEGF-F variants can bind to either VEGFR1 or VEGFR2 specifically.

1.2.3.1 VEGFR1 Signalling

VEGFR1 binds VEGF-A with a 10-fold higher affinity than VEGFR2 [73], but only undergoes weak tyrosine autophosphorylation due to the presence of a repressor sequence in the juxtamembrane domain [74]. The downstream signalling following VEGFR1 activation is poorly characterised, with often conflicting results. For example, Phospholipase-C(PLC)γ is activated by VEGFR1 and associates with Tyr1169 in VEGFR1 expressing NIH-3T3 cells [75]. However, VEGFR1 expressing Porcine Aortic Endothelial (PAE) cells have no PLCγ activity in response to PlGF [76]. Therefore, the signalling properties of VEGFR-1 are likely to vary between cell types. VEGFR-1 has been shown to interact with the p85 subunit of PI(3)K by yeast two hybrid screening [77] and *in vitro* phosphopeptide binding assays [78], as well as adaptor molecules such as Nck, Crk, and Grb2 [79] and the phosphatase SHP-2 [80]. In addition, VEGFR1 is capable of activating the MAPK pathway and inducing plasminogen activator production [76], although this was observed in VEGFR1 overexpressing PAE cells and has yet to be adequately shown in cells expressing endogenous levels of receptors.

In vivo studies have produced clear evidence for the biological role of VEGFR1. Mice that express a truncated form of VEGFR1 that lacks the tyrosine kinase domain develop normally [81], this contrasts dramatically to VEGFR1 knockout mice which die early in development due to increased production of endothelial cell progenitors [82]. This indicates that during development VEGFR1 is a negative regulator of angiogenesis, and this is further supported by the presence of a splice variant of VEGFR1 that lacks the TM domain and kinase domain during development. This soluble form of VEGFR1 is therefore deficient in signalling, but is expressed in a number of tissues in the embryo, and is believed to act as a

catalytically inert decoy receptor that binds to VEGF-A and thus inhibits VEGF-A induced angiogenesis [83, 84].

It is the ability of VEGFR1 to bind VEGF-A with higher affinity than VEGFR2 that may contribute to the ability of PlGF to potentiate VEGF-A driven angiogenesis. PlGF acts to displace VEGF-A from VEGFR1, thus increasing the amount of VEGF-A available to mediate signalling via VEGFR2 [85]. In addition to this indirect mechanism for mediating signalling via VEGFR2, the kinase activity of VEGFR1 has been shown to potentiate VEGFR2 signalling in pathological angiogenesis [85-87]. In particular, the presence of PlGF and functional tyrosine kinase activity of VEGFR1 has been shown to transphosphorylate VEGFR2 in mouse capillary endothelial cells (CECs). Following treatment of CECs with PlGF and the VEGFR2 specific ligand VEGF-E, a 13-fold increase in VEGFR2 phosphorylation was observed compared to a 4-fold increase observed when only VEGF-E was used as a stimulus, and a similar amplification was seen in human umbilical vein endothelial cells (HUVECs) [85]. This demonstrates that signalling through VEGFR1 is able to directly influence VEGFR2 signalling as well as indirectly control signalling through the regulation of VEGF-A concentrations at the plasma membrane.

VEGFR1 signalling has also been linked with the induction of MMP-9 in lung ECs and to facilitate lung metastases [88]. This activation of proteases may indicate a role for VEGFR1 signalling in the initial stages of angiogenesis and cell migration. Indeed, VEGFR1 blocking antibodies prevented migration but not proliferation of HUVECs in response to VEGF-A [89]. In line with this role, the migration of monocytes/macrophages in response to PlGF and VEGF-A stimulation is mediated by VEGFR1. Monocytes do not express VEGFR2, and hence the increased intracellular calcium levels and enhanced migration observed after PlGF stimulation

are mediated by VEGFR1 [90]. This response was also observed to be suppressed in monocytes from mice expressing the kinase-deleted VEGFR1 [81], confirming the potential role for VEGFR1 signalling in EC migration and recruitment of monocytes during angiogenesis. In addition, VEGFR1 signalling has also been shown to play a clear role in recruitment of endothelial progenitor cells (EPCs) from bone marrow [91] and recruitment of EPCs to the sites of tumourigenesis [92], a mechanism by which tumour-associated PlGF was able to increase tumour vasculogenesis and therefore enhance tumour size.

Therefore, VEGFR1 signalling plays a clear role in vascular development as a negative regulator during embryogenesis, but acts as a positive regulator during pathological angiogenesis in the adult. This is achieved through the regulation of multiple cell types such as monocytes and macrophages, smooth muscle cells, EPCs and vascular endothelial cells. VEGFR1 acts to enhance the effects of VEGFR2 signalling by being able to rapidly increase the concentration of VEGF-A available to VEGFR2 and by transactivation of VEGFR2. However, the precise signalling pathways activated directly by VEGFR1 in endothelial cells and how these contribute to endothelial cell function during angiogenesis remain unclear.

1.2.3.2 VEGFR2 Signalling

The major mediator of the mitogenic, angiogenic and permeability enhancing effects of VEGF is accepted to be VEGFR2. VEGFR2 is activated upon ligand-stimulated dimerisation and autophosphorylation of several tyrosine residues in the cytoplasmic domain [93]. The most prominent autophosphorylation sites in VEGFR2 were identified as being Tyr951, 1054, 1059, 1175 and 1214 [94]. These sites are believed to facilitate VEGF-A induced phosphorylation of a range of proteins in endothelial

cells. For example, Tyr1175 is the most important site in the VEGF-A dependent activation of Phospholipase C-γ and downstream pathways [95], and mice expressing receptors mutated at this residue died *in utero* due to vascular defects [96]. In addition to Phospholipase C-γ, VEGFR2 tyrosine residues have been shown to be required for the recruitment of the SH2 domain-containing proteins Grb2 [97], Nck [98] and Sck [99], Ras-GTPase activating protein [98], Src family GTPases [100] and protein tyrosine phosphatases [97]. Hence, VEGFR2 has the ability to recruit and activate a wide spectrum of signalling molecules and subsequent downstream pathways, allowing VEGFR2 to influence EC survival, proliferation, differentiation and migration as well as vessel permeability.

VEGFR2 activation is required for the anti-apoptotic effects of VEGF-A observed in HUVECs. HUVEC survival required active Akt downstream of VEGFR2 signalling and was inhibited by Wortmannin treatment, demonstrating that VEGF-promoted EC survival is mediated by the PI-3 kinase/Akt pathway [52]. In addition, survival signals through VEGFR2 require VE-Cadherin and the formation of stable adherens junctions that contain VEGFR2 and VE-Cadherin in complex with β-catenin and PI3K. Disruption of these complexes by expression of a truncated form of VE-Cadherin unable to bind β-catenin, or by VE-Cadherin antibodies, resulted in decreased Akt phosphorylation in response to VEGF-A and increased apoptosis [101].

The role of VEGFR2 in promoting EC mitogenesis has been clearly outlined and thoroughly investigated. Activation of the Raf/Mek/Erk pathway by VEGF-A has been shown in a variety of endothelial cells [76, 102] and the association of VEGFR2 with Grb2, either directly via Tyr1214 [103] or indirectly via the adaptor protein Shc [97], indicates that Ras may be involved in the activation of the Raf/Mek/Erk cascade

and regulation of the VEGF-induced mitogenic response. However, this may not necessarily be the case as Akt has been shown to phosphorylate endothelial nitric acid synthase (eNOS) in a Ca^{2+} dependent manner [104], leading to an increase in cGMP levels that may contribute to the activation of the MAPK cascade. In fact, this response has been shown to be partially prevented in HUVECs by treatment with Nitric Oxide Synthase (NOS) inhibitors [105].

Recruitment of Phospholipase C-γ to Tyr1175 of VEGFR2 is essential for EC mitogenesis. Activation of Phospholipase C-γ leads to the generation of inositol phosphate (IP3) and diacylglycerol (DAG) which in turn activate protein kinase C(PKC)s, the net result of which is the induction of Ras-independent Raf activation and subsequent ERK activation [106]. This role for PKC signalling in ECs is further supported by the ability of PKC inhibitors to inhibit mitogenic signalling initiated by VEGF-A [107]. It has also been shown that VEGF-A can selectively activate the Ca^{2+} sensitive PKC isoforms γ and β2 in bovine aortic endothelial (BAE) cells [106, 108] and PKCε in HUVECs [109].

In addition to EC proliferation and survival, VEGFR2 signalling is directly linked to the regulation of EC migration. The ability of VEGFR2 to influence EC migration appears to be primarily regulated by phosphorylation of focal adhesion kinase (FAK). VEGFR2 signalling results in the activation of FAK, a process which requires PKC signalling, and the subsequent recruitment of FAK and paxillin to new focal adhesions [110]. As with many other aspects of VEGFR2 signalling, this activation of FAK is dependent on signalling via Tyr1175 of VEGFR2. Tyr1175 phosphorylation recruits the adaptor protein Shb and subsequently activates both FAK and PI3-kinase, and hence regulates the formation of stress fibres and focal adhesions as well as cell migration [111]. Furthermore, VEGFR2 has been shown to

regulate the activation of both Rho and Rac small GTPases via a mechanism involving subunits of heterotrimeric G-proteins such as Gq/11 [112]. Therefore, signalling via VEGFR2 is clearly able modulate actin dynamics in EC migration.

1.3 THE EXTRACELLULAR MATRIX, INTEGRINS AND ANGIOGENESIS

EC interaction with the extracellular matrix (ECM) allows contact with surrounding tissues and prevents vessel collapse, as well as being required to facilitate EC proliferation and migration, and is therefore also critical for efficient angiogenesis.

In quiescent vessels, the ECM consists of a basement membrane which mediates interactions between ECs and surrounding pericytes, and an interstitial matrix which is located in between vascular cells. The basement membrane is typically composed of collagen IV, laminin and heparin sulphate proteoglycans, and the interstitial matrix is comprised of fibrillar collagens, fibronectin and elastin [113]. Upon the induction of angiogenesis there are clear changes in the make-up of the vascular ECM and EC-ECM interactions, with EC having to detach from some ECM components whilst degrading and reattaching to others. At the early stages of angiogenic sprouting, VSMCs must first detach from the vessel. This allows the underlying ECs to degrade and invade the basement membrane and initiate tube formation. Proteolysis of ECM components does not merely clear a path for EC migration but can alter the composition of the ECM by either exposing cryptic epitopes or changing the structure of ECM components, processes which can induce EC migration [114]. In addition, ECs secrete components of a provisional matrix consisting of fibronectin, fibrin and other components, which guide EC migration [115]. In turn, once tube formation has occurred the initial ECM needs to reform and interact with the new vessel in order to stabilise the vessel. Therefore, ECs need to

be able to sense and respond accordingly to these changes in ECM components at differing stages of angiogenesis.

1.3.1 Integrins in vascular development

Endothelial cell interaction with the ECM is primarily mediated by integrins: A family of heterodimeric transmembrane proteins consisting of α and β subunits which mediate cell-ECM and cell-cell contacts [116]. Overall there are over 18α and 8β subunits which can heterodimerise in over 24 different combinations, and hence allow binding to the entire range of ECM ligands with varying degrees of selectivity and specificity [117]. Following ligand binding, integrins are able to recruit numerous adaptor proteins as, due to the lack of intrinsic enzymatic activity, integrins must associate with other proteins in order to elicit signals. These include non-receptor tyrosine kinases such as Focal Adhesion Kinase (FAK), Integrin-linked Kinase (ILK) and Src-family kinases, which act to initiate intracellular signalling cascades. In addition to kinases, integrins are also able to recruit molecules that form a structural link between membrane receptors and the actin cytoskeleton, such as talin, α-actinin and filamin [118-120], therefore being able to directly influence actin dynamics and cell migration.

A number of these integrin heterodimers have been linked with the regulation of angiogenesis, indeed sixteen integrins are reportedly involved in angiogenesis with seven being expressed by ECs (α1β1, α2β1, α3β1, α5β1, α6β1, αvβ3 and αvβ5) [121], and hence integrins can influence many aspects of EC behaviour. As is suggested from this list of integrin heterodimers expressed in ECs, β1 containing integrins may play an important role angiogenesis. Antagonists of α1β1 or α2β1 inhibit VEGF-A-induced angiogenesis [122], whereas ligation of α5β1 potentiates

αvβ3-mediated EC migration [123], indicating that cross-talk between αvβ3 and α5β1 integrins may be important in angiogenesis.

1.3.1.1 αvβ3 integrin and angiogenesis

Whilst β1 containing integrins clearly play an important role in EC biology, the integrin αvβ3 is probably the best characterised integrin in terms of a role in angiogenesis. αvβ3 has been shown to localise at the tips of sprouting vessels [124] and to lamellopedia during endothelial cell migration [125]. *In vitro,* αvβ3 has been shown to mediate endothelial cell attachment, spreading and migration [126] and to be localised at endothelial cell capillary sprout tips during wound repair [127]. This role for αvβ3 in angiogenesis has been further supported by the observation that neutralising antibodies to αvβ3 inhibit bFGF-induced vessel sprouting in the Chick Chorioallantoic Membrane (CAM), but have no effect on pre-existing vessels [124], with similar results being observed for small molecule antagonists of αvβ3 [128]. In addition, αvβ3 expression is stimulated by hypoxia [129], nitric oxide [130] and pro-angiogenic growth factors such as VEGF-A [131] and FGF-2 [132], all of which further implicates αvβ3 as being involved in the angiogenic response.

αvβ3 mediates cell attachment to vitronectin, fibronectin, von-Willebrand factor, osteopontin, tenascin and thrombospondin [133]. Ligand binding initiates integrin signalling, termed 'outside-in signalling', which has been shown to mediate gene expression, cell survival, proliferation and differentiation. In terms of αvβ3 and EC, ligand binding to αvβ3 results in a suppression of p53 activity and the cell-cycle inhibitor $p21^{WAF1/CIP1}$ as well as increasing the Bcl-2/Bax ratio, the net result of which is an anti-apoptotic activity [134]. αvβ3 ligation has also been shown to be required

for sustained MAP-kinase activity in bFGF driven angiogenesis within the chick chorionallantoic membrane [135].

Studies with antibodies and small molecule antagonists of αvβ3 would suggest that active αvβ3 is required for efficient angiogenesis. However, this interpretation is no longer totally valid since studies involving genetic deletions of integrin subunits raised issues as to the exact role of αvβ3 in angiogenesis. Studies of αv knockout mice, i.e. mice that do not express αvβ3, αvβ5 and three other αv containing integrins, demonstrated that most animals die *in utero* with extensive brain and intestinal blood vessel abnormalities [136], suggesting that αv integrins are essential for blood vessel formation in these tissues. This phenotype is very similar to that observed in β8 subunit knockout mice [137], whereas in β3 [138], β5 [139] or both β3 and β5 [140] knockout mice vascular development is normal, therefore blood vessel defects in the αv knockout can be explained by loss of the αvβ8 heterodimer. Indeed, this apparent lack of requirement of αvβ3 for angiogenesis is further supported by the observation that patients suffering from the disease Glanzmann thrombasthenia; a congenital bleeding disorder caused by the lack or dysfunction of either αIIb or β3 integrin subunits, show defects in platelet aggregation, prolonged bleeding times and cutaneous and gastrointestinal bleeding [138], but suffer no defects in the formation of the vasculature [141]. Therefore, active αvβ3 would appear to not be required as such for neovascularisation, and in fact may be a negative regulator of angiogenesis. Genetic deletion studies suggest that αvβ3 inhibits angiogenesis by suppressing endothelial cell survival mediated by VEGFR2 [138] and in fact deletion of either β3 or both β3 and β5 results in an increase in tumour angiogenesis mediated by an upregulation of VEGFR2 [140, 142]. Therefore, it is clear that αvβ3 integrin is able to both positively and negatively regulate

angiogenesis. However, under what circumstances αvβ3 acts in a positive fashion and when it acts negatively remains to be understood. One interesting observation is that unligated integrins can induce cellular apoptosis; a process known as integrin mediated death [143]. In particular it was shown that cells over-expressing αvβ3, when attached to a matrix that did not ligate αvβ3, initiated apoptosis via activation of the initiator caspase, caspase 8 [143]. Therefore, the presence of unligated αvβ3 may overcome the survival signals that affect endothelial cells, if expressed at high enough levels, and thus negatively regulate angiogenesis by inducing endothelial cell apoptosis, a regulatory mechanism that would not be present in β3 knockout mice.

What is clear however is that angiogenesis is a finely balanced process and disruption by inhibiting, deleting or over-expressing αvβ3 integrin can obviously have dramatic effects on the angiogenic response.

1.4 INTEGRIN AND GROWTH FACTOR RECEPTOR CROSSTALK

The fact that genetic deletion of β3 results in an associated upregulation of VEGFR2 [140, 142] demonstrates that there is a direct interplay between integrins and growth factor receptors in angiogenesis.

It is clear that growth factors such as VEGF-A can modulate integrin expression, with an increase in the expression and promigratory function of αvβ3 in endothelial cells being induced by treatment with VEGF-A [131]. In addition, VEGF-A can directly influence integrin activity and signalling, as shown by the promotion of adhesion and migration of ECs via αvβ3 or αvβ5 following VEGF-A treatment [144]. The converse is also true, the effect of growth factors on cultured cells is dependent on integrin ligand binding and signalling. For example, optimal cell stimulation with

VEGF-A requires αvβ3 integrin-mediated cell adhesion to an appropriate extracellular matrix. When cells are cultured on vitronectin, tyrosine phosphorylation of VEGFR2 and the mitogenic activity of VEGF-A are both increased. Anti-αv and anti-β3 antibodies inhibit this VEGFR2 phosphorylation, and in addition to this β3 Integrin is necessary for the activation of PI(3)K following VEGF-A stimulation [145]. This observation is not limited to the VEGF receptors as binding to vitronectin via αvβ3 enhances the biological effects of PDGF-β [146] in ECs; and Insulin-like growth factor-1 (IGF-1) signalling in smooth muscle cells depends on αvβ3 integrin ligation [147]. This suggests that αvβ3 is the critical integrin involved in the co-ordinated activation of tyrosine kinase receptors. However, the combination of integrin and growth factor receptor required for biological responses may be cell type specific. For example, EGF-stimulated kidney epithelial cells depend on β1 Integrins [148], whereas EGF-stimulated smooth muscle cells depend on αvβ3 integrin [149]. This also raises the possibility that particular integrin/growth factor receptor interactions are required for specific cellular functions. In the case of cell adhesion and migration of a melanoma cell line expressing αvβ5, insulin growth factor (IGF) pre-stimulation was required, whereas cells expressing αvβ3 supported growth factor independent adhesion and migration. Therefore, in this cell type, growth factor stimulated cell migration was dependent on the presence of αvβ5 integrin [150].

When the biochemical links between growth factor receptors and integrins is considered the synergistic nature of the two becomes clearer. Coimmunoprecipitations of growth factor receptors and integrins have yielded a number of interactions, including αvβ3 with PDGFR and VEGFR2 [151] and α6β4 and α6β1 integrins with ErbB-2 receptor in human breast carcinoma cells following EGF stimulation [152]. In terms of cross talk between signalling pathways activated

by both Integrins and growth factor receptors, both are able to activate ERK. However, cells plated in the absence of growth factors can only induce a transient activation of ERK [153] and it has been established that in order to achieve a sustained ERK activation, stimulation by both integrins and growth factors is required [154]. A similar situation has been observed for signalling pathways other than the MAPK cascade; indeed growth factor activation of c-jun N-terminal kinase (JNK), PI(3)K and PKB/Akt is suppressed in non-adherent cells [155, 156]. Furthermore, Rac can be activated by growth factors in suspended cells, but it fails to couple to a downstream effector. Activation of integrins targets active Rac to membranes where it is able to interact with effectors, hence co-ordinated signalling by integrins and growth factor receptors is required for functional Rac activation [157].

Therefore, synergistic signalling by integrins and growth factors is important in cell proliferation, survival and migration and hence in endothelial cells plays a crucial role in angiogenesis. This conversely explains why the two play such crucial roles in the regulation of each other's actions.

1.5 RECEPTOR ENDOCYTOSIS

In mammalian cells, receptors residing at the cell surface can be internalised by membrane invagination and pinching off from the plasma membrane such that vesicles form and subsequently deliver their cargo to specific intracellular sites. This internalisation of receptors can occur via a clathrin-dependent mechanism or a clathrin-independent mechanism, for example by the formation of caveolae.

During clathrin-dependent endocytosis (Reviewed in [158]), clathrin and receptors targeted for internalisation accumulate at the plasma membrane through recruitment by the adaptor protein 2 (AP2) complex. The AP2 complex is a hetero-

tetramer consisting of two large (α and β) and two small (σ2 and μ2) subunits, where the β subunit interacts with clathrin [159] and the μ2 subunit binds endocytic signals within membrane associated proteins [160]. These endocytic signals fall into two groups: those that contain an essential tyrosine residue found within either an NPXY or YXXΦ motif, or those characterised by a di-leucine sequence (Reviewed in [161]). Subsequent polymerisation of clathrin results in the formation of clathrin-coated pits (CCPs) containing membrane receptors, which invaginate further and constrict, through a process driven by a number of proteins including dynamin, amphipysin and endophilin, forming a membrane neck. Dynamin self assembles into structures consisting of rings and spirals [162] and forms a collar around the membrane neck. The GTPase activity of dynamin is required for pinching off of invaginated pits and the formation of coated vesicles [163], and this can be activated by dynamin itself via an intramolecular GAP activated upon self-assembly [164] or through the interaction with numerous different components such as PIP2 [165], sorting nexin-9 [166] and amphipysin [167].

Alternatively, cell surface receptors can be internalised via caveolae, which are invaginations of the plasma membrane associated with the protein caveolin-1 [168]. Caveolae are a type of cholesterol-enriched membrane microdomain (CEMM) that are flask shaped and relatively immobile, and thus are not considered to be involved in constitutive endocytic trafficking [169]. Caveolin-1 can be phosphorylated at Tyr14 in response to growth factors and Src kinase activity [170, 171], and this is thought to stimulate caveolae-mediated endocytosis [172]. Caveolae-mediated endocytosis occurs via a dynamin-2 dependent mechanism [173, 174] and occurs in the presence of inhibitors of clathrin-mediated endocytosis, indicating that these two mechanisms of endocytosis are distinct.

Following internalisation, newly formed endosomes fuse with pre-existing early sorting endosomes marked by early endosomal antigen (EEA)-1 [175] and by Rab4 [176]. From this compartment there are three possible destinations for post-endocytic trafficking of receptors: the plasma membrane, late endosomes or the perinuclear recycling compartment (PNRC) (Reviewed in [177]), such that internalised receptors are either targeted for degradation or recycled back to the plasma membrane.

1.5.1 Growth factor receptor endocytosis

The accepted mechanism for growth factor receptor tyrosine kinase (RTK) internalisation and attenuation of signalling following ligand binding comes from numerous studies of the epidermal growth factor receptor (EGFR). Levels of EGFR remain relatively constant in unstimulated cells, but upon treatment with EGF EGFR is rapidly internalised and accumulates within multi-vesicular bodies (MVBs) where it is subsequently sorted for lysosomal degradation [178]. EGFR associates with AP2 via an internalisation signal flanked by tyrosine 974 [179] and ligand-dependent internalisation can be inhibited by expression of a GTPase-defective mutant of dynamin, indicating that internalisation of EGFR occurs via a clathrin-dependent mechanism. In addition to EGFR, the internalisation of numerous other RTKs, such as fibroblast growth factor receptor (FGFR) [180], platelet-derived growth factor receptor (PDGFR) [181], keratinocyte growth factor receptor (KGFR) [182] and hepatocyte growth factor receptor (HGFR) [183] has been shown to be mediated by a clathrin-dependent mechanism.

In addition, it is now clear that ubiquitination of RTKs plays a major role in receptor internalisation and sorting. In particular, the Cbl family of ubiquitin ligases is

involved in these processes. Activation of EGFR recruits growth factor receptor-binding protein 2 (Grb2), which in turn mediates the association of Cbl with the receptor and formation of this complex is required for receptor internalisation to occur [184]. However, the ubiquitin ligase activity of Cbl may not be required to facilitate receptor internalisation as the interaction of Cbl with the adaptor protein CIN85 has been shown to be sufficient to promote receptor internalisation [185]. CIN85 interacts with endophilins, regulatory components of CCPs [186] which bind to lipid bilayers and interact with many other components of CCPs such as dynamin and amphipysin [187]. If the interaction between Cbl-CIN85 and endophilins is disrupted, the EGFR internalisation and degradation in response to EGF is perturbed, without altering the ability of Cbl to ubiquitinate EGFR [186]. This mechanism is clearly important in EGFR endocytosis and has also been shown to influence internalisation of HGFR, PDGFR and c-Kit [188, 189], and therefore may represent a general mechanism for clathrin-dependent internalisation of RTKs.

1.5.2 Integrin endocytosis

A number of integrin β subunits contain the internalisation signal NPXY that is associated with clathrin-dependent internalisation, but in general there are few studies demonstrating a requirement for clathrin in integrin endocytosis. The NITY motif found in the cytoplasmic region of the β3 subunit is involved in the internalistion of IgG-β3 chimeras [190], and internalisation of β1 integrins in complex with the L1 adhesion molecule in neurons is prevented by treatment with the clathrin-dependent endocytosis inhibitor monoansyl cadaverine (MDC) [191]. However, α5β1 is still internalised under conditions of potassium depletion that reorganises the AP2 complex and inhibits clathrin-mediated endocytosis [192], and disruption of the

NPXY motif in the β3 subunit of αvβ3 and αIIbβ3 does not perturb the rapid internalisation of either integrin [193, 194]. Perhaps uniquely amongst integrins, αvβ5 has been visualised at CCPs and associates with clathrin-coated membrane domains via the β5 subunit [195], therefore clathrin-mediated endocytosis appears to regulate internalisation of αvβ5.

In contrast to αvβ5 a number of other integrins, including αvβ3 and α5β1 have been shown to associate with caveolin-1 [196, 197] and therefore are likely to be internalised via caveolae. Indeed, α2β1 has been shown to be localised at caveolae in response to antibody induced clustering, from which it is subsequently internalised in a PKC-α dependent manner [198]. PKC-α has been linked to the pinching off of caveolae [199] and furthermore is required in conjunction with dynamin to mediate the internalisation of β1 integrins [200]. In addition, integrins have been shown to associate with CEMMs that contain caveolin [201] and in particular, cholesterol depletion inhibited the internalisation of αLβ2 in leukocytes [202].

1.6 ENDOCYTIC RECYCLING PATHWAYS: REGULATORS OF CELL MIGRATION

The endocytic receptor recycling system was identified early on as potentially important in cellular processes that require polarisation and rapid movement of membrane and transmembrane proteins, such as cell migration [203, 204]. Directed migration requires cells to polarise key proteins according to the direction of travel, and this polarisation is achieved by active endo-exocytic recycling, which constantly retargets proteins to particular regions of the plasma membrane, in combination with a diffusion barrier within the plasma membrane itself.

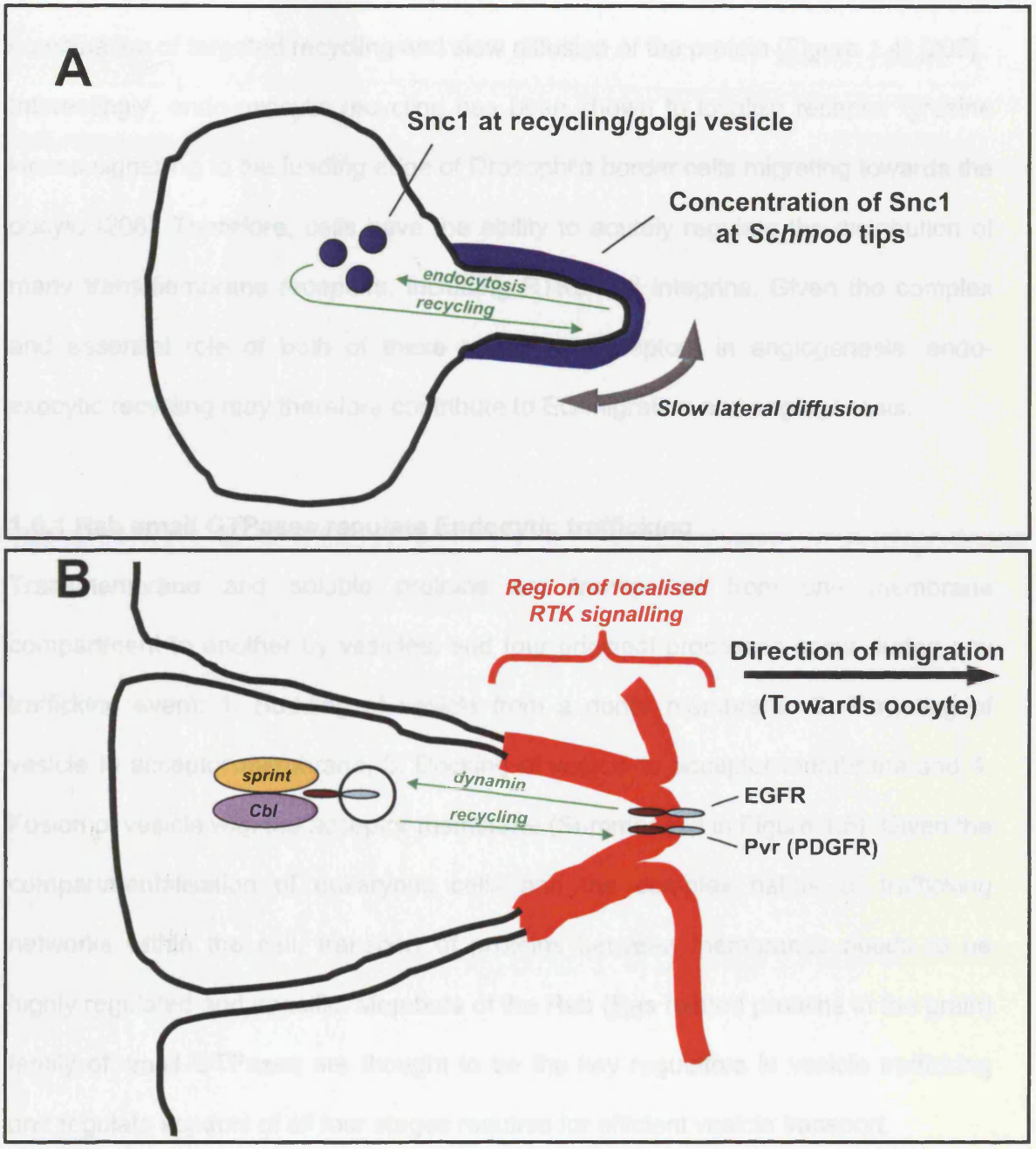

Figure 1.4: Contribution of endo-exocytosis to the generation of polarity in yeast and *Drosophila*

(A) Localisation of the yeast SNARE protein Snc1 is maintained at Schmoo tips by a combination of targeted endo-exocytosis and slow diffusion in the plasma membrane

(B) RTK signalling is localised to the leading edge of migrating Drosophila border cells by an endo-exocytic cycle that requires dynamin, Cbl and Sprint (Homologous to mammalian Rin1, a Rab5 GEF).

For example, in yeast the SNARE protein snc1 is maintained at schmoo tips by a combination of targeted recycling and slow diffusion of the protein (Figure 1.4) [205]. Interestingly, endo-exocytic recycling has been shown to localise receptor tyrosine kinase signalling to the leading edge of Drosophila border cells migrating towards the oocyte [206]. Therefore, cells have the ability to acutely regulate the distribution of many transmembrane receptors, including RTKs and integrins. Given the complex and essential role of both of these groups of receptors in angiogenesis, endo-exocytic recycling may therefore contribute to EC migration and angiogenesis.

1.6.1 Rab small GTPases regulate Endocytic trafficking

Transmembrane and soluble proteins are transported from one membrane compartment to another by vesicles, and four principal processes occur during any trafficking event: 1. Budding of vesicle from a donor membrane, 2. Targeting of vesicle to acceptor membrane, 3. Docking of vesicle to acceptor membrane and 4. Fusion of vesicle with the acceptor membrane (Summarised in Figure 1.5). Given the compartmentalisation of eukaryotic cells and the complex nature of trafficking networks within the cell, transport of proteins between membranes needs to be highly regulated and specific. Members of the Rab (Ras related proteins in the brain) family of small GTPases are thought to be the key regulators in vesicle trafficking and regulate aspects of all four stages requires for efficient vesicle transport.

Rab proteins are members of the wider Ras family of small GTPases with over 70 human Rab and Rab like proteins having so far been identified, of which the functions of 36 have been determined [207]. Rab proteins are found on all compartments of the endomembrane system, the plasma membrane, mitochondria

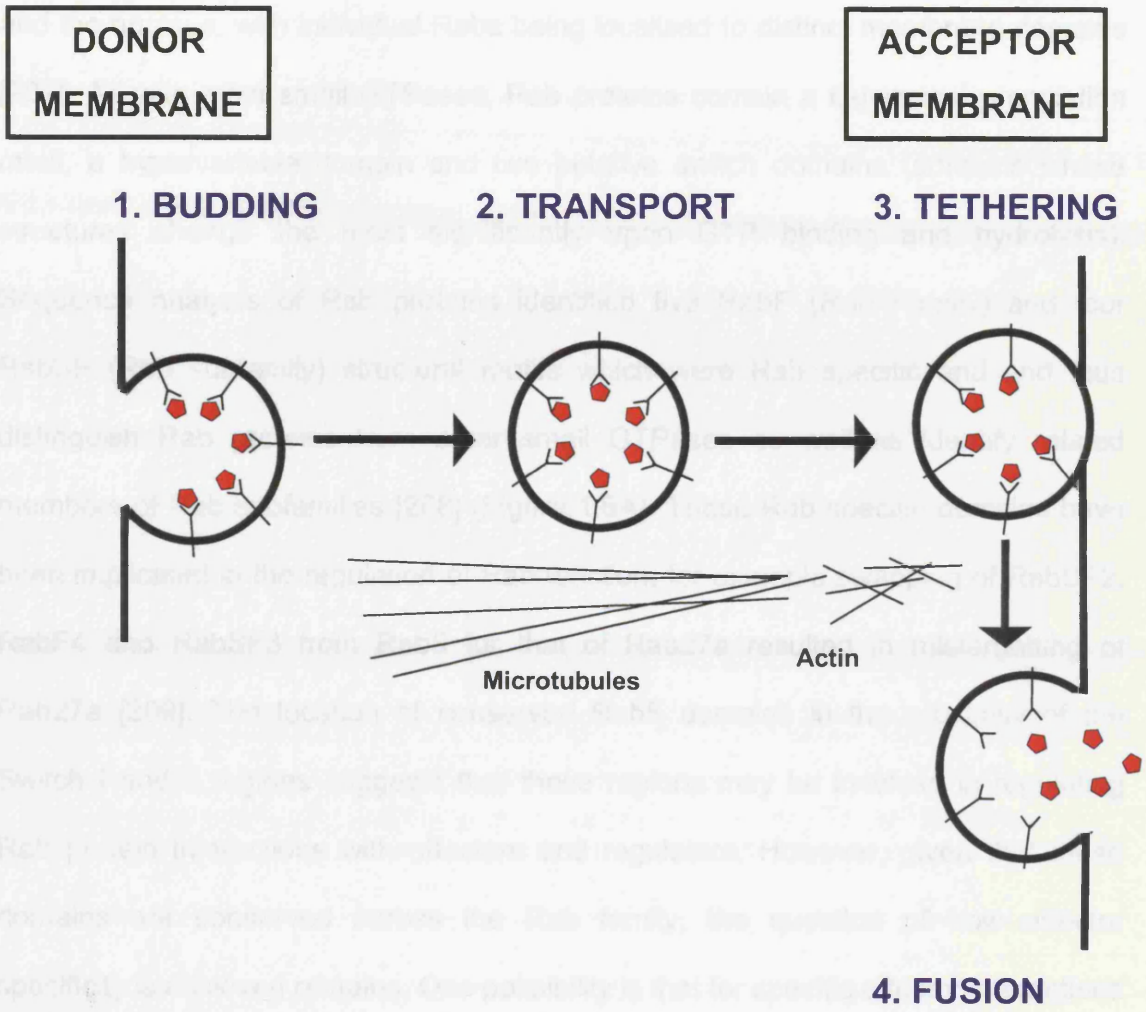

Figure 1.5: Stages of vesicular transport

Vesicular transport between one membrane compartment and another consists of four principal processes that occur during any trafficking event: 1. Budding of vesicle from a donor membrane, 2. Targeting of vesicle to acceptor membrane, 3. Docking of vesicle to acceptor membrane and 4. Fusion of vesicle with the acceptor membrane

and the nucleus, with individual Rabs being localised to distinct membrane domains [207]. As with most small GTPases, Rab proteins contain a C-terminal prenylation motif, a hypervariable domain and two putative switch domains (domains whose structures change the most significantly upon GTP binding and hydrolysis). Sequence analysis of Rab proteins identified five RabF (Rab Family) and four RabSF (Rab subfamily) structural motifs which were Rab specific and and thus distinguish Rab proteins from other small GTPases as well as identify related members of Rab subfamilies [208] (Figure 1.6A). These Rab specific domains have been implicated in the regulation of Rab function, for example swapping of RabSF2, RabF4 and RabSF3 from Rab5 for that of Rab27a resulted in mistargetting of Rab27a [209]. The location of conserved RabF domains in the proximity of the Switch I and II regions suggests that these regions may be involved in regulating Rab protein interactions with effectors and regulators. However, given that these domains are conserved across the Rab family, the question of how effector specificity is achieved remains. One possibility is that for specific effector interactions binding to both RabF and RabSF domains is required. Indeed Rab3 binds to Rabphilin-3 via a binding surface consisting of both Switch regions plus RabSF1, RabSF3 and RabSF4 [210], suggesting that Rab isoforms might interact with specific effectors via subfamily specific regions.

1.6.1.1 Regulation of Rab function

As with other members of the Rab family of small GTPases, Rabs can be considered as molecular switches cycling between a GDP bound inactive state and a GTP bound active state, and it is the active GTP bound form that interacts with downstream effector proteins [211]. Conversion from the GDP to the GTP bound

form occurs due to nucleotide exchange catalysed by GDP/GTP exchange factors (GEFs), with the opposite conversion being due to nucleotide hydrolysis facilitated by a GTP-activating protein (GAPs). In addition to this nucleotide binding/hydrolysis cycle, Rab proteins participate in a membrane association/ disassociation cycle that is also critical for Rab function (summarised in figure 1.6B). For a Rab to be considered as truly active it must be both in the GTP form and membrane associated [212], therefore both regulatory cycles must be tightly linked.

Newly synthesised Rabs are recognised and bound by Rab-Escort Protein (REP) [213], which presents them to Rab Geranyl Geranyl Transferase (RGGT). In turn, RGGT modifies Rabs via the covalent addition of a geranyl geranyl pyrophosphate to 2 cysteine residues at the C-Terminus [214], allowing Rab proteins to be inserted into membranes. The REP-Rab complex then dissociates from RGGT [215] and REP is then believed to deliver prenylated Rab protein to its target membrane [216].Once at their target membrane, Rab proteins are activated by a specific Guanine Nucleotide Exchange Factor (GEFs) and recruit a wide range of effector molecules through which they exert their effect on membrane trafficking. Rabs can then be returned to their inactive state by GTP hydrolysis catalysed by GTPase Activating Proteins (GAPs) and in their GDP bound form are removed from membranes by Rab GDP Dissociation Inhibitor (RabGDI) (Figure 1.6B). RabGDI is closely related to REP and both proteins show preference for GDP bound Rabs [217]. However, RabGDI is not involved in lipid modification of Rab proteins [216] as it cannot interact with RGGT [218] therefore its primary role seems to be that of maintaining a cytosolic pool of Rab proteins.

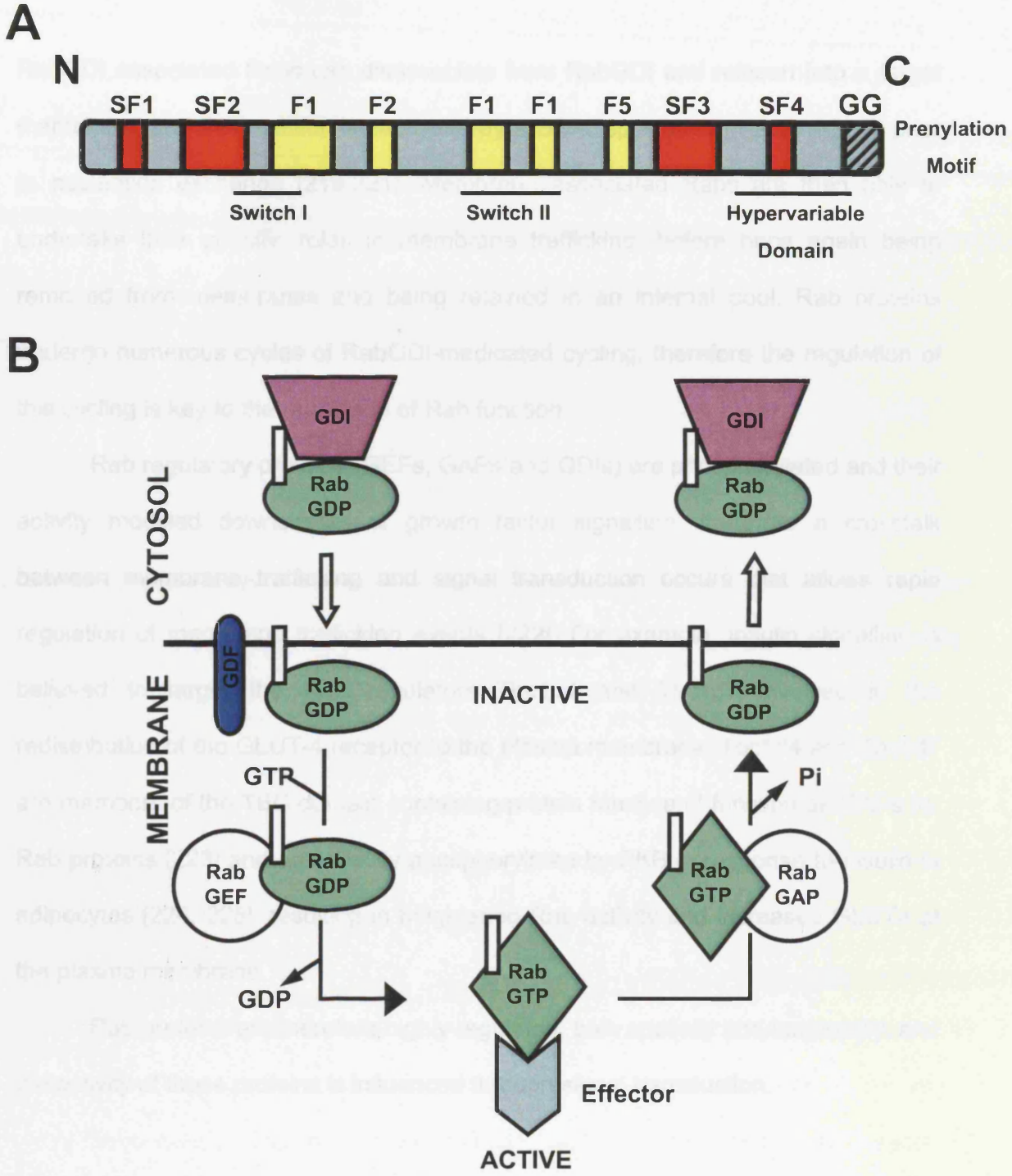

Figure 1.6: Rab protein domain structure and regulatory cycle

(A) Rab proteins are geranylgeranylated at the C-terminus and consist of 2 switch domains and a hypervariable domain. Rab proteins can be identified by the presence of Rab Family domains (F1-F5) and can be further characterised by the presence of Rab sub-family domains (SF1-SF4)

(B) The activity of Rab proteins is regulated by 2 cycles: Inactive, cytosolic GDP-Rabs associate with RabGDI and are inserted into membranes via interaction with RabGDFs. RabGDP is converted to active RabGTP by a RabGEF, and can then interact with effector molecules involved in mediating Rab function.. RabGTP is hydrolysed by a RabGAP to RabGDP which is subsequently extracted from the membrane by RabGDI. This regulatory cycle is critical for correct Rab function

RabGDI associated Rabs can disassociate from RabGDI and reinsert into a target membrane, and this reaction is catalysed by a GDI-displacement Factor (GDF) prior to nucleotide exchange [219-221]. Membrane associated Rabs are then able to undertake their specific roles in membrane trafficking, before once again being removed from membranes and being retained in an internal pool. Rab proteins undergo numerous cycles of RabGDI-medicated cycling, therefore the regulation of this cycling is key to the regulation of Rab function.

Rab regulatory proteins (GEFs, GAPs and GDIs) are phosphorylated and their activity modified downstream of growth factor signalling, therefore a crosstalk between membrane trafficking and signal transduction occurs that allows rapid regulation of membrane trafficking events [222]. For example, insulin signalling is believed to target the Rab regulators Tbc1d4 and Tbc1d7 involved in the redistribution of the GLUT-4 receptor to the plasma membrane. Tbc1d4 and Tbc1d7 are members of the TBC-domain containing protein family and function as GAPs for Rab proteins [223] and are directly phosphorylated by PKB in response to insulin in adipocytes [224, 225], resulting in heightened Rab activity and increased GLUT4 at the plasma membrane.

Rab proteins are therefore highly regulated, both spatially and temporally, and the activity of these proteins is influenced through signal transduction.

1.6.1.2 Rab effectors

The question then remains as to how Rab proteins influence the major steps involved in vesicle trafficking, and the key to this is the recruitment of numerous effector molecules to activated Rabs.

Rab proteins have been linked to the regulation of vesicle budding and formation [226-229] but to date only a single effector has been identified that has a clearly defined role in his process. TIP147 is a protein that associates with the tail of Mannose-6-phosphate receptors (MPRs) and is required for recycling of MPRs back to the Golgi from late endosomes [230]. In addition TIP47 associates with Rab9, which is also required for MPR recycling [231] and can therefore be described as a Rab9 effector. GTP Rab9 increases the association of TIP47 with late endosomes and the affinity of TIP47 for MPRs [232] resulting in the capture of MPRs in Rab9 positive vesicles that are subsequently trafficked back to the Golgi.

A number of Rab proteins have been shown to interact with microtubule or actin based motor proteins, and therefore facilitate vesicle movement along cytoskeletal tracks. Long range vesicle transport occurs along microtubules, with the cytoplasmic protein dynein moving vesicles towards the minus ends of microtubules and members of the kinesin family moving vesicles towards plus ends distributed at the cell periphery [233]. The first description of a Rab protein interacting with a motor protein demonstrated that a novel kinesin like protein Rabkinsin-6 binds specifically to Rab6-GTP at the Golgi. Over expression of Rabkinsein 6 resulted in the redistribution of Golgi toward the plus end of microtubules confirming the role of Rabkinesin 6 as a microtubule based motor [234].

Subsequently additional Rab6 effectors were identified that were dynactin binding and therefore demonstrated that, as well as interacting with kinesin motors, Rab6 could interact with the dynactin/dynein complex [235, 236] to mediate vesicle motility. In addition, dynein has been co isolated with Rab5 [237] and is recruited to Rab7 via the Rab7 effector protein Rab7 interacting lysosomal protein (RILP) [238]. Rab5 has also been shown to recruit the kinesin family member KIF16B to early

endosomes [239], illustrating that Rabs have the capability of interacting with multiple motor-proteins and how either microtubule plus or minus end directed trafficking is selected requires further investigation.

There are also many examples of Rab proteins regulating actin-based motility through interactions with Myosin motors. Four classes of Myosins (I, II, V, VI) have been implicated in the movement of ER, recycling endodomes, lysosomes, secretory granules and melanosomes in mammalian cells [240]. Myosin Va is recruited to melanosomes by Rab27a [241] via the association of melanophilin with Rab27a-GTP [240] resulting in the movement of melanosomes along actin filaments. In addition, Rab8 promotes actin dependent movement of vesicles from the Golgi to the plasma membrane [242] and Myosin VI has been shown to directly interact with optineurin [243] which itself interacts with Rab8 [244]. Therefore in addition to mediating vesicle formation Rab proteins are able to specifically recruit motor proteins in order to facilitate vesicle trafficking.

The final stage of vesicle transport is the tethering and fusion with acceptor membranes and it is this aspect of vesicle trafficking that Rab proteins are best known for. Rab-GTPs can bind either to long range tethers such as EEA-1 [245] or to short range multisubunit tethers such as the COG complex at the Golgi [246] or the HOPs complex in the late endocytic pathway [247]. The exocyst was the first multisubunit tethering complex to be identified as a Rab effector and is required for the tethering of secretory vesicles to the plasma membrane in yeast [248]. The exocyst subunit SEC15p directly binds to the Rab SEC4p in its GTP bound state [248] and mammalian SEC15 has been identified as an effector of Rab11 in mammalian cells [249], suggesting an important role for the exocyst complex in vesicle tethering in mammals.

Tethering factors act to co-ordinate membrane fusion following delivery of vesicles to acceptor membranes. Fusion is thought to be mediated by the pairing of SNAREs, with a SNARE on the transport vesicle (V-SNARE) pairing with its SNARE binding partner on the target membrane (t-SNARE) [250]. How this SNARE pairing acts to influence membrane fusion is still unclear, with the possibility that the pairing reaction itself drives membrane fusion and alternatively that downstream factors may be involved [251]. The tethering factor EEAI interacts with the t-SNARE syntaxin-13 and is required for homotypic early endosome fusion [252] and on COPII vesicles p115 interacts with a select set of COPII vesicle associated SNARES [253]. Therefore evidence suggests that Rab proteins do not interact directly with SNAREs to facilitate membrane fusion, but that they form membrane microdomains containing Rab effectors as well as proteins which interact with these complexes such as SNARES.

What is clear is that, through interactions with numerous effectors Rab proteins are able to regulate all aspects of vesicle trafficking including vesicle formation, transport, docking and fusion with acceptor membranes.

1.6.1.3 Rab4 and Rab11 regulate endocytic recycling

Following internalisation to early endosomes (EEs), receptors are either transported to multivesicular bodies and then to late endosomes for degradation, or are recycled back to the plasma membrane via one of two temporally and spatially distinct pathways. Receptor can exit EEs and return directly to the plasma membrane via a pathway regulated by the small GTPase Rab4; termed 'short-loop' recycling. Alternatively receptors are able to exit EEs and move on to the perinuclear recycling

compartment (PNRC), from which exocytosis can then occur via a pathway that can be regulated by Rab11; termed 'long-loop' recycling [254].

1.6.1.4 Rab4 regulators and effectors

Rab4 is localised to early/recycling endosomes where it contributes to cargo sorting and either recycling back to the plasma membrane [211] or trafficking along the degradative endosomal pathway [255] of internalised receptors.

Cycling between a membrane associated and cytosolic location is critical for correct Rab4 function. If Rab4 is fixed permanently on endosomal membranes a decrease in Rab4-mediated trafficking is observed [256]. Furthermore, RabGDI has been shown to dissociate Rab4 from vesicular membranes in a reconstituted system [257] although no GDF for Rab4 has thus far been described.

The regulation of Rab4 nucleotide exchange is unclear, with no GEF for Rab4 having yet been identified although two proteins have been identified which demonstrate GAP activity toward Rab4. Firstly, the p85 subunit of PI(3)K [258] and secondly the protein GAPcenA [259] activate the GTPase activity of Rab4. However, both these proteins were identified using in vitro GAP assays and demonstrated GAP activity towards other Rabs, therefore the importance of these GAPs in regulating Rab4 activity in the cell needs to be investigated.

A number of Rab4 effectors have been identified that regulate trafficking events from early endosomes. The adaptor protein CD2AP/CMS interacts specifically with Rab4-GTP and is involved in sorting from early to late endosomes, in particular in mediating ligand-induced degradation of EGFR [255, 260]. Therefore, interaction between Rab4-GTP and CD2AP may be the mechanism by which tyrosine kinase receptors are targeted for degradation rather than recycling. A role

for Rab4 effectors in cargo sorting at early endosomes is further supported by the observation that Rabenosyn-5, a Rab5 effector that regulates membrane tethering and fusion at the stage of vesicle entry into early endosomes [261], also binds directly to Rab4-GTP [262] thus binding both Rabs, and potentially acting to link endosomal Rab5 and Rab4 domains that are usually segregated. Overexpression of Rabenosyn-5 resulted in an increase in TfnR recycling from early endosomes to the plasma membrane, suggesting that association with Rabenosyn-5 mediates transition of cargo from Rab5 domains to Rab4 domains to allow rapid recycling of receptors back to the plasma membrane [262]. In addition to Rabenosyn-5, the Rab5 effector Rabaptin-5 is also capable of interacting with Rab4-GTP [263] suggesting that Rab proteins acting sequentially in transport through the endocytic network can share common effectors.

Rab4 has also been linked to the process of vesicle budding via its association with Rabaptin-4. Rabaptin-4 binds to γ1-adaptin, a component of the adaptor complex AP-1, at early endosomes in a Rab4-dependent fashion [264]. γ1-adaptin has been suggested to be involved in the formation of Clathrin-coated vesicles and returning TfnR to the plasma membrane via recycling endosomes [265]. Therefore, Rab4 activity mediates cargo sorting and potentially vesicle budding in early endosomes. In addition, GFP-Rab4 localises to endosomes on microtubules and interacts with the central portion of the cytoplasmic Dynein light chain intermediate chain-1 [266], suggesting that Rab4-mediated endosomal transport can occur via microtubule dependent transport involving the Dynein motor protein complex. In addition, Insulin activates Rab4 via activity of PKC-γ, and this GTP-Rab4 is then able to associate with the Kinesin-2 family member KIF3B, a motor protein involved in plus-end directed microtubule transport. The net result of this is delivery

of GLUT-4 receptors from Rab4 positive intracellular compartments to the plasma membrane [158]. However, GLUT-4 proteins cannot travel along microtubules all of the way to the plasma membrane. The final stages of GLUT-4 translocation involve the interaction of microtubule cargo with that of the actin cytoskeleton [267]. Insulin treatment results in the phosphorylation of Myosin Va by PKB and association with Rab4, suggesting that Rab4 acts to facilitate transfer of GLUT-4 containing vesicles from microtubule/KIF3B dependent motility to an F-Actin/Myosin Va dependent process in order to ensure the delivery of GLUT-4 to the plasma membrane.

Rab4 also has the potential to influence the selective association of vesicles with target membrane. As suggested, Rab proteins that act sequentially in endocytic trafficking may share effectors, therefore it is possible that an effector for both Rab4 and Rab11 exists to regulate early- to recycling-endosome transport. Indeed, Rab Coupling Protein (RCP) can interact directly and independently with Rab4-GTP and Rab11-GTP and expression of dominant negative RCP inhibit plasma membrane recycling of TfnR from recycling endosomes [268]. Therefore RCP may act as an intermediate between Rab4 and Rab11, interacting wih Rab4 at early endosomes and then binding to Rab11 on the acceptor membrane at recycling endosomes.

In terms of delivery of endosomes to the plasma membrane, a number of Rab4 effectors have been suggested to play a role in this process. The amount of Rab4-interacting protein Rabip4 [269] present at the plasma membrane in 3T3-L1 adipocytes increases following treatment with Insulin in parallel with an increase in the Insulin receptor GLUT4 at the plasma membrane [270]. Upon delivery to the plasma membrane, fusion of GLUT4-containing vesicles is dependent on the interaction between the v-SNAREs, VAMP-2 and VAMP-3, and their cognate t-SNAREs, Syntaxin-4 and SNAP-23 [271, 272]. GTP-Rab4 interacts directly with

Syntaxin-4 and this interaction is enhanced by GTP loading of Rab4 following Insulin stimulation [273]. Therefore it is possible that Rab4 facilitates the specific docking and fusion of recycled GLUT4-containing vesicles with the plasma membrane via interaction with the t-SNARE Syntaxin 4.

A number of Rab4 regulators and effectors have therefore been identified which mediate Rab4-dependent trafficking events and act at all stages of vesicle transport. However, a vast amount of work is still required in order to identify how Rab4 activity is regulated in terms of GDP/GTP and membrane cycling, as well as identifying more key effectors for the process of transport from early endosomes.

1.6.1.5 Rab11 regulators and effectors

Rab11 plays a role in trafficking from recycling endosomes either back to the plasma membrane [274] or to the trans-Golgi network (TGN) [275]. Currently no upstream regulators of Rab11 activity have been identified, but a number of Rab11 effectors have been characterised. Among the first Rab11 binding proteins to be identified was Rab11BP/Rabphilin-11 which binds specifically to Rab11-GTP [276]. Subsequently the Rab11-family of interacting proteins (Rab11-FIPs) that all share a highly homologous Rab11-binding domain (RBD) was identified [277]. The Class-I Rab11-FIPs (RCP, Rip11 and Rab1-FIP2) contain a C2-phospholipid binding domain at the N-terminus in addition to a C-terminal RBD, and are predominantly membrane bound and localised to recycling endosomes [278]. The Class II Rab11-FIPs (Rab11-FIP3 and Rab11-FIP4) contain an Ezrin-Radixin-Moesin (ERM) domain and have been localised to the TGN, recycling endosomes and centrosomes [277, 279, 280], and the Class III FIPs which currently contain only Rab11-FIP1 which has no homology to known protein domains [281]. Therefore the class of Rab11-FIP that

Rab11-GTP associates with will determine the nature of the transport event driven by Rab11 activity, for example truncation mutants of Class I FIPs inhibit recycling of internalised receptors whereas truncation mutants of Class II FIPs do not [279, 280]. A role for Class I FIPs in endosomal recycling is further supported by the observation that RCP associated with Rab11-GTP is required for efficient sorting of internalised TfnR for recycling back to the plasma membrane instead of transport through the degradative pathway [281] and that a Rab11-Rip11 complex regulates trafficking from apical recycling endosomes to the plasma membrane in polarised epithelial cells [282]. Furthermore, Rab11 complexed with Rab11-FIP2 associates with the motor protein Myosin Vb and drives plasma membrane recycling from recycling endosomes of internalised Transferrin receptor [283] and the chemokine receptor CXCR2 [284].

Myosin Vb has long been identified as an effector for Rab11 [285] and in addition to the Rab11-FIP2 mediated role in recycling outlined, has been linked to the Rab11-dependent plasma membrane trafficking of a number of other receptors. For example, Rab11 and Myosin Vb are critical regulators in the recycling of the M4 Muscarinic Acetylcholine receptor following agonist induced internalisation [286]. Therefore Rab11-GTP bound to Class I FIP effectors has been shown to play a role in sorting events at recycling endosomes and can interact with motor proteins required for transport to the plasma membrane.

The precise role for Class II Rab11-FIPs remains unclear. Rab11-FIP3 is required for maintaining the structural intensity of recycling endosomes, and this function is Rab11 dependent [287]. Rab11-FIP4 associates with Rab11 at recycling endosomes but does not play a role in recycling to the plasma membrane [288]. Therefore Class II FIPs are likely to be involved in trafficking from recycling

endosomes to the TGN given their observed localisation at this cellular compartment.

Rab11 can also interact with the tethering factor Sec15, a component of the exocyst complex, in a GTP dependent fashion [249]. In polarised epithelial cells the exocyst is variably localised to the Golgi apparatus, TGN and recycling endosomes, and is believed to promote targetting and fusion of vesicles to the plasma membrane [289, 290]. The interaction between Rab11 and Sec15 is required for polarised endocytic trafficking directed towards both poles of epithelial cells, as well as for basolateral to apical transcytosis [291] and, as such, the exocyst complex appears to be an important effector for Rab11. Interestingly, the Sec10 subunit of the exocyst can interact with the small GTPase Arf6 [292] and this association has been shown to influence post-endocytic recycling [289]. Furthermore, Arf6 is also able to bind Rab11 effectors such as the Class II FIPs Rab11-FIP3 and Rab11-FIP4 and influence trafficking from recycling endosomes [279], suggesting that there are dual effectors capable of co-ordinating the actions of both Arf and Rab GTPases.

Rab11 therefore has been shown to regulate sorting and trafficking events at recycling endosomes, resulting in either trafficking to the plasma membrane or the TGN. It achieves this through interactions with a number of effectors that mediate sorting, vesicle translocation and tethering, although as with Rab4, specific regulators of Rab11 activity still need to be identified and characterised.

1.6.2 Integrin trafficking and cell migration

It has been shown that a wide range of integrins accumulate in the PNRC prior to being returned in a Rab11-positive fashion (Reviewed in [293]), and therefore this recycling pathway would appear to be key in cell migration. Expression of a

truncated version of the Rab11-effector Rabphilin-11 reduced migration of HeLa cells [276] and numerous manipulations that disrupt this pathway perturb cell migration [284, 294, 295] demonstrating that Rab11 does indeed have a role in cell migration. The Rab11-dependent recycling pathway is clearly able to influence invasive cell migration, and has been shown to have a clear link to tumour development and disease progression. Rab11 is upregulated during skin carcinogenesis [296], linked to Barretts dysplasia [297] and is involved in hypoxia-promoted invasive migration of carcinoma cells [295]. In addition, the Rab11 family member Rab25 is upregulated in invasive versus non-invasive breast cancer cell lines and metastatic versus non-metastatic tumour cells [298]. Overexpression of Rab25 is further linked to the aggressiveness of breast and ovarian cancers [299], and physically associates with $\alpha5\beta1$ integrin. This association modulates trafficking of $\alpha5\beta1$, induces the assembly of three-dimensional cell-matrix adhesions and promotes invasive migration [300], illustrating the importance of both Rab25 and $\alpha5\beta1$ in invasive migration.

Recycling of the integrins $\alpha v\beta3$, $\alpha5\beta1$ and $\alpha6\beta4$ via the Rab11 pathway requires the inhibition of GSK3-β by phosphorylation by PKB/Akt [295, 301]. There are a number of ways in which inactivation of GSK3β could influence endocytic trafficking. Inhibition of GSK3 activity results in an increase in kinesin-based vesicle motility [302] and GSK3β has been shown to regulate microtubule stabilisation via phosphorylation of MAP1B [303]. Furthermore, inhibition of GSK3β leads to a reduction in Tau phosphorylation resulting in an increase in Tau binding to microtubules and promotion of microtubule assembly [304]. Therefore changes in GSK3β activity can impinge upon microtubule dynamics as well as potentially regulating microtubule motor proteins. In addition GSK3β may influence cytoskeletal dynamics through the regulation of Adenomatous polyposis coli (APC) protein. A

proportion of GSK3 in cells is found within a multiprotein complex in association with Axin, APC and β-Catenin. In this complex, GSK3 in active and phosphorylates the other protein components resulting in stabilisation of Axin and targetting of β-Catenin for degradation. Phosphorylation of GSK3 by PKB/Akt inhibits the phosphorylation of axin and β-catenin [305] and this leads to dissociation of the GSK3/Axin/β-Catenin/APC complex [306]. APC is a microtubule plus end-binding protein and promotes microtubule extensions at sights of cell protrusion [307], and has also been shown to interact with Kinesin-2 via an association with the Kinesin super family associated protein 3 (KAP3) [308]. Therefore APC can influence microtubule dynamics as well as motor proteins involved in microtubule-dependent vesicle motility. Furthermore, APC can be associated with the plasma membrane and this association is dependent on the actin cytoskeleton [309] and is involved in determining cell polarisation [310]. Therefore GSK3 could potentially regulate endocytic trafficking of integrins by influencing cytoskeletal dynamics and motor proteins involved in vesicular transport.

PKB/Akt also being shown to selectively regulate the recycling of β1 integrins, via the PNRC, by phosphorylation of ACAP1. When phosphorylated by PKB, ACAP1 regulated the stimulated recycling of β1 integrins in Hela cells by associating with the β1 tail at the recycling endosome, and knockdown of either PKB or ACAP1 inhibited cell migration in a wound healing assay [311]. In a separate study, expression of dominant negative ARF6 inhibited β1 integrin recycling in Hela cells and also inhibited β1 dependent migration towards a collagen I gradient [294]. Recycling of β1-integrins and directional cell motility is also regulated by PKCε [312]. PKCε specifically regulates β1 recycling via the phosphorylation of the intermediate filament protein Vimentin at the N-terminus in mouse embryo fibroblasts.

Phosphorylation of Vimentin results in the release of integrin-containing vesicles from Vimentin in the perinuclear region, allowing the trafficking if these vesicles back to the plasma membrane [313].

Whilst the influence of the Rab11 recycling pathway on integrin trafficking and cell migration has been substantially investigated, the role of the Rab4-dependent recycling pathway has been less extensively studied. What is clear however is that recycling via the Rab4-dependent pathway has a distinct influence on integrin trafficking and cell migration. In fibroblasts, PDGF stimulation and downstream signalling confers selectivity on integrin recycling by switching αvβ3 from the constitutive Rab11-dependent long loop recycling to Rab4-dependent short loop recycling [314]. αvβ3 must associate directly with the PKC-related kinase PKD1 for recycling via the Rab4 pathway to occur, and this association is required for polarisation of αvβ3 to the leading edge of migrating fibroblasts [193]. Interestingly, specific disruption of the Rab4/PKD1-dependent recycling of αvβ3 does not alter the overall ability of cells to migrate, but does compromise the ability of cells to migrate directionally and persistently [315].

Therefore the Rab11-dependent recycling pathway appears to traffic integrins, and other molecules, required for efficient invasive migration, whereas the Rab4-dependent pathway appears to be contributing specifically to the trafficking and distribution of αvβ3 integrin in fibroblasts.

1.6.3 Growth factor/chemokine receptor trafficking

Receptor mediated endocytosis was primarily considered a means of downregulating plasma membrane receptors following ligand binding. Indeed, ubiquitinylation of ligand occupied RTKs by the E3-ligase Cbl and their subsequent targeting for

degradation does indeed occur [316]. However, there is growing support for the hypothesis that endocytosis and recycling of growth factor receptors contributes to the complexity of signaling networks in eukaryotic cells [317]. Blockade of receptor internalisation has been shown to alter the pattern of MAPK activity following ligand binding to receptors such as the EGF [318], NGF [319] and HGF [320] receptors. The Transforming Growth Factor-β receptor (TGF-βR) is internalised in both the absence and presence of ligand [321] and this internalisation is required for signaling via SMADs [322]. TGF-βR is internalised to the Rab11 positive PNRC [321, 322] and recycled via a Rab11-dependent mechanism where the presence of ligand does not influence the rate of internalisation or recycling [321]. Furthermore, recycling pathways can resensitise receptors in order to prolong any signal output. For example, CXC chemokines and their cognate receptors are known to function in a range of migratory processes including the inflammatory response of leukocytes, tumour cell metastasis and angiogenesis [323-325]. Chemokine receptors are known to undergo ligand dependent internalisation followed by recycling, a process involved in receptor resensitisation [326]. Recently, the cytokine receptor CXCR2 was shown to recycle via the Rab11 pathway in a process involving the formation of a complex between CXCR2, the Rab11 effector Rab11-FIP2 and the myosin Vb motor protein [284]. Myosin Vb functionality was shown to be required for CXCR2 recycling and resensitisation, as well as chemotactic cell migration, indicating that recycling of chemokine receptors may be required during initial sensing of chemokine gradients and for subsequent directional migration.

Furthermore, the generation of cell polarity in a gradient of growth factor/chemokine is essential to a directional migratory response in chemotaxis. Indications from a Drosophila model of migration suggest that polarisation of growth

factor receptors is key to this process and depends on the ability to internalise and redistribute these receptors [206]. Border cells migrate toward the Drosophila oocyte in a chemotactic manner toward ligands for EGFR and PVR (homolgous to mammalian VEGFRs and PDGFRs). Cbl (an E3 ubiquitin ligase) mutants were found to show impaired border cell migration, concomitant with an inability to regulate the polarisation of PVR and EGFR signaling; this phenotype was also encountered in Border cells upon expression of dominant negative Shibire (Dynamin). PVR was shown to associate with Cbl and Sprint (homologous to mammalian RIN1, a Rab5-GEF), and this association is required to maintain restricted localisation of PVR and directional cell migration in this *in vivo* system [206].

In migrating fibroblasts of mammalian origin, the site of re-exocytosis of recycling receptors, has been reported to focus at the leading lamellae in response to EGF [327]. It is therefore likely that endocytic recycling contributes to cell migration in mammalian systems. The EGF receptor is internalised to early endosomes where it is able to encounter several proteins involved in downstream signalling [328-330]. EGF receptor not targeted for degradation is then trafficked to the PNRC where it can subsequently recycle back to the plasma membrane via a mechanism regulated by the Rab11 family effector Rab11-FIP2 [331]. The Arf GAP ASAP1 has also been found to regulate EGFR trafficking, and has a central role in cell spreading [332], indicating that EGFR trafficking can impact on focal adhesion and actino-myosin dynamics-both of which are key regulators of cell migration. Interestingly, the trafficking of EGFR has been shown to require RhoB [333]. Furthermore, RhoB and Rab11 are required for activation of Src [334], indicating that

recycling pathways may not control activity and localisation of exclusively transmembrane proteins, and that recycling pathways have multiple levels of control.

It is clear therefore that endocytic recycling pathways can influence and regulate processes such as cell migration and signalling. The fact that both integrins and growth factor receptors have been shown to be trafficked through these pathways suggests that endocytic recycling may provide a platform for influencing growth factor receptor/integrin cross-talk in endothelial cells.

1.7 HYPOTHESIS

αvβ3 integrin and VEGFR2 have been identified as key regulators of endothelial cell biology and angiogenesis, whereas the role of VEGFR1 remains unclear. Endocytic trafficking represents a mechanism by which the temporal and spatial distribution of receptors can be dynamically regulated, and as such can act to influence processes such as cell migration. However, little is known in regards to the endocytic trafficking of endogenous αvβ3 and VEGFR2 in primary cultured endothelial cells.

Therefore the hypothesis that will be investigated in this thesis is that angiogenic growth factors, such as VEGF-A and PlGF, regulate the endocytic recycling of αvβ3 integrin and VEGFR2, and that this recycling pathway mediates aspects of endothelial cell biology important in angiogenesis.

In particular, the characteristics of αvβ3 and VEGFR2 internalisation will be determined using a biotin-based approach utilising both immunoprecipitation and a capture-ELISA developed specifically for use in HUVECs. Furthermore, a capture ELISA will be used to measure recycling of internalised αvβ3 and VEGFR2 back to the plasma membrane, and the effect of VEGF-A and PlGF on this process

determined. Once the overall effect of growth factors on recycling has been identified, then the signalling effectors involved will be identified by utilising an RNAi based approach, in addition to pharmacological inhibition of VEGF receptors and other candidate regulatory molecules. Once a pathway involved in regulating recycling of either $\alpha v\beta 3$ or VEGFR2 has been identified, the effect of inhibiting this pathway on aspects of endothelial cell biology will be investigated. Cell migration will be assessed using Boyden chamber assays and then the behaviour of endothelial cells in three-dimensional assays that mimic aspects of angiogenesis will be determined.

Chapter Two: Materials and Methods

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2.1 ANTIBODIES, GROWTH FACTORS AND REAGENTS

Monoclonal mouse anti-human β3 integrin (clone VI-PL2) and anti-human α5 integrin was purchased from Pharmingen (San Diego, CA). Mouse anti-phospho Thr202/Tyr204 ERK p44/42 antibody and polyclonal rabbit anti-phospho Ser 21/9 GSK3-α/β were from New England Biolabs (NEB) (Beverley MA). Polyclonal rabbit anti-PKD1 (sc-639) and polyclonal rabbit anti-ERK1/2 (sc-93) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and anti-phospho-Ser^{916}PKD1 was as described in [335]. Mouse monoclonal anti-PECAM1 (9G11) and goat polyclonal anti-human VEGFR2 (AF357) were purchased from R&D Systems Inc. (MN, USA). Peroxidase labelled anti-phosphotyrosine antibody, PY-20H, and mouse anti-GSK3β were from BD Transduction laboratories (San Diego CA). Fluorescein-conjugated secondary antibodies were from Southern Biotechnology (AL, USA). EZ-link Sulfo-NHS-SS-biotin (21331) and enhanced chemiluminescence reagents (ECL) were from Pierce and Warriner Ltd (Chester, Cheshire, UK). Cell culture medium and Maxisorb 96 well plates were from Life Technologies (Rockville, USA), foetal calf serum (FCS) was from Biowest (S.A.S., France) and endothelial cell growth supplement was from Tebu-Bio, (France). GeneFECTOR transfection reagent was from Venn Nova (CA, USA). Human VEGF-A was from Peprotech Inc. (Rocky Hill NJ) and recombinant human PlGF was from R&D systems (Minneapolis MN). RNA oligonucleotide duplexes were from Dharmacon (CO, USA). Dynal sheep anti-mouse magnetic beads were form Invitrogen (UK), Streptavidin-conjugated horseradish peroxidase was from Amersham Biosciences (Bucks, UK). Primaquine diphosphate (16,039-3) was from Aldrich Chem. Co. (WI, USA) and Bovine Serum

Albumin (BSA) was from First Link (UK). SB216763 (EI-312) was from Affiniti. All other reagents were purchased from Sigma Chemical Co (Dorset, UK), VEGF receptor inhibitors were supplied by Simon Barry (Astra Zeneca, Macclesfield UK).

2.2 CELL CULTURE

2.2.1 Human Umbilical Vein Endothelial Cells (HUVECs)

HUVECs were isolated by collagenase digestion according to methods outlined by Marin *et al* (2001) from donor human umbilical cords. Cells were then cultured on gelatine coated plastic in Glutamax Media-199 (Life Technologies), supplemented with 20% FCS, 5 units/ml Heparin with 50μg/ml endothelial cell growth supplement (ECGS) (Totam Biologicals, Northampton). Cells were maintained at 37°C with 5% CO_2, and used between passages 3 and 6.

2.2.2 Human Dermal Fibroblasts (HDFs)

Human dermal fibroblasts (TCS) were grown in Dulbecco's modified Eagle's medium (DMEM) (Life technologies Inc.) with 10% foetal calf serum and 100U/ml penicillin and 100mg/ml streptomycin, and maintained at 37 °C with 10% CO_2

2.2.3 Transient transfection of HUVECs

HUVECs were transfected with RNAi duplexes using GeneFECTOR according to the manufacturer's instructions (Venn Nova, USA). The RNAi sequence targetting human PKD1 was 5'-AUACAUGAAAGCUGCAUUCUU-3', whereas a smartpool consisting of 4 separate duplexes was used to target Rab4a (NM_004578, Dharmacon Number M-008539-01), Rab4b (NM_016154, Dharmacon Number M-008780-01), Rab11a (NM_004663, Dharmacon Number M-004726-02), Rab11b

(NM_004218 Dharmacon Number M-004727-02), VEGFR1 (NM_002019, Dharmacon Number M-003136-02) and β3 integrin (NM_000212, Dharmacon Number M-004124-02). In addition, the individual duplexes that make up the Rab4a smartpool (Dharmacon Numbers D-008539-01,03,04 and 05) were used. For recycling assays, HUVECs were seeded into 150x20 Nunclon dishes, and at 70% confluency were washed 4 times with Opti-MEM media. For each dish, 60μl of Genefector in 1ml of Opti-MEM was added dropwise to 1ml of Opti-MEM containing 5μl of 20μM siRNA duplex/smartpool, and this mix was then added to the cell culture plate in a final volume of 12ml. Cells were incubated at 37°C for 4 hours and the media subsequently changes for complete Glutamax Media-199. The following day, transfected HUVECs were re-plated into the required number of 100x15 Nunclon dishes for experimentation 48 hours later. The 12:1 ratio of Genefector:20μM siRNA was maintained for all other transfections, but the overall volume changed depending on the surface area of the plates used.

2.3 RNA ISOLATION AND RT-PCR

Total cellular RNA was isolated from HUVECs using the Qiagen RNAeasy minikit in conjunction with QIAshredder columns for homogenisation. Reverse strand synthesis was undertaken using the Promega Improm-II reverse transcription system then 2μl of this cDNA was used in a PCR reaction consisting of 200μM dNTPs, 0.5μM forward primer, 0.5μM reverse primer, 1x Finnzyme Phusion HF buffer and 0.02U/μl Finnzyme Phusion high-fidelity DNA polymerase. The PCR cycling conditions were 30 cycles of Denaturation at 98°C for 10 seconds, Annealing at 60°C for 20 seconds and Extension at 72°C for 15 seconds. DNA products were

visualised by Agarose gel electrophoresis in the presence of Ethidium Bromide and images taken using a Biorad Gel-Doc system.

Primers were ordered from Invitrogen and their sequences were as follows:

Rab4A F	aaatcgaatcaggtgtcctg
RAb4A R	ctaagaaccacactcctcag
Rab4b F	aacatcgtgctcatcctctc
Rab4b R	ggaacattagctcattgtcctg
Rab11A F	acatcagcatattatcctccag
Rab11A R	tagatcactcttattgcccac
Rab11b F	ctacctattcaaagtcgtgctc
Rab11b R	ctccacgttctcatagctcag

Reference RNA from Human BE and SW480 cells was provided by Dr Kim Moran-Jones of the CRUK Beatson Institute.

2.4 PROTEIN ANALYSIS

2.4.1 Cell lysate preparation and immunoprecipitation

Confluent HUVECs were serum starved for 30 minutes at 37°C then treated for 20 minutes in the absence or presence of receptor tyrosine kinase inhibitor if required. Following this treatment, cells were stimulated with 50ng/ml VEGF-A or 25ng/ml PlGF depending on the experiment.

Cells were then lysed in 500µl, in the case of immunoprecipitation, or 100µl of a buffer containing 200mM NaCl, 75mM Tris, 15mM NaF, 1.5mM Na_3VO_4, 7.5mM

EDTA, 7.5mM EGTA, 1.5% Triton X-100, 0.75% Igepal CA-630, 50μg/ml leupeptin, 50μg/ml Aprotinin and 1mM 4-(2-Aminoethyl) benzynesulphonyl fluoride (AEBSF) and scraped from the dish with a rubber policeman. Lysates were passed through a 27G needle and clarified by centrifugation at 1400 x g for 5 minutes for immunoprecipitation, or 10,000 x g for 10 minutes in the preparation of whole cell lysates.

100μl of whole cell lysate was added to 50μl of 3x sample buffer including 50mM dithiothreitol and boiled for 5 minutes before analysis by Western blotting.

For immunoprecipitation, 57.5μl per condition of Dynal sheep anti-mouse magnetic beads were washed once in PBS containing 0.1% BSA then incubated for 2 hour tumbling at 4°C with 3μg mouse anti-β3 or mouse anti-α5 monoclonal antibodies. For VEGFR2, 57.5μl Dynal sheep anti-mouse magnetic beads per condition were incubated at 4°C with 24μg of mouse anti-goat IgG and 3.3μg goat anti-VEGFR2. Beads were subsequently washed with PBS containing 0.1% BSA before supernatant was added, and this was incubated for 2 hours at 4°C. Beads were then washed 4 times in 0.5X lysis buffer and immunoisolated material eluted by boiling for 5 minutes in 3x sample buffer including 50mM dithiothreitol.

2.4.2 Western Blotting

Samples were separated on an 8% or 12% polyacrylamide gel and transferred to PVDF membrane for 1 hour at 0.6mM/cm2 using a Biorad semi-dry transfer cell. Excess protein sites were blocked with 5% milk or bovine serum albumin (BSA) in TBS (20mM Tris-HCl pH7.4, 137mM NaCl) with 0.1% Tween-20, and blots probed with primary antibodies as described diluted 1 in 1000 overnight at 4°C. Following

subsequent probing with a relevant horseradish peroxidase conjugated secondary antibody, enzyme activity was detected with ECL.

2.4.3 Capture ELISA

Maxisorb 96 well plates (Life Technologies Inc.) were coated overnight with 5μg/ml anti-human β3 integrin, 5μg/ml anti-human α5 integrin or 2μg/ml anti-human VEGFR2 antibody in 0.05M Na_2CO_3 pH9.6 overnight at 4°C. Non-specific binding sites were blocked in PBS containing 0.1% Tween-20 (PBS-T) with 5% BSA for 1 hour at room temperature. Integrins or VEGF receptors were captured by overnight incubation of 50μl of cell lysate at 4°C. Unbound material was removed by extensive washing with PBS-T and wells were incubated for 1 hour at 4°C with streptavidin-conjugated horseradish peroxidase in PBS-T containing 1% BSA. Following further washing, biotinylated receptors were detected by chromogenic reaction with 0.56mg/ml ortho-phenylenediamine in a buffer containing 25.4mM Na_2HPO_4, 12.3mM citric acid, pH5.4 with 0.003% H_2O_2 at room temperature for 10 minutes. The reaction was stopped with 8M H_2SO_4 and absorbance read at 490nm.

2.5 CELL BASED ASSAYS

2.5.1 Internalisation and Recycling Assays

2.5.1.1 Internalisation

Serum starved cells were surface labelled with 0.2mg/ml NHS-SS-Biotin for 30 minutes at 4°C. Labelled cells were then washed twice in ice cold PBS and transferred to serum free Glutamax-199 in the absence or presence of 0.6mM primaquine (PQ) and 50ng/ml VEGF-A or 25ng/ml PlGF at 12°C, before being immediately placed at 37°C for the times indicated to allow internalisation of surface

proteins. At the indicated times, medium was aspirated and the cells rapidly placed onto ice and washed twice with ice-cold PBS. Biotin was removed from proteins remaining at the cell surface by reduction with the membrane impermeant reducing agent Sodium 2-mercaptoethanesulphonate (MesNa). A solution of 20mM MesNa in 50mM Tris, 100mM NaCl was adjusted to pH8.6 with 10M NaOH and immediately added to the cells, with reduction being allowed to proceed for 20 minutes at 4°C with gentle rocking. MesNa was quenched by addition of 20mM Iodoacetamide (IAA) for 10 minutes and the cells lysed as before. Levels of biotinylated integrins and VEGFR2 were determined by immunoprecipitation and Western blot, in addition to capture ELISA.

2.5.1.2 Recycling

Serum starved cells were surface labelled with 0.2mg/ml NHS-SS-Biotin for 30 minutes at 4°C as before. Following surface labelling, 5ml of serum-free Glutamax Media-199 pre-adjusted to 12°C was added to the cells, before the cells were immediately placed in an incubator at 37°C and internalisation allowed to occur for 15 minutes. Cells were then transferred onto ice and washed twice with ice-cold PBS and biotin removed from proteins remaining at the cell surface remaining by reduction with MesNa. The internalised fraction was then chased from the cells by returning them to 37°C in serum free Glutamax Media-199 in the absence or presence of 50ng/ml VEGF-A, 25ng/ml PlGF, 20mM LiCl or 10mM SB216763 for 15 minutes. Cells were returned to ice and biotin removed from recycled proteins by a second reduction with MesNa and unreacted MesNa was quenched with 20mM IAA for 10 minutes and the cells lysed as before. Levels of biotinylated integrin or

VEGFR2 were determined by capture ELISA and expressed as a proportion of the levels found in cells that had not been warmed to 37°C during the chase period.

2.5.2 Collagen tube formations

80-90% confluent HUVECs were placed in Glutamax Media-199 supplemented with 2% FCS and 5 units/ml Heparin overnight. Cells were then seeded onto a collagen gel consisting of 8-parts collagen (Vitrogen), 1-part 10X M-199, 0.5-parts 0.53M NaHCO3, 0.2-parts 0.5M NaOH and 0.3 parts sterile H_2O at 100000 cells per well in medium-199 with 0.2% BSA. Cells were incubated at 37°C for 3 hours to allow adherence, the media subsequently removed, and a top layer of collagen added. Gels were incubated at 37°C for a further hour before medium-199 containing 0.2% BSA and 50ng/ml VEGF-A or 25ng/ml PlGF as required was added. Plates were incubated for either 24 or 36 hours, following which the media was aspirated and the gels fixed in 2.5% glutaraldehyde in PBS. Tubes were then visualised by phase contrast microscopy and quantified using Image J.

2.5.3 Coculture tube formations

HUVECs and HDFs were seeded at 30000 cells/ml each in 24-well dishes and cultured in Glutamax Media-199 supplemented with 20% FCS, 5 units/ml Heparin with 50μg/ml endothelial cell growth supplement (ECGS). Cells were maintained at 37°C with 5% CO_2 and the media changed every 48 hours. SB216763, compound Y or PlGF were added on day 4 and day 6 of co-culture growth, and following 7 days cells were fixed in 4% paraformaldehyde and permeabilised with 0.2% Triton X-100 in PBS for 5 minutes. Non-specific binding sites were blocked with PBS containing 1% BSA for 1 hour at room temperature. Cells were incubated with anti-PECAM-1

primary antibody diluted 1:200 in 1% PBS-BSA at room temperature for 1 hour. Detection was by Cy2-conjugated secondary antibodies diluted 1:200. Tubes were visualised using an Olympus Confocal laser scanning microscope and quantified using Image J.

2.5.4 Boyden chamber motility assays

Boyden chambers were coated with 25μg/ml fibronectin or 2% BSA in PBS at 4°C overnight. Following this, chambers and wells were washed in PBS and placed within Glutamax-199 containing 20% FCS, 5 units/ml Heparin with 50μg/ml ECGS and 20ng/ml VEGF-A. HUVECs were added at 100000 cells per chamber and incubated at 37°C for 6 hours, after which time media was aspirated and any non-migratory cells removed from the upper surface of the membrane with a cotton bud. Cells were fixed in 10% formaldehyde in PBS for 30 minutes at room temperature and subsequently washed with PBS, before being stained with 1% toluidine blue in PBS for 1 hour. As a reference for calculating the % of migrating cells, 100000 cells per well of HUVECs were seeded into individual wells of a 24 well dish coated with 25μg/ml fibronectin and left to adhere for 6 hours at 37°C before fixing and staining as for the Boyden chambers. Membranes and wells were extensively washed with water before the dye was extracted with 100μl 2% SDS and the absorbance of this read at 630nm.

Chapter 3: Vascular endothelial growth factors (VEGFs) regulate αvβ3 recycling in Human Umbilical Vein Endothelial Cells (HUVECs)

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3.1 INTRODUCTION

Angiogenesis, the formation of new blood vessels from pre-existing ones, is critical in a number of physiological and pathological processes. In the normal adult, angiogenesis occurs during the ovarian cycle and in processes such as wound healing. However, angiogenesis also contributes to tumour pathology and is known to be essential for establishing a nutrient supply to allow continued growth of neoplasms [25]. The endothelial cell-specific growth factor, vascular endothelial growth factor A (VEGF-A) is regarded as one of the most important positive regulators of new blood vessel growth, and it is clear that both pathological and physiological angiogenesis appear to rely heavily on VEGF-A. The major mediator of the mitogenic and angiogenic enhancing effects of VEGF-A is accepted to be VEGFR2. Indeed, the presence of both VEGF-A and its receptor VEGFR2 is essential for both embryonic development [43] and tumour vascularisation [44, 45]. Furthermore, the mechanism of action and downstream signalling of VEGFR2 are now well-established to involve ligand-stimulated dimerisation and autophosphorylation of several tyrosine residues in the cytoplasmic domain of VEGFR2, which in turn recruits a range of signalling adaptors and kinases [93]. By contrast, the role of VEGFR1 signalling in angiogenesis remains unclear [336]. Although there are a number of reports indicating that activation of VEGFR1 by PlGF promotes endothelial cell migration, the signalling cascades and cellular processes downstream of this receptor are largely unknown. Indeed, VEGFR1 has low tyrosine kinase activity [337] and many workers consider that PlGF does not promote

receptor autophosphorylation indicating that the pathways through which PlGF/VEGFR1 acts to influence endothelial cells are likely to be somewhat unconventional. One line of evidence suggests that occupation of VEGFR1 with PlGF may initiate molecular 'cross-talk' that amplifies the efficacy of VEGFR2 [85].

Integrin-mediated interaction of endothelial cells with the extracellular matrix (ECM) is also critical for angiogenesis. αvβ3 integrin is a key regulator of endothelial cell function, and the fact that that this heterodimer is likely to have both facilitory and inhibitory roles in normal and pathological angiogenesis highlights the need for a greater understanding of the interplay between growth factor receptor signalling and integrin function in endothelial cells. Growth factor signalling can control integrin function by influencing the way that the heterodimers are handled by the endosomal and receptor recycling pathways. In particular, we have recently shown that αvβ3 integrin trafficking is controlled by both the PKB/GSK3 axis and protein kinase D1 (PKD1) in fibroblasts and that this influences the migratory behaviour of these cells [193, 301]. Additionally, the endosomal and recycling pathway is now known to play a key role in defining the amplitude, duration and spatial organisation of signalling downstream of a number of growth factor receptors [338]. Taken together these factors nominate a role for the endosomal system in coordinating the interplay between ECM and growth factor receptors during angiogenesis.

Here I show that αvβ3 and α5β1 integrins are rapidly internalised and recycle back to the plasma membrane. Moreover, treatment with PlGF or VEGF-A stimulates delivery of αvβ3 (but not α5β1) from an internal pool to the plasma membrane, via a mechanism that requires VEGFR1, GSK3β and Rab4a.

3.2 RESULTS

3.2.1 αvβ3 and α5β1 integrins are expressed in HUVECs and engage in exo-endocytic cycling

We have previously demonstrated that PDGF rapidly increases levels of αvβ3 integrin at the plasma membrane of mouse 3T3 fibroblasts, and that this is owing to an increase in the recycling of αvβ3 integrin to the plasma membrane from early endosomes via a Rab4-dependent mechanism. Therefore, I wished to test whether PlGF and VEGF-A regulate integrin recycling in primary cultured HUVECs.

To achieve this, I utilised a biotin-based recycling assay which had previously been developed in the laboratory and used for the determination of recycling in fibroblasts and tumour cell lines. Initially it was necessary for me to validate this assay for the detection of integrin recycling in endothelial cells, and to fine tune the parameters of the technique for application to primary cultured cells. Firstly, I determined whether αvβ3 and α5β1 integrins were expressed at the plasma membrane in HUVECs and whether antibodies raised against the α5 and β3 subunits could specifically recognise their respective heterodimers α5β1 and αvβ3. To do this, HUVECs were surface labelled on ice with sulfo-NHS-SS-Biotin, a membrane impermeant protein labelling reagent which will react with free primary amino groups thus forming covalent adducts with basic residues in protein side chains exposed at the plasma membrane. Following extensive washing to remove unreacted NHS-SS-Biotin, the cells were lysed in a Triton X100-containing buffer and α5 or β3 integrins immunoprecipitated from the lysates using antibodies conjugated to magnetic beads. Where appropriate an isotype-matched control antibody (RG16) was employed to determine the non-specific binding of biotinylated proteins to the beads. Immunoprecipitated material was then subjected to SDS-

PAGE followed by Western blotting, and biotinylated proteins detected using streptavidin-conjugated HRP. This analysis revealed that anti-α5 integrin coated beads immunoprecipitated two biotinylated proteins, one corresponding to the α5 chain itself (≈150kDa) and the other to its associated β1 subunit (≈120kDa) (Fig 3.1A). Additionally, the anti-β3 coated beads immunoprecipitated a protein corresponding to the β3 chain (≈80kDa) itself and its associated αv subunit (≈140kDa) (Fig 3.1A). The isotype control antibody did not precipitate any detectable biotinylated proteins (Fig 3.1A) and, importantly, there were no additional associated proteins in the integrin immunoprecipitations, indicating the specificity and selectivity of this approach as a method for detecting particular integrin heterodimers.

To determine whether αvβ3 and α5β1 are internalised in HUVECs, cells were serum starved and surface-labelled with NHS-SS-Biotin on ice as before. The cells were then warmed to 37°C for the indicated times to allow internalisation to occur, and then returned to ice. The NHS-SS-Biotin reagent contains a disulphide bond within a linker region which connects the reactive NHS moiety to the biotin, and this is susceptible to cleavage by reducing agents such as sodium 2-mercaptoethanesulfonate (MesNa) or glutathione (GSH). Thus to remove biotin from proteins remaining at the cell surface the cells were incubated with the membrane impermeant reducing agent MesNa for 15 minutes on ice. The biotinylated fraction of proteins will then represent those that had been internalised and thus protected from this reducing agent.

Cell surface reduction of HUVECs that had not been warmed to 37°C led to the removal of most of the biotin label from the integrins (Fig 3.1B; upper panel), indicating that under these circumstances the heterodimers had not been internalised

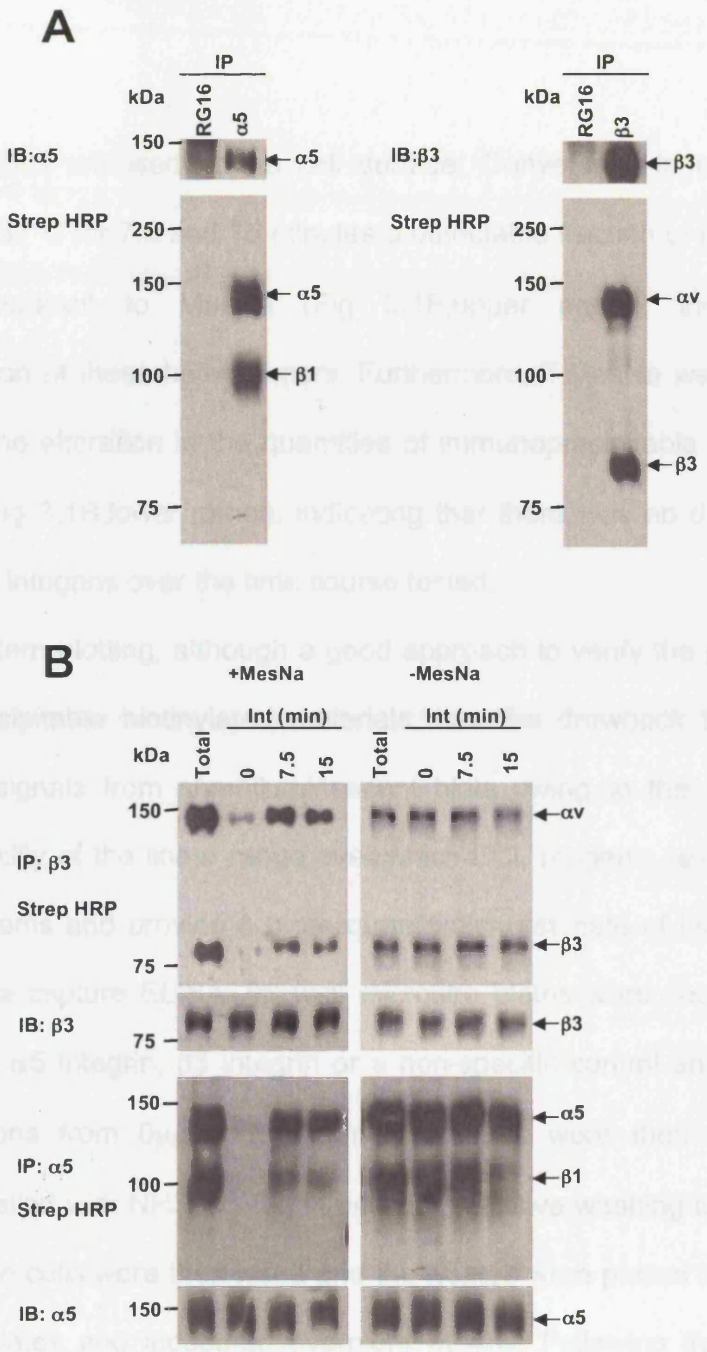

Figure 3.1: Internalisation of integrins in HUVECs

(**A**) Serum-starved HUVECs were surface-labelled with 0.2 mg/ml NHS-S-S-Biotin for 30 min at 4°C, lysates were prepared and immunoprecipitated (IP) with antibodies against α5, β3 or RG16 isotype control and immobilised material analysed by immunoblotting (IB) with anti-α5 or anti-β3, or by streptavidin-conjugated HRP. (**B**) Surface labelled cells were warmed to 37°C for the times indicated. Biotin was released from proteins remaining at the cell surface by MesNa treatment (left panels) and biotinylated integrin determined by immunoprecipitation of either β3 or α5 followed by probing of Westerns with Strep-HRP

and were thus exposed at the cell surface. Conversely, in cells that had been warmed to 37°C for 7.5 and 15 minutes a detectable fraction of both $\alpha5\beta1$ and $\alpha v\beta3$ became resistant to MesNa (Fig 3.1B;upper panel), indicating the likely internalisation of these heterodimers. Furthermore, if MesNa were omitted from the incubation no alteration in the quantities of immunoprecipitable $\alpha5\beta1$ or $\alpha v\beta3$ were detected (Fig 3.1B;lower panel), indicating that there was no degradation of those biotinylated integrins over the time course tested.

Western blotting, although a good approach to verify the purity and quality of immunoprecipitable biotinylated materials, has the drawback that it is difficult to quantitate signals from chemiluminescent blots owing to the unpredictability and irreproducibility of the linear range over which ECL reagents respond. To overcome these problems and provide a more quantitiative estimate of internalised integrin, I developed a capture ELISA. 96 well microtitre plates were coated with antibodies recognising $\alpha5$ integrin, $\beta3$ integrin or a non-specific control antibody at increasing concentrations from 0μg/ml to 8μg/ml. HUVECs were then serum-starved and surface-labelled with NHS-SS-Biotin prior to extensive washing to remove unreacted reagent. The cells were then lysed and the lysates were placed into antibody-coated microtitre plates and incubated overnight at 4°C. Following this, the plates were washed with PBS containing 0.1% Tween-20 in order to remove any proteins not bound by the antibodies. Biotinylated proteins captured by the specific antibodies were detected by streptavidin-HRP and the addition of the substrate o-phenylene diamine (OPA) which is converted to a yellow coloured product in the presence of HRP and H_2O_2. Reactions were stopped after approximately 15 minutes with 8M HCl and the absorbance values at 490nm measured using a spectrophotometer.

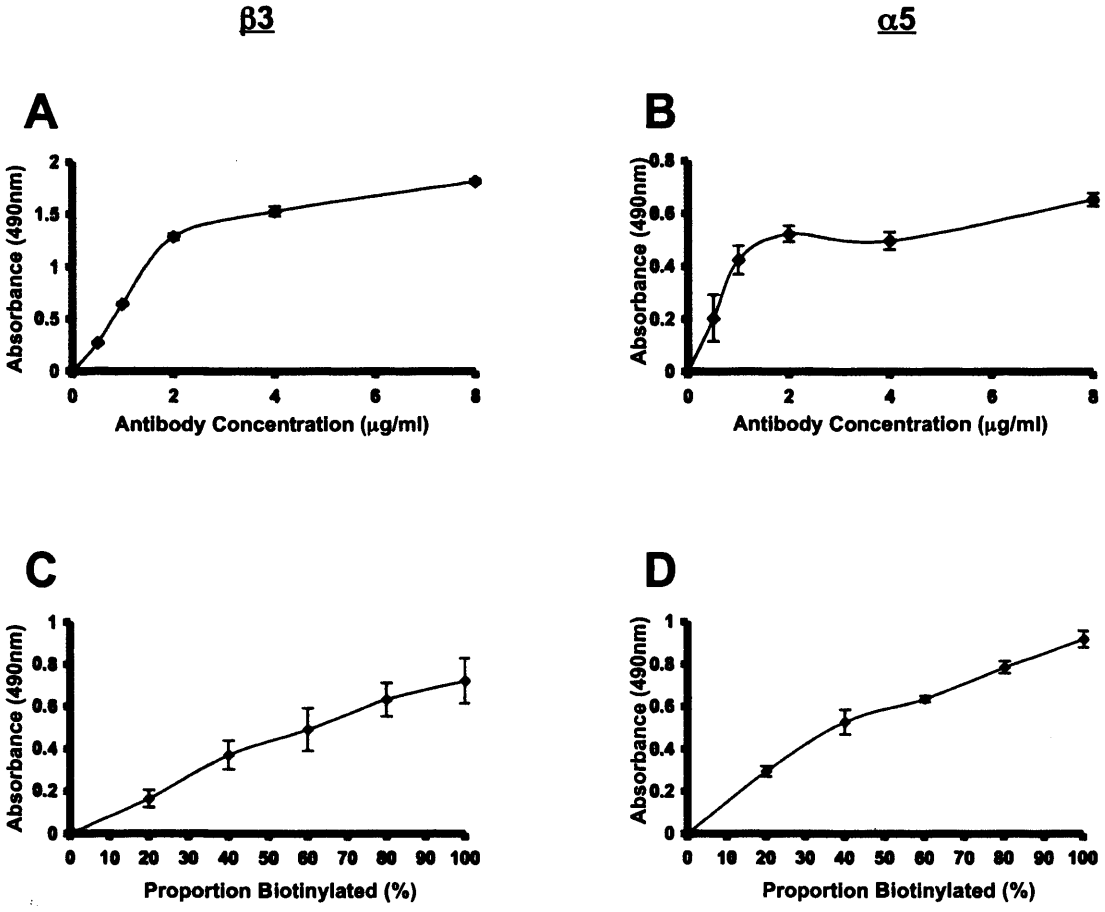

Figure 3.2: Validation of integrin capture-ELISA

(**A,B**) Serum-starved HUVECs were surface-labelled with 0.2 mg/ml NHS-S-S-Biotin for 30 min at 4°C, lysates were prepared and added to microtitre wells coated with either anti-human β3 (**A**) or anti-human α5 (**B**) integrin monoclonal antibodies at increasing concentrations, before incubation overnight at 4°C and ELISA development.

(**C,D**) Serum-starved HUVECs were surface-labelled with 0.2 mg/ml NHS-S-S-Biotin for 30 min at 4°C, lysates were prepared and serial diluted with unlabelled lysates before being added to microtitre wells coated with 5μg/ml of either anti-human β3 (**C**) or anti-human α5 (**D**) integrin monoclonal antibodies.

For both α5- and β3-coated microtitre plates the absorbance values measured increased with antibody quantity up to a coating concentration of 2μg/ml, with minimal increase observed at antibody concentrations higher than this (Fig 3.2A,B). In view of this, an antibody concentration of 5μg/ml was used to coat plates in all future experiments analysing α5 and β3 biotinylation.

It was important to determine whether the capture-ELISA responded linearly to the quantity of biotinylated integrins that was present in the microtitre wells. To do this lysates from NHS-SS-Biotin-labelled cells were mixed in various proportions with lysates from unlabelled HUVECs and then added to anti-α5 or anti-β3 coated 96 well microtitre plates and incubated overnight at 4°C before washing and developing as described earlier. This analysis revealed that within the working range of the assay (i.e. 0-0.6AU) the absorbance values change in direct proportion to the quantity of biotinylated integrin added to the microtitre well (Fig 3.2C,D).

To use the capture-ELISA to quantify integrin internalisation, 5 sets of experimental replicates are required, these are termed: Total, Blank (zero time point for internalisation), 5 minute internalisation, 10 minute internalisation and 15 minute internalisation. HUVECs were serum starved and surface labelled as before, then the 5, 10 and 15 minute internalisation groups were warmed at 37°C for the indicated time periods in the absence or presence of the recycling inhibitor primaquine (PQ), before being returned to ice and subjected to MesNa reduction. Following extensive washing to remove residual reducing agent, HUVECs were lysed and the lysates added to an anti-β3 coated 96 well microtitre plate and incubated overnight at 4°C prior to development as outlined earlier. The primary absorbance data (Fig 3.3A) were then subjected to the following manipulations:

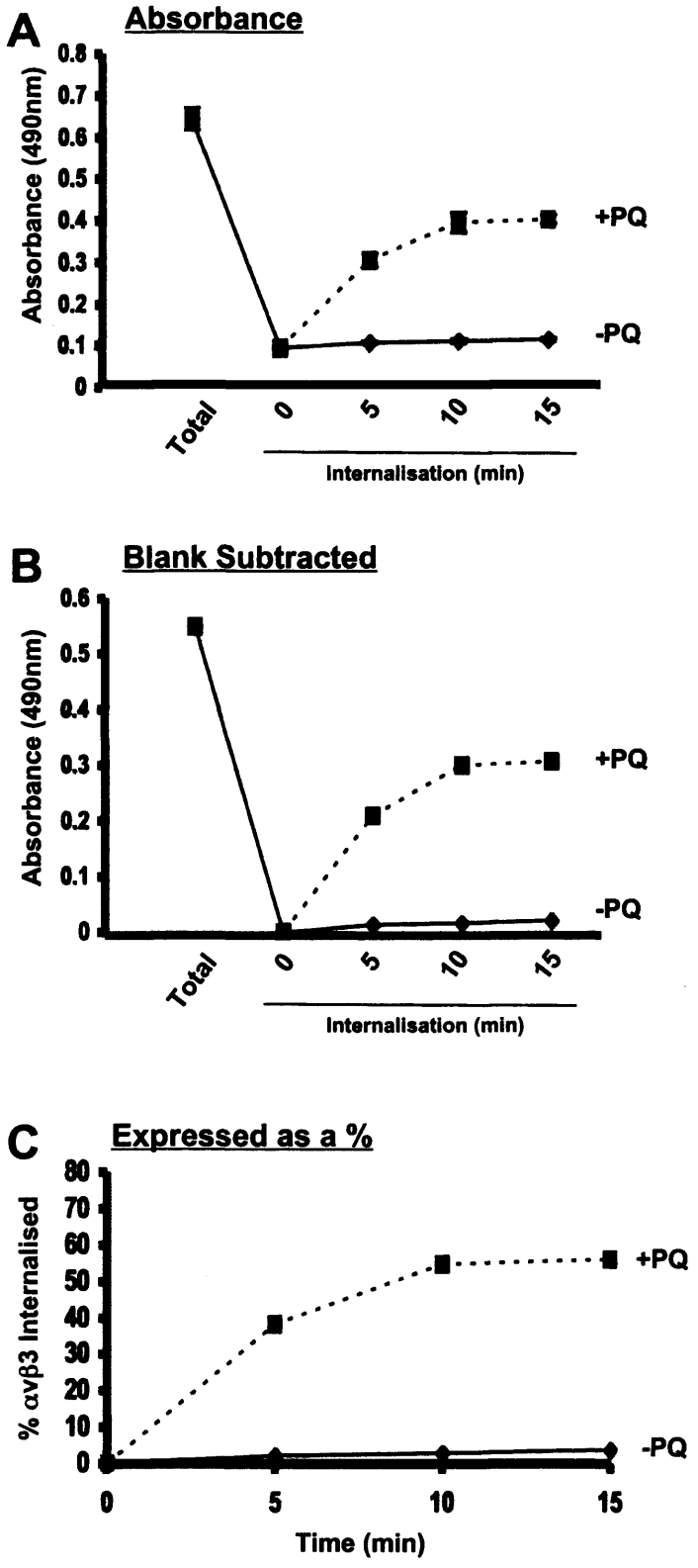

Figure 3.3: Data processing of integrin internalisaton capture-ELISA

Serum-starved HUVECs were surface-labelled with 0.2 mg/ml NHS-S-S-Biotin for 30 min at 4 °C and Surface labelled cells were warmed to 37 °C in the absence or presence of 0.6 mM PQ for the times indicated. Biotin was released from proteins remaining at the cell surface and biotinylated integrin determined by capture-ELISA using microtitre wells coated with anti-human β3 integrin monoclonal antibodies. From the initial absorbance values obtained (**A**), blank values were subtracted (**B**) and the amount of integrin internalised expressed as a percentage of the total surface integrin labelled (**C**).

The absorbance values of the blank were subtracted from all groups to remove background levels of absorbance (Figure 3.3B) and the amount of internalised integrin was expressed as a percentage of the total labelled integrin (Figure 3.3C). From this it is clear that αvβ3 was endocytosed in HUVECs, with the amount of internalised integrin reaching a maximum at ≈10-15 minutes following incubation of cells at 37°C. Moreover, it is clear that addition of the receptor recycling inhibitor primaquine strongly promoted the rate of internalisation and the quantity of biotinylated integrin retained within cells, thus supporting the conclusion that this assay is capable of measuring integrin endocytosis in HUVECs.

I proceeded to use the capture-ELISA approach to examine the endocytosis of αvβ3 and α5β1 integrins in HUVECs. In serum starved HUVECs, αvβ3 and α5β1 integrins are internalised resulting in internal pools representing approximately 10% and 20% of initial surface integrin respectively (Fig 3.4A,B). Moreover, inclusion of the receptor-recycling inhibitor primaquine (PQ) during the internalisation period effected a marked increase in the internalisation of both integrins (Fig 3.4A,B), demonstrating that αvβ3 and α5β1 integrins recycle back to the plasma membrane shortly after internalisation. In fibroblasts, PDGF does not affect the endocytic rates of either αvβ3 or α5β1 integrins. Internalisation assays performed in the presence of PQ and either PlGF or VEGF-A over the time course shown demonstrated that PlGF and VEGF-A do not affect endocytic rates or total levels of αvβ3 or α5β1 integrins in HUVECs over the time course investigated (Fig 3.5). Moreover the total quantity of β3 and α5 did not change during the internalisation period, indicating that the integrins were not appreciably degraded over the course of the internalisation assays (Fig 3.5 inset).

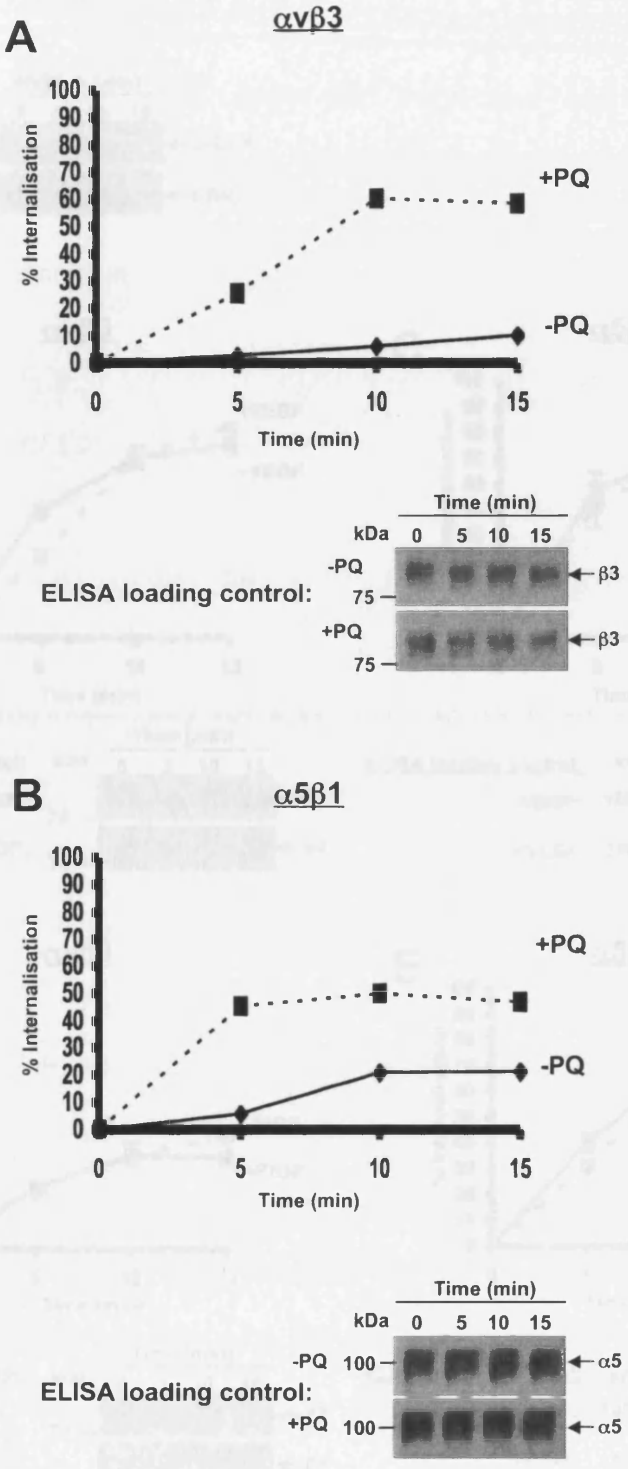

Figure 3.4: Internalisation of integrins in HUVECs

Serum-starved HUVECs were surface-labelled with 0.2 mg/ml NHS-S-S-Biotin for 30 min at 4°C and Surface labelled cells were warmed to 37°C in the absence or presence of 0.6 mM PQ for the times indicated. Biotin was released from proteins remaining at the cell surface and biotinylated integrin determined by capture-ELISA using microtitre wells coated with anti-human β3 (**A**) or anti human-α5 (**B**) integrin monoclonal antibodies. ELISA loading controls were performed by subjecting a fraction of lysate to SDS-PAGE and immunoblotting with either anti-human β3 (**A**) or anti-human α5 (**B**) monocloanl antibodies.

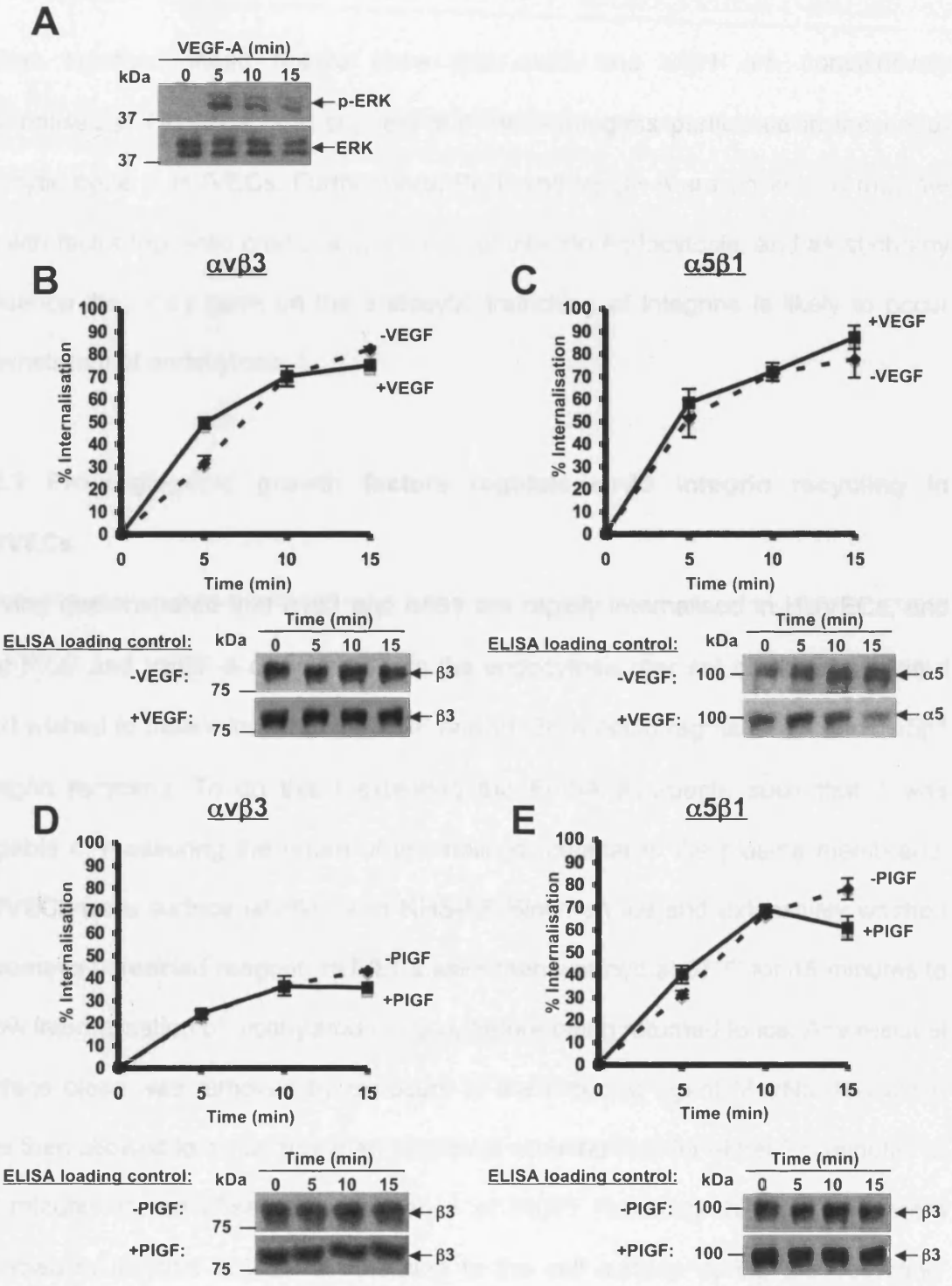

Figure 3.5: VEGFs do not regulate integrin internalisation

Surface-labelled cells were warmed to 37 °C in the presence of 0.6 mM PQ in the absence and presence of 50 ng/ml VEGF-A (**B,C**) or 25ng/ml PIGF(**D,E**) for the times indicated. Internalised integrin was determined by capture ELISA using microtitre wells coated with anti-human β3 (**B,D**) or anti-human α5 (**C,E**) integrin monoclonal antibodies. Response to VEGF-A and ELISA loading was determined by SDS-PAGE and immunoblottng with anti-phospho-ERK (**A**), anti-human β3 (**B,D**) or anti-human α5 (**C,E**)

Taken together, these results show that αvβ3 and α5β1 are constitutively internalised in HUVECs, and suggest that these integrins participate in the endo-exocytic cycle in HUVECs. Furthermore, PlGF and VEGF-A are unlikely to mediate growth-factor triggered changes in the rate of integrin endocytosis, and as such any influence they may have on the endocytic trafficking of integrins is likely to occur downstream of endocytosis.

3.2.2 Pro-angiogenic growth factors regulate αvβ3 integrin recycling in HUVECs

Having demonstrated that αvβ3 and α5β1 are rapidly internalised in HUVECs, and that PlGF and VEGF-A do not regulate the endocytosis (per se) of either integrin, I next wished to determine whether PlGF and VEGF-A could regulate αvβ3 and α5β1 integrin recycling. To do this I extended the ELISA approach, such that it was capable of measuring the return of internalised material to the plasma membrane. HUVECs were surface labelled with NHS-SS-Biotin on ice and extensively washed to remove unreacted reagent. HUVECs were then warmed at 37°C for 15 minutes to allow internalisation of biotinylated integrin, before being returned to ice. Any residual surface biotin was removed by exposure to the reducing agent MesNa. Recycling was then allowed to occur during an additional warming step for either 7.5 minutes or 15 minutes in the absence or presence of PlGF. Following this the cells were returned to ice and any biotin returning to the cell surface during the 'recycling' period was removed by a second exposure to MesNa. The cells were then lysed and the amount of labelled integrin remaining was determined by capture ELISA as outlined earlier. These experiments were composed in the following way: As for the internalisation assays described earlier there was a 'Total' group which was labelled

and remained on ice throughout the experiment, and a 'Blank' group which was labelled and subjected to MesNa exposure whilst remaining on ice throughout. In addition to this, a group representing the 'Internal Pool' of biotinylated integrin is required as a reference point. This group was subjected to a single warming period of fifteen minutes to allow internalisation to occur, followed by MesNa treatment to remove residual surface biotin. This 'internal pool' group then remained on ice and was not re-warmed during the 'recycling' period. Recycling was determined by re-warming cells to 37°C for the indicated 'recycling periods' and then re-exposure to MesNa on ice. The initial absorbance values measured demonstrated that HUVECs were efficiently surface labelled with NH-SS-Biotin (Fig 3.6A – Total) and as before exposure to MesNa removed most of this label (Fig 3.6A – Blank), indicating that little internalisation of αvβ3 occurred at 4°C. The 'internal pool' of biotinylated integrin was detectable as being approximately 5-10% of the total labelled surface integrins (Fig 3.6A – Internal Pool) and following the recycling period a decrease in absorbance value from the internal pool was observed (Fig 3.6A,B). The background or 'blank' values were then subtracted from all determinants to yield the values indicated in Fig 3.6C,D, then these 'recycling' values were expressed as a percentage of the internal pool (Fig 3.6E). From this it is apparent that approximately 30% of internalised αvβ3 was constitutively returned to the plasma membrane over a 15 minute time period (Fig 3.6E Red). Moreover, the addition of PlGF promoted the recycling of αvβ3 such that the rate at which this integrin returned to the plasma membrane was increased by approximately 2-fold (Fig 3.6E Blue).

I then proceeded to assess the influence of PlGF and other agents on the recycling of αvβ3 and α5β1 using the assay described above.

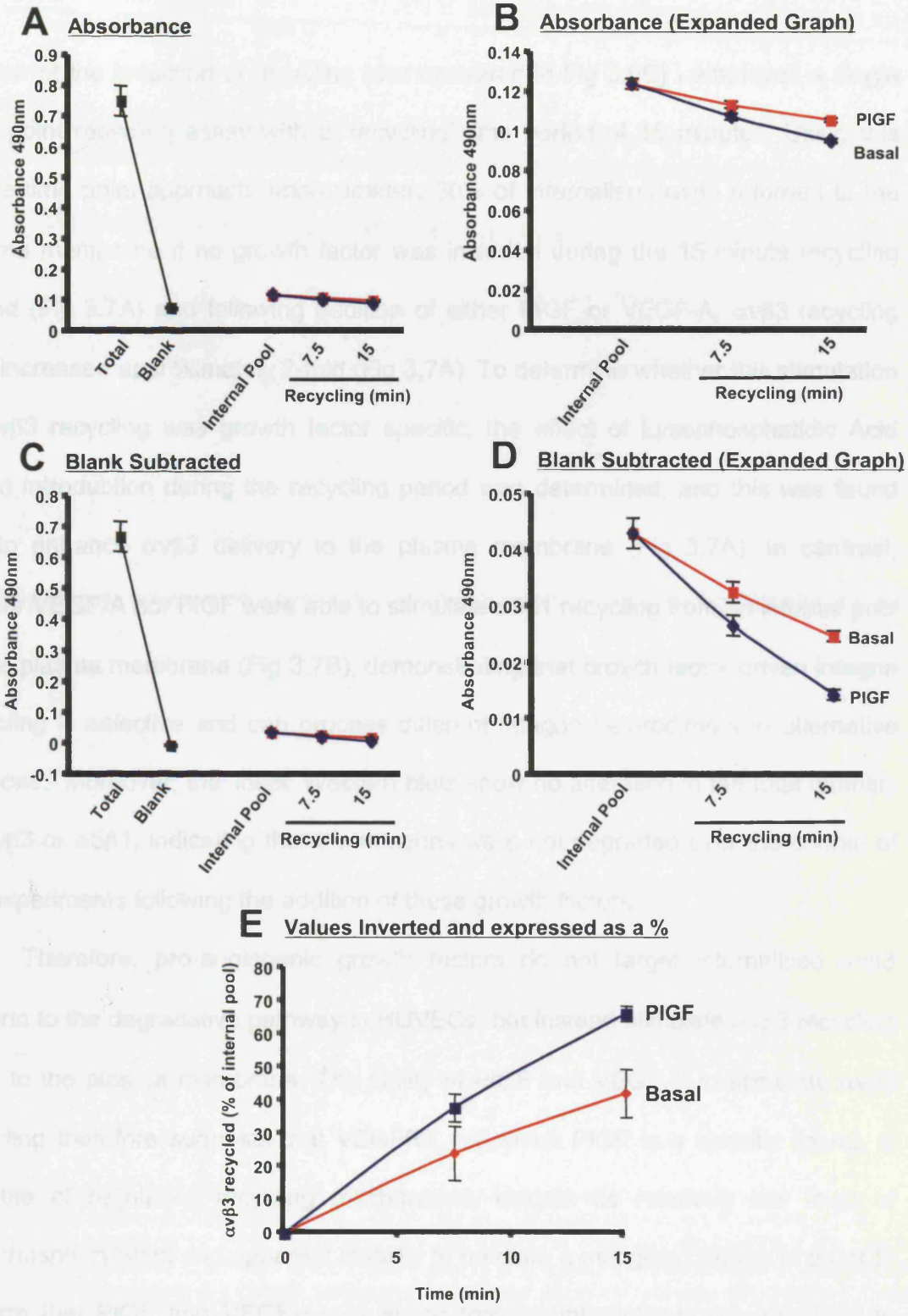

Figure 3.6: Data processing of integrin recycling capture-ELISA
HUVECs were surface-labelled with NHS-SS-Biotin and internalisation allowed to proceed for 15 min at 22°C. Biotin was removed from receptors remaining at the cell surface by treatment with MesNa at 4°C. Cells were then warmed to 37°C for 7.5 or 15 minutes in the absence (basal) or presence 25ng/ml PlGF, to allow recycling to the plasma membrane, followed by a second reduction with MesNa. Cells were lysed and biotinylated integrin determined by capture-ELISA using microtitre wells coated with anti-human-β3 integrin monoclonal antibodies. From the initial absorbance values obtained (**A,B**), blank values were subtracted (**C,D**) and the amount of integrin recycled expressed as a percentage of the internal pool of labelled integrin(**E**).

In view of the induction of recycling (demonstrated in Fig 3.6E) I employed a single time point recycling assay with a 'recycling' time period of 15 minutes. Using this single time point approach, approximately 30% of internalised αvβ3 returned to the plasma membrane if no growth factor was included during the 15-minute recycling period (Fig 3.7A) and following addition of either PlGF or VEGF-A, αvβ3 recycling was increased approximately 2-fold (Fig 3.7A). To determine whether this stimulation of αvβ3 recycling was growth factor specific, the effect of Lysophosphatidic Acid (LPA) introduction during the recycling period was determined, and this was found not to enhance αvβ3 delivery to the plasma membrane (Fig 3.7A). In contrast, neither VEGF-A nor PlGF were able to stimulate α5β1 recycling from an internal pool to the plasma membrane (Fig 3.7B), demonstrating that growth factor-driven integrin recycling is selective and can process different integrin heterodimers in alternative fashions. Moreover, the 'inset' Western blots show no alteration in the total quantity of αvβ3 or α5β1, indicating that the integrins were not degraded over the course of the experiments following the addition of these growth factors.

Therefore, pro-angiogenic growth factors do not target internalised αvβ3 integrin to the degradative pathway in HUVECs, but instead stimulate αvβ3 recycling back to the plasma membrane. The ability of PlGF and VEGF-A to stimulate αvβ3 recycling therefore suggests that VEGFR1, for which PlGF is a specific ligand, is capable of regulating recycling mechanisms, despite its relatively low level of autophosphorylation and apparent inability to mediate a mitogenic signal. In order to confirm that PlGF and VEGF-A are acting through interactions with VEGFR1 to regulate αvβ3 recycling, RNAi knockdown of VEGFR1 was used to determine the role of this receptor in regulating recycling.

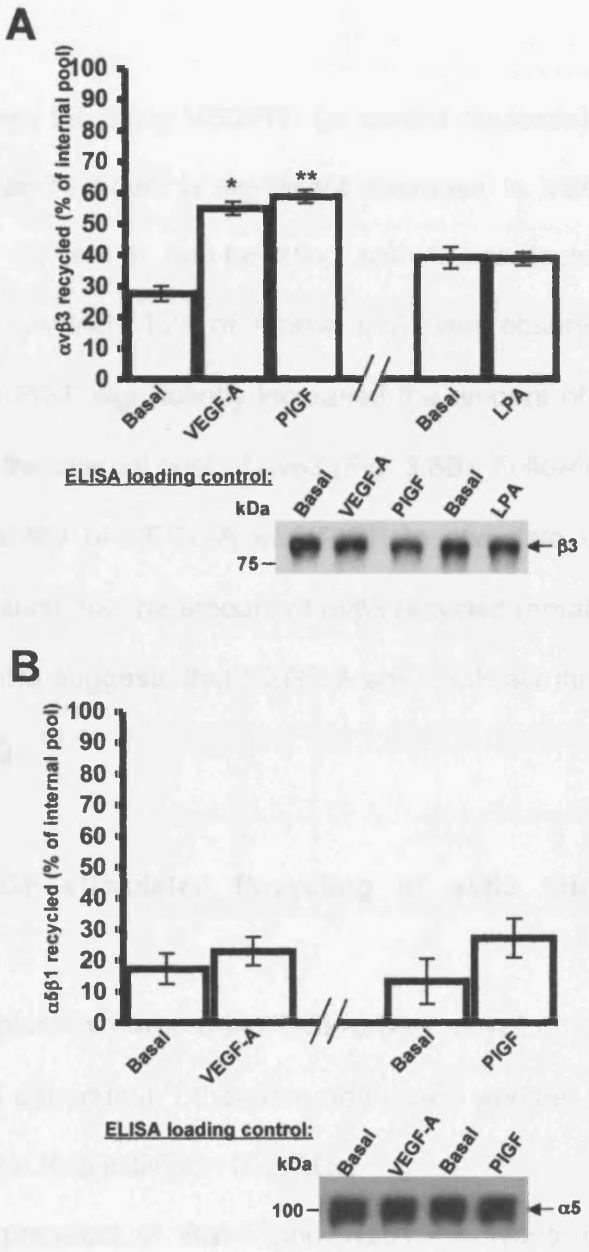

Figure 3.7: VEGF-A and PIGF promote recycling of αvβ3 but not α5β1
HUVECs were surface-labelled with NHS-SS-Biotin and internalisation allowed to proceed for 15 min at 22°C. Biotin was removed from receptors remaining at the cell surface by treatment with MesNa at 4°C. Cells were then warmed to 37°C for 15 minutes in the absence (basal) or presence of 50ng/ml VEGF-A, 25ng/ml PIGF, or LPA, to allow recycling to the plasma membrane, followed by a second reduction with MesNa. Cells were lysed and biotinylated integrin determined by capture-ELISA using microtitre wells coated with anti-human-β3 (**A**) or anti-human-α5 (**B**) integrin monoclonal antibodies. The proportion of integrin recycled to the plasma membrane is expressed as % of the pool of integrin labelled during the internalisation period. Values are mean±s.e.m., n>10 separate experiments. (*, ** $p<0.00001$ determined using students t-test)

Oligonucleotide duplexes targetting VEGFR1 (or control duplexes) were transfected into HUVECs and after 48 hours a significant decrease in VEGFR1 levels was observed (Fig 3.8A). In control non-targeting siRNA transfected cells, a basal recycling level of approximately 10% of internal αvβ3 was observed and treatment with either VEGF-A or PlGF significantly increased the amount of αvβ3 recycled to approximately 60% of the internal pool of αvβ3 (Fig 3.8B). Following suppression of VEGFR1 levels the ability of VEGF-A and PlGF to stimulate αvβ3 recycling is completely abrogated such that the amount of αvβ3 recycled remains at basal levels (Fig 3.8B). This therefore suggests that VEGF-A and PlGF act through VEGFR1 to promote αvβ3 recycling.

3.2.3 VEGF and PlGF stimulated Recycling of αvβ3 integrin is Rab4a-dependent

Previous work in fibroblasts showed that PDGF-driven recycling of αvβ3 from early endosomes was Rab4 dependent. I therefore addressed whether recycling of αvβ3 is similarly dependent on Rab activity in HUVECs.

Firstly, the expression of Rab4 and Rab11 isotypes in HUVECs was determined by PCR. This demonstrated that Rab4a and Rab11a were expressed in HUVECs whereas Rab4b and Rab11b were not (Fig 3.9A). An RNAi based approach was used to suppress the levels of Rab4 in HUVECs. Pooled siRNAs targetting Rab4a and Rab4b were transfected into HUVECs and the levels of Rab4 determined 48 hours following this. Oligonucleotides that targetted Rab4a significantly reduced the overall levels of Rab4, whereas targetting of Rab4b did not, confirming that Rab4a is the predominant isoform in HUVECs (Fig 3.9BA).

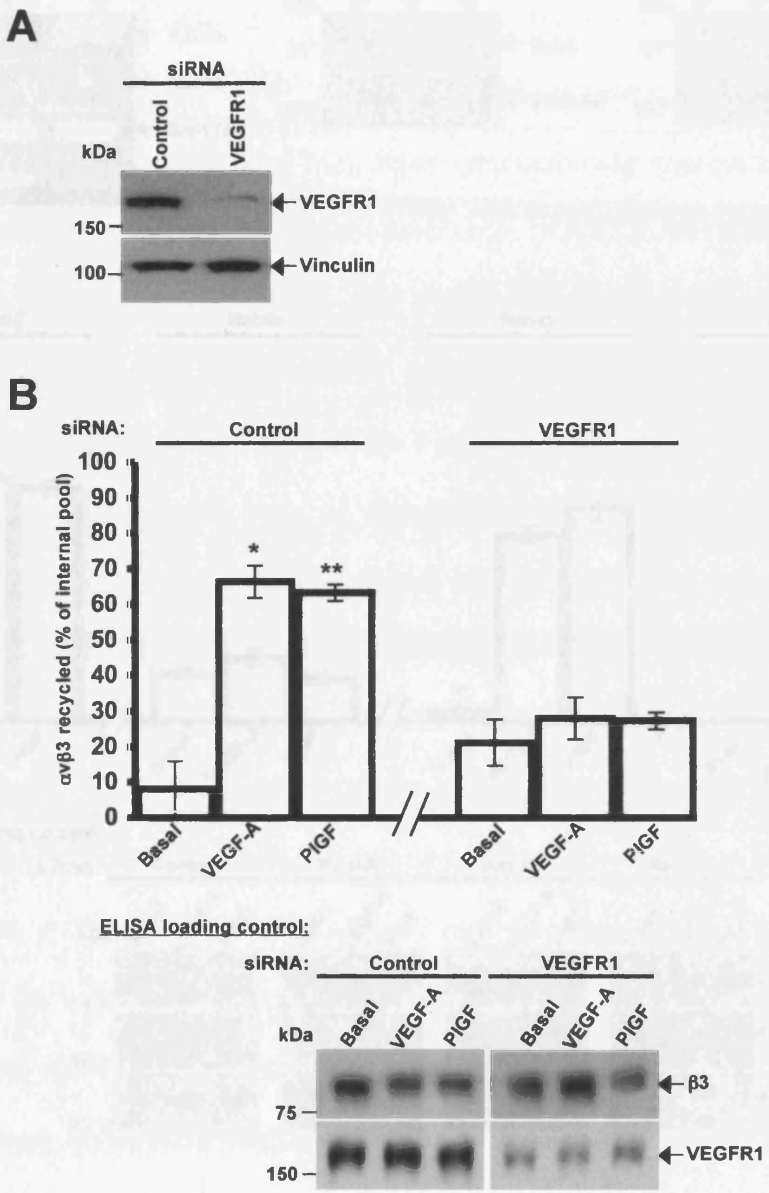

Figure 3.8: VEGFR1 is required for VEGF-A or PlGF driven αvβ3 recycling

(A) HUVECs were transfected with RNAi duplexes targetting VEGFR1, and after 48 hours lysates were prepared and immunoblotted for VEGFR1 or vinculin as a loading control.

(B) HUVECs were transfected with either non-targeting control RNAi duplexes or RNAi duplexes targeting VEGFR1 48 hours prior to experimentation. Serum-starved HUVECs were surface labelled with 0.2mg/ml NHS-SS-Biotin for 30 minutes at 4°C, and internalisation of cell surface proteins allowed to proceed for 15 minutes. Non-internalised biotin was removed by a brief exposure to MesNa at 4°C, and internalised integrin chased to the cell surface for 15 minutes at 37°C in the presence or absence of 50ng/ml VEGF-A or 25ng/ml PlGF. Cells were then re-exposed to MesNa and biotinylated integrin detected by capture ELISA using microtitre wells coated with anti-human β3 monoclonal antibody.Values are mean±s.e.m. for at least 3 experiments. (*, ** $p<0.00001$ determined using students t-test)

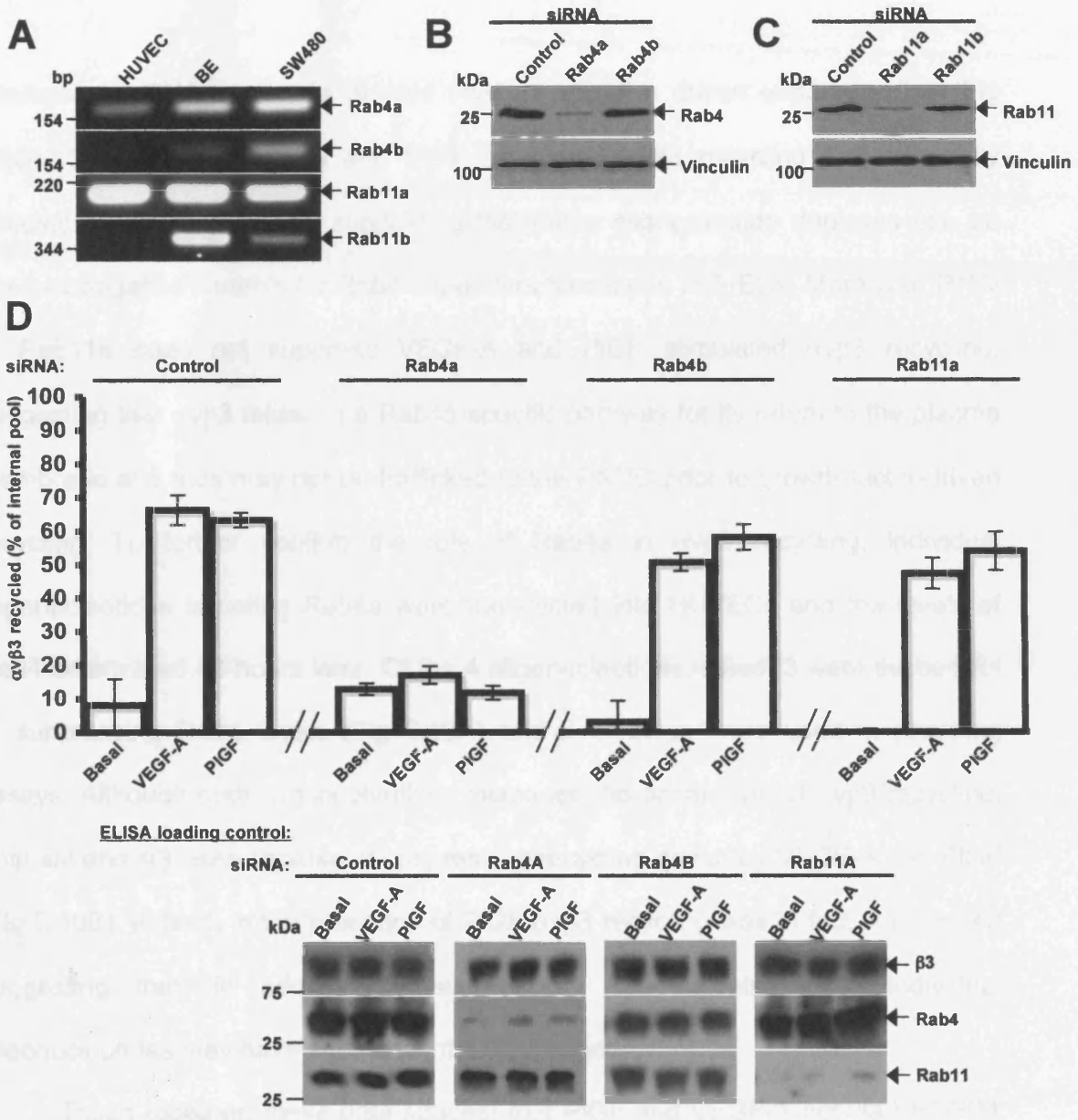

Figure 3.9: VEGF-A and PlGF driven αvβ3 recycling is Rab4a-dependent

(A) RNA was extracted from HUVECs and used as a template for PCR reactions using primers specific towards Rab4a, Rab4b, Rab11a and Rab11b mRNA sequences. RNA from the human BE and SW40 cell lines were used as controls.

(B) HUVECs were transfected with RNAi duplexes targetting either Rab4a, Rab4b, Rab11a or Rab11b and after 48 hours the level of Rab4 or Rab11 protein was determined following preparation of lysates and Western blotting.

(C) HUVECs were transfected with RNAi duplexes targetting Rab4a, Rab4b or Rab11a 48 hours prior to experimentation. Serum-starved HUVECs were surface labelled with 0.2mg/ml NHS-SS-Biotin for 30 minutes at 4°C, and internalisation of cell surface proteins allowed to proceed for 15 minutes. Non-internalised biotin was removed by a brief exposure to MesNa at 4°C, and internalised integrin chased to the cell surface for 15 minutes at 37°C in the presence or absence of 50ng/ml VEGF-A or 25ng/ml PlGF. Cells were then re-exposed to MesNa and biotinylated integrin detected by capture ELISA using microtitre wells coated with anti-human β3 monoclonal antibody.Values are mean±s.e.m. for at least 3 experiments.

Interestingly, RNAi of Rab4a ablated PlGF or VEGF-A driven αvβ3 recycling (Fig 3.9D). Conversely, VEGF-A and PlGF stimulated αvβ3 recycling was minimally affected by RNAi of Rab4b suggesting that these olignucleotide duplexes can be used as negative controls for Rab4-dependent function in HUVECs. Moreover, RNAi of Rab11a does not suppress VEGF-A and PlGF stimulated αvβ3 recycling, suggesting that αvβ3 relies on a Rab4a-specific pathway for its return to the plasma membrane and thus may not be trafficked to the PNRC prior to growth factor-driven recycling. To further confirm the role of Rab4a in αvβ3 recycling, individual oligonucleotides targeting Rab4a were transfected into HUVECs and the levels of Rab4 determined 48 hours later. Of the 4 oligonucleotides tested, 3 were successful at suppressing Rab4 levels (Fig 3.10A) and 2 of these were used in recycling assays. Although both oligonucleotides increased the basal level of αvβ3 recycling, both si2 and si3 were capable of suppressing recycling driven by VEGF-A and PlGF (Fig 3.10B). Indeed, in the presence of PlGF αvβ3 recycling was in fact suppressed suggesting that, in addition to suppressing Rab4 levels, these individual oligonucleotides may have additional off target effects.

Taken together, these data suggest that PlGF and VEGF-A act via VEGFR1 to promote the recycling of αvβ3 integrin from an internal pool to the plasma membrane and that this occurs via a Rab4a-dependent mechanism.

3.2.5 GSK3β inactivation (not PKD1 activation) is required for stimulation of αvβ3 recycling in HUVECs

Previously, we have shown that in fibroblasts PDGF stimulation results in the phosphorylation of protein kinase D1 (PKD1) at Ser^{916}, and that this event is required

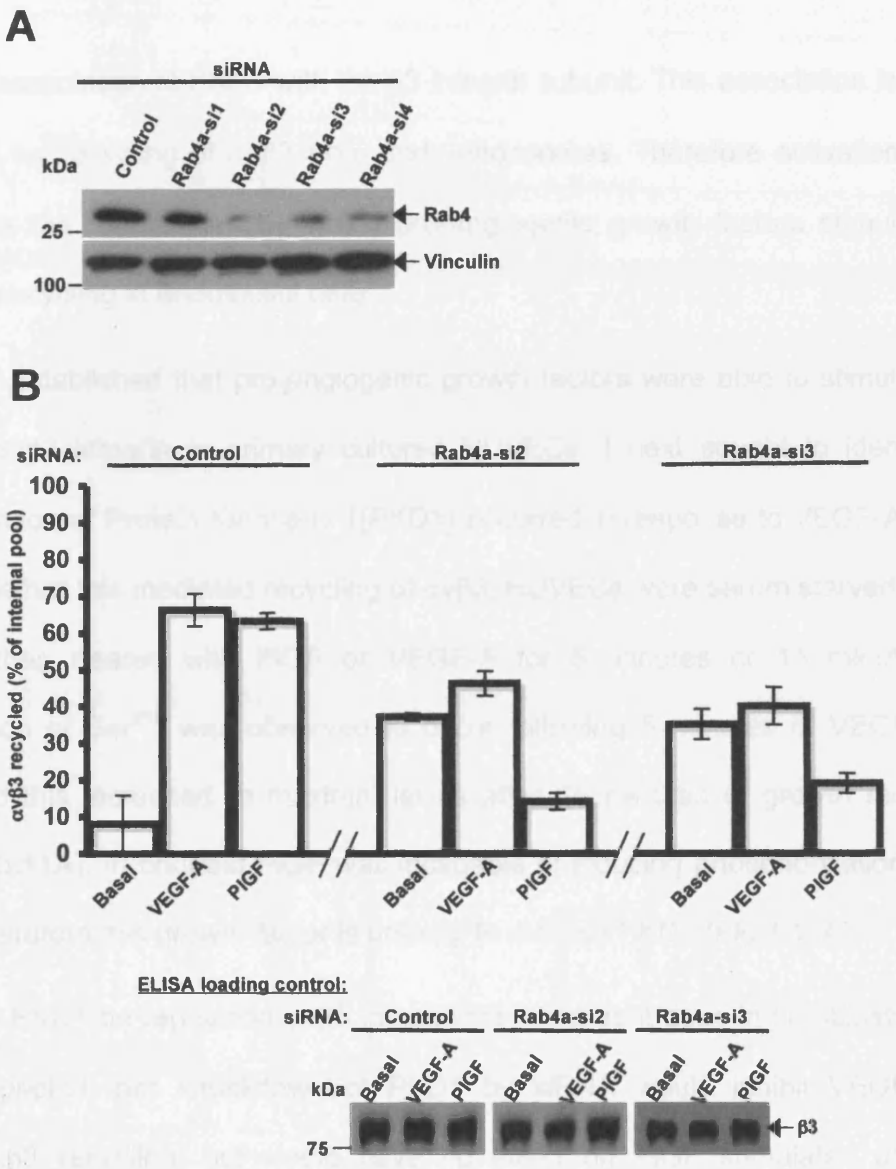

Figure 3.10: Individual RNAi duplexes targetting Rab4a abrogate VEGF-A/PIGF-driven αvβ3 recycling
(A) HUVECs were transfected with individual RNAi duplexes targetting Rab4a, and after 48 hours the level of Rab4 protein was determined following preparation of lysates and Western blotting.
(B) HUVECs were transfected with either Rab4a-si2 or Rab4a-si3 RNAi duplexes targetting Rab4a48 hours prior to experimentation. Serum-starved HUVECs were surface labelled with 0.2mg/ml NHS-SS-Biotin for 30 minutes at 4°C, and internalisation of cell surface proteins allowed to proceed for 15 minutes. Non-internalised biotin was removed by a brief exposure to MesNa at 4°C, and internalised integrin chased to the cell surface for 15 minutes at 37°C in the presence or absence of 50ng/ml VEGF-A or 25ng/ml PIGF. Cells were then re-exposed to MesNa and biotinylated integrin detected by capture ELISA using microtitre wells coated with anti-human β3 monoclonal antibody.Values are mean±s.e.m. for at least 3 experiments.

for the direct association of PKD1 with the β3 integrin subunit. This association is, in turn, required for recycling of αvβ3 from early endosomes. Therefore activation of PKD1 may be the mechanism by which pro-angiogenic growth factors stimulate αvβ3 integrin recycling in endothelial cells.

Having established that pro-angiogenic growth factors were able to stimulate recycling of αvβ3 integrin in primary cultured HUVECs, I next sought to identify whether activation of Protein Kinase D-1(PKD1) occurred in response to VEGF-A or PlGF, and whether this mediated recycling of αvβ3. HUVECs were serum starved for 30 minutes then treated with PlGF or VEGF-A for 5 minutes or 15 minutes. Phosphorylation of Ser^{916} was observed to occur following 5 minutes of VEGF-A treatment and this increased to maximal levels after 15 minutes of growth factor addition (Fig 3.11A). In contrast PlGF was incapable of inducing phosphorylation of Ser^{916}, and therefore this growth factor is unlikely to activate PKD1 (Fig 3.11A).

Should PKD1 be regulating αvβ3 integrin recycling as it does in fibroblasts, it would be expected that knockdown of PKD1 by siRNA would inhibit VEGF-A stimulated αvβ3 recycling, but would have no effect on PlGF stimulated αvβ3 recycling. To test this, HUVECs were transfected with either PKD1 siRNA duplexes or control non-targeting siRNA duplexes 48 hours prior to experimentation, after which time a significant knockdown of total PKD1 protein levels was observed (Fig 3.11B). Following PKD1 knockdown, PlGF drove αvβ3 recycling by approximately 4 fold, whereas VEGF-A increased recycling approximately 2.5 fold (Fig 3.11C), indicating that knockdown of PKD1 did not inhibit PlGF stimulated αvβ3 recycling but resulted in a partial inhibition of αvβ3 recycling in response to the presence of VEGF-A.

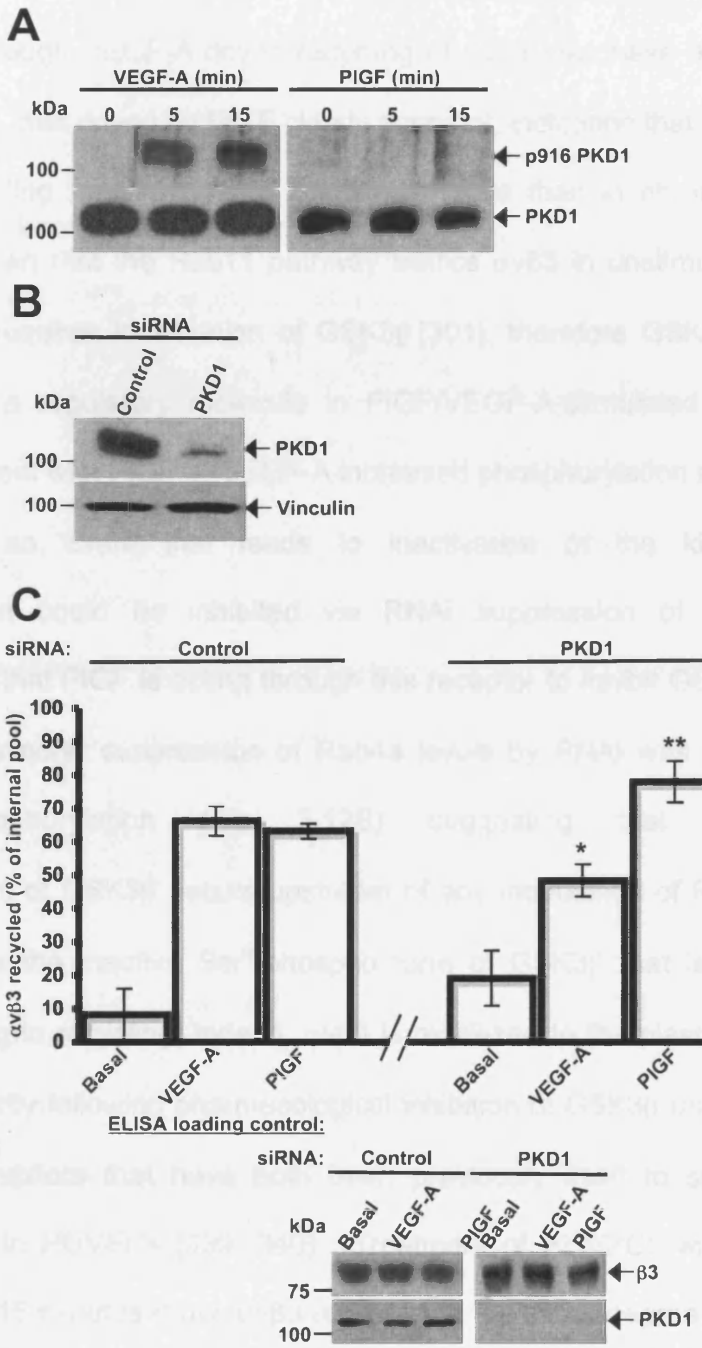

Figure 3.11: PKD1 is not required for VEGF-A or PlGF driven αvβ3 recycling

(A) Serum-starved HUVECs were challenged with 25ng/ml PlGF or 50ng/ml VEGF-A for 5 or 15 minutes, or allowed to remain quiescent (basal). Lysates were prepared and immunoblotted for phospho-PKD1 and total-PKD loading control.

(B,C) HUVECs were transfected with RNA duplexes targetting PKD1 48 hours prior to experimentation; a Western blot for PKD1 is presented to confirm the efficacy of the RNAi (**B**). Serum-starved HUVECs were surface labelled with 0.2mg/ml NHS-SS-Biotin for 30 minutes at 4°C, and internalisation of cell surface proteins allowed to proceed for 15 minutes. Non-internalised biotin was removed by a brief exposure to MesNa at 4°C, and internalised integrin chased to the cell surface for 15 minutes at 37°C in the presence or absence of 50ng/ml VEGF-A or 25ng/ml PlGF. Cells were then re-exposed to MesNa and biotinylated integrin detected by capture ELISA using microtitre wells coated with anti-human β3 monoclonal antibody.Values are mean±s.e.m. for at least 3 experiments. (* p=0.004, **p<0.00001 determined using students t-test)

Therefore, although VEGF-A-driven recycling of αvβ3 may have a component that involved PKD1, that driven by PlGF clearly does not, indicating that it is controlled by different signalling mechanisms in endothelial cells than in fibroblasts. We have previously shown that the Rab11 pathway traffics αvβ3 in unstimulated fibroblasts and that this requires inactivation of GSK3β [301], therefore GSK3β is a possible candidate for a regulatory molecule in PlGF/VEGF-A-stimulated αvβ3 recycling. Indeed, treatment with PlGF or VEGF-A increased phosphorylation of GSK3β at Ser^9 (Fig 3.12A), an event that leads to inactivation of the kinase, and this phosphorylation could be inhibited via RNAi suppression of VEGFR1 levels demonstrating that PlGF is acting through this receptor to inhibit GSK3β activity (Fig 3.12B). Furthermore, suppression of Rab4a levels by RNAi was unable to inhibit GSK3β phosphorylation (Fig 3.12B) suggesting that PlGF-stimulated phosphorylation of GSK3β occurs upstream of any modulation of Rab4a activity. In fibroblasts it is the inactive Ser^9-phospho form of GSK3β that is responsible for promoting integrin recycling; indeed, αvβ3 is mobilised to the plasma membrane of fibroblasts shortly following pharmacological inhibition of GSK3β using either LiCl or SB216763, inhibitors that have both been previously used to specifically inhibit GSK3 activity in HUVECs [339, 340]. Treatment of HUVECs with either LiCl or SB216763 for 15 minutes drove αvβ3 recycling to the same degree as did treatment with VEGF-A or PlGF (Fig 3.12C). Furthermore, LiCl was still able to stimulate recycling of αvβ3 following suppression of VEGFR1 levels, suggesting that inhibition of GSK3β activity by PlGF and VEGF-A occurs downstream of VEGFR1 binding to regulate αvβ3 recycling in HUVECs.

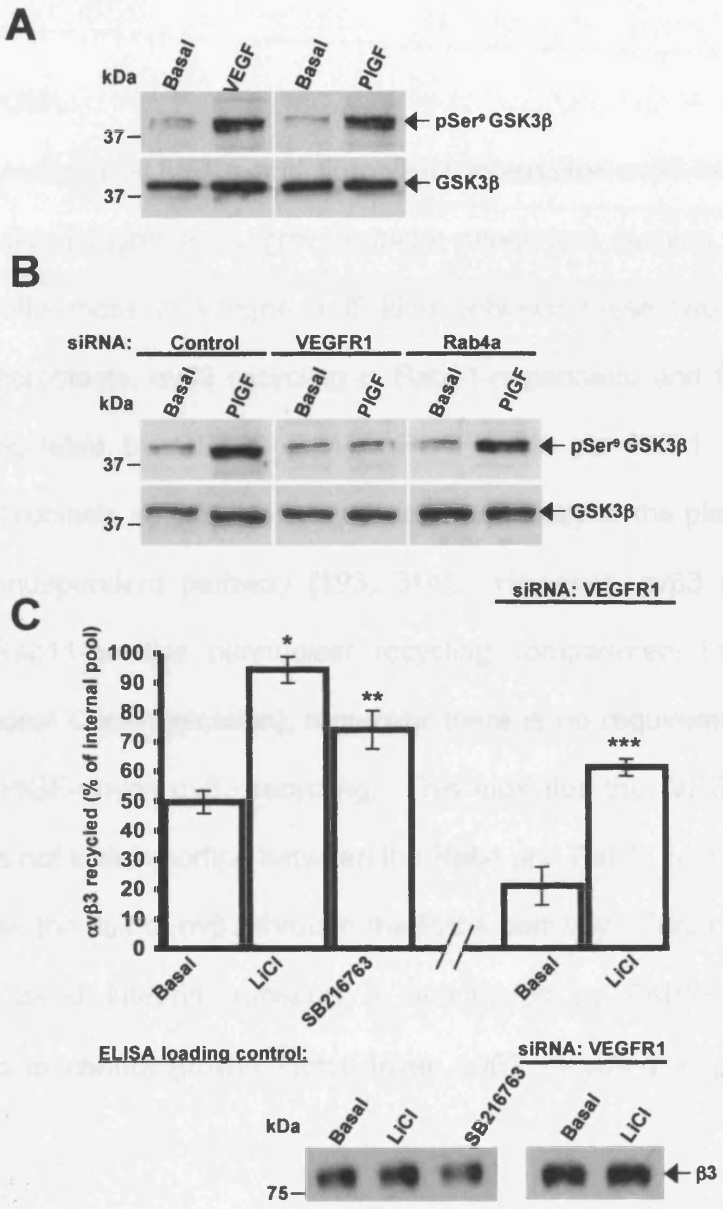

Figure 3.12: GSK3β inactivation downstream of VEGFR1 regulates growth factor-driven αvβ3 integrin recycling

(A) Serum-starved HUVECs were challenged with 25ng/ml PlGF or 50ng/ml VEGF-A for 5 minutes, or allowed to remain quiescent (basal). Lysates were prepared and immunoblotted with anti-phospho Ser9 GSK3β and appropriate loading controls.

(B) HUVECs were transfected with RNA duplexes targetting VEGFR1 or Rab4a and after 48 hours serum-starved HUVECs were challenged with 25ng/ml PlGF or 50ng/ml VEGF-A for 5 minutes, or allowed to remain quiescent (basal). Lysates were prepared and immunoblotted with anti-phospho Ser9 GSK3β and appropriate loading controls.

(C) Untransfected (Left side of graph) HUVECs, or HUVECs transfected with RNAi duplexes targetting VEGFR1 (Right side of graph) were surface labelled with 0.2mg/ml NHS-SS-Biotin for 30 minutes at 4°C, and internalisation of cell surface proteins allowed to proceed for 15 minutes. Non-internalised biotin was removed by a brief exposure to MesNa at 4°C, and internalised integrin chased to the cell surface for 15 minutes at 37°C in the presence or absence of either 20mM LiCl (Sigma; L-8895) or 10mM SB216763 (Affiniti; EI-312) Cells were then re-exposed to MesNa and biotinylated integrin detected by capture ELISA using microtitre wells coated with anti-human β3 monoclonal antibody.Values are mean±s.e.m. for at least 3 experiments. (*p<0.00001, **p=0.0018 *** p<0.00001 determined using students t-test)

3.3 DISCUSSION

Both primary cultured HUVECs and fibroblasts internalise αvβ3 constitutively and then recycle the integrin in a growth factor-dependent fashion, but there are fundamental differences in integrin trafficking between these two cell types. In unstimulated fibroblasts, αvβ3 recycling is Rab11-dependent, and flux through this pathway is regulated by GSK3β [301]. PDGF acts via PKD1 to switch αvβ3 trafficking in fibroblasts so that the integrin returns directly to the plasma membrane via a Rab11-independent pathway [193, 314]. However, αvβ3 does not traffic through the Rab11-positive perinuclear recycling compartment in HUVECs [Jim Norman; Personal Communication], moreover there is no requirement for PKD1 in VEGF-A and PlGF-driven αvβ3 recycling. This indicates that VEGF-A and PlGF-signalling does not switch sorting between the Rab4 and Rab11 recycling routes, but acts to increase the flux of αvβ3 through the Rab4 pathway. Furthermore, whereas in fibroblasts basal integrin transport is maintained by PKB/GSK-3β, GSK3β-signalling acts to control growth factor-driven αvβ3 recycling in primary cultured HUVECs.

GSK3β has been proposed to influence blood vessel growth through the control of endothelial cell migration [341], and the data presented here suggests that this is due, at least in part, to the regulation of αvβ3 recycling stimulated by growth-factors. In migrating astrocytes, GSK-3β has a clear role in microtubule dynamics and cell polarisation. In response to signalling through cdc42, GSK3β is phosphorylated and inactivated. This cdc42-dependent phosphorylation of GSK3β occurs specifically at the leading edge of migrating cells, the net result being the association of APC with the plus ends of microtubules, an association that is essential for cell polarisation [342]. Interestingly GSK3β inhibition has been shown to

promote the development of neuronal polarity via the regulation of polarised vesicular trafficking. In this case however, inhibition of GSK-3β is not achieved through phosphorylation by Akt/PKB [343]. GSK-3β controlled recycling may, therefore, be responsible for targeting αvβ3 and associated proteins to the leading edge of migrating endothelial cells and maintaining cell polarity during migration. It would be interesting to determine the localisation of phosphorylated-GSK3β following PlGF treatment in endothelial cells and whether expression of GSK3-S9A (a non-phosphorylatable form of GSK3β) is able to alter the distribution of αvβ3, cell polarity and migration of endothelial cells.

The observation that PlGF treatment of HUVECs is able to stimulate αvβ3 integrin recycling and induce phosphorylation of GSK3β at Ser^9 suggests that signalling through VEGFR1 is directly able to influence endosomal trafficking. However, PlGF may be influencing VEGFR2 signalling as VEGFR1 has been shown to transactivate VEGFR2 and potentiate signalling in pathological angiogenesis [85]. Therefore, it is important to determine whether VEGFR1 is mediating αvβ3 integrin recycling directly or indirectly through transactivation of VEGFR2.

Chapter 4: VEGFR2 trafficking in HUVECs

Chapter 4: VEGFR2 trafficking in HUVECs

4.1 INTRODUCTION

Relatively little is known about the endosomal trafficking of VEGF receptors and how this may regulate the surface distribution of receptors. VEGFR1 overexpressed in NIH-3T3 cells has been shown to be rapidly internalised and degraded following VEGF-A binding. This process involves the recruitment of Cbl and a CIN85-related adaptor protein CD2AP (CD2-associated protein) [344] and therefore is thought to occur via a clathrin-dependent mechanism. CD2AP has been shown to mediate delivery of cargo to late endosomes via association with Rab4 and Cbl [260], therefore there is a possibility that VEGFR1 may also be able to return to the plasma membrane via a Rab4-dependent recycling pathway.

VEGFR2, however, has been shown to be internalised via a caveloar pathway [345] and co-localises with caveolin-1 in intracellular organelles reminiscent of caveosomes [346]. This same study showed that VEGFR2 is found at the plasma membrane, in endosomes and in the perinuclear region of HUVECs and co-localises with EEA-1, caveolin-1 and dynamin-2. Inactivation of dynamin-2 GTPase function resulted in a loss of co-localisation of VEGFR2 with EEA-1, demonstrating that dynamin-2 is required for VEGFR2 internalisation [346]. A recent study however showed that tryphostin A23, which inhibits clathrin-mediated endocytosis, was able to increase the plasma membrane levels of VEGFR2 in unstimulated HUVECs [347]. Therefore the exact mode of VEGFR2 internalisation is still under debate.

Many studies have focused on internalisation pathways with the end result being VEGFR2 degradation. It is becoming clear, however, that receptor endocytosis is important in controlling downstream signalling, for example inhibition of EGF-

receptor endocytosis suppresses EGF-stimulated ERK1/2 activation [318] and TGFβ-receptors continue to signal from endosomal compartments [322]. Furthermore, in Drosophila, polarisation of the growth factor receptors EGFR and PVR (homologous to mammalian VEGFRs and PDGFRs) is key for chemotactic migration of Border cells towards the Drosophila oocyte, and this process depends on the ability to internalise and redistribute these receptors [206].

Therefore it remains possible that trafficking of VEGFR2 is able to control the duration, magnitude and localisation of VEGF-signalling as well as merely suppressing it. Post-endocytic trafficking of VEGFR2 and any influence this may have on VEGFR2 function, however, remains poorly studied. A number of studies have identified that VEGFR2 is present in early endosomes in the absence of ligand [346, 348], and indeed that VEGF-A stimulation results in VEGFR2 exiting early endosomes and trafficking to late endosomes [348]. Therefore, given the reported association of VEGFR2 with αvβ3 integrin in endothelial cells it is possible that VEGF receptors may be internalised and recycled as has been outlined for αvβ3.

4.2 RESULTS

4.2.1 VEGFR2 participates in the endo- exocytic cycle in HUVECs

Having established that VEGF-A and PlGF, acting through VEGFR1, were able to regulate recycling of $\alpha v\beta 3$ in HUVECs, I next wished to investigate the endocytic trafficking of VEGFR2. In order to do this I adapted the biotin-based assay to detect VEGFR2 internalisation and recycling in HUVECs.

HUVECs were serum starved and surface labelled with NHS-SS-Biotin and, following extensive washing to remove unreacted reagent, were lysed. VEGFR2 was immunoprecipitated from lysates using anti-VEGFR2 antibody conjugated to magnetic beads, and an isotype matched control antibody was used to determine the non-specific binding of proteins to the magnetic beads. Immunoprecipitated material was then subjected to SDS-PAGE followed by Western blotting, and biotinylated proteins were detected using streptavidin-conjugated HRP. The analysis demonstrated that immunoprecipitation of VEGFR2 using anti-VEGFR2 coated magnetic beads yielded a single biotinylated protein that corresponded in size to VEGFR2 (250kDa). No other biotinylated proteins were detected, demonstrating the specificity of the anti-VEGFR2 antibody used and the suitability of this approach to detecting biotinylated VEGFR2 (Fig 4.1A).

To determine whether VEGFR2 was constitutively internalised in HUVECs, cells were serum starved then surface the time periods indicated then returned to ice prior to surface reduction with MesNa in order to remove any biotin remaining at the cell surface. Treatment of cells that had not been warmed with MesNa removed the majority of biotin labelling from VEGFR2 (Fig 4.1B), whereas in cells warmed for 7.5

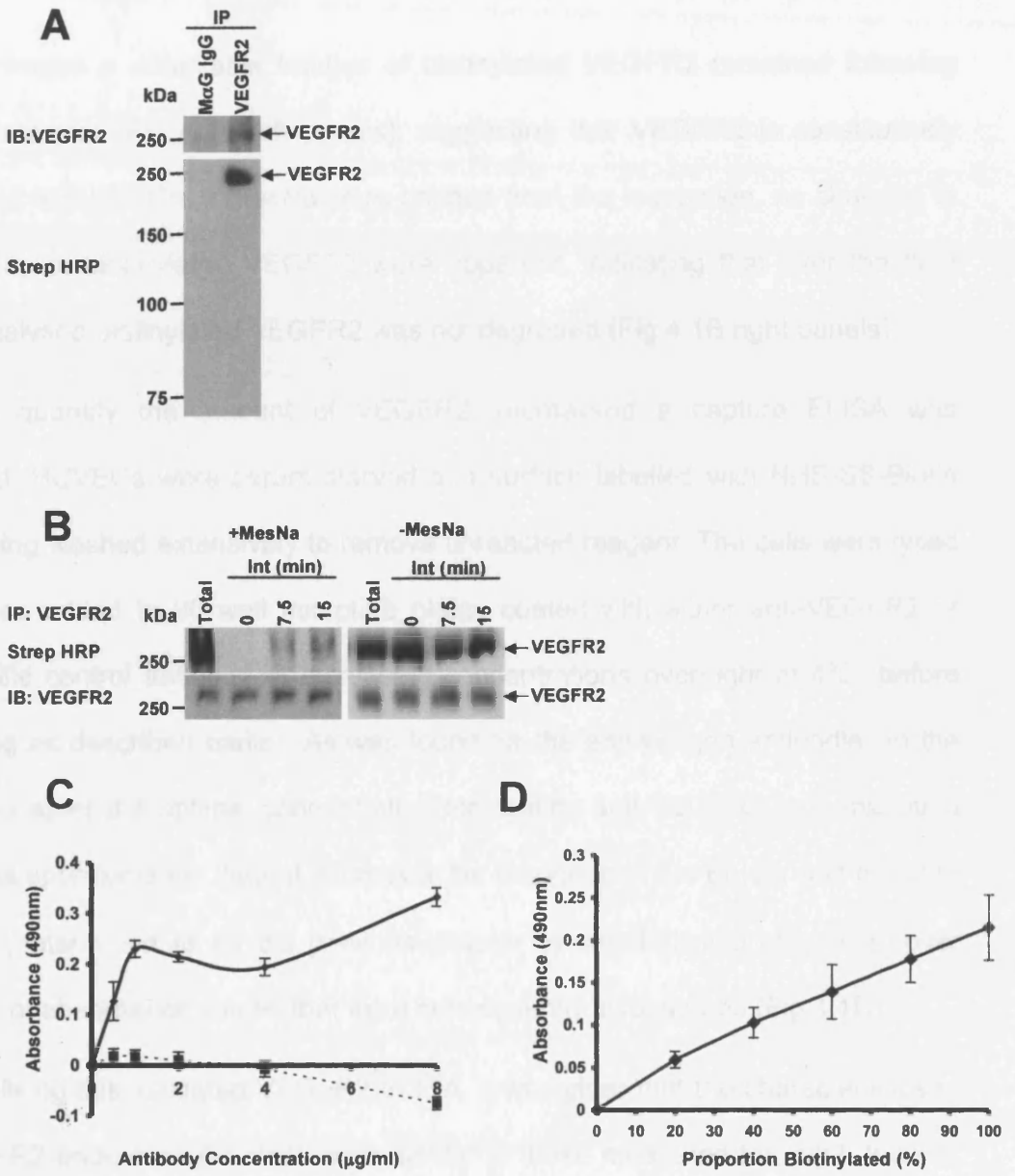

Figure 4.1: Internalisation of VEGFR2 in HUVECs

(**A**) Serum-starved HUVECs were surface-labelled with 0.2 mg/ml NHS-S-S-Biotin for 30 min at 4°C, lysates were prepared and immunoprecipitated (IP) with antibodies against VEGFR2 or mouse anti-goat isotype control and immobilised material analysed by immunoblotting (IB) with anti-VEGFR2, or by addition of streptavidin-conjugated HRP.

(**B**) Surface labelled cells were warmed to 37°C for the times indicated. Biotin was released from proteins remaining at the cell surface by MesNa treatment (left panels) and biotinylated VEGFR2 determined by immunoprecipitation of VEGFR2 followed by probing of Westerns with Streptavidin-HRP

(**C,D**) Serum-starved HUVECs were surface-labelled with 0.2 mg/ml NHS-S-S-Biotin for 30 min at 4°C, lysates were prepared and added to microtitre wells coated with increasing concentrations of anti-VEGFR2 antibody (C) or were serial diluted with unlabelled lysates before being added to microtitre wells coated with 2μg/ml of anti-VEGFR2 polyclonal anibody.

and 15 minutes a detectable fraction of biotinylated VEGFR2 remained following MesNa treatment (Fig 4.1B left panels), suggesting that VEGFR2 is constitutively internalised in HUVECs. If MesNa were omitted from the incubation, no changes to the quantity of biotinylated VEGFR2 were apparent, indicating that over the time course analysed biotinylated VEGFR2 was not degraded (Fig 4.1B right panels).

To quantify the amount of VEGFR2 internalised a capture ELISA was developed. HUVECs were serum starved and surface labelled with NHS-SS-Biotin before being washed extensively to remove unreacted reagent. The cells were lysed and lysates added to 96 well microtitre plates coated with either anti-VEGFR2 or non-specific control antibody at increasing concentrations overnight at 4°C, before developing as described earlier. As was found for the anti-integrin antibodies in the previous chapter the optimal concentration for coating anti-VEGFR2 onto microtitre plates was approximately 2μg/ml. Moreover the response of the ELISA was found to be linear (determined as for the previous chapter by serial dilution of lysates) over the range of absorbance values that were subsequently investigated (Fig 4.1D).

Utilising this validated VEGFR2 ELISA, it was clear that the characteristics of the VEGFR2 endo-exocytic cycle were similar to those measured for αvβ3. Indeed, VEGFR2 was constitutively internalised and recycled in HUVECs, as demonstrated by an accumulation of internalised VEGFR2 following treatment with primaquine compared to untreated cells (Fig 4.2A). Furthermore, addition of exogenous VEGF-A did not influence the rate at which the receptor was endocytosed or the overall levels of receptor over the time period analysed, despite stimulation of downstream signalling in the form of ERK phosphorylation (Fig 4.2B).

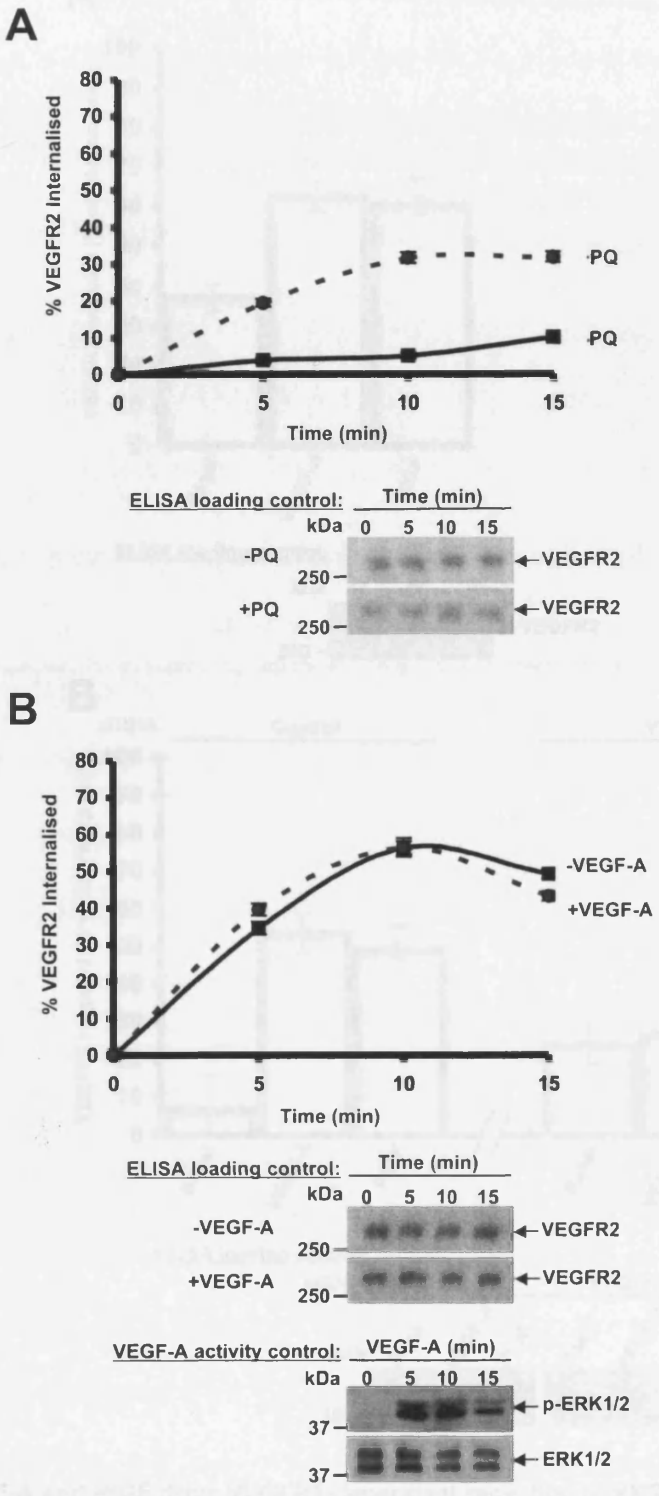

Figure 4.2 Internalisation of VEGFR2

(A,B) Serum-starved HUVECs were surface-labelled with 0.2 mg/ml NHS-S-S-Biotin for 30 min at 4°C and cells were warmed to 37°C in the absence or presence of 0.6 mM PMQ or 50ng/ml VEGF-A for the times indicated. Biotin was released from proteins remaining at the cell surface, and biotinylated VEGFR2 determined by capture-ELISA using microtitre wells coated with anti-human VEGFR2 polyclonal antibody.

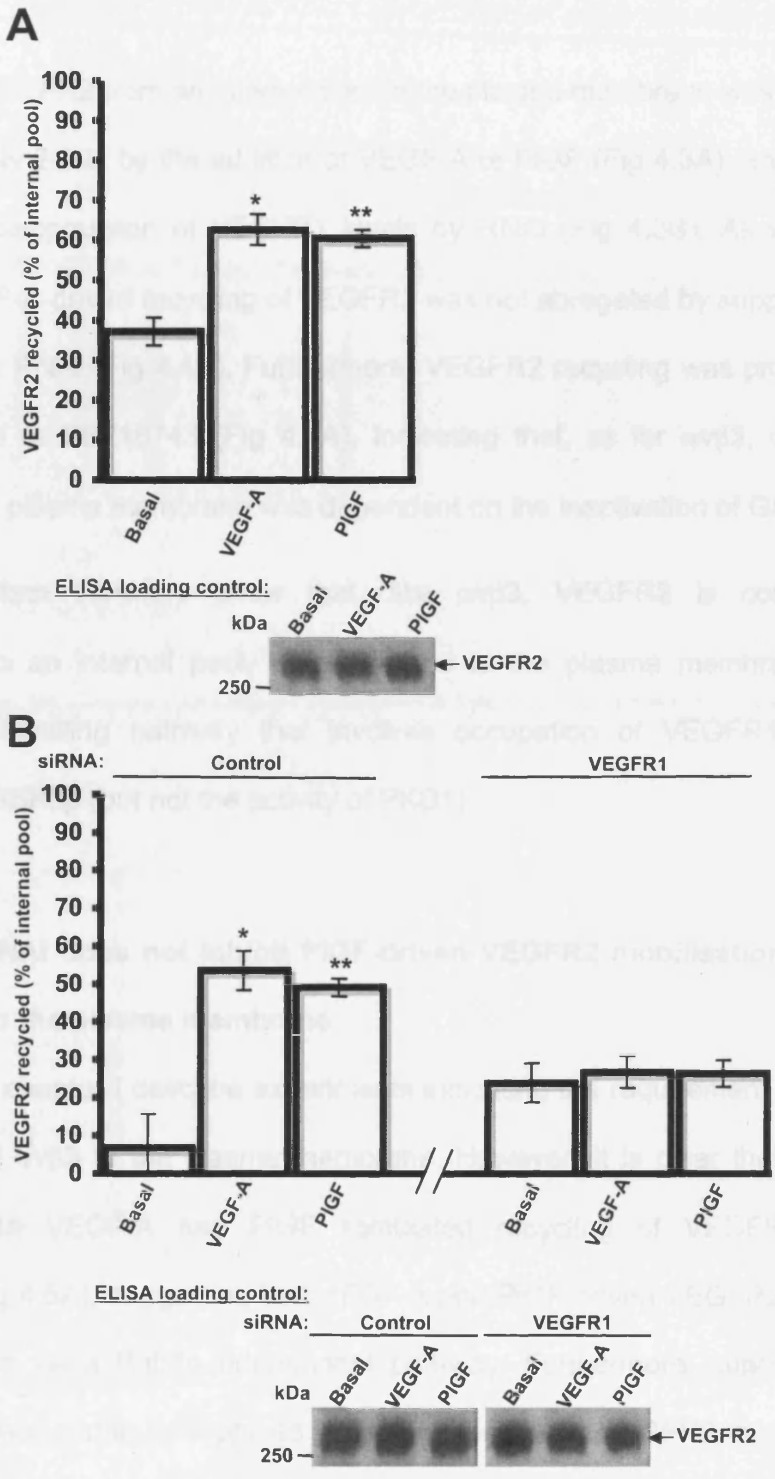

Figure 4.3: VEGF-A and PlGF drive VEGFR1-dependent recycling of VEGFR2
HUVECs were left untransfected (**A**) or were transfected with RNAi duplexes targetting VEGFR1 48 hours prior to experimentation (**B**). Serum-starved HUVECs were surface labelled with 0.2mg/ml NHS-SS-Biotin for 30 minutes at 4°C, and internalisation of cell surface proteins allowed to proceed for 15 minutes. Non-internalised biotin was removed by a brief exposure to MesNa at 4°C, and internalised proteins chased to the cell surface for 15 minutes at 37°C in the presence or absence of 50ng/ml VEGF or 25ng/ml PlGF. Cells were then re-exposed to MesNa and biotinylated VEGFR2 detected by capture ELISA using microtitre wells coated with anti-human VEGFR2 monoclonal antibody. Values are mean±s.e.m. for at least 3 experiments. (*,** $p<0.0002$ determined using students t-test)

The return of VEGFR2 from an internal pool to the plasma membrane was increased by approximately 2-fold by the addition of VEGF-A or PlGF (Fig 4.3A), and this was attenuated by suppression of VEGFR1 levels by RNAi (Fig 4.3B). As with αvβ3, PlGF and VEGF-A-driven recycling of VEGFR2 was not abrogated by suppression of PKD1 levels by RNAi (Fig 4.4A). Furthermore, VEGFR2 recycling was promoted by addition of LiCl or SB216743 (Fig 4.4A), indicating that, as for αvβ3, delivery of VEGFR2 to the plasma membrane was dependent on the inactivation of GSK3β.

These data therefore show that, like αvβ3, VEGFR2 is constitutively internalised into an internal pool, which returns to the plasma membrane under control of a signalling pathway that involves occupation of VEGFR1 and the inactivation of GSK3β (but not the activity of PKD1).

4.2.2 Rab4a RNAi does not inhibit PlGF-driven VEGFR2 mobilisation from an internal pool to the plasma membrane

In the previous chapter I describe experiments indicating the requirement for Rab4a in the return of αvβ3 to the plasma membrane. However, it is clear that following RNAi of Rab4a VEGF-A and PlGF stimulated recycling of VEGFR2 is not suppressed (Fig 4.5A), suggesting that VEGF-A and PlGF-driven VEGFR2 recycling is able to occur via a Rab4a-independent pathway. Furthermore, suppression of Rab11a was also unable to suppress growth factor driven VEGFR2 recycling (Fig 4.5A), suggesting that like αvβ3 VEGFR2 does not traffic through a Rab11-dependent recycling pathway either. Therefore, whilst both αvβ3 and VEGFR2 appear to be trafficked via recycling mechanisms that have similar kinetics and dependency on VEGFR1 and GSK3 inhibition the routes by which these receptors return to the plasma membrane clearly differ in their requirement for Rab4a.

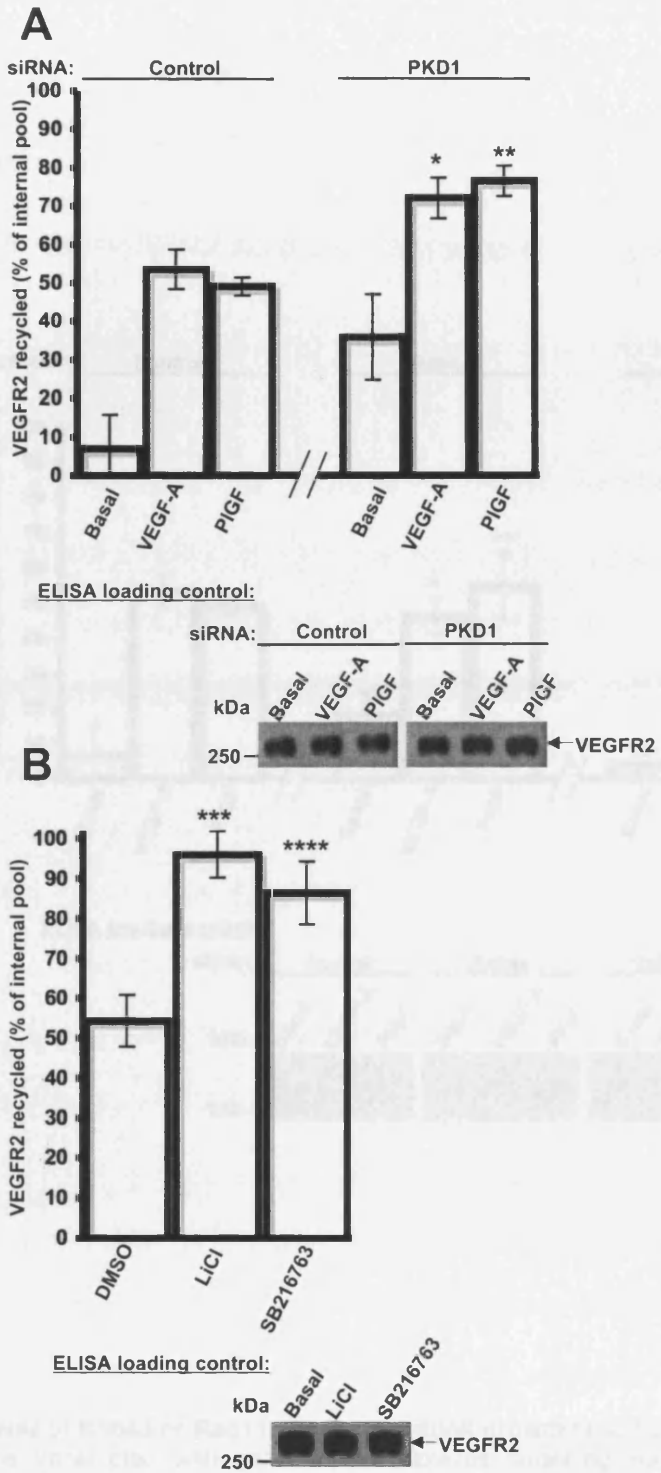

Figure 4.4: VEGFR2 recycling does not require PKD1 and is stimulated by GSK3 inhibition
HUVECs were transfected with RNA duplexes targetting PKD1 48 hours prior to experimentation (**A**), or remained untransfected (**B**) Serum-starved HUVECs were surface labelled with 0.2mg/ml NHS-SS-Biotin for 30 minutes at 4°C, and internalisation of cell surface proteins allowed to proceed for 15 minutes. Non-internalised biotin was removed by a brief exposure to MesNa at 4°C, and internalised VEGFR2 chased to the cell surface for 15 minutes at 37°C in the presence or absence of 50ng/ml VEGF-A, 25ng/ml PlGF (**A**), 20mM LiCl (Sigma; L-8895) or 10mM SB216763 (Affiniti; EI-312) (**B**). Cells were then re-exposed to MesNa and biotinylated integrin detected by capture ELISA using microtitre wells coated with anti-human VEGFR2 polyclonal antibody.Values are mean±s.e.m. for at least 3 experiments. (*p=0.0077, **p=0.0027, ***p<0.00001, ****p=0.0024 determined using students t-test)

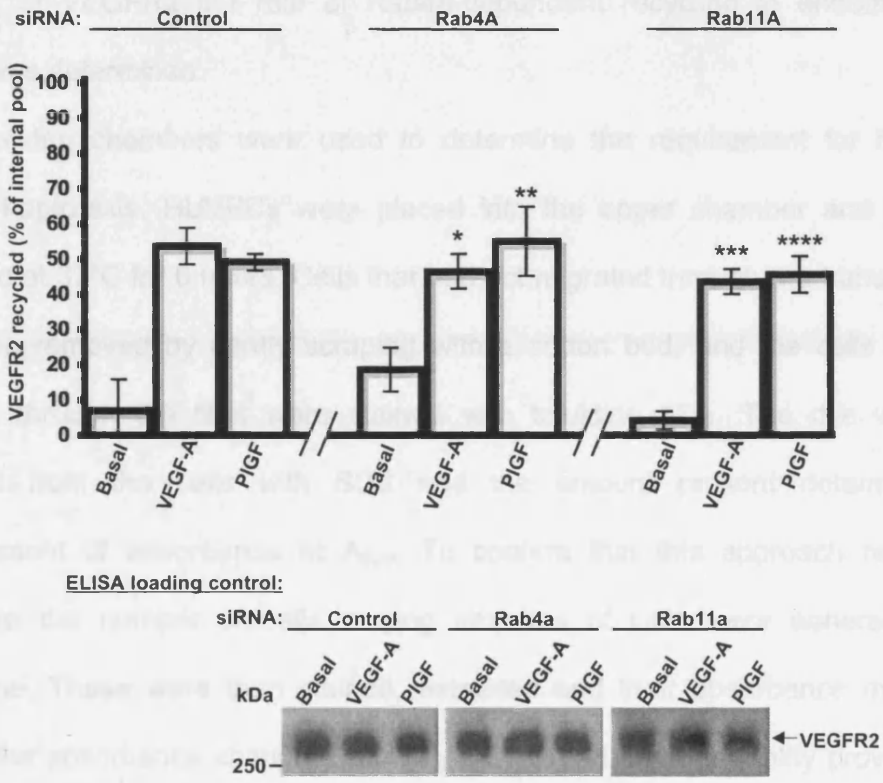

Figure 4.5: RNAi of Rab4a or Rab11a does not inhibit growth factor-driven VEGFR2 recycling
HUVECs were transfected with either RNAi duplexes targetting Rab4a or Rab11a 48 hours prior to experimentation. Serum-starved HUVECs were surface labelled with 0.2mg/ml NHS-SS-Biotin for 30 minutes at 4°C, and internalisation of cell surface proteins allowed to proceed for 15 minutes. Non-internalised biotin was removed by a brief exposure to MesNa at 4°C, and internalised VEGFR2 chased to the cell surface for 15 minutes at 37°C in the presence or absence of 50ng/ml VEGF-A or 25ng/ml PlGF. Cells were then re-exposed to MesNa and biotinylated VEGFR2 detected by capture ELISA using microtitre wells coated with anti-human VEGFR2 monoclonal antibodies. Values are mean±s.e.m. for at least 3 experiments. (*p=0.0007 , **p=0.0024, ***,****p<0.00001 determine using students t-test)

4.2.3 RNAi of Rab4a does not inhibit VEGF-driven cell migration and cord formation in a collagen gel assay

Having identified that Rab4a knockdown is able to suppress VEGF-A and PlGF-driven recycling of $\alpha v\beta 3$, and that both VEGF-A and PlGF were also able to stimulate recycling of VEGFR2 the role of Rab4a-dependent recycling in endothelial cell biology was determined.

Boyden chambers were used to determine the requirement for Rab4a in HUVEC haptotaxis. HUVECs were placed into the upper chamber and the cells incubated at 37°C for 6 hours. Cells that had not migrated through the transwell filter were then removed by gently scraping with a cotton bud, and the cells that had migrated through the filter were stained with toluidine blue. The dye was then extracted from the cells with SDS and the amount present determined by measurement of absorbance at A_{620}. To confirm that this approach responded linearly to the number of cells varying amounts of cells were adhered to the membrane. These were then stained, extracted and their absorbance measured. Indeed, the absorbance changes were linearly related to cell quantity provided that the cell number was between 5-100x10^3 cells per well (Fig 4.6A). Therefore for subsequent assays 80 x10^3 cells were added to each well.

When the transwell filters were coated with fibronectin, the migration of HUVECs was increased approximately 3-fold by comparison to BSA-coated filters. Moreover the addition of VEGF-A or PlGF did not alter this, indicating that these HUVECs display strong haptotaxis towards fibronectin and that this is unaffected by the presence of angiogenic growth factors. Importantly, siRNA knockdown of Rab4a did not inhibit the haptotaxis of HUVECs (in the absence or presence of VEGF-A or

PlGF), in fact it appeared to slightly to increase this index of HUVEC migration (Fig 4.6B).

When HUVECs were seeded in collagen gels and grown in serum free media for 24 hours, VEGF-A was required for HUVECs to survive and subsequently form cell aggregates and cords (Fig 4.7A). PlGF alone was unable to mediate cell survival and stimulate cord formation (Fig 4.7A). However, if HUVECs were incubated for 36 hours in collagen gels in the presence of VEGF-A clear individual cords were formed (Fig 4.7B). Suprisingly, HUVECs transfected with Rab4a siRNA duplexes formed cords that were approximately 40% longer than cells transfected with control siRNA (Fig 4.7C). Taken together these data suggest that although Rab4a is clearly required for PlGF-driven recycling of $\alpha v\beta 3$, this GTPase is not required for HUVEC haptotaxis or for the ability of cells to form cords in response to VEGF-A.

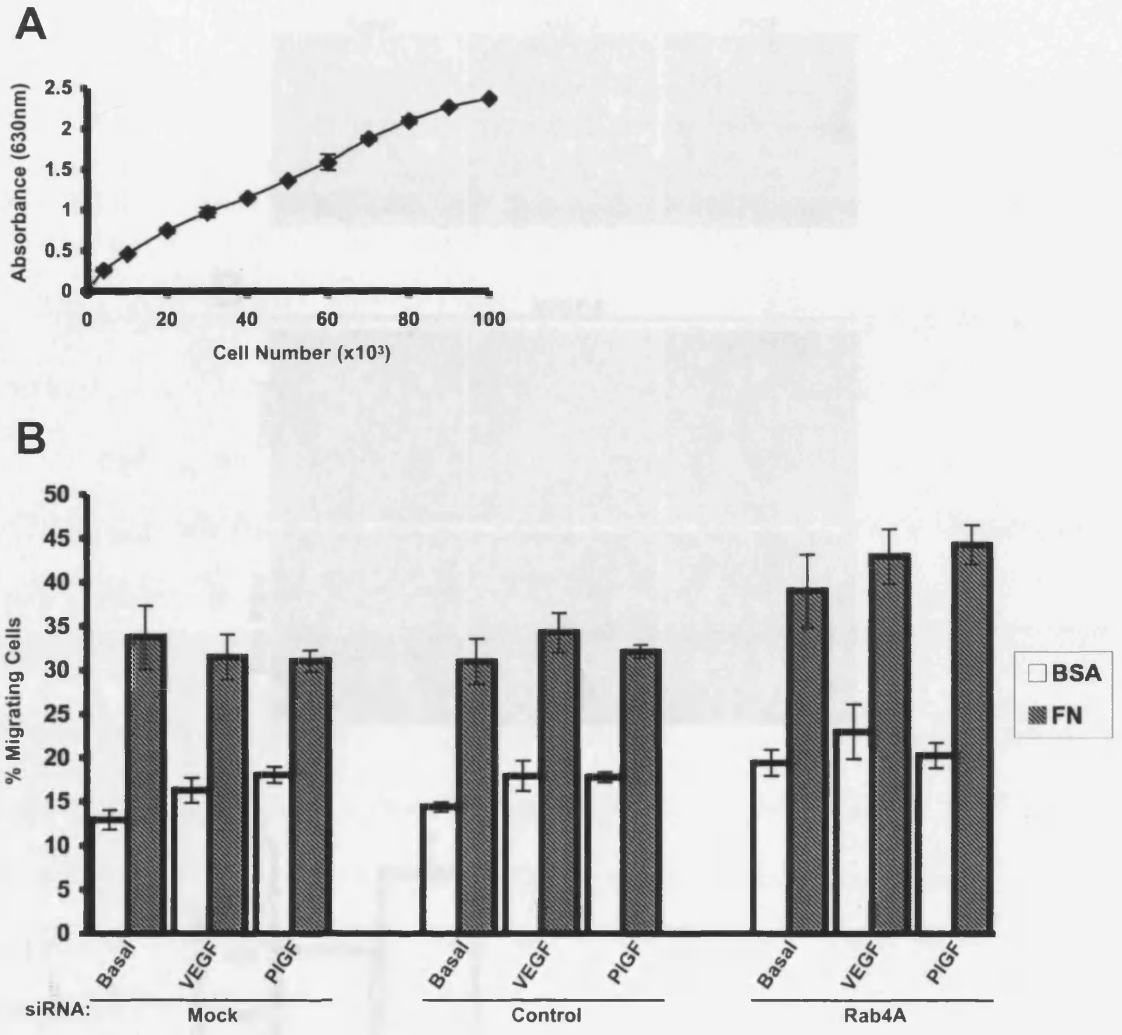

Figure 4.6: RNAi knockdown of Rab4a does not alter HUVEC haptotaxis in a Boyden chamber assay
(**A**) Increasing numbers of HUVECs were seeded onto transwell filters and allowed to attach for 1 hour before being fixed in 10% formaldehyde and stained with toluidine blue. Dye was extracted with 2% SDS and the absorbance of this extract measured at AU_{630}
(**B**) HUVECs were transfected with either RNAi duplexes targeting Rab4a, non-targeting control duplexes (control) or treated with GeneFector alone (Mock) 48 hours prior to experimentation. Cell migration was assessed using a Boyden chambers coated with BSA or FN over a time period of 6 hours in the absence or presence of 50ng/ml VEGF-A or 25ng/ml PIGF. Experiments were performed in triplicate with six determinants per condition and values expressed as a percentage of the total cells adhering.

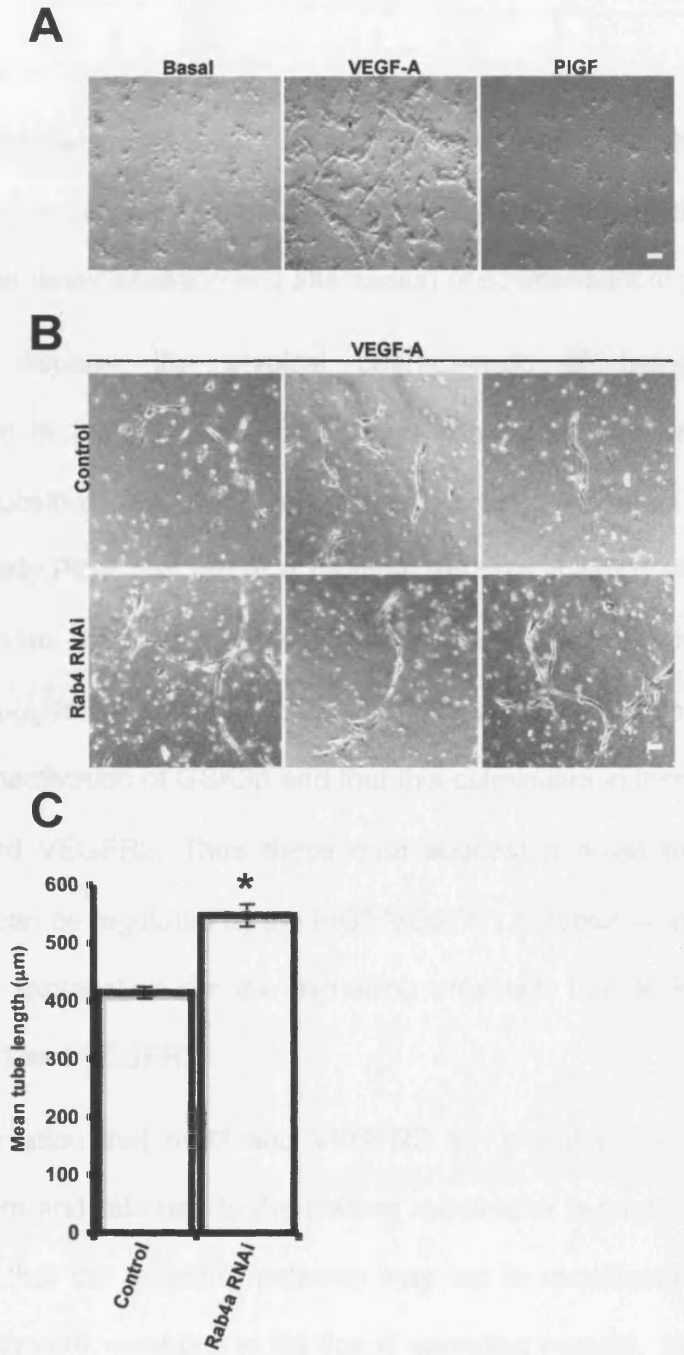

Figure 4.7: RNAi of Rab4a increases endothelial cell cord length in a collagen gel assay
(A) HUVECs were seeded onto collagen gels and grown in serum free media in the absence (basal) or presence of 50ng/ml VEGF-A or 25ng/ml PIGF for 24 hours. Tubes were then fixed in 10% Glutaraldehyde and analysed using a Hoffmans objective. Bar 100μm
(B) HUVECs were transfected with Rab4a targetting siRNA duplexes or lamin tragetting control duplexes 48 hours prior to experimentation, then seeded onto collagen gels and grown in serum free media in the presence of 50ng/ml VEGF-A for 36 hours. Cords were then fixed in 10% Glutaraldehyde and analysed by phase contrast microscopy. Bar 100μM
(D) Tube lengths were measured using Image J. Values are mean±s.e.m. for at least 3 experiments. (*$p<0.00001$)

4.3 DISCUSSION

Many growth factor receptors reside primarily at the plasma membrane, and their internalisation and subsequent degradation is precipitated by growth factor binding leading to receptor desensitisation and attenuation of downstream signalling [178].

VEGFR2 displays the atypical characteristic of being continuously internalised, even in the absence of ligand, to the same compartment as αvβ3 integrin (Rab4a positive early endosomes) [Jim Norman; Personal communication and [333]]. Crucially PlGF and VEGF-A promote the rate at which VEGFR2, as well as αvβ3, returns to the plasma membrane and this requires the presence of VEGFR1. This suggests that in HUVECs, VEGFR1 commands signalling pathways that lead to the inactivation of GSK3β and that this culminates in increased recycling of both αvβ3 and VEGFR2. Thus these data suggest a novel means by which integrin function can be regulated by the PlGF/VEGFR1 receptor-ligand pair and also may provide an explanation for the signalling crosstalk that is known to occur between VEGFR1 and VEGFR2.

The observation that αvβ3 and VEGFR2 are possibly co-trafficked by the endosomal system and returned to the plasma membrane in response to the same stimuli suggests that the recycling pathway may act to coordinate the delivery of growth factor and matrix receptors to the tips of sprouting vessels. In this regard it is notable that VEGFR2 and αvβ3 integrin may associate physically [151]. Therefore it is possible that the association formed between them occurs within endosomes, thus providing opportunities for the integrin and RTK to influence each other's trafficking and function. However, if Rab4a is removed the trafficking of αvβ3 and VEGFR2 is uncoupled such that the two receptors are no longer handled by the endosomal system in the same way. Upon removal of the Rab4a, internalised αvβ3 is unable to

recycle whereas VEGFR2 still returns to the plasma membrane, and can therefore be recycled via an alternative (Rab4a-independent) recycling pathway. It is possible that this involves the small GTPase RhoB which has been shown to colocalise with VEGFR2 in endosomes distinct to those which are associated with Rab4 [348]. RhoB has previously been shown to mediate delivery of Src-containing endosomes to the plasma membrane [334]. RhoB-positive endosomes contain actin polymerising molecules such as Scar1 and these endosomes associated with newly polymerised actin in a Src-dependent fashion [334]. Therefore Src controls translocation of RhoB-positive endosomes to the plasma membrane. Indeed, as pharmacological inhibition of Src inhibits the recycling of VEGFR2 [348] it is possible that it is this RhoB/Src dependent trafficking system that returns VEGFR2 to the plasma membrane following the addition of PlGF to HUVECs.

Chapter 5: PlGF promotes the branching of endothelial tubule-like structures via a Rab4a- and αvβ3-dependent mechanism.

Chapter 5: PlGF promotes the branching of endothelial tubule-like structures via a Rab4a- and αvβ3-dependent mechanism.

5.1 INTRODUCTION

In order to accurately replicate the processes involved in blood-vessel formation, and how growth factors, pharmacological agents, antibodies etc influence this *in vitro*, a number of assays have been evaluated over the past few years. During early stages of angiogenesis endothelial cells align into solid, multicellular, cord like structures which are interconnected forming a polygonal network [349]. Use of collagen gels (as in chapter 4) or Matrigel provides HUVECs with basement membrane proteins within which they undergo morphological changes that closely imitate pre-capillary cord formation *in vivo* [350], and as such are a useful tool for analysing the initial stages of vascular morphogenesis. However, HUVECs grown in collagen gels do not acquire lumen and when these are implanted in mice no perfusion is observed [351], demonstrating that the structures formed by HUVECs in collagen gels cannot be considered functional.

A more recent assay for evaluating endothelial cell tube formation involves the co-culture of HUVECs with human diploid fibroblasts of dermal origin [352]. In this Organotypic assay no artificial matrix is required as the fibroblasts secrete the appropriate matrix components that allow tubule formation. Tubules form over a period of up to 14 days and during that period 3 distinct stages of development occur. Firstly, during the 'early' stage (4-6 days) endothelial cells proliferate and have random motility. Next, the 'migratory' phase (7-9 days) is entered where endothelial cells adopt a bipolar morphology, migrate directionally, form tubes and sprout from existing vessels. Finally, tubules reach an 'established' phase (14-16 days) where

vessels become thicker [353]. Crucially, tubules form in the absence of additional growth factors as both cell types produce sufficient VEGF-A to mediate survival and other growth factors such as FGF are present in the foetal calf serum used to supplement growth media [352]. The tubules formed using this assay contain lumen and superficially resemble the microvascular bed [352]. Furthermore, upon implantation in mice these vessels become perfused and incorporated into the murine vasculature [351], demonstrating their functionality.

Use of this co-culture assay provides an *in vitro* assay that can accurately model *in vivo* angiogenesis, and allows the analysis of both stimulatory and inhibitory agents. I therefore utilised this co-culture assay in order to determine the influence of PlGF-driven recycling on endothelial cell structure formation in a quasi three dimensional setting.

5.2 RESULTS

5.2.1 PlGF-driven Rab4/GSK3β-dependent recycling alters HUVEC tube morphology in co-culture assays

HUVECs co-cultured with HDFs for 5 days formed numerous separate structures as identified by immunofluorescence staining for the endothelial cell specific marker PECAM-1 (Fig 5.1A), despite coverage with human dermal fibroblasts (HDFs) being incomplete (Fig 5.1B). If co-cultures were allowed to form for 7 or 10 days, fibroblasts covered the full surface area of the well and endothelial cell structures became thicker and more apparent (Fig 5.1C,D,E,F). These tubule-like structures were branched and cross-linked into a network; indeed structures that lack branch points or cross bridges to adjacent tubes were rare (Fig 5.1C). If co-cultures were incubated for 4 days and subsequently treated with PlGF for 3 days, a clear increase in cross-linking and network complexity of HUVEC structures was observed (Fig 5.2B). To quantify this morphological change I measured the distance between branch points along the tubule-like structures (white marks Fig 5.2A,B). Following treatment with PlGF, an increase in network complexity was reflected by a 35% decrease in the mean branch interval from 185μm to 122μm (Fig 5.2C).

To determine whether PlGF was able to drive branch formation through stimulation of Rab4a-dependent recycling, an RNAi based approach was used to suppress the levels of Rab4a in HUVECs seeded in co-cultures. HUVECs transfected with oligonucleotides that targetted Rab4a were clearly still able to form long endothelial cell structures (Fig 5.3B). Therefore, knockdown of Rab4a did not globally inhibit HUVEC survival, migration or sprouting.

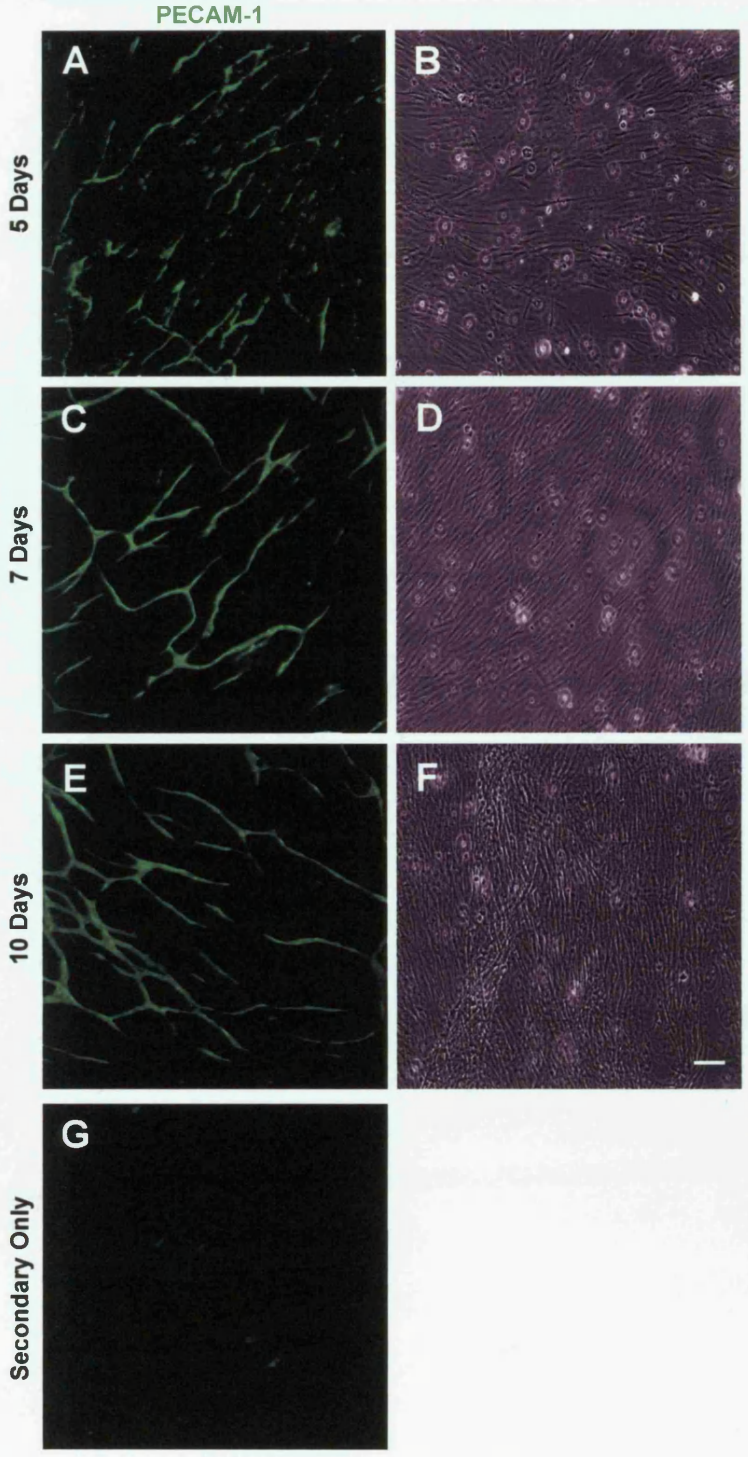

Figure 5.1: HUVECs form branched networks of tubule-like structures in an organotypic coculture assay

HUVECs were seeded in a coculture with human dermal fibroblasts. Cocultures were fed every 48 hours for either 5 (**A,B**), 7 (**C,D**) or 10 days (**E,F**), after which time cells were fixed in 4% paraformaldehyde and PECAM-1 visualised by indirect immunofluorescence (**A,C,E**) along with phase contrast pictures of the cocultures being taken (**B,D,F**). Bar 100μm

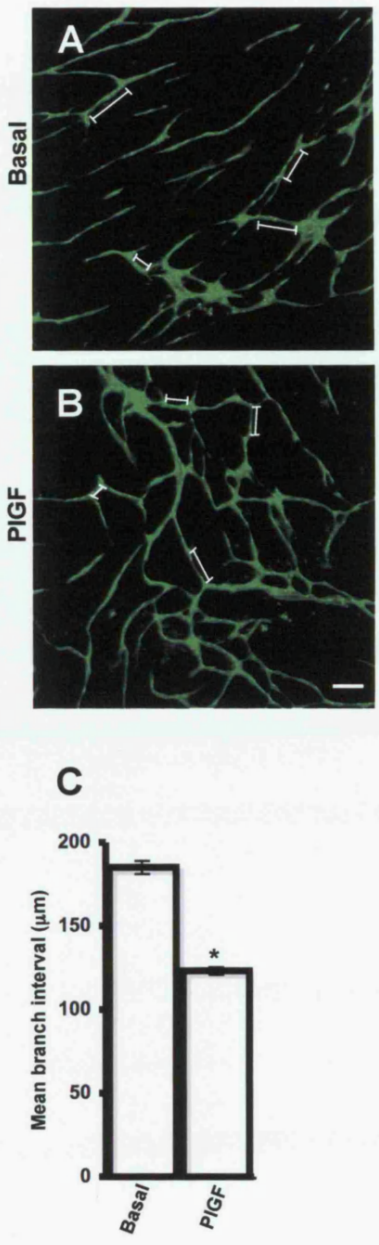

Figure 5.2: PIGF promotes HUVEC-structure branching and network complexity in an organotypic coculture assay

HUVECs were seeded in a coculture with human dermal fibroblasts. Cocultures were fed every 48 hours for a total of 7 days (**A**), with 20ng/ml PIGF being added on day 4 and day 6 (**B**). Cells were fixed in 4% paraformaldehyde and PECAM-1 visualised by indirect immunofluorescence. Bar 100μm

(**C**) Images were analysed using Image J, and the mean branch interval length measured. Values are mean±s.e.m. for at least 4 experiments. ($p<0.0000001$)

However, the structures formed have few branch points and were rarely seen to be cross-linked to one another. Indeed, when Rab4a was knocked down by RNAi the mean branch interval increased by approximately 40% from 185μm to 260μm (Fig 5.3G), suggesting that in the absence of Rab4a HUVEC tubule-like structure branching was inhibited. Addition of exogenous PlGF was unable to rescue the loss of branching observed following Rab4a knockdown (Fig 5.3E), demonstrating that PlGF-driven branch formation was Rab4a-dependent. As a further negative control for this RNAi of Rab4a, HUVECs were transfected with oligonucleotides targetting Rab4b which is not expressed by HUVECs (see Fig 3.3A). Transfection with these oligonucleotides did not alter the mean branch interval observed either in the absence or presence of PlGF (Fig 5.3C,F,G) suggesting that the changes observed with oligonucleotides targeting Rab4a are specific to suppression of Rab4 levels.

5.2.2 RNAi of β3 integrin abrogates PlGF-driven branching in co-culture assays

The observation that Rab4a RNAi inhibits PlGF-driven branch formation suggests that cycling of αvβ3 integrin may be required for sprouting and branch formation. Therefore, an RNAi approach to suppress the levels of αvβ3 was used to determine this. Indeed, if αvβ3 levels were suppressed by RNAi a clear change in HUVEC tubule-like structure morphology, similar to that seen following RNAi of Rab4a, was observed. Control siRNA transfected HUVECs form tube-like structures with numerous branch points (Fig 5.4A). HUVECs were clearly able to form tube like structures following knockdown of β3 levels, and these structures had fewer branch points (Fig 5.4B) such that there was a 40% increase in basal branch intervals (Fig 5.4E).

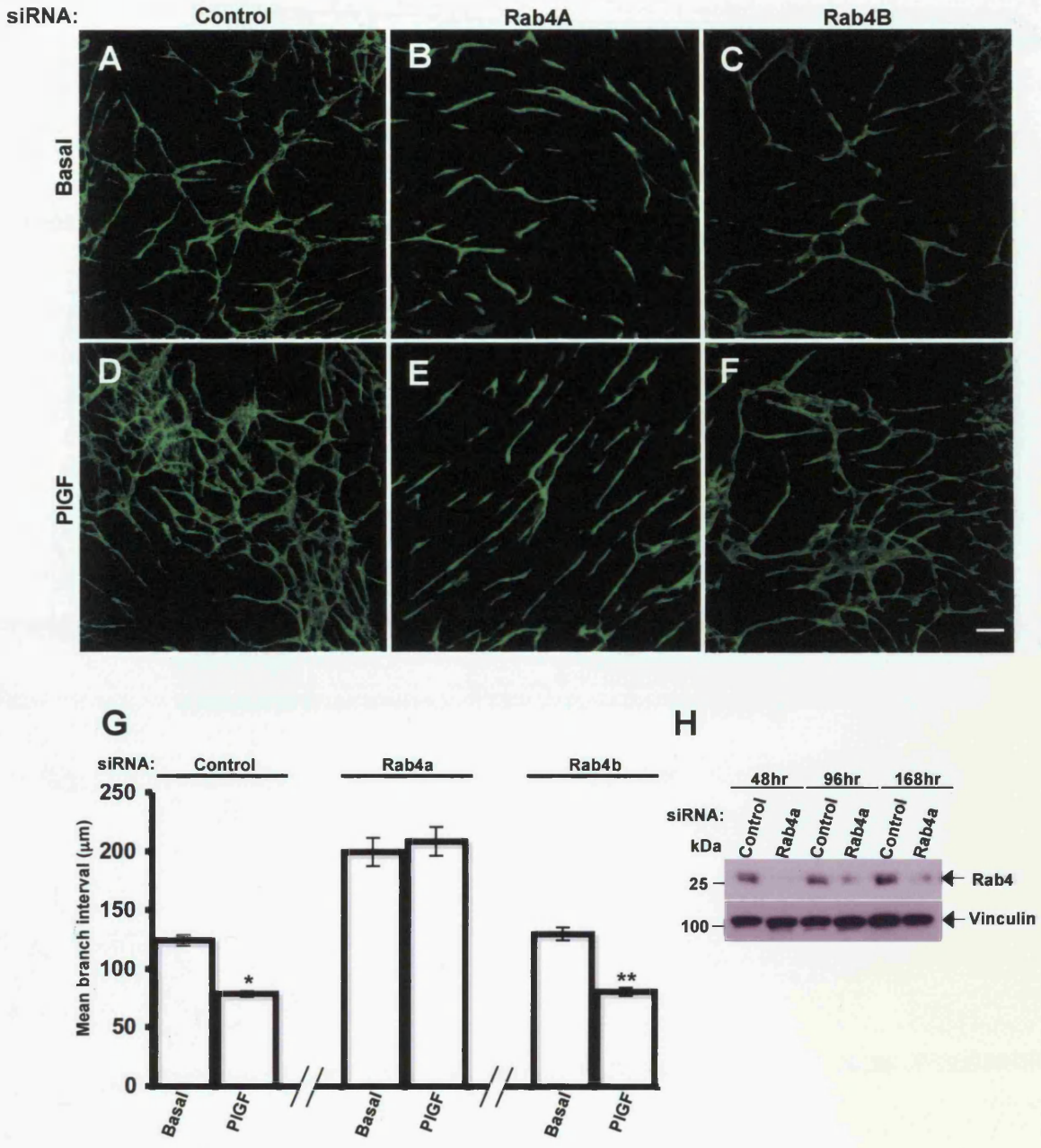

Figure 5.3 RNAi of Rab4a inhibits PlGF promoted vessel branching in a coculture tube formation assay
HUVECs were transfected with RNAi duplexes targetting Rab4a (**B,E**) or Rab4b (**C,F**) 24 hours before seeding in a coculture with human dermal fibroblasts. Cocultures were fed every 48 hours for a total of 7 days, with 25ng/ml PlGF (**D,E,F**) being added on day 4 and day 6. Cells were fixed in 4% paraformaldehyde and PECAM-1 visualised by indirect immunofluorescence. Bar 100μm
(**G**) The mean branch interval length was measured using Image J. Values are mean±s.e.m. for at least 4 experiments. (*,** $p<0.000001$)
(**H**) HUVECs were transfected with RNAi duplexes targetting Rab4a or non-targeting control duplexes and after 48 hours, 96 hours and 168 hours lysates were prepared and the levels of Rab4 protein determined by Western blot.

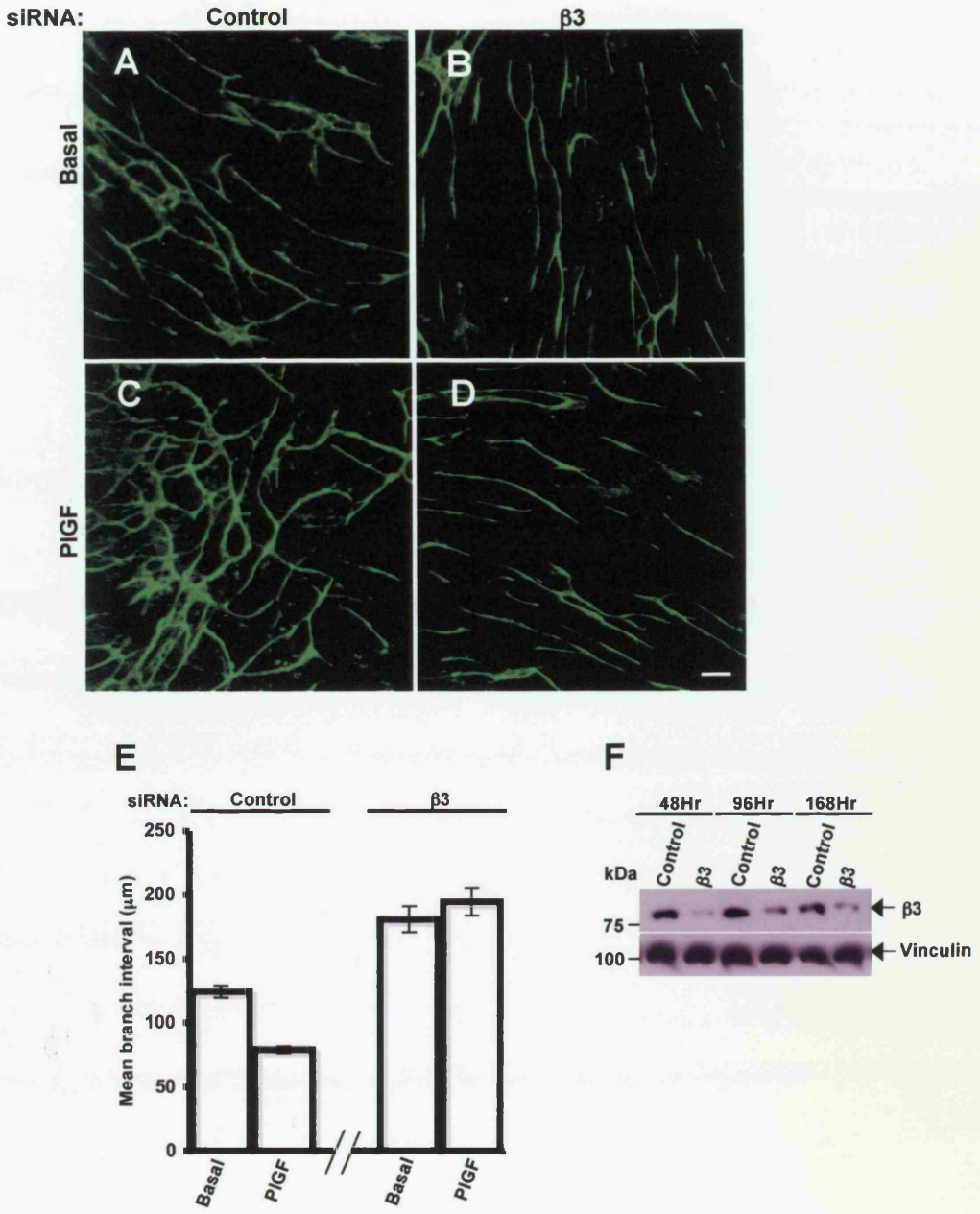

Figure 5.4 RNAi of β3 inhibits PlGF promoted branch formation in a coculture assay
HUVECs were transfected with control RNAi duplexes (**A,C**) or duplexes that target β3 (**B,D**) 24 hours before seeding in a coculture with human dermal fibroblasts. Cocultures were fed every 48 hours for a total of 7 days, with 25ng/ml PlGF (**C,D**) being added on day 4 and day 6. Cells were fixed in 4% paraformaldehyde and PECAM-1 visualised by indirect immunofluorescence. Bar 100μm
(**E**) The mean branch interval length was measured using Image J. Values are mean±s.e.m. for at least 4 experiments.
(**H**) HUVECs were transfected with control RNAi duplexes or duplexes targetting β3 and after 48 hours, 96 hours and 168 hours lysates were prepared and the levels of β3 protein determined by Western blot.

Moreover, the ability of PlGF to stimulate tubule-like structure branching was completely opposed by suppression of β3 levels (Fig 5.4D,E), indicating that although sprouting and elongation clearly do not require the activity of αvβ3 integrin, the ability of PlGF to drive (via Rab4a) branching and cross linkage and thus increase the complexity of the vascular network clearly requires αvβ3 functionality to be intact.

5.2.3 VEGFR1 is required for PlGF-driven vessel branching in co-culture assays

Having identified that PlGF can drive branching of tubule-like structures via a Rab4a- and αvβ3-dependent mechanism, the question of whether PlGF was signalling through VEGFR1 was addressed. Firstly, an RNAi based approach was used to suppress levels of VEGFR1 in HUVECs prior to seeding them into co-cultures. It was clear that knockdown of VEGFR1 did not globally affect the ability of HUVECs to survive in this assay, to aggregate or to arrange themselves into tube-like structures (Fig 5.5B). As with suppression of αvβ3 or Rab4a an approximate 40% increase in basal branch intervals was observed following knockdown of VEGFR1, and no decrease in branch interval is seen following addition of PlGF (Fig 5.5D,E). To further confirm that PlGF was signalling through VEGFR1 in order to regulate HUVEC tubule-like structure branching, a blocking antibody to VEGFR1 was used in co-culture assays. When used at a concentration of 20μg/ml this antibody completely opposed PlGF-stimulated phosphorylation of GSK3β at Ser^9 (Fig 5.6A). Addition of anti-VEGFR1 alone did not alter the ability of HUVECs to form vessel like structures, nor did it alter basal branch intervals as was observed in the RNAi based approach (Fig 5.6C,F). However, the presence of anti-VEGFR1 was able to inhibit PlGF-driven

branching and the consequent measured decrease in mean branch interval (Fig 5.6E,F). This therefore confirms that PlGF is acting through VEGFR1 to mediate HUVEC vessel branching in a co-culture assay.

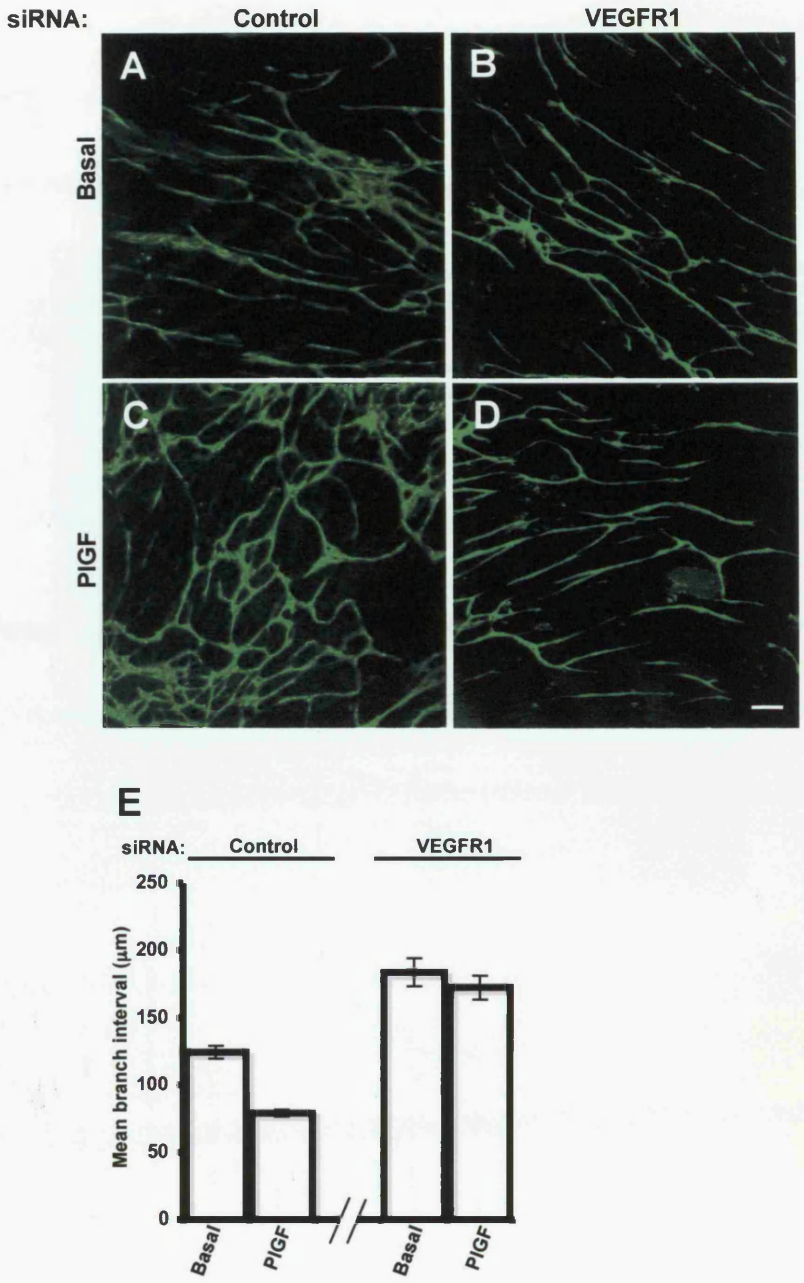

Figure 5.5 RNAi of VEGFR1 inhibits PlGF promoted branch formation in a coculture assay
HUVECs were transfected with control RNAi duplexes (**A,C**) or duplexes that target VEGFR1 (**B,D**) 24 hours before seeding in a coculture with human dermal fibroblasts. Cocultures were fed every 48 hours for a total of 7 days, with 25ng/ml PlGF (**C,D**) being added on day 4 and day 6. Cells were fixed in 4% paraformaldehyde and PECAM-1 visualised by indirect immunofluorescence. Bar 100μm
(**E**) The mean branch interval length was measured using Image J. Values are mean±s.e.m. for at least 4 experiments.

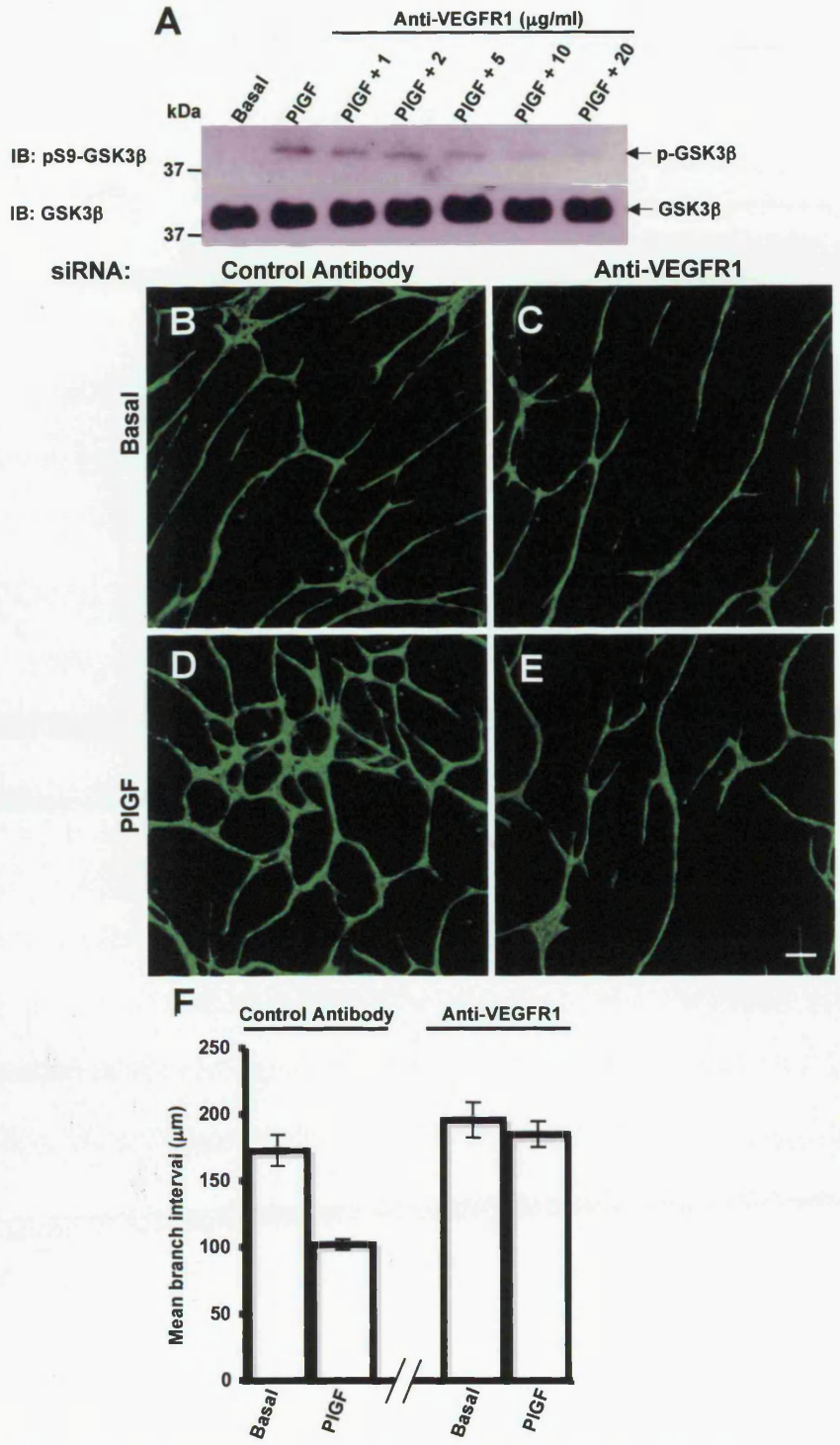

Figure 5.6: VEGFR1 blocking antibody inhibits PlGF stimulated branch formation in a coculture assay
(**A**) Serum-starved HUVECs were treated with anti-VEGFR1 blocking antibody at the concentrations indicated for 30 minutes before being challenged with 25ng/ml PlGF for 5 minutes. Lysates were prepared and the levels of phospho-GSK3β and total GSK3β were determined by Western blot.
(**B,C,D,E**) HUVECs were seeded in a coculture with human dermal fibroblasts. Cocultures were fed every 48 hours for a total of 7 days, with PlGF (**D,E**) or anti-VEGFR1 (**C,E**) being added on day 4 and day 6. Cells were fixed in 4% paraformaldehyde and PECAM-1 visualised by indirect immunofluorescence. Bar 100μm
(**F**) Images were analysed using Image J and the mean branch interval length measured. Values are mean±s.e.m. for at least 4 experiments.

5.3 DISCUSSION

These data identify the novel ability of PlGF to alter the morphology of endothelial cell structures formed when HUVECs are co-cultured with HDFs, such that the endothelial cell network is more complex and extensive with structures displaying more branch points and interconnecting cross bridges. The ability of PlGF to promote branching is completely opposed by knockdown of Rab4a, VEGFR1 or αvβ3, indicating that in order to achieve this morphology PlGF signalling via VEGFR1 is likely to involve Rab4a-dependent recycling of αvβ3.

Stimulation of HUVECs with PlGF is able to stimulate the formation of stress fibres, and this is likely to occur downstream of Rho activation [Jim Norman; Personal Communication]. Rho activation may occur downstream of PlGF-driven αvβ3 recycling and its subsequent engagement with the ECM. Following integrin engagement, a transient depression of Rho activation occurs in response to the activity of Src, FAK and p190 Rho GAP [354, 355]. However, at later time points activation of Rho is apparent [356], and this may be due to the activity of protein tyrosine phosphatase(PTP)α. PTPα is found in focal adhesions [357] and associates with αvβ3 but not α5β1 and activates the Src family kinase Fyn [358]. PTPα null cells exhibit decreased Rho-GTP levels [359], therefore Fyn may activate a Rho-GEF resulting in increased Rho-GTP levels. This 'late' occurrence of Rho activation following αvβ3 ligand binding suggests that PlGF-driven stress fibre formation may result from αvβ3 engagement that is a consequence of the ability of PlGF to drive integrin recycling. Indeed, engagement of αvβ3 in astrocytes stimulated the formation of focal adhesions and stress fibres, suggesting that Rho is activated following αvβ3 ligand binding in these cells [360]. Furthermore, overexpression of β3 in chinese hamster ovary (CHO) cells preferentially activated Rho when plated onto

fibronectin when compared to β1-overexpressing cells [361]. Preliminary studies have shown that PlGF-driven stress fibre formation is opposed by treatment with a small molecule antagonist of β3 and RNAi knockdown of VEGFR1, Rab4a and β3 levels, demonstrating that PlGF-driven stress fibre formation requires integrin function downstream of growth factor receptor activation and Rab4a-dependent recycling. A requirement for integrin signalling acting downstream of growth factors to mediate cellular responses has previously been shown. For example, PDGF-driven proliferation of oligodendrocytes [362] and VEGF-A-driven HUVEC migration in conjunction with phosphorylation of p38 and FAK [363] requires αvβ3 engagement to vitronectin.

5.3.1 Rho and vascular development

Rho has been linked to angiogenesis in a number of studies. For example expression of dominant negative (DN) Rho inhibited neovascularisation in a model of mouse-skin angiogenesis [364], and this was due to differences in EC organisation and vessel morphology rather than through differences in EC number, as DN Rho or inhibition of its downstream effector ROCK abrogated the ability of ECs to form pre-capillary cords [364]. Furthermore, expression of DN Rho impaired sprouting of capillary ECs from spheroids [365]. However, levels of Rho activity clearly need to be tightly regulated for efficient vascular remodelling to occur. Inhibition of the ERK/MAPK pathway results in an increase in Rho/ROCK signalling [366] and in a recent study by Mavria et al [353], that utilises a similar co-culture assay as I have used, inhibition of the ERK/MAPK pathway results in increased ROCK signalling and increased phospho-myosin light chain 2 (pMLC2) which leads to actomyosin contractility and vessel retraction [353].

5.3.2 Branching morphogenesis and Rho/ROCK signalling

Branching morphogenesis of epithelial and endothelial tubes is an important feature of the development of tube based organs such as the lung, kidney and blood vessels, and allows an increase in the total cellular area available for processes such as gas or nutrient exchange to occur [367]. In branching epithelium such as the ureteric bud (UB) of the mammalian kidney, 3 types of branching have been identified: simple bifurcation, trifurcation and lateral branching [368]. The development of branching epithelia requires specific components of the extracellular matrix [369] and specific integrins have been linked to a branched morphology. In the submandibular gland, antibodies against the integrin α6 subunit perturb branching morphogenesis [370] and in the kidney UB the laminin binding integrins α6β1, α6β4 and α3β1 are required for UB branching [371].

Therefore, it is possible that branching morphogenesis of the vasculature will be regulated by αvβ3 integrin engagement and signalling. As discussed earlier, αvβ3 engagement can activate Rho that, via ROCK signalling, phosphorylates MLC2 and induces cell contractility, and a role for Rho signalling and contractility in branching morphogenesis has been suggested in a number of studies. UB branching is inhibited by treatment with butadiene monoxime (BDM) (a myosin inhibitor which blocks the tension producing activity of myosin on actin [372]) and with the ROCK inhibitor Y27632, a treatment which results in the loss of actin filaments associated with growing branch tips [373]. Rho activation by cytotoxic necrotising factor (CNF)-1 accelerated epithelial branch formation in cultured embryonic lung rudiments [374] and lung bud formation was decreased by half in lungs treated with Y27632 associated with a loss of MLC phosphorylation and actin staining [375]. Interestingly, CNF-1 treatment promoted extension and elongation of vascular capillary networks

surrounding lung buds, and ROCK inhibition resulted in disorganisation and disruption of capillary network formation [375].

Taken together these studies indicate that Rho/ROCK signalling is able to influence epithelial branching morphogenesis and potentially vascular branching. I have shown that PlGF is able to promote endothelial tubule-like structure branching through VEGFR1, and that this process requires Rab4a-dependent recycling of αvβ3 integrin. This pathway is likely to result in activation of Rho and formation of stress fibres, and such provides the contractile force required to facilitate tubule branching.

5.3.3 PlGF and branching morphogenesis

A recent study by Kearney et al has shown that VEGFR1 is a positive modulator of vascular sprouting and branching morphogenesis [376]. Using VEGFR1-null embryonic stem (ES) cells induced to differentiate and form primitive vessels, it was shown that VEGFR1-null ES cell cultures could form a vascular plexus but had decreased sprout formation (both in terms of the rate of sprouting and the number of branch points) and formed less-complex vessel networks. This was not due to defect in cell division, and the authors attributed the changes observed to VEGFR1 negatively regulating the bioavailability of VEGF-A. In light of my data, I would suggest that the inhibition of sprouting and branch morphogenesis observed with VEGFR1-null ES cells may be owing to a loss of Rab4a-regulated recycling of αvβ3 trafficking, engagement and signalling. Furthermore, in mice engineered to constitutively overexpress PlGF in the skin, an increase in vascularisation was observed characterised by enlarged vessels and a decrease in distance between branches [377], similar to what I have observed in this *in vitro* study. Moreover, in a study using bovine retinal endothelial cells, both VEGF-A and PlGF were reported to

increase the number of capillary connections in a co-culture assay, and the response to both growth factors could be opposed by treatment with an anti-VEGFR1 antibody [378]. Interestingly, PlGF expression is induced by the transcription factor BF-2, which itself is restricted to stromal cells of the embryonic renal cortex and mediates the growth of the ureteric bud (UB) [379]. PlGF will therefore be highly expressed in stromal cells surrounding the UB, and addition of PlGF to kidney organ cultures increased the number of terminal buds by 40%, indicating that PlGF drives UB branching. The regulation of branching morphogenesis by PlGF may therefore represent not only an important mechanism in vascular development, but also in the development of other tubule networks.

Chapter 6: Growth factor-driven recycling of αvβ3 and VEGFR2 occurs independently of VEGF-receptor autophosphorylation

Chapter 6: Growth factor-driven recycling of αvβ3 occurs independently of VEGF-receptor autophosphorylation

6.1 INTRODUCTION

Due to the upregulation of angiogenesis in pathological disorders, targeting this process using antiangiogenic therapy is an attractive proposition. In terms of cancer, most current chemotherapeutic drugs are aimed towards having a direct effect on tumour cells themselves. This creates problems for treatment as firstly, normal cells may be susceptible to the drug's action and secondly, drug-resistance may develop due to the genetic instability of cancer cells. Therefore, targeting cells involved in tumour angiogenesis would negate the possibility of the development of drug resistance, as normal cells themselves are being targeted. In addition, as all solid tumours are angiogenesis-dependent the need for tumour-specific treatment is reduced.

VEGF-A is the most commonly upregulated angiogenic factor in tumours. Levels of VEGF-A are elevated following activation of oncoproteins such as Ras [380-382], loss of tumour-suppressor function [383] and the onset of hypoxia [384], all situations commonly found in tumours. Targeting the signalling properties of VEGF-A may therefore act to inhibit tumour angiogenesis and induce tumour blood-vessel regression, although any effect on the normal vasculature would have to be ascertained.

M528642 is a novel potent inhibitor of VEGF-receptor tyrosine kinase activity *in vitro*. M528642 is a selective inhibitor of the VEGF receptors and inhibits VEGFR1, VEGFR2 and VEGFR3 recombinant kinases with an IC50 value of < 2nM, but >1μM

against a range of other tyrosine and serine/threonine kinases including EGFR, ErbB2, Aurora, MEK, AKT, CDK2.

Therefore, to elucidate the signalling pathways involved in growth factor regulation of recycling pathways the effect of M528642 on the VEGF/PlGF dependent stimulation of $\alpha v\beta 3$ integrin recycling was determined in primary cultured HUVECs.

6.2 RESULTS

6.2.1 M528642 inhibits VEGFR2 autophosphorylation and downstream signalling

To investigate further the role of VEGF signalling on the regulation of αvβ3 integrin recycling, the effect of the small molecule VEGF receptor tyrosine kinase inhibitor M528642 on receptor recycling was analysed.

In serum starved HUVECs phosphorylation of ERK1/2 was at low levels (Fig 6.1A). Following stimulation with 50ng/ml VEGF-A for 5 minutes, substantial phosphorylation of ERK1/2 was observed (Fig 6.1A). Treatment with 2nM M528642 partially inhibited VEGF-A stimulated ERK1/2 phosphorylation, whereas complete inhibition was observed following treatment with either 5nM or 10nM M528642 (Fig 6.1A), indicating that for significant inhibition of VEGF-A signalling in HUVECs, M528642 should be used at a minimum final concentration of 5nM. For future use, a concentration of 10nM was used, and at this concentration inhibition of VEGF-A-stimulated phosphorylation of VEGFR2 was also observed (Fig 6.1B).

Surprisingly, although PlGF is a potent stimulus to αvβ3 recycling (chapter 3), this growth-factor did not stimulate ERK phosphorylation (Fig 6.1B), indicating that mitogenic signalling via VEGFR1 occurs at a negligible level in this system. This inability of PlGF to activate ERK1/2 also indicates that PlGF, and therefore VEGFR1, is unable to transactivate VEGFR2 and activate the ras/raf/mek/erk pathway.

In order to determine whether M528642 was also inhibiting VEGFR1 autophosphosphorylation and downstream signalling, phosphorylation of p38 MAPK in response to PlGF was analysed. Following serum starvation levels of phospho-p38 are low, but following treatment for 5 minutes with PlGF a clear phosphorylation of p38 was observed.

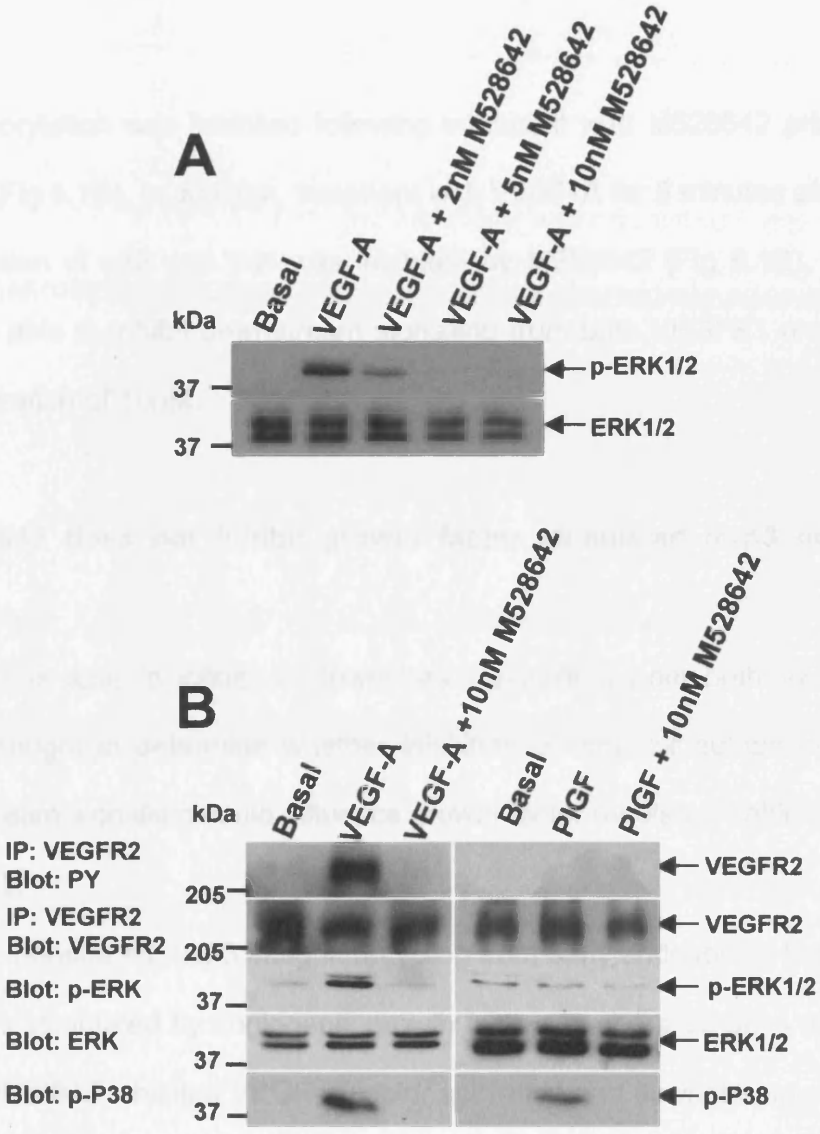

Figure 6.1: M528642 inhibit VEGFR2 autophosphorylation and downstream signalling
(**A**)Serum-starved HUVECs were treated with increasing concentrations of M528642 or a vehicle control for 15 minutes before being challenged with 50ng/ml VEGF-A or allowed to remain quiescent (basal).Lysates were prepared and subjected to SDS-PAGE and the levels of phospgo-ERK or total ERK determined by Western blot
(**B**) Serum-starved HUVECs were treated with 10nM M582642 or a vehicle control for 15 minutes. Cells were then challenged with 25ng/ml PlGF or 50ng/ml VEGF-A, or allowed to remain quiescent (basal). Lysates were prepared and immunoprecipitated (IP) with antibodies against VEGFR2 and immobilised material analysed by immunoblotting with anti-phosphotyrosine (PY) or anti-VEGFR2 (top panels). Lysates were also immunoblotted for phospho-ERK, total ERK and phospho-P38 (lower panels).

p38 phosphorylation was inhibited following treatment with M528642 prior to PlGF stimulation (Fig 6.1B). In addition, treatment with VEGF-A for 5 minutes also induced phosphorylation of p38 and this was inhibited by M528642 (Fig 6.1B). Therefore, M528642 is able to inhibit downstream signalling from both VEGFR1 and VEGFR2 at a concentration of 10nM.

6.2.2 M528642 does not inhibit growth factor stimulated αvβ3 or VEGFR2 recycling.

As M528642 is able to influence downstream signalling from both VEGFR1 and VEGFR2 I sought to determine whether inhibition of receptor autophosphorylation and downstream signalling could influence growth factor regulated trafficking of αvβ3 and VEGFR2.

As demonstrated, αvβ3 integrin recycling from early endosomes to the plasma membrane is stimulated by angiogenic growth factors such as VEGF-A and PlGF in HUVECs. M528642 inhibited VEGF-receptor activation and downstream signalling to phospho-ERK at a concentration of 10nM, and therefore this compound may be expected to inhibit growth factor-stimulated αvβ3 integrin and VEGFR2 recycling.

Surprisingly, treatment with 10nM M528642 was totally unable to oppose either VEGF-A or PlGF-stimulated αvβ3 integrin recycling, and this compound even appeared to enhance PlGF driven αvβ3 integrin recycling at this concentration (Fig 6.2). Furthermore, addition of 100nM M528642 also failed to suppress either VEGF-A- or PlGF-driven αvβ3 integrin recycling.

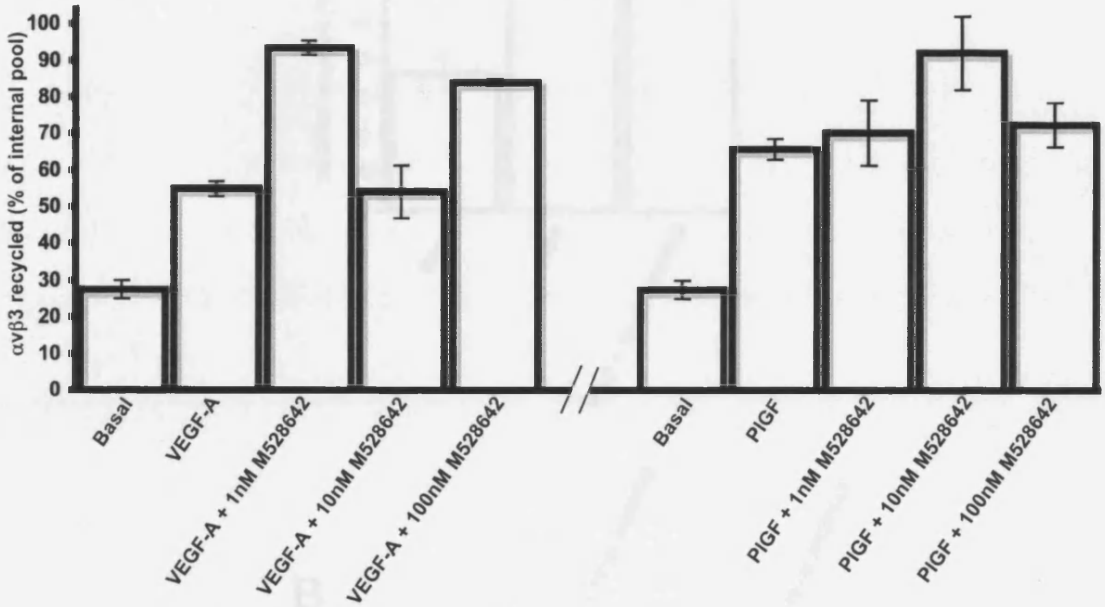

Figure 6.2 M528642 does not inhibit VEGF-A and PlGF promoted αvβ3 recycling
Serum-starved HUVECs were surface labelled with 0.2mg/ml NHS-SS-Biotin for 30 minutes at 4°C, and internalisation of cell surface proteins allowed to proceed for 15 minutes in the absence or presence of either M528642 at the concentrations indicated. Non-internalised biotin was removed by a brief exposure to MesNa at 4°C, and internalised integrin chased to the cell surface for 15 minutes at 37°C in the presence or absence of either 50ng/ml VEGF-A or 25ng/ml PlGF and M528642 VEGF RTK inhibitor at the indicated concentrations. Cells were then re-exposed to MesNa and biotinylated integrin detected by capture ELISA using microtitre wells coated with anti-human β3 monoclonal antibody. Values are mean±s.e.m. for at least 3 experiments.

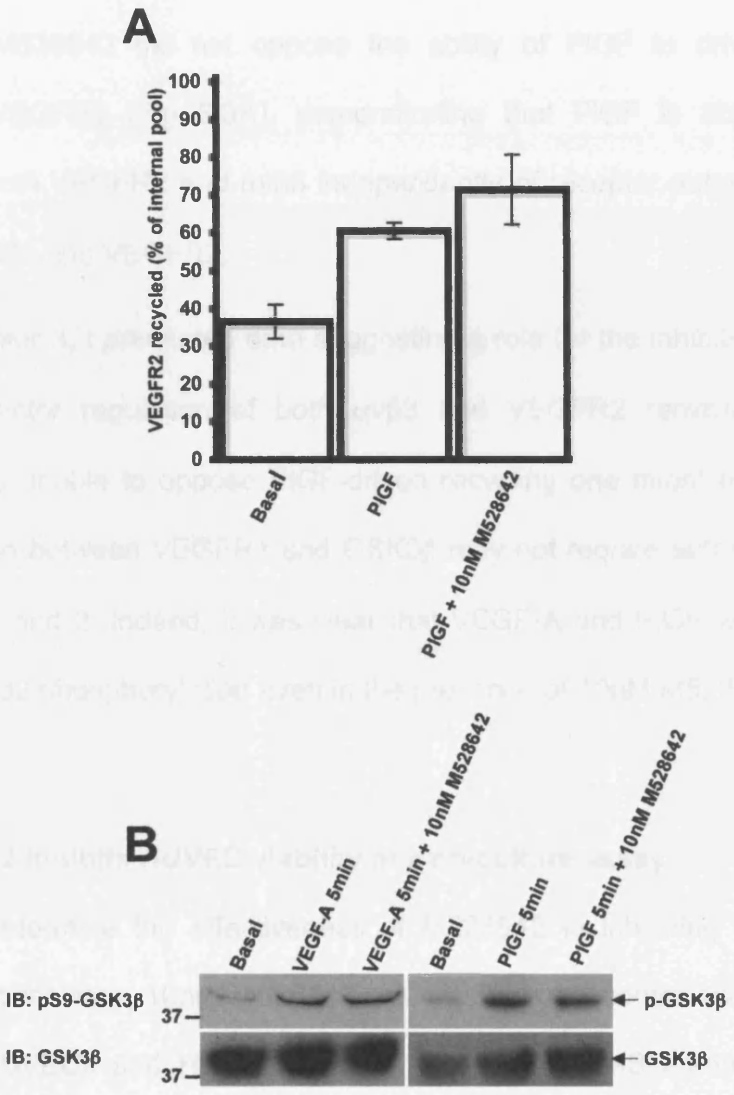

Figure 6.3 M528642 does not inhibit PlGF promoted VEGFR2 recycling or phosphorylation of GSK3β
(**A**) Serum-starved HUVECs were surface labelled with 0.2mg/ml NHS-SS-Biotin for 30 minutes at 4°C, and internalisation of cell surface proteins allowed to proceed for 15 minutes in the absence or presence of 10nM M528642 VEGF RTK inhibitor. Non-internalised biotin was removed by a brief exposure to MesNa at 4°C, and internalised integrin chased to the cell surface for 15 minutes at 37°C in the presence or absence of 25ng/ml PlGF and 10nM M528642 VEGF RTK inhibitor. Cells were then re-exposed to MesNa and biotinylated integrin detected by capture ELISA using microtitre wells coated with anti-human VEGFR2 polyclonal antibody. Values are mean±s.e.m. for at least 3 experiments.
(**B**) Serum-starved HUVECs were treated with 10nM M528642 VEGF RTK inhibitor or a vehicle control for 15 minutes. Cells were then challenged with 25ng/ml PlGF for either 5 minutes, or allowed to remain quiescent (basal). Lysates were prepared and subjected to SDS PAGE before being analysed by immunoblotting (IB) with anti-phospho Ser9 GSK3β and appropriate loading controls.

In addition, M528642 did not oppose the ability of PlGF to drive recycling of internalised VEGFR2 (Fig 6.3A), demonstrating that PlGF is able to stimulate recycling of both VEGFR2 and αvβ3 independently of receptor autophosphorylation of both VEGFR1 and VEGFR2.

In chapter 3, I presented data suggesting a role for the inhibition of GSK3β in the growth factor regulation of both αvβ3 and VEGFR2 recycling. Given that M528642 was unable to oppose PlGF-driven recycling one might hypothesise that communication between VEGFR1 and GSK3β may not require autophosphorylation of VEGFRs 1 and 2. Indeed, it was clear that VEGF-A and PlGF were still able to promote GSK3β phosphorylation even in the presence of 10nM M528642 (Fig 6.3B).

6.2.3 M528642 inhibits HUVEC viability in a co-culture assay

In order to determine the effectiveness of M528642 in inhibiting endothelial cell function and behaviour, 10nm M528642 or DMSO carrier control was added to co-cultures of HUVECs and HDFs on day 4. On day 7, DMSO treated co-cultures exhibited long HUVEC tube-like structures with numerous branch points (Fig 6.4A). As outlined in chapter 5, addition of exogenous PlGF results in a more complex network of endothelial cell structures as illustrated by an increased number of branch points (Fig 6.4C). Addition of 10nM M528642 completely inhibited HUVEC viability such that only few cells and no endothelial structures were observed at all in PECAM-1 stained co-cultures, despite no effect on HDF growth being observed (Fig 6.4C,F). This therefore demonstrates, at least in this simple cell assay, that M528642 has the ability to preferentially inhibit endothelial cell growth and survival.

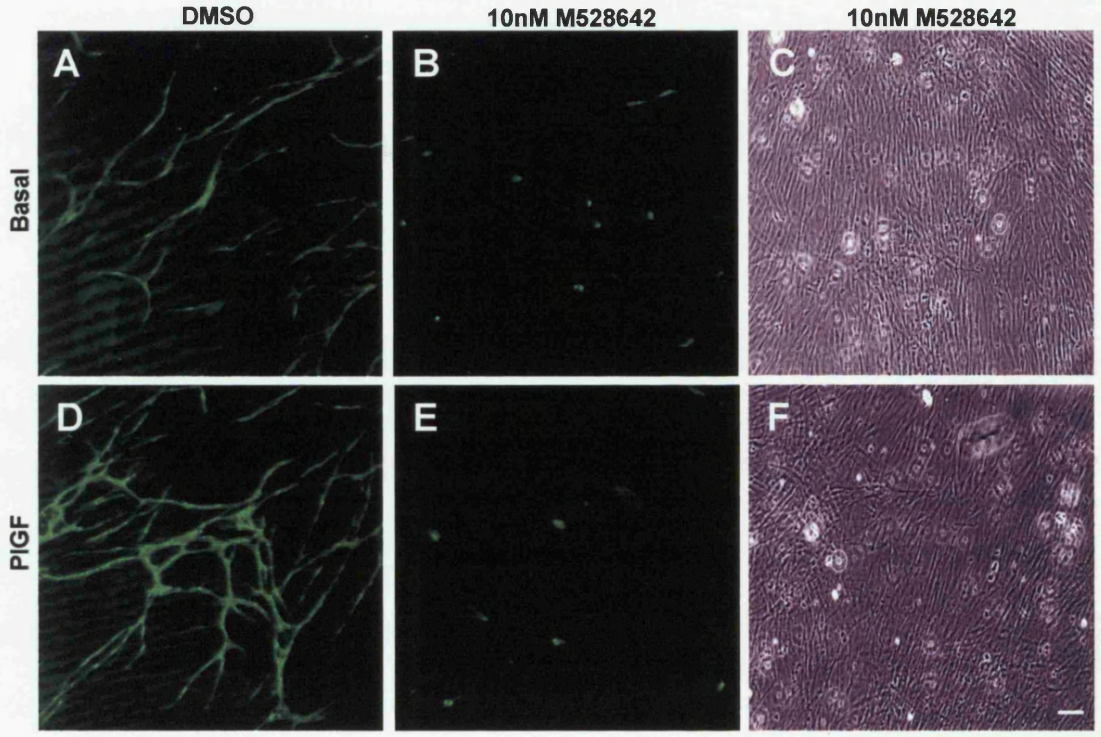

Figure 6.4 M528642 inhibit HUVEC tube formation in a coculture assay
HUVECs were seeded in a coculture with human dermal fibroblasts. Cocultures were fed every 48 hours for a total of 7 days (A) with 20ng/ml PIGF (C,D) and 10nM M528642 (B,D) being added on day 4 and day 6. Cells were fixed in 4% paraformaldehyde and PECAM-1 visualised by indirect immunofluorescence. Phase contrast images of co-cultures grown in the presence of M528642 were also taken to illustrate fibroblast growth. Bar 100μm

6.3 DISCUSSION

These data show that M528642, an inhibitor of VEGFR1, VEGFR2 and VEGFR3 kinase domains, is able to inhibit autophosphorylation of VEGFR2 at a concentration of 10nM, and downstream signalling from both VEGFR1 and VEGFR2 at the same dose. However, M528642 is did not inhibit VEGF-A- and PlGF- driven recycling of αvβ3 and VEGFR2 and, in fact, acts to potentiate PlGF driven αvβ3 recycling.

In chapter 3 I described a role for phosphorylation at Ser^9 and inactivation of GSK3β in αvβ3 integrin recycling. Inhibition of VEGFR1 and VEGFR2 kinase activity in the presence of PlGF did not inhibit phosphorylation of GSK3β at Ser^9, further supporting the theory that PlGF/VEGFR1 is signalling through GSK3β to mediate endosomal recycling of αvβ3. Furthermore, these data indicate that the observed inactivation of GSK3β by PlGF/VEGFR1 is not due to transactivation of VEGFR2 and suggest that phosphorylation of GSK3β following treatment with PlGF or VEGF-A occurs via a VEGF-receptor tyrosine kinase-independent signal. This type of signalling is not unprecedented, in the presence of genistein (a specific inhibitor of tyrosine kinases), PDGF induction of the immediate-early gene egr-1 occurs normally despite no measurable tyrosine kinase activity [385], indicating that alternative signalling pathways downstream of PDGF receptors may be activated independently from receptor autophosphorylation. Further work is required, however, to elucidate the novel mechanism by which VEGFR1 is able to phosphorylate GSK3β. The C-terminus of VEGFR1 has been reported to activate cdc42 (a known upstream regulator of GSK3β [342]) when fused with the N-terminus of the EGF receptor and expressed in HUVECs [386]. This could be opposed by treatment with the PI3K inhibitor wortmannin, which is able to oppose growth-factor driven αvβ3 recycling in HUVECs (Marnie Roberts, unpublished data), or pertussis

toxin [386]. Therefore, the ability of VEGFR1 to activate cdc42 involves pertussis toxin-sensitive G protein signalling, and it would be interesting to determine if this means of signalling is dependent on VEGFR1 kinase activity.

The presence of large quantities of unligated integrins at the plasma membrane has been shown to induce cellular apoptosis; a process known as integrin mediated death [143]. In particular it was shown that cells expressing $\alpha v\beta 3$, when attached to a matrix that did not ligate $\alpha v\beta 3$, initiated apoptosis via activation of the initiator caspase, caspase 8 [143]. Therefore, the presence of unligated $\alpha v\beta 3$ may overcome the survival signals that affect endothelial cells and thus negatively regulates angiogenesis by inducing endothelial cell apoptosis. M528642 is clearly able to inhibit VEGF-receptor signalling with a high degree of specificity and appears to inhibit HUVEC viability in a co-culture assay. M528642 is an extremely potent compound *in vivo* at inhibiting tumour growth [Simon Barry, personal communication], yet is unable to inhibit VEGF-A/PlGF driven recycling of $\alpha v\beta 3$ and VEGFR2. It is maintenance of this pathway that may contribute towards the efficacy of M528642 as rapid recycling rates may act to increase levels of unligated receptors at the cell surface. In addition, it may also be of interest that both Rab4 and Rab11 (but not Rab5) are likely to be amongst the transcripts that are actively recruited to ribosomes and translated during the early stages of TRAIL-induced apoptosis [Dr Martin Bushell, Personal communication], suggesting that recycling mechanisms may need to be maintained for an efficient apoptotic response.

Chapter 7: Discussion

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7.1 SUMMARY

Here I demonstrate that PlGF/VEGF-A signalling is linked to the adhesive/migratory machinery of endothelial cells via the regulation of Rab4a-dependent αvβ3 and VEGFR2 recycling by a mechanism that requires VEGFR1. αvβ3 and VEGFR2 are rapidly and constitutively internalised and then return to the plasma membrane in HUVECs. Stimulation of HUVECs with either VEGF-A or PlGF does not alter the internalisation rates of αvβ3 or VEGFR2, but does promote the recycling of αvβ3 and VEGFR2 to the plasma membrane.

In fibroblasts, growth factor-driven recycling of αvβ3 is dependent on the activity of PKD1 and its direct association with the β3 cytodomain [173]. However, PKD1 is not required for PlGF-driven recycling of αvβ3 in HUVECs. PlGF/VEGF-A stimulation of HUVECs resulted in phosphorylation and inactivation of GSK3β, and this inactivation of GSK3β is able to drive the recycling of both αvβ3 and VEGFR2. Knockdown of Rab4a by RNAi opposed PlGF/VEGF-A-stimulated recycling of αvβ3, but was unable to oppose PlGF/VEGF-A-driven recycling of VEGFR2, demonstrating that VEGFR2 is able to recycle via an alternative Rab4a-independent recycling pathway. This may involve the small GTPase RhoB, as RhoB has been shown to colocalise with VEGFR2-containing endosomes distinct to those that are Rab4 positive [333] (summarised in Figure 7.1)

PlGF is also able to stimulate branching of endothelial structures in an organotypic co-culture assay. This was opposed by RNAi knockdown of Rab4a, αvβ3 and VEGFR1, as well as the introduction of an anti-VEGFR1 blocking antibody, suggesting that the morphological effects of PlGF on HUVEC tubule-like

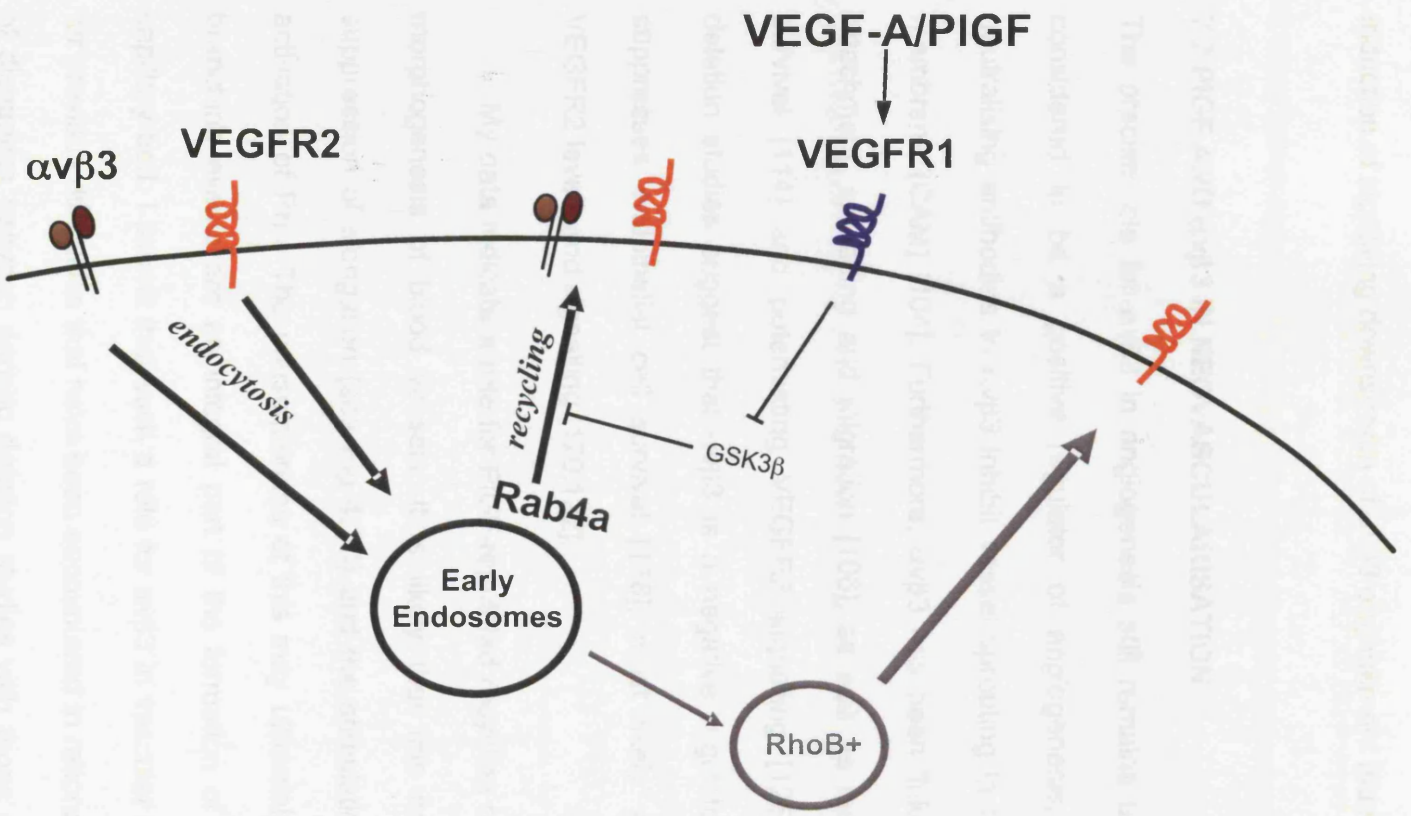

Figure 7.1 Summary of growth factor driven recycling of αvβ3 integrin and VEGFR2
αvβ3 integrin and VEGFR2 are both constitutively internalised and from this internal pool return to the plasma membrane. PlGF/VEGFR1 phosphorylates and inactivates GSK3β leading to an increase in this recycling of αvβ3 and VEGFR2. Recycling of both receptors occurs independently of receptor autophosphorylation and PKD1 activity, and recycling of αvβ3 is Rab4a-dependent. VEGFR2, on the other hand, is able to traffic via an alternative recycling pathway independent to Rab4a and this may be controlled by the small GTPase RhoB. αvβ3 may act to recruit VEGFR2 into the Rab4a/GSK3β-dependent recycling pathway such that their delivery to membrane domains is coordinated.

networks may result from PlGF/VEGFR1-mediated recycling of αvβ3 integrin and the induction of signalling downstream of αvβ3 engagement (summarised in figure 7.2).

7.2 PlGF AND αvβ3 IN NEOVASCULARISATION

The precise role for αvβ3 in angiogenesis still remains unclear. αvβ3 has been considered to be a positive regulator of angiogenesis, due to the fact that neutralising antibodies to αvβ3 inhibit vessel sprouting in the chick chorioallantoic membrane (CAM) [104]. Furthermore, αvβ3 has been linked with endothelial cell attachment, spreading and migration [106], as well as mediating endothelial cell survival [114] and potentiating VEGFR2 signalling [125]. Conversely, genetic deletion studies suggest that αvβ3 is a negative regulator of angiogenesis and suppresses endothelial cell survival [118], most likely via the suppression of VEGFR2 levels and signalling [120,122].

My data indicate a role for PlGF-regulated recycling of αvβ3 in the branching morphogenesis of blood vessels. It is likely that this may be mediated by a suppression of elongation (see Fig 4.7C) and the stimulation of branching via the activation of Rho. The consequences of this may ultimately be pro-angiogenic as branching events are an integral part of the formation of a complex, networked capillary bed. I submit that such a role for αvβ3 in vascular branching may account for previous difficulties that have been encountered in rationalising the interpretation of disparities between genetic deletion studies with those experiments that target αvβ3 pharmacologically. Blood vessels can clearly form in the absence of αvβ3, and this is associated with an increase in VEGFR2 levels and an increase in ERK/MAPK

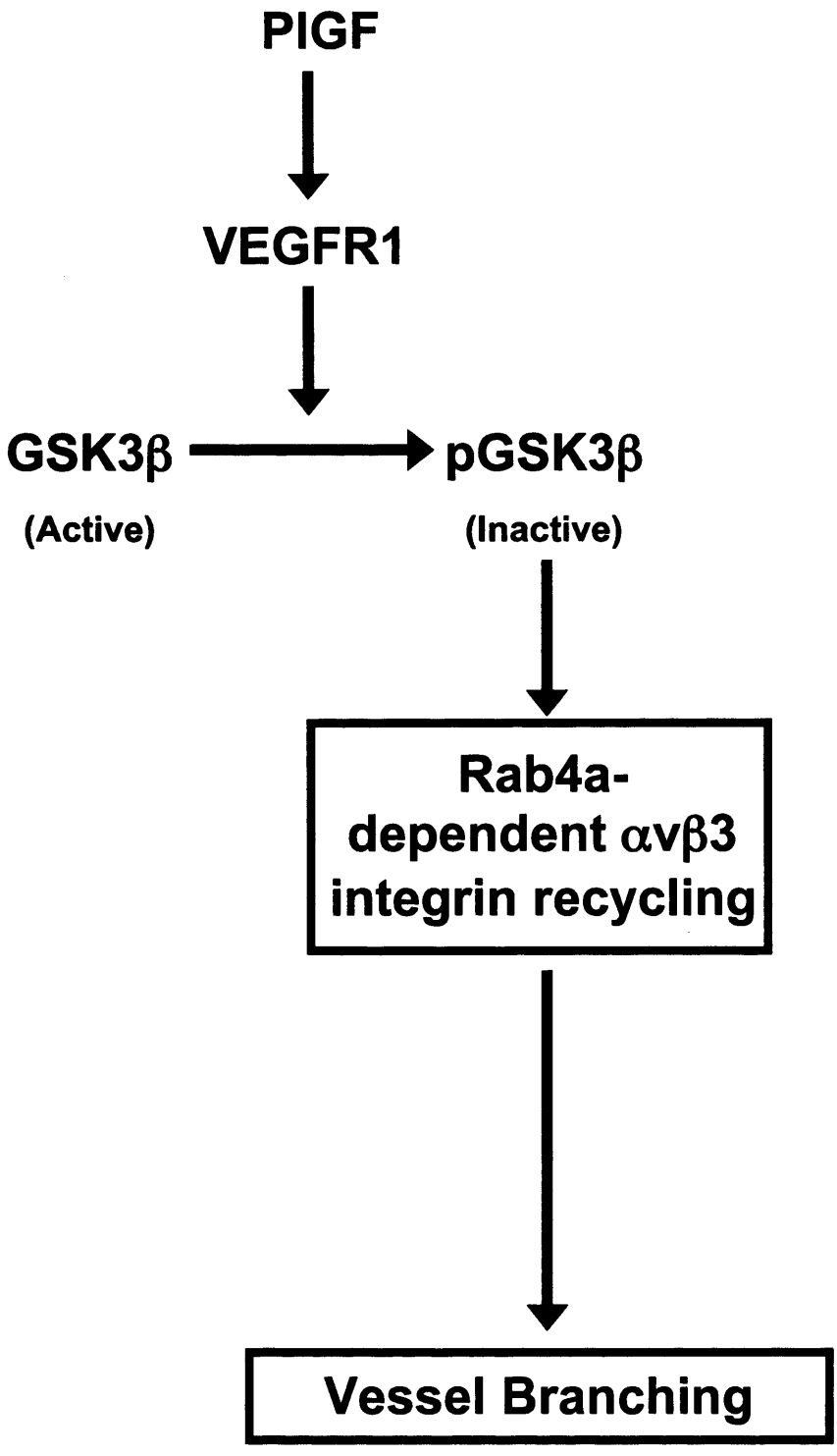

Figure 7.2 Mechanism by which PlGF influences branching morphogenesis of endothelial tubules
PlGF/VEGFR1 phosphorylates and inactivates GSK3β leading to stimulation of Rab4a-dependent recycling of αvβ3. This recycling, and subsequent engagement and signalling, of αvβ3 is required for endothelial tubule-like structure branching, which may involve the formation of focal adhesions and stress fibres in order to generate the contractile force required for branching.

signalling in endothelial cells [120,122]. ERK/MAPK signalling has been linked to the stimulation of vessel sprouting and elongation through the suppression of Rho/ROCK signalling [251], and as such genetic deletion of β3 would result in unchecked vessel elongation and an apparent increase in vessel growth.

These genetic deletion studies demonstrate that αvβ3 integrin is acting to temper VEGFR2 levels, and here I demonstrate that that αvβ3 and VEGFR2 are recycled by mechanisms that require VEGFR1 and exhibit similar kinetics. Therefore it is possible that αvβ3 may be acting as a 'chaperone' for VEGFR2 to enable the trafficking of the RTK through a Rab4a-dependent recycling pathway, indeed αvβ3 and VEGFR2 colocalise at early endosomes in HUVECs [Jim Norman; Personal communication]. This suggests that coordinated trafficking and delivery to specific membranes of both αvβ3 and VEGFR2 may be important in angiogenesis. Recycling membrane is trafficked to sites of cell protrusion during phagocytosis [278] and neurite outgrowth [279], and it is probable that the recycling events contribute to the delivery of αvβ3 and VEGFR2 to sites of protrusion during vessel growth. VEGFR2 is expressed at higher levels in tip cells of vascular sprouts, where it is highly localised to filopodia [280], and acts to guide endothelial sprouting through detection of extracellular VEGF-A gradients.

Therefore, the coordinated activity of both VEGFR2 and αvβ3 may be required to facilitate vessel elongation and branching and the formation of a networked capillary bed in angiogenesis. Any disruption to this coordinated trafficking (e.g. inhibition of αvβ3 attachment and signalling, genetic deletion of β3 or disruption of Rab4a-dependent recycling) results in the formation of abnormal vasculature, characterised by an inhibition of vascular branching morphogenesis.

7.3 VEGFR1 REGULATES VEGFR2 SIGNALLING

The role of PlGF and its receptor VEGFR1 in angiogenesis remains in debate. VEGFR1 is able to influence the angiogenic response via the recruitment of cell types other than endothelial cells to sites of neovascularisation, but it is unclear whether VEGFR1 acts as a functional receptor in endothelial cells, or whether its primary role is to act as an inert 'decoy' and therefore regulate the availability of VEGF-A for VEGFR2.

Here, PlGF acts through binding to VEGFR1 and is shown to be capable of eliciting cellular responses such as stimulation of integrin and VEGFR2 recycling and tubule-like structure branching, and as such is clearly able to influence endothelial cell behaviour. The observation that VEGFR1 is able to mobilise an internal pool of VEGFR2 to the plasma membrane suggests that the plasma membrane levels of VEGFR2 can be rapidly increased via VEGFR1-driven recycling. Hence, VEGFR1 may control the levels and distribution of VEGFR2 via stimulation of Rab4a-dependent recycling.

7.4 FUTURE PERSPECTIVES

7.4.1 Signalling pathways regulating Rab4a-dependent recycling

Having identified a pathway linking PlGF/VEGFR1 to $\alpha v\beta 3$ and VEGFR2 mobilisation, a number of questions need to be addressed in order to confirm and explain the mechanism for each stage outlined in figure 7.2. Firstly, the question of how VEGFR1 is communicating with GSK3β needs to be addressed, and this can be achieved by using a proteomics based approach. Immunoprecipitation of VEGFR1 and GSK3β following treatment with or without PlGF would provide samples which can be trypsin digested and the constituents identified by mass spectrometry or by

protein-array. Ideally I would wish to identify any overlap in molecules that are physically associated with VEGFR1 and GSK3β in the presence of PlGF, or identify molecules that can be linked via known signalling pathways potentially resulting in inactivation of GSK3β. An alternative approach would be to utilise M528642 in conjunction with either RNAi of β3 or inhibition of β3 signalling using a small molecule antagonist and use phospho-proteomics, such as a Kinexus screens [281], to identify proteins phosphorylated following PlGF stimulation. These approaches may not be viable with primary cultured HUVECs due to the large quantities of protein required in order to obtain reliable results, therefore the initial proteomic screens may need to be undertaken in a cell line system such as immortalised HUVECs or human microvascular endothelial cells (HMVECs). Any targets identified using this approach will then have to be subsequently confirmed in the primary cultured HUVECs.

An *in silico* approach can also be utilised to acquire potential targets identified by previous proteomics based approaches and publications. For example, Pathwaystudio (Ariadne genomics, MD) produces biological pathways based on protein-protein interactions and signalling events utilising databases produced by Ariadne themselves or by searching online references. Once candidate signalling molecules have been identified the effects of RNAi knockdown of these on αvβ3 and VEGFR2 recycling will be determined.

7.4.2 Role of VEGFR2 recycling in angiogenesis

The regulation and role of PlGF/VEGF-A-driven VEGFR2 recycling, however, remains relatively unclear compared to that of αvβ3. VEGFR2 localises to the tip of sprouting vessels and is involved in detecting gradients of VEGF-A and directing the

vessel accordingly [280]. Therefore PlGF/VEGF-A-driven recycling may act to localise VEGFR2 to the leading edge of directionally migrating cells and to the tips of sprouting vessels. At sprout tips VEGFR2 may colocalise with $\alpha v\beta 3$ such that the balance between ERK/MAPK and Rho/ROCK signalling is maintained to allow the appropriate ratio of vessel elongation and sprouting to retraction during angiogenesis.

VEGFR2 can be trafficked through a Rab4a-independent pathway that may involve the activity of RhoB. The involvement of RhoB can be addressed by suppression of RhoB levels in endothelial cells by RNAi and the influence of this on PlGF/VEGF-A-stimulated recycling of $\alpha v\beta 3$ and VEGFR2 determined. This would determine whether RhoB mediates trafficking of VEGFR2, but not $\alpha v\beta 3$, further outlining the specific nature of recycling pathways in endothelial cells. Potentially VEGFR2 could be trafficked through both a Rab4a-dependent pathway associated with $\alpha v\beta 3$, and a Rab4a-independent pathway mediated by RhoB. Ideally, some functional disparity between the two pathways would be apparent allowing them to be distinguished. For example if the Rab4a pathway mediates cell polarity, directional migration and VEGFR2 delivery to sprout tips, then the RhoB pathway would not impinge upon these aspects of endothelial cell behaviour. Rather RhoB may influence an alternative aspect of VEGFR2 regulation such as resensitisation in the presence of ligand, and as such may be involved in the regulation of prolonged responses to VEGF-A. The influence of RNAi of Rab4a and RhoB on endothelial cell migration, survival, proliferation and downstream signalling in response to PlGF and VEGF-A, as well as the cellular localisation of VEGFR2, will therefore be directly compared in order to determine the exact role of these two recycling pathways in regulating VEGFR2 and endothelial cell biology.

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