SIGNALLING INTERACTIONS BETWEEN PLATELETS AND LYMPHATIC ENDOTHELIAL CELLS, LINKED TO LYMPHANGIOGENESIS

By

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ABSTRACT

The platelet receptor CLEC-2 is the only known endogenous ligand for the transmembrane receptor podoplanin, which is expressed on lymphatic endothelial cells (LEC) as well as a number of other cell types. Both CLEC-2 and podoplanin are required for normal lymphangiogenesis as mouse embryos lacking either protein develop a phenotype in which blood is detected in the lymphatic vessels. This thesis examines the role of the podoplanin-CLEC-2 interaction in the migratory and tube-forming capabilities of LEC.

Addition of platelets or antibody-mediated podoplanin crosslinking both inhibited migration of LEC in transfilter migration assays in the presence, but not absence, of vascular endothelial growth factor (VEGF)-C. Similarly, platelets and podoplanin crosslinking reduced stability of LEC networks formed in co-cultures with fibroblasts. We also found that siRNA-mediated knockdown of podoplanin negated the pro-migratory effects of VEGF-C and VEGF-A. Furthermore, we obtained evidence that podoplanin signalling may involve RhoA and Rho-kinase, and that the effect of podoplanin might be linked to its phosphorylation by protein kinase A downstream of VEGF receptor signalling. These data suggest that the interaction of podoplanin and CLEC-2 prevents connection between blood and lymphatic vessels through reductions in LEC migration and stability of cell-cell interactions.

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ABBREVIATIONS

ACD	Acid citrate dextrose
ADAMTS1/3	A disintegrin and metalloprotease with thrombospondin motifs 1/3
ADP	Adenosine diphosphate
Ang	Angiopoietin
ANOVA	Analysis of variance
ApoE	Apolipoprotein E
BMP-9	Bone morphologica protein-9
BSA	Bovine serum albumin
CCBE1	Collagen and calcium-binding domain protein 1
CCL	CC chemokine ligand
CD40L	CD40 ligand
CDC	Center for Disease Control and Prevention
CLEC-2	C-type lectin receptor 2
COUP-TFII	Chicken ovalbumin upstream promoter transcription factor 2
CRP-XL	Crosslinked collagen-related peptide
CXCL4	CXC chemokine ligand 4
CXCR2	CXC chemokine receptor 2
DA	Ductus arteriosus
E	Embryonic day
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EDTA	Ethylene diamine tetraacetic acid
EGF	Epidermal growth factor
EMT	Epithelial to mesenchymal transition
ERK1/2	Extracellular signal-regulated kinase ½
ERM	Ezrin/radixin/moesin
F-actin	Filament actin
FAK	Focal adhesion kinase
FcγRIIA	Fc-gamma receptor 2a
FCS	Foetal calf serum
FGFb	Basic fibroblast growth factor
FOXC2	Forkhead box protein C2
FRC	Fibroblastic reticular cell
G-actin	Globular actin
GAP	GTPase activating protein
GDI	Guanosine nucleotide dissociation inhibitor
GDP	Guanosine diphosphate
GEF	Guanosine nucleotide exchange factor
GP	Glycoprotein
GTP	Guanosine triphosphate
HDF	Human dermal fibroblasts
HEV	High endothelial venule

HMEC-1	Human microvascular endothelial cells
HRP	Horseradish peroxidase
HUVEC	Human umbilical vein endothelial cells
ICAM1	Intracellular adhesion molecule 1
IFNγ	Interferon gamma
IL	Interleukin
ILK	Integrin-linked kinase
IP	Immunoprecipitation
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
LEC	Lymphatic endothelial cells
LYVE-1	Lymphatic vessel hyaluronan receptor-1
MAPK	Mitogen-activated protein kinase
MDCK	Madin-Darby canine kidney
MFI	Median fluorescent intensity
MK	Megakaryocytes
NO	Nitric oxide
PAF	Platelet-activating factor
PBS	Phosphate buffered saline
PDGF	Platelet-derived growth factor
PE	Phycoerythrin
PGI_2	Prostacyclin
PI3K	Phosphoinositide 3-kinase
PKA	Protein kinase A
PKC	Protein kinase C
PLCγ2	Phospholipase C-γ2
PlGF	Placental growth factor
Prox1	Prospero homeobox 1
PRP	Platelet-rich plasma
PTB	Phosphotyrosine binding
PVDF	Polyvinylidene difluoride
ROCK	Rho kinase
SEM	Standard error of the mean
SH2	Src homology 2
SHP1/2	Src-homology phosphatase 1/2
SLP-76	SH2-containing leukocyte protein of 76 kDa
S1P	Sphingsine-1-phosphate
S1PR1	Sphingosine-1-phospate receptor 1
SOX18	Sex determining region Y-related HMG-box 18
STAT3/5	Signal transducer and activator of transcription 3/5
TBS	Tris-buffered saline
TBST	Tris-buffered saline with Tween 20
TGFβ	Transforming growth factor β
Th17	T helper 17
TNFα	Tumour necrosis factor alpha

TSAd	T cell-specific adaptor
VCAM1	Vascular cell adhesion molecule 1
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
vWF	von Willebrand factor
WASP	Wiskott-Aldrich syndrome protein
WAVE	WASP family Verprolin-homologous
WB	Western blot

CHAPTER ONE

GENERAL INTRODUCTION

1.1. Overview

This thesis centres on the migratory and tube-forming capabilities of lymphatic endothelial cells, and how platelets may influence these processes within the context of lymphangiogenesis. Therefore, the following introductory chapter will describe: i) the lymphatic system and its formation; ii) endothelial cells and the process of cell migration; iii) vascular endothelial growth factor signalling within the context of cell migration; iv) the role of platelets in lymphatic development; v) the role of podoplanin in lymphangiogenesis and cell migration.

1.2. The lymphatic system and lymphangiogenesis

1.2.1. Functions of the lymphatic system

The lymphatic system is a complex network of vessels that was first described by Gasparo Aselli (Asselius, 1627), but its functions were not fully understood until the twentieth century. The lymphatic system has been found to have three main functions: i) to return protein-rich fluid exuded from blood vessels to the circulation; ii) to absorb dietary lipids; iii) to transport leukocytes and antigen-presenting cells to secondary lymphoid organs.

Lymph is taken up from the tissue by lymphatic capillaries and moves from there to the pre-collector vessels. From the pre-collector vessels, lymph travels to the collecting vessels and is returned to the circulation via the thoracic duct, which connects the lymphatic vessels to the inferior vena cava (Liersch and Detmar, 2007). As lymph travels around the body it passes through lymph nodes, where immune responses can be initiated by the presence of

foreign particles taken up by antigen-presenting cells. Dietary lipids are absorbed from the digestive system into the lacteal vessels in the form of chylomicrons (Alitalo et al., 2005). The lacteal vessels in the intestinal villi merge to form larger lymphatic vessels and the chyle is transported through the lymphatic system until it is returned to the blood vascular system at the subclavian vein.

1.2.2. Structure of the lymphatic system

The blood and lymphatic vascular systems are separate once fully developed, with the only connections between the two systems in the neck and the thoracic duct (Oliver and Detmar, 2002). These connections allow exuded fluid to be returned to the blood vascular system. The lymphatic systems consists of blind-ended capillaries, collecting vessels, lymph nodes, trunk vessels and the thoracic duct (Witte et al., 2001). Lymphatic capillaries are 30-80 µm in diameter (Alitalo, 2011) and consist of lymphatic endothelial cells (LEC) that are connected to the surrounding extracellular matrix (ECM) by anchoring filaments, which also regulate the opening of the capillary lumen (Liersch and Detmar, 2007). As interstitial pressure increases the anchoring filaments exert tension on the LEC, which results in dilation of the capillary lumen and opening of the cell junctions. These responses enable the capillaries to take up fluid, cells and macromolecules. The cell junctions in lymphatic capillaries have been described as “button-like” and allow fluid and leukocytes to enter the vessel lumen (Baluk et al., 2007; Pfeiffer et al., 2008; Dejana et al., 2009). Similarly, the discontinuous basement membrane seen in lymphatic capillaries provides entry points for dendritic cells (Pflicke and Sixt, 2009). In contrast to the capillaries, the lymphatic collecting vessels are surrounded by a layer of smooth muscle cells, have a basement membrane and continuous cell junctions

(Dejana et al., 2009; Alitalo, 2011). Collecting vessels have also been found to have bileaflet valves, which prevent backflow of lymph (Daroczy, 1984) and separate the collecting lymphatic vessels into smaller sections, known as lymphangions (Schulte-Merker et al., 2011). The combination of continuous cell junctions and bileaflet valves makes collecting lymphatic vessels resemble small veins.

1.2.3. Embryonic lymphatic development

In the early 1900s, Florence Sabin proposed that lymphatic vessels develop from embryonic veins and that the peripheral lymphatics develop by endothelial sprouting from the primary lymph sacs (Sabin, 1902, 1904). This is known as the centrifugal hypothesis of lymphangiogenesis and contrasts the centripetal hypothesis suggested by Huntington and McClure, in which the lymphatic vascular network is derived from the mesenchyme and is independent of the blood vascular system (Huntington and McClure, 1910). As knowledge of lymphatic development has improved, the centrifugal method of lymphangiogenesis has become accepted.

Lymphatic development begins at week 6-7 in human embryos and around embryonic day (E) 9.5 in mice, which is after the blood vascular system has formed and is functional. At this time, endothelial cells in the cardinal vein begin to express Prospero homeobox 1 (Prox1; Figure 1.1) which is thought to be promoted by the transcription factor sex determining region Y-related HMG-box 18 (SOX18; François et al., 2008). Prox1 then interacts with the

nuclear receptor chicken ovalbumin upstream promoter transcription factor 2 (COUP-TFII) to initiate differentiation of cells on the dorsal side of the cardinal vein; these LEC begin to display a characteristic phenotype (for example expressing podoplanin as described later; Srinivasan et al., 2010). Deletion of Prox1 in mice prevents formation of lymphatic vessels, although the blood vasculature develops normally (Wigle and Oliver, 1999). The lymphatic defect arises from endothelial cells being unable to develop a LEC phenotype (Wigle et al., 2002). Similarly, Prox1 heterozygous mice on most genetic backgrounds die perinatally, suggesting a haplo-insufficient effect of Prox1 (Wigle and Oliver, 1999; Harvey et al., 2005). Prox1 heterozygous mice that do not die perinatally are prone to developing adult-onset obesity, which has been shown to be connected to a defect in vascular integrity of the mesenteric lymphatic vessels (Harvey et al., 2005). While SOX18 is likely to be required for the initial induction of Prox1, it is not continuously expressed so does not have a role in maintaining the LEC phenotype (François et al., 2008). Similarly, COUP-TFII is only required during the initial process of LEC identification and is not required for maintenance (Lin et al., 2010).

As LEC differentiate they begin to express a number of specific markers that are not expressed by blood vascular endothelial cells, including vascular endothelial growth factor receptor (VEGFR) 3, lymphatic vessel hyaluronan receptor-1 (LYVE-1) and the transmembrane glycoprotein podoplanin (Gp38; Kaipainen et al., 1995; Banerji et al., 1999; Breiteneder-Geleff et al., 1999). When these newly-formed LEC have fully differentiated, they migrate away from the cardinal vein in a vascular endothelial growth factor C (VEGF-C)

dependent process (Karkkainen et al., 2004). The LEC form primary lymphatic sacs, which are thought to be separated from the vein by platelet aggregates (Bertozzi et al., 2010; Uhrin et al., 2010; Figure 1.1). The lymphatic vascular network develops from these sacs by further sprouting and remodelling (Adams and Alitalo, 2007). During the later stages of lymphatic development, the different types of lymphatic vessels form and valves develop in the larger collecting vessels (Petrova et al., 2004). There is also evidence of roles for the transcription factor forkhead box protein C2 (FOXC2), angiopoietins (Ang), neuropilin-2 and ephrin-B2 in the later stages of lymphatic development (Gale et al., 2002; Yuan et al., 2002; Mäkinen et al., 2005). FOXC2-deficient mice fail to develop lymphatic valves and it is thought that FOXC2 is also necessary for the maintenance of the valves (Petrova et al., 2004). The angiopoietin-2 knockout mouse displays lymphatic hypoplasia, although this can be rescued by Ang-1 (Gale et al., 2002), while Ang-1 has been shown to promote the growth of lymphatic vessels in adult tissue (Morisada et al., 2005; Tammela et al., 2005). Neuropilin-2 is believed to be required for the sprouting of new lymphatic vessels from existing lymphatics, as the knockout mice display a reduction in lymphatic capillaries and small vessels while the thoracic duct and collecting vessels develop normally (Yuan et al., 2002). Finally, mice lacking the C-terminal PDZ interaction site of ephrin-B2 display defective lymphatic remodelling in the skin, collecting vessel hyperplasia and an absence of valves (Mäkinen et al., 2005).

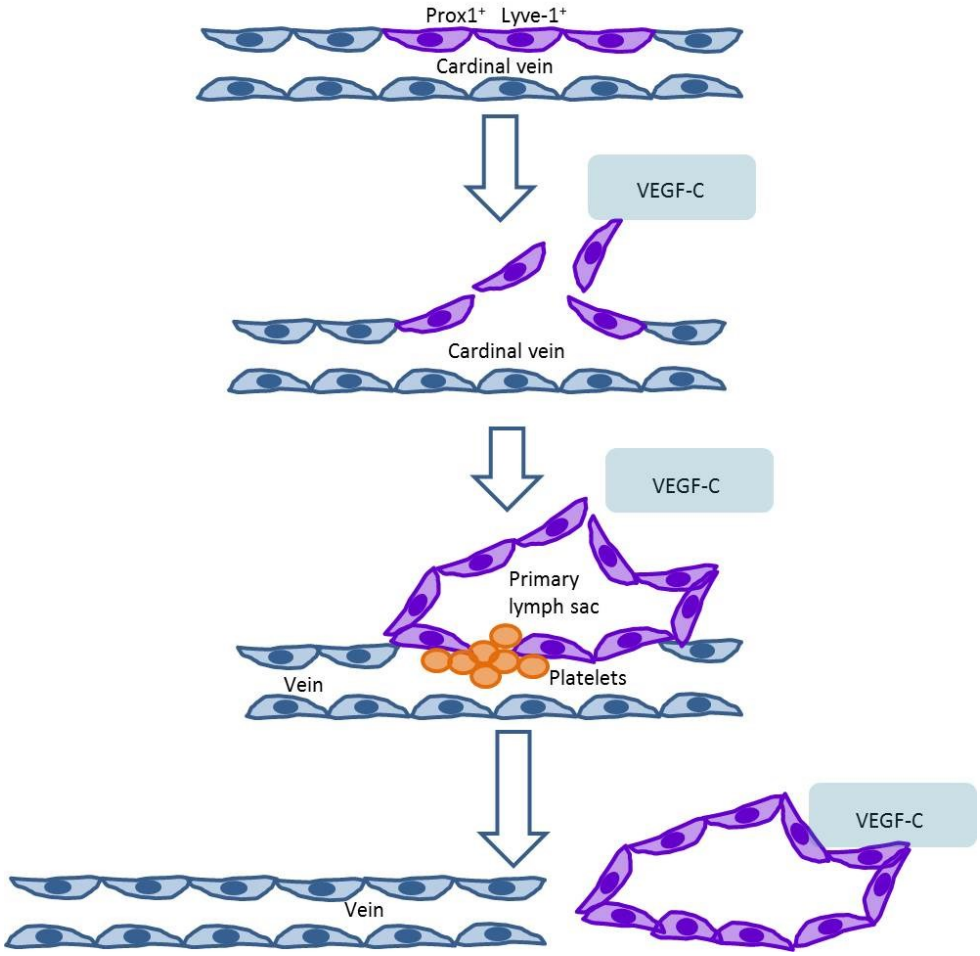


Figure 1.1: Embryonic lymphatic development. At around embryonic day (E) 9.5 in mice and week 6-7 in humans, a subpopulation of cells in the cardinal vein begin expressing the transcription factor Prox1, which interacts with the nuclear receptor COUP-TFII to initiate differentiation into LEC. The new LEC migrate away from the cardinal vein in a process that is dependent on VEGF-C, which is produced by the surrounding mesenchyme. These migrating cells form the primary lymph sacs and current knowledge suggests that platelet aggregates form at the connection between the lymph sacs and cardinal vein to separate the two vascular systems. Once the lymphatic sacs are separated from the blood vascular system they undergo further sprouting and remodelling to form the full lymphatic vascular network. Redrawn from Tammela and Alitalo, 2010.

1.2.4. Lymphangiogenesis in adults

In addition to occurring in embryonic development, lymphangiogenesis can arise in adults in response to inflammation, during wound healing and in tumours. During acute inflammation, such as in bacterial infections, proinflammatory cytokines and lipopolysaccharide promote expression of VEGF-C, while other lymphangiogenic factors, such as VEGF-D, are secreted by granulocytes and macrophages (Baluk et al., 2005; Kang et al., 2009). Lymphangiogenesis in response to acute inflammation can be beneficial: it promotes the mobilisation of macrophages and dendritic cells and allows resolution of tissue oedema (Baluk et al., 2005; Kataru et al., 2009; Huggenberger et al., 2011). Lymphatic growth can first be detected seven days after bacterial infection and a full network can be seen around two weeks after infection; lymphatic vessels have also been shown to be more abundant than blood vessels in this model (Baluk et al., 2005). Lymphatic hyperplasia has been found in a number of different inflammatory conditions, including psoriasis, ulcerative colitis and in murine models of inflammatory arthritis (Kunstfeld et al., 2004; Kaiserling et al., 2003; Zhang et al., 2007). There appears to be an important role for VEGF-A in inflammation-associated lymphatic development: in ultraviolet B-irradiated mouse skin, VEGF-A was associated with growth of hyperpermeable lymphatic vessels and it can be detected at elevated levels in psoriatic skin samples (Kajiya et al., 2006; Detmar et al., 1994). In some inflammatory conditions, VEGF-C mediated lymphangiogenesis seems to be beneficial. VEGF-C induced lymphangiogenesis in mice with inflammatory arthritis (Zhang et al., 2007) and this was associated with resolving inflammation through increased lymphatic drainage (Proulx et al., 2007; Polzer et al., 2008).

Metastasis to regional lymph nodes is an important stage in tumour dissemination and an indicator of disease progression. Studies utilising animal models of cancers have discovered that tumours are able to induce lymphangiogenesis by secreting VEGF-C (Mandriota et al., 2001; Skobe et al., 2001; Hirakawa et al., 2007) or VEGF-D (Stacker et al., 2001). This promotes spreading of the tumour to the draining lymph nodes. In addition, tumours are believed to secrete factors that enhance lymphatic expansion in the draining lymph nodes, which further encourages metastasis (Hirakawa et al., 2005, 2007; Harrell et al., 2007). Once metastatic cancer cells have entered the draining lymph node, they continue to promote lymphatic expansion, resulting in further metastasis to distant lymph nodes (Cueni and Detmar, 2008).

Lymphangiogenesis can also be observed during wound healing, particularly in the skin. Optimal healing in skin wounds is associated with the occurrence of both angiogenesis and lymphangiogenesis at the edge of the wound (Paavonen et al., 2000; Saaristo et al., 2004). Lymphatic vessels form in wounds after blood vessels have formed and there is also evidence of sprouting from existing lymphatic vessels at the edge of the wound (Paavonen et al., 2000). However, there are fewer of the lymphatic vessels than blood vessels in the wounds and the lymphatic vessels regress earlier (Paavonen et al., 2000).

The work within this thesis centres on the control of lymphatic endothelial cell migration and tube formation as parts of the lymphangiogenic process. Therefore, the next section will

outline the differences between lymphatic and vascular endothelial cells and introduce the process of cell migration.

1.3. Endothelial cells

1.3.1. Functions of endothelial cells

Endothelial cells originate in the splanchnopleuric mesoderm as mesenchymal stem cells, which develop into haemangioblasts (reviewed in Sumpio et al., 2002). These haemangioblasts ultimately differentiate into endothelial or haematopoietic cells. In an adult human there are 1-6 x 10^{13} endothelial cells, which cover a surface area of 1-7m^2 (Cines et al., 1998). Historically, it was believed that the only purpose of the endothelium was as a physical barrier between the blood and tissue. However, it is now accepted that the endothelium is a heterogeneous organ and has multiple functions, including roles in inflammatory and immune responses.

One of the main functions of endothelial cells is to regulate vascular permeability. There is variation in permeability between different parts of the vasculature, with the specialised endothelium of the blood-brain barrier being highly impermeable to most molecules, while the lymphatic capillaries are significantly more permeable. The differing permeabilities are partly regulated by distinct types of cell junctions: the blood-brain barrier has tight junctions between cells to prevent non-selective uptake of molecules into the brain (Rubin and Staddon, 1999), but the lymphatic capillaries have button-like junctions that allow the

movement of fluid and leukocytes (Baluk et al., 2007; Pfeiffer et al., 2008; Dejana et al., 2009).

Endothelial cells also have an important role in maintaining blood flow. This is achieved by releasing a combination of vasodilators, such as nitric oxide (NO) and prostacyclin (PGI_2), and vasoconstrictors, including endothelin and platelet-activating factor (PAF; Durand and Gutterman, 2013). Endothelial cells constitutively secrete NO, although its production can be altered in response to chemical or physical stimuli. NO tends to keep smooth muscle cells in a relaxed state and prevents platelets adhering to the endothelium (Mendelsohn et al., 1990; Loscalzo and Welch, 1995; Durand and Gutterman, 2013). In contrast, PGI_2 has no effect on platelet adhesion, but prevents platelet activation by working synergistically with NO (Radomski et al., 1987; Stamler et al., 1989; Korbut et al., 1990; Mollace et al., 1991). When the endothelium is injured it becomes a prothrombotic surface. Endothelial cells begin to express tissue factor, which accelerates the activation of clotting factors VII, X and IX (Drake et al., 1989; Chu, 2011), and platelets come into contact with the subendothelial matrix, especially collagen, which results in their activation. Circulating platelets are able to interact with these adhered activated cells, creating a haemostatic plug that will develop into a stable fibrin clot (reviewed by Cines et al., 1998).

Endothelial cells have been shown to be necessary for the adaptive immune response, where they are involved in the presentation of antigens to T cells and the recruitment of inflammatory cells (Pober et al., 1996; Butcher and Picker, 1996). More recent experiments

have shown that endothelial toll-like receptor signalling alone can induce an immune response in mice (Andonegui et al., 2009). During inflammation, adhesion receptors are upregulated by cytokines including interferon gamma (IFNγ), interleukin (IL)-1β and tumour necrosis factor alpha (TNFα; Springer, 1995). This facilitates recruitment of circulating lymphocytes, which receive further signals that promote firm adhesion and, ultimately, transmigration (Ahmed et al., 2011). Similarly, in the innate immune response neutrophils initially adhere to the endothelium before transmigrating into inflamed tissue (Tull et al., 2009). Leukocyte rolling and adhesion have also been found to be increased in mice lacking adiponectin, which was associated with increased expression of E-selection and VCAM-1 on vascular endothelial cells (Ouedraogo et al., 2007). Finally, endothelial cells have a role in the formation of new blood and lymphatic vessels, as described previously (see section 1.2.3).

1.3.2. Differences between vascular and lymphatic endothelial cells

There are a number of differences between blood and lymphatic vessels and the endothelial cells that form these vessels. LEC express a number of specific markers that are not present in vascular endothelial cells, although some of these markers can be detected on other cell types. Such markers include Prox1, a transcription factor that is essential for the differentiation of cardinal vein endothelial cells into LEC, the hyaluronan receptor LYVE-1, VEGFR3 and podoplanin (Wigle et al., 2002; Banerji et al., 1999; Kaipainen et al., 1995; Breiteneder-Geleff et al., 1999). The structure of blood and lymphatic capillaries is also different. LEC have little or no basement membrane and are not supported by pericytes or

smooth muscle cells. Instead, LEC are attached to the surrounding ECM by anchoring filaments and these filaments are also necessary to stabilise the lymphatic capillary (Liersch and Detmar, 2007). The lack of supporting cells, combined with loose intercellular junctions, ensures that the lymphatic capillaries are much more permeable than blood capillaries (Tammela and Alitalo, 2010), which have tight intercellular junctions and are surrounded by pericytes and smooth muscle cells.

1.3.3. Cell migration

Cell migration is an essential part of both angiogenesis and lymphangiogenesis. Migration of endothelial cells is dependent on actin, a cytoskeletal component. Actin is composed of globular subunits of 43 kDa (G-actin) that are converted into helical filaments, known as F-actin. The process of converting G-actin to F-actin requires the hydrolysis of ATP, which is bound to G-actin (Disanza et al., 2005). Actin-dependent endothelial cell migration comprises six key processes: i) sensing of motogenic signals by filopodia; ii) formation of lamellipodia; iii) attachment of the lamellipodia to the ECM; iv) contraction of the cell body by stress fibres; v) detachment of the rear of the cell; and vi) recycling of adhesion and signalling proteins (Lamalice et al., 2007). These processes occur as part of different migratory mechanisms: chemotaxis; haptotaxis; and mechanotaxis (Lamalice et al., 2007). Chemotaxis is the migration of cells towards a soluble chemoattractant gradient. The major chemoattractants involved in chemotaxis are VEGFs, basic fibroblast growth factor (FGFb) and angiopoietins. Haptotaxis is migration towards immobilised ligands, such as ECM

components, while mechanotaxis is migration in responses to mechanical forces, such as shear stress.

As cells begin to migrate, filopodia are extended from the edge of the cell in a process regulated by Rho GTPase family member Cdc42 and Wiskott-Aldrich syndrome protein (WASP). Filopodia are thin structures that contain parallel bundles of F-actin (Mattila and Lappalainen, 2008). The filopodia are able to sense promigratory stimuli, via receptors for a range of signalling molecules and ECM components (Mattila and Lappalainen, 2008). The detection of pro-migratory signals triggers the formation of lamellipodia at the leading edge of the cell (Small et al., 2002). As lamellipodia form, actin is polymerised by proteins including the Arp2/3 complex and Rac1, another member of the Rho GTPase family (Small et al., 2002). This is followed by cell attachment to the ECM via focal adhesions (1.3.3.1) and integrins (1.3.3.2), then contraction of stress fibres that propels the cell forwards. Finally, the rear of the cell detaches from the ECM as focal adhesions are disassembled and recycled.

1.3.3.1. Focal adhesions

Focal adhesions are the sites of connection between endothelial cells and the ECM, where the cell membrane is attached to both the actin cytoskeleton and ECM components. Focal adhesions are multiprotein complexes that are known to associate with around 150 different proteins (Zaidel-Bar et al., 2007). Such proteins include cytoskeletal-binding and adapter proteins, kinases, phosphatases and small GTPases (Kuo et al., 2011). Focal adhesions can

be found at the ends of actin bundles, either at the cell periphery or within the cell body (Huttenlocher and Horwitz, 2011). They have been observed in many cell types, including endothelial cells (Kano et al., 1996), and are believed to form once focal adhesion kinase (FAK) has been recruited to the adhesion site. In some cases, the initial sites of cell adhesion are filopodia (Mattila and Lappalainen, 2008). The presence of FAK promotes the recruitment of actin-binding proteins, including paxilin, talin, vinculin and α-actinin, which link the actin cytoskeleton to integrins (Huot et al., 1998; Romer et al., 2006; Mattila and Lappalainen, 2008). α-actinin is particularly important in the formation of focal adhesions (Choi et al., 2008), while vinculin is not essential for focal adhesion development but strengthens the interaction between integrins, talin and actin (Huttenlocher et al., 1996; Galbraith and Sheetz, 1997; Xu et al., 1998). In addition to actin-binding proteins, integrins, syndecans, Src-kinase (a non-receptor tyrosine kinase; Hunter and Sefton, 1980), and integrin-linked kinase (ILK) can also be found within the focal adhesion complex (Turner, 2000; Zamir and Geiger, 2001; Frame, 2004; Mitra et al., 2005; Legate et al., 2006).

Forward movement requires focal adhesions to initially attach to the ECM, which creates the force necessary to pull the cell body forwards (Nagano et al., 2012). Similarly, focal adhesions must undergo disassembly in order for directional migration to progress. Therefore, there is a continuous cycle of formation and turnover of focal adhesions at the leading edge of the cell during migration and disassembly of focal adhesions at the rear of the cell (Webb et al., 2002; Broussard et al., 2008). The assembly of focal adhesions is regulated by Rho GTPases (Vicente-Manzanares et al., 2009; Parsons et al., 2010), while FAK

and Src are involved in adhesion disassembly (Nagano et al., 2012). Fibroblasts derived from FAK-deficient embryos have larger adhesions that do not efficiently turn over, resulting in an inhibition of cell migration (Ilić et al., 1995; Sieg et al., 2000; Hsia et al., 2003; Webb et al., 2004).

1.3.3.2. Integrins

Integrins are one of the components of focal adhesions; their function is to bind cells to matrix proteins or other cells. The integrin family consists of 18 α-subunits and eight β-subunits that combine to form 24 heterodimers (Hynes, 2002). The large extracellular domain of an integrin heterodimer binds to the ECM, while the smaller cytoplasmic domain is connected to the actin cytoskeleton (Hynes, 2002). There is also a transmembrane domain that consists of around 20 amino acids (Banno and Ginsberg, 2008). Different integrins have different, but often overlapping, binding specificities. This specificity is determined by the integrin's extracellular domain and known ligands within the ECM include fibronectin, collagen and laminin. While integrins may have overlapping binding specificities, they appear to have distinct functions. Knockout mice have been created for most integrins and these all display different phenotypes, ranging from developmental defects to haemostatic problems (reviewed by Hynes, 2002).

The majority of integrins are not constitutively active and are expressed on the cell surface in an inactive form. Images obtained using electron microscopy suggest that inactive integrins exist in a bent conformation that changes to an extended conformation upon activation

(Weisel et al., 1992; Du et al., 1993; Takagi et al., 2002; Nishida et al., 2006; Zhu et al., 2007a). However, there is also evidence to suggest that the bent form can also bind to ligands (Xiong et al., 2001; Adair et al., 2005), which has led to the hypothesis that some integrins may be able to signal in their bent conformation (Xiong et al., 2003; Adair et al., 2005). Integrin activation and signalling can take two forms: inside-out or outside-in. Inside-out signalling involves the binding of ligands to other receptors on the cell surface, which generates signals leading to activation of integrins by separating their cytoplasmic tails (Abram and Lowell, 2009). This separation causes the integrins to unfold their extracellular domain (Luo et al., 2007), which increases their ligand-binding capacity and promotes clustering (Abram and Lowell, 2009). Inside-out signalling has been shown to be important in platelet activation (Caswell et al., 2009). Outside-in signalling is induced by integrin-ligand binding, which causes the cytoplasmic domains to separate and triggers a downstream signalling cascade by allowing cytoskeletal components and signalling molecules to interact with the cytoplasmic domains(Zhu et al., 2007b). In endothelial cells, outside-in signalling is thought to reinforce the connections between focal adhesions and the actin cytoskeleton (Yuan and Rigor, 2010).

As with focal adhesions, cell migration requires the assembly and disassembly of integrin-ligand interactions. Migration speed varies depending on which integrin is involved, for example $\alpha_5\beta_1$ will more readily turn over when in contact with fibronectin than $\alpha_v\beta_3$ will (Truong and Danen, 2009; Huveneers and Danen, 2009). Integrins are internalised from the plasma membrane via several different types of endomytosis (Lobert et al., 2010). These integrins can then be recycled in either a Rab11 or Rab4-dependent process (Caswell et al.,

2009). Ligand density and affinity, receptor concentration and cytoskeletal associations will also affect migration speed (Huttenlocher et al., 1996; Palecek et al., 1997). Release of the rear of a migrating cell is thought to be caused by contractile forces breaking the connection between integrins and the ECM or cytoskeleton (Huttenlocher and Horwitz, 2011). In leukocytes, active downregulation of integrin adhesion also appears to be necessary. In neutrophils, signalling via β_2 is thought to regulate β_3 integrin's attachment to and detachment from vitronectin (Hendey et al., 1996). Neutrophil-expressed β2 integrin is also thought to have roles in neutrophil apoptosis (Mayadas and Cullere, 2005). Activation of RhoA and its downstream effector, Rho-kinase (ROCK), has been shown to be involved in both integrin-dependent attachment of leukocytes to a surface and the detachment of the rear of these cells (Alblas et al., 2001; Liu et al., 2002). Similarly, RhoB and RhoC are involved in leukocyte adhesion; this is thought to be htrough activation of lymphocyte function-associtaed antigen 1 (Giagulli et al., 2004). Data published by Alblas et al. showed that detachment of leukocytes was through RhoA-induced contraction of actomyosin filaments and that ROCK signalling contributed to attachment via $\alpha_4\beta_1$ and β_2 integrins (Alblas et al., 2001). This was confirmed by Liu and colleagues, who also proposed that activation of ROCK and actomyosin assembly reduces integrin avidity, thereby promoting cell detachment (Liu et al., 2002).

1.3.3.3. RhoA signalling

The Rho GTPases are part of the Ras superfamily of GTP-binding proteins. There are many of these small proteins (Figure 1.2), but of particular interest to this project are: the Rho-

related subfamily, which consists of RhoA, RhoB and RhoC (Wennerberg and Der, 2004); Cdc42; Rac1. In particular, signalling downstream of the cytosolic RhoA is important in cell migration (Nobes and Hall, 1999) and is described further below, along with the roles of Cdc42 and Rac1 in cell migration.

Rho GTPases alternate between an inactive guanosine diphosphate (GDP)-bound and an active guanosine triphosphate (GTP)-bound form; the GTP-bound state has an increased affinity for downstream effector molecules. Activation of Rho GTPases is promoted by guanosine nucleotide exchange factors (GEFs) that catalyse the exchange of GDP for GTP (Cherfils and Chardin, 1999). Conversely, GTPase activating proteins (GAPs) and guanosine nucleotide dissociation inhibitors (GDIs) inhibit Rho GTPase activation. GDIs prevent GDP being exchanged for GTP by sequestering Rho-GDP in the cytoplasm (Olofsson, 1999). GAPs promote the Rho GTPase's intrinsic GTPase activity, which enhances conversion of Rho-GTP to Rho-GDP (Moon and Zheng, 2003).

Rac1 is involved in the polymerisation of actin and is required in the initial stages of tube formation and lumen development by vascular endothelial cells (Bayless and Davis, 2002; Connolly, 2002; Cascone et al., 2003) as well as in endothelial cell migration (Ridley et al., 2003). In its activated form, Rac1 activates WASP family Verprolin-homologous (WAVE) proteins (Ismail et al., 2009; Lebensohn and Kirschner, 2009), which promotes lamellipodia formation (Ridley et al., 2003). Additionally, activating Rac1 in specific areas of a cell (namely the front and sites of integrin clustering) is essential for directional migration (Ridley

et al., 2003). Rac1 activation can also be detected during the formation of cell networks by LEC, but it is not essential in this process, and the activation of Rac1 appears to be slower than the activation of RhoA (Navarro et al., 2008). Mice lacking Rac1 in endothelial cells have blood-filled lymphatic vessels, which are thought to be caused by an impairment in the differentiation of cardinal vein endothelial cells into LEC, combined with aberrant motility in the LEC that do properly differentiate (D'Amico et al., 2009). Rac1-deficient endothelial cells also display reduced migration and tube formation in the presence of VEGF or sphingosine-1-phosphate (S1P; Tan et al., 2008).

Cdc42 is important in the early stages of cell migration, where it regulates the extension of filopodia by the migrating cell. The precise mechanism by which Cdc42 induces filopodia formation is still unknown, although it has been suggested that this could involve actin polymerisation by mDia2 (Ladwein and Rottner, 2008). Lack of active Cdc42 prevents wound healing as the cells are unable to polarise (Nobes and Hall, 1999). This result was confirmed in a later study that also found that activation of Cdc42 reduces the migration of individual cells, as determined by using Boyden chamber assays (Valtcheva et al., 2013). Cdc42 has previously been shown to be involved in lymphangiogenesis, with increased Cdc42 activity promoting the conversion of actin stress fibres to cortical F-actin (Valtcheva et al., 2013), a concentrated region of actin filaments that interacts with membrane-bound proteins and intracellular signalling molecules. There is also evidence of cross-talk between Cdc42 and RhoA. Active Cdc42 is thought to inhibit RhoA in vascular and lymphatic endothelial cells (Ispanovic et al., 2008; Navarro et al., 2011; Valtcheva et al., 2013). If Cdc42 is constitutively

activated it inhibits RhoA, which leads to inadequate cell polarisation and an associated decrease in migration (Navarro et al., 2011).

Rho GTPases are thought to be involved in cell migration through their regulation of actin polymerisation, stress fibre formation and focal adhesion assembly (van Nieuw Amerongen and van Hinsbergh, 2001; Aepfelbacher et al., 1997). In particular, regulation of RhoA is thought to be important as either inhibiting or activating RhoA can reduce cell migration (Arthur et al., 2000; Arthur and Burridge, 2001; Nobes and Hall, 1999; Ridley et al., 1995; Takaishi et al., 1994). ROCK is a widely-studied downstream effector protein of RhoA. Activation of RhoA-ROCK signalling leads to stress fibre formation (Amano et al., 1996, 1997). After activation by RhoA, ROCK simultaneously phosphorylates the regulatory light chain of myosin II and inhibits myosin phosphatase (Huttenlocher and Horwitz, 2011). These events promote actin bundling and myosin-mediated cell contraction, which is necessary for movement of the cell body and detachment of the trailing edge of the cell (Huttenlocher and Horwitz, 2011).

Having considered the role of Rho GTPase signalling in endothelial cell migration, another important signalling pathway will be introduced: the vascular endothelial growth factor pathway. The main downstream effector proteins will be considered, along with the evidence for the importance of VEGF signalling in cell migration and lymphangiogenesis.

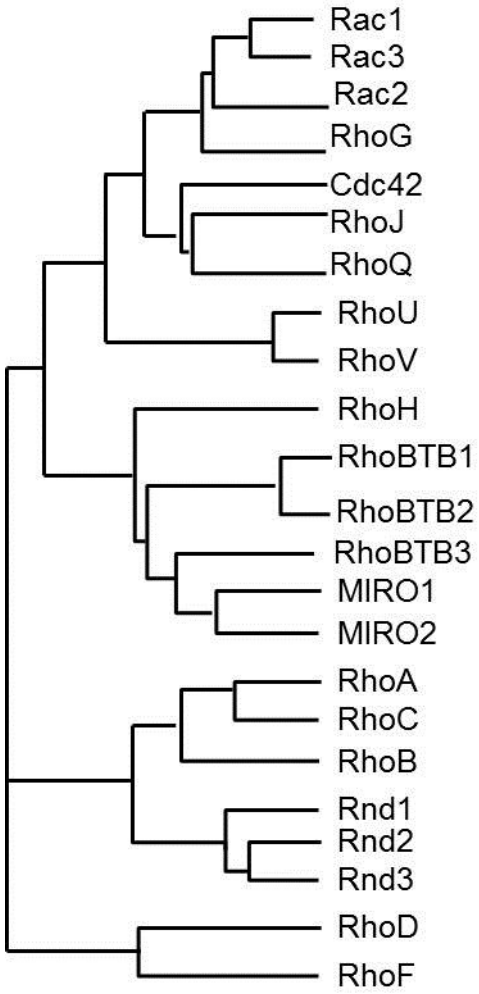


Figure 1.2: Phylogenetic tree of mammalian Rho GTPases. The Rho GTPases can be subdivided into six families. The classical subfamilies are the RhoA, Rac1 and Cdc42 groups. Filopodia extension is regulated by Cdc42, while Rac1 is involved in actin polymerisation. RhoA is involved in stress fibre formation, which is mediated by the downstream effector protein ROCK. Adapted from Tybulewicz and Henderson, 2009.

1.4. Vascular endothelial growth factors

The mammalian VEGF family consists of five glycoproteins (VEGF-A, VEGF-B, VEGF-C, VEGF-D and placental growth factor [PlGF]) that are able to bind to one of three receptors (VEGFR1, VEGFR2 and VEGFR3). There are also VEGF homologues produced by Orf viruses (collectively known as VEGF-E) and a homologue in snake venom (VEGF-F; Lohela et al., 2009). VEGFs have been shown *in vitro* to have roles in cell migration and proliferation (Mäkinen et al., 2001b; Pepper et al., 1992). Different members of the VEGF family are essential for angiogenesis and lymphangiogenesis (Leung et al., 1989; Cao et al., 1998), which will be discussed later (1.4.3).

1.4.1. Expression of vascular endothelial growth factor receptors by endothelial cells

The VEGFs bind to the VEGFRs with overlapping specificities (Figure 1.3). VEGF-A binds to VEGFR1 and VEGFR2, while VEGF-B and PlGF only bind to VEGFR1. VEGF-E and VEGF-F specifically bind VEGFR2. Although VEGF-A has a greater affinity for VEGFR1 than VEGFR2, most of this factor's signalling is via VEGFR2 (Shibuya, 2013). This is due to VEGFR1 having weaker kinase activity than VEGFR2 (Shibuya, 2013). VEGF-C is able to bind to both VEGFR2 and VEGFR3, but has a higher affinity for VEGFR3 (Mäkinen et al., 2001b). Murine VEGF-D is specific for VEGFR3 (Baldwin et al., 2001), but human VEGF-D can also bind to VEGFR2 after it has been processed (Achen et al., 1998). VEGFR1 and VEGFR2 can both be found on LEC, but only VEGFR2 is thought to be important in VEGF-C or VEGF-A mediated promotion of migration and proliferation (Kriehuber et al., 2001; Dellinger and Brekken, 2011). Similarly, VEGFR2 is thought to be more important in VEGF-A mediated migration of vascular

endothelial cells than VEGFR1 (Shibuya, 2013). While VEGFR3 is often used as a lymphatic marker, it is known to expressed by blood vessel endothelial cells, but at a much lower level than LEC (Kriehuber et al., 2001).

When ligands bind to VEGFRs, the receptors dimerise. Both VEGFR2 and VEGFR3 can readily form homodimers and these receptors have also been shown to form R2/R3 heterodimers (Dixelius et al., 2003; Nilsson et al., 2010). There is also evidence of R1/R2 heterodimers (Sato et al., 2000; Cudmore et al., 2012). Treatment with VEGF-C increases the number of VEGFR2/R3 heterodimers in cultured LEC, but is not able to promote the formation of VEGFR2 homodimers (Nilsson et al., 2010).

There are also a number of co-receptors that are important for VEGFR signalling, including heparan sulphate proteoglycans and neuropilins (Esko and Selleck, 2002; Geretti et al., 2008). While neuropilins are known to be required for correct signalling downstream of VEGFRs, they do not have any signalling motif in their cytoplasmic domain (Geretti et al., 2008) and are thought to promote the VEGFRs' tyrosine kinase signalling and enhance binding of VEGFs to their receptors (Lohela et al., 2003; Soker et al., 1998). In particular, neuropilin-2 is thought to be required for correct signalling downstream of VEGFR3 as mice deficient in this receptor either lack or have reduced numbers of lymphatic vessels (Yuan et al., 2002). The interaction between VEGFR3 and neuropilin-2 is not ligand-dependent and there does not appear to be an interaction between neuropilin-2 and VEGFR2 (Kärpänen et al., 2006).

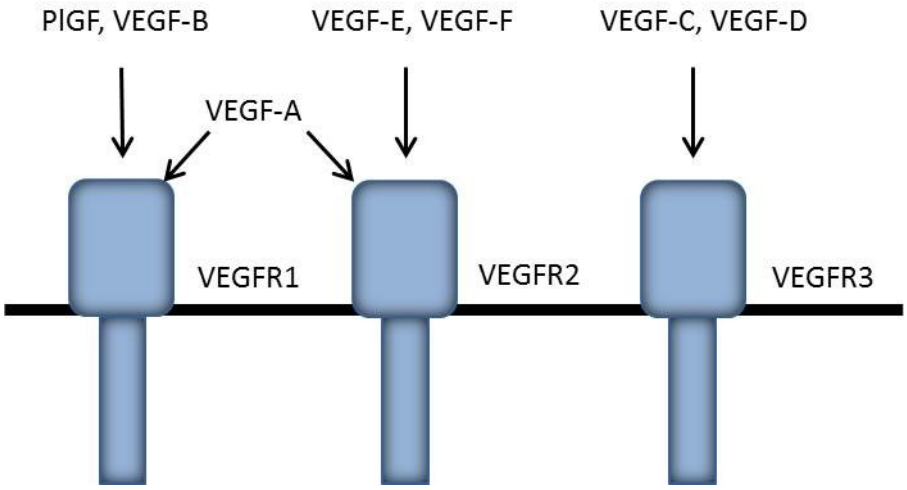


Figure 1.3: Specificity of vascular endothelial growth factors for their receptors. There are five members of the VEGF family and three receptors for these growth factors. Placental growth factor (PlGF) and VEGF-B both selectively bind to VEGFR1, while VEGF-E and VEGF-F are specific for VEGFR2. VEGF-A, which is important in angiogenesis, can bind to VEGFR1 and VEGFR2. The two VEGF family members associated with lymphangiogenesis in humans, VEGF-C and VEGF-D, bind preferentially to VEGFR3, but are able to bind to VEGFR2 in their fully processed forms. While mouse VEGF-C can also bind to both receptors, murine VEGF-D is specific for VEGFR3.

1.4.2. Signalling downstream of VEGFs

Vascular endothelial growth factors can exist as monomers, but only trigger receptor phosphorylation in their dimeric form (Toivanen et al., 2009). The balance between the monomeric and dimeric forms of VEGF-C and VEGF-D can be shifted towards the dimeric by replacing an unpaired cysteine (Cys137 in VEGF-C and Cys117 in VEGF-D) with an alanine (Toivanen et al., 2009; Leppänen et al., 2010). This stabilises the dimeric form of the growth factors, which leads to an increase in their biological activity (Toivanen et al., 2009; Leppänen et al., 2010). Although VEGFs primarily form homodimers, heterodimers consisting of VEGF-A and PlGF have been detected (Cao et al., 1996; Xu et al., 2006; Schomber et al., 2007). These VEGF-A:PlGF heterodimers are thought to be functionally inactive (Eriksson et al., 2002).

VEGFRs dimerise upon ligand binding, which leads to activation of the tyrosine kinase in the intracellular domain of the receptor and autophosphorylation (Shibuya and Claesson-Welsh, 2006). The phosphorylation of tyrosine residues creates binding sites for the Src homology 2 (SH2) or phosphotyrosine binding (PTB) domains of other signalling molecules (Fournier et al., 1999; Ratcliffe et al., 2002; Claesson-Welsh, 2003). Activation of VEGFRs triggers downstream signalling cascades that can influence migration, proliferation and cell survival (Shibuya and Claesson-Welsh, 2006). At present, it is unclear whether VEGFR homodimers trigger different signalling cascades to heterodimers.

Phosphorylation of tyrosine 1175 (Tyr1175) in VEGFR2 creates a binding site for phospholipase C-γ2 (PLCγ2), which results in increased proliferation via signalling from mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinase 1/2 (ERK1/2; (Takahashi et al., 2001; Meyer et al., 2003). This tyrosine residue is essential for normal VEGFR2 signalling and exchanging it for a phenylalanine is lethal, as is removing VEGFR2 completely (Sakurai et al., 2005) Two other tyrosine residues that are important in endothelial cell migration are Tyr951 and Tyr1214 (Zeng et al., 2001; Lamalice et al., 2004). The interaction between phosphorylated Tyr951 and T cell-specific adaptor (TSAd) is thought to be involved in the formation of stress fibres in response to VEGF-A, which leads to increased migration (Matsumoto et al., 2005). Tyr1214 has also been implicated in actin remodelling in response to VEGF-A, through activation of Cdc42 and MAPK (Lamalice et al., 2004).

Phosphorylation of VEGFR3 results in activation of the MAPK pathway via protein kinase C (PKC) and phosphoinositide 3-kinase (PI3K)-dependent activation of Akt (Joukov et al., 1998; Mäkinen et al., 2001b; Deng et al., 2015). Activation of this pathway promotes both survival and migration of the target cell (Roy et al., 2006). Activation of Akt is primarily by VEGFR2/3 heterodimers, while VEGFR3 homodimers activate ERK1/2 (Deng et al., 2015); this study also found that both the ERK and Akt pathways are important in LEC migration. There is also evidence suggesting that PLCγ, Src-homology phosphatase 2 (SHP2) and signal transducer and activator of transcription-3 and -5 (STAT3/5) are involved in signalling downstream of VEGFR3 (Korpelainen et al., 1999; Wang et al., 2004). Activated VEGFR3 has been shown to

interact with the SH2 domain of PLCγ and SHP2, which triggers a signalling cascade that results in cell migration and proliferation (Wang et al., 2004).

Negative regulation of VEGFRs is also required to limit the responses of the cell. This reduction in kinase activity can be through dephosphorylation by phosphatases, receptor internalisation or lysosome degradation (Olsson et al., 2006). VEGFR2 is known to be regulated by the phosphatases SHP1 and SHP2 (Guo, 2000; Gallicchio et al., 2005) and can be degraded following internalisation, in a process dependent on PKC (Singh et al., 2005). Internalisation of VEGFR3 requires ephrin-B2, and this transmembrane protein has also been shown to be important in VEGFR2 internalisation (Wang et al., 2010; Sawamiphak et al., 2010). Vascular endothelial phosphotyrosine phosphatase regulates VEGFR3 signalling and inhibition of this phosphatase increases VEGF-C induced ERK and Akt signalling (Deng et al., 2015). VEGFR3 signalling can also be reduced by the protease A disintegrin and metalloprotease with thrombospondin motifs 1 (ADAMTS1), which binds directly to VEGF-C, thereby preventing VEGF-C binding to VEGFR3 (Inagaki et al., 2014).

1.4.3. Role of VEGF-C and VEGFR3 in lymphangiogenesis

VEGF-C is expressed by the mesenchyme surrounding the cardinal vein during embryonic lymphangiogenesis (Kukk et al., 1996; Karkkainen et al., 2004) and lack of this growth factor results in a lack of lymphatic vessels, leading to embryonic lethality (Karkkainen et al., 2004). The VEGF-C heterozygous mouse also has lymphatic defects: lymphatic vessels do not form in

the skin and their development in other parts of the body is delayed (Karkkainen et al., 2004). A similar phenotype can be observed in mice that have a mutation inactivating VEGFR3 (Karkkainen et al., 2001). Interestingly, deletion of VEGF-D does not create a lymphatic defect, but administration of VEGF-D to VEGF-C deficient mice can increase lymphatic sprouting (Karkkainen et al., 2004). Conversely, overexpression of VEGF-C can increase lymphatic growth in adult tissues and is also thought to promote lymphatic function (Enholm et al., 2001; Saaristo et al., 2002b, 2004), although there may also be detrimental effects on the blood vessels (Saaristo et al., 2002a).

VEGF-C has multiple splice variants and its affinity for VEGFR3 increases with each processing step. Only mature, fully processed VEGF-C is able to bind to VEGFR2 (Joukov et al., 1997). The 29/31 kDa form of VEGF-C has little signalling activity, but is cleaved by the protease ADAMTS3, which creates the active 21/23 kDa VEGF-C (Jeltsch et al., 2014). This processing is promoted by collagen and calcium-binding domain protein 1 (CCBE1) and knocking out this protein gives a similar phenotype to the VEGF-C knockout (Jeltsch et al., 2014; Le Guen et al., 2014).

Deletion of VEGFR3 in mice results in embryonic lethality and defects in blood and lymphatic vessels (Dumont et al., 1998). Soluble VEGFR3 or VEGFR3-blocking antibodies prevent the maturation of lymphatic capillaries during embryonic development and the early postnatal period, but have no effect on established vessels (Mäkinen et al., 2001a; Karpanen et al., 2006). However, administration of VEGFR2- or VEGFR3-blocking antibodies to adult mice

prevents lymphangiogenesis in areas of wound healing (Goldman et al., 2007). Both of the blocking antibodies prevent LEC migration when administered from day 10 to day 60 post-injury, leading to a lack of functional vessels (Goldman et al., 2007). However, vessels are able to develop if the antibodies are only administered from day 17 onwards (Goldman et al., 2007).

Now that the two major signalling pathways in lymphatic endothelial cells, VEGF-C and RhoA signalling, have been considered, the role of platelets in the development of the lymphatic system will be introduced. The following section will describe the process of platelet formation, the physiological functions of platelets and the signalling pathway thought to be important in lymphatic development.

1.5. Platelets in lymphatic development

Platelets are small anuclear cells that range from 1-3 μm in diameter. There are 1.5-4x10^8/ml blood in a healthy adult. Circulating platelets last for 7-10 days in humans and 4-5 days in rodents before being degraded. Platelets have long been known to be important in haemostasis and thrombosis, and are now known to be involved in inflammation, immune responses, vascular integrity, angiogenesis, and lymphangiogenesis, as outlined below (1.5.2).

1.5.1. Platelet development

Platelets form from megakaryocytes (MK), in a process that takes around five days in humans and 2-3 days in rodents. With a diameter of 50-100µm, MK are both the largest and least expressed of all bone marrow cells, accounting for only 0.01% of cells (Nakeff and Maat, 1974). MK develop from haematopoietic stem cells, which are primarily found in the bone marrow but can be detected in other tissues, including the spleen (Morita et al., 2011). The formation of MK is thought to be regulated by thrombopoietin and its receptor, c-Mpl (Bartley et al., 1994; Kaushansky et al., 1994), although mice lacking either of these factors are still able to produce platelets (Choi et al., 1995; Ito et al., 1996). The newly-formed MK undergo cycles of endomitosis, which results in them becoming polyploidal. During this maturation the cells also increase in size, develop platelet-specific granules and increase the amount of cytoskeletal proteins in the cytoplasm (Machlus and Italiano, 2013).

Once fully matured, MK begin to extend long processes known as proplatelets. Each MK can produce 10-20 proplatelets, which gradually elongate and branch. The formation of proplatelets is dependent on reorganisation of β_1-tubulin and mice lacking this have a platelet count that is approximately 40% of normal (Lecine et al., 2000; Schwer et al., 2001). The platelets that are produced by the β_1-tubulin knockout mouse have both structural and functional defects. Mutations in human β_1-tubulin have also been detected and result in macrothrombocytopoenia (Kunishima et al., 2009). As the proplatelets are extending, organelles and granules are transported from the body of the MK into the proplatelets. These granules are able to move bidirectionally within the proplatelet until they become

trapped at the tip, where they will become part of a platelet (Richardson et al., 2005). Each MK is then believed to release thousands of platelets into the circulation (Patel et al., 2005).

In order for platelets to be released into the blood, polyploidal MK localise to the bone marrow sinusoidal endothelial cells and extend their proplatelets through the endothelial cells, into the vessel. MK are able to detect the vascular niche, an area that expresses fibrinogen and collagen IV and promotes the final stages of MK maturation (Avecilla et al., 2004). Of particular importance is fibrinogen, which binds to integrin $\alpha_{IIb}\beta_3$ on MK and promotes the extension of proplatelets (Larson and Watson, 2006). Similarly, S1P and its receptor, S1PR1, are known to mediate proplatelet extension. The proplatelets in the blood vessels are exposed to high concentrations of S1P, which promotes the release of platelets from their tips (Zhang et al., 2012). Finally, shear stress induced by blood flow encourages both the extension of proplatelets and the release of proplatelet fragments from the body of the MK (Junt et al., 2007; Dunois-Lardé et al., 2009).

1.5.2. Functions of platelets

The role of platelets in haemostasis is well-characterised. When the blood vessel is injured, components of the subendothelial matrix, including collagen, become exposed. In low shear conditions, such as in the veins, integrin $\alpha_2\beta_1$ can bind to the exposed collagen. However, under high shear it is von Willebrand factor (vWF) that binds to the exposed subendothelial collagen and then binds to glycoprotein (GP) Ib-IX-V (Modderman et al., 1992). These

interactions quickly dissociate, so are not able to mediate firm adhesion. The initial interactions between vWF and GP-Ib-IX-V allow GPVI to bind to collagen. This activates GPVI and promotes the conversion of $\alpha_{IIb}\beta_3$ and $\alpha_2\beta_1$ to their active conformations; this enables the integrins to bind to vWF and collagen, respectively (Ruggeri et al., 1983; Saelman et al., 1994). The combination of these events leads to stable platelet adhesion. Interestingly, $\alpha_2\beta_1$ does not trigger tyrosine kinase signalling (Gibbins, 2004), which is essential for platelet activation and it was this knowledge that led to the discovery of GPVI. It is now believed that $\alpha_2\beta_1$ stabilises the adherent platelets and that signalling via GPVI causes platelet activation (Moroi et al., 2000; Siljander et al., 2004). Once the platelets have been activated, they undergo spreading by extending filopodia and lamellipodia over the exposed matrix. Following this spreading, actin stress fibres form to further stabilise the adherent cells (Calaminus et al., 2007). Platelets then secrete the contents of their α-granules and dense granules. Secreted factors include adenosine diphosphate (ADP), thromboxane A_2, vWF and fibrinogen (Ren et al., 2008; Li et al., 2010), which all act to promote aggregation, where more platelets are recruited from the circulation. These cells become activated by thromboxane A_2 and ADP and adhere to deposited platelets through the binding of $\alpha_{IIb}\beta_3$ to fibrinogen and vWF, forming a "sandwich" (Offermanns, 2006). The activated platelets expose phosphatidyl serine on their surface, which promotes immobilisation of clotting factors and the generation of thrombin (Offermanns, 2006). This, in turn, activates more circulating platelets and facilitates the conversion of fibrinogen to fibrin. This stabilises the growing thrombus and ultimately occludes the original injury.

Like many other cell types, platelets are able to release microvesicles upon activation. These membrane vesicles are 0.1-1μm in size and contain many platelet membrane proteins. Platelet microvesicles express a number of platelet markers, including integrins α_{IIb} and β_3, and GPIb (Abrams et al., 1990; Scharf et al., 1992). They are thought to have a role in thrombosis and the development of some autoimmune diseases (Sellam et al., 2009; Bal et al., 2010; Boilard et al., 2010). There is also evidence to suggest that platelet-derived microvesicles induce angiogenesis and regulate inflammation (Kim et al., 2004; Cloutier et al., 2013).

Activated platelets may promote inflammation by binding circulating white blood cells in the bloodstream, or when they have been deposited at the vessel wall (Smyth et al., 2009). For instance, surface-deposited platelets captured flowing neutrophils and supported unstable rolling adhesions, and after further activation with ADP or thrombin the platelets caused neutrophils to display stable integrin-mediated binding (Stone and Nash, 1999). The stable adhesion of neutrophils was via signalling downstream of CXC chemokine receptor 2 (CXCR2). There is also evidence for platelet involvement in atherosclerosis. Blocking platelet GPIb or GPIIb/IIIa reduced atherosclerosis in the apolippoprotein E (ApoE) knockout mouse model (Massberg et al., 2002), while atherosclerosis was promoted by the infusion of activated platelets into these mice (Huo et al., 2003). Further evidence for platelet involvement in inflammation came with the report that monocytes were preferentially recruited to platelets attached to inflamed endothelium through binding to platelet P-selectin (Kuckleburg et al., 2011). The monocytes were found to be activated by CC-chemokine ligand 2 (CCL2), which

was released by endothelial cells in response to platelet-released CXC chemokine ligand 4 (CXCL4; Kuckleburg et al., 2011). There is also evidence to suggest that the interaction of CD40 with its ligand (CD40L) can promote the recruitment of leukocytes to inflammatory sites through CCL2 signalling (Karmann et al., 1995; Semple et al., 2011). Platelet-expressed CD40L has also been implicated in the progression of atherosclerosis through preventing the recruitment of regulatory T cells to the plaque (Lievens et al., 2010).

Platelets are thought to influence the immune response as they express Toll-like receptors (Shiraki et al., 2004; Andonegui et al., 2005; Aslam et al., 2006), CD40 and CD40L, MHC class I molecules and Fc-gamma receptor 2a (FcγRIIA), a receptor for IgG (Karas et al., 1982). They are also able to secrete serotonin, histamine, IL-1 and soluble CD40L, which are all known to mediate immune responses (Humphrey and Jaques, 1953; Henn et al., 1998; Lindemann et al., 2001). There is evidence for both beneficial and harmful actions of platelets in mediating the immune response. Platelet microvesicles are believed to be involved in the development of rheumatoid arthritis, as they have been detected in the synovial vessels of rheumatoid arthritis patients (Boilard et al., 2010). Similarly, platelets have been shown to become activated in systemic lupus erythematosus and increased numbers of platelet aggregates and microvesicless can be found in blood taken from these patients (Joseph et al., 2001; Sellam et al., 2009). However, platelets may aid the immune response during infections. Platelets have the ability to bind to certain strains of bacteria and aggregates will form around the bacteria, which is thought to lead to a prevention of bacterial growth through the release of mediators from the platelets (Kastrup et al., 2008; Kraemer et al., 2011). Platelets have also been

shown to bind to and contribute to the destruction of red blood cells infected with the malaria parasite, *Plasmodium falciparum* (McMorran et al., 2012).

Platelets have long been known to be involved in maintaining vascular integrity. Experiments in which rabbits were made thrombocytopenic showed evidence of endothelial thinning, which correlates with the observance of petechiae in these animals (Kitchens and Weiss, 1975; Aursnes and Pedersen, 1979). The secretion of VEGF and thrombin from α-granules has been associated with angiogenesis (Smyth et al., 2009). Models of angiogenesis have also determined that adhesion via GPIbα is required for the development of functional blood vessels, but that the GPVI-FcR γ-chain complex is not essential for angiogenesis (Kisucka et al., 2006). This study showed that mice lacking the extracellular domain of GPIbα developed leaky blood vessels in the cornea angiogenesis assay, in which FGFb was implanted into the corneas to stimulate the formation of blood vessels (Kisucka et al., 2006). More recently, platelets have been implicated in lymphangiogenesis (Abtahian et al., 2003; Bertozzi et al., 2010b; Uhrin et al., 2010; Suzuki-Inoue et al., 2010), which will be discussed further in the following sections.

1.5.3. CLEC-2 and its signalling pathway

The C-type lectin receptor CLEC-2 is a type II transmembrane protein that was originally identified as the receptor for rhodocytin, a snake venom toxin. Rhodocytin was found to induce platelet activation via a GPVI-independent mechanism (Navdaev et al., 2001) that

required the activation of Src kinase (Inoue et al., 1999; Suzuki-Inoue et al., 2006). CLEC-2 is highly expressed by platelets and MK (Senis et al., 2007), but has also been detected on liver sinusoidal endothelial cells and Kupffer cells (Chaipan et al., 2006; Tang et al., 2010), mouse, but not human, neutrophils, and macrophages (Kerrigan et al., 2009; Chang et al., 2010). CLEC-2 expression on these cell types is much lower than platelet expression (Chaipan et al., 2006; Kerrigan et al., 2009). mRNA for CLEC-2 has also been detected in monocytes and dendritic cells, but there is no evidence for expression of CLEC-2 protein on these cell types (Colonna et al., 2000).

CLEC-2 has a cytoplasmic domain that consists of 31 amino acids and contains a conserved amino acid sequence (YxxL), known as a hemITAM motif (Figure 1.4). This motif is similar to the immunoreceptor tyrosine-based activation motif (ITAM) motif found in the GPVI/FcRγ complex, which is phosphorylated by the Src family kinases Fyn and Lyn upon crosslinking of the receptor (Watson et al., 2005). Rhodocytin has been shown to induce the phosphorylation of the hemITAM motif in CLEC-2, which creates binding sites for the SH2 domains of Syk (Suzuki-Inoue et al., 2006; Fuller et al., 2007). The tyrosine residue of the hemITAM is essential for CLEC-2 signalling, as are the arginine residues in the SH2 domains of Syk, suggesting that Syk mediates CLEC-2 signalling (Fuller et al., 2007). CLEC-2 can be found on the platelet surface as a monomer or dimer (Hughes et al., 2010b). CLEC-2 activates Syk in its dimeric form, such that the two SH2 domains of each Syk molecule bind to the phosphorylated tyrosines in the two cytoplasmic domains of a CLEC-2 dimer (Watson et al., 2009; Hughes et al., 2010b). Unlike the GPVI/FcRγ ITAM, the CLEC-2 hemITAM is primarily

phosphorylated by Syk, although Src family kinases are essential for signalling further downstream of CLEC-2 (Spalton et al., 2009; Séverin et al., 2011). Downstream of Syk there is phosphorylation of SH2-containing leukocyte protein of 76 kDa (SLP-76) and activation of PLCγ2, both of which have been shown to be essential for rhodocytin-induced platelet activation (Suzuki-Inoue et al., 2006; Fuller et al., 2007). CLEC-2 signalling is known to be inhibited by molecules containing immunoreceptor tyrosine-based inhibitory motifs (ITIMs); one protein known to contain two ITIM motifs is G6b-B (Mori et al., 2008), a splice variant of the immunoglobulin superfamily member G6b (de Vet et al., 2001). There is also evidence to suggest that CLEC-2 signalling is negatively regulated by the protein tyrosine phosphatase Shp2 (Mazharian et al., 2013) and this phosphatase is known to associate with G6b-B via the ITIM motif in G6b-B (Mazharian et al., 2012).

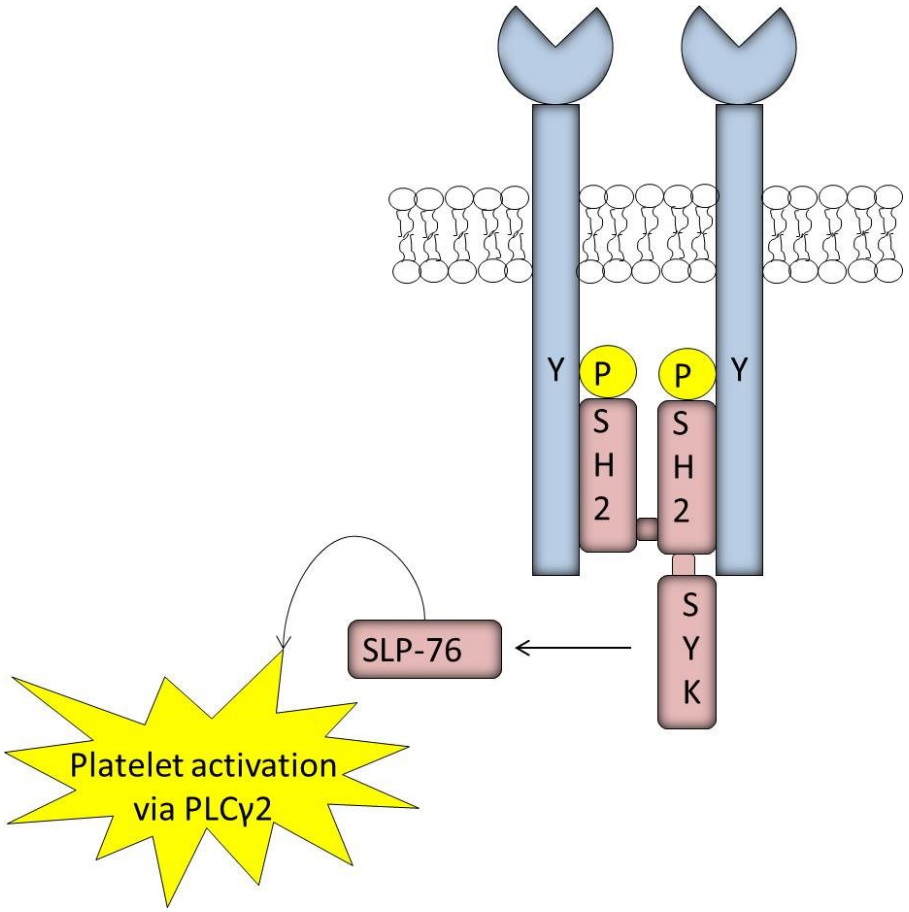


Figure 1.4: Signalling downstream of CLEC-2 in platelets. CLEC-2 exists as a dimer within lipid rafts on the surface of platelets. When ligands, such as rhodocytin or podoplanin, bind to CLEC-2 the conserved tyrosine residue within the cytoplasmic domain is phosphorylated. This creates a binding site for the SH2 domains of Syk, such that each Syk molecule is able to bind to a CLEC-2 dimer. The binding of Syk to CLEC-2 triggers a signalling pathway that involves the activation of SLP-76 and PLCγ2. This pathway leads to platelet activation and secretion.

HemITAM receptors, including CLEC-2, are found in lipid rafts. These rafts contain numerous signalling molecules, including Src family kinases and adaptor proteins (Locke et al., 2002; Wonerow et al., 2002; Pollitt et al., 2010). CLEC-2 has been shown to be transported to lipid rafts and undergoes tyrosine phosphorylation after activation by rhodocytin (Pollitt et al., 2010). The same study also demonstrated that disruption of lipid rafts prevents phosphorylation of CLEC-2 and further signalling downstream.

The actin cytoskeleton is thought to have a role in CLEC-2 mediated platelet activation. Inhibition of actin polymerisation through treatment with cytochalasin D prevented tyrosine phosphorylation of the hemITAM motif (Pollitt et al., 2010). This resulted in the inhibition of tyrosine phosphorylation of Syk and PLCγ2 and reduced aggregation in response to rhodocytin. Interestingly, in platelets treated with cytochalasin D and then stimulated with GPVI agonists – collagen-related peptide and convulxin – tyrosine phosphorylation of Syk and PLCγ2 could be detected (Pollitt et al., 2010), suggesting that the requirement of actin polymerisation is specific to the CLEC-2 signalling pathway.

Secondary mediators are also important in platelet activation via CLEC-2. Inhibition of the ADP receptor $P2Y_{12}$ resulted in a minor inhibition of CLEC-2 mediated aggregation, while blocking the formation of thromboxane A_2 using indomethacin resulted in a much greater inhibition of aggregation (Pollitt et al., 2010). Indomethacin also reduced phosphorylation of CLEC-2.

At present, the only known endogenous ligand for CLEC-2 is the transmembrane protein podoplanin, which was identified following the observation that podoplanin-expressing tumour cells caused platelet activation (Suzuki-Inoue et al., 2007). The evidence of a role for podoplanin in lymphatic development will be discussed in detail later (1.6).

1.5.4. Physiological roles of CLEC-2

CLEC-2 is thought to have several physiological functions, including roles in closure of the ductus arteriosus (DA), brain development, haemostasis, lymph node development and maintenance, maintaining the integrity of high endothelial venules (HEV), and lymphatic development and maintenance (1.5.5). Platelets appear to have a role in the closure of the DA in premature neonates as this fails to close in children with thrombocytopenia (Echtler et al., 2010). The same study also showed that DA closure was impaired in mice with defects in platelet production and adhesion (Echtler et al., 2010). The possibility of a role for CLEC-2 in brain development arose with the development of the CLEC-2 deficient mice, which were found to have brain haemorrhages from E12.5 (Tang et al., 2010; Finney et al., 2012). This is thought to be due to the interaction between CLEC-2 and podoplanin, which is expressed on the epithelium of the choroid plexus (Williams et al., 1996).

There is controversy surrounding the potential role for CLEC-2 in haemostasis and thrombosis. Mice that have had CLEC-2 depleted through antibody treatment have been shown to have increased tail bleeding in a filter blotting assay (May et al., 2009), but when a

different type of tail bleeding assay was performed, neither the antibody-treated nor CLEC-2 knockout mice were found to have increased tail bleeding (Hughes et al., 2010a; Suzuki-Inoue et al., 2010; Bender et al., 2013). It is possible, however, that there is functional overlap between CLEC-2 and GPVI, with respect to haemostasis. Mice deficient in both of these receptors display defects in both haemostasis and arterial thrombosis that are not seen in the single-knockouts (Bender et al., 2013). However, further work would be required to confirm any functional redundancy.

More recently, work has focused on the role of CLEC-2 in the development and maintenance of the lymph nodes. Stillborn *Clec1b*$^{-/-}$ mice have been found to lack mesenteric and inguinal lymph nodes (Bénézech et al., 2014). The same study also found that CLEC-2 deficiency did not affect the initial development of mesenteric lymph nodes, but that CLEC-2 is required for lymph node development in the later stages of embryogenesis (Bénézech et al., 2014). However, lymph node development may not require platelet- or megakaryocyte-expressed CLEC-2: lymph nodes were present in platelet-specific CLEC-2 knockout mice and the architecture of these lymph nodes was similar to that observed in wildtype littermates (Bénézech et al., 2014). In contrast, platelet-expressed CLEC-2 is thought to be required for the maintenance of existing lymph nodes. This conclusion was drawn from the observation that platelet-specific CLEC-2 knockout mice have smaller mesenteric lymph nodes, which, like the Peyer's patches in these mice, contained red blood cells (Bénézech et al., 2014). Further examination of these small lymph nodes discovered that they were fibrotic and disorganised (Bénézech et al., 2014).

As well as being involved in maintaining lymph nodes, the podoplanin-CLEC-2 interaction also appears to be involved in maintaining the integrity of HEVs within the lymph nodes. In one study, podoplanin was depleted postnatally in mice, which led to a bleeding phenotype in the mesenteric and cervical lymph nodes (Herzog et al., 2013). Imaging of the affected lymph nodes revealed extravasated red blood cells around HEVs (Herzog et al., 2013). Interestingly, there were no extravasated cells around non-HEV vessels in the same mice (Herzog et al., 2013). Use of fibroblastic reticular cell (FRC)-specific and endothelial-specific podoplanin knockout mice showed that podoplanin expression on FRCs, not LEC, was required for HEV integrity (Herzog et al., 2013). Further experiments confirmed that normal HEV integrity was maintained by the interaction of FRC-expressed podoplanin and platelet-expressed CLEC-2, as platelet-specific CLEC-2 knockout mice also developed bleeding in lymph nodes (Herzog et al., 2013).

1.5.5. Platelets in lymphatic development and maintenance

During lymphatic development, platelet aggregates are believed to form at the connection points between blood and lymphatic vessels, which results in separation of the two systems (Bertozzi et al., 2010; Uhrin et al., 2010; Figure 1.1). The recent interest in the role for platelets in the correct formation of lymphatic vessels began with the creation of mice lacking Syk, SLP-76 or PLCγ2, which all develop a blood-lymphatic mixing phenotype (Cheng et al., 1995; Turner et al., 1995; Wang et al., 2000; Abtahian et al., 2003; Figure 1.5). This phenotype is thought to arise from misconnections between the blood and lymphatic vessels

(Abtahian et al., 2003). In particular, the SLP-76 knockout mouse suggested a role for platelets as this protein is only expressed by haematopoietic cells and administration of bone marrow from these knockout mice into wildtype mice resulted in blood-lymphatic mixing in the intestines (Abtahian et al., 2003). As all of these proteins are known to be downstream of CLEC-2 in platelets, a CLEC-2 knockout mouse was created. This also displayed a similar phenotype, in which blood could be detected within the lymphatic vessels (Bertozzi et al., 2010b; Suzuki-Inoue et al., 2010). The role for platelets in the development of this phenotype was confirmed by the creation of a platelet-specific CLEC-2 knockout (Finney et al., 2012; Figure 1.5).

CLEC-2 is also thought to be required for the maintenance of the lymphatic system and its continued separation from the blood vascular system. Replacing the haematopoietic cells of wildtype mice with CLEC-2-deficient cells caused blood to enter the intestinal lymphatic vessels; similar effect was seen after injecting neonatal wildtype mice with an anti-CLEC-2 antibody (Hess et al., 2014). Further examination of these mice found that blood probably enters the intestinal lymphatics from the mesenteric lymphatics and that the initial site of blood entry is the thoracic duct, where the lymphatic system connects to the subclavian vein (Hess et al., 2014). Platelet aggregates and thrombi were detected in close proximity to the lymphovenous valve (LVV) in wildtype, but not CLEC-2 knockout, mice; even though there was blood entering the thoracic duct in the knockout mice (Hess et al., 2014). Such thrombi were found to compensate for a functioning LVV, as mice that were completely lacking or lacking a functional LVV were able to maintain separation of the blood and lymphatic systems

(Hess et al., 2014). The requirement for CLEC-2 mediated haemostasis in maintenance of the lymphatic vasculature is not fully understood, but it has been suggested that haemostasis may be necessary to prevent increases in pressure from forcing venous blood into lymphatic vessels (Boulaftali et al., 2014). Interestingly, CLEC-2-deficient embryos have blood-filled lymphatic sacs from E11.5, which is before the development of the LVV (Bertozzi et al., 2010b; Uhrin et al., 2010). Therefore, it has been suggested that CLEC-2 mediated intervascular haemostasis may also be necessary at earlier points in lymphatic development (Boulaftali et al., 2014).

There is still debate concerning how platelets regulate lymphatic development. It has been suggested that CLEC-2 mediated platelet activation stimulates the release of angiogenic factors from α-granules that may affect LEC (Bertozzi et al., 2010a) and that integrin $\alpha_{IIb}\beta_3$ is essential for correct separation of the blood and lymphatic vessels (Uhrin et al., 2010). However, there is controversy surrounding both of these hypotheses. Glanzmann thrombasthenia patients – whose platelets lack α-granules or dense granules – have not been found to have any lymphatic defects (Nurden et al., 2012). Similarly, mice lacking either α_{IIb} or β_3 integrin subunits do not develop the blood-lymphatic mixing phenotype (Hodivala-Dilke et al., 1999; Emambokus and Frampton, 2003). An alternative theory is that the interaction of CLEC-2 and podoplanin modulates the migratory and tube-forming capabilities of LEC, and it is this hypothesis that underlies the work presented in this thesis. Therefore, the final section in this chapter will consider the current knowledge of podoplanin's expression and function, and how this may be involved in lymphangiogenesis.

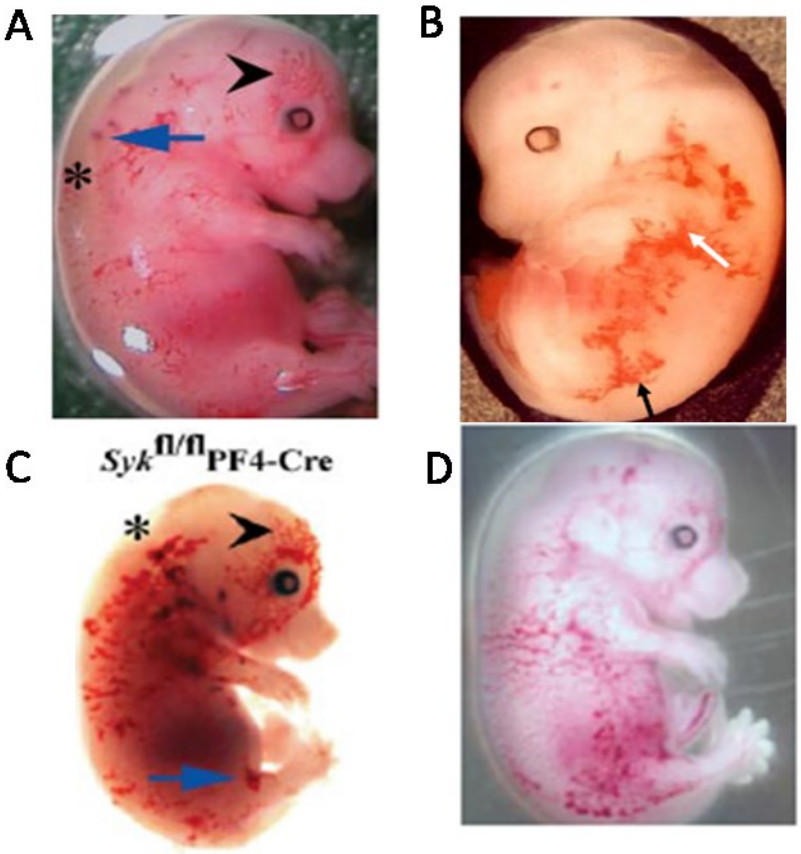


Figure 1.5: Mice lacking CLEC-2, podoplanin or associated signalling proteins develop a blood-lymphatic mixing phenotype. A) Platelet-specific CLEC-2 knockout mice develop a phenotype in which there is blood in the lymphatic vessels. A similar phenotype is seen in mice lacking podoplanin (B), Syk (C) or SLP-76 (D). This figure was constructed from images published by Bertozzi et al., 2010; Uhrin et al., 2010; and Finney et al., 2012.

1.6. Podoplanin

1.6.1. Structure and expression of podoplanin

Podoplanin is a mucin-type glycosylated transmembrane protein of 43kDa. It is expressed by several cell types including type I alveolar cells (Rishi et al., 1995), choroid plexus epithelial cells (Williams et al., 1996), kidney podocytes (Breiteneder-Geleff et al., 1997), LEC (Wetterwald et al., 1996) and fibroblastic reticular cells (Farr et al., 1992). More recently, evidence has emerged of podoplanin expression by T helper 17 (Th17) cells (Peters et al., 2011) and inflammatory macrophages (Hou et al., 2010; Kerrigan et al., 2012). Podoplanin was first detected on LEC in 1996 and was known as E11 antigen (Wetterwald et al., 1996), but later became known as podoplanin after its detection on kidney podocytes (Breiteneder-Geleff et al., 1997). Podoplanin is highly expressed by LEC (Petrova et al., 2002; Hirakawa et al., 2003) and this expression is induced by Prox1, with podoplanin being able to be detected on Prox1-positive cells from E11.5 (Hong et al., 2002; Schacht et al., 2003). Overexpression of Prox1 in vascular endothelial cells causes them to differentiate into podoplanin-expressing lymphatic cells (Petrova et al., 2002) and there is also evidence suggesting that IL-3 induces expression of podoplanin in vascular endothelial cells (Gröger et al., 2004).

Podoplanin has one transmembrane domain, a short cytoplasmic tail and a heavily glycosylated extracellular domain (Martín-Villar et al., 2005). Glycosylation of the extracellular portion of podoplanin has been shown to be essential for correct lymphatic development, as mice lacking the T-synthase enzyme necessary for O-glycosylation have blood within their lymphatic vessels (Fu et al., 2008). Podoplanin has been detected at the

plasma membrane and appears to be concentrated at cell protrusions, such as filopodia (Martín-Villar et al., 2006). There is some doubt concerning how podoplanin is presented at the cell membrane. Two groups have reported that podoplanin is found within lipid rafts in mouse alveolar epithelial cells and when overexpressed in canine kidney cells (Barth et al., 2010; Fernández-Muñoz et al., 2011). However, other groups have reported that podoplanin is associated with tetraspanins, specifically CD9 (Nakazawa et al., 2011; Iwasaki et al., 2013). While tetraspanin microdomains may include lipids, such as palmitate, cholesterol and gangliosides (Charrin et al., 2003; Odintsova et al., 2003), they are believed to be distinct from lipid rafts. This hypothesis is based upon differences in sensitivity to temperature changes and cholesterol depletion (Hemler, 2005). Additionally, the proteins associated with either tetraspanin microdomains or lipid rafts are not thought to overlap (Hemler, 2005).

1.6.2. Functions of podoplanin

Although podoplanin has been found to be expressed in many different tissues, its function is still largely unknown. Recent work has identified potential roles for podoplanin in development of the heart and lungs, development and maintenance of secondary lymphoid organs, cancer progression, the immune response, and lymphatic development (1.6.2.1). A role for podoplanin in cardiac development was suggested by the discovery that podoplanin-deficient mouse embryos display hypoplasia of the atrial septum, left atrium dorsal wall and pulmonary vein (Douglas et al., 2009). The cells responsible for cardiac development have increased E-cadherin and decreased RhoA when podoplanin is knocked out (Mahtab et al., 2008, 2009). This suggests that these cells are unable to go through epithelial-mesenchymal

transition (EMT), thereby reducing their migration and resulting in hypoplasia (Mahtab et al., 2008, 2009).

Podoplanin-knockout mice die shortly after birth due to a failure in lung inflation, which is believed to arise from a defect in type I alveolar cells (Ramirez et al., 2003). Under normal developmental conditions these cells are highly proliferative during early and mid-gestation, but do not proliferate as extensively in the latter stages of embryonic development. However, type I alveolar cells that are podoplanin-deficient continue proliferating at a high rate throughout development (Ramirez et al., 2003). This is thought to be due to decreased expression of the cell cycle regulator p21 (Millien et al., 2006).

In addition to its role in lymphatic development (see 1.6.2.1), podoplanin may also be involved in the development and maintenance of secondary lymphoid organs. This idea was first postulated by Peters *et al.* (2011), who found that podoplanin-deficient mice had a decreased number of lymph nodes. Those nodes that did form were found to be disorganised. Similarly, the spleens of these animals appeared disorganised. However, the mechanism underlying the development of this phenotype is not currently known.

Podoplanin has recently been shown to function within the immune system, where it promotes the motility of dendritic cells (Acton et al., 2012). This study found that podoplanin on LEC and FRCs interacted with CLEC-2 on dendritic cells, which led to an increase in

dendritic cell motility from peripheral areas of the lymph node to the T cell zone. The requirement of the podoplanin-CLEC-2 interaction was confirmed with the use of CLEC-2 deficient dendritic cells and siRNA-mediated knockdown of podoplanin; both of these alterations resulted in decreased dendritic cell migration (Acton et al., 2012). There have also been reports of podoplanin expression on some T cells and macrophages (Hou et al., 2010; Peters et al., 2011; Kerrigan et al., 2012). Podoplanin expression by these cell types appears to be associated with inflammatory states, such as in experimental autoimmune encephalomyelitis (Peters et al., 2011), but further work is needed to fully understand the role of podoplanin in these conditions.

Podoplanin-positive lymphatic vessels have been detected within several tumour types, including ovarian (Birner et al., 2000), cervical (Birner et al., 2001), pancreatic (Rubbia-Brandt et al., 2004; Sipos et al., 2004), and breast tumours (Schoppmann et al., 2001). It is believed that these vessels are involved in tumour cell invasion and migration (Ji et al., 2007) and the observation of lymphangiogenesis in cancer is usually associated with poor prognosis (Dadras et al., 2003; Maula et al., 2003). In some cancers, podoplanin appears to be upregulated on the cancer cells themselves. Podoplanin has been detected in tumour cells from patients with Kaposi's sarcoma (Breiteneder-Geleff et al., 1999), mesothelioma (Kimura and Kimura, 2005), and squamous cell carcinoma (Kato et al., 2005; Martín-Villar et al., 2005; Wicki et al., 2006). The expression of podoplanin by tumour cells is thought to promote invasion and metastasis by those cells (Mishima et al., 2006; Kunita et al., 2007), and in some tumour cells podoplanin has been found at the leading edge of invasive tumours (Wicki et al., 2006). It is

thought that podoplanin increases the motility of tumour cells, possibly through remodelling the actin cytoskeleton or by RhoA-mediated effects (Martín-Villar et al., 2006; Moustakas and Heldin, 2007), which will be described later (section 1.6.3).

1.6.2.1. Podoplanin in lymphatic development

The requirement for podoplanin in normal lymphatic development was first suggested by Schacht *et al.*, who observed cutaneous lymphoedema in the neck and lower limbs of newborn podoplanin-deficient mice (Schacht et al., 2003). Further analysis of these animals found a lack of lymphatic capillaries in the skin and intestines, and defective lymphatic patterning in areas where vessels did develop. Importantly, there was no defect in the development or function of blood vessels in these mice (Schacht et al., 2003). Interestingly, mice heterozygous for podoplanin were viable and survived to adulthood, although they appeared to have dilated lymphatic vessels in the ear skin and intestine (Schacht et al., 2003). At a similar time to this study, another group reported that loss of the haematopoietic proteins Syk or SLP-76 prevented the separation of the blood and lymphatic vasculature (Abtahian et al., 2003), but the connection between these proteins and podoplanin was not understood.

After the discovery of CLEC-2 on platelets and confirmation of podoplanin as its binding partner (Suzuki-Inoue et al., 2006, 2007), further work focussed on how the interaction between platelets and lymphatic endothelial cells might influence lymphatic development.

One group generated podoplanin-knockout mice on a different genetic background and found that 20% of these mice survived and were able to reach adulthood (Uhrin et al., 2010). Blood was detected within the lymphatic vessels of the podoplanin-deficient embryos (Figure 1.5), but any microvascular defects appeared to resolve in the early postnatal period, although some of the surviving mice later developed chylothorax or chylous ascites (Uhrin et al., 2010). The same study suggested that the separation of the two vascular systems requires podoplanin-mediated platelet aggregation – a hypothesis that was supported by a different group (Bertozzi et al., 2010b). The Bertozzi study also reported that platelets do not affect the viability, proliferation or migration of LEC *in vitro* (Bertozzi et al., 2010b). This report conflicts with data published by Finney *et al.* (2012), who showed that platelets from wildtype mice significantly inhibit LEC migration and network formation on Matrigel and that platelets from CLEC-2 or Syk-deficient mice have a weaker inhibitory effect. This study also showed that antibody-mediated crosslinking of podoplanin inhibited LEC migration and confirmed that platelets do not affect LEC survival (Finney et al., 2012).

A soluble Fc fusion version of podoplanin has also been created. This protein consists of the extracellular domain of human podoplanin linked to an immunoglobulin Fc domain (Cueni et al., 2010). Podoplanin-Fc was shown to reduce the migration of LEC towards type I collagen and inhibit lymphangiogenesis in both *in vitro* and *in vivo* assays (Cueni et al., 2010). However, when this protein was overexpressed in the skin of mice, it caused disseminated intravascular coagulation, which proved fatal in several of the animals. This was thought to occur through podoplanin-Fc in the serum interacting with platelet CLEC-2 (Cueni et al.,

2010). The same group also created a mutated mouse podoplanin-Fc, where the threonine at position 34 was exchanged for an alanine (known as mPdpnT34A-Fc). This particular mutation was chosen as threonine 34 has been shown to be crucial for podoplanin to bind to CLEC-2 (Kato et al., 2003). The mutated fusion protein reduced, but did not prevent, podoplanin binding to and activating CLEC-2 and it also reduced the severity and frequency of disseminated intravascular coagulation (Bianchi et al., 2014). mPdpnT34A-Fc had anti-lymphangiogenic effects similar to those observed with the human podoplanin-Fc and the authors suggested that these effects were through podoplanin-Fc blocking the interaction of endogenous podoplanin and an unidentified ligand, rather than a CLEC-2 dependent mechanism (Bianchi et al., 2014). This appears to be the first indication that podoplanin is able to interact with a ligand other than CLEC-2 in the process of lymphangiogenesis.

1.6.3. Signalling downstream of podoplanin

The cytoplasmic domain of podoplanin consists of only nine amino acids (Martín-Villar et al., 2005). Within this domain is a conserved sequence of three basic amino acids that are necessary for podoplanin to interact with the ezrin/radixin/moesin (ERM) protein family, specifically ezrin and moesin (Scholl et al., 1999; Martín-Villar et al., 2006). ERM proteins connect membrane-bound proteins to the actin cytoskeleton and are involved in signalling pathways that control cell migration and adhesion (Bretscher et al., 2002), and podoplanin has been shown to co-localise with ezrin in a fibroblast cell line (Acton et al., 2014). The presence of these three basic amino acids is required for cells to undergo EMT, which is observed when podoplanin is overexpressed in Madin-Darby canine kidney (MDCK) cells

(Martín-Villar et al., 2006). Overexpression of podoplanin promoted the migration of MDCK cells, while cells transfected with a podoplanin construct lacking its extracellular domain displayed erratic movements when observed under time-lapse microscopy (Martín-Villar et al., 2006). The same study showed that overexpression of podoplanin increased the amount of active RhoA, and that the cytoplasmic domain and its basic amino acids were necessary for this increase in RhoA activity (Martín-Villar et al., 2006). The increase in RhoA activity was associated with an increase in phosphorylation of ERM proteins, leading the authors to conclude that podoplanin phosphorylates ERM proteins via RhoA and its effector protein, ROCK (Martín-Villar et al., 2006).

Studies by a different group confirmed that podoplanin is necessary for efficient cell migration and network formation, showing that siRNA-mediated knockdown of podoplanin in LEC reduced the ability of these cells to migrate across a wound or form networks on Matrigel (Navarro et al., 2008, 2011). Podoplanin knockdown was associated with a reduction in active RhoA and treating LEC with an inhibitor of Rho was able to prevent network formation (Navarro et al., 2008). In contrast to the Martín-Villar study (2006), which found that overexpression of podoplanin increased ERM phosphorylation, this later study found that podoplanin knockdown had no effect on the amount of phosphorylated nor basal ERM proteins (Navarro et al., 2008). There was no effect on the distribution of ERM proteins after podoplanin knockdown, although the presence of phosphorylated ERM proteins in microvilli appeared to be reduced after podoplanin knockdown (Navarro et al., 2008).

Podoplanin knockdown has also been shown to impair polarisation by LEC, which is thought to underlie the defect in migration observed with these cells (Navarro et al., 2011). This study confirmed the requirement for podoplanin in RhoA activation, showing that podoplanin-knockdown LEC have decreased levels of active RhoA. The lack of active RhoA was associated with an inability of LEC to polarise and a reduction in migration. This study also found that there was an increase in active Cdc42 after podoplanin knockdown (Navarro et al., 2011). Through expression of constitutively active and dominant-negative Cdc42 constructs, the authors conclude that directional LEC migration is dependent on regulation of both Cdc42 and RhoA, with cells requiring a low level of active Cdc42 and a higher level of RhoA-GTP for correct migration (Navarro et al., 2011).

Podoplanin has been shown to interact with another transmembrane glycoprotein, CD44, particularly in cell surface protrusions (Martín-Villar et al., 2010). This association was found in cells that endogenously expressed podoplanin and cells in which podoplanin had been overexpressed. The interaction between CD44 and podoplanin could be detected in the trailing edge of migrating cells, but could not be found at cell junctions, even though both proteins were expressed in these areas (Martín-Villar et al., 2010). Removal of podoplanin's cytoplasmic domain did not prevent its interaction with CD44, suggesting that the interaction was not via ERM proteins (Martín-Villar et al., 2010). The ectodomain of podoplanin, however, is essential for interaction with CD44. This study also reported that CD44-podoplanin complexes were more frequently detected in epidermal growth factor (EGF)-stimulated cells, suggesting that the interaction may promote motility (Martín-Villar et

al., 2010). This was confirmed by further experiments where podoplanin, CD44, or both were knocked down in the HN5 cancer cell line. These experiments found that decreasing podoplanin or CD44 gave less directional movement, as determined by a reduction in wound healing, and that reducing both proteins resulted in a more striking inhibition of migration (Martín-Villar et al., 2010).

Further studies have also examined the connection between podoplanin, ERM proteins and the actin cytoskeleton. Fibroblastic cells overexpressing podoplanin showed increased phosphorylated ERM proteins and myosin light chain in the cell cortex (Acton et al., 2014). These cells had a contractile morphology and the phenotype was found to require ezrin phosphorylation (Acton et al., 2014). The contracted morphology of these cells was reversed by treatment with inhibitors RhoA/C, ROCK or myosin II (Acton et al., 2014). Similarly, treatment with soluble recombinant CLEC-2-Fc relaxed the podoplanin-expressing NIH/3T3 cells and caused ezrin to be redistributed from the plasma membrane to the cytoplasm (Acton et al., 2014). Co-transfection of podoplanin and CD44 also reduced contraction; the interaction between these proteins was suggested by the reduction in CD44 expression after podoplanin knockdown (Acton et al., 2014). Further experiments in the same study showed that CLEC-2 sequesters FRC-expressed podoplanin in lipid rafts, where interaction with CD44 causes relaxation of the cells. This is thought to have a role in lymph node expansion during inflammation: the influx of CLEC-2 expressing dendritic cells during acute inflammation increases inhibition of podoplanin, allowing the FRC networks to stretch and expand, as required (Acton et al., 2014).

The ability of FRCs to contract appears to be required for maintaining normal lymph node size and a normal number of FRCs (Astarita et al., 2015). The FRCs of mice treated with an anti-podoplanin antibody showed increased proliferation and were larger than FRCs from control-treated mice, which was noted as being consistent with a "blasting phenotype" (Astarita et al., 2015). Mice that specifically lacked podoplanin in FRCs had larger lymph nodes and greater total cellularity than wildtype littermates. These mice also developed a bleeding phenotype that is believed to be due to a lack of HEV integrity (Astarita et al., 2015). The FRC network in mice treated with anti-podoplanin antibody had a "finer appearance" and greater space between fibres than those seen in control-treated mice; similar effects were observed in the FRC-specific podoplanin knockout mice (Astarita et al., 2015). There was also an increased T-cell response to LPS in mice treated with anti-podoplanin antibody; suggesting that podoplanin may have a role in restraining the T-cell response (Astarita et al., 2015).

More recently, two serine residues in the cytoplasmic tail of podoplanin have been reported to regulate cell motility. These two serines, which are conserved between mouse and human podoplanin, can be phosphorylated by protein kinase A (PKA), but not PKC (Krishnan et al., 2013). This study used embryonic fibroblasts from podoplanin-deficient mice as a model in which to overexpress different podoplanin constructs. They found that expression of podoplanin increased cell migration and that mutating the conserved serines to alanine residues improved migration further. They suggested that PKA phosphorylation of serine residues inhibits cell migration (Figure 1.6) and confirmed this by overexpressing

phosphomimetic podoplanin constructs (Krishnan et al., 2013). The cells expressing the phosphomimetic construct did not migrate more than the control transfected cells (Krishnan et al., 2013).

While there have been several publications on how podoplanin might signal and how it may be involved in lymphatic development, there is still much that is not understood. As previous studies have shown that platelet-expressed CLEC-2 is essential for lymphatic development and that platelets modulate the migration of LEC (Finney et al., 2012), the work within this thesis will expand the knowledge of how these two proteins interact and of their function in LEC.

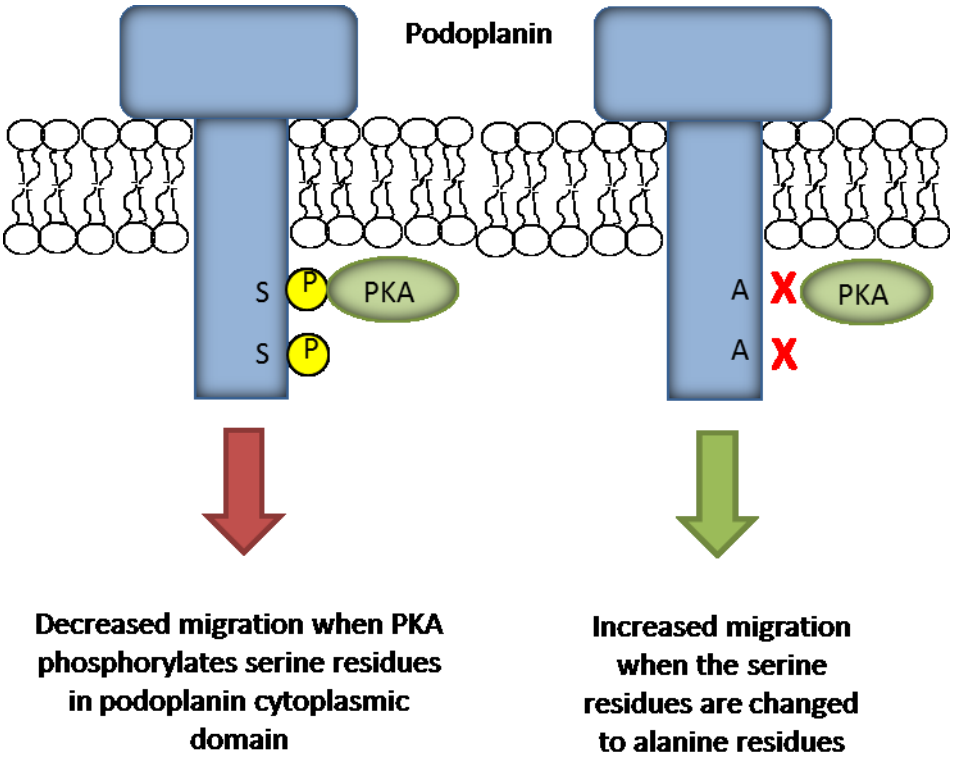
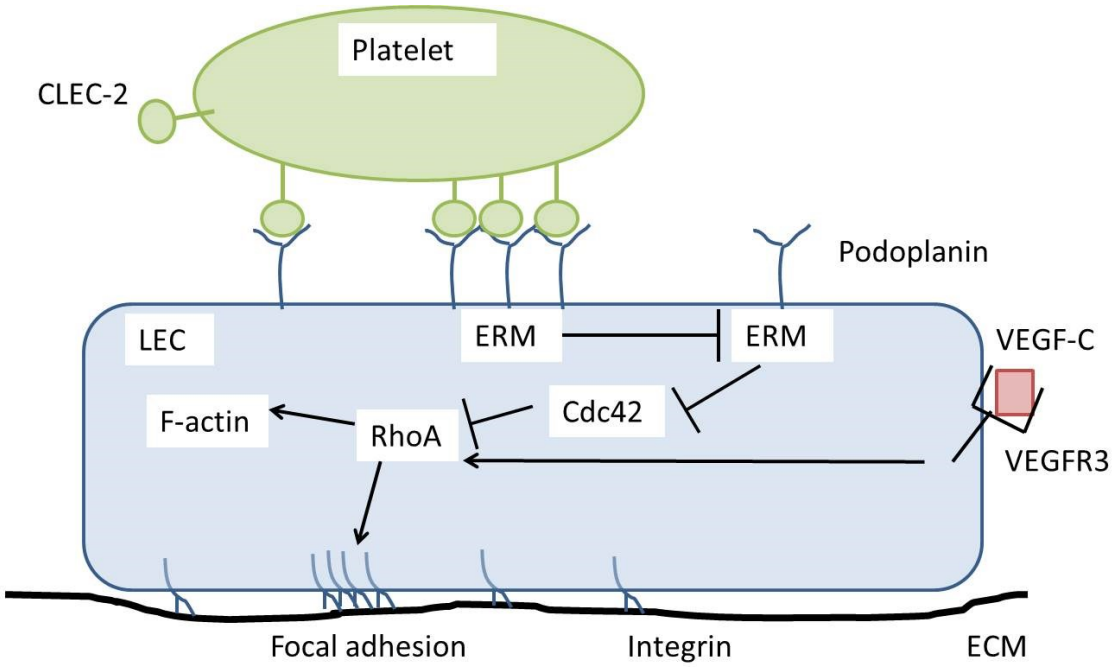


Figure 1.6: Role of conserved serines in the cytoplasmic tail of podoplanin and PKA in cell migration. When PKA phosphorylates the serine residues in the intracellular domain of podoplanin, cell migration is reduced. If these serine residues are mutated to alanines, which cannot be phosphorylated by PKA, then migration is increased. Summary of data reported by Krishnan et al., 2013.

1.7. Hypothesis and aims

Mice deficient in podoplanin, CLEC-2 or associated signalling proteins all develop a phenotype in which blood can be detected within the lymphatic vessels, suggesting that there are points of misconnection between the two systems (Abtahian et al., 2003; Bertozzi et al., 2010b; Uhrin et al., 2010; Finney et al., 2012). In particular, Finney *et al.* showed that platelets from wildtype mice inhibit VEGF-C mediated LEC migration and that platelets from CLEC-2 deficient mice have a weaker inhibitory effect (Finney et al., 2012). Therefore, the hypothesis of this project is that platelet CLEC-2 binds to and clusters podoplanin on LEC, which alters signalling pathways within LEC and results in an inhibition of migration. This hypothesis is illustrated in Figure 1.7. The change in signalling within LEC may be through podoplanin's association with ERM proteins, effects on RhoA signalling and changes in VEGFR signalling. With this in mind, the aims of this PhD project were:

- To assess the effect of platelets on LEC migration using transfilter migration and wound healing assays;
- To assess the effect of crosslinking podoplanin on LEC migration;
- To develop an *in vitro* assay to assess tube formation by LEC;
- To use this assay to assess the effect of platelets or podoplanin crosslinking on the tube-forming capabilities of LEC;
- To assess signalling pathways downstream of podoplanin in LEC.



ECM: extracellular matrix; ERM: ezrin/radixin/moesin

Figure 1.7: Summary of signalling proteins that may be involved in LEC migration. Platelet-expressed CLEC-2 is thought to cluster podoplanin on LEC. This clustering may alter signalling through ERM proteins, which could lead to changes in Cdc42 or RhoA activation. Similarly, signalling downstream of VEGFR3 is likely to be important in LEC migration. The changes in migration may be due to effects on the actin cytoskeleton or the turnover of integrins and focal adhesions.

CHAPTER TWO

MATERIALS AND METHODS

2.1. Ethics statement

This study was carried out with the ethical approval of the Science, Technology, Engineering and Mathematical Ethical Review Committee of the University of Birmingham. All blood donors were healthy adult volunteers who had given written informed consent. Human umbilical cords were obtained from the Human Biomaterials Resource Centre (University of Birmingham) after informed consent was given by donors.

2.2. Source of antibodies

Antibodies were used for a number of applications, including flow cytometry, Western blotting, co-immunopreceiptations and functional assays. The antibodies used are detailed in Table 2.1.

2.3. Cell culture

2.3.1. Source of cells

Human dermal LEC and human dermal fibroblasts (HDF) were from PromoCell (Heidelberg, Germany). Human umbilical vein endothelial cells (HUVEC) were isolated in-house from donated umbilical cords obtained from the Human Biomaterials Resource Centre. An immortalised microvascular endothelial cell line (HMEC-1) was obtained from the Centre for Disease Control and Prevention (CDC; Atlanta, Georgia, USA); they were initially developed

and described by Dr Edwin Ades (Ades et al., 1992). All cells were cultured at 37°C and 5% CO_2.

2.3.2. Lymphatic endothelial cells

LEC were maintained in flasks containing MV2 medium (PromoCell) that was supplemented with: 5% foetal calf serum (FCS), 5 ng/ml EGF, 10 ng/ml FGFb, 20 ng/ml insulin-like growth factor, 0.5 ng/ml $VEGF_{165}$, 1 µg/ml ascorbic acid and 0.2 µg/ml hydrocortisone (all PromoCell). 2.5 µg/ml amphotericin (Gibco, Paisley, UK), 100 U/ml penicillin (Sigma, Poole, UK) and 100 µg/ml streptomycin (Sigma) were added, except during siRNA transfections. Cells were labelled as passage two on arrival and used in experiments from passage 3-7. During expansion, cells were cultured to passage four and then frozen. Aliquots of frozen cells were thawed and passaged 1:3 every 3-4 days.

2.3.3. Vascular endothelial cells

HUVEC and HMEC-1 were cultured in Medium 199 (Gibco) that was supplemented with 20% FCS, 2.5 µg/ml amphotericin, 100 U/ml penicillin, 100 µg/ml streptomycin, 1 ng/ml EGF, 28 µg/ml gentamycin and 1 µg/ml hydrocortisone (all Sigma). When HUVEC were used in experiments they were either newly isolated or had undergone one passage. HMEC-1 cells were passaged 1:3 every 3-4 days.

Table 2.1: Antibodies. PE is phycoerythrin; IP is co-immunoprecipitation; WB is Western blot.

Primary	Clone	Host species	Conjugation	Use	Source
α2 integrin	Gi9	Mouse	Unconjugated	IP	Immunotech
β1 integrin	Mab13	Rat	Unconjugated	IP	BD Biosciences
CD9	C9.BB	Mouse	Unconjugated	IP, WB	Prof Fedor Berdichevski, Univ. Birmingham
CLEC-2	AYP1	Mouse	488	Flow cytometry	Produced in-house
Podoplanin	18H5	Mouse	Unconjugated	Functional assay	Santa Cruz Biotechnology
	NZ-1.3	Rat	Unconjugated	Functional assay, IP, WB	eBioscience
	NZ-1.3	Rat	PE	Flow cytometry	eBioscience
VEGFR2	IMC-1121b	Human	Unconjugated	Functional assay, IP	ImClone
	89106	Mouse	PE	Flow cytometry	R&D Systems
VEGFR3	IMC-3C5	Human	Unconjugated	Functional assay, IP	ImClone
	54733	Mouse	PE	Flow cytometry	R&D Systems
Isotype controls					
Human IgG	-	-	Unconjugated	IP	Sigma
MOPC 21	-	Mouse	Unconjugated	IP	Sigma
Mouse IgG_1	-	-	PE	Flow cytometry	Immunotools
	-	-	488	Flow cytometry	Produced in-house
Rat IgG_{2a}	-	-	Unconjugated	Functional assay	eBioscience
	-	-	PE	Flow cytometry	eBioscience
Secondary					
Mouse IgG_1	-	-	Alkaline phosphatase	Functional assay	Sigma
Rat IgG_{2a}	-	-	Unconjugated	Functional assay	eBioscience

2.3.4. Human dermal fibroblasts

HDF were cultured in fibroblast growth medium 2 (PromoCell), which was supplemented with: 2% FCS, 1 ng/ml FGFb, 5 µg/ml insulin (all Promocell), 2.5 µg/ml amphotericin, 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were labelled as passage two on arrival and were used up to passage ten. HDF were expanded to passage four and then frozen. Thawed aliquots of HDF were grown in 75cm^2 flasks and were passaged 1:3 every 5-7 days.

2.3.5. Detachment of cells from flasks

Medium was removed from 25cm^2 flasks and the cells were washed with 2ml 0.02% ethylene diamine tetraacetic acid (EDTA) (Sigma), before 2ml 0.25% trypsin (Sigma) was added. The flask was incubated at 37°C until the cells had detached. Trypsin was then neutralised with 4ml of the appropriate medium and the cells were pelleted (400 x *g*, 5 minutes). For passaging, cells were split between three 25cm^2 flasks or transferred into one 75cm^2 flask. For an experiment, cells were resuspended in 1ml culture medium, 20 µl was transferred to a counting slide (Nexcelom, Massachusetts, USA) and the number of cells was counted using a cell counter (Cellometer Auto T4, Nexcelom). Cells were then diluted to the required concentration using the appropriate medium.

For 75cm^2 flasks, medium was removed and the cells were washed with 4ml of 0.02% EDTA, followed by the addition of 4ml of 0.25% trypsin. The rest of the procedure was the same as for 25cm^2 flasks.

2.3.6. Freezing and thawing of LEC and HDF

Cells were detached from the flask as described above (2.3.5). After centrifuging, the supernatant was aspirated and cells were resuspended in 0.5ml Cryo-SFM (PromoCell) per 25cm^2. Cell suspensions were transferred into cryovials labelled with the date, cell type, passage number and batch number. Vials were placed in a cell cryopreservation device and kept at -80°C until frozen. For longer-term storage, vials were transferred to a liquid nitrogen tank.

Before thawing, 25cm^2 flasks were coated with 1% gelatin in phosphate buffered saline (PBS; both Sigma) and kept at 37°C. 8ml of the appropriate medium was warmed to 37°C. The vial of cells was defrosted by agitating in warm water, then the cells were added to the warmed medium. The cells were pelleted and then resuspended in 1ml of warmed medium. The gelatin was removed from the flask and replaced with 4ml warmed medium. The cells were then transferred to the flask and kept at 37°C. After 4 hours, the medium was replaced.

2.4. siRNA knockdown of podoplanin

2.4.1. Source of duplexes

Two siRNA duplexes targeting podoplanin and a non-specific control were purchased from Sigma (Table 2.2). These duplexes were provided lyophilised and were resuspended in sterile water to give a concentration of 100µM. The duplexes were further diluted to 20µM with sterile water and aliquots were stored at -80°C. The podoplanin duplexes were labelled 1 or

2 to distinguish them from each other. The control duplex has no homology to any known genes.

Table 2.2: siRNA duplexes

siRNA	Duplex sequence	Catalogue number	Company
Podoplanin siRNA-1	GUCUAGUUUGGUCUAUCUU[dT][dT] AAGAUAGACCAAACUAGAC[dT][dT]	SASI_Hs01_00094891	Sigma
Podoplanin siRNA-2	GUCUAGUUUGGUCUAUCUU[dT][dT] AAGAUAGACCAAACUAGAC[dT][dT]	SASI_Hs01_00192618	Sigma
Universal negative control #1		SIC001	Sigma

2.4.2. Transfection of LEC with siRNA

$5x10^4$ LEC were seeded onto wells of 24-well plates in antibiotic-free MV2 medium. The cells were incubated overnight at 37°C, with the transfection performed the following day when cells were approximately 80% confluent. Duplexes to treat three wells were prepared by diluting in Optimem (Gibco, Paisley, UK) to give a final concentration of 10-70nM. In a separate tube, a solution of 10% RNAiMAX lipofectamine (Invitrogen, Paisley, UK) in Optimem was prepared. The duplexes and RNAiMAX were incubated at room temperature for 10 minutes. Duplex and RNAiMAX were combined and incubated for a further 10 minutes at room temperature. The duplexes were then diluted with 1.6ml Optimem. Meanwhile, the cells were washed with 500µl Optimem. 500µl diluted duplex was added to each well and the plate was incubated at 37°C and 5% CO_2 for four hours. The transfection medium was then replaced with antibiotic-free MV2 and the plate was incubated at 37°C and 5% CO_2 for between 24 and 72 hours. In later experiments, cells were transfected with 30nM siRNA duplex and used in transfilter assays 48 hours after transfection. Knockdown efficiency was determined by flow cytometry (see Section 2.5.1).

2.5. Flow cytometry

2.5.1. Staining of LEC for podoplanin, VEGFR2, VEGFR3 or CD31

LEC were detached from flasks with trypsin, as described above (2.3.5), but were resuspended in sterile PBS. $4x10^4$ LEC were transferred into FACS tubes in a volume of 75µl. Cells were stained with 0.125µg of phycoerythrin (PE)-conjugated anti-podoplanin (NZ-1.3) or PE-conjugated rat IgG_{2a}. Tubes were protected from light and incubated on ice for 40

minutes. The samples were diluted with 300µl PBS before being analysed on a FACSCalibur flow cytometer (BD Bioscience, Oxford, UK). A minimum of 2,500 events were collected per sample. The data was analysed using Summit v4.3 (Dako, Colorado, USA). This confirmed that podoplanin was present on the surface of the PromoCell LEC (Figure 2.1). Each new batch of LEC received from PromoCell was checked for podoplanin expression before being used in a functional assay. In later experiments, LEC were detached from 12- or 24-well plates using Accutase (Gibco).

Expression of VEGFR2, VEGFR3 and CD31 was determined following siRNA-mediated knockdown of podoplanin. Cells were detached from a 24-well plate using Accutase, then divided between FACS tubes. LEC were stained with PE-conjugated antibodies against CD31 (clone 9G11, 1:7.5 dilution), VEGFR2 (89106, 1:7.5) or VEGFR3 (54733, 1:7.5). The isotype control was a PE-conjugated mouse IgG_1 (1:7.5). Samples were treated and analysed as described above.

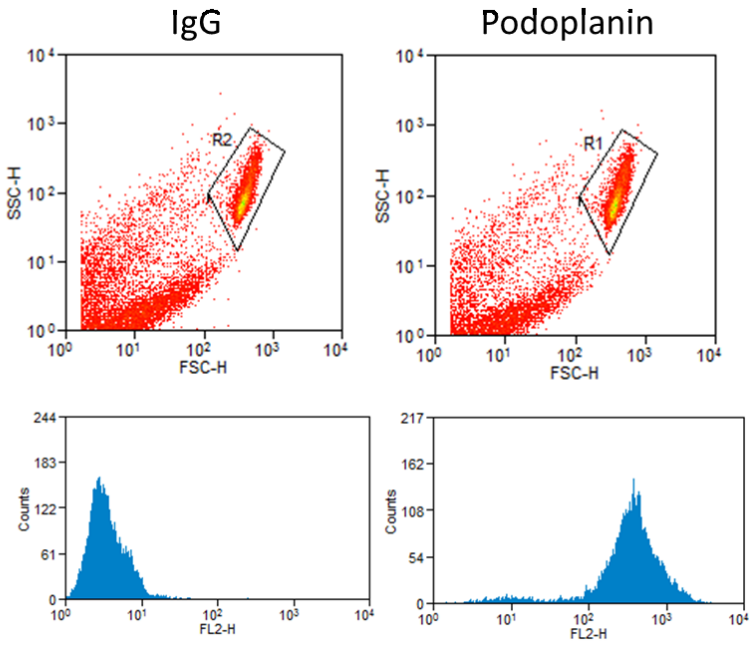


Figure 2.1: Light scatter plots and fluorescent intensity plots from flow cytometry to analyse expression of podoplanin on LEC. LEC were stained with PE-conjugated rat IgG_{2a} or anti-human podoplanin. Cells were gated based upon size and granularity, analysed by forward and side scatter intensity. Median fluorescence intensity was used to quantify podoplanin staining. Left panel: representative scatter plot and histogram of LEC stained with rat IgG_{2a}. Right panel: representative scatter plot and histogram of LEC stained with the anti-podoplanin antibody.

2.6. Preparation of washed platelets and platelet microvesicles

2.6.1. Preparation of washed human platelets using prostacyclin

Whole blood was taken from healthy volunteers into 4% sodium citrate (Sigma, 1:9 volume). Coagulation was prevented by the addition of 10% acid citrate dextrose (ACD: 120mM sodium citrate, 110mM glucose, 80mM citric acid; Hughes et al., 2010). The blood was centrifuged in 5ml tubes at 200 x *g* for 20 minutes. The resulting platelet-rich plasma (PRP) was transferred to a 50ml tube. 10 µg/ml PGI_2 was added to the PRP, which was gently mixed and centrifuged at 1000 x *g* for 10 minutes. The plasma was discarded and the platelets washed with 25ml modified Tyrode's buffer (134mM NaCl, 0.34mM Na_2HPO_4, 2.9mM KCl, 12mM $NaHCO_3$, 20mM HEPES, 5mM glucose, 1mM $MgCl_2$; pH 7.3) with 3ml ACD and 10 µg/ml PGI_2 added, before centrifugation for 10 minutes at 1000 x *g*. The resulting platelet pellet was resuspended in 1ml modified Tyrode's buffer and counted using a cell counter (Coulter, High Wycombe, UK). Platelets were diluted to the required concentration using modified Tyrode's and were allowed to rest for at least 30 minutes before use so that the effects of prostacyclin were reversed.

2.6.2. Preparation of washed human platelets using theophylline

Whole blood was taken from healthy volunteers into citrate phosphate dextrose adenine at a ratio of 1:8. Theophylline buffer was added to the blood at a final concentration of 7mM. The blood was divided between tubes and centrifuged at 200 x *g* for 10 minutes. The PRP was collected and diluted with 10ml Ca^{2+}/Mg^{2+}-free PBS (Sigma). A further 7mM theophylline buffer was added to the PRP and the tube was centrifuged at 1,000 x *g* for 10 minutes.

Platelets were resuspended in 1ml Ca^{2+}/Mg^{2+}-free PBS and counted, then diluted to the required concentration. Platelets did not have to be rested before use.

2.6.3. Preparation of platelet releasate

Washed human platelets were isolated as described above and resuspended at a concentration of $1x10^9$/ml. 130µl platelets were stimulated with 300nM rhodocytin (activating ligand for CLEC-2; produced by Dr JA Eble, University of Frankfurt) under stirring conditions for five minutes. The platelets were pelleted by centrifuging at 20,800 x *g* for five minutes at 37°C. The resulting supernatant was then used in transfilter migration assays.

2.6.4. Preparation of washed mouse platelets

Mice used in this thesis were either *Clec1b*$^{fl/fl}$PF4-Cre$^+$ (hereafter referred to as *Clec1b*$^{fl/fl}$PF4-Cre) or *CLEC1b*$^{fl/fl}$PF4-Cre$^-$ (referred to as *Clec1b*$^{fl/fl}$). They did not or did express CLEC-2, respectively (Finney et al., 2012). [redacted]. Whole blood was removed from the vena cava into 10% ACD. Blood was centrifuged in a microcentrifuge at 425 x *g* for six minutes. The PRP and some of the erythrocytes were transferred to a clean 1.5ml tube. These tubes were centrifuged at 200 x *g* for six minutes. The PRP was transferred to a clean tube. 200µl modified Tyrode's was added to the remaining red blood cells, which were centrifuged for a further six minutes at 200 x *g*. The PRP from this step was added to that obtained previously. The pooled PRP was diluted with modified Tyrode's to give a final volume of 1ml. 1µg/ml PGI_2 was added and the PRP

was centrifuged at 1,000 x *g* for six minutes. The supernatant was discarded and the pellet resuspended in 200µl modified Tyrode's. The platelets were counted and diluted to the required concentration. Platelets were allowed to rest for at least 30 minutes before use.

2.6.5. Preparation of human and mouse microvesicles

$1x10^8$ washed platelets were obtained using the methods detailed above (2.6.2 and 2.6.4). Platelets were stimulated with 1µg/ml crosslinked collagen-related peptide (CRP-XL; produced by Dr RW Farndale, University of Cambridge) and incubated at 37°C on a roller-mixer for 30 minutes. Platelets were pelleted by centrifuging at 2,000 x *g* for 20 minutes. The supernatant was transferred to a clean tube and then centrifuged at 13,000 x *g* to remove any residual platelets. The supernatant containing microvesicles was transferred to a clean tube and used in an assay or stored at -80°C.

2.6.5.1. Staining of microvesicles for CD41 and CLEC-2

Microvesicles prepared from mouse platelets were checked for expression of CD41 and CLEC-2. 50µl of whole blood or platelet-derived microvesicles were stained with 1/100 mouse IgG-488 or CLEC-2-488. Tubes were protected from light and incubated at room temperature for 20 minutes. 150µl filtered PBS was added to each sample before analysis on an Accuri C6 flow cytometer (BD Biosciences).

2.7. Transfilter cell migration assays

2.7.1. Selection of VEGF-C concentration and assay timepoint

LEC were detached from flasks (2.3.5) and resuspended in MV2 medium at a concentration of $1.5x10^5$/ml. 700µl MV2 medium containing 150ng/ml VEGF-C or 300ng/ml VEGF-C was applied to wells of a 24-well plate. $3x10^4$ LEC in a volume of 200µl were pipetted into cell culture inserts with 8µm pores (BD Biosciences) which were added to the wells. The plate was incubated for 24 or 48 hours at 37°C and 5% CO_2. Medium was removed from both chambers of the well and the inserts were washed twice with 1ml sterile PBS. A fixative/staining solution of 2% formaldehyde (Sigma) and 2µg/ml bisbenzimide (fluorescent nuclear stain; Sigma) in sterile PBS was added to the well (700µl below the insert and 200µl on top of the insert). The plate was protected from light and incubated at room temperature for 15 minutes. The inserts were then washed twice with sterile PBS. The membrane was cut out of each insert and mounted onto a microscope slide using Hydromount (National Diagnostics, Georgia, USA). Slides were protected from light and stored at 4°C until analysis.

Transfilter assays were analysed on an invert fluorescent microscope (Zeiss, Hertfordshire, UK) using a 40x objective. The number of cells visible above and below the insert (Figure 2.2) was counted in 12 separate fields for each filter. Percentage transmigration was calculated as follows:

$$\text{Percentage migration} = \frac{Number\ of\ migrated\ cells}{Total\ number\ of\ cells} \times 100$$

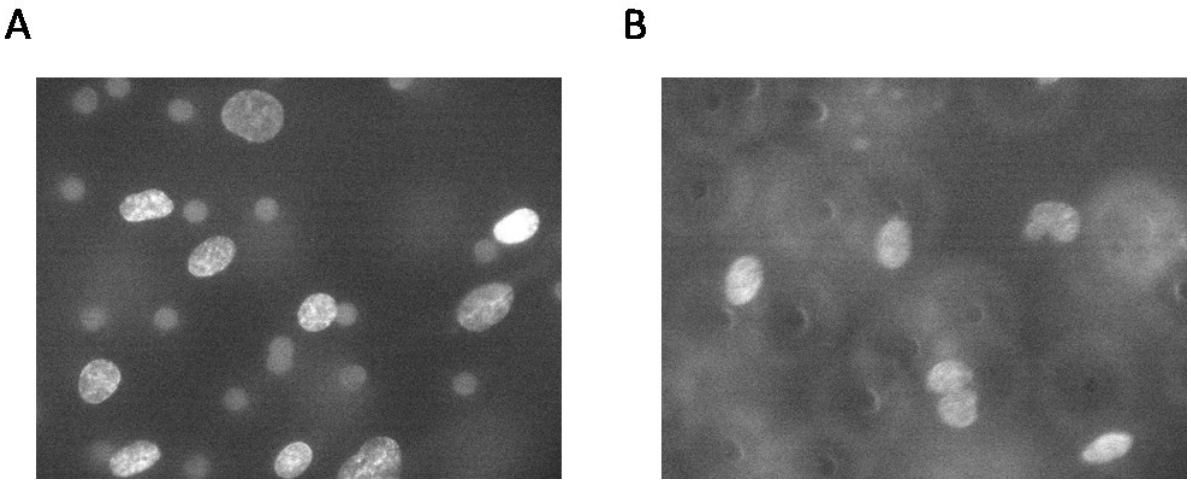


Figure 2.2: Analysis of the transfilter migration assay. LEC were fixed onto the cell culture inserts with 2% formaldehyde and the nuclei were stained with 2µg/ml bisbenzimide. An invert fluorescent microscope was used to visualise the cells. To calculate percentage migration, cells on the upper (A) and lower (B) side of each insert were counted. The number of cells on the lower side of the insert was expressed as a percentage of the total number of adherent cells.

These experiments showed that after 48 hours incubation, there was no difference in migration between the three concentrations of VEGF-C (Figure 2.3). At 24 hours, however, increased migration was seen with 300ng/ml VEGF-C compared to either 150ng/ml or no VEGF-C. Therefore, 300ng/ml VEGF-C and a timepoint of 24 hours were selected for future experiments. This data was originally published as part of an MRes thesis (Langan, 2011), but is included here as a justification of the experimental conditions selected.

2.7.2. Determination of platelet number and addition time

LEC were seeded onto inserts in the presence or absence of VEGF-C as described above and were incubated at 37°C for an hour before $1x10^4$ – $1x10^8$ washed human platelets were added in a volume of 100µl. Non-platelet controls were treated with 100µl modified Tyrode's buffer. After 24 hours, the filters were washed, fixed and stained as described above. These experiments showed that platelets had no effect on LEC migration in the absence of VEGF-C, but that they inhibited LEC migration in a count-dependent manner in the presence of VEGF-C (Figure 2.4). The effect of platelets was significant when $1x10^7$ or $1x10^8$ were applied. Therefore, future experiments used $1x10^8$ platelets.

Separate experiments were performed to determine whether the addition of washed platelets at the start would prevent LEC adhering to the inserts. In these experiments LEC were seeded onto the inserts with 300ng/ml VEGF-C added to the wells. $1x10^8$ washed human platelets were added either immediately after LEC or an hour later. The cells were

fixed and stained after 24 hours incubation at 37°C. These experiments showed that the time of platelet addition had no effect on the number of cells on the insert or on the inhibitory effect of the platelets themselves (Figure 2.5). Therefore, further experiments maintained the hour-long incubation between seeding LEC and the addition of platelets. The data for platelet number and time of addition were originally presented in an MRes thesis and have been included to explain the choice of experimental conditions.

2.7.3. Determination of the podoplanin crosslinking strategy

In initial experiments, the transfilter migration assay was performed with the addition of 2µg/ml anti-human podoplanin or 2µg/ml rat IgG alone. The antibodies were added an hour after the LEC had been seeded and percentage transmigration was determined after 24 hours. Neither the anti-podoplanin antibody nor the rat IgG inhibited LEC migration (Figure 2.6A), so further experiments were performed to determine the effect of crosslinking these antibodies. 2µg/ml rat IgG or anti-human podoplanin was crosslinked by addition of an anti-rat IgG_{2a} at ratios of 1:1, 7.5:1 or 15:1. For crosslinking, the primary antibody was added 30 minutes after LEC had been seeded and the secondary antibody was added after a further 30 minutes. Percentage transmigration was assessed after 24 hours. This showed that treatment with the rat IgG and secondary antibody had no effect on LEC migration, but that applying the anti-human podoplanin with the crosslinking secondary inhibited LEC migration in the presence of VEGF-C (Figure 2.6B). The inhibition increased as the ratio of secondary to primary antibody was increased. Therefore, in further crosslinking experiments 2µg/ml anti-human podoplanin and 30µg/ml anti-rat IgG_{2a} were used.

2.7.4. Transfilter assays with the addition of inhibitors, growth factors or antibodies

Transfilter assays were used to assess LEC migration under a range of conditions, including migration in the presence of inhibitors of Rho (CT04; Cytoskeleton Inc., Colorado, USA), ROCK (Y27632; Calbiochem, Nottingham, UK) and PKA (H-89; Calbiochem). In experiments where inhibitors were combined with crosslinking, LEC were seeded onto the filters and incubated at 37°C for 30 minutes, before crosslinking was carried out as described above (2.7.3). After a further 30-minute incubation at 37°C the inhibitors were added.

The effect of different growth factors on LEC migration was also assessed. Migration in the presence of 300ng/ml VEGF-C, 10ng/ml FGFb (Peprotech, London, UK) or 20ng/ml EGF (Sigma) was compared. These experiments were performed at 12, 16 and 18-hour timepoints.

The effects of VEGFR2 and VEGFR3-blocking antibodies (IMC-1121b and IMC-3C5, respectively) on LEC migration were also assessed in the presence of 300ng/ml VEGF-C or 30ng/ml VEGF-A. Both antibodies were used at a concentration of 5µg/ml and percentage transmigration was assessed after 24 hours.

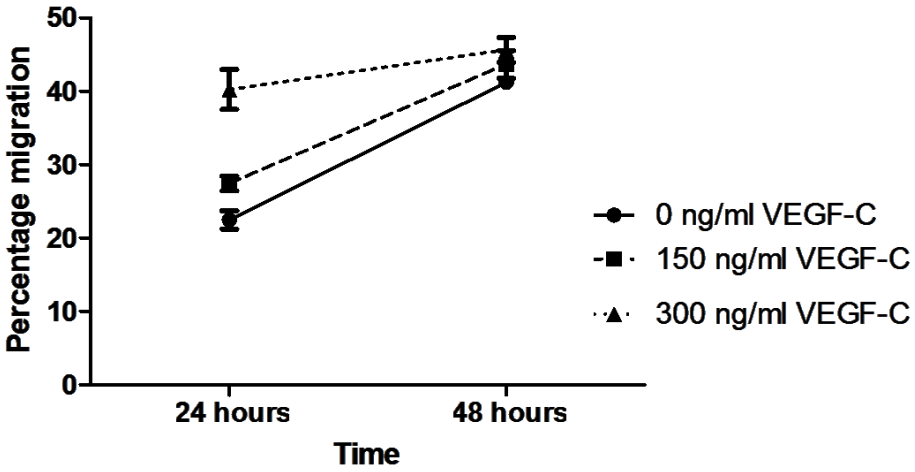


Figure 2.3: Effect of timepoint and VEGF-C concentration in the transfilter migration assay. LEC were seeded onto inserts with 8µm pores in culture medium with or without VEGF-C and incubated at 37°C for 24 or 48 hours. Data are mean ± SEM of two independent experiments. This data was originally published as part of an MRes thesis (Langan, 2011).

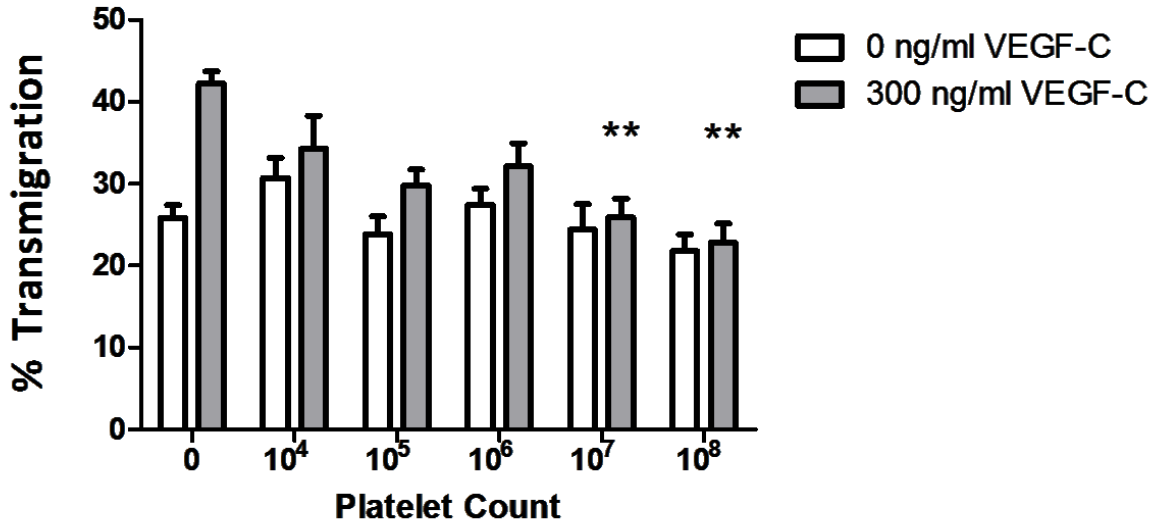


Figure 2.4: Effect of washed human platelets on LEC migration. LEC were seeded onto cell inserts in culture medium with or without 300ng/ml VEGF-C and incubated at 37°C for an hour. $1x10^4$-$1x10^8$ washed human platelets were added to the filter and percentage transmigration was assessed after 24 hours. ANOVA showed an effect of both platelets and VEGF-C (both $p<0.01$). ** = $p<0.01$ compared to no platelets by Dunnett's test. Data are mean ± SEM of four independent experiments. This data was originally presented as part of an MRes thesis (Langan, 2011).

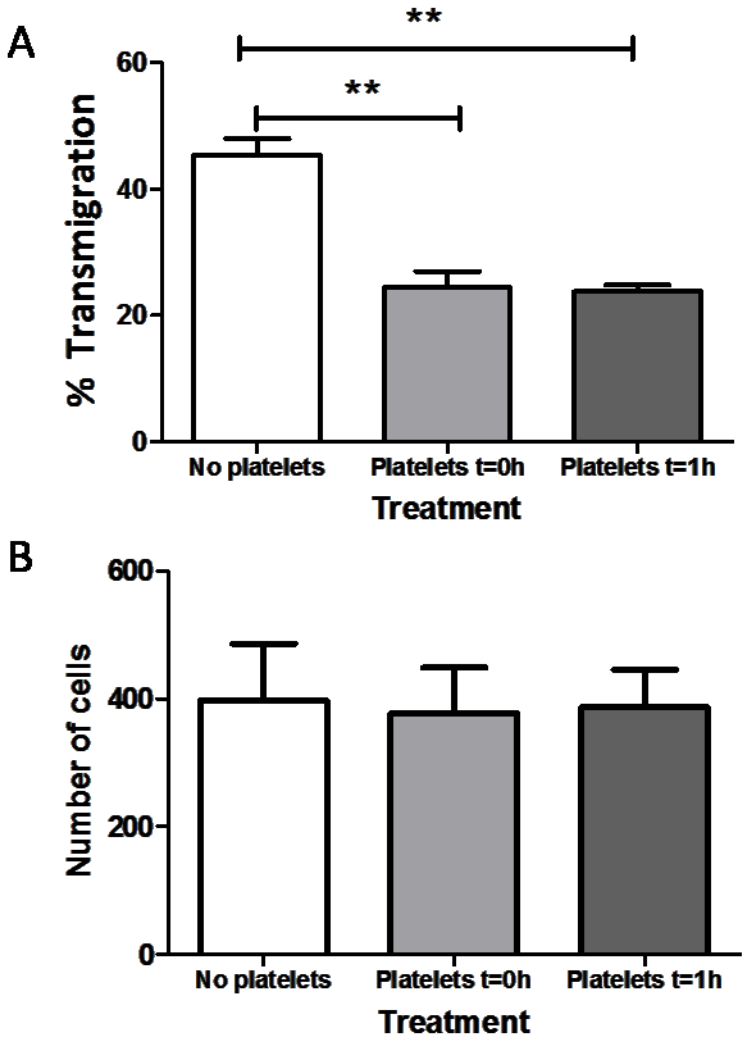


Figure 2.5: Effect of time of platelet addition on LEC transmigration and number of cells adhering to the filter. LEC were seeded onto filters in culture medium with 300ng/ml VEGF-C. $1x10^8$ platelets were added at the same time as LEC (t=0h) or after the plate had been incubated at 37°C for an hour (t=1h). Percentage transmigration was assessed after 24 hours. A) ANOVA showed that the presence of platelets was significant ($p<0.01$). ** = $p<0.01$ by Bonferroni post-test. B) The cells in the upper and lower field of each filter were counted. These were added together to give the number of adhered cells in 12 fields per insert. ANOVA showed no effect of treatment on adhered cells ($p = 0.982$). Data are mean ± SEM of three independent experiments and were originally reported in an MRes thesis (Langan, 2011).

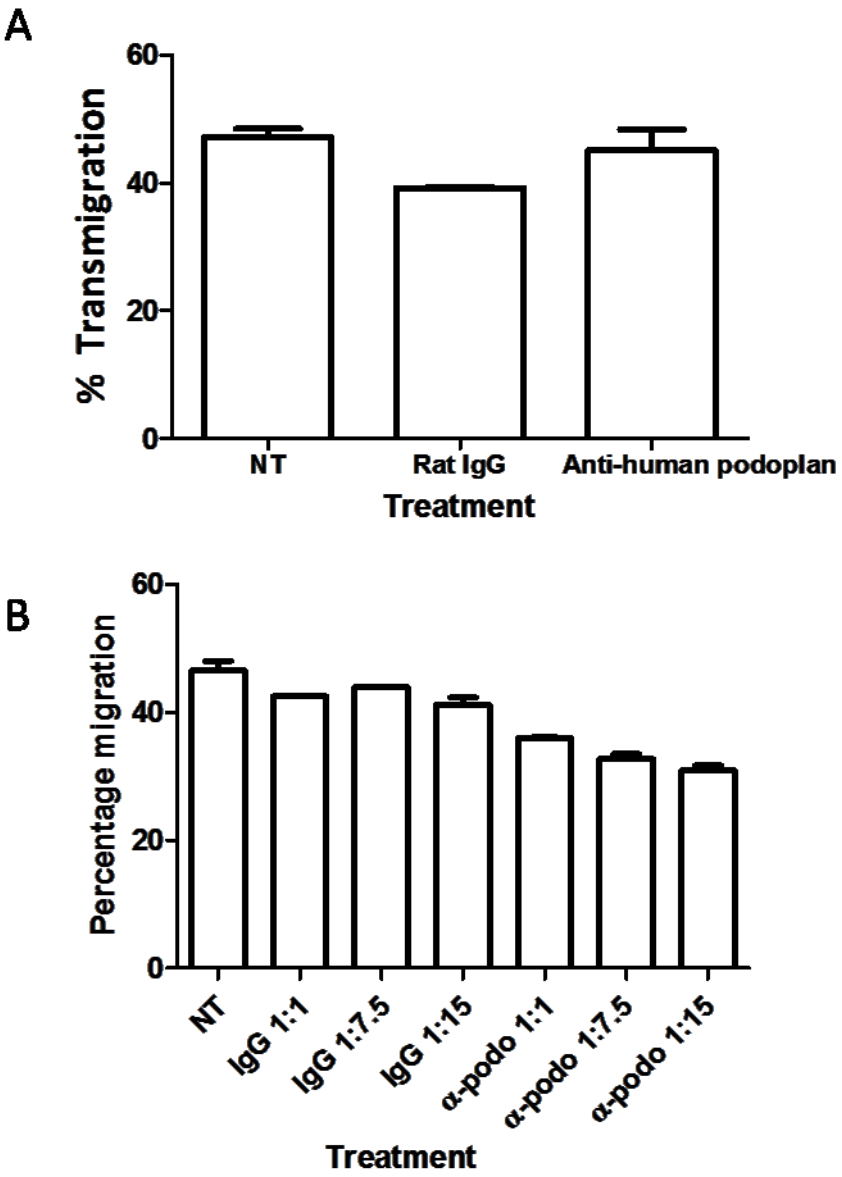


Figure 2.6: Effect of antibodies and antibody-mediated crosslinking on LEC migration. LEC were seeded onto cell culture inserts in culture medium containing 300ng/ml VEGF-C. A) Inserts were incubated for an hour at 37°C before the addition of 2µg/ml rat IgG or anti-human podoplanin (NZ-1.3). Percentage transmigration was assessed after 24 hours. Data are mean ± SEM of two independent experiments. B) Plates were incubated at 37°C for 30 minutes before the addition of 2µg/ml rat IgG or anti-human podoplanin. After a further 30 minutes incubation an anti-rat secondary antibody was added at the indicated ratios. Percentage transmigration was assessed after 24 hours. Error bars represent mean ± SEM of at least two independent experiments. This data was previously submitted as part of an MRes thesis (Langan, 2011).

2.8. Scratch wound assay

$1x10^5$ LEC were seeded onto wells of a 12-well plate that had been covered with 1% gelatin or left untreated. Before treatment with gelatin, lines were drawn on the underside of each well to divide it into three equal sections. The plate was incubated at 37°C and 5% CO_2 until the LEC had formed a confluent monolayer. Phase contrast images were taken and the monolayers were scratched with a 200µl pipette tip, with the wound being made in the central section of the well. Detached cells were washed away with 1ml sterile PBS. 1ml culture medium with or without 300ng/ml VEGF-C was added to each well and phase contrast images were taken using the 4x objective on an invert microscope (Olympus, Southend-on-Sea, UK). These images represent t=0 hours. Further images were taken at points between 16 and 48 hours.

2.8.1. Scratch wound assay with the addition of platelets

LEC were seeded onto 12-well plates and allowed to grow to confluence. Washed human platelets were isolated as described above (section 2.6.2) and diluted to $1x10^9$/ml. To assess the effect of platelet addition before wounding, $2x10^8$ platelets and 800µl Ca^{2+}/Mg^{2+}-free sterile PBS were added to wells along with 1ml culture medium with or without VEGF-C at a final concentration of 300ng/ml. The plate was incubated for an hour at 37°C. Medium and any free platelets were removed from all wells and the monolayers were scratched. Detached cells were washed away with 1ml Ca^{2+}/Mg^{2+}-free sterile PBS, before culture medium with or without 300ng/ml VEGF-C was added to each well. To determine the effect of platelets after wounding, $2x10^8$ washed platelets were added to monolayers immediately

after wounding, using the same medium as above. Images were taken at regular intervals between 0 and 48 hours.

2.8.2. Scratch wound assay with podoplanin crosslinking

LEC were seeded and wounded as described above (section 2.8). After detached cells had been washed away, 1ml culture medium with or without 300ng/ml VEGF-C was added to each well. The plate was imaged, then 2µg/ml anti-human podoplanin (clone NZ-1.3) was added to chosen wells. The plate was incubated at 37°C for 30 minutes. Crosslinking was induced in some of the antibody-treated wells by adding 30µg/ml anti-rat IgG_{2a}. The plate was kept at 37°C and images were taken at regular intervals between 16 and 48 hours. Four images were taken of each well. Images were taken at approximately the same location, using the lines drawn on the base of the plate as a guide.

The area of the wound in the different images was analysed in ImageJ. To do this, the polygon selection tool was used to draw around the perimeter of the wound and the area of the wound was calculated (Figure 2.7). The wound areas were expressed as a percentage of the wound at the start of the experiment. This was calculated as follows:

$$\text{Percentage wound area} = \frac{Area\ of\ wound}{Area\ of\ wound\ at\ t = 0\ hours} \times 100$$

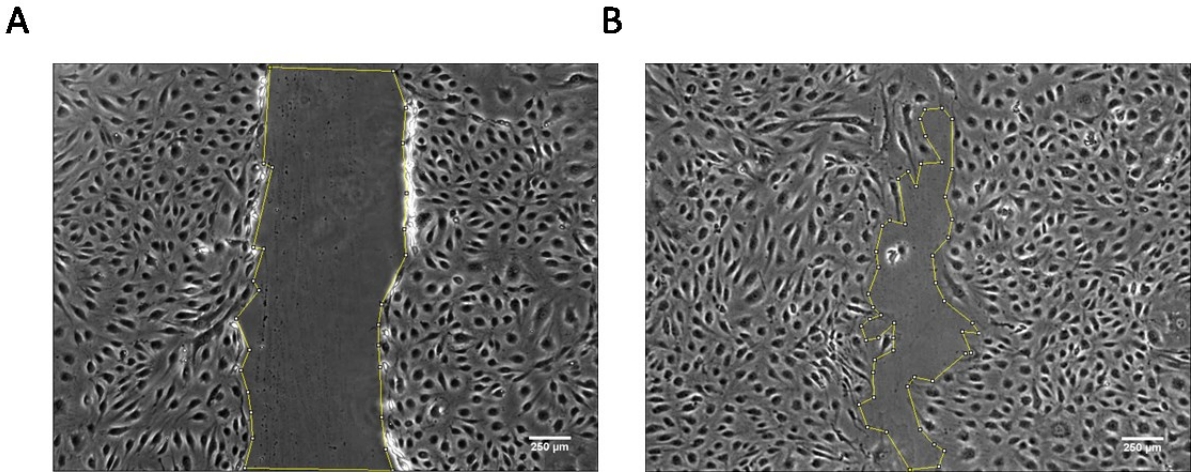


Figure 2.7: Analysis of the scratch wound assay. Phase contrast images were taken using a 4x objective. The polygon selection tool on ImageJ was used to mark the perimeter of the wound and the area of the wound was calculated by ImageJ. Wound areas were expressed as a percentage of the wound at t=0 hours. (A) Image of wound taken at t-0 hours. (B) Image of wound taken at t-16 hours.

2.9. Formation of endothelial "tubes" in co-cultures with fibroblasts

$3x10^4$ human dermal fibroblasts were seeded onto wells of a 12-well plate and maintained at 37°C and 5% CO_2 until confluent (typically four days). LEC were incubated with 5µM Cell Tracker Green (Life Technologies, Paisley, UK) in 10ml of the appropriate culture medium for 30 minutes at 37°C. The cells were centrifuged at 400 x *g* for 5 minutes, then resuspended in 4-12ml medium and incubated for a further 30 minutes at 37°C. $3x10^4$ endothelial cells were seeded onto each confluent well of fibroblasts. In initial experiments, the co-cultures were imaged every day to assess the appropriate timepoint for later experiments. At the end of each experiment, the co-cultures were washed twice with PBS and fixed with ice-cold 70% ethanol for 30 minutes at room temperature. The cultures were washed with PBS and stored at 4°C in 1ml PBS until post-staining.

2.9.1. Addition of platelets

To test whether platelets prevented the formation of tubes, LEC were stained with Cell Tracker Green and seeded onto the fibroblasts in the presence of 300ng/ml VEGF-C as described above (2.9). The plate was incubated at 37°C for an hour, then $2x10^8$ washed human platelets were added to the assay. The co-cultures were imaged after three days.

In separate experiments, the ability of platelets to disrupt pre-formed tubes was tested. LEC were stained and seeded onto fibroblasts in the presence of VEGF-C, as described above. The co-culture was incubated at 37°C and 5% CO_2 for three days, before fluorescent images were

taken. $2x10^8$ washed human platelets were added to some of the cultures and the plate was incubated at 37°C for 24 hours. The co-cultures were then imaged again. In other experiments, platelets isolated from *Clec1b*$^{fl/fl}$ or *Clec1b*$^{fl/fl}$PF4-Cre mice were added instead of human platelets.

2.9.2. Addition of crosslinking antibodies

LEC were seeded onto fibroblasts as described above (2.9). The plate was incubated at 37°C for 30 minutes, after which 2µg/ml rat IgG or anti-human podoplanin (clone NZ-1.3) was added. The plate was incubated for 30 minutes at 37°C, then 30µg/ml anti-rat IgG_{2a} was added to the wells to induce crosslinking. The plates were kept at 37°C and 5% CO_2 for three days before fluorescent images were taken. In separate experiments, the effect of crosslinking on pre-formed tubes was assessed. In these experiments, LEC were co-cultured with fibroblasts for three days. Fluorescent images were taken before the crosslinking antibodies were added, as described above. The plate was incubated for a further 24 hours at 37°C before more fluorescent images were taken. The cultures were fixed with ice-cold 70% ethanol and stored at 4°C until post-staining.

2.9.3. Post-staining of co-cultures

Fixed co-cultures were washed twice with 1ml PBS. 0.4µg/ml anti-human podoplanin (clone 18H5) in 1% bovine serum albumin (BSA; Sigma) was added to each well and the plate was incubated at 37°C for 45 minutes. Cultures were washed three times with PBS, before 1:500

alkaline phosphatase-conjugated anti-mouse (Sigma) in 1% BSA was added. The plate was incubated at 37°C for 45 minutes. Cultures were washed twice with 1ml PBS, then three times with 1ml distilled water. The tubes were visualised by adding 500µl SigmaFast BCIP/NBT substrate (Sigma) and incubating at room temperature for 25 minutes. The reaction was stopped by washing the cultures with 1ml distilled water. The plates were then protected from light and allowed to dry at room temperature.

2.9.4. Analysis of co-culture experiments

All co-culture images were obtained using an invert microscope (Olympus). Fluorescent images of the Cell Tracker Green were taken using the 10x objective, while bright field images of the alkaline phosphatase substrate were taken with the 4x objective. Bright field images were analysed using ImageJ 1.49 (NIH, Maryland, USA) and the associated angiogenesis plugin (Angiogenesis Analyzer; Carpentier et al., 2012).

First, the background was removed from each image using the "Threshold" function in ImageJ (Figure 2.8A). Then the image was converted to RGB Colour format, again using ImageJ. The Angiogenesis Analyzer plugin was then used to analyse the network formation in the images (using the "Analyze HUVEC Phase Contrast" option). This created another image displaying the tree that the plugin had created (Figure 2.8A); this included identification of isolated sections (dark blue), branches (green) and junctions. The plugin also generated a data table based on the tree and from this, total tube length was chosen for comparison between

treatments. Total tube length was used as the parameter for comparing treatments as this same parameter could also be determined in images that were analysed manually, thus allowing comparison of the two different analyses.

This plugin was not suitable for the fluorescent images due to the uneven illumination of these images. Therefore, the 10x objective fluorescent images were analysed manually. The segmented line tool in ImageJ was used to draw along each tube and then measure the length of each (Figure 2.8B). The length of the tubes in each image was added together to give the total tube length. There is a potential for bias in this analysis, which could have been avoided by giving the data to another person to analyse. To avoid bias, in the experiments with mouse platelets, I was not aware of the genotype of the platelets until after the analysis had been completed.

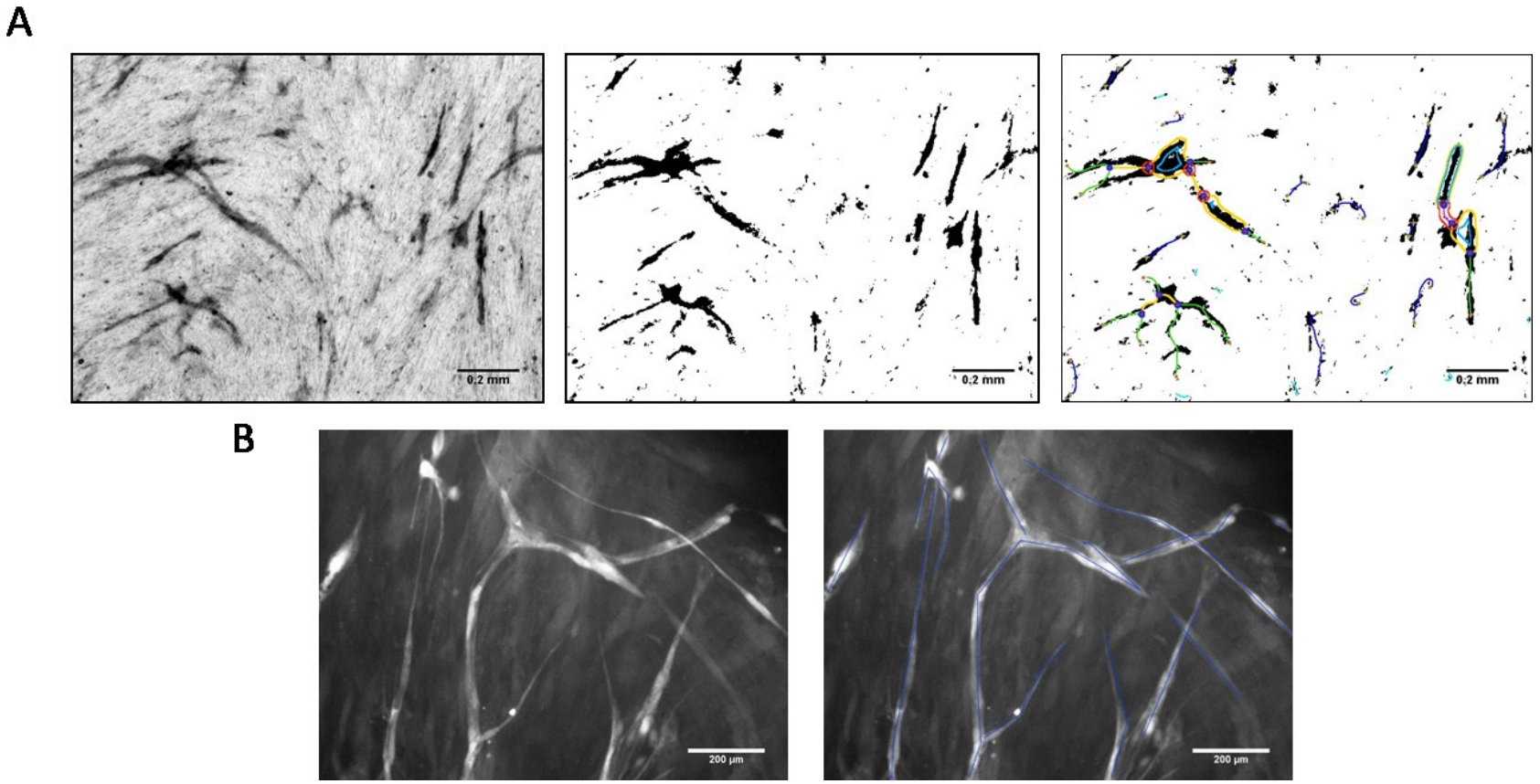


Figure 2.8: Analysis of the co-culture assay. A) Representative bright field image before (left) and after (centre) thresholding to remove the background. After the Angiogenesis Analyzer plugin had finished analysing each image, it produced a tree diagram (right) showing the objects included in the analysis. Isolated sections: dark blue; branches: green; turquoise: fragments; master junction: blue circle with red centre; mesh area: yellow with internal turquoise line. B) Fluorescent images that had been taken using the 10x objective were analysed manually using ImageJ. The segmented line tool was used to draw along each tube; the length of the tube was then measured. The total length of the tubes in each image was calculated from these measurements. Left: representative fluorescent image before analysis. Right: annotated image to show the sections of tube that were measured in this image.

2.10. RhoA activation assay

RhoA activation was assessed using a G-LISA RhoA assay (Cytoskeleton Inc.). This assay is based on a sandwich ELISA and gives results in terms of absorbance at 490nm. There is a RhoA-GTP binding protein attached to the wells of the provided plate. Any RhoA-GTP in a sample will bind to the plate and is then detected using an anti-RhoA antibody and horseradish peroxidase (HRP)-labelled secondary antibody. In these experiments, each condition was tested in duplicate, alongside a blank sample and positive control Rho protein that was provided as part of the assay kit. All samples, and the assay plate, were kept on ice, unless otherwise stated. While the assay does not give an actual concentration of RhoA in each sample, it gives readouts in absorbance, allowing quantitative comparison between samples.

2.10.1. Timecourse of response to VEGF-C

$4.2x10^5$ LEC were seeded onto 6-well culture inserts with 3µm pores (BD Biosciences) and were incubated at 37°C for two hours. Cells were stimulated with 300ng/ml VEGF-C for 5, 15, 30 or 60 minutes at room temperature. The basal samples were prepared immediately. At the end of the required incubation, LEC were washed twice with ice-cold Ca^{2+}/Mg^{2+}-free PBS and lysed on ice for five minutes with 100µl cell lysis buffer (including protease inhibitor cocktail) provided in the G-LISA assay kit. Cells and lysate were scraped from the filters and transferred into cold 1.5ml tubes. The samples were centrifuged at 10,000 x *g* for one minute. The lysates were stored at -80°C.

Before the G-LISA assay was performed, the amount of protein in each cell lysate was quantitated. 10µl of each lysate was applied to wells of a 96-well plate; 10µl lysis buffer was used as a negative control. 290µl Precision Red protein detection reagent (Cytoskeleton Inc.) was added to each well and the plate was incubated for one minute at room temperature. Absorbance was read at 600nm using a VersaMax spectrophotometer. The value obtained with the negative control was subtracted from all other values. The resulting values were multiplied by 3.75 to give a protein concentration in mg/ml. In order to be suitable for use in the G-LISA assay, samples had to have a protein concentration of at least 0.4mg/ml.

100µl ice-cold distilled water was added to the required wells of the provided 96-well assay plate. Lysates were made up to equal concentrations in 60µl volumes in the provided lysis buffer and the RhoA control protein was diluted 1:5 in lysis buffer to give a 60µl sample. 60µl binding buffer was added to each tube; the tubes were vortexed and placed on ice. Water was removed from the plate, then 50µl of each sample was applied in duplicate. The plate was incubated at 4°C on an orbital shaker for 30 minutes. The wells were washed twice with 200µl wash buffer, then incubated with 200µl antigen-presenting buffer for two minutes at room temperature. The plate was then washed three times with 200µl wash buffer. 50µl anti-RhoA (1:250 in antibody dilution buffer) was added to each well and the plate was incubated for 45 minutes at room temperature on an orbital shaker. Wells were washed as before, then 50µl secondary antibody (1:62.5 in antibody dilution buffer) was added to each well. The plate was incubated on an orbital shaker for 45 minutes at room temperature. The wells were washed three times with 200µl wash buffer. Equal volumes of the HRP detection

reagents were mixed and 50µl of this solution was applied to each well. The plate was incubated at 37°C for 15 minutes, then the reaction was stopped by the addition of 50µl HRP stop buffer. Absorbance was then measured at 490nm using a VersaMax spectrophotometer (Molecular Devices, Wokingham, UK).

2.10.2. Effect of crosslinking of podoplanin

$4.2x10^5$ LEC were seeded onto 6-well filters in the presence of MV2 medium and the plate was incubated at 37°C for an hour. Crosslinking was induced in some wells by the addition of 2µg/ml anti-human podoplanin followed by 30µg/ml anti-rat IgG_{2a} 30 minutes later. Wells were treated with 300ng/ml VEGF-C or fresh MV2 medium for five minutes, before the cells were lysed as described above (see section 2.10.1). In separate experiments, LEC were seeded in the presence of MV2 medium or 300ng/ml VEGF-C. The plate was incubated at 37°C for an hour, before podoplanin crosslinking was induced by the addition of antibodies. The plate was incubated at 37°C for a further 10 hours, after which the cells were lysed and lysates were stored at -80°C.

2.11. Co-immunoprecipitation and Western blotting to analyse binding partners of podoplanin

2.11.1. Surface biotinylation of LEC and co-immunoprecipitation

LEC were seeded onto 10cm dishes and allowed to grow to confluence in MV2 medium. Once confluent, LEC were treated with 300ng/ml VEGF-C or MV2 medium for 24 hours. 20µl protein G sepharose beads were incubated with 750µl 1% Triton X-100 lysis buffer and 1µg non-specific human IgG_1, mouse IgG (MOPC) or 1µg antibody against the following (full details given in Table 2.1): α_2-integrin, VEGFR2, VEGFR3, podoplanin, ADAM10 and CD9. The tubes were incubated overnight at 4°C.

The following day, the LEC were washed three times with 10ml PBS, then incubated with 1mg/ml Sulfo-NHS-LC-biotin (Pierce, Paisley, UK) for 30 minutes at room temperature. The reaction was quenched with 2.5ml 300mM glycine. Cells were washed with PBS, scraped from the dishes and aliquoted into 1.5ml tubes, before being centrifuged at 2,650 x *g* for three minutes. The supernatant was aspirated and the cell pellets lysed in 1% Brij97 lysis buffer (1% Brij 97, 10mM Tris, 150mM NaCl, 2mM $CaCl_2$, 2mM $MgCl_2$, 0.02% sodium azide) containing protease inhibitor cocktail (Sigma). The tubes were incubated on ice for 30 minutes while lysis completed; the cells were pelleted by centrifuging at 20,800 x *g* for 10 minutes. Meanwhile, the antibody-coupled beads were washed twice with 1ml 1% Brij97 lysis buffer. The LEC lysates were incubated with the antibody-coupled beads for 90 minutes at 4°C. The bead-bound samples were then washed four times with 1ml 1% Brij97 and transferred to clean 1.5ml tubes. 50µl of 2x non-reducing sample buffer was added to each

tube and the samples were stored at -20°C, to be analysed by Western blotting at a later date. The SDS in the non-reducing sample buffer elutes the samples from the protein G sepharose beads.

2.11.2. Western blot: Odyssey exposure

Samples were boiled for five minutes, then run on 4-12% Tris-glycine gradient gels (Novex, Paisley, UK) at 125V. Gels and blotting membrane (Millipore, Watford, UK) were pre-incubated in Western transfer buffer (24mM Tris, 191mM glycine, 20% methanol) for 30 minutes at room temperature. The proteins were transferred onto the membrane by running at 30V for 90 minutes. The membranes were blocked with 5% milk powder in Tris-buffered saline with Tween 20 (TBST; 20mM Tris, 137mM NaCl, 0.1% Tween) for an hour at room temperature. The membranes were washed twice with TBST, then incubated with anti-human CD9 antibody (C9.BB; 1:20 in 3% BSA) overnight at 4°C. Membranes were rinsed with TBST, then washed three times for 10 minutes with high-salt TBST (0.5M NaCl in TBST). The secondary antibodies, streptavidin-800 and anti-mouse-680, were prepared at a 1:10,000 dilution in 3% BSA. The membranes were incubated with the secondary antibodies for two hours at room temperature. The membranes were washed five times for five minutes with high-salt TBST, followed by a final 5-minute wash with Tris-buffered saline (TBS; 20mM Tris, 137mM NaCl). The membranes were blotted dry then visualised using an Odyssey imaging system (LI-COR Biosciences, Cambridge, UK).

2.12. Statistical analysis

Graphs were created in GraphPad Prism 5.0. Error bars represent the standard error of the mean (SEM). Statistical tests were performed in Minitab 13.3. Data expressed as percentage transmigration was first arcsine transformed before being tested for significance. The effects of individual treatments were compared using paired or unpaired t-tests as appropriate. Where effects of multiple conditions needed to be analysed, one- or two-way Analysis of Variance (ANOVA) was performed with post-hoc tests where appropriate; Bonferroni's test to compare between treatments or Dunnett's test to compare treatments to control.

CHAPTER THREE

THE EFFECT OF THE PODOPLANIN-CLEC-2 INTERACTION ON LYMPHATIC ENDOTHELIAL CELL MIGRATION

3.1. Introduction

CLEC-2 and podoplanin are known to be required for correct development of the lymphatic vasculature (Schacht et al., 2003; Bertozzi et al., 2010b; Suzuki-Inoue et al., 2010; Uhrin et al., 2010; Finney et al., 2012), but the mechanism underlying their role is not currently understood. Platelet-expressed CLEC-2 is known to interact with podoplanin, a transmembrane protein expressed by LEC (Wetterwald et al., 1996; Suzuki-Inoue et al., 2007). Podoplanin is thought to be required for efficient migration of epithelial cells and this requirement may be connected to signalling downstream of podoplanin affecting the actin cytoskeleton and RhoA signalling (Martín-Villar et al., 2006; Moustakas and Heldin, 2007).

It has been suggested that activation of platelets through CLEC-2 prompts the release of soluble angiogenic mediators that promote lymphatic development (Bertozzi et al., 2010b). However, lymphatic defects have not been reported in Glanzmann thrombasthenia patients, who lack α-granules and dense granules in platelets (Nurden et al., 2012). A separate study has shown that platelets from wildtype (*Clec1b*$^{fl/fl}$) mice inhibit VEGF-C mediated migration of LEC and that the platelets from CLEC-2 deficient (*Clec1b*$^{fl/fl}$PF4-Cre) mice have a significantly weaker inhibitory effect (Finney et al., 2012). This suggests that the role of platelets in lymphangiogenesis is through a direct interaction between CLEC-2 and podoplanin.

Platelets, like many other cell types, are able to release microvesicles when they are activated. Human platelet microvesicles range from 0.1-1µm in size and have been shown to express GPIb and integrins α_{IIb} and β_3 (Abrams et al., 1990; Scharf et al., 1992). During the course of this thesis, data obtained by our group showed that microvesicles derived from human platelets expressed CLEC-2 (Gitz et al., 2014). Therefore, we hypothesised that platelet microvesicles would also be able to inhibit LEC migration.

The studies in this chapter aimed to further assess the roles of CLEC-2 and podoplanin in regulating the migration of LEC. Two *in vitro* migration assays were used: the transfilter assay and the scratch wound assay. We hypothesised that the interaction between platelet CLEC-2 and LEC-expressed podoplanin would modulate LEC migration. To test this, both platelets and antibody-mediated crosslinking of podoplanin were used in the two migration assays. We also assessed whether platelet-derived microvesicles could modulate LEC migration.

3.2. Results

3.2.1. Effect of platelets on the migration of LEC, HMEC-1 and HUVEC

The transfilter migration assay was initially characterised as part of an MRes thesis (Langan, 2011) and the results of this characterisation are detailed in the Materials and Methods chapter. Our previous experiments used platelets that had been isolated using the prostacyclin method. As this isolation method requires the platelets to be rested for at least

30 minutes before use, we also isolated platelets using the phosphodiesterase inhibitor theophylline and compared the two platelet preparations in transfilter migration assays. We found that both types of washed platelets were able to inhibit VEGF-C stimulated LEC migration and that there was no difference between the two preparations (Figure 3.1). Therefore, future experiments used the theophylline method of platelet isolation as these platelets did not need to be rested before they could be used in an assay.

To investigate whether the inhibitory effect of platelets required expression of podoplanin, transfilter assays were also performed with HMEC-1, an immortalised endothelial cell line that was previously shown to express podoplanin (Nisato et al., 2004), and HUVEC, which do not (Gröger et al., 2004; Sölder et al., 2012). We first tested whether our supply of HMEC-1 expressed podoplanin, and found this present but at a much lower level than LEC (Figure 3.2). Transfilter assays using HMEC-1 had to be re-characterised, as we found that these cells migrated faster than LEC. As such, there was little difference between basal and VEGF-C stimulated HMEC-1 migration after 4 or 24 hours, but the difference was apparent after 12 and 20 hours (Figure 3.3A). A timepoint of 12 hours was selected for further experiments with HMEC-1 as the difference between basal and VEGF-C stimulated migration was greatest then.

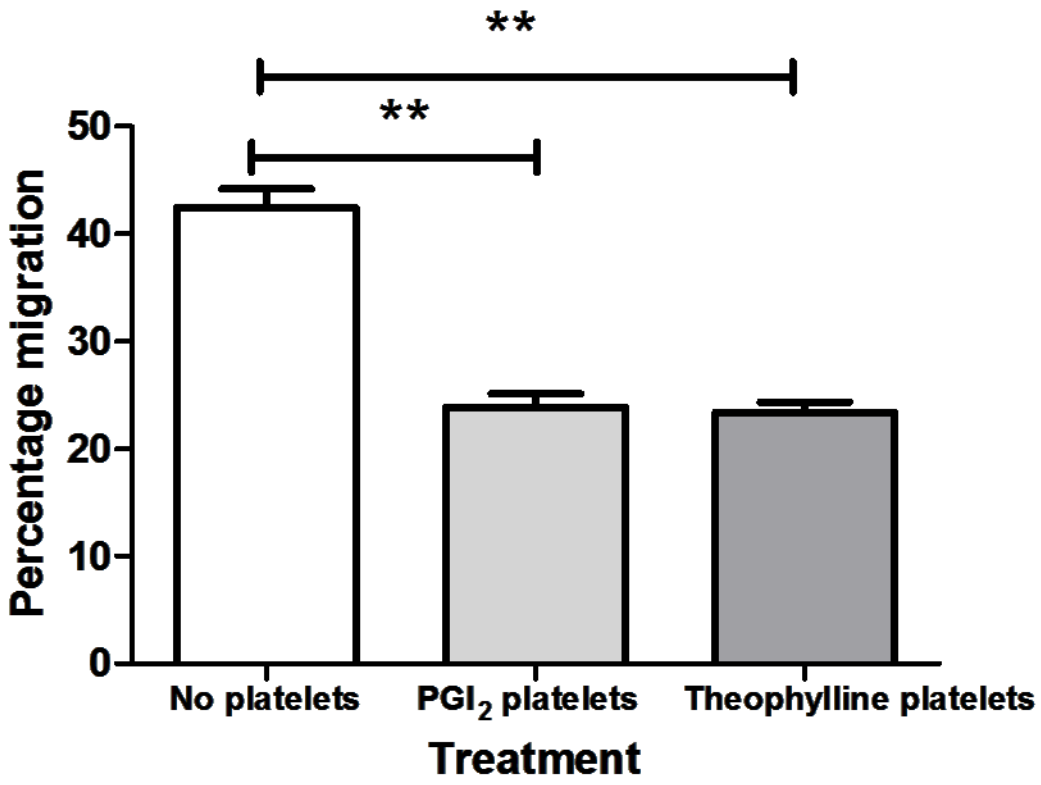


Figure 3.1: Effects of platelets obtained using different isolation methods on LEC migration. LEC were seeded onto culture inserts with 8µm pores in culture medium containing 300ng/ml VEGF-C. After incubation for an hour at 37°C, $1x10^8$ washed platelets were added to the filter. The platelets had been isolated using either the prostacyclin (PGI_2) or theophylline method. Percentage transmigration was assessed after 24 hours. ANOVA showed that the effect of platelets was significant ($p<0.01$). ** = $p<0.01$ by Bonferroni post-hoc test. Data are mean ± SEM of six independent experiments.

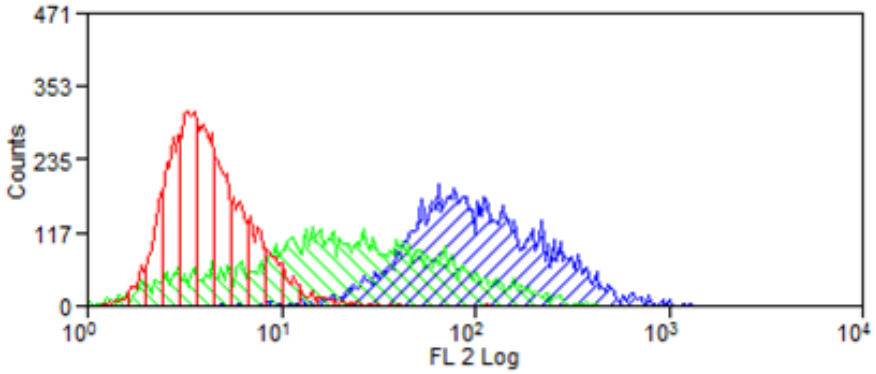


Figure 3.2: Podoplanin expression on LEC and HMEC-1. LEC and HMEC-1 were detached from flasks and stained with 0.125µg/ml PE-conjugated anti-human podoplanin or rat IgG. Samples were incubated on ice for 40 minutes before being analysed on a FACSCalibur flow cytometer. Red: IgG-LEC; Green: podoplanin-HMEC-1; Blue: podoplanin-LEC. Traces are representative of two independent experiments.

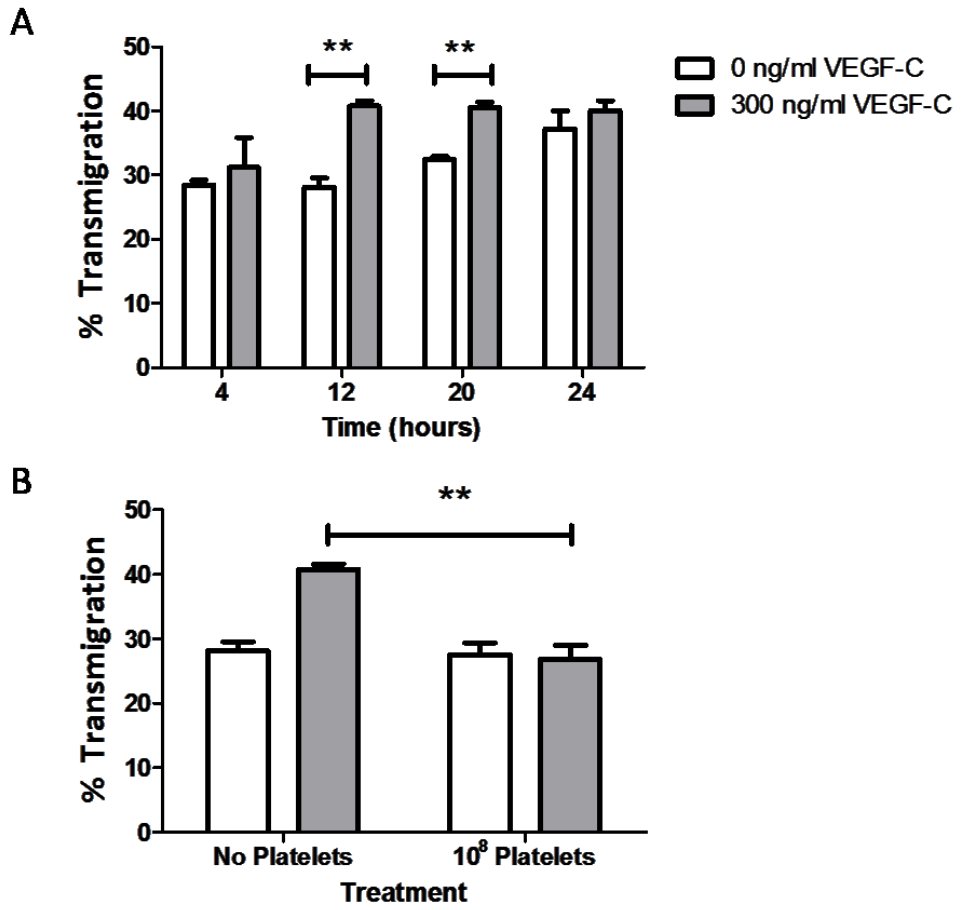


Figure 3.3: Characterisation and effect of platelets on the migration of the HMEC-1 endothelial cell line. (A) HMEC-1 were seeded onto filters with 8µm pores in the presence of culture medium with or without 300ng/ml VEGF-C. The plate was incubated for 4, 12, 20 or24 hours, after which the cells were fixed and percentage transmigration was determined. ANOVA showed that both time ($p<0.05$) and VEGF-C ($p<0.01$) had significant effects on migration. ** = $p<0.01$ by Bonferroni post-test. (B) HMEC-1 were seeded onto filters for an hour before 100µl sterile Ca^{2+}/Mg^{2+}-free PBS or $1x10^8$ washed human platelets was added. After 12 hours, percentage transmigration was assessed. ANOVA showed that there was a significant effect of both VEGF-C ($p<0.05$) and platelets ($p<0.05$). ** = $p<0.01$ by Bonferroni post-test. Both data sets are mean ± SEM of three independent experiments

The ability of platelets to modulate HMEC-1 migration was then tested. HMEC-1 were allowed to settle on the culture inserts for an hour before the addition of 100µl Ca^{2+}/Mg^{2+}-free sterile PBS or $1x10^8$ washed human platelets to the upper culture medium. Platelets were able to inhibit VEGF-C stimulated migration of HMEC-1, but had no effect on unstimulated migration (Figure 3.3B).

We next performed transfilter assays with HUVEC. In our hands, these cells do not express podoplanin. Instead of stimulating with VEGF-C, HUVEC were stimulated with VEGF-A as this growth factor is known to be important in angiogenesis and in the migration of vascular endothelial cells (Leung et al., 1989; Koch et al., 1994). We found that platelets had no effect on HUVEC migration in the absence of VEGF-A (Figure 3.4). However, $1x10^8$ platelets were able to significantly inhibit VEGF-A mediated migration of HUVEC (Figure 3.4).

To allow direct comparison of different endothelial cells, further experiments were carried out with LEC in which $1x10^8$ platelets inhibited migration by 46 ± 6% (mean ± SEM of four independent experiments). This number of platelets caused a 34 ± 6% inhibition in HMEC-1 migration and a 23 ± 4% reduction in HUVEC migration (mean ± SEM of three or four independent experiments, respectively). This trend in the effects of platelets is consistent with the differences in levels of podoplanin expression.

In order to assess whether platelets were preventing LEC migration by releasing a soluble mediator, transfilter assays were performed with platelet releasate. Washed platelets were isolated from whole human blood and 130µl of these cells were stimulated with 300nM rhodocytin under stirring conditions at 37°C for five minutes. This agonist was chosen as it activates platelets via CLEC-2, as does podoplanin (Suzuki-Inoue et al., 2006, 2007). The stimulated platelets were pelleted by centrifugation and the resulting supernatant was applied to the transfilter assay. We found that while the platelets inhibited VEGF-C mediated LEC migration, the platelet releasate had only a very small and non-significant effect (Figure 3.5). Thus, our data were consistent with the concept that the inhibitory effect of platelets was through interaction of CLEC-2 and podoplanin. To test this further, we decided to use siRNA to reduce podoplanin expression in LEC and use transfilter assays to assess whether this would alter the effect of platelets on LEC migration.

3.2.2. Effect of siRNA transfection on surface expression of podoplanin

Before we could assess the impact of podoplanin knockdown on LEC migration, the efficacy of knockdown had to be characterised. LEC were seeded onto wells of a 24-well plate in culture medium without penicillin, streptomycin or amphotericin. The plate was incubated overnight at 37°C and 5% CO_2. The next day, when cells were around 80% confluent, the LEC were transfected with non-specific or podoplanin siRNA duplexes. The podoplanin duplexes were used at 50, 70 or 90nM and the non-specific duplex was used at 50nM. As a negative control, LEC were treated with lipofectamine but no duplex. After transfection, LEC were maintained in culture medium with antibiotics and podoplanin expression was determined

by flow cytometry at 24, 48 and 72 hours after transfection. We found that both of the podoplanin duplexes were able to reduce podoplanin expression by approximately 50% and that this level of knockdown was consistent across the different timepoints (Figure 3.6A). We also found that podoplanin expression increased over time in cells treated with lipofectamine or transfected with non-specific siRNA (Figure 3.6A).

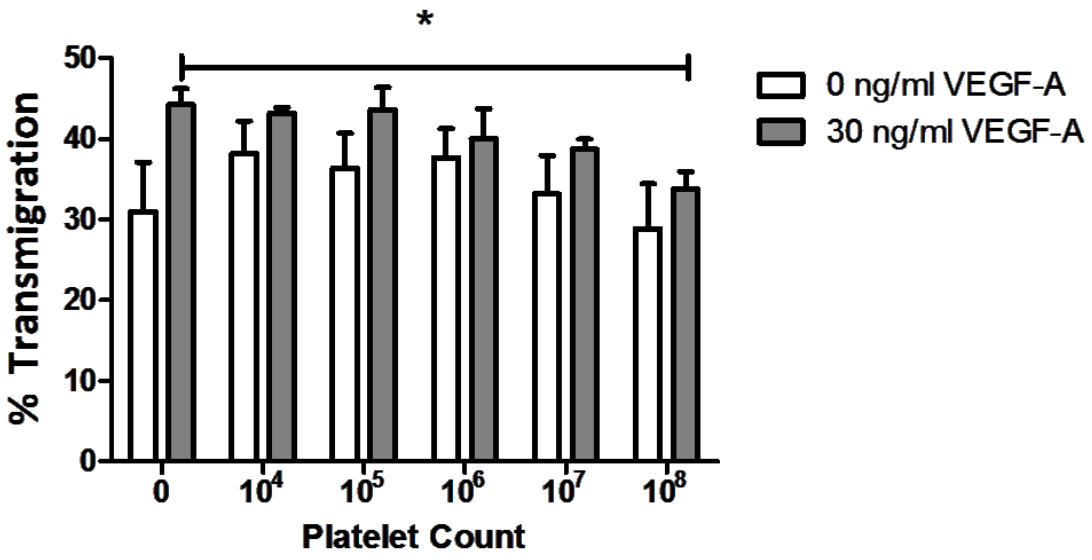


Figure 3.4: Effect of platelets on HUVEC migration. $3x10^4$ HUVEC were seeded onto cell culture inserts in medium with or without 30ng/ml VEGF-A. The inserts were incubated for an hour before different numbers of washed human platelets were added. 100µl sterile PBS was added to wells without platelets. Percentage transmigration was assessed after 24 hours. ANOVA showed that VEGF-A and platelets both had a significant effect (both $p<0.01$). * $p<0.05$ compared to no platelets and 30ng/ml VEGF-A by Dunnett's test. Data are mean ± SEM of four independent experiments.

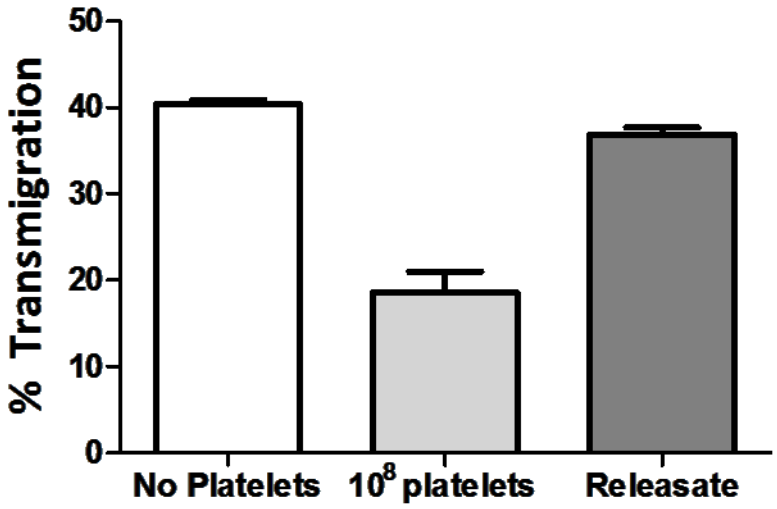


Figure 3.5: Effect of releasate from rhodocytin-stimulated platelets on LEC migration in the presence of VEGF-C. LEC were seeded onto cell culture inserts in medium containing 300ng/ml VEGF-C. $1x10^8$ washed human platelets or the releasate from platelets stimulated with 300nM rhodocytin were added to the filters. Percentage transmigration was assessed after 24 hours. Data are mean ± SEM of two independent experiments.

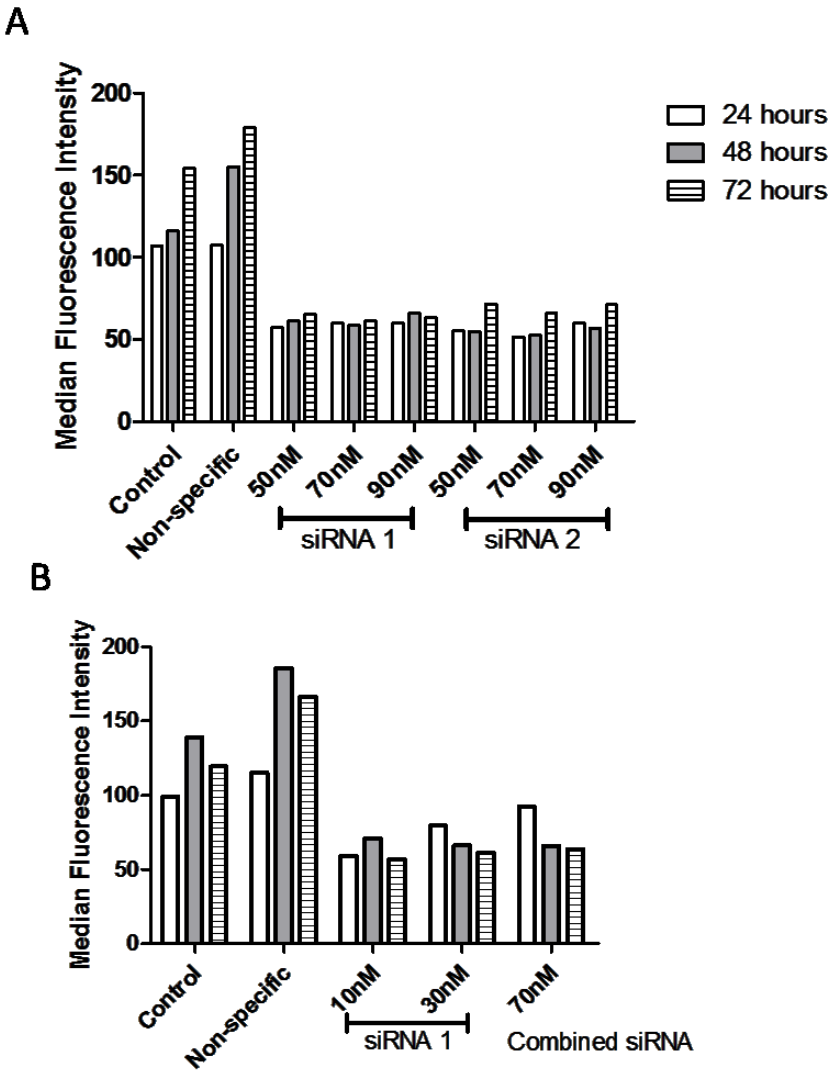


Figure 3.6: Characterisation of siRNA-mediated knockdown of podoplanin. LEC were seeded onto wells of a 24-well plate in medium without antibiotics and incubated overnight at 37°C and 5% CO_2. Transfections were performed the following day when cells were around 80% confluent. (A) LEC were transfected with 50, 70 or 90nM of two different podoplanin duplexes. The controls were transfection with 50nM non-specific siRNA duplex or treatment with lipofectamine. Podoplanin expression was determined by flow cytometry 24, 48 or 72 hours after transfection. Data are representative of two independent experiments. (B) LEC were transfected with 10 or 30nM of one podoplanin duplex or with 70nM of the combined podoplanin duplexes. Controls were transfection with 30nM non-specific siRNA or treatment with lipofectamine. Podoplanin expression was determined by flow cytometry after 24, 48 or 72 hours.

As there was little difference in podoplanin knockdown between 50, 70 and 90nM of duplex, we transfected LEC with lower concentrations of duplex to determine whether 50nM was the saturating concentration. We also combined the two duplexes (at a final concentration of 70nM) to assess whether this would improve the knockdown efficiency. Podoplanin expression was determined by flow cytometry at 24, 48 and 72 hours after transfection. We found that 10 or 30nM of a single duplex resulted in around a 50% reduction in podoplanin expression at every timepoint (Figure 3.6B). Combining the two duplexes did not further reduce podoplanin expression at any of the tested timepoints (Figure 3.6B). Therefore, in future knockdown experiments we used 30nM of one siRNA duplex.

While characterising the podoplanin knockdown, we found that the expression of podoplanin on the surface of non-transfected and non-specific siRNA-transfected LEC increased over time (Figure 3.6). The cells transfected with specific siRNA did not show an increase in podoplanin expression with time. We thus investigated the kinetics of podoplanin expression and the possibility that the trypsin used to dissociate the cells caused loss followed by recovery. We performed experiments in which LEC were detached from plates with trypsin, seeded into different wells, and then detached at chosen times using different dissociation reagents prior to staining for flow cytometry. We compared trypsin, Accutase and cell dissociation buffer (CDB). Accutase is a dissociation reagent that consists of a mixture of proteolytic and collagenolytic enzymes. It is supposed to increase the viability of cells after detachment, compared to trypsin. The CDB is a non-enzymatic solution based on Hanks' balanced salt solution, with additional EDTA, glycerol and sodium citrate.

This solution is thought to detach cells without modifying cell surface proteins. This experiment showed that podoplanin expression increased over three days from a relatively low value after six hours, and the increase was evident for each of the dissociation reagents used (Figure 3.7). However, we also found that dissociating LEC with Accutase or CDB gave a higher MFI for podoplanin than trypsin dissociation (Figure 3.7). Thus, trypsin appeared to remove more podoplanin than the other agents when applied at each time point during "recovery".

Therefore, we performed transfilter assays to check whether the difference in podoplanin expression following different methods of dissociation had any functional effect. LEC were detached with trypsin, Accutase or CDB and seeded onto culture inserts in medium containing 300ng/ml VEGF-C. The plate was incubated at 37°C and 5% CO_2 for an hour before the addition of $1x10^8$ washed human platelets to selected wells. Percentage transmigration was assessed after 24 hours. We found that dissociation reagent had no effect on VEGF-C-stimulated migration and that platelets were able to inhibit this migration in all tested conditions (Figure 3.8). Therefore, we chose to use trypsin to dissociate LEC for functional assays and Accutase to dissociate LEC for flow cytometry.

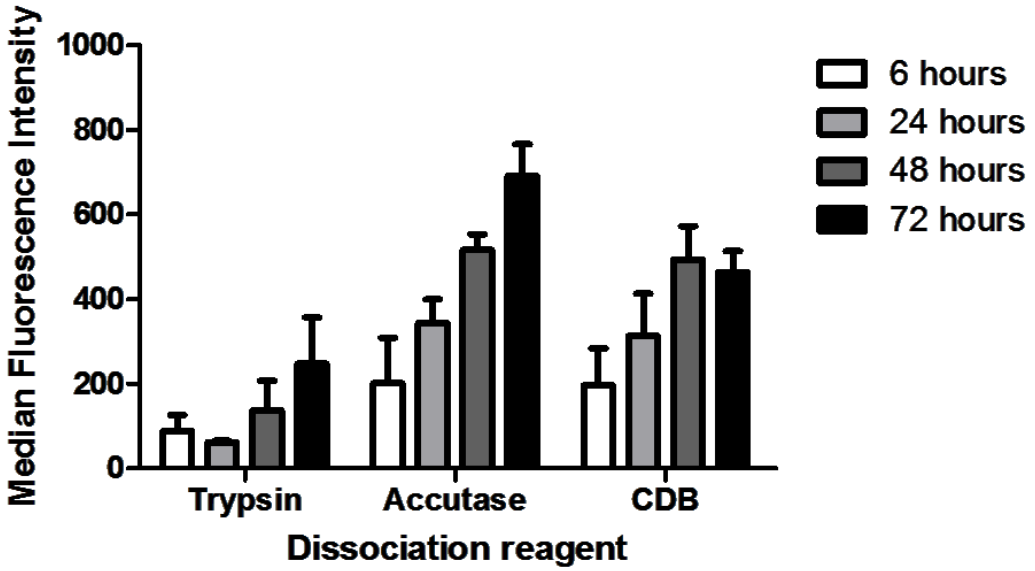


Figure 3.7: Effect of dissociation reagent on podoplanin expression. LEC were detached using trypsin and seeded onto wells of a 24-well plate then incubated at 37°C and 5% CO_2 for 6-72 hours. At the indicated time points, LEC were detached from the wells using trypsin, Accutase or non-enzymatic cell dissociation buffer (CDB). The cells were stained with 0.125µg/ml PE-conjugated anti-human podoplanin and analysed by flow cytometry. The median fluorescence intensity was used to quantify podoplanin expression. Data are mean ± SEM of two independent experiments.

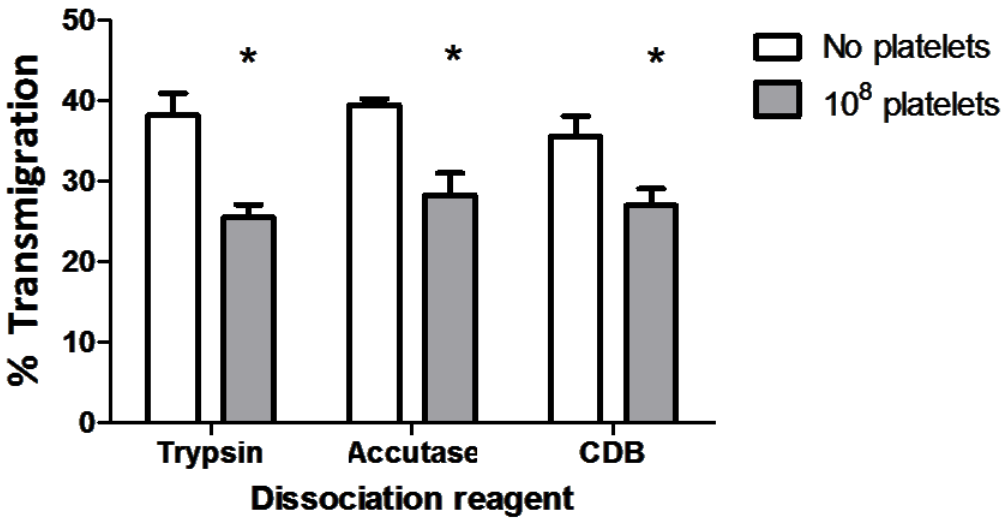


Figure 3.8: Effect of dissociation reagent on LEC migration in the presence of VEGF-C and washed human platelets. $3x10^4$ LEC were detached with trypsin, Accutase or CDB and seeded onto culture inserts in medium containing 300ng/ml VEGF-C. The plate was incubated at 37°C and 5% CO_2 for an hour before the addition of $1x10^8$ washed human platelets to selected wells. Percentage transmigration was determined after 24 hours. ANOVA showed a significant effect of platelets ($p<0.05$), but not dissociation reagent. * = $p<0.05$ compared to no platelets by paired t-test. Data are mean ± SEM of three independent experiments.

3.2.3. Effect of podoplanin knockdown on LEC migration

Next, we assessed the effect of podoplanin knockdown on VEGF-C stimulated migration. LEC were transfected with 30nM podoplanin or non-specific siRNA duplexes. The negative controls were treated with lipofectamine but not transfected. After 48 hours incubation, knockdown efficiency was determined by flow cytometry and $3x10^4$ transfected LEC were seeded onto culture inserts in medium with or without 300ng/ml VEGF-C. The plate was incubated at 37°C for an hour, then $1x10^8$ washed human platelets were added to chosen wells. Percentage migration was assessed after 24 hours. We found that basal migration without VEGF-C was not modified by addition of platelets or by transfection with non-specific or podoplanin siRNA (Figure 3.9A). VEGF-C was able to promote the migration of non-transfected LEC and LEC that had been transfected with a non-specific siRNA, and platelets were able to inhibit this increase in migration (Figure 3.9A). However, when podoplanin had been knocked down, VEGF-C was no longer able to increase migration, and washed platelets no longer inhibited migration in the presence of VEGF-C (Figure 3.9A). Thus, the pro-migratory effect of VEGF-C and the inhibitory effects of platelets were lost when podoplanin expression was reduced. We also confirmed the degree of knockdown in these experiments and found podoplanin expression to be 50% of control levels after transfection with podoplanin siRNA (Figure 3.9B).

3.2.4. Effect of crosslinking podoplanin on LEC migration

Having shown the ability of platelets to modulate LEC migration was linked to expression of podoplanin, we wanted to test whether the effects might be through clustering of

podoplanin. As noted in Section 2.7.3, ligation of podoplanin by antibody alone did not modify migration. We thus crosslinked podoplanin using an anti-human podoplanin antibody (clone NZ-1.3) in conjunction with an anti-rat secondary antibody. We found that crosslinking podoplanin inhibited VEGF-C stimulated migration, but had no effect in the absence of VEGF-C (Figure3 .10). As a control, the combination of a rat non-specific IgG and the secondary antibody had no effect on migration (Figure 3.10). Similarly, treatment with the anti-podoplanin antibody or rat IgG alone did not alter LEC migration (Figure 3.10).

Next, we assessed whether the effect of podoplanin crosslinking was specific to responses to VEGF-C as opposed to other growth factors. We first characterised the transfilter assay using FGFb and EGF alongside VEGF-C. In initial experiments we found that all growth factors could stimulate migration and that the difference between basal migration and growth factor-stimulated migration was more evident after 12 hours than 16 or 18 hours (Figure 3.11A). Therefore, further experiments used a 12-hour incubation period and podoplanin was crosslinked in the presence of the different growth factors. We found that VEGF-C, FGFb and EGF all promoted migration but that crosslinking podoplanin was only able to inhibit VEGF-C stimulated migration (Figure 3.11B).

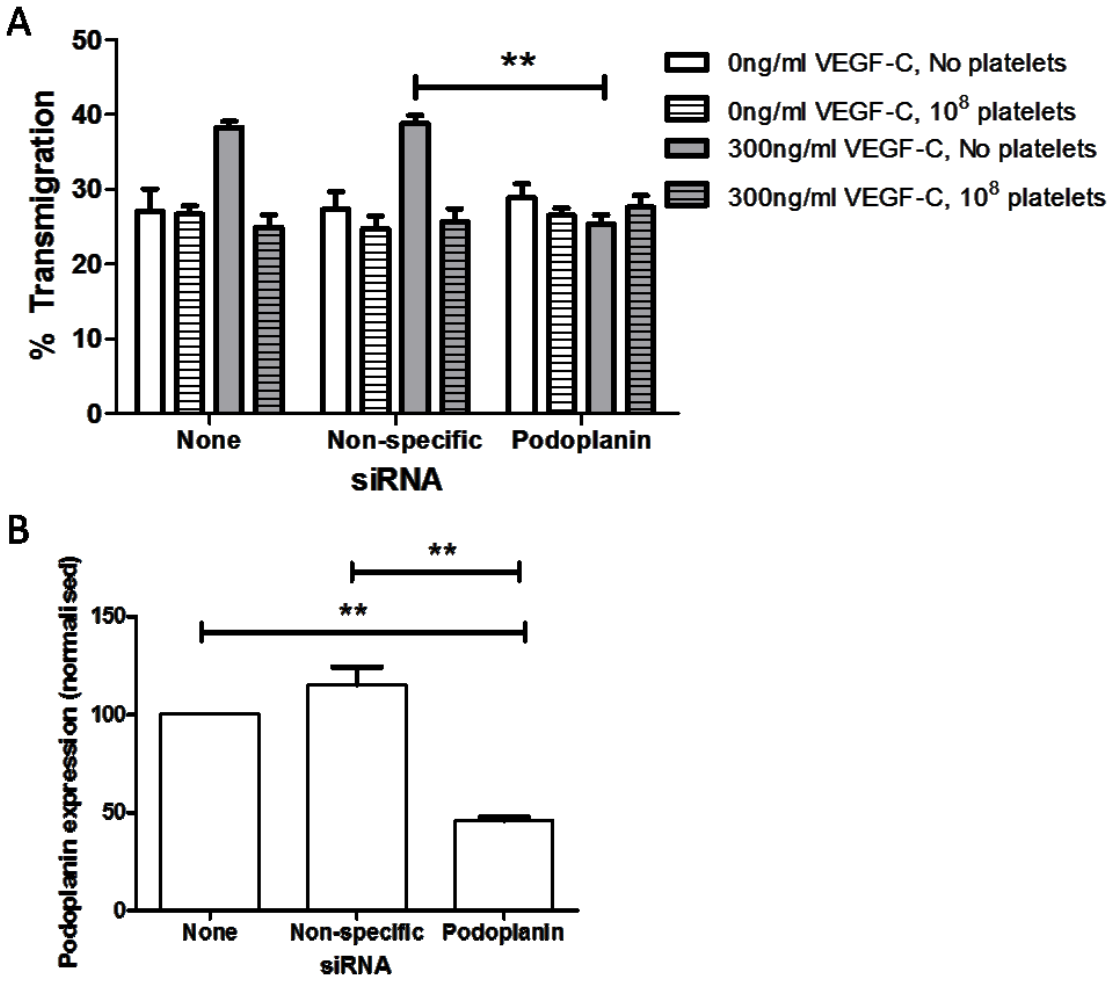


Figure 3.9: Effect of podoplanin knockdown on transfilter migration of LEC. LEC were seeded onto 24-well plates in medium without antibiotics and incubated at 37°C overnight. LEC were transfected with 30nM podoplanin or non-specific siRNA or were treated with lipofectamine without duplexes. Cells were incubated at 37°C for 48 hours. (A) $3x10^4$ LEC were seeded onto culture filters in medium with or without 300ng/ml VEGF-C. The plate was incubated for an hour before $1x10^8$ washed human platelets were added to chosen wells. Percentage transmigration was assessed after 24 hours. ANOVA showed a significant effect of platelets ($p<0.05$). ** = $p<0.01$ by paired t-test. Data are mean ± SEM of three independent experiments. (B) Podoplanin expression was determined by flow cytometry. The median fluorescent intensity (MFI) was expressed as a percentage of the MFI obtained with the non-transfected control. ANOVA found a significant effect of transfection. ** = $p<0.01$ by Bonferroni post-test. Data are mean ± SEM of three independent experiments.

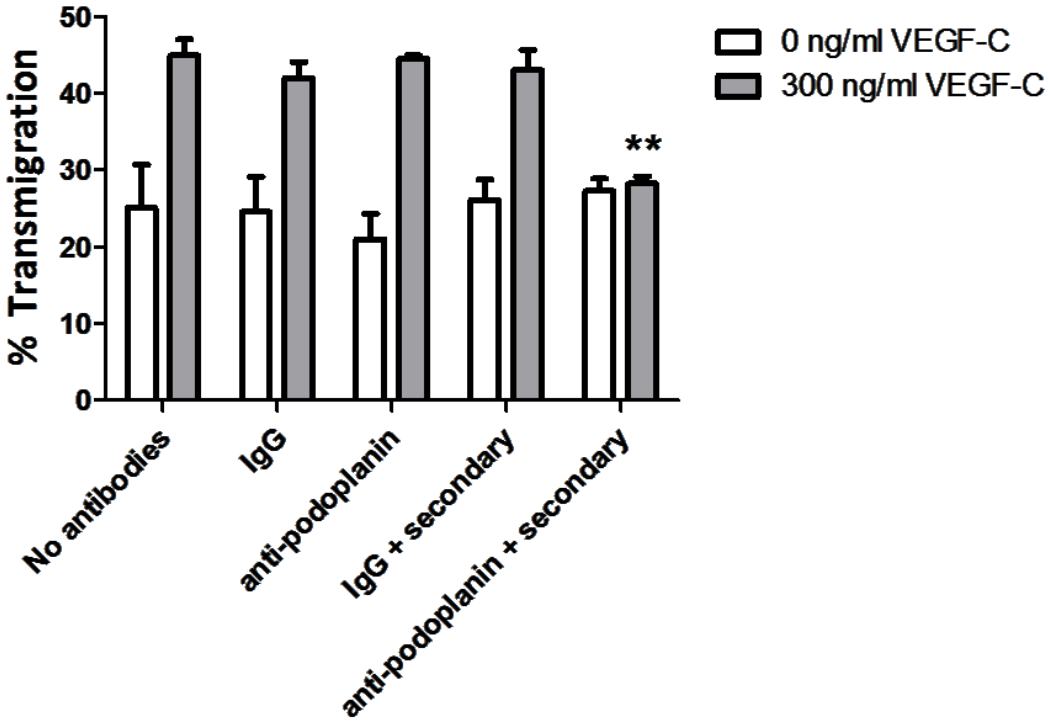


Figure 3.10: Effect of crosslinking podoplanin on LEC migration. LEC were seeded onto cell culture inserts in culture medium with or without 300ng/ml VEGF-C and incubated at room temperature for 30 minutes. 2µg/ml anti-human podoplanin antibody or rat IgG was added and after a further 30-minute incubation 30µg/ml secondary antibody was added. 200µl sterile PBS was added to the wells without antibodies. Percentage transmigration was assessed after 24 hours. ANOVA showed that treatment with VEGF-C had a significant effect ($p<0.01$). ** = $P<0.01$ compared to no antibodies by Dunnett's test. Data are mean ± SEM of three independent experiments.

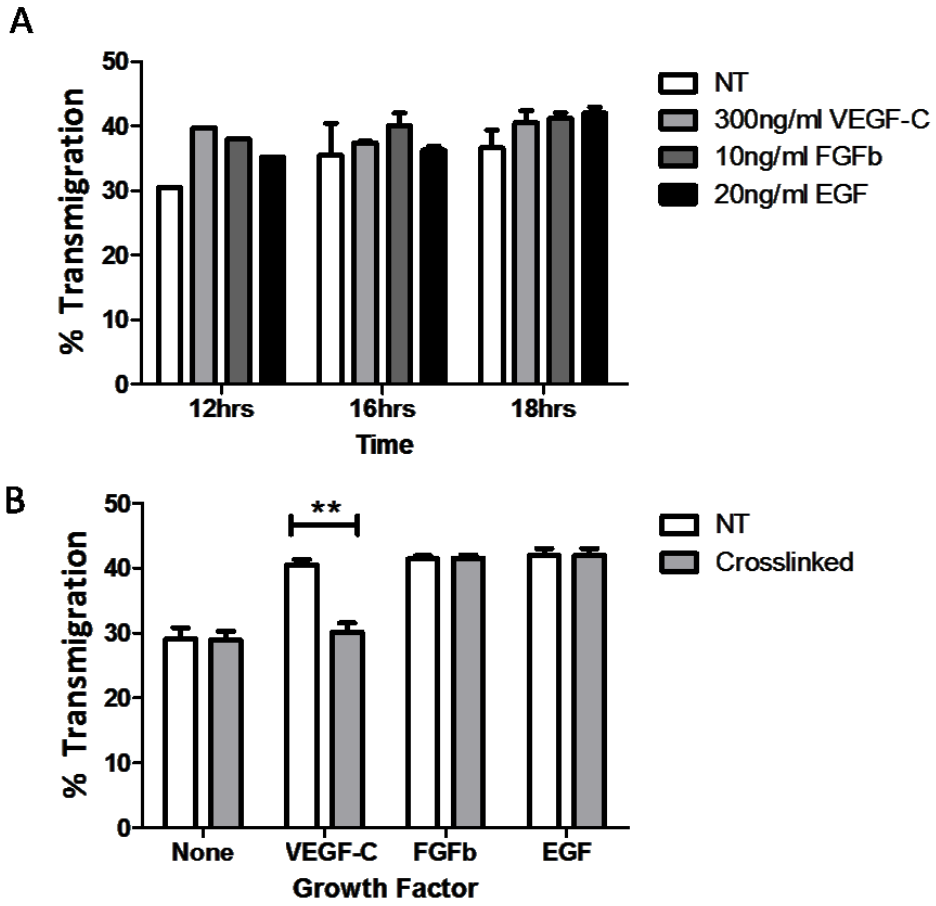


Figure 3.11: Effect of different growth factors and of podoplanin crosslinking on LEC migration. (A) LEC were seeded onto filters in the presence of culture medium or medium that had been supplemented with 300ng/ml VEGF-C, 10ng/ml FGFb or 20ng/ml EGF. The filters were incubated at 37°C and 5% CO_2 for 12-18 hours. Error bars represent mean ± SEM of at least two independent experiments. (B) LEC were seeded onto filters in the presence of culture medium or medium that had been supplemented with 300ng/ml VEGF-C, 10ng/ml FGFb or 20ng/ml EGF. Podoplanin was crosslinked using an anti-podoplanin antibody and appropriate secondary at a ratio of 1:15. The wells without crosslinking were treated with 200µl sterile PBS. ** = $p<0.01$ by paired t-test. Data are mean ± SEM of at least three independent experiments.

3.2.5. Effect of platelet-derived microvesicles on LEC migration

Transfilter assays were used to test whether platelet-derived microvesicles could modulate LEC migration. $1x10^8$ washed human platelets or the microvesicles derived from $1x10^8$ washed platelets were added to LEC seeded on filters. In accordance with previous experiments, we found that washed human platelets inhibited LEC migration. Interestingly, we also found that microvesicles derived from CRP-XL stimulated platelets inhibited LEC migration (Figure 3.12).

To assess the role of CLEC-2 in the microvesicle-dependent inhibition of migration, the transfilter experiments were repeated with platelets and microvesicles prepared from *Clec1b*$^{fl/fl}$ or *Clec1b*$^{fl/fl}$PF4-Cre mice. LEC were seeded onto cell culture inserts and $1x10^8$ washed mouse platelets or the microvesicles from $1x10^8$ platelets stimulated with 1µg/ml CRP-XL were added. In agreement with data previously published by our group (Finney et al., 2012), platelets from *Clec1b*$^{fl/fl}$ mice inhibited LEC migration and platelets from *Clec1b*$^{fl/fl}$PF4-Cre mice had a weaker inhibitory effect (Figure 3.13A). We also found that platelet microvesicles from *Clec1b*$^{fl/fl}$ mice or *Clec1b*$^{fl/fl}$PF4-Cre mice inhibited LEC migration (Figure 3.13B). Similar to the platelet data, microvesicles from *Clec1b*$^{fl/fl}$PF4-Cre mice had a significantly weaker inhibitory effect than *Clec1b*$^{fl/fl}$ microvesicles.

Flow cytometry was used to test whether microvesicles derived from mouse platelets expressed CLEC-2. Beads of known size (300nm, 500nm and 1µm, respectively) were used to

identify the microvesicles and determine their approximate size. This analysis showed that mouse platelets from *Clec1b*$^{fl/fl}$ mice expressed both CD41 and CLEC-2 (Figure 3.14A and B). However, we were unable to detect either CD41 (Figure 3.14C) or CLEC-2 (Figure 3.14D) on microvesicles isolated from mouse platelets.

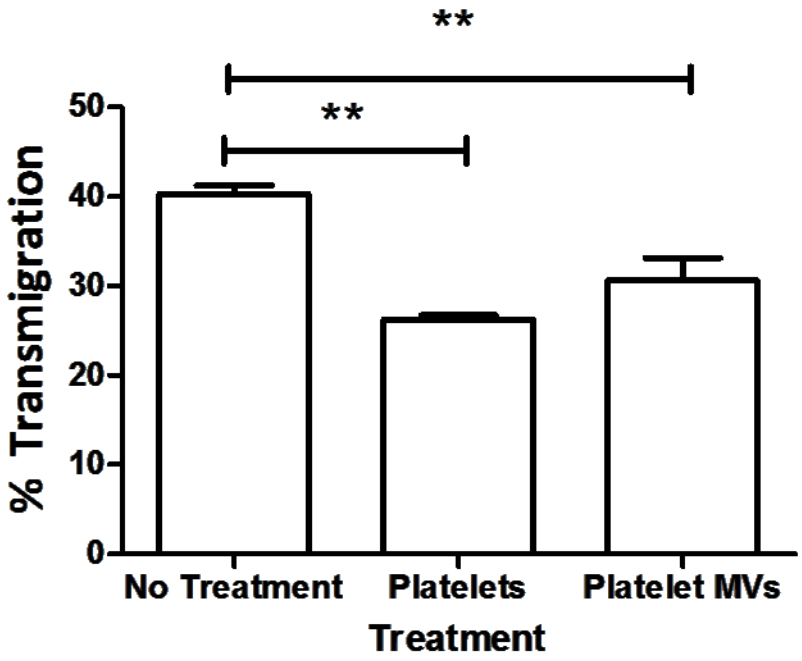


Figure 3.12: Effect of human platelet-derived microvesicles on VEGF-C stimulated LEC migration. LEC were seeded onto culture inserts in culture medium containing 300ng/ml VEGF-C and incubated for an hour at 37°C. $1x10^8$ washed human platelets or the microvesicles from $1x10^8$ platelets stimulated with 1µg/ml CRP-XL were added to each filter. The non-treated filters received 100µl Ca^{2+}/Mg^{2+}-free sterile PBS and percentage transmigration was assessed after 24 hours. ANOVA showed that the effect of treatments were significant ($p<0.05$). ** = $p<0.01$ by Bonferroni test. Data are mean ± SEM of four independent experiments.

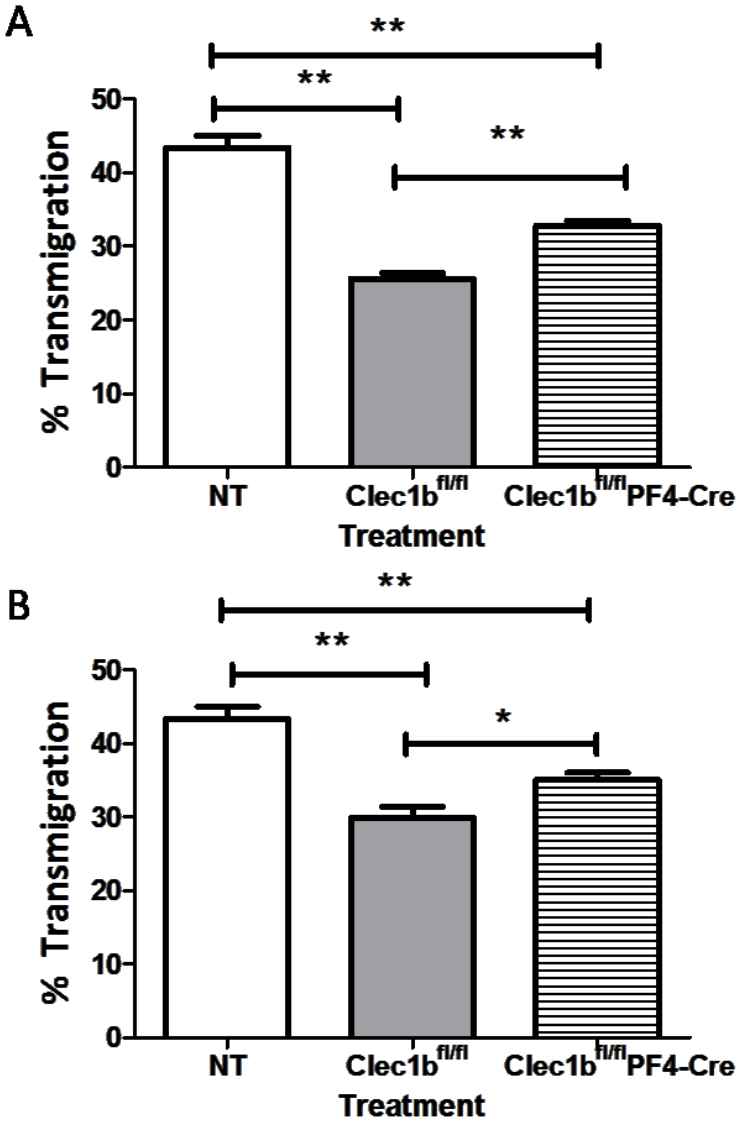


Figure 3.13: Effect of mouse platelets and platelet-derived microvesicles on VEGF-C stimulated LEC migration. LEC were seeded onto cell culture filters in medium containing 300ng/ml VEGF-C and incubated for an hour at 37°C and 5% CO_2. (A) $1x10^8$ platelets from *Clec1b*$^{fl/fl}$ or *Clec1b*$^{fl/fl}$PF4-Cre mice were added to the filter. Wells without platelets received 100µl Ca^{2+}/Mg^{2+}-free sterile PBS. Percentage migration was assessed after 24 hours. ANOVA showed that platelets had a significant effect ($p<0.01$). ** = $p<0.01$ by Bonferroni post-test. (B) Microvesicles derived from *Clec1b*$^{fl/fl}$ or *Clec1b*$^{fl/fl}$PF4-Cre mice were added to the filters. Wells without platelets received 100µl Ca^{2+}/Mg^{2+}-free sterile PBS. Percentage migration was assessed after 24 hours. ANOVA showed a significant effect of platelets ($p<0.01$). * = $p<0.05$; ** = $p<0.01$, by Bonferroni post-test. Data in both graphs are mean ± SEM of at least four independent experiments.

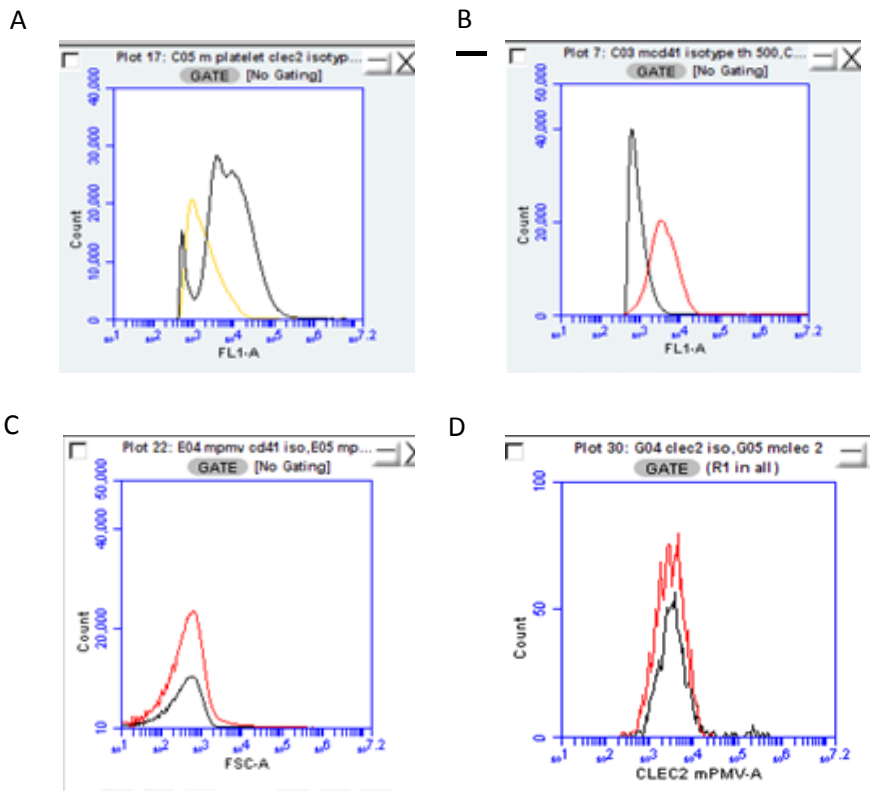


Figure 3.14: Expression of CD41 and CLEC-2 on mouse platelets and platelet-derived microvesicles. 50µl whole blood or platelet-derived microvesicles were stained with 1:100 mouse IgG-488, CD41-488 or CLEC-2-488. Samples were protected from light and incubated at room temperature for 20 minutes before being analysed on an Accuri C6 flow cytometer. (A) Expression of CD41 on mouse platelets. Yellow: IgG; Black: CD41. (B) Expression of CLEC-2 on mouse platelets. Black: IgG; Red: CLEC-2. (C) Expression of CD41 on platelet-derived microvesicles. Black: IgG; Red: CD41. (D) Expression of CLEC-2 on platelet-derived microvesicles. Black: IgG; Red: CLEC-2. Plots are representative of those obtained in two independent experiments.

3.2.6. The scratch wound assay as an alternative model of endothelial cell migration

All of the migration data reported so far was obtained using the transfilter migration assay. We decided to assess the effect of platelets and podoplanin crosslinking in a different assay of cell migration and chose the scratch wound assay as our model. Initially, the experimental timecourse and effect of pre-treating the plates with gelatin were assessed. These experiments found that pre-treatment with gelatin slowed migration, such that the wounds only healed by 20% in 24 hours (Figure 3.15A and Figure 3.16A). In comparison, wound closure after 24 hours was 50% on plates that had not been treated with gelatin (Figure 3.15B and Figure 3.16B). With these plates, the wound fully healed in 48 hours. Therefore, further experiments used plates that had not been pre-treated with gelatin. Addition of VEGF-C to these experiments did not accelerate wound healing (Figure 3.15 and Figure 3.16). Lack of effect was confirmed in further experiments that only compared basal and VEGF-C stimulated migration (Figure 3.17). Nevertheless, both unstimulated and VEGF-C stimulated cultures were used in subsequent experiments, as the transfilter assay had shown that the effect of platelets differed between these conditions.

Having characterised the assay, platelets were added to determine whether they would modulate LEC wound recovery. This variant of the assay was performed in two different ways: i) platelets were incubated with the monolayer for an hour before wounding; ii) the monolayer was scratched and washed before platelets were added. Both protocols were performed in the presence and absence of VEGF-C and images were taken from 0-40 hours. When the monolayer was scratched before platelets were added, subsequent addition of

platelets initially slowed wound healing, but by 40 hours healing was similar with or without platelets (Figure 3.18). This pattern was seen in either the presence (Figure 3.18A) or absence (Figure 3.18B) of VEGF-C. Conversely, if platelets were incubated with LEC before the monolayer was injured, the wound healed faster than in the absence of platelets (Figure 3.19). The effect of platelets was small, but significant, in the presence of VEGF-C (Figure 3.19A) and was more pronounced in the absence of VEGF-C (Figure 3.19B).

To help assess whether platelets might be physically obstructing wound healing rather than causing changes by binding podoplanin, antibody-mediated podoplanin crosslinking was used to assess the effect of podoplanin clustering on wound healing. In these experiments, the monolayer was wounded before podoplanin was crosslinked. To maintain consistency with previous measurements, these experiments were performed in culture medium with or without VEGF-C. We found that addition of the anti-human podoplanin alone had no effect on wound healing of either unstimulated or VEGF-C stimulated LEC (Figure 3.20). Crosslinking podoplanin had no effect in the presence of 300ng/ml VEGF-C (Figure 3.20A) and significantly promoted LEC wound recovery in the absence of VEGF-C (Figure 3.20B), compared to no antibodies but not compared to anti-podoplanin antibody alone.

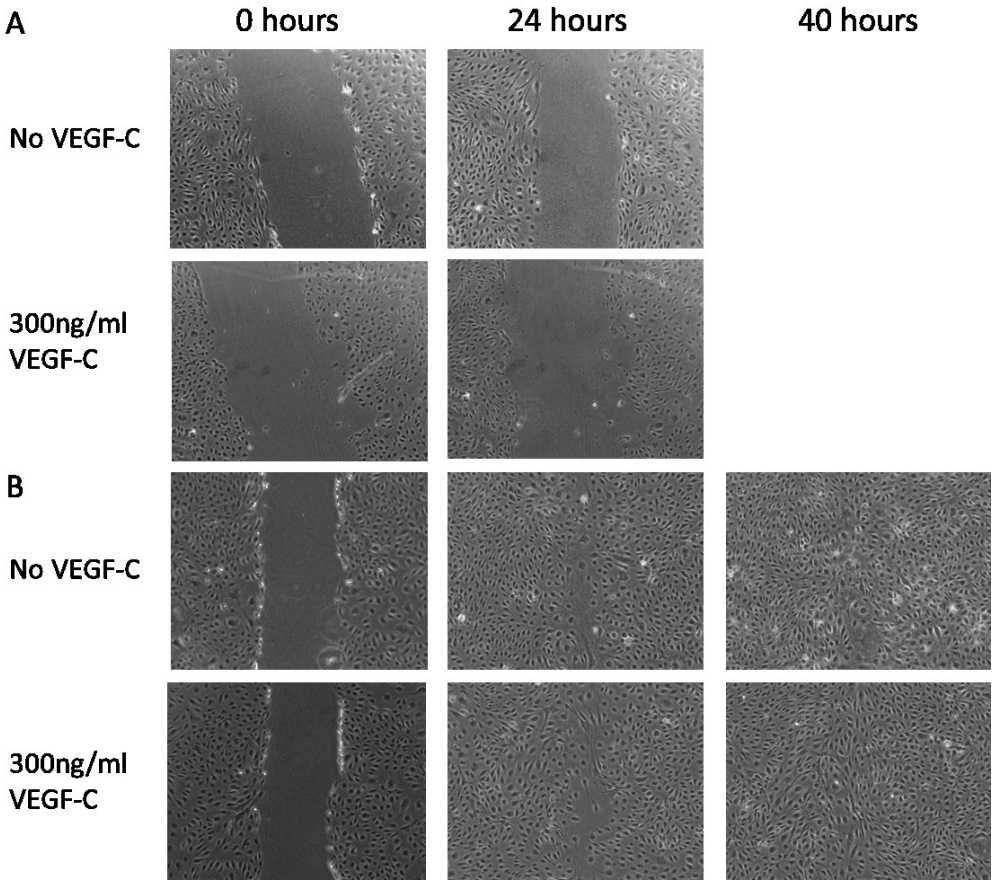


Figure 3.15: Characterisation of the scratch wound assay. LEC were seeded onto plates that had been coated with 1% gelatin (A) or left untreated (B) and allowed to grow to confluence. The monolayers were wounded and phase contrast images were taken with a 4x objective to monitor wound closure. Images are representative of three independent experiments.

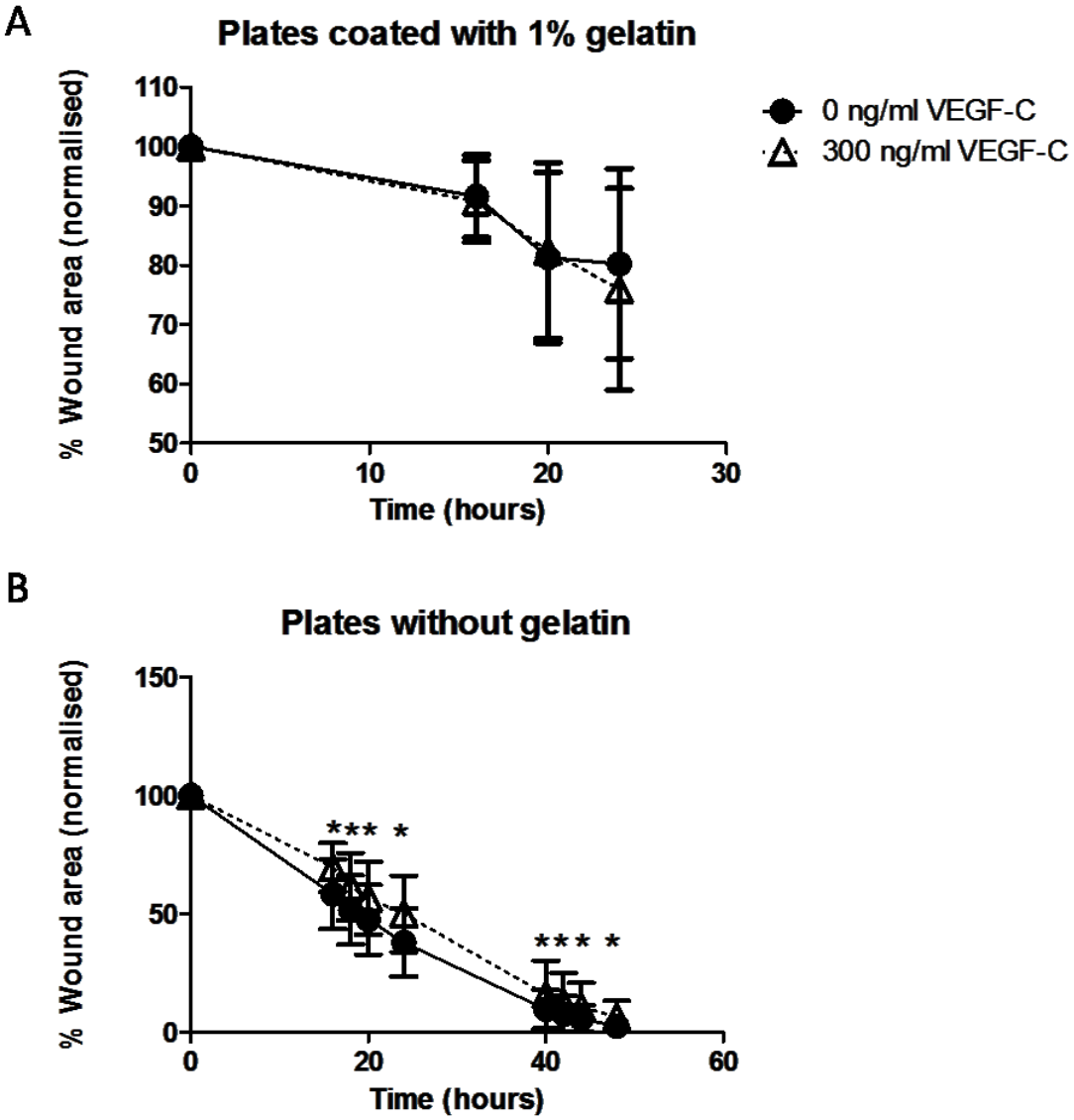


Figure 3.16: Effects of coating and VEGF-C on the time course of scratch wound recovery. Phase contrast images obtained during the scratch wound assay were analysed using ImageJ. The area of the wound in each image was calculated and expressed as a percentage of the original wound. (A) Quantitation of scratch area on plates that had been pre-treated with 1% gelatin. ANOVA found no effect of time or VEGF-C ($p>0.05$). (B) Quantitation of wound area on plates that were not pre-treated with gelatin. ANOVA showed an effect of time on wound area ($p<0.01$). * = $p<0.05$ compared to wound area at time 0 hours by Dunnett's test for both data sets. Data are mean ± SEM of three independent experiments.

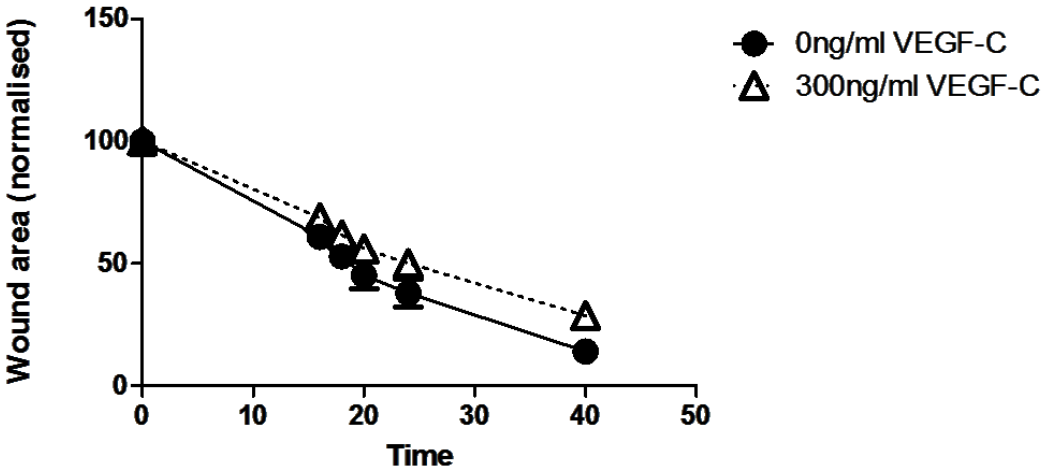


Figure 3.17: Effect of VEGF-C treatment on LEC wound recovery. LEC were grown on uncoated plates until confluent. The monolayer was wounded, and then the cells were maintained in culture medium with or without 300ng/ml VEGF-C. Images were taken between 16 and 40 hours and used to determine percentage wound area. ANOVA showed that VEGF-C treatment did not affect wound recovery. Data are mean ± SEM of at least ten independent experiments.

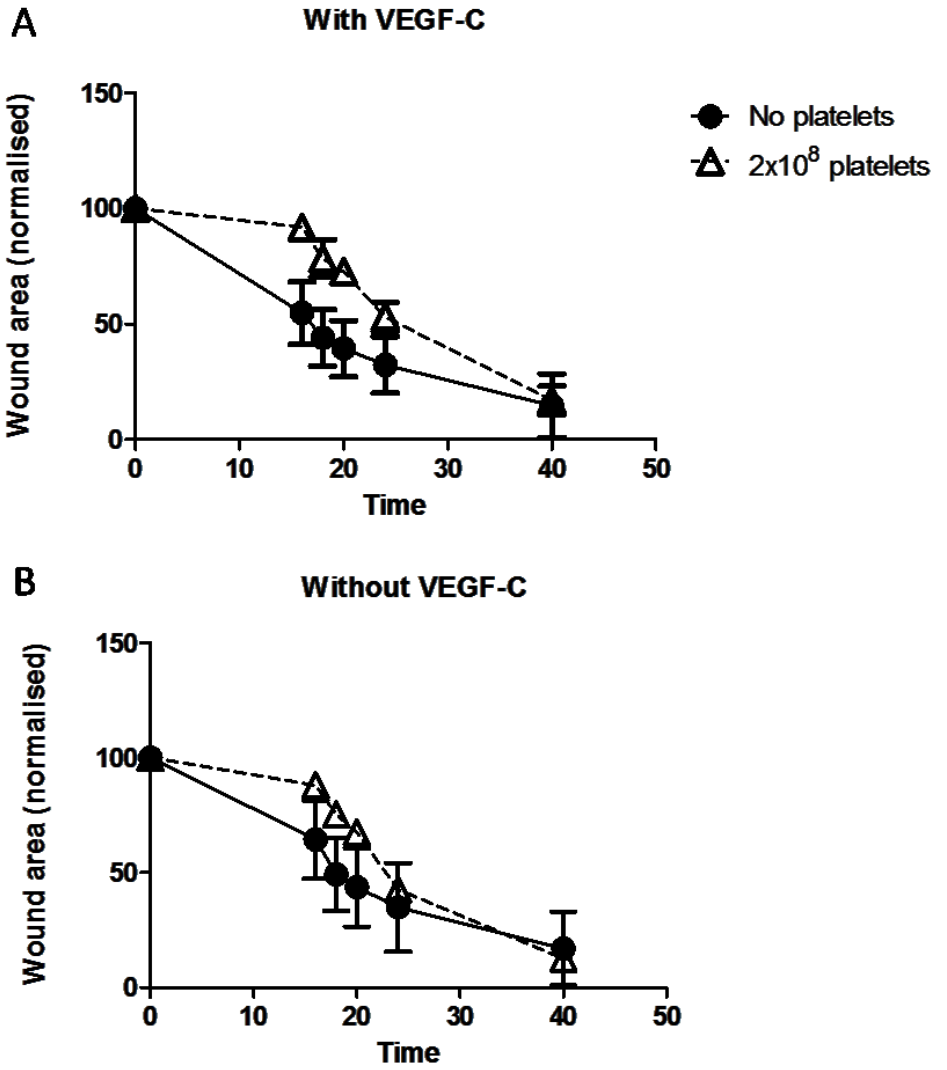


Figure 3.18: Effect of adding platelets after wounding on LEC wound recovery. Monolayers of LEC were wounded and the detached cells washed away before 200µl Ca^{2+}/Mg^{2+}-free sterile PBS or $2x10^8$ washed human platelets were added. Phase contrast images were used to quantify wound area. (A) Quantitation of wound area in the presence of 300ng/ml VEGF-C. ANOVA showed that the effects of time and platelets were significant ($p<0.01$ and $p<0.05$, respectively). (B) Quantitation of wound area in culture medium without VEGF-C. ANOVA showed that the effect of time was significant ($p<0.01$) but that the effect of platelets was not. Data are mean ± SEM of two independent experiments.

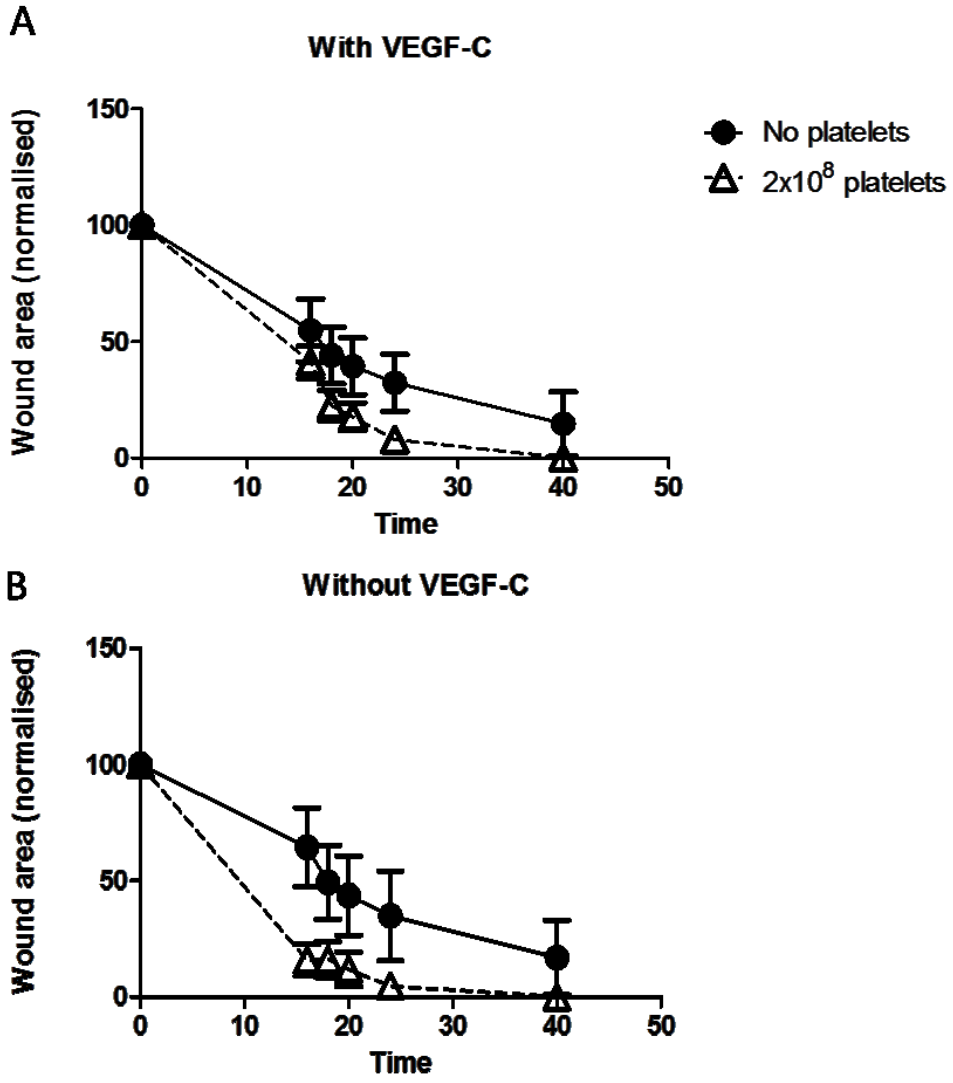


Figure 3.19: Effect of pre-incubation with platelets on LEC wound recovery
$2x10^8$ washed human platelets or 200µl Ca^{2+}/Mg^{2+}-free sterile PBS were incubated with LEC for an hour before the monolayer was wounded. Phase contrast images were taken using a 4x objective for up to 40 hours after wounding. From these images, percentage wound area was calculated. (A) Quantitation of wound healing in culture medium containing 300ng/ml VEGF-C. ANOVA showed that the effects of time and platelets were significant (both $p<0.01$). (B) Quantitation of wound area in culture medium without VEGF-C. ANOVA showed that the effects of time and platelets were significant (both $p<0.01$). Data are mean ± SEM of at least four independent experiments.

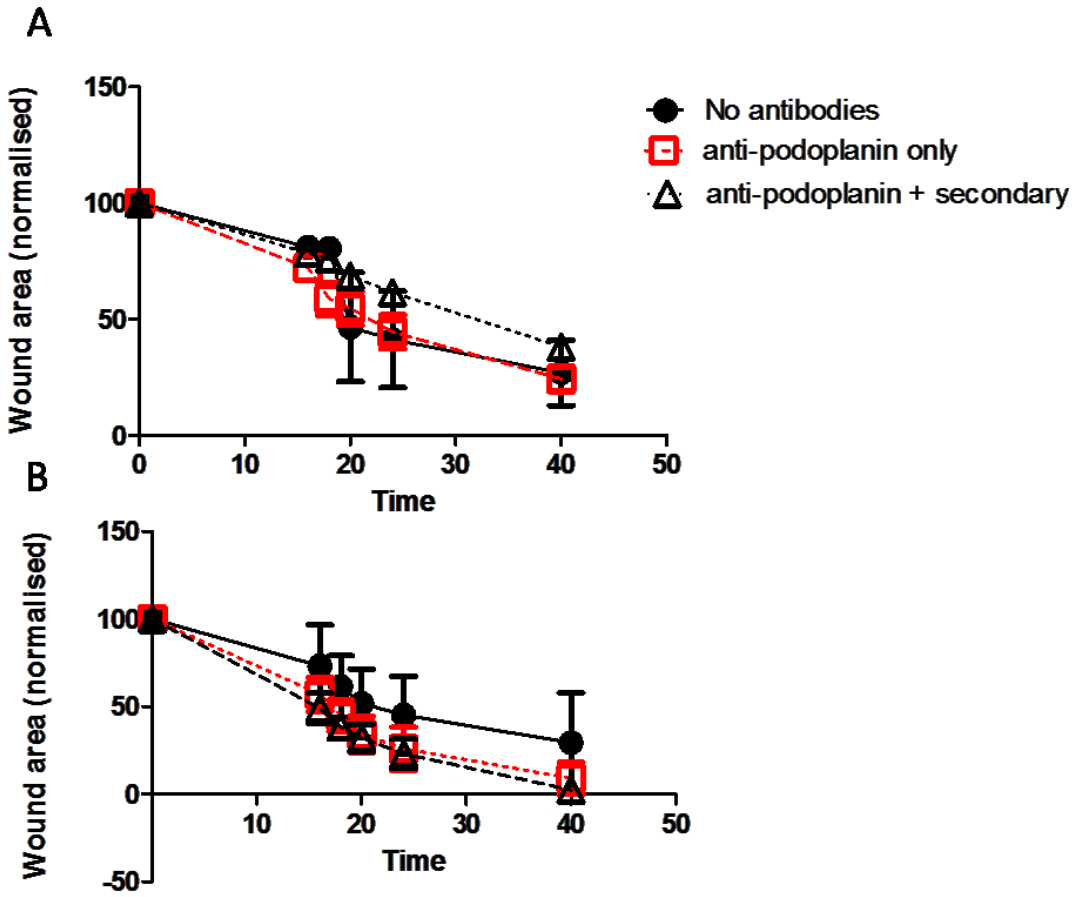


Figure 3.20: Effect of podoplanin crosslinking on LEC wound recovery. Monolayers of LEC were wounded before the addition of 2µg/ml anti-human podoplanin, with or without subsequent crosslinking (1:15 anti-human podoplanin and rat IgG2a). (A) Quantitation of wound area in culture medium containing 300ng/ml VEGF-C. ANOVA found no effect of treatment. (B) Quantitation of wound area in culture medium without VEGF-C. ANOVA showed an effect of treatment ($p<0.05$). Crosslinking was found to promote wound recovery compared to no antibodies, but not primary antibody alone, by Bonferroni test ($p<0.05$). Data are mean ± SEM of three independent experiments.

3.3. Discussion

In this chapter, two *in vitro* migration assays were used to assess the effect of the interaction of platelet CLEC-2 and LEC-expressed podoplanin on the migration of lymphatic endothelial cells. We previously found that washed human platelets, isolated using prostacyclin, inhibited LEC migration in the presence of VEGF-C (Langan, 2011). Here, we showed that platelets isolated using theophylline had the same inhibitory effect. We also showed that washed human platelets were able to inhibit VEGF-C mediated migration of HMEC-1. This is consistent with the hypothesis that the inhibitory effect of platelets is via the interaction of podoplanin and CLEC-2 as both LEC and HMEC-1 express podoplanin. However, there may be a podoplanin-independent aspect to platelet-mediated inhibition of LEC migration. This is suggested by the finding that, in high numbers, platelets were able to inhibit VEGF-A stimulated migration of HUVEC. These blood vascular endothelial cells do not express podoplanin (Gröger et al., 2004; Sölder et al., 2012). At present, it is not known whether this effect on HUVEC is through platelets binding to the endothelial cells or through the release of soluble mediators from platelet granules. However, the presence of a podoplanin-independent effect of platelets concurs with previously reported data that showed that *Clec1b*$^{fl/fl}$PF4-Cre platelets were able to inhibit LEC migration, although they had a weaker effect than *Clec1b*$^{fl/fl}$ platelets (Finney et al., 2012).

To further assess the role of podoplanin in LEC migration, we knocked down podoplanin using siRNA and then performed transfilter assays in the presence of VEGF-C. We found that the reduction in podoplanin expression negated the pro-migratory effect of VEGF-C and that

platelets could not further inhibit LEC migration in podoplanin-knockdown cells. This provided further evidence that the effect of platelets is through the interaction of CLEC-2 and podoplanin. It also suggested that podoplanin is necessary for the promotion of migration by VEGF-C, which will be more fully explored later in this thesis (see Chapter 6).

When characterising the podoplanin knockdown, we found that there was a stable 50% reduction in podoplanin expression, regardless of siRNA duplex used, concentration of the selected duplex or incubation time after transfection. This suggests that podoplanin is a stably-expressed protein that has a relatively long half-life. Other groups have similarly struggled to reduce podoplanin in LEC, with reductions from 34-50% reported previously (Schacht et al., 2003; Navarro et al., 2008). Furthermore, we found that cell surface expression of podoplanin increased over time in non-transfected cells but not in those with reduced podoplanin expression. Thus newly-synthesised podoplanin that would be expressed at the cell surface was continually suppressed by transfection. We hypothesised that trypsin used to detach the LEC from flasks might initially lower expression and that recovery would normally occur, but not after transfection. Thus, we analysed podoplanin expression at different times after seeding, comparing detachment with trypsin, Accutase or CDB. We found that detachment with Accutase or the non-enzymatic CDB reduced podoplanin expression less than trypsin. However, the difference in podoplanin expression observed after different methods of detachment did not impact on the effect of platelets on LEC migration. These data suggest that trypsin cleaves a proportion of the podoplanin expressed on the cell surface, so for further flow cytometry experiments LEC were detached

from flasks or plates with Accutase. The lack of functional effect suggests that either: the amount of podoplanin present after trypsin detachment reaches a threshold required for platelet-mediated effects or; some of the podoplanin molecules expressed at the cell surface do not signal. Further evidence for the latter comes from the transfilter data obtained with podoplanin-knockdown, since podoplanin expression was reduced by 50%, but there was a complete loss of VEGF-promoted migration.

To show that podoplanin alone could indeed regulate migration and to test the hypothesis that platelets exert their effects on LEC migration by clustering podoplanin, we used antibodies to crosslink podoplanin on LEC. We found that crosslinking podoplanin had no effect on LEC migration in the absence of VEGF-C, nor in the presence of FGFb or EGF, but crosslinking inhibited the increase in migration caused by the presence of VEGF-C. We also found that neither the anti-podoplanin antibody nor non-specific rat IgG alone inhibited LEC migration. Similarly, combining the rat IgG with the secondary antibody did not reduce LEC migration. Therefore, both the presence of the specific anti-podoplanin antibody and its crosslinking with a secondary antibody were required to inhibit LEC migration. This supports our hypothesis that the effect of platelets is through clustering of podoplanin. However, it also highlights a paradox with regards to the role of podoplanin in cell migration. Other groups have previously reported that overexpression of podoplanin increased the migration of MDCK cells (Martín-Villar et al., 2006) and that siRNA-mediated knockdown of podoplanin in LEC reduced migration (Navarro et al., 2011) in the presence of VEGF-A. These studies, therefore, imply that podoplanin has a promigratory effect. Indeed, we found that

knockdown of podoplanin resulted in impaired migration in the presence of VEGF-C. On the other hand, our data showed that crosslinking podoplanin had an inhibitory effect on migration. Thus it seems that podoplanin is necessary to obtain the full response to VEGF-C but there is an alteration in signalling downstream of podoplanin when the receptor is clustered. The signalling pathways underlying these effects of podoplanin on migration will be explored further in later chapters.

Although we hypothesised that platelets inhibit LEC migration through clustering podoplanin and modulating signalling within LEC, others have suggested that platelets release soluble mediators that are necessary for normal lymphatic development (Bertozzi et al., 2010b; Osada et al., 2012). In particular, it was reported that platelets activated with poly(PHG) released bone morphogenetic protein-9 (BMP-9), which inhibited LEC proliferation, migration and tube formation (Osada et al., 2012). The same study also reported that transforming growth factor β (TGFβ) and platelet factor 4 inhibited LEC migration and proliferation. To test whether platelets release soluble mediators that influence LEC migration, we activated washed platelets with 300nM rhodocytin and the resulting supernatant was used in transfilter migration assays. We chose to stimulate platelets with rhodocytin as this activates platelets via CLEC-2 (Suzuki-Inoue et al., 2006). Therefore, this should induce the release of the same soluble mediators as podoplanin-induced platelet activation. We found that this rhodocytin-induced platelet releasate had no effect on LEC migration. The discrepancy between our data and the results published by Osada and colleagues (2012) may be due to the platelet agonist used. Osada used poly(PHG), which

acts on GPVI, while I used the CLEC-2 agonist rhodocytin. Osada et al. state that they chose not to use rhodocytin to activate platelets for supernatant preparation as it is known to bind integrin $\alpha_2\beta_1$ (Navdaev et al., 2001), which is expressed by LEC (Hong et al., 2004). Therefore, they chose a GPVI agonist as GPVI is not expressed on LEC. However, here platelets were pre-treated with rhodocytin before the supernatant was prepared, so that the amount of rhodocytin present in the platelet releasate would be negligible and unlikely to directly act on LEC. It is possible that platelet activation via the two different agonists resulted in the release of different soluble mediators, which had differing effects on LEC migration. As, in my hands, platelet releasate had no effect on LEC migration, I have not further assessed the effects of compounds that could be released from platelet granules. However, this could potentially be done in later work. In particular, this work could establish whether platelets release mediators that are also able to inhibit HUVEC migration, as we found that $1x10^8$ platelets inhibited HUVEC migration. HUVEC do not express podoplanin, suggesting that the effect of platelets in this case is via a different mechanism. The effect of platelets on HUVEC migration was weaker than that on LEC or HMEC-1 migration. Therefore, it is possible that the additional inhibitory effect seen on HMEC-1 and LEC migration is through the interaction of podoplanin and CLEC-2.

Although platelets inhibited LEC migration, it was unclear when platelets would be able to interact with LEC, as platelets are not normally found in lymph. However, data from our group suggests that platelet-derived microvesicles express CLEC-2 (Gitz et al., 2014). As these microvesicles are small in size (0.1-1µm) it is feasible that they are able to access

tissues and lymph when platelets are not able to. We found that microvesicles derived from human platelets were able to inhibit VEGF-C mediated LEC migration. To further assess the role of CLEC-2 in this inhibition of migration, microvesicles were prepared from platelets isolated from *Clec1b*$^{fl/fl}$ or *Clec1b*$^{fl/fl}$PF4-Cre mice. First we showed that platelets from both *Clec1b*$^{fl/fl}$PF4-Cre and *Clec1b*$^{fl/fl}$ mice inhibited LEC migration and that *Clec1b*$^{fl/fl}$ platelets had a stronger inhibitory effect. This result agrees with previously-published data (Finney et al., 2012). Here we found that microvesicles from *Clec1b*$^{fl/fl}$ mice inhibited LEC migration. Interestingly, microvesicles derived from *Clec1b*$^{fl/fl}$PF4-Cre platelets also inhibited LEC migration and the effect of these microvesicles was less than for the vesicles from *Clec1b*$^{fl/fl}$ mice. However, we were unable to confirm that the microvesicles isolated from mouse platelets expressed CLEC-2, or indeed the platelet marker CD41 using flow cytometry. It is possible that the microvesicles were too small to allow efficient antibody binding, as we were able to show that the mouse platelet-derived microvesicles were smaller than those isolated from human platelets.

The data described in this chapter suggest that platelet-derived microvesicles inhibit LEC migration, but do not definitively show this is via presentation of CLEC-2. Further mechanistic studies would need to be performed in order to fully understand their effect. For example, the inhibitory effect of the microvesicles could be confirmed by using ultracentrifugation to pellet the microvesicles and then using the remaining supernatant in the migration assay (Falati et al., 2003). However, continuing studies in our group suggest that a large proportion of the smallest microvesicles remain after such a step. There are few

reports of the isolation of microvesicles from washed mouse platelets, with two reports from the same group (Boilard et al., 2010, 2011). This group used flow cytometry to determine the size and concentration of the isolated microvesicles. This method, however, only assesses larger vesicles due to the availability of reference beads. The smallest of the beads that we had available was 500nm. Therefore, it would also be advisable to perform further analyses on the size of the mouse platelet-derived microvesicles and the number of microvesicles that can be obtained from a known number of platelets. Both of these questions could be addressed by analysing the microvesicles using nanoparticle tracking analysis equipment (Nanosight; Amesbury, UK). If further studies are likely to use microvesicles isolated from knockout mice it would be useful to know how microvesicle size and number in mice compares to humans. This information could show how comparable mouse models are to human microvesicle production and suggest whether accurate conclusions about human microvesicles could be drawn from data obtained with mice.

This chapter also used the scratch wound assay as an alternative model of LEC migration. In contrast to the transfilter assay, the presence of VEGF-C did not appear to promote wound healing by LEC. While there are not many studies reporting the wound recovery assay in both the presence and absence of VEGF-C, one group has reported that VEGF-C promotes LEC migration in this assay (Yin et al., 2011), which conflicts with our observations. Another study reported that platelets had no effect on LEC wound recovery in the absence of VEGF-C when added after injury of the monolayer (Bertozzi et al., 2010b). We found the effect of platelets to be dependent on when they were added to the assay. When platelets were

added to the assay after the LEC monolayer had been injured there was an inhibition in wound healing. Whether this is truly due to the interaction of CLEC-2 and podoplanin or is caused by platelets physically occupying the wound area, thereby preventing wound healing, is unclear. The physical blockade appears to be more likely, because when we pre-incubated platelets with the LEC monolayer for an hour before wounding, the platelets promoted wound recovery.

To again assess the effect of podoplanin clustering without the need for platelets, we crosslinked podoplanin using antibodies in the wound recovery assay. Crosslinking podoplanin had no significant effect on VEGF-C stimulated wound recovery, but significantly increased wound recovery in the absence of VEGF-C. on balance, we conclude that the effects of platelets, at least through podoplanin ligation, are negligible in the wound assay.

The discrepancies between the data obtained with the wound assay compared to the transfilter data may be due to the assays modelling different types of migration: the transfilter assay models the migration of individual sparsely-seeded cells, while the scratch assay shows migration of cells from a monolayer, tending to act as a sheet. Additionally, the effect of VEGF-C was different in the two assays. VEGF-C had no effect on migration in the wound recovery assay but promoted transfilter migration. Other groups using this assay found that VEGF promoted vascular endothelial cell migration (Gao et al., 2013; Zang et al., 2013) and that VEGF-C promoted LEC migration (Yin et al., 2011). The differences between

in vitro migration assays will be returned to later in this thesis (Chapter4), after the description of a third assay that was used to model network formation by LEC.

In summary, we have shown in this chapter that platelets are able to inhibit the VEGF-C simulated migration of LEC and HMEC-1. The effect of platelets can be recreated using antibodies to crosslink podoplanin, which suggests that platelet CLEC-2 is clustering podoplanin on LEC. Similarly, the effect of platelets is lost after podoplanin knockdown. Furthermore, we have shown that the releasate from rhodocytin-stimulated platelets does not have any inhibitory effect, implying that the inhibitory effect of platelets is not via a soluble mediator released after CLEC-2 mediated platelet activation. We have also presented evidence to suggest that platelet-derived microvesicles have the potential to inhibit LEC migration. Finally, we characterised a wound recovery assay and used this as a second model of LEC migration. However, we found that this was not dependent on VEGF-C or podoplanin ligation, which may be due to the type of migration that it models.

CHAPTER FOUR

THE ROLE OF THE PODOPLANIN-CLEC-2 INTERACTION IN TUBE FORMATION BY LYMPHATIC ENDOTHELIAL CELLS

4.1. Introduction

Tube formation is an important process in the development of both the blood and lymphatic vascular systems. As such, assays that model tube formation by endothelial cells are widely used to study both angiogenesis and lymphangiogenesis. Work on tube formation by LEC has only previously used the Matrigel network formation assay. In this assay, LEC are seeded onto plates coated with Matrigel, which contains growth factors including EGF, FGFb, platelet-derived growth factor (PDGF) and TGFβ. The endothelial cells are cultured on the Matrigel for a relatively short period of time (6-18 hours), where they rapidly form 2-D networks before being imaged. Using this assay, Navarro *et al*. showed that siRNA-mediated knockdown of podoplanin reduced network formation by LEC and that transfection of the same siRNA into blood vascular endothelial cells had no effect on the network-forming capabilities of these cells (Navarro et al., 2008). A different study showed that human platelets inhibited LEC network formation in this assay (Osada et al., 2012). Additionally, mouse platelets have been used with human LEC in the Matrigel assay. This showed that platelets from *Clec1b*$^{fl/fl}$ mice inhibited network formation and that platelets isolated from *Clec1b*$^{fl/fl}$PF4-Cre mice had a weaker inhibitory effect (Finney et al., 2012). Therefore, there is evidence to suggest that the interaction of podoplanin and CLEC-2 negatively regulates tube formation by LEC.

Rather than using the Matrigel network formation assay, which only generates short-lived structures, we chose to characterise a different assay that has been used in the study of angiogenesis. In this assay fibroblasts are cultured until confluent and endothelial cells are

then co-cultured with the fibroblasts for several days. This tube formation assay was first described by Bishop and colleagues, who found that co-culturing fibroblasts and HUVEC resulted in the formation of tubes of HUVEC within seven days in the absence of growth factor stimulation (Bishop et al., 1999). They also reported that addition of 10ng/ml VEGF to the co-cultures increased the formation of tube-like structures and that the structures formed appeared to have lumens. The protocol outlined in that study is slightly different to the one we have used, as the Bishop study seeded the two cell types on the same day (Bishop et al., 1999). In contrast, we grew a confluent monolayer of HDF, then added LEC. This protocol is more consistent with that reported by later studies and is thought to reduce the time required to detect tube formation (Mavria et al., 2006; Kaur et al., 2011). It has not been reported for use with LEC before.

We hypothesised that platelets or antibody-mediated crosslinking of podoplanin would disrupt or prevent tube formation by LEC. To test this, we first characterised the co-culture tube formation assay to find the optimum conditions for LEC. We then used this assay to examine the effect of the podoplanin-CLEC-2 interaction on LEC tube formation.

4.2. Results

4.2.1. Characterisation of the co-culture tube formation assay

We first characterised how long the HDF and LEC would need to be cultured in order for tubes to form. HDF were grown to confluence before LEC that had been stained with Cell Tracker Green were added to the wells. The co-cultures were maintained in culture medium, either with or without the addition of 300ng/ml VEGF-C, at 37°C and 5% CO_2. Fluorescent images were taken at 24-hour intervals. They showed that LEC were able to form tube-like structures when co-cultured with HDF in either the presence or absence of VEGF-C. Tube-like structures could be detected in VEGF-C stimulated cultures after 24 hours and were present in non-stimulated cultures from 48 hours (Figure 4.1). LEC appeared elongated after 24 hours in co-cultures that had not been treated with VEGF-C. Tube formation appeared to be maximal by 72 hours, and there was no further growth by 144 hours (Figure 4.1). Indeed, the tubes appeared to be disintegrating by 144 hours. Therefore, timepoints up to 72 hours were selected for further analysis of tube formation by LEC.

Next, the endothelial cell seeding density was varied. The protocol we initially followed, which was developed using HUVEC, used $3x10^4$ endothelial cells per well of a 12-well plate. To determine whether this was the optimal seeding density, LEC were seeded onto HDF monolayers at three different densities ($3x10^4$, $6x10^4$ and $9x10^4$ per well) and the cultures were maintained in MV2 medium that had not been supplemented with VEGF-C. Images were taken using the 4x objective on a fluorescent microscope after 72 hours co-culture. These experiments showed that there was little difference in tube formation when $3x10^4$ or

$6x10^4$ LEC were seeded (Figure 4.2). Interestingly, when $9x10^4$ LEC were seeded onto the HDF monolayer, a relatively high level of background staining was observed (Figure 4.2). There also appeared to be clusters or "islands" of LEC rather than tubes, suggesting that the endothelial cells were beginning to form a sheet-like monolayer. Therefore, future experiments maintained a seeding density of $3x10^4$ LEC per well.

The final stage of characterisation involved determining the most appropriate way to analyse the images obtained in the co-culture experiments. Two analysis protocols were tested: i) manual analysis of fluorescent images; ii) post-staining of the co-cultures using an anti-podoplanin antibody, followed by automated analysis. The two analysis protocols are described fully in the Materials and Methods chapter (section 2.9.4). The images used for analysis were of co-cultures maintained in medium with or without 300ng/ml VEGF-C. All images were taken with a 4x objective, either using fluorescence or bright field microscopy. In these images, there appeared to be more tubes detected in the absence of VEGF-C by the post-staining protocol compared to the fluorescence protocol (Figure 4.3A), which was confirmed when the images were analysed numerically (Figure 4.3B and C). Addition of VEGF-C increased tube length, which was confirmed by both of the analyses. We found that the analysis of the fluorescent images tended to show greater variability between experiments (Figure 4.3B), which resulted in only the post-stained analysis showing a significant difference between basal and VEGF-C stimulated tube formation (Figure 4.3C).

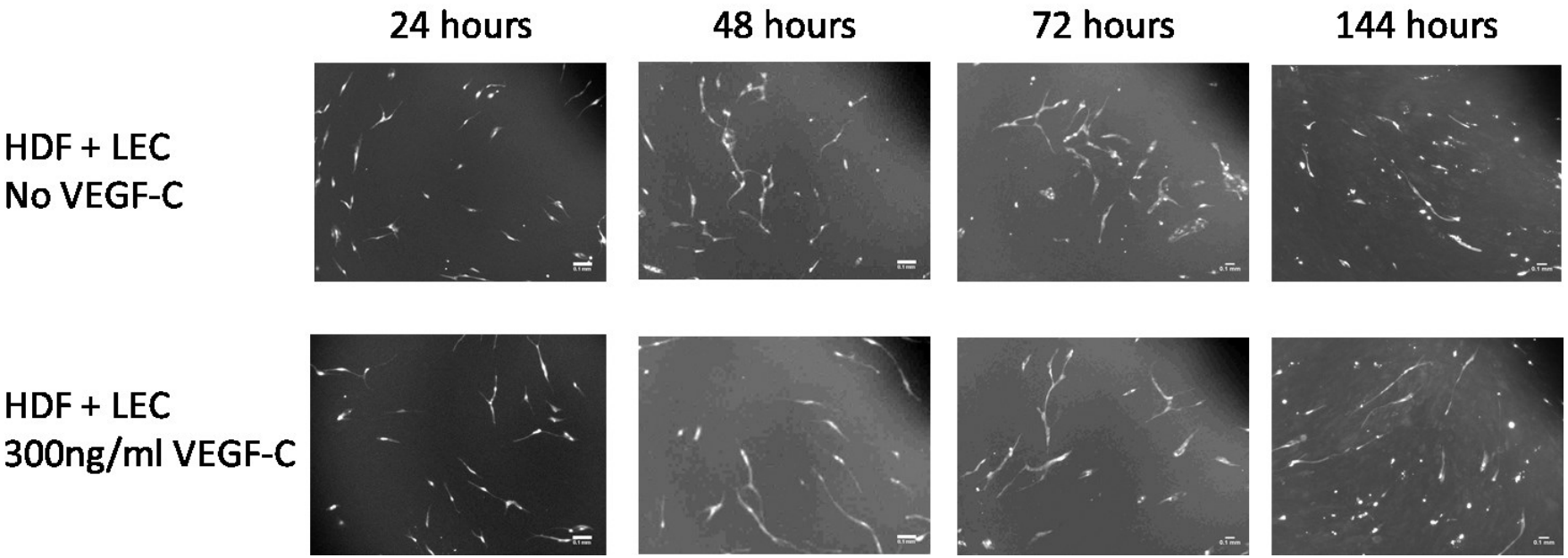


Figure 4.1: Time course of tube formation. HDF were grown to confluence on 12-well plates. $3x10^4$ LEC were stained with 5µM Cell Tracker Green and seeded onto the HDF monolayer in the presence of MV2 medium with (lower panel) or without (upper panel) 300ng/ml VEGF-C. Cultures were maintained at 37°C and 5% CO_2 and imaged using the 4x objective of a fluorescent microscope at 24 hour intervals. Images are representative of three independent experiments. Scale bars represent 100µm.

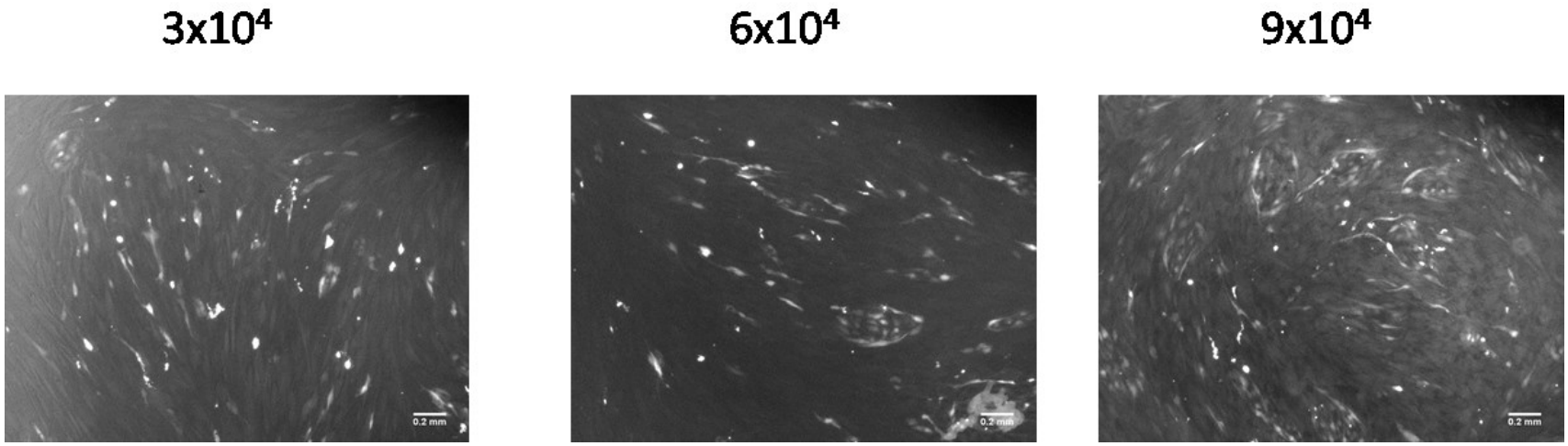


Figure 4.2: Effect of cell seeding density on tube formation by LEC. LEC were stained with 5µM Cell Tracker Green and seeded onto HDF monolayers at densities of $3x10^4$, $6x10^4$ or $9x10^4$ cells per well. Co-cultures were maintained in MV2 medium that had not been supplemented with VEGF-C. Images were taken after 72 hours with a 4x objective. Images are representative of two independent experiments. Scale bars represent 200µm.

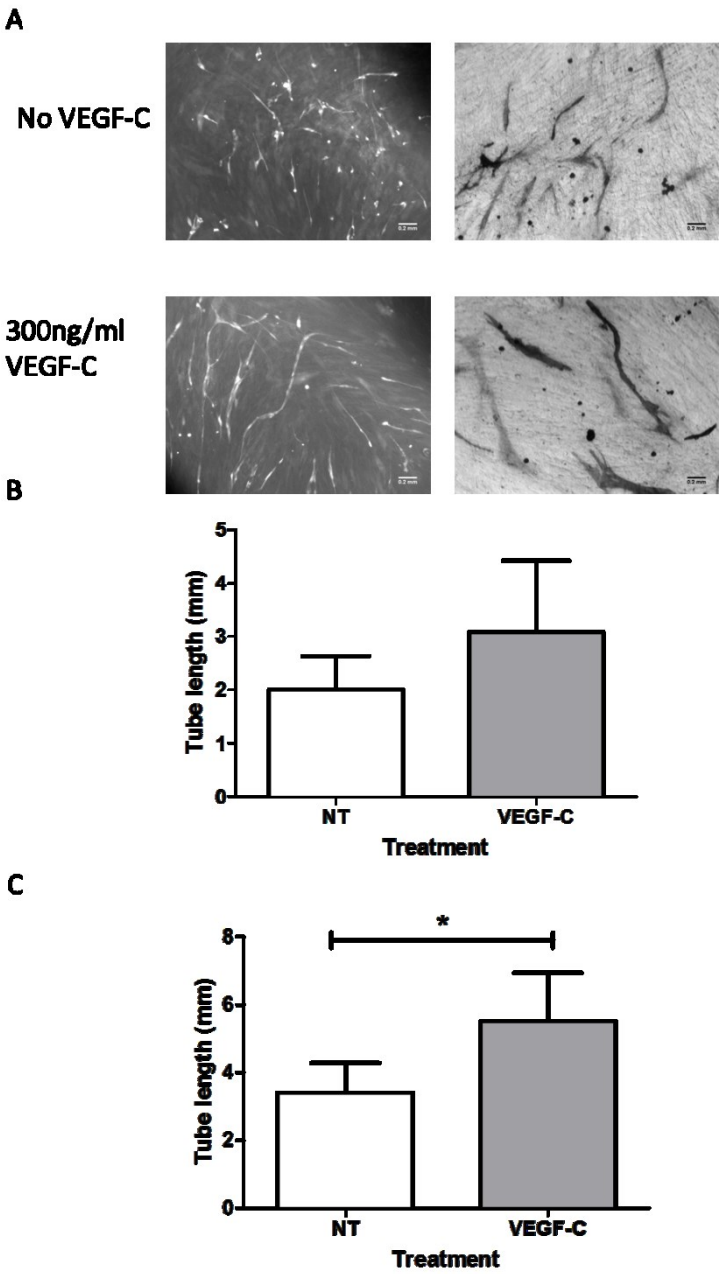


Figure 4.3: Comparison of analysis protocols for the co-culture tube formation assay. (A) Fluorescent images (top panels) were taken of basal or VEGF-C stimulated co-cultures using a 4x objective. These same cultures were then fixed with 70% ethanol and post-stained with an anti-human podoplanin antibody and alkaline phosphatase-conjugated secondary. The post-stained plates were imaged using bright field microscopy and a 4x objective. Images are representative of three independent experiments. Scale bars represent 200µm. (B) The length of tubes in fluorescent images was analysed by manually drawing along the length of each tube and measuring it in ImageJ. (C) Post-stained images were analysed using the Angiogenesis Analyzer plugin for ImageJ. * $P<0.05$ by paired t-test. Data in both graphs are mean ± SEM of three independent experiments.

However, there were occasions where the post-stained images did not reproduce tube networks seen with the "live" fluorescent images of the same co-culture. An example of this is shown in Figure 4.4. In the fluorescent images, there were clear tubes in the co-culture (Figure 4.4; left panel). In contrast, the post-stained images from both conditions show smaller, fragmented structures (Figure 4.4; right panel). The reason for the occasional discrepancies between the two analyses is not clear. Therefore, in order to maintain consistency in analysis, we decided to use the manual measurement of tubes in fluorescent images. Another advantage of this analysis is that it could be repeated at intervals for a given culture.

4.2.2. Effect of platelets on co-culture tube formation

Having characterised the co-culture assay, we added washed human platelets to assess whether they would have any effect on tube formation by LEC. This experiment was performed in two different ways: i) LEC were co-cultured with HDF for three days before the addition of platelets; ii) LEC and platelets were added to the HDF monolayer on the same day. Therefore, we were able to examine whether platelets disrupted pre-formed tubes or prevented the formation of LEC tubes. First, HDF and LEC were co-cultured in the presence of VEGF-C for three days before fluorescent images were taken using a 10x objective. Washed human platelets were then added to selected co-cultures and the plate was incubated at 37°C and 5% CO_2 for 24 hours, after which time further fluorescent images were taken. These experiments confirmed that LEC were able to form tubes when co-cultured with HDF in the presence of VEGF-C (Figure 4.5). The addition of washed human

platelets resulted in disruption of the previously-formed tubes, with LEC appearing rounded (Figure 4.5).

Next, we assessed whether platelets could prevent the formation of LEC tubes. This was done by adding the LEC and washed platelets to the co-culture on the same day. Fluorescent images were taken after three days of co-culture. This showed that the addition of washed platelets prevented the formation of tubes, although the LEC appeared to be more elongated in these co-cultures (Figure 4.6; right) compared to those where platelets were added after the formation of the tubes (Figure 4.5; bottom right).

In order to compare the approaches, tubes in the images were manually measured as described previously (section 2.9.4). This showed that platelets were able to promote the disintegration of LEC tubes (Figure 4.7A). We also found that platelets displayed a tendency to prevent tube formation (Figure 4.7B), but the effect of platelets was not significant in this protocol. For further experiments we chose to use the model of tube disintegration as this protocol showed a significant effect of platelets.

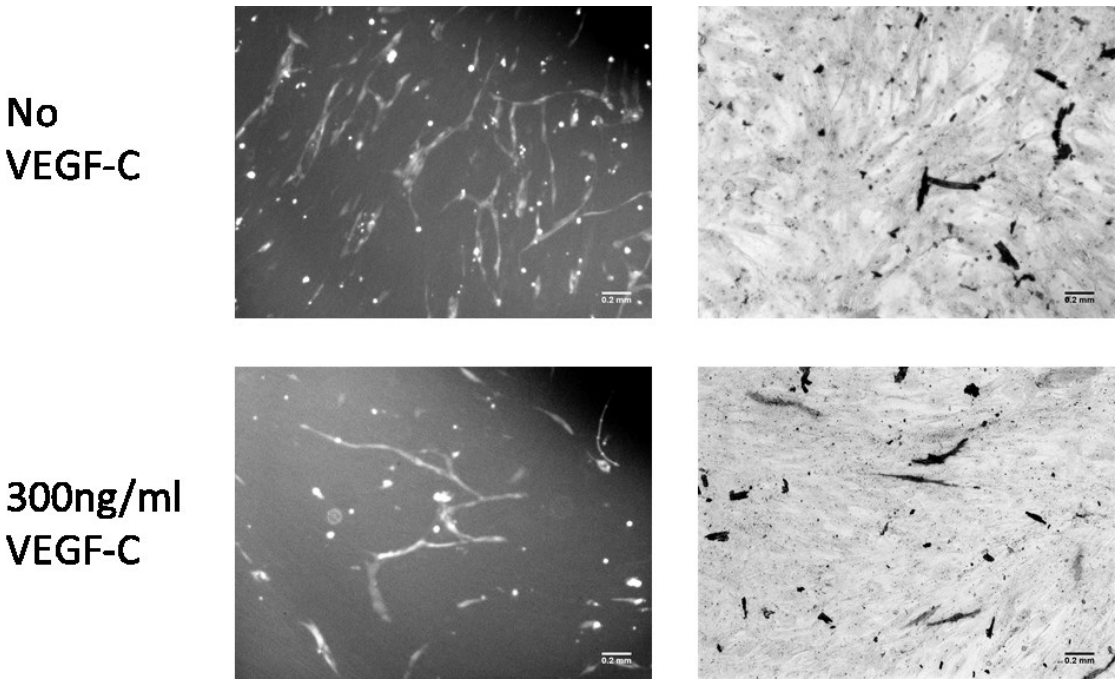


Figure 4.4: Observed occasional differences in tubes detected in fluorescent versus post-stained co-cultures. LEC and HDF were co-cultured in medium with or without 300ng/ml VEGF-C before fluorescent images (left panel) were taken using a 4x objective. The same co-cultures were fixed and then post-stained with an anti-podoplanin antibody and alkaline phosphatase-conjugated secondary. Bright field images were taken of the post-stained cultures (right panel). Images are representative of a minimum of six images taken per well. Scale bars represent 200µm.

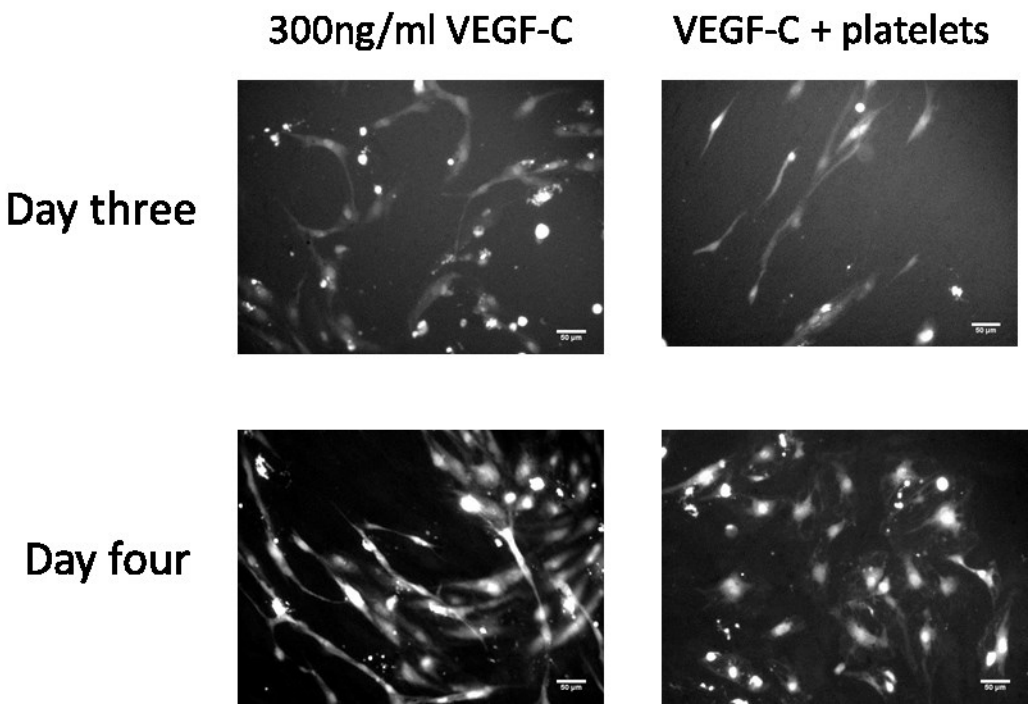


Figure 4.5: Effect of washed human platelets on pre-formed networks of LEC. HDF were grown to confluence on 12-well plates. $3x10^4$ LEC were stained with 5µM Cell Tracker Green and then cultured with the HDF in the presence or absence of VEGF-C for three days. Fluorescent images were taken of all wells. $2x10^8$ washed human platelets were added to selected wells. The co-cultures were incubated at 37°C and 5%CO_2 for a further 24 hours, then fluorescent images were taken. Images are representative of those obtained in three independent experiments. Scale bars represent 50µm.

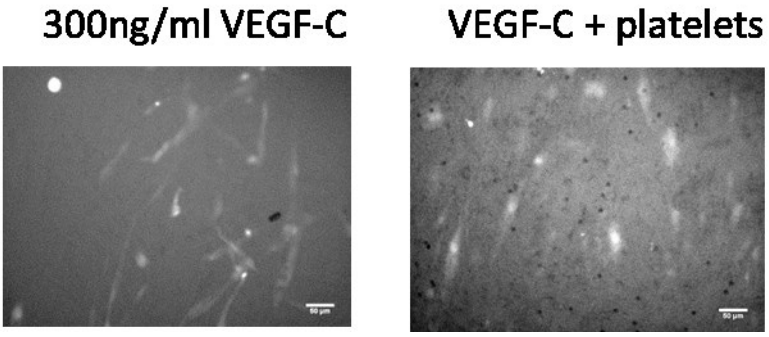


Figure 4.6: Effect of platelets on network formation by LEC. $3x10^4$ LEC were seeded onto confluent monolayers of HDF in the presence of VEGF-C and incubated at 37°C and 5% CO_2 for an hour. $2x10^8$ washed human platelets were added to selected wells. The co-cultures were incubated at 37°C for three days before fluorescent images were taken using a 10x objective. Images are representative of those taken in three independent experiments. Scale bars represent 50µm.

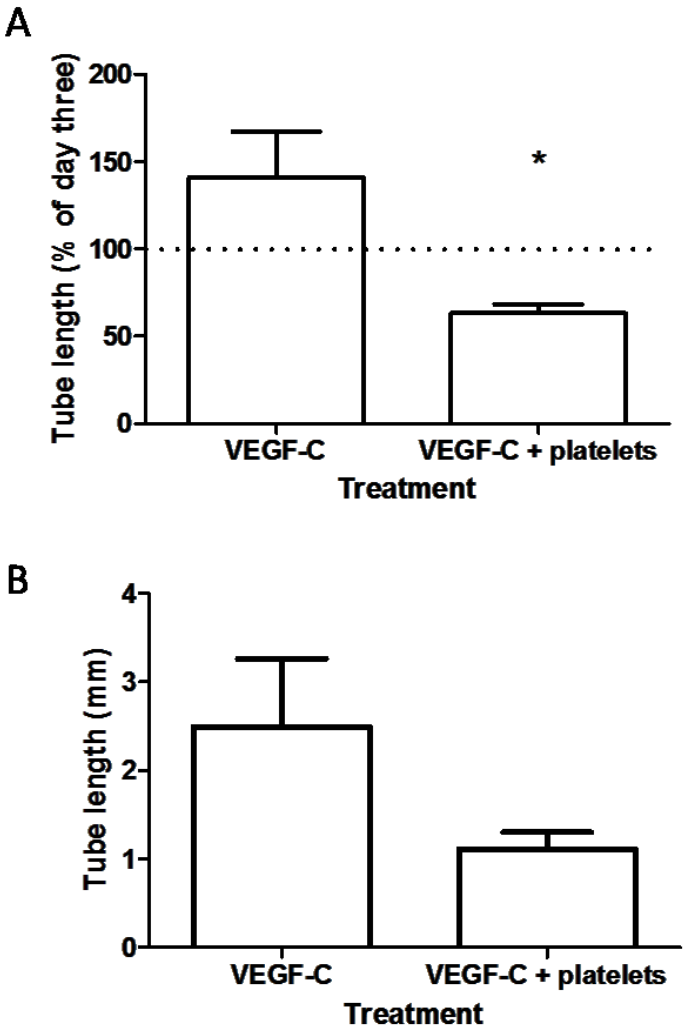


Figure 4.7: Analysis of the co-culture assay in the presence of platelets. The tubes observed in the fluorescent images of the co-cultures were manually measured, as described in the Materials and Methods chapter. (A) Analysis of co-cultures in which networks were allowed to form before platelets were added. Values are expressed relative to the value on day three. * = $p<0.05$ compared to VEGF-C by paired t-test. (B) Analysis of co-cultures in which platelets were added on the same day as LEC. Values are absolute tube length. Paired t-test found no significant difference. Both data sets are mean ± SEM of three independent experiments.

In order to determine the requirement for CLEC-2 in platelet-induced disintegration of tubes, we repeated the co-culture assay with platelets isolated from *Clec1b*$^{fl/fl}$ or *Clec1b*$^{fl/fl}$PF4-Cre mice. The addition of either *Clec1b*$^{fl/fl}$ or *Clec1b*$^{fl/fl}$PF4-Cre platelets disrupted the pre-formed tubes, although elongated LEC could still be seen (Figure 4.8A; central and right panels). Analysis of these images showed that both *Clec1b*$^{fl/fl}$PF4-Cre and *Clec1b*$^{fl/fl}$ platelets caused significant disintegration of tubes in the presence of VEGF-C (Figure 4.8B). However, there was no significant difference in tube length when *Clec1b*$^{fl/fl}$ or *Clec1b*$^{fl/fl}$PF4-Cre platelets were compared.

4.2.3. Effect of crosslinking podoplanin on co-culture tube formation

Having shown that platelets were able to disrupt networks formed by LEC, we assessed whether antibody-mediated crosslinking of podoplanin would have a similar effect. LEC were stained with Cell Tracker Green and seeded onto confluent monolayers of HDF in culture medium containing 300ng/ml VEGF-C. The cells were cultured at 37°C and 5% CO_2 for three days before fluorescent images were taken using a 4x objective ("Day three"). Cultures were treated with either 2µg/ml rat IgG or anti-human podoplanin before the addition of 30µg/ml secondary antibody, which induced crosslinking of podoplanin where the anti-podoplanin had been used. The cells were cultured for a further 24 hours before further fluorescent images were taken. These images showed that networks of LEC formed in all of the co-cultures before antibodies were added (Figure 4.9; top row). These networks could still be detected in the non-treated or IgG-treated co-cultures 24 hours later (Figure 4.9; bottom left and centre). However, when podoplanin had been crosslinked the networks disintegrated within 24 hours, although there were still fluorescent LEC detected in the co-

cultures (Figure 4.9; bottom right). Quantification of tube length in these co-cultures confirmed that crosslinking podoplanin caused the disintegration of the networks (Figure 4.10). Combining a rat IgG with the secondary antibody also appeared to have a small effect on the length of the tubes, but this was much weaker than the effect of crosslinking and was not statistically significant (Figure 4.10).

We also assessed whether crosslinking podoplanin could prevent the formation of LEC networks. In these experiments, LEC were seeded onto HDF in the presence of 300ng/ml VEGF-C. LEC were allowed to settle for an hour before treatment with antibodies. After podoplanin had been crosslinked, the cells were co-cultured for three days at 37°C and 5% CO_2. At the end of this incubation, fluorescent images were taken using a 10x objective. We found that networks of LEC were maintained in all of the tested conditions (Figure 4.11A) and quantification of tube length confirmed that there was no significant difference in tube length (Figure 4.11B).

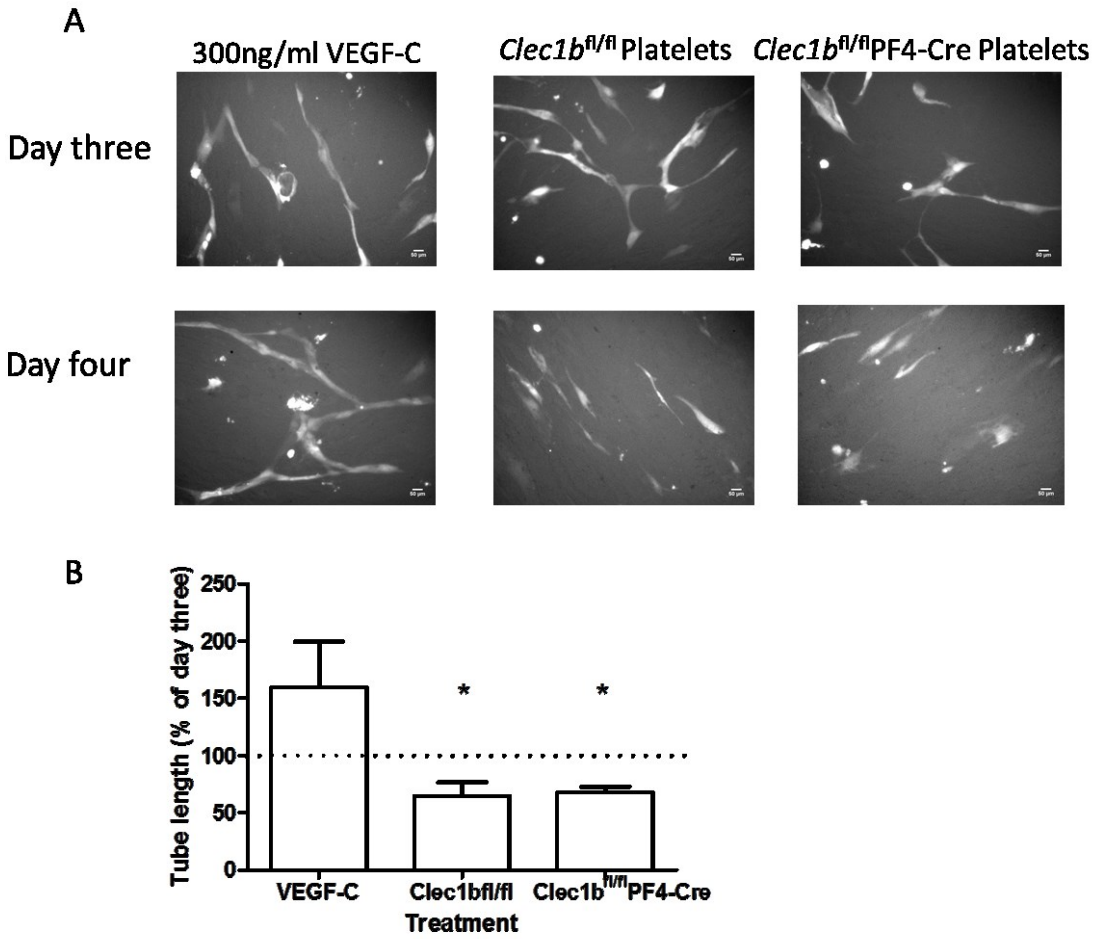


Figure 4.8: Effect of mouse platelets on pre-formed LEC networks. (A) HDF and LEC were co-cultured for three days. Fluorescent images were taken, then $2x10^8$ washed platelets from *Clec1b*$^{fl/fl}$ or *Clec1b*$^{fl/fl}$PF4-Cre mice were added to the co-culture. After 24 hours incubation with platelets, further fluorescent images were taken. Images are representative of those taken in four independent experiments. Scale bars represent 50µm. (B) Analysis of tube length in the same experiments. Dotted line represents normalised tube length at day three. ANOVA showed an effect of platelets ($p<0.05$). * = $p<0.05$ compared to VEGF-C by Dunnett's post-test. Data are mean ± SEM of four independent experiments.

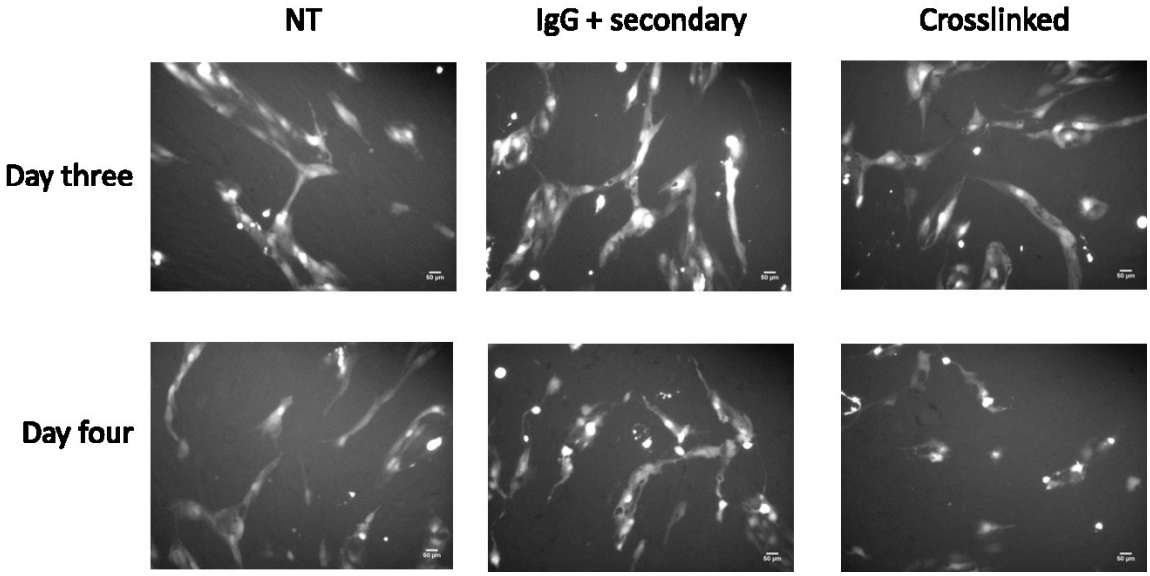


Figure 4.9: Effect of podoplanin crosslinking on the stability of LEC networks. $3x10^4$ LEC were stained with 5µM Cell Tracker Green, then co-cultured with HDF for three days. Fluorescent images were taken using a 10x objective before podoplanin crosslinking was induced using an anti-podoplanin antibody and anti-rat secondary antibody. As controls, cultures were either treated with sterile PBS or a rat IgG along with the secondary antibody. The cultures were incubated at 37°C and 5% CO_2 for 24 hours before further fluorescent images were taken. Images are representative of those taken in at least two independent experiments.

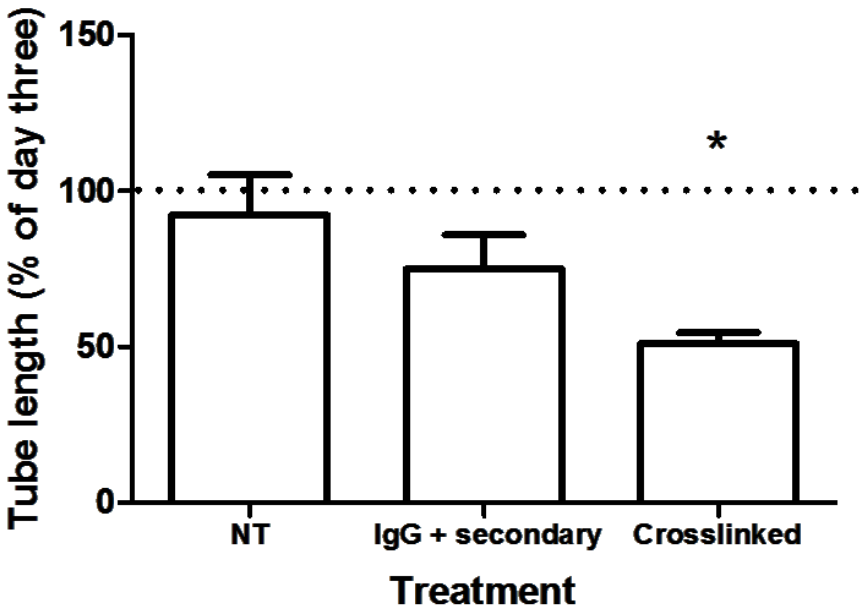


Figure 4.10: Measurement of tube length in co-cultures before and after treatment with antibodies. Fluorescent images taken before and after antibody treatment were manually analysed for tube length. Tube length was expressed as a percentage of that seen before treatment on day three. ANOVA showed that treatment was significant ($p<0.05$). * = $p<0.05$ by compared to no treatment (NT) by Dunnett's post-test. Data are mean ± SEM of 2-5 independent experiments.

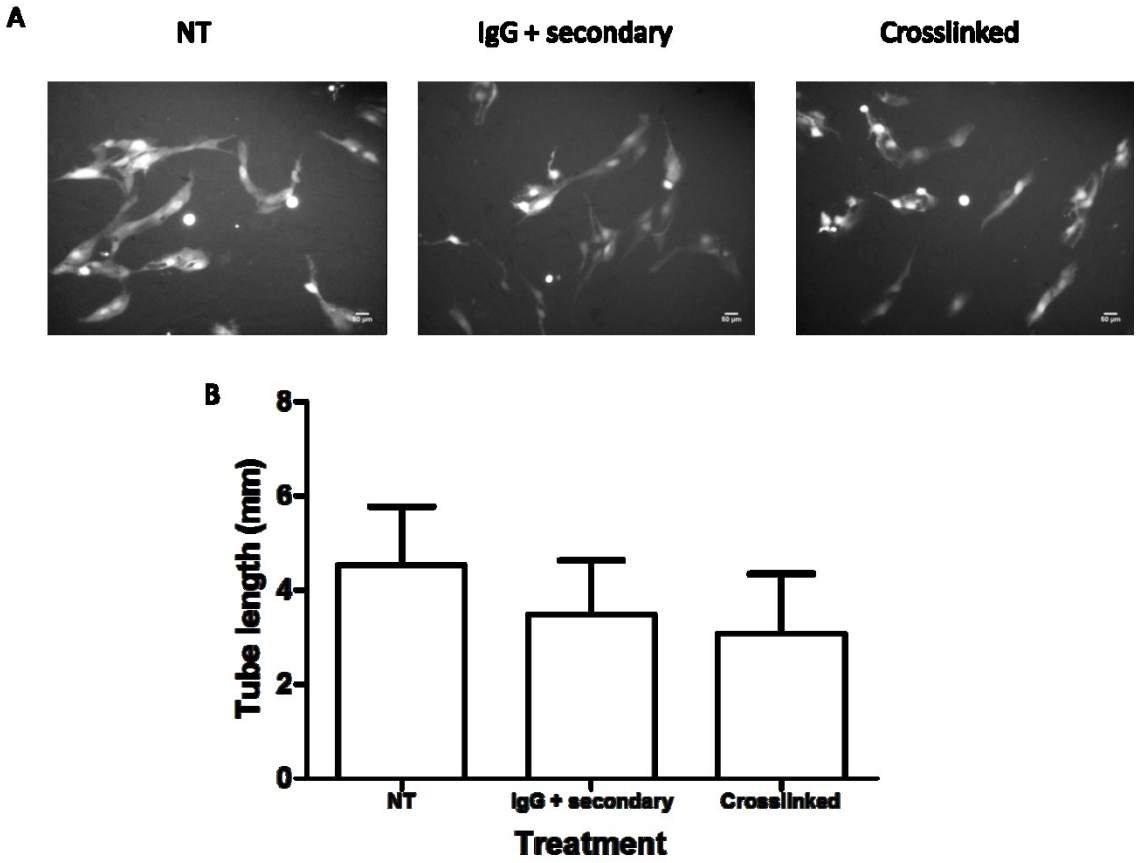


Figure 4.11: Effect of podoplanin crosslinking on network formation by LEC. $3x10^4$ LEC were stained with 5µM Cell Tracker Green and seeded onto monolayers of HDF in culture medium containing 300ng/ml VEGF-C. After incubation for an hour at 37°C and 5% CO_2, podoplanin was crosslinked using an anti-podoplanin antibody and appropriate secondary. The controls were treated with sterile PBS or a rat IgG along with the secondary antibody. The cultures were maintained at 37°C for three days. (A) Fluorescent images of the co-cultures were taken using a 10x objective. Images are representative of those taken in four independent experiments. Scale bars represent 50µm. (B) The length of the tubes in each image was manually measured. One-way ANOVA determined that treatment had no effect on tube length. Data are mean ± SEM of four independent experiments.

4.3. Discussion

This chapter has described the characterisation of a "tube" formation assay, in which LEC were co-cultured with HDF, and the use of this assay to determine the effects of platelets and of ligation of podoplanin on network formation by LEC. These studies showed for the first time that LEC are able to form tube-like structures when co-cultured with HDF and that VEGF-C promoted network formation in this assay. In the initial description of this assay, VEGF-A was found to promote the formation of tubes by HUVEC (Bishop et al., 1999). We showed for the first time that human platelets inhibited the formation of LEC networks and also promoted the disintegration of pre-existing networks. The inhibition of network formation may be linked to the inhibitory effect of platelets on LEC migration, described earlier in this thesis. The inhibitory effect of human platelets on LEC network formation on Matrigel has previously been described by another group (Osada et al., 2012). However, the disintegration of formed networks by platelets suggests the interaction of podoplanin and CLEC-2 may also cause LEC to dissociate from each other, for instance by reducing the stability of cell-cell interactions.

Platelets from *Clec1b*$^{fl/fl}$ or *Clec1b*$^{fl/fl}$PF4-Cre mice promoted the disintegration of LEC tubes. Interestingly, there was no difference in the degree of disruption of LEC networks between the two types of platelets. In contrast, data obtained with the transfilter migration assay showed a clear difference in the inhibitory effect of *Clec1b*$^{fl/fl}$ and *Clec1b*$^{fl/fl}$PF4-Cre platelets on motility. Previously-published studies showed that *Clec1b*$^{fl/fl}$ platelets prevented LEC network formation on Matrigel and that *Clec1b*$^{fl/fl}$PF4-Cre platelets partially prevented

network formation (Finney et al., 2012). The apparent discrepancy between the current data and the results reported by Finney *et al.* may be due to the differences between the co-culture and Matrigel assays, which is explored further below. In addition, the Matrigel assay examines network formation, while the co-culture tube formation assay can examine both tube formation and disruption. Another explanation for the discrepancy in the co-culture and transfilter results is that the formation of LEC networks is likely to involve motility of LEC, which is assessed in the transfilter assay. In contrast the dissociation of pre-formed networks may not require LEC migration. Therefore, this tube disruption protocol could be modelling a different phenomenon, which would explain the lack of difference between *Clec1b*$^{fl/fl}$ and *Clec1b*$^{fl/fl}$PF4-Cre platelets. In either case, it appears that the effects of platelets are partially dependent on CLEC-2. However, there may be additional mechanisms independent of CLEC-2 underlying the effect of platelets on both LEC migration and network formation.

To examine the role of podoplanin ligation in LEC network formation without the influence of any other platelet effects, antibodies were used to crosslink podoplanin. These experiments showed that podoplanin crosslinking caused the disintegration of pre-formed LEC networks, but that crosslinking did not significantly inhibit the initial formation of these networks. This suggests that the effect of platelets on network formation may be largely independent of the CLEC-2-podoplanin interaction, while the podoplanin clustering by CLEC-2 may be more important in the dissolution of established networks. Comparing the current results to the Matrigel assay data, in which *Clec1b*$^{fl/fl}$PF4-Cre platelets had a weaker effect

than *Clec1b*$^{fl/fl}$ platelets on formation of networks (Finney et al., 2012), suggests that the two assay systems are measuring different phenomena.

To our knowledge, there are no published studies where this co-culture assay has been used to assess tube formation by LEC. There have been many studies that used the Matrigel network formation assay with LEC (Navarro et al., 2008; Finney et al., 2012; Osada et al., 2012). However, there are limitations to that assay, namely that the networks form on a surface of deposited growth factors and that the networks only persist for a matter of hours (Francescone et al., 2011). Indeed, cells appear to die soon after. In comparison, the co-culture assay forms endothelial networks on a surface of HDF and these networks can be maintained for several days (Bishop et al., 1999). There is also evidence to suggest that the structures formed in this assay have lumens (Bishop et al., 1999). Therefore, we would argue that the co-culture assay is a more appropriate model of tube formation by endothelial cells *in vivo*.

In summary, the data in this chapter has shown that LEC form networks of tube-like structures when co-cultured with HDF. Washed human platelets prevented the formation of these structures and promoted the dissolution of pre-existing networks. This dissolution of networks appeared to be partially dependent on CLEC-2. Crosslinking podoplanin also caused network disintegration, but did not affect the formation of LEC networks. Having shown that the interaction of CLEC-2 and podoplanin has effects on migration and network

stability for LEC, the next chapters explore signalling pathways that may underlie these effects, starting with links to the RhoA pathway.

CHAPTER FIVE

THE ROLE OF RHOA SIGNALLING IN LYMPHATIC ENDOTHELIAL CELL MIGRATION

5.1. Introduction

RhoA has long been known to be regulate cell migration and is involved in several migratory processes, including actin polymerisation, stress fibre formation and assembly of focal adhesions (Aepfelbacher et al., 1997; van Nieuw Amerongen and van Hinsbergh, 2001). It is thought that there needs to be precise regulation of RhoA as cell migration can be reduced by both inhibitors and activators of RhoA (Takaishi et al., 1994; Ridley et al., 1995; Nobes and Hall, 1999; Arthur et al., 2000; Arthur and Burridge, 2001). Similarly, activation of ROCK, a signalling protein downstream of RhoA, leads to stress fibre formation (Amano et al., 1996, 1997). ROCK activation is also necessary for movement of the cell body and detachment of the rear of the migrating cell (Huttenlocher and Horwitz, 2011).

Although the cytoplasmic domain of podoplanin is very short, it is known to contain a sequence of three amino acids that allow it to bind to ERM proteins (Scholl et al., 1999; Martín-Villar et al., 2006). ERM proteins connect membrane-bound proteins to the actin cytoskeleton and are therefore involved in cell migration (Bretscher et al., 2002). Overexpression of podoplanin in MDCK cells has been shown to promote migration, which was associated with an increase in active RhoA and ERM phosphorylation (Martín-Villar et al., 2006). The authors of that study suggested that podoplanin recruited ERM proteins, leading to activation of RhoA and ROCK, which results in stabilisation and phosphorylation of ERM proteins as well as increased migration (Martín-Villar et al., 2006). Additionally, another group reported that knockdown of podoplanin reduced LEC migration and that this

inhibition of migration was associated with a reduction in active RhoA (Navarro et al., 2008, 2011).

With these studies in mind, the work in this chapter aimed to assess the role of RhoA and its effector, ROCK, in LEC migration. This was achieved through the use of inhibitors of Rho and ROCK in the transfilter assay. We hypothesised that VEGF-C treatment, which promotes migration, would increase active Rho and that crosslinking podoplanin, which inhibits migration, would reduce this. To test this hypothesis we used an ELISA-based RhoA activation assay that detects active, GTP-bound RhoA.

5.2. Results

5.2.1. Effect of RhoA inhibition on LEC migration

To assess the effect of RhoA signalling in LEC migration, an inhibitor of Rho, CT04, was used. This is a cell-permeable inhibitor based on the C3 transferase enzyme that is able to inhibit RhoA, RhoB and RhoC. Initially, a dose response experiment was performed to determine which concentration would be most effective. These experiments were performed in both the presence and absence of VEGF-C. We found that CT04 was able to inhibit both basal and VEGF-C stimulated migration and that this inhibition was significant from 1µg/ml CT04 (Figure 5.1). Higher concentrations of the inhibitor had a stronger inhibitory effect until 4µg/ml, after which there was no further effect when CT04 dose was increased. Therefore, further experiments used CT04 at a concentration of 4µg/ml.

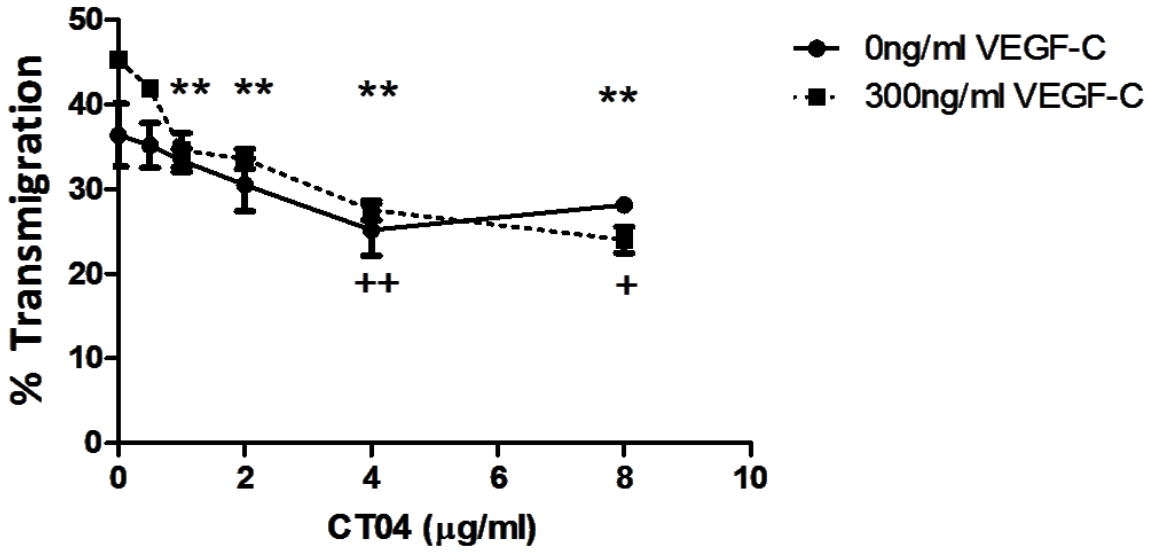


Figure 5.1: Titration of the Rho inhibitor CT04. LEC were seeded onto filters in culture medium with or without 300ng/ml VEGF-C and incubated at 37°C for an hour before CT04 (0.5-8μg/ml) was added. Percentage transmigration was determined after 24 hours. ANOVA showed a significant effect of treatment with CT04 in the absence ($p<0.005$) and presence ($p<0.001$) of VEGF-C. ** = $p<0.01$ compared to 300ng/ml VEGF-C, no CT04 by Dunnett's test. + = $p<0.05$, ++ = $p<0.01$ compared to 0ng/ml VEGF-C, no CT04 by Dunnett's test. Data are mean ± SEM of at least two independent experiments.

Next, we assessed the effect of combining the Rho inhibitor with podoplanin crosslinking. In these experiments, podoplanin was crosslinked using antibodies before the CT04 was added. In the presence of VEGF-C, crosslinking or CT04 each decreased migration similarly, but combination of CT04 with crosslinking did not further depress migration (Figure 5.2). In the absence of VEGF-C, no treatments modified migration significantly (Figure 5.2). These results indicate that while RhoA signalling is required for increased migration induced by VEGF-C, suppression of RhoA does not further impair migration after podoplanin has been crosslinked. The effect of RhoA inhibition appears less for unstimulated LEC. When data for 4μg/ml CT04 from the separate experiments on unstimulated LEC (Figure 5.1 and Figure 5.2) were combined, CT04 caused 20 ± 6% inhibition of migration (mean ± SEM from six independent experiments; $p<0.05$ by paired t-test). These results suggest that RhoA contributes to basal migration and more so, the increase induced by VEGF-C, and that the inhibitory effect of podoplanin crosslinking may involve alteration of RhoA signalling in LEC.

5.2.2. Effect of Rho kinase inhibition on LEC migration

We furthered assessed the role of RhoA signalling in LEC migration using an inhibitor of ROCK, Y27632. Initial experiments tested two different concentrations of the inhibitor. This showed that 100μM Y27632 inhibited LEC migration in the presence of VEGF-C (Figure 5.3) and this concentration was used in further experiments.

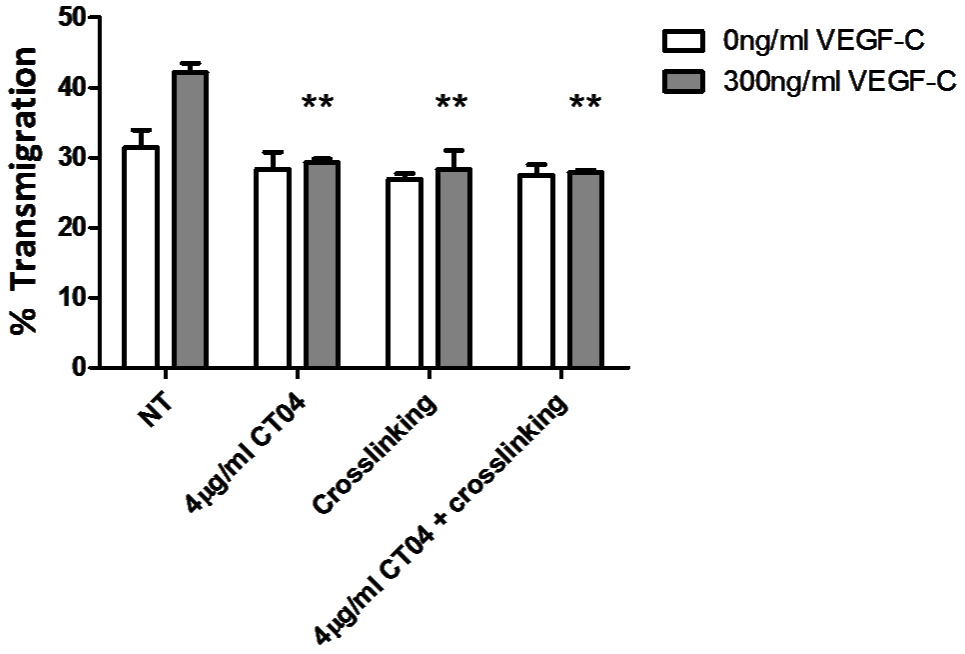


Figure 5.2: Effect of RhoA inhibition and podoplanin crosslinking on LEC migration. LEC were incubated on cell culture inserts for an hour in culture medium with or without 300ng/ml VEGF-C. Podoplanin crosslinking was induced with antibodies, before the Rho inhibitor, CT04, was added. Percentage transmigration was assessed after 24 hours. ANOVA showed a significant effect of treatment in the presence of VEGF-C ($p<0.01$), but not in the absence of VEGF-C. Bonferroni post-test showed significant effects of each treatment compared to no treatment (** = $p<0.01$), but no difference between treatments. Data are mean ± SEM of three independent experiments.

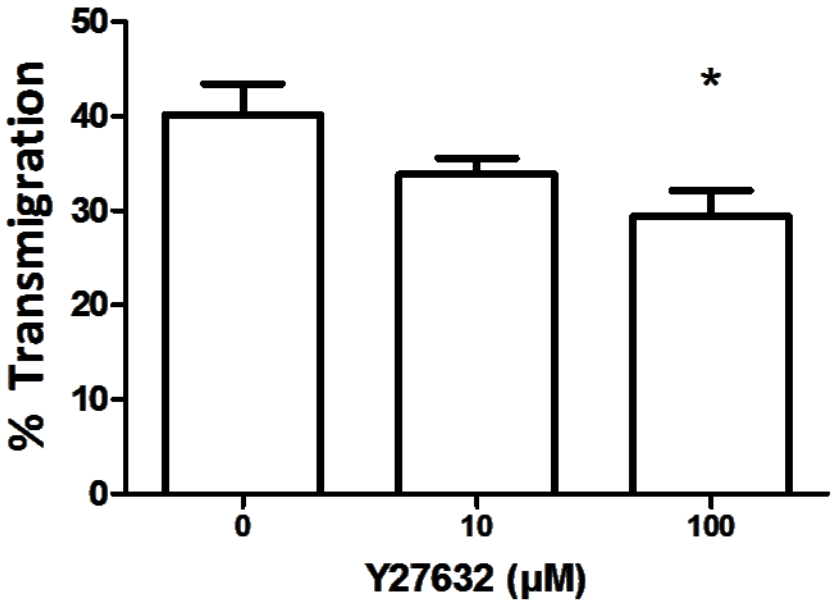


Figure 5.3: Titration of the ROCK inhibitor, Y27632. LEC were seeded onto cell culture inserts in the presence of 300ng/ml VEGF-C and allowed to settle for an hour. 10 or 100µM Y27632 was added to the filter and 100µl sterile PBS was added to the negative control. Percentage transmigration was assessed after 24 hours. ANOVA showed that Y27632 had a significant effect ($p<0.05$). * = $p<0.05$ compared to no treatment by Dunnett's test. Data are mean ± SEM of five independent experiments.

Next, we combined ROCK inhibition with podoplanin crosslinking in the transfilter assay. This experiment was originally performed as part of a preliminary study (Langan, 2011), but the n-value was increased during the course of this project. As with the Rho inhibitor experiments, podoplanin was crosslinked using antibodies before the inhibitor was added. Percentage transmigration was assessed after 24 hours. In the presence of VEGF-C, crosslinking or Y27632 each inhibited migration, but combining Y27632 with crosslinking did not further depress migration (Figure 5.4). This supports the result obtained with the Rho inhibitor and suggests that podoplanin crosslinking alters signalling involving both RhoA and ROCK in LEC.

5.2.3. RhoA activation in LEC

Having obtained evidence to suggest that crosslinking podoplanin modulates RhoA signalling, we hypothesised that there may be a change in RhoA activation. Firstly, we assessed the effect of VEGF-C treatment on RhoA activation. In keeping with the transfilter assay, LEC were seeded onto cell culture inserts before the application of VEGF-C for 5-60 minutes. We found that VEGF-C increased active RhoA in 5-15 minutes, but after 30 or 60 minutes exposure to VEGF-C, activation of RhoA reduced again (Figure 5.5). As there was little difference in active RhoA after five or fifteen minutes, we selected an incubation time of five minutes for future experiments.

We next assessed the effect of crosslinking podoplanin on RhoA activation. In the first experiments, podoplanin was crosslinked using the same antibody-mediated strategy as in

the migration assays. After podoplanin had been crosslinked, the medium on the LEC was replaced with fresh culture medium or 300ng/ml VEGF-C. The cells were incubated for five minutes before the lysates were prepared and analysed. This confirmed that VEGF-C treatment increased active RhoA, but also showed that crosslinking podoplanin had no effect on RhoA activation in the presence of VEGF-C (Figure 5.6A). There appeared to be a slight increase in active RhoA after crosslinking in the absence of VEGF-C (Figure 5.6A).

To make the time course consistent with the transfilter migration assay, we also incubated the LEC with culture medium or 300ng/ml VEGF-C for an hour before inducing podoplanin crosslinking. The cells were then incubated at 37°C for a further ten hours before the lysates were prepared. This protocol showed that a long incubation with VEGF-C increased active RhoA (Figure 5.6B). We also found that crosslinking podoplanin had no effect on RhoA activation, regardless of whether VEGF-C was present (Figure 5.6B). These results suggested that while podoplanin may interact with RhoA signalling, crosslinking podoplanin does not lead to a change in the amount of active RhoA. As each experiment was only performed twice, we were not able to statistically analyse the data.

RhoA activation was analysed in only a low number of experiments because of limitations of the assay kit, which provided reagents to analyse a maximum of 96 samples and was very expensive. As each sample needed to be run in duplicate and the positive control had to be included in each experiment, this reduced the number of samples that could be tested. Therefore, we decided it would be better to perform experiments with three different

conditions, each with an n-value of two, than with two conditions three times. However, when the data for 5 minutes treatment with VEGF-C was combined from the two experiments we found that VEGF-C increased active RhoA by 77 ± 92% (mean ± SEM of four independent experiments; $p>0.05$ by paired t-test).

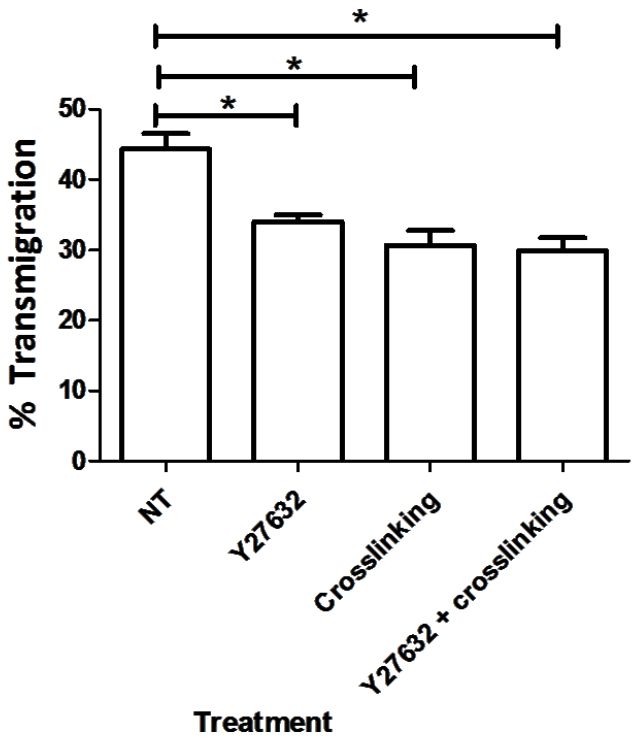


Figure 5.4: Effect of ROCK inhibition and podoplanin crosslinking on LEC migration. LEC were cultured on cell culture inserts for an hour in culture medium containing 300ng/ml VEGF-C. Podoplanin crosslinking was induced with antibodies before the addition of 100µM Y27632. Percentage transmigration was assessed after 24 hours. ANOVA showed that the treatments had a significant effect on migration ($p<0.05$). * = $p<0.05$ by Bonferroni post-test. Data are mean ± SEM of at least three independent experiments. A similar data set was reported in an MRes thesis (Langan, 2011), but the above data set has an increased n-value.

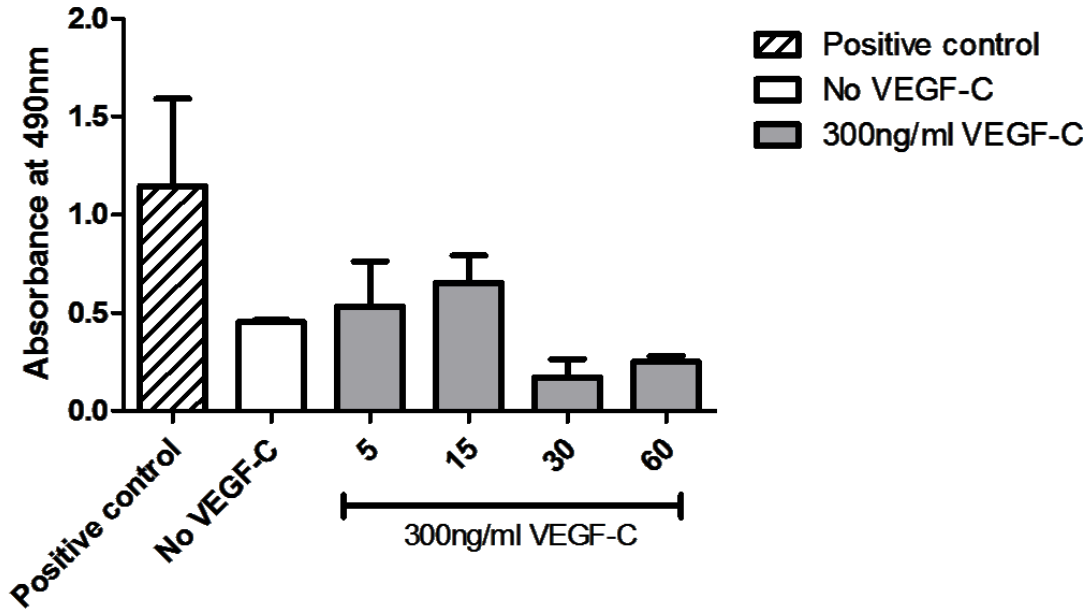


Figure 5.5: Effect of VEGF-C on activation of RhoA. LEC were seeded onto cell culture inserts and incubated at 37°C for two hours. The cells were stimulated with 300ng/ml VEGF-C for the indicated timepoints before lysates were prepared from the cells. The cells that were not treated with VEGF-C were lysed immediately. Lysates were used in a G-LISA RhoA activation assay. The positive control was provided with the assay and is a constitutively active RhoA of unknown concentration. Data are mean ± SEM of two independent experiments.

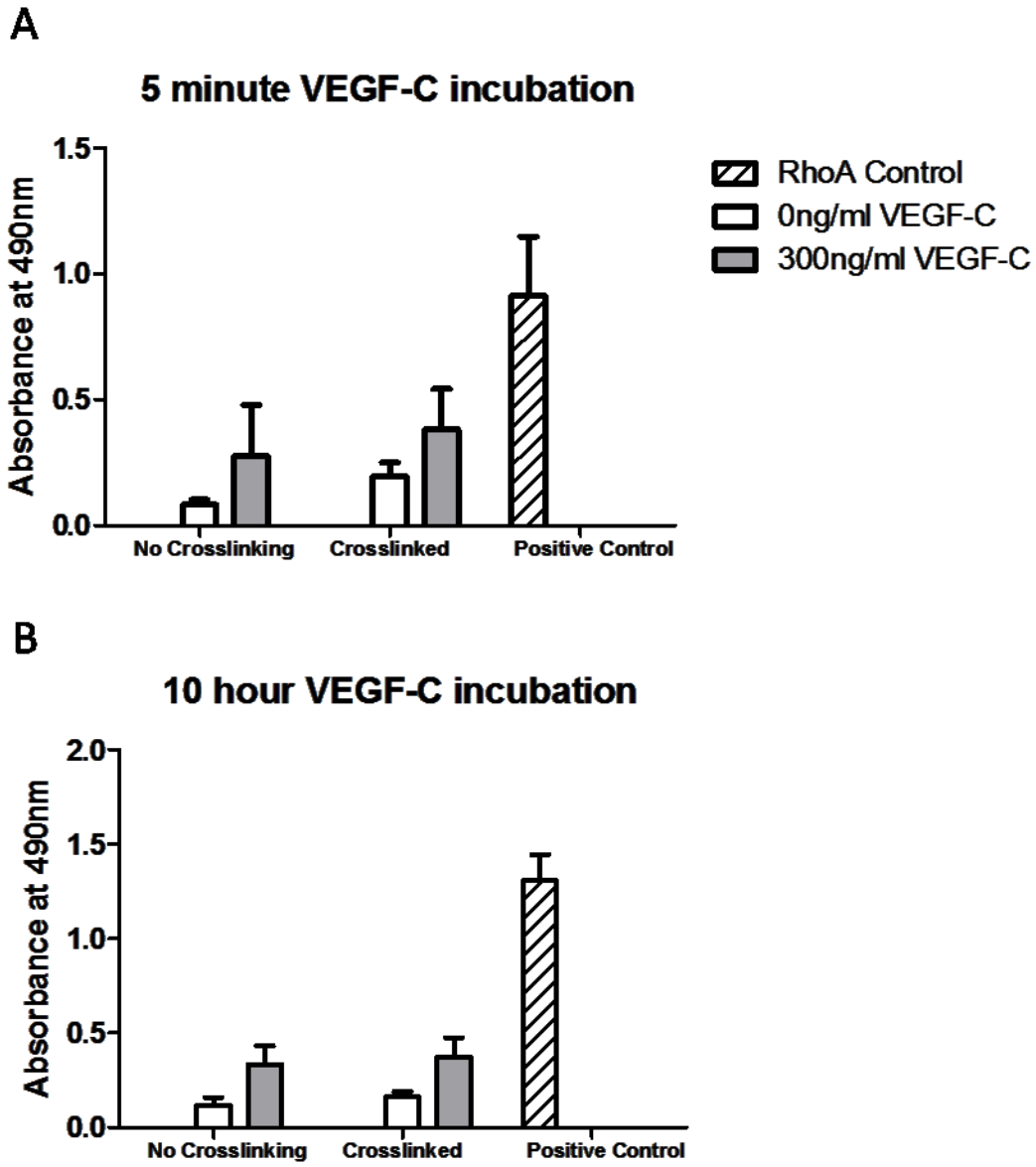


Figure 5.6: Effect of podoplanin crosslinking and VEGF-C on RhoA activation in LEC. (A) LEC were seeded onto cell culture inserts and incubated at 37°C for an hour before podoplanin crosslinking was induced with antibodies. LEC were treated with culture medium with or without 300ng/ml VEGF-C for five minutes before lysates were prepared. (B) LEC were seeded onto filters in the culture medium with or without VEGF-C and incubated for an hour before podoplanin was crosslinked. After crosslinking, the cells were incubated for a further 10 hours at 37°C before the lysates were prepared. Data in both graphs represent mean ± SEM of two independent experiments.

5.3. Discussion

In this chapter Rho and ROCK inhibitors and a RhoA activation assay were used to assess the role of RhoA signalling in LEC migration. I previously showed that the ROCK inhibitor Y27632 inhibited VEGF-C mediated LEC migration and that combining this inhibitor with washed human platelets did not give any further inhibitory effect (Langan, 2011). Here, I showed that the same was true when the ROCK inhibitor was combined with antibody-mediated crosslinking of podoplanin. This suggests that the inhibitory effect of platelets and of podoplanin crosslinking may involve ROCK signalling. Similarly, an inhibitor of Rho (CT04) was able to inhibit VEGF-C stimulated LEC migration but combining this inhibitor with podoplanin crosslinking did not result in a stronger inhibitory effect. Again, this suggests that podoplanin crosslinking may affect the Rho signalling pathway. Thus, while crosslinking reduces migration, additional inhibition of the RhoA pathway has no further effect. The data obtained with both the Rho and ROCK inhibitors is consistent with that reported by Martín-Villar *et al*. (2006), who showed that overexpression of podoplanin in MDCK cells increased the migration of these cells. They also showed that inhibition of either Rho or ROCK reduced the migration of podoplanin-expressing MDCK cells (Martín-Villar et al., 2006).

When characterising the transfilter assay with the Rho inhibitor, I found that the inhibitor reduced migration in both the presence and absence of VEGF-C. However, in later experiments the Rho inhibitor only reduced VEGF-C stimulated migration. This discrepancy is likely to be due to the comparatively small number of experiments performed. Further analysis of the data showed that CT04 inhibited unstimulated LEC migration by about 20%,

which is statistically significant. Thus, Rho activation played a role in basal as well as stimulated migration.

Having obtained data that suggested podoplanin crosslinking affected RhoA signalling, I used an ELISA-based assay to determine whether VEGF-C stimulation and podoplanin crosslinking altered the amount of active RhoA in LEC. This assay was chosen over the more established pulldown assay as it required significantly less cellular material: lysates were prepared from cells on 6-well culture inserts rather than from 20cm^2 culture dishes (van Nieuw Amerongen et al., 2000). The G-LISA assay is also easier to quantify as it gives results in terms of absorbance, rather than producing Western blots. It was previously found that overexpression of podoplanin in MDCK cells increased the amount of detectable RhoA-GTP in the absence of VEGF-C (Martín-Villar et al., 2006) and that LEC had low basal levels of RhoA-GTP, which appeared to be dependent on the level of expression of podoplanin (Navarro et al., 2008). We found that RhoA-GTP could be detected in the absence of VEGF-C. Initial experiments showed that 300ng/ml VEGF-C increased the amount of active RhoA in LEC after 5-15 minutes. However, at later timepoints (30 and 60 minutes) this increase in active RhoA was lost. A transient effect of VEGF-C on RhoA-GTP levels in LEC was reported by others, who found that the amount of active RhoA initially increased, but decreased again after 45 minutes incubation with 50ng/ml VEGF-C (Navarro et al., 2011). Our subsequent data suggested that crosslinking podoplanin could increase active RhoA in the absence of VEGF-C. However, crosslinking podoplanin had no effect on RhoA-GTP levels in VEGF-C

stimulated cells. Thus, there were no early effects on RhoA activation after crosslinking of podoplanin.

We had hypothesised that RhoA activation would be reduced upon podoplanin crosslinking, as crosslinking causes an inhibition of migration. Therefore, to make the RhoA assay time course more consistent with the transfilter migration assay, the RhoA activation assay was repeated with a longer incubation period. In these experiments, LEC were treated with VEGF-C for an hour before crosslinking was induced; the lysates were prepared after a further ten hours incubation at 37°C. This showed that VEGF-C increased active RhoA, suggesting that RhoA activation was biphasic, with early and much later responses. Nevertheless, crosslinking podoplanin still did not affect RhoA activation in LEC. Therefore, we concluded that although podoplanin crosslinking appears to modify RhoA signalling, this is not through altering the amount of active RhoA. Instead it may be through effects on proteins downstream of ROCK. These could include ERM proteins, LIM domain kinase 1 and myosin light chain phosphatase (Riento and Ridley, 2003).

In summary, we have shown in this chapter that podoplanin crosslinking may inhibit migration through interactions with the RhoA signalling pathway. In particular, RhoA and ROCK appear to operate downstream of podoplanin crosslinking. However, we do not believe that the effect of podoplanin crosslinking is through changes in the amount of active RhoA in LEC. Therefore, in the next chapter we will assess the role of the VEGF signalling pathway in LEC migration.

CHAPTER SIX

THE INTERACTION OF PODOPLANIN AND VEGF SIGNALLING IN LYMPHATIC ENDOTHELIAL CELL MIGRATION

6.1. Introduction

During embryonic lymphangiogenesis, VEGF-C is produced by the mesenchyme surrounding the developing vessels (Kukk et al., 1996; Karkkainen et al., 2004). Mice lacking VEGF-C die before birth and lack lymphatic vessels, while VEGF-C heterozygous mice are viable but do not form lymphatic vessels in the skin (Karkkainen et al., 2004). VEGF-C is known to bind to two receptors: VEGFR2 and VEGFR3; both of these receptors are expressed by LEC (Kriehuber et al., 2001; Dellinger and Brekken, 2011). However, VEGF-C has a greater affinity for VEGFR3 than VEGFR2 (Mäkinen et al., 2001b). Deletion of VEGFR3 causes defects in both blood and lymphatic vessels, which are embryonic lethal (Dumont et al., 1998). Genetic mutations that inactivate VEGFR3, the major receptor for VEGF-C, result in a similar phenotype to VEGF-C heterozygosity (Karkkainen et al., 2001).

Work described earlier in this thesis (Chapter 3) and in a previous publication (Finney et al., 2012) suggested that the interaction of CLEC-2 and podoplanin inhibited VEGF-C stimulated LEC transmigration. We have also shown that podoplanin knockdown negated the pro-migratory effect of VEGF-C (see Section 3.2.3). Therefore, we assessed whether podoplanin knockdown altered surface expression of VEGFR2 or VEGFR3.

The signalling pathway downstream of podoplanin is not well understood. There is evidence to suggest that podoplanin interacts with ERM proteins and CD44 (Martín-Villar et al., 2006, 2010); the interaction with CD44 is thought to be required for the promotion of migration by

podoplanin (Martín-Villar et al., 2010). More recently, it has been suggested that two serine residues in the cytoplasmic domain of podoplanin regulate cell motility. These serine residues can be phosphorylated by PKA and mutation of these serines to alanines improved migration of podoplanin-expressing cells (Krishnan et al., 2013). The authors suggested that PKA phosphorylation of the serine residues inhibited cell migration (Figure 1.6; Krishnan et al., 2013).

Podoplanin has been reported to interact with tetraspanins (Nakazawa et al., 2011; Iwasaki et al., 2013). The interaction with the tetraspanin CD9 was first identified in a fibrosarcoma cell line (Nakazawa et al., 2011) and tetraspanins were later found to be abundantly expressed in LEC (Iwasaki et al., 2013). The Iwasaki study also reported that CD9 was required for migration and network formation by LEC. This suggests that the podoplanin-tetraspanin interaction may be required for lymphangiogenesis.

With the above studies in mind, we assessed whether the interaction of podoplanin with VEGF signalling pathways had a role in transfilter migration of LEC. We assessed which VEGFRs were involved in LEC migration, explored the role of PKA in LEC migration and used co-immunoprecipitation experiments to assess whether podoplanin and VEGFRs were associated with tetraspanins.

6.2. Results

6.2.1. Role of VEGFR2 and VEGFR3 in LEC migration

As LEC are known to express both VEGFR2 and VEGFR3 (Kriehuber et al., 2001; Dellinger and Brekken, 2011), we assessed which of these receptors is important in LEC migration. This was done by performing transfilter assays in which LEC were stimulated with VEGF-A, VEGF-C or VEGF-C (Cys156Ser). These growth factors act on VEGFR1 and VEGFR2, VEGFR2 and VEGFR3, and VEGFR3 only, respectively. We found that all three growth factors promoted LEC migration over 24 hours (Figure 6.1), suggesting that both VEGFR2 and VEGFR3 can modify this response.

To test the relative importance of signalling via VEGFR2 or VEFGR3 in LEC migration, we performed transfilter assays with the addition of VEGFR2- and VEGFR3-blocking antibodies. LEC were seeded onto inserts in culture medium with 30ng/ml VEGF-A, 300ng/ml VEGF-C or without additional growth factors. The plates were incubated at 37°C and 5% CO_2 for an hour before the addition of 5µg/ml blocking antibodies against VEGFR2 (clone IMC-1121b), VEGFR3 (clone IMC-3C5) or a combination of the two antibodies. Percentage transmigration was assessed after 24 hours. In the absence of VEGF-C or VEGF-A, none of the treatments reduced LEC migration (Figure 6.2). We found that in the presence of VEGF-A, the VEGFR2 antibody inhibited LEC migration, but the VEGFR3-blocking antibody alone did not have a significant effect (Figure 6.2A). The combination of the antibodies had a similar effect to ant-VEGFR2 alone. When LEC were stimulated with VEGF-C, each antibody tended to reduce migration but only the combination of the two blocking antibodies gave statistically

significant inhibition (Figure 6.2B). Thus, it appears that VEGFR2 alone transduced the effect of VEGF-A, while both VEGFR2 and VEGFR3 transduced VEGF-C.

We next assessed whether podoplanin crosslinking would inhibit LEC migration stimulated by the different forms of VEGF; VEGF-A and VEGF-C (Cys156Ser), as well as VEGF-C as shown in Section 3.2.4. LEC were seeded onto culture inserts in medium with or without growth factors. The plate was incubated at room temperature for 30 minutes, then 2µg/ml anti-human podoplanin antibody was added to chosen wells. Crosslinking was induced in these same wells by the addition of 30µg/ml anti-rat secondary antibody after a further 30 minute incubation. Percentage transmigration was determined after 24 hours. We found that crosslinking podoplanin inhibited migration in the presence of each of the three tested growth factors (Figure 6.3).

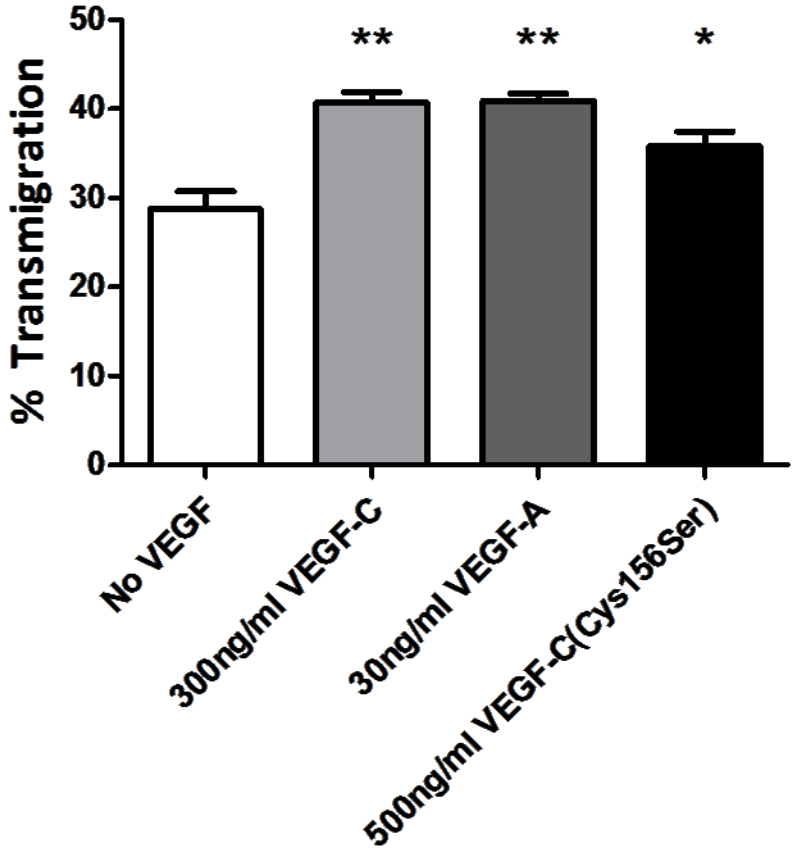


Figure 6.1: Effect of VEGFs on LEC migration. $3x10^4$ LEC were seeded onto cell culture inserts in medium without growth factors or with 300ng/ml VEGF-C, 30ng/ml VEGF-C or 500ng/ml VEGF-C(Cys156Ser). Percentage transmigration was assessed after 24 hours. ANOVA found an effect of treatment ($p<0.01$). ** = $p<0.01$ & * = $p<0.05$ compared to no VEGF by Dunnett's test. Data are mean ± SEM of four independent experiments.

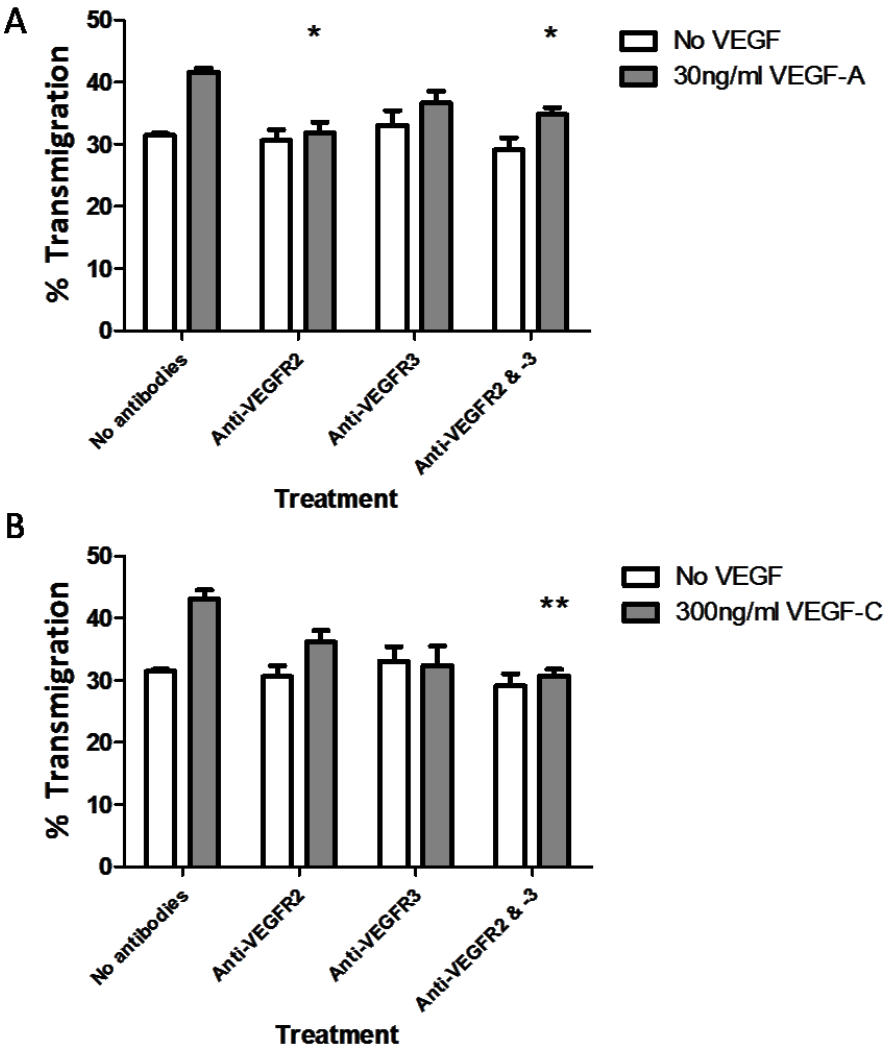


Figure 6.2: Effect of VEGFR-blocking antibodies on LEC migration in the presence or absence of vascular endothelial growth factors. $3x10^4$ LEC were seeded onto cell culture inserts in culture medium without or with 30ng/ml VEGF-A (A) or 300ng/ml VEGF-C (B). Cells were incubated for an hour at 37°C and 5% CO_2 before the addition of 5µg/ml anti-VEGFR2 or anti-VEGFR3 antibodies or a combination of the two antibodies. Percentage transmigration was assessed after 24 hours. (A) ANOVA showed a significant effect of both VEGF-A ($p<0.01$) and antibody treatment ($p<0.05$). * = $p<0.05$ compared to no antibody by Dunnett's test. (B) ANOVA showed that both VEGF-C ($p<0.01$) and antibody treatment ($p<0.05$) had a significant effect. ** = $p<0.01$ compared to no antibody by Dunnett's test. Both data sets are mean ± SEM of four independent experiments.

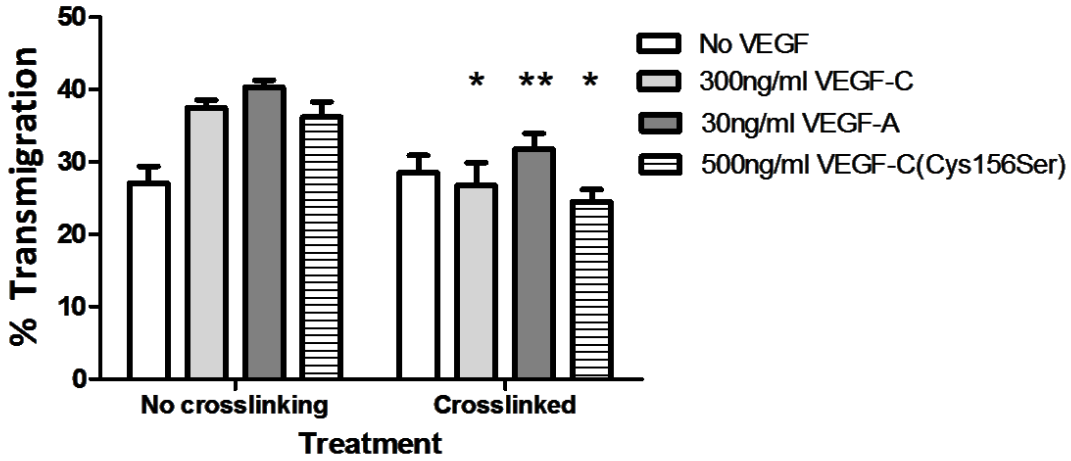


Figure 6.3: Effect of crosslinking podoplanin on LEC migration in the presence of different vascular endothelial growth factors. $3x10^4$ LEC were seeded onto cell culture inserts in medium without additional growth factors or with 300ng/ml VEGF-C, 30ng/ml VEGF-A or 500ng/ml VEGF-C (Cys156Ser). The plate was incubated at room temperature for 30 minutes. 2µg/ml anti-human podoplanin antibody was added to selected wells. Crosslinking was induced in these wells by the addition of 30µg/ml anti-rat secondary antibody. Percentage transmigration was determined after 24 hours. ANOVA showed that both VEGF treatment and crosslinking had significant effects ($p<0.05$ and $p<0.01$, respectively). * = $p<0.05$ and ** = $p<0.01$, compared to no crosslinking by Bonferroni post-test. Data are mean ± SEM of at least three independent experiments.

6.2.2. Effect of podoplanin knockdown on VEGF-A stimulated LEC migration

Earlier in this thesis, we showed that podoplanin knockdown negated the pro-migratory effect of VEGF-C and that platelets could not further inhibit the migration of podoplanin-knockdown LEC (see Section 3.2.3). We have also found that VEGF-A is able to promote LEC migration and that podoplanin crosslinking inhibits this (Figure 6.3). Therefore, we assessed the impact of podoplanin knockdown on VEGF-A mediated migration. LEC were transfected with podoplanin or non-specific siRNA duplexes or treated with lipofectamine alone. At 48 hours after transfection, $3x10^4$ LEC were seeded onto culture inserts in medium with or without 30ng/ml VEGF-A. The plate was incubated at 37°C and 5% CO_2 for 24 hours, after which percentage transmigration was determined. We found that the migration of LEC transfected with podoplanin siRNA was not promoted by VEGF-A (Figure 6.4). In comparison, the cells transfected with non-specific siRNA or treated with lipofectamine migrated more in the presence of 30ng/ml VEGF-A. Podoplanin knockdown had no effect on basal migration (Figure 6.4).

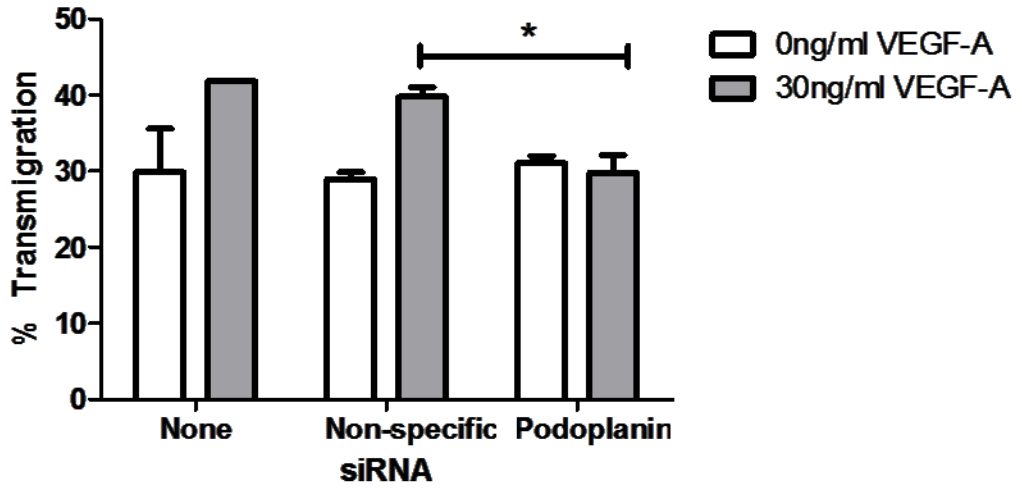


Figure 6.4: Effect of podoplanin knockdown on VEGF-A stimulated migration of LEC. LEC were seeded onto 24-well plates in medium without antibiotics. The next day, the LEC were transfected with 30nM non-specific or podoplanin siRNA. The negative controls were treated with lipofectamine but not transfected with any duplex. At 48 horus after transfection, $3x10^4$ LEC were seeded onto culture inserts in medium with or without 30ng/ml VEGF-A. Percentage transmigration was assessed after 24 hours. ANOVA showed a significant effect of VEGF-A treatment ($p<0.05$). * = $p<0.05$ by Bonferroni post-test. Error bars represent mean ± SEM of at least two independent experiments.

6.2.3. Effect of podoplanin knockdown on expression of VEGFRs

Having shown that podoplanin was required for the pro-migratory effects of both VEGF-C and VEGF-A, we began to assess the reason underlying this. Initially, we checked the expression of VEGFR2 and VEGFR3. LEC were transfected with siRNA targeting podoplanin or non-specific siRNA and incubated at 37°C and 5% CO_2 for 48 hours. Expression of VEGFR2, VEGFR3, podoplanin and CD31 was then determined by flow cytometry. Initial experiments indicated that podoplanin expression was reduced by around 50% following transfection with podoplanin siRNA (Figure 6.5A) and that the same siRNA duplexes did not alter CD31 expression (Figure 6.5B) but might cause a slight reduction in expression of VEGFR2 and VEGFR3 (Figure 6.5C&D). However, when we quantified expression in three independent experiments, there was no significant difference in expression of VEGFR2 or VEGFR3 (Figure 6.6) between podoplanin-knockdown and control transfected LEC.

6.2.4. Effect of PKA inhibition on LEC migration

Having shown that podoplanin knockdown did not affect the expression of VEGFR2 or VEGFR3, we decided to investigate the role of PKA in LEC migration. An inhibitor of PKA (H-89) was used in transfilter assays, either alone or in combination with podoplanin crosslinking. These experiments were performed in culture medium with or without VEGF-C. In the absence of VEGF-C, PKA inhibition increased migration of LEC (Figure 6.7A). Podoplanin crosslinking alone had no effect on unstimulated migration, but it negated the pro-migratory effect of the PKA inhibitor (Figure 6.7A). In the presence of VEGF-C, the PKA inhibitor did not further increase migration (Figure 6.7B). Podoplanin crosslinking inhibited

migration as expected, but combining this with the PKA inhibitor prevented the inhibition of migration.

6.2.5. Assessment of podoplanin interacting with other cell surface proteins

We also assessed whether podoplanin and VEGFRs interacted with tetraspanins in LEC. In order to examine this, LEC were surface biotinylated then lysed using 1% Brij97 lysis buffer. This lysis buffer was chosen as it is known to keep the interaction between tetraspanins and their associated proteins intact (Berditchevski et al., 1996; Rubinstein et al., 1996). The lysates were then immunoprecipitated using protein G sepharose beads that had been bound, in separate tubes, to mouse IgG or antibodies against: β1 integrin, α2 integrin, podoplanin, VEGFR2, VEGFR3, ADAM10 or CD9. The proteins were eluted from the beads, separated by SDS-PAGE and analysed by Western blots for neutravidin or CD9. We found that a 25kDa band, representing CD9, could be detected in the CD9, ADAM10 and podoplanin immunoprecipitations (Figure 6.8A), but not in those for VEGFR2 or VEGFR3. This suggests that both podoplanin and ADAM10 are tetraspanin-associated. The neutravidin blot detected a number of proteins in the podoplanin and ADAM10 co-immunoprecipitations (Figure 6.8B). The pattern of bands seen in the podoplanin and ADAM10 co-immunoprecipitations is often seen with tetraspanins and their associated proteins. Within these bands we were able to detect CD9 (25kDa), podoplanin (42kDa) and ADAM10 (80kDa). There was also a faint band around the expected molecular weight for CD9 in the VEGFR3 lane (Figure 6.8B). However, when the experiment was repeated, we were not able to detect this band (Figure 6.9B) and the association between podoplanin and

CD9 appeared much weaker (Figure 6.9A and B). We also performed the co-immunoprecipitation experiment on VEGF-C stimulated LEC and found no association between VEGFR2 or VEGFR3 and CD9 (Figure 6.9C and D). We also found the association between podoplanin and CD9 to be slightly weaker than in basal conditions (Figure 6.9C and D).

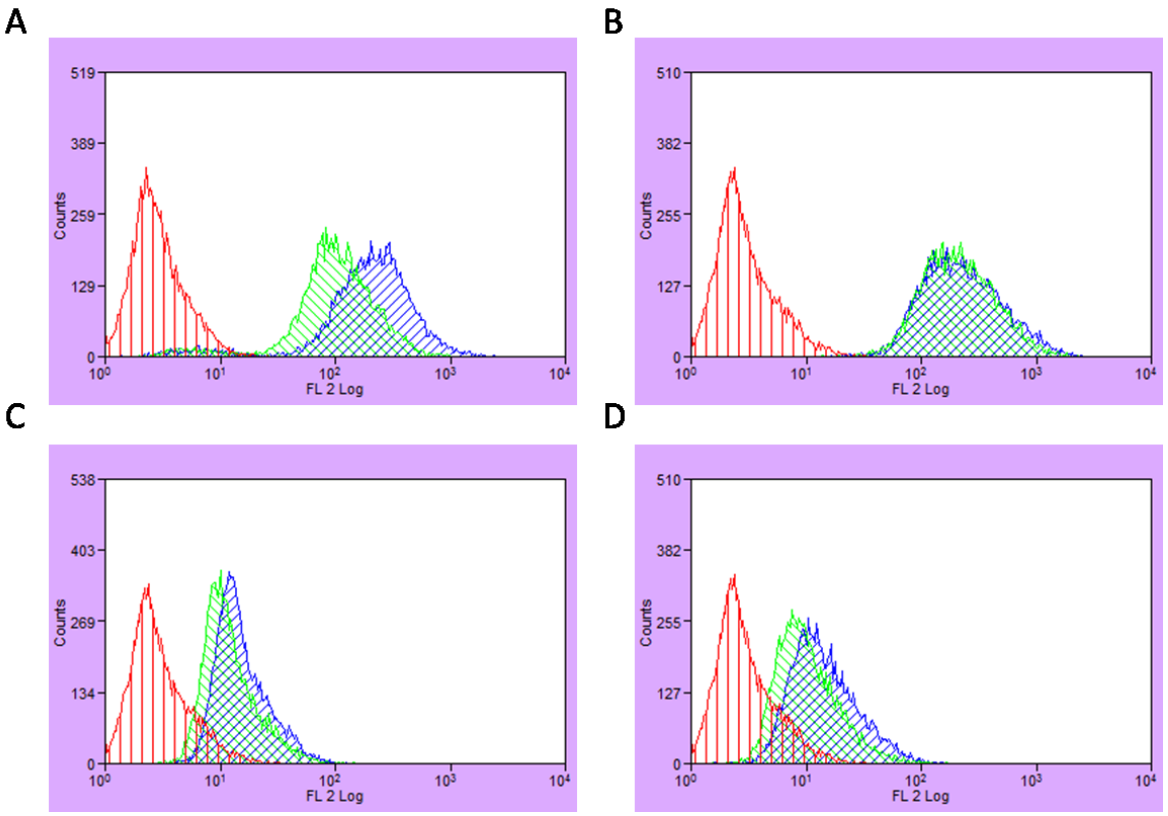


Figure 6.5: Effect of transfection of podoplanin siRNA duplexes on expression of podoplanin, CD31, VEGFR2 and VEGFR3. LEC were seeded onto 24-well plates in culture medium without antibiotics. The following day cells were transfected with podoplanin or non-specific siRNA duplexes. The transfected cells were incubated for 48 hours before being analysed by flow cytometry. The expression of podoplanin (A), CD31 (B), VEGFR2 (C) and VEGFR3 (D) was determined. In all plots, red = IgG, blue = non-specific siRNA transfection, green = podoplanin siRNA transfection. Plots are from one of three independent experiments.

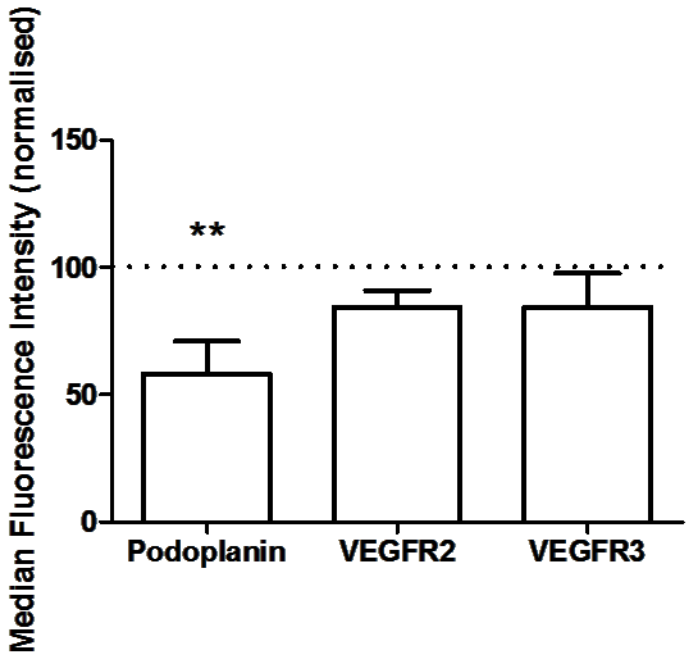


Figure 6.6: Quantitation of VEGFR2 and VEGFR3 expression following podoplanin knockdown. LEC were transfected with non-specific or podoplanin siRNA. 48 hours after transfection, LEC were stained for podoplanin, VEGFR2 or VEGFR3 and analysed by flow cytometry. The median fluorescence intensity (MFI) was expressed as a percentage of the MFI of the LEC transfected with non-specific siRNA. ** = $p<0.01$ compared to non-specific siRNA by paired t-test. Data are mean ± SEM of at least three independent experiments.

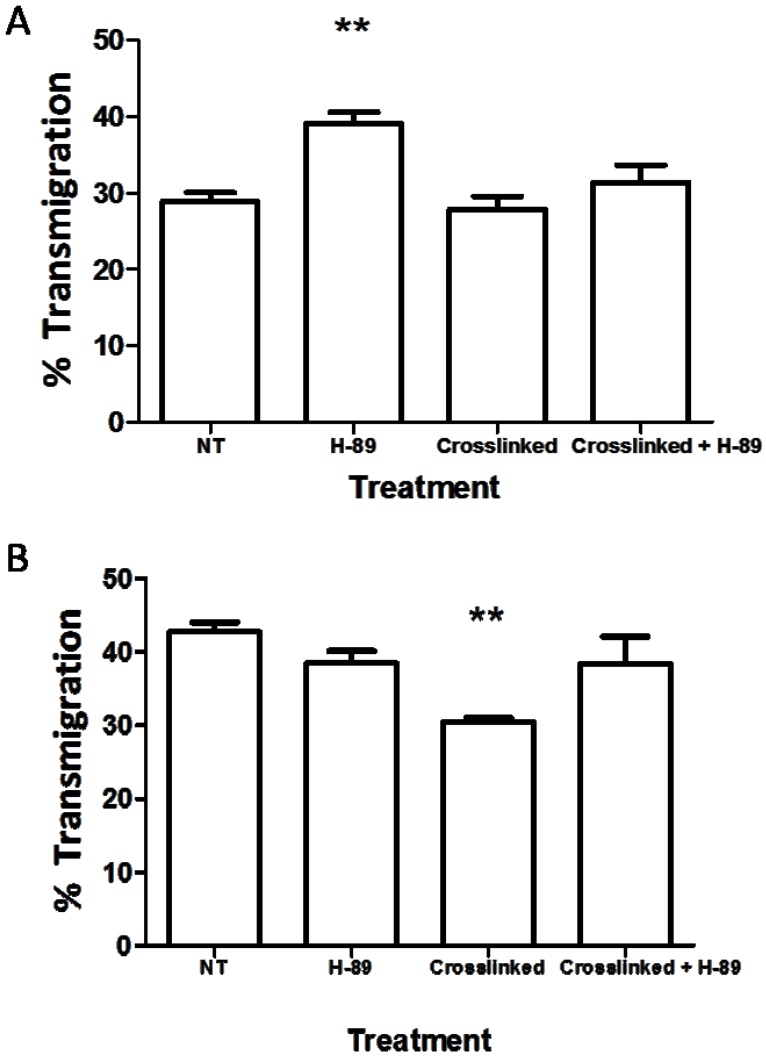


Figure 6.7: Effect of PKA inhibition on basal and VEGF-C stimulated migration of LEC. $3x10^4$ LEC were seeded onto culture inserts in medium without (A) or with (B) 300ng/ml VEGF-C. After incubation at room temperature for 30 minutes, 2µg/ml anti-human podoplanin antibody was added to selected wells. Following a further 30-minute incubation, crosslinking of podoplanin was induced in these wells by the addition of 30µg/ml anti-rat secondary antibody. The plate was incubated for another 30 minutes before 50nM H-89 was added. Percentage transmigration was determined after 24 hours. (A) ANOVA showed an effect of treatment ($p<0.05$). ** = $p<0.01$ by Dunnett's test. (B) ANOVA showed a significant effect of treatment ($p<0.05$). ** = $p<0.01$ by Dunnett's post-test. Data in both graphs are mean ± SEM of at least three independent experiments.

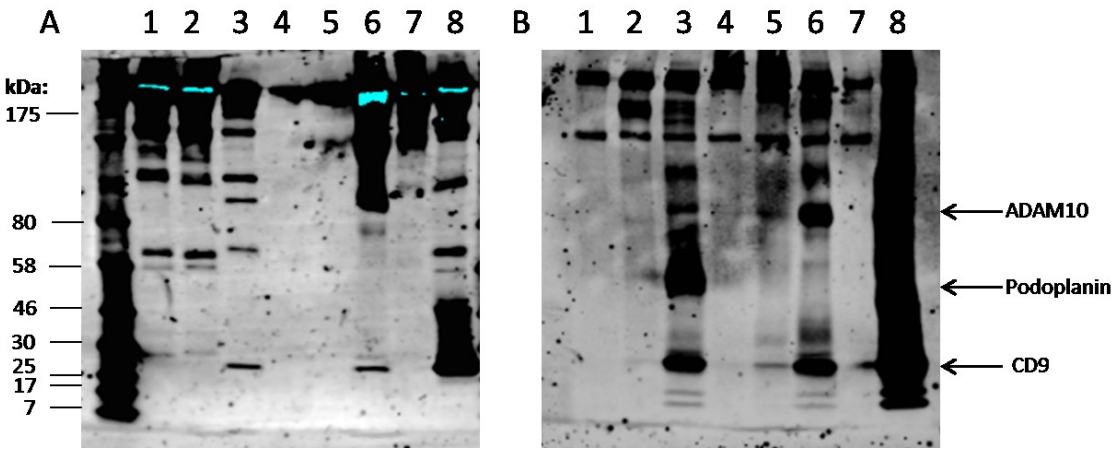


Figure 6.8: Co-immunoprecipitation of surface biotinylated LEC. LEC were surface biotinylated and lysates were prepared using 1% Brij97 lysis buffer. The lysates were immunoprecipitated with protein G sepharose beads that had been bound to mouse IgG (1) or antibodies against: β_1 integrin (2), podoplanin (3), VEGFR2 (4), VEGFR3 (5), ADAM10 (6), α_2 integrin (7) or CD9 (8). The samples were analysed by Western blots for CD9 (A) and neutravidin (B). (A) A band at 25kDa, representing CD9, was detected in the podoplanin, ADAM10 and CD9 co-immunoprecipitations. (B) In the neutravidin blot, a similar pattern of bands was seen in the podoplanin, ADAM10 and CD9 co-immunoprecipitations. The bands at 25, 45 and 80kDa represent CD9, podoplanin and ADAM10, respectively.

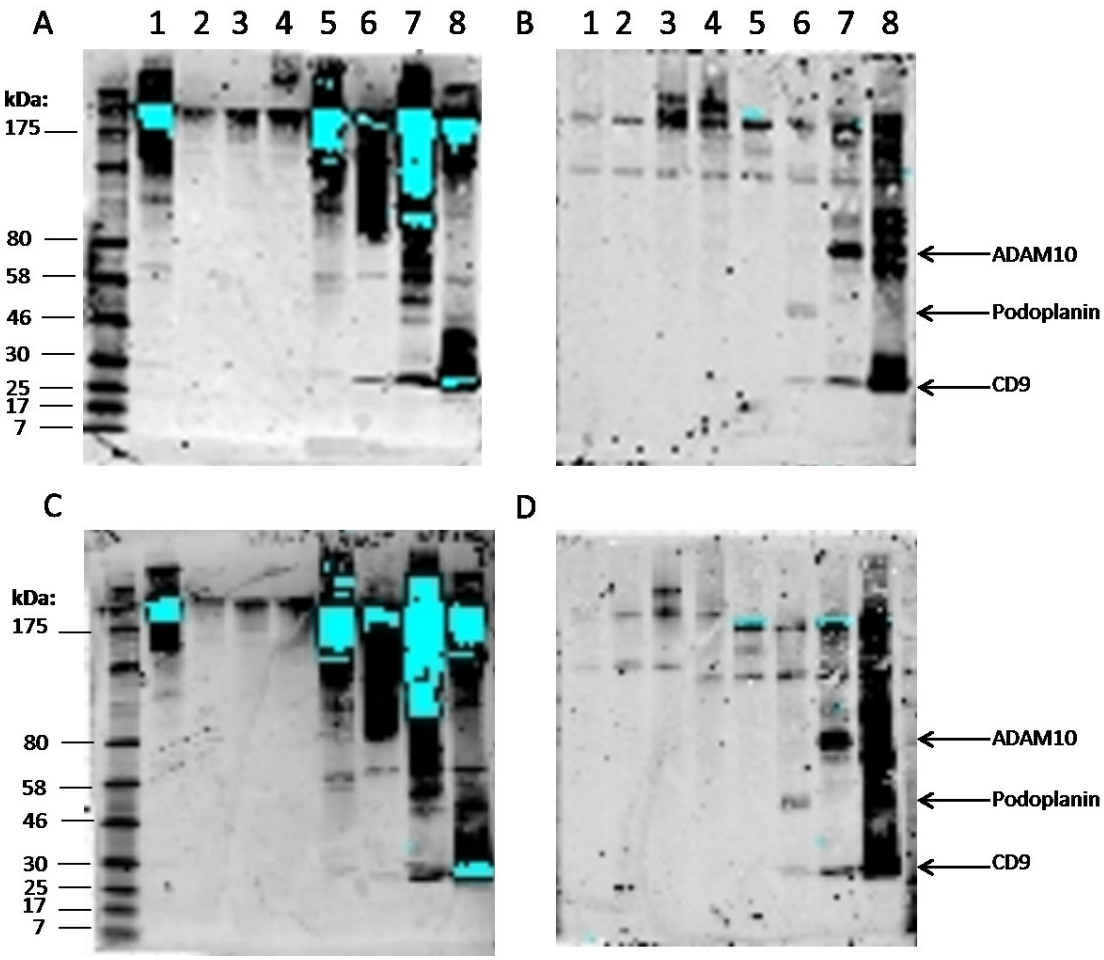


Figure 6.9: Effect of VEGF-C treatment on co-expression on LEC surface markers with tetraspanin CD9. LEC were grown to confluence in 10cm dishes then treated with culture medium with or without 300ng/ml VEGF-C for 24 hours. Cells were surface biotinylated and lysates were prepared using 1% Brij97 lysis buffer. Lysates were co-immunoprecipitated with protein G sepharose beads bound to mouse (1) or human (2) IgG, or antibodies against: VEGFR2 (3), VEGFR3 (4), α_2 integrin (5), podoplanin (6), ADAM10 (7) or CD9 (8). (A) Western blot for CD9 on lysates prepared from basal LEC. (B) Neutravidin Western blot of basal LEC. (C) CD9 blot of lysates from VEGF-C stimulated LEC. (D) Neutravidin blot of VEGF-C stimulated LEC.

6.3. Discussion

In this chapter we showed that VEGF-A, VEGF-C and VEGF-C (Cys156Ser) were all able to promote LEC migration, which suggests that both VEGFR2 and VEGFR3 are likely able to stimulate migration. More specifically, VEGFR2 was important in VEGF-A mediated migration, as the promotion of migration was prevented by administration of a VEGFR2-blocking antibody. For VEGF-C stimulated LEC migration, both VEGFR2 and VEGFR3 were important and appeared to complement each other. We found that blocking either receptor had lesser effects, but blocking both simultaneously significantly and completely inhibited the effect of VEGF-C on migration. This is consistent with previous reports that VEGF-A can bind to VEGFR1 and VEGFR2, but not VEGFR3, and that VEGF-C binds both VEGFR2 and VEGFR3. Heterodimers between VEGFR2 and VEGFR3 are also known to form in LEC (Dixelius et al., 2003), which may additionally explain why blocking both receptors is necessary to prevent the increase in migration associated with VEGF-C treatment. We also showed that crosslinking podoplanin inhibited LEC migration in the presence of each of the three tested VEGF isoforms, which suggests that podoplanin crosslinking may interact with signalling pathways downstream of both VEGFR2 and VEGFR3.

To further assess the role of podoplanin in LEC migration, we knocked down podoplanin using siRNA and then performed transfilter assays in the presence of VEGF-A. We found that the reduction in podoplanin expression negated the pro-migratory effect VEGF-A, in a manner similar to that seen with VEGF-C (see Chapter3). Thus podoplanin appears to be necessary for promotion of migration in response to VEGF isoforms. This again suggests that

podoplanin may be involved in signalling downstream of VEGFR2 or VEGFR3. These data are consistent with reports from other groups. Navarro *et al.* (2011)reported that siRNA-mediated podoplanin knockdown reduced LEC migration in the scratch wound assay. These experiments were performed in the presence of VEGF-A, making their finding consistent with that reported here. However, they did not report any data where podoplanin-knockdown LEC were used in migration assays without VEGF-A.

Having shown that podoplanin knockdown negated the pro-migratory effects of VEGF-A and VEGF-C, we assessed expression of the two LEC-expressed VEGFRs: VEGFR2 and VEGFR3. We found that there was no significant reduction in expression of either receptor following podoplanin knockdown. Work by other members of the group assessed other signalling molecules downstream of podoplanin, including ERM proteins and Akt. They found no evidence that podoplanin crosslinking modulated the phosphorylation response of these proteins after treatment with VEGF-C (Leyre Navarro-Núñez, personal communication).

We also assessed the role of PKA in LEC migration as a previous study had reported a role for PKA in podoplanin signalling and cell migration in fibroblasts (Krishnan et al., 2013). We found that inhibiting PKA promoted LEC migration in the absence of other growth factors, but could not further increase VEGF-C stimulated migration. We also found that PKA inhibition prevented the reduction in migration usually seen with podoplanin crosslinking in the presence of VEGF-C. Interestingly, the promotion of migration induced by the PKA inhibitor in unstimulated LEC was lost after podoplanin crosslinking. A link between

podoplanin crosslinking, PKA activation and cell migration thus seems likely. That the inhibitory effect of podoplanin crosslinking is through the action of PKA is supported by the previous finding, that PKA can phosphorylate two serine residues in the cytoplasmic domain of podoplanin (Krishnan et al., 2013). This study also found that mutating the serine residues to alanines increased the migration of podoplanin-expressing cells (Krishnan et al., 2013). Thus, it is conceivable that podoplanin is phosphorylated by PKA and that crosslinking podoplanin enables this serine phosphorylation, resulting in decreased migration. The interaction of podoplanin with platelet CLEC-2 may similarly promote serine phosphorylation.

Our data from the PKA experiments suggest that two different scenarios arise, depending on whether LEC have been stimulated with VEGF isoforms. In the absence of VEGF, serine residues in the cytoplasmic tail of podoplanin are phosphorylated by PKA (Figure6.10A). This results in a low level of migration, which we consider to be basal. When the PKA inhibitor is added, podoplanin cannot be phosphorylated, so migration increases (Figure 6.10B). However, when podoplanin is crosslinked, it may lock the serine residues into a conformation that is difficult to dephosphorylate. Therefore, the PKA inhibitor does not increase LEC migration in the absence of VEGF after podoplanin has been crosslinked. However, when VEGF is present a different situation arises. In the absence of podoplanin crosslinking, signals from VEGFR2 or VEGFR3 may be able to inhibit PKA, thus preventing podoplanin phosphorylation (Figure 6.10C). The result of this is that LEC migrate more than in the absence of VEGF. When the PKA inhibitor is added to VEGF-stimulated LEC, it cannot

further promote migration as podoplanin is not phosphorylated, potentially through PKA inhibition via VEGF-C (Figure 6.20C). If podoplanin has been crosslinked, it could lock the phosphorylated serines in a conformation that prevents dephosphorylation. This means that VEGF is no longer able to promote migration (Figure 6.10D). However, when the PKA inhibitor is also added, PKA receives inhibitory signals from both the inhibitor and VEGFR signalling. The combination of these signals may be enough to overcome the effect of podoplanin crosslinking promoting phosphorylation, which would explain the recovery in migration seen in Figure 6.7. Further experiments to assess the phosphorylation state of podoplanin after these different treatments would help test whether these hypotheses are correct.

Finally, we examined whether podoplanin and VEGFRs interacted with tetraspanins in LEC. Podoplanin has previously been reported to be associated with the tetraspanin CD9 in a fibrosarcoma cell line (Nakazawa et al., 2011). A separate study found that several tetraspanins, including CD9, could be detected in LEC and that knockdown of CD9 reduced VEGF-C mediated LEC migration and network formation (Iwasaki et al., 2013). We found that podoplanin was tetraspanin-associated in LEC, but could not find any interaction between CD9 and the two VEGFRs. We can be confident that podoplanin is tetraspanin-associated as the bands in both the neutravidin and CD9 blots are similar to those seen in the ADAM10 lane. ADAM10, an ectodomain sheddase, is known to be highly tetraspanin-associated (André et al., 2006; Arduise et al., 2008; Haining et al., 2012). However, from the experiments described in this thesis, we cannot say whether podoplanin is associated with

CD9 alone or with other tetraspanins. We used CD9 as the tetraspanin in the co-immunoprecipitation experiments as it is highly expressed in many cell types, including LEC (Klein-Soyer et al., 2000; Erovic et al., 2003; Iwasaki et al., 2013). The lysis buffer used was selected to keep the tetraspanin microdomain intact. Therefore, it is possible that other tetraspanins were pulled down along with CD9 and podoplanin, and that podoplanin may actually be directly associated with one of these tetraspanins rather than CD9. Similarly, it is conceivable that VEGFR2 and VEGFR3 are associated with a lowly-expressed tetraspanin, so were not detected in these experiments. These receptors are expressed at a much lower level than podoplanin in LEC, which may also have contributed to our failure to detect them in these experiments. Having shown that podoplanin is tetraspanin-associated, future experiments could focus on determining which tetraspanin podoplanin is associated with. This could be done through tagging a range of different tetraspanins with a FLAG epitope and haemagglutinin-tagging podoplanin. Chemical crosslinking followed by co-immunoprecipitations under stringent lysis conditions would reveal which tetraspanins podoplanin is associated with. This protocol has previously been used to determine which tetraspanins are associated with ADAM10 (Haining et al., 2012). It would also be interesting to determine whether knockdown of tetraspanins alters the migration and tube formation of LEC, and whether this alters the effect of platelets. There is already evidence to suggest that tetraspanin knockdown will alter LEC function, as knockdown of CD9 in LEC reduced both migration and network formation (Iwasaki et al., 2013).

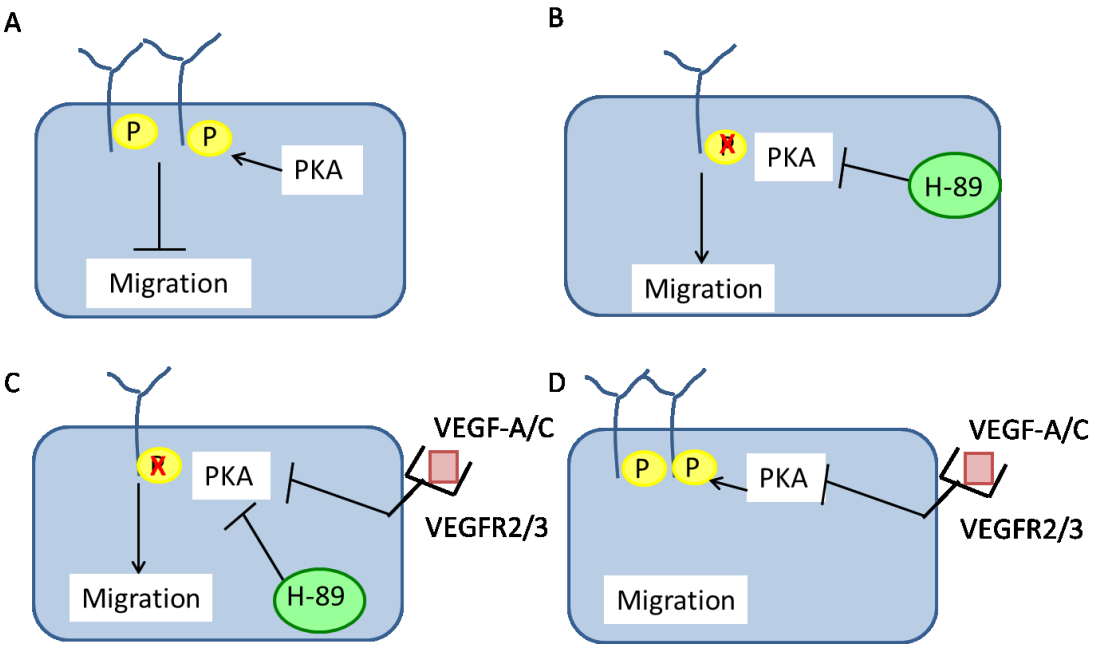


Figure 6.10: Proposed mechanism of podoplanin, PKA and VEGF interaction in LEC migration. (A) In the absence of VEGF, PKA phosphorylates serine residues in the cytoplasmic tail of podoplanin, thereby reducing migration. The same situation arises in cells where podoplanin has been crosslinked. (B) In the absence of VEGF, the PKA inhibitor (H-89) prevents the phosphorylation of serine residues. This leads to an increase in migration. However, the PKA inhibitor is not able to overcome the effects of crosslinked podoplanin, so migration of these cells is low. (C) In the presence of VEGF, PKA is inhibited by signalling downstream of VEGFR2 or VEGFR3. Podoplanin is not phosphorylated, so LEC migrate more efficiently. Adding H-89 will also prevent podoplanin phosphorylation, but cannot further increase migration. (D) Finally, when podoplanin is crosslinked in the presence of VEGF, migration is reduced. This may be due to crosslinking "locking" podoplanin in its phosphorylated state, which signals from VEGFRs are not able to overcome. However, when the PKA inhibitor is also added, there are enough inhibitory signals to overcome the effect of podoplanin crosslinking, which results in increased migration.

In summary, we have shown that signalling downstream of both VEGFR2 and VEGFR3 is important in LEC migration and that crosslinking podoplanin may interact with signalling downstream of both receptors. The presence of podoplanin is required for the promotion of migration by VEGF-A or VEGF-C, and a 50% reduction in surface expression of podoplanin is sufficient to negate the pro-migratory effect of these growth factors. There is also a role for PKA in podoplanin signalling; podoplanin clustering may facilitate serine phosphorylation by PKA or stabilise phosphorylation, leading to an inhibition of migration. Finally, podoplanin appears to be tetraspanin-associated in LEC, although further experiments are required to determine which tetraspanins podoplanin associate with and whether VEGFRs are also associated.

CHAPTER SEVEN

GENERAL DISCUSSION

7.1. Summary of main findings

This thesis has investigated the role of the podoplanin-CLEC-2 interaction in LEC migration and network formation and explored the signalling pathways that may be underlying the observed effects. LEC migration was assessed using the transfilter migration and scratch wound recovery assays, while network formation was assessed using an assay in which LEC were co-cultured with HDF. Assessment of signalling pathways involved the use of inhibitors or blocking antibodies in the robust transfilter assays and examination of RhoA activation using an ELISA-based assay. The main findings of this thesis showed that:

- Platelets inhibit VEGF-C and VEGF-A stimulated transfilter migration of LEC. The effect of platelets on VEGF-promoted LEC migration was count-dependent and platelets had no effect in the absence of these growth factors. The effect of platelets was partly CLEC-2 dependent: platelets isolated from mice lacking CLEC-2 were able to inhibit LEC migration, but had a weaker effect than wildtype. Similarly, platelets were able to inhibit HMEC-1 and HUVEC migration, but their effects were weaker, corresponding with progressively lower levels of podoplanin expression on these cell types. Platelet-derived microvesicles appeared to have a similar inhibitory effect to platelets.

- Crosslinking podoplanin with antibodies also inhibited VEGF-A and VEGF-C mediated LEC migration in the transfilter assay, but had no effect in the absence of these growth factors. An anti-human podoplanin antibody alone had no effect on LEC

migration. The inhibitory effect of podoplanin crosslinking was specific to migration promoted by VEGF isoforms; crosslinking had no effect on migration promoted by FGFb or EGF.

- Knockdown of podoplanin using siRNA negated the pro-migratory effects of VEGF-C and VEGF-A. Platelets were not able to further inhibit LEC migration after podoplanin knockdown. This lack of effect of VEGF-C or VEGF-A was not due to a reduction in the receptors for these growth factors, VEGFR2 and VEGFR3. A proportion of podoplanin also appeared to be stably expressed at the cell surface, as shown by a consistent 50% retention of surface expression after siRNA transfection. However, this fraction did not appear to be fully functional as there was a complete loss of response to VEGF-A or VEGF-C.

- Co-culture of LEC with HDF resulted in network formation by LEC within 72 hours. Platelets and podoplanin crosslinking caused disintegration of pre-formed networks of LEC. Neither treatment prevented network formation over prolonged periods. Mouse platelets were able to disrupt LEC networks, but there was no significant difference between the effects of platelets from *Clec1b*$^{fl/fl}$ or *Clec1b*$^{fl/fl}$PF4-Cre mice.

- Inhibition of Rho or ROCK inhibited VEGF-C stimulated LEC migration, but combining either treatment with podoplanin crosslinking had no further effect. This suggests that the inhibition in LEC migration seen with platelets or podoplanin crosslinking may involve signalling through RhoA and ROCK. We also found that VEGF-C increased active RhoA in LEC, but that crosslinking podoplanin did not inhibit this

effect. This led us to conclude that while the RhoA/ROCK signalling pathway may be downstream of podoplanin, the inhibition of migration is not through a change in RhoA-GTP levels.

- Combining PKA inhibition with podoplanin crosslinking prevented the inhibition of VEGF-C stimulated migration usually seen after podoplanin crosslinking. Inhibition of PKA also promoted LEC migration in the absence of VEGF-C; an effect that was also inhibited by podoplanin crosslinking. Thus, PKA appeared to play a role in regulation of migration by podoplanin in LEC.

- Immunoprecipitation indicated that podoplanin was associated with the tetraspanin CD9 in LEC. While the experiments performed for this thesis could not determine whether other tetraspanin-podoplanin interactions were present, we can state that the association between podoplanin and tetraspanins was comparable to that of ADAM10, which is known to be highly tetraspanin-associated (André et al., 2006; Arduise et al., 2008; Dornier et al., 2012; Haining et al., 2011; Prox et al., 2012).

7.2. Relation of results to previous or concurrent studies by others

Work previously published in an MRes thesis (Langan, 2011) showed that platelets were able to inhibit VEGF-C mediated LEC migration in a count-dependent manner and that they had no effect in the absence of VEGF-C. This finding does not appear to have been reported by others, although another group has reported an effect of platelets on LEC migration in the absence of VEGF-C (Osada et al., 2012). That same study also reported that platelets

promoted HUVEC migration. In our hands, however, platelets were able to inhibit VEGF-A mediated HUVEC migration, although percentage inhibition was lower than that observed with LEC. We have also reported here that platelets from both *Clec1b*$^{fl/fl}$ and *Clec1b*$^{fl/fl}$PF4-Cre mice inhibit VEGF-C stimulated LEC migration. This is consistent with a previous report (Finney et al., 2012). Another group showed that wildtype, but not CLEC-2 deficient mouse platelets had a significant inhibitory effect on LEC migration (Osada et al., 2012). However, as with the data reported using human platelets, these experiments were performed in the absence of VEGF-C. That group also assessed LEC migration after a much shorter timepoint (six hours, rather than the 24 hours used in this thesis), which may explain the differences to the results reported in this thesis.

We also showed that platelet-derived microvesicles were able to inhibit LEC migration. To our knowledge, this result has not previously been published. However, others in our group have shown that human platelet microvesicles express CLEC-2 (Gitz et al., 2014). To determine whether the effect platelet microvesicles was via CLEC-2, we isolated microvesicles from *Clec1b*$^{fl/fl}$ and *Clec1b*$^{fl/fl}$PF4-Cre mouse platelets. This showed that both types of microvesicles were able to inhibit LEC migration. While the microvesicles from *Clec1b*$^{fl/fl}$PF4-Cre platelets appeared to have a weaker inhibitory effect, this was not found to be statistically significant. However, we were only able to perform a relatively low number of experiments due to availability of mice, which is likely to have affected the statistical power. Interestingly, we were unable to detect either CLEC-2 or CD41 on mouse platelet-derived microvesicles, although both markers could be detected on mouse platelets. There

are no previous reports of staining mouse platelet-derived microvesicles with anti-CD41 antibodies for flow cytometry, so it may be possible that these microvesicles are too small to detectably stain with antibodies.

It has previously been shown that crosslinking podoplanin using an anti-human podoplanin antibody and appropriate secondary inhibits VEGF-C stimulated LEC migration (Langan, 2011; Finney et al., 2012). Work presented in this thesis confirmed these previous reports and showed that podoplanin crosslinking does not inhibit basal migration or migration promoted by FGFb or EGF. It has previously been suggested that podoplanin has a pro-migratory function. This conclusion was drawn from the observation that overexpression of podoplanin in MDCK cells promoted the directional migration of these cells (Martín-Villar et al., 2010). Another group reported that podoplanin knockdown in LEC reduced migration (Navarro et al., 2011). Together, these studies imply a pro-migratory role for podoplanin in LEC. Our data, however, show that if podoplanin is clustered (by platelets or crosslinking antibodies) then migration is reduced. This suggests that there is a change in signalling downstream of podoplanin upon clustering.

We also knocked down podoplanin using siRNA. Perhaps surprisingly, we found that reducing surface expression of podoplanin negated the pro-migratory effect usually seen with VEGF-C or VEGF-A. We also found that platelets had no further inhibitory effect. Previous studies, in which podoplanin was knocked down with siRNA, showed that this reduction in podoplanin caused an inhibition of migration (Navarro et al., 2011). However,

as all the reported data was obtained in the presence of VEGF-A, we cannot say whether podoplanin knockdown truly inhibited migration or whether, as in our hands, it actually negated the pro-migratory effect of VEGF-A. Our data suggest that podoplanin interacts with VEGF signalling, but we found no evidence to suggest that podoplanin knockdown altered expression of VEGFR2 or VEGFR3.

The data presented in this thesis showed that LEC can form networks after co-culture with HDF. To the best of our knowledge, this assay has not previously been reported using LEC, but is well-established as a model of tube formation by vascular endothelial cells (Bishop et al., 1999; Mavria et al., 2006; Kaur et al., 2011). We also showed that platelets and podoplanin crosslinking both caused the disintegration of pre-formed networks of LEC. Human platelets have previously been shown to have an inhibitory effect on LEC network formation using the Matrigel assay (Osada et al., 2012), which is supported by our data. Similarly, we showed that platelets from *Clec1b*$^{fl/fl}$ and *Clec1b*$^{fl/fl}$PF4-Cre mice were able to cause disintegration of LEC networks. However, in this co-culture model of "tube" formation, we found only a slight difference between *Clec1b*$^{fl/fl}$ and *Clec1b*$^{fl/fl}$PF4-Cre platelets. This is consistent with data reported by Finney et al. (2012), who showed that *Clec1b*$^{fl/fl}$PF4-Cre platelets had a weaker inhibitory effect on network formation. Finally, podoplanin knockdown has been shown to inhibit LEC network formation on Matrigel (Navarro et al., 2008). However, we did not knock down podoplanin in this assay as it would have required a large number of cells and the cells were co-cultured for 3-4 days, in which time surface expression of podoplanin could increase.

RhoA has previously been implicated in the migration and network formation of lymphatic endothelial cells; it is also thought to be involved in signalling downstream of podoplanin. A study by Martín-Villar at al. (2006) showed that overexpression of podoplanin in epithelial cell lines increased the amount of active RhoA in these cells and a later study by Navarro and colleagues showed that podoplanin knockdown inhibited network formation by LEC, which was associated with impaired RhoA activation (Navarro et al., 2008). A later study by the same group reported that podoplanin knockdown reduced directional migration of LEC and decreased RhoA-GTP levels in response to VEGF-A (Navarro et al., 2011). Interestingly, they also showed that RhoA-GTP levels varied after VEGF-A treatment of control-transfected LEC: active RhoA decreased after five minutes, increased by 15 minutes then decreased again by 45 minutes. Data we obtained using Rho and ROCK inhibitors in the transfilter assay suggested that both RhoA and ROCK may be involved in LEC migration downstream of podoplanin. However, unlike the previously published studies, we could not find any evidence to suggest that the inhibition of migration was due to a change in RhoA-GTP levels. We found the effect of VEGF-C treatment on RhoA-GTP levels to be consistent with the reported effect of VEGF-A (Navarro et al., 2011). In our hands, VEGF-C initially increased active RhoA, but this decreased after 30 minutes.

There are numerous reports concerning the requirement of VEGF-C for correct development of the lymphatic vasculature. Deletion of either VEGF-C or its major receptor, VEGFR3, results in lymphatic defects that can be embryonically lethal (Dumont et al., 1998; Karkkainen et al., 2001, 2004). While VEGF-C predominantly signals through VEGFR3, the

fully processed form of VEGF-C can also bind to VEGFR2 (Joukov et al., 1997); these two receptors are also known to form heterodimers (Dixelius et al., 2003; Nilsson et al., 2010). In accordance with this, we found that both VEGF-A and VEGF-C were able to promote LEC migration and that blocking antibodies against VEGFR2 and VEGFR3 were able to inhibit this promoted migration. We also found that crosslinking podoplanin was able to inhibit both VEGF-A and VEGF-C stimulated migration. This result, combined with the observation that podoplanin knockdown prevented the increase in migration usually seen with VEGF-C or VEGF-A treatments, suggested that podoplanin interacts with signalling downstream of VEGFRs. In the transfilter migration experiments, we found that platelets and podoplanin crosslinking had different effects on VEGF-stimulated and basal migration. It is difficult to compare these results with other studies as the majority of other reports publish migration and network formation in either the absence of VEGFs (Navarro et al., 2008; Bertozzi et al., 2010b; Osada et al., 2012) or presence the presence of VEGF-A (Navarro et al., 2011) or VEGF-C (Finney et al., 2012). To further investigate the interaction of podoplanin and VEGF signalling, other members of the group assessed phosphorylation of proteins thought to be downstream of podoplanin or VEGFRs. These experiments examined the effect of VEGF-C treatment and podoplanin crosslinking, but could not find an effect of podoplanin crosslinking on phosphorylation of downstream mediators, including ERM proteins and Akt.

The signalling pathway downstream of podoplanin is not well understood. There is a report that suggests that PKA may be involved in signalling downstream of podoplanin (Krishnan et al., 2013). This study showed that when two serine residues in the cytoplasmic domain of

podoplanin were changed to alanine residues, PKA was no longer able to phosphorylate podoplanin. The serine to alanine mutation was also associated with an increase in migration of fibroblasts (Krishnan et al., 2013). Consistent with this report, we found that inhibition of PKA promoted LEC migration in the absence of VEGF-C, but was not able to further increase VEGF-C stimulated migration. Furthermore, we found that combining PKA inhibition with podoplanin crosslinking prevented the inhibition of migration usually seen with podoplanin crosslinking. This suggests that the inhibitory effect of podoplanin crosslinking is through PKA-mediated phosphorylation of the serine residues in the cytoplasmic domain of podoplanin.

The data using the PKA inhibitor H-89 suggests a scenario in which phosphorylation of the serine residues in the cytoplasmic tail of podoplanin is a key regulator of migration. We hypothesised that in the absence of VEGF, these serine residues are phosphorylated, which keeps migration at a basal level. When LEC were treated with H-89, migration increased, suggesting that PKA was phosphorylating these residues. This is consistent with the data reported by Krishnan and colleagues (2013) where transfection of a podoplanin construct without the serine residues gave an increase in migration. Here, crosslinking of podoplanin may have prevented dephosphorylation of the serine residues, since H-89 did not increase the migration of LEC that had been treated with crosslinking antibodies. When LEC were stimulated with VEGF isoforms, it is possible that mediators downstream of VEGFR2 or VEGFR3 inhibited PKA, which caused an increase in LEC migration as podoplanin was no longer phosphorylated. Then, when the PKA inhibitor was added, it could not further

promote migration as podoplanin was already in its non-phosphorylated state. When podoplanin was crosslinked, the effect of VEGF was lost; this may have been due to podoplanin becoming "locked" into its phosphorylated state. When VEGF treatment was combined with administration of the PKA inhibitor, increased migration was retained (both tending to inhibit podoplanin phosphorylation). In this case, however, the "locking" effect of crosslinking could not maintain podoplanin phosphorylation in the face of the combined effects of H-89 and VEGFRs on PKA, and migration remained high.

Our finding that podoplanin is associated with tetraspanins is also consistent with a previous report (Nakazawa et al., 2011). Tetraspanins have been shown to be present in LEC and CD9 has been reported to be involved in both lymphangiogenesis and signalling downstream of VEGFR3 (Iwasaki et al., 2013). While we were able to show that podoplanin interacted with tetraspanins in LEC, we could not detect an interaction between VEGFR3 or VEGFR2 and tetraspanins.

7.3. Physiological relevance of the work presented

The data reported in this thesis have demonstrated that the interaction of podoplanin and CLEC-2 inhibits VEGF-C mediated migration and tube stability of LEC. Both of these proteins have previously been shown to be essential for correct development of the lymphatic system (Schacht et al., 2003; Bertozzi et al., 2010b; Suzuki-Inoue et al., 2010; Uhrin et al., 2010) and previous studies have also confirmed that CLEC-2 expression on platelets is

essential for normal lymphatic development (Finney et al., 2012). Our data suggest that platelets bind to and cluster podoplanin, which leads to a change in both migration and tube stability. The interaction of CLEC-2 and podoplanin may, therefore, prevent lymphatic endothelial cells from further migration and reconnecting with blood vessels once the two vascular systems have separated. On the other hand, the podoplanin-CLEC-2 interaction may disrupt the stability of newly-formed lymphatic vessels in close proximity to the blood vessels, thus promoting separation of the two systems. Furthermore, we have described data that suggest that platelet-derived microvesicles may also be able to inhibit LEC migration. As microvesicles are small in size it is possible that they would be able to diffuse from the blood vessels into the lymphatic vasculature, where they could exert their effects on LEC.

The work presented here has focused on the process of lymphangiogenesis. The main period of lymphangiogenesis is during embryonic development. To the best of our knowledge, there are no reports of human conditions in which neonates have blood-filled lymphatic vessels. Given that many of the mice that display this defect die before birth, it is likely that the same would occur in humans. However, there are instances where lymphangiogenesis takes place in adults, namely during wound healing and in tumour development. If we fully understood the role of the podoplanin-CLEC-2 interaction in the development of lymphatic vessels, it might be possible to mimic this interaction to promote lymphangiogenesis in wound healing situations. Conversely, it might also be possible to block the interaction between these proteins, which according to our data would disrupt

preformed networks of LEC. This could be of benefit in targeting tumours that develop their own lymphatic vasculature, as anti-angiogenic compounds are already used in the pharmacological treatment of cancers.

This thesis has also shown that podoplanin may be involved in signalling downstream of VEGFR2 and VEGFR3. We showed that both VEGF-A and VEGF-C promoted LEC migration, which was inhibited by podoplanin crosslinking. Additionally, knockdown of podoplanin negated the pro-migratory effect of both of these growth factors. This suggests that there is an interaction in the signalling pathways downstream of podoplanin and VEGFRs. While VEGF-C is known to be essential for lymphatic development (Dumont et al., 1998; Karkkainen et al., 2001, 2004), our data suggest that VEGF-A may also be involved. There may also be an interaction between podoplanin, VEGF signalling and PKA. We have suggested that the inhibitory effect of podoplanin crosslinking requries PKA-mediated phosphorylation of serine residues in the cytoplasmic domain of podoplanin. Signalling downstream of VEGFR2 or VEGFR3 may act to dephosphorylate podoplanin or inhibit PKA, thus preventing phosphorylation. With further understanding of how podoplanin, VEGF signalling and PKA interact, it may be possible to target this interaction to promote or inhibit lymphatic vessel development, as appropriate.

The RhoA signalling pathway also appears to regulate LEC migration. Our data showed that inhibition of Rho or ROCK could not further inhibit migration of LEC when podoplanin had been crosslinked. This suggested that podoplanin crosslinking was acting via Rho and ROCK.

However, we also found that this change in migration was not due to an alteration in RhoA activity. It may be possible to assess the effect of podoplanin crosslinking on mediators downstream of Rho and ROCK, but as this signalling pathway is present in numerous cell types it would be difficult to target it specifically in LEC.

Finally, we found that podoplanin interacts with tetraspanins in LEC. Podoplanin appears to be highly tetraspanin-associated, but from our data we cannot state which tetraspanin(s) in addition to CD9 podoplanin is associated with. Improved understanding of this interaction may provide further insights into the role of podoplanin in lymphangiogenesis. Our data confirms reports that podoplanin is associated with tetraspanins (Nakazawa et al., 2011; Iwasaki et al., 2013). The tetraspanin CD9 has also been reported to be required for lymphangiogenesis, specifically LEC migration and network formation, and may be required for signalling downstream of VEGFR3 (Iwasaki et al., 2013). Thus it may be that podoplanin and VEGFRs are maintained within the same tetraspanin microdomains and that this is necessary for these proteins to interact. Targeting this interaction, however, may prove challenging as tetraspanins are known to function in an overlapping and compensatory manner.

7.4. Future studies

The data presented in this thesis give rise to further studies that could be performed in order to better understand the role of podoplanin and CLEC-2 in the development of the lymphatic vasculature. Some suggestions for future studies are:

- Assessment of the role of platelet-derived microvesicles in LEC migration and network formation. Our data suggest an effect of microvesicles on LEC migration, but this was in medium that may have also contained soluble mediators. Therefore, to confirm that the effects seen were due to microvesicles themselves, the prepared microvesicles could be ultra-centrifuged and the resulting supernatant or peller compared for activity in the transfilter migration assay. Centrifugation has been used by others to remove microvesicles, but data from our group suggests that smaller microvesicles remain in the supernatant. Additionally, platelet-derived microvesicles could be used in the co-culture assay of network formation to determine whether they have similar effects to platelets in this assay. We could also fluorescently label the microvesicles and test whether they bind to LEC. This could be done using either flow cytometry or immunofluorescent microscopy.

- Examination of the role of platelet releasate on LEC migration. Data presented in this thesis showed that the releasate from rhodocytin-stimulated platelets did not inhibit LEC migration. This contrasts with data published by another group who used poly(PHG) to stimulate platelets (Osada et al., 2012). Therefore, releasates could be

prepared from platelets stimulated with a range of agonists and the effect of these releasates on LEC migration could be compared.

- Assessment of the RhoA signalling pathway in LEC. Our data suggested that podoplanin crosslinking acts via RhoA and ROCK, but that this was not through an alteration in RhoA activation. Therefore, further experiments could assess effect of podoplanin crosslinking on the phosphorylation of other proteins downstream of podoplanin or RhoA. Phosphorylation of ERM proteins, LIM domain kinase 1 or myosin light chain phosphatase could be examined.

- Studies of the roles of podoplanin phosphorylation and PKA could be pursued further. It would be desirable to directly assess serine phosphorylation of podoplanin in various treatment regimes, although we currently do not have adequately sensitive antibodies. In addition, we could attempt siRNA-mediated knockdown of PKA to see how this influences migration, although this enzyme is likely to have effects on targets other than podoplanin.

- Assessment of the role of the tetraspanin-podoplanin interaction in LEC migration and network formation. Initially, the tetraspanins with which podoplanin associates could be determined. This could be achieved by FLAG epitope-tagging a number of different tetraspanins and haemagglutinin-tagging podoplanin. Chemical crosslinking followed by co-immunoprecipitation experiments under stringent lysis conditions would show which tetraspanins podoplanin is associated with. Following from this, these tetraspanins could be knocked down in LEC and these cells used in migration or

network formation assays with or without the addition of platelets or podoplanin crosslinking.

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