Defining the role of myeloid cells in the regulation of developmental, tumour and inflammation-stimulated lymphangiogenesis

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Doctor of Philosophy

Emma Gordon

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Enrolled through the Department of Medicine, Faculty of Health Sciences, The University of Adelaide

Research conducted in the Division of Haematology, Centre for Cancer Biology, SA Pathology, Adelaide.

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Abstract

Lymphatic vessels are an integral component of the cardiovascular system. These specialised vessels are essential for the return of interstitial fluid to the bloodstream, immune cell trafficking and the absorption of fats from the digestive tract. Despite the crucial role played by lymphatic vessels in homeostasis and human disease, little is known about the signals that regulate lymphatic vascular growth and development (lymphangiogenesis). The aim of this project was to investigate the role of two lineages of immune cells, macrophages and mast cells, in embryonic, tumour and inflammation-stimulated lymphangiogenesis.

The first aim of this study was to determine the expression pattern of LYVE-1, a marker expressed on lymphatic vessels and a sub-population of macrophages, during early embryonic development. LYVE-1 expression was documented for the first time in the yolk sac blood vasculature and in early embryonic arteries, inter-somitic veins and endothelial cells of the lung and endocardium. These results have important implications for the use of LYVE-1 as a specific marker of lymphatic vascular endothelium.

The major aim of this project was to investigate the role of cells of the macrophage lineage in lymphangiogenesis. Macrophages expressing LYVE-1 were intimately associated with developing lymphatic vessels in the mouse embryo. Characterisation of this sub-population of LYVE-1-positive macrophages revealed that they shared a gene expression profile with Tie2-expressing monocytes. Lineage tracing studies illustrated that, while localised in close association with lymphatic vessels, macrophages did not trans-differentiate to lymphatic endothelial cells during embryonic or tumour-stimulated lymphangiogenesis. These data provide strong support to exclude myeloid cells as a source of lymphatic endothelial progenitor cells. Characterisation of lymphatic vascular development in macrophage-
deficient mice revealed that macrophages play a key role in shaping the dermal lymphatic vasculature during development by regulating lymphatic endothelial cell proliferation.

The final aim of this study was to investigate the role of mast cells during embryonic and inflammation-stimulated lymphangiogenesis. While mast cells appeared to be dispensable for the construction of the lymphatic vasculature, they were found to play a key role in UVB irradiation-induced lymphangiogenesis. Hyperplastic lymphatic vessels were a striking feature of UVB irradiated tissue in mast cell-deficient mice, revealing a novel role for mast cells in restraining the magnitude of lymphangiogenesis stimulated by this inflammatory insult.

In conclusion, these studies provide strong evidence to exclude cells of the myeloid/macrophage lineage as a pool of lymphatic endothelial progenitor cells during development and in the tumour microenvironment. This work has discovered that macrophages define the calibre of dermal lymphatic vessels during development by restraining lymphatic endothelial cell proliferation. This is in contrast to previous studies which reported macrophages stimulate lymphangiogenesis in settings of inflammation. In addition, a previously undescribed role for mast cells in the regulation of inflammation-stimulated lymphangiogenesis was identified. Taken together, these data illustrate that the sources of signals driving embryonic and disease-stimulated lymphangiogenesis are likely to be distinct. As myeloid cells regulate lymphangiogenesis in disease states, the results of this project have important implications when considering the targeting of myeloid cells for lymphangiogenic therapies.
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Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution to Emma Gordon and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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Emma Gordon
The following publications have resulted from work performed by the candidate during the period of this candidature:

**Gordon, E., Gale, N., Harvey ,N.** Expression of the hyaluronan receptor LYVE-1 is not restricted to the lymphatic vasculature; LYVE-1 is also expressed on embryonic blood vessels. *Developmental Dynamics* 2008 Jul;237(7):1901-1909.

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Abbreviations

%: percentage
°C: degrees Celsius
adj: adjusted
AP: alkaline phosphatase
APC: antigen presenting cell
BEC: blood endothelial cell
BMCMC: bone marrow-derived cultured mast cell
BSA: bovine serum albumin
CAM: chorioallantoic membrane
cDNA: complimentary DNA
cm: centimeter
CO₂: carbon dioxide
CV: cardinal vein
DA: dorsal aorta
DAB: 3,3-diaminobenzidine
DAPI: 4',6-diamidino-2-phenylindole
DLV: dermal lymphatic vessel
DMEM: Dulbecco’s Modified Eagles Medium
DNA: deoxyribonucleic acid
dNTP: deoxynucleoside triphosphate
DTT: dithiothreitol
E: embryonic day
EBM-2: Endothelial Basal Medium-2
EDTA: ethylenediaminetetraacetic acid
EGM-2: EBM-2 supplemented with Endothelial Growth Media-2 MV SingleQuots
FBS: foetal bovine serum
Fig: figure
h: hour
H₂O: water
HA: hyaluronic acid
HBBS: Hanks balanced salt solution
HCl: hydrochloric acid
HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HHF: HBBS with 10 mM HEPES and 5% FBS
JLS: jugular lymph sac
K₃Fe(CN)₆: potassium ferricyanide
K₄Fe(CN)₆: potassium ferrocyanide
kb: kilobase
kJ/m²: kilojoules per metre squared
LEC: lymphatic endothelial cell
LLC: Lewis lung carcinoma
LS: lymph sac
LVAP: Lymphatic Vessel Analysis Protocol
M: molar
MAC: macrophage
MACS: magnetic activated cell sorting
mAmp: milliampere
M-CSF: macrophage colony-stimulating factor
mg: milligrams
MgCl₂: magnesium chloride
min: minute
ml: millilitre
mm: millimetre
mM: millimolar
MMP: matrix metalloproteinase
MQ: milliQ
mRNA: messenger RNA
n: number of replicates
NaCl: sodium chloride
NaH₂PO₄: sodium dihydrogen phosphate
ng: nanogram
NIH: National Institute of Health
nm: nanometers
nM: nanomolar
NT: neural tube
O₂: oxygen
OD: optical density
PAGE: polyacrylamide gel electrophoresis
PBS: phosphate buffered saline
PBS-T: 0.1% Triton X-100/PBS
PBS-Tw: 0.1% Tween20/PBS
PCR: polymerase chain reaction
pen/strep: penicillin/streptomycin
PFA: paraformaldehyde
pH: hydrogen ion concentration
PLB: protein loading buffer
pmol: picomol
PVDF: polyvinylidine difluoride
RNA: ribonucleic acid
RPMI: Roswell Park Memorial Institute Media
RT: room temperature
RT-PCR: reverse transcription polymerase chain reaction
SDS: sodium dodecyl sulphate
sec: second
TAM: tumour-associated macrophage
TBE: Tris/boric acid/EDTA buffer
TBS-T: Tris buffered saline/0.1% Tween 20
TEM: Tie2-expressing monocyte
Tris: Tris (hydroxymethyl) aminomethane
UVB: ultraviolet B
v/v: volume per volume
V: volts
w/v: weight per volume
WEHI: Walter and Eliza Hall Institute
WT: wild-type
xg: x gravity
X-gal: 5-bromo-4-chloro-3-indolyl-β-D-galactosidase
α: alpha
β-gal: β-galactosidase
λ: lambda
μg: microgram
μl: microliter
μm: micrometre
μM: micromolar
1. Introduction
1.1. The lymphatic vascular system

Due to the crucial role of the blood vasculature in development, homeostasis and disease, vasculogenesis (the formation of new blood vessels by the de novo production of endothelial cells) and angiogenesis (the formation and remodelling of new blood vessels from existing blood vessels) have been the focus of intensive research in past decades. In contrast, the lymphatic vasculature has received much less attention. The growth and development of new lymphatic vessels (lymphangiogenesis) has recently become the focus of intense research due to the recognition of the role of lymphatics in an expanding catalogue of human diseases, together with the discovery of molecular markers that allow lymphatic vessels to be distinguished and manipulated.

Lymphatic vessels are a vital component of the cardiovascular system and serve several functions critical for embryonic development and adult homeostasis. These include the uptake and return of interstitial fluid and protein to the bloodstream, the absorption of lipids from the digestive tract and the trafficking of cells of the immune system (Oliver and Alitalo 2005). Individuals with dysfunctional lymphatic vessels suffer from chronic edema and impaired inflammatory responses, making these vessels, like blood vessels, essential for life. As well as being required for homeostasis, excessive lymphangiogenesis is involved in numerous pathologies such as tumour metastasis, and multiple inflammatory disorders including asthma, arthritis, psoriasis and organ rejection. A better understanding of lymphatic vessels and the molecular signals which influence their growth and development will allow for novel therapies for these diseases to be generated. This chapter reviews the structure and function of the lymphatic vasculature, the origins of the lymphatic system, the genetic pathways known to regulate lymphangiogenesis, the role of lymphatic vessels in disease, and how cells of the immune system interface with lymphatic vessels.
1.2. Lymphatic vessels – a historical perspective

The lymphatic vessels were first described by the ancient Greeks around 300 b.c. (Lord 1968). Their lipid transport properties enabled them to be identified as distinct from the blood vasculature; mesenteric lymphatic vessels in suckling young were visualised by the milk they carried. Further studies describing the lymphatic vasculature were documented in the 1500’s, when anatomists described the connection of the thoracic duct to the venous vasculature, and that the lymphatic system was comprised of a connected system of vessels (Lord 1968). The specialised function of the lymphatic system was first described by Gaspar Aselli (Asellius 1627), who recognised the absorptive nature of the vessels while studying a dog that had consumed a fatty meal. Work from William Hunter and his students William Cruikshank and William Hewson (Yoffey 1970) identified the lymphatic vasculature in fish, birds and mammals. Hewson reported that lymph nodes, the organising centres of the immune response, were absent in the fish and turtle and limited in birds, but prominent in mammals. This revealed the increasing complexity of the lymphatic system across phyla, and set the scene for subsequent embryonic studies on lymphangiogenesis.

1.3. Lymphatic vessel structure and function

The lymphatic system is composed of a vascular network of thin-walled capillaries and larger collecting vessels lined by a continuous layer of endothelial cells (Fig 1.1) (Oliver and Harvey 2002). Lymphatic capillaries lack support structures which are associated with blood vessels, such as a basement membrane, pericytes and smooth muscle cells. This allows them to be highly permeable, thereby facilitating the uptake of interstitial fluid and protein. Fluid, macromolecules and leukocytes enter blind ended lymphatic capillaries via openings between discontinuous button-like junctions found throughout the initial lymphatics (Baluk et al. 2007). These buttons differ from the zipper-like junctions found on larger, collecting lymphatics and blood vessels, and allow opening and closing of capillary junctions without disrupting vessel integrity. Capillaries connect to extracellular matrix components via
Figure 1.1. Schematic representation of the anatomy of the lymphatic vascular system.

The initial lymphatic capillaries, which lack smooth muscle coverage and a basement membrane, are found in the superficial layer of the dermis. Interstitial fluid, macromolecules and cells enter lymphatic capillaries through endothelial gaps in response to an increase in interstitial pressure. After pressure returns to normal, gaps are sealed by overlapping oak-leap shaped lymphatic endothelial cells. Lymphatic capillaries drain to pre-collecting lymphatic vessels in the deep dermis, which in turn drain to collecting lymphatic vessels in the subcutaneous tissue. Collecting vessels are specialised for the transport of lymph and are surrounded by basement membrane and smooth muscle cells to allow for propulsion of lymph, which occurs in a uni-directional manner due to the presence of intraluminal valves. Collecting vessels drain via the lymph nodes to lymphatic trunks which connect to the thoracic duct, which ultimately meet with the great veins of the neck to facilitate fluid return to the bloodstream.
lymphatic capillary
collecting lymphatic vessel
smooth muscle cell
valve
lymph node
anchoring filaments
lymphatic capillary
collecting lymphatic vessel
smooth muscle cell
valve
lymph node
subclavian vein
thoracic duct
skin
subcutaneous tissue
anchoring filaments, which is essential for fluid uptake. During tissue swelling, anchoring filaments become taut, allowing for opening of the vessel lumen to facilitate the uptake of tissue fluid (Tammela and Alitalo 2010). After fluid uptake occurs and interstitial fluid returns to normal volume, anchoring filaments slacken and lymphatic endothelial cells return to their normal position (Shayan et al. 2006).

The lymphatic capillaries connect to pre-collecting lymphatic vessels in the deep dermis, which have segments containing valves, loose basement membrane and smooth muscle cell coverage. These segments alternate with sections that resemble lymphatic capillaries (Shayan et al. 2006). Pre-collecting vessels drain to larger collecting lymphatic vessels located in the subcutaneous tissue, which are remodelled from the lymphatic capillary plexus. Collecting vessels are characterised by a layer of perivascular smooth muscle cells, the presence of a basement membrane, and continuous ‘zipper-like’ endothelial junctions (Oliver and Harvey 2002; Baluk et al. 2007). The contractile ability of specialised smooth muscle cells on collecting vessels creates intrinsic wall motion. This, along with compression by the associated skeletal muscle, enables lymph propulsion, with valves present to prevent fluid backflow (von der Weid and Zawieja 2004). Collecting vessels drain via lymph nodes to lymphatic trunks which connect to the thoracic duct, ultimately meeting with the great veins of the neck to facilitate fluid return to the bloodstream (Fig 1.1) (Jeltsch et al. 2003). The connection of the collecting vessels with lymph nodes and other secondary lymphoid organs, such as Peyer’s patches and tonsils, is important in inflammatory processes. Leukocytes and antigen presenting cells (APCs) display antigen epitopes to lymphocytes within these organs, where upon activation, lymphocytes clonally expand and develop an antigen-specific immune response (Cupedo and Mebius 2005).
The origin of the lymphatic vascular system remains controversial, and has been proposed to occur via two mechanisms. (A) Lymphatic endothelial cells have a venous origin. In this model, Prox1 is expressed in a polarised population of the cardinal vein, and these Prox1-positive cells subsequently bud from the cardinal veins in a polarised manner to form the primitive lymphatic vascular plexus. (B) Lymphatic endothelial cells have a local origin. This model proposed that Prox1-positive lymphangioblasts derived from the mesenchyme form the primitive lymphatic plexus, that subsequently connects back to the large veins. Evidence from avian and *Xenopus* studies suggest that both models work concurrently during embryogenesis. DA – dorsal aorta, CV – cardinal vein, LS – lymph sac.
(A) The Venous Origin

(B) The Local Origin
1.4. The origin of the lymphatic system

1.4.1. Lymphatic endothelial cells have a venous origin

More than 100 years ago, Florence Sabin first proposed the model suggesting lymphatic endothelial cells arise from the embryonic veins (Fig 1.2A) (Sabin 1904). Evidence supporting Sabin’s model was recently provided by studies investigating Prox1 expression in mice (Wigle and Oliver 1999; Oliver and Harvey 2002; Wigle et al. 2002; Srinivasan et al. 2007), with further support for the model being observed in vivo in zebrafish (Yaniv et al. 2006) and Xenopus (Ny et al. 2005). The homeobox transcription factor Prox1 is the earliest marker of lymphatic endothelial cell identity during mouse development (see 1.5.1) (Wigle and Oliver 1999). Analysis of early mouse embryos revealed that Prox1 is initially expressed in a polarised manner in the anterior cardinal veins at E9.5 (Wigle and Oliver 1999). At E10.5, Prox1-positive cells start to bud from the cardinal veins in a polarised manner, and form jugular lymph sacs by E11.5-E12.5. By E12.5 the number of lymphatic endothelial cells further increases and these cells progressively acquire the expression of additional markers of lymphatic endothelial cells. By E14.5, Prox1 and LYVE-1 expression is reduced in the veins and becomes more restricted to lymphatic endothelial cells (reviewed in (Oliver and Harvey 2002)). The lymphatic vasculature further extends through the embryo via budding from the lymph sacs and sprouting from existing lymphatic vessels (Fig 1.3).

Recent work from Srinivasan and colleagues (Srinivasan et al. 2007) used Cre/loxP-based lineage tracing studies in the mouse to determine the origin and fate of Prox1 expressing lymphatic endothelial cells. They observed that endothelial cells in the cardinal veins were the first Prox1 expressing endothelial cells in the embryo at E9.5, and that without venous-derived Tie2 progeny, the number of embryonic lymphatic endothelial cells was significantly reduced. To determine whether there was also a hematopoietic contribution to the
Figure 1.3. Development of the mammalian lymphatic vasculature.

(A) Coup-TFII expression specifies endothelial cells as venous and not arterial in nature. (B) VEGFR-3 is expressed on the venous population and LYVE-1 is expressed in a salt-and-pepper distribution pattern in the cardinal veins (CV). (C) Sox18 is expressed in a polarised population of endothelial cells in the CV, specifying competence to initiate the switch to become a lymphatic endothelial cell. (D) Sox18 induces expression of Prox1, which specifies commitment to lymphatic endothelial cell fate. LYVE-1 becomes restricted to a polarised population of cells in the cardinal veins. (E) Prox1-positive cells bud from the CV in a polarised manner in response to VEGF-C. VEGFR-3 expression is down-regulated on the CV and becomes specific to lymphatic endothelial cells, which also express Neuropilin-2, making them more responsive to VEGF-C. These signals are required to form the jugular lymph sacs (LS). (F) Separation of the blood and lymphatic vasculatures is mediated by platelet aggregation at the site of separation, with this process dependent on CLEC-2 expressed on platelets, and podoplanin expression on lymphatic endothelial cells. Signalling via the Syk tyrosine kinase and the adaptor protein Slp-76 in platelets is required for separation of the vascular systems. (G) The expansion of the lymphatic vascular system continues, driven by VEGF-C/VEGFR-3 signalling and possibly other as yet unidentified signals. (H) Remodelling from lymphatic capillaries to collecting lymphatic vessels is the final stage in the development of the lymphatic system. This requires recruitment of smooth muscle cells and basement membrane components, formation of intraluminal valves and development of continuous intraendothelial junctions.
A

Coup-TFII

B

Lyve-1

VEGFR3

C

Sox18

D

Prox1

E

Proliferation

Migration

VEG-C

Neuropilin-2

Ccbe1

F

Syk, SLP-76

Podoplanin/CLEC-2

Fiaf

G

Differentiation

VEGF-C

H

Ephrin-B2

Angpt1/2 and Tie1/2

FoxC2

Integrin-α9

Adapted from Tammela and Alitalo, 2010
developing lymphatic vasculature, hematopoietic cells expressing Runx1 were labelled with lacZ by crossing a tamoxifen-inducible Cre line, under the control of the Runx1 promoter, with the Rosa26R reporter line (Samokhvalov et al. 2007), and administering tamoxifen at E9.5. While cells expressing Runx1 were often found adjacent to the developing lymphatic vasculature, these cells did not co-express lymphatic markers. Runx1-/- embryos displayed blood filled lymphatics, however this was suggested to be secondary to a primary angiogenic defect (Srinivasan et al. 2007). However, recent studies suggest this is rather due to a loss of hematopoietic derived Syk signalling, which is essential for the separation of the blood and lymphatic vascular systems (see 1.7.1) (Bohmer et al. 2010). While the study by Srinivasan and colleagues provided evidence that lymphatic endothelial cells are primarily derived from the embryonic veins, it did not rule out a role for hematopoietic cells in the growth and patterning of the developing lymphatic vasculature.

1.4.2. Lymphatic endothelial cells have a local origin

In contrast to Sabin, Huntington and McClure (Huntington 1910) proposed a model of lymphatic development where lymphatic endothelial cells arise de novo from the mesenchyme to form lymphatic vessels that subsequently connect to the large veins (Fig 1.2B). Evidence supporting this model has been documented in Xenopus (Ny et al. 2005), where Prox1-positive mesodermal precursor cells, termed lymphangioblasts, were observed to trans-differentiate from vascular progenitor cells and contribute to lymphatic vessel formation. In the mouse, scattered cells found in the mesenchyme in regions of new lymphatic vessel growth co-express the leukocyte marker CD45 and lymphatic markers LYVE-1 and Prox1 (Buttler et al. 2006; Buttler et al. 2008). It was suggested that these cells with macrophage characteristics may trans-differentiate into lymphatic endothelial cells, however lineage tracing evidence for these hypothesis was not provided in these studies.

Evidence for a dual origin of lymphatic endothelial cells from the veins and mesenchyme has been documented in avian models, where cells of the quail paraxial mesoderm that
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were grafted into chick embryos were observed to integrate into the endothelium of the jugular lymph sac (Schneider et al. 1999). While the deep parts of the jugular lymph sac were determined to have a venous origin (via intravenous application of Dil-conjugated acetylated low-density lipoprotein into day 4 avian embryos), scattered Prox1-positive cells were hypothesised to be lymphangioblasts which contribute to the superficial parts of the jugular lymph sac and the dermal lymphatic vessels (Wilting et al. 2006). However, the true nature of these cells remains unresolved. While these studies provide evidence of contribution to the developing lymphatic vasculature via mesenchymal cells, they hypothesise a dual origin of lymphatic vessels from veins and mesenchyme-derived lymphangioblasts. This suggests that the two proposed models from Sabin and Huntington and McClure may work concurrently during embryonic lymphangiogenesis in a range of vertebrate models.

While the role of lymphangioblasts remains controversial in the field of lymphangiogenesis, evidence provided by Srinivasan and colleagues nevertheless revealed that lymphatic endothelial cells primarily have a venous origin during embryonic development in mammals (Srinivasan et al. 2007). However, whether or not cells of the myeloid lineage contribute to the generation of the lymphatic vasculature by acting as a source of lymphatic endothelial progenitor cells had not been directly examined.

1.5. Genes involved in lymphatic endothelial cell fate specification

1.5.1. Prox1

The discovery of molecular markers and genes important for lymphatic development has allowed new insights into the field of lymphatic vascular biology. The homeobox transcription factor Prox1 was first cloned by homology to the Drosophila gene prospero (Oliver et al. 1993) and has since been found to be a key regulator in lymphangiogenesis
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(Wigle and Oliver 1999; Wigle et al. 2002). To date, Prox1 is the most reliable marker of lymphatic endothelial cell identity. Interestingly, unpublished data from K. Betterman and N. Harvey recently identified Prox1 expression in venous valves, suggesting that Prox1 may be expressed in a population of endothelial cells in the mature blood vasculature. As well as in endothelial cells, Prox1 is also expressed in the eye, muscle, liver, pancreas and inner ear (Oliver et al. 1993). Additionally, recent studies identified Prox1 in a population of hematopoietic cells in the mouse, where it was suggested to act as an antagonist of self-renewal (Buttler et al. 2006; Hope et al.). In the vasculature, Prox1 is first expressed in a polarised subset of venous endothelial cells at approximately embryonic day (E) 9.5. These cells bud away from the veins to form the primitive lymph sacs by E12.5 (Fig 1.3). In Prox1-/- embryos, which die at around E15, this sprouting is reduced and lymphatic cell fate fails to be specified (Wigle and Oliver 1999) (Table 1.1).

Expression of Prox1 in blood endothelial cells is sufficient to specify lymphatic endothelial cell identity; ectopic expression in blood vascular endothelial cells has been shown to result in up-regulation of lymphatic markers and down-regulation of blood endothelial markers (Hong et al. 2002; Petrova et al. 2002). In order to maintain lymphatic endothelial cell identity, Prox1 must be constantly expressed or de-differentiation to a blood endothelial cell phenotype occurs (Johnson et al. 2008). In addition to the death of Prox1-/- mice before birth, the loss of one copy of Prox1 causes most mice to die shortly after birth. These mice display phenotypes characteristic of lymphatic abnormalities (Harvey et al. 2005). Those Prox1+/- mice that do survive develop adult-onset obesity due to abnormal lymph leakage from mispatterned and ruptured lymphatic vessels (Harvey et al. 2005). These results suggest that development of the lymphatic vasculature is highly sensitive to changes in Prox1 dosage. Prox1 expression has recently been shown to be dynamically regulated during embryonic development and adulthood, with substantial reduction in Prox1 protein levels in adult lymphatic endothelial cells compared to their embryonic counterparts.
Table 1.1. Genes that mediate lymphatic specification and maturation.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Reference</th>
<th>Gene&lt;sup&gt;-/-&lt;/sup&gt; lymphatic phenotype</th>
<th>Lethality</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prox1</td>
<td>Wigle and Oliver, 1999</td>
<td>No lymphatic vessels, no lymphatic endothelial cell specification</td>
<td>Yes, E14.5</td>
</tr>
<tr>
<td>Sox18</td>
<td>Francois et al., 2008</td>
<td>No lymphatic vessels, no differentiation of lymphatic endothelial cells from cardinal vein</td>
<td>Yes, E14.5</td>
</tr>
<tr>
<td>Coup-TFII</td>
<td>Srinivasan et al., 2010,</td>
<td>Required for venous specification and lymphatic endothelial cell specification at time of Prox1 expression, defective angiogenesis and heart development</td>
<td>Yes, E10.5</td>
</tr>
<tr>
<td></td>
<td>You et al., 2005, Pereira et al., 1999</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LYVE-1</td>
<td>Gale et al., 2007</td>
<td>No lymphatic phenotype</td>
<td>Viable</td>
</tr>
<tr>
<td>Vegfr3</td>
<td>Dumont et al., 1998</td>
<td>Failure to remodel blood vascular plexus</td>
<td>Yes, E9.5</td>
</tr>
<tr>
<td>Vegfc</td>
<td>Karkkainen et al., 2004</td>
<td>No lymphatic vessels, failure to migrate from cardinal vein</td>
<td>Yes, E17-19</td>
</tr>
<tr>
<td>Vegfd</td>
<td>Baldwin et al., 2005</td>
<td>No lymphatic phenotype</td>
<td>Viable</td>
</tr>
<tr>
<td>Vegfa</td>
<td>Ferrara et al., 1996, Carmeliet et al., 1996</td>
<td>Vegfa&lt;sup&gt;+/−&lt;/sup&gt; mice fail to develop blood islands, endothelial cells and major vessel tubes, and have reduced numbers of red blood cells</td>
<td>Yes, E11-12</td>
</tr>
<tr>
<td>Vegfr2</td>
<td>Shalaby et al., 1995</td>
<td>Failure to undergo vasculogenesis and hematopoiesis</td>
<td>Yes, E8.5-9.5</td>
</tr>
<tr>
<td>Neuropilin-2</td>
<td>Yuan et al., 2002</td>
<td>Transient hypoplasia of lymphatic capillaries</td>
<td>Yes, perinatal</td>
</tr>
<tr>
<td>Ccbe1</td>
<td>Hogan et al., 2009</td>
<td>Fish: No lymphatic</td>
<td>Fish: Yes in 25/28</td>
</tr>
<tr>
<td>Genes</td>
<td>Authors</td>
<td>Description</td>
<td>Timepoint</td>
</tr>
<tr>
<td>-------</td>
<td>---------</td>
<td>-------------</td>
<td>-----------</td>
</tr>
<tr>
<td>Syk/Slp76</td>
<td>Abtahian et al., 2003</td>
<td>Vessels, failure to sprout from veins</td>
<td>Lethal from 10-20 days</td>
</tr>
<tr>
<td>Podoplanin/CLEC2</td>
<td>Carramolino et al., 2010, Schacht et al., 2003, Fu et al., 2008, Bertozzi et al., 2010, Uhrin et al., 2010</td>
<td>Abnormal lymph transport, failure to separate blood and lymphatic vasculature, failure to accumulate platelets at site of lymphatic-blood separation</td>
<td>Yes, perinatal</td>
</tr>
<tr>
<td>Efnb2</td>
<td>Wang et al, 2010, Makinen et al., 2005, Adams et al., 1999</td>
<td>Angiogenic defects, failure to internalise VEGFR-2/3 and initiate signalling, if lack PDZ binding domain display ectopic mural cell coverage, absent valves</td>
<td>Yes, perinatal</td>
</tr>
<tr>
<td>Angiopoietin 2</td>
<td>Gale et al., 2002, Dellinger et al., 2008</td>
<td>Hypoplastic lymphatic vessels, failure to develop collecting vessel phenotype, ectopic smooth muscle coverage</td>
<td>Yes, perinatal</td>
</tr>
<tr>
<td>Tie2</td>
<td>Takakura et al., 1998, Dumont et al., 1994, Sato et al., 1995</td>
<td>Failure to remodel blood vascular plexus, defective haematopoiesis and heart development</td>
<td>Yes, E10.5-12.5</td>
</tr>
<tr>
<td>Tie1</td>
<td>Puri et al., 1995</td>
<td>Edema, haemorrhage, defective blood vessel integrity</td>
<td>Yes, E13.5</td>
</tr>
<tr>
<td>FoxC2</td>
<td>Petrova et al., 2004</td>
<td>Abnormal patterning, ectopic mural cell coverage, absence of valves</td>
<td>Yes, E12.5</td>
</tr>
<tr>
<td>Integrin-a9</td>
<td>Bazigou et al., 2009, Huang et al., 2000</td>
<td>Respiratory failure, chylothorax, defective valve formation</td>
<td>Yes, perinatal</td>
</tr>
<tr>
<td>Gene</td>
<td>Author</td>
<td>Description</td>
<td>Observation</td>
</tr>
<tr>
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</tr>
<tr>
<td>Adrenomedullin</td>
<td>Fritz-Six et al., 2008</td>
<td>Abnormal jugular lymph sacs due to decrease in endothelial cell proliferation</td>
<td>Yes, E14.5-E15.5</td>
</tr>
<tr>
<td>Aspp1</td>
<td>Hirashima et al., 2008</td>
<td>Delayed lymphatic vessel formation, mispatterned collecting lymphatic vessels</td>
<td>No</td>
</tr>
<tr>
<td>Emilin-1</td>
<td>Danussi et al., 2008, Zanetti et al., 2004</td>
<td>Lymphatic vessel hyperplasia and enlargement, irregular patterning of vessels, mild lymphedema, decreased lymph drainage, enhanced lymph leakage</td>
<td>No</td>
</tr>
</tbody>
</table>
This down-regulation may suggest that once the lymphatic vascular plexus is formed, reduced levels of Prox1 are sufficient to maintain lymphatic endothelial cell identity. Nevertheless, Prox1 acts as a master switch that suppresses blood endothelial cell identity while programming lymphatic endothelial cell identity.

While it is established that Prox1 is required for the specification and maintenance of lymphatic endothelial cell fate, both the factors which regulate Prox1 gene expression and protein activity, and in turn, the genes and proteins which are regulated by Prox1, remain largely undefined. Recent studies demonstrated that the SRY[sex determining region] box 18 (Sox18) transcriptional factor and the nuclear hormone receptor Coup-TFII are required to initiate Prox1 expression in a polarised population of cells within the embryonic cardinal veins, indicating that they are necessary for the upstream programming of lymphatic endothelial cell fate (Francois et al. 2008; Srinivasan et al. 2010) (see 1.5.2, 1.5.3). Two studies have recently identified a role for microRNAs in the regulation of Prox1 expression (Kazenwadel et al. 2010; Pedrioli et al. 2010). miR-181 and miR-31 bind to and suppress Prox1 expression, and as a result, are capable of re-programming lymphatic endothelial cell identity towards blood endothelial cell identity when ectopically expressed. These data suggest that miR-181 and/or miR-31 expression in blood endothelial cells is sufficient to silence Prox1, and as a result, act to maintain a blood endothelial cell phenotype.

While the direct target genes of Prox1 in lymphatic endothelial cells have yet to be determined, recent evidence suggests that Prox1 induces integrin-α9 expression in endothelial cells, stimulating their motility (Mishima et al. 2007). Fibroblast growth factor receptor 3 (FGFR-3) has also been identified as a Prox1 target gene. Fgfr-3 transcriptional activation may be mediated by direct binding of Prox1 to response elements in the Fgfr-3 promoter. This results in FGFR-3 up-regulation in newly formed lymphatic vessels, with maintenance of FGFR-3 throughout development (Shin et al. 2006). Given the fact ectopic
expression of *Prox1* in blood endothelial cells is sufficient to induce a switch to a lymphatic endothelial cell phenotype (Hong *et al*. 2002; Petrova *et al*. 2002), it is likely multiple genes involved in lymphatic endothelial cell specification are regulated by *Prox1*. The identity of these *Prox1* target genes remains to be elucidated.

### 1.5.2. *Sox18*

A link between *Sox18* and the lymphatic vasculature was made when it was observed that mutations in *Sox18* cause the human syndrome hypotrichosis-lymphoedeme-telangiectasia (Irrthum *et al*. 2003). Francois and colleagues (Francois *et al*. 2008) discovered that *Sox18* is expressed in a polarised manner in the cardinal veins prior to *Prox1* expression (Fig 1.3). It was demonstrated that *Sox18* binds to the *Prox1* promoter, activating *Prox1* expression to specify lymphatic endothelial cell fate. Furthermore, over-expression of *Sox18* in blood endothelial cells resulted in the induction of *Prox1*. In mice lacking *Sox18*, *Prox1* expression was absent and lymphatic endothelial cell fate was not specified, resulting in embryonic lethality in some mouse strains (Francois *et al*. 2008) (Table 1.1). This variability in strain phenotype was demonstrated to be due to *Sox7* and *Sox17*, which are not usually expressed in lymphatic endothelial cells, being activated to compensate for the loss of *Sox18* in a strain specific manner (Hosking *et al*. 2009). At present, the signals leading to the initiation of *Sox18* expression remain undefined, as while *Sox18* is required for *Prox1* expression in the cardinal veins, *Sox18* is also expressed in the embryonic arteries, even though these vessels do not express *Prox1* or give rise to lymphatic endothelial cells (Francois *et al*. 2008).

### 1.5.3. *Coup-TFII*

Given that *Sox18* is expressed not only in the cardinal veins but also in the embryonic arteries, it was apparent that there was additional factor(s) expressed in veins which are also required for lymphatic endothelial cell specification. The orphan nuclear receptor *Coup-TFII* is absolutely required for venous identity in the blood vasculature (Fig 1.3) and is
necessary to suppress the expression of arterial markers *Neuropilin-1* and *Notch* in veins (You *et al*. 2005). *Coup-TFII*<sup>−/−</sup> mice are embryonic lethal, displaying defects in angiogenesis and heart development, and failure to remodel the primitive capillary plexus (Pereira *et al*. 1999) (Table 1.1). In *Tie2-Cre;Coup-TFII*<sup>flox/flox</sup> mice, where *Coup-TFII* is ablated only in endothelial cells, venous fate was lost and very few lymphatic endothelial cells were present (You *et al*. 2005; Srinivasan *et al*. 2007). Two possibilities were proposed; that venous endothelial cell identity was critical for the induction of *Prox1* expression, or, that *Coup-TFII* regulates *Prox1* expression in co-operation with *Sox18* (Srinivasan *et al*. 2007). Support for the latter hypothesis was provided by subsequent studies, which demonstrated that *Coup-TFII* and *Prox1* physically interact in lymphatic endothelial cells to form a stable complex that controls lymphatic endothelial cell identity *in vitro*. This complex regulates the expression of lineage specific genes including *VEGFR-3*, *Fgfr-3* and *Neuropilin 2* (Lee *et al*. 2009; Yamazaki *et al*. 2009). Using mouse genetic experiments, it was determined that interaction between *Coup-TFII* and *Prox1* is required *in vivo* for lymphatic endothelial cell fate specification at the time that *Prox1* is first expressed in the veins (Srinivasan *et al*. 2010). Furthermore, both *Sox18* and *Coup-TFII* act together to induce *Prox1* expression in the cardinal veins, as *Coup-TFII* alone is not sufficient to induce lymphatic endothelial cell fate *in vivo*; there is delay between *Coup-TFII* expression in the veins and the detection of the first *Prox1*-positive lymphatic endothelial cells (Srinivasan *et al*. 2010). *Coup-TFII* appears to have a more subtle role after the initial specification and formation of primitive lymphatic structures, suggesting it may not regulate *Prox1* expression after the initial specification of lymphatic endothelial cell fate. Alternatively, after a transient interaction with *Coup-TFII* on the *Prox1* promoter, *Prox1* has been hypothesised to be recruited to a different site of its promoter to further regulate its own expression.

### 1.5.4. LYVE-1

Lymphatic vessel endothelial receptor-1 (LYVE-1), a homolog of the hyaluronic acid (HA) receptor CD44, was first identified in lymphatic vessels of adult tissues, and is one of the
most commonly utilised markers to identify lymphatic endothelium (Banerji et al. 1999; Jackson 2009). LYVE-1 expression has also been reported on a population of macrophages (Schledzewski et al. 2006; Gordon et al. 2008; Gordon et al. 2010) and on endothelial sinusoids of lymph nodes, liver and spleen (Banerji et al. 1999; Mouta Carreira et al. 2001; Prevo et al. 2001; Nonaka et al. 2007; Gordon et al. 2008). During development, LYVE-1 is expressed in the cardinal veins prior to Prox1 (Fig 1.3), and was originally thought to provide the first indicator of lymphatic endothelial cell competence prior to induction of Sox18 and Prox1 (Fig 1.3) (Wigle et al. 2002). However, work described in Chapter 3 of this thesis has demonstrated LYVE-1 is also expressed on the arterial endothelium in the early mouse embryo, suggesting LYVE-1 is not simply a marker of competence to become a lymphatic endothelial cell (Gordon et al. 2008). In the adult, LYVE-1 expression on lymphatic vessels becomes restricted to capillaries, and is down-regulated on mature, collecting lymphatic vessels as vascular remodelling proceeds (Makinen et al. 2005).

The physiological role of LYVE-1 and the functional significance of its expression in the lymphatic vasculature remain an enigma. LYVE-1−/− mice (Valenzuela et al. 2003) display no overt defects in embryonic development, or in lymphatic vessel development and function (Table 1.1) (Gale et al. 2007). In addition, these mice have normal levels of HA in the tissue and blood, indicating that LYVE-1 is dispensable for HA trafficking (Gale et al. 2007). However, these results are mitigated by subsequent studies which indicate that LYVE-1 is functionally masked in lymphatic endothelium (Nightingale et al. 2009). Interestingly, membrane glycoprotein CRS binding protein-1 (CSRBPB-1), which has been found to be identical to LYVE-1, has been implicated in interstitial fluid flow. CRSPB-1−/− mice display morphological and functional abnormalities in lymphatic capillaries, which are in a constantly contracted, open configuration in some tissues, resulting in increased interstitial fluid flow (Huang et al. 2006). However, CRSPB-1−/− mice remain grossly normal, suggesting the presence of factors that compensate for the function of this protein.
In studies of immune function, LYVE-1 also appears to be dispensable. LYVE-1⁻/⁻ mice display normal trafficking of immune cells and normal resolution of oxazolone-induced skin inflammation (Gale et al. 2007). When transplanted with B16F10 melanomas and Lewis lung carcinomas, LYVE-1⁻/⁻ mice exhibited normal tumour growth (Gale et al. 2007), suggesting the possibility of compensation by related receptors such as CD44. To examine whether CD44 was able to compensate for the loss of LYVE-1, LYVE-1⁻/⁻CD44⁻/⁻ mice were analysed and found to be indistinguishable from wild-type mice under normal conditions (Luong et al. 2009). However, while resolution of carrageenan injection-induced inflammation (which is accompanied by leukocyte infiltration and edema) was comparable to wild-type mice, LYVE-1⁻/⁻CD44⁻/⁻ mice exhibited increased edema formation (Luong et al. 2009). Therefore, a role for LYVE-1 in restraining inflammation cannot be ruled out. This is supported by findings of Johnson and colleagues (Johnson et al. 2007), who observed LYVE-1 was down-regulated by pro-inflammatory cytokines, suggesting this receptor may play a role during inflammation.

1.6. Lymphatic factors involved in proliferation and migration of the lymphatic vasculature.

1.6.1. VEGFR-3

The third identified member of the family of transmembrane tyrosine kinase vascular endothelial growth factor receptors, vascular endothelial growth factor receptor-3 (VEGFR-3, also known as Flt4) (other members are VEGFR-1 and VEGFR-2, see 1.6.3) was one of the first lymphatic-specific markers to be characterised (Kaipainen et al. 1995; Karkkainen et al. 2001). Its high affinity ligands VEGF-C (vascular endothelial growth factor-C) and VEGF-D (vascular endothelial growth factor-D) were identified soon after (Joukov et al. 1996; Achen et al. 1998). VEGFR-3 is expressed on both blood vessels and budding
lymphatic endothelial cells at E9.5, but becomes restricted to lymphatic endothelial cells as lymphatic vascular maturation proceeds (Fig 1.3). VEGFR-3 is also expressed on a population of tumour associated macrophages (Skobe et al. 2001; Schoppmann et al. 2002). VEGFR-3 is stimulated by binding of the ligands VEGF-C and VEGF-D (Fig 1.4); over-expression of these ligands in transgenic mouse models is sufficient to induce VEGFR-3 activation and stimulate lymphangiogenesis (Veikkola et al. 2001). Additionally, a soluble form of VEGFR-3 (VEGFR-3 trap) is sufficient to inhibit VEGF-C and VEGF-D signalling, inhibiting lymphangiogenesis and leading to regression of the embryonic lymphatic vasculature (Makinen et al. 2001). Treatment of newborn mice with VEGFR-3 trap induces regression of lymphatic capillaries up to 2 weeks after birth, but 4 weeks after birth, despite sustained VEGFR-3 inhibition, the lymphatic vasculature appears normal (Karpanen et al. 2006). These results indicate VEGFR-3 is essential for the development of the lymphatic vascular tree after the formation of the lymph sacs, but is not required for maintenance of the lymphatic vasculature during adulthood.

In addition to its role in lymphangiogenesis, VEGFR-3 has been shown to play a role in blood vascular development. Vegfr3−/− mice die at E9.5 due to defects in blood vessel remodelling, indicating an early blood vascular role (Table 1.1) (Dumont et al. 1998). While mice lacking Vegfr3 undergo vasculogenesis and angiogenesis, these vessels have abnormal lumens, leading to vascular leakage and embryonic lethality (Dumont et al. 1998). Additionally, blockade of VEGFR-3 in tumour models decreases angiogenesis (Laakkonen et al. 2007). Tammela and colleagues (Tammela et al. 2008) recently demonstrated that VEGFR-3 is highly expressed in angiogenic blood vessel sprouts, and that blockade of its signalling results in decreased sprouting, vascular density, vessel branching and endothelial cell proliferation in tumour models and in the postnatal retinal vasculature. This study also examined the role of Notch, which has been demonstrated to suppress vascular sprouting via its ligand Dll4 (Hellstrom et al. 2007). Expression of VEGFR-3 was up-regulated after
The VEGF/VEGFR signalling pathway regulates both angiogenesis and lymphangiogenesis. The VEGF family bind to their respective receptors, as indicated by arrows, to stimulate signalling. Ephrin-B2 interaction with VEGFR-2 and VEGFR-3 is required for receptor internalisation, signalling and subsequent angiogenesis and lymphangiogenesis, respectively. Neuropilin-2 binds to VEGF-C, and via its co-receptor VEGFR-3, initiates signalling which is important for lymphatic endothelial tip cell formation. Angpt2 and Angpt1 regulate blood and lymphatic endothelial cell proliferation and vessel remodelling, a process thought to be mediated via receptor Tie2. However, recent studies have revealed that Tie1 may also be involved in this signalling pathway. Blockade of these pathways may be achieved using blocking antibodies to ligands or receptors, traps to prevent ligand binding, or kinase inhibitors to prevent intracellular signalling.
Adapted from Hirakawa, 2009 and Tammela and Alitalo, 2010
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Notch inhibition (Tammela et al. 2008), suggesting Notch acts to suppress VEGFR-3 induced sprouting. These results suggest that targeting both VEGFR-3 and Notch may lead to novel anti-angiogenic therapies (Padera and Jain 2008).

1.6.2. VEGF-C/D

VEGF-C and VEGF-D bind to VEGFR-3 (see 1.6.1) and VEGFR-2 (Fig 1.4), and are critical lymphangiogenic factors, inducing the migration, proliferation and survival of lymphatic endothelial cells (Fig 1.3) (reviewed in (Tammela et al. 2005)). The full length VEGF-C and VEGF-D human proteins are 48% homologous, and after processing by plasmin and other unknown proteases, they have a higher affinity for both VEGFR-2 and VEGFR-3 than their unprocessed forms (Joukov et al. 1997; Stacker et al. 1999). Vegfc−/− mice display a complete absence of the lymphatic vasculature; despite the fact lymphatic endothelial cells are initially specified in the cardinal veins, they fail to migrate away from the veins to form primary lymph sacs (Table 1.1) (Karkkainen et al. 2004). If Vegfc is over-expressed, lymphatic vessels are hyperplastic (Jeltsch et al. 1997), whereas if only one copy of Vegfc is lost, the lymphatic vasculature is present but vessels are severely hypoplastic (Karkkainen et al. 2004). As well as being important for expansion of the mammalian lymphatic vasculature, in zebrafish, VEGF-C signalling is required for lymphatic endothelial cell budding from the primitive venous endothelium to form the thoracic duct (Kuchler et al. 2006; Yaniv et al. 2006). Therefore VEGF-C is important for the development of the lymphatic vasculature across phyla.

Unlike VEGF-C, VEGF-D does not appear to be essential for the development of the lymphatic vasculature, as Vegfd−/− mice do not display phenotypic changes associated with a defect in lymphatic function (Table 1.1) (Baldwin et al. 2005). However, over-expression of Vegfd is sufficient to rescue the hypoplastic vessels observed in Vegfc−/− mice (Haiko et al. 2008). Thus, VEGF-D is able to somewhat rescue defects resulting from Vegfc deficiency.
Interestingly, compound deletion of *Vegfc* and *Vegfd* does not phenocopy *Vegfr3* mice, with these mice surviving with no vascular defects after E9.5 (Haiko *et al.* 2008). This suggests there may be yet unidentified factors that are able to signal via VEGFR-3.

### 1.6.3. VEGFR-2/VEGF-A

VEGF-A (vascular endothelial growth factor-A) is usually associated with vasculogenesis and angiogenesis via signalling through receptor VEGFR-2 (vascular endothelial growth factor receptor-2) (Fig 1.4). This signalling pathway promotes proliferation, survival, sprouting and migration of blood endothelial cells (Lohela *et al.* 2009). VEGF-A also binds to VEGFR-1 (vascular endothelial growth factor-1) with low affinity (Fig 1.4), however this interaction does not stimulate angiogenesis and the downstream signalling is poorly understood (Lohela *et al.* 2009). The VEGF-A/VEGFR-2 signalling pathway is essential in vascular development; deletion of a single allele of *Vegfa* results in early embryonic lethality at mice die at E11-12 (Table 1.1). Mutant mice fail to develop blood islands, endothelial cells and major vessel tubes, and produce reduced numbers of red blood cells (Carmeliet *et al.* 1996; Ferrara *et al.* 1996). Additionally, *Vegfr2* mice are embryonic lethal at E8.5-9.5 due to a lack of vasculogenesis and haematopoiesis (Table 1.1) (Shalaby *et al.* 1995), confirming the importance of this signalling axis during development.

The VEGF-A/VEGFR-2 pathway is also implicated in lymphangiogenesis. VEGF-A has been shown to promote lymphatic vessel growth in the developing lung (Mallory *et al.* 2006) and in mouse models of inflammation (Cursiefen *et al.* 2004; Kunstfeld *et al.* 2004; Kajiya *et al.* 2009; Huggenberger *et al.* 2010). If VEGFR-2 signalling is stimulated in the dermis by over-expression of VEGF-E, a specific activator of VEGFR-2 (Fig 1.4), lymphatic vessel enlargement with very little sprouting is observed (Wirzenius *et al.* 2007). This process is independent of VEGF-C and VEGF-D, as blocking these factors had no effect on the lymphatic phenotype (Wirzenius *et al.* 2007). Therefore, the VEGF-A/VEGFR-2 pathway may provide a novel anti-lymphangiogenic therapeutic target in some settings.
Recent work identified a soluble form of VEGFR-2 (sVEGFR-2) that is able to specifically bind to VEGF-C, but not VEGF-A, and block its activity during developmental and inflammation-stimulated lymphangiogenesis, without affecting hemangiogenesis (Albuquerque et al. 2009). In the absence of sVEGFR-2 at birth, spontaneous lymphatic invasion of the normally alymphatic cornea was observed, and skin lymphatic vessels were hyperplastic (Albuquerque et al. 2009). Furthermore, administration of sVEGFR-2 enhanced corneal transplant survival and suppressed lymphangioma cellular proliferation via the inhibition of neo-lymphangiogenesis. The exact role of this soluble receptor in different disease states remains to be seen, but modulation of its activity has potential as a novel therapeutic to treat lymphatic diseases.

1.6.4. Neuropilin-2

Neuropilin-2 (Nrp2) was originally identified as a receptor for class III semaphorins and a mediator of axonal guidance, but has subsequently been implicated in lymphangiogenesis due to its ability to bind VEGF-C/D (Fig 1.4). Its expression in the vascular system is restricted to the venous and lymphatic endothelium (Yuan et al. 2002). In Nrp2-deficient mice, development of the lymphatic vasculature is abnormal; Nrp2−/− mice display hypoplasia of lymphatic capillaries during development which results in perinatal lethality (Table 1.1) (Yuan et al. 2002). Arteries, veins and large collecting lymphatic vessels develop normally in Nrp2−/− mice, indicating that Nrp2 is selectively required for the construction of small lymphatic vessels and capillaries (Yuan et al. 2002).

Upon stimulation by VEGF ligands, Nrp2 has been shown to be capable of interacting with both VEGFR-2 (Favier et al. 2006) and VEGFR-3 (Karpanen et al. 2006), resulting in receptor internalisation in vitro. A recent study demonstrated that Nrp2 is required for lymphatic sprout formation but not other aspects of lymphangiogenesis (Xu et al. 2010). Nrp2 was observed to genetically interact with VEGFR-3 to mediate VEGF-C stimulated
lymphatic sprouting (Xu et al. 2010). Regulation of sprouting by VEGFR-3 is in agreement with previous work that demonstrated when VEGFR-3, but not VEGFR-2, is selectively activated, lymphatic sprouting is induced, whereas VEGFR-2 activity results in enlargement, but not sprouting of lymphatic capillaries (Veikkola et al. 2001; Wirzenius et al. 2007). Inhibition of Nrp2 using blocking antibodies or genetic ablation affects tip cell behaviour at the leading edge of lymphatic vessels (Xu et al. 2010), increasing the frequency at which lymphatic sprouts stall/retract. This implies that Nrp2 signalling is required to allow for sprout extension. These results also suggest Nrp2 acts as an attractive cue in vascular development, as opposed to in axonal guidance, where it has been documented to act as a repulsive cue (Chen et al. 2000). Nrp2 blocking antibodies have also been shown to decrease tumour metastasis (Caunt et al. 2008), suggesting that, like VEGFR-3, Nrp2 may provide a novel target for future anti-metastatic drugs. Given that Nrp2 specifically modulates lymphatic endothelial tip cell behaviour, blocking this receptor should specifically target newly growing vessels, while leaving mature vessels unaffected. This would be advantageous to targeting growth factor signalling such as VEGF-C/VEGFR-3; inhibition of these factors may also target existing, mature vessels which may lead to undesirable side effects.

1.6.5. Ccbe1

A recent genetic screen in zebrafish led to the discovery of a new regulator of lymphangiogenesis, Ccbe1 (collagen and calcium-binding EGF domain-1) (Hogan et al. 2009). This ligand, with an as yet undiscovered receptor, is required for lymphangioblast budding from the vein, with its deletion leading to a complete absence of lymphatic vessels in zebrafish (Table 1.1) (Hogan et al. 2009). This indicates that it is an indispensable extracellular guidance molecule. The phenotype of Ccbe1−/− mutants is similar to what is observed when VEGF-C is inactivated (Kuchler et al. 2006) and the expression patterns of both ligands is overlapping, suggesting a connection between the two signalling pathways in the regulation of lymphangioblast budding and migration. Genetic mapping of human
patients with generalised lymphatic vessel dysplasia identified mutations in Ccbe1 (Alders et al. 2009), indicating this gene is important for the normal development of the lymphatic vasculature in zebrafish and in mammals.

1.7. Genes required for separation of the blood and lymphatic networks.

1.7.1. Syk and Slp-76

The lymphatic vasculature arises from the cardinal veins, however as development proceeds, the two vascular systems become separate (Fig 1.3). In the adult mouse, lympho-venous connections are maintained in the renal, hepatic and adrenal veins and in the lymph nodes, however the main connection between the blood and lymphatic vasculatures is at the site of the subclavian vein, where the thoracic duct returns lymph to the bloodstream (Maby-El Hajjami and Petrova 2008). The separation of blood and lymphatic vessels during embryogenesis is regulated by signalling mediated via the tyrosine kinase Syk and adaptor protein Slp-76; mice lacking these proteins display a striking phenotype of blood filled lymphatic vessels (Table 1.1) (Abtahian et al. 2003). Most Slp-76-deficient mice die by the age of 3 weeks due to intestinal vascular abnormalities that result in arterio-venous shunting of blood. Sebzda and colleagues (Sebzda et al. 2006) found that defects caused by Slp-76-deficiency can be rescued by expression of Slp-76 in a subset of hematopoietic cells positive for GATA1 (expressed in platelets, erythrocytes and eosinophils), providing evidence for a hematopoietic contribution to vascular development and the separation of blood and lymphatic vessels.

Recent work demonstrated that M2-polarised myeloid cells (which are classically pro-angiogenic, have an immune suppressive phenotype, and release cytokines to promote a Th2 immune response (Schmid and Varner 2010)) contain a subset of cells defined by the expression of Syk (Bohmer et al. 2010). These cells were found to be important in
developmental lymphangiogenesis. When the \( \text{Syk}^{-/} \) M2-population of myeloid cells were isolated, they produced elevated lymphangiogenic and angiogenic factors when compared to \( \text{Syk}^{+/} \) M2-myeloid cells (Bohmer et al. 2010). When transplanted into wild-type mice in a dorsal skin fold chamber model, \( \text{Syk}^{-/} \) myeloid cells stimulated lymphangiogenesis by increasing both the number of filopodia and the calibre of lymphatic vessels (Bohmer et al. 2010). It was hypothesised that this increase in lymphatic vessel area leads to direct contact between blood and lymphatic vessels, causing the formation of blood-lymphatic endothelial junctions and hence the blood-lymphatic shunts observed in \( \text{Syk}^{-/} \) embryos (Bohmer et al. 2010).

Another factor recently shown to be involved in separation of the blood and lymphatic lineage is fasting-induced adipose factor (Fiaf) (Backhed et al. 2007). The role of Fiaf appears to be organ-specific, with defects in blood-lymphatic separation in \( \text{Fiaf}^{-/} \) mice restricted to the small intestine. This suggests there may be different factors involved in lymphatic/blood vascular separation in different organs.

### 1.7.2. Podoplanin and CLEC-2

Podoplanin, first characterised as a glomerular filtration-regulating protein, has been shown to be a specific marker, with respect to endothelial cells, for lymphatic endothelium (Breiteneder-Geleff et al. 1999). \( \text{Podoplanin}^{-/} \) mice die at birth due to respiratory failure, and display lymphatic defects resulting from diminished lymphatic flow, vessel dilation and congenital lymphedema (Table 1.1) (Schacht et al. 2003). As well as being important for lymphatic development, podoplanin promotes tumour motility by increasing tumour-stimulated lymphangiogenesis and metastasis to regional lymph nodes (Cueni et al. 2010). Blockade of podoplanin using a Fc-trap has recently been shown to inhibit lymphangiogenesis \textit{in vitro} and \textit{in vivo} in the mouse cornea, suggesting that this protein could provide a novel anti-lymphangiogenic therapeutic target (Cueni et al. 2010). Taken together, these studies reveal podoplanin is a key regulator of lymphangiogenesis, but until
recently, the exact role and mechanism of action of this protein during lymphatic development has remained elusive.

Interestingly, mice lacking endothelial cell O-glycans, such as podoplanin, display disorganised, blood-filled lymphatics at birth (Fu et al. 2008). Although podoplanin−/− mice were previously observed to have abnormal lymphatic vessels, they were not reported to have misconnections between the blood and lymphatic vasculatures (Schacht et al. 2003). Re-evaluation of these mice by Fu and colleagues found that podoplanin−/− mice, like O-glycan-deficient mice, indeed have disorganised and blood-filled lymphatic vessels, confirming that podoplanin is required for the separation of the blood and lymphatic vessels during development (Fu et al. 2008). Recent studies have demonstrated that platelet activation by podoplanin is crucial in regulating the separation of the blood and lymphatic systems (Bertozzi et al. 2010; Carramolino et al. 2010; Uhrin et al. 2010). Platelet aggregates accumulate at the site of the separation zone of the developing jugular lymph sac and cardinal vein (Fig 1.3), however this does not occur in podoplanin−/− embryos, which display blood filled lymphatics (Uhrin et al. 2010). Blood-lymphatic mixing was also observed after inhibiting platelet aggregation, or treatment with podoplanin blocking antibodies (Uhrin et al. 2010), and mice deficient in platelets display blood filled lymphatics (Carramolino et al. 2010). Platelets interact with lymphatic endothelial cells via the podoplanin receptor C-type lectin-like receptor 2 (CLEC-2); genetic experiments revealed loss of CLEC-2 on platelets prevented their binding to podoplanin on lymphatic endothelial cells, resulting in blood-filled lymphatics (Bertozzi et al. 2010). Bertozzi and colleagues also demonstrated that activation of platelets by podoplanin stimulates Slp-76 signalling within platelets (Bertozzi et al. 2010). These results reveal a signalling pathway between podoplanin on lymphatics and CLEC-2 on platelets, which activates Slp-76 signalling to regulate the separation of the blood and lymphatic vasculatures.
1.8. Genes involved in the maturation of the lymphatic vasculature.

1.8.1. Ephrin-B2

As well as being important in controlling axonal guidance and blood vessel remodelling (Adams 2002), ephrins are involved in the remodelling and maturation of the lymphatic vasculature. Mice that lack the PDZ interaction domain of ephrin-B2 (Efnb2), a transmembrane ligand for Eph receptors, display abnormalities in lymphatic vascular remodelling, including defects in valve formation, ectopic smooth muscle cell coverage of capillaries, and persistent LYVE-1 expression on collecting lymphatic vessels (Makinen et al. 2005).

Ephrin-B2 and its receptor EphB4 had been demonstrated to be essential for embryonic blood vessel formation; Efnb2<sup>−/−</sup> and EphB4<sup>−/−</sup> mice die during embryogenesis due to angiogenic defects (Table 1.1) (Wang et al. 1998; Adams et al. 1999). However the mechanisms underlying these defects have, until recently, remained unclear. Recent work revealed that ephrin-B2 is a positive regulator of internalisation and signalling of VEGFR-2 and VEGFR-3, and is essential for angiogenesis and lymphangiogenesis (Fig 1.4) (Sawamiphak et al. 2010; Wang et al. 2010). These studies revealed that interaction between ephrin-B2 and VEGFR-2/VEGFR-3 is essential for tip cell elongation; Efnb2<sup>−/−</sup> mice displayed reduced blood and lymphatic vessel sprouting via inhibition of filopodial protrusions (Sawamiphak et al. 2010; Wang et al. 2010). Furthermore, over-expression of ephrin-B2 in the endothelium resulted in ectopic filopodial protrusions, confirming ephrin-B2 as a positive regulator of filopodial extension and cell motility (Wang et al. 2010). While stimulation of cultured endothelial cells with ephrin-B2 using ephrin-B2/Fc or EphB4/Fc fusion proteins resulted in VEGFR-3 internalisation in the absence of VEGF-C, signalling was not activated. This revealed that functional co-operation between the VEGF-C/VEGFR-3 and ephrin-B2/VEGFR-3 pathways is required to mediate lymphangiogenesis (Wang et al. 2010). Given that ephrin-B2 controls both angiogenic and lymphangiogenic signalling,
targeting this ligand may provide novel anti-angiogenic and anti-lymphangiogenic therapies to inhibit tumour development and metastasis.

1.8.2. Angiopoietin 1 and 2 and Tie1 and 2

The importance of Angiopoietin 1 (Angpt1), Angiopoietin 2 (Angpt2) and their receptors Tie1 and Tie2 in blood vascular development is well established; this signalling pathway is implicated in vessel sprouting, remodelling and endothelial cell quiescence during developmental and pathological angiogenesis (reviewed in (Augustin et al. 2009)). The first indication that this signalling plexus was involved in lymphangiogenesis came from investigation of the phenotype of Angpt2\(^{-/-}\) mice (Gale et al. 2002). During development, Angpt2\(^{-/-}\) mice displayed minor defects in the blood vasculature, such as mispatterned kidney and retinal vasculatures. However the lymphatic vasculature defects in these mice were severe. Angpt2\(^{-/-}\) pups displayed a failure to develop a lymphatic collecting vessel phenotype, impaired smooth muscle cell recruitment to collecting lymphatic vessels and hypoplastic lymphatic capillaries, resulting in perturbed lymphatic function (Table 1.1) (Gale et al. 2002; Dellinger et al. 2008). Interestingly, lymphatic vascular defects but not blood vascular defects in Angpt2\(^{-/-}\) mice were rescued by over-expression of Angpt1, suggesting that Angpt2 plays an agonistic role in lymphatic vessels (Gale et al. 2002; Dellinger et al. 2008). In agreement with a role for Angpt1 in the stimulation of lymphangiogenesis during development, over-expression of Angpt1 in adult tissues using transgenic mice or viral vectors stimulated lymphatic endothelial cell proliferation, promoting vessel enlargement and the formation of new lymphatic vessel sprouts (Morisada et al. 2005; Tammela et al. 2005). These results suggest that signalling via the angiopoietin/Tie pathway is required for embryonic lymphatic vessel assembly and maturation (Fig 1.3).

Signalling of Angpt1 and Angpt2 has, until recently, been suggested to occur via the tyrosine kinase receptor Tie2, due to the reported expression of this receptor on lymphatic endothelium (Morisada et al. 2005; Tammela et al. 2005). Tie2-deficient mice die between
E10.5 and E12.5 due to a failure to remodel the maturing blood vascular plexus (Table 1.1) (Dumont et al. 1994; Sato et al. 1995). Tie2−/− mice also display defective haematopoiesis and development of the heart endocardium (Dumont et al. 1994; Takakura et al. 1998). Despite its importance in the developing blood vasculature, subsequent studies have failed to detect expression of Tie2 on both initial and collecting lymphatics (Srinivasan et al. 2007; Dellinger et al. 2008), suggesting that angiopoietins may signal through an alternative receptor to regulate lymphangiogenesis. Expression of Tie1 has been reported on lymphatic vessels, with this receptor capable of stimulation by Angpt1, and forming heterodimers with Tie2 (Saharinen et al. 2005). Tie1−/− mice are embryonic lethal and die around E13.5 due to edema, haemorrhage and defective blood vessel integrity (Table 1.1) (Puri et al. 1995). However, until recently the role of Tie1 in lymphatic development was unknown. Recent publications have described a role for Tie1 in normal lymphatic vascular development (D’Amico et al. 2010; Qu et al. 2010), demonstrating that it is dispensable for lymphatic endothelial cell competence, but essential for the normal formation of the jugular lymph sacs (D’Amico et al. 2010). Reduction in Tie1 levels led to dilated, disorganised lymphatic vessels, and abnormal regression of the lymphatic vasculature due to an increase in lymphatic endothelial cell apoptosis (Qu et al. 2010). Although preliminary, these results suggest Angpt1 and Angpt2 may act via Tie1 to regulate the development and remodelling of the lymphatic vasculature, instead of Tie2 as originally reported (Fig 1.4).

### 1.8.3. FoxC2

FoxC2 is a forkhead transcription factor involved in the specification of the lymphatic capillary versus collecting lymphatic vessel phenotype, and is highly expressed in developing lymphatic vessels as well as lymphatic valves in the adult (Fig 1.3) (Dagenais et al. 2004; Petrova et al. 2004). In Foxc2−/− mice, the initial development of the lymphatic vasculature occurs normally. However, during later stages of development, as initial lymphatic capillaries remodel into large collecting vessels around postnatal day 2 in the mouse (Shayan et al. 2006), collecting lymphatic vessels fail to form valves, and capillaries
are ectopically covered by basement membrane components and smooth muscle cells (Table 1.1) (Petrova et al. 2004). The human autosomal disease lymphedema-distichiasis is caused by heterozygous loss-of-function of Foxc2 (Fang et al. 2000). While the lymphatic vessels in lymphedema-distichiasis patients are normal and lymph is taken up by the vessels, backflow of lymph occurs. This is presumably due to defective patterning of vessels, abnormal presence of smooth muscle cells, and most importantly, lack of valves (Petrova et al. 2004).

A recent study identified a transcription factor, nuclear factor of activated T cells (NFATc1), using genome wide analysis of binding sites and co-immunoprecipitation studies, that interacts with FoxC2 (Norrmen et al. 2009). This co-operative transcriptional regulation controls the expression of a set of genes involved in capillary maturation and valve formation (Norrmen et al. 2009). Interestingly, if NFATc1 is inhibited in Foxc2-/- mice, expression of Angpt2 is up-regulated in lymphatic endothelium (Norrmen et al. 2009). This suggests that Angpt2 may stimulate the recruitment of smooth muscle cells to collecting vessels downstream of FoxC2 and NFATc1.

1.8.4. Integrin-α9

Mice deficient in integrin-α9 die around postnatal day 6 to 12 due to respiratory failure, edema, and lymphocytic infiltration in the chest wall around the lymphatic vessels (Table 1.1) (Huang et al. 2000). This revealed that this receptor was important for the development of the lymphatic system. A recent study has revealed a role for integrin-α9 in the assembly of valves in collecting lymphatic vessels (Bazigou et al. 2009). Up-regulation of integrin-α9 and fibronectin-EIIIA in the extracellular matrix leads to lymphatic valve leaflet formation, which allows for opening and closing of valves in response to changes in pressure (Bazigou et al. 2009). In vitro experiments suggested that assembly of the integrin-α9-EIIIA complex directly regulated fibronectin fibril assembly, which is essential for the extracellular matrix core of valve leaflets. Mice containing an endothelial-specific deletion of integrin-α9 display
defective valve formation (Bazigou et al. 2009), but unlike Foxc2\(^{-/-}\) or Efnb2\(^{-/-}\) mice, have no other lymphatic malformations. This reveals that integrin-\(\alpha 9\) is specifically required for the formation of valves. These results may also provide insight into valve formation in the blood vasculature; the discovery of Prox1 in venous valves (K. Betterman and N. Harvey, unpublished) suggests that valve formation may be regulated by a common genetic program between blood and lymphatic vessels.

1.8.5. Adrenomedullin

Vasodilator and diuretic peptide adrenomedullin (AM) is required for normal mouse lymphatic vascular development. Mice defective in AM, or components of its receptor complex (calcitonin receptor-like receptor and receptor activity-modifying protein 2 [RAMP2]), both display edema and embryonic lethality around mid-gestation (Fritz-Six et al. 2008). While the exact cause of embryonic lethality was not determined, loss of AM led to hypoplastic jugular lymph sacs, but no defects in peripheral skin or retroperitoneal lymphatic vessels (Table 1.1) (Fritz-Six et al. 2008). This suggests that AM controls the proliferation of developing lymphatic vessels in a tissue specific manner during embryogenesis. AM has also been shown to decrease lymphatic endothelial cell permeability and lymphatic flow in vitro and in vivo by reorganisation of the tight junction protein ZO-1 and the adherens protein VE-Cadherin (Dunworth et al. 2008), revealing a role for AM in regulating the lymphatic endothelial cell barrier. The exact role of AM in lymphatic vessel remodelling remains to be elucidated, as studies from another group determined the edema in RAMP2 gene targeted mice is due to hyperpermeable blood vessels, not lymphatic defects (Ichikawa-Shindo et al. 2008). The differences in these findings may be due to different genetic background of the mice utilised in each study.

1.8.6. Aspp1

Apoptosis stimulating protein of p53 (Aspp1) was recently reported to play an important role during embryonic lymphangiogenesis (Hirashima et al. 2008). Aspp1\(^{-/-}\) mice display
subcutaneous edema and disorganised lymphatic vessels during embryonic development, however some functionality of lymphatic vasculature was resolved in adults despite abnormal patterning of the collecting the lymphatic vessels (Table 1.1) (Hirashima et al. 2008). Loss of p53 did not affect lymphangiogenesis (Hirashima et al. 2008), suggesting Aspp1 acts via other mechanisms in lymphatic endothelial cells.

1.8.7. Emilin-1

The connection of the lymphatic vasculature to the associated extracellular matrix is essential for normal lymphatic function. Emilin-1 is an elastic microfibril-associated protein expressed on lymphatic vessels in vivo and in vitro (Danussi et al. 2008). Emilin-1/− mice have an irregular pattern of superficial and visceral lymphatic vessels and a reduction of anchoring filaments, therefore have impaired connections to the extracellular matrix (Danussi et al. 2008). These mice display functional defects such as lymphedema, decreased lymph drainage and enhanced lymph leakage, demonstrating the importance of lymphatic endothelial cell connections to the extracellular matrix.

1.9. Lymphatic vessels and disease

1.9.1. Lymphatic insufficiency

Aberrations in lymphatic vascular development and/or function are responsible for many human diseases. These include diseases of lymphatic vascular insufficiency, including primary and secondary lymphedema, and malignancies of the lymphatic endothelium, such as lymphangiosarcoma and Kaposi’s sarcoma (Alitalo et al. 2005; Oliver and Alitalo 2005). Lymphedema is due to stagnation of proteins and associated fluid accumulation in the interstitium, and currently no curative treatments exist (Tammela and Alitalo 2010). Cases of primary lymphedema are rare, however genetic mapping studies have associated many of these malignancies to dysfunction of genes essential for normal lymphatic development.
(discussed in 1.5, 1.6, 1.7 and 1.8), including Vegfr3 (Connell et al. 2009), Foxc2 (Petrova et al. 2004), integrin-α9 (Ma et al. 2008), Sox18 (Irrthum et al. 2003) and Ccbe1 (Alders et al. 2009). Secondary lymphedema is the cause of 99% of cases of lymphedema in the world, and is a result of damage to the lymphatic vessels (Radhakrishnan and Rockson 2008). The principle cause of secondary lymphedema in the third world is filariasis, which is an infection of the lymphatic vessels by parasitic worms, leading to scaring and impaired function of the lymphatic system (Pfarr et al. 2009). Breast cancer surgery is the most common cause of secondary lymphedema in the developed world, as surgery performed to remove auxiliary lymph nodes often disrupts lymphatic network connectivity and function, leading to impaired afferent lymphatic flow and lymphedema (Alitalo et al. 2005; Radhakrishnan and Rockson 2008). Recent work in a mouse model demonstrated that lymphatic vascular dysfunction can result in obesity (Harvey et al. 2005), a discovery that correlates with long-standing observations of adipose tissue accumulation in lymphedema patients (Brorson 2003). This suggests the possibility of a novel causative factor for obesity onset in the human population.

Stimulation of the growth of new lymphatic vessels as a treatment for lymphedema patients is the focus of novel therapeutic research. Tammela and colleagues (Tammela et al. 2007) recently demonstrated that treatment of lymph-node excised mice with lymphangiogenic growth factors VEGF-C and VEGF-D, together with a lymph node transplant, induced growth of lymphatic capillaries which matured into functional collecting lymphatic vessels. While these studies are preliminary, they demonstrate that the manipulation of lymphatic growth factors may provide novel therapeutics for patients in the treatment of lymphedema.

1.9.2. Inflammatory disease

Lymphatic vessels act as the principle route of transport for soluble antigens and APCs to reach the lymph nodes and other secondary lymphoid organs. Lymphatic vessels express
secondary lymphoid chemokine CCL21, which attracts activated APCs expressing chemokine receptor CCR7 (Kerjaschki et al. 2004; Ohl et al. 2004). Once they have reached the subcapsular sinapse and have moved to the paracortex, APCs localise around high endothelial venules. This localisation allows presentation of antigen to B and T cells, which enter the lymph node via the high endothelial venules from the blood, inducing a lymphocyte response (Alvarez et al. 2008).

Recent research has discovered lymphangiogenesis is a feature of many inflammatory diseases. Mouse models of multiple chronic inflammatory diseases, such as rheumatoid arthritis, asthma, psoriasis and organ rejection, are all associated with increased lymphangiogenesis (Cursiefen et al. 2004; Kerjaschki et al. 2004; Kunstfeld et al. 2004; Baluk et al. 2005; Kajiya et al. 2006; Zhang et al. 2007; Polzer et al. 2008; Kajiya et al. 2009; Huggenberger et al. 2010). Depending on the tissue and inflammatory stimulus, lymphatic vessels can act to; promote the pathology associated with inflammation by acting as a route of transport for inflammatory cells, or, help to resolve inflammation by acting to clear pro-inflammatory cells and fluid from the site. Blockade of the VEGFR-3/VEGF-C signalling pathway has been shown to regulate lymphangiogenesis, and resulting pathology, in inflammatory settings (Chen et al. 2004; Cursiefen et al. 2004; Baluk et al. 2005; Huggenberger et al. 2010). Precisely defining the role of lymphatic vessels in inflammatory disorders in a model specific fashion will allow for the development of novel therapeutics via the manipulation of lymphangiogenic signalling pathways.

1.9.3. Tumour Metastasis

Lymphatic vessels are inextricably linked with the progression of many forms of cancer due to the fact that they are exploited by tumour cells as a route of metastasis (Alitalo et al. 2005). Tumours can utilise lymphatic vessels as a route of metastasis by invading pre-existing lymphatic vessels (Fig 1.5A), or by producing growth factors which elicit a
Figure 1.5. Tumours can exploit lymphatic vessels as routes of metastasis.

(A) Tumour cells can break away from the primary tumour site to invade pre-existing lymphatic vessels, from where they can metastasise to distant sites. (B) Alternatively, tumours and tumour-associated macrophages can secrete lymphangiogenic growth factors VEGF-C, VEGF-D and VEGF-A, which induces sprouting of adjacent lymphatic vessels toward the primary tumour site. These newly formed lymphatic vessels in the tumour environment are dilated and highly permeable, allowing access of tumour cells into the vessel lumen and subsequent metastasis.
lymphangiogenic response (Fig 1.5B) (Mandriota et al. 2001; Skobe et al. 2001; Stacker et al. 2001; Padera et al. 2002; Maula et al. 2003; He et al. 2005). The first tumour cells metastasise to what is known as the sentinel lymph node, from which further dissemination may occur to other nodes and organs (Pepper 2001). Lymphatic vessels can exist within tumours or at the tumour periphery (Ji 2006), and there is evidence in human tumours that vessels at both locations are important for metastasis (Skobe et al. 2001; Schoppmann et al. 2002). The targeting of lymphatic vessels is an area of great interest in the development of new anti-metastatic therapies. Understanding of the difference between lymphatic endothelial cells in different vascular environments and across tumour types, and determining the different mechanisms between tumour-stimulated and normal lymphangiogenesis, are some of the challenges that need to be explored to further develop effective and specific therapeutic strategies.

The VEGF-C/D/VEGFR-3 signalling axis has been demonstrated to be important for tumour associated lymphangiogenesis and hence metastasis. In mouse models, an increase in VEGF-C/D leads to increased tumour lymphangiogenesis and tumour dissemination to regional lymph nodes (Mandriota et al. 2001; Skobe et al. 2001; Stacker et al. 2001; Roberts et al. 2006). Multiple studies in human tumours, such as malignant melanoma, colorectal carcinoma and gastric cancer, have detected an association between increased expression levels of VEGF-C/D and poor prognosis (reviewed in (Achen et al. 2005; Rinderknecht and Detmar 2008). Given the correlation between VEGF-C/D expression and lymphatic invasion and secondary metastasis, inhibition of the VEGF-C/D/VEGFR-3 pathway is a promising target for the development of new anti-metastatic therapeutics. Inhibition of the VEGF-C/D/VEGFR-3 signalling axis using blocking antibodies leads to a 60-70% reduction in lymph node metastasis in mouse tumour models (He et al. 2002; Lin et al. 2005; Roberts et al. 2006). Therefore inhibition of this pro-lymphangiogenic pathway may provide a novel therapeutic target to prevent tumour metastases in human patients.
1.10. The role of macrophages in lymphangiogenesis and angiogenesis

1.10.1. Macrophages as a source of endothelial progenitor cells during lymphangiogenesis

Macrophages are cells of the myeloid lineage (Fig 1.6) which have been proposed to play a role during developmental and pathological lymphangiogenesis. Some data in the literature has suggested that macrophages are a source of endothelial progenitor cells during development. Although cells with lymphatic and macrophage characteristics have been suggested to contribute to the developing lymphatic endothelium in a variety of vertebrate models (Ny et al. 2005; Buttler et al. 2006; Wilting et al. 2006; Buttler et al. 2008), work from others has determined that hematopoietic cells are not required for the formation of the lymphatic vasculature (Srinivasan et al. 2007).

In models of adult pathological neo-lymphangiogenesis, macrophages have been suggested to contribute to the genesis of lymphatic vessels via a process of “trans-differentiation” to lymphatic endothelial cells (Fig 1.7) (Kerjaschki et al. 2004; Maruyama et al. 2005; Kerjaschki et al. 2006; Maruyama et al. 2007). In vitro experiments revealed that CD11b-positive macrophages are capable of forming tube-like structures that express markers of lymphatic endothelium, such as LYVE-1 and podoplanin (Maruyama et al. 2005). Furthermore, in vivo studies in mouse models of corneal injury and diabetic wound healing found lymphatic vessels with cells that were positive for LYVE-1 and the macrophage marker F4/80, suggesting that new lymphatic vessels are formed by the incorporation of macrophages into vessels (Maruyama et al. 2005; Maruyama et al. 2007). However, lineage tracing analysis was lacking in these studies of inflammatory-stimulated lymphangiogenesis, therefore whether macrophages or bone marrow-derived cells were truly ‘trans-differentiating’ into lymphatic endothelial cells could not definitively be determined.
Figure 1.6. Origins of myeloid and lymphoid cell types.

Myeloid and lymphoid lineages are derived from a common pluripotent hematopoietic stem cell progenitor. Lymphoid stem cells give rise to lymphoid cells, comprising of B and T cells. Myeloid-derived cells include erythrocytes, platelets, basophils, eosinophils, neutrophils, macrophages and dendritic cells. Mast cells are thought to be derived from a multipotent progenitor cells distinct from myeloid stem cells. However, given mast cells express transcription factors required for myeloid cell development, the relationship between these lineages remains controversial. Environmental stimuli determine the fate of each cell type, with each committed cell exhibiting a unique phenotype in the peripheral blood until recruited to tissues by chemoattractants. Once in their tissue of residence, each cell type may change its gene expression profile in response to local signals.
Figure 1.7. A model for the role of macrophages in lymphangiogenesis.

It has been suggested that macrophages contribute to lymphangiogenesis by; acting as a source of lymphatic endothelial progenitor cells which incorporate into growing vessels, or, by providing growth and patterning signals such as VEGF-A and VEGF-C/D that direct the expansion of the existing lymphatic vasculature. Macrophages are recruited to sites of lymphatic vessel growth during tumour progression, inflammation and possibly development, by the presence of factors such as VEGF-A, VEGF-C and M-CSF in the local environment.
Inflammatory Stimuli → Development → Homing → Tumour → Source of factors

Trans-differentiation

Lymphatic Vessel
A human study using gender mismatched bone-marrow transplants detected the presence of donor-derived lymphatic progenitor cells, by detecting the Y-chromosome via in situ hybridisation, incorporated into newly formed lymphatic vessels, but not existing lymphatic vessels, after renal transplant rejection (Kerjaschki et al. 2004; Kerjaschki et al. 2006). Transplant rejection is characterised by chronic inflammation and lymphatic endothelial cell proliferation. This work suggested a contribution of lymphatic endothelial progenitor cells to newly formed lymphatic vessels, but not existing lymphatic vessels, in highly inflammatory settings (Kerjaschki et al. 2006). In contrast to these findings during inflammation-stimulated lymphangiogenesis, He and colleagues (He et al. 2004) did not find significant incorporation of bone marrow-derived cells into lymphatic vessels in mouse models of tumour or VEGF-C-stimulated lymphangiogenesis. The differences in these results may potentially be due to differences in the models of postnatal lymphangiogenesis studied, or perhaps due to differences in the markers employed to identify lymphatic endothelial cells.

At the time the studies for this thesis commenced, whether or not macrophages and bone marrow-derived cells were a source of lymphatic endothelial progenitor cells during developmental and/or postnatal lymphangiogenesis was controversial. One of the aims of this project was to resolve these discrepancies in the field by using a genetic lineage tracing approach to determine whether cells of the myeloid lineage comprise a source of lymphatic endothelial progenitor cells during development and tumour-stimulated lymphangiogenesis.

1.10.2. Macrophages as a source of endothelial progenitor cells during angiogenesis

The contribution of bone marrow-derived endothelial progenitor cells to the blood vascular endothelium is another much debated topic in vascular research. Results from mouse studies have led to the hypothesis that bone marrow-derived cells mobilise to the tumour environment, where they are able to differentiate into endothelial cells (reviewed in (Shojaei
Endothelial progenitor cells were initially defined as VEGFR-2 expressing cells from the bone marrow, which are mobilised in response to VEGF-A to surround newly formed vessels (Asahara et al. 1999). There is evidence that these cells actively contribute to the tumour vasculature, as disruption of the mobilisation of endothelial progenitor cells to tumours via blockade of VEGF-A results in decreased tumour size and blood flow (Shaked et al. 2006; Nolan et al. 2007). However, whether endothelial progenitor cells contribute to the vascular endothelium may be both context and tumour stage dependent (Nolan et al. 2007; Shojaei et al. 2008). A study by Nolan and colleagues found that the early tumour vasculature is comprised of differentiated, bone marrow-derived mature endothelial progenitor cells, whereas late stage tumour vasculature is mainly comprised of non-bone marrow-derived endothelium that originated from the tumour periphery (Nolan et al. 2007). Whether endothelial progenitor cells are derived from the myeloid cell lineage remains controversial. A study by Yang and colleagues demonstrated that Gr1-positive, CD11b-positive myeloid cells act as blood endothelial progenitor cells. When injected into the tumour environment, these cells incorporated directly into the tumour endothelium and acquired endothelial cell properties (Yang et al. 2004).

In contrast to the data suggesting endothelial progenitor cells contribute to the vascular endothelium (Yang et al. 2004; Shaked et al. 2006; Nolan et al. 2007), recent studies using lineage tracing and chimeric mouse models have demonstrated that VEGFR-2 expressing bone marrow-derived cells do not contribute to the vascular endothelium at sites of VEGF-A or tumour-induced angiogenesis, and that these cells are not required for tumour progression (Gothert et al. 2004; Purhonen et al. 2008). Results from these studies demonstrated that bone marrow-derived cells were mobilised to sites of angiogenesis, where they shared a close spatial association with blood vessels but did not contribute to the vessel wall (Purhonen et al. 2008). Other studies using GFP expressing bone marrow chimeras also did not observe any integration of bone marrow-derived cells into the vessel
Chapter 1: Introduction

wall in primary or metastatic Lewis Lung Carcinoma (LLC) tumours, even when tumours were engineered to over-express VEGF-A, which was coupled with a massive infiltration of hematopoietic cells (Wickersheim et al. 2009). Instead of acting as endothelial progenitors, bone marrow-derived cells were determined to be trafficking leukocytes or peri-endothelial myeloid cells which did not incorporate into the vessel wall (Wickersheim et al. 2009).

The contrasting data in the field of endothelial progenitor research may be due to different tumour models utilised, differences in the classification of ‘trans-differentiated’ endothelial cells, or as suggested by Nolan and colleagues (Nolan et al. 2007), different stages of tumour development being analysed. While the role of endothelial progenitor cells in angiogenesis remains controversial, most evidence suggests that incorporation of bone marrow-derived progenitor cells into blood vessels is an extremely rare event, if it occurs at all. Given the low frequency of this trans-differentiation event, the biological significance of such an occurrence is unlikely to be significant.

1.10.3. Macrophages as a source of growth and patterning signals during inflammation-stimulated lymphangiogenesis

While the ability of macrophages to trans-differentiate into endothelial cells is still a controversial topic in vascular research, multiple studies suggest that macrophages play a pro-lymphangiogenic role in states of inflammation. Whether the end result of lymphangiogenesis is anti-inflammatory, by helping to clear fluid and inflammatory cells from the site of infection, or pro-inflammatory, by acting as a route of transport for inflammatory cells to reach the inflamed site, appears to be model dependent. Macrophages recruited to sites of inflammation have been demonstrated to promote the growth of new lymphatic vessels via the production of growth factors VEGF-A, VEGF-C and VEGF-D (Fig 1.7) (Chen et al. 2004; Cursiefen et al. 2004; Baluk et al. 2005; Schledzewski et al. 2006; Jeon et al. 2008; Kataru et al. 2009; Kim et al. 2009). While VEGF-C/D act via VEGFR-3 to directly simulate lymphangiogenesis, VEGF-A produced by macrophages
recruited to the inflamed cornea appears to acts as a chemoattractant to promote recruitment of VEGF-C/D producing macrophages, as opposed to directly promoting lymphangiogenesis by binding to and signalling via VEGFR-2 (Cursiefen et al. 2004).

In *Mycoplasma pulmonis* infection, lymphangiogenesis induced by macrophage-derived VEGF-C/D decreases pathology; newly formed lymphatic vessels help to clear fluid and inflammatory cells from the site of inflammation (Baluk et al. 2005). A soluble VEGFR-3 Ig ligand trap was shown to decrease lymphangiogenesis in this model, leading to bronchial lymphedema and exaggerated airflow obstruction (Baluk et al. 2005). Therefore, manipulation of VEGF-C/D produced by macrophages can mediate lymphangiogenesis in inflammatory disorders (Baluk et al. 2005). In a bacterial-induced model of acute inflammation in the skin, decreasing the number of macrophages, or blockade of VEGF-C/D or VEGF-A, has been shown to prevent inflammatory lymphangiogenesis and delay antigen clearance and inflammation resolution (Kataru et al. 2009). Therefore, as in airway inflammation (Baluk et al. 2005), VEGF-C,-D and –A produced by macrophages reduces pathogenesis associated with acute inflammation via stimulation of new lymphatic vessels (Kataru et al. 2009). These studies highlight the important role of lymphatic vessels in clearing immune cells and fluid to reduce pathology.

In contrast to the above data (Baluk et al. 2005; Kataru et al. 2009), other groups have demonstrated that lymphangiogenesis stimulated by macrophage-derived VEGF-C/D exacerbates inflammation. Macrophage production of VEGF-C/D in a mouse model of LPS-induced peritonitis is the causative factor for aberrant lymphangiogenesis and lymphatic dysfunction, which results in pathology including fibrosis and peritoneal fluid accumulation. Therefore macrophages contribute to pathogenesis in this setting (Kim et al. 2009). Studies of corneal transplant rejection found if the influx of inflammatory cells via the lymphatics is
inhibited by blockade of lymphangiogenesis (via inhibition of the VEGFR-3 signalling pathway), a delayed type hypersensitivity response is prevented, which in turn decreases transplant rejection (Chen et al. 2004). Additional studies in the cornea revealed that increasing VEGFR-3 concentration stimulates an influx of VEGF-A producing macrophages via the lymphatics, which are able to further stimulate lymphangiogenesis and angiogenesis (Chung et al. 2009). Inhibiting either macrophage infiltration or macrophage-derived pro-lymphangiogenic factors would potentially provide novel therapeutic targets for these inflammatory disorders.

1.10.4. Macrophages as a source of growth and patterning signals during tumour-stimulated lymphangiogenesis

To utilise lymphatic vessels as a route of metastasis, tumours can invade adjacent, pre-existing lymphatic vessels (Fig 1.5A), or produce growth factors which elicit a lymphangiogenic response (Fig 1.5B). Lymphangiogenic factors produced by tumours and tumour-associated macrophages (TAMs) stimulate the growth of peri-tumoural lymphatic vessels (Alitalo et al. 2005). These newly formed lymphatic vessels are abnormal, often being dilated and highly permeable, therefore allowing tumour cells to easily invade and metastasise (He et al. 2005; Williams et al. 2010).

Depending on the microenvironment, macrophages are phenotypically specialised to fulfil distinct roles. TAMs are derived from circulating inflammatory cells, and depending on the extracellular environment, acquire a “classical” M1 or “alternative” M2 activation state (Coffelt et al. 2010). In growing tumours, TAMs are largely M2 polarised, favouring a tissue remodelling and pro-angiogenic phenotype, and provide an array of pro-angiogenic factors (Lin et al. 2006; Coffelt et al. 2010). As well as their defined role in angiogenesis (see 1.10.5), it has been determined TAMs express high levels of VEGF-C/D in human cervical cancer and mouse models of gastric cancer, inducing tumour-associated
lymphangiogenesis (Schoppmann et al. 2002; Iwata et al. 2007). Recently, a population of alternatively activated macrophages were observed to promote lymphangiogenesis, tumour growth and metastasis in mouse LLC tumours via production of VEGF-C (Zhang et al. 2009). In addition to enhancing tumour metastasis via lymphatic vessels, TAMs enhance other tumour-associated pathologies. When mice are transplanted with human advanced ovarian cancer cells, tumour progression is associated with ascites formation due to a profound lymphangiogenic response adjacent to the tumour (Jeon et al. 2008). It was determined that VEGF-C/D produced by macrophages is the contributing factor to this aberrant lymphangiogenic response, as blockade of VEGFR-3 prevented ascites formation (Jeon et al. 2008). Therefore, TAMs not only increase the risk of metastases, but also increase other pathologies associated with an aberrant lymphangiogenic response around the tumour site.

Recruitment of macrophages to sites of tumour growth can occur via numerous chemoattractants; macrophages expressing VEGFR-3 are recruited in response to VEGF-C produced by tumour cells in a mouse model of melanoma (Skobe et al. 2001). Alternatively, macrophage infiltration is decreased by blocking macrophage colony-stimulating factor (M-CSF), which in turn decreases tumour-stimulated angiogenesis and lymphangiogenesis while not affecting healthy vasculature outside of the tumour (Kubota et al. 2009). In a study of human invasive breast cancer and melanoma, VEGF-C producing TAMs had a positive association with metastasis (Dadras et al. 2005; Schoppmann et al. 2006). However, VEGF-C is produced by macrophages as well as tumour cells in melanoma, and the relative contribution towards lymphangiogenesis by growth factors produced by each cell type is yet to be elucidated (Dadras et al. 2005). In summary, recent work in the field suggests that targeting macrophages or macrophage-derived factors will lead to novel lymphangiogenic therapeutics for the treatment of inflammatory disorders and prevention of tumour metastasis.
1.10.5. Macrophages as a source of growth and patterning signals during developmental and tumour-stimulated angiogenesis

The role of macrophages in the promotion of angiogenesis in tumour and inflammatory models has been well established (Espinosa-Heidmann et al. 2003; Sakurai et al. 2003; Pollard 2004). As well as producing pro-lymphangiogenic factors VEGF-C/D, myeloid cells can produce VEGF-A, which has been demonstrated to be important during tumour-induced angiogenesis (Stockmann et al. 2008). In tumours with a conditional deletion of myeloid-derived VEGF-A, tumour progression was retarded and the blood vasculature was found to be less tortuous, with vessels displaying an increase in pericyte coverage and a decrease in vessel length. However, there was no detectable change in the amount of total VEGF-A expressed in the tumour microenvironment (Stockmann et al. 2008), revealing that even a small change in VEGF-A concentration is sufficient to affect angiogenesis and tumour growth. This study demonstrates the contribution of myeloid-derived angiogenic and lymphangiogenic factors cannot be determined by simply examining their magnitude of expression, but needs to be carefully analysed using myeloid-specific knockout mice.

In addition to the production of VEGF-A, macrophages can produce factors such as matrix metalloproteinases (MMPs) that influence angiogenesis (Bergers et al. 2000; Cho et al. 2007). In pancreatic islet tumours, production of MMP-9 by myeloid cells induces the angiogenic switch, characterised by activation and expansion of the tumour and tumour-associated vasculature, making sequestered VEGF-A available to induce angiogenesis (Bergers et al. 2000). MMP-9 producing CD11b-positive Gr1-positive myeloid cells have been shown to promote increased vascular density and maturation in tumours, with the deletion of Mmp9 in these cells ablatting their pro-angiogenic effect (Yang et al. 2004). The importance of macrophage-derived MMP-9 is further demonstrated by a study which found that tumours cannot grow or stimulate vasculogenesis in Mmp9-deficient mice (Ahn and Brown 2008). However, when these mice were transplanted with macrophages from Mmp9-
sufficient mice, tumour growth and vasculogenesis was restored (Ahn and Brown 2008). While MMPs are clearly important for the tumour angiogenic response, the mechanisms by which myeloid-derived MMPs influence lymphangiogenesis are still largely undefined. MMP regulation of lymphangiogenesis does occur, with broad spectrum MMP inhibitors found to inhibit the lymphangiogenic response associated with lung cancer (Nakamura et al. 2004). A recent study by Bruyere and colleagues revealed an important role for MMP-2 in lymphangiogenesis (Bruyere et al. 2008). When MMPs were inhibited, or Mmp2 depleted, lymphatic endothelial cells displayed less outgrowth in an in vitro aortic ring assay (Bruyere et al. 2008). Furthermore, Mmp-2/- mice displayed impaired formation of lymphangioma (Bruyere et al. 2008). While MMPs were implicated in lymphangiogenesis in this study, whether MMP-2 was myeloid-derived remains to be determined.

While the importance of TAMs in angiogenesis and tumour development is well established (Murdoch et al. 2008), an alternative population of Tie2-expressing monocytes has been observed to infiltrate tumours, where they associate with the tumour vasculature and promote tumour growth (De Palma et al. 2005). This population of Tie2-expressing monocytes (TEMs) do not incorporate within the vascular endothelium, and possess a gene expression profile distinct from endothelial cells, suggesting they promote angiogenesis by production of growth factors (De Palma et al. 2005; Pucci et al. 2009). TEMs display a distinct gene expression profile from TAMs, and despite being a minor population of tumour-associated macrophages, when TEMs are isolated and then implanted with tumour cells in vivo, they drive the angiogenic response to a greater degree than TAMs (De Palma et al. 2005; Pucci et al. 2009). It is thought that Angpt2 expressed on tumour endothelium acts as a chemoattractant for TEMs. In states of hypoxia, Tie2 expression is increased on TEMs, suggesting that in this highly pro-angiogenic environment, TEMs become more responsive to Angpt2, resulting in increased recruitment to the tumour site, increased tumour angiogenesis and enhanced tumour progression (Murdoch et al. 2007). This is supported by
the finding that tumour-derived TEMs express higher levels of Tie2 than circulating TEMs isolated from the peripheral blood (Venneri et al. 2007). While TEMs have been demonstrated to be important in angiogenesis, their role in lymphangiogenesis has not been investigated.

In further support of a role for macrophages during vascular development, recent work demonstrated that macrophages are required for the vascularisation of the brain (Fantin et al. 2010). In this model, macrophages act downstream of VEGF-A-mediated tip cell formation, and are spatially associated with neighbouring tip cells to act as chaperones to promote vascular fusion, and thereby increase vascular complexity (Fantin et al. 2010). These macrophages, like TEMs which promote the angiogenic switch in cancer, express cell surface markers Tie2 and Neuropilin-1 (De Palma et al. 2005; Pucci et al. 2009; Fantin et al. 2010), however the signals that induce this macrophage lineage are yet to be determined. Taken together, these results demonstrate how heterogeneous populations of macrophages play specific roles in developmental angiogenesis and in the angiogenic switch during cancer. Therefore, macrophages could be targeted for anti-angiogenic therapies in cancer, or pro-angiogenic therapies to promote vascularisation in ischemic disease.

1.11. Mast Cells

Mast cells are derived from the myeloid lineage and are found in most tissues, being most abundant at interfaces of the environment, such as the skin, airways and digestive tract (Abraham and St John 2010). This localisation of mast cells makes them one of the first cell types to interact with allergens, toxins and antigens from the environment. While mast cells have traditionally been thought to arise from a common myeloid progenitor, recent work has described mast cell progenitors which were proposed to arise directly from multipotent
progenitors during adult haematopoiesis (Fig 1.6) (Chen et al. 2005). The relationship between mast cell progenitor cells, multipotent progenitors and common myeloid progenitors is yet to be determined. Mast cells express transcription factors required for granulocyte/macrophage lineage development such as PU.1, and share expression of GATA-1 and GATA-2 with erythrocyte/megakaryocyte lineage cells. Furthermore, PU.1 and GATA-2 are required for mast cell development (Walsh et al. 2002). This suggests mast cells share a close developmental relationship with these two other myeloid lineages.

Mast cell progenitor cells originate from the bone marrow and migrate to their tissue of residence, where they mature in situ. Upon stimulation, mast cells are able to proliferate, differentiate and degranulate, releasing a wide variety of inflammatory mediators (Galli et al. 2008). Classically, mast cells are important in generating and maintaining innate and adaptive immune responses, and in the development of adaptive immunity and tolerance (Galli et al. 2008; Abraham and St John 2010). However, they have more recently been identified as negative regulators of the inflammatory response (Grimbaldeston et al. 2007; Biggs et al. 2010). Understanding how mast cells regulate both positive and negative inflammatory responses is currently an area of considerable interest.

1.11.1. Mast cells as inflammatory regulators

Mast cells have classically been viewed as a pro-inflammatory cell type, and are involved in the inflammatory response and subsequent clearance of parasite and bacterial infections, acting to increase host defence (reviewed in (Galli et al. 2008; Pejler et al. 2010)). However, mast cells can also act to increase pathology associated with inflammatory disorders. In mouse models of arthritis, mast cells are a contributing factor to pathogenesis, with mast cell-deficient mice displaying resistance to arthritis which is reversed after engraftment of these mice with wild-type mast cells (Lee et al. 2002). Recent work has demonstrated this effect is via fibroblast exposure to mast cell protease 6 (mMCP-6) in the inflamed joint, which results in an increase in neutrophil chemotactic factors (Shin et al. 2009).
Additionally, mast cells have been shown to promote pathological severity of psoriasis via production of pro-inflammatory mediators (Kakurai et al. 2009; Kneilling and Rocken 2009; Theoharides et al. 2010). Interestingly, lymphatic vessel enlargement and density increases pathology in mouse models of arthritis and psoriasis (Kunstfeld et al. 2004; Zhang et al. 2007; Polzer et al. 2008; Guo et al. 2009; Huggenberger et al. 2010), yet whether mast cells promote lymphangiogenesis in these settings has not been investigated.

As well as acting as positive regulators of inflammation, recent work has revealed that mast cells are able to negatively regulate the inflammatory response. Mast cells were observed to limit pathology in the skin resulting from contact hypersensitivity (characterised by infiltration of inflammatory cells to the challenged site and tissue edema) and chronic ultraviolet B (UVB) irradiation (characterised by substantial tissue damage due to induction of inflammatory responses at the site of irradiation) (Grimbaldeston et al. 2007). After chronic UVB irradiation, mast cell-deficient mice displayed increased leukocyte infiltration, epidermal hyperplasia and epidermal necrosis (Grimbaldeston et al. 2007). When these mice were engrafted with wild-type mast cells, pathology was substantially reduced via vitamin D(3) dependent induction of anti-inflammatory IL-10 by mast cells (Grimbaldeston et al. 2007; Biggs et al. 2010). Recent studies have found that UVB-induced pathology is associated with the formation of enlarged, dysfunctional lymphatic vessels in the skin due to an increase in VEGF-A (Kajiya et al. 2006; Kajiya et al. 2009), yet whether mast cells act to limit lymphangiogenesis in this setting, subsequently limiting pathology, remains to be seen.

1.1.1.2. Mast cells and tumour-stimulated angiogenesis and lymphangiogenesis

Characterising the role of mast cells in regulation of tumour-stimulated vessel growth is of great interest, given their association with tumours and the angiogenic switch. Mast cells have been observed to localise around tumours via recruitment by chemoattractants (Conti et al. 1997), where they can produce pro-angiogenic factors including VEGF-A, MMP-9 and Angpt1; mast cell recruitment and subsequent degranulation has been shown to contribute
to the angiogenic switch in mouse models of tumour formation (Coussens et al. 1999; Nakayama et al. 2004; Soucek et al. 2007; Theoharides et al. 2007). In addition, the presence of mast cells around human tumours has been shown to be a poor prognostic indicator (Crivellato et al. 2008). A role for AM, an established regulator of lymphangiogenesis (see 1.8.5) (Fritz-Six et al. 2008), in mast cell stimulation of angiogenesis has been reported. Blocking of AM with antibodies or with siRNA decreased mast cell-induced angiogenesis and tumour cell growth in in vitro assays, suggesting that AM is a key molecule in the cross-talk between tumour cells and mast cells (Zudaire et al. 2006).

While much work has examined mast cell regulation of tumour-stimulated angiogenesis, the effect of mast cells on tumour-associated lymphangiogenesis remains largely uncharacterised. Work by Brideau and colleagues determined that endostatin, a potent inhibitor of angiogenesis and tumour growth, inhibits mast cell migration and adhesion to tumours, leading to inhibition of tumour lymphangiogenesis (Brideau et al. 2007). As mast cells were shown to express high levels of VEGF-C in this model, this decrease in lymphangiogenesis was presumably due to a decrease in mast cell-derived VEGF-C. Recent studies found that cultured mast cells produce VEGF-A, VEGF-C and VEGF-D, and are able to stimulate angiogenesis in a chick chorioallantoic membrane assay (Detoraki et al. 2009). However, this study neglected to investigate the effect of mast cells on lymphangiogenesis. Questions remain in the field as to whether mast cells are able to regulate lymphangiogenesis in settings of developmental and inflammation-stimulated lymphangiogenesis. This project aimed to investigate this possibility using a variety of techniques in mast cell-deficient mice.
1.12. Summary and Project Aims

The source and identity of embryonic lymphatic endothelial progenitor cells has been actively debated for more than a century. Moreover, it is not known whether the reservoirs of lymphatic endothelial progenitor cells utilised during embryogenesis and pathological lymphangiogenesis are the same, or different. Work in mouse models of inflammation has suggested that macrophages contribute to the generation of lymphatic vessels via a process of trans-differentiation to lymphatic endothelial cells. In addition, macrophages have been shown to produce pro-lymphangiogenic growth factors in settings of inflammation. At the time that this project was initiated, the issue of whether macrophages comprise a pool of lymphatic endothelial progenitor utilised for embryonic or inflammation-stimulated lymphangiogenesis was a key question in the field. In addition, the question of whether macrophages are required for normal growth and patterning of the embryonic lymphatic vasculature was unexplored. Another intriguing question, given that mast cells act as key regulators of inflammation and have been ascribed a pro-angiogenic role in the tumour micro-environment, was whether mast cells regulate lymphangiogenesis. The primary aims of this study were to:

1. Precisely document the localisation and molecular identity of macrophages with respect to the developing lymphatic vasculature during embryogenesis (Chapter 3 and Chapter 4).

2. Determine whether cells of the myeloid lineage contribute to lymphatic vascular genesis during embryonic development and in the tumour microenvironment by acting as a source of lymphatic endothelial progenitor cells (Chapter 4).

3. Determine whether macrophages provide growth and patterning signals that direct the patterning and expansion of the lymphatic vasculature, and to evaluate the nature of these signals (Chapter 5).

4. Investigate the role of mast cells in developmental and inflammation-stimulated lymphangiogenesis (Chapter 6).
2. Materials and Methods
2.1. **Materials**

All chemical reagents were of analytical grade and purchased from Sigma unless stated otherwise.

2.2. **RNA analysis**

2.2.1. **Total RNA preparation**

RNA was isolated from purified primary cells using RNAsafe (Ambion, TX) or TRIzol® reagent (Invitrogen, CA). RNA was isolated from wild-type or $PU.1^{-/-}$ skin samples following homogenisation in TRIzol® reagent. All RNA preparation was conducted according to the manufacturer's instructions (Invitrogen, Ambion).

2.2.2. **RNA quantification**

RNA concentration was measured using a NanoDrop1000 spectrophotometer (Thermo Scientific), assuming that an optical density (OD) of 1.0 correlates to 40 μg/ml of RNA. For samples used in Microarray studies, RNA quality was assessed using a Bioanalyser (Agilent Technologies).

2.2.3. **Microarray analysis**

Total RNA from F4/80$^+$LYVE-1$^-$ and F4/80$^+$LYVE-1$^+$ isolated macrophages was purified using RNAsafe according to the manufacturer’s instructions. Total RNA from wild-type and $PU.1^{-/-}$ skin was purified using TRIzol® reagent according to the manufacturer’s instructions. RNA quality was assessed using a Bioanalyzer picochip (Agilent Technologies). 100 ng of pooled RNA from three biological replicates was submitted to the Adelaide Microarray Centre for gene expression analysis. Affymetrix Mouse Gene 1.0 ST Arrays were used according to the Genechip Whole Transcript (WT) Sense Target Labelling Assay (P/N 701880). Microarray data was analysed using Partek Genomics Suite (Partek...
Differential gene expression was assessed by ANOVA with the p value adjusted using step-up multiple test correction (Benjamini 1995) to control the false discovery rate. Adjusted p values of < 0.05 were considered to be significant.

### 2.3. DNA analysis

#### 2.3.1. Primer design

All primers were purchased from Geneworks. Oligonucleotides were received as dried pellets and were resuspended at 100 pmol in sterile milliQ (MQ) H₂O. All primers are given in the 5’ to 3’ direction.

**Genotyping**

<table>
<thead>
<tr>
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<th>Forward 5’-3’</th>
<th>Reverse 5’-3’</th>
</tr>
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<tbody>
<tr>
<td>Gtrosa²⁶&lt;sup&gt;tmSor&lt;/sup&gt; WT</td>
<td>AAAGTCGCTCTGAGTTGTTAT</td>
<td>GGAGCGGGAGAAATGGATATG</td>
</tr>
<tr>
<td>Gtrosa²⁶&lt;sup&gt;tmSor&lt;/sup&gt; MUT</td>
<td>AAAGTCGCTCTGAGTTGTTAT</td>
<td>GCGAAGAGTTTGTCTCAACC</td>
</tr>
<tr>
<td>Lyzs&lt;sup&gt;tm1(cre)Ifo&lt;/sup&gt; WT</td>
<td>CTTGGGCTGCAAGAATTTCCT</td>
<td>TTACAGTCGGCCAGGCTGAC</td>
</tr>
<tr>
<td>Lyzs&lt;sup&gt;tm1(cre)Ifo&lt;/sup&gt; MUT</td>
<td>CTTGGGCTGCAAGAATTTCCT</td>
<td>CCCAGAAATGCCAGATTACG</td>
</tr>
</tbody>
</table>

**mRNA expression (real time RT-PCR):**

<table>
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<tr>
<th></th>
<th>Forward 5’-3’</th>
<th>Reverse 5’-3’</th>
</tr>
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<tbody>
<tr>
<td>Acta2</td>
<td>GCATCCACGAAACCACCTAT</td>
<td>TGGAAGGTAGACAGCGAAGG</td>
</tr>
<tr>
<td>Actb</td>
<td>GATCATTGCTCTGACTGAC</td>
<td>GTCATAGTCGGCGCTAGAAGCAT</td>
</tr>
<tr>
<td>Angpt1</td>
<td>CAGCATCTGGAGCATGTGAT</td>
<td>AACATCTGTCACTTCGGG</td>
</tr>
<tr>
<td>Angpt2</td>
<td>GATCTTCTCCAGCCCTAC</td>
<td>CAGCAAGTCGTTCCAATCT</td>
</tr>
<tr>
<td>CD163</td>
<td>AGCGAATGACTTTCCCTCAAGAGGA</td>
<td>GTGTGCTCTGAATGACCCCTTT</td>
</tr>
<tr>
<td>CD34</td>
<td>TCCCCATCAGTCTCCATCAA</td>
<td>CAGTTGGAAGTCTGTGT</td>
</tr>
<tr>
<td>Emr1</td>
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<td>GGTCACTTCTTCCATGGT</td>
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<tr>
<td>Lyve1</td>
<td>TGGTGTATCTCTCGCTCTC</td>
<td>TTCTGCGTGACTCTGCTG</td>
</tr>
</tbody>
</table>
### 2.3.2. cDNA synthesis

Complementary DNA (cDNA) was randomly primed from 0.2-1 μg of total RNA using Superscript™ III First-Strand Synthesis SuperMix (Invitrogen) with a mixture of oligo dT and random hexamer primers. Reactions were performed on a Mastercycler (Eppendorf), using the following parameters: addition of oligo dT, random hexamer primers and annealing buffer to RNA followed by 5 min at 65°C, 1 min on ice, addition of 1st Strand Reaction mix and enzyme mix, 10 min at 25°C, 5 min at 50°C, 5 min at 85°C. All reactions were held at 4°C.
2.3.3. **Real time RT-PCR**

Real time RT-PCR was subsequently performed in triplicate using RT² Real Time SYBR Green/ROX PCR Master Mix (SA Biosciences) as per the manufacturer’s instructions. Samples were run on a Rotor-Gene 6000 machine (Corbett Research) using the following parameters: 2 min at 50°C, 15 min at 95°C, followed by 40 cycles of 95°C for 15 sec, 60°C for 25 sec and 72°C for 10 sec, followed by a 3 min extension at 72°C. Melt curve was achieved by a ramp from 72°C to 99°C with 5 sec for each step. Reactions were performed in triplicate and the messenger RNA (mRNA) expression levels normalised against the internal control gene beta actin (actB) using the \( \Delta\Delta C_T \) method as previously described (Livak and Schmittgen 2001). Data was analysed using the Rotor-Gene 6000 Series Software (Corbett Research).

2.4. **Tissue culture and cellular analysis**

2.4.1. **Cell lines and culture**

EL4 lymphoma or Lewis lung carcinoma (LLC) carcinoma cells (obtained from C. Bonder) were grown in Roswell Park Memorial Institute (RPMI) Media (Gibco) with 10% Foetal Bovine Serum (FBS), 2 mM glutamine and 100 μg/ml penicillin/streptomycin (pen/strep). Cell culture was carried out in a Class II Biological Safety Cabinet (Euroclone). Cell density was calculated using a haemocytometer and viability determined by trypan blue (0.8% w/v in phosphate buffered saline [145 mM NaCl, 7.5 mM Na₂HPO₄, 2.5 mM NaH₂PO₄] [PBS], pH 7.4) dye exclusion. When cells were nearly confluent, media was aspirated, cells rinsed in PBS and detached by incubation in trypsin (Lonza). Following treatment with trypsin for 5 min, cells were removed to a tube containing complete medium and pelleted for 5 min at 394xg. The cell pellet was washed 3x in PBS and resuspended at 10⁶ cells per 100 μl of PBS ready for injection (see 2.6.3).
2.4.2. Cryopreservation and thawing of cells

Cells in log phase were harvested, pelleted by centrifugation at 400xg for 5 min and resuspended in complete medium at 2x10^6 cells/ml. 500 μl of cryoprotectant (20% FBS/20% dimethyl sulphoxide, 60% media) was added to a 500 μl aliquot of cells in cryopreservation tubes (Nunc). Cells were frozen by controlled rate freezing and stored in liquid nitrogen tanks.

Cells at a concentration of 2x10^6 cells/ml were thawed rapidly in a 37°C water bath and then resuspended in 10 ml warm media. Cells were centrifuged at 400xg, washed again in media before being seeded into a T25 tissue culture flask.

2.4.3. Primary cell culture

Macrophages and lymphatic endothelial cells were freshly isolated for each experiment from embryonic skin as described in 2.4.5 and 2.4.7. Tissue culture plates were coated with 0.5 mg/ml fibronectin (Roche) in PBS for 45 min. Cells were plated onto fibronectin coated dishes at approximately 1x10^5 cells/ml in Endothelial Basal Medium-2 (EBM-2) supplemented with Endothelial Growth Media-2 MV SingleQuots (containing hydrocortisone, hEGF, FBS, VEGF, hFGF-B, R3-IGF-1, ascorbic acid and gentamicin/amphotericin-B) (EGM-2) (Lonza) and grown for up to 7 days in a 37°C humidified 5% CO₂ atmosphere.

2.4.4. Preparation of embryonic skin suspension

Preparation of embryonic skin suspension was performed as previously described (Gordon et al. 2010; Kazenwadel et al. 2010). Embryonic skin was dissected from wild-type C57Bl/6 embryos at embryonic day (E) 15.5 or 16.5 in Hanks Balanced Salt Solution (HBBS) with 10 mM 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid (HEPES) and 5% FBS (HHF) and digested in 5 ml of digestion buffer (Dulbecco’s Modified Eagles Medium (DMEM)/20% FBS with Collagenase II [Worthington Biochemical Corporation, 2.5 mg/ml], Collagenase IV
[Worthington Biochemical Corporation, 2.5 mg/ml] and Deoxyribonuclease I [Worthington Biochemical Corporation, 1 mg/ml]) per 5-8 embryos at 37ºC for 30 min with frequent re-suspension of tissue. Digested skin was filtered through a 40 μm Nylon Cell Strainer (BD Falcon) and rinsed with an equal volume of HHF. Cells were pelleted at 394g for 5 min, washed with HHF and resuspended in an appropriate volume of HHF.

### 2.4.5. Macrophage isolation

Macrophages were isolated from E15.5 and E16.5 skin using a magnetic cell separation procedure (MACS®) as previously described (Gordon et al. 2010). Embryonic mouse skin cell suspensions were incubated with rat anti-mouse F4/80 antibody (see 2.5.4 for antibody concentration) (Invitrogen) at 4ºC for 5 min, washed in 10 ml of MACS buffer (PBS pH 7.2 with 0.5% bovine serum albumin [BSA] and 2 mM ethylenediaminetetraacetic acid [EDTA]) per 1x10^8 cells and pelleted by centrifugation at 394xg for 5 min. Cells were resuspended in 80 μl MACS buffer with 20 μl anti-rat magnetic MicroBeads (Miltenyi Biotech) per 1x10^7 cells and incubated at 4ºC for 15 min. Cells were washed in 10 ml of MACS buffer, pelleted by centrifugation at 394xg for 5 min and resuspended in 500 μl MACS buffer before separation on a MiniMACS column (Miltenyi Biotech) to purify F4/80-positive cells by positive selection. To obtain a pure F4/80-positive (F4/80^+) population, cells were purified on a second MiniMACS column. Approximately 2x10^6 F4/80^+ macrophages were obtained from an initial suspension of 1x10^8 cells. The F4/80-negative (F4/80^-) fraction was retained for use in 2.4.7.

### 2.4.6. Isolation of different populations of macrophages

F4/80-positive, LYVE-1-negative (F4/80^+LYVE-1^-) and F4/80-positive, LYVE-1-positive (F4/80^+LYVE-1^+) macrophages were isolated from E15.5 skin using a magnetic cell separation procedure (MACS®) as previously described (Gordon et al. 2010). Embryonic mouse skin cell suspensions were incubated with biotin-conjugated rat anti-mouse F4/80 antibody (see 2.5.4 for antibody concentration) (Caltag Laboratories) at 4ºC for 5 min,
washed in 10 ml of MACS buffer and pelleted by centrifugation at 394xg for 5 min. Cells were resuspended in 80 μl MACS buffer plus 20 μl anti-biotin multisort magnetic MicroBeads (Miltenyi Biotec) per 1x10⁷ cells and incubated at 4°C for 15 min. Cells were washed in 10 ml of MACS buffer, pelleted by centrifugation at 394xg for 5 min and resuspended in 500 μl MACS buffer before separation on a MiniMACS column. To obtain a pure F4/80⁺ population, cells were purified on a second MiniMACS column.

To remove anti-biotin multisort magnetic beads, cells were pelleted by centrifugation at 394xg for 5 min, resuspended in 1 ml MACS buffer plus 20 μl multisort release reagent (Miltenyi Biotec) and incubated at 4°C for 5 min. Cells were separated on a MiniMACS column, with the negative fraction containing released cells. The negative fraction was incubated with rabbit anti-mouse LYVE-1 (see 2.5.4 for antibody concentration) (Angiobio) at 4°C for 5 min, and cells were washed in 2 ml of MACS buffer and pelleted by centrifugation at 394xg for 5 min. Cells were resuspended in 80 μl MACS buffer plus 20 μl anti-rabbit magnetic MicroBeads (Miltenyi Biotec) per 1x10⁷ cells and incubated at 4°C for 15 min. Cells were washed in 2 ml of MACS buffer, pelleted by centrifugation at 394xg for 5 min and resuspended in 500 μl MACS buffer before separation on a MiniMACS column. F4/80⁺LYVE-1⁻ macrophages were purified in the negative fraction and F4/80⁺LYVE-1⁺ macrophages were purified in the positive fraction. Approximately 6x10⁴ F4/80⁺LYVE-1⁻ macrophages and 4x10⁴ F4/80⁺LYVE-1⁺ macrophages were obtained from an initial suspension of 1x10⁷ cells.

### 2.4.7. Lymphatic endothelial cell isolation

Lymphatic endothelial cells were isolated from E15.5 and E16.5 skin using a magnetic cell separation procedure (MACS®) as previously described (Kazenwadel et al. 2010). Macrophages were isolated from embryonic mouse skin as in 2.4.5. Hematopoietic cells were depleted from the cell mixture as follows. The F4/80⁻ fraction was incubated with anti-mouse F4/80 antibody (see 2.5.4 for antibody concentration) (Invitrogen) and rat anti-mouse
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CD45 antibody (see 2.5.4 for antibody concentration) (BD Pharmingen) at 4°C for 5 min to remove any remaining hematopoietic cells and macrophages. Cells were washed in 10 ml of MACS buffer per 1x10⁸ cells and pelleted by centrifugation at 394xg for 5 min. Cells were resuspended in 80 μl MACS buffer with 20 μl anti-rat magnetic MicroBeads per 1x10⁷ cells and incubated at 4°C for 15 min. Cells were washed in 10 ml of MACS buffer per 1x10⁸ cells, pelleted by centrifugation at 394xg for 5 min and resuspended in 500 μl MACS buffer before separation on a MiniMACS column to purify a F4/80⁻, CD45⁻ population of cells by negative selection.

Lymphatic endothelial cells (LEC) were subsequently purified from the negative population as follows. Cells were incubated with rabbit anti-mouse LYVE-1 (see 2.5.4 for antibody concentration) (Angiobio) at 4°C for 5 min and cells were washed in 10 ml of MACS buffer per 1x10⁸ cells and pelleted by centrifugation at 394xg for 5 min. Cells were resuspended in 80 μl MACS buffer with 20 μl anti-rabbit magnetic MicroBeads per 1x10⁷ cells and incubated at 4°C for 15 min. Cells were washed in 10 ml of MACS buffer per 1x10⁸ cells, pelleted by centrifugation at 394xg for 5 min and resuspended in 500 μl MACS buffer before separation on a MiniMACS column to purify LYVE-1⁺ LEC by positive selection. To obtain a pure LYVE-1⁺ population, cells were purified on a second MiniMACS column. Approximately 1x10⁶ LYVE-1⁺ LEC were obtained from an initial suspension of 1x10⁸ cells.

**2.4.8. Blood endothelial cell isolation**

Blood endothelial cells (BEC) were isolated from E16.5 skin using a magnetic cell separation procedure (MACS®) as previously described (Kazenwadel et al. 2010). Macrophages, hematopoietic cells and lymphatic endothelial cells were isolated as in 2.4.5 and 2.4.7. BEC were subsequently purified from the LYVE-1⁻ fraction by Jan Kazenwadel as follows. Cells were incubated with rat anti-mouse CD31 (see 2.5.4 for antibody concentration) (Biolegend) at 4°C for 5 min and cells were washed in 10 ml of MACS buffer per 1x10⁸ cells and pelleted by centrifugation at 394xg for 5 min. Cells were resuspended in
80 μl MACS buffer with 20 μl anti-rat magnetic MicroBeads per 1x10^7 cells and incubated at 4°C for 15 min. Cells were washed in 10 ml of MACS buffer per 1x10^8 cells, pelleted by centrifugation at 394xg for 5 min and resuspended in 500 μl MACS buffer before separation on a MiniMACS column to purify CD31^+, LYVE-1^- BEC by positive selection. To obtain a pure CD31^+ population, cells were purified on a second MiniMACS column. Approximately 3.5x10^6 CD31^+ BEC were obtained from an initial suspension of 1x10^8 cells.

2.4.9. Preparation of macrophage conditioned media

Macrophages were obtained from embryonic skin suspensions as described in 2.4.5. Approximately 2x10^6 macrophages were grown at 37°C/5% CO_2 in a T25 flask in EGM-2 and 100 μg/ml macrophage colony-stimulating factor (M-CSF) (PeproTech Inc) for 72 h until approximately 50% confluent. To obtain skin cell conditioned media, approximately 3x10^6 digested skin cells were grown at 37°C/5% CO_2 in a T25 flask in DMEM with 20% FBS and 100 μg/ml pen/strep for 24 h until approximately 90% confluent. To obtain macrophage-depleted skin cell conditioned media, after macrophage and hematopoietic depletion as in 2.4.5 and 2.4.7, approximately 3x10^6 cells were grown at 37°C/5% CO_2 in a T25 flask in DMEM with 20% FBS and 100 μg/ml pen/strep for 24 h until approximately 90% confluent. All conditioned media was removed and filtered through a 0.45 μm low protein binding filter (Nalgene) and stored in aliquots at -20°C until use.

2.4.10. Lymphatic endothelial cell culture with conditioned media

Freshly isolated lymphatic endothelial cells were plated in a fibronectin coated 96 well plate in EGM-2 at 8x10^4 cells/ml with 100 μl per well. If lymphatic endothelial cells were co-cultured with macrophages, 5x10^4 LEC were plated in each well with macrophages plated at 2.5x10^4 cells per well. 24 h after cells were plated, conditioned media was added at 20-50% of total volume with EGM-2. The proliferation of cells was assessed at time points between 1 day and 6 days (see 2.4.13 and 2.4.15).
2.4.11. Lymphatic endothelial cell culture with growth factors and soluble receptors

<table>
<thead>
<tr>
<th>Name</th>
<th>Supplier</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>M-CSF</td>
<td>PeproTech Inc</td>
<td>1 - 5 μg/ml</td>
</tr>
<tr>
<td>Soluble Mouse VEGFR-3 receptor</td>
<td>R&amp;D Systems</td>
<td>500 ng/ml</td>
</tr>
</tbody>
</table>

Freshly isolated lymphatic endothelial cells were plated in a fibronectin coated 96 well plate EGM-2 at 8x10^4 cells/ml with 100 μl per well. 24 h after cells were plated, growth factors and/or soluble receptors were added in EGM-2, or in 50% conditioned media with EGM-2, and the proliferation of cells was assessed between 24 h and 96 h (see 2.4.13).

2.4.12. Transient transfections of siRNA

siRNA constructs were obtained to target the following sequences (5’-3’direction):

- negative control siRNA: ACACTAAGTACGTCGTATTAC
- mTie1: AATGCTAGCACACGATATCTT
- mTEK: AACCAGCTGTGCAGTTTAACT
- mAngpt2: GCACCGCTACGTGCTTAGAT

All cells were transfected with SureFECT transfection Reagent (SA Biosciences, Qiagen). One day prior to transfection, lymphatic endothelial cells were isolated as described in 2.4.7 and plated in a fibronectin coated 96 well or 24 well plate at 8x10^4 cells/ml. For siRNA transfection in a 96 well plate, 0.3 μl SureFECT reagent (SA Biosciences, Qiagen) was diluted in 40 μl OptiMem serum-free media (JRH). 20 nM of each gene specific siRNA was diluted into 38 μl of OptiMem diluted SureFECT. The pooled SureFECT and siRNA complexes were incubated at room temperature (RT) for 20 min, then added to the cells. 160 μl of EGM-2 was added, and cells were assessed between 24 h and 96 h.
For siRNA transfection in a 24 well plate, 1.5 μl SureFECT reagent was diluted in 100 μl OptiMem serum-free media and 30 nM of each gene specific siRNA was diluted into 91 μl of OptiMem diluted SureFECT. The pooled SureFECT and siRNA complexes were incubated at RT for 20 min, then added to the cells. 400 μl of EGM-2 was added, and cells were assessed between 24 h and 96 h.

2.4.13. Assessment of cell proliferation

Cell proliferation was assessed using the Cell Titer Aqueous One Solution Proliferation Assay (Promega) according to the manufacturer’s instructions. 20 μl of Cell Titer 96® One Solution Reagent was added to cells in a 96 well plate after 24 h to 96 h of culture, with 100 μl of culture medium per well. The plate was incubated at 37°C/5% CO₂ for 4 h and the colour change was measured as optical density at 490 nm using an EL808 Ultra Microplate reader (Bio-tek Instruments, Inc.). Increased optical density at 490 nm correlated with an increase in the number of viable cells.

2.4.14. Assessment of cell proliferation after co-culture

Embryonic LEC were isolated and stained with CellVue Claret (CVC) Far Red Fluorescent Cell linker (Sigma) to assess proliferation of LEC after co-culture. Cells were stained according to manufacturer’s instructions. LEC were washed in HBBS, pelleted at 500xg for 5 min and resuspended in 100 μl Diluent C (Sigma) per 10⁶ cells. 0.2 μl CVC diluted in 100 μl Diluent C per 10⁶ cells was added to the cell suspension and incubated at RT for 3-4 min with frequent resuspension. 200 μl FBS was immediately added to cells and incubated for 1 min. Cells were pelleted at 500xg for 10 min, resuspended in 1 ml EGM-2, and transferred to a fresh tube to minimise carryover of excess dye. Cells were pelleted at 500xg for 5 min, washed 3 times in 1 ml of EGM-2, and resuspended in EGM-2 for viability assessment and plating into a fibronectin coated 24 well plate. 5x10⁴ LEC were plated in each well, with or without cells for co-culture, which were plated at 2.5x10⁴ cells per well.
2.4.15. Flow cytometry

CVC stained lymphatic endothelial cells, with or without macrophages, were rinsed in PBS and detached by incubation in trypsin. Following treatment with trypsin for 5 min, cells were removed to a tube, rinsed with HHF and pelleted for 5 min at 400xg. Cells resuspended in 200 μl HHF and blocked with 1.5 μl gamma globulin (Jackson ImmunoResearch Laboratories) for 30 min on ice with frequent resuspension. To appropriate controls or experimental samples, cells were incubated with 1.25 μl rat anti-mouse CD31 PE-Cy7 (Biolegend) in 100 μl HHF for 30 min on ice with frequent resuspension. Cells were washed twice with 2 ml HHF and resuspended in HHF at a final volume of 200 μl. Cells were analysed after 3 to 6 days. Samples were analysed on a FC-500 flow cytometer (Beckman Coulter) with CD31-positive, CVC-positive cells gated for analysis. Proliferation index was calculated using Proliferation Wizard module in ModFit LT (Verity Software House).

2.4.16. Cell staining (immunofluorescence)

Freshly isolated primary cells were grown on fibronectin coated chamber slides for 24 h to 96 h prior to staining. Cells were fixed in 4% phosphate-buffered paraformaldehyde (PFA)/PBS for 30 min at RT, washed in PBS and incubated in blocking solution (1% BSA, 0.3% Triton X-100, 0.01% thimerosal [Sigma] in PBS) for 30 min. Primary antibodies in blocking solution were added for 2 h at RT (see 2.5.4 for antibody details and dilutions). Cells were then washed 3 times in PBS and secondary antibodies were added in blocking solution for 1 h at RT. Cells were washed again in PBS and mounted in Prolong Gold Antifade reagent with DAPI (4’,6-diamidino-2-phenylindole) (Invitrogen). Images were obtained using a Nikon C1 laser scanning confocal microscope (Nikon) equipped with three solid lasers; a Radius 405nm, Coherent 488 Sapphire laser and Coherent Compass 215 M-10/532nm. All image acquisitions were performed at RT. Alternatively, immunofluorescence was analysed using an Olympus microscope (model BX51) with UPlanApo objectives, fitted with a camera (model DP70; Olympus) and processed with Olysia Bioreport software (Olympus). Images were processed using Adobe Photoshop CS2 Version 9.0.2.
2.5. Protein analysis

2.5.1. Protein extraction

For direct immunoblotting: cells were resuspended in TRIzol® and protein extracted. After RNA extraction, protein was recovered from the remaining organic phase according to manufacturer’s instructions (Invitrogen).

2.5.2. SDS-PAGE and protein transfer

Resolving polyacrylamide gels of 8% were prepared with the following reagents: 8% acrylamide/bis, 3.75 mM Tris hydroxymethyl aminomethane (Tris) pH 8.8, 0.1% sodium dodecyl sulphate (SDS), 0.05% ammonium persulfate, 0.05% TEMED (Sigma). The resolving gels were cast with a MiniPROTEAN cast and stand (Bio-Rad), and overlaid with a stacking gel (8% acrylamide/bis, 1.25 mM Tris-HCl pH 6.8, 0.1% SDS, 0.05% ammonium persulfate, 0.1% TEMED) into which a comb of 10 wells was inserted. The assembled gel was placed in a Mini-PROTEAN Tetra cell tank (Bio-Rad) and submerged in protein electrophoresis buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3). Samples were prepared for electrophoresis making up to a volume of 30 μl with 1% SDS and adding 10 μl 4x protein loading buffer (PLB) (PLB - 100 mM Tris-HCl pH 6.8, 200 mM dithiothreitol (DTT), 4% SDS, 0.2% bromophenol blue, 20% glycerol). 5 μl Kaleidoscope marker (Bio-Rad) and prepared samples were loaded into the wells of the protein gel and run at 200 V.

When the dye front reached the bottom of the gel, separated proteins were transferred to a polyvinylidene difluoride (PVDF) (Perkin Elmer) membrane using a Criterion Blotter wet transfer apparatus (Bio-Rad). Prior to transfer, the protein gel was soaked in protein transfer buffer (50 mM Tris, 380 mM glycine) for 15 min. PVDF membrane was soaked in Methanol for 5 min, rinsed 3 times in MQ H₂O and soaked in protein transfer buffer for 15 min. The transfer apparatus was assembled as follows: two sheets of whatman filter paper presoaked
in protein transfer buffer were placed on the cathode (+) of the apparatus. The gel was placed on top of the whatman and a same-sized piece of PVDF in transfer buffer was placed on top of the gel, followed by and another two pieces of whatman. Proteins were transferred to the membrane for 2 h at 300 mAmms.

2.5.3. Immunoblotting

PVDF membranes were blocked in 5% skim milk powder in 0.1% Tween20/PBS (PBS-Tw) for 1-2 h and incubated with primary antibodies diluted in 5% skim milk powder in PBS-Tw overnight at 4°C (see 2.5.4 for antibody details and dilutions). After washing in PBS-Tw, the membrane was incubated with anti-rabbit, anti-rat and/or anti-goat Alkaline Phosphatase (AP) conjugated antibodies (GE Healthcare) and anti-mouse Cy5 (GE Healthcare) diluted in 5% skim milk powder in PBS-Tw for 1 h at RT (see 2.5.4 for antibody details and dilutions). After washing in PBS-Tw, detection of bound AP was achieved using ECF Western Blot substrate (GE Healthcare) and blots were directly scanned on a Typhoon imager (GE Healthcare). Densitometry was performed with ImageQuant TL software (GE Healthcare).

2.5.4. Other antibodies

Primary antibodies used for immunoblotting, Immunostaining and magnetic sorting are below.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Species raised in</th>
<th>Clone and Supplier</th>
<th>Dilution for immuno-blot</th>
<th>Dilution for immuno-staining</th>
<th>Dilution for magnetic sorting</th>
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The following antibodies were used for secondary detection at 1:1000: Alexa Fluor® 488, 555 or 647-conjugated donkey anti-rabbit IgG1, Alexa Fluor® 488 or 555-conjugated goat anti-rabbit IgG1, Alexa Fluor® 488 or 555-conjugated donkey anti-rat IgG1, Alexa Fluor®
488 or 555-conjugated goat anti-rat IgG1, Alexa Fluor® 546-conjugated goat anti-hamster IgG1, Alexa Fluor® 488, 555 or 647-conjugated donkey anti-goat IgG1 (all from Molecular Probes).

The following antibodies were used for secondary detection for immunoblots: alkaline phosphotase-conjugated goat anti-rat IgG (1:1000, Invitrogen), alkaline phosphotase-conjugated rabbit anti-goat IgG (1:1000, Invitrogen), alkaline phosphotase-conjugated goat anti-rabbit IgG (1:5000, GE Healthcare), Cy5-conjugated goat anti-mouse IgG (1:2000, GE Healthcare).

Direct conjugation of rabbit antibodies to Alexa Fluor® 555 was performed using the Zenon Rabbit IgG Labelling Kit (Molecular Probes) according to manufacturer’s instructions.

2.6. Mouse manipulation

2.6.1. Mouse Experiments

Experiments were performed in compliance with the ethical guidelines of the National Health and Medical Research Council of Australia or with the “Guide for the Care and Use of Laboratory Animals” prepared by the Institute of Laboratory Animal Resources, National Research Council, and published by the National Academy Press. Experiments were approved and conducted in accordance with the SA Pathology Animal Ethics Committee, The University of Adelaide Animal Ethics Committee, Walter and Eliza Hall Institute (WEHI) Animal Ethics Committee and University of Cincinnati Institutional Animal Care and Use Committee regulations. LysMCre mice were generated by Dr I Forster (University of Cologne, Germany) (Clausen et al. 1999) and obtained from Dr Andrew Roberts (WEHI, Melbourne). ROSA26R Cre reporter mice were generated by Professor Philippe Soriano (Fred Hutchinson Cancer Research Centre, USA) (Soriano 1999) and obtained from
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Professor Richard Harvey (The Victor Chang Cardiac Research Institute, Sydney). 

LysMCre\textsuperscript{+/−};Rosa26R\textsuperscript{+/−} mice were generated by breeding LysMCre\textsuperscript{−/−} with Rosa26R\textsuperscript{−/−} mice and progeny were genotyped according to established protocols (Clausen et al. 1999; Soriano 1999). Female C57BL/6 mice and c-Kit mutant genetically mast cell–deficient WBB6F1-Kit\textsuperscript{W/W−} (Kit\textsuperscript{W/W−}) mice and the congenic normal WBB6F1-Kit\textsuperscript{+/−} (Kit\textsuperscript{+/−}) mice were obtained from The Jackson Laboratory. The following embryos were obtained from collaborators: PU.1\textsuperscript{−/−} mice (Dakic et al. 2005) and Rag2\textsuperscript{−/−} mice were generated as described previously (Shinkai et al. 1992). Wnt7b\textsuperscript{Δt} (Lobov et al. 2005), Wnt7b\textsuperscript{floX} (Rajagopal et al. 2008), Ang2\textsuperscript{bacZ} (Gale et al. 2002) c-fmsEGFP (Sasmono et al. 2003), Z/EG (Novak et al. 2000), Csf1r-ICre (Deng et al. 2010) and Csf1r\textsuperscript{−/−} (Li et al. 2006) mice have been previously described.

2.6.2. Genotyping of LysMCre and Rosa26R mice

2.6.2.1. Isolation of DNA from tissue

Small portions of tails from mice used in experiments were removed. DNA was isolated from tails of newborn mouse pups or embryos for genotyping as follows. Tails were incubated in Genomic DNA digestion buffer (20 mM Tris pH 8.0, 5 mM EDTA, 400 mM NaCl, 1% SDS) (700 μl for pups, 500 μl for embryos) with 0.5 mg/ml Proteinase K (Roche) at 55°C overnight with agitation. Solutions were placed at 37°C for 1 h prior to phenol/chloroform extraction. 600 μl phenol/chloroform (1:1) was added to the tube and vortexed thoroughly for 15 sec. The aqueous and solvent layers were separated by centrifugation at 9300xg for 10 min. The upper aqueous phase was transferred to a new tube and DNA precipitated with 1 volume of isopropanol. DNA was pelleted by centrifugation at 15700xg for 10 min and the pellet washed in 70% ethanol. The pellet was air dried and resuspended in sterile MQ H\textsubscript{2}O (100 μl for pups, 40 μl for embryos).

2.6.2.2. DNA quantification

DNA concentration was measured using a NanoDrop1000 spectrophotometer (Thermo Scientific), assuming that an OD of 1.0 correlates to 50 μg/ml of DNA.
2.6.2.3. **Polymerase Chain Reaction (PCR)**

DNA was synthesised using 1 μl cDNA product, 50 units of Taq polymerase (New England Bio Labs), 20 μM forward and reverse primers, 0.2 mM dNTPs in 1x Taq PCR buffer (New England Bio Labs) in a total volume of 20 μl sterile MQ H₂O. Reactions were performed on a Mastercycler (Eppendorf), with the following conditions: 2 min at 95°C, followed by 30 cycles of 95°C for 30 sec, 1 min at 67°C (for Rosa26R genotyping) or 1 min at 63°C (for LysMCre genotyping) and 72°C for 1 min/kb, followed by a final 2 min extension at 72°C. All reactions were held at 4°C.

2.6.2.4. **Electrophoresis of DNA**

DNA was analysed by electrophoresis in 1% agarose gels in 0.5x TBE (Tris boric acid) buffer (44.5 mM Tris, 44.5 mM boric acid, 1 mM EDTA). Prior to electrophoresis, 10x DNA loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol) was added to a final concentration of 1x to samples, then loaded onto gels immersed in TBE buffer. Electrophoresis was performed at 100 V for 40 min. DNA was stained with ethidium bromide solution (2 μg/ml) and visualised under ultraviolet light on a transilluminator at 254 nm (BTS-20.M, UVItec).

2.6.3. **EL4 and LLC Solid Tumour Model**

EL4 lymphoma or LLC carcinoma cells (obtained from C. Bonder) were grown in RPMI (Gibco) with 10% FCS, 2 mM glutamine and 100 μg/ml pen/strep. Cells were grown until nearly confluent, washed 3 times in PBS and resuspended at 10⁶ cells per 100μl. LysMCre⁺/₋;Rosa26R⁺/₋ compound heterozygous mice were anaesthetised with isofluorane/O₂, and 10⁶ tumour cells were injected subcutaneously on one flank. Mice were observed daily for approximately 1-2 weeks and bi-dimensional measurements using callipers were taken of tumours every two days until 1 cm diameter subcutaneous tumours formed. At completion, mice were humanely killed and tumours and the associated dermis were dissected prepared for cryosectioning as in 2.6.5.
2.6.4. Chronic UVB Irradiation Model

2.6.4.1. Preparation and adoptive transfer of BMCMCs into mast cell-deficient mice

As previously described (Grimbaldeston et al. 2007) bone marrow cultured mast cells (BMCMCs) were obtained by culturing bone marrow cells from femurs and tibias of female mice in DMEM with 20% (v/v) medium conditioned by the growth of the WEHI-3 mouse myelomonocytic cell line (containing interleukin-3 to a final concentration of 3 ng/ml) for 4–6 weeks, at which time >95% of the cells were identified as mast cells by May Grunwald-Giemsa staining (Sigma) and by flow cytometric analysis (c-Kit+ FcεRI+). For mast cell engraftment studies, BMCMCs derived from WT C57BL/6 (WT BMCMCs) or C57BL/6-Mcpt4−/− (Mcpt4−/− BMCMCs) mice were transferred by intradermal injection (2 injections into each ear of 10⁶ cells in 25 μl DMEM/each injection) into 4–6 week old KitW/Wv mice. Ultraviolet B (UVB) experiments were initiated 6 weeks after intradermal injection transfer of BMCMCs. All experiments were performed by Boris Fedoric and Michele Grimbaldeston.

2.6.4.2. UV Irradiation

Mice were irradiated using, a bank of 6 Philips FS40T12 lamps (Ultraviolet Resources International), emitting a broad 270-380 nm band of UVB, with peak emission at 310 nm. The peak emission comprised approximately 65% of the energy emitted. The intensity and spectral output of the UVB lamps were measured using a UVX spectrophotometer with a UVX-31 sensor (UV products). A new sheet of clear PVC plastic film (0.23 mm thick) was taped to the top of each perspex cage prior to irradiation to screen wavelengths less than 290 nm. The lamps were held 15 cm above the cages. Fourteen week old mice were placed in individual compartments of perspex cages and were conscious and had full range of movement during irradiation.

For protective exposures followed by high dose chronic exposures, the entire wild-type, KitW/Wv or WT BMCMC→KitW/Wv mouse (including both ears) was irradiated first with a UVB
dose of 1.2 kJ/m² for 4 exposures, followed with 2 kJ/m² for 6 exposures, followed with 4 kJ/m² for 12 exposures, followed with 8kJ/m² for 22 exposures (i.e. equivalent to 0.5, 1, 2 and 4 erythema dosage as previously determined (Grimbaldeston et al. 2007)) every 2 days for a total of 44 exposures (total cumulative dose = 240.5 kJ/m² UVB). For high dose chronic exposures, the entire wild-type, Kit<sup>W/Wv</sup> or WT BMCMC→ Kit<sup>W/Wv</sup> mouse (including both ears) was irradiated first with a UVB dose of 4 kJ/m² for 12 exposures, followed with 8kJ/m² for 30 exposures (i.e. 2 and 4 erythema dosage as previously determined (Grimbaldeston et al. 2007)), every 2 days for a total of 42 exposures (total cumulative dose = 288 kJ/m² UVB). For low dose chronic exposures, the entire wild-type, Kit<sup>W/Wv</sup>, WT BMCMC→ Kit<sup>W/Wv</sup> or Mcpt4<sup>-/-</sup> BMCMC→ Kit<sup>W/Wv</sup> mouse (including both ears) was irradiated with a UVB dose of 4 kJ/m² for 12 exposures (i.e. equivalent to 2 erythema dose as previously determined (Grimbaldeston et al. 2007)), every 2 days for a total of 12 exposures (total cumulative dose = 48 kJ/m² UVB). Ear thicknesses were measured using a micrometer (Ozaki MFG. CO., LTD., Itabashi, Tokyo, Japan) before each UVB irradiation. Mice were killed by CO₂ inhalation 24 h after the final UVB exposure and samples of ear were taken for analysis. All experiments were performed by Boris Fedoric and Michele Grimbaldeston.

### 2.6.5. Preparation of tissue for sectioning

Embryos or adult tissue were dissected and fixed in 4% PFA/PBS overnight at 4°C. Following fixation, tissues were washed extensively in PBS and tissues used for whole mount immunostaining were stored in PBS containing 0.01% thimerosal at 4°C. Embryos or tissues to be frozen were cryoprotected in 30% sucrose/PBS overnight at 4°C with gentle agitation, then frozen in TissueTek® O.C.T. compound (Sakura Finetek), prior to cryosectioning 10 μm or 60 μm transverse sections onto Polysine glass slides (Thermo Scientific) using a cryostat (Leica CM1950). Tumours and UVB irradiated ears were cut in half vertically and both halves were embedded in O.C.T. with the cut side facing the bottom
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of the block. This enabled a cross-sectional orientation of both halves of the ear from the base to the tip in a single section, or of the tumour through the centre.

2.6.6. X-gal staining

Embryos or tissues were dissected from mice, rinsed 3 times for 5 min in ice cold PBS and fixed for 1 h in 4% PFA/PBS on ice. Following fixation, tissues were washed with PBS and incubated in permeabilising solution (2 mM MgCl₂, 0.01% sodium deoxycholate, 0.02% Igepal) for 15 min on ice. After aspiration, fresh permeabilising solution was added for a further 15 min at RT. Permeabilising solution was removed and tissues were incubated in staining solution (2.5 nM 5-bromo-4-chloro-3-indolylo-D-galactosidase (X-gal), 5 mM K₃Fe(CN)₆, 5 mM K₄Fe(CN)₆, 2 mM MgCl₂, 2% Igepal in PBS) overnight at 30°C. After several washes with PBS, tissues were fixed overnight in 4% PFA/PBS at 4°C. Tissues were then washed extensively with PBS and analysed using an Olympus dissecting microscope (SZX7) and photographed using an Olympus DP20-5E digital camera, with associated software. Image acquisition was performed at RT. Alternatively, tissues were processed for cryosectioning (see 2.6.5).

2.6.7. Immunostaining on whole tissue (colour)

Tissues were blocked with 0.5% BSA and 0.1% Triton X-100 in PBS overnight at 4°C, followed by incubation with primary antibody overnight at 4°C. After several washes with 0.1% Triton X-100/PBS (PBS-T), samples were incubated overnight at 4°C with biotin-conjugated goat anti-rabbit or goat anti-rat IgG (Vector Laboratories, CA). After thorough washing in PBS-T, samples were incubated overnight with avidin:biotin:peroxidase complex (Vector Elite, Vector Laboratories), washed extensively with PBS-T and then developed with a 3,3-diaminobenzidine (DAB) substrate kit (Vector Laboratories), according to the manufacturer's instructions. Immunostaining was analysed using an Olympus dissecting microscope (SZX7) and photographed using an Olympus DP20-5E digital camera, with associated software. Image acquisition was performed at RT.
2.6.8. Toluidine Blue staining

For visualisation of mast cells, 10 μm sections of ears cut in a cross sectional orientation were rinsed in H₂O for 5 min and then flooded with 0.1% Toluidine Blue (Sigma) in 1 normal hydrochloric acid (1N HCl) pH 1.0, for 1 min. Slides were washed with H₂O to remove excess Toluidine Blue, air dried, rinsed in xylene and mounted with DePeX mounting medium (containing dibutyl phthalate) (BDH Chemicals).

2.6.9. Immunostaining on whole tissue (fluorescence)

Tissues were blocked overnight at 4°C in blocking solution (5% normal goat serum [Jackson ImmunoResearch], 0.2% BSA, 0.3% Triton X-100, 0.01% thimerosal in PBS) followed by overnight incubation at 4°C with one or more primary antibodies, diluted in blocking solution (see 2.5.4 for antibody details and dilutions). If goat antibodies were used, blocking solution comprised of 1% BSA, 0.3% Triton X-100, 0.01% thimerosal in PBS. Tissues were washed extensively in PBS-T and incubated overnight at 4°C in respective secondary antibodies diluted in PBS-T and/or with directly conjugated primary antibody (see 2.5.4 for antibody details and dilutions). The following day, tissues were washed extensively in PBS-T and mounted in Prolong Gold Antifade reagent (Molecular Probes). Images were obtained using a confocal microscope (Radiance 2100; Bio-Rad Laboratories) equipped with three lasers, an Argon ion 488 nm, a Green HeNe 543 nm and a Red Diode 637 nm and an inverted microscope (IX70; Olympus) with UApo objectives. All image acquisitions were performed at RT. Images were processed using Adobe Photoshop CS2, Version 9.0.2.

2.6.10. Immunostaining on frozen sections (fluorescence)

Sections were washed in Tris-buffered saline (200 mM Tris pH7.5, 300 mM NaCl, 0.1% Tween 20) (TBS-T) and incubated in blocking buffer (60% maleate buffer [100 mM maleic acid, 150 mM NaCl, pH 7.4], 20% normal goat serum, 2% Blocking Reagent [Roche]) for 1 h at RT. Primary antibody was added overnight in blocking buffer (see 2.5.4 for antibody details and dilutions). After several washes in TBS-T, sections were incubated with
respective secondary antibodies diluted in blocking buffer for 3 h at RT, or with directly conjugated primary antibody (see 2.5.4 for antibody details and dilutions). After several washes in TBS-T and a rinse in MQ H₂O, sections were mounted in Prolong Gold Antifade reagent (Molecular Probes). Images were obtained using a confocal microscope, as in 2.6.9. Alternatively, immunofluorescence was analysed using an Olympus microscope (model BX51) with UPlanApo objectives, fitted with a camera (model DP70; Olympus) and processed with Olysia Bioreport software (Olympus).

2.7. Image Analysis

Quantification of vasculature was performed on immunostained whole tissue or sections using Lymphatic Vessel Analysis Protocol (LVAP) (Shayan et al. 2007) and NIH ImageJ software (Abramoff MD 2004).

2.7.1. Quantification of macrophage identity around lymphatic vasculature

For analysis of embryonic sections, four images of 60 μm sections (599.04 x 599.04 μm) per embryo, from three embryos, were quantified for expression of lymphatic and macrophage markers. 2 images were taken from the jugular lymph sac region and 2 images were taken from the dermal lymphatic vessel region for each embryo. For whole mount dermal skin analyses, four images (599.04 x 599.04 μm) per embryo, of three independent embryos, were quantified for expression of lymphatic and macrophage markers. Images were taken in the dorsal skin of the embryo.

2.7.2. Quantification of lymphatic vessels in the dermis

For whole mount dermal skin analyses, lymphatic vessel width was measured from three images (599.04 x 599.04 μm or 1198.08 x 1198.08 μm) per embryo, from at least three embryos per genotype, to determine the average vessel width. For each image, vessel width was measured at least once between each branch point. For proliferation analyses,
the number of proliferating cells was quantified from 6 images (599.04 x 599.04 μm) per embryo, from four embryos per genotype, to determine the number of proliferating cells/image. For branch point analyses, the number of branches was quantified from 3 images (1198.08 x 1198.08 μm) per embryo, from at least three embryos per genotype, to determine the number of branch points/image. All images were taken in the dorsal skin of the embryo.

2.7.3. Quantification of lymphatic vessels in the pleural cavity and submucosa

For pleural cavity and submucosa analyses, lymphatic vessel width and branch points were measured from two images (506 x 473 pixels) per embryo, from three embryos per genotype, to determine the average vessel width in pixels and number of branch points/image. For each image, vessel width was measured at least once between each branch point.

2.7.4. Quantification of jugular lymph sac size

The area of the jugular lymph sac region was quantified from three images of 10 μm sections (329 x 437 μm) per embryo, from three embryos per genotype. Analysed sections were taken in the anterior lymph sac region (L1), the mid lymph sac region (L2) and the posterior lymph sac region (L3).

2.7.5. Quantification of lymphatic vessels in whole mount ear skin

For whole mount ear skin analysis, lymphatic vessel width and branch points were measured from three images (1198.08 x 1198.08 μm) per embryo, from at least two embryos per genotype, to determine the average vessel width in pixels and number of branch points/image. For each image, vessel width was measured at least once between each branch point. Images were taken of three independent areas in the periphery of the ear.
2.7.6. Quantification of the vasculature in UVB irradiated ears

For analysis of UVB irradiated ears, vessel width, the number of vessels, and the area of the image occupied by the ear was analysed. At least six images (1198.08 x 1198.08 μm) per ear, from the listed number of ears per genotype, were quantified to determine the average vessel width and the number of vessels/image. To determine the vessel density, the number of vessels/image was divided by the total area occupied by the ear/image. Images from each ear spanned from the base to the tip of the ear pinnae. For each image, vessel width was measured perpendicular to the epidermis.

2.8. Statistical Analysis

To determine significance, a two-tailed t-Test (Two-Sample Assuming Equal Variances) was conducted using Microsoft Excel. A p value of <0.05 was considered statistically significant. For microarray analysis, differential gene expression was assessed by ANOVA with the p value adjusted using step-up multiple test correction (Benjamini 1995) to control the false discovery rate. Adjusted p values < 0.05 were considered to be significant. For ear swelling response, the interaction between each group was evaluated using one way ANOVA repeated measures with Tukey’s post test. A p value of < 0.05 was considered to be significant. Ear swelling statistical analysis was performed by Boris Fedoric.
3. Characterising the expression of hyaluronan receptor LYVE-1 during embryonic vascular development.
3.1. **Introduction**

Hyaluronic acid (HA) is a glycosaminoglycan that is a critical component of extracellular matrix within all tissues, which depending on its chain length and interaction with binding proteins and receptors, fulfils a range of functions from danger signal to adhesive substratum (Jackson 2009). LYVE-1 is a transmembrane receptor that was identified on the basis of shared structural and functional similarity with the HA receptor CD44 (Banerji *et al.* 1999; Prevo *et al.* 2001). As with CD44, LYVE-1 was shown to bind both soluble and immobilized HA, yet unlike CD44, which is found on leukocytes that traffic through lymphatic vessels, LYVE-1 co-localises with HA on the luminal face of the lymph vessel wall (Banerji *et al.* 1999). Initial functional studies demonstrated that LYVE-1 is able to act as an endocytic receptor for HA, suggesting that LYVE-1 is likely to be important for facilitating HA transport from tissues, via the lymph, to lymph nodes where it is subsequently degraded (Prevo *et al.* 2001).

Initial investigations in mouse and human tissues documented that the expression of LYVE-1 was restricted to lymphatic vascular endothelium and to the endothelial sinusoids of lymph nodes, liver and spleen (Banerji *et al.* 1999; Mouta Carreira *et al.* 2001; Prevo *et al.* 2001; Nonaka *et al.* 2007). Previous investigations of early embryonic lymphatic vascular development documented that LYVE-1 was expressed in endothelial cells of the embryonic cardinal veins at E9.5-10.5, prior to the induction of Prox1 expression in a polarised population of venous endothelial cells (Wigle *et al.* 2002). This observation led to the proposal that LYVE-1 expression in the embryonic cardinal veins signifies competence of the venous endothelium to receive an inductive signal that results in lymphatic endothelial cell fate specification (Wigle *et al.* 2002). In adults, LYVE-1 expression on collecting vessels decreases while remaining high on lymphatic capillaries (Makinen *et al.* 2005). As a result of these studies, LYVE-1 is currently one of the markers most often utilised to identify lymphatic endothelium during development and in inflammatory-stimulated
lymphangiogenesis. In addition to being expressed on lymphatic vessels, recent studies have reported that LYVE-1 is also expressed outside the endothelial cell compartment on a sub-population of macrophages, a pool of cells that has been proposed to contribute to inflammation-stimulated lymphangiogenesis (Cursiefen et al. 2004; Maruyama et al. 2005; Schledzewski et al. 2006; Maruyama et al. 2007).

A specific role for LYVE-1 in lymphatic vascular development is yet to be determined, as LYVE-1−/− mice have an apparently normal phenotype, with no defects in lymphatic vessel structure or trafficking, or secondary lymphoid tissue structure or cellularity (Gale et al. 2007). While mice deficient in CSRPB-1, which is identical to LYVE-1, display morphological and functional differences in lymphatic capillaries in some tissues, with lymphatic vessels being in a constantly open, contracted state (Huang et al. 2006), these mice remain grossly normal, suggesting compensatory factors for the function of this protein. To examine whether CD44 was able to compensate for the loss of LYVE-1, LYVE-1−/−CD44−/− mice were analysed and found to be indistinguishable from wild-type mice under normal conditions (Luong et al. 2009). However, while resolution of carrageenan injection-induced inflammation, which is accompanied by leukocyte infiltration and edema, was comparable to wild-type mice, LYVE-1−/−CD44−/− mice exhibited increased edema formation (Luong et al. 2009), not ruling out a role for LYVE-1 in controlling inflammation. This is supported by the fact that pro-inflammatory cytokines can down-regulate LYVE-1 expression via internalisation and degradation of the receptor in lysosomes and by switching off gene expression (Johnson et al. 2007).

Work in our laboratory discovered that LYVE-1 is expressed on blood vessels in the embryonic yolk sac, raising the question as to whether LYVE-1 expression was truly restricted to lymphatic vessels during embryogenesis. As LYVE-1 is often utilised as a marker specific for the lymphatic vasculature during development and inflammation, it is
critical to document the normal pattern of expression and identity of LYVE-1-positive endothelium. The aim of this chapter was to document the expression of LYVE-1 in the embryonic blood and lymphatic vasculatures during development.

3.2. LYVE-1 is expressed on blood vessels of the embryonic yolk sac.

In the course of studying yolk sac vascular development, we made the observation that LYVE-1 was expressed extensively throughout the yolk sac vasculature (Fig 3.1). This study therefore set out to characterise the identity of these vessels. LYVE-1 expression was evident on the yolk sac vasculature at all stages investigated between embryonic day (E) 9.5 and E18.5 (Fig 3.1 – Fig 3.4), as well as on yolk sac macrophages from E11.5 onwards (Fig 3.1, arrowheads and inset panels). All LYVE-1-positive vessels in the yolk sac vasculature co-expressed the pan-endothelial marker CD31 (Fig 3.1), confirming that LYVE-1-positive structures were endothelial in nature. In order to determine whether LYVE-1-positive vessels were blood or lymphatic in nature, yolk sacs were co-stained with a variety of established markers of lymphatic and blood vascular endothelial cells. Analysis of the expression of definitive markers of lymphatic endothelium such as Prox1 (Wigle and Oliver 1999) and the membrane glycoprotein, podoplanin (Wetterwald et al. 1996; Breiteneder-Geleff et al. 1999), revealed that the none of the yolk sac vessels were positive for either lymphatic marker at any stage of yolk sac vascular development analysed, from E9.5 to E18.5 (Fig 3.2). These results indicated that LYVE-1-positive vessels were not lymphatic in nature. Podoplanin expression was however, observed in the yolk sac endoderm (Fig 3.2, inset panels). While podoplanin expression is restricted to lymphatic endothelial cells and not blood endothelial cells, it is also expressed on a wide variety of epithelial cell types and on tissues derived from embryonic ectoderm and endoderm (Wetterwald et al. 1996; Williams et al. 1996).
Whole mount immunostaining and confocal microscopy of mouse yolk sacs during development. LYVE-1 is expressed uniformly on the yolk sac capillary plexus at E9.5, with the endothelial nature of this staining confirmed by co-staining with CD31. As remodelling of the capillary plexus into mature arteries, veins and capillaries is initiated at E11.5, LYVE-1 expression on a subset of larger vessels is decreased (white arrows). LYVE-1 expression is retained on some large vessels throughout embryogenesis (open arrows). LYVE-1 expression is also obvious on yolk sac macrophages from E11.5 onwards (arrowheads, inset panels at higher magnification). Scale bars: 100 μm.
Figure 3.2. LYVE-1-positive vessels in the yolk sac are not lymphatic in nature.

Whole mount immunostaining and confocal microscopy of mouse yolk sacs during development. LYVE-1-positive vessels in the mouse yolk sac do not express definitive markers of the lymphatic endothelium such as Prox1 or podoplanin at any stage of development (E9.5-E18.5). Thus, these vessels are not lymphatic in nature. Podoplanin expression is observed in the yolk sac endoderm (red cell surface staining, inset panels). Scale bars: 100 μm.
Figure 3.3. VEGFR-3 expression is dynamic during the development and remodelling of the yolk sac vascular plexus.

Whole mount immunostaining and confocal microscopy of mouse yolk sacs during development. LYVE-1-positive capillaries co-express VEGFR-3, consistent with previous reports of VEGFR-3 expression on embryonic blood vessels and angiogenic capillaries. As maturation of the yolk sac capillary plexus proceeds, VEGFR-3 expression, like LYVE-1 expression, is decreased on large vessels. VEGFR-3 expression is also diminished on the yolk sac capillaries as development proceeds, and by E18.5 is largely absent, except on some sprouting blood vessels (white arrow). Scale bars: 100 μm.
Figure 3.4. LYVE-1 expression is reduced on arteries during yolk sac vascular remodelling.

Whole mount immunostaining and confocal microscopy of mouse yolk sacs during development. LYVE-1-negative vessels were confirmed as arterial in nature by their expression of Nrp1 (white arrows, LYVE-1/Nrp1 panels), together with their recruitment of α smooth muscle α-actin-positive, perivascular smooth muscle cells (white arrows, LYVE-1/SMA, CD31/SMA panels). LYVE-1 expression was retained on large veins throughout embryogenesis, as determined by lack of Nrp1 expression and loose smooth muscle coverage (open arrows). Scale bars: 100 μm.
LYVE-1-positive capillaries at early stages of embryonic development were also positive for VEGFR-3 (Fig 3.3), although VEGFR-3 levels progressively decreased on the large vessels of the yolk sac, followed by the capillary plexus, as development proceeded (Fig 3.3). Within the embryo, VEGFR-3 expression has been documented on blood vessels at early stages of embryogenesis, but becomes progressively restricted to lymphatic vessels as development proceeds (Kaipainen et al. 1995). Our data is consistent with this mode of VEGFR-3 down-regulation on blood vessels. By E18.5, VEGFR-3 expression was only visible on the angiogenic sprouts of the yolk sac blood vasculature (Fig 3.3, arrow), findings that are in accordance with those recently published by Tammela and colleagues (Tammela et al. 2008), who found VEGFR-3 is highly expressed in angiogenic sprouts in the embryonic inter-somitic vessels and postnatal retina. Blockade of VEGFR-3 signalling resulted in decreased sprouting, vascular density, vessel branching and endothelial cell proliferation. Taken together, these results demonstrate that the mouse yolk sac is devoid of lymphatic vessels, unlike the avian chorioallantoic membrane (CAM) which contains lymphatic vessels characterised by expression of VEGFR-2 and VEGFR-3, and that possess the ability to proliferate in response to VEGF-C (Wilting et al. 2000). In addition, these data provide the first evidence that LYVE-1 is expressed widely in the blood vascular compartment of the embryonic mouse yolk sac throughout embryonic development.

3.3. LYVE-1 expression is decreased on arteries during yolk sac vascular remodelling.

While investigating LYVE-1 expression in the yolk sac vasculature, it was observed that from E11.5 onwards, in conjunction with angiogenic remodelling of the capillary plexus, LYVE-1 expression was decreased on mature vessels morphologically resembling arteries (Fig 3.1, white arrows). These vessels retained CD31 expression. In order to define the identity of LYVE-1-negative vessels, co-staining with markers of arterial and venous endothelium was performed. Neuropilin-1 (Nrp1) has previously been demonstrated to be
expressed preferentially in arteries rather than veins (Herzog et al. 2001; Moyon et al. 2001). Smooth muscle α-actin (SMA), a marker of smooth muscle cells which are recruited to provide pericellular support to vascular endothelium, was also investigated. While vascular smooth muscle cells are recruited to both arteries and veins, arteries display greater smooth muscle cell investment. LYVE-1-negative vessels were confirmed to be arteries by virtue of their expression of Nrp1 (Fig 3.4, white arrows, Fig 3.5) and their recruitment of tightly associated smooth muscle cells (Fig 3.4, white arrows, Fig 3.5). LYVE-1 expression was maintained on large veins at E16.5 and E18.5 (Fig 3.4, open arrows, Fig 3.5), which displayed looser smooth muscle cell recruitment. LYVE-1 first became down-regulated on arteries as smooth muscle cell recruitment commenced (Fig 3.4), as has been described for large collecting lymphatic vessels (Makinen et al. 2005). This suggests smooth muscle cell association triggers down-regulation of LYVE-1 on endothelial cells, or that LYVE-1 down-regulation precedes smooth muscle cell recruitment. However, given smooth muscle cell recruitment is normal in LYVE-1−/− mice (Gale et al. 2007), the former is more likely. Whether this occurs at a genetic level, or via LYVE-1 internalisation, remains to be elucidated. Taken together, these data illustrate that LYVE-1 expression in the mouse yolk sac vasculature is dynamic; at E9.5, LYVE-1 is expressed ubiquitously throughout the yolk sac capillary plexus, then as the yolk sac vasculature is remodelled, LYVE-1 expression is retained on large veins and capillaries but is progressively decreased on arteries until none remains on arterial endothelial cells by E16.5.

3.4. LYVE-1 is expressed on intra-embryonic blood vessels during early embryogenesis.

Given the prominent expression of LYVE-1 in the yolk sac blood vasculature, LYVE-1 expression on the intra-embryonic blood vasculature at early stages of embryonic development was investigated. Whole mount immunostaining of E9.5 - E11.5 embryos revealed prominent LYVE-1 expression on the inter-somitic vessels of E9.5 and E10.5
Figure 3.5. LYVE-1 expression is maintained on veins and down-regulated on arteries during remodelling of the yolk sac capillary plexus.

Single channel panels at E11.5 and E16.5 of images from Fig 3.1 and 3.4 clearly demonstrate the expression of arterial marker Nrp1 and tight recruitment of smooth muscle α-actin positive smooth muscle cells to LYVE-1-negative vessels during yolk sac vascular development. LYVE-1-positive vessels were confirmed as arterial in nature by CD31 expression and loose smooth muscle cell association. Dashed lines denote position of arteries at E16.5 in LYVE-1 panels. Scale bars: 100 μm.
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embryos, as well as in the heart and umbilical vessels (Fig 3.6). LYVE-1 expression was also evident on macrophages present in the dermis from E10.5 onwards. Interestingly, LYVE-1 expression on the intra-embryonic blood vasculature was transient, as by E11.5, LYVE-1 expression on inter-somitic vessels was no longer obvious by whole mount immunostaining (Fig 3.6). Staining of stage matched embryos with CD31 further suggested the endothelial nature of the LYVE-1 staining (Fig 3.6). This is the first demonstration of LYVE-1 expression on inter-somitic blood vessels. While previous work has described LYVE-1 expression on endothelial cells of the cardinal veins at E10.5 as Prox1-positive lymphatic endothelial cells bud away (Wigle et al. 2002), LYVE-1 expression has not been documented on any other intra-embryonic blood vascular endothelial cells.

In order to more precisely determine the identity of the blood vessels in which LYVE-1 was expressed at early stages of embryogenesis, immunostaining analyses of transverse sections of wild-type E9.5 and E10.5 embryos were performed. Arterial and venous endothelial cell identity was confirmed by the expression of Neuropilin-1 and Neuropilin-2 (Nrp2), respectively. Nrp1 is preferentially expressed in arteries, while Nrp2 is expressed preferentially in veins (Herzog et al. 2001). Experiments confirmed that LYVE-1 was prominently expressed on inter-somitic blood vessels at E9.5 and E10.5 (Fig 3.7), particularly on inter-somitic veins as observed by co-staining with Nrp2 (Fig 3.7E) but not Nrp1 (Fig 3.7C, G).

In addition to LYVE-1 expression on inter-somitic veins, LYVE-1 was also observed in select endothelial cells of the cardinal veins at E9.5 and E10.5 (Fig 3.8B, C, E, F), prior to the initiation of Prox1 expression (Fig 3.8A, D). Unexpectedly, LYVE-1 expression was observed in select cells of the dorsal aortae, as identified by CD31 (Fig 3.8B, C, 3.9B, C, E, F) or Nrp1 (Fig 3.9A, Fig 3.10B, E). Consistent with observations in the yolk sac vasculature, intra-embryonic arterial LYVE-1 expression was observed before significant
Figure 3.6. LYVE-1 is expressed on intra-embryonic blood vessels at early stages of embryogenesis.

Whole mount DAB immunostaining of embryos from E9.5 to E11.5. At E9.5 and E10.5, LYVE-1 expression is visible on the inter-somitic blood vessels (black arrowheads), umbilical vessels (dashed arrows), and endocardium (black arrows), in addition to the yolk sac vasculature. CD31 staining of stage matched embryos demonstrates the endothelial vasculature of the developing embryo. At E11.5, LYVE-1 expression was no longer obvious on inter-somitic vessels. LYVE-1 expression was visible in dermal macrophages (punctate staining) from E10.5 onwards.
Figure 3.7. LYVE-1 is expressed on inter-somitic veins during early embryogenesis.

Immunostaining of 10 μm transverse embryonic sections analysed by confocal microscopy. Prominent LYVE-1 expression was observed on inter-somitic blood vessels, particularly on inter-somitic veins. Arterial and venous endothelial cells were confirmed as such at a molecular level by the expression of Nrp1 (C, G) and Nrp2 (E), respectively. LYVE-1 positive endothelial cells were confirmed as endothelial in nature and not of monocyte/macrophage lineage, by virtue of the fact that they were not c-fmsEGFP-positive (C). Panels B, D, F and H are single channel LYVE-1 images of panels A, C, E and G respectively. Dashed line on embryo indicates region of section. Scale bars: 100 μm. NT, neural tube, V, inter-somitic veins, A, inter-somitic arteries. Immunostaining was performed by Natasha Harvey.
Figure 3.8. LYVE-1 is expressed on the cardinal veins and anterior dorsal aortae during early embryogenesis.

Immunostaining of 10 μm transverse embryonic sections analysed by confocal microscopy. LYVE-1-positive endothelial cells are obvious in the dorsal aortae (B, C, E, F, white arrows) and cardinal veins (B, C, E, F) of wild-type embryos at E9.5 and E10.5. Adjacent tissue sections were stained with Prox1 and CD31 (A, D) to illustrate lymphatic and blood vascular morphology. Prox1 expression is also visible in the neural tube (A). Arterial and venous endothelial cell identity in the dorsal aortae and cardinal veins were confirmed as such at a molecular level by the expression of CD31 and smooth muscle cell association. Panels C and F are single channel LYVE-1 images of panels B and E, respectively. Dashed line on embryo indicates region of section. Scale bars: 100 μm. DA, dorsal aorta, CV, cardinal vein, NT, neural tube. Immunostaining was performed by Natasha Harvey.
Figure 3.9. LYVE-1 is expressed on the posterior dorsal aortae during early embryogenesis.

Immunostaining of 10 μm transverse embryonic sections analysed by confocal microscopy. LYVE-1-positive endothelial cells are obvious in the posterior dorsal aortae (B, C, E, F, white arrows) of wild-type (B-F) and c-fmsEGFP embryos (A) at E9.5 and E10.5. Arterial endothelial cell identity in the dorsal aortae was confirmed as such at a molecular level by the expression of Nrp1, CD31 and smooth muscle cell association, respectively. Panels C and F are single channel LYVE-1 images of panels B and E, respectively. Dashed line on embryo indicates region of section. Scale bars: 100 μm. Immunostaining was performed by Natasha Harvey.
Figure 3.10. LYVE-1-positive macrophages do not integrate into the arterial or venous endothelium.

Immunostaining of 10 μm transverse embryonic sections analysed by confocal microscopy. LYVE-1-positive endothelial cells are obvious in the anterior dorsal aortae and cardinal veins (B, C, E, F, white arrows) of c-fmsEGFP embryos at E9.5 and E10.5. Arterial and venous endothelial cell identity in the dorsal aortae and cardinal veins were confirmed as such at a molecular level by the expression of Nrp1, Nrp2 and CD31, respectively. LYVE-1-positive endothelial cells were confirmed as endothelial in nature and not of monocyte/macrophage lineage, by virtue of the fact that they were not c-fmsEGFP-positive. Rare LYVE-1-positive, c-fmsEGFP-positive macrophages were observed (C, F, open arrow). Dashed lines indicate the positions of the dorsal aortae and cardinal veins. Dashed line on embryo indicates region of section. Scale bars: 100 μm. DA, dorsal aorta, CV, cardinal vein, NT, neural tube. Immunostaining of A, B, D, E was performed by Natasha Harvey.
smooth muscle cell recruitment to the dorsal aortae (Fig 3.8B, C, E, F, 3.9B, C, E, F); by E11.5 smooth muscle cells uniformly surround the dorsal aortae and arterial LYVE-1 expression is no longer obvious (Fig 3.11). LYVE-1 expression was, however, still observed on the cardinal veins at E11.5, as identified by Nrp2 staining, coupled with Prox1-positive lymphatic endothelial cells budding from the veins in a polarised manner (Fig 3.11).

3.5. LYVE-1-positive macrophages do not integrate into the arterial or venous endothelium.

Following the observation that LYVE-1 is expressed on a population of macrophages in the yolk sac, embryo and adult mouse (Maruyama et al. 2005; Schledzewski et al. 2006; Gordon et al. 2010) (Fig 3.1), it was next investigated whether the select LYVE-1-positive cells within the arterial or venous endothelium were of macrophage, rather than endothelial origin. To test this hypothesis, transgenic c-fmsEGFP mice that express EGFP under control of the macrophage colony-stimulating factor (CSF-1) receptor, c-fms, promoter were utilised (Sasmono et al. 2003). Immunostaining of E9.5 and E10.5 c-fmsEGFP embryos revealed that the LYVE-1-positive cells present in the blood vasculature were not GFP-positive and therefore were not monocyte/macrophage derived, but were endothelial as confirmed by co-expression of Nrp1 (Fig 3.9A, 3.10B, E, white arrows) or Nrp2 (Fig 3.10C, F, white arrows). While very few GFP-positive macrophages were observed at these early developmental stages, some GFP-positive macrophages did express LYVE-1 (Fig 3.10C, F, open arrow) yet were not found within the blood vessel walls. Some macrophages appeared to be in circulation within the dorsal aortae and cardinal veins, although as these cells did not express endothelial markers, they were not endothelial in nature (Fig 3.10A). In summary, these results confirm that LYVE-1 is expressed on select endothelial cells within the cardinal veins and dorsal aortae in early-stage mouse embryos.
Figure 3.11. LYVE-1 becomes restricted to the cardinal veins and lymphatic vessels by E11.5.

Immunostaining of 10 µm transverse embryonic sections analysed by confocal microscopy. By E11.5, LYVE-1 was not apparent in the dorsal aortae, but was expressed around the cardinal veins and in budding lymphatic endothelial cells (A, B). Adjacent tissue sections were stained with Prox1 (C) to illustrate lymphatic morphology. Arterial and venous endothelial cell identity in the dorsal aortae and cardinal veins were confirmed as such at a molecular level by the expression of Nrp1 and Nrp2, respectively. LYVE-1-positive endothelial cells were confirmed as endothelial in nature and not of monocyte/macrophage lineage, by virtue of the fact that they were not F4/80-positive (A, B). Dashed line on embryo indicates region of section. Scale bars: 100 µm. DA, dorsal aorta, CV, cardinal vein.
3.6. **LYVE-1 as a marker of lymphatic endothelial cell fate.**

Expression of LYVE-1 in the cardinal veins has previously been observed to precede that of Prox1 (Wigle *et al.* 2002) and suggested to signify competence of venous endothelial cells to receive signals resulting in lymphatic endothelial cell specification. In agreement with this model of lymphatic endothelial cell fate, results from this study observed LYVE-1 expression in endothelial cells of the cardinal veins at E9.5 and E10.5 that preceded that of Prox1 (Fig 3.8A-C, 3.10A-C). In fact, very few Prox1-positive endothelial cells were obvious in embryos analysed at E9.5. In contrast to the model proposed by Wigle and colleagues (Wigle *et al.* 2002), LYVE-1 expression in the cardinal veins was not uniform on all endothelial cells, but was rather expressed in a salt and pepper distribution pattern. Once lymphatic endothelial cell identity had been specified by Prox1 expression at E10.5, LYVE-1 expression was higher on the lymphatic-committed venous endothelium (Fig 3.8D-F, compare staining in DA to CV in F). The continuity of LYVE-1 expression on cardinal veins endothelial cells mirrored that of Prox1 at E10.5 (Fig 3.8D, E), and at E11.5, LYVE-1 was expressed in Prox1-positive lymphatic endothelial cells budding from the cardinal veins (Fig 3.11). Data suggest that while LYVE-1 expression precedes Prox1 in select blood vascular endothelial cells, LYVE-1 is further up-regulated or maintained following the specification of lymphatic endothelial cell fate.

3.7. **LYVE-1 is expressed on embryonic endocardium, liver sinusoids and lung capillaries.**

To further determine the identity of the cell type expressing LYVE-1 in the embryonic heart (observed in whole mount embryos, Fig 3.6), transverse sections of wild-type embryos at a variety of developmental stages were stained with anti-LYVE-1 antibody. These analyses revealed that LYVE-1 was expressed in endothelial cells lining both the atria and ventricles of the heart (Fig 3.12D, E). As observed in intra-embryonic blood vessels of the cardinal veins and dorsal aortae (Fig 3.8, 3.9, 3.10), endocardial LYVE-1 expression was not
Figure 3.12. LYVE-1 is expressed on embryonic endocardium, liver sinusoids and lung capillaries from E10.5.

Immunostaining of 10 μm transverse embryonic sections analysed by confocal microscopy. LYVE-1 is expressed on endocardial cells of the heart from E10.5 onwards (D, E, white arrows). LYVE-1 is expressed on sinusoidal endothelial cells of the liver from E10.5 onwards (J-L) and on lung capillaries from E12.5 onwards (F, K, L). LYVE-1-positive endothelial cells were confirmed as blood and not lymphatic in nature by staining of adjacent tissue sections with Prox1 and CD31. None of the endothelial cells lining the heart (A, B), liver sinusoids (G, H) or lung capillaries (C, H, I), were Prox1-positive, with the exception of lung lymphatic vessels (C, open arrow), which also expressed high levels of LYVE-1 (F, open arrow). Myocardial (A, B) and hepatocyte (G, H) identity were confirmed by Prox1 expression. Scale bars: 100 μm. A, atrium, V, ventricle, Lu, lung, Li, liver. Immunostaining was performed by Natasha Harvey.
uniform on all CD31-positive endocardial cells (Fig 3.12D, E, 3.13A, B, arrows). Expression of LYVE-1 in the endocardium of the heart has not been previously described, and persisted through all stages of embryonic development analysed. As has previously been described (Mouta Carreira et al. 2001; Prevo et al. 2001; Nonaka et al. 2007), expression of LYVE-1 was obvious at all stages of embryogenesis analysed in CD31-positive sinusoidal endothelial cells of the liver (Fig 3.12J-L, 3.13D-F) and CD31-positive endothelial cells of the lung (Fig 3.12F, K, L, 3.13C, E, F). Interestingly, the level of LYVE-1 expressed on lung capillary endothelial cells was much lower than that observed in endothelial cells of lymphatic vessels within the lung (Fig 3.12F, 3.13C), as well as being lower than the level observed on liver sinusoidal endothelium (Fig 3.12J-L, 3.13D-F).

To determine whether LYVE-1 positive endothelia in the heart, liver and lung were lymphatic in nature, adjacent sections were stained with Prox1. Prox1-positive endothelial cells were not observed in the endocardium (Fig 3.12A, B), the liver sinusoids (Fig 3.12G, H), or the lung vasculature (Fig 3.12C, H, I), with the exception of lymphatic vessels in the lung (Fig 3.12C, open arrow denotes lymphatic vessel). As previously reported, Prox1 was observed in non-endothelial myocardium of the heart (Oliver et al. 1993), where it is required for muscle structure and growth of the heart (Risebro et al. 2009). Additionally, Prox1 was expressed in the non-endothelial liver hepatoblasts (Oliver et al. 1993; Dudas et al. 2004), where it has been demonstrated to regulate liver morphogenesis and hepatocyte function and commitment (Sosa-Pineda et al. 2000; Papoutsi et al. 2007; Kamiya et al. 2008). Taken together, these results reveal that LYVE-1 stains endothelial cells in the heart, liver and lung, and suggests the mechanisms for controlling LYVE-1 expression in endothelial cells are tissue and cell-type specific.
Figure 3.13. Single channel images confirm that LYVE-1 is expressed on embryonic endocardium, liver sinusoids and lung capillaries from E10.5.

Single channel panels of images from figure 3.12 clearly demonstrate the expression of LYVE-1 on endocardial cells of the heart from E10.5 onwards (A, B, white arrows). LYVE-1 is expressed on sinusoidal endothelial cells of the liver from E10.5 onwards (D-F) and on lung capillaries from E12.5 onwards (C, E, F). Lymphatic vessels in the lung express higher levels of LYVE-1 (C, open arrow). A-C and D-F are single channel images of D-F and J-L of Fig 3.12, respectively. Scale bars: 100 μm. A, atrium, V, ventricle, Lu, lung, Li, liver. Immunostaining was performed by Natasha Harvey.
3.8. Discussion

The aim of this chapter was to precisely document the embryonic expression pattern of LYVE-1 with respect to the developing vasculature. While this receptor has typically been described as being restricted to lymphatic endothelium, recent reports have documented its expression on a population of macrophages as well as endothelial sinusoids of lymph nodes, liver and spleen (Banerji et al. 1999; Mouta Carreira et al. 2001; Prevo et al. 2001; Nonaka et al. 2007; Gordon et al. 2010). Detailed analyses performed in this study provide the first evidence that LYVE-1 is expressed extensively throughout the embryonic blood vasculature (in yolk sac blood vessels throughout embryonic development, in early stage inter-somitic blood vessels and in early stage isolated arterial endothelial cells) as well as in the endocardium. These data have important implications for the use of LYVE-1 as a specific marker of the lymphatic vasculature in the settings of embryogenesis and neo-lymphangiogenesis in the adult.

LYVE-1, Prox1 and VEGFR-3 are the most commonly used markers for the lymphatic vasculature (Baluk and McDonald 2008). VEGFR-3 expression has been documented on early embryonic blood vessels and a population of stem cells (Kaipainen et al. 1995), indicating that use of this marker to identify lymphatic endothelium must be interpreted with caution. A recent study demonstrated that VEGFR-3 is also expressed on tip cells of sprouting blood vessels where it regulates vascular network formation (Tammela et al. 2008), revealing that this receptor is important for angiogenesis, as well as lymphangiogenesis. Other commonly used markers to identify lymphatic vessels include podoplanin, which is also expressed on podocytes, epithelial cells of the lung, mesothelial cells and myoepithelial cells, and Nrp2, which is expressed on veins (Dobbs et al. 1988; Breiteneder-Geleff et al. 1999; Herzog et al. 2001). To date, Prox1, the master regulator of lymphatic vessel identity (Wigle and Oliver 1999), has proven to be the most reliable marker of lymphatic endothelium. Taken together, published work and results from this study
indicate a previously unrecognised endothelial cell heterogeneity, and suggest that a combination of endothelial cell markers should be utilised to precisely assess vascular endothelial cell identity.

Our initial observation of LYVE-1-positive vessels in the yolk sac (Fig 3.1) raised the possibility that these vessels were lymphatic in nature. The avian embryo contains lymphatic vessels both within the chorioallantoic membrane (CAM) and the embryo (Wilting et al. 2000), identified by their expression of VEGFR-3 and VEGFR-2, and their ability to proliferate in response to VEGF-C (Wilting et al. 2000). While studies in this chapter also identified VEGFR-3 on the early LYVE-1-positive vessels in the yolk sac, expression of VEGFR-3 was progressively down-regulated during development (Fig 3.3). This observation is in accordance with the reported expression and role of VEGFR-3 during early embryonic blood vascular development (Kaipainen et al. 1995; Dumont et al. 1998) and angiogenesis (Valtola et al. 1999; Tammela et al. 2008). Extensive analysis of LYVE-1-positive vessels in the yolk sac demonstrated they did not express podoplanin or Prox1 at any stage of development (Fig 3.2), but that they did express blood vascular markers such as Nrp1 and were invested with vascular smooth muscle α-actin (Fig 3.4, 3.5). To the best of our knowledge, this work provides the first formal demonstration that the mouse yolk sac, unlike the avian CAM, does not contain lymphatic vessels.

In addition to expression on blood vessels in the yolk sac, LYVE-1 was also observed on a population of CD31-negative cells (Fig 3.1). LYVE-1-positive macrophages have been shown to have a pro-angiogenic and pro-lymphangiogenic role during inflammation (Maruyama et al. 2005; Schledzewski et al. 2006; Cho et al. 2007; Jeon et al. 2008; Attout et al. 2009; Kubota et al. 2009). The data presented in this chapter illustrate that the yolk sac is devoid of lymphatic vessels, suggesting that this population of macrophages may be playing a role during blood vascular development/remodelling in the yolk sac. Macrophages
found in the yolk sac pre-date those derived from bone-marrow hematopoietic cells, (Herbomel et al. 1999; Lichanska and Hume 2000) and have recently been established to be important during brain angiogenesis by Fantin and colleagues (Fantin et al. 2010). However, the expression of LYVE-1 on this macrophage population was not investigated in this study. The role and functional significance of embryonic LYVE-1-positive macrophages is the focus of studies presented in Chapter 4 of this thesis.

LYVE-1 expression is observed in the cardinal veins prior to the induction of polarised Prox1 expression, and has been thought to signify competence of the venous endothelium to receive lymphatic endothelial cell specification signals (Oliver and Harvey 2002; Tammela and Alitalo 2010). The results presented in this chapter demonstrated that LYVE-1 is expressed on select intra-embryonic arterial endothelial cells during early embryogenesis, as well as on cells of the embryonic cardinal veins (Fig 3.8-3.10). This suggests that LYVE-1 expression may potentially be considered an indicator of immature vessels, rather than as an indicator per se, of endothelial competence to receive signals that specify lymphatic endothelial cell fate. In agreement, analysis of yolk sac vascular identity demonstrated LYVE-1 expression is restricted to immature vessels that are not invested with smooth muscle (Fig 3.4, 3.5). Vessels lacking LYVE-1 expression as development proceeded were confirmed to be arterial in nature, as determined by Nrp1 expression and smooth muscle α-actin cell recruitment. This inverse relationship between LYVE-1 expression and smooth muscle coverage is in agreement with data of Makinen and colleagues (Makinen et al. 2005), who observed that Ephrin-B2 mutant mice had defective smooth muscle cell recruitment to collecting lymphatic vessels, with these vessels maintaining abnormally high LYVE-1 expression. While it is possible that the reduction in LYVE-1 expression on arterial endothelial cells and collecting lymphatic vessels plays a direct role in promoting the recruitment of smooth muscle cells, this is unlikely as no vascular defects have been described in LYVE-1−/− or LYVE-1−/−CD44−/− mice (Gale et al.
Chapter 3: Results

Arterial LYVE-1 down-regulation is more likely to occur as a consequence of smooth muscle association with the arterial endothelium.

The mechanism by which mural cells are recruited to larger lymphatic vessels, leading to the maturation of the vascular plexus, is unresolved. The angiopoietin family of growth factors and receptors has been implicated in the remodelling of the lymphatic vascular plexus (reviewed in (Augustin et al. 2009), as Angpt2−/− mice retain LYVE-1 expression on collecting lymphatic vessels and fail to recruit smooth muscle coverage (Gale et al. 2002; Dellinger et al. 2008). FoxC2 is also involved in lymphatic vessel maturation; in the absence of Foxc2, collecting lymphatics lack valves and capillaries have ectopic smooth muscle coverage (Petrova et al. 2004). FoxC2 has been postulated to repress expression of platelet derived growth factor B (Pdgf-b) in lymphatic capillaries, a gene which is essential for the recruitment of mural cells to blood vessels (Lindblom et al. 2003; Petrova et al. 2004). Recent work has demonstrated that NFATc1 acts co-operatively with FoxC2, and upon maturation, collecting vessels down-regulate FoxC2, in turn down-regulating Prox1, VEGFR-3 and LYVE-1 (Norrmen et al. 2009). Furthermore, down-regulation of NFATc-1 and FoxC2 leads to an increase in Angpt2 expression (Norrmen et al. 2009), suggesting that Angpt2 acts downstream of NFATc1 and FoxC2 to stimulate smooth muscle cell recruitment to collecting vessels. Results from this chapter observed that LYVE-1 expression is down-regulated on arteries during yolk sac blood vascular maturation (Fig 3.4, 3.5), however the exact role and functional significance of the down-regulation of LYVE-1 on the remodelling vascular plexus remains to be elucidated.

At E9.5 and E10.5, LYVE-1 distribution was non-uniform in both the cardinal veins and the dorsal aortae of the embryo. It is possible that the heterogeneity in LYVE-1 expression observed is due to subtle heterogeneity in the phenotype of individual arterial and venous endothelial cells. Variability in LYVE-1 expression was also observed between tissues, with
the endocardium, liver sinusoidal endothelium and lung capillary endothelial cells all
displaying different levels of LYVE-1 protein on the surface of endothelial cells (Fig 3.12,
3.13). Studies by Johnson and colleagues documented that cell surface LYVE-1 expression
is reversibly lost following exposure to the pro-inflammatory cytokine TNFα (Johnson et al.
2007); however this had no effect on HA binding activity. Whether the variability observed in
embryonic vascular LYVE-1 expression could reflect differences in the local concentration
of TNFα or other developmentally expressed cytokines/growth factors remains to be
explored. A recent study described a mechanism whereby LYVE-1 is functionally silenced
by sialylation in a reversible fashion (Nightingale et al. 2009), once again demonstrating the
complexity of this receptor, its regulation, and its functional role in vascular development.
Future challenges will be to determine the factors that regulate LYVE-1 expression and
endothelial cell phenotypes within blood and lymphatic vessels.

In conclusion, this study has provided a detailed analysis of the expression of the
hyaluronan receptor LYVE-1 in the embryonic vasculature. LYVE-1-positive vessels in the
yolk sac were determined to be blood vascular in nature, formally demonstrating for the first
time that the mouse yolk sac is devoid of lymphatic vessels. LYVE-1 expression was also
observed on yolk sac macrophages, with studies investigating the role of LYVE-1-positive
macrophages presented in Chapter 4. In addition, results demonstrated for the first time that
LYVE-1 is expressed on endothelial cells within the dorsal aorta and inter-somitic veins, as
well as in the endocardium of the heart. Variability in LYVE-1 expression levels across
different tissues was observed, with future challenges being to determine what regulates
this heterogeneous expression. These results have important implications for the use of
LYVE-1 as an exclusive marker of lymphatic endothelium during development and
inflammation-stimulated lymphangiogenesis, demonstrating it must be used in conjunction
with other established lymphatic markers to precisely identify vascular identity.
4. Investigating whether macrophages comprise a pool of lymphatic endothelial progenitor cells during embryonic and tumour-stimulated lymphangiogenesis.
4.1. Introduction

The embryonic origin of lymphatic endothelial cells has traditionally been a controversial topic in lymphangiogenesis research. Two schools of thought exist; the first, initially suggested by Florence Sabin, proposes that lymphatic endothelial cell progenitors arise from the embryonic veins (Sabin 1904). Evidence for this model has been provided by studies of Prox1 in the mouse and zebrafish (Wigle and Oliver 1999; Wigle et al. 2002; Yaniv et al. 2006). A recent study by Srinivasan and colleagues, published after the commencement of the work presented in this thesis, utilised lineage tracing to demonstrate that lymphatic endothelial cells arise primarily from the veins in the mouse embryo (Srinivasan et al. 2007). The second model, first proposed by Huntington and McClure (Huntington 1910), suggests that lymphatic endothelial cells arise de novo from the mesenchyme to form lymphatic vessels that subsequently connect to the large veins (Oliver 2004). Recent studies in the avian and Xenopus has suggested lymphatic endothelial cells have a dual origin from the veins and from mesenchymal progenitor cells (Ny et al. 2005; Wilting et al. 2006). The mechanism of embryonic lymphatic vascular growth following progenitor cell specification is also un-resolved, as it is not clear whether lymphatic vessels grow via sprouting and proliferation of established lymphatic endothelial vessels, or via a contribution from circulating progenitor cells. In addition, whether postnatal lymphangiogenesis in response to inflammatory stimuli including the tumour microenvironment (neo-lymphangiogenesis) occurs by recapitulating embryonic events such as budding from veins, via the remodelling of existing lymphatic vessels, or via the recruitment of circulating progenitor cells, has not been established.

In recent in vitro studies and in vivo models of wound healing in the mouse cornea that induce adult pathological neo-lymphangiogenesis, macrophages were suggested to contribute to the genesis of lymphatic vessels via a process of “trans-differentiation” to
lymphatic endothelial cells (Maruyama et al. 2005; Maruyama et al. 2007). Lineage tracing data was, however, lacking in these studies, and markers utilised to identify lymphatic endothelial cells, such as LYVE-1 and VEGFR-3, are not specific to the lymphatic endothelium, but are also expressed on cells of the macrophage lineage (Skobe et al. 2001; Schoppmann et al. 2002; Schledzewski et al. 2006; Gordon et al. 2008). A study analysing human samples with gender-mismatched recipient-derived bone marrow cells demonstrated a contribution of lymphatic endothelial progenitor cells to newly formed lymphatic vessels after renal transplant rejection, suggesting progenitor cells derived from the circulation contribute to lymphangiogenesis in this setting (Kerjaschki et al. 2006). In contrast, using xenograft experiments, He and colleagues (He et al. 2004) did not find significant incorporation of fluorescent bone-marrow derived cells into lymphatic vessels during tumour or VEGF-C-stimulated lymphangiogenesis. Rather, tumour-induced lymphatic vessels were found to originate from the pre-existing lymphatic network. These conflicting data may potentially be explained by different models of pathological neo-lymphangiogenesis examined, or to the differences in markers used to identify lymphatic endothelial cells.

This study set out to resolve the long debated issue of whether macrophages comprise a source of lymphatic endothelial progenitor cells during development and disease. The aims of this chapter were: 1) to precisely document the localisation and molecular identity of macrophages with respect to the developing lymphatic vasculature during embryogenesis, and 2) to definitively establish whether cells of the myeloid lineage constitute a source of lymphatic endothelial progenitor cells either during embryogenesis, or in the tumour microenvironment, using genetic lineage tracing studies.
4.2. A population of LYVE-1-positive macrophages is intimately associated with the developing lymphatic vasculature.

The first aim of this chapter was to investigate the localisation of macrophage populations with respect to the lymphatic vasculature. c-fmsEGFP transgenic mice that express EGFP in cells of the myeloid lineage (Sasmono et al. 2003) were utilised for analysis of macrophage localisation and identity during embryogenesis. At embryonic day (E) 11.5, when Prox1 cells first bud away from the cardinal veins (Wigle and Oliver 1999), few GFP-positive cells were observed in the embryo (Fig 4.1A, B). In some instances, a close spatial association between budding lymphatic endothelial cells and macrophages was observed. Analysis at E14.5 revealed numerous GFP-positive macrophages within the embryo compared to E11.5 (compare Fig 4.1A to 4.1C and F), suggesting a dramatic expansion of the macrophage population between these embryonic stages. At E14.5, a discrete population of embryonic macrophages was observed that co-expressed the hyaluronan receptor LYVE-1, also expressed by lymphatic endothelial cells (Banerji et al. 1999). Intriguingly, this LYVE-1-positive macrophage population was intimately localised with the developing embryonic lymphatic vasculature (Fig 4.1C-I). In some cases, LYVE-1-positive, GFP-positive macrophages were integrated into jugular lymph sacs (Fig 4.1D-E, arrowheads) and dermal lymphatic vessels (Fig 4.1G-I, arrowheads). When the localisation of macrophages with respect to the developing lymphatic vasculature was quantified, it was determined that LYVE-1-positive macrophages were significantly more likely to be found around lymphatic vessels than in the rest of the embryo (Fig 4.1J). In contrast, LYVE-1-negative, GFP-positive, F4/80-positive macrophages were not preferentially associated with lymphatic vessels (Fig 4.1J). The close spatial association of LYVE-1-positive macrophages with the lymphatic vasculature suggested that these cells may play a role in lymphatic vascular development.
Figure 4.1. LYVE-1-positive macrophages share a close spatial association with lymphatic vessels.

Confocal images of 10 μm sections demonstrated a close spatial association of GFP-positive macrophages with Prox1-positive lymphatic endothelial cells budding from the cardinal vein of E11.5 c-fmsEGFP embryos (A, B). Confocal z-stack images of 60 μm sections demonstrated LYVE-1-positive, GFP-positive macrophages in close association with, and within (arrowheads, D-E, G-I), the jugular lymph sac (JLS) and dermal lymphatic vessel (DLV) endothelium of E14.5 c-fmsEGFP embryos. Panel I illustrates a 90° rotation of panel H in order to view the lymphatic vessel depicted in panel H in cross section. Blue and white arrowheads denote two macrophages that are integrated within this dermal lymphatic vessel, both of which express GFP, LYVE-1 and F4/80. LYVE-1-positive macrophages were significantly more abundant in areas around the developing lymphatic vasculature (J). Boxed regions in C, D and G are shown at higher magnification in D, E and H, respectively. Scale bars: A, F - 100 μm, B, D, G - 50 μm, C - 150 μm, E, H - 25 μm. Data are representative of at least four independent experiments. Data shown represent the mean ± SEM. p values were calculated using Student’s paired t-test. *p ≤ 0.05.
Macrophage identity at E14.5

Around developing lymphatic vasculature

Not around developing lymphatic vasculature

Percentage of macrophages

- 100%
- 80%
- 60%
- 40%
- 20%
- 0%

F4/80+ LYVE-1+
GFP+ LYVE-1+
GFP+ F4/80+

* Significant difference
As macrophages have been shown to influence blood vascular patterning by initiating a death switch in the hyaloid vasculature (Lobov et al. 2005; Rao et al. 2007), it was investigated whether macrophages might be recruited to areas of the developing lymphatic vasculature to actively program apoptosis of lymphatic endothelial cells, or alternatively, to engulf corpses of cells undergoing apoptosis. Staining to detect activated caspase-3 indicated very few apoptotic cells in or around the jugular lymph sacs (Fig 4.2), and rare apoptotic cells in the endothelium were not associated with macrophages (Fig 4.2C, D, blue arrowhead). This suggests that macrophages do not actively program a death switch in lymphatic endothelial cells during development which is important for lymphatic vessel remodelling, and are not recruited to the jugular lymph sacs to engulf cell corpses.

4.3. LYVE-1-positive macrophages are abundant in the dermis.

Next, the identity of macrophages within the dermis was investigated, as the skin is a tissue rich in lymphatic vessels. Whole mount immunostaining analyses revealed a large number of LYVE-1-positive macrophages in the embryonic skin (Fig 4.3). When quantified at E15.5, by analysis of immunostaining, it was determined approximately 55% of the total dermal macrophage population (GFP-positive) was LYVE-1-positive, and approximately 85% of F4/80-positive macrophages was LYVE-1-positive (Fig 4.3E). These LYVE-1-positive macrophages were observed to contact approximately 67% of lymphatic endothelial sprouts (Fig 4.3B, D, arrowheads). These results demonstrate that LYVE-1-positive macrophages comprise the major population of macrophages in the dermis, and that macrophages interact closely with sprouting lymphatic vessels. Coupled with the observation that LYVE-1-positive macrophages integrate within lymphatic vessels (Fig 4.1), the data is suggestive of a role for LYVE-1-positive macrophages in lymphatic development.
Figure 4.2. Macrophages are not recruited to actively program apoptosis of lymphatic endothelial cells during development.

Confocal z-stack images of 60 μm transverse sections through the c-fmsEGFP jugular lymph sac. Immunostaining demonstrated LYVE-1-positive macrophages are not recruited to act as phagocytes. Panel D is a single channel image of panel C illustrating a caspase-3-positive apoptotic cell (blue arrowhead). Boxed regions in A and B are shown at higher magnification in B and C respectively. Scale bars: A - 100 μm, B - 50 μm, C, D - 25 μm.
Figure 4.3. LYVE-1-positive macrophages are abundant in the dermis.

Whole mount immunostaining of the dermis of c-fmsEGFP embryos at E14.5 and E15.5 revealed numerous LYVE-1-positive macrophages in the dermis (A-D). Lymphatic vessel sprouts often contacted LYVE-1-positive macrophages (arrowheads), suggesting this population of macrophages may play a role in the patterning of the dermal lymphatic vasculature. Quantification indicated over 50% of GFP-positive macrophages in the dermis are LYVE-1-positive and over 80% of F4/80-positive macrophages are LYVE-1-positive (E). Scale bars: A, C - 100 μm, B, D - 50 μm. Data are representative of three independent experiments. Data shown represent the mean ± SEM.
Macrophage Identity at E15.5

E14.5

E15.5

Percentage of macrophages

F4/80+ LYVE-1+ | GFP+ LYVE-1+ | GFP+ F4/80+
4.4. **Macrophages associated with lymphatic vessels do not express Prox1.**

While macrophages were observed to be closely associated with budding lymphatic endothelial cells at E11.5, and integrating into/transiting through the developing lymphatic vasculature at E14.5, whether they were trans-differentiating into lymphatic endothelial cells was unresolved. In order to establish whether LYVE-1-positive macrophages integrated into lymphatic vessels co-expressed markers of lymphatic endothelial cell identity, expression of the definitive marker of lymphatic endothelial cell identity, Prox1 (Wigle and Oliver 1999), was analysed. At E11.5, LYVE-1-positive, GFP-positive macrophages within the cardinal veins or associated with budding lymphatic endothelial cells did not express Prox1 (Fig 4.4A-D, arrows). In addition, at E14.5, LYVE-1-positive, GFP-positive macrophages within jugular lymph sacs (Fig 4.4E-H, arrows) or dermal lymphatic vessels (Fig 4.4I-L, arrows) did not express Prox1. These data suggest LYVE-1-positive macrophages are unlikely to be undergoing trans-differentiation to lymphatic endothelial cells, and highlights the importance of using multiple markers to precisely assess lymphatic endothelial cell identity.

4.5. **Lymphatic endothelial progenitor cells arise independently of the myeloid cell lineage during embryogenesis.**

In order to exclude the possibility that expression of the *c-fmsEGFP* transgene might be extinguished in macrophages undergoing trans-differentiation to lymphatic endothelial cells, and thereby to definitively determine whether cells of the monocyte/macrophage lineage comprise a pool of lymphatic endothelial progenitor cells, direct lineage tracing experiments were performed. For these experiments, *LysMCre* mice (Clausen *et al.* 1999), expressing Cre recombinase in the endogenous *LysM* locus which is expressed selectively in cells of the myeloid lineage, were crossed with *ROSA26R* Cre reporter mice (Soriano 1999) (Fig 4.5A). In the progeny of these crosses, *LysM* driven Cre will excise a stop sequence flanked by *loxP* sites, initiating expression of *LacZ* in the myeloid lineage (Fig 4.5A). The advantage
Figure 4.4. LYVE-1-positive macrophages are within lymphatic vessels, but do not express Prox1.

Confocal z-stack images of 60 μm sections of E11.5 c-fmsEGFP embryos demonstrated GFP-positive macrophages (arrows) are integrated within the cardinal vein and share a close spatial association with budding lymphatic endothelial cells, but do not express Prox1 (A-D, white circles in D illustrate location of integrated macrophages in C). At E14.5, LYVE-1-positive macrophages that are within the jugular lymph sac (JLS) and dermal lymphatic vessel (DLV) endothelium do not express Prox1 (E-L, white circles in H and L illustrate location of integrated macrophages (arrows) in G and K, respectively). Panels D, H and L illustrate single channel Prox1 images of C, G and K, respectively. Boxed regions in A, B, E, F and J are shown at higher magnification in B, C, F, G and K, respectively. Scale bars: A, E, I - 100 μm, B, F, J - 50 μm, C, D, G, H, K, L - 25 μm. Data are representative of at least four independent experiments.
Figure 4.5. Generation and analysis of Cre expression in LysMCre;Rosa26R mice.

Generation of LysMCre;Rosa26R mice for lineage tracing studies (A). Cells in which LysM is active will undergo excision of stop site flanked by loxp sites, allowing for expression of LacZ, due to expression of Cre recombinase under the control of the LysM promoter. Analyses of 10 μm sections at E14.5 revealed a low proportion of F4/80-positive (B-E) and CD11b-positive (F-I) macrophages express LacZ, as detected with an antibody against β-galactosidase (β-gal). No β-gal-positive cells expressed lymphatic endothelial cell marker Nrp2. Panels E and I illustrate single channel β-gal images of D and H, respectively. Boxed regions in B, C, F and G are shown at higher magnification in C, D, G and H, respectively. Scale bars: B, E - 150 μm, C, G - 100 μm, D, E, H, I - 50 μm. Data are representative of at least four independent experiments.
A

\[ \text{LysMCre} \times \text{Rosa26R} \]

\[ \text{Clausen et al., 1999} \times \text{Soriano, 1999} \]

\[ \begin{align*}
\text{Cre} & \quad \text{LysM} \\
\text{stop} & \quad \text{LoxP} \\
\text{LacZ} & \quad \text{LacZ}
\end{align*} \]

\[ \text{LysMCre;Rosa26R} \]

\[ \begin{align*}
\text{F4/80/\(\beta\)-gal/Nrp2} & \quad \beta\text{-gal} \\
\text{CD11b/\(\beta\)-gal/Nrp2} & \quad \beta\text{-gal}
\end{align*} \]
of lineage tracing is that reporter gene expression will be present in all descendents of progenitors that expressed LysM.

Cells were traced using immunostaining with an antibody against β-galactosidase (β-gal), as unlike X-gal staining, immunostaining allowed for simultaneous analyses of β-gal-positive cells with established lymphatic and macrophage markers. The Cre excision efficiency of embryonic macrophages was assessed by co-staining embryonic tissue with β-gal together with established macrophage markers, followed by confocal microscopy. Cre-mediated excision in embryonic macrophages was determined to be low; approximately 40% of F4/80-positive (Fig 4.5B-E) or CD11b-positive (Fig 4.5F-I) macrophages were positive for reporter gene expression in LysMCre+/--;ROSA26R+/− mice at E14.5. The finding that LysM driven Cre resulted in reporter gene expression in only a proportion of embryonic macrophages is in agreement with work by others, which found LysM is switched on only after the onset of liver haematopoiesis (Lichanska et al. 1999; Fantin et al. 2010), although this had not been established prior to our analyses. Despite low labelling efficiency, initial analyses were undertaken to determine whether β-gal-positive cells were observed within lymphatic vessels. Nrp2 was utilised to detect lymphatic endothelial cells to avoid cross-reactivity, as Prox1, LYVE-1 and β-gal antibodies were all raised in rabbit. No β-gal-positive cells were observed to be integrated within the jugular lymph sacs, and Nrp2 expression was not detected in any CD11b-positive, F4/80-positive, β-gal-positive cells derived from the myeloid lineage that were closely associated with jugular lymph sacs (Fig 4.5B-I).

Due to low reporter gene expression in LysMCre+/--;ROSA26R+/− mice, a second lineage tracing approach was taken. For these experiments, Csf1r-iCre mice (Deng et al. 2010), expressing Cre recombinase under control of the Csf1r/c-fms promoter, expressed selectively in cells of the myeloid lineage, were crossed with Z/EG Cre reporter mice (Novak et al. 2000) to generate Csf1r-iCre;Z/EG mice (Fig 4.6A). In the progeny of these crosses, in
Figure 4.6. Lineage tracing in Csf1r-iCre;Z/EG embryos reveals that cells of the myeloid lineage do not comprise a pool of lymphatic endothelial progenitor cells during development.

Generation of Csf1r-iCre;Z/EG mice for embryonic lineage tracing (A). All cells of the myeloid lineage expressing Csf1r will undergo excision of a β-geo cassette flanked by loxP sites, allowing for expression of Z/EG (GFP), due to expression of Cre recombinase under the control of the Csf1r/c-fms promoter. Confocal z-stack images of 60 μm sections demonstrated the localisation of LYVE-1-positive, GFP-positive macrophages in close association with, and within (arrows, C, F), the jugular lymph sac (JLS) and dermal lymphatic vessel (DLV) endothelium of E14.5 Csf1r-iCre;Z/EG embryos. LYVE-1-positive macrophages integrated within the JLS or DLV endothelium do not express Prox1 (D, G, white circles illustrate location of integrated macrophages (arrows) in C and F). Panels D and G illustrate single channel Prox1 images of C and F, respectively. Boxed regions in B and E are shown at higher magnification in C and F, respectively. Scale bars: B, E - 100 μm, C, D, F, G - 25 μm. Data are representative of three independent experiments.
A) 

\[ \text{Csf1r-iCre} \rightarrow \text{Cre} \rightarrow \beta\text{geo} \rightarrow \text{Z/EG} \rightarrow \text{Csf1r-iCre; Z/EG} \]

Deng et al., 2010

Novak et al., 2000

B - G) 

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Legend:
- c-fms
- Cre
- βgeo
- loxP
- Z/EG
- Csf1r-iCre; Z/EG
Chapter 4: Results

all myeloid cells expressing Csf1r/c-fms, Cre-mediated excision of a β-geo cassette flanked by loxP sites initiates expression of Z/EG (Fig 4.6A). Quantification of Cre excision efficiency at E14.5 revealed 76% of F4/80-positive cells were positive for reporter gene expression. The expression of GFP, LYVE-1 and Prox1 was then assessed in embryonic macrophages using fluorescent immunostaining and confocal microscopy. In concordance with results from c-fmsEGFP mice and LysMCre^{+/+};ROSA26R^{+/-} mice, Prox1 expression was not detected in any LYVE1-positive, GFP-positive cells derived from the myeloid lineage, either within, or closely associated with, jugular lymph sacs (Fig 4.6B-D) or dermal lymphatic vessels (Fig 4.6E-G). Taken together, this lineage tracing data provides strong evidence to exclude cells of the myeloid lineage as a source of lymphatic endothelial progenitor cells during embryogenesis.

4.6. Lymphatic endothelial progenitor cells arise independently of the myeloid cell lineage in the tumour microenvironment.

While LysMCre^{+/+};ROSA26R^{+/-} mice were not ideal for embryonic lineage tracing studies due to low Cre excision efficiency during embryogenesis (see section 4.5), their use to label adult macrophages was well established (Clausen et al. 1999). Quantification of immunostaining analyses of bone marrow (Fig 4.7A-D) and adult skin from the flank (Fig 4.7E-G) determined that Cre excision efficiency in these mice was substantially higher in adulthood, with approximately 95% of F4/80-positive cells positive for β-gal in the bone marrow and adult skin. These results are in agreement with the previously described excision efficiency of 83-98% (Clausen et al. 1999). Therefore, these mice were used for adult lineage tracing studies.

To directly trace the fate of cells of the monocyte/macrophage lineage during tumour lymphangiogenesis, adult LysMCre^{+/+};ROSA26R^{+/-} mice were implanted subcutaneously with Lewis lung carcinoma (LLC) or EL4 lymphoma cells. Following 10-14 days of tumour...
Figure 4.7. Expression of Cre in LysMCRe;Rosa26R adult mice is sufficient for use in adult lineage tracing studies.

Immunostaining analysis of bone marrow (A-D) and 10 μm sections of dermis from the flank (E-G) of LysMCRe+/--;Rosa26R+/-- adult mice revealed approximately 95% of F4/80-positive macrophages are positive for β-gal, revealing these mice can be used for adult lineage tracing studies. B, C and D are single panel images of A. F and G are single panel images of E. Scale bars: E-G - 100 μm. Data are representative of at least three independent experiments.
A F4/80\beta-gal/DAPI

B F4/80

C \beta-gal

D DAPI

E F4/80/\beta-gal

F F4/80

G \beta-gal
growth, peri- and intra-tumoural lymphangiogenesis was examined, and the localisation of myeloid cells was investigated with respect to both the pre-existing and newly formed lymphatic vasculature. Both tumour types were associated with an influx of CD11b-positive and F4/80-positive macrophages, and stimulated the growth of both peri-tumoural and intra-tumoural lymphatic vessels, as visualised by LYVE-1 staining (Fig 4.8). CD11b-positive macrophages shared a close spatial association with lymphatic vessels (Fig 4.8C, F, L, arrows). Unlike during embryogenesis, LYVE-1-positive macrophages were rare, and were not spatially associated with lymphatic vessels (Fig 4.8E, arrowhead). This suggests that LYVE-1-positive macrophages may play a different role during development as opposed to tumour progression.

In order to trace the fate of myeloid cells in the tumour environment, β-gal expression was assessed in combination with lymphatic markers. While an influx of β-gal-positive inflammatory cells was observed both surrounding and within LLC and EL4 tumours, the integration of β-gal-positive cells derived from the myeloid lineage into either peri- or intra-tumoral lymphatic vessels was a rare event (Fig 4.9, 4.10). Those β-gal-positive cells that were within lymphatic vessels did not express Prox1 (Fig 4.9D, H, 4.10D, H) demonstrating that cells of the myeloid lineage were not undergoing a trans-differentiation event to generate lymphatic endothelial cells. These data provide strong evidence to illustrate that lymphatic endothelial cells arise independently of the monocyte/macrophage lineage during tumour-stimulated lymphangiogenesis.


Due to the observation that LYVE-1-positive, but not LYVE-1-negative myeloid cells selectively integrated within the embryonic lymphatic vasculature during development, this study sought to characterise the gene expression profiles of embryonic LYVE-1-positive and LYVE-1-negative macrophages in order to further investigate their function. Primary
Subcutaneous LLC and EL4 tumour models displayed both existing dermal lymphatic vessels (A-C, G-I) and newly formed peri/intra-tumoural lymphatic vessels (D-F, J-L), as detected with anti-LYVE-1 antibody. Stimulation of lymphangiogenesis was coupled with a large influx of CD11b-positive, F4/80-positive macrophages. Macrophages often shared a close spatial association with lymphatic vessels (C, F, L, arrows). Boxed regions in A, B, D, E, G, H, J, K are shown at higher magnification in B, C, E, F, H, I, K, L, respectively. Scale bars: A, D, G, J - 100 μm, B, E, H, K - 50 μm, C, F, I, L - 25 μm. Data are representative of at least eight independent experiments per tumour type.
Figure 4.9. Myeloid cells do not comprise a pool of lymphatic endothelial progenitors during LLC-stimulated lymphangiogenesis.

Lineage tracing in adult LysMCre$^{+/-}$;ROSA26R$^{-/-}$ mice revealed very few β-gal-positive cells derived from the myeloid lineage integrated within Prox1-positive, CD31-positive pre-existing dermal (A-D) or newly generated peri/intra-tumoral (E-H) lymphatic vessels induced by the subcutaneous LLC tumour model. Prox1 expression was not observed in any β-gal-positive cells within lymphatic vessels (C, D, G, H). White circles in panels D and H illustrate location of macrophages within lymphatic vessels (arrows) in C and G, respectively. Panels D and H illustrate single channel Prox1 images of C and G, respectively. Scale bars: A, E - 100 μm, B, F - 50 μm, C, D, G, H - 25 μm. Data are representative of eight independent experiments.
CD31/β-gal/Prox1

A BC D E F GH

dermis tumour
dermis
dermis

LLC
Figure 4.10. Myeloid cells do not comprise a pool of lymphatic endothelial progenitors during EL4-stimulated lymphangiogenesis.

Lineage tracing in adult LysMCre^{+/--};ROSA26R^{+/--} mice revealed very few β-gal-positive cells derived from the myeloid lineage integrated within Prox1-positive, CD31-positive pre-existing dermal (A-D) or newly generated peri/intra-tumoral (E-H) lymphatic vessels induced by the subcutaneous EL4 tumour model. Prox1 expression was not observed in any β-gal-positive cells within lymphatic vessels (C, D, G, H). White circles in panels D and H illustrate location of macrophages within lymphatic vessels (arrows) in C and G, respectively. Panels D and H illustrate single channel Prox1 images of C and G, respectively. Scale bars: A, E - 100 μm, B, F - 50 μm, C, D, G, H - 25 μm. Data are representative of eight independent experiments.
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Macrophages were isolated from E15.5 embryonic skin, as LYVE-1-positive macrophages were abundant in this tissue (Fig 4.3). Using a magnetic cell separation procedure (MACS®), F4/80-positive macrophages were isolated from single cell suspensions of embryonic skin using a biotin-labelled anti-F4/80 antibody and anti-biotin Multisort MACS® beads. Following cleavage of the Multisort beads from the F4/80-positive macrophages, cells were separated on the basis of LYVE-1 expression using an anti-LYVE-1 antibody and anti-rabbit MACS® beads to obtain a F4/80-positive, LYVE-1-negative population and a F4/80-positive, LYVE-1-positive population (Fig 4.11A). Purity of the isolated cell fractions was determined using real time RT-PCR (Fig 4.11B). LYVE-1-positive cells expressed significantly more Lyve-1 than the LYVE-1-negative fraction, while F4/80 (Emr1) expression was not significantly different between the macrophage populations. This confirmed the success of the magnetic cell sorting strategy in isolating LYVE-1-positive and LYVE-1-negative macrophages.

4.8. The gene expression profile of embryonic LYVE-1-positive macrophages resembles that of Tie2-expressing monocytes (TEMs).

In order to characterise the gene expression profiles of LYVE-1-positive and LYVE-1-negative macrophages, microarray and real time RT-PCR analyses were employed. It was hypothesised LYVE-1-positive macrophages would produce elevated levels of lymphangiogenic growth and patterning factors due to their close association with the developing lymphatic vasculature. RNA and cDNA was immediately prepared from freshly isolated cells (as described in 4.7) and subjected to analysis. In order to obtain sufficient amount of sample for microarray analysis, nine independent cell separations were performed and pooled to obtain a final number of three samples. Due to limited sample availability, real time RT-PCR validation of microarray candidates was not performed for all genes. Microarray analysis revealed Lyve-1 was elevated 15.42 fold in LYVE-1-positive macrophages compared to LYVE-1-negative macrophages (Fig 4.12). Interestingly, LYVE-
Figure 4.11. Method for isolation of LYVE-1-positive macrophages from the embryonic dermis.

(A) MACS® magnetic bead cell isolation protocol developed for the isolation of LYVE-1-positive and LYVE-1-negative macrophages. Macrophages were isolated from the E15.5 dermis on the basis of F4/80 expression, then magnetic beads were cleaved and the isolated macrophage population was subsequently separated on the basis of LYVE-1 expression. (B) Real time RT-PCR analysis revealed the LYVE-1-positive population expressed significantly higher amounts of Lyve-1, whereas no change in F4/80 expression between the LYVE-1-positive and LYVE-1-negative populations was observed. Data shown represent the mean ± SEM of four independent cell isolations. p values were calculated using Student’s paired t-test. *p ≤ 0.05.
A) 1) Macrophages labelled with rat anti-mouse biotin-labelled F4/80 antibody and anti-biotin MACS multisort microbeads
   2) Multisort MACS beads cleaved
   3) Macrophages labelled with rabbit anti-mouse LYVE-1 antibody and anti-rabbit MACS microbeads

**KEY**
- F4/80^+^LYVE-1^-^ macrophage
- F4/80^+^LYVE-1^+^ macrophage
- biotinylated F4/80 antibody
- anti-biotin multi-sort MACS bead
- rabbit LYVE-1 antibody
- anti-rabbit MACS bead

B)  

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**F4/80 (Emr1)**

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<tr>
<td>F4/80^+^LYVE-1^+^</td>
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**Lyve-1**
Figure 4.12. Differential expression of genes in LYVE-1-positive macrophages versus LYVE-1-negative macrophages.

Following the extraction of RNA from MACS® isolated macrophages, microarray and real time RT-PCR analysis revealed genes differentially regulated in LYVE-1-positive, F4/80-positive macrophages compared to LYVE-1-negative, F4/80-positive macrophages. The gene expression profile of LYVE-1-positive, F4/80-positive macrophages closely resembled that of Tie2-expressing monocytes (TEMs) (Pucci et al., 2009). For microarray analysis, n=3 replicates comprised of samples pooled from nine independent cell isolations. For microarray, p values were calculated using step-up multiple test correction (Benjamini, 1995) to control the false discovery rate. Adjusted p values (Adj. p) of ≤ 0.05 were considered to be significant. For real time RT-PCR, n=3 independent cell isolations. p values were calculated using Student’s paired t-test. *p ≤ 0.05.
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* Elevated in LYVE-1-positive macrophages
* Decreased in LYVE-1-positive macrophages
1-positive macrophages did not express elevated levels of established lymphangiogenic growth factors 

\textit{Vegfc} (Jeltsch \textit{et al.} 1997), \textit{Vegfd} (Stacker \textit{et al.} 1999) or of angiogenic growth factor 

\textit{Vegfa} (Ferrara \textit{et al.} 1996) when compared to the LYVE-1-negative population (Fig 4.12). This suggested LYVE-1-positive macrophages do not influence the lymphatic vasculature by providing established pro-lymphangiogenic growth factors.

In addition to investigating well established lymphangiogenic growth factors \textit{Vegfc}, \textit{Vegfd} and \textit{Vegfa}, this study sought to characterise the expression of pro-angiogenic matrix metalloproteases (MMPs) (Kessenbrock \textit{et al.} 2010) in LYVE-1-positive macrophages. As elevated MMP levels increases bioavailability of growth factors such to stimulate developmental lymphangiogenesis (Nakamura \textit{et al.} 2004), it was envisioned that LYVE-1-positive macrophages may produce increased levels of MMPs compared to LYVE-1-negative macrophages. However, there was no difference in expression levels between the macrophage populations of candidate pro-lymphangiogenic MMPs including \textit{Mmp}2 (Bruyere \textit{et al.} 2008), \textit{Mmp}8 (Palavalli \textit{et al.} 2009), \textit{Mmp}9 (Bergers \textit{et al.} 2000) or \textit{Mmp}12 (Cho \textit{et al.} 2007) (Fig 4.12), all of which have established roles in angiogenesis. As LYVE-1 has been shown to be rapidly down-regulated and gene expression switched off in lymphatic endothelial cells \textit{in vitro} after exposure to TNF-\alpha (Johnson \textit{et al.} 2007), it was predicted that LYVE-1-negative macrophages may express increased levels of this pro-inflammatory cytokine. However, expression of \textit{TNF-\alpha} was unchanged between the LYVE-1-positive and LYVE-1-negative populations (Fig 4.12), suggesting expression of LYVE-1 on macrophages is not regulated by autocrine production of TNF-\alpha.

Recent work has demonstrated that a distinct sub-populations of macrophages play a specialised role in physiological processes including inflammation, angiogenesis and tissue remodelling (Venneri \textit{et al.} 2007; Pucci \textit{et al.} 2009; Fantin \textit{et al.} 2010). This population of Tie2-expressing monocytes (TEMs) are distinct from tumour-associated macrophages.
(TAMs), which have an established role in neo-angiogenesis (Murdoch et al. 2008; Pucci et al. 2009; Coffelt et al. 2010). Analysis of the genes selectively enriched in the LYVE-1-positive versus LYVE-1-negative population revealed significant overlap with the gene signature of "embryonic/foetal TEMs" and tumour derived TEMs (Pucci et al. 2009), shown to have a role in developmental (Fantin et al. 2010) and pathological (Venneri et al. 2007; Pucci et al. 2009) angiogenesis. Genes commonly expressed at increased levels in these populations included Lyve1, Mrc1, Igf1, Cd163, Stab1, Nrp1, Plxnd1 and Sema6d, while genes commonly down-regulated in LYVE-1-negative and Tie2-negative embryonic macrophages included Sell, Angpt1 and Egf (Fig 4.12). While Tie2 expression was not statistically significant between the populations, expression was elevated in LYVE-1-positive macrophages as detected by real time RT-PCR. It may be that at the developmental stage analysed in this study, Tie2 expression in LYVE-1-positive macrophages has not been switched on at maximum levels, or that the LYVE-1-positive population encompass only part of the Tie2-positive macrophage population.

4.9. Discussion
The origin of the embryonic lymphatic vascular system has been debated for 100 years and remains controversial in the field, with the two models being proposed; 1) lymphatic endothelial cells arise from the veins (Sabin 1904), or, 2) lymphatic endothelial cells arise de novo from the mesenchyme (Huntington 1910). The focus of this chapter was to determine whether cells of the myeloid lineage contribute to the developing lymphatic vasculature by acting as a source of lymphatic endothelial progenitor cells. Extensive lineage tracing evidence illustrated that the lymphatic vasculature of both the mouse embryo and the tumour microenvironment arises independently of the myeloid cell lineage, thereby excluding monocytes/macrophages as a reservoir of lymphatic endothelial progenitor cells in these settings. This study also aimed to characterise the gene expression profile of LYVE-1-positive dermal macrophages, which shared an intimate association with
lymphatic vessels, and found that their gene signature is suggestive of a role in tissue morphogenesis and remodelling events.

After the commencement of this study, work by Srinivasan and colleagues in the mouse embryo provided evidence to validate Florence Sabin’s hypothesis that the majority of lymphatic vessels in the early vertebrate embryo originate from the veins (Srinivasan et al. 2007). However, studies in model systems including the avian (Wilting et al. 2000; Wilting et al. 2006) and Xenopus embryo (Ny et al. 2005) have provided evidence to support a dual origin from venous and mesenchymal progenitor cell components. Lineage tracing data presented in this chapter provides strong evidence to exclude cells of the monocyte/macrophage lineage as a source of lymphatic endothelial progenitor cells during embryogenesis in the mouse (Fig 4.6), in agreement with the data of Srinivasan and colleagues (Srinivasan et al. 2007).

Evidence supporting the model where lymphatic endothelial cells arise de novo from the mesenchyme during development (Huntington 1910) has come from findings that CD45, LYVE-1 and Prox1 expressing cells are observed in the mesenchyme of mouse embryos, and were suggested to trans-differentiate into lymphatic endothelial cells and contribute to the lymphatic endothelium (Buttler et al. 2006; Buttler et al. 2008). However, work in this chapter and by others has demonstrated that hematopoietic cells do not contribute to lymphatic endothelium during development (Srinivasan et al. 2007; Gordon et al. 2010). The differences in the conclusions between the studies are likely due to the lineage tracing approach utilised in this chapter being more stringent than the circumstantial localisation observed by Buttler and colleagues. Taken together, these results indicate the importance of utilising a lineage tracing approach to determine cell fate.
In addition to developmental analyses, lineage tracing data analysing two different models of tumour development, both of which stimulate lymphangiogenesis, also excluded cells of the monocyte/macrophage lineage as a source of lymphatic endothelial progenitors in the tumour microenvironment (Fig 4.9, 4.10). This finding is in contrast to the suggestions of others that in settings of inflammation-stimulated neo-lymphangiogenesis, macrophages possess the ability to trans-differentiate to lymphatic endothelial cells (Maruyama et al. 2005; Kerjaschki et al. 2006; Maruyama et al. 2007; Attout et al. 2009). While we observed myeloid-derived LYVE-1-positive cells apparently integrated into lymphatic vessels, lineage tracing analyses revealed that none of these cells expressed Prox1. Other groups claiming that macrophages or hematopoietic derived endothelial progenitor cells integrate within lymphatic vessels and trans-differentiate into lymphatic endothelial cells during neo-lymphangiogenesis did not examine Prox1 expression (Religa et al. 2005; Maruyama et al. 2007; Jiang et al. 2008; Attout et al. 2009) but instead analysed expression of cell surface markers including LYVE-1 and VEGFR-3, also reported to be expressed on cells of the macrophage lineage (Skobe et al. 2001; Schoppmann et al. 2002; Schledzewski et al. 2006; Baluk and McDonald 2008; Gordon et al. 2008). Recent work identified a population of podoplanin-positive bone marrow-derived progenitor cells that express Prox1 mRNA, and incorporate into lymphatic vessels in the cornea, and in models of wound healing and melanoma (Lee et al. 2010). When these podoplanin-positive, CD11b-positive cells were injected into wounded mice, they were observed to incorporate within newly formed lymphatic vessels, identified using LYVE-1, where they down-regulated CD45 expression as detected by immunostaining (Lee et al. 2010). However, whether these apparently integrated progenitor cells expressed Prox1 protein was not investigated, therefore whether these cells are truly trans-differentiating to lymphatic endothelial cells, or rather just sitting within or transiting through the vessel wall, remains to be elucidated. Results from all these studies confirm that when evaluating hematopoietic cell contribution to developing lymphatic endothelium, multiple markers specific for lymphatic endothelial cell identity must be
utilised. Prox1 is required for initiation and maintenance of lymphatic endothelial cell fate (Wigle and Oliver 1999; Johnson et al. 2008), and is able to suppress genes characteristic of blood endothelial cell identity (Oliver 2004). Therefore Prox1 is a master regulator of lymphatic endothelial cell fate. For this reason, it was used as a definitive marker of lymphatic endothelial cell identity for experiments in this thesis.

Interestingly, Zumsteg and colleagues (Zumsteg et al. 2009) recently published a study incorporating lineage tracing of myeloid cells using CD11bCre;Z/EG mice into which prostate adenocarcinoma TRAMP-C1 cells were implanted. In contrast to data presented in this chapter, rare GFP-positive, LYVE-1-positive, Prox1-positive cells were observed integrated into tumour lymphatic vessels (Zumsteg et al. 2009). The differences between our findings and those of Zumsteg and colleagues may potentially be explained by variations in tumour type, or differences in Cre lines utilised for lineage tracing. Differences may also be attributed to the tumour stage analysed, as it has been shown in the setting of tumour-induced angiogenesis that endothelial progenitor cells contribute significantly to early vessel growth, whereas at later stages, sprouting from pre-existing blood vessels is the predominant angiogenic mechanism (Nolan et al. 2007). Nonetheless, extensive lineage tracing data in LysMCre<sup>+</sup>;ROSA26R<sup>+</sup> mice utilising two mouse tumour models found no evidence for the trans-differentiation of myeloid cells into lymphatic endothelial cells within the peri-tumoural or intra-tumoural lymphatic vasculature (Fig 4.9, 4.10). Our data are in accordance with He and colleagues (He et al. 2004) who found that transplanted bone marrow cells did not contribute to the generation of LLC tumour-stimulated lymphatic endothelium.

The contribution of bone marrow-derived endothelial progenitor cells to the blood vascular endothelium is another controversial topic in vascular research. One line of thought is that bone marrow-derived cells mobilise to the tumour environment, where they are able to
differentiate into endothelial cells. The other view is these cells provide angiogenic factors that influence the developing vasculature (reviewed in (Shojaei et al. 2008). There is conflicting evidence that these cells significantly contribute to tumour vascularisation (Shaked et al. 2006), with the role of endothelial progenitor cells possibly being both context and tumour stage dependent (Nolan et al. 2007; Shojaei et al. 2008). Recent studies using lineage tracing and chimeric mouse models have demonstrated that VEGFR-2 expressing bone marrow-derived cells do not contribute to the vascular endothelium at sites of VEGF-A or tumour-induced angiogenesis, and that these cells are not required for tumour progression (Gothert et al. 2004; Purhonen et al. 2008). In agreement, in this chapter, as well as finding no integration of myeloid cells into tumour-associated lymphatic vessels, no incorporation of progenitor cells into the blood vessel wall was observed. Other studies using GFP expressing bone marrow chimeras also did not observe any integration of bone marrow-derived cells into the vessel wall in primary or metastatic LLC tumours, even when tumours were engineered to over-express VEGF, coupled with a massive infiltration of hematopoietic cells (Wickersheim et al. 2009). It may be hypothesised that over-expression of VEGF-C or VEGF-D may lead to increased mobilisation of myeloid cells to the tumour site. However, it is unlikely this would affect the incorporation of myeloid cells into the lymphatic vascular endothelium.

While macrophages were not observed to trans-differentiate into lymphatic endothelial cells, they were integrated within the lymphatic vasculature. The role of these cells within lymphatic vessels remains unclear. We hypothesise that the integration of macrophages within lymphatic endothelium may reflect transient macrophage migration across the lymphatic endothelium (into, or out of vessels) or alternatively, a place of residence important for fulfilling an immune surveillance role (ie. by surveying the passing contents of lymph). Discriminating between these possibilities will rely on the use of intravital microscopy to analyse macrophage migration in real time. Whether or not macrophages are
able to trans-differentiate to lymphatic endothelial cells in other settings of inflammation remains to be established, although the data from this study suggests that this is unlikely to be a common event.

As LYVE-1-positive macrophages do not express high levels of *Vegfa*, *Vegfc*, *Vegfd* or *Mmp9* (Fig 4.12), they do not fit the classical profile of TAMs, which have a defined role in neo-angiogenesis (Venneri *et al.* 2007; Murdoch *et al.* 2008; Pucci *et al.* 2009; Coffelt *et al.* 2010; Fantin *et al.* 2010; Schmid and Varner 2010). Gene profiling analyses of LYVE-1-positive versus LYVE-1-negative macrophages isolated from the embryonic dermis illustrated that the LYVE-1-positive macrophage population has a phenotype closely related to CD11c*Mrc*Tie2*+* monocytes/foetal macrophages (TEMs) (De Palma *et al.* 2005; Pucci *et al.* 2009). Expression of the endothelial marker Tie2 by this subset of monocytes that are closely associated with the tumour vasculature may have led to the hypothesis that monocytes act as endothelial progenitor cells. Shared expression of genes also results in few tools to discriminate between the populations. Although TEMs express commonly used endothelial cell marker Tie2, they do not incorporate into the vascular endothelium (De Palma *et al.* 2003). TEMs also possess a gene expression profile distinct from endothelial lineage cells, with robust expression of hematopoietic markers *CD45* and *F4/80* (*Emr1*) and low expression of endothelial genes *Vegfr2* and *VE-Cadherin* (Pucci *et al.* 2009). Specific ablation of TEMs inhibits tumour angiogenesis, but does not affect the infiltration of TAMs (De Palma *et al.* 2005), suggesting TEMs and TAMs comprise distinct macrophage populations. As was observed in this chapter with the LYVE-1-positive macrophage population (Fig 4.8), TEMs comprise a minor proportion of tumour infiltrating macrophages. However, when TEMs are isolated and then implanted with tumour cells *in vivo*, they drive the angiogenic response to a greater degree than TAMs (De Palma *et al.* 2005). As well as tumour-derived TEMs, TEMs derived from peripheral blood have also been demonstrated to drive the angiogenic response (Venneri *et al.* 2007). We hypothesise that LYVE-1-positive
macrophages associated with lymphatic vessels may contribute to events such as lymphatic vascular morphogenesis and remodelling.

While the genetic profile of LYVE-1-positive macrophages closely resembles that of TEMs, analysis using microarray and real time RT-PCR demonstrated that they do not appear to express statistically significantly elevated levels of Tie2 (Fig 4.12). As a hallmark of TEMs is the expression of Tie2 (De Palma et al. 2005; Pucci et al. 2009), it was surprising not to observe a significant increase of this in LYVE-1-positive macrophages. It may be that at the developmental stage analysed in this chapter, LYVE-1-positive macrophages have not been induced to express elevated levels of Tie2 by the appropriate stimuli. For example, expression of Tie2 on macrophages has been shown to be up-regulated in response to hypoxia (Murdoch et al. 2007). Alternatively, expression of Tie2 in monocytes may differ across tissue types, with only a small proportion of LYVE-1-positive macrophages expressing Tie2 in the dermis. It may be that the LYVE-1-positive macrophages are precursors to the more mature TEMs that arise during adult tumour states. A lineage tracing approach could be used to test this hypothesis.

In conclusion, this study has provided evidence to illustrate that lymphatic endothelial cells arise independently of the myeloid lineage during embryogenesis and in the tumour microenvironment, therefore excluding monocytes/macrophages as a source of lymphatic endothelial progenitor cells in these settings. It has also provided the first characterisation of LYVE-1-positive macrophages during development, demonstrating that they closely resemble Tie2-expressing monocytes which have an established role in angiogenesis. These data suggest that macrophages may be providing signals during development that contribute to lymphatic vascular morphogenesis and remodelling. Subsequent studies presented in Chapter 5 aimed to determine whether macrophages are required for normal
lymphatic development, and to characterise the macrophage-derived signals driving embryonic lymphangiogenesis.
5. Analysing the role of macrophages in embryonic lymphatic vascular patterning and proliferation.
5.1. Introduction

The studies documented in Chapter 4 of this thesis revealed an intimate association of macrophages with the developing lymphatic vasculature. While lineage tracing studies determined that macrophages do not comprise a pool of lymphatic endothelial progenitor cells, Chapter 5 sought to determine whether macrophages play a role during lymphatic development by providing growth and patterning signals that guide lymphangiogenesis.

In models of adult neo-lymphangiogenesis, macrophages recruited to sites of inflammation have been demonstrated to promote the growth of new lymphatic vessels via the production of lymphangiogenic growth factors VEGF-A, VEGF-C and VEGF-D (Cursiefen et al. 2002; Cursiefen et al. 2004; Alitalo et al. 2005; Baluk et al. 2005; Schledzewski et al. 2006; Jeon et al. 2008; Kim et al. 2009; Kubota et al. 2009). Inhibition of macrophage infiltration, via blockade of macrophage chemoattractants or chemical depletion of macrophages, has been shown to decrease the pathology associated with inflammatory and tumour-stimulated lymphangiogenesis (Cursiefen et al. 2004; Kataru et al. 2009; Kubota et al. 2009). In addition, blocking the activity of macrophage-derived VEGF-C and VEGF-D is sufficient to decrease pathological lymphangiogenesis (Chen et al. 2004; Baluk et al. 2005; Jeon et al. 2008). These results demonstrate that macrophages largely contribute to pathogenesis associated with acute inflammation via the production of lymphangiogenic growth factors.

Macrophages also have an established pro-angiogenic role in vivo during development and in states of neo-angiogenesis promoted by inflammatory stimuli and tumours (Bergers et al. 2000; Espinosa-Heidmann et al. 2003; Sakurai et al. 2003; Pollard 2004; Stockmann et al. 2008). While macrophages may not comprise a major source of VEGF-A in tumours, conditional deletion of macrophage-derived VEGF-A leads to abnormal tumour vasculature (Stockmann et al. 2008). In addition to production of VEGF-A which acts directly on the
blood vasculature, macrophages can produce growth factors such as matrix metalloproteases (MMPs) which act indirectly to increase bioavailability of VEGF-A, thereby stimulating angiogenesis (Cho et al. 2007; Bourlier et al. 2008). Recent work has demonstrated that macrophages promote cell fusion downstream of VEGF-mediated tip cell induction in the vascularisation of the developing brain (Fantin et al. 2010). These Tie2-positive macrophages share a similar gene expression profile to those that have been shown to promote the angiogenic switch in cancer (Pucci et al. 2009; Fantin et al. 2010). Macrophages are also involved in remodelling the complex developing vasculature in the eye via the canonical WNT pathway; macrophage-derived Wnt7b promotes the programmed cell death of vascular endothelial cells (Lobov et al. 2005). This death switch is regulated by angiopoietin 2, which acts in a dual manner to suppress survival signals in vascular endothelial cells, and stimulate macrophage production of Wnt7b (Rao et al. 2007). These studies establish a crucial role for macrophages in various aspects of vascular development, and suggest that they could be targeted for novel angiogenic therapies.

The studies described above have suggested that macrophages are involved in lymphangiogenesis and angiogenesis, however the requirement and specific role of macrophages in embryonic lymphangiogenesis had not yet been precisely determined. The aim of this chapter was to determine whether macrophages are required for embryonic lymphangiogenesis, and to identify and characterise macrophage-derived signals that influence lymphangiogenesis.

5.2. Macrophage-deficient mice have hyperplastic dermal lymphatic vessels.

To determine whether cells of the myeloid lineage are required for normal embryonic lymphangiogenesis, the process of lymphatic vascular development was investigated in $PU.1^{-/-}$ embryos. Transcription factor PU.1 is a hematopoietic-specific member of the ets
family, and is required for the development of multiple hematopoietic lineages. Mice lacking the domain containing PU.1 are devoid of macrophages, granulocytes and lymphocytes, and die around birth due to severe septicaemia (Scott et al. 1994; McKercher et al. 1996; Back et al. 2004; Dakic et al. 2005). PU.1⁻/⁻ mice do contain erythrocytes and megakaryocytes, with PU.1 being expressed at low levels in erythroid progenitor cells, but becoming down-regulated upon erythrocyte terminal differentiation (Back et al. 2004). In addition, PU.1 has also been shown to act as a tumour suppressor in myeloid leukemia (Cook et al. 2004).

Initial immunostaining of PU.1⁻/⁻ embryos revealed that these mice were completely devoid of F4/80-positive macrophages (Fig 5.1B, 5.3J-L), confirming the ideal nature of this model in which to investigate the requirement of macrophages for embryonic lymphangiogenesis. Assessment of the development and patterning of the lymphatic vasculature in embryonic PU.1⁻/⁻ skin by whole mount immunostaining at E16.5 revealed strikingly enlarged lymphatic vessels in comparison to wild-type counterparts (Fig 5.1A, B, D, E, G). Enlarged dermal lymphatic vessels was also confirmed by analysis of embryonic sections (Fig 5.1H, I). This data suggested a role for macrophages during the patterning of the lymphatic vasculature in the embryonic dermis.

As PU.1 is required for the differentiation of lymphocytes and granulocytes as well as macrophages (McKercher et al. 1996; Back et al. 2004), lymphatic vascular patterning was investigated Rag2⁻/⁻ embryos deficient in mature B and T lymphocytes. Rag2⁻/⁻ embryos contain immature B and T lymphocytes, but these cells fail to mature and rearrange their immunoglobulin or T cell receptor loci due to a lack of V(D)J activity (Shinkai et al. 1992). No other abnormalities have been observed in Rag2⁻/⁻ mice. Whole mount immunostaining of embryonic Rag2⁻/⁻ skin revealed that these mice indeed contain an abundance of F4/80-positive macrophages (Fig 5.1C). Rag2⁻/⁻ embryos displayed lymphatic vessels of an
Figure 5.1. Dermal lymphatic vessel hyperplasia in macrophage deficient mice.

Whole mount immunostaining and confocal microscopy of E16.5 dorsal skin from mice of the indicated genotype revealed that Prox1 (A-C) and Nrp2 (D-F) -positive dermal lymphatic vessels are enlarged in macrophage-deficient $PU.1^{-/}$ (B, E) and $Csf1r^{-/}$ embryos (F) compared to WT (A, D) and $Rag2^{-/-}$ (C) counterparts. The absence of macrophages in $PU.1^{-/}$ embryos was confirmed by F4/80 immunostaining (B). Vessel enlargement is restricted to the lymphatic vasculature; patterning and calibre of CD31-positive blood vessels (D, E, F) in embryonic $PU.1^{-/}$ (E) and $Csf1r^{-/}$ skin (F) is indistinguishable from wild-type (D). Vessel diameter was quantified using LVAP and ImageJ software (G). Vessel hyperplasia in $PU.1^{-/}$ skin was confirmed by analysis of embryonic sections (H, I). Scale bars: A-F - 150 μm, H-I - 100 μm. Data are representative of at least six independent experiments, with the exception of $Csf1r^{-/}$ analysis, n=3. Data shown represent the mean ± SEM. p values were calculated using Student’s paired t-test. *p ≤ 0.05. Immunostaining of A-E was performed by Natasha Harvey.
**Figure G**

Bar graph showing the average vessel width (μm) for different genotypes: WT, PU.1-/-, Rag2-/-, Csfr1-/-.

**Figure H**

Images of LYVE-1/CD31 staining in WT and PU.1-/- genotypes.

**Figure I**

Images of Prox1/F4/80 staining in WT, PU.1-/-, Rag2-/-, and Csfr1-/- genotypes.

**Figure J**

Images of CD31/Nrp2 staining in WT, PU.1-/-, Rag2-/-, and Csfr1-/- genotypes.
indistinguishable calibre to wild-type counterparts, indicating that mature lymphocytes are not required for normal embryonic dermal lymphangiogenesis (Fig 5.1C, G).

To confirm observations that a lack of macrophages was responsible for the enlarged dermal lymphatic vessels observed in \textit{PU.1}^{−/−} embryos, a second macrophage-deficient mouse model was analysed. CSF-1 is the primary regulator of mononuclear phagocyte production. CSF-1 signalling is mediated via the CSF-1 receptor, CSF-1R (Dai et al. 2002). Csf1r^{−/−} embryos have a paucity of myeloid cells (Li et al. 2006). Enlarged lymphatic vessels were also observed in the dermis of Csf1r^{−/−} embryos (Fig 5.1F, G), further supporting a role for macrophages in the patterning of the dermal lymphatic vasculature.

To investigate whether a lack of granulocytes was likely to contribute to the phenotype observed in \textit{PU.1}^{−/−} and Csf1r^{−/−} mice, immunostaining of wild-type E14.5 sections and E16.5 dermis with antibodies directed against granulocytes was performed. Staining revealed very few granulocytes compared to the abundance of macrophages (Fig 5.2), suggesting that monocyte/macrophage deficiency, as opposed to lymphocyte or granulocyte deficiency, was responsible for lymphatic vessel enlargement in \textit{PU.1}^{−/−} and Csf1r^{−/−} embryonic skin. Taken together, these data suggest that macrophages regulate dermal lymphatic vessel calibre during embryonic development.

5.3. Macrophages play a tissue/context dependent role in lymphatic vascular development.

In order to ascertain whether the phenotype of lymphatic vessel enlargement in myeloid cell-deficient mice extended to all embryonic tissues, lymphatic vascular development and patterning in a range of \textit{PU.1}^{−/−} organs was investigated. The jugular lymph sacs at E14.5 were examined, as they are the initial lymphatic structures to be formed after Prox1-positive lymphatic endothelial cells bud away from the cardinal veins at E9.5 (Wigle and Oliver
Figure 5.2. Very few granulocytes reside adjacent to jugular lymph sacs or dermal lymphatic vessels.

Immunostaining for granulocytes through transverse sections of the jugular region (A, B) and dermal lymphatic vessels (C, D) of an E14.5 wild-type embryo. Whole mount immunostaining and confocal microscopy of E16.5 wild-type dorsal skin (E, F). Very few granulocytes (arrows), as detected with anti-Ly6G (A-D) or anti-Gr1 (E, F) antibodies, were observed at embryonic stages analysed. Granulocytes were not spatially associated with lymphatic vessels. n=2. Scale bars: 100 μm.
1999). In contrast to the phenotype in the skin, the jugular lymph sacs of E14.5 $PU.1^{-/-}$ embryos were significantly smaller than those of wild-type littermates (Fig 5.3A-M), despite the surrounding tissue being completely devoid of F4/80-positive macrophages as observed by immunostaining (Fig 5.3J-L). This suggests that macrophages may play a pro-lymphangiogenic role in this embryonic microenvironment. Lymphatic vessel patterning was also examined in other organs of $PU.1^{-/-}$ mice. No alteration in lymphatic vessel calibre or branching was observed in the pleural cavity (Fig 5.4A-D) or submucosa (Fig 5.4E-H) of E16.5 embryos compared to their wild-type counterparts. These data reveal that the role played by macrophages during embryonic lymphangiogenesis may be tissue/context dependent.

5.4. An increase in lymphatic endothelial cell proliferation leads to hyperplastic dermal lymphatic vessels in macrophage-deficient mice.

We next sought to determine the mechanism responsible for increased dermal lymphatic vessel calibre in $PU.1^{-/-}$ skin. Work of others had described PU.1 expression to be restricted to macrophages, lymphocytes and granulocytes (McKercher et al. 1996; Back et al. 2004; Dakic et al. 2005). In order to investigate whether PU.1 plays a cell autonomous role in this setting, it was assessed whether PU.1 was expressed in blood or lymphatic endothelial cells. Immunostaining analyses revealed that PU.1 was not apparent in blood or lymphatic endothelial cells but was observed in CD45-positive hematopoietic cells and LYVE-1-positive macrophages (Fig 5.5A-F). These data illustrate that PU.1 plays a non-cell autonomous role in lymphatic vascular patterning.

To further define the nature of the phenotype in the $PU.1^{-/-}$ dermis, the branching of lymphatic vessels and the proliferation of lymphatic endothelial cells was investigated. No significant difference in the number of lymphatic vessel branch points in dermal lymphatic vessels was observed (Fig 5.6A-C). This suggested that macrophages do not influence
Figure 5.3. *PU.1*\(^{-/-}\) embryos exhibit hypoplastic jugular lymph sacs.

Immunostaining of transverse sections through the jugular region of E14.5 wild-type (A-C) and *PU.1*\(^{-/-}\) (D-F) embryos with antibodies against Prox1 and CD31. The absence of macrophages in *PU.1*\(^{-/-}\) embryos was confirmed by immunostaining with an anti-F4/80 antibody (J-L). Dashed white lines outline the margins of the jugular lymph sac (JLS) at each level analysed (L1-L3). JLS size was quantified using ImageJ software (M). Scale bars: 100 µm. At least 3 independent embryos of each genotype were analysed. Data shown represent the mean ± SEM. p values were calculated using Student’s paired t-test. *p ≤ 0.05.
Figure 5.4. *PU.1*−/− embryos display normal lymphatic vasculature in the pleural cavity and submucosa.

Whole mount DAB immunostaining of E16.5 wild-type and *PU.1*−/− pleural cavity (A, B) and submucosa (E, F) with an antibody against LYVE-1. Lymphatic vessel calibre and branching in organs of *PU.1*−/− embryos was indistinguishable from WT (C, D, G, H). Vessel diameter was quantified using LVAP and ImageJ software. At least 3 independent embryos of each genotype were analysed. Data shown represent the mean ± SEM. p values were calculated using Student’s paired t-test. *p ≤ 0.05. DAB staining was performed by Natasha Harvey.
WT

PU.1−/−

Pleural Cavity Submucosa

AB

EF

C

Branching

WT

PU.1−/−

0

20

30

40

50

Branchpoints / image

D

Vessel Width

WT

PU.1−/−

0

10

20

30

40

Branchpoints / image

G

Vessel Width

WT

PU.1−/−

0

6

8

10

12

14

D

average vessel width (pixels)

H

average vessel width (pixels)
Figure 5.5. **PU.1 is not expressed in endothelial cells.**

Immunostaining and confocal microscopy of E14.5 wild-type embryo with antibodies against CD45, LYVE-1, CD31 and PU.1 (A-F). PU.1 is expressed in CD45-positive hematopoietic cells (B, arrows, C) and LYVE-1-positive macrophages (E, arrow), but not in lymphatic or blood vascular endothelial cells. Panels C and F are a single channel images of panels B and E, respectively. White circle in F illustrates location of PU.1-positive, LYVE-1-positive macrophage. Scale bars: A, D - 100 μm, B, C, E, F - 25 μm.
Figure 5.6. Lymphatic endothelial cell proliferation is increased in the *PU.1*−/− embryonic dermis.

Whole mount immunostaining of dorsal skin isolated from E16.5 wild-type and *PU.1*−/− embryos and stained with antibodies against Nrp2 and PH3. White dots denote branch points (A, B). Arrows illustrate PH3-positive, Nrp2-positive mitotic lymphatic endothelial cells (D, E). Scale bars: A, B - 150 μm. D, E - 100 μm. At least 4 independent embryos of each genotype were analysed. At least 3 (for branching analysis) or 6 (for proliferation analysis) fields of view were quantified per embryo. Data shown represent the mean ± SEM. p values were calculated using Student’s paired t-test. *p ≤ 0.05. Immunostaining was performed by Natasha Harvey.
lymphatic vessel sprouting in the dermis during development, as has been documented in the early postnatal retina (Gerhardt et al. 2003). To investigate lymphatic endothelial cell proliferation, skin was stained with antibodies against phosphohistone 3 (PH3) together with endothelial cell markers. These analyses revealed there was a significant increase in the number of PH3-positive lymphatic endothelial cells in $PU.1^{-/-}$ lymphatic vessels (Fig 5.6D-F). Taken together, this data suggests macrophages control either proliferation, survival or apoptosis of lymphatic endothelial cells.

Two potential mechanisms for the hyperplasia of lymphatic vessels in $PU.1^{-/-}$ mice were hypothesised; 1) macrophages provide a direct pro-death signal to embryonic lymphatic endothelial cells which normally restrains lymphatic vessel calibre, or, 2) macrophages provide signals that act to restrain lymphatic endothelial cell proliferation under normal conditions.

### 5.5. Macrophage-derived Wnt7b does not initiate apoptosis in dermal lymphatic endothelial cells.

To investigate whether macrophages direct a death-inducing signal to lymphatic endothelial cells, this study investigated the role of Wnt7b and angiopoietin 2 (Angpt2) in the regulation of dermal lymphatic vessel patterning. Recent work has demonstrated that macrophages direct postnatal regression of the retinal vasculature via an Angpt2 co-ordinated Wnt7b-initiated death switch (Lobov et al. 2005; Rao et al. 2007). In order to determine whether macrophage-derived Wnt7b delivers a death switch signal to lymphatic endothelial cells, dermal lymphatic vascular patterning was investigated in embryos harbouring a targeted inactivation of $Wnt7b$ selectively in macrophages ($Csf1r-iCre;Wnt7b^{floxed}$, Rao, Pollard and Lang, unpublished). No alterations in lymphatic vessel calibre were observed in these embryos (Fig 5.7A, B, E), nor in embryos homozygous for a hypomorphic $Wnt7b$ mutation ($Wnt7b^{d1/d1}$) (Lobov et al. 2005) (Fig 5.7D, E), suggesting that macrophage-derived Wnt7b
Figure 5.7. Macrophage-derived Wnt7b does not initiate apoptosis in embryonic dermal lymphatic endothelial cells.

Lymphatic vessel calibre and patterning was investigated using whole mount immunostaining for the indicated markers and confocal microscopy of dorsal skin from Wnt7b\textsuperscript{flox/-} (A), Csf1r-\textsuperscript{iCre};Wnt7b\textsuperscript{flox/-} (B), wild-type (C) and Wnt7b\textsuperscript{d1/d1} (D) embryos at E16.5. Neither lymphatic vessel patterning nor calibre was affected in Wnt7b mutant mice. Lymphatic vessel width was quantified using LVAP and ImageJ software (E). Scale bars: 150 μm. Data are representative of at least three independent experiments. Data shown represent the mean ± SEM. p values were calculated using Student's paired t-test. *p \leq 0.05. Immunostaining was performed by Natasha Harvey.
Prox1/Nrp2/CD31

A. Wnt7b^{flox/-}

B. Wnt7b^{flox/-}; Cre

SMA/Nrp2

C. WT

D. Wnt7b^{d1/d1}

E. Vessel Width

- Wnt7b^{flox/-}
- Wnt7b^{flox/-}; Cre
- WT
- Wnt7b^{d1/d1}

average vessel width (μm)
Chapter 5: Results

does not transmit a death signal to lymphatic endothelial cells during embryonic lymphatic vascular development. This also provided evidence to suggest that instead of regulating cell death via a Wnt7b-dependent mechanism, macrophages were providing signals that normally act to restrain lymphatic endothelial cell proliferation.

5.6. Angiopoietin 2 is a pro-survival signal in lymphatic endothelial cells.

To further investigate whether macrophages may orchestrate a death switch influenced by Angpt2 in embryonic lymphatic endothelial cells, removal of which could result in lymphatic vessel hyperplasia, lymphatic vessel patterning in an allelic series of \( PU.1;\text{Angpt2} \) mutant mice was examined. It was hypothesised that if Angpt2 was important for co-ordinating a death switch in lymphatic endothelial cells, removal of one or more copy of \( \text{Angpt2} \) might lead to increased hyperplasia of lymphatic vessels in \( PU.1^{-/-};\text{Angpt2}^{-/-} \) and \( \text{Angpt2}^{-/-} \) mice. In contrast, data revealed that removal of one allele of \( \text{Angpt2} \) in a \( PU.1^{-/-} \) background rescued the lymphatic hyperplasia phenotype, with lymphatic vessels displaying normal calibre in \( PU.1^{-/-};\text{Angpt2}^{-/-} \) mice (Fig 5.8A-C, E). In addition, the dermal lymphatic vessels of \( \text{Angpt2}^{-/-} \) mice were strikingly sparse and hypoplastic (Fig 5.8D, E). This is in agreement with previous data from Gale and colleagues, who demonstrated that \( \text{Angpt2}^{-/-} \) pups: 1) display a failure to develop a lymphatic collecting vessel phenotype, with impaired smooth muscle cell recruitment to collecting vessels, and 2) exhibit hypoplastic lymphatic capillaries (Gale et al. 2002; Dellinger et al. 2008). These observations suggest Angpt2 signalling has a pro-survival role in lymphatic endothelial cells during embryonic development.

Taken together with experiments performed in \( Wnt7b \) mutant mice, our data illustrated that macrophages do not initiate an Angpt2/Wnt7b mediated death switch in lymphatic endothelial cells that is responsible for the regulation of lymphatic vessel calibre. Accordingly, analysis of apoptotic cells in the dermis of wild-type and \( PU.1^{-/-} \) mice (Fig 5.8F, G) and the jugular lymph sac of \( c-fmsEGFP \) embryos (Chapter 4, Fig 4.2), revealed very
Figure 5.8. Angiopoietin 2 is a pro-survival signal in lymphatic endothelial cells.

Whole mount immunostaining and confocal microscopy of dorsal skin from an allelic series of E16.5 wild-type (A), PU.1+/−;Angpt2+/+ (B), PU.1+/−;Angpt2+/− (C) and PU.1+/−;Angpt2−/− (D) embryos demonstrates rescue of lymphatic vessel hyperplasia in PU.1+/−;Angpt2+/− skin (C compared to B) and pronounced lymphatic vessel hypoplasia in PU.1+/−;Angpt2−/− skin (D). The patterning and recruitment of vascular smooth muscle cells to the large arteries and veins in the dorsal skin of mutant mice (B-D) is indistinguishable from wild-type littermates (A). Nrp2-positive hair follicles are particularly pronounced in Angpt2−/− skin (D). Vessel width was quantified using LVAP and ImageJ software (E). Whole mount immunostaining and confocal microscopy of WT (F) and PU.1−/− (G) dorsal skin stained with antibodies against activated caspase-3 and VEGFR-3. Scale bars: A-D - 150 μm, F-G - 100 μm. Data are representative of at least four independent experiments. Data shown represent the mean ± SEM. p values were calculated using Student’s paired t-test. *p ≤ 0.05. Immunostaining was performed by Natasha Harvey
A SMA/Nrp2

E Vessel Width

F Caspase-3/VEGFR-3
few apoptotic lymphatic endothelial cells around the developing lymphatic vasculature. However, it appeared that caspase-3-positive cell corpses were more frequent in embryonic \textit{PU.1}^{-/-} skin, presumably due to lack of removal by phagocytic macrophages (Fig 5.8G). These data reveal that macrophages do not actively program the apoptosis of lymphatic endothelial cells in the dermis during development, nor are they likely to be recruited to the developing lymphatic vasculature to engulf apoptotic cell corpses. Rather, macrophages are likely to be providing signals that act to restrain the calibre of lymphatic vessels by regulating lymphatic endothelial cell proliferation.

5.7. Expression of lymphangiogenic factors are elevated in the skin of \textit{PU.1}^{-/-} mice.

In order to investigate the mechanism underlying increased proliferation of lymphatic endothelial cells in macrophage-deficient mice, the relative expression of known pro-lymphangiogenic factors was quantified in \textit{PU.1}^{-/-} skin compared to the skin of wild-type littermates using microarray analysis and real time RT-PCR. RNA and cDNA was prepared from the dorsal skin of wild-type and \textit{PU.1}^{-/-} embryos at E16.5. Validating the observation that macrophages are absent in the \textit{PU.1}^{-/-} skin (Fig 5.1), \textit{F4/80} (\textit{Emr1}) was significantly decreased by both array and real time RT-PCR in \textit{PU.1}^{-/-} skin (Fig 5.9). Data revealed that the expression of genes characteristic of lymphatic endothelial cells, including \textit{Prox1} and \textit{Nrp2} were elevated in \textit{PU.1}^{-/-} skin compared to wild-type littermates by both array and real time RT-PCR. Additional lymphangiogenic genes \textit{podoplanin} and \textit{Sox18} were also elevated by microarray analysis. Expression of the lymphatic marker \textit{LYVE-1} was elevated in wild-type skin, though to a lesser degree than \textit{Prox1}, by real time RT-PCR. This is presumably due to the fact that \textit{LYVE-1} is also expressed on macrophages, which are absent in the \textit{PU.1}^{-/-} dermis. The expression of genes characteristic of blood vascular endothelial cells (\textit{Nrp1}) or blood vessel-associated smooth muscle cells (\textit{Acta2}) was not significantly altered (Fig 5.9). These data reflect the fact that the hyperplasia observed in the skin of \textit{PU.1}^{-/-} mice was restricted to lymphatic vessels; the calibre of blood vessels and patterning of the major
Figure 5.9. Differential expression of genes in \textit{PU.1}^{-/-} skin compared to wild-type skin.

Following the extraction of RNA from E16.5 skin, microarray and real time RT-PCR analysis revealed factors differentially expressed in \textit{PU.1}^{-/-} skin compared to wild-type. Genes were classified as elevated or decreased in \textit{PU.1}^{-/-} skin by real time RT-PCR results. \(n=4\). For microarray, \(p\) values were calculated using step-up multiple test correction (Benjamini, 1995) to control the false discovery rate. \#Adjusted \(p\) values (Adj. \(p\)) of \(\leq 0.05\) were considered to be significant. For real time RT-PCR, \(p\) values were calculated using Student’s paired t-test. \*\(p \leq 0.05\).
<table>
<thead>
<tr>
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<th>Adj. p</th>
<th>fold change</th>
<th>p</th>
<th>Gene</th>
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arteries and veins appeared normal (Fig 5.1D, E). This is also the likely reason that expression of pan-endothelial marker CD31 (Pecam) was not significantly elevated in PU.1−/− skin.

In accordance with the increased proliferation of lymphatic endothelial cells observed in PU.1+/− mice, elevated levels of established pro-lymphangiogenic stimuli including Vegfc (Jeltsch et al. 1997), Vegfd (Figf) (Stacker et al. 1999), and Angpt2 (Gale et al. 2002) were observed in PU.1+/− skin (Fig 5.9). The expression of the angiogenic growth factor Vegfa was not significantly altered, again highlighting that the selective hyperplasia of lymphatic vessels. Confirming results that macrophage-derived Wnt7b is not involved in regulation of dermal lymphatic endothelial cell proliferation (Fig 5.7), Wnt7b expression was not significantly different between the wild-type and PU.1+/− dermis. When analysing receptors and ligands in the angiopoietin/Tie signalling axis, it was observed that Tie1 and Tie2 (Tek) were elevated in the PU.1+/− skin. The role of the angiopoietin/Tie signalling axis was the focus of future experiments (see 5.12).

PU.1+/− skin also contained elevated expression of established pro-angiogenic MMPs Mmp2 (Bruyere et al. 2008) and Mmp14. MMP-14 is a membrane bound matrix metalloproteinase that activates pro-MMP-2 (Sato et al. 1992), illustrating a mechanism by which the processing and activity of MMP-2, and potentially additional pro-lymphangiogenic proteins, could be further enhanced in macrophage-deficient skin. Broad spectrum MMP inhibitors have been shown to decrease the lymphangiogenic response associated with lung cancer (Nakamura et al. 2004), suggesting that an increase in MMP-2 and MMP-14 may contribute to dermal PU.1+/− lymphatic vessel hyperplasia. Pro-angiogenic Mmp9 (Bergers et al. 2000) levels were unchanged between wild-type and PU.1+/− dermis. As targeted deletion of MMP-9 affects blood vasculature (Yang et al. 2004; Ahn and Brown 2008) yet targeted deletion of MMP-2 affects lymphatic vasculature but not aortic vasculature (Bruyere et al. 2008), these
Chapter 5: Results

results may reflect a specific requirement of MMP-2 in lymphangiogenesis. Mmp8 expression was significantly decreased in PU.1⁻/⁻ mice, likely due to the fact MMP-8 is stored in secretory granules in neutrophils (Balbin et al. 2003; Dorweiler et al. 2008), which are absent in the PU.1⁻/⁻ dermis. Mmp8-deficient mice are more susceptible to inflammation and have impaired wound healing, with MMP-8 often mutated in melanoma (Gutierrez-Fernandez et al. 2007; Palavalli et al. 2009), yet how or whether this MMP regulates angiogenesis or lymphangiogenesis remains to be elucidated. Taken together, these data suggest that the increased proliferation of lymphatic endothelial cells in PU.1⁻/⁻ skin may occur as a direct result of the elevated expression and potentially increased activity of lymphangiogenic signals in the PU.1⁻/⁻ dermal microenvironment including Vegfc, Vegfd, Angpt2, Angpt1 and Mmp2.

5.8. Identification of novel factors potentially involved in lymphatic vessel hyperplasia in PU.1⁻/⁻ skin.

Increasing the amount of VEGF-C and VEGF-D in mice using genetic strategies or recombinant growth factors results in hyperplastic lymphatic vessels, which also display increased sprouting (Jeltsch et al. 1997; Stacker et al. 2001; Tammela et al. 2007). As PU.1⁻/⁻ mice did not display an increase in sprouting of the dermal lymphatic vasculature, it was predicted there may be other factors, in addition to VEGF-C/D, which contribute to lymphatic endothelial cell hyperproliferation and vessel hyperplasia. Therefore, the next aim of this work was to investigate novel candidate factors identified by microarray, which were either significantly elevated or decreased and that may contribute to the vessel hyperplasia observed in the PU.1⁻/⁻ dermis. Candidates were chosen for investigation and validated by real time RT-PCR based on their known angiogenic or lymphangiogenic activity and the degree to which they were differentially expressed in PU.1⁻/⁻ vs. wild-type skin. Platelet factor-4 (PF4), decreased 8.3 fold in PU.1⁻/⁻ skin by array, has been described as an inhibitor of tumour associated angiogenesis (Bikfalvi and Gimenez-Gallego 2004;
Yamaguchi et al. 2005), and has been shown to inhibit endothelial cell proliferation, migration and angiogenesis both in vitro and in vivo (Bikfalvi 2004). Cx3cr1, decreased 2.82 fold in PU.1\(^{−/−}\) skin by array, is normally expressed by macrophages, dendritic cells, T cells and fibroblasts (Blaschke et al. 2003), therefore it was expected this receptor would show decreased expression in the PU.1\(^{−/−}\) dermis. This receptor was of interest as Cx3cr1-deficient mice have enhanced neo-vascular response after injury in the cornea, due to an decrease in angiogenic inhibitors such as thrombospondin 1, thrombospondin 2 and ADAMTS1 (Lu et al. 2008). However, it has also been shown to have a pro-inflammatory response associated with an increase in Mmp2 and Vegfa expression (Blaschke et al. 2003; Ryu et al. 2008), therefore the exact role of this receptor in the angiogenic and lymphangiogenic process remains unknown. As with Cx3cr1, Ccr2, decreased 2.81 fold in PU.1\(^{−/−}\) skin by array, has been shown to have both pro-angiogenic, inflammatory effects (Salcedo et al. 2000; Fujii et al. 2006; Koga et al. 2008) and anti-angiogenic effects (Ochoa et al. 2007) depending on the setting. This suggests that both Ccr2 and Cx3cr1 may play context dependent roles in endothelial cell regulation. While all these factors may potentially contribute to the PU.1\(^{−/−}\) vessel hyperplasia, further analysis of their role was outside the scope of this thesis.

Many of the factors that were significantly decreased in PU.1\(^{−/−}\) skin are derived from mast cells, such as mast cell protease 4 (Mcpt4) (Tchougounova et al. 2005; Magnusson et al. 2009; Waern et al. 2009), mast cell chymase 1 (Cma1) (Miller and Pemberton 2002) and mast cell carboxypeptidase A3 (Cpa3) (Feyerabend et al. 2005). Upon further investigation, almost all mast cell chymases and proteases were significantly decreased in the PU.1\(^{−/−}\) dermis (Fig 5.9), indicating that PU.1\(^{−/−}\) mice are profoundly deficient in mast cells at E16.5. Although decreased expression of mast cell-derived factors may simply reflect a lack of mast cells in PU.1\(^{−/−}\) mice, this may also contribute to the PU.1\(^{−/−}\) phenotype. As mast cells are able to produce lymphangiogenic growth factors (Detoraki et al. 2009), the role of mast
cells in embryonic and inflammatory-stimulated lymphangiogenesis was investigated (Chapter 6).

5.9. **Macrophages are not the major source of lymphangiogenic growth factors VEGF-C and VEGF-D in the dermis.**

Macrophages have been hypothesised to drive lymphangiogenesis in settings of inflammation by producing pro-lymphangiogenic signals including VEGF-C and VEGF-D. Data illustrated above demonstrated that the expression levels of pro-lymphangiogenic factors including *Vegfc*, *Vegfd* and *Mmp2* were increased in the skin of macrophage-deficient mice (Fig 5.9), suggesting that macrophages do not constitute the major source of VEGF-C/D required for development and patterning of the lymphatic vasculature during embryogenesis. In order to investigate the relative expression levels of *Vegfc*, *Vegfd* and additional pro-lymphangiogenic stimuli in macrophages compared to the remaining cells in embryonic skin, F4/80-positive macrophages were isolated from single cell suspensions of embryonic dermis using MACS® technology. Macrophages were subjected to two rounds of purification on MACS columns to obtain a pure F4/80-positive population. RNA was prepared from these primary cells immediately to prevent changes in gene expression that may occur after cell culture. The expression of candidate genes was quantified in the macrophage population versus macrophage-depleted dermal cells using real time RT-PCR. In accordance with the *PU.1* array and real time RT-PCR data, the levels of *Vegfc*, *Vegfd* and *Mmp2* expressed by macrophages was less than 10% of the total levels of these pro-lymphangiogenic genes expressed in the embryonic dermis (Fig 5.10). In addition, macrophages were not the major source of pro-angiogenic *Vegfa*. In contrast, the expression of macrophage-specific genes including *F4/80 (Emr1)* and *Mmp8* was restricted to the F4/80-positive population (Fig 5.10) and *Mmp14* levels were comparable between populations.
Figure 5.10. Macrophages are not the major source of pro-lymphangiogenic growth factors in the embryonic dermis.

F4/80-positive macrophages were isolated from single cell suspensions of wild-type E16.5 skin using MACS® magnetic bead cell isolation technology. Following the extraction of RNA and synthesis of cDNA, real time RT-PCR was utilised to investigate relative gene expression in F4/80-positive versus F4/80-depleted skin fractions. Expression of F4/80 (Emr1) served as a control for the purity of cell fractionation. n=4 independent cell isolations for F4/80− and F4/80+ samples with the exception of Mmp14 and Vegfd analyses, n=3. Data shown represent the mean ± SEM. p values were calculated using Student’s paired t-test. *p ≤ 0.05.
**Lyve-1**

mRNA expression levels relative to Actb

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**F4/80 (Emr1)**

mRNA expression levels relative to Actb

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**Vegfc**

mRNA expression levels relative to Actb

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**Vegfd**

mRNA expression levels relative to Actb

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<tbody>
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**Vegfa**

mRNA expression levels relative to Actb

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**Mmp2**

mRNA expression levels relative to Actb

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**Mmp8**

mRNA expression levels relative to Actb

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**Mmp14**

mRNA expression levels relative to Actb

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Further investigation into the potential sources of pro-lymphangiogenic signals in the dermis revealed that in the wild-type setting, lymphatic endothelial cells express the majority of Angpt2 and Tie1 in the dermis, while Angpt1 and Vegfc were expressed at substantially higher levels in dermal cells depleted of blood and lymphatic endothelial cells and macrophages (Fig 5.11). These suggest that Angpt1 and Vegfc expression is increased in the dermal environment as a result of macrophage deficiency, though the precise cell types responsible for their production remain uncharacterised. Lymphatic endothelial cells were not the major source of Tie2 (Tek), suggesting that Angpt2 may signal through Tie1 on the lymphatic endothelium, instead of Tie2 as has been suggested (Tammela et al. 2005). Taken together, these data suggest that in contrast to settings of inflammation-stimulated neo-lymphangiogenesis, macrophages are not the major source of lymphangiogenic signals during embryonic lymphatic vascular development. However, macrophages do express low levels of pro-lymphangiogenic factors. Conditional deletion of VEGF-A in macrophages has been shown to affect tumour vasculature, despite no detectable change of VEGF-A expression (Stockmann et al. 2008). Therefore low gene expression does not necessarily rule out the importance, or the magnitude of active growth factors, provided by macrophages. In summary, these data illustrates that macrophages in the embryonic dermis normally restrain the expression of pro-lymphangiogenic growth factors and proteases produced by as yet uncharacterised cell population(s) resident in the dermal environment.

5.10. Isolation of dermal macrophages and lymphatic endothelial cells.

In order to investigate the mechanism by which embryonic dermal macrophages regulate the proliferation of lymphatic endothelial cells, in vitro cell culture assays were established. Primary embryonic lymphatic endothelial cells and macrophages have not previously been successfully been purified and cultured together. In order to recapitulate the in vivo setting as much as possible, primary macrophages and lymphatic endothelial cells were isolated
Figure 5.11. Expression of pro-lymphangiogenic genes in cell populations isolated from embryonic dermis.

F4/80-positive macrophages (MAC), lymphatic endothelial cells (LEC) and blood endothelial cells (BEC) were isolated from single cell suspensions of wild-type E16.5 skin using MACS® magnetic bead cell isolation technology. Following the extraction of RNA and synthesis of cDNA, real time RT-PCR was utilised to investigate relative gene expression in each population, as well as in dermal cells remaining following the depletion of MAC, LEC and BEC (depleted skin). n=3. Data shown represent the mean ± SEM. p values were calculated using Student’s paired t-test. *p ≤ 0.05.
from E16.5 embryonic skin, and were purified using a magnetic cell separation procedure (MACS®) and subjected to short term cell culture (Gordon et al. 2010) (Fig 5.12). Macrophages and lymphatic endothelial cells were subjected to two rounds of purification on MACS columns to obtain pure F4/80-positive and LYVE-1-positive populations (Fig 5.12). Purity of the isolated cell fractions was determined by immunostaining (Fig 5.13A) and real time RT-PCR (Fig 5.13B). As expected, lymphatic endothelial cells expressed Prox1 and LYVE-1, and macrophages expressed F4/80 and occasionally LYVE-1 (Fig 5.13A). Furthermore, lymphatic endothelial cells selectively expressed elevated levels of established lymphatic markers LYVE-1 and Prox1, and were negative for expression of established blood endothelial cell marker CD34 (Fig 5.13B). Likewise, macrophages expressed established macrophage factors F4/80 and LYVE-1, but not endothelial cell markers Prox1 and CD34 (Fig 5.13B). This data validated the success of the magnetic cell sorting strategy in isolating pure populations of lymphatic endothelial cells and macrophages.

As macrophages and lymphatic endothelial cells are cultured in different media with different supplements (see Chapter 2, Materials and Methods), a co-culture strategy was established. To investigate whether macrophages could grow in lymphatic endothelial cell media, cells were co-cultured EBM-2 medium supplemented with EGM-2 MV SingleQuots (EGM-2) and with 100 μg/ml M-CSF. Following culture, cells were fixed and immunostained to assess their identity. Macrophages retained F4/80 expression and lymphatic endothelial cells retained LYVE-1 and Prox1 expression (Fig 5.14A). As observed in the dermis, a sub-population of F4/80 macrophages was also LYVE-1-positive. The ability of isolated lymphatic endothelial cells to proliferate normally in co-culture media was also investigated. Increased cell density was observed between 1 to 3 days, as determined by increasing absorbance values at 490 nm in a Cell Titer Aqueous One Solution Proliferation Assay (Fig 5.14B), revealing lymphatic endothelial cells were able to proliferate in culture. The addition
Figure 5.12. Method for isolation of macrophages and lymphatic endothelial cells from the embryonic dermis.

Method used for isolation of macrophages (MAC), on the basis of F4/80 expression, and then lymphatic endothelial cells (LEC), on the basis of LYVE-1 expression. Cells were isolated from wild-type E15.5 or E16.5 dermis using a magnetic cell separation procedure (MACS®) as previously described (Gordon et al., 2010). Cells were subjected to two rounds of purification on MACS columns to obtain a pure F4/80-positive macrophage population and a pure LYVE-1-positive lymphatic endothelial cell population.
1) Macrophages labelled with rat anti-mouse F4/80 antibody and anti-rat MACS microbeads

2) Repeat separation to remove 10% contamination to get pure macrophage population

3) Remaining macrophages and haematopoietic cells labelled with rat anti-mouse F4/80 antibody and rat anti-mouse CD45 and anti-rat MACS microbeads

3) Lymphatic endothelial cells labelled with rabbit anti-mouse LYVE-1 antibody and anti-rabbit MACS microbeads

4) Repeat separation to remove 10% contamination to get pure lymphatic endothelial cell population

KEY
- F4/80+ macrophage
- rat F4/80 antibody
- anti-rat MACS bead
- CD45+ cell
- rat CD45 antibody
- LYVE-1+ LEC
- rabbit LYVE-1 antibody
- anti-rabbit MACS bead
Figure 5.13. High purity of isolated lymphatic endothelial cells and macrophages.

Immunostaining (A) and real time RT-PCR (B) of macrophages (MAC) and lymphatic endothelial cells (LEC) cells isolated using MACS® magnetic bead cell isolation technology (Fig 5.12). Results revealed a high degree of purity of isolated cell populations and success of the cell isolation procedure after 72 h of culture. Scale bars: 50 μm. n=3 independent cell isolations. Data shown represent the mean ± SEM.
B

mRNA expression levels relative to Actb

F4/80

LYVE-1

Prox1

CD34

A

merge

LYVE-1

Prox1

DAPI

merge

F4/80

LYVE-1

DAPI

macrophage

LEC

MAC

LEC

flow through

mRNA expression levels relative to Actb
Figure 5.14. Devising a co-culture strategy for macrophages and lymphatic endothelial cells.

(A) Immunostaining of macrophages and lymphatic endothelial cells (LEC) co-cultured in EGM-2 supplemented with M-CSF. Macrophages and lymphatic endothelial cells retained cellular identity. (B) Addition of macrophage supplement M-CSF did not affect lymphatic endothelial cell proliferation in culture. Increased absorbance at 490 nm correlates to an increase in the number of viable cells in culture. Scale bars: 50 μm. n=3 independent cell isolations. Data shown represent the mean ± SEM.
A

LEC macrophage co-culture

merge  F4/80  LYVE-1  DAPI

merge  F4/80  Prox1  DAPI

B

Proliferation of LEC

Absorbance at 490nm

Time after treatment

- no M-CSF
- 1 µg M-CSF
- 2 µg M-CSF
- 5 µg M-CSF

1 day  3 days
Chapter 5: Results

of macrophage supplement M-CSF did not appear to affect lymphatic endothelial cell proliferation (Fig 5.14B). Therefore it was determined for co-culture experiments, cells would be cultured in EGM-2 supplemented with 100 μg/ml M-CSF.

5.11. Macrophages regulate lymphatic endothelial cell proliferation in vitro.

This study next sought to investigate whether macrophages restrain lymphatic endothelial cell proliferation in vitro. In order to specifically measure lymphatic endothelial cell, but not macrophage, proliferation, lymphatic endothelial cells were stained with CellVue Claret and cultured with macrophages for up to 6 days. With each cell division, CellVue Claret fluorescence intensity is decreased. Upon completion of culture, CD31-positive lymphatic endothelial cells were analysed for relative CellVue Claret intensity (Fig 5.15A) by determining the proliferation index using the Proliferation Wizard module in ModFit LT (Verity Software House). The proliferation index is a measure of CellVue Claret intensity; an increase in proliferation index correlates to an increase in the number of cell divisions. When lymphatic endothelial cells were cultured with macrophages, lymphatic endothelial cell proliferation was stimulated (Fig 5.15B). Therefore embryonic macrophages possess the ability to provide pro-proliferative signals to lymphatic endothelial cells in vitro. This result was unexpected, as lymphatic endothelial cell proliferation is increased in vitro in PU.1⁻⁻ skin. This suggested macrophage regulation of lymphatic endothelial cell proliferation during development in vivo is likely to be mediated via an indirect mechanism that involves additional cell types in the embryonic dermis. In order to assess whether soluble factors in the skin bind to and block macrophage-derived pro-lymphangiogenic growth factors in vivo, conditioned media produced from whole skin depleted of macrophages was added to lymphatic endothelial cells co-cultured with macrophages. Unexpectedly, conditioned media did not affect the pro-proliferative effect of macrophages on lymphatic endothelial cells (Fig 5.15C). Therefore, the differential effects of macrophages on endothelial cell proliferation in
Figure 5.15. Effect of macrophage co-culture on lymphatic endothelial cell proliferation in vitro.

For analysis of co-culture of lymphatic endothelial cells (LEC) with macrophages, LEC were stained with CellVue Claret (CVC) and CD31 (A). With each LEC cell division, CVC intensity decreases. The proliferation index is a measure of CVC intensity; an increase in proliferation index correlates to an increase in the number of cell divisions. Values are expressed as fold change in the proliferation index compared to A) LEC, or B) LEC macrophage co-culture. Macrophages were able to stimulate LEC proliferation in vitro (B), which was unchanged by the addition of whole skin conditioned media (C). A is one representative experiment of n=3, indicating the gating of LEC for analysis and plot of CVC intensity of gated cells. (B) n=3 independent cell isolations, data shown represent the mean ± SEM. (C) n=1 independent cell isolation, data shown represent the mean. p values were calculated using Student’s paired t-test. *p ≤ 0.05.
A

<table>
<thead>
<tr>
<th>LEC</th>
<th>LEC macrophage co-culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gating</td>
<td>Gating</td>
</tr>
<tr>
<td>CVC intensity</td>
<td>CVC intensity</td>
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</table>

B

Proliferation of LEC

![Bar chart showing proliferation of LEC over time](image)

C

Proliferation of LEC

![Bar chart showing proliferation of LEC over time](image)
vivo and in vitro are not mediated by soluble factors produced by additional cell types in the skin.

In addition to co-culture studies, the effect of macrophage conditioned media on lymphatic endothelial cell proliferation was investigated to determine whether soluble factors produced by macrophages could influence proliferation in vitro. Lymphatic endothelial cells were cultured with conditioned media harvested from macrophages, whole dermal cell suspensions (to recapitulate conditions in the wild type dermis), or dermal cell suspensions depleted of macrophages (to recapitulate conditions in \( PU.1^{-/-} \) dermis). In contrast to what was observed in co-culture experiments, the addition of macrophage or dermal conditioned media to embryonic lymphatic endothelial cells did not result in significantly elevated lymphatic endothelial cell proliferation, as determined by the Cell Titer Aqueous One Solution Proliferation Assay (Fig 5.16). Blocking VEGF-C and VEGF-D signalling using a recombinant mouse VEGFR-3/Fc chimera had no effect on proliferation when cultured with conditioned media (Fig 5.16). These data suggest that macrophage-mediated lymphatic endothelial cell proliferation in this in vitro setting may rely on cell-cell contact.

5.12. Tie1 mediates lymphatic endothelial cell proliferation in vitro.

Given the rescue of dermal lymphatic vessel hyperplasia in \( PU.1^{+/-};Angpt2^{+/-} \) embryos, the phenotype of lymphatic vessel hypoplasia in \( Angpt2^{-/-} \) embryos, and the results observed from real time RT-PCR and microarray analysis, signalling via the angiopoietin/Tie axis was investigated. To establish siRNA transfection assays, primary lymphatic endothelial cells were transfected with fluorescently tagged siRNA (Fig 5.17A). Following the demonstrated success of transfection, siRNA directed against Tie1, Tie2 (Tek) or Angpt2 was introduced to primary embryonic lymphatic endothelial cells. Validation of knockdown by each siRNA was assessed by real time RT-PCR and Western blot for Tie2, however due to the lack of effective antibodies against mouse Angpt2 and Tie1, assessment of knockdown for these
Figure 5.16. Effect of conditioned media on lymphatic endothelial cell proliferation *in vitro*.

Culture of primary embryonic dermal lymphatic endothelial cells with macrophage or skin cell conditioned media, or skin conditioned media depleted of macrophages, did not result in elevated lymphatic endothelial cell proliferation. Addition of soluble VEGFR-3/Fc chimera did not affect proliferation. Increased absorbance at 490 nm correlates to an increase in the number of viable cells in culture. n=3 independent cell isolations. Data shown represent the mean ± SEM. p values were calculated using Student’s paired t-test. *p ≤ 0.05.
Proliferation of LEC

- Absorbance at 490nm
- VEGFR-3
- untreated
- macrophage conditioned media
- skin conditioned media
- macrophage depleted-skin conditioned media
- Time after culture: 24h, 48h

The graph shows the absorbance at 490nm for different conditions of LEC proliferation. The x-axis represents the presence (+) or absence (-) of VEGFR-3, and the y-axis represents absorbance. The graph includes data points for untreated, macrophage conditioned media, skin conditioned media, and macrophage depleted-skin conditioned media, with time points at 24h and 48h.
Lymphatic endothelial cells were successfully transfected with fluoroscein-tagged siRNA (A). Degree of knockdown of target genes *Tie2* (*Tek*) (B), *Tie1* (D) and *Angpt2* (E) was determined using real time RT-PCR after 72 h of culture. Reduction in Tie2 protein (C) was determined using Western blot analysis after 72 h of culture. Scale bars: 50 μm. n=3 independent cell isolations. Data shown represent the mean ± SEM. p values were calculated using Student's paired t-test. *p ≤ 0.05.*
mRNA expression levels relative to Actb

**A**

LEC

**B**

* Tie2 (Tek) *

- mRNA expression levels relative to Actb

**C**

- neg siRNA
- Tie2 siRNA

- β-actin

**D**

* Tie1 *

- mRNA expression levels relative to Actb

**E**

* Angpt2 *

- mRNA expression levels relative to Actb
genes was restricted to real time RT-PCR. When siRNA directed against Tie2 alone or in combination with Tie1 was introduced to lymphatic endothelial cells, expression of Tie2 decreased 60-80% as detected by real time RT-PCR (Fig 5.17B), with Tie2 protein reduced by 75% as detected by Western blot (Fig 5.17C). siRNA against Tie1, alone or in combination with Tie2, decreased Tie1 expression 60-80% (Fig 5.17D). Transfection with siRNA against Angpt2 decreased Angpt2 expression levels 65-80% (Fig 5.17E). Interestingly, transfection with siRNA against Tie1 decreased Angpt2 mRNA levels by approximately 60%, and transfection with siRNA against Angpt2 decreased Tie1 mRNA levels approximately 40%. This suggests a possible regulation loop between Angpt2 and Tie1.

As knockdown efficiency of targeted genes had been confirmed, the resultant effect on lymphatic endothelial cell identity and proliferation was assessed. Immunostaining of transfected cells revealed no change in expression of lymphatic endothelial cell markers CD31, Prox1, LYVE-1 or VEGFR-3 (Fig 5.18). This indicates lymphatic endothelial cell identity remains intact after siRNA mediated knockdown of Tie1, Tie2 and Angpt2. To measure proliferation of lymphatic endothelial cells after knockdown, transfected cells were cultured for 96 hours and proliferation was measured using the Cell Titer Aqueous One Solution Proliferation Assay. Reduction of Tie1, but not Tie2 or Angpt2 expression, resulted in a reduction of proliferation of approximately 20% compared to negative control siRNA (Fig 5.19). These data illustrate that signalling via the Tie1 receptor mediates pro-proliferative signalling events in lymphatic endothelial cells in vitro, supporting recently published data which revealed a critical role for Tie1 activity during lymphatic vascular development in vivo (D'Amico et al. 2010; Qu et al. 2010).
Figure 5.18. Lymphatic endothelial cell identity is maintained after siRNA transfection.

Immunostaining of transfected cells after 72 h of culture revealed siRNA mediated knockdown of Tie2 (Tek), Tie1 or Angpt2 did not affect expression or cellular localisation of lymphangiogenic factors Prox1 (A, B, D, E, G, H, J, K), CD31 (A, C, D, F, G, I, J, L), LYVE-1 (B, E, H, K) or VEGFR-3 (C, F, I, L). Scale bars: 100 μm. Data are representative of two independent experiments.
neg

A

B

C

Tie2

D

E

F

siRNA target

G

H

I

Tie1

J

K

L

Angpt2
Figure 5.19. Tie1 mediates lymphatic endothelial cell proliferation *in vitro*.

siRNA-mediated knockdown of *Tie1* in primary embryonic dermal lymphatic endothelial cells, but not *Tie2* or *Angpt2*, resulted in significantly decreased lymphatic endothelial cell proliferation when compared to negative control after 96 h of culture. Increased absorbance at 490 nm correlates to an increase in the number of viable cells in culture. Values are expressed as fold change compared to absorbance of lymphatic endothelial cells transfected with negative control siRNA. n=3 independent cell isolations. Data shown represent the mean ± SEM. p values were calculated using Student’s paired t-test. *p ≤ 0.05.
Fold Change in Absorbance at 490 nm

siRNA target  neg  Tie1  Tie2  Angpt2  Tie1/Tie2

Proliferation of LEC
5.13. Discussion

The role of macrophages and cells of the myeloid lineage in lymphangiogenesis has been an area of intense interest in recent years. Previous studies have suggested that cells of the myeloid lineage contribute to lymphangiogenesis by either; 1) constituting a pool of lymphatic endothelial progenitor cells, or, 2) providing a critical source of pro-lymphangiogenic growth factors. Work presented in this thesis has shown that lymphatic endothelial cells arise independently of the myeloid lineage during embryogenesis and in the tumour environment (Chapter 4) (Gordon et al. 2010). However, the close association of macrophages with lymphatic vessels, together with their established pro-inflammatory role in settings of inflammation, led us to investigate whether macrophages are required for the normal development of the lymphatic system. Our findings revealed that macrophage-deficient mice had abnormal jugular lymph sac development (Fig 5.3) and dermal lymphatic patterning due to an increase in dermal lymphatic endothelial cell proliferation (Fig 5.1 and Fig 5.6). The study determined that dermal macrophages are not a principal source of pro-lymphangiogenic signals during embryonic development (Fig 5.10), but act instead to restrain the calibre of dermal lymphatic vessels via a Wnt7b-independent pathway (Fig 5.7).

Our work also highlighted the important pro-lymphangiogenic roles of angiopoietin 2 and Tie1, with in vitro data suggesting that Tie1 acts to regulate lymphatic endothelial cell proliferation.

Analysis of lymphatic vascular development in macrophage-deficient \( PU.1^{-/-} \) and \( Csf1r^{-/-} \) mice revealed pronounced dermal lymphatic vessel hyperplasia in these mutants compared to their wild-type counterparts (Fig 5.1). This data is in contrast to the proposal that macrophages are the major source of pro-lymphangiogenic stimuli in settings of inflammation, where profound infiltration of macrophages accompanies a robust expansion of lymphatic vessels, presumably due to an increase in VEGF-C, VEGF-D and VEGF-A expression (Kataru et al. 2009). These functional lymphatic vessels assist in clearing
antigens and enhancing resolution inflammation. However, macrophages do not always stimulate growth of functional lymphatic vessels, as in states of chronic inflammation, hyperplastic, non-functional lymphatic vessels are formed after macrophage infiltration (Kajiya et al. 2009; Huggenberger et al. 2010). Whether the lymphatic vessels in PU.1−/− skin are non-functional remains to be elucidated, although edema was not a feature of PU.1−/− embryos suggesting this is unlikely to be the case.

Relative quantification analyses of pro-lymphangiogenic gene expression in macrophages compared to macrophage-depleted dermal cells revealed that cells of the macrophage lineage contribute less than 10% of the total Vegfc, Vegfd and Mmp2 levels expressed in the embryonic dermis (Fig 5.10). However, a role for macrophage-derived lymphangiogenic factors cannot be discounted based purely on their magnitude of expression. A recent study by Stockmann and colleagues (Stockmann et al. 2008) illustrated that conditional deletion of myeloid-derived VEGF-A has a profound effect on tumour angiogenesis. The vasculature of tumours lacking myeloid-derived VEGF-A was normalised, being less tortuous, with increased pericyte coverage and a decrease in vessel length. Despite the vascular normalisation observed in these tumours, there was an absence of a detectable change in the amount of total VEGF-A expressed in the tumour microenvironment (Stockmann et al. 2008). Therefore, despite the fact macrophages are not a major source of lymphangiogenic growth factors Vegfc, Vegfd and Mmp2 in the dermis, the loss of these myeloid-derived factors may still impact embryonic lymphangiogenesis. Definitive analysis of the role of macrophage-derived factors in embryonic and inflammation-stimulated lymphangiogenesis will rely on the generation of conditional knockout mice. Future studies in our laboratory aim to dissect the role of macrophage-derived Vegfc during development and inflammation-stimulated lymphangiogenesis.
In vitro co-culture experiments demonstrated that primary embryonic macrophages are able to stimulate primary embryonic lymphatic endothelial cell proliferation (Fig 5.15), suggesting that embryonic macrophages have the potential to promote lymphangiogenesis in this context. Macrophage-mediated proliferation in this in vitro setting may rely on cell-cell contact, as it was found that macrophage-conditioned media did not significantly promote lymphatic endothelial cell proliferation (Fig 5.16). Nevertheless, macrophage regulation of lymphatic endothelial cell proliferation in vitro is distinct to that which was observed in vivo. During inflammatory states, the mechanism by which macrophages promote lymphangiogenesis and whether the newly formed lymphatic vessels are functional, is dependent on both the inflammatory stimuli and tissue (Alitalo et al. 2005). Therefore, macrophages appear to play tissue-specific specialised lymphangiogenic roles. This would explain both the hypoplasia of jugular lymph sacs observed in PU.1<sup>−/−</sup> embryos (Fig 5.3), and the lack of lymphatic phenotype observed in the pleural cavity and submucosa (Fig 5.4). The distinct and varied role of macrophages may also reflect the distinct population of macrophages present in each tissue, as different populations have been shown to play distinct roles during developmental and tumour-stimulated angiogenesis (Pucci et al. 2009; Fantin et al. 2010).

Our studies determined that the hyperplasia of lymphatic vessels in the dermis of macrophage-deficient mice occurred not as a result of the absence of a macrophage initiated endothelial cell death switch (Fig 5.7), as occurs in the postnatal hyaloid blood vasculature (Lobov et al. 2005), but instead as a result of lymphatic endothelial cell hyperproliferation (Fig 5.6). Lymphatic endothelial cell hyperproliferation in macrophage-deficient skin is potentially due to increased expression, proteolytic processing and/or activity of pro-lymphangiogenic signals in the dermal microenvironment including Vegfc, Vegfd and Mmp2. Precisely how macrophages control the expression and activity of these pro-lymphangiogenic signals in the skin remains to be elucidated. Of particular interest was
the finding that the hyperplasia observed in dermal lymphatic vessels of *PU.1<sup>−/−</sup>* mice did not extend to dermal blood vessels (Fig 5.1). Both the calibre and the patterning of arteries, veins and capillaries in embryonic macrophage-deficient skin was indistinguishable from wild-type. In concordance with this data, the expression of pro-angiogenic genes important for blood vascular development, including *Vegfa*, was not altered in macrophage-deficient skin (Fig 5.9). In contrast, recent work has demonstrated that yolk sac-derived macrophages are essential for normal brain blood vascularisation (Fantin *et al.* 2010). Analysis of *PU.1<sup>−/−</sup>* hindbrains in this study revealed vascular abnormalities, with no defects observed for tip cell induction or sprouting, but a decrease in vascular complexity resulting from mis-alignment of tip cells for fusion. Macrophages were found to act downstream of VEGF-A mediated sprout formation by interacting with tip cells to promote vascular fusion (Fantin *et al.* 2010). Therefore, as described above, macrophages play a tissue-specific role in regulating vascular development, likely mediated by sub-populations of macrophages present in each tissue microenvironment.

Results from this study revealed abnormal developmental lymphangiogenesis in two genetic macrophage-deficient mouse strains, *PU.1<sup>−/−</sup>* and *Csf1r<sup>−/−</sup>* (Fig 5.1). Recent work by Suda and colleagues (Kubota *et al.* 2009) demonstrated that *op/op* mice, which have a reduced number of monocyte/macrophage lineage cells (Cecchini *et al.* 1994), exhibited a transient defect in postnatal lymphatic vascular development. In contrast to findings presented in this chapter, which did not observe any abnormalities in lymphatic vessel branching in *PU.1<sup>−/−</sup>* mice (Fig 5.6), reduced lymphatic vessel branching was observed in selected *op/op* tissues such as the postnatal retina, trachea and ears. It was not possible to examine postnatal lymphatic vessel development in *PU.1<sup>−/−</sup>* mice as they die around birth (Scott *et al.* 1994; McKercher *et al.* 1996; Back *et al.* 2004). Therefore, it was not plausible to determine whether the differences observed in lymphatic vascular phenotypes between *op/op* and *PU.1<sup>−/−</sup>* mice are due to different tissue types or developmental stage analysed, genetic
background of each mouse line, or retention of some macrophages in op/op mice. Regardless, results presented in this chapter confirmed pronounced dermal lymphatic vessel hyperplasia during embryogenesis in two independently generated lines of \( PU.1^{-/-} \) mice (McKercher et al. 1996; Dakic et al. 2005), as well as \( Csf1r^{-/-} \) mice (Dai et al. 2002). This demonstrates that in the absence of myeloid cells such as macrophages, dermal lymphatic vascular patterning is abnormal.

Recent work demonstrated that a subset of myeloid cells defined by a M2-polarised population of Syk expressing cells were important for developmental lymphangiogenesis (Bohmer et al. 2010). Syk is crucial for the separation of the blood and lymphatic systems during development, with lymphatic hyperplasia and incomplete separation of the blood and lymphatic system observed in \( Syk^{-/-} \) embryos (Sebzda et al. 2006). When the \( Syk^{-/-} \) M2-polarised myeloid population of cells were isolated from the skin, they were observed to produce elevated levels of lymphangiogenic and angiogenic factors (Bohmer et al. 2010). When this population of cells was isolated from the foetal liver, it was not found to express high levels of angiogenic factors compared to cells from \( Syk^{+/+} \) mice. When transplanted, skin-derived \( Syk^{-/-} \) cells stimulated lymphangiogenesis \textit{in vivo} by increasing the number of filopodia, similar to what was observed when mice were transplanted with recombinant VEGF-C (Tammela et al. 2005). However, \( Syk^{-/-} \) cells derived from foetal liver gave rise to lymphatic vessels with increased calibre without an increase in sprouting. This data again demonstrates that myeloid cells from different tissue compartments of the embryo have a different capacity to stimulate lymphangiogenesis. The significance of these Syk expressing myeloid cells in the context of our study remains to be elucidated, as no defects in the separation of the blood and lymphatic vascular systems was observed in \( PU.1^{-/-} \) embryos.

This study revealed a role for the angiopoietin/Tie signalling axis during lymphatic development, as revealed by knock-out mouse models (Fig 5.8), real time RT-PCR
analyses (Fig 5.9) and microarray analyses (Fig 5.9). Microarray analyses also revealed a potential role for myeloid lineages other than macrophages, such as mast cells, in regulating lymphangiogenesis (Fig 5.9), as investigated further in Chapter 6. Angpt2-deficient mice have defects in blood and lymphatic vessel development and remodelling (Gale et al. 2002); pups fail to undergo proper postnatal remodelling of the lymphatic vasculature and exhibit a collecting vessel phenotype, and dermal vessels prematurely recruit smooth muscle cells (Dellinger et al. 2008). In agreement with findings from this study, at postnatal day 0, Angpt2−/− pups have formed a primary plexus of lymphatic vessels in the dermis, but these vessels are hypoplastic (Fig 5.8) (Dellinger et al. 2008). When the first exon of Angpt2 is replaced with Angpt1 cDNA (Angpt2 knock-out, Angpt1 knock-in mice), the lymphatic vasculature forms and remolds normally, suggesting that activation of Tie receptors is required for normal lymphatic development (Gale et al. 2002; Dellinger et al. 2008). While expression of Tie2 has been reported on lymphatic vessels in the ear and intestine (Morisada et al. 2005; Tammela et al. 2005), a role for Tie2 in lymphatic development remains to be elucidated. Recent reports have failed to detect expression of Tie2 on both initial and collecting lymphatics (Srinivasan et al. 2007; Dellinger et al. 2008). This is also supported by the findings that no change in lymphatic endothelial cell proliferation was observed after reduction of Tie2 levels using siRNA targeting (Fig 5.19). This study also observed low levels of Tie2 in lymphatic endothelial cells compared to blood endothelial cells (Fig 5.11), suggesting that this receptor is likely to be more important for blood vascular development.

Targeted knockdown of Tie1 using siRNA suggested a role for the Tie1 receptor during lymphatic endothelial cell proliferation (Fig 5.19). While it has previously been reported that Tie1 is expressed on lymphatic vessels, is of capable being phosphorylated by Angpt1 and can form heterodimers with Tie2 (Iljin et al. 2002; Saharinen et al. 2005), until recently, a role for Tie1 in lymphatic vascular development had not been described. While the role of
Tie1 in lymphatic endothelial cell proliferation was being investigated in studies presented in this chapter, two groups described a role for Tie1 in normal lymphatic vascular development (D'Amico et al. 2010; Qu et al. 2010). In agreement with the finding that Tie1 is expressed in isolated lymphatic endothelial cells (Fig 5.11), Tie1 was observed in Prox1-positive lymphatic endothelial cells throughout development and during adulthood (Qu et al. 2010). Reduction in Tie1 levels using genetic targeting led to dilated and disorganised lymphatic vessels in all tissues analysed, and interestingly, an increase in lymphatic endothelial cell apoptosis leading to abnormal regression of the vasculature (Qu et al. 2010). This suggests that the decreased numbers of lymphatic endothelial cells observed in culture after Tie1 siRNA knockdown compared to negative control (Fig 5.19) may be due to an increase in apoptosis of lymphatic endothelial cells, in addition to a decrease in proliferation. Analysis of cell death in cells transfected with Tie1 siRNA could be used to distinguish between these possibilities.

The exact role of Tie1 in blood vascular development is still under investigation, as depending on the mechanism by which Tie1 levels were reduced in the mouse, it had either no effect on arterial and venous vasculature (Qu et al. 2010), or was essential for blood vessel integrity (D'Amico et al. 2010). Even when blood vascular malformations were observed, these occurred after lymphatic vessel abnormalities (D'Amico et al. 2010), indicating that the lymphatic phenotype in Tie1-deficient mice was not secondary to blood vascular defects. Findings presented in this chapter did not observe a synergistic effect of reducing lymphatic endothelial cell proliferation when both Tie1 and Tie2 were knocked down (Fig 5.19). This suggests that while Tie1 is capable of forming heterodimers with Tie2 (Saharinen et al. 2005), it signals alone to influence lymphatic endothelial cell proliferation and/or survival. Unexpectedly, our results revealed no change in lymphatic endothelial proliferation or survival in vitro after reduction of Angpt2 levels (Fig 5.19). Another member of the angiopoietin family, Angpt1, has been shown to activate both Tie1 and Tie2 in vitro.
(Saharinen et al. 2005), and to promote lymphatic vessel sprouting, proliferation and vessel hyperplasia in vivo (Tammela et al. 2005). This suggests that Angpt1 may signal through Tie1 or Tie2 to compensate for the loss of Angpt2. Future studies will investigate the role of Angpt1 in lymphatic endothelial cell proliferation and survival by performing siRNA mediated knockdown experiments.

In conclusion, this study has determined that cells of the myeloid lineage are essential for normal embryonic lymphatic vascular development. They provide signals that regulate dermal lymphatic vessel calibre by controlling pro-lymphangiogenic growth factor expression, hence restraining lymphatic endothelial cell proliferation. Myeloid cell regulation of lymphangiogenesis was not uniform in all tissues, suggesting that sub-populations of macrophages play tissue-specific roles in regulating vascular development. A novel role for the receptor Tie1 in regulating lymphatic endothelial cell proliferation was also identified. Whether macrophages signal via this receptor remains unresolved and will require further study. Defining the precise identity, role and relative contribution of myeloid cell-derived signals during developmental and inflammation stimulated neo-lymphangiogenesis will be the focus of future work.
6.1. Introduction

Mast cells are a population of myeloid cells with an evolutionary conserved role in pathogen surveillance. While they have been suggested to arise from a multipotent progenitor cell independently of the common myeloid progenitor cell in the adult, mast cells share expression of transcription factors essential for the development of other myeloid cell lineages, such as macrophages and megakaryocytes (Walsh et al. 2002; Chen et al. 2005). Mast cells are found at sites of the body that interface with the environment, such as the skin, airways and digestive tract, and localise in the close vicinity of blood vessels, lymphatic vessels, nerve fibres and immune cells (Abraham and St John 2010). After mast cell precursors have migrated from the bone marrow to their resident tissue, they mature to fulfil specific roles. When stimulated by pathogens, allergens or toxins, mast cells proliferate, differentiate and degranulate, releasing a wide range of inflammatory mediators that include serine proteases, chymases and other kinds of proteases (Pejler et al. 2010). The mechanism of action of these inflammatory mediators is dependent on the tissue type and inflammatory stimulus.

Mast cells have a dual role in regulating the inflammatory process, as they can act to promote and suppress the inflammatory response (Galli et al. 2008). They have classically been viewed as a pro-inflammatory cell type, promoting the initiation and magnitude of inflammation in response to pathogens, and in settings of allergic and autoimmune disease (Malaviya and Abraham 2001; Grimbaldeston et al. 2006; Sayed et al. 2008; Magnusson et al. 2009). Promotion of inflammation by mast cells may act to limit pathology by clearing bacterial infections, or may contribute to pathogenesis in cases where inflammation is positively correlated with pathology (Lee et al. 2002; Nakae et al. 2007; Pejler et al. 2010). However, recent studies revealed that mast cells can also negatively regulate the inflammatory response, as they limit the skin pathology associated with contact hypersensitivity responses (Grimbaldeston et al. 2007) and chronic ultraviolet B (UVB;
wavelengths 280-320 nm) irradiation (Grimbaldeston et al. 2007; Biggs et al. 2010). Mast cells also contribute to immunosuppression after *Anopheles* mosquito bites (Depinay et al. 2006) and during induction of tolerance to skin allografts (Lu et al. 2006). The exact mechanisms by which mast cells negatively regulate the immune response are yet to be fully elucidated.

Mast cells have recently been shown to play a role in tumour progression, with mast cell localisation around human tumours associated with poor prognosis (Crivellato et al. 2008). In addition, mast cell accumulation is associated with the expansion and survival of B-cell tumours, multiple myeloma, epithelial carcinogenesis and colon cancer in the mouse (Coussens et al. 1999; Nakayama et al. 2004; Gounaris et al. 2007; Soucek et al. 2007). Promotion of tumour growth by mast cells occurs via induction of the angiogenic switch, characterised by activation and expansion of the tumour and tumour-associated vasculature, which is essential for tumour growth and metastatic dissemination. Induction of the angiogenic switch may occur via the release of a range of mast cell-derived pro-angiogenic mediators such as VEGF-A, IL-8, MMP-9, MMP-2, Angpt1, FGF-2, TGF-β and TNF-α (Coussens et al. 1999; Nakayama et al. 2004; Soucek et al. 2007; Theoharides et al. 2007; Crivellato et al. 2008). However, little is known about mast cell regulation of lymphangiogenesis despite their established production of lymphangiogenic growth factors VEGF-C and VEGF-D (Detoraki et al. 2009). A recent study by Brideau and colleagues demonstrated that blocking mast cell migration and adhesion to squamous cell carcinomas led to decreased tumour-associated lymphangiogenesis, and as a result, decreased lymph node metastasis (Brideau et al. 2007). However, the mechanisms by which mast cells regulate lymphangiogenesis are still largely undefined.
The aims of this chapter were to determine whether mast cells are required for normal lymphatic vascular development and remodelling, and to investigate whether mast cells regulate lymphangiogenesis during inflammation in the adult mouse.


Microarray and real time RT-PCR analysis of $PU.1^{-/-}$ mice compared to their wild-type counterparts revealed a significant decrease in numerous mast cell-derived factors in the $PU.1^{-/-}$ dermis, such as mast cell protease 4 ($Mcpt4$), mast cell chymase 1 ($Cma1$) and mast cell carboxypeptidase A3 ($Cpa3$) (Fig 5.9). To determine whether mast cells were present or absent in $PU.1^{-/-}$ embryos, sections from E16.5 and E18.5 $PU.1^{-/-}$ embryos and their wild-type counterparts were stained with Toluidine Blue, a dye that binds to the cytoplasmic granules of maturing mast cells. Toluidine Blue staining revealed that differentiated mast cells were absent in $PU.1^{-/-}$ embryos (Fig 6.1E, F, I, J). Analysis of wild-type sections revealed that very few mast cells were present in the embryo at E14.5, however some immature granular cells were detected in the dermis (Fig 6.1A, B). Both the number and maturity of mast cells in the dermis increased progressively during development (Fig 6.1A, B, C, D, G, H). Though less numerous than in the dermis, mast cells were observed in a wide variety of tissues at E18.5, being most abundant in the muscle and adrenal gland (Fig 6.2). To the best of our knowledge, this is the first analysis of mast cell localisation during embryogenesis in the mouse. While this study did not investigate the presence of mast cell progenitor cells during development, our data reveals that granular/differentiated mast cells are first detected in the embryo at E14.5 and progressively increase in number and maturity until birth.
Figure 6.1. *PU.1*−/− mice lack mast cells which are present in the dermis of wild-type embryos during development.

Toluidine blue staining of 10 μm of WT and *PU.1*−/− embryonic tissue sections revealed rare immature mast cells at E14.5 (A, B, arrows), numerous maturing mast cells at E16.5 (C, D), and abundant granular mast cells at E18.5 in WT dermis (G, H). No mast cells were observed in *PU.1*−/− embryos (E, F, I, J). Inset panels show higher magnification of mast cells.

Scale bars: 100 μm. JLS, jugular lymph sac.
Figure 6.2. Mast cells are present in multiple tissues of E18.5 embryos.

Toluidine Blue staining of 10 μm wild-type embryonic tissue sections revealed the presence of mast cells in the gut, diaphragm, kidney and pancreas (arrows). Mast cells were most abundant in the adrenal gland and muscle (arrows). Inset panels show higher magnification of mast cells. No mature mast cells were observed in the liver or lung. Scale bars: 100 μm.
6.3. Mast cell-deficient c-Kit mutant mice exhibit normal adult lymphatic vascular patterning in adult skin.

In order to determine whether mast cells play important roles during embryonic lymphatic vascular development and inflammation-stimulated lymphangiogenesis, these processes were investigated in mast cell-deficient c-Kit mutant mice. c-Kit mutant mice, WBB6F1-Kit^{W/Wv} (Kitamura et al. 1978; Galli et al. 2005) were utilised as a mast cell-deficient mouse model, as these mice have profoundly reduced numbers of mast cells (Galli et al. 2005). Kit^{W} contains a mutation encoding a truncated KIT protein not expressed on the cell surface due to lack of a transmembrane domain, and Kit^{Wv} contains a point mutation that decreases the kinase activity of the receptor. As well as being mast cell-deficient, Kit^{W/Wv} mice are profoundly deficient in melanocytes and neutrophils, and display several other abnormalities such as anaemia, sterility, and a decrease in the interstitial cells of Cajal in the intestinal tract (Chervenick and Boggs 1969; Galli et al. 2005; Grimbaldeston et al. 2005; Zhou et al. 2007).

Lymphatic vascular patterning in the adult ear skin of Kit^{W/Wv} mice was indistinguishable from that of wild-type counterparts; no discernible differences in vessel width or branching were obvious in mutant mice, as analysed by whole mount immunostaining and confocal microscopy (Fig 6.3). Additionally, no abnormalities in blood vascular patterning were observed in mast cell-deficient mice (Fig 6.3A, B). As mature mast cells are not present during the crucial stages of lymphatic development in the embryo, E9.5 to E14.5 (Oliver and Harvey 2002) (Fig 6.1), and given that no defects were observed in the adult lymphatic vasculature of Kit^{W/Wv} mice, it is unlikely that mast cells are required for normal embryonic lymphangiogenesis. Due to the time constraints of this PhD project, the breeding and analysis of embryonic Kit^{W/Wv} mice was not undertaken. Nevertheless, these data suggest that mast cells are not required for normal lymphatic vessel development or maturation.
Figure 6.3. Mast cell-deficient c-Kit mutant mice exhibit normal lymphatic vessel patterning in adult ear skin.

Whole mount immunostaining and confocal microscopy of WT and mast cell-deficient Kit<sup>W/Wv</sup> ears revealed that the width (C) and branching (D) of lymphatic vessels in Kit<sup>W/Wv</sup> mice (B) was indistinguishable from WT counterparts (A). Lymphatic vessel diameter and branching was quantified using LVAP and Image J software. Scale bars: 150 μm. Data are representative of one mouse of each genotype. Data shown represent the mean ± SD. Immunostaining was performed by Natasha Harvey.
CD31/SMA/LYVE-1

A  WT  

B  Kit

Average vessel width (pixels)

C  Vessel Width

D  Branching

Branch points/field of view
under steady-state conditions, and illustrate that the primary cause of the lymphatic vascular defects observed in $PU.1^{-/-}$ mice is not the absence of mast cells.

6.4. **mMCP-4-deficient mice exhibit normal embryonic and adult lymphatic vascular patterning.**

Mouse mast cell protease 4 (mMCP-4) (encoded by *Mcpt4*) has an established role in regulating pathology in diseases associated with aberrant lymphangiogenesis, such as tumour development, arthritis and airway inflammation (Tchougounova *et al.* 2005; Zhang *et al.* 2007; Magnusson *et al.* 2009; Waern *et al.* 2009). mMCP-4 is the closest mouse homologue to human chymase, with similar substrate specificity, tissue distribution and serglycine-binding properties (Pejler *et al.* 2010). *Mcpt4*−/− mice display less severe collagen-induced arthritis, increased susceptibility to airway inflammation, defective fibronectin and pro-MMP-9 processing, reduced basal intestinal permeability and protection from abdominal aortic aneurysm (Tchougounova *et al.* 2005; Groschwitz *et al.* 2009; Magnusson *et al.* 2009; Sun *et al.* 2009; Waern *et al.* 2009). However, *Mcpt4*−/− mice have no alterations in mast cell activity, morphology or activity of other mast cell proteases (Tchougounova *et al.* 2003). Given the published data, this study sought to define the role of mMCP-4 during embryonic and inflammation-stimulated lymphangiogenesis.

In order to investigate the role of mMCP-4 in embryonic lymphatic vascular development, the dermal lymphatic vasculature of *Mcpt4*−/− embryos was investigated (Tchougounova *et al.* 2003). No abnormalities in lymphatic vascular patterning in the dermis of *Mcpt4*−/− embryos were observed at E16.5; lymphatic vessel branching and calibre were indistinguishable from wild-type counterparts (Fig 6.4). When compared to wild-type, the *Mcpt4*−/− dermis displayed normal infiltration of LYVE-1-positive macrophages (Fig 6.4C, D) and CD45-positive hematopoietic cells (Fig 6.4E, F). The patterning of the lymphatic vasculature in the ear skin of adult mice was also investigated using whole mount immunostaining and confocal
Figure 6.4. *Mcpt4*−/− embryos exhibit normal embryonic dermal lymphatic vessel patterning.

Whole mount immunostaining and confocal microscopy of E16.5 dorsal skin revealed that the width and branching of lymphatic vessels in embryonic *Mcpt4*−/− skin (B, D, F, G, H) was indistinguishable from WT counterparts (A, C, E, G, H). In addition, no substantial differences in the infiltration of LYVE-1-positive macrophages and CD45-positive hematopoietic cells to the dermis of *Mcpt4*−/− embryos were observed compared to WT (compare A-E to C-F). The CD31 (A-D) or CD45 (E, F) channel was removed to allow for clear analysis of lymphatic vessel diameter and branching. Vessel diameter and branching were quantified using LVAP and Image J software. Scale bars: 100 μm. Data are representative of at least three embryos for each genotype. Data shown represent the mean ± SEM.
**Figure 1:**

**Vessel Width**

**G**

- WT
- Mcpt4-/-

Average vessel width (μm)

- WT: 16
- Mcpt4-/-: 12

**Branching**

**H**

- WT
- Mcpt4-/-

Branch points / field of view

- WT: 35
- Mcpt4-/-: 30

**Prox1/NRP2**

**A**

- E16.5 WT
- Mcpt4-/-

**Prox1/LYVE-1**

**B**

- E16.5 WT
- Mcpt4-/-

**Prox1/LYVE-1**

**C**

- E16.5 WT
- Mcpt4-/-

**Prox1/NRP2**

**D**

- E16.5 WT
- Mcpt4-/-

**Prox1/LYVE-1**

**E**

- E16.5 WT
- Mcpt4-/-

**Prox1/NRP2**

**F**

- E16.5 WT
- Mcpt4-/-

**Prox1/NRP2**

**G**

- E16.5 WT
- Mcpt4-/-

**Prox1/LYVE-1**

**H**

- E16.5 WT
- Mcpt4-/-

**Prox1/NRP2**

**I**

- E16.5 WT
- Mcpt4-/-

**Prox1/LYVE-1**

**J**

- E16.5 WT
- Mcpt4-/-

**Prox1/NRP2**

**K**

- E16.5 WT
- Mcpt4-/-

**Prox1/LYVE-1**

**L**

- E16.5 WT
- Mcpt4-/-

**Prox1/NRP2**
microscopy; lymphatic vessel branching and calibre in adult Mcpt4−/− mice was indistinguishable from wild-type counterparts (Fig 6.5). Additionally, blood vascular patterning in the dermis during embryogenesis and in the adult appeared normal when analysed by immunostaining with pan-endothelial marker CD31 (Fig 6.4, 6.5). These results illustrate that mMCP-4 is not required for the development and/or remodelling of the dermal lymphatic vasculature.

6.5. Mast cells limit the angiogenic and lymphangiogenic response to chronic high dose UVB irradiation.

This study next sought to determine whether mast cells regulate inflammation-stimulated lymphangiogenesis. Data presented in Chapters 4 and 5 of this thesis, together with the work of others, has demonstrated that myeloid cells play distinct roles during embryonic and inflammation-stimulated lymphangiogenesis (Baluk et al. 2005; Kerjaschki et al. 2006; Maruyama et al. 2007; Jeon et al. 2008; Kataru et al. 2009; Kim et al. 2009; Gordon et al. 2010). Therefore, our finding that mast cells are unlikely to be required for developmental lymphangiogenesis does not preclude them from playing an important role in inflammation-stimulated lymphangiogenesis.

The model of inflammation-stimulated lymphangiogenesis chosen for this study was that induced by UVB irradiation. Excessive exposure to solar ultraviolet radiation, including UVB radiation (wavelengths between 280 and 320 nm) results in substantial tissue damage such as sunburn and photoaging (accelerated aging of the skin), via the induction of innate inflammatory responses at the site of irradiation (Kligman 1996; Gonzalez et al. 1999). Mice treated with multiple exposures of UVB irradiation over weeks to months display more mast cells at the site of irradiation, which is a feature also observed in photoaged human skin (Kligman 1996; Grimaldeston et al. 2006). A recent study observed that mast cells act as negative regulators of the inflammatory response after chronic UVB irradiation, limiting
Figure 6.5. *Mcpt4*⁻⁄⁻ mice exhibit normal lymphatic vessel patterning in adult ear skin.

Whole mount immunostaining and confocal microscopy of adult ear skin revealed that the calibre and branching of lymphatic vessels in *Mcpt4*⁻⁄⁻ mice (B, C, D) was indistinguishable from WT (A, C, D). Lymphatic vessel diameter and branching was quantified using LVAP and Image J software. Scale bars: 150 μm. Data are representative of at least two embryos for each genotype. Data shown represent the mean ± SD.
pathologies such as epidermal hyperplasia, epidermal necrosis and leukocyte infiltration (Grimbaldeston et al. 2007).

In order to assess the role of mast cells in UVB irradiation-induced lymphangiogenesis, Kit\(^{W/Wv}\) mice were compared to wild-type mice and also to Kit\(^{W/Wv}\) mice engrafted with wild-type bone marrow-derived cultured mast cells (WT BMCMC→ Kit\(^{W/Wv}\)), as wild-type mice, unlike Kit\(^{W/Wv}\) mice, are pigmented, giving protection against UVB irradiation (Galli and Kitamura 1987; Grimbaldeston et al. 2005). For initial experiments, mice were given a protective dose of UVB irradiation of 4 exposures of 1.2 kJ/m\(^2\) (equivalent to 0.5 erythema dose to induce slight redness without defined margins), followed by 6 exposures of 2 kJ/m\(^2\) (equivalent to 1 erythema dose to induce distinct homogeneous redness with defined margins), followed by 12 exposures of 4 kJ/m\(^2\) (equivalent to 2 erythema dose to induce frank redness), followed by 22 exposures of 8 kJ/m\(^2\) (equivalent to 4 erythema dose to induce very intense redness) (Grimbaldeston et al. 2007). Mice were irradiated with this regime of UVB to determine at which dose Kit\(^{W/Wv}\) mice display increased ear swelling compared to WT BMCMC→ Kit\(^{W/Wv}\) mice, and therefore at which dose mast cells begin to act as negative regulators of the inflammatory response, if at all. It was determined Kit\(^{W/Wv}\) mice began to display increased ear swelling compared to WT BMCMC→ Kit\(^{W/Wv}\) mice when the dosage was increased to 4 kJ/m\(^2\) (Fig 6.6A). However, after the irradiation regime of 8 kJ/m\(^2\) was completed, ear thickness was not significantly different between Kit\(^{W/Wv}\) and WT BMCMC→ Kit\(^{W/Wv}\) mice, suggesting mast cells are not as protective after this high UVB dose.

To assess the effect of UVB irradiation on lymphangiogenesis, the lymphatic vasculature of irradiated adult ear skin was initially analysed upon completion of the UVB exposure regime using whole mount immunostaining and confocal microscopy. However, due to the severe pathology and significantly increased thickness of the irradiated ears, analysis of irradiated
WT, Kit^{W/Wv} or WT BMCMC→ Kit^{W/Wv} mice were treated with 4 exposures of 1.2kJ/m², 6 exposures of 2kJ/m², 12 exposures of 4kJ/m² + 22 exposures of 8kJ/m² and ear swelling response measured. WT BMCMC→ Kit^{W/Wv} mice displayed significantly reduced ear swelling compared to Kit^{W/Wv} mice, until treated with high dose, 8 kJ/m² UVB irradiation, after which ear thickness became comparable (A). Whole mount immunostaining and confocal microscopy of ears revealed that when compared to WT (B), Kit^{W/Wv} mice (C) and WT BMCMC→ Kit^{W/Wv} mice (D) have abnormal lymphatic vasculature. Scale bars: 150 μm. Data shown represent the mean + SEM of the indicated number of mice per genotype from one independent UVB irradiation experiment. p values were calculated using one way ANOVA repeated measures with Tukey’s post test. *p < 0.01, **p < 0.001, N.S., not significant. UVB irradiation treatment and analysis of ear swelling response was performed by Boris Fedoric and Michele Grimbaldeston. Immunostaining was performed by Natasha Harvey.
Ear swelling response following chronic UVB exposure

![Graph showing ear swelling response following chronic UVB exposure.](image)

**A**

- WBBF1 (Kit\(^{+}\)) \(n=5\)
- WBBF1 (Kit\(^{-}\)) \(n=4\)
- WBBF1 (Kit\(^{-}\)) + WT BMCMC \(n=3\)

**B** WT

**C** Ki\(^{+/+}\)

**D** WT BMCMC

CD31/SMA/LYVE-1
skin using this methodology proved to be sub-optimal (Fig 6.6C, D). Tissue sections of irradiated ears were therefore prepared and subjected to immunostaining in order to precisely determine the effects of UVB irradiation on angiogenesis and lymphangiogenesis. A dramatic lymphangiogenic and angiogenic response after chronic UVB irradiation was observed in \( Kit^{W/Wv} \) and WT BMCMC→ \( Kit^{W/Wv} \) mice compared to WT mice (Fig 6.7). In correlation with the significant difference in ear swelling (Fig 6.6), quantification of vessel diameter revealed that the width of both lymphatic vessels and blood vessels was increased in \( Kit^{W/Wv} \) and WT BMCMC→ \( Kit^{W/Wv} \) mice compared to WT (Fig 6.7, 6.8). There was no significant difference in vessel width, vessel number or vessel density between \( Kit^{W/Wv} \) mice compared to WT BMCMC→ \( Kit^{W/Wv} \) mice (Fig 6.8), in agreement with the finding that there was no significant difference in ear swelling upon completion of the UVB exposure regime (Fig 6.6). While the number of both blood and lymphatic vessels was increased in \( Kit^{W/Wv} \) and WT BMCMC→ \( Kit^{W/Wv} \) mice compared to WT (Fig 6.8), there was a decrease in lymphatic vessel density (Fig 6.8). The increase in lymphatic vessel calibre coupled with decreased lymphatic vascular density in \( Kit^{W/Wv} \) and WT BMCMC→ \( Kit^{W/Wv} \) mice compared to WT may reflect an attempt by the lymphatic vasculature to compensate for increased interstitial pressure in response to chronic inflammation.

6.6. Mast cells limit the lymphangiogenic and angiogenic response to chronic high dose UVB irradiation without a preceding low UVB dose.

Results thus far suggested that mast cells are able to somewhat restrain the magnitude of ear swelling when high dose UVB irradiation was preceded by a low dose of UVB irradiation (1.2 kJ/m\(^2\) and 2 kJ/m\(^2\)). To investigate whether mast cells restrict the lymphangiogenic response without a preceding low dose of UVB irradiation, mice were treated with a regime of 12 exposures at 4 kJ/m\(^2\), followed by 30 exposures at 8 kJ/m\(^2\) (Fig 6.9). As has been described previously (Grimbaldeston et al. 2007), Toluidine Blue staining revealed that \( Kit^{W/Wv} \) mice displayed some mast cells in the ear pinnae after chronic UVB irradiation (Fig
Figure 6.7. Mast cell-deficient mice display a robust angiogenic and lymphangiogenic response to low dose UVB irradiation followed by chronic high dose UVB irradiation.

10 μm cryosections of UVB irradiated ears were immunostained with lymphatic and blood vascular markers. Lymphatic vessels and blood vessels were visualised at the tip, middle and base of the ear after UVB treatment of 4 exposures of 1.2kJ/m², 6 exposures of 2kJ/m², 12 exposures of 4kJ/m² + 22 exposures of 8kJ/m² in WT (A-C), Kit<sup>W/Wv</sup> (D-F) or WT BMCMC→ Kit<sup>W/Wv</sup> (G-I) mice. Irradiated ears imaged with a dissecting microscope indicate the lack of pigment in Kit<sup>W/Wv</sup> and WT BMCMC→ Kit<sup>W/Wv</sup> mice and reveal visible pathology induced by UVB treatment. When compared to WT, Kit<sup>W/Wv</sup> mice and WT BMCMC→ Kit<sup>W/Wv</sup> mice exhibit abnormal blood and lymphatic vasculature. Scale bars: 150 μm. Data are representative of WT n=3, Kit<sup>W/Wv</sup> n=2 and WT BMCMC→ Kit<sup>W/Wv</sup> n=1 from one independent UVB irradiation experiment.
Figure 6.8. Mast cells restrict the angiogenic and lymphangiogenic response to low dose UVB irradiation followed by chronic high dose UVB irradiation.

Quantification of vessel width (A), vessel number (B) and vessel density (C) from 10 μm cryosections of ears (Fig 6.7) after UVB treatment regimen of 4 exposures of 1.2kJ/m², 6 exposures of 2kJ/m², 12 exposures of 4kJ/m² + 22 exposures of 8kJ/m² in WT, Kit<sup>W/Wv</sup> or WT BMCMC→Kit<sup>W/Wv</sup> mice. Quantification was performed using LVAP and ImageJ software. WT n=3, Kit<sup>W/Wv</sup> n=2 and WT BMCMC→Kit<sup>W/Wv</sup> n=1 from one independent UVB irradiation experiment. At least six images were quantified per mouse. Data shown represent the mean ± SD.
4 exposures of 1.2 kJ/m², 6 exposures of 2 kJ/m², 12 exposures of 4 kJ/m² + 22 exposures of 8 kJ/m²

**Vessel Width**

- **WT**
- **Kit**
- **WT BMCMC**

**Vessel Number**

- **WT**
- **Kit**
- **WT BMCMC**

**Vessel Density**

- **WT**
- **Kit**
- **WT BMCMC**

Legend:
- Blue: Lymphatic Vessels
- Red: Blood Vessels
Figure 6.9. Mast cells limit the ear swelling response associated with chronic high dose UVB irradiation.

WT, Kit<sup>W/Wv</sup> or WT BMCMC→ Kit<sup>W/Wv</sup> mice were treated with 12 exposures of 4kJ/m<sup>2</sup> + 30 exposures of 8kJ/m<sup>2</sup> and ear swelling response measured. WT BMCMC→ Kit<sup>W/Wv</sup> mice displayed significantly reduced ear swelling compared to Kit<sup>W/Wv</sup> mice. Data shown represent the mean + SEM of the indicated number of mice per genotype from one independent UVB irradiation experiment. p values were calculated using one way ANOVA repeated measures Tukey’s post test. **p < 0.001, N.S., not significant. UVB irradiation treatment and analysis of ear swelling response was performed by Boris Fedoric and Michele Grimbaldeston.
Ear swelling response following chronic UVB exposure

- WBBF1 (Kit<sup>wt</sup>) n=4
- WBBF1 (Kit<sup>W/Wv</sup>) n=4
- WBBF1 (Kit<sup>W/Wv</sup>) + WT BMC n=5

Boris Fedoric
6.10E, F). However, quantification revealed that Kit<sup>W/Wv</sup> mice still displayed reduced numbers of mast cells when compared to WT and WT BMCMC→ Kit<sup>W/Wv</sup> mice (Fig 6.10G).

Both Kit<sup>W/Wv</sup> and WT BMCMC→ Kit<sup>W/Wv</sup> mice displayed an increase in blood and lymphatic vessel diameter and number, but not density, when compared to WT mice (Fig 6.11, 6.12). When WT BMCMC→ Kit<sup>W/Wv</sup> mice were compared to Kit<sup>W/Wv</sup> mice, reduced ear swelling accompanied by decreased lymphatic vessel diameter was observed (Fig 6.9, 6.12). This suggests that mast cells provide some protection against the magnitude of inflammation induced by this treatment regime. A trend towards reduced blood vessel diameter was also observed in WT BMCMC→ Kit<sup>W/Wv</sup> mice compared to Kit<sup>W/Wv</sup> mice. No significant differences in the number or density of lymphatic or blood vessels were observed between WT BMCMC→ Kit<sup>W/Wv</sup> mice and Kit<sup>W/Wv</sup> mice (Fig 6.12). Taken together, these data demonstrate that mast cells significantly limit inflammation-induced lymphatic vessel enlargement following this high dose UVB treatment regime.

The increase in lymphatic vessel diameter that was a feature of Kit<sup>W/Wv</sup> and WT BMCMC→ Kit<sup>W/Wv</sup> mice was envisioned to be due to either; elevated lymphatic endothelial proliferation within vessels, or, vessel dilation due to “stretching” of lymphatic endothelial cells. Preliminary analyses revealed an increase in the number of lymphatic endothelial cell nuclei within the large vessels of Kit<sup>W/Wv</sup> and WT BMCMC→ Kit<sup>W/Wv</sup> mice compared to the vessels of WT mice (Fig 6.13A-C). This observation suggested that the increased lymphatic vessel diameter observed following chronic UVB irradiation is likely a result of elevated lymphatic endothelial cell proliferation. To assess the proliferation of lymphatic endothelial cells in WT, Kit<sup>W/Wv</sup> and WT BMCMC→ Kit<sup>W/Wv</sup> mice, ear sections were immunostained with an antibody directed against phosphohistone 3 (PH3), a marker of mitotic cells. Proliferating cells were abundant in the epidermis of Kit<sup>W/Wv</sup> and WT BMCMC→ Kit<sup>W/Wv</sup> mice compared to WT (Fig 6.13D-F), in agreement with the epidermal hyperplasia described following UVB
Figure 6.10. Analysis of mast cells in ear pinnae before and after chronic high dose UVB irradiation.

Toluidine Blue staining to detect mast cells in 10 μm sections of WT, Kit<sup>W/Wv</sup> or WT BMCMC→Kit<sup>W/Wv</sup> mice treated with 12 exposures of 4kJ/m<sup>2</sup> + 30 exposures of 8kJ/m<sup>2</sup>. Mast cells were abundant in the ears of WT (A, B) and WT BMCMC→Kit<sup>W/Wv</sup> (E, F) mice. A limited number of mast cells was observed in Kit<sup>W/Wv</sup> ears (C, D) after UVB treatment, however these mice remained mast cell-deficient when compared to WT and WT BMCMC→Kit<sup>W/Wv</sup> mice, as determined by analysis of mast cell numbers in ear pinnae before and after UVB irradiation (G). Data shown represent the mean. WT n=4, Kit<sup>W/Wv</sup> n=3, WT BMCMC→Kit<sup>W/Wv</sup> n=4 from one independent UVB irradiation experiment. p values were calculated using Mann-Whitney U test. *p ≤ 0.05. Analysis of mast cell numbers in ear pinnae was performed by Michele Grimbaldeston.
Mast cell numbers in ear pinnae

G

WT

Kit<sup>W/W-v</sup> WT BMCMCs

**Michele Grimaldeston**
Figure 6.11. Mast cell-deficient mice display a robust angiogenic and lymphangiogenic response to chronic high dose UVB irradiation.

10 μm cryosections of UVB irradiated ears were immunostained with lymphatic and blood vascular markers. Lymphatic vessels and blood vessels were visualised at the tip, middle and base of the ear after UVB treatment of 12 exposures of 4kJ/m² + 30 exposures of 8kJ/m² in WT (A-C), Kit^{W/Wv} (D-F) or WT BMCMC→ Kit^{W/Wv} (G-I) mice. Irradiated ears imaged with a dissecting microscope indicate the lack of pigment in Kit^{W/Wv} and WT BMCMC→ Kit^{W/Wv} mice and display visible pathology induced by UVB treatment. When compared to WT, Kit^{W/wv} mice and WT BMCMC→ Kit^{W/Wv} mice exhibit abnormal blood and lymphatic vasculature. Scale bars: 150 μm. Data are representative of WT n=3, Kit^{W/Wv} n=2 and WT BMCMC→ Kit^{W/Wv} n=3 from one independent UVB irradiation experiment.
CD31/SMA/LYVE-1

Tip  | Middle  | Base

ABC  | DEF     | GHI

WT  Kit

W/Wv WT BMCMC/g314/g46/g76/g87/W/Wv
Figure 6.12. Mast cells restrict the angiogenic and lymphangiogenic response to chronic high dose UVB irradiation.

Quantification of vessel width (A), vessel number (B) and vessel density (C) from 10 μm cryosections of ears (Fig 6.11) after UVB treatment regimen of 12 exposures of 4kJ/m² + 30 exposures of 8kJ/m² in WT, Kit$^{W/Wv}$ or WT BMCMC→ Kit$^{W/Wv}$ mice. Quantification was performed using LVAP and ImageJ software. WT n=3, Kit$^{W/Wv}$ n=2 and WT BMCMC→ Kit$^{W/Wv}$ n=3 from one independent UVB irradiation experiment. At least six images were quantified per mouse. Data shown represent the mean ± SD. For samples where n>3, p values were calculated using Student’s paired t-test. *p ≤ 0.05.
12 exposures of 4 kJ/m² + 30 exposures of 8kJ/m²

**Vessel Width**

- Lymphatic Vessels
- Blood Vessels

**Vessel Number**

**Vessel Density**
Figure 6.13. Increased lymphatic vessel calibre is due to elevated lymphatic endothelial cell proliferation.

To determine whether an increase in lymphatic vessel width was due to an increase in endothelial cell proliferation or to vessel dilation, the number of nuclei per lymphatic vessel, as visualised by DAPI and LYVE-1 staining, was quantified in 10 μm sections. Results revealed an increase in the number of lymphatic endothelial cell nuclei in the vessels of irradiated Kit^{W/Wv} and WT BMCMC→Kit^{W/Wv} ears when compared to WT lymphatic vessels (A-C). Tissue sections were stained with an anti-phosphohistone 3 (PH3) antibody to visualise mitotic cells (D-I). While proliferating cells were rarely observed in large diameter lymphatic vessels (H, white arrow), they were commonly observed in the epidermis (E, F). White dashed lines distinguish epidermal borders. Scale bars: 100 μm. Data are representative of at least two mice for each genotype.
<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>Kit&lt;sup&gt;W/Wv&lt;/sup&gt;</th>
<th>WT BMCMC&lt;sup&gt;→Kit&lt;sup&gt;W/Wv&lt;/sup&gt;&lt;/sup&gt;</th>
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<td><strong>LYVE-1/DAPI</strong></td>
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<td><strong>CD31/PH3/NRP2</strong></td>
<td><img src="D" alt="Image" /></td>
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irradiation (Grimbaldeston et al. 2007). However, very few proliferating lymphatic endothelial cells were apparent in WT, Kit<sup>W/Wv</sup> and WT BMCMC→Kit<sup>W/Wv</sup> mice, suggesting that the majority of lymphatic endothelial cell proliferation is likely to be an early event following UVB irradiation-induced inflammation.

### 6.7. Mast cell regulation of lymphangiogenesis after short term UVB irradiation.

As mast cells were established to be potent at limiting the ear swelling associated with UVB irradiation after 12 exposures of 4 kJ/m<sup>2</sup> (Fig 6.6, 6.9), this study next sought to determine whether the enhanced protection at this time point was associated with restraining the lymphangiogenic response. In addition, given the established role of mMCP-4 in regulating the pathology of inflammatory disorders (Magnusson et al. 2009; Waern et al. 2009), we sought to investigate the role of mast cell-derived mMCP-4 in limiting the lymphangiogenic response. To this end, UVB irradiation-induced inflammation was investigated in Kit<sup>W/Wv</sup> mice engrafted with Mcpt4<sup>−/−</sup> mast cells (Mcpt4<sup>−/−</sup> BMCMC→Kit<sup>W/Wv</sup>). After 12 exposures of 4 kJ/m<sup>2</sup>, ear swelling was moderately reduced in WT BMCMC→Kit<sup>W/Wv</sup> mice and Mcpt4<sup>−/−</sup> BMCMC→Kit<sup>W/Wv</sup> when compared to Kit<sup>W/Wv</sup> mice (Fig 6.14). However, this decrease was not as significant as previous experiments, thought to be due to fewer viable mast cells being successfully engrafted in recipient mice (B. Fedoric and M. Grimbaldeston, data not shown). Mcpt4<sup>−/−</sup> BMCMC→Kit<sup>W/Wv</sup> mice displayed slightly reduced ear swelling compared to Kit<sup>W/Wv</sup> mice, yet increased ear swelling compared to WT BMCMC→Kit<sup>W/Wv</sup> mice. This suggests that mast cell-derived mMCP-4 may contribute to the negative immunomodulatory properties of mast cells.

When lymphatic and blood vessels were analysed, all mice lacking pigment (Kit<sup>W/Wv</sup>, WT BMCMC→Kit<sup>W/Wv</sup> and Mcpt4<sup>−/−</sup> BMCMC→Kit<sup>W/Wv</sup>) displayed an increase in lymphatic vessel diameter compared to WT, while blood vessel diameter remained largely unchanged (Fig 6.15, 6.16). Given that blood vessel diameter was increased after long term UVB exposure
Figure 6.14. Mast cells limit the ear swelling response associated with short term UVB irradiation.

WT, Kit$$^{W/Wv}$$, WT BMCMC → Kit$$^{W/Wv}$$ or MCP-4$$^{-/-}$$ BMCMC → Kit$$^{W/Wv}$$ mice were treated with 12 exposures of 4kJ/m$$^2$$ and ear swelling response measured. WT BMCMC → Kit$$^{W/Wv}$$ mice displayed reduced ear swelling compared to Kit$$^{W/Wv}$$ mice. MCP-4$$^{-/-}$$ BMCMC → Kit$$^{W/Wv}$$ mice displayed an ear swelling response that was less pronounced than Kit$$^{W/Wv}$$ mice, but more pronounced than WT BMCMC → Kit$$^{W/Wv}$$ mice. Data shown represent the mean ± SEM of the indicated number of mice per genotype from one independent UVB irradiation experiment. For samples where n>3, p values were calculated using one way ANOVA repeated measures with Tukey's post test. **p < 0.001, N.S., not significant. UVB irradiation treatment and analysis of ear swelling response was performed by Boris Fedoric and Michele Grimbaldesdon.
Ear swelling response following low dose chronic UVB exposure

- WBBF1 (Kit<sup>WT</sup>) n=5
- WBBF1 (Kit<sup>W/Wv</sup>) n=4
- WBBF1 (Kit<sup>W/Wv</sup>) + WT BMCMC n=2
- WBBF1 (Kit<sup>W/Wv</sup>) + Mcpt4<sup>−/−</sup> BMCMC n=5

4kJ/m²

Boris Fedoric
Figure 6.15. Mast cell-deficient mice display a robust angiogenic and lymphangiogenic response to short term UVB irradiation.

10 μm cryosections of UVB treated ears were immunostained with lymphatic and blood vascular markers. Lymphatic vessels and blood vessels were visualised at the tip, middle and base of the ear after UVB treatment of 12 exposures of 4kJ/m² in WT (A-C), Kit\textsuperscript{W/Wv} (D-F), WT BMCMC→Kit\textsuperscript{W/Wv} (G-I) or Mcpt4\textsuperscript{-/-} BMCMC→Kit\textsuperscript{W/Wv} (J-L) mice. Treated ears imaged with a dissecting microscope indicate the lack of pigment of Kit\textsuperscript{W/Wv}, WT BMCMC→Kit\textsuperscript{W/Wv} and Mcpt4\textsuperscript{-/-} BMCMC→Kit\textsuperscript{W/Wv} mice, and display visible pathology induced by UVB irradiation. When compared to WT, Kit\textsuperscript{W/Wv} mice, WT BMCMC→Kit\textsuperscript{W/Wv} mice and Mcpt4\textsuperscript{-/-} BMCMC→Kit\textsuperscript{W/Wv} mice exhibit abnormal blood and lymphatic vasculature. Scale bars: 150 μm. Data are representative of WT n=4, Kit\textsuperscript{W/Wv} n=3, WT BMCMC→Kit\textsuperscript{W/Wv} n=1 and Mcpt4\textsuperscript{-/-} BMCMC→Kit\textsuperscript{W/Wv} n=4 from one independent UVB irradiation experiment.
Figure 6.16. Mast cells restrict the angiogenic and lymphangiogenic response to short term UVB irradiation.

Quantification of vessel width (A), vessel number (B) and vessel density (C) from 10 μm cryosections of ears (Fig 6.15) after UVB treatment of 12 exposures of 4kJ/m² in WT, Kit<sup>W/Wv</sup>, WT BMCMC→ Kit<sup>W/Wv</sup> or Mcpt4<sup>−/−</sup> BMCMC→ Kit<sup>W/Wv</sup> mice. Quantification was performed using LVAP and ImageJ software. WT n=4, Kit<sup>W/Wv</sup> n=3, WT BMCMC→ Kit<sup>W/Wv</sup> n=1 and Mcpt4<sup>−/−</sup> BMCMC→ Kit<sup>W/Wv</sup> n=4 from one independent UVB irradiation experiment. At least six images were quantified per mouse. Data shown represent the mean ± SD. For samples where n>3, p values were calculated using Student’s paired t-test. *p ≤ 0.05.
12 exposures of 4 kJ/m²

**Vessel Width**

- **Average vessel width (µm)**
  - WT: 4.5 ± 0.5
  - W/Wv: 5.0 ± 0.6
  - W/Wv + WT MC: 5.5 ± 0.7
  - W/Wv + Mcpt4⁻/⁻ MC: 6.0 ± 0.8

**Vessel Number**

- **Number of vessels / field of view**
  - WT: 20 ± 2
  - W/Wv: 30 ± 3
  - W/Wv + WT MC: 40 ± 4
  - W/Wv + Mcpt4⁻/⁻ MC: 50 ± 5

**Vessel Density**

- **Number of vessels / mm²**
  - WT: 50 ± 5
  - W/Wv: 60 ± 6
  - W/Wv + WT MC: 70 ± 7
  - W/Wv + Mcpt4⁻/⁻ MC: 80 ± 8

**Legend**:
- Blue bars represent lymphatic vessels
- Red bars represent blood vessels

*Significant differences at p < 0.05
(Fig 6.8, 6.12), our results suggest UVB-induced lymphatic vessel hyperplasia precedes that of blood vessels. When compared to mice that were exposed to 8 kJ/m² (Fig 6.8, 6.12), mice irradiated with 12 exposures of 4 kJ/m² had reduced vessel width (Fig 6.16), presumably due to the shorter and lower dose treatment regime. PH3 immunostaining revealed that mitotic cells were commonly observed in the epidermis, but few proliferating lymphatic endothelial cells were seen at the completion of the irradiation regime (Fig 6.17). This suggests the majority of lymphatic endothelial cell proliferation, resulting in vessel hyperplasia, occurs after the initial UVB exposures. These results support those by Kajiya and colleagues (Kajiya et al. 2006), who observed numerous proliferating lymphatic endothelial cells after a single treatment of UVB irradiation equivalent to 1 erythema dose.

6.8. Chronic UVB irradiation-induced skin pathology is associated with hematopoietic cell and macrophage infiltration.

Studies so far had established that the pathology associated with chronic UVB irradiation correlated with lymphatic vessel hyperplasia, a response that was limited by the presence of mast cells. Macrophage recruitment during inflammation has been demonstrated to promote angiogenesis and lymphangiogenesis via the production of pro-lymphangiogenic and angiogenic factors (Baluk et al. 2005; Jeon et al. 2008; Kataru et al. 2009; Kim et al. 2009). Therefore, we investigated whether mast cells might limit hematopoietic cell and macrophage infiltration to the site of irradiation, and thereby act indirectly to restrain lymphatic vessel hyperplasia. CD45-positive hematopoietic cells were observed to infiltrate UVB irradiated ears in all mice, after both chronic high dose UVB exposure (Fig 6.18A-C) and short term, lower dose exposure (Fig 6.18G-J). Many of these hematopoietic cells were classified as macrophages due to co-expression of CD11b (Fig 6.18D-F) and F4/80 (Fig 6.18A-J). Increased hematopoietic cell infiltration was observed in Kit<sup>W/Wv</sup> mice and engrafted Kit<sup>W/Wv</sup> mice compared to WT mice, which correlated with increased ear swelling. These results suggest that increased numbers of inflammatory cells are recruited to the site
Figure 6.17. Short term UVB-induced pathology in mast cell-deficient mice is associated an increase in epidermal proliferation.

10 μm sections of WT (A), Kit<sup>W/Wv</sup> (B), WT BMCMC→ Kit<sup>W/Wv</sup> (C) and Mcpt4<sup>−/−</sup> BMCMC→ Kit<sup>W/Wv</sup> (D) ears after UVB treatment with 12 exposures of 4kJ/m² were immunostained and imaged with confocal microscopy. To determine whether the increase in lymphatic vessel calibre was due to elevated lymphatic endothelial cell proliferation, sections were stained with an anti-phosphohistone 3 (PH3) antibody to visualise mitotic cells. Proliferating cells were commonly observed in the epidermis (white arrows) but not in the vasculature. White dashed lines distinguish epidermal borders. Scale bars: 100 μm. Data are representative of at least two mice for each genotype.
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Figure 6.18. UVB-induced pathology is associated with hematopoietic cell and macrophage infiltration.

10 μm sections of ears following UVB treatment of 12 exposures of 4kJ/m² + 30 exposures of 8kJ/m² (A-F) or 12 exposures of 4kJ/m² (G-J) were immunostained and imaged with confocal microscopy. Staining revealed that compared to WT (A, D, G), Kit
ttW/Wv (B, E, H), WT BMCMC→ KitttW/Wv (C, F, I) and Mcpt4+/− BMCMC→ KitttW/Wv (J) displayed pronounced infiltration of hematopoietic cells and macrophages in the ear tissue following UVB exposure. Scale bars: 100 μm. Data are representative of at least two mice for each genotype.
12 exposures of 4 kJ/m² + 30 exposures of 8 kJ/m²

WT BMCMC

WT

Mcpl4-/- BMCMC

CD45/LYVE-1/F4/80

DE F

12 exposures of 4 kJ/m²

CD45/LYVE-1/F4/80

AB C

GH I J
of UVB irradiation, where they likely release pro-inflammatory and pro-lymphangiogenic factors to increase inflammation and lymphatic vessel hyperplasia. These results are in agreement with previous studies which utilised flow cytometry to demonstrate increased numbers of granulocytes, macrophages, and CD4-positive T cells in the ear skin of UVB irradiated Kit\(^{W/Wv}\) mice when compared to identically treated WT or WT BMCMC→ Kit\(^{W/Wv}\) mice (Grimbaldeston et al. 2007; Biggs et al. 2010). Future experiments will utilise flow cytometry to accurately quantify the number of infiltrating inflammatory cells in Kit\(^{W/Wv}\) mice compared to engrafted Kit\(^{W/Wv}\) mice after chronic high dose (8 kJ/m\(^2\)) and low dose (4 kJ/m\(^2\)) UVB irradiation.

### 6.9. Discussion

The focus of this chapter was to investigate the role of mast cells in embryonic and inflammation-stimulated lymphangiogenesis. Embryonic studies established that mMCP-4 is not required for developmental lymphangiogenesis. In addition, analyses of lymphatic vascular patterning in adult mast cell-deficient mice suggested that mast cells are not required for the development and remodelling of the lymphatic vasculature. Chronic UVB irradiation studies uncovered a novel role for mast cells in the regulation of lymphangiogenesis. Mast cell-deficient mice displayed hyperplastic lymphatic vessels and increased infiltration of inflammatory cells following chronic UVB irradiation, suggesting that mast cells may normally act to limit the inflammatory response to this insult.

Despite intense research on the role of mast cells in settings of inflammation, few studies have investigated embryonic mast cell development. A study of mast cell development in the rat embryo found the first mast cells arise at E15 in the head mesenchyme near the ventral portion of the developing brain, and that the number of mast cells in the embryo subsequently increases progressively until birth (Combs et al. 1965). Mast cells observed in the embryo were found to be of an immature status compared to mast cells found in the
adult rat, as determined by granule formation and morphologic characteristics (Combs et al. 1965). Data presented in this chapter established that immature mast cells are present in the dermis at E14.5, and that the number, distribution and maturity of mast cells progressively increases until E18.5 (Fig 6.1, 6.2) in accordance with development in the rat. While the hematopoietic origin of mast cells has since been established (Kitamura et al. 1981; Chen et al. 2005; Abraham and St John 2010), the role of mast cells during embryonic development remains undefined.

This study determined that Mcpt4−/− mice undergo normal dermal lymphatic and blood vascular development (Fig 6.4). Additionally, Mcpt4−/− mice and mast cell-deficient KitW/Wv mice displayed normal blood and lymphatic patterning in the adult ear (Fig 6.3, 6.5). As lymphatic vascular development is unperturbed in mast cell-deficient KitW/Wv mice, and very few mast cells are present in the embryo during crucial developmental stages when the lymphatic vasculature is formed (E9.5-E14.5) (Fig 6.1), mast cells are unlikely to be required for normal embryonic lymphangiogenesis. However, the possibility remains that compensatory factors may rescue/restore potential lymphatic vascular abnormalities in mast cell-deficient mice after birth. As no genotyping strategy has been described in the literature for KitW/Wv mice (KitW/Wv mice lack pigment and are therefore identified via phenotyping after birth), the analysis of lymphatic vascular development in KitW/Wv embryos was not plausible within the timeframe remaining on this project. Future investigations aim to develop a genotyping strategy for KitW/Wv mice, and to determine whether these mice display normal or aberrant lymphatic vascular development.

Interestingly, mast cells, and in particular mMCP-4, have shown to be important in settings of homeostasis, with mMCP-4 recently shown to regulate intestinal epithelial cell migration and barrier formation (Groschwitz et al. 2009). Therefore, this chymase plays a role in tissue homeostasis under conditions that are not associated with mast cell degranulation in
response to inflammatory stimuli. In addition, Mcpt4−/− mice display excessive fibronectin accumulation due to a failure to process pro-MMP-9 and pro-MMP-2 (Tchougounova et al. 2003; Tchougounova et al. 2005). Fibronectin is important for the formation of valve leaflets during embryonic lymphangiogenesis (Bazigou et al. 2009). Additionally, the fibronectin binding protein integrin α4β1, a newly discovered marker for proliferating lymphatic endothelium, regulates growth factor and tumour-induced adhesion, migration, invasion and proliferation of lymphatic endothelial cells (Garmy-Susini et al. 2010). Findings presented in this chapter did not observe any developmental or remodelling lymphatic abnormalities in embryonic or adult Mcpt4−/− mice, suggesting that the increased fibronectin accumulation in these mice does not alter lymphatic vessel patterning at the developmental stages analysed.

Although mast cells and mMCP-4 were found to be dispensable for the normal development and maturation of lymphatic vessels, their established role as immunoregulators of the inflammatory response suggested they may play a role in regulating lymphangiogenesis during inflammation. Indeed, the transplant of wild-type mast cells into KitW/Wv mice resulted in reduced ear swelling and a reduction in lymphatic vessel and blood vessel calibre following chronic high dose UVB irradiation when compared to mast cell-deficient KitW/Wv mice (Fig 6.9, 6.11, 6.12). Therefore, in agreement with their role as negative immunomodulators of the inflammatory response (Grimbaldeston et al. 2007), mast cells act to negatively regulate vessel hyperplasia induced by UVB exposure. Whether mast cells regulate lymphangiogenesis in other settings of inflammation where pathology is associated with the lymphangiogenic response remains to be elucidated (Baluk et al. 2005; Zhang et al. 2007; Guo et al. 2009). Interestingly, mast cells have been shown to enhance the inflammatory response during both inflammatory arthritis and airway inflammation (Lee et al. 2002; Nakae et al. 2007; Magnusson et al. 2009), therefore it may be hypothesised that mast cells promote lymphangiogenesis in these settings. While mast cells exacerbate
airway inflammation, mMCP-4 protects against allergic airway inflammation in an ovalbumin-based model (Waern et al. 2009). This suggests that while mast cells as a whole contribute to pathology, their individual granule components may possess activities which counteract the overall harmful influence of the mast cells. Future studies will investigate the role of mast cells and their individual granule components in regulating neo-lymphangiogenesis in a disease-specific fashion.

Though lymphatic vessels were strikingly increased in calibre in mast cell-deficient mice compared to wild-type at the earliest time point following UVB irradiation analysed in this study (12 exposures of 4 kJ/m²) (Fig 6.15, 6.16), very few proliferating lymphatic endothelial cells were detected (Fig 6.17). Lymphatic endothelial cell hyperproliferation resulting in vessel hyperplasia is therefore likely to occur at earlier time points following UVB exposure. This is in agreement with results from Kajiya and colleagues, who observed proliferating endothelial cells in lymphatic vessels after a single UVB exposure equivalent to 1 erythema dose (Kajiya et al. 2006). Future studies will examine lymphatic endothelial cell proliferation at earlier time points following UVB irradiation, such as 1-5 exposures of 4 kJ/m². These data will provide further insight as to how mast cells regulate lymphatic vessel calibre.

Whether lymphatic vessels are anti-inflammatory, by helping to clear fluid and inflammatory cells from the site of inflammation, or pro-inflammatory, by acting as a route of transport for inflammatory cells to reach the site of the inflammatory stimulus, appears to be dependent on both the tissue type and the stimulus. Previous studies of chronic and acute UVB irradiation found enlarged, functionally impaired and hyperpermeable lymphatic vessels formed after irradiation when compared to non-irradiated mice, a process mediated via VEGF-A signalling through VEGFR-2 (Kajiya et al. 2006). Due to their non-functionality, these vessels were established to contribute to UVB induced pathology. Subsequent studies determined that stimulation of lymphatic endothelial cell proliferation and formation
of functional lymphatic vessels by VEGF-C signalling through VEGFR-3 attenuates inflammation after UVB exposure, counteracting the pro-inflammatory effects of VEGF-A (Kajiya et al. 2009). Recent work using transgenic mouse models has revealed the same mechanism of lymphatic vessel hyperplasia in a model of chronic skin inflammation which mimics the human disease psoriasis (Huggenberger et al. 2010). Interestingly, mast cells have been shown to produce VEGF-C and VEGF-D in this setting (Theoharides et al. 2007; Detoraki et al. 2009). It is conceivable that during chronic UVB irradiation, the wild-type mast cells in KitW/Wv engrafted mice produce VEGF-C and/or VEGF-D to induce a switch to produce functional, anti-inflammatory lymphatic vessels which are less dilated than their KitW/Wv counterparts. Future studies will utilise intravital lymphangiography to determine the functionality of wild-type, KitW/Wv and WT BMCMC→KitW/Wv lymphatic vessels following UVB irradiation. Despite the fact that VEGF-A stimulation of VEGFR-2 forms enlarged lymphatic vessels during chronic skin inflammation (Kajiya et al. 2009; Huggenberger et al. 2010), there remains the possibility that the enlarged lymphatic vessels in KitW/Wv mice are due to excessive VEGF-C stimulation of VEGFR-3. Measuring the amounts of VEGF-A and VEGF-C in these mice, and the use of functional and blocking antibodies, could be used to distinguish between these possibilities.

Recent studies have identified soluble forms of VEGFR-1 (sVEGFR-1) responsible for the avascular cornea (Ambati et al. 2006), and VEGFR-2 (sVEGFR-2), responsible for the alymphatic cornea (Albuquerque et al. 2009). sVEGFR-1 binds and blocks VEGF-A activity in the cornea (Ambati et al. 2006), whereas sVEGFR-2 is able to specifically bind to VEGF-C, but not VEGF-A, and block its activity during developmental and inflammation-stimulated lymphangiogenesis (Albuquerque et al. 2009). In the skin, sVEGFR-2 is found in hair follicles, the epidermis and dermis of wild-type mice, and in its absence, lymphatic vessels are dilated and hyperplastic, but not increased in density (Albuquerque et al. 2009). It has been demonstrated that mast cells express Vegfr-1 and Vegfr-2 mRNA, and present both
receptors on their cell surface (Detoraki et al. 2009). However, it is not known whether mast cells can synthesise the soluble receptor forms. It is conceivable that mast cells produce sVEGFR-2 which acts as a sink for VEGF-C, possibly produced by infiltrating inflammatory cells, hence limiting lymphangiogenesis in chronic inflammation. If the enlarged lymphatic vessels observed in mast cell-deficient mice are due to an increase in VEGF-A, it may also be possible that mast cells produce sVEGFR-1 which acts as a sink for excess VEGF-A, limiting both lymphangiogenesis and angiogenesis. Additionally, mast cells produce factors such as MMPs that may regulate processing of the receptors to their soluble forms. Investigating the presence of sVEGFR-2, sVEGFR-1 and blocking their activity in wild-type, Kit<sup>W/Wv</sup> and WT BMCMC→ Kit<sup>W/Wv</sup> mice would distinguish between these possibilities.

While the VEGF family of growth factors and receptors are likely to play a role in the UVB-induced lymphatic vessel hyperplasia observed in mast cell-deficient mice, other ligand/receptor pathways have been implicated in regulating vessel calibre. The angiopoietin family of ligands and their Tie receptors have been established to be important in regulating lymphatic vessel calibre (Gale et al. 2002; Dellinger et al. 2008; Gordon et al. 2010), however whether this pathway is activated after UVB irradiation remains to be seen. Recent studies have identified an alternative pathway for regulating lymphangiogenesis and angiogenesis via Ephrin-B2, a transmembrane ligand for Eph receptors; Efnb2<sup>−/−</sup> mice display defective angiogenesis and lymphangiogenesis (Sawamiphak et al. 2010; Wang et al. 2010). It is tempting to speculate that mast cells may limit the lymphangiogenic response by expressing Eph receptors, which bind, to and sequester, available Ephrin-B2. This would prevent the interaction between Ephrin-B2 and VEGFR-2/3, impeding receptor internalisation and signalling via VEGFs. Future studies aim to investigate the expression of angiopoietins, ephrins and their respective receptors on mast cells and on UVB irradiation-induced hyperplastic lymphatic vessels.
While $Kit^{WWv}$ mice are profoundly deficient in mast cells (Galli et al. 2005), some dermal mast cells were observed in $Kit^{WWv}$ mice following chronic UVB irradiation (Fig 6.10) (Grimbaldeston et al. 2007). This suggests that chronic UVB irradiation leads to the promotion of local mast cell influx, proliferation and/or survival, even in the presence of c-Kit mutations. How these Kit-deficient mast cells regulate lymphangiogenesis compared to wild-type mast cells remains to be elucidated. Mast cells have been demonstrated to exhibit heterogeneity in the expression of chymases, tryptases and/or carboxypeptidases depending on their tissue localisation (Weidner and Austen 1993). It may be conceived that under appropriate UVB stimuli, Kit-deficient mast cells are recruited from other tissues to the skin, where they synthesise pro-lymphangiogenic factors leading to enlarged lymphatic vessels. Isolation and analysis of Kit-deficient mast cells compared to wild-type mast cells is required to investigate this possibility.

A striking increase in the number of CD45-positive, CD11b-positive and/or F4/80-positive cells recruited to the site of UVB irradiation was observed in $Kit^{WWv}$ mice compared to wild-type counterparts (Fig 6.18). While there appeared to be a decrease in inflammatory cell infiltration in WT BMCMC→$Kit^{WWv}$ mice compared to $Kit^{WWv}$ mice, accurate quantification will require flow cytometric analysis. These results are in agreement with reports which observed increased numbers of inflammatory cells in the ear skin of chronic low dose UVB-irradiated $Kit^{WWv}$ mice when compared to identically treated wild-type or WT BMCMC→$Kit^{WWv}$ mice (Grimbaldeston et al. 2007; Biggs et al. 2010). Macrophage recruitment to the site of inflammation has been demonstrated to promote angiogenesis and lymphangiogenesis in many settings (Baluk et al. 2005; Jeon et al. 2008; Kataru et al. 2009; Kim et al. 2009). The influx of inflammatory cells at the site of chronic UVB irradiation is likely due to increased routes of transport via enhanced lymphangiogenesis, and possibly impaired drainage of inflammatory cells due to dysfunctional lymphatic vessels (Kajiya et al. 2009). However whether these cells further enhance the lymphangiogenic response via
production of growth factors VEGF-A, VEGF-C and/or VEGF-D remains to be determined. Analysis of lymphangiogenic factors produced by infiltrating inflammatory cells, chemically or genetically depleting macrophages in Kit\textsuperscript{W/Wv} mice prior to irradiation, and the use of blocking antibodies to negate VEGF-A/C/D could provide answers to these questions.

The role of the established pro-angiogenic proteases MMP-2 and MMP-9 is of considerable interest, as mast cell proteases, specifically mMCP-4, are required to process these MMPs to their active forms (Tchougounova \textit{et al.} 2005). In turn, active MMP-9 has an established role in the promotion of tumour angiogenesis and lymphangiogenesis (Bergers \textit{et al.} 2000; Bruyere \textit{et al.} 2008; Kessenbrock \textit{et al.} 2010). By this mechanism, it would be expected that Kit\textsuperscript{W/Wv} and Mcpt4\textsuperscript{−/−} BMCMC→ Kit\textsuperscript{W/Wv} mice would have less active MMP-9, and therefore a decreased angiogenic and lymphangiogenic response. As this was not observed in our experiments, it may be hypothesised mast cells are not the major source of MMPs in this setting of chronic UVB irradiation. It may be that MMP-9 produced by the influx of CD45 or CD11b-positive cells (Fig 6.18) increases bioavailability of VEGF-A (Ahn and Brown 2008), leading to the increased angiogenic and lymphangiogenic response observed. Analysis of MMP-9 levels in irradiated mice will provide clues as to whether this protease plays a role in regulating lymphangiogenesis and UVB irradiation-induced pathology.

In conclusion, this study has determined that mast cells are unlikely to be required for the development and maturation of the lymphatic vasculature. However, the data presented has illustrated that mast cells act to reduce inflammation and restrain the calibre of blood and lymphatic vessels following chronic UVB irradiation. Whether mast cells regulate the lymphangiogenic response directly, via production of growth factors and proteases, or indirectly, via factors that influence infiltration and activity of other inflammatory cells such as macrophages, remains to be elucidated and will be the focus of future work. We hypothesise that mast cells regulate lymphangiogenesis in additional pathological settings.
including psoriasis, asthma, arthritis and the tumour environment. Examining the role of mast cells and mast cell-derived factors in these settings may provide opportunities to control lymphangiogenesis in a disease-specific manner, and develop novel ways to treat these disorders.
7. General Discussion
7.1. General Overview

The origin of the lymphatic system has been a controversial topic in lymphangiogenesis research, and prior to the commencement of this study, two models of development were actively debated. The first, proposed by Florence Sabin, suggested that lymphatic vessels arise from the embryonic veins via a process of sprouting and migration towards the periphery of the embryo (Sabin 1904). The second, proposed by Huntington, McClure and others (Huntington 1910), proposed a mesenchymal origin, where lymphatic endothelial cells arise de novo in the mesenchyme to form lymphatic vessels that subsequently connect to the large veins. Recent work in a number of animal models has suggested that lymphatic endothelial cells have a dual origin from venous and mesenchymal components (Buttler et al. 2006; Wilting et al. 2006). Recent literature proposed that macrophages may constitute a pool of lymphatic endothelial progenitor cells in settings of inflammation (Maruyama et al. 2005; Kerjaschki et al. 2006; Maruyama et al. 2007), as well as a crucial source of growth and patterning signals that influence inflammation-stimulated lymphangiogenesis (Cursiefen et al. 2002; Cursiefen et al. 2004; Baluk et al. 2005; Schledzewski et al. 2006; Jeon et al. 2008; Kim et al. 2009; Kubota et al. 2009). This PhD project sought to investigate whether macrophages and cells of the myeloid lineage comprise a pool of lymphatic endothelial progenitor cells, or provide growth and patterning signals important for developmental, tumour and inflammation-stimulated lymphangiogenesis.
7.2. Major Findings

7.2.1. Expression of the hyaluronan receptor LYVE-1 is not restricted to lymphatic vessels during embryogenesis; LYVE-1 is also expressed in the embryonic blood vasculature.

Chapter 3 presents an extensive study of the expression of hyaluronan receptor LYVE-1, a commonly used marker for the lymphatic endothelium, during mouse embryonic development (Gordon et al. 2008). Our serendipitous discovery that LYVE-1 is expressed on the blood vessels of the developing mouse yolk sac led to further characterisation of LYVE-1 expression in the mouse embryo, which demonstrated that LYVE-1 is expressed on early stage inter-somitic veins and isolated arterial endothelial cells, as well as on the endocardium. This was the first report of LYVE-1 expression on early embryonic arterial and inter-somitic blood vessels, and suggests a previously unrecognised plasticity of immature endothelial cells. As previously reported, LYVE-1 expression was also observed on a population of macrophages and on endothelial sinusoids of lymph nodes, liver and spleen (Banerji et al. 1999; Mouta Carreira et al. 2001; Prevo et al. 2001; Nonaka et al. 2007). Given LYVE-1 is commonly used to identify lymphatic vessels in human tumours, in order to accurately correlate lymphangiogenesis with tumour progression and prognosis, it is critical to use a combination of endothelial cell markers to precisely assess vascular endothelial cell identity.

7.2.2. LYVE-1-positive macrophages are closely associated with developing lymphatic vessels and resemble Tie2-expressing monocytes.

Chapter 4 sought to investigate the localisation and molecular identity of LYVE-1-positive macrophages during development. While LYVE-1-positive macrophages had been described prior to the commencement of these studies (Schledzewski et al. 2006), they had not been characterised in the developing mouse embryo. It was determined that LYVE-1-positive macrophages were rare at E11.5 and abundant at E14.5, revealing a dramatic
expansion of the macrophage population between these embryonic stages. Additionally, LYVE-1-positive macrophages were significantly more likely to be found around the lymphatic vasculature, suggesting they play a role in developmental lymphangiogenesis. The development of a novel macrophage isolation method allowed for analysis of sub-populations of macrophages based on LYVE-1 expression. Gene expression profiling determined LYVE-1-positive macrophages share a profile similar to CD11c^Mrc^Tie2^ monocytes (TEMs), which have been shown to play a role in developmental (Fantin et al. 2010) and pathological (Venneri et al. 2007; Pucci et al. 2009) angiogenesis. Results from Chapter 4 provided the first gene expression analysis of LYVE-1-positive macrophages, and due to their shared gene expression profile with TEMs, suggested these cells may contribute to events such as lymphatic vascular morphogenesis and remodelling.

7.2.3. **Lymphatic endothelial cells arise independently of the myeloid lineage during embryonic and tumour-stimulated lymphangiogenesis.**

In addition to examining the role and function of LYVE-1-positive macrophages in the developing mouse embryo, Chapter 4 sought to definitively establish whether LYVE-1-positive macrophages and cells of the myeloid lineage constitute a source of lymphatic endothelial progenitor cells either during embryogenesis, or in the tumour microenvironment, using genetic lineage tracing studies. Using two independent lineage tracing models and stringent immunostaining analyses, results determined that lymphatic endothelial cells arise independently of the myeloid lineage, thus providing evidence against the model of macrophage trans-differentiation (Fig 7.1). These results are in agreement with those of Srinivasan and colleagues, published after the commencement of this study (Srinivasan et al. 2007), who concluded the majority of lymphatic endothelial cells in the mouse embryo are derived from the venous lineage. While some groups have since suggested that macrophages act as lymphatic endothelial progenitor cells in the tumour microenvironment (Zumsteg et al. 2009), data from this thesis are in accordance those which found that pre-existing lymphatic endothelium contributes to the generation of
Figure 7.1. Macrophages do not trans-differentiate into lymphatic endothelial cells, but are required for the normal patterning of the lymphatic vasculature.

Lineage tracing data revealed macrophages do not trans-differentiate into lymphatic endothelial cells during development or in settings of tumour-stimulated lymphangiogenesis. The role of macrophages during settings of inflammation-stimulated lymphangiogenesis was not investigated in this study. While macrophages do not act as lymphatic endothelial cell progenitor cells, they were demonstrated to be essential for the patterning of the lymphatic vasculature, with macrophages expressing LYVE-1 closely associated with developing lymphatic vessels. The importance of myeloid-derived VEGF-C/D and as yet uncharacterised lymphangiogenic factors in the development and patterning of lymphatic vessels will be the focus of future studies. Expression of Tie1 was reported on lymphatic endothelial cells, and a previously undescribed role for Tie1 in the regulation of lymphatic endothelial cell proliferation was revealed. Both the identity and the source of the ligand(s) for Tie1 are yet to be determined, but are hypothesised to be a member of the angiopoietin family.
tumour-stimulated lymphatic vessels (He et al. 2004). Our results provide extensive evidence to demonstrate that lymphatic endothelial cells arise independently of the myeloid lineage during developmental and tumour-stimulated lymphangiogenesis (Gordon et al. 2010).

7.2.4. **Macrophages define the calibre of dermal lymphatic vessels during embryonic development by regulating lymphatic endothelial cell proliferation.**

While Chapter 4 demonstrated that macrophages do not trans-differentiate into lymphatic endothelial cells during developmental or tumour-stimulated lymphangiogenesis, there remained the question as to whether macrophages provide growth and patterning signals that direct the development of the lymphatic vasculature, as has previously been suggested to occur during neo-lymphangiogenesis (Cursiefen et al. 2002; Cursiefen et al. 2004; Alitalo et al. 2005; Baluk et al. 2005). Therefore, the work documented in Chapter 5 sought to investigate whether developmental lymphangiogenesis occurs normally in the absence of myeloid cells. Using a series of genetically targeted mice, our work established that myeloid cells are essential for normal lymphatic development by acting to restrain the calibre of dermal lymphatic vessels (Fig 7.1). In addition, a previously undescribed role for the receptor Tie1 in regulating lymphatic endothelial cell proliferation was identified using in vitro cell culture studies. These results are in support of work from two groups published after completion of these studies, which identified Tie1 as a key regulator of lymphatic vascular development in vivo (D'Amico et al. 2010; Qu et al. 2010).

7.2.5. **Mast cells regulate UVB irradiation-induced lymphangiogenesis.**

Chapter 6 sought to identify the role of another myeloid cell lineage, the mast cell, in developmental and inflammation-stimulated lymphangiogenesis. This study was conceived by results from microarray data in Chapter 5 which revealed $PU.1{^{−/−}}$ mice are profoundly deficient in mast cell-derived proteases and chymases. This was confirmed by staining
which revealed that \textit{PU.1}\textsuperscript{−/−} embryos lack mast cells. Normal lymphatic vascular patterning in mast cell-deficient adult mice suggested mast cells are not required for normal lymphangiogenesis. However, after chronic UVB exposure, mast cell-deficient mice displayed an increased ear swelling response, which correlated with hyperplastic lymphatic vessels and an influx of CD45-positive hematopoietic cells to the site of irradiation (Fig 7.2). Mast cells have previously been shown to increase tumour-stimulated lymphangiogenesis (Brideau \textit{et al.} 2007), therefore the results documented in Chapter 6 are the first to suggest that mast cells act to limit the lymphangiogenic response. In addition, our results suggest that the future targeting of mast cells for novel anti-inflammatory therapies must be performed in a model-specific manner.

\textbf{7.3. Future Directions}

Investigation as to whether or not macrophages comprise a pool of lymphatic endothelial progenitor cells was initially prompted by studies in the literature which suggested that macrophages are able to trans-differentiate into lymphatic endothelial cells (Maruyama \textit{et al.} 2005; Kerjaschki \textit{et al.} 2006; Maruyama \textit{et al.} 2007; Zumsteg \textit{et al.} 2009). Work documented in Chapter 4 (Gordon \textit{et al.} 2010) together with the work of others has provided no \textit{in vivo} evidence to support this claim either during development (Srinivasan \textit{et al.} 2007; Gordon \textit{et al.} 2010) or in the settings of LLC or EL4 tumour-stimulated lymphangiogenesis (He \textit{et al.} 2004; Gordon \textit{et al.} 2010). However, whether macrophages act as lymphatic endothelial progenitor cells in other models of neo-lymphangiogenesis promoted by alternative stimuli cannot be excluded. The contribution of macrophages to the lymphatic endothelium, and the biological relevance of this proposed contribution, needs to be investigated in a model-specific fashion. In addition, results presented in Chapter 5 have indicated that the role of potential macrophage-derived lymphangiogenic factors may be model or tissue specific. Our finding that macrophages act to restrain the calibre of dermal lymphatic vessels during development is in contrast to published data suggesting that
Figure 7.2. Mast cells regulate lymphatic vessel calibre in settings of UVB irradiation-induced neo-lymphangiogenesis.

Mast cells regulate the calibre of lymphatic vessels in settings of chronic UVB irradiation-induced inflammation. In the absence of mast cells, there is an increase in the infiltration of CD45-positive hematopoietic cells to the site of inflammation, and lymphatic vessels are hyperplastic. It is hypothesised hematopoietic cells provide pro-lymphangiogenic growth factors which promote lymphatic endothelial cell proliferation and vessel hyperplasia. The factors produced by mast cells which restrict the infiltration of hematopoietic cells are as yet unknown, and will be the focus of future studies. Mast cells or mast cell-derived factors, such as sVEGFR-1 or sVEGFR-2, may also act to block the effects of hematopoietic-derived factors. The precise mechanism by which mast cells influence neo-lymphangiogenesis will be the focus of future studies.
Chronic UVB irradiation

No Mast Cells

Mast Cells

lymphatic vessel

VEGF-A/C/D

sVEGFR-1?
sVEGFR-2?

?
macrophage-derived factors promote neo-lymphangiogenesis during inflammation (Cursiefen et al. 2004; Jeon et al. 2008; Kataru et al. 2009; Kim et al. 2009). This may have implications when targeting macrophages or macrophage-derived factors for therapies, as treatments which inhibit the lymphangiogenic response in one setting may promote lymphangiogenesis in another. Therefore, the targeting of macrophages or macrophage-derived factors in the future will need to be assessed in a disease-specific manner in order to achieve the desired response.

While results documented in this thesis found that macrophages are not the major source of VEGF-C or VEGF-D in the dermis during development, the role of these macrophage-derived factors cannot be ruled out by their magnitude of expression alone. A recent report utilised a conditional deletion of VEGF-A in myeloid cells to determine that although myeloid cells are not the major source of VEGF-A in the tumour microenvironment, deletion of myeloid-derived VEGF-A is sufficient to decrease the tumourigenic vascular response and retard tumour progression (Stockmann et al. 2008). Our collaborators are in the process of generating gene-targeted mice harbouring a floxed Vegfc allele, which will form the basis of future studies in our laboratory which aim to determine the contribution of myeloid-derived VEGF-C to developmental, tumour and inflammation-stimulated lymphangiogenesis.

In vitro cell culture experiments revealed a previously unrecognised role for Tie1 in the regulation of lymphatic endothelial cell proliferation, supporting recent publications which described a role for Tie1 in regulating lymphatic vascular development in vivo (D'Amico et al. 2010; Qu et al. 2010). Future studies will investigate the signalling pathways activated downstream of Tie1, as well as the effect of Tie1 siRNA knockdown on lymphatic endothelial cell functions including cell migration and tube formation. These assays are currently being developed in our laboratory for primary lymphatic endothelial cells. Little is currently known about the mechanism of Tie1 activation or its downstream targets, however
in vitro experiments have demonstrated that Tie1 is phosphorylated by Angpt1 and Angpt4. Tie1 activation is further increased after the formation of heterodimers with Tie2 (Saharinen et al. 2005), and Tie1 has also been shown to modulate Tie2 activity in cultured endothelial cells (Yuan et al. 2007; Singh et al. 2009). However, results presented in Chapter 5 along with other in vivo studies have not detected significant Tie2 expression in lymphatic endothelial cells (Dellinger et al. 2008). Additional studies are required to determine whether Tie1 acts in conjunction with Tie2, and to investigate the identity of angiopoietins or alternative ligands which may mediate receptor activation. This will allow for elucidation of the downstream effectors in this pathway which regulate lymphatic endothelial cell behaviours such as proliferation, migration and tube formation.

Work presented in this thesis has provided the first evidence to illustrate that mast cells play an important role in the regulation of lymphatic vessel calibre following UVB irradiation. The mechanism of action or signalling pathways engaged by this population of myeloid cells on lymphatic endothelial cells will be the focus of future studies. Mast cells play a unique role in limiting the pathology of chronic UVB irradiation (Grimbaldeston et al. 2007; Biggs et al. 2010), presumably at least in some part via regulating the neo-lymphangiogenic response. As mast cells typically act as promoters of the inflammatory response (Malaviya and Abraham 2001; Grimbaldeston et al. 2006; Sayed et al. 2008; Magnusson et al. 2009), whether or not they promote lymphangiogenesis in other settings remains to be elucidated. Additionally, whether mast cells act directly or indirectly to regulate lymphangiogenesis will be the focus of future studies. It was observed in our model that mast cells limit the infiltration of CD45-positive hematopoietic cells and macrophages to the site of UVB irradiation. Macrophages have an established role in promoting inflammatory lymphangiogenesis (Cursiefen et al. 2002; Cursiefen et al. 2004; Alitalo et al. 2005; Baluk et al. 2005; Schledzewski et al. 2006; Jeon et al. 2008; Kim et al. 2009; Kubota et al. 2009). It is tempting to speculate that mast cells might somehow restrict inflammatory cell infiltration.
to the site of UVB-induced inflammation, thereby acting indirectly to limit the lymphangiogenic response via limiting immune cell-derived pro-lymphangiogenic stimuli.

7.4. Concluding Remarks

In conclusion, this study has provided extensive lineage tracing evidence to illustrate that lymphatic endothelial cell arise independently of the myeloid lineage. In addition, we have demonstrated that cells of the myeloid lineage provide signals that define the calibre of dermal lymphatic vessels by restraining the proliferation of dermal lymphatic endothelial cells during development. A novel role for mast cells in the regulation of inflammatory lymphangiogenesis has been demonstrated, with the mechanism behind this to be the focus of future studies. Work presented in this thesis supports the model first suggested by Florence Sabin, which proposed that lymphatic endothelial cells are of venous origin and not derived from progenitor cells of mesenchymal origin. This contributed to clarifying a long debated question in the field of lymphatic vascular biology. Defining the precise role and contribution of myeloid-derived signals during developmental and pathological lymphangiogenesis in a model-dependent manner will provide important clues as to how to target these signals for potential lymphangiogenic therapies.

Many recent studies have demonstrated that targeting immune cell trafficking, activity or secreted factors inhibits tumour and inflammation-stimulated angiogenesis and lymphangiogenesis in vivo (reviewed in (Karpanen and Alitalo 2008; Murdoch et al. 2008; Jurisic and Detmar 2009)), yet how the immune system as a whole responds to these therapies is yet to be established. Potential off-target effects or compensatory factors may reduce the therapeutic potential of these treatments in some settings, and therefore need to be assessed in a careful manner. There is evidence to suggest that after chemotherapy and radiotherapy, tumours produce elevated levels of macrophage chemoattractants, resulting in increased macrophage infiltration and production of growth factors, an effect which
assists in tumour recovery (McDonnell et al. 2003; Ahn and Brown 2008). Subsequent studies have suggested blocking macrophage infiltration enhances chemotherapy potency (Fischer et al. 2007). Therefore, multi-faceted therapies incorporating conventional treatment with novel therapies which block myeloid cell recruitment and/or function are likely to have considerable therapeutic potential in the future.
8. Bibliography


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