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BMP-SMADs: a new take on lymphatic vessel development

BMP-SMADs: een nieuwe kijk op lymfevat ontwikkeling

Thesis

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List of used abbreviations

A:	artery
AIDS:	acquired immune deficiency syndrome
Ao:	aorta
At:	atrium
ALK1:	activin receptor-like kinase 1
ALS:	amyotrophic lateral sclerosis
APC:	antigen presenting cell
AVC:	atrioventricular canal
BM:	basement membrane
BMP:	bone morphogenetic proteins
BRE:	BMP-SMAD response element
BSA:	bovin serum albumin
Bv:	bicuspid valve
CCBE1:	collagen and calcium binding EGF domains 1
CLEC2:	C-type lectin-like receptor 2
CV:	cardinal vein
COUPTFII:	chicken ovalbumin upstream promotor transcriptional factor II
CX37:	Connexin 37
DA:	dorsal aorta
DEPC:	diethylpyrocarbonate
DLL:	Delta-like
dOFT:	distal outflow tract
E:	embryonic day
EC:	endothelial cell
ECM:	extracellular matrix
Efnb2	Eph related tyrosine receptor ligand B2
EIIA:	extra domain A
ELISA:	enzyme-linked immunosorbent assay
EndoMT:	endothelial to mesenchymal transition
eNOS:	endothelial nitric oxide synthase
FGF:	fibroblast growth factor
FN:	fibronectin
HES:	Hairy and Enhancer of Split-1 Hairy/enhancer-of-split related with YRPW motif protein
HERP:	HES-related repressor protein
HEY:	Hairy/enhancer-of-split related with YRPW motif protein
HH:	hedgehog
L:	lymphatic vessel
LA:	left atrium
LEC:	lymphatic endothelial cell
LN:	lymph node
EndMT:	endothelial-to-mesenchymal transition
(e)GFP:	(enhanced) green fluorescent protein
FISH:	fluorescent in situ hybridisation
FOXC2:	forkhead box protein c2
HHT:	hereditary hemorrhagic telangiectasia
iAVC:	inferior atrioventricular canal cushion
ICAM1:	intercellular adhesion molecule 1
ID:	inhibitor of differentiation
ISH:	in situ hybridisation
ITGA9:	integrin alpha 9
IVIS:	*in vivo* imaging system
IVS:	inter-ventricular septum
KO:	knockout

LS:	lymphatic sac
LSS:	laminar shear stress
LV:	left ventricle
LVC:	lymphatic valve forming EC
LVV:	lympho-venous valves
MCP1	monocyte chemotactic protein 1
MMP:	matrix metalloproteinases
NFAT:	calcineurin/Nuclear Factor of Activated T-cells
NICD:	notch intracellular domain
NRP2:	neuropilin-2
OCT:	optimal cutting temperature
OFT:	outflow tract
ON:	overnight
OSS:	oscillatory shear stress
P:	postnatal day
PAH:	pulmonary arterial hypertension
PBS:	phosphate buffered saline
PECAM:	platelet endothelial cell adhesion marker
PFA:	paraformaldehyde
pH3:	phospho histone H3
PDPN:	podoplanin
PDGF	platelet derived growth factor
PDGFR	platelet derived growth factor receptor
pOFT:	proximal outflow tract
PROX1:	Prospero-related homeobox-1
pSMAD:	phosphorylated SMAD
RA:	right atrium
RT:	room temperature
RV:	right ventricle
S:	somites
sAVC:	superior atrioventricular canal cushion
SMAD:	Sma and mothers against decapentaplegic homolog
SMC:	smooth muscle cell
SOX18:	SRY-related HMG-box 18
SP:	septum primum
TBS:	tris buffered saline
TGFβ:	transforming growth factor beta
Tq	turquoise
TV:	tricuspid valve
Tx:	tamoxifen
V:	vein
VCAM1:	vascular cell adhesion marker 1
VE:	vascular endothelial
VEGF:	vascular endothelial growth factor
VEGFR:	vascular endothelial growth factor receptor
VWF:	von Willebrand factor

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Summary

Millions of people suffer from malfunctioning lymphatic vessels resulting in diseases such as lymphedema, that cause tissue swelling and inflammation. In healthy conditions, this lymphatic vasculature plays important roles in the uptake and drainage of excessive fluid, macromolecules and immune cells from the interstitial space. Valves in the collecting lymphatic vessels safeguard a unidirectional lymph flow and prevent lymph backflow. Neither cures nor effective treatments are available for patients with lymphedema or other lymphatic-related diseases. This is in part because our understanding of the molecular mechanisms that regulate lymphatic vessel development and function remains limited. Recently, Bone Morphogenetic Protein (BMP) signaling has been implicated in the development of lymphatic vessels and valves, yet the direct involvement of the intracellular SMAD1 and SMAD5 effector proteins that act in BMP signaling, have not been studied. Here we make use of mouse models to study the spatial-temporal dynamics and function of SMAD1/5 in the developing lymphatic vasculature. These *in vivo* studies are strengthened by *in vitro* experiments, using cultured human dermal lymphatic endothelial cells (dLECs), and transcriptomic profiling.

We studied *in vivo* the context dependent activation of the BMP-SMAD mediated signaling pathway in endothelial cells (ECs) of blood and lymphatic vessels (**Chapter 3**). These studies reveal the highly dynamic nature of BMP-SMAD action in a spatial-temporal manner in different subtypes of vessels. Such results can provide insight in vascular bed and/or organ-specific diseases, and phenotypic heterogeneity within populations of cells, here ECs.

The next part of the study, which builds on the previous work, documents the role of BMP-SMAD1/5 during the development and functioning of the lymphatic vasculature (**Chapter 4**), which provides also the basis for subsequent work (**Chapter 5**). The genetic inactivation (double knockout, dKO) of *Smad1/5* in lymphatic endothelium in mouse embryos results in dilated lymphatic sacs, while early postnatal dKO of *Smad1/5* in LECs leads to smaller, underdeveloped mouse pups with multiple lymphatic defects. Furthermore, this work also incorporates assays for uptake and drainage by lymphatic vessels in mice and altogether shows novel SMAD1/5 functions in different lymphatic vessel beds.

Subsequently, I carried out a more in-depth morphometric analysis to substantiate SMAD1/5 roles in lymphatic endothelium. I demonstrate that *Smad1/5*-KO mice show delayed valve development, supernumerary valves, dilated lymphatic vessels that lack smooth muscle cell coverage, and altered localization patterns of key lymphatic markers. Using RNA-Sequencing in dLECs, we uncover a novel mechanism in which BMP9-SMAD1/5-mediated signaling regulates mRNA expression of different WNT pathway components in lymphatic endothelium. Furthermore, under oscillatory shear stress (OSS) conditions, SMAD1/5 dampens WNT/β-catenin signaling. Likewise, *in vivo*, SMAD1/5 normally suppresses WNT/β-catenin signaling and promotes lymphangion stabilization. In *Smad1/5*-dKO mice, drug-mediated degradation of β-catenin rescues, in part, the observed lymphatic phenotype. Hence, this complex study shows that SMAD1/5 are important context-dependent attenuators of WNT/β-catenin signaling that contribute to stabilization of postnatal lymphatic collecting vessels.

In conclusion, this PhD thesis on the role of SMAD1/5 in lymphatic endothelium provides new fundamental insights, novel biological effects and action modes of BMP signalling. These results can foster new translational studies aiming at improving diagnosis (incl. predictive diagnosis) and timely treatment of subclinical lymphedema.

Nederlandse samenvatting

Miljoenen mensen lijden aan slecht functionerende lymfevaten, wat vaak leidt tot aandoeningen zoals lymfoedeem. Het lymfevat netwerk speelt een grote rol in de opname en afvoer van overtollig vocht, macromoleculen en immuuncellen vanuit de interstitiële ruimte en voorkomt zo weefselzwelling. Kleppen in collecterende lymfevaten verzekeren lymfestroming in één richting. Tot op heden zijn er geen medicijnen of effectieve behandelingen beschikbaar om patiënten met lymfoedeem of andere lymfatische aandoeningen te genezen. Ondanks het groot maatschappelijk belang van deze aandoeningen, is onze kennis over de moleculaire mechanismen die de ontwikkeling en functie van lymfevaten sturen nog steeds relatief beperkt. Recent werd aangetoond dat de BMP signaalweg belangrijk is voor de ontwikkeling van lymfevaten en kleppen, maar de betrokkenheid van de intracellulaire BMP-effector eiwitten SMAD1 en SMAD5 staat nog ter discussie. In deze studie maken we gebruik van muismodellen om SMAD1/5 signalering te bestuderen tijdens de ontwikkeling van het lymfevaten netwerk. Deze *in vivo* studie wordt ondersteund door *in vitro* experimenten in humane dermale lymfatische endotheelcellen (dLECs) en aanvullende transcriptoom analyse.

Wij bestudeerden de *in vivo*, context-afhankelijke activatie van de BMP-SMAD gemedieerde signaalweg in endotheelcellen (ECs) van bloed- en lymfevaten (**Hoofdstuk 3**). Onze resultaten tonen de context-afhankelijke en dynamische aard van BMP-SMAD gemedieerde gen transcriptie aan in verschillende subtypes van vaten. Zulke resultaten verschaffen mogelijk meer inzicht in verschillende vasculaire of orgaanspecifieke aandoeningen, en het fenotypisch heterogeen karakter in populaties van cellsn zoals ECs.

De volgende stap in ons onderzoek, dat voortbouwt op het vorige luik, documenteert de rol van BMP-SMAD1/5 gemedieerde signalering tijdens de ontwikkeling en het functioneren van lymfevaten (**Hoofdstuk 4**) en is ook cruciaal voor het volgende onderzoeksluik (in **Hoofdstuk 5**). Muizenembryo's waarin *Smad1/5* beide conditioneel geïnactiveerd (dubbele knockout, dKO) werden, vertonen verwijde lymfatische zakken, terwijl kort na de geboorte deze muizen groeiachterstanden en lymfevatendefecten vertonen. Bovendien omvatte dit onderzoek ook verschillende testen om de opname en afvoer van lymfe in lymfevaten te bestuderen. Alles samen onthult dit onderzoek nieuwe en diverse functies van SMAD1/5 in meerdere subtypes lymfevaten.

Daarna voerde ik een meer diepgaande morfometrische analyse uit van de rol van SMAD1/5 in lymfatisch endotheel. Smad1/5-KO muizen vertonen een vertraagde lymfevatklep ontwikkeling, meer kleppen, verwijde lymfevaten, afwezigheid van gladde spiercellen en veranderde lokalisatiepatronen voor belangrijke lymfevat markers. Dit onderwoek ontmaskerde een nieuw mechanisme waarbij BMP9-SMAD1/5 gemedieerde signalering mRNA-expressie van verschillende WNT-componenten reguleert in dLECs. Dit inzicht werd verkregen door middel van ondermeer RNA-Sequencing. Bovendien tonen we aan dat SMAD1/5 WNT/β-catenine signalering dempt bij turbulente stroomcondities. We tonen aan dat in de muis SMAD1/5 normaal WNT/β-catenine signalering onderdrukt en zo de stabilisatie van het lymfangion bevordert. Bovendien herstelt geïnduceerde degradatie van β-catenine in Smad1/5-dKO muizen de geobserveerde defecten. Deze studie toont aan dat SMAD1/5 belangrijke context-afhankelijke mediatoren zijn van de WNT/β-catenine signaalweg en bijdragen aan de stabilisatie van het lymfevat netwerk na de geboorte.

Samengevat biedt dit proefschrift over de rol van SMAD1/5 in lymfevatontwikkeling nieuwe fundamentele inzichten in biologische processen en actiemechanismen van BMP-signalering. Deze kennis is belangrijk voor het stimuleren van nieuwe translationele studies met als ultiem doel diagnoses en behandelingen van (sub)klinisch lymfoedeem te verbeteren.

Chapter 1

Introduction

Multicellular adult animals, including vertebrates, demand an internal regulatory system to provide nutrients and oxygen and remove metabolic waste products to/from cells and tissues. To overcome this demand vertebrates, including humans, have developed two transport systems that consist of highly ramified, tubular networks to maintain body homeostasis: the circulatory blood system and the unidirectional lymphatic vasculature. The lymphatic vasculature is mainly responsible for the uptake of extravasated fluids from the capillary beds and the transport of dietary lipids from the intestines. Moreover, it is an important conduit for immune cells. It plays key roles in health and disease, including in tumor biology, inflammation and fat metabolism. Worldwide millions of people suffer from lymphatic defects [1]. Despite its importance for health and disease, studies of the genetic and molecular regulations of the development of the lymphatic system remained largely unexplored until about twenty years ago.

1.1. The lymphatic vasculature

The lymphatic vasculature is part of the lymphatic system, which comprises in addition to the elaborate network of lymphatic vessels also lymphoid organs and lymph nodes (LNs). Here, the emphasis will be on the intricate lymphatic vessel network. Erasistratus of Cheos first mentioned lymphatic vessels 304-250 BC. After his initial description, it took 2,000 years to "rediscover" the lymphatic vasculature [2]. Gaspare Aselli characterized the "milky vein" lymphatic vasculature in more detail, followed by the discovery of the thoracic duct by Thomas Bartholin half way the 17th century. Over centuries scientists argued about the origin of the lymphatic vasculature. Early 20th century, Florence Sabin was the first to report that lymphatic vessels originate from the venous vessels. Ink injection experiments in pig embryos revealed that the jugular lymph sacs develop from the anterior cardinal veins [3]. The venous origin of the lymphatics was later also confirmed by more advanced lineage tracing experiments, including genetic approaches, and as the result of experiments involving targeted gene deletion [4].

Functions in health

Lymphatic vessels are crucial for homeostasis, thereby maintaining normal blood and tissue overall size/volume. Firstly, lymphatic vessels (re)absorb extravasated fluid from the interstitial space. Organs and tissues demand oxygen and nutrients to function properly. To provide oxygen and nutrients the fluid from the blood capillary bed extravasates into the interstitial space. However, only 90% of interstitial fluid flows back into the venous system [5]. To prevent accumulation of fluid in interstitial spaces, lymphatic vessels play an eminent role. Per day, in a healthy adult person, the lymphatic vessels transport up to 2-4 liters of interstitial fluid containing macromolecules and catabolic waste products to the venous system [5].

Secondly, the lymphatic system plays a pivotal role in the normal functioning of the immune system. Lymphatic vessels are in close contact with other lymphoid organs and LNs. Antigen presenting cells (APCs) from the interstitial space travel via the lymphatic vessels to the LN where they remain. Here the initial immune response results in the release of lymphocytes in lymph [5], which is then transported to the blood vessels. Lymphatic vessels can regulate inflammatory responses by fluid transport of extravasated leukocytes and APCs. The lymphatic system contributes to the decrease of inflammation induced edema and to the initiation of an immune response. During inflammation the LNs and lymphatic vasculature becomes red and

enlarged due to swelling. This is often in response to bacterial (e.g. *Streptococcus* or *Staphylococcus*), viral or fungal infections [6].

Lastly, lymphatic vessels in the intestines, the so-called lacteals, are responsible for the uptake and transport of dietary fats from the intestinal tract towards the liver. Most nutrients from the intestinal tract are absorbed by the blood vessels and are immediately transported to the portal vein, but fats indeed take a different route. Bile from the liver and pancreatic enzymes cover fats in the intestines and hence reduce their size and compact them into smaller micelles, which are taken up by enterocytes. Next, lipids and proteins will cover the micelles, which triggers the micelles to enter into the lymphatic lacteals. The combination of lymph and emulsified fat is called chyle [5,7,8].

Organization of the lymphatic vasculature

Over the last decades, our understanding of how lymphatic vessel hierarchy and structure is established, has expanded tremendously. Interstitial fluid enters the blind ending lymphatic capillary network. Next, lymph from the capillary network drains into larger pre-collector vessels and collector vessels (Figure 1.1). The inner lining of all lymphatic vessels consist of lymphatic endothelial cells (LECs). The lymphatic capillary network consist of one layer of LECs, which are connected by discontinuous and button-like intercellular junctions and anchored by filaments to the extracellular matrix (ECM) at the abluminal side of the endothelium, and only have a minimum basement membrane (BM) [9]. High-resolution imaging of lymphatic capillaries further shows that the LECs in here have an oak-leaf like shape with overlapping flaps, and lack junctions at their tips [10]. When interstitial pressure increases, the ECM stretches. Fluid can then enter the lymphatic capillaries more easily at the tip of the LEC flaps (Figure 1.1). This does not affect the intercellular junctions on both sides of the flaps. The anchoring filament proteins Emilin-1 and Fibrillin help to open lymphatic capillaries when the interstitial pressure increases. Yet, Emilin-1 and Fibrillin-1 deficient mice show neither defects nor reduced lymph drainage [11,12].

Collecting vessels are more quiescent and have continuous zipper-like junctions that prevent leakage of lymph. They have a BM, intraluminal valves and a thin layer of smooth muscle cells (SMCs). Intraluminal valves ensure a unidirectional flow of lymph. The contraction of SMCs, muscles and arteries help propel the lymph into the direction of LNs and finally into the blood stream. Collecting lymphatic vessels enter the LN via afferent lymphatic vessels. In the LN, lymph is drained through three different zones, the subcapsular, trabecular and medullary sinuses. Lymph exits the LN through efferent lymphatic vessels. From the LNs the lymph is drained towards the thoracic duct and the right lymphatic duct back into the left and right subclavian veins, respectively. The lymphatic network is present in nearly all tissues and organs except for the retina, bone marrow and bone tissue [9,13].

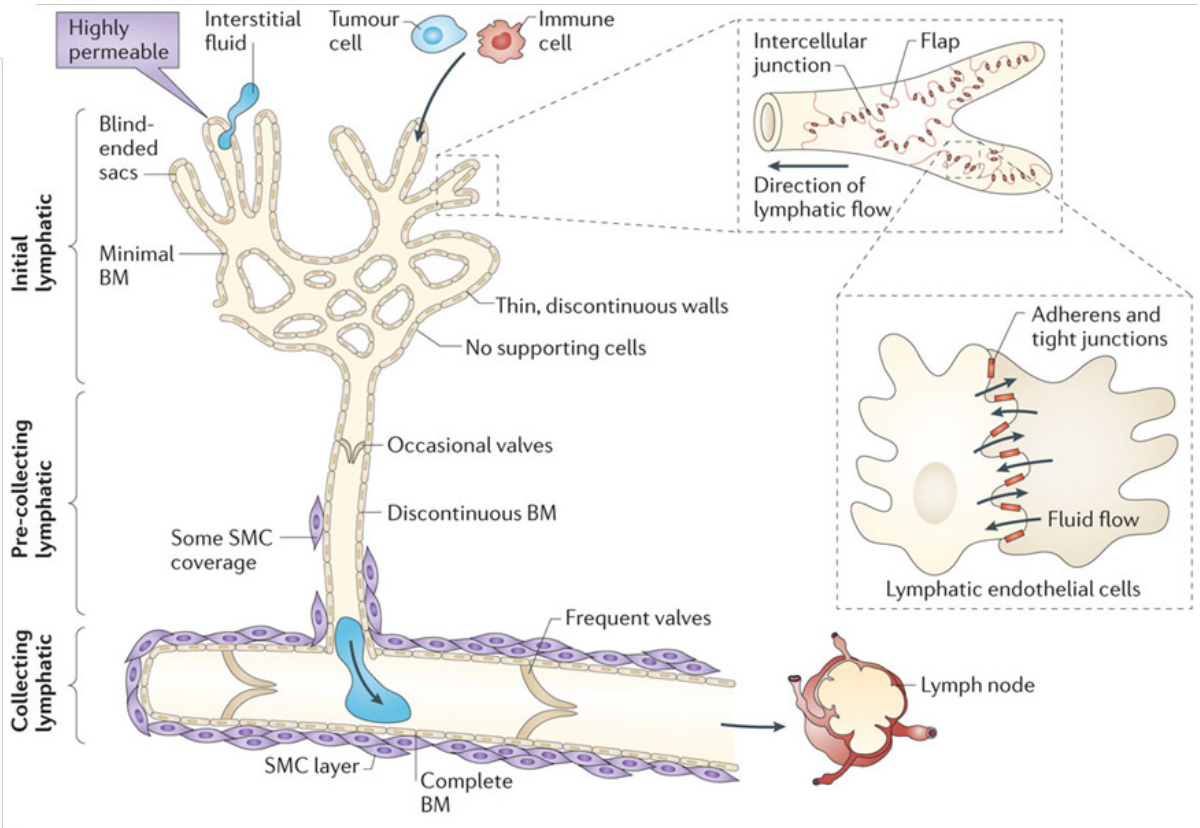

Figure 1.1: Lymphatic vessel hierarchy.

Lymphatic vessels are divided in different subtypes: initial or capillary lymphatic vessels, pre-collector and collector lymphatic vessels. Capillary lymphatics are characterized by blind-ending vessels, minimal basement membrane (BM) and button-like junctions. (Pre-)Collector lymphatic vessels receive lymph from the capillary bed and have a basement membrane, lymphatic valves, zipper-like junctions and are covered by smooth muscle cells (SMCs). Figure taken from [9].

Pathologies

The lymphatic vasculature is often involved in pathological settings, such as edema, tissue inflammation and cancer metastasis. Notwithstanding the great socio-economic impact of these lymphatic diseases many challenges remain with regard to understanding the complex features of lymphatic function. Lymphedema is the major lymphatic disease. People with lymphedema suffer from chronic tissue swelling in the face, arms, legs or abdominal wall, each being associated with disability and increased inflammation risks. Lymphedema can have a primary (genetic) and secondary (acquired) origin. It results from accumulation of interstitial fluids because of lymphatic dysfunction. Lymph stasis, lymphatic dysplasia, malformation and misconnection, or obstruction of lymphatic vessels and/or absence of functional lymphatic valves and smooth muscle cell recruitment, can underlie lymphatic dysfunction [15]. The World Health Organization estimates that more than 150 million people worldwide suffer from secondary lymphedema. The National Institute of Health (US) published that the incidence of primary lymphedema is as high as 1 in 300 live births. Furthermore, 1.4 billion people in 73 countries around the globe are threatened by lymphatic filariasis or elephantiasis. Currently, over 120 million people are infected with the parasite, filarial worms, responsible for lymphatic filariasis, of which at least 40 million are disfigured and incapacitated by the disease. Moreover, the incidence of secondary lymphedema among cancer survivors is 20-40%. In the US, lymphedema is estimated to affect more than 10 million people, which is more than acquired immune deficiency syndrome (AIDS), Parkinson's disease, muscular dystrophy, multiple sclerosis and amyotrophic lateral sclerosis

(ALS) combined. In Europe, precise numbers of patients with lymphatic-related deaths have not been published, but cardiovascular-related deaths, in which lymphatic diseases are included, in Belgium alone shows that 1,921/100,000 discharged patients died in 2016. Cardiovascular-related deaths in all EU member states are estimated to have reached 1.9 million deaths in 2015. Other diseases impacted by the lymphatic system include heart disease, AIDS, diabetes and rheumatoid arthritis [14]. Unfortunately, therapies that help patients with lymphatic malfunctions are limited. Here, I will summarize a few well-known diseases related to lymphatic dysfunction.

Lymphedema-distichiasis syndrome is an example of a primary lymphedema that is caused by mutations in *FOXC2* [16]. People suffering from lymphedema-distichiasis develop lymphedema of the limbs, legs and feet because of impaired lymphatic valve development. The growth of extra eyelashes (distichiasis) in the inner lining of the eyelid is a characteristic of this syndrome. Another example of a primary lymphedema is hypotrichosis-lymphedema-telangiectasia. This is a congenital disorder caused by mutations in the SRY-related HMG-box 18 gene (*SOX18*) [17] and is characterized by lymphedema, telangiectasias and hypotrichosis or alopecia, which causes hair loss. Telangiectasias are small dilated vessels towards the surface of the dermal layers of the skin. The syndrome has been reported in both patients with autosomal dominant and recessive inheritance patterns [17,18]. Milroy's disease is caused by mutations in the gene encoding Vascular Endothelial Growth Factor Receptor-3 (*VEGFR3*). Besides lymphedema, lymphatic defects are also observed in intestines where they cause steatorrhea (excess fat in feces) due to ineffective transport of chylomicrons. Moreover, a shortage in lymphocytes causes secondary symptoms, like lymphopenia and impaired cell mediated immunity [19]. Emberger syndrome is a rare genetic syndrome characterized by lymphedema in the lower limbs and genitals and myelodysplasia. Patients suffering from Emberger syndrome have an enhanced risk of developing acute myeloid leukaemia [20]. Research has shown that the GATA-binding protein-2 (GATA2) is prominently present in lymphatic valves and, when absent in patients or mice, these develop a similar phenotype as Emberger syndrome. GATA2 as transcription factor was found to regulate in the mouse the genes encoding several important players in lymphatic development, like Prospero-related homeobox-1 (*Prox1*), Integrin-alpha 9 (*Itga9*) and Eph-related tyrosine receptor ligand B2 (*EfnB2*), compatible with its role during valve development [21–23]. Lastly, genetic mutations in Collagen and calcium binding EGF domains 1 (*CCBE1*) and Vascular Endothelial Growth Factor C (*VEGFC*) have also been related to cases with primary lympehedema [92,93].

Secondary lymphedema can be caused by conditions or procedures that damage the lymphatic vasculature or LNs, thereby impairing lymph flow. Lymphedema often results from being unable to compensate for a cut in lymphatic vessels or removal of LNs during surgery. Similarly, radiotherapy can damage lymphatic vessels or LNs and therefore also affect the lymph flow. Infections of the LNs, accumulation of cancer cells or parasites in lymph vessels or LNs can all underlie lymphedema [24].

As mentioned above, lymphatic vessel and valve defects may underlie lymphedema. Several lymphatic-related genes are important for lymphatic vessel and valve development. In mice, genetic inactivation of these genes results often in edema. Loss of Forkhead box protein C2 (encoded by *Foxc2*; see above) or *Efnb2*, an arterial/lymphatic specific endothelium marker, prevents the formation of lymphatic valves [25,26]. In the same study, it was shown that *Prox1* deletion decreases VE-cadherin localization at cell-cell contacts, but without affecting the total

amount of VE-Cadherin [25]. VE-cadherin functions include anchoring the actin cytoskeleton to cell-cell junctions. The loss of VE-cadherin causes less well-organized lymphatic vessels. Inactivation of *Itga9* and/or the lack of the extra domain A (EDA or EIIIA) of Fibronectin (FN) in mice affects the elongation of lymphatic valve leaflets and result in non-functional valves [27,28]. Similarly, connexin 43/47 (encoded by *Cx43/47*) deficiency in mice leads to abnormal lymphatic valve development. These gap junction proteins are normally abundantly present in lymphatic valves. Interestingly, *CX47* mutations have been described in lymphedema patients [29].

The spreading of tumor cells of many different types of cancer, such as melanoma, colorectal cancer, breast and lung cancer often requires the co-development of new lymphatic vessels. Tumor cells use the lymphatic vasculature as a conduit to LNs and the blood circulation. From within the lymph node and the blood the tumor cells can metastasize to distant organs/tissues. Cancer metastasis correlates with a poor prognosis once LNs become populated and, therefore, is often also used as a measure of determining tumor stage [30]. The role of lymphatic vessels during cancer metastasis was shown in a in a mouse model for breast carcinoma. In this study lymphangiogenesis was induced in regions of primary tumor growth, this resulted in increased LN metastasis and a significant lower life expectancy [31].

More recently, the lymphatic vasculature has for the first time been documented in the central nervous system and functions in draining cerebrospinal fluid and clearing macromolecules from the brain [32]. This is potentially a major finding. The lymphatic vasculature is a major route for clearance of macromolecules, such as toxins. So far, it was thought, under normal homeostasis, that clearance of cerebral amyloid β happens via blood vessels [33], however, new evidence suggest that the lymphatic vasculature plays a key role in the clearance of Amyloid β [34,35]. Based on these new findings, investigation of neurodegenerative diseases, such as Alzheimer's disease, in which the accumulation of Amyloid β leads to the neurodegenerative pathology, and the role the lymphatic vasculature might play could lead to new major breakthroughs in the medical field.

1.2 Lymphatic vessel development in vertebrates

The development of lymphatic vessels has been documented primarily in mouse and zebrafish. During mouse embryogenesis the formation of the lymphatic vasculature is preceded by blood vessel formation, and the first LECs have venous origin. The development of the blood vascular network is mediated by two distinct processes, vasculogenesis and angiogenesis (Figure 1.2 [36]). Because of the similarity and interdependence of blood and lymphatic vessel development I address here first, and briefly, vasculogenesis and angiogenesis.

Vasculogenesis

The development of the circulatory system starts soon after initiation of gastrulation, concomitant with somite formation. The first vascular structure develops through vasculogenesis (Figure 1.2 [37]). The earliest event in early vasculogenesis in mice (E7.0) (also studied in amniotes like quail) is the differentiation of hemangioblast cells in the extra-embryonic mesoderm of the yolk sac. These hemangioblast cells are the precursors of endothelial cells (ECs) and hematopoietic cells that form the blood islands; they never form in the amnion. The fusion (coalescence) between these yolk-sac blood islands results in a vascular plexus, which connects to the embryonic blood vessels that form *de novo* from E7.5 onwards [38]. As soon as there is a beating heart tube and blood circulation, this network further remodels into an arteriovenous vascular system [39–41]. The dorsal aorta and cardinal vein are two major blood vessels generated by vasculogenesis. Ligands of the Vascular Endothelial Growth Factor (VEGF), Hedgehog (Hh), Fibroblast Growth Factor (FGF) and BMP signaling pathways, respectively, are vital for the induction/formation of the hematovascular lineage and early EC differentiation. Their interaction with liganded VEGFR1/2 is crucial for both development and remodeling of blood islands [42–44].

Angiogenesis

After the establishment of a primitive plexus, angiogenesis results in the formation of new vessels from pre-existing ones, accompanied by vessel remodeling through endothelial sprouting and splitting of the lumen of vessels (intussusceptive angiogenesis). The formation of a new vessel sprout is triggered by pro-angiogenic signals such as VEGF, secreted from (and its gene transcription activated in) a hypoxic environment [45,46]. These signals elicit the selection of a tip cell in a nearby vessel, which initiates a chain of events, including early, local degradation of ECM. The leading tip cell will grow towards the hypoxic region and guide the following stalk cells that elongate the forming sprout. When two sprouts meet, often assisted by macrophages, they can anastomose. Perfusion can happen before or after anastomosis. Emerging sprouts and vessels become supported by mural cells (Figure 1.3). Phalanx cells are covered by other perivascular cells (pericytes) that keep the ECs quiescent. As a result of the paracrine multi-factor controlled vasculogenesis, angiogenesis and the vessel remodeling, cell-cell interaction and hemodynamic cues, a functional and well-structured vascular tree is formed[47].

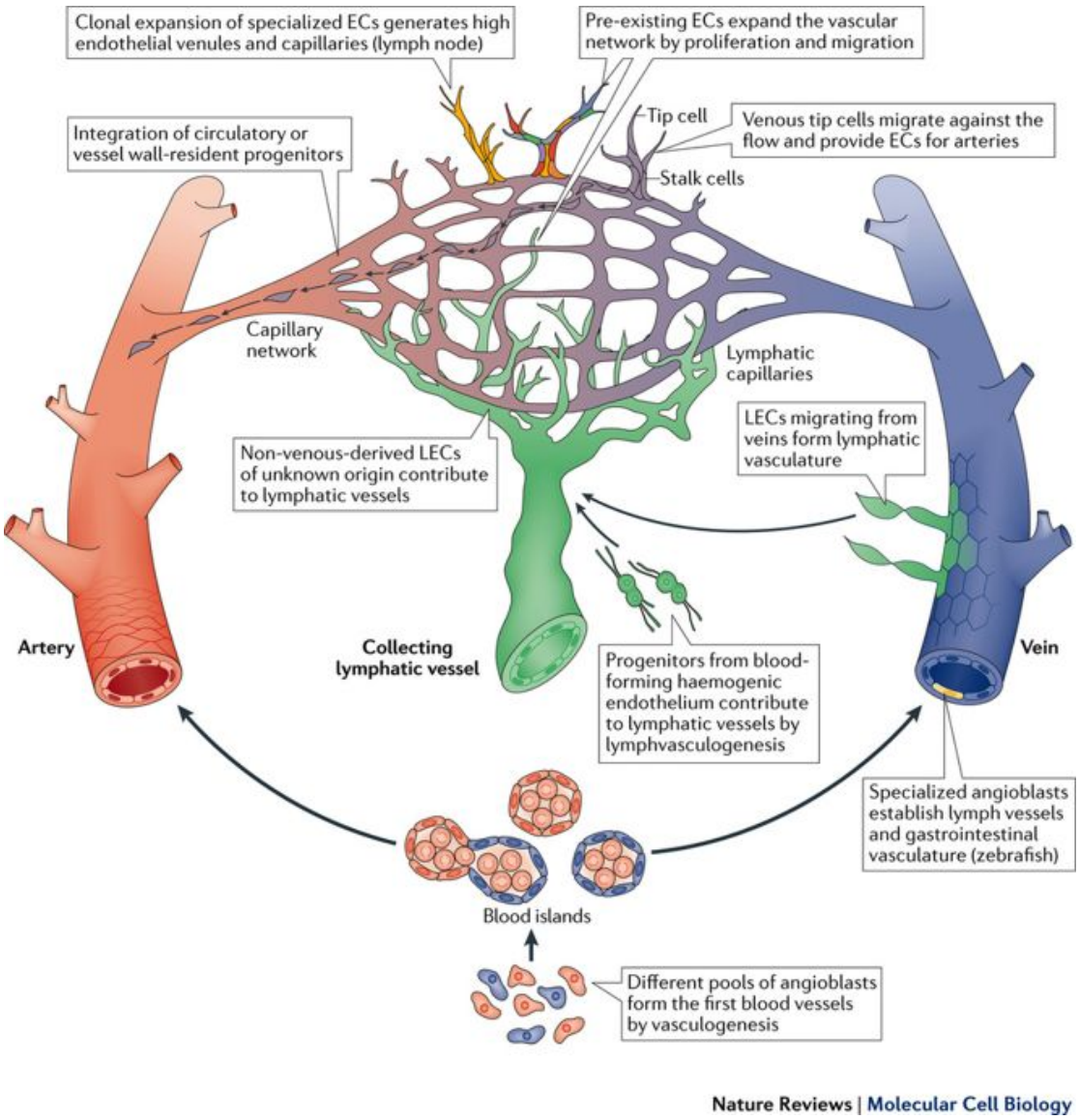

Figure 1.2: Origin of the lymphatic vasculature.
Vasculogenesis is the *de novo* differentiation of endothelial cell (EC) progenitors (angioblasts) in the developing embryo. These cells aggregate in blood islands that will coalesce into a primitive vascular plexus. Angiogenesis refers to the expansion of the vascular network from pre-existing vessels. Subsequently, the arterio-venous specification leads to the formation of the venous and arterial blood circulation, and the vessels will remodel by arteriogenesis. The first lymphatic endothelial cells (LECs) bud from the cardinal vein through *trans*differentiation of venous ECs (in zebrafish, mammals) and the intersomitic veins (in mammals). These LECs will form the lymphatic sac. Lastly, blood-forming hemogenic endothelium contributes to the lymphatic vasculature of certain organs by lymphvasculogenesis [36]. For more details, see Chapters 1.2 and 1.3.

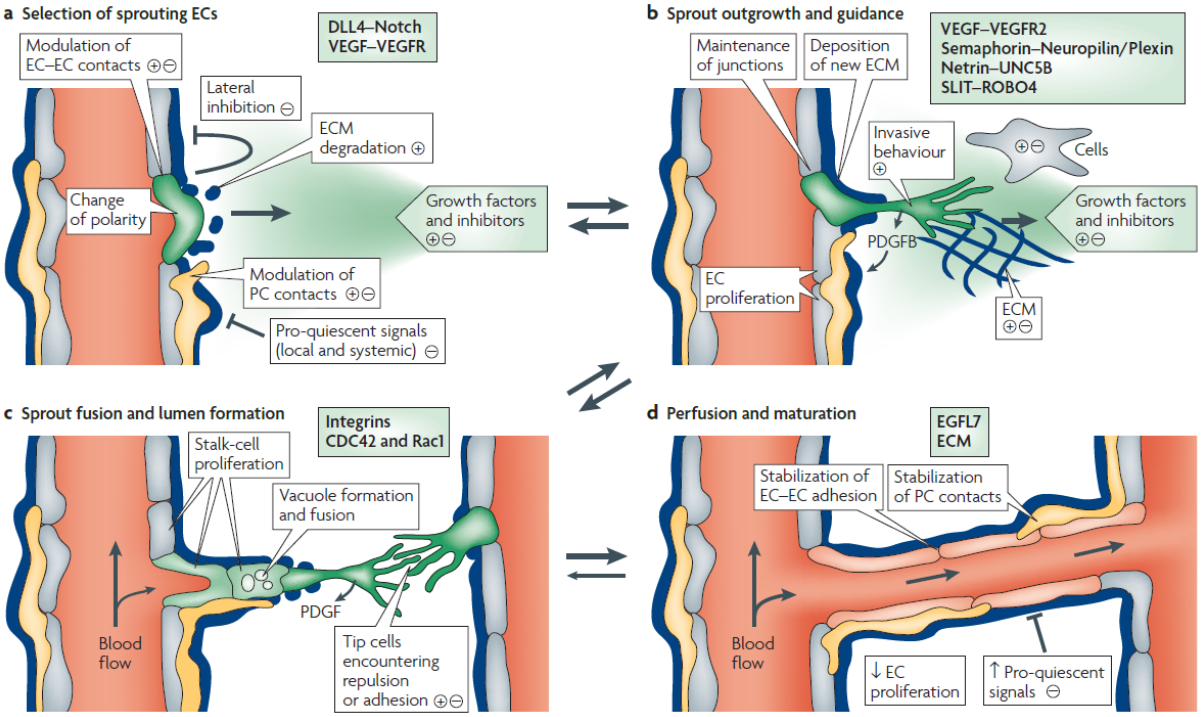

Figure 1.3 : A schematic representation of sprouting angiogenesis.
The balance between sprouting tip and non-tip cells is mediated by pro-angiogenic factors (+) that e.g. cause ECM degradation, and anti-angiogenic factors (-) that contribute in lateral inhibition. In pro-angiogenic conditions selected ECs sprout (green), while they will actively inhibit their neighboring cells to become a tip cell and boost stalk cell features (grey) (A). The sprouting cell regionally degrades matrix components and migrates towards an environment enriched pro-angiogenic factors such as VEGF and may be repelled from other areas. The sprouting tip cell will release platelet-derived growth factor that attract pericytes to new tip cells (B). By either adhesive or repulsive interactions tip cells might fuse, whereas the adjacent stalk cells undergo lumen formation (C). The lumen formation will lead to a continuous lumen and blood that supplies oxygen and nutrients to the surrounding tissues. Consequently, the flow will contribute in the stabilization of the vessel, matrix deposition and recruitment of peri-endothelial cells (D). Taken from [48].

Molecular regulation and dynamics of tip and stalk cells

The tip and stalk cells are the most prominent EC phenotypes during sprouting angiogenesis. Every EC can become a tip, stalk or eventually phalanx cell depending on the context. Tip and stalk cells are dynamically changing phenotype during sprouting angiogenesis, achieved by continuous monitoring of other surrounding cells and intra- and extra-cellular cues, but also influenced by metabolic status of the cells [49]. Consequently, ECs must be able to swiftly regulate the induction/up-regulation or repression/down-regulation of phenotype-specific transcripts. VEGF-A/VEGFR2/VEGFR3-mediated signaling triggers tip cell selection through enrichment of Delta-like 4 (DLL4), a ligand of Notch. DLL4 signals to adjacent ECs and instructs them to become stalk cells via DLL4/Notch1-mediated lateral inhibition that leads, through Hairy and Enhancer of Split-1 (HES1) and Hairy/enhancer-of-split related with YRPW motif protein 1 (HEY1), to down regulation of the levels of VEGFR2 and DLL4[50,51]. VEGF is also involved in stalk cell proliferation, and its crosstalk with the DLL4-Notch axis is required for a balance between tip and stalk cells. If unbalanced, this results in excessive sprouts (hypersprouting) or reduced vessel formation [52–54]. However, VEGF is not the only regulator of sprouting angiogenesis and tip versus stalk cell phenotype (see section 1.4).

Intussusceptive angiogenesis

The primitive vascular plexus may expand through sprouting angiogenesis and intussusceptive angiogenesis. Intussusceptive angiogenesis does not involve sprouting of ECs in the surrounding tissues, but results in expansion of the vessel network through transluminal tissue pillar formation and vessel splitting. There are three phases that can be distinguished: intussusceptive microvascular growth, arborization and branching remodeling [55].

The mechanisms that underlie the formation of pillars are not completely understood. Indicators of induction of pillar formation are intraluminal endothelial protrusions followed by EC contacts, cell junction reorganization, recruitment of myofibroblasts and pericytes to the pillar, and collagen deposition [55]. Hemodynamic cues, such as blood flow, play a major role in pillar formation. This was shown by clamping one of the developing branches of an artery. Increased blood flow leads almost immediately to the formation of pillars and *vice versa* [56]. Changes in flow are sensed by ECs and such signal is often transduced by Platelet Endothelial Cell Adhesion Marker (PECAM) and vascular endothelial (VE) cadherin, eventually leading to the transcription of endothelial Nitric Oxide Synthase (eNOS) and growth factor encoding genes. Here the VEGF/VEGFR2 axis is involved, but also basic Fibroblast Growth Factor (bFGF) and Platelet Derived Growth Factor B (PDGF-B) play a role in stabilizing the vessel through stimulating recruitment of pericytes [57,58]. Additionally, Notch1 inhibition correlates with increased recruitment of mononuclear cells and increased levels of CXCL12 and CXCR4, resulting in excessive intussusceptive angiogenesis [59].

Arteriovenous specification

As development continues, new vascular beds develop to support the growing need for oxygen and nutrients in different organs and tissues and these vessels remodel into an elaborate network of arteries, veins and capillaries. At the molecular level Chicken Ovalbumin Upstream Promoter-Transcription Factor 2 (COUPTFII) plays a key role in balancing EC fate. TCOUPTFII regulates gene expression of venous EC fate by blocking activation of the arterial VEGF-Notch cascade [60], while heterodimers with LEC-enriched PROX1 and COUPTFII instruct LEC fate [61]. The COUPTFII homodimers have a repressing effect on the Notch target genes *Hey1/2.* Importantly, arteriovenous identity is also hemodynamically regulated [61,62]. Arterial and venous vessels acquire different structural and functional features to accommodate the different hemodynamic pressure in the circulatory system (Figure. 1.4).

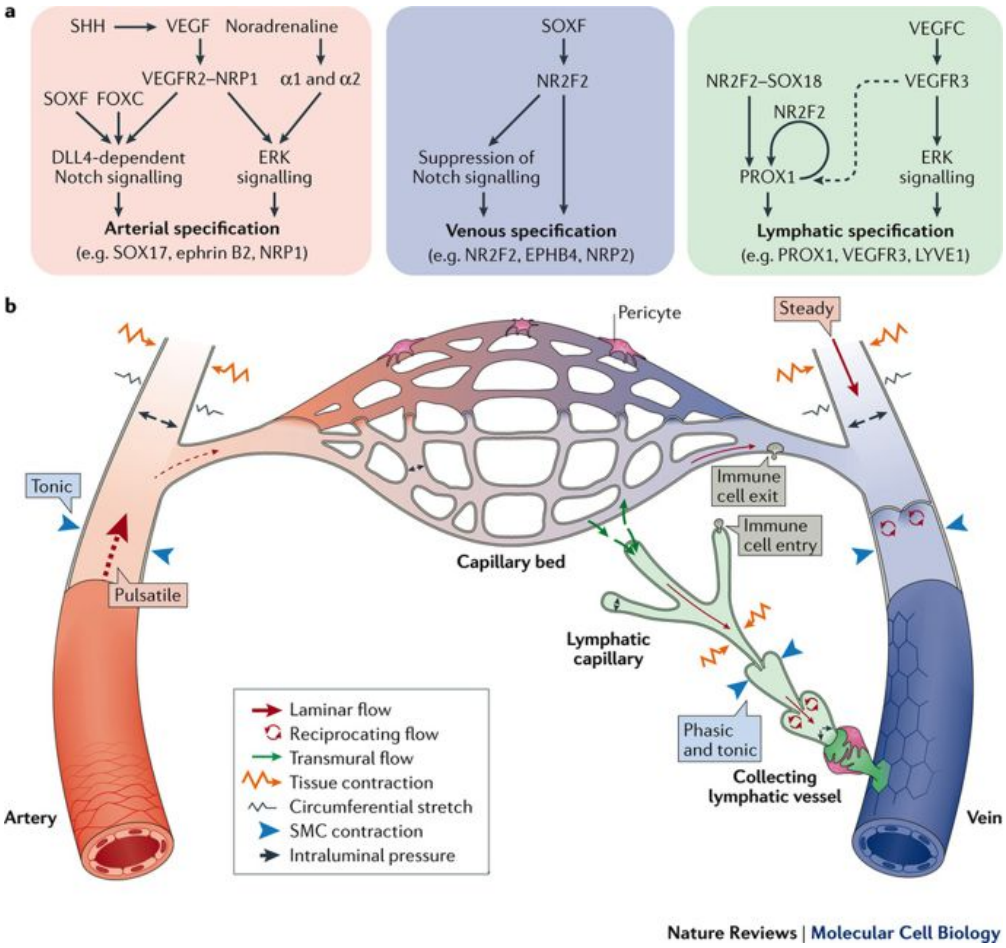

Figure 1.4: Molecular mechanisms underlying EC specification.
Three signaling networks that regulate arterial, venous and lymphatic vessel identity in zebrafish and mice (A). Different types of mechanical forces shape the blood and lymphatic depending on its function (arterial, venous or lymphatic), size and location. These mechanical forces include: flow, pressure, organ function, cell-cell interaction and cell-ECM adhesion. Arteries are under constant high pressure, maintaining a constant blood flow towards the blood capillary beds. The pressure in the venous and lymphatic vasculature is lower and both have valves that prevent back flow. At valve sites fluid flow is non-stable (B) [36].

Lymphangiogenesis

Lymphatic commitment

As mentioned above, the development of the lymphatic vasculature starts within the venous vasculature around E9.5 in mice. A subpopulation of venous ECs in the dorsal cardinal vein become LEC progenitors [4,63–65], detectable by the appearance of Prox1. Currently, few transcription factors have been described as critical for LEC specification and differentiation, Also, how they interact with other signaling pathways, such as the Vegfc-Vegfr3 and Notch pathway, is still not documented in much detail. As venous and lymphatic ECs are closely related, some of these transcription factors may have become shared. For instance, mutations in a member of the SOX gene family, Sry-related HMG-box 18 (*SOX18)*, in humans is linked with hypotrichosis lymphedema telangiectasia [17]. In mice, *Sox18* is mainly expressed in hair follicles and vascular ECs [66]. Indeed, point mutations in *Sox18* lead to cardiovascular and hair follicle defects [67,68]. Around E7.5 in the mouse, Sox18 becomes enriched in allantois and yolk sac blood islands [68]. From E9.0 the CV becomes positive; however, this is not maintained in LEC from E14.5 onwards [69]. Yet, in early lymphatic commitment and specification *Sox18* expression remains important for generating LEC progenitors through activation of *Prox1* in the CV [69] (Figure.1.4a and 1.5).

Sox18 is an upstream activator of *Prox1* by directly binding to 4kb-sequence of the *Prox1* promotor region that has two conserved SoxF-binding sites [69]. Interestingly, Sox7 and Sox17 synthesis similarly leads to activation of *Prox1* [70]. The activation of *Prox1* by Sox18 in venous EC of the CV (and not in arteries) suggests that arteries either fail to stimulate the production of factors that may induce *Prox1* or lack repressors that inhibit *Prox1* [69]. MAPK/ERK signaling regulates lymphatic vessel sprouting through the modulation of *Vegfr3* in mice [71], but might also regulate *Sox18* in the CV [72]. Hyperactivation of MAPK/ERK results in increased levels of Sox18 in veins and the dorsal aorta and subsequently induction of *Prox1* in these regions. This provides evidence that the MAPK/ERK signaling pathway blocks arterial repressors for *Sox18* or induces the expression of venous *Sox18* (co-)activators in the arteries [72].

Other co-activators of the LEC phenotype include COUPTFII (Figure. 1.4a and 1.5). *COUPTFII* regulates the expression of *Prox1* by direct binding to its promoter [61]. *COUPTFII* is a steroid/thyroid hormone receptor and mainly expressed in mesenchymal tissues and the venous endothelium during development [73]. It is important for maintaining venous and promoting lymphatic identity through the inhibition of arterial markers [60,74]. Deletion of *COUPTFII* results in a decrease of LEC progenitors in mice [74]. Srinivasan and co-workers showed that *COUPTFII* promotes LEC specification through the activation of *Prox1* by binding directly to a 9.5kb-long fragment upstream of the *Prox1* open reading frame [61]. In addition, COUPTFII plays a role in maintaining PROX1 levels through development and postnatal sprouting of dermal lymphatic capillaries via direct regulation of the Neuropilin-2 gene (*Nrp2*) [75,76]. Importantly, *COUPTFII* remains expressed in LEC, but is not required for LEC quiescence [76].

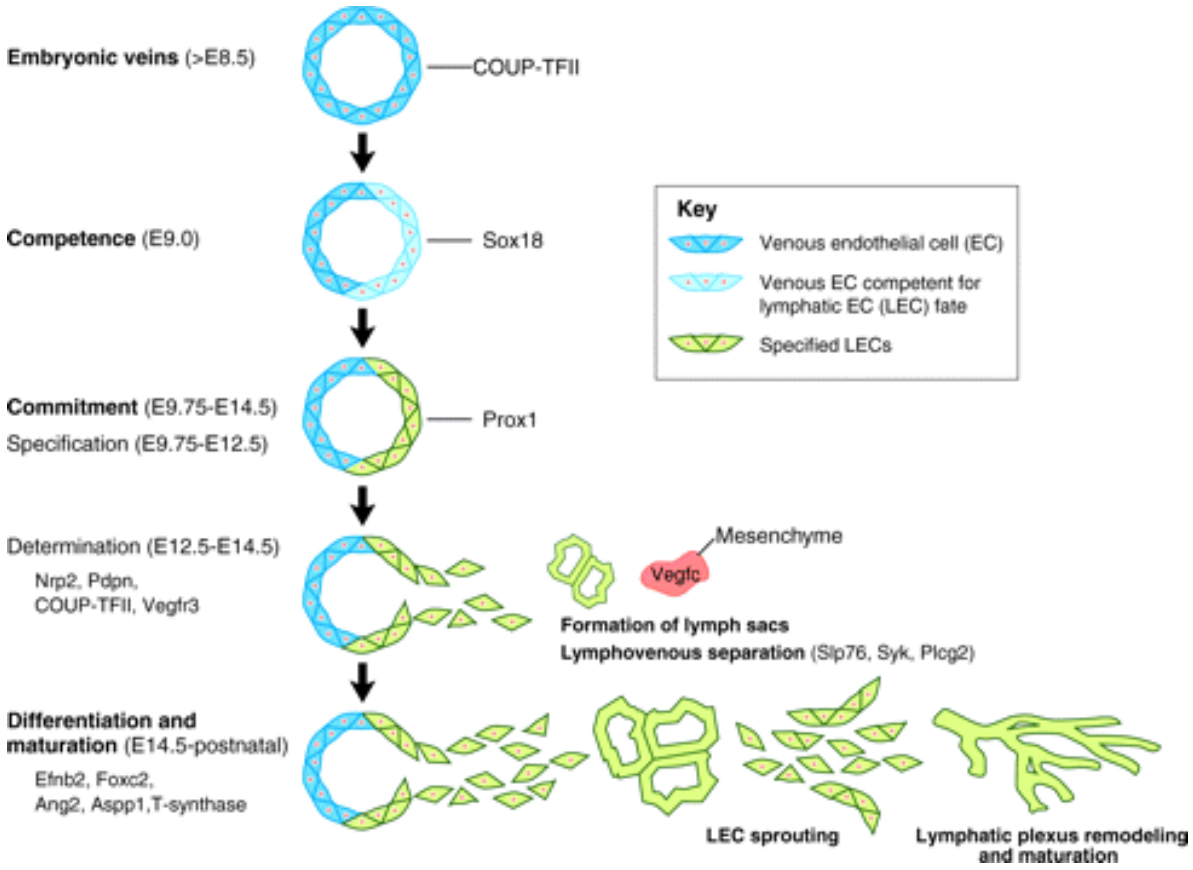

Figure 1.5: Schematic representation of embryonic lymphatic vessel development in mice.
Venous ECs of the cardinal vein have high levels of COUP-TFII (dark blue) from E8.5. From E9.0 onwards, the venous ECs become competent for LEC fate (light blue) and become SOX18^{+}, once LECs are clearly specified (green) PROX1 becomes enriched in these cells. From E12.5 onward LECs will migrate into the mesenchyme depending on levels of VEGFC (red) and form lymphatic sacs and lymphovenous valves that separate the blood and lymphatic vasculature. Finally, through sprouting lymphangionesis and maturation, a fully functional lymphatic vasculature arises. Adapted from [77].

Prox1 expression in venous ECs initiates LEC specification at E9.5 in mice [78] (Figure 1.5). Prox1 was the first LEC marker ever identified, yet its expression is not limited to LECs. The central nervous system, lens, heart, liver and pancreas also express *Prox1* [65,79]. General genetic inactivation of *Prox1* in mice results in the absence of the entire lymphatic vasculature, while conditional deletion of *Prox1* at different times during mouse embryogenesis showed that LECs lose their identity and upregulate more venous EC markers [65,80]. In addition, *Prox1* expression is required for the budding of LECs from the CV [81]. Heterozygote embryos for *Prox1* have edema, blood-filled lymphatics, and lack lymphovenous valves [82,83]. Moreover, haplo-insufficiency of *Prox1* mostly leads to perinatal death, due to severe lymphatic dysfunction, while surviving adult mice have leaky lymphatic vessels [83]. This indicates that *Prox1* is not only important for LEC commitment, but also for LEC differentiation. Today it is widely accepted that *Prox1* is a central regulatory gene in the stepwise process leading to the development of the lymphatic vasculature and it is clear that for these purposes its expression is tightly regulated.

When LEC commitment is initiated, LEC progenitors are triggered to bud from the CV (Figure 1.5). *Prox1* expression then no longer requires COUPTFII [61]. Prox1 induces on its turn expression of several genes, including *Vegfr3*. Different studies showed that ectopic expression of *Prox1 in vitro* results in increased *Vegfr3* [84,85]. *Vegfr3* expression is demonstrated, and its ligand

Vegfc is produced, in blood and lymphatic vessels. Functional inactivation of *Vegfr3* in mice results in abnormal remodeling of the early blood vasculature around E10.0 [86]. However, lymphatic vessels express *Vegfr3* only from E10.5 onwards. Absence of the Vegfc/Vegfr3 signaling axis results in a failure of Prox+ LEC progenitors to bud form the CV [87]. Interestingly, *Vegfr3* is needed for proper regulation of *Prox1* through a feedback loop required for the identity and correct numbers of LEC progenitors[88] (Figure 1.4). Furthermore, post-translational regulation of PROX1 through sumoylation significantly changes it activity to regulate Vegfr3 and through microRNAs (miR-181 and miR-31) *in vitro* [20,89–91]. Thus, LEC-commitment and identity highly depend on proper Prox1 levels regulated by different factors.

Lymphatic differentiation and migration

Upon budding of LEC progenitors from the CV, they migrate in the surrounding mesenchyme and form primary lymphatic structures, called lymph sacs (Figure 1.5). This process depends on VegfC/Vegfr3 signaling and *Nrp-2* expression [81]. During this process LEC progenitors mature and express other genes, such as *Podoplanin* (*Pdpn)*[81]. Importantly, not all LEC progenitors migrate away from the CV. Indeed, some stay behind and contribute to the formation of the lymphovenous valves. As mentioned, Vegfc is a critical regulator of the budding of LECs from the CV. Studies have shown that budding LECs remain inter-connected and form strings that eventually form the luminized lymphatic sacs [64,81]. In addition, Ccbe1 plays a major role in the budding of LECs. *CCBE1* has been associated with Hennekam syndrome, such patients suffer from severe lymphedema, lymphangiectasias and cardiovascular symptoms [92,93].

Initially, the whole mature lymphatic vasculature was believed to develop from the lymph sacs and primitive lymphatic plexus [3,78,94]. However, 3D reconstructions have revealed that, simultaneously with the formation of the lymph sacs, LECs additionally accumulate near the intersomitic blood vessels where they form the peripheral longitudinal lymphatic vessel. This vessel gives rise to the formation of the superficial lymphatic vasculature, while the lymph sacs contribute to the formation of the thoracic duct, the largest lymphatic vessel [64]. Finally, these vessel structures will differentiate in an elaborate network of lymphatic capillaries and (pre-) collector vessels. Similar to the blood vasculature, the sprouting lymphatic vessels have tip cells that sprout towards chemo-attractants, like Vegfc. Vegfc also activates through Vegfr2 Calcineurin/Nuclear Factor of Activated T-cells (NFAT) signaling in LECs. Calcineurin is a serine/threonine phosphatase and regulates nuclear translocation of NFAT factors, activating lymphatic sprouting, maturation of collecting lymphatic vessels and regulates also the formation of a defined boundary of valve-forming cells [95,96].

Lympho-venous separation

To evolve into two distinct tubular systems, the venous and lymphatic vasculature need to be separated. Yet, multiple connections remain close to the junction of the jugular and subclavian veins, and these connections are physically separated by lympho-venous valves (LVVs) that provide unidirectional entrance from the lymphatic vasculature to the venous system[82]. These LVVs are initiated by Prox1+ LEC progenitors that did not participate in the budding from the CV[82]. As mentioned, *Prox1* heterozygous mice lack LVVs [82,83]. In addition, platelet-specific deletion of platelet activation receptor C-type lectin-like receptor 2 (encoded by *Clec2*) or lack of Pdpn (its ligand) leads to LVV defects and cause fluid backflow and blood-filled lymphatic vessels [97–99]. Pdpn is a small O- and N-glycosylated transmembrane protein present from E11.5 onwards in LECs. In

addition, mice deficient in Spleen tyrosine kinase (*Syk*), Lymphocyte cytosolic Protein 2 (*Slp-76*) and Phospholipase C Gamma 2 (*Plcγ2*), respectively, have similar defects [98,100,101]. Clec2 activation induces *Syk*, *Slp-76* and *Plcγ2* and results in platelet activation, including the release of platelet alpha-granules, important for proper lympho-venous separation. Valve formation in lymphatic vessels will be discussed in more detail in section 1.3.

Lymphvasculogenesis

In addition to the classic formation of new lymphatic vessels from venous EC of the cardinal vein by lymphangiogenesis, the lymphatic vasculature in some organs has a more heterogeneous origin. By lineage tracing and high-resolution imaging of the dermal lymphatic vasculature in development it has been shown that LECs have a *Tie2* endothelial lineage origin[4]. In agreement with this finding the cervical and thoracic dermal LECs have also a venous origin, yet later studies found isolated clusters of LECs in the lumbar region of the skin that were Tie2-negative [102]. In fact, cell sorting of all skin LECs revealed that 30% of LECs had a non-venous origin. These clusters are able to become rapidly incorporated in the developing lymphatic plexus. This new process was termed lymphvasculogenesis (Figure 1.2) [102]. So far, the exact origin of these cell populations are not identified, these lymphatic progenitors could come from a hematopoietic origin, similar to monocytes and macrophages [103–105].

Clusters of LECs from an unknown origin were not exclusively found in dermal lymphatics, but also in the mesenteric and heart lymphatic plexus [106–108]. In contrast to dermal LEC progenitors, the clusters in the heart and mesentery could be traced by using a *Tie2-Cre* line with a R26R-EYFP reporter or *Pdgfb-CreER*^{T2} line with a R26-mTmG reporter. Interestingly, the origin of these cells was not venous, but hemogenic instead. Lineage tracing, using a *Pdgfb-CreER*^{T2} line, labels all hemogenic vessels including the dorsal aorta and yolk sac, but not the venous-derived LECs. Remarkably, the clusters of LEC were found to be PDGFb+, suggestive of a hemogenic origin [109]. Additionally, the hemogenic marker cKIT (KIT Proto-Oncogene, Receptor Tyrosine Kinase) was present in these clusters [106]. Both dermal and mesenteric non-venous derived LECs, initially, have low *Lyve1* expression. As the clusters assemble, form vessels and become integrated with the venous derived lymphatic vessels, they will co-express *Vegfr2/3*, *Prox1*, *Nrp2* and *Pdpn* [102,106].

Currently, it is not completely clear which progenitor cell type contributes to the development of the Tie2-Cre linage negative, lymphatic vasculature of the heart[107]. Lineage tracing using a hematopoietic transgene, such as based on Vav1-Cre control, similarly to the mesentery, suggests the hematopoietic progenitors to contribute to the development of the lymphatic vasculature of the heart. However, studying non-EC sources, including WT1+ pro-epicardial organ, MESP1+ or NKX2.5+ mesoderm, and WNT1+ neural crest, could provide additional insight in the underlying process for the development of the heart lymphatics [107].

1.3 Lymphatic vessel maturation

Subsequent functional differentiation of the premature lymphatic vessels results in major differences in vessel type and segments *e.g*. the formation of lymphatic valves, different EC cell junctions, BM composition and mural cell coverage.

Valvulogenesis

The formation of lymphatic valves is vital for the maturation of the collecting lymphatic vessels and adequate functioning of the lymphatic vasculature. Onset of valve development depends on the maturation stage of the lymphatic vessel and its location. Lymphatic valvulogenesis can be summarized in a few stages (Figure 1.6). Several factors, including disturbed flow [110] (see section 1.2, mechanotransduction in lymphatic vessels), trigger and regulate the formation of lymphatic valves. In the mouse, formation of lymphatic valves is initiated around E16 in the larger lymphatic vessels, such as the thoracic duct, when Prox1 and Foxc2 become enriched at one side of the lymphatic vessel wall, which renders these LECs more permissive to become a valve forming region.

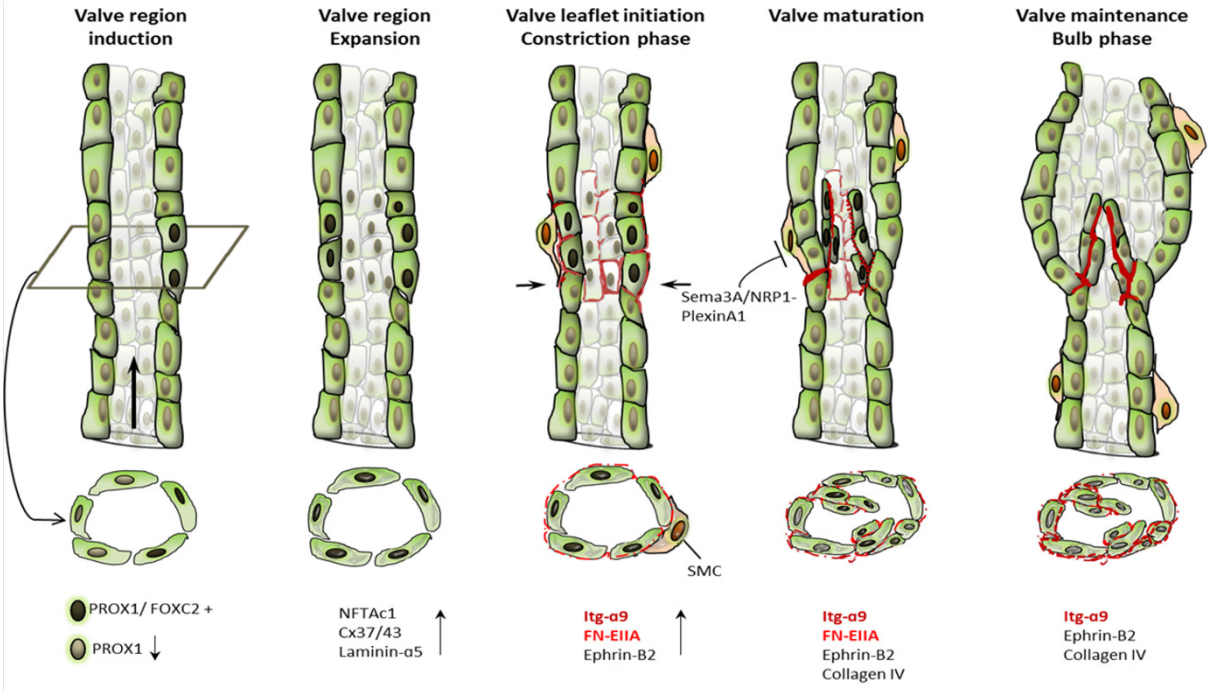

Figure 1.6: Schematic representation of lymphatic valve development.
Valvulogenesis can be divided in different sequential steps. The top and bottom rows represent, respectively, a longitudinal view of a lymphatic vessel and a transverse section at the level of the plane indicated in the top panel. From left to right: (1) a new '*valve induction region'* is marked by the clustering of Prox1/Foxc2+ LECs at one side of the collecting vessel wall; (2) the '*valve region expansion'* phase is recognized by an annular presence of Prox1/Foxc2, and valve-forming cells condense circumferentially and reorient perpendicular to the direction of flow; (3) the '*valve leaflet initiation'* phase is identified by the ring and cells start to protrude into the vessel lumen; and finally, (4) '*valve maturation*' and (5) '*maintenance (bulb)' phases* both show clear semilunar bicuspid leaflets, a lymphatic bulb downstream of the valves becomes apparent in the maintenance phase and SMCs cover the lymphangion, but not the valve area.

During the valve region expansion phase, a cluster of Prox1+ lymphatic valve forming cells (LVCs) forms a ring-like constriction. Flow mediates PROX1 enrichment [111]. Importantly, Foxc2 interacts with Calcineurin/Nfatc1, as was shown by co-immunoprecipitation assays and mouse studies [28]. Developing lymphatic valves activate Foxc2-calcineurin/NFATc1 signaling, as indicated by the increase of nuclear NFATc1 in LVCs. Cx37 is also required for the assembly and delamination of LVCs in the valve leaflet initiation stage, but functions also during postnatal maintenance of valves [112]. Cx37 facilitates the rearrangement of LVCs to this ring-like constriction that is composed of 3-4 rows of cells.

During the valve leaflet initiation stage, LVCs start to secrete ECM components, such as Laminin-α5, Collagen IV and Fibronectin-EIIA. Hereafter, Prox1 and Foxc2 actions become less prominent. Then, LVCs starts to protrude into the lumen of the lymphatic vessel. In the valve maturation phase, the cells align along each other and elongate further in the direction of the lymph flow. Interestingly Foxc2 and Prox1 become asymmetrically distributed towards the upstream and downstream region of the LVCs. The upstream side of the leaflets has LVCs that have high-Prox1 and low-FoxC2 levels, whereas the downstream side of the leaflets has LVCs that show the opposite. After elongation, these cells shape the valve leaflets. Itgα9 anchors LVCs to the leaflet matrix and supports deposition of the before mentioned ECM components. The secretion of ECM components will also lead to the thickening of the leaflets via increased eNOS levels [113]. Yet another important transcription factor is Gata2. *Gata2*-GFP knock-in mice revealed GFP localization in both arterial, venous and lymphatic ECs [114]. Moreover, LVCs are enriched in Gata2 [20]. Additionally, Semaphorin-3A is shown to modulate valve leaflet formation via interaction with NRP-1 and Plexin-A1 on LVCs and repellence of SMCs, thereby maintaining valve areas [115].

Absence of one of the aforementioned key lymphatic vessel and valve signature genes results in a dysfunctional lymphatic vessel plexus or valve. For example, in humans, a point mutation in *FOXC2* results in lymphedema distichiasis [16,116]. Foxc2 is a member of the forkhead/winged-helix family of transcription factors. *Foxc2*$^{+/-}$ mice display a similar phenotype as human patients, such as an extra row of eyelashes, increased LNs and retrograde lymph flow [117]. Foxc2 is likely an important and conserved regulator of lymphatic vessel patterning and valve development during the maturation of collecting lymphatic vessels [27,28]. During the maturation of the lymphatic vasculature *Prox1*, *Lyve1* and *Vegfr3* become downregulated in the lymphangion of the collecting lymphatic vasculature. Interestingly, *Foxc2*$^{-/-}$ mice have excessive levels of these markers [28]. Similar to the *Foxc2*$^{-/-}$ mice, depletion of Calcineurin in ECs is sufficient to affect valve region expansion [25]. In fact, depletion of Calcineurin at any given stage during lymphatic valve development results in valve abnormalities. Therefore, the Foxc2-calcineurin/NFATc1 signaling cascade is crucial for valve development and maintenance. In addition, *Cx37*$^{-/-}$ mice do not form clusters of LVCs and consequently lack valves [25,118]. Moreover, conditional inactivation of *Gata2* in ECs results in edema, hemorrhages and high embryonic lethality due to defective lympho-venous separation. *Gata2*$^{-/-}$ lymphatic vessels have increased coverage of SMCs and have underdeveloped valves. Remarkably, Gata2 was found to regulate expression of key lymphatic valve signature genes, such as *Prox1*, *Foxc2*, *NFATC1* and *Itga9* [20]. It is hypothesized that Gata2 binding to the *Prox1* locus is required to sustain high levels of Prox1 in LVCs [119].

Altogether, valves become important regulators of flow by preventing backflow of lymph. Dysfunctional underdeveloped valves or lymphatic capillary filtration and other lymph conduit

perturbations could lead to lymph stasis, a symptom in which lymph flow is stopped within part or the entire lymphatic network and may give rise to lymphedema [27,120–123]. Reversely, lymph flow is a major regulator of valve development. *In vitro* flow studies have shown that oscillatory flow regulates the expression of both *Cx37* and Calcineurin/NFATc1 signaling in LECs through Prox1 and Foxc2 dependent mechanisms [25]. This highlights the impact of shear stress on LEC function in development. Shear stress will be discussed later in this introduction.

Smooth muscle cell (SMC) recruitment

Valves in collecting lymphatic vessels are important structures for lymph drainage and prevention of backflow of lymph. The role of SMCs in lymph drainage is less understood, yet it is suggested that SMC contractions play a major role in promoting lymph drainage in the larger lymphatic vessels[124]. SMCs are innervated by different types of neurons that trigger SMC contraction and propel lymph drainage. Normally SMCs are only covering the lymphangion, not the capillaries or the valve regions. Patients with severe lymphedema, such as in lymphedema distichiasis, caused by mutations in *FOXC2*, have valve defects, but additionally also show ectopic recruitment of SMCs to lymphatic capillaries [27]. In the mouse ear, SMCs colonize collecting lymphatic vessels from P14 onwards when Lyve1 levels drop. In the lymphatic capillaries, which do not recruit SMCs, Lyve1 remains high [22,110,125–127]. SMC-LEC interaction may govern lymphatic vessels specification into collector or capillary vessel types [126,127].

In co-cultures of LECs and SMCs, the recruitment of SMCs to LECs stimulates the release and proteolytic processing of LEC-derived Reelin, a matrix protein. *Reelin*$^{-/-}$ mice have reduced SMC coverage and sustained Lyve1 [126]. Upon stimulation by SMCs, Reelin is needed to induce the gene encoding monocyte chemotactic protein 1 (Mcp1), which suggests an autocrine positive feedback mechanism for Reelin-mediated control of EC factor gene expression upstream of SMC recruitment [126]. Additionally, Pdgfb is required for peri-EC recruitment to the blood and lymphatic vasculature [128,129]. Indeed, upregulation of *PDGFB* in human dermal LECs underlies the ectopic SMC coverage, as a consequence of lack of *FOXC2* expression [27,130]. Conversely, Foxc2 is highly localized in developing collecting lymphatic vessels where SMCs are recruited. Remarkably, overexpression of *Pdgfb* in mouse LECs is not sufficient to obtain ectopic coverage of SMCs in capillaries [129]. Importantly, Pdgfb binding to the heparan sulphate proteoglycan Perlecan, as well as Collagen IV, promotes SMC recruitment. Altogether, the molecular mechanisms that control SMC recruitment specifically to the lymphangion, and not to capillaries and valve forming regions, are complex.

Regulation of lymphatic vessels by ECM

The ECM is a highly organized network of macromolecules composed of collagens, glycosaminoglycans, which include hyaluronan, and proteoglycans. ECM provides structural support to tissues and regulates cellular responses in development and homeostasis. An example of specialized ECM can be found in the vascular BM that interacts with (L)EC and is produced by (L)ECs and Peri-ECs. BM includes mainly Collagen-IV, FN, Laminins and heparan sulfate proteoglycans. The collecting lymphatic vessels have a continuous BM and recruitment of SMC coincides with the assembly of BM [28,126]. In addition to the ECM surrounding the lymphatic vasculature, valves have a small ECM core, which contains mainly Collagen-IV, Laminin-α5 and

FN-EIIIA, on which LVCc attach. Lymphatic capillaries have a discontinuous BM, containing gaps that allow entry of fluid and other macromolecules in the lymphatic vasculature, yet the composition of capillary BM as compared to larger collecting vessels has not been extensively investigated. ECM helps to direct EC sprouting and growth by providing adhesive gradients. Several key proteins for sprouting lymphangiogenesis, such as Vegfc and Ccbe1, (in)directly bind to heparan sulphate in ECM enhancing lymphatic vessel sprouting [64,92,131]. During lymphatic vessel maturation Itga9 and FN play an important role in vessel stabilization and valve morphogenesis. *Itga9* is abundantly expressed in collecting lymphatic vessels and valves [112]. *Itga9*$^{-/-}$ mice suffer from rudimentary valves with a disorganized ECM. Further studies revealed that FN-EIIA as a ligand of integrin-α9 [112]. Anchorage of capillary LECs to the ECM is facilitated by Emilin-1, a major component of the anchoring filaments. Loss of *Emilin-1* results in hyperplastic lymphatic capillaries which are unresponsive to interstitial pressure [11]. Lymphatic capillaries also highly express the glycosaminoglycan *Lyve1*, yet *Lyve1*-deficient mice do not display an obvious phenotype [132]. Dynamic remodeling of ECM is essential for development, wound healing and organ homeostasis. Matrix metalloproteinases (MMPs) regulate cellular responses to the ECM. For example, MMP-mediated degradation of the ECM supports early angiogenesis. Surrounding the lymphatic vasculature, two MMPs (MMP2 and MMP9) are located. A role for MMP9 in lymphangiogenesis was not confirmed, but MMP2 supports lymphangiogenic sprouting [133,134].

LECs sense changes in ECM properties, which triggers intracellular responses, such as cytoskeletal rearrangements, activating signaling pathways and gene expression alterations. Recently it was shown that ECM stiffness impacts lymphatic vessel morphogenesis. During lymphangiogenesis the environment of the cardinal vein becomes less stiff. This induces a *Gata2*-dependent transcriptional program that makes LECs more responsive to VEGFC and guides their migration away from the cardinal vein. This was supported by transcriptome analysis comparing soft and stiff matrix [135]. In the lymphatic capillary bed, ECM plays also an important role. Capillaries have overlapping intercellular junctions (primary valves) through which interstitial fluid enters upon increased fluid pressure. The ECM swells when interstitial pressure is high and pulls via the anchoring filaments, containing Emilin-1 and Fibrillin, the primary valves open. This fluid is directed towards the larger collecting vessels, where intraluminal valves are formed and prevent backflow and peri-endothelial cells further support a unidirectional flow.

Mechanotransduction in lymphatic vessels

Mechanotransduction is the mechanism through which cells convert biomechanical stimuli, such as ECM stiffness, interstitial fluid pressure, fluid pressure-induced cell stretching and fluid flow-induced shear stress (also see Figure 1.4b), in electrochemical and biological activity. The lymphatic vasculature is important for fluid homeostasis. The blood and lymphatic vasculature are exquisitely sensitive to alterations in flow. However, different vascular beds experience very different flow patterns. The lymphatic system is a low flow system [136,137]. Flow-induced shear stress can be defined as a force that may act parallel, as in laminar shear, or tangential, as in oscillatory shear, to cells. Flow-induced shear stress is critical for the development of heart, venous and lymphatic valves. Fluid flow is highest in arteries (2-8 mm/s), then veins (1-2 mm/s), followed by lymphatic vessels (0.9 mm/s). These vessels are sensitive to different levels of shear stress. Following the same order, arteries undergo 100 dyn/cm^2 shear mediated stress, veins 5 dyn/cm^2 and lymphatic vessels 0.4-0.9 dyn/cm^2. This highlights that LECs

are very sensitive to small changes in shear stress [138,139]. LECs process flow-induced shear stress in different ways. Firstly, transmembrane receptors like Integrins, Cadherins and G-protein coupled receptors (GPCRs) can undergo a flow-induced conformational switch [140–143]. Secondly, mechanosensitive ion channels can also respond to increased pressure or stretch, like the PIEZO channels [122,144,145]. The primary cilium contains many of these ion channels, to mediate mechanotransduction in response to flow [146,147]. Thirdly, gap junctions are transmembrane channels that can open or close upon mechanical stimuli and are composed of Connexins. In the vasculature Cx37, Cx43 and Cx47 have been shown to be important in formation of flow mediated valve formation [118,148].

1.4 Major signaling pathways

This section will discuss some major signaling pathways important for "lymphatic" vessel development, maturation and maintenance.

The VEGF family

The VEGF pathway is an extensively studied pathway with major functions in sprouting (lymph)angiogenesis. This pathway is often misregulated in pathological conditions . In mammals, the VEGF family exists of five secreted ligands (VEGF-A, -B, -C, -D, and placental growth factor) and three receptors (VEGFR-1, -2, and -3), and engages several co-receptors. VEGF binding to its receptor complexes results in autophosphorylation of tyrosine residues that recruit downstream Src-homolog-2-containing adaptors and kinases. This pathway may then regulate processes, such as EC migration, survival, proliferation and tube formation.

Many tissues and cells express VEGF and VEGFR. From the VEGF family, VEGFC and VEGFR3 are crucial for the development of the lymphatic vasculature [54,87]. Yet, VEGFC-VEGFR3-mediated signaling has also been shown to have pro-angiogenic functions. However, for angiogenesis, the VEGFA ligand is more involved and interacts mostly with VEGFR1/2. The tyrosine kinase activity of VEGFR2 is the major mediator of VEGFA signaling during sprouting angiogenesis. VEGFR1 has very low kinase activity as compared to VEGFR2 and therefore, amongst other functions, acts as a decoy receptor to limit the effects of the VEGF-VEGFR2 mediated signaling cascade in ECs [149]. The VEGF signaling pathway is also modulated through co-receptors like Nrp1 and Nrp2. Nrp1 enhances VEGF binding activity to VEGFR1/2 and Nrp2 does this for VEGFC binding to VEGFR3 [149]. Studies have shown that Nrp2 mediates VEGFC-VEGFR3 induced lymphatic sprouting. In addition, VEGF may regulate EC integrity through Semaphorin3A and $Nrp1^{+}$ monocytes [75,150]. Altogether, the VEGF pathway is a critical player in the (lymph)angiogenesis field and holds many promising therapeutic applications for a variety of (non) vascular diseases.

The Notch pathway

The Notch-mediated signaling pathway is an important signaling system that is implicated in cell fate, proliferation, apoptosis and differentiation processes. In mammals, the Notch pathway

exists of four Notch transmembrane receptors (Notch-1, -2, -3, and -4) and five membrane bound ligands (DLL-1, -3 and -4; Jagged-1, and -2) [151]. In brief, binding of the ligand to its receptors triggers the canonical Notch signaling pathway that results in intracellular proteolytic cleavage and release of the Notch intracellular domain (NICD) that subsequently translocates to the nucleus. There, NICD forms a transcriptional complex with RBP-Jκ and Mastermind-like proteins and regulates downstream targets, such as hairy and enhancer (*HES-1, -5, -7*), Hairy/enhancer-of-split related with YRPW motif protein (*HEY-1, -2*), and HES-related repressor protein (*HERP-1, -3*) [151,152].

The Notch pathway plays major roles in vessel biology, for example in arterial specification, lateral inhibition of new sprouts during sprouting angiogenesis and EC quiescence. Using both *in vitro* and *in vivo* approaches it has been established that the DLL4-Notch signaling axis is a negative regulator of VEGF signaling. Indeed Notch inhibition results in ectopic sprouts [51,54]. Moreover, heterozygous deletion of *Dll4* or inhibition of Notch1 cleavage with γ-secretase inhibitors resulted in a similar phenotype [51]. As aforementioned COUPTFII is a key regulator of venous or lymphatic EC fate through repressing Notch signaling and inducing *Prox1* expression in venous ECs, therefore it was hypothesized that the Notch pathway might also play a role in lymphatic vessel development. Interestingly, *in vivo* blockage of RBPJκ in mice did not result in a LEC fate defect [61]. On the other hand, *in vitro* experiments with cultured LECs showed that increased Notch receptor activity represses *Prox1* and *CouptfII* through HEY proteins [153]. Additionally, blocking Notch signaling *in vitro* promotes LEC sprouting in a *Vegfr2/3*-dependent way [154]. Yet, treating neonatal mouse tail dermis, ears, and retinas with blocking antibodies against Notch1 and DLL4 resulted in an opposite effect as compared to the *in vitro* study [155].

Murtomaki *et al.* (2013) showed that inactivation of *Notch1* in LECs in mice results in an increased number of LEC progenitors and enlarged lymph sacs [156]. These studies identify a role for Notch signaling during LEC specification and sprouting lymphangiogenesis. A later study revealed that Notch-1 remains present within lymphatic vessels until E16.5 and from E18.5 onwards becomes more restricted in regions were valves form. Deletion of *Notch1* in LECs resulted in increased numbers of Prox1-high cells, which is specific for valve LECs, disturbed cell orientation, and decreased Itga9 in valve forming regions. Blocking all Notch signaling, using a dominant-negative Mastermind-like transgene, resulted in a decreased number of valves, failure of LVCs to cluster, and decreased FN-EIIA localization in valve forming regions [157]. This shows that the Notch pathway plays an additional role in lymphatic valve development. Altogether, these data suggest that Notch signaling achieves its different functions via different interaction partners and target genes; it highlights the complexity and context dependency of the regulation of the lymphatic vasculature and that more studies are required to better understand the role of Notch signaling therein.

The WNT pathway

WNT signaling is complex, involving 19 ligands, 10 receptors and at least three different downstream cascades [158]. The best-characterized pathway is the canonical WNT signaling pathway in which β-catenin is the central mediator. In the absence of WNT ligands, cytosolic β-catenin is phosphorylated and targeted for degradation by different kinases of the β-catenin destruction complex, which includes kinases like GSK3β or CK1. Upon binding of a canonical WNT

ligand to a heteromer composed of a Frizzled receptor (FZD) and specific members of LRP co-receptors, GSK3β becomes functionally inactive. Consequently, β-catenin accumulates in the nucleus where it interacts with T-cell factors (TCFs, not to be confused with some E2-type basic helix-loop-helix factors such as Tcf4, better named E2-2) to regulate transcription of WNT target genes. In addition, β-catenin also acts at the cell membrane to link Cadherins to the cytoskeleton, which results in altered cell-cell adhesion, plasma membrane dynamics, cell shape and migration [159,160].

The WNT/β-catenin pathway is critical for lymphatic valve development and promotes patterning of the lymphatic vasculature [161,162]. The regulation of *Prox1* during the valve region induction phase, is not entirely dependent on OSS, but additionally requires signaling input [25,110]. In zebrafish embryos, it was shown that WNT/β-catenin regulates *prox1* [163], but this was detected during early lymphangiogenesis. Another study, however, highlighted that WNT/β-catenin regulates *Prox1* during lymphatic valve development in mice [161,162]. They also show that Prox1 associates with WNT/β-catenin to regulate *Gata2* and *Foxc2 in vitro*. Furthermore, Prox1 and WNT/β-catenin signaling also interact via a feedback loop, which could explain the eventual higher levels of Prox1 in LVCs (see major Chapter 5 for more information).

The BMP pathway

The BMP signaling pathway was reported for the first time by Senn more than a century ago, in 1889. Nicholas Senn described the use of antiseptic decalcified bone implants in the treatment of osteomyelitis and some bone deformities [165]. Then Pierre Lacroix suggested that there was a hypothetical substance, Osteogenin, which might induce bone growth [166]. However, BMPs were identified by Marshall R. Urist, in 1965. He showed that demineralized, acid-extracted segments of long bones could initiated new bone formation when implanted on appropriate carriers in muscle pouches in rabbits [167]. Meanwhile, it has become clear that BMP signaling is vital during embryonic development and in adult tissue homeostasis. Although BMPs were first identified as osteogenic factors, it is now known that BMPs fulfil several other functions, also in soft tissues [152]. Many disorders, including vascular diseases (see section 1.4; functions in health and vascular disease), are linked to misregulation of the levels and functions of BMPs or its downstream effectors, making it highly important to study these. Altogether, BMP signaling and its intracellular signal transduction cascade regulate many cellular processes, both in embryos and adult organisms, including cell proliferation vs. differentiation, cell survival vs. apoptosis, cell migration and accompanying cell shape changes, and cellular homeostasis altogether. Their roles in cardiovascular development are well studied [152,168], but their function(s) in lymphatic development is (are) much less understood.

BMPs are secreted growth/differentiation factors of the Transforming Growth Factor type β (TGF-β) superfamily. Briefly, the BMP signaling cascade is initiated when ligands bind to two different serine/threonine kinase receptors, the types I and II receptors [168]. The type-I receptor is phosphorylated by liganded type-II receptors. Type-I receptors are also known as Activin receptor-like kinases (ALKs), due to the way they were identified in the field, using low-stringency hybridization in cDNA libraries and degenerate PCR approaches. Different ALKs bind and are activated by different TGF-β members: in general ALK1, ALK2, ALK3 and ALK6 are activated by BMPs; ALK4, ALK5 and ALK7 are activated by TGF-β, nodal and activins. However, ALK1 may also

bind TGF-β, and ALK2 may also bind Activin A. Type II receptors include Activin receptor type IIA (ActRIIA), type IIB, (ActRIIB), BMP type II receptor (BMPRII), TGF-β receptor type II (TGFβRII) and AMH type II receptor (AMHRII). ActRIIA was the first receptor shown to also bind BMP-7. Hence, different ligands have different affinities for different combinations of receptors, which potentially results in a complex diversity/specificity of signaling. In addition, co-receptors like Endoglin and Betaglycan (for TGF-β), and Cripto (for Nodal), do attenuate ligand-receptor interactions as well. Upon ligand binding and type-I receptor phosphorylation, the type-I receptor phosphorylates intracellular substrates. The (often referred to as) "canonical" BMP signaling cascade involves the phosphorylation and hence activation in the cytoplasm of specific transcription factors, the receptor-regulated (R-)SMADs. When activated, two of these R-SMADs form a trimeric complex with the common mediator (co)-SMAD4 (which is not a receptor kinase substrate). Then the complexes accumulate in the nucleus where they bind to DNA and, often in co-operation with other transcription factors, regulate transcription of pathway-dependent target genes. Besides canonical SMAD signaling there is also non-canonical signaling, which notably is not automatically necessarily SMAD-independent (as this is often not investigated thoroughly), which involves activation of MAP kinases (MAPKs), and also Rho-like small GTPase and PI3K/AKT pathways [169]. An overview on canonical SMAD signaling is provided in Figure 1.7.

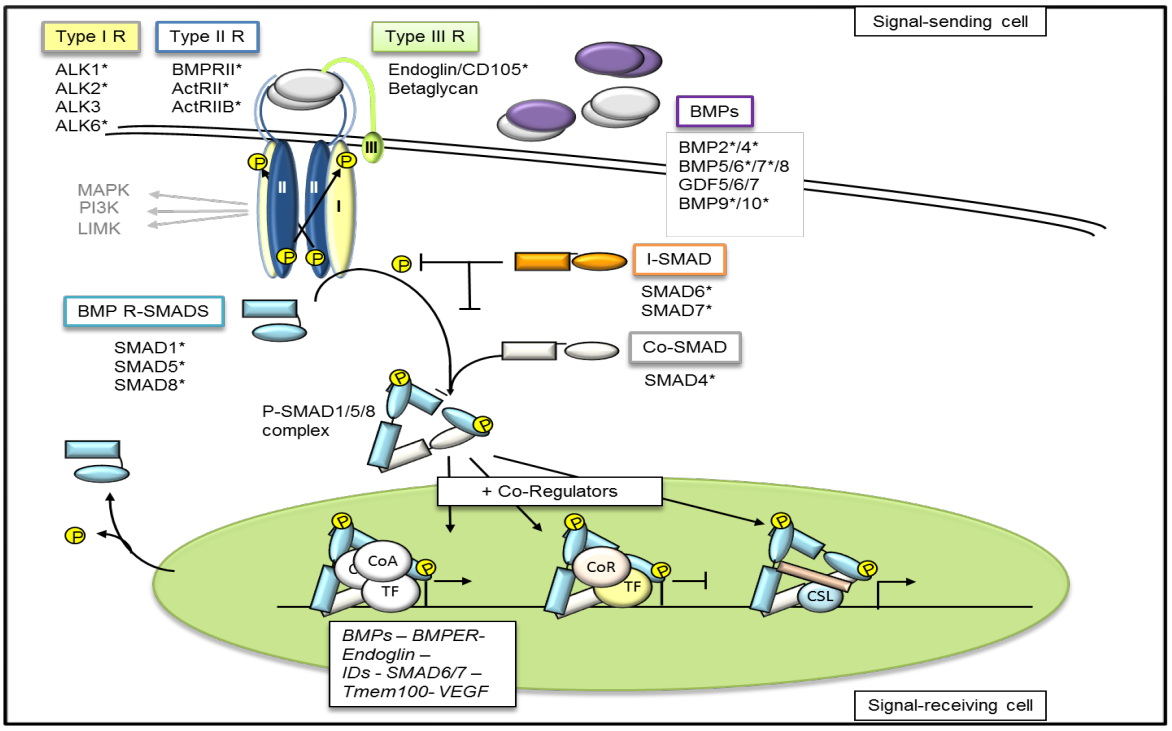

Figure 1.7: Simplified overview of the canonical BMP signaling pathway.
Dimerized BMP ligands (grey/purple) signal via heterotetrameric receptor complexes (blue/yellow) leading to the phosphorylation and activation of intracellular R-SMADs (light blue). R-SMADs will form a trimeric complex with co-SMAD4 (white) and translocate to the nucleus. In the nucleus the resulting SMAD complex incorporates different DNA-binding cofactors that confer target gene selectivity and regulate gene transcription. Inhibitory (i)SMADs (orange) compete with SMAD1/5/8(9) for activation or lead to SMAD1/5/8(9) degradation and hence inhibit BMP signaling output. Taken from [152].

SMADs are divided into three groups; the R-SMADs, I-SMADs and Co-SMADs respectively. R-SMADs are divided in 2 subgroups. The first group consists of SMAD1/5/9, the latter formerly known as SMAD8, and these are known to be activated by ALK1/2/3/6 and hence mediate primarily BMP signals. The second group contains SMAD2/3 and is activated by ALK4/5/7. SMAD6/7 are part of the inhibitory SMADs (Figure 1.7).

R-SMADs consist of two conserved globular domains (MH1 and MH2) separated by a Pro-rich sequence-variable linker region. Both domains interact with many other proteins. The functions of MH1 in receptor-activated R-SMADs include specific DNA-binding and nuclear import, while MH2, which in one-hybrid fusion (of MH2 to a DNA-binding domain of a heterologous transcription factor) displays transcription activation of appropriate reporters, but also interacts with numerous Smad-interacting proteins (SIPs), including many transcription factors. Receptor-mediated phosphorylation at and close to the C-terminus of each R-Smad creates there a pS–X–pS motif, triggering relief of auto-inhibitory (MH1 with MH2) interaction in each SMAD monomer, at the same time enabling complex formation between 2 R-SMADS with one SMAD4. In the nucleus, such Smad complex binds to different co-factors, including DNA-binding transcription factors, which confers eventual target gene transcription. The linker region in activated SMADs can become the target for post-translational modifications, including combinatorial GSK3β-MAPK mediated phosphorylation (resulting in integration of e.g. BMP with WNT signalling) and proteasomal degradation through SMAD ubiquitin regulatory factors-1 and -2 (Smurf-1, -2) [170]. As SMADs show an overall low affinity for DNA-binding, the aforementioned co-factors contribute in regulating transcription by strengthening the interaction between SMADs and DNA and/or changing their activity (e.g. pulling their activation into repression) [171]. Other functions of SMADs include their direct regulation of components needed for microRNA (miR) maturation [172] and chromatin remodeling [173].

Functions in health and vascular disease

Studies have linked impaired BMP signaling to disorders such as hereditary haemorrhagic telangiectasia (HHT), Pulmonary arterial hypertension (PAH), and fibrodysplasia ossificans progressiva (FOP). In HHT patients, a lack of capillaries results in high-pressure blood flow from the arteries directly into the thinner walled, less elastic veins, resulting in enlarged blood vessels and haemorrhages. Mutations in the genes encoding Endoglin (*Eng*), Activin receptor like type 1 (*Acvrl1*) and *Smad4* respectively result in type1, type 2 and juvenile HHT subtypes [174].

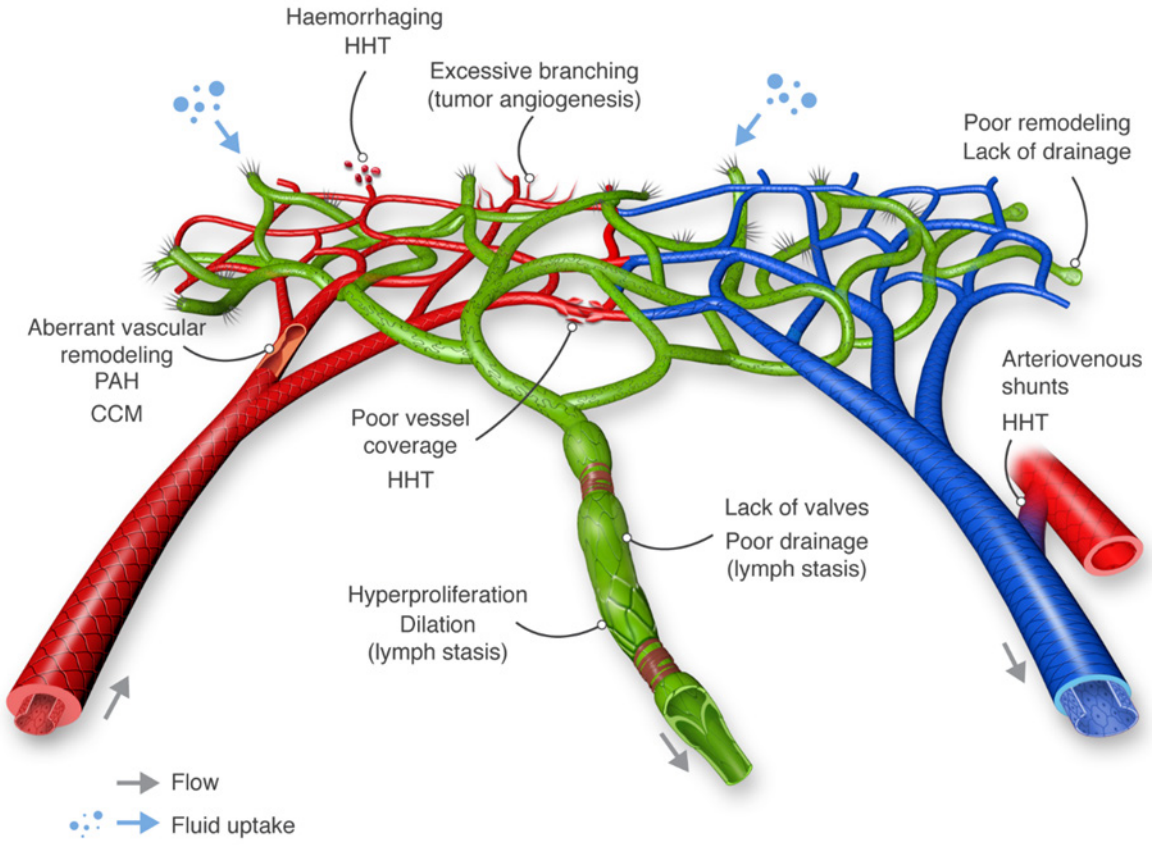

Figure 1.8: The blood and lymphatic vasculature in health and disease.
The circulatory cardiovascular system consists of a hierarchical network of arteries/ arterioles (red), a capillary network, and venules/veins (blue). Unidirectional lymphatic vessels (green) drain extravasated fluid and cells from the blind-ending capillary bed into collecting vessels. Unbalanced BMP signaling may result in different blood and lymphatic vessel defects, such as pulmonary arterial hypertension (PAH), hereditary hemorrhagic telangiectasia (HHT) and cerebral cavernous malformation (CCM). PAH (mutations in *BMPR2*, *ACVRL1*, *SMAD9*) characterized by aberrant vascular remodeling that can lead to obstruction of small arteries. HHT (mutations in *ENG*, *ACVRL1*, *SMAD4*, *GDF2* (*BMP9*)) is characterized by arteriovenous shunts, poor vessel coverage and hemorrhages. CCM has been associated with increased BMP6 signaling. At this moment BMP related lymphatic defects (hyper-proliferation, dilation, lack of valves, poor drainage) are only observed in mouse and zebrafish models with impaired BMP2, BMP9 and ALK1 signaling [175–177] (taken from [174]).

PAH is a progressive disorder characterized by increased pressure in the pulmonary artery. Studies revealed that most commonly, BMPR2 mutations result in PAH (Figure. 1.8) [171,178]. FOP is a rare connective tissue disorder and results in the transformation of ECs, skeletal muscles and connective tissue into bone. FOP is caused by a mutation in the activin receptor type A1 (*Acvr1*) gene encoding the BMP type 1 receptor (ALK2) [179]. Our laboratory and others have shown that SMAD1/5 play an essential role during angiogenesis in the blood vasculature [180,181]. An endothelium specific KO for *Smad1* or *Smad5* showed to be viable and asymptomatic, which could indicate that SMAD1 and SMAD5 can compensate for each other [182]. Generation of an EC specific double KO for both *Smad1* and *Smad5* in the mouse showed their requirement during blood vessel development [180]. EC specific *Smad1/5*-KO mice die around day E9,5 due to severe cardiovascular defects. When three out of four alleles of *Smad1/5* are lacking, then embryos die slightly later (E12,5-E14,5) and display cardiovascular, as well as lymphatic defects, like edema.

In addition, EC specific inactivation of *Smad1/Smad5* affects tip cell polarity and Notch mediated stalk cell/tip cell balance. The affected stalk cell function resulted in hyper sprouting from the dorsal aorta [180]. Downstream of BMP/SMAD signaling, the Inhibitor of differentiation (ID) family members, which are helix-loop-helix (HLH) proteins, inhibit cell differentiation and stimulate cell cycle processes [152]. *In vitro*, ID1 stimulates EC migration and tube formation, and stabilizes HES1. HEY1 induces ID protein degradation, thereby antagonizing *Bmp*/*Id1*-induced migration of ECs. Absence of ID proteins in SMAD1/5 deficient ECs results in a high amount of tip like cells and a hyper sprouting phenomenon because of insufficient Notch-mediated HES1 level [180]. The function of SMAD9 is poorly defined. So far, *Smad9* null and conditional KO mice showed that adult *Smad9* homozygous mutants are viable and fertile [183]. Also, *Smad9* homozygous mice lacking one allele for Smad1 or Smad5 did not result in an overt phenotype. Defects seen in *Smad1* or *Smad5* null embryos were not aggravated in the absence of *Smad9* [183]. Interestingly, it has been shown that SMAD9 has an indispensable function in the processing of miR-21 and miR-27 required for BMP-induced growth suppression [172].

Few studies have highlighted the importance of the BMP pathway in lymphatic vessel development and function. Interestingly, recently altered BMP signaling has been identified in patients with lymphatic malformations [184]. Furthermore, it has become clear from animal models that BMP signaling not only regulates the blood vasculature, but also the lymphatic vasculature. Interestingly, *Bmp2* and *Bmp9* either repress or induce important processes during lymphatic vessel development and maturation in mice [175–177,185]. For example, BMP9 is shown to control lymphatic vessel maturation and valve formation [176,185]. *Bmp9* knockout (KO) neonates display enlarged lymphatic vessels in the mesentery and lymphatic valve initiation is impaired due to insufficient down regulation of *Prox1* and *Lyve1* expression, but not *Foxc2*. Moreover, they showed that *Bmp9* deficiency causes an abnormal lymphatic vasculature patterning and a decrease in drainage of fluid extravasated from the blood capillary beds [176]. The link between ALK1, a type I receptor for BMP9 and -10, and early postnatal lymphatic development was demonstrated in a paper from Niessen *et al.* [177]. They show that *in vivo* blockage of ALK1, using decoy receptors or ALK1 specific antibodies, causes defective lymphatic development in multiple organs in neonatal mice. They show that ALK1 is important for lymphatic vascular development, remodeling, and postulate a link between ALK1 and VEGFR3, causing LECs to undergo the apoptotic program [177]. BMP9 is not the only BMP-ligand regulating lymphatic vessel development, but BMP2 also plays a role in embryonic lymphatic development in a zebrafish model [175]. Here the authors give evidence that BMP signaling increases the number of venous endothelial cells at the expense of lymphatic endothelial cell specification. The authors show that *Sox18* expression, which is known to induce *Prox1*, was unchanged and therefore it is possible that BMP2 signaling modulates *Prox1* transcripts at a post-transcriptional level. Indeed, miR31 and miR181a, function downstream of BMP2 signaling and can modulate PROX-1 levels [91,175]. In conclusion, these data illustrate that BMP signaling is important for both embryonic and postnatal stages of blood and lymphatic vessel development. But unlike in the blood endothelium it remains unclear whether SMADs mediate the BMP induced response in lymphatic endothelium. This study attempts to further investigate the role of BMP effectors “SMAD1 and SMAD5” during lymphatic development.

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Chapter 2

Objectives

We reported edema and blood-filled lymphatic vessels in EC specific *Smad1/5*-KO mouse embryos with one active allele from either *Smad1* or *Smad5* [176]. It was unclear whether these lymphatic defects were primary or secondary to underlying cardiovascular defects. Few other studies have highlighted the importance of the BMP signaling cascade by means of ligand and receptor KO mice in lymphatic vessel biology [171–173,180]. From these studies, it still remained open whether SMAD1/5 are indispensable for BMP-mediated regulation of lymphatic vessel development, maturation and maintenance. The general aim of my PhD was to investigate the role of BMP-SMAD1/5 mediated signaling in postnatal lymphatic vessels in the mouse (under Ethical approval P107/2011 and P077/2016).

The three main objectives of my PhD research were:

- **Objective 1:** To document within the developing and postnatal lymphatic vasculature the spatiotemporal dynamics of BMP-SMAD signaling. To gear the functional study of SMAD1/5 mediated signaling in postnatal lymphatic vessels (Obj. 2), I profiled different postnatal vascular beds and BMP-SMAD signaling therein. Thereto I made use of *BRE::gfp* mice in which GFP presence reports BMP-SMAD mediated signaling due to the BMP-SMAD response element (BRE) of *Id1* that drives activation on a minimal promoter and GFP to spatiotemporally document where SMAD1/5 mediated signaling occurs in the lymphatic system in different tissues.

- **Objective 2:** Defining the function of BMP-SMAD1/5 in lymphatic vessel development, maturation and maintenance. The establishment of a tamoxifen inducible LEC specific *Smad1/5*-KO mouse model during my pre-doc year was instrumental to study the role of SMAD1/5 in the developing postnatal lymphatic vessel system. In this model, mice injected with tamoxifen undergo Cre-mediated recombination of 'floxed' alleles. I characterized these mice phenotypically (marker analysis by immunofluorescence (IF), immunohistochemistry (IHC), confocal analysis and morphometric analysis) and introduced functional testing of the lymphatic system to complement the phenotypic analysis.

- **Objective 3:** Finally, to further unravel the mechanism of BMP-SMAD mediated signaling in LECs, candidate target genes of SMAD1/5 were identified in cultured dermal lymphatic endothelial cells by RNA-Sequencing approaches. Validation of selected target genes in vitro in gain- and loss-of-function studies, were complemented with rescue experiments to validate working hypotheses also *in vivo*.

Altogether, we anticipated that these objectives could lead to new insights and/ or therapeutic approaches for lymphatic-related diseases, such as lymphedema.

Chapter 3

BMP-SMAD signalling output is highly regionalized in cardiovascular and lymphatic endothelial networks

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Because parts of the results in this publication have been published in the theses of K. Beets (BMP-SMAD signalling output in embryonic vessels) and N. Criem (in embryonic heart), the contribution of these authors has been explicitly mentioned in the respective legends. To maintain focus on vessels, the figure on BMP-SMAD signalling in the embryonic heart is not included. It can be consulted on https://bmcdevbiol.biomedcentral.com/track/pdf/10.1186/s12861-016-0133-x. Additional unpublished BMP-SMAD signalling and pSMAD1/5/9 data in lymphatic vessels are also provided in Chapter 4, Figures 4.2 and 4.3.

3.1 Summary

Bone morphogenetic protein (BMP) signalling has emerged as a fundamental pathway in endothelial cell biology and deregulation of this pathway is implicated in several vascular disorders. To better understand the *in vivo* context-dependency of BMP-SMAD signalling, we investigated differences in BMP-SMAD transcriptional activity in different vascular beds during mouse embryonic and postnatal stages using the *BRE::gfp* BMP signalling reporter mouse. A mosaic pattern of GFP was present in various angiogenic sprouting plexuses and in endocardium of cardiac cushions and trabeculae in the heart. High calibre veins seemed to be more *BRE::gfp* transcriptionally active than arteries, and ubiquitous activity was present in embryonic lymphatic vasculature. Postnatal lymphatic vessels showed however only discrete micro-domains of transcriptional activity. The *BRE::gfp* mouse allows to investigate selective context-dependent aspects of BMP-SMAD signalling. Our data reveals the highly dynamic nature of BMP-SMAD mediated transcriptional regulation in time and space throughout the vascular tree, supporting that BMP-SMAD signalling can be a source of phenotypic diversity in some, but not all, healthy endothelium. This knowledge can provide insight in vascular bed or organ-specific diseases and phenotypic heterogeneity within an endothelial cell population.

3.2 Introduction

The formation of the cardiovascular and lymphatic network is crucial for development and physiology. The cardiovascular system fuels nearly every tissue with oxygen and nutrients and removes waste products, while the lymphatic system is important for the drainage of extravasated fluid, the uptake of fat and is a vital part of the immune system [1]. Blood vessel development by sprouting from pre-existing vessels is called sprouting angiogenesis. In hypoxic environments angiogenic sprouts with tip and stalk cells emerge. Sprouts anastomose to form new functional vessels that supply oxygen to the initially hypoxic environment [1]. From the cardinal vein some venous endothelial cells (ECs) differentiate into lymphatic ECs (LECs), that migrate to form lymphatic sacs which in turn sprout to form a lymphatic network similar to angiogenesis events [2]. Failure to establish a (lymphatic) vascular network leads to severe embryonic defects at mid- to late gestation, whereas misregulation after birth can lead to diseases such as cancer, chronic and inflammatory disorders and oedema [3–5].

ECs form the inner cellular lining of blood and lymphatic vessels and the heart, and differ in protein expression, morphology and function depending on the vascular bed. Exposure to external and internal cues as well as epigenetic programming results in EC macro-heterogeneity and micro-heterogeneity [6–8]. This means that the endothelium acquires site- and organ-specific structural and functional properties, which are extensively reviewed in Aird *et al.* [7–9].

BMP signalling has emerged as a fundamental pathway of EC identity by regulating cardiovascular and lymphatic development [10]. BMPs are members of the transforming growth factor beta (TGFβ) family with more than 20 BMP members identified. BMP ligands reported to function in ECs are BMP2/4/6/7/9/10 [11]. BMPs bind to heteromeric transmembrane receptor complexes that consist of type I (ALK1/2/3/6) and type II receptors (BMPR2, ACTR2A, ACTR2B) and often also a co-receptor (Endoglin, Betaglycan). Ligand binding and phosphorylation of the GS-domain of the type I receptor by the type II receptor leads to recruitment and phosphorylation

of the intracellular effectors SMAD1, SMAD5 and SMAD9 (pSMAD1/5/9) [12]. Activated pSMADs form a complex with the common SMAD, SMAD4, and translocate to the nucleus where they stimulate transcription of specific BMP target genes such as the inhibitors of differentiation (*IDs*), *HEY1* and *SMAD6/7*; and repress e.g. *Apelin* [13]. BMPs can also regulate other (non-canonical) pathways that do not involve SMAD proteins [14,15].

BMP signalling is highly tuned by extracellular and intracellular modulators, but also by signalling interplay with other signalling pathways. Furthermore, BMPs are known to trigger expression of different target genes in a dose-dependent manner [16,17], a landmark of morphogens. In addition, hemodynamic changes can induce BMP signalling and activate SMAD proteins in ECs [18,19]. Recently, excessive BMP6 has been implicated in cerebral cavernous malformation [20]. Moreover, other regionalized vascular disorders such as hereditary hemorrhagic telangiectasia (HHT) and pulmonary arterial hypertension (PAH) are mainly caused by mutations in the BMP receptors *ACVRL1* (encoding ALK1) or *ENG* (encoding Endoglin) and *BMPR2* respectively [21–24]. The question remains how mutations in components of the same BMP pathway can cause such organ-specific diseases. A better understanding of the heterogeneity in BMP signalling output in different vascular beds may provide this insight and perhaps even the opportunity for disease-specific therapy.

Phosphorylated SMAD1/5/9 are routinely used to monitor BMP transcriptional activity, however this may confound interpretation, because pSMADs also play a role in chromatin remodelling and miRNA biogenesis [15]. To investigate the transcriptional activity of BMP-SMAD signalling many BMP reporter mice have been generated [25–30]. In this study we examined the *BRE::gfp* reporter mouse in which BMP response elements (BRE), derived from the *ID1*-promotor, drive the expression of enhanced green fluorescent protein (eGFP) [25]. The substantial decrease in GFP levels observed in *Smad5*-deficient *BRE::gfp* embryos corroborate the BMP-SMAD sensitivity of this reporter [25]. A commonality between all BRE-based reporters is that BRE activity does not completely overlap with pSMAD1/5/9 signalling domains [26,27,29,31] because the onset of reporter activity first requires *de novo* mRNA and protein synthesis and GFP maturation, and the half-life of the reporter protein may deviate from pSMAD1/5/9 [29,32,33]. Moreover, pSMAD1/5/9 can also bind with different affinities and regulate other DNA-sequences like e.g. MEME2 [34]; pSMAD1/5/9 also has non-transcriptional functions [15]. Additionally, the *BRE::gfp* reporter is heterozygous, and it is becoming apparent that gene expression in general occurs with bursts of monoallelic expression instead of constant biallelic expression [35,36]. Nonetheless, the relevance of the *BRE::gfp* reporter mouse became apparent in our previous study. Discrete GFP localisation patterns in angiogenic endothelium of *BRE::gfp* embryos, with an otherwise widespread pSMAD1/5/9 localisation, singled out those cells that underwent ID-mediated BMP-SMAD and Notch co-signalling essential for robust stalk cell fate [37].

In this study we aimed to further document regional differences in BMP-SMAD dependent transcriptional activity in murine endothelium of blood vessel, lymphatic vessels and the heart at embryonic and postnatal stages. We defined regions with stereotypic mosaic and continuous *BRE::gfp* localisation patterns, yet also GFP-negative regions were found in areas where BMP-SMAD signalling has been reported, compatible with the morphogen functions of BMP ligands. Our data support that BMP-SMAD signalling can play a role in phenotype switching and endothelial cell heterogeneity.

3.3 Results

3.3.1 Co-localisation of *BRE::gfp* transcriptional activity and GFP in endothelium

To study BMP transcriptional activity in vessel development, we used the previously generated *BRE::gfp* reporter strain [25]. We took advantage of the unstable nature of *gfp* mRNA, the sensitivity and single cell resolution of *in situ* hybridisation (ISH) on the one hand and the direct co-observation of GFP fluorescence on the other hand to validate whether GFP protein localisation reflects well the *BRE::gfp* transcriptional activity in endothelium. We show that there is a near to absolute correlation between the *gfp* mRNA expression and direct GFP fluorescence localisation in vascular beds like *e.g.* the cardinal vein (**Fig. 3.1A-D**). In other endothelial linings such as in the heart ventricles there is a good overlap between mRNA and protein, yet a fraction of the cells express only *gfp* mRNA or only GFP protein. This is indicative of onset of transcriptional activity while GFP protein translation and maturation is still taking off in the former cells, while transcriptional activity has already terminated but GFP protein is still present in the latter cells (**Fig. 3.1E-G**). This pattern suggests the dynamic turning "on" and "off" of transcriptional activity. Overall, we conclude that GFP protein patterns report with fidelity *BRE::gfp* transcriptional activity patterns.

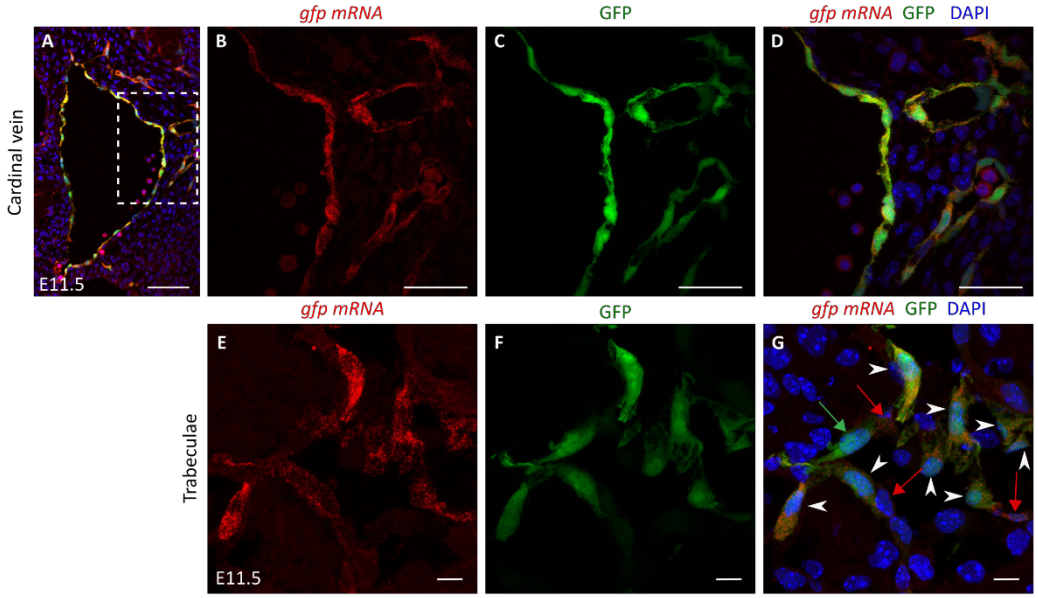

Figure 3.1: GFP reporter protein faithfully recapitulating transcriptional activation of the *BRE::gfp* transgene.
In situ hybridisation for *gfp* mRNA (red) and direct GFP fluorescence (green) on cryosections through the cardinal vein (A-D) and the ventricular trabeculae (E-G) at E11.5. DAPI is used to stain nuclei. The boxed area in A is enlarged in panels B-D. The corresponding low magnification view of the trabeculae in the ventricle is not provided because of insufficient intensity of the ISH signal. White arrowheads indicate ECs double positive for *gfp* mRNA and direct GFP fluorescence. The red and green arrows depict ECs only positive for *gfp* mRNA or GFP fluorescence respectively. Scale bars: 100µm (A); 50µm (B-D); 10µm (E-G). Performed by N. Criem.

3.3.2 *BRE::gfp* transcriptional activity is present in a mosaic pattern during embryonic angiogenesis

To closely examine *BRE::gfp* transcriptional activity in the rapidly expanding vascular plexus, we analysed embryonic day (E)9.5 (22 somites) and E10 (30 somites) *BRE::gfp* mouse embryos. Tip and stalk cell formation as well as anastomosis during sprouting angiogenesis can then be investigated. In the roof of the hindbrain, sprouts are formed from the perineural vascular plexus at opposite lateral sides of the embryo that then anastomose medially in a caudal fashion from the level of the otic vesicles onwards [37].

Whole-mount immunostainings of *BRE::gfp* embryos showed that GFP was mainly present in and around the heart region at 22 somites (s), with little *BRE::gfp* transcriptional activity in the Endomucin-positive blood vessels (**Fig. 3.2A-C**). However, in 30s embryos a scattered GFP pattern co-localised particularly within the main vessels of e.g. the head and the intersomitic vessels (**Fig. 3.2D-F**). The hindbrain roof was excised from these embryos and the dorsal vascular plexus was flat-mounted (**Fig. 3.2G, top panel**) [37]. In the two-dimensional vascular plexus at 22s *BRE::gfp* transcriptional activity occurs in a scattered or mosaic pattern throughout the plexus, which is in accordance with previous observations [37]. Remarkably, some tip cells were found to express low levels of GFP while the mosaic pattern in non-tip cells was more intense throughout the plexus (Figure 4.2G, bottom panels). This is in contrast with earlier observations where GFP was not detected in tip cells [37], which likely reflects higher affinity of the current chicken polyclonal anti-GFP antibody than the previously used mouse monoclonal anti-GFP antibody. At E9.75 the vessels from the superficial vascular plexus start to invade the hindbrain, thus forming a multi-layered vascular network [38].

Depth coding of the dorsal hindbrain region at 30s revealed the superficial and deeper plexus (**Fig. 3.2H**). The latter had slender vessels with slim tip cells, whereas the superficial plexus had thicker honeycomb-like vessels and broader tip cells, as seen at 22s (**Fig. 3.2G-J**). The GFP localisation pattern in both plexuses was mosaic and comparable to the plexus of the 22s embryo, yet, the deeper plexus seemed to be enriched in GFP-positive ECs (**Fig. 3.2J**) compared to the superficial plexus (**Fig. 3.2I**). Additionally, a string of GFP-positive non-endothelial cells was observed in the midline at both stages. This correlates with the presence of BMP6 and BMP7 in the dorsal midline at E9.5-E10.5 [39,40]. In general, the two distinct dorsal vascular plexuses demonstrate heterogeneity in BMP-SMAD signalling in an angiogenic vascular bed.

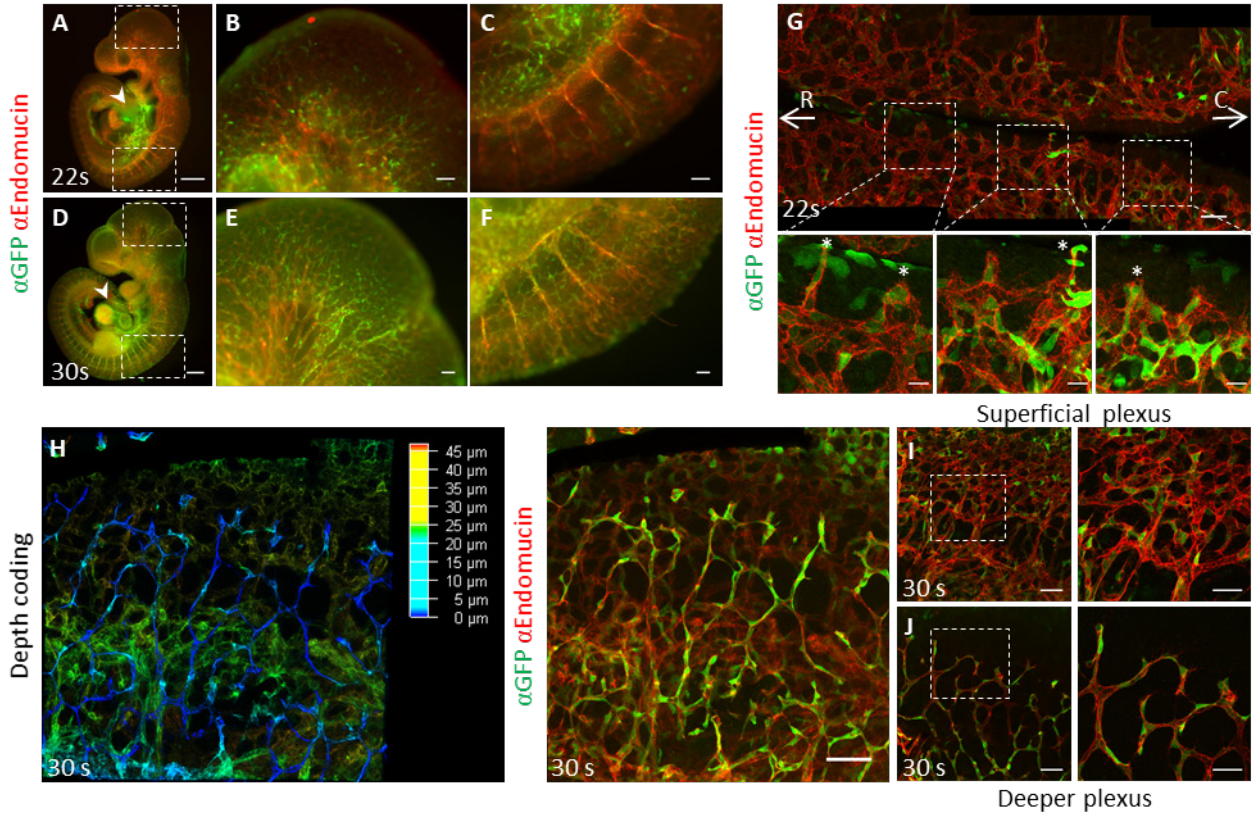

Figure 3.2: Mosaic *BRE::gfp* transcriptional activity in midgestation mouse hindbrain vasculature. Immunodetection of Endomucin and GFP in *BRE::gfp* embryos of 22s (A-C) and 30s (D-F). Arrowheads indicate the heart region. Boxed areas represent the head region (B, E) and the intersomitic vessels (C, F). (G) Overview of a 22s dorsal vascular plexus (Endomucin and GFP) in a flat-mounted hindbrain roof. Rostral is to the left, caudal to the right. Boxed areas (bottom panels) show sprouts at the midline. Asterisks depict GFP-positive tip cells. (H) Depth coding of a 30s dorsal vascular plexus and the original picture (right panel). Blue and green colours represent more ventral and dorsal tissues respectively. The vascular plexus at 30s consists of a superficial (I) and deeper plexus (J). Boxed areas are enlarged in the right panels. Scale bars: 500µm (A; D); 100µm (B-C; E-F); 75µm (G, top; H; I-J, left panels); 50µm (I-J, right panels); 25µm (G, bottom). Performed by K. Beets.

3.3.3 Spatiotemporal changes in *BRE::gfp* activity during retinal angiogenesis

The retina is commonly used to investigate postnatal blood vessel development. The primary plexus develops from the optic nerve towards the peripheral margin. Around postnatal day (P) 5 the primary vascular plexus invades the deeper retinal layers perpendicularly where after the outer plexus forms again radially. Different aspects of vessel formation can be studied because vascular sprouting happens at the periphery while remodelling occurs simultaneously in the centre [41,42].

The GFP localisation pattern was diverse but stereotypic throughout the retinal stages investigated and the GFP levels decreased over time. At P4, the vasculature has sprouted halfway across the retina (**Fig. 3.3A**). The sprouting front displayed relatively strong GFP signals compared to the central plexus (**Fig. 3.3B-C**). Many tip cells at the sprouting front as well as the arteries and veins in the centre were GFP-positive, whereas the intermediate capillary bed displayed a more mosaic GFP distribution (**Fig. 3.3B-C**). Moreover, arteries seemed weaker GFP-positive compared to the strong GFP-positive veins (**Fig. 3.3C**). At P8 the sprouting front

developed into a vascular border. Comparable to P4, GFP-positive arteries and veins were observed, along with a mosaic distribution of GFP in the capillaries (**Fig. 3.3D-E**). At this stage the matured vessels in the centre have sprouted into the retina to form the perpendicular vessels and the outer plexus. Approximately half of the perpendicular vessels seemed GFP-positive, though weaker than the veins of the primary plexus (**Fig. 3.3E-F**). Moreover, the less ramified outer plexus also displayed a mosaic GFP pattern (**Fig. 3.3G**). Overall, fewer GFP-positive cells were present in the different plexuses at P10, although the mosaic distribution was maintained in the primary and outer plexus (**Fig. S3.1**). These data indicate a dynamic nature of BMP-SMAD signalling over time.

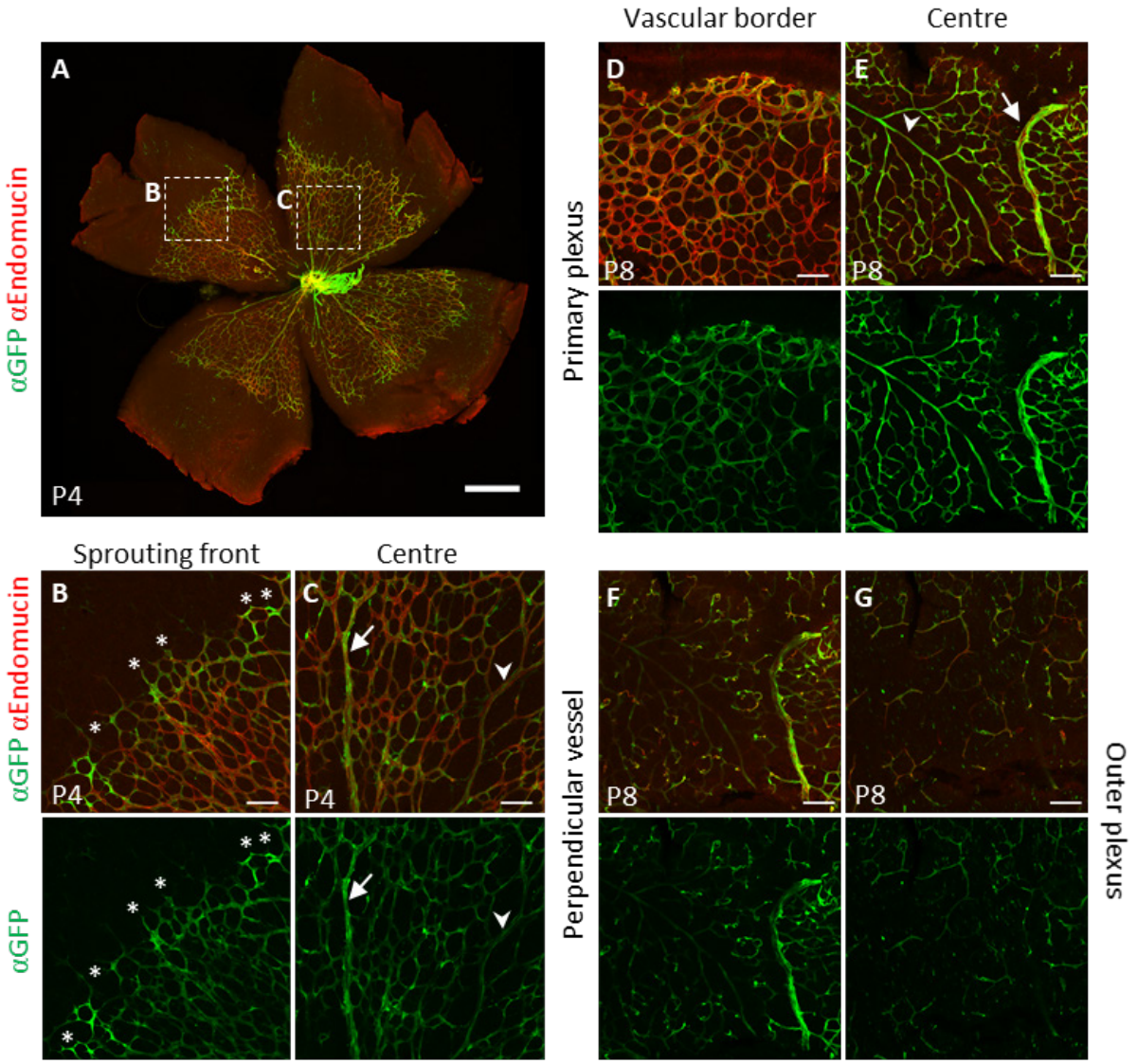

Figure 3.3: Different *BRE::gfp* transcriptional activity patterns in the postnatal vasculature.
(A) Overview of a P4 retina with immunodetection of Endomucin and GFP. Boxed areas show the sprouting front (B) and the centre (C) of the retinal plexus. Single staining for GFP is shown in the bottom panels. Asterisks depict GFP-positive tip cells, the arrows point to the veins, while the arrowheads show the arteries. (D-G) The retina at P8, immunostained for Endomucin and GFP, has a multi-layered vascular plexus consisting of the vascular border (D) and centre (E) of the primary plexus, the perpendicular vessels (F) and outer plexus (G). Single GFP staining is shown in the lower panels. Scale bars: 500µm (A); 75µm (B-G). Performed by M.W. Staring.

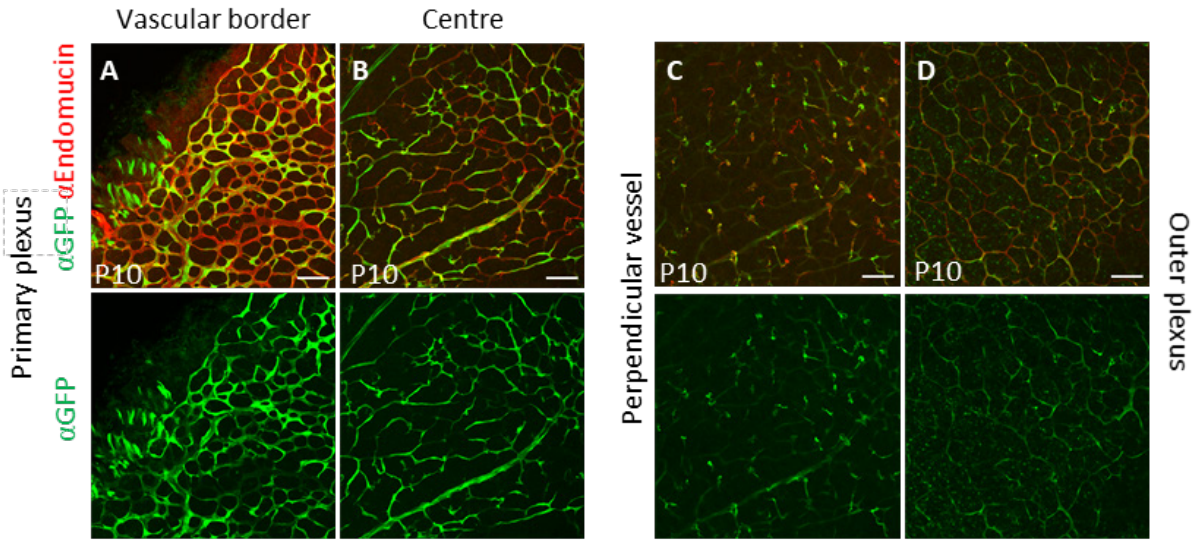

Figure S3.1: *BRE::gfp* localisation patterns in the P10 retina.
(A-D) Immunodetection of Endomucin and GFP in the retina at P10. The retina has a multi-layered vasculature that consist of the vascular border (A) and centre (B) of the primary plexus, the perpendicular vessels (C) and outer plexus (D). Single staining for GFP is shown in the lower panels. Scale bars: 75µm. Performed by M.W. Staring.

3.3.4 Levels of *BRE::gfp* activity differ in embryonic and postnatal blood and lymphatic vessels

Little information is available on BMP-SMAD signalling in different lymphatic beds. At E9.75 the first LECs differentiate from venous ECs in the cardinal vein [1]. These LECs bud of, migrate and assemble into lymphatic sacs by E11.5, which will remodel into a functional lymphatic network. We found interesting spatial-temporal differences in *BRE::gfp* transcriptional activity during lymphangiogenesis. In the embryo most blood and lymphatic vessels have a widespread GFP localisation pattern, while in postnatal tissues like the mesentery, intestinal villi and the ear skin many blood vessels appeared to have reduced GFP signals and the lymphatic vessels had discrete and unique GFP localisation patterns.

In general, the blood vessels, including the cardinal vein, and lymphatic vessels displayed a continuous GFP localisation pattern at E11.5-E14.5 (**Fig. 3.4A-D, 4.2**). However, the aorta showed a mosaic GFP localisation pattern (**Fig. 3.4A**). Interestingly, nearly all PROX1-positive LECs budding from the cardinal vein were GFP-positive, yet still weakly positive for the blood vessel marker Endomucin in E10.5 embryos (**Fig. 3.4A**). Likewise, at E12.5 some GFP-positive ECs were PROX1- and Endomucin-positive (**Fig. 3.4B**), yet in few sections at E14.5 the GFP-positive cardinal vein adjacent to the lymphatic sac was Endomucin-positive on the medial side, while the lateral side, closest to the lymphatic sac, was PROX1-positive (**Fig. 3.4C**). Blood and lymphatic vessels in dorsal skin biopsies were also ubiquitously GFP-positive at E14.5 and E16.5 (**Fig. 3.4D-E** and Supplementary Figure 4C, not included in this thesis). However, the GFP signal appeared more uniform in the blood vessels, whereas the LECs showed different levels of GFP among neighbouring cells (**Fig. 3.4D-E**). Other GFP-positive cord-like structures were observed at E14.5 and E16.5 in dorsal skin biopsies, but these were excluded as vessels because they were Collagen Type IV-negative (Supplementary Figure 5, not included in this paper). Similar structures have been described as Schwann cells of sensory nerves [43].

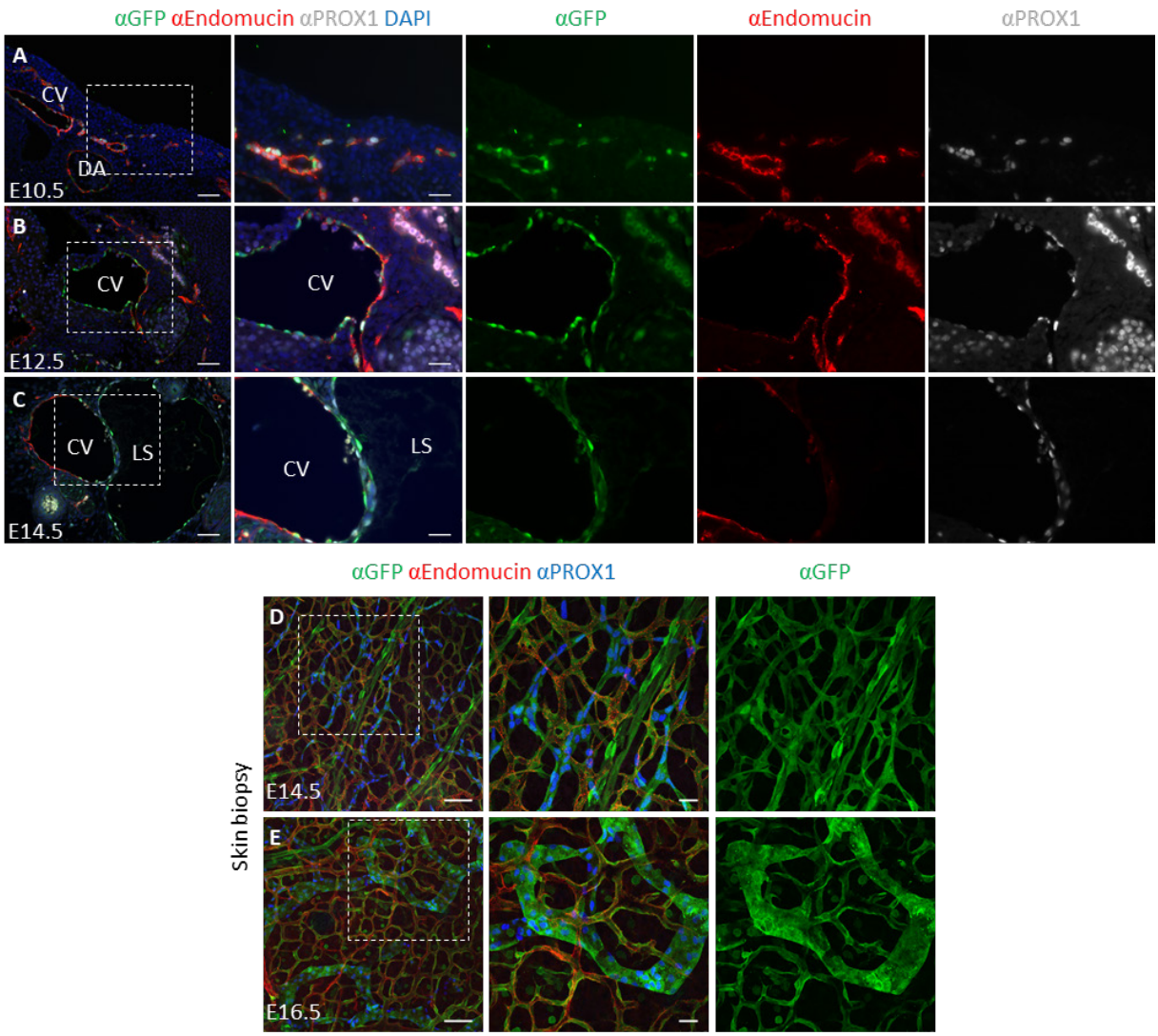

Figure 3.4: Spatial-temporal changes in *BRE::gfp* transcriptional activity in blood and lymphatic vessels.
(A-C) Transverse sections at the level of the neck of E10.5 (A), E12.5 (B) and E14.5 (C) *BRE::gfp* embryos with immunodetection of GFP, the blood vessel marker Endomucin and the lymphatic vessel marker PROX1. DAPI is used to stain nuclei. Boxed areas are enlarged in the right panels, with single GFP, Endomucin and PROX1 staining next to it. Skin biopsies of E14.5 (D) and E16.5 (E) *BRE::gfp* embryos with immunodetection of GFP, Endomucin and PROX1. Boxed areas are shown in the middle (and right) panels with single staining for GFP in the right panel. CV: cardinal vein; DA: dorsal aorta; LS: lymphatic sac. Scale bars: 75µm (D-E, left panels); 50µm (A-C, left panels; D-E, right panels); 25µm (A-C, right panels). Performed by K. Beets.

Since the lymphatic network is still expanding after birth, we investigated the postnatal mesentery, intestines and ear skin. Different types of lymphatic vessels occur in each of these tissues. The mesentery contains collecting vessels, while lacteals resorb lipids from the intestines and lymphatic capillaries drain lymph from ear tissue. Interestingly, many venous ECs appeared GFP-positive in P4 and P6 mesentery (**Fig. 3.5A-B**), whereas only few GFP-positive arterial ECs were observed (**Fig. 3.5A-B**) with also some GFP-positive peri-endothelial cells covering the arteries (Figure 4.6B). The developing lymphatic vessels were predominantly GFP-negative,

although *BRE::gfp* transcriptional activity was specifically present in some valve forming regions (**Fig. 3.5B**). At P10, the arterial ECs and peri-endothelial cells appeared GFP-negative and also fewer venous ECs were GFP-positive, whereas the lymphatic vessels still showed GFP in the valve regions (**Fig. 3.5C**). Remarkably, the vessels had reduced *BRE::gfp* transcriptional activity over time from P4 to P10 (**Fig. 3.5A-C**). Most intestinal villi comprise a LYVE1-positive lymphatic vessel, called the lacteal, which is surrounded by an Endomucin-positive blood capillary. Only few ECs of the blood capillaries and lacteals were GFP-positive (**Fig. 3.5D-E**). Other non-endothelial cells, such as goblet cells, were also GFP-positive in the villus (**Fig. 3.5D-E**). In the ear skin *BRE::gfp* transcriptional activity was absent from the lymphatic capillary bed at P6 and P10, with the exception of a single GFP-positive LEC at some branch points (**Fig. 3.5F-I**).

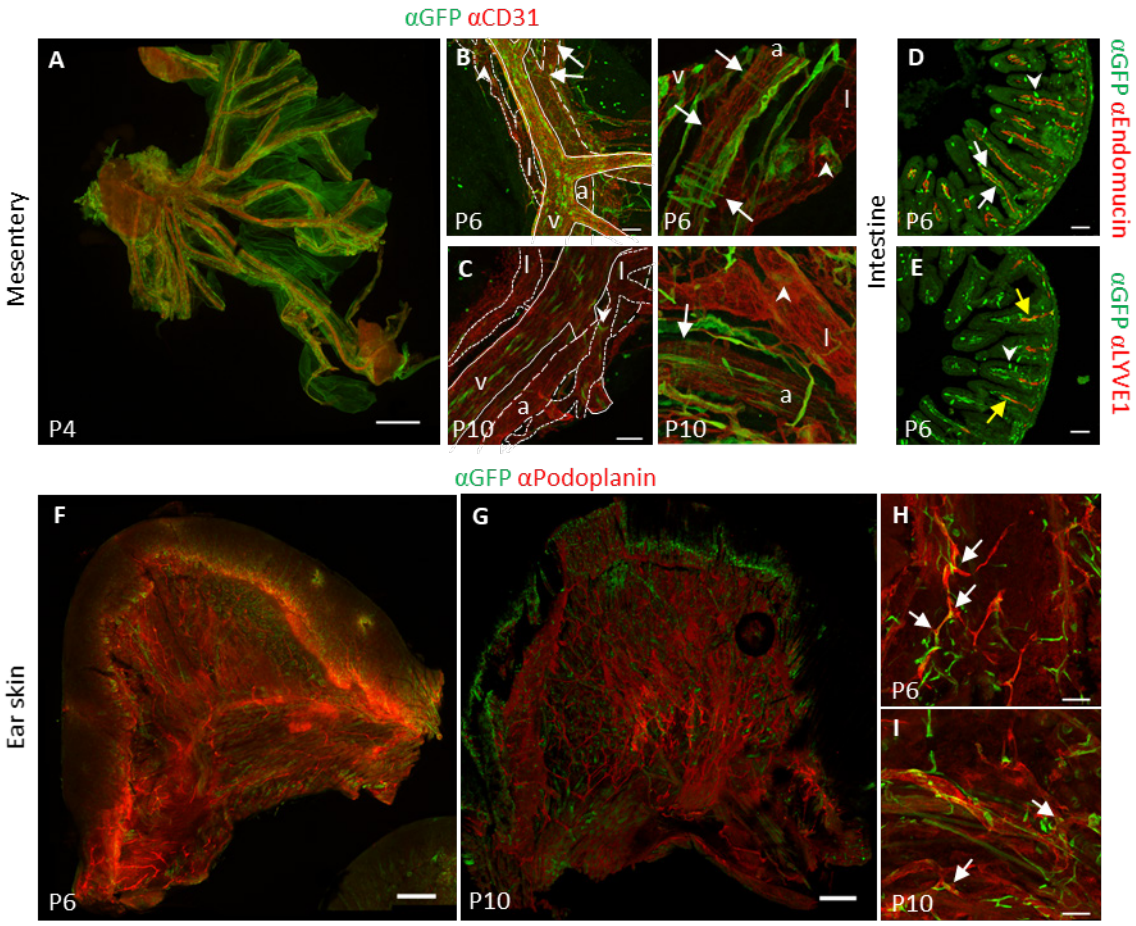

Figure 3.5: *BRE::gfp* transcriptional activity in postnatal blood and lymphatic vessels.
(A) Stitch of a P4 mesentery with immunodetection of GFP and the pan-endothelial marker CD31. Arteries, veins and collecting lymphatic vessels in a P6 (B) and P10 (C) mesentery are depicted. Arrows point to GFP-positive peri-endothelial cells. Arrowheads show GFP-positive ECs in the vicinity of a valve. Sections of P6 intestines with immunodetection of GFP and Endomucin (D) or the lymphatic vessel marker LYVE1 (E). White arrows point to some GFP-positive cells in a capillary, yellow arrows show GFP-positive LECs in a lacteal. Arrowheads show some GFP-positive goblet cells. Stitch of P6 (F) and P10 (G) dorsal ear skin with immunodetection of GFP and the lymphatic marker Podoplanin. GFP-positive LECs at the branch points are depicted by arrows at P6 (H) and P10 (I). A: artery; L: lymphatic vessel; V: vein. Scale bars: 1mm (A); 500µm (F-G); 75µm (B-E; H-I). Performed by M.W. Staring.

3.3.5 *BRE::gfp* transcriptional activity does not correlate with proliferation

Inhibitors of differentiation or IDs are helix-loop-helix proteins that interact with and inhibit basic helix-loop-helix transcription factors. Through induction of these IDs, the BMP pathway promotes EC migration and tube formation [44]. This led to the hypothesis that GFP-positive ECs would be proliferative. Several embryonic and postnatal tissues were investigated for co-localisation of GFP and phospho histone 3 (pH3) in the blood and lymphatic system. PH3 is only present in the M-phase of the cell cycle [45]. There was rarely overlap between pH3 and GFP-positive blood or lymphatic ECs in the neck region at E12.5 and E14.5 (**Fig. 3.6A-D**) or in dorsal skin biopsies from E14.5 and E16.5 embryos (**Fig. 3.6E-H**).

When pH3 seemed to be present in a GFP-positive ECs, the proliferating cell was usually not in the same focal plane as the EC (supplementary Figure 6A, not included in this thesis), or showed mainly low to no levels of GFP (data not shown). In addition, skin biopsies of E14.5 and E16.5 embryos were immunostained for Ki67 which marks all active phases of the cell cycle. Proliferation decreased between E14.5 and E16.5, but eventhough many non-endothelial cells were proliferative only few GFP-positive ECs and LECs were Ki67-positive (Supplementary Figure 6B-C, not included in this thesis). Also in the heart there was almost no co-localisation observed between pH3 and GFP (**Fig. 3.6I**). Furthermore, ECs of the sprouting front and the centre of the retina were not pH3-positive at P3, however, at this stage non-endothelial cells were more proliferative at the sprouting front than at the centre (**Fig. 3.6J**). Overall, proliferation decreased by P8 but shifted towards the ECs, as now pH3-positive ECs could be observed (**Fig. 3.6K**). Some of these proliferating cells seemed more GFP-positive than others. This is an intriguing difference in co-localisation pattern which suggests another context dependent role for BMP-SMAD signalling.

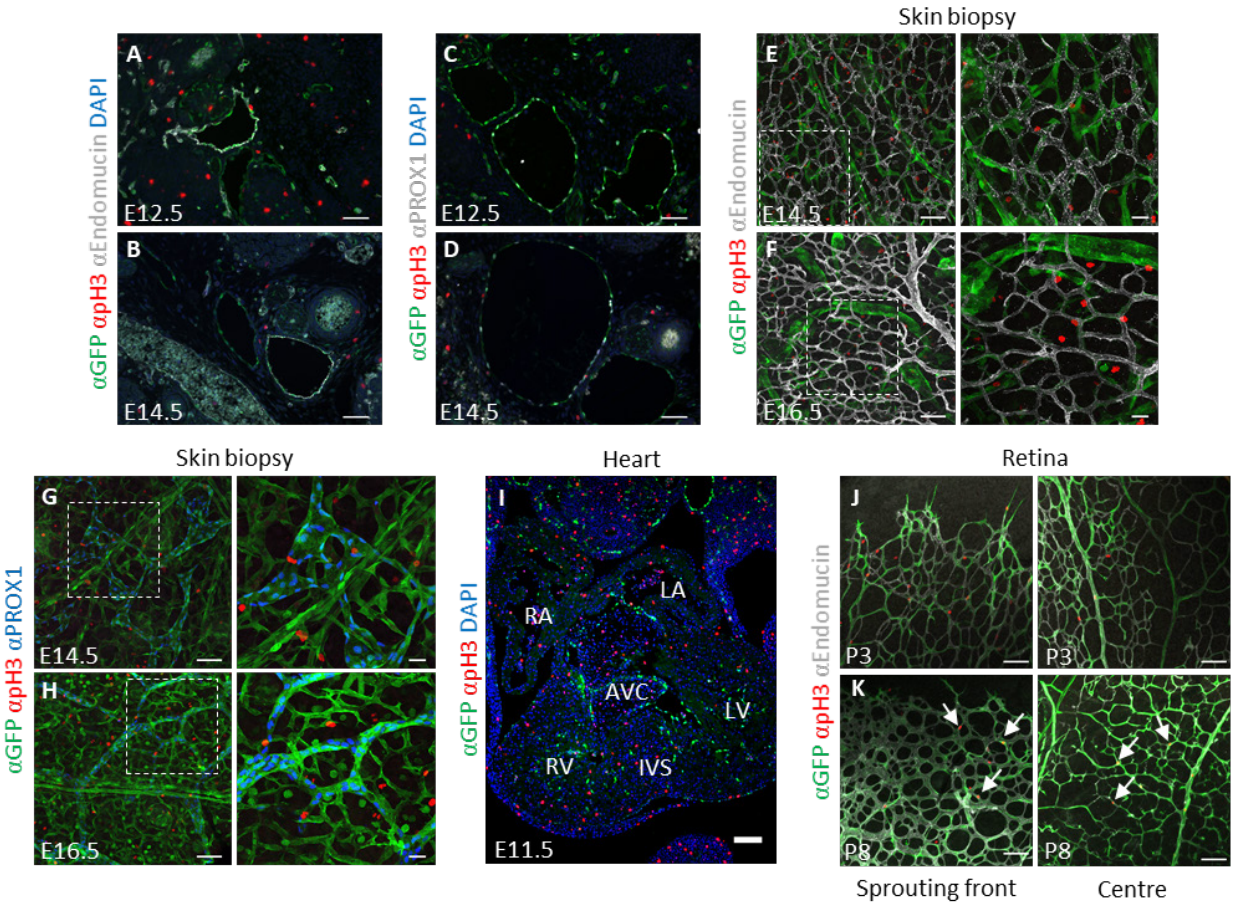

Figure 3.6: *BRE::gfp* transcriptionally active ECs are rarely proliferative.
Transverse sections of E12.5 (A, C) and E14.5 (B, D) *BRE::gfp* mouse embryos with immunodetection of the proliferation marker (pH3), GFP and Endomucin (A-B) or PROX1 (C-D). Dorsal skin biopsies obtained from E14.5 (E, G) and E16.5 (F, H) *BRE::gfp* embryos show pH3, GFP and Endomucin (E-F) or PROX1 (G-H) staining. Boxed areas are enlarged in the right panels. (I) Overview of an E11.5 heart with immunodetection of GFP and pH3. DAPI is used to stain nuclei. The sprouting front (left panels) and centre (right panels) of P3 (J) and P8 (K) retinas with immunodetection of GFP, Endomucin and pH3. Arrows point to pH3-positive ECs. AVC: atrioventricular canal; IVS: inter-ventricular septum; LA: left atrium; LV; left ventricle; RA: right atrium; RV: right ventricle. Scale bars: 100µm (I); 75µm (E-H, left panels; J-K); 50µm (A-D); 25µm (E-H, right panels). Performed by N. Criem, K. Beets and M.W. Staring.

3.4 Discussion

Spatial-temporal information on output of important signalling pathways in the vasculature may help to increase our understanding of how mutations in components of the same pathway can cause organ-specific vascular disorders and provide a window of opportunity for designing disease-specific therapy. In the past decade, many BRE-reporters have been generated in zebrafish and mice [25–30]. The *BRE::gfp* reporter used in this study is not the most sensitive, as some other BMP reporters show broader patterns of transcriptional activity. However, this precisely allows to zoom in on selective processes and dose-dependent BMP actions. Our study shows that GFP patterns in endothelium faithfully report transcriptional activation of the *BRE::gfp* transgene, and are remarkably robust. However the BMP-SMAD transcriptional output is highly dynamic in time and space, in the different cardiovascular and lymphatic beds of *BRE::gfp* mice.

Mosaic GFP localisation patterns were observed in different regions of the developing vascular tree and heart. It was found in the dorsal vascular plexuses of midgestation mouse embryos and in the capillary bed of P4-P10 retinas. In addition, at E9.5-E11.5 the endocardial cells of the atrio-ventricular canal (AVC) and outflow tract (OFT) cushions and those lining the atrial and ventricular trabeculae also displayed a mosaic GFP pattern. Such a mosaic pattern of transcriptional activity suggests a role for BMP-SMAD in EC plasticity and micro-heterogeneity. The multi-layered vascular network of the dorsal hindbrain and retina develop in a similar fashion, with the deeper plexus of the dorsal hindbrain resembling morphologically more the capillary network in the primary plexus of the retina [38]. Previously, we showed that the *BRE::gfp* pattern singled out stalk cell competent cells in the dorsal vascular plexus that were undergoing BMP and Notch co-signalling and that loss of BMP-SMAD signalling in endothelium resulted in a stalk cell defect [37].

We also observed weak GFP-positive tip cells in the dorsal hindbrain and retinal plexuses. These tip cells might have been former stalk cells that have taken over the tip cell position [46], with traces of non-degraded GFP. Alternatively, BMP6 and BMP7 synthesized by cells at the midline [39,40] may function as pro-angiogenic guidance cues that trigger an alternative BMP-SMAD signalling pathway in the tip cells. Circulatory BMP9 is likely to promote stalk cell competence through activating the mosaic transcriptional activity observed in the rest of the dorsal vascular plexus. In the retina, BMP9 and BMP10 are important for postnatal vascular remodelling [47]. Remarkably, BMP10 was unable to induce BRE activity *in vitro*, suggesting that the GFP signals in the centre of the retina, where vessel maturation and remodelling occurs, were the result of BMP9 signalling. However, also BMP2, 4, 6 and 7 have been shown to play significant roles in retinal neurogenesis and vascularisation [48]. Retinal vascularisation is preceded and stimulated by the development of a vast network of neuronal cells [49], the latter also depending on BMP-SMAD signalling [48,50]. The retinal ECs reciprocally promote differentiation of the neuronal plexus [49]. Our data do not allow to distinguish the precise source and type of BMP signal, yet, BMP-SMAD transcriptional activity seems more imperative at the sprouting front than in the centre where the vascular plexus is maturing.

Many BMPs have been reported to regulate cardiac cushion development [51,52]. In the AVC cushions, BMP2 stimulates ECs to undergo EndMT [53], while BMP4 is important in the OFT for proliferation and growth of endocardial cushions rather than EndMT [54]. Studies with KO mice

reveal that BMP2, ALK2, ALK3, BMPR2, SMAD4 and SMAD6 are important for the development of the AVC cushions and to a lesser extent OFT cushions [53,55–62]. Our study shows that BMP signalling induces mosaic transcriptional activity in cushion endocardium, likely to maintain an intact cushion epithelium while a few cells can undergo EndMT. Whether the GFP-positive cells or rather their neighbours are subsequently triggered to undergo EndMT remains to be elucidated. Taken together, in cushion endocardium and in angiogenic endothelium, the mosaic - perhaps stochastic - transcriptional BMP-SMAD activity seems to serve as a source of phenotypic diversity. The exquisite fine-tuning of the BMP pathway, which also involves negative feedback mechanisms, may also generate switch modes of activation states. Whether the mosaicism in BMP-SMAD transcriptional activity is static or dynamic, with *BRE::gfp* activity switching between 'on' and 'off' states, cannot be addressed directly in our model due to limitations in the resolution of real-time intravital microscopy, combined with the need for potentially long windows of observation. Dynamic mosaicism in expression has recently been demonstrated for von Willebrand factor (VWF), and also *in vitro* for ESM1 and ephrin-B2, in some but not all vascular beds. This appears to be a phenotype switching strategy for adaptive homeostasis [63].

Remarkably, during embryonic development the lymphatic vessels showed widespread *BRE::gfp* transcriptional activity, yet in pups GFP-positive ECs were restricted to the valve forming regions of collecting lymphatic vessels in the mesentery. This is in agreement with the role of BMP9 in lymphatic valve development [64]. In the lymphatic capillary bed of the ear skin, an occasional GFP-positive cell would localise at branch points. Furthermore, GFP-positive endocardial cells were observed on the atrial side of the tricuspid and bicuspid heart valve leaflets, but also in the inflow tract. All these patterns correspond with endothelium undergoing fluid shear stress, which can induce BMP-SMAD signalling. Hemodynamic alterations have been reported to induce BMP4 and activate SMAD1/5 in the aorta [18,65,66], and to mediate arteriogenesis [19].

Mature ECs are characterized by a slow proliferation rate. For example, in adult ear skin only 0.2% of the LECs are reported to be Ki67-positive, whereas approximately 30% of LECs are Ki67-positive in embryonic skin at E16.5-E17.5 [67]. The role of BMP signalling in EC proliferation is thought to be highly context dependent [13]. Because *BRE::gfp* signals peaked around midgestation and progressively decreased in postnatal stages in the vascular tree, we reasoned that correlations between BMP-SMAD transcriptional output and proliferation may become apparent in specific vascular beds. Remarkably, we found that proliferating pH3-positive ECs were almost invariably GFP-negative in the different vascular beds analysed, except in the P10 retina where more often double positive cells were observed.

A recurrent theme was – like in zebrafish embryos [68] – that the abundance of GFP-positive ECs was higher in veins than in arteries in several embryonic and postnatal tissues. For instance, the ECs in the cardinal vein showed a continuous GFP localisation pattern, whereas the aorta had a mosaic GFP pattern. In the mesentery almost no GFP-positive ECs were observed in the arteries, yet some of its peri-endothelial cells were GFP-positive. In contrast, many ECs of the veins were GFP-positive. ALK1, Endoglin and BMPR2 play a role in the establishment and maintenance of mural cell coverage on mature vessels [69,70], primarily in arteries. Mutations in BMPR2 lead to PAH which is characterized by abnormal proliferation of ECs and smooth muscle cells (SMCs) in arterioles [13,71], whereas a deletion of BMPR2 leads to insufficient recruitment and decreases *PDGFRβ* expression in mural cells [71]. BMP2/BMPR2 signalling negatively regulates PDGFBB

induced proliferation of pulmonary arterial SMCs in a pSMAD1/5/9 independent manner [71]. It is likely that veins express more BMP2 and hence limit the number of SMC coverage, but also the differences in shear stress in both vessel types may underlie the above differences.

3.5 Conclusion

We experienced that the *BRE::gfp* reporter is an exquisitely useful tool to get grip on the complex BMP-SMAD transcriptional signalling contexts *in vivo*. The GFP signals are robust, reproducible and highly regionalised; they correlate well with known areas of BMP signalling in the endothelium and reveal new microdomains of BMP signalling. Our study underscores the regionalised and heterogeneous nature of BMP signalling in the circulatory and lymphatic vasculature of embryos and pups, with striking shifts in transcriptional output over time in different endothelium types. This study highlights that extrapolation of results obtained in one vascular bed to another, or generalisation, should be done with extreme care. Examining other BMP signalling reporters and intercrossing them can likely shed light on yet other facets of the complex BMP-SMAD signalling output. Knowledge on differential signalling output is highly valuable to better understand the ontogeny of BMP-linked diseases and may lead to improved disease-tailored therapies.

3.6 Experimental procedures

Mice and tissue collection

BRE::gfp transgenic mice and endothelium-specific *Smad1;Smad5*-KO (*Tie2cre*$^{+/0}$*;Smad1*$^{fl/fl}$*;Smad5*$^{fl/fl}$) mice were used. Genotyping of the transgenic *BRE::gfp*, *Cre, Smad1* and *Smad5* floxed or recombined alleles was done as described [2537]. All embryos and postnatal organs were dissected in ice-cold diethylpyrocarbonate **(**DEPC)-treated phosphate buffered saline (PBS) and fixed overnight (ON) in 4% paraformaldehyde (PFA) in PBS at 4°C. Afterwards they were rinsed with PBS and saline and stored in 70% ethanol until processing. Fixed E9.5-12.5, E14.5 and E16.5 embryos and P6 intestines were processed for paraffin sectioning. Skin tissue from embryonic day (E)14.5 and E16.5 *BRE::gfp* embryos was dissected after fixation (Supplementary Figure 4C). Layers of muscle and tissue were carefully removed from the skin, leaving the superficial lymphatic network intact. From each embryo two skin biopsies were harvested. Retinas were collected from fixed eyes by removing the cornea and carefully lifting the retina from the remaining eyeball. Ears were collected from postnatal pups and separated into a ventral and dorsal side of which the latter was analysed. For each analysis a minimum of three animals was examined.

Immunohistochemistry

Whole-mount procedure: Embryos, skin biopsies, retinas, mesentery and ear skins were rehydrated and blocked in 2% bovine serum albumin (BSA) in Tris buffered saline (TBS) for 3h at room temperature (RT). Tissues were incubated ON with primary antibodies in 2% BSA in TBS at 4°C, except for the embryos which were kept at RT. This was followed by blocking for 3h in 2% BSA in TBS and incubation with the secondary antibody ON (Alexa antibodies, Jackson Immunology). The list of primary antibodies and the used dilutions are provided in supplementary material. After whole-mount immunostaining of E9.5 (22 ± 2 somites) and E10 (30 ± 2 somites) embryos the forebrain and the abdomen caudally from the forelimb bud were transversally removed. All ventral tissues including the heart were removed and the neural tube was then cut open at the ventral side. The hindbrain was mounted on a glass slide with the ventral side facing up.
The mesentery was excised from the intestines after whole-mount immunostaining, and the retina was cut into a four-leaf clover before mounting on a glass slide.

Paraffin sections: Transversal and sagittal sections (6-8µm) of paraffin embedded tissues were processed for immunohistochemistry using an automated platform (Ventana Discovery Ultra, Roche). Immunofluorescent triple detection of pSMAD1/5/9, GFP and MF20 was done manually. The list of primary antibodies, as well as the conditions used, are provided in supplementary material. Antigen retrieval was done by submerging the slides in Tris-EDTA buffer (10mM Tris Base, 1mM EDTA, 0.01% Tween20, pH9.0) for 30 min at 96°C. For pSMAD1/5/9, endogenous peroxidases were inactivated in 3%H_2O_2 in Methanol for 30 min and the antibody signal was amplified using the Perkin Elmer TSA Biotin system kit (NEL700A001KT).

Primary antibody (name)	Concentration for whole-mount/paraffin/cryosections	Company	Catalogue #
CD31	1/100	BD Pharmingen	553370
Collagen type IV	1/100	AbdSerotec	2150-1470
Endomucin	1/100	Santa Cruz	sc65495
GFP	1/100; 1/500	Abcam	ab13970
Isolectin B4*	1/100	Sigma-Aldrich	L2140-1MG
Ki67	1/100	Abcam	ab16667
LYVE1	1/100	Abcam	ab14917
pH3	1/100	Abcam	ab5176
Podoplanin	1/100	R&D systems	AF3244
PROX1	1/100 biotin	R&D systems	AF2727
MF20	1/100	R&D systems	MAB4470
pSMAD1/5/9	1/300	Cell Signaling	#13820

***In situ* hybridization**

Embryos were dissected in DEPC-treated PBS and fixed ON in 4% PFA in PBS at 4°C. Afterwards they were washed three times 30 min in DEPC-treated PBS, immersed in 15% sucrose and snap frozen in Optimal Cutting Temperature (OCT) compound (Richard-Allan Scientific #6502) with liquid nitrogen. The *GFP* fluorescent *in situ* hybridisation (ISH) probe was custom designed with the probe designer tool from Stellaris (LGC biosearch technologies). The coding sequence of the *pEGFP-N2* plasmid (accession number U57608.1) was used for probe design. Fluorescent ISH (FISH) was performed according to the manufacturer's protocol (Stellaris) with the addition of an extra permeabilisation step with 1% TritonX100 (Sigma T8787) in PBS. After FISH, the sections were immunofluorescently stained for CD31 (Suppl. Table 2). Images were acquired using a Nikon A1R Eclipse Ti confocal microscope.

3.7 Declarations

Ethics

All animal procedures were approved by the ethical committee and performed according to the guidelines of the Animal Welfare Committee of KU Leuven, Belgium (P107/2011, P209/2013).

Author's contribution

KB, MWS, NC, LU and AZ conceived and designed the study; KB, MWS and NC generated all the presented data; NS and LU contributed with some of the immunohistochemistry analyses at an early stage of the project; *BRE::gfp* mice were provided by SMCSL and EM provided technical and mouse husbandry support; KB drafted the manuscript, with help of MWS, NC, SMCSL and AZ. All authors discussed the work, read and approved the final manuscript.

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Supplemental information

Additional supplemental information not found within this Chapter is available in the online version of the paper. https://bmcdevbiol.biomedcentral.com/track/pdf/10.1186/s12861-016-0133-x

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Chapter 4

Absence of *Smad1/5* in mouse LECs results in a dysfunctional lymphatic vessel network

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Manuscript (tentative title) in preparation

Note: this *manuscript in preparation* has been modified to bring together better the results from Chapters 3 and 5

Chapter 5

BMP-SMADs SMAD1/5 promote lymphatic vessel stabilization by regulating WNT signaling

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Manuscript submitted for publication

Chapter 6

General discussion

Curriculum Vitae

Personal information

Name:	Michael (William) Staring
Email:	michael.staring@gmail.com
Date of birth:	24 February 1987 (Sassenheim, The Netherlands)
Address:	Diestsesteenweg 304, Tienen, Belgium
Nationality:	Dutch

Education

2018-present	PhD student Institute: Erasmus University Rotterdam Department of Cell Biology Promotor: Prof. Dr. Danny Huylebroeck
2014-present	PhD student Institute: KULeuven Departments: VIB center for Brain and Disease Research, Department Cardiovascular research and the Department of Human Genetics Promotor: Prof. Dr. An Zwijsen
2013-2014	Predoctoral thesis Institute: KULeuven Laboratory of developmental signaling Promotor: Prof. Dr. An Zwijsen
2010-2012	Bachelor in Life Sciences Institute: University of Applied Sciences Leiden
2007-2009	Bachelor Veterinary Medicine Institute: University of Antwerp (not completed)
1999-2007	High school: Rijnlands Lyceum Sassenheim, The Netherlands VWO Profiel: Sciences HAVO Profiel: Sciences VMBO Profiel: Agriculture

Certificates

2017	Writing Skills for Biomedical Researchers, KU Leuven
2015	Effective writing for life sciences research course, University of Oxford
2015	Animal Science, FELASA C, KU Leuven
2011-2012	Animal Science, FELASA B, University of Applied Sciences Leiden

List of publications

M. W. Staring*, A. de Jaime-Soguero, H. Peacock, E. Maas, A. Francis, L. Umans, A. Luttun, W.F.J. van Ijcken, E.A.V. Jones, D. Huylebroeck, F. Lluis, E. Mulugeta, A. Zwijsen, " SMAD1/5 promote lymphatic vessel remodelling by limiting canonical WNT signaling" Manuscipt submitted

K. Beets*, **M. W. Staring*,** N. Criem*, E. Maas, N. Schellinx, S. M. C de Sousa Lopes, L. Umans and A. Zwijsen, "BMP-SMAD signalling output is highly regionalized in cardiovascular and lymphatic endothelial networks.," BMC Dev. Biol., vol. 16, no. 1, p. 34, Oct 2016

Scholarships/ Prizes

2018	Flanders Science Communication award with IPPO
2018	BSCDB travel grant
2018	FWO travel grant
2018	FLOF scholarship, department Cardiovascular Sciences, KULeuven, 9 months.
2014	Selected for a promo movie for VSB-foundation based on my pre-doctoral year: https://www.vsbfonds.nl/20389.aspx
2013	Received a VSB scholarship for one year
2013	Selected as student of the year at the University of Applied Sciences, Leiden
2012	Received an Erasmus scholarship

Experience

- Co-founder and Secretary: Infopunt Proefdieronderzoek
- Co-organizer: VIB PhD symposium 2016 (70 PhD students)
- Supervisor of Bachelors student:
    - Gilles America; UC Leuven Limburg; February - June 2017
    - Jeroen Vanderlinden; UC Leuven Limburg; February – June 2015
    - Iris Janssens; Thomas More Geel; February – June 2014
    - Koen Demaegd; KULeuven, Medicine; 2014
- Volunteer at "VIB wetenschap op stap" 2015 at 'Sancta Maria basisschool'
- Volunteer at the "kinderuniversiteit" 18 October 2014, workshop 'de heldenklas'
- Tutor at University of Applied Sciences Leiden, 2011

Course

Training at VIB	Single cell analysis	21/09/2016
	Essentials of image editing	17/05/2016
	Graphpad Prism	13/05/2016
	Basic statistics theory	02/05/2016
	Initiation GIMP and Inkscape	28/04/2016
	Short and concise scientific writing seminar	05/11/2015
Training by Infrafrontier	Mouse blood and lymphatic vessel phenotyping training course at CBATEG, facultat de Veterinaria de Barcelona, Barcelona, Spain	17/11/2014-20/11/2014

Skills

Languages:	Dutch (native speaker), English (Fluent)
IT:	MS Word, Excel, Powerpoint, qBASE+, ImageJ, GIMP, Inkscape, Graphpad Prism, LAIS

Oral presentations

- TRR81 Winterschool "Chromatin Changes in Differentiation and Malignancies" 2019, Kleinwalsertal, Austria, 28/02/2019.
  Title: SMAD1/5 stabilize lymphatic endothelium and valve development by regulating the WNT-β-catenin pathway

- NAV congress 2018, Lunteren, the Netherlands, 04/01/2019
  Selected for a "Bolk voordracht". Title: SMAD1/5 stabilize lymphatic endothelium and valve development by regulating the WNT-β-catenin pathway

- Joint BSCDB-FWO-IUAP meeting, Leuven, 24/11/2015
  Title: Lymphatic valve leaflet formation depends on BMP-SMAD signaling

- VIB PhD symposium, Leuven, 24/09/2015
  Title: Maturation of lymphatic vessels depends on BMP-SMAD1/5 mediated signaling in the mouse

- IUAP meeting, Brussels, 28/05/2014
  Title: BMP signaling in postnatal lymphatic vessel maturation

Poster presentations

- GRC lymphatics 2018, Lucca Barga, Italy, 11/03/2018 – 16/03/2018
  Title: Lymphatic valve leaflet development depends on BMP-SMAD mediated signaling in mice

- VIB metabolism in cancer and stromal cells, Leuven, 8/09/2015 - 10/09/2015
  Title: Differential BMP-SMAD signaling in circulatory and lymphatic vasculature

- VIB seminars, Blankenberge, 30/03/2015 - 01/04/2015
  Title: Maturation of lymphatic vessels depends on BMP-SMAD1/5 mediated signaling in the mouse

- PhD symposium, Leuven, 25/09/2014
  Title: BMP-SMAD mediated signaling during postnatal lymphangiogenesis in mice

- IUAP, Liege, 09/12/2013
  Title: BMP-SMAD mediated signaling is important for normal lymphangiogenesis in the mouse

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"If you can imagine it, you can achieve it. If you can dream it, you can become it". An inspiring quote from the American writer William Arthur Ward, which really captures my track as a PhD student. In addition, hard work, perseverance and, most of all, teamwork was key for this journey. Writing these last pages of my PhD thesis makes me increasingly aware and grateful of everything and everyone that has been involved to help me accomplish this massive academic milestone.

An, as promoter, mentor, biggest believer and support throughout many years, I would like to express the most appreciation and respect to you. The never-ending commitment to your team and all its previous members is amazing. I have really enjoyed learning from you and appreciated the many thoughtful cards and messages in difficult and festive times. We also shared many hours of debates, discussions, chats, one-to-ones, meetings and spring parties. At times, even an extremely busy and demanding schedule did not stop you from finding a way to discuss my project, latest results and often many other topics not related to lab work at all. Overall, I think you are a great example for students, lab members and other scientist and not only for your approach to science, but also for your personal skills. Altogether, I am proud and immensely thankful to have obtained my PhD under your supervision!

Danny, you officially became my promoter during the second half of my PhD track. However, you already showed specific interest in my personal development and that of the lymphatic project from much earlier stages. I want to thank you for all the support and opportunities you have given me over the many years we worked together. I deeply appreciate your clear (yet, sometimes long) and interesting takes on science. Reaching the end of my PhD track now, I believe it is that curiosity, together with the efforts and teamwork provided in Leuven and Rotterdam, that resulted in a fantastic outcome. I am honored to have obtained my PhDs under the supervision of both you and An and I am sure our paths will cross again.

Very early, but briefly, in my PhD adventure I had the pleasure to have **Lieve** as my co-promoter. I really want to underline my gratitude towards you. You have co-shaped my scientific career in a very early stage and with the help of fundamental skills you have taught me, I was able to obtain my PhD.

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In the early phase of my PhD we shared our office with the group of **Guy Froyen** in VIB. You all have been an example for me and I wish you the best in your life. After that, **Lorna**, **Annick** and **Enrico** moved in. Thank you all for your expertise in histology and the pleasant working environment. At this time, we also enjoyed a close collaboration with Danny's group in Leuven, named "Celgen", in this period **Ruben**, **Agata**, **Veronique**, **Eve**, **Elke S**, **Kathleen**, **Andrea, Lieve, Annick** and **Flore**. Thank you for creating a great lab working environment and open platform to discuss a lot of preliminary data. I also enjoyed all the social activities. My special thanks here go to **Annick**. I want to specifically thank you for being such a great support. You have made the final stage of my PhD workable. I am happy we will publish our work together. I wish you best of luck in the future. This publication would also not have been the same without the collaboration we shared with **Fredi** and **Anchel**. I have greatly appreciated your dedication to this project and your input during the process, on both the science and private aspects. Similarly, this manuscript would not have been the same with the input of **Liz** and **Hanna**. I thank you for openness and training in the world of *in vitro* flow experiments. I have learned a lot from you. Good luck with the final stages of your manuscripts. Next, I would like to thank **Aernout** for all your discussions and interest in this project. I enjoyed learning about your work in the last years and it definitely positively shaped my project. **Petra**, we have had a lot of long conversations sitting behind the FACs sorter. I have enjoyed these sessions a lot. Good luck in the future and I am sure once or twice a cupcake will be sent in your direction. Finally, **Willeke** (my paranymph) I am very happy I was able to make you

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I have always enjoyed extracurricular activities. One of them, I am particularly proud of. **Jeroen**, **Annerieke**, **Liesbeth**, **Julie**, **Maarten**, **Anneke**, **Vinoy** from IPPO thank you for including me. I have learned a lot about science communication from you. I hope together we can continue to improve science communication.

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