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ENDOTHELIAL - MURAL CELL INTERPLAY IN REGULATION OF BLOOD- AND LYMPH VESSEL DEVELOPMENT AND FUNCTION

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Endothelial - mural cell interplay in regulation of blood- and lymph vessel development and function

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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This thesis is dedicated to my family

献给我的家人

POPULAR SCIENCE SUMMARY

The blood- and lymphatic vasculatures together compose the circulatory system of the human body. Our physiology depends on proper vascular function, as evidenced by the impact of vascular dysmorphogenesis or dysfunction in multiple diseases. To understand mechanisms in regulation of vascular development and function would open new possibilities for vascular targeting in patients. The vascular wall is composed of endothelial cells (ECs) that enclose the blood, as well as perivascular cells closely attached to the ECs on the abluminal side. The interaction of these two cell types is critical to regulate vessel generation and functional maintenance in the context of neonatal development, wound healing, cancer and cardiovascular diseases etc. This thesis aims to understand molecular mechanisms controlling the interaction of these two cell types, with the focus on PDGFRβ signalling, a molecular machinery that is highly involved in endothelial-mural cell interplay. We revealed that PDGFRβ signalling regulates the interaction of the lymphatic endothelium with perivascular smooth muscle cells (SMC). We also showed that SMC directly controls lymphatic vessel contraction. Furthermore, we revealed the interaction of PDGFRβ with PDGFB and other molecules including PDGFD and neuropilin1 in a coordinated manner that together regulate endothelial cell and perivascular cell interplay in growing blood vessels. From a technical perspective, we developed a novel imaging model in high resolution using the cornea of living mice that enables detailed analysis of the migrating motions of these two cell types during vascular network formation. Together, this thesis extended the understanding of endothelial and perivascular cells interplay that may contribute to the discovery of future treatment of cardiovascular diseases.

血管系统和淋巴管系统共同构成了人体的循环系统。受损伤的血管或淋巴管功能是许多相关疾病的根本原因。因此，探索血管以及淋巴管系统的发育规律将为发现新的心血管疾病治疗靶点和开发新的治疗方法作出基础性的贡献。血管壁由构建血管腔的“内皮细胞”和血管周围紧密包裹的“血管周细胞”组成。两种细胞类型之间的相互作用对于新生儿循环系统发育，成年人伤口愈合，以及在癌症和心血管疾中调节血管生成和维持功能维持有重要作用。这篇博士论文旨在探索控制这两种细胞类型相互作用的分子机制，并重点探究血小板衍生生长因子受体 B（PDGFRβ）在血管和淋巴管发育水平的信号通路。我们首先应用多种经过基因编辑的小鼠作为动物模型，揭示了 PDGFRβ 信号直接控制淋巴管平滑肌细胞（一种淋巴管特异型周细胞）的生成，以及淋巴管平滑肌细胞在皮肤淋巴管节律性收缩中的重要作用。此外，我们还发现了 PDGFRβ 与其他分子（包括血小板衍生生长因子 D（PDGFD）和神经纤毛蛋白 1（neuropilin1）协同调节内皮细胞和血管周细胞在血管生长中的相互作用。从技术创新的角度，我们开发了一种新的高分辨率活体显微镜成像技术：利用麻醉小鼠的眼角膜分析炎症诱导的血管网络发育的动态过程。亚细胞水平的分辨率使得该技术可以清晰的记录并分析血管内皮细胞与血管周细胞的迁移运动以及相互作用。综上，这篇博士论文拓展了对内皮细胞和血管周细胞相互作用机制的理解，并为发现心血管疾病的药物靶点和治疗方法开辟了新方向。

ABSTRACT

Blood- and lymphatic vascular development and homeostasis depend on correct endothelial- mural cell interaction, and dysregulation thereof is apparent in multiple human diseases. However the mechanisms controlling the formation of these respective vascular systems are not fully understood. Previously, the binding of EC-derived Platelet derived growth factor B (PDGFB) to its receptor, PDGFRβ on mural cells, was shown to play an important role in recruitment of mural cells to blood capillaries. Whether PDGFB carries similar functions in the lymphatic vasculature is still unclear. In addition, potential regulation of PDGFRβ signalling by other PDGFs, in the context of endothelial - mural cell interaction, is not fully understood.

This thesis focuses on PDGFRβ signalling, mediated by PDGFB and PDGFD, in regulation of endothelial - mural cell interplay in blood and lymphatic vessel development. By generation of several genetic modified mouse models, including inducible targeting of PDGFB in the lymphatic endothelium, we reveal that PDGFB is required for recruitment of smooth muscle cells (SMCs) to collecting lymphatic vessels. In addition we show that SMCs play no major role in the establishment of lymphatic vessel identities but that these cells are responsible for the recorded pulsatile contraction of dermal collecting vessels. Furthermore, our data suggest that it is unlikely that pathological SMC recruitment to capillaries is caused by altered PDGFB expression alone, but that it also relies on extra cellular matrix composition. Besides PDGFB, we also demonstrated a potential involvement of PDGFD in regulation of EC-pericyte interplay. We found that, although PDGFB and PDGFD evoked similar PDGFRβ activation, these ligands promoted differential pericyte behavioural responses in 3-dimesional angiogenesis assays. This may be related to our discovery of an interaction between PDGFD and Neuropilin-1 (NRP1).

A part of this thesis was also dedicated to record vascular development from a new level of both imaging and time resolution. By using the wounded mouse cornea as a live imaging site, we developed an *in vivo* imaging approach that allows for documentation of vascular morphogenesis over time, at subcellular resolution. We used this method to analyse EC migratory behaviour and highlighted directional migration against blood flow. We also characterized vessel patterning with respect to mural cell distribution during sprouting angiogenesis in the inflamed cornea. Furthermore, we recorded temporal and spatial aspects of VEGFA-induced vessel permeability by intra vital live imaging and revealed distinct artery-venous properties.

Taken together, this thesis contributes to the understanding of the roles of endothelial- and mural cells in the context of blood and lymphatic vascular development. Our findings shed new light on mechanisms regulating cardiovascular homeostasis in development and disease.

LIST OF SCIENTIFIC PAPERS

I. **Smooth muscle cell recruitment to lymphatic vessels requires PDGFB and impacts vessel size but not identity**
Yixin Wang, Yi Jin, Maarja Andaloussi Mäe, Yang Zhang, Henrik Ortsäter, Christer Betsholtz, Taija Mäkinen, Lars Jakobsson.
Development (2017) 144, 3590-3601 doi:10.1242/dev.147967

II. **Characterization of multi-cellular dynamics of angiogenesis and vascular remodelling by intravital imaging of the wounded mouse cornea**
Yixin Wang, Yi Jin, Bàrbara Laviña and Lars Jakobsson.
Submitted.

III. **Neuropilin 1 binds PDGF-D and is a co-receptor in PDGF-D–PDGFRβ signaling**
Lars Muhl, Erika Bergsten Folestad, Hanna Gladh, Yixin Wang, Christine Moessinger, Lars Jakobsson and Ulf Eriksson.
Journal of Cell Science (2017) 130, 1365-1378 doi:10.1242/jcs.200493

Papers not included in the thesis

I. **Endoglin prevents vascular malformation by regulating flow-induced cell migration and specification through VEGFR2 signalling.**
Jin, Y., L. Muhl, M. Burmakin, Y. Wang, A. C. Duchez, C. Betsholtz, H. M. Arthur and L. Jakobsson
Nat Cell Biol (2017), 19(6): 639-652.

II. **Defects in β-Cell Ca^{2+} Dynamics in Age-Induced Diabetes**
Luosheng Li, Aleksandra Trifunovic, Martin Köhler, Yixin Wang, Jelena Petrovic Berglund, Christopher Illies, Lisa Juntti-Berggren, Nils-Göran Larsson and Per-Olof Berggren
Diabetes (2014), 63(12): 4100-4114.

CONTENTS

LIST OF ABBREVIATIONS

AKT	Protein kinase B
ALK	Activin receptor-like kinase
ANG	Angiopoietin
BEC	Blood endothelial cell
BMPs	Bone morphogenetic proteins
CCL21	Chemokine ligand 21
Celsr1	Cadherin EGF LAG seven-pass G-type receptor 1
CNS	Central nervous system
DC	Dendritic cell
DLL4	Delta-like 4
E	Embryonic day
EC	Endothelial cell
ECM	Extra cellular matrix
EMILIN1	Elastin microfibril interfacer 1
ERK	Extracellular signal–regulated kinases
FN	Fibronectin
FOXC2	Forkhead box protein C2
hHSC	Human hepatic stellate cell
HSPG	Heparan sulfate proteoglycan
JAK	Janus tyrosine kinase
JNK	C-Jun N-terminal kinase
LD	Lymphedema-distichiasis
LEC	Lymphatic endothelial cell
LYVE-1	Lymphatic vessel hyaluronan receptor 1
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemotactic protein 1
MHC	Myosin heavy chain

MLC	Myosin light chain
NFAT	Nuclear factor of activated T-cells
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NG2	Neural/Glial antigen 2
NO	Nitric Oxide
NRP	Neuropilin
PCP	Planar cell polarity
PDGF	Platelet derived growth factor
PDGFR	Platelet derived growth factor receptor
PI3K	Phosphoinositide 3-kinase
PLC-γ	Phosphoinositide phospholipase C-γ
PROX1	Prospero related homeobox-1
PYK-2	Potein tyrosine kinase 2 beta
SEMA	Semaphorin
SMA	Smooth muscle actin
SMAD	Sma mothers against decapentaplegic
SMC	Smooth muscle cell
SOX18	SRY(sex determining region Y) box 18
STAT	Signal transducer and activator of transcription
TGF-β	Transforming growth factor beta
VANGL2	Van Gogh-like 2
VE-cadherin	Vascular endothelial cadherin
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
4-OHT	4-hydroxytamoxifen

1 INTRODUCTION

The complex mammalian bodies require a sophisticated transportation system to sustain oxygenation, nutrition, maintenance of liquid balance and immune surveillance. This is achieved mainly via the network of blood and lymphatic vessels. The blood flows from the heart, via arteries and arterioles to the capillaries, where liquid, gas and nutrients are exchanged between blood and tissue. The metabolic products are accordingly transferred from tissue to the capillary blood, and via venules and veins back to the heart. In addition to the blood vasculature, the lymphatic vasculature is crucial in maintaining tissue liquid homeostasis. Tissue liquid drains through blind-ended capillaries into larger, smooth muscle covered collecting vessels that merge and connect to the subclavian vein, thereby entering the blood circulation.

1.1 Development of the blood vasculature

In early embryonic development, the formation of the blood vasculature is initiated by a process denoted vasculogenesis, where a subpopulation of cells within the mesoderm differentiates into endothelial precursor cells which assemble into the primary blood vessel plexus in the yolk sac (Carmeliet 2005). Formation of the dorsal aorta and the cardinal vein were directly via assembly of endothelial precursor cells (Cleaver and Melton 2003, Adams and Alitalo 2007). In addition circulating bone-marrow-derived endothelial precursor cells are also suggested to contribute to enlargement of vessels (Rafii, Lyden et al. 2002, Grunewald, Avraham et al. 2006, Shibuya 2006, Adams and Alitalo 2007). Circulation of blood is established in the primary plexus upon vessel loop formation with the contribution of cardiac contraction and entry of erythrocytes (Jones 2011). The vessel network is further developed into a more complex vasculature composed of endothelial cells (EC) and closely associated mural cells: pericytes and smooth muscle cells (SMCs). The mechanisms of vasculogenesis not only play a key role in early vessel development, but also contribute to pathological vessel generation e.g. tumour formation in adulthood (Rafii, Lyden et al. 2002, Adams and Alitalo 2007).

However, a large proportion of blood vessels, e.g. in the central nervous system, extend and develop into a network via the process of angiogenesis, where ECs migrate out from the pre-existing vasculature forming a new vessel structure. One important regulatory factor that promotes EC activation and migration is vascular endothelial growth factor A (VEGFA), which together with VEGFB, VEGFC, VEGFD and placental growth factor (PlGF) composes the VEGF family. Binding of VEGFs to their receptors: vascular endothelial growth factor receptor 1, -2, and -3 (VEGFR1, -2, and -3), activates multiple downstream pathways that regulate various cellular functions (Adams and Alitalo 2007).

1.2 Development of the lymphatic vasculature

The development of lymphatic vessels initiates 6 to 7 weeks post fertilisation in humans and at embryonic days (E) 9.0 to 9.5 in mice (Alitalo, Tammela et al. 2005). At this stage, a group of ECs within the anterior cardinal vein start to express lymphatic vessel hyaluronan receptor 1 (LYVE-1) (Oliver 2004). From E9.5 to E11, a subpopulation of LYVE-1+ ECs start to express several key transcription factors e.g. prospero related homeobox-1 (PROX1), podoplanin and neuropilin 2 (NRP-2) that were established to be major regulators for lymphatic vascular development (Breiteneder-Geleff, Soleiman et al. 1999, Yuan, Moyon et al. 2002, Oliver 2004). By expression of VEGFR3, the subpopulation of LYVE-1+, PROX1+, podoplanin+, NRP2+, VEGFR3+ ECs respond to VEGFC, and migrates out from the cardinal vein to form the primary lymphatic sac. Notably, platelet aggregation is suggested to be important to seal off the blood vessels during this process and defective platelet aggregation causes blood filled lymphatic vessels (reviewed in (Schulte-Merker, Sabine et al. 2011)). Until E14.5, lymphatic endothelial cells (LECs) accordingly proliferate and migrate out from the primary lymphatic sac, and establish the lymphatic vascular network via budding and sprouting (Tammela and Alitalo 2010, Yang and Oliver 2014).

Although a major part of the mammalian lymphatic tree originates from veins, a large proportion of superficial dermal lymphatic vessels and part of the mesenteric lymphatic vasculature are derived from progenitors of a non-venous origin (Martinez-Corral, Ulvmar et al. 2015, Stanczuk, Martinez-Corral et al. 2015). These LECs express typical

lymphatic markers including PROX1, VEGFR3 and NRP-2 but not LYVE-1 and contribute to the establishment of lymphatic vasculature via assembly into isolated endothelial clusters that develop into a network. This alternative mode of lymphatic vessel establishment is defined as a lymphvasculogenesis. This knowledge sheds new light on the complexity of lymphatic vasculature origin and may provide novel targets for treatments of cancer, lymphedema or tissue trauma.

1.3 Key factors/signalling pathways regulating vascular development

1.3.1 VEGF / VEGFR signalling

VEGF/VEGFR signalling regulates essential pathways during the process of angiogenesis. In blood endothelial cells (BECs), binding of VEGFA to its membrane receptor tyrosine kinase VEGFR2 results in BEC proliferation, migration and vessel sprouting. However, the pro-angiogenic effect of VEGFA-VEGFR2 interaction is counteracted by VEGFA binding to VEGFR1, which has higher VEGF affinity but less tyrosine-kinase activity and in turn considered as an inhibitory control of angiogenesis (Jakobsson, Franco et al. 2010, Krueger, Liu et al. 2011, Boucher, Clark et al. 2017, Pitulescu, Schmidt et al. 2017). Interestingly, a recent study also showed the involvement of pericytes in regulating VEGFA/VEGFR2 signalling via expression of soluble VEGFR1 functioning as a VEGFR2 antagonist (Eilken, Dieguez-Hurtado et al. 2017). In the process of sprouting angiogenesis, VEGFA regulates EC migration, promoted by their extending filopodia (Gerhardt, Golding et al. 2003). In addition to the impact of differential VEGFR2 levels and degree of activation, several other EC signalling cascades have been shown to be central in the establishment and extension of new sprouts, including Delta-like 4/Notch and CXCL12/CXCR4 (Hellstrom, Phng et al. 2007, Jakobsson, Franco et al. 2010, Nakayama, Nakayama et al. 2013, Xu, Hasan et al. 2014, Hasan, Tsaryk et al. 2017, Jin, Muhl et al. 2017, Pitulescu, Schmidt et al. 2017). The precise involvement of these cascades in the dynamic process of angiogenesis has been challenging to dissect. Recent studies, using lineage tracing and live imaging in zebrafish, clearly indicate that leading tip cells contribute to artery formation, a process that in turn relies on Notch activation (Hasan, Tsaryk et al. 2017, Pitulescu, Schmidt et al. 2017).

In the lymphatic vasculature, VEGFC/VEGFR3 signalling is essential for LEC migration and sprouting formation. The binding of receptor tyrosine kinase VEGFR3 and VEGFR2, predominantly as homodimers, to their ligands VEGFC and VEGFD activates downstream signalling pathways controlling LEC proliferation, migration and survival. LECs of mice lacking expression of VEGFC fail to migrate from veins to develop the primary lymph sac (Karkkainen, Haiko et al. 2004, Coso, Bovay et al. 2014). The importance of VEGFC for human physiology is also reflected by the fact that a point mutation in *VEGFC* causes lymphedema in patients (Gordon, Schulte et al. 2013). In contrast, VEGFD deletion does not affect lymphangiogenesis, while VEGFD is able to compensate the loss of VEGFC during lymphatic vessel sprouting in zebra fish (Astin, Haggerty et al. 2014). Mice lacking VEGFR3 has early cardiovascular defects and die at (E) 9.5 (Dumont, Jussila et al. 1998). However, transgenic mice expressing a soluble form of VEGFR3, functioning as a blocker of VEGFC/VEGFR3 signalling display inhibited lymphangiogenesis and lymphedema-like phenotypes, highlighting the functional importance of VEGFC/VEGFR3 signalling (Makinen, Jussila et al. 2001). Unlike other VEGFs, both VEGFC and VEGFD require post-secretion proteolytic processing to potentiate receptor binding (Joukov, Sorsa et al. 1997, Vaahtomeri, Karaman et al. 2017). Upon ligand binding, phosphorylated VEGFR3 recruits signal transduction protein kinases extracellular signal–regulated kinases 1/2 (ERK1/2), protein kinase B (AKT), protein tyrosine kinase 2 beta (PYK-2), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), c-Jun N-terminal kinase 1/2 (JNK1/2) and activate their downstream signalling (Dixelius, Makinen et al. 2003, Alitalo, Tammela et al. 2005, Salameh, Galvagni et al. 2005). Notably, the binding affinity of VEGFC to VEGFR3 is enhanced by the co receptor NRP-2 which contributes to VEGFR3 internalization (Xu, Yuan et al. 2010).

1.3.2 Mural cell – Endothelial cell interaction

Vascular function and integrity relies on the interaction between mural cells and the endothelium. Mural cells can be divided into pericytes and vascular smooth muscle cells (vSMCs). Pericytes, which are mainly recruited to the capillaries of the blood vasculature, serve as the major cell type in regulation of blood vessel integrity and morphogenesis. Although pericytes are described in different tissues with variable

morphologies, they are mainly defined as vascular mural cells that make focal contact with the ECs and share their basement membrane (BM) (Armulik, Abramsson et al. 2005). Pericytes can be characterized by several markers including neural/glial antigen 2 (NG2), platelet derived growth factor receptor beta (PDGFRβ), anabyl aminopeptidase N (CD13) and desmin (Armulik, Genove et al. 2011). During embryonic development, pericytes in the central nervous system are derived from ectoderm whereas non-CNS pericytes in different organs have distinct origins (Armulik, Genove et al. 2011). Loss of pericytes in the micro vessel wall results in blood brain barrier leakage, severe retinal deterioration, glomerulosclerosis and proteinuria etc. (Lindblom, Gerhardt et al. 2003, Armulik, Genove et al. 2010, Daneman, Zhou et al. 2010).

Besides pericytes, vSMCs are also specialized mural cells recruited mainly to arteries and veins in the blood vasculature and the collecting vessels in the lymphatic vasculature (Figure1). Their contractility enables vSMCs to regulate blood pressure and distribution, however their contractility in the lymphatic vessels are tissue type dependent (Muthuchamy, Gashev et al. 2003). In addition to vessel physiology, vSMCs contribute to the production of extra cellular matrix (ECM) (Rensen, Doevendans et al. 2007). Typically vSMCs are characterized by the expression of smooth muscle actin, CD13, PDGFRβ, desmin etc. although the expression of markers for vSMCs varies depending on developmental stages and environmental cues (Rensen, Doevendans et al. 2007). vSMCs have multiple origins during different developmental stages. In the central nervous system (CNS), a major source of vSMCs, similar to pericytes, is from the neural crest. The mesoderm and mesothelium give rise to the vSMCs at coelomic vasculature. In addition, dorsal aorta contains vSMCs derived from the secondary heart field, neural crest and somite (Armulik, Genove et al. 2011). Alterations in vSMCs morphology and function result in cardiovascular diseases including systemic hypertension, asthma, obstructive bladder disease etc (Owens, Kumar et al. 2004).

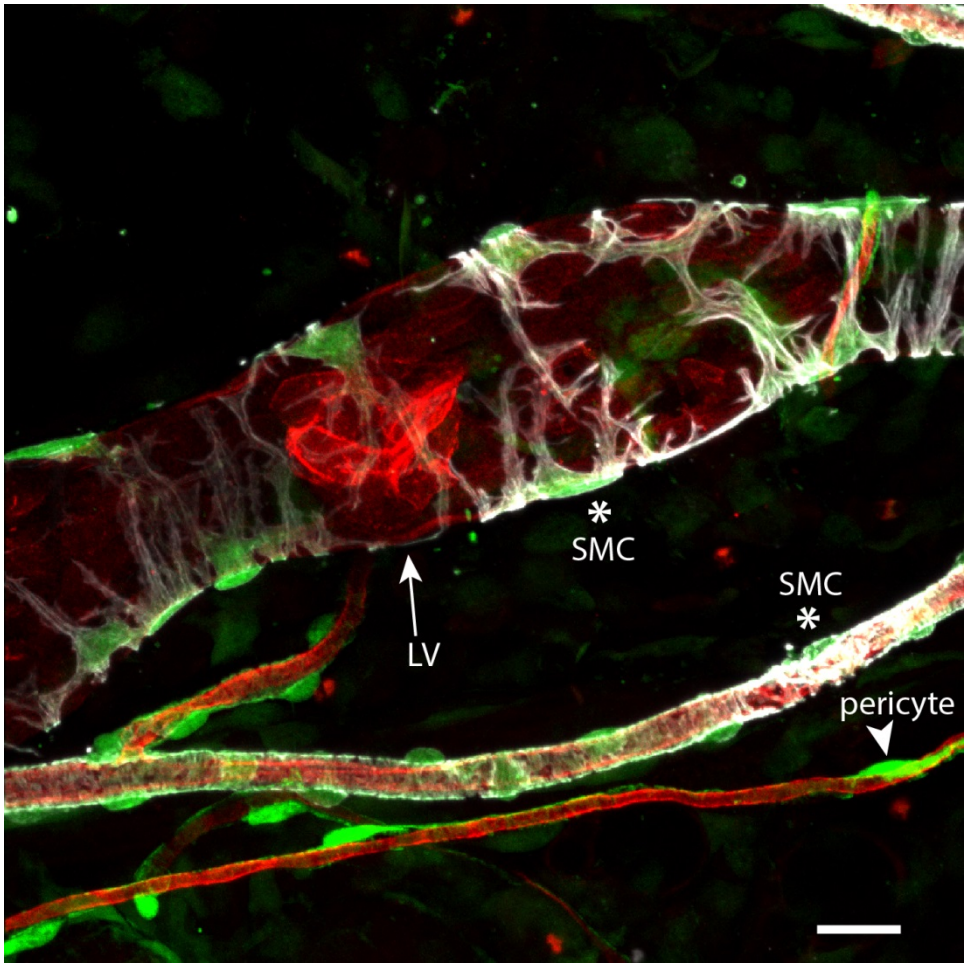

Figure1. PDGFRβ expressing mural cells of blood- and lymphatic vessels. Immunostaining of dermal dorsal ear skin of a *Pdgfrβ-GFP* mouse with antibodies against Podocalyxin (red), α-SMA (grey). PDGFRβ^{+} (GFP, green);α-SMA^{+} (grey) double positive SMCs (asterisk) are found on the lymphatic vessel (identified by the lymphatic valve (LV), arrow) and the arteriole while PDGFRβ^{+};α-SMA^{-} pericytes are located on the blood capillaries. Scale bar indicates 20µm.

1.3.3 PDGFB / PDGFRβ

In the blood vasculature, mural cells are recruited to the vasculature via binding of platelet derived growth factor B (PDGFB) to the cell surface receptor platelet derived growth factor receptor β (PDGFRβ) (Hellstrom, Kalen et al. 1999, Tallquist, French et al. 2003). PDGFRs are important tyrosine kinase receptors that form homodimers –αα, –ββ or heterodimers –αβ on the cell membrane. The interaction of PDGFs and PDGF receptors mediates autophosphorylation of the receptors and activate downstream pathways, typically janus tyrosine kinase (JAK)/ signal transducer and activator of

transcription (STAT), phosphoinositide 3-kinase (PI3K), phosphoinositide phospholipase C-γ (PLC-γ) or mitogen-activated protein kinase (MAPK), which subsequently lead to mural cell migration, proliferation and ECM synthesis (Figure2) (Boor, Ostendorf et al. 2010). The PDGF family is composed of four gene products PDGFA, -B, -C, and –D together forming five combinations of homodimers or heterodimers PDGF-AA, PDGF-BB, PDGF-AB, PDGF-CC and PDGF-DD (Fredriksson, Li et al. 2004). The dimerization of ligands of PDGF receptors provides PDGF signalling with high flexibility in controlling mural cell and EC interaction since binding affinity of ligands to the PDGF receptors vary depending on the forms of dimers e.g. all forms except PDGF-DD activate PDGFRα while PDGFRβ are specifically activated by PDGF-BB and PDGF-DD (Fredriksson, Li et al. 2004).

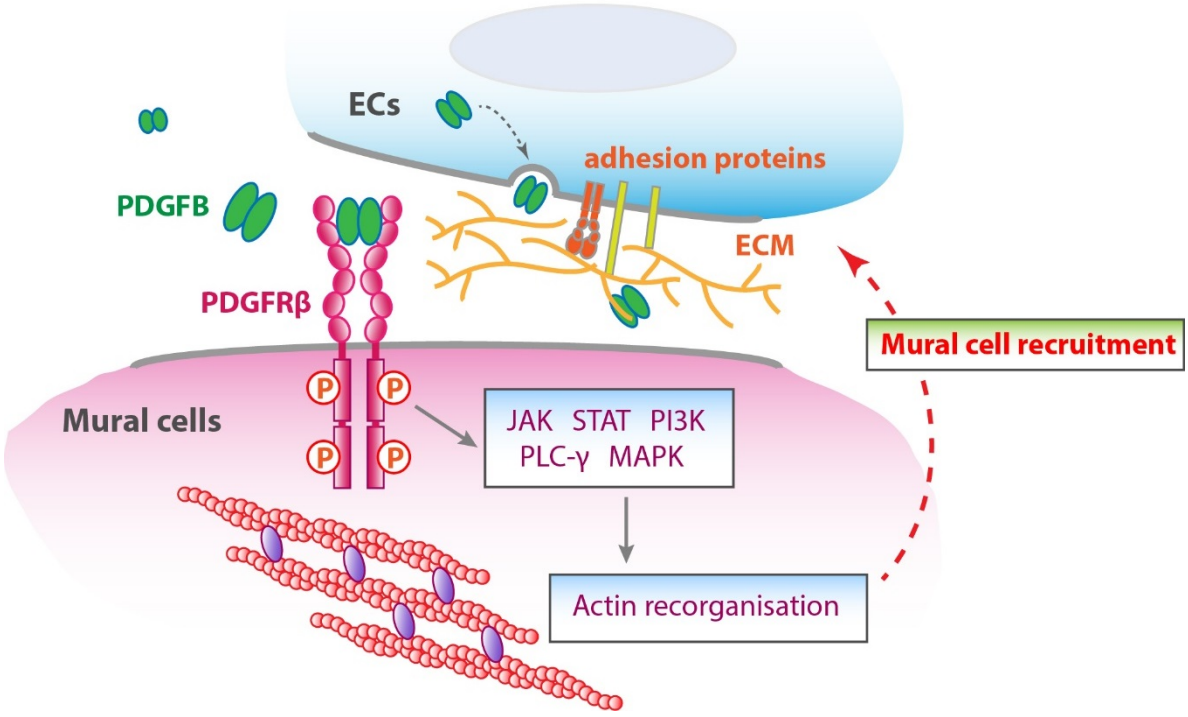

Figure2. An overview of mural cell recruitment mediated by PDGFB/PDGFRβ signalling.

In mammalian tissues, PDGFB is produced by many cells including ECs, megakaryocytes, and neurons (Heldin and Westermark 1999, Andrae, Gallini et al. 2008). PDGFB gene promotor contains several regulatory elements (e.g. Sis proximal

element(SPE)) that bind to multiple transcription factors such as Sp1, Sp3 transcription factors, AP-1-like and Ets like transcription factors etc., suggesting a wide range of regulatory mechanisms (Heldin and Westermark 1999). Regulation of PDGFB secretion is believed to be constitutive whereas the transcriptional regulation of the *PDGFB* gene is affected by growth factors, oxygen tension, thrombin and cytokines (Makela, Alitalo et al. 1987, Abboud 1995, Heldin and Westermark 1999, Fruttiger, Calver et al. 2000, Nishishita and Lin 2004, Andrae, Gallini et al. 2008). Moreover, PDGFB production in cultured ECs was shown to be promoted by fluid shear stress (Mitsumata, Fishel et al. 1993). PDGFB deletion in mouse causes mural cell loss from the vasculature during embryonic development, which leads to severe haemorrhage and perinatal lethality (Leveen, Pekny et al. 1994). Via binding to heparan sulfate proteoglycans (HSPGs), PDGFB is retained to the ECM and this is suggested to be highly involved in mural cell recruitment (Lindblom, Gerhardt et al. 2003). By knocking out the binding motif of PDGFB to heparan sulfate that enables the retention of PDGFB to the basement membrane, pericytes are partially dissociated from the vascular bed, leading to long term vessel defects including increased permeability of mouse blood brain barrier, severe retinal deterioration, glomerulosclerosis, and proteinuria (Lindblom, Gerhardt et al. 2003, Armulik, Genove et al. 2010). Other than the vasculature, e.g. in human dermal fibroblasts, PDGFB was shown to potentiate production of hyaluronan, which is a glycosaminoglycan normally found in soft connective tissues and its accumulation implicates the phase of rapid tissue development during embryonic stage (Laurent and Fraser 1992, Li, Asteriou et al. 2007). These observations highlight the contribution of PDGFB signalling in regulation of endothelial-pericyte interaction and its importance in vascular development and homeostasis.

1.3.4 Angiopoietins / TIE2

The angiopoietin (ANG)/TIE signalling axis is crucial for regulation of angiogenesis and vascular homeostasis. It consists of two tyrosine kinase receptors, TIE1 and TIE2, expressed by the endothelium, and three secreted ligands ANG1, ANG2, and ANG4 (mouse orthologue of human ANG4 is denoted Ang3), among which ANG1 is secreted by various cell types including vascular mural cells and fibroblasts, whereas ANG2 is

primarily produced by ECs (Huang, Bhat et al. 2010). Previous studies suggested that ANG1 serves as a TIE2 agonist while ANG2 normally serves as an ANG1 antagonist however in lymphatic vessels, ANG2 is considered as a TIE2 agonist (Maisonpierre, Suri et al. 1997, Gale, Thurston et al. 2002, Dellinger, Hunter et al. 2008, Huang, Bhat et al. 2010). Deletion of either ANG1 or TIE2 in mice is embryonically lethal (Dumont, Gradwohl et al. 1994, Sato, Tozawa et al. 1995, Suri, Jones et al. 1996). Overexpression of *Ang1* or injection of recombinant ANG1 in mice showed increased blood vessel branching and remodelling, highlighting the role of mural cell-derived ANG1 in regulating vessel development (Thurston, Suri et al. 1999, Uemura, Ogawa et al. 2002, Armulik, Genove et al. 2011). Cardiac specific knockout of ANG1 demonstrated that the failure of vascular development also related to defective flow (Jeansson, Gawlik et al. 2011). However, ANG1 seems not to be involved in pericyte maintenance in the quiescent vasculature by conditional knockout of ANG1 (Armulik, Genove et al. 2011, Jeansson, Gawlik et al. 2011).

Transgenic overexpression of ANG2 reproduces the phenotype of ANG1 knockout mice, highlighting its inhibitory role on ANG1/TIE signalling (Maisonpierre, Suri et al. 1997). *Ang2*-null mice displayed defective remodelling of both the blood vasculature and the lymphatic vasculature. The mice had peripheral lymphedema and hypoplasia, which may be a consequence of prematurely recruited vSMCs, insufficient downregulation of LYVE-1 in collecting vessels or reduced capillary density and branching (Gale, Thurston et al. 2002, Dellinger, Hunter et al. 2008). However, the major defects of lymphatic vessel, but not the blood vessels, could be rescued by the *Ang1* knock-in mice, suggesting a differential role for ANG2 in blood versus lymphatic vessel (Gale, Thurston et al. 2002). By using *Ang2* knockout mice and treatment with anti-ANG2 antibodies, recent studies showed that ANG2 also affects vascular endothelial cadherin (VE-cadherin) phosphorylation, and accordingly impacts button-like junction formation, valve formation, and lymphatic vessel permeability (Zheng, Nurmi et al. 2014). Although the antibody treated mice showed deregulated SMC recruited to lymphatic vessels, PDGFB expression levels were not affected. The mechanisms of regulation of SMC recruitment via ANG2 therefore remain unclear.

1.3.5 Transforming growth factor beta (TGF-β)

Transforming growth factor beta (TGF-β) is expressed by both ECs and mural cells but the activation of TGF-β is suggested to require participation of both cell types (Sato and Rifkin 1989). Via binding to TGF-β receptors e.g. activin receptor-like kinase (ALK)-1 or -5, it promotes proliferation and differentiation of both ECs and mural cells. Activated Alk-5 results in phosphorylation of downstream Sma mothers against decapentaplegic (SMAD) 2/3 and promotes mesenchymal cell differentiating into vSMCs while ALK-1 activation triggers SMAD1/5 that results in cell proliferation and migration (Ota, Fujii et al. 2002, Chen, Kulik et al. 2003, Armulik, Genove et al. 2011). In ECs, ALK-1 and ALK-5 show sophisticated interplay upon activation by TGF-β or bone morphogenetic proteins (BMPs).

TGF-β plays a complex but generally inhibitory role in regulating lymphangiogenesis. LEC specific conditional knockout of TGF-β causes severe defects in lymphatic vessel sprouting and branching in a Neuropilin2 dependent manner and at the same time causes an increase in LEC proliferation during vessel patterning (James, Nalbandian et al. 2013). TGF-β signalling prevents lymphangiogenesis during wound healing in mice and it inhibits LEC migration towards VEGFC *in vitro* (Oka, Iwata et al. 2008, Avraham, Daluvoy et al. 2010). TGF-β also exhibits an anti-inflammatory role as suggested by the fact that TGF-β knockout mice display inflammatory complications with organ failure and death (Shull, Ormsby et al. 1992, Tammela and Alitalo 2010). However, how TGF-β signalling affects mural cell recruitment and differentiation in the lymphatic system is not fully understood.

1.3.6 Neuropilin

Neuropilins (NRP), including NRP1 and NRP2, are widely expressed none-tyrosine kinase transmembrane co-receptors that play multiple roles in both neural and vascular systems (Klagsbrun, Takashima et al. 2002, Fantin, Lampropoulou et al. 2015). As co-receptors of Plexins, NRP1 and NRP2 bind to several classes of semaphorins (SEMA) that are key regulators of neuronal guidance and axon growth (Pellet-Many, Frankel et al. 2008, Fantin, Lampropoulou et al. 2015). In the vascular system, NRP1 and NRP2

are found in both blood and lymphatic vessels, however the expression of NRP1 and NRP2 is enriched at arteries and veins respectively (Stalmans, Ng et al. 2002, Yuan, Moyon et al. 2002, Gu, Rodriguez et al. 2003, Adams and Alitalo 2007, Bouvree, Brunet et al. 2012, Jurisic, Maby-El Hajjami et al. 2012, Zachary 2014). *Nrp1* knockout mice die between E10.5 and E13.5, from severe defects in both blood and nervous systems (Kitsukawa, Shimizu et al. 1997, Kawasaki, Kitsukawa et al. 1999). Although it was suggested that NRP1 interacts with VEGFR2 and enhances receptor phosphorylation and downstream signalling, NRP1 can also function in a VEGFA-independent manner that does not require VEGFR2 activation (Fantin, Herzog et al. 2014, Zachary 2014). Besides its role in VEGF signalling, NRP1 also mediates PDGFR signalling *in vitro*. NRP1 is suggested to physically interact with PDGFB and knockdown of *Nrp1* in aortic SMCs prevents PDGF mediated SMC migration (Banerjee, Sengupta et al. 2006, Pellet-Many, Frankel et al. 2011). In human hepatic stellate cells (hHSC), NRP1 enhances PDGFRβ autophosphorylation on Tyr857 kinase domain and accordingly promotes downstream Rac1 activation (Cao, Yaqoob et al. 2010). However, the functional evidence of this interaction *in vivo* is currently limited. In the lymphatic vasculature, correct interaction of SEMA3a/NRP1 is required during vessel maturation and function. Mice either lacking SEMA3a or exposed to antibody-mediated inhibition of SEMA3a/NRP1 binding, showed defective lymphatic valve formation (Bouvree, Brunet et al. 2012, Jurisic, Maby-El Hajjami et al. 2012). These studies highlighted the diverse role of NRP1 in regulation of both blood and lymphatic vessel development.

Less is known about the function and signalling of NRP2. *Nrp2* knock out mice lacked an obvious vascular phenotype, but displayed reduced number of lymphatic capillaries in the heart and lungs at birth (Yuan, Moyon et al. 2002). Mechanistically this may be explained by the interaction between NRP2 and VEGFC thereby contributing to VEGFR3 internalization and lymphatic vessel sprouting (Xu, Yuan et al. 2010). The relatively modest involvement of NRP2 in lymphangiogenesis compared to that of VEGFR3, points to a partial impact on downstream signalling pathways (Vaahtomeri, Karaman et al. 2017).

1.4 Transcriptional regulation, identities and function of the lymphatic vasculature

1.4.1 PROX1

PROX1 is one of the first transcription factors that distinguishes LEC identity from that of blood EC identity at around E9.5 to E10 in mice and its activation is directly controlled by upstream SOX18 (SRY(sex determining region Y) box 18) (Francois, Caprini et al. 2008). *Prox1* knockout mouse embryos display LEC migrational arrest, failure to further express LEC specific markers e.g. VEGFR3, LYVE-1 and have a blood vessel phenotype (Wigle, Harvey et al. 2002). In addition to determining the LEC fate, PROX1 together with the transcription factor forkhead box protein C2 (FOXC2) contribute to lymphatic valve development by promoting expression of gap junction proteins connexin37 and activation of calcenurin/ nuclear factor of activated T-cells (NFAT) signalling (Sabine, Agalarov et al. 2012). During lymphatic vessel maturation, PROX1 expression is gradually reduced and only maintained at valve regions during postnatal development (Norrmen, Ivanov et al. 2009).

1.4.2 FOXC2

FOXC2 is highly involved in controlling remodelling and maturation of lymphatic vessels. FOXC2, together with PROX1 and VEGFR3 are highly expressed in LECs of developing collecting vessels but are downregulated postnatally, leaving only high expression at lymphatic valve regions (Norrmen, Ivanov et al. 2009). Lymphedema-distichiasis (LD) patients harbour *FOXC2* loss of function mutations. Most of the patients are diagnosed with distichiasis and develop normal or more lymphatic vessels, but with lymph backflow (Brice, Mansour et al. 2002). This mutation was also suggested to be highly associated with defective valve function of superficial and deep veins of the lower limb (Mellor, Brice et al. 2007). *Foxc2* knockout mice appear to have a normal initiation of lymphatic vessels but display lymphatic defects including SMC recruitment to the capillaries, increased overall expression of *Pdgfb* mRNA and capillaries being surrounded with a thick layer of collagen IV, altogether causing abnormal lymphatic drainage (Petrova, Karpanen et al. 2004). FOXC2 was also shown

to physically interact with NFATc1 and to be involved in maintenance of LEC quiescence via stabilization of intercellular junction and cytoskeleton in collaboration with shear stress (Norrmen, Ivanov et al. 2009, Sabine, Bovay et al. 2015). These studies indicate a major role of FOXC2 in defining and maintaining collecting vessel and capillary identity.

1.4.3 Junction proteins and lymphatic vessel identities

To certify resorption of interstitial fluid, lymphatic collecting vessels and capillaries (alternatively denoted initial lymphatic vessels) display unique identities. In capillaries, discontinuous button like junctions enable entry of fluid and cells, mediated by a pressure gradient from interstitium (high) to the capillaries (low) (Schulte-Merker, Sabine et al. 2011). The binding of anchoring filaments within LECs to the molecules of the ECM assures persistent vessel lumen structure even upon increased interstitial tissue pressure (Leak and Burke 1968, Schulte-Merker, Sabine et al. 2011). Lymph (the liquid inside the lymphatic vessel) enters so called precollectors, with mixed identities of capillary and collecting vessel. These vessels in turn connect to the collecting vessels, which are composed of LECs connected by zipper-like cell junctions that prevent liquid exchange between vessels and surrounding tissue. Interestingly, although button-like and zipper like junctions display distinct morphology, both contain the same components, including VE-cadherin, occludin, claudin-5, zonula occludens-1, etc. (Baluk, Fuxe et al. 2007). Moreover, lymphatic capillaries can be distinguished from collecting vessels by their expression of LYVE-1 and chemokine ligand 21 (CCL21). The role of LYVE-1 in capillary function is not yet understood. LYVE-1 deficiency in mice does not cause defects of either lymphatic vessel development or dendritic cell (DC) migration to lymph nodes (Gale, Prevo et al. 2007). CCL21 on the other hand serves as a guidance molecule for DC migration and is secreted and immobilized in interaction with heparan sulfate within the ECM (Weber, Hauschild et al. 2013, Ulvmar and Makinen 2016). This highlights the importance of the lymphatic vasculature for immune surveillance.

1.4.4 Valve development and Basement Membrane proteins of collecting vessels

Lymphatic collecting vessels have bi-leaflet luminal valve structures that prevent lymph backflow of liquid leading to uni-direction flow that is critical for tissue drainage. The development of lymphatic valves of mesenteric collecting vessels is initiated between E15 to E16 through the assembly of a subgroup of LECs expressing high levels of FOXC2 and PROX1, that together with flow control the transcription of connexin37/calceneurin, in turn defining the valve territory (Sabine, Agalarov et al. 2012). During valve development LECs reorient and migrate along the vessel wall, controlled by planar cell polarity (PCP) signalling core proteins cadherin EGF LAG seven-pass G-type receptor 1 (CELSR1), Van Gogh-like (VANGL2) as well as integrin α9, to form the bi-leaflet structure during E17 to E18 (Bazigou, Xie et al. 2009, Tatin, Taddei et al. 2013).

In the blood vasculature, ECM proteins are relatively equally deposited around the arteries, veins and capillaries. However, ECM proteins are predominantly present around collecting vessels with only scattered appearance in capillaries. Several ECM proteins (e.g. collagen IV, laminins, EMILIN1, fibronectin (FN), HPSGs, reelin etc.) are expressed by LECs of valves and collecting vessels. So far, two BM proteins are indicated to be directly involved in signalling that controls lymphatic valve development. The interaction of FN-EIIIA with integrin α9 regulates FN fibril assembly during valve formation (Bazigou, Xie et al. 2009). EMILIN1 also interacts with integrin α9 and lack of EMILIN1 results in reduced valve number and compromised lymphatic function due to defective LEC proliferation and migration (Danussi, Del Bel Belluz et al. 2013). Together, these studies suggest an indispensable role of ECM proteins in activating integrin α9 signalling in control of lymphatic valve formation.

Mechanisms controlling ECM deposition, and its impact on lymphatic endothelial biology, other than valve formation, is less studied. One example of a well understood factor is reelin, an ECM glycoprotein. reelin is suggested to regulate collecting vessel maturation and patterning in an autocrine fashion, where reelin secretion is mediated by direct contact between SMCs and the lymphatic endothelium. Reelin in turn triggers production of monocyte chemotactic protein 1 (MCP-1), a factor that promotes SMC migration and proliferation (Lutter, Xie et al. 2012). The involvement of other ECM components and their functional roles in lymphangiogenesis remain to be clarified.

1.4.5 Lymphatic function, drainage and contribution of SMCs

The lymph vasculature returns 20%-50% of the plasma volume and 50-200% of plasma protein daily from peripheral tissue to circulation (Taylor 1990, von der Weid and Muthuchamy 2010). Muscle contraction and tissue/body movement promote drainage but the relative contribution of collecting vessel SMC contraction is still not clarified. The contractility of SMCs depends on the different combination of muscle specific- and non-muscle specific myosin heavy chains (MHC) and myosin light chains (MLC) that together allow distinct contractile properties (von der Weid and Muthuchamy 2010). For example, thoracic ducts in rats display weaker and more irregular phasic contraction than mesenteric lymphatic vessels (Muthuchamy, Gashev et al. 2003). This is probably due to higher expression level of SMB, a MHC isoform with higher ATPase activity, in the mesenteric vessels than in the thoracic ducts (Eddinger 1998, Babu, Warshaw et al. 2000).

Although the origin of lymphatic SMCs remains unclear, the SMCs contain both smooth and striated muscle contractile proteins and provide pumping forces for lymph drainage (Muthuchamy, Gashev et al. 2003, Schulte-Merker, Sabine et al. 2011). The phasic contraction of SMCs is strictly regulated by oscillation of nitric oxide (NO) production from the endothelium and calcium ion influx into SMCs (Karaki, Ozaki et al. 1997, Kunert, Baish et al. 2015). Opening of the valve and a previous contraction allows lymph flow which increases shear stress and vessel stretching that activates NO production. Accumulation of NO leads to relaxation of the vessel wall and increased vessel diameter, which leads to gradually decreased shear stress. Filling with lymph and local degradation of NO stretches the vessel wall, which results in opening of voltage-, stretch-, ion- activated Ca^{2+} channels in the SMCs and the rapid Ca^{2+} influx leads to SMC contraction to mediate the lymph flow (Wang, Nepiyushchikh et al. 2009, Kunert, Baish et al. 2015). These physiological processes highlight the important functional role of SMCs in lymphatic contraction and drainage. Under inflammatory conditions, vessel contractility might also be affected by external factors e.g. prostanoids, histamine, and serotonin etc.

1.5 An *in vivo* live imaging approach to study angiogenesis

Angiogenesis is a highly dynamic process and studies thereof therefore benefit from live imaging technologies. *In vivo* live imaging provides several challenges in addition to those of *in vitro* live imaging but also allow more relevant analysis of angiogenic processes, due to the preserved microenvironment, growth factors, cellular interactions etc. Current animal models, frequently used for the study of angiogenesis and lymphangiogenesis, usually require genetic modification to introduce expression of endogenous fluorescent reporters. Such models include zebrafish and several tissues in rodents, e.g. cornea, cranium, and skin (Staton, Reed et al. 2009).

Among these imaging models, the mouse cornea has been used as a live imaging site in many studies of developmental biology, tumour biology and drug testing. The cornea is composed of several layers including the epithelium, Bowman's layer, stroma, Dua's layer, Descemet's membrane and the endothelial layer (Navaratnam, Utheim et al. 2015). The cornea is transparent and non-vascularized but vessels can be recruited from the vasculature of the limbus, via angiogenesis. This response can be triggered following various types of stimuli, e.g. micropellet transplantation, suture implantation, cornea transplantation and alkali burn (Jo, Mailhos et al. 2006, Rogers, Birsner et al. 2007, Kilarski, Samolov et al. 2009, Staton, Reed et al. 2009, Yuen, Wu et al. 2011, Kang, Ecoiffier et al. 2016). Injury-induced corneal neovascularization can be categorized into superficial vascularization, vascular pannus, and deep stromal vascularization, depending on the severity and duration of the inflammation (Lee, Wang et al. 1998). Using the mouse cornea as a live imaging site has several obvious advantages. The newly formed vessels are quantifiable and easily treated with drugs. The method can also be applied to different transgenic mouse lines. Some of the methods, e.g. suture implantation, allow for the study of vessel regression, particularly apparent upon suture removal. The overall challenges include reaching single cell resolution, sufficiency and specificity of endogenous reporting constructs, surgical precision, and other technical difficulties to acquire high quality longitudinal images and overcoming breathing motions.

2 AIMS OF THIS THESIS

Mural cell–blood endothelial cell interaction involves PDGFB/PDGFRβ signalling during angiogenesis. SMCs of lymphatic collecting vessels contribute to vessel function however to what extent their recruitment is regulated by PDGFB/PDGFRβ signalling in physiological and pathological conditions is not fully understood. This thesis aims to investigate the mechanism controlling mural cell-EC contact in the vasculature from a developmental as well as technical perspective. The specific aims include:

1. To describe the mechanisms and functional relevance of SMC recruitment to the lymphatic vasculature (Paper I).

2. To develop a method that allows for characterization of dynamic aspects of vascular morphogenesis and function in the living mouse. Utilize this method to describe EC/mural cell behaviour during angiogenesis and vascular remodelling *in vivo* (Paper II).

3. To investigate the interaction of PDGFD and NRP1 and its role in PDGFRβ mediated EC-pericyte interplay (Paper III).

3 RESULTS AND DISCUSSION

3.1 Smooth muscle cell recruitment to lymphatic vessels requires PDGFB and impacts vessel size but not identity (Paper I)

3.1.1 PDGFB/PDGFRβ signalling in the lymphatic vasculature

To characterize the expression of *Pdgfb* in lymphatic endothelium, we used a transgenic mouse line (*Pdgfb-CreER^{T2}-IRES-egfp;R26-mTmG*) expressing an inducible variant of the Cre recombinase under the control of the *Pdgfb* promoter, a *Pdgfb* promoter driven GFP, and the conditional allele for inducible expression of membrane bound GFP under control of the *R26* promoter. We showed that LECs of collecting vessels but not capillaries express *Pdgfb* as indicated by GFP expression. We also showed that perivascular SMCs express *Pdgfrb*. This was evident by the presence of GFP in transgenic mice carrying a *Pdgfrb-GFP* construct. Together, these data suggest that PDGFB/PDGFRβ signalling is involved in spatial recruitment of SMCs to the collecting lymphatic vessels.

3.1.2 LEC specific deletion of PDGFB causes defective SMC recruitment and vessel dilation of the lymphatic vasculature

To further examine the role of PDGFB in regulation of SMC recruitment to the lymphatic vessels, we generated the mouse line *Prox1-CreER^{T2};Pdgfbflox/flox;R26R-eYFP* (herein denoted *Pdgfb^{iLECKO}*) that allows for inducible LEC specific deletion of PDGFB in the lymphatic endothelium, together with YFP expression indicating Cre-mediated recombination. In dermal ear vasculature, we showed that LEC specific deletion of PDGFB (induced P4-P7, analysed at P21) causes severe reduction of collecting vessels covered by perivascular SMC in the *Pdgfb^{iLECKO}* (1.5%) versus the control (44.2%) and enlarged vessel diameter. Similar observation was also found in large diameter lymphatic vessels in the mesentery with increased vessel diameter and reduced SMC coverage when inducing PDGFB deletion before initial SMC recruitment. Interestingly, although deletion of PDGFB caused reduced SMC coverage in the hind limb, it did not affect vessel diameter, suggesting tissue specific regulation of collecting vessel diameter. To investigate whether PDGFB is required also for the maintenance of SMC coverage and if

SMC coverage in turn affects vessel morphology, we deleted PDGFB after initial SMCs had been recruited to the hind limb vessels (at P1 and P2). Also in this case SMC coverage of collecting vessels were reduced but without altered diameter, suggesting at least a partial role for PDGFB in maintenance of SMC coverage. The data in Paper I provide direct genetic evidence for the requirement of LEC-derived PDGFB in recruitment of SMCs to collecting vessels, in turn restricting their diameter.

3.1.3 SMCs are required for collecting vessel contraction but do not affect main capillary or collecting vessel identities

To further understand the involvement of SMCs in establishment of vessel identities, we examined the dermal ear lymphatic endothelium in absence of SMCs in *Pdgfb*^{iLECKO} mice. We showed that deposition of BM proteins, including collagen IV and laminins, was unchanged comparing the *Pdgfb*^{iLECKO} and control. Collecting vessels of *Pdgfb*^{iLECKO} mice also displayed normal valve formation. Furthermore, the expression level of VEGFR3, VE-cadherin, LYVE-1, CCL21 and ECM proteins were not changed in the *Pdgfb*^{iLECKO} compared to control. However, the LECs of *Pdgfb*^{iLECKO} mice were larger than those of control. Several studies have reported a link between SMC recruitment and LYVE-1 downregulation, however without ruling out possible systemic or secondary effects (Petrova, Karpanen et al. 2004, Dellinger, Hunter et al. 2008, Lutter, Xie et al. 2012, Meinecke, Nagy et al. 2012). In Paper I we show that SMCs are not directly involved in establishing collecting vessel- capillary hierarchy and that the contribution by perivascular SMCs to the BM is only minor. Previous studies have shown that perivascular SMCs assist vessel contraction in large diameter vessels such as popliteal and mesenteric vessels, and that this contraction promotes function. The contractility of dermal collecting vessels and the contribution of SMCs to their function were less understood. By subcutaneous injection of fluorescent tracers that perfuse the lymphatic vasculature, followed by live imaging, we revealed that dermal collecting vessels contract with variable amplitude and frequency. *Pdgfb*^{iLECKO} mice showed defective contraction of the ear skin vasculature highlighting the contribution of perivascular SMCs to lymphatic vessel function.

3.1.4 LEC-specific overexpression of PDGFB is not sufficient for ectopic SMC investment to the capillaries

Aberrant SMC recruitment to the lymphatic capillaries was previously linked to upregulation of PDGFB and considered one of the causes of lymphedema (Petrova, Karpanen et al. 2004, Meinecke, Nagy et al. 2012). To examine whether PDGFB is sufficient to drive SMC recruitment to the lymphatic vessels, we generated the mouse line *Prox1CreER^{T2};R26-hPDGFB* that allows for inducible LEC specific overexpression of human PDGFB. Surprisingly, we found no PDGFRβ+, αSMA+ mural cells recruited to the capillaries besides the increased SMC coverage at collecting vessels upon Cre induced *hPDGFB* overexpression. Our observation therefore suggests that other mechanisms, in addition to differential regulation of *PDGFB* expression, are involved in mediating ectopic SMC recruitment to the capillaries under pathological conditions.

3.1.5 Recruitment of SMCs to collecting vessels is promoted by binding of PDGFB to ECM proteins

In blood vessels, perivascular mural cell recruitment relies on the presence of PDGFB and its binding to heparan sulfate chains of ECM molecules. This was demonstrated by gene targeting of the heparan sulfate binding motif within PDGFB in mice (denoted *Pdgfb $^{ret/ret}$* mice) causing defective pericyte investment to the vasculature, increased permeability in the CNS, severe retinal deterioration, and proteinuria (Lindblom, Gerhardt et al. 2003, Armulik, Genove et al. 2010). We therefore looked into whether correct retention of PDGFB to the ECM proteins was required for SMC recruitment to lymphatic vessels. Indeed, *Pdgfb $^{ret/ret}$* mice at 10 weeks of age displayed decreased SMC investment to the collecting vessels and enlarged vessel diameter, suggesting a requirement for ECM retention of PDGFB for SMC recruitment to the lymphatic vasculature. Further analyses of ECM composition in collecting vessels and capillaries, showed a more abundant deposition of collagen IV, laminins, and perlecan in the endothelium of collecting vessels compared to capillaries. Interestingly, it has been demonstrated that domain III-2 of perlecan can directly bind PDGFB but not PDGFRβ *in vitro*. This suggests that perlecan may act to retain PDGFB in the ECM (Gohring, Sasaki et al. 1998). Taken together, our data in Paper I suggest that binding of PDGFB to ECM proteins serve to restrict SMC recruitment to collecting vessels and not capillaries (Figure3).

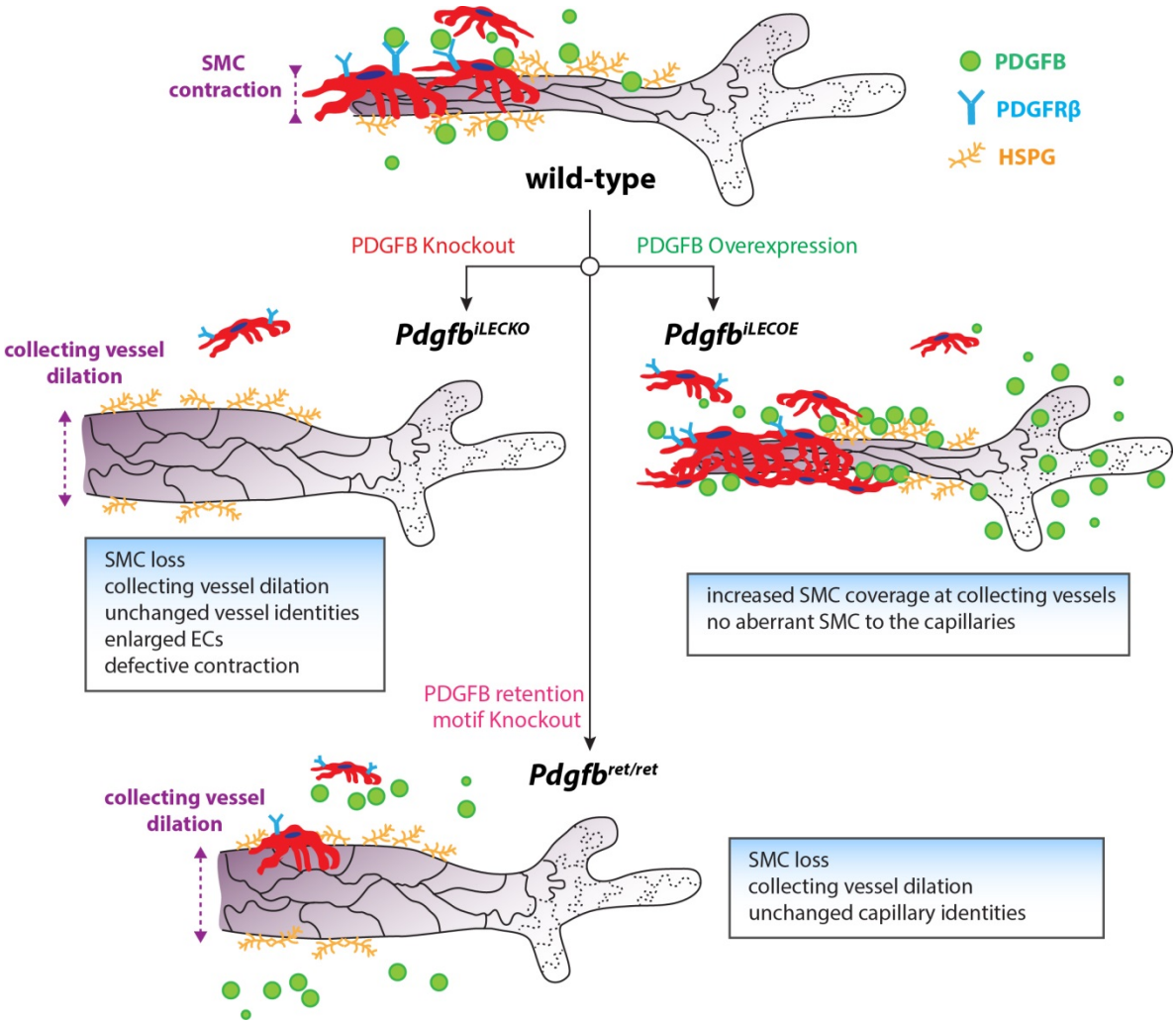

Figure3. An overview of PDGFB mediated EC/SMC interaction within lymphatic vessels in wild-type and three genetic modified mouse models.

3.2 Characterization of multi-cellular dynamics of angiogenesis and vascular remodelling by intravital imaging of the wounded mouse cornea (Paper II)

3.2.1 Establishment of an in vivo live imaging model to assess vascular morphogenesis.

As the only non-vascularized tissue, the cornea provides a unique site for *in vivo* live imaging with several benefits, including high transparency and ease of access, both for the microscope objective and for local application of drugs. Upon surgical silk knot implantation, as previously described, vessels start to sprout from the limbus towards the suture location (Kilarski, Samolov et al. 2009, Yuen, Wu et al. 2011). We first tested the *in vivo* live imaging approach on a reporter mouse line, *Cdh5(PAC)CreER^{T2};R26R-eYFP*, that allows for inducible EC-specific YFP expression. Delivery of Tamoxifen (20mg/ml) by gavage one week before suture implantation enables YFP expression in almost all ECs in the neovasculature of the cornea. This can also be achieved by direct application of 5µl 4-hydroxytamoxifen (4-OHT) (20mg/ml) to the cornea during suture implantation, which provides a local Cre-driven recombination that limits systemic consequences of induced gene deletion. By using a 25x water immersion objective, the same vascular bed within an area of 0.98mm x 0.73mm could be recorded with Z stacks daily under short anaesthesia (1 hour) for up to 5 days. To examine rapid processes such as filopodia dynamics, mice were anaesthetized for up to 6 hours allowing for continuous recording of Z stacks.

3.2.2 Analysis of EC migratory behaviour in perfused vessels at single cell resolution.

To investigate the migratory behaviours of ECs in lumenized, blood perfused vessels, we used double transgenic mice (herein denoted *Claudin5-GFP;Ng2DsRED*) with EC-specific expression of GFP and pericyte-specific expression of DsRED. The condensed GFP localization in the EC soma allows for recognition of individual ECs in the vessel wall of lumenized vessels. By combining epi-fluorescence and bright field imaging, EC migration in relation to blood flow direction could be identified and analysed. We found that 67.3% of ECs within lumenized vessels did not move. However although 32.7% of the cells did change location 13.7% demonstrated clear migration while 19.0% were unmeasurable due to proliferation, overlap with neighbouring cells or apoptosis. When analysing EC migration in relation to blood flow, 84.6% out of the migrating ECs migrated against flow direction compared to 15.4% ECs migrating with flow.

Interestingly, further characterizing the migration speed we found that out of the ECs migrating with the flow, they did so faster in venules than in arterioles. These observations suggest vessel specific EC behaviour, at least in this model of inflammation induced angiogenesis

Vessel pruning is a critical step during vascular remodelling. Benefitting from the single cell level resolution in this model, we studied vessel pruning from day 6 to day 8 post suture implantation in *Cdh5(PAC)-CreER^{T2};R26R-eYFP* mice that also received a fluorescent tracer administered via the tail vein. The observed EC migratory behaviours at the pruning sites reminded of those previously described in zebra fish (Lenard, Daetwyler et al. 2015). Altogether, these observations in Paper II highlight the potential of this model to study details of vessel morphogenesis *in vivo*.

3.2.3 Mural cells of sprouting vessels of the sutured mouse cornea.

To examine the morphology of angiogenic vessels in the wounded mouse cornea, we used *Cdh5(PAC)CreER^{T2};R26R-eYFP;Ng2DsRED* mice to simultaneously visualize the endothelium and its perivascular mural cells. Similar to the NG2+ pericyte coverage described previously in other tissues in development (Murfee, Skalak et al. 2005, Murfee, Rehorn et al. 2006), we found a more abundant NG2+ pericyte recruitment to arterioles than to venules in the wounded mouse cornea, indicated by live imaging and immunofluorescence staining. We also confirmed that pericytes expressed PDGFRβ by utilizing *Pdgfrβ-GFP* mice. Furthermore, by using the *Claudin5-GFP;Ng2DsRED* mice, we recorded vascular expansion from day 6 to day 9 post suture implantation and revealed an intensive pericyte coverage in the established capillary network compared to the sprouting tips that were devoid of pericytes. Interestingly, fluorescent tracer injection into the tail vein indicated that sprouting vessel tips lumenize prior to pericyte coverage. Together, these observations expose that mural cell recruitment is relatively conserved, comparing inflammation-induced vessel morphogenesis and developmental vascular morphogenesis.

3.2.4 Permeability of the remodelling vasculature.

To investigate permeability of the newly formed vasculature in the wounded mouse cornea, we injected fluorescent tracer into the tail vein, followed by direct application of 300ng VEGFA (100ng/µl) or PBS as control to the mouse cornea at post suture

implantation day 8. The vasculature was then continuously imaged with high resolution Z stacks every 5 minutes. Live imaging revealed tracer leakage from vessels initiating at 15 minutes post VEGFA application with progression to severe multifocal leakage at 50 minutes. Interestingly, measurement of intravascular tracer intensity indicated a more severe leakage at the venules compared to the arterioles. These data highlight a vessel type specific permeability of newly formed vascular beds and the potential of this method in providing a spatial temporal resolution of vessel permeability.

3.3 Neuropilin 1 binds PDGFD and may function as co-receptor in PDGFD–PDGFRβ signaling (Paper III)

3.3.1 Distinct binding of PDGFD to NRP1 requires its C-terminal Arg^{370} residue and is assisted by heparin.

The interaction of NRP1 to PDGFs was previously reported however the specific binding site was not known (Banerjee, Sengupta et al. 2006, Ball, Bayley et al. 2010, Cao, Yaqoob et al. 2010). Herein by alignment the amino acid sequence, we revealed a common C-terminal arginine residue of PDGFD that is shared by $VEGFA_{121}$, $VEGFA_{165}$ and VEGFE but not by PDGFB. This motif of VEGF isoforms was previously reported to be involved in binding to NRP1 (Cebe-Suarez, Grunewald et al. 2008, Delcombel, Janssen et al. 2013). Furthermore, we mapped the binding site of NRP1 to PDGFD at b1 and b2 domain, and showed the dosage dependent binding of NRP1 to PDGFD, which is similar to the binding of NRP1 to VEGFs. On the other hand PDGFB did not bind NRP1. By analysis of the binding abilities of recombinant NRP1 with the supernatant from fibroblast cells overexpressing mutated PDGFD (lacking the C-terminal), we confirmed the requirement of the C-terminal residue on PDGFD in its binding to NRP1. Together, these results suggested a distinct role of PDGFD comparing to PDGFB in the interaction with NRP1, mediated by its C-terminal arginine residue. Previously heparin or HSPGs were reported to be involved as a co-factor for the binding of VEGFA to NRP1 (Mamluk, Gechtman et al. 2002). Here we show that heparin also enhances the binding of VEGF-A_{165} and VEGF-A_{189} to NRP1 as well as the PDGFD core protein (the activated form) but not full length PDGFD or PDGFB, suggesting the requirement of protein cleavage to reveal the heparin binding site for activation of PDGFD.

3.3.2 Co-localisation of NRP1 with PDGFRβ signalling is mediated by C-terminal Arg^{370} of PDGFD.

To investigate whether PDGFD alters in PDGFRβ downstream signalling in a different manner in comparison to PDGFB, we stimulate fibroblasts with PDGFB and PDGFD respectively. Immunofluorescence of fibroblast cells showed PDGFRβ internalization by both growth factors. However the co-localization of PDGFRβ with NRP1 as well as NRP1 internalization were only found in PDGFD but not PDGFB treated cells. We further confirmed the requirement of PDGFD in mediating NRP1-PDGFRβ interaction

via co-immunoprecipitation, although PDGFD and PDGFB showed no difference in PDGFRβ phosphorylation as well as downstream signalling. Together, our results suggest that NRP1 can be involved in PDGFD-PDGFRβ signalling.

We then investigated whether the interaction of NRP1 with PDGFRβ requires C-terminal Arg^{370} of PDGFD by comparing the PDGFRβ-NRP1 co-clustering in fibroblast cells upon treatment with mutate PDGFD or wildtype PDGFD (supernatant of PDGFD overexpressing fibroblast cells). Our results showed the requirement of C-terminal Arg^{370} of PDGFD in mediating NRP1-PDGFRβ co-localisation. However siRNA knockdown of either *PDGFRB* or *NRP1* in fibroblasts suggested that although PDGFRβ is required for NRP1 internalisation upon interaction with PDGFD, the activation of PDGFRβ was independent of NRP1.

3.3.3 PDGFD mediated NRP1 translocation modulates VEGFA-VEGFR2 signalling.

To investigate the involvement of PDGFD mediated NRP1 activation in modulating VEGFR2 signalling, we stimulate human umbilical vein endothelial cells (HUVECs) with PDGFB or PDGFD to interfere NRP1-VEGFR2 interaction in the presence of VEGFA. We showed that PDGFD but not PDGFB inhibits NRP1-VEGFR2 co-clustering and translocate NRP1 to EC junctions, comparing to VEGFA that only mediates NRP1 internalization. Other studies suggested the importance of the availability of NRP1 pool in regulation of VEGFR2 or TGF-β signalling (Koch 2012, Aspalter, Gordon et al. 2015, Kofler and Simons 2016). Our data in the same line suggests a direct role of PDGFD in modulating VEGFR2 signalling via interfering NRP1distribution.

3.3.4 PDGFD enhances pericyte-EC interaction via assisting intercellular interaction of NRP1 and PDGFRβ.

To understand the functional role of PDGFD in regulating PDGFRβ signalling, we performed *ex vivo* sprouting assay using mouse embryo explant or aortic ring cultures containing endogenous reporters for EC (GFP) and pericytes (DsRed) respectively. We found that although both PDGFB and PDGFD stimulation increased vessel sprouting speed as well as pericytes detachment, PDGFD retains a higher number of pericytes at the endothelium comparing to PDGFB. Interestingly, by measuring the migration speed of pericytes that retained or detached to the endothelium, we found an increased migration speed of pericytes when detaching from the sprouts than those retained at the sprouts

upon stimulation of PDGFD. However PDGFB stimulation showed similar migration speed of pericytes in both situations. These observations suggest a potential transcellular activation of PDGFRβ signalling of pericytes via NRP1 expressesd by neighbouring ECs in presence of PDGFD but not PDGFB.

To further dissect the mechanisms of differential effect of pericytes-EC interaction upon PDGFD and PDGFB, we performed co-culture of ECs and an EC cell line (porcine aortic EC (PAE)) that overexpresses PDGFRβ in stimulation of PDGFB or PDGFD, followed by NRP1 immunoprecipitation. We showed that PDGFD but not PDGFB enables co-immunoprecipitation of PDGFRβ with NRP1 although both growth factors activate PDGFRβ. Importantly this is a process that requires participation of NRP1 since the co-immunoprecipitation of NRP1 and PDGFRβ were only observed in co-cultured PDGFRβ-expressing cells and ECS but not in pericytes culture alone. We also showed co-localisation of NRP1 and PDGFRβ via immunofluorescence staining in HUVEC/human pericytes co-culture upon PDGFD stimulation. These observations point to a different signal transduction of NRP1 mediated PDGFD-PDGFRβ in the context of pericyte-EC interaction in comparison to PDGFB-PDGFRβ signalling.

Taken together, our study in Paper III reveals the direct binding of NRP1 to PDGFD that modulates the distribution and availability of NRP1 possibly in mediating other signalling pathways where NRP1 is involved. We also demonstrat the role of NRP1 as a co-receptor that mediates PDGFD-PDGFRβ signalling that is involved in intercellular communication between endothelial cells and pericytes.

4 FUTURE PERSPECTIVES

Compromised regulation of endothelial – mural cell interaction is evident in many diseases. The present thesis extended the understanding of endothelial – mural cell interaction in the context of both blood and lymphatic vasculature, with a specific focus on the role of PDGF/PDGFRβ signalling. It opens up new directions and technical possibilities to explore the regulatory mechanisms of vascular development and function.

The results from Paper I revealed the regulatory role of PDGFB in mediating smooth muscle cell recruitment to certain part of lymphatic vessels. Interestingly, we also observed that PDGFB-mediated smooth muscle cell recruitment was tissue type dependent. Considering that the perivascular muscle cells are composed of different sub types, it would be of great interest to investigate the heterogeneity of perivascular muscle cells in the lymphatic system via screening of muscle specific marker expression via immunofluorescence, or single cell sequencing in different tissues. This would provide essential contributions to understand the initiation and progression of lymphedema, displaying pathological thickening of lymph vessel walls, in turn potentially linked to altered perivascular muscle cell populations (Ogata, Fujiu et al. 2015).

To understand the requirement of PDGFB in recruitment of SMCs to the lymphatic endothelium, we generated the mouse model (*Pdgfb*^{iLECKO}) that permits inducible LEC specific deletion of PDGFB. We did not observe any obvious phenotype of lymphedema in these mice even under severe loss of SMCs and enlarged vessel diameter. However we only kept the mice until one month's age, while the development of lymphedema in humans may take years even after acute condition e.g. surgery or radio therapy etc. It is therefore of interest to stretch the endpoint of analysis to evaluate the lymphatic function in this model. In that case the altered morphogenesis of collecting vessels might challenge the plasticity of the lymphatic system. Furthermore, it would be beneficial to characterize the long-term impact of loss of SMCs on LEC biology since perivascular SMCs not only serve as motors for contraction but also provide signalling cues to the endothelium.

Paper II of the thesis described an *in vivo* live imaging approach using the wounded mouse cornea to study vascular development and EC behaviour. The unique features of this method allows longitudinal documentation of vessel patterning, detailed analysis of EC migration, quantified at individual cell level and quantitative measurement of vessel

permeability at spatial temporal resolution. In our recently published study, this method was used to investigate directional migration of BECs lacking endoglin and revealed their defective directional migration against blood flow. Since we also showed the possibilities to induce local Cre-mediated recombination, this tool would be beneficial for studies using mice with conditional gene deletion, to minimize systemic effect.

In Paper III we showed that PDGFD could mediate intercellular interaction of NRP1 and PDGFRβ that in turn could assist pericyte-EC communication during sprouting angiogenesis. Although PDGFD is not generally required for mural cell recruitment the degree of its pathophysiological contribution is not fully clarified. Considering the previously reported disorganized NG2+ pericytes in the vasculature of the developing heart in *Pdgfd*-/- mice, it will be interesting to perform similar *ex vivo* sprouting assays (Paper III, figure 6) using antibodies blocking PDGFD in mice and evaluate the impact on pericyte and EC communication.

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