Role of the VEGFC/VEGFR3 pathway, beyond developmental lymphangiogenesis

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ACADEMIC DISSERTATION

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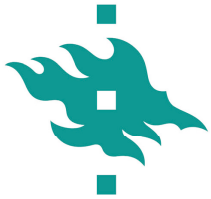

To be publicly discussed, with the permission of the Faculty of Medicine, University of Helsinki, in Haartman Institute, Lecture Hall 1, Haartmaninkatu 3, Helsinki, on May 26, 2017, at 12 noon.

Helsinki 2017

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ISBN 978-951-51-3158-4 (paperback)
ISBN 978-951-51-3159-1 (PDF)
ISSN 2342-3161 (paperback)
ISSN 2342-317X (PDF)
Unigrafia Helsinki 2017

To Eeva

It ain't about how hard you hit.
It's about how hard you can get
hit and keep moving forward
–Rocky Balboa

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ABBREVIATIONS

ADAMTS3	A disintegrin and metalloproteinase with thrombospondin motifs 3
Ang	Angiopoietin
BEC	Blood vascular endothelial cell
BMP	Bone morphogenic protein
CCBE1	Collagen and calcium binding EGF domains1
CCL21	Chemokine (C-C motif) ligand 21
CLEC-2	C-type lectin receptor 2
COUP-TFII	Chicken Ovalbumin Upstream Promoter transcription factor 2
CVDs	Cardiovascular diseases
Dll4	Delta like ligand 4
E day	Embryonic day
EMPs	Erythroid-myeloid progenitors
FGF	Fibroblast growth factor
Foxc2	Forkhead box protein C2
HEV	High endothelial venule
Hey	Hairy/enhancer-of-split related to YRPW motif protein
HFD	High fat diet
IAL	Inflammation associated lymphangiogenesis
LDL	Low-density lipoprotein
LEC	Lymphatic endothelial cell
Lyve1	Lymphatic vessel endothelial Hyaluronan receptor 1
Notch	Notch homolog 1, translocation-associated (Drosophila)
Nrarp	NOTCH-regulated ankyrin repeat protein
Nrp	Neuropilin
P Day	Postnatal day
PlGF	Placenta growth factor
Prox1	Prospero homeobox 1
RCT	Reverse cholesterol transport
Sox18	SRY-Box 18
TGF-β	Transforming growth factor-beta
TIE	Tyrosine kinase Ig and EGF homology domains
VEGF	Vascular endothelial growth factor

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on these original publications, referred to in the text by their Roman numerals:

I Tammela T*, Zarkada G*, **Nurmi H**, Jakobsson L, Heinolainen K, Tvorogov D, Zheng W, Franco CA, Murtomäki A, Aranda E, Miura N, Ylä-Herttuala S, Fruttiger M, Mäkinen T, Eichmann A, Pollard JW, Gerhardt H and Alitalo K. VEGFR-3 controls tip to stalk conversion at vessel fusion sites by reinforcing Notch signalling.
Nature Cell Biology 13(10):1202-13 (2011)

II Vuorio T, **Nurmi H**, Moulton K, Kurkipuro J, Robciuc MR, Ohman M, Heinonen SE, Samaranayake H, Heikura T, Alitalo K and Ylä-Herttuala S. Lymphatic vessel insufficiency in hypercholesterolemic mice alters lipoprotein levels and promotes atherogenesis.
Arteriosclerosis Thrombosis Vascular Biology 34(6):1162-70. (2014)

III **Nurmi H**, Saharinen P, Zarkada G, Zheng W, Robciuc MR* and Alitalo K*. VEGF-C is required for intestinal lymphatic vessel maintenance and lipid absorption.
EMBO Molecular Medicine 7(11):1418-25. (2015)

IV Fang S*, **Nurmi H***, Heinolainen K, Chen S, Salminen E, Saharinen P, Mikkola H and Alitalo K Critical requirement of VEGF-C in transition to fetal erythropoiesis
Blood 128(5):710-20 (2016)

* These authors contributed equally to the study

Publication I was included in the thesis of Georgia Zarkada (University of Helsinki)

Publication II will be included in the thesis of Taina Vuorio (University of Eastern Finland)

ABSTRACT

There are two vascular systems in the body, for blood and lymph, and both are indispensable for embryonic development. The closed circuit of blood vessels is responsible for oxygen and nutrient delivery to all compartments of the body and removal of CO_2 and waste products from tissues. The open-ended lymphatic system works as a return route for fluid, immune cells and dietary lipids into the blood circulation. The heart is the central engine for blood flow whereas lymphatic flow is achieved by skeletal and smooth muscle contraction around the lymphatic vessels. Differences between the blood vascular and lymphatic systems are apparent, from the molecular to the functional level. However, the two types of vessels also share a number of signaling molecules and building blocks of vessels.

The purpose of the studies presented in this thesis was to expand our knowledge about Vascular Endothelial Growth Factors (VEGFs) and their receptors in development and pathological conditions. In the first study, we found, surprisingly, that VEGFR-3, the receptor for the principal lymphatic vessel growth factor, VEGF-C, is crucial for the normal patterning of the developing blood vessels and that endothelial deletion of *Vegfr3*, but not VEGFR-3-blocking antibodies, leads to excessive blood vessel sprouting and branching. Furthermore, macrophages that express the VEGFR-3 and VEGFR-2 ligand VEGF-C localized to the vessel branchpoints, and *Vegfc* heterozygous mice exhibited inefficient angiogenesis characterized by decreased vascular branching. Our second study focused on the pathological aspect of the atherosclerosis. By using the transgenic mouse that overexpress soluble VEGFR-3 (VEGF-C/D trap) we could link the impaired lymphatic vessels to lipoprotein metabolism, increased plasma cholesterol levels, and enhanced atherogenesis. In the third project we focused on the role of lymphangiogenic growth factors in the steady-state homeostasis of lymphatic vessels in adult mice. Our findings from this study indicated that the lymphangiogenic growth factors provide trophic and dynamic regulation of the intestinal lymphatic vasculature, which could be especially important in the dietary regulation of adiposity and cholesterol metabolism. Finally in the last study we discovered a new role of VEGF-C in the embryonic development. To our surprise, we found a striking VEGF-C dependent phenotype with defective fetal liver erythropoiesis that resulted severe anemia in the *Vegfc* deteled embryos.

These studies have revealed a new viewpoint regarding the VEGF-C/VEGFR-3 pathway in embryonic and pathological conditions. With these results, our understanding of the VEGF family members has expanded beyond the blood and lymphatic vessel. Hopefully, this new knowledge will improve the possibilities to target the VEGF-C/VEGFR-3 pathway for the treatment of human diseases such as atherosclerosis, obesity, and vascular diseases.

REVIEW OF THE LITERATURE

Introduction

Vascular endothelial growth factors (VEGFs) and their receptors are the main regulators of endothelial cell growth, proliferation and migration leading to vessel growth. In general terms, the VEGF/VEGFR-2 pathway is crucial for blood vessel growth *(angiogenesis)* during embryonic development, whereas lymphatic vessel growth is dependent of VEGF-C/VEGFR-3 signaling. Studies using transgenic mouse models during the past three decades have revealed that these signaling molecules are required for normal vascular development. As an example, deletion of VEGF-C causes embryonic lethality around embryonic day (E) 16.5 and detailed inspection of these embryos revealed that they lack lymphatic vessels (Karkkainen et al., 2004). However, constitutive deletion of *Vegfr3*, the main VEGF-C receptor for lymphangiogenesis, arrested cardiovascular development at E9.5, leading to embryonic lethality even before the beginning of differentiation of the first lymphatic endothelial cells (Dumont et al., 1998). Thus VEGFR-3 has a dual role in blood and lymphatic vasculatures.

While the vascular developmental aspects of these key players have been confirmed in groundbreaking studies using knockout mice, the roles of these same molecules have been studied in several pathological conditions. In cancer and lymphedema, our knowledge of the molecular interactions required for new vessel growth has increased rapidly. The aim of the studies presented in this thesis has been to investigate more thoroughly the other roles that the VEGF-C/VEGFR-3 pathway has during development and in pathological conditions. Studies presented here indicate embryonic function of VEGF-C/VEGFR-3 beyond the lymphatic vessel regulation. In adult mice, our purpose was to clarify how these molecules regulate homeostasis under quiescent and challenged or pathological conditions. Mainly, all of the studies have been done using transgenic mouse models, which provide a way of validating the physiological and pathological interactions related to human diseases.

Development of vascular networks

The role of the blood circulatory system is to carry oxygen, nutrients, different types of blood cells and signaling molecules, such as hormones, to the cells and remove carbon dioxide and waste products from the peripheral tissues. This constant flow is based on the pumping mechanism of the heart, which together with other mechanisms like smooth muscle cell contractions in the arteries keeps blood flow and pressure at a certain level. Main components of the blood are erythrocytes, which are responsible for gas exchange and various types of leukocytes, including the immune cells of the body. The acellular part of the blood is called plasma, which can be described as a protein-rich fluid. The blood vessel network can be divided by the size of the vessels to large arteries, arterioles, capillaries, venules and veins. Altogether the length of the blood vessel system is 100 000 kilometers and the area covered by the vessel endothelium is about half of an ice hockey rink All tissues in the body, except the cornea and the cartilage are vascularized (Cines et al., 1998).

Lymphatic vasculature differs from blood vasculature in a few important aspects (Figure 1). First, lymphatic vasculature participates in the maintenance of the fluid balance in the body, in immune cell trafficking and in dietary lipid absorption from the gut. Second the lymphatic vessel network is not closed like the blood circulatory system. Lymphatic capillaries are blind-ended; they can be found in most of the tissues. Third, flow in lymphatic vessels is not driven by a particular organ, unlike in the blood circulatory system. Instead, it is controlled by the pulsatile activity of the lymphatic collector vessels, which contain valves to prevent lymph backflow and which are responsible for returning the lymph back to blood circulation (Bazigou and Makinen, 2013). At the cellular level blood endothelial cells (BECs) differ from lymphatic endothelial cells (LECs) by their molecular signature and by their interactions with the environment (Lohela et al., 2009).

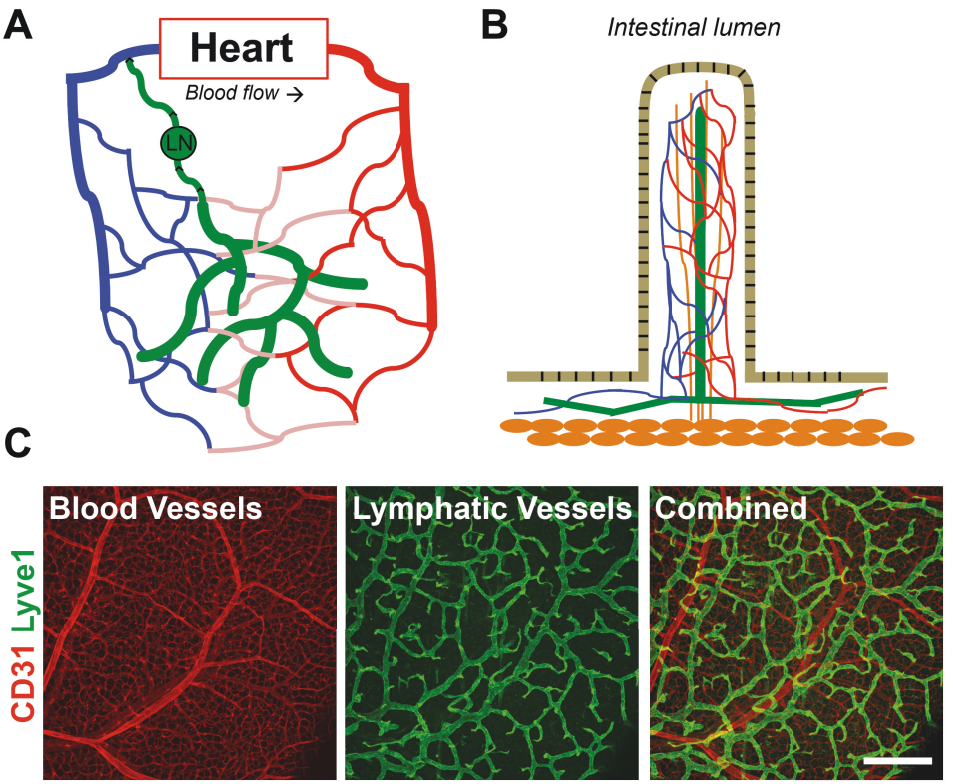

Figure 1. Differences between blood and lymphatic vessels. A) Simplified schematic of fundamental differences between blood and lymphatic vessels. Blood vessels are a closed system with constant pumping from the heart, and lymphatic vessels are blind-ended, unidirectional vessels with valve structures in the collector vessels. B) Blind-end lymphatic vessels in the gut known as lacteals are responsible for dietary lipid absorption from the intestinal lumen. Blood vessels are presented here in red and blue, and lymphatic vessels in green. An important cell type responsible for barrier function in the intestine, the enterocytes, is shown in brown and smooth muscle cells in orange. C) Immunofluorescence images from the intestinal wall of mice. Scale bar 400 μm.

Blood vascular system

In mouse embryos, the first functional organ system is the cardiovascular system. In mice, this system can be distinguished already at E7.0 by expression of VEGFR-2 in angioblasts, which are mesodermal cells that differentiate to primitive endothelial cells (Dumont et al., 1995). These cells are responsible for formation of blood islands in the yolk sac, where the precursor cells of the endothelia and the hematopoietic cells interact (Medvinsky et al., 2011). These cells then form the primitive vascular plexus of the embryo in a process called vasculogenesis (Risau and Flamme, 1995). Later, the primary means for new vascular formation is angiogenesis, which can be described as formation of new vessels from existing ones via sprouting or splitting (Djonov et al., 2000). Several different vascular growth factors plus other signaling molecules, hypoxia and blood flow are responsible for the angiogenic steps that are necessary for proper vascular formation during development (Udan et al., 2013). This process includes the migration and proliferation of endothelial cells and branching and remodeling of the vasculature. The main molecular driver behind angiogenesis is the VEGF/VEGFR-2 pathway. Blood vessel growth and its directionality are mediated by a VEGF gradient, which reflects the hypoxia gradient or cellular source (e.g. macrophage) of VEGF (Ruhrberg et al., 2002). The endothelial cells will subsequently differentiate to obtain arterial or venous identity, which is regulated by expression changes of, for instance Eph receptors and Notch ligands (Chong et al., 2011). Still later, some specific venous ECs will differentiate to LECs starting from the cardinal vein (Yang and Oliver, 2014).

During the postnatal period angiogenesis continues in the growing tissues mainly via sprouting from previously established vascular beds. During the sprouting angiogenesis, ECs are highly responsive to external signals. The ECs have the ability to upregulate and downregulate signaling cascades during the vascular expansion (Jakobsson et al., 2009). The VEGFR-Notch pathway is well known in this process. The “Tip” cells are responsible for interaction with VEGFs via VEGFRs and the interaction induces Notch ligand, like Dll4, expression in these cells; the Notch signaling in the “stalk” cells form a stabilizing mechanism for gradient-dependent sprouting (Hellstrom et al., 2007; Tammela et al., 2008). Tip and stalk cells show differences in gene expression, with tip cells expressing somewhat more VEGFR-2 (Gerhardt et al., 2003) and stalk cells more decoy-receptor VEGFR-1 (Krueger et al., 2011).

Lymphatic vasculature

Lymphatic vasculature was long considered a passive “drainage” of fluid and other components that had escaped from blood circulation. In the recent decades, this view has changed dramatically. The master regulator of the differentiation of lymphatic vessels from blood vessels is the Prospero Homeobox 1 (*Prox1)* gene (Wigle and Oliver, 1999). Although Prox1 can be found in some blood vessels, e.g. in venous valves (Bazigou et al., 2011) and cardiac valves (Rodriguez-Niedenfuhr et al., 2001), it has been shown to be a key transcription factor for the early steps of LEC differentiation from the embryonic veins. This has been shown also in the experimental setups where overexpression of *Prox1* in BECs alters their expression pattern toward LECs (Hong et al., 2002; Petrova et al., 2002).

During development several other molecules have been indicated to play a role in the early steps of lymphangiogenesis. The COUP-TFII and SOX18 transcription factors are responsible for Prox1 activation at E9.5 (Koltowska et al., 2013). On the other hand, Prox1 expression is responsible for VEGFR-3 upregulation in the common cardinal vein (Wigle et al., 2002). At E9.5 VEGFR-3 is highly expressed in the developing blood vasculature, and its complete absence leads to embryonic death one day later, at E10.5, because of cardiovascular defects (Dumont et al., 1998). From the cardinal vein, LECs start their budding to form the primitive lymph sac around E10-E10.5. This step is dependent on VEGF-C and its absence leads to failure of lymph sac formation (Hagerling et al., 2013; Karkkainen et al., 2004). Recent findings have highlighted the importance of VEGF-C processing throughout this process. The proteolytic activation of VEGF-C requires cooperation of the secreted factor collagen and calcium binding EGF domains 1 (CCBE1) plus a disintegrin and metalloproteinase with thrombospondin motifs 3 (ADAMTS3) (Bos et al., 2011; Bui et al., 2016; Janssen et al., 2016; Jeltsch et al., 2014; Le Guen et al., 2014).

Growth of the lymphatic vessels starts mostly from embryonic veins; it is directed towards the VEGF-C growth factor gradient in embryonic lymphangiogenesis (Yang and Oliver, 2014). However, recent fascinating findings have provided strong evidence that in several tissues there are other cellular sources that constitute non-venous origins of lymphatic vessels. Lymphatic vessel formation in the heart (Klotz et al., 2015), mesentery (Stanczuk et al., 2015) and skin (Martinez-Corral et al., 2015) is based on a process called lymphvasculogenesis, where assembly of cells of hemogenic origin forms lymphatic vessel structures. When the primary lymphatic plexus has been established, it will go through maturation, which includes hierarchical transformation that leads to blind-end primary capillaries to pre-collectors and collecting vessels (Yang and Oliver, 2014). An important step in this phase is the formation of lympho-venous valves, which prevent blood backflow to the lymphatic system (Hess et al., 2014). Collecting lymphatic vessels recruit smooth muscle cells (SMCs) and form a surrounding basement membrane simultaneously as the lymphatic valves are formed to the collectors via a Foxc2 transcription factor-dependent pathway (Norrmen et al., 2009). Upon maturation, the expression patterns of mature lymphatic vessel markers change from the developmental stage where LYVE-1, Prox-1, and VEGFR-3 are all fairly uniformly expressed in the primitive lymphatic plexuses. After maturation, the valve regions have higher expression of VEGFR-3 and Prox1 than the intervening lymphangions, the functional unit of a lymphatic vessel that locates between two valves, in the collector vessels, whereas LYVE-1 expression is decreased in the collectors and only maintained in the capillaries (Norrmen et al., 2009). Lymphatic capillaries change their junctions from zipper-like to button-like after birth (Baluk et al., 2007; Zheng et al., 2014). Angiopoietin (Ang2) signaling regulates this change and it also contributes to vessel network formation during embryonic development (Zheng et al., 2014).

The lymphatic vessel network spreads throughout the skin and into most of the internal organs. New studies have indicated that the lymphatic vessels extend also to the eye and to the central nervous system (Aspelund et al., 2014; Louveau et al., 2015). There are three main functions for which lymphatic vessels are responsible: dendritic cell trafficking from tissues to lymph nodes

(Miteva et al., 2010; Randolph et al., 2005), regulation of tissue fluid balance (Dongaonkar et al., 2009), and absorption of dietary lipids (Iqbal and Hussain, 2009). The immune cell trafficking is closely related to the events of inflammation and will be discussed later in more detail. Regulation of tissue fluid homeostasis is an important function that has been from reviewed from a physiological perspective (Wiig and Swartz, 2012). The absorption of dietary lipids and lipid-soluble vitamins is the third well-described function of lymphatic vessels in normal conditions (Iqbal and Hussain, 2009). The main role of the lymphatic vessels in the intestine is to take up and transport chylomicrons, which have been packed and processed by the enterocytes, to the blood circulation via the thoracic duct (Dixon, 2010). A few studies have now shown that lymphatic vessels can maintain sodium balance via the VEGF-C/VEGFR-3 pathway (Machnik et al., 2009; Wiig et al., 2013; Zhang et al., 2015). These findings however have not, however, yet convinced all researchers in the field.

Vascular endothelial growth factor family

Five main VEGFs and their three receptors with specific ligand binding and signaling patterns have been described to control blood and lymphatic endothelial cell proliferation and migration (Figure 2). Their molecular interactions are discussed below.

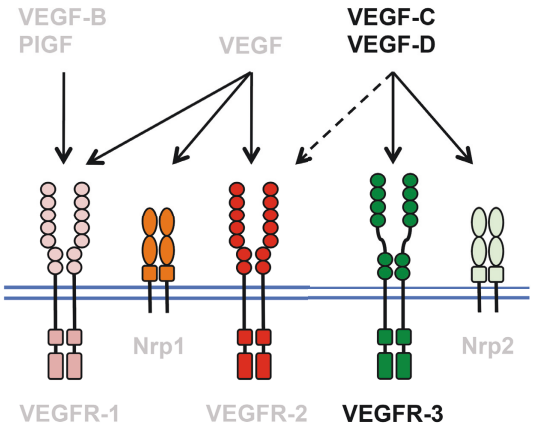

Figure 2. VEGF family. Main components of the VEGF family without soluble forms of the receptors or occasionally occurring heterodimers. The VEGF-C/VEGFR-3 axis is highlighted among other described interactions.

Ligands

VEGF, VEGF-B and PIGF

VEGF was discovered and named vascular permeability factor (VPF) in 1983 (Senger et al., 1983) and it was cloned six years later (Ferrara and Henzel, 1989). It is the best-characterized angiogenic factor. Deletion of a single allele of *Vegf* leads to embryonic lethality around E11.5 because of defects in blood vessel development (Carmeliet et al., 1996; Ferrara et al., 1996). VEGF binds to VEGFR-1 and VEGFR-2 (de Vries et al., 1992; Quinn et al., 1993) and to the neuropilin receptors 1 and 2 (Gluzman-Poltorak et al., 2000; Soker et al., 1998). Its expression can be detected already at E7.0 in the developing embryo (Dumont et al., 1995), highlighting the importance of this growth factor in early

development. *Vegf* gene expression dosage is critical; even a moderate increase of its expression leads to embryonic death at E12.5 because of defective heart development. VEGF acts as a paracrine factor, but autocrine signaling has been reported in endothelial cells (Lee et al., 2007). One of the principal regulators controlling the expression of *Vegf* is hypoxia via the hypoxia-inducible factor (HIF)-1, but many inflammatory cytokines, hormones, and growth factors can also induce *Vegf* gene activation (Chung and Ferrara, 2011). The main role of the VEGF is to boost EC growth and survival (Alon et al., 1995; Benjamin et al., 1999; Benjamin and Keshet, 1997). This occurs mainly via the MAPK pathway, the PI3K-Akt pathway, and downstream proteins mediating anti-apoptotic signals (Gerber et al., 1998a; Gerber et al., 1998b). Studies have shown that VEGF is a potent inducer of vascular permeability (Dvorak et al., 1995) and inflammation (Larcher et al., 1998). Overexpression of VEGF via , for example, a viral vector induces robust and uncontrolled angiogenesis in several models (Detmar et al., 1998; Kenyon et al., 1996; Leung et al., 1989; Pettersson et al., 2000). Because of the vascular leakage and uncontrolled robust angiogenesis, the clinical use of the VEGF is very limited. Besides its roles in angiogenesis, VEGF functions in other processes in various tissues. It can boost bone formation (Midy and Plouet, 1994), affect lung maturation (Compernolle et al., 2002), and enhance neuronal proliferation (Zachary, 2005). VEGF can induce lymphatic vessel hyperplasia via VEGFR2, which is expressed at low levels in the lymphatic vessels (Nagy et al., 2002; Wirzenius et al., 2007).

VEGF-B, originally named as VRF (VEGF-related factor) was discovered in 1996 (Grimmond et al., 1996; Olofsson et al., 1996). VEGF-B deficiency does not affect normal mouse development (Aase et al., 2001). VEGF-B is robustly expressed in the heart, skeletal muscle, and some other tissues such as brown fat in embryos and adults (Aase et al., 1999; Nash et al., 2006). VEGF-B does not function as a traditional angiogenic factor comparable to VEGF. This has been shown in studies where it is overexpressed in several tissues without causing angiogenesis or vascular growth (Bhardwaj et al., 2003; Lahteenvuo et al., 2009; Li et al., 2008). However, VEGF-B does induce growth of coronary vasculature that protects the heart from myocardial infarction (Bry et al., 2010; Huusko et al., 2012; Karpanen et al., 2008; Kivela et al., 2014). VEGF-B has been linked to vascular remodeling in adipose tissue, adipose tissue metabolism, and insulin sensitivity via its function as a competitive ligand for the VEGF/VEGFR-1 pathway (Hagberg et al., 2010; Hagberg et al., 2012; Robciuc et al., 2016).

PlGF is one of the oldest members of the VEGF-family discovered over aquarter of a century ago (Maglione et al., 1991). Similarly to VEGF-B, it binds to VEGFR-1 and NRP-1 (Migdal et al., 1998; Park et al., 1994). PlGF-deficient mice are viable, but unlike the VEGF-B deficient mice, they have defects in angiogenesis and arteriogenesis in conditions, such as wound healing and inflammation, which have not been reported in the VEGF-B mice (Carmeliet et al., 2001; Freitas-Andrade et al., 2012). PlGF also differs from VEGF-B in its potential to induce angiogenesis (Luttun et al., 2002), inflammation (Oura et al., 2003), and vascular permeability (Odorisio et al., 2002) Because of these findings, it seems that in the pathological situations the inhibition of PlGF may be beneficial (De Falco, 2012).

VEGF-C and VEGF-D

VEGF-C and VEGF-D was isolated and cloned roughly twenty years ago as a ligands that can bind to VEGFR-3 and VEGFR-2 (Achen et al., 1998; Joukov et al., 1996). VEGF-C and VEGF-D growth factors differ from other VEGF family members by their proteolytic processing. They are expressed as precursor proteins, which can only bind to VEGFR-3 (Joukov et al., 1996; Lee et al., 1996). In humans both proteins are cleaved by furin or the proprotein convertases PC5 and PC7, which increase their binding activity towards VEGFR-3 (McColl et al., 2003; McColl et al., 2007; Siegfried et al., 2003). In humans, both proteins can be further processed by N-terminal cleavage to produce the fully processed forms (VEGF-C/D-ΔNΔC), which can bind to and activate VEGFR-2 and VEGFR-3 (Achen et al., 1998; Joukov et al., 1996; Joukov et al., 1997). The mouse VEGF-C-ΔNΔC can also bind to VEGFR-2 (Joukov et al., 1996), whereas VEGF-D-ΔNΔC cannot (Baldwin et al., 2001).

Two different VEGF-C mRNA splice variants have been reported in mice and three in humans, but the shorter forms are poorly understood (Lee et al., 1996). VEGF-C is the main lymphangiogenic growth factor during development. During the development of the mouse, lack of *Vegfc* leads to complete lymphatic vessel growth arrest and failure of the primary LECs to bud out from the cardinal vein, resulting embryonic death around E16.5 (Karkkainen et al., 2004). *Vegfc* heterozygous mice die during the first weeks after birth because of lipid absorption problems; a few mice may survive in certain genetic backgrounds to weaning age (P21). However, also these mice develop peripheral edema because of hypoplastic cutaneous lymphatic vessels (Karkkainen et al., 2004). The expression of VEGF-C starts at about E8 near the areas where the primary budding of LECs begins from veins (Karkkainen et al., 2004; Kukk et al., 1996). VEGF-C has also a role in coronary artery development in the heart (Chen et al., 2014a; Chen et al., 2014b). In the developing brain, VEGF-C expression triggers VEGFR-3+ neural progenitor proliferation in the olfactory bulb and in glial precursor cells in the optic nerve (Le Bras et al., 2006). Studies in zebrafish have indicated that VEGF-C is crucial for the growth of axons of motor neurons via VEGFR-3 signaling (Kwon et al., 2013). In humans, VEGF-C expression has been analyzed both during the fetal period and in adult tissues (Partanen et al., 2000). Especially strong expression was observed in endocrine organs such as the thyroid, the adrenal medulla, and the pancreas (Partanen et al., 2000).

VEGF-C signaling via VEGFR-3 is crucial for LEC growth and survival via PI3 kinase-dependent Akt activation and PKC-dependent p42/p44 MAPK signaling (Makinen et al., 2001b). Adenoviral overexpression of VEGF-C in the airways induces sprouting lymphangiogenesis (Baluk et al., 2005), and the same outcome can be seen when it is overexpressed in the skin of transgenic mice (Jeltsch et al., 1997). Lymphatic vessel growth in the tumors is mostly VEGF-C-dependent and VEGF-C has been implicated in tumor metastasis to the sentinel lymph nodes and other organs (Hirakawa et al., 2007; Skobe et al., 2001a; Valtola et al., 1999).

VEGF-D was originally named as c-fos-induced growth factor (FIGF) for fibroblastic cells, and after its binding affinity to VEGFR-2 and VEGFR-3 was discovered it was re-named as VEGF-D (Achen et al., 1998; Orlandini et al., 1996). VEGF-D is dispensable during normal lymphatic vessel growth, even

though it is expressed in several tissues during development (Avantaggiato et al., 1998; Baldwin et al., 2005). VEGF-D has been reported to affect angiogenesis in both zebrafish and mice (Duong et al., 2014; Song et al., 2007). Expression of VEGF-D has been detected in several tissues, but its function in different tissues is poorly known (Achen et al., 1998). The lymphangiogenic effect of VEGF-D has been shown in several pathological situations, such as in allergic corneal lymphangiogenesis (Lee et al., 2015), tumor growth (Kopfstein et al., 2007; Van den Eynden et al., 2007) and in inflammation (Gordon et al., 2010). There is speculation that inflammation-associated lymphangiogenesis (IAL) is more VEGF-D- than VEGF-C-dependent (Bui et al., 2016). VEGF-D-induced lymphangiogenesis has been shown by using transgenic mice overexpressing VEGF-D or by viral delivery of this growth factor (Byzova et al., 2002; Veikkola et al., 2001). VEGF-D can furthermore induce angiogenesis when delivered into certain tissues using adenoviral vectors (Anisimov et al., 2009; Rutanen et al., 2004).

Receptors

VEGFR-1 and VEGFR-2

VEGFR-1, also known as Fms-like tyrosine kinase 1 (Flt1), was discovered in 1990 (Shibuya et al., 1990) and two years later it was confirmed to be a receptor for VEGF (de Vries et al., 1992). It comprises an extracellular domain made up seven immunoglobulin homology domains, a trans-membrane domain and a split intracellular tyrosine kinase domain, which is responsible for intracellular signaling after ligand binding and receptor dimerization (Tanaka et al., 1997). *Vegfr1* deficiency leads to embryonic lethality around E9.0 because of the unorganized overgrowth of blood vascular endothelium (Fong et al., 1995). This and other findings suggest that VEGFR-1, which has weak tyrosine kinase activity, is not responsible for angiogenesis, rather it acts as a decoy-receptor for VEGF (Fong et al., 1995; Fong et al., 1999; Hiratsuka et al., 1998). During development VEGFR-1 is expressed mainly in hemangioblasts and in the adults in the endothelium, monocytes, dendritic cells and hematopoietic stem cells (Shibuya, 2001). Recently, VEGFR-1 was found to have an important role in adipose tissue metabolism in mice fed a high-fat diet (Robciuc et al., 2016).

VEGFR-2 is the main receptor for VEGF and it is responsible for developmental and pathological angiogenesis (Chung and Ferrara, 2011). It was found as fetal liver kinase-1 (Flk1) in mice (Matthews et al., 1991) and kinase-insert domain receptor (KDR) in humans (Terman et al., 1991). VEGF/VEGFR-2 signaling promotes endothelial cell survival, migration, proliferation, and sprouting (Gille et al., 2001). In addition to VEGF binding to VEGFR-2, the processed lymphangiogenic factors VEGF-C-ΔNΔC in mice and humans and VEGF-D-ΔNΔC in humans can activate endothelial cell signaling via VEGFR-2 (Achen et al., 1998; Joukov et al., 1997). *Vegfr2*-deficient embryos die around E9.0 because of impaired vasculogenesis and hematopoiesis (Shalaby et al., 1995). VEGFR-2 is expressed from E7.0 onwards in several compartments of the developing embryo (Millauer et al., 1993) and its expression can be detected in mature endothelial cells in adults throughout the lifespan (Matsumoto and Claesson-Welsh, 2001). Besides endothelial cells, VEGFR-2 is expressed for instance, hematopoietic cells (Ziegler et al., 1999) and some neuronal cells

(Yang and Cepko, 1996). Low VEGFR-2 expression can be detected in LECs and a selective VEGFR-2 ligand is known to induce lymphatic vessel hyperplasia, but not sprouting (Nagy et al., 2002; Wirzenius et al., 2007).

VEGFR-3

VEGFR-3, also called Fms-like tyrosine kinase 4 (Flt4) was isolated in 1993 from human leukemia cells (Pajusola et al., 1992). It differs structurally from VEGFR-2, as it is proteolytically cleaved in the extracellular domain, but a disulfide bond (-S-S-) keeps the fragments together (Pajusola et al., 1994). The ligands for VEGFR-3 are VEGF-C and VEGF-D. VEGFR-3 has a dual role in embryonic blood and lymphatic vessel development. VEGFR-3 expression starts at E8.5 in blood vascular endothelium and *Vegfr3* deficiency leads to embryonic death because of cardiovascular defects at E9.5 (Dumont et al., 1998). Later, during embryonic development and postnatal stages, VEGFR-3 expression is mainly observed in LECs and blocking of this pathway inhibits lymphatic vessel growth (Karpanen et al., 2006b; Makinen et al., 2001a; Zarkada et al., 2015). Deletion of VEGF-C and VEGF-D causes embryonic lethality around E16.5 because of lymphatic vessel defects, indicating that VEGFR-3 activity in the early embryonic stages is probably not controlled by these two VEGFR-3 ligands (Haiko et al., 2008). VEGFR-3 can form heterodimers with VEGFR-2, but detailed *in vivo* function of this heterodimer is incompletely known (Dixelius et al., 2003; Nilsson et al., 2010).

Besides LECs, some BECs express VEGFR-3 in adult tissues. VEGFR-3 can be found in fenestrated vessels in several organs, like the liver, spleen, and endocrine organs, as well as in high endothelial venules (HEVs) (Lymboussaki et al., 1998; Partanen et al., 2000). It has a role in non-endothelial cells such as dendritic cells (Hamrah et al., 2004), hematopoietic cells (Thiele et al., 2012) and in the corneal epithelia cells (Cursiefen et al., 2006). The variety in VEGFR-3 expression indicates its pivotal role in several diverse actions maintaining body homeostasis.

Other key molecules in vessel growth during development

Neuropilin (Nrp-1) and Nrp-2 were found to be involved in neuronal guidance via acting as receptors for semaphorins (Chen et al., 1997; Kolodkin et al., 1997). However, a few years later it was established that VEGFs can bind to neuropilins, which act as co-receptors for VEGFRs (Fuh et al., 2000; Makinen et al., 1999; Migdal et al., 1998; Soker et al., 1998; Whitaker et al., 2001). *Nrp1* deficiency leads to embryonic lethality at E13.5 because of vascular defects (Kawasaki et al., 1999). Nrp2 is not necessary for vascular development, however Nrp2-deficient mice show abnormalities in lymphatic capillaries (Yuan et al., 2002). Combined deletion of both alleles of Nrp1 and Nrp2 leads to embryonic death already at E8.5, indicating an early requirement for interplay between these two molecules (Takashima et al., 2002). There is complicated interplay between VEGFs and Nrps. Some VEGF isoforms bind both Nrps and some only one of them (Gluzman-Poltorak et al., 2000; Soker et al., 2002). VEGF-B and PlGF can only bind to Nrp1 (Makinen et al., 1999; Migdal et al., 1998) and VEGF-C and VEGF-D can bind to both neuropilins (Karpanen et al., 2006a). The current view holds that Nrp1 is responsible for the interactions with VEGFR-2 signaling to promote angiogenesis (Soker et al., 2002), while Nrp2

interacts with VEGFR-3 to enhance lymphangiogenesis (Karpanen et al., 2006a).

The TIE receptors (Tyrosine kinase Ig and EGF homology domains) and their ligands angiopoietins (Angs) are another crucial growth factor family for vascular development (Augustin et al., 2009; Eklund and Saharinen, 2013). There are two Tie receptors (Tie1 and Tie2) (Dumont et al., 1992; Partanen et al., 1992). Tie receptor expression is restricted for the most part to the endothelial cells with a few exceptions, e.g. hematopoietic stem cells (Batard et al., 1996). The ligands for Tie2 are called Angiopoietins (Ang1, Ang2, and Ang3/4), whereas Tie1 is an orphan receptor, contributing to the Tie-Ang signaling via Tie2 (Saharinen et al., 2005). Recent discoveries have shown that Tie1 is required for full-scale Tie2 activation *in vivo* (D'Amico et al., 2014; Korhonen et al., 2016). Early characterization with *Tie1*- and *Tie2*-deficient mice has revealed the obligatory role of TIE receptors in embryonic vascular development (Dumont et al., 1994; Patan, 1998; Puri et al., 1995; Sato et al., 1995). *Ang1* deficiency in mice phenocopy the *Tie2* KOs (Suri et al., 1996) Tie-Ang signaling is based on a cellular microenvironment. Tie receptors are located in the cellular junctions of endothelial cells (Fukuhara et al., 2008; Saharinen et al., 2008). Mesenchymal cells express Ang1 and this acts in a paracrine matter with Tie2 to supports EC survival and this interaction has an pivotal role in a stabilization of the endothelial cell junctions and cytoskeleton interaction via actin (Gamble et al., 2000; Saharinen et al., 2008). Ang2 is a weaker ligand for Tie2 and it can be seen as an antagonist for Tie2, resulting in dose-dependent inhibitory effects on the Ang1-Tie2 pathway (Fiedler et al., 2004). Ang2 expression increases in situations where vascular stability is compromised, as in VEGF stimulation, hypoxia, or inflammation-based cytokine upregulation (Mandriota and Pepper, 1998). In addition, Ang2 has a mandatory role in the LEC junction maturation (Zheng et al., 2014).

Integrins are located on the cell surface and they are responsible for binding of matrix proteins like collagen and fibronectin. They form heterodimers and contain α- and β-subunits (Silva et al., 2008). Integrins interact with well-known vascular pathways. α v β 3-integrin is required for full VEGFR-2 activation via VEGF (Masson-Gadais et al., 2003), and in the Ang/Tie pathway destabilization of ECs is β 1-integrin-dependent (Hakanpaa et al., 2015). In the lymphatic system, the VEGF-C/VEGFR-3 pathway seems to require $\alpha 5 \beta 1$ interaction for proper activation (Zhang et al., 2005). β 1-Integrin contact with VEGFR-3 can induce receptor phosphorylation followed by cell migration independently of the ligand (Wang et al., 2001).

Other growth factors/ligands have been indicated in the past decade to play a part in vessel development. Examples include platelet-derived growth factor pathway (Armulik et al., 2011), transforming growth factor-β (TGF-β) – bone morphogenic protein (BMP) signaling (Cai et al., 2012), and fibroblast growth factors (FGFs) (Presta et al., 2005). Apart from these, there are individual molecules that have a role in vessel growth and maturation.

VEGF-C/VEGFR-3 pathway in pathological conditions

Lymphatic vessels are involved in several human diseases. Below, I describe selected pathological conditions in which lymphatic vessels and the VEGF-C/VEGFR-3 pathway have a crucial function in disease pathogenesis. These conditions include cancer and its metastasis, lymphedema, inflammation and diseases related to lipid metabolism.

Cancer

Numerous studies in humans and rodents have shown the importance of angiogenesis in tumor growth. For this reason, several anti-angiogenic treatments have been developed for inhibition of tumor vascularization (Welti et al., 2013). However, the main reason for cancer mortality is metastasis, and a key indicator for patient survival is the sentinel lymph node metastases (Tuttle, 2004), highlighting the importance of lymphatic vessels in cancer biology. However, investigations of the lymphatic vessel involvement in tumor growth have not yet led to clinical therapeutic applications.

The same growth factors and receptors that regulate developmental lymphangiogenesis are also involved in tumor lymphangiogenesis and regulate lymphatic vessel invasion by tumor cells. Several human tumor lines express high levels of VEGF-C and VEGF-D (He et al., 2004; Hirakawa et al., 2007; Sleeman and Thiele, 2009). This indicates that tumor cells actively attract lymphatic vessels towards the growing tumor. Studies performed with mice have shown that blocking antibodies against VEGFR-3 inhibit tumor metastasis to lymph nodes, and in some tumors they also decrease angiogenesis (Laakkonen et al., 2007; Rutkowski et al., 2013; Tammela et al., 2008). Several upstream factors can promote the VEGF-C/D-VEGFR-3 axis in tumor lymphangiogenesis. Prostaglandins have been shown to modulate VEGF-C levels in the tumor microenvironment (Su et al., 2004; Timoshenko et al., 2006). Hormones such as erythropoietin (Lee et al., 2011) and adrenomedulin (Karpinich et al., 2013) increase tumor and sentinel lymph node lymphangiogenesis. Although their exact molecular mechanisms are not well known, VEGF-C/D-VEGFR-3 is very likely involved. Chemokines have also been shown to modulate metastasis to lymphatic vessels. Tumor cells secrete VEGF-C and the resulting increase in VEGFR-3 signaling in LECs leads to increased CCL21 secretion by the LECs, which can stimulate tumor cell migration toward lymphatic vessels (Issa et al., 2009). In the tumor microenvironment, lymphangiogenic factors induce sprouting lymphangiogenesis, whereas beyond the primary tumor the downstream collecting lymphatic vessels expand in response to these factors (He et al., 2004). This has been related to increased VEGF-C and VEGF-D secretion from invasive tumor cells (Hoshida et al., 2006; Karnezis et al., 2012).

Based on the animal models and human studies, blocking of the VEGF-C/D-VEGFR-3 pathway can inhibit metastasis (Alitalo, 2011; Stacker et al., 2014). Several inhibitors and targets are under investigation as candidate drugs that could inhibit the VEGF-C/D-VEGFR-3 pathway (Stacker et al., 2014) and antibodies that block VEGFR-3 have been tested in phase I clinical trials in humans.

Lymphedema

Lymphedema is defined by abnormal accumulation of interstitial fluid in tissues, usually in the limbs, but lymphedema it is not restricted to the extremities. Lymphedemas are divided into two different types based on their origin; primary lymphedema is caused by mutations related to lymphatic vascular development genes and secondary lymphedema occurs after surgical operations or other types of damage that compromise lymphatic vessel function.
Mutations in about twenty different genes have been associated with different types of primary lymphedemas. Mutations of *VEGFR3* are the most common reason for lymphedema (Brouillard et al., 2014). In a subset of these mutations, lymphedema is only one symptom caused by the abnormal gene function (Connell et al., 2013). Missense kinase-inactivating mutations in *Vegfr3* have been described in so-called *Chy* mice (Karkkainen et al., 2000). These mice were used as a model for primary lymphedema characterization and treatment because their mutation and phenotype resembles those in a large subset of human primary lymphedema patients (Brouillard et al., 2014; Irrthum et al., 2000; Karkkainen et al., 2001). Mutations of *VEGFR3* are the most common reason for Milroy's disease and its subtypes, which are congenital type of lymphedemas that involve lower limb swelling (Irrthum et al., 2000; Karkkainen et al., 2000). Whereas the *Chy* mice lack lymphatic vessels in the skin, in Milroy's disease patients the initial lymphatic vessels are present but they are non-functional (Karkkainen et al., 2001; Mellor et al., 2010). Rare mutations of *Vegfc* can also cause a Milroy-like disease, which is impossible to distinguish clinically from the *Vegfr3* mutations (Gordon et al., 2013). Another gene closely to related to the VEGF-C/VEGFR-3 axis is the *Ccbe1.* It has been shown that Ccbe1 is important factor for VEGF-C processing (Jeltsch et al., 2014). Mutations of this gene have been shown to cause the Hennekam-syndrome. Patients with the Hennekam-syndrome have early-onset lymphedema, lymphangiectasia and mental retardation (Alders et al., 2009). *Foxc2* mutations cause late-onset primary lymphedema with some other characteristic symptoms like distichiasis (Brice et al., 2002). Mice that lack the *Foxc2* gene have irregular lymphatic vessel patterning, abnormal pericyte interaction and defects in valve formation in the collecting lymphatic vessels (Petrova et al., 2004). VEGFR-3 has been shown to genetically interact with *FoxC2* in LECs (Petrova et al., 2004). Recently it has been shown that *Foxc2* is important for vessel stabilization by mechanotransductive signals induced by lymph flow (Sabine et al., 2015). Besides these genes, several others are involved in normal lymphatic vessel development and mutations of these genes cause primary lymphedema in humans (Aspelund et al., 2016; Brouillard et al., 2014; Connell et al., 2013).

An acquired lymphedema not related to genetic mutations is called secondary lymphedema, which is due to physical disruption, radiation damage, infection, or prolonged inflammation followed by fibrosis. A common form of secondary lymphedema can be found in a breast cancer patient who has undergone a surgical procedure in which the axillary LNs have been removed. Up to 30% of these patients develop lymphedema of the arm (Petrek and Heelan, 1998). In these individuals, lymphatic vessels commonly dilate and become dysfunctional (Ikomi et al., 2006). The scale of the breast cancer operation correlates with the severity of the condition. In addition, in geographically restricted secondary lymphedema cases in the tropics, e.g. in Africa and South-America, parasitic

nematodas, transmitted by mosqitoes, can target lymphatic vessels and lymph nodes, preventing the normal flow of lymph and causing tissue swelling and fibrosis (Shenoy, 2008). Currently, only a very limited number of treatment options are available for lymphedema patients via interfering the VEGF-C/VEGFR-3 pathway.

Inflammation associated lymphangiogenesis (IAL)

Lymphangiogenesis is known to take place during inflammatory processes (Kim et al., 2014). This context-dependent and dynamic process can eventually have a major role in tissue healing (Kim et al., 2014). IAL occurs in response to a variety of inflammatory stimuli. For example, upon Gram-negative bacterial infection, several cell types produce high levels of lymphangiogenic growth factors because of bacterial lipopolysaccharide and cytokine exposure. Upon cytokine-induced recruitment, macrophages and granulocytes enhance further VEGF-C expression in the inflamed tissue (Baluk et al., 2005) Lymphangiogenesis in inflammatory settings facilitates the resolution of tissue edema, and it promotes macrophage and DC mobilization to draining LNs (Huggenberger et al., 2011; Kataru et al., 2009). However, IAL modulation has a great impact on the inflammation clearance. In the skin, blocking of the VEGF-C/VEGFR-3 pathway prolongs tissue swelling in the oxazolone-induced hypersensitivity model, and, on the other hand, VEGF-C overexpression accelerates inflammation clearance (Huggenberger et al., 2011; Huggenberger et al., 2010). In inflammatory bowel disease (IBD) models, exogenous VEGF-C attenuates progression of the pathogenesis (D'Alessio et al., 2014). In contrast, reduction of macrophages that produce lymphangiogenic growth factors boosts the inflammation (Becker et al., 2016). It has further been shown that during mycoplasma-induced airway inflammation, lymphatic vessels remodel their junctions (Baluk et al., 2005). Fourteen days after infection with *Mycoplasma pulmonis,* the sprouting lymphatic vessels had zipper-type junctions instead of buttons, but the change was reversed by antibody treatment against VEGFR-3 (Baluk et al., 2005). Thus, the lymphatic vessels show great plasticity during inflammation.

IAL seems to be involved also in tissue rejection after organ transplantation. An extensive lymphangiogenic response is associated with transplant rejection (Kerjaschki et al., 2004). Blocking of pathological lymphangiogenesis has been shown to be an effective treatment for preventing rejection of, for example, heart (Nykanen et al., 2010) and cornea transplants (Hos et al., 2015). The clinical potential of lymphatic vessels needs to be recognized, especially in the transplantation field.

Lymphatic vessels in obesity and lipid homeostasis

Obesity is the result of an imbalance between calorie uptake and energy consumption. The first evidence showing an interaction between the genes that control lymphatic vessel development and those that contribute to the onset of adiposity and obesity came from studies of *Chy* mice (Karkkainen et al., 2001) and Prox1+/− mice (Harvey et al., 2005), respectively. The latter phenotype was recently reversed by expression of a *Prox1* transgene in lymphatic endothelium, which prevented the late-onset obesity (Escobedo et al., 2016). Prox1+/- mice have dysfunctional lymphatic vessels and continuous leakage of chyle, which has been shown to be adipogenic *in vitro*, into the interstitial space (Harvey et

al., 2005) The obese phenotype has not been observed in other mice with lymphatic vessel dysfunction such as the Chy, K14-VEGFR3-Ig, or *Vegfc* heterozygous mice (Aspelund et al., 2016). *Prox1* is known to be expressed in many other metabolically active tissues, including the heart and skeletal muscle (Risebro et al., 2009), which could explain the obesity phenotype. Lymph has been shown to promote adipogenesis in the Prox1+/- mice (Escobedo et al., 2016; Harvey et al., 2005) and Chy mice have more adipocytes in subcutaneous tissue (Karkkainen et al., 2001). These findings suggest that lymphatic vessel dysfunction and lymph leakage promotes adipogenesis, but clear molecular evidence is still lacking.

It was recently reported that lymphatic vessels function in reverse cholesterol transport (RCT) (Lim et al., 2013; Martel et al., 2013). In these studies, lymphatic vessel dysfunction correlated with the impaired RCT in genetic and surgical models, demonstrating the essential role that lymphatic vessels have in cholesterol metabolism. This finding could link dysfunctional lymphatic vessels to the progression of atherosclerosis (Randolph and Miller, 2014). Previous studies have indicated that hypercholesterolemic mice have lymphatic vessel dysfunction and degeneration (Lim et al., 2009). A recent study has also proposed that soluble VEGFR-2 extracellular domain has a crucial role in controlling VEGF-C levels in atheroma-related lymphangiogenesis in mice (Taher et al., 2016). In humans, expression of lymphatic vessel markers increases in the areas where the plaques are located, indicating counter play between lesion formation and subsequent lymphatic vessel growth (Grzegorek et al., 2014). Thus, clearly, the lymphatic vessels are involved in lipid metabolism and related disorders, but more thorough investigations are required to uncover the molecular pathways that would provide clinical benefit.

Other diseases related to VEGF-C/VEGFR-3 pathway

Lymphatic vessels and therefore also the VEGF-C/VEGFR-3 pathway are becoming the focus of new interest in other diseases as well. Below, I briefly describe some of the new hot topics in the field.

The recently discovered meningeal lymphatic vessels (Aspelund et al., 2015; Louveau et al., 2015) have triggered intense discussion of their function, especially in pathological settings. In neurodegenerative Alzheimer's disease and neuroinflammatory multiple sclerosis, the immune system has been shown to play a pivotal role, and the lymphatic vessels have been speculated to be involved in both (Louveau et al., 2016). However experimental evidence from the pre-clinical models does not yet exist.

Lymphatic vessel growth in the eye, mainly in the cornea, has been investigated in great detail during the past years (Bock et al., 2013). Corneal (blood and lymphatic) vessel growth occurs in several pathological conditions such as in corneal crafts and in aging-related diseases (Bock et al., 2013). New evidence shows that corneal transplantations are more successful after blocking the VEGF-C/VEGFR-3 pathway (Hos et al., 2015). After the discovery of Schlemm's canal as a VEGFC/VEGFR-3-dependent drainage pathway from the anterior chamber of the eye (Aspelund et al., 2014; Park et al., 2014), intense research has tried to validate its role in intra-ocular pressure regulation.

The C-type Lectin Receptor 2 (CLEC-2) was recently found to be responsible for the maturation of collecting lymphatic vessels in mouse embryos by inducing blood clotting when blood tries to enter lymphatic vessels at the lymphovenous border (Sweet et al., 2015). In pathological settings, this may be relevant in the case of a surgical trauma of the thoracic duct, in heart failure, and in lymphomas (Doerr et al., 2005). Under pathologically elevated venous pressures, lymphovenous clot formation may become more crucial for maintaining blood/lymph separation. If lymphovenous hemostasis is inhibited by antiplatelet/anticoagulant treatment or pathological hemostatic defects, dysfunction of the lymphatic vessels may be exacerbated, contributing to lymphedema (Welsh et al., 2016).

Thus, specific drug and treatment candidates can be used to treat lymphatic vessels in certain pathological conditions such as cancer and lymphedema. In cardiovascular diseases, obesity and inflammation, lymphatic vessels have also been found to be affected and in the near future this knowledge will hopefully translate into new clinical applications. Recent findings on lymphatic vessels in the central nervous system have raised hopes for new potential applications in diseases where the lymphatic vascular system has not yet been targeted.

AIMS OF THE STUDIES

The specific aims of my thesis studies have been listed below.

I. To clarify how VEGFR-3 contributes to angiogenesis

II. To determine the role of the VEGF-C/VEGFR-3 pathway in atherosclerosis using mice in which this pathway is interfered

III. To elucidate whether VEGF-C is necessary for lymphatic vessel maintenance in adult mice, in addition to its crucial role during lymphatic vessel development

IV. To investigate erythropoiesis in Vegfc gene deleted mice based on their pale appearance relative to their littermates

MATERIALS AND METHODS

The materials and methods are described in detail in the original publications. Tables of the most relevant materials and methods are provided below, followed by detailed protocols of the methods used most.

Materials

Table I. Mouse lines

Csf1$^{op/op}$	Mice with a spontaneous osteopetrosis mutation	(Wiktor-Jedrzejczak et al., 1982)	I
Foxc2$^{+/-}$	*Foxc2* gene deletion	(Iida et al., 1997)	I
K14-Vegfr3-Ig	Over-expression of the human VEGFR3-Ig protein under the K14 promoter in skin	(Makinen et al., 2001a)	II
LDLR$^{-/-}$*/ApoB*$^{100/100}$	Mice expressing an APO-B100 transgene and are deficient in low-density lipoprotein receptor	(Veniant et al., 1998)	II
PdgfbiCreER^{T2}	Inducible Cre recombinase under the control of *Pdgfb* gene promoter	(Claxton et al., 2008)	I
Rosa26-CreER^{T2}	Inducible Cre recombinase under the control of *Rosa26* gene promoter	(Ventura et al., 2007)	III,IV
Rosa26-R	Reporter mice expressing β-calactosidase (LacZ) gene after Cre-recombination	(Soriano, 1999)	I
Rosa26$^{LSL\text{-}tdTomato}$	Expression of TdTomato gene after Cre-recombination	(Madisen et al., 2010)	IV
Vav-Cre	Constitutive Cre recombinase under the control of *Vav* gene promoter	(de Boer et al., 2003)	IV
Vegfc$^{+/LacZ}$	Vegfc heterozygous mice in which β-calactosidase (LacZ) gene has been inserted into the Vegfc locus	(Karkkainen et al., 2004)	I,III,IV
Vegfc $^{flox/flox}$	Mice with conditionally targeted Vegfc alleles	(Aspelund et al., 2014)	III,IV
Vegfd$^{+/-}$	Heterozygous *Vegfd* gene deleted mice	(Baldwin et al., 2005)	I,III
Vegfr2$^{+/LacZ}$	β-calactosidase (LacZ) gene has been use to replace one allele of the *Vegfr2* gene	(Shalaby et al., 1995)	I,III,IV
Vegfr3$^{+/LacZ}$	β-calactosidase (LacZ) gene has been used to replace one allele of the *Vegfr3 gene*	(Dumont et al., 1998)	I,III,IV
Vegfr3$^{+/KD}$	Mice have a missensepoint mutation in the tyrosine kinase domain of Vegfr3 (A3157T)	(Karkkainen et al., 2001)	I,II
Vegfr3 $^{flox/flox}$	Vegfr3 conditionally targeted mice	(Haiko et al., 2008)	I,III,IV
NMRI,CD1,C57bl,FVB/n	Wild type mice	Taconic, Envigo	I,III,IV

Table II. Antibodies

Activated Caspase 3, mouse	Rabbit polyclonal	R&D Systems, AF835	IV
BrdU	Mouse monoclonal, Alexa Fluor 594 conjugated	Invitrogen, B35132, clone MoBU-1	I
c-Kit, mouse	Goat, biotinylated	R&D Systems, AF1356	IV
CD3, mouse	Rabbit monoclonal	Abcam, ab16669	II
CD45, mouse	Rat polyclonal	BD Pharmigen, 550566	IV
Collagen IV, mouse	Rabbit polyclonal	Cosmo Bio (Lb, 1403)	I
Dll4, mouse	Goat polyclonal	R&D systems AF1389	I
E-Cadherin, mouse	Purified mouse	BD Pharmigen, 610181	IV
Endomucin, mouse	Rat monoclonal	Santa Cruz, sc-65495	I
F4/80, mouse	Rat monoclonal	AbD Serotec, MCA497R	I, IV
FITC	Rabbit polyclonal	Invitrogen, 71-1900	I
FOXC2, mouse		(Furumoto et al., 1999)	I
GFP	Rabbit polyclonal	Torrey Pined Biolabs TP401	I
hF4-3C5, human	Mouse humanized monoclonal	ImClone systems (Persaud et al., 2004)	I
LYVE1, mouse	Rabbit polyclonal	Reliatech, 103-PA50AG	II
LYVE1, mouse	Rabbit polyclonal	(Karkkainen et al., 2004)	III,IV
mMQ (Macrophages)	Rabbit polyclonal	ACSC, AIA31240	II
PECAM-1, mouse	Rat monoclonal	BD Biosciences 553370, clone MEC 13.3	I, II, III,IV
PECAM-1, mouse	Hamster monoclonal	Millipore, MAB1398Z	IV
Prox-1, human	Goat polyclonal	R&D systems, AF2727	III
SMA, mouse	Mouse Cy3-conjugated	Sigma, C6189	III
TIE2, mouse	Rat monoclonal	eBioscience, CD202b, Clone:TEK4	I
VEGFC, human	Rabbit polyclonal, #6	(Baluk et al., 2005)	I
VEGFR2, mouse	Goat polyclonal	R&D systems, AF644	IV
VEGFR2, mouse	Rat monoclonal	(Prewett et al., 1999); CD101	I
VEGFR3, mouse	Goat polyclonal	R&D Systems, AF 743	I, III, IV
VEGFR3, mouse	Rat monoclonal	(Pytowski et al., 2005) Clone mF4-31C1	I
Ter-119, mouse	Rat polyclonal	BD Pharmigen, 550565	IV
Von Willebrand Factor	Rabbit polyclonal	DAKO, A0082	IV
Conjugated secondary antibodies	Chicken, Donkey, Goat – 488,594,647 labeled	Invitrogen	I.II.III,IV

Table III. Cell lines, recombinant viruses, recombinant proteins and inhibitors

CELL LINES			
B16-F10-Luc-G5	Luciferase expressing mouse melanoma cells	Caliper Life	I
HDBEC	Human dermal blood vascular endothelial cells	Promo Cell	I
LLC	Mouse Lewis lung carcinoma cells	ATCC	I
mESC	Mouse embryonic stem cells derived from blastocysts	(Holmborn et al., 2004)	I
VIRAL VECTORS			
$AdVEGF_{165}$	Human VEGF165 - Adenovirus	(Enholm et al., 2001)	I
$AdVEGFB_{167}$	Human VEGFB167 - Adenovirus	(Lahteenvuo et al.,	I

		2009)	
pMX-Dll4 ECTM-EGFP	pMX retrovirus that encodes mouse Dll4-ECTM-EGFP	(Zheng et al., 2011a)	I
AAV-sVEGFR3	Mouse sVEGFR3 - AAV	(Aspelund et al., 2014)	IV
RECOMBINANT PROTEINS			
Dll4-Fc	Mouse Dll4 fused to Fc domain of human IgG	(Zheng et al., 2011b)	I
Jagged 1	A Notch activating synthetic peptide	(Weijzen et al., 2002)	I
sc-Jagged1	Scrambled sequence for Jagged1. peptide	(Weijzen et al., 2002)	I
$VEGF_{165}$	Recombinant human VEGF	R&D systems	I
$VEGFC_{\Delta N \Delta C}$	Fully processed form of human VEGFC	(Karpanen et al., 2006a)	I
SMALL MOLECULE INHIBITOR			
Sunitinib	Multi targeted receptor tyrosine kinase inhibitor	Pfizer	III

Methods

Table IV. Summary of methods

Adenoviral transduction of mice	I
Cell culture	I, IV
Cell transfections and transductions	I
Characterization of a new transgenic mouse strain	III
Clinical chemistry	II
FACS analysis	IV
Functional analysis of lymphatic vessels	II, III
Histological analysis of mouse aortas	II
Immunoluoresence/immunohistochemistry	I - IV
Immunoprecipitation and immunoblotting	I
In Vivo studies	I - IV
Microscopy	I - IV
Mouse embryo stimulation with growth factors/gene deletions	I, IV
Lipid analysis	III
PI3K activity assay	I
Polymerase chain reaction	I - IV
Production and purification of Dll4-Fc	I
Real-time quantitative PCR	I, III, IV
Statistical analysis	I - IV
Tumor experiments	I
X-gal staining of the tissues	I, III, IV

Cell culture

Primary human blood vascular endothelial cell (hBEC) experiments.
hBECs (PromoCell) were maintained in endothelial cell growth medium (ECGM, PromoCell) with supplements provided by the manufacturer. For the stimulation experiments, hBECs were starved in serum-free ECGM for 4 h and stimulated for 1 or 2 h with different growth factors in starvation medium. For the silencing

experiments, hBECs were transfected with human VEGFR3 or non-targeted siRNA (Thermo Scientific Dharmacon siGENOME ON-TARGETplus SMARTpool reagents), using Oligofectamine (Invitrogen). Gene expression was examined 48 h post-transfection by qRT-PCR of cell lysates in RLT buffer (Qiagen). Alternatively, cells were lysed in PLCLB lysis buffer (150mM NaCl, 5% glycerol, 1% Triton X-100, 1.5 M MgCl2, 50 mM HEPES, pH 7.5, 1mM Na3VO4, phenylmethylsulphonyl fluoride, leupeptin and aprotinin) for immunoprecipitation.

Methocult CFU assay
Freshly isolated cells from E14.5 fetal livers, yolk sac (YS) and E10.5 embryos were plated in duplicates in 1 ml of MethoCult GF M3434 medium or SF M3436 medium according to the manufacturer's instructions (Stemcell Techonologies). Colonies were studied and counted at days 3, 10, and 14.

Functional analysis of the lymphatic vessels

Peripheral lymphatic vessel function was determined by injecting Evans Blue (30 µl, 30 mg/ml in 0.7% sodium chloride (NaCl)) into footpads of anesthetized mice. After 30 min, the mice were euthanized and transport of the dye to the thoracic duct was evaluated. For lipid absorption test in the gut, mice were pre-treated with WR1339 (Tyloxapol;Sigma) at 500 mg/kg intraperitoneal (i.p) for lipase inhibition one hour prior to oil Gavage.

Histological analysis of the mouse aortas

Mice were euthanized with carbon dioxide (CO2) or decapitation or perfused with phosphate-buffered saline (PBS) after over dose of anesthetics through the left ventricle. Aortas were dissected from the aortic arch to bifurcation and fixed in 4% PFA-PBS overnight. Aortas were opened longitudinally and attached to a black surface. For hematoxylin-eosin, modified Movat's pentachrome and immunohistochemical stainings, tissue samples were collected in 4% PFA-PBS for overnight fixation, processed to paraffin blocks and cut into 4 or 7 µm sections. For Oil-red-O staining, tissue samples were snap-frozen in liquid nitrogen and cut into 10 µm frozen sections. Cross-sectional lesion areas were quantified from aortic roots stained with hematoxylin-eosin. Percentage of the lesion area in the intima was calculated from tissue sections at the aortic sinus level, defined by the presence of three valve cusps. Vascular outward remodeling was determined by measuring the area within the internal elastic lamina.

Immunofluorescence/Immunohistochemistry

Tissue sections ranging between 10 and 100 µm in thickness were fixed overnight with 4% PFA or with cold acetone immediately before the staining, washed with PBS and blocked with TNB (PerkinElmer). Sections were washed with TNT buffer and the primary antibodies were detected with the appropriate Alexa 488, 594 or 647 secondary antibody conjugates (Molecular Probes/Invitrogen). Whole mount tissues were fixed overnight in 4 % paraformaldehyde (PFA), followed by washeing in PBS and staining with antibodies in donkey immune mix. For analysis of the retinal microvasculature, tissues were stained with biotinylated Griffonia simplicifolia lectin (Vector

Laboratories), followed by immunostaining. All fluorescently labeled samples were mounted with Vectashield containing 4,6-diamidino-2-phenylindole (DAPI, Vector Laboratories).

In vivo studies

Cre-recombinase activation
For induction of CreERT2 mediated recombination in embryos, the mother was injected at the indicated days with two consecutive intragastric doses of 4-OH tamoxifen (4-OHT) (Sigma) (25 mg/ml dissolved in 100 µl of ethanol/olive oil). In the neonatal mice, the Cre-mediated recombination was induced between P1 and P5 by daily intragastric administration of 4-OHT (2 µl at 25 mg/ml dissolved in ethanol). Recombination in adult mice (7–8 weeks old) was done by intragastric tamoxifen (Sigma, dissolved in 100 µl of corn oil at 2 mg/ml) administration during five consecutive days. In some experiments, Cre was induced by subcutaneous implantation of sustained tamoxifen-release pellets.

Tumor experiments
B16-F10-Luc2-G5 mouse melanoma or mouse LLC cells were maintained in DMEM, supplemented with 2 mM L-glutamine, penicillin (100 U/ml), streptomycin (100 µg/ml), and 10% fetal calf serum (Promo Cell). For B16-F10-Luc2-G5 cells, zeocin was added at a final concentration of 0.3 mg/ml as a selection marker. The B16 and LLC syngeneic tumor grafts were established by injecting 2-4*10^6 cells into the subcutaneous space of the abdominal flank.

High Fat Diet (HFD) studies
For the HFD experiments, the mice were treated with tamoxifen at the age of 7-8 weeks. The diet (60% fat, D12492, Research Diets Inc.) was started approximately 4 weeks later and continued for an additional 8 weeks. The glucose tolerance test (1g/kg; GTT) and the insulin tolerance test (0.75 U/kg; ITT) were performed after 6 hours of fasting. Feces were collected for lipid analysis. Mouse body composition was analyzed by dual-energy x-ray absorptiometric (DEXA) densitometry (Lunar PIXImus 2, GE Heathcare) after 8 weeks of HFD. For lipid absorption test, mice were pre-treated with WR1339 (Tyloxapol;Sigma) 500 mg/kg i.p. for lipase inhibition one hour prior to oil Gavage.

Treatment with the receptor tyrosine kinase (RTK)-inhibitor Sunitinib was done by daily intra-gastric administration at a concentration of 60 mg/kg for 14 days. Sunitinib was diluted to 1,8% NaCl, 0,1 % Tween20, 0,5 carboxy-methyl cellulose solution. Control-treated mice were administered the same solution without Sunitinib.

Stress anemia model
Phenylhydrazine hydrochloride (PHZ; 60 mg/kg, i.p) or 5-fluorouracil (5-FU; 150 mg/kg, intraperitoneally) was injected into adult mice at the age of 13 and 16 weeks, respectively. Peripheral blood (PB) was collected from the saphenous vein into heparinized capillaries (Hirschmann laborgerate) every 3 days after PHZ or 5-FU treatment. Analysis was then performed using Exigo automated blood analyzer (Boule Medical AB).

Microscopy

Immunofluorescence images were taken with a compound fluorescent microscope (Zeiss Axioplan 2, Carl Zeiss; 5x objective with numerical aperture (NA) 0.15; 10x 0.30) or a confocal microscope (Zeiss LSM 510Meta, objectives 10x with NA 0.45, oil objectives 10x with NA 1.3 and 63x with NA 1.4 or with a Zeiss LSM780 (10x with NA:0.45 20X with NA:0.80). Three-dimensional projections were digitally reconstructed from confocal z stacks. Bright-field sections were viewed with a Leica DM LB microscope (Leica Microsystems), and images were captured with an Olympus DP50 color camera (Olympus Soft Imaging Solutions GMBH).

Mouse embryo stimulation

E10.5-E11.5 NMRI wild-type embryos were excised from amnionic sacs and placed in Dulbecco's modified Eagle's medium (DMEM) containing 0.2% bovine serum albumin (BSA) on ice. The embryos were injected through the outflow tract with 0.5 ml of DMEM containing recombinant proteins. Embryos were placed in DMEM containing the same concentration of growth factors, incubated at 37^{o}C for 20 min and then lysed in lysis buffer. Insoluble materials were removed by centrifugation at 14 000g for 15 min.

Real-time quantitative PCR

Total RNA from tissues or cultured cells was isolated using the RNeasy Mini Kit (Qiagen) or NucleoSpin RNA II Kit (Macherey-Nagel). Homogenization was carried out using rotor-stator homogenization, followed by on-column DNase digestion (RNase-Free DNase Set, 79254). Quality control of samples was carried out using a Nanodrop ND-1000 spectrophotometer. RNA was reverse-transcribed using the DyNAmo cDNA Synthesis Kit or according to the manufacturer's instructions. Three qRT-PCR reactions were carried out from every *in vitro* transcription reaction using TaqMan Gene Expression Assays (Applied Biosystems) and the DyNAmo Probe qPCR Kit (F-450S, Finnzymes) or the iQ Supermix Kit. qRT-PCR was carried out using a BIO-RAD C1000 Thermal cycler according to a standardized protocol.

Statistical analysis

Quantitative data were compared between groups by two-tailed unpaired t-test or one-way ANOVA followed by post hoc tests for multiple comparisons. Values are expressed as mean +/- SEM or +/- SD. P-value < 0.05 was considered significant**.**

X-gal staining of the tissues

Tissues were dissected and immersed into cold washing buffer (1M MgCl2, 1% deoxycholate, 2% Nonidet P-40, 0.1 M Phosphate buffer pH 7.3) and then fixed in fixing solution (25% glutaraldehyde, 100mM EGTA pH 7.3, 1M MgCl2, 0,1M phosphate buffer pH 7.3) for 4 h at RT, washed 3x15 minutes with washing buffer, stained with X-Gal solution overnight at 37^{o}C for β-galactosidase activity. Alternatively, fixing was done at +4^{o}C. and the staining at RT. Tissues were then washed for 8-24 h in washing buffer and fixed with 4% PFA overnight at

+4^{o}C. Next day, samples were washed with PBS and processed for paraffin embedding.

A detailed description of the methods can be found in the original publications

RESULTS AND DISCUSSION

Study I: VEGFR-3 is required for blood vessel formation via Notch signaling

New blood vessels rise mainly from pre-existing ones via sprouting during angiogenesis (Chung and Ferrara, 2011). The irreplaceable key players in this process are VEGF (Carmeliet et al., 1996; Ferrara et al., 1996) and its primary receptor VEGFR-2 (Gille et al., 2001). Hierarchical growth of the developing vascular bed is crucial for proper vascular function. Hierarchy is based on specification of the tip and stalk cells of the sprouts (Jakobsson et al., 2009). This is controlled by VEGF induced delta-like 4 (Dll4) expression in the tip cells and Notch1 activation in the stalk cells (Jakobsson et al., 2009). The role of VEGFR-3 in blood vessel development is evident from embryonic studies, where *Vegfr3* deletion led embryonic death as early as E9.5 (Dumont et al., 1998). Whereas in intrauterine development VEGFR-3 expression is later restricted lymphatic vessels (Kaipainen et al., 1995), has been shown that inhibition of VEGFR-3 can inhibit also pathological angiogenesis to some extent (Tammela et al., 2008). On the other hand VEGFR-3 has been linked to sprouting angiogenesis via Notch signaling (Tammela et al., 2008) and it has been proposed to have a suppressing role that prevents overactivation of VEGFR-2 in the blood vessel (Matsumura et al., 2003).

In this study, we show that genetic deletion of *Vegfr3* from BECs leads to blood vascular hyperplasia with increased vessel sprouting, branching, and cell proliferation in the neonatal mouse retina angiogenesis model (Figure 1, I). This was also confirmed by careful examination of the developing vascular beds in the Chy-mice (Karkkainen et al., 2001), which have reduced signaling capacity in the VEGFR-3 pathway (Figure 2, I). These results conflict with previous findings that show that blocking of the VEGFR-3 pathway with antibodies inhibits angiogenesis (Tammela et al., 2008).

Phosphorylation of the VEGFR-2 was increased when VEGFR-3 was deleted from the endothelial cells, explaining the increased vascular phenotype of the *Vegfr3*-deleted mice (Figure 3, I). Our model indicates that when the VEGFR-3-mediated signaling is inactive, the potent VEGFR-2 signaling pathway is more active, resulting in increased endothelial cell growth. Loss of VEGFR-3 signaling from endothelial cells decreases expression of the Notch target genes *Hey1*, *Hey2,* and *Nrarp,* and a rescue experiment with Jagged1, a small Notch agonist peptide, reversed the effect of *Vegfr3* gene deletion, confirming the interplay between VEGFR-3 and Notch1 (Figure 4, I). It seems that VEGFR-3 suppresses VEGFR-2 signaling, and this balances the Notch cascade. However, if the VEGFR-3 pathway is blocked by genetic deletion, Notch-based suppression is not functional in the stalk cell compartment. VEGF-C stimulation of VEGFR-3 in BECs led to Notch activation and decreased sensitivity to VEGF signaling (Benedito et al., 2009; Tammela et al., 2008). However, this phenotype was only obtained by genetic deletion of *Vegfr3,* whereas blocking antibodies against VEGFR-3 did not produce similar effects (Tammela et al., 2008). This indicates that blocking of the receptor or its genetic deletion lead to different outcomes in the developing vascular plexuses. Blocking of VEGFR-3 with antibodies or kinase inhibitors can only target the active part of VEGFR-3

signaling, whereas the passive part of the signaling can be eliminated only via genetic deletion of the *Vegfr3*.

Thus, although VEGF-C is the main factor responsible for lymphatic vessel growth (Karkkainen et al., 2004), we show in this study that reduced levels of VEGF-C, by using the *Vegfc*$^{+/LacZ}$ mouse, cause a delay in normal blood vessel development in the neonatal retina, including decreased branching and Notch target gene expression (Figure 5, I). This phenotype differs from the endothelial cell *Vegfr3* deletion phenotype, which instead causes increasing sprouting of developing vascular bed. VEGF-C expression was found to be restricted to macrophages in the retina, being especially prominent at the fusion sites of the developing vessels. The fully processed form of VEGF-C can bind to VEGFR-2 (Joukov et al., 1996; Joukov et al., 1997). Manipulating the *Vegfc* levels did not entirely recapitulate the receptor deficiency, but instead led to growth retardation in the vascular plexuses. In both cases, the Notch activation pattern and normal tip/stalk cell contribution were altered.

Later studies have deepened our understanding about the interplay between VEGFR2 and VEGFR3 in angiogenesis. In 2012, Notch-dependent VEGFR3 upregulation was reported to allow angiogenesis without VEGF-VEGFR2 signaling (Benedito et al., 2012); however, a contradictory study revealed that VEGFR2 is required independently of VEGFR3 signaling for endothelial Dll4 upregulation and angiogenic sprouting (Zarkada et al., 2015). This later study speculates about the discrepant findings and concludes that major differences exist in some technical aspects (Zarkada et al., 2015). The differences between these studies could be explained by the incomplete VEGFR2 deletion and the specificity of the tools (e.g. the tyrosine kinase inhibitor) used to inactivate VEGFR3 in the previous studies.

Study I provides more thorough understanding of how VEGFR-3 regulates the conserved VEGF/VEGFR-2 pathway in angiogenesis, but further analysis is required to elucidate the exact molecular interactions contributing to the angiogenesis between VEGFRs and the Notch pathway. VEGFR3 signaling in BECs could be regulated by specific intra-cellular interactions of tyrosine residues that activate downstream signaling differentially depending of the context. This could lead to development of new clinical innovations by manipulating the VEGFR-3 in pathological angiogenesis.

Study II: Lymphatic vessel deficiency in mice promotes atherosclerosis

Cardiovascular diseases (CVDs) and cancer are the leading causes of mortality in Western countries. The progression of atherogenesis has been characterized well during the past decades. High low-density lipoprotein (LDL) levels in the blood predisposes to lipid accumulation to the intima of arteries, which later is followed by macrophage infiltration and active proliferation of the smooth muscle cells (Lusis, 2000). Active angiogenesis has been shown to occur at sites of atheroma development (Moulton, 2006), but so far the role of lymphatic vessels in this process has been poorly described. In this study, our aim was to investigate how atheromas develop upon lymphatic vessel insufficient mouse strains in a hypercholesterolemic mouse strain fed HFD.

First, we were able to show that in *Chy* mice (Karkkainen et al., 2001) cholesterol levels are significantly higher than in their WT littermates prior to HFD and K14-VEGFR-3-Ig (Makinen et al., 2001a) mice showed a similar increased cholesterol level trend in comparison with their littermates after the HFD (Figure 1, II). When the K14-VEGFR3-Ig mice was then crossed with a hypercholestrolemic $LDLR^{-/-}/ApoB^{100/100}$ background, they showed a similar pattern triglyceride levels prior and after the HFD (Figure 2, II). The lipoprotein profile in the K14-VEGFR3-Ig; $LDLR^{-/-}/ApoB^{100/100}$ mice were compared with that in the $LDLR^{-/-}/ApoB^{100/100}$ mice. An increased LDL fraction was observed in K14-VEGFR3-Ig; $LDLR^{-/-}/ApoB^{100/100}$ mice (Figure 3, II) and the subsequent *en face* analysis of the large arteries indicated larger atheromas in the aorta in these mice. The most striking differences were observed when the mice were challenged with HFD in young or middle age and especially in the early stages of the disease (Figure 4, II). This indicates that lymphatic vessels and elevated lipoprotein levels have a strong impact on the onset of the atheroma formation.

The other atheroma mouse model $ApoE^{-/-}$ has been shown to have impaired lymphatic drainage and abnormal lymphatic vessels in the skin (Lim et al., 2009). This shows that there is a connection between elevated cholesterol levels and lymphatic dysfunction. This was the first indication that high lipoprotein levels and lymphatic vessels interact. Later, it was discovered that the lymphatic vessels have an important role in reverse cholesterol transport (RCT) and this transport function was meditated by SR-B1 (Lim et al., 2013). The lymphatic vessel-deficient *Chy* mice have also been shown to have impaired RCT, and the difference seems to be meditated by macrophages (Martel et al., 2013). Another study revealed that immune cell trafficking, one important function of lymphatic vessels that is crucial for atheroma formation, is compromised in K14-VEGFR3-Ig mice (Thomas et al., 2012).

It is thus obvious that dysfunctional lymphatic vessels affect elevated lipoprotein levels in the blood circulation and that RCT is impaired when lymphatic vessels are lacking or poorly functional. Our results show that the lymphatic vessels are present in the areas where the plaques are develop and that soluble VEGFR3 prevents lymphatic neovascularization (Figure 6, II). The more rapidly advancing atheromas in the K14-VEGFR3; $LDLR^{-/-}/ApoB^{100/100}$ mice might be generated because the dysfunctional lymphatic vessels cannot clear lipids from the developing plaques.

So far, human studies have shown increased levels of VEGF-C, VEGF-D, and LYVE-1 in postmortem atheroma samples compared with healthy controls (Grzegorek et al., 2014). This indicates that cytokine secretion activates the lymphatic system and this later causes increased lymphangiogenesis via directional sprouting and vessel growth towards the inflamed atheroma plaque combined with lymphocyte/macrophage trafficking in atherosclerosis (Grzegorek et al., 2014). Preliminary studies from mice propose that extracellular vesicles could be used as biomarkers for inflammatory progression and as an indicator for lymphatic vessel dysfunction (Milasan et al., 2016).

Open questions remain regarding the interplay between lymphatic vessels and CVDs. For example, how do lymphatic endothelial cells contribute to plaque development? Is increased lymphangiogenesis in the atheroma lesions

beneficial for disease prevention, and how does RCT act in atheroma plaques compared with healthy tissues? These questions should be answered by using more sophisticated models in order to determine whether lymphatic vessels can actively clear the adverse lipid particles from the atheroma and to what extent? In murine models, however, it is not possible to mimic all of the aspects of atherosclerosis, as we know that the disease progression and also the tissue itself differ from those in humans.

Overall our study revealed that lymphatic vessels are an active player in the progression of atherosclerosis and they can be seen as a potential target for clinically relevant treatments of atherosclerosis.

Study III: Intestinal lymphatic vessels need constant VEGF-C/VEGFR-3 pathway signaling for maintenance and function

Already more than a decade ago it was shown that VEGF-C is indispensable for lymphatic vessel development (Karkkainen et al., 2004). VEGF-C driven pathological lymphangiogenesis has been under profound investigation especially in the field of lymphedema and cancer metastasis research, where VEGF-C or its inhibition may provide possibilities for disease therapy (Alitalo, 2011). However attempts to understand the role of VEGF-C expression in quiescent tissues have not been made to the same degree, mainly because tools for this have been lacking. One of the main tasks of lymphatic vessels is the lipid absorption from the gut. This task is carried out by special blind-ended capillaries, called lacteals, which differs from the other lymphatic capillaries, for example in their junctions, which are zipper-like (Bernier-Latmani et al., 2015; Choe et al., 2015). Lacteals are located in the villus, the epithelial cell covered extension in the intestinal wall, where they are covered by blood capillary network (Kamba et al., 2006). The entering of the chylomicron particles in the lacteals has been shown to use both paracellular and transport meditated delivery strategies (Dixon, 2010). In this decade there have been reports about unexpected function of lymphatic vessels in the homeostasis. We generated the conditional Cre-Lox *Vegfc* mouse to address the question about the role of VEGF-C in adult tissues (Aspelund et al., 2014). In this study, we wanted to clarify the role of *Vegfc* in adult mice, especially in the gut, where lymphatic vessels are under constant stress because of active nutrient absorption (Dixon, 2010).

We first showed that the *Vegfc* targeted conditional alleles are effectively deleted pre- and postnatally in the intestine used as the model organ. Our findings showed that developing lymphatic vessels are dependent on VEGF-C in all stages of their development. The surprising finding however was that the intestinal lymphatic vessels, both lacteals and the vessel plexus in the intestinal wall, goes through atrophy when *Vegfc* expression is eliminated in the adult and this phenotype was aggravated in the *Vegfd* deficient background (Figure 1, III and Figure 3). The atrophy was restricted to the initial lymphatic vessels of the gut and it did not occur for example in the skin even after prolonged deletion periods.

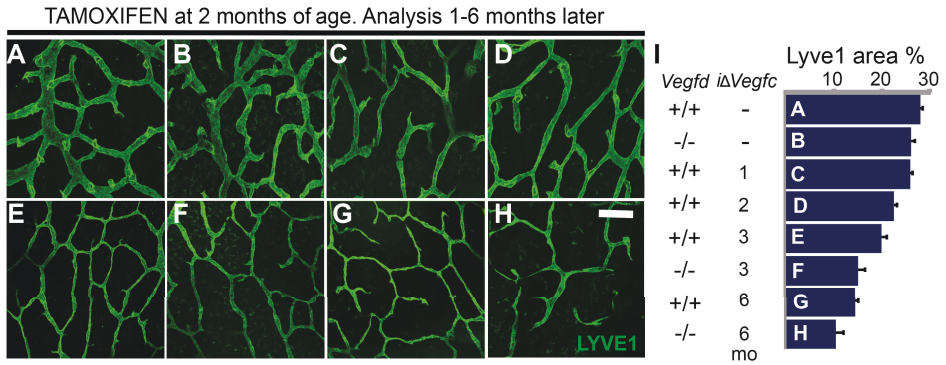

Figure 3. VEGF-C is required for intestinal lymphatic vessel maintenance in adult mice. A-H) Lymphatic vessels staining with LYVE1 after global *Vegfc* deletion in the intestinal wall of adult mice. Detailed information about genotypes and deletion durations are provided in Study III. I) Quantification of LYVE1 areas in A–H. Duration of the *Vegfc* deletion is indicated in months (mo), and *Vegfd* indicates the VEGF-D genotype. Scale bar 400 µm. Data are represented as mean +/- SEM. *P > 0.05 vs. WT.

Dynamic control of the lymphatic vessel maintenance in the gut was later confirmed by using a *Dll4* conditional mouse model, where lacteals start to regress in adult mice after lymphatic vessel specific *Dll4* deletion (Bernier-Latmani et al., 2015). The simultaneous finding from our group indicates that *Vegfr3* deletion in adult tissues reduces lymphatic vessel caliper in the skin and trachea (Zarkada et al., 2015) suggests that lymphatic vessel signaling mechanism differ between different organs. This finding is important regarding the possible clinical applications, as it emphasizes the consideration of how different vascular plexuses behave under inhibition of the VEGF-C/VEGFR3 signaling pathway.

A second important finding was that in adult animals, *Vegfc* expression is restricted to the smooth muscle cell compartment (Figure 2, III). Previous *Vegfc* expression studies have been carried out only in embryos (Karkkainen et al., 2004; Le Bras et al., 2006), and this was the first time that we were able to locate the exact source of VEGF-C expression in the gut. The intestinal lymphatic vessel atrophy was a slow process and by itself did not affect the overall well-being of the mouse, despite the fact that the intestinal lymphatic vessels have a crucial role in fat absorption from the gut. We challenged these mice with HFD and observed that the weight gain of the *Vegfc* deficient mice was slower and their obesity related metabolic parameters were improved when compared to their littermates, which have normal lymphatic vessels. An obvious consequence of the lymphatic phenotype was that the cholesterol and free fatty acid levels in the stools were increased because of their impaired absorption in the gut (Figure 3, III). Lacteal shortening and intestinal wall lymphatic vessel diminution were shown to be crucial for proper lipid absorption, however we could not exclude additional effects of the VEGF-C in the intestine. To our surprise, lipid accumulation was not observed in the intestinal stroma, suggesting a signaling mechanism that regulates lipid uptake to enterocytes, adjusting it to further transport level inside the intestinal villus.

These findings as such highlight the differences between lymphatic vessels in different parts of the body and on the other hand raise the question of the pathophysiology of the intestinal lymphatic vessels. It has been shown in mouse models that inflammatory bowel disease (IBD) affects the gut lymphatic vessels and by blocking expansion of these vessels via interrupting the VEGF-C signaling the symptoms become more severe (D'Alessio et al., 2014). The role of lymphatic vessel expansion during IBD was described already decades ago (Van Kruiningen and Colombel, 2008), but comprehensive understanding has been lacking about the molecular dynamics underlying this event.

Additional studies are required to reveal how vascular growth factors and intestinal lymphatic vessels are involved in gut homeostasis and in the pathological process beyond nutrient absorption.

Study IV: VEGF-C functions in embryonic erythropoiesis

Twenty years ago, it was shown that especially VEGF (Ferrara et al., 1996) and VEGFR-2 (Shalaby et al., 1995) are essential in the early steps of hematopoiesis development. In mammals there are three waves of hematopoiesis, namely the primitive, transient and definitive stages (Tober et al., 2007) and a host of factors regulate these processes (Palis, 2014). However open questions still remain in the regulation mechanisms that influence the primitive blood cell migration, proliferation and differentiation in several hematopoietic sites in the developing embryo. In mouse embryos, constitutive *Vegfc* deletion causes embryonic lethal phenotype around E16 (Karkkainen et al., 2004). Heterozygous Vegfc mice survive, but not in Mendelian ratio or in all backgrounds, for example in the C57Bl/6 background for unknown reason (Karkkainen et al., 2004). Our aim was to clarify if *Vegfc* has other necessary functions during embryogenesis beyond the lymphatic vessel development. By using a Cre-Lox conditional *Vegfc* mouse (Aspelund et al., 2014) we tackled this question by inducing *Vegfc* deletion as early as on embryonic day (E) 7.5 in the C57bl/6 background.

Besides an evident edema phenotype, we observed a tremendous decrease in liver size by day E13.5, which was further found to occur because of decreased erythropoiesis involving increased apoptosis of erythrocytes and their supporting macrophages (Figure 1, IV and Figure 4). When we analyze the peripheral blood of these embryos, we detected increased amounts of nucleated red blood cells in the circulation and overall signs of immature erythropoiesis (Figure 2, IV). This was later confirmed with the aid of flow cytometry and qPCR analysis (Figure 3 IV). These findings were similar to observations regarding the impairment of erythropoiesis shown previously in CCBE1 (Zou et al., 2013) and ADAMTS3 (Janssen et al., 2016) deleted mice. Both of these molecules are actively involved in the proteolytic processing of VEGF-C (Aspelund et al., 2016).

By using the conditional Vegfc deletion, we next investigated the sensitive time period when Vegfc is required for normal erythropoiesis. To our surprise we did not observe deficient erythropoiesis when *Vegfc* deletion was induced at E10.5 or later. Further, our investigation showed that VEGF-C has a crucial role in

fetal liver colonization of the erythroid-myeloid progenitors (EMPs) and later, in the expansion of the macrophage population in the liver (Figure 4, IV). It has been previously reported that α4β1 and α5β1 integrins are important for erythropoiesis (Eshghi et al., 2007; Ulyanova et al., 2011). Our data indicated that α4 integrin levels were reduced in erythromyeloid progenitors (EMPs) after VEGF-C deletion.

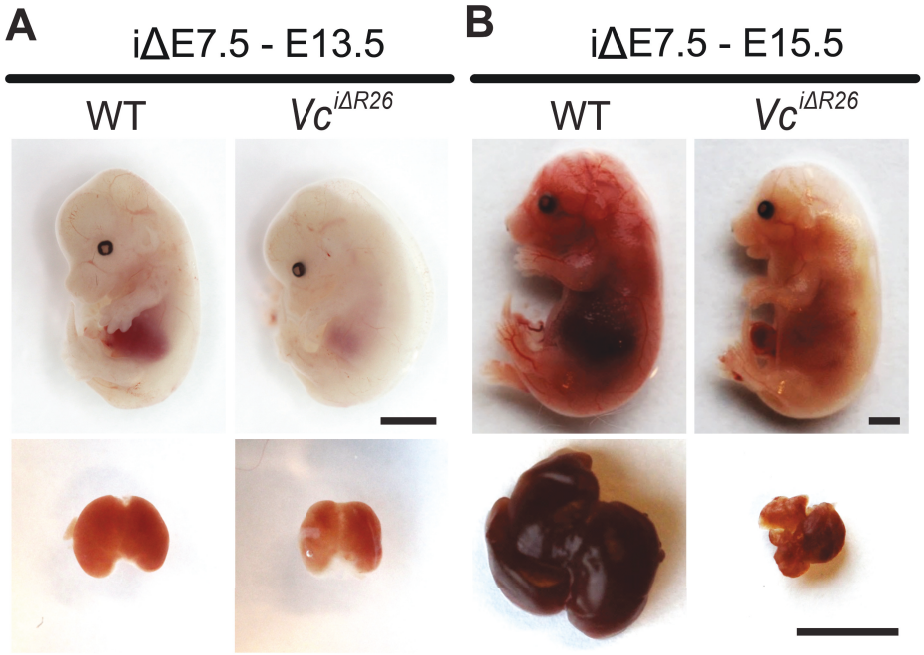

Figure 4. Decreased fetal liver size after global deletion of *Vegfc* at E7.5. A-B) Representative images of embryos and their livers at E13.5 and E15.5 after global *Vegfc* deletion ($Vc^{i\Delta R26}$) at E7.5.

We summarized our findings in a model in which VEGF-C regulation of erythropoiesis happens in a yolk sac, in embryonic blood circulation, and later in the fetal liver. Vegfc acts via influencing α4 integrin levels and fetal liver colonization of EMPs, and, later on, erythrocyte maturation (Figure 5, IV). Taken together, our data indicate that *Vegfc* is a major regulator in fetal erythropoiesis, but not in adult erythropoiesis in homeostatic conditions. Further studies are required to determine whether the VEGF-C/VEGFR-3 signaling pathway regulates the bone marrow stem cell niche in the adult mouse. These studies will require specific experiments that challenge the bone marrow, as it is the main regulator of blood cell production in adult animals.

These findings revealed an unexpected role of the VEGF-C in the regulation of embryonic erythropoiesis. It has not earlier been shown that this molecule, which is responsible for lymphatic vessel expansion during development (Karkkainen et al., 2004) has such an important role in blood cell formation. The signaling cascade behind this event was surprising, as it seems to indicate a rather minor role of the well-known VEGF-C-VEGFR2/VEGFR3 pathway in this process. A scheduled deletion scheme enabled this investigation, and it might

be that more precise investigation of the VEGFR2/VEGFR3 pathway will pinpoint some similarities regarding the erythropoiesis phenotype.

In adult animals, erythropoiesis is restricted mainly to the bone marrow. Further investigation is required to analyze the possible role of VEGF-C in hematopoietic stem cell regulation under hematopoietic stress.

CONCLUDING REMARKS

Studies described in this thesis have deepened our knowledge regarding the VEGF-C/VEGFR-3 pathway in embryo development and pathological conditions. Previously it was well established that VEGF-C is necessary for lymphatic vessel development (Karkkainen et al., 2004) and that VEGFR-3 is necessary for normal embryonic blood vessel development even prior its function in lymphatic vessel growth (Dumont et al., 1998). Findings reported in this thesis have brought new insights to the essential functions of VEGF-C and VEGFR-3.

In the first study of this dissertation, we show how VEGFR-3 is a critical modulator of normal angiogenesis during developmental angiogenesis and in the last study we show that VEGF-C has a previously unidentified role in the regulation of the embryonic erythropoiesis. Both of these studies revealed new and previously unknown roles of these molecules during development. These findings expand our view in terms of the basic research and the new information might turn out to be later useful for clinical therapeutics development. Targeting VEGFR-3 in pathological angiogenesis may be advantageous compared with targeting the traditional VEGF/VEGFR-2 pathway, which strongly affects healthy and normal blood vessels by e.g. increasing leakage. The VEGF-C/VEGFR-3 axis may have important functions in the pathogenesis of obesity and cardiovascular disease. The second study of this dissertation highlights the role of lymphatic vessels in the formation of atheromas. Based on these results, the deficiency on the lymphatic vessel function enhances atherosclerosis. This brings up the question for the possible medical opportunities to increase lymphatic vessel function to prevent the plaque formation or even to reduce already existing plaques. We could also show that constant VEGF-C/VEGFR-3 signaling is needed for proper lipid absorption from the intestine. When interruption occurs in the VEGF-C/VEGFR-3 pathway, lipid absorption is decreased by the atrophy that develops in the intestinal lymphatic vessels. Further studies are required for better understanding of how lymphatic vessels and the VEGF-C/VEGFR-3 pathway contribute also to intestinal inflammation.

These results have increased our understanding of the functions of lymphangiogenic growth factors. Especially Studies II, III, and IV have revealed previously unknown interactions and phenomena requiring further investigations to gain a comprehensive overview of the role of the VEGF-C/VEGFR-3 signaling pathway in these biological incidents.

My humble wish is that these findings will benefit the research community and eventually the well-being of human patients with various diseases.

ACKNOWLEDGMENTS

This work was carried out during 2009-2016 in the Translational Cancer Biology Laboratory/Program, which is a part of the Research Program Unit at the Faculty of Medicine of the University of Helsinki, Finland.

I am deeply grateful to my supervisor, Professor Kari Alitalo of the Finnish Academy of Sciences, for providing me with the opportunity to work in his laboratory and for his constant support throughout the thesis process. His never-ending interest in new findings and contagious enthusiasm for challenges create an atmosphere that truly drives young scientists to achieve (almost) impossible goals. The infrastructure created by Professor Alitalo is unique in Finland and it enabled the high-quality *in vivo* studies included here.

Professors Päivi Ojala and Timo Otonkoski from the University of Helsinki are kindly acknowledged for their participation in my thesis committee. Professor Klaus Elenius from the University of Turku and Professor Juha Partanen from the University of Helsinki are thanked for reviewing and commenting on the manuscript of this thesis. Carol Ann Pelli is thanked for editing the language of this thesis. The Doctoral Program of Biomedicine and its current and past directors have provided a multitude of training opportunities, for which I am most grateful.

I have been privileged to work with several excellent scientists. I thank Dr. Tuomas Tammela for trusting in my abilities to work in this laboratory and for bringing me into this exciting project. I owe a debt of gratitude to my first really hands-on supervisor Dr. Georgia Zarkada for showing me what it means to work in a top-quality scientific environment. I thank Dr. Wei Zheng for his unbelievable patience in creating new techniques that have been useful for the whole laboratory and especially for me.
There are two scientists who have been irreplaceable during my thesis project. Dr. Marius Robciuc joined the lab in 2012 and became my close collaborator. His enthusiasm and never-ending passion for research have greatly influenced me. Another exceptional scientist is Dr. Shentong Fang, who introduced me to a previously unfamiliar field. Her dedication to science is extremely high, setting an example for others. I warmly thank both of these scientists for their contributions to this thesis and especially for excellent collaboration.

In the past eight years, I have worked with numerous researchers who have influenced this thesis. I acknowledge all co-authors in our laboratory, in the University of Eastern Finland, and in other universities in Finland and abroad. I am extremely grateful to all past and current members of the Alitalo laboratory for creating an environment where scientific thinking can flourish.

This work would have not been possible without professional technical help from our own skilled laboratory engineers and technicians led by laboratory manager Tapio Tainola. I thank all of you for making my life much easier. I acknowledge the Biomedicum Imaging Unit (BIU) for providing imaging equipment and helpful personnel. I thank the Laboratory Animal Center for taking excellent care of the experimental mice.

I have been financially supported by personal grants from the following foundations: Ida Montin Foundation, Biomedicum Helsinki Foundation, Paolo Foundation, Orion Research Foundation, and Finnish Cultural Foundation. The Doctoral Program of Biomedicine and the Leducq Foundation have also provided funding via the University of Helsinki.

I thank my boys, my friends, and my band of brothers for cheering me up when I have felt defeated and for joining the celebration when science has been fruitful. My mother and father have supported me in every step I've taken in life, even though I am not sure they really know what I have been doing in the past eight years. From them, I have learned that you need to work hard to achieve something. I thank you both for that. I also thank my sister's family, my other friends and relatives, and the Koskinen clan for support on this bumpy road.

There is one person I cannot thank enough, the brave woman who shares her life with me, my dear Eeva. During this project I have encountered several obstacles that would not have been overcome without your constant support. All of the patience and foresight that you have shown during this project have made me understand what it means to have someone stand beside you no matter what. I just hope that I can provide you with the same steadfastness and security now and always.

Helsinki, April 2017

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