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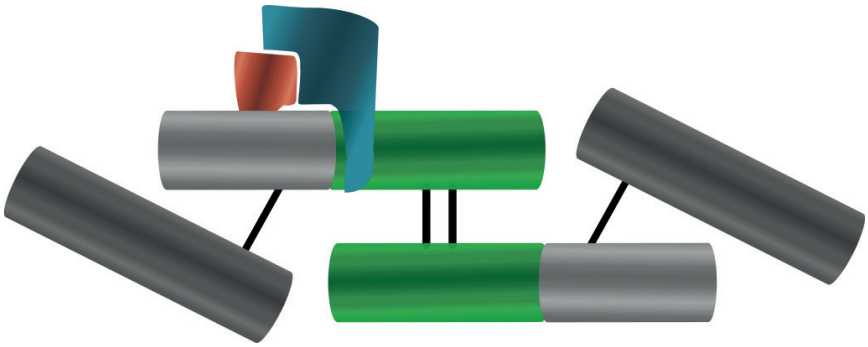
SAWAN KUMAR JHA

MECHANISM OF VEGF-C ACTIVATION AND EFFECT ON LYMPHATIC VESSEL GROWTH AND REGENERATION



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Mechanism of VEGF-C Activation and Effect on Lymphatic Vessel Growth and Regeneration

Sawan Kumar Jha

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To everyone who has been part of my life

'*Few are those who see with their own eyes and feel with their own hearts*'
- Albert Einstein

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LIST OF ORIGINAL PUBLICATIONS

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I. Jeltsch M, **Jha SK**, Tvorogov D, Anisimov A, Leppanen VM, Holopainen T, Kivela R, Ortega S, Karpanen T, and Alitalo K. CCBE1 enhances lymphangiogenesis via a disintegrin and metalloprotease with thrombospondin motifs-3-mediated vascular endothelial growth factor-C activation. Circulation *129*, 1962–1971 (2014).

II. **Jha SK**, Rauniyar, K, Karpanen T, Leppanen VM, Brouillard P, Vikkula M, Alitalo K, and Jeltsch M. Efficient activation of the lymphangiogenic growth factor VEGF-C requires the C-terminal domain of VEGF-C and the N-terminal domain of CCBE1. Sci. Rep. *7*, 4916 (2017).

III. **Jha SK***, Rauniyar K*, Chronowska E, Mattonet K, Maina EW, Koistinen H, Stenman, UH, Alitalo K, and Jeltsch M. KLK3/PSA and cathepsin D activate VEGF-C and VEGF-D. eLife. 8, e44478 (2019). * Equal contribution.

ABBREVIATIONS

aa	amino acid
AAV 9	adeno-associated virus serotype 9
ADAMTS3	A disintegrin and metalloproteinase with thrombospondin motifs 3
BEC	blood endothelial cell
CCBE1	Collagen and calcium-binding EGF domain-containing protein 1
cDNA	complementary DNA
CHO	chinese hamster ovary
C-terminal	carboxy terminal
CV	cardinal vein
EC	endothelial cells
ECM	extracellular matrix
FLT4	Fms related receptor tyrosine kinase 4
HS	Hennekam Syndrome
HSPG	heparan sulfate proteoglycan
HUVEC	human umbilical venous endothelial cell
HSPG	heparan sulfate proteoglycan
Ig	immunoglobulin
K14	Keratin 14
KLK	Kallikrein
KLK3	Kallikrein related peptidase 3
LEC	lymphatic endothelial cell

LV	lymphatic vessel
MD	Milroy disease
mRNA	messenger RNA
Nrp	Neuropilin
N-terminal	amino terminal
PSA	Prostate-specific antigen
SMC	smooth muscle cell
TK	tyrosine kinase
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor

ABSTRACT

Lymphangiogenesis, the growth of the lymphatic vasculature, is a crucial process during embryonic development, and - if compromised by genetic damage - can lead to hereditary lymphedema. Although the molecular mechanisms that regulate the growth, development, and maintenance of the lymphatic vasculature have been researched with increasing intensity over the last 25 years, the therapeutic regeneration of lymphatic vessels is still a work in progress in the treatment of conditions such as lymphedema. Vascular endothelial growth factor-C (VEGF-C) is the primary growth factor responsible for the growth and development of the lymphatic vasculature. VEGF-C is activated by a complex process, which is indispensable for its ability to induce lymphangiogenesis via its primary receptor, VEGFR-3. The understanding of this process is a key factor for the development of VEGF-C as a drug target. The goal in my studies has been to increase our insights into VEGF-C activation at the molecular level, to identify its regulatory factors, and to establish its role in the lymphangiogenic process.

Absence of the collagen- and calcium-binding EGF domains 1 (CCBE1) protein interrupts the lymphangiogenic process at about the same developmental stage when VEGF-C is first required. We utilized cell-based assays and adeno-associated viral-based gene transduction to investigate the role of CCBE1 on VEGF-C activation. In study I, we identified A disintegrin and metalloprotease with thrombospondin motifs-3 (ADAMTS3) as a protease that cleaves and activates VEGF-C, resulting in the major mature form of VEGF-C. We showed that CCBE1 acts as a cofactor in this process by enhancing the ability of ADAMTS3 to activate VEGF-C. Correspondingly, CCBE1 augmented the lymphangiogenic potential of VEGF-C *in vivo*.

The presence of N- and C- terminal domains and their proteolytic cleavage characterize both CCBE1 and VEGF-C. In study II, we investigated the role of these domains for VEGF-C activation and the lymphangiogenic process. Our study demonstrated a requirement for the C-terminal domain of VEGF-C for the robust activation of VEGF-C both in vitro and in vivo. Moreover, we identified that the N- and C-terminal domains of CCBE1 have independent roles in the process of VEGF-C activation. The C-terminal domain accelerates the proteolytic cleavage, while the N-terminal domain aids in the assembly of the VEGF-C/ADAMTS3/CCBE1 cleavage complex by mobilizing VEGF-C to the endothelial cell surface.

In study III, we searched for additional proteases that can cleave VEGF-C. We identified kallikrein-related peptidase 3 (KLK3) in seminal plasma and cathepsin D in

saliva as proteases that cleave and activate VEGF-C. In human seminal plasma, we found substantial amounts of VEGF-C, which became activated concurrently with the semen liquefaction process. The newly identified VEGF-C cleavage sites are conserved in VEGF-D and we found that KLK3 and cathepsin D were able to activate VEGF-D as well. We also found that cleaved forms of VEGF-C and VEGF-D differ in their abilities to activate VEGFR-2 and VEGFR-3. When their N-termini were progressively shortened, the ability of VEGF-D to bind to and activate VEGFR-3 was decreased, while VEGF-C lost preferentially its ability to bind to and activate VEGFR-2.

These findings contribute to the existing knowledge on the mechanisms of VEGF-C activation and the functional consequences thereof, and provide new opportunities to target VEGF-C for therapeutic purposes.

REVIEW OF THE LITERATURE

1 Introduction

The term "growth factors" describes a structurally diverse group of extracellular signaling molecules that stimulate cell proliferation. Growth factors trigger cellular responses via often cell-type-specific receptors, which translate the extracellular signal into an intracellular signal. The arguably most important growth factor family for vascular endothelial cells - and therefore for blood and lymphatic vessels - is the VEGF family. The signal of VEGFs is mediated by the VEGF receptors, which constitute a subfamily within the receptor tyrosine kinases (RTKs). While the growth of blood vessels is largely dependent on the VEGF(-A)/VEGFR-2 signaling pathway, lymphatic vessels depend mainly on the VEGF-C/VEGFR-3 pathway. The discovery of several lymphedema-causing mutations led to the identification of several genes involved in the regulation of lymphatic vessel growth and development. Most notably, mutations in the collagen and calcium-binding EGF domains 1 (CCBE1) gene were identified in a subset of hereditary lymphedema cases, and in mice, the CCBE1 gene deletion phenotype resembled very closely the VEGF-C gene deletion phenotype. This phenotypic similarity indicated a close link of CCBE1 to VEGF-C, but mechanistically, their relationship remained unclear.

Many in-vitro and in-vivo studies have focused on the lymphatic endothelial cells, the lymphatic vasculature, and its function. Subsequently, studies have identified the role of the lymphatic vasculature for etiology and progression in diseases like cancer, inflammation, and lymphedema. However, the importance of VEGF-C activation for lymphatic vessel growth and development had largely been overlooked. Unlike the hemangiogenic VEGF-A, VEGF-C needs to undergo activation by proteolysis in order to achieve receptor activating potential. Since the lack of VEGF-C activation underlies several hereditary lymphedema conditions, it seems reasonable to pay attention to it when considering VEGF-C as a therapeutic target.

This study aimed to investigate the mechanism of VEGF-C activation, its regulation and role in lymphatic vessel growth and regeneration. Our results show that CCBE1 is required for the activation of VEGF-C through the protease ADAMTS3 during developmental lymphangiogenesis, as well as for the activation of VEGF-C via KLK3/PSA, which might be triggered during tumor lymphangiogenesis. These novel insights into the molecular mechanisms of VEGF-C-mediated lymphangiogenesis

provide cues for targeting VEGF-C in both pro-lymphangiogenic and anti-lymphangiogenic therapeutic strategies.

2 The vascular system

In vertebrates, the vascular system is fundamental for the transport of nutrients, gases, cells, hormones, signaling mediators, metabolic waste products, and fluid throughout the body. Broadly, the mammalian vascular system can be divided into the circulatory system (the vessels that carry blood) and the lymphatic system (the vessels that carry lymph), although the demarcation between these two systems can be for some individual vessel types blurry.

2.1 The cardiovascular system

The circulatory (or blood vascular) system consists of a closed network of blood vessels and the heart. Arteries carry blood from the heart into the peripheral tissues, and veins return blood from these tissues to the heart. The blood capillaries are the smallest vessels that connect the arterial and venous vasculature, where the bidirectional exchange of gases, nutrients, and waste occurs between the blood and the interstitial space. Blood vessels come in different types: as arteries, arterioles, veins, venules and capillaries based on their function and hierarchy. The wall of the arteries consists of three layers: the innermost layer (tunica intima) consisting of endothelial cells (ECs) sitting on the basement membrane, the middle layer (tunica media) comprising smooth muscle cells and elastins, and the outermost layer (tunica externa) composed of collagen bundles and fibroblasts. Blood capillaries, on the other hand, are simple structures composed of a single layer of ECs, the basal lamina, and a sparse pericyte coverage. Blood capillaries penetrate almost all organs of the body, but most extensively organs that are metabolically active like skeletal muscle, liver, and kidney. Veins are similar in structure to arteries except for their sparse coverage by smooth muscle and connective tissues, and for the presence of valves to prevent backflow of blood.

2.2 The lymphatic vascular system

The lymphatic vascular system, also initially ambiguously referred to as the secondary vascular system, is a blind-ended network of vessels. While its appreciation and scientific discovery has been lagging compared to the cardiovascular system, the importance of the lymphatic system has become increasingly clear over the last quarter of a century due to its involvement in many physiological and pathological processes.

The lymphatic capillaries or initial lymphatics are composed of oak leaf-shaped lymphatic endothelial cells (LECs), which are covered by a discontinuous basement membrane (BM), that lacks perivascular cells (pericytes/SMCs). Abluminally, the capillary LECs are attached via elastic anchoring filaments to the interstitium, which renders them responsive to interstitial pressure (Leak, 1968, 1970; Leak and Burke, 1966). The junctions between capillary LECs are discontinuous and button-like (Baluk et al., 2007). Interstitial liquid entering the lymphatic capillaries flows as lymph through collecting lymphatic vessels, which, unlike capillaries, feature perivascular cells, a basement membrane, continuous zipper-like junctions, and lymphatic valves (Tammela and Alitalo, 2010). The lymphatic valves ensure the directionality of the lymph propulsion, which is driven by an alliance of external forces (skeletal muscle contraction, respiration, and blood vessel pulsation) and internal forces (SMCs contraction, reviewed in (Moore and Bertram, 2018).

2.2.1 Function of lymphatic vessels

Lymphatic vessels (LVs) are present in most vascularized tissues but are absent in bone, brain, cartilage, and cornea. LVs maintain tissue fluid homeostasis by absorbing the excess fluid, macromolecules, and cells from the extracellular space and returning them into the blood circulation. LVs are an essential component of the immune surveillance: they transport antigens to lymph nodes and serve as a conduit for immune cells (Randolph et al., 2017). Another critical function of the specialized LVs in the intestinal villi (lacteals) is in the absorption of dietary lipids in the form of chylomicrons (Dixon, 2010). Intriguingly, the recent identification of lymphatic vessels in the meningeal layer of the brain (Aspelund et al., 2015; Louveau et al., 2015) and the lymphatic nature of the ring-shaped vascular structure surrounding the anterior eye ball (Schlemm's canal) (Aspelund et al., 2014) has energized the field of lymphatic biology. Recent report suggests that meningeal LVs at the base of skull also absorb cerebrospinal fluid (CSF) from the meninges (Ahn et al., 2019) which is in-line with their role in maintaining fluid balance, but interestingly, some lymphatic functions also extend to stem cells niche: e.g. they maintain hair-follicle stem cell behavior during tissue regeneration (Gur-Cohen et al., 2019; Peña-Jimenez et al., 2019).

2.2.2 The development of the lymphatic system

The development of the lymphatic vasculature initiates after the establishment of the blood vasculature. The molecular processes during the development are largely conserved among vertebrates, and researchers mostly use zebrafish and mice as models to study pathways involved in lymphatic growth and development (Butler et al., 2009; Semo et al., 2016). In mice, LECs differentiate from a subpopulation of

SOX18 positive ECs in the cardinal vein (CV) at E9.0, which later express Prox1 (Francois et al., 2008). However, the exact molecular switch that induces the differentiation remains unclear. A study by Srinivasan et al. suggests cooperation between SOX18 and orphan nuclear receptor COUP-TFII for the activation of *Prox1* in the CV around E9.5 (Srinivasan et al., 2010). PROX1 positive LEC progenitors bud off from the dorsal CV and sprout laterally in clusters or as individual cells to form primitive lymph sacs (François et al., 2012; Wigle et al., 2002). PROX1 is not only required to establish but also to maintain LEC identity. Conditional ablation of *Prox1* during embryonic development reverses the identity of LECs to BECs (Johnson et al., 2008), and exogenous PROX1 expression in BECs induces LECs identity (Hong et al., 2002; Kim et al., 2010). The migration and proliferation of LECs to form the primary lymph sacs at E10.5-E12.5 are dependent on VEGF-C/VEGFR3 signaling, and ablation of *Vegfc* in mice leads to a failure of LECs to migrate, and, consequently, the lymph sacs do not develop (Karkkainen et al., 2004). The role of VEGF-C during lymphatic development will be discussed later in detail.

2.2.3 Lymphangiogenesis versus lymphvasculogenesis

Two opposing theories were proposed for the origin of the lymphatic vessels in the beginning of the 20th century. Florence Sabin, in 1902, proposed that the first mammalian lymphatic vessels develop from the venous ECs by sprouting (Sabin, 1902). In contrast, Huntington and McClure, in 1908, proposed that mesenchymal precursor cells ("lymphangioblasts") would differentiate in-situ into the first lymphatic vessels. In 1932, van der Jagt observed that both mechanisms contribute to the development of the lymphatic system in the sea turtle (Van Der Jagt, 1932). The venous origin theory since then has been supported by many studies in mice (Hägerling et al., 2013; Srinivasan et al., 2007; Wigle and Oliver, 1999; Yang et al., 2012) and zebrafish (Küchler et al., 2006; Yaniv et al., 2006). On the other hand, studies in birds supported the mesenchymal precursor theory (Papoutsi et al., 2001). Recently, the use of lineage tracing models to study the organ-specific development of lymphatic vasculature confirmed that the lymphatics are of heterogeneous origin not only in birds and turtles but also in mammals. Organs with non-venous contributions to the lymphatic vasculature include the skin (Tie2 lineage negative precursor) (Martinez-Corral et al., 2015), mesentery (hemogenic endothelium precursor) (Stanczuk et al., 2015), and the heart (cKit lineage hemogenic-derived precursor) (Klotz et al., 2015).

2.2.4 Remodeling and maturation of lymphatic vessels

The expansion of the lymphatic network from the primitive lymph sacs occurs by sprouting lymphangiogenesis and results in the tree-like structure with the lymphatic

capillaries, pre-collectors, and collectors. The separation of the lymph sacs and the CV is mediated by platelets, which accumulate to seal off the original venolymphatic communication (Schulte-Merker et al., 2011). The remodeling and maturation of the lymphatic vasculature start with Foxc2 expression in LECs around E15.5. Foxc2 is indispensable for lymphatic remodeling, especially in the establishment of the collecting lymphatic vessels. Foxc2 deficient mice lack lymphatic valves in the collecting vessels and their lymphatic capillaries show an abnormally high pericyte coverage (Norrmén et al., 2009; Petrova et al., 2004). EphrinB2, which is expressed in LECs of collecting LVs, is another regulator of lymphatic remodeling and aids in the establishment of a hierarchy in the lymphatic network with functions including lymphatic valve formation, sprouting from lymphatic plexuses, and SMCs coverage (Makinen et al., 2005). Furthermore, ANG2 contributes to lymphatic maturation, as its deficiency in mice leads to lymphatic hypoplasia, lack of collecting lymphatic vessels and valves, and abnormal SMCs ensheathing of lymphatic capillaries (Dellinger et al., 2008; Gale et al., 2002). Interestingly, ANG2 also regulates the zipper to button transition in the initial lymphatics during the development (Zheng et al., 2014). Integrin-α9 is required for the proper structural formation of the lymphatic valves, and the ablation of *Itga9* results in disrupted lymphatic valve formation, which results in abnormal lymph flow (Bazigou et al., 2009).

3 Molecular regulation of angiogenesis and lymphangiogenesis

The regulation of the proliferative aspects of angiogenesis and lymphangiogenesis is mainly regulated by the VEGF/VEGFR signaling axes. However, many other factors and processes are required to ultimately form a well-established, hierarchical network of functional blood and lymphatic vessels. Interference with any of the regulatory processes can have significant impacts on the functionality of the networks and results in disease.

3.1 Vascular endothelial growth factors - The ligands

The mammalian VEGF gene family consists of 5 members: VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placenta growth factor (PlGF) (Figure 1). VEGFs are mostly secreted glycoproteins characterized by the presence of a central VEGF homology domain (VHD) containing eight highly conserved cysteine residues. Six of these cysteines form a cystine-knot structure, while the other two participate in the dimer formation via disulfide bonds (Holmes and Zachary, 2005). VEGFs undergo post-transcriptional and post-translational modifications, which control their biochemical properties and functions. The VEGFs are ligands for the vascular endothelial growth

factor receptors (VEGFRs), and the signaling is supported by several co-receptors and accessory molecules, such as neuropilins (Pellet-Many et al., 2008), integrins (Malinin et al., 2012), and heparan sulfate proteoglycans (van Wijk and van Kuppevelt, 2014).

3.1.1 VEGF-A

VEGF-A (also known as VEGF) was initially identified as a vascular permeability factor (Senger et al., 1983) because of its ability to induce vascular leakage. VEGF-A was independently discovered as a specific mitogen for endothelial cells (Ferrara and Henzel, 1989; Leung et al., 1989), and later recognized as the ligand for VEGFR-1 and VEGFR-2 (Quinn et al., 1993; de Vries et al., 1992). VEGFR-2 is the central signaling receptor for VEGF-A, despite the higher binding affinity of VEGFR-1 for VEGF-A (Peach et al., 2018). Binding of VEGF-A to VEGFR-2 induces angiogenesis

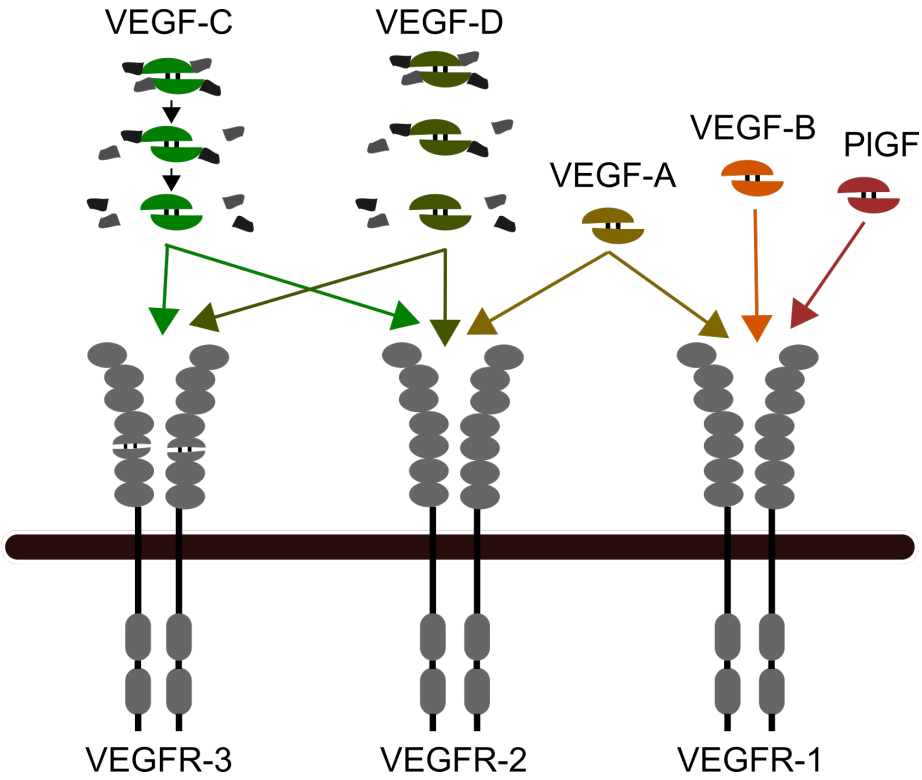

Figure 1: *VEGFs and VEGF receptors (VEGFRs). The major ligands and their interacting receptors on the endothelial cells are shown. VEGFR-1 is expressed mostly by blood vascular endothelial cells (BECs) and VEGFR-3 by lymphatic endothelial cells (LECs). VEGFR-2, on the other hand, is expressed by both LECs and BECs. Both VEGF-C and VEGF-D are activated by proteolytic excision of N- and C-terminal propeptides.*

and vasculogenesis via endothelial cell proliferation, sprouting, and migration (Apte et al., 2019).

3.1.1.1 Molecular properties and structure of VEGF-A

In most animals, the VEGF-A gene undergoes alternative splicing, which generates isoforms of variable length. The most common human forms include VEGF-A_{121}, VEGF-A_{165}, VEGF-A_{189}, and VEGF-A_{206}. Interestingly, isoforms (e.g. VEGF-A_{165b}) have also been identified which appear to inhibit VEGF-A signaling (Bates et al., 2013; Woolard et al., 2004). However, other studies have questioned the occurrence and inhibitory function of these VEGF-A_{xxxb} isoforms (Bridgett et al., 2017; Catena et al., 2010; Harris et al., 2012). Due to the different lengths of their C-terminal domains, the VEGF-A isoforms differ in their ability to bind heparan sulfates, neuropilin-1 (Nrp-1) and neuropilin-2 (Nrp-2) (Sarabipour and Gabhann, 2018; Soker et al., 1998). The shortest common isoform VEGF-A_{121} lacks almost completely heparin-binding and is therefore freely diffusible, while the longer isoforms VEGF-A_{189} and VEGF-A_{206} are strongly heparin-binding and therefore largely immobilized on heparan sulfate proteoglycans (HSPGs). The most prevalent form - VEGF-A_{165} - is an intermediate form with both heparin-binding and diffusible properties (Houck et al., 1991, 1992; Park et al., 1993).

VEGF-A isoform-specific deletions in mice suggest an essential role of the isoforms in vascular patterning. Exclusive expression of VEGF-A_{120} (equivalent to human VEGF-A_{121}) in the absence of VEGF-A_{164} and VEGF-A_{188} (equivalent to human VEGF-A_{165} and VEGF-A_{189}, respectively) results in neonatal death. These mice have relatively sparse and dilated blood vessels in most organs, but the most significant effect is observed in the heart, and the mice die because of heart failure (Carmeliet et al., 1999). Additionally, these mice show reduced vascular complexity and abnormally short and misdirected extensions of tip cell filopodia (Ruhrberg et al., 2002). Investigation of the vascular patterning in the retina of these mice revealed that VEGF-A_{164} contains all cues for normal vascular growth and remodeling. While the exclusive expression of VEGF-A_{120} resulted in a generalized impaired vessel growth, exclusive expression of VEGF-A_{188} resulted only in arterial growth defects (Stalmans et al., 2002). These findings were consistent with the bone growth phenotype of VEGF-A_{188} isoform expressing mice, which showed stunted bone growth resulting from apoptosis of chondrocytes (Maes et al., 2004). These isoform-specific expression studies indicate the importance of extracellular matrix (ECM)-binding properties of VEGF-A for the guidance of vascular growth, and demonstrate organ-specific vascular responses to specific isoforms.

Importantly, many functions of VEGF-A require neuropilins, and the VEGF-A sequences responsible for neuropilin binding overlap with the sequences that define the ECM-binding properties of VEGF-A (Krilleke et al., 2009). Neuropilins will be discussed in a separate section later. In addition to diversification by differential splicing, the longer isoforms of VEGF-A can be proteolytically cleaved by plasmin (Houck et al., 1992; Keyt et al., 1996; Plouët et al., 1997), urokinase (Plouët et al., 1997) and MMP-3 (Lee et al., 2005). However, the physiological significance of these proteolytically cleaved forms remains elusive. It has been speculated that by proteolytic cleavage, sequestered (and therefore inactive) VEGF-A might be released quickly (e.g. for wound healing purposes, (Roth et al., 2006).

3.1.1.2 VEGF-A is essential for vasculogenesis and angiogenesis

The deletion of even a single allele of VEGF-A in mice leads to embryonic lethality caused by defective angiogenesis and blood island formation (Carmeliet et al., 1996; Ferrara et al., 1996). On the other hand, an increase in expression of VEGF-A even by 2-3 fold can lead to embryonic death at E12.5-E14, resulting from severe developmental defects of the heart (Miquerol et al., 2000). Hence, the dosage of VEGF-A seems to be important during embryonic development, where its expression can be detected in mice as early as E7.5 (Dumont et al., 1995).

3.1.1.3 VEGF-A regulation and expression

The expression of VEGF-A is crucially regulated at the transcriptional level. VEGF-A is a hypoxia-responsive gene, and hypoxia-inducible factor (HIF-1) induces the transcription of VEGF-A mRNA and promotes its stabilization (Pugh and Ratcliffe, 2003). At the same time, VEGF-A is also regulated by a HIF1 independent mechanism, for example by nutrient-sensitive transcriptional coactivator protein PGC-1α (peroxisome-proliferator-activated receptor-gamma coactivator-1α) (Arany et al., 2008).

3.1.1.4 Biological function of VEGF-A

Transgenic or adenoviral expression of VEGF-A induces significant angiogenesis, but also vascular leakage and inflammation (Baluk et al., 2005; Detmar et al., 1998; Larcher et al., 1998; Thurston, 2002). The effect of VEGF-A on lymphangiogenesis has been controversial. The application of VEGF-A-encoding adenovirus in mice showed enlargement of the lymphatic vessels, with no effect on sprouting (Nagy et al., 2002; Saaristo et al., 2002; Wirzenius et al., 2007). Moreover, VEGF-A also induced lymphatic vessel growth in a corneal inflammatory model. This effect was shown to be indirect via VEGFR-1-mediated recruitment of macrophages, which secrete VEGF-C (Cursiefen et al., 2004). Forced VEGF-A expression in tumor models

results in enhanced tumor lymphangiogenesis and lymphatic metastasis (Björndahl et al., 2005; Hirakawa et al., 2005). Thus, VEGF-A-mediated lymphangiogenesis seems to occur - mostly or always indirectly - during pathological situations, but there is little if any evidence for a physiological role of VEGF in lymphangiogenesis.

3.1.1.4 Non-endothelial targets of VEGF-A

In addition to its role in vessel growth and regulation, several roles of VEGF-A in non-vascular contexts have been described. E.g. in the central nervous system, VEGF-A signaling promotes the survival, growth, and migration of neuronal cells (Mani et al., 2010; Rosenstein et al., 2003; Ruiz de Almodovar et al., 2011), and it promotes pain transmission by sensitizing VEGFR-1- and VEGFR-2-expressing neurons (Hulse et al., 2014; Lin et al., 2010; Nesic et al., 2010; Selvaraj et al., 2015; Yang et al., 2018). VEGF-A has also been shown to increase osteoblast activity and migration, suggesting a role in bone formation (Hiltunen et al., 2003; Mayr-wohlfart et al., 2002). Finally, in cancer, VEGF-A does not only act on vascular endothelial cells to establish the tumor vasculature, but it can act further directly on the cancer cells. E.g. it can promote TAZ activation and contribute to stemness in breast cancer cells via the Nrp2 pathway (Elaimy et al., 2018), and it can signal via VEGFR-2 on leukemic cells to promote growth and survival in an autocrine manner (Dias et al., 2000).

3.1.2 VEGF-B and Placenta growth factor (PlGF)

VEGF-B, also known as VEGF related factor (VRF) and PlGF VEGFR-1 ligands (Olofsson et al., 1998; Park et al., 1994). In humans, VEGF-B exists in two different isoforms (VEGF-B_{167} and VEGF-B_{186}), whereas four different PlGF isoforms exist in humans (PlGF1-4), but only one in mice (De Falco, 2012). Both VEGF-B and PlGF are largely dispensable for embryonic development in mice: the targeted deletion of *Vegfb* results only in a mild cardiac conduction defect (Aase et al., 2001), and the *Plgf* null mice show impaired blood vessel growth only under pathological conditions, such as ischemia, inflammation, wound healing and cancer (Carmeliet et al., 2001). VEGF-B is a less potent angiogenic growth factor than VEGF-A, and its overexpression in muscle or adventitial tissue via adenoviral vector has no effect on blood vessel growth (Bhardwaj et al., 2003; Rissanen et al., 2003). In contrast, VEGF-B induces angiogenesis in adipose tissue (Robciuc et al., 2016), and also acts as a coronary growth factor, induces physiological cardiac hypertrophy and protects the heart from myocardial ischemia (Bry et al., 2010; Huusko et al., 2012; Kivelä et al., 2014). The angiogenic potential of VEGF-B likely results from the competition of VEGF-B with VEGF-A for the decoy receptor VEGFR-1, displacing VEGF-A and hence making it better available for VEGFR-2 (Anisimov et al., 2013; Kivelä et al., 2019). In contrast, PlGF induces significant angiogenesis and vascular permeability (Luttun et al., 2002;

Odorisio et al., 2002), probably because it can activate VEGFR-1 better than VEGF-B (Anisimov et al., 2013).

3.1.3 VEGF-C

VEGF-C was identified and purified as a VEGFR-3-specific ligand from the conditioned media of the human prostate cancer cell line PC3 (Joukov et al., 1996). Later during the same year, murine VEGF-C was cloned from the human glioma cell line G61 and given the name VEGF-related protein (VRP) (Lee et al., 1996).

3.1.3.1 Molecular properties and structure of VEGF-C

VEGF-C, unlike VEGF, VEGF-B, and PlGF is characterized by the presence of N- and C-terminal propeptides flanking the VEGF homology domain (VHD) (Figure 2). While the N-terminal domain has no homology to other known proteins, the C-terminal domain of VEGF-C contains a repetitive cysteine-rich motif that resembles the motif in the silk-like protein produced by larval salivary glands of the midge *Chironomus tentans* (Joukov et al., 1996). The C-terminal domain is critical for VEGF-C function, and a mutant lacking the C-terminus (*vegfc*^{um18}) showed a secretion defect and halted lymphatic growth (Villefranc et al., 2013). Similar mutations were identified in patients with Milroy-like primary lymphedema (Balboa-Beltran et al., 2014; Gordon et al., 2013). Because of the secretion defect in these mutants, the physiological role of C-terminus of VEGF-C could not be established.

To better understand the role of the VEGF-C propeptides, a chimeric protein was generated, where the N- and C- terminal propeptides of VEGF-C flank the VHD of VEGF-A ("VEGF-CAC"; C refers to VEGF-C and A to VEGF-A). Adenoviral delivery of this chimera induced a widening of lymphatic vessels and an extensive branching of blood capillaries compared to $VEGF_{165}$ (Keskitalo et al., 2007), suggesting a role of propeptide in modulating VHD activity. Additionally, chimeric proteins generated by swapping the C-terminal propeptide of VEGF-C with heparin-binding domains of VEGF-A ($VEGF\text{-}CA_{65}$ and $VEGF\text{-}CA_{89}$) generated a sparse network of wider lymphatic capillaries, which preferably formed at locations with a high heparan sulfate concentrations such as basement membranes (Tammela et al., 2007a). Viral vectors expressing VEGF-C from a wild type full-length cDNA induce lymphangiogenesis distinct from viruses that deliver mature VEGF-C from a truncated cDNA (ΔNΔC-VEGF-C). The former induces a large mesh of narrower lymphatic capillaries, whereas ΔNΔC-VEGF-C induces a sparse but dilated network of lymphatic sprouts (Tammela et al., 2007a) resembling the effect of $VEGF_{120}$ on blood vessels (Lee et al., 2005).

The activity of VEGF-C, unlike many other growth factors, depends strictly on the proteolytic removal of its two propeptides flanking the VEGF-homology domain (VHD). The proteolytic removal of the propeptides is sequential and dictates VEGF-C binding affinity towards VEGFR-2 and VEGFR-3 (Joukov et al., 1997). The proteolytic processing involves first the cleavage of the C-terminal domain to yield polypeptides of 29/31 kDa, and then the removal of N-terminal propeptide and along with it, the C-terminal domain to generate a mature VEGF-C - 21/23 kDa form (Joukov et al., 1997). The incremental processing increases the affinity of VEGF-C towards VEGFR-3, and the final mature form also effectively activates VEGFR-2 (Joukov et al., 1997). The constitutive cleavage between the VHD and the C-terminal domain upon VEGF-C secretion has been shown to be mediated by proprotein convertases (PCs; Figure 2) (Siegfried et al., 2003).

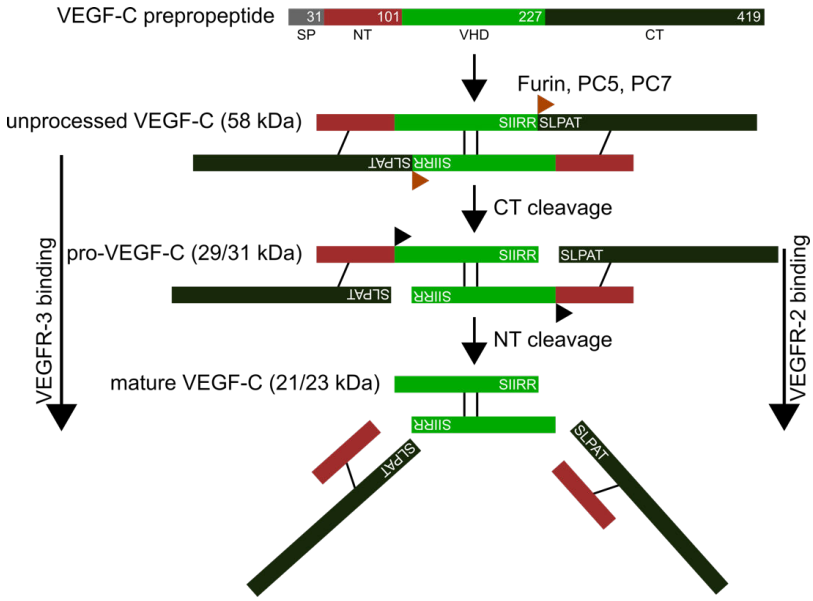

Figure 2: *Schematic of VEGF-C biosynthesis and proteolytic processing. VEGF-C is synthesized as inactive prepropeptide. The prepropeptide consists of signal peptide (SP), N-terminal propeptide (NT), VEGF homology domain (VEGF) and C-terminal propeptide (CT). The processing includes removal of C-terminal propeptide (marked by brown triangle) and N-terminal propeptide (marked by black triangle) (modified from Rauniyar et al., 2018).*

The cleavage of VEGF-C by PCs does not remove the C-terminal propeptide, because the C-terminal propeptide remains covalently linked via disulfide bonds to the N-terminal propeptide. This intermediate species (referred to as pro-VEGF-C) showed only a minimal activity towards VEGFR-2 and VEGFR-3 (McColl et al., 2003). In line with this, several studies have shown that the unprocessed VEGF-C and pro-VEGF-C are less active in vivo and in vitro (Anisimov et al., 2009; Joukov et al., 1997; Khatib et al., 2010; McColl et al., 2003). In order to gain full receptor activation potential, the VEGF-C polypeptide chain needs to be cleaved between the N-terminal propeptide and the VHD. This final, activating cleavage releases both the N- and C-terminal propeptides. However, before our present studies the only protease, that was known to perform this final, activating cleavage, was plasmin. Due to its role in blood clot dissolution (Chapin and Hajjar, 2015) and its expression pattern (Bugge et al., 1995), most researchers ruled out plasmin as an endogenous protease that activates VEGF-C during development.

Both VEGF-C and VEGF-D dimers are stabilized by intermolecular disulfide bridges (Leppanen et al., 2010, 2011). However, at the same time, the amino acid sequences of VEGF-C and VEGF-D feature one extra cysteine residue in the VHD; this is located at the dimer interface in proximity to the interchain disulfide bridges (Leppanen et al., 2011; Toivanen et al., 2009). Mutation of this cysteine residue into an alanine residue resulted in a significant rise of dimer stability and receptor activation potential (Anisimov et al., 2009; Toivanen et al., 2009). It is not well understood why this cysteine is conserved throughout the animal kingdom despite its destabilizing function. The structure and binding of VEGF-C and VEGF-D are described in the section *Binding of VEGF-C and VEGF-D to VEGFR-2 and VEGFR-3*.

3.1.3.2 VEGF-C expression and its regulation

VEGF-C expression can be detected as early as E8.5 in developing mouse embryos in the jugular region and later at E10.5 in the mesenchyme region close to the area of lymphatic sprout formation (Karkkainen et al., 2004). In the adult mice, in which the lymphatic endothelium is quiescent, the expression of VEGF-C mRNA decreases with levels remaining highest in lung and heart and somewhat lower levels in liver and kidney (Kukk et al., 1996). VEGF-C expression also occurs in the aorta and pulmonary artery (Chen et al., 2014a), endocrine glands, such as the thyroid, adrenal medulla and pancreas (Partanen et al., 2000), platelets (Wartiovaara et al., 1998), and SMCs in ensheathing the arteries and surrounding the lacteals in the intestine (Nurmi et al., 2015). Transcription of VEGF-C is unaffected by hypoxia (Enholm et al., 1997), and the *VEGFC* gene lacks any hypoxia-regulated binding sites in its promoter (Chilov et al., 1997). However, in certain tumor cells, hypoxia was shown to increase VEGF-C expression by an internal ribosome entry site (IRES) mediated mechanism (Morfoisse

et al., 2014). Inflammation and radiation damage are potent triggers of VEGF-C expression (Baluk et al., 2005; Nolan et al., 2013; Ristimäki et al., 1998). Macrophages critically regulate VEGF-C during inflammation (Machnik et al., 2009; Suh et al., 2019). Interstitial fluid pressure was also shown to increase the expression of VEGF-C in a tail injury model (Goldman et al., 2007a). This could also be involved in interstitial flow-mediated lymphangiogenic response (Planas-Paz and Lammert, 2013). In addition its role during development of the lymphatic vasculature, VEGF-C also plays a critical role in maintaining the integrity and function of some lymphatic networks in the adult; for example, of the meningeal lymphatics (Antila et al., 2017) and the lacteals (Nurmi et al., 2015).

3.1.3.3 VEGF-C is essential for development of the lymphatic system

VEGF-C is the primary lymphangiogenic molecule which is indispensable during lymphatic development. The deletion of VEGF-C in mice leads to embryonic death around E15.5-E17.5. The primary phenotype after *vegfc* deletion in mice is the failure of specified LECs to emigrate from the cardinal vein to form the primitive lymph sacs (Hägerling et al., 2013; Karkkainen et al., 2004). Haploinsufficiency of VEGF-C, on the other hand, leads to hypoplastic and partially functional lymphatic vessels (Karkkainen et al., 2004). While the role of VEGF-C is evolutionarily conserved during the development of lymphatic vessels (Küchler et al., 2006; Ny et al., 2005), VEGF-C is also critical for the development of specific blood vessels, such as the coronary arteries in the heart. Ablation of VEGF-C causes a reduction in the peri truncal coronary vessels, a complete absence of aortic epicardial vessels (ASVs), and reduces dorsal and lateral coronary growth (Chen et al., 2014b, 2014a). Interestingly, in zebrafish, loss of VEGF-C ablates the formation of intersegmental vessels (ISVs) and central arteries and also negatively impacts the development of liver buds from the endoderm (Ober et al., 2004). Both LECs and BECs express VEGFR-2, a secondary receptor for VEGF-C (Kriehuber et al., 2001), and thus BECs can grow and migrate in response to VEGF-C (Makinen et al., 2001a). However, how VEGF-C mediated signaling effects are largely confined to the VEGFR-3 pathway instead of VEGFR-2, will be discussed in the *VEGFR* section.

3.1.3.4 VEGF-C/VEGFR-3 signaling

VEGF-C/VEGFR-3 signaling is essential for the growth, survival, and migration of LECs. In endothelial cell culture, VEGF-C/VEGFR-3 signaling acts via the conventional downstream routes; for example PI3K/AKT and MAPK/ERK pathways (Deng et al., 2015; Makinen et al., 2001a; Salameh et al., 2005). Furthermore, the VEGF-C/VEGFR-3 mediated PI3K/AKT pathway activation is essential for the development of normal lymphatic vasculature (Zhou et al., 2010). Stimulation of

LECs with VEGF-C induces a distinct phosphorylation pattern of the tyrosine (Y) residues in VEGFR-3, (Y1063, Y1068, Y1230, Y1231, Y1337 and Y1363) (Dixelius et al., 2003).Phosphorylation of Y1063 in VEGFR-3 regulates JNK mediated survival signals whereas Y1230, Y1232 and Y1337 regulate PI3K/AKT and MAPK/ERK mediated proliferation, migration and survival (Salameh et al., 2005).

3.1.3.5 Biological function of VEGF-C

The lymphangiogenic potential of VEGF-C has been studied in several transgenic and viral- mediated delivery models (Rauniyar et al., 2018). Transgenic overexpression of VEGF-C in the skin of mice induces lymphatic hyperplasia with no effect on blood vessel (Jeltsch et al., 1997). A similar result was seen when recombinant VEGF-C protein was applied to the chick chorioallantoic membrane (CAM), but additionally, mild angiogenesis was detected in the areas of the highest VEGF-C concentrations (Oh et al., 1997). Adenovirus- or adeno associated virus (AAV)-mediated delivery of VEGF-C resulted in lymphangiogenesis, and hyperplastic blood vessels, which were tortuous and leaky (Rissanen et al., 2003). However, there was no evidence of sprouting of the VEGF-C induced blood vessels (Saaristo et al., 2002). To separate VEGFR-2-mediated effects from VEGFR-3-mediated effects, a mutant form of VEGF-C, VEGF-C_{C156S}, was developed, which binds almost exclusively to VEGFR-3 (Joukov et al., 1998). This mutant confirmed that most of the VEGF-C effects are mediated via the VEGFR-3 pathway (Veikkola et al., 2001). The therapeutic potential of VEGF-C has been demonstrated in various disease models, e.g. lymphedema (Honkonen et al., 2013a; Karkkainen et al., 2001; Saaristo et al., 2002; Szuba et al., 2002a; Tammela et al., 2007b; Visuri et al., 2015; Yoon et al., 2003), diabetic wound healings (Saaristo et al., 2006), inflammation (Hagura et al., 2014), and the aorta denudation model of arterial restenosis (Hiltunen et al., 2003). In fact, adenoviral delivery of the VEGFR-3 specific mutant, VEGF-C_{C156S}, showed an exclusive effect on lymphatic vessels (Saaristo et al., 2002; Visuri et al., 2015). Nevertheless, the wild-type form of VEGF-C was more effective in enhancing lymphatic growth and function in a direct quantitative comparison (Visuri et al., 2015). These results suggest the cooperation of VEGFR-2 in efficient VEGF-C signaling.

VEGF-C is also extensively studied in the context of tumor biology because of its potential to induce lymphatic metastasis. High levels of VEGF-C have been detected in several cancers and were associated with poor disease prognosis (Chen et al., 2012). The role of VEGF-C/VEGFR-3 pathway in tumors will be discussed in the section on *tumor lymphangiogenesis*.

3.1.3.6 Non-endothelial targets of VEGF-C

Similar to VEGF-A, VEGF-C has also been studied for its non-endothelial targets, especially in the central nervous system: in the embryonic brain VEGF-C induces the proliferation of VEGFR-3-expressing neuronal precursor cells in the optic nerve and olfactory bulb (Le Bras et al., 2006). It promotes neurogenesis by activating quiescent neural stem cells (NSCs) in the hippocampus of adult mice (Han et al., 2015), and also acts as a neurotrophic factor for dopaminergic neurons partly through the direct effect on the neurons (Piltonen et al., 2011). In zebrafish, VEGF-C/VEGFR-3 signaling regulates the growth of axons of motor neurons (Kwon et al., 2013) and endodermal development (Ober et al., 2004). In addition to neural target cells, VEGF-C also affects hematopoiesis by regulating megakaryopoiesis (Thiele et al., 2012) and fetal erythropoiesis (Fang et al., 2016).

3.1.4 VEGF-D

VEGF-D, also known as a c-Fos-induced growth factor (FIGF), is the closest paralog of VEGF-C (Figure 3).

3.1.4.1 Molecular properties and structure of VEGF-D

Similar to VEGF-C, it undergoes posttranslational processing and also binds to heparin/HSPGs via its C-terminal domain (Harris et al., 2013). The VEGF-D C-terminal domain is cleaved by proprotein convertases furin, PC5, and PC7 and at the N-terminal domain by plasmin (McColl et al., 2003, 2007; Siegfried et al., 2003). Two differently processed forms of VEGF-D are produced by VEGF-D-transfected 293T cells - the major mature form (^{89}FAATFY...SIIRR^{205}) and the minor mature form (^{100}KVIDEE...SIIRR^{205}) (Stacker et al., 1999a). Plasmin cleavage of VEGF-D yields two polypeptides - one similar to the major mature form and another one amino acid shorter than the minor mature form, suggesting that the plasmin cleavage is physiologically relevant (McColl et al., 2003). Only the longer of the two mature human VEGF-D forms (the major mature form) can bind and activate both VEGFR-2 and VEGFR-3 (Achen et al., 1998; Stacker et al., 1999a), while minor mature human VEGF-D only activates VEGFR-2 (Leppanen et al., 2011). However, unlike human VEGF-D, which does bind and activate human VEGFR-2, mouse VEGF-D may not be able to bind or activate mouse VEGFR-2 (Baldwin et al., 2001).

3.1.4.2 VEGF-D expression and its regulation

VEGF-D is widely expressed during mouse embryonic development (Avantaggiato et al., 1998; Baldwin et al., 2005). In developing embryo, lungs are the major site of VEGF-D expression; in adult mice VEGF-D mRNA was sufficiently detected in the heart, lung, skeletal muscle, colon and intestine (Achen et al., 1998; Stacker et al.,

1999a). Limited data is available on the regulation of VEGF-D expression. Interleukin 7 increases the expression of VEGF-D in breast and lung cancer cells, ultimately enhancing the lymphangiogenesis in these models (Al-Rawi et al., 2005; Ming et al., 2009). VEGF-D at the protein level was reduced by transforming growth factor β (Cui et al., 2014).

3.1.4.3 VEGF-D is dispensable for lymphatic development

VEGF-D, unlike VEGF-C, is dispensable for the development of lymphatic vessels (Baldwin et al., 2005), and mouse embryo with a compound deletion of *Vegfc* and *Vegfd* appear similar to those with a deletion of *Vegfc* (Haiko et al., 2008). However, *Vegfd*-deleted mice show postnatally minor alterations in the lymphatics surrounding the bronchioles in the lungs (Baldwin et al., 2005) and in the initial lymphatics in the skin of adult male mice (Paquet-Fifield et al., 2013). Transgenic expression of VEGF-D in the skin could however rescue the phenotype induced by the deletion of VEGF-C in mouse skin (Haiko et al., 2008).

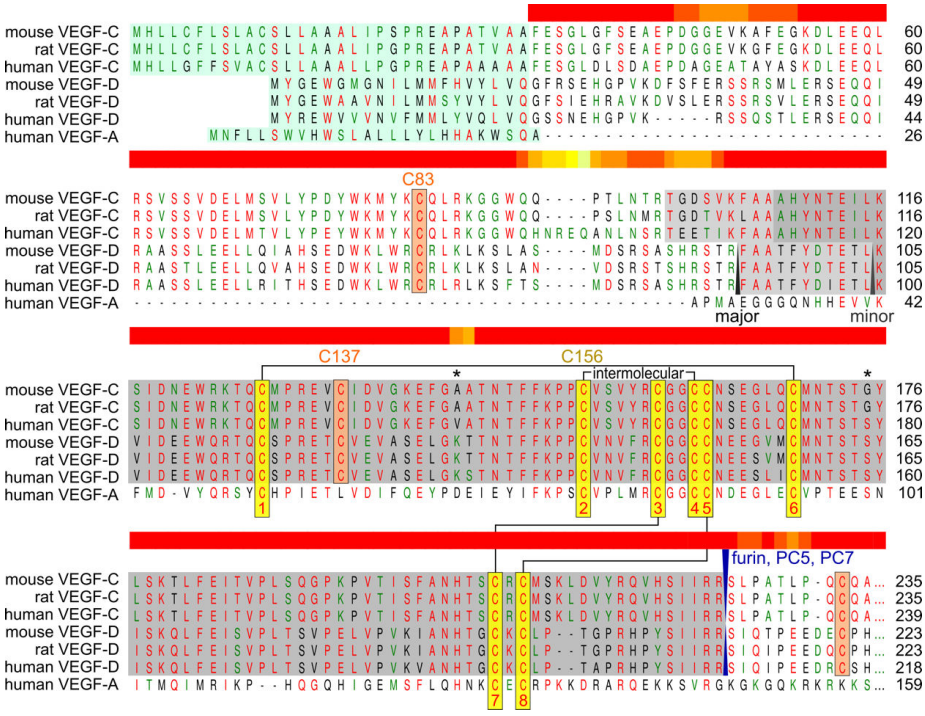

Figure 3: *Alignment of VEGF-C and VEGF-D amino acid sequences from mouse, rat and human species, and comparison with human VEGF-A. The aa sequence of the mature form of mouse and human VEGF-C are identical except the two residues marked by asterisks*. Adapted from Rauniyar et al., 2018.*

3.1.4.4 Functions of VEGF-D in mammals and fish

Recent studies indicate substantial differences in the molecular regulation of lymphatic development between different animal clades. In zebrafish, VEGF-D binds to VEGFR-2 (zKdr) but not VEGFR-3 (zFlt4), suggesting that VEGFR-2 is the primary receptor of VEGF-D in zebrafish (Vogrin et al., 2019). Previously, Vegfd was shown to be essential for the development of the facial lymphatics in zebrafish (Bower et al., 2017), where it signals via zKdr (Astin et al., 2014; Vogrin et al., 2019). Vegfd can also rescue the loss of the orthodox Kdr ligand Vegfaa in zebrafish (Rossi et al., 2016), confirming its ability to signal efficiently via zKdr. Moreover, knockdown of Vegfd in *Xenopus* limits LEC migration and sprouting (Ny et al., 2008). Also, the compound deletion of Vegfd and Sox18 induces arteriovenous fusion in zebrafish and uncontrolled angiogenesis in mice (Duong et al., 2014).

3.1.4.5 Biological function of VEGF-D

In-line with the results from the zebrafish studies, VEGF-D was shown to stimulate lymphangiogenesis and in various mammalian systems, it stimulated concurrent angiogenesis and lymphangiogenesis. Transgenic overexpression of VEGF-D in the skin of mice leads to lymphatic hyperplasia with no apparent effect on the blood vasculature (Veikkola et al., 2001). Adenoviral and adeno-associated viral (AAV9) delivery of a mature form of VEGF-D (ΔNΔC-VEGF-D) in rat cremaster muscle and mouse skeletal muscle induced both lymphangiogenesis and angiogenesis (Anisimov et al., 2009; Byzova et al., 2002; Rissanen et al., 2003). Adenoviral mediated local delivery of ΔNΔC-VEGF-D in the myocardium was shown to be therapeutically beneficial (Hartikainen et al., 2017; Rutanen et al., 2004), raising hopes for its usage in the treatment of refractory angina pectoris. However, many of these studies used a form of mature VEGF-D, in which the N-terminus differs from the major and minor mature forms of endogenous VEGF-D. Because the N-terminus is paramount for the determination of receptor binding specificity (Leppanen et al., 2011), it is difficult to judge the significance of these in-vivo results.

3.1.5 Binding of VEGF-C and VEGF-D to VEGFR-2 and VEGFR-3

Although the amino acid sequences of human VEGF-C and VEGF-D are about 50% similar (pairwise Needleman-Wunsch global alignment) (Figure 3), the two factors differ significantly in their receptor binding characteristics. The crystal structure of VEGF-C in complex with the growth factor binding domains of VEGFR-2 and VEGFR-3 revealed the critical requirement of amino acid residues in the N-terminal helix for the interaction with VEGFR-2 (Leppanen et al., 2010), and VEGFR-3 (Leppanen et al., 2013). Most of the amino acid residues associated with VEGF-C and VEGF-D binding to VEGFR-2 and VEGFR-3 are conserved (Leppanen et al., 2010,

2011, 2013). The N-terminal helix of the VHD of VEGF-D critically influences its affinity for VEGF-2 versus VEGFR-3. The removal of N-terminal residues (resulting in the minor mature form, starting with residues KVIDE) results in a near-complete loss of the VEGFR-3 activation potential of VEGF-D, while maintaining activity towards VEGFR-2 intact. This was confirmed in vivo using AAVs encoding the major and the minor mature form of VEGF-D, respectively. The crystal structure of VEGF-D unveiled the role of the amino acid residues ^{92}TFY...ETL^{99} (Figure 3) for VEGFR-3 binding (Leppanen et al., 2011). Recently, an elegant mutational study confirmed the importance of ^{93}FYD...IET^{98} (Figure 3) in VEGFR-3 binding and of ^{93}FYD...WQR^{108} (Figure 3) in both VEGFR-2 and VEGFR-3 binding. However, the homologous regions in VEGF-C are not required for binding to VEGFR-2 or VEGFR-3 (Davydova et al., 2016).

3.2 Vascular endothelial growth factor receptors (VEGFRs)

VEGFRs are class V receptor tyrosine kinases, which consists of seven extracellular immunoglobulin (Ig)-like loops, a transmembrane domain, a juxtamembrane domain, a tyrosine kinase domain, and a C-terminal tail (Koch et al., 2011). VEGFs, exert their function by inducing dimerization and transphosphorylation of the VEGFRs. The signaling of VEGFs is supported and regulated by several other factors including co-receptors. The VEGF/VEGFR signaling axis regulates primarily the processes of proliferation, survival, and migration of ECs (Koch and Claesson-Welsh, 2012a).

3.2.1 VEGFR-1

VEGFR-1 (also known as Flt1, Fms-like tyrosine kinase 1) is the receptor for VEGF-A (de Vries et al., 1992), PlGF (Park et al., 1994) and VEGF-B (Olofsson et al., 1998). The affinity of VEGF for VEGFR-1 is much higher than the affinity for VEGFR-2. In contrast, the receptor tyrosine kinase activity of VEGFR-1 is much weaker than the VEGFR-2 tyrosine kinase activity (Waltenberger et al., 1994). Alternative splicing of VEGFR-1 produces a soluble isoform, sVEGFR-1, which contains the extracellular ligand-binding domain (Kendall and Thomas, 1993). sVEGFR-1 inhibits angiogenesis by acting as an extracellular trap for VEGF-A (Carmeliet et al., 2001; Gerhardt et al., 2003). VEGFR-1 can heterodimerize with VEGFR-2 in endothelial cells (Autiero et al., 2003; Cudmore et al., 2012). However, the physiological significance of VEGFR-1/VEGFR-2 heterodimers remains unclear.

Flt1-/- embryos die around E8.5-E9.5 due to blood vessel hyperplasia and the presence of endothelial-like cells inside the blood vessels (Fong et al., 1995). However, mice with a targeted deletion (TK-/-) of the tyrosine kinase domain of *Flt1* showed only a minor defect in VEGF-A-mediated macrophage migration (Hiratsuka et al., 1998). To

further demonstrate the role of VEGFR-1 during the embryonic development, knock-in mutant mice (TM-TK-/-) lacking both transmembrane and tyrosine kinase domain of *Flt1* was generated. Interestingly, only half of the TM-TK-/- mice survived, whereas changing of the genetic background of mice to increase VEGFR-2 level resulted in almost complete survival of most of the mice. The embryonic lethality in these mice was because of a defect in the growth and survival of endothelial cells (Hiratsuka et al., 2005). Although, the phenotype of TM-TK-/- mice is difficult to interpret, it appears that VEGFR-1 is a negative regulator of VEGF function in angiogenesis.

Although VEGFR-1 is largely specific for endothelial cells, VEGFR-1 expression and function has been described also in some non-endothelial cells, predominantly in hematopoietic cells. VEGFR-1 is e.g. expressed by monocytes and macrophages, which respond to VEGF-A with migration (Barleon et al., 1996; Sawano et al., 2001). VEGFR-1 promotes also the cell cycle and motility in a subset of hematopoietic stem cell populations (Hattori et al., 2002).

3.2.2 VEGFR-2

VEGFR-2 (also known as Flk1, fetal liver kinase 1 in mice and KDR in humans) is the receptor for VEGF-A (Quinn et al., 1993), mature VEGF-C (human and mouse), and VEGF-D (human) (Joukov et al., 1997; Stacker et al., 1999a). VEGFR-2 is the primary angiogenic receptor and induces endothelial cell proliferation, survival, sprouting, migration and vessel permeability (Koch and Claesson-Welsh, 2012b).

Ligand binding to VEGFR-2 has been extensively studied. The ligand binding site locates to the second and third Ig-like domains of VEGFR-2, with the second Ig-like domain being the main determinant for the ligand binding (Fuh et al., 1998; Leppanen et al., 2010; Shinkai et al., 1998). The role of Ig-like domains 4-7 is to regulate homodimerization but they don't have any direct contribution to ligand binding (Hyde et al., 2012; Kendrew et al., 2011). An electron microscopic study of the VEGF/VEGFR-2 complex showed that receptors without ligand are monomeric, but that ligand binding stimulated receptor-receptor interaction via Ig-like domain 7 (Ruch et al., 2007). In another study, VEGFR-2 existed as a dimer also in the absence of the ligand, and ligand binding induced a further conformational change in the transmembrane domain of the receptor (Sarabipour et al., 2016).

Vegfr2-/- embryos die at about E8.5-E9.5 due to impaired vasculogenesis and hematopoiesis (Shalaby et al., 1995, 1997). The expression of VEGFR-2 in embryonic vasculature starts already at E7.0 (Millauer et al., 1993). In adults, VEGFR-2 is expressed at lower level than in embryos, and it is important for EC survival signals

(Lee et al., 2007; Maharaj et al., 2006; Matsumoto and Claesson-Welsh, 2001). Prominent expression of VEGFR-2 in tip cells relative to the stalk cells during retinal vascular development, presumably helps the tip cells respond to a VEGF-A gradient by migrating towards the highest VEGF-A concentration (Gerhardt et al., 2003). VEGFR-2 is expressed in LECs (both in capillaries and collectors) (Saaristo et al., 2002), and *Lyve-1* specific deletion of *Vegfr2* leads to lymphatic vessel hypoplasia both in adults and embryos (Dellinger et al., 2013). Furthermore, adenoviral delivery of VEGF-A induced hyperplasia of the lymphatic vessels (Nagy et al., 2002; Wirzenius et al., 2007).

VEGFR-2 is also expressed in hematopoietic cells (Katoh et al., 1995; Ziegler et al., 1999), neurons (Ogunshola et al., 2002) and neural stem cells (Maurer et al., 2003).

3.2.3 VEGFR-3

VEGFR-3 (also known as Fms-like tyrosine kinase, Flt4) is the main receptor for VEGF-C and VEGF-D. VEGFR-3, unlike other VEGFRs, undergoes proteolytic processing of the fifth Ig-like domain (Lee et al., 1996; Pajusola et al., 1993, 1994), and yields 120-kDa and 75-kDa fragments linked by disulfide bridges. However, mutating the cysteine residue 445 in Ig-like domain 5, which prevents the processing, does not affect VEGFR-3 activity (Tvorogov et al., 2010). In humans, two alternative splice variants exist for VEGFR-3: a short and a long variant (Hughes, 2001; Pajusola et al., 1993).

The crystal structure of VEGF-C in complex with VEGFR-3 identified the direct involvement of Ig-like domain 2 in VEGF-C binding, whereas the Ig-like domain 1 protruded away from the binding site (Leppanen et al., 2013). In contrast, the Ig-like domain 1 was required for VEGF-D binding (Leppanen et al., 2011), suggesting that it modulates the stability of ligand-binding to Ig-like domain 2. Also the Ig-like domains 4-7 are critical for dimerization and receptor activation (Leppanen et al., 2013). An antibody directed against Ig-like domain 5 not only inhibited VEGFR-3 homodimerization but also its heterodimerization with VEGFR-2 (Tvorogov et al., 2010).

Deletion of *Vegfr3* leads to embryonic death at E10-E12.5 due to the defect in the remodeling of the primary vascular plexus, whereas vasculogenesis is not affected (Kaipainen et al., 1995). In order to understand the role of VEGFR-3 during lymphatic development, mutant mice were generated that lack the ligand-binding domain or feature an inactivating mutation in the tyrosine kinase domain. The lymph sacs developed normally in the absence of the ligand-binding domain of VEGFR-3, but the mice lacked any other lymphatic structures. In contrast, mice having an inactive kinase

domain failed to form any lymph sacs. The development of the blood vasculature was normal in both mutants (Zhang et al., 2010). Transgenic overexpression of soluble VEGFR-3 in the skin led to regression of lymphatic vessels, but the mice survived and later, regenerated new lymphatic vessels (Makinen et al., 2001b).

The expression of VEGFR-3 can be found already at E8.5 in BECs (in the angioblasts of head mesenchyme and veins). Later, VEGFR-3 becomes restricted to LECs in the developing lymphatic vessels (Kaipainen et al., 1995). In addition to LECs, VEGFR-3 is also expressed in BECs; for example high endothelial venules (Kaipainen et al., 1995), tumor vasculature (Laakkonen et al., 2007), and in fenestrated vessels in liver, kidney, and endocrine glands (Partanen et al., 2000). VEGFR-3 is also highly expressed in actively sprouting BECs, for example in the developing retinas and in tumors (Tammela et al., 2008). Endothelial cell-specific deletion of *Vegfr3* induces angiogenic sprouting in the retinal vasculature. An increase in VEGFR-2 and a decrease in Notch activity was found to modulate this hypervascularization effect (Tammela et al., 2011; Zarkada et al., 2015). Additionally, when *Vegfr3* is deleted in adult mice, the mice developed vascular leakage via modulation of VEGF/VEGFR2 signaling (Heinolainen et al., 2017).

VEGFR-3 is expressed in several non-endothelial cells, for example in corneal epithelium (Cursiefen et al., 2006), osteoblasts (Orlandini et al., 2006), neuronal progenitors (Le Bras et al., 2006) and, conjunctival monocytic cells (Hamrah et al., 2004).

3.2.4 Possible role of VEGFR-2/VEGFR-3 heterodimers

The formation of VEGFR-2/VEGFR-3 heterodimers has been debated, but a complete understanding of VEGFR-2/VEGFR-3 interaction is lacking. The presence of VEGFR-2/VEGFR-3 heterodimers was described in several studies in vitro (Alam et al., 2004; Goldman et al., 2007b; Harris et al., 2013). Both VEGF-C and VEGF-A were shown to induce VEGFR-2/VEGFR-3 heterodimerization in endothelial cells. VEGF appeared to stimulate heterodimerization about 25-fold and VEGF-C 100-fold in otherwise untreated endothelial cells (Nilsson et al., 2010). In embryoid bodies, VEGF-C induced VEGFR-2/VEGFR-3 heterodimers localized to tip cells in response to VEGF-C, where they may stimulate sprouting angiogenesis (Nilsson et al., 2010). VEGF-C can also induce VEGFR-2/VEGFR-3 heterodimerization in LECs, which interestingly leads to a differential VEGFR-3 phosphorylation pattern (Dixelius et al., 2003). Overexpression of WT, ligand binding domain, or kinase mutant VEGFR-3 in HUVECs followed by stimulation with VEGF-A_{165} induced the formation of a VEGFR-2/VEGFR-3 complex, which reduced VEGFR-2 mediated ERK signaling (Zhang et al., 2010), while in another report, VEGF-C was shown to stimulate the

VEGFR-2/VEGFR-3 dimerization, and activate AKT signaling in human dermal lymphatic endothelial cells (Deng et al., 2015).

4. Molecules that regulate the VEGF-VEGFR pathway

While the intrinsic affinity of the VHD of the VEGFs is the major determinant of VEGFR binding, several extracellular and cell surface molecules modulate the VEGF/VEGFR interaction.

4.1 Neuropilins

Neuropilins (Nrp1 and Nrp2) are single-pass transmembrane glycoproteins, which lack tyrosine kinase activity and have a short cytoplasmic tail containing a PDZ domain (Guo and Kooi, 2015). The extracellular region in both neuropilins consists of three domains a1/a2, b1/b2, and c (Wild et al., 2012). Neuropilins were initially identified as receptors for class 3 semaphorins, which play a critical role in controlling axon guidance (Ekpe et al., 2018). In-vitro biochemical studies have demonstrated that many VEGFs interact with Nrp1 and/or Nrp2. However, there is no definitive answer to the question of the in-vivo significance of many of these interactions. The major neuropilin function seems to be the stabilization of the VEGF/VEGFR interaction and regulation of the intracellular VEGFR turnover.

4.1.1 Neuropilin 1

Nrp1 modulates VEGFR-2 mediated vascular permeability (Becker et al., 2005; Fantin et al., 2017). It interacts with VEGFR-2 in the presence of VEGF-A_{165}, which results in enhanced binding of VEGF-A_{165} to VEGFR-2 (Soker et al., 1998, 2002). This interaction requires the presence of the heparin-binding domain of VEGF-A_{165} (Fuh et al., 2000). Interestingly, VEGF-A_{121}, which interacts very weakly with heparan sulfates, can bind to Nrp1, but unlike VEGF-A_{165}, this interaction doesn't form a complex with Nrp1 and VEGFR-2 (Pan et al., 2007). Nrp1 can also bind to VEGF-C (Kärpänen et al., 2006). Both VEGF-C and VEGF-A_{165} bind to the b1/b2 domains of Nrp1 (Kärpänen et al., 2006; Mamluk et al., 2002). Additionally, Nrp1 knockdown in LECs decreases VEGF-C-mediated AKT activation (Deng et al., 2015). Yet, blocking the interaction between VEGF-C and Nrp1 did not have any effect on VEGF-C function (Caunt et al., 2008). However, semaphorin 3A/Nrp1 signaling was shown to influence the remodeling of the lymphatic vessels. Blocking semaphorin 3A binding to Nrp-1 around E12.5-E16.5 resulted in increased SMCs coverage of the collecting vessels and abnormal morphology of the lymphatic valves (Jurisic et al., 2012).

Nrp1 is expressed in SMCs of collecting lymphatic vessels (Jurisic et al., 2012), and ECs of arteries (Herzog et al., 2001). The ablation of Nrp1 causes defects in guidance of neuronal extensions (Kitsukawa et al., 1997) and vascular development in mice (Gerhardt et al., 2004; Kawasaki et al., 1999), leading to embryonic death by E13.5 due to the vascular defects. However, targeted deletion of the cytoplasmic domain of Nrp1 does not seem to affect angiogenesis per se, but only arteriogenesis and arterial-venous patterning (Fantin et al., 2011; Lanahan et al., 2013).

4.1.2 Neuropilin 2

Neuropilin2 (Nrp2) is expressed by neurons in the developing brain (Chen et al., 2000), and in venous and lymphatic ECs (Yuan et al., 2002). Nrp2 expression in lymphatic ECs is strongest in the tip cells of the emerging lymphatic sprouts (Xu et al., 2010). In Nrp2-deficient mice, lymph sacs and collecting lymphatics form largely normally, but the formation of smaller lymphatic vessels and capillaries is compromised, suggesting that Nrp2 is involved lymphangiogenic sprouting. However, most of the mice survived without any aberrant phenotype. The surviving mice seem to regenerate most lymphatics postnatally (Yuan et al., 2002). Moreover, blocking Nrp2 binding to VEGF-C in mice during early postnatal period constrains lymphatic sprouting by inhibiting tip cell formation (Xu et al., 2010).

Both VEGF-C and VEGF-D bind Nrp2, and the binding is regulated by heparin (Kärpänen et al., 2006). The binding of VEGF-C to Nrp2 is enhanced by the N-terminal domain of VEGF-C (Kärpänen et al., 2006) and requires the removal of the C-terminal propeptide, which unmasks binding epitopes at the C-terminal end of the mature VEGF-C (Parker et al., 2015). Once VEGF-C and VEGF-D bind Nrp2, it induces co-internalization of VEGFR-3 and Nrp2 (Kärpänen et al., 2006). However, the physiological significance of the process remains speculative. Interestingly, a blocking antibody that prevents Nrp2 and VEGF-C interaction inhibits LECs migration without affecting their proliferation (Caunt et al., 2008). A similar effect could be observed in NRP2 silenced endothelial cells (Favier et al., 2006). In addition to VEGF-C binding, Nrp2 also interacts with VEGFR-2 and VEGFR-3, which is dependent on the presence of the ligands VEGF-A and VEGF-C (Favier et al., 2006).

4.2 Integrins

Integrins are structurally related heterodimeric extracellular matrix (ECM) protein receptors composed of α and β subunits (Silva Rita et al., 2008). Although integrins lack enzymatic activity, they play essential roles in fundamental functions of the vasculature, such as cell growth, survival, and migration.

Integrin $\alpha5\beta3$ is the most abundant integrin in ECs. It interacts with VEGFR-2 in a ligand-dependent or independent system, where ligand binding enhances VEGFR-2 activity, and thereby induces mitogenic signals in ECs and activates Src (Borges et al., 2000; Mahabeleshwar et al., 2006; Soldi et al., 1999). Integrin $\alpha5\beta3$ also regulates VEGFR-2 level in ECs (Reynolds et al., 2004).

The role of Integrin $\alpha9$ is well established during lymphatic development. Prox1 induces integrin $\alpha9$ expression in embryonic stem cell derived ECs and HUVECs (Harada et al., 2009; Mishima et al., 2007). Deletion of the integrin $\alpha9$ gene in mice results in postnatal death from chylothorax (Huang et al., 2000), mainly because it alters the shape of lymphatic valves (Bazigou et al., 2009). In a solid-phase binding assay, VEGF-C and VEGF-D bound to integrin $\alpha9\beta1$, which induced migration of microvascular endothelial cells (Vlahakis et al., 2005). Thus, at least for lymphatic valve morphogenesis during development, the fibronectin-integrin $\alpha9$ complex seems to be critical (Bazigou et al., 2009).

Stimulation of dermal microvascular endothelial cells with extracellular matrix (ECM) components - fibronectin or collagen enhanced VEGFR-3 kinase activity through its association with $\beta1$ integrin in a ligand-independent system (Wang et al., 2001). VEGF-C also induced the interaction between integrin $\alpha5\beta1$ and VEGFR-3 via fibronectin, promoting the growth and survival of LECs (Zhang et al., 2005). Moreover, $\beta1$integrin regulates VEGFR-3 activity by responding to mechanical forces during development (Lorenz et al., 2018; Planas-Paz et al., 2011). Recently, integrin-linked kinase (ILK), a known interaction partner of integrin, was shown to modulate VEGFR-3 activity in a pressure-dependent fashion by controlling its interaction with $\beta1$ integrin (Urner et al., 2019).

4.3 Extracellular matrix

Extracellular matrix (ECM) acts as a dynamic foundation for the functioning of cells in tissues by providing structural support. Integrins and cell surface proteoglycans mediate large part of ECM signaling events. ECM provides also spatiotemporal regulation of growth factor availability and acts as a storage for heparan sulfate binding proteoglycans (HSPGs) (Ferrara, 2010; Schultz and Wysocki, 2009).

The ECM mostly influences ECs through integrins, which regulates ECs growth, survival and migration (Mettouchi, 2012). The vascular basement membranes (BM), thin sheet-like structural components below the EC layer, are composed of ECM proteins and proteoglycans. The primary structural components of endothelial BM are laminin and type IV collagen intertwined in the mesh of heparan sulfate proteoglycans (HSPGs) proteins perlecan and nidogen (Witjas et al., 2019). The laminin α4 chain,

which is expressed by blood capillaries (Iivanainen et al., 1997), maintains the structural stability of the blood vessels by retaining BM integrity (Thyboll et al., 2002). LECs express less ECM proteins than BECs, which probably explains the irregular BM coverage around the lymphatic capillaries (Hirakawa et al., 2003; Podgrabinska et al., 2002; Vainionpää et al., 2007).

HSPGs consist of a core protein covalently linked to heparan sulfate glycosaminoglycan chains. HSPGs are found on the cell surface and in the extracellular matrix. HSPGs can act as coreceptors for several growth factors and regulate their physiological functions (Sarrazin et al., 2011). Endothelial HSPGs are critical for PDGF-B mediated organized recruitment of pericytes to the blood vessel wall (Armulik et al., 2011). The heparin-binding domain of growth factors is rich in basic amino acid residues with a net positive charge, which mediates their binding to HSPGs (Billings and Pacifici, 2015).

Physiological angiogenesis requires a balance between the availability of VEGF-A in the pericellular matrix and the soluble phase of tissue fluids. Of the various VEGF isoforms, VEGF-A_{165} is the only isoform that fulfills these requirements and it is the only isoform that suffices for the normal angiogenic process (Gerhardt et al., 2003; Martino et al., 2015). VEGF-A_{164} promotes directed sprouting of endothelial tip cells, while VEGF-A_{120} induces mostly only proliferation of the ECs (Gerhardt et al., 2003; Ruhrberg et al., 2002; Stenzel et al., 2011; Wijelath et al., 2006). All the isoforms of VEGF-A, except VEGF-A_{121}, have a heparin-binding affinity and thus can tether to the cell surface or ECM (Ferrara, 2010; Gitay-Goren et al., 1992; Park et al., 1993). Typical binding proteins for VEGF-A in the ECM are laminin (Ishihara et al., 2018) and fibronectin (Wijelath et al., 2006). To clarify the effect of matrix binding on VEGFR-2 signaling, VEGF-A_{165} was incorporated in the collagen matrix and exposed to the ECs. Matrix incorporation of VEGF-A_{165} induced different responses; long-term VEGFR-2 phosphorylation, differential VEGFR-2 downstream signaling, and enhanced VEGFR-2 clustering and distribution (Chen et al., 2010). Furthermore, MMP9 and plasmin can release VEGF-A from the ECM and regulate its bioavailability (Bergers et al., 2000; Houck et al., 1992; Lee et al., 2005).

In contrast to VEGF, the matrix binding properties of VEGF-C and VEGF-D have been studied less. VEGF-C was shown to bind HSPGs from LECs, which regulated VEGF-C mediated growth and sprouting of LECs. Further, deletion of Ndst1 enzyme, which is responsible for the sulfation of HSPGs affected VEGF-C mediated survival of LECs and hence lymphangiogenesis. Syndecan-4 was shown to be abundantly expressed in LECs, in which it interacts with VEGFR-3 in the presence of VEGF-C, enhancing VEGF-C activity (Johns et al., 2016). Nevertheless, the study was carried

out under pathological conditions, such as wound healing and cancer, and hence the role of HSPGs during developmental lymphangiogenesis remains unexplored.

5. Collagen and calcium-binding EGF domains 1 (CCBE1)

CCBE1 (collagen and calcium-binding EGF domains 1) is a secreted extracellular matrix protein, which contains N-terminally three epidermal growth factor (EGF)-like sequences and C-terminally two collagen-like sequences (Figure 4). CCBE1 was initially identified as the mutant gene in zebrafish (*full of fluid)*, which lacks lymphatic vessels (Hogan et al., 2009). Later, mutations in the CCBE1 gene were identified in a subset of patients with Hennekam Syndrome (HS) (Alders et al., 2009). HS, also known as lymphedema-lymphangiectasia mental retardation syndrome, is composed of generalized lymphedema, intestinal lymphangiectasia, mental retardation and characteristic facial features (Balkom et al., 2002).

The N-terminal EGF-like domains of CCBE1 harbor the majority of known patient mutations, while the C-terminal collagen-like domain (Alders et al., 2013) is less affected. Interestingly, some of the mutants, for instance, mutants C67S (C75S in human) and R150C (R158C in human), that affected the N-terminal domain were able to rescue the *ccbe1* knockdown phenotype in zebrafish (Alders et al., 2009). During development, ablation of CCBE1 results in the inability of ECs to bud from the cardinal veins resulting in an absence of lymph sacs in both zebrafish and mice (Bos et al., 2011; Hogan et al., 2009). Both VEGF-C and CCBE1 are required for the budding of LECs from the cardinal vein (Bos et al., 2011; Karkkainen et al., 2004), but the developmental block in the two differs. The *Ccbe1-/-* embryos form irregular and dilated sprouts still attached to the CV, whereas in the *Vegfc-/-* embryos, the specified LECs in the CV completely fail to egress (Hägerling et al., 2013). During lymphatic development, both the EGF- and collagen-like domains of CCBE1 are crucial. While the phenotypes of *Ccbe1* and *Ccbe1*-collagen-domain deletion mutants are similar, the animals deleted for the *Ccbe1*-EGF-like-domain were able to form isolated and enlarged lymphatic structures, suggesting that although the LECs were able to emigrate from the cardinal veins, they failed to organize into lymphatic vessels (Roukens et al., 2015).

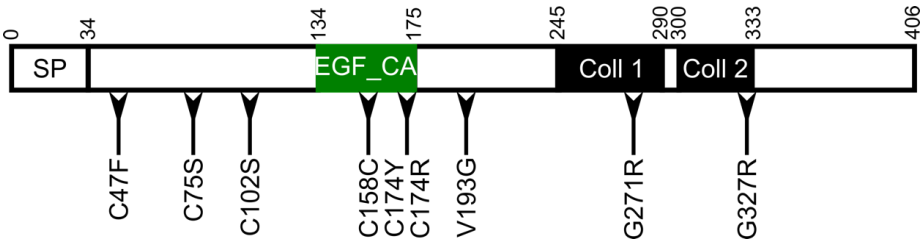

Figure 4: *Schematic view of the CCBE1 domains. CCBE1 is an extracellular matrix protein and contains signal peptide (SP), EGF_CA (EGF-like calcium binding domain) and two Collagen-like repeats (Coll1 and Coll2). Most of the mutations in Hennekam Syndrome are localized to the N-terminal part of CCBE1. Domain information retrieved from Uniprot and Alders et al., 2013.*

CCBE1 was not observed in ECs, but in their surrounding region associated with lymphatic vasculature growth (Bos et al., 2011; Hägerling et al., 2013). CCBE1 binds to the ECM components, most strongly to vitronectin, and enhances the lymphangiogenic activity of VEGF-C in a corneal micropocket assay (Bos et al., 2011). VEGF-C and CCBE1 showed co-operative activity during the budding of LECs from the cardinal veins in the double heterozygous *Ccbe1+/-; Vegfc+/-* mice (Hägerling et al., 2013). The expression of CCBE1 in zebrafish is known to be regulated by transcription factors E2F7 and E2F8 that bind to its promoter, thereby modulating lymphangiogenesis (Weijts et al., 2013).

Beyond lymphangiogenesis, CCBE1 has not been studied extensively. It was suggested as a tumor suppressor in ovarian and breast cancer cell lines (Barton et al., 2010; Mesci et al., 2017). However, CCBE1 is clearly important for the development of the heart. It is expressed in early cardiac progenitors and its morpholino-induced knockdown showed that it is important for heart development in chics; the main effects were seen in the cardiac progenitor cells and in the differentiation of cardiomyocytes (Furtado et al., 2014). Interestingly, like VEGF-C – also CCBE1 has an important role in fetal erythropoiesis (Zou et al., 2013) and sinus venosus mediated coronary vasculature development (Bonet et al., 2018), suggesting that these effects of CCBE1 may be - similar to embryonic lymphangiogenesis - mediated through VEGF-C.

6. Proteases

6.1 Plasmin and Thrombin

Plasmin and thrombin are serine proteases produced as inactive precursors, plasminogen, and prothrombin, respectively. Thrombin cleaves fibrinogen into fibrin

to generate the fibrin clot (Coagulation) (Göbel et al., 2018), whereas plasmin dissolves the fibrin clot (Fibrinolysis) to soluble fragments (Chapin and Hajjar, 2015). Both coagulation and fibrinolysis are necessary for wound healing. Blood coagulation initiates the immediate process of hemostasis to prevent blood loss (Reinke and Sorg, 2012). The hemostatic process involves thrombin activation of platelets (Brass, 2003) and the aggregation of platelets at the injury site (Wang et al., 2014) after thrombin formation (Monroe Dougald M. et al., 2002). Plasmin and thrombin, which are essential mediators of the wound healing process, and are also known to cleave and thereby activate VEGF-C and VEGF-D (Lim et al., 2019; McColl et al., 2003). Platelets contain VEGF-C, which is secreted in response to its activation (Wartiovaara et al., 1998). Moreover, VEGF-C is required for the platelet mediated induction of lymphangiogenesis during wound healing (Lim et al., 2019). Because plasmin and thrombin are two major proteases that regulate the wound healing process and also cleave VEGF-C and VEGF-D in vitro, their role in inducing the activation of VEGF-C and VEGF-D in vivo remains speculative. However, direct evidence of the involvement of plasmin and thrombin in these functions in vivo would require extensive analysis, including genetic models.

6.2 A disintegrin and metalloproteinase with thrombospondin motifs 3 (ADAMTS3)

Proteinases of the ADAMTSs family are secreted multi-domain zinc metalloproteases. They share common motifs in their N-terminal protease domain (signal peptide, pro-domain, metalloproteinase domain, disintegrin domain), but feature C-terminally heterogeneous ancillary domains (Kelwick et al., 2015). ADAMTSs are synthesized as precursors, which become active by proteolytic removal of the prodomain (Brocker et al., 2009). The ADAMTS family consists of 19 members, subdivided into seven families based on sequence similarity (Porter et al., 2005).

ADAMTS3, along with ADAMTS2 and ADAMTS14, belongs to procollagen N-proteinase subfamily (commonly called *aminoprocollagen peptidase*) due to its ability to cleave the N-terminal propeptide of procollagen (Bekhouche and Colige, 2015). All three procollagen N-proteinases share a high level of sequence similarity (Colige et al., 2002). The expression of ADAMTS2, -3 and -14 is tissue specific. ADAMTS2 is expressed in skin, bone, tendons and aorta (Colige et al., 1997), ADAMTS3 in cartilage and nervous system (Fernandes et al., 2001; Goff et al., 2006), while ADAMTS14 expression mostly overlaps with ADAMTS2 expression (Goff et al., 2006). ADAMTS2 cleaves procollagen types I, II and III (Colige et al., 2005; Goff et al., 2006; Wang et al., 2003). Alternatively, ADAMTS3 can also process procollagen II in cartilage and procollagen I in dermatosparactic fibroblasts (Fernandes et al., 2001;

Goff et al., 2006), suggesting tissue-specific roles for ADAMTS2, -3 and -14. Mutations in ADAMTS2 which affect the amino procollagen cleavage have been described in Ehlers-Danlos syndrome Type VIIC in human and dermatosparaxis in cattle (Colige et al., 1999). *Adamts2* gene-deleted mice are viable but suffer from reduced processing of aminoprocollagen I and III, which results in fragile skin resulting from the deposition of aminoprocollagen I and reduced parenchymal density in their lungs (Goff et al., 2006). The developmental expression pattern of ADAMTS3 in mouse tissues (like developing cerebral cortex and urinary bladder wall) suggested that it had functions that were not related to procollagens (Goff et al., 2006).

6.3 KLK3

Kallikreins (KLKs) exist as plasma kallikreins and tissue kallikreins. KLK1, KLK2 and KLK3 (or PSA) are the classical kallikreins (tissue kallikreins/glandular kallikreins), which share a high sequence similarity (62-67%) and are secreted as inactive proenzymes (Lawrence et al., 2010). Both KLK3 and KLK2 are expressed and secreted by prostate epithelium in response to androgen (Young et al., 1991).

The substrate and subsequent functions of KLK1, KLK2 and KLK3 have been widely studied. KLK1 is a trypsin-like kininogenase enzyme, which proteolytically activates low molecular weight kinin into bradykinin (BK) and lys-bradykinin (kallidin), and affects several physiological processes, such as smooth muscle contraction, vascular permeability, vascular cell growth, electrolyte balance etc, (Madeddu et al., 2007). KLK2 has a very low kininogenase activity while KLK3 lacks any kininogenase activity (Deperthes et al., 1997). Semenogelins and fibronectin are substrates for KLK2 that activate seminal fluid liquefaction (Deperthes et al., 1996). Interestingly, KLK2 also cleaves the pro-form of PSA into the mature form (Kumar et al., 1997; Takayama et al., 1997). However, the extent of this cleavage remains unclear.

KLK3 is a well-known prostate cancer biomarker for disease diagnosis and monitoring (Hong, 2014). However, the variability of KLK3 levels in humans makes it a controversial biomarker, and many studies focus on the sensitivity and specificity of its detection. Genetic variation in KLK3 levels is associated with prostate cancer risk (Kote-Jarai et al., 2011). KLK3 gene in some individuals shows heterozygous deletion, which correlates its low level in these individuals (Rodriguez et al., 2013).

KLK3 is the most highly expressed KLK in the prostate tissues and in the seminal fluid (Shaw and Diamandis, 2007). It cleaves semenogelin which is prerequisite for seminal clot liquefaction (Lilja, 1985; Lilja et al., 1987), an important step inducing free motility of sperm inside the female reproductive system. Recently, genetic variation in the KLK3 gene was identified in infertile men (Gupta et al., 2017). This

did not affect semen PSA levels, suggesting that it altered the proteolytic activity of KLK3. The role of KLK3 in cancer progression has been controversial (Ishii et al., 2004; Peternac et al., 2006). There is no evidence of in vivo tumorigenic role of KLK3 and further analyses have suffered from the lack of a KLK3 homologue in mice. Interestingly, KLK3 was claimed to be an antiangiogenic molecule in vitro (Fortier et al., 2003, 1999; Mattsson et al., 2008, 2009) and in vivo (Fortier et al., 2003). This effect seems to be independent of its serine protease activity in vivo (Fortier et al., 2003), but dependent on its enzymatic activity in vitro in ECs (Mattsson et al., 2008). Galectin-3, a substrate for KLK3 has also been shown to regulate EC migration, also affects growth and differentiation (Saraswati et al., 2011).

Although VEGF-C was identified as a substrate for KLK4 in a tetrapeptide positional scanning synthetic combinatorial libraries screening (Matsumura et al., 2005), the validation of this in-silico analysis was never performed.

6.4 Cathepsin D

Cathepsin D is a lysosomal aspartyl protease mostly found intracellularly, but it can also be secreted, whereby it degrades several proteins in an acidic environment. Cathepsin D cleaves several substrates, e.g. fibroblast growth factor (FGF), insulin, insulin-like growth factor binding protein (IGFBP), collagen, fibronectin etc. (Benes et al., 2008). Cathepsin D affects tumor growth, progression, and metastasis (Berchem et al., 2002; Glondu et al., 2001, 2002; Hu et al., 2008). The expression and secretion of cathepsin D is increased in breast cancer cells and is estrogen dependent (Liaudet-Coopman et al., 2006). Thrombin mediated upregulation of cathepsin D enhances angiogenesis in chick choriallantoic membrane (CAM) assay and human umbilical vein endothelial cell (HUVECs) (Hu et al., 2008). Cathepsin D is also vital in mediating neuronal cell homeostasis by balancing neuronal cell survival and death and has been implicated in neurodegenerative disease like Neuronal Ceroid Lipofuscinoses (NCL) (Shacka and Roth, 2007; Vidoni et al., 2016).

7 Lymphangiogenesis in health and diseases

Lymphatic vessels in adults are mostly quiescent with few exceptions; for example, the ovaries and mammary glands during the female reproductive cycle, the lacteals in the intestine, and during the hair growth cycle. While many pathological conditions cause a reactivation of lymphangiogenesis (e.g. inflammation, wound healing, and cancer), some pathologies are also characterized by a lack of lymphangiogenesis, most notably primary lymphedema.

7.1 Lymphedema

Lymphedema is a pathological condition where the function of the lymphatic vessels is compromised, resulting in the accumulation of protein-rich fluid, inflammation, decreased immune response, fibrosis, and subcutaneous fat deposition. The compromise in the function of the lymphatic vessels can result from an abnormal lymphatic development or obstruction in the regular lymph flow. Lymphedema is typically recognized as a swelling of the extremities. It can be classified as primary (hereditary) or secondary (acquired), depending on the mode of disease pathogenesis (Warren et al., 2007).

Primary lymphedema is a rare developmental abnormality caused by mutations in genes that are required for the development and function of the lymphatic vasculature. So far, mutations in 28 genes explain about 30% of primary lymphedema cases (Brouillard et al., 2014). These genes encode proteins involved mainly in VEGFR-3 signaling, including some transcription factors, such as GATA2, FOXC2 or SOX18, and they usually cause reduced activity via the VEGFR-3 pathway (Brouillard et al., 2014, 2017; Kazenwadel et al., 2015). Missense point mutations in the FLT4 gene that encodes VEGFR-3 protein are among the most common mutations and a cause of Milroy disease (MD; hereditary lymphedema type I, OMIM 153100) (Connell et al., 2009). These mutations have been shown to abolish VEGFR-3 activity in vitro (Karkkainen et al., 2000). Patients with MD exhibit primarily edema of the lower limbs (Mellor et al., 2010). While VEGFR-3 missense mutations in mice with the *Chy* allele of Flt4 cause lymphatic aplasia in the skin (Karkkainen et al., 2001), patients with MD have lymphatic hypoplasia, which causes lymphatic insufficiency (Mellor et al., 2010). Most of the mutations in FLT4 were identified as autosomal dominant (Connell et al., 2009). However, a few de-novo mutations resulting in sporadic congenital lymphedema have also been identified, suggesting no family history requirement for the MD diagnosis (Carver et al., 2007; Ghalamkarpour et al., 2006). Autosomal dominant mutations in *Vegfc* have been identified in Milroy-like disease with variable disease penetrance (Balboa-Beltran et al., 2014; Fastré et al., 2018; Gordon et al., 2013). *Ccbe1* was found to be mutated in a subset of patients with the Hennekam Syndrome that features lymphatic dysplasia and intestinal lymphangiectasia (Alders et al., 2009). Additionally, the *FAT4*, *FOXC2*, *PTPN14*, *SOX18*, *PIEZO1*, *GATA2*, *EPHB4*, and *GJC2* genes have been identified as causative in primary lymphedema (Grada and Phillips, 2017).

Secondary lymphedema is the most common form of lymphedema where the normal lymphatic vessels are disrupted by infection, surgery, trauma, radiation etc. Secondary lymphedema affects millions of patients worldwide. Breast cancer-associated lymphedema is common for example among breast cancer survivors who undergo

axillary lymph node dissection or radiotherapy (Rockson and Rivera, 2008). Lymphatic filariasis is common in tropical regions and is caused by nematode parasites (mostly *Wuchereria bancrofti*); it affects more than 120 million people. The lodging of the parasites inside the lymphatics leads to an obstruction of the lymphatic flow, and, thus, lymphedema (Pfarr et al., 2009). Currently, no curative treatment exists for secondary lymphedema and the symptomatic treatment consists mostly of physiotherapy, compression garments, and liposuction.

7.2 Tumor lymphangiogenesis

Tumor metastasis is responsible for most of the cancer-associated deaths. Metastasis is a multi-step process, in which the tumor cells escape from the primary tumor to form a daughter tumor at a distance. In many types of tumors, the cells spread from the primary sites to the lymph nodes and, lymph node metastasis has been attributed to tumor aggressiveness and poor prognosis (Stacker et al., 2014). The role of lymph node metastasis in tumor cell dissemination to distant organs was initially based on correlation analysis (Podgrabinska and Skobe, 2014). Recent studies in mice have showed dissemination of tumor cells from lymph nodes to distant organs via the high endothelial venules in lymph nodes (Brown et al., 2018; Pereira et al., 2018). Nevertheless, not all tumor cells disseminate through the lymphatic route (Naxerova et al., 2017). Lymphatic vessels can be located in the tumor periphery (peritumoral) or, more rarely, within the tumor. Intratumoral lymphatics can be non-functional and dysmorphic due to the high physical pressure inside the tumor (Leu et al., 2000; Padera et al., 2002). While most of the tumor-associated lymphatic vessels are located peritumorally, there is no good statistics for human cancers and thus, the debate on the role of intratumoral lymphangiogenesis during tumor metastasis is not entirely settled (Ji, 2006).

The mechanism of tumor lymphangiogenesis has been extensively studied. VEGF-C/D-VEGFR-3 signaling axis is the major signaling pathway that contributes to tumor lymphangiogenesis. VEGF-C levels have been studied in several human tumor types. VEGF-C level correlates with lymph node metastasis, poor prognosis and survival (Akagi et al., 2000; Kinoshita et al., 2001; Möbius et al., 2007; Tsurusaki et al., 1999). VEGF-C may in some cases regulates leukemic cell growth and proliferation (Dias et al., 2002). VEGF-C levels also correlate with poor disease prognosis as well as poor drug responsiveness in acute myeloid leukemia (de Jonge et al., 2008, 2010).VEGF-C and VEGF-D are primarily expressed by tumor cells and tumor-associated stromal cells, such as fibroblasts and macrophages (Dieterich and Detmar, 2016).

Overexpression of VEGF-C and VEGF-D induces lymphatic metastasis in several tumor models (Hirakawa et al., 2007; Mandriota et al., 2001; Mattila et al., 2002;

Skobe et al., 2001; Stacker et al., 2001), where the effect can result in intratumoral lymphangiogenesis, peritumoral lymphangiogenesis, lymph node metastasis, and distant metastasis. Moreover, inhibition of VEGF-C/D-VEGFR-3 signaling axis using soluble VEGFR-3 or monoclonal antibodies against VEGFR-3, VEGF-C, Nrp2 has been shown to inhibit lymphatic metastasis in several preclinical models (Caunt et al., 2008; Gogineni et al., 2013; Lin et al., 2005; Roberts et al., 2006). Additional growth factors like VEGF-A, angiopoietins, hepatocyte growth factor (HGF), fibroblast growth factor (FGF), and insulin-like growth factor (IGF) have also been shown to induce lymph node metastasis in experimental tumor models (Dieterich and Detmar, 2016).

In addition to their role in the development of tumor metastasis and immunosuppressive tumor environment (Lund et al., 2012), lymphatic vessels associated together with the so-called immunoscore correlate with a better outcome of colon carcinoma patients (Galon et al., 2012). Furthermore, VEGF-C associated lymphangiogenesis promotes immunosurveillance and exacerbates the response to immunotherapy in glioblastoma (Song et al., 2020) and melanoma (Fankhauser et al., 2017), mostly via the recruitment of primed T cells into the tumor.

8 Therapeutic Lymphangiogenesis

8.1 Pro-lymphangiogenic therapy

Pro-lymphangiogenic therapy may be beneficial to treat patients with secondary lymphedema. VEGF-C has been used as a therapeutic candidate in the form of recombinant protein, adenovirus, and naked plasmid in various preclinical models of lymphedema, where it has resolved lymphedema (Karkkainen et al., 2001; Saaristo et al., 2002; Szuba et al., 2002b; Tammela et al., 2007b; Visuri et al., 2015; Yoon et al., 2003). Interestingly, a combination of lymph node transfer and targeted adenoviral delivery of VEGF-C has been shown to be a promising approach for the regeneratation of functional lymphatic network that replaces damaged lymphatic vessels (Honkonen et al., 2013b; Lähteenvuo et al., 2011). In fact, adenoviral VEGF-C (“Lymfactin”) has successfully completed human Phase I studies (https://herantis.com/) and patients are being recruited for a Phase II study. The anti-inflammatory drugs ketoprofen (NCT03783715) and bestatin may also reduce skin thickness and cutaneous pathology in patients with primary and secondary lymphedema (Rockson et al., 2018). However, the clinical trials for bestatin (Ubenimex) were discontinued after phase II as neither the primary nor the secondary objectives were reached (Eiger BioPharmaceuticals, 2018).

8.2 Anti-lymphangiogenic therapy

The development of an anti-lymphangiogenic drugs for cancer therapy has not resulted in any tangible results yet. The blocking antibody against VEGFR-3, IMC-3C5 (NCT01288989) showed only minor effects on the tumor in human patients (Saif et al., 2016). Similar clinical trials were initiated in 2012 for the partially neutralizing VEGF-C-targeting antibody VGX-100 (NCT01514123) both alone, or in combination with the VEGF-A-neutralizing antibody bevacizumab, but there has not been further progress on this.

Despite the lack of success stories, combination of treatments targeting VEGF-C/VEGFR-3 and VEGF-A/VEGFR-2 signaling are still considered as a viable strategy for the control of tumor angiogenesis, lymphangiogenesis and metastasis (Matsumoto et al., 2013). The use of VEGF-C/VEGFR-3 targeting is supported by recent evidence showing that VEGF-C expression is increased in response to anti-VEGF therapy (Li et al., 2014).

However, anti-VEGF-C agents are also used outside oncology. E.g. soluble VEGFR-3 (OPT-302) is currently in Phase I/II clinical trial for the treatment of neovascular wet acute form of age-associated macular degeneration (AMD) in combination with Ranibizumab (anti-VEGF, NCT03345082) or for the treatment of persistent diabetic macular edema in combination with Aflibercept (soluble VEGFR-1, NCT03397264).

AIMS OF THE STUDY

The main aim of the study was to understand the mechanism of VEGF-C activation during lymphangiogenesis and identify the molecular regulators for VEGF-C.

The specific aims of the thesis are:

I. To study the mechanism of CCBE1 mediated VEGF-C regulation during lymphangiogenesis
II. To investigate the localization, distribution, and roles of the different domains of VEGF-C, CCBE1, and ADAMTS3 during VEGF-C/VEGFR-3 signaling.
III. To identify additional proteases responsible for VEGF-C activation, and comparing their effect on VEGFR-2 and VEGFR-3 activation.

MATERIALS AND METHODS

The section includes a summary of the materials and methods from my thesis. The detailed protocol of the methods is described in the original publications.

MATERIALS

Mouse lines

Mouse line	Description	Source	Used in
Vegfr3$^{EGFP/Luc}$	EGFP-luciferase fusion protein at the 3'-UTR of *Vegfr3* gene under IRES element	(Martínez-Corral et al., 2012)	I
K14-VEGF-C-CT	Overexpression of C-terminal propeptide of VEGF-C in basal epidermal keratinocytes	II	II
K14-VEGF-ΔC	Overexpression of VEGF-C lacking the C-terminal propeptide in basal epidermal keratinocytes	II	II
C57Bl/6JRccHsd, FVB/N	Wild-type mice	Envigo, Harlan	I, III

Cell lines

Cell line	Description	Source	Study used in
Ba/F3-hVEGFR-3/EpoR	Murine pro-B cells expressing chimeric human VEGFR-3	(Achen et al., 2000)	I, II, III
Ba/F3-mVEGFR-2/EpoR	Murine pro-B cells expressing chimeric mouse VEGFR-2	(Stacker et al., 1999b)	I, II, III
PAE-VEGFR-2-StrepIII	Porcine aortic endothelial (PAE) cells expressing strepIII-tagged VEGFR-2	(Anisimov et al., 2013)	III

PAE-VEGFR-2	Porcine aortic endothelial (PAE) cells expressing VEGFR-2	(Waltenberger et al., 1994)	III
PAE-VEGFR-3-StrepIII	Porcine aortic endothelial (PAE) cells expressing strepIII-tagged VEGFR-3	(Leppanen et al., 2013)	I, II, III
PAE-VEGFR-3	Porcine aortic endothelial (PAE) cells expressing VEGFR-3	(Pajusola et al., 1994)	I, II, III
PAE-VEGFR-3/neuropilin-2	Porcine aortic endothelial (PAE) cells expressing VEGFR-3 and neuropilin 2	(Kärpänen et al., 2006)	I
293T	Human embryonic kidney cells	ATCC	I, II, III
CHO DG44	Chinese hamster ovary derived epithelial cells	Invitrogen	I, II, III
Sf9	Insect cells (*Spodoptera frugiperda*)	Invitrogen	I, II, III
Schneider S2 cells	Insect cells (*D. melanogaster*)	Invitrogen	I, II, III
NIH-3T3	Mouse embryo derived fibroblast cells	ATCC	I, II
Cos-7	Monkey kidney derived fibroblast cells	ATCC	II
293T-CCBE1-StrepIII	293T cells expressing Strep III tagged CCBE1	Study I	I, II, III
293F-ADAMTS3-V5-H6	293F cells expressing V5- and his-tagged ADAMTS3	Study I	I
LEC	Human dermal lymphatic endothelial cells	Promocell	II
HUVEC	Human umbilical vein endothelial cells	Promocell	II
HUVEC-VEGFR-3-GFP	Human umbilical vein endothelial cells expressing GFP-tagged VEGFR-3	(Ghalamkarpour et al., 2009)	I

Recombinant proteins

Recombinant protein	Description	Source	Study used in
ΔNΔC-VEGF-C	C-terminal his-tagged human VEGF-C with aa residues 103-215 (minor form)	(Kärpänen et al., 2006)	I, II, III
pro-VEGF-C	Untagged VEGF-C expressed from full-length cDNA	I, II, III	I, II, III
pro-VEGF-D	C-terminally his-tagged human full-length VEGF-D	(Achen et al., 1998)	I, III
ΔNΔC-VEGF-D	C-terminally his-tagged human major mature form of VEGF-D (aa residues 89-205)	(Achen et al., 1998)	III
CCBE1-StrepIII	StrepIII-tagged full-length CCBE1	I,	I, II, III
CCBE1-175-V5	C-terminally his-tagged CCBE1 (aa residues 1-175)	I, II	I, II
CCBE1-CollD-V5-H6	C-terminally V5- and his-tagged CCBE1 (aa residues 207-406)	II	II
ADAMTS3-V5-H6	C-terminally V5- and his-tagged full-length ADAMTS3	I	I, II
VEGFR-2 /Fc D1-3	C-terminally IgG1Fc-tagged human VEGFR-2 extracellular domains 1-3	(Leppanen et al., 2010)	I, III
VEGFR-3/Fc D1-7	C-terminally IgG1Fc-tagged human VEGFR-3 extracellular domains 1-7	(Leppanen et al., 2013)	I, II, III

VEGFR-3/Fc D1-3	C-terminally IgG1Fc-tagged human VEGFR-3 extracellular domains 1-3	(Leppanen et al., 2013)	I, II
VEGFR-3/Fc D4-7	C-terminally IgG1Fc-tagged human VEGFR-3 extracellular domains 4-7	(Leppanen et al., 2013)	II
Collagen type I	Collagen 1 HC, rat tail	BD Biosciences, #354249	II
Fibronectin	Fibronectin purified from human plasma	Sigma, #F08952	II
Gelatin	Gelatin purified from porcine skin	Sigma, #G1890	II
Vitronectin/Fc	C-terminally IgG1Fc-tagged vitronectin	(Hakanpaa et al., 2015)	II
KLK3	KLK3 (isoform B) purified from human seminal plasma	(Wu et al., 2004)	III
Cathepsin D	His-tagged cathepsin D (aa residues 21- 412)	R&D Systems, #1014-AS-010	III

Recombinant AAV9

AAV9	**Payload**	**Source**	**Study used in**
VEGF-C	Full-length VEGF-C	(Karkkainen et al., 2001)	I, III
ΔNΔC-VEGF-C	Mature VEGF-C form (aa residues 108-223)	(Anisimov et al., 2009)	I, III
mCCBE1-V5	V5-tagged mouse CCBE1	I	I
HSA	Human serum albumin (control)	(Anisimov et al., 2009)	I
KLK3-form (VEGF-C)	Mature VEGF-C (KLK3-cleaved	III	III

	form)		
Cathepsin D form (VEGF-C)	Mature VEGF-C (cathepsin D-cleaved form)	III	III
Control (S1/S2)	Scrambled sequence		III

Antibodies

Antibody	**Type**	**Source**	**Study used in**
anti-VEGF-C antiserum-6, human	Rabbit polyclonal	(Baluk et al., 2005)	I, II, III
anti-VEGF-C antiserum-882, human	Rabbit polyclonal	(Joukov et al., 1997)	III
anti-VEGF-C, mouse	Mouse monoclonal	Santa Cruz Biotechnology, #sc-374628	III
anti-VEGF-C, human	Goat polyclonal	R&D Systems, #AF752	I, II
anti-KLK3, human	Mouse monoclonal, #5C7	(Stenman et al., 1999)	III
anti-VEGF-D, human	Goat polyclonal	R&D Systems, #AF286	I, III
anti-phosphotyrosine	Mouse monoclonal	Millipore/Merck, clone #4G10	I, II, III
anti-VEGFR-2, human	Goat polyclonal	R&D Systems, #AF357	III
anti-VEGFR-3, human	Mouse monoclonal	(Dumont et al., 1998)	I, II, III
anti-VEGFR-3, human	Rabbit polyclonal	Santa Cruz Biotechnology, #sc-321	
anti-CCBE1,	Rabbit polyclonal	Atlas antibodies,	I, II, III

human		#HPA041374	
anti-ADAMTS3, human	Goat polyclonal	Santa Cruz Biotechnology, #sc-21486	
anti-Penta-His	Mouse monoclonal	Qiagen, #34660	I, II, III
anti-Lyve-1, mouse	Rabbit polyclonal	(Karkkainen et al., 2004)	I, II, III
anti-CD31, mouse	Rat monoclonal	BD Biosciences, Clone MEC 13.3	I, III
anti-CD31, mouse	Armenian hamster monoclonal	Milipore/Merck, #MAB1398Z	II
Strep-Tactin-HRP	Strep-Tactin-HRP conjugate	IBA, #2-1502-001	I, II, III
anti-V5 tag	Mouse monoclonal	Invitrogen/ThermoFischer Scientific, #46-0705	I, II
anti-Prox1, human	Goat polyclonal	R&D Systems, #AF2727	I, II
anti-CD45, mouse	Rat polyclonal	BD Pharmingen, #550566	I
anti-Actin, Smooth muscle, mouse	Mouse Cy3-conjugated	Sigma, C6189	I

METHODS

Method	**Study used in**
AAV9 transduction of mice	I, III
Bioluminescent optical imaging	I
Cell culture, transfection and transduction	I, II, III
Molecular (plasmid) cloning	I, II, III
Coomassie and silver staining	I, III

Immunofluorescence/immunohistochemistry	I, II, III
Immunoprecipitation and immunoblotting	I, II, III
Lentivirus production and shRNA	I
Mass spectrometry and N-terminal sequencing	I, II, III
Matrix binding assays	II
Metabolic labeling	I, II, III
Microscopy	I, II, III
Phosphorylation assays	I, II, III
Protein binding assay/ELISA	II
Protein expression and purification	I, II, III
Real-time quantitative PCR	I, II
Statistical analysis	I, II, III
Western blotting	I, II, IIII

AAV9 transduction experiment

Adeno-associated viral serotype 9 (AAV9) vectors (*Table Recombinant AAV9*) were injected into tibialis anterior (TA) muscles (i.m.) of mice at doses between $4x10^{10}$ to $6x10^{10}$ viral particles/muscle. Two (Study II) to Three (Study I) weeks after transduction, mice were terminally anesthetized with an intraperitoneal injection of ketamine and xylazine. TA muscles were embedded in OCT and snap-frozen in a liquid nitrogen-cooled solution of 2% pentane in 2-methyl butane. A small piece of TA muscles was snap-frozen in an eppendorf tube for protein/mRNA analysis.

Immunoprecipitation and Western blotting

Immunoprecipitation was performed to analyze the (co)interacting partner of proteins of interest. Immunoprecipitations were performed either from the cell lysates (for phosphorylation assay) or from cell supernatants. Immunoprecipitation was performed by precipitating proteins with mix of protein A/G sepharose (GE Healthcare) and antibodies overnight at 4°C. The precipitated proteins were extracted by boiling the sepharose beads in Laemmli sample buffer and separated by SDS-PAGE. Proteins were then transferred to membranes (polyvinylidene fluoride membranes or nitrocellulose) and incubated with primary antibodies against indicated proteins and

compatible secondary antibodies. Western blot bands were imaged using the Li-COR Odyssey Fc or cDigit Imaging system (Li-COR Biosciences) and quantified using Fiji ImageJ (NIH) or Image StudioLite (Li-COR Biosciences).

Protein expression and purification

Recombinant proteins were purified from insect (S2 and Sf9 cells) and mammalian cells (293T and CHO cells). While stable cell pools or baculovirus-based methods were used for protein expression and purification from insect cells, stable cell pools or clones were used for mammalian cells. The proteins were either expressed with tags (StrepIII and Hexahistidine) or untagged (only full-length VEGF-C). The purification of tagged proteins was based on affinity-based purification, whereas the purification of untagged VEGF-C was accomplished by heparin affinity chromatography followed by cation exchange chromatography. For all purifications, the final step was size-exclusion chromatography.

Phosphorylation assay

Phosphorylation assays were performed to detect the activity of VEGFR-2 and VEGFR-3. Near confluence cells expressing VEGFR-2 or VEGFR-3 were serum-starved overnight in DMEM/0.2%BSA. The cells were stimulated using recombinant proteins for 5-30 minutes and lysed with 1% Triton-X100 or mix of 0.5% Triton-X100 and 0.5% NP-40 in 50mM Tris pH 8 containing 1mM Na_3VO_4, 1mM NaF, 2mM PMSF, 2µg/ml leupeptin and 0.07U/ml aprotinin. Proteins from the lysates were immunoprecipitated using appropriate antibodies and analyzed by Western blotting.

Metabolic labeling

Metabolic labeling is an alternative method to Western blotting for protein detection, which incorporates radiolabeled amino acids (in this case, cysteine and methionine). In our study, metabolic labeling was performed to reduce the non-specific signals from the precipitating antibody bands during the immunoprecipitation. For metabolic labeling, cells were incubated with [^{35}S]-cysteine/[^{35}S]-methionine (EasyTag Express Protein Labeling Mix, PerkinElmer) for 24 to 48 hours. The proteins were precipitated from the supernatant or lysates using antibodies and resolved using SDS-PAGE. The gels were then vacuum dried and exposed to phosphoimager plates, which were scanned using a Typhoon 9400 scanner (Amersham Biosciences; GE Healthcare).

Cell culture, transfection and stable cell line generation

Cells were grown in appropriate media according to the manufacturer's instructions. Cells were transfected with expression constructs using jetPEI transfection reagent

(Polypus-transfection Inc.) and grown in the presence of DMEM/0.2%BSA or metabolic labeling medium, 24 hours post-transfection.

For the generation of stable cell lines, cells were transfected with expression plasmids and grown for 48 hours. The cells were then selected in the presence of selection antibiotics. Visible clones were isolated by the cloning ring technique and expanded. Protein expression by the stable cell lines was confirmed by Western blotting, and the clones with the highest expression levels were used for further studies.

Quantitative PCR

mRNA was analyzed from mouse tissues or cells. Total RNA was extracted from cells and tissues with the Nucleospin RNA II kit (Macherey-Nagel) followed by cDNA synthesis using the iScript cDNA synthase kit (Bio-Rad) or High Capacity cDNA Reverse Transcription Kits (Applied Biosystems). qPCR analysis was performed using the SYBR Green qPCR kit or Taq man probe kit (Thermo Fischer Scientific) with Bio-Rad CFX96 Real-time System. Gene expression was normalized to HPRT or GAPDH, and relative fold changes were quantified using the $2^{-\Delta\Delta Ct}$ method.

Ba/F3-VEGFR-EpoR assays

Ba/F3-VEGFR-EpoR cells are murine bone marrow-derived cells that express the ligand-binding domain of VEGFR and the transmembrane and cytoplasmic domain from the mouse erythropoietin receptor (EpoR). These chimeric receptor-containing Ba/F3 cells are dependent on the ligands (e.g. VEGF-C for VEGFR-2 and VEGFR-3) for their growth and survival in the absence of IL-3. For the assay, Ba/F3 cells were grown in the presence of conditioned cell culture medium or recombinant proteins for 48 hrs, followed by the MTT (Sigma) assay.

Histochemistry and Immunofluorescence

Frozen tissues sections of 8-10 μm were fixed in ice-cold acetone and stained with the primary antibodies and appropriate secondary antibodies. DAPI with VECTASHIELD (Vector laboratories) was used to stain nuclei.

For the whole mount staining, mouse ears were separated into dorsal and ventral halves and fixed in 4% PFA for 1 hour at room temperature. The tissues were blocked with donkey immunomix (5% donkey serum, 0.2% BSA, 0.05% sodium azide, 0.3% Triton X-100 in PBS) and stained with primary and appropriate fluorescently labelled secondary antibodies. The ears were washed extensively with PBS/0.3% Triton X-100 after each antibody incubation.

Fluorescent images were taken with an Axioimager Z2 upright epifluorescence microscope and a Zeiss LSM 780 confocal microscope. Images were processed and quantified using Fiji Image J.

Statistical analysis

Data in the study were analyzed using GraphPad Prism statistical analysis software (Versions 6, 7, and 8). Data in the studies are presented as mean±SD or mean±SEM. The data were compared for the differences using two-tailed unpaired t-test or one-way ANOVA followed by post hoc tests (Tukey test, Games-Howell test). Data were considered significant at $P<0.05$.

RESULTS AND DISCUSSION

I. CCBE1 induces lymphangiogenesis via ADAMTS3 regulated proteolytic processing of VEGF-C

CCBE1 increases the lymphangiogenic potential of VEGF-C, and its deficiency results in a phenotype similar to Vegfc deficient embryos (Bos et al., 2011), implying that CCBE1 may function in the VEGF-C/VEGFR-3 signaling pathway.

To study the effect of CCBE1 on pro-VEGF-C, 293T cells were cotransfected with VEGF-C and CCBE1 expression constructs. The coexpression of CCBE1 enhanced the processing of VEGF-C to the fully processed 21 kDa mature form, which improved the growth and survival of IL-3-depleted Ba/F3 cells expressing chimeric VEGFR-2/EpoR or VEGFR-3/EpoR - receptor. Notably, the secretion of VEGF-C was also increased upon by the CCBE1 coexpression. Although there is no clear demarcation between cell types expressing VEGF-C and CCBE1 during mouse development, both are expressed near the region where the first lymphatic sprouts appear (Bos et al., 2011; Hägerling et al., 2013; Karkkainen et al., 2004). Furthermore, in vivo, the combination of CCBE1 and VEGF-C stimulated lymphangiogenesis and angiogenesis better than VEGF-C alone. These findings have subsequently been confirmed by independent studies (Bui et al., 2016; Guen et al., 2014).

The primary structure of CCBE1 did not fit into any protease family. CCBE1 enhancement of VEGF-C processing was limited to HEK 293T cells in our analysis of a set of cell lines, suggesting the involvement of a selectively expressed CCBE1-interacting protease. Mass spectrometric analysis has been commonly used to identify such protein-protein interactions (Smits and Vermeulen, 2016). Mass spectrometric analysis of partially purified CCBE1 from 293T supernatant identified a disintegrin and metalloproteinase with thrombospondin motifs 3 (ADAMTS3) as the main protease bound to CCBE1. Cotransfection studies confirmed the processing of VEGF-C by ADAMTS3, which was enhanced in the presence of CCBE1. The cleavage site in VEGF-C was at the junction between the N-terminal propeptide and VHD (^{109}FAA↓AHYNT^{116}; Figure 5), which was identical to the site of processing identified previously in cultures of PC-3 prostate carcinoma (Joukov et al., 1997). While the biosynthesis of VEGF-D is very similar to VEGF-C (Achen et al., 1998), it was not affected by ADAMTS3. The difference in the processing of VEGF-C and VEGF-D is attributed to the distinct amino acid residues preceding the VHD (Figure 3 and Figure 5).

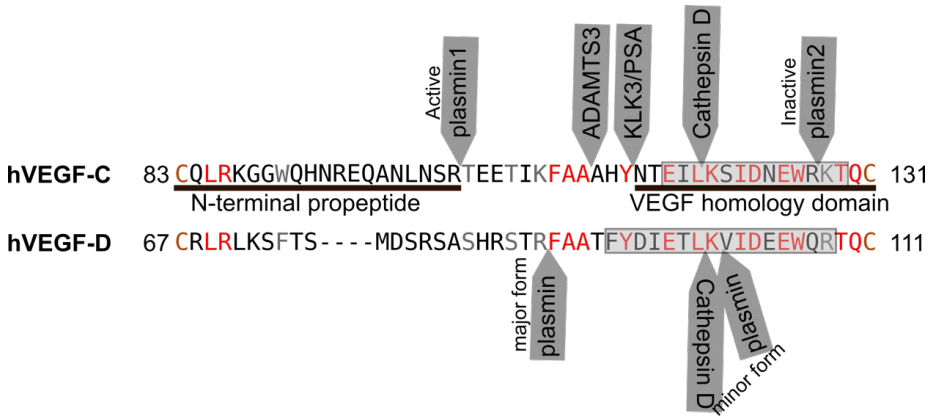

Figure 5: *Representation of the amino acid sequences of human VEGF-C and VEGF-D. The proteases (identified in our study and known before) that cleaves VEGF-C and VEGF-D between the N-terminal propeptides and VHD are shown. The N-terminal alpha-helix of VEGF-C and VEGF-D are boxed.*

ADAMTS3 was initially identified as a procollagenase (Bekhouche and Colige, 2015). Interestingly, mutations in the *ADAMTS3* gene were recently identified in patients affected by HS, confirming the importance of ADAMTS3 during lymphatic vasculature development (Brouillard et al., 2017). Hence, ADAMTS3 was the first known protease that activates VEGF-C during the development of the lymphatic vasculature. However, the loss of *Adamts3* in mice compromised the development of lymphatic vessels without any visible effect on procollagen processing (Janssen et al., 2016). Thus, the essential function of ADAMTS3 is in lymphangiogenesis and not processing procollagens in connective tissue (Janssen et al., 2016).

Because CCBE1 enhanced VEGF-C mediated lymphangiogenesis in the cornea (Bos et al., 2011) via its N-terminal domain (residues 1-191), we generated a recombinant protein comprising amino acid residues 1-175 of CCBE1 ($CCBE_{1\text{-}175}$). When co-applied with pro-VEGF-C, $CCBE_{1\text{-}175}$ increased the phosphorylation of VEGFR-3 in porcine aortic endothelial cells (PAE) stably expressing VEGFR-3 (PAE-VEGFR-3) more than pro-VEGF-C alone. We could precipitate both pro- and mature VEGF-C by VEGFR-3- and anti-phosphotyrosine antibodies from the PAE cells after their covalent cross-linking in the presence of $CCBE_{1\text{-}175}$. Importantly, binding of pro-VEGF-C to VEGFR-3 was increased in the presence of $CCBE_{1\text{-}175}$ in PAE cells expressing VEGFR-3 or both VEGFR-3 and Nrp2. Pro-VEGF-C was not only compromised in its ability to induce VEGFR-3 phosphorylation, but it also competitively inhibited the effect of mature VEGF-C on VEGFR-3 activation.

ADAMTS3 has a thrombospondin (TSP) motif, which contains a conserved binding motif for CD36, which is expressed on the endothelial cell surface (Son et al., 2018; Tortorella et al., 2000). We found ADAMTS3 mRNA in LECs and HUVECs (in study II). The activation of VEGFR-3 by VEGF-C in the presence of $CCBE_{1-175}$ was very rapid in our study. The rapid activation could be due to the ability of $CCBE_{1-175}$ to move pro-VEGF-C from the liquid phase to the EC surface, where rapid processing occurred by the ADAMTS3 protease. Thus, $CCBE_{1-175}$ may concentrate necessary components for VEGF-C processing on the PAE cell surface, where ADAMTS3 is thought to be bound via its TSP motif to the CD36 receptor. Indeed, we could also detect the interaction between CCBE1 and ADAMTS3 in our assay. Moreover, the EGF domain of CCBE1 ($CCBE_{1-191}$) also binds to ECM components (Bos et al., 2011), which would enable the ECM to act as a secondary processing compartment. Interestingly, the TSP motif in the closely related protease ADAMTS4 resulted in substrate processing by interacting with the substrate (Tortorella et al., 2000), which in the case of ADAMTS3 would be the VEGF-C/CCBE1 complex.

We also found that the coexpression of Nrp2 and VEGFR-3 was enough for pro-VEGF-C binding to VEGFR-3, and that $CCBE_{1-175}$ enhanced this binding. However, pro-VEGF-C on its own inhibited VEGFR-3 signaling and hence in-situ activation of VEGF-C on the LECS surface is most likely responsible for the subsequent activation of inactive pro-VEGF-C, while bound to VEGFR-3. In addition to VEGF-C, ADAMTS3 also cleaved CCBE1. CCBE1 used in our study was $CCBE_{1-175}$, which encodes the N-terminal EGF-like domains of CCBE1 and resembles the size of CCBE1 polypeptide after ADAMTS3 cleavage. However, whether this cleavage is a prerequisite for the VEGF-C processing remains open for investigation.

In conclusion, the activation of VEGF-C is an essential process during the development as lack of any of the activating factor CCBE1/ADAMTS3 compromises the development of the lymphatic vasculature. We have shown that ADAMTS3 can activate VEGF-C with the assistance of CCBE1 and that a trimeric complex of CCBE1/VEGF-C/ADAMTS3 is required for the efficient processing and activation of pro-VEGF-C. Based on the results in study I, we have proposed a model (Figure 6; model 1) of VEGF-C activation. However, in our study, we could not explain the molecular mechanism by which CCBE1 increases VEGF-C secretion and mobilization, and especially the individual contributions of the two domains of CCBE1 to its activity.

II. The role of the different CCBE1 and VEGF-C domains in VEGF-C-mediated lymphangiogenesis

The biological activity of pro-VEGF-C suggests that the role of its C-terminal domain is similar to that of the heparin-binding domain of VEGF-A, despite having a lower affinity for heparin. However, the C-terminal domain of VEGF-C is much larger and energetically more expensive to synthesize than the heparin-binding of VEGF-A and we speculated that its high conservation throughout the whole animal kingdom might reflect some additional function.

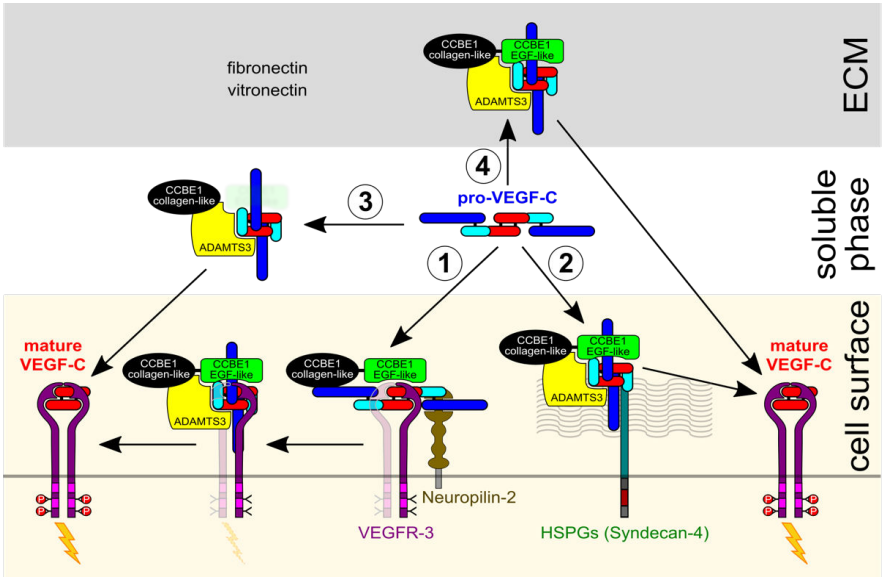

Figure 6: *Model of VEGF-C activation based on the thesis. Four models of VEGF-C activation are shown. 1. Activation of VEGF-C bound to VEGFR-3, 2. Activation of HSPG-bound VEGF-C, 3. Activation of VEGF-C in soluble phase and 4. Activation of ECM bound VEGF-C. Adapted from Jha et al., 2017.*

To understand the role of the C-terminal domain of VEGF-C, we purified different recombinantly expressed forms of VEGF-C, including VEGF-C-NT (the N-terminal propeptide of VEGF-C), VEGF-C-CT (the C-terminal propeptide of VEGF-C), ΔNΔC-VEGF-C (the VHD of VEGF-C) and pro-VEGF-C (full-length VEGF-C). We studied the ability of these different VEGF-C forms to bind ECM deposited by NIH-3T3 and Cos7 fibroblast cells. Both VEGF-C-CT and VEGF-C-FL bound to the ECM deposited by fibroblast cells, and the bound proteins were released in the presence of heparin or ADAMTS3.

Fibronectin is one of the most widely expressed ECM proteins, and VEGF-A had been previously shown to specifically bind to fibronectin (Wijelath et al., 2006). Both LECs and BECs express comparable levels of fibronectin (Podgrabinska et al., 2002). In our assay, we found a concentration-dependent binding of pro-VEGF-C to fibronectin. Zebrafish with a mutant *vegfc* gene, that produces a VEGF-C protein without the C-terminal domain, failed to develop the earliest lymphatic vessels, which was attributed to a secretion defect in the mutant (Villefranc et al., 2013). In another study, it was shown that a cleavage of the C-terminal propeptide is not a prerequisite for the CCBE1-enhanced N-terminal processing of VEGF-C (Bui et al., 2016). Hence, we hypothesized that the C-terminal propeptide of pro-VEGF-C is required for pro-VEGF-C localization to the ECM and cell surface. The localization of pro-VEGF-C could be essential for the gradient formation during lymphatic growth similar to what has been reported in the case of VEGF-A and angiogenesis (Gerhardt et al., 2003).

To better understand the in vivo role of the C-terminal domain of VEGF-C, we developed transgenic mice overexpressing VEGF-C-CT (K14-VEGF-C-CT; contains only C-terminal propeptide) and VEGF-C-ΔC (K14-VEGF-C-ΔC; lacks C-terminal propeptide) under the keratin 14 (K14) promoter, which targets transgene expression to the basal epidermis of the skin. Surprisingly, K14-VEGF-C-CT mice showed a sparser network of lymphatic vessels in the dermis than WT mice. Analysis of the skin of compound K14-VEGF-C-ΔCxK14-VEGF-C-CT mice revealed that they have stronger hyperplasia of the lymphatic vessels than K14-VEGF-C-ΔC mice. The functional analysis of the lymphatic vessels by fluorescent microlymphangiography was consistent with the observed lymphatic vessel phenotype. In order to explain the in vivo findings, we analyzed supernatants of cell cultures transfected with expression constructs for the different forms of VEGF-C. Constructs encoding VEGF-C-ΔC resulted in a minimal expression of the mature form of VEGF-C, but complementation of VEGF-C-ΔC with VEGF-C-CT increased the amount of mature VEGF-C. Notably, expression of the CT domain increased the precipitation of both pro- and mature forms of VEGF-C by VEGFR-3/Fc protein over that obtained by VEGF-C-ΔC transfection alone.

K14-VEGF-C-CT mice showed reduced complexity in lymphatic vessel patterning and branching. We speculated the reduction of the lymphatic network complexity could result from competition between the CT propeptide of K14-VEGF-C-CT mice with the endogenous VEGF-C, ultimately mobilizing the endogenous VEGF-C into the soluble phase. Interestingly, this phenotype resembles the blood vasculature patterning phenotype observed in mice that express only the VEGF-A_{120} isoform (Ruhrberg et al., 2002). Accordingly, several studies have shown that the matrix binding of VEGF-A maintains complexity of vascular branching and patterning

whereas the soluble form of VEGF-A is mostly responsible for the proliferation of endothelial cells and result in hyperplasia of the blood vasculature (Carmeliet et al., 1999; Gerhardt et al., 2003; Lee et al., 2005; Ruhrberg et al., 2002). The K14-VEGF-C-ΔC mice showed hyperplastic lymphatic vessels in the skin, presumably because the transgene-encoded protein had an increased ability to activate VEGFR-3 (Joukov et al., 1997). The compound K14-VEGF-C-ΔCxK14-VEGF-C-CT mice showed the strongest lymphatic hyperplasia suggesting requirement for the CT domain for full VEGF-C activity in lymphangiogenesis, which was confirmed in the in vitro complementation assay.

CCBE1 expression was detected in human dermal lymphatic vessels (Hasselhof et al., 2016) and in a subset of Prox1 expressing LECs (Facucho-Oliveira et al., 2011). Our analysis confirmed the expression of CCBE1 both at the protein and mRNA level, and ADAMTS3 at the mRNA level in LECs. However, during development, the expression of CCBE1 occurred near the developing vessels rather than is the ECs (Bos et al., 2011). Hence, further in vivo studies are required for understanding of the expression dynamics of CCBE1. One major problem with CCBE1 detection at the protein level is because of the lack of specific antibodies against CCBE1.

The phenotype of the CCBE1ΔEGF knock-in embryos suggests a possible role of the EGF-like domains in establishing guidance cues for the LECs (Roukens et al., 2015). Our previous study (Study I) also suggested the requirement of the EGF-like domains of CCBE1 ($CCBE_{1\text{-}175}$) in the regulation of VEGF-C mediated VEGFR-3 activity. To further investigate the role of $CCBE_{1\text{-}175}$, we stimulated both PAE and PAE-VEGFR-3 cells with $CCBE_{1\text{-}175}$ and pro-VEGF-C. After stimulation, we observed a reduction in the amount of pro-VEGF-C in the supernatant of both PAE or PAE-VEGFR-3 cells, suggesting that more pro-VEGF-C was sequestered to the cell surface in the presence of $CCBE_{1\text{-}175}$. Interestingly, CCBE1, ADAMTS3 and VEGF-C, when expressed in 293T cells, remain mainly bound to the cell surface. One reason for this may be that VEGF-C binds cell surface heparan sulfate proteoglycans produced by LECs (Yin et al., 2011). The combination of $CCBE_{1\text{-}175}$ with pro-VEGF-C also significantly enhanced the effect of pro-VEGF-C on VEGFR-3 activity in Ba/F-VEGFR-3/EpoR assay.

$CCBE_{1\text{-}175}$ rapidly immobilizes pro-VEGF-C to the surface of the endothelial cells irrespective of the presence of VEGFR-3. Similar to VEGF-C, CCBE1 and ADAMTS3 also localized to the cell surface, which would rapidly stimulate the formation of CCBE1/VEGF-C/ADAMTS3 trimeric complex on the surface of the ECs, ultimately resulting in efficient VEGF-C activation. The presence of coreceptors, such as β1 integrin (Zhang et al., 2005), Nrp2 (Study I), and syndecan-4 (Johns et al., 2016) on the surface of LECs could promote stability of the complex, and CCBE1

binding to vitronectin (Bos et al., 2011) would further increase local concentration of CCBE1 in the ECM to induce proper lymphatic vessel assembly and sprouting. Nrp2 is more abundant in tip cells than stalk cells in the growing lymphatic vessels (Xu et al., 2010). Furthermore, the Nrp2 deficient mice have a defect in sprouting lymphangiogenesis but have normal lymph sacs (Yuan et al., 2002), which partially resembles to the phenotype of CCBE1ΔEGF knock-in embryos (Roukens et al., 2015). Nevertheless, the activation of pro-VEGF-C by $CCBE_{1\text{-}175}$ also required the presence of ADAMTS3 in our cell-based assays, which could activate VEGF-C in situ, on the cell surface. The CT domain of CCBE1 (CCBE1-CollD), on the other hand is required for the cofactor-like acceleration of the enzymatic activity of ADAMTS3 (Roukens et al., 2015). Hence, the CCBE1-CollD mediated activation of pro-VEGF-C would happen in solution rather than on the cell surface.

We also identified a heterozygous missense substitution (R565Q) in ADAMTS3 in an individual affected with lymphedema. The mutation is located in the TSP-1 motif, which is highly conserved among different species as well among the ADAMTS family members. We first analyzed the effect of the mutant ADAMTS3 on VEGF-C processing in cell culture. As expected, VEGF-C processing was similar to that of wild-type ADAMTS3, suggesting an indirect effect of the mutant on VEGF-C activity. Since we observed interaction between ADAMTS3 and CCBE1 in our previous study (Study I), we hypothesized that CCBE1 interaction can mediate the effect of the mutant ADAMTS3. For this, constructs expressing CCBE1 and the mutant ADAMTS3 or wild-type ADAMTS3 were transfected into 293T cells. We found weaker binding of CCBE1 to the mutant ADAMTS3 than to wild-type ADAMTS3. Conversely, the amount of CCBE1 in the supernatant was higher in the presence of mutant ADAMTS3, corresponding to the level of CCBE1 in supernatant when only CCBE1 was transfected.

The mutation identified in our study is localized to highly conserved TSP-1 motif of ADAMTS family. CCBE1 was recently identified as a proteoglycan with chondroitin sulfate modification (Bui et al., 2016). The increase of CCBE1 in the supernatants in the presence of mutant ADAMTS3 R565Q is most likely due to the reduced cell surface association of CCBE1 in the presence of this mutant. Furthermore, ADAMTS3 R565Q did not catalyze processing of CCBE1, as observed in study I. Likewise, the ADAMTS3 R565 homologous mutant of ADAMTS13 (R398H), inhibits the processing of von Willebrand factor (VWF) in congenital thrombocytopenic purpura (Levy et al., 2001).

In study II, our aim was to extend our understanding on the individual domains of VEGF-C and CCBE1. Our in vitro study suggested the ability of the EGF-like domains of CCBE1 to modulate VEGF-C activity by translocating soluble pro-VEGF-C to the

cell surface. Even more interestingly, we observed binding of the N-terminal domain of CCBE1 to the ligand-binding domain (D1-3) of VEGFR-3 suggesting that CCBE1 could provide a decoy ligand for VEGFR-3. However, further studies are needed for the understanding of this observation. VEGF-C belongs to the VEGF family of growth factors. VEGF-A is one of the best studied growth factors in terms of biochemistry, therapeutics, signaling properties, and in vivo function. The understanding of the regulation and biochemistry of VEGF-A has contributed to the development of therapies targeting VEGF-A (Apte et al., 2019). In a similar fashion, more understanding of the VEGF-C biochemistry is likely to be instrumental in all future attempts to target it for therapeutic purposes.

Based on study I and II we propose a model of VEGF-C activation (Figure 6). The model shows four different modes of VEGF-C activation; activation of VEGF-C bound to VEGFR-3, activation of HSPG-bound VEGF-C, activation of VEGF-C in soluble phase and activation of ECM bound VEGF-C. We also speculate that distinct model of VEGF-C activation might differentiate the ability of VEGF-C to form gradient versus the proliferation of LECs.

III. KLK3/PSA and cathepsin D activate VEGF-C

The importance of VEGF-C activation by ADAMTS3 has been firmly established (Study I, II) (Bui et al., 2016; Guen et al., 2014; Janssen et al., 2016). In the contrast, the significance of VEGF-C (and VEGF-D) activation by plasmin has remained elusive, although there were speculations that plasmin cleavage would be relevant during inflammation and wound healing (Bui et al., 2016). We were interested in yet other proteases that might activate VEGF-C. Since ADAMTS3 was not able to activate VEGF-D, also the question about the primary activating protease for VEGF-D was of interest.

A study aimed to identify novel substrates for KLK4 reported VEGF-C as a potential target (Matsumura et al., 2005). However, this prediction had not been validated in vitro or in vivo. VEGF-C was initially purified and cloned from a prostate cancer cell line, PC-3 (Joukov et al., 1996). Other prostate cancer cells were also found to express high levels of VEGF-C, which correlated with lymph node metastasis (Jennbacken et al., 2005). KLK3 is the major protein secreted by the prostate epithelium (Shaw and Diamandis, 2007). Hence, we analyzed kallikrein-related peptidases for their abilities to activate VEGF-C. KLK3 cleaved pro-VEGF-C, resulting in mature VEGF-C, which supported the survival of Ba/F3-VEGFR-3/EpoR and Ba/F3-VEGFR-2/EpoR cells, suggesting that the generated VEGF-C species was biologically active. The cleavage

site of the KLK3 processed VEGF-C was three amino acid residues C-terminal of the ADAMTS3 cleavage site (^{109}FAAAHY↓NT^{116}; Figure 5). The amino acid sequence around the cleavage sites for both ADAMTS3 and KLK3 is 100% conserved among all mammals and birds, suggesting evolutionary conservation of VEGF-C activation.

The textbook substrate of KLK3 is seminogelin, cleavage of which is required for the seminal clot liquefaction (Robert et al., 1997). The activation of VEGF-C by KLK3 suggested that VEGF-C is involved in human reproduction since KLK3 is a principal constituent of semen. VEGF-A, a member of the VEGF family, is expressed in the prostate and is present in semen (Brown et al., 1995). VEGFR-2, and VEGFR-3 are also expressed by spermatozoa (Obermair et al., 1999), suggesting their possible role in sperm function and reproduction. We detected significant levels of VEGF-C in liquefied human seminal plasma using several antibodies raised against VEGF-C whereas we could not detect VEGF-D. Western blot analysis showed the presence of bands similar to both pro-VEGF-C and mature VEGF-C. The binding of VEGF-C from the semen to VEGFR-2/Fc was weaker than to VEGFR-3/Fc. Seminal plasma stimulated the phosphorylation of VEGFR-3 in PAE-VEGFR-3 cells, suggesting that active VEGF-C species were present in seminal plasma. Mature VEGF-C polypeptides was observed mostly in the liquefied semen sample, and less in the non-liquefied sample. Furthermore, the levels of mature VEGF-C tended to increase in the presence of acidic pH suggesting that the activation of VEGF-C happens in the acidic environment inside the female reproductive system.

Similarly to VEGF-C, latent TGFβ1 has been shown to be cleaved mostly by KLK14, generating activated TGFβ1 in seminal plasma (Emami and Diamandis, 2010). TGFβ1 from seminal plasma plays a critical role in modulating the immune response and embryo implantation (Robertson et al., 2002). Seminal plasma triggers a series of inflammatory reactions by recruiting several inflammatory cells into the uterus post-coitus (Robertson, 2005). The inflammatory response triggers expression of VEGF-C (Hamrah et al., 2003; Krebs et al., 2012; Ristimäki et al., 1998). Hence, VEGF-C could be involved in post-coital immunomodulation. KLK3 mediated activation of VEGF-C may also have important roles in tumor progression by regulating both angiogenesis and lymphangiogenesis. However, there is no clear evidence on the role of KLK3 in tumors (Ishii et al., 2004; Peternac et al., 2006). However, there is a possibility that VEGF-C activation is the missing link between KLK3 and tumor progression. Such a connection could be studied in a xenograft-based mouse model of prostate cancer (Lin et al., 2014), as the mouse lacks KLK3 and KLK3 orthologs.

CCBE1 is a part of the seminal plasma proteome (Jodar et al., 2015). We detected large amounts of CCBE1 by western blotting in seminal plasma. We also showed that CCBE1 was able to significantly enhance the KLK3 mediated VEGF-C processing,

making KLK3 the second protease that is regulated by CCBE1. The cleavage sites for KLK3 and ADAMTS3 are located only three amino acid residues apart. Hence, the sequence around the cleavage site could play an important role in mediating CCBE1 related activity. The regulation of KLK3 by CCBE1 suggests additional physiological relevance of CCBE1 in VEGF-C activation. In contrast, plasmin appeared to be unaffected by CCBE1 (Study I).

The binding of VEGF-C to VEGFR-3 and VEGFR-2 requires the N-terminal helix of VEGF-C (Leppanen et al., 2010, 2013), and its removal at the secondary cleavage site of plasmin (^{126}WR↓KT^{129}) renders VEGF-C inactive (Study I). Alternatively, partial removal of the N-terminal helix of VEGF-D (referred to as minor mature VEGF-D; ^{100}KVID→) largely abolishes its binding to VEGFR-3 without significantly affecting VEGFR-2 affinity (Leppanen et al., 2011). To understand the role of the N-terminal helix of VEGF-C in receptor binding, we generated a hypothetical VEGF-C (^{120}KSID→) form homologous to the minor VEGF-D form (VEGF-C_{DMH}). VEGF-C_{DMH} purified from S2 cells binds normally to VEGFR-3 but only with low affinity to VEGFR-2. However, when produced and purified from 293T cells, it did not bind to either VEGFR-2 or VEGFR-3. This difference could be because of the presence of four extra amino acid residues in the linker that remained from the cloning procedure. We also compared the ability of equimolar amounts of ADAMTS3-, KLK3-, plasmin-cleaved VEGF-C, and VEGF-C_{DMH} to activate VEGFR-3 and VEGFR-2. The phosphorylation of both VEGFR-2 and VEGFR-3 decreased with the successive cleavages of the N-terminus. In the -terminal sequencing analysis, we also identified substantial amounts of VEGF-C_{DMH} (^{120}KSID→) when purifying the longer mature form of VEGF-C (ΔNΔC-VEGF-C), suggesting that VEGF-C_{DMH} could be a physiologically relevant species.

We then found that human saliva has a significant proteolytic effect on VEGF-C. We subjected human saliva to cation exchange chromatography and analyzed the fractions for VEGF-C cleaving activity followed by mass spectrometry of the major VEGF-C cleaving fraction. Based on the top hits from the mass spectrometric analysis, cathepsin D was selected for further analysis because its cleavage context was similar to that of VEGF-D and the VEGF-C_{DMH} form. Cathepsin D not only cleaved pro-VEGF-C and pro-VEGF-D, but also the minor mature form of VEGF-C and the major mature form of VEGF-D, which we call here "secondary activation". Interestingly, the cleavage of both pro-VEGF-D and major mature VEGF-D was faster than the cleavage of VEGF-C. Cathepsin D-processed pro-VEGF-C activated both VEGFR-2 and VEGFR-3 in the Ba/F3 assay, while the secondary activation reduced the activity of VEGF-C towards VEGFR-2. Similarly, processing of pro-VEGF-D increased the

VEGFR-2 phosphorylation, but as expected, secondary activation of VEGF-D decreased its activity towards both VEGFR-2 and VEGFR-3.

We did not detect any cathepsin D cleaved VEGF-C form, VEGF-C_{DMH}, in the supernatants of transfected 293T cells. This could be because of the abundance of ADAMTS3 in 293T cells cleave VEGF-C, thereby removing the recognition sequence for cathepsin D binding in VEGF-C. This suggest that cathepsin D and ADAMTS3 have non-overlapping function in VEGF-C activation. "Secondary activation" of the mature forms of VEGF-C and VEGF-D further suggests that cathepsin D can modulate VEGF-C affinity to VEGFR-2 and VEGFR-3 in vivo. In particular, because cathepsin D efficiently processes the major form of VEGF-D, it may be important for VEGF-D activation.

To study the effect of the KLK3- and cathepsin D-cleaved VEGF-C in vivo, we generated AAV9 vectors expressing the cathepsin D- and KLK3-cleaved forms of VEGF-C from truncated cDNAs. The vectors were injected into the mouse tibialis anterior muscle, which were analyzed two weeks later. The effects obtained in lymphatic and blood vessels were consistent with the VEGFR-3 and VEGFR-2 activity assays, indicating that cathepsin D cleaved VEGF-C is less active than the KLK3 cleaved form, indicating the importance of N-terminal helix in governing the activation potential of VEGF-C for VEGFR-2 and VEGFR-3. Our studies show that the sequential shortening of the N-terminal alpha helix reduces VEGF-C's relative affinity for VEGFR-2 and VEGFR-3, ultimately abolishing all receptor activity with the secondary plasmin cleavage (Plasmin2, Figure 5)

In study III, we identified two additional proteases, KLK3 and cathepsin D, that culd provide context-specific or even organ-specific VEGF-C activation. The possible roles of these proteases in the regulation of the possible non-endothelial functions of VEGF-C remains an open area of research. KLK3 is a well-known prostate cancer biomarker (Lilja et al., 2008). Cathepsin D is an aspartic protease expressed in various tissues and it has a wide range of substrates (Benes et al., 2008). Both KLK3 and cathepsin D could possible also activate VEGF-C and VEGF-D to promote lymphatic metastasis. Besides, the KLK3 processing of VEGF-C might have significance in human reproduction. Further studies are needed to demonstrate the significance of KLK3 and cathepsin D in the processing of VEGF-C and VEGF-D for both physiological and pathological functions of VEGF-C.

My study (Study I, II and III) provides an insight into the importance of VEGF-C activation in the growth and development of lymphatic vasculature. VEGF-C alone is not a very potent lymphangiogenic agent and requires co-factor CCBE1 and the proteases-ADAMTS3 and KLK3. Hence, the possibility to develop a combination

based therapeutic approach to boost VEGF-C activation might be beneficial in the treatment of lymphedema. VEGF-C, on the other hand, has long been considered to be a target for tumor-associated lymphatic metastasis. The blocking antibody against VEGFR-3 and neutralizing antibody against VEGF-C has clinically been tested, but with no success. Hence, targeting the molecules involved in the activation of VEGF-C might provide an alternative strategy to inhibit VEGF-C related function.

CONCLUSIONS AND FUTURE PROSPECTS

My thesis study has focused on the growth factor VEGF-C, which is arguably the most important growth factor for the lymphatic system. We identified ADAMTS3, KLK3, and cathepsin D as proteases that regulate VEGF-C processing and activation. Among these, ADAMTS3 is required during embryonic development, while KLK3 and cathepsin D likely mediate niche-specific VEGF-C functions. Our findings unveil the roles of individual domains of VEGF-C and CCBE1 during VEGF-C activation and establish that the matrix binding property of VEGF-C is crucial for its function. Our study suggests that the formation of the VEGF-C/ADAMTS3/CCBE1 multiprotein complex on the surface of the lymphatic endothelial cells (LECs) is the rate-limiting step for VEGF-C activation. Based on this, we have proposed a model of VEGF-C activation, which can be utilized to expand the therapeutic repertoire for lymphatic associated diseases.

Although our research has increased our knowledge of VEGF-C activation and its importance for lymphatic vessel growth, there are still substantial gaps in our understanding of this complicated mechanism. It would be interesting to test the relevance of KLK3- and cathepsin D-mediated VEGF-C activation in physiological and pathological conditions. Perhaps, specific proteases play a role in organ-specific activation of VEGF-C. A fertile research opportunity could be the role of seminal plasma VEGF-C in reproduction. Additionally, CCBE1 affects only KLK3 and ADAMTS3 mediated activation of VEGF-C. While we have a hypothetical explanation for why the CCBE1 acceleration of VEGF-C cleavage is limited to ADAMTS3 and KLK3 while excluding plasmin, experimental evidence for our steric hindrance model is lacking. We also have shown that ADAMTS3 is able to cleave CCBE1, thus separating its two domains. This cleavage might further provide complexity to the VEGF-C regulation during lymphatic development.

Lymphatic research has gained momentum over the past decades, which has resulted in an amazing amount of new data about lymphatic development. However, the relative lack of in-depth mechanistic insights into the lymphangiogenic process still restricts the identification of targets for therapeutic intervention. Even today, most of the genes causing hereditary lymphedema are unknown, which indicates that there are still ample opportunities to identify novel molecular players and interventional targets.

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