VEGF-C: The evolutionary origin, activation, and potential as a drug target

Khushbu Rauniyar

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Doctoral Programme in Integrated Life Science
Doctoral School of Health Sciences
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Supervisor

Michael Jeltsch, PhD
Associate Professor
Drug Research Program (Faculty of Pharmacy) and IndiviDrug Research Program (Faculty of Medicine)
University of Helsinki and Wihuri Research Institute
Finland

Thesis Committee

Pipsa Saharinen, PhD
Professor
Faculty of Medicine
University of Helsinki
Finland

Caroline Heckman, PhD
Research Director
Institute for Molecular Medicine Finland
University of Helsinki
Finland

Reviewers

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Finland

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Uppsala University
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Germany

Custos

Kari Keinänen, PhD
Professor
Faculty of Biological and Environmental Sciences
University of Helsinki
Finland

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LIST OF ORIGINAL PUBLICATIONS

This thesis includes the following four original publications, which are denoted by Roman numerals (I-IV) in the text. Reprints of the original publications have been made at the end of the thesis with the permission of the copyright holders.

I. Jha SK, **Rauniyar K**, Kärpänen T, Leppänen 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).

II. 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).

III. **Rauniyar K***, Akhondzadeh S*, Gąciarz A , Künnapuu J, and Jeltsch M. Bioactive VEGF-C from E. coli. *Sci. Rep.* 12(1), 18157 (2022).

IV. **Rauniyar K**, Bokharie H, and Jeltsch M. Expansion and collapse of VEGF diversity in major clades of the animal kingdom. *Angiogenesis*. 10.1007/s10456-023-09874-9 (2023).

* Equal contribution

ABBREVIATIONS

AAV	adeno-associated viral vector
ADAMTS3	a disintegrin and metalloproteinase with thrombospondin motifs 3
BEC	blood vascular endothelial cell
BR3P	balbiani ring 3 protein
CCBE1	collagen and calcium-binding EGF domains 1
cDNA	complementary DNA
CHO	Chinese hamster ovary
CSF	cerebrospinal fluid
CT	carboxy-terminal
EC	endothelial cell
ECM	extracellular matrix
EGF	epidermal growth factor
Flt4	fms-like tyrosine kinase
HIF	hypoxia-inducible factor
HKLLS	Hennekam lymphangiectasia–lymphedema syndrome
HSPG	heparan sulfate proteoglycan
HUVEC	human umbilical venous endothelial cell
Ig	immunoglobulin
ISF	interstitial fluid
K14	keratin 14
KDR	kinase-insert domain receptor
LEC	lymphatic endothelial cell
LN	lymph node
LVA	lymphaticovenous anastomosis
LYVE1	lymphatic vessel endothelial hyaluronan receptor 1
MAPK	mitogen-activated protein kinase
MBP	maltose binding protein
MD	Milroy’s disease
mRNA	messenger RNA
Nrp	neuropilin

NT	amino-terminal
PDGF	platelet-derived growth factor
PI3K-AKT	phosphatidylinositol-3-kinase and protein kinase B
PlGF	placental growth factor
PROX1	prospero-related homeobox protein 1
PSA	prostate-specific antigen
PVF	PDGF/VEGF-like factor
RTK	receptor tyrosine kinase
SEMA3	class III semaphorin
SMC	smooth muscle cell
SOX18	SRY-box transcription factor 18
sVEGFR	soluble vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
VEGF	vascular endothelial growth factor
VHD	VEGF homology domain
VLNT	vascularized lymph node transfer
WGD	whole genome duplication

ABSTRACT

Lymphatic vessels are an integral part of the immune system that defends us against bacteria and viruses. These vessels are a drainage system for returning interstitial tissue fluid and immune cells into the blood circulation. A dysfunctional lymphatic system causes swelling of tissues due to excess fluid, known as lymphedema. The primary factor that stimulates the growth of lymphatic vessels (lymphangiogenesis) is vascular endothelial growth factor-C (VEGF-C). Although this has been known since 1997, many mechanistic details of VEGF-C's action have only been recently uncovered. VEGF-C needs to be activated by the removal of its C- and N-terminal propeptides before it can generate new vessels, and this activation requires the CCBE1 helper protein and one of several proteases. VEGF-C can be a drug target for diseases involving the lymphatics, such as lymphedema, cancer, and heart diseases. However, the main limitation is our incomplete understanding of the actual mechanisms of VEGF-C activation for developing a functional lymphatic vasculature. The primary aim of my studies has been to identify additional puzzle pieces in VEGF-C activation, which will prove instrumental in future attempts to therapeutically target VEGF-C.

In study I, we have characterized the role of the individual domains of VEGF-C and CCBE1 in the activation of VEGF-C. Our study determined the crucial role of the C-terminal domain of VEGF-C for efficient VEGF-C activation. In addition, we established differential roles of C- and N-terminal domains of CCBE1 for VEGF-C activation. The N-terminal domain is essential for mobilizing VEGF-C to the endothelial cell surface to form the VEGF-C/ADAMTS3/CCBE1 cleavage complex, whereas the C-terminal domain aids ADAMTS3-mediated VEGF-C processing. We have also proposed a model of VEGF-C activation where VEGF-C can be activated when bound to VEGFR-3/HSPGs on the cell surface or to ECM as well as in the soluble phase.

In study II, we discovered additional proteases activating VEGF-C. We identified both VEGF-C and VEGF-D as substrates for KLK3 and cathepsin D. The presence of KLK3, VEGF-C, and CCBE1 in sperm plasma suggested a role of KLK3-activated VEGF-C in reproduction. KLK3 and cathepsin D cleavage of VEGF-C and VEGF-D generated N-terminally different forms that varied in their affinities towards VEGFR-2 and VEGFR-3. Cathepsin D-cleaved VEGF-C was VEGFR-3 specific and lost its angiogenic properties, whereas cathepsin-D-activated VEGF-D was VEGFR-2 specific and lost its lymphangiogenic properties. Identification of novel VEGF-C activating proteases has opened ways to therapeutically target VEGF-C in reproduction and metastatic cancer besides lymphedema treatment.

In study III, we described for the first time a method to produce biologically active VEGF-C from *E. coli* using a combination of redox-modified Origami strain and maltose binding

protein (MBP) tag. There are no reports of successful attempts to produce active VEGF-C in *E. coli* without the need for refolding from inclusion bodies. Such bacterial VEGF-C could be a readily available cost- and time-effective source of VEGF-C for *in vitro* studies.

In study IV, we analyzed the occurrence of PDGF/VEGF homologs in different animal clades. We identified Cnidaria as the simplest animals containing PDGF/VEGF-like proteins. Almost all cnidarian PDGF/VEGFs contain the BR3P repeat characteristic for the C-terminal domain of VEGF-C. This finding suggests a VEGF-C-like protein as the earliest PDGF/VEGF to emerge during evolution. Interestingly, we showed a complete absence of PlGF in Amphibia and VEGF-B in crocodiles and birds. In addition, the presence of VEGF-F was not limited to venomous reptiles but could also be found in non-venomous lizards and gekkos. Due to recent advancements in our understanding of the development of fish vasculature, we also analyzed the heterogeneity of PDGF/VEGF molecules in fishes, including verification of gene prediction with mRNA sequencing data. This study provides a basis for choosing appropriate animal models for vascular biology research.

With these studies, we primarily advance the understanding of VEGF-C biology and thus lay the groundwork for developing VEGF-C into a therapeutic target for diseases involving the lymphatic system.

REVIEW OF THE LITERATURE

1. Introduction

The importance of the cardiovascular system was recognized long before medicine started to become a science, and the concept of blood and the heart are deeply rooted in perhaps all human cultures. Contrary to this, explicit recognition of a separate lymphatic vasculature happened only about 400 years ago by Gaspare Aselli. Moreover, only within the last 25 years have lymphatics become a subject of extensive research due to the recognition that they play essential roles in many - if not all - disease processes. The cell type that characterizes the lymphatic system is the lymphatic endothelial cell (LEC). LECs form the lumen-facing inner layer of all lymphatic vessels. Lymphatic capillaries consist exclusively of this single cell layer, which sits on top of a discontinuous basement membrane. Larger lymphatic vessels are, similar to larger blood vessels, multi-layered.

The master molecule responsible for the growth of lymphatic vessels is vascular endothelial growth factor (VEGF)-C. The lymphatic system does not develop at all in the absence of VEGF-C. For the same reason, it is also essential for *in vitro* growth of LECs and for *in vitro* and *in vivo* approaches to engineer lymphatic vessels and networks for therapeutic purposes. Having been translated from its messenger RNA (mRNA), VEGF-C exists in an inactive form (unprocessed VEGF-C). The unprocessed VEGF-C must undergo stepwise proteolytic processing before it is active, requiring at least three proteases.

The proteolytic processing changes the properties of VEGF-C. Most notably, the affinities for its various interaction partners changes, including the affinities for its receptors, vascular endothelial growth factor receptor (VEGFR)-2 and VEGFR-3. Many essential insights into the production and activation of VEGF-C have only been gained in the last few years, and there are still many unknowns.

Often, VEGF-C is treated as a single defined molecular species ignoring the fact that there are many different forms of VEGF-C resulting mainly from the use of alternative proteases for the final activating proteolytic cleavage. The pharmacologic targeting of VEGF-C necessitates an intimate understanding of its biology, irrespectively whether the goal is to increase VEGF-C signaling (to treat, e.g., lymphedema) or to block VEGF-C signaling (to prevent, e.g., lymphogenic metastasis in cancer patients).

This study aimed to investigate the phylogeny of VEGF-C and identify the proteases involved in the differential activation of VEGF-C and its roles. In addition, this study also provides a method for producing bioactive VEGF-C (a cysteine-rich growth factor) from a bacterial host, which is time and cost-effective for *in vitro* studies involving VEGF-C.

While the focus of this literature overview is on the lymphatic aspects of VEGF-C signaling, several recent research results indicate that the properties of VEGF-C are also relevant to the cardiovascular system.

2. The circulatory system

In mammals, the circulatory system is broadly divided into the blood vascular system and the lymphatic vascular system (Figure 1).

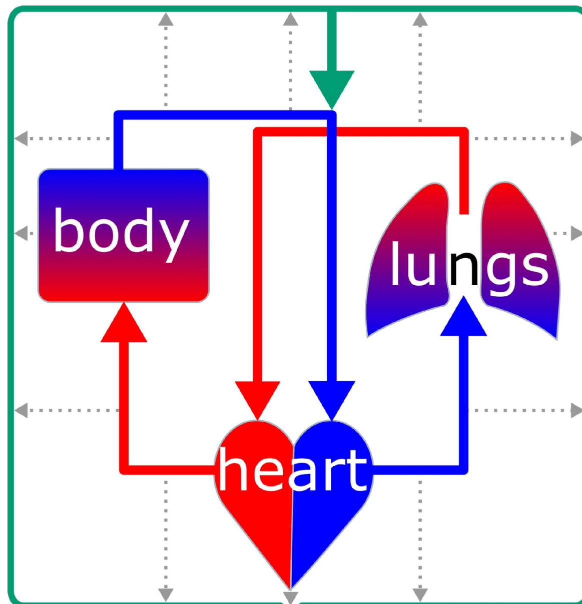

Figure 1. ***Highly simplified schematic view of the two vascular systems in mammals and birds.*** *The high-pressure blood vascular system leaks fluid into the interstitial space, from where it is taken up by the lymphatics and returned into the blood vascular system.*

2.1 The blood vascular system

The blood vascular system contains a closed loop of blood vessels: arteries, veins, and capillaries. Oxygenated blood is carried by the arteries to the capillaries, where a two-way exchange of gases and nutrients occurs between the blood and tissues. The deoxygenated blood is then collected by veins and transported back to the heart and lungs.

The blood vessels, except capillaries, are composed of three different layers: the outermost layer (adventitia) that provides structural support and shape to the vessels and is composed of

collagen bundles and fibroblasts; the middle layer (tunica media) that regulates the internal diameter of the vessels and is composed of smooth muscle cells (SMCs) and elastin; and the innermost layer (tunica intima) that is lined by a monolayer of endothelial cells (ECs) that facilitate smooth movement of blood. The EC lining is associated with a basement membrane, a thin layer of extracellular matrix (ECM) that can vary depending on the type of vessel and tissue environment. The major components of this ECM include laminins, collagens, fibronectin, and heparan sulfate proteoglycans (HSPGs). Capillaries are composed of a thin sheet of ECs, basement membrane, and scattered perivascular cells (pericytes) that are embedded within the walls of capillaries.

2.2 The lymphatic vascular system

While the vertebrate cardiovascular system forms a closed circulatory system, the lymphatic system is a one-way conduit consisting of a vascular network of blind-ended capillaries, collecting vessels, large ducts, and lymphoid organs.

2.2.1 Structure and function of lymphatic vessels

The lymphatic capillaries or initial lymphatics are the site of lymph formation and consist of a single endothelial layer of oak leaf-shaped LECs that rest on a permeable discontinuous basement membrane (Baluk et al., 2007; Breslin et al., 2018; Castenholz, 1984). These capillaries have a larger diameter compared to blood capillaries and contain flap-like minivalves formed by the overlap of LECs that allows the flow of interstitial fluid (ISF) only in one direction. The overlapping EC junctions and lack of pericytes and SMCs facilitate the uptake of large macromolecules. These vessels are associated with the interstitium by anchoring filaments that connect LECs to the ECM and respond to increased interstitial pressure by pulling LECs apart (Breslin et al., 2018). The initial lymphatics drain the ISF as lymph into the collecting lymphatic vessels, which feature a smooth muscle layer, pericytes, and one-way bicuspid valves. Mostly, a precollector that lacks smooth muscle but has one-way valves is present between the initial and collecting lymphatics. The valves in the collecting lymphatics are spaced at intervals forming chambers, which form a contractile unit called lymphangion responsible for pumping the lymph against the pressure gradient (Mislin, 1976; Smith, 1949). The lymph enters the lymph nodes (LNs) through afferent lymphatics and exits through efferent lymphatics into larger lymph trunks (thoracic duct and right lymphatic duct) and empties into the subclavian veins (Tammela and Alitalo, 2010). The number of lymph nodes varies considerably, with some animals having no lymph nodes, aquatic birds having only two, mice having 22, and humans having approximately 450 lymph nodes (Haley, 2017).

Lymphatic vessels mainly serve as a drainage system that returns excess interstitial tissue fluid and inflammatory cells back into the blood circulation, thereby maintaining body fluid

balance. Lymphatic vessels also help in immune surveillance by transporting immune cells and antigens to LNs, fostering immune response (Randolph et al., 2017). Apart from these well-known functions, lymphatic vessels have been shown to play versatile roles in tissue- and organ-specific manner (Wilting and Becker, 2022). Intestinal lymphatic vessels, known as lacteals, play a vital role in the absorption of dietary lipids and fat-soluble vitamins in the form of chylomicrons and maintain gut immunity and homeostasis (Dixon, 2010). Recently, meningeal lymphatics have gathered attention because of their potential for cerebrospinal fluid (CSF) and brain ISF macromolecular clearance as well as immune cell egression from the CSF (Ahn et al., 2019; Aspelund et al., 2015a; Louveau et al., 2015). In addition, Schlemm's canal, a channel lined by the endothelium that surrounds the cornea and helps in aqueous humor drainage, has similarities with lymphatic vessels (Aspelund et al., 2014). The roles of lymphatic vessels have also started to emerge in stem cell niches and the hair follicle regeneration cycle (Gur-Cohen et al., 2019; Peña-Jimenez et al., 2019; Petrova and Koh, 2020).

2.2.2 Lymphatic vessel development and growth

Lymphatic vessels originate only after the establishment of the cardiovascular system. There are two theories for the origin of lymphatic vessels. Florence Sabin proposed in 1902 a venous origin for lymphatic vessels when she observed the lymphatic vessels in pig embryos using intradermal ink injections (Sabin, 1902, 1904). The contrasting theory by Huntington and McClure proposed in 1908 that lymph sacs originate from mesenchymal precursor cells (lymphangioblasts) independently of veins and, during development, form venous connections (Huntington and McClure, 1908). Studies in mice and zebrafish have confirmed the venous origin theory (Hagerling et al., 2013; Küchler et al., 2006; Srinivasan et al., 2007; Yaniv et al., 2006), and studies in *Xenopus* tadpoles and birds have supported the mesenchymal precursor theory (Ny et al., 2005; Papoutsi et al., 2001; Schneider et al., 1999; Wilting et al., 2006). Recent studies using lineage tracing experiments have confirmed a heterogeneous origin for lymphatic vessels. There is evidence for the non-venous origin of lymphatics, where lymphatic vessels form via the assembly of cells of hemogenic origin in the heart and mesentery and non–Tie2-lineage cells in the skin, based on a process called lymphvasculogenesis (Klotz et al., 2015; Martinez-Corral et al., 2015; Stanczuk et al., 2015).

The master regulator of LECs' commitment and differentiation is Prospero-related homeobox protein 1 (PROX1) (Wigle and Oliver, 1999). In mice, a subset of SRY-box transcription factor 18 (SOX18)-positive ECs in the cardinal vein differentiate into LECs upon stimulation by yet unidentified signal(s) at E9.0 (Francois et al., 2008; Johnson et al., 2008). Later, around E9.5, SOX18 and nuclear hormone receptor COUP-TFII are responsible for PROX1 activation (Koltowska et al., 2013; Srinivasan et al., 2010). These PROX1-positive cells, which also express lymphatic vessel endothelial hyaluronan receptor 1 (LYVE1) and VEGFR-3, migrate and proliferate to form primary lymph sacs upon stimulation by VEGF-C

(E10-10.5) (François et al., 2012; Wigle and Oliver, 1999). The peripheral lymphatic vasculature then forms by centrifugal sprouting from lymph sacs, which undergo remodeling and maturation to form large collecting lymphatic vessels by accumulating mural cells and forming valves. After maturation, the uniform expression pattern of PROX1, LYVE1, and VEGFR-3 changes, leading to higher expression of PROX1 and VEGFR-3 in the valve regions and LYVE1 expression in the capillaries (Norrmén et al., 2009). A recent study in zebrafish anal fin suggests that some LECs can transdifferentiate to form blood vessels later in development (Das et al., 2022).

3. Molecular regulators of the blood and lymphatic vascular system

The blood and lymphatic vascular systems are tightly regulated and depend on the interactions between many signaling molecules, mainly VEGFs and their receptors. In addition, several other factors are involved in the normal growth and development of the vascular systems, for example, cell proliferation, differentiation, migration, cell adhesion, and cell signaling. Many of the molecular regulators of these two vascular systems have gathered therapeutic interest due to their involvement in diseases.

3.1 Vascular endothelial growth factor (VEGF)

In humans, five different genes encode members of the VEGF family: VEGF-A, placenta growth factor (PlGF), VEGF-B, VEGF-C, and VEGF-D (Figure 2). Viruses and reptiles also contain VEGF-like genes, collectively called VEGF-E and VEGF-F, respectively. VEGFs are secreted glycoproteins and are characterized by a central VEGF homology domain (VHD). The VHD consists of a receptor-binding domain and typically exhibits eight highly conserved cysteine residues, six of which give rise to a cystine-knot structure, and the remaining two form intermolecular disulfide bonds (Holmes and Zachary, 2005). Individual VEGFs are further defined by the presence of additional sequences specific to them. Some of these sequences act as propeptides whose cleavage alters the biological properties of the molecule. VEGF-C and VEGF-D are special among VEGFs because they have exceptionally long amino (N)- and carboxy (C)-terminal propeptides that fold into their own domains and must be proteolytically cleaved/removed in order for the growth factor to become active.

VEGFs can be broadly classified as hemangiogenic (VEGF-A, PlGF, and VEGF-B) or lymphangiogenic (VEGF-C and VEGF-D). One major difference between these two groups of VEGFs is that hemangiogenic VEGFs can interact with VEGFR-1, whereas lymphangiogenic VEGFs can interact with VEGFR-3. The angiogenic receptor VEGFR-2 can be activated by some members from both groups (VEGF-A, VEGF-C, and VEGF-D).

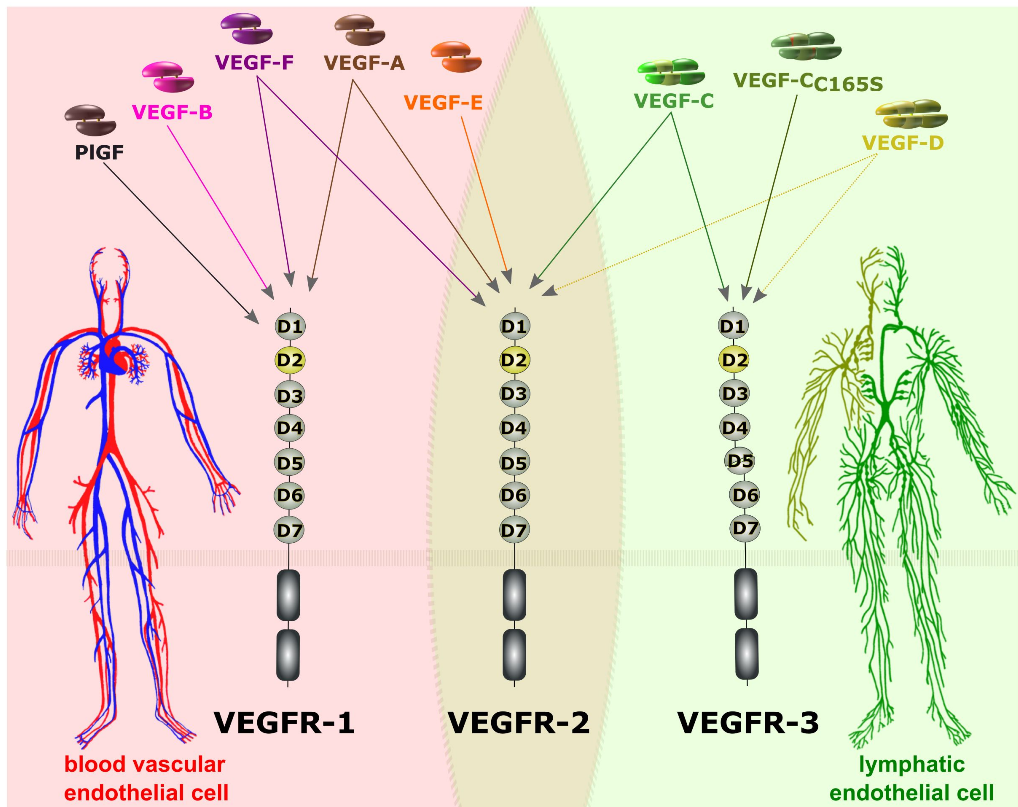

Figure 2. ***Vascular endothelial growth factors (VEGFs) and their interacting receptors (VEGFRs).*** *VEGFR-1 is expressed on blood vascular endothelial cells, VEGFR-3 on lymphatic endothelial cells, and VEGFR-2 on both, promoting both angiogenesis and lymphangiogenesis, respectively. The extracellular domains of the receptors are denoted as D1-D7. The domain D2 of all three VEGFRs is required for interactions with their respective VEGF ligands. (Modified from Rauniyar et al., 2018)*

3.1.1 Hemangiogenic VEGFs

3.1.1.1 VEGF-A

VEGF-A, also known as VEGF or vascular permeability factor (VPF), was discovered by Senger et al. in 1983 (Senger et al., 1983) and cloned in 1989 (Ferrara and Henzel, 1989; Leung et al., 1989). It exists as an antiparallel dimer that is covalently linked by two disulfide bonds, each pole of the homodimer containing receptor binding sites (Muller et al., 1997; Wiesmann et al., 1997). Its main roles in angiogenesis, vasculogenesis, hematopoiesis, and

vascular permeability are mediated through VEGFR-2 signaling. Although it has a high binding affinity to VEGFR-1, signaling through VEGFR-1 is weaker compared to VEGFR-2 signaling. Hence, VEGFR-1 is considered a decoy receptor for VEGF-A, which limits its availability for VEGFR-2 and regulates VEGF-A/VEGFR-2 signaling (Anisimov et al., 2013; Hiratsuka et al., 2005; Shibuya, 2006a). VEGF-A is expressed in mice at a very early embryonic stage of E7.0 and is indispensable for angiogenesis and vasculogenesis (Dumont et al., 1995). Mice with a single allele deletion die already at E11.5 (Carmeliet et al., 1996; Ferrara et al., 1996). Moderate overexpression of VEGF-A can also lead to death at E12.5-E14 (Miquerol et al., 2000), showing the importance of tight regulation of VEGF-A levels.

Human VEGF-A exists in different isoforms produced by alternative splicing: VEGF-A_{121}, VEGF-A_{165}, VEGF-A_{189}, VEGF-A_{206}, VEGF-A_{145}, VEGF-A_{148}, VEGF-A_{162}, and VEGF-A_{183} reviewed in (Takahashi and Shibuya, 2005). The respective murine isoforms are one amino acid shorter than the human isoforms (Takahashi and Shibuya, 2005). These isoforms differ in their ability to bind HSPGs, VEGFRs, integrins, and co-receptors neuropilin-1 (Nrp1) and neuropilin-2 (Nrp2) and exhibit different expression patterns in tissues. The shortest of the major isoforms VEGF-A_{121} does not bind to HSPGs. However, VEGF-A_{189} and VEGF-A_{206}, which are longer isoforms, have strong binding affinity to HSPGs and are sequestered in the ECM and at the cell surface. The most common isoform VEGF-A_{165} binds as well to HSPGs but also has diffusible properties (Park et al., 1993). In addition, an inhibitory form of VEGF-A, VEGF-A_{165b}, has been described (Bates et al., 2013; Woolard et al., 2004), the occurrence of which is questioned in some studies (Bridgett et al., 2017; Catena et al., 2010; Harris et al., 2012). All these isoforms have distinct roles in vascular development and patterning as well as arterial development, which have been studied in mice. Deletion of heparin-binding VEGF-A isoforms in mice leads to neonatal death due to impaired myocardial angiogenesis and ischemic cardiomyopathy (Carmeliet et al., 1999). These mice also exhibit a decrease in capillary branch formation and abnormally directed extensions of EC filopodia (Ruhrberg et al., 2002). Exclusive expression of the heparin-binding isoform VEGF-A_{189} resulted in arterial growth defects (Stalmans et al., 2002) and stunted bone growth (Maes et al., 2004). These mice studies with different isoforms suggest a role of VEGF-A binding to ECM and cell surface for the guidance of EC sprouting and initiation of vascular branching. The longer ECM-bound VEGF-A isoforms can be released by proteolytic cleavage by plasmin, urokinase, or matrix metalloproteinases at their C-terminus (Künnapuu et al., 2021; Lee et al., 2005; Park et al., 1993; Plouët et al., 1997).

The expression of VEGF-A is regulated principally by the hypoxia-inducible factor-1 (HIF-1), whereas various other stimuli such as inflammatory cytokines, growth factors, hormones, and tumor promoters can also induce VEGF-A expression (Chung and Ferrara, 2011; Pugh and Ratcliffe, 2003; Takahashi and Shibuya, 2005). VEGF-A is mainly involved in increasing EC growth and survival through mitogen-activated protein kinase (MAPK) and

phosphatidylinositol-3-kinase and protein kinase B (PI3K-AKT) pathways. Overexpression of VEGF-A in transgenic mice leads to uncontrolled angiogenesis and, thus, vascular leakage and inflammation (Baluk et al., 2005a; Detmar et al., 1998; Larcher et al., 1998). The lymphangiogenic effects of VEGF-A are considered secondary, e.g., in a corneal inflammatory model, VEGF-A induced lymphatic vessel growth due to recruitment of macrophages that secrete VEGF-C (Cursiefen et al., 2004). However, some studies in tumor models have shown a VEGF-C-independent increase in lymphangiogenesis and lymphatic metastasis by overexpressing VEGF-A (Björndahl et al., 2005; Hirakawa et al., 2005). In addition to its endothelial roles, VEGF-A can also boost bone formation by increasing osteoblast migration (Mayr-Wohlfart et al., 2002; Midy and Plouet, 1994), affect lung maturation by producing surfactant proteins (Compernolle et al., 2002), and promote neuronal survival, growth, and migration (Mani et al., 2010; Rosenstein et al., 2003; Zachary, 2005). VEGF-A can also signal in an autocrine manner via co-receptor Nrp2 to increase the survival and proliferation of cancer stem cells through transcriptional coactivator with pdz-binding motif (TAZ) activation (Elaimy et al., 2018).

3.1.1.2 VEGF-B

VEGF-B was identified in 1996 and alternatively termed VEGF-related factor (VRF) (Grimmond et al., 1996; Olofsson et al., 1996a). It exists in two isoforms produced by alternative splicing: the heparin-binding form VEGF-B_{167} and the more soluble form VEGF-B_{186} (Olofsson et al., 1996b, 1996a). VEGF-B is normally secreted as a homodimer but can form heterodimers when co-expressed with VEGF-A_{165} (Olofsson et al., 1996b). VEGF-B is abundantly expressed in the heart, skeletal muscle, neuronal cells, and brown adipose tissues (Aase et al., 1999; Li et al., 2001; Nash et al., 2006). Although it signals via VEGFR-1 and Nrp1 (Makinen et al., 1999; Olofsson et al., 1998), it is not considered a primary angiogenic factor because it is dispensable for embryonic development in mice. *Vegfb*$^{-/-}$ mice appear normal and fertile; however, several studies have reported reduced heart size, coronary artery dysfunction, and abnormal atrial conduction (Aase et al., 2001; Bellomo et al., 2000). On the other hand, overexpression of VEGF-B does not have any angiogenic effect (Bhardwaj et al., 2003; Li et al., 2008; Lähteenvuo et al., 2009), but it can have a protective effect on the heart by inducing development and function of coronary vasculature (Bry et al., 2010; Huusko et al., 2012; Kivelä et al., 2014). In recent years, VEGF-B has gained much attention due to its potent neurotropic effect (Li et al., 2009; Poesen et al., 2008) and its role in lipid metabolism (Karpanen et al., 2008) and regulating fatty acid uptake and transportation by upregulating the expression of fatty acid transport proteins (Hagberg et al., 2010).

3.1.1.3 PlGF

PlGF was discovered in 1991 and is abundantly expressed in the placenta (Maglione et al., 1991). Human PlGF exists in four different isoforms (PlGF1-4), whereas mice have only one splice isoform, PlGF2 (Cao et al., 1997; Maglione et al., 1993; Takahashi and Shibuya, 2005; Yang et al., 2003). PlGF signals via VEGFR-1 and co-receptor Nrp1, resembling VEGF-B (Migdal et al., 1998; Park et al., 1994) and is redundant for embryonic blood vessel development in mice. However, PlGF differs from VEGF-B despite signaling through the same receptor, VEGFR-1 (Anisimov et al., 2013). *Plgf*-deficient mice have decreased angiogenesis, vascular permeability, and arteriogenesis during pathological conditions such as inflammation, ischemia, and cancer (Carmeliet et al., 2001; Freitas-Andrade et al., 2012). PlGF can induce angiogenesis and collateral vessel growth and stimulate the migration of monocytes, EC growth, and vasodilatation (Clauss et al., 1996; Iwasaki et al., 2011; Odorisio et al., 2006; Pipp et al., 2003). It can also attract myeloid progenitors to the growing collateral vessel, hence proving its therapeutic potential.

3.1.2 Lymphangiogenic growth factors

3.1.2.1 VEGF-C

VEGF-C, also known as VEGF-related protein (VRP), was discovered in 1996 and cloned from the human prostatic carcinoma cell line PC3 (Joukov et al., 1996). Mouse VEGF-C was cloned from the human glioma cell line G61 (Lee et al., 1996). VEGF-C is very different from the angiogenic growth factors since it is secreted as an inactive preproprotein. It consists of an N-terminal domain with no homology to any other proteins, a VHD, and a C-terminal domain with repetitive cysteine-rich motif characteristic of the Balbiani ring 3 protein (BR3P) (Joukov et al., 1996) (Figure 3). Proteolytic cleavage of its N- and C-terminal domains regulate the binding pattern and affinity towards VEGFR-2 and VEGFR-3, and hence its activity. The proteolytic processing is sequential, involving cleavage of the C-terminal propeptide first, followed by removal of the N-terminal domain (Joukov et al., 1997). The C-terminal domain is constitutively cleaved by proprotein convertases furin, PC5, and PC7. After cleavage, the C-terminal propeptide remains covalently bound to the N-terminal domain by disulfide bonds, resulting in pro-VEGF-C (Siegfried et al., 2003). This pro-VEGF-C has increased affinity to VEGFR-3, but only fully processed mature VEGF-C (21/23 kDa) can bind to and activate both VEGFR-2 and VEGFR-3. Removal of the N-terminal propeptides is mediated by proteases such as plasmin, thrombin, a disintegrin and metalloproteinase with thrombospondin motifs 3 (ADAMTS3), which will be discussed in the later sections (Jeltsch et al., 2014; Lim et al., 2019; McColl et al., 2003).

VEGF-C exists as an antiparallel homodimer that is stabilized by two disulfide bonds formed between Cys165 and Cys156 (Leppänen et al., 2010). The dimer stability is affected because of the close proximity of one unpaired cysteine residue, Cys137, at the dimer interface.

Mutating this Cys137 into alanine has been shown to increase dimer stability and biological activity (Anisimov et al., 2009) and hence is sometimes used in the production of recombinant VEGF-C. VEGF-C expression levels decrease in adults due to the quiescent lymphatic endothelium. Higher expression levels are observed in the heart, lungs, mesenchymal cells, and vascular SMCs (Karkkainen et al., 2004; Kukk et al., 1996). In addition, VEGF-C is also expressed in endocrine glands, aorta and pulmonary artery, platelets, and lacteals (Chen et al., 2014a; Nurmi et al., 2015; Partanen et al., 2000; Wartiovaara et al., 1998). Unlike VEGF-A, its transcription is not regulated by HIF (Chilov et al., 1997; Enholm et al., 1997); however, VEGF-C expression was shown to increase in tumor cells under hypoxic conditions (Morfoisse et al., 2014). In addition, during inflammation, an increase in VEGF-C levels was shown to be driven by inflammatory cells (Baluk et al., 2005b).

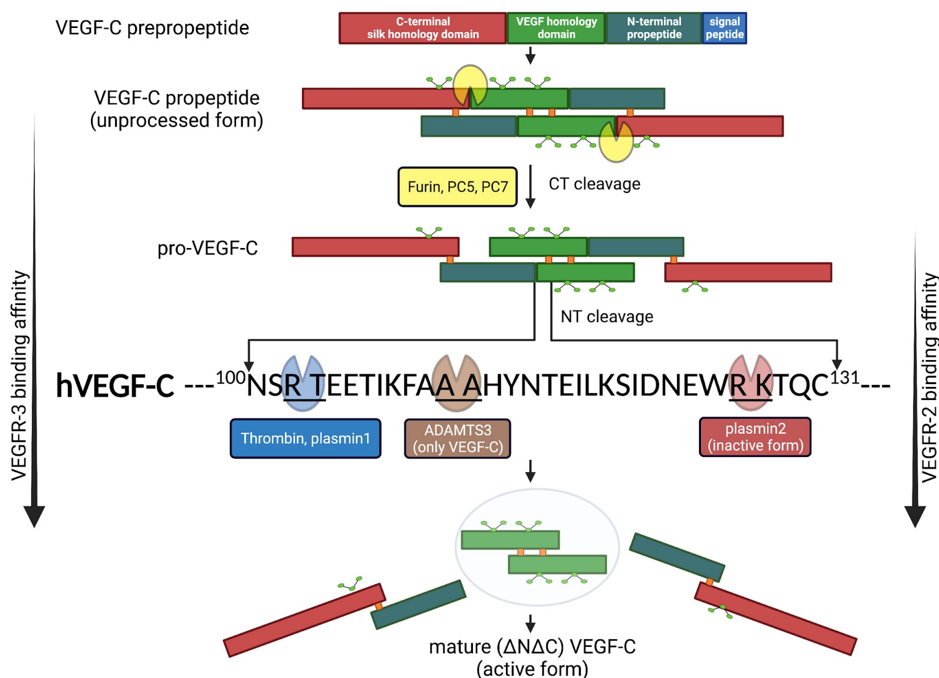

Figure 3. ***Biosynthesis and proteolytic processing of VEGF-C.*** *After synthesis of the inactive prepropeptide, VEGF-C is processed into its mature, active form by sequential removal of its C-terminal (CT) and N-terminal (NT) propeptides, which increases the binding affinity of VEGF-C to VEGFR-2/3. The N-glycosylation sites in VEGF-C are shown by ball-ended v-shaped green sticks. PC5 and PC7 refer to proprotein convertases.*

VEGF-C is the major lymphangiogenic growth factor that signals through the VEGF-C/VEGFR-3 axis and the downstream signaling pathways PI3K/AKT and MAPK/ERK (Deng et al., 2015; Mäkinen et al., 2001; Salameh et al., 2005). It is crucial for the growth, survival, and migration of LECs. It is expressed in developing mouse embryos as early as E8.5 (Karkkainen et al., 2004). Complete deficiency of VEGF-C during embryonic development in mice leads to lethality around E16.5 due to growth arrest of the lymphatic vessels. The LECs are unable to bud out from the cardinal vein and migrate to form lymph sacs (Hägerling et al., 2013; Karkkainen et al., 2004). Heterozygous *Vegfc*$^{+/-}$ mice show lymphatic hypoplasia and edema leading to death during the first few weeks of birth, suggesting VEGF-C as a crucial growth factor for the development of lymphatics (Karkkainen et al., 2004). On the other hand, overexpression of VEGF-C in the skin of transgenic mice and in the airways induces lymphatic hyperplasia (Jeltsch et al., 1997). VEGF-C is considered crucial for coronary arteries development in the heart, and hence deletion of VEGF-C leads to lack of epicardial coronary vessels and hypoplastic peritruncal coronary vessels (Chen et al., 2014b). In zebrafish, VEGF-C is important for precursor cell division and controls PROX1 expression required for LEC commitment (Koltowska et al., 2015). VEGF-C deficiency in zebrafish affects its endoderm development (Ober et al., 2004). VEGF-C/VEGFR-3 signaling plays a vital role in the development of facial and meningeal lymphatics in zebrafish (Hogan and Schulte-Merker, 2017). VEGF-C has also been shown to be crucial for cardiac lymphatic development in adult zebrafish (Harrison et al., 2019). Hence, the role of VEGF-C in lymphangiogenesis and angiogenesis is evolutionarily conserved.

VEGF-C can also induce angiogenesis via its secondary receptor, VEGFR-2. Adenoviral delivery of VEGF-C also results in hyperplastic leaky blood vessels. Studies have shown that VEGF-C can stimulate neovascularization in the mouse cornea and rabbit ischemic hindlimb model (Cao et al., 1998; Witzenbichler et al., 1998). To confirm that the VEGF-A-like effects of VEGF-C, such as increased vascular permeability, are mediated through VEGFR-2 and not VEGFR-3, a VEGF-C point mutant (VEGF-C_{C156S}) which specifically binds and activates only VEGFR-3 was developed (Joukov et al., 1998). This mutant was lymphangiogenic but had no effects on the blood vessels.

Genetic alterations in the human *Vegf-c* gene have been associated with Milroy-like lymphedema (Balboa-Beltran et al., 2014; Gordon et al., 2013; Mukenge et al., 2020). In addition, a truncation mutant in zebrafish (*Vegf-c*^{um18}*)* lacking the C-terminal domain of VEGF-C shows a secretion defect and blockage in its paracrine activity required for tip cell positioning in the developing blood endothelial sprouts (Villefranc et al., 2013). Hence, the C-terminal domain proves to be crucial for the VEGF-C function, which is further investigated in the study included in this thesis. To analyze the role of N- and C-terminal propeptides, a study investigated the effect of a chimera (VEGF-CAC) produced by fusing both VEGF-C propeptides to the VHD of VEGF-A. Adenoviral delivery of this chimera to

immunodeficient mouse ear skin led to more potent branching of blood capillaries compared to VEGF-A (Keskitalo et al., 2007), suggesting that the propeptides enhance VHD activity. In addition, the lymphangiogenesis pattern induced with adenoviral vectors expressing full-length and truncated VEGF-C differs in that the former induces a large network of narrower lymphatic capillaries, whereas the latter induces sparse dilated lymphatic sprouts (Tammela et al., 2007a).

The role of VEGF-C in therapeutic lymphangiogenesis has been well studied in disease models such as lymphedema (Honkonen et al., 2013; Karkkainen et al., 2001; Saaristo et al., 2002; Szuba et al., 2002; Tammela et al., 2007b; Visuri et al., 2015; Yoon et al., 2003), inflammation (Hagura et al., 2014), and diabetic wound healing (Saaristo et al., 2006). Furthermore, the therapeutic potential of VEGF-C in regulating tumor lymphangiogenesis has been well-studied (Chen et al., 2013; Su et al., 2006; Wang et al., 2012). Several tumor cells show an increased expression level of VEGF-C (Salven et al., 1998), and hence the VEGF-C/VEGFR-3 axis could be targeted for anti-tumor therapies (Ding et al., 2012; Khromova et al., 2012), which will be discussed in the section 8 *VEGF-C as a therapeutic target.* VEGF-C is also involved in macrophage-mediated blood pressure regulation (Beaini et al., 2019; Machnik et al., 2009). Non-endothelial roles of VEGF-C have been studied mainly in the central nervous system. VEGF-C stimulates the proliferation of neural progenitor cells expressing VEGFR-3 and is required during embryonic brain development (Le Bras et al., 2006). VEGF-C also serves as a neurotrophic factor that can protect embryonic dopaminergic neurons via various mechanisms (Piltonen et al., 2011). In addition, VEGF-C can activate adult hippocampal neural stem cells (NSCs) and hence promote neurogenesis (Han et al., 2015). The hematopoietic function of VEGF-C in regulating fetal erythropoiesis and megakaryocytic lineage is also well-studied (Fang et al., 2016; Thiele et al., 2012).

Recently, the lymphatic system was characterized in the CNS (meningeal lymphatics) (Aspelund et al., 2015b; Louveau et al., 2015), which develops postnatally around the skull and spinal canal. VEGF-C is essential for the maintenance of the meningeal lymphatic vessel network in adults, which is required for the clearance of interstitial fluid, waste products, and macromolecules from the brain. Overexpression of VEGF-C via viral vectors was able to induce meningeal lymphangiogenesis. Conversely, studies have shown reduced meningeal lymphatic vessels in response to inhibitors of the VEGF-C/VEGFR-3 signaling (Antila et al., 2017). Manipulation of the meningeal lymphatics could provide therapeutic benefits in patients with neuropathological conditions.

3.1.2.2 VEGF-D

VEGF-D is the closest paralog of VEGF-C. It was initially termed FIGF (c-fos-induced growth factor) (Orlandini et al., 1996) but was later renamed VEGF-D (Achen et al., 1998; Yamada et al., 1997). Similar to VEGF-C, VEGF-D also undergoes proteolytic cleavage of

its N- and C-terminal propeptides to generate molecular diversity (Joukov et al., 1997; Stacker et al., 1999). However, unlike mature VEGF-C, which binds to and activates both VEGFR-2 and VEGFR-3, maximally processed VEGF-D (the minor mature form, ^{100}KVIDE...SIIRR^{205}) can only bind to VEGFR-2 and not VEGFR-3 (Leppänen et al., 2011). This explains the fact that mature VEGF-D has stronger angiogenic potential compared to VEGF-C (Rissanen et al., 2003). However, mouse VEGF-D has been reported not to activate mouse VEGFR-2 (Baldwin et al., 2001). This divergence in VEGF-D function may have resulted after the evolution of the placental mammals around 65-66 million years ago (O'Leary et al., 2013). The longer major mature form of VEGF-D (^{89}FAATF...SIIRR^{205}) can activate both VEGFR-2 and VEGFR-3 (McColl et al., 2003).

VEGF-D is expressed abundantly in the lungs during embryonic development in mice (Avantaggiato et al., 1998; Baldwin et al., 2005). In adults, its expression can be seen in the heart, skeletal muscle, lung, small intestine, and colon (Achen et al., 1998; Stacker et al., 1999). Its expression is regulated by c-Fos and Fra-1 transcription factors (Debinski et al., 2001; Orlandini et al., 1996). VEGF-D expression in fibroblasts can also be induced by cadherin 11-mediated cell-cell contact (Orlandini and Oliviero, 2001). In tumor settings such as lung and breast cancers, the expression of VEGF-D was shown to be induced by interleukin 7 (Al-Rawi et al., 2005; Ming et al., 2009).

Although VEGF-D is in many ways similar to VEGF-C, it is dispensable for mouse lymphatic development, unlike VEGF-C. *Vegf-d* knockout mice exhibit only a minor decrease in lymphatic vessel density surrounding the lung bronchioles (Baldwin et al., 2005). Deletion of both *Vegf-c* and *Vegf-d* shows an aggravated intestinal lymphatic phenotype compared to *Vegf-c* deletion alone (Nurmi et al., 2015). Transgenic overexpression of VEGF-D in mouse skin induces lymphatic hyperplasia (Veikkola et al., 2001). Adenoviral delivery of VEGF-D in the ischemic rabbit hind limb skeletal muscle, rat cremaster muscle, inflammatory mouse respiratory tract, and rabbit carotid artery models induces lymphangiogenesis and angiogenesis (Anisimov et al., 2009; Bhardwaj et al., 2003; Byzova et al., 2002; Kholová et al., 2007; Rissanen et al., 2003). Several studies have suggested the therapeutic effects of adenoviral VEGF-D in the myocardium (Hartikainen et al., 2017; Rutanen et al., 2004).

Zebrafish VEGF-D exclusively binds to VEGFR-2 (zKdr) and not VEGFR-3, suggesting zKdr as the primary receptor for its signaling (Vogrin et al., 2019). It is crucial for facial lymphatics development in zebrafish (Bower et al., 2017), and its overexpression leads to defects in the blood vessels (Song et al., 2007). Knockdown of *Vegfd* in Xenopus tadpoles impaired the sprouting and migration of LECs transiently (Ny et al., 2008). These findings suggest a conserved role of VEGF-D during developmental lymphangiogenesis in mammals, zebrafish, and frogs.

3.1.3 Others

3.1.3.1 VEGF-E

VEGF-E, also known as Orf-virus VEGF, is a collective name for a family of related VEGF-like proteins. It was discovered from the genome of Orf virus, a zoonotic parapoxvirus that affects ungulates and occasionally humans, causing highly vascularized lesions (Lyttle et al., 1994; Meyer et al., 1999; Ogawa et al., 1998; Wise et al., 1999). Despite the lack of a heparin-binding basic region, it can bind to and autophosphorylate specifically VEGFR-2, resulting in a strong angiogenic response similar to VEGF-A_{165}. However, unlike VEGF-A, VEGF-E has only minor effects on inflammation and vascular permeability, probably due to the absence of VEGFR-1 binding (Meyer et al., 1999; Ogawa et al., 1998; Shibuya, 2006b), suggesting its clinical use in ischemic diseases with fewer side effects compared to VEGF-A. Transgenic overexpression of VEGF-E in mouse skin induced vascularization without edematous lesions (Kiba et al., 2003). Adenoviral delivery of VEGF-E in mouse skin induced hyperplasia of the lymphatics without any lymphatic sprouting (Wirzenius et al., 2007). In addition, viral-expressed VEGF-E has been shown to increase keratinocyte proliferation and epidermal regeneration in proliferative skin lesions caused by Orf virus infection (Wise et al., 2012). These findings suggest the possible use of VEGF-E in promoting the re-epithelialization of wounds.

3.1.3.2 VEGF-F

VEGF-like proteins isolated from snake venom are collectively termed VEGF-F. It was first purified from *Vipera aspis* venom, and due to its hypotensive effect, it was termed hypotensive factor (HF) (Komori et al., 1999). Several snake venom VEGFs have been isolated and studied since then, most of them showing effects like increased vascular permeability, EC proliferation, angiogenesis, and hypotension reviewed in (Ferreira et al., 2021). However, they have varied structures and functions among different species, and hence VEGF-Fs can signal via VEGFR-1 and/or VEGFR-2 (Aloui et al., 2009; Brown et al., 2007; Chen et al., 2005; Takahashi et al., 2004; Yamazaki et al., 2009; Zhong et al., 2015). Their VHD is highly conserved, with eight cysteine residues forming a cystine knot; however, the C-terminal domain has undergone great variation during molecular evolution (Yamazaki et al., 2009).

3.2 Vascular endothelial growth factor receptors (VEGFRs)

VEGFRs belong to the class V cell surface receptor tyrosine kinases (RTKs). All three VEGFRs (VEGFR-1, VEGFR-2, and VEGFR-3) share structural similarities and consist of an extracellular domain composed of seven immunoglobulin (Ig)-like loops, a transmembrane domain, and an intracellular tyrosine kinase domain followed by a C-terminal

tail. VEGFs bind to VEGFRs causing homo- or heterodimerization of the receptors that trigger the kinase activity followed by intracellular signaling and cellular response.

3.2.1 VEGFR-1

VEGFR-1, commonly known as Flt1 (Fms-like tyrosine kinase 1), was identified in 1990 (Shibuya et al., 1990), and the ligands for this receptor (VEGF-A, VEGF-B, and PlGF) were discovered later (Olofsson et al., 1998; Park et al., 1994; de Vries et al., 1992). Different biological activities of VEGFR-1 ligands are due to the difference in interaction with the ligand binding second Ig-like domain (D2) of VEGFR-1 and the third domain (D3) that provides additional binding sites (Anisimov et al., 2013; Davis-Smyth et al., 1996, 1998). This membrane-bound receptor is expressed in angioblasts at E8.5 during embryonic development, and later its expression is abundant in blood vascular endothelial cells (BECs), monocytes, macrophages, pericytes, dendritic cells, osteoclasts, trophoblasts, and hematopoietic stem cells. It also exists in a soluble isoform (sVEGFR-1), which is produced by alternative splicing and contains only the first six Ig-like extracellular domains (D1-6) without the transmembrane- and the tyrosine kinase domain (Kendall and Thomas, 1993; Kendall et al., 1996).

Both soluble and membrane-bound isoforms of VEGFR-1 have a stronger affinity to VEGF-A compared to VEGFR-2; however, the tyrosine kinase activity of VEGFR-1 is weaker than that of VEGFR-2 (Seetharam et al., 1995; Waltenberger et al., 1994). Despite its weaker kinase activity, VEGFR-1 can mediate the migration of monocytes and macrophages (Barleon et al., 1996; Clauss et al., 1996; Sawano et al., 2001). VEGFR-1 can act as a dual regulator of angiogenesis. sVEGFR-1 can trap and suppress VEGF-A levels through its ligand binding domains and hence negatively regulate vascular development during embryogenesis (Goldman et al., 1998; Kendall and Thomas, 1993) as well as VEGF-A-mediated pathological angiogenesis (Takayama et al., 2000). However, in adults, it can transduce weak proliferative and migratory signals to ECs (Shibuya, 2001, 2006c, 2006a). Under pathological conditions such as tumors overexpressing VEGFR-1-specific ligand, PlGF, it can act as a positive regulator of angiogenesis (Hiratsuka et al., 2001). However, inhibition of VEGFR-1 signaling via anti-PlGF antibodies could not reduce tumor angiogenesis (Bais et al., 2010).

Deletion of *Vegfr-1* in mice leads to embryonic lethality around E8.5-E9 due to mispatterned blood vessels caused by BECs overgrowth and increased hemangioblast commitment (Fong et al., 1995, 1999). In contrast, deletion of the *Vegfr-1* tyrosine kinase domain resulted in normal angiogenesis with a minor defect in macrophage migration (Hiratsuka et al., 1998). These studies further support VEGFR-1 as a negative regulator of angiogenesis and suggest it as a decoy receptor that regulates the availability of VEGF-A. However, several studies have confirmed the signaling role of the VEGFR-1 kinase domain for pathological

angiogenesis and macrophage migration. Inhibition of VEGFR-1 exhibited decreased pathological neovascularization and anti-inflammatory effects (Luttun et al., 2002). In addition, the formation of VEGFR-1/VEGFR-2 heterodimers has been suggested to positively or negatively modulate VEGFR-2 function (Carmeliet et al., 2001; Huang et al., 2001; Kendall et al., 1996; Rahimi et al., 2000; Zeng et al., 2001).

3.2.2 VEGFR-2

VEGFR-2, commonly known as Flk1 (fetal liver kinase 1) in mice (Matthews et al., 1991) and KDR (kinase-insert domain receptor) in humans (Terman et al., 1991), is the major receptor responsible for angiogenesis. The ligands for VEGFR-2 are VEGF-A (Quinn et al., 1993), mature VEGF-C, and mature human VEGF-D (Figure 2) (Joukov et al., 1997; Stacker et al., 1999). The Ig-like extracellular domains D2 and D3 are responsible for ligand binding (Fuh et al., 1998; Leppänen et al., 2010; Shinkai et al., 1998), whereas domains D4–D7 regulate the homodimerization of VEGFR-2 upon ligand binding (Hyde et al., 2012; Kendrew et al., 2011). Several studies have suggested that VEGFR-2 can exist as a monomer or a dimer in the absence of a ligand; however, ligand binding changes the conformation of the transmembrane domain (Ruch et al., 2007; Sarabipour et al., 2016), resulting in increased phosphorylation of the kinase domain.

VEGFR-2 is expressed as early as E7.0 in mouse embryos (Millauer et al., 1993), and its expression level lowers in mature ECs in adults (Matsumoto and Claesson-Welsh, 2001). In addition, it is also expressed in hematopoietic cells (Ziegler et al., 1999), retinal progenitor cells, and neuronal cells (Yang and Cepko, 1996). VEGFR-2 is indispensable for the proliferation, survival, migration, and sprouting of ECs (Gille et al., 2001; Koch and Claesson-Welsh, 2012). Deletion of *Vegfr2* in mouse embryos is lethal around E8.5-E9.0 caused by defective blood vessel formation and hematopoiesis (Shalaby et al., 1995, 1997). Its expression is elevated in physiological and pathological angiogenesis in adults. Upregulation of VEGFR-2 in the endothelial tip cell compared to stalk cells guides filopodia toward the VEGF-A concentration gradient and induces vascular sprouting (Gerhardt et al., 2003). VEGFR-2 expression is also found in LECs (Saaristo et al., 2002), and overexpression of VEGFR-2-specific ligand, VEGF-E, induced lymphatic hyperplasia without sprouting, suggesting the role of VEGFR-2 signaling in stimulating lymphatic vessel enlargement (Wirzenius et al., 2007).

Recombinant sVEGFR-2 has been used to inhibit VEGF-A-mediated tumor angiogenesis and growth in several *in vitro* and *in vivo* studies (Davidoff et al., 2000; Huang et al., 1998; Roeckl et al., 1998; Tseng et al., 2002). The presence of sVEGFR-2 has been confirmed in mouse and human plasma (Ebos et al., 2004) and suggested as a prognostic biomarker in several cancers and ischemic diseases (Becker et al., 2010; Jürgensmeier et al., 2013; Shenavandeh et al., 2017; Thielemann et al., 2013; Wieczór et al., 2016). Inhibitors of

VEGFR-2 or the VEGF-A/VEGFR-2 pathway have been in clinical trials and clinical use to target VEGFR-2 signaling required for primary tumor growth and vascularization reviewed in (Crawford and Ferrara, 2009; Ferrara, 2009; Fontanella et al., 2014; Huang et al., 2012; Peng et al., 2017).

3.2.3 VEGFR-3

VEGFR-3, commonly known as Flt4 (Fms-like tyrosine kinase), is the primary lymphangiogenic receptor (Pajusola et al., 1992). VEGF-C and VEGF-D are the ligands for this receptor (Figure 2) (Joukov et al., 1996; Lee et al., 1996). The major ligand binding site is located at D2, and the presence of D3 increases the ligand binding affinity (Leppänen et al., 2013). However, for VEGF-D binding, D1 is also required to stabilize the interaction between ligand and D2 (Leppänen et al., 2011). Similar to other VEGFRs, D4-D7 are crucial for receptor dimerization (Leppänen et al., 2013), which was further confirmed by the inability to form dimers in the presence of an antibody against D5 (Tvorogov et al., 2010). It shows structural dissimilarity with other VEGFRs as it undergoes proteolytic cleavage in its D5, but the cleavage products remain attached to each other by a disulfide bridge (Pajusola et al., 1993, 1994). Alternative splicing of VEGFR-3 results in two isoforms, the longer one being more abundant and having 65 amino acid residues more than the shorter isoform at the carboxy-terminal end (Hughes, 2001; Pajusola et al., 1993).

VEGFR-3 serves dual functions for both blood and lymph vessel embryonic development. Its expression in BECs starts already at E8.5 (Kaipainen et al., 1995), and deletion of *Vegfr3* results in embryonic lethality around E9.5 due to cardiovascular defects (Dumont et al., 1998). However, later during embryogenesis and postnatal stages, its expression decreases in BECs and becomes restricted to LECs (Kaipainen et al., 1995). VEGFR-3 expression can also be detected in high endothelial venules, fenestrated vessels in the liver, spleen, and endocrine organs, monocytes/macrophages, osteoblasts, and neural progenitor cells (Le Bras et al., 2006; Orlandini et al., 2006; Partanen et al., 2000; Schoppmann et al., 2002; Skobe et al., 2001a). It is crucial for the sprouting, migration, proliferation, and survival of LECs during lymphatic development. VEGFR-3 has been shown to play a functional role in tumor angiogenesis and postnatal retinal angiogenesis (Laakkonen et al., 2007; Partanen et al., 1999; Tammela et al., 2008; Valtola et al., 1999). *Vegfr3* deficiency inhibits lymphatic vessel growth (Karkkainen et al., 2004; Karpanen et al., 2006a; Mäkinen et al., 2001) and also results in vascular leakage by modulating VEGF-A/VEGFR-2 signaling (Heinolainen et al., 2017). However, the lack of VEGFR-3 ligands, VEGF-C and VEGF-D, leads to embryonic lethality caused by defective lymphatic vessels later during E16.5; hence, these ligands do not regulate VEGFR-3 activity in early embryonic development (Haiko et al., 2008).

VEGFR-3 can also form heterodimers with VEGFR-2 (Alam et al., 2004; Goldman et al., 2007; Harris et al., 2013), and both VEGF-A and VEGF-C are able to facilitate this

heterodimerization (Nilsson et al., 2010). VEGFR-3/VEGFR-2 heterodimers induced by VEGF-C were shown to localize to tip cells and stimulate sprouting angiogenesis (Nilsson et al., 2010). VEGF-A-stimulated heterodimers were shown to downregulate VEGFR-2-mediated ERK signaling (Zhang et al., 2010). However, there is a lack of understanding of the role of these heterodimers.

3.3 Other molecules involved in the VEGF/VEGFR regulation

3.3.1 Neuropilins

Neuropilins (Nrp1 and Nrp2) are transmembrane non-tyrosine kinase glycoproteins originally identified as class III semaphorin (SEMA3) receptors, signaling axon guidance (Chen et al., 1997; Fujisawa et al., 1995; He and Tessier-Lavigne, 1997; Kolodkin et al., 1997; Takagi et al., 1991). Nrps were later discovered to bind VEGFs and serve as co-receptors for VEGFRs that do not mediate direct signaling effects but are important to stabilize VEGF/VEGFR interaction (Fuh et al., 2000; Makinen et al., 1999; Migdal et al., 1998; Soker et al., 1998). The extracellular part of Nrps consists of a1/a2 domains for SEMA3 binding, b1/b2 domains for interaction with SEMA3 and VEGFs, and c domain involved in Nrps interaction with other receptors (Renzi et al., 1999; Wild et al., 2012) (Figure 4).

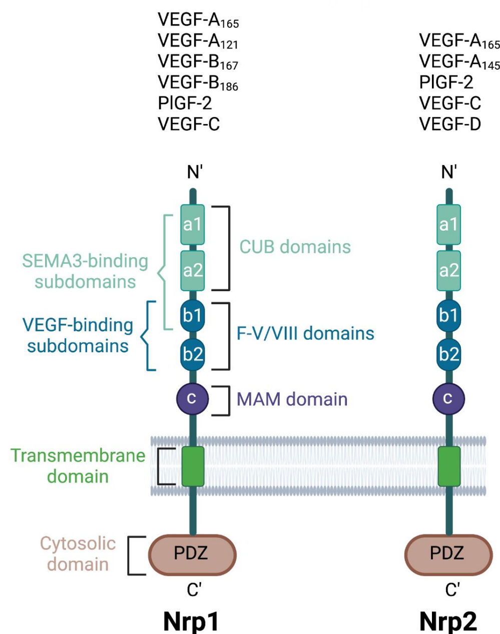

Figure 4. ***Structure of neuropilins and their interaction with ligands.*** *Nrp1 and Nrp2 contain two CUB (for complement C1r/C1s, Uegf, Bmp1) domains (a1, a2), two coagulation factor V/VIII homology domains (b1, b2), and a MAM (meprin/A5-protein/PTPmu) domain (c) in their extracellular region. The subdomains interacting with the VEGF and semaphorin ligands are shown. PDZ is an acronym for the first letters of three proteins in which the domain was discovered [post synaptic density protein (PSD95), Drosophila disc large tumor suppressor (DlgA), and zonula occludens-1 protein (zo-1)].*

Nrps interact with different isoforms of several VEGFs and form complexes with VEGFRs. Nrp1 binds to VEGF-A_{165}, VEGF-A_{121}, VEGF-B_{167}, VEGF-B_{186}, PlGF-2, and VEGF-C (Karpanen et al., 2006b; Makinen et al., 1999; Migdal et al., 1998; Ober et al., 2004; Pan et al., 2007; Soker et al., 1998), whereas ligands for Nrp2 are VEGF-A_{165}, VEGF-A_{145}, PlGF-2, VEGF-C, and VEGF-D (Gluzman-Poltorak et al., 2000; Karkkainen et al., 2001) (Figure 4). Nrp1 has been shown to promote VEGF-A_{165} interaction with VEGFR-2 and modulate VEGF-A_{165}-induced angiogenesis (Soker et al., 1998, 2002). Deficiency of *Nrp1* leads to embryonic lethality around E13.5 due to defective nervous and vascular systems (Kawasaki et al., 1999; Kitsukawa et al., 1995, 1997). However, *Nrp2* deficiency is not lethal but results in smaller lymphatic vessels and capillaries without affecting the lymph sac and collecting lymphatics (Yuan et al., 2002). The ablation of both *Nrp1* and *Nrp2* leads to early embryonic death around E8.5 due to more severe vascular defects, suggesting that an early interplay between both is necessary for normal vascular development (Takashima et al., 2002).

The interaction of VEGFs with Nrp1 and Nrp2 is dependent on the heparin-binding domains of VEGFs (Fuh et al., 2000; Karpanen et al., 2006b). Upon Nrp2 binding, VEGF-C and VEGF-D have been shown to promote co-internalization of both Nrp2 and VEGFR-3, but the physiological significance remains unknown (Karpanen et al., 2006b). Blocking VEGF-C/Nrp2 interaction or silencing Nrp2 in ECs has shown defects in LECs migration but not proliferation (Caunt et al., 2008; Favier et al., 2006). Furthermore, monomeric soluble Nrp1 produced by alternative splicing was shown to be capable of trapping VEGF-A_{165}, thereby inhibiting pathological angiogenesis in tumors (Gagnon et al., 2000; Yamada et al., 2001). Conversely, tumor cells expressing Nrp1 induced enhanced angiogenesis and tumor progression by stimulating VEGF-A_{165}/VEGFR-2 signaling or increasing the availability of VEGF-A_{165} (Miao et al., 2000; Parikh et al., 2004).

3.3.2 Integrins

Integrins are transmembrane $\alpha\beta$ heterodimeric cell adhesion receptors. In vertebrates, the integrins family is composed of non-covalently linked 18 α and eight β subunits, resulting in 24 different heterodimers (Hynes et al., 2002; Silva et al., 2008). Based on their ligand binding properties, they can be grouped into collagen-binding, leukocyte, laminin-binding, and RGD-recognizing receptors reviewed in (Barczyk et al., 2009). Upon binding to their

specific extracellular ligands, integrins mediate intracellular signaling events that regulate cell adhesion, proliferation, survival, growth, and apoptosis reviewed in (Eliceiri, 2001; van der Flier and Sonnenberg, 2001; Takada et al., 2007).

Several integrin knockout studies in mice have suggested varied roles of specific integrins, for example, integrin α_9 in lymphatic development (Huang et al., 2000), integrin α_V in vasculogenesis (Bader et al., 1998), integrin $\alpha_9\beta_1$ in lymphangiogenesis (Huang et al., 2000; Vlahakis et al., 2005), integrin $\alpha_{IIb}\beta_3$ in thrombus formation (Chen et al., 2002; Hodivala-Dilke et al., 1999), and integrin β_2 in immune responses (Graham et al., 1993; Zuchtriegel et al., 2020). Integrin $\alpha_V\beta_3$ is abundantly present in ECs and induces VEGFR-2-mediated mitogenic signals in ECs by activating Src (Borges et al., 2000; Soldi et al., 1999). Blocking antibodies or cyclic RGD peptides against $\alpha_V\beta_3$ have been shown to inhibit angiogenesis, retinal neovascularization, and tumor growth (Brooks et al., 1994a, 1994b; Drake et al., 1995; Friedlander et al., 1996; Hammes et al., 1996). β_3 knockout studies have revealed increased tumor growth and angiogenesis (Taverna et al., 2004, 2005), accelerated wound healing (Reynolds et al., 2005), enhanced atherosclerosis and inflammation (Weng et al., 2003), suggesting its role in the suppression of these processes. Integrin α_9 has also been widely studied in the context of lymphatic development. PROX1 deletion was shown to decrease integrin α_9 expression in human umbilical venous endothelial cells (HUVECs) and LECs, suggesting its role in regulating lymphangiogenesis (Mishima et al., 2007). Furthermore, integrin α_9 deletion in mice leads to death around 6-12 days after birth due to congenital bilateral chylothorax (Huang et al., 2000). $\beta 1$ integrins have been shown to be important mechanosensors for LEC proliferation and VEGFR-3 phosphorylation during increased ISF accumulation and cell stretching, suggesting the role of $\beta 1$ integrins in lymphatic development and fluid homeostasis (Planas-Paz et al., 2012).

VEGF-A was shown to induce the expression of collagen receptors–$\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrins–in ECs, suggesting their role in promoting VEGF-A-mediated angiogenesis (Senger et al., 1997). VEGF-C and VEGF-D can directly bind to $\alpha_9\beta_1$ integrin in a dose-dependent manner in a solid-phase binding assay, indicating a mechanism by which $\alpha_9\beta_1$ integrin modulates lymphangiogenesis (Vlahakis et al., 2005). VEGF-A is also a direct ligand for $\alpha_9\beta_1$ integrin, suggesting that VEGF-A-mediated adhesion and migration of HUVECs are dependent on this integrin (Vlahakis et al., 2007). Hence, $\alpha_9\beta_1$ can be a potential therapeutic target for inhibiting both pathological angiogenesis and lymphangiogenesis.

3.3.3 Extracellular matrix

The ECM consists of dynamic, interlinked macromolecular networks outside the cells with tissue-specific composition. In addition to providing structural support to the cells, ECM is crucial for directing physiological functions, eliciting signal transduction, and regulating gene transcription by binding to growth factors and interacting with cell-surface receptors

(Frantz et al., 2010). The major components of the ECM include proteoglycans and fibrous proteins such as collagen, fibronectins, elastins, and laminins (Bosman and Stamenkovic, 2003; Frantz et al., 2010; Theocharis et al., 2016). The ECM also regulates the growth, survival, and migration of ECs via integrins (Mettouchi, 2012). The ECM receptor integrins are responsible for the adhesion of cells to the ECM components and hence are involved in signaling events. The vascular basement membrane is also composed of ECM proteins such as laminin $\alpha4$ and proteoglycans, which provide structural stability to the blood vessels (Thyboll et al., 2002; Witjas et al., 2019). Several growth factors bind to ECM proteins and heparin or HSPGs that are present in ECM, establishing a stable reservoir of growth factors required for developmental patterning processes. This is also crucial for regulating the distribution and activation of growth factors and their presentation to cells. These ECM-bound growth factors can be released by the degradation of glycosaminoglycans in the HSPGs (Ferrara, 2010; Schultz and Wysocki, 2009).

The presence of heparin-binding domain in all VEGF-A isoforms, except VEGF-A_{121}, enables them to bind to multiple proteins, such as laminin and fibronectin, and HSPGs in the ECM (Ferrara, 2010; Gitay-Goren et al., 1992; Ishihara et al., 2018; Park et al., 1993; Wijelath et al., 2006). The ECM-embedded VEGF-A can be released by plasmin, factor VII-activating protease (FSAP), urokinase-type plasminogen activator (uPA), and matrix metalloproteinases (MMPs), which provides a balance between VEGF-A bioavailability in the soluble form and in the pericellular matrix, which is required for physiological angiogenesis (Bergers et al., 2000; Houck et al., 1992a; Lee et al., 2005; Plouët et al., 1997; Uslu et al., 2019). Several mice studies have shown that VEGF-A_{120}, which lacks a matrix binding domain, can only induce EC proliferation as opposed to directed sprouting of ECs by longer matrix binding isoform VEGF-A164 (Gerhardt et al., 2003; Martino et al., 2015; Ruhrberg et al., 2002; Stenzel et al., 2011; Wijelath et al., 2006). Furthermore, matrix-bound VEGF-A_{165}, when compared to soluble VEGF-A, showed prolonged VEGFR-2 activation, increased VEGFR-2 clustering and its association with $\beta1$ integrin, and differential downstream signaling after VEGFR-2 activation (Chen et al., 2010).

The effects of ECM binding on VEGF-C and VEGF-D have been relatively less studied. Studies have shown that pro-VEGF-C binds to the ECM protein fibronectin and cell surface HSPGs via its heparin-binding C-terminal domain, which is required for efficient VEGF-C activation and regulation of LECs growth and sprouting (Johns et al., 2016). Syndecan-4, a major HSPG expressed abundantly in lymphatic endothelium, acts as a co-receptor for VEGF-C-mediated pathological lymphangiogenesis. Genetic deletion of the Ndst1 enzyme, responsible for heparan sulfate biosynthesis, or syndecan-4 led to inhibition of VEGF-C-mediated pathological lymphangiogenesis by impairing VEGF-C/VEGFR-3 interaction (Johns et al., 2016).

4. Proteases and proteins involved in VEGF-C activation

4.1 Collagen and calcium-binding EGF domains 1

CCBE1 is a secreted ECM protein containing three epidermal growth factor (EGF)-like repeats at the N-terminus and two collagen-like repeats at the C-terminus. It was discovered by genetic mapping as a mutant gene from zebrafish that lacked lymphatic vasculature (Hogan et al., 2009). Later, using homozygosity mapping, several mutations in the human *Ccbe1* gene were identified as a cause of Hennekam lymphangiectasia–lymphedema syndrome (HKLLS), which is an autosomal recessive hereditary disorder characterized by generalized lymphedema, intestinal lymphangiectasia, and facial anomalies (Alders et al., 2009; Hennekam et al., 1989). Most of the identified point mutations in *Ccbe1* localize to its N-terminal domain, and only two of these mutations affect the C-terminal domain, all resulting in compromised CCBE1 protein (Alders et al., 2013; Connell et al., 2010; Frosk et al., 2015).

Ablation of *Ccbe1* in zebrafish and mice leads to a lack of lymph sacs due to the inability of ECs to egress from the cardinal veins, resembling the *Vegfc* knockout phenotype (Bos et al., 2011; Hogan et al., 2009). In double heterozygous *Vegfc+/-*, *Ccbe1+/-* mice, both proteins showed cooperative function during LECs budding and migration (Hägerling et al., 2013). Although both VEGF-C and CCBE1 are essential for the angiogenic sprouting and budding of LECs from cardinal veins, there is no evidence of direct interaction between CCBE1 and VEGF-C (Hogan et al., 2009). Despite lacking enzymatic activity, CCBE1 was shown to enhance VEGF-C-mediated lymphangiogenesis in a corneal micropocket assay (Bos et al., 2011). CCBE1 was also demonstrated to bind to the ECM components, mainly vitronectin. Studies have shown that CCBE1 acts as a helper protein that enhances ADAMTS3-mediated activation of VEGF-C (Jeltsch et al., 2014). In addition, the absence of *Ccbe1* was shown to impair VEGF-C/VEGFR-3 signaling (Le Guen et al., 2014).

Both N- and C-terminal domains of CCBE1 have been studied. The C-terminal domain can act functionally similar to a co-enzyme (Le Guen et al., 2014). Furthermore, functional analysis of both CCBE1 domains using domain deletion mutants revealed that the collagen-domain-deleted mutant failed to assist VEGF-C activation *in vivo* and copied the *Ccbe1* knockout phenotype. However, deletion of the EGF-like domain had only partial effects on VEGF-C activation and could form rudimentary lymphatics (Roukens et al., 2015).

CCBE1 expression is regulated in zebrafish by transcription factors E2F7 and E2F8 (Weijts et al., 2013). It is expressed in early cardiac progenitors and has a role in the development of the heart. *Ccbe1* knockdown leads to defects in cardiogenesis (Furtado et al., 2014). Similar to VEGF-C, it is crucial for coronary vasculature formation and fetal erythropoiesis, indicating that these effects are mediated via VEGF-C (Bonet et al., 2018; Zou et al., 2013).

CCBE1 has also been linked with cancers since it has been shown to suppress tumor growth in various cancer cell lines (Barton et al., 2010; Mesci et al., 2017).

4.2 Plasmin and Thrombin

Plasmin and thrombin are trypsin-like serine proteases and components of the blood fibrinolytic/coagulation system. Thrombin exists as an inactive proenzyme, prothrombin, which upon activation, promotes blood coagulation and fibrin clot formation, whereas plasmin, produced as inactive precursor plasminogen, degrades the fibrin clot into soluble products (Chapin and Hajjar, 2015; Göbel et al., 2018; Licari and Kovacic, 2009). Both plasmin and thrombin play a crucial role in hemostasis and are essential for the wound-healing process (Reinke and Sorg, 2012). During wound healing, thrombin recruits platelets to the hemostatic plug, and platelets release VEGF-C, which is cleaved and activated by plasmin and thrombin, resulting in VEGF-C-mediated lymphangiogenesis (Brass, 2003; Monroe et al., 2002; Wang et al., 2014; Wartiovaara et al., 1998).

Both VEGF-C and VEGF-D were identified as substrates for plasmin using a scintillation proximity assay (McColl et al., 2003, 2004). In addition, plasmin plays a role in modulating the angiogenic effects of VEGF-A during wound healing by releasing VEGF-A from the ECM or cell surface (Houck et al., 1992b; Plouët et al., 1997). Plasmin was shown to cleave both N- and C-terminal propeptides from the VHD of VEGF-D, resulting in active VEGF-D that can activate both VEGFR-2 and VEGFR-3. N-terminal cleavage of pro-VEGF-D by plasmin occurs at two different sites, which are compatible with the mature VEGF-D forms purified from 293EBNA cells (McColl et al., 2003; Stacker et al., 1999). However, prolonged incubation of both VEGF-C and VEGF-D with plasmin renders them inactive. In a later study, plasmin could only activate VEGF-D but not VEGF-C, whereas thrombin effectively activated both VEGF-C and VEGF-D, further confirmed by inhibition of VEGF-C activation by specific thrombin inhibitor in a dose-dependent manner (Lim et al., 2019). Furthermore, *in vivo* studies using different wound models showed a concrete link between hemostasis and lymphangiogenesis and identified VEGF-C but not VEGF-D as the main driver of lymphangiogenesis during wound healing (Lim et al., 2019).

4.3 A disintegrin and metalloproteinase with thrombospondin motifs

The ADAMTS family consists of 19 secreted multi-domain metalloproteinases, with a common N-terminal protease domain containing a signal peptide, prodomain, metalloproteinase domain, and disintegrin domain followed by heterogenous C-terminal ancillary domains. ADAMTS are secreted as zymogens that undergo proteolytic cleavage of their prodomain to become active (Apte, 2004; Kelwick et al., 2015) (Figure 5). ADAMTS3,

together with ADAMTS2 and ADAMTS14, was identified as a procollagen N-propeptidase responsible for the proteolytic activation of procollagens (Fernandes et al., 2001; Le Goff et al., 2006). Later, it was discovered that CCBE1 can increase the processing of pro-VEGF-C in co-transfected 293T cells but lacks enzymatic activity. Mass spectrometric analysis of CCBE1 produced from a 293T cell line overexpressing CCBE1 led to the identification of ADAMTS3 as the endogenous protease responsible for VEGF-C activation (Jeltsch et al., 2014; Le Guen et al., 2014).

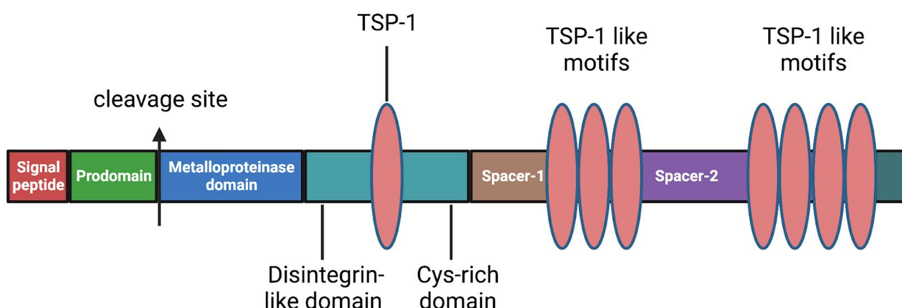

Figure 5. *Structural organization of ADAMTS3.* *It contains an N-terminal signal peptide, a prodomain followed by a furin-cleavage site for its activation, a catalytic metalloproteinase domain, a disintegrin-like domain, a cysteine-rich domain, and multiple thrombospondin type 1 motifs (TSP-1) at the C-terminus.*

However, the indispensable role of ADAMTS3 in embryonic lymphangiogenesis was identified from *Adamts3* knockout studies in mice. *Adamts3*+/- mice were fertile, but *Adamts3*-/- mice died around E15.0 due to cutaneous lymphedema and liver degeneration starting around E13.0-13.5 (Janssen et al., 2016), which resembles the phenotype of *Ccbe1*-deficient mice (Bos et al., 2011). However, there were no defects in procollagen processing, indicating that ADAMTS3 expression during embryonic development is unrelated to collagen biology (Janssen et al., 2016). Later, ADAMTS3 was also identified as a protease that cleaves and inactivates Reelin, an ECM glycoprotein that is responsible for embryonic brain development and regulation of lymphatic vascular development by recruiting SMCs to the lymph vessels (Hattori and Kohno, 2021; Lutter et al., 2012; Ogino et al., 2017). Reelin-deficient mice have been shown to exhibit abnormal collecting lymphatic vessels. A decrease in Reelin signaling in the adult brain has been associated with the pathogenesis and deterioration of neuropsychiatric diseases. It has been shown that excess Reelin signaling can cause abnormal hippocampal development. Hence, one could speculate that mental retardation in HKLLS linked to *ADAMTS3* deficiency might result from failure of Reelin inactivation.

Several human lymphatic phenotypes have been linked to *ADAMTS3* deficiency. A biallelic missense mutation in the *ADAMTS3* gene was shown to abolish VEGF-C activation and cause HKLLS3 (Brouillard et al., 2017). Another loss-of-function variant of *Adamts3*, a homozygous nonsense *Adamts3* mutation, was also reported to cause HKLLS3 (Scheuerle et al., 2018).

ADAMTS3-mediated VEGF-C activation is likely to occur only during the embryonic stage because the expression of ADAMTS3 becomes restricted to the cartilage and central nervous system in adults (Fernandes et al., 2001; Le Goff et al., 2006). In addition, it was also shown that loss of *Adamts3* in zebrafish does not affect lymphatic function, but a simultaneous loss of *Adamts3* and *Adamts14* was required to observe lymphatic defects, suggesting some redundant functions (Wang et al., 2020). A recent study provided evidence that ADAMTS2 and ADAMTS14 could efficiently activate VEGF-C, and *Adamts2* deficiency, but not *Adamts14,* in adult mice leads to skin lymphedema. Furthermore, *Adamts2*- and/or *Adamts14*-KO mice showed decreased lymphangiogenesis in an acute neolymphangiogenesis model, suggesting that ADAMTS3 can be substituted by ADAMTS2 and ADAMTS14 during adulthood (Dupont et al., 2022).

4.4 Kallikrein-related peptidases

KLKs are a family of highly conserved 15 serine proteases in humans with a broad spectrum of physiological roles (Lawrence et al., 2010). For instance, KLK1 is involved in regulating blood pressure, smooth muscle contraction, vascular permeability, and vascular cell growth (Bhoola et al., 1992). KLK2 and KLK3 are crucial for semen liquefaction (Pampalakis and Sotiropoulou, 2007), KLK4 affects tooth maturation (Lu et al., 2008), and KLK5, KLK7, and KLK14 are involved in skin desquamation (Borgoño et al., 2007). KLK3 is the most abundant secreted protein in the prostate and is better known as a prostate cancer biomarker, prostate-specific antigen (PSA) (Hong, 2014; Shaw and Diamandis, 2007). However, the diagnostic/prognostic role of KLK3 is controversial due to the variation in the levels of human KLK3. KLK3 cleaves semenogelins required for the liquefaction of the seminal fluid clot to release motile spermatozoa (Lilja, 1985; Lilja et al., 1987). KLK3 is also released into the blood circulation in prostate cancer and some benign conditions, but the vast majority of it is inactive because it is in complex with protease inhibitors (Lilja, 2008; Lilja et al., 2008; Stenman et al., 1994).

KLK3 expression is regulated by steroid hormones, mainly androgen (Young et al., 1991). Several studies have shown that KLK3 is upregulated in ovarian cancer but downregulated in breast cancers (Avgeris et al., 2012; Borgoño and Diamandis, 2004). Other studies using cellular and xenograft models suggest KLK3 as a growth promoter of prostate cancer cells (Niu et al., 2008; Srinivasan et al., 2019; Williams et al., 2011). KLK3 has been suggested to inhibit angiogenesis by some early studies, likely because of its proteolytic activity.

However, recent studies show KLK3 involvement in promoting cancer cell growth, invasion, metastasis, and angiogenesis by proteolytically activating several growth factors and releasing angiogenic or lymphangiogenic factors by degrading the ECM components (Borgoño and Diamandis, 2004; Koistinen et al., 2021). Studies with transgenic mice expressing active KLK3 in the prostate could not provide any evidence for the involvement of KLK3 in tumor growth (Williams et al., 2010). Gene deletion studies of *Klk3* in mice are not possible due to a lack of a *Klk3* ortholog in mice, and hence there is little evidence for the role of KLK3 in cancer progression *in vivo* (Lawrence et al., 2010). Genetic variations in the human *KLK3* gene have been associated with an increased risk of prostate cancer and infertility in men (Gupta et al., 2017; Kote-Jarai et al., 2011).

In a peptide library scan, KLK4 was identified to proteolytically activate pro-KLK3. In addition, VEGF-C was discovered as a potential substrate for KLK4 from a protein database search for sequences with predicted KLK4 cleavage sites, but this in-silico analysis was not validated in the study (Matsumura et al., 2005).

4.5 Cathepsin D

Cathepsin D is a soluble aspartyl endopeptidase with ubiquitous distribution and is secreted as inactive preprocathepsin D. The main function of cathepsin D was considered to degrade lysosomal proteins in an acidic environment (Benes et al., 2008). Later, it was shown to mediate tumor growth and metastasis as well as act as a paracrine factor for ECs (Berchem et al., 2002; Glondu et al., 2001, 2002; Hu et al., 2008). Cathepsin D expression is upregulated by estrogen in breast cancer cells and has been proposed to be an independent prognostic factor of breast cancer (Liaudet-Coopman et al., 2006; Tandon et al., 1990). It is also upregulated by thrombin and induces angiogenesis in the chick chorioallantoic membrane assay and HUVECs (Hu et al., 2008). Downregulation of cathepsin D has been shown to inhibit tumor growth and metastasis in breast cancer cells (Glondu et al., 2002). A mutant cathepsin D that lacks its enzymatic activity was also shown to still stimulate tumor growth, indicating a possible extracellular interaction with yet unidentified cell surface receptors (Glondu et al., 2001). Cathepsin D has a dual role in apoptosis as it has been shown to either prevent or induce apoptosis via different mechanisms depending upon environmental conditions. Several studies on cathepsin D knockout mice models provide evidence that cathepsin D can inhibit apoptosis under physiological conditions, whereas it can promote apoptosis induced by cytotoxic agents (Liaudet-Coopman et al., 2006). Cathepsin D-mediated cleavage of neuronal proteins that cause neurodegenerative diseases is necessary for neuronal cell homeostasis (Shacka et al., 2007; Vidoni et al., 2016).

5. Use of recombinant VEGF-C in vascular biology

Since active VEGF-C is only generated after proteolytic cleavage of its N- and C-terminal propeptides (Joukov et al., 1997), recombinant VEGF-C is mostly produced from propeptides-deleted mutant VEGF-C complementary DNA (cDNA) without the sequences coding for both propeptides (ΔNΔC-VEGF-C). Most commercial suppliers and scientific publications have used truncated cDNA to produce recombinant VEGF-C. However, thus produced pre-activated VEGF-C can differ N-terminally from the endogenously activated VEGF-C due to differences in the cleavage context of signal peptides (Künnapuu et al., 2021). We have observed that slight differences in the N-terminal sequences of different mature VEGF-C forms produced by different proteases can affect their affinity and activation potential toward VEGFR-2 and -3 (Study II). Hence, interpretation of the study results using recombinant VEGF-C can be facilitated only by N-terminal sequencing of the VEGF-C form used, which is usually not performed. Furthermore, after the identification of VEGF-C activating proteases (Jeltsch et al., 2014; Lim et al., 2019; McColl et al., 2003), it appears to be a mistake continuing to produce recombinant VEGF-C forms with no physiological counterpart. In addition, it should be noted that these pre-activated VEGF-C forms might not exist as a single species *in vivo*.

To date, recombinant VEGF-C has been produced for structural and functional studies, mostly using insect or mammalian expression systems (Jeltsch et al., 2006; Leppänen et al., 2010; Oh et al., 1997). Most often, cystine knot proteins tend to aggregate and form inclusion bodies when expressed in *Escherichia coli* cytoplasm (von Einem et al., 2010; de Marco, 2009; Tuan et al., 1996). However, angiogenic growth factors (VEGF-A, VEGF-B, and PlGF) have been purified from bacterial hosts from the inclusion bodies by solubilization and refolding (Christinger et al., 1996, 2004; Pizarro et al., 2010; Seyedarabi et al., 2013; Siemeister et al., 1996), but the exact refolding efficacies and conditions have not been reported (Iyer et al., 2001; Scrofani et al., 2000). Since VEGF-C, unlike VEGF-A, is one of the cysteine-richest long proteins (Leppänen et al., 2010), its production in a prokaryotic host is challenging due to the difficulty in the formation of correct disulfide bonds. VEGF-C produced in bacterial expression systems is offered by some suppliers but with a compromise in its bioactivity (BioVision, 2010). In addition, the presence of an unpaired cysteine residue Cys137 in the VHD of truncated cDNA leads to interference in proper disulfide bond formation (Chiu et al., 2014).

Various strategies have been utilized for promoting correct disulfide bond formation in *E. coli*, including co-expressing enzymes involved in disulfide bond formation and isomerization (e.g., CyDisCo system) (Hatahet et al., 2010; de Marco, 2009; Nguyen et al., 2011), changing the intracellular redox environment (e.g., *E. coli* strains Origami, AD494, or SHuffle) (Bessette et al., 1999), and/or utilizing the oxidizing environment of the periplasm

(Berkmen, 2012; Matos et al., 2014). However, there are no reports of successful attempts to produce active VEGF-C in *E. coli* without the need for refolding from inclusion bodies.

6. Phylogeny of VEGFs

VEGFs are crucial for angiogenesis and lymphangiogenesis in vertebrates (Risau, 1997; Takahashi and Shibuya, 2005). However, in several invertebrates, despite the lack of these two processes, PDGF/VEGF-like factors (PVFs) are present, suggesting a very early emergence of VEGFs during evolution (Kipryushina et al., 2015). The role of VEGF homologs in invertebrates remains largely unknown, and only a few have been studied functionally, including *Drosophila melanogaster* (*D. melanogaster*) PVFs (Heino et al., 2001; Read, 2018; Zheng et al., 2017) and *Caenorhabditis elegans* (*C. elegans*) PVF (Dalpe et al., 2013; Tarsitano et al., 2006). It is important to understand the ancestral roles or functions of VEGF homologs in less complex organisms in order to provide new insights into their possible roles in vertebrates. Most mammalian genes are part of gene families, the origin of which can often be traced back to whole genome duplications (WGDs) (Dehal and Boore, 2005; Kasahara et al., 2007). Earlier phylogenetic analyses showed that two major duplications in the vertebrate lineages led to the emergence of VEGF family members: two lineages evolving into VEGF-A, PlGF, and VEGF-B after the first duplication and VEGF-C and VEGF-D after the second duplication (He et al., 2014). The third whole genome duplication in the teleost lineage is responsible for multiple VEGF paralogs in most fish species, including zebrafish (Macqueen and Johnston, 2014).

Since VEGF-A was discovered first among all other VEGFs and was found crucial for blood vessel development, it is considered the prototype member of the VEGF family (Carmeliet et al., 1996; Ferrara et al., 1996). Since then, different VEGF homologs have been identified in different animals. Alignment of the VHD of VEGFs revealed that VEGFs and PDGFs are two separate branches of the PDGF/VEGF superfamily (Holmes and Zachary, 2005). There are very few studies describing the phylogenetic relationship between VEGF family members, each with some shortcomings (Dormer and Beck, 2005; He et al., 2014; Holmes and Zachary, 2005; Kasap, 2005; Kipryushina et al., 2015). Some older analyses suffer from limited data available at the time, while other study results lack adequate biological context. The existence of VEGF-E has been suggested by a single horizontal gene transfer from host to virus (Hughes et al., 2010). Further, in an analysis by He et al., pseudocowpox VEGF has been used as an outgroup to root the tree, which is questionable as pseudocowpox VEGF is likely a result of host-to-virus horizontal gene transfer (He et al., 2014; Lyttle et al., 1994).

7. Physiological and pathological lymphangiogenesis

Physiological lymphangiogenesis during embryonic development is crucial. However, the lymphatic vasculature in adults is in a quiescent state except in the female reproductive cycle, hair growth cycle, and intestinal lacteals, where tissue regeneration is required. In pathological conditions such as tumor metastasis, inflammation, and immune response, lymphangiogenesis is actively involved in disease progression, whereas lymphangiogenesis is insufficient, e.g., in lymphedema.

7.1 Tumor metastasis

Tumor metastasis is the major cause of cancer mortality, where tumor cells detach from the primary tumor and disseminate to regional lymph nodes and distant organs, mostly through blood and lymphatic vessels (Alitalo and Carmeliet, 2002; Podgrabinska and Skobe, 2014). The lymphatic route via the high endothelial venules in lymph nodes is easier for metastasis due to its greater permeability (Brown et al., 2018; Pereira et al., 2018). Generally, the lymphatic vessels located peritumorally and occasionally intratumorally are important for tumor metastasis (Leu et al., 2000; Padera et al., 2002). Sentinel and regional lymph node metastasis is a major indicator of disease progression in carcinoma patients (Stacker et al., 2014).

Several studies on experimental and human tumors indicate that tumor cells regulate the invasion of lymphatic vessels via the VEGF-C/VEGFR-3 signaling axis (Karpanen et al., 2001; Mandriota et al., 2001; Skobe et al., 2001b). There are multiple associations between high levels of VEGF-C and tumor-induced lymphangiogenesis and metastasis (Akagi et al., 2000; Karpanen et al., 2001; Kinoshita et al., 2001; Möbius et al., 2007; Skobe et al., 2001b; Tsurusaki et al., 1999). VEGF-C and VEGF-D, secreted by cancer cells and corresponding stromal cells like macrophages and fibroblasts, facilitate metastasis by stimulating lymphatic vessel sprouting at the tumor margin (Dieterich and Detmar, 2016; He et al., 2005). However, the association between VEGF-D and lymph node metastasis is unclear, with some tumors showing increased VEGF-D levels and others showing no correlation or downregulated VEGF-D (Ishikawa et al., 2003; Kawakami et al., 2003; Kurebayashi et al., 1999; Nakamura et al., 2003; O-charoenrat et al., 2001; White et al., 2002). Furthermore, upregulation of VEGFR-3 has been shown to be involved in tumor angiogenesis (Clarijs et al., 2002; Kubo et al., 2000; Partanen et al., 1999). Mice studies have shown that inhibiting VEGFR-3 signaling using blocking antibodies could be beneficial in suppressing VEGF-C-dependent tumor lymphangiogenesis and metastatic spread to the lymph nodes and even angiogenesis (Laakkonen et al., 2007; Rutkowski et al., 2013; Tammela et al., 2008). In addition, the use of soluble VEGFR-3 to block its interaction with VEGF-C in several mouse tumor models has been shown to decrease tumor lymphangiogenesis (Crnic et al., 2004; He et al., 2002; Karpanen et al., 2001; Krishnan et al., 2003).

In several tumor models, growth factors such as VEGF-A, hepatocyte growth factor, insulin-like growth factor, and angiopoietins are also involved in inducing tumor metastasis (Dieterich and Detmar, 2016). Additional upstream factors affecting the VEGF-C/D-VEGFR-3 axis have been shown to promote tumor lymphangiogenesis. Prostaglandins can affect the levels of VEGF-C in the tumor microenvironment (Su et al., 2004; Timoshenko et al., 2006). Erythropoietin and adrenomedullin can promote sentinel lymph node metastasis (Karpinich et al., 2013; Lee et al., 2011). Moreover, chemokines such as CCL21, secreted by LECs in response to increased VEGF-C/VEGFR-3 signaling in tumor cells, can stimulate the migration of tumor cells toward lymph vessels (Issa et al., 2009). Tumor-associated lymphangiogenesis by VEGF-C has been considered important for shaping the immune microenvironment of tumor cells. Studies on mouse models of melanomas have demonstrated that VEGF-C-induced tumor lymphangiogenesis increases the efficacy of immunotherapy. This beneficial effect was shown to be due to increased infiltration of naive T-cells in response to increased VEGF-C expression (Fankhauser et al., 2017).

7.2 Inflammation

Lymphangiogenesis plays a crucial role in inflammatory responses by regulating tissue fluid clearance and recruiting macrophages (Kim et al., 2014). Leukocytes are transported from the site of inflammation to the peripheral lymphoid organs via the lymphatic vessels. In inflammatory processes, lymphangiogenesis resolves tissue edema and promotes the mobilization of macrophages and dendritic cells into the afferent lymphatic vessels via the chemokine receptor CCR7 (Huggenberger et al., 2011; Kataru et al., 2009; Ohl et al., 2004). In addition, other receptors, such as common lymphatic endothelial and vascular endothelial receptor-1 (CLEVER-1) and mannose receptors, are shown to control inflammatory cell traffic in the lymphatic vessels (Irjala et al., 2001; Salmi et al., 2004).

Several studies indicate that inflammatory cells – mainly macrophages – express VEGF-C, VEGF-D, and VEGFRs and hence stimulate lymphangiogenesis in inflamed tissues (Mimura et al., 2001; Schoppmann et al., 2002; Skobe et al., 2001a). There are reports suggesting the ability of macrophages to transdifferentiate into LECs and incorporate into lymphatic endothelium, thereby inducing lymphangiogenesis (Kerjaschki, 2005; Kerjaschki et al., 2006; Maruyama et al., 2005). VEGF-C and VEGFR-3 were also found to be expressed by dendritic cells in the inflamed mouse cornea (Hamrah et al., 2003). VEGF-A was found to enhance VEGF-C-induced lymphangiogenesis by recruiting macrophages to the inflamed mouse cornea (Cursiefen et al., 2004). Depletion of intestinal monocyte/macrophages that express VEGF-C/D promotes intestinal inflammation (Becker et al., 2016). During inflammation, proinflammatory cytokines can upregulate VEGF-C levels and lymphatic vessel growth via NF-κB, a transcription factor that increases the production of inflammatory cytokines and chemokines (Ristimäki et al., 1998).

Lymphatic hyperplasia has been shown to be associated with human kidney transplant rejection, psoriatic lesions, chronic airway inflammation in a mouse model, and inflammatory neovascularization in a cornea model (Baluk et al., 2005b; Chen et al., 2004; Cursiefen et al., 2004; Hamrah et al., 2003; Kerjaschki et al., 2004; Kunstfeld et al., 2004). Blocking the VEGF-C/VEGFR-3 pathway using VEGFR-3 decoy prolongs mucosal edema in an oxazolone-induced hypersensitivity model (Huggenberger et al., 2011). Inhibiting pathological lymphangiogenesis with a VEGF-C/D trap has been shown to prevent graft rejection in heart and cornea transplants (Hos et al., 2015; Nykänen et al., 2010). On the other hand, overexpression or exogenous administration of VEGF-C stimulates inflammation clearance and halts the progression of pathogenesis in inflammatory bowel disease (D'Alessio et al., 2014; Huggenberger et al., 2010, 2011).

Lymphangiogenesis can exacerbate certain inflammatory conditions due to increased lymphatic drainage, which could increase systemic exposure to pathogens and inflammatory mediators, thereby eliciting an unwanted immune response. On the other hand, the beneficial effects of lymphangiogenesis in many inflammatory conditions could be due to improved lymph flow and decreased edema. Hence, therapeutic approaches modulating inflammatory lymphangiogenesis should be carefully designed considering the context of inflammation.

7.3 Lymphedema

Lymphedema is generally a chronic, debilitating pathological condition characterized by swelling of the extremities due to compromised lymphatic vessel function. Acute lymphedema may result from excessive fluid leakage from damaged veins in venous diseases like deep vein thrombosis and varicose veins, which overwhelms the lymphatic system. Insufficient lymphatic drainage results in the accumulation of ISF in tissues, fibrosis, inflammation, decreased immunity, and impaired wound healing. Lymphedema can etiologically be classified as primary or secondary lymphedema (Warren et al., 2007).

Primary or hereditary lymphedema is a rare congenital disorder resulting from mutations in genes responsible for lymphatic vessel development and function. To date, mutations in 28 genes have been identified, which explains about one-third of the primary lymphedema cases, either isolated or as part of a complex syndrome. Almost all of these genes encode proteins involved in the VEGFR-3 or its downstream signalings, such as *FLT4, VEGFC, CCBE1, ADAMTS3, FOXC2, PTPN11, SOX18, or GATA2* (Brouillard et al., 2014, 2017, 2021; Kazenwadel et al., 2015). The most common cause of hereditary lymphedema type 1A or Milroy's disease (MD) is missense kinase-inactivating mutations in the *FLT4* gene, resulting in decreased VEGFR-3 activity (Connell et al., 2009; Karkkainen et al., 2000). Mutation in the *VEGFC* gene is the cause of hereditary lymphedema type 1D (MD-like phenotype) (Gordon et al., 2013). MD patients exhibit chronic and disfiguring edema in the lower limbs, probably due to non-functional initial lymphatic vessels (Mellor et al., 2010).

Similar missense *VEGFR3* mutations in the germline of *Chy* mice have been studied for characterizing and treating human primary lymphedema (Karkkainen et al., 2001). However, these mice have lymphatic aplasia in the skin compared to MD patients with lymphatic hypoplasia. In addition to autosomal dominant mutations in *VEGFR3*, some de-novo VEGFR3 mutations have also been identified in MD patients without a family history of congenital lymphedema (Carver et al., 2007; Ghalamkarpour et al., 2006). MD-like phenotype has also been observed in a subset of patients with *VEGFC* mutations, due to defects in VEGF-C secretion or loss of function (Balboa-Beltran et al., 2014; Fastré et al., 2018; Gordon et al., 2013). Furthermore, mutations in *CCBE1, ADAMTS3, and FAT4* are causative of HKLLS, characterized by early-onset lymphedema, facial anomalies, intestinal lymphangiectasia, and neurocognitive impairments (Alders et al., 2009, 2014; Brouillard et al., 2017; Scheuerle et al., 2018). *FOXC2* mutations have been linked to pubertal onset Lymphedema-distichiasis syndrome (Brice et al., 2002; Petrova et al., 2004). Additionally, mutations in *KIF11, ITGA9, GJA1, PTPN14, IKBKG, RASA1, KRAS*, and several other genes have been identified to be involved in primary lymphedema (Aspelund et al., 2016; Grada and Phillips, 2017).

Secondary or acquired lymphedema is caused by damaged or obstructed lymphatics as a result of surgery, trauma, infection, therapeutic interventions such as radiation therapy, obesity, or prolonged inflammation. The most common form of secondary lymphedema globally is lymphatic filariasis (or elephantiasis), caused by infection with parasites, mainly *Wuchereria bancrofti.* The parasites cause scarring of lymph tissues, valve destruction, lymphangiectasis, and decreased lymphatic vessel contractility, permanently damaging the lymphatic system (Lourens and Ferrell, 2019; Pfarr et al., 2009; Shenoy, 2008). A type of tropical lymphedema, Podoconiosis, results from the interaction between different environmental and genetic factors. The most common cause is exposure to irritants in the volcanic red clay soil, which penetrate through the skin leading to inflammation and blockage of lymphatic drainage (Deribe et al., 2018). Breast cancer-associated lymphedema, which occurs after surgical excision of axillary lymph nodes during mastectomy combined with radiation therapy, is the most common form of lymphedema in developed countries (Cormier et al., 2010; Ozaslan and Kuru, 2004; Rockson, 2018).

Currently, there are very limited symptomatic treatment options, such as manual lymphatic drainage, bandage, exercise, and debulking surgeries. Curative treatment options are lacking except for lymph node transplants in patients with advanced secondary lymphedema (Gould et al., 2018; Thompson et al., 2021). A combination of prolymphangiogenic therapy (VEGF-C) with surgical interventions such as lymphaticovenous anastomosis (LVA) and vascularized lymph node transfer (VLNT) has been proposed for lymphedema treatment (Rockson, 2021). VEGF-C gene therapy (Lymfactin®) combined with VLNT was well tolerated in phase I clinical trial, reducing the excess arm volume in patients with breast

cancer-related upper-arm lymphedema (Leppäpuska et al., 2022). Although results from the phase II trial were inconclusive, it holds potential for lymphedema management.

8. VEGF-C as a therapeutic target

VEGF-C can be a drug target for diseases involving the lymphatics, such as lymphedema and cancer. However, it is important to note that the treatment of lymphedema asks for a diametrically opposing strategy (promoting VEGF-C action) (Figure 6a) compared to the inhibition of cancer metastasis (inhibiting VEGF-C action) (Figure 6b).

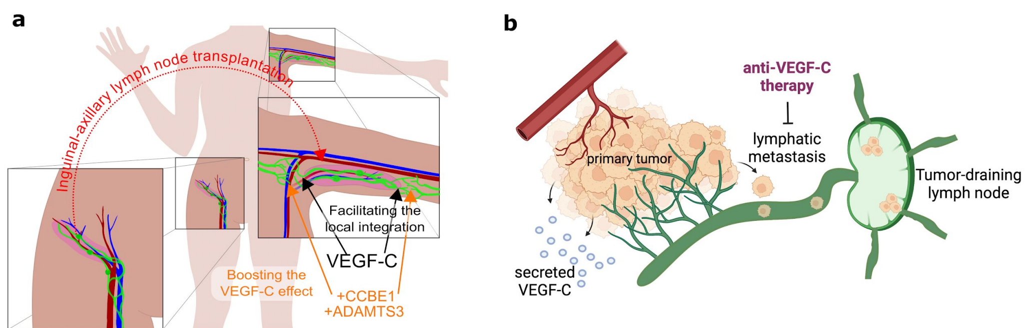

Figure 6. VEGF-C as a drug target. *(a) Promoting VEGF-C action in breast-cancer-associated lymphedema patients. (b) Blocking VEGF-C action to inhibit lymphatic vessels-mediated tumor metastasis.*

8.1 VEGF-C inhibition

Several pathological and clinical observations have suggested lymphatics as the most common pathway for initial metastasis in many solid tumors (Alitalo and Carmeliet, 2002). Studies have reported a positive correlation between the upregulation of VEGF-C expression and enhanced tumor metastasis in various experimental and human tumors (Achen et al., 2005; He et al., 2005; Stacker et al., 2002). Anti-lymphangiogenic therapy inhibiting the VEGF-C/VEGFR-3 axis has been considered in many preclinical studies for inhibiting metastatic spread through lymphatic vessels (Crnic et al., 2004; He et al., 2002; Karpanen et al., 2001; Krishnan et al., 2003; Laakkonen et al., 2007; Skobe et al., 2001b).

Soluble VEGFR-3 has been successfully demonstrated to function as a VEGF-C trap and block tumor metastasis to lymph nodes in various mouse tumor models. In addition, neutralizing antibodies against VEGFR-3 have also been shown to suppress metastasis (Persaud et al., 2004; Pytowski et al., 2005; Roberts et al., 2006; Tvorogov et al., 2010). However, clinical trials on inhibiting the VEGF-C/VEGFR-3 pathway have not been very

promising. A blocking antibody against VEGFR-3, IMC-3C5, had a minimal antitumor response in colorectal cancer patients in phase I clinical trial (Saif et al., 2016). Another phase I clinical trial evaluated the humanized monoclonal antibody against VEGF-C, VGX-100, in patients with solid tumors either as monotherapy or in combination with anti-VEGF-A antibody, bevacizumab (Falchook et al., 2014). The combination therapy showed modest antitumor activity, but further studies are warranted. Furthermore, an increase in VEGF-C expression in response to anti-VEGF-A therapy supports the use of combination therapy (Li et al., 2014). Currently, there are no clinically approved anti-lymphangiogenic therapies for inhibiting tumor lymphangiogenesis. However, there are several multi-targeted RTK inhibitors successfully used in the treatment of various cancers, e.g., imatinib, sorafenib, sunitinib, and pazopanib (Ferguson and Gray, 2018). Recently, an oral RTK inhibitor, anlotinib, that targets all VEGFRs, PDGFRs, FGFRs, and c-Kit, has been approved as a third-line treatment for refractory advanced non-small-cell lung cancer and second-line treatment for soft tissue sarcoma (Gao et al., 2020).

Additionally, combination therapies of soluble VEGFR-3, OPT-302, an inhibitor of VEGF-C/D, with ranibizumab or aflibercept (anti-VEGF therapies) were effective in the management of neovascular age-related macular degeneration, and phase 3 clinical trials, ShORe and COAST, have started (Opthea, 2021; Slakter et al., 2022). In addition, Phase 1b/2a clinical trial on combination therapy of OPT302 with aflibercept for the treatment of patients with diabetic macular edema was recently completed (Le et al., 2021). These ocular diseases result in loss of visual acuity due to the formation of edema caused by abnormal neoangiogenesis and neolymphangiogenesis. Hence, combination treatment with anti-VEGF-A/C/D therapies could provide additional benefits over the current standard of care (Gucciardo et al., 2020).

8.2 VEGF-C promotion

Prolymphangiogenic therapies using VEGF-C to treat secondary lymphedema have been explored in a large number of preclinical studies. Virus-mediated VEGF-C gene therapy was first observed to be effective for secondary lymphedema treatment in transgenic mice with *VEGFR3* inactivating mutation (Karkkainen et al., 2001). This observation was supported by several follow-up studies using recombinant VEGF-C, VEGF-C hydrogels combined with adipose-derived stem cells, naked plasmid, and adenoviral delivery of VEGF-C in various preclinical models of lymphedema (Hwang et al., 2011; Jin et al., 2009; Lähteenvuo et al., 2011; Szuba et al., 2002; Tammela et al., 2007b; Visuri et al., 2015; Yoon et al., 2003). Delivery of the VEGFR-3-specific VEGF-C isoform (VEGF-C_{C156S}) via adeno-associated viral vectors (AAV) has resulted in improved lymphangiogenesis without any effects on blood vessels in various porcine and mouse models (Saaristo et al., 2002; Visuri et al., 2015).

In addition, targeted antibody-mediated VEGF-C delivery, for e.g., human VEGF-C fused to F8 antibody (angiogenesis-marking extradomain A of fibronectin), and targeted VEGF-C-loaded nanoparticles in various mice models have led to improved lymphatic drainage and marked expansion of lymphatic vessels (Goodlett et al., 2021; Schwager et al., 2018). Furthermore, functional lymphatic regeneration was observed in animal models treated with VEGF-C incorporated in nanofibrillar collagen scaffolds (BioBridge™) alone or in conjunction with lymph node transplantation or adipose-derived stem cells (Hadamitzky et al., 2016; Nguyen et al., 2022). BioBridge™ implantation in patients who underwent VLNT and/or LVA increased the effectiveness of these physiologic procedures (Nguyen et al., 2021; Rochlin et al., 2020).

Following positive results from numerous preclinical trials, phase I and II clinical trials with Lymfactin® (an adenoviral type 5-based vector expressing human VEGF-C) were initiated (Hartiala et al., 2020a, 2020b). The drug was well-tolerated in the phase I trial; however, the final report of the phase II trial has not been published, except in the company press report where the study was announced to be inconclusive (Herantis Pharma Plc, 2021). In addition, a novel approach using nucleoside-modified VEGF-C mRNA encapsulated in lipid nanoparticles showed durable, organ-specific functional lymphangiogenesis in a mouse lymphedema model, further confirming the potential of VEGF-C therapy for lymphedema (Szőke et al., 2021). Recently, the Theralymph project has been initiated, which aims to develop innovative regenerative gene therapy using non-integrative multi-gene delivery LentiFlash® technology to restore lymphatic flow in breast cancer-associated lymphedema patients (Theralymph, 2020).

AIMS OF STUDY

This study aimed to explore the molecular regulators involved in VEGF-C activation and to determine the origin of VEGF-C using phylogenetic analyses.

The specific aims to achieve the above goals are as follows:

I. To explore the roles of N- and C-terminal domains of CCBE1 and VEGF-C, respectively.

II. To identify novel proteases involved in activating VEGF-C and to characterize the receptor binding and activation potential of differently activated VEGF-C forms.

III. To produce bioactive VEGF-C from *E. coli* by combining maltose-binding protein tag and redox-modified cytoplasm of Origami (DE3) strain.

IV. To determine the occurrence of PDGF and VEGF growth factors in the animal kingdom and to study the evolutionary relationship between them.

MATERIALS AND METHODS

Materials and methods used in this thesis are described in detail in the original articles. The summary of methods used is as follows:

Method	**Original article**
Mammalian cell culture, transfection, and stable cell line generation	**I, II**
Bacterial cell culture and transformation	**III**
AAV9 transduction of mice	**II**
Molecular cloning	**I, II, III**
Protein production and purification	**I, II, III**
Extracellular matrix binding assay	**I, II**
Metabolic labeling	**I, II**
Protein binding assay/ELISA	**I, II**
Mass spectrometry and N-terminal sequencing	**I, II**
Real-time quantitative PCR	**I, II**
Western blotting	**I, II, III**
Microscopy	**I, II**
Immunoprecipitation	**I, II, III**
Phosphorylation assay	**I, II, III**
Ba/F3-VEGFR-EpoR assay	**I, II, III**
Coomassie and silver staining	**I, II, III**
BLAST	**IV**
T-coffee 12.00 (Multiple sequence alignment tool)	**IV**

PhyML 3.0 (Tree building software)	**IV**
PAL2NAL (tool to convert protein alignments into mRNA alignments), HyPhy (detection of purifying selection/conservation), DIVAA (quantification of sequence diversity)	**IV**
Statistical analysis	**I, II, III, IV**

Brief description of methods

Phosphorylation assay

Cells expressing VEGFR-2 or VEGFR-3 were grown to near confluence and then serum-starved overnight. Cells were treated with recombinant proteins for 5-30 minutes, lysed with protease-inhibitor containing lysis buffer, and immunoprecipitated with the appropriate antibodies. Samples were resolved by SDS-PAGE and analyzed by western blotting/ECL.

Immunoprecipitation and Western blotting

For immunoprecipitation, cell lysates or supernatants were mixed with respective antibodies and protein A/G sepharose (GE Healthcare) overnight at 4°C. The protein A/G sepharose beads were washed several times and boiled with Laemmli sample buffer. The precipitated proteins were separated by SDS-PAGE and then transferred to membranes (polyvinylidene fluoride membranes or nitrocellulose). The membranes were incubated with appropriate primary and secondary antibodies. Finally, the imaging of the protein bands on the membrane was performed with Li-COR Odyssey Fc or cDigit Imaging system (Li-COR Biosciences), and protein quantification was done using Fiji ImageJ (NIH) or Image StudioLite (Li-COR Biosciences).

Ba/F3-VEGFR-EpoR assays

Ba/F3 cells (20,000 cells/well) stably expressing the VEGFR-3/EpoR chimera or VEGFR-2/EpoR chimera were added to a 96-well cell culture plate containing serial dilutions of samples (cell culture supernatants, lysates, or recombinant proteins). The cell viability was determined after 48 hours by adding MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (Sigma), 0.5 mg/ml). After two hours, cell lysis solution (10% SDS, 10 mM HCl) was added and incubated overnight at 37 °C. Finally, the absorbance was measured at 540 nm.

Cell culture, transfection, and stable cell line generation

Cells were grown in an appropriate medium with necessary supplements according to the available instructions. Cells were transfected using JetPei transfection reagent (Polypus-transfection Inc.) or Effectene (for S2 cells, Qiagen, Venlo, The Netherlands). For mammalian cells, the medium was changed to DMEM containing 0.2% BSA after 24 hours of transfection, and the conditioned supernatants/lysates were collected 48-72 hours after transfection.

For stable cell line generation, the appropriate antibiotic selection was started 48-72 hours after transfection and continued for 3-4 weeks. Clones were selected using the cloning ring method, or cell pools were selected and expanded. Expression of the protein of interest was confirmed by SDS-PAGE/Western blotting, and the stable cell lines were frozen for future use.

Protein production and purification

Recombinant proteins were produced from stably transfected mammalian cell lines (293T and CHO) and insect cell lines (S2 and Sf9), as well as from transformed bacterial cells (several *E. coli* strains). Affinity chromatography was used for the purification of the tagged proteins (Hexahistidine, StrepIII, or MBP tags), followed by size exclusion chromatography (SEC). Untagged full-length VEGF-C was purified using heparin affinity chromatography followed by cation exchange chromatography and SEC.

Metabolic labeling

Cells transfected with the desired expression constructs were metabolically labeled after 24 hours by adding [35S]-cysteine/[35S]-methionine (PerkinElmer, Waltham, MA). The samples (cell lysates and culture medium) were collected after 24 to 48 hours or after the appropriate chase time. The samples were resolved using SDS-PAGE after immunoprecipitation with the appropriate antibodies. The gels were vacuum dried and exposed to phosphoimager plates. The signals were visualized using a Typhoon 9400 scanner (Amersham Biosciences; GE Healthcare). Quantification of signals was performed using the ImageJ software (National Institutes of Health, Bethesda, MD).

Statistical analysis

The data obtained were analyzed using GraphPad Prism (versions 6, 7, 8, GraphPad Software Inc., USA). The significance of differences was determined using one-way ANOVA with Dunnett's or Tukey's multiple comparisons tests and unpaired Student's t-test. Data were considered statistically significant when the p-value was below 0.05. The results obtained were presented as mean±SD or mean±SEM.

RESULTS AND DISCUSSION

I. Different domains of CCBE1 and VEGF-C contribute to lymphangiogenic signaling by different mechanisms

The VEGF-C C-terminus has been compared to the heparin-binding domain of VEGF-A; however, it is unusually long and has less affinity towards heparin compared to VEGF-A. The role of the CT-propeptide in lymphatic development has been suggested by studies on a truncated VEGF-C that lacks the C-terminal domain (*vegfc*^{um18}) as well as identical mutations in Milroy-like lymphedema patients (Balboa-Beltran et al., 2014; Gordon et al., 2013; Villefranc et al., 2013). These mutants show secretion defects and hence do not completely imply all the functions of VEGF-C. Since the CT domain of VEGF-C is well conserved evolutionarily, we presumed that it might have some undiscovered roles.

Previous studies showing activation of pro-VEGF-C directly on the EC surface (Jeltsch et al., 2014) led us to investigate the association of different domain-deletion VEGF-C mutants to the ECM. To study this, we checked the binding of VEGF-C-CT (C-terminal domain of VEGF-C), VEGF-C-NT (N-terminal domain of VEGF-C), ΔNΔC-VEGF-C (VEGF-C lacking both N- and C-terminal domains), and pro-VEGF-C to ECM deposited by NIH-3T3 cells. Only pro-VEGF-C and VEGF-C-CT showed binding to the ECM, suggesting the role of the CT domain of VEGF-C in localizing pro-VEGF-C to the ECM. The most abundant ECM protein, fibronectin, is expressed by both BECs and LECs, and VEGF-A has been shown to bind fibronectin (Podgrabinska et al., 2002; Wijelath et al., 2006). Hence, we analyzed the binding of pro-VEGF-C to ECM proteins such as fibronectin and collagen I. Pro-VEGF-C bound to both ECM proteins, but the binding was concentration-dependent only for fibronectin. The bound VEGF-C could be released from the ECM when incubated with ADAMTS3 or heparin. Studies using a CT cleavage-resistant VEGF-C mutant have shown that CCBE1-assisted ADAMTS3 cleavage of the NT domain of VEGF-C does not require complete removal of its CT domain (Bui et al., 2016). Hence, our hypothesis was that efficient activation of VEGF-C requires localization to the ECM and cell surface, which is mediated by the CT propeptide.

Further, we conducted *in vivo* studies to explore the lymphangiogenic potential of the CT domain of VEGF-C. We produced transgenic mice overexpressing either VEGF-C-CT or VEGF-C-ΔC (VEGF-C without the C-terminus) in the basal keratinocytes of the epidermis using the keratin 14 (K14) promoter. As expected, K14-VEGF-C-ΔC mice showed lymphatic hyperplasia when compared to wild-type (WT) mice. However, in K14-VEGF-C-CT mice, the number of lymphatic capillaries was decreased compared to the WT mice, which was unexpected. Surprisingly, K14-VEGF-C-ΔC x K14-VEGF-C-CT double transgenic mice showed the highest lymphatic hyperplasia, suggesting a role of the CT domain in inducing

efficient lymphangiogenesis. To confirm these findings, we used recombinant VEGF-C-CT and VEGF-C-ΔC to perform a bioassay that measures the survival of Ba/F3 cells expressing a chimeric VEGFR-3/EpoR receptor mediated by ligand binding. VEGF-C-CT showed almost complete inhibition of VEGFR-3 activation in this *in vitro* assay, complementing the *in vivo* results. Hence, we assumed that inhibition of VEGF-C activation by the CT domain of VEGF-C most probably occurs due to the competition with the CT domain in pro-VEGF-C during cleavage complex assembly. Further complementation assays showed that VEGF-C-ΔC, when expressed alone, results in decreased VEGF-C activation but coexpression with the CT domain of VEGF-C lifts the activation block resulting in an increased amount of mature VEGF-C. This suggests that the presence of the CT domain of VEGF-C is required for cleavage of the NT domain of VEGF-C to generate mature active VEGF-C.

Since our observations indicated that VEGF-C activation occurs on the cell surface, we checked the localization of CCBE1 and ADAMTS3, the obligatory components of the VEGF-C cleavage complex. CCBE1 expression has been shown in PROX1-positive human dermal LECs (Facucho-Oliveira et al., 2011; Hasselhof et al., 2016); however, it is expressed near the developing vessels, and not ECs, during early developmental stages (Bos et al., 2011). We confirmed that CCBE1 is primarily located on the LECs surface using stainings on mouse tissues and also at the protein and mRNA levels. We could also confirm the presence of ADAMTS3 in LECs at the mRNA level. Hence, the localization of VEGF-C, CCBE1, and ADAMTS3 to the cell surface hints at the requirement of this trimeric complex for activating VEGF-C efficiently. Some studies have also shown the association between CCBE1 and the ECM component, vitronectin (Bos et al., 2011). This association might be required to provide stability to the trimeric complex by increasing the local concentration of CCBE1. In addition, coreceptors such as Nrp2, β1-integrin, and syndecan-4 present on the surface of LECs could also stabilize the trimeric complex (Jeltsch et al., 2014; Johns et al., 2016; Zhang et al., 2005).

Furthermore, using solid-phase binding assays, we showed that CCBE1 could bind to the VEGF-C-binding domains of VEGFR-3. Studies have shown the role of EGF domains of CCBE1 in regulating VEGF-C-mediated VEGFR-3 signaling and in establishing guidance cues for LECs (Roukens et al., 2015). Due to the interaction of both VEGF-C and CCBE1 with VEGFR-3, we investigated their interplay using plain PAE cells and PAE cells expressing VEGFR-3. Stimulation of these cells with pro-VEGF-C in the presence of the NT domain of CCBE1 (CCBE1-175) decreased pro-VEGF-C level in the conditioned supernatant of both cells, suggesting the role of CCBE1-175 in redistributing pro-VEGF-C from soluble phase to cell surfaces. Sequestration of pro-VEGF-C to the cell surface in plain PAE cells describes the binding of pro-VEGF-C to the HSPGs. In addition, recombinant CCBE1-175 increased pro-VEGF-C effects on VEGFR-3 activation. Surprisingly, we observed competition between CCBE1 and pro-VEGF-C for VEGFR-3 binding. Whether

VEGF-C acts in a non-directional manner or forms a gradient likely depends on the presence of CCBE1 and ADAMTS3. VEGF-C activation on LEC surfaces would be responsible for a non-directional signal, whereas ECM-bound VEGF-C activation could provide a growth factor gradient, which has been proposed to be important for functional network formation and patterning. This instructional gradient is absent in mice lacking the NT domain of CCBE1, resulting in unorganized networks (Roukens et al., 2015). In our cell-based studies, we used PAE and Ba/F3 cells that express low amounts of endogenous ADAMTS3. However, in NIH-3T3 cells expressing VEGFR-3, which do not express endogenous ADAMTS3, there was no increase in receptor phosphorylation by pro-VEGF-C, even in the presence of the NT-domain of CCBE1. Hence, the presence of ADAMTS3 is necessary for VEGF-C activation by CCBE1.

Furthermore, we studied the properties of a heterozygous *Adamts3* R565Q mutant which was discovered in a lymphedema patient. This missense mutation is located in the TSP-1 motif of ADAMTS3, which is well-conserved in ADAMTS family members and is responsible for the cell surface association of ADAMTS3 (Tortorella et al., 2000). We could not detect any direct negative effect of this mutant on the activation of pro-VEGF-C. ADAMTS13 mutation (R398H) is responsible for congenital thrombotic thrombocytopenic purpura by preventing the cleavage of von Willebrand factor (Levy et al., 2001). Since studies have shown an interaction between ADAMTS3 and CCBE1 (Jeltsch et al., 2014), we hypothesized that disturbance in this interaction could be possible for the mutant ADAMTS3 effects. When we co-transfected plasmids expressing WT or mutant ADAMTS3 and CCBE1 in 293T cells, we could detect a dramatically reduced association between mutant ADAMTS3 and CCBE1 as compared to WT ADAMTS3. We showed an increase in CCBE1 amounts in the supernatant of transfected cells in the presence of mutant ADAMTS3. This observation is likely due to the decrease in interaction between CCBE1 and mutant ADAMTS3 on the cell surface, resulting in a shift of cell surface-bound CCBE1 to the supernatant. Studies have shown that the C-terminal domain of CCBE1 has chondroitin sulfate modifications (Bui et al., 2016). Hence, our findings on the R565Q mutant suggest that altered cell surface localization might contribute to the disease mechanism. However, since this mutant is heterozygous and cannot completely block the interaction between ADAMTS3 and CCBE1, it is unlikely to explain the lymphedema phenotype independently.

Based on current experimental evidence, we have suggested a model for the activation of VEGF-C (Figure 7) in which all three components of the VEGF-C/ADAMTS3/CCBE1 trimeric cleavage complex are crucial. Pro-VEGF-C should be mobilized to stimulate lymphangiogenesis. Although pro-VEGF-C activation can occur in the soluble phase (Bui et al., 2016), the co-localization of pro-VEGF-C, ADAMTS3, and CCBE1 suggests a major proportion of VEGF-C activation takes place in the ECM and on the cell surface. Since pro-VEGF-C and CCBE1 are bound to the ECM, pro-VEGF-C activation can occur in the ECM in the presence of protease/CCBE1 complex. The mature VEGF-C thus released from

the ECM, which still has a relatively low affinity for the ECM, might be important for gradient formation required for further directing and organizing the lymphangiogenic response. Pro-VEGF-C activation on the cell surface can occur when bound to the VEGFR-3 or HSPGs (Jeltsch et al., 2014; Johns et al., 2016). Activation of VEGFR-3-bound VEGF-C can provide immediate signaling, whereas HSPG-bound VEGF-C has to be translocated to the VEGFR-3 to stimulate signaling. Hence, we suggest that VEGF-C activation in different locations might be necessary for determining the migration versus proliferation/survival-promoting effects of VEGF-C. Delineating the complexity of VEGF-C activation is necessary to target VEGF-C therapeutically for diseases involving the lymphatics, such as lymphedema and cancer (as explained in the section 'VEGF-C as a therapeutic target').

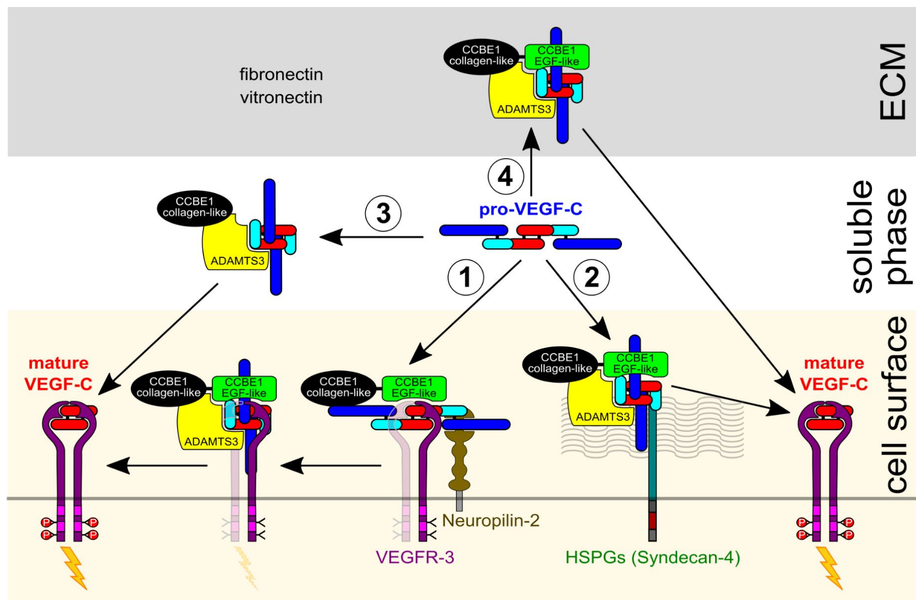

Figure 7. Four different models for VEGF-C activation. (1 and 2) VEGF-C activation on the cell surface (VEGFR-3 bound or HSPG-bound VEGF-C). (3) VEGF-C activation in the soluble phase, and (4) VEGF-C activation in the ECM (fibronectin-bound VEGF-C). *Adapted from Study I.*

II. Identification of novel VEGF-C and VEGF-D activating proteases

It is well established that ADAMTS3 protease is responsible for the activation of pro-VEGF-C during embryonic development (Janssen et al., 2016; Jeltsch et al., 2014). However, ADAMTS3 has a very restricted expression pattern after birth, and a recent study has identified ADAMTS2 and ADAMTS14 as VEGF-C activating proteases during adulthood (Dupont et al., 2022). In contrast, proteases activating VEGF-D remained unknown despite featuring a proteolytic activation very similar to VEGF-C (Bui et al., 2016; Stacker et al., 1999). Plasmin has been shown to activate VEGF-C and VEGF-D *in vitro*, but it can be safely assumed that plasmin is not a physiological activator (McColl et al., 2003). VEGF-C was originally cloned from prostate-derived cell line PC-3 (Joukov et al., 1996), and high levels of VEGF-C are expressed by several other prostate cancer cell lines (Jennbacken et al., 2005). A study by Matsumura et al., using a peptide library scan, predicted VEGF-C as KLK4 substrate, but the prediction remained unvalidated (Matsumura et al., 2005). In addition, kallikrein-related peptidases, mainly KLK3, are abundantly expressed by the prostate epithelium (Shaw and Diamandis, 2007). Hence, based on these observations, we investigated the ability of multiple KLKs to activate VEGF-C.

We found that the major kallikrein-related peptidase in human semen, KLK3 (also known as PSA), is able to specifically activate both VEGF-C and VEGF-D. KLK3-processed VEGF-C and VEGF-D were biologically active, as shown by cell-based assays using Ba/F3-VEGFR-3/EpoR and Ba/F3-VEGFR-2/EpoR cells. Edman degradation of the KLK3-activated VEGF-C revealed a cleavage site between Tyr-114 and Asn-115, resulting in a unique VEGF-C species. The KLK3-cleaved VEGF-C is three amino acids shorter at the N-terminus compared to the active VEGF-C produced by ADAMTS3 cleavage (Figure 8). Furthermore, we found that the amino acid sequences surrounding the ADAMTS3 and KLK3 cleavage sites (^{108}KFAA↓AHY↓N^{115}) are 100% conserved in mammals and birds during evolution.

Figure 8. VEGF-C and VEGF-D cleavage sites of different proteases. *KLK3 cleaves hVEGF-C between Tyr-114 and Asn-115 and hVEGF-D between Tyr-94 and Asp-95.*

Cathepsin D cleaves hVEGF-C between Leu-119 and Lys-120 and hVEGF-D between Leu-99 and Lys-100.

KLK3 is mainly responsible for seminal clot liquefaction by cleaving seminogelins and has a role in reproduction (Robert et al., 1997). Hence, we hypothesized that KLK3-cleaved VEGF-C could have a possible role in reproductive biology. VEGF-A has been detected in human seminal plasma and is considered important by some researchers for implantation and sperm motility (Brown et al., 1995; Iyibozkurt et al., 2009; Obermair et al., 1999; Torry et al., 2007). Studies have also shown the expression of VEGFR-2 and VEGFR-1 by spermatozoa, suggesting their role in sperm function (Obermair et al., 1999). In our study, we could detect significant amounts of both pro-VEGF-C and mature VEGF-C in human seminal plasma. The amounts of mature VEGF-C increased in liquefied seminal plasma, suggesting activation of pro-VEGF-C by KLK3 during the liquefaction process. Furthermore, the activation of VEGF-C increased in acidic pH, indicating the possibility of VEGF-C activation in an acidic vaginal environment. The lymphangiogenic effect of VEGF-C has been shown to be important for ovarian follicle maturation and uterine implantation (Red-Horse, 2008; Rutkowski et al., 2013). We also investigated the potential of seminal VEGF-C for receptor binding and activation. Seminal VEGF-C had a weaker binding affinity to VEGFR-2 compared to VEGFR-3, and it could phosphorylate VEGFR-3, suggesting the presence of an active VEGF-C species. However, we could not detect VEGF-D in the human seminal plasma, which could also result from insensitive anti-VEGF-D antibodies to detect such low amounts. In contrast, we could show substantial amounts of CCBE1 in human seminal plasma, similar to a seminal plasma proteome study (Jodar et al., 2016). We further showed that KLK3-mediated VEGF-C activation is enhanced by CCBE1, providing additional significance of CCBE1 in the activation of VEGF-C.

Seminal plasma also contains TGFβ1, which gets activated by KLK14 during liquefaction, similar to VEGF-C (Emami and Diamandis, 2010). Seminal TGFβ1 has a crucial role in immunomodulation and uterine implantation (Robertson et al., 2002). Similarly, seminal VEGF-C might have a role in modulating the immune response, as several studies show that VEGF-C is expressed by the inflammatory cells recruited by seminal plasma in the uterus postcoitus (Hamrah et al., 2003; Kalkunte et al., 2009; Krebs et al., 2012). However, the absence of KLK3 and KLK3 orthologs in mice makes it difficult to address the function of KLK3-activated VEGF-C in seminal plasma (Pavlopoulou et al., 2010).

Several studies have argued about the involvement of KLK3 in cancer progression; however, there is no clear evidence in favor of or against the cancer-promoting effect of KLK3 (Ishii et al., 2004; LeBeau et al., 2010; Mattsson et al., 2008; Peternac et al., 2006). Its expression levels seem to increase in the early stages of prostate cancer, whereas it might slow down prostate cancer progression at the later stages (Magdolen et al., 2012). Likewise, the expression of VEGF-C in prostate cancer cells is also controversial, with both supporting and

opposing studies (Jennbacken et al., 2005; Mori et al., 2010; Yang et al., 2014). In this study, we provide a link between KLK3, VEGF-C, and cancer progression.

To explain the weaker affinity of seminal VEGF-C towards VEGFR-2 compared to VEGFR-3, we studied the effect of partial/complete removal of the NT helix of VEGF-C and VEGF-D on receptor binding and activation. Studies have shown that incomplete removal of the NT helix of VEGF-D (minor mature VEGF-D, ^{100}KVID→) dramatically decreases its binding affinity towards VEGFR-3, but VEGFR-2 binding is unaffected (Leppänen et al., 2011). However, complete removal of the NT helix of VEGF-C (obtained by secondary plasmin cleavage, ^{126}WR↓KT^{129}) renders it inactive (Jeltsch et al., 2014). Hence, we cloned a putative VEGF-C form (^{120}KSID→) corresponding to the minor mature VEGF-D form (VEGF-C_{DMH} for 'D Minor Homology') and characterized its receptor binding and activation potential. VEGF-C_{DMH} purified from S2 cells had a low affinity to VEGFR-2 but bound strongly to VEGFR-3. Furthermore, we compared the receptor binding potential of VEGF-C_{DMH} and different N-terminally truncated VEGF-C species obtained by ADAMTS3, KLK3, and plasmin cleavage. Progressive shortening of the N-terminal helix resulted in decreased affinity of VEGF-C towards its receptors, preferentially towards VEGFR-2.

We then searched for additional VEGF-C activating proteases in other bodily fluids, including saliva. Using ion exchange chromatography, we concentrated the VEGF-C activating components of human saliva and subjected the most active fractions to mass spectrometric analysis. Among the top hits, we considered cathepsin D as a possible candidate due to its cleavage context (L↑K) matching VEGF-C_{DMH}. Recombinant cathepsin D was able to cleave both pro-VEGF-C and pro-VEGF-D, and the resulting cathepsin D-cleaved VEGF-C/D activated both VEGFR-2 and VEGFR-3. In addition, cathepsin D could cleave the minor mature VEGF-C and major mature VEGF-D forms (secondary activation). However, the secondary activation decreased the affinity of VEGF-C towards VEGFR-2 and VEGF-D towards both VEGFR-2 and VEGFR-3. This ability of cathepsin D to modulate the affinity of VEGF-C/D towards their receptors adds a layer of complexity to VEGF-C/D signaling. More interestingly, cathepsin D-mediated VEGF-D activation was much more rapid compared to VEGF-C activation, suggesting VEGF-D activation as the more relevant function of cathepsin D. Furthermore, the cathepsin D-cleaved VEGF-C_{DMH} form was not detected in transfected 293T cell supernatants, most probably due to the removal of the sequences necessary for cathepsin D recognition by endogenous ADAMTS3 in these cells.

Additionally, we studied the *in vivo* effects of active VEGF-C species produced by KLK3 and cathepsin-D cleavage to confirm the *in vitro* results. We transduced the mouse tibialis anterior muscle with recombinant AAV9 vectors expressing deletion mutants corresponding to differentially cleaved VEGF-C forms. As expected, these novel VEGF-C forms stimulated angiogenesis and lymphangiogenesis, compatible with the binding and receptor phosphorylation results. KLK3-cleaved VEGF-C showed a stronger response compared to

cathepsin-D-cleaved VEGF-C, confirming the requirement of the N-terminal helix of VEGF-C for efficient receptor activation. In our study, we show that progressive shortening of the NT helix affects the affinity of VEGF-C for its receptors, and complete removal of the NT helix by extended plasmin cleavage abolishes its activity.

Several studies have investigated the role of cathepsin-D as a cancer biomarker and its mitogenic role in cancer metastasis. Cathepsin D has been shown to increase tumor invasion, metastasis, and angiogenesis, and increased cathepsin D levels have been linked to the risk of breast cancer recurrence. Hence, cathepsin-D inhibition has been a target for cancer therapy (Glondu et al., 2002). The overlapping expression of cathepsin-D and VEGF-C in cancer metastasis and the ability of cathepsin-D to cleave VEGF-C/D suggests a possible mechanism for tumor progression via lymphatic metastasis. However, extensive gene-targeted mouse model studies are necessary to validate this hypothesis.

The bottom line from this study suggests that different proteases might activate VEGF-C for specific niche functions, such as ADAMTS3 for developmental lymphangiogenesis, KLK3 for reproduction, and KLK3 and cathepsin D for tumor metastasis. Further studies are required to establish the physiological and pathological significance of KLK3 and cathepsin-D in VEGF-C activation.

III. Production of bioactive VEGF-C from *E. coli*

Prokaryotic expression hosts have been widely used for the production of recombinant proteins due to fast turnaround times and cost-effectiveness, ease of use, and relatively higher yields compared to mammalian cells. However, the lack of post-translational modifications, such as glycosylation, limits its use in some instances (Rosano and Ceccarelli, 2014). Not all eukaryotic proteins are easy to produce in *E. coli*. Notably, cystine knot proteins do not fold correctly in the unmodified *E. coli* cytoplasm. Barriers to successful cystine knot protein production in *E. coli* are the absence of enzymes catalyzing the formation and isomerization of disulfide bonds and the unfavorable redox environment (von Einem et al., 2010; de Marco, 2009). VEGF-C is one of the cysteine-richest long proteins (> 400 aa) in the human proteome, with a cysteine content between 7.2 and 8.6% (Leppänen et al., 2010). Attempts to produce active VEGF-C in the cytoplasm of *E. coli* have mostly resulted in the formation of inclusion bodies. Although other members of the VEGF family have been produced in *E. coli* by solubilization and refolding from inclusion bodies (Christinger et al., 1996, 2004; Seyedarabi et al., 2013), reported refolding efficacies are typically very low (Iyer et al., 2001; Scrofani et al., 2000). For lymphangiogenic VEGFs, only eukaryotic expression systems have been used for protein production (Jeltsch et al., 2006; Leppänen et al., 2010, 2011; Oh et al., 1997), which further shows the difficulties in producing VEGF-C from *E. coli*.

Bacterial VEGF-C from truncated cDNA is commercially available, but its biological activities are low and not well-documented (BioVision, 2010). The low biological activity might be explained by the unpaired cysteine residue in the VHD of VEGF-C from truncated cDNA, which is thought to impede correct disulfide bond formation (Chiu et al., 2014). In our study, we observed a difference in the rate of mammalian VEGF-C secretion when VEGF-C was produced from full-length versus truncated cDNA. The CT domain of VEGF-C, which is cysteine-rich, appeared to be responsible for the slower secretion of full-length VEGF-C. Bacterial VEGF-C produced from both full-length and truncated cDNA resulted in inclusion body formation, similar to other cystine-knot proteins (von Einem et al., 2010; de Marco, 2009). There are two major approaches to ensure the proper folding of cysteine-rich proteins in bacterial cytoplasm: co-expressing enzymes required for disulfide bond formation and isomerization (Hatahet et al., 2010; Nguyen et al., 2011) and modifying the redox environment of the bacterial cytoplasm (Bessette et al., 1999). In our study, we tested several combinational approaches, of which one approach was successful to produce bioactive VEGF-C from *E. coli* without the need for refolding from inclusion bodies.

VEGF-C contains two glycosylation sites in the VHD, and before using *E. coli* for VEGF-C production, we analyzed whether the lack of glycosylation might affect the receptor binding of VEGF-C. We produced single and double glycosylation mutants of VEGF-C and checked their binding to VEGFR-2 and VEGFR-3. Both single glycosylation mutants retained their receptor binding affinity; however, double glycosylated mutants were not expressed, similar to other secreted proteins (Rasmussen, 1992). Many studies have successfully produced secreted cystine-rich proteins in *E. coli* using protein disulfide isomerases (PDIs) for correct disulfide bond formation (Hatahet et al., 2010; Nguyen et al., 2011). However, our attempts failed to produce bacterial VEGF-C with PDI assistance, using the CyDisCo expression system, which co-expresses Erv1 (a sulfhydryl oxidase) and human PDI for oxidative protein folding and isomerization, respectively (Nguyen et al., 2011). This failure could be because of the unusually high probability of forming incorrect inter- and intramolecular disulfide bonds with 18 cysteines in the VEGF-C dimer. Our attempts to produce VEGF-C by targeting it to the oxidizing environment of periplasm also failed, probably due to very high cystine content. This approach has been used successfully for smaller proteins with relatively fewer disulfide bonds (Berkmen, 2012; Dagar et al., 2017; Matos et al., 2014).

We then used two approaches in parallel: using a solubility enhancing tag such as maltose binding protein (MBP) fused to VEGF-C (Lebendiker and Danieli, 2017) and refolding from solubilized inclusion bodies. MBP-tagged VEGF-C expressed in *E. coli* strain BL21 could be seen in the soluble cytoplasmic fraction, but it was inactive. On the other hand, we could obtain bioactive VEGF-C by refolding solubilized inclusion bodies. We optimized the folding conditions using successive screens.

Interestingly, MBP-tagged VEGF-C, when expressed in the cytoplasm of redox-modified *E. coli* strain Origami (DE3), was biologically active. The Origami strain contains a mutation in

both glutathione reductase and thioredoxin reductase, thereby providing a more favorable environment for correct disulfide bond formation in VEGF-C. Four different forms of MBP-tagged VEGF-C (minimal, major, and minor mature VEGF-C forms and FL-VEGF-C) were produced in the *E. coli* Origami strain. All three mature VEGF-C forms were biologically active in the Ba/F3-VEGFR-3/EpoR assay. Since MBP-tagged minimal mature VEGF-C showed the strongest activity, this form was used for further characterization. Minimal mature VEGF-C also stimulated phosphorylation of both VEGFR-2 and VEGFR-3 expressed by PAE cells. Surprisingly, untagged mature forms of VEGF-C were also active when expressed in the *E. coli* Origami strain. However, their activity was minimal when compared to the MBP-tagged VEGF-C forms.

Unfortunately, purification of MBP-tagged minimal mature VEGF-C became challenging due to the extensive proteolytic degradation in the *E. coli* Origami strain. Proteolytic processing of the hexahistidine tag from MBP-VEGF-C fusion protein was apparently responsible for the low yield of active VEGF-C purified using Ni affinity chromatography. Despite the absence of a hexahistidine tag, the endogenous affinity of VEGF-C to the Ni sepharose, as shown previously for VEGF-A (Mohanraj et al., 1995), allowed us to purify very limited amounts of VEGF-C. The majority of the active VEGF-C remained unpurified in the flowthrough, which showed almost the same level of activity as the lysate. Compared to IMAC, we could increase the purification efficiency 6-fold using amylose affinity chromatography, but nevertheless, the yield remained low. The use of protease inhibitors during protein production and purification steps in the Origami strain did not rescue the degradation of hexahistidine and MBP tags.

The purified fraction with the highest activity contained a partially cleaved MBP tag fused to VEGF-C, as observed by the size of the fraction in SEC and SDS-PAGE/Western blotting. We also analyzed whether the removal of the MBP tag would increase the activity of purified mature VEGF-C. Cleavage of the MBP tag from the fusion protein using TEV protease did not increase the bioactivity of VEGF-C as compared to MBP-tagged VEGF-C. The N-terminal MBP tag did not interfere with VEGFR-3 binding or activation, which can be explained by the X-ray structure of VEGF-C in complex with VEGFR-2/3 (Leppänen et al., 2010, 2013). The X-ray structure of the complex supports the idea that the N-terminal MBP tag would point away from the receptor, whereas the C-terminal end of the VHD would point towards the cell surface and hence interfere with the receptor binding (Jeltsch et al., 2014).

The total yield of purified active VEGF-C remained low in our study due to the inefficient binding of the proteolytically processed VEGF-C fusion protein to both Ni sepharose and amylose resins. We believe that optimization of the purification methods, e.g., using ion-exchange chromatography or immobilized VEGFR-3, would increase the recovery of VEGF-C from the bacterial lysates. In addition, combinational approaches such as co-expressing PDIs in the Origami strain or using eukaryotic chaperones that are known to aid in protein folding (Kase et al., 2010; Ozawa et al., 2001) could also be explored to

improve the yield since the proteins might be most sensitive to degradation in the unfolded state.

This study describes for the first time a method to produce biologically active VEGF-C from *E. coli* using a combination of redox-modified Origami strain and maltose binding protein (MBP) tag. Such bacterial VEGF-C could be a readily available cost- and time-effective source of VEGF-C for *in vitro* applications, such as LECs culture.

IV. Phylogenetic analysis of the PDGF/VEGF growth factor family

VEGFs and PDGFs together form a highly conserved subgroup of cystine-knot growth factors, mainly required for blood and lymphatic vascular systems (Vitt et al., 2001). However, invertebrates such as *C. elegans,* which lack vascular systems, also have PVFs. Animal models such as mice have been extensively used in biomedical research since more than 98% of mouse genes have corresponding human orthologs (Mural et al., 2002). In this study, we explored the prerequisites for studying PDGF/VEGFs by performing a comprehensive analysis of their occurrence in all animal clades. The evolutionary relationships between the members of the PDGF/VEGF growth factor family have only been addressed by some older analyses, which exhibit limitations due to insufficient sequence data available at that time (Dormer and Beck, 2005; He et al., 2014; Holmes and Zachary, 2005; Kasap, 2005; Kipryushina et al., 2015). We have proposed a likely phylogenetic tree based on our analyses and provided some useful insights into the evolutionary aspects of the PDGF/VEGF family.

We searched for PDGF/VEGF homologs in the NCBI database by combining 13 query sequences with 52 animal clades. The majority of the resulting blast hits (90.5%) were programmatically classified as PDGF/VEGF family members, and the remaining hits were manually classified. The sampling bias in underrepresented clades with limited sequence data was made clear by considering the total number of animal species, sequenced genomes, and protein sequences in each animal clade. A summary of the results is shown in Figure 9. Our results, together with the phylogenetic tree of the animal kingdom, predict the emergence of the first PDGF/VEGF-like molecule (proto-PDGF/VEGF) before the deuterostome/protostome split (DPS), predating the Cambrian, about 540 MYA. We identified that the simplest animals with PDGF/VEGF are Cnidaria and compared their amino acid sequences with human VEGFs, PDGFs, and invertebrate PVFs. Surprisingly, all but one Cnidarian VEGFs contained long NT and CT domains with three to five BRP3 motif repeats at the CT end of the VHD, which is characteristic of the present-day lymphangiogenic growth factors VEGF-C and VEGF-D. However, cysteine residues forming

the intermolecular disulfide bonds in mammalian PDGFs/VEGFs were often absent, similar to the invertebrate PVFs. This result challenges the commonly held opinion about VEGF-A being the VEGF prototype.

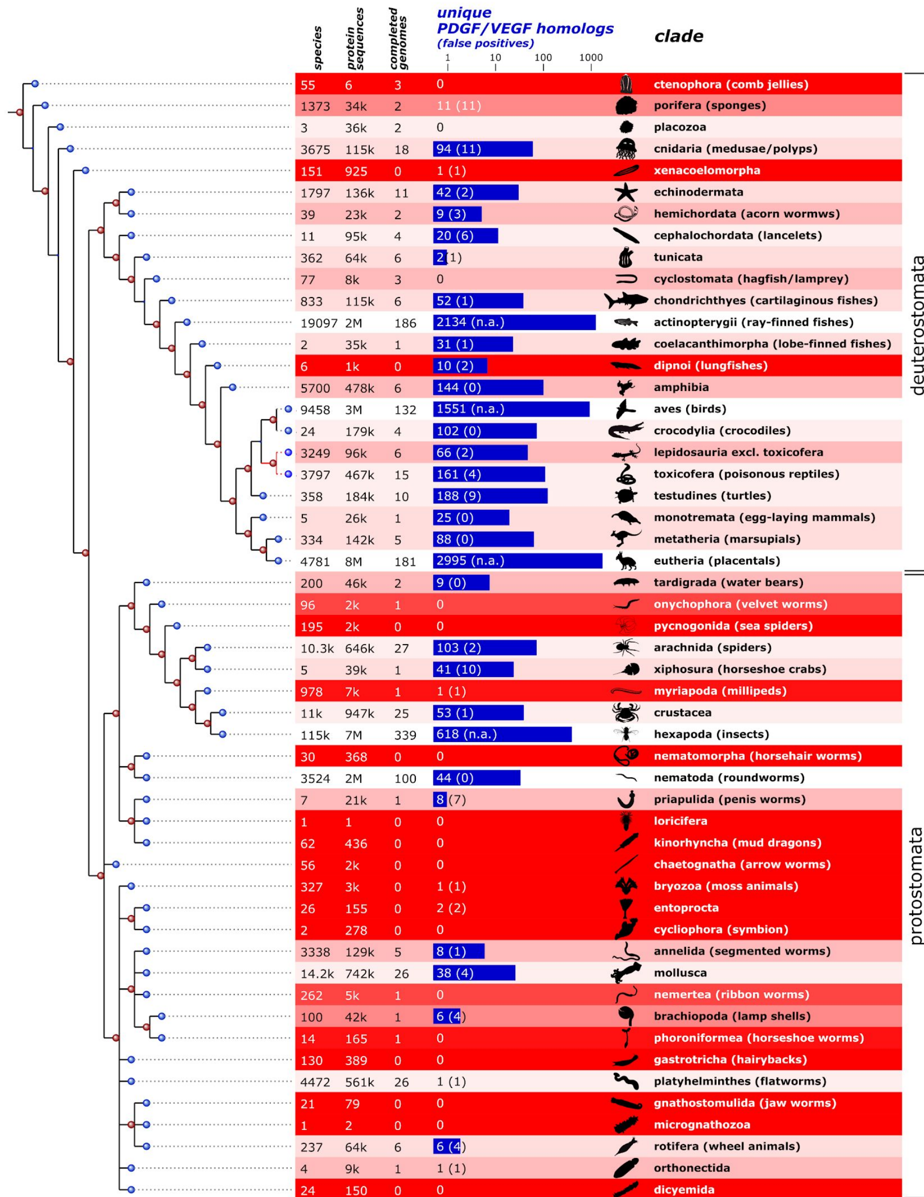

Figure 9. Quantitative representation of the PDGF/VEGF-like blast hits from 52 animal clades. *The total number of blast hits in each clade is indicated in blue, and false positive hits are represented in parentheses. False positives were manually excluded only in clades with <500 species. Most protostome phyla were underrepresented in the NCBI sequence databases. The darkness of the red color indicates the reliability of the analysis results, with darker being less reliable. On the left is the consensus tree of life aligned with the animal clades. Adapted from Study IV.*

The three WGDs only partially explain the emergence of novel PDGF/VEGF family members (Dehal and Boore, 2005; Glasauer and Neuhauss, 2014) (Figure 10). The diversification of proto-PDGF/VEGF likely occurred after the DPS since the diversification pattern is prominent only in the deuterostome branch. Species like Echinodermata that diverged soon after the DPS already contain both VEGF-A-like (proto-VEGFA) and VEGF-C-like (proto-VEGFC) proteins, suggesting that the first diversification event took place before the first vertebrate whole genome duplication (VGD1). In addition, proto-PDGF also emerged before VGD1 and became established in the cephalochordate branch prior to its divergence. This is in line with the function of PDGF in the stabilization of blood vessels, as cephalochordates have a pressurized vascular system (Hellström et al., 1999). VGD1 must be responsible for a proto-VEGFA and proto-VEGFC duplication, but there is no evidence that the duplicated genes were established in the genome. In contrast, VGD1 likely established the two PDGF subgroups from proto-PDGF duplication.

Duplication of the proto-VEGFC and proto-VEGFA genes likely occurred during VGD2, resulting in the VEGF-C/VEGF-D subfamily and VEGF-A/PlGF/VEGF-B subfamily, respectively. The most parsimonious explanation for the emergence of more than two members in the subfamily soon after the VGD2 is a limited duplication (VEGF-B/PlGF duplication) in the common ancestors of all Actinopterygii. Similarly, but much more recently, a limited duplication in the common ancestor of all Lepidosauria is likely responsible for the emergence of VEGF-F. The third WGD occurred in the common ancestors of the teleost fish lineage 350 MYA (Christoffels et al., 2004), presumably resulting in ten *vegf* and eight *pdgf* genes. We could detect at least 13 functional *pdgf/vegf* genes in the most researched teleost, i.e., in zebrafish. Salmonids underwent one additional WGD 88 MYA (Macqueen and Johnston, 2014), theoretically resulting in 36 different *pdgf/vegf* genes. However, all these genes are not anymore active today. For the salmonid *Salmo trutta,* we could identify 26 active *pdgf/vegf* genes using the Ensemble gene prediction pipeline. For 21 of these genes, we identified the mRNA transcripts from the PhyloFish mRNA database (Pasquier et al., 2016).

Since the first proto-PDGF/VEGF diversification took place before the DPS, the PVFs in the protostome branch are difficult to classify. Genomic assemblies for only six invertebrate phyla were found in significant numbers to draw any conclusions, and all of them except flatworms feature one (nematodes) or more (insects, mollusks, segmented worms) *Pvf* genes.

Interestingly, these *Pvf* genes are evolutionarily conserved until today. For instance, *C. elegans* PVF1 is able to activate human VEGFR-1 and VEGFR-2 (Tarsitano et al., 2006). In addition, PVFs commonly appear in most genome-sequenced species of mollusks, crustaceans, insects, and spiders. Most protostome animals, except mollusks and segmented worms (Annelida), lack a cardiovascular system. There are very limited defined biological roles of invertebrate PVFs in vascular development. The hemolymph system of Drosophila is considered as an open vascular system and it has been shown that Drosophila PVF1 is important for blood cell migration (Kipryushina et al., 2015).

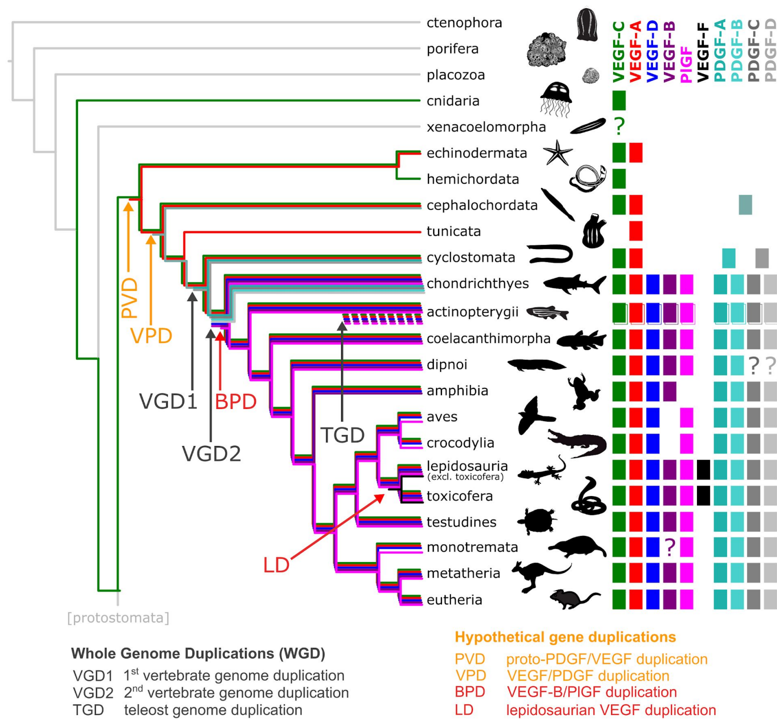

Figure 10. Existence of PDGF/VEGF family members in the deuterostomes. *The three WGDs only partially explain the emergence of PDGF/VEGF genes. Several limited gene duplication events explain the emergence of PlGF, VEGF-B (PlGF/VEGFB duplication), and VEGF-F (Lepidosauria duplication). Lineage-specific gene loss events explain the absence of PlGF, VEGF-B, and VEGF-C genes in Amphibia, Archosauria (aves, crocodylia), and Tunicata, respectively. Adapted from Study IV.*

On the other hand, all the animals in the deuterostome branch contained VEGF-A- and VEGF-C-like proteins except Tunicata. The reduction in the body plan complexity in Tunicata might be responsible for the loss of VEGF-C-like sequences and an overall

reduction in the VEGF paralogs (Chang et al., 2015). Strikingly, some members of the VEGF family showed lineage-specific gene loss, even without a reduction in the body plan complexity. For instance, PlGF orthologs are absent in Amphibia, and VEGF-B orthologs are absent in Archosauria, which includes extant birds, crocodiles, and dinosaurs. We found protein sequences in birds annotated as 'VEGF-B', but they were wrongly annotated in the database as they lack the characteristic exon-intron structure and overlapping reading frames of VEGF-B (Olofsson et al., 1996a). Our phylogenetic analysis shows that they are closer to VEGF-A (or PlGF) than to any VEGF-B. Secondary gene loss events of PlGF and VEGF-B might have been similarly well tolerated during evolution as they are tolerated when experimentally performed in mice (Aase et al., 2001; Bellomo et al., 2000). The precise role of VEGF-B in mammals remains controversial (Li et al., 2012), and the loss of VEGF-B in birds during evolution might have been beneficial to provide high metabolic turnover during flight. However, despite their relative redundancy in mice, these genes have been conserved over 500 MYA, which demands an explanation.

As a counterpoint to the absence of PlGF and VEGF-B orthologs in amphibians and birds, we found that VEGF-F has a more widespread occurrence than generally thought. Since VEGF-F was discovered as a venom component of vipers (Komori et al., 1999), the general impression was that VEGF-F is found only in venomous reptiles. Surprisingly, we identified VEGF-F in non-venomous reptiles such as lizards and gekkos. Hence, we believe that VEGF-F gene duplication likely occurred early in the lepidosaurian lineage before venom was invented. However, the reason for proto-VEGF-F emergence and persistence in gekkos and lizards and its function in this non-viper branch of the VEGF-F tree is unknown.

Some viruses have captured VEGFs (VEGF-E) from their respective hosts in a single host-to-virus gene transfer event during viral evolution (Lyttle et al., 1994). We found VEGF-like sequences in the genomes of the orf virus, bovine pustular stomatitis virus, pseudocowpoxvirus, and megalocytivirus. In our phylogenetic tree, VEGF-E clusters were well separated from the vertebrate VEGFs, suggesting no recent host-to-virus gene transfer. Our analysis of all VEGF-E sequences suggested their single origin from VEGF-A due to sequence homology. Although the host range of these viruses is non-overlapping (parapoxviruses infect mammals and megalocytiviruses infect fish) (Haller et al., 2014; Subramaniam et al., 2012), all viral VEGF-Es likely originate from a single acquisition, probably from a mammalian host.

The occurrence of VEGFs is considerably heterogeneous among fishes. Both bony and cartilaginous fish feature the same five VEGF family members as mammals, while jawless fish lack PlGF, VEGF-B, and VEGF-D orthologs. Recent advancements in the understanding of developmental pathways of zebrafish vasculature (Das et al., 2022) led us to inspect pseudogenization and duplications of PDGF/VEGF genes in and outside the teleost lineage. We analyzed the RNAseq data in the FishPhylo database to investigate how well the PDGF/VEGF ohnologs withstood inactivation/pseudogenization. Despite the heterogeneity,

we could identify PDGF/VEGF ohnologs in most fishes, except for *vegfbb* in the teleost lineage and *pdgfba* in five out of six salmonid species. Salmonids were found to maintain some of the PDGF/VEGF ohnologs originating after Salmonid genome duplication. Interestingly, Holostei fish contained two conserved *vegfc* genes, indicating either an individual gene duplication event or persistence of the VEGF-C gene duplication from VGD2. VEGF-C gene duplications were frequently seen among several fish species. We detected strong purifying selection, mainly in the receptor binding domain of VEGF-C, followed by the silk homology domain (SHD). It is interesting to note that despite being very long, the ECM-binding SHD has been conserved until today. In addition to orchestrating the proteolytic activation of VEGF-C (study I), one further potential function of the SHD might be to keep VEGF-C inactive by steric hindrance. Other possible role of the SHD might be in the establishment of VEGF-C gradients by interacting with the ECM, similar to VEGF-A (Ruhrberg et al., 2002), which is thought to be important for developmental lymphangiogenesis. Diversity in the PDGF/VEGF gene family of fishes makes fish models (e.g., zebrafish model) difficult to interpret but also provides room for discovering morphological and physiological differences unknown in terrestrial vertebrates.

CONCLUDING REMARKS

The studies included in my thesis mainly focus on the origin and activation of the primary lymphangiogenic growth factor VEGF-C. We established the functions of both C- and N-terminal domains of VEGF-C and CCBE1. Our findings explain the critical role of ECM binding of VEGF-C for its function and the formation of a trimeric cleavage complex (VEGF-C/ADAMTS3/CCBE1) for efficient VEGF-C activation. We have also proposed a model for VEGF-C activation based on our results, which could be utilized for exploring the combinational therapeutic approaches to target VEGF-C. Furthermore, we identified novel VEGF-C/D activating proteases, KLK3/PSA and cathepsin D, which are likely required for the niche-specific functions of VEGF-C. We suggest a physiological role of KLK3-activated VEGF-C in reproduction and pathological roles of KLK3- and cathepsin-D-activated VEGF-C in tumor progression. It would be interesting to test these hypotheses in relevant animal models. This could help to expand the number of indications for VEGF-C targeting drugs.

In addition, we have developed for the first time a method to produce biologically active VEGF-C from *E. coli*, which would provide a readily available source for *in vitro* VEGF-C research due to its time- and cost-effectiveness. Our final study on the expansion and collapse of PDGF/VEGF growth factors in all animal clades has provided new insights into the evolutionary pathways and origin of these growth factors. With this study, we have challenged a few commonly held opinions, such as VEGF-A being the VEGF prototype, VEGF-B being important for cardiac neovascularization, and VEGF-F being limited to the viper family. We show that VEGF-C is the phylogenetically oldest VEGF, that VEGF-B is absent in birds and crocodiles, and that VEGF-F can be found in non-venomous reptiles such as geckos and lizards. Such information is valuable when choosing an animal model for vascular biology research, but it immediately raises further questions: E.g., What might be the purpose of VEGF-F in non-venomous reptiles? Why did birds and crocodiles lose VEGF-B while it was maintained in mammals?

Advancements in the field of lymphatic research have gained momentum in the last decades. However, there are still gaps in understanding the mechanistic details of lymphangiogenesis, which restricts identifying new targets for therapeutic purposes. Our study results could be utilized to plan future studies that enable efficient therapeutic targeting of VEGF-C.

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