CCBE1 enhances lymphangiogenesis via ADAMTS3-mediated VEGF-C processing

Master's Thesis

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Abstract

Lymphangiogenesis is the process that leads to the formation of lymphatic vessels from pre-existing vessels. Vascular endothelial growth factor C (VEGF-C), the major lymphangiogenic growth factor, is produced as an inactive precursor and needs to be proteolytically processed into a mature form in order to activate its receptors VEGFR-3 and VEGFR-2. A deficiency of VEGF-C during embryonic lymphangiogenesis results in embryonic lethality due to the lack of lymphatic vasculature. Hennekam lymphangiectasia-lymphedema syndrome (OMIM 235510) is in a subset of patients associated with mutations in the collagen- and calcium-binding EGF domains 1 (*CCBE1*) gene. CCBE1 and VEGF-C act at the same stage during embryonic lymphangiogenesis and their deficiency results in similar lymphatic defects. The mechanism behind the lymphatic phenotype caused by *CCBE1* mutations is unknown. The aim of this study was to investigate the potential link between VEGF-C and CCBE1 that could contribute to the lymphatic phenotype.

In this study, 293T cells were used to observe the effect of CCBE1 on VEGF-C processing. The co-transfection of constructs coding for CCBE1 and VEGF-C showed processing of the inactive pro-VEGF-C into the active, mature form. However, this processing was efficient only in 293T cells. When CCBE1 from 293T supernatant was purified, A disintegrin and metalloproteinase with thrombospondin type 1 motif 3 (ADAMTS3) co-purified with CCBE1. The levels of pro-VEGF-C and active VEGF-C were monitored by immunoblotting or immunoprecipitating metabolically labeled supernatant with specific antibodies or receptors followed by autoradiography. The activity of the processed VEGF-C was verified by proliferation of Ba/F3 cells stably expressing VEGFR-3/EpoR or VEGFR-2/EpoR chimeras. Furthermore, a VEGFR-3 phosphorylation assay was performed in PAE (Porcine Aortic Endothelial) cells to study details of the CCBE1-mediated regulation of VEGF-C.

We found that CCBE1 increases the proteolytic processing of pro-VEGF-C, thereby resulting in increased activity of VEGF-C. CCBE1 itself has no effect on VEGF-C activity but regulates VEGF-C by modulating the activity of the ADAMTS3 protease. We also found that both pro- and mature- VEGF-C can bind to VEGFR-3 but only mature form is able to induce VEGFR-3-mediated signaling. In addi-

tion to cleaving VEGF-C, ADAMTS3 was found to directly or indirectly mediate CCBE1 cleavage. The N-terminal amino acid sequence of the ADAMTS3-processed VEGF-C confirmed that ADAMTS3 is the protease responsible for the activation of VEGF-C by 293 cells. Hence, we have identified a mechanism that regulates VEGF-C activity. This mechanism suggests the possible use of CCBE1 as a therapeutic means to treat diseases that involve the lymphatic system.

Keywords: VEGF-C, CCBE1, ADAMTS3, lymphangiogenesis, endothelium.

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Acronyms

ADAMTS3 A disintegrin and metalloproteinase with thrombospondin type 1 motif-3

Ang1 Angiopoietin 1

Ang2 Angiopoietin 2

BEC Blood vascular endothelial cell

BSA Bovine serum albumin

CCBE1 Collagen and calcium binding EGF domain 1

COUP-TFII Chicken ovalbumin upstream promoter-transcription factor II

EC Endothelial cell

Efnb2 Ephrin B2

Foxc2 Fork head box C2

HIF Hypoxia inducible factor

HUVEC Human umbillical vein endothelial cell

IgG Immunoglobulin G

IL-3 Interleukin-3

LPS Lipopolysaccharide

LEC Lymphatic endothelial cell

mRNA messenger RNA

Nrp Neuropilin

NFATc1 Nuclear factor of activated T cell

PBS Phosphate buffered saline

PlGF Placental growth factor

PDGF Platelet-derived growth factor

PAGE Polyacrylamide gel electrophoresis

Pdpn Podoplanin

PAE Porcine aortic endothelium

Prox1 Prospero-related homeobox domain 1

SMS Smooth muscle cell

TLR Toll-like receptor

TGF Transforming growth factor

TBS Tris buffered saline

VEGF Vascular endothelial growth factor

VEGF-C Vascular endothelial growth factor C

VEGFR Vascular endothelial growth factor receptor

VHD VEGF homology domain

1 Introduction

1.1 The vascular system

The primary mode of gas and nutrient transport in primitive animals is diffusion. In vertebrates, the vascular system is required for the transport of nutrients, gases, cells, hormones, metabolic waste products and fluids throughout the body. The mammalian vascular system includes vessels carrying blood - the circulatory system - and vessels carrying lymph - the lymphatic vascular system.

The circulatory system comprises a regular and closed network of arteries, veins and capillaries. Arteries and veins mainly carry the blood, whereas in the capillaries, the exchange of matter between blood and tissues takes place (Pugsley and Tabrizchi, 2000).

In contrast, the lymphatic vascular system is an open network of lymphatic capillaries, collecting vessels, the thoracic duct and interspersed lymph nodes (Jussila and Alitalo, 2002).

1.1.1 The structure of the lymphatic system

Lymph originates in the thin-walled lymphatic capillaries by draining the interstitial spaces. It flows through the collecting vessels and finally enters the subclavian vein to re-join the blood circulation. The vascular tissues of the human body are well equipped with lymphatic capillaries (Schmid-Schönbein, 1990), but avascular tissues like the cornea and cartilage together with a few vascular tissues like the brain and bone marrow lack lymphatic vessels (Oliver and Detmar, 2002). Lymphatic capillaries are composed of a single layer of overlapping lymphatic endothelial cells (LECs), which are attached to the adjacent extracellular matrix via filaments, which are involved in the regulation of fluid entry into the lumen and which are operated by interstitial pressure (Leak and Burke, 1966, 1968). The lumen of lymphatic capillaries is larger compared to the lumen of blood capillaries. The lymph flow in the collecting lymphatic vessels is maintained by contractions from the smooth muscle cells (SMCs) surrounding the lymphatic vessel, but normal physiological processes like respiration and skeletal muscle movement make important contributions to the

lymph propulsion. Valves in the lymphatic vessels prevent the back-flow of lymph (Alitalo et al., 2005).

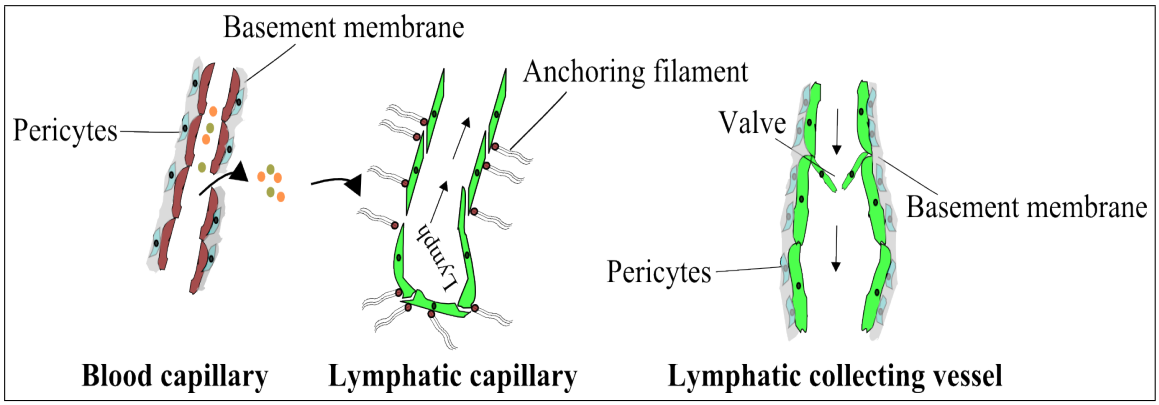

Figure 1: **The structure of lymphatic vessels**. Blood capillaries feature a continuous basement membrane and pericytes but lymphatic capillaries lack both. The lumina of lymphatic capillaries are larger than those of blood capillaries. This facilitates fluid and macromolecule entry in response to high interstitial pressure. Lymphatic collecting vessels contain a basement membrane, pericytes, smooth muscle cells and luminal valves. Smooth muscle cells propel the lymph and the valves prevent the backflow of the lymph. Modified from Norrmén et al., 2011.

Lymphatic vessels have accessory organs; the lymphoid organs. There are three types of lymphoid organs. Primary lymphoid organs are the thymus and the bone marrow and function in the formation and maturation of lymphocytes. Secondary lymphoid organs are spleen, lymph nodes, appendix, tonsils and payer's patches; and they have critical roles in modulating the immune response (Margaris and Black, 2012). Tertiary lymphoid organs are organized structures formed from aggregates of B and T-cells in response to chronic inflammation and are structurally similar to the lymph nodes (Neyt et al., 2012). The lymph nodes are complex organs in which macrophages, lymphocytes and dendritic cells interact with each other and encounter invading pathogens before they enter the blood circulation (Ohtani and Ohtani, 2008; Swartz, 2001).

1.1.2 The function of the lymphatic system

One of the important functions of the lymphatic system is to maintain tissue fluid homeostasis. The lymphatic capillaries take up surplus interstitial fluid, which has

leaked from the blood vessels due to the blood pressure. This fluid is then transported back into the blood vascular system (Casley-Smith, 1985).

Beside its role in tissue fluid homeostasis, the lymphatic system has well-established roles in the immune defense where T cells and B cells encounter antigen in coordination with antigen presenting cells at the lymph node; thereafter transmitting the immune encountered cells to the systemic circulation (Kesler et al., 2013; Mackay et al., 1990). One example of innate immune defense orchestrated by the lymphatic system occurs through Toll-like receptor (TLR-4) signaling. LECs express high levels of TLR-4, which can be activated by lipopolysacharides (LPS). This results in the generation of chemokines that recruit macrophages, which induce lymphangiogenesis by secreting the lymphatic specific growth factors VEGF-C and -D (Kang et al., 2009). Furthermore, emerging evidence of the lymphatic system's role in inflammatory responses by modulating lymphangiogenic factors VEGF-C and VEGFR-3 has encouraged researchers to develop therapeutic strategies targeting the lymphatics to control chronic inflammations (Huggenberger et al., 2010).

Besides the role in fluid homeostasis and immune defense, the lymphatic system plays a third, important function in the digestive tract. Intestinal absorption of dietary lipids in the form of chylomicrons is the role of specialized lymphatic vessels in the small intestine called lacteals (Choi et al., 2012).

1.2 Lymphangiogenesis

1.2.1 Development of the lymphatic system

The development of the lymphatic system in the mouse starts at E10.5, much later than the development of the cardiovascular system, which starts around E6.5-7 (Wigle and Oliver, 1999). Two opposing theories were proposed in the beginning of the 20th century. The centrifugal sprouting theory by Sabin advocates the formation of lymphatic vessel from primary lymph sacs, which itself originate from embryonic veins (Sabin, 1902), whereas the centripetal sprouting theory by McClure and Huntington proposes lymph sac formation without a blood vascular origin (Huntington and McClure, 1910). However, results have been published in support of both centrifugal (Dumont et al., 1998; Hägerling et al., 2013; Srinivasan et al., 2007; Wigle

and Oliver, 1999; Wigle et al., 2002) and centripetal sprouting (Papoutsi et al., 2001; Schneider et al., 1999). Interestingly, a *Xenopus laevis* based genetic model suggests that both centripetal and centrifugal processes can coexist in the development of the lymphatic vasculature. The mode of development of the lymphatic vasculature appears to depend on the model organism being studied (Ny et al., 2005).

1.2.2 Lymphatic endothelial cell specification

Transcription factors like Prox1, CoupTFII, Sox18 and FoxC2 play vital roles in the lymphatic vasculature development; the first three being important in specification of the lymphatic fate while the latter has a role in the formation of lymphatic valves (François et al., 2011; Petrova et al., 2004).

Prospero-Related Homeobox Domain 1 (Prox1), a specific marker for LECs, is an important factor in deciding the identity of LECs and later in the migration of LECs, but it is not required for the initial budding of endothelial cells from the anterior cardinal veins. *Prox1* null embryos lack lymph sacs and hence have no lymphatic vasculature (Wigle and Oliver, 1999). They don't show any significant expression of lymphatic markers but show expression of BEC-specific markers (Wigle et al., 2002). This was consistent with the results obtained from two independent studies overexpressing Prox1 in primary blood vascular endothelial cells. The results indicated upregulation of various lymphatic specific genes and downregulation of blood vascular specific genes supporting the notion that Prox1 could reprogram blood vascular endothelial cells to resemble lymphatic endothelial cells (Hong et al., 2002; Johnson et al., 2008; Kim et al., 2010).

Sox-18 is required for the differentiation of lymphatic endothelial cells from the cardinal vein and also induces the expression of Prox1 along with other lymphatic markers (François et al., 2008). Similarly, conditional deletion of *Nr2f2* in mice during the early stage of lymphatic development restricts lymph sac formation and is essential during later stage of lymphatic development to maintain LECs identity. The gene product of *Nr2f2*, COUP-TFII, exerts its function by regulating the expression of neuropilin2 (Nrp2), a co-receptor for VEGF-C (Tsai et al., 2010). Additionally, COUP-TFII forms homodimers resulting in the continuation of the venous identity, while COUP-TFII heterodimerization with Prox1 appears to be

involved in the differentiation into the lymphatic direction (Aranguren et al., 2013).

Apart from the importance of transcription factors in deciding the lymphatic fate, NOTCH1 expression tightly regulates the specification of BECs into LECs through the suppression of Prox1 and upregulation of blood vasculature-specific VEGFR-2 and Nrp1 (Kang et al., 2010). It has also been shown that inhibition of NOTCH signaling, both in vivo and in vitro, promotes lymphangiogenesis (Zheng et al., 2011).

1.2.3 Regulation of the expansion of the lymphatic vascular network

The co-localization of VEGF-C and its receptor VEGFR-3 during the process of lymphatic development suggested a role of the VEGFR-C/VEGFR-3 signaling axis in the regulation of the lymphatic vasculature development (Kukk et al., 1996). Studies of *Vegfc* -/- embryos highlighted the role of VEGF-C in the budding and migration of LECs from the anterior cardinal vein. VEGF-C deletion prevents the migration of LECs thereby resulting in a complete lack of the lymphatic vasculature. VEGF-C apparently has no role in the specification of the lymphatic fate (Hägerling et al., 2013; Kärkkäinen et al., 2004). This was consistent with the role of VEGF-C in the lymphatic development of zebra fish (Küchler et al., 2006).

The expansion of the lymphatic vascular network is mostly dependent upon VEGF-C/VEGFR-3 signaling (Mäkinen et al., 2001b) and interstitial pressure (Planas-Paz et al., 2011). The use of VEGF-C traps like soluble VEGFR3/IgG-Fc chimeras in mouse embryos after E14.5 results in hypoplastic lymphatic vessels (Mäkinen et al., 2001b). Also in adults, VEGF-C seems to be the major stimulant for lymphangiogenesis (Kärpanen and Alitalo, 2008). Surgical removal of lymph nodes in the treatment of malignant tumors has been a common practice, which frequently results in lymphedema as a side effect. The adenoviral delivery of VEGF-C/VEGF-D to mice with removed axillary lymph node and lymphatic vessels resulted in a regain of functional lymphatic vessels, demonstrating one of the therapeutic strategies to treat lymphedema conditions in adults (Tammela et al., 2007).

Neuropilin-2 (Nrp2), a co-receptor for VEGF-C, is expressed by ECs of veins and lymphatic vessels. *Nrp2* mutant mice show defects in the growth of small lymphatic vessels and capillaries during development (Yuan et al., 2002). This was later sup-

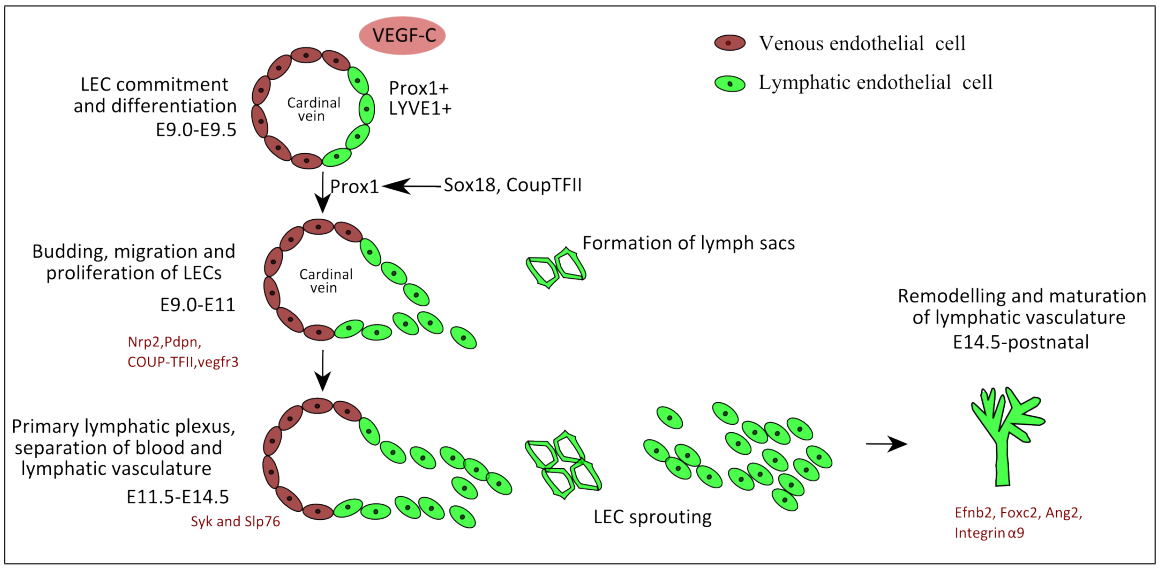

Figure 2: **The development of lymphatic vessels**. COUP-TFII and Sox18, expressed in the venous endothelial cells, stimulate Prox1 expression and hence lymphatic fate specification. The separation of LECs from venous endothelial cells requires SH2 domain containing leukocyte protein of 76kDa (slp76) and spleen tyrosine kinase (syk). The differentiating LECs form the primary lymph sacs after VEGF-C stimulated emigration from their venous origin. Important molecules involved in the later stages of lymphatic development are neuropilin 2 (Nrp2), forkhead box C2 (Foxc2), podoplanin (Pdpn), angiopoietin 2 (Ang2), ephrin B2 (Efnb2) and integrin $\alpha 9$. Figure modified from Alitalo et al., 2005; Oliver and Srinivasan, 2010 and Tammela and Alitalo, 2010.

ported by an independent experiment where blocking of Nrp2 binding to VEGF-C prevented sprouting of endothelial cells during the lymphatic development. The lymphangiogenic activity of Nrp2 is accomplished by its interaction with VEGFR-3 when stimulated by VEGF-C (Xu et al., 2010). The stimulation of human lymphatic endothelial cells with VEGF-C/VEGF-D leads to the endocytosis of VEGFR-3 and Nrp2, probably regulating the receptor based signaling process (Kärpanen et al., 2006).

1.2.4 Remodeling and maturation of lymphatic vessels

Several proteins have been identified that assist in the process of remodeling and maturation. The forkhead transcription factor Foxc2 is expressed in LECs during

the development of lymphatic vessels, and mutations in *FOXC2* are associated with lymphedema-distichiasis syndrome in humans. The disease is characterized by lower limb lymphedema and distichiasis (a double row of eye lashes; Dagenias et al., 2004) . The study of *Foxc2* -/- mice revealed defects in lymphatic vascular patterning and a role of Foxc2 in keeping the lymphatic capillary network free of pericytes and SMCs (Petrova et al., 2004). Later, another study described the interaction between Foxc2 and NFATc1 (Nuclear factor of activated T cells) downstream of VEGFR-3 signaling being responsible for lymphatic vessel maturation; thus supporting the earlier evidence (Kulkarni et al., 2009).

The role of TGFβ (transforming growth factor β) in lymphatic vascular development is not yet clear. One of the functions of TGFβ could be to enhance lymphatic sprouting and lymphatic network formation, resulting in enhanced lymphangiogenesis as shown in the skin of mice during development (James et al., 2013). A mutation in the C-terminal PDZ domain of ephrinB2, the ligand for ephrin type-B receptor 2 tyrosine kinase, results in a failure of lymphatic vessel remodeling and lymphatic luminal valve formation (Mäkinen et al., 2005).

Additionally, the role of Ang/Tie signaling in lymphatic development has been studied in detail. The endothelial cell specific tyrosine receptors, Tie1 and Tie2 are expressed by lymphatic endothelial cells (D'Amico et al., 2010; Saharinen et al., 2005). *Tie1* -/- mice show defects in the lymph sac patterning and edema formation (D'Amico et al., 2010). *Ang2* -/- mice show defects in the formation of large collecting lymphatic vessel and poor association with SMCs; highlighting the role of Ang2 in maturation of lymphatic vessels during the later stages of embryonic development (Gale et al., 2002). Ang1, the ligand for Tie2, can induce lymphangiogenesis in vivo in adult mice (Tammela et al., 2005), but can also rescue the phenotype from *Ang2* -/- mice (Gale et al., 2002).

The development of the lymphatic system is a complex process with probably many unidentified players and mechanisms.

1.2.5 Lymphangiogenesis in adults

Lymphatic endothelial cells in adults are normally quiescent. The roles and mechanisms of angiogenesis in adults are well understood but lymphangiogenesis in adults

is less well understood.

Adult lymphangiogenesis is involved in tumor metastasis, which will be discussed in the section *Tumor lymphangiogenesis*.

1.3 Molecular regulation of lymphangiogenesis

The assembly of lymphatic endothelial cells into lymphatic vessels requires a balance between VEGF-C and VEGFR-3 along with other regulatory factors and processes. Hence, it is interesting to study regulatory factors, which could play vital roles in the physiology of lymphangiogenesis.

1.3.1 Vascular endothelial growth factors

The mammalian VEGF gene family consists of 5 members: VEGF (also known as VEGF-A), VEGF-B, VEGF-C, VEGF-D and placental growth factor (PlGF). The VEGF family of growth factors comprises the ligands for vascular endothelial growth factor receptors (VEGFRs). VEGFs are the major molecular players regulating the processes of angiogenesis and lymphangiogenesis. The signaling between the growth factors and their receptors is complex and often crosstalk between different members of the VEGF family and their receptors occurs (Norrmén et al., 2011). The binding of VEGFs to their receptors is guided by the differential affinities of the VEGFs for different receptors (Kärpanen and Alitalo, 2008). VEGFs belong to the VEGF/PDGF (platelet derived growth factor) family of growth factors and contain the central VEGF homology domain (VHD) characterized by the presence of eight conserved cysteine residues participating in intra- and inter- molecular disulfide bonds (Fairbrother et al., 1998).

1.3.2 VEGF

Vascular endothelial growth factor (VEGF) is the major mitogen for endothelial cells and is involved in both normal, developmental and pathological angiogenesis (Ferrara et al., 1998; Miquerol et al., 2000). It is the major angiogenic and vasculogenic factor (Carmeliet et al., 1996) and regulates endothelial cell proliferation

and migration (Bernatchez et al., 1999). VEGF can be produced by all the cells of the body (Ferrara et al., 1992) and hypoxia is the major inducer for VEGF expression (Liu et al., 1995), regulated via the transcriptional activator HIF-1α (Forsythe et al., 1996). Additionally, the expression of VEGF is stimulated by inflammatory cytokines, oncogenes and growth factors (Ferrara et al., 2003).

The effect of VEGF on lymphangiogenesis has been controversial. Adenoviral delivery of VEGF-A_{164} to adult mice resulted in formation of giant lymphatic vessels with reduced function (Nagy et al., 2002) but the mechanism causing this phenotype was not described. However, the effect of VEGF on lymphangiogenesis has been suggested to be indirect. In the mouse model of inflammatory corneal neovascularization, VEGF can recruit macrophages to the inflammatory tissues via VEGFR-1 and the recruited macrophages could induce lymphangiogenesis by secreting VEGF-C (Cursiefen et al., 2004). Similarly, VEGF overexpressing tumor models in mice showed peri-tumoral lymphangiogenesis and lymph node metastasis, further reflecting the potential role of VEGF in lymphangiogenesis (Björndahl et al., 2005; Hirakawa et al., 2005).

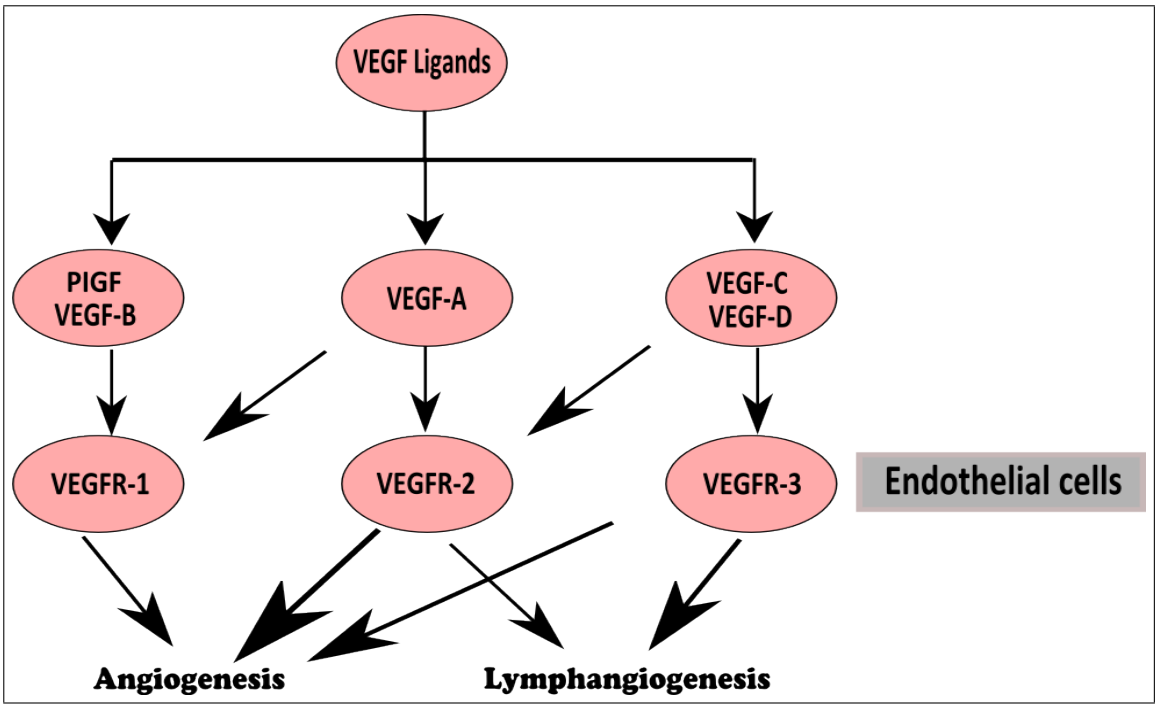

Figure 3: **Growth factors of the VEGF family and their receptors**

1.3.3 VEGF-B

VEGF-B, also known as VEGF related factor (VRF), has two isoforms (VEGF-B_{167} and VEGF-B_{186}) as a result of alternative splicing (Olofsson et al., 1998). Recent studies in mice show a potential role of VEGF-B in cardiac conduction (Aase et al., 2001) and prevention of ischaemic heart disease (Kivelä et al., 2014). However, more studies are needed to understand the role of VEGF-B in order to be able to utilize it as a therapeutic target.

1.3.4 PlGF

The name PlGF comes from its discovery from human placenta (Maglione et al., 1991), but it is also expressed in heart, lungs and skeletal muscle (Falco, 2012). PlGF stimulates endothelial cell growth (Maglione et al., 1993), but its deficiency has no effect on embryonic angiogenesis (Carmeliet et al., 2001). In contrast to the embryonic effect, PlGF seems to have a significant angiogenic effect under ischaemic conditions in heart inflammation (Carmeliet et al., 2001; Luttun et al., 2002), and it also shows an arteriogenic effects which are mediated by monocytes (Pipp et al., 2003).

1.3.5 VEGF-C, the major lymphangiogenic factor

VEGF-C (or VEGF-related protein, VRP) was identified in 1996 by two independent groups (Joukov et al., 1996; Lee, 1996). The 419 amino acid residue protein features long N- and C-terminal extensions flanking the central VEGF homology domain (VHD). The C-terminal domain of VEGF-C contains a repetitive cysteine pattern resembling the Balbiani ring 3 domains protein (BR3P). VEGF-C expression starts in embryos at E8.5 and reaches its peak at E12.5 during the formation of the primary lymph sacs after LEC emigration from the embryonic veins (Kukk et al., 1996). VEGF-C mRNA is expressed in lymph nodes, heart, kidney, muscle, placenta, ovary and small intestine (Lee, 1996).

Proteolytic processing. VEGF-C is synthesized as a preproprotein, consisting of an N-terminal signal sequence, an N-terminal propeptide, the VEGF homology

domain (VHD) and the C-terminal propeptide (Joukov et al., 1996). VEGF-C undergoes stepwise proteolytic processing (**Figure 4**) which has a significant effect on the affinity of VEGF-C towards its two known receptors VEGFR-3 and VEGFR-2. The mature form (referred to as 21/23 form) exists as homodimer and binds both receptors (Joukov et al., 1997; Stacker et al., 1999). The mature form contains two N-glycosylation sites (out of the three N-glycosylation sites in pro-VEGF-C) and nine cysteine residues (Joukov et al., 1997). The proteolytic processing of VEGF-C is not completely understood. The limited information about the processing is based on transfection studies in cell lines. The proprotein convertases furin, PC5 and PC7 cleave VEGF-C at the C-terminus into pro-VEGF-C. This happens probably before secretion (Siegfried et al., 2003). Similarly, the serine protease plasmin can cleave VEGF-C at the N-terminus generating a mature form of VEGF-C capable of binding to both VEGFR-2 and -3 (McColl et al., 2003).

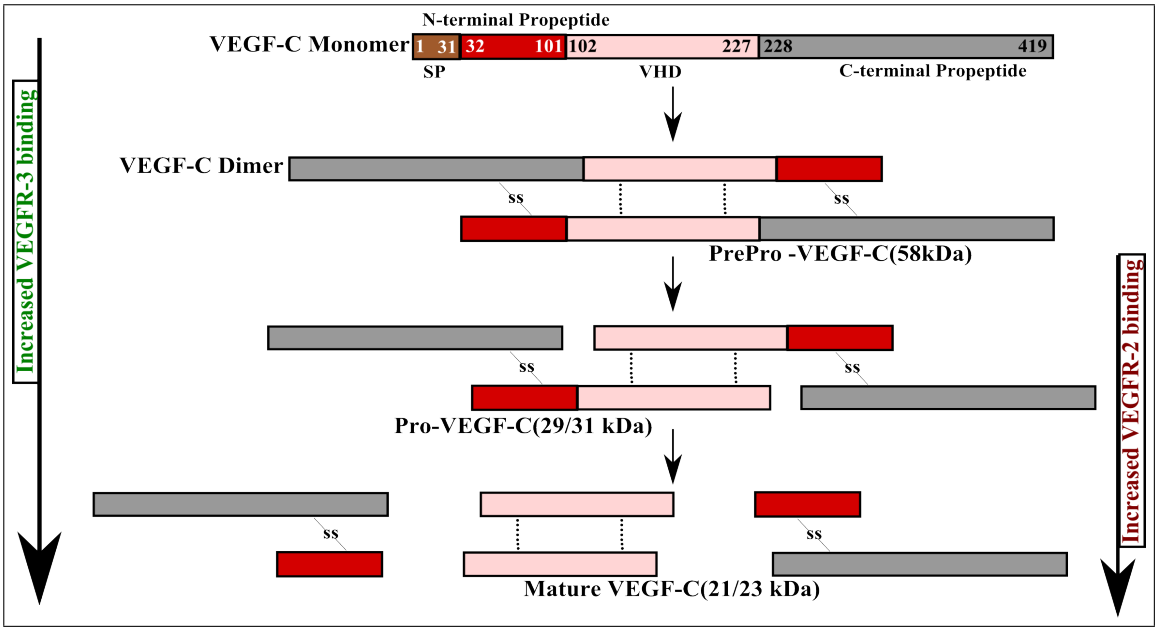

Figure 4: **Model of proteolytic processing of VEGF-C**. VEGF-C domain structure starting from the N-terminus: Signal peptide (SP), N-terminal propeptide, VEGF homology domain (VHD) and C-terminal propeptide. The processing occurs in a stepwise manner starting from unprocessed (prepro-VEGF-C) via pro-VEGF-C to the mature VEGF-C, which has angiogenic in addition to lymphangiogenic activity.

Biological activity. The role of VEGF-C in lymphangiogenesis is well established. The importance of VEGF-C during embryonic lymphangiogenesis has been discussed in the section *Development of the lymphatic system*. VEGF-C induces hyperplasia of lymphatic vessels in the skin of transgenic mice and the chick chorioallantoic membrane (Jeltsch et al., 1997; Oh et al., 1997; Veikkola et al., 2001). Homozygous deletion of *Vegfc* in mice is lethal for the embryos and heterozygous deletion results in lymphatic hypoplasia and lymphedema (Kärkkäinen et al., 2004). Mutation of Cys-156 into serine strongly reduces binding of VEGF-C to VEGFR-2 (Joukov et al., 1998). In zebrafish, *Vegfc* is both angiogenic and vasculogenic during early vascular development (Ober et al., 2004). In transgenic mice, induced overexpression of VEGF-C stimulates angiogenesis and lymphangiogenesis in embryos, but the effect is restricted to the lymphatic vessel in adults (Lohela et al., 2008). VEGFR-3, the receptor for VEGF-C, is expressed in all endothelial cells during the early stages of development but becomes limited to the LECs later in development (Kukk et al., 1996). VEGFR-2, the second receptor for VEGF-C, is expressed in both LECs and BECs in vitro (Kriehuber et al., 2001) and in agreement with this, both LECs and BECs grow and migrate in response to VEGF-C (Mäkinen et al., 2001a).

VEGF-C mRNA levels are not regulated by hypoxia (Enholm et al., 1997). However, VEGF-C protein levels have been shown to be regulated via an internal ribosome entry site (IRES) under hypoxia (Morfoisse et al., 2014). VEGF-C expression has also been shown to increase in the presence of pro-inflammatory cytokines (Ristimäki et al., 1998).

1.3.6 VEGF-D, the "other" lymphangiogenic factor

VEGF-D (c-fos induced growth factor, FIGF) is structurally similar to VEGF-C and is also similarly processed (Orlandini et al., 1996; Stacker et al., 1999). Human VEGF-D, like VEGF-C, binds to both VEGFR-2 and VEGFR-3 (Achen et al., 1998), but binding of VEGF-D to VEGFR-2 is absent in mice (Baldwin et al., 2001). VEGF-D stimulates the migration of capillary endothelial cells (Achen et al., 1998) and induces lymphangiogenesis and angiogenesis in adult tissues (Byzova et al., 2002). Unlike VEGF-C, VEGF-D deficient mice have no significant lymphatic phenotype (Baldwin et al., 2005). Hence, VEGF-C appears to be the major lymphan-

giogenic factor and VEGF-D might act as a redundant factor. This has been shown in the double *Vegfc -/-* :*Vegfd -/-* mice whose lymphatic phenotype resembles the one of the *Vegfc -/-* mice (Haiko et al., 2008).

1.3.7 Vascular endothelial growth factor receptors (VEGFRs)

VEGFRs belong to the class III receptor tyrosine kinases and consist of seven extracellular immunoglobulin (Ig) homology domains, a single transmembrane domain and an intracellular tyrosine kinase domain. They are indispensable for vascular development in general as well as for maintaining the vascular integrity in the adult (Olsson et al., 2006).

VEGFR-1. VEGFR-1 (Fms-like tyrosine kinase 1, Flt1) is normally expressed on blood vascular endothelial cells, macrophages, dendritic cells and hematopoietic stem cells (Shibuya, 2001). VEGFR-1 has a strong affinity for VEGF (de Vries et al., 1992), PlGF (Park, 1994) and VEGF-B (Olofsson et al., 1998). VEGFR-1 can also crosstalk with VEGFR-2 by forming heterodimers (Autiero et al., 2003).

VEGFR-2. VEGFR-2 (fetal liver kinase 1, Flk1 in mice and KDR in humans) is the major angiogenic receptor and is involved in survival, growth, sprouting and migration of endothelial cells (Gille et al., 2001). Mice deficient in *Vegfr2* show embryonic lethality as a result of disrupted vasculogenesis and hematopoiesis (Shalaby et al., 1995). Compared to VEGFR-1, VEGFR-2 has a stronger tyrosine kinase activity when stimulated with VEGF (Waltenberger et al., 1994). VEGFR-2 has affinities for VEGF-A, VEGF-E, pro/mature VEGF-C and mature VEGF-D (Shibuya and Claesson-Welsh, 2006).

VEGFR-3. During biosynthesis, VEGFR-3 (Flt4) becomes proteolytically cleaved within its fifth Ig homology domain (Pajusola et al., 1994). However, mutations preventing the processing have no effect on its kinase activity (Tvorogov et al., 2010). VEGFR-3 homodimerizes after binding its ligands VEGF-C or VEGF-D and it also can heterodimerize with VEGFR-2 with consequential differences for its signaling capacity (Dixelius et al., 2003). In the early stage of development (before the forma-

tion of lymphatic vessels), VEGFR-3 is expressed on blood vessels and has a vital role in the early stages of cardiovascular development (Dumont et al., 1998). Once the lymphatic vessels arise, VEGFR-3 expression becomes restricted to the lymphatic compartment (Kaipainen et al., 1995) and has a role in the survival and maintenance of lymphatic vessels (Kärkkäinen et al., 2004). However, VEGFR-3 expression can be upregulated in blood vessels during pathological conditions (Paavonen et al., 2000; Partanen et al., 1999).

1.4 Lymphangiogenesis in disease

The function of the lymphatic system is compromised in many diseases. Disorders can be characterized by either lymphatic insufficiency or pathological lymphatic growth like in tumor lymphangiogenesis or inflammation.

1.4.1 Lymphedema

Lymphedema is the condition of lymphatic insufficiency due to aberrant, dysfunctional or absent lymphatic vessels, obstruction of the normal flow of lymph or increased capillary filtration leading to accumulation of interstitial fluid, thereby increasing the interstitial pressure. It visually manifests as swelling of the face, arms, legs or the abdominal wall. Lymphedema can be classified into primary lymphedema (hereditary) or secondary (acquired) lymphedema (Norrmén et al., 2011).

Primary lymphedemas are rare diseases. **Table 1** summarizes various types of primary lymphedema with features and molecular mechanisms.

Table 1: **Selected hereditary lymphedema conditions**. ∗: not assigned yet; M: Milroy-type lymphedema

Disease	**Gene (protein)**	**Characteristics**	**Time of onset**	**Molecular mechanisms**	**Reference**
Hereditary Lymphedema Type 1A (M)	*FLT4* (VEGFR-3)	Lymphedema mainly of lower extremities	Congenital	Mutations in VEGFR-3 compromise LEC growth, function and proliferation	(Kärkkäinen et al., 2000)
Hereditary Lymphedema Type 1B (M)	6q16.2-q22.1	Lymphedema mainly of lower extremities	Childhood to puberty	?	(Malik, 2008)
Hereditary Lymphedema Type 1C (M)	*GJC2* (connexin 47)	Lymphedema of the extremities	Childhood to puberty	Connexin communication between LECs and their environment is disturbed	(Kanady, 2011)
∗ (M)	*VEGFC* (VEGF-C)	Lymphedema of the lower extremities	Congenital or in early childhood	Reduced activity of VEGF-C because of mutation	(Gordon et al., 2013)
Meige Lymphedema, hereditary Lymphedema Type 2	?	Lymphedema mainly of the lower extremities	puberty	?	(Rezaie, 2008)
Lymphedema-Distichiasis Syndrome	*FOXC2* (FOXC2)	Lymphedema of the lower limbs, extra eye lashes and varicose veins	puberty or later	Lymphatic valve malformation and abnormal pericyte recruitment to the lymphatic capillaries	(Petrova et al., 2004)
Hennekam Syndrome	*CCBE1* (CCBE1)	Lymphedema of the extremities, lymphangiectasia of the intestine and mental retardation	congenital	Mutations in CCBE1 impede VEGF-C activation	(Alders et al., 2009)

Milroy disease (OMIM 153100), a type of congenital lymphedema, has been found to be associated with missense mutations in the *FLT4* gene (Connell et al., 2009; Evans et al., 2003; Ferrell et al., 1998; Kärkkäinen et al., 2000) and results in hypoplastic or aplastic lymphatic capillary networks (Mellor et al., 2010). *In vitro* and *in vivo* studies have shown that mutations in the tyrosine kinase domain of VEGFR-3 can compromise VEGFR-3 signaling (Kärkkäinen et al., 2000, 2001). Recently, a frameshift mutation in *VEGFC* has also been identified with a phenotype similar to Milroy disease and the mutant protein had no lymphangiogenic activity in the zebrafish model (Gordon et al., 2013).

Mutations in the transcription factor *FOXC2* can cause Lymphedema-distichiasis syndrome (Finegold et al., 2001), and *SOX18* can cause Hypotrichosis-lymphedema-telangiectasia syndrome (Irrthum et al., 2003). Hennekam lymphangiectasia-lymphedema syndrome is in a subset of patients associated with point mutations in the *CCBE1* gene (Alders et al., 2009).

Secondary lymphedema represents the major fraction of lymphedema incidence worldwide and can result from radiation, surgery or infection, causing a defective lymphatic vasculature (Warren et al., 2007). Filariasis, the infection caused by nematodes like *Wuchereria bancrofti* and *Brugia malayi* (Dreyer et al., 2000) affects millions of lives in tropical areas and it can result in secondary lymphedema. Lymphatic filariasis features blockage of normal lymph flow, which is caused by the accumulation of adult worms in the lymphatic vasculature (Pfarr, 2009). Another common form of secondary lymphedema can result as a complication from postmastectomy radiotherapy (PMRT) and/or lymph node removal (Hinrichs et al., 2004).

There are not many options for treating lymphedema except physiotherapy or compression treatment (Warren et al., 2007). However, the discoveries of the genetic causes of lymphedema have paved the way for the development of therapeutic strategies.

1.4.2 Tumor lymphangiogenesis and metastasis

Metastasis is the complex process by which tumor cells spread from the primary tumor to a distant, secondary site with often fatal consequences for the patient (Fidler,

2003). Tumor metastasis can occur via two routes: through the blood vasculature (Zetter, 1998) or via the lymphatic vasculature, followed by later dissemination of tumor cells into the blood vasculature (Alitalo et al., 2005). The role of the blood vasculature in tumor progression has been well established, but the contribution of the lymphatic vasculature is poorly understood (Skobe et al., 2001). The advent of lymphatic-specific markers has played a significant role in studying lymphangiogenesis in cancer progression with large numbers of articles claiming its importance. Clinical studies of cancer have often identified lymph node metastasis as a common event in the carcinoma development of breast, prostate, and colon as well as in melanoma (Kawada and Taketo, 2011). Lymph node metastasis has also been used as a clinical prognostic marker for tumor aggressiveness (Nathanson, 2003).

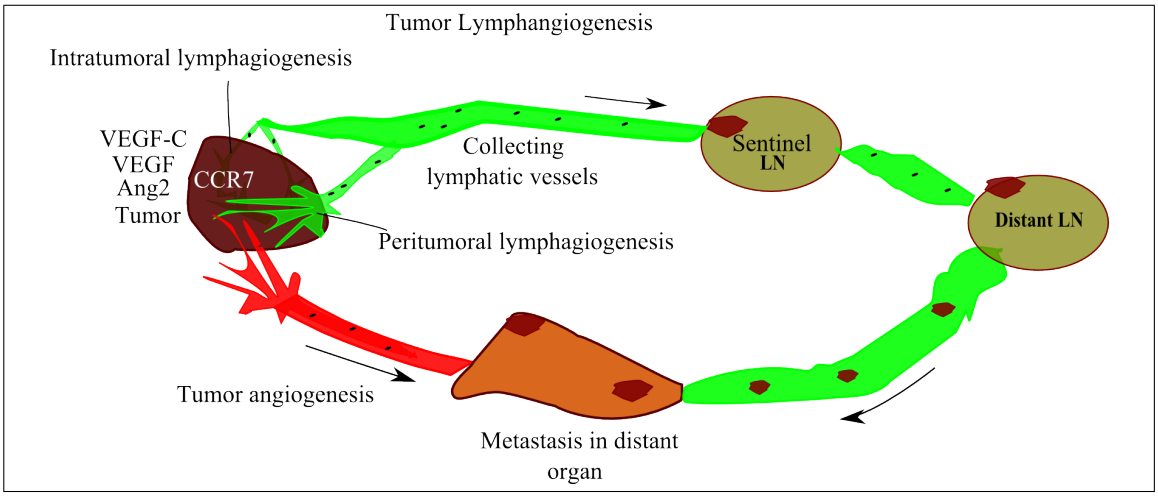

Figure 5: **Summary of lymphatic metastasis**. The tumor secretes VEGF-C and VEGF-D. Tumor cells express inflammatory cytokine receptors like CCR7 to stimulate lymphangiogenesis, lymphatic vessel hyperplasia and tumor cell dissemination into local lymphatic vessels and lymph nodes. VEGF, Ang2 and macrophages contribute to the metastatic process. The disseminated tumor cells can translocate further to distant lymph nodes and even drain back into blood vessels, from where they can colonize still other cites.

In vivo studies have shown the presence of lymphatic vessels at the tumor periphery, where they play a role in the passaging of tumor cells (Deutsch et al., 1992; Leu et al., 2000). In contrast, there is an absence of functional lymphatics within solid tumors because of the physical conditions (e.g. pressure) within tumors (Jain and Fenton, 2002; Padera et al., 2002). However, there have been a few studies

that suggested the presence of proliferative intra-tumoral lymphatic vessels in some human cancers (Beasley et al., 2002; Dadras et al., 2003; Maula et al., 2003), but it is commonly believed that intra-tumoral lymphatic vessels are not a prerequisite for metastasis (Wong et al., 2005). The lymphatic vessels exceed blood vessels in terms of permeability and hence lymphatic vessels are more likely to be penetrated by tumor cells (Alitalo et al., 2005).

VEGF-C is produced by many solid tumors and its expression correlates with the metastatic potential of the tumor (Mandriota et al., 2001; Mattila et al., 2002; Skobe et al., 2001). Furthermore, clinical studies in melanoma patients have shown poor prognosis for patients with high expression of VEGF-C mRNA (Goydos and Gorski, 2003). The significant upregulation of VEGF-C in tumors accompanies the development of resistance to anti-VEGF therapy (Li et al., 2014). Recently, studies in patients with mantle cell lymphoma and in a comparable mouse model elucidated the expression of VEGF-C by tumor associated macrophages as a major stimulator of lymphangiogenesis (Song et al., 2013). Contrary to VEGF-C, the influence of its homolog VEGF-D on metastasis remains controversial. Both upregulation of VEGF-D (Stacker et al., 2001; Von Marschall et al., 2005) and downregulation (O-charoenrat et al., 2001) have been observed in different tumors.

Lymphatic endothelial cells produce the chemokines CCL21 (Gunn et al., 1998) and CXCL12 (Müller et al., 2001). These chemokines are ligands for the receptors CCR7 and CXCR4, which are broadly expressed by tumor cells. Inhibition of CXCL12/CCL21 interactions resulted in a significant reduction in lymph node and lung metastasis (Müller et al., 2001).

The establishment of the role of VEGF-C/VEGF-D and VEGFR-3 signaling in tumor progression transformed this axis into one of the major targets for therapeutic intervention. Several studies using neutralizing antibodies against VEGF-D (Achen et al., 2000) and VEGFR-3 (Chaudary et al., 2011; Roberts et al., 2006; Shimizu et al., 2004), siRNA targeted against VEGF-C (Chen et al., 2005) and soluble VEGFR-3 fusion proteins (Lin et al., 2005) have shown significant effects on the inhibition of tumor lymphangiogenesis and hence on the metastasis.

1.5 Targeting lymphangiogenesis for therapy

Therapeutic strategies targeting the lymphatic system can be either pro-lymphangiogenic (for the treatment of lymphatic insufficiency) or anti-lymphangiogenic (to blunt a disease-promoting lymphangiogenic response).

1.5.1 Pro-lymphangiogenic therapy

Stimulating lymphatic growth to treat lymphatic insufficiency diseases like primary lymphedema would treat the underlying cause of the disease and hence will be complementary to the traditional symptomatic treatment strategies. An adeno-viral vector encoding VEGF-C significantly stimulated lymphangiogenesis when injected into adult mice (Enholm et al., 2001). Similarly, VEGF-C gene therapy in the Chy-mouse model of primary lymphedema enhanced the expression of VEGFR-3, thereby leading to the growth of functional lymphatic vessels (Kärkkäinen et al., 2001). The delivery of VEGF-C and Ang2 using a hydrogel HAMC (hyaluronan and methylcellulose) delivery system into the nodal removal site was able to reconstitute lymphatic function and significantly reduce edema (Baker et al., 2010).

1.5.2 Anti-lymphangiogenic therapy

In metastasis, tumor cells can either travel via the lymphatic or the blood vasculature. Lymphatic metastasis is common in many cancer types. Several preclinical therapies have been devised based on anti-lymphangiogenesis like siRNA against VEGF-C (Chen et al., 2005), monoclonal antibodies against VEGF-C (e.g. VGX-100; Falchook et al., 2013), VEGFR31-Ig (Zhang et al., 2010), monoclonal antibodies against (e.g.VD1, Achen et al., 2000) and monoclonal antibodies against VEGFR-3 (Roberts et al., 2006). The successful development of Avastin as anti-angiogenic drug has spurred the search for an anti-lymphangiogenic therapy to treat tumor metastasis. One advantage of anti-lymphangiogenic therapy could be the absence of on-target side effects due to the relatively low lymphangiogenic activity in adults.

1.6 CCBE1 as a novel regulator of lymphangiogenesis

Collagen and calcium binding EGF domains 1 (CCBE1) is a secreted protein with 406 AA residues (in humans) containing N-terminal EGF-like repeats and a C-terminal domain with two collagen-like repeats (Hogan et al., 2009). Hennekam lymphangiectasia-lymphedema syndrome (OMIM 235510), short Hennekam syndrome or HS, is a rare autosomal recessive disorder characterized by lymphedema, lymphangiectasia and mental retardation (Van Balkom et al., 2002). Mutations in CCBE1 are associated with the Hennekam syndrome in a subset of patients, but the molecular mechanism behind the lymphatic vasculature abnormalities remained unclear (Alders et al., 2009). Mutations found in CCBE1 mostly occur in the N-terminal EGF-like repeats which contain conserved cysteine residues and are thought to be crucial for the integrity of the protein (Alders et al., 2009).

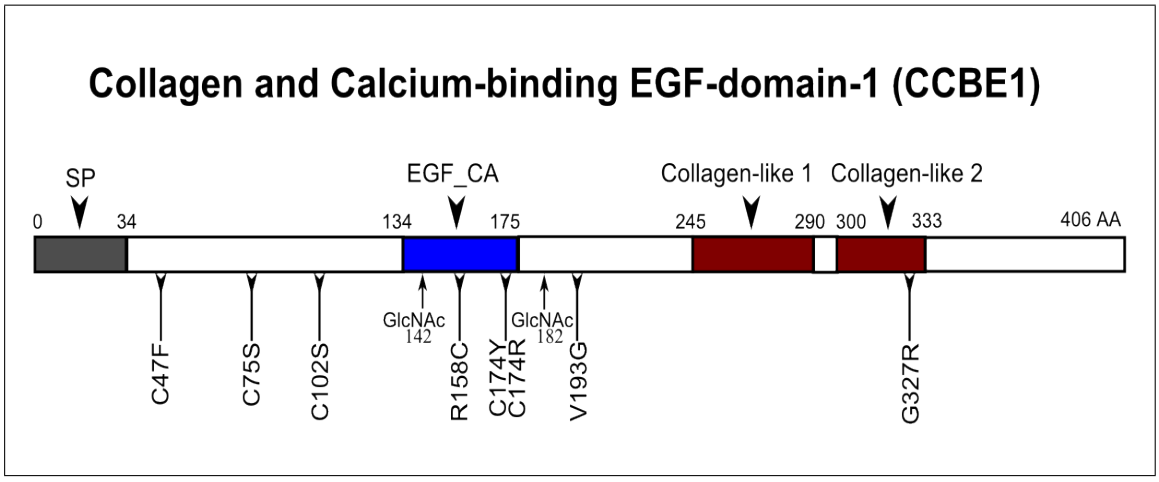

Figure 6: **Schematic view of the structure of CCBE1**. Mutations in CCBE1 as seen in Hennekam Syndrome. *SP*: signal peptide, *GlcNAc*: potential N-linked glycosylation site, *EGF_CA*: EGF-like calcium binding domain. Domain information retrieved from Uniprot (http://www.uniprot.org/uniprot/Q6UXH8).

CCBE1 is probably not produced by endothelial cells but it is essential for embryonic lymphangiogenesis based on studies in zebrafish and mice (Bos et al., 2011; Hogan et al., 2009). *Ccbe1* -/- mice have fewer differentiated LECs and the primary lymph sacs do not form. This suggests a possible role of CCBE1 in LEC budding, sprouting, migration and lymph sac assembly (Bos et al., 2011). Although VEGF-C and CCBE1 display similar phenotypic features during the development of the lymphatic vasculature, the cause for this similarity remained largely unknown (Hogan

et al., 2009).

CCBE1 has also been shown to act like a tumor suppressor in ovarian cancer, as its loss promoted cellular migration and survival (Barton et al., 2009). Additionally, evidence suggesting CCBE1 as a marker of cardiac precursors during early stages of mouse development implies a possible function of CCBE1 during heart morphogenesis (Facucho-Oliveira et al., 2011).

1.7 A disintegrin and metalloproteinase with thrombospondin type I motif 3 (ADAMTS3)

A disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) proteases are structurally similar to the ADAMs proteases. ADAMs are typically transmembrane proteases, whereas ADAMTSs are typically secreted proteases (Kuno et al., 1997). The schematic structure of human ADAMTS3 is shown in **Figure 7**.

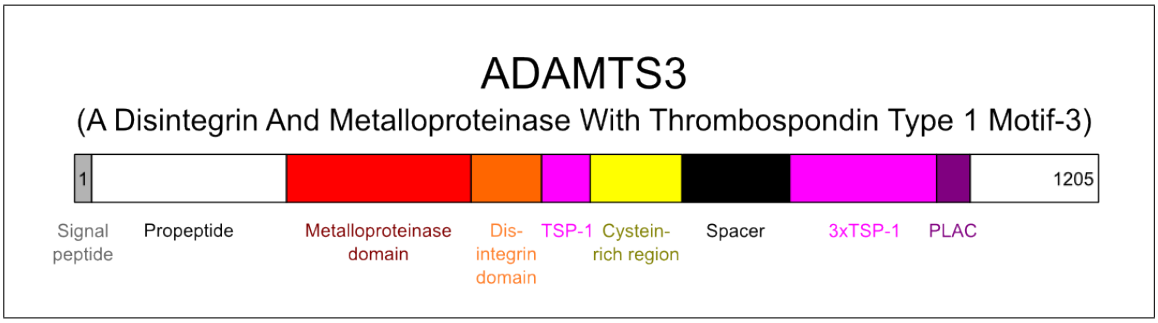

Figure 7: **Schematic view of the structure of ADAMTS3**. TSP-1: Thrombospondin 1; PLAC: Protease and lacunin. Domain information retrieved from Uniprot (http://www.uniprot.org/uniprot/O15072).

The ADAMTS family consist of 19 members (Stanton et al., 2011). ADAMTSs have at least one furin recognition site. Upon cleavage at this site, the ADAMTS proteases become activated (Brocker et al., 2009). Moreover, some results suggest further processing of ADAMTSs in the C-terminal region, which might be important for the regulation of ADAMTSs activity and availability in the extracellular matrix (RodrÍguez-Manzaneque et al., 2000). ADAMTSs are classified into seven major subfamilies (Llamazares et al., 2003). The subfamilies are shown in **Table 2**.

The group consisting of ADAMTS-2, -3 and -14 is also referred to as the procollagen-

Table 2: **The subfamilies of human ADAMTS proteins**. Modified from Llmazares et al., 2003 and Porter et al., 2005; ∗: not assigned.

Subfamilies of ADAMTS	**ADAMTS**	**Function**
Aggrecanases	ADAMTS-1 ADAMTS-4 ADAMTS-5 ADAMTS-8 ADAMTS-15	Cleave the major cartilage proteoglycan aggrecan
Procollagen N-proteinases	ADAMTS-2 ADAMTS-3 ADAMTS-14	Process procollagen into mature collagen
GON-ADAMTSs	ADAMTS-9 ADAMTS-20	Potential role in organ morphogenesis
Orphan ADAMTSs	ADAMTS-6 ADAMTS-7 ADAMTS-10 ADAMTS-12	?
vWFCP	ADAMTS-13	von Willebrand cleaving protease
*	ADAMTS-16 ADAMTS-18	?
*	ADAMTS-17 ADAMTS-19	?

N-proteinase group. They share a high degree of homology among each other (Colige et al., 2002) and remove the N-terminal propeptide from procollagens, which is a prerequisite for collagen assembly into mature fibrils (Lee et al., 2012). ADAMTS3, also known as procollagen II N-peptidase, seems to be the main enzyme that processes procollagen II in human cartilage (Fernandes et al., 2001). Mutations in ADAMTS2 (type I procollagenase) cause Ehlers-Danlos syndrome in humans and dermatosparaxis in cattle (Colige et al., 1999). Apart from procollagen, no other substrates have been identified for ADAMTS3 until now .

2 The aim of the study

VEGF-C is secreted as a 29/31 kDa form (pro-VEGF-C), which needs to be proteolytically processed to remove the N-terminal propeptides in order to release the active, mature form, which consists largely of the VHD domain. This proteolytic modification results in a significant increase of its affinity to the angiogenic receptor, VEGFR-2, and the lymphangiogenic receptor, VEGFR-3. It has been more than a decade since VEGF-C was discovered, but the knowledge about its proteolytic processing is still incomplete. In order to gain more insight into lymphangiogenesis and in order to design better therapeutic interventions it is paramount to understand the mechanism(s) of VEGF-C activation. Because of the similar phenotypes of the *Vegfc* and *Ccbe1* gene deleted mice and the lymphangiogenic potential of CCBE1, we speculated that CCBE1 and VEGF-C could interact with each other at the level of proteolytic activation.

The specific aims of the study were:

I. To study the effect of CCBE1 on VEGF-C.

II. To understand the mechanism involved in CCBE1-mediated enhancement of VEGF-C processing.

3 Materials and methods

3.1 Clonings

3.1.1 pCHOKE-B-hCCBE1-V5-H6

pCHOKE-B3-hCCBE1-V5-H6 (Michael Jeltsch, maxiprep#1482) and pCHOKE-B-hCCBE1-V5-H6 (Michael Jeltsch, maxiprep#1462) were digested with SfiI and SphI (NEB) and dephosphorylated using calf intestinal phosphatase (Finnzymes/Thermo Scientific). Inserts and vectors were electrophoretically separated on agarose gels and fragments were visualized under UV light following ethidium bromide staining. The correct fragments were excised from the gel and purified with the QIAEX II Gel Extraction Kit (Qiagen). The fragments were ligated using the Rapid Ligation Kit (Roche). The ligated vector was transformed into NEB 5-alpha chemically competent cells (NEB). 50μl of each transformation reaction were plated on Luria-agar plates containing 100μg/ml ampicillin followed by overnight incubation at 37°C. Clones were picked, inoculated in Luria broth containing 100μg/ml ampicillin and cultured overnight at 37°C for plasmid isolation using the QIAprep Spin miniprep kit (Qiagen). The miniprep was sequenced with a ABI 3130xl Genetic Analyzer (Applied Biosystems) and a DNA maxiprep (Nucleo Bond Xtra Maxi, BIOTOP) was prepared for the correct clone.

3.1.2 Constructs for ADAMTS proteases

ADAMTS1 (GenBank BC040382) and ADAMTS2 (Genbank BC046456) cDNAs were received as ready expression vectors (pCMV-SPORT6). ADAMTS14 cDNA (GenBank BC140263) was received as a gateway entry clone (pENTR223.1) and transferred into the gateway destination vector (pEF-DEST51) using the LR recombinase system (Invitrogen) according to manufacturer's protocol. ADAMTS3 was subcloned into pCI-neo (Promega). For this, the insert was derived from pCRII-V5-H6-hADAMTS3 (Michael Jeltsch, maxiprep #1542) by Blp1 & PmeI digest, and the vector from pCI-neo (Michael Jeltsch, maxiprep# 1530) by BlpI and SmaI digest. The cloning steps used were similar to the ones used for the preparation of pCHOKE-B-hCCBE1-V5-H6.

3.1.3 Constructs for the VEGF-C/VEGF-D chimera

The VEGF-C/VEGF-D chimera version 2 (CDC-V2) construct was created by overlapping polymerase chain reaction (PCR) mutagenesis. Two different PCR products were obtained; one using 5'-CATGGAGACAGACACACTCCTGCTATG-3' as the forward primer and 5'-GATCTCTGTATTATAATGTGCTGCAGCAAATTTTATAGTCTCTTC-3' as the reverse primer, and the other using 5'-GCACATTATAATACAGAGATCCTAAAAGTTATAGATGAAGAATGGCA-3' as the forward primer and 5'-CCGGATGCTAGCGTTTAAACGAATT-3' as the reverse primer. For both PCRs pSec-TagI-CDC-V1 (Michael Jeltsch, maxiprep, #1432) was used as template. Both PCR products were then used as the template for a second PCR using 5'-CATGGAGACAGACACACTCCTGCTATG-3' as forward and 5'-CCGGATGCTAGCGTTTAAACGAATT-3' as reverse primer. The final PCR product was inserted into pSec-TagI-CDC-V1 using BspeI and BsgI as restriction sites.

Other expression clones that were used in this study were: pCHOKE-B-hVEGF-C-FL-H6 (Michael Jeltsch, maxiprep#1438) and pSecTagI-CDC-V1 (Michael Jeltsch, maxiprep #1432).

3.2 Antibodies

Anti-VEGF-C antiserum (Baluk et al., 2005), anti-VEGF-C-antibody (R&D Systems, AF752), anti-VEGF-D antibody (R&D Systems, AF286), anti-V5 antibody (Invitrogen, #46-0705), anti-ADAMTS3 antibody (Sigma, #A6477), anti-ADAMTS3 antibody (Santa Cruz, sc-21486, #L2303), anti-phosphotyrosine antibody 4G10 (Merck Millipore) and PY20 (BD Transduction Laboratories) were used for both immunoprecipitation and Western blotting. Anti-VEGFR-3 antibody (Santa Cruz, sc-321), chimeric VEGFR-3/IgGFc (Mäkinen et al., 2001b) and streptactin sepharose (IBA) were used for immunoprecipitation. Similarly, streptactin-HRP conjugate (IBA) and streptavidin-HRP conjugate (R&D Systems, #890803) were used for Western blots.

3.3 Cell culture

293T, 293F, PAE-VEGFR-3 (Pajusola et al., 1994), PAE-VEGFR-3/Neuropilin-2 (Kärpanen et al., 2006), Ba/F3-hVEGFR-3/EpoR (Achen et al., 1998), Ba/F3-mVEGFR-2/EpoR (Achen et al., 2000) and NIH-3T3 cells were grown in D-MEM (Lonza), PC-3 cells in Ham's F-12 (Lonza), DU-4475 cells in RPMI 1640 (Lonza), CHO cells in α-MEM (Life Technologies) supplemented with 10% fetal bovine serum (FBS; Biowest, #1800-500; 20% FBS for DU-4475), 2mM L-glutamine, penicillin (100U/ml) and streptomycin(100U/ml).

3.4 Stable cell line generation, protein expression and purification

3.4.1 Stable cell line generation

293F cells were transfected with the mammalian expression plasmid pCI-neo-hADAMTS3-V5-H6 and pCHOKE-B-hCCBE1-V5-H6. After 48 hours, the transfected cells were split to new cell culture dishes at different dilutions (1:10, 1:20, 1:100, 1:300). The cells were then selected with G-418 (Roche) at a concentration of 800μg/ml. Isolated visible colonies were then transferred to new dishes with the help of cloning rings. The stable expression of CCBE1 and ADAMTS3 was confirmed by Western blotting.

3.4.2 ADAMTS3 purification

For the purification of his-tagged ADAMTS3, stably transfected cells were grown in 60 petri dishes and at confluence, the regular medium was replaced by D-MEM supplemented with 2% FCS, 30μM $ZnCl_2$ and 0.1U heparin/ml. After 5 days, conditioned medium was collected and centrifuged at 13000 rpm for 20 minutes at 4°C. The medium was then dialyzed against phosphate buffer using Spectra/Por dialysis membranes (Spectrum Laboratories. Inc, CA) having a molecular weight cut off (MWCO) of 15 kDa. The dialysis buffer was exchanged every 12 hrs four times. The pH of the dialyzed medium was adjusted to 8 with 3M Tris/HCl pH 10.5 and the NaCl concentration was adjusted to 450mM followed by addition of

150 μl Ni^{2+}-NTA-Agarose (Qiagen) slurry per 50 ml of the dialyzed media. Binding was performed by gentle rotation overnight at 4°C. The Ni^{2+}-NTA resin was rescued by centrifugation at low speed (500g) for 5 minutes and loaded to a chromatography column (Pharmacia Biotech) connected to an Äkta Explorer FPLC-apparatus (Pharmacia Biotech/GE Healthcare). The column was washed with 50 column volumes of 3x PBS containing 10% glycerol followed by washes with 10mM and 20mM imidazole. The elution was performed with 3x PBS containing 10% glycerol and 250mM imidazole. Eluates were collected in 0.5ml fractions. The peak fractions were dialyzed against 1x TBS and sterilized by filtration using Millex-GV PVDF Durapore 0.22 μm syringe filter units (Millipore). The proteins were aliquoted and stored at -70°C.

3.4.3 CCBE1 purification

For the purification of CCBE1, 293T cells stably expressing a strepIII-tagged CCBE1 were grown in 200ml of 10% FBS or D-MEM, 0.2% BSA medium for 6 days. For the purification of ADAMTS3, the conditioned medium was processed as described above except that streptactin sepharose (IBA) was used for binding, 100mM Tris/HCl pH 8, 150mM NaCl, 1mM EDTA for washing, and washing buffer containing 2.5mM desthiobiotin for elution.

3.5 Ba/F3-VEGFR/EpoR assays

The capacity of human VEGF-C to bind to its receptor VEGFR-3 and VEGFR-2 after processing with CCBE1 and/or ADAMTS3 was analyzed by Ba/F3-VEGFR/EpoR assays. These assays use murine bone marrow derived Ba/F3 cells expressing chimeric receptors consisting of the extracellular (EC) ligand binding domain of VEGFR-2 or VEGFR-3 and the transmembrane and cytoplasmic domain of mouse erythropoietin receptor (EpoR). Ba/F3 cells require interleukin-3 (IL-3) for their growth and survival. Ligands of the chimeric receptors are able to eliminate the dependency of Ba/F3-VEGFR/EpoR cells on IL-3, providing a survival signal through the cytoplasmic domain of the Epo receptor (Blau et al., 1997).

For the assay, Ba/F3 cells were washed twice with PBS and seeded at 20,000

cells/well to 96-well microtiter plates (Nunc, Thermo Scientific). Conditioned media of VEGF-C with or without CCBE1 and/or ADAMTS3 were used to supplement the cell culture media at different concentrations. After 48hrs, a viability assay was performed by adding 10μl of 5mg/ml of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (Sigma) in PBS to each well. The plates were incubated for 2 hours at 37°C followed by addition of 100μl of lysis buffer (10% SDS, 10mM HCl). The absorbance was measured at 540nm after incubation at 37°C over night in the dark.

3.6 Cell transfections and metabolic labeling

Cell transfection. Cells were transfected or co-transfected with expression constructs using jetPEI transfection reagent (Polypus-transfection Inc) according to the manufacturer's recommendations. 24 hrs after transfection, cells were washed twice with PBS and grown either in D-MEM/0.2 % BSA or in metabolic labeling medium (see below) for 18 hrs.

Metabolic labeling. Metabolic labeling by incorporating radiolabeled amino acids cysteine and methionine was used as a sensitive alternative to Western blotting when immunoprecipitation resulted in non-specific signals from the precipitating antibody. The cells were grown in 8μl of Easy Tag Express Protein Labeling Mix (35^S, PerkinElmer) per ml of cysteine/methionine deficient D-MEM medium. The supernatants were harvested 48hrs after transfection. The harvested supernatants were centrifuged at high speed for 15 minutes at 4°C and precipitated with the respective antibodies and protein A sepharose or streptactin for strepIII-tagged proteins. The binding was performed overnight at 4°C and the samples were separated by PAGE. The gels were then vacuum-dried for 2 hrs at 80°C and exposed to a phosphoimager plate or X-ray film. Phosphoimager plates were read on a Typhoon 9400 scanner (Amersham Biosciences/GE Healthcare).

3.7 Immunoprecipitation, SDS-PAGE and Western blotting

Cell supernatants or lysates were precipitated using 50μl of a 50% slurry of protein A/G Sepharose (PAS/PGS; GE Healthcare) in PBS and gently mixed overnight at 4°C. The antibody dilutions were used as recommended by the supplier. The immunoprecipitation reactions were centrifuged at 500g for 4 minutes and washed three times with ice cold 0.1% Tween-20 in PBS. Then 30μl of reducing 2x Laemmli standard buffer were added to the sepharose and after boiling at 95°C for 5 minutes, the samples were separated by SDS-PAGE (10% or 4-20% Mini-Protean TGX^{TM} Gels; Bio-Rad). Membranes and gels were equilibrated in semi-dry Western blot transfer buffer for 15 min. Proteins were then blotted to Protran nitrocellulose membrane (PerkinElmer) on a Trans-Blot SD Semi-Dry Transfer Cell apparatus (Bio-Rad) in transfer buffer at 37V for 40 min. Membranes were then blocked in 5%BSA-TBS for 1 hr on an orbital shaker at RT. After blocking, the membranes were incubated with the primary antibody against the indicated proteins at the indicated dilutions (in 5% BSA-0.1% Tween-TBS) overnight at 4°C with gentle agitation. The membranes were then washed with 0.1% Tween-TBS at RT followed by incubation with the appropriate secondary HRP-conjugated antibodies in 10% skim milk-0.05% Tween-TBS at RT for 1 hr. Membranes were finally washed (0.1% Tween-TBS for 6 times, 8 mins each wash) and proteins were visualized on an X-ray film by ECL using Supersignal West Pico maximum sensitivity substrate (Thermo Scientific). Autoradiographies and Western blots were quantified using the ImageJ Software (NIH, Bethesda, MD).

3.8 VEGFR-3 phosphorylation

PAE cells expressing VEGFR-3-StrepIII or VEGFR-3/Neuropilin-2 were grown to confluence and starved overnight in serum free D-MEM. ΔNΔC-VEGF-C (Kärpanen et al., 2006), pro-VEGF-C (Veli-Matti Leppänen, unpublished), CCBE1Δ175 (Veli-Matti Leppänen, unpublished) were diluted to final concentrations of 0.02, 0.4, 5μg/ml in 1ml D-MEM/0.1% BSA and incubated at 37°C for 30 minutes. Cells were stimulated with the incubated samples for 10 min. After the stimulation, the cells were lysed in 1% Triton X-100 in 50mM Tris pH 8 or RIPA buffer containing 50mM NaF, 10μg/ml leupeptin, 10μg/ml aprotinin, and 5mM Na_3VO_4. After lysis, the

samples were immunoprecipitated with streptactin sepharose, followed by Western blotting with Anti-phosphotyrosine (pY) antibody 4G10.

Similarly, cells were starved for cross-linking overnight and washed twice with PBS followed by stimulating the PAE-VEGFR-3-StrepIII and PAE-VEGFR-3/Neuropilin2 cells with ΔNΔC-VEGF-C at 100ng/ml, pro-VEGF-C at 1μg/ml and CCBE1Δ175 at 50μg/ml at 37°C for 3.5 min. Then 5μM DTSSP (Thermo Scientific) in an ice-cold 5mM sodium citrate (pH 6) was added and the stimulated cells were incubated for 6.5 min at 37°C. The cells were then washed with ice-cold TBS, lysed with 1% Triton X-100 in 50mM Tris pH 8 and immunoprecipitated with anti-VEGFR-3 antibodies or pY antibody followed by Western blotting with anti-VEGF-C antibody or streptavidin-HRP.

3.9 Processing of recombinant pro-VEGF-C with ADAMTS3

2.3μg of pro-VEGF-C (purified from insect cells) was incubated with either 30μg of ADAMTS3 expressed by insect cells or 8.3μg of ADAMTS3 expressed by 293T cells. The samples were incubated at 37°C for 12hrs, 24 hrs or 48 hrs. The cleaved VEGF-C was later analyzed by Western blotting using anti-VEGF-C antiserum.

3.10 Protein mass spectrometry and N-terminal sequencing

Both mass spectrometry of purified CCBE1-StrIII and N-terminal sequencing by Edman degradation of the ADAMTS3-cleaved VEGF-C were performed by the Proteomics core unit, Institute of Biotechnology, University of Helsinki. To avoid overdigestion, ADAMTS3 was titrated to identify the optimal amount of ADAMTS3 required to cleave VEGF-C. The product of activation of 25μg VEGF-C with 60μg ADAMTS3 in TBS for 24 hours was selected for sequencing.

3.11 Statistical analysis

One-way Anova was used to test for the significance of the Ba/F3 assay results. Tukey's test was used as post-hoc test and Graphpad Prism (Graphpad Software, Inc) was used as analysis software. Error bars represent the standard deviations.

4 Results

4.1 CCBE1 enhances VEGF-C processing

The CCBE1-coding expression construct, when transfected into 293T cells, resulted in the appearance of a protein of approx. 50 kDa both in the cellular lysate and conditioned media. In addition to the 50 kDa form, CCBE1 appeared as a diffuse band around 100 kDa in the conditioned media (**Figure 8 A**).

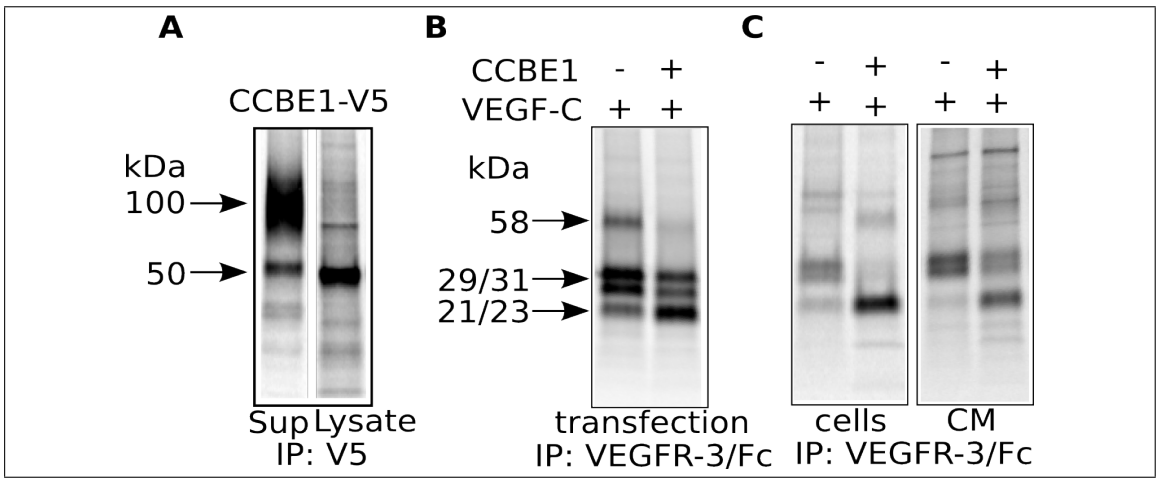

Figure 8: **CCBE1 enhances VEGF-C processing**. A) Autoradiography showing CCBE1 at approx. 45-50 kDa in the lysate and the supernatant. The additional diffuse band of approx. 100 kDa appears only in the supernatant. B) Co-transfection of the construct coding for CCBE1 increased the processing of pro-VEGF-C into the mature (21/23 kDa) form of VEGF-C. C) CCBE1 also enhances VEGF-C processing *in trans*. Two cell populations individually transfected with constructs coding for CCBE1 or VEGF-C or their respective conditioned media were mixed and VEGF-C processing was analyzed.

Three major bands were observed after the transfection of VEGF-C into 293T cells; unprocessed VEGF-C at 58 kDa, pro-VEGFC at 29/31 kDa and the fully processed mature form at 21/23 kDa (**Figure 8 B, lane 1**). When constructs coding CCBE1 and VEGF-C were co-transfected into 293T cells, the majority of the unprocessed VEGF-C and pro-VEGF-C was converted into mature VEGF-C (**Figure 8 B, lane 2**). The immunoprecipitation of VEGF-C was performed with VEGFR-3/Fc.

CCBE1 is believed to be a protein of the extracellular matrix. It's expression appears

to be non-endothelial and not overlapping with VEGF-C expression (Hogan et al., 2009). Thus, we looked for the possibility of VEGF-C processing *in trans*. Two different cell populations expressing VEGF-C or CCBE1 were mixed. Similarly, in another experiment, conditioned media from CCBE1 and VEGF-C expressing cells were mixed and incubated. In both of these experiments, CCBE1 was found to enhance VEGF-C processing (**Figure 8 C**).

4.2 The processed form of VEGF-C activates VEGFR-3 and VEGFR-2

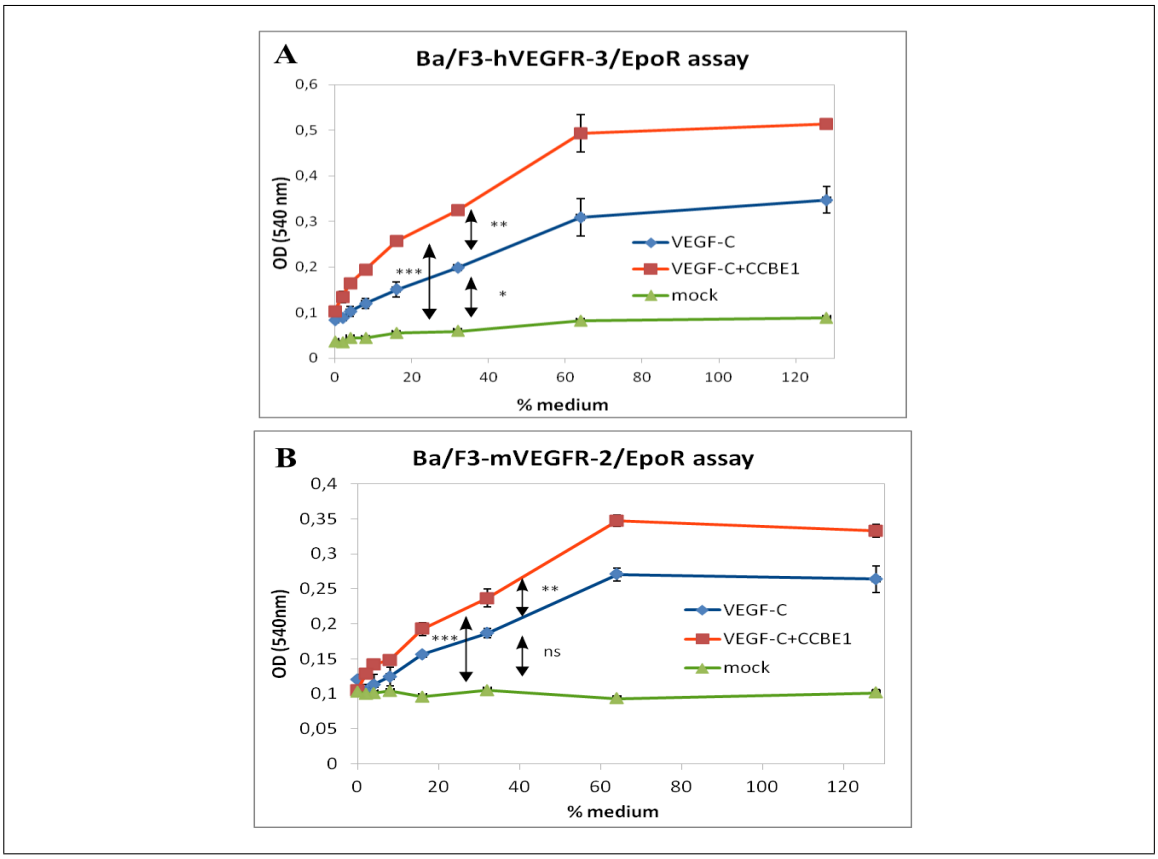

Figure 9: **The processed VEGF-C can activate VEGFR-3 and VEGFR-2**. A) A Ba/F3-hVEGFR-3/EpoR assay showing increased survival in the presence of conditioned media from cells co-transfected with VEGF-C and CCBE1 coding constructs. B) The same as A, but for Ba/F-mVEGFR-2/EpoR cells. $*$: P-value<0.05, $**$: P-value<0.01, $***$: P-value<0.001, ns: no significant.

In order to verify the function of the processed VEGF-C, a Ba/F3 assay was performed using conditioned media from cells cotransfected with VEGF-C/CCBE1 expression constructs (**Figure 9A and 9B**). The survival of Ba/F3-hVEGFR-3/EpoR cells and Ba/F3-mVEGFR-2/EpoR was significantly increased even with the pro-VEGF-C compared to the mock. This was probably caused by the VEGF-C processing (enhancement) mediated by endogenous CCBE1 or ADAMTS3 produced by the Ba/F3 cells or as a result of a impure preparation of VEGF-C.

4.3 Processing of VEGF-C by CCBE1 is efficient in 293T cells

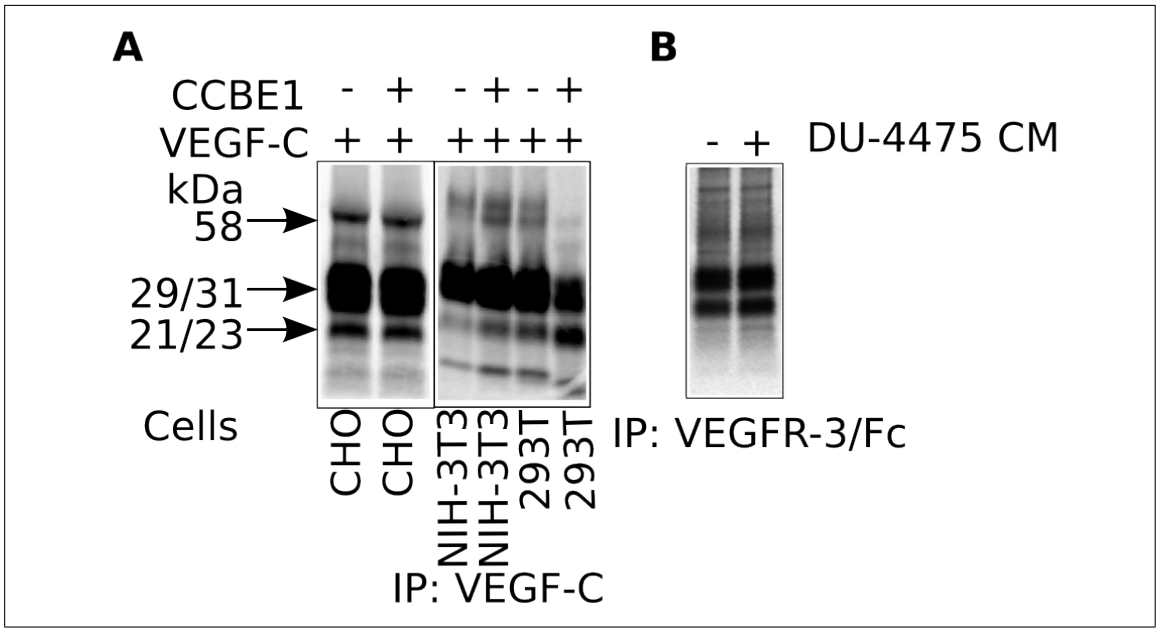

Figure 10: **Among all cell lines tested, CCBE1-dependent VEGF-C processing is efficient only in 293T cells from all cell lines tested**. A) and B) Autoradiography showing inefficient processing of VEGF-C in CHO, NIH-3T3 and DU-4475 cells compared to processing in 293T cells. Note that DU-4475 do endogenously express large amounts of CCBE1.

The processing of VEGF-C was analyzed in the cell lines CHO, DU-4475 and NIH-3T3. DU-4475 endogenously expresses CCBE1 (data not shown). However, there was no effect on VEGF-C processing by DU-4475 cells or conditioned supernatant from DU-4475 cells (**Figure 10B**). Similarly, CHO cells and NIH-3T3 cells were

co-transfected with expression constructs coding for VEGF-C and CCBE1 and the processing was less efficient compared to mock- or CCBE1- co-transfected 293T cells (**Figure 10A**).

Because CCBE1 is not a protease, it appeared likely that CCBE1 assists in the cleavage of VEGF-C by a protease that is expressed in 293T cells. To test this hypothesis, CCBE1 was partially purified from 293T cell supernatant. The mass spectrometric analysis of the tryptic fragments of CCBE1 revealed ADAMTS3 as the major protease, that co-purified with CCBE1.

4.4 VEGF-C is processed by ADAMTS3

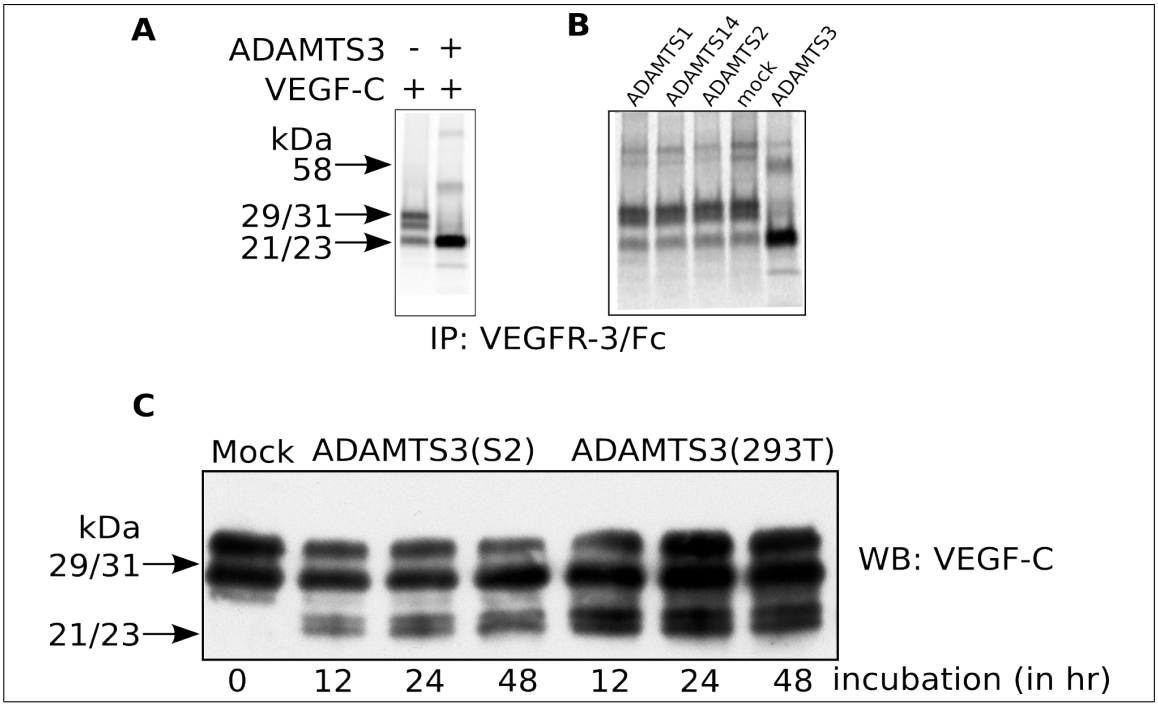

Figure 11: **ADAMTS3 cleaves VEGF-C**. A) Autoradiography showing efficient cleavage of VEGF-C when co-transfected with a construct coding for ADAMTS3. B) Autoradiography showing no detection of ADAMTS1, -2 and -14 -mediated processing of VEGF-C, compared to ADAMTS3 C) Western blot showing cleavage of VEGF-C by ADAMTS3 purified from insect (S2) cells and mammalian (293T) cells demonstrating protease activity after purification.

VEGF-C was efficiently processed by ADAMTS3 when co-transfected (**Figure 11A**). ADAMTS-2 and -14 belong to the same family of procollagen-N-proteinases (Lee

et al., 2012) and ADAMTS1 has been implicated in ovarian lymphangiogenesis (Brown et al., 2006). Therefore, we tested the effect of these proteases on VEGF-C.

However, the co-transfection of constructs coding for ADAMTS1, ADAMTS2 and ADAMTS14 with VEGF-C didn't affect the processing of VEGF-C (**Figure 11B**). The processing of VEGF-C by ADAMTS3 was further confirmed by incubating the purified proteins together (**Figure 11C**). Also in this system, VEGF-C was cleaved by ADAMTS3.

Interestingly, when constructs coding ADAMTS3 and CCBE1 were co-transfected, it resulted in cleavage of CCBE1 into a fragment of about 25kDa (**Figure 12**). The 25kDa size corresponds to the tagged C-terminal collagen-like domain of CCBE1, which is recognized by streptactin. Also antibodies against ADAMTS3 could precipitate the 25 kDa fragment of CCBE1.

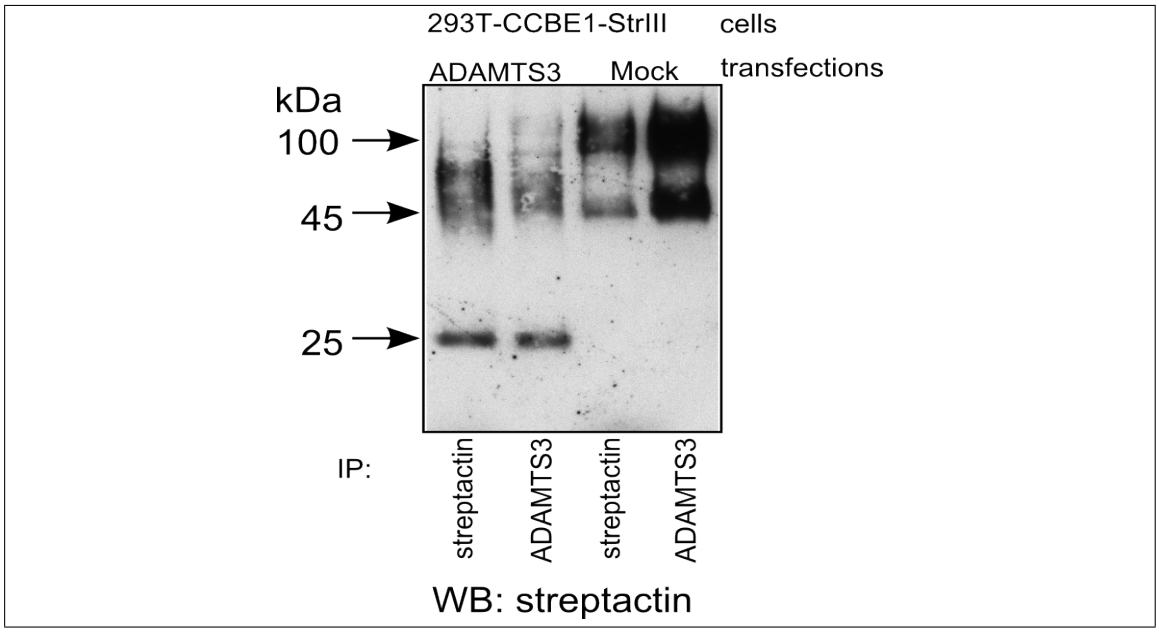

Figure 12: **ADAMTS3 transfection results in CCBE1 cleavage**. Western blot showing CCBE1 cleavage in the presence of ADAMTS3. Note, that the 25kDa fragment of CCBE1 is precipitated both with streptactin and ADAMTS3 antibodies.

4.5 CCBE1 enhances ADAMTS3 mediated VEGF-C cleavage

ADAMTS3 cleaves VEGF-C. In order to look for a possible cooperation of ADAMTS3 and CCBE1 in VEGF-C processing, the amount of ADAMTS3 and CCBE1 used for VEGF-C processing were titrated. For titration, conditioned media were mixed in different ratios. When CCBE1, VEGF-C and ADAMTS3 were mixed in a ratio of 60:30:1, ADAMTS3 did cleave VEGF-C more efficiently when CCBE1 was present compared to when CCBE1 was absent (**Figure 13A**). Additionally, the same conditioned media were used for a Ba/F3-hVEGFR-3/EpoR assay and the results were consistent with the Western blotting results (**Figure 13B**).

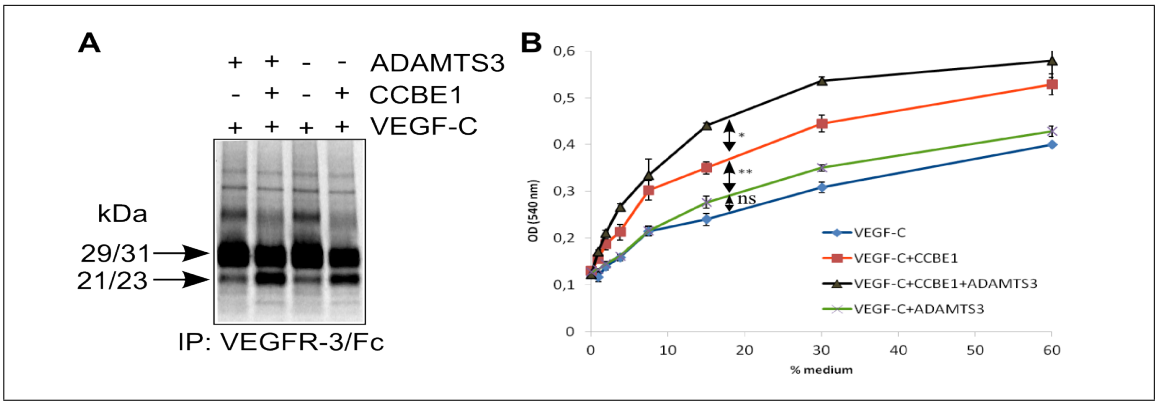

Figure 13: **CCBE1 enhances ADAMTS3-mediated VEGF-C cleavage**. A) Autoradiography showing that the effect of ADAMTS3 and CCBE1 together on VEGF-C processing is larger than other combinations (VEGF-C, VEGF-C+CCBE1 and VEGF-C+ADAMTS3). The conditioned media from separately transfected cells were mixed in a ratio of VEGF-C:CCBE1:ADAMTS3=60:30:1. B) Ba/F3-hVEGFR-3-EpoR assays with the samples from panel A. A significant increase in cell survival results when VEGF-C-, ADAMTS3- and CCBE1- media are mixed together compared to VEGF-C-media, VEGF-C-media mixed with CCBE1-media, or VEGF-C-media mixed with ADAMTS3-media. Note that the increase in survival of cells after mixing ADAMTS3- and VEGF-C-media was not significant compared to VEGF-C-medium alone. This likely results from the low concentration of ADAMTS3 (only about 1%) in the mix of conditioned media and the absence of CCBE1. $*$: P-value<0.05, $**$: P-value<0.01, $***$: P-value<0.001, ns: no significant.

4.6 Potential activation of VEGF-D by CCBE1

VEGF-D is similar in structure to VEGF-C. This led to the question, whether VEGF-D also is a substrate for ADAMTS3. No change in the ratio of pro-VEGF-D and mature VEGF-D was observed, when expression constructs coding for VEGF-D and CCBE1 were co-transfected (**Figure 14A**). Both VEGF-C and VEGF-D have a similar cleavage motif at the beginning of the VEGF homology domain (VHD) after the N-terminal propeptide. Hence, to study the nature of substrate recognition by ADAMTS3, two chimeric constructs were made exchanging domains between VEGF-C and VEGF-D (CDC-V1 and CDC-V2; see **Figure 15**).

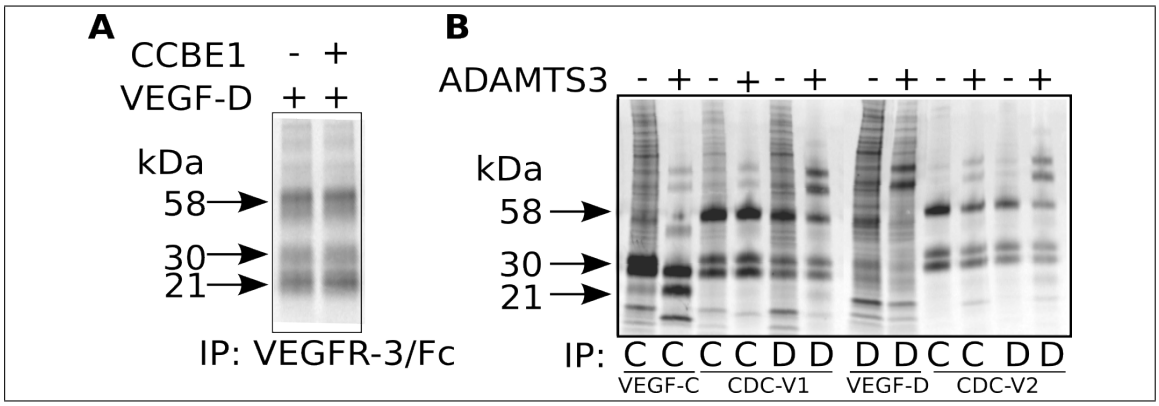

Figure 14: **No detection of CCBE1-mediated VEGF-D processing**. A) The autoradiography shows no change in the ratios of the different VEGF-D forms after transfection with expression construct coding for CCBE1 compared to mock. B) The autoradiography shows no significant differences in the processing of VEGF-D, CDC-V1 and CDC-V2 when transfected with a construct coding for ADAMTS3. VEGF-C was used as a positive control. Note, that bands higher than 58kDa are detected, which could represent aggregated protein. C: Anti-VEGF-C (R&D, AF752) and D: Anti-VEGF-D (R&D, AF286).

No increases of the amount of mature protein were observed when an expression construct coding for ADAMTS3 was co-transfected. The precipitation of chimeras from the conditioned media after transfection was done with antibodies against both VEGF-C and VEGF-D to detect all possible cleavage products. Surprisingly, high molecular weight bands were seen for all proteins when ADAMTS3 was present indicating possible ADAMTS3-mediated aggregation (**Figure 14B**).

N-terminal sequencing of the mature form of recombinant VEGF-C processed by

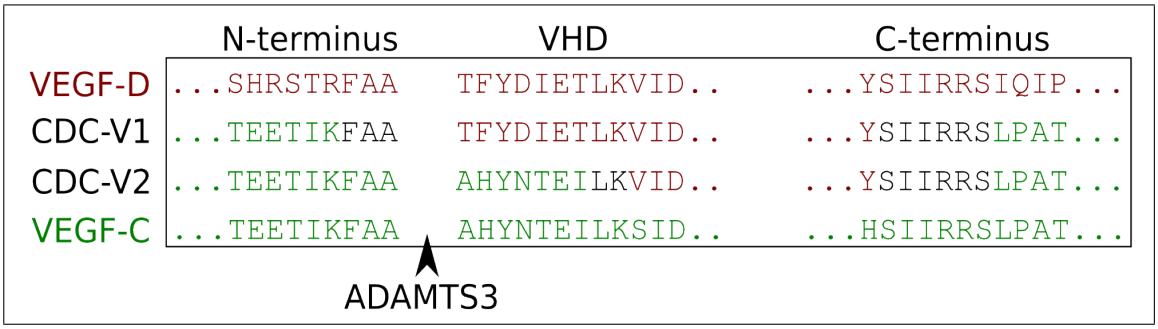

Figure 15: **Cleavage site comparison between VEGF-C, VEGF-D and VEGF-C/VEGF-D chimeras**

purified ADAMTS3 was performed and the N-terminal sequence was found to be AHYNT (**Figure 15**). This N-terminal form of VEGF-C had already been described (Joukov et al., 1997) and corresponds to the mature form of VEGF-C as produced by 293EBNA cells.

4.7 The N-terminal domain of CCBE1 enhances the processing of pro-VEGF-C into active VEGF-C

ADAMTS3 acts on CCBE1 and probably separates the N-terminal EGF-like and the C-terminal collagen-like domain. The difficulty to produce and purify large amounts of full-length CCBE1 prompted us to study the ability of the individual CCBE1 domains to aid in the processing of VEGF-C. The phosphorylation of VEGFR-3 in PAE cells stably expressing human VEGFR-3 was strongly enhanced when applying pro-VEGF-C together with CCBE1Δ175 compared to pro-VEGF-C alone (compare lane 2 and 3, **Figure 16A**). The strength of phosphorylation was similar to that obtained from mature VEGF-C (compare lane 1 and 3, **Figure 16A**).

When VEGFR-3 was precipitated and probed with a VEGF-C antibody, both pro- and mature VEGF-C were found to be bound to VEGFR-3 (**Figure 16B**) when pro-VEGF-C was used together with CCBE1 for the stimulation. In contrast, pro-VEGF-C alone was only bound to VEGFR-3 in presence of neuropilin-2 (**Figure 16D**). In order to identify the VEGF-C form bound to the phosphorylated VEGFR-3, the incubated samples were crosslinked during the phosphorylation step and then precipitated with phosphotyrosine antibody followed by probing with streptavidin-HRP (VEGF-C was biotinylated). The cross linking shows that the VEGFR-3

activation occurs only with the mature VEGF-C (**Figure 16C**) and pro-VEGF-C alone is not bound to the phosphorylated VEGFR-3.

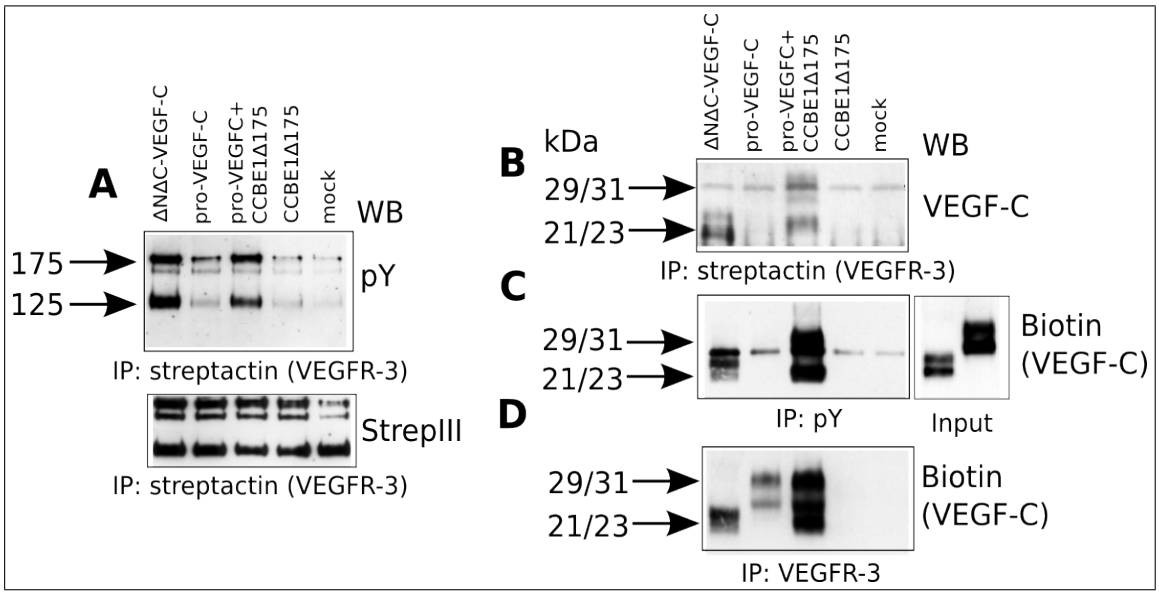

Figure 16: **The N-terminal domain of CCBE1 regulates VEGF-C mediated VEGFR-3 phosphorylation**. PAE cells stably expressing VEGFR-3 were stimulated with pro-VEGF-C alone or with the N-terminal domain of CCBE1 (CCBE1Δ175). A) Strong phosphorylation of VEGFR-3 is seen upon stimulation with CCBE1Δ175 and pro-VEGF-C compared to pro-VEGF-C alone. The lower panel shows the loading control. B) Cross-linking during stimulation shows binding of pro-VEGF-C and mature VEGF-C to VEGFR-3 when probed with the VEGF-C antibody. C) On the right is the input of VEGF-C used for the cross-linking experiment. Differences in the migration between lanes 1 and 3 (see Figure 16A to C) can result from differential N-glycosylation, the presence of the His-tag in ΔNΔC-VEGF-C or differences between cleavage and cDNA truncation. Cross-linking shows binding of the mature form of VEGF-C to the phosphorylated VEGFR-3. D) PAE cells stably expressing additionally Nrp2 were used for stimulation and cross linked as in C. In these cells, pro-VEGF-C is able to bind to VEGFR-3 without the help of CCBE1 but is not able to induce phosphorylation of VEGFR-3 (Michael Jeltsch, unpublished data).

5 Discussion

5.1 CCBE1 regulates VEGF-C processing

VEGF-C has to be proteolytically processed into the mature form that is mainly composed of the VEGF homology domain (VHD). This processing involves two sequential steps: removal of the C-terminal propeptide to give rise to pro-VEGF-C and finally the removal of the N-terminal propeptide to yield mature VEGF-C. It is this activation process that governs the differential specificity and affinity of VEGF-C for VEGFR-3 and VEGFR-2 (Joukov et al., 1997). Hence, understanding this activation mechanism is important. It is known that the secretory proprotein convertases furin mediates the C-terminal processing of VEGF-C (Siegfried et al., 2003). However, the processing of VEGF-C at its N-terminus is not clearly understood and has not been investigated in detail. One of the aims of this study was to search for the proteins involved in this proteolysis. Studies have shown involvement of CCBE1, a putative extracellular matrix protein in the regulation of embryonic lymphangiogenesis (Alders et al., 2009; Hogan et al., 2009). In addition, CCBE1 was shown to have a direct lymphangiogenic potential (Bos et al., 2011). Studies in mice showed enhanced lymphangiogenic activity of VEGF-C when CCBE1 and pro-VEGF-C are acting together (Michael Jeltsch, unpublished data). This encouraged a search for a possible link between VEGF-C and CCBE1.

This study shows that CCBE1 enhances VEGF-C processing when expressed together. The processed mature VEGF-C could be precipitated both with VEGFR-3/Fc and VEGFR-2/Fc, confirming that fully processed VEGF-C has both lymphangiogenic and angiogenic potential. Also in the Ba/F3 assays, mature VEGF-C was able to significantly increase the growth and survival of both VEGFR-2/EpoR and VEGFR-3/EpoR expressing Ba/F3 cells. The processing enhancement of VEGF-C in the presence of CCBE1 was efficient only in 293T cells. Although there is a large amount of endogenous CCBE1 secreted by DU-4475 cells, the conditioned media of these cells didn't influence the processing of VEGF-C. The mass spectrometry of partially purified CCBE1 showed the presence of ADAMTS3 as its interacting partner, which was later confirmed by a functional assay. We found that ADAMTS3 efficiently processes VEGF-C into its active, mature form. Since, ADAMTS-2 and -14 belong to the same procollagenase family as ADAMTS3 (Lee

et al., 2012) and ADAMTS1 has a significant role in ovarian lymphangiogenesis (Brown et al., 2006), they were also tested for their involvement in VEGF-C processing. However, no significant processing was detected.

VEGF-D, a structural homolog of VEGF-C, and VEGF-C itself are activated by plasmin (McColl et al., 2003). VEGF-C and VEGF-D have a similar cleavage motif at the beginning of the VEGF homology domain (VHD) after the N-terminal propeptide. Surprisingly, ADAMTS3 and CCBE1 had no effect on VEGF-D processing. In a further study using a chimeric form of VEGF-C and VEGF-D, no effect on processing was detected. The differential substrate recognition of VEGF-C and VEGF-D by ADAMTS3 might be due to differences between the charge environments preceding the VHD domain. Nevertheless, further studies need to be conducted to confirm the cleavage site nature and requirements for ADAMTS3 cleavage.

5.2 The interplay between CCBE1 and ADAMTS3 governs VEGF-C processing and signaling

Titrations were performed using different ratios of VEGF-C, CCBE1 and ADAMTS3. Importantly, ADAMTS3 was efficient at the very low concentration of about 1% provided CCBE1 was present, and the resulting products could significantly increase receptor activation. Hence, it is likely that CCBE1 could act as mediator to enhance VEGF-C processing through ADAMTS3. To confirm the identity of ADAMTS3-processed VEGF-C, N-terminal sequencing was performed and revealed the presence of the VHD domain with an N-terminus identical to the one described before (Joukov et al., 1997).

CCBE1 was difficult to express and purify in large amounts when we used the full length cDNA. This prevented us from gaining insight into the properties of its full-length form and behaviour in the cellular context. However, the N-terminal domain of CCBE1 could be purified and was used for interaction studies in PAE cells. The phosphorylation of VEGFR-3 was greatly increased when PAE cells were stimulated with the N-terminal domain of CCBE1 together with pro-VEGF-C in a short stimulation period. Cross linking during stimulation showed binding of both pro- and mature form of VEGF-C to VEGFR-3. It has been shown earlier that pro-

VEGF-C can bind to VEGFR-3 (Joukov et al., 1997; Lee, 1996). In our study, we show that pro-VEGF-C is bound to VEGFR-3 only in the presence of CCBE1, but that binding of the mature form of VEGF-C does not require CCBE1. We also show that mature VEGF-C binds to the phosphorylated receptor and hence is responsible to mediate VEGFR-3 signaling in the endothelial cells. Interestingly, binding of pro-VEGF-C to the PAE-VEGFR-3 cells in the absence of CCBE1 was observed only if the cells expressed additionally neuropilin-2. It is known that pro-VEGF-C binds to VEGFR-3 in the presence of neuropilin-2 (Kärpanen et al., 2006). ADAMTS3 contains thrombospondin motifs that likely bind to the CD36 receptor present on endothelial cells (Li et al., 1993). Further, our study shows that ADAMTS3 is specific for VEGF-C compared to its closest homologues. ADAMTS3 itself is a complex molecule and possesses multiple domains with different functions thought to be involved in cell to cell and cell to matrix interactions. Based on the domain structure, one could speculate that the receptor binding of ADAMTS3 is important in addition to its protease function. Additionally, the efficient activity of CCBE1 on VEGF-C on the PAE-VEGFR-3 cell surface compared to the activity of CCBE1 when incubated with VEGF-C in solution reflects the necessity of cellular surface or extracellular matrix (CCBE1 is thought to be the component of extracellular matrix) for the localization and concentration of VEGF-C activity *in vivo*. Our studies, indicate that ADAMTS3 is also able to regulate CCBE1 itself, but further functional studies are needed to confirm this aspect of ADAMTS3 function.

Based on this study, we propose a model (**Figure 17**) by which CCBE1 could regulate the VEGF-C mediated response to enhance its lymphangiogenic effect. According to our model, CCBE1 concentrates pro-VEGF-C to VEGFR-3, where ADAMTS3 or other proteases can cleave VEGF-C into the mature form. This mature form of VEGF-C has the maximal potential to activate VEGFR-3. Alternatively, ADAMTS3 could cleave VEGF-C without CCBE1 assistance but the presence of CCBE1 ensures the convergence of VEGF-C towards its receptor or other structures; hence ensuring its role as a lymphangiogenic factor. This mechanism is a likely scenario for developmental lymphangiogenesis where VEGF-C and CCBE1 act at the same developmental stage.

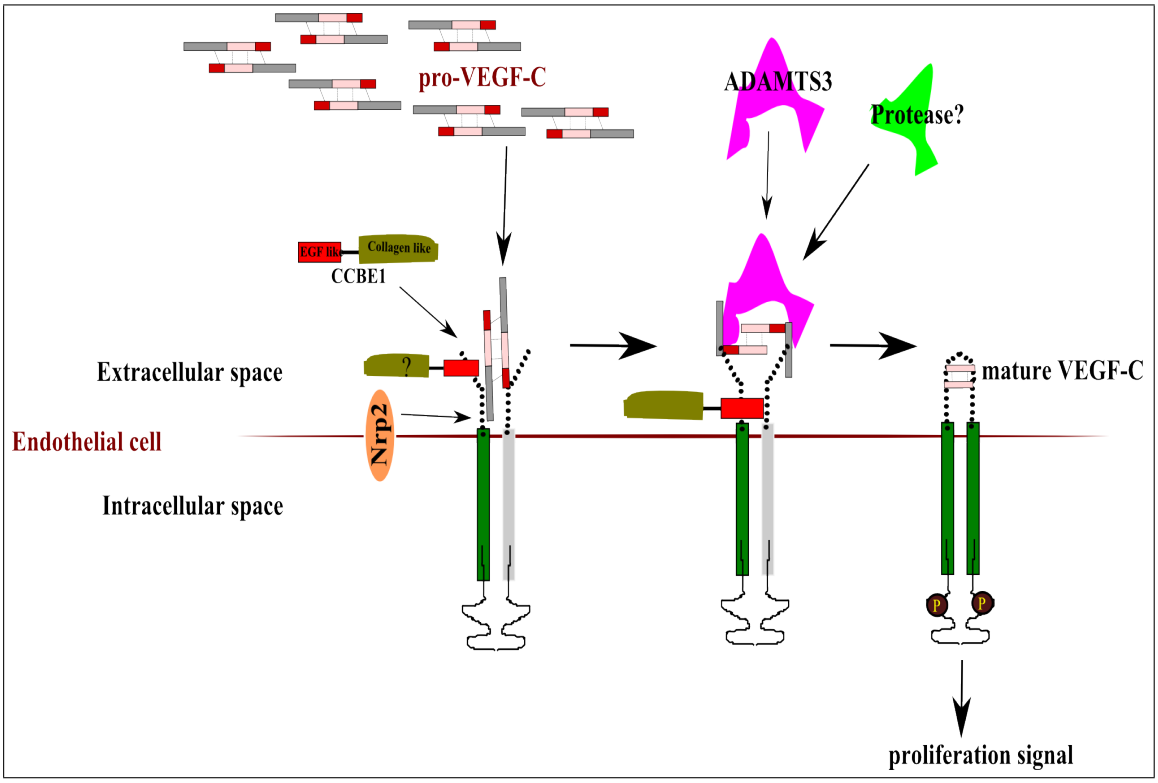

Figure 17: **A model for the regulation of VEGF-C activation**. Pro-VEGF-C can bind to VEGFR-3 in the presence of the N-terminal domain of CCBE1. ADAMTS3 or other unknown proteases process the bound pro-VEGF-C into the mature form. One half of the dimeric receptor in the complex is drawn transparently showing that dimerization may or may not be required for the initial binding of pro-VEGF-C.

6 Future aspects of study

This study shows the mechanism by which CCBE1 may cause the lymphatic phenotype of Hennekam syndrome. It also shows that CCBE1 itself is not a stimulator of lymphangiogenesis but that it regulates VEGF-C function. Plasmin is known to activate VEGF-C but such activation is most likely important only in pathological situations, e.g. during wound healing. Therefore, it would be interesting to investigate the relevance of this mechanism in the context of pathological processes like cancer. It would also be interesting to study whether cleavage of CCBE1 is required for its cleavage enhancing activity. This could be accomplished by creating CCBE1 mutants resistant to ADAMTS3 cleavage and then studying the effect of mutant

CCBE1 on VEGF-C processing and its effect on LECs.

Since CCBE1 was found to modulate lymphangiogenesis, it would be interesting to validate CCBE1 as a therapeutic target since there is no causal treatment available to primary lymphedema patients.

7 Conclusion

The aim of this study was to identify the mechanism involved in CCBE1-mediated lymphangiogenesis. Here we identify ADAMTS3 as a protease that activates VEGF-C. Moreover, this study implies a model by which CCBE1 can regulate VEGF-C to enhance its lymphangiogenic effect in vivo. CCBE1 itself has no lymphangiogenic potential but regulates VEGF-C requiring at least ADAMTS3 protease to exert its effect.

Lymphatic diseases can be either characterized by deficiency or by enhanced lymphangiogenic activity. Hence, there is a need to stimulate lymphangiogenesis in some diseases and to suppress it in others, and for the development of therapeutic strategies to accomplish these goals. This study provides a model and suggests CCBE1 as a potential target for lymphangiogenesis modulation.

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