Title: METHODS AND USES RELATED TO ADAMTS3

Abstract: The present invention relates to the utilization of ADAMTS3 metalloprotease. More specifically, the present invention relates to a method of producing fully processed mature VEGF-C polypeptide by a cell and a method of cleaving VEGF-C polypeptides. Furthermore, the present invention relates to different uses of ADAMTS3, ADAMTS3 inhibitors or compositions comprising ADAMTS3 or ADAMTS3 inhibitors, and a method of treating a lymphatic disorder. In addition, the invention relates to a method of producing VEGF-Ch Binding molecules.
METHODS AND USES RELATED TO ADAMTS3

FIELD OF THE INVENTION

The present invention relates to the utilization of ADAMTS3 metalloprotease. More specifically, the present invention relates to a method of producing fully processed mature VEGF-C polypeptide by a cell and a method of cleaving VEGF-C polypeptides. Furthermore, the present invention relates to different uses of ADAMTS3, ADAMTS3 inhibitors or compositions comprising ADAMTS3 or ADAMTS3 inhibitors, and a method of treating a lymphatic disorder. In addition, the invention relates to a method of producing VEGF-C binding molecules.

BACKGROUND OF THE INVENTION

Disorders or diseases that involve the lymphatic system include for example lymphedema, lipedema, many inflammatory processes (like type 1 diabetes, chronic airway diseases, psoriasis, metabolic disease, obesity) and cancer. Primary lymphedema is a chronic disabling and disfiguring condition of localized lymph fluid retention and swelling caused by a dysfunctional lymphatic system. It is a lifelong condition severely affecting the quality of life.

Primary lymphedema, a rare inherited disease, generally results from abnormal development of the lymphatic vessels. The symptoms of primary lymphedema may occur at birth or later in life.

Acquired or secondary lymphedema results from damage to the lymph system. In Western countries, it is most commonly caused by lymph node dissection, surgery and/or radiation therapy, in which damage to the lymphatic system is caused during the treatment of cancer, particularly breast cancer. It has been estimated that in the U.S. approximately 110,000 breast cancer patients suffer from lymphedema due to axillary lymph node dissection and/or radiation, and nearly 15,000 new patients develop the breast-cancer-associated lymphedema each year.

In addition to cancer therapies causing problems of the lymphatic system, also tumor progression is very closely linked to the lymphatic system. Primary tumors may occur in almost any tissues of the body. In cancer metastasis malignant cells escape from the tumor via lymph vessels or blood vessels. These cells move via the lymphatic system or the bloodstream to other parts of the body. They may arrest at a distant lymph node to form lymph node
metastases or extravasate through the blood vessel wall into the surrounding tissue followed by multiplication at the new location to form daughter tumors.

Therapeutic lymphangiogenesis, regeneration of lymphatic vessels, is an attractive prospect for treating various diseases that involve the dysfunctional lymphatic system. Despite the recent explosion of knowledge on the molecular mechanisms governing lymphangiogenesis the exact morphogenetic mechanisms of initial lymphangiogenesis remain incompletely understood.

Vascular endothelial growth factor C (VEGF-C) is the main driver of lymphangiogenesis in embryonic development and in various lymphangiogenic processes in adults (Alitalo, 2011, Nature Medicine 17: 1371–1380). VEGF-C acts by activating VEGFR-3 and - in its proteolytically processed mature form - also VEGFR-2. Deletion of the Vegfc gene in mice results in failure of lymphatic development due to the inability of newly differentiated lymphatic endothelial cells to migrate from the central veins to sites where the first lymphatic structures form (Karkkainen et al, 2003, Nature Immunology 5: 74–80; Hägerling et al, 2013, EMBO J 32: 629-644). This phenotype could be rescued by the application of recombinant VEGF-C (Karkkainen et al, 2003, ibid.). For the rescue, a “mature” recombinant form of VEGF-C was used, which lacked the N- and C-terminal propeptides. In cells secreting endogenous VEGF-C, these propeptides need to be proteolytically cleaved off from the central VEGF homology domain (VHD) in order for VEGF-C to reach its full signaling potential (Joukov et al, 1997, EMBO J 16: 3898–3911). VEGF-C can activate the main angiogenic receptor VEGFR-2 significantly only when both propeptides are cleaved off (Joukov et al, 1997, ibid.) and hence, the mature VEGF-C stimulates also angiogenesis.

Mutations in the VEGF-C receptor VEGFR-3 have been shown to result in hereditary lymphedema known as Milroy disease (Karkkainen et al, 2000, Nature Genetics 25: 153–159). On the other hand, the Hannekam lymphangiectasia–lymphedema syndrome is linked to mutations in the collagen-and calcium-binding EGF domains 1 (CCBE1) gene (Alders et al, 2009, Nature Genetics 41: 1272–1274), but it is unclear how the mutant CCBE1 causes the lymphatic phenotype. In any case, a genetic interaction of CCBE1 and VEGF-C has been suggested owing to the similarity of the phenotype of the CCBE1 -/- and the VEGF-C -/- mice and the more severe phenotype of the double heterozygous CCBE1+/--;VEGF-C+/- mice (Hägerling et al, 2013, ibid.).
Although CCBE1 has been suggested to augment the pro-lymphangiogenic activity of VEGF-C in a corneal micropocket assay (Bos et al. 2011, Circulation Research 109: 486–491), its true role in lymphangiogenesis has remained obscure. One of the reasons is that, since the production of native full-length CCBE1 was impossible, the CCBE1 protein used in the experiments was an artificial truncated CCBE1 protein fused with an Fc domain of human IgG.

Specific means, e.g. molecules interacting with VEGF-C growth factor or regulating the function of VEGF-C, are needed for increasing or blocking VEGF receptor signaling. Indeed, tools are needed for the modulation of lymphangiogenesis in disorders of the lymphatic system. For example, there is no curative treatment available for lymphedema and no specific drug to suppress lymphatic metastasis. Current practise of lymphedema is limited to symptomatic treatment and palliative care. And in cancer treatment, metastasis prevention would be preferable to the current metastasis treatment. Hence, there is a need for drugs that can modulate the lymphatic system.

BRIEF DESCRIPTION OF THE INVENTION

An object of the present invention is thus to provide specific methods and tools for modulating VEGF-C or its function. Also, the purpose is to develop therapies for treating lymphatic disorders with these specific tools.

The invention is based on the finding that ADAMTS-3 proteolytically cleaves the largely inactive VEGF-C precursor (pro-VEGF-C) resulting in full activation of VEGF-C, which leads to increased VEGF-C receptor VEGFR-3 and VEGFR-2 signaling, lymphangiogenesis and angiogenesis in vivo and that the cleavage activity of ADAMTS-3 is controlled by collagen- and calcium-binding EGF domains 1 (CCBE1) protein.

In different lymphatic disorders VEGFR-3 and/or VEGFR-2 receptor signaling is either impaired or increased too much. Defective receptor signaling leads to insufficient lymphangiogenesis (e.g. in lymphedema and lipedema), whereas increased receptor signaling results in too much lymphangiogenesis (e.g. in cancer, promoting lymphatic metastasis, and inflammatory processes, such as chronic airway diseases, psoriasis, type 1 diabetes, atherosclerosis or obesity-associated inflammation). Thus, the present invention provides therapeutic tools for the modulation of lymphangiogenesis and angiogenesis in a variety of diseases that are associated either with a dysfunctional or a co-opted lymphatic vasculature.
Furthermore, the invention is based on the realization that CCBE1 works via promoting the proteolytic cleavage of the largely inactive VEGF-C (i.e. the precursor 29/31-kDa form of VEGF-C) by ADAMTS3 protease, resulting in mature VEGF-C. Indeed, the invention reveals that ADAMTS3 protease can be used as a tool for increasing the VEGF-C receptor signaling by specifically cleaving the largely inactive VEGF-C and therefore, for modulating lymphangiogenesis and angiogenesis in subjects with a dysfunctional lymphatic system. On the other hand, the invention reveals that suppression or inhibition of ADAMTS3 can be used for down-regulating lymphangiogenesis and angiogenesis where appropriate, e.g. when inhibition of tumor progression and metastasis is attempted. According to the invention ADAMTS3 or a suppressor of ADAMTS3 can be used either alone or in combination with other therapeutically effective agents and/or treatments.

Advantages of the arrangements of the invention are that patients with lymphatic disorders may receive specific and effective treatments. Indeed, by the methods and uses of the present invention (i.e. utilizing CCBE1, a combination of CCBE1 and VEGF-C, ADAMTS3 or a combination of ADAMTS3 and any other therapeutic agent) it is possible to cleave or to modulate the cleavage of the precursor VEGF-C into the fully processed (i.e. mature) form, and thereby achieve the positive effects of VEGF-C, e.g. increased lymphangiogenesis and/or angiogenesis, in a target tissue. Furthermore, undesired lymphangiogenesis and/or angiogenesis can be suppressed by utilizing specific antibodies, binding proteins or aptamer oligonucleotides (i.e. specific against CCBE1, CCBE1 and VEGF-C, ADAMTS3, or ADAMTS3 and any other therapeutic target).

The objects of the invention are achieved by a method and an arrangement, which are characterized by what is stated in the independent claims. The specific embodiments of the invention are disclosed in the dependent claims.

In one aspect, the present invention relates to a method of producing fully processed mature VEGF-C polypeptide, wherein the method comprises the steps of

- optionally providing VEGF-C polypeptide in an incompletely processed or unprocessed form,
- providing ADAMTS3 polypeptide, and
- cleaving the incompletely processed or unprocessed form of VEGF-
C polypeptide by ADAMTS3,
  thereby producing mature VEGF-C.
In another aspect, the present invention relates to a method of
  optionally providing VEGF-C polypeptide in a incompletely pro-
  cessed or unprocessed form,
  providing ADAMTS3 polypeptide, and
  cleaving the incompletely processed or unprocessed form of VEGF-
  C polypeptide by ADAMTS3,
  thereby producing mature VEGF-C.
One further aspect of the present invention relates to a use of
  ADAMTS3 for producing fully processed mature VEGF-C polypeptide in cell
  culture.
  Another further aspect of the invention relates to a use of ADAMTS3
  for cleaving VEGF-C polypeptide.
  Still, the present invention relates to ADAMTS3 or a composition
  comprising ADAMTS3 for use as a medicament.
  Still, the present invention relates to ADAMTS3 or a composition
  comprising ADAMTS3 for use in the treatment of a lymphatic disorder.
  Still, the present invention relates to use of ADAMTS3 or a compo-
  sition comprising ADAMTS3 for the manufacture of a medicament for treatment of a lymphatic disorder.
  Still, the present invention relates to an inhibitor of ADAMTS3 or a
  composition comprising an inhibitor of ADAMTS3 for use as a medicament.
  Still, the present invention relates to an inhibitor of ADAMTS3 or a compo-
  sition comprising an inhibitor of ADAMTS3 for use in the treatment of a lymphatic disorder.
  Still, the present invention relates to use of an inhibitor of ADAMTS3
  or a composition comprising an inhibitor of ADAMTS3 for the manufacture of a
  medicament for treatment of a lymphatic disorder.
  Still, the present invention relates to a method of treating a lymphatic
  disorder by administering to a subject in need thereof ADAMTS3, an inhibitor
  of ADAMTS3 or a composition comprising ADAMTS3 or an inhibitor of
  ADAMTS3.
  Still further, the present invention relates to a method of producing a
  VEGF-C binding molecule against a fully processed mature VEGF-C polypep-
tide, wherein the method comprises the steps of

- cleaving the incompletely processed or unprocessed form of VEGF-C polypeptide by ADAMTS3 polypeptide to obtain a fully processed mature VEGF-C;

- immunizing a subject with the fully processed mature VEGF-C immunogen or screening a library with the fully processed mature VEGF-C; and

- generating VEGF-C binding molecules against the fully processed mature VEGF-C.

Other aspects, specific embodiments, objects, details and advantages of the invention are set forth in the following drawings, detailed description and examples.

BRIEF DESCRIPTION OF THE DRAWINGS

In the following the invention will be described in greater detail by means of preferred embodiments with reference to the attached drawings, in which

Figure 1 demonstrates that CCBE1 coexpression increases VEGF-C proteolytic processing to the mature, fully active VEGF-C. 293T cells were transfected with CCBE1 alone (Figure 1A) or with VEGF-C +/-CCBE1 (Figure 1D), incubated with radioactive amino acids and the medium supernatants (sup) and cell lysates were analysed by precipitation with a V5 antibody (IP: V5) and the soluble VEGF-C receptor (VEGFR-3/Fc) followed by autoradiography. Note that both intra- and extracellular CCBE1 is detected, and CCBE1 secretion is, unlike collagens, not dependent on ascorbate supplementation to the cell culture medium. Figure 1B shows a schematic view of the biosynthesis and processing of VEGF-C. Shown is the schematic structure of the VEGF-C precursor and the various processed forms (Karpanen & Alitalo, 2008, Annual Review of Pathology: Mechanisms of Disease 3: 367–397). Arrows point to the corresponding bands in panel D. Figure 1D demonstrates that VEGF-C precipitates with VEGFR-3/Fc contain uncleaved, partially cleaved and cleaved VEGF-C. Co-expression with CCBE1 decreases the amount of uncleaved VEGF-C and increases the amount of activated VEGF-C. Figure 1C illustrates that supernatants from cultures expressing both CCBE1 and VEGF-C have higher activity in promoting the growth of Ba/F3-VEGFR-3/EpoR cells than supernatants from cultures expressing only VEGF-C. Statistically significant difference of P<0.05 is marked with * and of P<0.001 with ***, n.s. indicates not
statistically significant differences; n = 4.

Figure 2 demonstrates that CCBE1 facilitates VEGF-C secretion. Figure 2A shows VEGF-C immunoprecipitation from supernatants and lysates of cells transfected with VEGF-C with and without CCBE1, as shown. The molecular weights on the left show the mobility of the major VEGF-C forms. Note that the amount of the mature 21 kDa form of VEGF-C increases (8-fold) in the supernatant and the amounts of the uncleaved VEGF-C and the 29/31 kDa polypeptide are reduced (by 74% and 51%, correspondingly) upon CCBE1 cotransfection (compare lanes 1, 2 to 3, 4). The 14 kDa fragment resulting from the N-terminal cleavage of VEGF-C is detected only in the supernatants of the cotransfected cells. Cotransfection with CCBE1 facilitates the secretion of VEGF-C as the intracellular VEGF-C polypeptides are reduced by 80% in the cell lysates of the CCBE1-cotransfected cells (compare lanes 5, 6 to 7, 8). Figure 2B shows that conditioned medium from cultures expressing both CCBE1 and VEGF-C have increased activity in promoting the growth of Ba/F3 cells expressing mouse (m) or human (h) VEGFR-2/EpoR chimeras than supernatants from cultures expressing only VEGF-C. The curves were statistically different from each other at all shown data points except for x=0 (P<0.05 marked with * and at P<0.01 marked with **; n = 3).

Figure 3 demonstrates that collagen-domain-truncated, Fc-fused CCBE1 does not affect the VEGFR-3 stimulating activity of VEGF-C. hVEGFR-3/EpoR-Ba/F3 cells were stimulated with increasing concentrations of human VEGF-C₃N₃C (mature VEGF-C; Figure 3A) or full-length VEGF-C (VEGF-C precursor; Figure 3B) in the presence of a constant dose (5 µg/ml) of truncated CCBE1-Fc (“A”) or negative control protein, which consists only of the Fc part (“B”). Cells were incubated in stimulation conditions for a total of 3 days. At the end of this period, thiazolyl blue tetrazolium bromide (MTT, Sigma-Aldrich) was added to cell cultures and the amount of living cells in each culture (a direct score of cell proliferation) was measured by OD540 optical density (yellow MTT is reduced by mitochondrial enzymes of living cells producing dark-blue material).

Figure 4 shows that VEGF-C cleavage is enhanced by cells expressing CCBE1 in trans. Separate cell populations were transfected with VEGF-C or CCBE1 and then mixed for the metabolic labeling period, as indicated. Note that when CCBE1 is (co-)transfected, the amounts of the mature VEGF-C (21 kDa) increase and the full-length (58 kDa) or partially cleaved
(29/31 kDa) VEGF-C decrease in the supernatant (Figure 4A), while the amounts in the cell lysates remain unchanged (Figure 4B). Note also a small difference in the migration of VEGF-C between the stably and transiently transfected cells, resulting from the different glycosylation pattern of VEGF-C produced by the stably transfected 293S GnTI− cells (Chaudhary et al., 2012, Nature Protocols 7: 453–466).

Figures 5 show that CCBE1 and VEGF-C do not interact in a stable manner, CCBE1 is produced by 293T, PC3 and DU-4475 cell lines, but DU-4475-produced CCBE1 does not promote VEGF-C cleavage. (A) Media conditioned with strep-tagged CCBE1 and full-length VEGF-C were mixed, but no retention of VEGF-C could be detected on the streptactin column (second lane). The faint signal results from non-specific binding of VEGF-C to the column (compare to the fourth lane, for which only media conditioned with full-length VEGF-C was applied to the column). (B) The ConA-bound fraction of conditioned cell culture medium was analyzed by SDS-PAGE/Western blotting. Note that the 293T and PC3 cell lines express only small amounts of endogenous CCBE1, whereas the DU-4475 cells express large amounts. Two species of CCBE1 can be detected in the supernatant, migrating around 45 and 100 kDa, respectively. In the transfected 293T cells, a short CCBE1 form of 25 kDa is observed. (C) Addition of conditioned medium (CM) from CCBE1-producing 293T cells to VEGF-C-producing cells promotes cleavage of VEGF-C, while CM from DU-4475 cells does not. This effect was observed whether serum-containing (10% FCS) or serum-free (0.2% BSA) medium was used for the conditioning. Figures 5 show that the VEGF-C cleavage-enhancing activity of CCBE1 is secreted into the medium, i.e. is a soluble component of the conditioned supernatant of CCBE1-producing 293T cells. This demonstrates that the activity is not restricted to the cell-surface or the extracellular matrix, but could be administered therapeutically e.g. as a soluble protein.

Figure 6 demonstrates that CCBE1 enhances lymphangiogenesis in vivo. Immunostaining of mouse tibialis anterior muscle transduced by AAV9 encoding the indicated factors and stained for the indicated antigens. Note that the full-length VEGF-C alone induces only a mild lymphangiogenic response, but its co-transduction with CCBE1 results in a strong response as detected by LYVE-1 and Prox-1 staining of lymphatic vessels. As a positive control, AAV9 encoding ΔNΔC-VEGF-C (an equivalent of the fully processed, mature VEGF-C) was used. The response to serum albumin was comparable to that of
CCBE1 alone. Statistically significant difference of $P<0.05$ is marked with *, of $P<0.01$ with ** and of $P<0.001$ with ***, n.s. indicates that the differences are not statistically significant; n $\geq 5$.

Figure 7 demonstrates that CCBE1 co-transduction with VEGF-C stimulates angiogenesis. Immunohistochemistry for endothelial (PECAM-1) and smooth muscle cell (SMA) markers in tibialis anterior muscles. Quantification of the stained areas is shown on the right. Statistically significant difference of $P<0.05$ is marked with *, of $P<0.01$ with ** and of $P<0.001$ with ***, n $\geq 5$.

Figure 8 illustrates recruitment of CD45+ leukocytes by CCBE1/VEGF-C co-transduction. Prox1 transcription factor was used as the marker for lymphatic endothelial cells. Analysis was done as in Figure 7.

Figure 9 illustrates VEGFR-3-luciferase reporter signals in mice injected with the indicated AAV9 vectors into the tibialis anterior muscle. Note that the co-transduction with VEGF-C and CCBE1 results in a strong luciferase signal indicating a major lymphangiogenesis response, while full-length VEGF-C and CCBE1 result only in minor luciferase activity.

Figure 10 shows that in a metabolic labeling-pulse-chase analysis, wild-type VEGF-C secretion peaks at 2h, whereas a VEGF-C mutant without the C-terminal propeptide ($\Delta C$-VEGF-C) peaks already between 15 and 45 minutes.

Figure 11 shows the effect of the N- and C-terminal propeptides of VEGF-C on growth factor processing. Note that the amounts of both the mature 21 kDa form of VEGF-C and the 14 kDa N-terminal propeptide are reduced by competition with the C- and N-terminal propeptides (Figure 11A).

Figure 11B illustrates the proteolytic processing of VEGF-D and the VEGF-D/VEGF-C chimera (CDC) in the presence or absence of CCBE1 cotransfection. C-pp, C-terminal propeptide; N-pp, N-terminal propeptide; HSA, human serum albumin; $\Delta N\Delta C$, C-and N-terminally truncated form of VEGF-C comparable to mature VEGF-C (Kärpänen et al. 2006, Faseb J, 20:1462-1472).

Figure 12 demonstrates that in ADAMTS3-transfected cells CCBE1 is very efficiently cleaved. (A) ADAMTS3-transfection supernatants of stable VEGF-C-expressing 293T cells were immunoprecipitated with VEGFR-3/Fc. In the supernatant of mock-transfected cells, only modest amounts of mature VEGF-C are seen in the supernatant. CCBE1 transfection alone results in a partial conversion of pro-VEGF-C into mature VEGF-C, while ADAMTS3 transfection results in the complete disappearance of pro-VEGF-C and highest
amounts of mature VEGF-C. (B) Conditioned medium of VEGF-C-expressing cells was mixed with conditioned media from CCBE1-, ADAMTS3- and mock-transfected cells. When ADAMTS3-conditioned supernatant was used at a concentration of 1%, its effect on VEGF-C cleavage was indistinguishable from the mock sample. While CCBE1 alone resulted in appreciable activation of VEGF-C (presumably due to the endogenous ADAMTS3 produced by 293T cells), the highest levels of active VEGF-C were observed when both ADAMTS3 and CCBE1 were present. Fold increase of mature VEGF-C (marked by the red frame) is given relative to medium of untransfected cells. (C) Supernatants of VEGF-C-expressing CHO cells were mixed with sups of 293T cells expressing CCBE1 and ADAMTS3 and untransfected 293T cells at ratio of 15:16:2:47, incubated for 24h and assayed for their ability to promote the survival of Ba/F3-hVEGFR-3/EpoR cells. The curves were statistically different from each other at all data points except for those without fill and the comparison VEGF-C versus VEGF-C+ADAMTS3 (P<0.05 marked with * and at P<0.001 marked with **; n = 3). (D) ADAMTS3 can cleave CCBE1, separating the C-terminal collagen-like domain from the N-terminal domain. The C-terminal domain of CCBE1 can be co-precipitated using an antibody directed against ADAMTS3. Note, that full-length CCBE1 non-specifically binds to protein G sepharose and that the detection of any co-precipitating full-length CCBE1 is therefore not possible.

Figure 13 shows the effect of the N-terminal domain of CCBE1 on the induction of phosphorylation by pro-VEGF-C. VEGFR-3 expressing PAE cells were stimulated with pro-VEGF-C together with the N-terminal domain of CCBE1 (CCBE1Δ175) or the mature VEGF-C. (A) Pro-VEGF-C induces only a marginal activation of the receptor by itself, while in the presence of CCBE1Δ175 it induces a robust phosphorylation response. (B) VEGFR-3-bound mature VEGF-C was detected when CCBE1Δ175 had been present during the stimulation with pro-VEGF-C, indicating that CCBE1-enhanced processing takes place during the stimulation period. (C) Cross-linking of VEGF-C during VEGFR-3 stimulation shows, that mature VEGF-C is bound to the phosphorylated VEGFR-3. Note, that the apparent molecular weight of the mature VEGF-C produced by CCBE1-enhanced cleavage of pro-VEGF-C and the recombinant ΔNΔC-VEGF-C differ, because ΔNΔC-VEGF-C is produced from a truncated, histidine-tagged cDNA resulting in non-native N- and C-termini of the protein and differential glycosylation. (D) An experiment similar to that of
(C) done with VEGFR-3 plus neuropilin-2 expressing cells. The asterisks in (B) and (C) mark non-specific signals with the same electrophoretic mobility as the 29 kDa fragment of pro-VEGF-C.

Figure 14 shows that pro-VEGF-C can inhibit mature VEGF-C. (A) 10 minutes pre-incubation of LECs with pro-VEGF-C reduces their ability to respond to mature VEGF-C. Arrows denote the 125 and 175 kDa fragments of VEGFR-3. Western blotting was performed on separate gels. (B) Mature VEGF-C, but not pro-VEGF-C, induces the phosphorylation of Erk, Akt and eNOS. eNOS detection was performed on the stripped pErk membrane, and actin detection was performed on the stripped pAkt membrane. (C) HUVECs stably expressing VEGFR-3-GFP fusion protein were used for live cell fluorescence imaging after the addition of pro-VEGF-C or ΔNΔC-VEGF-C. ΔNΔC-VEGF-C resulted in a rapid internalization of VEGFR-3 (first row), which can be blocked to a large extent by the anti-VEGFR-3 antibody 3C5 (second row). Pro-VEGF-C does not change significantly the cell surface localization of VEGFR-3 (third row). Arrows denote the concentration of VEGFR-3 in endosomes after 40 minutes of stimulation.

Figure 15 shows a schematic view of VEGFR-3 activation by VEGF-C. Pro-VEGF-C binding to VEGFR-3 is assisted by the N-terminal domain of CCBE1. Pro-VEGF-C is then proteolytically processed in-situ and the mature VEGF-C initiates VEGFR-3 signaling. Alternatively, in the presence of CCBE1, pro-VEGF-C might initiate signaling without the need of proteolytic processing. Note, that the presence of the transparently shown molecules is hypothetical: VEGFR-3 could be either monomeric or dimeric during the initial binding of VEGF-C Furthermore; it is unknown whether the removal of the C-terminal domain of CCBE1 is a prerequisite for the CCBE1 action.

Figure 16 shows plasmin cleavage of VEGF-C and the activity of plasmin-cleavage products in the Ba/F3-VEGFR-3/EpoR assay. (A) Pro-VEGF-C is cleaved by plasmin, but the cleavage is not enhanced by CCBE1 (B). (C) While low amounts of plasmin resulted in a strong activation of VEGF-C, high amounts led to inactivation of VEGF-C.

Figure 17 shows potential cleavage of VEGF-D by ADAMTS3, and VEGF-C by other ADAMTS family members. (A) VEGF-C is cleaved by recombinant ADAMTS3. (B) VEGF-D was incubated with or without ADAMTS3, but no difference in the ratio between mature VEGF-C and the pro-VEGF-C form were observed. The presence of CCBE1 had again no effect. (C) Align-
ment of the cleavage context for plasmin and ADAMTS2/3 substrates. The cleavage contexts of the four known procollagen targets of ADAMTS2 were aligned with VEGF-C and VEGF-D sequences. The ADAMTS2 cleavage sites are indicated by a yellow triangle, the ADAMTS3 cleavage site by a black triangle, plasmin cleavage sites are indicated by red triangles and the furin cleavage site by a blue triangle. The numbers denote the theoretical isoelectric point of the stretch comprising the 10 amino acid residues N-terminal to the first plasmin cleavage site. Background color-coding for the amino residues: magenta, acidic; red, basic; green: ADAMTS2/3 cleavage motif; yellow, exceptions to the cleavage motif. (D) Transfection of ADAMTS1 and the procollagenase-type ADAMTS2 and ADAMTS14 into VEGF-C expressing 293F cells did not result in increased generation of mature VEGF-C as detected by VEGF-C antiserum after VEGFR-3/Fc precipitation.

Figure 18 shows comparison of expression levels of ADAMTS2, ADAMTS3 and ADAMTS14 by quantitative PCR. (A) Comparison of individual ADAMTS expression levels between different cell types relative to CHO =1. (B) Comparison within one cell type relative to ADAMTS2 = 1. The cell lines that do process VEGF-C into its mature form do all express ADAMTS3, while those cells that are unable to process VEGF-C into its mature form do express very low amounts of ADAMTS3 or none at all.

Figure 19 shows suppression of VEGF-C processing by ADAMTS3 shRNA and effect of the N- and C-terminal propeptides of VEGF-C on growth factor processing. (A) The processing of pro-VEGF-C into mature VEGF-C was reduced in 293T cells by lentiviral ADAMTS3 shRNA compared to the non-target shRNA. Note that the baseline processing of VEGF-C is increased in the cells transduced with non-targeting control shRNA compared to the non-transduced cells. (B) Alignment of the amino acid sequence of the chimeras and their parent proteins. VEGF-C-derived sequences are shown in green, VEGF-D-derived sequences in red. The boxed region marks where the proteolytic cleavage occurs in VEGF-C and VEGF-D.

DETAILED DESCRIPTION OF THE INVENTION

The present invention demonstrates a novel type of regulation of a VEGF-C. We have explored the link between CCBE1 and VEGF-C using both in vitro and in vivo assays and report that ADAMTS3 cleaves VEGF-C. CCBE1 affects lymphangiogenesis by enhancing the cleavage of VEGF-C by the
ADAMTS3 metalloprotease, which removes the N-terminal propeptide from pro-VEGF-C, resulting in the mature, fully active VEGF-C.

We were able to demonstrate that CCBE1 interacts with the metalloproteinase ADAMTS3 as shown by mass spectrometry and a functional assay. ADAMTS3 cleavage of pro-VEGF-C was enhanced by CCBE1. CCBE1 does not cleave VEGF-C, but it greatly enhances the activation of pro-VEGF-C. Based on our results we propose the model of VEGFR-3 activation shown in Figure 15. First, CCBE1 enables pro-VEGF-C binding to VEGFR-3. After binding, pro-VEGF-C becomes a substrate for proteases such as ADAMTS3, and the in-situ generated mature VEGF-C initiates signaling.

Our results prove ADAMTS3 and CCBE1 as critical factors of VEGF-C cleavage, and provide ADAMTS3 and/or CCBE1 as tools for therapeutics. ADAMTS3 is also reported for the production of anti-VEGF-C antibody.

**VEGF-C**

According to the present invention, the fully processed mature VEGF-C is obtained by the cleavage ability of ADAMTS3. This fully processed mature VEGF-C is able to activate its receptors.

As used herein, the term "VEGF-C" refers to any VEGF-C, such as any VEGF-C polypeptide or VEGF-C polynucleotide including for example any variants of VEGF-C and recombinant VEGF-Cs.

As used herein, the term "VEGF-C polypeptide" refers to any known form of VEGF-C including prepro-VEGF-C, partially processed VEGF-C, and fully processed mature VEGF-C. During its biosynthesis, the full-length form of VEGF-C (58 kDa) first undergoes a proteolytic cleavage in the C-terminal part, resulting in the 29/31 kDa intermediate form held together via disulfide bonds, and a subsequent cleavage at two alternative sites in the N-terminus, yielding the mature, fully active 21 kDa or 23 kDa form of VEGF-C. This process is known to be inefficient, as the majority of VEGF-C protein does not become activated. However, the difference in the lymphangiogenic potential between the mature and the 29/31 kDa intermediate forms is remarkable (Anisimov et al, 2009, Circulation Research 104:1302–1312).

In some embodiments, the VEGF-C polypeptide in accordance with the present invention is the full-length, or prepro, form of VEGF-C. In some further non-limiting embodiments, the prepro-VEGF-C polypeptide lacks a sig-
nal sequence and, thus, may comprise amino acids 32-419 of the sequence depicted in SEQ ID NO: 3, for instance. A person skilled in the art realizes that there are alternative cleavage sites for signal peptidases and that other proteases may process the N-terminus of VEGF-C without affecting the activity thereof. Consequently, the VEGF-C polypeptide may differ from that comprising or consisting of amino acids 32-419 of SEQ ID NO: 3.

Alternatively or additionally, the VEGF-C polypeptide may be in the form of a partly processed VEGF-C, such as that comprising amino acids 32-227 covalently linked to amino acids 228-419 of the amino acid sequence depicted in SEQ ID NO: 3. Again, owing to alternative cleavage sites for signal peptidases and other proteases, the partially processed VEGF-C polypeptide may have an amino acid composition different from that of the non-limiting example described above without deviating from the present invention and its embodiments.

As used herein “an incompletely processed or unprocessed form of VEGF-C” refers to any other VEGF-C forms than the fully processed form. Therefore, incompletely processed or unprocessed forms of VEGF-C include but are not limited to a full-length form, prepro-form, prepro-form lacking a signal sequence and partly processed VEGF-C form.

As used herein “a fully processed mature VEGF-C” refers to an active form of VEGF-C. An active form of VEGF-C is able to activate VEGFR-3 and/or VEGFR-2 and thus, also the signalling cascade from these receptors. In some embodiments, the VEGF-C polypeptide is in the fully processed, or mature, form thereof such as that comprising amino acids 112-227 or 103-227 of the amino acid sequence depicted in SEQ ID NO: 3. Further, the VEGF-C polypeptide may be in any other naturally occurring or engineered form. If desired, different forms of VEGF-C polypeptides may be used in any combination. In a specific embodiment, the VEGF-C polypeptide is a mammalian VEGF-C polypeptide, more preferably human.

It is also contemplated that any of the VEGF-C polypeptides described herein may vary in their amino acid sequence as long as they retain their biological activity, particularly their capability to bind and activate VEGFR-2 and/or VEGFR-3. In some embodiments, the VEGF-C polypeptide may be a conservative sequence variant of any VEGF-C polypeptide described herein or it may comprise an amino acid sequence that is at least 85%, 90%, 95%, 96%, 97%, 98%, 99% or more identical to the amino acid sequence depicted in SEQ ID NO: 3, or any biologically relevant fragment thereof.
As used herein, the % identity between the two sequences is a function of the number of identical positions shared by the sequences (i.e. % identity = # of identical positions/total # of positions x 100), taking into account the number of gaps, and the length of each gap, which need to be introduced for optimal alignment of the two sequences. The comparison of sequences and determination of identity percentage between two sequences can be accomplished using mathematical algorithms available in the art. This applies to both amino acid and nucleic acid sequences.

The cleavage of VEGF-C by ADAMTS3 results in the fully processed mature form of VEGF-C. In one embodiment of the invention the fully processed mature VEGF-C is in the form of a polypeptide comprising amino acids 112-227 of SEQ ID NO: 3 or an amino acid sequence having at least 80% amino acid sequence identity therewith. In another embodiment of the invention, the fully processed mature VEGF-C polypeptide consists of amino acid residues 112-227 of SEQ ID NO: 3. The fully processed mature VEGF-C formed by the ADAMTS3 cleavage is identical to a naturally occurring mature VEGF-C. VEGF-C, which is produced from a truncated cDNA, is not identical to the naturally occurring mature VEGF-C. When VEGF-C is produced from a full-length cDNA, most of the resulting protein is in the inactive pro-VEGF-C form. Many cell line used for protein production (like CHO cells) are almost completely unable to produce mature VEGF-C and even 293T cells (which do the best job in producing mature VEGF-C from a full length cDNA from all cell lines we tested) process VEGF-C very inefficiently. Therefore, the present invention provides very specific tools (i.e. ADAMTS3) for cleaving VEGF-C to a form, which is identical to the naturally occurring mature VEGF-C. Inefficient or even absent VEGF-C cleavage of the cells may be enhanced by the present invention, thereby increasing amount of fully processed mature VEGF-C.

As used herein, the term "VEGF-C polynucleotide" refers to any polynucleotide, such as single or double-stranded DNA or RNA, comprising a nucleic acid sequence encoding any VEGF-C polypeptide. For instance, the VEGF-C polynucleotide may encode a full-length VEGF-C and comprise or consists of nucleic acids 524-1687 of a nucleic acid sequence depicted in SEQ ID NO: 4. In some other embodiments, the VEGF-C polynucleotide may encode intermediate forms of VEGF-C and comprise or consists of either nucleic acids 737-1687 or 764-1687 of the nucleic acid sequence depicted in SEQ ID NO: 4. In some further embodiments, the VEGF-C polynucleotide may encode mature forms of
VEGF-C and comprise or consists of either nucleic acids 737-1111 or 764-1111 of the nucleic acid sequence depicted in SEQ ID NO: 4. None of the above embodiments contains sequences encoding a signal peptide or a stop codon but other embodiments may comprise such sequences. In some still further embodiments, the C-terminus of the mature forms may be shortened without losing receptor activation potential.

Conservative sequence variants of said nucleic acid sequences are also contemplated. In connection with polynucleotides, the term “conservative sequence variant” refers to nucleotide sequence modifications, which do not significantly alter biological properties of the encoded polypeptide. Conservative nucleotide sequence variants include variants arising from the degeneration of the genetic code (silent mutations).

Nucleotide substitutions, deletions and additions are also contemplated. Accordingly, multiple VEGF-C encoding polynucleotide sequences exist for any given VEGF-C polypeptide, any of which may be used therapeutically as described herein.

In some further embodiments, the VEGF-C polynucleotide may comprise a nucleic acid sequence which is at least 85%, 90%, 95%, 96%, 97%, 98%, 99% or more identical to the VEGF-C nucleic acid sequences described above, as long as it encodes a VEGF-C polypeptide that has retained its biological activity, particularly the capability to bind and activate VEGFR-2 and VEGFR-3.

In some embodiments, any VEGF-C polynucleotide described herein comprises an additional N-terminal nucleotide sequence motif encoding a secretory signal peptide operably linked to the polynucleotide sequence. The secretory signal peptide, typically comprised of a chain of approximately 5 to 30 amino acids, directs the transport of the polypeptide outside the cell through the endoplasmic reticulum, and is cleaved from the secreted polypeptide. Suitable signal peptide sequences include those native for VEGF-C, those derived from another secreted proteins, such as CD33, Ig kappa, or IL-3, and synthetic signal sequences.

A VEGF-C polynucleotide may also comprise a suitable promoter and/or enhancer sequence for expression in the target cells, said sequence being operatively linked upstream of the coding sequence. If desired, the promoter may be an inducible promoter or a cell type specific promoter, such as an endothelial cell specific promoter. Suitable promoter and/or enhancer se-
quences are readily available in the art and include, but are not limited to, EF1, CMV, and CAG.

Furthermore, any VEGF-C polynucleotide described herein may comprise a suitable polyadenylation sequence operably linked downstream of the coding sequence.

VEGF-C of the present invention may be an animal, mammalian or human VEGF-C. In a specific embodiment of the invention, VEGF-C is a human VEGF-C. In one embodiment of the invention, the incompletely processed or unprocessed VEGF-C polypeptide is a recombinant polypeptide. As used herein, “a recombinant polypeptide” refers to a polypeptide produced by recombinant DNA techniques. For example, VEGF-C may be in a form of a fusion protein of VEGF-C and any other protein.

**ADAMTS3**

ADAMTS3 (i.e. ADAM with thrombospondin type 1 motif, 3) is a member of the ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) protein family. Members of the family share several distinct protein modules, including a propeptide region, a metalloproteinase domain, a disintegrin-like domain, and a thrombospondin type 1 (TS) motif. Individual members of this family differ in the number of C-terminal TS motifs, and some have unique C-terminal domains. The protein encoded by ADAMTS3 gene is the major procollagen II N-propeptidase. Endothelial cells express ADAMTS3 (Aung et al., 2011, Physiol Genomics 43:917-929), most of which remains likely cell-surface associated due to a thrombospondin motif, which contains the high-affinity SVTCG binding site that confers CD36 binding (Li et al., 1993, J Biol Chem 268:16179-16184).

As used herein, the term “ADAMTS3” refers to a full-length ADAMTS3 polypeptide or to a polynucleotide encoding said full-length ADAMTS3, unless clearly stated otherwise. ADAMTS3 of the invention may be an animal, mammalian or human ADAMTS3. In one embodiment, ADAMTS3 is a human ADAMTS3. In some embodiments, the full-length ADAMTS3 polypeptide comprises an amino acid sequence depicted in SEQ ID NO: 11. In one embodiment, the ADAMTS3 polypeptide is without a signal peptide and comprises as amino acid sequence depicted in SEQ ID NO: 12. ADAMTS3 polypeptide may also be a truncated ADAMTS3 polypeptide. In one embodiment of the invention ADAMTS3 has a sequence of SEQ ID NO: 11 or SEQ ID NO: 12.
It is evident to a person skilled in the art that the ADAMTS3 polypeptide to be used in accordance with the present invention may vary from the polypeptide depicted in SEQ ID NO: 11 or SEQ ID NO: 12 as long as it retains its biological activity. An exemplary way of determining whether or not a ADAMTS3 variant has maintained its biological activity is to determine its ability to cleave full-length VEGF-C. This may be performed e.g. by incubating cells expressing full-length VEGF-C with the ADAMTS3 variant in question and concluding that the ADAMTS3 variant has retained its biological activity if VEGF-C cleavage is enhanced. Said VEGF-C cleavage may be determined e.g. by metabolic labelling and protein-specific precipitation, such as immunoprecipitation, according to methods well known in the art.

In some embodiments, the ADAMTS3 polypeptide comprises an amino acid sequence which is a conservative sequence variant of SEQ ID NO: 11 or SEQ ID NO: 12. In some other embodiments, the ADAMTS3 polypeptide comprises an amino acid sequence that is at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or more identical to the amino acid sequence depicted in SEQ ID NO: 11 or SEQ ID NO: 12.

As used herein, the term “ADAMTS3 polynucleotide” refers to any polynucleotide, such as single or double-stranded DNA or RNA, comprising a nucleic acid sequence encoding an ADAMTS3 polypeptide. In some embodiments, the ADAMTS3 polynucleotide comprises a nucleic acid sequence depicted in SEQ ID NO: 13, SEQ ID NO: 14, with 5' and 3' untranslated regions (UTRs), or a conservative sequence variant thereof. In some preferred embodiments, the ADAMTS3 polynucleotide has a nucleic acid sequence of SEQ ID NO: 13 or SEQ ID NO: 14 or a conservative sequence variant thereof.

Multiple ADAMTS3 encoding polynucleotide sequences exist for any ADAMTS3 polypeptide, any of which may be used in accordance with the present invention. In some further embodiments, the ADAMTS3 polynucleotide may be at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or more identical to the nucleic acid sequence depicted in SEQ ID NO: 13 or SEQ ID NO: 14, as long as it encodes a ADAMTS3 polypeptide that has retained its biological activity.

An ADAMTS3 polynucleotide may also comprise a suitable promoter and/or enhancer sequence for expression in the target cells, said sequence being operatively linked upstream of the coding sequence. If desired, the promoter may be an inducible promoter or a cell type specific promoter, such as
an endothelial cell specific promoter. Suitable promoter and/or enhancer sequences are readily available in the art and include, but are not limited to, EF1, CMV, and CAG.

Furthermore, any ADAMTS3 polynucleotide described herein may comprise a suitable polyadenylation sequence operably linked downstream of the coding sequence.

In one embodiment of the invention, the ADAMTS3 polypeptide is a recombinant polypeptide. For example, ADAMTS3 may be in a form of a fusion protein of ADAMTS3 and any other protein.

**ADAMTS3 inhibitors**

According to the present invention ADAMTS3 inhibitors can be used for decreasing the level of active mature VEGF-C. These inhibitors negatively modulate i.e. inhibit or suppress lymphangiogenesis and they are needed for example in cancer therapy or anti-inflammatory therapy.

As used herein "an inhibitor or ADAMTS3" refers to any molecule which specifically binds to ADAMTS3 and thereby inhibits the function of ADAMTS3. Molecules inhibiting the function of ADAMTS3 are not limited to anti-ADAMTS3-antibodies, but also include any molecules such as polypeptides, polynucleotides or small molecules having binding affinities to ADAMTS3 (either to ADAMTS3 polypeptide or polynucleotides (deoksiribonucleotides or ribonucleotides)). In one embodiment of the invention the inhibitor of ADAMTS3 is an anti-ADAMTS3-antibody or an ADAMTS3 binding polypeptide, polynucleotide or small molecule. As used herein, “a small molecule” refers to a molecule is a low molecular weight (<900 Daltons) organic compound that regulates a biological process, herein binds specifically to ADAMTS3.

As used herein “antibodies” refer to proteins produced by immune cells or by recombinant techniques in response to foreign materials known as antigens. Antibodies of the present invention bind to ADAMTS3 and either mark them for destruction or inhibit their function by binding to them.

As used herein “binding polypeptides” refer to polypeptides which bind to ADAMTS3 and thereby inhibit or suppress their function.

Any processes described herein under the chapter “Antibody and binding molecule production” can be used for screening or producing ADAMTS3 inhibitors. An exemplary way of determining whether or not a molecule inhibits ADAMTS3 is to determine its ability inhibit the cleavage of full-
length VEGF-C by ADAMTS3. This may be performed e.g. by incubating cells having ADAMTS activity and expressing full-length VEGF-C with the ADAMTS3 inhibitor in question and concluding that the ADAMTS3 inhibitor indeed functions if VEGF-C cleavage is decreased or inhibited. Said VEGF-C cleavage may be determined e.g. by metabolic labelling and protein-specific precipitation, such as immunoprecipitation, followed by PAGE analysis and suitable protein detection, according to methods well known in the art.

**Administration**

The cleavage of the incompletely processed or unprocessed VEGF-C by ADAMTS3 occurs primarily outside the cell, e.g. on the cell surface or in the extracellular matrix. Therefore, ADAMTS3, ADAMTS3 inhibitor and/or VEGF-C may be provided therein. According to the present invention the incompletely processed or unprocessed VEGF-C is optionally provided to a target tissue or cell culture. Indeed, if the cell microenvironment already contains large amounts of VEGF-C (specifically incompletely processed or unprocessed VEGF-C), it is not always necessary to provide additional VEGF-C. In such a case only addition of ADAMTS3 may be sufficient to increase the VEGF-C receptor activation. However, if further increased VEGF-C receptor activation is needed, incompletely processed or unprocessed VEGF-Cs may be provided to the cell culture or tissue simultaneously, separately or sequentially with ADAMTS3. As used herein “cell culture” refers to a group of cells (i.e. comprising at least two cells) in *in vitro*, *ex vivo* or *in vivo* conditions.

ADAMTS3 may be administered to a target tissue or cell culture in various ways, for instance by gene administration or gene therapy, protein administration or protein therapy, or any desired combination thereof. Administration of ADAMTS3 by different ways or routes may be simultaneous, separate, or sequential.

Depending on the use or method of the invention, ADAMTS3 may be the only agent to promote the VEGF-C cleavage or the only therapeutically effective agent (i.e. having an ability to ameliorate any harmful effects of lymphatic disorders). ADAMTS3 may also be administered together with other agents, such as agents promoting the VEGF-C cleavage or therapeutically effective agents. In one embodiment of the invention, the composition further comprises other therapeutically effective agents. For co-administration of ADAMTS3 and any other agent the route and method of administration may be
selected independently. Further, co-administration of ADAMTS3 and any other agent promoting the VEGF-C cleavage or any other therapeutically effective agent may be simultaneous, separate, or sequential. In one embodiment of the invention, the composition is used concurrently with other therapeutic agents or therapeutic methods.

As used herein, the terms "providing a gene or polynucleotide" or "gene therapy" refer to the transfer of a ADAMTS3 polynucleotide into selected target cells or tissues in a manner that enables expression thereof in an amount, which enables VEGF-C to be sufficiently (i.e. in any desired amount) cleaved, or alternatively in a therapeutically effective amount. In accordance with the present invention, gene provision or therapy may be used to replace a defective gene, or supplement a gene product that is not produced in a desired amount in a cell or in a therapeutically effective amount or at a therapeutically useful time in a subject with lymphatic disorder.

As used herein, the term "subject" refers to a subject, which is selected from a group consisting of an animal, a mammal or a human. In one embodiment of the invention, the subject is a human or an animal.

As used herein, the terms "providing a protein or polypeptide" or "protein therapy" refer to the administration of an ADAMTS3 polypeptide in an amount, which enables VEGF-C to be sufficiently (i.e. in any desired amount) cleaved in a cell, or alternatively in therapeutically effective amount to a subject, particularly a mammal or a human, with a lymphatic disorder for which therapy is sought. Herein, the terms "polypeptide" and "protein" are used interchangeably to refer to polymers of amino acids of any length. Preferably a polypeptide is administered to a target tissue or cell culture.

As used herein, the term "therapeutically effective amount" refers to an amount of ADAMTS3 with which the harmful effects of a lymphatic disorder (e.g. lymphedema) are, at a minimum, ameliorated. The harmful effects of a lymphatic disorder include any detectable or noticeable effects of a subject such as swelling, aching, discomfort, heaviness or tightness of the affected tissue.

For gene provision or therapy, "naked" ADAMTS3 polynucleotides described above may be applied in the form of recombinant DNA, plasmids, or viral vectors. Delivery of naked polynucleotides may be performed by any method that physically or chemically permeabilizes the cell membrane. Such methods are available in the art and include, but are not limited to, electro-
poration, gene bombardment, sonoporation, magnetofection, lipofection, liposome-mediated nucleic acid delivery, and any combination thereof.

In some other embodiments, ADAMTS3 polynucleotides may be incorporated into a viral vector under a suitable expression control sequence. Suitable viral vectors for such gene therapy include, but are not limited to, retroviral vectors, such as lentivirus vectors, adeno-associated viral vectors, and adenoviral vectors. Preferably, the viral vector is a replication-deficient viral vector, i.e. a vector that cannot replicate in a mammalian subject. A non-limiting preferred example of such a replication-deficient vector is a replication-deficient adenovirus. Suitable viral vectors are readily available in the art. In the specific embodiment of the invention, the ADAMTS3 is overexpressed by adenoviral or adeno-associated viral vectors.

Delivery of therapeutic ADAMTS3 polynucleotides to a subject, preferably a mammalian or a human subject, may be accomplished by various ways well known in the art. For instance, viral vectors comprising ADAMTS3 encoding polynucleotide(s) may be administered directly into the body of the subject to be treated, e.g. by an injection into a target tissue having compromised lymphatic vessels.

Delivery of ADAMTS3 polynucleotides to in vivo cells (e.g. endothelial, endothelial-associated cells, epithelial cells) results in the expression of the polypeptides in vivo and is, thus, often referred to as in vivo gene therapy. Alternatively or additionally, delivery of the present polypeptides may be effected ex vivo by use of viral vectors or naked polypeptides. Ex vivo gene therapy means that target cells, preferably obtained from the subject to be treated, are transfected (or transduced with viruses) with the present polynucleotides ex vivo and then administered to the subject for therapeutic purposes. Non-limiting examples of suitable target cells for ex vivo gene therapy include endothelial cells, endothelial progenitor cells, smooth muscle cells, leukocytes, and especially stem cells of various kinds. ADAMTS3 polynucleotides may also be delivered to any in vitro cells. In one embodiment the target cell is an in vitro, in vivo or ex vivo cell.

In gene provision or therapy, expression of ADAMTS3 may be either stable or transient. Transient expression is often preferred. A person skilled in the art knows when and how to employ either stable or transient gene therapy.

In addition to gene provision or therapy, also protein provision or therapy aims at VEGF-C cleavage and furthermore increased VEGF-C recep-
tor signaling. For protein provision or therapy, ADAMTS3 may be obtained for example by standard recombinant methods. Recombinant techniques are well known to a person skilled in the art. In one embodiment of the invention ADAMTS3 polypeptide is a recombinant polypeptide. In one embodiment of the invention the VEGF-C and/or ADAMTS3 polypeptide have/has been recombinantly produced from a polynucleotide in the same cell, in which the fully processed mature VEGF-C is produced or the VEGF-C polypeptide is cleaved. In another embodiment of the invention the VEGF-C and/or ADAMTS3 polypeptide have/has been recombinantly produced from a polynucleotide in another cell than in which the fully processed mature VEGF-C is produced or the VEGF-C polypeptide is cleaved. A desired polynucleotide may be cloned into a suitable expression vector and expressed in a compatible host according to methods well known in the art. Examples of suitable hosts include but are not limited to bacteria (such as E. coli), yeast (such as S. cerevisiae), insect cells (such as Sf9 cells), and preferably mammalian cell lines. Expression tags, such as His-tags, hemagglutinin epitopes (HA-tags) or glutathione-S-transferase epitopes (GST-tags), may be used to facilitate the purification of VEGF-C. If expression tags are to be utilized, they have to be cleaved off prior to administration to a subject in need of the recombinant protein.

In one embodiment of the invention ADAMTS3 protein is administered directly to the target tissue (e.g. tissue with lymphatic problems). Furthermore, ADAMTS3 proteins may be administered to in vivo, ex vivo or in vitro cells.

In the methods or uses of the invention, the ADAMTS3 and/or VEGF-C polypeptides or polynucleotides may be naturally occurring purified polypeptides or isolated polynucleotides. Indeed, an incompletely processed or unprocessed VEGF-C and/or ADAMTS3 may be provided as polynucleotides to the cell or polypeptides to cell culture by administration.

VEGF-C may be administered to a target tissue cell culture or cell in a same way as ADAMTS3 described under this chapter “administration”.

According to the present invention ADAMTS3 inhibitor is provided to a target tissue or cell culture. ADAMTS3 inhibitor may be administered to a target tissue or cell culture in various ways, for instance by gene administration or gene therapy, protein administration or protein therapy, or by administration of the molecule (in case not a nucleotide or protein) or any desired combina-
tion thereof. Administration of ADAMTS3 inhibitor by different ways or routes may be simultaneous, separate, or sequential.

Depending on the use or method of the invention, ADAMTS3 inhibitor may be the only agent to inhibit the VEGF-C cleavage or the only therapeutically effective agent (i.e. having an ability to ameliorate any harmful effects of lymphatic disorders). ADAMTS3 inhibitor may also be administered together with other agents, such as agents decreasing the VEGF-C cleavage or therapeutically effective agents. In one embodiment of the invention, the composition further comprises other therapeutically effective agents. For co-administration of ADAMTS3 inhibitor and any other agent the route and method of administration may be selected independently. Further, co-administration of ADAMTS3 inhibitor and any other agent inhibiting the VEGF-C cleavage or any other therapeutically effective agent may be simultaneous, separate, or sequential. In one embodiment of the invention, the composition is used concurrently with other therapeutic agents or therapeutic methods.

ADAMTS3 inhibitor polynucleotide may be administered into selected target cells or tissues in a manner that enables expression thereof in an amount, which inhibits the VEGF-C cleavage by the cells. In accordance with the present invention, gene provision or therapy may be used to inhibit a function of a gene product that is overproduced by a cell.

ADAMTS3 inhibitor polypeptide may be administered in an amount, which inhibits the VEGF-C cleavage by a cell. Preferably a polypeptide is administered to a target tissue or cell culture.

Therapeutically effective amount of ADAMTS3 inhibitor refers to an amount of ADAMTS3 inhibitor with which the harmful effects of a lymphatic disorder (e.g. cancer or inflammatory disorder) are, at a minimum, ameliorated. The harmful effects of a lymphatic disorder include any detectable or noticeable effects of a subject such as swelling, aching, discomfort, heaviness or tightness of the affected tissue.

For ADAMTS3 inhibitor the same methods and arrangements as for ADAMTS3 gene or protein therapy, described above, apply. Both gene and protein therapies aim at decreased VEGF-C cleavage and furthermore decreased VEGF-C receptor signaling.

Small molecules may be provided to a cell by any known method suitable for that molecule.

Amounts and regimens for therapeutic administration of ADAMTS3
or inhibitors of ADAMTS3 according to the present invention can be determined readily by those skilled in the clinical art of treating a lymphatic disorder. Generally, the dosage of the ADAMTS3 or ADAMTS3 inhibitor treatment will vary depending on considerations such as: age, gender and general health of the patient to be treated; kind of concurrent treatment, if any; frequency of treatment and nature of the effect desired; extent of tissue damage or extent of cancer or inflammation; duration of the symptoms; and other variables to be adjusted by the individual physician. For instance, when viral vectors are to be used for gene delivery, the vector is typically administered, optionally in a pharmaceutically acceptable carrier, in an amount of $10^7$ to $10^{13}$ viral particles, preferably in an amount of at least $10^9$ viral particles. On the other hand, when ADAMTS3 inhibitor or protein therapy is to be employed, a typical dose is in the range of 0.01 to 20 mg/kg, more preferably in the range of 0.1 to 10 mg/kg, most preferably 0.5 to 5 mg/kg.

A desired dosage can be administered in one or more doses at suitable intervals to obtain the desired results. A typical non-limiting daily dose may vary from about 50 mg/day to about 300 mg/day.

Indeed, only one administration of ADAMTS3 or ADAMTS3 inhibitor may have therapeutic effects. However, in one embodiment of the invention, ADAMTS3 or ADAMTS3 inhibitors are administered several times during the treatment period. ADAMTS3 or ADAMTS3 inhibitors may be administered for example from 1 to 20 times, 1 to 10 times or two to eight times in the first 2 weeks, 4 weeks, monthly or during the treatment period. The length of the treatment period may vary, and may, for example, last from a single administration to 1-12 months or more.

**Pharmaceutical compositions**

The present invention provides not only therapeutic methods and uses for treating disorders and conditions related to impaired lymphatic vasculature but also to pharmaceutical compositions for use in said methods and therapeutic uses. Such pharmaceutical compositions comprise ADAMTS3, either alone or in combination with other agents such as a therapeutically effective agent or agents and/or a pharmaceutically acceptable vehicle or vehicles.

Also, the present invention provides not only therapeutic methods and uses for treating disorders and conditions related to increased lymphatic
vasculature but also to pharmaceutical compositions for use in said methods and therapeutic uses. Such pharmaceutical compositions comprise inhibitors of ADAMTS3, either alone or in combination with other agents such as a therapeutically effective agent or agents and/or a pharmaceutically acceptable vehicle or vehicles.

A pharmaceutically acceptable vehicle may for example be selected from the group consisting of a pharmaceutically acceptable solvent, diluent, adjuvant, excipient, buffer, carrier, antiseptic, filling, stabilising agent and thickening agent. Optionally, any other components normally found in corresponding products may be included. In one embodiment of the invention the pharmaceutical composition comprises ADAMTS3 and a pharmaceutically acceptable vehicle. In another embodiment of the invention the pharmaceutical composition comprises an inhibitor of ADAMTS3 and a pharmaceutically acceptable vehicle.

For instance, the pharmaceutically acceptable vehicle may be a sterile non-aqueous carrier such as propylene glycol, polyethylene glycol, or injectable organic ester. Suitable aqueous carriers include, but are not limited to, water, saline, phosphate buffered saline, and Ringer's dextrose solution.

A variety of administration routes may be used to achieve an effective dosage to the desired site of action as well known in the art. Thus, suitable routes of administration for include, but are not limited to, subconjunctival delivery, intragastrical delivery, parenteral delivery (e.g. intravenous injection), enteral delivery (e.g. orally), local administration, topical administration (e.g. dermally or transdermally), as known to a person skilled in the art.

The pharmaceutical composition may be provided in a concentrated form or in a form of a powder to be reconstituted on demand. Furthermore, the pharmaceutical composition may be in any form, such as solid, semisolid or liquid form, suitable for administration. A formulation can be selected from a group consisting of, but not limited to, for example solutions, emulsions, suspensions, tablets, pellets and capsules. In case of lyophilizing, certain cryoprotectants are preferred, including polymers (povidones, polyethylene glycol, dextran), sugars (sucrose, glucose, lactose), amino acids (glycine, arginine, glutamic acid) and albumin. If solution for reconstitution is added to the packaging, it may consist e.g. of sterile water, sodium chloride solution, or dextrose or glucose solutions.
Means and methods for formulating the present pharmaceutical preparations are known to persons skilled in the art, and may be manufactured in a manner which is in itself known, for example, by means of conventional mixing, granulating, dissolving, lyophilizing or similar processes.

Optional therapeutically effective agents

ADAMTS3 may be administered to cell culture or a subject in combination with other agents increasing the VEGF-C receptor signalling or other therapeutically effective agents. In one embodiment of the invention, the method comprising administration of ADAMTS3 and optionally VEGF-C further comprises a step of providing CCBE1 polypeptide after the step of providing ADAMTS3 polypeptide. ADAMTS3 inhibitor may be administered to cell culture or a subject in combination with other agents decreasing or inhibiting the VEGF-C receptor signalling or other therapeutically effective agents. In addition to ADAMTS3 or ADAMTS3 inhibitor, a pharmaceutical composition of the invention may comprise at least one, two, three, four or five other therapeutically effective agents. In one embodiment of the invention, the other therapeutically effective agent to be used with ADAMTS3 is CCBE1 and/or incompletely processed or unprocessed VEGF-C.

As used herein, the term “CCBE1” refers to a full-length collagen-and calcium-binding EGF domains 1 (CCBE1) polypeptide or to a polynucleotide encoding said full-length CCBE1. Preferably, CCBE1 is a mammalian CCBE1, preferably human. In some embodiments, the full length CCBE1 polypeptide comprises an amino acid sequence depicted in SEQ ID NO: 1, preferably without a signal peptide. Presumably, the signal peptide of human CCBE1 consists of amino acids 1-34 of SEQ ID NO: 1 but when produced in mammalian cells, the signal peptide is automatically cleaved off correctly.

It is evident to a person skilled in the art that the CCBE1 polypeptide to be used in accordance with the present invention may vary as long as it retains its biological activity. An exemplary way of determining whether or not a CCBE1 variant has maintained its biological activity is to determine its ability to promote the cleavage of full-length VEGF-C. This may be performed e.g. by incubating suitable cells (e.g. VEGFR-3 expressing PAE cells) expressing full-length VEGF-C with the CCBE1 and ADAMTS3 variant in question and concluding that the CCBE1 variant has retained its biological activity if VEGF-C cleavage is enhanced. Said VEGF-C cleavage may be determined e.g. by
metabolic labelling and protein-specific precipitation, such as immunoprecipitation, according to methods well known in the art.

In connection with polypeptides, the variants refers to amino acid sequence modifications, which arise from amino acid substitutions with similar amino acids well known in the art (e.g. amino acids of similar size and with similar charge properties) and which do not significantly alter the biological properties of the polypeptide in question. Amino acid deletions and additions are also contemplated and used. In one embodiment of the invention, the CCBE1 polynucleotide comprises a polynucleotide sequence as depicted in SEQ ID NO: 2.

As used herein, the term “CCBE1 polynucleotide” refers to any polynucleotide, such as single or double-stranded DNA or RNA, comprising a nucleic acid sequence encoding a CCBE1 polypeptide. In some preferred embodiments, the CCBE1 polynucleotide comprises a coding sequence (CDS) for full-length CCBE1, or a conservative sequence variant thereof.

Any of the embodiments and features described above may apply independently to ADAMTS3 and optional other agents (such as VEGF-C and/or CCBE1) and may be used in any desired combination. Thus, at least ADAMTS3 may be delivered by gene administration or therapy or protein administration or therapy. It is also contemplated that VEGF-C and/or CCBE1 may be administered using both gene therapy and protein therapy. Also any of the embodiments and features described above may apply independently to ADAMTS3 inhibitors and optional other agents and may be used in any desired combination.

Lymphatic disorders

As set forth above, the present therapeutic methods and uses relate to the treatment of disorders and conditions that involve impaired lymphatic vasculature.

By "lymphatic disorder" is meant any clinical condition affecting the lymphatic system. Disorders with impaired lymphatic system include but are not limited to lymphedemas. Therapy of these disorders can be based on increasing the function of ADAMTS3. Disorders that activate the lymphatic vasculature such as any types of tumours (benign, malignant), cancers (primary, metastasis) and inflammatory diseases are included within “the lymphatic disorder”. Therapy of these disorders can be based on inhibiting the function of
ADMTS3. In one embodiment of the invention the lymphatic disorder is a lymphedema. Non-limiting examples of lymphedemas include lymphedema, such as primary lymphedema (e.g. Milroy’s syndrome, Meige’s lymphedema, and lymphedema tarda), secondary lymphedema, lipedema, lymphangiectasia or Gorham’s disease. In one embodiment of the invention the lymphatic disorder is an inflammatory disorder, tumor or cancer. In another embodiment the inflammatory disorder is selected from a group consisting of type 1 diabetes, atherosclerosis, chronic airway diseases, psoriasis, metabolic disease and obesity.

As used herein, the term “treatment” or “treating” refers to administration of at least ADAMTS3 to a subject, preferably a mammal or human subject, for purposes which include not only complete cure but also prophylaxis, amelioration, or alleviation of disorders or symptoms related to a lymphatic disorder. Therapeutic effect of administration of ADAMTS3 may be assessed by monitoring symptoms such as swelling, aching, discomfort, heaviness, or tightness of the affected tissue.

Alternatively, as used herein, the term “treatment” or “treating” refers to administration of at least an inhibitor of ADAMTS3 to a subject, preferably a mammal or human subject, for purposes which include not only complete cure but also prophylaxis, amelioration, or alleviation of disorders or symptoms related to a lymphatic disorder. Therapeutic effect of administration of ADAMTS3 inhibitor may be assessed by monitoring symptoms such as swelling, aching, discomfort, heaviness, or tightness of the affected tissue.

Before classifying a human or animal patient as suitable for the therapy of the present invention, for example increased or decreased VEGFR3 signalling may be assayed. After these preliminary studies and based on the results deviating from the normal, the clinician may suggest ADAMTS3 or ADAMTS3 inhibitor treatment for a patient.

**Antibody and binding molecule production**

According to the present invention ADAMTS3 is also used in methods for producing specific antibodies or binding molecules against a fully processed, mature and active VEGF-C. The specific antibodies or binding polypeptides result from using a fully processed mature VEGF-C, which is obtained by ADAMTS3 cleavage, as an antigen or immunogen. Purification of the VEGF-C antigen/immunogen after ADAMTS3 cleavage can be performed by
subtractive IMAC (immobilized metal ion affinity chromatography). Both the uncleaved VEGF-C and ADAMTS3 are histidine-tagged and after cleavage the material is passed over an IMAC column. The cleavage byproducts and ADAMTS3 are retained on the column whereas VEGF-C will pass through the column given the appropriate conditions.

The fully cleaved VEGF-C ADAMTS3 polypeptide so prepared is distinct from the VEGF-C polypeptide prepared from recombinant cDNA fragment encoding the same amino acid residues. Variation in signal sequence cleavage and/or disulphide bonding of the two antiparallel polypeptides contribute to the differences and activity of the VEGF-C dimer.

Molecules inhibiting the function of an active VEGF-C are not limited to antibodies, but also include any polypeptides having binding affinities to a fully processed mature VEGF-C. As used herein “the VEGF-C binding molecule” refers to any molecule which specifically binds to a fully processed mature VEGF-C. These molecules include anti-VEGF-C-antibodies and any other VEGF-C binding factors, such as a VEGF-C binding polypeptide or small molecule. In one embodiment of the invention the VEGF-C binding molecule is an anti-VEGF-C-antibody or a VEGF-C binding polypeptide, or small molecule. As used herein, “a small molecule” refers to a molecule is a low molecular weight (<900 Daltons) organic compound that regulates a biological process and according to the present invention binds specifically to a fully processed mature VEGF-C.

Such antibodies or binding polypeptides against VEGF-C negatively modulate, i.e. inhibit or suppress lymphangiogenesis, as is needed for example in cancer therapy or anti-inflammatory therapy.

As used herein “antibodies” refer to proteins produced by immune cells or by recombinant techniques in response to foreign materials known as antigens. Antibodies of the present invention bind to fully processed mature VEGF-C’s and either mark them for destruction or inhibit their function by binding to them.

As used herein “binding polypeptides” refer to polypeptides which bind to fully processed mature VEGF-Cs and thereby inhibit or suppress their function.

Traditional antibody production involves preparation of antigen samples and their injection into laboratory or farm animals so as to evoke high expression levels of antigen-specific antibodies in the serum, which can then
be recovered from the animal. The target antigen (e.g., peptide or hapten) may be either synthesized or purified, an appropriate immunogenic carrier protein is conjugated to the antigen in order to create the immunogen, and depending on whether polyclonal or monoclonal antibodies are produced, either serum or hybridoma supernatant is screened for antibody titer and isotype. Polyclonal antibodies are recovered directly from serum (bleeds). Monoclonal antibodies are produced by fusing antibody-secreting spleen cells from immunized mice with immortal myeloma cell to create monoclonal hybridoma cell lines that express the specific antibody in cell culture supernatant.

In one embodiment, the process of creating a usable specific antibody comprises at least steps of preparing an immunogen, immunizing a subject and collecting the antibodies. Furthermore, for example hybridoma creation, purification of the antibodies as well as labeling of the antibodies may be utilized. In another embodiment of the invention, a method of producing an anti-VEGF-C-antibody against a fully processed mature VEGF-C polypeptide comprises the steps of cleaving the incompletely processed or unprocessed form of VEGF-C polypeptide by ADAMTS3 polypeptide to obtain fully processed mature VEGF-C; preparing a fully processed mature VEGF-C immunogen; immunizing a subject with the fully processed mature VEGF-C immunogen, and collecting the anti-VEGF-C-antibody against the fully processed mature VEGF-C.

According to the present invention also antibody or non-antibody libraries as well as recombinant antibody or non-antibody technologies can be used for producing specific antibodies or polypeptides against a fully processed mature VEGF-C, which is obtained by ADAMTS3 cleavage.

Combinatorial antibody libraries enable a generation of purely human monoclonal antibodies for medical use. In these antibody libraries, mRNAs from B lymphocytes, coding for millions or even billions of the antibody heavy and light chains, are rescued by PCR technology and expressed as functional structures most often on phage surfaces that allow isolation of clones with particular characteristics, e.g., binding to a target molecule (such as a fully processed mature VEGF-C), cell type, or tissue. The libraries can be created from immune individuals, biased for clones against the immunogen, or be as diverse as possible, producing so-called naive libraries from which theoretically antibodies to any antigen may be isolated. Libraries can also be created fully synthetically.
Recombinant antibodies (rAb) are created using recombinant DNA. Chimeric antibodies, humanized antibodies, and antibodies from gene libraries can all be considered recombinant antibodies. Libraries of antibody genes are created and displayed on the surfaces of cells, phages or ribosome display is used. Displayed libraries are incubated with antigen during iterative rounds of "panning" to enrich for antigen-specific antibodies. Selected antibodies are screened for desirable characteristics. Genes for the selected antibodies are sequenced and undergo affinity maturation to further increase antibody function. Genes for highest performing antibodies are transferred into protein expression systems for larger scale antibody production.

Non-antibody (e.g. polypeptide, or oligonucleotide/aptamer) libraries enable finding specific polypeptides or oligonucleotides/aptamers for medical uses. Libraries of polypeptides or oligonucleotides are displayed and further incubated with fully processed mature VEGF-C’s of fragments thereof during multiple rounds of "panning" to enrich for specific polynucleotides or oligonucleotides.

In one embodiment of the invention the process of creating a usable specific binding polypeptide comprises at least steps of screening a library with fully processed mature VEGF-C's and collecting the specific molecules capable of binding to fully processed mature VEGF-C's. In another embodiment of the invention, a method of producing an anti-VEGF-C-antibody or polypeptide against a fully processed mature VEGF-C polypeptide comprises the steps of cleaving the incompletely processed or unprocessed form of VEGF-C polypeptide by ADAMTS3 polypeptide to obtain fully processed mature VEGF-C; screening a library with the fully processed mature VEGF-C, and collecting the VEGF-C binding molecule against the fully processed mature VEGF-C. In one embodiment of the invention, the library is an antibody, polypeptide, nucleotide or small molecule library.

The present invention relates to a method of producing an anti-VEGF-C-antibody or VEGF-C binding polypeptide against a fully processed mature VEGF-C polypeptide. The incompletely processed or unprocessed form of the VEGF-C polypeptide is cleaved by ADAMTS3. After immunization of an animal or screening of an antibody or non-antibody library, anti-VEGF-C-antibodies or VEGF-C binding molecules such as polypeptides against the fully processed mature VEGF-C are obtained.

In one embodiment of the invention, the subject to be immunized
with the fully processed mature VEGF-C immunogen can be selected from a
group consisting of a human or an animal such as a rabbit, a mouse, rat,
chicken, lama, camel, dromedaries, alpacas or sharks. Optionally, a carrier
protein of the immunogen may be selected from the group consisting of key-
hole limpet hemocyanin (KHL), blue carrier immunogenic protein, bovine se-
rum albumin (BSA) and ovalbumin (OVA).

The methods and materials used in the antibody or binding mole-
cule production include any conventional methods (such as conventional hy-
bridoma technology or recombinant antibody or non-antibody technologies) in
the field and these are well known to a skilled person.

It will be obvious to a person skilled in the art that, as technology
advances, the inventive concept can be implemented in various ways. The in-
vention and its embodiments are not limited to the examples described below
but may vary within the scope of the claims.

EXAMPLES

Materials and methods

Cloning. The genes expressed via the recombinant aden-o-
associated virus (rAAV9) vector were cloned into the psubCAG-WPRE plasmid
(Paterna et al, 2000, Gene Therapy 7: 1304–1311), which is a derivative of
psubCMV-WPRE, where the CMV promoter has been replaced with the com-
posite CAG promoter, consisting of the chicken β-actin promoter, cytomegalov-
319). Cloning of full-length mVEGF-C, ANΔC-mVEGF-C and HSA into AAV-
vector (psubCAG-WPRE) was described earlier (Anisimov et al, 2009, ibid.).
mCCBE1, fused to a V5 tag (mCCBE1-V5), was cloned as follows: A partial
CCBE1 coding DNA sequence (CDS; Genebank #BC152322, Image clone ID
40140631) was cloned as a SacI/XbaI fragment into pVK1 (a pUC19-derived
vector (Anisimov et al, 2007, Molecular Breeding 19: 241–253)). The missing
nucleotides were amplified from brown adipose tissue mRNA with primers 5’-
GCCGCTAGCCGCCACCATGGGTGCGCGCGCCT-3’ (SEQ ID NO: 5) and 5’-
GGAGCTTGGGACAAATGTC-3’ (SEQ ID NO: 6) and above CDS was com-
pleted by inserting the NheI/Sacl fragment resulting in vector pVK1-CCBE1. A
PCR-amplified V5-tag (obtained with primers 5’-
ACCAGGAGCACCAGGAAGAC-3’ (SEQ ID NO: 7) and 5’-
GCCTCTAGAAGCGCTAGGTCAGTGCTGTCAGGGCCGAG-
CAGAGGGTTAGGGATAGGGCTTCCTGGATATAAATTCTTGAGG-3' (SEQ ID NO:8)) was added to the CDS as an Eco81I/Xba1 fragment. From the resulting vector, the complete CDS was excised as an Mlu1 fragment and cloned into psubCAG-WPRE.

For the in-vitro studies, an identical vector was constructed, in which the mouse CCBE1 CDS was replaced by the human CCBE1 CDS (Genebank #NM_133459), and for the co-immunoprecipitation study, a StreptIII-tag (Junttila et al, 2005, PROTEOMICS 5: 1199–1203) was inserted immediately following the CCBE1 CDS. The mammalian expression constructs for VEGF-C and ΔC-VEGF-C construct have been described before (Joukov et al, 1997, ibid.). The chimeric VEGF-C/VEGF-D (CDC) expression construct was assembled by overlapping PCR into the pMosaic vector (Jeltsch et al, 2006, J. Biol. Chem. 281: 12187–12195). The insert comprised sequences coding for amino acid residues Phe32-Ala111 and Ser228-Ser419 from human VEGF-C and intervening residues Thr92-Arg206 from human VEGF-D (version 1) and amino acid residues Phe32-Ala111 and Ser228-Ser419 from human VEGF-C and intervening residues Thr92-Arg206 from human VEGF-D (version 1).

cDNA clones for the ADAMTS constructs were obtained from the Mammalian Gene Collection. ADAMTS1 (GenBank BC040382) and ADAMTS2 (GenBank BC046456) were expression-ready (pCMV-Sport6); the ADAMTS3 cDNA clone (pCR-XL-TOPO, BC130287,) was subcloned as an EcoRI fragment into the expression vector pCI-neo (Promega) and pAc5.1/V5His (Invitrogen) adding sequences encoding for a C-terminal V5 and hexahistidine tag. The ADAMTS14 cDNA clone (pENTR223.1, GenBank BC140263) was transferred into the expression vector pEF-DEST51 using the Gateway recombination system.

The constructs for recombinant VEGF-C expression in insect cells employed the pMT-Ex vector (a modified version of pMT-BiP-V5His-C (Kärpänen et al, 2006, The FASEB Journal 20: 1462–1472)) and comprised sequences coding for the signal peptide of the Drosophila BiP, for amino acid residues Phe32-Ala111 for the N-terminal propeptide, Ser228-Ser419 for the C-terminal propeptide and Thr112-Arg227 for the ΔNΔC form of VEGF-C, followed by sequences coding for a hexahistidine tag. The construct for the expression of full-length VEGF-D comprised amino acid residues 22-354 in the same context.
The construct for the expression of VEGF-C from its full-length cDNA was based on pFastBac1 (Invitrogen). In this construct the CDS of VEGF-C was modified by swapping the sequences coding for its endogenous signal peptide against sequences coding for the melittin signal peptide and by adding sequences coding for a C-terminal hexahistidine tag.

For the expression of the N-terminal domain of CCBE1, residues 1-175 of human CCBE1 were cloned into the pFastBac (Invitrogen) baculovirus expression vector with a C-terminal Factor Xa cleavage site (IEGR) and a hexahistidine tag.

**Antibodies.** Anti-VEGF-C antiserum (Baluk et al. 2005, J Clin Invest 115:247-257), anti-VEGF-C antibody (R&D Systems, , Minneapolis, MN, AF752), anti-V5 antibody (Invitrogen, Carlsbad, CA, #46-0705), anti-phosphotyrosine antibody 4G10 (Merck Millipore, Billerica, MA) and PY20 (BD Transduction Laboratories, Franklin Lakes, NJ), and anti-CCBE1 antibody (Atlas Antibodies AB, Stockholm, Sweden, #HPA041374) were used for both immunoprecipitation and detection after Western blotting. Anti-VEGF-D antibody VD1 (Achen et al. Eur J Biochem 2000; 267:2505-2515), anti-VEGFR-3 antibody (Santa Cruz, Dallas, TX, sc-321), chimeric VEGFR-3/IgGFc (Mäkinen et al. 2001, Nat Med, 7:199-205) or streptactin sepharose (IBA, Göttingen, Germany) were used for immunoprecipitation. Anti-VEGF-D antibody (R&D Systems, AF286) and streptactin-HRP conjugate (IBA) and streptavidin-HRP conjugate (R&D systems, #890803) were used for detection after Western blotting. Antibody hF4-3C5 was used to block VEGFR-3 activation and generously provided by ImClone Systems (Persaud et al. 2004, J Cell Sci, 117:2745-2756).

**rAAV production.** rAAV9 viruses were made by a three-plasmid transfection method and purified by ultracentrifugation using discontinuous iodixanol gradient, as described (Anisimov et al, 2009, ibid.). To generate the AAV9 serotype, we used serotype-determining helper plasmid p5E18-VP2/9, instead of p5E18-VP2/8 (Michelfelder et al, 2011, PLoS ONE 6: e23101).

**Protein expression and purification.** S2 cells were transfected using Effectene (Qiagen, Venlo, The Netherlands). Stable cell pools were selected for 3 weeks with 400 μg/ml hygromycin starting 2 days after transfection. For protein production, the cells were adapted to suspension culture and induced for 4-5 days with 1 mM CuSO₄. After batch-binding to Ni²⁺NTA sepharose from the pH-adjusted conditioned supernatant, the Ni²⁺NTA se-
pharose was loaded onto a column, washed with 20 mM imidazole, and eluted with a step-gradient of 250 mM imidazole. The protein was further size-separated on a Superdex 200 column with PBS as a running buffer.

**Cell culture and generation of stable cell lines.** 293T, 293S GnTi− and NIH-3T3 cells were grown in D-MEM 10% FCS. PC-3 cells were grown in Ham’s F-12 10% FCS, DU-4475 cells in RPMI 1640 20% FCS and S2 cells in HyClone SFX-Insect (ThermoScientific, Rockford, IL) or Insect-Xpress (Lonza Group, Basel, Switzerland).

293T, 293GPG, 293S GnTi+, PAE-VEGFR-3 (Leppanen et al. 2013, Proc Natl Acad Sci USA 110: 12960-12965) and NIH-3T3 cells were grown in D-MEM 10% FCS. PC-3 cells were grown in Ham’s F-12 10% FCS, DU-4475 cells in RPMI 1640 20% FCS, CHO cells in α-MEM/10% FCS or EX-CELL ACF CHO Medium and S2 cells in HyClone SFX-Insect (ThermoScientific, Rockford, IL) or Insect-Xpress (Lonza Group, Basel, Switzerland). 293T cells were infected with retrovirus, that had been produced using standard methods in 293GPG cells using the pMX-hCCBE1-StrepIII-IRES-EGFP construct, cultured for one week and sorted once with FACS for EGFP. 293T cells were transfected with the expression plasmid pCI-neo-hADAMTS3-V5-H6, selected with G418 and clonal lines established by the ring cloning technique. Stable expression of CCBE1 and ADAMTS3 were confirmed by Western blotting. PAE-VEGFR-3 cells have been described before (Leppanen et al. ibid.). LECs, BECs and HUVECs were purchased from Promocell (Heidelberg, Germany) and maintained according to the instructions of the supplier. The Ba/F3-hVEGFR-2/EpoR cell line was generated similar to the Ba/F3-mVEGFR-2/EpoR (Stacker et al. 1999, J Biol Chem 274:34884-34892); however, pCl-neo was used instead of pEF-BOS. The junctional amino acid sequences of the chimera were ...FFIIEGAQEKTNLEGs (SEQ ID NO: 9) (end of VEGFR-2 part) – (start of mEpoR part) LILTLLLILVTLILLL/SHRRTLQKIGWPGIPSPE... (SEQ ID NO: 15) This chimeric construct was electroporated with one 30 ms 1400V pulse (Neon transfection device, Invitrogen) into Ba/F3 cells. Cells were grown in medium containing 2 ng/ml mIL-3 for 36 hours, after which they were split and selection was started for three weeks with 1.2 mg/ml G418. Cells were maintained with sub-optimal mIL-3 concentration (0.4 ng/ml) and optimal VEGF-A and VEGF-C concentrations (200 and 300 ng/ml, respectively).
Transfections, metabolic labeling and protein analysis. 293T and 293S GnTI− cells were (co)transfected with expression constructs coding for the indicated proteins. 24 hours after the transfection, the cells were metabolically labeled with [35S]-cysteine/[35S]-methionine (PerkinElmer, Waltham, MA) and 48 hours later, conditioned cell culture medium and cell lysates were harvested. For the short labeling experiments, harvesting was performed already after 24 hours. Alternatively, in order to produce unlabeled protein for Western blotting, the culture medium of the cells was exchanged against DMEM 0.2% BSA and the supernatant and cell lysates were harvested 48 hours later.


Samples were washed and resolved by 4-20% SDS-PAGE. For autoradiography, gels were dried and exposed to phosphoimager plates or X-ray film. For the immunodetection, proteins were transferred to nitrocellulose. Specific signals were detected with anti-VEGF-C antiserum, anti-V5 antibody or anti-hCCBE1 antibody in combination with ECL. Quantitation of the autoradiography and Western blots was performed from the laser scanner read-outs or scanned X-ray film using the ImageJ software (NIH, Bethesda, MD).

Protein expression and purification. S2 cells were transfected using Effectene (Qiagen, Venlo, The Netherlands). Stable cell pools were selected for 3 weeks with 400 μg/ml hygromycin starting 2 days after transfection. For protein production, the cells were adapted to suspension culture and induced for 4-5 days with 1 mM CuSO4. After batch-binding to Ni2+NTA sepharose from the pH-adjusted conditioned supernatant, the Ni2+NTA sepharose was loaded onto a column, washed with 20 mM imidazole, and eluted with a step-gradient of 250 mM imidazole. The protein was further size-separated on a Superdex 200 column with PBS as a running buffer.

Recombinant baculovirus was produced using the FactBac system (Invitrogen, Carlsbad, CA) according to the instructions of the manufacturer.
Sf9 cells were infected and conditioned medium harvested 4 days post infection. Protein purification was performed as above. CCBE1Δ175 was purified in Hepes-buffered saline supplemented with 2 mM CaCl₂.

StrepIII-tagged CCBE1 was enriched from 200 ml conditioned medium of 293T cells expressing strepIII-tagged CCBE1 using streptactin sepharose (IBA) according to the instructions of the manufacturer and the eluted peak fraction was dialyzed against TBS. Histagged ADAMTS3 was purified from 1.5 litres of conditioned medium of stably transfected 293F cells (D-MEM supplemented with 2%FCS, 30µM ZnCl₂ and 0.1U heparin/ml). The collected medium was dialyzed against phosphate buffered saline, pH-adjusted to 8.0 and batch-bound protein was eluted with a step-gradient of 250 mM imidazole from the Ni²⁺NTA sepharose (IBA) after washing with 20 mM imidazole and dialyzed against 1xTBS.

**Ba/F3-VEGFR/EpoR assays.** The Ba/F3-hVEGFR-3/EpoR (Achen et al, 2000, ibid.) and Ba/F3-mVEGFR-2/EpoR (Stacker et al, 1999b) bioassays were performed with conditioned cell culture medium essentially as described (Mäkinen et al, 2001, ibid.). The Ba/F3-hVEGFR-2/EpoR cell line was generated similar as the cell lines above; however, pCI-neo was used instead of pEF-BOS. The junctional amino acid sequences of the chimera were ...FFLIEGAQEFKTNLEGS (end of VEGFR-2 part) – (start of mEpoR part) LILTL-LSLILVLISLLTTVLALLSHRRLQQKIQPGIIPSPESEF... (SEQ ID NOs: 9 and 10, respectively) This chimeric construct was electroporated with one 30 ms 1400V pulse (Neon transfection device, Invitrogen) into Ba/F3 cells. Cells were grown in medium containing 2 ng/ml mIL-3 for 36 hours, after which they were split and selection was started for three weeks with 1.2 mg/ml G418. Cells were maintained with sub-optimal mIL-3 concentration (0.4 ng/ml) and optimal VEGF-A and VEGF-C concentrations (200 and 300 ng/ml, respectively).

**Stimulation of receptor phosphorylation in PAE-VEGFR-3 cells and LECs.** Near confluence PAE-VEGFR-3 cells or LECs were washed with PBS and starved overnight in D-MEM 0.2%BSA. ΔNΔC-VEGF-C, pro-VEGF-C and CCBE1Δ175 were diluted to 0.02, 0.4 and 5 µg/ml in 1 ml D-MEM/0.1% BSA and incubated at 37°C for 30 minutes. The cells were stimulated for 10 or 30 minutes to detect phosphorylation of VEGFR-3 or downstream signaling proteins and then washed with ice-cold PBS. To cross-link proteins, cells were washed twice with PBS and purified proteins were applied in PBS (ΔNΔC-VEGF-C 100 ng/ml, pro-VEGF-C 1000 ng/ml and CCBE1Δ175 at 25-50
μg/ml). DTSSP (ThermoScientific, Waltham, MA) was added to a final concentration of 2 mM after 3.5 minutes and crosslinking was performed for 6.5 minutes at 37°C. Cells were once washed with ice-cold TBS, lysed with 1% Triton X-100 and the immunoprecipitated fraction or the total lysate was analyzed by SDS-PAGE/Western.

**HUVEC receptor trafficking.** HUVECs stably transfected with the pMXs-VEGFR-3-GFP vector (Ghalamkarpour A et al. *J Med Genet.* 2009; 46:399-404) cells were grown on glass-bottom microwells (MatTek Co.) for 24 h. The FCS concentration was reduced to 0.5%, and after 12 h, the cells were placed on a Zeiss LSM 5 DUO Confocal microscope and treated with pro-VEGF-C or ΔNΔC-VEGF-C (100 ng/ml) at 36°C and 5% CO2. The GFP signal was recorded at a wavelength of 488 nm. The blocking antibody hF4-3C5 was used at 5μg/ml.

**Pulse chase.** 293T cells were transfected and grown for 36 hours on 6-cm dishes to near confluency. Cells were starved for 30 min in met-/cys-deficient D-MEM, 5% dialyzed FCS, after which cells were metabolically labeled for 2 hours with [35S]-cysteine/[35S]-methionine. Thereafter, cells were washed with warm PBS and 5 ml of chase medium was added (D-MEM, 10% FCS + 2 mM cold L-methionine + 2mM cold L-Cysteine). At the indicated time points, the dishes were placed on ice and the medium was removed for analysis.

**Competition of VEGF-C cleavage by VEGF-C propeptides.** Purified histidine-tagged proteins were included in the labeling medium of the VEGF-C/CCBE1-cotransfected 293T cells at a concentration of 25 μg/ml. The labeling medium was conditioned from 30-72 hours after transfection, depleted from histidine-tagged proteins with Ni2+NTA sepharose, and VEGF-C was immunoprecipitated, separated by PAGE and visualized by exposure to X-ray film. Inhibition of the N-terminal cleavage of VEGF-C was measured by quantitating the 14 kDa N-terminal cleavage product from the laser-scanner read-out.

**Cleavage of pro-VEGF-C and pro-VEGF-D.** Indicated amounts of plasmin (Sigma P1867) were incubated for 18 hours with 2.5 μg of pro-VEGF-C. 1.15 μg of pro-VEGF-C were incubated in TBS as indicated with 4.15 μg 293T-cell-derived ADAMTS3 or 15 μg of S2-cell-derived ADAMTS3. 1.7 μg of pro-VEGF-D were incubated with 7.5 μg recombinant 293T-cell-derived ADAMTS3 in TBS for 30 hours either with or without 10 μl recombinant CCBE1. Analysis of the cleavage reaction was performed by SDS-
PAGE/Western. All incubations were at 37°C and recombinant CCBE1 was included as indicated at a concentration of approximately 2.5 µg/ml.

**Co-immunoprecipitation analysis of strep-tagged CCBE1.** 293T cells were transfected with either CCBE1-strepIII or full-length VEGF-C constructs. Conditioned media were used in a Strep-Tactin (Qiagen) pull-down either separately or as a mix of CCBE1 and VEGF-C. Mixed media were incubated for 10 min at RT before being applied to the pull-down. Precipitates were analyzed with anti-CCBE1 antibody and anti-VEGF-C antiserum after SDS-PAGE and Western blotting. Input represents 25 µl of full-length VEGF-C conditioned medium, which was loaded as a positive control.

**ADAMTS3/CCBE1 co-immunoprecipitation.** 293T-CCBE1-StrepIII cells were transfected with ADAMTS3 or mock expression constructs. Conditioned media were immunoprecipitated with ADAMTS3 antibody (Santa Cruz sc-21486) and protein G sepharose or used in a streptactin pull-down. Precipitates were analyzed with streptactin-HRP conjugate after SDS-PAGE and Western blotting.

**Mass spectroscopy.** Enriched CCBE1 protein was subjected to liquid chromatography-mass spectrometry as previously described (Varjosalo et al. 2013, Nat Methods, 10: 307-314). In short, mass spectrometry analysis was performed on an Orbitrap Elite ETD mass spectrometer (Thermo Scientific, Waltham, MA) using the Xcalibur version 2.7.1 coupled to a Thermo Scientific nLCII nanoflow system (Thermo Scientific) via a nanoelectrospray ion source. Peak extraction and subsequent protein identification was achieved using Proteome Discoverer software (Thermo Scientific). Calibrated peak files were searched against human protein databases by a SEQUEST search engine. Database searches were limited to tryptic peptides with a maximum of 1 missed cleavage; carbamidomethyl cysteine and methionine oxidation were set as fixed and variable modifications, respectively.

**Quantitative PCR for ADAMTS2, -3 and -14.** Total RNA was isolated from the cells with NucleoSpin RNA II kit according to the protocol (Macherey-Nagel) and cDNA synthesis was performed using iScript cDNA synthesis kit (Bio-Rad). QPCR was carried out using SYBR green chemistry with Bio-Rad CFX96 Real-Time System. All data were normalized to GAPDH and quantification was performed using the 2-DDCT method. The following primers were used: hADAMTS2 (fwd 5' - AAATCTACCATGACGAGTCC -3' SEQ ID
Downregulation of ADAMTS3 by lentiviral shRNA. Constructs expressing ADAMTS3 shRNA (TRCN0000050571) and non-target control shRNA were from the RNAi Consortium shRNA library (Moffat et al. 2006, Cell, 124:1283-1298). Lentivirus, generated by using the standard methods according to the RNAi Consortium, was used to transduce 293T cells. After selection of stable pools, the efficiency of VEGF-C cleavage was assayed as described in the Materials and Methods section under Transfection, metabolic labeling and protein analysis.

In vivo experiments. Tibialis anterior muscles of FVB/N male mice were injected with 1:1 mixed solutions of rAAV9s encoding m(mouse)CCBE1-V5, full-length mVEGF-C or HSA. The AAV9-HSA and AAV9-ΔNΔC-mVEGF-C single vectors were used as negative and positive controls, correspondingly. Total concentration of the vector particles in a single injected dose was $6 \times 10^10$. Three weeks after transduction the mice were sacrificed by CO$_2$ overdose. The tibialis anterior muscles were isolated, embedded into O.C.T. (Sakura Finetek Europe, Alphen aan den Rijn, The Netherlands), sectioned (10 μm thickness)
and stained for the lymphatic (LYVE-1, Prox-1) and blood vascular (PECAM-1) as well as smooth muscle cell/pericyte (smooth muscle actin, SMA) and leukocyte markers (CD45), followed by Alexa-conjugated secondary antibodies (Molecular Probes, Invitrogen). Fluorescent images were obtained in an Axioplan 2 microscope (Carl Zeiss AG, Oberkochen, Germany); the objectives were: 10x NA=0.3 WD 5.6 and 20x NA=0.5 WD 2.0; the camera was a Zeiss AxioCam-HRm 14-bit greyscale CCD; the acquisition software was Zeiss AxioVision 4.6. Quantification of the stained areas was done using ImageJ software. Prox-1 positive nuclei were counted manually. The detection of luciferase activity in EGFP/Luc Vegfr3EGFP/Luc mice was performed as previously described (Martínez-Corrall et al, 2012, PNAS 109: 6223-6228). The National Board for Animal Experiments of the Provincial State Office of Southern Finland approved all animal experiments carried out in this study.

Any inhibitors of ADAMTS3, such as an anti-ADAMTS3 antibody, are administered to a mouse model. Also, ADAMTS3 proteins or polynucleotides/aptamers (such as ADAMTS3 expression constructs or viral vectors described earlier in the materials and methods) are administered to a mouse model. Administration or injection is carried out and the tibialis anterior muscles are isolated and studies as described in the above paragraph (under in vivo experiments).

**Statistical analysis.** Significance of the differences was determined using one way-ANOVA. When equal variances were assumed, Tukey’s test was used as a post-hoc test. When variances were not assumed as equal, Games-Howell test was used as a post-hoc test. The EC50 of the Ba/F3 assays were calculated using logistic regression. Error bars in figures denote the standard deviation.

**Production of anti-VEGF-C-antibodies or VEGF-C binding polypeptides against a fully processed mature VEGF-C polypeptide**

The purified, incompletely processed or unprocessed form of VEGF-C polypeptide is cleaved by an ADAMTS3 polypeptide produced by insect cells and purified from conditioned cell culture supernatant. If the C-terminal cleavage is incomplete, similarly obtained furin can be used to complete the cleavage. The mature VEGF-C polypeptide is purified by subtractive IMAC, followed by one or more conventional chromatography steps (e.g. gel filtration, affinity chromatography). An antibody library, a polypeptide library (such as an SH3
domain library) or an oligonucleotide/aptamer library is screened with the purified, mature VEGF-C. Antibodies, polypeptides or oligonucleotides/aptamers binding to the fully processed mature VEGF-C are collected as a group or individually. Groups can be optionally subjected to another round of screening.

5 **Results**

**CCBE1 enhances VEGF-C processing and secretion, resulting in increased VEGFR-3 activation.** CCBE1 was detected as a protein of 40-55 kDa molecular weight in both cell lysates and conditioned media of 293T cells transfected with a CCBE1 expression vector (Figure 1A). In the supernatant, part of the CCBE1 migrated as a diffuse, glycosylated band corresponding to a putative CCBE1 dimer. Transfected VEGF-C was expressed as the uncleaved 58 kDa precursor, C-terminally processed 29/31 kDa and fully processed 21 kDa mature form (Figure 1D, lane 1). However, when VEGF-C and CCBE1 were cotransfected, the amounts of the uncleaved VEGF-C and the 29/31 kDa polypeptide were reduced dramatically and the mature, fully activated VEGF-C became the major species (Figure 1D, compare lanes 1 to 3). These results indicated that CCBE1 accelerates both the N-terminal and the C-terminal proteolytic processing of VEGF-C.

In order to be able to analyze the effect of CCBE1 cotransfection on the intracellular VEGF-C forms, we used a shorter labeling period. Cotransfection with CCBE1 facilitated the secretion of VEGF-C as the intracellular amount was reduced by 80% in the lysates of the cotransfected cells (Figure 2A, compare lanes 5, 6 to 7, 8).

The conditioned medium from the CCBE1/VEGF-C cotransfected cultures stimulated the growth and survival of Ba/F3-VEGFR-3/EpoR and Ba/F3-VEGFR-2/EpoR cells better than the supernatant of cells transfected with VEGF-C alone, while CCBE1 alone showed very little activity. This confirmed that the enhanced secretion and cleavage resulted in increased levels of active VEGF-C protein in the cultures (Figure 1C and Figure 2B). Notably, also the supernatants of cells expressing CCBE1 alone resulted in a slight increase in the survival of Ba/F3-VEGFR-3/EpoR cells, presumably because of increased processing and activation of endogenous VEGF-C made by the cells.

**Truncated CCBE1 does not enhance VEGF-C stimulated VEGFR-3 activity.** CCBE1 was truncated as disclosed by Bos et al. (2011,
ibid.), i.e. so that collagen-binding domain was removed and the rest of the CCBE1 molecule was fused to Fc domain of human IgG for better protein stability. The truncated CCEB1 was purified to homogeneity and tested in the established in vitro system together with purified full-length or mature VEGF-C protein. We run the experiment as follows. hVEGFR-3/EpoR-Ba/F3 cells were stimulated by increasing concentrations of human VEGF-CΔNΔC (mature VEGF-C) (Figure 3A) or full-length VEGF-C (VEGF-C precursor) (Figure 3B) in presence of constant dose (5 μg/ml) of truncated CCBE1-Fc or negative control protein, which is Fc part. Cells were incubated in stimulation conditions for a total of 3 days. At the end of this period, thiazolyl blue tetrazolium bromide (MTT, Sigma-Aldrich) was added to cell cultures and the numbers of alive cells in each culture (a direct score of cell proliferation) was measured by OD540 optical density (yellow MTT is reduced by mitochondrial enzymes of alive cells producing dark-blue material). The results demonstrate clearly that the truncated CCBE1 is not able to enhance the FGFR-3 stimulation activity of VEGF-C.

**CCBE1 can enhance VEGF-C processing in trans.** In the developing mouse embryo and zebrafish, CCBE1 is expressed adjacent to developing lymphatic structures, but not by the endothelium itself (Hogan et al, 2009, Nature Genetics 41: 396–398). Thus, we wanted to determine if CCBE1 production in trans by other cells can also enhance VEGF-C processing. We transfected separate cultures of 293T cells with VEGF-C or CCBE1, and mixed the cell populations 24 hours after transfection. Alternatively, we mixed CCBE1-transfected cells with cells stably expressing VEGF-C. As in the co-transfection experiments, CCBE1 increased the efficiency of the extracellular processing of VEGF-C (Figure 4A). In contrast, the intracellular processing and secretion of VEGF-C was not affected when adjacent cells produced an excess of CCBE1 (Figure 4B).

**CCBE1 enhances the lymphangiogenic activity of VEGF-C in vivo.** In order to investigate if CCBE1 enhances lymphangiogenesis in vivo, we transduced mouse tibialis anterior muscles with adeno-associated virus (AAV) vectors expressing CCBE1 (AAV9-CCBE1) alone, together with an AAV9-VEGF-C vector in a 1:1 ratio, or AAV9-human serum albumin (HSA) as a negative control. AAV9 encoding an activated form of VEGF-C (ΔNΔC-VEGF-C) was used as a positive control to mimic the fully proteolytically processed (mature) form of VEGF-C.
Two weeks after the AAV transduction, the skeletal muscles were analyzed by immunohistochemistry using markers for endothelial cells (PECAM-1), lymphatic endothelial cells (LYVE-1, Prox1) and leukocytes (CD45). In this assay, both full-length VEGF-C and ΔNΔC-VEGF-C stimulated lymphangiogenesis, although the latter ("mature" form) gave a considerably stronger response at the same viral dose, whereas only ΔNΔC-VEGF-C stimulated angiogenesis (Figure 6 and Figure 7, bottom row). This suggested that the proteolytic processing of full-length VEGF-C was inefficient in the AAV9 transduced muscle.

However, when full-length VEGF-C was co-transduced with CCBE1, lymphangiogenesis was significantly enhanced, as shown by the LYVE-1 and Prox-1 staining (Figure 6). We also observed significantly more angiogenesis (Figure 7), indicating that CCBE1 enhances the proteolytic processing of VEGF-C also *in vivo*. An increased number of CD45+ leukocytes was observed after co-transduction of full-length VEGF-C/CCBE1 (Figure 8), suggesting that VEGF-C activation increases also the leukocyte recruitment via VEGFR-3.

To corroborate these findings, we used AAV9-transduced heterozygous Vegfr3EGFP/Luc mice (Martínez-Corrales et al, 2012, ibid.) to monitor lymphangiogenesis by optical bioluminescent imaging *in vivo*. We detected strong luciferase signals in mice co-transduced with the AAVs encoding VEGF-C and CCBE1, whereas weaker signals were detected in mice transduced with VEGF-C or CCBE1 alone, and no bioluminescent signals in mice transduced with HSA (Figure 9).

**VEGF-C propeptides are involved in CCBE1 mediated proteolysis.** Our attempts to demonstrate a physical interaction of VEGF-C and CCBE1 were unsuccessful. We thus assumed that the CCBE1-VEGF-C interaction is weak, short-lived and perhaps indirect. We tried to identify the structural element in VEGF-C that is responsible for the CCBE1-mediated accelerated secretion and cleavage. When we compared the secretion kinetics between wild-type VEGF-C and a VEGF-C mutant devoid of its C-terminal propeptide in a pulse-chase experiment, the labeled VEGF-C mutant reached its secretion peak already between 15 and 45 minutes, whereas wild-type VEGF-C reached its secretion peak only after two hours (Figure 10). This suggested that the C-terminal propeptide regulates the secretion efficiency. We tried to inhibit the CCBE1-enhanced processing of VEGF-C by adding high concentra-
tions of the purified VEGF-C N-terminal or C-terminal propeptide or VHD. Approximately 79% inhibition of VEGF-C processing was observed with the C-terminal propeptide and about 43% inhibition with the N-terminal propeptide, while the VHD of or the BSA control did not alter the ratios of the proteolytic fragments produced by the transfected cells (Figure 11A), confirming that the VEGF-C propeptides are involved in the CCBE1 mediated cleavages. Although VEGF-D is very similar to VEGF-C in structure and proteolytic processing (Leppänen et al, 2011, Blood 117: 1507–1515; Stacker et al, 1999a, J. Biol. Chem. 274: 32127–32136), CCBE1 cotransfection did not accelerate the proteolytic processing of human full-length VEGF-D or a chimeric factor where the VEGF-D VHD is flanked by the N- and C-terminal propeptides of VEGF-C (Figure 11B, lanes 3-6).

VEGF-C and CCBE1 are processed by the ADAMTS3 procollagenase. Our attempts to demonstrate a physical interaction of VEGF-C and CCBE1 were unsuccessful (Figure 5A). Next, we stably expressed CCBE1 in 293T cells, purified the protein and subjected it to tryptic digestion followed by mass spectrometry. The most abundant co-purified protease was ADAMTS3. Efficient N-terminal processing of pro-VEGF-C was obtained when ADAMTS3 was expressed together with VEGF-C in 293T cells (Figure 12A). In order to analyze if CCBE1 enhances the ADAMTS3-mediated VEGF-C cleavage, the amounts of ADAMTS3 used for VEGF-C cleavage were titrated. When CCBE1-, VEGF-C- and ADAMTS3-conditioned media were mixed in a ratio of 60:30:1, the ADAMTS3-mediated cleavage of VEGF-C was more efficient in the presence of CCBE1 than without (Figure 12B), and the corresponding medium had significantly increased activity in the VEGFR-3/EpoR-expressing Ba/F3 cells (Figure 12C). When the media of the ADAMTS3 co-transfected samples were precipitated with ADAMTS3 antibodies or streptactin and analyzed in Western blotting with antibodies recognizing the C-terminus of CCBE1, the specific CCBE1 band migrated at 25 kDa, which corresponds to the collagen-like domain of CCBE1 (Figure 12D), indicating that ADAMTS3 cleaves CCBE1 between the EGF and collagen homology domains. Interestingly, the DU-4475 cells produced only uncleaved CCBE1 (Figure 5B), which did not promote VEGF-C activation (Figure 5C).

VEGF-C cleavage by plasmin is not influenced by CCBE1. As previously published (McColl et al. 2003, J Exp Med; 198:863-868), VEGF-C was efficiently cleaved by plasmin (Figure 16A). The fragments obtained with
low amounts of plasmin activated VEGFR-3, but this activity was lost at high plasmin concentrations (Figure 16B). CCBE1 did not affect the efficiency of plasmin cleavage (Figure 16C). Edman degradation of the final products revealed the N-terminal sequence KTQC and a complete lack of the N-terminal helix, which is incompatible with VEGFR-3 activation (Leppanen et al. 2013 ibid.).

**ADAMTS3 produced by 293T cells processes VEGF-C to the mature form.** The N-terminus of the 21/23 VEGF-C generated by incubation with recombinant, purified ADAMTS3 was identical to that reported for mature VEGF-C produced by 293 cells (Joukov et al. 1997, ibid.) (Figure 17A). VEGF-D was not cleaved by ADAMTS3 under the same conditions (Figure 17B), despite featuring a similar cleavage motif (Figure 17C).

Apart from ADAMTS3, two other proteases, ADAMTS2 and ADAMTS14, belong to the procollagenase subfamily of ADAMTS proteases (Porter et al., 2005, Biochem J 386:15-27). Interestingly, the ADAMTS1 gene deletion in mice results in deficient ovarian lymphangiogenesis (Brown et al., 2006, Dev Biol. 300:699-709). However, unlike ADAMTS3, ADAMTS1, 2 or 14 did not cleave VEGF-C (Figure 17D). Interestingly, the cleavage motif of ADAMTS3 in VEGF-C is the same as the ADAMTS2 motif in procollagens (FA[AP]↓, Dombrowski et al. 1988, J Biol Chem 263: 16545-16552), which were so far the only known substrates of ADAMTS3 (Fernandes et al., 2001, J Biol Chem 276:31502-31509).

We found that the cell lines that produce active, mature VEGF-C (293T, 293T-CCBE1 and PC-3 cells) express ADAMTS3, while the cell lines that were unable or extremely inefficient in producing active VEGF-C (CHO, NIH-3T3), expressed very little or no ADAMTS3 (Figures 18A and B). Furthermore, when ADAMTS3 was silenced in 293T cells by using lentiviral shRNA, the VEGF-C cleavage was inhibited (Figure 19A).

VEGF-C/VEGF-D chimeras generated by propeptide swapping were not subject to ADAMTS3 cleavage (Figures 11B and 19B). Interestingly however, 79 % of VEGF-C processing was inhibited by the purified C-terminal propeptide and 43% by the N-terminal propeptide, whereas the VHD or BSA gave no inhibition (Figure 11A), indicating that the VEGF-C propeptides are necessary, but not sufficient for VEGF-C recognition by ADAMTS3.

**The N-terminal domain of CCBE1 enhances pro-VEGF-C cleavage to the mature form.** Because of the difficulty to express sufficient
amounts of full-length CCBE1, we investigated if the isolated N-terminal domain of CCBE1 (CCBE1Δ175) can increase VEGF-C activity. We stimulated VEGFR-3 transfected PAE cells with pro-VEGF-C, which resulted in very little VEGFR-3 phosphorylation when compared to mature VEGF-C (Figure 13A, lanes 1 and 2). When the recombinant CCBE1Δ175 was added with pro-VEGF-C, VEGFR-3 phosphorylation was strongly increased (Figure 13A, third lane). Analysis of VEGFR-3 coprecipitated proteins of the pro-VEGF-C stimulated cells indicated that both pro-VEGF-C and mature VEGF-C are bound to the receptor in the presence of CCBE1Δ175 (Figure 13B, compare lanes 2 and 3). To identify what form of VEGF-C was bound to the phosphorylated VEGFR-3 receptor, we applied purified CCBE1Δ175 and biotinylated, purified pro-VEGF-C to cultures of PAE-VEGFR-3 cells in PBS for 210 s and cross-linked VEGFR-3 associated proteins for 390 s. Precipitation and analysis of tyrosyl phosphorylated proteins indicated that mature VEGF-C was bound to activated VEGFR-3, when both CCBE1Δ175 and pro-VEGF-C were used for the stimulation (Figure 13C). Pro-VEGF-C alone did not co-precipitate with VEGF-C, unless VEGFR-3 was co-expressed with neuropilin-2 (Figure 13D). However, even then, pro-VEGF-C induced very little phosphorylation of VEGFR-3 (data not shown).

Pro-VEGF-C can act as a competitive inhibitor of mature VEGF-C. We next analyzed the ability of pro-VEGF-C to inhibit VEGFR-3 activation by mature VEGF-C. Indeed, pre-incubation of LECs with high amounts of pro-VEGF-C abolished their ability to respond to mature VEGF-C (Figure 14A). Unlike mature VEGF-C, pro-VEGF-C did not stimulate the endocytosis of VEGFR-3 or the phosphorylation of the Erk, Akt or eNOS downstream signaling proteins in BECs or LECs (Figure 14B and C).
CLAIMS

1. A method of producing fully processed mature VEGF-C polypeptide, wherein the method comprises the steps of optionally providing VEGF-C polypeptide in an incompletely processed or unprocessed form,

   providing ADAMTS3 polypeptide, and

   cleaving the incompletely processed or unprocessed form of VEGF-C polypeptide by ADAMTS3,

   thereby producing mature VEGF-C.

2. A method of cleaving VEGF-C polypeptide, wherein the method comprises the steps of optionally providing VEGF-C polypeptide in a incompletely processed or unprocessed form,

   providing ADAMTS3 polypeptide, and

   cleaving the incompletely processed or unprocessed form of VEGF-C polypeptide by ADAMTS3,

   thereby producing mature VEGF-C.

3. The method according to claim 1 or 2, wherein the incompletely processed or unprocessed VEGF-C polypeptide is a recombinant polypeptide.

4. The method according to any one of claims 1-3, wherein ADAMTS3 polypeptide is a recombinant polypeptide.

5. The method according to any one of claims 1-4, wherein the VEGF-C and/or ADAMTS3 polypeptide have/has been recombinantly produced from a polynucleotide.

6. The method according to any one of the previous claims, wherein the fully processed mature VEGF-C is in the form of a polypeptide comprising amino acids 112-227 of SEQ ID NO: 3 or an amino acid sequence having at least 80% amino acid sequence identity therewith.

7. The method according to any one of the previous claims, wherein ADAMTS3 comprises amino acids of SEQ ID NO: 11 or an amino acid sequence having at least 80% amino acid sequence identity therewith.

8. The method according to any one of the previous claims, wherein the incompletely processed or unprocessed VEGF-C polypeptide is provided as a polypeptide or polynucleotide to cell culture by administration.

9. The method according to any one of the previous claims, wherein the ADAMTS3 polypeptide is provided as a polypeptide or polynucleotide to
cell culture by administration.

10. The method according to any one of the previous claims, where-
in the incompletely processed or unprocessed VEGF-C polypeptide and the
ADAMTS3 polypeptide are provided as a polypeptide(s) and/or polynucleo-
tide(s) to cell culture by administration.

11. The method according to any one of the previous claims, where-
in the incompletely processed or unprocessed form of VEGF-C is a full length
VEGF-C, a prepro-VEGF-C, a pre-pro form lacking a signal sequence or a
partly processed VEGF-C.

12. The method according to any one of the previous claims, where-
in VEGF-C is a human VEGF-C.

13. The method according to any one of the previous claims, where-
in ADAMTS3 is a human ADAMTS3.

14. The method according to anyone of the previous claims further
comprising a step of providing CCBE1 polypeptide after the step of providing
ADAMTS3 polypeptide.

15. Use of ADAMTS3 for producing fully processed mature VEGF-C
polypeptide in cell culture.

16. Use of ADAMTS3 for cleaving VEGF-C polypeptide.

17. ADAMTS3 or a composition comprising ADAMTS3 for use in the
treatment of a lymphatic disorder.

18. An inhibitor of ADAMTS3 or a composition comprising an inhibi-
tor of ADAMTS3 for use as a medicament.

19. An inhibitor of ADAMTS3 or a composition comprising an inhibi-
tor of ADAMTS3 for use in the treatment of a lymphatic disorder.

20. A method of treating a lymphatic disorder by administering to a
subject in need thereof ADAMTS3, an inhibitor of ADAMTS3 or a composition
comprising ADAMTS3 or an inhibitor of ADAMTS3.

21. The method according to claim 20 or the composition for use
according to claim 19, wherein the composition further comprises a pharma-
ceutically acceptable vehicle.
22. The method according to claim 20 or 21 or the composition for use according to any one of claims 17-19 or 21, wherein the composition further comprises other therapeutically effective agents.

23. The method or the composition for use according to claim 22, wherein in the presence of ADAMTS3 the other therapeutically effective agent is CCBE1 and/or an incompletely processed or unprocessed VEGF-C.

24. ADAMTS3 or the composition for use or the method according to any one of claims 17 or 20-23, wherein the lymphatic disorder is a lymphedema.

25. ADAMTS3 or the composition for use or the method according to claim 24, wherein the lymphedema is selected from the group consisting of primary lymphedema, Milroy's syndrome, Meige's lymphedema, lymphedema tarda, secondary lymphedema, lipedema, lymphangiectasia and Gorham's disease.

26. The inhibitor of ADAMTS3 or the composition for use or the method according to any one of claims 19-22, wherein the lymphatic disorder is an inflammatory disorder, tumor or cancer.

27. The inhibitor of ADAMTS3 or the composition for use or the method according to claim 26, wherein the inflammatory disorder is selected from a group consisting of type 1 diabetes, atherosclerosis, chronic airway diseases, psoriasis, metabolic disease and obesity.

28. The method according to anyone of the previous claims 20-27, wherein the subject is a human or an animal.

29. A method of producing a VEGF-C binding molecule against a fully processed mature VEGF-C polypeptide, wherein the method comprises the steps of

   cleaving the incompletely processed or unprocessed form of VEGF-C polypeptide by ADAMTS3 polypeptide to obtain a fully processed mature VEGF-C;

   immunizing a subject with the fully processed mature VEGF-C immunogen or screening a library with the fully processed mature VEGF-C; and

   generating VEGF-C binding molecules against the fully processed mature VEGF-C.
30. The method according to claim 29, wherein the VEGF-C binding molecule is an anti-VEGF-C-antibody or a VEGF-C binding polypeptide or small molecule.

31. The method according to claim 29 or 30, wherein the library is an antibody, polypeptide, nucleotide or small molecule library.
Figures 1A to 1D.
Figures 2A and B.
Figure 3A.

Figure 3B.
**Figure 4A.**

**Figure 4B.**

**Figures 5A to C.**
Figure 7.
Figure 8.
Figure 9.

Figure 10.
Figure 11A.

Figure 11B.
Figures 12A to D.
Figures 13A to D.
Figures 14A and B.
Figure 14C.
Figure 15.
Figures 16A-C.

Figures 17A, B and D.
Figure 17C.
Figures 18A and B.
Figures 19A and B.
**INTERNATIONAL SEARCH REPORT**

**A. CLASSIFICATION OF SUBJECT MATTER**

**INV.** A61K38/48 C12N9/64

**ADD.**

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

A61K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, Sequence Search, BIOSIS, EMBASE, WPI Data

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

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<th>Relevant to claim No.</th>
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<td>X</td>
<td>WO 00/53774 A2 (NEUROCININE BIOSCIENCES INC [US]; KELNER GREGORY S [US]; CLARK MELODY [US]) 14 September 2000 (2000-09-14) figure 9; sequence 9 page 29, line 23 - page 31, line 6</td>
<td>17-23, 26,28</td>
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<td>A</td>
<td>WO 2004/009773 A2 (LUDWIG INST CANCER RES [US]; MCCOLL BRADLEY [AU]; BALDWIN MEGAN [AU]) 29 January 2004 (2004-01-29) page 19, line 1 - line 2; claim 3; figure 3c</td>
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Further documents are listed in the continuation of Box C.

See patent family annex.

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**Date of the actual completion of the international search**

21 April 2015

**Date of mailing of the international search report**

29/04/2015

Name and mailing address of the ISA/

European Patent Office, P.B. 5618 Patentlaan 2 NL - 2230 HV Rijswijk

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Authorized officer

Lanzrein, Markus

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<td>KARI ALITALO: &quot;The lymphatic vasculature in disease&quot;, NATURE MEDICINE, vol. 17, no. 11, 7 November 2011 (2011-11-07), pages 1371-1380, XP055149482, ISSN: 1078-8956, DOI: 10.1038/nm.2545 figure 1</td>
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