 doi:10.20944/preprints202101.0304.v1

Review

The proteolytic activation of vascular endothelial growth factors

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Abstract: Specific proteolytic cleavages turn on, modify, or turn off the activity of vascular endothelial growth factors (VEGFs). Proteolysis is most prominent among the lymph-angiogenic VEGF-C and VEGF-D, which are synthesized as precursors that need to undergo enzymatic removal of their C- and N-terminal propeptides before they can activate their receptors. The activating cleavage of VEGF-C is mediated by at least five different proteases: plasmin, ADAMTS3, prostate-specific antigen, cathepsin D, and thrombin. All of these proteases except for ADAMTS3 can also activate VEGF-D. Processing by different proteases results in distinct forms of the "mature" growth factors, which differ in affinity and receptor activation potential. The “default” VEGF-C-activating enzyme ADAMTS3 does not activate VEGF-D and therefore, VEGF-C and VEGF-D do function in different contexts. VEGF-C itself is also regulated in different contexts by different proteases. During embryonic development, ADAMTS3 activates VEGF-C. In contrast, thrombin and plasmin likely activate VEGF-C/-D during tissue injury-induced lymphangiogenesis, and PSA and cathepsin D perhaps during tumor-associated pathological lymphangio-genesis. Additionally, cathepsin D from saliva might activate latent VEGF-C/-D upon wound licking, thereby accelerating healing. Similar to tyrosine kinase receptors and VEGFs themselves, these activating proteases could be targeted to modulate angiogenesis and lymphangiogenesis in relevant diseases.

Keywords: Vascular Endothelial Growth Factors (VEGFs); VEGF-A; PlGF; VEGF-B; VEGF-C; VEGF-D; angiogenesis; lymphangiogenesis; CCBE1; proteases; ADAMTS3; plasmin; cathepsin D; KLK3; prostate-specific antigen (PSA); thrombin; wound healing; metastasis; proteolytic activation; vascular biology

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Introduction

In vertebrates, the family of vascular endothelial growth factors (VEGFs) comprises typically five genes: VEGF-A (in older literature often referred to simply as "VEGF"), Placenta Growth Factor (PlGF), VEGF-B, VEGF-C, and VEGF-D. In addition to these orthodox VEGFs, several genes coding for VEGF-like molecules have been discovered in venomous reptiles (collectively named VEGF-F) [1] and in several viruses of the poxvirus and iridovirus families (collectively named VEGF-E) [2–5]. In vertebrates, the VEGF growth factors are central to the development and maintenance of the cardiovascular system and the lymphatic system. Non-vertebrates also feature VEGF-like molecules, but their functions are less well defined.

The subdivision of the vertebrate vascular system into the cardiovascular and the lymphatic system is reflected at the molecular level by a subdivision of the VEGF family into VEGFs acting primarily on blood vessels (VEGF-A, PlGF and VEGF-B) and VEGFs acting mostly on lymphatic vessels (VEGF-C and VEGF-D). This specificity results from the expression pattern of the three VEGF receptors (VEGFRs). VEGFR-1 and VEGFR-2 are expressed on blood vascular endothelial cells (BECs), while lymphatic endothelial cells (LECs) express VEGFR-2 and VEGFR-3 (Figure 1).

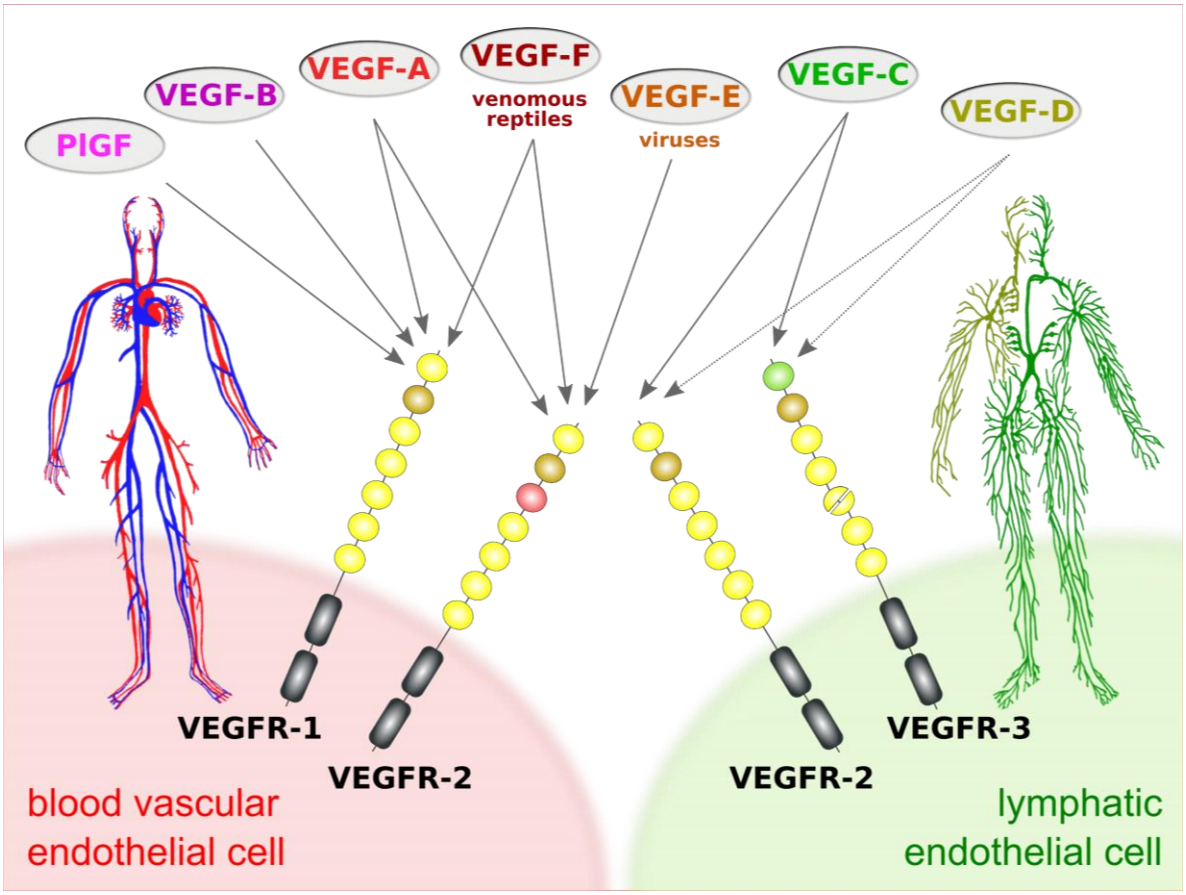

Figure 1. VEGFs act on blood and/or lymphatic vessels depending on their affinities towards VEGF receptors 1, -2, and -3. Growth factors that are able to activate VEGFR-2 can in principle promote both the growth of blood vessels (angiogenesis) and lymphatic vessels (lymphangiogenesis) since VEGFR-2 is expressed on both blood and lymphatic endothelium. Please note that VEGF-E and VEGF-F are not of human origin: The genes for VEGF-E can be found in viral genomes and VEGF-F is a snake venom component.

The biology of the VEGFs and their signaling pathways has been extensively discussed elsewhere [6,7]. From all VEGF family members, only VEGF-A and VEGF-C are essential in the sense that

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constitutive ablation of their genes in mice results in embryonic lethality [8–10]. VEGF-A was also the first gene, where the ablation of a single allele resulted in embryonic lethality [8,9]. While the primary function and importance of the cardiovascular system is obvious also to the layperson, the tasks of the lymphatic system escape even some life science professionals. Its major three tasks are:

1. Tissue drainage for fluid balance and waste disposal
2. Immune surveillance including hosting and trafficking of immune cells
3. Uptake of dietary long-chain fatty acids and other highly lipophilic compounds in the gut

Considerable effort has been devoted to the mechanisms and effects of receptor binding and downstream signaling of the VEGFs. Less is known about the processes upstream of receptor binding such as secretion, release, and proteolytic processing. In this review, we want to briefly give an overview of what is known about the proteolytic processing of VEGFs with a focus on the lymphangiogenic VEGFs.

Evolutionarily, the importance of proteases has been remarkable. Proteolytic processing is often used to regulate protein activity and to create variation in a protein's function. This has been suggested by phylogenetic and functional studies in all kingdoms of life, including viruses [11], plants [12] and animals [13]. Not surprisingly, proteases are used to regulate and to create functional variety in the VEGF family and thus can be regarded as signaling molecules [14].

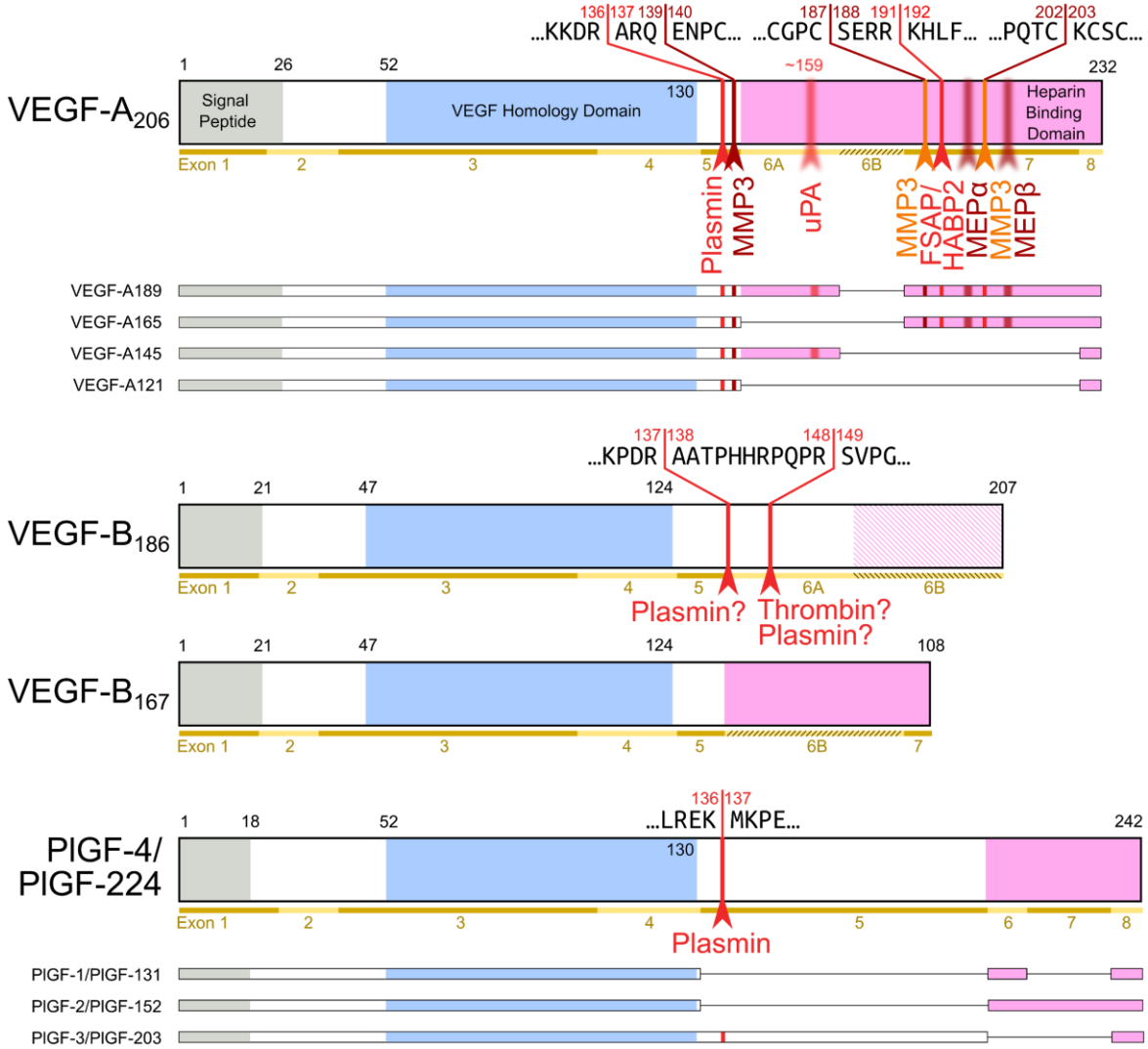

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Figure 2. Most diversity among the hemangiogenic VEGFs is achieved by alternative splicing. Nevertheless, proteolytic processing of VEGF-A [15] and PlGF [16] can convert the longer, heparin-binding isoforms into more soluble shorter species. VEGF-B is a special case, as alternative splicing results in two isoforms that translate the same nucleotide sequence in two different frames resulting in a heparin-binding and a soluble isoform [17,18]. Due to the near-perfect cleavage context [19], thrombin has been suspected to be the responsible protease for VEGF-B_{186} cleavage [20]. Prothrombin is indeed expressed by 293T cells [21], in which the cleavage has been demonstrated [17]. Plasmin cleaves VEGF-B_{186} at least at four different sites, of which the two most likely predicted sites are indicated. Importantly, the interaction epitope for neuropilin-1 binding [17] is removed by the predicted plasmin cleavage between Arg137 and Ala138. Cleavages, where only the approximate position is known are indicated by semi-transparent, blurry arrows. For the plasmin cleavage of VEGF-A, only the most sensitive site is shown as prolonged incubation results in progressing degradation of VEGF-A [22], and the same is likely the case for VEGF-B_{186} [17]. For VEGF-A and PlGF, the numbering is according to the longest shown isoform. VEGF-A is cleaved not only by MMP3 but also in a similar fashion by MMP7, MMP9, MMP19, and - less efficiently - by MMP1 and MMP16 [22].

Proteolytic processing of the hemangiogenic VEGFs

Among the hemangiogenic VEGFs, protein diversification within a single VEGF family member relies more on differential mRNA splicing than on proteolytic processing (reviewed in [15]). mRNA splicing generates several isoforms of VEGF-A, which differ by the extent of the C-terminal, predominantly basic heparin-binding domain (HBD) [23–25]. The HBD mediates the interaction of VEGF-A with the extracellular matrix, cell surface heparan proteoglycans (HSPGs), and neuropilin-1. The interaction with HSPGs involves both a sequence-specific binding epitope and electrostatic effects of a predominantly basic amino acid sequence. Only a few isoforms are completely devoid of heparin-binding properties under physiological conditions and therefore fully soluble. Mice expressing only the major soluble isoform (VEGF-A_{121}) are born, but show severe cardiovascular defects and die from cardiac failure [26].

The matrix-binding properties of the larger VEGF-A isoforms are important for the formation of growth factor gradients which are assumed to be essential for efficient organ vascularization [27,28]. VEGF-A_{189} and VEGF-A_{206} are sequestered in the extracellular matrix (or on cell surface HSPGs) and at least VEGF-A_{189} has been shown not to participate in receptor activation [29]. Proteases such as plasmin, urokinase-type plasminogen activator (uPA), and factor VII-activating protease (FSAP) can release and thus activate the ECM-bound, longer VEGF-A isoforms [30,24,31,32]. The cleavage of the main isoform VEGF-A_{165} can also be mediated by various matrix metalloproteinases (MMPs), especially MMP-3, resulting in smaller, non-heparin-binding products [22]. While such cleavages do liberate VEGF-A and are necessary for the mitogenic activity of VEGF-A_{189} [29], they were reported to reduce the mitogenicity of VEGF-A_{165} [33]. Unfortunately, there is little insight into the nature of the molecular handover of HSPG- and ECM-bound VEGF-A to VEGFR-2 or to the VEGFR-2/neuropilin signaling complex [7]. The isoform composition and the location where the cleavage happens are likely important determinants of the net effect. The release of cell surface HSPG-bound VEGF-A is perhaps more likely to result in productive signaling compared to the release of ECM-bound VEGF-A. Complementary to the ECM release by proteolytic cleavage of VEGF-A, enzymatic degradation of the ECM-binding sites, e.g. of HSPGs by heparinases or binding site competition by heparin or heparan sulfate achieves the same release, but without loss of the HBD [30].

Of the four human PlGF isoforms, PlGF-2 and -4 contain also a C-terminal heparin-binding domain. At least the PlGF-2 HBD can be removed by plasmin [16]. VEGF-B_{167} contains as well a heparin-binding domain homologous to the one in VEGF-A_{165}, but it is unknown whether this domain is subject to proteolytic removal. A yet unknown protease unmasks the neuropilin-1 binding site of the longer VEGF-B_{186} isoform, but its target site is absent in VEGF-B_{167} [17]. The cleavage context suggests

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that thrombin is able to unmask the neuropilin-1 binding epitope (see Figure 2) [19]. Plasmin cleavage at the same site is likely but does not result in neuropilin-1 binding due to additional cleavages that remove the sequences that are important for neuropilin-1 binding [17].

The lymphangiogenic growth factors VEGF-C and VEGF-D

The hemangogenic VEGFs are rendered inactive either by means of ECM-association or - as in the case for VEGF-A_{189} - by their C-terminal auxiliary domain. Preventing receptor activation by means of inhibitory domains is as well characteristic of the lymphangiogenic VEGFs. Upon secretion, VEGF-C and VEGF-D are kept inactive by their N- and C-terminal propeptides. Hence, the secreted forms are referred to as pro-VEGF-C and pro-VEGF-D. The removal of the propeptides requires two concerted proteolytic cleavages and happens in a very similar fashion for both VEGF-C and VEGF-D (see Figure 3):

1. The first cleavage is constitutively executed by protein convertases before secretion. This intracellular cleavage occurs between the central VEGF homology domain (VHD) and the C-terminal propeptide, but it does not result in the removal of the C-terminal propeptide because it remains covalently attached to the rest of the molecule by disulfide bonds [34–36].
2. The second, extracellular cleavage activates the protein. This cleavage occurs between the N-terminal propeptide and the VHD [34] and can be mediated by a variety of different proteases. The protease that mediates VEGF-C activation in the embryonic development of the mammalian lymphatic system is ADAMTS3 [37–39]. ADAMTS3 is specific for VEGF-C and does not activate VEGF-D. All other activating proteases target both VEGF-C and VEGF-D: plasmin [39,40], prostate-specific antigen (KLK3/PSA), cathepsin D (CatD) [41], and thrombin [42]. The resulting forms of VEGF-C and VEGF-D are referred to as active, mature, or short forms. However, they differ from each other at their N-terminus, because different proteases cleave at different positions within the linker between the N-terminal propeptide and the VHD (see Figure 4).

Figure 3. Two proteolytic cleavages are needed to activate VEGF-C and VEGF-D. The first cleavage, by protein convertases, is constitutive and intracellular. The second is highly regulated and happens after secretion of the pro-forms. Many different enzymes have been shown to catalyze the second cleavage, but the primary activating protease of VEGF-C in mammalian developmental lymphangiogenesis is ADAMTS3. Immunoprecipitation (IP) of transfected 293T cells with a VEGFR-

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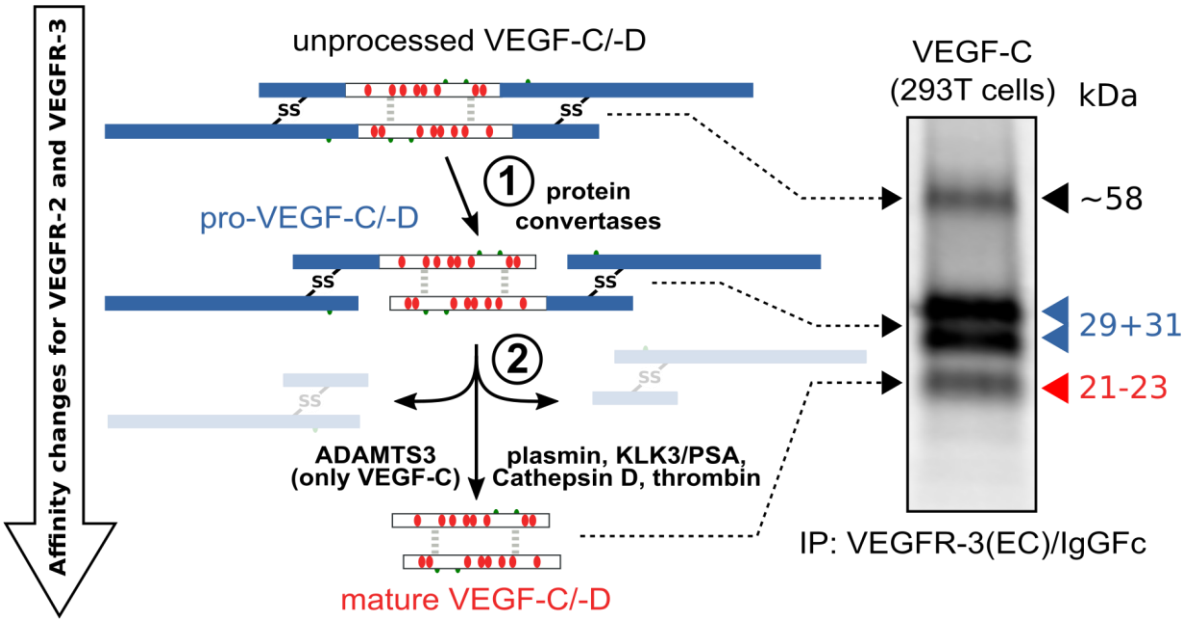

3(EC)/IgGFc fusion protein pulls down the 58 kDa full-length VEGF-C, the pro-VEGF-C peptides of 31 kDa and 29 kDa, and the mature VEGF-C.

Interestingly, pro-VEGF-C is able to competitively block the receptor activation of active, mature VEGF-C. Its propeptides do not preclude VEGF receptor binding but only interfere with receptor activation. Apart from VEGFR-3, pro-VEGF-C binds also the co-receptor neuropilin-2. C-terminal propeptide processing exposes two terminal arginines (R226,227) which contribute to the conserved binding site for neuropilins [43]. Because it is not entirely clear whether pro-VEGF-C is completely incapable of receptor activation or whether it has some residual activity, pro-VEGF-C is either a partial agonist or an antagonist of mature VEGF-C [39].

Plasmin and thrombin

The first protease that was discovered to activate both VEGF-C and VEGF-D is the serine protease plasmin. Plasmin is able to remove both the N- and the C-terminal propeptides of VEGF-D to create a mature form containing only the VEGF homology domain [40]. One of plasmin's main functions is to degrade fibrin, the main component of blood clots. Thrombin is the newest addition to the group of VEGF-C/D-activating enzymes. In addition to its classical role in the conversion of soluble fibrinogen into insoluble fibrin fibrils during blood clotting, it plays a crucial role in early wound healing [42] by activating VEGF-C, which is released from α-granules upon platelet aggregation [44]. Hence both thrombin and plasmin act concertedly to maintain a supply of active VEGF-C over the complete wound healing period. However, in the absence of tissue damage, inactive prothrombin is not converted into thrombin and inactive plasminogen not into plasmin. Therefore, the activation of VEGF-C by thrombin or plasmin seems to be restricted to situations with tissue damage.

Figure 4. Human VEGF-C and -D are processed in a very similar fashion. However, ADAMTS3, which is essential for lymphatic development, cannot activate VEGF-D (at least not in mammals). mRNA splice isoforms of VEGF-C have only been reported in mice [45], but these do not contain the

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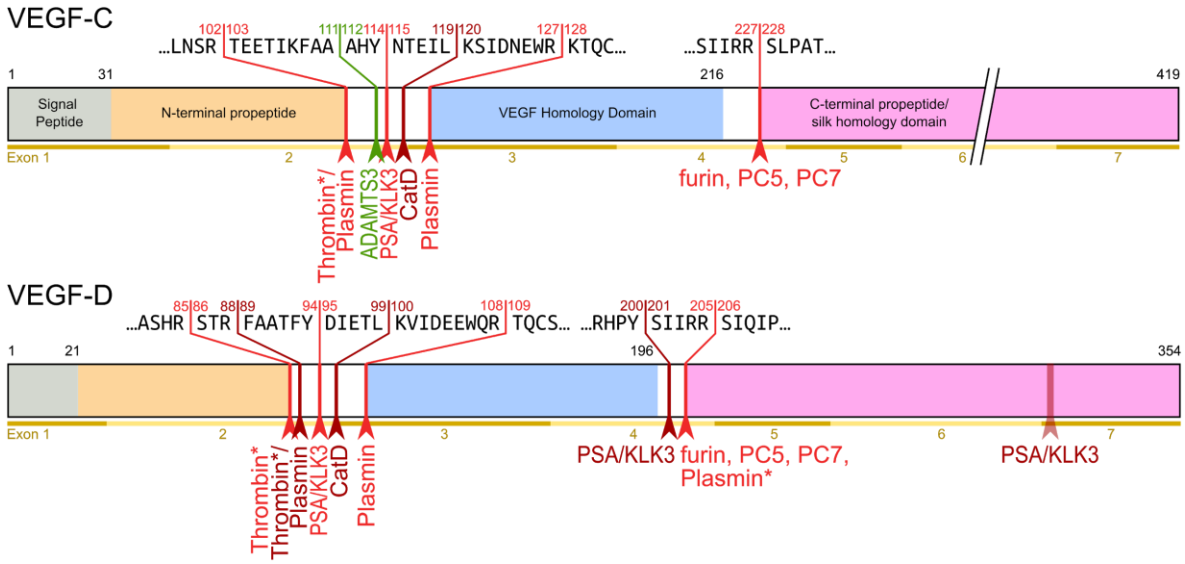

full VEGF homology domain and are therefore not shown here. *Cleavage site is only predicted based on the amino acid context.

ADAMTS3 and the cofactor CCBE1

ADAMTS3 was identified in the search for the endogenous protease that activates VEGF-C. Although plasmin had been identified as a VEGF-C-activating protease [40], it was never seriously considered as a physiological activator of VEGF-C due to its role in fibrin clot degradation and since neither for plasminogen knock-out mice [46] nor for human homozygous functional ablations [47] any lymphatic phenotype had been reported.

In 2009, Alders et al. had shown by homozygosity mapping, that mutations in the human *CCBE1* gene can cause Hennekam Syndrome, which is characterized by generalized lymphatic dysplasia [48]. When the same *Ccbe1* gene was ablated in zebrafish or mice [49,50], the phenotype was closely phenocopying the *Vegfc* knock-out [10]. Because it lacks any protease signature, CCBE1 was assumed to be somehow essential for the VEGF-C/VEGFR-3 signaling pathway, but not to be the VEGF-C-activating protease itself.

Co-transfection of CCBE1 with VEGF-C demonstrated that CCBE1 was able to enhance the proteolytic processing of VEGF-C in 293T cells, and ADAMTS3 was identified as the responsible protease by mass spec analysis of a partially purified CCBE1 from a CCBE1-overexpressing 293T cell line [39]. Based on in-vitro data and its high homology to ADAMTS2, ADAMTS3 had been thought to function in the proteolytic maturation of procollagens [51]. At least in mice, *Adamts3* deletion does not lead to deficiencies in collagen fibril assembly but instead aborts lymphatic development [38]. Subsequent publications confirmed that both ADAMTS3 and CCBE1 are required for successful pro-VEGF-C activation but interestingly, a direct interaction between VEGF-C and CCBE1 has never been demonstrated [37,52,53].

It seems that CCBE1 facilitates the encounter of VEGF-C with ADAMTS3, which is doing the actual cleavage of VEGF-C. From all VEGF-C-activating enzymes, only ADAMTS3 and PSA/KLK3 have been shown to be influenced by CCBE1.

Species-specific differences

Based on sequence similarity, in-vitro substrate, and domain organization, ADAMTS2, -3, and -14 form the *aminoprocollagen peptidase* subgroup within the ADAMTS protein family. Species-specific

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differences in the function of these proteases can be seen in vertebrates: In zebrafish, Adamts3 and Adamts14 compensate for each other [54] but such compensation does not happen in mice [38]. Whether the fact that human ADAMTS14 can activate VEGF-C in vitro [55] reflects species differences among mammals or whether it is an observation without a physiological equivalent is still unknown.

Also on the growth factor side, important species differences have been reported. In mice, VEGF-D is dispensable for the development of the lymphatic system [56], while this is not the case in zebrafish [54], where VEGF-D can partially compensate for the loss of VEGF-C [57]. However, even murine VEGF-D reportedly differs from human VEGF-D in its inability to interact with mouse VEGFR-2 [58]. Exactly the opposite was reported for zebrafish, where the VEGF-D-VEGFR-3 interaction was reported to be entirely absent [59], implying that lymphangiogenesis might happen in zebrafish independently of VEGFR-3. While direct demonstrations of the substrate specificities of the zebrafish Adamts proteases are still missing, it appears clear that zebrafish data is not easily extrapolated to mammals and that, unfortunately, the same might be true for the extrapolation of mouse data to humans.

Which cell types provide ADAMTS3 and CCBE1?

Since VEGF-C, ADAMTS3, and CCBE1 are all secreted proteins, immunohistochemistry cannot reveal their cellular origin. In the establishment of the early zebrafish lymphatics, *Pdgfra*-positive fibroblast populations appeared to be the source for *Vegfc*, *Adamts3*, *Adamts14*, and *Ccbe1* as identified by single-cell RNA sequencing [55]. While in-vitro data supports the notion that fibroblasts are perhaps the dominating source for CCBE1 also for mammals [53], smooth muscle cells appear to make a significant contribution [10,60]. In some contexts, also blood vascular endothelial cells appear to be an important source of VEGF-C [61,62] as well as CCBE1 [63,64]. However, these are crude approximations of the true cellular heterogeneity and in non-homeostatic situations like inflammation or cancer, other cell types, e.g. immune cells like macrophages, are likely major producers of both VEGF-C and VEGF-C-activating proteases [65–67]

Enigmatic propeptides

The evolutionary origins of both propeptides of VEGF-C and VEGF-D are unclear. Unless assuming horizontal gene transfer, they have been conserved for hundreds of millions of years and can be found in virtually all invertebrate VEGF homologs [68–70]. Apart from the VEGFs, the only homologous sequences were found among larval silk proteins of the mosquito genus Chironomus [34,71], resulting in the nickname “silk homology domain” for the C-terminal propeptide.

Because the C- and the N-terminal propeptides of VEGF-C and VEGF-D are linked by disulfide bonds, the first, constitutive cleavage by the protein convertase furin (or PC5 or PC7) does not remove any of the propeptides from VEGF-C or VEGF-D. Both propeptides are released simultaneously with the activating cleavage between the N-terminal propeptide and the VEGF homology domain (see Figures 3 and 4). With 80 and 192 amino acid residues, respectively, the N- and C-terminal propeptides of VEGF-C are significantly longer than typical propeptides. They also fold independently and are therefore also often referred to as N- and C-terminal *domains*. According to the current understanding, the propeptides serve multiple functions.

1. ECM-association and cell surface (HSPG) binding are mediated by the heparin-binding C-terminal propeptide [53,72]
2. Both propeptides collaborate in regulating receptor binding and activation [34,39]. While pro-VEGF-C binds VEGFR-3, it cannot (or only marginally) activate VEGFR-3. Thus pro-VEGF-C, as well as the individual VEGF-C propeptides, are competitive inhibitors of mature VEGF-C [39].
3. The presence of the C-terminal propeptide is required for efficient cleavage of the N-terminal propeptide by ADAMTS3 [53]

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Analogous to VEGF-A, the heparin-binding properties are likely necessary for the correct spatio-temporal distribution of the growth factor and its activity. When the VEGF-C propeptides are grafted upon VEGF-A, the resulting blood vasculature was denser compared with VEGF-A-induced vasculature [73]. Vice versa, when the C-terminal domain of VEGF-C was replaced by the heparin-binding domain of VEGF-A, less, but larger lymphatic vessels were generated which localized preferentially to HSPG-rich structures like basement membranes [74]. The heparin-binding of VEGF-C is somewhat weaker compared to that of VEGF-A. Although the majority of heparin-binding properties are mediated by the C-terminal propeptide, also mature VEGF-C is a heparin-binding growth factor (NaCl elution concentrations: VEGF-A_{165} 0.8M, pro-VEGF-C 0.435M, mature VEGF-C 0.265M) [72]. This might explain why both mature and pro-VEGF-C have a local effect and do not diffuse far [55]. That the C-terminal domain mediates the association or embedding of VEGF-C in the extracellular matrix has been speculated shortly after its discovery [34], and was recently directly demonstrated in vitro [53]. Thus, pro-VEGF-C might be similar in this respect to latent TGF-β [75].

Changing receptor preferences with KLK3 and cathepsin D

Based on N-terminal sequencing, two different mature, active forms had been identified for both VEGF-C and VEGF-D [34,76]. In the supernatant of 293 cells, the shorter mature form of VEGF-C was the dominant ("major") form, while for VEGF-D, the longer mature form was dominant. While this indicated early on that different proteases are involved in the activation of VEGF-C and VEGF-D, it remained unknown which proteases were involved. In 2011, Leppänen et al. reported that the shorter ("minor") form of active VEGF-D was not able to activate VEGFR-3 [77]. This was surprising as the activation of VEGFR-3 is considered to be a prerequisite of being lymphangiogenic. This finding indicated for the first time that a lymphangiogenic growth factor could be converted into an angiogenic growth factor by proteolysis. At the same time, it explained why VEGF-D had been identified in some experimental settings as an exceptionally strong angiogenic growth factor [78]. While other research confirmed the disparity between VEGF-C and VEGF-D in terms of protease utilization for activation [37], the exact nature of the VEGF-D activating proteases remained unknown until 2019 when Jha et al. tested whether their newly discovered VEGF-C-activating proteases PSA and Cathepsin D (CatD) could also activate VEGF-D [41]. In fact, CatD was able to generate the VEGFR-2-specific mature form of VEGF-D, which Leppänen et al. had described in 2011 [77]. Despite this, it still remains to be shown which protease activates VEGF-D in vivo and whether there is a "physiological protease" equivalent to the VEGF-C-activating ADAMTS3. Perhaps, VEGF-D is solely activated in non-homeostatic situations like tissue damage. Nevertheless, also without any pathological challenge, VEGF-D knock-out mice display subtle alterations in some lymphatic networks [56,79]. These minor phenotypes could result from a lack of activated VEGF-D, but equally well from a lack of pro-VEGF-D (assuming it has some low level of activity) or possibly VEGF-C/VEGF-D heterodimers.

When comparing the effects of different VEGF-C- and VEGF-D-activating proteases [41], two trends are visible, which are summarized in Figure 5:

1. The shorter the N-terminus of the resulting mature growth factor, the lower is its receptor binding affinity and receptor activation potential.

2. Despite this similarity, the shortening affects VEGF-C and VEGF-D very differently. While VEGF-C loses rapidly its potential to activate VEGFR-2 (e.g. through activation by ADAMTS3 or PSA), VEGF-D maintains much of its VEGFR-2 binding and activation potential. Vice versa, VEGF-D rapidly loses its VEGFR-3 binding and activation potential, whereas VEGF-C maintains much of it when processed to a similar degree.

3. Both VEGF-C and VEGF-D are completely inactivated with respect to their receptor tyrosine kinase activity by complete removal of their N-terminal helices, which e.g. can be achieved by prolonged exposure to plasmin.

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Plasmin activation of VEGF-C, which has been shown previously and independently by two different groups [39,40], was not detected in a recent study [42]. Possibly, cleavage products might not have been recognized by the antibody due to low sensitivity or an absent epitope. Alternatively or additionally, the internal FLAG-tag preceding the cleavage site, which was used to prevent detection failure due to isoform-specific VEGF-C antibodies, might have interfered with the activation.

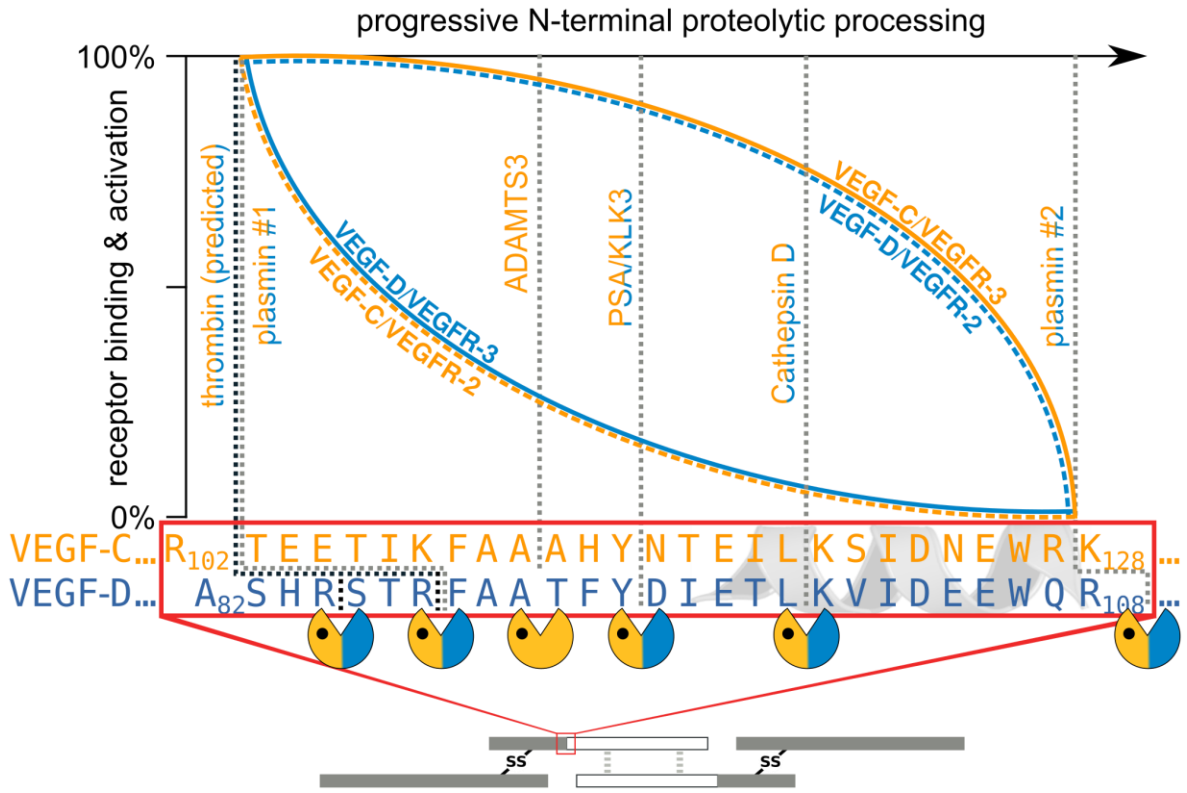

Figure 5. Biochemically, the group of lymphangiogenic activator enzymes is diverse. It includes a metalloproteinase (ADAMTS3), serine proteases (PSA/KLK3, thrombin and plasmin) and an aspartic protease (cathepsin D). VEGF-C and VEGF-D share all activating enzymes except for the most important one: ADAMTS3. ADAMTS3 is exclusive for VEGF-C and required for the physiologic activation of VEGF-C during developmental lymphangiogenesis [37–39]. VEGF-C and VEGF-D are differently affected by proteolytic processing. With progressing processing, VEGF-C largely maintains its lymphangiogenic properties but loses quickly its angiogenic properties. VEGF-D behaves exactly in the opposite way: processing with Cathepsin D almost completely abolishes its lymphangiogenic properties and fully unmasks its angiogenic properties [41]. Extensive exposure of both VEGF-C and VEGF-D with plasmin abolishes all VEGFR-2 and VEGFR-3 binding properties.

Secondary processing and inactivation

At least in vitro, the longer forms of activated VEGF-C and VEGF-D can undergo additional cleavages, which further shorten the N-terminus and modify the receptor binding capabilities. E.g. CatD can remove the lymphangiogenic potential from plasmin-activated VEGF-D and the angiogenic potential from ADAMTS3-activated VEGF-C [41]. Finally, a cleavage by plasmin can inactivate VEGF-C and VEGF-D. However, such secondary (or tertiary) processing has not yet been demonstrated in vivo.

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Other cleavages

The activating, N-terminal cleavage of VEGF-D has also been proposed to be mediated by the protein convertases furin or PC5 [35]. While this is certainly a possibility, it seems unlikely that this represents a major mechanism of VEGF-D activation in vivo. The mature VEGF-D produced in furin-deficient Lovo cells upon transfection with furin could well be due to any other endogenous protease in Lovo cells. The requirement for furin in this system might occur if C-terminal furin-processing was a prerequisite for the activating N-terminal cleavage, although such a prerequisite seems not to exist for VEGF-C [37]. Vice versa, plasmin [40] or PSA [41] have not only been shown to perform the N-terminal processing of VEGF-D, but also the C-terminal processing. However, this is as well unlikely relevant, since the protein convertases cleave constitutively inside the cell before VEGF-D ever has the chance to encounter plasmin or PSA.

Possible involvement in reproduction and wound healing

That VEGF-C is a possible substrate for kallikrein-like peptidases had been proposed before [80], but the identification of KLK3 (aka prostate-specific antigen, PSA) was nevertheless surprising especially because KLK3 is largely confined to sperm plasma. The presence of both VEGF-C and CCBE1 and a VEGF-C-activating protease in sperm plasma is too tempting not to speculate about a possible function of VEGF-C for reproductive biology, but such has not been confirmed yet. Mutations in KLK3 do affect male fertility [81], but this is unsurprising since the main biological function of KLK3 is the degradation of gel-like seminogelins, which releases the sperm cells [82]. VEGF-A, which is also present in sperm plasma [83,84], had a modest effect on sperm motility [85], and therefore similar experiments were attempted with VEGF-C with very variable results (unpublished data by the author), perhaps due to logistically challenging experimental setup.

Similar to KLK3, also cathepsin D was identified after an exhaustive analysis of bodily fluids for possible VEGF-C-cleaving activities [41]. VEGF-C had been previously shown to accelerate wound healing [86,87], and - at least in cell culture - VEGF-C had been shown to be deposited by virtue of its C-terminal domain into the extracellular matrix and is released by proteases [53]. Thus, it appears possible that Cathepsin D provided by wound licking might activate latent ECM-embedded pro-VEGF-C, thus providing an instant angiogenic, lymphangiogenic, and immunologic stimulus for wound healing. Compared to a single gene in humans, *KLK1* was several times duplicated in rodents leading to at least 23 *KLK1* orthologs (some of which being pseudogenes), and some researchers believe this expansion to be driven by the evolutionary pressure to heal bite wounds rapidly and efficiently [88].

Activating VEGF-C and VEGF-D in cell culture

While there are several cell lines, that endogenously express VEGF-C or VEGF-D (most notably PC-3, from which VEGF-C was originally identified [89]), almost all experiments that require the expression of these growth factors have been performed by cDNA transfection. When the full-length wild-type cDNAs are used, the inactive pro-forms are dominating in the cell culture supernatant of most cell lines (see Figure 3). Cells that express both CCBE1 and ADAMTS3 (such as various cell lines derived from 293 cells) will process at least some of the pro-VEGF-C into mature, active VEGF-C. This is sufficient to give background bands for both pro-VEGF-C and mature VEGF-C even in the absence of added proteases. If these background bands are missing from a western blot, the detection is likely not very sensitive or something interferes with the physiological activation of VEGF-C by ADAMTS3. The degree of processing is relatively difficult to predict and appears to depend on cell density, stress level, cell culture medium, and - most importantly - VEGF-C expression levels. In any case, the processing is inefficient, and the 293T cell line that was used to generate the gel image in Figure 3, is among the cell lines that most efficiently activate VEGF-C.

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Truncated cDNAs are used to recombinantly express pre-activated VEGF-C and VEGF-D

Therefore, when larger amounts of active VEGF-C are required, the solution has been to express a mutant VEGF-C cDNA, from which the sequences coding for the propeptides have been deleted ("ΔNΔC-VEGF-C"). All recombinant, commercially available VEGF-C and VEGF-D proteins are produced in this fashion. However, because the cleavage context of the signal peptide is disturbed, the N-terminus of the resulting protein can differ from the endogenously activated VEGF-C and only N-terminal sequencing can reveal which form of VEGF-C is present. With few exceptions (R&D Systems), this information is not provided by vendors. The same is true for many scientific publications that use truncated cDNAs to express VEGF-C or VEGF-D. While it is possible to predict the signal peptidase's likely cleavage position, only N-terminal sequencing can give a definite answer. Many of the early experiments involving recombinant VEGF-D have used a truncated cDNA that results in a VEGF-D form, which is an intermediate between the VEGFR-2-monospecific and the VEGFR-2/VEGFR-3 bispecific endogenous VEGF-D forms, making it difficult to interpret the data [58]. However, after the recent identification of the cleaving proteases, it became possible to generate specific mature forms by co-transfection of the protease with the full-length wildtype growth factor cDNA [39,41]. However, when using pre-activated forms of VEGF-C or VEGF-D, one should remember that it is unclear whether these really exist as independent species in vivo. Pro-VEGF-C efficiently binds VEGFR-3 in the context of neuropilin-2 and the "in-situ" activation of pro-VEGF-C while being bound to VEGFR-3 might be the normal mode of activation [39,53]. Interestingly, a transgenic mouse expressing pre-activated (ΔNΔC-VEGF-C) VEGF-C under the control of the keratin-14 promoter did not show the characteristic lymphatic phenotype in the skin as mice expressing VEGF-C from a full-length cDNA under the same promoter (unpublished data by the author)[90].

Modulation of proteolytic processing

Protease inhibitors have a veritable track record as drugs, targeting e.g. viral proteases in AIDS and other viral infections [91], neutrophil elastase in lung diseases [92], and angiotensin-converting enzyme in cardiovascular diseases [93]. The opposite approach - promoting proteolysis - has also resulted in life-saving treatments, e.g. the use of tissue plasminogen activator (tPA) to dissolve blood clots in the immediate treatment of ischemic stroke [94].

Given the importance of the lymphatic system in many diseases [95], both VEGF-C and VEGF-D are likely worthwhile drug targets. The concept of pro-lymphangiogenic therapy to treat lymphedema has progressed to clinical trials using adenoviral VEGF-C gene therapy [96]. However, because a continuous low-level supply with VEGF-C appears necessary to maintain the structure and functionality of heavily engaged lymphatic networks [60,97,98], a highly specific VEGF-C activating protease might equally be suitable if it can activate, endogenous ECM-embedded VEGF-C. Such VEGF-C activation might both act via stimulating lymphatic pumping [99] and also by inducing a compensatory expansion of the lymphatic network [100].

In inflammatory and infectious diseases, the lymphatic network has to manage the fluid balance during inflammatory swelling. But perhaps more importantly, there is increasing evidence that both innate and adaptive immunological responses are crucially dependent on the lymphatics during all stages of an immune response [101,102]. Thus the activation of VEGF-C could be used as a generic means to boost any immune response similar to an adjuvant.

Proteolytic activation of VEGF-C and VEGF-D in cancer

The crucial role of tumor-associated lymphatics for metastasis was recognized early on [103–106] and VEGF-C/VEGF-D inhibition has been proposed to therapeutically block metastasis. Since the tumor-promoting effects of VEGF-C and VEGF-D likely require proteolytic processing, inhibition could not only target the growth factors or receptors, but also the activating proteases. In vitro, VEGF-C-expressing MCF-7 and MDA-MB-435 cells, which have been used for xenograft tumor models, are

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inefficient in activating the growth factor [103,105]. Therefore, it is assumed that the activating proteases are supplied in these xenograft models by the stromal tumor compartment, perhaps by fibroblasts, inflammatory or endothelial cells [107,108]. Harris et al. generated a mutated form of VEGF-D, which is resistant to proteolytic activation, and showed that this mutant was unable to promote tumor growth and lymph node metastasis in a mouse tumor model [109].

Tumor cells can migrate and form distant metastases via two distinct pathways: via blood vessels (hematogenic spread), and via lymphatics (lymphogenic spread). Both of these pathways can be stimulated by VEGF-C and VEGF-D. By stimulating lymphangiogenesis into the tumor periphery (and occasionally also into the tumor) these growth factors maximize the access of tumor cells to the lymphatic vasculature. VEGF-C further appears to actively prepare the downstream lymph nodes for arriving cancer cells [110]. If the tumor happens to express suitable proteases, it is likely that VEGF-C (and even more so VEGF-D) can be activated into forms that mimic VEGF-A, but which are not inhibited by current anti-angiogenic treatments [111]. Such angiogenic redundancy might be one of the reasons why VEGF-A treatment is much less universal as originally anticipated, and why in amenable cancers, initial treatment success is usually followed by the development of resistance [112].

However, anti-lymphangiogenic therapy is a double-edged sword [113] because tumor-associated lymphatics are crucially important for the immune response against the tumor. When VEGF-C action was blocked in a mouse tumor model that was treated with immunotherapy, the mice receiving the anti-VEGF-C treatment died earlier than those that did not receive the treatment [114]. Similarly, in a mouse glioblastoma model, VEGF-C was able to amplify the CD8+ T cell response against the tumor [115]. To be able to successfully target VEGF-C in cancer, a thorough understanding of the underlying molecular mechanisms is needed, which might finally allow us to separate the metastasis-enhancing from the immune response-enhancing function of VEGF-C. However, at this moment, we do not even know which proteases are activating VEGF-C in human cancers. A high-probability guess is that different proteases are involved depending on the cancer type.

Blocking VEGF-C and VEGF-D activation

Proteolytic processing of VEGF-C and VEGF-D has been so far experimentally blocked only by mutagenesis of the cleavage sites. Furin and related protein convertases cleave VEGF-C after the double arginines (R226,227). Joukov et al. reported that mutating these arginines into serines (R226,227S) largely blocked VEGF-C processing [34]. This is surprising since the N-terminal cleavage site should have been still subject to proteolytic attack because the first, constitutive C-terminal cleavage and the second, N-terminal cleavage were subsequently shown to occur independently from each other [37]. To generate an even more activation-resistant form of VEGF-D, Harris et al. mutated in addition to the C-terminal cleavage site also the major N-terminal cleavage site and reported similar to Joukov et al. almost complete abrogation of VEGF-D activation [109]. It is unclear why the unmutated minor N-terminal cleavage site in this protein did not result at least in partial activation, since for the same 293 EBNA cell line, the minor N-terminal cleavage site had been shown to account for approximately 20% of the activated protein [76]. However, for a therapeutic effect, the activation block does not need to be complete since pro-VEGF-C acts as an antagonist of mature VEGF-C and therefore a low level of activation might be acceptable [39].

Genetic lesions affecting proteolytic processing of VEGF-C

Mutations that disrupt the VEGF-C/VEGFR-3 signaling pathway result in hereditary lymphedema. While the most common type of hereditary lymphedema is caused by a mutation in the VEGF-C receptor [116,117], any components of the signaling pathway can be affected, including the proteolytic activation of VEGF-C. Thus, in human lymphedema patients, disease-causing mutations have been found in VEGF-C itself [118,119], in its activating protease ADAMTS3 [52,53], and in the cofactor CCBE1 [48,120]. Notwithstanding our progress in the understanding of the molecular basis

of lymphedema, for a large fraction of primary lymphedema, the underlying genetic lesions are still unknown [121].

Outlook: Molecular nudging

With first successes in Crispr-Cas therapeutic clinical trials, genetic deficiencies within the VEGF-C/VEGFR-3 signaling pathways appear at least theoretically amenable for repair. However, at the moment even the cutting-edge trials limit themselves to cells that can be easily modified ex-vivo (blood diseases like sickle cell disease and β-thalassemia) [122] or to very localized targets [123]. We are still far from a systemic repair of solid tissues, which would be needed since the lymphatic system penetrates almost all organs of our bodies. Since at least a fraction of the VEGF-C appears to originate from blood vascular endothelial cells, a vascular-targeted repair appears possible [124]. If sufficiently specific, the systemic delivery of regulatory factors like CCBE1 or ADAMTS3 might alternatively result in a widespread low-level activation ("molecular nudging") of endogenous VEGF-C and a therapeutic effect. While such interventions don't reverse developmental routes already taken, they still might result in a significant improvement of life quality.

For cancer, being the prototype of a moving drug target, molecular nudging is not likely to have any impact. While a multitargeted anti-VEGF-A/-C/-D therapy might result in improved survival, any progress in this area will likely be incremental since using alternative tumor angiogenesis factors is only one of many escape mechanisms that tumors can deploy [112].

Author Contributions

Writing—original draft preparation, M.J.; writing—review and editing, J.K., M.J.; visualization, H.B., M.J.; supervision, project administration, and funding acquisition, M.J. All authors have read and agreed to the published version of the manuscript.

Funding

This research was funded by the Päivikki and Sakari Sohlberg Foundation, the Novo Nordisk Foundation, and the Academy of Finland (#337430). M.J. was supported by the Paulo Foundation and the Einar and the Karin Stroem Foundation for Medical Research; H.B. was supported by a Fellowship from the Finnish National Agency for Education.

Conflicts of Interest

The authors declare no conflicts of interest.

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