The lymphangiogenic growth factors VEGF-C and VEGF-D

Part 1: Fundamentals and embryonic development

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Summary

VEGF-C and VEGF-D are the two central signaling molecules that stimulate the development and the growth of lymphatic system. Both belong to the VEGF protein family which plays important roles in the growth of blood vessels (angiogenesis) and lymphatic vessels (lymphangiogenesis). In mammals the VEGF family comprises five members: VEGF, PlGF, VEGF-B, VEGF-C and VEGF-D. The family was named after its first discovered member VEGF ("Vascular Endothelial Growth Factor"). VEGF-C and VEGF-D form functionally and structurally a subgroup within this family. They differ from the other VEGFs by their peculiar biosynthesis: they are produced as inactive precursors and need to be activated by proteolytic removal of their long N- and C-terminal propeptides. Unlike the other VEGFs, VEGF-C and VEGF-D are direct stimulators of lymphatic growth. They exert their lymphangiogenic function via VEGF receptor-3, which is expressed in the adult organism almost exclusively on lymphatic endothelial cells. In this review we give an overview of the VEGF protein family and their receptors with the emphasis on the lymphangiogenic VEGF-C and VEGF-D, and we discuss their biosynthesis and their role in embryonic lymphangiogenesis.

Keywords: VEGF-C, VEGF-D, growth factors, lymphangiogenesis

Die lymphangiogenen Wachstumsfaktoren VEGF-C und VEGF-D Teil 1: Grundlagen und Embryonalentwicklung

Zusammenfassung

VEGF-C und VEGF-D sind die zwei zentralen Signalmoleküle, die für die Ent-wicklung und das Wachstum des Lymphgefäßsystems verantwortlich sind. Beide gehören zur VEGF-Proteinfamilie, deren Mitglieder hauptsächlich im Wachstum von Blutgefässen (Angiogenese) und Lymphgefässen (Lymphangiogenese) ihre Funktionen haben. Die VEGF-Familie umfasst in Säugetieren fünf Mitglieder: VEGF, PlGF, VEGF-B, VEGF-C und VEGF-D. Benannt wurde diese Familie nach ihrem zuerst entdeckten Mitglied VEGF („Vascular Endothelial Growth Factor"). VEGF-C und VEGF-D bilden funktionell und strukturell eine Untergruppe innerhalb der VEGF-Familie. Sie unterscheiden sich von den anderen VEGFs durch ihre besondere Biosynthese: sie werden als inaktive Vorgängermoleküle produziert, für deren Aktivierung ihre langen N- und C-terminalen Propeptide enzymatisch abgespalten werden müssen. Im Gegensatz zu den anderen VEGFs sind VEGF-C und VEGF-D direkte Stimulatoren für das Wachstum lymphatischer Gefäße. Ihre lymphangiogene Wirkung enfalten VEGF-C und VEGF-D über den VEGF-Rezeptor-3 (VEGFR-3), der im erwachsenen Organismus fast nur auf den Endothelzellen der Lymphvaskulatur zu finden ist. In diesem Artikel geben wir einen Überblick über die VEGF-Proteinfamilie und deren Rezeptoren mit dem Schwerpunkt auf den lymphangiogenen Mitgliedern VEGF-C und VEGF-D, über ihre Biosynthese und ihre Rolle in der Embryonalentwicklung.

Schlüsselwörter: VEGF-C, VEGF-D, Wachstumsfaktoren, Lymphangiogenese

The VEGF family of proteins and their receptors

VEGF receptors

Endothelial cells form the innermost layer of all blood and lymphatic vessels, and these cells play the central role during vascular growth. Vascular growth requires the interaction of various growth factors and receptors. Among those, the VEGF protein family with their receptors is dominant with VEGF receptor-2 (VEGFR-2) for the growth of blood vessels and VEGFR-3 for the growth of lymphatic vessels. Other important molecules that are not covered in this review are (among others) the Tie receptors and their angiopoietin ligands, which play complex, partly context-dependent roles in the preservation, stabilization and remodeling of blood vessels [1, 2], the PDGF receptors with their PDGF ligands, which are necessary for the stabilization of the vessel wall by pericytes and vascular smooth muscle [3], and the Eph receptors with their ephrin ligands, which are involved in the determination of the venous and arterial identity of blood vessels [4].

The signaling of the VEGF molecules affects the growth and function of endothelial cells via VEGF receptors. Only endothelial cells express VEGF receptors [the few exceptions are listed in the supplement of the review by Olsson et al.; 5]. The VEGF receptors are tyrosine kinase receptors. Their N-terminal domain protrudes from the cell membrane into the extracellular space and has an affinity for one or more specific VEGFs (binding partners or ligands). The C-terminal part executes its catalytic function in the cytoplasm, when the extracellular portion becomes occupied with its binding partner (see Figure 1). In case of the VEGF receptors, this activation of the catalytic function is achieved because each VEGF molecule features two binding sites for its receptor (bivalency). The two binding sites of the VEGF molecule bind two VEGF receptor molecules and thereby the intracellular, catalytic do-

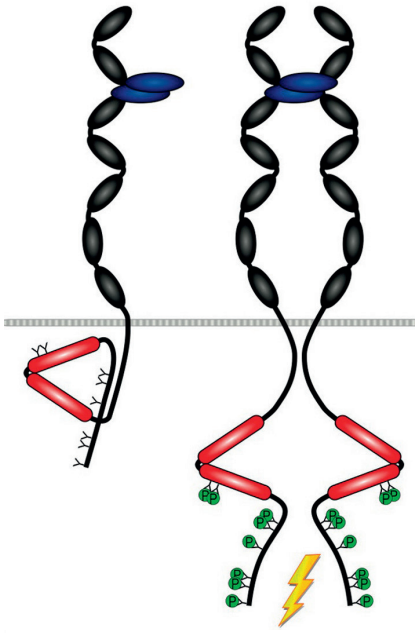


Figure 1.

Model of the activation of tyrosine kinase receptors by a bivalent ligand. Simultaneous binding of a ligand (e.g. VEGF-C, shown in blue) to the extracellular domains of two receptor molecules (e.g. VEGFR-3, shown in gray black) positions the intracellular catalytic domains (shown in red) in such a way that they can phosphorylate each other. This changes the three-dimensional structure of the intracellular receptor part and exposes affinities to other intracellular signaling molecules (second messengers) that are activated by docking to the phosphorylated tyrosine residues. Such activations propagate in a cascade fashion until the signal enters the cell nucleus where it modifies the transcriptional activity of target genes. The intracellular signaling of VEGF receptors is reviewed in detail by Olsson et al. [5].

mains of the receptors are positioned in such a way that they activate each other through the transfer of phosphate groups from ATP to specific tyrosine residues. This changes the threedimensional structure of the intracellular domain and this change allows interaction with and activation of other intracellular signaling molecules, which ultimately causes a change in gene expression and thus a change of cell behavior [6]. Apart from the VEGF receptors, most VEGFs bind to additional cell membrane-bound molecules (so-called co-receptors), for example neuropilins [7]. However, these interactions are typically of lower affinity.

The VEGF growth factors

Mammals have five different VEGFs: VEGF-A, VEGF-B, VEGF-C, VEGF-D and PlGF (placental growth factor).

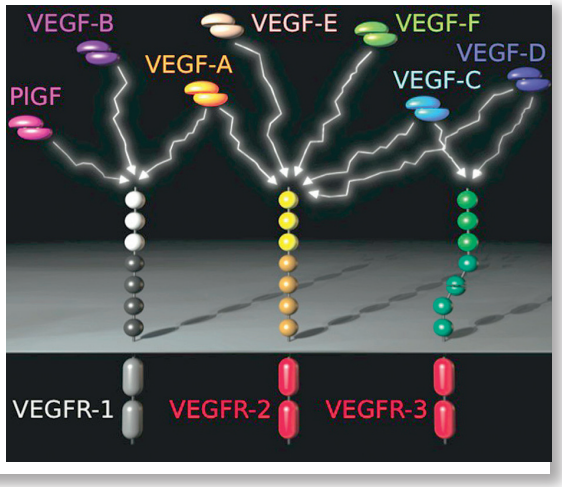
VEGF-A. VEGF-A was the first VEGF growth factor that was discovered and it is often simply referred to as VEGF. In the early literature it is also known as vascular permeability factor (VPF) because of its property to increase the permeability of blood vessels [8]. Its main function is in the stimulation of blood vessel growth (angiogenesis). VEGF-A is a medically relevant drug target due its enabling role for tumor growth. Blocking blood vessel growth by the anti-VEGF-A antibody bevacizumab ("Avastin") showed already more than 10 years ago that anti-angiogenesis represents a useful addition to the therapeutic options against certain forms of cancer. VEGF-A has two different receptors on endothelial cells: VEGF receptor-1 (VEGFR-1) and VEGFR-2.

VEGF-B and PlGF. In contrast to VEGF-A, VEGF-B and PlGF are only able to interact with VEGFR-1. Almost all the important functions of VEGF-A are mediated via the signal transduction of VEGFR-2; and accordingly, VEGF-B and PlGF are only weakly angiogenic. VEGFR-1, in contrast to VEGFR-2, has even an inhibitory function by binding the potently angiogenic VEGF-A, but not triggering a similarly strong reaction as VEGFR-2 does [9-13]. VEGF-B and PlGF, however, seem to have specific functions for angiogenesis of the heart muscle [14, 15], for certain pathological processes [16] and fatty acid metabolism [17].

VEGF-C and VEGF-D. VEGF-C and VEGF-D can both interact with VEGFR-2 and VEGFR-3. Their main task is the stimulation of the growth of lymphatic vessels (lymphangiogenesis). VEGF-C was discovered in 1996 as a binding partner of VEGF receptor-3, for which no ligand had been found yet [18]. A little later, murine VEGF-C was described independently, but the authors named the molecule VRP [VEGF-related protein; 19]. Soon thereafter, the

Figure 2.

Schematic representation of the VEGF growth factors and VEGF receptors. The VEGF receptors are transmembrane receptors with an extracellular domain consisting of seven immunoglobulin (Ig) homology domains and an intracellular split-kinase domain. The outer three Ig homology domains (shown in different color) are sufficient to interact with the respective ligands. The VEGFs consist of two individual polypeptide chains, which are connected by disulfide bridges. Typically, two identical protein chains connect to form a VEGF molecule (homodimer), but also two different protein chains can connect to form a so-called heterodimer (e.g. PlGF and VEGF-A). Similarly, two different VEGF receptors can ligate to form heterodimers and such a ligation can be mediated by a ligand; for example, VEGF-C can ligate VEGF receptor-2 and VEGF receptor-3 resulting in a VEGFR-2/3 heterodimer. Such receptor heterodimers may have special functions [64, 86]. Among the VEGF receptors, VEGF receptor-3 is exceptional, as it is the only one whose extracellular domain is proteolytically cleaved [87].



specific lymphangiogenic properties of VEGF-C were demonstrated in various animal models [20, 21]. Because in some of these models, the angiogenic properties of VEGF-C became apparent [21-23], a VEGF-C mutant was developed that interacts exclusively with VEGF receptor-3 and thus no longer has angiogenic potency [the so-called C156S VEGF-C mutant; 24]. With this mutant, the lymphangiogenic function of VEGF-C could be separated from the angiogenic function and independently studied.

VEGF-D is the second specific lymphangiogenic growth factor. It was identified and described independently by three different research teams, once under the acronym FIGF [c-fos-induced growth factor; 25] and twice as VEGF-D [26, 27].

VEGF-E and VEGF-F. In addition to these five mammalian VEGFs there are VEGF-E and VEGF-F. VEGF-E is the collective name for proteins closely related to the VEGFs that were detected in the genome of certain pathogenic viruses and that are involved in the disease etiology [28-32]. The collective term VEGF-F denotes homologous proteins that have been identified as accessory components of snake venoms [33-37]. Their function is probably to increase the permeability of blood vessels in order to potentiate the effects of the primary venom components.

The structure of VEGF molecules

VEGF family members are molecules that feature a central domain homologous to VEGF. This domain is referred to as VEGF homology domain (VHD, shown in red in Figure 3). This homology can be identified at all levels of the protein structure (from the amino acid sequence up to the three-dimensional structure of the protein). The VHD contains the receptor binding domain. In addition, most VEGFs feature accessory domains that further determine the specific properties of individual VEGFs, e.g. the affinity of VEGF-A to the co-receptors Neuropilin-1 and -2 [38], or of VEGF-C to the co-receptor Neuropilin-2 [39, 40].

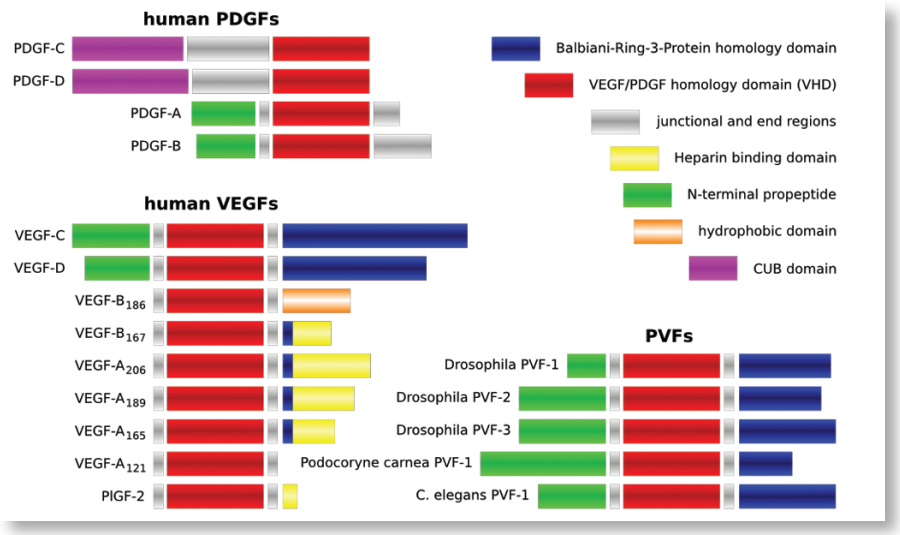


Figure 3.

Schematic representation of the domain structure of selected members of the VEGF and PDGF protein families. The PDGF family is so closely related to the VEGF family that the two are sometimes grouped together as PDGF/VEGF family. In fact, in the invertebrates, the two families cannot be distinguished from each other and are referred to as PVFs [PDGF/VEGF-like growth factors; 85]. The comparison of human VEGFs with these PVFs allows to draw conclusions about the structure of ancestral PDGF/VEGF-like molecules and these appear more similar to today's lymphangiogenic VEGF-C and VEGF-D than to the haemangiogenic VEGF-A. The PVFs of the fruit fly *Drosophila* function in the migration of hemocytes and the PVFs of the jellyfish *Podocoryne carnea* plays a role in the formation of the tentacles and the gastrointestinal vascular apparatus. The function of the nematode *C. elegans*' PVF (PVF-1) is unknown.

All of the VEGF family members are composed of two polypeptide chains (dimeric proteins). During the biosynthesis of the polypeptide chains they align in an antiparallel fashion with a hydrophobic contact area facing each other, and become covalently linked with two disulfide bridges. The resulting shape resembles roughly a flattened ellipsoid. At each end of this ellipsoid, there is one epitope that can bind a matching VEGF receptor. Both epitopes are composites of parts from both polypeptide chains. This composite nature of the receptor binding epitopes explains why monomeric VEGF (VEGF with only one polypeptide chain) is biologically inactive: it cannot ligate two receptors [41].

Alternative splicing

Like almost all secreted proteins, the VEGFs are glycoproteins. Most of them are produced in different forms. The diversity is produced either by alternative splicing or by posttranslational modification of the protein (e.g. by proteolytic cleavage). Through alternative splicing of the VEGF-A mRNA, a variety of different VEGF-A isoforms are produced that differ primarily in their affinity for heparan sulfate proteoglycans (HSPGs), which occur primarily on cell surfaces and in the extracellular matrix [see Figure 3; 12, 41]. The interaction with HSPGs immobilizes the so-called "heparin-binding" VEGF-A isoforms. Thus a concentration gradient can form, which can be used by blood vessels for orientation and directed growth [42, 43].

The activation of VEGF-C and VEGF-D

Splice isoforms have been also described for VEGF-C and VEGF-D, but their functions are unknown [19]. VEGF-C and VEGF-D get their diversity

mainly by enzymatic (proteolytic) cleavage of their precursor molecules (see Figure 4).

The affinity of the VEGF-C precursor molecules for the VEGF receptor-3 is modest and its affinity is even lower for VEGF receptor-2. With increasing processing, the affinities for both receptors increase, and fully processed mature VEGF-C and VEGF-D have in addition to their lymphangiogenic also strong angiogenic potencies [44-47]. How the activation of VEGF-C and VEGF-D is controlled in-vivo, is not precisely known. It is believed that the availability and activity of specific proteases is central to this control: The activation of VEGF-C and VEGF-D by specific proteases would therefore be one of the crucial factors determining whether VEGF-C and VEGF-D act as lymphangiogenic or angiogenic effectors. In addition to regulating the activity of VEGF-C, the C-terminal propeptides of VEGF-C and VEGF-D have other functions: similar to the heparin-binding domain of VEGF-A, they endow the molecules with heparin affinity [48]. It is also interesting that the C-terminal propeptide contains a repetitive arrangement of cysteine residues, which is otherwise almost exclusively known from the salivary proteins of silk weaving mosquito larvae of the genus Chironomus; hence the name "Silk homology domain" for the C-terminal propeptide [18, 49]. However, the whys and hows of this similarity are unknown.

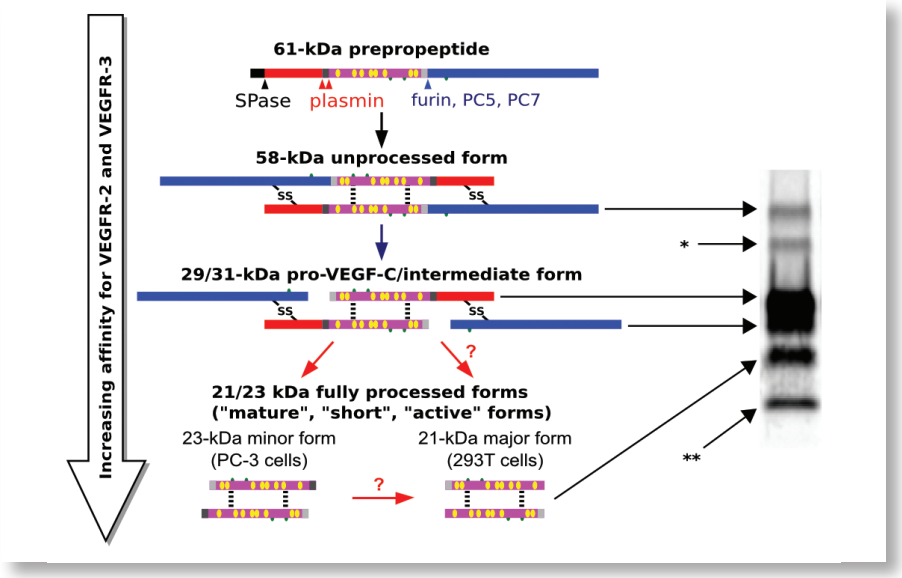


Figure 4.

The enzymatic maturation of VEGF-C. VEGF-C is produced as a precursor molecule. Its signal peptide is removed during translocation into the endoplasmic reticulum (ER). In the ER, the protein folding takes place. On the way through the Golgi apparatus, the second enzymatic cleavage of the VEGF-C polypeptide chain takes place. After this, the two halves of the protein are still held together by disulfide bridges. This intermediate form (pro-VEGF-C) is secreted and subsequently, the mature, active forms are generated by two alternative, extracellular proteolytic cleavages.

The second intracellular proteolytic cleavage is mediated by the enzymes furin, PC5 or PC7 [88]. The enzymes that catalyze the following activating extracellular proteolytic cleavages are not well defined. Cleavage by plasmin can give rise to VEGF-C forms which are similar or identical to the mature VEGF-C [89], and VEGF-C activation by plasmin seems therefore important for certain situations such as wound healing [90, 91]. However, it is questionable whether plasmin is the physiologically relevant enzyme for VEGF-C activation during physiological expansion of the lymphatic system.

Depending on the cell type, two different mature forms of VEGF-C are produced. 293 cells produce a form that is about 9 amino acid residues shorter than the form produced by PC-3 cells [44]. It is unknown, whether these two forms differ in function; both bind and activate VEGF receptor-2 and VEGF receptor-3. It is also unknown, whether the 21-kDa major, mature form is produced by processing of the 23-kDa minor, mature form or whether both mature forms are generated directly from pro-VEGF-C by independent proteolytic processing.

On the right, a typical band pattern is shown, which can be observed after electrophoretic separation of VEGF-C which was expressed from a full-length cDNA. The asterisk marks a minor 43-kDa form [44] and the double asterisk the N-terminal propeptide.

The enzymatic maturation of VEGF-D occurs analogous to that of VEGF-C [45]. However, there is a critical difference between the two different mature forms of VEGF-D: the shorter of the two has lost its affinity for VEGF receptor-3, and is thus only angiogenic and not anymore lymphangiogenic [92].

Vasculogenesis or angiogenesis?

Two different mechanisms can lead to the formation of new blood vessels: vasculogenesis and angiogenesis. Vasculogenesis is the differentiation of progenitor cells (angioblasts or lymphangioblasts) into endothelial cells and the formation of a primitive vascular network while angiogenesis refers to the growth of new blood vessels from pre-existing vessels. Vasculogenesis plays mainly a role during the early development of the vascular system, whereas angiogenesis is the main mechanism for vascular growth during the later phases of embryonic development and in the adult organism.

In mammals, the lymphatic system forms by angiogenic processes emanating from the large veins [50, 51]. The initially disputed fact that also vasculogenesis can contribute to the development of the lymphatic system was shown e.g. in birds [52] and frogs [53].

To form new vessels, endothelial cells have to execute a complex program: they need to switch from their resting state back into active cell cycling. One of the main triggers of cell cycle re-entry for blood vessel endothelial cells is the lack of oxygen (hypoxia), which necessarily results from avascular growth. Molecular oxygen sensors activate a genetic switch that activates the angiogenesis program

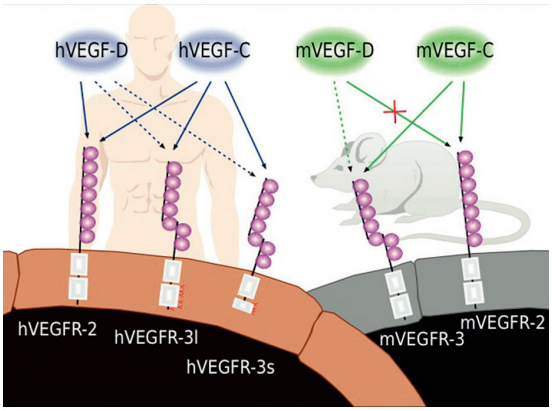


Figure 5.

Major molecular regulators of lymphangiogenesis differ between mice and humans. Mouse VEGF-D cannot activate mouse VEGF receptor-2 and mice have no second short splice isoform of VEGF receptor-3. The function of the short splice isoform is unknown. The latter difference is due to a retroviral integration that is specific to humans (or at least higher primates). For these reasons, it is unclear to what extent experimental knowledge about the lymphatic vasculature and its diseases is applicable to humans, when it was gained from the common model organisms [82].

[54]. The triggers for the expansion of the lymphatic system are less well known, but the interstitial pressure seems to play an important role for embryonic lymphangiogenesis [55] and the inflammatory status for pathologic lymphangiogenesis [56].

Differences between the blood and lymphatic system

The pressure within the blood vessel system leads to leakage of blood plasma, which thereby becomes tissue fluid. The main function of the lymphatic vessels is to return this excess tissue fluid back into the blood circulation. After uptake by the lymphatics, the tissue fluid becomes lymph fluid. On its way back into the blood vessel system, the lymph passes through the lymph nodes, which are base stations of the immune system. In the lymph nodes, immune cells which specifically recognized antigens, mature and multiply. Another function of the lymphatic vessels is limited to the colon: the uptake and transport of dietary fats with long chain fatty acids and fat-soluble vitamins [57].

Blood vessels and lymphatic vessels are constructed differently. Blood endothelial cells are connected to each other via tight junctions and adherens junctions and have a continuous basement membrane on their tissue-facing (basolateral) side. In contrast, lymphatic endothelial cells are only loosely connected by overlaps, and their basement membrane is incomplete. They are connected to the underlying tissue by elastic fibers (anchoring filaments). These fibers mechanically open the overlaps at elevated tissue pressure and thus ensure the flow of tissue fluid into the lymphvascular lumen [58-60].

Blood and lymphatic endothelium also differ in the expression of various markers: both blood and lymphatic endothelial cells express the general endothelial marker PECAM-1 (platelet endothelial cell adhesion molecule 1), but they express a different set of VEGF receptors: blood endothelial cells express VEGFR-1 and -2, while lymphatic endothelial cells express VEGFR-2 and -3. Fenestrated endothelium [61], high endothelial venules [HEVs; 62, 63] and the blood vessels of tumors [64] are exceptions in that they do express the lymphatic marker VEGF receptor-3.

The mechanisms of the directional growth are similar for blood vessels and nerve cell axons [65]: a specialized cell on the tip of the vascular sprout (tip cell) determines the direction of growth of subsequent cells (stalk cells) by extending filopodia that sense growth factor concentration gradients [66-68].

VEGF-C and VEGF-D in embryonic development

VEGF-C and VEGF-D have two receptors: VEGFR-2 and VEGFR-3. VEGFR-2 is the primary receptor on endothelial cells of blood vessels (BECs) and stimulates their growth, while VEGFR-3 exerts the same function on lymphatic endothelial cells (LECs). Accordingly, VEGF-C and VEGF-D can act both as angiogenic and lymphangiogenic growth factors. VEGFR-3 was discovered before VEGF-C and VEGF-D and therefore, VEGFR-3 was for some time an "orphan receptor", i.e. a receptor without known binding partners (ligands). However, soon after the discovery of VEGFR-3, the specific expression pattern of VEGFR-3 suggested, that its function was closely related to the lymphatic system. In the early stages of embryonic development all endothelial cells express VEGFR-3, but with advancing age, its expression becomes more and more restricted to lymphatic endothelial cells [63]. Finally VEGFR-3 expression is so specific for lymphatic endothelial cells, that it can be used as a marker for identification [69].

Genetically engineered mice that do not express VEGFR-3, die between the 9th and 10th day of embryonic development (E9.5) from failures in the organization and maturation of blood vessels [70]. This confirmed the essential role of VEGFR-3 in the development of the cardiovascular system, since at this time, the development of the lymphatic system has not started yet.

Mice that not express the VEGFR-3 ligand VEGF-C die about three days later (E12.5) due to generalized edema. In these mice, the lymphatic system does not develop [71].

Interestingly, neither the development of the blood vascular system nor of the lymphatic vascular system is af-

fected by the lack of the second lymphangiogenic growth factor VEGF-D [72]. The absence of both VEGFR-3 ligands (VEGF-C and VEGF-D) during embryonic development does not lead to the same severe disorders in the development of the vascular system as the absence of VEGF receptor-3 [73]. Therefore, there might be yet unknown ligands for VEGF receptor-3. Alternatively, VEGFR-3 might be activated independently of a ligand [74, 75].

A molecule that synergizes with VEGF-C and which is required for the development of the lymphatic system and lymphangiogenesis in general is CCBE1 (collagen and calcium binding EGF domains 1 protein). The blockade of lymphatic development appears in CCBE1-deficient and VEGF-C-deficient mice around the same time and is phenotypically very similar. However, it is unclear what exact role CCBE1 plays for the lymphatic system [76-78]. Mutations in the human CCBE1 gene can be responsible for Hennekam syndrome, a rare genetic disease whose main symptoms include lymphedema and lymphangiectasia of the intestine [79].

Differences between mice and humans

Because we owe a considerable part of our knowledge about the molecular mechanisms of lymphangiogenesis to laboratory mice, it is necessary to mention two important differences between mice and humans with respect to the VEGF-C/VEGF-D/VEGF receptor-3 signaling pathway (depicted in Figure 5): While mature human VEGF-D can activate the angiogenic receptor VEGFR-2, this is not the case for mouse VEGF-D [80]. It is therefore believed that VEGF-D could have a different function for mice and humans. Furthermore, there are two splice variants of human VEGF receptor-3 [a short and a long isoform; 81], whereas in mice, only one isoform can be detected. The appearance of two VEGFR-3 splice variants can be attributed to a retroviral insertion into the FLT4 gene [82], and the two isoforms differ in the signals, which are generated upon stimulation with VEGF-C [83, 84].

The second part of this review will focus on the roles that VEGF-C and VEGF-D play in various diseases that involve the lymphatic system. For some of these diseases mouse models exist. However, due to the above mentioned differences between mice and humans, one should be careful when extrapolating from preclinical studies in animal models to clinical trials with human patients.

Bibliography

1. Thomas M, Augustin HG: The role of the Angiopoietins in vascular morphogenesis. Angiogenesis 2009;12:125-137.

2. Saharinen P, Bry M, Alitalo K: How do angiopoietins Tie in with vascular endothelial growth factors? Curr Opin Hematol 2010;17:198-205.

3. Andrae J, Gallini R, Betsholtz C: Role of platelet-derived growth factors in physiology and medicine. Genes Dev 2008;22:1276-1312.

4. Adams RH, Eichmann A: Axon guidance molecules in vascular patterning. Cold Spring Harb Perspect Biol 2010;2:a001875.

5. Olsson A-K, Dimberg A, Kreuger J, Claesson-Welsh L: VEGF receptor signalling - in control of vascular function. Nat Rev Mol Cell Biol 2006;7:359-371.

6. Lemmon MA, Schlessinger J: Cell signaling by receptor tyrosine kinases. Cell 2010;141:1117-1134.

7. Koch S, Claesson-Welsh L: Signal transduction by vascular endothelial growth factor receptors. Cold Spring Harb Perspect Med 2012;2:a006502.

8. Keck PJ, Hauser SD, Krivi G, Sanzo K, Warren T, Feder J et al.: Vascular permeability factor, an endothelial cell mitogen related to PDGF. Science 1989;246:1309-1312.

9. de Vries C, Escobedo JA, Ueno H, Houck K, Ferrara N, Williams LT: The fms-like tyrosine kinase, a receptor for vascular endothelial growth factor. Science 1992;255:989-991.

10. Plouet J, Moukadiri H: Characterization of the receptor to vasculotropin on bovine adrenal cortex-derived capillary endothelial cells. J Biol Chem 1990;265:22071-22074.

11. Shibuya M, Claesson-Welsh L: Signal transduction by VEGF receptors in regulation of angiogenesis and lymphangiogenesis. Exp Cell Res 2006;312:549-560.

12. Ferrara N: Vascular endothelial growth factor: basic science and clinical progress. Endocr Rev 2004;25:581-611.

13. Ho VC, Duan LJ, Cronin C, Liang BT, Fong GH: Elevated vascular endothelial growth factor receptor-2 abundance contributes to increased angiogenesis in vascular endothelial growth factor receptor-1-deficient mice. Circulation 2012;126:741-752.

14. Lähteenvuo JE, Lähteenvuo MT, Kivelä A, Rosenlew C, Falkevall A, Klar J, et al.: Vascular endothelial growth factor-B induces myocardium-specific angiogenesis and arteriogenesis via vascular endothelial growth factor receptor-1- and neuropilin receptor-1-dependent mechanisms. Circulation 2009;119:845-856.

15. Luttun A, Tjwa M, Moons L, Wu Y, Angelillo-Scherrer A, Liao F et al.: Revascularization of ischemic tissues by PlGF treatment, and inhibition of tumor angiogenesis, arthritis and atherosclerosis by anti-Flt1. Nat Med 2002;8:831-840.

16. Li X, Tjwa M, Van Hove I, Enholm B, Neven E, Paavonen K et al.: Reevaluation of the role of VEGF-B suggests a restricted role in the revascularization of the ischemic myocardium. Arterioscler Thromb Vasc Biol 2008;28:1614-1620.

17. Hagberg CE, Falkevall A, Wang X, Larsson E, Huusko J, Nilsson I et al.: Vascular endothelial growth factor B controls endothelial fatty acid uptake. Nature 2010;464:917-921.

18. Joukov V, Pajusola K, Kaipainen A, Chilov D, Lahtinen I, Kukk E et al.: A novel vascular endothelial growth factor, VEGF-C, is a ligand for the Flt4 (VEGFR-3) and KDR (VEGFR-2) receptor tyrosine kinases. EMBO J 1996;15:290-298.

19. Lee J, Gray A, Yuan J, Luoh SM, Avraham H, Wood WI: Vascular endothelial growth factor-related protein: a ligand and specific activator of the tyrosine kinase receptor Flt4. Proc Natl Acad Sci USA 1996;93:1988-1992.

20. Jeltsch M, Kaipainen A, Joukov V, Meng X, Lakso M, Rauvala H et al.: Hyperplasia of lymphatic vessels in VEGF-C transgenic mice. Science 1997;276:1423-1425.

21. Oh SJ, Jeltsch MM, Birkenhäger R, McCarthy JE, Weich HA, Christ B et al.: VEGF and VEGF-C: specific induction of angiogenesis and lymphangiogenesis in the differentiated avian chorioallantoic membrane. Dev Biol 1997;188:96-109.

22. Cao Y, Linden P, Farnebo J, Cao R, Eriksson A, Kumar V et al.: Vascular endothelial growth factor C induces angiogenesis in vivo. Proc Natl Acad Sci USA 1998;95:14389-14394.

23. Eichmann A, Corbel C, Jaffredo T, Br©*ant C, Joukov V, Kumar V et al.: Avian VEGF-C: cloning, embryonic expression pattern and stimulation of the differentiation of VEGFR2-expressing endothelial cell precursors. Development 1998;125:743-752.

24. Joukov V, Kumar V, Sorsa T, Arighi E, Weich H, Saksela O et al.: A recombinant mutant vascular endothelial growth factor-C that has lost vascular endothelial growth factor receptor-2 binding, activation, and vascular permeability activities. J Biol Chem 1998;273:6599-6602.

25. Orlandini M, Marconcini L, Ferruzzi R, Oliviero S: Identification of a c-fos-induced gene that is related to the platelet-derived growth factor/vascular endothelial growth factor family. Proc Natl Acad Sci USA 1996;93:11675-11680.

26. Yamada Y, Nezu J, Shimane M, Hirata Y: Molecular cloning of a novel vascular endothelial growth factor, VEGF-D. Genomics 1997;42:483-488.

27. Achen MG, Jeltsch M, Kukk E, Mäkinen T, Vitali A, Wilks AF et al.: Vascular endothelial growth factor D (VEGF-D) is a ligand for the tyrosine kinases VEGF receptor 2 (Flk1) and VEGF receptor 3 (Flt4). Proc Natl Acad Sci USA 1998;95:548-553.

28. Lyttle DJ, Fraser KM, Fleming SB, Mercer AA, Robinson AJ: Homologs of vascular endothelial growth factor are encoded by the poxvirus orf virus. J Virol 1994;68:84-92.

29. Ogawa S, Oku A, Sawano A, Yamaguchi S, Yazaki Y, Shibuya M: A novel type of vascular endothelial growth factor, VEGF-E (NZ-7 VEGF), preferentially utilizes KDR/Flk-1 receptor and carries a potent mitotic activity without heparin-binding domain. J Biol Chem 1998;273:31273-31282.

30. Meyer M, Clauss M, Lepple-Wienhues A, Waltenberger J, Augustin HG, Ziche M, et al.: A novel vascular endothelial growth factor encoded by Orf virus, VEGF-E, mediates angiogenesis via signalling through VEGFR-2 (KDR) but not VEGFR-1 (Flt-1) receptor tyrosine kinases. EMBO J 1999;18:363-374.

31. Wise LM, Veikkola T, Mercer AA, Savory LJ, Fleming SB, Caesar C, et al.: Vascular endothelial growth factor (VEGF)-like protein from orf virus NZ2 binds to VEGFR2 and neuropilin-1. Proc Natl Acad Sci USA 1999;96:3071-3076.

32. Wise LM, Inder MK, Real NC, Stuart GS, Fleming SB, Mercer AA: The vascular endothelial growth factor (VEGF)-E encoded by orf virus regulates keratinocyte proliferation and migration and promotes epidermal regeneration. Cell Microbiol 2012;14:1376-1390.

33. Komori Y, Nikai T, Taniguchi K, Masuda K, Sugihara H: Vascular endothelial growth factor VEGF-like heparin-binding protein from the venom of Vipera aspis aspis (Aspic viper). Biochemistry 1999;38:11796-11803.

34. Junqueira de Azevedo IL, Farsky SH, Oliveira ML, Ho PL: Molecular cloning and expression of a functional snake venom vascular endothelium growth factor (VEGF) from the Bothrops insularis pit viper. A new member of the VEGF family of proteins. J Biol Chem 2001;276:39836-39842.

35. Gasmi A, Abidi F, Srairi N, Oijatayer A, Karoui H, Elayeb M: Purification and characterization of a growth factor-like which increases capillary permeability from Vipera lebetina venom. Biochem Biophys Res Commun 2000;268:69-72.

36. Gasmi A, Bourcier C, Aloui Z, Srairi N, Marchetti S, Gimond C et al.: Complete structure of an increasing capillary permeability protein (ICPP) purified from Vipera lebetina venom. ICPP is angiogenic via vascular endothelial growth factor receptor signalling. J Biol Chem 2002;277:29992-29998.

37. Yamazaki Y, Matsunaga Y, Tokunaga Y, Obayashi S, Saito M, Morita T: Snake venom Vascular Endothelial Growth Factors (VEGF-Fs) exclusively vary their structures and functions among species. J Biol Chem 2009;284:9885-9891.

38. Neufeld G, Kessler O, Herzog Y: The interaction of Neuropilin-1 and Neuropilin-2 with tyrosine-kinase receptors for VEGF. Adv Exp Med Biol 2002;515:81-90.

39. Kärpänen T, Heckman CA, Keskitalo S, Jeltsch M, Ollila H, Neufeld G et al.: Functional interaction of VEGF-C and VEGF-D with neuropilin receptors. FASEB J 2006;20:1462-1472.

40. Xu Y, Yuan L, Mak J, Pardanaud L, Caunt M, Kasman I et al.: Neuropilin-2 mediates VEGF-C-induced lymphatic sprouting together with VEGFR3. J Cell Biol 2010;188:115-130.

41. Grunewald FS, Prota AE, Giese A, Ballmer-Hofer K: Structure-function analysis of VEGF receptor activation and the role of coreceptors in angiogenic signaling. Biochim Biophys Acta 2010;1804:567-580.

42. Gerhardt H, Golding M, Fruttiger M, Ruhrberg C, Lundkvist A, Abramsson A et al.: VEGF guides angiogenic sprouting utilizing endothelial tip cell filopodia. J Cell Biol 2003;161:1163-1177.

43. Ruhrberg C, Gerhardt H, Golding M, Watson R, Ioannidou S, Fujisawa H et al.: Spatially restricted patterning cues provided by heparin-binding VEGF-A control blood vessel branching morphogenesis. Genes Dev 2002;16:2684-2698.

44. Joukov V, Sorsa T, Kumar V, Jeltsch M, Claesson-Welsh L, Cao Y et al.: Proteolytic processing regulates receptor specificity and activity of VEGF-C. EMBO J 1997;16:3898-3911.

45. Stacker SA, Stenvers K, Caesar C, Vitali A, Domagala T, Nice E et al.: Biosynthesis of vascular endothelial growth factor-D involves proteolytic processing which generates non-covalent homodimers. J Biol Chem 1999;274:32127-32136.

46. Anisimov A, Alitalo A, Korpisalo P, Soronen J, Kaijalainen S, Leppänen V-M et al.: Activated forms of VEGF-C and VEGF-D provide improved vascular function in skeletal muscle. Circ Res 2009;104:1302-1312.

47. Rissanen TT, Markkanen JE, Gruchala M, Heikura T, Puranen A, Kettunen MI et al.: VEGF-D is the strongest angiogenic and lymphangiogenic effector among VEGFs delivered into skeletal muscle via adenoviruses. Circ Res 2003;92:1098-1106.

48. Harris NC, Davydova N, Roufail S, Paquet-Fifield S, Paavonen K, Karnezis T, et al.: The Propeptides of VEGF-D Determine Heparin Binding, Receptor Heterodimerization, and Effects on Tumor Biology. J Biol Chem 2013;288:8176-8186.

49. Case ST, Cox C, Bell WC, Hoffman RT, Martin J, Hamilton R: Extraordinary conservation of cysteines among homologous Chironomus silk proteins sp185 and sp220. J Mol Evol 1997;44:452-462.

50. Wigle JT, Oliver G: Prox1 function is required for the development of the murine lymphatic system. Cell 1999;98:769-778.

51. Oliver G: Lymphatic vasculature development. Nat Rev Immunol 2004;4:35-45.

52. Papoutsi M, Tomarev SI, Eichmann A, Prols F, Christ B, Wilting J: Endogenous origin of the lymphatics in the avian chorioallantoic membrane. Dev Dyn 2001;222:238-251.

53. Ny A, Koch M, Schneider M, Neven E, Tong RT, Maity S et al.: A genetic Xenopus laevis tadpole model to study lymphangiogenesis. Nat Med 2005;11:998-1004.

54. Lendahl U, Lee KL, Yang H, Poellinger L: Generating specificity and diversity in the transcriptional response to hypoxia. Nat Rev Genet 2009;10:821-832.

55. Planas-Paz L, Strilic B, Goedecke A, Breier G, Fassler R, Lammert E: Mechanoinduction of lymph vessel expansion. EMBO J 2012;31(4):788-804.

56. Carmeliet P: Angiogenesis in life, disease and medicine. Nature 2005;438: 932-936.

57. Nordskog BK, Phan CT, Nutting DF, Tso P: An examination of the factors affecting intestinal lymphatic transport of dietary lipids. Adv Drug Deliv Rev 2001;50:21-44.

58. Casley-Smith JR, Florey HW: The structure of normal small lymphatics. Q J Exp Physiol Cogn Med Sci 1961;46:101-106.

59. Leak LV, Burke JF: Fine structure of the lymphatic capillary and the adjoining connective tissue area. Am J Anat 1966;118:785-809.

60. Jeltsch M, Tammela T, Alitalo K, Wilting J: Genesis and pathogenesis of lymphatic vessels. Cell Tissue Res 2003;314:69-84.

61. Partanen TA, Arola J, Saaristo A, Jussila L, Ora A, Miettinen M et al.: VEGF-C and VEGF-D expression in neuroendocrine cells and their receptor, VEGFR-3, in fenestrated blood vessels in human tissues. FASEB J 2000;14:2087-2096.

62. Lacorre D-A, Baekkevold ES, Garrido I, Brandtzaeg P, Haraldsen G, Amalric F et al.: Plasticity of endothelial cells: rapid dedifferentiation of freshly isolated high endothelial venule endothelial cells outside the lymphoid tissue microenvironment. Blood 2004;103:4164-4172.

63. Kaipainen A, Korhonen J, Mustonen T, van Hinsbergh VW, Fang GH, Dumont D et al.: Expression of the fms-like tyrosine kinase 4 gene becomes restricted to lymphatic endothelium during development. Proc Natl Acad Sci USA 1995;92:3566-3570.

64. Tammela T, Zarkada G, Wallgard E, Murtomaki A, Suchting S, Wirzenius M et al.: Blocking VEGFR-3 suppresses angiogenic sprouting and vascular network formation. Nature 2008;454:656-660.

65. Carmeliet P: Blood vessels and nerves: common signals, pathways and diseases. Nat Rev Genet 2003;4:710-720.

66. Gerhardt H, Ruhrberg C, Abramsson A, Fujisawa H, Shima D, Betsholtz C: Neuropilin-1 is required for endothelial tip cell guidance in the developing central nervous system. Dev Dyn 2004;231:503-509.

67. Fantin A, Vieira JM, Gestri G, Denti L, Schwarz Q, Prykhozhij S et al.: Tissue macrophages act as cellular chaperones for vascular anastomosis downstream of VEGF-mediated endothelial tip cell induction. Blood 2010;116:829-840.

68. Jakobsson L, Bentley K, Gerhardt H: VEGFRs and Notch: a dynamic collaboration in vascular patterning. Biochem Soc Trans 2009;37:1233-1236.

69. Petrova TV, Bono P, Holnthoner W, Chesnes J, Pytowski B, Sihto H et al.: VEGFR-3 expression is restricted to blood and lymphatic vessels in solid tumors. Cancer Cell 2008;13:554-556.

70. Dumont DJ, Jussila L, Taipale J, Lymboussaki A, Mustonen T, Pajusola K et al.: Cardiovascular failure in mouse embryos deficient in VEGF receptor-3. Science 1998;282:946-949.

71. Karkkainen MJ, Haiko P, Sainio K, Partanen J, Taipale J, Petrova TV et al.: Vascular endothelial growth factor C is required for sprouting of the first lymphatic vessels from embryonic veins. Nat Immunol 2004;5:74-80.

72. Baldwin ME, Halford MM, Roufail S, Williams RA, Hibbs ML, Grail D et al.: Vascular endothelial growth factor D is dispensable for development of the lymphatic system. Mol Cell Biol 2005;25:2441-2449.

73. Haiko P, Makinen T, Keskitalo S, Taipale J, Karkkainen MJ, Baldwin ME et al.: Deletion of vascular endothelial growth factor C (VEGF-C) and VEGF-D is not equivalent to VEGF receptor 3 deletion in mouse embryos. Mol Cell Biol 2008;28:4843-4850.

74. Zhang X, Groopman JE, Wang JF: Extracellular matrix regulates endothelial functions through interaction of VEGFR-3 and integrin alpha5beta1. J Cell Physiol 2005;202:205-214.

75. Galvagni F, Pennacchini S, Salameh A, Rocchigiani M, Neri F, Orlandini M et al.: Endothelial cell adhesion to the extracellular matrix induces c-Src-dependent VEGFR-3 phosphorylation without the activation of the receptor intrinsic kinase activity. Circ Res 2010;106:1839-1848.

76. Hogan BM, Bos FL, Bussmann J, Witte M, Chi NC, Duckers HJ et al.: Ccbe1 is required for embryonic lymphangiogenesis and venous sprouting. Nat Genet 2009;41:396-398.

77. Bos FL, Caunt M, Peterson-Maduro J, Planas-Paz L, Kowalski J, Karpanen T et al.: CCBE1 is essential for mammalian lymphatic vascular development and enhances the lymphangiogenic effect of vascular endothelial growth factor-C in vivo. Circ Res 2011;109:486-491.

78. Hagerling R, Pollmann C, Andreas M, Schmidt C, Nurmi H, Adams RH, et al.: A novel multistep mechanism for initial lymphangiogenesis in mouse embryos based on ultramicroscopy. EMBO J 2013;32:629-644.

79. Alders M, Hogan BM, Gjini E, Salehi F, Al-Gazali L, Hennekam EA et al.: Mutations in CCBE1 cause generalized lymph vessel dysplasia in humans. Nat Genet 2009;41:1272-1274.

80. Baldwin ME, Catimel B, Nice EC, Roufail S, Hall NE, Stenvers KL et al.: The specificity of receptor binding by vascular endothelial growth factor-d is different in mouse and man. J Biol Chem 2001;276:19166-19171.

81. Pajusola K, Aprelikova O, Armstrong E, Morris S, Alitalo K: Two human FLT4 receptor tyrosine kinase isoforms with distinct carboxy terminal tails are produced by alternative processing of primary transcripts. Oncogene 1993;8:2931-2937.

82. Hughes DC: Alternative splicing of the human VEGFGR-3/FLT4 gene as a consequence of an integrated human endogenous retrovirus. J Mol Evol 2001;53:77-79.

83. Borg JP, deLapeyriere O, Noguchi T, Rottapel R, Dubreuil P, Birnbaum D: Biochemical characterization of two isoforms of FLT4, a VEGF receptor-related tyrosine kinase. Oncogene 1995;10:973-984.

84. Dixelius J, Makinen T, Wirzenius M, Karkkainen MJ, Wernstedt C, Alitalo K et al.: Ligand-induced vascular endothelial growth factor receptor-3 (VEGFR-3) heterodimerization with VEGFR-2 in primary lymphatic endothelial cells regulates tyrosine phosphorylation sites. J Biol Chem 2003;278:40973-40979.

85. Tarsitano M, De Falco S, Colonna V, McGhee JD, Persico MG: The C. elegans pvf-1 gene encodes a PDGF/VEGF-like factor able to bind mammalian VEGF receptors and to induce angiogenesis. FASEB J 2006;20:227-233.

86. Nilsson I, Bahram F, Li X, Gualandi L, Koch S, Jarvius M, et al.: VEGF receptor 2/-3 heterodimers detected in situ by proximity ligation on angiogenic sprouts. EMBO J 2010;29:1377-1388.

87. Pajusola K, Aprelikova O, Pelicci G, Weich H, Claesson-Welsh L, Alitalo K: Signalling properties of FLT4, a proteolytically processed receptor tyrosine kinase related to two VEGF receptors. Oncogene 1994;9:3545-3555.

88. Siegfried G, Basak A, Cromlish JA, Benjannet S, Marcinkiewicz J, Chretien M et al.: The secretory proprotein convertases furin, PC5, and PC7 activate VEGF-C to induce tumorigenesis. J Clin Invest 2003;111:1723-1732.

89. McColl BK, Baldwin ME, Roufail S, Freeman C, Moritz RL, Simpson RJ et al.: Plasmin activates the lymphangiogenic growth factors VEGF-C and VEGF-D. J Exp Med 2003;198:863-868.

90. Saaristo A, Tammela T, Timonen J, Yla-Herttuala S, Tukiainen E, Asko-Seljavaara S et al.: Vascular endothelial growth factor-C gene therapy restores lymphatic flow across incision wounds. FASEB J 2004;18:1707-1709.

91. Saaristo A, Tammela T, Farkkilä A, Kärkkäinen M, Suominen E, Yla-Herttuala S et al.: Vascular endothelial growth factor-C accelerates diabetic wound healing. Am J Pathol 2006;169:1080-1087.

92. Leppänen V-M, Jeltsch M, Anisimov A, Tvorogov D, Aho K, Kalkkinen N et al.: Structural determinants of vascular endothelial growth factor-D receptor binding and specificity. Blood 2011;117:1507-1515.

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