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Abstract—The therapeutic potential of vascular endothelial growth factor (VEGF)-C and VEGF-D in skeletal muscle has been of considerable interest as these factors have both angiogenic and lymphangiogenic activities. Previous studies have mainly used adenoviral gene delivery for short-term expression of VEGF-C and VEGF-D in pig, rabbit, and mouse skeletal muscles. Here we have used the activated mature forms of VEGF-C and VEGF-D expressed via recombinant adeno-associated virus (rAAV), which provides stable, long-lasting transgene expression in various tissues including skeletal muscle. Mouse *tibialis anterior* muscle was transduced with rAAV encoding human or mouse VEGF-C or VEGF-D. Two weeks later, immunohistochemical analysis showed increased numbers of both blood and lymph vessels, and Doppler ultrasound analysis indicated increased blood vessel perfusion. The lymphatic vessels further increased at the 4-week time point were functional, as shown by FITC-lectin uptake and transport. Furthermore, receptor activation and arteriogenic activity were increased by an alanine substitution mutant of human VEGF-C (C137A) having an increased dimer stability and by a chimeric CAC growth factor that contained the VEGF receptor-binding domain flanked by VEGF-C propeptides, but only the latter promoted significantly more blood vessel perfusion when compared to the other growth factors studied. We conclude that long-term expression of VEGF-C and VEGF-D in skeletal muscle results in the generation of new functional blood and lymphatic vessels. The therapeutic value of intramuscular lymph vessels in draining tissue edema and lymphedema can now be evaluated using this model system. **(*Circ Res*. 2009;104: 1302-1312.)**

Key Words: VEGF-C ■ VEGF-D ■ adeno-associated virus ■ angiogenesis ■ lymphangiogenesis ■ skeletal muscle

Blood and lymphatic vessels integrate to form the circulatory system, which provides oxygen and nutrients to the tissues and removes carbon dioxide and waste metabolites. Endothelial cells (ECs) form a surface barrier between the intra- and extravascular spaces, and EC number determines the functional capacity of the vasculature in each tissue. Vascular endothelial growth factors (VEGFs) are considered to be the main mitogenic and survival factors for ECs, obligatory for embryonic angio- and lymphangiogenesis and capable of activating ECs in the adult.[1–3] In mammals, the 5 known VEGFs are VEGF-A, -B, -C, -D, and placenta growth factor. Of these, VEGF-C and VEGF-D activate primarily lymphatic ECs, which express VEGF receptor (VEGFR)-3.[4,5] After biosynthesis, full-length (fl) VEGF-C and VEGF-D undergo proteolytic cleavage of their C- and N-terminal propeptide domains.[6,7] This releases the mature growth factor (ΔNΔC, here short form [sf]), with an increased affinity toward VEGFR-3 and toward VEGFR-2, which is expressed mainly in blood vessels. VEGFR-2–binding has been assumed to be responsible for the angiogenic properties of VEGF-C and VEGF-D in a number of experimental conditions.[8–10] However, no systematic studies have addressed the capacity of the proteolytically activated, mature VEGF-C/-D forms to stimulate angiogenesis versus lymphangiogenesis in vivo.

The ability to stimulate both VEGFR-2 and VEGFR-3 in blood and lymphatic vessels has made VEGF-C and VEGF-D attractive candidates for therapeutic improvement in many types of vascular insufficiencies, including skeletal and cardiac muscle ischemia, lymphedema, and lymphatic vessel hypoplasia.[11] Adenoviral gene transfer was effective in delivering vascular growth factors to pig myocardium,[12] to rabbit and mouse skeletal muscle,[9,13] and to the periadventitial tissue of rabbit carotid arteries.[14] Transgene expression was found to be robust but transient, lasting only 1 to 3 weeks, yet it resulted in significant improvement of tissue perfusion and capillary size. However, declining levels of transgene expression lead to significantly decreased perfusion and to pruning of newly formed blood vessels.[9,12] In contrast to adenovirus, recombinant adeno-associated virus (rAAV) is an effective vehicle for delivering transgenes into many types

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of tissues and organs including muscle, liver, and brain.[15,16] Although rAAV transgene expression levels are lower compared to adenovirus, they are permanent in the muscle.[17–19] This feature of rAAV may provide a vessel maintenance function when vascular growth factors are expressed. These properties of the rAAV vectors prompted us to study the long-term angiogenic versus lymphangiogenic properties of the full-length versus short forms of VEGF-C, VEGF-D, and, for comparison, VEGF-A or a chimeric CAC growth factor that contained the VEGF-A VEGFR-binding domain flanked by VEGF-C propeptides, all delivered via rAAV into skeletal muscle of mice. We addressed in particular the effects of the activated, mature short forms of VEGF-C and VEGF-D.

Materials and Methods

An expanded Materials and Methods section containing detailed description of cell survival assay and analysis of protein expression, rAAV vector preparation, protein purification, isothermal titration calorimetry, VEGFR-2 and VEGFR-3 ELISA assays, immunohistochemistry, Doppler ultrasound measurements of perfusion in the transduced muscles, and FITC-lectin microlymphography are available in the online data supplement at http://circres.ahajournals.org.

Muscle Transduction by the rAAV Vectors

Six- to 7-week-old female FVB/NJ, ICR, and C57BL/6J mice (3 to 4 per group) were anesthetized with xylazine-ketamine (xylazine, Rompun, Bayer; ketamine, Ketalar, Pfizer), and 5×10^{10} rAAV particles (in 30 μL volume) were injected into each *tibialis anterior* (*t.a.*) muscle. All mouse experiments were approved by the Provincial State Office of Southern Finland and carried out in accordance with institutional guidelines.

Fluorescent Angiography of Blood Vessels via Cardiac Perfusion

Blood vessels were directly visualized by cardiac perfusion with the lipophilic carbocyanine dye 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI).[20] After perfusion, the *t.a.* muscle was isolated, and 120-μm sections were analyzed by confocal microscopy using the excitation wavelength of 543 nm.

Statistical Analysis

We evaluated statistical significance by analysis of variance using the Dunnett (2-sided) test as a post hoc test, with $P<0.05$ regarded as significant. Where pairwise comparisons between experimental groups were made, the Tukey test was used. Where indicated, Student's *t* test was also used. The results are presented as mean values±SD, unless otherwise indicated. All experiments were reproduced at least 3 times.

Results

Expression and Analysis of Recombinant VEGF-C and VEGF-D

The regions encoding the proteolytically processed mature forms of human and mouse VEGF-C and VEGF-D, denoted VEGF-Csf and VEGF-Dsf, were cloned downstream of a signal sequence placed under the CMV promoter in the rAAV8 vector. Figure 1A shows the amino acid sequences of human and mouse VEGF-Csf. The residues that differ between the mouse and human sequences are marked in blue and green (alignment of sequences from 7 animal species is shown in Figure I in the online data supplement). Two residues (valine 146 and serine 179) in hVEGF-Csf were substituted to alanine (V146A) and glycine (S179G), respectively, that are found in the mVEGF-Csf protein. Alternatively, the cysteine 137 residue in hVEGF-Csf (marked red) that is not conserved in VEGF-A, VEGF-B, or placenta growth factor was substituted to alanine.

Production and receptor binding of the vector-encoded growth factors was analyzed from the medium of transfected 293T cells, precipitated with VEGFR-2-Ig or VEGFR-3-Ig (Figure 1B). The full-length cDNAs were also expressed and their products migrated as full-length, partially processed and fully processed polypeptides (Figure 1C), as described previously.[6,7] Note that the VEGF-Dsf and VEGF-Dfl bands appear relatively weaker when compared to the corresponding VEGF-C bands. This may reflect their significantly lower affinity to the receptors used for precipitation.[2]

VEGF-C137A Provides Increased Dimer Stability and Receptor Activity

Before the in vivo experiments, recombinant proteins were assayed for stimulation of receptor dimerization-mediated cell proliferation using mouse BaF3 pro-B cells expressing either VEGFR-2-Epo or VEGFR-3-Epo chimeric receptor (see the expanded Materials and Methods section for details). Dilutions of transfected 293T cell media containing similar amounts of the growth factors were used. Whereas the full-length growth factors were processed to several forms in the conditioned medium, the short forms had increased activity (see asterisks and brackets in Online Figure II). Overall, the results also indicated that VEGF-D is less active than VEGF-C in stimulating VEGFR-3 and especially VEGFR-2 activation (Online Figure II). These results are consistent with previously published data on VEGFR-2/VEGF-D interaction[2,5,21] and with the data reported for phosphorylation of VEGFR-2 stably expressed by porcine aortic endothelial cells.[22]

Of all the mutant human VEGF-C:s, the strongest stimulation of BaF3 cell proliferation was obtained with hVEGF-Csf C137A (Figure 1D and 1E). Compared to the parental growth factor (hVEGF-Csf), this protein was particularly active toward VEGFR-2/BaF3 cells (Figure 1D). The G175 residue in the mouse sequence also seemed to provide increased activity over the human S179 (hVEGF-Csf S179G versus hVEGF-Csf in Figure 1D), whereas replacement of V146 of hVEGF-Csf to A (corresponding to A142 in mouse VEGF-C) resulted in unaltered or diminished activity (Figure 1D and 1E).

According to a model based on the structures of the other VEGF family proteins,[23–25] we predicted that the location of the C137 residue is in the dimer interface of hVEGF-Csf (Figure 2A). Despite its increased activity in BaF3 cell proliferation assays, isothermal titration calorimetry indicated that hVEGF-Csf C137A binds VEGFR-3 with an affinity similar to that of the wild-type protein (K_d 280±30 nmol/L and K_d 310±50 nmol/L, respectively; Figure 2B). Further analysis used an ELISA-based competition assay, where plates were coated with $VEGF_{165}$ (for VEGFR-2–binding) or hVEGF-Csf (for VEGFR-3) and then incubated with

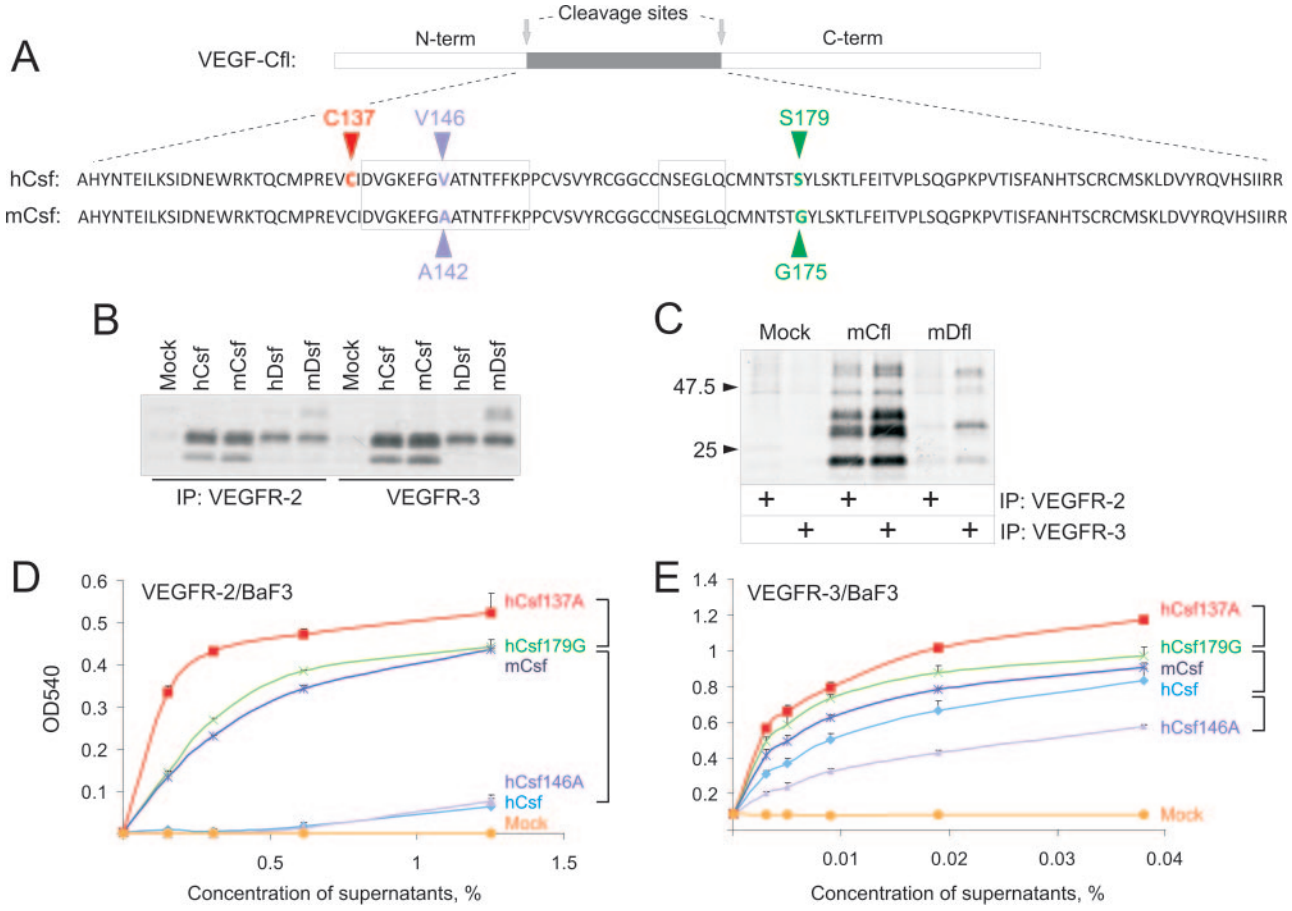

Figure 1. Biochemical characterization of recombinant growth factors. A, Alignment of the human (h) and mouse (m) VEGF-Csf (Csf) sequences. The amino acid residue differences (V146/A142, S179/G175) are indicated in blue and green and the mutated residue (C137) in red. B, Soluble VEGFR-2 and VEGFR-3 precipitation of human and mouse factors followed by SDS-PAGE analysis under reducing conditions. Empty plasmid was used as the mock control. C, Coprecipitation of mouse full-length (VEGF-Cfl) and VEGF-Dfl with VEGFR-2 or VEGFR-3. D, MTT cell survival assay with VEGFR-2/BaF3 cells for the VEGF-Csf native and mutant molecules produced in transfected 293T cells. Mouse and human VEGF-Csf are denoted by mCsf and hCsf, respectively. V146A, S179G, and C137A mutants of hVEGF-Csf are denoted as hCsf146A, hCsf179G, and hCsf137A, respectively. E, The same assay as in D but using VEGFR-3/BaF3 cells. In D and E, statistically significant ($P<0.05$) differences between the activities of certain proteins over range of studied concentrations are indicated by square brackets.

VEGFR-2-Ig or VEGFR-3-Ig proteins preincubated with dilutions of a blocking hVEGF-Csf or its C137A mutant. The amounts of receptor fusion proteins bound to the plates were then quantified by using horseradish peroxidase–conjugated antibodies. The results indicated that wild-type and mutant hVEGF-Csf do not differ significantly in binding to VEGFR-2 (EC_{50} 2.6±0.3 and 2.7±0.6 nmol/L, respectively) or VEGFR-3 (EC_{50} 2.2±0.6 and 1.3±0.3 nmol/L, respectively; $P>0.05$ for both comparisons, Student's t test) (Figure 2C). However, a difference was obtained in nonreducing conditions where hVEGF-Csf C137A migrated as a dimer, whereas the other 2 hVEGF-Csf mutants and the wild-type protein also contained some monomers. When treated with the reducing agents 2-ME or dithiothreitol (DTT) (Figure 2D and 2E), hVEGF-Csf C137A retained a dimeric structure in conditions in which the native hVEGF-Csf protein was monomeric (1 mmol/L DTT). Thus, the increased activity of the hVEGF-Csf C137A mutant protein in the cellular bioassay probably resulted from an increased stability of its dimeric structure.

rAAV8-VEGF-C/-D Transgenes Induce Both Angiogenesis and Lymphangiogenesis

The plasmid vectors used in the in vitro studies were subsequently packaged into rAAV8 and injected into mouse *t.a.* muscle. Two or 4 weeks after the injection, the muscles were processed for immunohistochemical staining of platelet endothelial cell adhesion molecule (PECAM)-1 (endothelial cells), MECA32 (blood vascular endothelial cells only), LYVE-1 (lymphatic endothelial cells), smooth muscle actin (SMA) (smooth muscle cells and pericytes), and CD45 (leukocytes). Additional immunostaining was done for PROX-1 and VEGFR-3 to confirm the lymphatic vessel phenotype. Three different mouse strains (FvB/NJ, C57Bl/6J, and ICR) were used in the experiments to validate the key findings because several studies have demonstrated significant interstrain differences in their responses to the angiogenic growth factors basic fibroblast growth factor and VEGF.[26,27]

In muscles of the FvB/NJ and ICR mice, only small changes were observed in the blood or lymphatic vessels 2 weeks after injection of the vectors (data not shown). Anal-

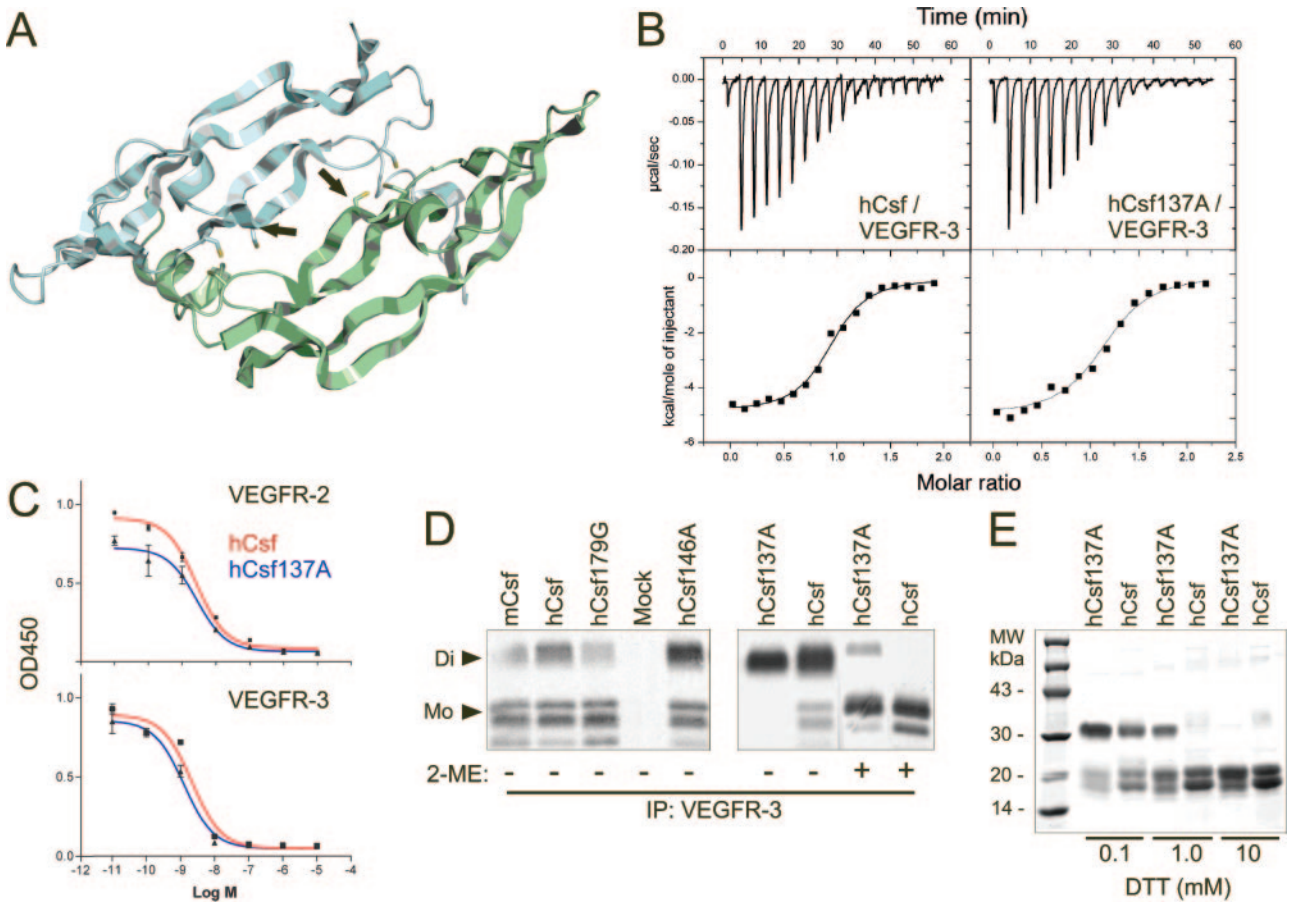

Figure 2. Biochemical properties of wild-type vs mutant VEGF-Csf and VEGF-Dsf. A, Three-dimensional computer model (SWISS-MODEL[45]) shows the putative location of the C137A mutation in the VEGF-Csf structure. The mutant residue (shown by red in Figure 1A) is marked with arrows. The 2 antiparallel polypeptide chains of hVEGF-Csf homodimer are shown in gray and green. The hVEGF-Csf model is based on the known crystal structure of VEGF amino terminal residues 8 to 109[23] and other VEGF family proteins.[24,25] B, Analysis of the VEGFR-3–binding affinities of human VEGF-Csf and its C137A mutant by isothermal titration calorimetry. The proteins were produced as described in Materials and Methods. C, ELISA-based competitive binding assay with VEGF-Csf and its C137A mutant. D, VEGFR-3 coprecipitation of [^{35}S]-labeled human VEGF-Csf mutants or wild-type proteins, which were obtained from the media of 293T cells transfected with the corresponding plasmids (dimer [Di], monomer [Mo]). Mock represents the supernatant of 293T cells transfected with empty plasmid. E, SDS-PAGE of purified VEGF-Csf and its C137A mutant treated with various concentrations of DTT. The proteins were stained with Coomassie blue.

ysis at 4 weeks, however, revealed both an angiogenic and a lymphangiogenic response to the short forms of the factors (Figure 3 and Online Figures III and IV). Double immunostaining of PECAM-1 and mVEGF-Dsf suggested a paracrine vascular effect around the growth factor producing myofibers (Online Figure V). Full-length mVEGF-C induced only some lymphangiogenesis, but no angiogenesis, whereas mVEGF-Dfl had no effect. The lymphangiogenic response was confirmed in parallel samples stained for PROX-1 (data not shown). Blood vessels (MECA32) with their associated perivascular smooth muscle (SMA) were notably increased in skeletal muscles injected with rAAV-mVEGF-Csf, which was also the most active among the wild-type factors in the VEGFR-2/BaF3 cellular assay (Figure 1 and Online Figure II). rAAV-VEGF$_{165}$, used as a control, induced only angiogenesis (Figure 3).

The vascular response in C57Bl/6J skeletal muscle was more rapid, being clear by 2 weeks after the injection of the vectors. Because all mice of this strain injected with the corresponding concentrations of the control rAAV8-VEGF$_{165}$ vector died or had to be euthanized (because of VEGF$_{165}$-induced vascular leakage[28]), we instead used a chimeric VEGF-A called CAC that contains the VEGF$_{165}$ residues 37 to 135 (VEGF homology domain) flanked with the propeptides of VEGF-C.[29] The mice tolerated treatment with this factor without overt symptoms despite the fact that rAAV-CAC induced significantly stronger angiogenic and arteriogenic responses than hVEGF-Csf, hVEGF-Dsf, or mVEGF-Dsf in the skeletal muscles (MECA32 and SMA panels in Figure 4A). A vector encoding the hVEGF-Csf C137A mutant was also tested. Its arteriogenic effects were significantly stronger when compared to the parental factor hVEGF-Csf (Figure 4A), although its lymphangiogenic activity was not significantly different (see the LYVE-1 graph in Figure 4A: $P>0.05$, when hCsf137A is compared to any other experimental group). These effects were even more enhanced at week 11, the latest time point analyzed (data not shown). In all muscle samples analyzed, we observed a strong accumulation of CD45-positive cells with a tendency to concentrate in areas of neovessel formation (Figure 4A and data not shown).

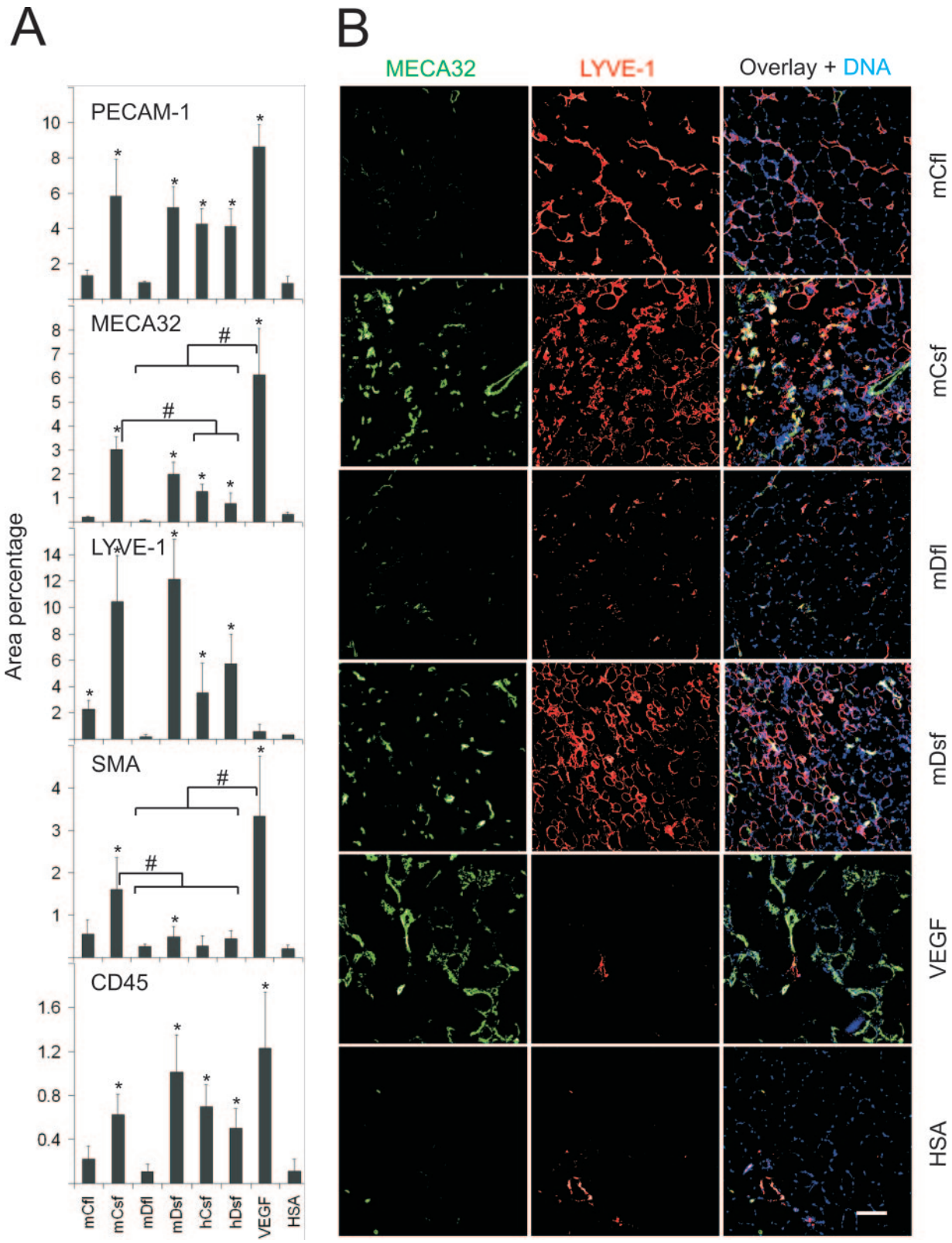

Figure 3. Blood vascular and lymphatic endothelial staining of the target muscles 4 weeks after gene transduction. Immunostaining for PECAM-1 (endothelial cells), MECA32 (blood vessel endothelial cells), LYVE-1 (lymphatic endothelial cells), SMA (smooth muscle cells and pericytes), and CD45 (leukocytes) frozen sections of the *t.a.* muscles of FVB/NJ mice. Full-length forms of mouse VEGF-Cfl and VEGF-Dfl were tested along with mouse and human VEGF-Csf and VEGF-Dsf. A, Quantification of the immunostaining was performed as described in Materials and Methods. *$P<0.05$, when compared to the HSA control, #statistical significance at $P<0.05$ between the indicated experimental groups indicated by brackets. B, Representative images of mouse factor immunostaining are shown. The corresponding PECAM-1/SMA immunostaining patterns are shown in Online Figure III. Note that only mVEGF-Csf and $VEGF_{165}$ induced significant recruitment of smooth muscle cells. Scale bars here and in all other figures are 100 μm, unless otherwise indicated.

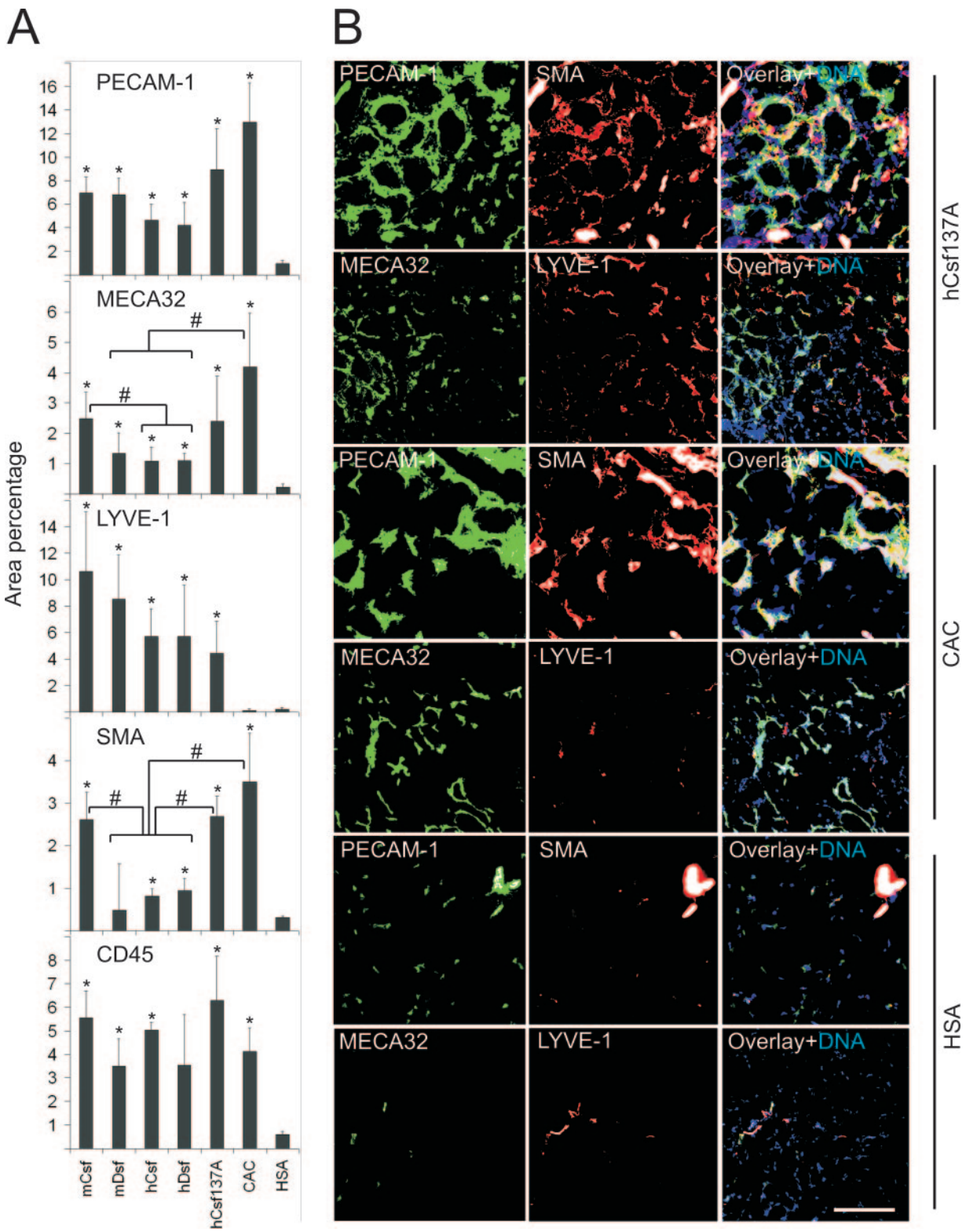

Figure 4. Blood vascular, lymphatic endothelial, and smooth muscle staining 2 weeks after gene transduction. Double-immunostaining of the indicated antigens in frozen muscle sections from C57Bl/6J mice. A, Quantification of the immunostaining was performed as in Figure 3. B, Representative images of hVEGF-Csf C137A-, CAC-, and HSA-treated muscle samples. Note that whereas hVEGF-Csf C137A induces growth of both blood (MECA32-positive) and lymphatic (LYVE-1-positive) vessels, CAC induces only blood vessels. Note that some of the inflammatory cells were also PECAM-1–positive.

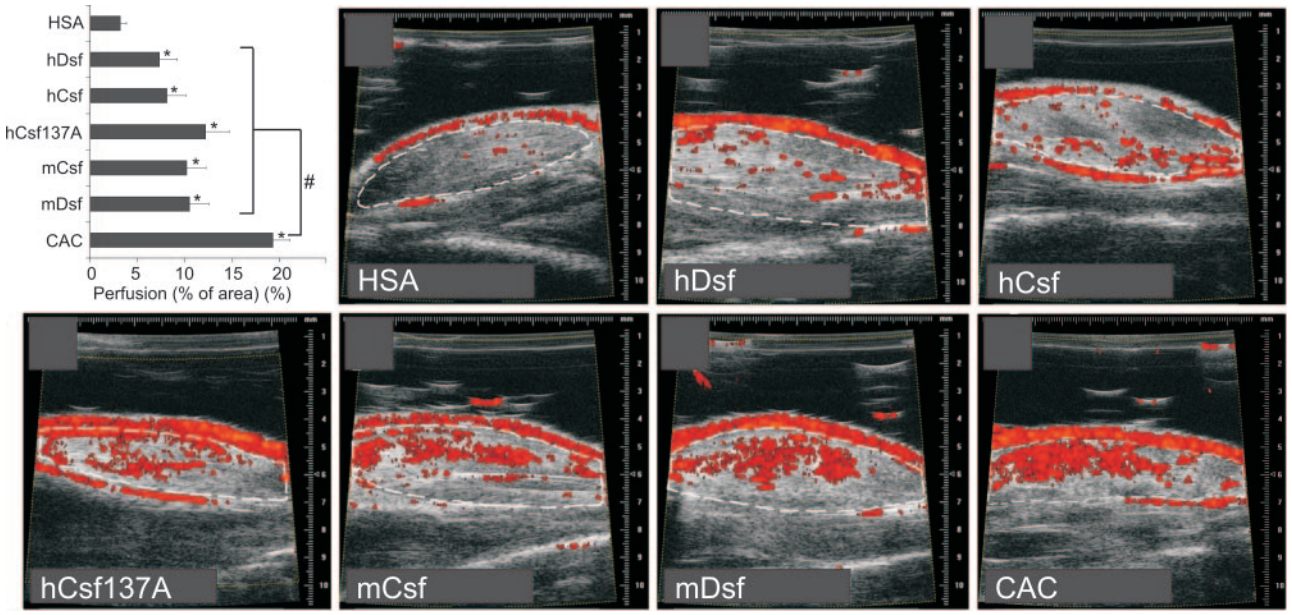

Figure 5. Muscle perfusion 2 weeks after transduction of rAAV vectors. C57Bl/6J mice were separated into 7 groups, and 4 animals per group were injected with vectors encoding the indicated growth factors. Blood flow in *t.a.* muscle was quantified by Doppler ultrasound. Significance values were determined between the test groups and the negative control group (HSA). The bars indicate ±SEM. *Statistically significant differences to HSA control; #statistical significance between CAC and any other experimental group. Representative 2D images from the scanning of the muscles transduced with the various vectors are indicated.

Increased Blood Vessel Perfusion in the Muscles Expressing VEGF-C/-D

To observe any changes in blood perfusion in the treated muscles, we used Doppler ultrasound analysis to detect functional blood vessels with diameters of ≥30 μm. In C57Bl/6J skeletal muscles treated for 2 weeks with the various factors, significantly increased perfusion was recorded (Figure 5). The strongest effect was observed in muscles treated with rAAV-CAC ($P<0.05$). rAAV-hVEGF-C C137A also showed a strong angiogenic effect, although not statistically different from the effects of wild-type VEGF-C/-Dsf proteins. The effect of mVEGF-Dsf seemed to be as strong as that of mVEGF-Csf, whereas the former was significantly weaker in inducing SMA-positive perivascular cells (Figure 4A).

To further image the morphology and perfusion of vessels, the lipophilic dye DiI was injected to the left ventricle of the heart. This fluorescent dye is incorporated into blood vascular endothelial cell membranes, whereas unbound dye in the vessel lumen is washed away by subsequent perfusion fixation.[20] Confocal microscopy of thick muscle sections demonstrated that the AAV-mediated VEGF-C/-D expression leads to widening of preexisting vessels (Figure 6). Increased neovessel formation by sprouting was also observed in the hVEGF-Csf 137A–treated samples and in the positive control (CAC). The CAC-induced vessels differed greatly from those induced by VEGF, which resulted in local angioma-like vascular patterns in the treated muscles (compare CAC and VEGF in Figure 6).

VEGF-C– and VEGF-D–Induced Intramuscular Lymphatic Vessels Are Functional

Comparison of MECA32, LYVE-1, and PROX-1 immunostaining after 4 weeks of transgene expression in FvB/NJ mice demonstrated that the majority of the induced endothelium within the muscles was of lymphatic origin (Figure 3 and data not shown). In comparison, neither $VEGF_{165}$ nor CAC induced lymphatic vessel growth (Figures 3 and 4).

We used FITC-conjugated *Lycopersicon esculentum* lectin[30,31] to determine whether the newly formed lymphatic vessels were functional, ie, capable of absorbing macromolecules (such as FITC-lectin) and conducting fluid flow. Indeed, FITC-lectin injected into the distal end of the *t.a.* muscle was uniformly distributed within the newly formed lymphatic vessels 45 minutes after injection, although some was also present in between the myofibers (Figure 7), demonstrating that the vessels were functional.

Discussion

We show here that rAAV-delivered activated forms of VEGF-C and VEGF-D induce both angiogenesis and lymphangiogenesis in skeletal muscle and that the latter response tends to predominate at the 4-week time point used in our experiments.

Previously, adenovirally delivered human $VEGF\text{-}D^{\Delta N\Delta C}$ or $VEGF_{165}$ was reported to induce transient angiogenic effects (increased vessel density and perfusion) in mouse skeletal muscle.[9] These angiogenic effects peaked at 4 to 7 days after injection and then sharply decreased, reaching negligible levels at 2 weeks. The effects of $VEGF_{165}$ were slightly more prolonged. Lymphatic vessels were only very weakly affected by hVEGF-D in this system. For the present studies, we used rAAV-delivered transgene expression, because it was previously reported to provide more prolonged transgene expression combined with lower immunogenicity.[17–19,32] Using immunohistochemical analysis, we could observe a significant increase in lymphatic as well as blood vessels at 2- and

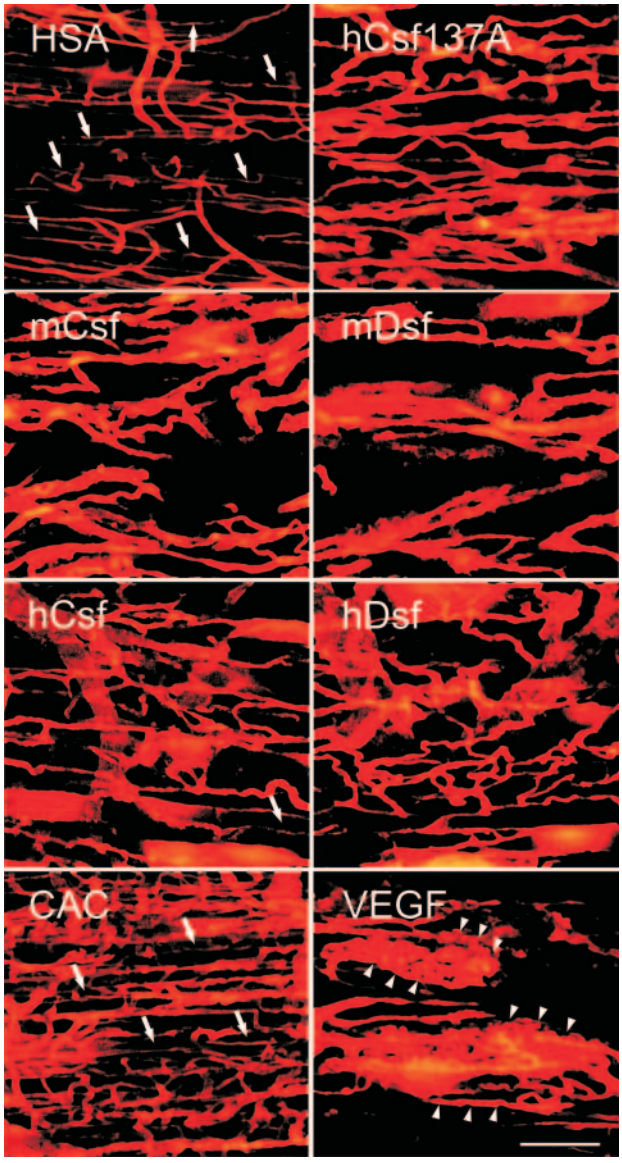

Figure 6. Comparison of perfusion staining of vessels induced by VEGF-C/-D, CAC, and VEGF. Intramuscular blood vessels were stained by perfusion with the lipophilic dye DiI 2 weeks after rAAV transduction. Note that both VEGF-C and VEGF-D increase vessel density and size. In CAC-treated muscles, vessel density is markedly increased and vessel morphology most similar to that of muscle injected with the control vector (HSA), whereas VEGF induced the formation of angioma-like structures (arrowheads). Capillary-sized vessels are indicated by arrows.

4-week time points, although the overall magnitude of the response depended on the mouse strain and in particular on the vascular growth factor used. Both effects were more profound when the activated short forms were used, as compared with the full-length forms. Full-length mVEGF-D was not angiogenic or lymphangiogenic in our assays, and full-length mVEGF-C was only able to induce formation of lymphatic vessels, apparently originating from the preexisting lymphatics found in between the myofibers.[33]

hVEGF-Dsf is significantly less active than hVEGF-Csf at inducing proliferation of VEGFR-2/BaF3 and VEGFR-3/BaF3 cells. Furthermore, neither mVEGF-Dfl nor mVEGF-Dsf was active in the VEGFR-2/BaF3 assay, consistent with the previously reported lack of mVEGF-D/mVEGFR-2 interaction.[21] However, our in vivo data (immunohistochemistry for PECAM-1, MECA32, and LYVE-1, ultrasound analysis of muscle perfusion, visualization of blood vessels with DiI, and studies of the functionality of newly formed lymphatic vessels by FITC-lectin uptake and transport) conclusively demonstrate that the angiogenic activity of rAAV-delivered VEGF-Dsf is comparable to that of VEGF-Csf, although mVEGF-Dsf shows significantly weaker smooth muscle cell recruiting activity than mVEGF-Csf, at least in the FvB/NJ and C57Bl/6J mice.

One explanation for the apparent discrepancy between in vitro and in vivo data are that human or mouse VEGF-Dsf binds to VEGFR-3 in vitro with at least 20-fold lower affinity than VEGF-Csf.[2,5,21] Thus, to obtain equal stimulatory activity of VEGF-C and VEGF-D on receptor-bearing BaF3 cells in vitro, the proteins have to be added into the cell cultures in significantly different quantities, as here demonstrated using the BaF3 cell-based assays. Yet, in vivo, rAAV-infected myofibers provide continuous expression and supply of the transgenic protein, which may accumulate in high enough local concentrations to stimulate endothelial cell growth.

In the present study, as well as in previous reports, VEGF-D was shown to induce an angiogenic response.[9,13,14] Although mVEGF-D does not activate mouse VEGFR-2,[21,22] it is possible that VEGFR-3 is involved in the angiogenic response.[10,34,35] On the other hand, we observed accumulation of CD45- and F4/80-positive cells, especially in the C57Bl/6J mice, that could produce angiogenic factors at sites of rAAV transgene expression. The accumulation of bone marrow–derived inflammatory cells capable of producing a range of biologically active molecules (including VEGF, transforming growth factor-β, platelet-derived growth factor-B, and others), can provide additional angiogenic and arteriogenic signals at sites of adult angiogenesis.[36–38] Furthermore, inflammatory cells and especially macrophages can also promote lymphangiogenesis by synthesis and secretion of VEGF-C and VEGF-D.[39–41]

Our functional tests confirmed that the newly generated blood and lymphatic capillaries were perfused and thus functional. Transgene expression in muscle tissue at 4 weeks after vector injection was consistent with morphological and functional changes observed at that time. However, only some myofibers expressed the transgenes (A Anisimov, unpublished data, 2008), similar to what has been reported for rAAV8-LacZ–injected canine muscle, where the initially high numbers of β-galactosidase–positive myofibers were significantly reduced 4 to 8 weeks after injection.[42] It has been reported that the majority of stably transduced host cells apparently maintain recombinant AAV genome as extrachromosomal episomes.[43]

The strongest stimulator of BaF3 cell proliferation was the hVEGF-Csf C137A mutant, which showed improved dimer stability in multiple assays. Evidently, disulfide-reducing agents that lead to full dissociation of the native hVEGF-Csf only partially affected hVEGF-Csf C137A homodimers. It should be noted that the cysteine residue corresponding to C137 in human VEGF-C is not conserved outside of the VEGF-C/-D subfamily, and according to our structural

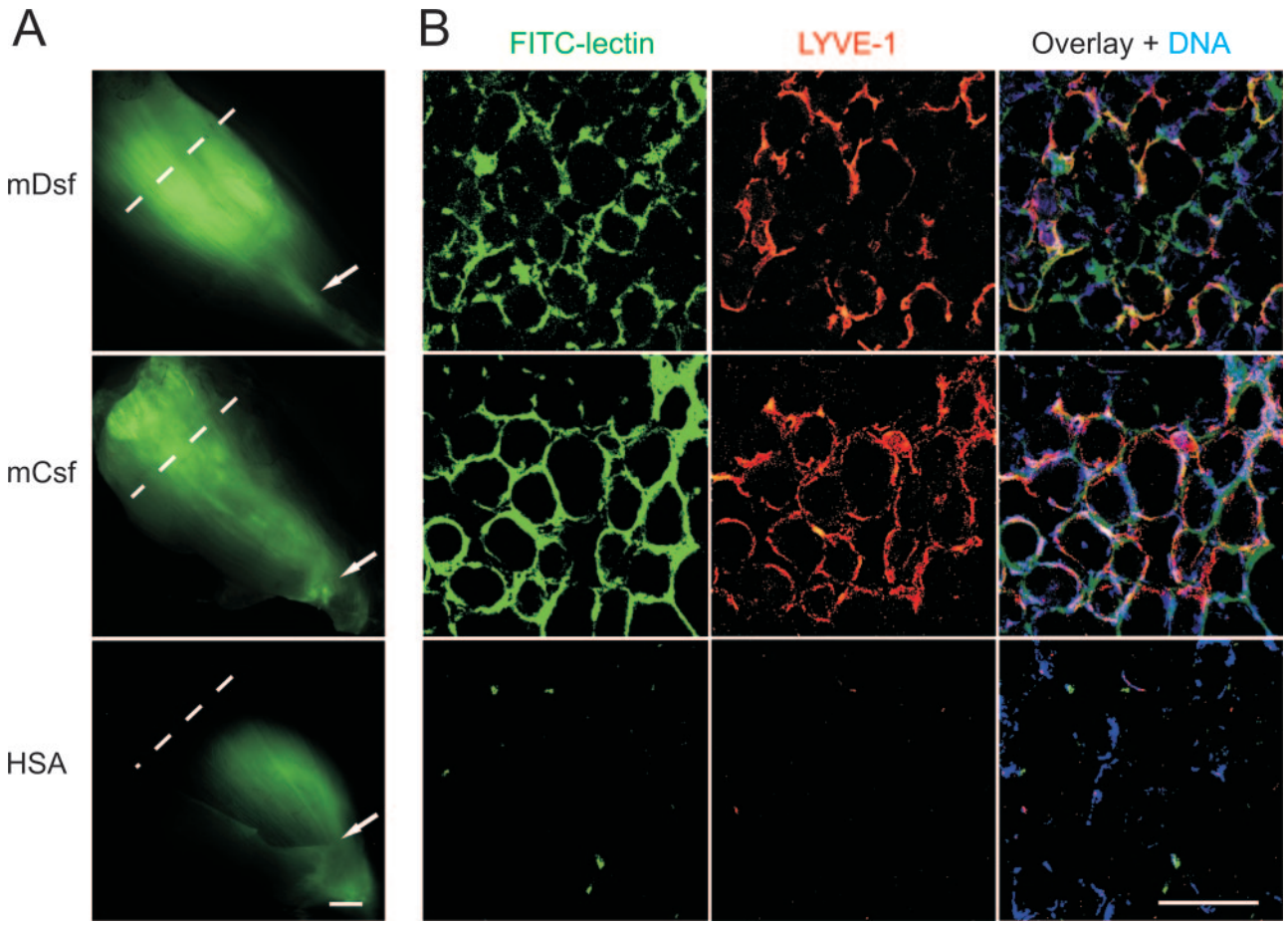

Figure 7. Mouse VEGF-Csf and VEGF-Dsf induce formation of functional lymphatic vessels. A, Lymphatic dye uptake and transfer were imaged 45 minutes after FITC-lectin injection into the distal (lower) part of *t.a.* muscles 4 weeks posttransduction, with rAAVs encoding the indicated proteins. The stained sections were prepared from the regions indicated by the punctuated lines. Arrows indicate sites of FITC-lectin injection. The scale bar indicates 1 mm. B, FITC-lectin, nuclear (Hoechst 33258), and anti–LYVE-1 immunostaining were performed using muscle sections obtained from the muscle areas indicated in A.

model, it is located next to the putative intermolecular disulfide bridge. We therefore suggest that this cysteine residue makes the intermolecular disulfide bond unstable, resulting in noncovalent homodimers of wild-type VEGF-Csf.[6] Replacing cysteine 137 with alanine may lead to the stabilization of the intermolecular disulfide bond and ultimately to a more stable hVEGF-Csf C137A homodimer. As mentioned previously, this more stable dimeric form of VEGF-C can bind to and activate the VEGFR-3 and VEGFR-2 receptors more efficiently and the corresponding change in VEGF-D has a similar effect.[44]

Our immunohistological data demonstrate that compared to hVEGF-Csf, the hVEGF-CsfC137A mutant shows significantly increased arteriogenic activity (SMA graph in Figure 4A) but not increased lymphangiogenic activity. In previous studies of adenovirally transfected mouse and rabbit skeletal muscles, human VEGF-D was also demonstrated to possess arteriogenic activity.[9,13] The differences between previously published data and our own might be attributable to differences in target cells and to varying levels of transgene expression. Unexpectedly, all C57Bl/6J mice transduced with rAAV8-VEGF$_{165}$ died within 10 days, whereas mice transduced with corresponding doses of rAAV8-CAC survived and showed strong angiogenesis in the injected muscles. CAC was also a stronger angiogenic inducer than human VEGF-C or VEGF-D, but it did not promote lymphangiogenesis. Thus, this chimeric factor has interesting properties that might be exploited for potential gene therapy.

In conclusion, we have found that rAAV-delivered activated VEGF-C and VEGF-D and an engineered VEGF-C variant can all induce blood and lymphatic vessels in mouse skeletal muscle. The high arteriogenic activity of the hVEGF-Csf C137A growth factor seems to be attributable to its enhanced dimer stability resulting from the formation of stable interchain disulfide bonds. The most active arteriogenic factors also demonstrated a trend toward higher angiogenic activity, and the newly generated blood and lymphatic vessels were determined to be functional. Future goals now will include determining the therapeutic benefit of these factors in conditions of ischemia or muscle edema.

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Disclosures

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Supplemental material

Materials and Methods

Construction of the rAAV vectors

Full-length mouse VEGF-C and VEGF-D (mVEGF-Cfl and mVEGF-Dfl, respectively) were cloned into blunted MluI and NheI restriction sites of psubCMV-WPRE rAAV expression vector.[1] A pGA4 vector construct encoding IgK-signal peptide (21 amino acid residues) followed by the short form of mouse VEGF-C (mVEGF-Csf, residues 108-223) was synthesized by GeneArt (www.geneart.com) and cloned into the MluI restriction site of psubCMV-WPRE. The IgK-signal peptide was similarly joined to a fragment encoding residues 112-227 of human VEGF-C (hVEGF-Csf) and inserted into psubCMV-WPRE. The V146A, S179G and C137A mutants of hVEGF-Csf were produced by site-directed mutagenesis. The mouse interleukin-3 signal peptide (33 amino acid residues) was fused to the short form of mouse VEGF-D (mVEGF-Dsf, residues 94-210) or human VEGF-D (hVEGF-Dsf, residues 89-205) by PCR amplification. The resulting fragment was then cloned into the MluI site of psubCMV-WPRE. rAAV vectors encoding human VEGF-A_{165} isoform ($VEGF_{165}$) and the chimeric factor CAC have been described previously.[2]

Recombinant AAV vector preparation

The recombinant AAVs (rAAVs, serotype 8) were produced as described previously[3,4] with the following modifications. Instead of pDG plasmid, a combination of adenovirus helper plasmid pBS-E2A-VA-E4[5] and the serotype helper plasmid p5e18-VD2/8[6] were employed. The rAAV preparations were purified by ultracentrifugation using an iodixanol step gradient.[7] The rAAV-containing layer was

directly used for determination of virus titer by real-time PCR, for *in vitro* testing and mouse injections.

MTT cell survival assay and analysis of protein expression by IP

VEGFR-2/BaF3 and VEGFR-3/BaF3 cells are derived from the IL-3-dependent mouse pro-B cell line, BaF3[8]. These express a chimeric receptor consisting of the extracellular domain of mouse VEGFR-2[9,10] or human VEGFR-3 fused in-frame to the transmembrane and intracellular domains of mouse erythropoietin receptor.[11] In interleukin-3-deficient media, both cells can be rescued by the addition of the respective ligands. Supernatants containing the growth factors of interest were produced by transfection of 293T cells using jetPEI transfection reagent (Polyplus-transfection). Proteins were metabolically labeled with [^{35}S]Cys/Met (Promix, NEN Radiochemicals). 50 µl volumes of serial dilutions of supernatants, as well as positive and negative controls, were applied in the wells of 96-well plates in triplicate. Subsequently, 20,000 VEGFR/BaF3 cells in 50 µl were added to each well and the cells were incubated at 37^{o}C for 48 h. MTT substrate was added, and the cells were incubated at 37^{o}C for 2 h. Lysis buffer (10% SDS, 10 mM HCl) was added, and the plates were incubated at 37^{o}C overnight for color development. Quantification was done by absorbance at 540 nm. For analysis of protein expression, supernatants containing [^{35}S]-labelled growth factors were precipitated with VEGFR-2-Ig or VEGFR-3-Ig and Protein A Sepharose, and analysed using SDS-PAGE and autoradiography.

Protein purification

For the *in vitro* binding assays, hVEGF-Csf was prepared as described previously[12]. C-terminal 6xHis-tagged hVEGF-Csf C137A mutant (residues 112-215) was expressed in Sf9 insect cells using the Bac-to-Bac baculovirus expression system (Gibco). Soluble VEGFR-3 (domains 1-3 fused to IgGFc) was prepared as described previously.[13] Soluble VEGFR-2 (domains 2-3 fused to IgGFc) was prepared similarly except that it was expressed in Sf9 insect cells using the Bac-to-Bac baculovirus expression system (Gibco). The cDNA coding for the human $VEGF_{165}$ isoform was inserted into the pMT/V5-HisA expression vector (Invitrogen). $VEGF_{165}$ was purified from conditioned supernatant from S2 cells stably transfected with the resulting vector (pMT-$hVEGF_{165}$) by heparin affinity chromatography followed by cation exchange chromatography and gel filtration.

Isothermal titration calorimetry

Calorimetric titrations of the soluble VEGFR-3 with hVEGF-Csf C137A, or WT (wild type), were carried out at 25°C using a VP-ITC calorimeter (MicroCal). Prior to titration, the protein samples were degassed for 5 min. 10 mM HEPES, 100 mM sodium chloride, pH 7.5 was used to control for heat dilution effects. VEGFR-3 was used in the calorimeter cell at a concentration of 6 μM, and the VEGF-Csf ligands in the syringe at a concentration of 0.14-0.17 mM. Data were processed using MicroCal Origin 7.0 software.

VEGFR-2 and VEGFR-3 ELISA assays

For the enzyme linked immunoassays, hVEGF-Csf at 0.8 μg/ml (VEGFR-3 assay) and $VEGF_{165}$ at 0.9 μg/ml (VEGFR-2 assay) in PBS were used to coat MaxiSorp immunoplates (Thermo Fischer Scientific). To determine the relative binding

affinities, 0.2 μg/ml of soluble human VEGFR-2-Fc was preincubated with 10 pM to 10 μM hVEGF-Csf C137A (or hVEGF-Csf) in 0.5 % BSA and 0.1 % Tween-20, and then transferred onto the $VEGF_{165}$ plate for the competition assay. Similarly, soluble 0.3 μg/ml human VEGFR-3-Fc was preincubated with hVEGF-Csf C137A (or hVEGF-Csf) and then transferred onto the hVEGF-Csf plate. After extensive washing with PBS, 0.1 % Tween-20, the bound receptor was quantified by HRP-conjugated anti human antibody (DakoCytomation) together with SureBlue peroxidase substrate (KPL). To determine the IC_{50} values for the one-site binding model, nonlinear regression analysis was performed using Prism v3.02 (GraphPad Software).

Immunohistochemistry

Muscles were isolated and frozen in O.C.T (TissueTek, Sakura Finetek). Cryosections (8 μm) were cut, acetone-fixed, and immunostained using the following antibodies: rat anti-PECAM-1 (Pharmingen), rat anti-CD45 (Pharmingen), rat F4-80 (Serotec), mouse anti-smooth muscle actin (SMA)-Cy3 (Sigma), rabbit anti-human PROX-1 and rabbit anti-mouse LYVE-1 antisera, and Hoechst 33258 for DNA staining. Secondary antibodies were Alexa Fluor-conjugated (Molecular Probes, Eugene, OR). Microvessel area density was quantified using ImageJ software (NIH).

***In vivo* Doppler ultrasound measurements of perfusion in transduced muscles**

Doppler ultrasound was used to analyze blood perfusion in the transduced *t.a.* muscles of anesthetized mice two weeks post-transduction. Mice were anesthetized with xylazine (Rompun, Bayer)-ketamine (Ketalar, Pfizer) and their hindlegs were shaved. For analysis, the mouse was gently fixed on a platform heated to 36°C and the analysed area was covered by ultrasound gel. Muscle area was scanned using the

VEVO 770 Micro-Ultrasound System (VisualSonics Inc.). B-Mode images were acquired to confirm anatomic boundaries of target muscle in Doppler images. To evaluate the potential effect of heart rate on Doppler signals, electrocardiographic (ECG) measurements were performed immediately following Doppler analysis. No significant differences in heart rate were observed between the mice (data not shown). Image stacks were generated by three-dimensional scanning. The fraction of surface area covered by perfused blood vessels was quantified as "perfused vessels (% of area)" in Doppler images using the VisualSonics software. The region of interest was manually restricted to the tibialis anterior muscle.

FITC-lectin microlymphography

2 µl of FITC-lectin (Vector Laboratories) was injected into the lower (distal) region of the *t.a.* muscle of anesthetized mice. After 45 min the mice were sacrificed, and the muscles were prepared, photographed, frozen in O.C.T, and sectioned. Parallel sections were stained with antibodies recognizing markers of lymphatic endothelium, PROX-1 or LYVE-1, followed by incubation with secondary antibodies conjugated with Alexa Fluor-594.

Supplemental figure legends

Online Figure I. A. Alignment of VEGF-C sequences. The VEGF-C sequences of the different vertebrates indicated show considerable conservation in the region of proteolytically processed, mature human VEGF-C (hVEGF-Csf). Residues differing between species are marked in red. Species: Human – homo sapiens; monkey – macaca mulatta (rhesus monkey); dog – canis lupus familiaris; cow – bos taurus;

mouse – mus musculus; rat – rattus norvegicus; chicken – gallus gallus. **B. Schematic representation of the VEGF proteins used in this study.** The CAC protein consist of the N- and C-terminal propeptide domains (NT and CT, respectively) of human VEGF-C fused to the VEGF homology domain (VHD, amino acid residues 37 to 135) of human VEGF-A.[2] Growth factor abbreviations are described in the legend of Figure 1.

Online Figure II. Receptor activation by various forms of VEGF-C and VEGF-D. MTT cell survival assay was carried out using VEGFR-3/BaF3 and VEGFR-2/BaF3 cells as described in Materials and Methods. Supernatant produced by empty vector-transfected 293T-derived cells was used as a mock control. Interleukin-3 (IL-3, 2 ng/ml) was used as a positive control for cellular viability/proliferation. Note that all VEGF-D forms are inactive, when used in the concentrations corresponding to the highest VEGF-C (either sf or fl) concentrations shown (for example, compare OD540 readings for VEGF-D and VEGF-C stimulation of VEGFR-2/BaF3 and VEGFR-3/BaF3 cells obtained at 2.5% of and 0.3% ligand concentrations, respectively). Statistically significant differences between the activities of the various growth factor forms over range of sub-saturating concentrations are indicated by the appropriately colored asterisks and brackets (* indicates $P<0.05$).

Online Figure III. Blood vascular, lymphatic, endothelial and smooth muscle immunostaining two weeks after transduction of the indicated vectors into *t.a.* muscles of ICR mice. Double-immunostaining of the indicated antigens were carried out using frozen muscle sections. **A.** Quantification of the immunostaining as described in Materials and Methods. **B.** Representative images obtained from the

mVEGF-Csf-, mVEGF-Dsf-, and HSA vector-transduced muscle samples. Note that in the ICR mouse strain the inflammatory (CD45-positive) cells are more numerous also in the negative control (HSA), in contrast to the other two mouse strains tested. * - statistically significant ($P<0.05$) differences between experimental groups and control (HSA).

Online Figure IV. Double immunostaining of transduced muscles for PECAM-1 and SMA. Samples are described in the legend of Figure 3. Quantification of the immunostaining is shown in Figure 3A.

Online Figure V. Mouse VEGF-Dsf induces a strong paracrine angiogenic effect in skeletal muscle. PECAM-1 immunostaining labels blood vessels and shows a rich vascular network surrounding myofibres positive for mVEGF-Dsf (mDsf). The scale bar indicates 50 μm.

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Human   AHYNTEILKSIDNEWRKTQCMPREVCIDVGKEFGVATNTFFKPPCVSVYRCGGCCNSEGLQCMNTSTSYLSKTLFEITVPLSQGPKPVTISFANHTSCRCMSKLDVYRQVHSIIRR
Monkey  AHYNAEILKSIDNEWRKTQCMPREVCIDVGKEFGVATNTFFKPPCVSVYRCGGCCNSEGLQCMNTSTSYLSKTLFEITVPLSQGPKPVTISFANHTSCRCMSKLDVYRQVHSIIRR
Dog     AHYNAEILKSIDNEWRKTQCIPREVCIDVGKEFGAATNTFFKPPCVSVYRCGGCCNSEGLQCMNTSTSHLSKTLFEITVPLSQGPKPVTISFANHTSCRCMSKLDVYRQVHSIIRR
Cow     AHYNTEILRSIDNEWRKTQCMPREVCIDVGKEFGAATNTFFKPPCVSVYRCGGCCNSEGQQCMNTSTSYLSKTLFEITVPLSQGPKPVTISFANHTSCRCMSKLDVYRQVHSIIRR
Mouse   AHYNTEILKSIDNEWRKTQCMPREVCIDVGKEFGAATNTFFKPPCVSVYRCGGCCNSEGLQCMNTSTGYLSKTLFEITVPLSQGPKPVTISFANHTSCRCMSKLDVYRQVHSIIRR
Rat     AHYNTEILKSIDNEWRKTQCMPREVCIDVGKEFGAATNTFFKPPCVSVYRCGGCCNSEGLQCMNTSTGYLSKTLFEITVPLSQGPKPVTISFANHTSCRCMSKLDVYRQVHSIIRR
Chicken AHYNAEILKSIDTEWRKTQCMPREVCVDVGKEFGATTNTFFKPPCVSIYRCGGCCNSEGLQCMNISTNYISKTLFEITVPLSHGPKPVTVSFANHTSCRCMSKLDVYRQVHSIIRR
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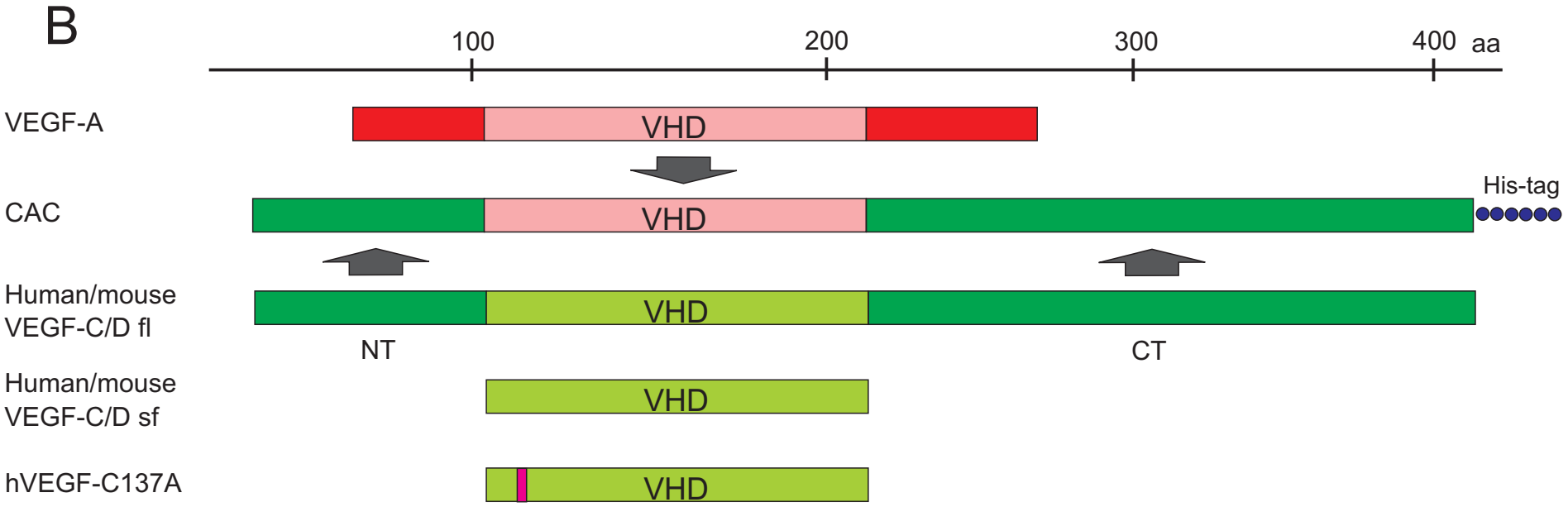
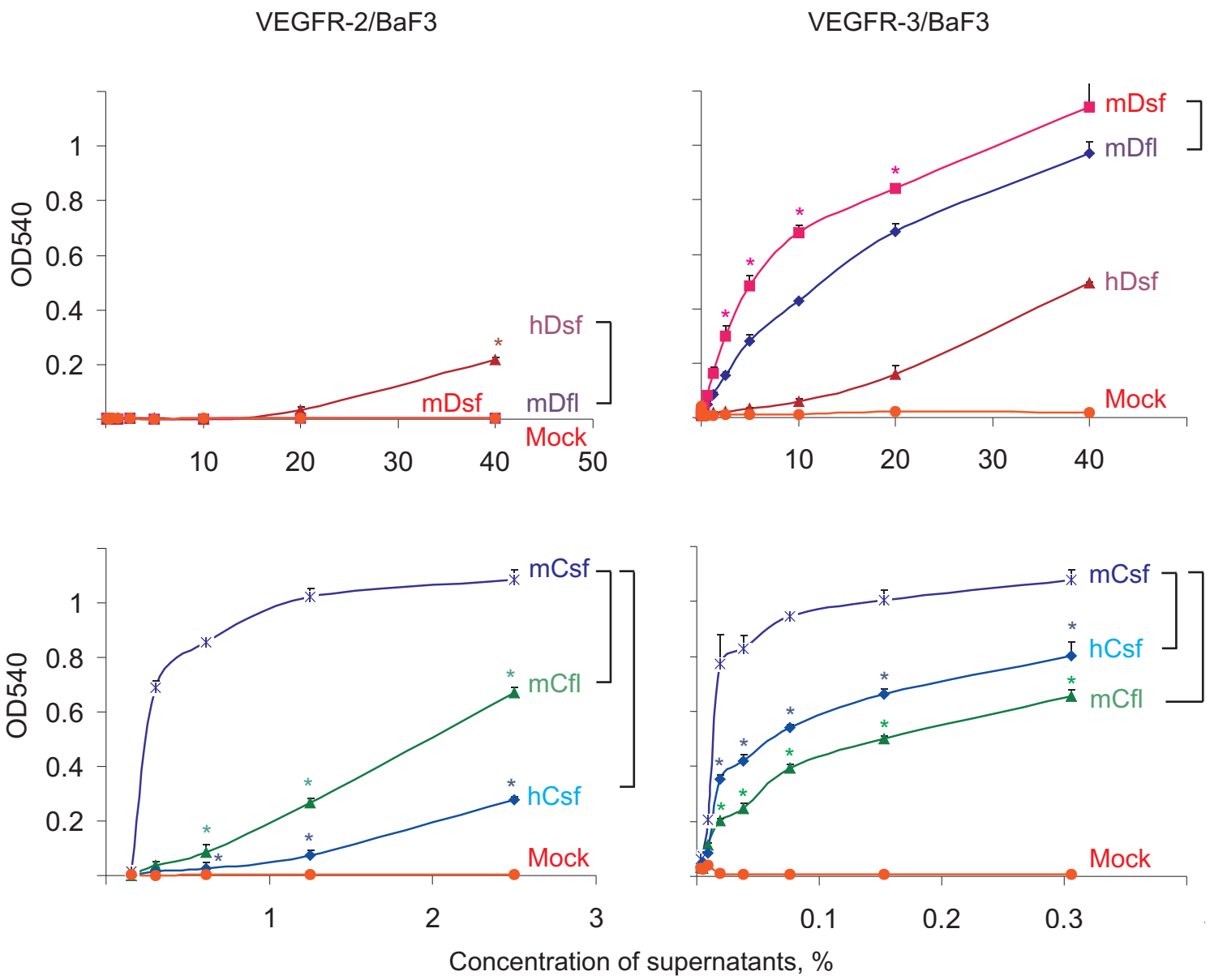
B





Anisimov et al., Online Fig. II