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Objective—The endogenous role of the VEGF family member vascular endothelial growth factor-B (VEGF-B) in pathological angiogenesis remains unclear.

Methods and Results—We studied the role of VEGF-B in various models of pathological angiogenesis using mice lacking VEGF-B (VEGF-$B^{-/-}$) or overexpressing VEGF-B_{167}. After occlusion of the left coronary artery, VEGF-B deficiency impaired vessel growth in the ischemic myocardium whereas, in wild-type mice, VEGF-B_{167} overexpression enhanced revascularization of the infarct and ischemic border zone. By contrast, VEGF-B deficiency did not affect vessel growth in the wounded skin, hypoxic lung, ischemic retina, or ischemic limb. Moreover, VEGF-B_{167} overexpression failed to enhance vascular growth in the skin or ischemic limb.

Conclusion—VEGF-B appears to have a relatively restricted angiogenic activity in the ischemic heart. These insights might offer novel therapeutic opportunities. (***Arterioscler Thromb Vasc Biol*. 2008;28:1614-1620**)

Key Words: VEGF-B ■ angiogenesis ■ arteriogenesis ■ collateral growth ■ cardiac ischemia ■ limb ischemia

Vascular endothelial grow factor (VEGF) is a key regulator of angiogenesis in health and disease by binding to VEGF receptor-2 (VEGFR-2),[1] but the angiogenic activity of its homologue VEGF-B, which binds VEGFR-1 (Flt-1), is less defined.[2,3] By contrast, genetic and inhibition studies revealed that Flt-1, and its other specific ligand PlGF, stimulate pathological angiogenesis only.[4,5]

See accompanying article on page 1575

VEGF-B is produced as 2 different isoforms: a heparin-binding VEGF-B_{167} and diffusible VEGF-B_{186} isoform.[3,6,7] VEGF-B is widely expressed in many tissues and cell types, including cardiac and skeletal myocytes and endothelial and mural cells.[3,6,7] In vitro, VEGF-B stimulates endothelial cell growth and proliferation.[8] By contrast, loss-of-function studies failed to reveal a consistent role for VEGF-B in pathological angiogenesis. Indeed, in 2 lines, loss of VEGF-B did not cause vessel defects in the embryo or healthy adult mouse, whereas recovery of coronary flow was impaired after transient occlusion ex vivo but, apparently, not because of reduced coronary vessel growth.[9,10] Moreover, loss of VEGF-B failed to reduce angiogenesis in the cornea[9] or ischemic retina.[11] One study proposed a role for VEGF-B in pulmonary hypertension,[12] but this finding was contested by others.[13] The effects of VEGF-B deficiency on revascularization of the infarcted myocardium in vivo remain unknown. Gain-of-function studies also showed inconsistent effects. Indeed, overexpression of VEGF-B stimulates angiogenesis in skin wounds and ischemic limbs[14–16] or promotes hypertrophy of remote myocardium after myocardial infarction,[17] whereas VEGF-B had negligible effects after adenoviral gene transfer in rabbit carotid arteries or normoxic hindlimbs.[18,19] The therapeutic potential of VEGF-B (VEGF-B_{167}) to promote revascularization of ischemic myocardium remains unknown. Overall, the precise role of VEGF-B in pathological angiogenesis remains to be established.

As these controversies might be attributable to differences in genetic background or phenotyping methodology, we

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characterized the angiogenic role of VEGF-B in different mouse models of disease. By using VEGF-B$^{-/-}$ mice on a pure C57BL/6 inbred background, and various strategies to overexpress VEGF-B, we found that VEGF-B promotes vessel growth in the ischemic heart but not in other organs.

Methods

For detailed Methods, please see the supplemental materials (available online at http://atvb.ahajournals.org).

Animals, Models, Histology, Immunohistochemistry, and Morphometric Analyses

VEGF-B$^{-/-}$ mice[9] were back-crossed onto a C57BL/6 background for 8 generations. C57BL/6 and NMRI nu/nu mice (all 8 to 12 weeks old) were obtained from Charles River Laboratories (Les Ocins, France) and adult SCID mice were from Taconic M&B Europe (Ry, Denmark). LacZ-tagged Flt1 mice, expressing βgalactosidase (β-Gal) under the control of the Flt-1 promotor were kindly provided by J. Rossant (Toronto, Ontario, Canada).[20] Animal experiments were approved by local committees. All experimental approaches are described in detail in the supplemental methods.

Production and Administration of VEGF-B Protein, Plasmid, and Adenovirus

Recombinant human VEGF-B$_{167}$ (rhVEGF-B$_{167}$) protein was obtained from Amrad Corporation and delivered systemically via osmotic minipumps. Adenoviruses, constructed by cloning the murine PlGF-2, or the human VEGF-B$_{167}$ or VEGF-B$_{186}$ cDNA into the pACCMVpLpA plasmid, were intradermally or intravenously injected. A plasmid expressing murine VEGF-B$_{167}$ (pcDNA3.mVEGF-B$_{167}$) or an empty pcDNA3 plasmid was administered via muscle electroporation. These experimental methods are described in more detail in the supplemental methods.

Statistics

Data (mean±SEM) were analyzed using 2-tailed Student *t* test, with $P<0.05$ considered statistically significant.

Results

Loss of VEGF-B Impairs Revascularization After Myocardial Infarction

To study the potential of VEGF-B to stimulate revascularization of the ischemic heart (infarct and border zone), we used a previously established model of acute myocardial infarction (MI) in wild-type (WT) and VEGF-B$^{-/-}$ mice.[21] At 7 days after MI, the density of both thrombomodulin positive (TM$^+$) capillaries and smooth muscle α-actin positive (SMA$^+$) covered vessels in the infarct area of VEGF-B$^{-/-}$ mice was only 65% of that of WT mice (n=14; $P<0.05$ in all groups; Figure 1A and 1B). Revascularization of the ischemic border zone was also impaired in VEGF-B$^{-/-}$ mice (TM$^+$ vessels: 340±39 vessels/mm^2 in WT versus 234±38 vessels/mm^2 in VEGF-B$^{-/-}$ mice; n=5; $P<0.05$). The number of macrophages, infiltrating into the infarct area, was normal (Mac3$^+$ area/infarct area: 0.22±0.06% in VEGF-B$^{-/-}$ mice versus 0.22±0.05% in WT mice; n=12; P=NS). Thus, loss of VEGF-B impairs angiogenesis and vessel maturation in the ischemic heart.

We next analyzed whether administration of recombinant human (rh) VEGF-B$_{167}$ protein rescued the impaired myocardial revascularization in VEGF-B$^{-/-}$ mice, and therefore administered rhVEGF-B$_{167}$, the predominant isoform in cardiac and skeletal muscle.[7] Continuous systemic delivery of a

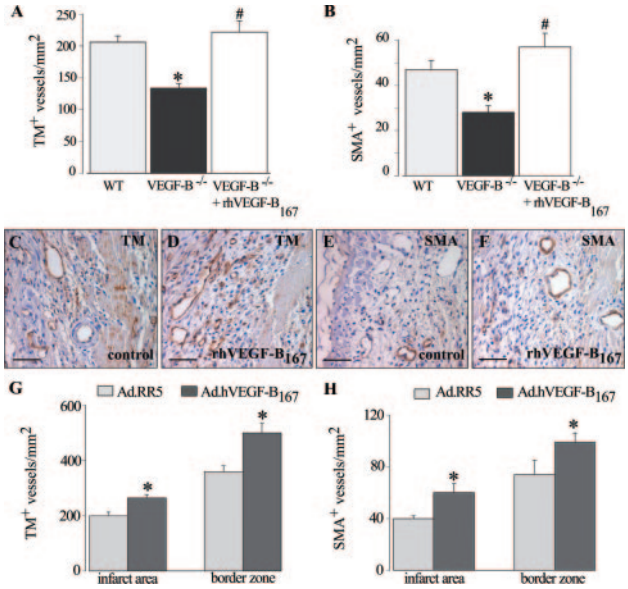

Figure 1. A and B, Morphometric analysis revealed a reduced number of TM$^+$ and SMA$^+$ vessels in the infarcted area of VEGF-B$^{-/-}$ mice, as compared to WT animals (*$P<0.05$) or to VEGF-B$^{-/-}$ mice, treated with rhVEGF-B$_{167}$ ($^{\#}P<0.05$ vs no treatment). C–F, Immunostaining showed increased TM$^+$ and SMA$^+$ vessels after delivery of rhVEGF-B$_{167}$ to WT mice. G and H, Systemic injection of Ad.hVEGF-B$_{167}$ stimulates the growth of TM$^+$ and SMA$^+$ vessels in the infarct area and border zone of WT mice (*$P<0.05$). Scale bars: 50 μm.

daily dose of 1.5 μg rhVEGF-B$_{167}$ for 1 week to VEGF-B$^{-/-}$ mice normalized the impaired revascularization of the infarct (n=5; $P<0.05$; Figure 1A and 1B), and increased the growth of TM$^+$ vessels in the ischemic border zone by 1.5-fold ($P<0.05$, n=5).

VEGF-B$_{167}$ Therapy Enhances Ischemic Myocardial Revascularization

We then used 3 different techniques to investigate whether delivery of VEGF-B stimulated revascularization of ischemic hearts in WT mice. First, we administered VEGF-B$_{167}$ protein. Pilot studies revealed that delivery of rVEGF$_{167}$ via osmotic minipumps significantly increased the VEGF-B$_{167}$ blood plasma levels (Note I, please see supplemental materials). VEGF-B$_{167}$ protein therapy indeed increased the density of capillaries and arterioles in the infarct area (TM$^+$ vessels/mm^2: 206±10 after vehicle versus 285±33 after rhVEGF-B$_{167}$; SMA$^+$ vessels/mm^2: 47±4 after vehicle versus 62±11 after rhVEGF-B$_{167}$; n=5; $P<0.05$; Figure 1C through 1F), and stimulated vessel growth in the ischemic border by 1.5-fold and 1.3-fold, respectively ($P<0.05$; n=5).

Second, similar results were obtained after intravenous injection of an adenoviral vector encoding hVEGF-B$_{167}$ (Ad.hVEGF-B$_{167}$), known to transduce hepatocytes, which then release the transgene product into the circulation for up to 21 days (Note II, please see supplemental materials). Compared to control Ad.RR5 virus, VEGF-B$_{167}$ gene transfer increased the densities of TM$^+$ and SMA$^+$ vessels in the infarct and in the ischemic border (n=9; $P<0.05$ versus Ad.RR5; Figure 1G and 1H) at 7 days after MI. VEGF-B$_{167}$ gene transfer did not stimulate macrophage recruitment (Mac3$^+$ area/infarct area:

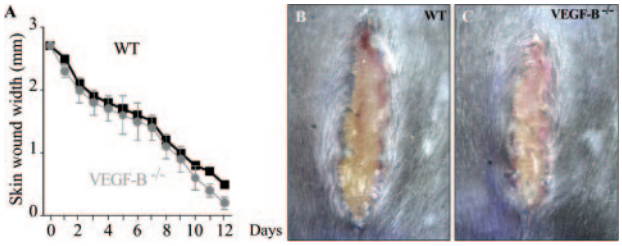

Figure 2. A–C, Skin wound healing is comparable in WT and VEGF-$B^{-/-}$ mice as analyzed by measurement of the wound width (A), and illustrated by macroscopic inspection (B and C).

0.20±0.05% after Ad.hVEGF-B_{167} versus 0.19±0.06% after Ad.RR5; n=9; *P*=NS).

Third, implantation of VEGF-B_{167}-expressing myoblasts, but not control $LacZ^+$ myoblasts, in the ischemic border zone induced vessel growth in the ischemic myocardium (supplemental Figure I). Hence, using different techniques (delivery of protein or gene transfer) and routes of administration (locally or systemically), VEGF-B_{167} therapy enhanced vessel growth in ischemic hearts of WT mice.

VEGF-B Does Not Affect Vessel Growth in Skin, Lung, or Retina

To further study the role of VEGF-B in pathological angiogenesis, we analyzed skin wound healing, using a linear skin incision model. Daily measurements revealed no defects in the rate or extent of skin wound healing in VEGF-$B^{-/-}$ mice (Figure 2A through 2C). Consistent herewith, loss of VEGF-B failed to affect the number of endothelial cell–lined and mural cell–covered vessels in the granulation tissue at 5 days after wounding ($CD31^+$ vessels/mm^2: 270±16 in WT mice versus 290±20 in VEGF-$B^{-/-}$ mice; SMA^+ vessels/mm^2: 67±9 in WT mice versus 62±7 in VEGF-$B^{-/-}$ mice; n=5; *P*=NS). Macrophage infiltration was also normal ($F4/80^+$ area as % of total granulation tissue area: 4.59±0.25 in WT mice versus 4.55±0.92 in VEGF-$B^{-/-}$ mice; n=5; *P*=NS). Additional studies using adenoviral vectors to locally overexpress $VEGFB_{167}$ or VEGF-B_{186} in the skin confirmed that VEGF-B does not affect the skin vasculature (supplemental Figure II).

Furthermore, loss of VEGF-B failed to affect mural cell recruitment and vessel remodeling in hypoxic lungs (supplemental Figure IIIA through IIIC) or neovascularization in ischemic retinas (supplemental Figure IIIE through IIIF). Together, VEGF-B plays a negligible role in vessel growth, maturation, and remodeling in normal or wounded skin, in hypoxic lungs, or in ischemic retinas.

Loss of VEGF-B Does Not Affect Revascularization of Ischemic Limbs

We next analyzed whether VEGF-B affects revascularization of ischemic limbs.[21] In an established mouse model of hind-limb ischemia, the ischemic gastrocnemius muscle is revascularized by capillary angiogenesis, whereas collateral vessel growth occurs in the adductor muscle.[21] In the ischemic gastrocnemius muscle, vessel densities in the regenerating areas were comparable in VEGF-$B^{-/-}$ mice at 7 days after ischemia (Table). In addition, laser Doppler perfusion analysis and endurance/graded treadmill exercise tests confirmed normal revascularization of ischemic limbs in VEGF-$B^{-/-}$ mice after ischemia (Table; supplemental Figure IVA and IVB). Moreover, to exclude the possibility that VEGF-B would only act as a modifier of PlGF, we analyzed mice lacking both VEGF-B and PlGF. However, compared to WT or VEGF-$B^{-/-}$ mice, limb reperfusion was comparably reduced in mice lacking PlGF alone or in mice lacking both VEGF-B and PlGF (supplemental Figure IVC and IVD; Table), indicating that VEGF-B is redundant for limb reperfusion even in conditions of genetically crippled limb revas-

Table. Negligible Role of VEGF-B in Revascularization After Limb Ischemia

			WT Mice			
	WT Mice	VEGF-$B^{-/-}$ Mice	Empty Plasmid	pmVEGF-B_{167}	Ad.RR5	Ad.hVEGF-B_{167}
Angiogenesis						
Capillary-to-myocyte ratio	0.96±0.06	0.97±0.06	ND	ND	1.16±0.06	1.19±0.10
Total limb perfusion						
Laser Doppler (% of non-ligated)	90±5	90±11	86±9	82±6	92±5	94±4
Treadmill exercise test						
Endurance (% of baseline)	56±6	50±7	ND	ND	ND	ND
Grade exercise (% of baseline)	57±4	52±6	ND	ND	ND	ND
Collateral growth						
Lumen area, μm^2						
Main collateral artery	2810±300	2480±150	2890±190	2630±280	2294±425	2415±511
2nd collateral branch	740±75	790±50	760±60	790±65	704±190	688±163
3rd collateral branch	120±10	140±7	96±3	98±6	105±13	92±13
Collateral side branches (n/mm^2)						
2nd collateral branches	3±0.8	3±0.7	2±0.3	3±0.4	4±1.8	3±1.4
3rd collateral branches	5±1.0	6±1.5	8±1.2	9±1.6	7±1.6	10±3.6
Total perfusion area, $\mu m^2/mm^2$	2730±700	3000±800	3140±360	3460±670	3098±569	3020±524

Analysis was performed as described in methods. ND indicates not determined; *P*=NS for WT/control mice vs VEGF-$B^{-/-}$/VEGF-B–treated animals.

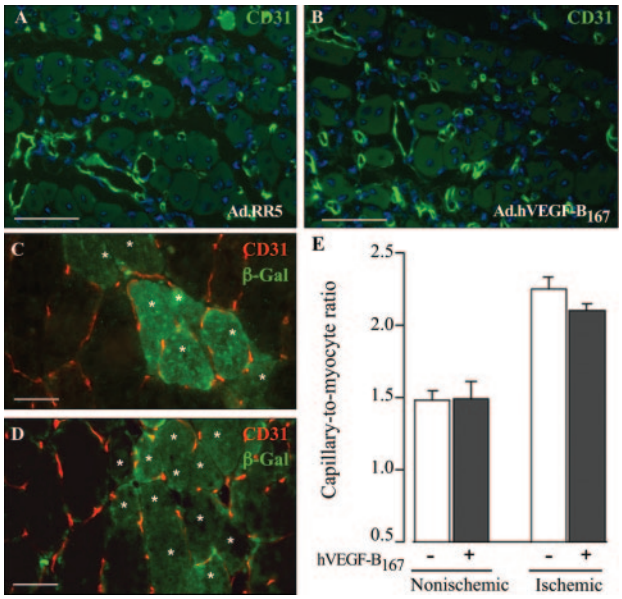

Figure 3. A and B, Immunolabeling for CD31 showed comparable vessel densities in regenerating gastrocnemius muscles of Ad.RR5 or Ad.hVEGF-B_{167} injected mice. C and D, Double staining for β-Gal (green cells, marked by an asterisk) and CD31 revealed an identical vascularization pattern in gastrocnemius muscles, injected with control or VEGF-B_{167} overexpressing myoblasts. E, Capillary-to-myocyte ratio is comparable in normal or ischemic limbs, injected with control or VEGF-B_{167} overexpressing myoblasts. Scale bars: 50 μm.

cularization in $PlGF^{-/-}$ mice.[22] When analyzing in the adductor muscle the number and lumen size of preexisting collateral vessels, we found, again, no genotypic differences at 7 days after ischemia (Table; supplemental Figure IVE and IVF). Macrophage recruitment around the collaterals (supplemental Figure IVG through IVI) as well as in vitro macrophage activation were also unaffected by, respectively, loss or addition of VEGF-B (supplemental Figure IVG through IVI). Thus, endogenous VEGF-B is redundant for the revascularization of ischemic limbs.

VEGF-B_{167} Therapy Does Not Enhance Revascularization in Ischemic Limbs

As our findings above do not exclude the possibility that overexpression of VEGF-B_{167} might enhance collateral growth or capillary angiogenesis in ischemic limbs, as suggested by others,[15,16] we overexpressed VEGF-B_{167} in ischemic hindlimbs of WT mice using various established methods. To analyze the effect of VEGF-B_{167} overexpression on both collateral growth and capillary angiogenesis in the ischemic limb, we intravenously injected the adenoviral vector Ad.hVEGF-B_{167}, similar as done for our MI experiments and resulting in increased VEGF-B plasma levels (Note II, please see supplemental materials). At 7 days after ligation, Ad.hVEGF-B_{167}, gene transfer did not improve angiogenesis in the gastrocnemius muscle (Table; Figure 3A and 3B) and failed to increase the number and size of preexisting collateral vessels in the adductor muscle (Table). VEGF-B_{167} therapy did not stimulate infiltration of macrophages (numbers around the main collateral vessel: 14 ± 2 after Ad.RR5 versus 12 ± 1 after Ad.hVEGF-B_{167} gene transfer, n=5; P=NS), and failed to improve limb perfusion (Table). Also, VEGF-B_{167} therapy failed to stimulate ischemic limb revascularization at later time points, thus excluding a delayed effect (Note III, please see supplemental materials).

To further assess any possible effect of VEGF-B on ischemic limb revascularization (in particular capillary angiogenesis), we implanted VEGF-B_{167}–expressing mouse myoblasts into the anterior tibialis muscles (supplemental Figure I). In healthy (nonligated) muscle, no vessel growth was induced at the engraftment site of VEGF-B_{167}–expressing myoblasts, as compared to control $LacZ^{+}$ myoblasts (Figure 3E). To assess whether VEGF-B_{167} affects vessel growth in ischemic skeletal muscle, myoblasts were implanted immediately after ligation of the femoral artery. Capillary density was increased in the ischemic anterior tibialis muscle at 28 days postligation, but implantation of VEGF-B_{167}–expressing myoblasts did not further enhance angiogenesis (Figure 3C through 3E). To exclude a transient effect on vessel growth, legs were also harvested 7 days after ischemia induction and myoblast implantation, again not resulting in increased vessel densities (not shown).

In addition, in vivo electroporation of a plasmid expressing murine VEGF-B_{167} (pmVEGF-B_{167}) in the adductor muscle (to analyze collateral growth) was ineffective (Note IV, please see supplemental materials; Table). Overall, using various established strategies, VEGF-B_{167} therapy failed to stimulate revascularization of ischemic limbs.

Upregulation of VEGF-B_{167}, but not of Flt-1, in the Ischemic Myocardium

In an effort to obtain some insight in the cardio-restricted properties of VEGF-B, we analyzed, by ELISA, the levels of VEGF-B in cardiac and skeletal muscle of WT mice. Compared to skeletal muscle, the heart expressed higher levels of VEGF-B in baseline conditions (pg/mg protein: 272 ± 8 in heart versus 174 ± 6 in skeletal muscle; n=6; $P<0.05$). At 4 days after MI, levels of VEGF-B were increased by 1.8 ± 0.3 -fold and 2.0 ± 0.3-fold in the infarct and infarct border zone, respectively (n=6; $P<0.05$). At 7 days, the fold upregulation of VEGF-B was 1.4 ± 0.1 and 1.5 ± 0.1, in the respective areas (n=3; P=NS). By contrast, at 4 and 7 days after limb ischemia, VEGF-B protein expression did not increase in the ischemic skeletal muscle (fold increase: 0.9 ± 0.1 at day 4 and 0.8 ± 0.1 at day 7; n=3 to 6; P=NS). This cardio-restricted upregulation of VEGF-B expression was specific, as the PlGF levels were increased in both ischemic myocardium and limb (not shown). Thus, VEGF-B expression is upregulated in ischemic hearts only, consistent with the cardio-restricted phenotype of VEGF-$B^{-/-}$ mice (for discussion, see below).

To analyze the expression of Flt-1, we performed RT-PCR using specific primers and found more abundant Flt-1 transcripts in the heart than skeletal muscle in baseline conditions (trancript levels of Flt-1 per 1000 copies of GADPH: 1.29 ± 0.11 in the myocardium versus 0.40 ± 0.04 in skeletal muscle, n=5; $P<0.05$). However, Flt-1 mRNA expression did not increase in the heart or skeletal muscle at 4 and 7 days after induction of ischemia (not shown). To identify the cell types expressing Flt-1 in vivo, we subjected LacZ-tagged Flt-1 reporter mice to MI or limb ischemia. Immunostaining

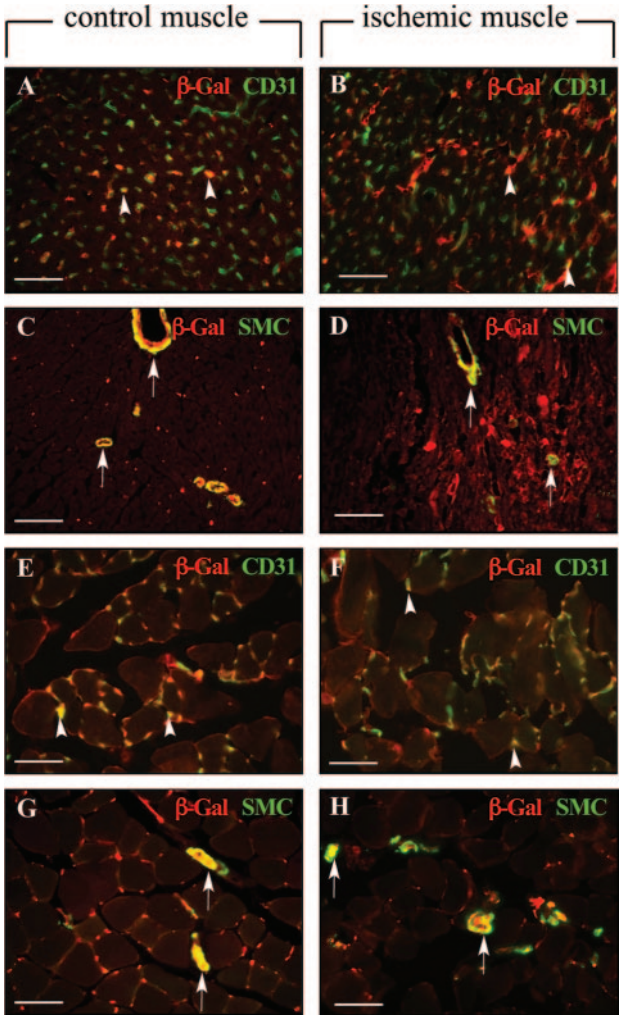

Figure 4. A-H, Double immunostaining of β-Gal and CD31 (A, B, E, F) or SMA (C, D, G, H) in normal and ischemic heart (A–D) or skeletal muscle (E–H) sections of LacZ-tagged Flt-1 mice revealed labeling of Flt-1 expressing cells in most $CD31^+$ cells (arrowheads) and in all SMA^+ cells (arrows). Scale bars: 50 μm.

for β-Gal revealed a similar labeling pattern for Flt-1 in both heart and skeletal muscle in ischemic as well as in nonischemic conditions. Indeed, double immuno-labeling studies showed expression of Flt-1 in SMA^+ SMCs and most $CD31^+$ ECs of nonischemic (Figure 4A, 4C, 4E, and 4G) and ischemic heart or limb tissue at 4 (Figure 4B, 4D, 4F, and 4H) and 7 days (not shown) after induction of ischemia. In the ischemic heart and skeletal muscle, Flt-1 expression also colocalized with some of the $CD45^+$ infiltrating leukocytes (not shown). Thus, the cardio-restricted activity of VEGF-B is unlikely explained by differences in Flt-1 expression.

Discussion

We studied loss- and gain-of-function of VEGF-B in pathological angiogenesis by using various genetic and experimental approaches. The principal finding is that VEGF-B has a relatively restricted role in pathological angiogenesis and, even more remarkably, predominantly in the ischemic myocardium.

The precise role and therapeutic potential of VEGF-B in the revascularization of the ischemic myocardium in vivo has not been studied thus far. By stimulating the ingrowth of new vessels in the ischemic infarct borders, VEGF-B resembles other angiogenic factors such as VEGF and PlGF (and others), which have, quantitatively and qualitatively, similar effects in rodent models of MI (reviewed in[4]). VEGF-B differs, however, from these agents in its selectivity to stimulate angiogenesis primarily in ischemic myocardium and not in other tissues. These loss- and gain-of-function findings in the mouse, together with findings that VEGF-B promotes compensatory hypertrophy of the remote myocardium after myocardial infarction,[17] warrant further consideration of the therapeutic potential of VEGF-B for promoting functional recovery of MI.

In contrast to its role in the ischemic heart, various types of under- and overexpression studies indicate that VEGF-B is dispensable for vessel growth in the skin, lung, retina, and particularly the ischemic limb. Indeed, although others reported a minor role of VEGF-B in skin, retina, or lung,[11–14] the negligible role of VEGF-B in ischemic limbs was unexpected, given that VEGF-B stimulates revascularization of the ischemic heart, another type of muscle. To confirm that the lack of an effect of VEGF-B was not attributable to a particular experimental condition, we used different complementary, nonoverlapping strategies. First, loss of VEGF-B did not impair revascularization of ischemic limbs, neither did it aggravate the revascularization defect in $PlGF^{-/-}$ mice, indicating that VEGF-B was ineffective alone and as a modifier of its homologue PlGF. Gain-of-function of VEGF-B, achieved via various strategies, also failed to stimulate the revascularization of ischemic limbs. This failure was not attributable to insufficient expression, as injection of the same dose of a VEGF-B expressing adenovirus or implantation of the same engineered VEGF-B myoblasts sufficed to stimulate vessel growth in the ischemic heart. Also, each of these strategies have previously been used in ischemic limbs to demonstrate the angiogenic activity of other well-known angiogenic factors, such as VEGF and PlGF ([21,23]; unpublished Tjwa M and Carmeliet P, 2008). In contrast to our findings, others reported that $VEGF\text{-}B_{167}$ promotes ischemic limb revascularization.[15,16] The precise explanation for this discrepancy remains unknown, and might be attributable to experimental differences in gene transfer method, ischemic limb model or methods of analysis.

A remarkable observation is that VEGF-B has angiogenic properties, which substantially differ from those of its homologues VEGF and PlGF. Of all angiogenic members of the VEGF family, VEGF is the most widely active, affecting angiogenesis in health and disease.[1,4] PlGF, by contrast, is redundant for embryonic vascularization and vessel maintenance in healthy conditions, but involved in the angiogenic switch in disease conditions.[21,22] VEGF-B is not only dispensable in development and health, but also in most conditions of pathological angiogenesis, except for the ischemic heart. The selectivity and specificity of each of these VEGF family members is remarkable, when comparing it, for instance, to the largely redundant and overlapping angiogenic activity of the 24 FGF family members.[24] Though beyond the

scope of the present study, it remains outstanding why VEGF-B has such a restricted angiogenic activity in the ischemic heart. The lack of its angiogenic activity in noncardiac tissues cannot be simply attributed to absent expression of VEGF-B or its receptor Flt-1, because both are constitutively expressed in these tissues, including in skeletal muscle.[7,21,22] Interestingly, however, VEGF-B levels were upregulated by ischemia in the ischemic heart but not in the ischemic muscle, which might possibly explain, at least in part, why angiogenesis was impaired in VEGF-B$^{-/-}$ mice in the ischemic myocardium, but not in the ischemic limb. Another possible mechanism might be that the endothelial cells in the heart have distinct tissue-specific characteristics as compared to endothelial cells in other tissues, similar to the distinct differentiation properties of endothelial cells in the nervous system, endocrine organs, lymphoid tissue, etc.[25] It is indeed known that coronary endothelial cells are derived from unique progenitors, ie, the epicardium-derived progenitor cells, and require distinct angiogenic signals (such as thymosin-β4).[26] Perhaps, also, the intracellular signaling pathway, induced by VEGF-B, is distinct in these coronary endothelial cells. Because VEGF and PlGF transmit specific angiogenic signals through Flt-1,[5] VEGF-B could also have a different angiogenic activity profile. Further hypothetical explanations for the selective angiogenic activity of VEGF-B in the heart might include differences in the composition of extracellular matrix proteins (which modulate the activity of VEGF-B[27]), or in a role for Neuropilin-1, another VEGF-B receptor (reviewed in[6]).

Regardless of the mechanisms, few other tissue-selective angiogenic molecules have been identified thus far, including EG-VEGF, BDNF, and others.[1] Such tissue-specific angiogenic signals are medically relevant, as they offer attractive therapeutic opportunities. Indeed, the potent activity of VEGF and its associated risk of adverse effects (bleeding, leakage, hypotension, malignancy, etc), for instance, has precluded systemic administration of this angiogenic agent for the revascularization of ischemic tissues.[1,4] In contrast, a molecule such as VEGF-B, which would more selectively stimulate vessel growth in the heart, and primarily in ischemic conditions, would be expected to stimulate vessels in the specific target organ without causing adverse effects. Our observations that VEGF-B did not cause any noticeable side effects of general toxicity, hemorrhage, edema, or hypotension (not shown) are consistent with such a model. Overall, these mouse genetic findings support a—therapeutically attractive—model whereby VEGF-B selectively promotes angiogenesis in the ischemic myocardium.

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Disclosures

None.

References

1. Ferrara N, Kerbel RS. Angiogenesis as a therapeutic target. *Nature*. 2005; 438:967–974.
2. Grimmond S, Lagercrantz J, Drinkwater C, Silins G, Townson S, Pollock P, Gotley D, Carson E, Rakar S, Nordenskjold M, Ward L, Hayward NK, Weber G. Cloning and characterization of a novel human gene related to vascular endothelial growth factor. *Genome Research*. 1996;6:124–131.
3. Olofsson B, Pajusola K, Kaipainen A, von Euler G, Joukov V, Saksela O, Orpana A, Pettersson R, Alitalo K, Eriksson U. Vascular endothelial growth factor B, a novel growth factor for endothelial cells. *Proc Natl Acad Sci USA*. 1996;93:2576–2581.
4. Carmeliet P. Angiogenesis in life, disease and medicine. *Nature*. 2005; 438:932–936.
5. Tjwa M, Luttun A, Autiero M, Carmeliet P. VEGF and PlGF: two pleiotropic growth factors with distinct roles in development and homeostasis. *Cell Tissue Res*. 2003;314:5–14.
6. Nash AD, Baca M, Wright C, Scotney PD. The biology of vascular endothelial growth factor-B (VEGF-B). *Pulm Pharmacol Ther*. 2006;19: 61–69.
7. Li X, Aase K, Li H, von Euler G, Eriksson U. Isoform-specific expression of VEGF-B in normal tissues and tumors. *Growth Factors*. 2001;19: 49–59.
8. Olofsson B, Korpelainen E, Pepper MS, Mandriota SJ, Aase K, Kumar V, Gunji Y, Jeltsch MM, Shibuya M, Alitalo K, Eriksson U. Vascular endothelial growth factor B (VEGF-B) binds to VEGF receptor-1 and regulates plasminogen activator activity in endothelial cells. *Proc Natl Acad Sci USA*. 1998;95:11709–11714.
9. Aase K, von Euler G, Li X, Ponten A, Thoren P, Cao R, Cao Y, Olofsson B, Gebre-Medhin S, Pekny M, Alitalo K, Betsholtz C, Eriksson U. Vascular endothelial growth factor-B-deficient mice display an atrial conduction defect. *Circulation*. 2001;104:358–364.
10. Bellomo D, Headrick JP, Silins GU, Paterson CA, Thomas PS, Gartside M, Mould A, Cahill MM, Tonks ID, Grimmond SM, Townson S, Wells C, Little M, Cummings MC, Hayward NK, Kay GF. Mice lacking the vascular endothelial growth factor-B gene (Vegfb) have smaller hearts, dysfunctional coronary vasculature, and impaired recovery from cardiac ischemia. *Circ Res*. 2000;86:E29–E35.
11. Reichelt M, Shi S, Hayes M, Kay G, Batch J, Gole GA, Browning J. Vascular endothelial growth factor-B and retinal vascular development in the mouse. *Clin Experiment Ophthalmol*. 2003;31:61–65.
12. Wanstall JC, Gambino A, Jeffery TK, Cahill MM, Bellomo D, Hayward NK, Kay GF. Vascular endothelial growth factor-B-deficient mice show impaired development of hypoxic pulmonary hypertension. *Cardiovasc Res*. 2002;55:361–368.
13. Louzier V, Raffestin B, Leroux A, Branellec D, Caillaud JM, Levame M, Eddahibi S, Adnot S. Role of VEGF-B in the lung during development of chronic hypoxic pulmonary hypertension. *Am J Physiol Lung Cell Mol Physiol*. 2003;284:L926–L937.
14. Mould AW, Greco SA, Cahill MM, Tonks ID, Bellomo D, Patterson C, Zournazi A, Nash A, Scotney P, Hayward NK, Kay GF. Transgenic overexpression of vascular endothelial growth factor-B isoforms by endothelial cells potentiates postnatal vessel growth in vivo and in vitro. *Circ Res*. 2005;97:e60–e70.
15. Wright CE. Effects of vascular endothelial growth factor (VEGF)A and VEGFB gene transfer on vascular reserve in a conscious rabbit hindlimb ischaemia model. *Clin Exp Pharmacol Physiol*. 2002;29:1035–1039.
16. Silvestre JS, Tamarat R, Ebrahimian TG, Le-Roux A, Clergue M, Emmanuel F, Duriez M, Schwartz B, Branellec D, Levy BI. Vascular endothelial growth factor-B promotes in vivo angiogenesis. *Circ Res*. 2003;93:114–123.
17. Tirziu D, Chorianopoulos E, Moodie KL, Palac RT, Zhuang ZW, Tjwa M, Roncal C, Eriksson U, Fu Q, Elfenbein A, Hall AE, Carmeliet P, Moons L, Simons M. Myocardial hypertrophy in the absence of external stimuli is induced by angiogenesis in mice. *J Clin Invest*. 2007;117: 3188–3197.
18. Bhardwaj S, Roy H, Gruchala M, Viita H, Kholova I, Kokina I, Achen MG, Stacker SA, Hedman M, Alitalo K, Yla-Herttuala S. Angiogenic responses of vascular endothelial growth factors in periadventitial tissue. *Hum Gene Ther*. 2003;14:1451–1462.
19. Rissanen TT, Markkanen JE, Gruchala M, Heikura T, Puranen A, Kettunen MI, Kholova I, Kauppinen RA, Achen MG, Stacker SA, Alitalo

K, Yla-Herttuala S. VEGF-D is the strongest angiogenic and lymphangiogenic effector among VEGFs delivered into skeletal muscle via adenoviruses. *Circ Res*. 2003;92:1098–1106.
20. Fong GH, Klingensmith J, Wood CR, Rossant J, Breitman ML. Regulation of flt-1 expression during mouse embryogenesis suggests a role in the establishment of vascular endothelium. *Dev Dyn*. 1996;207:1–10.
21. Luttun A, Tjwa M, Moons L, Wu Y, Angelillo-Scherrer A, Liao F, Nagy JA, Hooper A, Priller J, De Klerck B, Compernolle V, Daci E, Bohlen P, Dewerchin M, Herbert JM, Fava R, Matthys P, Carmeliet G, Collen D, Dvorak HF, Hicklin DJ, Carmeliet P. Revascularization of ischemic tissues by PlGF treatment, and inhibition of tumor angiogenesis, arthritis and atherosclerosis by anti-Flt1. *Nat Med*. 2002;8:831–840.
22. Carmeliet P, Moons L, Luttun A, Vincenti V, Compernolle V, De Mol M, Wu Y, Bono F, Devy L, Beck H, Scholz D, Acker T, DiPalma T, Dewerchin M, Noel A, Stalmans I, Barra A, Blacher S, Vandendriessche T, Ponten A, Eriksson U, Plate KH, Foidart JM, Schaper W, Charnock-Jones DS, Hicklin DJ, Herbert JM, Collen D, Persico MG. Synergism between vascular endothelial growth factor and placental growth factor contributes to angiogenesis and plasma extravasation in pathological conditions. *Nat Med*. 2001;7:575–583.
23. von Degenfeld G, Banfi A, Springer ML, Wagner RA, Jacobi J, Ozawa CR, Merchant MJ, Cooke JP, Blau HM. Microenvironmental VEGF distribution is critical for stable and functional vessel growth in ischemia. *Faseb J*. 2006;20:2657–2659.
24. Rusnati M, Presta M. Fibroblast growth factors/fibroblast growth factor receptors as targets for the development of anti-angiogenesis strategies. *Curr Pharm Des*. 2007;13:2025–2044.
25. Aird WC. Phenotypic heterogeneity of the endothelium: I. Structure, function, and mechanisms. *Circ Res*. 2007;100:158–173.
26. Smart N, Rossdeutsch A, Riley PR. Thymosin beta4 and angiogenesis: modes of action and therapeutic potential. *Angiogenesis*. 2007;10:229–241.
27. Ikuta T, Ariga H, Matsumoto K. Extracellular matrix tenascin-X in combination with vascular endothelial growth factor B enhances endothelial cell proliferation. *Genes Cells*. 2000;5:913–927.

SUPPLEMENTAL INFORMATION

SUPPLEMENTAL NOTES

Note I: Osmotic minipumps, delivering either 1.5 µg/day recombinant mouse VEGF-B_{167} protein (rmVEGF-B_{167}, a kind gift from Dr. A. Nash (Amrad Corporation, Melbourne, Australia) or saline vehicle, were implanted subcutaneously in WT mice, subjected to myocardial infarction, and the blood plasma levels of mVEGF-B_{167} were measured at 4 days after pump implantation by using a modified version of a previously established home-made ELISA [1]. This mouse VEGF-B_{167} ELISA is more sensitive compared to the human VEGF-B_{167} ELISA, and we therefore chose to analyze the biodistribution of mVEGF-B_{167}. Administration of rmVEGF-B_{167} via osmotic minipumps increased the blood plasma levels of mVEGF-B_{167} from below the detection threshold of the ELISA (<0.13 ng/ml) after saline treatment towards 1.5 ± 0.2 ng/ml after rmVEGF-B_{167} therapy (N=4; P<0.05), indicating that our delivery strategy indeed increased the circulating levels of mVEGF-B_{167}. We were unable to find increased levels of mVEGF-B_{167} in heart extracts (not shown), likely due to the high endogenous levels of mVEGF-B_{167} in the heart [2].

Note II: Systemic adenoviral VEGF-B_{167} gene delivery is known to transduce hepatocytes that then release the transgene product. Adenoviral vectors encoding hVEGF-B_{167} (Ad.hVEGF-B_{167}) or control vectors (Ad.RR5) were injected intravenously at $3x10^9$ p.f.u. per mouse, immediately after ligation of the LAD or femoral artery. In mice, treated with a control adenovirus (Ad.RR5) [3], hVEGF-B plasma levels were below the threshold of detection (<0.50 ng/ml). However, after intravenous injection of Ad.hVEGF-B_{167}, at the time of LAD ligation, circulating hVEGF-B_{167} levels were as high as 5 ng/ml at 7 days post-MI (*N*=10; *P*<0.05). In addition, after ligation of the femoral artery, the circulating levels of hVEGF-B_{167} increased for periods up to 3 weeks (ng/ml plasma: 4.1 ± 0.6, 4.7 ± 0.4, 2.6 ± 0.9, 1.8 ± 0.5 ng/ml at 3, 7, 14 and 21 days post-ligation; *N*=8; *P*<0.05 *versus* undetectable levels after Ad.RR5 at all days), while by 28 days post-ligation, hVEGF-B_{167} levels reached the detection threshold of the assay.

Note III: A delayed effect of Ad.hVEGF-B_{167} gene transfer on revascularization of ischemic limbs was excluded since, also at 28 days after ischemia, VEGF-B_{167} therapy failed to improve total limb perfusion, angiogenesis in the gastrocnemius muscle (capillary-to-myocyte ratio: 1.71 ± 0.07 after Ad.RR5 *versus* 1.77 ± 0.14 after Ad.hVEGF-B_{167}; *N*=5;

P=NS), and size and number of collateral vessels in the adductor muscle (lumen area in μm^2: main collateral, 2^{nd} and 3^{rd} collateral branch: 2,040 ± 470, 785 ± 54 and 92 ± 14 after Ad.RR5 *versus* 2,390 ± 530, 678 ± 125 and 97 ± 16 after Ad.hVEGF-B_{167}; number of collateral side branches per mm^2, 2^{nd} and 3^{rd} collateral branch: 3 ± 0.4 and 12 ± 2.4 after Ad.RR5 *versus* 3 ± 0.4 and 8 ± 0.9 after Ad.hVEGF-B_{167}; total perfusion area in $\mu m^2/mm^2$: 3,020 ± 190 after Ad.RR5 *versus* 2,940 ± 620 after Ad.hVEGF-B_{167}; *N*=5; *P*=NS).

Note IV: To selectively analyze the effects on collateral growth, we locally delivered VEGF-B_{167} in the adductor muscle via *in vivo* electroporation of a plasmid expressing murine VEGF-B_{167} (pmVEGF-B_{167}) – a technique enabling stable overexpression of a transgene for several weeks starting from 1 day after electroporation [4, 5] – we achieved a 6-fold increase in the expression levels of mVEGF-B in the adductor muscle at 8 days after electroporation (pg/mg protein: 651 ± 122 after pmVEGF-B_{167} *versus* 119 ± 15 after empty plasmid; *N*=6; *P*<0.05). One day after electroporation (i.e. when the transgene is already expressed [5]), mice were subjected to limb ischemia and sacrificed 7 days later to analyze collateral growth in the adductor muscle. Compared to control, pmVEGF-B_{167} electroporation failed to increase the number or size of the pre-existing collateral vessels (Table 1), indicating that VEGF-B_{167} therapy did not stimulate collateral vessel growth in the limb. Laser Doppler analysis also failed to show better perfusion of the ischemic limbs after local pmVEGF-B_{167} electroporation (Table 1).

SUPPLEMENTAL TABLES

Table I: Negligible role of VEGF-B in pulmonary vessel remodeling after hypoxia

	WT mice		**VEGF-B$^{-/-}$ mice**	
	normoxia	**hypoxia**	**Normoxia**	**hypoxia**
Vessel remodeling (per 10^3 alveoli)				
Non-muscularized vessels	6.8 ± 0.4	3.4 ± 0.01*	7.6 ± 0.4	3.8 ± 0.3*
Partially-muscularized vessels	3.0 ± 0.1	4.5 ± 0.3*	3.8 ± 0.4	4.4 ± 0.4
Muscularized vessels	1.0 ± 0.3	4.6 ± 0.9*	1.1 ± 0.2	5.2 ± 0.2*
SMA$^-$ vessels	9.3 ± 0.7	4.9 ± 0.1*	10.3 ± 0.3	4.6 ± 1.2*
SMA$^{\pm}$ vessels	1.5 ± 0.2	4.3 ± 0.8*	2.6 ± 0.3	4.5 ± 0.2*
SMA$^+$ vessels	0.1 ± 0.1	2.7 ± 0.6*	0.1 ± 0.1	2.0 ± 0.6*
Arterial media thickness (μm)	1.53 ± 0.1	2.01 ± 0.08*	1.50 ± 0.07	2.15 ± 0.12*
RV hypertrophy [RV/(LV+S)]	0.20 ± 0.01	0.26 ± 0.01*	0.20 ± 0.02	0.25 ± 0.01*
Hematocrit (%)	32 ± 2.5	43 ± 1.4*	35 ± 0.7	45 ± 2.6*

Pulmonary vessel remodeling, induced by chronic exposure to hypoxia and resulting in pulmonary hypertension, was examined in WT and VEGF-B$^{-/-}$ mice. The density of pulmonary vessels was determined, while discriminating between non-muscularized vessels (with only an internal elastic membrane (IEL)), partially-muscularized vessels (IEL and partial external elastic membrane (EEL)) and muscularized vessels (complete IEL and EEL). To evaluate right ventricle (RV) hypertrophy, the dry weight of the RV was measured and divided by the total dry weight of the left ventricle plus septum (LV+S). Values are means ± SEM. * P<0.05 *versus* normoxia; P=NS for WT mice *versus* VEGF-B$^{-/-}$ mice.

SUPPLEMENTAL FIGURE LEGENDS

Figure I: As an alternative method to deliver VEGF-B to cardiomyocytes, we implanted in the myocardial wall of the infarct border, mouse myoblasts, transduced *ex vivo* with a retrovirus, to constitutively produce mouse VEGF-B_{167}, since a similar strategy using VEGF-A-expressing myoblasts was previously shown to stimulate vessel growth in muscle [6-8]. ***A,B,*** Immunofluorescent staining for VEGF-B (green) confirmed the absence of VEGF-B in control myoblasts expressing only LacZ (*A*) and production of mVEGF-B_{167} in myoblasts expressing both VEGF-B and LacZ (*B*). Nuclei are counterstained with Dapi (blue). The production of mVEGF-B_{167} by engineered myoblasts in vivo was further confirmed by determining mVEGF-B expression levels in cell lysates and muscle extracts. Indeed, myoblasts, transduced with a retrovirus encoding mVEGF-B_{167}, secreted 26 ± 2 ng/10^6 cells/24h of VEGF-B, while control LacZ-expressing myoblasts failed to express detectable amounts of VEGF-B. At 3 days after implantation of myoblasts into the gastrocnemius muscle, increased amounts of VEGF-B protein were found in the muscles implanted with the VEGF-B_{167}-expressing myoblasts (299 ± 67 pg/mg protein *versus* 22 ± 2 pg/mg protein in control muscles; N=6; P<0.05). Further in vitro experiments revealed that the secreted VEGF-B protein was bio-active (not shown). ***C,D,*** Double immunofluorescent staining for ß-galactosidase (site of myoblast engraftment) and CD31 (blood vessels) in the ischemic myocardium revealed no signs of vessel growth around the implantation site of control myoblasts (*C*), but a robust angiogenic induction around the site of VEGF-B_{167}-expressing myoblast implantation (*D*). Thus, intramyocardial implantation of VEGF-B_{167}-expressing myoblasts enhanced vessel growth in the ischemic myocardium at 28 days post-MI, while control $LacZ^+$ myoblasts failed to promote angiogenesis. Scale bars: 50 µm.

Figure II: We showed previously that adenoviral gene transfer of PlGF into the skin of ears enlarged pre-existing vessels with subsequent stabilization by acquisition of a pericyte coat [9]. We therefore injected adenoviruses, expressing hVEGF-B_{167} (Ad.hVEGF-B_{167}), hVEGF-B_{186} (Ad.hVEGF-B_{186}) or mPlGF-2 (Ad.mPlGF) intradermally into the ear skin**.** ***A-I,*** Ears were whole-mount immunostained for CD31 (endothelial cells; brown) in panels *A,D,G*, for CD31 (green) and SMA (smooth muscle cells; red) in panels *B,E,H*, and for F4/80 (macrophages, green) and SMA (red) in panels *C,F,I*. As expected, gene transfer of mPlGF-2 increased the density, tortuosity and size of pre-existing vessels, and

stimulated their coverage by mural cells (*A,B*). In contrast, Ad.hVEGF-B_{167} or Ad.hVEGF-B_{186} gene transfer minimally enlarged the pre-existing vessels without increase in number, tortuosity or mural cell coverage (*D,E,G,H*). Consistent herewith, we found that many F4/80+ macrophages had infiltrated after Ad.mPlGF gene transfer, while only few macrophages were present after Ad.hVEGF-B_{167} or Ad.hVEGF-B_{186} gene transfer (*C,F,I*). A control virus (Ad.CMV) failed to affect any of these parameters (not shown). Scale bars: 100 µm in panels *A,B,D,E,G,H* and 50 µm in *C,F,I*.

Figure III: ***A-D,*** To further analyze the effects of VEGF-B on vessel remodeling and recruitment of mural cells, we examined in VEGF-$B^{-/-}$ mice the remodeling of pulmonary vessels in response to chronic hypoxia [10]. Hart's elastin staining revealed no genotypic differences between WT and VEGF-$B^{-/-}$ mice in pulmonary vessel structure in normoxia (*A,B*; see also Table S1). In WT mice, continuous hypoxic conditions for 4 weeks increased the number of thick-walled muscularized vessels by ~4.6-fold (*C*; Table S1). A comparable 4.7-fold increase in the number of thick-walled muscularized vessels was observed in VEGF-$B^{-/-}$ mice (*D*; Table S1), suggesting that loss of VEGF-B did not impair mural cell recruitment. Similar results were obtained when hypoxic vessel remodeling was analyzed by immunostaining for SMA (Table S1). Consistent herewith, no genotypic differences were observed in the development of right ventricle (RV) hypertrophy, which normally is caused by pulmonary hypertension (Table S1). Compared to WT mice, VEGF-$B^{-/-}$ mice were as sensitive to chronic hypoxia, as evidenced by the similar increase in hematocrit levels (Table S1). The lack of an effect of VEGF-B on pulmonary hypertension is consistent with earlier findings by Louzier *et al*. [11]. ***E,F,*** To further analyze the effects of VEGF-B on angiogenesis, we studied in VEGF-$B^{-/-}$ mice retinal neovascularization in response to ischemia, using an established model [10]. Neovascularization of the ischemic retina, analyzed by H&E staining on cross-sections, was comparable in WT (*E*) and VEGF-$B^{-/-}$ mice (*F*; arrows indicate neovessels). Indeed, compared to WT mice, loss of VEGF-B failed to reduce the number of endothelial cells, forming new intravitreal vessel sprouts (per retinal cross-section: 102 ± 13 in WT mice *versus* 118 ± 10 in VEGF-$B^{-/-}$ mice; *N*=5; *P*=NS) or the number of neovascular tufts (per retinal cross-section: 42 ± 4 in WT mice *versus* 50 ± 8 in VEGF-$B^{-/-}$ mice; *N*=5; *P*=NS; Figure S3*E,F*). These findings are consistent with findings by Reichelt *et al*. [12]. Scale bars: 50 µm in panels *E,F* and 25 µm in panels *A-D.*

Figure IV: ***A-D,*** At 7 days after ligation, laser Doppler perfusion analysis on ischemic limbs also failed to show any genotypic differences (*A,B*; see also Table 1). Ischemic limb perfusion was also measured in mice lacking both VEGF-B and PlGF – the rationale for this experiment being that limb perfusion was reduced in $PlGF^{-/-}$ mice [10] and that, possibly, the consequences of VEGF-B deficiency might be more apparent when limb revascularization was already impaired by prior loss of PlGF. However, at 7 days after ischemia, laser Doppler imaging revealed that, compared to WT or $VEGF\text{-}B^{-/-}$ mice (Table 1; $P<0.05$), limb perfusion was comparably reduced in mice lacking PlGF alone or in mice lacking both VEGF-B and PlGF (*C,D*; % perfusion of non-ligated limb: 47 ± 5% in $PlGF^{-/-}$ mice *versus* 55 ± 9% in $VEGF\text{-}B^{-/-}$:$PlGF^{-/-}$ mice; $N=5$; *P*=NS). Please note the color scale: from blue (low perfusion) to red (high perfusion). ***E,F,*** H&E staining of transverse sections through the adductor muscles revealed a comparable size of the main collateral vessel in WT (*E*) and $VEGF\text{-}B^{-/-}$ mice (*F*). ***G-I,*** Morphometric analysis revealed a similar amount of $F4/80^{+}$ macrophages around the collaterals in WT and $VEGF\text{-}B^{-/-}$ adductor muscles (*G*), as also illustrated by microscopic pictures (*H,I*). In addition, VEGF-B failed to stimulate the production by cultured macrophages of TNF-alpha, previously implicated in collateral growth [9] (data not shown). Please note that the black staining inside the vessels results from bismuth-gelatin filling of the vessels, which enables a better macroscopic visualization of the collaterals. Scale bars: 50 µm.

SUPPLEMENTAL METHODS

Animal models

Myocardial ischemia model: Myocardial ischemia was induced by ligation of the left anterior descending (LAD) coronary artery in female mice as described [3, 9]. Seven days after LAD ligation, hearts were harvested, sectioned and analysed for vessel densities and/or macrophage infiltration in the infarct area and border zone.

Mouse skin wound model: A standardized 15 mm full-thickness skin incision was made on the back of mice, taking care not to damage the underlying muscle, as described [10]. Wound healing was quantified by daily measuring the width and the length of the wound. Analysis of vessel densities and inflammation was performed in skin sections, harvested 5 days after wounding.

Mouse ear skin assay: Mouse ears, injected with the PlGF or VEGF-B adenoviral vectors, were dissected, fixed in 1% phosphate buffered paraformaldehyde and whole-mount immunostained for endothelial cells, smooth muscle cells and macrophages using fluorescently conjugated secondary antibodies (Alexa 488 or 546, Molecular Probes).

Mouse ischemic retinopathy model: Mice at postnatal day 7 were exposed to hyperbaric (80%) oxygen for 5 days, as described [10]. After returning to normoxia for another 5 days, the eyes were harvested, fixed in 1% paraformaldehyde, paraffin embedded and sectioned. After H&E staining, the number of endothelial cells and vascular tufts in the vitreous cavity were counted, as described [10, 13].

Pulmonary vascular remodeling: Eight-weeks old male mice were placed in a chamber under normobaric hypoxia (10% O_2) or in normal air (21% O_2; control) as described [14]. After 4 weeks, the right ventricular (RV) wall was dissected from the left ventricle (LV) and septum (S), and dried at 55°C before weighing. Alternatively, lungs were perfusion-fixed, dissected and sectioned as described [14]. Pulmonary vascular remodeling was assessed by counting the number of non-muscularized (only IEL), partially muscularized (IEL plus incomplete EEL) and fully muscularized (IEL and complete EEL) peripheral vessels (located distal to the bronchi) per 10^3 alveoli, as described [14].

Ischemic hindlimb model: In male mice, the right femoral artery and vein (proximal to the popliteal artery) and the cutaneous vessels branching from the caudal femoral artery side branch were ligated, avoiding damage of the femorale nerve [9]. Seven days after ligation, functional perfusion measurements of the total limb were performed using a Lisca PIM II camera (Gambro). Perfusion, averaged over 3 images per mouse in the total hindlimb, was expressed as a ratio of right (ischemic) to left (normal) limb. Limb motor

function was determined via treadmill running exercise (Simplex II, Columbus Instruments), after a one-day conditioning training session [15]. The test protocol included a graded exercise test at constant inclination of 10 degrees with increases in belt speed of 2 m/min every 5 minutes. Exhaustion was defined as failure to abandon the shock grid within 15 seconds. After training, the femoral artery was occluded, and at 7 days later, mice were re-tested on the treadmill. Recovery of function was expressed as a ratio to the baseline exercise time. For histology, the gastrocnemius and adductor muscles were harvested at 7 or 28 days after femoral artery ligation. Vessel densities in the regenerating gastrocnemius muscle were determined morphometrically by analyzing the capillary-to-myocyte ratio. Remodeling of collateral vessels and macrophage recruitment in the upper hindlimb were analyzed as described [9]. In brief, collateral side branches were categorized as smaller or larger than 300 μm^2. Total perfusion area was calculated using the total sum of the side branch luminal areas.

Transplantation of VEGF-B_{167}-expressing myoblasts: A MFG retrovirus encoding mouse VEGF-B_{167} cDNA, generated as described [6], was used to transduce LacZ-expressing myoblasts. LacZ-expressing myoblasts were used for transplantation to visualize the engrafted cells. Specific expression of the VEGF-B gene was verified by immunofluorescent staining of myoblasts using antibody, recognizing mouse VEGF-B_{167} (R&D Systems). The amount of gene product, secreted by the VEGF-B producing myoblasts *in vitro*, was quantified by ELISA. The biological activity of medium conditioned by VEGF-B producing myoblasts was compared to medium of VEGF-A expressing cells or control myoblasts, using a HUVEC proliferation assay (Cell Titer Aqueous One Solution Assay, Promega). Myoblasts, co-expressing VEGF-B_{167} and LacZ or control LacZ-myoblasts, were injected into the anterior tibial muscle ($5x10^5$ cells/injection) in normal conditions or immediately after femoral artery ligation and transection in adult male SCID mice, as described [8, 16]. At 28 days following surgery, tibialis and myocardial muscles were harvested and double labeled for endothelial cells and muscle fibers expressing the LacZ reporter gene, using a polyclonal rabbit antibody to LacZ (Eppendorf 5-Prime). Capillaries and muscle fibers were counted and expressed as capillary-to-muscle fiber ratio. Myoblasts were also injected in the infarct border zone ($5x10^5$ cells/injection) of female SCID mice, immediately after LAD ligation, and hearts were harvested for histology at 28 days post-MI.

Histology, immunohistochemistry and morphometric analyses

All tissues were fixed in 1% phosphate buffered paraformaldehyde and embedded in paraffin. Serial parasagittal (hearts) or transverse (skin, retina, lung, limb muscle) sections were cut at 8 µm thickness and stained for H&E or Hart's elastin. Immunostainings were performed using primary antibodies for endothelial cells (rabbit anti-thrombomodulin (TM), gift from Dr. R.W. Jackman, Boston, MA; rat anti-CD31, Becton Dickinson), smooth muscle cells (mouse anti-smooth muscle alpha-actin (SMA), Sigma), macrophages (rat anti-Mac3 and rat anti-F4/80, both Becton Dickinson), and ß-Gal (rabbit anti- ß-Gal, Cappel). Following primary antibody incubation, sections were incubated with peroxidase-labeled IgGs (Dako), followed by amplification with the proper tyramide signal amplification systems (Perkin Elmer, Life Sciences). Morphometric analyses of the vessel densities and macrophage positive areas in the ischemic, granulation or wound border tissues were performed using a Zeiss Axioplan microscope with KS300 image analysis software.

Production and administration of VEGF-B protein, plasmid and adenovirus

The recombinant human VEGF-B167 (rhVEGF- B167) protein was obtained from Amrad Corporation (gift from Dr. A. Nash), and its activity was tested using the Ba/F3 pre-B cell viability assay as described [17] (data not shown). Continuous delivery of 1.5 µg/mouse/day rhVEGF-B167 protein was achieved by subcutaneously implanting osmotic minipumps (Alzet, type 2001), as described [3].

Adenoviruses were constructed by cloning the murine PlGF-2, or the human VEGF-B_{167} or VEGF-B_{186} cDNA into the pACCMVpLpA plasmid, using previously published methods [3, 9]. RNA analysis and immunoblotting of extracts and conditioned medium of cells, transduced with the respective adenovirus, revealed that the transduced cells produced murine PlGF-2, hVEGF-B_{167} or hVEGF-B_{186} protein (not shown). For the ear assays, $1x10^9$ pfu adenovirus (Ad.mPlGF, Ad.hVEGF-B_{167}, Ad.hVEGF-B_{186} or control Ad.CMV) was injected intradermally in the ears of female NMRInu/nu mice. In the ischemic hind limb model, $3x10^9$ pfu adenovirus (Ad.hVEGF-B_{167} or control Ad.RR5) was intravenously injected in the tail vein of male C57BL/6 mice, immediately after ligation of the femoral artery.

A plasmid expressing murine VEGF-B_{167} (pcDNA3.mVEGF-B_{167}) or an empty pcDNA3 plasmid was administered via muscle electroporation as described [4, 5]. Briefly, one day before ligation of the femoral artery, 15 µg of expression plasmid, encoding

mVEGF-B_{167}, or 15 µg empty plasmid (both at 1mg/ml in 0.9% NaCl) was injected into the adductor muscle (total volume of 15 µl over 3 injection sites) using a Hamilton syringe, and electrotransfer (five electric pulses of 100V with a fixed pulse duration of 20 ms and an interval of 200 ms; Electro Square Porator ECM 830, BTX, Harvard Bioscience), with Tweezertrode 520 electrodes (BTX, Harvard Bioscience) was performed.

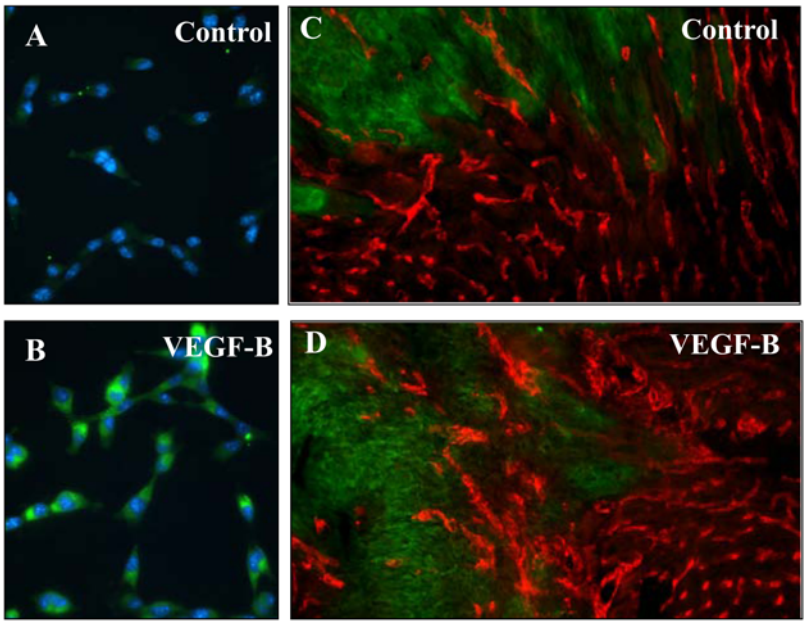
RT-PCR for Flt-1 and ELISA for human and mouse VEGF-B

Quantitative real-time RT-PCR and protein extraction were performed as described [10]. For determining mouse VEGF-B_{167} levels a modified version of a previously established ELISA [1] was used. Plates were coated with a monoclonal antibody to murine VEGF-B (#MAB751, R&D Systems) and mrVEGF-B_{167} (gift from Dr. A. Nash) was used as standard. Human VEGF-B_{167} plasma levels were determined with a home-made ELISA using antibodies (#MAB3372 as capture antibody and #AF751 as detection antibody) and rhVEGF-B_{167} (#751-VE-025) from R&D Systems.

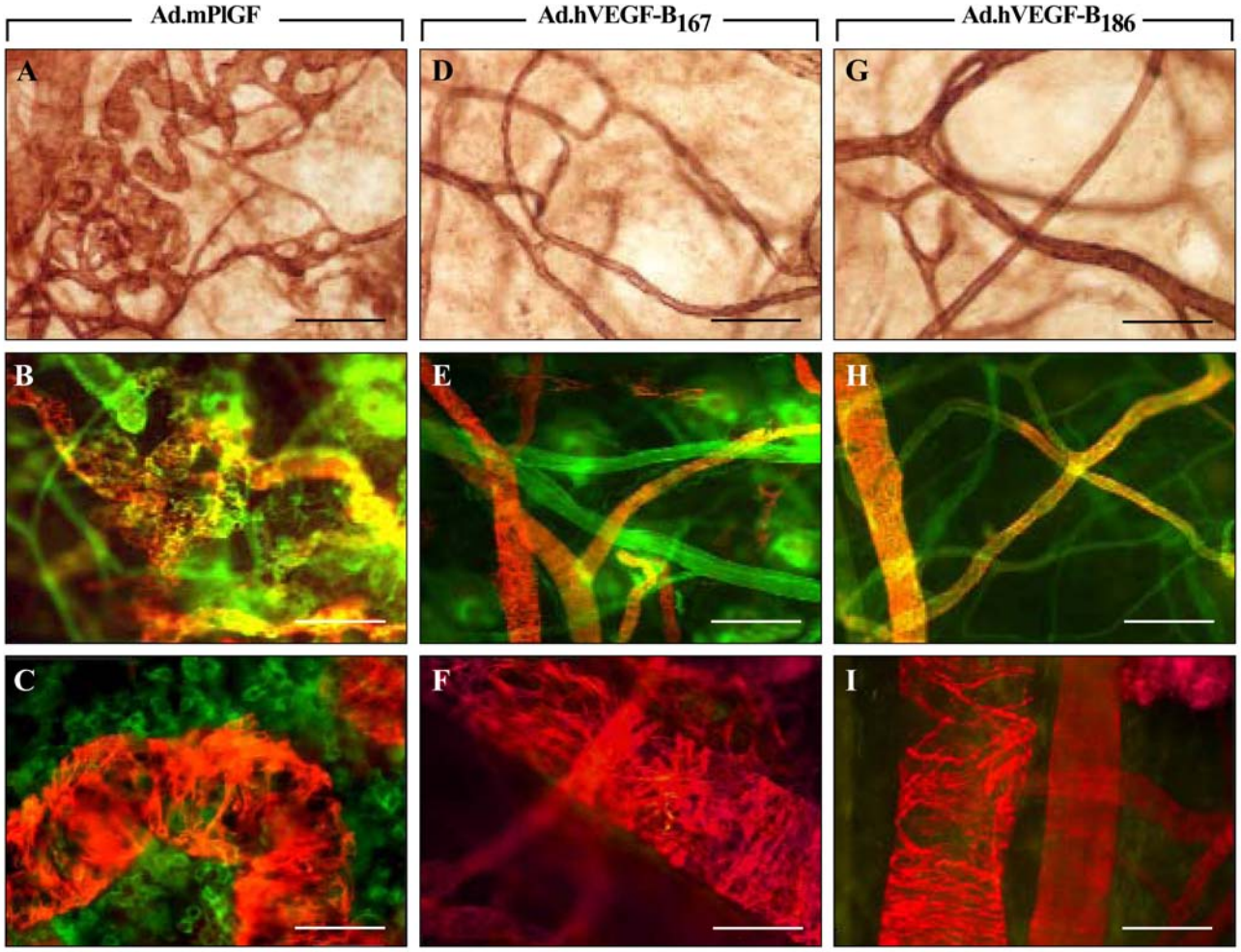
SUPPLEMENTAL REFERENCES

1. Leppanen P, Koota S, Kholova I, Koponen J, Fieber C, Eriksson U, Alitalo K, Yla-Herttuala S. Gene transfers of vascular endothelial growth factor-A, vascular endothelial growth factor-B, vascular endothelial growth factor-C, and vascular endothelial growth factor-D have no effects on atherosclerosis in hypercholesterolemic low-density lipoprotein-receptor/apolipoprotein B48-deficient mice. *Circulation.* 2005;112:1347-1352.
2. Li X, Aase K, Li H, von Euler G, Eriksson U. Isoform-specific expression of VEGF-B in normal tissues and tumors. *Growth Factors.* 2001;19:49-59.
3. Heymans S, Luttun A, Nuyens D, Theilmeier G, Creemers E, Moons L, Dyspersin GD, Cleutjens JP, Shipley M, Angellilo A, Levi M, Nube O, Baker A, Keshet E, Lupu F, Herbert JM, Smits JF, Shapiro SD, Baes M, Borgers M, Collen D, Daemen MJ, Carmeliet P. Inhibition of plasminogen activators or matrix metalloproteinases prevents cardiac rupture but impairs therapeutic angiogenesis and causes cardiac failure [see comments]. *Nat Med.* 1999;5:1135-1142.
4. Jang HS, Kim HJ, Kim JM, Lee YS, Kim KL, Kim JA, Lee JY, Suh W, Choi JH, Jeon ES, Byun J, Kim DK. A novel ex vivo angiogenesis assay based on electroporation-mediated delivery of naked plasmid DNA to skeletal muscle. *Mol Ther.* 2004;9:464-474.
5. Qian HS, Liu P, Huw LY, Orme A, Halks-Miller M, Hill SM, Jin F, Kretschmer P, Blasko E, Cashion L, Szymanski P, Vergona R, Harkins R, Yu J, Sessa WC, Dole WP, Rubanyi GM, Kauser K. Effective treatment of vascular endothelial growth factor refractory hindlimb ischemia by a mutant endothelial nitric oxide synthase gene. *Gene Ther.* 2006;13:1342-1350.
6. Springer ML, Chen AS, Kraft PE, Bednarski M, Blau HM. VEGF gene delivery to muscle: potential role for vasculogenesis in adults. *Mol Cell.* 1998;2:549-558.
7. Ozawa CR, Banfi A, Glazer NL, Thurston G, Springer ML, Kraft PE, McDonald DM, Blau HM. Microenvironmental VEGF concentration, not total dose, determines a threshold between normal and aberrant angiogenesis. *J Clin Invest.* 2004;113:516-527.
8. von Degenfeld G, Banfi A, Springer ML, Wagner RA, Jacobi J, Ozawa CR, Merchant MJ, Cooke JP, Blau HM. Microenvironmental VEGF distribution is critical for stable and functional vessel growth in ischemia. *Faseb J.* 2006;20:2657-2659.
9. Luttun A, Tjwa M, Moons L, Wu Y, Angelillo-Scherrer A, Liao F, Nagy JA, Hooper A, Priller J, De Klerck B, Compernolle V, Daci E, Bohlen P, Dewerchin M, Herbert JM, Fava R, Matthys P, Carmeliet G, Collen D, Dvorak HF, Hicklin DJ, Carmeliet P. Revascularization of ischemic tissues by PlGF treatment, and inhibition of tumor angiogenesis, arthritis and atherosclerosis by anti-Flt1. *Nat Med.* 2002;8:831-840.
10. Carmeliet P, Moons L, Luttun A, Vincenti V, Compernolle V, De Mol M, Wu Y, Bono F, Devy L, Beck H, Scholz D, Acker T, DiPalma T, Dewerchin M, Noel A, Stalmans I, Barra A, Blacher S, Vandendriessche T, Ponten A, Eriksson U, Plate KH, Foidart JM, Schaper W, Charnock-Jones DS, Hicklin DJ, Herbert JM, Collen D, Persico MG. Synergism between vascular endothelial growth factor and placental growth factor contributes to angiogenesis and plasma extravasation in pathological conditions. *Nat Med.* 2001;7:575-583.
11. Louzier V, Raffestin B, Leroux A, Branellec D, Caillaud JM, Levame M, Eddahibi S, Adnot S. Role of VEGF-B in the lung during development of chronic hypoxic pulmonary hypertension. *Am J Physiol Lung Cell Mol Physiol.* 2003;284:L926-L937.

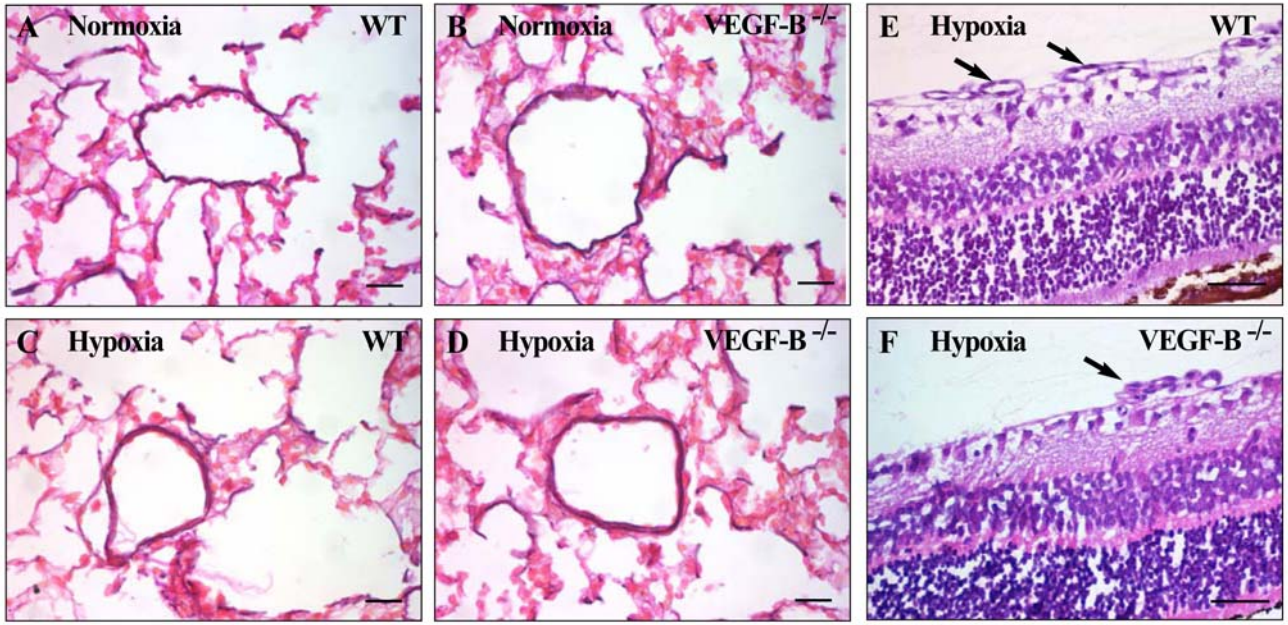
12. Reichelt M, Shi S, Hayes M, Kay G, Batch J, Gole GA, Browning J. Vascular endothelial growth factor-B and retinal vascular development in the mouse. *Clin Experiment Ophthalmol.* 2003;31:61-65.
13. Smith LE, Wesolowski E, McLellan A, Kostyk SK, D'Amato R, Sullivan R, D'Amore PA. Oxygen-induced retinopathy in the mouse. *Invest Ophthalmol Vis Sci.* 1994;35:101-111.
14. Brusselmans K, Compernolle V, Tjwa M, Wiesener MS, Maxwell PH, Collen D, Carmeliet P. Heterozygous deficiency of hypoxia-inducible factor-2alpha protects mice against pulmonary hypertension and right ventricular dysfunction during prolonged hypoxia. *J Clin Invest.* 2003;111:1519-1527.
15. Umans L, Cox L, Tjwa M, Bito V, Vermeire L, Laperre K, Sipido K, Moons L, Huylebroeck D, Zwijsen A. Inactivation of Smad5 in endothelial cells and smooth muscle cells demonstrates that Smad5 is required for cardiac homeostasis. *Am J Pathol.* 2007;170:1460-1472.
16. Banfi A, Springer ML, Blau HM. Myoblast-mediated gene transfer for therapeutic angiogenesis. *Methods Enzymol.* 2002;346:145-157.
17. Makinen T, Olofsson B, Karpanen T, Hellman U, Soker S, Klagsbrun M, Eriksson U, Alitalo K. Differential binding of vascular endothelial growth factor B splice and proteolytic isoforms to neuropilin-1. *Journal of Biological Chemistry.* 1999;274:21217-21222.



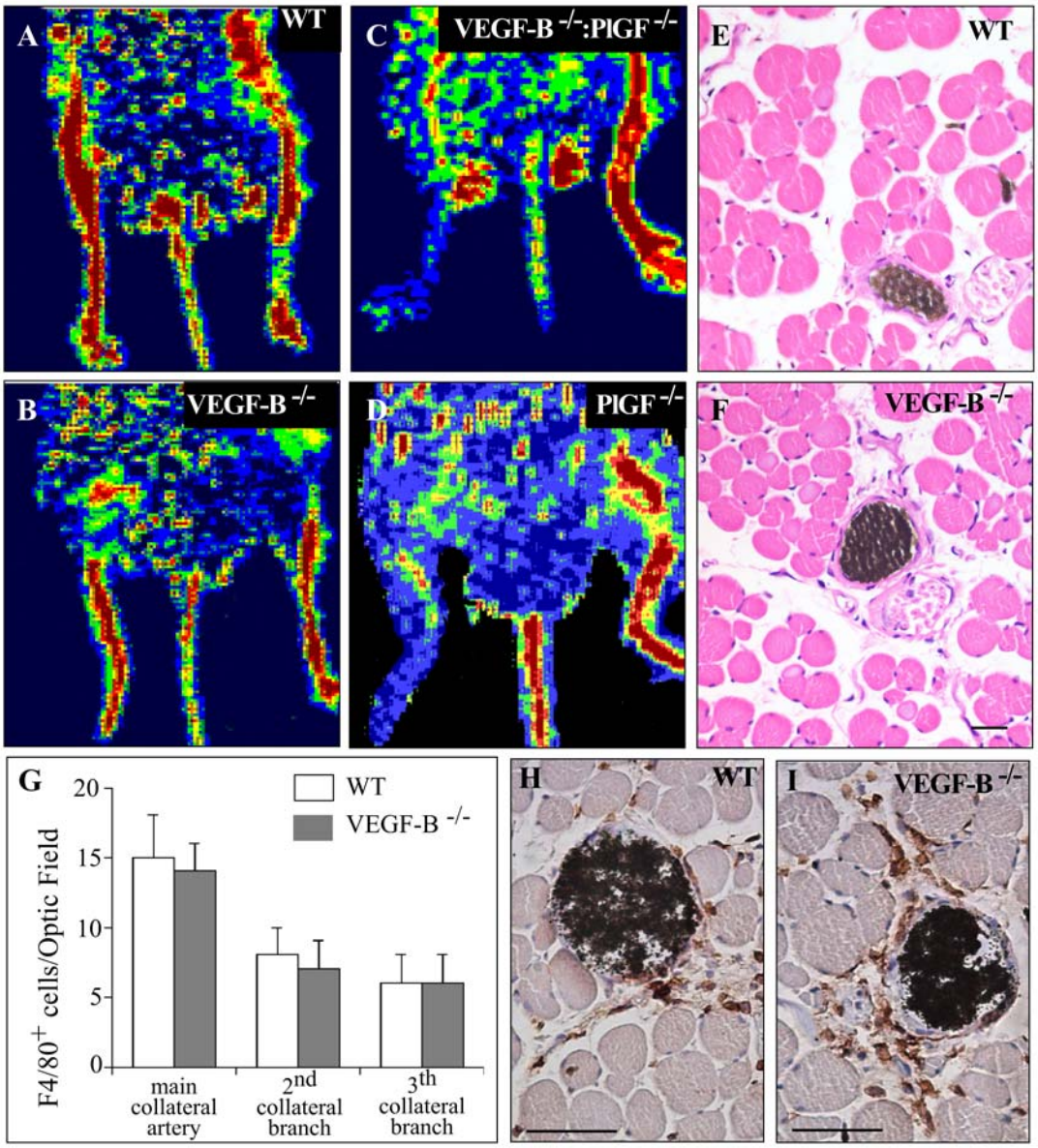
Supplement Figure I



Supplement Figure II



Supplement Figure III



Supplement Figure IV