Mice Lacking the Vascular Endothelial Growth Factor-B Gene (Vegfb) Have Smaller Hearts, Dysfunctional Coronary Vasculature, and Impaired Recovery From Cardiac Ischemia

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Abstract—Vascular endothelial growth factor-B (VEGF-B) is closely related to VEGF-A, an effector of blood vessel growth during development and disease and a strong candidate for angiogenic therapies. To further study the in vivo function of VEGF-B, we have generated Vegfb knockout mice (Vegfb−/−). Unlike Vegfa knockout mice, which die during embryogenesis, Vegfb−/− mice are healthy and fertile. Despite appearing overtly normal, Vegfb−/− hearts are reduced in size and display vascular dysfunction after coronary occlusion and impaired recovery from experimentally induced myocardial ischemia. These findings reveal a role for VEGF-B in the development or function of coronary vasculature and suggest potential clinical use in therapeutic angiogenesis. The full text of this article is available at http://www.circresaha.org. (Circ Res. 2000;86:e29-e35.)

Key Words: angiogenesis ■ cardiac ischemia ■ coronary vasculature

Vascular endothelial growth factor-B (VEGF-B)1–3 is a secreted growth factor that has strong sequence homology with VEGF-A, a primary regulator of angiogenesis in development, corpus luteum formation, wound healing, and cancer.4 VEGF-B can form stable heterodimers with VEGF-A5 and is generally coexpressed with VEGF-A.5,6 VEGF-B can bind to two of the VEGF-A receptors, VEGFR-17 and neuropilin-1,8 suggesting that it may regulate the bioavailability and/or action of VEGF-A.9 Although VEGF-B has been reported to behave as an endothelial cell mitogen,2 part of the mitogenic activity reported may be due to VEGF-B/VEGF-A heterodimers.5

Several mouse models have been generated by gene knockout technology where the genes encoding Vegf-A or its receptors have been mutated. Both Vegfa−/− and Vegfa−/− mice are unable to survive to term due to a general impairment of blood vessel formation in the early embryo.5,10 Vegfb12010 mice, where only two of the three major Vegf-A isoforms have been knocked out, die postnatally after cardiac failure due to widespread myocardial ischemia.11 Vegfr1−/− mice die as embryos due to defects in angiogenesis,12 but partial knockout mice, where only the tyrosine kinase-encoding portion of the Vegfr1 gene is deleted, develop normal vasculature.13

To study the in vivo role of Vegf-B, we have generated a knockout mouse line and found that, unlike the Vegf-A–related knockout, Vegfb−/− mice appear outwardly normal and fertile. Because Vegfb transcripts are expressed predominantly in the heart during murine embryogenesis and adult life,1,14–16 suggesting a specific role for Vegf-B during cardiac development, we have concentrated on studying the cardiac phenotype in these mice. Vegfb−/− hearts are reduced in size compared with hearts of Vegfb+/+ littermates and display clinical symptoms of impaired recovery from experimentally induced ischemia. The results suggest an essential role for Vegf-B in establishment of a fully functional coronary vasculature and highlight the potential of this cytokine for application in the emerging field of therapeutic angiogenesis.

Materials and Methods

Generation of Vegfb+/− Mice

All mice used for the present study were supplied by the Animal Resources Centre (Western Australia), and their treatment was in

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From the QCF Transgenic Laboratory and Human Genetics Laboratory, Joint Experimental Oncology Program, the Queensland Institute of Medical Research (D.B., G.U.S., C.A.P., M.G., A.M., M.M.C., I.D.T., S.M.G., S.T., N.K.H., G.F.K.), and the Department of Pathology, University of Queensland (M.C.C.), Brisbane, Australia; Griffith University (J.P.H.), Gold Coast Campus, Southport, Australia; Department of Cardiothoracic Surgery (P.S.T.), Imperial College School of Medicine, National Heart and Lung Institute, London, UK; and Centre for Molecular and Cellular Biology (C.W., M.L.), University of Queensland, Brisbane, Australia.
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accordance with the National Health and Medical Research Council (NH&MRC) guidelines for the care of experimental animals.

Targeted inactivation of the Vegfb gene was achieved by replacing exons 3 to 7 (Figure 1a) with a promoter-less β-geo cassette. The β-geo gene was preceded by an internal ribosomal entry site to give cap-independent translation of β-geo.17 Targeted 129/SvJ ES cells (C1368) were injected into C57BL/6 blastocysts to produce chimeras. Progeny of germline-transmitting chimeras were genotyped by polymerase chain reaction (PCR) amplification of tail-tip DNA using PCR1, 5'-TTT GAT GGC CCC AGC CAC-3', PCR2, 5'-CCC CCA GCT GAC TGC TCG-3', and PCR3, 5'-CTA GTG GAT CCC CCG GGC-3' (Figure 1b).

β-Gal Staining and Immunohistochemistry

Frozen sections and whole embryos were stained for β-Gal as described.18 For quantitation of capillary density, transverse sections of the left ventricle (LV) were cut at comparable levels in Vegfb+/+ and Vegfb−/− P30 hearts (30 days postpartum) (4 hearts each), immunostained with anti–PECAM-1 (clone M13, Pharmingen), and the capillaries counted using ImagePlus software on 7 randomly chosen fields (×40 magnification, ~0.06 mm² per field) in the epicardial, endocardial, and midmyocardial portion of the LV. Because no difference between genotypes was found within each portion, capillary density data were averaged for each heart. Coronary vessels were counted as anti–smooth muscle α-actin (FITC conjugated, clone 1A4, Sigma)–stained vessels in whole sections.

Heart Weight and LV Thickness

Vegfb+/+, Vegfb+/−, and Vegfb−/− mice of either 129/SvJ or C57BL/6J×129/SvJ background were weighed. After dissection, the hearts were trimmed of surrounding tissue and weighed. A subset of the P25 hearts was fixed in formalin and microdissected to obtain a similar angle of section. LV thickness was measured on sections with a stage micrometer (n=10 Vegfb+/+ hearts; n=16 Vegfb+/− hearts; n=14 Vegfb−/− hearts).

Langendorff Perfusion

Hearts were isolated from mice anesthetized with 60 mg/kg sodium pentobarbital. Vegfb+/+ (161±7 g wet heart weight [WHW], n=15), Vegfb+/− (152±6 mg WHW, n=14), and Vegfb−/− mice (155±7 mg WHW, n=16) hearts were perfused in the Langendorff mode as described.19

For ischemia, baseline measurements were recorded from Vegfb+/+ (n=8), Vegfb+/− (n=8), and Vegfb−/− hearts (n=8) after 30 minutes of stabilization. Global normothermic ischemia was initiated for 20 minutes before 30 minutes of aerobic reperfusion. To examine reactive hyperemia, a subset of hearts (n=7 for Vegfb+/+, n=6 for Vegfb+/−, and n=8 for Vegfb−/−) was perfused as described above and after stabilization was subjected to a single 20-second period of zero flow followed by reperfusion at 90 mm Hg perfusion pressure. The coronary flow response was recorded, peak hyperemic flows were measured in individual experiments, and percentage of flow-debt repayment over the initial 60 seconds of reperfusion was calculated as follows:

\[
\text{Repayment} = \frac{-\text{total flow for 60 s reperfusion}}{-\text{total flow for 60 s preocclusion}} \times 100\% 
\]

where total coronary flows were measured in mL/g and were calculated by digital integration of coronary flow for the 60 seconds before and 60 seconds after occlusion using the Chart V3.5.6 program (AD Instruments, Castle Hill, Australia), and flow-debt was calculated as basal coronary flow (mL/60 seconds/g)×20 seconds of occlusion.

Statistical Analyses

Body/heart weight, LV thickness, and capillary density data were analyzed using unpaired Student’s t tests. Body/heart weight data were also analyzed using the generalized linearization equation.20 Hyperemia data were analyzed via one-way ANOVA and functional parameters by two-way ANOVA for repeated measures. Where significant effects were detected, the Tukey’s HSD post hoc test was used. In all tests, significance was accepted at P<0.05.

An expanded Materials and Methods section is available online at http://www.circresaha.org.

Results

Generation of the Vegfb−/− Mouse

The Vegfb knockout mice generated with the modified Vegfb locus shown in Figure 1a were produced in normal mendelian ratios, were healthy and fertile, and did not display any overt phenotype. The genotype of mice was determined by PCR amplification of tail-tip DNA from P10 pups (Figure 1b). Rather than producing Vegf-B, this modified locus results in β-Gal expression under the control of the endogenous Vegfb promoter (herein referred to as Vegf-B/β-Gal). Because the Vegfb−/− mice generated in this manner had no obvious developmental defects, we assumed that Vegf-B/β-Gal expression in these mice accurately reflects the endogenous Vegfb expression pattern.

Cardiac Vegf-B/β-Gal Expression Pattern in the Vegfb−/− Mouse

Using β-Gal staining, Vegf-B/β-Gal expression was first detected in the heart at E10.5 (embryonic day 10.5), it became prominent at E12.5 (Figure 2a) and further increased thereafter (Figure 2b). Throughout development, Vegf-B/β-Gal expression appeared to be restricted to the myocardium (Figures 2c through 2g) and subepicardium (Figures 2g and 2h) and remained undetectable in endothelial cells, including those of the endocardium and coronary endothelium. Endocardial derivatives, such as the valve leaflets were always devoid of Vegf-B/β-Gal expression. During development, the
highest concentration of Vegf-B/β-Gal–expressing cells was seen in the right ventricular myocardium and right aspect of the interventricular septum (Figures 2i and 2j) in correlation with the massive early postnatal coronary capillary and vessel growth. The prevalence of Vegf-B/β-Gal expression switches from the RV to the LV in the early neonatal period (Figures 2i and 2j) reflects the predominant early postnatal capillarization of this chamber. In the juvenile heart, the ventricular prevalence of expression is lost, and the density of Vegf-B/β-Gal–expressing cells is similar in the ventricles and the atria (data not shown).

The great arteries in the heart expressed low levels of Vegf-B/β-Gal at all stages of development (eg, Figure 2b) and in juvenile mice (data not shown). Vegf-B/β-Gal was undetectable in the tunica intima and media of coronary vessels (Figures 2j, arrowheads, and 2k and 2l), although we found Vegf-B/β-Gal expression in other vessels in the body (eg, the intralobar component of the pulmonary arteries) (data not shown).

Postnatal Heart Growth in Vegfb−/− Mice

Although histological examination of all organs revealed no differences between genotypes, Vegfb−/− hearts frequently appeared marginally smaller than their Vegfb+/+ and Vegfb+/− littermates (Figure 3a). We recorded the total body and heart weight of 122 animals including male and female Vegfb+/+, Vegfb−/−, and Vegfb+/− mice at several ages between P3 and P91. These mice were grouped as P3 to P9 mice (Vegfb−/−, n = 15; Vegfb+/−, n = 20; and Vegfb+/+, n = 13) and P25 or older (Vegfb−/−, n = 20; Vegfb+/−, n = 28; and Vegfb+/+, n = 27). We found no consistent genotype-dependent decrease in body weight; however, heart weight was always reduced in Vegfb−/− mice. To account for the inherent interlitter and intralitter variability in body weight, due to sex, age, and genetic background, we used heart/body ratios to display the results. Although we found no significant difference in heart/body ratio in relation to sex or genetic background, statistical analysis revealed a dramatic increase in heart/body ratio from P3-9 to P25 (or older) in all animals regardless of genotype (Figure 3b). There were no differences in percentage of heart/body weight ratios among genotypes in P3-9 mice (Vegfb+/+, 0.64±0.02; Vegfb−/−, 0.59±0.03; and Vegfb+/−, 0.66±0.03), but we found a significant (P<0.05) decrease in percentage of heart/body weight ratio in P25 (or older) Vegfb−/− (0.78±0.02) mice compared with Vegfb+/+ (0.87±0.04) and Vegfb+/− (0.89±0.02) mice (Figure 3b). When familial (litter/parents) correlation among mice was taken into account, this significant difference remained (data not shown).
significant differences between P30 Vegfb−/−, Vegfb+/−, and Vegfb+/+ hearts from same-sex littermates, illustrating a slightly reduced Vegfb−/− heart size. b, Percentage of body/weight ratio is significantly reduced in the juvenile (>P25) Vegfb−/− mice but not in early postnatal mice (P3-9), indicating the impaired growth of the Vegfb−/− hearts in the first few weeks after birth. Values are mean±SEM. *P<0.05 vs Vegfb+/+ mice; P<0.01 vs Vegfb−/− mice.

not shown). No significant difference was found between Vegfb+/+ and Vegfb−/− mice at any stage.

The dramatic increase in heart weight during the first few weeks after birth is well documented and appears to be mainly due to massive growth of the coronary capillaries and vessels, but cardiomyocyte proliferation and hypertrophy also contribute.22 No difference in the size of myocardial cells was found in histological sections in Vegfb+/+, Vegfb+/−, or Vegfb−/− hearts (data not shown). LV thickness was significantly decreased in P25 Vegfb−/− (0.80±0.03 mm, n=14) compared with Vegfb+/+ (0.89±0.03 mm, n=10) and Vegfb+/− (0.91±0.02 mm, n=16) hearts (*P=0.059 versus Vegfb+/+ and P<0.05 versus Vegfb−/−). Analysis of capillary density using standard morphometric measures found no significant differences between P30 Vegfb+/+ (2321±255 capillaries/mm²) and Vegfb−/− (2334±253 capillaries/mm²) hearts. Vessel density measures in adjacent heart sections also showed no differences between Vegfb+/+ (270±10 vessels/section) and Vegfb−/− (275±14 vessels/section) hearts.

Reactive Hyperemic Responses in Vegfb+/+, Vegfb+/−, and Vegfb−/− Hearts

Baseline contractile function and coronary flow were equivalent in Langendorff-perfused hearts from all three groups under normoxic conditions (see Table online, http://www.circresaha.org). To test whether alterations in vascular function would be more evident during active responses to modified myocardial O₂ delivery, we exposed hearts to transient (20 seconds) coronary occlusion and studied the hyperemic response on reperfusion. The reactive hyperemic responses differed subtly between groups (Figure 4). Although peak hyperemic flow was comparable in all three groups of hearts (32 to 36 mL·min⁻¹·g⁻¹) (Figure 4a), overall flow-debt repayment during the initial 60 seconds of reperfusion (during which flow recovered to preocclusion levels) was significantly lower in Vegfb−/− mice (~60%) versus the other two groups (~100%) (Figure 4b). There were no differences in repayment between Vegfb+/+ and Vegfb−/− hearts. These findings indicate that the functional status of the coronary vasculature is impaired in Vegfb−/− mice.

Responses to Ischemia-Reperfusion in Vegfb+/+, Vegfb+/−, and Vegfb−/− Hearts

As noted, baseline functional parameters were comparable in hearts from Vegfb+/+, Vegfb+/−, and Vegfb−/− mice (see Table online, http://www.circresaha.org). Global normothermic ischemia completely abolished contractile function in all hearts within 2 to 3 minutes and caused a rapid rise in diastolic pressure. Time to onset of contracture and peak-developed contracture are indicators of the severity of ischemic injury. Although no difference was found in the rate of contracture development, peak contracture during ischemia was greater in Vegfb−/− compared with Vegfb+/+ and Vegfb−/− hearts (Figure 5a). Diastolic pressure was significantly elevated in Vegfb−/− hearts compared with Vegfb+/+ and Vegfb−/− hearts during reperfusion and recovered minimally (~73 mm Hg) relative to the other two groups (~35 mm Hg) (Figure 5a). Recovery of contractile function was slightly depressed throughout reperfusion in Vegfb−/− hearts, with the rate-pressure product being significantly lower at 30 minutes compared with Vegfb+/+ and Vegfb−/− hearts (Figure 5b). Coronary flow
larization in any individual organ. Although expression patterns with developmental processes of vasculature, although no attempts were made to correlate to reflect a paracrine action of Vegf-B on the developing pattern of Vegf-B expression has previously been interpreted major site of Vegf-B expression. The general developmental expression to processes of vascularization in the heart, the expression is not detected until E10.5. We can first detect scripts are produced in the heart from E8.5, Vegf-B protein vs coronary flow (c) are shown. Values are mean ±SEM. *P<0.05 vs Vegfb+/+ hearts.

Discussion

In the present study, we have used promoter trap LacZ expression in the Vegfb−/− mouse to correlate Vegf-B/β-Gal expression to processes of vascularization in the heart, the major site of Vegf-B expression. The general developmental pattern of Vegf-B expression has previously been interpreted to reflect a paracrine action of Vegf-B on the developing vasculature, although no attempts were made to correlate expression patterns with developmental processes of vascularization in any individual organ. Although Vegfb transcripts are produced in the heart from E8.5, Vegf-B protein expression is not detected until E10.5. We can first detect Vegf-B/β-Gal at E10.5 in the interventricular septum and propose that Vegf-B/β-Gal (and therefore Vegf-B) production increases substantially at this time point, in spatial and temporal correlation with the commencement of coronary endothelial growth in the heart. Indeed, Vegf-B/β-Gal levels increase both throughout development and after birth, closely correlating with the progression of cardiac angiogenesis. Vegf-B/β-Gal is conspicuous in the subepicardium where heart angiogenesis has been shown to commence. It is more densely expressed in the ventricles than the atria and correlates with the degree of coronary angiogenesis that is more advanced in the ventricles than in the atria at fetal stages. After birth, Vegf-B/β-Gal expression increases further in the LV, at a time when substantial capillary growth occurs predominantly in this chamber. The disparity between LV and RV capillary growth rates decreases several days after birth. Accordingly, by P25, Vegf-B/β-Gal expression becomes even throughout the heart with levels of expression similar in RV, LV, and the atria. Despite the correlation between capillarization and Vegf-B/β-Gal expression, such expression was not found in the smooth muscle cells of the differentiated coronary vessels. It is therefore likely that Vegf-B may exert its paracrine action on the microvasculature surrounding the expressing myocardium, rather than on the endothelium of the coronary vessels.

We find that Vegfb−/− hearts appear morphologically and functionally normal in the unstressed animal but do not undergo the same extent of postnatal growth as those of Vegfb−/− and Vegfb+/− animals. Postnatal heart growth appears to be mainly due to the substantial increase in the coronary microvasculature and vessels. This increase has been attributed to the action of Vegf-A16 and Vegf-A18 because mice lacking these Vegf-A isoforms die as a consequence of severe heart ischemia due to an almost total absence of postnatal capillary and coronary vessel growth. Postnatal ablation of Vegf-A (and possibly Vegf-B) function by administering a soluble Flt-1 receptor (mFlt(1–3)-IgG) is also lethal. In the heart, this treatment leads to cardiomyocyte necrosis and massive capillary and vessel density reduction. Because Vegf-B is coexpressed with Vegf-A in the myocardium of the ventricles, can form biologically active heterodimers with Vegf-A, and also binds Flt-1, it is likely that the abnormal coronary angiogenesis described above is a result of interference with the normal function of both Vegf-A and Vegf-B. We tested whether the observed Vegfb−/− reduction in heart weight was a consequence of impaired growth of the vascular network by measures of coronary capillary and vessel density. We found no significant differences between Vegfb−/− and Vegfb+/− hearts, although additional studies measuring lumen size, patency, and permeability of capillaries and vessels in the heart will reveal whether any structural abnormalities in the vascular network of Vegfb−/− hearts may be responsible for reduced volume of this organ. Alternatively, the observed microcardia could be attributed to an effect of Vegf-B on cardiomyocyte growth. Vegf-B effect on heart muscle could be mediated by the Vegf-B receptor, neuropilin-1, which is expressed in the developing cardiac muscle. However, this is unlikely, because cardiomyocytes do not appear affected in size or function in the Vegfb−/− heart, and we cannot rule out a direct effect of Vegf-B ablation on myocytes. It is worth noting, nevertheless, that cardiomyocytes are normal in the Vegf−/− mouse, where neuropilin-1 ligand Vegf-A165 has been ablated. A slight decrease in left ventricular thickness in the Vegfb−/− heart may indicate that some developmental hypoplasia, resulting from suboptimal vascularization, could be responsible for the observed microcardia.

Ablating Vegfb expression reduced the ability to repay coronary flow after a transient coronary occlusion. This occurred despite baseline coronary flow in the Vegfb−/− heart appearing normal, which was not unexpected given that
moderate impairment of vascularization or vascular function that might result from deletion of the Vegfb gene could be compensated by enhanced intrinsic vasodilatation. Impairment of flow-debt repayment, despite similar peak flows, suggests inhibition of flow-mediated dilatation, which occurs subsequent to the immediate hyperemic response, indicating that the functional status of the coronary vasculature is impaired in some way in Vegfb<sup>−/−</sup> hearts. Reactive hyperemia is thought to be mediated by the combined actions of nitric oxide (NO) and adenosine, with potential involvement of K<sub>ATP</sub> channels. The prolongation of the hyperemic response is thought to be at least partially NO dependent. Thus, one possible mechanism contributing to this change is an impaired NO production. However, deletion of the endothelial NO synthase gene fails to alter peak hyperemic flow, flow repayment, and adenosine responses in murine hearts.

Heart rate was almost identical in hearts of all genotypes before and after ischemia, and no significant differences existed for heart rate between any groups at any time. Interestingly, deletion of Vegfb reduced functional recovery from ischemia-reperfusion and appeared to worsen contracture during ischemia. The mechanism of contracture is not well understood but may involve rigor bond formation as a result of impaired glycolytic ATP formation. During reperfusion, diastolic dysfunction was significantly greater in knockout mice; the difference was wholly due to a change in contractile force and not rate. Recovery of the rate-pressure product was slightly reduced wholly due to a change in contractile force and not rate. The mechanism of contracture is not well understood but may involve rigor bond formation as a result of impaired glycolytic ATP formation.

In the present study, we have shown that, despite heart morphology and function being normal in Vegfb<sup>−/−</sup> mice, the response to coronary occlusion and myocardial recovery from ischemia are compromised. Thus, although Vegf-B may play a redundant role in establishing the coronary vasculature, our results define a unique role in the development and maintenance of function in response to ischemic insult.

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References


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Supplementary information: expanded Materials and Methods

Generation of Vegfb<sup>−/−</sup> mice

Targeted inactivation of the Vegfb gene was achieved using a standard gene replacement targeting vector (Figure 1a) with exons 3-7 of the Vegfb gene removed and replaced by a promoter-less β-geo cassette so that β-geo is transcribed under the control of the Vegfb promoter. The β-geo structural gene was preceded by an internal ribosomal entry site signal sequence to give cap-independent translation of the β-geo fusion protein<sup>16</sup>. Targeted 129/SvJ ES cells (C1368) were injected into C57BL/6J blastocysts and the chimeric embryos transferred to pseudopregnant females for development to term. Resulting chimeras were mated with C57BL/6J animals. The progeny of germline-transmitting chimeras, identified by coat colour transmission, were genotyped for the targeted Vegfb locus by PCR amplification of DNA from tail tip biopsies using the primers shown in Figure 1 (PCR1, 5′-ttt gat ggc ccc agc cac-3′; PCR2, 5′-ccc cca gct gac tgc tgg-3′; PCR3, 5′-ctg gat gat ccc ccc ggc-3′).

Histology, western blotting and immunohistochemistry

Standard methods for histology, western blotting and immunostaining were used. Anti-PECAM-1 (CD31) (clone M13, Pharmingen, USA) and FITC conjugated anti-SM α-actin (clone 1A4, Sigma, USA) antibodies were used as endothelial cell markers according to the manufacturers instructions (Fig. 1). Anti-VEGF-A polyclonal antibodies (sc507, Santa Cruz, USA) and anti-VEGFR-1 polyclonal antibodies (sc316, Santa Cruz, USA) were used at a
dilution of 1/1000 for western blots of heart and brain protein extract from Vegfb<sup>+/+</sup>, Vegfb<sup>+/−</sup>
and Vegfb<sup>−/−</sup> mice.

All tissues used for immunohistochemistry were cryosectioned (6-10μm sections). For quantitation of capillary density, we found PECAM-1 immunostaining with Avidin Biotin block (DAKO, USA) gave the most reliable results. Transverse sections of the ventricles were cut at comparable levels in Vegfb<sup>+/+</sup> and Vegfb<sup>−/−</sup> P30 hearts (4 hearts each), immunostained with PECAM-1 and the number of capillaries counted with the aid of ImagePlus software on 7 randomly chosen fields (40x magnification, circa 0.06mm<sup>2</sup> per field) in the epicardial, endocardial and midmyocardial portion of the LV. Coronary vessels were counted as Smooth Muscle α-actin (SMA) stained vessels in whole transverse sections contiguous to those used for capillary counts.

β-Gal staining

Frozen sections and whole embryos were incubated for at least 7 hours at 30-37°C in a staining solution containing 5mM potassium ferrocyanide, 5mM potassium ferriyanide, 2mM MgCl<sub>2</sub> and 10μg/ml of X-Gal in phosphate buffered saline (PBS) pH 7.3 as previously described<sup>17</sup>. In the Vegfb<sup>−/−</sup> mice we found a punctate cytoplasmic stain resulting from the β-geo expression rather than the more common diffuse appearance typical of LacZ reporter gene expression. While currently we have no explanation for this appearance, it has been observed in other cases where the β-geo fusion gene has been used instead of LacZ.

Heart and body weight measures
Inbred 129/SvJ or C57BL/6J x 129/SvJ hybrid mice were killed by cervical dislocation and weighed. The hearts were trimmed to the same degree from surrounding tissue and weighed.

**Left ventricular thickness**

A subset of P25 hearts was fixed in formalin and carefully microdissected to inspect the internal relief. The microdissection was longitudinal across the left ventricle. The hearts were subsequently carefully oriented in paraffin blocks to obtain a similar angle of section. Left ventricular thickness was measured with a stage micrometer in the midportion of the LV at three different levels in one section per heart. The average between the three measures was used for statistical analysis.

**Langendorff perfused murine heart model**

Hearts were isolated from adult male and female *Vegfb^{+/+} (161±7 mg wet heart weight, n=15), Vegfb^{+/−} (152±6 mg wet heart weight, n=14) and Vegfb^{−/−} mice (155±7 mg wet heart weight, n=16) and were perfused in the Langendorff mode as described by us for other species. Mice were anaesthetised with 60 mg/kg sodium pentobarbital administered intraperitoneally, a thoracotomy was performed and hearts rapidly excised into ice-cold perfusion fluid. The aorta was rapidly cannulated and the coronary circulation of all hearts was perfused at a constant pressure of 80 mmHg (unless stated otherwise) with modified Krebs-Henseleit buffer containing: NaCl, 120 mM; NaHCO₃, 25 mM; KCl, 4.7 mM; CaCl₂, 2.5 mM; Mg₂SO₄ 1.2 mM; KH₂PO₄ 1.2 mM, glucose, 15 mM; and EDTA, 0.5 mM. The buffer was equilibrated with 95% O₂, 5% CO₂ at 37°C, giving a pH of 7.4 and PO₂ ≥640 mmHg in the perfusate reservoir, and a PO₂ of ~600 mmHg at the tip of the aortic cannula.
over a 1-5 ml/min flow range. Buffer was filtered through a 5.0 μ filter, and all perfusate delivered to the heart was filtered through an in-line 0.45 μm Sterivex-HV filter cartridge (Millipore, Bedford, MA, USA). The left ventricle was vented with a small polyethylene apical drain and a fluid-filled balloon constructed of polyvinyl chloride plastic film was inserted into the left ventricle via the mitral valve. The balloon was connected to a P23 XL pressure transducer (Viggo-Spectramed, Oxnard, CA, USA) by fluid filled polyethylene tube, permitting continuous measurement of left ventricular pressure. Balloon volume was controlled by a Hamilton 500 μl threaded plunger syringe calibrated to 5.29 μl per revolution (Hamilton Co., Reno, NV, USA). The balloon was initially inflated by ~20 μl to give an end-diastolic pressure of 4 mmHg and then maintained at a constant volume throughout the experiment to permit comparison of diastolic and systolic function throughout the time course. Coronary flow rate was continuously monitored via a cannulating Doppler flow-probe (1N probe; Transonic Systems Inc, Ithaca, NY, USA) located in the aortic perfusion line and connected to a T206 flowmeter (Transonic Systems Inc, Ithaca, NY, USA). All data (ventricular and perfusion pressures, coronary flow) were recorded at a sampling speed of 1 KHz on a 4 channel MacLab data acquisition system (ADInstruments, Castle Hill, Australia) connected to an Apple 7300/180 computer. The ventricular pressure signal was digitally processed to yield peak systolic pressure, diastolic pressure, ±dP/dt, and heart rate. Hearts were instrumented for functional measurements as described below. After instrumentation hearts were immersed in warmed perfusate inside a 10 ml water jacketed bath maintained at 37°C. The temperature of coronary perfusion fluid was continuously assessed by a needle thermistor located at the entry into the aortic cannula. All hearts were stabilised for a 30 min period prior to experimentation. Baseline functional data for Langendorff perfused hearts are presented in Table 1.
Experimental protocol

For the ischemia protocol, baseline functional measurements were recorded after 30 min stabilisation and global normothermic ischemia was then initiated by clamping the aortic cannula for 20 min. Hearts were then reperfused for 30 min. Studies were performed in hearts from Vegfb\(^{+/+}\) (n=8), Vegfb\(^{+/−}\) (n=8) and Vegfb\(^{−/−}\) mice (n=8). To examine reactive hyperaemia a subset of hearts (n=7 for Vegfb\(^{+/+}\), n=6 for Vegfb\(^{+/−}\), n=8 for Vegfb\(^{−/−}\)) were perfused as described above, and after stabilisation were subjected to a single 20 s period of zero flow followed by reperfusion at 90 mmHg perfusion pressure. The coronary flow response was recorded, peak hyperemic flows were measured in individual experiments, and % flow-debt repayment over the initial 60 s of reperfusion was calculated as:

\[
\text{Repayment} = \frac{\text{total flow for 60 s reperfusion} - \text{total flow for 60 s pre-occlusion}}{\text{flow-debt}} \times 100\%
\]

where total coronary flows were measured in ml/g and were calculated by digital integration of coronary flow for the 60 s prior to and 60 s post-occlusion using the Chart V3.5.6 program (ADInstruments, Castle Hill, Australia), and flow-debt was calculated as basal coronary flow (ml/60 s/g) x 20 s of occlusion.

Statistical analyses

Body/heart weight data were analysed using unpaired t-test (ANOVA). They were also analyzed using the Generalized Estimation Equation (GEE) procedure which enables estimation of correlations such as those stemming form within litter effects. All results are expressed as mean±SEM. Functional parameters under baseline conditions and during ischemia-reperfusion were statistically analyzed by a two-way analysis of variance for repeated measures. Peak hyperemic flows and % flow-debt repayment data was analyzed via
a one-way analysis of variance. For both ischemia-reperfusion and hyperemia studies, the Tukeys HSD post-hoc test was employed where significant effects were detected. In all tests significance was accepted at the 95% confidence level (P<0.05).
Table 1. Baseline functional data for Langendorff perfused hearts under normoxic conditions.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>$Vegfb^{+/+}$ (n=15)</th>
<th>$Vegfb^{+/c}$ (n=14)</th>
<th>$Vegfb^{-/-}$ (n=16)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Systolic Pressure</td>
<td>160±16</td>
<td>152±11</td>
<td>168±12</td>
</tr>
<tr>
<td>(mmHg)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diastolic Pressure</td>
<td>2±1</td>
<td>1±1</td>
<td>3±1</td>
</tr>
<tr>
<td>(mmHg)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart Rate (bpm)</td>
<td>358±8</td>
<td>363±14</td>
<td>350±19</td>
</tr>
<tr>
<td>+dP/dt (mmHg/s)</td>
<td>5150±460</td>
<td>4471±428</td>
<td>5196±418</td>
</tr>
<tr>
<td>-dP/dt (mmHg/s)</td>
<td>-3940±460</td>
<td>-3446±298</td>
<td>-4627±569</td>
</tr>
<tr>
<td>Coronary Flow (ml/min/g)</td>
<td>16.6±1.5</td>
<td>17.2.0±1.8</td>
<td>17.8±2.4</td>
</tr>
</tbody>
</table>
**Figure legend**

**Figure 1.** Representative sections used for capillary and vessel density counts. (a), PECAM-1 stained endocardial region of the LV, showing the endothelial network (brown). (b), Smooth muscle α-actin (SMA) stained section showing coronary vessels (flourescent green). Original magnification 40X. Bar=100μm.
Figure 1 (Online Suppl. Info)