Contribution of VEGF-B-induced endocardial endothelial cell lineage in physiological vs pathological cardiac hypertrophy

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Abstract

Background and aims. Preclinical studies have shown the therapeutic potential of VEGF-B in revascularization of the ischemic myocardium, but the associated cardiac hypertrophy and adverse side effects remain a concern. To understand the importance of endothelial proliferation and migration for the beneficial vs adverse effects of VEGF-B in the heart, we explored the cardiac effects of autocrine vs paracrine VEGF-B expression in transgenic and gene-transduced mice.

Methods. We used single cell RNA sequencing (scRNA seq) to compare cardiac endothelial gene expression in VEGF-B transgenic mouse models. Lineage tracing was used to identify the origin of a VEGF-B-induced novel endothelial cell (EC) population, and AAV-mediated gene delivery to compare the effects of VEGF-B isoforms. Cardiac function was investigated using echocardiography, magnetic resonance imaging, and micro-computed tomography.

Results. Unlike in physiological cardiac hypertrophy driven by a cardiomyocyte-specific VEGF-B transgene (αMHC-VEGF-B), autocrine VEGF-B expression in cardiac endothelium (aP2-VEGF-B) was associated with septal defects and failure to increase perfused sub-endocardial capillaries postnatally. Paracrine VEGF-B led to a robust proliferation and myocardial migration of a novel cardiac EC lineage (VEGFB-iECs) of endocardial origin, whereas autocrine VEGF-B increased proliferation of VEGFB-iECs, but failed to promote their migration and efficient contribution to myocardial capillaries. The surviving aP2-VEGF-B offspring showed an altered ratio of secreted VEGF-B isoforms and developed massive pathological cardiac hypertrophy with a distinct cardiac vessel pattern. In the normal heart, we found a small VEGFB-iEC population that was only minimally expanded during myocardial infarction but not during physiological cardiac hypertrophy associated with mouse pregnancy.

Conclusions. Paracrine and autocrine secretion of VEGF-B induce expansion of a specific endocardium-derived EC population with distinct angiogenic markers. However, autocrine VEGF-B signaling fails to promote VEGFB-iEC contribution to myocardial capillaries, predisposing to septal defects, and inducing a mismatch between angiogenesis and myocardial growth, resulting in pathological cardiac hypertrophy.

Novelty and Significance

What is known?

- VEGF-B has greater translational potential than VEGF, which induces vascular leakage and tissue inflammation.
- VEGF-B gene transfer to the heart induces angiogenesis and physiological cardiac hypertrophy.
- VEGF-B has two isoforms, which have distinct biochemical and functional properties.

What new information does this article contribute?

- VEGF-B gene transfer to the heart leads to vascular expansion of a unique endocardium-derived endothelial cell population that is slightly expanded after myocardial infarction, but not during pregnancy.
- VEGF-B production in cardiac endothelial cells leads to cardiac septal defects, pathological angiogenesis, and cardiac hypertrophy.
- Posttranslational regulation of VEGF-B isoforms differs in cardiomyocytes versus cardiac endothelial cells.

Induction of myocardial vessel growth could alleviate cardiomyocyte damage during myocardial ischemia. However, attempts to use VEGF-A for this purpose have been halted because it promotes vascular leakage and inflammation. In contrast, VEGF-B provides a promising therapeutic tool in the heart as it causes very little vascular leakage or inflammation. Furthermore, very high doses of VEGF-B are well tolerated because it functions indirectly via endogenous VEGF-A signaling. Our present study shows that VEGF-B production by cardiac endothelial cells (ECs) leads to cardiac pathology. Comparison of the vascular phenotypes in the two transgenic models revealed that VEGF-B overexpression induces a novel cardiac EC lineage (VEGFB-iECs) that contributes robustly to sub-endocardial vessels upon paracrine expression, but very little upon autocrine expression. In contrast, the VEGFB-iECs were not expanded in cardiac hypertrophy associated with mouse pregnancy and they showed only slight expansion during myocardial infarction caused by coronary vessel ligation. The endocardium-derived VEGFB-iECs should provide a vascular marker for the targeting and monitoring of therapeutic attempts to salvage myocardial tissue in critical ischemia. Furthermore, our findings on the cell type-specific regulation of VEGF-B isoforms provide a valuable basis for further translational development of VEGF-B.

Non-standard Abbreviations and Acronyms

AAV9	Adeno-associated virus 9
aP2	Adipocyte protein 2
BAT	Brown adipose tissue
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CAG	CMV early enhancer/chicken β-actin (CAG) promoter
Cdh5	Cadherin 5
CFs	Cardiac fibroblasts
CMCs	Cardiomyocytes
Cre-ER^{T2}	Cre recombinase fused to a triple mutant form of the human estrogen receptor
DEGs	Differentially expressed genes
ECG	Electrocardiography
ECs	Endothelial cells
EDTA	Ethylenediaminetetraacetic acid
EdU	5-Ethynyl-2'deoxyuridine
EF	Ejection fraction
EGTA	Egtazic acid
ELISA	Enzyme-linked immunosorbent assay
FABP4	Fatty acid binding protein 4
FACS	Fluorescence-activated cell sorting
FBs	Fibroblasts
FS	Fractional shortening
HCl	Hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	Horseradish peroxidase
HSC70	Heat shock cognate protein
HW/BW	Heart weight / body weight
HW/TL	Heart weight / tibial length
I.p.	Intraperitoneal
IR	Infrared
KCl	Potassium chloride
KH_2PO_4	Potassium dihydrogen phosphate
$KHCO_3$	Potassium bicarbonate
LAD	Left anterior descending
LV	Left ventricle
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulfate
MI	Myocardial infarction

MRI	Magnetic resonance imaging
Na_2HPO_4	Disodium hydrogen phosphate
$Na_4P_2O_7$	Tetrasodium pyrophosphate
NaCl	Sodium chloride
NaF	Sodium fluoride
$NaHCO_3$	Sodium bicarbonate
NRP-1	Neuropilin-1
OCT	Optimal cutting temperature compound
P	Pregnant
PBS	Phosphate-buffered saline
PD	Post-delivery
PFA	Paraformaldehyde
PVDF	Polyvinylidene difluoride
RFP	Red fluorescent protein
RT	Room temperature
RT-qPCR	Quantitative real-time PCR
ScRNA seq	Single cell RNA sequencing
SMA	Smooth muscle actin
SVF	Stromovascular fraction
sWAT	Subcutaneous white adipose tissue
TG	Transgenic
UMAP	Uniform Manifold Approximation and Projection
VEGF-A	Vascular endothelial growth factor A
VEGF-B	Vascular endothelial growth factor B
VEGFB-iECs	VEGF-B induced endothelial cells
VEGFR-1	Vascular endothelial growth factor receptor 1
VEGFR-2	Vascular endothelial growth factor receptor 2
VEGFRs	Vascular endothelial growth factor receptors
VEGFs	Vascular endothelial growth factors
WB	Western blot
WPRE	Woodchuck hepatitis virus post-transcriptional regulatory element
WT	Wild-type
αMHC	Myosin heavy chain alpha
μCT	Micro-computed tomography

Introduction

Ischemic heart disease is the number one cause of mortality worldwide[1,2]. Cardiac ischemia induced by occlusion of a coronary vessel endangers a massive sudden loss of cardiomyocytes (CMCs) that exceeds the regenerative capacity of the myocardium[3]. Due to their angiogenic properties, vascular endothelial growth factors (VEGFs) and their receptors (VEGFRs) provide promising therapeutic tools for alleviation of cardiac ischemia prior to CMC damage[4]. Attempts to use VEGF-A gene transfer as a therapeutic tool to induce angiogenesis[5–9] have been hindered by VEGF-induced vascular leakage and tissue inflammation[10,11]. More recent studies using VEGF-B gene transduction have shown that VEGF-B activates the endogenous VEGF/VEGFR-2 signaling pathway activity[12–15], without resulting in significant tissue inflammation or vascular leakage[13,16].

The *Vegfb* gene encodes two protein isoforms, VEGF-B_{186} and VEGF-B_{167}, which are secreted by CMCs in the mammalian heart. Both isoforms bind to VEGFR-1, expressed on the surface of cardiac endothelial cells (ECs)[17]. After its secretion, VEGF-B_{186} undergoes proteolytic cleavage, resulting in VEGF-B_{127} isoform[18]. Unlike VEGF-B_{186}, VEGF-B_{167} and VEGF-B_{127} bind also to the neuropilin-1 (NRP-1) co-receptor[18]. Previous studies have shown that a CMC-specific αMHC-VEGF-B transgene that produces both VEGF-B_{186} and VEGF-B_{167} isoforms expands the cardiac vasculature and leads to a physiological-like cardiac hypertrophy[12–15]. Transgenic (TG) rats expressing the αMHC-VEGF-B transgene have normal cardiac function and show an improved cardiac ejection fraction (EF%) and fractional shortening (FS%) after myocardial infarction (MI) caused by ligation of the left anterior descending (LAD) coronary artery[15]. Furthermore, expression of VEGF-B_{186} in mice via adeno-associated virus (AAV) vector delivery decreased scarring and improved cardiac perfusion after MI[14], and VEGF-B_{167} gene transfer provided protection against non-ischemic heart failure in a canine cardiac tachypacing model[19,20]. However, in one study, excessive levels of VEGF-B_{186}, and in particular of its cleaved VEGF-B_{127} form, predisposed the hypertrophic heart to arrhythmias after MI or dobutamine treatment, which both increase ectopic ventricular activity[21,22].

The metabolic effects of VEGF-B overexpression are of interest when considering its therapeutic potential in the setting of cardiovascular disease. In obese and insulin-resistant mice, VEGF-B gene transfer promoted weight loss and attenuated metabolic complications[23]. In rats, VEGF-B also decreased coronary lipoprotein lipase activity and cardiac lipid metabolite accumulation and augmented cardiac insulin action, suggesting that it may be cardio-protective in diabetes[24]. However, in long-term experiments, TG mice expressing only the human VEGF-B_{167} isoform in CMCs showed an increased death rate, apparently because of mitochondrial lipotoxicity[25].

As these contrasting results indicate, further development of VEGF-B as a therapeutic tool requires knowledge of its optimal site of expression and associated adverse effects, and of the properties of its two isoforms. In the present study, we report that paracrine VEGF-B signaling induces a novel endocardium-derived cardiac EC population that is marked by expression of a unique set of transcripts. We have evaluated here this EC population in two different models of cardiac hypertrophy and signaling, to better understand how VEGF-B can be safely used in the

heart. EC proliferation is stimulated by the binding of VEGF-A to its receptor VEGFR-2. EC migration during sprouting angiogenesis occurs towards higher concentrations of VEGF-A in a gradient generated by VEGF-A expressing cells[26]. We wanted to establish a cardiac model of autocrine VEGF-B signaling that would stimulate EC proliferation, but not directed migration. For this, we expressed the VEGF-B transgene as an autocrine ligand in the coronary endothelium, under control of adipocyte protein 2 (aP2/FABP4) promoter[23] and compared its effects to those in the cardiomyocyte-specific paracrine model (αMHC-VEGF-B)[13], with particular attention to coronary vessel growth and cardiac hypertrophy.

Our results indicate that autocrine VEGF-B signaling that lacks the paracrine VEGF-B gradient for EC migration, induces a scattered pattern of novel VEGFB-iECs, which however fail to contribute to sub-endocardial angiogenesis as much as in the paracrine model. The autocrine VEGF-B signaling led to septal defects already during embryogenesis. The surviving TG mice enabled us to characterize the pathological angiogenesis in postnatal mice. We also studied this novel EC population in other models of cardiac hypertrophy and observed that it was not induced during increased cardiac demand in pregnancy, and was only minimally expanded by MI. These results indicate that VEGF-B can enhance cardiac angiogenesis in normal and ischemic heart in a unique way that should allow the development of safe VEGF-B-based therapies for cardiac ischemia patients.

Methods

See detailed methods section in the Supplemental material.

Mouse models. All animal experimental procedures were approved by the National Animal Experiment Board following the regulations of the EU and national legislation. The Supplemental material contains detailed information on the TG rodent lines, experimental procedures, and treatments used in the study. The numbers of mice used in each experiment are indicated in the respective figure legends.

Cardiac echography, electrocardiography (**ECG**), **and magnetic resonance imaging** (**MRI**)**.** Analysis of cardiac function was performed under isoflurane anesthesia using the Vevo 2100 Ultrasound system (FujiFilm VisualSonics Inc). Parameters listed in Supplemental tables 1 and 2 were calculated using the 2D M-mode. Lead II ECG signals were acquired using limb electrodes and the processing of digital signals was performed using the previously published program in Matlab (MathWorks, Natick, MA)[27]. Cardiac MRI was carried out as described earlier[28].

Ligation of the LAD coronary artery. Induction of cardiac ischemia and subsequent MI was achieved by ligating left anterior descending (LAD) coronary artery in adult mice as described earlier[14].

Statistical Analysis. Values are indicated as mean ± SEM. Prism 9 software was used for statistical analyses (GraphPad Software, San Diego, CA). All mouse (n) numbers and statistical

tests used are indicated in figure legends, where absence of asterisk indicates non-significance, (*) indicates P-value ≤ 0.05, (**) ≤ 0.01, and (***) ≤ 0.001.

Data availability

All methods and study materials are available to other researchers upon reasonable request. All single cell RNA sequencing data will be deposited to the GEO database.

Results

Production and analysis of TG mice expressing VEGF-B in coronary endothelium. Endogenous cardiac EC proliferation and migration processes are driven predominantly by paracrine signaling via VEGF[4,29]. We aimed to create a model that short-circuits the signals for EC migration along the paracrine growth factor gradient and to retain the signals for EC proliferation. Accordingly, we employed aP2-VEGF-B TG mice that express the mouse VEGF-B gene under the aP2/fatty acid binding protein 4 (FABP4) promoter (**Figure 1A**)[23], which is activated in coronary ECs starting at embryonic day E14.5[30]. At this timepoint in wild-type (WT) mice, the FABP4 expressing cells arise by angiogenic sprouting from the first wave of ECs that colonize the heart and later form the coronary vasculature. During further development, all cardiac blood vascular ECs start to express aP2/FABP4[30]. As shown by the comparison of VEGF-B RNA expression between WT and TG mice in **Figure 1B**, we detected highly elevated levels of VEGF-B RNA in the coronary ECs isolated by FACS from ten weeks old aP2-VEGF-B mice (**Supplemental figure 1**), but not in CMCs or cardiac fibroblasts (CFs) isolated from the same mice. VEGF-B immunostaining of cardiac tissue sections confirmed specific expression of the transgene in cardiac ECs (**Figure 1C**). Western blotting (WB) analysis of the cardiac lysates and isolated cardiac ECs from the aP2-VEGF-B mice showed expression of the VEGF-B_{186} isoform, but, surprisingly, no expression of VEGF-B_{167} (**Figure 1D**)**.** The mouse VEGF-B_{186} isoform was detected also in sera from the TG mice at an average concentration of 10-15 ng/ml (**Supplemental figure 2A**), whereas VEGF-B concentration in sera from WT mice was below the ELISA detection level.

At 10 weeks of age, the aP2-VEGF-B TG hearts were considerably bigger than the hearts of their WT littermates, as indicated by the heart weight to body weight ratios (HW/BW) (**Figure 1E**). However, there was no difference in the body weights or the weights of epididymal or subcutaneous fat depots, spleens, kidneys, or lungs, between the aP2-VEGF-B mice and their WT littermates (**Supplemental figure 2B**). Immunohistochemical analysis confirmed that the aP2-VEGF-B mice have larger CMCs and a greater myocardial vessel area fraction than their WT littermates (**Supplemental figure 2C, D**).

Development of cardiac pathology in the aP2-VEGF-B mice. Upon genotyping of the offspring from matings between the aP2-VEGF-B heterozygous and WT mice at four weeks of age, we observed that only about 15-20% of the mice were aP2-VEGF-B TG, instead of the expected 50%. In contrast, at embryonic day 18.5 (E18.5), both genotypes were equally represented (**Supplemental figure 2E**). Peripartum observation of the pups indicated that about half of the

TG pups die or are eaten by the mothers during the first hours after birth. This differed significantly from the αMHC-VEGF-B mice that express the transgene in CMCs and are born in normal Mendelian ratios (**Supplemental figure 2E**). **Supplemental figure 2F** shows a comparison of cardiac hypertrophy development in the aP2-VEGF-B and αMHC-VEGF-B pups at P0, P7, P14, and P28. As can be seen from the data, at P0, there is no significant difference in HW/BW ratios between the two, but both the αMHC-VEGF-B mice[14] and the aP2-VEGF-B mice show clear hypertrophy already at P7 (**Supplemental figure 2F**). We then used μCT imaging to compare the TG hearts of pups that died postnatally at P0 to the hearts of their WT littermates. Intriguingly, we observed cardiac ventricular septal defects of varying severity in the TG pups (**Figure 1F** and **Supplemental figure 3A, B**). Furthermore, echocardiography demonstrated an impaired cardiac function in the surviving TG pups at P14. We also found decreased left ventricular ejection fraction in the adult TG mice (**Figure 1G**), which was confirmed by magnetic resonance imaging (**Figure 1H, Supplemental figure 2G**)**.** Electrocardiography[27] of the adult TG mice showed a prolonged PQ-interval and increased amplitude and widening of the QRS-complex (**Supplemental figure 2H**). These findings indicated that the autocrine VEGF-B signaling in the developing heart leads to septal defects, and, in the surviving TG mice, to cardiac hypertrophy with left ventricular (LV) dysfunction, thus providing a model of pathological cardiac hypertrophy.

VEGF-B TG mice amplify a unique cardiac EC population. To analyze cellular mechanisms that contribute to the physiological (αMHC-VEGF-B) and pathological (aP2-VEGF-B) cardiac hypertrophy, we subjected the cardiac ECs to single cell RNA sequencing (scRNA seq) analysis. Isolated cardiac ECs of adult WT, aP2-VEGF-B, and αMHC-VEGF-B mice yielded several cell types that were grouped into 15 different clusters based on their exclusive expression of marker genes (**Figure 2A and Supplemental figure 4A**). Interestingly, comparison of the clusters between the WT and TG mice identified a cell population (cluster 6) that was significantly increased in both TG models (**Figure 2A, Supplemental figure 4B**). This EC population showed high expression of a distinct set of transcripts (*Plvap, Cd24a, Chst2, Exoc3l2, Col13a1, Ces2e, Esm1,* and *Foxf1*), of which all except *Plvap* were exclusively expressed in the cluster 6 EC population. Thus, we designated these cells as VEGF-B-induced ECs (hereafter VEGFB-iECs; **Figure 2B, Supplemental figure 4C**). Further analysis showed that the VEGFB-iECs markers *Plvap, Cd24a, Esm1,* and *Ces2e* represent some of the most differentially expressed genes (DEG) among all cardiac ECs isolated from the TG vs WT mice (**Figure 2C**). These findings indicated that significant VEGF-B-specific transcriptomic changes occur in the cardiac EC population in the two TG models that express VEGF-B in the heart. We also performed scRNA seq of the adult cardiac stromovascular cell fraction (SVF) from the aP2-VEGF-B, αMHC-VEGF-B, and WT hearts to address possible VEGF-B-induced effects in other cell types. Analysis of the scRNA seq data showed 23 cell clusters that were annotated based on their expression of specific markers (**Supplemental figure 5A, B**). These included cardiac ECs, fibroblasts (FBs), and immune cells. We also captured a small population that likely represents skewed small CMCs that managed to fit within the beads used in the scRNA pipeline. Analysis of cell numbers/cluster did not indicate major differences between the WT and VEGF-B TG mice, apart from a slightly increased number of captured macrophages in the VEGF-B TG mice (**Supplemental figure 5C**). Also in the SVF cell analysis, the ECs showed increased expression of VEGFB-iEC marker transcripts in aP2-VEGF-B mice and even more prominently in αMHC-VEGF-B mice

(**Supplemental figure 5D**). Analysis of differential gene expression in cardiac FBs in the TG and WT mice did not indicate major differences, with the exception that very low levels of VEGF-B mRNA were detected in cardiac FBs in aP2-VEGF-B mice (**Supplemental figure 5E**).

Because scRNA seq analysis suggested that there is an increased number of macrophages in the VEGF-B TG vs WT hearts, we performed immunofluorescence analysis of Cd45 leukocyte and Cd206 M2 macrophage markers in cardiac sections. However, immunofluorescence analysis of cardiac sections did not indicate any significant differences between the VEGF-B TG mice or their WT littermates (**Supplemental figure 6A, B**). For further confirmation, we used RT-qPCR to check the cardiac expression of F4/80, a well-known and widely used marker of murine macrophage populations, and the inflammation markers *Vcam1*, *Il6*, *Il1-β*, and *Tnfα* (**Supplemental figure 6C**). We also analyzed the cardiac stress markers *Nppa* and *Nppb* (**Supplemental figure 6D**), but found no differences in these transcripts between the TG and WT mice.

Immunohistochemical staining of cardiac sections confirmed that Plvap marks only the endocardium in WT mice[31] (**Figure 3A**). Staining of sections from aP2-VEGF-B hearts revealed a similar pattern, plus a weaker spot-wise staining in a limited number of sub-endocardial vessels (**Figure 3A,** quantified in **Supplemental Figure 7B**). Interestingly, the αMHC-VEGF-B mice showed an additional striking pattern of expression, in a gradient-like fashion with the strongest staining in capillary-sized vessels in the immediate sub-endocardial area, gradually fading towards the mid-myocardial and epicardial areas (**Figure 3A**). To accurately localize the expanded EC population in the heart, we stained thick cardiac sections from PBS-perfused hearts with antibodies against the protein encoded by the *Cd24* marker. In the WT hearts, weak Cd24 staining decorated 5-10% of the Podocalyxin-positive endothelium (**Supplemental figure 7A, B**). In the aP2-VEGF-B mice, both sub-endocardial and sub-epicardial regions showed increased staining (in 25 and 15% of the ECs, respectively), while in the αMHC-VEGF-B mice, Cd24 staining was observed mostly in the sub-endocardial region (26% of the ECs, **Supplemental figure 7A, B**). In addition to the staining of Cd24 in the ECs, we observed occasional Cd24-positive cells within the vascular lumen. Since Cd24 is expressed in a majority of immature cells of most if not all major hematopoietic lineages[32,33], these cells were likely erythrocytes that escaped PBS perfusion. Quantifications across different regions of the heart showed a consistent increase in vessel lumen size in aP2-VEGF-B mice, whereas the αMHC-VEGF-B hearts showed a significant increase of lumens only in the mid-myocardium (**Supplemental figure 7C**). For additional confirmation of the location of the VEGFB-iECs, we co-stained Col13a1 and Plvap in cardiac sections from WT and VEGF-B TG mice. We observed clear overlap of both markers in the sub-endocardial ECs (**Supplemental figure 8A**). Overall, the superimposed expression patterns of Plvap, Cd24, and Col13a1 markers suggested that the coronary endothelium responds to VEGF-B by activation of ECs first in the sub-endocardial region, and then by a more widespread activation in the coronary vasculature.

We next validated that the expanded VEGFB-iEC-containing vessels are functional. We injected LE-lectin via the tail vein into αMHC-VEGF-B and WT littermate mice, allowed it to circulate for 10 min, and then sacrificed the mice and stained cardiac sections for Plvap. Decoration of the

cardiac Plvap-positive endothelium with lectin fluorescence confirmed that the VEGFB-iEC vessels are functional (**Supplemental figure 8B)**.

To analyze the endothelial identity of VEGFB-iECs, we checked the expression of capillary, arterial, and venous markers in them by using our scRNA seq dataset. We found increased expression of the capillary marker *Car4* and minimal expression of arterial marker *Gja4* or venous marker *Nr2f2* (**Supplemental figure 9A**). We further confirmed the capillary endothelial identity by immunofluorescence staining and found that the Plvap-positive ECs were negative for the venous marker Nr2f2 (Coup-tfII) and unlike arterial ECs, they had no adjacent smooth muscle actin (SMA) positive smooth muscle cells (**Supplemental figure 9B**). The VEGFB-iECs can thus be classified as capillary ECs.

To explore if the VEGFB-iEC markers are cardiac-specific and to compare their expression between the aP2-VEGF-B and αMHC-VEGF-B mice, we used RT-qPCR to quantify these transcripts in the heart, lungs, skeletal muscles (tibialis anterior), and livers from the TG and WT control mice (**Supplemental figure 9C**). We found that the VEGFB-iEC marker transcripts are significantly more abundant in aP2-VEGF-B than WT hearts, and most abundant in the αMHC-VEGF-B hearts (**Supplemental figure 9C**). Presumably because the aP2 promoter is weakly active also in skeletal muscle[34], six of the eight markers were more abundant in the tibialis anterior muscle in the aP2-VEGF-B mice than in the WT mice (**Supplemental figure 9C**). Thus, our results show that VEGF-B induces expansion of capillary-like ECs that exhibit specific markers, and that paracrine VEGF-B signaling is a stronger inducer of the VEGFB-iECs than autocrine VEGF-B signaling.

VEGFB-iECs are derived from the endocardium. In the heart, the expression of Plvap is restricted to the endocardium in WT mice[31] (**Figure 3A**). Furthermore, our scRNA seq analysis and immunohistochemical staining of Plvap plus SMA in the TG mice indicated that the arterial and VEGFB-iEC markers are mutually exclusive (**Supplemental figure 9A, B**). This suggested that VEGFB-iECs are endocardium-derived. To lineage trace the endocardial cells, we crossed αMHC-VEGF-B mice with BmxCreER^{T2}; Rosa26-LSL-tdTomato mice that express the inducible recombinase in endocardial ECs and in a subset of arterial ECs. We then activated the lineage tracer by tamoxifen in BmxCreER^{T2}; Rosa26-tdTomato; αMHC-VEGF-B mice and their BmxCreER^{T2}; Rosa26-tdTomato littermates at postnatal day 1 (P1) or at postnatal day 5 (P5) and analyzed the mice at six weeks of age (**Figure 3B**). Immunofluorescence analysis of cardiac sections from BmxCreER^{T2}; Rosa26-tdTomato; αMHC-VEGF-B mice showed a vascular pattern that indicated expansion of the tdTomato+ (RFP+) endothelium, most extensively in the sub-endocardial vessels, with a descending gradient towards the epicardial side of the LV wall (**Figure 3B**), whereas the BmxCreER^{T2}; Rosa26-tdTomato littermates showed labelling mainly in the endocardium and arterial ECs (**Figure 3B**). Apart from the endocardial cells and very few other scattered ECs, we did not observe any other Plvap-positive ECs in WT hearts. However, when lineage tracing was activated in BmxCreER^{T2}; Rosa26-tdTomato; αMHC-VEGF-B pups at P1, these mice showed 87.62% overlap between tdTomato (RFP) and Plvap staining at six weeks. Induction at P5 resulted in 40.07% overlap at six weeks, indicating that the VEGFB-iECs are induced during the early postnatal period (**Figure 3B**). Because recent studies have shown that

a subset of coronary arteries forms by angiogenic extension of endocardium-derived Dll4-positive vascular tunnels in the neonatal heart[35,36], we stained also Dll4 in adult ap2-VEGF-B, αMHC-VEGF-B, and WT mice. We found Dll4 in arteries coated by SMA-positive smooth muscle cells, whereas the VEGFB-iECs, identified by Plvap staining, did not show Dll4 staining (**Supplemental figure 10A**).

We were intrigued by our result showing the early postnatal endocardial contribution to the VEGFB-iECs, as the endocardium is believed not to express VEGFR-2[37], while our mechanistic hypothesis was that the VEGFB-iECs are induced by displacement of the endogenous VEGF-A from VEGFR-1 to VEGFR-2. We thus stained the endocardium for VEGFR-2 and Cd31 in P1 and in adult hearts. We detected weak VEGFR-2 staining only in some segments of the endocardial layer at P1 (**Supplemental figure 10B**). Cardiac scRNA seq data showed substantial expression of VEGFR-2 (*Kdr*) in adult endocardium and elevated expression in VEGFB-iECs. Immunostaining showed weak VEGFR-2-positive endocardial segments that were mostly located within the endocardium in between juxtaposed trabeculations that may experience only moderate shear stress from the ventricular pumping of circulating blood (**Supplemental figure 10C, D**).

To test the function of VEGFR-2 in the induction of VEGFB-iECs, we generated BmxCreERT2; Rosa26-tdTomato; αMHC-VEGF-B; VEGFR-2$^{fl/fl}$ mice and their BmxCreERT2; Rosa26-tdTomato; αMHC-VEGF-B; VEGFR-2$^{wt/wt}$ littermates, deleted VEGFR-2 in the endocardium by administering tamoxifen daily during P1-P3 and analyzed the mice at four to six weeks of age (**Supplemental figure 11A**). In contrast to the control αMHC-VEGF-B mice (**Figure 3B**), the VEGFR-2-deleted αMHC-VEGF-B mice showed very few Plvap-positive, but tdTomato (RFP) negative VEGFB-iECs in the sub-endocardial region (**Supplemental figure 11A**). Lineage tracing showed 87.62% overlap between RFP and Plvap staining in six-week-old mice induced at P1 (**Figure 3B**), indicating that only few (≃12%) VEGFB-iECs were induced prior to P1. Such cells could account for the observed VEGFB-iECs observed in the αMHC-VEGF-B mice in which VEGFR-2 had been deleted. Furthermore, the tdTomato-positive ECs in the undeleted hearts extended deeper into the myocardium than in the VEGFR-2-deleted hearts (**Supplemental figure 11A**), indicating that endocardial VEGFR-2 deletion decreases endocardial contribution to the expanding coronary vasculature. These results support the model that the VEGF-B transgene-induced activation of the endocardium during the early postnatal stages drives the formation of VEGFB-iECs that perfuse the sub-endocardial myocardium.

VEGFB-iECs do not expand during physiological cardiac hypertrophy in pregnant mice. Normal pregnancy in mice involves a marked increase in blood volume and cardiac output that is associated with growth of heart size by day 18 post-coitum and reversion of cardiac hypertrophy by day seven post-delivery (PD)[38]. To know if expansion of VEGFB-iECs was triggered during endogenous physiological cardiac hypertrophy, we isolated cardiac ECs from αMHC-VEGF-B mice and WT littermates at day 18 of pregnancy (P) and seven days PD and subjected them to scRNA seq analysis. At these timepoints, we recovered more VEGFB-iECs from the αMHC-VEGF-B mice than their WT littermates, but the results indicated that pregnancy as such did not alter the VEGFB-iECs (**Figure 4A**). Analysis of the number of cells per cluster and EC transcript expression confirmed that VEGFB-iECs were not amplified by pregnancy or after delivery (**Figure**

4B, C). Furthermore, HW/TL analysis confirmed cardiac hypertrophy at day 18 of pregnancy in WT mice and showed that VEGF-B transgene expression does not increase the physiological cardiac hypertrophy in the pregnant mice (**Figure 4D**). WB analysis showed decreased expression of the VEGF-B transgene-encoded protein by day 18 of pregnancy and 7 days post-delivery, and a parallel increase in the soluble form of VEGFR-1 (**Figure 4E**). In agreement with published data[39], transcripts related to fatty acid metabolism were upregulated on day 18 of pregnancy in both αMHC-VEGF-B TG and their WT littermate hearts (**Figure 4F**). These hearts expressed also low levels of markers of endothelial to mesenchymal transition (EndMT), inflammation, and cardiac stress (**Figure 4F**).

The VEGF-B-induced cardiac EC population is slightly expanded in mice subjected to MI. Li et al.[40] have shown that *Plvap* transcripts are induced in ECs during MI caused by ligation of the LAD coronary artery. Thus, Plvap provides an endothelial marker of cardiac neovasculogenesis[40]. To investigate if the MI-induced ECs reported by Li et al. are related to the VEGFB-iECs, we ligated the LAD in αMHC-VEGF-B mice and their WT littermates and analyzed the cardiac ECs by scRNA sequencing two days after ligation (**Figure 5A**). Of interest, whereas most of the control and αMHC-VEGF-B mice survived the LAD operation, nine out of 11 operated aP2-VEGF-B mice died within one day after the operation, thus they were not analyzed further (**Supplemental figure 12A**).

Masson trichrome staining revealed significant thinning of the LV wall in the surviving aP2-VEGF-B mice two days after LAD operation (**Figure 5B**). In echocardiography, EF% and FS% were significantly decreased in both WT and αMHC-VEGF-B mice post the LAD operation (**Supplemental table 1**). ScRNA seq comparison of all four groups indicated that the VEGFB-iECs were expanded by 4.3% by MI in the WT mice but not further increased in the αMHC-VEGF-B mice (**Figure 5C, D**). The slight increase in VEGFB-iECs cell numbers in the LAD operated WT mice was not reflected as a significant increase in the total level of the VEGFB-iEC marker transcripts in the heart (**Figure 5E**). In addition, no further increase in VEGFB-iEC marker transcripts occurred in the LAD operated vs non-operated αMHC-VEGF-B mice (**Figure 5E**). In agreement with the previously published data[40], we observed that the MI treatment induced an overall increase of *Plvap* transcripts in cardiac ECs and that the elevated *Plvap* expression was further increased in TG mice subjected to MI (**Figure 5E**). To further validate our findings, we performed RT-qPCR for VEGFB-iEC marker transcripts using cardiac lysates from all four groups, which confirmed findings from the scRNA seq analysis (**Figure 5F**). Taken together, the VEGFB-iEC population is not expanded during pregnancy and is only slightly expanded after MI. These findings suggest that VEGFB-iECs have translational potential that can be employed via gene transduction of VEGF-B in ischemic heart.

VEGF-B isoforms are differentially regulated at post-transcriptional level. As shown in **Figure 1D**, the αMHC-VEGF-B TG mice express both VEGF-B polypeptide isoforms in CMCs, whereas the aP2-VEGF-B mice express only the VEGF-B_{186} isoform in the cardiac ECs. Yet, RT-qPCR analysis indicated that the aP2-VEGF-B mice express similar overall levels of the 186 and 167 transcripts in the cardiac ECs (**Supplemental figure 12B, C**). This raised a question about the mechanism behind the differential expression of the VEGF-B isoform-encoded proteins in the

CMCs vs ECs of TG mice. To answer this question, we compared the VEGF-B RNA vs protein expression in mice injected with AAV9s encoding VEGF-B_{167}, VEGF-B_{186}, or their combination. We chose the AAV9 vector because it transduces cardiac cells very effectively[41]. After one week of vector transduction, the VEGF-B polypeptides were analyzed from blood plasma of mice treated with heparin to increase the release of the tissue-sequestered VEGF-B_{167} into blood. The results indicated that only the full-length 186 isoform and its proteolytically processed 127 aa fragment are produced by AAV9-VEGF-B_{186}, whereas no signal was obtained from parallelly treated mice expressing AAV9-VEGF-B_{167}, unless a threefold higher concentration of AAV9-VEGF-B_{167} was used (**Supplemental figure 12D**). We furthermore confirmed that the VEGF-B antibody recognizes both isoforms equally (**Supplemental figure 12E**).

We then analyzed if variation in AAV9 transduction or transgene RNA expression can explain the differences between the VEGF-B isoform levels. We first estimated the concentration of intraperitoneal (i.p.) transfected AAV in different tissues using qPCR amplification of the non-transcribed WPRE element in the vector. The highest AAV9 transduction levels were detected in the heart, spleen, subcutaneous white adipose tissue (sWAT), brown adipose tissue (BAT), and liver (**Supplemental figure 12F**). Analysis of transcripts encoding the two VEGF-B isoforms in liver and heart at one, two, and four weeks after vector transduction indicated that there is a direct correlation between the transcript expression level and the delivered AAV9 dose (**Supplemental figure 12G**). Yet, the VEGF-B_{186} isoform was expressed at a much higher level than the VEGF-B_{167} isoform in the heart at all three timepoints (**Supplemental figure 12H**). This suggested that VEGF-B_{167} was degraded at a faster rate than VEGF-B_{186} regardless of the cell of origin.

All mice treated with the same dose of AAV9-VEGF-B vector showed similar kinetics of cardiac EC proliferation that peaked at the two-week timepoint, declining thereafter (**Supplemental figure 13A, B**). Interestingly however, the mice transduced with AAV9-VEGF-B_{167} showed a trend of less cardiac EC proliferation than VEGF-B_{186} mice (**Supplemental figure 13A, B**), suggesting a faster turnover of the VEGF-B_{167} isoform. Mice injected with either vector showed cardiac hypertrophy at two and four weeks after gene delivery (**Supplemental figure 13C**), yet echocardiography did not indicate significant differences in EF% or FS% between the groups (**Supplemental figure 13D, Supplemental table 2**). To investigate if the two VEGF-B isoforms induce different downstream signaling cascades, we injected WT mice with 19.8*10^^{11}vp of AAV9 encoding either VEGF-B_{186}, VEGF-B_{167}, or a scrambled control. Two weeks later, we analysed VEGFR-1, VEGFR-2, and NRP-1 and the phosphorylation of VEGFR-1, ERK1/2, and AKT by gel electrophoresis of cardiac lysates. We found no significant difference in the phosphorylation of VEGFR-1 between the different groups (**Supplemental figure 14A, B**). However, soluble VEGFR-1 (sVEGFR-1) and the premature form of VEGFR-2[42] were significantly more abundant in the AAV9-VEGF-B_{186} and AAV9-VEGF-B_{167} hearts than in control hearts. In line with our previous findings, the mature form of VEGFR-2[42] showed a trend of higher expression in mice treated with either AAV9-VEGF-B isoform (**Supplemental figure 14A, B**). NRP-1 and the mature form of VEGFR-1 were increased in the AAV9-VEGF-B_{186} treated mice in comparison to the two other groups, perhaps because of the more abundant expression of VEGF-B_{186} than VEGF-B_{167} (**Supplemental figure 14A, B**). Probing of VEGFR-2 downstream signaling by phospho-ERK1/2

or phospho-AKT analysis did not reveal any significant differences between the groups at this timepoint (**Supplemental figure 14A, B**).

Both VEGF-B isoforms expand VEGF-B-iECs indirectly via VEGFR-2 activation. Analysis of VEGFB-iECs markers by RT-qPCR in heart lysates showed that the AAV9-VEGF-B vectors increase VEGFB-iECs transcript levels minimally at the one-week timepoint (**Supplemental figure 15A**), and more robustly at two and four weeks (**Supplemental figure 15A**). *Flt-1* gene deletion has been shown to increase VEGFR-2 signaling activity by depleting VEGFR-1 that acts as a decoy receptor for VEGF-A[12,43], thus promoting increased binding of endogenous VEGF-A to VEGFR-2[12]. To further check if expansion of the VEGFB-iEC cluster in adult mice is mediated by displacement of the endogenous VEGF-A from VEGFR-1 to VEGFR-2, we deleted VEGFR-1 in Cdh5-CreER^{T2}; VEGFR-1$^{fl/fl}$ mice[44] for two weeks, followed by analysis of the VEGFB-iEC markers. In addition, we included to the analysis mice with constitutive deletion of VEGFR-1 tyrosine kinase domain (VEGFR-1TK$^{-/-}$[45]). The results showed that, with the exception of *Col13a1*, all markers were increased in mice deleted of endothelial VEGFR-1 for two weeks but not in the mice lacking only the VEGFR-1 tyrosine kinase domain (**Supplemental figure 15B, C**). However, there were only a few Plvap-positive VEGFB-iECs in the immediate sub-endocardium in adult WT mice treated with AAV9-VEGF-B$_{186}$ vector vs scrambled control (**Supplemental figure 16A**).

To further confirm our findings, we deleted VEGFR-2 using Cdh5-CreER^{T2}[12,46], followed by administration of AAV9-VEGF-B$_{186}$ for two weeks (**Supplemental figure 16B**). The results confirmed that deletion of VEGFR-2 in ECs inhibits both the VEGF-B$_{186}$-induced cardiac hypertrophy[12] and the increase in VEGFB-iECs markers (**Supplemental figure 16C, D**). Accordingly, we conclude that amplification of VEGFB-iECs requires VEGFR-2 activity. We believe that increased VEGF-B signaling in the adult heart targets the VEGFR-2-positive endocardial cells and the vascular endothelium, and leads to preferential expansion of VEGFB-iECs as they have more abundant VEGFR-2 expression. The possibility remains that upon ischemia/cardiac damage, some of the already established VEGFB-iECs display a strong contribution to VEGF-A-induced angiogenesis as suggested by Figure 6 in our previous study[14].

Discussion

We demonstrate, using two TG models and gene transduction, that both paracrine and autocrine signaling by VEGF-B in the heart induces expansion of a unique endocardium-derived cardiac EC population during the early postnatal period. Although the VEGFB-iECs are similarly induced in both VEGF-B TG models, the gradient induced by paracrine VEGF-B secretion in αMHC-VEGF-B mice leads to a well-organized VEGFB-iEC vessel gradient descending from the sub-endocardial vessels towards the sub-epicardium in the heart. In contrast, autocrine production of VEGF-B in cardiac ECs in aP2-VEGF-B mice disrupts this pattern and results in a distinct phenotype, marked by septal defects in newborn pups, massive cardiac hypertrophy in the surviving mice, and decreased cardiac function. Comparative analysis of mice from different experiments in which we deleted endocardial VEGFR-2, or endothelial VEGFR-1, as well as from mice lacking only VEGFR-1 tyrosine kinase domain, or mice expressing AAV9-VEGF-B$_{186}$ but no VEGFR-2, indicate that the expansion of the VEGFB-iECs requires VEGFR-2 activity. In addition,

we demonstrate that AAV-VEGF-B-mediated gene delivery to adult heart causes a significant increase in VEGFB-iEC transcripts but only minimal expansion of VEGFB-iECs in the immediate sub-endocardial region. Furthermore, our comparison of the paracrine and autocrine models revealed significant cell-specific differences in the relative expression levels and decay rates of the VEGF-B_{167} and VEGF-B_{186} isoforms, which should be important for the development of a pro-angiogenic VEGF-B gene therapy.

One of the characteristic markers of the VEGFB-iECs is Plvap[47], which is constitutively expressed only in the endocardium and few venous cells in the normal heart. Plvap is upregulated by VEGF-A signal transduction[31,48,49], in agreement with our finding that both TG and viral vector-mediated expression of VEGF-B results in increased signaling by endogenous VEGF-A via VEGFR-2[12]. Plvap forms multimers that form the spokes of the wheel-patterned fenestrations that characterize endothelia with high permeability in e.g. kidney glomerulus, choroid plexus, adrenal gland, and in a number of pathological conditions[49]. A previous study by Li et al. reported increased Plvap expression in the ischemic mouse and human heart and described it as a novel endothelial-specific marker of cardiac neovasculogenesis[40].

The second most common marker of VEGFB-iECs is Cd24a, a glycosyl-phosphatidylinositol-linked surface glycoprotein, which acts as a ligand for P-selectin, a leukocyte adhesion receptor on activated ECs[50]. *Cd24* silencing has been reported to decrease human umbilical vein EC-migration and downregulated the expression of VEGF-A via inhibiting the phosphorylation and nuclear translocation of STAT3[51].

Esm1 expression marks VEGFB-iECs and the tip cells of angiogenic vessel sprouts, which have been shown to play a role in angiogenesis, inflammation, and vascular permeability and it is required for optimal response to VEGF-A stimuli and VEGF-induced vascular permeability[52]. Lineage tracing experiments have shown that the tip cells in angiogenic sprouts are derived from venous ECs, from which Esm1-positive ECs migrate into nearby vessels where they join with ECs of arterial identity[52–54]. In our model, the expanded VEGFB-iECs showed only capillary markers, thus resembling angiogenic capillary EC populations[55].

The Foxf1 transcription factor, another VEGFB-iEC marker, has also a critical role in embryonic vasculature development and in vascular sprouting[56,57]. EC deletion of *Foxf1* reduced EC proliferation, increased apoptosis, inhibited VEGF-A signaling, and decreased expression of EC genes that are critical for vascular development, such as *Vegfr-1, Vegfr-2*, and *Pecam1*[56]. The *Exoc3l2* marker gene is involved in targeting of exocytic vesicles to the cell surface[58]. Interestingly, upregulation of Exoc3l2 was reported in response to VEGF-A stimulation of primary cultures of human ECs, and *Exoc3l2* silencing showed inhibition of VEGFR-2 phosphorylation and VEGF-directed migration of cultured ECs[58].

Col13, a transmembrane collagen[59,60], has been concluded to enhance angiogenesis through a mechanism involving β1-integrins and the JNK pathway[61]. Here, we confirmed the selective expression of Col13a1 only in the VEGFB-iECs by immunofluorescent staining, whereas the steady-state endothelium in the WT heart had very little or no expression of Col13a1. It thus

seems that several of the VEGFB-iEC markers represent transcripts that are regulated by VEGF-A during angiogenic processes, yet their assessment in the context of VEGF-A stimulation of cardiac neovascularization is made difficult by the toxicity of angiogenesis-inducing levels of VEGF-A.

The VEGFB-iECs were not affected during pregnancy and they showed only a 4.3% increase in WT mice subjected to LAD ligation. Interestingly, recent analyses of transcriptomic changes in ischemic human hearts and in mice after MI[62–65], do not indicate significant expansion of VEGFB-iEC marker transcripts. Thus, the unique ability of VEGF-B to expand the VEGFB-iEC population likely underlies the protective effects of VEGF-B in ischemic heart.

Coronary vessel ECs arise from two distinct sources; the sinus venosus and the endocardium[66]. The onset of endocardial contribution to coronary vessel formation has been debated recently. Some studies reported that a second wave of coronary ECs arises from the endocardium postnatally[67], while other studies challenged the idea of postnatal contribution and proposed prenatal contribution instead[35,68]. Concerning this, the results of Tang et al.[36] are in agreement with a study showing that chemokine signaling mediated by the Cxcl12-Cxcr4 ligand-receptor pair is responsible for endocardium-derived EC migration and their contribution to coronary arteries[35]. Our study shows that VEGF-B expression in CMCs or cardiac ECs boosts the endocardium-derived supply of VEGFB-iECs during the early postnatal period. We conclude that VEGFB-iECs have a capillary-like EC phenotype without arterial markers, such as Dll4. Thus, the VEGFB-iECs represent a unique EC population that can be employed to improve perfusion in the sub-endocardial myocardium, with anastomoses to other coronary vessels.

In contrast to earlier reports[37], we find VEGFR-2-positive segments in the adult endocardium. Laminar blood flow-induced shear-stress has been shown to upregulate Krüppel-Like Factor 2 (*Klf-2*), which inhibits VEGFR-2 transcription[69]. As shown in our previous report[15], VEGFR-2 immunofluorescence in arteries that have high laminar shear stress is very low in adult mouse heart despite significant expression of *Kdr* transcripts in arterial ECs as shown in our scRNA seq data of adult cardiac ECs. Building on that concept, VEGFR-2 expression may show dynamic regulation as the shear stress in endocardial segments between trabeculae where we detected VEGFR-2 can differ from that in the endocardium facing the endocardial cavity. In addition, the VEGFB-iECs in adult mice showed increased expression of *Kdr*, which should render them more susceptible to the angiogenic effect caused by VEGF-B-mediated displacement of VEGF-A from VEGFR-1 to VEGFR-2. Accordingly, these VEGFR-2 expressing endocardial cells and the few established VEGFB-iECs in WT mice could preferentially respond to VEGF-B signaling, leading to an increase of VEGFB-iECs in the adult sub-endocardium.

We also found that the mechanisms of VEGF-B isoform regulation differ between CMCs and ECs. In particular, the VEGF-B_{167} protein was below our detection threshold in aP2-VEGF-B TG hearts, despite comparable levels of transcripts encoding the two isoforms in the same samples. This indicates that autocrine VEGF-B secretion by ECs results in lower levels of VEGF-B_{167} than its paracrine secretion from CMCs. Our data shows that similar levels of the isoform-specific RNAs encoded by the AAV vectors also result in more abundant expression of VEGF-B_{186} than VEGF-

B_{167} protein in the heart, yet both proteins increased proliferation of cardiac ECs with similar time-dependent kinetics. Thus, VEGF-B_{186} induced more proliferation of ECs than VEGF-B_{167}, presumably because VEGF-B_{186} has higher concentration. However, comparison of VEGF-B isoforms through probing of VEGFR-2 downstream signaling did not reveal any significant differences between the groups at two weeks post-AAV delivery. Our results indicate post-transcriptional regulation of VEGF-B isoform expression in the heart in a cell type-specific manner. VEGF-B_{167} contains heparin sulfate binding domain sequestering it largely to the cell surface[18], which increases VEGF-B_{167} concentration on the cell surface, resulting in a more rapid but apparently shorter-lived stimulation of ECs than with VEGF-B_{186}. A likely reason for such difference is that unlike VEGF-B_{167}, secreted VEGF-B_{186} escapes cell surface binding because it does not bind NRP-1 or heparan sulfate until it is cleaved by, as yet unknown protease(s) available in the EC microenvironment.

CMCs produce VEGF-B, which binds VEGFR-1 and NRP-1 on the surface of ECs, inducing a shift of endogenous VEGF-A to VEGFR-2[12,70–72]. VEGF-A signaling was shown to induce angiogenic sprouting in the early postnatal retina, where tip cell migration depends on a gradient of the heparan sulfate-binding VEGF-A_{165} isoform, whereas the VEGF-A_{121} isoform that does not bind to heparan sulfate and therefore does not form a concentration gradient, promoted only EC proliferation in a concentration-dependent manner[26]. Consistent with these phenotypes, the cardiac blood vessel density in the sub-endocardial area, where the vascular effect of paracrine VEGF-B was most prominent in the αMHC-VEGF-B mice, was lower in the autocrine aP2-VEGF-B mice, but because of increased EC proliferation, the vessels were larger than in the WT or αMHC-VEGF-B mice. We hypothesize that the cardiac septal defects in the autocrine aP2-VEGF-B mouse model arise from lack of a VEGF-B gradient for angiogenic sprouting, whereas proliferation is retained in the VEGF-B-producing ECs. In part via an angiocrine effect on other cardiac cells, the aP2-VEGF-B mice then develop a massive postnatal heart size with a scattered pattern of expanded VEGFB-iECs-positive vessels. We thus posit that the mismatch between CMC growth and the vascular growth pattern leads to the septal defects and pathological cardiac phenotype.

Interestingly, circulating VEGF-B levels have been found to be inversely correlated with ongoing LV remodeling in patients with heart failure[73,74]. Furthermore, VEGF-B_{186} gene transfer was reported to decrease scarring and improve perfusion after LAD-induced MI[14]. Also, VEGF-B_{167} gene transfer provided protection against non-ischemic heart failure in a canine cardiac tachypacing model[19,20]. Although numerous reports have indicated that increasing VEGF-B in the heart has translational potential for treatment of cardiac ischemia and heart failure, one should proceed to clinical studies with caution[19–22,75], as one study has reported ventricular arrhythmias in pigs subjected to VEGF-B_{186} adenoviral gene transfer before MI[21]. In addition, constitutive overexpression of human VEGF-B_{167} in CMCs caused increased death of adult TG mice apparently because of mitochondrial lipotoxicity[25]. Despite these concerns, VEGF-B holds therapeutic potential due to its ability to redirect endogenous levels of VEGF-A to VEGFR-2 without inducing vascular leakage. By understanding the molecular and cellular mechanisms of VEGF-B action via increased endogenous VEGF-A-VEGFR-2 signaling, VEGF-B could eventually be safely tested as a time- and concentration-controlled therapeutic modality in cardiac

ischemia. VEGF-B could be especially beneficial to combat age-related health issues as murine studies with minimal increase of VEGF-A signaling have suggested that counteracting age-related VEGF-A signaling insufficiency promotes healthy aging and extends life span[76].

Author contributions

IS, MR, PP, RK, and KA designed the study. IS, MR, PP, KAH, DR, AT, YvW, and SA acquired the data. IS analyzed data and prepared the figures. IS and KA interpreted the data. KA supervised the study and provided funding. IS, RK, and KA wrote the manuscript. IS and PP performed all experiments to address reviewers' comments. IS, PP, and KA wrote the reviewers' response letter. LE contributed to the generation of aP2-VEGF-B transgenic mouse line and provided Col13a1 antibodies. EM contributed to the LAD experiments and TAC experiments in revision phase. PS contributed to vascular leakage experiments in revision phase. MRI acquisition was performed in the laboratory of SYH. Validating expression of VEGF-B in different cardiac cell types was performed by DR in SE lab. All authors have seen, commented, and accepted the manuscript.

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Disclosures

The authors declare no conflict of interest related to the contents of this manuscript.

Supplemental Material

Supplemental detailed methods
Supplemental figures S1–S16
Supplemental tables S1–S3

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Figure Legends

Figure 1. Septal defects and massive pathological cardiac hypertrophy caused by autocrine VEGFB expression in coronary ECs. (A) Illustration of the aP2-VEGF-B transgene encoding both VEGF-B isoforms (VEGF-B_{186} and VEGF-B_{167}). **(B)** RT-qPCR analysis of mV*egf-b* RNA in ECs, CFs, and CMCs isolated from the aP2-mVEGF-B mice (n=3 WT, 4 aP2). **(C)** Immunohistochemical staining of aP2 (FABP4) and VEGF-B in cryosections from the hearts of adult aP2-VEGF-B and their WT littermate mice. Scale bars 50 μm. **(D)** WB analysis of VEGF-B and β-actin in heart lysates and isolated ECs from adult aP2-VEGF-B, αMHC-VEGF-B and WT mice. Quantifications show the fold expression normalized to β-actin. # indicates a background band. **(E)** Macroscopic images showing the cardiac hypertrophy phenotype in aP2-VEGF-B mice and quantification of HW/BW ratio indicating significant hypertrophy (n=9 WT, 7 aP2). Scale bar 2 mm. **(F)** *Ex vivo* μCT scans of aP2-VEGF-B and littermate hearts on P0. Arrows point to the septal defects. **(G)** Echocardiography parameters from aP2-VEGF-B heart and WT littermates at P14 (n=8 WT, 4 aP2) and at 10 weeks (n=9 WT, 7 aP2). **(H)** Cardiac MRI values from aP2-VEGF-B and WT littermate hearts (n=5). *P, unpaired two-tailed t-test. Welch correction was used when variance was significantly different.

Figure 2. Single cell RNA sequencing of cardiac ECs from VEGF-B TG mice reveals expansion of a unique EC population. (A) UMAP plot showing clusters obtained from integrated

analysis of cardiac ECs isolated from aP2-VEGF-B, αMHC-VEGF-B, and WT adult mice. **(B)** Dot plot showing the expression percentage of VEGFB-iECs and EC markers in aP2-VEGF-B, αMHC-VEGF-B, and WT mice. **(C)** Volcano plots showing the highest DEGs across all cardiac ECs from aP2-VEGF-B or αMHC-VEGF-B vs WT mice.

Figure 3. VEGFB-iECs are derived from the endocardium and reside in the sub-endocardium. (A) Immunohistochemical staining of Plvap and Podocalyxin in cardiac cryosections from aP2-VEGF-B, αMHC-VEGF-B and WT mice. Scale bars 500 μm and 100 μm, respectively. **(B)** Immunohistochemical staining of RFP and Plvap in 200 μm thick cardiac sections from six-week-old BmxCreERT2; Rosa26-tdTomato; αMHC-VEGF-B and their BmxCreERT2; Rosa26-tdTomato littermates that were induced with tamoxifen on P1 or P5. Scale bar 100 μm.

Figure 4. Cardiac hypertrophy in pregnant mice does not cause expansion of VEGFB-iECs. (A) UMAP plot showing clusters obtained from integrated analysis of cardiac ECs from non-pregnant female αMHC-VEGF-B, and WT littermate mice in comparison to 18 days pregnant mice and 7 days after delivery. **(B)** Bar-plot showing comparison of the percentage of each cell population across all 6 groups. **(C)** Violin plots showing expression of VEGFB-iECs cluster markers in all 6 groups. **(D)** HW/TL comparison in 18-day pregnant vs non-pregnant mice, and at 7 days post-delivery from WT and αMHC-VEGF-B TG mice (n=6 WT, 9 WT_P, 8 αMHC, 8 αMHC_P, 2 WT_PD, 3 αMHC_PD). **(E)** WB analysis of heart lysates from all 6 groups for hVEGF-B, VEGFR-1, and VEGFR-2. **(F)** Dot plot showing average expression and expression percentage of markers of fatty acid metabolism, EndMT, inflammation, and cardiac stress across all 6 groups. *P, unpaired two-tailed t-test.

Figure 5. VEGFB-iECs are not expanded by MI in the TG mice. (A) Diagram indicating the LAD ligation site and the timeline of the experiments. **(B)** Masson trichrome staining of cardiac sections obtained from adult aP2-VEGF-B, αMHC-VEGF-B and WT mice. Scale bars 500 μm. **(C)** UMAP plot showing clusters obtained from integrated analysis of cardiac ECs isolated from WT and αMHC-VEGF-B mice under normal conditions and 2 days post LAD ligation. **(D)** Bar plot showing comparison of the percentage of each cell population across all 4 groups. **(E)** Violin plots showing expression of VEGFB-iECs markers in all 4 groups. **(F)** RT-qPCR data showing expression of VEGFB-iECs marker transcripts in heart lysates. *P, one-way ANOVA with Dunnett's correction.