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

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#### 1 Abstract

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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.

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9 Methods. We used single cell RNA sequencing (scRNA seq) to compare cardiac endothelial gene 10 expression in VEGF-B transgenic mouse models. Lineage tracing was used to identify the origin 11 of a VEGF-B-induced novel endothelial cell (EC) population, and AAV-mediated gene delivery to 12 compare the effects of VEGF-B isoforms. Cardiac function was investigated using 13 echocardiography, magnetic resonance imaging, and micro-computed tomography.

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15 **Results.** Unlike in physiological cardiac hypertrophy driven by a cardiomyocyte-specific VEGF-B 16 transgene (αMHC-VEGF-B), autocrine VEGF-B expression in cardiac endothelium (aP2-VEGF-17 B) was associated with septal defects and failure to increase perfused sub-endocardial capillaries 18 postnatally. Paracrine VEGF-B led to a robust proliferation and myocardial migration of a novel 19 cardiac EC lineage (VEGFB-iECs) of endocardial origin, whereas autocrine VEGF-B increased 20 proliferation of VEGFB-iECs, but failed to promote their migration and efficient contribution to 21 myocardial capillaries. The surviving aP2-VEGF-B offspring showed an altered ratio of secreted 22 VEGF-B isoforms and developed massive pathological cardiac hypertrophy with a distinct cardiac 23 vessel pattern. In the normal heart, we found a small VEGFB-iEC population that was only 24 minimally expanded during myocardial infarction but not during physiological cardiac hypertrophy 25 associated with mouse pregnancy.

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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.

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#### **1** Novelty and Significance

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#### 3 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.
- 8 VEGF-B has two isoforms, which have distinct biochemical and functional properties.
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### 10 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.
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19 Induction of myocardial vessel growth could alleviate cardiomyocyte damage during myocardial 20 ischemia. However, attempts to use VEGF-A for this purpose have been halted because it 21 promotes vascular leakage and inflammation. In contrast, VEGF-B provides a promising 22 therapeutic tool in the heart as it causes very little vascular leakage or inflammation. Furthermore, 23 very high doses of VEGF-B are well tolerated because it functions indirectly via endogenous 24 VEGF-A signaling. Our present study shows that VEGF-B production by cardiac endothelial cells 25 (ECs) leads to cardiac pathology. Comparison of the vascular phenotypes in the two transgenic 26 models revealed that VEGF-B overexpression induces a novel cardiac EC lineage (VEGFB-iECs) 27 that contributes robustly to sub-endocardial vessels upon paracrine expression, but very little 28 upon autocrine expression. In contrast, the VEGFB-iECs were not expanded in cardiac 29 hypertrophy associated with mouse pregnancy and they showed only slight expansion during 30 myocardial infarction caused by coronary vessel ligation. The endocardium-derived VEGFB-iECs 31 should provide a vascular marker for the targeting and monitoring of therapeutic attempts to 32 salvage myocardial tissue in critical ischemia. Furthermore, our findings on the cell type-specific 33 regulation of VEGF-B isoforms provide a valuable basis for further translational development of 34 VEGF-B. 35

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1	Non-standard Abbreviations and Acronyms		
2	AAV9	Adeno-associated virus 9	
3	aP2	Adipocyte protein 2	
4	BAT	Brown adipose tissue	
5	BCA	Bicinchoninic acid	
6	BSA	Bovine serum albumin	
7	CAG	CMV early enhancer/chicken β-actin (CAG)	
8		promoter	
9	Cdh5	Cadherin 5	
10	CFs	Cardiac fibroblasts	
11	CMCs	Cardiomyocytes	
12	Cre-ER <sup>T2</sup>	Cre recombinase fused to a triple mutant form	
13		of the human estrogen receptor	
14	DEGs	Differentially expressed genes	
15	ECG	Electrocardiography	
16	ECs	Endothelial cells	
17	EDTA	Ethylenediaminetetraacetic acid	
18	EdU	5-Ethynyl-2'deoxyuridine	
19	EF	Ejection fraction	
20	EGTA	Egtazic acid	
21	ELISA	Enzyme-linked immunosorbent assay	
22	FABP4	Fatty acid binding protein 4	
23	FACS	Fluorescence-activated cell sorting	
24	FBs	Fibroblasts	
25	FS	Fractional shortening	
26	HCI	Hydrochloric acid	
27	HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic	
28		acid	
29	HRP	Horseradish peroxidase	
30	HSC70	Heat shock cognate protein	
31	HW/BW	Heart weight / body weight	
32	HW/TL	Heart weight / tibial length	
33	l.p.	Intraperitoneal	
34	IR	Infrared	
35	KCI	Potassium chloride	
36	KH <sub>2</sub> PO <sub>4</sub>	Potassium dihydrogen phosphate	
37	KHCO <sub>3</sub>	Potassium bicarbonate	
38	LAD	Left anterior descending	
39	LV	Left ventricle	
40	MgCl <sub>2</sub>	Magnesium chloride	
41	MgSO <sub>4</sub>	Magnesium sulfate	
42	MI	Myocardial infarction	

1	MRI	Magnetic resonance imaging
2	Na <sub>2</sub> HPO <sub>4</sub>	Disodium hydrogen phosphate
3	Na <sub>4</sub> P <sub>2</sub> O <sub>7</sub>	Tetrasodium pyrophosphate
4	NaCl	Sodium chloride
5	NaF	Sodium fluoride
6	NaHCO₃	Sodium bicarbonate
7	NRP-1	Neuropilin-1
8	OCT	Optimal cutting temperature compound
9	Р	Pregnant
10	PBS	Phosphate-buffered saline
11	PD	Post-delivery
12	PFA	Paraformaldehyde
13	PVDF	Polyvinylidene difluoride
14	RFP	Red fluorescent protein
15	RT	Room temperature
16	RT-qPCR	Quantitative real-time PCR
17	ScRNA seq	Single cell RNA sequencing
18	SMA	Smooth muscle actin
19	SVF	Stromovascular fraction
20	sWAT	Subcutaneous white adipose tissue
21	TG	Transgenic
22	UMAP	Uniform Manifold Approximation and Projection
23	VEGF-A	Vascular endothelial growth factor A
24	VEGF-B	Vascular endothelial growth factor B
25	VEGFB-iECs	VEGF-B induced endothelial cells
26	VEGFR-1	Vascular endothelial growth factor receptor 1
27	VEGFR-2	Vascular endothelial growth factor receptor 2
28	VEGFRs	Vascular endothelial growth factor receptors
29	VEGFs	Vascular endothelial growth factors
30	WB	Western blot
31	WPRE	Woodchuck hepatitis virus post-transcriptional
32		regulatory element
33	WT	Wild-type
34	αMHC	Myosin heavy chain alpha
35	μCΤ	Micro-computed tomography
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#### 1 Introduction

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3 Ischemic heart disease is the number one cause of mortality worldwide<sup>1,2</sup>. Cardiac ischemia 4 induced by occlusion of a coronary vessel endangers a massive sudden loss of cardiomyocytes 5 (CMCs) that exceeds the regenerative capacity of the myocardium<sup>3</sup>. Due to their angiogenic 6 properties, vascular endothelial growth factors (VEGFs) and their receptors (VEGFRs) provide 7 promising therapeutic tools for alleviation of cardiac ischemia prior to CMC damage<sup>4</sup>. Attempts to use VEGF-A gene transfer as a therapeutic tool to induce angiogenesis<sup>5-9</sup> have been hindered 8 9 by VEGF-induced vascular leakage and tissue inflammation<sup>10,11</sup>. More recent studies using VEGF-B gene transduction have shown that VEGF-B activates the endogenous VEGF/VEGFR-10 2 signaling pathway activity<sup>12–15</sup>, without resulting in significant tissue inflammation or vascular 11 12 leakage<sup>13,16</sup>.

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The Vegfb gene encodes two protein isoforms, VEGF-B<sub>186</sub> and VEGF-B<sub>167</sub>, which are secreted 14 15 by CMCs in the mammalian heart. Both isoforms bind to VEGFR-1, expressed on the surface of 16 cardiac endothelial cells (ECs)<sup>17</sup>. After its secretion, VEGF-B<sub>186</sub> undergoes proteolytic cleavage, 17 resulting in VEGF-B<sub>127</sub> isoform<sup>18</sup>. Unlike VEGF-B<sub>186</sub>, VEGF-B<sub>167</sub> and VEGF-B<sub>127</sub> bind also to the neuropilin-1 (NRP-1) co-receptor<sup>18</sup>. Previous studies have shown that a CMC-specific αMHC-18 19 VEGF-B transgene that produces both VEGF-B<sub>186</sub> and VEGF-B<sub>167</sub> isoforms expands the cardiac vasculature and leads to a physiological-like cardiac hypertrophy<sup>12–15</sup>. Transgenic (TG) rats 20 21 expressing the aMHC-VEGF-B transgene have normal cardiac function and show an improved cardiac ejection fraction (EF%) and fractional shortening (FS%) after myocardial infarction (MI) 22 23 caused by ligation of the left anterior descending (LAD) coronary artery<sup>15</sup>. Furthermore, 24 expression of VEGF-B<sub>186</sub> in mice via adeno-associated virus (AAV) vector delivery decreased 25 scarring and improved cardiac perfusion after MI<sup>14</sup>, and VEGF-B<sub>167</sub> gene transfer provided 26 protection against non-ischemic heart failure in a canine cardiac tachypacing model<sup>19,20</sup>. However, 27 in one study, excessive levels of VEGF-B<sub>186</sub>, and in particular of its cleaved VEGF-B<sub>127</sub> form, predisposed the hypertrophic heart to arrhythmias after MI or dobutamine treatment, which both 28 29 increase ectopic ventricular activity<sup>21,22</sup>.

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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<sup>23</sup>. 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<sup>24</sup>. However, in long-term experiments, TG mice expressing only the human VEGF-B<sub>167</sub> isoform in CMCs showed an increased death rate, apparently because of mitochondrial lipotoxicity<sup>25</sup>.

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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 1 migration during sprouting angiogenesis occurs towards higher concentrations of VEGF-A in a 2 gradient generated by VEGF-A expressing cells<sup>26</sup>. We wanted to establish a cardiac model of 3 autocrine VEGF-B signaling that would stimulate EC proliferation, but not directed migration. For 4 5 this, we expressed the VEGF-B transgene as an autocrine ligand in the coronary endothelium, 6 under control of adipocyte protein 2 (aP2/FABP4) promoter<sup>23</sup> and compared its effects to those in the cardiomyocyte-specific paracrine model (αMHC-VEGF-B)13, with particular attention to 7 8 coronary vessel growth and cardiac hypertrophy. 9 10 Our results indicate that autocrine VEGF-B signaling that lacks the paracrine VEGF-B gradient 11 for EC migration, induces a scattered pattern of novel VEGFB-iECs, which however fail to 12 contribute to sub-endocardial angiogenesis as much as in the paracrine model. The autocrine 13 VEGF-B signaling led to septal defects already during embryogenesis. The surviving TG mice

- 14 enabled us to characterize the pathological angiogenesis in postnatal mice. We also studied this
- 15 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 16 17 results indicate that VEGF-B can enhance cardiac angiogenesis in normal and ischemic heart in
- 18 a unique way that should allow the development of safe VEGF-B-based therapies for cardiac
- 19 ischemia patients.
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#### 21 Methods

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23 See detailed methods section in the Supplemental material.

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25 Mouse models. All animal experimental procedures were approved by the National Animal 26 Experiment Board following the regulations of the EU and national legislation. The Supplemental 27 material contains detailed information on the TG rodent lines, experimental procedures, and 28 treatments used in the study. The numbers of mice used in each experiment are indicated in the 29 respective figure legends.

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31 Cardiac echography, electrocardiography (ECG), and magnetic resonance imaging (MRI). 32 Analysis of cardiac function was performed under isoflurane anesthesia using the Vevo 2100

33 Ultrasound system (FujiFilm VisualSonics Inc). Parameters listed in Supplemental tables 1 and 2 34 were calculated using the 2D M-mode. Lead II ECG signals were acquired using limb electrodes 35 and the processing of digital signals was performed using the previously published program in

- 36 Matlab (MathWorks, Natick, MA)<sup>27</sup>. Cardiac MRI was carried out as described earlier<sup>28</sup>.
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38 Ligation of the LAD coronary artery. Induction of cardiac ischemia and subsequent MI was 39 achieved by ligating left anterior descending (LAD) coronary artery in adult mice as described 40 earlier<sup>14</sup>.

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42 Statistical Analysis. Values are indicated as mean ± SEM. Prism 9 software was used for 43 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  $\leq 0.05$ , (\*\*)  $\leq 0.01$ , and (\*\*\*)  $\leq 0.001$ .
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#### 4 Data availability

5 All methods and study materials are available to other researchers upon reasonable request. All 6 single cell RNA sequencing data will be deposited to the GEO database.

- 7
- 8 Results
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10 Production and analysis of TG mice expressing VEGF-B in coronary endothelium. Endogenous cardiac EC proliferation and migration processes are driven predominantly by 11 paracrine signaling via VEGF<sup>4,29</sup>. We aimed to create a model that short-circuits the signals for 12 13 EC migration along the paracrine growth factor gradient and to retain the signals for EC 14 proliferation. Accordingly, we employed aP2-VEGF-B TG mice that express the mouse VEGF-B 15 gene under the aP2/fatty acid binding protein 4 (FABP4) promoter (Figure 1A)<sup>23</sup>, which is activated in coronary ECs starting at embryonic day E14.5<sup>30</sup>. At this timepoint in wild-type (WT) 16 17 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 18 blood vascular ECs start to express aP2/FABP4<sup>30</sup>. As shown by the comparison of VEGF-B RNA 19 expression between WT and TG mice in Figure 1B, we detected highly elevated levels of VEGF-20 21 B RNA in the coronary ECs isolated by FACS from ten weeks old aP2-VEGF-B mice 22 (Supplemental figure 1), but not in CMCs or cardiac fibroblasts (CFs) isolated from the same 23 mice. VEGF-B immunostaining of cardiac tissue sections confirmed specific expression of the 24 transgene in cardiac ECs (Figure 1C). Western blotting (WB) analysis of the cardiac lysates and 25 isolated cardiac ECs from the aP2-VEGF-B mice showed expression of the VEGF-B<sub>186</sub> isoform, 26 but, surprisingly, no expression of VEGF-B<sub>167</sub> (**Figure 1D**). The mouse VEGF-B<sub>186</sub> isoform was 27 detected also in sera from the TG mice at an average concentration of 10-15 ng/ml 28 (Supplemental figure 2A), whereas VEGF-B concentration in sera from WT mice was below the 29 ELISA detection level.

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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**).

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**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

1 TG pups die or are eaten by the mothers during the first hours after birth. This differed significantly 2 from the aMHC-VEGF-B mice that express the transgene in CMCs and are born in normal 3 Mendelian ratios (Supplemental figure 2E). Supplemental figure 2F shows a comparison of cardiac hypertrophy development in the aP2-VEGF-B and  $\alpha$ MHC-VEGF-B pups at P0, P7, P14. 4 5 and P28. As can be seen from the data, at P0, there is no significant difference in HW/BW ratios 6 between the two, but both the αMHC-VEGF-B mice<sup>14</sup> and the aP2-VEGF-B mice show clear 7 hypertrophy already at P7 (Supplemental figure 2F). We then used µCT imaging to compare the 8 TG hearts of pups that died postnatally at P0 to the hearts of their WT littermates. Intriguingly, we 9 observed cardiac ventricular septal defects of varying severity in the TG pups (Figure 1F and 10 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 11 12 in the adult TG mice (Figure 1G), which was confirmed by magnetic resonance imaging (Figure 13 **1H, Supplemental figure 2G**). Electrocardiography<sup>27</sup> of the adult TG mice showed a prolonged 14 PQ-interval and increased amplitude and widening of the QRS-complex (Supplemental figure 15 **2H**). These findings indicated that the autocrine VEGF-B signaling in the developing heart leads 16 to septal defects, and, in the surviving TG mice, to cardiac hypertrophy with left ventricular (LV) 17 dysfunction, thus providing a model of pathological cardiac hypertrophy.

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19 VEGF-B TG mice amplify a unique cardiac EC population. To analyze cellular mechanisms 20 that contribute to the physiological ( $\alpha$ MHC-VEGF-B) and pathological (aP2-VEGF-B) cardiac 21 hypertrophy, we subjected the cardiac ECs to single cell RNA sequencing (scRNA seq) analysis. 22 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 23 24 genes (Figure 2A and Supplemental figure 4A). Interestingly, comparison of the clusters 25 between the WT and TG mice identified a cell population (cluster 6) that was significantly 26 increased in both TG models (Figure 2A, Supplemental figure 4B). This EC population showed 27 high expression of a distinct set of transcripts (Plvap, Cd24a, Chst2, Exoc3l2, Col13a1, Ces2e, 28 Esm1, and Foxf1), of which all except Plvap were exclusively expressed in the cluster 6 EC 29 population. Thus, we designated these cells as VEGF-B-induced ECs (hereafter VEGFB-iECs; 30 Figure 2B, Supplemental figure 4C). Further analysis showed that the VEGFB-iECs markers 31 Plvap, Cd24a, Esm1, and Ces2e represent some of the most differentially expressed genes 32 (DEG) among all cardiac ECs isolated from the TG vs WT mice (Figure 2C). These findings 33 indicated that significant VEGF-B-specific transcriptomic changes occur in the cardiac EC 34 population in the two TG models that express VEGF-B in the heart. We also performed scRNA 35 seq of the adult cardiac stromovascular cell fraction (SVF) from the aP2-VEGF-B, αMHC-VEGF-36 B, and WT hearts to address possible VEGF-B-induced effects in other cell types. Analysis of the 37 scRNA seq data showed 23 cell clusters that were annotated based on their expression of specific 38 markers (Supplemental figure 5A, B). These included cardiac ECs, fibroblasts (FBs), and 39 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 40 did not indicate major differences between the WT and VEGF-B TG mice, apart from a slightly 41 42 increased number of captured macrophages in the VEGF-B TG mice (Supplemental figure 5C). 43 Also in the SVF cell analysis, the ECs showed increased expression of VEGFB-iEC marker 44 transcripts in aP2-VEGF-B mice and even more prominently in aMHC-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).

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5 Because scRNA seq analysis suggested that there is an increased number of macrophages in 6 the VEGF-B TG vs WT hearts, we performed immunofluorescence analysis of Cd45 leukocyte 7 and Cd206 M2 macrophage markers in cardiac sections. However, immunofluorescence analysis 8 of cardiac sections did not indicate any significant differences between the VEGF-B TG mice or 9 their WT littermates (Supplemental figure 6A, B). For further confirmation, we used RT-qPCR 10 to check the cardiac expression of F4/80, a well-known and widely used marker of murine macrophage populations, and the inflammation markers Vcam1, II6, II1- $\beta$ , and  $Tnf\alpha$ 11 12 (Supplemental figure 6C). We also analyzed the cardiac stress markers Nppa and Nppb 13 (Supplemental figure 6D), but found no differences in these transcripts between the TG and WT 14 mice.

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16 Immunohistochemical staining of cardiac sections confirmed that Plvap marks only the 17 endocardium in WT mice<sup>31</sup> (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 18 19 (Figure 3A, quantified in Supplemental Figure 7B). Interestingly, the αMHC-VEGF-B mice 20 showed an additional striking pattern of expression, in a gradient-like fashion with the strongest 21 staining in capillary-sized vessels in the immediate sub-endocardial area, gradually fading 22 towards the mid-myocardial and epicardial areas (Figure 3A). To accurately localize the 23 expanded EC population in the heart, we stained thick cardiac sections from PBS-perfused hearts 24 with antibodies against the protein encoded by the Cd24 marker. In the WT hearts, weak Cd24 25 staining decorated 5-10% of the Podocalyxin-positive endothelium (Supplemental figure 7A, B). 26 In the aP2-VEGF-B mice, both sub-endocardial and sub-epicardial regions showed increased 27 staining (in 25 and 15% of the ECs, respectively), while in the αMHC-VEGF-B mice, Cd24 staining 28 was observed mostly in the sub-endocardial region (26% of the ECs, Supplemental figure 7A, 29 B). In addition to the staining of Cd24 in the ECs, we observed occasional Cd24-positive cells 30 within the vascular lumen. Since Cd24 is expressed in a majority of immature cells of most if not all major hematopoietic lineages<sup>32,33</sup>, these cells were likely erythrocytes that escaped PBS 31 32 perfusion. Quantifications across different regions of the heart showed a consistent increase in 33 vessel lumen size in aP2-VEGF-B mice, whereas the  $\alpha$ MHC-VEGF-B hearts showed a significant 34 increase of lumens only in the mid-myocardium (Supplemental figure 7C). For additional 35 confirmation of the location of the VEGFB-iECs, we co-stained Col13a1 and Plvap in cardiac 36 sections from WT and VEGF-B TG mice. We observed clear overlap of both markers in the sub-37 endocardial ECs (Supplemental figure 8A). Overall, the superimposed expression patterns of 38 Plvap, Cd24, and Col13a1 markers suggested that the coronary endothelium responds to VEGF-39 B by activation of ECs first in the sub-endocardial region, and then by a more widespread 40 activation in the coronary vasculature.

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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).

3

4 To analyze the endothelial identity of VEGFB-iECs, we checked the expression of capillary. 5 arterial, and venous markers in them by using our scRNA seg dataset. We found increased 6 expression of the capillary marker Car4 and minimal expression of arterial marker Gja4 or venous 7 marker Nr2f2 (Supplemental figure 9A). We further confirmed the capillary endothelial identity 8 by immunofluorescence staining and found that the Plvap-positive ECs were negative for the 9 venous marker Nr2f2 (Coup-tfII) and unlike arterial ECs, they had no adjacent smooth muscle 10 actin (SMA) positive smooth muscle cells (Supplemental figure 9B). The VEGFB-iECs can thus 11 be classified as capillary ECs.

12

13 To explore if the VEGFB-iEC markers are cardiac-specific and to compare their expression 14 between the aP2-VEGF-B and  $\alpha$ MHC-VEGF-B mice, we used RT- $\alpha$ PCR to quantify these 15 transcripts in the heart, lungs, skeletal muscles (tibialis anterior), and livers from the TG and WT 16 control mice (Supplemental figure 9C). We found that the VEGFB-iEC marker transcripts are 17 significantly more abundant in aP2-VEGF-B than WT hearts, and most abundant in the αMHC-18 VEGF-B hearts (Supplemental figure 9C). Presumably because the aP2 promoter is weakly 19 active also in skeletal muscle<sup>34</sup>, six of the eight markers were more abundant in the tibialis anterior 20 muscle in the aP2-VEGF-B mice than in the WT mice (Supplemental figure 9C). Thus, our 21 results show that VEGF-B induces expansion of capillary-like ECs that exhibit specific markers, 22 and that paracrine VEGF-B signaling is a stronger inducer of the VEGFB-iECs than autocrine 23 VEGF-B signaling.

24

25 **VEGFB-iECs are derived from the endocardium.** In the heart, the expression of Plvap is 26 restricted to the endocardium in WT mice<sup>31</sup> (Figure 3A). Furthermore, our scRNA seg analysis 27 and immunohistochemical staining of Plvap plus SMA in the TG mice indicated that the arterial 28 and VEGFB-iEC markers are mutually exclusive (Supplemental figure 9A, B). This suggested 29 that VEGFB-iECs are endocardium-derived. To lineage trace the endocardial cells, we crossed 30 aMHC-VEGF-B mice with BmxCreER<sup>12</sup>; Rosa26-LSL-tdTomato mice that express the inducible recombinase in endocardial ECs and in a subset of arterial ECs. We then activated the lineage 31 tracer by tamoxifen in BmxCreER<sup>T2</sup>; Rosa26-tdTomato; αMHC-VEGF-B mice and their 32 BmxCreER<sup>T2</sup>; Rosa26-tdTomato littermates at postnatal day 1 (P1) or at postnatal day 5 (P5) and 33 34 analyzed the mice at six weeks of age (Figure 3B). Immunofluorescence analysis of cardiac sections from BmxCreER<sup>T2</sup>; Rosa26-tdTomato; αMHC-VEGF-B mice showed a vascular pattern 35 36 that indicated expansion of the tdTomato+ (RFP+) endothelium, most extensively in the sub-37 endocardial vessels, with a descending gradient towards the epicardial side of the LV wall (Figure 38 **3B**), whereas the BmxCreER<sup>T2</sup>; Rosa26-tdTomato littermates showed labelling mainly in the 39 endocardium and arterial ECs (Figure 3B). Apart from the endocardial cells and very few other 40 scattered ECs, we did not observe any other Plvap-positive ECs in WT hearts. However, when lineage tracing was activated in BmxCreER<sup>T2</sup>; Rosa26-tdTomato; αMHC-VEGF-B pups at P1, 41 these mice showed 87.62% overlap between tdTomato (RFP) and Plvap staining at six weeks. 42 43 Induction at P5 resulted in 40.07% overlap at six weeks, indicating that the VEGFB-iECs are 44 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<sup>35,36</sup>, 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

5 figure 10A).

6

7 We were intrigued by our result showing the early postnatal endocardial contribution to the 8 VEGFB-iECs, as the endocardium is believed not to express VEGFR-2<sup>37</sup>, while our mechanistic 9 hypothesis was that the VEGFB-iECs are induced by displacement of the endogenous VEGF-A 10 from VEGFR-1 to VEGFR-2. We thus stained the endocardium for VEGFR-2 and Cd31 in P1 and 11 in adult hearts. We detected weak VEGFR-2 staining only in some segments of the endocardial 12 layer at P1 (Supplemental figure 10B). Cardiac scRNA seq data showed substantial expression 13 of VEGFR-2 (Kdr) in adult endocardium and elevated expression in VEGFB-iECs. 14 Immunostaining showed weak VEGFR-2-positive endocardial segments that were mostly located within the endocardium in between juxtaposed trabeculations that may experience only moderate 15 16 shear stress from the ventricular pumping of circulating blood (Supplemental figure 10C, D).

17

18 To test the function of VEGFR-2 in the induction of VEGFB-iECs, we generated BmxCreERT2; Rosa26-tdTomato; αMHC-VEGF-B; VEGFR-2<sup>fl/fl</sup> mice and their BmxCreERT2; Rosa26-tdTomato; 19 αMHC-VEGF-B; VEGFR-2<sup>wt/wt</sup> littermates, deleted VEGFR-2 in the endocardium by administering 20 21 tamoxifen daily during P1-P3 and analyzed the mice at four to six weeks of age (Supplemental 22 figure 11A). In contrast to the control αMHC-VEGF-B mice (Figure 3B), the VEGFR-2-deleted 23 aMHC-VEGF-B mice showed very few Plvap-positive, but tdTomato (RFP) negative VEGFB-iECs 24 in the sub-endocardial region (Supplemental figure 11A). Lineage tracing showed 87.62% 25 overlap between RFP and Plvap staining in six-week-old mice induced at P1 (Figure 3B), 26 indicating that only few (~12%) VEGFB-iECs were induced prior to P1. Such cells could account 27 for the observed VEGFB-iECs observed in the  $\alpha$ MHC-VEGF-B mice in which VEGFR-2 had been 28 deleted. Furthermore, the tdTomato-positive ECs in the undeleted hearts extended deeper into 29 the myocardium than in the VEGFR-2-deleted hearts (Supplemental figure 11A), indicating that 30 endocardial VEGFR-2 deletion decreases endocardial contribution to the expanding coronary 31 vasculature. These results support the model that the VEGF-B transgene-induced activation of 32 the endocardium during the early postnatal stages drives the formation of VEGFB-iECs that 33 perfuse the sub-endocardial myocardium.

34

35 VEGFB-iECs do not expand during physiological cardiac hypertrophy in pregnant mice. 36 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 37 by day seven post-delivery (PD)<sup>38</sup>. To know if expansion of VEGFB-iECs was triggered during 38 39 endogenous physiological cardiac hypertrophy, we isolated cardiac ECs from αMHC-VEGF-B 40 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 aMHC-41 42 VEGF-B mice than their WT littermates, but the results indicated that pregnancy as such did not 43 alter the VEGFB-iECs (Figure 4A). Analysis of the number of cells per cluster and EC transcript 44 expression confirmed that VEGFB-iECs were not amplified by pregnancy or after delivery (Figure

1 **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 2 3 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-4 5 delivery, and a parallel increase in the soluble form of VEGFR-1 (Figure 4E). In agreement with published data<sup>39</sup>, transcripts related to fatty acid metabolism were upregulated on day 18 of 6 7 pregnancy in both aMHC-VEGF-B TG and their WT littermate hearts (Figure 4F). These hearts 8 expressed also low levels of markers of endothelial to mesenchymal transition (EndMT), 9 inflammation, and cardiac stress (Figure 4F).

10

11 **The VEGF-B-induced cardiac EC population is slightly expanded in mice subjected to MI.** 12 Li et al.<sup>40</sup> have shown that *Plvap* transcripts are induced in ECs during MI caused by ligation of 13 the LAD coronary artery. Thus, Plvap provides an endothelial marker of cardiac 14 neovasculogenesis<sup>40</sup>. To investigate if the MI-induced ECs reported by Li et al. are related to the 15 VEGFB-iECs, we ligated the LAD in  $\alpha$ MHC-VEGF-B mice and their WT littermates and analyzed 16 the cardiac ECs by scRNA sequencing two days after ligation (**Figure 5A**). Of interest, whereas 17 most of the control and  $\alpha$ MHC-VEGF-B mice survived the LAD operation, nine out of 11 operated

18 aP2-VEGF-B mice died within one day after the operation, thus they were not analyzed further

- 19 (Supplemental figure 12A).
- 20

21 Masson trichrome staining revealed significant thinning of the LV wall in the surviving aP2-VEGF-22 B mice two days after LAD operation (Figure 5B). In echocardiography, EF% and FS% were 23 significantly decreased in both WT and aMHC-VEGF-B mice post the LAD operation 24 (Supplemental table 1). ScRNA seq comparison of all four groups indicated that the VEGFB-25 iECs were expanded by 4.3% by MI in the WT mice but not further increased in the αMHC-VEGF-26 B mice (Figure 5C, D). The slight increase in VEGFB-iECs cell numbers in the LAD operated WT 27 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 28 29 transcripts occurred in the LAD operated vs non-operated αMHC-VEGF-B mice (Figure 5E). In 30 agreement with the previously published data<sup>40</sup>, we observed that the MI treatment induced an 31 overall increase of *Plvap* transcripts in cardiac ECs and that the elevated *Plvap* expression was 32 further increased in TG mice subjected to MI (Figure 5E). To further validate our findings, we 33 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-34 35 iEC population is not expanded during pregnancy and is only slightly expanded after MI. These 36 findings suggest that VEGFB-iECs have translational potential that can be employed via gene 37 transduction of VEGF-B in ischemic heart.

38

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<sub>186</sub> isoform in the cardiac ECs. Yet, RTqPCR 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

1 CMCs vs ECs of TG mice. To answer this guestion, we compared the VEGF-B RNA vs protein expression in mice injected with AAV9s encoding VEGF-B<sub>167</sub>, VEGF-B<sub>186</sub>, or their combination. 2 3 We chose the AAV9 vector because it transduces cardiac cells very effectively<sup>41</sup>. After one week 4 of vector transduction, the VEGF-B polypeptides were analyzed from blood plasma of mice 5 treated with heparin to increase the release of the tissue-sequestered VEGF-B<sub>167</sub> into blood. The results indicated that only the full-length 186 isoform and its proteolytically processed 127 aa 6 7 fragment are produced by AAV9-VEGF-B<sub>186</sub>, whereas no signal was obtained from parallelly 8 treated mice expressing AAV9-VEGF-B<sub>167</sub>, unless a threefold higher concentration of AAV9-9 VEGF-B<sub>167</sub> was used (**Supplemental figure 12D**). We furthermore confirmed that the VEGF-B 10 antibody recognizes both isoforms equally (Supplemental figure 12E).

11

12 We then analyzed if variation in AAV9 transduction or transgene RNA expression can explain the 13 differences between the VEGF-B isoform levels. We first estimated the concentration of 14 intraperitoneal (i.p.) transfected AAV in different tissues using gPCR amplification of the non-15 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 16 17 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 18 19 correlation between the transcript expression level and the delivered AAV9 dose (Supplemental 20 figure 12G). Yet, the VEGF-B<sub>186</sub> isoform was expressed at a much higher level than the VEGF-B<sub>167</sub> isoform in the heart at all three timepoints (Supplemental figure 12H). This suggested that 21 22 VEGF-B<sub>167</sub> was degraded at a faster rate than VEGF-B<sub>186</sub> regardless of the cell of origin.

23

24 All mice treated with the same dose of AAV9-VEGF-B vector showed similar kinetics of cardiac 25 EC proliferation that peaked at the two-week timepoint, declining thereafter (Supplemental figure **13A**, **B**). Interestingly however, the mice transduced with AAV9-VEGF-B<sub>167</sub> showed a trend of 26 27 less cardiac EC proliferation than VEGF-B<sub>186</sub> mice (Supplemental figure 13A, B), suggesting a 28 faster turnover of the VEGF-B<sub>167</sub> isoform. Mice injected with either vector showed cardiac hypertrophy at two and four weeks after gene delivery (Supplemental figure 13C), yet 29 echocardiography did not indicate significant differences in EF% or FS% between the groups 30 31 (Supplemental figure 13D, Supplemental table 2). To investigate if the two VEGF-B isoforms 32 induce different downstream signaling cascades, we injected WT mice with 19.8\*10<sup>^11</sup>vp of AAV9 33 encoding either VEGF-B<sub>186</sub>, VEGF-B<sub>167</sub>, or a scrambled control. Two weeks later, we analysed 34 VEGFR-1, VEGFR-2, and NRP-1 and the phosphorylation of VEGFR-1, ERK1/2, and AKT by gel 35 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 36 VEGFR-1 (sVEGFR-1) and the premature form of VEGFR-2<sup>42</sup> were significantly more abundant 37 38 in the AAV9-VEGF-B<sub>186</sub> and AAV9-VEGF-B<sub>167</sub> hearts than in control hearts. In line with our previous findings, the mature form of VEGFR-2<sup>42</sup> showed a trend of higher expression in mice 39 treated with either AAV9-VEGF-B isoform (Supplemental figure 14A, B). NRP-1 and the mature 40 form of VEGFR-1 were increased in the AAV9-VEGF-B<sub>186</sub> treated mice in comparison to the two 41 42 other groups, perhaps because of the more abundant expression of VEGF-B<sub>186</sub> than VEGF-B<sub>167</sub> 43 (Supplemental figure 14A, B). Probing of VEGFR-2 downstream signaling by phospho-ERK1/2

1 or phospho-AKT analysis did not reveal any significant differences between the groups at this 2 timepoint (**Supplemental figure 14A, B**).

3

4 Both VEGF-B isoforms expand VEGF-B-iECs indirectly via VEGFR-2 activation. Analysis of 5 VEGFB-iECs markers by RT-gPCR in heart lysates showed that the AAV9-VEGF-B vectors increase VEGFB-iECs transcript levels minimally at the one-week timepoint (Supplemental 6 7 figure 15A), and more robustly at two and four weeks (Supplemental figure 15A). Flt-1 gene 8 deletion has been shown to increase VEGFR-2 signaling activity by depleting VEGFR-1 that acts as a decoy receptor for VEGF-A<sup>12,43</sup>, thus promoting increased binding of endogenous VEGF-A 9 10 to VEGFR-2<sup>12</sup>. To further check if expansion of the VEGFB-iEC cluster in adult mice is mediated 11 by displacement of the endogenous VEGF-A from VEGFR-1 to VEGFR-2, we deleted VEGFR-1 in Cdh5-CreER<sup>T2</sup>; VEGFR-1<sup>1/fl</sup> mice<sup>44</sup> for two weeks, followed by analysis of the VEGFB-iEC 12 markers. In addition, we included to the analysis mice with constitutive deletion of VEGFR-1 13 tyrosine kinase domain (VEGFR-1TK<sup>-/-45</sup>). The results showed that, with the exception of Col13a1, 14 all markers were increased in mice deleted of endothelial VEGFR-1 for two weeks but not in the 15 16 mice lacking only the VEGFR-1 tyrosine kinase domain (Supplemental figure 15B, C). However, 17 there were only a few Plvap-positive VEGFB-iECs in the immediate sub-endocardium in adult WT mice treated with AAV9-VEGF-B<sub>186</sub> vector vs scrambled control (**Supplemental figure 16A**). 18 19

20 To further confirm our findings, we deleted VEGFR-2 using Cdh5-CreER<sup>T212,46</sup>, followed by administration of AAV9-VEGF-B<sub>186</sub> for two weeks (Supplemental figure 16B). The results 21 22 confirmed that deletion of VEGFR-2 in ECs inhibits both the VEGF-B<sub>186</sub>-induced cardiac 23 hypertrophy<sup>12</sup> and the increase in VEGFB-iECs markers (Supplemental figure 16C, D). 24 Accordingly, we conclude that amplification of VEGFB-iECs requires VEGFR-2 activity. We 25 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-26 27 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 28 29 contribution to VEGF-A-induced angiogenesis as suggested by Figure 6 in our previous study<sup>14</sup>.

30

#### 31 Discussion

32

33 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 34 35 EC population during the early postnatal period. Although the VEGFB-iECs are similarly induced 36 in both VEGF-B TG models, the gradient induced by paracrine VEGF-B secretion in aMHC-VEGF-B mice leads to a well-organized VEGFB-iEC vessel gradient descending from the sub-37 38 endocardial vessels towards the sub-epicardium in the heart. In contrast, autocrine production of 39 VEGF-B in cardiac ECs in aP2-VEGF-B mice disrupts this pattern and results in a distinct 40 phenotype, marked by septal defects in newborn pups, massive cardiac hypertrophy in the 41 surviving mice, and decreased cardiac function. Comparative analysis of mice from different 42 experiments in which we deleted endocardial VEGFR-2, or endothelial VEGFR-1, as well as from 43 mice lacking only VEGFR-1 tyrosine kinase domain, or mice expressing AAV9-VEGF-B<sub>186</sub> but no 44 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<sub>167</sub> and VEGF-B<sub>186</sub> isoforms, which should be important for the development of a proangiogenic VEGF-B gene therapy.

7

8 One of the characteristic markers of the VEGFB-iECs is Plvap<sup>47</sup>, which is constitutively expressed 9 only in the endocardium and few venous cells in the normal heart. Plvap is upregulated by VEGF-10 A signal transduction<sup>31,48,49</sup>, in agreement with our finding that both TG and viral vector-mediated 11 expression of VEGF-B results in increased signaling by endogenous VEGF-A via VEGFR-2<sup>12</sup>. 12 Plvap forms multimers that form the spokes of the wheel-patterned fenestrations that characterize 13 endothelia with high permeability in e.g. kidney glomerulus, choroid plexus, adrenal gland, and in a number of pathological conditions<sup>49</sup>. A previous study by Li et al. reported increased Plvap 14 expression in the ischemic mouse and human heart and described it as a novel endothelial-15 specific marker of cardiac neovasculogenesis<sup>40</sup>. 16

17

18 The second most common marker of VEGFB-iECs is Cd24a, a glycosyl-phosphatidylinositol-19 linked surface glycoprotein, which acts as a ligand for P-selectin, a leukocyte adhesion receptor 20 on activated ECs<sup>50</sup>. *Cd24* silencing has been reported to decrease human umbilical vein EC-21 migration and downregulated the expression of VEGF-A via inhibiting the phosphorylation and 22 nuclear translocation of STAT3<sup>51</sup>.

23

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<sup>52</sup>.
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<sup>52-54</sup>. In our model, the expanded VEGFB-iECs showed only capillary markers,
thus resembling angiogenic capillary EC populations<sup>55</sup>.

31

32 The Foxf1 transcription factor, another VEGFB-iEC marker, has also a critical role in embryonic vasculature development and in vascular sprouting<sup>56,57</sup>. EC deletion of *Foxf1* reduced EC 33 proliferation, increased apoptosis, inhibited VEGF-A signaling, and decreased expression of EC 34 genes that are critical for vascular development, such as Vegfr-1, Vegfr-2, and Pecam1<sup>56</sup>. The 35 *Exoc3/2* marker gene is involved in targeting of exocytic vesicles to the cell surface<sup>58</sup>. Interestingly, 36 upregulation of Exoc3l2 was reported in response to VEGF-A stimulation of primary cultures of 37 38 human ECs, and Exoc3/2 silencing showed inhibition of VEGFR-2 phosphorylation and VEGF-39 directed migration of cultured ECs<sup>58</sup>.

40

41 Col13, a transmembrane collagen<sup>59,60</sup>, has been concluded to enhance angiogenesis through a 42 mechanism involving  $\beta$ 1-integrins and the JNK pathway<sup>61</sup>. Here, we confirmed the selective 43 expression of Col13a1 only in the VEGFB-iECs by immunofluorescent staining, whereas the 44 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

3 cardiac neovascularization is made difficult by the toxicity of angiogenesis-inducing levels of

- 4 VEGF-A.
- 5

6 The VEGFB-iECs were not affected during pregnancy and they showed only a 4.3% increase in 7 WT mice subjected to LAD ligation. Interestingly, recent analyses of transcriptomic changes in 8 ischemic human hearts and in mice after MI<sup>62–65</sup>, do not indicate significant expansion of VEGFB-9 iEC marker transcripts. Thus, the unique ability of VEGF-B to expand the VEGFB-iEC population

10 likely underlies the protective effects of VEGF-B in ischemic heart.

11

12 Coronary vessel ECs arise from two distinct sources; the sinus venosus and the endocardium<sup>66</sup>. 13 The onset of endocardial contribution to coronary vessel formation has been debated recently. 14 Some studies reported that a second wave of coronary ECs arises from the endocardium 15 postnatally<sup>67</sup>, while other studies challenged the idea of postnatal contribution and proposed prenatal contribution instead<sup>35,68</sup>. Concerning this, the results of Tang et al.<sup>36</sup> are in agreement 16 with a study showing that chemokine signaling mediated by the Cxcl12-Cxcr4 ligand-receptor pair 17 18 is responsible for endocardium-derived EC migration and their contribution to coronary arteries<sup>35</sup>. 19 Our study shows that VEGF-B expression in CMCs or cardiac ECs boosts the endocardium-20 derived supply of VEGFB-iECs during the early postnatal period. We conclude that VEGFB-iECs 21 have a capillary-like EC phenotype without arterial markers, such as DII4. Thus, the VEGFB-iECs 22 represent a unique EC population that can be employed to improve perfusion in the sub-23 endocardial myocardium, with anastomoses to other coronary vessels.

24

25 In contrast to earlier reports<sup>37</sup>, we find VEGFR-2-positive segments in the adult endocardium. 26 Laminar blood flow-induced shear-stress has been shown to upregulate Krüppel-Like Factor 2 (*Klf-2*), which inhibits VEGFR-2 transcription<sup>69</sup>. As shown in our previous report<sup>15</sup>, VEGFR-2 27 28 immunofluorescence in arteries that have high laminar shear stress is very low in adult mouse 29 heart despite significant expression of Kdr transcripts in arterial ECs as shown in our scRNA seq 30 data of adult cardiac ECs. Building on that concept, VEGFR-2 expression may show dynamic 31 regulation as the shear stress in endocardial segments between trabeculae where we detected 32 VEGFR-2 can differ from that in the endocardium facing the endocardial cavity. In addition, the 33 VEGFB-iECs in adult mice showed increased expression of Kdr, which should render them more 34 susceptible to the angiogenic effect caused by VEGF-B-mediated displacement of VEGF-A from 35 VEGFR-1 to VEGFR-2. Accordingly, these VEGFR-2 expressing endocardial cells and the few 36 established VEGFB-iECs in WT mice could preferentially respond to VEGF-B signaling, leading 37 to an increase of VEGFB-iECs in the adult sub-endocardium.

38

39 We also found that the mechanisms of VEGF-B isoform regulation differ between CMCs and ECs.

40 In particular, the VEGF-B<sub>167</sub> protein was below our detection threshold in aP2-VEGF-B TG hearts,

41 despite comparable levels of transcripts encoding the two isoforms in the same samples. This

42 indicates that autocrine VEGF-B secretion by ECs results in lower levels of VEGF-B<sub>167</sub> than its

43 paracrine secretion from CMCs. Our data shows that similar levels of the isoform-specific RNAs

44 encoded by the AAV vectors also result in more abundant expression of VEGF-B<sub>186</sub> than VEGF-

1 B<sub>167</sub> protein in the heart, yet both proteins increased proliferation of cardiac ECs with similar time-2 dependent kinetics. Thus, VEGF-B<sub>186</sub> induced more proliferation of ECs than VEGF-B<sub>167</sub>, 3 presumably because VEGF-B<sub>186</sub> has higher concentration. However, comparison of VEGF-B 4 isoforms through probing of VEGFR-2 downstream signaling did not reveal any significant 5 differences between the groups at two weeks post-AAV delivery. Our results indicate post-6 transcriptional regulation of VEGF-B isoform expression in the heart in a cell type-specific manner. 7 VEGF-B<sub>167</sub> contains heparin sulfate binding domain sequestering it largely to the cell surface<sup>18</sup>, 8 which increases VEGF-B<sub>167</sub> concentration on the cell surface, resulting in a more rapid but 9 apparently shorter-lived stimulation of ECs than with VEGF-B<sub>186</sub>. A likely reason for such 10 difference is that unlike VEGF-B<sub>167</sub>, secreted VEGF-B<sub>186</sub> 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 11 12 in the EC microenvironment.

13

14 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<sup>12,70-72</sup>. VEGF-A signaling was shown to induce 15 angiogenic sprouting in the early postnatal retina, where tip cell migration depends on a gradient 16 17 of the heparan sulfate-binding VEGF-A<sub>165</sub> isoform, whereas the VEGF-A<sub>121</sub> isoform that does not bind to heparan sulfate and therefore does not form a concentration gradient, promoted only EC 18 19 proliferation in a concentration-dependent manner<sup>26</sup>. Consistent with these phenotypes, the 20 cardiac blood vessel density in the sub-endocardial area, where the vascular effect of paracrine 21 VEGF-B was most prominent in the αMHC-VEGF-B mice, was lower in the autocrine aP2-VEGF-22 B mice, but because of increased EC proliferation, the vessels were larger than in the WT or 23 aMHC-VEGF-B mice. We hypothesize that the cardiac septal defects in the autocrine aP2-VEGF-24 B mouse model arise from lack of a VEGF-B gradient for angiogenic sprouting, whereas 25 proliferation is retained in the VEGF-B-producing ECs. In part via an angiocrine effect on other 26 cardiac cells, the aP2-VEGF-B mice then develop a massive postnatal heart size with a scattered 27 pattern of expanded VEGFB-iECs-positive vessels. We thus posit that the mismatch between 28 CMC growth and the vascular growth pattern leads to the septal defects and pathological cardiac 29 phenotype.

30

31 Interestingly, circulating VEGF-B levels have been found to be inversely correlated with ongoing LV remodeling in patients with heart failure<sup>73,74</sup>. Furthermore, VEGF-B<sub>186</sub> gene transfer was 32 reported to decrease scarring and improve perfusion after LAD-induced MI<sup>14</sup>. Also, VEGF-B<sub>167</sub> 33 gene transfer provided protection against non-ischemic heart failure in a canine cardiac 34 tachypacing model<sup>19,20</sup>. Although numerous reports have indicated that increasing VEGF-B in the 35 heart has translational potential for treatment of cardiac ischemia and heart failure, one should 36 proceed to clinical studies with caution<sup>19–22,75</sup>, as one study has reported ventricular arrhythmias 37 in pigs subjected to VEGF-B<sub>186</sub> adenoviral gene transfer before MI<sup>21</sup>. In addition, constitutive 38 39 overexpression of human VEGF-B<sub>167</sub> in CMCs caused increased death of adult TG mice 40 apparently because of mitochondrial lipotoxicity<sup>25</sup>. Despite these concerns, VEGF-B holds 41 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 42 43 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 44

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

3 VEGF-A signaling insufficiency promotes healthy aging and extends life span<sup>76</sup>.

4

### 5 Author contributions

6 IS, MR, PP, RK, and KA designed the study. IS, MR, PP, KAH, DR, AT, YvW, and SA acquired 7 the data. IS analyzed data and prepared the figures. IS and KA interpreted the data. KA 8 supervised the study and provided funding. IS, RK, and KA wrote the manuscript. IS and PP 9 performed all experiments to address reviewers' comments. IS, PP, and KA wrote the reviewers' 10 response letter. LE contributed to the generation of aP2-VEGF-B transgenic mouse line and 11 provided Col13a1 antibodies. EM contributed to the LAD experiments and TAC experiments in 12 revision phase. PS contributed to vascular leakage experiments in revision phase. MRI acquisition 13 was performed in the laboratory of SYH. Validating expression of VEGF-B in different cardiac cell 14 types was performed by DR in SE lab. All authors have seen, commented, and accepted the 15 manuscript.

16

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## 42 **Disclosures**

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

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# 2 Supplemental Material

- 3 Supplemental detailed methods
- 4 Supplemental figures S1–S16
- 5 Supplemental tables S1–S3
- 6

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#### 25 Figure Legends

26

27 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 28 29 encoding both VEGF-B isoforms (VEGF-B<sub>186</sub> and VEGF-B<sub>167</sub>). (B) RT-qPCR analysis of mVegf-b RNA in ECs, CFs, and CMCs isolated from the aP2-mVEGF-B mice (n=3 WT, 4 aP2). (C) 30 31 Immunohistochemical staining of aP2 (FABP4) and VEGF-B in cryosections from the hearts of 32 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 33 34 mice. Quantifications show the fold expression normalized to  $\beta$ -actin. # indicates a background 35 band. (E) Macroscopic images showing the cardiac hypertrophy phenotype in aP2-VEGF-B mice 36 and quantification of HW/BW ratio indicating significant hypertrophy (n=9 WT, 7 aP2). Scale bar 37 2 mm. (F) Ex vivo µCT scans of aP2-VEGF-B and littermate hearts on P0. Arrows point to the 38 septal defects. (G) Echocardiography parameters from aP2-VEGF-B heart and WT littermates at 39 P14 (n=8 WT, 4 aP2) and at 10 weeks (n=9 WT, 7 aP2). (H) Cardiac MRI values from aP2-VEGF-40 B and WT littermate hearts (n=5). \*P, unpaired two-tailed t-test. Welch correction was used when 41 variance was significantly different.

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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.

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6 Figure 3. VEGFB-iECs are derived from the endocardium and reside in the sub-7 endocardium. (A) Immunohistochemical staining of Plvap and Podocalyxin in cardiac 8 cryosections from aP2-VEGF-B, αMHC-VEGF-B and WT mice. Scale bars 500 µm and 100 µm, 9 respectively. (B) Immunohistochemical staining of RFP and Plvap in 200 µm thick cardiac 10 sections from six-week-old BmxCreERT2; Rosa26-tdTomato; αMHC-VEGF-B and their 11 BmxCreERT2; Rosa26-tdTomato littermates that were induced with tamoxifen on P1 or P5. Scale 12 bar 100 µm.

13

14 Figure 4. Cardiac hypertrophy in pregnant mice does not cause expansion of VEGFB-iECs.

15 (A) UMAP plot showing clusters obtained from integrated analysis of cardiac ECs from non-16 pregnant female  $\alpha$ MHC-VEGF-B, and WT littermate mice in comparison to 18 days pregnant mice 17 and 7 days after delivery. (B) Bar-plot showing comparison of the percentage of each cell 18 population across all 6 groups. (C) Violin plots showing expression of VEGFB-iECs cluster 19 markers in all 6 groups. (D) HW/TL comparison in 18-day pregnant vs non-pregnant mice, and at 20 7 days post-delivery from WT and aMHC-VEGF-B TG mice (n=6 WT, 9 WT P, 8 aMHC, 8 21 αMHC P, 2 WT PD, 3 αMHC PD). (E) WB analysis of heart lysates from all 6 groups for hVEGF-22 B, VEGFR-1, and VEGFR-2. (F) Dot plot showing average expression and expression percentage 23 of markers of fatty acid metabolism, EndMT, inflammation, and cardiac stress across all 6 groups.

24 \*P, unpaired two-tailed t-test.

25

26 Figure 5. VEGFB-iECs are not expanded by MI in the TG mice. (A) Diagram indicating the 27 LAD ligation site and the timeline of the experiments. (B) Masson trichrome staining of cardiac 28 sections obtained from adult aP2-VEGF-B, αMHC-VEGF-B and WT mice. Scale bars 500 μm. (C) 29 UMAP plot showing clusters obtained from integrated analysis of cardiac ECs isolated from WT 30 and  $\alpha$ MHC-VEGF-B mice under normal conditions and 2 days post LAD ligation. (D) Bar plot 31 showing comparison of the percentage of each cell population across all 4 groups. (E) Violin plots 32 showing expression of VEGFB-iECs markers in all 4 groups. (F) RT-gPCR data showing 33 expression of VEGFB-iECs marker transcripts in heart lysates. \*P, one-way ANOVA with 34 Dunnett's correction.