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Lack of cardiac and high-fat diet induced metabolic phenotypes in two independent strains of *Vegf-b* knockout mice

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Vascular endothelial growth factor-B (VEGF-B) has been implicated to play a significant role in coronary vessel growth and endothelial uptake and transport of fatty acids in heart and skeletal muscle. Additionally, recent studies have shown that *Vegf-b* deficiency protects from high-fat diet (HFD)-induced diabetes and insulin resistance. We compared the cardiac function and the effects of HFD on body composition and glucose metabolism in two available *Vegf-b* knockout (*Vegf-b*$^{-/-}$ strains) mouse strains side by side with their respective littermate controls. We found no differences in HFD-induced weight gain, glucose tolerance or insulin resistance between the *Vegf-b*$^{-/-}$ strains and their littermate control mice. Furthermore, there was no difference in basal cardiac function and cardiac expression of genes involved in glucose or fatty acid metabolism between the *Vegf-b*$^{-/-}$ strains and their littermate control mice. We conclude that VEGF-B is dispensable for normal cardiac function under unstressed conditions and for HFD-induced metabolic changes.

As the prevalence of cardiovascular diseases increases together with obesity and type 2 diabetes, targeting a growth factor involved in cardiac metabolism, angiogenesis and whole body metabolism could have significant therapeutic implications. Vascular endothelial growth factor-B (VEGF-B), the most abundant VEGF in the heart[1], mediates its signaling via binding to VEGF-Receptor 1 (VEGF-R1/Flt-1)[2] and neuropilin 1 (Nrp1)[3] receptor. VEGF-B has been suggested to play an important role in cardiac metabolism[4,5], but the mechanisms are still largely unknown. Long-term *Vegf-b*$_{167}$ over-expression in the heart of transgenic mice elevated ceramide levels and decreased triglyceride (TG) levels[4]. In contrast, *Vegf-b* knockout mice showed decreased fatty acid (FA) and increased glucose uptake in the heart[5]. More recently VEGF-B was shown to stimulate coronary vessel growth and arterialization[6–9] and to induce a shift from FA oxidation towards glucose oxidation in the heart[10].

To investigate the role of VEGF-B two constitutive *Vegf-b*$^{-/-}$ knockout models have been generated. The *Vegf-b* deficient mice of both strains were healthy and fertile, and had minimal phenotypes. The mice published by Bellomo *et al.*[11] showed decreased heart size, reduced left ventricle (LV) wall thickness and impaired recovery from experimentally induced myocardial ischemia via transient coronary occlusion *ex vivo*[11] (here referred to as Bellomo mice), while the other *Vegf-b*$^{-/-}$ strain (Aase mice) displayed a prolonged PQ interval in electrocardiograms, indicating a delayed atrioventricular impulse conduction[12]. Despite these minor differences in the phenotypes, both publications on the *Vegf-b*$^{-/-}$ models speculated that *Vegf-b* has a protective role in the normal or ischemic heart. Such an idea is supported by experiments with adenoviral VEGF-B gene delivery and VEGF-B protein delivery, indicating angiogenic effects in the ischemic heart[7,8].

Recent reports have drawn attention to the findings showing that the Aase *Vegf-b*$^{-/-}$ mice gain more weight than control mice by shunting lipids more effectively to white adipose tissue (WAT)[5], and that VEGF-B neutralization results in better glucose and insulin responses[13]. But as suggested by Carmeliet *et al.*[14] confirmation of the anti-diabetic, yet obese, phenotype in another *Vegf-b*$^{-/-}$ strain would strengthen these findings. Further concern was raised by the results of Kivelä *et al.*[10], who found no difference in cardiac or skeletal muscle fatty acid uptake

between wildtype and *Vegf-b*$^{-/-}$ rats. Here we report the first direct side by side comparison of body composition, glucose metabolism and cardiac function in both of the available *Vegf-b*$^{-/-}$ strains[11,12] under identical unstressed environmental conditions. Our results show that, despite different targeting constructs and previously observed findings, *Vegf-b* deficiency causes only minor, if any, changes in cardiac phenotype and high-fat diet (HFD)-induced metabolic responses.

Results

Confirmation of *Vegf-b* deletion and sequence of the *Vegf-b* targeting constructs. We first confirmed that both littermate control groups expressed similar levels of VEGF-B (Fig. 1a and b). To confirm that neither *Vegf-b*$^{-/-}$ strain expresses VEGF-B, we analyzed the VEGF-B mRNA expression in the heart (Fig. 1). The VEGF-B exon 1-2 RT-PCR indicated at least 10,000 fold decreased VEGF-B mRNA concentration in the hearts of both gene targeted mouse strains (Fig. 1a). However, an unexpectedly high amount of signal was obtained from the Aase strain by RT-PCR using primers from VEGF-B exons 6 and 7 (Fig. 1b). To rule out the possibility of a NeoR-VEGF-B exon 6-7 fusion protein expression in the Aase mice, the inserted NeoR knockout construct and its junctions with the *Vegf-b* gene were sequenced (Fig. 1c). Sequence analysis verified the presence of polyadenylation signals and a stop codon in the NeoR construct, as expected, rendering the generation of NeoR-VEGF-B exon 6-7 fusion protein highly unlikely (Fig. 1c). Further analysis of the junction sites confirmed the correct insertion of the targeting construct in exon 3 and upstream of exon 5 and that the NeoR construct contained the NeoR gene preceded by a PGK promoter. Because the 3′ homology arm used to create the Aase *Vegf-b*$^{-/-}$ mice contains the sequence of exon 6 but not exon 7 sequences[12], the presence of the exon 6-7 mRNA, detected by RT-PCR, further verifies a *bona fide* insertion of the targeting construct in the genomic DNA. Absence of any residual VEGF-B exon 6-7 expression in the Bellomo *Vegf-b*$^{-/-}$ mice implies that the β-geo construct has successfully replaced exons 3-6 (note that exon 6 is named exon 7 in the original report by Bellomo[11]). Sequencing of both the 3′ and 5′ insertion sites of the β-geo cassette in the Bellomo *Vegf-b*$^{-/-}$ mice showed that the construct was properly integrated into the *Vegf-b* genomic DNA (Fig. 1c).

No cardiac phenotype of *Vegf-b*$^{-/-}$ strains on standard diet. To compare the cardiac phenotypes of Bellomo and Aase *Vegf-b*$^{-/-}$ mice on standard diet, we performed echocardiography to determine the left ventricular volume (LV Vol) in diastole (d) and systole (s), the thickness of LV wall (LVAW; d, s, LVPW; d, s) and LV mass, LV internal diameter (LVID; d, s) and functionality of the heart as indicated by the ejection fraction (EF) and fractional shortening (FS; Table 1). The results showed no differences in these parameters when the *Vegf-b*$^{-/-}$ mice were compared to their respective controls or to each other (Table 1). Furthermore, no differences were found between the electrocardiography recordings between the study groups (Table 1).

Response to a Western-type diet. To investigate possible differences in body weight and glucose metabolism as reported for the Aase *Vegf-b*$^{-/-}$ mice[5,13], we analyzed the response of the Bellomo *Vegf-b*$^{-/-}$ mice to a Western-type diet (WD; 42% calories from fat), starting at 14 weeks of age. The control group was maintained on a standard diet (SD). Upon WD, no difference was observed in body weight in the Bellomo *Vegf-b*$^{-/-}$ mice versus their littermate controls. However, the SD

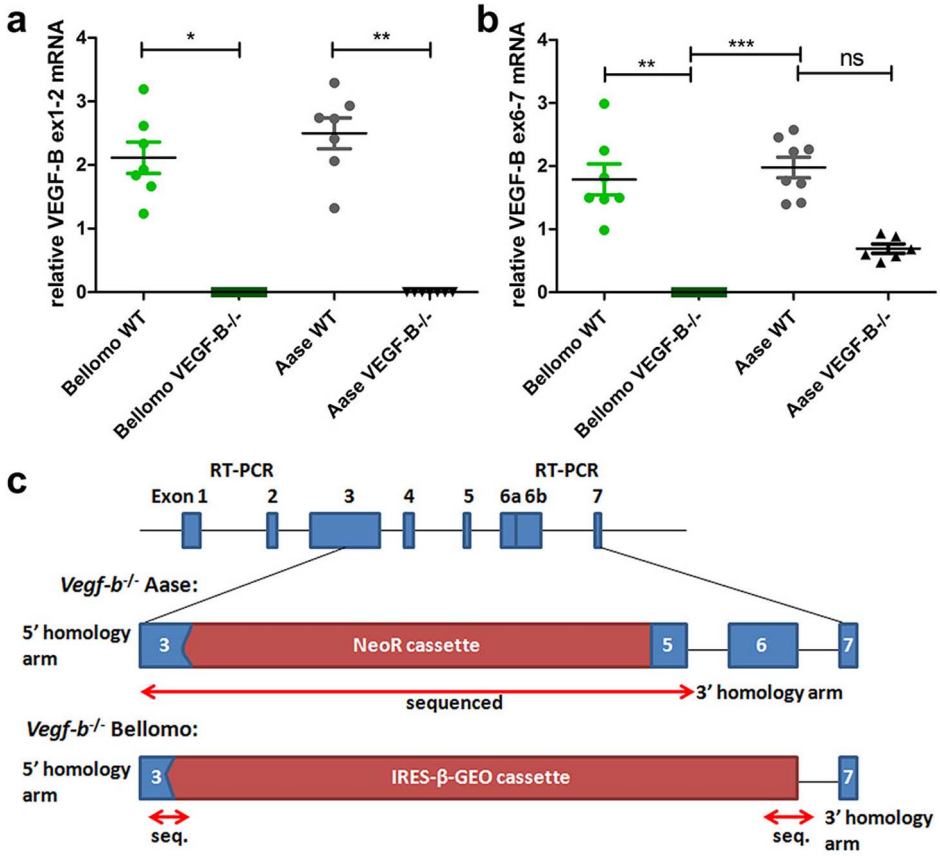

Figure 1 | VEGF-B mRNA expression and structure of the targeting constructs. (a) VEGF-B exon 1-2 mRNA expression. (b) VEGF-B exon 6-7 mRNA expression. (c). Overview of the Aase and Bellomo *Vegf-b* targeting constructs in the corresponding *Vegf-b*$^{-/-}$ mice. Red arrow indicates the sequenced area. n=7, Kruskal-Wallis with Dunn's post hoc test (a and b). *p<0.05, **p<0.01 ***p<0.001 ns= non significant. Results are expressed as mean ± S.E.M.

Table 1 | Cardiac phenotype of Bellomo and Aase *Vegf-b*$^{-/-}$ mice

Phenotype	Parameter	Aase WT	Aase *Vegf-b*$^{-/-}$	Bellomo WT	Bellomo *Vegf-b*$^{-/-}$
Heart function	EF (%)	56.18 ± 9.45	65.57 ± 13.72	62.75 ± 6.09	66.77 ± 9.90
	FS (%)	29.31 ± 5.94	36.57 ± 9.86	33.66 ± 4.29	36.91 ± 7.13
Heart size	LVAW; d (mm)	0.84 ± 0.14	0.93 ± 0.15	0.95 ± 0.10	0.92 ± 0.13
	LVAW; s (mm)	1.21 ± 0.14	1.37 ± 0.33	1.38 ± 0.14	1.45 ± 0.21
	LVID; d (mm)	4.24 ± 0.33	3.95 ± 0.14	4.05 ± 0.16	3.94 ± 0.17
	LVID; s (mm)	3.01 ± 0.48	2.50 ± 0.36	2.69 ± 0.23	2.49 ± 0.38
	LVPW; d (mm)	0.72 ± 0.12	0.80 ± 0.14	0.77 ± 0.11	0.79 ± 0.10
	LVPW; s (mm)	1.08 ± 0.16	1.20 ± 0.28	1.20 ± 0.17	1.23 ± 0.118
	LV Vol; d (μl)	80.88 ± 14.52	67.90 ± 5.67	72.24 ± 6.70	67.73 ± 6.69
	LV Vol; s (μl)	36.40 ± 14.31	22.92 ± 8.64	27.05 ± 5.60	22.99 ± 9.27
	LV mass	124.92 ± 23.39	129.88 ± 15.01	132.93 ± 12.09	125.94 ± 22.15
ECG	PQ interval (ms)	43.97 ± 2.41	42.59 ± 3.65	43.80 ± 1.25	43.30 ± 1.12
	QRS interval (ms)	10.41 ± 0.48	10.06 ± 0.20	10.43 ± 0.46	10.86 ± 0.27
	QT interval (ms)	37.50 ± 3.52	41.11 ± 2.63	41.25 ± 2.36	39.93 ± 0.97
	P amplitude (mV)	1.250 ± 0.25	0.8376 ± 0.24	1.221 ± 0.12	1.261 ± 0.12
	R amplitude (mV)	10.11 ± 0.59	10.43 ± 0.75	9.495 ± 0.74	10.08 ± 0.45
	Heart rate (bpm)	366.0 ± 32.17	400.3 ± 20.08	439.1 ± 18.27	441.6 ± 19.11

Heart function determined by ejection fraction (EF) and fractional shortening (FS). Heart size in diastole (d) and systole (s) represented by left ventricular anterior wall (LVAW) thickness, left ventricular internal diameter (LVID), left ventricular posterior wall (LVPW) thickness, left ventricle (LV) volume and LV mass in Aase and Bellomo VEGF-B$^{-/-}$ and littermate control mice. ECG recordings are shown as PQ, QRS and QT interval, heart rate in beats per minute (bpm) and amplitudes of P and R peak. Results are expressed as mean ± S.E.M. n=4–9.

Bellomo *Vegf-b*$^{-/-}$ mice were significantly heavier between 12-21 weeks of age (Fig. 2a, $p=0.0375$ at 13 weeks and $p=0.0171$ at 21 weeks on SD), but the difference disappeared by 23 weeks of age. Fat analysis after WD by magnetic resonance imaging (MRI) showed no changes in body fat percentage (Fig. 2b) or amount of fat normalized to body size (Fig. 2c). Furthermore, neither the intraperitoneal glucose tolerance test (IPGTT; Fig. 2d, e, g and h) nor the intraperitoneal insulin tolerance test (IPITT; Fig. 2f and i) showed differences between the littermate control and *Vegf-b*$^{-/-}$ mice on SD or WD. Plasma TG, cholesterol and serum free FA levels did not differ between the littermate controls and Bellomo *Vegf-b*$^{-/-}$ mice before or after SD or WD (Table 2). There was a similar significant increase in cholesterol levels with age regardless of the diet ($p<0.0001$) in the littermate controls and Bellomo *Vegf-b*$^{-/-}$ mice. TGs and free FAs remained unchanged. Food intake was similar in both strains on both diets (Table 2).

Response to a high-fat diet. We also studied both *Vegf-b*$^{-/-}$ strains on a 60% HFD, including their respective littermate controls, and analyzed their response to IPGTT and IPITT according to the published protocol[13] (Fig. 3). No differences in glucose tolerance were observed between any of the strains after 15 weeks on HFD (Fig. 3a, b). Similarly, IPITT did not reveal any major changes in insulin sensitivity except that the Aase littermate control mice showed lower glucose levels at 20 minutes (Fig. 3c, Aase WT versus Aase *Vegf-b*$^{-/-}$ $p<0.01$, Aase WT versus Bellomo WT $p<0.05$) and 40 minutes (Fig. 3c, Aase WT versus Aase *Vegf-b*$^{-/-}$ $p<0.05$) after the insulin injection. These results were not surprising considering that the body weight gain of these mice was similar at the time of IPGTT (15 weeks on 60% HFD or WD; Fig. 3d and data not shown). Additionally, no difference was detected in insulin stimulated GLUT4 translocation between Bellomo *Vegf-b*$^{-/-}$, Aase *Vegf-b*$^{-/-}$ and littermate control hearts (Fig. 3e) or skeletal muscles (Fig. 3 f) on SD.

Cardiac gene expression profile on high-fat diet. Differences in the expression levels of mRNAs encoding key mitochondrial and fatty acid transport proteins (FATPs) between the *Vegf-b* knockout and control mice were reported[5]. They found that the fatty acid transport protein 4 (*Fatp4*) and *Fatp3* are regulated by VEGF-B in the heart. However, in a subsequent study using *Vegf-b* transgenic and knockout rats, *Vegf-b* regulation of *Fatp3* was not detected[5,10]. In the present study, we did not detect differences in either FATP3 or FATP4 mRNA levels between any of the *Vegf-b*$^{-/-}$ strains after HFD (Table 3) or in Bellomo *Vegf-b*$^{-/-}$ on SD and WD (data not shown). We also analyzed expression of a set of metabolic genes involved in glucose metabolism, FA oxidation, FA synthesis and transcriptional regulation of metabolism in the heart (Table 3) but *Vegf-b* deficiency did not have any significant impact on the expression level of these key genes of metabolic pathways on HFD.

Discussion

This is the first time *Vegf-b* knockout strains have been available for direct comparison of body composition, cardiac function and glucose and insulin tolerance in identical environmental circumstances. The strength of this side by side comparison of mouse lines is that external factors which affect the sensitive tolerance tests used, such as housing conditions, stress and handling, or methodological differences were minimized. Both strains were tested for body composition, glucose and insulin tolerance and cardiac function.

We report the surprising finding that neither of the two *Vegf-b* knockout mice showed differences in body weight gain, amount of fat or insulin resistance upon WD or HFD feeding. The only difference found was that on SD, the Bellomo *Vegf-b*$^{-/-}$ mice were significantly heavier at the age 12–20 weeks compared to littermate controls, but this difference tended to disappear with aging. This finding is similar as to the 15% weight gain of Aase *Vegf-b*$^{-/-}$ mice on SD at 16–18 weeks of age[5].

Previously, Bellomo *et al.* reported smaller hearts and decreased LV thickness in Bellomo *Vegf-b*$^{-/-}$ mice as compared with control mice[11] while Aase *et al.* reported no differences in heart size and LV thickness but instead a prolonged PQ interval in Aase *Vegf-b*$^{-/-}$ mice in basal state[12]. Here we did not observe any differences in the cardiac structural or functional parameters measured between or within the two *Vegf-b*$^{-/-}$ strains. This shows that in basal state under the same unstressed circumstances the cardiac phenotype and functionality is not affected by *Vegf-b* deficiency in contrast to previous findings[11,12].

At the molecular level, the expression of FATP3 and FATP4 was not changed in *Vegf-b*$^{-/-}$ hearts after HFD. In rats, only FATP4 was found regulated by *Vegf-b*[10]. This suggests that previously observed FATP changes likely reflected the metabolic state of the mice rather than the deficiency of the *Vegf-b* gene. Nevertheless it has been shown that *Flt-1-TK*$^{-/-}$ and *Nrp-1-EC*$^{-/-}$ mice have decreased *Fatp* expression on SD as well[5], showing that VEGF-B signaling may be important in FATP regulation.

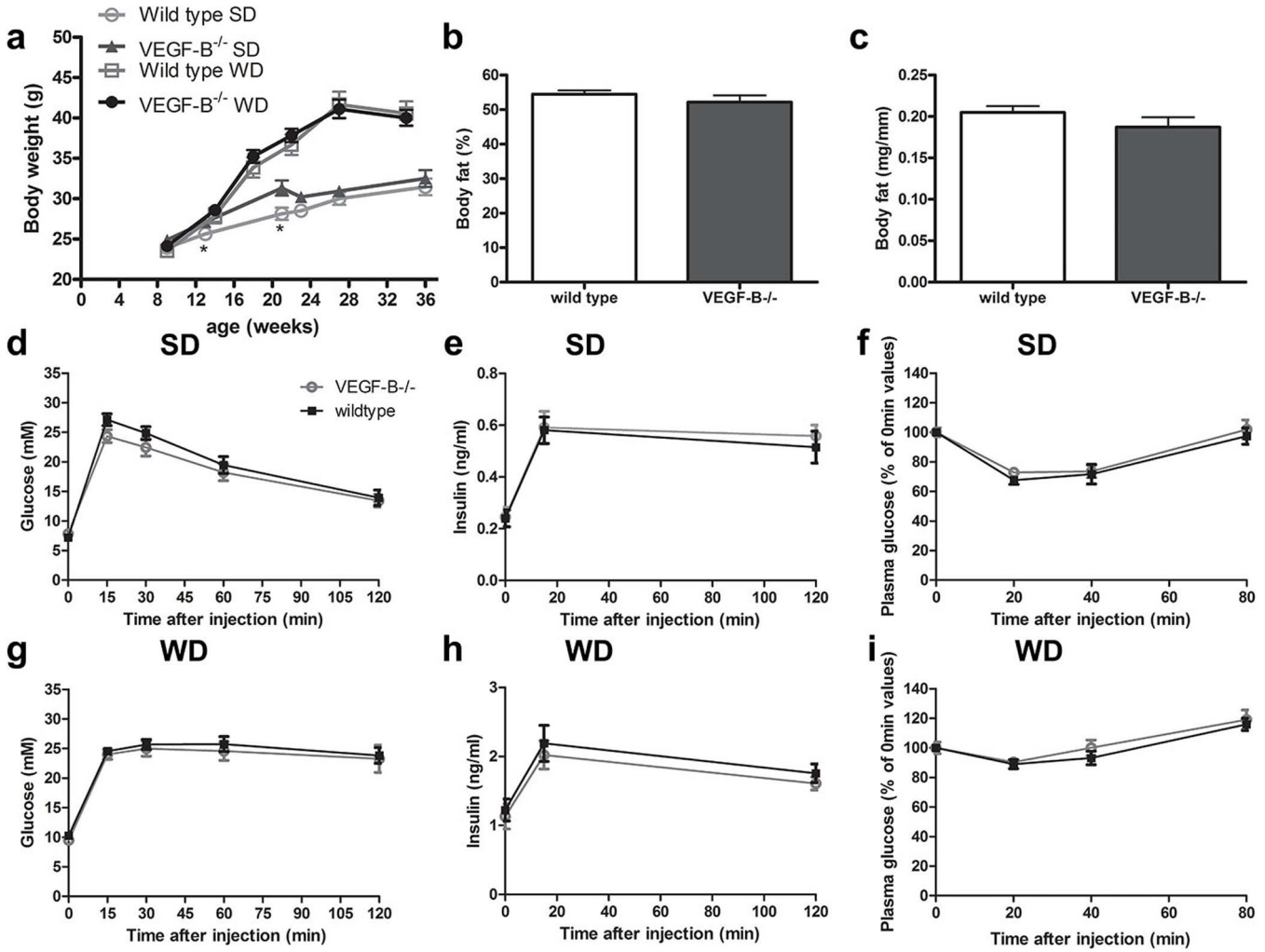

Figure 2 | Body composition and glucose metabolism in Bellomo *Vegf-b*$^{-/-}$ mice on Western-type diet. (a). Body weight during the course of Western-type diet (WD) and standard diet (SD) feeding. (b). Body fat percentage and (c) body fat normalized to body size after WD. (d). Glucose and (e) insulin levels during intraperitoneal glucose tolerance test (IPGTT) on SD. (g). Glucose and (h) insulin levels during IPGTT on WD. The glucose dose used was 2 mg/g body weight. (f). Intraperitoneal insulin tolerance test (IPITT) with the insulin dose of 0.25 mU/g body weight on SD and (i) on WD in Bellomo *Vegf-b*$^{-/-}$ and littermate control mice. The statistical analyses were performed with one-way ANOVA (a) or Student's t-test (b to i). Results are expressed as mean ± S.E.M. n=8-12.

Aase *Vegf-b*$^{-/-}$, Bellomo *Vegf-b*$^{-/-}$and Bellomo littermate control mice displayed similar glucose metabolism responding equally during IPGTT and IPITT. Interestingly, the Aase littermate controls responded differently than Bellomo littermate controls during IPITT. Thus it is tempting to speculate that the reason for this minor difference is the background of the mice studied. When considering the production and backcrossing of these mice it is known that both strains have been produced similarly by injecting targeted ES cells of 129/Ola[12] (new nomenclature: 129P2) or 129/SvJ[11] (new nomenclature: 129X1) into C57BL or C57BL/6J blastocysts, respectively. The resulting Bellomo *Vegf-b*$^{-/-}$ chimeras were backcrossed into C57BL background from that moment while for the Aase mice the back-

Table 2 | Free FA, TG and cholesterol levels and food intake of Bellomo *Vegf-b*$^{-/-}$ and littermate control mice on a Western-type diet

Age, diet, genotype	free FA (mM)	TG (mM)	Cholesterol (mM)	Food intake (g/day)
13w, SD,				
wild type	0.34 ± 0.04	0.9 ± 0.09	3.5 ± 0.09	-
Vegf-b$^{-/-}$	0.35 ± 0.04 n.s.	1.0 ± 0.12 n.s.	3.6 ± 0.11 n.s.	-
31w, SD,				
wild type	0.27 ± 0.03	0.93 ± 0.06	4.84 ± 0.20 ***	3.2 ± 0.3
Vegf-b$^{-/-}$	0.25 ± 0.01 n.s.	0.85 ± 0.07 n.s.	5.20 ± 0.18 n.s.,***	3.3 ± 0.2 n.s.
31w, WD,				
wild type	0.28 ± 0.03	1.15 ± 0.05	7.09 ± 0.35 ***	3.5 ± 0.2
Vegf-b$^{-/-}$	0.25 ± 0.02 n.s.	1.15 ± 0.03 n.s.	7.53 ± 0.27 n.s.,***	3.2 ± 0.2 n.s.

n.s. non-significant compared to wild type of matching age and diet, *** $p<0.001$ compared to 13 week, standard diet, two-way ANOVA. Results are presented as means ± S.E.M. n=10–12

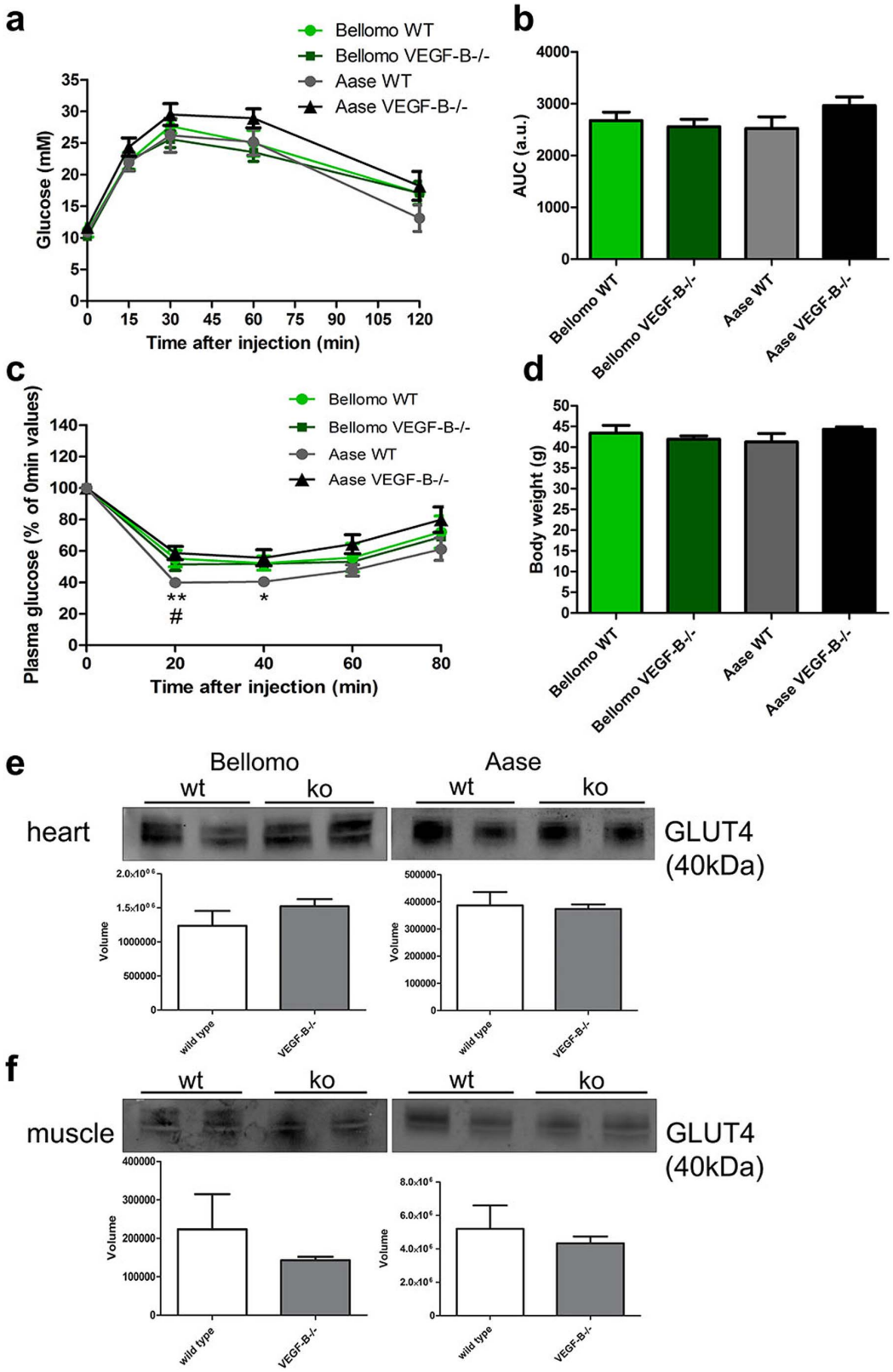

Figure 3 | **Body weight and glucose metabolism in both *Vegf-b*$^{-/-}$ strains.** (a). Response to intraperitoneal glucose tolerance test (IPGTT) after 15 weeks on 60% HFD. (b) Area under IPGTT curve. The glucose dose was 1 mg/g body weight. (c). Intraperitoneal insulin tolerance test (IPITT) after 17 weeks of 60% HFD. The insulin dose was 0.75 mU/g body weight. (d). Body weight on 60% HFD at the time of IPGTT. (e). Western blot analysis of GLUT4 protein in the plasma membrane fraction of the heart and (f) skeletal muscle (n=2).Blot images were cropped for comparison. Densitometry analysis of GLUT4 expression in wild type (white bars) and *Vegf-b*$^{-/-}$ (grey bars) mouse heart (e) or skeletal muscle (f) was performed using ImageQuant software. The statistical analyses were performed with one-way ANOVA (a, b and d) or Student's t-test (e and f). In (c) Kruskal-Wallis with Dunn's post test with significant differences indicated by: # Aase WT versus Bellomo WT $p<0.05$, ** Aase WT versus Aase *Vegf-b*$^{-/-}$ $p<0.01$ and * Aase WTversus Aase *Vegf-b*$^{-/-}$ $p<0.05$ in (c). Results are expressed as mean ± S.E.M. n=6–13.

Table 3 | Cardiac expression of glucose and FA metabolism related genes measured by RT-PCR

pathway/metabolism	gene	Aase WT	Aase *Vegf-b*$^{-/-}$	Bellomo WT	Bellomo *Vegf-b*$^{-/-}$
glucose	*Pdha1*	2.729 ± 0.32	3.051 ± 0.16	3.231 ± 0.26	3.093 ± 0.30
	Pdk4	3.037 ± 0.39	3.737 ± 0.48	2.986 ± 0.31	2.422 ± 0.15
	Glut4(Slc2a4)	2.603 ± 0.28	3.064 ± 0.18	2.969 ± 0,32	3.005 ± 0.23
FA oxidation	*Acadm*	1.144 ± 0.07	1.203 ± 0.16	0.977 ± 0.11	1.104 ± 0.08
	Acadl	1.244 ± 0.12	1.293 ± 0.17	1.022 ± 0.12	1.181 ± 0.09
	Cpt1β	1.106 ± 0.08	1.321 ± 0.24	0.939 ± 0.14	1.023 ± 0.10
FA transport	*Fatp3(Slc27a3)*	1.070 ± 0.06	1.304 ± 0.16	0.912 ± 0.12	0.926 ± 0.12
	Fatp4(Slc27a4)	1.303 ± 0.11	1.445 ± 0.22	0.912 ± 0.10	1.015 ± 0.08
FA synthesis	*Fasn*	1.110 ± 0.08	1.135 ± 0.15	0.891 ± 0.11	1.005 ± 0.08
co-factors	*Pgc-1α*	2.521 ± 0.45	3.026 ± 0.16	3.350 ± 0.36	2.954 ± 0.39
	Ncor1	2.497 ± 0.40	3.145 ± 0.26	3.496 ± 0.26	3.527 ± 0.58
	Pparα	2.545 ± 0.43	2.859 ± 0.12	3.192 ± 0.36	3.213 ± 0.26

Pdha1,pyruvate dehydrogenase alpha 1; *Pdk4*, pyruvate dehydrogenase kinase, isozyme 4; *Slc2a4/Glut4*, glucose transporter 4; *Acadm*, acyl-Coenzyme A dehydrogenase, C-4 to C-12 straight chain; *Acadl*, acyl-Coenzyme A dehydrogenase, long chain; *Cpt1β*, carnitine palmitoyltransferase I beta; *Slc27a3/Fatp3*, fatty acid transport protein 3; *Slc27a4/Fatp4*, fatty acid transport protein 4; *Fasn*, fatty acid synthase; *Pgc-1α*, peroxisome proliferator-activated receptor gamma, coactivator 1 alpha; *Ncor1*, nuclear receptor co-repressor 1; *Pparα*, peroxisome proliferator-activator receptor alpha. Messenger RNA expression of indicated genes was normalized to B2M or PPIA. Results are expressed as mean ± S.E.M. n=5–7.

crossing has been less extensive as indicated by the observation of grey coated mice in the progeny (18.2% of Aase *Vegf-b*$^{-/-}$ and Aase littermate controls, unpublished observation M.H.D.), that is consistent with 129 background in these mice. Within the 129 strains significant genetic differences exist[15]. For example 129T2 mice have lower insulin levels than C57BL/6 mice, yet they show no differences in glucose tolerance[16] whereas 129X1 mice display impaired glucose tolerance but unaltered insulin levels, suggesting that the background is a key determinant of metabolic phenotyping[17]. This emphasizes the importance of thorough backcrossing of genetically modified strains before metabolic phenotyping can be performed.

Overall we conclude here that *Vegf-b* is dispensable for cardiac function, cardiac metabolism and HFD-induced metabolic state under otherwise unstressed conditions.

Methods

Experimental animals. Bellomo *Vegf-b*$^{-/-}$ mice containing a beta-geo cassette[11] in C57Bl/6J and Aase *Vegf-b*$^{-/-}$ mice expressing neomycin resistance gene (NeoR)[12] in C57Bl/6N[5] background were used in this study (see constructs Fig. 1c). Within the strain, heterozygous mice were crossbred to obtain homozygotes and wild-type littermate controls. Wild type littermate controls were included for both strains in all experiments. Male mice were fed a Western-type diet containing 42% of calories from fat (TD.88137 Harlan Teklad, Indianapolis, IN, USA) from 14 weeks of age or a HFD containing 60% of calories originating from fat (Research Diets, New Brunswick, NJ, USA) from 5 weeks of age onwards. A group of Bellomo *Vegf-b*$^{-/-}$ mice with littermate controls was maintained on a SD as an additional control. All animals had *ad libitum* access to water and food and were maintained in standard housing conditions in the Laboratory Animal Center of the University of Eastern Finland. All animal procedures were approved by the Animal Experiment Board in Finland and carried out according to the guidelines of the Experimental Animal Committee of the University of Eastern Finland.

PCR and sequencing. Genomic DNA from Aase *Vegf-b*$^{-/-}$ and wild type littermate control mice was isolated using the ChargeSwitch gDNA Micro Tissue kit (Thermo Scientific, Waltham, MA, USA). The junction sites of the NeoR construct in the mouse genomic DNA and the inserted construct itself were amplified with the Phusion Flash High Fidelity Master Mix (Thermo Scientific, Waltham, MA, USA) using forward sequencing primers: Aase F4 (5′-cagtggtgccatggatagacg-3′), New Aase KO (5′-gctattcggctatgactgggcac-3′), NEO 3′end (5′-gaccgctatcaggacatagcgttg-3′), and reverse sequencing primers: New Aase KO (5′-gtgcccagtcatagccgaatagc-3′) mVEGFB upstream from exon 5 (5′-ggctctactctcagtcacctggtcc-3′) and mVEGFB exon 5 (5′-cacctgtctggcttcacagcac-3′). All primers were from Oligomer (Helsinki, Finland). The PCR products were sub cloned into pBluescript II SK (Stratagene, Santa Clara, CA, USA) and sequenced with the Applied Biosystems Dye Terminator (v.3.1) sequencing kit (Haartman Institute sequencing unit, Helsinki). The obtained sequencing results were analyzed by aligning them to reference sequences.

Genomic DNA from the hearts of Bellomo *Vegf-b*$^{-/-}$ mice was isolated using standard Proteinase K based DNA extraction protocol. PCR for the junction sites of the IRES-beta-GEO construct within the mouse genomic DNA was performed with Phusion Flash High Fidelity enzyme (Thermo Scientific, Waltham, MA, USA) using various primer pairs. The PCR products were sequenced with Applied Biosystems 3130xl Genetic Analyzer using the following primers: PCR1 from the original Bellomo paper forward: (5′-ccggagctcggcctccag-3′), IRES reverse (5′-gttccgctgcctgcaaagggtc-3′), b-geo forward (5′-gacggcgaggatctcgtcgtg-3′) and mVEGFB intr6-7 reverse (5′-gactgagtcactcactatatgtgaagc-3′). Primers were from Eurofins MWG Operon (Ebersberg, Germany). The obtained sequence data were aligned to reference sequences to verify the correct insertion of the construct.

Echocardiography and electrocardiography. Echocardiography and ECG measurements were done with a high-resolution imaging system Vevo 2100 (VisualSonics Inc., Toronto, ON, Canada) with a high-frequency ultrasound probe (MS400) operating at 18–38 MHz. The measurements were done as described earlier[6].

Bodyweight, magnetic resonance imaging and food intake. Bodyweight was monitored during the experiment every two to three weeks. MRI was performed on a 4.7 T magnet (Magnex Scientific, UK) interfaced with Varian $^{\text{UNITY}}$INOVA console (Varian Inc. Palo Alto, CA) using 60 mm quadrature volume transceiver (Rapid Biomed, Rimpar, Germany) on 32 week old Bellomo *Vegf-b*$^{-/-}$ mice on Western-type diet. Chemical-shift-selective 3D gradient echo sequence (TR/TE 100/12 ms, resolution of 200 μm in transversal plane and 800 μm in B0 direction)[18] was applied to obtain coverage of the whole body. MR images were post-processed and automatic water fat analysis was performed with lab built analysis program running on Matlab platform[18]. The percentage of adipose tissue was calculated as fat area normalized to water + fat area. Furthermore, the fat mass per unit length (mg/mm of mouse length) was calculated. At sacrification three different WAT pads (perigonadal, subcutaneous and renal) were dissected and weighed, together with heart, liver, kidney, pancreas and intrascapular brown adipose tissue. For food intake animals were caged separately and pre-weighed food was placed in the food choppers and measured every three days. The daily intake was calculated by food intake divided by the measurement time.

Tolerance tests. For IPGTT, animals on Western-type diet were fasted overnight and injected with 2 mg/g glucose (Sigma-Aldrich, St Louis, MO, USA) intraperitoneally. IPITT were performed on non-fasted mice with 0.25 mU/g insulin (Actrapid, Novo Nordisk, Denmark). On a 60% HFD IPGTT was performed on mice fasted for 2 h with 1 mg/g glucose or IPITT after 1 h fasting with 0.75 mU/g insulin[13]. Pooled data from ≥3 experiments are reported. Blood was collected from the tail vein in non-anesthetized mice.

GLUT4 protein western blot. Heart and skeletal muscle samples were obtained fresh from sacrificed mice following overnight fasting and 3 to 4 hours of refeeding. Plasma membrane fractions of heart and skeletal muscle lysates were isolated by centrifugating with 1000 g in a buffer containing 25 mM Tris, pH 7,4, 0,1 mM EDTA, protease inhibitors and 0,25 M sucrose. Protein concentration was determined by BCA kit (Thermo Scientific, Waltham, MA, USA). Five μg of protein was run on SDS-PAGE and blotted to nitrocellulose membrane (BioRad, Hercules, CA, USA). Primary GLUT4 antibody C-20 and HRP-conjugated secondary antibodies were used for antibody staining (Santa Cruz Biotechnologies, CA, USA). Immunolabeling was detected with ECL Plus detection system (GE Healthcare, Fairfield, CT, USA).

Blood, serum and plasma metabolites. Glucose measurements were performed with an Ascensia Elite XL (Bayer, Leverkusen, Germany) glucose meter. Glucose values presented in the IPGTT graph on Western-type diet of Bellomo *Vegf-b*$^{-/-}$ mice were determined microfluorimetrically[18]. For other blood metabolites plasma or serum samples were taken from the saphenous vein after 17–18 h fasting. Serum free FAs were determined using a colorimetric assay (WAKO, Osaka, Japan). Plasma triglyceride (TG) levels and total cholesterol levels were measured colorimetrically (Microlab 200 analyzer, Merck, Darmstadt, Germany). Plasma insulin levels were determined by rat insulin ELISA kit (Crystal Chem, Downers, IL, USA).

Gene expression analysis. RNA was isolated from heart samples with TRI-Reagent (Sigma-Aldrich, St Louis, MO, USA). RNA samples were DNAse treated by DNAse Free kit (Thermo Fisher Scientific, Waltham, MA, USA) and reverse transcribed using Revertaid™ (Thermo Fisher Scientific, Waltham, MA, USA). Quantitative PCR was performed on a StepOnePlus Real-Time PCR system (Thermo Fisher Scientific, Waltham, MA, USA). Relative mRNA expression of genes were measured using specific Assays-on-Demand systems (Thermo Scientific, Waltham, MA, USA). Expression levels were normalized to peptidylprolyl isomerase A (*PPIA*) or beta-2-microglobilin (*B2M*).

Statistical tests. Data are reported as means ± S.E.M. Data were checked for normal distribution. In the case of multiple groups and when normally distributed, the data were analyzed by one-way ANOVA with Bonferroni's Multiple comparison post test or two-way ANOVA. When not normally distributed Kruskal-Wallis test with Dunn's post test was used. In comparing two groups two-tailed Student's t-test was used. The statistical analyses and symbols used are reported in the figure legends. Differences were considered statistically significant at $p<0.05$.

1. Nash, A. D., Baca, M., Wright, C. & Scotney, P. D. The biology of vascular endothelial growth factor-B (VEGF-B). *Pulm Pharmacol Ther.* **19**, 61–9 (2006).
2. Olofsson, B. *et al*. Vascular endothelial growth factor B (VEGF-B) binds to VEGF receptor-1 and regulates plasminogen activator activity in endothelial cells. *Proc Natl Acad Sci U S A.* **95**, 11709–14 (1998).
3. Makinen, T. *et al*. Differential binding of vascular endothelial growth factor B splice and proteolytic isoforms to neuropilin-1. *J Biol Chem.* **274**, 21217–22 (1999).
4. Karpanen, T. *et al*. Overexpression of vascular endothelial growth factor-B in mouse heart alters cardiac lipid metabolism and induces myocardial hypertrophy. *Circ Res.* **103**, 1018–26 (2008).
5. Hagberg, C. E. *et al*. Vascular endothelial growth factor B controls endothelial fatty acid uptake. *Nature.* **464**, 917–21 (2010).
6. Huusko, J. *et al*. The effects of VEGF-R1 and VEGF-R2 ligands on angiogenic responses and left ventricular function in mice. *Cardiovasc Res.* **86**, 122–30 (2010).
7. Lähteenvuo, J. E. *et al*. Vascular endothelial growth factor-B induces myocardium-specific angiogenesis and arteriogenesis via vascular endothelial growth factor receptor-1- and neuropilin receptor-1-dependent mechanisms. *Circulation.* **119**, 845–56 (2009).
8. Li, X. *et al*. Reevaluation of the role of VEGF-B suggests a restricted role in the revascularization of the ischemic myocardium. *Arterioscler Thromb Vasc Biol.* **28**, 1614–20 (2008).
9. Bry, M. *et al*. Vascular endothelial growth factor-B acts as a coronary growth factor in transgenic rats without inducing angiogenesis, vascular leak, or inflammation. *Circulation.* **122**, 1725–33 (2010).
10. Kivelä, R. *et al*. VEGF-B-induced vascular growth leads to metabolic reprogramming and ischemia resistance in the heart. *EMBO Mol Med.* **6**, 307–21 (2014).
11. Bellomo, D. *et al*. Mice lacking the vascular endothelial growth factor-B gene (vegfb) have smaller hearts, dysfunctional coronary vasculature, and impaired recovery from cardiac ischemia. *Circ Res.* **86**, E29–35 (2000).
12. Aase, K. *et al*. Vascular endothelial growth factor-B-deficient mice display an atrial conduction defect. *Circulation.* **104**, 358–64 (2001).
13. Hagberg, C. E. *et al*. Targeting VEGF-B as a novel treatment for insulin resistance and type 2 diabetes. *Nature.* **490**, 426–30 (2012).
14. Carmeliet, P., Wong, B. W. & De Bock, K. Treating diabetes by blocking a vascular growth factor. *Cell Metab.* **16**, 553–5 (2012).
15. Wong, G. T. Speed congenics: Applications for transgenic and knock-out mouse strains. *Neuropeptides.* **36**, 230–6 (2002).
16. Andrikopoulos, S., Blair, A. R., Deluca, N., Fam, B. C. & Proietto, J. Evaluating the glucose tolerance test in mice. *Am J Physiol Endocrinol Metab.* **295**, E1323–32 (2008).
17. Goren, H. J., Kulkarni, R. N. & Kahn, C. R. Glucose homeostasis and tissue transcript content of insulin signaling intermediates in four inbred strains of mice: C57BL/6, C57BLKS/6, DBA/2, and 129X1. *Endocrinology.* **145**, 3307–23 (2004).
18. Pirinen, E. *et al*. Enhanced polyamine catabolism alters homeostatic control of white adipose tissue mass, energy expenditure, and glucose metabolism. *Mol Cell Biol.* **27**, 4953–67 (2007).

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Author contributions

M.H.D. directed and performed most of the experiments and wrote the manuscript. E.P. directed and performed part of the experiments and helped writing the manuscript. J.H. performed cardiac phenotyping, maintained the mouse strains and participated in writing the manuscript. R.K. analysed the Bellomo mouse sequencing and participated in writing the manuscript. D.S. designed the Aase mouse sequencing and interpreted all the sequencing results. K.A. acquired and initiated work on the Bellomo strain, directed part of the work, contributed to the design of the study and revised the manuscript. S.Y.H. contributed to the design of the study and directed all the work. All authors reviewed the manuscript.

Additional information

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