A Vegfc-Emilin2a-Cxcl8a Signaling Axis Required for Zebrafish Cardiac Regeneration

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Abstract

Background: Ischemic heart disease following the obstruction of coronary vessels leads to the death of cardiac tissue and the formation of a fibrotic scar. In contrast to adult mammals, zebrafish can regenerate their heart after injury, enabling the study of the underlying mechanisms. One of the earliest responses following cardiac injury in adult zebrafish is coronary revascularization. Defects in this process lead to impaired cardiomyocyte repopulation and scarring. Hence, identifying and investigating factors that promote coronary revascularization holds great therapeutic potential.

Methods: We used wholemount imaging, immunohistochemistry and histology to assess various aspects of zebrafish cardiac regeneration. Deep transcriptomic analysis allowed us to identify targets and potential effectors of Vegfc signaling. We employed newly generated loss- and gain-of-

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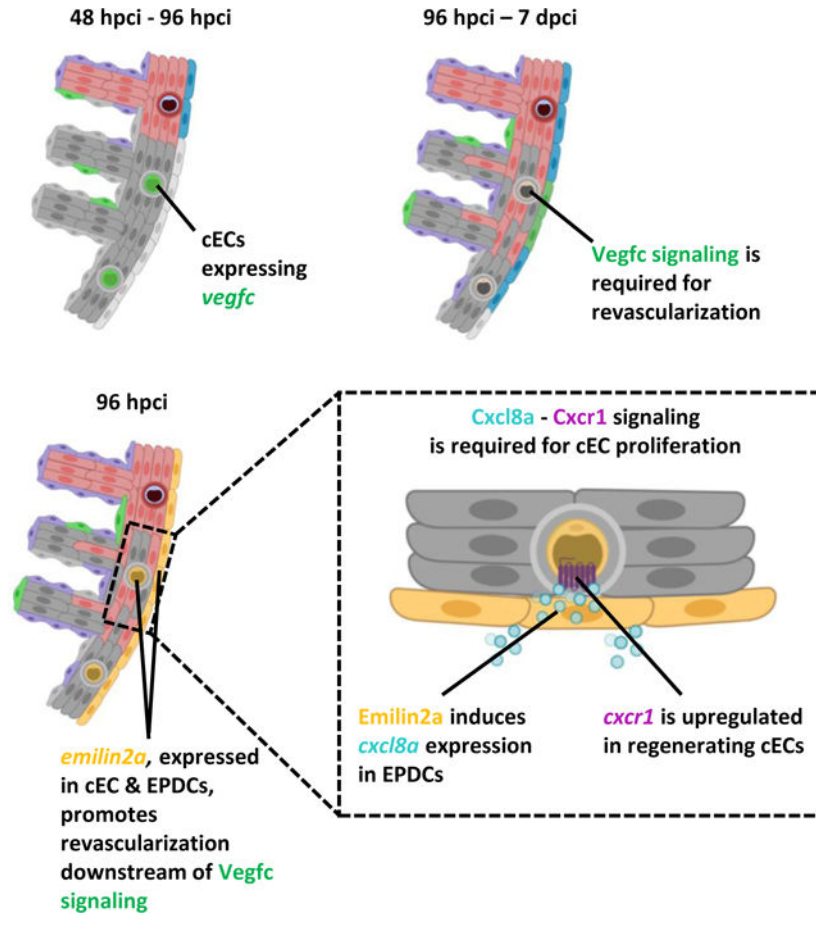
function genetic tools to investigate the role of Emilin2a and Cxcl8a-Cxcr1 signaling in cardiac regeneration.

Results: We first show that regenerating coronary endothelial cells upregulate *vegfc* upon cardiac injury in adult zebrafish, and that Vegfc signaling is required for their proliferation during regeneration. Notably, blocking Vegfc signaling also significantly reduces cardiomyocyte dedifferentiation and proliferation. Using transcriptomic analyses, we identified *emilin2a* as an effector of Vegfc signaling, and found that manipulation of *emilin2a* expression can modulate coronary revascularization as well as cardiomyocyte proliferation. Mechanistically, Emilin2a induces the expression of the chemokine gene *cxcl8a* in epicardium-derived cells, while *cxcr1*, the Cxcl8a receptor gene, is expressed in coronary endothelial cells. We further show that Cxcl8a-Cxcr1 signaling is also required for coronary endothelial cell proliferation during cardiac regeneration.

Conclusions: These data show that after cardiac injury, coronary endothelial cells upregulate *vegfc* to promote coronary network re-establishment and cardiac regeneration. Mechanistically, Vegfc signaling upregulates epicardial *emilin2a* and *cxcl8a* expression to promote cardiac regeneration. These studies aid in understanding the mechanisms underlying coronary revascularization in zebrafish, with potential therapeutic implications to enhance revascularization and regeneration in injured human hearts.

Graphical Abstract

48 hpci – 96 hpci — cECs expressing *vegfc*

96 hpci – 7 dpci — Vegfc signaling is required for revascularization

96 hpci — *emilin2a*, expressed in cEC & EPDCs, promotes revascularization downstream of Vegfc signaling

Cxcl8a - Cxcr1 signaling is required for cEC proliferation

Emilin2a induces *cxcl8a* expression in EPDCs

cxcr1 is upregulated in regenerating cECs

INTRODUCTION

Ischemic heart disease arises from reduced blood flow to the heart caused by narrowed coronary arteries. Blockage of coronary arteries results in myocardial infarction (MI), leading to the death of downstream tissues. These events lead to cardiac fibrosis and remodeling[1], thereby imposing a major medical challenge given the inability of the adult mammalian heart to regenerate[2]. Following MI in humans, new capillaries sprout from pre-existing ones via angiogenesis to help re-oxygenate the injured tissue[3,4]. In addition, epicardial collaterals develop through the enlargement and widening of preformed ones to help the ischemic area in a process termed arteriogenesis[4]. Patients in which collateral arteries form to a larger extent exhibit improved outcome following MI in comparison to those with a lesser extent of collateral formation[5]. These observations have motivated researchers to develop angiogenic and arteriogenic therapies to treat MI; however, clinical trials have thus far proven ineffective[6]. Therefore, understanding the mechanisms that regulate coronary network re-establishment in a regenerative setting might ultimately lead to beneficial clinical outcomes. Over the last two decades, the zebrafish (*Danio rerio*) has been used extensively as a model organism to study cardiac regeneration, owing to the remarkable ability of its heart to regenerate[7,8,9,10]. Recent studies have shown that coronary revascularization of the injured tissue is essential for a complete regenerative response[11].

Following cardiac injury in zebrafish, coronary endothelial cells rapidly enter the cell cycle and revascularize the injured tissue[11]. Regenerating coronaries sprout from pre-existing vessels superficially, as well as intraventricularly, forming a scaffold for cardiomyocytes (CMs) to repopulate the injured tissue[12]. Blocking this process leads to reduced CM proliferation and repopulation of the injured tissue, as well as increased scarring[11,12]. Given the importance of coronary revascularization, identifying molecules that aid this process holds great therapeutic potential.

Recent studies have highlighted the importance of angiocrine factors in tissue homeostasis and repair[13]. Angiocrine molecules are secreted by endothelial cells and can have various effects on neighboring tissues[12]. Amongst the many angiocrine molecules, vascular endothelial growth factor C (Vegfc) displays a significant upregulation in its gene expression levels following both ventricular cryoinjury and resection[14,15], and intraperitoneal administration of VEGFC can improve cardiac output and performance following MI in adult mouse hearts[16]. Vegfc regulates lymphangiogenesis during mouse and zebrafish development[17,18,19,20,21] as well as in the adult zebrafish heart[22,23,24]. VEGFC also regulates blood vessel angiogenesis in different developmental settings[25,26,27,28,29]. For instance, addition of VEGFC stimulates angiogenic sprouting in chick embryos and mouse corneas[25]. In zebrafish, knockdown of *vegfc* using morpholinos reduces intersegmental vessel sprouting[30,31]. Conversely, overexpressing a constitutively active form of human VEGFC in zebrafish embryos led to intersegmental vessel hypersprouting, further highlighting a role for *vegfc* in regulating blood vessel angiogenesis[32]. Recent studies have also suggested an important role for VEGFC in regulating coronary development[27,33]. Cardiac ventricles from *Vegfc* mutant mice display a significant reduction in the coverage and branching of subepicardial coronaries[27,33]. Moreover, cohort studies on patients with coronary artery disease further indicate that patients with high levels of circulating VEGFC exhibit a better prognosis[28]. Furthermore, intramyocardial administration of VEGFC promoted the formation of collateral arteries following MI in pigs[26]. However, the mechanisms by which VEGFC signaling induces coronary revascularization or collateral formation following cardiac injury remain elusive.

Here, we show that after cardiac injury in adult zebrafish, Vegfc signaling promotes coronary endothelial cell (cEC) and CM proliferation by positively regulating the expression of *emilin2a*, a gene encoding an extracellular matrix (ECM) component. Using tissue specific gain-of-function tools, we show that Emilin2a is also a pro-regenerative factor that induces cEC proliferation and scar resolution. Mechanistically, Emilin2a induces the expression of the chemokine gene *cxcl8a* in epicardium-derived cells (EPDCs), while its receptor *cxcr1* is expressed in regenerating cECs. Our results further indicate that Cxcl8a-Cxcr1 signaling is likewise required for coronary revascularization and scar resolution. Overall, these results highlight the role of the angiocrine molecule Vegfc in regulating matrisome-associated factors to create a more permissive environment for cardiac regeneration in adult zebrafish.

METHODS

All supporting data are available within the article and its Supplemental Materials. Detailed materials and methods can be found in the Supplemental Materials. Please see the Major Resources Table in the Supplemental Materials.

DATA AVAILABILITY

RNAseq data from this study have been deposited in the Gene Expression Omnibus (GEO) database under accession number GSE168175.

RESULTS

vegfc expression is upregulated in coronary endothelial cells after cardiac injury in zebrafish

During zebrafish embryogenesis, *vegfc* is expressed in the dorsal aorta and it regulates developmental angiogenesis[51,52]. In adult zebrafish, *vegfc* expression is induced after cardiac injury[14,15]. To gain insight into the temporal pattern of *vegfc* expression during cardiac regeneration, we performed RT-qPCR analyses on zebrafish ventricles at 48 and 96 (hpci) as well as at 7 days post cryoinjury (dpci). We observed that *vegfc* was upregulated at these time points when compared with sham operated ventricles, showing the highest expression levels at 96 hpci (Figure 1A), which coincides with the peak of cEC proliferation[12]. To identify which cell type(s) express *vegfc* during cardiac regeneration, we performed *in situ* hybridization on wild-type (WT) ventricles and observed *vegfc* expression at 96 hpci in the periphery of the injured tissue, where regenerating coronaries and EPDCs are located (Figure 1B). To better define the source of *vegfc* expression during cardiac regeneration, we performed RT-qPCR analyses on sorted cECs and EPDCs using the *Tg(−0.8flt1:RFP)* and *TgBAC(tcf21:mCherry)* lines, respectively. These analyses showed an upregulation in *vegfc* expression in sorted cECs at 96 hpci in comparison with sham operated hearts (96 hps) (Figure 1C), but not in sorted EPDCs (Figure S1A and S1B). We also analyzed *vegfc* expression in sorted adult CMs using the *Tg(myl7:DsRed)* line, and observed very low expression before and after cardiac injury (Figure S1C). Altogether, these results indicate that *vegfc* expression is upregulated by cECs during zebrafish cardiac regeneration.

Vegfc signaling promotes coronary endothelial cell proliferation after cardiac cryoinjury in zebrafish

vegfc upregulation in cECs after cardiac injury suggests a role during regeneration. To test this hypothesis, we utilized a *Tg(hsp70l:sflt4)* line. Upon heat shock, the expression of the soluble receptor sFlt4 is induced, which acts as a decoy to block Vegfc signaling[29]. Together with this line, and in order to visualize coronaries, we used the *Tg(−0.8flt1:RFP)* and *Tg(flt1:Mmu.Fos-GFP)* lines, which specifically label cECs in the zebrafish ventricle (Figure S1D). After optimization of the heat shock regimen (Figure S1E), we performed daily heat shocks on *Tg(hsp70:sflt4); Tg(−0.8flt1:RFP)* and *Tg(−0.8flt1:RFP)* (controls), and analyzed their ventricles (Figure 1D). We quantified cEC proliferation at 96 hpci and found that blocking Vegfc signaling resulted in a significant reduction in cEC proliferation

(Figures 1D and 1E); it also resulted in reduced coronary vessel coverage of the injured tissue at 7 dpci (Figures 1F and 1G), a stage when the injured tissue is extensively repopulated by regenerating coronaries in control zebrafish[11]. In addition, we observed a decrease in the expression levels of *cxcr4a* and *apln* (Figure S1F), two genes that have been shown to regulate coronary regeneration in zebrafish[12].

During cardiac regeneration, CMs dedifferentiate and proliferate to replenish the injured tissue[53,54,55,56], and this process is regulated in part by cECs[11,12]. Hence, we reasoned that altering revascularization by blocking Vegfc signaling could lead to defects in CM regeneration. To test this possibility, we performed heat shock treatments on cryoinjured *Tg(hsp70l:sflt4)* and non-transgenic siblings until 96 hpci, when revascularization is very active, and analyzed CM dedifferentiation and proliferation at 7 dpci. We found that blocking Vegfc signaling resulted in a reduction in both CM dedifferentiation and proliferation in the injury border zone (Figures 2A–D and S1G). To gain more insight into these CM phenotypes, we analyzed the expression of *vegfr1*, *vegfr2* and *vegfr3* in sorted CMs and observed very low or no detectable mRNA levels before or after injury (Figures S1H and S1I), indicating that they are unlikely due to reduced Vegfc signaling in CMs. Next, to test whether reducing coronary revascularization by blocking Vegfc signaling impacts scar resolution, we induced the expression of *sflt4* until 96 hpci, when cEC proliferation is at its peak[12], and analyzed the scar area at 30 and 90 dpci. We found that ventricles in which Vegfc signaling was blocked displayed a significantly larger scar area when compared with non-transgenic siblings subjected to the same treatment at both 30 (Figures S1J and S1K) and 90 (Figures 2E and 2F) dpci, in line with recent findings using *vegfc+/um18* zebrafish[22] as well as *Tg(hsp70l:sflt4)* zebrafish[23].

Vegfc regulates lymphangiogenesis during development[21,57] and cardiac regeneration in zebrafish[22,23]. To rule out that the regeneration phenotypes observed upon global Vegfc signaling interference are due to lymphatic defects, we performed injuries on 3 months old (~27–28 mm in length) zebrafish, in which, based on reporter expression, lymphatics are only observed in the bulbus arteriosus and not yet present in the ventricle[22,23]. To further test for the presence of lymphatic vessels at these stages and during regeneration, we performed intramyocardial injections of Qdots which are taken up and cleared by lymphatics[23]. These experiments were performed in animals carrying the *Tg(lyve1b:DsRed)* transgene which marks lymphatic vessels. Using this approach, we confirmed the absence of lymphatics in untouched 3 months old zebrafish ventricles and during regeneration experiments in WT animals as well as after blockade of Vegfc signaling (Figures S2A and S2B). Similar to our previous experiments with older (i.e., 8 months old) animals (Figures 1D and 1E), we observed a significant decrease in cEC proliferation at 96 hpci in 3 months old *Tg(hsp70l:sflt4)* zebrafish when compared with controls (Figures S2C and S2D). These data indicate that reduced revascularization upon blocking Vegfc signaling is independent of the presence or absence of ventricular lymphatics. However, in order to work with zebrafish devoid of ventricular lymphatics, we used animals 27–28 mm in length for the rest of the study.

Since Flt4 (Vegfr3) is the main receptor for both Vegfc and Vegfd[58,59], it is possible that the cardiac regeneration defects in *Tg(hsp70l:sflt4)* zebrafish are at least partially due to

alterations in Vegfd signaling. To test this possibility, we performed cryoinjuries on WT, hypomorphic *vegfc⁻/⁻* [32] and *vegfd⁻/⁻* [22] zebrafish, and analyzed cEC proliferation in injured ventricles at 96 hpci. Notably, cEC proliferation was reduced in *vegfc⁻/⁻* but not *vegfd⁻/⁻* ventricles (Figures S2E and S2F), consistent with previous findings that *vegfd⁻/⁻* zebrafish do not exhibit cardiac regeneration defects[22,24]. These data indicate that defects observed in cEC regeneration upon *sflt4* overexpression are due to blocked Vegfc signaling, although one cannot completely rule out an effect on other Vegf signaling pathways. Overall, these findings indicate that blocking Vegfc signaling impairs coronary revascularization as well as CM dedifferentiation and proliferation during cardiac regeneration in zebrafish.

Vegfc signaling induces expression of the extracellular matrix gene *emilin2a* to promote coronary revascularization

To gain mechanistic insight into how Vegfc signaling regulates coronary regeneration, we performed transcriptomic analyses of cryoinjured ventricles after blocking Vegfc signaling using the *Tg(hsp70l:sflt4)* line and non-transgenic siblings as controls. We extracted injured ventricles at 24 hpci, and after comparing their transcriptome (Figure 3A), cross-referenced the significantly downregulated genes with those upregulated during WT cardiac regeneration in zebrafish, from a published dataset[15] (Figure S3A). We identified 10 candidate genes (Figure S3A). Next, we performed RT-qPCR analysis to check their expression levels in WT and *Tg(hsp70l:sflt4)* ventricles at 24 hpci (time point when the RNAseq was performed) and 96 hpci (time point when we observed the reduced cEC phenotype) (Figures S3B and S3C). Amongst these candidate genes, *emilin2a* was the only one to display a consistent and significant downregulation when blocking Vegfc signaling (Figures 3B, S3B and S3C). To test whether *emilin2a* is a target of Vegfc signaling, we injected *vegfc* mRNA into one-cell stage WT embryos and found that *emilin2a* expression was significantly increased at 48 hpf (Figure S3D). As Vegfc signaling regulates endothelial sprouting[51,52], we reasoned that the changes in *emilin2a* expression could occur as a consequence of reduced revascularization. To test this possibility, we analyzed *emilin2a* expression in ventricles from *Tg(hsp70l:dn-vegfaa)* zebrafish, a heat shock inducible transgenic line expressing a dominant negative form of Vegfaa that can block revascularization[11,34]. We performed 1 hour heat shocks every day for 3 days on WT, *Tg(hsp70l:sflt4)* and *Tg(hsp70l:dn-vegfaa)* zebrafish, and analyzed *emilin2a* expression by RT-qPCR on uninjured ventricles. While *emilin2a* expression was significantly downregulated in *Tg(hsp70l:sflt4)* ventricles, it remained unchanged in *Tg(hsp70l:dn-vegfaa)* ventricles (Figure S3E), further indicating that *emilin2a* is a target of Vegfc signaling, and not merely an endothelial marker. To explore the possibility that the *VEGFC-EMILIN2* axis is also at play in human endothelial cells, we knocked down *VEGFC* in cultured human umbilical vein endothelial cells (HUVECs) and observed a significant reduction in *EMILIN2* expression (Figure 3C). Altogether, these results indicate that *EMILIN2* is an effector of VEGFC signaling in zebrafish as well as in human ECs.

EMILIN2 is a glycoprotein that belongs to the EMI domain endowed (EDEN) superfamily of proteins which consists of EMILIN1, EMILIN2, MMRN1 and MMRN2[60]. It has previously been shown that *emilin2a* is expressed in the dorsal aorta of zebrafish embryos at 24 hpf [61]; however, its role in heart regeneration remains unknown.

We next analyzed the expression pattern of *emilin2a* at different time points after cryoinjury and found that *emilin2a* was strongly upregulated in the injured tissue at 96 hpci (Figure 3D), which coincides with the peak of *vegfc* expression (Figure 1A) and cEC proliferation[12]. Using *in situ* hybridization on ventricular sections, we observed *emilin2a* expression in the periphery of the injured tissue at 96 hpci, similar to *vegfc* expression, while no expression was detectable in uninjured ventricles (Figure 3E). We further analyzed this expression by RT-qPCR on sorted cECs (*−0.8flt1*:RFP$^+$) and sorted EPDCs (*tcf21*.mCherry$^+$) and found *emilin2a* expression induced in both cell types after injury (Figure 3F), while very low expression was observed in CMs before and after cardiac injury (Figure S3F). We also compared *emilin2a* expression between both cells types (EPDCs and cECs) and found that EPDCs expressed higher levels than cECs at 96 hpci (Figure 3G).

Previous studies have shown that stimulating HUVECs with recombinant EMILIN2 enhanced their migration in a scratch assay[62]. We also observed a significant upregulation of *EMILIN2* at 6 hours after scratch (Figure S3G), when cells are actively migrating. Furthermore, the loss of EMILIN2 significantly reduced microvessel sprouting from cultured mouse aortic rings, suggesting an angiogenic role[63,64]. These data led us to hypothesize that Emilin2a regulates different aspects of revascularization during cardiac regeneration in zebrafish. To test this hypothesis, we generated an *emilin2a* mutant allele using the CRISPR/Cas9 technology. The zebrafish genome contains two *emilin2* genes, *emilin2a* and *emilin2b*[61]. Because of the existence of *emilin2b* in the zebrafish genome, we generated a full locus deletion (FLD) allele in order to avoid possible transcriptional adaptation[65,66] (Figures S4A–S4E). *emilin2a* FLD mutants lack *emilin2a* expression (Figure S4F) and display no obvious defects as larvae (Figure S4G). Furthermore, the gross morphology and coronary coverage of *emilin2a$^{-/-}$* adult ventricles were comparable with those of *emilin2a$^{+/+}$* ventricles when uninjured (Figure S4H). Upon cryoinjury, *emilin2a$^{-/-}$* ventricles displayed a significant reduction in cEC proliferation at 96 hpci (Figures 3H and 3I) and impaired coronary vessel coverage at 7 dpci (Figure S5A and S5B), while devoid of lymphatics (Figure S5C). In addition, *emilin2a$^{-/-}$* ventricles exhibited a significant reduction in CM proliferation at 7 dpci (Figures 3J and 3K) and displayed a larger scar at 30 (Figure S5D and S5E) and 90 (Figures 3L and 3M) dpci. Altogether, these results indicate that *emilin2a* is required for cEC proliferation and scar resolution during cardiac regeneration in zebrafish.

Previously published transcriptomic analyses have shown that, unlike in zebrafish, *emilin2a* is not upregulated following cryoinjury in medaka (*Oryzias latipes*)[15], a teleost species that cannot regenerate its heart and lacks a coronary network[67] (Figure S5F). Furthermore, our data showing that *emilin2a* mutant ventricles retain a larger fibrotic scar at 90 dpci as well as the HUVEC data mentioned earlier[62] (Figure S3G) led us to hypothesize that Emilin2a can enhance cardiac regeneration. To test this hypothesis, we generated a transgenic zebrafish line expressing *emilin2a* under a heat shock promoter. We quantified cEC proliferation at 96 hpci in *Tg(hsp70l:emilin2a); Tg(−0.8flt1:RFP)* ventricles in comparison with controls (i.e., *Tg(−0.8flt1:RFP)* zebrafish subjected to the same treatment) (Figure 4A). We found that overexpressing *emilin2a* significantly increased cEC proliferation at 96 hpci (Figure 4A and 4B) and marginally increased coronary vessel coverage at 7 dpci (Figure S6A and S6B), despite the absence of ventricular lymphatics (Figure S6C). Moreover, overexpressing

emilin2a resulted in an increase in CM proliferation at 7 dpci (Figure 4C and 4D) and a reduced scar area at 30 dpci (Figure 4E and 4F), in comparison with non-transgenic siblings.

In view of these results, we reasoned that the coronary revascularization phenotype in *Tg(hsp70l:sflt4)* zebrafish hearts might be rescued by overexpressing *emilin2a*. We utilized the HOT-CRE system[68] and generated a *Tg(hsp70l:LCL-emilin2a-p2A-mCherry)* zebrafish line, which in combination with the *TgBAC(tcf21:CreERT2)* line[38], allows spatial and temporal control of *emilin2a* expression in EPDCs (Figure S6D–S6G). Next, we overexpressed *emilin2a* in EPDCs while blocking Vegfc signaling and observed a significant increase in cEC proliferation in comparison with hearts in which *emilin2a* expression was not induced (Figures 4G and 4H). Notably, we also observed an increase in CM proliferation (Figure S6H and S6I) and a significant decrease in scar area at 30 dpci (Figures 4I and 4J). Altogether, these findings indicate that *emilin2a*, whose expression is stimulated by Vegfc signaling in EPDCs and cECs, is required for, and can enhance, heart regeneration.

emilin2a induces *cxcl8a* expression in epicardium-derived cells

Previous studies indicate that in HUVECs and human gastric tumors, EMILIN2 exerts its angiogenic role via CXCL8[62,64], a chemokine that signals through the G-protein coupled receptors CXCR1 and CXCR2 to regulate various aspects of wound healing[69]. Notably, other studies have highlighted the ability of CXCL8 to act as a pro-angiogenic factor in *in vitro* settings[70,71,72]. Thus, Emilin2a might induce *cxcl8a* expression during cardiac regeneration in zebrafish. To test this possibility, we first analyzed *cxcl8a* expression levels in *emilin2a*$^{-/-}$ ventricles at 96 hpci and observed a significant reduction when compared with *emilin2a*$^{+/+}$ ventricles (Figure 5A). Moreover, we observed *cxcl8a* upregulation in *Tg(hsp70l:emilin2a)* ventricles at 96 hpci when compared with non-transgenic siblings (Figure S7A). To further test whether *cxcl8a* is downstream of Vegfc signaling, we analyzed *cxcl8a* expression after blocking Vegfc signaling and found a significant downregulation in *Tg(hsp70l:sflt4)* ventricles at 96 hpci (Figure S7B). Conversely, injecting *vegfc* mRNA into one-cell stage zebrafish embryos resulted in an increase in *cxcl8a* expression (Figure S7C). In the regenerating heart, we observed *cxcl8a* expression in cells covering the injured tissue (Figure 5B). To determine the source of this expression, we performed RT-qPCR analyses on sorted EPDCs and sorted cECs, and found increased *cxcl8a* expression in regenerating EPDCs (Figure 5C), but not cECs, at 96 hpci when compared with sham operated controls (Figures S7D and S7E). Overall, these findings indicate that downstream of Vegfc signaling, the ECM protein Emilin2a induces *cxcl8a* expression, and that EPDCs are a source of Cxcl8a during zebrafish cardiac regeneration.

Cxcl8a-Cxcr1 signaling promotes coronary revascularization.

In view of the injury-induced upregulation of *cxcl8a* expression, we wanted to test whether Cxcl8a plays an important role during cardiac regeneration, and thus generated *cxcl8a* mutants using the CRISPR/Cas9 technology (Figures S8A and S8B). In silico analysis predicts that the protein encoded by the mutant transcript lacks the C-terminal 31 amino acids which are essential for Cxcl8a dimerization and receptor binding[73]. We analyzed cEC proliferation in *Tg(flt1:Mmu.Fos-GFP); cxcl8a*$^{+/+}$ and *Tg(flt1:Mmu.Fos-GFP); cxcl8a*$^{-/-}$ siblings at 96 hpci, and found a significant reduction in the mutants (Figures 5D and 5E).

Moreover, *cxcl8a*$^{-/-}$ ventricles displayed a significant reduction in coronary vessel coverage at 7 dpci (Figures 5F and 5G), while devoid of lymphatics (Figure S8C). In addition, *cxcl8a*$^{-/-}$ ventricles displayed a marginally larger scar at 30 (Figures S8D and S8E), and 90 (Figures 5H and 5I) dpci. Altogether, these data indicate that Cxcl8a plays an important role in coronary revascularization during cardiac regeneration in zebrafish.

In view of the reduced cEC proliferation phenotype in *cxcl8a* mutants, we reasoned that its receptor(s) might be expressed in cECs during heart regeneration. Indeed, we detected *cxcr1* expression at the periphery of the injured tissue at 96 hpci by *in situ* hybridization (Figure 6A). We also observed a significant upregulation of *cxcr1* expression in sorted cECs at 96 hpci, while no *cxcr2* expression was detected (Figure 6B). Importantly, pharmacological inhibition (with SB225002)[74] of Cxcr1 reduced coronary vessel coverage at 7 dpci (Figures S8F and S8G). Furthermore, in order to investigate the role of *cxcr1* during cardiac regeneration, we performed cryoinjuries on *cxcr1*$^{-/-}$ ventricles and observed a significant reduction in cEC proliferation at 96 hpci (Figures 6C and 6D), as well as defective coronary vessel coverage at 7 dpci (Figures 6E and 6F). Next, we checked whether *cxcr1*$^{-/-}$ ventricles were able to regenerate. Although they displayed similar scar size as *cxcr1*$^{+/+}$ sibling controls at 30 dpci (Figures S8H and S8I), *cxcr1*$^{-/-}$ ventricles displayed significantly larger scars at 90 dpci (Figures 6G and 6H). Altogether, these findings suggest that Cxcl8a signals through Cxcr1 in regenerating cECs to promote revascularization and support cardiac regeneration in zebrafish.

DISCUSSION

Following cardiac injury, the zebrafish heart mounts a fast angiogenic response that is essential for the regenerative process[11]. Here, we show that the regenerating coronary endothelium upregulates *vegfc* which is required for coronary revascularization after cardiac injury, independent of the presence or absence of lymphatics in the ventricle. We further show that reducing coronary revascularization by blocking Vegfc signaling impaired key aspects of cardiac regeneration including CM dedifferentiation and proliferation, as well as scar resolution. Mechanistically, Vegfc signaling induces *emilin2a* and *cxcl8a* expression, mostly in EPDCs, to promote coronary revascularization during cardiac regeneration in zebrafish.

One of the factors contributing to the inability of the adult mammalian heart to regenerate is its poor revacsularization[75]. In contrast, models with cardiac regenerative capacity like the zebrafish display a robust and fast angiogenic response to cardiac injury that supports heart regeneration[11]. Supporting this notion, perturbing coronary revascularization in regenerating zebrafish hearts impairs CM proliferation and prevents scar resolution[11]. On the other hand, inducing collateral artery formation following MI in adult mice results in a significant improvement in cardiac function[76]. Similarly, stabilizing vessel-like structures in the non-regenerative medaka heart following cardiac injury reduces scarring[15]. Therefore, improving revascularization in non-regenerating hearts should stimulate their regenerative response, and a better understanding of the mechanisms underlying revascularization in the regenerating zebrafish heart may help identify new therapeutic avenues.

Our data show that blocking Vegfc signaling results in reduced coronary revascularization, reduced CM proliferation, and the retention of a larger scar. Coronaries provide a scaffold for CMs during cardiac regeneration as well as during development[12]. Therefore, we hypothesize that the reduced CM dedifferentiation and proliferation phenotypes observed upon blocking Vegfc signaling occur as a consequence of reduced revascularization; however, tissue specific loss- and gain-of-function approaches will be needed to test this hypothesis. Our data show that Vegfc is a crucial angiocrine factor secreted by cECs to promote cardiac regeneration. Recent studies have emphasized how endothelial cells in different organs play important roles in regulating tissue homeostasis and repair by secreting angiocrine factors[13]. For instance, liver sinusoidal ECs govern the balance between liver regeneration and fibrosis by the preferential activation of the CXCR7 and CXCR4 receptors, respectively[77]. Similarly, following pneumonectomy, pulmonary capillary ECs secrete angiocrine factors, such as MMP14, that promote alveolar epithelial regeneration[78]. Importantly, it has been shown that EC-derived VEGFC stimulates the proliferation of neural stem cells during development and regeneration[79,80]. Likewise, our study illustrates the angiocrine role of Vegfc in the context of zebrafish cardiac regeneration to promote the proliferation of cECs and subsequently CMs. We further describe a mechanism by which Vegfc signaling is required for cardiac regeneration by stimulating the expression of the ECM gene *emilin2a*. During zebrafish development, Vegfc acts in both an autocrine and paracrine manner to induce venous as well as lymphatic sprouting by inducing cell cycle arrest via Vegfr3[32,81]. Similarly, our data showing *emilin2a* expression by cECs and EPDCs during cardiac regeneration suggest that Vegfc acts in both an autocrine and paracrine manner to promote *emilin2a* expression. Importantly, we found that global overexpression of *emilin2a* stimulates coronary revascularization and reduces scarring after cardiac injury. Previous studies have shown that *emilin2a* expression is not induced following cardiac injury in non-regenerative organisms such as medaka[15] and adult mice[82]. These observations, as well as our data highlighting the importance of *emilin2a* in promoting cardiac regeneration in zebrafish, indicate that Emilin2a enhance the regeneration process. Furthermore, we show that the regulation of *emilin2a* expression by Vegfc signaling is also present in human endothelial cells, encouraging future investigations into the role of VEGFC and EMILIN2 in promoting collateral formation in patients after MI.

Our data reveal how alterations in the ECM molecule Emilin2a affect coronary revascularization and cardiac regeneration in zebrafish. Indeed, recent studies have emphasized the role of the ECM in regulating different processes essential for cardiac regeneration including angiogenesis[83,84,85]. Interestingly, we found a number of ECM genes downregulated upon blocking Vegfc signaling (Table S4), including fibronectin 1b (*fn1b*) which has been previously implicated in angiogenesis[86,87,88] as well as in zebrafish cardiac regeneration[89]. We speculate that Vegfc signaling alters the expression of several ECM genes, thereby creating a microenvironment that facilitates coronary revascularization following cardiac injury. Further studies are needed to explore the potential of various ECM proteins in promoting cardiac regeneration.

To better understand how Emilin2a exerts its role as a pro-regenerative factor, we identified the pro-inflammatory chemokine Cxcl8a as a potential target. These results are in line with data showing that EMILIN2 induces CXCL8 expression to promote the migration of

HUVECs[58]. Our data reveal that *cxcl8a* is expressed in EPDCs in the injured tissue while *cxcr1* is expressed in regenerating cECs, suggesting an interaction between these cell types to regulate coronary revascularization. Recent studies have shown that EPDCs are a source of important signals that regulate coronary regeneration in zebrafish[12] and mouse[90]. CXCL8 has been shown in different settings to act as a pro-angiogenic factor via the activation of the G-protein coupled receptors CXCR1/2[70,71,72]. Our data showing reduced cEC proliferation in both *cxcl8a* and *cxcr1* mutants suggest that in the context of cardiac regeneration, Cxcl8a mediates its angiogenic role by activating Cxcr1 expressed by cECs. Accordingly, previous reports have implicated CXCR1 in promoting the proliferation of HUVECs in culture and endothelial cells in chick embryos, via different signaling pathways including Rho, Rac and MAPK activation[91]. Here, we provide evidence for a cellular crosstalk regulated by Cxcl8a-Cxcr1 signaling to promote cEC proliferation and cardiac regeneration.

Overall, our data highlight the importance of Vegfc signaling in promoting cardiac regeneration in zebrafish. We propose that Vegfc signaling induces the expression of the ECM component Emilin2a and chemokine Cxcl8a, as well as other factors, to provide a milieu that promotes cardiac regeneration. Our findings, along with data showing the importance of VEGFC signaling in promoting coronary development in mammals[33,38], lay a strong foundation to investigate the potential of a VEGFC-EMILIN2-CXCL8 signaling axis to enhance collateral formation and thereby improve cardiac function following myocardial infarction in mammals.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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NON-STANDARD ABBREVIATIONS AND ACRONYMS

MI	myocardial infarction
cECs	coronary endothelial cells
EPDCs	epicardium-derived cells
CMs	cardiomyocytes
ECM	extracellular matrix
hpci	hours post cryoinjury

dpci	days post cryoinjury
4-OHT	4-hydroxytamoxifen
EtOH	ethanol
HS	heat shock

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NOVELTY AND SIGNIFICANCE

What is known?

- The adult zebrafish heart mounts a fast angiogenic response following cardiac injury, and blocking this process impedes cardiac regeneration.

- *vegfc* expression is upregulated following cardiac injury in adult zebrafish

- Vegfc signaling is required for lymphangiogenesis during development and cardiac regeneration.

What new information does this article contribute?

- Vegfc signaling is required for coronary revascularization during cardiac regeneration in adult zebrafish.

- We identify two new effectors of Vegfc signaling: the genes encoding the extracellular matrix protein Emilin2a and the chemokine Cxcl8a, and show that they are both required for coronary revascularization during cardiac regeneration in adult zebrafish.

- Vegfc signaling promotes the expression of *emilin2a*, which in turn promotes *cxcl8a* expression in epicardium-derived cells to orchestrate a coronary-epicardium cross-talk necessary for cardiac regeneration.

Ischemic heart disease is a leading cause of death worldwide due to the inability of the adult mammalian heart to replace lost tissue. However, patients with improved collateral artery formation display improved clinical prognosis. Hence, identifying angiogenic factors in a regenerative setting holds promising therapeutic potential. We show that *vegfc* expression is upregulated in regenerating coronaries following cardiac injury in adult zebrafish and that Vegfc signaling is required for coronary revascularization. To understand the underlying mechanisms, we performed transcriptomic analysis and identified the extracellular matrix gene *emilin2a* and the chemokine gene *cxcl8a* as targets of Vegfc signaling. Mechanistically, we show that Emilin2a promotes the expression of *cxcl8a* in epicardium-derived cells to induce coronary revascularization and cardiac regeneration in adult zebrafish. These findings reveal an angiocrine role for Vegfc in promoting the expression of several factors that provide a milieu suitable for coronary revascularization and cardiac regeneration.
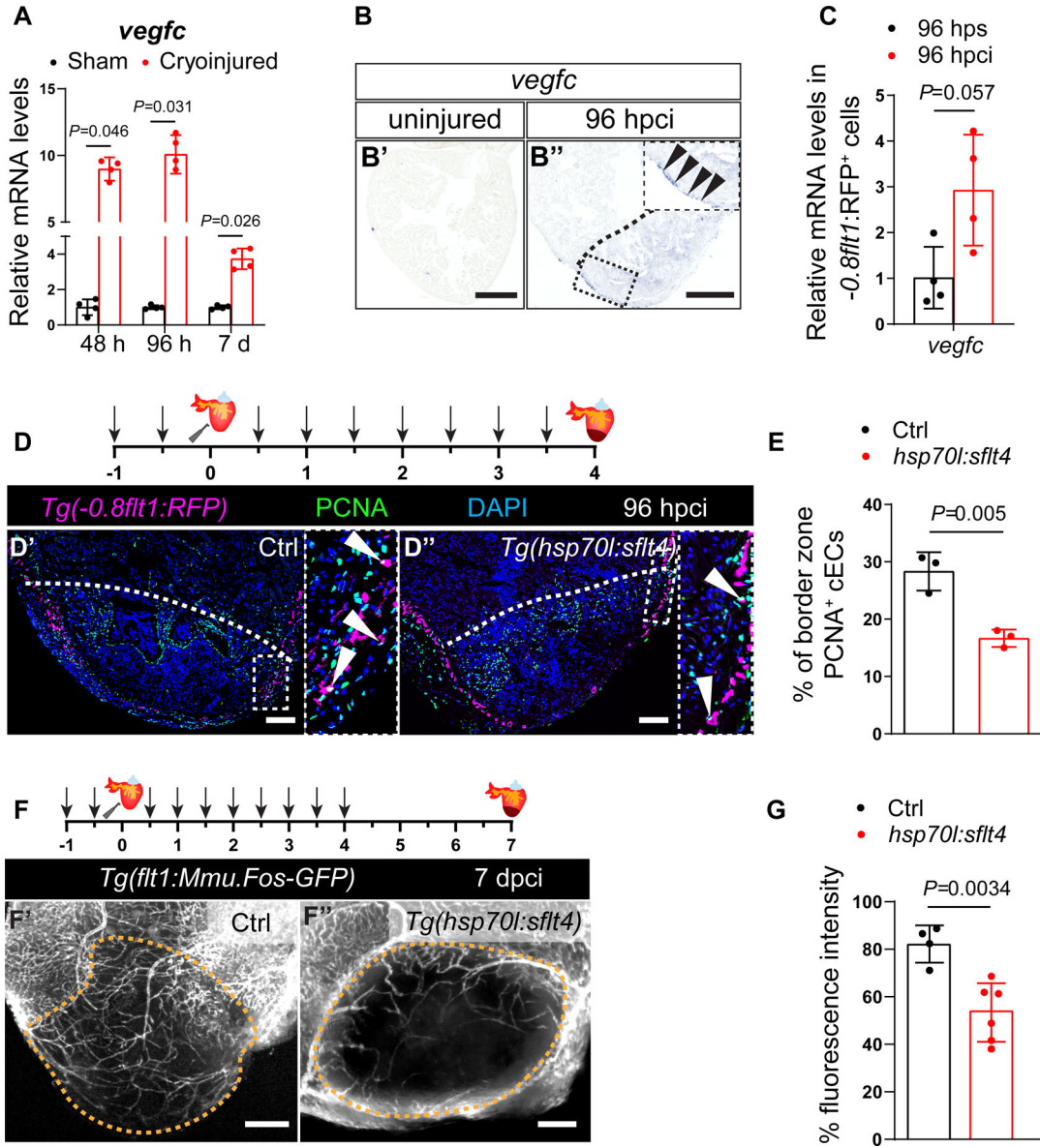
Figure 1: Vegfc signaling promotes coronary endothelial cell proliferation after cardiac injury in zebrafish.

A. RT-qPCR analysis of *vegfc* mRNA levels at 48 and 96 hpci and 7 dpci in the injured tissue normalized to sham-operated hearts (n=4); h: hpci, d: dpci. **B.** *in situ* hybridization for *vegfc* expression on sections of uninjured **(B')** and 96 hpci **(B")** hearts. Arrowheads point to *vegfc* expression. **C.** RT-qPCR analysis of *vegfc* mRNA levels at 96 hpci normalized to sham-operated hearts (96 hps) in sorted *−0.8flt1*:RFP+ cells (cECs) (n=4). **D.** Schematic representation of heat shock treatments (arrows) and cardiac cryoinjury. Immunostaining of sections of cryoinjured hearts of sibling *Tg(0.8flt1:RFP)* (Ctrl) **(D')** and *Tg(hsp70l:sflt4); Tg(−0.8flt1:RFP)* **(D")** zebrafish at 96 hpci; sections stained for RFP (coronaries, magenta), PCNA (proliferation marker, green), and DNA (DAPI, blue). Arrowheads point to PCNA+ cECs. **E.** Percentage of PCNA+ cECs in the border zone of *Tg(−0.8flt1RFP)* (Ctrl) (n=3) and *Tg(hsp70l:sflt4); Tg(−0.8flt1:RFP)* (n=3) ventricles at 96

hpci. **F.** Schematic representation of heat shock treatments (arrows) and cardiac cryoinjury. Wholemount images of cryoinjured hearts of sibling *Tg(flt1:Mmu.Fos-GFP)* (Ctrl) **(F')** and *Tg(hsp70l:sflt4); Tg(flt1:Mmu.Fos-GFP)* **(F")** zebrafish at 7 dpci. **G.** Percentage of GFP fluorescence intensity in the injured tissue of *Tg(flt1:Mmu.Fos-GFP)* (Ctrl) (n=4) and *Tg(hsp70l:sflt4); Tg(flt1:Mmu.Fos-GFP)* (n=6) ventricles at 7 dpci. Black **(B)**, white **(D)**, and orange **(F)** dotted lines delineate the injured tissue. Statistical tests: Non-parametric Mann-Whitney test **(A,C)**, Student's t-test **(E,G)**. Scale bars: 100 μm **(B,D,F)**. Ct values of RT-qPCR data are listed in table S3.
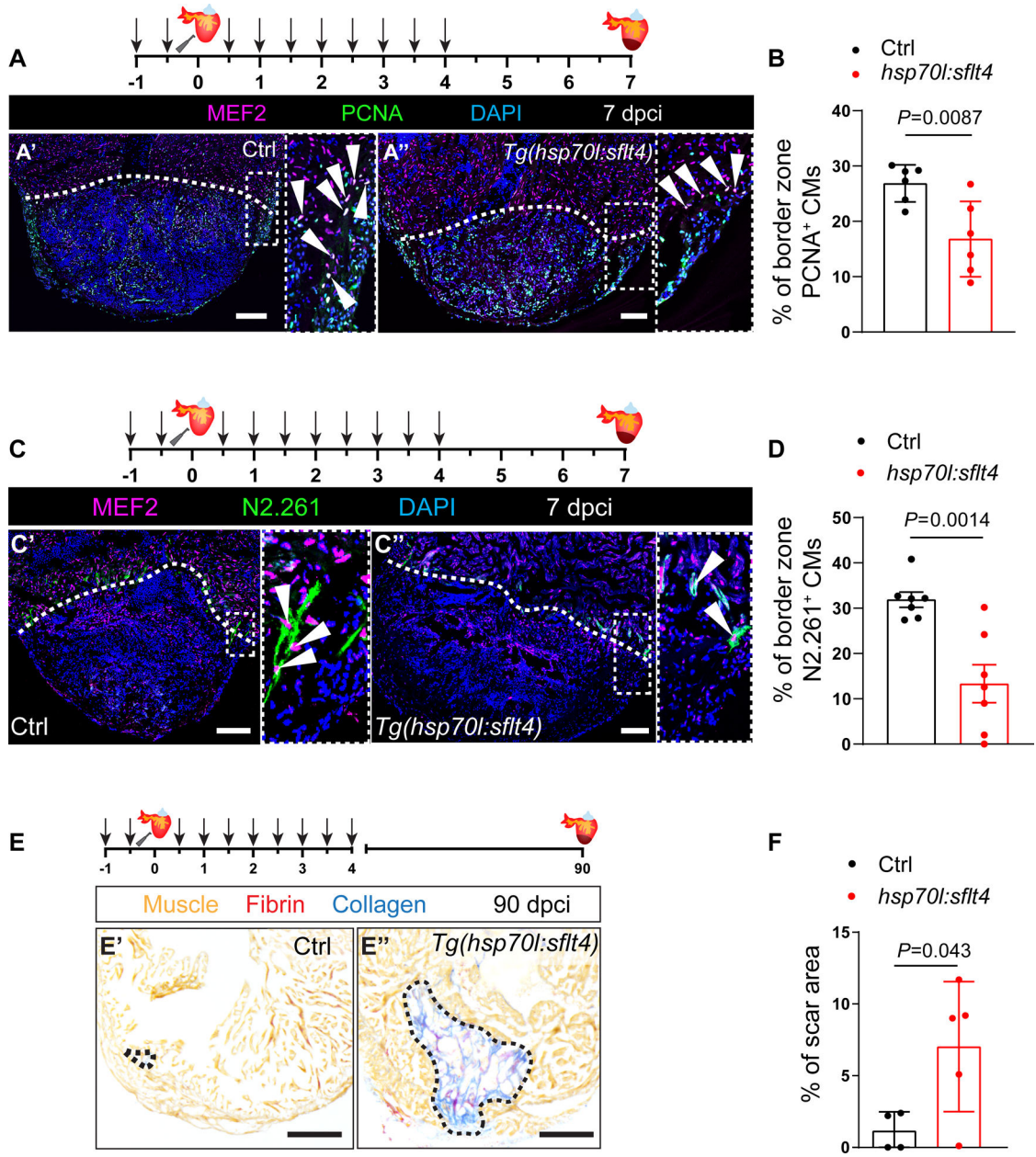
Figure 2: Blocking Vegfc signaling leads to reduced cardiomyocyte dedifferentiation and proliferation after cardiac injury in zebrafish.

A. Schematic representation of heat shock treatments (arrows) and cardiac cryoinjury. Immunostaining of sections of cryoinjured hearts of non-transgenic sibling (Ctrl) **(A')** and *Tg(hsp70l:sflt4)* **(A")** zebrafish at 7 dpci; sections stained for MEF2 (CMs, magenta), PCNA (proliferation marker, green), and DNA (DAPI, blue). Arrowheads point to PCNA+ CMs. **B.** Percentage of PCNA+ CMs in the border zone of non-transgenic sibling (Ctrl) (n=6) and *Tg(hsp70l:sflt4)* (n=6) ventricles at 7 dpci. **C.** Schematic representation of heat shock treatments (arrows) and cardiac cryoinjury. Immunostaining of sections of cryoinjured hearts of non-transgenic sibling (Ctrl) **(C')** and *Tg(hsp70l:sflt4)* **(C")** zebrafish at 7 dpci; sections stained for MEF2 (CMs, magenta), N2.261 (embryonic myosin heavy chain, green),

and DNA (DAPI, blue). Arrowheads point to N2.261$^+$ CMs. **D.** Percentage of N2.261$^+$ CMs in the border zone of non-transgenic sibling (Ctrl) (n=7) and *Tg(hsp70l:sflt4)* (n=7) ventricles at 7 dpci. **E.** Schematic representation of heat shock treatments (arrows) and cardiac cryoinjury. AFOG staining of sections of non-transgenic sibling (Ctrl) **(E')** and *Tg(hsp70l:sflt4)* **(E")** ventricles at 90 dpci. Orange, Muscle; red, Fibrin; blue, Collagen. **F.** Percentage of scar area relative to ventricular area in non-transgenic sibling (Ctrl) (n=4) and *Tg(hsp70l:sflt4)* (n=5) ventricles at 90 dpci. White **(A,C)**, and black **(E)** dotted lines delineate the injured tissue. Statistical test: Student's t-test **(B,D,F)**. Scale bars: 100 μm **(A,C,E)**.
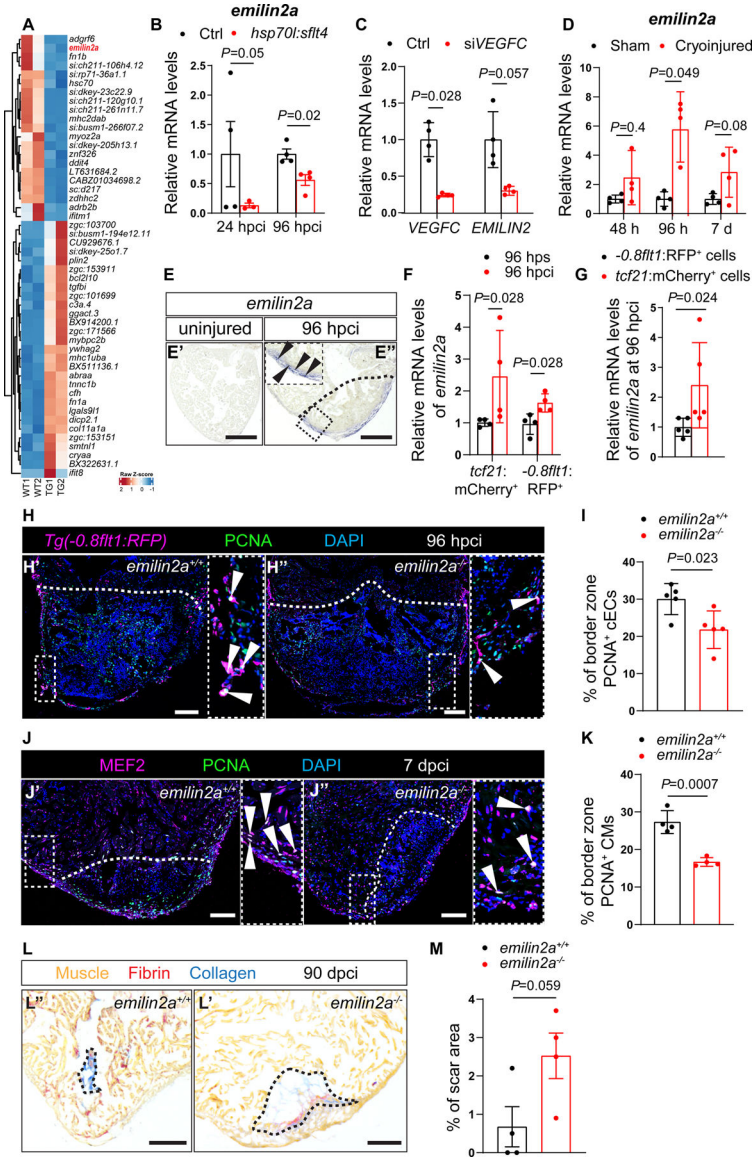
Figure 3: Vegfc signaling stimulates *emilin2a* expression to promote coronary endothelial cell proliferation in regenerating zebrafish hearts.
A. Heat map showing differentially expressed genes in non-transgenic sibling (WT) versus *Tg(hsp70l:sflt4)* hearts at 24 hpci. Genes are broadly divided into two clusters: one that contains genes that are upregulated in *Tg(hsp70l:sflt4)* ventricles and the other that contains genes that are downregulated in *Tg(hsp70l:sflt4)* ventricles. **B.** RT-qPCR analysis of *emilin2a* mRNA levels at 24 and 96 hpci in *Tg(hsp70l:sflt4)* ventricles normalized to non-transgenic sibling (Ctrl) hearts (n=4). **C.** RT-qPCR analysis of *VEGFC* and *EMILIN2* mRNA levels in HUVECs after *siVEGFC* treatment (n=4) in comparison with scrambled control (n=4). **D.** RT-qPCR analysis of *emilin2a* mRNA levels at 48 and 96 hpci and 7 dpci in injured tissue normalized to sham-operated hearts (n=4); h: hpci, d: dpci. **E.** *in situ* hybridization for *emilin2a* expression on sections of uninjured **(E')** and 96 hpci **(E")** hearts. Arrowheads point to *emilin2a* expression. **F.** RT-qPCR analysis of *emilin2a* mRNA levels in sorted *tcf21*:mCherry+ cells (EPDCs) (n=4) and sorted *−0.8flt1*:RFP+ cells (cECs) (n=4)

at 96 hpci normalized to 96 hps. **G.** RT-qPCR analysis of *emilin2a* mRNA levels in sorted *tcf21*:mCherry$^+$ cells (EPDCs) (n=5) normalized to sorted *−0.8flt1*:RFP$^+$ cells (cECs) (n=5) at 96 hpci. **H.** Immunostaining of sections of cryoinjured hearts of *Tg(flt1:Mmu.Fos-GFP); emilin2a$^{+/+}$* (**H'**) and *Tg(flt1:Mmu.Fos-GFP); emilin2a$^{-/-}$* (**H''**) sibling zebrafish at 96 hpci; sections stained for GFP (coronaries, magenta), PCNA (proliferation marker, green), and DNA (DAPI, blue). Arrowheads point to PCNA$^+$ cECs. **I.** Percentage of PCNA$^+$ cECs in the border zone of *Tg(flt1:Mmu.Fos-GFP); emilin2a$^{+/+}$* (n=5) and *Tg(flt1:Mmu.Fos-GFP); emilin2a$^{-/-}$* (n=5) ventricles at 96 hpci. **J.** Immunostaining of sections of cryoinjured hearts of *emilin2a$^{+/+}$* (**J'**) and *emilin2a$^{-/-}$* (**J''**) sibling zebrafish at 7 dpci; sections stained for MEF2 (CMs, magenta), PCNA (proliferation marker, green), and DNA (DAPI, blue). Arrowheads point to PCNA$^+$ CMs. **K.** Percentage of PCNA$^+$ CMs in the border zone of *emilin2a$^{+/+}$* (n=4) and *emilin2a$^{-/-}$* (n=4) ventricles at 7 dpci. **L.** AFOG staining of sections of *emilin2a$^{+/+}$* (**L'**) and *emilin2a$^{-/-}$* (**L''**) ventricles at 90 dpci. Orange, Muscle; red, Fibrin; blue, Collagen. **M.** Percentage of scar area relative to ventricular area in *emilin2a$^{+/+}$* (n=4) and *emilin2a$^{-/-}$* (n=4) ventricles at 90 dpci. Black (**E,L**), and white (**H,J**) dotted lines delineate the injured tissue. Statistical tests: Non-parametric Mann-Whitney test (**B,C,D,F,G**), Student's t-test (**I,K,M**). Scale Bars: 100 μm (**E,H,J,L**). Ct values of RT-qPCR data are listed in table S3.
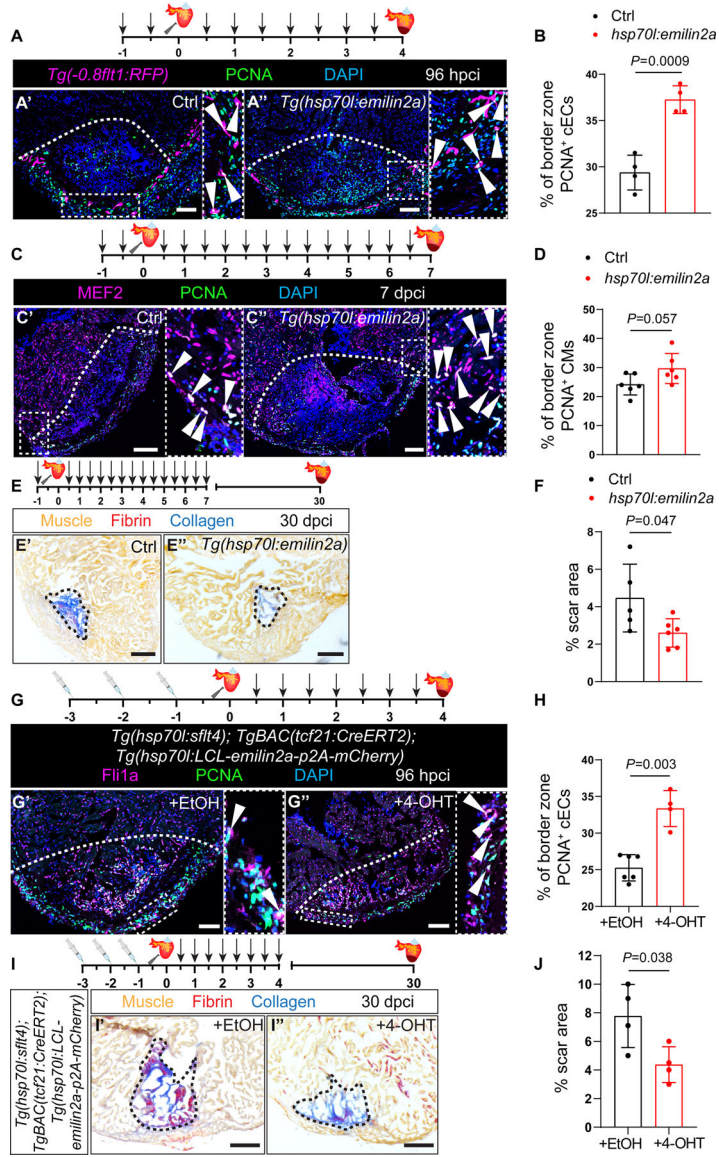
Figure 4: *emilin2a* **overexpression increases coronary endothelial cell proliferation and can rescue Vegfc signaling block in regenerating zebrafish hearts.**
A. Schematic representation of heat shock treatments (arrows) and cardiac cryoinjury. Immunostaining of sections of cryoinjured hearts of *Tg(0.8flt1:RFP)* (Ctrl) **(A')** and *Tg(hsp70l:emilin2a); Tg(−0.8flt1:RFP)* **(A'')** sibling zebrafish at 96 hpci; sections stained for RFP (coronaries, magenta), PCNA (proliferation marker, green), and DNA (DAPI, blue). Arrowheads point to PCNA+ cECs. **B.** Percentage of PCNA+ cECs in the border zone of *Tg(−0.8flt1RFP)* (Ctrl) (n=4) and *Tg(hsp70l:emilin2a); Tg(−0.8flt1:RFP)* ventricles (n=4) at 96 hpci. **C.** Schematic representation of heat shock treatments (arrows) and cardiac cryoinjury. Immunostaining of sections of cryoinjured hearts of non-transgenic sibling (Ctrl) **(C')** and *Tg(hsp70l:emilin2a)* **(C'')** zebrafish at 7 dpci; sections stained for MEF2 (CMs, magenta), PCNA (proliferation marker, green), and DNA (DAPI, blue). Arrowheads point to PCNA+ CMs. **D.** Percentage of PCNA+ CMs in the border zone of non-transgenic sibling (Ctrl) (n=6) and *Tg(hsp70l:emilin2a)* (n=6) ventricles at 7 dpci. **E.** Schematic representation

of heat shock treatments (arrows) and cardiac cryoinjury. AFOG staining of sections of non-transgenic sibling (Ctrl) **(E')** and *Tg(hsp70l:emilin2a)* **(E")** ventricles at 30 dpci. Orange, Muscle; red, Fibrin; blue, Collagen. **F.** Percentage of scar area relative to ventricular area in non-transgenic sibling (Ctrl) (n=5) and *Tg(hsp70l:emilin2a)* (n=6) ventricles at 30 dpci. **G.** Schematic representation of Ethanol (EtOH) or 4-hydroxytamoxifen (4-OHT) injections followed by cardiac cryoinjury and heat shock treatments (arrows). Immunostaining of sections of cryoinjured hearts of *Tg(hsp70l:sflt4); TgBAC(tcf21:CreERT2); Tg(hsp70l:LCL-emilin2a-p2A-mCherry)* zebrafish injected with EtOH **(G')** or 4-OHT **(G")** at 96 hpci; sections stained for Fli1a (endothelial cells, magenta), PCNA (proliferation marker, white), and DNA (DAPI, blue). Arrowheads point to PCNA$^+$ cECs. **H.** Percentage of PCNA$^+$ cECs in the border zone of *Tg(hsp70l:sflt4); TgBAC(tcf21:CreERT2); Tg(hsp70l:LCL-emilin2a-mCherry)* at 96 hpci injected with EtOH (n=6) or 4-OHT (n=4). **I.** Schematic representation of Ethanol (EtOH) or 4-hydroxytamoxifen (4-OHT) injections followed by cardiac cryoinjury and heat shock treatments (arrows). AFOG staining of sections of cryoinjured hearts of *Tg(hsp70l:sflt4); TgBAC(tcf21:CreERT2); Tg(hsp70l:LCL-emilin2a-p2A-mCherry)* zebrafish injected with EtOH **(I')** or 4-OHT **(I")** at 30 dpci. Orange, Muscle; red, Fibrin; blue, Collagen. **J.** Percentage of scar area relative to ventricular area in *Tg(hsp70l:sflt4); TgBAC(tcf21:CreERT2); Tg(hsp70l:LCL-emilin2a-mCherry)* at 30 dpci injected with EtOH (n=4) or 4-OHT (n=4). White **(A,C,G)**, and Black **(E,I)** dotted lines delineate the injured tissue. Statistical test: Student's t-test **(B,D,F,H,J)**. Scale Bars: 100 μm **(A,C,G)**, 200 μm **(E,I)**.

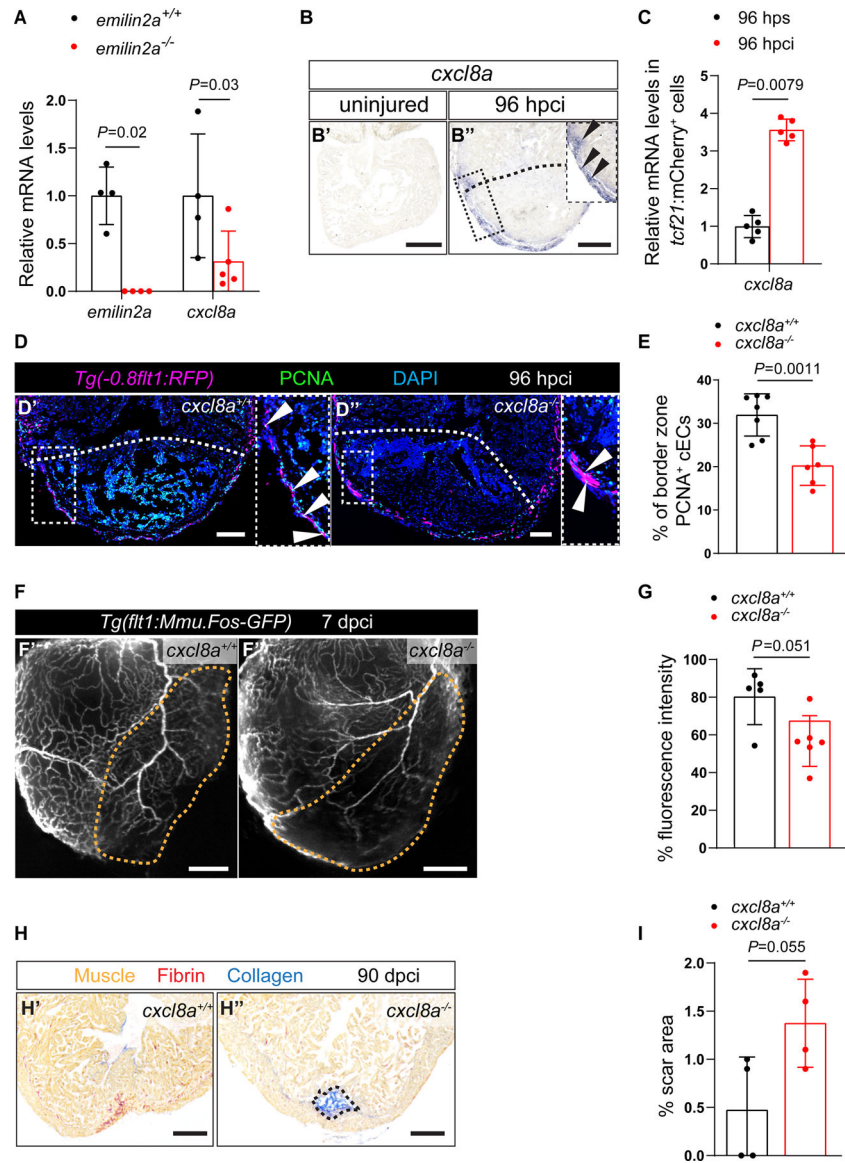**Figure 5: Emilin2a induces *cxcl8a* expression in EPDCs to promote coronary endothelial cell proliferation after cardiac injury in zebrafish.**

A. RT-qPCR analysis of *emilin2a* and *cxcl8a* mRNA levels in *emilin2a*$^{-/-}$ ventricles normalized to *emilin2a*$^{+/+}$ ventricles at 96 hpci (n=4–5). **B.** *in situ* hybridization for *cxcl8a* expression on sections of uninjured (**B'**) and 96 hpci (**B''**) hearts. Arrowheads point to *cxcl8a* expression. **C.** RT-qPCR analysis of *cxcl8a* mRNA levels in sorted *tcf21*:mCherry^{+} (EPDCs) (n=5) at 96 hpci normalized to 96 hps. **D.** Immunostaining of sections of cryoinjured hearts of *Tg(flt1:Mmu.Fos-GFP); cxcl8a*$^{+/+}$ (**D'**) and *Tg(flt1:Mmu.Fos-GFP); cxcl8a*$^{-/-}$ (**D''**) sibling zebrafish at 96 hpci; sections stained for GFP (coronaries, magenta), PCNA (proliferation marker, green), and DNA (DAPI, blue). Arrowheads point to PCNA^{+} cECs. **E.** Percentage of PCNA^{+} cECs in the border zone of *cxcl8a*$^{+/+}$ (n=7) and *cxcl8a*$^{-/-}$ (n=6) ventricles at 96 hpci. **F.** Wholemount images of ventricles of *Tg(flt1:Mmu.Fos-GFP); cxcl8a*$^{+/+}$ (**F'**) and *Tg(flt1:Mmu.Fos-GFP); cxcl8a*$^{-/-}$ (**F''**) sibling zebrafish at 7 dpci. **G.** Percentage of GFP fluorescence intensity in the injured tissue of *Tg(flt1:Mmu.Fos-GFP);*

$cxcl8a^{+/+}$ (n=5) and $Tg(flt1:Mmu.Fos-GFP); cxcl8a^{-/-}$ (n=6) ventricles at 7 dpci. **H.** AFOG staining of sections of $cxcl8a^{+/+}$ **(H')** and $cxcl8a^{-/-}$ **(H'')** ventricles at 90 dpci. Orange, Muscle; red, Fibrin; blue, Collagen. **I.** Percentage of scar area relative to ventricular area in $cxcl8a^{+/+}$ (n=4) and $cxcl8a^{-/-}$ (n=4) ventricles at 90 dpci. Black **(B,H)**, white **(D)** and orange **(F)** dotted lines delineate the injured tissue. Statistical tests: Non-parametric Mann-Whitney test **(A,C,G)**, Student's t-test **(E,I)**. Scale Bars: 200 μm **(B,F,H)**, 100 μm **(D)**. Ct values of RT-qPCR data are listed in table S3.
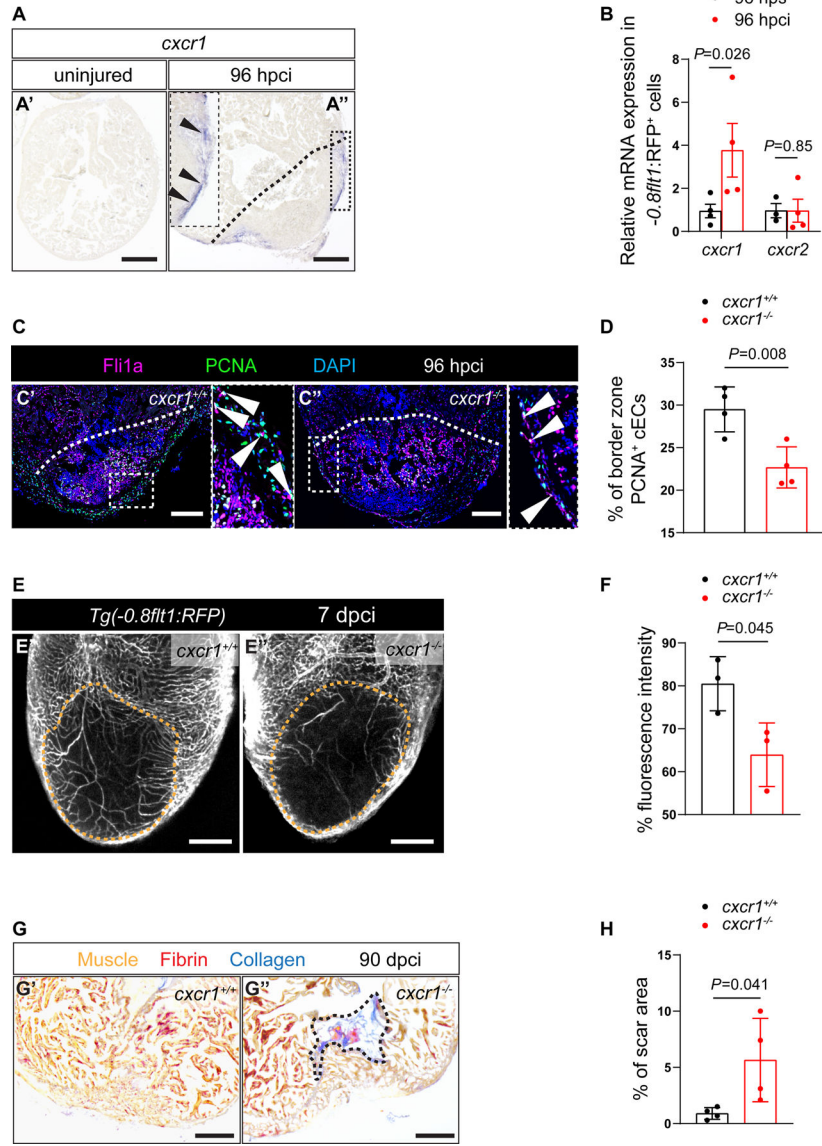
Figure 6: Cxcl8a-Cxcr1 signaling promotes coronary endothelial cell proliferation after cardiac injury in zebrafish.

A. *in situ* hybridization for *cxcr1* expression on sections of uninjured **(A')** and 96 hpci **(A")** hearts. Arrowheads point to *cxcr1* expression. **B.** RT-qPCR analysis of *cxcr1* and *cxcr2* mRNA levels in sorted *−0.8flt1*:RFP+ cells (cECs) (n=3–4) at 96 hpci normalized to 96 hps. **C.** Immunostaining of sections of cryoinjured hearts of *cxcr1+/+* **(C')** and *cxcr1−/−* **(C")** sibling zebrafish at 96 hpci; sections stained for Fli1a (endothelial cells, magenta), PCNA (proliferation marker, green), and DNA (DAPI, blue). Arrowheads point to PCNA+ cECs. **D.** Percentage of PCNA+ cECs in the border zone of *cxcr1+/+* (n=4) and *cxcr1−/−* (n=4) ventricles at 96 hpci. **E.** Wholemount images of ventricles of *Tg(−0.8flt1:RFP); cxcr1+/+* **(E')** and *Tg(−0.8flt1:RFP); cxcr1−/−* **(E")** sibling zebrafish at 7 dpci. **F.** Percentage of RFP fluorescence intensity in the injured tissue of *Tg(−0.8flt1:RFP); cxcr1+/+* (n=3) and *Tg(−0.8flt1:RFP); cxcr1−/−* (n=3) ventricles at 7 dpci. **G.** AFOG staining of sections of *cxcr1+/+* **(G')** and *cxcr1−/−* **(G")** ventricles at 90 dpci. Orange, Muscle; red, Fibrin;

blue, Collagen. **H.** Percentage of scar area relative to ventricular area in *cxcr1$^{+/+}$* (n=4) and *cxcr1$^{-/-}$* (n=4) ventricles at 90 dpci. Black **(A,G)**, white **(C)** and orange **(E)** dotted lines delineate the injured tissue. Statistical tests: Non-parametric Mann-Whitney test **(B)**, Student's t-test **(D,F,H)**. Scale Bars: 200 μm **(A,E,G)**, 100 μm **(C)**. Ct values of RT-qPCR data are listed in table S3.