



# Vegfaa instructs cardiac muscle hyperplasia in adult zebrafish

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**During heart development and regeneration, coronary vascularization is tightly coupled with cardiac growth. Although inhibiting vascularization causes defects in the innate regenerative response of zebrafish to heart injury, angiogenic signals are not known to be sufficient for triggering regeneration events. Here, by using a transgenic reporter strain, we found that regulatory sequences of the angiogenic factor *vegfaa* are active in epicardial cells of uninjured animals, as well as in epicardial and endocardial tissue adjacent to regenerating muscle upon injury. Additionally, we find that induced cardiac overexpression of *vegfaa* in zebrafish results in overt hyperplastic thickening of the myocardial wall, accompanied by indicators of angiogenesis, epithelial-to-mesenchymal transition, and cardiomyocyte regeneration programs. Unexpectedly, *vegfaa* overexpression in the context of cardiac injury enabled ectopic cardiomyogenesis but inhibited regeneration at the site of the injury. Our findings identify Vegfa as one of a select few known factors sufficient to activate adult cardiomyogenesis, while also illustrating how instructive factors for heart regeneration require spatiotemporal control for efficacy.**

heart | vegfa | angiogenesis | regeneration

**F**or patients with heart failure, new approaches that can replace lost or dysfunctional cardiac tissue through regeneration offer the possibility to improve clinical outcomes. Unlike adult mammals, zebrafish and neonatal mice mount strong regenerative responses following injury and have emerged as model systems to understand mechanisms of heart regeneration (1–3). For zebrafish and neonatal mice, cardiac injury results in the deployment of a developmental growth program and proliferation of spared cardiomyocytes (CMs) (2, 4, 5). Thus, a major strategy for enhancing innate regenerative responses has been to identify CM mitogens. However, heart regeneration is more complex than CM proliferation alone and requires an orchestration of interactions between the multiple cell types that make up the heart. For instance, disruption of signaling between epicardium, endocardium, and CMs by interference with FGF, retinoic acid, NF- $\kappa$ B, or Notch signaling pathways can vitiate zebrafish heart regeneration (6–10). Similarly, alterations to nerve or macrophage responses in neonatal mice or adult zebrafish impair heart regeneration (11–14). A detailed understanding of the mechanisms that coordinate tissue–tissue interactions during regeneration may inspire novel ways to stimulate regeneration in the adult mammalian heart.

During cardiac development and regeneration, angiogenesis shadows tissue growth. In developing mouse and zebrafish hearts, thickening of the ventricular wall by CM hyperplasia is tightly coupled to coronary angiogenesis (15, 16). Accordingly, during zebrafish heart regeneration, impairment of angiogenesis compromises the regenerative program and results in scarring (6, 15, 17). Recent work established angiogenesis as an early event during heart regeneration, while also implicating the Vegfa paralog Vegfaa as a required endothelial mitogen (17). The extent to which an angiogenic factor like Vegfa can influence cardiac repair in mammals on its own is a question of therapeutic significance that has been asked many times, with varying results (18). In a context of innate heart regeneration, this topic is unexplored.

Here, we examined whether and how induction of angiogenesis by increased expression of *vegfaa* can influence cardiac growth. We describe endogenous expression of zebrafish *vegfaa* and demonstrate the effects of ectopic *vegfaa* during multiple stages of cardiac growth, including injury-induced regeneration. Although, to our knowledge, Vegfaa has not been previously described as a cardiac mitogen in the uninjured heart, we found that overexpression of *vegfaa* drives CM hyperplasia and activates regenerative programs in the epicardium and endocardium. Together, these data place *vegfaa* in an exceptional group of secreted factors capable of inducing a cardiac regenerative program.

## Results

***vegfaa* Is Expressed in Epicardium During Zebrafish Cardiac Growth and Homeostasis.** Transcriptional profiling of the zebrafish heart and candidate gene analysis have demonstrated that *vegfaa* is rapidly up-regulated in the zebrafish heart following injury (17, 19). To define spatiotemporal patterns of *vegfaa* expression during heart regeneration, we generated a BAC transgenic line with *vegfaa* regulatory sequences directing the expression of a fluorescent reporter, EGFP [*TgBAC(vegfaa:EGFP)<sup>pd26b</sup>*, hereafter referred to as *vegfaa:EGFP*]. Mirroring previous reports of *vegfaa* expression, *vegfaa:EGFP* fish displayed EGFP fluorescence in developing somites, indicating that *vegfaa* regulatory sequences faithfully report domains of endogenous *vegfaa* (SI Appendix, Fig. S1 A and B) (20–24). During cardiac development, the expression domain for *Vegfa* varies among

## Significance

The human heart is unable to meaningfully recover lost cardiac muscle after injury. As a result, injuries such as myocardial infarction cause irreversible damage that increases the risk for heart failure. Replacing lost or dysfunctional cardiac tissue can potentially reverse or prevent heart failure. Here we demonstrate that experimentally increasing expression of the angiogenic factor *vegfaa* alone is able to induce a cardiac growth program in zebrafish. We also show evidence that the site of *vegfaa* expression is important for patterning the cardiac growth response. This work identifies Vegfa as a growth factor capable of activating genetic programs for heart regeneration and has generalizable implications for therapeutic administration of cardiac growth factors.

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The authors declare no conflict of interest.

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Data deposition: The fastq files for the RNASeq data of Vegf overexpression vs. controls are available on National Center for Biotechnology Information (NCBI) BioProject, <https://www.ncbi.nlm.nih.gov/bioproject> (accession no. PRJNA475163) and on NCBI Sequence Read Archive, <https://www.ncbi.nlm.nih.gov/sra> (accession no. SRP150028).

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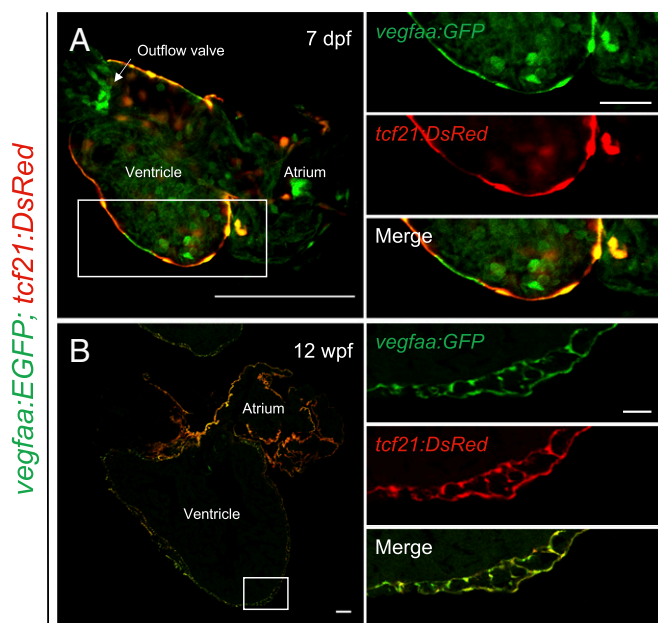
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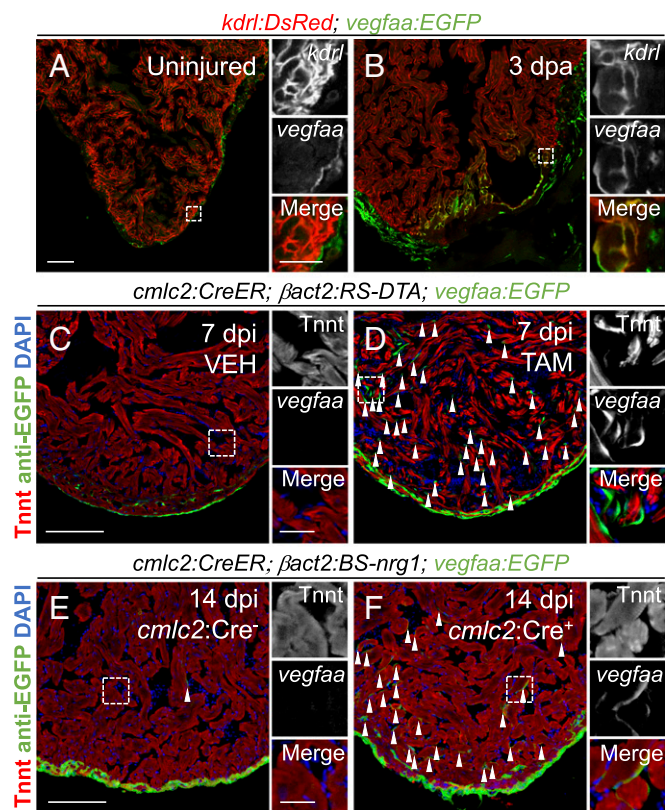
species. Whereas assessment in mouse indicates that *Vegfa* is expressed by CMs and the endocardium, *Vegfa* is expressed by CMs and the epicardium during development of the quail heart (25, 26). In *vegfaa:EGFP* zebrafish, we did not detect EGFP in CMs at any stage of zebrafish development. Instead, *vegfaa:EGFP* hearts displayed colocalization of EGFP with the epicardial reporter *tcf21:DsRed* during development and homeostasis (Fig. 1). EGFP signal was also present in epicardial-derived cells and *tcf21*-negative cardiac valves, and, in rare instances, endocardial cells. Our data indicate that the epicardium is the major source of *Vegfaa* under homeostatic conditions.

#### *vegfaa* Expression Is Dynamic During Zebrafish Heart Regeneration.

To determine which cardiac cells increase *vegfaa* expression during heart regeneration, we analyzed *vegfaa:EGFP* fish following amputation of the ventricular apex. Compared with the uninjured heart, we noted more EGFP<sup>+</sup> cells on the surface of the heart. Additionally, we visualized more EGFP<sup>+</sup> cells within the wound (SI Appendix, Fig. S1 C and E). These results are consistent with a previous report demonstrating a greater than twofold increase in *vegfaa* transcripts following cryoinjury in zebrafish (17). To better define the cellular sources of *vegfaa* during regeneration, we crossed *vegfaa:EGFP* fish to *kdr1:dsRed* fish that mark endothelial cells, including the ventricular endocardium (8). EGFP fluorescence colocalized with DsRed fluorescence following apical resection, whereas these markers rarely colocalized in the uninjured heart (Fig. 2 A and B). We also observed *vegfaa:EGFP* expression following genetic ablation of CMs, indicating that endocardial expression of *vegfaa* is not unique to the mode of injury (Fig. 2 C and D) (27, 28). *vegfaa* was also induced by global cardiac expression of the CM mitogen *nrg1* in the absence of injury, further relating *vegfaa* induction to muscle hyperplasia (Fig. 2 E and F) (28). Unlike with apical resection, *vegfaa*-positive endocardial cells were observed throughout the heart after CM ablation or *nrg1* induction, consistent with the global proliferative responses in these models. Together, our



**Fig. 1.** *vegfaa* expression during zebrafish heart development and homeostasis. Tiled images of *vegfaa:EGFP; tcf21:DsRed* cardiac sections during (A) larval and (B) adult stages. Sections are immunostained for EGFP. Boxes correspond to the region magnified in adjacent panels. (Scale bars: A and B, Left, 100  $\mu$ m; A and B, Right, 25  $\mu$ m.)



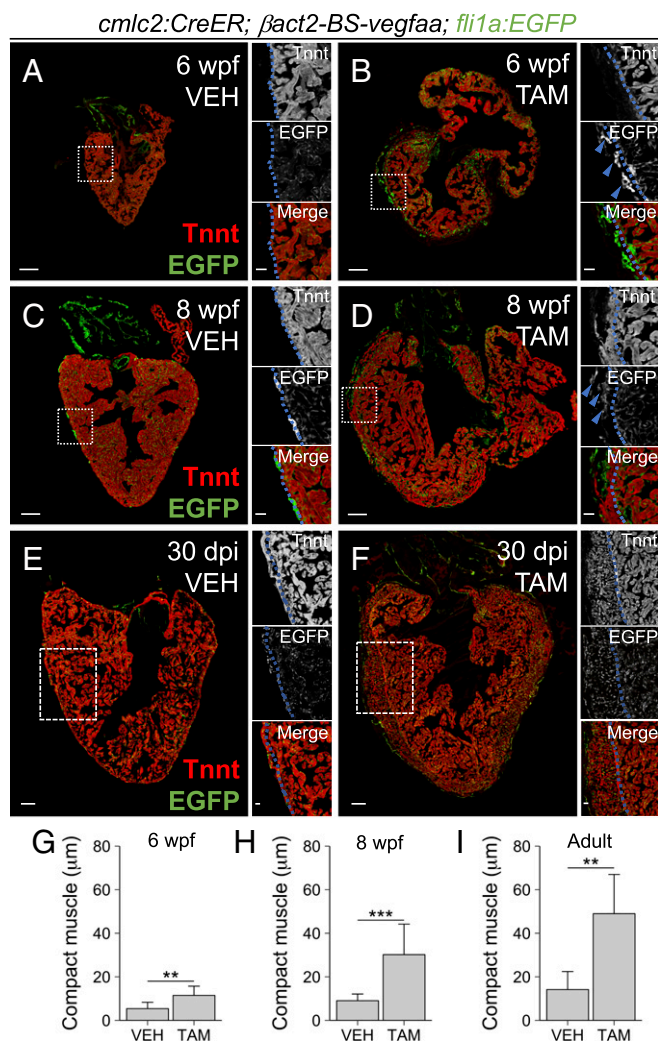
**Fig. 2.** *vegfaa* expression during adult zebrafish heart regeneration. (A and B) Colocalization of *vegfaa:EGFP* and *kdr1:DsRed* expression in hearts without injury and at 3 dpa. (C and D) *vegfaa:EGFP* expression in an uninjured heart and a heart 7 d post CM ablation (dpi). (E and F) *vegfaa:EGFP* expression in an uninjured heart and a heart after 14 d (dpi) of *nrg1* overexpression. Arrows indicate EGFP<sup>+</sup> endocardial cells. Boxes correspond to the region magnified in adjacent panels. [Scale bars: A, C, and E, 100  $\mu$ m (Left) and 25  $\mu$ m (Right); scale bars correspond to B, D, and F, respectively.]

results demonstrate the emergence of an endocardial source of *vegfaa* during zebrafish heart regeneration.

#### Induced *vegfaa* Overexpression Hypervascularizes the Zebrafish Heart and Rapidly Leads to Cardiomegaly.

To determine the influence of *vegfaa* on cardiac growth, we generated a new transgenic strain of zebrafish to conditionally overexpress *vegfaa*, *Tg( $\beta$ actin2:loxP-mTagBFP-STOP-loxP-vegfaa)<sup>pd262</sup>*, hereafter referred to as  *$\beta$ act2:BS-vegfaa*. We crossed  *$\beta$ act2:BS-vegfaa* fish with *cmlc2:CreER* fish to enable conditional overexpression of *vegfaa* from CMs by administration of tamoxifen (SI Appendix, Fig. S2 A–C). Further, we crossed *cmlc2:CreER;  $\beta$ act2:BS-vegfaa* fish with *fli1a:EGFP* fish to visualize EGFP<sup>+</sup> endothelial cells following *vegfaa* overexpression. When we induced *vegfaa* overexpression at 30 d post fertilization (dpf), a time point before the development of coronary vessels, we observed precocious and enhanced coronary vasculogenesis by 6 wk post fertilization (wpf; SI Appendix, Fig. S2 D, F, and G). Thus, *vegfaa* overexpression is sufficient to induce ectopic coronary vasculature in the zebrafish heart, similar to what has been observed in mice (29).

In addition to increased vasculature, we noticed that hearts overexpressing *vegfaa* as juveniles were dysmorphic, with larger chambers. When examined in cross-section at 6 wpf, we found hearts overexpressing *vegfaa* to have CMs dispersed sparsely in areas of ectopic vasculature (Fig. 3 A and B). Overall, the maximal thickness of the Tnnt<sup>+</sup> compact muscle in hearts overexpressing *vegfaa* at 6 wpf was more than twice that of control hearts ( $11.47 \pm 4.23 \mu$ m vs.  $5.40 \pm 2.90 \mu$ m,  $P = 0.003$ , Student's *t* test; Fig. 3G). By 8 wpf, the precocious compact muscle layer became even more



**Fig. 3.** Effects of cardiac *vegfaa* overexpression in the absence of injury. (A–D) Tiled images of *cmlc2:CreER;  $\beta$ act2:BS-vegfaa; fli1a:EGFP* hearts at 6 wpf and 8 wpf after treatment with vehicle or tamoxifen at 30 dpf. (E and F) Tiled images of adult *cmlc2:CreER;  $\beta$ act2:BS-vegfaa; fli1a:EGFP* hearts 30 dpi with vehicle or tamoxifen. Sections are immunostained for Tnnt. Boxes correspond to the region magnified in adjacent panels. Blue lines approximate the CM primordial layer and blue arrows denote ectopic vasculature. (G–I) Maximum thickness of the Tnnt<sup>+</sup> compact layer at 6 wpf after treating 30-dpf fish with vehicle ( $n = 11$ ) or tamoxifen ( $n = 6$ ); at 8 wpf after treating 30-dpf fish with vehicle ( $n = 11$ ) or tamoxifen ( $n = 9$ ); and at 30 dpi after treating adult fish with vehicle ( $n = 5$ ) or tamoxifen ( $n = 6$ ). Error bars indicate 5D (\*\* $P < 0.01$  and \*\*\* $P < 0.001$ , Student's  $t$  test). (Scale bars: A–F, Left, 100  $\mu$ m; A–F, Right, 25  $\mu$ m.)

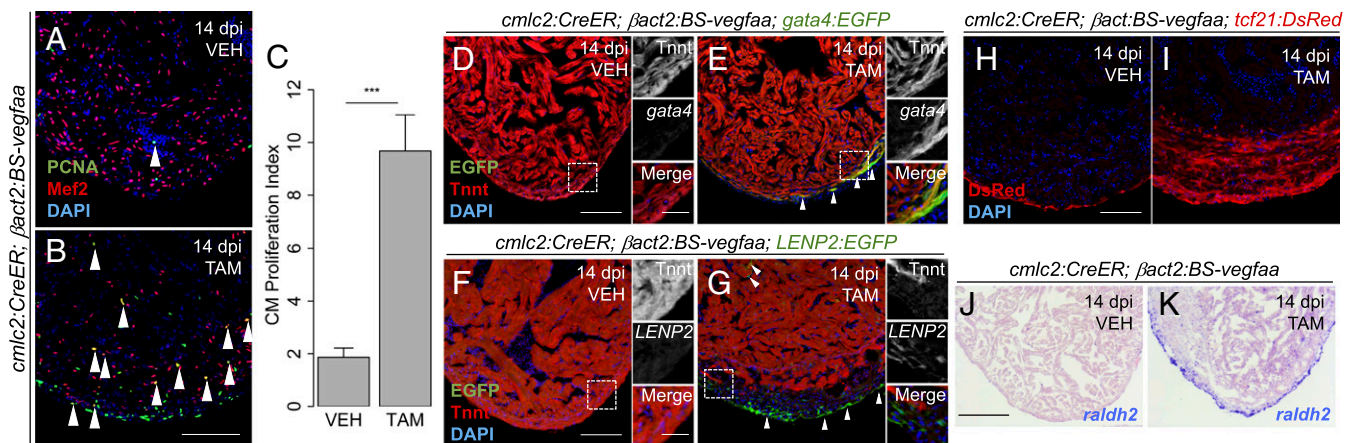
pronounced (Fig. 3 C and D). Maximal compact muscle thickness was increased by more than threefold with *vegfaa* overexpression compared with control hearts ( $30.19 \pm 14.00 \mu\text{m}$  vs.  $9.04 \pm 3.06 \mu\text{m}$ ,  $P < 0.001$ , Student's  $t$  test; Fig. 3H). Together, these results reveal that *vegfaa* overexpression can stimulate vascular growth and expansion of the muscular ventricular wall during zebrafish heart development.

To determine the extent to which *vegfaa* overexpression could affect the adult heart, we induced *vegfaa* overexpression in mature, 4-mo-old zebrafish and assessed animals 30 d later (SI Appendix, Fig. S2E). Strikingly, induced cardiac *vegfaa* overexpression caused expansion of the coronary vasculature, atrial growth, and thickening of the compact muscle layer in the ventricle (Fig. 3 E and F). Importantly, ectopic blood vessels were filled with nucleated blood cells, suggestive of functional perfusion (SI Appendix, Fig. S2 H and I). Similar to 4 wk of overexpression during development, the

maximal thickness of the compact muscle layer after 30 d of *vegfaa* overexpression in the adult heart was more than three times that of control hearts ( $49.00 \pm 18.00 \mu\text{m}$  vs.  $14.16 \pm 8.26 \mu\text{m}$ ,  $P = 0.003$ , Student's  $t$  test; Fig. 3I). Thus, ectopic *vegfaa* is sufficient to stimulate cardiac growth in zebrafish at juvenile and adult stages.

***vegfaa* Induces an Ectopic Heart Regeneration Program.** Zebrafish heart regeneration proceeds by induced cell cycle entry and successful division of a predominantly mononucleate, diploid population of CMs (4, 5, 30). Accompanying this proliferative response is a pronounced activation and expansion of the epicardial cells, which cover and integrate into the cardiac wound (6, 31, 32). To determine whether *vegfaa* could induce cardiomegaly through a regenerative program, we assayed these hallmarks. First, we evaluated CM cell cycle reentry by determining the percentage of Mef2<sup>+</sup>/PCNA<sup>+</sup> cells after *vegfaa* overexpression, a proxy for CM proliferation used in many studies (1). Although we did not detect a significant increase in the total number of Mef2<sup>+</sup> CMs by 14 d post incubation (dpi) with tamoxifen ( $1,397 \pm 243$  Mef2<sup>+</sup> cells per section vs.  $1,113 \pm 215$  Mef2<sup>+</sup> cells per section in control hearts,  $P = 0.40$ , Mann–Whitney  $U$  test), we identified a significant increase in the number of cycling CMs. In vehicle-treated hearts,  $1.86 \pm 0.35\%$  of Mef2<sup>+</sup> CMs were Mef2<sup>+</sup>/PCNA<sup>+</sup> (mean  $\pm$  SEM,  $n = 10$ ). By contrast, CM proliferation was sharply increased in hearts overexpressing *vegfaa*, with  $9.67 \pm 1.37\%$  of CMs positive for PCNA, an approximately fivefold increase (mean  $\pm$  SEM,  $n = 8$ ;  $P < 0.001$ , Mann–Whitney  $U$  test; Fig. 4 A–C), indicating that cardiomegaly directed by Vegfaa is hyperplastic. Interestingly, we observed trabecular and compact CM cycling during *vegfaa* overexpression. However, CM cycling and ectopic muscle was most prominent in the compact layer, suggesting a strong effect of *vegfaa* overexpression to expand the compact layer. We also noted a large increase in Mef2<sup>+</sup>/PCNA<sup>+</sup> nonmyocardial cells on the outer surface of the heart (Fig. 4 A and B). By using a *tcf21:DsRed* transgene to visualize epicardial responses, we observed a massive thickening of the *tcf21:DsRed*<sup>+</sup> tissue layer, indicative of epicardial expansion (Fig. 4 H and I). The maximal thickness of the *tcf21*<sup>+</sup> cell layer after 2 wk of *vegfaa* overexpression ( $72.46 \pm 38.25 \mu\text{m}$ , mean  $\pm$  SD,  $n = 5$ ) was increased by more than threefold compared with control hearts ( $21.25 \pm 5.27 \mu\text{m}$ , mean  $\pm$  SD,  $n = 4$ ;  $P = 0.03$ , Student's  $t$  test).

In addition to structural features of regeneration, we assessed *vegfaa*-overexpressing hearts for markers of regenerative signaling. Regenerating CMs, for example, can be marked by activation of regulatory sequences of the cardiogenic transcription factor *gata4* and sarcomere disassembly (4, 33). Animals overexpressing *vegfaa* had *gata4* expression in the compact muscle that was not observed in control animals (Fig. 4 D and E). Additionally, we noted that the expanded compact muscle layer in *vegfaa*-overexpressing hearts stains less prominently for Tnnt and is generally less organized compared with the trabecular muscle (Fig. 3 D and F). As a part of the epicardial response during heart regeneration, epicardial cells undergo epithelial-to-mesenchymal transition (EMT); correspondingly, we observed induction of the EMT markers *snai2*, *twist1*, and *pdgfrb* by in situ hybridization in hearts following *vegfaa* overexpression (SI Appendix, Fig. S3). Notably, these markers were induced in the region corresponding to expansion of *tcf21*<sup>+</sup> epicardial cells and their derivatives (Fig. 4 H and I). Hence, in zebrafish, *vegfaa* overexpression results in epicardial expansion and EMT, similar to what has been reported following overexpression of *Vegfa* in the mouse heart (34). Additionally, during zebrafish heart regeneration, the epicardium and the endocardium are activated to secrete paracrine factors, like retinoic acid, that support cell-cycle reentry of spared CMs (8). We noted increased expression in these tissues of the enzyme responsible for retinoic acid synthesis, *raldh2*, with *vegfaa* overexpression (Fig. 4 J and K). Finally, we recently identified an enhancer element upstream of the *lep* gene that is



**Fig. 4.** Cardiac *vegfaa* overexpression elicits an ectopic heart regeneration program. (A and B) Tiled images of *cmlc2:CreER; βact2:BS-vegfaa* hearts 14 dpi with vehicle or tamoxifen. Arrowheads indicate Mef2<sup>+</sup>/PCNA<sup>+</sup> nuclei. Mef2<sup>+</sup>/PCNA<sup>+</sup> nuclei are likely to be cycling epicardial cells, endothelial cells, fibroblasts, or inflammatory cells. (C) CM proliferation 14 dpi with vehicle (*n* = 10) or tamoxifen (*n* = 8). Error bars indicate SEM (\*\**P* < 0.001, Mann-Whitney *U* test). (D and E) *cmlc2:CreER; βact2:BS-vegfaa; gata4:EGFP* hearts 14 dpi with vehicle or tamoxifen. Arrowheads indicate *gata4*<sup>+</sup> CMs. (F and G) Images of *cmlc2:CreER; βact2:BS-vegfaa; LEMP2:EGFP* hearts 14 dpi with vehicle or tamoxifen. Arrowheads indicate *LEMP2*<sup>+</sup> cells. Tnnt marks CMs. Boxes correspond to the region magnified in adjacent panels. (H and I) Images of *cmlc2:CreER; βact2:BS-vegfaa; tcf21:DsRed* hearts 14 dpi with vehicle or tamoxifen. (J and K) In situ hybridization for *raldh2* on ventricular sections. Violet staining indicates expression. [Scale bars: B, H, and I, 100 μm (also apply to A, I, and K, respectively); D and F, 100 μm (Left) and 25 μm (Right) (also apply to E and G, respectively).]

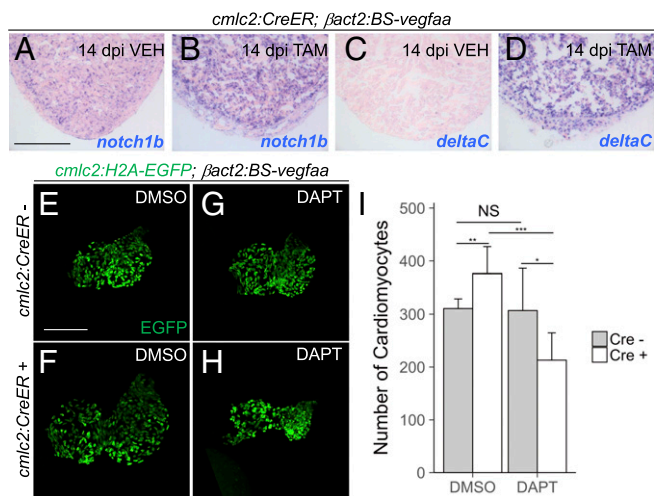
activated in endocardial and epicardial tissues during regeneration, but not during embryogenesis (19). We found that *vegfaa* overexpression was sufficient to trigger expression via this enhancer (Fig. 4 F and G). Together, our data strongly suggest that *vegfaa* overexpression directs cardiac growth by induction of a regenerative program.

**Features of the Vegfaa-Instructed Regeneration Program.** To understand how *vegfaa* overexpression induces a regenerative program, we compared transcriptional profiles of hearts overexpressing *vegfaa* vs. control hearts (SI Appendix, Fig. S4A). As might be expected, gene-set enrichment analysis revealed a significant up-regulation of genes related to “endothelial migration.” However, we also observed enrichment of genes related to “immune response” and “epithelial cell migration” (SI Appendix, Fig. S4 B–D). Gene sets down-regulated with *vegfaa* overexpression were related to “mitochondrion,” “energy derivation by oxidation of organic compounds,” and “generation of precursor metabolites and energy,” suggesting that *vegfaa* overexpression results in changes to cardiac energy utilization (SI Appendix, Fig. S4 E–G).

As a possible mediator of endothelial responses, we assessed Notch signaling. Endothelial Notch signaling is induced during zebrafish heart regeneration and is known to modulate CM proliferation (9, 35). Similarly, during mouse heart development, Notch inhibition leads to defects in CM proliferation (36, 37). Consistent with increased Notch signaling, our transcriptional profiling experiments showed hearts overexpressing *vegfaa* to have increased expression of Notch ligands and Notch receptors (Dataset S1). In situ hybridizations for *notch1b* and *deltaC* confirmed strong induction with *vegfaa* overexpression (Fig. 5 A–D).

To determine the mechanism by which *vegfaa* induces CM hyperplasia, we first examined whether Vegfaa effects were the result of increased coronary vasculature vs. other tissues. To disentangle contributions from the coronary circulation, we studied the effects of *vegfaa* overexpression on CM numbers in zebrafish embryos, not yet having formed coronary vasculature (38). We treated *βact2:BS-vegfaa;cmlc2:H2B-EGFP* and *cmlc2:CreER; βact2:BS-vegfaa;cmlc2:H2B-EGFP* fish with 4-HT at 2 dpf and then DMSO until 7 dpf. We found that *vegfaa* overexpression caused a ~21% expansion of CM numbers (376.4 ± 50.6 CMs vs. 310.7 ± 18.2 CMs, *P* = 0.002, Student’s *t* test; Fig. 5 E, F, and I), suggesting that Vegfaa is able to induce CM hyperplasia independently of the coronary vasculature.

To test whether the effects on CM numbers caused by *vegfaa* overexpression depend on Notch signaling, we employed the γ-secretase inhibitor DAPT (39). As demonstrated previously, inhibition of Notch signaling with the small molecule DAPT does not directly affect CM numbers and results in hearts with small ventricles (306.9 ± 79.3 CMs vs. 310.7 ± 18.2 CMs, *P* = 0.89, Student’s *t* test; Fig. 5 G–I) (40). However, *vegfaa* overexpression did not increase CM numbers in the presence of DAPT, even lowering counts by ~30% (213 ± 51.6 CMs vs. 306.9 ± 79.3 CMs, *P* = 0.01, Student’s *t* test; Fig. 5 F, G, and I).



**Fig. 5.** Dependence of *vegfaa*-induced CM hyperplasia on Notch signaling. (A–D) In situ hybridization for *notch1b* and *deltaC* on cardiac sections from *cmlc2:CreER; βact2:BS-vegfaa* animals 14 dpi with vehicle or tamoxifen. Violet staining indicates expression. (E–H) Maximum-intensity projections of 7 dpf hearts from *cmlc2:H2A-EGFP; βact2:BS-vegfaa* fish with or without an additional *cmlc2:CreER* transgene. Animals were first treated with 4-HT, followed by DMSO (*n* = 9 for Cre<sup>−</sup> animals and *n* = 10 for Cre<sup>+</sup> animals) or DAPT (*n* = 8 for Cre<sup>−</sup> animals and *n* = 9 for Cre<sup>+</sup> animals). (I) Number of CMs at 7 dpf for treatment groups shown in E–H. Error bars indicate SD (\**P* < 0.05, \*\**P* < 0.01, and \*\*\**P* < 0.001, Student’s *t* test). NS, not significant. [Scale bars: A and E, 100 μm (also apply to B–D and F–H, respectively).]

These data suggest that the positive effects of *vegfaa* on CM proliferation may act in part through Notch signaling.

**Ectopic *vegfaa* Misdirects Cardiogenesis After Injury.** As *vegfaa* overexpression on its own increased indicators of regeneration, we predicted that supplementing Vegfaa after cardiac injury would accelerate muscle regeneration. To test this idea, we partially resected ventricles 1 wk after inducing myocardial *vegfaa* expression. Fish overexpressing *vegfaa* displayed a  $\sim 72\%$  increased percentage of proliferating CMs at the wound ( $23.87 \pm 2.24\%$ , mean  $\pm$  SEM,  $n = 12$ ) compared with control animals ( $13.89 \pm 2.91\%$ , mean  $\pm$  SEM,  $n = 8$ ;  $P = 0.02$ , Mann–Whitney  $U$  test; Fig. 6 *B, E*, and *G*). Importantly, we also noted an increased percentage of cycling CMs more globally throughout the heart, including away from the wound. In animals overexpressing *vegfaa*, the number of CMs throughout the ventricle that were positive for PCNA was nearly double that in control hearts ( $9.08 \pm 0.86\%$  vs.  $4.47 \pm 1.08\%$ , mean  $\pm$  SEM,  $P = 0.004$ , Mann–Whitney  $U$  test; Fig. 6 *C, F*, and *H*). Thus, *vegfaa* overexpression increases CM proliferation during heart regeneration.

To determine effects on muscle regeneration, we induced *vegfaa* overexpression, performed apical resection, and assessed hearts histologically at 30 d post amputation (dpa), when regeneration of the myocardial wall is typically complete (3). Surprisingly, animals globally overexpressing *vegfaa* had persistent gaps and scarring at the resection site (Fig. 6 *J, K, M*, and *N* and *SI Appendix, Fig. S5*). Blinded, semiquantitative scoring of regenerative responses, as performed previously (7, 11), confirmed this impaired regeneration (mean regeneration score of  $2.71 \pm 0.59$  with *vegfaa* overexpression vs.  $1.14 \pm 0.36$  in control animals;  $P < 0.001$ , Kruskal–Wallis test; Fig. 6*O*). However, we still noted consistent tissue growth away from the wound, with thicker ventricular walls in *vegfaa*-overexpressing hearts (Fig. 6 *I, J, L*, and *M*). Thus, global *vegfaa* overexpression prevents regeneration at the resection site despite organ-wide neocardiogenesis, indicating a classic, instructive role for *vegfaa* signaling in tissue morphogenesis during regeneration.

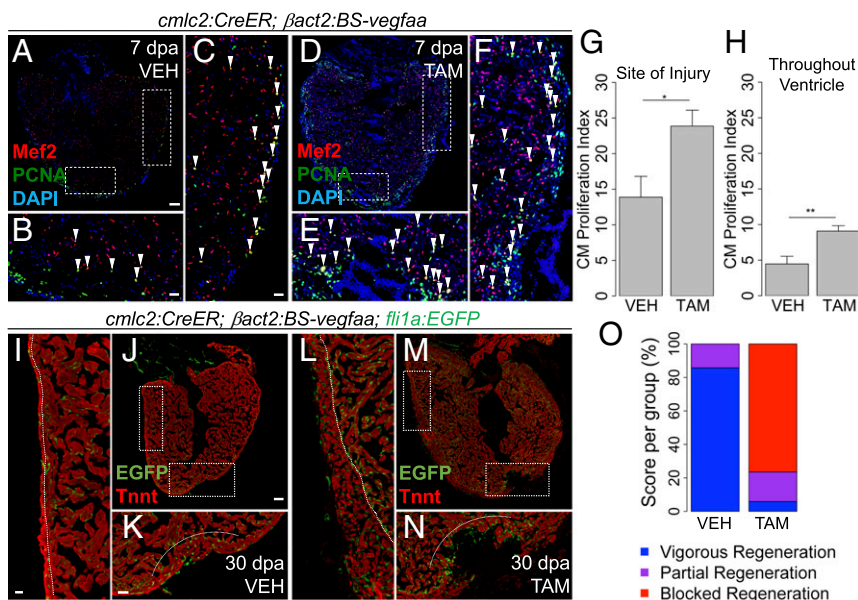
## Discussion

During organogenesis, a dialogue between parenchymal cells and organ-specific vascular endothelium coordinates angiogenesis with organ growth. For instance, *Vegfa* expression in mice by alveolar epithelial cells and by CMs guides the development of

the pulmonary and coronary vasculatures, respectively (41, 42). Reciprocally, specialized vascular endothelium secretes angiocrine factors to support organogenesis. Signals from the endothelium are required for growth of the liver bud and differentiation of pancreatic islets during development (43, 44). More recently, an appreciation of the cross-talk between vascular endothelium and parenchymal cells has been extended to tissue regeneration. Angiocrine factors support hepatic regeneration and expansion of basal cells and alveolar cells during lung regeneration (45). Here, we extend the symbiotic link between angiogenesis and tissue growth to the context of innate heart regeneration in zebrafish.

We identify *Vegfa* as an instructive factor for hyperplastic cardiac growth. Endocardial expression of *vegfaa* is spatiotemporally related to regions of regenerative growth after injury. However, global misexpression of *vegfaa* in cardiac muscle after apical resection abrogates local tissue regeneration despite concomitant ventricular wall neocardiogenesis. In contrast to other manipulations that block heart regeneration in zebrafish, ectopic *vegfaa* expression formally misdirects growth rather than inhibiting it (7–9, 17). Ultimately, our investigation of *vegfaa* roles during animal development and regeneration indicate that cardiomyogenic growth and morphogenesis are linked but distinct: a crucial concept in regeneration of intricately patterned structures like limbs, but not intuitive for heart regeneration.

Although *Vegfa* has been suggested to stimulate low levels of CM cycling, but past literature does not predict such marked effects on cardiomyogenesis (46–48). While our data in larvae suggest that the coronary vasculature is not required for *Vegfa* to induce CM proliferation, the cardiac endothelium is likely a key mediator of *Vegfaa* effects. Not only are the primary receptors for *Vegfa*, *kdrl* and *flt1*, expressed by the endocardium and vascular endothelium (Fig. 2*B*), but *vegfaa*-induced CM hyperplasia depends on Notch signaling (Fig. 5 *E–I*) (8, 17). Prior work has shown Notch pathway elements to be induced in the endocardium during development and regeneration in zebrafish (9, 10, 40). Notch inhibition reduces indicators of CM proliferation and impairs heart regeneration after resection or cryoinjury. Interestingly, hyperactivation of Notch signaling was reported to increase CM proliferation after cryoinjury but to reduce CM proliferation after resection injury. This discrepancy may be the result of different genetic tools or injury contexts, yet neither study reported activation of overt ectopic myocardial growth (9, 10). Thus, in addition to



**Fig. 6.** Effect of *vegfaa* overexpression on heart regeneration. (*A–E*) Tilescan images of *cmlc2:CreER*; $\beta$ *act2:BS-vegfaa* hearts at 7 dpa after treatment with vehicle or tamoxifen. Boxes mark the regions at the injury site (*B* and *E*) and away from the injury site (*C* and *F*). Arrowheads indicate *Mef2*<sup>+</sup>/*PCNA*<sup>+</sup> nuclei. *PCNA*<sup>+</sup> nuclei that do not colocalize with *Mef2* are likely to be cycling epicardial cells, endothelial cells, fibroblasts, or inflammatory cells. (*G* and *H*) CM proliferation adjacent to the wound (*G*) and throughout the ventricle (*H*) at 7 dpa and after treatment with vehicle ( $n = 8$ ) or tamoxifen ( $n = 12$ ). Error bars indicate SEM (\* $P < 0.05$  and \*\* $P < 0.01$ , Mann–Whitney  $U$  test). (*I–N*) *cmlc2:CreER*; $\beta$ *act2:BS-vegfaa*;*flil1a:EGFP* ventricles at 30 dpa. *Tnnt* marks CMs. Boxes mark the regions at the injury site (*K* and *N*) and away from the injury (*I* and *L*). Dashed lines in *K* and *N* approximate the resection plane. Dashed lines in *I* and *L* indicate the boundary of trabecular muscle and compact muscle. (*O*) Regeneration scores at 30 dpa after treatment with vehicle ( $n = 14$ ) or tamoxifen ( $n = 17$ ;  $P < 0.001$ , Kruskal–Wallis test). [Scale bars: *A* and *J*, 100  $\mu$ m (also apply to *D* and *M*, respectively); *B*, *C*, *I*, and *K*, 25  $\mu$ m (also apply to *E*, *F*, *L*, and *N*, respectively).]

inducing Notch signaling, Vegfaa is likely to trigger additional factors that promote cardiomyogenesis. Future work can be directed to identify how and which subsets of the cardiac endothelium might mediate *vegfaa* effects on cardiac growth.

Our work suggests that Vegfa signaling can be manipulated for therapeutic heart regeneration. In fact, Vegfa signaling might even account for some of the stimulatory effects on CM cell cycling recently reported with chronic hypoxia, as *Vegfa* is classically induced in hypoxic tissue (49–51). Although we show that misexpression of *vegfaa* can disrupt regenerative morphogenesis, a more targeted application of Vegfa should better direct and augment regenerative responses. In support of this concept, localized injection of virus overexpressing *Vegfa* and plasmids encoding *Vegfa* are reported to increase ventricular function when they are injected into the border zone following experimental myocardial infarction in mammals (18). Unfortunately, despite promising preclinical findings in mammalian models, human clinical trials with VEGFA have not been successful, likely related to the low doses of VEGFA used (18). Indeed, our work suggests a threshold effect for Vegfa. In zebrafish, *vegfaa* is expressed at low levels by the epicardium, but only with increases in expression above

these levels did we observe ectopic cardiogenesis and regenerative responses. Thus, our findings help highlight the need for attention to factor dosage, duration, and site of delivery to translate regenerative biology to therapeutics. Indeed, newer strategies that allow for controlled delivery of Vegfa and other factors via modified RNA, engineered nanofibers, or specialized regulatory elements may be needed to achieve therapeutic heart regeneration (19, 34, 52).

## Materials and Methods

Procedures for zebrafish transgenesis and ventricular resection have been previously described (7, 32). Immunostaining and in situ hybridization procedures are described in refs. 7 and 8. Compact muscle thickness, CM proliferation, and regeneration scores were measured as previously described (7, 30). Detailed materials and methods are provided in the *SI Appendix*.

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