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Primordial cardiomyocytes orchestrate myocardial morphogenesis and vascularization but are dispensable for regeneration

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eLife Assessment

This **important** work identifies *phlda2* as a specific marker for primordial cardiomyocytes in the adult zebrafish heart and demonstrates their essential role in myocardial morphogenesis and coronary vascularization, but not in heart regeneration. The conclusions are well supported by single-cell transcriptomics, new genetic tools, and cell-specific ablation experiments. Overall, the evidence is **solid** and provides insight into the difference between developmental and regenerative cardiac programs. This work will be of interest for those studying cardiac development and regeneration.

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Abstract

The vertebrate heart is composed of heterogeneous cardiomyocyte (CM) populations, however, the roles of distinct CM subpopulations in heart development and repair remain poorly defined. Here, using single-cell RNA sequencing analysis of adult zebrafish heart, we identified a unique CM subpopulation marked by the expression of *phlda2*, which is associated with anaerobic metabolism and different from mature CMs that are enriched for oxidative phosphorylation genes. We demonstrated *phlda2*⁺ cells constituted a primordial CM compartment localized between compact and trabecular muscles. Genetic ablation of *phlda2*⁺ CMs during development severely disrupted heart morphogenesis, leading to defective myocardial trabeculation and compaction, and impaired coronary vascularization. Surprisingly, despite their essential roles in development, the depletion of *phlda2*⁺ CMs didn't impair myocardial restoration and revascularization following ventricular resection. We further found that primordial CMs failed to regenerate after either surgical amputation or genetic ablation, indicating a limited regenerative capacity. Our findings identify primordial CMs as an organizer for heart morphogenesis but not essential for regeneration, revealing a fundamental difference between developmental and regenerative programs in the vertebrate heart.

Introduction

The vertebrate myocardium is not a homogeneous tissue but consists of cardiomyocyte (CM) subpopulations that are functionally and molecularly different^{8,9}. Recent single-cell transcriptomic studies have revealed this cellular heterogeneity in both mammalian and zebrafish hearts, showing clusters of CMs with specialized metabolic, structural, and contractile properties^{10–14}. Although these studies provide the evidence of cellular diversity of the myocardium, the functional significance of these CM subpopulations during heart development and disease remains a central question in cardiac biology.

Humans, like all mammals, lack natural ability to efficiently replace lost myocardium with new contractile tissue, a deficiency that results in the leading cause of morbidity and mortality^{15–17}. However, zebrafish possess a remarkable capacity to effectively regenerate the lost CMs after injury^{2,6,18–21}, facilitated by non-myocardial cells including epicardial cells^{3,5}, immune cells^{22,23}, endothelial cells^{24–26} and nerve cells²⁷, making zebrafish an excellent model for exploring natural heart regeneration. Previous studies have shown that spared CMs dedifferentiate and proliferate to replace the lost myocardium^{2,16,28}. However, it remains unclear whether CM subpopulations contribute equally to myocardial restoration. Furthermore, although the regeneration process has been generally accepted to recapitulate the developmental program²⁹, it is unknown whether all CM subpopulations play similar roles in heart morphogenesis and regeneration. Therefore, identifying and characterizing the roles of CM subpopulations are critical for understanding the mechanisms of heart development and repair.

Adult zebrafish myocardium is composed of three main types of CMs with different spatial arrangements: the inner mass of trabecular CMs, the outer layer of compact CMs, and a single-cell-thick layer of primordial CMs that located beneath the compact layer^{30–32}. While the trabecular and compact muscles have been extensively studied^{30,33–36}, the function of primordial CMs remains elusive due to the lack of genetic tools for their manipulation. Primordial CMs are thought to retain immature characteristics³⁷, but their molecular identity and functional contributions to myocardial morphogenesis and regeneration have not been fully elucidated. Here, we performed single-cell RNA sequencing (scRNA-seq) analysis of adult zebrafish heart to define CM subpopulations, and investigated the contribution of primordial CMs to myocardial morphogenesis and regeneration. Our studies reveals that primordial CMs are an essential organizer for myocardial morphogenesis and coronary vascularization, but not required for myocardial restoration and revascularization, highlighting regeneration goes beyond the reactivation of developmental programs.

Methods

Zebrafish and heart injuries

4–10-month-old outbred EK or EK/AB zebrafish were used for ventricular resection surgeries. To deplete *phlda2*-expressing cells, *phlda2:mCherry-NTR* transgenic zebrafish were used at the age of 6–8 weeks (juvenile) and 4–12 months (adult). Animal density was maintained at ~4 fish/L in all experiments. The *phlda2:mCherry-NTR* animals and control wild type siblings were treated with vehicle or 10 mM metronidazole (Mtz) (MilliporeSigma) in a 1.5 L mating tank for 12 hours/day to ablate *phlda2*⁺ cells. Transgenic strains described elsewhere include: *gata4:EGFP*¹; *cmlc2:EGFP*²; *tcf21:mCherry*³; and *deltaC:EGFP*⁴. All transgenic strains were analyzed as hemizygotes. All animal procedures were performed in accordance with Emory University guidelines.

Constructs, zebrafish transgenes

phlda2:EGFP

The *phlda2* translational start codon in the BAC clone CH211-193F12 was replaced with *EGFP* by Red/ET recombineering technology (GeneBridges). 5' and 3' homologous arms flanking the *EGFP* cassette for recombination were a 248-base-pair (bp) fragment upstream of the start codon and a 364-bp fragment downstream, respectively. BACs for transgenesis were purified with a midi-prepare 100 kit (Qiagen) and co-injected with PI-SceI into one-cell-stage zebrafish embryos. The full name of this transgenic line is Tg (*phlda2:EGFP*)^{em21}.

phlda2:mCherry-NTR

The *phlda2* translational start codon in the BAC clone CH211-193F12 was replaced with *mCherry-NTR* by Red/ET recombineering. 5' and 3' homologous arms flanking the *mCherry-NTR* cassette for recombination were a 248-bp fragment upstream of the start codon, and a 364-bp fragment downstream, respectively. The full name of this transgenic line is Tg (*phlda2:mCherry-NTR*)^{em22}.

Histological methods and quantification

Histological analyses were performed on 10 μm cryosections or whole-mount paraformaldehyde-fixed hearts. Sectioned ventricular tissues were imaged using a Zeiss LSM800 confocal scanning microscope with ZEN V3.7. F.

To quantify trabecular muscle loss, three medial longitudinal sections were selected from each heart and imaged. Single optical slices of the ventricle were acquired using a 20x objective lens (1024 $\times$ 1024 pixels) at the same ventricular position in sections from *cmhc2:EGFP* hearts. Trabecular muscle loss was quantified as the ratio of *cmhc2:EGFP*⁺ cell area to the total heart area within a region extending 100 μm from the ventricular wall. Quantification of *gata4:EGFP*⁺ expression in adult fish was performed as described⁵.

To quantify coronary expansion in developing hearts from *deltaC:EGFP* fish, whole-mounted specimens were selected, and ventricle images were captured using a 20x objective lens (1,024 $\times$ 1,024 pixels). The coronary vessel lengths were measured by ImageJ software. To quantify the vessel broken, the total number of vessel fragments were counted. To quantify coronary vessel expansion in regenerating hearts from *deltaC:EGFP* fish, images of ventricles were captured using a 20x objective lens (1,024 $\times$ 1,024 pixels). The GFP signals were measured in pixels by ImageJ software for signals in the injury site.

To quantify *gata4:EGFP*⁺ expression in developing hearts from *gata4:EGFP* fish, whole-mounted specimens were selected and ventricle images were captured using a 20x objective lens (1,024 $\times$ 1,024 pixels). The GFP signals were measured in pixels and mean intensity by ImageJ software in the injury site. To quantify *gata4:EGFP*⁺ expression in regenerating hearts from *gata4:EGFP* fish, images of ventricles were captured using a 20x objective lens (1,024 $\times$ 1,024 pixels). The GFP signals were measured in pixels by ImageJ software for signals in the injury site.

AFOG staining

A triple staining with Aniline blue, acid Fuchsin and Orange-G (AFOG) was performed as previously described⁶. Briefly, cryosections were fixed in Bouin's solution (Sigma Aldrich) for 2 h at 60°C, and one hour at room temperature. The slides were washed for 30 min in tap water and then treated with 1% phosphomolybdic acid (Sigma Aldrich) and 0.25% phosphotungstic acid solution (Sigma Aldrich) for 5 min. They were rinsed with distilled water, and incubated for 5 min with AFOG solution containing 3 g acid Fuchsin (Sigma Aldrich), 2 g Orange G (Sigma Aldrich), 1 g Anilin Blue (Sigma Aldrich) in distilled water, with pH adjusted to 1.1. After 5-min wash with distilled water, the sections were dehydrated in graded series of ethanol and two changes of xylene, then mounted with Permount mounting medium (Fisher Chemical). Degree of regeneration was scored in a blind-test assessment of AFOG stained sections at 30 dpa. Three sections with evident injury were selected per heart. The criteria for scoring were based on the amount of persisting collagen (blue staining) and the wound closure with a new myocardium. The hearts without persisting collagenous scar were classified as fully regenerated hearts. Hearts with scar remnants that were covered by a new myocardium was considered as incomplete regeneration. The absence of a new myocardium around the wound was considered as a blocked regeneration.

Single-cell RNA-sequencing

To prepare *cmhc2*⁺ cells for single-cell RNA-sequencing analyses, *cmhc2:EGFP* transgenic fish were raised to adult stages. Adult hearts were collected at four months old. The heart samples were digested with 0.26 U/mL LiberaseTM Thermolysin Medium (TM) based on a previously published protocol⁷. Dissociated cells were spun down and live EGFP⁺ cells were sorted by flow cytometry. Isolated cells were sent to Emory Integrated Genomics Core (EIGC) center for 10x single-cell RNA-sequencing. Single-cell RNA-seq libraries were prepared using the Chromium Single Cell 3' Library & Cell Bead Kit v3.1 (Cat. No. 1000128, 1000127, 120262; 10x Genomics) according to the manufacturer's protocol. Libraries were sequenced with an Illumina NextSeq550 using mid-output 150-cycle kits according to manufacturer specifications. The newly generated scRNA-seq data were

demultiplexed, aligned, and quantified using Cell Ranger Single-Cell Software. Preliminary filtered data generated from Cell Ranger were used for downstream analysis by the Seurat R package according to standard workflow.

The scRNA-seq dataset has been submitted to Gene Expression Omnibus (GEO). To review, please use the GEO accession number GSE312374 with the token gbulwsiivdqlven.

Statistical analysis

All data are presented as mean $\pm$ standard error of the mean (SEM). All statistical analyses were performed using GraphPad Prism 7 software. The Mann-Whitney Rank-Sum test was used for assessing statistical differences between the 2 groups. Results with p values < 0.05 were considered statistically significant. Statistical details of experiments can be found in the figures and figure legends.

Results

Characterization of cardiomyocyte heterogeneity in adult zebrafish heart

To characterize the cellular heterogeneity of adult zebrafish myocardium, we performed scRNA-seq with isolated CMs. The cells activating the regulatory sequences of the CM maker *cmlc2* were purified from the hearts of adult *cmlc2:EGFP* animals². We applied stringent quality filtering and discarded a small number of *EGFP*[−]/*cmlc2*[−] cells, and obtained high-quality transcriptomes of 1668 CMs. Unsupervised clustering of these cells identified 3 different clusters (Fig. 1A [↗](#)), each with a distinct gene expression patterns. Cluster 1 and 2 showed an enrichment of genes associated with mature CMs, such as *ckmt2a* (Fig. 1B [↗](#)) and *ckmt2b* (Fig. S1A [↗](#)). However, due to their significant overlap, it remains unclear whether these two clusters represent distinct cellular identities of CMs. In contrast, Cluster 3 was marked by prominent expression of *phlda2* (Fig. 1C [↗](#)), as well as *actn1* (Fig. 1D [↗](#)) indicative of an immature cellular state³⁷, and *notch3* (Fig. S1B [↗](#)), reflecting elevated Notch signaling activity³⁴. To gain insights into the biological characteristics of these cluster, we performed Gene Ontology (GO) enrichment analysis with specifically enriched transcripts in each CM cluster (Fig. 1E [↗](#)). Clusters 1 and 2 were enriched for GO terms related to aerobic respiration and oxidative phosphorylation, suggesting the mature CMs which efficiently produce the energy to sustain the constant contraction^{38,39}. Cluster 3 showed reduced enrichment for the metabolic pathways, suggesting an immature status. Notably, Cluster 3 was also enriched for GO terms related to muscle development and the morphogenesis of epithelial sheets. This suggests that the cells in this cluster may play a role in heart development and exhibit an epithelial-like structure with polarity.

The *phlda2:EGFP*⁺ cells represent primordial cardiomyocytes in adult zebrafish hearts

Primordial layer is a single-CM thickness, characteristics of the embryonic ventricle, and high notch activity^{30,34,37}. To determine whether Cluster 2 is primordial CMs, we generated *phlda2:EGFP* and *phlda2:mCherry-NTR* BAC transgenic animals, containing sequences of 64,684 bp upstream of the *phlda2* translation initiation codon and 51,710 bp downstream of the stop codon. We observed prominent fluorescence signals in the ventricular wall covering the surface of the ventricle (Fig. 2A [↗](#)). As predicted by scRNA-seq result, *phlda2:EGFP*⁺ expression occupied a subset of cells marked by *cmlc2:mCherry*⁺ fluorescence in adult hearts (Fig. 2B [↗](#)), indicating that *phlda2*⁺ cells are CMs. Moreover, *phlda2*-directed fluorescence signals did not co-localize with reporter transgenes marking coronary vessels or epicardial cells^{3,4} (Fig. 2C [↗](#) and 2D [↗](#)). Examination of juvenile hearts revealed similar expression in the primordial CM layer (Fig. S1D [↗](#)), indicating that *phlda2* transgenic reporters label the primordial cardiac wall throughout the development.

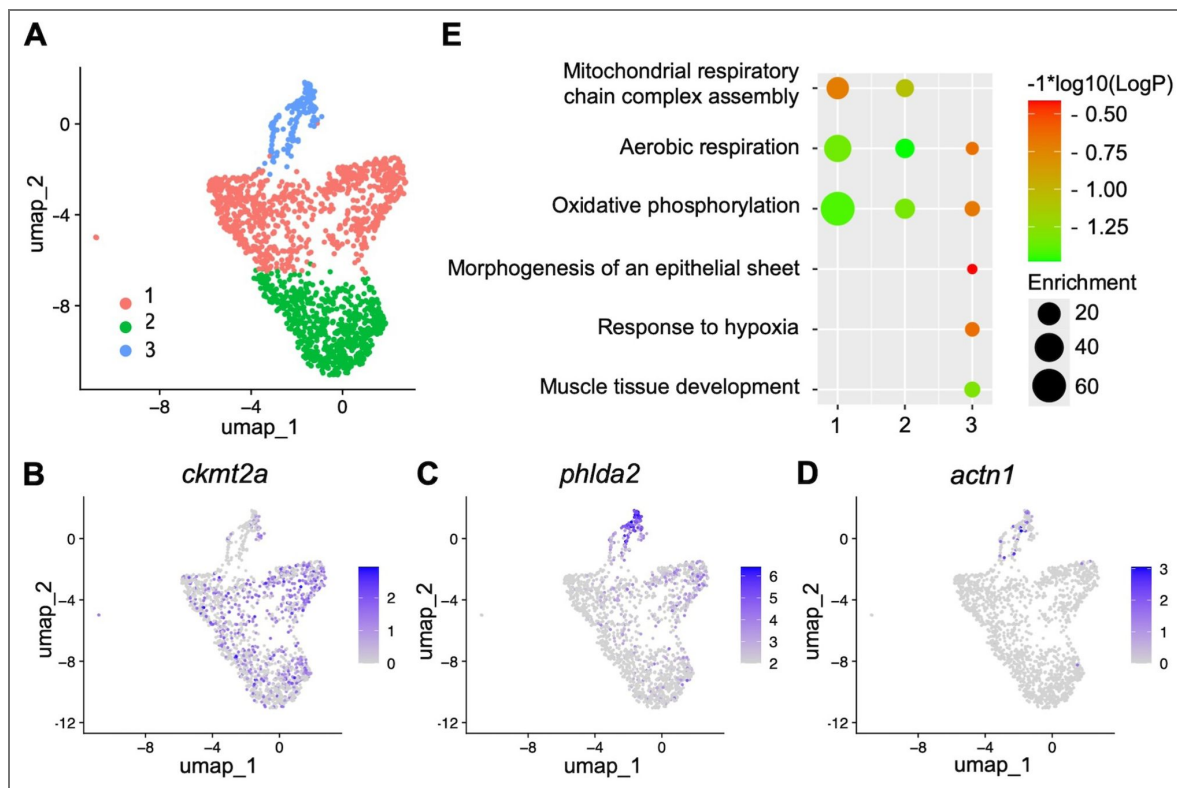


Figure 1. scRNA-seq experiments reveal distinct cardiomyocyte clusters in adult zebrafish hearts.

(A) Uniform manifold approximation and projection (UMAP) clustering of *cm/c2⁺* single-cells from adult hearts. (B-D) Feature plot of *ckmt2a* (B), *phlda2* (C), *actn1* (D), expression in cardiomyocyte clusters of adult hearts. (E) Identification of cardiomyocyte clusters based on gene ontology analysis.

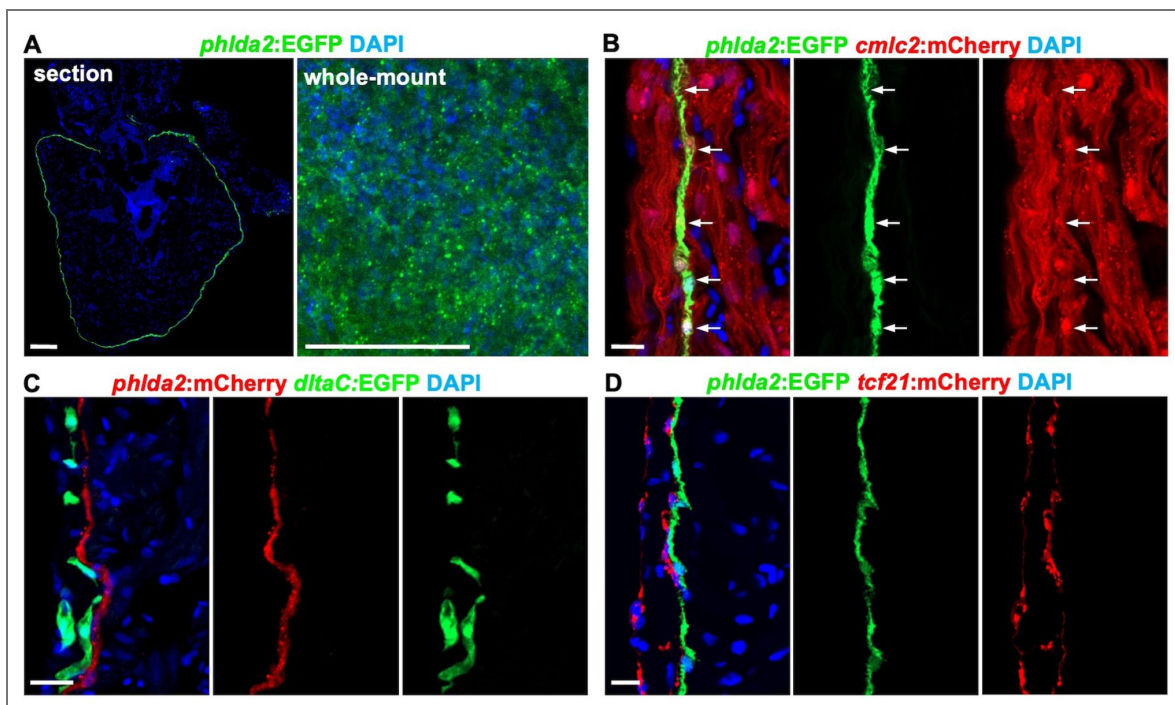


Figure 2. *phlda2:EGFP*⁺ cells specifically label primordial cardiomyocytes in adult zebrafish hearts.

(A) *phlda2*⁺ cells visualized in whole cardiac sections and whole-mount heart from adult *phlda2:EGFP* animals. n=8. Scale bar, 100 μ m. (B) Confocal slices indicating *phlda2*⁺ cells in uninjured *phlda2:EGFP;cmhc2:mCherry* ventricles. White arrows represent *phlda2*⁺/*cmhc2*⁺ cells. n=8. Scale bar, 10 μ m. (C) Confocal slices indicating *phlda2*⁺ cells in adult *phlda2:mCherry;deltaC:EGFP* ventricles. n=12. Scale bars, 20 μ m. (D) Confocal slices indicating *phlda2*⁺ cells in adult *phlda2:EGFP;tcf21:mCherry* ventricles. n=10. Scale bars, 10 μ m. All data are representative of two independent experiments.

Primordial cardiomyocytes are required for morphogenesis of myocardium and coronary vascularization

The zebrafish myocardium consists of an inner trabecular layer, a thin primordial layer, and an outer compact layer. Previous multiclonal studies revealed that primordial cardiomyocytes can give rise to trabecular CMs, which later breach the primordial layer to contribute to the compact myocardium³⁰. It was reported that nearly 60% of CMs within both the trabecular and outer myocardium originated from primordial cells labeled at the embryonic stage and analyzed at 21 dpf⁴⁰. However, these observations only demonstrate a potential lineage relationship. Whether this cell population is required for the formation or structural integrity of these myocardial layers remains unknown. A major barrier to addressing this question has been the lack of genetic tools for the specific ablation of primordial cells. To address this, we used the bacterial NTR system for inducible cell ablation⁴¹. We generated a new transgenic line and incubated *phlda2:mCherry-NTR* adults with the prodrug metronidazole (Mtz)⁴¹. This treatment depleted approximately 96.9% of ventricular *phlda2*⁺ cells without apparent effects on animal survival (Fig.S1E). We incubated juvenile *phlda2:mCherry-NTR;cmlc2:EGFP* animal and control *cmlc2:EGFP* siblings for 3 days with Mtz, and then assess the hearts for histological analysis at 10 and 30 days after treatment (dpt). We found primordial CM depletion led to structural abnormality in the heart. The compact muscle was disorganized when compared to controls, and trabecular muscle formation was severely impaired showing an approximately 54.7% reduction closed to the ventricular wall (Fig.3A–3B). In juvenile zebrafish ventricles, *gata4:EGFP*⁺ CMs form the initial clones of compact muscle, which then gradually expand and converge with others to eventually encapsulate the ventricle and create a contiguous wall of compact muscle³⁵. We analyzed hearts of *phlda2:mCherry-NTR;gata4:EGFP* fish and identified the ablation of primordial cells showed loss of sarcomere organization compared with control siblings at 10 dpt (Fig.3C). Moreover, primordial cell-depleted hearts exhibited a 16.9% reduction in *gata4:EGFP* fluorescence intensity and a 48.9% reduction in the fluorescence area (Fig.3D–3E). During zebrafish heart development, the ventricle becomes vascularized after the compact myocardium has formed post-embryonically⁴². This process is closely associated with myocardial maturation and likely depends on proper organization of the compact layer to provide structural and molecular cues for vessel growth and integrity. To determine whether ablation of primordial CMs affects vascularization during heart development, we examined coronary vessel formation in *phlda2:NTR;deltaC:EGFP* fish, in which *deltaC:EGFP* has been utilized to visualize coronary endothelial cells^{4,43}. We found that coronary vessels were fragmented and poorly developed in primordial cell-ablated hearts, although the total vessel length showed no reduction (Fig.3F–H). These findings indicate that primordial CMs are required for myocardial growth and vascular formation.

Primordial cardiomyocytes are not essential for myocardial regeneration and coronary revascularization

Our data indicated primordial CMs play essential roles during heart development. Next, we examined their contribution to heart regeneration. The *gata4* expression is induced in regenerating CMs and represent the proliferating CMs with reactivation of a cardiac developmental program^{2,5,35}. We treated adult *phlda2:mCherry-NTR;gata4:EGFP* fish and control *gata4:EGFP* siblings with Mtz for 12 hours per day over three consecutive days, starting 6 days before resection of the ventricular apex, and collected hearts at 7 days post-amputation (dpa). We imaged and quantified *gata4:EGFP*⁺ signals at the injury site and didn't detect significant difference in *EGFP*⁺ signals between *phlda2*⁺ cell-depleted fish and control siblings (Fig.4A–4B). In addition, with *cmlc2:EGFP* animals, we found that ablation of primordial CMs did not affect the regeneration index score. Myocardial regeneration proceeded normally, with new muscle effectively replacing the injured tissue by 30 dpa (Fig.4C–4D). To further assess the requirement of primordial CMs for coronary revascularization, we depleted primordial cells in *phlda2:NTR;deltaC:EGFP* fish and didn't observe found obvious difference of coronary vessel density in the regenerating area of control siblings (Fig.4E–4F). Previous studies have shown

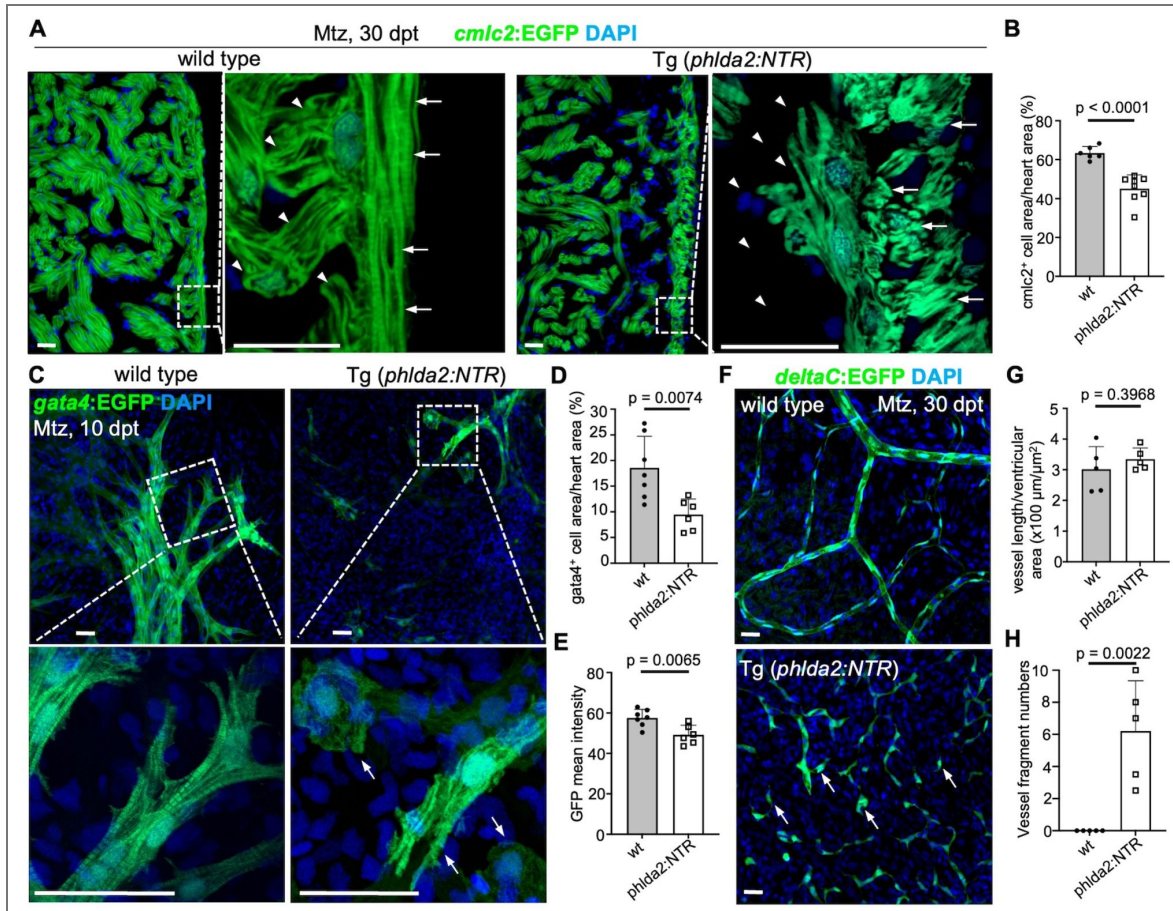


Figure 3. Primordial cardiomyocytes are required for morphogenesis of myocardium and coronary vascularization.

(A) Compact and trabecular muscle in ventricular sections from Mtz-treated *cmlc2:EGFP* and *cmlc2:EGFP;phlda2:mCherry-NTR* animals. Animals were treated with Mtz during the juvenile stage and analyzed 30 dpt. Boxed area is enlarged with high magnification. Arrows represent the compact muscle and arrowheads represent trabecular muscle. $N = 6-8$. Scale bar, 20 μm . (B) Quantification of *cmlc2*⁺ cell area relative to heart area within 100 μm of the heart wall from experiments in (A). Mann-Whitney rank-sum test. (C) Visualization of *gata4*⁺ cardiomyocytes in whole-mounted juvenile *gata4:EGFP* and *gata4:EGFP;phlda2:mCherry-NTR* hearts after Mtz treatment. Boxed area is enlarged with high magnification. Arrowheads represent EGFP signals in *phlda2* cell-depleted hearts. $n=6-7$. Scale bars, 20 μm . (D-E) Quantification of the percentage of EGFP⁺ pixels (D) and EGFP intensity (E) on the ventricular surface from experiments in (D) and (E). Mann-Whitney rank-sum test. (F) Coronary vessels in whole-mounted *deltaC:EGFP* and *deltaC:EGFP;phlda2:mCherry-NTR* hearts. Animals were treated with Mtz during the juvenile stage and analyzed at 30 dpt. Arrows represent the broken vessel fragments. $n=5$. Scale bars, 20 μm . (G-H) Quantification of vessel length/heart area (G) and vessel fragments (H) on the ventricular surface from experiments in (F). All data are representative of three independent experiments.

that zebrafish hearts can fully regenerate without remaining scars by 30 dpa. We also performed AFOG staining, which labels intact muscle in orange, collagen in blue, and fibrin in red^{6,44}. Results showed minimal scar area in primordial cell-depleted hearts, with no obvious difference compared to sibling controls at 30 dpa (Fig. 4G–4H). Overall, these findings demonstrate that primordial CMs are not essential for heart regeneration, despite their critical role in development.

Primordial cardiomyocytes exhibit limited regenerative capacity

Previous studies have indicated that in adult zebrafish heart, existing CMs can robustly regenerate within several weeks after resection surgery or genetic ablation¹⁸. We next assessed the regeneration capacity of primordial CMs. We first examined the *phlda2:mCherry-NTR* hearts at various time points after partial resection of the ventricular apex. The primordial layers were lost at the injury site, even 60 dpa at which time the heart regeneration was complete (Fig. 5A). We next asked whether this limited regenerative capacity reflects an intrinsic limitation of the cells themselves. To test it, we treat the *phlda2:mCherry-NTR* animals without resection with Mtz to ablate primordial CMs. We didn't observe the recovery of *phlda2:mCherry* fluorescence, indicating the failure of the primordial regeneration (Fig. 5B). Since primordial CMs did not regenerate after either apical resection or direct ablation, we therefore asked whether a regenerative cue could overcome this limitation. We performed apical resection after the ablation of primordial CMs, and found that primordial CMs still failed to regenerate (Fig. 5C), suggesting that their limited regenerative capacity is intrinsic rather than environment dependent. Finally, we found that *phlda2:EGFP*⁺ cells in the injury site did not colocalize with *gata4:EGFP*⁺ cells, suggesting they have limited proliferative capacity and do not contribute to the new muscle (Fig. 5D). We conclude that the primordial CMs have intrinsically limited regenerative potential, explaining its absence and functional dispensability in the regenerated heart.

Discussion

In this study, we characterized CM subpopulations in the adult zebrafish heart using scRNA-seq and identified *phlda2* as a marker of primordial CMs, which exhibit transcriptional features of an immature cellular state and functions associated with morphogenesis of an epithelial sheet. Using newly generated *phlda2* reporter and ablation lines, we revealed that primordial CMs reside beneath the compact layer and play essential roles in ventricular morphogenesis and coronary vascularization. However, unlike other CM subtypes, these cells display very limited regenerative capacity and are dispensable for myocardial regeneration. Our findings identified primordial CMs as a crucial organizer for heart morphogenesis but plays no roles during regeneration, revealing a difference between developmental and regenerative programs in the vertebrate heart.

During heart development, multicolor clonal analyses have revealed that the primordial CM lineage contributes to trabecular formation, and trabecular CMs later breach this layer to form the compact myocardium^{30,45,46}. However, direct functional evidence has been lacking due to the absence of specific genetic tools. In this study, we generated a *phlda2:mCherry-NTR* line to specifically deplete primordial CMs. The results show that the depletion of these cells caused a marked reduction in the outer trabecular muscle, whereas inner trabeculae remained largely intact, thereby supporting previous reports that primordial CMs, positioned outside the trabecular layer, contribute to the formation of the trabeculae³⁰. Furthermore, we observed a disorganized compact myocardium and disrupted *gata4* expression after the loss of primordial CMs, indicating a crucial role for primordial CMs in coordinating myocardial structural organization and maturation. The loss of these cells likely disrupts the structural and molecular cues required for compact muscle maturation, resulting in an immature ventricular wall.

Coronary vessel formation was also severely affected by the loss of primordial CMs, leading to fragmented and poorly developed vasculature. The coronary vasculature normally invades the compact myocardium during post-embryonic development, a process tightly coupled with myocardial maturation^{24,42}. Thus, the vascular defects we observed could arise indirectly from the disorganization of the compact myocardium, which fails to provide a proper scaffold for vessel growth. Alternatively, primordial CMs themselves may produce paracrine factors that promote

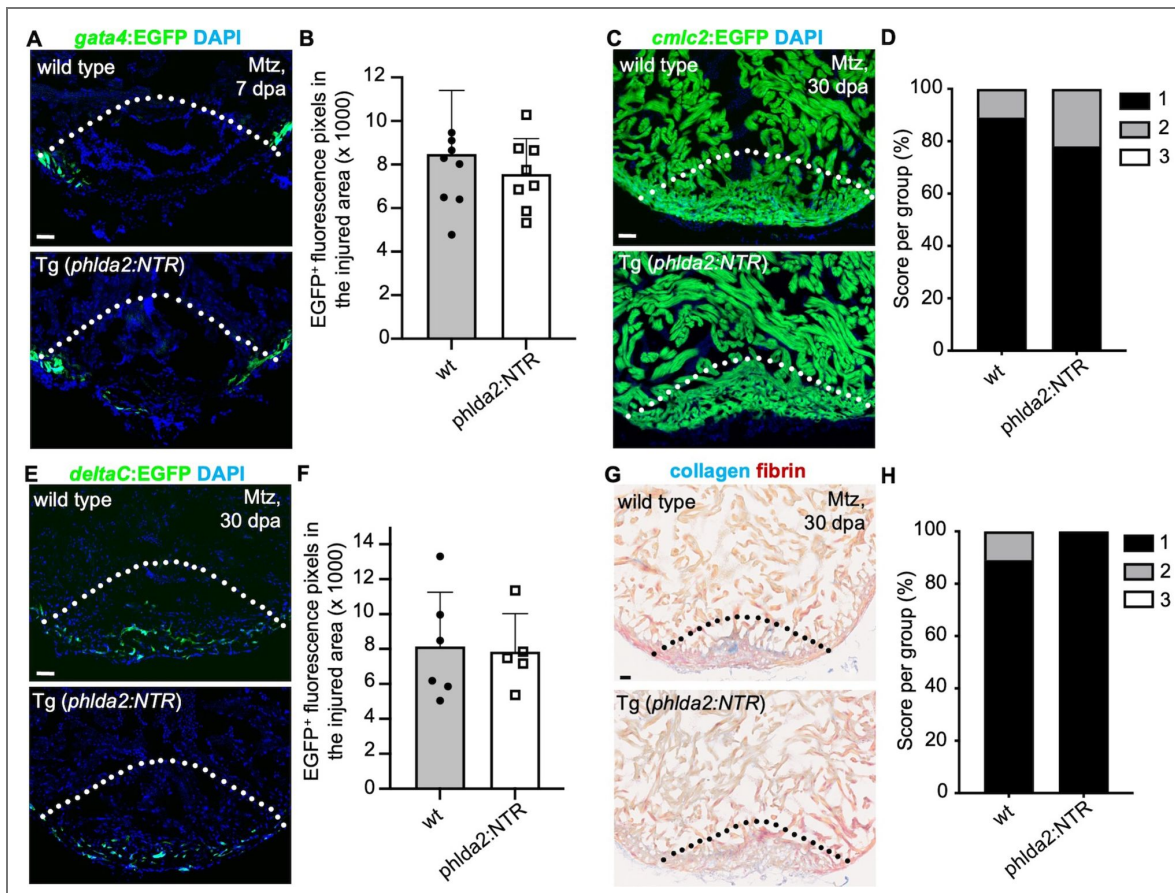


Figure 4. Primordial cardiomyocytes are not essential for myocardial regeneration and coronary revascularization.

(A) Section images of Mtz-treated *gata4:EGFP* or *gata4:EGFP;phlda2:mCherry-NTR* ventricles at 7 dpa assessed for *gata4:EGFP*⁺ CMs in the injury site. Adult animals were treated with Mtz before amputation. Dashed line indicates amputation plane. N = 8. Scale bars, 20 μ m. (B) Quantification of EGFP⁺ pixels from experiments in (A). Scale bars, 20 μ m. Mann-Whitney rank-sum test. (C) Section images of Mtz-treated *cmlc2:EGFP* or *cmlc2:EGFP;phlda2:mCherry-NTR* ventricles at 30 dpa assessed for muscle recovery. Adult animals were treated with Mtz before amputation. Dashed line indicates amputation plane. N = 9 - 10. Scale bar, 20 μ m. (D) Quantification of regeneration indices from experiments in (C). Myocardial regeneration is categorized as follows: 1 = complete regeneration of a new myocardial wall; 2 = partial regeneration; and 3 = a strong block in regeneration. (E) Section images of Mtz-treated *deltaC:EGFP* or *deltaC:EGFP;phlda2:mCherry-NTR* ventricles at 30 dpa assessed for coronary vessels in the injury site. Adult animals were treated with Mtz before amputation. Dashed line indicates amputation plane. N = 5 - 6. Scale bars, 20 μ m. (F) Quantification of EGFP⁺ pixels from experiments in (E). Scale bars, 20 μ m. Mann-Whitney rank-sum test. (G) Section images of Mtz-treated *wt siblings* or *phlda2:mCherry-NTR* ventricles at 30 dpa assessed for AFOG staining collagen/fibrin deposition in the injury site. Adult animals were treated with Mtz before amputation. Dashed line indicates amputation plane. N = 9. Scale bars, 20 μ m. (H) Quantification of scar score from experiments in (G). The scar score is categorized as follows: 1 = hearts without persisting collagenous scar; 2 = hearts with scar remnants that were covered by a new myocardium; 3 = the absence of a new myocardium around the wound. The Chi-squared test was performed. All data are representative of three independent experiments.

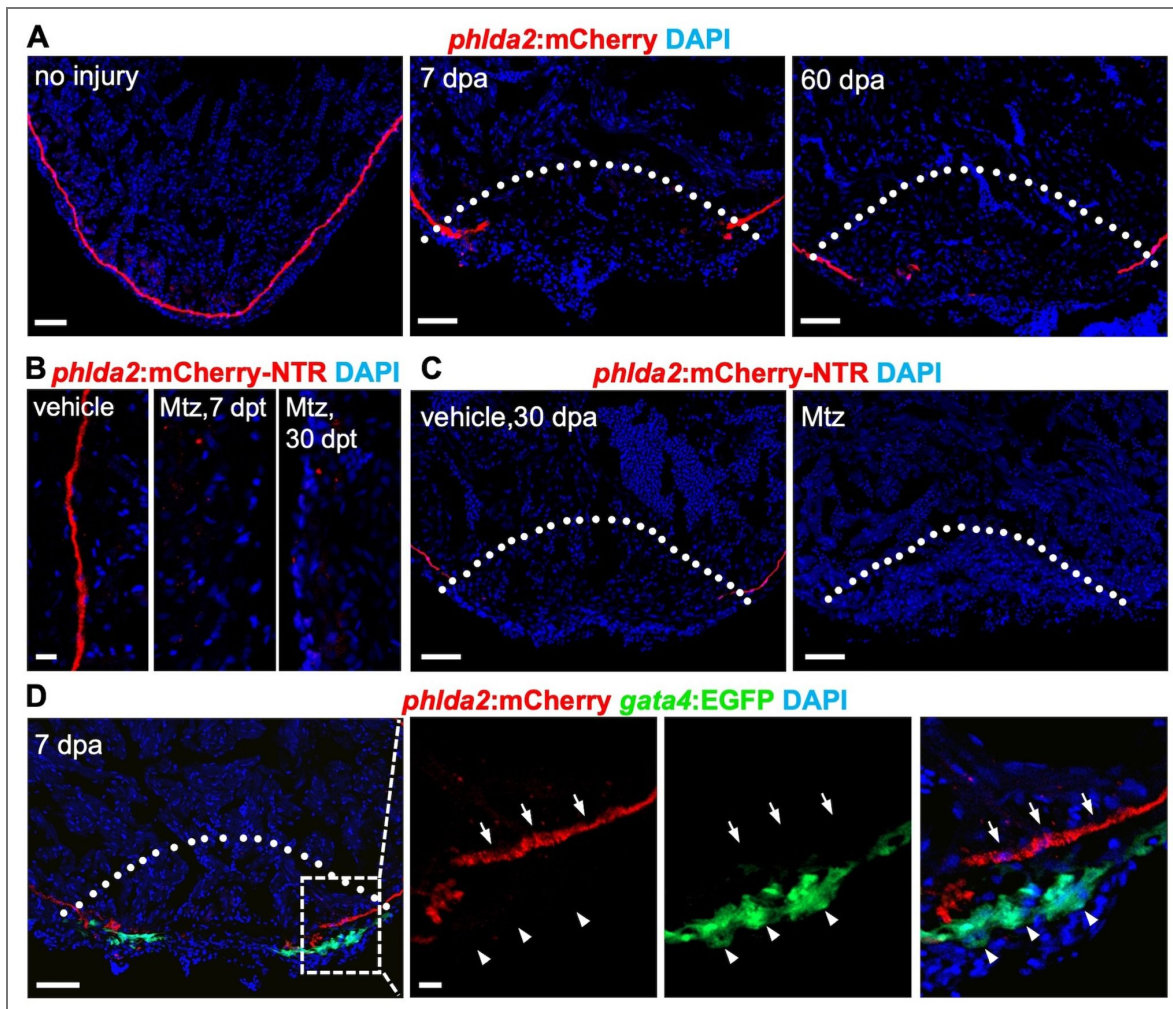


Figure 5. Primordial cardiomyocytes have limited regenerative capacity.

(A) Section images of *phlda2:mCherry-NTR* ventricles without injury and during regeneration at 7, and 60 dpa. $n = 11-13$. Dashed line indicates amputation plane. Scale bar, 20 μm . (B) Section images of *phlda2:mCherry-NTR* ventricles without Mtz treatment and after 7 or 30 days of Mtz treatment. $n = 12-15$. Scale bar, 20 μm . (C) Section images of ventricles of vehicle- or Mtz-treated *phlda2a:mCherry-NTR* at 30 dpa. Animals were treated with Mtz during the juvenile stage and amputated as adults. Dashed line indicates amputation plane. $n = 12$. Scale bars, 20 μm . (D) Section images of *phlda2:mCherry-NTR;gata4:EGFP* ventricles at 7 dpa assessed for *phlda2*⁺ cells and *gata4:EGFP*⁺ CMs in the injury site. Box area is shown in higher magnification. Arrows represent *phlda2*⁺ cells; arrowheads represent *gata4*⁺ cells. Dashed line indicates amputation plane. $n = 12$. Scale bar, 20 μm . All data are representative of two independent experiments.

vessel growth and stability. Consistently, our scRNA-seq analysis revealed that *vegfaa* was highly expressed in primordial CMs (Fig. S1C). VEGF signaling plays a pivotal role in vascular development, acting as an important endothelial mitogen that stimulates endothelial cell tubulogenesis, and maintains vessel stability and integrity^{25,47,48}. These findings suggest that primordial CMs may serve as a critical architectural and signaling hub to coordinate the morphogenesis of both myocardial and vascular structures during heart development.

In contrast to their essential role in heart development, primordial CMs appear to play no role in cardiac regeneration. Depletion of these cells did not affect myocardial or vascular regeneration, nor did it impair scar resolution. Furthermore, primordial CMs failed to regenerate after either surgical amputation or genetic ablation, even under strong regenerative conditions. Their inability to regenerate in the injured tissue, together with the lack of overlap with *gata4*⁺ proliferative CMs, indicates that these cells possess intrinsically limited proliferative capacity. They are different from trabecular and compact CMs, which efficiently contribute to the formation of new myocardium. This intrinsic restriction likely explains why the loss of primordial CMs does not impair the heart regeneration, suggesting that zebrafish cardiac regeneration is primarily driven by other CM subsets with greater regenerative potential.

Our study demonstrates that primordial CMs are essential for heart morphogenesis but dispensable for regeneration. These findings emphasize the functional diversity among CM subtypes and broaden our knowledge of CM heterogeneity in the zebrafish heart. Importantly, our results show that heart regeneration does not simply reactivate the developmental programs across all cardiomyocytes. Instead, regeneration depends on the CM subsets with high proliferative and regenerative capacity, rather than engaging all CMs equally. This work provides new insights into heart development and regeneration and may inspire new ideas to enhance cardiac repair in mammals.

Data availability

The scRNA-seq dataset has been submitted to Gene Expression Omnibus (GEO). To review, please use the GEO accession number GSE312374 (Sun et al., 2025 [DOI](#)).

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Additional information

Author contributions

Conceptualization, J.S., and J.W.; methodology, J.S., and J.W.; investigation, J.S., L.C., and J.W.; formal analysis, J.S., L.C., and J.W.; visualization, J.S., L.C., and J.W.; writing, J.S. and J.W.; funding acquisition, J.W.; supervision, J.W. All authors commented on the manuscript.

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Peer reviews

Reviewer #1 (Public review):

Summary:

This manuscript addresses an important question in cardiac biology: whether distinct cardiomyocyte (CM) subpopulations play specialized roles during heart development and regeneration. Using single-cell RNA sequencing and newly generated genetic tools, the authors identify *phlda2* as a specific marker of primordial cardiomyocytes in the adult zebrafish heart. They further show that these primordial CMs function are essential for myocardial morphogenesis and coronary vascularization but are dispensable for myocardial regeneration or revascularization after injury. These findings indicate that heart regeneration doesn't simply recapitulate developmental processes.

Strengths:

A major strength of the study is the generation of a *phlda2* BAC reporter, which provides a specific and reliable marker for primordial cardiomyocytes. The lack of genetic tools has previously limited functional analysis of this CM population. By using *phlda2* regulatory elements to generate reporter and NTR-based ablation lines, the authors can visualize and selectively manipulate primordial CMs in vivo. This enables a direct functional interrogation rather than relying on lineage tracing or correlative evidence. Through genetic ablation, the authors convincingly demonstrate that primordial CMs are essential for myocardial morphogenesis and coronary vascular organization during development but are not necessary for heart regeneration.

Weaknesses:

- (1) The manuscript would benefit from clarifying whether the primordial cardiomyocytes ablation affects epicardial cell behaviors during heart development, given that the well-established role of the epicardium in supporting coronary vessel growth, it is possible that the vascular phenotypes observed after primordial CM ablation may be affected, at least in part, by altered epicardial cells.
- (2) Because primordial cardiomyocytes form a dense, single-cell-thick layer covering the ventricular surface, it would be informative to determine whether their loss alters the spatial distribution or inward migration of coronary endothelial cells or epicardial cells.
- (3) The manuscript carefully examines the relationship between primordial CMs and *gata4*⁺ cardiomyocytes during regeneration. However, their relationship during heart development should be more fully addressed.
- (4) As loss of cardiomyocytes is known to induce *gata4*:GFP activation during regeneration, it would be important to determine whether ablation of primordial cardiomyocytes alone triggers *gata4*:GFP expression in neighboring cardiomyocytes. This analysis would further support the conclusion that primordial cardiomyocytes are not required for regenerative responses.

<https://doi.org/10.7554/eLife.110256.1.sa3>

Reviewer #2 (Public review):

Summary:

In the manuscript "Primordial Cardiomyocytes orchestrate myocardial morphogenesis and vascularization but are dispensable for regeneration", Sun et al. identify a novel marker of primordial cardiomyocytes and use it to visualize and ablate the population during development and regeneration. The role of the primordial layer has not been investigated because the tools to manipulate this population have not existed. The manuscript is straightforward, easy to understand, and addresses an important question that has not been explored.

While the manuscript provides important insights into the role of primordial CMs, backed by a convincing methodology, the authors should clarify their requirements for heart development and maturation. Specifically, is the primordial layer required for the fish to survive? Do primordial CMs regenerate when ablated during development, and do the defects observed (in trabecular and compact CMs and coronary vessels) resolve after 10 days post-treatment when they were detected?

Strengths:

The major strengths are the identification of a marker that enables manipulation of primordial cardiomyocytes and the tools generated by the team.

Weaknesses:

The major weakness is not considering the longer-term consequences of primordial layer ablation during development, as it is unclear whether the animals succumb to the acute cardiac defects observed or fully recover.

<https://doi.org/10.7554/eLife.110256.1.sa2>

Reviewer #3 (Public review):

Summary:

The authors performed single-cell RNA sequencing of adult zebrafish hearts and identified markers for distinct cardiomyocyte subpopulations. One marker, *phlda2*, marks primordial cardiomyocytes. They generated transgenic reporter lines to characterize *phlda2* expression patterns and a *phlda2*-NTR ablation line to determine the functional requirement of primordial cardiomyocytes during heart regeneration. They found that *phlda2*⁺ primordial cardiomyocytes are essential for myocardial morphogenesis and coronary vessel development. Interestingly, when *phlda2*⁺ primordial cardiomyocytes are ablated during heart regeneration, *gata4*⁺ cortical cardiomyocytes, coronary vessel revascularization, and scar tissue formation are not affected.

Strengths:

The authors identified a new primordial cardiomyocyte marker, *phlda2*. They further demonstrated that primordial cardiomyocytes are important for heart morphogenesis but dispensable for heart regeneration. Their findings reveal a potential difference between heart development and regeneration programs.

Weakness:

Despite the interesting findings, the authors did not provide supplemental data for their scRNAseq to demonstrate the data quality and support their conclusions, and some results are not well described.

<https://doi.org/10.7554/eLife.110256.1.sa1>

Author response:

We thank the reviewer for the thoughtful and constructive evaluation of our work and for recognizing its potential interest to researchers working on cardiac development and regeneration. We are planning to address the specific concerns as noted by the reviewers in the following way:

Public Reviews:

Reviewer #1 (Public review):

Summary:

This manuscript addresses an important question in cardiac biology: whether distinct cardiomyocyte (CM) subpopulations play specialized roles during heart development and regeneration. Using single-cell RNA sequencing and newly generated genetic tools, the authors identify phlda2 as a specific marker of primordial cardiomyocytes in the adult zebrafish heart. They further show that these primordial CMs function are essential for myocardial morphogenesis and coronary vascularization but are dispensable for myocardial regeneration or revascularization after injury. These findings indicate that heart regeneration doesn't simply recapitulate developmental processes.

Strengths:

A major strength of the study is the generation of a phlda2 BAC reporter, which provides a specific and reliable marker for primordial cardiomyocytes. The lack of genetic tools has previously limited functional analysis of this CM population. By using phlda2 regulatory elements to generate reporter and NTR-based ablation lines, the authors can visualize and selectively manipulate primordial CMs in vivo. This enables a direct functional interrogation rather than relying on lineage tracing or correlative evidence. Through genetic ablation, the authors convincingly demonstrate that primordial CMs are essential for myocardial morphogenesis and coronary vascular organization during development but are not necessary for heart regeneration.

Weaknesses:

(1) The manuscript would benefit from clarifying whether the primordial cardiomyocytes ablation affects epicardial cell behaviors during heart development, given that the well-established role of the epicardium in supporting coronary vessel growth, it is possible that the vascular phenotypes observed after primordial CM ablation may be affected, at least in part, by altered epicardial cells.

We thank the reviewer for this thoughtful comment and agree that primordial cardiomyocyte ablation may indirectly affect coronary vessel growth through changes in epicardial cell behavior. Therefore, we will perform additional analyses to examine epicardial cell behaviors, including epicardial coverage and migration following primordial cardiomyocyte ablation using the established epicardial reporter line tcf21:nucEGFP during heart development.

(2) Because primordial cardiomyocytes form a dense, single-cell-thick layer covering the ventricular surface, it would be informative to determine whether their loss alters the spatial distribution or inward migration of coronary endothelial cells or epicardial cells.

We thank the reviewer for this important comment. We will analyze the spatial distribution and inward migration of coronary endothelial and epicardial cells after primordial cardiomyocyte ablation using high-resolution imaging and quantitative analysis

(3) The manuscript carefully examines the relationship between primordial CMs and *gata4*⁺ cardiomyocytes during regeneration. However, their relationship during heart development should be more fully addressed.

We appreciate the suggestion and will carefully investigate the relationship between primordial cardiomyocytes and *gata4*⁺ cardiomyocytes during heart development.

(4) As loss of cardiomyocytes is known to induce *gata4*:GFP activation during regeneration, it would be important to determine whether ablation of primordial cardiomyocytes alone triggers *gata4*:GFP expression in neighboring cardiomyocytes. This analysis would further support the conclusion that primordial cardiomyocytes are not required for regenerative responses.

We acknowledge the reviewer's comments and will test whether primordial cardiomyocyte ablation induces *gata4*:GFP activation in neighboring cardiomyocytes in the adult heart.

Reviewer #2 (Public review):

Summary:

In the manuscript "Primordial Cardiomyocytes orchestrate myocardial morphogenesis and vascularization but are dispensable for regeneration", Sun et al. identify a novel marker of primordial cardiomyocytes and use it to visualize and ablate the population during development and regeneration. The role of the primordial layer has not been investigated because the tools to manipulate this population have not existed. The manuscript is straightforward, easy to understand, and addresses an important question that has not been explored.

While the manuscript provides important insights into the role of primordial CMs, backed by a convincing methodology, the authors should clarify their requirements for heart development and maturation. Specifically, is the primordial layer required for the fish to survive?

We thank the reviewer for this important question. We will examine the survival of fish following primordial cardiomyocyte ablation during development.

Do primordial CMs regenerate when ablated during development, and do the defects observed (in trabecular and compact CMs and coronary vessels) resolve after 10 days post-treatment when they were detected?

We thank the reviewer for this valuable comment. We will perform additional analyses to determine whether primordial cardiomyocytes regenerate after ablation during development and to assess the extent and dynamics of their recovery. We will also evaluate whether the defects in trabecular and compact myocardium and coronary vasculature persist or resolve in adult hearts following primordial cardiomyocyte ablation during development.

Reviewer #3 (Public review):

Summary:

*The authors performed single-cell RNA sequencing of adult zebrafish hearts and identified markers for distinct cardiomyocyte subpopulations. One marker, *phlda2*, marks primordial cardiomyocytes. They generated transgenic reporter lines to characterize *phlda2* expression patterns and a *phlda2*-NTR ablation line to determine the functional requirement of primordial cardiomyocytes during heart regeneration. They found that *phlda2*⁺ primordial cardiomyocytes are essential for myocardial morphogenesis and coronary vessel development. Interestingly, when *phlda2*⁺*

primordial cardiomyocytes are ablated during heart regeneration, gata4+ cortical cardiomyocytes, coronary vessel revascularization, and scar tissue formation are not affected.

Strengths:

The authors identified a new primordial cardiomyocyte marker, phlda2. They further demonstrated that primordial cardiomyocytes are important for heart morphogenesis but dispensable for heart regeneration. Their findings reveal a potential difference between heart development and regeneration programs.

Weakness:

Despite the interesting findings, the authors did not provide supplemental data for their scRNAseq to demonstrate the data quality and support their conclusions, and some results are not well described.

We appreciate the reviewer's comment. We will include supplemental data to demonstrate the quality of our single-cell RNA sequencing. Additionally, we will provide more detailed descriptions of the key results in the main text and figure legends to clearly support our conclusions regarding primordial cardiomyocytes and their roles in heart morphogenesis and regeneration.

<https://doi.org/10.7554/eLife.110256.1.sa0>