Published in final edited form as:

Dev Dyn. 2014 November ; 243(11): 1477–1486. doi:10.1002/dvdy.24171.

Regenerative responses after mild heart injuries for cardiomyocyte proliferation in zebrafish

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Abstract

Background—The zebrafish heart regenerates after various severe injuries. Common processes of heart regeneration are cardiomyocyte proliferation, activation of epicardial tissue and neovascularization. In order to further characterize heart regeneration processes, we introduced milder injuries and compared responses to those induced by ventricular apex resection, a widely used injury method. We used scratching of the ventricular surface and puncturing of the ventricle with a fine tungsten needle as injury inducing techniques.

Results—Scratching the ventricular surface induced subtle cardiomyocyte proliferation and responses of the epicardium. Endothelial cell accumulation was limited to the surface of the heart. Ventricular puncture induced cardiomyocyte proliferation, endocardial and epicardial activation and neo-vascularization, similar to the resection method. However, the degree of the responses was milder, correlating with milder injury. Sham operation induced epicardial *aldh1a2* expression but not *tbx18* and WT1.

Conclusions—Puncturing the ventricle induces responses equivalent to resection at milder degrees in a shorter time frame and would be used as simple injury model. Scratching the ventricle did not induce heart regeneration and would be used for studying wound responses to epicardium.

Keywords

Zebrafish; Heart; Regeneration; Cardiomyocytes; Epicardial cells; Endothelial cells

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COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

INTRODUCTION

Adult zebrafish possess significant ability to regenerate the heart after damage, which contrasts to the very limited regenerative ability of the mammalian heart after immediate postnatal period. Although adult mammalian cardiomyocytes proliferate, the degree of proliferation is too little to restore the myocardium after injury, such as ischemic damage. Thus, the injured heart does not regenerate in mammals, leading to cardiac failure, and in severe cases, to death. With established genomics, genetics, molecular and bioinformatic tools, the zebrafish heart regeneration provides an excellent experimental platform to study how the heart responds to injury and regenerates.

Although the zebrafish heart shares anatomical similarities to the mammalian heart, there are several differences. The zebrafish heart possesses one ventricle and one atrium, a simpler structure compared to the four-chambered mammalian heart. In addition, the zebrafish heart lacks coronary arteries found in the mammalian heart. Instead, they possess small vessels on the surface of the heart and in the compact muscle layer of the ventricle. Thus, rather than coronary artery ligation to induce cardiac ischemia in mammals, resection of the ventricular apex has been established to induce injuries to the zebrafish heart. Even after removing approximately 20% of the ventricle, zebrafish survive and regenerate the heart. Recently, several other injury methods have been reported. These include cryoprobe-induced injury, genetic ablation of cardiomyocytes by inducible transgenic expression of toxin and cardiac-specific nitroreductase transgene coupled with cytotoxic pre-drug Metronidazole treatment.

Among these injury methods, processes of heart regeneration after resection of the ventricular apex are best characterized. Immediately after resection, a blood clot forms and seals the wound. The accumulated fibrin clot is cleared during myocardial regeneration. Two major processes for myocardial regeneration are restoration of lost myocardium and neo-vascularization of regenerating area. The former is achieved by proliferation of pre-existing cardiomyocytes, rather than resident stem or progenitor cells. Cardiomyocytes undergo partial de-differentiation and proliferate to restore the injured myocardial layer, and the contribution of stem or progenitor cells is little, if any. Local migration of cardiomyocytes into the injury site is coupled with cardiomyocyte proliferation and is essential for myocardial repair. Neo-vascularization of the regenerating myocardial layer is essential for heart regeneration. The epicardial tissue, which is a thin layer tissue enveloping the heart, plays important roles in neovascularization. Immediately after ventricular resection epicardial cells are activated and migrate into the myocardial layer after epithelial-mesenchymal transition. They contribute to peri-vascular cells rather than cardiomyocytes and support neo-vascularization and heart regeneration. Within 1-2 months, the resected heart restores the anatomical structure and function.

Several markers (genes/proteins) have been used to monitor responses of cardiomyocytes and epicardial cells during heart regeneration. In response to injury, cardiomyocytes de-differentiate, lose their tight connection to each other and disassemble the sarcomeric structure. Although these alterations are visible under transmission electron microscopy, such an analysis is labor intensive. Instead, cell cycle markers, such as proliferating cell nuclear antigen (PCNA, an S phase marker) or phospho histone H3 (mitosis marker),

coupled with myocytes markers (myocyte enhancer factor-2C, MEF2C or myosin heavy chain, MHC) are widely used to detect proliferating cardiomyocytes. The epicardial tissue expresses developmental genes in response to injury. After ventricular resection, organ-wide epicardial activation takes place, and the epicardial cells express *aldehyde dehydrogenase 1a2* (*aldh1a2*, also known as *retinaldehyde dehydrogenase 2*, *raldh2*) and *t-box 18* (*tbx18*). Fluorescent reporters driven by regulatory sequences of *wilms tumor 1b* (*wt1b*), *tbx18* and *tcf21* are also activated in response to ventricular resection. The epicardial response becomes more restricted to the area around the injury during heart regeneration.

Other reported injury methods induce more severe damage to the heart. For instance, cryoprobe-induced injury involves severe inflammation and regeneration requires 90 – 120 days. Transgenic induction of toxin expression in cardiomyocytes ablates approximately 60% cardiomyocytes. Although these methods induce a significant degree of damage, the heart regenerates by cardiomyocyte proliferation and neo-vascularization, similar to the heart after resection.

Interestingly, it has been shown that the heart responds to sham operations, i.e., opening the pericardiac cavity without heart injury. Sham-operated hearts exhibit upregulation of *aldh1a2* in the epicardium, indicating that these cells sense environmental changes around the heart and that such responses might not be specific to regeneration. In order to characterize how the heart responds to injury, we compared heart response to milder injuries. We introduced injury by scratching the surface of the ventricle or by puncturing the ventricle using a fine needle and compared the responses to those induced by resection. Our results show that scratching only induced responses at the surface of the ventricle without significant heart regeneration responses. In contrast, puncturing induced similar responses compared to resection, and would be used as a milder injury method with shorter time frame of heart regeneration.

RESULTS AND DISCUSSION

Fibrin accumulation and clearance after surface scratch and ventricular puncture

Studies to date show that the zebrafish heart regenerates after various types of injuries, such as ventricular resection, cryoprobe-induced injury and genetic ablation of cardiomyocytes by transgenic induction of toxin. These methods induce significant degrees of injury, and induce activation of epicardial tissue, neo-vascularization and cardiomyocyte proliferation, suggesting that these processes are common for heart regeneration.

To understand how the heart responds to injury, we sought to identify which known responses of the regenerating heart are associated with cardiomyocyte proliferation, a major driving force of heart regeneration. For this purpose, we introduced milder injuries to the heart and compared responses to those induced by ventricular resection, a widely used heart injury method. We tested two methods, scratching the surface of the ventricle and puncturing the ventricle using a fine tungsten needle.

We first examined damage to the myocardial layer by visualizing cardiomyocytes by *cmlc2a* mRNA in situ hybridization and anti-MHC immunostaining. One day after scratching, we

detected slightly reduced *cmhc2a* expression close to the surface of the heart (Fig. 1B), compared to the uniform strong signal in the sham-operated heart (Fig. 1A). The scratched area showed fibrin clot accumulation at 3 and 7 day post injury (dpi) (red color, denoted by arrowheads, Fig. 1I, L), which is likely derived from bleeding of coronary vessels at the heart surface. The fibrin clot was undetectable at 14 dpi (Fig. 1O).

As a second method, we punctured the ventricle with a fine tungsten needle, which induced evident damage to the myocardial layer. The myocardial tissue, where the needle passed through, exhibited a reduction in *cmhc2a* and MHC signals at 1 dpi (Fig. 1C, G), indicating a loss of cardiomyocytes. The fibrin clot accumulated in the myocardial layer close to the surface at 3 and 7 dpi (Fig. 1J, M). The fibrin clot was reduced by 14 dpi (Fig. 1P) and undetectable at 30 dpi (Fig. 1R).

Compared to these methods, ventricular resection, a widely used injury method, induced significant damage, which was evident by the loss of cardiomyocytes (Fig. 1D, H). As previously reported, MHC-positive cardiomyocytes gradually replaced the fibrin clot (Fig. 1K, N, Q). As the resected heart is known to require 30-60 days to regenerate, significant amount of the myocardial layer was still damaged at 14 dpi (Fig. 1Q) and residual fibrin was still detectable in some sections at 30 dpi (Fig. 1S).

These results demonstrate that the scratch and puncture methods induced significantly lower damage compared to the conventional resection method. The scratch method induced injury to the surface of the heart and the damage to the myocardial layer is subtle. The puncture method induced damage that affects internal areas of the myocardial layer.

Cardiomyocyte proliferation occurs in response to mild heart damages

As the scratch method and the puncture method induced significantly milder injury than that by resection to the heart, we next examined whether these methods induce cardiomyocyte proliferation. It has been demonstrated that cardiomyocyte proliferation is high at 7-14 days after resection. Because injuries by the scratch and puncture methods are milder than those by resection, we monitored cardiomyocyte proliferation at 3, 7 and 14 dpi using anti-PCNA and anti-Mef2 (detected as white nuclei, denoted by yellow arrowheads in Fig. 2).

Without injury, we detected a few proliferating cardiomyocytes in the heart, consistent with a report showing that the adult zebrafish heart continues to grow. The number of proliferating cardiomyocytes in sham-operated hearts at 3, 7 and 14 day time points did not show differences compared to uninjured heart (Fig. 2A, A', E, E', I, I', M), indicating that sham operation did not induce cardiomyocyte proliferation. At 3 dpi, hearts injured by all three methods exhibited a subtle increase in the number of proliferating cardiomyocytes, compared to sham-operated hearts. (Fig. 2B, B', C, C', D, D', M, $p < 0.05$ for sham vs scratch, $p < 0.01$ for sham vs puncture, $p < 0.01$ for sham vs resection). At 7 dpi, as previously demonstrated, hearts injured by resection showed a significant increase in the number of proliferating cardiomyocytes (Fig. 2H, H', M, $p < 0.01$, compared to 3 dpi). Hearts injured by the puncture method also showed increased number of proliferating cardiomyocytes (Fig. 2G, G', M, $p < 0.05$, compared to 3 dpi), however, hearts injured by the scratch method did not show significant alteration of the number of proliferating cardiomyocytes compared to 3

dpi (Fig. 2E, E', M). At 14 dpi, the number of proliferating cardiomyocytes remained high in the resected hearts (Fig. 2L, L', M, $p < 0.01$, compared to 7 dpi), as previously shown. The punctured heart showed a slight reduction in the number of proliferating cardiomyocytes, compared to the number at 7 dpi, without statistical significance (Fig. 2K, K', M). The scratched heart also showed a slight reduction in the number of proliferating cardiomyocytes, and the number did not show statistical differences to that of sham-operated hearts at 14 dpi (Fig. 2J, J', M). Throughout our analysis, an increase in cardiomyocyte proliferation was detected within and close to the injury site, which is consistent with our previous report showing cardiomyocyte migration into the injury site. This observation was in contrast to an increase in cardiomyocyte proliferation around and far away from the injury site with significant cryoprobe-induced heart injury.

These results demonstrate that scratching the heart induced very low levels of increase in the number of proliferating cardiomyocytes, likely due to subtle effects on the myocardium close to the surface (Fig. 1B). However, the number of proliferating cardiomyocytes did not increase during the monitored period. In contrast, puncturing the heart induced milder increases in the number of proliferating cardiomyocytes compared to the resected heart. The results also indicate the correlation between cardiomyocyte proliferation and the severity of myocardial damage.

Endothelial cell response

Neo-vascularization is a critical process to support proliferating cardiomyocytes for heart regeneration. Thus, we next examined whether milder injury induced vascularization of the injury site. For this purpose, we used the *fli1-EGFP*; *cmlc2a-mCherry* double transgenic line that allows for visualization of the endothelial cells (green) and the myocardial tissue (magenta).

First, we examined these reporter signals in sham-operated hearts. Thick *fli1-EGFP* signals were detected in the compact zone of the myocardial layer, which is the condensed part at the outer layer of the myocardium (white arrowheads, Fig. 3A, A'). These thick *fli1-EGFP* signals, which represent coronary vessels, were detected within the myocardial *cmlc2a-mCherry* signals, illustrating an intact myocardial layer and endothelium. We also detected thinner *fli1-EGFP* signals in the trabecular zone, a spongy inner layer (white arrows, Fig. 3A, A'). These thinner *fli1-EGFP* signals likely represent the endocardial tissue and were detected broadly on the surface of the trabecular zone.

One day after injury, the scratched heart lacked the *fli1-EGFP* signal from the surface of the injury site; however, the *fli1-EGFP* signals appeared to be unaffected inside the myocardial layer (Fig. 3B, B'). Seven days after injury, we observed accumulated *fli1-EGFP* signals at the surface of the myocardial layer of the scratched heart (yellow arrowheads, Fig. 3F, F'). Therefore, scratching induced only injury to the surface and endothelial cells accumulated at the surface injury site.

Contrary to this, puncturing induced a response in the myocardial layer. One day after injury, endothelial cells were missing from the surface (Fig. 3C, C'). At 7 dpi, endothelial cells accumulated inside the myocardial layer where the *cmlc2a-mCherry* signals are sparse

(yellow arrowheads, Fig. 3G, G') compared to the uninjured area. Similar, but more significant responses were observed in resected hearts, in which thick *fli1-EGFP* signals were scattered in a broad area where the *cmlc2a-mCherry* signals are sparse (yellow arrowheads, Fig. 3H, H'). Thus, the thick *fli1-EGFP* signals within the sparse *cmlc2a-mCherry* signals represent neo-vascularization of the injured area.

These data, together with cardiomyocyte proliferation assay data (Fig. 2), support the idea that scratching only affects the surface of the ventricle, and the myocardial layer shows only a subtle increase in proliferation. In contrast, puncturing the ventricle triggered cardiomyocyte proliferation and neo-vascularization, two critical responses in the myocardial layer for heart regeneration, to a milder degree than resection. The results here also suggest that the response of endothelial cells occurred independent of myocardial injury and regeneration.

Activation of *aldh1a2* in response to mild injuries

It has been demonstrated that *aldh1a2* is rapidly induced in the epicardial and endocardial tissue after ventricular resection in zebrafish. *Aldh1a2* encodes an enzyme that catalyzes the synthesis of retinoic acid from retinaldehyde. Retinoic acid synthesis by *aldh1a2* is required for cardiomyocyte proliferation and regeneration of the heart in zebrafish. Interestingly, epicardial, not endocardial, *aldh1a2* seems to have a second role. Expression of *aldh1a2* is induced in the epicardial tissue after opening the pericardiac sac, which affects the pericardiac environment. Thus, the epicardial *aldh1a2* expression is also a response to environmental change.

Given early and organ-wide activation of *aldh1a2* after resection, we first monitored expression of *aldh1a2* at 1 dpi. Consistent with previous reports, sham-operation induced broad epicardial expression of *aldh1a2* at the 1-day time point (arrowheads point to signals around the apex, arrows point to signals away from the apex, Fig. 4A, A'), compared to undetectable signals in hearts without operations (Fig. 4E, E'). At time points this early, *aldh1a2* induction in the endocardium was detected to different degrees by three injury methods, in addition to organ-wide epicardial expression (Fig. 4B, B', C, C', D, D'). With scratching, a weak signal of *aldh1a2* was detected close to the injury site (open arrowhead, Fig. 2B, B'). Both puncturing and resection induced more evident endocardial *aldh1a2* expression (Fig. 4C, C', D, D').

Consistent with a previous report, epicardial *aldh1a2* became patchy 3 days after sham-operation (Fig. 4F). Both the scratch and puncture methods induced stronger *aldh1a2* signals in the epicardial tissue (Fig. 4G, H), compared to the sham operated hearts. The stronger signals support the idea that *aldh1a2* expression involves both a response to pericardiac environmental change and a response to injury to the epicardial tissue. Endocardial *aldh1a2* was not detected by the scratch and puncture methods at 3 dpi, suggesting that endocardial *aldh1a2* diminished due to mild injury (Fig. 4G, H). This idea is supported by the observation that endocardial *aldh1a2* was still detectable in resected hearts at 3 dpi (Fig. 4I).

At 7 dpi, sham operated hearts did not exhibit evident epicardial *aldh1a2* expression (Fig. 4J), suggesting that the pericardiac environment was stabilized by this time point. Contrary

to this, hearts injured by all three methods still exhibited epicardial *aldh1a2* expression to different degrees, which correlated with the severity of the injury. This indicates that the epicardial *aldh1a2* expression at 7 dpi is associated with epicardial injury.

These data show that both epicardial and endocardial *aldh1a2* expression correlate with the degree of injury. The epicardial expression is induced organ-wide manner, and involves responses to pericardiac environmental change and epicardial injury. The evident endocardial *aldh1a2* signals in punctured hearts, which diminish faster than those in resected hearts, also support our idea that puncture-induced injuries represent milder injury that induces heart regeneration responses.

Epicardial *tbx18* and WT1 expression

A previous study has demonstrated that epicardial cells respond to injury and activate expression of developmental genes. Recent studies suggested that the epicardial tissue is not homogenous and implied that each epicardial marker gene labels a subset of epicardial cells in regenerating zebrafish heart. Among several epicardial genes, reporter signals of the *tbx18-EGFP* and *wt1b-EGFP* lines during heart regeneration have been examined by several groups. The EGFP reporter in these transgenic fish is driven by regulatory sequences of the *tbx18* gene or *wt1b* gene and was not detected in uninjured adult hearts, but it was induced in the epicardial cells after heart injury. Similarly, the *tbx18* mRNA was not detected in the uninjured adult heart but was induced in the epicardium after ventricular resection. Contrary to these reports, a previous report showed *wt1b* mRNA expression in adult hearts without injury. Because the *wt1b* mRNA expression was detected by RT-PCR, cell types that express *wt1b* in uninjured hearts remained unknown. Thus, we first examined expression of *tbx18* by in situ hybridization and WT1 by immunofluorescence in sham-operated hearts and found that both *tbx18* and WT1 were undetectable in the epicardium and myocardium without injuries (Fig 5A, A', E, E').

Three days after injury by scratching, we did not detect evident expression of *tbx18* and only faint expression of *tbx18* at the injury site was detected (Fig. 5B, B'). Contrary to this result, both puncture and resection induced more evident expression of *tbx18* in the epicardium (Fig. 5C, C', D, D'), and the signal was detected away from the injury site (arrows, Fig. 5C, D). Correlating with the difference in severity of injury, the puncture-induced expression of *tbx18* was weaker compared to resection.

WT1 was induced 3 days after scratching, and some of WT1-positive cells were also PCNA-positive (Fig. 5F, F'), indicating that epicardial cells respond to scratch injuries and proliferate. Compared to this, hearts injured by puncture showed stronger multi-layered WT1 signals (Fig. 5G, G'). Hearts injured by resection showed further stronger WT1 signals (Fig. 5H, H'). In both punctured and resected hearts, WT1 signals away from injury sites were also detected (arrows, Fig. 5G, H), which were not detected in scratched hearts. In addition to previous reports of participation of epicardial cells in heart regeneration, our data shows that epicardial cells proliferate in response to epicardial injury. The degree of multilayered structures from epicardial cells at the injury sites correlates with the degree of heart injuries, further supporting a role of epicardial cells in heart regeneration.

These results support the idea that puncturing the ventricle induces regenerative responses, similar to resection, at milder degree, but the scratching the ventricle does not. These data also support the idea that epicardial response involves both response to peri-cardiac environmental changes, visualized by *aldh1a2*, and response to heart injury, visualized by organ-wide marker expression (*tbx18* and WT1) and multi-layered WT1-positive epicardium at the injury site. Recent studies suggested that epicardial derived cells function as cellular scaffold for regenerating cardiomyocytes to promote their proliferation, in addition to supporting neovasculature formation through secretion of trophic factors. The weaker response of epicardial gene/protein expression and lower levels of cardiomyocyte proliferation after puncture than resection are in agreement with this idea.

The remarkable ability to regenerate the heart after various injuries made zebrafish an excellent model animal to study heart regeneration. With the identification of pre-existing cardiomyocytes as the cellular source of regenerated myocardium, it has been attracting more attention to what factors trigger, maintain and terminate heart regeneration. Several reports demonstrated that cardiomyocyte proliferation and myocardial regeneration are regulated by various factors. These include retinoic acid, interleukin11 and relaxin 3, sonic hedgehog, transforming growth factor β , insulin like growth factor and hypoxia-induced signaling. A recent publication that employed fluorescent ubiquitylation-based cell cycle indicator (FUCCI) to examine cardiomyocyte proliferation is an example of growing attention to seek factors for cardiomyocyte proliferation during heart regeneration. Our study showed that a milder injury with the puncture method could activate endocardial cells, epicardial cells, neovascularization and cardiomyocyte proliferation. The puncture method provides a simple and reproducible procedure to injure the heart, and the process of heart regeneration is quicker than those induced by other methods. In this point of view, the puncture method could have a potential to be applied to seek compounds to regulate heart regeneration.

EXPERIMENTAL PROCEDURES Zebrafish maintenance and heart surgery

Zebrafish were maintained under standard conditions at around 28°C with a 14 h light 10 h dark cycle. Adult zebrafish (6 to 15-months old) were used for experiments.

Adult zebrafish were anesthetized and the heart was exposed by manually dissecting the ventral body wall. For resection, we amputated ventricular apex as previously published. To injure the heart by puncturing, the heart was lifted with forceps, which was placed under the heart, and a 200 μm -diameter tungsten needle was inserted into the ventricle whose edge is sharpened for embryo surgery experiments. The injury was visually confirmed such that the edge of the needle comes out from the dorsal side of the ventricle, so that the needle passed through the ventricle. To injure the heart by scratching, the surface of the ventricle was scratched with the same sharp tungsten needle. Care and experimentation were done in accordance with the Institutional Animal Care and Use Committee of the University of Minnesota.

Histology, in situ hybridization and immunostaining

For histological examination, 14 µm thick cryosections were processed to visualize fibrin clots in combination with anti-MHC antibody staining .

In situ hybridization and immunostaining on 14 µm thickness sections were performed as previously described . Briefly, sections were washed with PBS with 0.1 % Triton X-100, and heat-induced antigen retrieval was performed with citrate buffer (pH6.0) by boiling for 40 min. The primary antibodies used were anti-MHC antibody (Developmental Studies Hybridoma Bank, MF20, 5.14 µg/ml), anti-PCNA antibody (Santa Cruz Biotechnology; sc-56, 1:100), anti-MEF2 antibody (Santa Cruz Biotechnology; sc-313, 1:50) and anti-WT1 antibody (Abcam; ab15249, 1:200). Secondary antibodies used were Alexa fluorophore-labelled anti-mouse or rabbit IgG (Invitrogen, Carlsbad, CA, 1:1,000). For chromogenic detection, biotinylated anti-mouse IgG (Vector laboratories, Burlingame, CA, 1:500) and a horseradish peroxidase streptavidin system (Vector laboratories) were used.

Cardiomyocyte proliferation assay

Sections around the center of the injury were stained with anti-PCNA and anti-MEF2 antibodies. Three sections around the center of the injury site were examined per fish. Confocal images at the injury sites (512 × 512 pixels, 212 µm × 212 µm) with x40 objective lens were acquired, and total number of PCNA + MEF2 double positive cells from the three images was used as number of proliferating cardiomyocyte per fish. Statistical significance was determined by one-way ANOVA and Tukey HSD test.

Imaging

Bright field images of heart sections were taken by using Nikon ACT1 software and DXM1200 digital camera (Nikon, Melville, NY, USA) or using Zeiss AxioVision software and AxioCam MRc5 digital camera (Carl Zeiss Microscopy). Fluorescent confocal images were obtained by using Zeiss LSM 710 laser scanning microscope system (Carl Zeiss Microscopy), and analyzed by ZEN2009 software (Carl Zeiss Microscopy).

Acknowledgments

We are grateful to the zebrafish core facility at the University of Minnesota for general help with breeding and maintenance of zebrafish lines. We thank Drs. Michael O'Connor, Thomas Neufeld, Laura Gammill and Yasushi Nakagawa for sharing equipment, Dr. Angel Raya for sharing *flil-EGFP; cmlc2a-mCherry* transgenic fish. MF20 (anti-MHC) antibodies were obtained from the Developmental Studies Hybridoma Bank, developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biology, Iowa City, IA 52242. This work is supported in part by the Minnesota Medical Foundation (4099-9216-12) and the National Institute of Arthritis and Musculoskeletal and Skin Diseases of NIH (R01AR064195).

REFERENCES

1. Bader D, Masaki T, Fischman DA. Immunochemical analysis of myosin heavy chain during avian myogenesis in vivo and in vitro. *J Cell Biol.* 1982; 95:763–770. [PubMed: 6185504]
2. Beltrami AP, Barlucchi L, Torella D, Baker M, Limana F, Chimenti S, Kasahara H, Rota M, Musso E, Urbanek K, Leri A, Kajstura J, Nadal-Ginard B, Anversa P. Adult cardiac stem cells are multipotent and support myocardial regeneration. *Cell.* 2003; 114:763–776. [PubMed: 14505575]

3. Bergmann O, Bhardwaj RD, Bernard S, Zdunek S, Barnabe-Heider F, Walsh S, Zupicich J, Alkass K, Buchholz BA, Druid H, Jovinge S, Frisen J. Evidence for cardiomyocyte renewal in humans. *Science*. 2009; 324:98–102. [PubMed: 19342590]
4. Bollig F, Mehringer R, Perner B, Hartung C, Schafer M, Scharl M, Volff JN, Winkler C, Englert C. Identification and comparative expression analysis of a second wt1 gene in zebrafish. *Dev Dyn*. 2006; 235:554–561. [PubMed: 16292775]
5. Chablais F, Jazwinska A. The regenerative capacity of the zebrafish heart is dependent on TGFbeta signaling. *Development*. 2012; 139:1921–1930. [PubMed: 22513374]
6. Chablais F, Veit J, Rainer G, Jazwinska A. The zebrafish heart regenerates after cryoinjury-induced myocardial infarction. *BMC Dev Biol*. 2011; 11:21. [PubMed: 21473762]
7. Choi WY, Gemberling M, Wang J, Holdway JE, Shen MC, Karlstrom RO, Poss KD. In vivo monitoring of cardiomyocyte proliferation to identify chemical modifiers of heart regeneration. *Development*. 2013; 140:660–666. [PubMed: 23293297]
8. Choi WY, Poss KD. Cardiac regeneration. *Curr Top Dev Biol*. 2012; 100:319–344. [PubMed: 22449849]
9. Colucci WS. Molecular and cellular mechanisms of myocardial failure. *Am J Cardiol*. 1997; 80:15L–25L.
10. Curado S, Anderson RM, Jungblut B, Mumm J, Schroeter E, Stainier DY. Conditional targeted cell ablation in zebrafish: a new tool for regeneration studies. *Dev Dyn*. 2007; 236:1025–1035. [PubMed: 17326133]
11. Detrich, IHW.; Westerfield, M.; Zon, LI. *The Zebrafish: Genetics, Genomics, and Informatics*. Elsevier Academic Press; San Diego: 2004.
12. Duester G. Retinoic acid synthesis and signaling during early organogenesis. *Cell*. 2008; 134:921–931. [PubMed: 18805086]
13. Fang Y, Gupta V, Karra R, Holdway JE, Kikuchi K, Poss KD. Translational profiling of cardiomyocytes identifies an early Jak1/Stat3 injury response required for zebrafish heart regeneration. *Proc Natl Acad Sci U S A*. 2013; 110:13416–13421. [PubMed: 23901114]
14. Gonzalez-Rosa JM, Martin V, Peralta M, Torres M, Mercader N. Extensive scar formation and regression during heart regeneration after cryoinjury in zebrafish. *Development*. 2011; 138:1663–1674. [PubMed: 21429987]
15. Gonzalez-Rosa JM, Peralta M, Mercader N. Pan-epicardial lineage tracing reveals that epicardium derived cells give rise to myofibroblasts and perivascular cells during zebrafish heart regeneration. *Dev Biol*. 2012; 370:173–186. [PubMed: 22877945]
16. Hu N, Yost HJ, Clark EB. Cardiac morphology and blood pressure in the adult zebrafish. *Anat Rec*. 2001; 264:1–12. [PubMed: 11505366]
17. Huang Y, Harrison MR, Osorio A, Kim J, Baugh A, Duan C, Sucov HM, Lien CL. Igf Signaling is Required for Cardiomyocyte Proliferation during Zebrafish Heart Development and Regeneration. *PLoS One*. 2013; 8:e67266. [PubMed: 23840646]
18. Itou J, Kawakami H, Burgoyne T, Kawakami Y. Life-long preservation of the regenerative capacity in the fin and heart in zebrafish. *Biol Open*. 2012a; 1:739–746. [PubMed: 23213467]
19. Itou J, Oishi I, Kawakami H, Glass TJ, Richter J, Johnson A, Lund TC, Kawakami Y. Migration of cardiomyocytes is essential for heart regeneration in zebrafish. *Development*. 2012b; 139:4133–4142. [PubMed: 23034636]
20. Jopling C, Sleep E, Raya M, Marti M, Raya A, Belmonte JC. Zebrafish heart regeneration occurs by cardiomyocyte dedifferentiation and proliferation. *Nature*. 2010; 464:606–609. [PubMed: 20336145]
21. Jopling C, Sune G, Faucherre A, Fabregat C, Izpisua Belmonte JC. Hypoxia induces myocardial regeneration in zebrafish. *Circulation*. 2012; 126:3017–3027. [PubMed: 23151342]
22. Kawakami Y, Capdevila J, Buscher D, Itoh T, Rodriguez Esteban C, Izpisua Belmonte JC. WNT signals control FGF-dependent limb initiation and AER induction in the chick embryo. *Cell*. 2001; 104:891–900. [PubMed: 11290326]
23. Kawakami Y, Rodriguez-Leon J, Koth CM, Buscher D, Itoh T, Raya A, Ng JK, Esteban CR, Takahashi S, Henrique D, Schwarz MF, Asahara H, Izpisua Belmonte JC. MKP3 mediates the

- cellular response to FGF8 signalling in the vertebrate limb. *Nat Cell Biol.* 2003; 5:513–519. [PubMed: 12766772]
24. Kikuchi K, Gupta V, Wang J, Holdway JE, Wills AA, Fang Y, Poss KD. tcf21+ epicardial cells adopt non-myocardial fates during zebrafish heart development and regeneration. *Development.* 2011a; 138:2895–2902. [PubMed: 21653610]
 25. Kikuchi K, Holdway JE, Major RJ, Blum N, Dahn RD, Begemann G, Poss KD. Retinoic acid production by endocardium and epicardium is an injury response essential for zebrafish heart regeneration. *Dev Cell.* 2011b; 20:397–404. [PubMed: 21397850]
 26. Kikuchi K, Holdway JE, Werdich AA, Anderson RM, Fang Y, Egnaczyk GF, Evans T, Macrae CA, Stainier DY, Poss KD. Primary contribution to zebrafish heart regeneration by gata4(+) cardiomyocytes. *Nature.* 2010; 464:601–605. [PubMed: 20336144]
 27. Kikuchi K, Poss KD. Cardiac regenerative capacity and mechanisms. *Annu Rev Cell Dev Biol.* 2012; 28:719–741. [PubMed: 23057748]
 28. Kim J, Wu Q, Zhang Y, Wiens KM, Huang Y, Rubin N, Shimada H, Handin RI, Chao MY, Tuan TL, Starnes VA, Lien CL. PDGF signaling is required for epicardial function and blood vessel formation in regenerating zebrafish hearts. *Proc Natl Acad Sci U S A.* 2010; 107:17206–17210. [PubMed: 20858732]
 29. Lepilina A, Coon AN, Kikuchi K, Holdway JE, Roberts RW, Burns CG, Poss KD. A dynamic epicardial injury response supports progenitor cell activity during zebrafish heart regeneration. *Cell.* 2006; 127:607–619. [PubMed: 17081981]
 30. Lien CL, Harrison MR, Tuan TL, Starnes VA. Heart repair and regeneration: recent insights from zebrafish studies. *Wound Repair Regen.* 2012; 20:638–646. [PubMed: 22818295]
 31. Lieschke GJ, Currie PD. Animal models of human disease: zebrafish swim into view. *Nat Rev Genet.* 2007; 8:353–367. [PubMed: 17440532]
 32. Mihatsch MJ, Bremer J. Acid fuchsin orange G-stain (AFOG) for glomerular protein deposits. *Clin Nephrol.* 1978; 9:259. [PubMed: 78783]
 33. Pasumarthi KB, Field LJ. Cardiomyocyte cell cycle regulation. *Circ Res.* 2002; 90:1044–1054. [PubMed: 12039793]
 34. Pfeffer MA, Braunwald E. Ventricular remodeling after myocardial infarction. Experimental observations and clinical implications. *Circulation.* 1990; 81:1161–1172. [PubMed: 2138525]
 35. Porrello ER, Mahmoud AI, Simpson E, Hill JA, Richardson JA, Olson EN, Sadek HA. Transient regenerative potential of the neonatal mouse heart. *Science.* 2011; 331:1078–1080. [PubMed: 21350179]
 36. Poss KD, Wilson LG, Keating MT. Heart regeneration in zebrafish. *Science.* 2002; 298:2188–2190. [PubMed: 12481136]
 37. Raya A, Consiglio A, Kawakami Y, Rodriguez-Esteban C, Izpisua-Belmonte JC. The zebrafish as a model of heart regeneration. *Cloning Stem Cells.* 2004; 6:345–351. [PubMed: 15671662]
 38. Raya A, Koth CM, Buscher D, Kawakami Y, Itoh T, Raya RM, Sternik G, Tsai HJ, Rodriguez-Esteban C, Izpisua-Belmonte JC. Activation of Notch signaling pathway precedes heart regeneration in zebrafish. *Proc Natl Acad Sci U S A* 100 Suppl. 2003; 1:11889–11895.
 39. Reese DE, Mikawa T, Bader DM. Development of the coronary vessel system. *Circ Res.* 2002; 91:761–768. [PubMed: 12411389]
 40. Schnabel K, Wu CC, Kurth T, Weidinger G. Regeneration of cryoinjury induced necrotic heart lesions in zebrafish is associated with epicardial activation and cardiomyocyte proliferation. *PLoS One.* 2011; 6:e18503. [PubMed: 21533269]
 41. Wang J, Karra R, Dickson AL, Poss KD. Fibronectin is deposited by injury-activated epicardial cells and is necessary for zebrafish heart regeneration. *Dev Biol.* 2013; 382:427–435. [PubMed: 23988577]
 42. Wang J, Panakova D, Kikuchi K, Holdway JE, Gemberling M, Burris JS, Singh SP, Dickson AL, Lin YF, Sabeh MK, Werdich AA, Yelon D, Macrae CA, Poss KD. The regenerative capacity of zebrafish reverses cardiac failure caused by genetic cardiomyocyte depletion. *Development.* 2011; 138:3421–3430. [PubMed: 21752928]

43. Wills AA, Holdway JE, Major RJ, Poss KD. Regulated addition of new myocardial and epicardial cells fosters homeostatic cardiac growth and maintenance in adult zebrafish. *Development*. 2008; 135:183–192. [PubMed: 18045840]
44. Woods IG, Kelly PD, Chu F, Ngo-Hazelett P, Yan YL, Huang H, Postlethwait JH, Talbot WS. A comparative map of the zebrafish genome. *Genome Res*. 2000; 10:1903–1914. [PubMed: 11116086]
45. Zhou B, Honor LB, He H, Ma Q, Oh JH, Butterfield C, Lin RZ, Melero-Martin JM, Dolmatova E, Duffy HS, Gise A, Zhou P, Hu YW, Wang G, Zhang B, Wang L, Hall JL, Moses MA, McGowan FX, Pu WT. Adult mouse epicardium modulates myocardial injury by secreting paracrine factors. *J Clin Invest*. 2011; 121:1894–1904. [PubMed: 21505261]

Key findings

- Scratching the ventricle induces epicardial responses with subtle cardiomyocyte proliferation
- Puncturing the ventricle induces similar responses to those by resection
- Responses by puncturing are milder and the repair processes are quicker compared to resection
- Puncturing the ventricle has a potential to apply to systematic analysis, such as compound screening, for cardiomyocyte proliferation

Fig. 1. Comparison of injury to the myocardial layer by different injury methods
(A-D) In situ hybridization of *cmlc2a* (purple) after various injuries to the heart. (E-S) Immunostaining of MHC (brown) coupled with fibrin detection (red) after various injuries to the heart. Sham operation (A, E), Scratch-induced injury (B, F, I, L, O), puncture-induced injury (C, G, J, M, P, R) and resection-induced injury (D, H, K, N, Q, S) were performed. Arrowheads point to fibrin clot. Time points after injury are indicated. Brackets indicate the injury sites. Scale bar, 50 μm.

Fig. 2. Cardiomyocyte proliferation after different damages to the heart
 (A-L, A'-L') Representative images of MEF2 (magenta) and PCNA (green) immunostaining at 3 dpi (A-D, A'-D'), 7 dpi (E-H, E'-H') and 14 dpi (I-L, I'-L') after sham operation (A, A', E, E', I, I'), scratch- (B, B', F, F', J, J'), puncture- (C, C', G, G', K, K') and resection- (D, D', H, H', L, L') induced injury. Yellow arrowheads point to proliferating cardiomyocytes, observed as white signals. A'-L' show close up images of the boxed areas in A-L. Brackets indicate the injury sites. Scale bar, 50 μ m.

(M) A graphic representation of the number of proliferating cardiomyocytes at indicated time points after different injury methods. Shown are average $\pm$ S.D. (n=3). CM: cardiomyocyte.

Fig. 3. Analysis of endothelial cells after different damages to the heart

The *fli1:EGFP* signal (green, endothelial cells) and the *cmlc2a:mCherry* signal (magenta, cardiomyocytes) at 1 day (A-D, A'-D') and 7 days (E-H, E'-H') after various injuries. A'-H' show close up images of boxed areas in A - H. White arrowheads and arrows in A' point to thick and thinner *fli1:EGFP* signals, respectively. For simplicity, only panel A' is labeled. Yellow arrowheads point to the *fli1:EGFP* signals at the surface of injury sites. Brackets indicate the injury sites. Scale bar, 50 μm. cz: compact zone, tb: trabecular zone.

Fig. 4. Expression of *aldh1a2* after various injuries

(A-D, A'-D', F-M) In situ hybridization of *aldh1a2* at 1 day (A-D, A'-D'), 3 day (F-I) and 7 day (J-M) after various injuries. (E) In situ hybridization of *aldh1a2* without injury. A'-E' show close up images of boxed areas in A - E. (A, A', F) Arrowheads and arrows point to evident signals in the epicardium at the apex and away from the apex, respectively. (B-D, B'-D', G-I, K-M) Arrowheads and arrows point to evident signals in the epicardium around injury sites and away from injury sites, respectively. Open arrowheads point to endocardial signals. Brackets indicate the injury sites. Scale bar, 50 μ m.

Fig. 5. Induction of *tbx18* and WT1 in the epicardium after various injuries

In situ hybridization of *tbx18* (A-D, A'-D') and immunofluorescence of WT1 (magenta) + PCNA (green) (E-H, E'-H') 3 days after various injuries. In A-D' arrowheads and arrows point to signals around and away from injury sites, respectively. In E-H' arrowheads point to proliferating epicardial cells, and arrows point to WT1 signal away from injury sites. Brackets indicate the injury sites. Scale bar, 50 μm.