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Acknowledgments, including funding information, should be gathered into a brief statement at the end of the references and notes and will be edited to conform to Science style.

STYLE EXAMPLES

Journals

1. N. Tang, *Atmos. Environ.* **14**, 819-834 (1980). [one author]

2. J. C. Smith, M. Field, *Proc. Natl. Acad. Sci. U.S.A.* **51**, 930-935 (1964). [two or more authors]

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2. G. Reuter, personal communication. [Must be accompanied with a letter of permission and must not be used to support a central claim, result, or conclusion.]

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1. A. M. Lindroth *et al*., *Science* **292**, 2077 (2001); published online 10 May 2001 (10.1126/science.1059745). [if not yet in the print version of *Science*, use "in press" instead of volume number, page number, and year]

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1. A. Smette *et al*., *Astrophys. J.,* in press (available at http://xxx.lanl.gov/abs/astro-ph/0012193). [if now published, omit the URL and provide only a standard reference]

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Supporting Online Material
www.sciencemag.org/cgi/content/full/1076249/DC1
Figs. S1 and S2
Tables S1 and S2
References

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Heart Regeneration in Zebrafish

Kenneth D. Poss,* Lindsay G. Wilson, Mark T. Keating*

Cardiac injury in mammals and amphibians typically leads to scarring, with minimal regeneration of heart muscle. Here, we demonstrate histologically that zebrafish fully regenerate hearts within 2 months of 20% ventricular resection. Regeneration occurs through robust proliferation of cardiomyocytes localized at the leading epicardial edge of the new myocardium. The hearts of zebrafish with mutations in the Mps1 mitotic checkpoint kinase, a critical cell cycle regulator, failed to regenerate and formed scars. Thus, injury-induced cardiomyocyte proliferation in zebrafish can overcome scar formation, allowing cardiac muscle regeneration. These findings indicate that zebrafish will be useful for genetically dissecting the molecular mechanisms of cardiac regeneration.

Injured human hearts do not regenerate. Instead, damaged myocardium is replaced by fibrotic scar tissue. Cardiomyocytes, the major structural cells of the heart, may undergo hypertrophy in the wound area to increase muscular mass. Although recent findings suggest that cardiomyocytes within the diseased human heart can proliferate (*1*), most evidence to date indicates that myocyte proliferation is not a significant component of the mammalian response to cardiac injury (*2*).

Teleost fish, including zebrafish, can regenerate spinal cord, retina, and fins (*3*, *4*). To determine whether zebrafish can also regenerate heart muscle, we surgically removed ~20% of the ventricular myocardium from 1- to 2-year-old adults (Fig. 1, A and B) and

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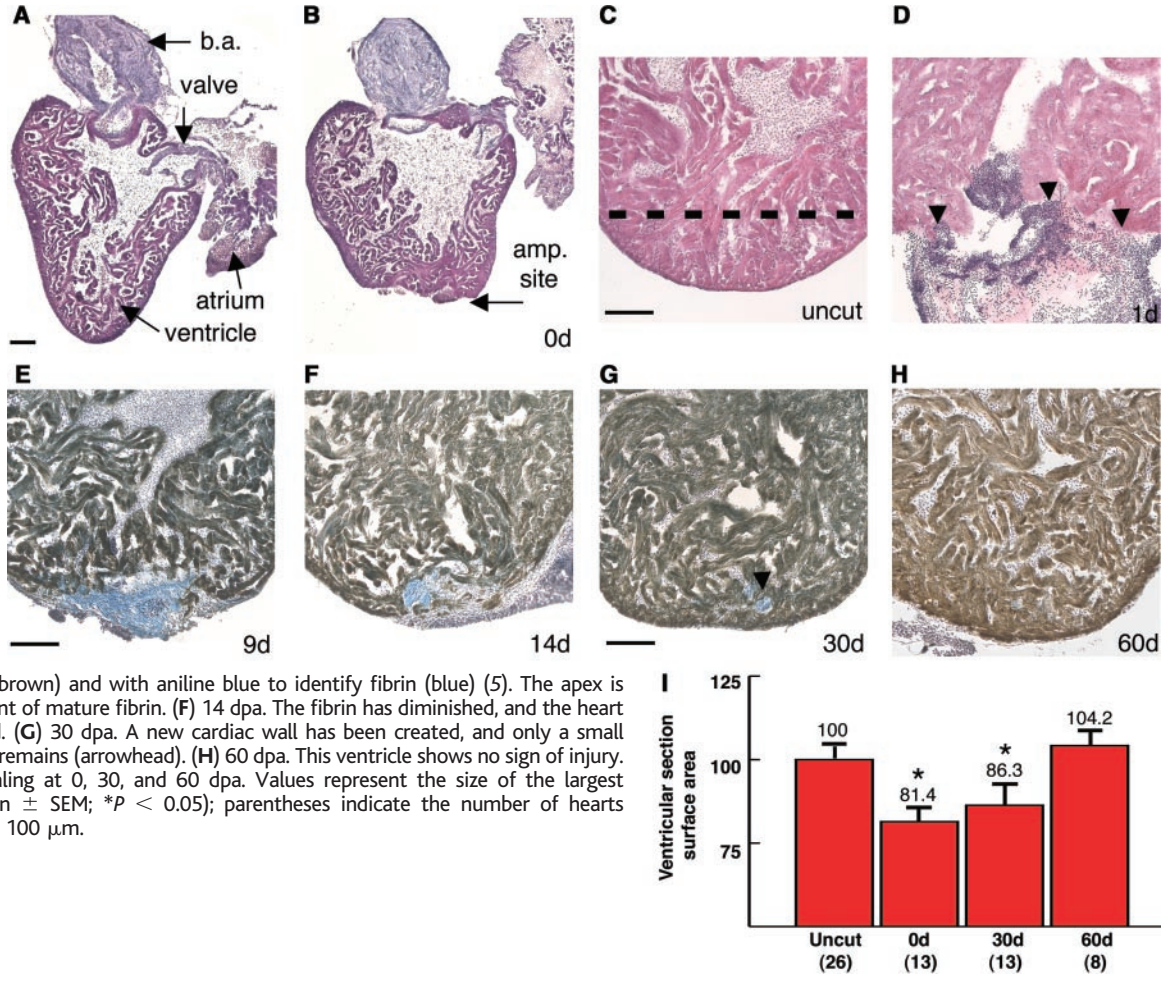

Fig. 1. Regeneration of ventricular myocardium in the resected zebrafish heart. Hematoxylin and eosin stain of the intact zebrafish heart before (**A**) and after about 20% ventricular resection (**B**) (*5*). b.a., bulbous arteriosus. (**C**) An intact ventricular apex at higher magnification, indicating the approximate amputation plane (dashed line). All images in this and subsequent figures display longitudinal ventricular sections of the amputation plane. (**D**) 1 dpa. The large clot is filled with nucleated erythrocytes (arrowheads). (**E**) 9 dpa. The heart section is stained for the presence of myosin heavy chain to identify cardiac muscle (brown) and with aniline blue to identify fibrin (blue) (*5*). The apex is sealed with a large amount of mature fibrin. (**F**) 14 dpa. The fibrin has diminished, and the heart muscle has reconstituted. (**G**) 30 dpa. A new cardiac wall has been created, and only a small amount of internal fibrin remains (arrowhead). (**H**) 60 dpa. This ventricle shows no sign of injury. (**I**) Quantification of healing at 0, 30, and 60 dpa. Values represent the size of the largest ventricular section (mean ± SEM; **P* < 0.05); parentheses indicate the number of hearts examined (*5*). Scale bars, 100 μm.

examined hearts histologically at eight time points after surgery (*5*). After several seconds of profuse bleeding from the ventricular lumen, a large clot of erythrocytes formed in the wound (Fig. 1, C and D). Beginning at 2 to 4 days postamputation (dpa), these erythrocytes were replaced by fibrin, which reached maximum levels at 7 to 9 dpa (Fig. 1E). For the first few days after ventricular resection, zebrafish appeared less active and less coordinated while swimming than sham-operated animals. After 1 week of recovery, however, they were indistinguishable from controls.

From 9 to 30 dpa, cardiac myofibers surrounded, penetrated, and eventually replaced the clot (Fig. 1, F and G). By 30 dpa, a contiguous wall of muscle had formed (Fig. 1G); by 60 dpa, the fibrin clot had completely disappeared, and the size and shape of most zebrafish ventricles appeared grossly normal by histology (Fig. 1H) (*5*). The contractile properties of beating hearts in situ at 60 dpa also appeared grossly normal through direct visual inspection (*6*).

To quantify the replacement of zebrafish heart muscle, we measured the surface area of longitudinal ventricular sections of injured hearts by digital imaging and computer quantification. Amputation removed ~19% of the ventricular section surface area (Fig. 1I). Measurements taken 30 dpa revealed partial recovery of ventricular section surface area, and, by 60 dpa, the surface area was completely recovered (Fig. 1I) (*5*).

The zebrafish ventricle is composed of a thin, external layer of compact myocytes penetrated by blood vessels and by internal myocytes organized into elaborate trabeculae (fig. S1). An antibody to β catenin, which is localized to adherens junctions (*7*, *8*), clearly demarcated these two types of myocardium. This method revealed that the initial wound area through 7 dpa consisted mainly of trabecular myocytes exposed to the clot. By 14 dpa, most of the wound was enveloped by a wall of compact myocytes, leaving only a small portion of medial trabecular myocardium exposed to the clot. By 30 dpa, and even more prominently at 60 dpa, a large, contiguous wall of compact muscle 10 to over 30 myocytes wide was created at the apex, replacing the resected compact and trabecular myocardium (fig. S1). These observations indicate that a new layer of compact myocytes was reestablished and then expanded after amputation.

The restoration of cardiac muscle could result from myocyte hypertrophy or hyperplasia. To assess zebrafish cardiomyocyte proliferation, we assayed cell cycle entry by measuring the nuclear incorporation of bromodeoxyuridine (BrdU), a marker of DNA synthesis. In uninjured hearts, about 3% of compact cardiomyocyte nuclei incorporated BrdU after a 7-day labeling period, with no trabecular cardiomyocyte incorporation (Fig. 2A and fig. S2). In contrast, in injured hearts ~17% of compact and trabecular myocytes near the amputation plane incorporated BrdU at 7 dpa (Fig. 2B). BrdU incorporation peaked at ~32% at 14 dpa, with most cycling myocytes localized to compact muscle at the lateral edges of the wound (Fig. 2C). At 30 dpa, the BrdU incorporation index in compact muscle was still 20% (Fig. 2D) but had considerably decreased to 7% (fig. S2) by 60 dpa. Cardiac myocytes also underwent mitosis, as defined by expression of phosphorylated histone-3, a marker of condensed chromatin. Although mitoses were rarely seen in the uninjured heart, we observed 3 to 10 cardiomyocyte mitoses per wound area at 14 dpa (Fig. 2E).

We next gave a pulse of BrdU to zebrafish that were from 7 to 14 dpa and assessed cardiomyocyte labeling at 14, 30, and 60 dpa. After the pulse, BrdU-positive cells continued to increase in number and were often found adjacent to one another in the regenerated myocardium, consistent with cardiomyocyte cell division (Fig. 2F). Nuclei in these myocytes were frequently punctate-labeled, suggesting BrdU dilution via karyokinesis (fig. S3).

Pulse-chase experiments also revealed that the leading edge of proliferation during regeneration was the new layer of outermost (epicardial) myocytes. Zebrafish labeled with BrdU at 30 and 60 dpa demonstrated proliferation in epicardial cardiomyocytes of the new compact muscle (Fig. 2D). By contrast, zebrafish labeled from 7 to 14 dpa with BrdU

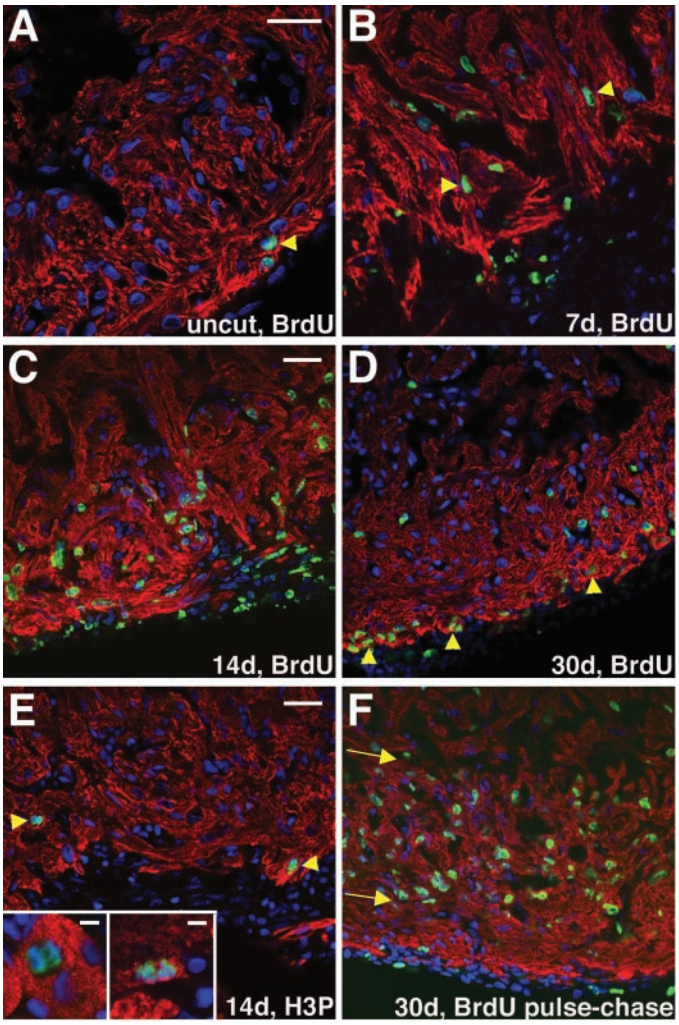

Fig. 2. Cardiomyocyte proliferation accompanies zebrafish heart regeneration. (**A**) Confocal image of a heart section of an unamputated fish labeled for 7 days with BrdU, stained for myosin heavy chain to identify cardiomyocytes (red), and stained with BrdU to detect cycling cells (green) and with 4′,6′-diamidino-2-phenylindole (DAPI) to detect nuclei (blue). A low percentage of compact myocytes incorporate BrdU over this period (arrowhead) (*5*). (**B**) 7 dpa (0- to 7-dpa BrdU labeling). BrdU incorporation occurs in trabecular myocytes at the amputation plane (arrowheads). Most hearts also showed labeling in compact myocytes adjacent to the wound area at this stage (not shown). (**C**) 14 dpa (7- to 14-dpa BrdU labeling). Many cardiomyocytes incorporate BrdU during this period, largely in compact muscle adjacent to the wound. (**D**) 30 dpa (23- to 30-dpa BrdU labeling). Myocyte BrdU incorporation continues as the compact muscular layer expands. Labeling is usually limited to the most epicardial myocytes of the apex (arrowheads). (**E**) Cardiomyocyte mitoses observed at 14 dpa in the wound area, with the use of an antibody to phosphorylated histone-3 (H3P; arrowheads indicate positive cells). Insets show a cardiomyocyte with sister chromatid segregation (left) and cardiomyocytes that have recently completed karyokinesis (right). Sections are stained for myosin heavy chain (red) and H3P (green) and stained with DAPI (blue). (**F**) 30 dpa (7- to 14-dpa BrdU labeling). Arrows indicate the area of incorporation. There is an increase in labeled cardiomyocytes in 30-dpa pulse-chase hearts. Several unlabeled layers of new myocytes are external to labeled myocytes, presumably resulting from cell division after the labeling period. Scale bars, 25 μm; 5 μm in (E) insets.

and given a 16- or 46-day chase showed substantial BrdU incorporation in the inner layers of the new compact myocardium, with extremely limited labeling in epicardial myocytes (Fig. 2F and fig. S3). These data indicate that, after dispersed cardiomyocyte proliferation in the wound area to restore the ventricular wall, zebrafish cardiac regeneration occurs via a gradient of proliferation that is greatest in epicardial myocytes. Regenerated myocytes are displaced inward as epicardial myocytes continue to proliferate and advance.

Fibrosis is the dominant histological reaction to injury in mammalian and amphibian hearts. This response typically follows fibrin deposition after cardiac injury (*9*). By contrast, zebrafish ventricles displayed only small deposits of collagen extending into the fibrin clot at 14 dpa (Fig. 3A). The extent of collagen deposition at 30 and 60 dpa varied from complete absence to small deposits (n = 13 hearts) (Fig. 3B).

Why do zebrafish respond to cardiac injury with regeneration, whereas fibrosis predominates in other vertebrates? We propose a model in which scarring complements regeneration, and it is the vigor of myocyte proliferation within a given species that determines the predominant response. This model predicts that inhibition of regeneration would lead to scarring. To test this model, we analyzed the cardiac injury response in *mps1* mutant zebrafish. These animals harbor a temperature-sensitive mutation in Mps1, a mitotic checkpoint kinase that is up-regulated in many proliferative cell types (*10*–*13*). At the restrictive temperature, this mutation potently blocks fin regeneration through failed cell proliferation (*13*). In situ hybridization of 14-dpa zebrafish hearts showed induction of *mps1* mRNA in a small number of myocytes near the wound, consistent with localization and function of Mps1 in regenerating cardiomyocytes (fig. S4). The *mps1* mutants formed normal fibrin clots by 8 to 9 dpa (*6*). In wild-type fish, cardiac myofibers invariably penetrated the clot and constructed a bridge of new muscle around the wound by 17 to 26 dpa (Fig. 3C) (n = 12). By contrast, the ventricular wall was not restored in *mps1* mutants (Fig. 3D) (n = 13); instead, the injured hearts retained fibrin deposits and developed large connective-tissue scars (Fig. 3, E and F) (n = 7) (*14*).

We conclude that, in response to mechanical injury, zebrafish hearts can regenerate without scarring. A new ventricular wall of compact myocardium is created in the injured heart by cardiomyocyte proliferation, a process that requires the cell-cycle regulator Mps1. Although cardiomyocyte proliferation in injured adult amphibian hearts has been documented (*15*–*19*), we suggest that zebrafish represent an improved model system for understanding heart regeneration. Zebrafish restore the ventricular myocardial wall, whereas published findings indicate little or no cardiac muscle regeneration after amphibian heart resection. Also, a wide array of molecular and genetic tools, including mutagenesis screens and complete genome sequence information, are available to those working with zebrafish. Thus, dissection of zebrafish cardiac muscle regeneration may illuminate factors that can stimulate a regenerative response in the mammalian heart.

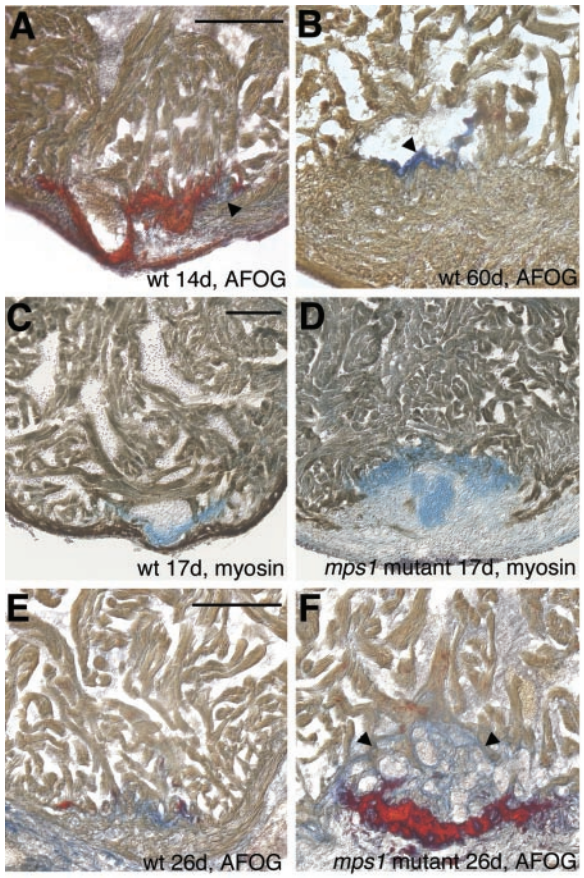

Fig. 3. Cardiomyocyte proliferation is required for scarless regeneration. 14-dpa (**A**) and 60-dpa (**B**) ventricles stained with acid fuchsin–orange G (AFOG; fibrin, orange/red; collagen, blue), which is highly sensitive for collagen (arrowheads). Regenerated ventricles contain minimal collagen. (**C** and **D**) 17-dpa ventricles double-stained for myosin heavy chain and with aniline blue (muscle, brown; fibrin and collagen, grayish blue). Wild-type ventricles display new compact muscular wall formation (C), whereas *mps1* mutant ventricles demonstrate no evidence of new muscle (D) (*14*). (**E** and **F**) 26-dpa hearts stained with AFOG. The wild-type cardiac injury response includes minor fibrin retention and collagen deposition (E), whereas extensive fibrosis (arrowheads) is observed in cardiac wounds of *mps1* mutants (F). Scale bars, 100 μm.

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Materials and Methods
Figs. S1 to S4

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