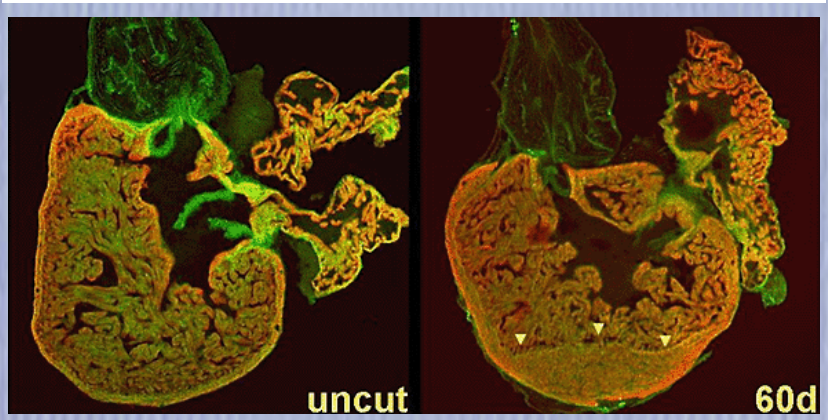
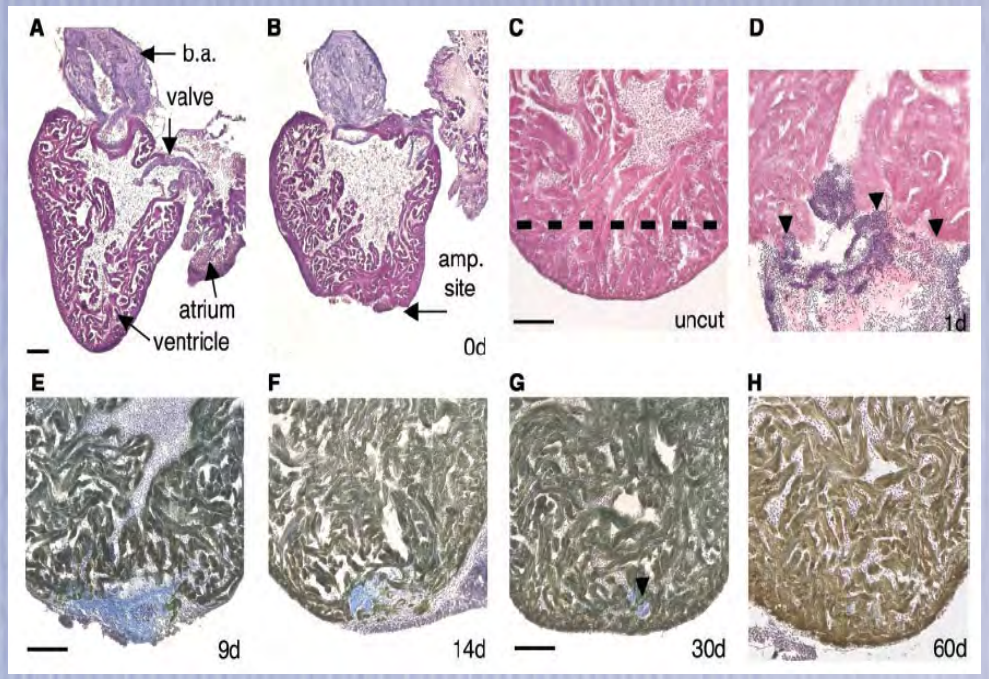
Heart Regeneration in Zebrafish

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.



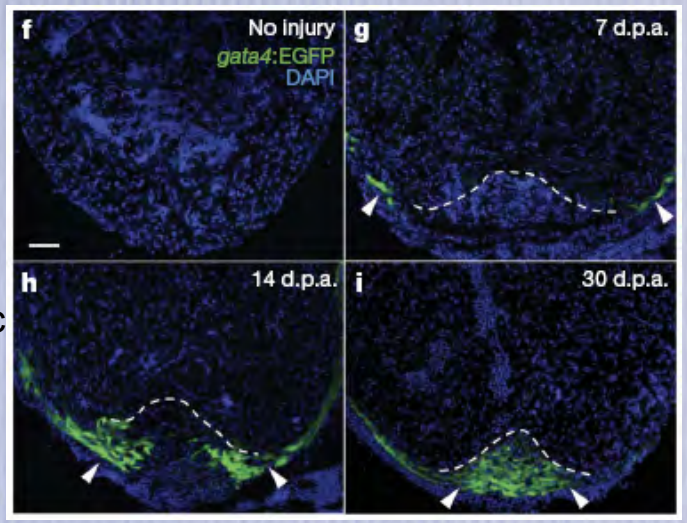


SCIENCE VOL 298 13 DECEMBER 2002

Primary contribution to zebrafish heart regeneration by *gata4*$^{+}$ cardiomyocytes

Kazu Kikuchi[1,2], Jennifer E. Holdway[1,2], Andreas A. Werdich[4], Ryan M. Anderson[5], Yi Fang[1,2], Gregory F. Egnaczyk[1,2,3], Todd Evans[6], Calum A. MacRae[4], Didier Y. R. Stainier[5] & Kenneth D. Poss[1,2]

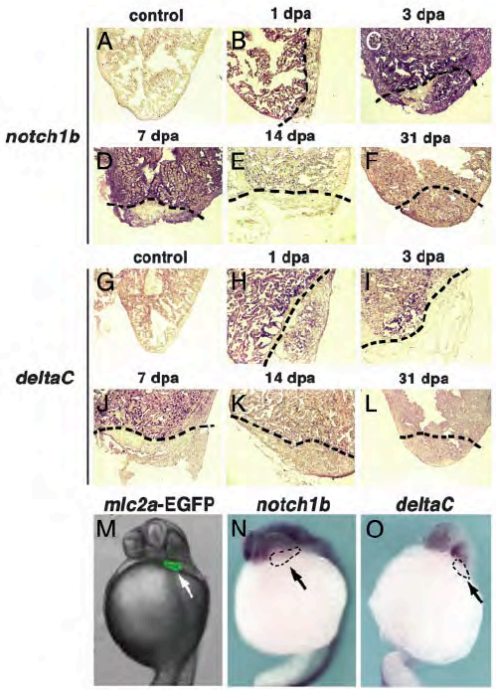
Cardiomyocytes are the source of the regenerating tissues and expressed a marker of regeneration called *gata4*—a transcription fac involved in normal development of the heart



NATURE | Vol 464 | 25 March 2010

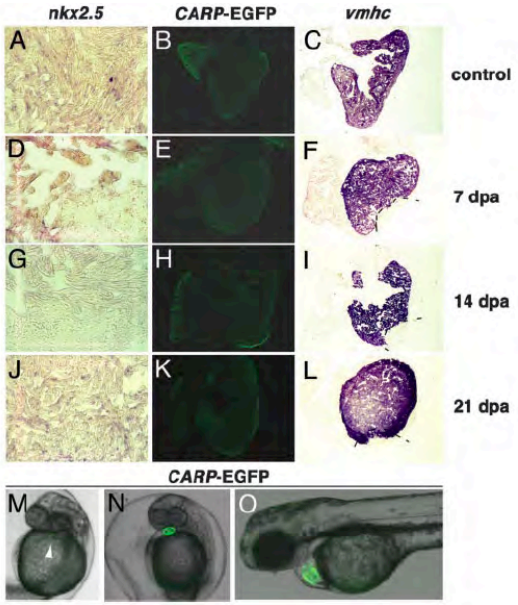
Activation of Notch signaling pathway precedes heart regeneration in zebrafish

Ángel Raya*†, Christopher M. Koth*†, Dirk Büscher*†, Yasuhiko Kawakami*†, Tohru Itoh*†, R. Marina Raya*, Gabriel Sternik*, Huai-Jen Tsai‡, Concepción Rodríguez-Esteban*, and Juan Carlos Izpisúa-Belmonte*§



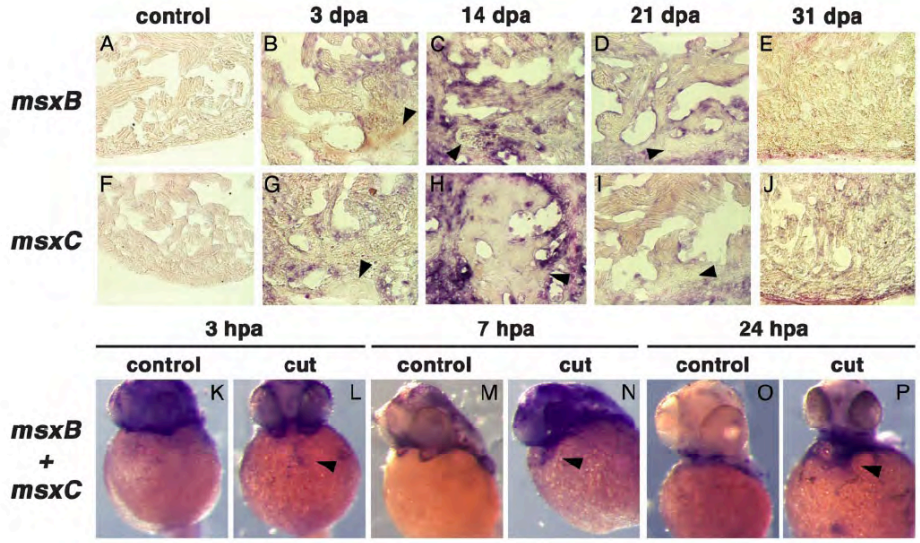
notch1b and ***deltaC*** are up-regulated during heart regeneration but not in the developing heart

Heart regeneration in zebrafish is accompanied by up-regulation of components of the Notch pathway, followed by members of the Msx family. These genes are not expressed during zebrafish heart development, indicating that **regeneration** involves the execution of a specific genetic program, rather than redeployment of a developmental program.



Markers of early cardiac development are not up-regulated during heart regeneration

msxC and msxB are expressed in the regenerating heart but not in 24- to 48-hpf embryos. However, both genes are expressed after removal of 50% of the developing heart



Many different hypothesis for the BrdU labeling results:

- First, differentiated, contracting CMs in existing myofibers could be stimulated to enter the cell cycle, divide, and reform the apex.
- Second, regeneration could proceed through the recruitment of undifferentiated progenitor cells that form new, proliferative CMs.
- A third conceivable mechanism for the origin of regenerative muscle is a chimera of these two mechanisms called “dedifferentiation”, in which existing muscle would downregulate contractile genes toward creation of undifferentiated or poorly differentiated cells.

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LETTERS

Zebrafish heart regeneration occurs by cardiomyocyte dedifferentiation and proliferation

Chris Jopling[1], Eduard Sleep[1,2]†, Marina Raya[1]†, Mercè Martí[1], Angel Raya[1,2,3]† & Juan Carlos Izpisúa Belmonte[1,2,4]

Although mammalian hearts show almost no ability to regenerate, there is a growing initiative to determine whether existing cardiomyocytes or progenitor cells can be coaxed into eliciting a regenerative response. In contrast to mammals, several non-mammalian vertebrate species are able to regenerate their hearts[1–3], including the zebrafish[4,5], which can fully regenerate its heart after amputation of up to 20% of the ventricle. To address directly the source of newly formed cardiomyocytes during zebrafish heart regeneration, we first established a genetic strategy to trace the lineage of cardiomyocytes in the adult fish, on the basis of the Cre/*lox* system widely used in the mouse[6]. Here we use this system to show that regenerated heart muscle cells are derived from the proliferation of differentiated cardiomyocytes. Furthermore, we show that proliferating cardiomyocytes undergo limited dedifferentiation characterized by the disassembly of their sarcomeric structure, detachment from one another and the expression of regulators of cell-cycle progression. Specifically, we show that the gene product of *polo-like kinase 1* (*plk1*) is an essential component of cardiomyocyte proliferation during heart regeneration. Our data provide the first direct evidence for the source of proliferating cardiomyocytes during zebrafish heart regeneration and indicate that stem or progenitor cells are not significantly involved in this process.

Regenerated cardiomyocytes are derived from differentiated, pre-existing cardiomyocytes

has been regenerated by cardiomyocytes. The exact source of these new cardiomyocytes is not yet known definitively. To address this question we developed and successfully implemented the 4-hydroxy-tamoxifen (4-OHT)-inducible Cre/*lox* approach in zebrafish to label regenerating cardiomyocytes genetically (for a detailed description of the lines generated and/or methodologies, see Methods and Supplementary Figs 1–9).

genetically labelled 48 h after fertilization. About 20% of the ventricle was removed, and cardiac regeneration was subsequently assessed at 7, 14 and 30 days after amputation. At 7 days after amputation, the remaining cardiac tissue was uniformly positive for green fluorescent protein (GFP) (Fig. 1a, b), with much of the missing tissue now replaced by a fibrin/collagen clot ($n = 5$ hearts) (Fig. 1c). At 14 days

($n = 7$ hearts; Fig. 1f). These results suggest that the regenerated cardiomyocytes arise from differentiated GFP-positive cardiomyocytes. These findings were substantiated at 30 days after amputation, when regeneration is nearly complete; all of the cardiomyocytes within the

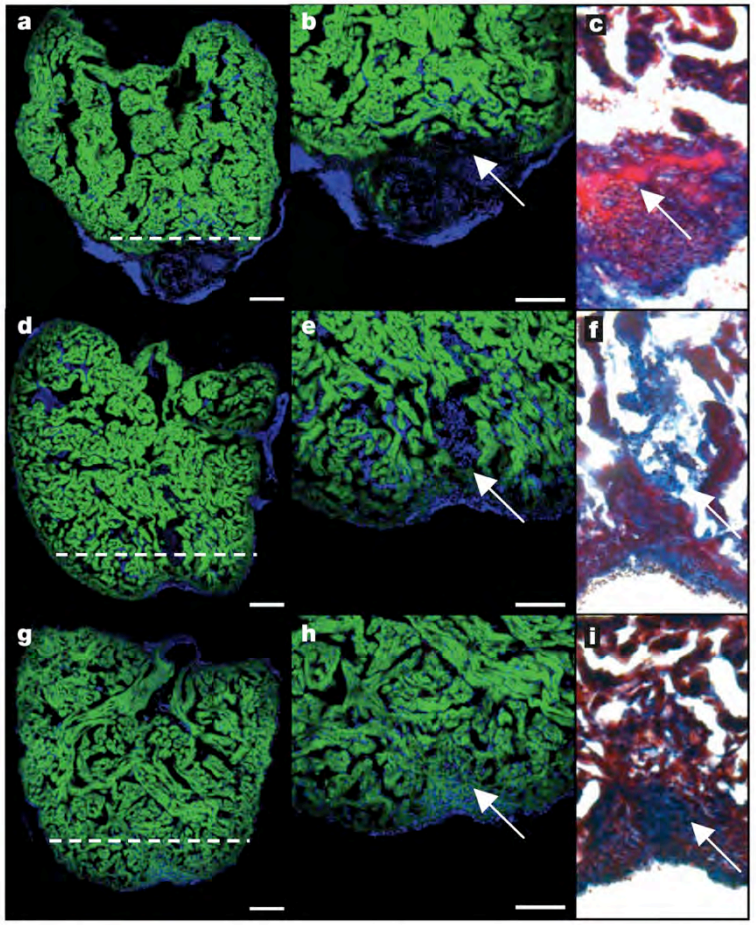

Figure 1 | Regenerated cardiomyocytes are derived from differentiated cardiomyocytes. Cardiomyocytes in transgenic zebrafish (tg-cmlc2a-Cre-Ert2: tg-cmlc2a-LnL–GFP) were genetically labelled at 48 h after fertilization by inducing Cre activity with tamoxifen. These embryos were then grown to adulthood (3 months or sexually mature), at which point the heart was amputated and allowed to regenerate for 7 (**a**–**c**), 14 (**d**–**f**) or 30 (**g**–**i**) days. The dashed white line represents the plane of amputation. At 7 days after amputation (**a**; enlargement in **b**) relatively little regeneration has occurred. Trichromic staining indicates that a fibrin clot has formed adjacent to the wound (**c**). By 14 days after amputation, GFP-positive cardiomyocytes have regenerated a substantial amount of new cardiac tissue (**d**; enlargement in **e**) and the fibrin clot was decreased in size (**f**). At 30 days after amputation, heart regeneration is virtually complete (**g**; enlargement in **h**) and all of the regenerated tissue is composed of GFP-positive cardiomyocytes. The clot has been replaced by a small scar (**h**). Scale bars, 100 μm (**a**, **d**, **g**) and 75 μm (**b**, **e**, **h**). Panels **c**, **f** and **i** are ×2 magnifications of the areas indicated with a white arrow in **b**, **e** and **h**.

Regenerating cardiomyocyte partially disassemble the contractile apparatus but not revert to an embryonic stage

lineage they regress[7,8]. An increase in the expression of the cardiac-progenitor-associated genes *nkx2.5* and *hand2* during zebrafish heart regeneration has been reported[9]. However, our own *in situ* hybridization analyses failed to detect any significant upregulation of either transcript (data not shown), confirming previous results from our laboratory[5]. Furthermore, genome-wide transcriptome data[10,11] also failed to detect significant changes in the expression of either transcript during zebrafish heart regeneration. These results argue against an extensive dedifferentiation of cardiomyocytes as a prerequisite for their proliferation in the context of heart regeneration.

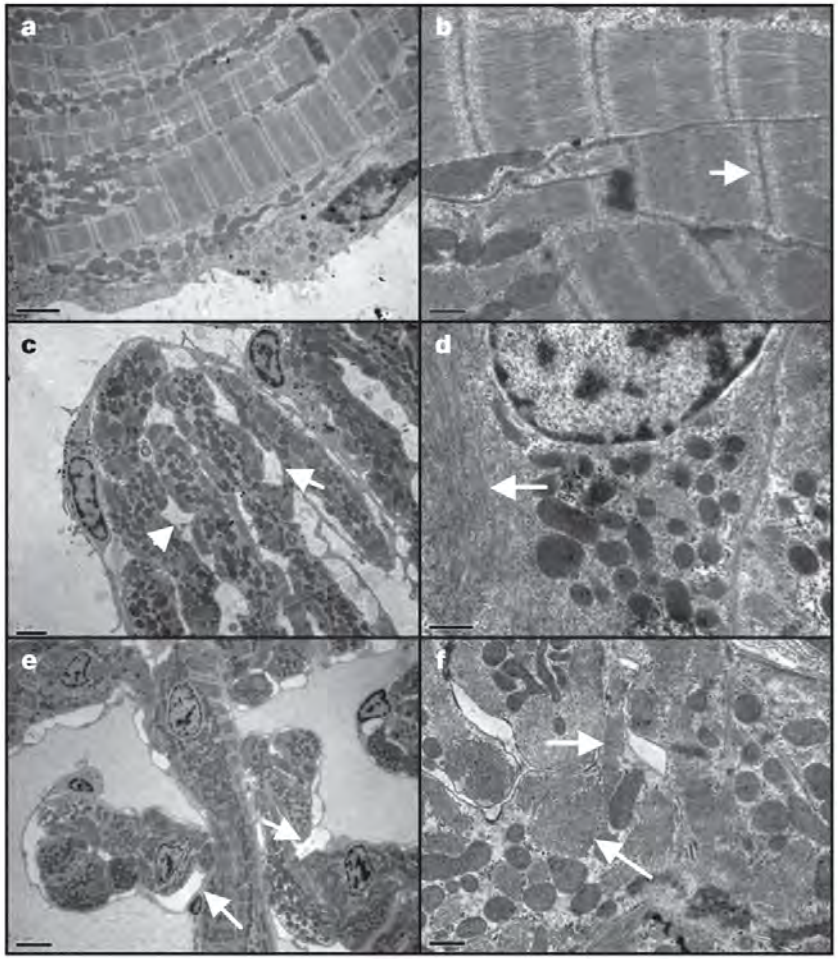

Figure 3 | Cardiomyocytes dedifferentiate, resulting in the disassembly of sarcomeric structure and detachment. Electron microscopy of sections of a control heart (**a**, **b**) and a regenerating heart at 5 days (**c**, **d**) and 7 days (**e**, **f**) after amputation. Cardiomyocytes in unamputated control samples show a tightly organized sarcomeric structure (**a**); at higher magnification (**b**) the Z-lines are clearly visible (arrow). At 5 days after amputation many of the cardiomyocytes have a disorganized sarcomeric structure (**c**) along with the appearance of intercellular spaces (arrows). Closer examination reveals a loss of Z-lines (**d**, arrow). At 7 days after amputation there is a similar loss of structure and appearance of intercellular spaces (**e**, arrows). At higher magnification (**f**) myosin fibres are visible (arrows); however, both longitudinal (upper arrow) and transverse (lower arrow) fibres are present within the same cardiomyocyte, indicating disorganized sarcomeric structure. Scale bars, 0.5 μm (**a**, **b**, **d**) and 2 μm (**c**, **e**, **f**).

546 Cell Reports 26, 546–554, January 15, 2019 © 2018 The Author(s).

Endocardial Notch Signaling Promotes Cardiomyocyte Proliferation in the Regenerating Zebrafish Heart through Wnt Pathway Antagonism

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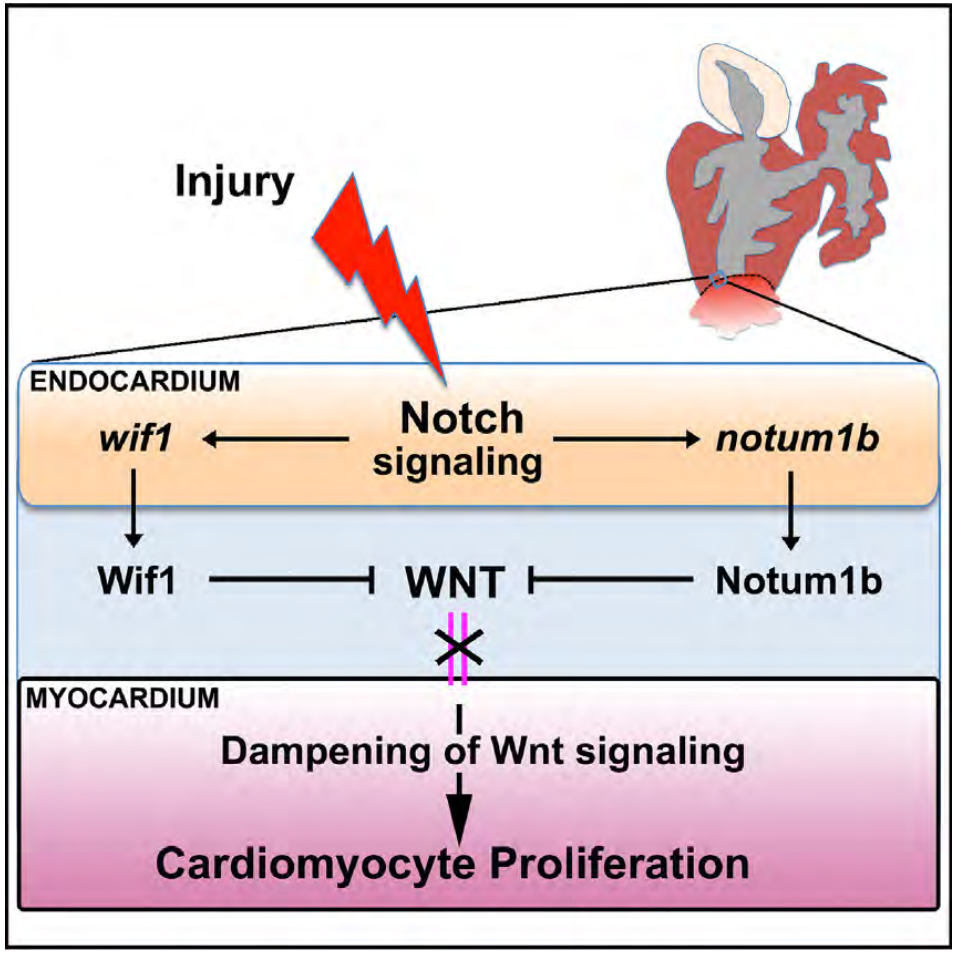
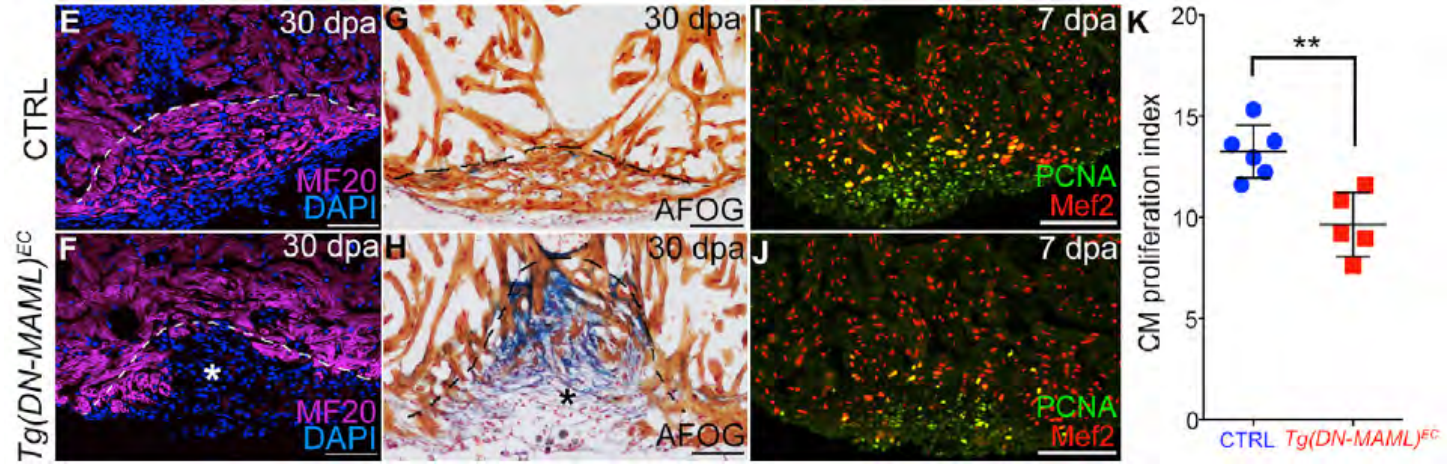
- Notch signaling supports cardiomyocyte proliferation by dampening myocardial Wnt activity during zebrafish heart regeneration

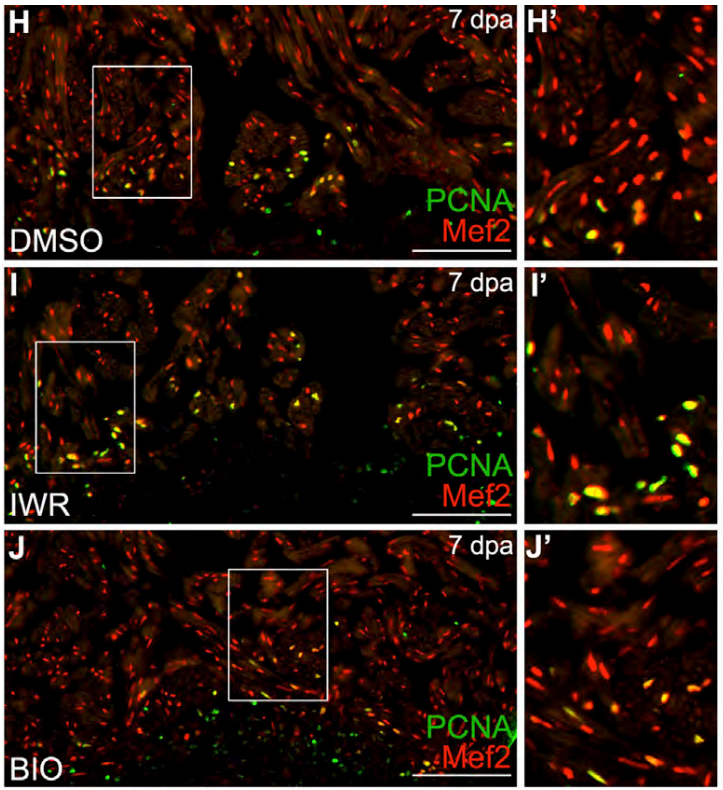
Notch receptors are upregulated in the endocardium (Munch et al., 2017; Raya et al., 2003; Zhao et al., 2014) and epicardium (Zhao et al., 2014) following ventricular apex amputation (Raya et al., 2003; Zhao et al., 2014) or cryoinjury (Munch et al., 2017). In both models, global suppression of Notch signaling impedes cardiomyocyte proliferation and induces scarring.

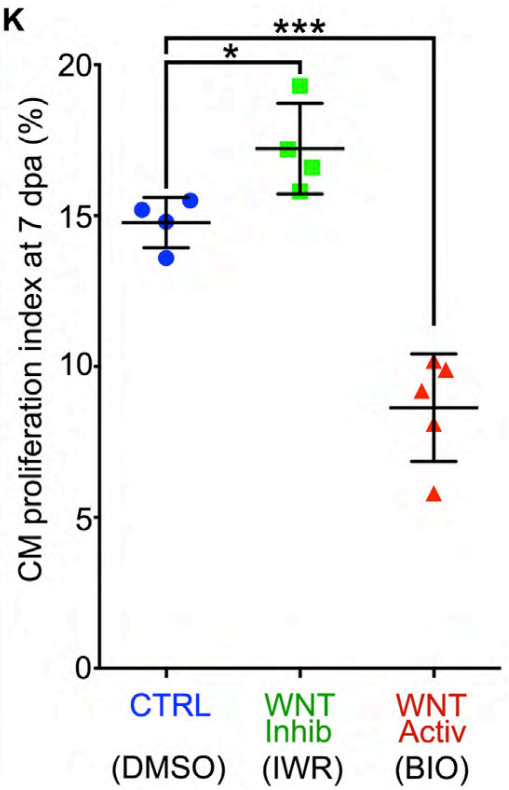
Endocardial-specific Notch inhibition dampens cardiomyocyte proliferation and leads to regenerative failures following apex amputation. Furthermore, we learned that the secreted Wnt antagonists, Wif1 and Notum1b, are significantly downregulated in Notch-suppressed hearts, suggesting that Notch-mediated Wnt pathway suppression is required to enable cardiomyocyte renewal.

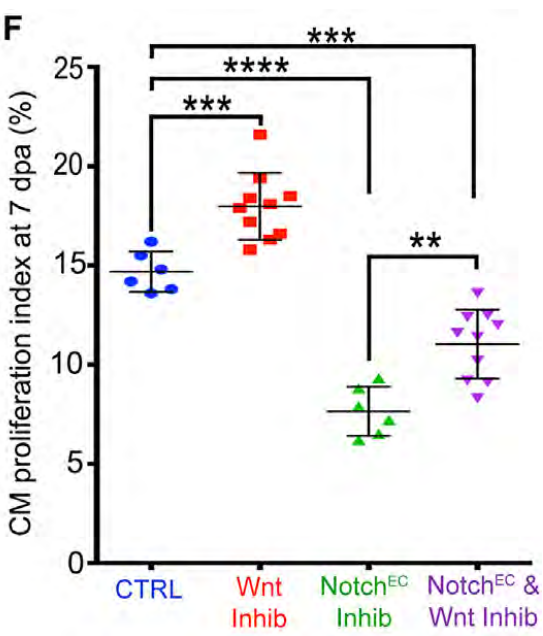




Notch inhibition dampens cardiomyocyte proliferation







Hyperactivation of Wnt signaling dampened cardiomyocyte proliferation and blocked heart regeneration.

Wnt pathway inhibition partially rescued the myocardial proliferation deficit of endocardial-specific Notch-suppressed hearts.

What about mammals?

LETTER

doi:10.1038/nature11682

Mammalian heart renewal by pre-existing cardiomyocytes

Samuel E. Senyo[1], Matthew L. Steinhauser[1], Christie L. Pizzimenti[1], Vicky K. Yang[1], Lei Cai[1], Mei Wang[4,5], Ting-Di Wu[2,3], Jean-Luc Guerquin-Kern[2,3], Claude P. Lechene[4,5] & Richard T. Lee[1,6]

Although recent studies have revealed that heart cells are generated in adult mammals, the frequency of generation and the source of new heart cells are not yet known. Some studies suggest a high rate of stem cell activity with differentiation of progenitors to cardiomyocytes[1]. Other studies suggest that new cardiomyocytes are born at a very low rate[2–4], and that they may be derived from the division of pre-existing cardiomyocytes. Here we show, by combining two different pulse–chase approaches—genetic fate-mapping with stable isotope labelling, and multi-isotope imaging mass spectrometry—that the genesis of cardiomyocytes occurs at a low rate by the division of pre-existing cardiomyocytes during normal ageing, a process that increases adjacent to areas of myocardial injury. We found that cell cycle activity during normal ageing and after injury led to polyploidy and multinucleation, but also to new diploid, mononucleate cardiomyocytes. These data reveal pre-existing cardiomyocytes as the dominant source of cardiomyocyte replacement in normal mammalian myocardial homeostasis as well as after myocardial injury.

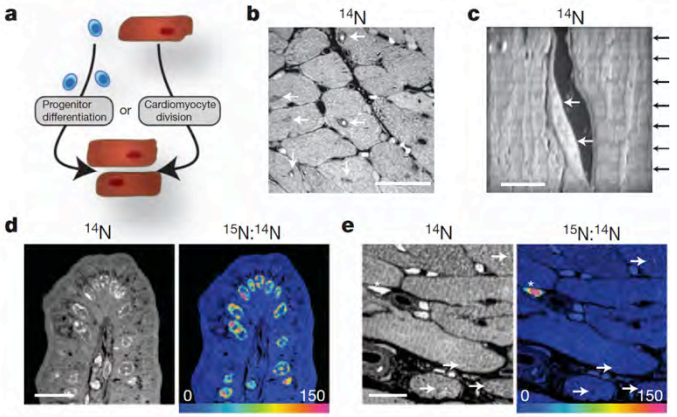


Figure 1 | Use of MIMS to study cardiomyocyte turnover. **a**, Primary question: are new cardiomyocytes derived from progenitors or from pre-existing cardiomyocytes? **b**, ^{14}N mass image. Subcellular details are evident, including cardiomyocyte nuclei (white arrows). Scale bar, 20 μm. **c**, MIMS resolves periodic sarcomeres (black arrows) in cardiomyocytes. Non-cardiomyocytes (white arrows) are seen outside cardiomyocyte borders. Scale bar, 5 μm. **d**, Right, ^{15}N:^{14}N hue–saturation–intensity image of small-intestinal epithelium after labelling with [^{15}N]thymidine. The scale ranges from blue, where the ratio is equivalent to natural ratio (0.37%, expressed as 0% above natural ratio (enrichment over natural ratio)), to red, where the ratio is 150% above natural ratio. ^{15}N labelling is concentrated in nuclei in a pattern resembling chromatin. Scale bar, 15 μm. **e**, Right, ^{15}N:^{14}N hue–saturation–intensity image of heart section (left ventricle). [^{15}N]Thymidine was administered for 1 week. Asterisk, rare $^{15}N^{+}$ interstitial cells. Cardiomyocyte nuclei (white arrows) are unlabelled. Scale bar, 15 μm.

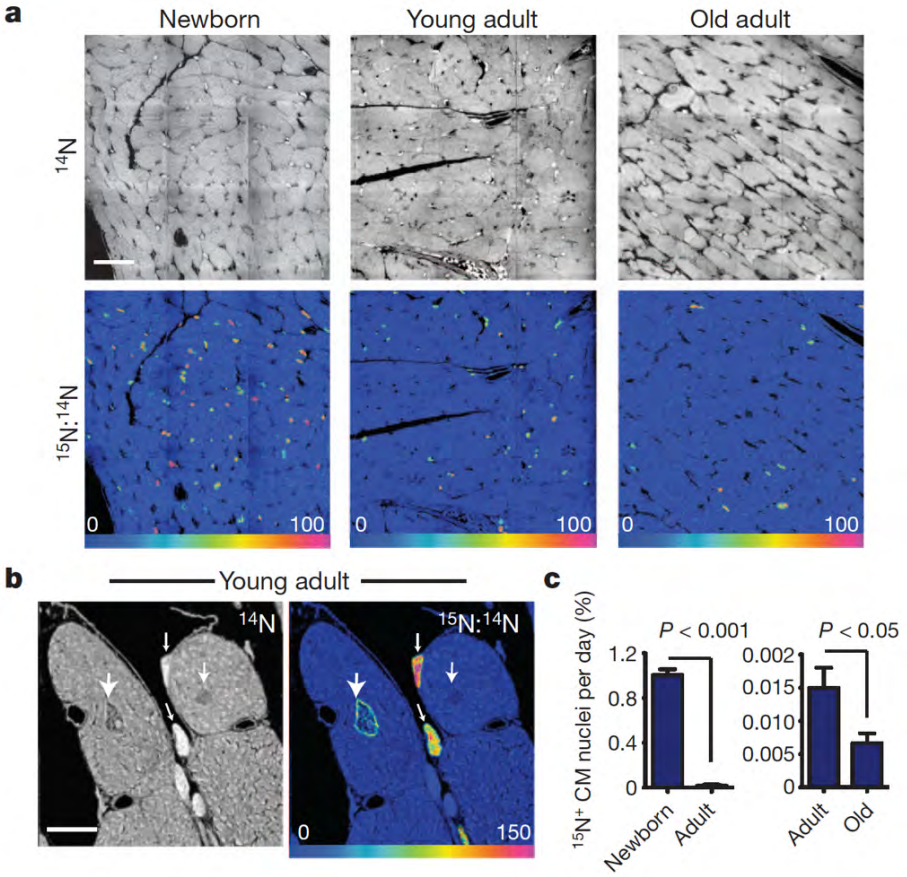


Figure 2 | Cardiomyocyte DNA synthesis decreases with age. **a**, [^{15}N]Thymidine was administered for 8 weeks to mice of different ages: newborn, starting at postnatal day 4; young adult, starting at 2 months; old adult, starting at 22 months. Top, ^{14}N mass images show histological details. Bottom, ^{15}N:^{14}N hue–saturation–intensity images show ^{15}N^{+} nuclei. Mosaics are constructed from nine tiles, 60 μm each. Scale bar, 30 μm. **b**, High-magnification analysis shows a cardiomyocyte from the young adult with nuclear ^{15}N labelling (large arrow), two labelled non-cardiomyocytes (small arrows) and an adjacent unlabelled cardiomyocyte nucleus (medium arrow). Scale bar, 10 μm. **c**, Age-related decline in cardiomyocyte DNA synthesis. Left, comparison of newborn with young adult. Right, scale reduced to compare young adult with old adult ($n = 3$ mice per group). Error bars indicate s.e.m.

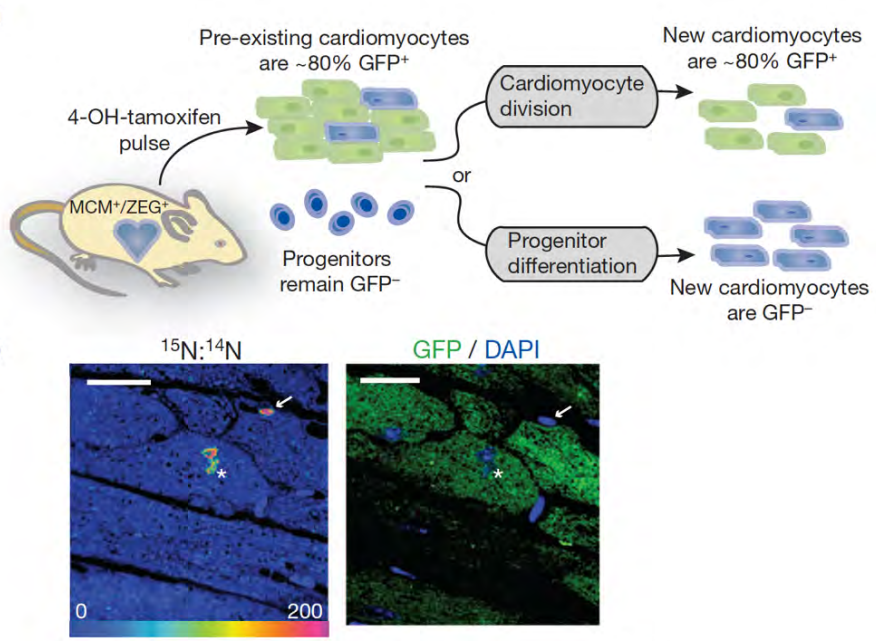
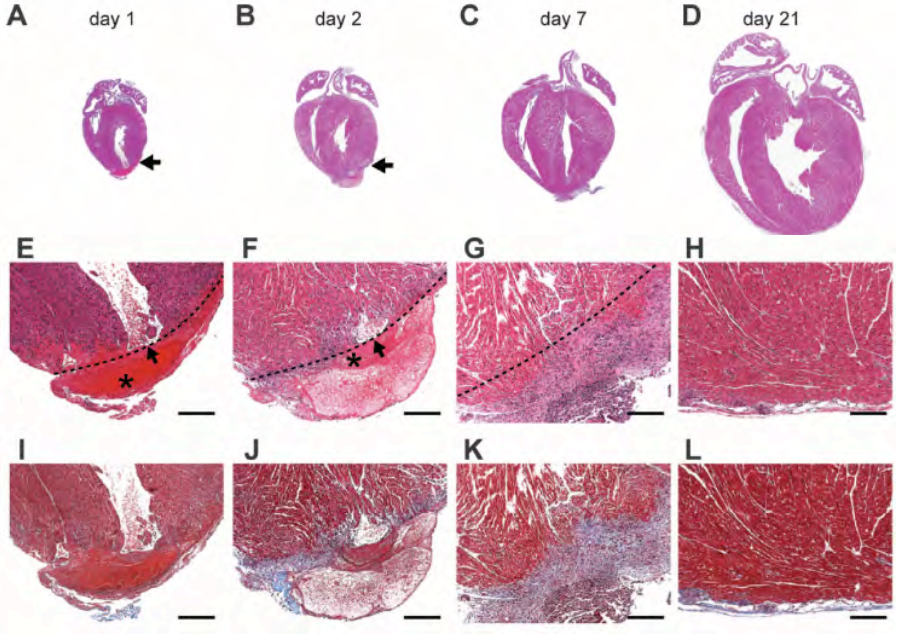


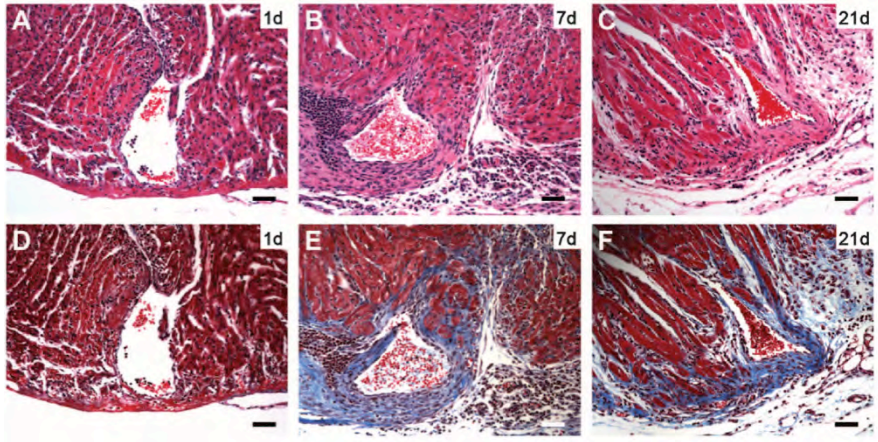
Figure 3 | New cardiomyocytes are derived from pre-existing cardiomyocytes during ageing. **a**, Experimental strategy. MerCreMer^{+}/ZEG^{+} (MCM^{+}ZEG^{+}) mice ($n = 4$) were treated for 2 weeks with 4-OH-tamoxifen to induce cardiomyocyte-specific GFP expression. [^{15}N]Thymidine was administered continuously during a 10-week chase, then cycling cells were identified by ^{15}N labelling. New cardiomyocytes (^{15}N^{+}) derived from pre-existing cardiomyocytes should express GFP at a rate similar to that of the surrounding quiescent (^{15}N^{-}) cardiomyocytes. New cardiomyocytes (^{15}N^{+}) derived from progenitors should be GFP^{-}. **b**, Left, ^{15}N:^{14}N hue–saturation–intensity image showing a [^{15}N]thymidine-labelled cardiomyocyte nucleus (white asterisk) and a ^{15}N^{+} non-cardiomyocyte (white arrow). Right, immunofluorescent image showing that the ^{15}N^{+} cardiomyocyte is GFP^{+}. Scale bars, 15 μm.

25 FEBRUARY 2011 VOL 331

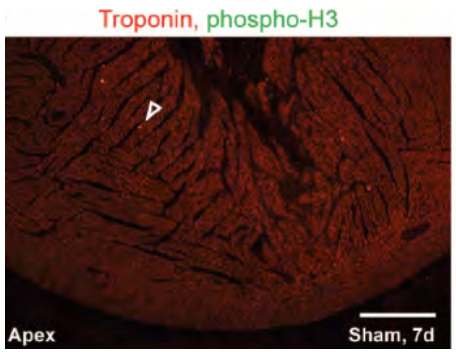
Transient Regenerative Potential of the Neonatal Mouse Heart

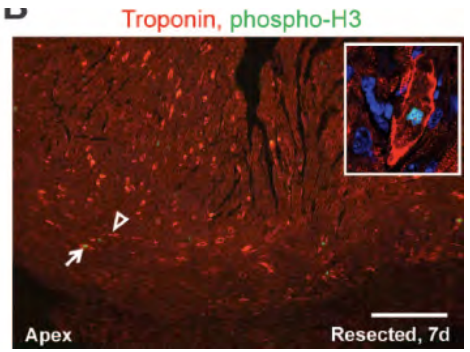
Enzo R. Porrello,[1] Ahmed I. Mahmoud,[2] Emma Simpson,[3] Joseph A. Hill,[1,2] James A. Richardson,[1,3] Eric N. Olson,[1]* Hesham A. Sadek[2]*





- Hearts of 1-day-old neonatal mice can regenerate after partial surgical resection, but this capacity is lost by 7 days of age.
- The regenerative response was characterized by cardiomyocyte proliferation with minimal hypertrophy or fibrosis.
- The majority of cardiomyocytes within the regenerated tissue originated from preexisting cardiomyocytes.





NATURE PROTOCOLS | VOL.9 NO.2 | 2014 | PROTOCOL

Surgical models for cardiac regeneration in neonatal mice

Ahmed I Mahmoud[1], Enzo R Porrello[2], Wataru Kimura[3], Eric N Olson[4] & Hesham A Sadek[3]

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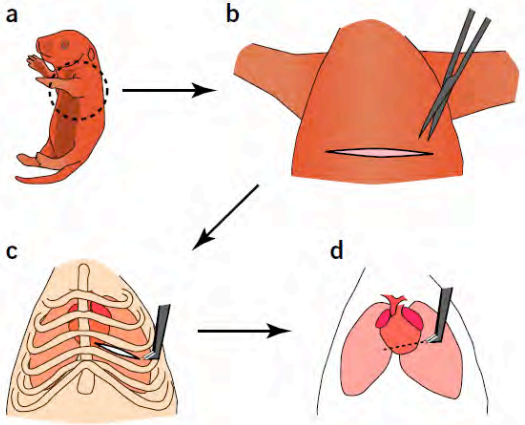
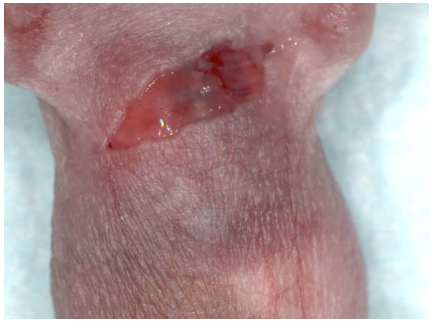
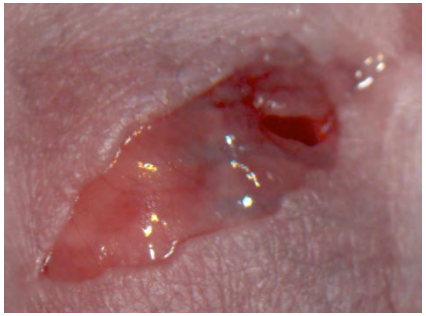
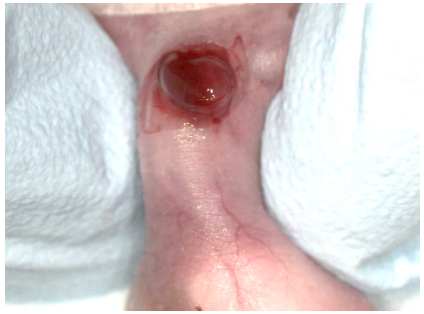


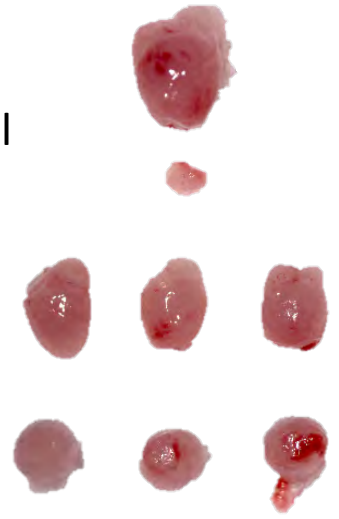
(Mahmoud et al., 2014)

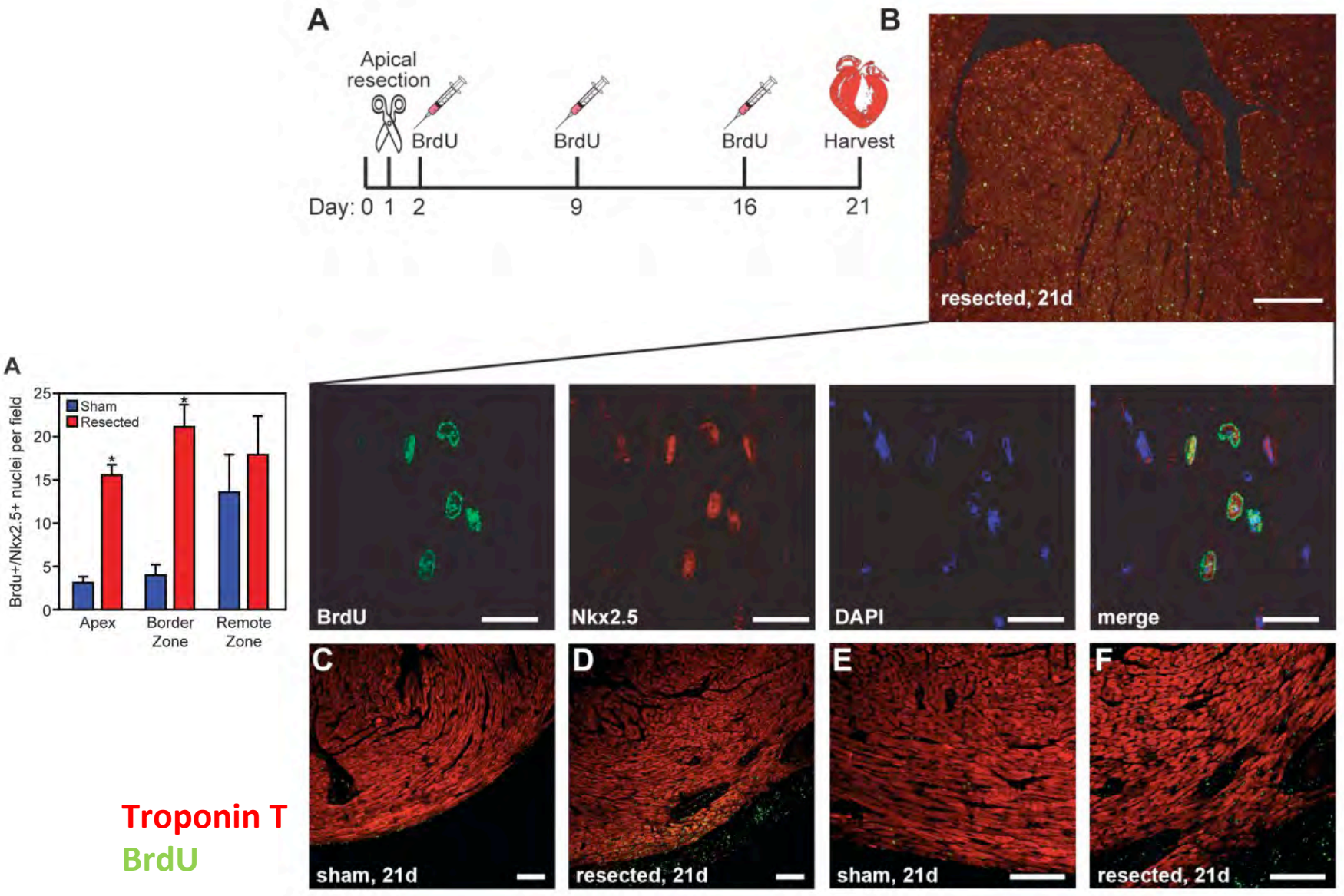
Apical resection surgical model







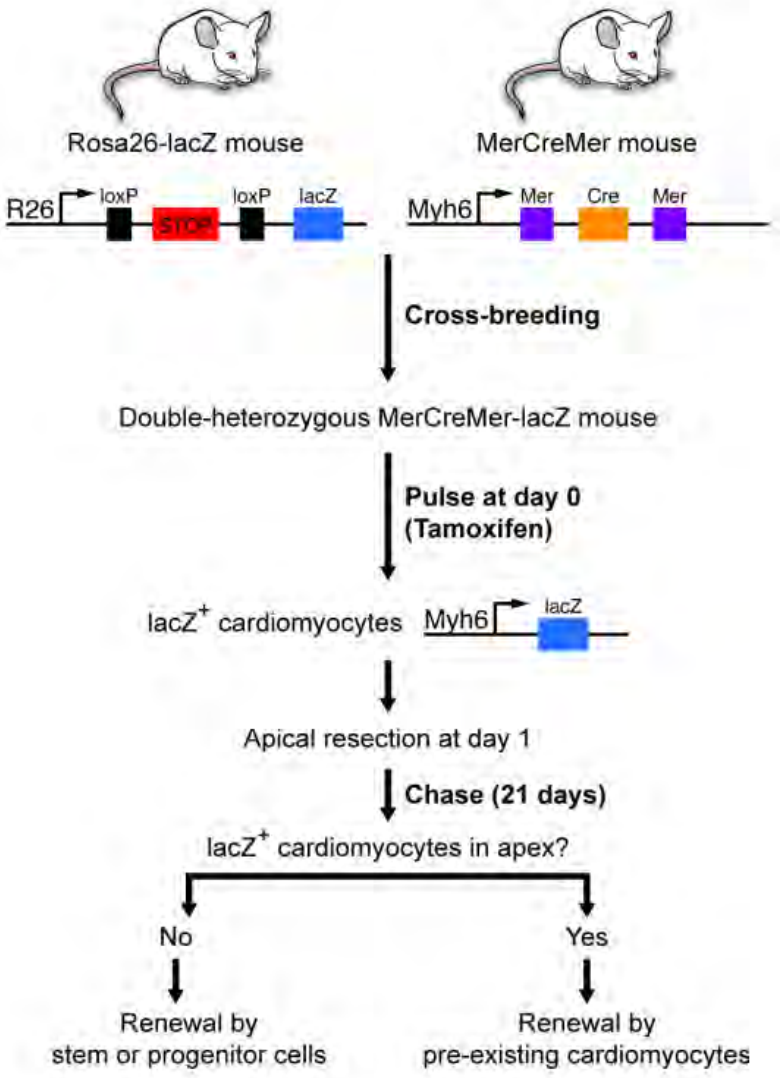


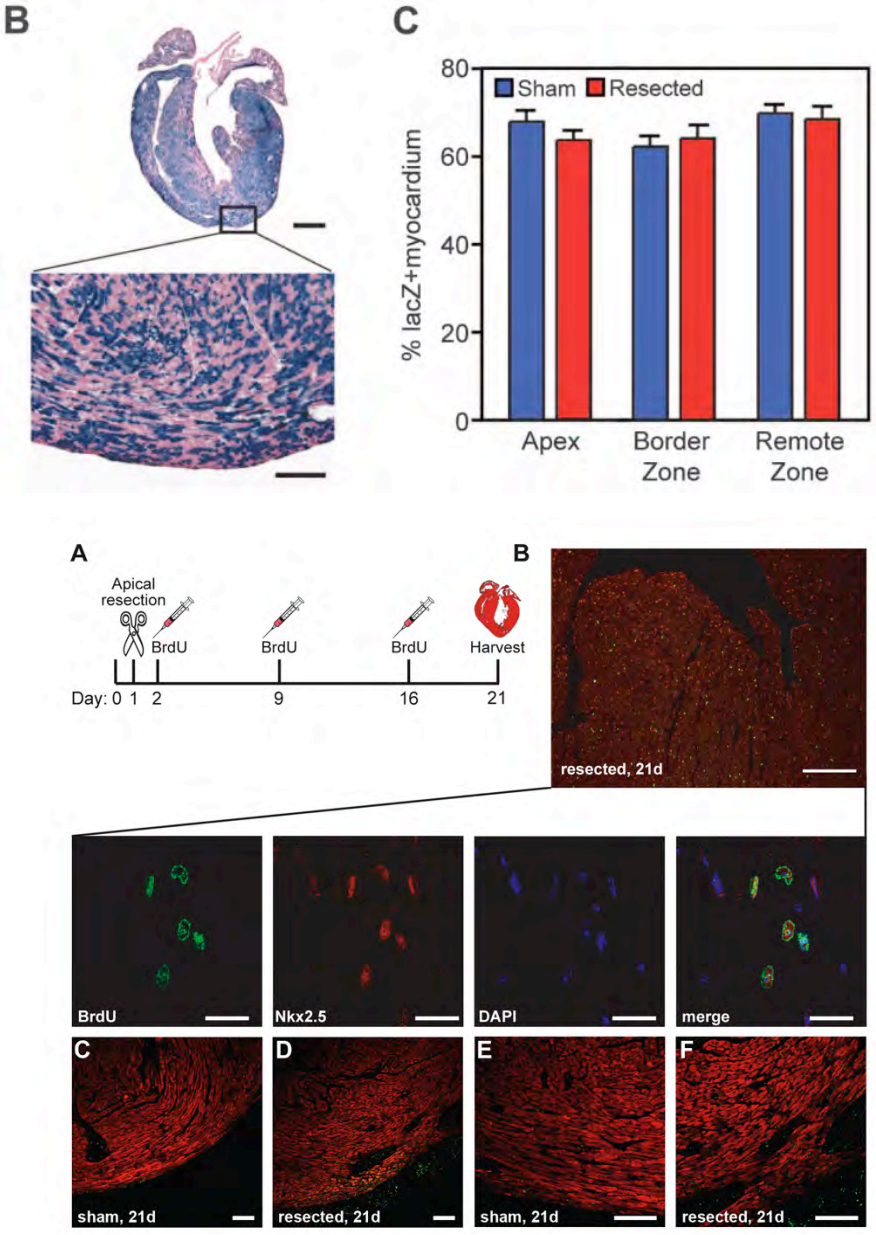


Newly formed cardiomyocytes

(Porrello et al., 2011)

Which cells do they derive from ?





(Porrello et al., 2011)

Stem Cell Research (2014) 13, 556–570

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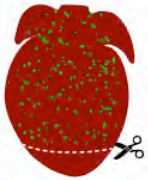
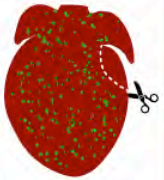
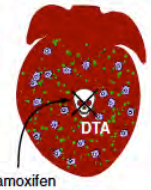
REVIEW

A neonatal blueprint for cardiac regeneration

Enzo R. Porrello[a], Eric N. Olson[b,*]

Newt 	**Apical Resection** 	**Basal Resection** 
Regeneration:	Yes (incomplete)	Yes (complete)
CM Proliferation:	Yes (global)	Yes (global)
Genetic Lineage:	N/A	N/A
Zebrafish 	**Apical Resection** 	**Genetic Ablation** 
Regeneration:	Yes	Yes
CM Proliferation:	Yes (sub-epicardial + localized)	Yes
Genetic Lineage:	Pre-existing CMS	Pre-existing CMS
Neonatal Mouse 	**Apical Resection** 	**Myocardial Infarction** 
Regeneration:	Yes	Yes
CM Proliferation:	Yes (global)	Yes (global)
Genetic Lineage:	Pre-existing CMS	Pre-existing CMS

ESC European Society of Cardiology

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