Development 140, 660-666 (2013) doi:10.1242/dev.088526

In vivo monitoring of cardiomyocyte proliferation to identify chemical modifiers of heart regeneration

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SUMMARY

Adult mammalian cardiomyocytes have little capacity to proliferate in response to injury, a deficiency that underlies the poor regenerative ability of human hearts after myocardial infarction. By contrast, zebrafish regenerate heart muscle after trauma by inducing proliferation of spared cardiomyocytes, providing a model for identifying manipulations that block or enhance these events. Although direct genetic or chemical screens of heart regeneration in adult zebrafish present several challenges, zebrafish embryos are ideal for high-throughput screening. Here, to visualize cardiomyocyte proliferation events in live zebrafish embryos, we generated transgenic zebrafish lines that employ fluorescent ubiquitylation-based cell cycle indicator (FUCCI) technology. We then performed a chemical screen and identified several small molecules that increase or reduce cardiomyocyte proliferation during heart development. These compounds act via Hedgehog, Insulin-like growth factor or Transforming growth factor β signaling pathways. Direct examination of heart regeneration after mechanical or genetic ablation injuries indicated that these pathways are activated in regenerating cardiomyocytes and that they can be pharmacologically manipulated to inhibit or enhance cardiomyocyte proliferation during adult heart regeneration. Our findings describe a new screening system that identifies molecules and pathways with the potential to modify heart regeneration.

KEY WORDS: Heart regeneration, Chemical screen, Cardiomyocyte, Cell proliferation, Epicardium, Endocardium, Zebrafish

INTRODUCTION

A fundamental cellular block to heart regeneration in adult mammals is the low capacity of mature mammalian cardiomyocytes to undergo proliferation after injury. Several studies support the notion that the endogenous proliferative capacity of cardiomyocytes is subject to enhancement in ways that enable new muscle regeneration. A recent study using radiocarbon-based tracing methods suggested that human cardiomyocytes retain some proliferative capacity through adulthood (Bergmann et al., 2009). Additionally, fetal and neonatal mice regenerate after cardiac injury through cardiomyocyte proliferation (Drenckhahn et al., 2008; Porrello et al., 2011). Finally, adult heart regeneration occurs robustly in certain non-mammalian vertebrates, most prominently in zebrafish, by proliferation of existing cardiomyocytes (Poss et al., 2002; Jopling et al., 2010; Kikuchi et al., 2010).

Signaling pathways that participate in zebrafish heart regeneration have been identified by candidate approaches, including the Fibroblast growth factor (Fgf), Platelet-derived growth factor and retinoic acid (RA) pathways (Lepilina et al., 2006; Kim et al., 2010; Kikuchi et al., 2011). However, none has been manipulated in a manner that enhances cardiomyocyte proliferation after injury. Moreover, a well-known strength of the zebrafish model system is the opportunity for mutagenesis or pharmacological screens (Driever et al., 1996; Haffter et al., 1996; Kaufman et al., 2009; Peterson and Fishman, 2011). However, owing to the lengthy and involved nature of both cardiac injury and detection methods in adult animals, no *in vivo* screening approach has been developed with which to dissect zebrafish heart regeneration. Here, we pursued a surrogate model for heart regeneration that utilizes the screening advantages of zebrafish embryos: small size, rapid development, and transparency. Our study defines new factors and a new approach to understanding, and potentially guiding, heart regeneration.

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Accepted 23 November 2012

MATERIALS AND METHODS

Zebrafish and injury models

Outbred EK strain zebrafish (4-12 months of age) were used for ventricular resection surgeries as described previously (Poss et al., 2002). Cardiomyocyte ablation experiments were performed in Z-CAT zebrafish bathed for 12 hours in 0.1 μM 4-hydroxytamoxifen, as described (Wang et al., 2011). Published transgenic lines used in this study were *shh*:EGFP (Shkumatava et al., 2004; Wills et al., 2008), *cmlc2:CreER* (Kikuchi et al., 2010), *bactin2:loxp-mCherry-STOP-loxp-DTA* (Wang et al., 2011) and *cmlc2:nuc-DsRed2* (Mably et al., 2003).

Constructs containing mCherry-zCdt1 or Venus-hGeminin (Sugiyama et al., 2009) were cloned behind the 5.1 kb *cmlc2* (*myl7* – Zebrafish Information Network) promoter (Rottbauer et al., 2002). The cassette contained I-*Sce*I sites and was co-injected with I-*Sce*I into one-cell-stage embryos. F1 fish containing each transgene were crossed to generate double transgenic *cmlc2:FUCCI* fish, which were incrossed to generate animals homozygous for both transgenes. These double homozygous transgenics were crossed to outbred EK or AB fish to produce double hemizygous embryos for experiments. The full names for the two transgenic lines used in the FUCCI system are *Tg(cmlc2:mCherry-zCdt1)*^{pd57} and *Tg(cmlc2:Venus-hGeminin)*^{pd58}.

The Hh signaling reporter line contains a transgene expressing enhanced green fluorescent protein (EGFP) under the control of a 900 bp *ptch2* promoter fragment, with an additional Gli transcription factor binding site (gbs) (GACCACCCA) (Sasaki et al., 1997) engineered into the 5′ end. The full name for this line is *Tg(GBS-ptch2:EGFP)*^{umz23} (M.-C.S., unpublished data).

Drug treatments

SAG (EMD Chemicals) was either purchased in solution or dissolved in water to a 10 mM stock solution. NBI-31772 (Calbiochem) was dissolved in DMSO for a 30 mM stock solution. SU5402 (Santa Cruz Biotechnology), γ-secretase inhibitor (Calbiochem), SB-431542 (Selleckchem), RA (Sigma-Aldrich) and DEAB (Sigma-Aldrich) were dissolved in DMSO to a final concentration of 10 mM. CyA (Toronto Research Chemicals), NVP-AEW541 (Cayman Chemical), and GSK-3 Inhibitor IX (BIO, Calbiochem) were dissolved in ethanol to stock solutions of 10 mM.

For embryo studies, *cmlc2:FUCCI* embryos were collected within a 30-minute window from male-female pairings. From 1 day post-fertilization (dpf) onwards, embryos were treated with n-phenylthiourea (PTU; Sigma-Aldrich) to block development of pigmentation. Drug treatment was performed at 3-4 dpf in 3 ml of buffered embryo water in 6-well plates. For adult studies, injured adult fish were treated in 20 ml of buffered fish water per animal in finger bowls. For embryos, all drugs were used at 5 µM except for NBI-31772, RA and BIO, which were used at 2.5 µM. In adults, SAG was used at 2.5 µM, NBI-31772 was used at 10 µM, CyA was used at 10 µM and NVP-AEW541 was used at 2 µM.

Histology

Initial screening for FUCCI signals was performed using a Leica M205 FA dissecting microscope. *z*-stacks were acquired from 50 µM-thick sections of paraformaldehyde (PFA)-fixed embryos using a Zeiss LSM 700 confocal microscope. Three-dimensional reconstructions were made using Imaris Version 7.3 software. Quantification of mCherry$^+$ and/or Venus$^+$ nuclei was also performed using surface analysis in Imaris software. Red$^+$green$^-$ and red$^+$green$^+$ nuclei were considered non-proliferating cells, and red$^-$green$^+$ nuclei were scored as proliferating. Quantification of total cardiomyocytes using the *cmlc2:nuc-DsRed2* line was performed similarly with single channel surface analysis using Imaris software. For EdU labeling, embryos were incubated in 500 µM 5-ethynyl-2′-deoxyuridine (EdU) for 3 hours at 28°C before fixation. Next, 25 µm-thick cryosections of the embryo were treated with reagent containing either 20 µM AlexaFluor-488 or 20 µM AlexaFluor-594 azide (Molecular Probes) for EdU detection. Images were acquired using a Zeiss LSM 700 confocal microscope.

In situ hybridization for *igf2b* and *tgfb3* were performed as described previously (Poss et al., 2002) using an InSituPro robot (Intavis). Digoxygenin-labeled cRNA probes were subcloned from embryonic zebrafish cDNA. The 0.6-kb *igf2b* probe was isolated using the primers 5′-CTCGAGGCCACCATGGAGGACCAACTAAAACA-3′ and 5′-GCGG-CCGCTCACTTGTGGCTAACGTAGT-3′. The 2.0-kb *tgfb3* probe was cloned using the primers 5′-ACCGGTGCCACCATGCATTTGGGC-AAAGGACT-3′ and 5′-GCGGCCGCGGTTTTGTCATTTTAATTAA-3′.

Immunofluorescence was performed as previously described (Kikuchi et al., 2011). *shh*:EGFP and *GBS-ptch2:EGFP* native fluorescence was enhanced using an antibody against GFP. Primary antibodies used in this study include anti-Mef2 (rabbit; Santa Cruz), anti-PCNA (mouse; Sigma), anti-Myosin heavy chain (F59, mouse; Developmental Studies Hybridoma Bank), anti-GFP (rabbit; Invitrogen) and anti-IGF-1Rβ (rabbit; Santa Cruz).

Cardiomyocyte proliferation was quantified from 10 µm-thick cryosections of PFA-fixed hearts as previously described, by counting Mef2$^+$ and PCNA$^+$ nuclei in injury sites (Kikuchi et al., 2011). In *cmlc2:FUCCI* adult hearts, cardiomyocytes in uninjured ventricles show little or no *cmlc2:*Venus-hGeminin fluorescence. Thus, all Venus-hGeminin$^+$ cardiomyocytes observed during regeneration were scored as proliferating (supplementary material Fig. S4).

RESULTS AND DISCUSSION

A transgenic system to visualize cardiomyocyte proliferation

To monitor proliferating cardiomyocytes in live zebrafish embryos, we adapted the fluorescent ubiquitylation-based cell cycle indicator (FUCCI) system, which employs two fusion proteins, mCherry-zCdt1 and Venus-hGeminin, which are expressed cyclically in the G1 and S/G2/M phases, respectively (Sakaue-Sawano et al., 2008; Sugiyama et al., 2009). Expression of these fusion proteins behind the *cardiac myosin light chain 2* (*cmlc2*) promoter in stable transgenic lines, *Tg(cmlc2:mCherry-zCdt1)*^{pd57}; *Tg(cmlc2:Venus-hGeminin)*^{pd58} (referred to hereafter as *cmlc2:FUCCI*), identified cardiomyocytes at different cell cycle stages (Fig. 1A). To test stage specificity, we assessed incorporation of 5-ethynyl-2′-deoxyuridine (EdU), a marker of DNA synthesis, in both lines. Whereas *cmlc2:*mCherry-zCdt1$^+$ cardiomyocytes did not incorporate EdU, a subset of *cmlc2:*Venus-hGeminin$^+$ cells had nuclear EdU staining (supplementary material Fig. S1). Visual examination of live *cmlc2:FUCCI* embryos indicated age-dependent differences in the number of proliferating (Venus-hGeminin$^+$) cardiomyocytes. We found that cardiomyocyte proliferation increased in frequency from

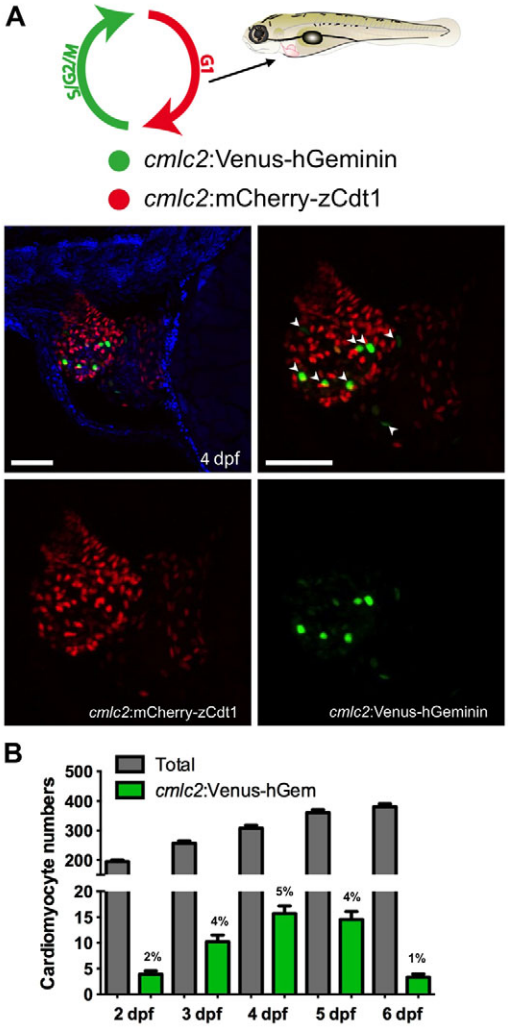

Fig. 1. FUCCI zebrafish for visualizing cardiomyocyte proliferation. (**A**) Representative maximum intensity projection of the heart of a 4 dpf *cmlc2:FUCCI* transgenic larva, visualizing non-proliferating (*cmlc2:*mCherry-zCdt1, red) and proliferating (*cmlc2:*Venus-hGeminin, green) cardiomyocytes, as depicted in the schematic at the top. Cardiomyocytes with only the Venus-hGeminin signal (arrowheads) were considered to be proliferating. DAPI, blue. Scale bars: 50 µm. (**B**) Time course of total (red$^+$ and green$^+$) and proliferating (green$^+$) cardiomyocytes from 2 to 6 dpf. Percentages of proliferating cardiomyocytes are indicated above bars. Data are represented as mean±s.e.m. *n*=13-24 embryos per stage.

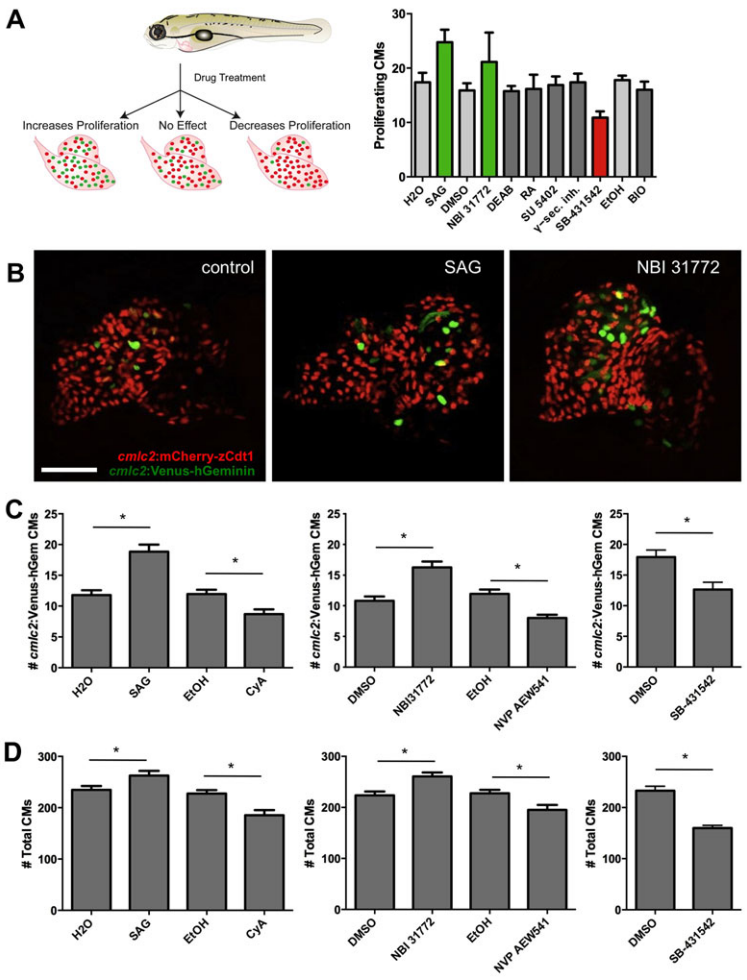

Fig. 2. Hedgehog and Igf signaling promote embryonic cardiomyocyte proliferation in zebrafish. (**A**) Small molecule screen to identify signaling pathways that affect cardiomyocyte (CM) proliferation between 3 and 4 dpf. Green bars, drugs that were identified as potential enhancers of proliferation; red bars, drug that was identified as a potential inhibitor. Data are represented as mean±s.e.m. *n*=8-12 embryos per condition. In the schematic, green and red circles represent proliferating and non-proliferating cells, respectively. (**B**) Representative maximum intensity projections of 4 dpf *cmlc2:FUCCI* embryos treated with Smoothened agonist (SAG) or the Igf agonist NBI-31772. Scale bars: 50 μm. Anterior is to the left in images. (**C**) Effects of treatments with SAG (5 μM), CyA (5 μM), NBI-31772 (2.5 μM), NVP AEW541 (5 μM) and SB-431542 (5 μM) on cardiomyocyte proliferation signals. *n*=30-48, mean±s.e.m. **P*<0.005, Student's *t*-test. Experiments for an individual graph were performed on a combined pool of embryos. Proliferation indices could vary between pools used for each individual graph. (**D**) Changes in proliferation were reflected by analogous changes in the total number of cardiomyocytes in *cmlc2:nucDsRed2* embryos at 4 dpf. *n*=23-35, mean±s.e.m. **P*<0.05, Student's *t*-test.

2% of total cardiomyocytes at 2 dpf, to 4-5% at 3-5 dpf (Fig. 1B). Thus, the *cmlc2:FUCCI* transgenic system sensitively monitors cardiomyocyte proliferation in live zebrafish embryos.

Identification of enhancers of cardiomyocyte proliferation during development

Although early embryonic zebrafish cardiomyocytes arise from progenitor cell differentiation, published evidence indicates that most or all cardiac growth after 2 dpf occurs through cardiomyocyte proliferation (Qu et al., 2008; de Pater et al., 2009; Gupta and Poss, 2012). To determine whether the *cmlc2:FUCCI* line could detect effects of small molecules on cardiomyocyte proliferation, we exposed 3 dpf embryos to a panel of compounds reported to target common developmental signaling pathways (Sun et al., 1999; Liu et al., 2001; Perz-Edwards et al., 2001; Chen et al., 2002; Frank-Kamenetsky et al., 2002; Inman et al., 2002; Meijer et al., 2003; Cuny et al., 2008; Yu et al., 2008; Rao et al., 2009). At 4 dpf, we visually inspected live, treated embryos for changes in the number of cardiomyocytes expressing Venus-hGeminin (supplementary material Fig. S2), before fixing embryos for quantitative analysis. Drugs targeting the β-catenin (BIO), RA (DEAB), Notch (γ-secretase inhibitor) and Fgf (SU5402) signaling pathways did not produce obvious effects on FUCCI signals in cardiomyocytes. By contrast, drugs targeting the Hedgehog (Hh), Insulin-like growth factor (Igf) and Transforming growth factor β (Tgfβ) signaling pathways each modified cardiomyocyte proliferation (Fig. 2A).

Hh signaling plays a role in specifying the number of myocardial progenitors in embryos, and reductions in Hh signaling cause defects in cardiac morphogenesis (Zhang et al., 2001; Washington Smoak et al., 2005; Lin et al., 2006; Goddeeris et al., 2007; Thomas et al., 2008; Hami et al., 2011). To our knowledge, Hh signaling has not been implicated in direct control of cardiomyocyte proliferation (Washington Smoak et al., 2005; Lavine et al., 2008). A 24-hour treatment of *cmlc2:FUCCI* embryos with 5 μM Smoothened agonist (SAG), an Hh pathway agonist (Frank-Kamenetsky et al., 2002), increased the number of proliferating cardiomyocytes by 60% (Fig. 2A-C). In other experiments, we treated 3 dpf embryos with 5 μM cyclopamine (CyA), a Smoothened antagonist (Chen et al., 2002). This regimen reduced cardiomyocyte FUCCI proliferation signals by 27% at 4 dpf (Fig. 2C). As an independent method to examine the effects of Hh pathway modulation on cardiomyocyte production, we counted the total number of cardiomyocyte nuclei per animal using the *cmlc2:nucDsRed2* line. SAG treatment increased animal cardiomyocyte numbers by 10%, whereas CyA reduced total cardiomyocyte numbers by 19% (Fig. 2D).

Recent studies have described positive effects of Igf and Tgfβ signaling on cardiomyocyte proliferation during embryonic development (Qi et al., 2007; Song et al., 2007; Brade et al., 2011; Li et al., 2011; Xin et al., 2011). These findings were reflected in our screen, as treatment of *cmlc2*:FUCCI embryos with the Igf signaling agonist NBI-31772 (2.5 μM) increased cardiomyocyte proliferation by 41%, whereas the Igf receptor antagonist NVP

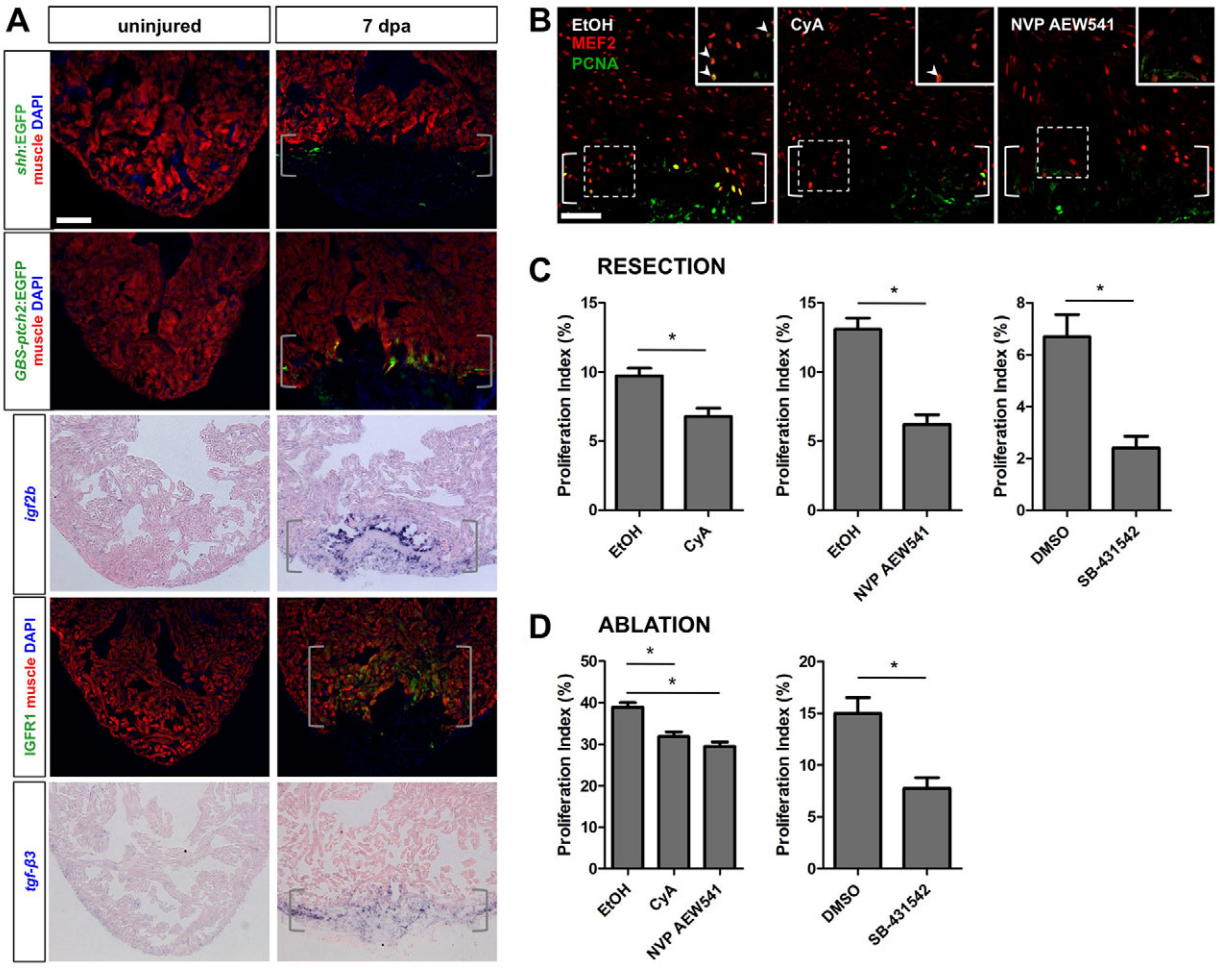

Fig. 3. Hedgehog, Igf and Tgfβ signaling are required for myocardial regeneration in zebrafish. (**A**) The Shh ligand *shha* (transgenic reporter) and regulator/response gene *ptch2* (transgenic reporter), Igf ligand *igf2b* (*in situ* hybridization) and receptor Igfr1 (immunofluorescence), and Tgfβ ligand *tgfb3* (*in situ* hybridization) each showed increased expression in the wound area by 7 dpa. Brackets indicate injury site. Scale bar: 100 μm. (**B**) Treatment with CyA (10 μM) or NVP AEW541 (2 μM) from 6 to 7 dpa decreased cardiomyocyte proliferation. Mef2, red; PCNA, green. Brackets indicate injury site. Insets: High magnifications of the boxed areas. Arrowheads indicate proliferating cardiomyocytes. Scale bar: 50 μm. (**C**) Quantification of the effects of CyA (10 μM), NVP AEW541 (2 μM) and SB-431542 (10 μM) on cardiomyocyte proliferation after resection injury. Fish were treated from 6 to 7 dpa. *n*=9-15, mean±s.e.m. **P*<0.01, Student's *t*-test. (**D**) Quantification of the effects of CyA, NVP AEW541 and SB-431542 on proliferation after genetic cardiomyocyte ablation. Fish were treated from 6 to 7 dpa. *n*=12-13, mean±s.e.m. **P*<0.001, Student's *t*-test. Data in each graph were obtained from different clutches of fish.

AEW541 (5 μM) reduced proliferation signals by 33%. Independent quantification of total cardiomyocyte numbers verified these effects, with agonist- and antagonist-treated embryos possessing 17% more and 14% fewer cardiomyocytes, respectively (Fig. 2B,C). We also found that the Tgfβ receptor inhibitor SB-431542 (5 μM), which specifically inhibits the Tgfβ/Activin pathways and not the closely related BMP pathway (Inman et al., 2002; Chablais and Jaźwińska, 2012), reduced numbers of proliferating cardiomyocytes by 30% in zebrafish embryos, as well as the total number of cardiomyocytes by 31% (Fig. 2C,D). Thus, a direct chemical screen employing a new FUCCI transgenic line revealed that Hh, Igf and Tgfβ signaling pathways promote cardiomyocyte proliferation during zebrafish heart development.

Hh, Igf and Tgfβ signaling pathways are active during heart regeneration

Adult zebrafish regenerate heart muscle efficiently after partial ventricular resection, cryoinjury or genetic cardiomyocyte ablation (Poss et al., 2002; Chablais et al., 2011; González-Rosa et al., 2011; Schnabel et al., 2011; Wang et al., 2011). We first examined expression patterns of Hh, Igf or Tgfβ pathway components before and after cardiac injury in adult zebrafish. Analysis of *shh:EGFP* transgenic fish revealed that *shh* regulatory sequences become activated in epicardial tissue adjacent to and within the injury site 7 days after partial ventricular resection (7 dpa), a time of robust muscle regeneration (Fig. 3A; supplementary material Fig. S3A). To examine whether injury activates Hh signaling during regeneration, we made a transgenic reporter strain that reflected expression of the Hh target gene *ptch2* [*Tg(GBS-ptch2:EGFP)*^{umz23}]. By 7 dpa, EGFP fluorescence was localized to cardiomyocytes in the area of regeneration (Fig. 3A). Igf signaling components were similarly induced by cardiac injury. We detected *igf2b* in endocardial cells in the injury site by 7 dpa using *in situ* hybridization, along with lower intensity signals in epicardial cells, coincident with Igfr1 immunofluorescence in injured and regenerating muscle (Fig. 3A; supplementary material Fig. S3C). The ligand gene *tgfb3* was also

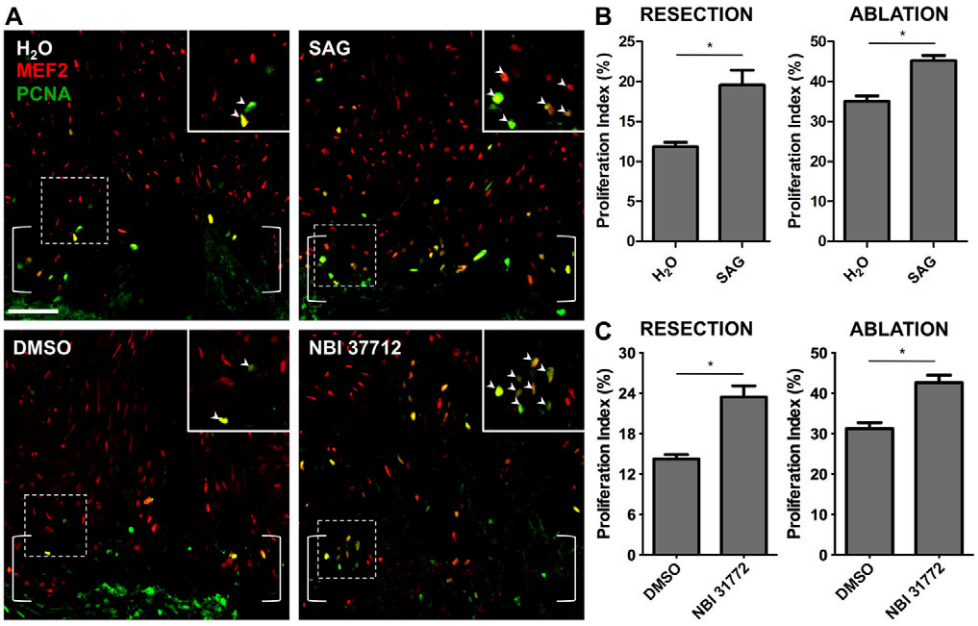

Fig. 4. Hedgehog or Igf pathway activation increases cardiomyocyte proliferation during regeneration in zebrafish. (**A**) Treatment with SAG (2.5 μM) or NBI-31772 (10 μM) from 6 to 7 dpa increased cardiomyocyte proliferation. Mef2, red; PCNA, green. Brackets indicate injury site. Insets: High magnification of the boxed areas. Arrowheads indicate proliferating cardiomyocytes. Scale bar: 50 μm. (**B**) Quantification of cardiomyocyte proliferation following treatment with SAG (2.5 μM) after resection (left) or ablation (right) injury models. Fish were treated from 6 to 7 dpa. *n*=9-15, mean±s.e.m. **P*<0.01, Student's *t*-test. (**C**) Quantification of cardiomyocyte proliferation following treatment with NBI-31772 after resection (10 μM) or ablation (5 μM) injury models. *n*=11-17, mean±s.e.m. **P*<0.05, Student's *t*-test.

induced by injury, with expression limited to the area of regeneration by 7 dpa, in cardiomyocytes but possibly other additional cell types (Fig. 3A; supplementary material Fig. S3B). Additionally, Chablais and Jazwinska recently reported that *tgfb1*, *tgfb2* and *tgfb3* are induced after cryoinjury to the zebrafish heart, along with the receptors *alk4* (*acvr1b* – Zebrafish Information Network), *alk5a* (*tgfbr1a* – Zebrafish Information Network) and *alk5b* (*tgfbr1b* – Zebrafish Information Network) (Chablais and Jaźwińska, 2012).

To examine whether the identified pathways are essential for cardiomyocyte proliferation during heart regeneration, we tested the effects of inhibitors. Animals were treated 6 days after resection of ~20% of the ventricle or diffuse genetic ablation of cardiomyocytes, and assessed for cardiomyocyte proliferation 24 hours later. Although we found that the *cmlc2:FUCCI* line can be used for analyzing adult cardiomyocyte proliferation (supplementary material Fig. S4), we used an independent approach to measure the proportions of cardiomyocytes expressing the proliferation marker PCNA (Fig. 3B). Following treatment with 10 μM CyA, 2 μM NVP AEW541 or 10 μM SB-431542, proliferation indices at 7 dpa were decreased compared with vehicle-treated controls by 30%, 53% and 64%, respectively (Fig. 3C). Similarly, proliferation indices decreased after genetic ablation by 18%, 24% and 48%, respectively (Fig. 3D). Recently, SB-431542 was shown to diminish cardiomyocyte proliferation after cryoinjury (Chablais and Jaźwińska, 2012). Together, these results indicate that the Hh, Igf and Tgfβ signaling pathways are necessary for normal heart regeneration.

Hh and Igf agonists increase cardiomyocyte proliferation during regeneration

Finally, we examined whether the agonists SAG and NBI-31772 identified from the embryo screen would enhance the proliferative response mediated by Hh or Igf components during heart regeneration. We found that these drugs do not noticeably affect proliferation in uninjured adult zebrafish ventricles as they do in embryonic hearts (data not shown). We treated animals for 24 hours from 6 to 7 days after injury and assessed cardiomyocyte proliferation. Remarkably, addition of 2.5 μM SAG or 10 μM NBI-31772 after partial ventricular resection each increased cardiomyocyte proliferation by 65%. These treatments also stimulated cardiomyocyte proliferation after genetic ablation of cardiac muscle, with SAG and NBI-31772 boosting indices by 30% and 36%, respectively, compared with vehicle treatment (Fig. 4A-C). Thus, our results indicate that compounds that promote cardiomyocyte proliferation in the injured adult zebrafish heart can be isolated from embryonic chemical screens.

Conclusions

FUCCI technology enables the visualization of cell proliferation events in live animals. Here, we developed a FUCCI-based screening system that identified modulators of adult heart regeneration, including compounds that augment proliferation of differentiated cardiomyocytes. Our data uncover new requirements for Hh, Igf and Tgfβ signaling during cardiac regeneration. Moreover, our results encourage larger-scale, unbiased screening efforts with *cmlc2:FUCCI* to find many new chemical enhancers of cardiomyocyte proliferation. Expanding the panel of pharmacological manipulations that regulate this process will be crucial for understanding how and why heart regeneration occurs naturally. Additionally, a subset of compounds identified from these screens in zebrafish are likely to represent new tools for probing, and possibly changing, the regenerative capacity of the injured mammalian heart.

Acknowledgements
We thank A. Sakaue-Sawano and A. Miyawaki for FUCCI plasmids; J. Burris, A. Eastes, N. Blake and P. Williams for zebrafish care; A. Dickson for artwork; and Poss laboratory members for comments on the manuscript.

Funding
K.D.P. is an Early Career Scientist of the Howard Hughes Medical Institute. This work was supported by fellowships from the American Heart Association (AHA) [to W.-Y.C., M.G. and J.W.]; a fellowship from the National Institutes of Health (NIH) [F32 HL106987 to W.Y.C.]; grants from the NIH [NS039994 to R.O.K.; HL081674 to K.D.P.]; and grants from the American Federation for Aging Research and the AHA [to K.D.P.]. Deposited in PMC for release after 6 months.

Competing interests statement
The authors declare no competing financial interests.

Supplementary material
Supplementary material available online at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.088526/-/DC1

References

Bergmann, O., Bhardwaj, R. D., Bernard, S., Zdunek, S., Barnabé-Heider, F., Walsh, S., Zupicich, J., Alkass, K., Buchholz, B. A., Druid, H. et al. (2009). Evidence for cardiomyocyte renewal in humans. *Science* **324**, 98-102.
Brade, T., Kumar, S., Cunningham, T. J., Chatzi, C., Zhao, X., Cavallero, S., Li, P., Sucov, H. M., Ruiz-Lozano, P. and Duester, G. (2011). Retinoic acid stimulates myocardial expansion by induction of hepatic erythropoietin which activates epicardial Igf2. *Development* **138**, 139-148.
Chablais, F. and Jaźwińska, A. (2012). The regenerative capacity of the zebrafish heart is dependent on TGFβ signaling. *Development* **139**, 1921-1930.
Chablais, F., Veit, J., Rainer, G. and Jaźwińska, A. (2011). The zebrafish heart regenerates after cryoinjury-induced myocardial infarction. *BMC Dev. Biol.* **11**, 21.
Chen, J. K., Taipale, J., Cooper, M. K. and Beachy, P. A. (2002). Inhibition of Hedgehog signaling by direct binding of cyclopamine to Smoothened. *Genes Dev.* **16**, 2743-2748.
Cuny, G. D., Yu, P. B., Laha, J. K., Xing, X., Liu, J. F., Lai, C. S., Deng, D. Y., Sachidanandan, C., Bloch, K. D. and Peterson, R. T. (2008). Structure-activity relationship study of bone morphogenetic protein (BMP) signaling inhibitors. *Bioorg. Med. Chem. Lett.* **18**, 4388-4392.
de Pater, E., Clijsters, L., Marques, S. R., Lin, Y. F., Garavito-Aguilar, Z. V., Yelon, D. and Bakkers, J. (2009). Distinct phases of cardiomyocyte differentiation regulate growth of the zebrafish heart. *Development* **136**, 1633-1641.
Drenckhahn, J. D., Schwarz, Q. P., Gray, S., Laskowski, A., Kiriazis, H., Ming, Z., Harvey, R. P., Du, X. J., Thorburn, D. R. and Cox, T. C. (2008). Compensatory growth of healthy cardiac cells in the presence of diseased cells restores tissue homeostasis during heart development. *Dev. Cell* **15**, 521-533.
Driever, W., Solnica-Krezel, L., Schier, A. F., Neuhauss, S. C., Malicki, J., Stemple, D. L., Stainier, D. Y., Zwartkruis, F., Abdelilah, S., Rangini, Z. et al. (1996). A genetic screen for mutations affecting embryogenesis in zebrafish. *Development* **123**, 37-46.
Frank-Kamenetsky, M., Zhang, X. M., Bottega, S., Guicherit, O., Wichterle, H., Dudek, H., Bumcrot, D., Wang, F. Y., Jones, S., Shulok, J. et al. (2002). Small-molecule modulators of Hedgehog signaling: identification and characterization of Smoothened agonists and antagonists. *J. Biol.* **1**, 10.
Goddeeris, M. M., Schwartz, R., Klingensmith, J. and Meyers, E. N. (2007). Independent requirements for Hedgehog signaling by both the anterior heart field and neural crest cells for outflow tract development. *Development* **134**, 1593-1604.
González-Rosa, J. M., Martín, V., Peralta, M., Torres, M. and Mercader, N. (2011). Extensive scar formation and regression during heart regeneration after cryoinjury in zebrafish. *Development* **138**, 1663-1674.
Gupta, V. and Poss, K. D. (2012). Clonally dominant cardiomyocytes direct heart morphogenesis. *Nature* **484**, 479-484.
Haffter, P., Granato, M., Brand, M., Mullins, M. C., Hammerschmidt, M., Kane, D. A., Odenthal, J., van Eeden, F. J., Jiang, Y. J., Heisenberg, C. P. et al. (1996). The identification of genes with unique and essential functions in the development of the zebrafish, Danio rerio. *Development* **123**, 1-36.
Hami, D., Grimes, A. C., Tsai, H. J. and Kirby, M. L. (2011). Zebrafish cardiac development requires a conserved secondary heart field. *Development* **138**, 2389-2398.
Inman, G. J., Nicolás, F. J., Callahan, J. F., Harling, J. D., Gaster, L. M., Reith, A. D., Laping, N. J. and Hill, C. S. (2002). SB-431542 is a potent and specific inhibitor of transforming growth factor-beta superfamily type I activin receptor-like kinase (ALK) receptors ALK4, ALK5, and ALK7. *Mol. Pharmacol.* **62**, 65-74.
Jopling, C., Sleep, E., Raya, M., Martí, M., Raya, A. and Izpisúa Belmonte, J. C. (2010). Zebrafish heart regeneration occurs by cardiomyocyte dedifferentiation and proliferation. *Nature* **464**, 606-609.
Kaufman, C. K., White, R. M. and Zon, L. (2009). Chemical genetic screening in the zebrafish embryo. *Nat. Protoc.* **4**, 1422-1432.
Kikuchi, K., Holdway, J. E., Werdich, A. A., Anderson, R. M., Fang, Y., Egnaczyk, G. F., Evans, T., Macrae, C. A., Stainier, D. Y. and Poss, K. D. (2010). Primary contribution to zebrafish heart regeneration by gata4(+) cardiomyocytes. *Nature* **464**, 601-605.
Kikuchi, K., Holdway, J. E., Major, R. J., Blum, N., Dahn, R. D., Begemann, G. and Poss, K. D. (2011). Retinoic acid production by endocardium and epicardium is an injury response essential for zebrafish heart regeneration. *Dev. Cell* **20**, 397-404.
Kim, J., Wu, Q., Zhang, Y., Wiens, K. M., Huang, Y., Rubin, N., Shimada, H., Handin, R. I., Chao, M. Y., Tuan, T. L. et al. (2010). PDGF signaling is required for epicardial function and blood vessel formation in regenerating zebrafish hearts. *Proc. Natl. Acad. Sci. USA* **107**, 17206-17210.
Lavine, K. J., Schmid, G. J., Smith, C. S. and Ornitz, D. M. (2008). Novel tool to suppress cell proliferation in vivo demonstrates that myocardial and coronary vascular growth represent distinct developmental programs. *Dev. Dyn.* **237**, 713-724.
Lepilina, A., Coon, A. N., Kikuchi, K., Holdway, J. E., Roberts, R. W., Burns, C. G. and Poss, K. D. (2006). A dynamic epicardial injury response supports progenitor cell activity during zebrafish heart regeneration. *Cell* **127**, 607-619.
Li, P., Cavallero, S., Gu, Y., Chen, T. H., Hughes, J., Hassan, A. B., Brüning, J. C., Pashmforoush, M. and Sucov, H. M. (2011). IGF signaling directs ventricular cardiomyocyte proliferation during embryonic heart development. *Development* **138**, 1795-1805.
Lin, L., Bu, L., Cai, C. L., Zhang, X. and Evans, S. (2006). Isl1 is upstream of sonic hedgehog in a pathway required for cardiac morphogenesis. *Dev. Biol.* **295**, 756-763.
Liu, X. J., Xie, Q., Zhu, Y. F., Chen, C. and Ling, N. (2001). Identification of a nonpeptide ligand that releases bioactive insulin-like growth factor-I from its binding protein complex. *J. Biol. Chem.* **276**, 32419-32422.
Mably, J. D., Mohideen, M. A., Burns, C. G., Chen, J. N. and Fishman, M. C. (2003). heart of glass regulates the concentric growth of the heart in zebrafish. *Curr. Biol.* **13**, 2138-2147.
Meijer, L., Skaltsounis, A. L., Magiatis, P., Polychronopoulos, P., Knockaert, M., Leost, M., Ryan, X. P., Vonica, C. A., Brivanlou, A., Dajani, R. et al. (2003). GSK-3-selective inhibitors derived from Tyrian purple indirubins. *Chem. Biol.* **10**, 1255-1266.
Perz-Edwards, A., Hardison, N. L. and Linney, E. (2001). Retinoic acid-mediated gene expression in transgenic reporter zebrafish. *Dev. Biol.* **229**, 89-101.
Peterson, R. T. and Fishman, M. C. (2011). Designing zebrafish chemical screens. *Methods Cell Biol.* **105**, 525-541.
Porrello, E. R., Mahmoud, A. I., Simpson, E., Hill, J. A., Richardson, J. A., Olson, E. N. and Sadek, H. A. (2011). Transient regenerative potential of the neonatal mouse heart. *Science* **331**, 1078-1080.
Poss, K. D., Wilson, L. G. and Keating, M. T. (2002). Heart regeneration in zebrafish. *Science* **298**, 2188-2190.
Qi, X., Yang, G., Yang, L., Lan, Y., Weng, T., Wang, J., Wu, Z., Xu, J., Gao, X. and Yang, X. (2007). Essential role of Smad4 in maintaining cardiomyocyte proliferation during murine embryonic heart development. *Dev. Biol.* **311**, 136-146.
Qu, X., Jia, H., Garrity, D. M., Tompkins, K., Batts, L., Appel, B., Zhong, T. P. and Baldwin, H. S. (2008). Ndrg4 is required for normal myocyte proliferation during early cardiac development in zebrafish. *Dev. Biol.* **317**, 486-496.
Rao, S. S., O'Neil, J., Liberator, C. D., Hardwick, J. S., Dai, X., Zhang, T., Tyminski, E., Yuan, J., Kohl, N. E., Richon, V. M. et al. (2009). Inhibition of NOTCH signaling by gamma secretase inhibitor engages the RB pathway and elicits cell cycle exit in T-cell acute lymphoblastic leukemia cells. *Cancer Res.* **69**, 3060-3068.
Rottbauer, W., Saurin, A. J., Lickert, H., Shen, X., Burns, C. G., Wo, Z. G., Kemler, R., Kingston, R., Wu, C. and Fishman, M. (2002). Reptin and pontin antagonistically regulate heart growth in zebrafish embryos. *Cell* **111**, 661-672.
Sakaue-Sawano, A., Kurokawa, H., Morimura, T., Hanyu, A., Hama, H., Osawa, H., Kashiwagi, S., Fukami, K., Miyata, T., Miyoshi, H. et al. (2008). Visualizing spatiotemporal dynamics of multicellular cell-cycle progression. *Cell* **132**, 487-498.
Sasaki, H., Hui, C., Nakafuku, M. and Kondoh, H. (1997). A binding site for Gli proteins is essential for HNF-3beta floor plate enhancer activity in transgenics and can respond to Shh in vitro. *Development* **124**, 1313-1322.
Schnabel, K., Wu, C. C., Kurth, T. and Weidinger, G. (2011). Regeneration of cryoinjury induced necrotic heart lesions in zebrafish is associated with epicardial activation and cardiomyocyte proliferation. *PLoS ONE* **6**, e18503.
Shkumatava, A., Fischer, S., Müller, F., Strahle, U. and Neumann, C. J. (2004). Sonic hedgehog, secreted by amacrine cells, acts as a short-range signal to direct differentiation and lamination in the zebrafish retina. *Development* **131**, 3849-3858.

Song, L., Yan, W., Chen, X., Deng, C. X., Wang, Q. and Jiao, K. (2007). Myocardial smad4 is essential for cardiogenesis in mouse embryos. *Circ. Res.* **101**, 277-285.

Sugiyama, M., Sakaue-Sawano, A., Iimura, T., Fukami, K., Kitaguchi, T., Kawakami, K., Okamoto, H., Higashijima, S. and Miyawaki, A. (2009). Illuminating cell-cycle progression in the developing zebrafish embryo. *Proc. Natl. Acad. Sci. USA* **106**, 20812-20817.

Sun, L., Tran, N., Liang, C., Tang, F., Rice, A., Schreck, R., Waltz, K., Shawver, L. K., McMahon, G. and Tang, C. (1999). Design, synthesis, and evaluations of substituted 3-[(3- or 4-carboxyethylpyrrol-2-yl)methylidenyl]indolin-2-ones as inhibitors of VEGF, FGF, and PDGF receptor tyrosine kinases. *J. Med. Chem.* **42**, 5120-5130.

Thomas, N. A., Koudijs, M., van Eeden, F. J., Joyner, A. L. and Yelon, D. (2008). Hedgehog signaling plays a cell-autonomous role in maximizing cardiac developmental potential. *Development* **135**, 3789-3799.

Wang, J., Panáková, D., Kikuchi, K., Holdway, J. E., Gemberling, M., Burris, J. S., Singh, S. P., Dickson, A. L., Lin, Y. F., Sabeh, M. K. et al. (2011). The regenerative capacity of zebrafish reverses cardiac failure caused by genetic cardiomyocyte depletion. *Development* **138**, 3421-3430.

Washington Smoak, I., Byrd, N. A., Abu-Issa, R., Goddeeris, M. M., Anderson, R., Morris, J., Yamamura, K., Klingensmith, J. and Meyers, E. N. (2005). Sonic hedgehog is required for cardiac outflow tract and neural crest cell development. *Dev. Biol.* **283**, 357-372.

Wills, A. A., Kidd, A. R., 3rd, Lepilina, A. and Poss, K. D. (2008). Fgfs control homeostatic regeneration in adult zebrafish fins. *Development* **135**, 3063-3070.

Xin, M., Kim, Y., Sutherland, L. B., Qi, X., McAnally, J., Schwartz, R. J., Richardson, J. A., Bassel-Duby, R. and Olson, E. N. (2011). Regulation of insulin-like growth factor signaling by Yap governs cardiomyocyte proliferation and embryonic heart size. *Sci. Signal.* **4**, ra70.

Yu, P. B., Deng, D. Y., Lai, C. S., Hong, C. C., Cuny, G. D., Bouxsein, M. L., Hong, D. W., McManus, P. M., Katagiri, T., Sachidanandan, C. et al. (2008). BMP type I receptor inhibition reduces heterotopic [corrected] ossification. *Nat. Med.* **14**, 1363-1369.

Zhang, X. M., Ramalho-Santos, M. and McMahon, A. P. (2001). Smoothened mutants reveal redundant roles for Shh and Ihh signaling including regulation of L/R symmetry by the mouse node. *Cell* **105**, 781-792.