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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.

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 μ M 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-SceI sites and was co-injected with I-SceI into one-cell-stage embryos. F1 fish containing each transgene were crossed to generate double transgenic *cmlc2:FUCCI* fish, which were inbred 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) (GACCACCA) (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).

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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 μ M except for NBI-31772, RA and BIO, which were used at 2.5 μ M. In adults, SAG was used at 2.5 μ M, NBI-31772 was used at 10 μ M, CyA was used at 10 μ M and NVP-AEW541 was used at 2 μ M.

Histology

Initial screening for FUCCI signals was performed using a Leica M205 FA dissecting microscope. *z*-stacks were acquired from 50 μ M-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 μ M 5-ethynyl-2'-deoxyuridine (EdU) for 3 hours at 28°C before fixation. Next, 25 μ m-thick cryosections of the embryo were treated with reagent containing either 20 μ M AlexaFluor-488 or 20 μ M 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). Digoxigenin-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'-GCGGCCGCTCACTTGTGGCTAACGTAGT-3'. The 2.0-kb *tgfb3* probe was cloned using the primers 5'-ACCGGTGCCACCATGCATTGGGC-AAAGGACT-3' and 5'-GCGGCCGCGGTTTGTTCATTTTAATTA-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-1R β (rabbit; Santa Cruz).

Cardiomyocyte proliferation was quantified from 10 μ m-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-

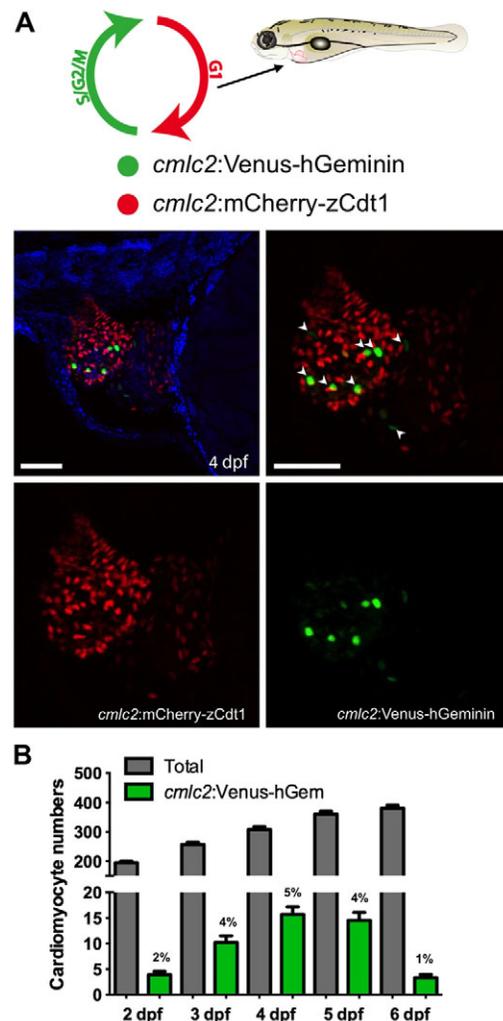


Fig. 1. FUCCI zebrafish for visualizing cardiomyocyte proliferation.

(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 μ m. (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 \pm s.e.m. $n=13$ –24 embryos per stage.

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

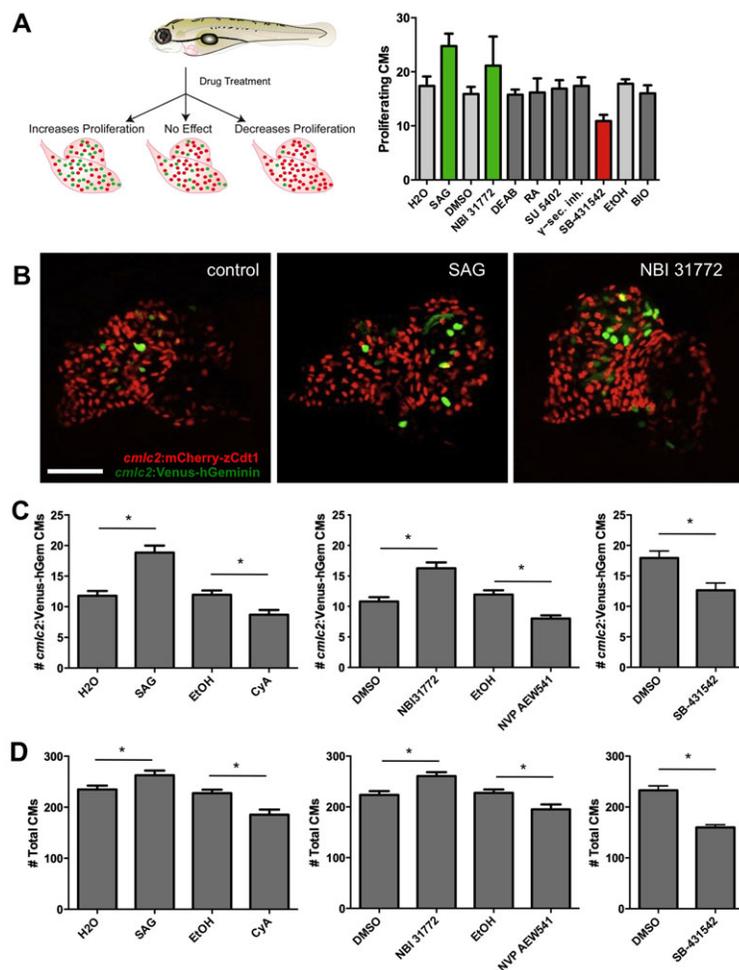


Fig. 2. Hedgehog and Igf signaling promote embryonic cardiomyocyte proliferation in zebrafish. (A) Small molecule screen to identify signaling pathways that affect cardiomyocyte (CM) proliferation between 3 and 4 dpf.

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 Smoothed agonist (SAG) or the Igf agonist NBI-31772. Scale bars: 50 μ m. Anterior is to the left in images. (C) Effects of treatments with SAG (5 μ M), CyA (5 μ M), NBI-31772 (2.5 μ M), NVP AEW541 (5 μ M) and SB-431542 (5 μ M) 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 (Tgfb) 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 μ M Smoothed 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 μ M cyclopamine (CyA), a Smoothed 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 Tgfb 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 μ M) increased cardiomyocyte proliferation by 41%, whereas the Igf receptor antagonist NVP

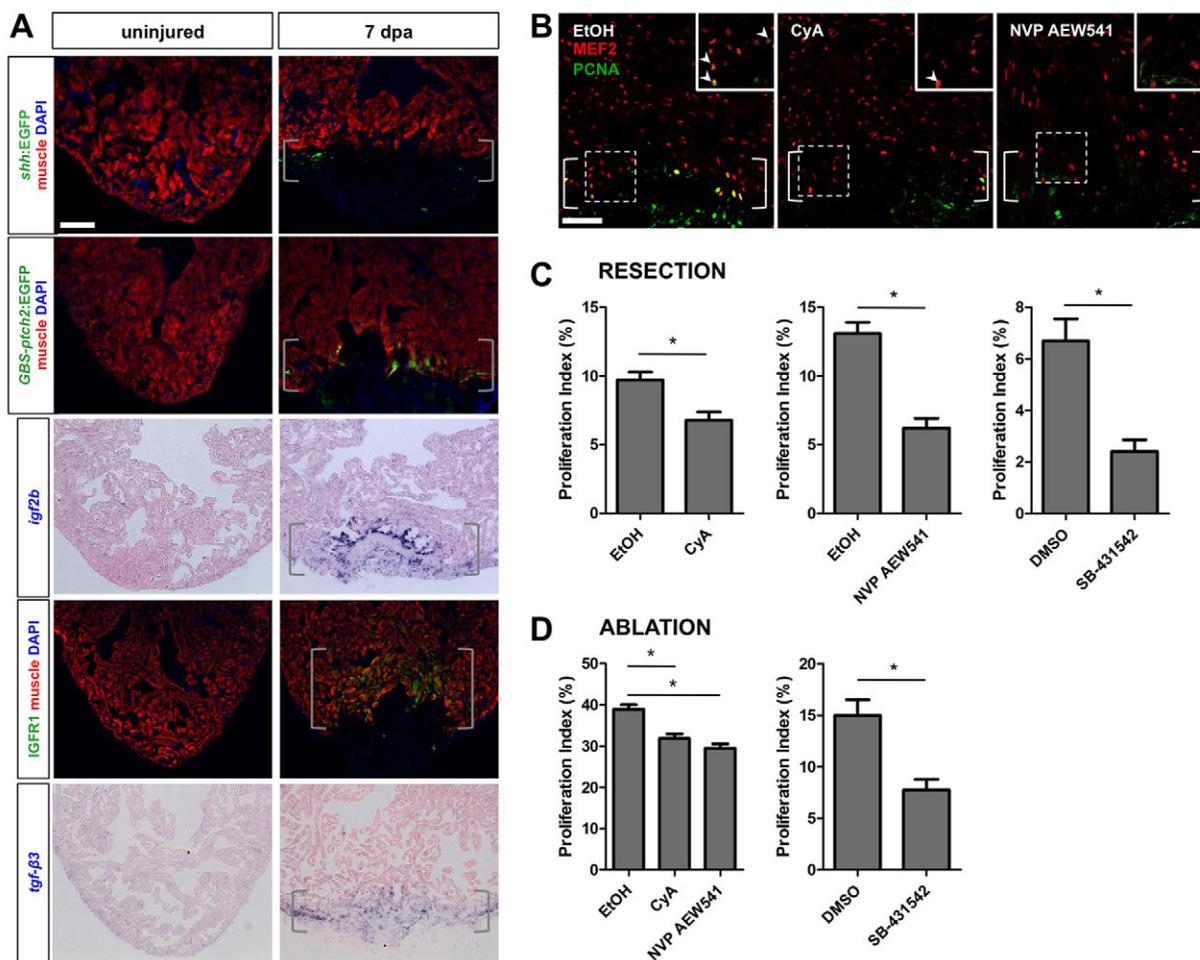


Fig. 3. Hedgehog, Igf and Tgf β signaling are required for myocardial regeneration in zebrafish. (A) The Shh ligand *shha* (transgenic reporter) and regulator/response gene *ptch2* (transgenic reporter), Igf ligand *igf2b* (*in situ* hybridization) and receptor Igfr1 (immunofluorescence), and Tgf β ligand *tgfb3* (*in situ* hybridization) each showed increased expression in the wound area by 7 dpa. Brackets indicate injury site. Scale bar: 100 μ m. (B) Treatment with CyA (10 μ M) or NVP AEW541 (2 μ M) 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 μ m. (C) Quantification of the effects of CyA (10 μ M), NVP AEW541 (2 μ M) and SB-431542 (10 μ M) on cardiomyocyte proliferation after resection injury. Fish were treated from 6 to 7 dpa. $n=9-15$, mean \pm 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 \pm s.e.m. * $P<0.001$, Student's *t*-test. Data in each graph were obtained from different clutches of fish.

AEW541 (5 μ M) 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 Tgf β receptor inhibitor SB-431542 (5 μ M), which specifically inhibits the Tgf β /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 Tgf β signaling pathways promote cardiomyocyte proliferation during zebrafish heart development.

Hh, Igf and Tgf β 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 Tgf β 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

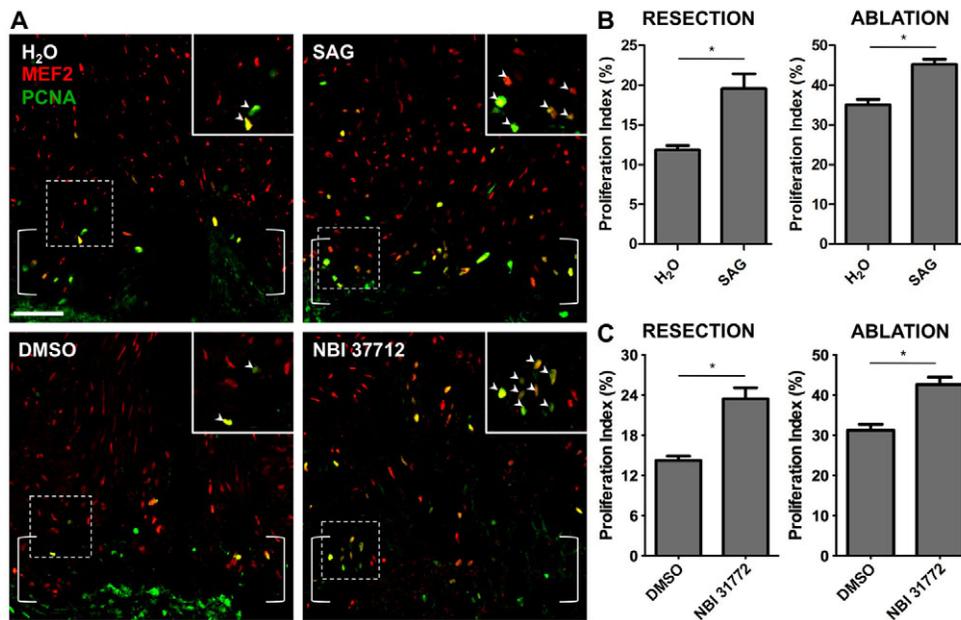


Fig. 4. Hedgehog or Igf pathway activation increases cardiomyocyte proliferation during regeneration in zebrafish. (A) Treatment with SAG (2.5 μ M) or NBI-31772 (10 μ M) from 6 to 7 dpa increased cardiomyocyte proliferation. Mef2, red; PCNA, green. Brackets indicate injury site. Insets: High magnification of the boxed areas. Arrowheads indicate proliferating cardiomyocytes. Scale bar: 50 μ m. (B) Quantification of cardiomyocyte proliferation following treatment with SAG (2.5 μ M) after resection (left) or ablation (right) injury models. Fish were treated from 6 to 7 dpa. $n=9-15$, mean \pm s.e.m. * $P<0.01$, Student's t -test. (C) Quantification of cardiomyocyte proliferation following treatment with NBI-31772 after resection (10 μ M) or ablation (5 μ M) injury models. $n=11-17$, mean \pm 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* (*tgfb1a* – Zebrafish Information Network) and *alk5b* (*tgfb1b* – Zebrafish Information Network) (Chablais and Jazwińska, 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 μ M CyA, 2 μ M NVP AEW541 or 10 μ M 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 Jazwińska, 2012). Together, these results indicate that the Hh, Igf and Tgf β 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 μ M SAG or 10 μ M 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 Tgf β 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.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material available online at

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