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 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; Shkumatava et al., 2004; Wills et al., 2008, cmlc2:CreER (Kikuchi et al., 2010), bactin2:loxp-nCherry-STOP-loxp-DTA (Wang et al., 2011) and cmlc2:muc-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 incrossed to generate animals homozygous for both transgenes. These double hemizygous transgensics 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)257 and Tg(cmlc2:Venus-hGeminin)258.

The Hh signaling reporter line contains a transgene expressing enhanced green fluorescent protein (EGFP) under the control of a 900 bp ptc2 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-ptic2:EGFP)am12 (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 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 paraffin-embedded fish 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-DeRed2 line was performed similarly with single channel surface analysis using Imaris software. For EdU labeling, embryos were incubated in 500 μM 5-ethyl-2'-deoxyuridine (EdU) for 3 hours at 28°C before fixation. Next, 25 μm-thick crosssections 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 tgfβ3 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'-GGCGG-CGCCATCTTGTCGGTCAACTGAGT-3'. The 2.0-kb tgfβ3 probe was cloned using the primers 5'-ACCGTGCCCACCATGGCATGGC-AAAAGACT-3' and 5'-GGCGGCCGGTTTGGCTTTCTATATTAA-3'.

Immunofluorescence was performed as previously described (Kikuchi et al., 2011). shb:EGFP and GBS-pitch2: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 showed 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 ubiquitination-based cell cycle indicator (FUCCI) system, which employs two fusion proteins, mCherry-

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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.
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...
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 Jazwinska, 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
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 Jazwinska, 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). We treated animals for 24 hours from 6 to 7 days after injury and assessed cardiomyocyte proliferation. Remarkably, addition of 2.5 \(\mu\)M SAG or 10 \(\mu\)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\(\beta\) 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.

**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 \(\mu\)M SAG or 10 \(\mu\)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.

**Fig. 4. Hedgehog or Igf pathway activation increases cardiomyocyte proliferation during regeneration in zebrafish.** (A) Treatment with SAG (2.5 \(\mu\)M) or NBI-31772 (10 \(\mu\)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 \(\mu\)m. (B) Quantification of cardiomyocyte proliferation following treatment with SAG (2.5 \(\mu\)M) 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 \(\mu\)M) or ablation (5 \(\mu\)M) injury models. \(n=11-17\), mean±s.e.m. *\(P<0.05\), Student’s t-test.
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Competing interests statement
The authors declare no competing financial interests.

Supplementary material
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References


**Fig. S1. Co-staining of cardiac FUCCI expression with EdU incorporation.** (A) Cells expressing the *cmlc2:mCherry-zCdt1* transgene (red) do not overlap with cells containing incorporated EdU (green). Images are representative maximum intensity projections from the same heart. DAPI, blue. Inset: High magnification of the boxed area. (B) A subset of cells expressing the *cmlc2:Venus-hGeminin* transgene (green) also contain incorporated EdU (red). DAPI, blue. Inset: High magnification of the boxed area. Arrowheads, co-expressing cells; dotted yellow line, outline of the heart. Note that many EdU⁺ cells in these images are not cardiomyocytes.
Fig. S2. Effects of drug treatment on cardiomyocyte proliferation can be visualized in live embryos with a stereomicroscope. 

*cmIc2:FUCCI* embryos treated with the indicated drugs from 3 to 4 dpf, exhibiting signal changes detectable via the green channel (dashed area). *cmIc2*: Venus-hGeminin, green. Bright-field image is overlaid.
Fig. S3. Complete time course panel of ligand expression. (A) *shha* regulatory sequences drive EGFP in the valves of uninjured hearts, and is upregulated in patchy areas (arrowheads) of the epicardium at 1 and 3 dpa. By 7 dpa, expression is detected in epicardial cells within the injury, as well as more globally in the epicardium surrounding the heart. Top: *shh*:EGFP, green; Myosin heavy chain, red; DAPI, blue; notched arrowheads, *shh*:EGFP expression; filled arrowheads, valve expression. Bottom: *shh*:EGFP expression. (B) *tgfb3* expression is detectable at low levels in the myocardium by 1 and 3 dpa and localizes to the injury site by 7 dpa. (C) After injury, the ligand *igf2b* is expressed in endocardial cells, and at lower levels in epicardial tissue, near the injury site.
Fig. S4. Cardiomyocyte proliferation can be visualized in adult cmlc2:FUCCI hearts. (A) Treatment with SAG (2.5 μM) from 6 to 7 days after resection injury increased levels of Venus-hGeminin+ cardiomyocytes. cmlc2:mCherry-zCdt1, red; cmlc2:Venus-hGeminin, green. Brackets indicate injury site. (B) Quantification of the effects of SAG treatment, using cmlc2:FUCCI fish. n=6, mean±s.e.m. *P<0.0001, Student’s t-test.