Hippo signaling impedes adult heart regeneration

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ABSTRACT
Heart failure due to cardiomyocyte loss after ischemic heart disease is the leading cause of death in the United States in large part because heart muscle regenerates poorly. The endogenous mechanisms preventing mammalian cardiomyocyte regeneration are poorly understood. Hippo signaling, an ancient organ size control pathway, is a kinase cascade that inhibits developing cardiomyocyte proliferation but it has not been studied postnatally or in fully mature adult cardiomyocytes. Here, we investigated Hippo signaling in adult cardiomyocyte renewal and regeneration. We found that unstressed Hippo-deficient adult mouse cardiomyocytes re-enter the cell cycle and undergo cytokinesis. Moreover, Hippo deficiency enhances cardiomyocyte regeneration with functional recovery after adult myocardial infarction as well as after postnatal day eight (P8) cardiac apex resection and P8 myocardial infarction. In damaged hearts, Hippo mutant cardiomyocytes also have elevated proliferation. Our findings reveal that Hippo signaling is an endogenous repressor of adult cardiomyocyte renewal and regeneration. Targeting the Hippo pathway in human disease might be beneficial for the treatment of heart disease.

KEY WORDS: Cell cycle, Proliferation, Regeneration, Mouse

INTRODUCTION
Whereas other organs have some regenerative capacity, heart muscle or cardiomyocytes fail to renew or regenerate sufficiently to repair the damaged heart. Although both cardiac stem cells and endogenous cardiomyocyte renewal have been described, these endogenous mechanisms are overwhelmed in the face of acute cardiomyocyte loss (Kikuchi and Poss, 2012). This clinical reality has prompted multiple efforts to supplement human damaged cardiomyocyte loss (Kikuchi and Poss, 2012). This clinical reality has prompted multiple efforts to supplement human damaged cardiomyocyte loss (Kikuchi and Poss, 2012). Although both cardiac stem cells and endogenous cardiomyocyte renewal have been described, these endogenous mechanisms are overwhelmed in the face of acute cardiomyocyte loss (Kikuchi and Poss, 2012).

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Hippo signaling in adult cardiomyocyte renewal and regeneration. We found that unstressed Hippo-deficient adult mouse cardiomyocytes re-enter the cell cycle and undergo cytokinesis. Moreover, Hippo deficiency enhances cardiomyocyte regeneration with functional recovery after adult myocardial infarction as well as after postnatal day eight (P8) cardiac apex resection and P8 myocardial infarction. In damaged hearts, Hippo mutant cardiomyocytes also have elevated proliferation. Our findings reveal that Hippo signaling is an endogenous repressor of adult cardiomyocyte renewal and regeneration. Targeting the Hippo pathway in human disease might be beneficial for the treatment of heart disease.

RESULTS
Hippo inhibits adult cardiomyocyte renewal
To test the role of Salv, Lats1Lats2 (hereafter Lats1/2) in the postnatal heart, Hippo pathway inactivation in the unstressed adult mouse heart induced cardiomyocyte renewal. Moreover, Hippo deficiency promoted efficient heart regeneration in both postnatal cardiac apex resection and adult myocardial infarction models revealing a crucial, inhibitory role for Hippo signaling in cardiomyocyte renewal and regeneration.

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capacity when Hippo signaling is deleted. In contrast to Hippo mutant hearts, control hearts only incorporated EdU in cardiac fibroblasts (Fig. 1A,B). Quantification of EdU-positive cells showed significant induction of DNA synthesis in Hippo-deficient hearts with a greater increase in Lats1/2 mutants compared with Salv CKO cardiomyocytes (Fig. 1B). Cell cycle re-entry was also quantified in isolated cardiomyocyte nuclei using fluorescence-activated cell sorting (FACS) analysis (Fig. 1C) (Bergmann et al., 2009). Both Lats1/2 CKO and Salv CKO cardiomyocyte nuclei had increased numbers of Ki-67 (Mki67)-expressing cardiomyocytes compared with controls (Fig. 1C; supplementary material Fig. S1A). Moreover, total cardiomyocyte number was increased with more mononuclear cardiomyocytes in Lats1/2 and Salv CKO hearts than in controls (Fig. 1D,E). Average cardiomyocyte size in these hearts was significantly smaller than that of controls (Fig. 1F). These results show that cardiomyocytes re-enter the cell cycle upon Hippo pathway disruption and support the hypothesis that Hippo signaling is a negative regulator of adult cardiomyocyte renewal.

In addition to de novo DNA synthesis and cell counting, we evaluated whether Salv CKO and Lats1/2 CKO cardiomyocytes progress through mitosis and cytokinesis using other methods. We performed ploidy analysis using FACS to sort nuclei isolated from control and Hippo-deficient cardiomyocytes (Fig. 1G). We reasoned that if DNA duplication occurred in the absence of karyokinesis, then total DNA content per nuclei in Hippo-deficient hearts would be greater than that in controls. Total DNA content was unchanged between control and both the Salv CKO and the Lats1/2 CKO cardiomyocyte nuclei, supporting the notion that Hippo-deficient nuclei re-enter the cell cycle and progress through karyokinesis (Fig. 1G).
We performed immunohistochemistry with the M-phase marker Aurora B kinase (Aurkb) to determine if cytokinesis occurred in Hippo-deficient adult cardiomyocytes. Aurkb expression in Lats1/2 and Salv CKO cardiomyocytes was clearly detectable at the cleavage furrow providing direct evidence for cytokinesis (Fig. 1H,I). In contrast to Hippo-deficient hearts, Aurkb expression was barely detected in control hearts (Fig. 1I).

**Hippo-deficient postnatal cardiomyocytes regenerate**

Resection of the cardiac apex in the first 6 days of life results in cardiac regeneration whereas resections performed at postnatal day (P) 7 or later results in fibrosis and scarring (Porrello et al., 2011). Moreover, the cells that contribute to ventricular wall repair, as determined by lineage-tracing experiments, are primarily derived from myosin heavy chain-expressing cardiomyocytes (Porrello et al., 2011). If Hippo signaling represses cardiomyocyte regenerative capacity beyond P6, then Hippo activity should be higher at P7 and later stages. Western blots indicated that Hippo activity, as measured by pYap expression, is low in the P2 regenerative phase hearts. By contrast, pYap levels sharply increase at the P10 and P21 non-regenerative stages, consistent with the hypothesis that Hippo signaling represses cardiac regeneration (Fig. 2A).

To test regenerative capability, we performed apex resection of uniform size at the normally non-regenerative P8 in control and Hippo-deficient hearts. To inactivate Salv, we injected mice with four tamoxifen doses prior to and after the resection (Fig. 2B). Both GFP fluorescence, detecting recombination in the mTmG reporter, and immunofluorescence with an anti-Salv antibody indicated efficient deletion of Salv in mutant myocardium at 4 and 21 days post resection (dpr) and in three-month-old adults after tamoxifen injection (Fig. 2C,D).

Evaluation of 21 dpr hearts by serial sectioning revealed severe scarring of control hearts in all but a few cases (Fig. 2E,F; Fig. 3D-F). By contrast, resected Hippo-deficient hearts efficiently regenerated the myocardium with reduced scar size (Fig. 2E,F; Fig. 3D,E). Lineage tracing indicated that the regenerated cardiac apex was derived primarily from pre-existing alpha myosin heavy chain-expressing cardiomyocytes, although this experiment does not rule out a small contribution from resident stem cells (Fig. 3A-C). In addition to the cardiomyocyte-specific Salv CKO, we also used the Nkx2.5 cre driver, which inactivates Salv during development and is known to efficiently delete Salv (Heallen et al., 2011). Nkx2.5<sup>cre</sup> Salv mutants also robustly repaired the heart (Fig. 2F; Fig. 3E). Echocardiography revealed that fractional shortening (FS) of resected control hearts was significantly reduced whereas resected Hippo-deficient FS resembled sham levels (Fig. 2G), indicating normal contractile function in these hearts. Histological staining revealed that some Salv CKO resected hearts appeared slightly more dilated compared with controls (Fig. 2E), although this was not evident by echocardiography. Further experiments are required to determine whether the mild dilation in Salv CKO hearts is progressive or transitory.

**Hippo-deficient adult and postnatal cardiomyocytes regenerate after infarction**

We next investigated whether Hippo-deficiency enhanced heart regeneration after myocardial infarction, an experimental system that produces persistent fibrotic scar. (F) Scar surface area measurements were obtained from multiple specimens and averages calculated. Statistical analysis was by unpaired Student’s t-test. *P<0.05, **P<0.01, ***P<0.001. Error bars represent s.e.m.
that more accurately models human cardiomyocyte loss secondary to coronary artery disease. We performed left anterior descending (LAD) coronary artery occlusion at both P8 and two months of age. In P8 hearts, following LAD occlusion we found that there was functional recovery and reduced scar size when analyzed at 21 days after occlusion (Fig. 4A-D). Histology also confirmed the recovery of myocardium with less scar tissue after LAD occlusion (Fig. 4E).

In adult hearts, we found similarly strong functional (Fig. 4F-H) and histological evidence for cardiomyocyte regeneration (Fig. 4I,J) after LAD occlusion. FS and ejection fraction (EF) evaluated by echocardiography indicated that by three weeks post LAD occlusion, adult Hippo-deficient hearts had recovered function to a level comparable to that of sham-operated animals, suggesting that Hippo-deficient cardiomyocytes have increased survival and/or proliferation after ischemic damage (Fig. 4F,G).

Hippo-deficient cardiomyocytes are more proliferative after injury

To investigate the reparative process in more depth, we evaluated 4 dpr (P12) Hippo-deficient hearts after apex resection. Four hours prior to harvest, hearts were pulsed with EdU to visualize cells that had entered the cell cycle. In control hearts, EdU-positive cells were primarily found in the GFP-negative, non-cardiomyocyte lineage near the resected zone and are likely to be infiltrating inflammatory cells and proliferating cardiac fibroblasts (Fig. 5A,C). Similar proliferating GFP-negative cells were also observed in Salv CKO hearts (Fig. 5B,D). In contrast to controls, both Salv CKO resected and sham-operated hearts had EdU/GFP double-positive cardiomyocytes within both the border zone, or a border zone equivalent region in shams, and distal heart regions (Fig. 5B,D-G).

To determine whether cardiomyocytes were progressing through cytokinesis after apex resection, we evaluated Aurkb activity. In the Hippo-deficient hearts, there was significantly more Aurkb staining, indicating that Hippo-deficient cardiomyocytes were progressing through cytokinesis (Fig. 5H). We also determined whether cell cycle progression genes that have been previously shown to be upregulated in developing Hippo-deficient hearts were required for elevated cardiomyocyte proliferation in postnatal Hippo-deficient cardiomyocytes. Small interfering RNA (siRNA) knockdown of Salv in neonatal cardiomyocytes resulted in enhanced cell cycle entry that was repressed by knocking down Aurkb, Birc5 and Ccne2 (Fig. 5I). We conclude that postnatal Hippo deficiency enhances the ability of cardiomyocytes to re-enter the cell cycle and progress through cytokinesis after apex resection.

To gain more insight into the timing of Hippo-deficient cardiomyocyte proliferation, we evaluated EdU incorporation at multiple stages. In P8 and P12 unstressed hearts, EdU incorporation was significantly elevated with increased numbers of cardiomyocytes (Fig. 5J,K). In addition, similar to what was observed in the adult heart, there were increased numbers of mononucleated cardiomyocytes in Hippo-deficient hearts (Fig. 5J,K).

DISCUSSION

Our findings indicate that Hippo is an endogenous inhibitor of adult cardiomyocyte renewal and regeneration. By inactivating Hippo pathway components in postnatal cardiomyocytes, we found that
Hippo-deficient cardiomyocytes regain proliferative and regenerative capacity. Our data provide new insight into the cellular mechanisms underlying cardiac regeneration and indicate that Hippo signaling is a viable target for therapeutic approaches to heart disease.

Data from human and mouse studies indicate that cardiomyocytes regenerate inefficiently at ~1% per year in young hearts (Bergmann et al., 2009; Kajstura et al., 2012; Mollova et al., 2013; Senyo et al., 2013). Our data, showing that Hippo-deficient adult cardiomyocytes re-enter the cell cycle and undergo cytokinesis, indicate that Hippo signaling is a major endogenous repressor for cardiomyocyte proliferation. Because cardiomyocyte renewal diminishes with age, it will be important to determine whether Hippo signaling is also involved in loss of renewal capacity during cardiac aging. Indeed, our preliminary results indicate that Hippo inactivation in eight-month-old hearts leads to cardiomyocyte cell cycle re-entry.

Fig. 4. Cardiac regeneration and functional recovery following myocardial infarction. (A) B-mode echocardiographic image from representative 21 day post-occlusion heart. LV, left ventricle; P, papillary muscle. (B,C) Ejection fraction (B) and fractional shortening (C) percentages of P8 LAD-O hearts. Occluded (O) and sham-operated (S) control (ctrl) and Salv CKO (CKO) hearts at 21-32 days post myocardial infarction (dpmi). ctrl/O (n=7), CKO/O (n=9), ctrl/S (n=6), CKO/S (n=9). Statistical analysis was by one-way ANOVA with Bonferroni’s multiple comparison test. (D) Infarct surface area measurements were obtained from multiple specimens and averages calculated. Statistical analysis was by unpaired Student’s t-test. Control (n=6), Salv CKO (n=8). (E) Serial transverse sections of 4-week post-occlusion hearts. (F,G) Ejection fraction (F) and fractional shortening (G) percentages of adult LAD-O hearts. 1 week: Ctrl Sham (n=4), Ctrl LAD-O (n=6), CKO Sham (n=3), CKO LAD-O (n=3); 2 weeks: Ctrl Sham (n=3), Ctrl LAD-O (n=5), CKO Sham (n=3), CKO LAD-O (n=2); 3 weeks: Ctrl Sham (n=7), Ctrl LAD-O (n=7), CKO Sham (n=3), CKO LAD-O (n=5). (H) Adult LAD-O infarct size 3 weeks post-occlusion measured as percentage fibrosis of LV myocardium (total fibrotic area/total myocardial area×100). Ctrl LAD-O (n=6), CKO LAD-O (n=3). Statistical analysis was by two-way ANOVA with Bonferroni’s multiple comparison test. (I) Illustration of transverse section planes presented in J. Cross indicates plane of occlusion. IVS, interventricular septum; LV, left ventricle; RV, right ventricle. (J) Serial transverse sections of representative 3-week post-occlusion adult hearts. All echocardiographic measurements were collected blind from genotype. Scale bars: 1 mm. *P<0.05, **P<0.01, ***P<0.001.
It is notable that cell cycle re-entry occurs as an organ-wide response in Hippo-deficient hearts at the 4 dpr stage we examined here. This is similar to zebrafish and neonatal heart regeneration in which apex resection induces cell cycle re-entry throughout the heart (Kikuchi and Poss, 2012). The timing of activated cell cycle re-entry varies depending on the heart region examined. In neonatal mouse hearts, cardiomyocyte cell cycle re-entry in the distal area peaks at 1 dpr but persists until 7 dpr whereas border zone cardiomyocyte cell cycle re-entry peaks at 7 dpr (Porrello et al., 2011). It will be important in the future to examine Hippo-deficient hearts carefully to determine the kinetics of cell cycle re-entry and perhaps to uncover the mechanisms underlying organ-wide cell cycle response. Our findings provide new insight into mammalian heart regeneration and lend support to the idea that endogenous cardiomyocytes can be manipulated in vivo to repair the heart. Recent work has shown that the Hippo effector Yap also promotes cardiomyocyte regeneration when overexpressed in late fetal and neonatal cardiomyocytes (Xin et al., 2013). Moreover, deleting Yap at the same immature cardiomyocyte stage results in reduced cardiomyocyte proliferation (Del Re et al., 2013). Our data support and extend those findings by revealing that, in addition to immature...
cardiomyocytes, Hippo deficiency enhances regeneration in mature cardiomyocytes. It will be important to investigate Yap target genes in the context of regeneration in order to understand the regenerative process in more depth.

Typically, a stressed cardiomyocyte re-enters the cell cycle but fails to proceed through cytokinesis, perhaps owing to an unyielding sarcomere structure or anillin localization defect (Engel et al., 2006; Kikuchi and Poss, 2012). Recently reported innovative approaches with therapeutic promise include in vivo reprogramming of cardiac fibroblasts to cardiomyocytes and delivering microRNAs directly to damaged heart (Efe et al., 2011; Bruneau, 2012; Jayawardena et al., 2012). It is conceivable that combining these different methods with molecules that can transiently reduce Hippo signaling in the heart might prove to be an effective method of regenerating adult human cardiomyocytes.

**MATERIALS AND METHODS**

**Mouse alleles and transgenic lines**

The Nkx2.5<sup>Cre</sup> transgenic line and floxed alleles for <i>ww45/Salvador</i> and <i>lats1-l2</i>/Warts have been described previously (Heallen et al., 2011). The <i>Myh6-cre/Esr1</i> transgenic line (The Jackson Laboratory) directs expression of a tamoxifen-inducible Cre in cardiomyocytes. The GitROSA<sup>26;</sup>Sortm4(ACB-TdTomato,-EGFP)Luo3 (abbreviated to <i>mTmG</i>) Cre reporter line (The Jackson Laboratory) expresses red fluorescence in the absence of Cre recombinease in all cell types. In the presence of Cre, theloxP-flanked mT cassette is deleted, red fluorescence is abolished, and downstream expression of eGFP (mG) green fluorescence is activated. <i>Myh6-cre/Esr1</i> mice were mated to <i>mTmG</i> mice to generate progeny that express cardiomyocyte-specific Cre that is tamoxifen-inducible and traceable via immunofluorescence imaging. DNA was extracted from tail biopsies for genotyping. Genotyping primers for <i>Myh6-cre/Esr1</i> and <i>mTmG</i> are as follows: 5′-AGTTGACACTGATCAGG-3′; 5′-ATACCAGGATCATGAAAGC-3′; 5′-TCATTGGGGGTCCCTGGC-3′; 5′-CGAGGGGATACCAAGCAATA-3′.

For Figs 1, 4 and 5, control was <i>Mhc cre-Ert</i>; <i>mTmG</i> and <i>Salv KO</i> were <i>Mhc cre-Ert</i>; <i>mTmG</i> and <i>Salv KO</i>; control was <i>Salv KO</i> and <i>Salv KO</i>. For Figs 1, 2 and Supplementary material Fig S1, <i>Lats1/2 KO</i> was <i>Mhc cre-Ert</i>; <i>mTmG</i>; <i>Lats1/2 KO</i>.

**DNA incorporation in the adult heart**

For adult cardiomyocyte renewal studies, 3- to 4-month-old animals of control or conditional knockout lines of <i>Salv</i> and <i>Lats1/2</i> were used. All mice were crossed with <i>Myh6-cre/Esr1</i> driver and the <i>mTmG</i> reporter lines for lineage tracing. For Cre activation, tamoxifen (0.5 mg) was administered at 0.5 and P6 (0.25 mg EdU 4 hours before harvest); for P12 samples, tamoxifen (0.5 mg) was administered at P8, P9, P10 and P11 (0.25 mg EdU 4 hours before harvest).

**Apex resection**

Surgical resection of the heart apex was performed on P8 mice as described by Porrello et al. (Porrello et al., 2011) using modified procedures. Vceryl sutures (6-0 absorbable) were used to close the thoracic cavity, and the entire procedure required ~12 minutes from the onset of hypothermia to recovery. Sham procedures excluded apex amputation. To increase survival rate and prevent maternal neglect and cannibalization, pups were fostered to nursing ICR mothers approximately the same age. Mice recovered up to 21 dpr, then were euthanized and dissected hearts were processed for histology and immunocytochemistry. Surface area measurements of resected apex tissue were calculated using a Zeiss StiefO Discovery.V12 stereoscope equipped with an AxioCam HRc digital camera to assay surgical reproducibility and determine amount of resected tissue of hearts categorized by scar severity. All measurements and functions were controlled by the Carl Zeiss Axiovision software program (Carl Zeiss Microimaging). Similarly, fibrotic scar size was measured using the procedures described above. Scar severity was categorized as follows: severe (transmural fibrosis of the apex), mild (trace fibrosis at the apex) or absent (fibrosis not detected). Lineage tracing of cardiomyocytes after heart apex resection was performed by crossing the <i>Salvador</i> floxed allele with <i>Myh6-cre/Esr1</i> and <i>mTmG</i> mice. Cre activity was induced with four consecutive intraperitoneal or subcutaneous injections of tamoxifen from P7 to P10. Following apex resection at P8, pups were injected with EdU (0.5 mg) 4 hours prior to heart excision. Hearts were excised at 4 and 21 dpr. For EdU pulse chase experiments, pups recovered for 4 dpr and EdU (0.25 mg/animal) was injected to label replicating DNA. Four hours after EdU injection, pups were euthanized and hearts excised. Fixation and tissue processing were performed as described below.

**LAD occlusion**

For P8 samples, surgical permanent occlusion of the left anterior descending coronary artery (LAD-O) was performed on P8 mice (Mahmoud et al., 2013). Nylon sutures (8-0 non-absorbable) were used to occlude the LAD. Proper occlusion was noted by blanching of the myocardium and also during dissection 3-4 weeks post occlusion via visual inspection. Vceryl sutures (6-0 absorbable) were used to close the thoracic cavity, and the entire procedure required ~12 minutes from the onset of hypothermia to recovery. Sham procedures excluded placement of a suture around the LAD. To increase survival rate and prevent maternal neglect and cannibalization, pups were fostered to nursing ICR mothers of litters approximately the same age. Mice recovered up to 3-4 weeks post-occlusion, were euthanized and dissected hearts were processed for histology and immunocytochemistry. Automated fibrotic scar size was measured using image segmentation MIQuant, open source code for Matlab (Nascimento et al., 2011).

For adult samples, LAD-O was performed as described for P8 with minor modifications. Tamoxifen (1.5 mg) was administered at three time points: 7 and 6 days pre-LAD-O and within 2 hours post-LAD-O. At 1, 2 and 3 weeks post-LAD-O, echocardiography was performed in the Baylor College of Medicine Mouse Phenotyping core using a VisualSonics 770 system equipped with a 30 MHz scanhead (RMV7007B). Mice recovered up to 3 weeks post-occlusion, were euthanized and dissected hearts were processed for histology and immunocytochemistry. Automated fibrotic scar size was measured as described for P8 LAD-O.

**Histology**

For trichrome staining, dissected hearts were immediately fixed with 10% formalin overnight at room temperature, dehydrated in an ethanol series, and paraffin embedded. Coronally sectioned tissues (7 μm) were deparaffinized in xylene, rehydrated and fixed in Bouin’s Fluid (EMS) at 36°C for 15 minutes. Following washes in deionized water, sections were sequentially stained with Weigert’s Iron Hematoxylin, Beibrich Scarlet-Acid Fuchsin solution, phoshotungstic/phosphomolybdic solution and Aniline Blue solution. Sections were dehydrated by ethanol, cleared in Xylene and slides were mounted.
siRNA knockdown

siRNA knockdown was performed on cultured mouse neonatal cardiomyocytes in vitro in 4-well plate format. At 80% confluency, cells were transfected with 1.5 μL Lipofectamine RNAiMax Transfection Reagent (Life Technologies) and 1.5 μL of predesigned siRNAs (10 μM; IDT, Coralville, Iowa) diluted in 50 μL of OPTI-MEM. Cells were stained for EdU following a 24-h incubation at normal growth conditions. siRNA duplex names: AurkB (MMC.RNAi.N011496.12.7), Birc5 (MMC.RNAi.N001012273.12.2), Ccne2 (MMC.RNAi.N001037134.12.1), Salv (MMC.RNAi.N022028.12.1), siRNA neg control (NC1 Negative Control Sequence).

Statistics

Differences between groups were examined for statistical significance using unpaired Student’s t-tests, ANOVA or χ² distribution. All error bars represent s.e.m. P<0.05 was regarded as significant.

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Author contributions

T.H. and Y.M. designed and performed experiments and analyzed data; J.L. and G.T. performed experiments and analyzed data; J.T.W. and R.L.J. provided reagents; J.F.M. designed and supervised experiments and analyzed data; T.H., Y.M. and J.F.M. wrote the manuscript.

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Supplemental material

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