Cardiac-Specific YAP Activation Improves Cardiac Function and Survival in an Experimental Murine MI Model

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Rationale: Yes-associated protein (YAP), the terminal effector of the Hippo signaling pathway, is crucial for regulating embryonic cardiomyocyte proliferation.

Objective: We hypothesized that YAP activation after myocardial infarction (MI) would preserve cardiac function and improve survival.

Methods and Results: We used a cardiac-specific, inducible expression system to activate YAP in adult mouse heart. Activation of YAP in adult heart promoted cardiomyocyte proliferation and did not deleteriously affect heart function. Furthermore, YAP activation after MI preserved heart function and reduced infarct size. Using adeno-associated virus subtype 9 (AAV9) as a delivery vector, we expressed human YAP (hYAP) in the adult murine myocardium immediately after MI. We found that AAV9:hYAP significantly improved cardiac function and mouse survival. AAV9:hYAP did not exert its salutary effects by reducing cardiomyocyte apoptosis. Rather, AAV9:hYAP stimulated adult cardiomyocyte proliferation. Gene expression profiling indicated that AAV9:hYAP stimulated expression of cell cycle genes and promoted a less mature cardiac gene expression signature.

Conclusions: Cardiac-specific YAP activation after MI mitigated myocardial injury, improved cardiac function, and enhanced survival. These findings suggest that therapeutic activation of YAP or its downstream targets, potentially through AAV-mediated gene therapy, may be a strategy to improve outcome after MI. (Circ Res. 2014;115:354-363.)

Key Words: heart failure ■ myocardial infarction ■ regeneration ■ survival

Myocardial infarction (MI) causes the loss of billions of cardiomyocytes (CMs). Morbidity and mortality after MI remain high despite modern medical and surgical management. The fundamental barrier to improving outcome is our inability to replace the lost CMs. To overcome this barrier, there has been tremendous interest in strategies to increase CM number by stimulating CM cell cycle re-entry, CM survival by inhibition of apoptosis, or CM differentiation from stem cells.

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The Hippo–Yes-associated protein (YAP) pathway, originally identified in Drosophila as an important regulator of tissue growth,1 has attracted recent interest as a potential means to enhance heart regeneration. In this highly conserved signaling pathway, YAP, a transcriptional coactivator, promotes organ growth and cellular proliferation by binding to sequence-specific transcription factors, most notably TEAD1 (TEA domain family member 1),2 thereby activating expression of cell cycle regulators and other target genes. YAP expression and activity are tightly controlled, as excessive YAP activity causes tissue overgrowth and cancer, whereas insufficient YAP activity causes tissue hypoplasia.3 The Hippo kinase cascade restrains YAP activity by catalyzing its phosphorylation on serine 127, which leads to its exclusion from the nucleus. Activation of YAP activity, either by inactivation of Hippo kinase cascade components3 or by forced expression of activated YAP lacking the Hippo kinase phosphorylation site,4,5 drives fetal CM proliferation, resulting in remarkable fetal overgrowth. On the contrary, cardiac-specific YAP deletion causes lethal cardiac hypoplasia.4,5 Thus, YAP is a potent stimulator of fetal CM proliferation.

Original received February 4, 2014; revision received May 13, 2014; accepted May 14, 2014. In April 2014, the average time from submission to first decision for all original research papers submitted to Circulation Research was 14.38 days.

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The online-only Data Supplement is available with this article at http://circres.ahajournals.org/lookup/suppl/doi:10.1161/CIRCRESAHA.115.303632/-/DC1.

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Circulation Research is available at http://circres.ahajournals.org

DOI: 10.1161/CIRCRESAHA.115.303632
In this study, we tested the hypothesis that adult-stage, cardiac-specific activation of YAP drives CM proliferation and improves outcome in MI. We found that long-term YAP activation did not cause cardiac hypertrophy. Transgenic or adenovirus-associated virus subtype 9 (AAV9)–mediated activation of YAP improved the heart function and survival after MI. These data show that cardiac-specific activation of YAP mitigates the injury caused by MI, and suggest that therapeutic activation of YAP or its downstream targets may be a strategy to improve outcome after MI.

Methods
Detailed Materials and Methods are provided in the Online Data Supplement. All animal procedures were approved by the Institutional Animal Care and Use Committee. MI, echocardiography, and MRI were performed and analyzed blinded to genotype and treatment targets by using collagenase perfusion to count dissociated CMs confirmed smaller average CM size in YAPGOF compared with control (Figure 2F). We confirmed this finding by using collagenase perfusion to count dissociated CMs confirmed smaller average CM size in YAPGOF compared with control (Figure 2F). We observed no change in sarcomere organization (Online Figure I), indicating that reduced CM size is not because of dedifferentiation. The measured CM number was higher in YAPGOF compared with control (Figure 2G).

Increased CM number in hearts after adult-stage YAP induction suggested that YAP stimulated CM cell cycle re-entry. We further tested this hypothesis by assessing CM proliferation after 4 weeks of YAP activation with 2 independent markers: phosphohistone H3 (pH3), an M-phase marker; and EdU uptake, an S-phase marker. In histological sections, the fraction of CMs positive for EdU or pH3 was, respectively, 4-fold and 5-fold higher in YAPGOF compared with controls (Online Figure II). To further confirm this finding and exclude potential artifacts related to difficulty in establishing CM boundaries in tissue sections, we repeated these measurements in dissociated CM preparations. These studies confirmed that a significantly higher fraction of CMs were labeled by EdU or pH3 in YAPGOF mice (Figure 2G and 2H). YAP significantly increased the mononuclear CM fraction (YAPGOF, 12.4% versus control, 7.1%), and EdU+ CMs were nearly exclusively mononuclear (Figure 2I). This observation is consistent with other reports that proliferating CMs are more likely to be mononuclear.

Results
Inducible CM-Specific YAP Expression Model
We generated Myh6-Cre; ROSA26R-rTATTA, TRE-YAP mice (referred to as YAPGOF), which express activated human YAP selectively in CMs under the control of Doxycycline (Figure 1A). The activated YAP protein contains the S127A mutation that inhibits its cytoplasmic sequestration by Hippo kinase phosphorylation. We used Myh6-Cre; ROSA26R-rTATTA lacking TRE-YAP as controls. To validate transgene expression in YAPGOF mice, we treated adult mice with doxycycline for 4 weeks and then analyzed YAP expression by Western blotting (Figure 1B–1D). Compared with controls, YAPGOF mice expressed elevated YAP in the heart but not in other organs (Figure 1B and 1C). Moreover, YAP expression was dependent on doxycycline administration (Figure 1D).

YAP Stimulates Adult CM Cell Cycle Activity
In Vivo
Doxycycline treatment from week of life 4 to 8 did not significantly change the size of YAPGOF hearts compared with their littermate controls (Figure 2A). At the same time, CM size was reduced (Figure 2B and 2C), suggesting that YAPGOF hearts contained more CMs (Figure 2D). We confirmed this finding by using collagenase perfusion to count dissociated CMs (Figure 2E). Morphometry on dissociated, rod-shaped CMs confirmed smaller average CM size in YAPGOF compared with control (Figure 2F). We observed no change in sarcomere organization (Online Figure I), indicating that reduced CM size is not because of dedifferentiation. The measured CM number was higher in YAPGOF compared with control (Figure 2G).

Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Term</th>
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<tr>
<td>AAV9</td>
<td>adenovirus-associated virus serotype 9</td>
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<tr>
<td>AAV9:Luci</td>
<td>AAV9 with luciferase expressed from the cardiac troponin T promoter</td>
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<tr>
<td>AAV9:YAP</td>
<td>AAV9 with human YAP[S127A] expressed from the cardiac troponin T promoter</td>
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<tr>
<td>CM</td>
<td>cardiomyocyte</td>
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<tr>
<td>EdU</td>
<td>5-ethyl-2′-deoxyuridine</td>
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<tr>
<td>MI</td>
<td>myocardial infarction</td>
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<tr>
<td>pH3</td>
<td>phosphohistone H3</td>
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<tr>
<td>RFP</td>
<td>red fluorescent protein</td>
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<tr>
<td>YAP</td>
<td>Yes-associated protein</td>
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<tr>
<td>YAPGOF</td>
<td>YAP gain-of-function mice (genotype Myh6-Cre; Rosa26R-rTATTA, TRE-YAP)</td>
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Figure 1. Cardiac phenotype of Yes-associated protein gain-of-function (YAPGOF) mice. A, Doxycycline (Dox)-regulated, cardiomyocyte (CM)-specific expression system. B, Cardiac YAP expression of Dox-treated mice was measured by Western blot. C, Reverse transcription polymerase chain reaction showing YAP overexpression in YAPGOF heart. D, Dox-dependent YAP expression in YAPGOF mice. rTATTA indicates reverse tetracycline activator protein.
absence of nuclear or cellular division (leading to polyploidy), nuclear division in the absence of cellular division (leading to multinucleation), and the infrequency of CM compared with nonmyocyte proliferation. To provide further independent evidence that activated YAP stimulates CM cell cycle activity that generates new CMs, we used an in vivo clonal analysis strategy (Figure 3A) similar to that recently used to demonstrate adult CM proliferation downstream of neuregulin. In Myh6-MerCreMer; Rosa26rtTA/; TRE-YAP; CAG-fs-RFP mice, low-dose tamoxifen treatment induced irreversible red fluorescent protein (RFP) labeling and expression of the doxycycline-dependent reverse tetracycline activator protein in a small fraction of CMs (Online Figure III). Doxycycline treatment selectively activated YAP expression in RFP-labeled CMs in the experimental group containing the TRE-YAP transgene, but not in controls lacking this transgene. If YAP stimulated CM proliferation and new myocyte formation, then we expected to find clusters containing ≥2 RFP+ CMs more frequently in the presence of the TRE-YAP transgene. Indeed, after 4 weeks on doxycycline, the number of RFP+ clusters containing ≥2 labeled CMs was significantly greater in the aYAP-expressing hearts (Figure 3B and 3C), indicative of CM proliferation.

Chance labeling of adjacent CMs by independent Cre recombination events in this single-color assay leads to a background of clusters with ≥2 cells that are not because of proliferation. To better account for these events, we refined the assay using a multicolor Cre reporter (Figure 3D–3F), where each Cre recombination event triggers expression of 1 of the 4 fluorescent reporters: cyan fluorescent protein, RFP, nuclear green fluorescent protein, and yellow fluorescent protein. We had difficulty detecting endogenous fluorescence of cyan fluorescent protein above autofluorescent background, and nuclear green fluorescent protein is only visible in cells when the plane of section goes through the nucleus. Therefore, we only

Figure 2. Yes-associated protein (YAP) stimulates adult cardiomyocyte (CM) proliferation. YAPGOF and control (Ctrl) mice were treated with doxycycline (Dox) from 4 to 8 weeks of life. Hearts were analyzed at 8 weeks. A, Heart weight-to-body weight (HW/BW) ratio was not significantly different between groups. B and C, CM cross-sectional area was lower in YAPGOF than in Ctrl mice. WGA-stained sections were used to measure CM cross-sectional area. *P<0.05. E to G, The size and number of CMs isolated from hearts by collagenase perfusion. n=4 to 8. Bar, 25 μm. D, The relative number of CMs in YAPGOF and Ctrl hearts based on heart weight and CM cross-sectional area. *P<0.05. E to G, The size and number of CMs isolated from hearts by collagenase perfusion. n=4 to 8. Bar, 25 μm. H and I, The fraction of CMs bearing proliferative markers 5-ethynyl-2′-deoxyuridine (EdU) or phosphohistone H3 (pH3) was determined in dissociated heart preparations. Yellow arrow indicates proliferating CM, and arrowhead indicates a nonmyocyte. n=5. Bar, 25 μm. J, Nucleation of all CMs with EdU CMs was determined in dissociated cardiomyocytes. The large majority of proliferating CMs in Ctrl and YAPGOF groups were mononuclear. DAPI indicates 4',6-diamidino-2-phenylindole; TNNI3, troponin I type 3; and WGA, wheat germ agglutinin.

Figure 3. Clonal analysis of Yes-associated protein (YAP)–induced adult cardiomyocyte (CM) proliferation. A to C, One-color clonal analysis of CM proliferation. A, Schematic of the experimental design. *Adjacent cells that were labeled by 2 separate recombination events, rather than by proliferation of a single-labeled cell. B, Representative images. Red arrows indicate a cluster of 3 red cells. C, Quantification showing that clusters of ≥2 red fluorescent protein–positive (RFP+) cells were found more frequently in YAP gain-of-function (YAPGOF) hearts. n=5 hearts per group. Bar, 50 or 10 μm. D to F, Two-color clonal analysis of CM proliferation. D, Experimental design. A bichromatic cluster caused by 2 independent labeling events. E, Representative images. Yellow arrows indicate bichromatic clusters; and red arrows, monochromatic clusters. The number of arrows sharing a common tail indicates the number of cells in that cluster. F, Quantification showing that monochromatic clusters with ≥2 cells were more common in YAPGOF. Bichromatic clusters were not significantly different between groups. n=4 hearts per group. Bar, 25 μm. WGA indicates wheat germ agglutinin; and YFP, yellow fluorescent protein.
considered clusters of cells as monochromatic or bichromatic (red and yellow arrows, respectively, Figure 3E). Bichromatic clusters of ≥2 cells involved multiple recombination events rather than proliferation. These clusters occurred at similar frequency in YAPGOF and control. In contrast, monochromatic clusters of ≥2 CMs potentially arose from proliferation. These clusters were more common in YAPGOF (Figure 3F), consistent with YAP stimulation of formation of new CMs through proliferation. This assay may underestimate the extent of YAP-driven CM proliferation, because it is likely that not all labeled CMs expressed YAP, and because random adjacent recombination continues to contribute to a subset of multicell clusters, increasing the baseline number of 2-cell clusters. Overall, the clonal assays supported the conclusion that a YAP stimulated productive CM cell cycle activity in the adult heart and indicated that YAP action is cell autonomous.

**Long-Term Induction of YAP Did Not Cause Cardiac Hypertrophy**

Long-term YAP ectopic activation in the liver caused liver tumors.10,11 To test whether long-term YAP activation is deleterious to the heart, we overexpressed YAP in the heart by treating YA PGOF mice with doxycycline for 4.5 months. Long-term YAP activation did not cause cardiac hypertrophy, as indicated by the heart size, heart-to-body weight ratio, and left ventricle wall thickness (Figure 4A–4C). Echocardiography showed that heart function of YAPGOF mice was similar to their control littermate at 2 and 3 months of doxycycline induction, but at 4 months, heart function was slightly lower than controls. The left ventricle wall thickness of control and YAPGOF mice was similar even after 4 months of YAP activation (Figure 4D). One month of YAP activation did not cause measureable cardiac fibrosis, whereas 4 months of YAP activation was accompanied by a slight, statistically significant increase in myocardial fibrosis (Figure 4E and 4F). Although YAP is an oncogene, no tumors were observed in YAPGOF mice. These results indicate that long-term YAP overexpression did not cause cardiac hypertrophy and that YAP activation for >3 months may have deleterious effects (fibrosis; mild reduction of heart function) that would need to be balanced against potential benefits.

**Cardiac-Specific Expression of Activated YAP Mitigated Myocardial Injury**

YAP stimulation of CM proliferation, and the tolerance of the heart to sustained YAP activation, prompted us to ask whether YAP activation after MI is beneficial. We designed our experiment to start human YAP (hYAP) induction 1 week after permanent left anterior descending ligation (Figure 5A), because it is likely that the major events in the immediate period after MI relate to cell death and we sought to focus on the pro proliferative activity of YAP. We permanently ligated the left anterior descending coronary artery in 8-week-old control and YAPGOF mice to induce MI. We then started doxycycline 1 week after surgery and continued it for 4 more weeks. At the end of the study, we measured heart function by cardiac MRI and then measured heart and body weight. The hearts were collected for histological analysis. The MRI data showed higher ejection fraction (ejection fraction %) in the YAPGOF group compared with controls (Figure 5B and 5C), indicating that YAP activation post-MI improves cardiac function. After MI, the heart typically undergoes hypertrophic remodeling to compensate for lost CMs, resulting in a higher heart-to-body weight ratio. In the long run, this cardiac hypertrophy is detrimental for cardiac function. We found that 1 month of YAP activation decreased heart-to-body weight ratio (Figure 5D), suggesting that YAP activation attenuates hypertrophic remodeling post-MI. We visualized the myocardial scar in picro-sirius red/fast green-stained cryosections, where fibrotic tissue stains red and viable myocardium stains green (Figure 5E).12 Compared with control mice, YAPGOF mice had smaller infarct size, and this was confirmed by quantitative analysis (Figure 5F). These data together suggest that activation of YAP after MI improved heart function and reduced infarct size.

To investigate the mechanisms underlying the beneficial activity of YAP, we measured CM apoptosis and proliferation in the post-MI hearts. At this time point 5 weeks after the MI, we did not detect significant apoptosis of CM by terminal deoxynucleotidyl transferase dUTP nick-end labeling staining in remote, infarct, or border zones, although we did find many terminal deoxynucleotidyl transferase dUTP

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**Figure 4. Cardiac-specific overexpression of Yes-associated protein (YAP) is well tolerated in adult mouse.** Cardiac-specific overexpression of YAP is well tolerated in adult mouse. A and B, Four months of YAP induction in adult heart did not grossly affect heart size, morphology (A), or heart weight-to-body weight (HW/BW) ratio (B). A, Bar, 5 mm. C and D, Echocardiographic analysis of prolonged YAP overexpression on adult heart function. Ctrl indicates control; Dox, doxycycline; FS, fractional shortening; LVPW,d, diastolic left ventricular posterior wall thickness; NS, not significant; and YAPGOF, YAP gain-of-function. *P<0.05. n=4. E and F, Effect of YAP overexpression on myocardial fibrosis, assessed by picro-sirius red/fast green staining. E, Representative sections. Fibrotic areas stain red. F, Quantification.
and serotype 9 is significantly cardiotropic. We generated AA V9:hY AP after MI. We induced MI and then injected virus directly into the myocardium at 3 positions along the margin of the ischemic area (Figure 6A). Experimentally, this was best done immediately after MI rather than 1 week later. In a short-term study, we administered EdU 4 days after MI and analyzed hearts 1 day later for viral expression and CM proliferation. In a long-term study, we followed the MI mice for 23 weeks to determine whether AA V9:hYAP treatment increased survival after MI. An additional control group was unoperated and received no AAV. By 5 days after MI and AAV9 delivery, AA V9:YAP drove elevated expression of YAP (Figure 6B). AA V9 is known to establish expression of its cargo for more than several months. We confirmed sustained expression of our AA V9 constructs by measuring luciferase activity 23 weeks after MI and AA V9:Luci delivery. As shown in Figure 6C, the luciferase signal was robust in the hearts of AA V9:Luci-treated mice, but was undetectable in mice treated with AA V9:YAP. Moreover, luciferase activity was only detected in the heart and not in liver, skeletal muscle, or other organs. These data demonstrated that direct myocardial injection of AA V9 with the troponin T (TNT) promoter successfully drove long-term overexpression of cargo genes specifically in the heart.

We determined the effect of AA V9:hYAP on murine survival after MI, because improved survival is a gold-standard metric of the efficacy of a therapeutic intervention for MI. During a 23-week follow-up period, we found that AA V9:hYAP enhanced survival after MI compared with AA V9:Luci (P=0.0327; Figure 6D). This indicated that AA V9:hYAP enhances the outcome after MI.

To evaluate whether AA V9:hYAP improved survival by preserving ventricular function, we performed echocardiography on mice at 2 and 4 weeks and cardiac MRI at the completion of the study at 23 weeks. At 2 and 4 weeks, MI hearts had significantly reduced systolic function compared with sham controls. At 2 weeks, the degree of cardiac dysfunction was comparable between AA V9:hYAP and AA V9:Luci treatment groups (Figure 6E). However, at 4 weeks the AA V9:hYAP-treated hearts had significantly better systolic function than AA V9:Luci controls (Figure 6E). At 23 weeks, cardiac MRI showed that systolic function of both groups was depressed and was not

AAV9 Delivery of hYAP

Because cardiac-specific transgenic YAP activation seemed promising, we moved forward to test the potential of YAP gene therapy for improving myocardial outcome after MI. AA V9 is a safe and efficient vector for in vivo gene transfer, and serotype 9 is significantly cardiotropic. We generated AA V9:cTNT::3Flag-hYAP (AA V9:hYAP), in which the CM-specific chicken troponin T promoter drives expression of triply Flag-tagged, activated human YAP[S127A] (Online Figure IVA). As a control, we generated AA V9:cTNT::Luciferase (AA V9:Luci), in which 3Flag-hYAP was replaced by luciferase.

We validated performance of the viral vectors by delivering them subcutaneously to 3- to 5-day-old neonatal mice. Uninjected wild-type neonatal mice were used as controls in these validation studies. Seven days after virus injection, hearts were analyzed by Western blotting and immunohistochemistry. Western blots showed a single endogenous YAP band in control mice and an additional band in AA V9:hYAP-treated mice, reflecting the larger, exogenous 3Flag-hYAP (Online Figure IVB). The luciferase protein was detected in hearts treated with AA V9:Luci but not in control hearts (Online Figure IVC). Immunofluorescence staining for Flag or luciferase demonstrated strong, specific signals in the AA V9:hYAP and AA V9:Luci group, respectively (Online Figure IVD and IVE). Together these data validate highly efficient AA V9-mediated YAP and luciferase expression in the heart.

AAV9:hYAP Improved Cardiac Function and Survival After MI

We next evaluated the effect of AA V9:hYAP on the outcome after MI. We induced MI and then injected virus directly into:

nick-end labeling–positive nonmyocytes. In contrast, the fraction of CMs labeled by EdU (administered twice during the fourth post-MI week) or pH3 was higher in YAPGOF compared with control in the tissue border and remote from the infarct (Figure 5G and 5H). These results indicate that YAP stimulates post-MI CM proliferation, leading to reduced infarct size and improved myocardial outcome after MI.

### Figure 5: Activated Yes-associated protein (YAP) improved myocardial outcome after myocardial infarction (MI)

**A.** Experimental design. **B and C.** MRI assessment of ventricular function. **B.** Representative images. **C.** Quantification. Bar, 5 mm. **D.** Heart weight-to-body weight (HW/BW) ratio was lower in YAPGOF. **E and F.** Scar size, measured as a percentage of ventricular circumference in sequential picro-sirius red- and fast green-stained sections. Bar, 5 mm. **G.** 5-Ethynyl-2′-deoxyuridine–positive (EdU) cardiomyocytes (CMs) were more frequent in YAPGOF border and remote zones. Representative section shows an EdU+ CM (arrow) in YAPGOF. Bar, 25 μm. **H.** Phosphohistone H3–positive (pH3′) CMs were more frequent in YAPGOF. Representative section shows a pH3′ CM (arrow). Bar, 10 μm. DAPI indicates 4',6-diamidino-2-phenylindole; DOX, doxycycline; EF, ejection fraction; TNNI3, troponin I type 3; and WGA, wheat germ agglutinin.
significantly different between the surviving 3 AAV9:Luci mice and 6 AAV9:hYAP mice, as assessed by ejection fraction and left ventricular end-systolic volume (Figure 6F). The difference between the 2- to 4-week echo data and the 23-week MRI data is likely to be attributable to survival bias; that is, the mice with the lowest ejection fraction were likely to have died before 23 weeks and thereby masked differences between groups when examined at the time of cardiac MRI. This conclusion was supported by subgroup analysis of the 4-week echo data: mice that died between 4 and 23 weeks had significantly reduced heart function compared with mice that survived to 23 weeks (Figure 6G).

YAP is an oncogene, and its activation in liver caused liver tumors.10,11 AAV9 is reported to transduce mainly heart, skeletal muscle, and liver,14 leading to the possibility that AAV9:hYAP might induce liver tumors. As shown in Figure 6C, the YAP signal was only detected in the heart, likely because of direct myocardial injection and our use of the cardiac-specific TNT promoter. To exclude AAV9:hYAP-induced liver tumors more fully, we examined livers from AAV9:Luci- and AAV9:hYAP-treated mice. The gross morphology and liver weight of AAV9-treated livers were indistinguishable from sham controls (Online Figure VA and VB). Moreover, liver sections stained with hematoxylin and eosin showed no difference among 3 groups (Online Figure VC). These data indicate that myocardial injection of AAV9:hYAP is not oncogenic, at least within the 23-week time frame of the study.

**AAV9:hYAP Induced CM Proliferation Without Affecting CM Apoptosis**

Because YAP regulates organ size by both promoting cell proliferation and suppressing cell death,10 we hypothesized that YAP activation after MI protects the heart through the same mechanisms. We tested this hypothesis by measuring cell proliferation and cell death in the hearts collected from the short-term study, that is, 5 days after MI and myocardial delivery of viral vector. We measured the fraction of CMs that passed through S phase during the EdU pulse that was administered 4 days after MI. In unoperated controls, EdU+ CMs were rare. In the border zone of MI mice, there were significantly more EdU+ CMs in the AAV9:hYAP group than in the AAV9:Luci group (arrow, Figure 7A and 7B; arrowheads indicate nonmyocytes). To investigate the effect of AAV9:hYAP on CM death, we used terminal deoxynucleotidyl transferase dUTP nick-end labeling and TNNI3 (troponin I type 3) costaining to measure the frequency of apoptotic CMs in the infarct border region. We measured the fraction of CMs that passed through S phase during the EdU pulse that was administered 4 days after MI. In unoperated controls, EdU+ CMs were rare. In the border zone of MI mice, there were significantly more EdU+ CMs in the AAV9:hYAP group than in the AAV9:Luci group (Figure 7C and 7D). There was no significant difference in apoptotic CM frequency between AAV9:hYAP and AAV9:Luci groups.
The proproliferative activity of YAP requires its interaction with TEAD transcription factors. Mutation of human Yap serine 94 to alanine blocks YAP interaction with TEAD and abolishes its proliferative activity. To determine whether TEAD interaction and YAP mitogenic activity are essential for YAP preservation of function in the MI-injured heart, we generated AAV9-YAP[S94A;S127A] (abbreviated AAV9:hYAPS94A) and compared it with AAV9:hYAP. One month after MI and AAV delivery, AAV9:YAP and AAV9:hYAPS94A drove equivalent levels of YAP expression (Online Figure VIA). AAV9:YAP-transduced hearts showed higher fractional shortening and lower heart weight-to-body weight ratio than AAV9:Luci control, consistent with our results reported above. However, AAV9:hYAPS94A-transduced mice were not distinguishable from AAV:Luci controls (Online Figure VIB and VIC). Furthermore, AAV9:hYAP- but not AAV9:hYAPS94A- treated mice had more EdU+ CMs than the AAV9:Luci control (Online Figure VID and VIE). These data together suggest that YAP-TEAD interaction and YAP mitogenic activity are required for preserving heart function after MI.

Transcriptional Profiling Highlights Biological Processes Modulated by AAV9:hYAP

To gain greater understanding of the effect of YAP on myocardial injury responses, we performed genome-wide transcriptional profiling. Five days after MI and AAV injection, we collected heart apexes for microarray analysis (Figure 8A). Compared with AAV9:Luci group, 774 genes and 350 genes were down- and upregulated in the AAV9:hYAP group, respectively (P<0.05; fold change >0.5 log2 scale; Online Table III and Figure 8B). Genes upregulated by YAP were related to cell cycle regulation, wound healing, and inflammation (Figure 8C). Downregulated genes were enriched for functional terms related to cardiomyopathy, heart development, and myofibril assembly. Interestingly, the other major category of functional terms enriched for downregulated genes was related to energy metabolism, tricarboxylic acid cycle, and hexose metabolic processes. We chose several differentially expressed genes from these categories for validation by quantitative reverse transcription polymerase chain reaction. Overall the agreement between microarray and quantitative reverse transcription polymerase chain reaction was excellent. Compared with MI+AAV9:Luci, the MI+AAV9:hYAP group had higher expression of genes linked to the inflammation (Ccl2, Ccl7, Mmp8, Il1b) and cell cycle regulation (Aurka, Ccnb1, Ccn2, Cdc20, Cdk1) and lower expression of genes related to sarcomeres (Mybpc3, Myh6, Myh7, Myl3; Figure 8D).

Prolonged myocardial inflammation after MI contributes to adverse myocardial remodeling. For example, upregulation of the inflammatory marker interleukin-6 was a predictor of adverse myocardial outcome after MI. To determine whether YAP effect on myocardial inflammatory markers was transient or sustained, we measured expression of eight inflammatory marker genes 1 month after MI, including Ccl2, Ccl7, Mmp8, and Il1b, which were upregulated by AAV:YAP at 5 days after MI compared with AAV:Luci (Figure 8D). These 4 inflammatory marker genes, as well as additional markers Icam1, Il10, and Tgfb1, were expressed at comparable levels between AAV:YAP and AAV:Luci at 1 month, suggesting resolution of the effect observed at 5 days (Figure 8E). Expression of Il6 was significantly downregulated by AAV:YAP (Figure 8E), consistent with the beneficial effect of AAV:YAP on myocardial wound healing.

Overall, our data show that Yap promotes a less mature myocardial gene expression profile with reduced expression of sarcomere and oxidative phosphorylation genes and increased expression of cell cycle genes. YAP enhanced the inflammatory response shortly after MI, but this response was short-lived and resolved by 1 month.

Discussion

The mammalian heart’s limited innate regenerative capacity, the vulnerability of the heart to myocardial insults, and the inadequacies of current heart disease treatment have led to a search for approaches to enhance adult heart regeneration. Enticing adult mammalian CMs to re-enter the cell cycle productively has proven to be a tremendous challenge, and approaches that increase or sustain fetal or neonatal CM proliferation have often failed to translate to the adult mammalian heart. Thus, approaches initially developed in model systems based on CMs that have not terminally exited the cell cycle, such as adult zebrafish or fetal or neonatal mouse, need to be critically assessed in an adult mammalian system.
The Hippo–YAP pathway is a critical regulator of organ size, and YAP activation through either YAP overexpression or Hippo loss of function enhances cell proliferation. Based on these data, we and others studied Hippo–YAP regulation of heart growth and showed that YAP robustly stimulates CM proliferation in fetal and newborn heart. In the current study, we induced YAP expression in adult CMs using 2 independent methods (inducible, CM-specific transgene and cardiac-specific AAV) and found that YAP promotes adult CM cell cycle re-entry. Moreover, we showed that YAP activation in the heart is well tolerated for ≤3 months and does not induce CM hypertrophy. After MI, YAP activation reduced scar size, improved heart function, and robustly enhanced survival. While this study was in preparation, 2 articles were published that reported on Hippo–YAP and postnatal heart regeneration. Xin et al reported that constitutive overexpression of activated YAP enhanced neonatal cardiac regeneration and that constitutive YAP activation reduced scarred and enhanced heart function after MI in 1-month-old mice. Heallen et al studied postnatal inactivation of Hippo kinase components Salv and Lats1/2. One role of Salv and Lats1/2 is to inhibit YAP; however, these genes also regulate several other CM responses, including hypertrophy, apoptosis, and autophagy. Adult-stage knockout of these genes stimulated CM proliferation, and adult-stage Salv knockout commencing 1 week before MI improved heart function and reduced scar size at 3 weeks post-MI. Our study is consistent with the proregenerative effects of YAP activation and advances the field by clearly showing that YAP drives CM cell cycle re-entry (as opposed to maintenance of a fetal proliferative state). Furthermore, we establish the long-term survival benefit of YAP activation. Critical for potential therapeutic translation, we further show that YAP retains efficacy when activated at the time of, or even 1 week after, MI.

We used transcriptional profiling to define major biological processes influenced by YAP activation after MI. Interestingly, upregulated genes were enriched for functional annotations related to inflammatory responses and wound healing. There is growing evidence that inflammatory responses play a complex role in myocardial injury responses. While aspects of sustained myocardial inflammation adversely affect myocardial outcome after MI, inflammatory responses are also essential for regenerative responses, including mammalian

Figure 8. Biological processes perturbed by Yes-associated protein (YAP) activation. A, Experimental design. Five days after myocardial infarction (MI) and adeno-associated virus subtype 9 with human YAP[S127A] expressed from the cardiac troponin T promoter (AAV9:hYAP) or AAV9 with luciferase expressed from the cardiac troponin T promoter (AAV9:Luci) injection, total RNA was prepared from heart apex. B, Differentially expressed genes (FC >0.5 in log2 scale; P<0.05) segregated samples by treatment group. C, GO term enrichment analysis of up- and downregulated genes. All GO terms in Biological Processes_FAT or KEGG Pathways with Benjamin–Hochberg P<1.0E-4 are shown, after removing closely related terms. D, Quantitative reverse transcription polymerase chain reaction (qRT-PCR) validation of microarray gene expression measurements. E, qRT-PCR measurement of inflammation genes 1 month after MI and AAV injection. P<0.05. ARVC indicates arrhythmogenic right ventricular cardiomyopathy; FC, fold change; GO, gene ontology; and KEGG, Kyoto Encyclopedia of Genes and Genomes.
heart regeneration. Our finding that YAP regulates wound healing is consistent with a recent report showing that YAP is essential for skin wound healing. YAP acutely upregulated inflammation genes, with expression normalizing by 1 month after MI. It will be interesting to assess whether this transiently enhanced inflammatory response contributes to or detracts from the overall beneficial activity of YAP.

Our data also suggest that YAP promotes a CM transcriptional profile notable for downregulation of muscle and oxidative metabolism genes characteristic of mature CMs and upregulation of cell cycle genes. This transcriptional signature suggests that YAP activation may promote a less mature CM state conducive to proliferation. YAP regulation of mitochondrial structure and function is intimately linked to its regulation of proliferation, suggesting a potential direct link between YAP activation, mitochondrial gene expression, and proliferation in the heart. Sarcomere disassembly has been suggested to be integral to CM proliferation. YAP induced downregulation of sarcomere genes such as Myh6, Myh7, Mybpc3, and Myd3, and this downregulation might contribute to YAP stimulation of CM proliferation. However, YAP-induced sarcomere gene downregulation seems to be selective to the context of MI, because we did not observe it in cultured neonatal rat CMs. Additional studies will be required to decipher how YAP coordinates regulation of genes related to muscle, metabolism, and proliferation.

Measurement of CM proliferation is a difficult challenge. Measurement of the fraction of CMs in S phase (EdU) or M phase (pH3) can overestimate the extent of new CM formation, because CMs can become polyploid or multinucleated. It has been suggested that measurement of aurora B kinase provides additional specificity by marking cells undergoing cytokinesis, but it is necessary to distinguish the subset of Aurora B cells with staining localized to the cleavage furrow (and therefore undergoing cytokinesis) from nuclear staining observed throughout mitosis. This distinction is difficult and subjective in tissue sections. Previously, a multicolor fluorescent reporter was used to study the clonal origins of the zebrafish heart, and another group used the cluster size of a sparsely activated monochromatic reporter to measure CM proliferation in response to an exogenous agent. Here, we combined these strategies and coupled sparse Cre activation of a doxycycline-inducible transgene with Cre activation of a multicolor reporter. This assay provided an independent method to measure productive, transgene-stimulated CM proliferation. Because some Cre-labeled cells may not overexpress YAP, this method may underestimate the extent of proliferation. However, the observation that single-color, multicell CM clusters are more frequent in YAPGF, whereas dual-color multicell clusters do not provide clear evidence that YAP stimulates productive CM proliferation. Furthermore, this method provides evidence that YAP’s mitogenic activity is cell autonomous.

Adult YAP activation drove modest levels of CM proliferation, whereas fetal YAP activation robustly augmented CM proliferation. This likely accounts for the modest beneficial effect of YAP on heart function and fibrosis after MI. Why is YAP mitogenic activity reduced in adult CMs? Potentially, changes in YAP protein–protein interactions in the adult heart might reduce its transcriptional activity. For instance, α-catenin and plakoglobin are strongly expressed in adult heart, and YAP interaction with these factors at cell–cell junctions may reduce its transcriptional potency (even when phosphorylation is inhibited by mutation of the Hippo kinase site). Alternatively, the well-known general block to adult CM proliferation may pose a nonspecific impediment to YAP-stimulated proliferation.

In conclusion, we demonstrated that YAP activation in the adult heart is well tolerated. We showed that YAP activation after MI improves cardiac function and survival, providing proof of concept that YAP or its downstream targets have therapeutic efficacy in improving the outcome after MI. YAP stimulates CM proliferation and modulates myocardial inflammatory responses, and these effects likely contribute to its efficacy in improving the outcome after MI.

Acknowledgments

We thank David Bennett at Small Animal Imaging Core in the Beth Israel Deaconess Medical Center for collecting the MRI data. We thank Vionnie Yu and David Scadden for sharing the CAG-fs-RFP mouse before publication. We are grateful to R.J. van der Geest (Leiden University Medical Centre, Leiden, The Netherlands) for providing the Mass software for the analysis of the MRI data.

Sources of Funding

Z. Lin was supported by an American Heart Association (AHA) postdoctoral fellowship. W.T. Pu was supported by National Institutes of Health HL116461 and HL100401, by an AHA Established Investigator Award, and by charitable contributions from Gail Federici Smith, Edward Marram, and Karen Carpenter.

Disclosures

None.

References

Activation of YAP in adult CMs, driven either by adult-stage activation of a cardiac-restricted transgene or by a CM-selective adenovirus subtype 9 vector, stimulates CM proliferation.

Novelty and Significance

What Is Known?

- Reduced cardiomyocyte (CM) number underlies many forms of heart failure. Therapeutic approaches that stimulate CM proliferation would be immensely beneficial.
- The evolutionarily conserved Hippo–Yes-associated protein (YAP) pathway critically regulates organ growth and has been shown to stimulate fetal and neonatal CM proliferation.
- Constitutive YAP activation from fetal through adult stages sustains CM cell cycle activity, enhances neonatal and juvenile heart regeneration, and attenuates cardiac dysfunction after myocardial infarction.
- Whether or not induced expression of YAP stimulates cell cycle re-entry of adult, terminally differentiated CMs, and the efficacy of therapeutic YAP activation in the adult heart shortly after myocardial infarction are largely unknown.

What New Information Does This Article Contribute?

- YAP activation in the heart did not cause cardiac hypertrophy and was well tolerated for 3 months.
- Activation of YAP in adult CMs, driven either by adult-stage activation of a cardiac-restricted transgene or by a CM-selective adenovirus subtype 9 vector, stimulates CM proliferation.
- Activation of YAP in adult CMs after myocardial infarction attenuates cardiac dysfunction and improves long-term survival.
- YAP activation after myocardial infarction upregulates genes related to inflammation and cell cycle and downregulates genes related to oxidative metabolism and muscle cells.
- Mosaic transgene activation in CMs labeled with a multicolor fluorescent reporter is an effective method to test the transgene’s cell-autonomous stimulation of CM proliferation.

In an effort to address the tremendous biomedical need for approaches to enhance the limited innate regenerative capacity of the mammalian heart, we studied the effect of YAP activation in the adult heart. Our results show that sustained YAP activation is well tolerated in the heart and that YAP drives productive adult CM proliferation. Furthermore, YAP activation shortly after myocardial infarction improved myocardial function and survival. Thus, activation of YAP or its downstream targets should be further evaluated as regenerative approaches to heart disease.
Cardiac-Specific YAP Activation Improves Cardiac Function and Survival in an Experimental Murine MI Model

Zhiqiang Lin, Alexander von Gise, Pingzhu Zhou, Fei Gu, Qing Ma, Jianming Jiang, Allan L. Yau, Jessica N. Buck, Katryna A. Gouin, Pim R.R. van Gorp, Bin Zhou, Jinghai Chen, Jonathan G. Seidman, Da-Zhi Wang and William T. Pu

Circ Res. 2014;115:354-363; originally published online May 15, 2014; doi: 10.1161/CIRCRESAHA.115.303632

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/115/3/354

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2014/05/15/CIRCRESAHA.115.303632.DC1
Cardiac specific YAP activation post myocardium infarction improved cardiac function and mouse survival

A. Detailed Materials and Methods
B. Supplemental References
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   Online Table II. Primers used in this study.
   Online Table III. Microarray data table.
D. Supplemental Figures
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   Online Figure II. Cardiomyocyte cell cycle activity induced by expression of activated YAP.
   Online Figure III. Tamoxifen titration to achieve infrequent cardiomyocyte labeling by Myh6-MerCreMer.
   Online Figure IV. AAV construction and validation.
   Online Figure V. Direct intramyocardial injection of AAV9-hYAP did not cause liver tumors.
   Online Figure VI. YAP-TEAD interaction is required for the beneficial activity of YAP after MI.
A. Detailed Materials and Methods.

Animal experiments

All animal procedures were approved by the Institutional Animal Care and Use Committee. TetO-YAP\(^1\), Rosa26\(^{fs-rTA2}\), and MHC\(\alpha\)-Cre\(^3\) alleles were previously described. Dox was administered at 1 mg/mL in drinking water. 5-ethynyl-2’deoxyuridine (EdU) was administered at 5 \(\mu\)g/g body weight IP. To induce MI, mice aged 8 weeks were subjected to LAD ligation as described previously.\(^4\)

Echocardiography was performed on a VisualSonics Vevo 2100 with Vevostrain software. Magnetic resonance imaging (MRI) was carried out at Small Animal Imaging Core in Beth Israel Hospital. For in vivo bioluminescent imaging, mice were administered Luciferin (150 \(\mu\)g/gram body weight IP), sedated using isoflurane, and imaged 10 min later on a Xenogen IVIS System.

MI, echocardiography, and MRI were performed blinded to genotype and treatment group.

Immunohistochemistry.

Hearts were fixed and embedded in OCT. 8 \(\mu\)m cryosections were used for immunostaining, using antibodies listed in Online Table 1. EdU was detected with the Click-iT EdU imaging kit (Invitrogen). TUNEL staining was performed using the Roche in situ death detection kit. Imaging was performed on a Fluoview 1000 confocal microscope, or a Nikon TE2000 epifluorescent microscope.

AAV9 packaging

3Flag-hYAP and Luciferase were separately cloned into ITR-containing AAV plasmid (Penn Vector Core P1967) harboring the chicken cardiac TNT promoter, to get pAAV.cTnT::3Flag-hYAP and pAAV.cTnT::Luciferase, respectively. AAV9 was packaged in 293T cells with AAV9:Rep-Cap and pHelper (pAd deltaF6, Penn Vector Core) and purified and concentrated by gradient centrifugation\(^5\). AAV9 titer was determined by quantitative PCR.
Gene Expression

Real time PCR was performed with Syber Green or Taqman detection using an ABI 7500 thermocycler. PCR primers are listed in Online Table II. Expression profiling of total RNA from heart apex was performed using Affymetrix 2.0 ST microarrays. The microarray data is available at GSE54612 and tabulated in Online Table III. GO term analysis used DAVID.  

Infarct size measurement

Heart sections were collected at regular intervals. Sections were stained with Sirius Red-Fast Green. Digital images were captured and infarct sizes were calculated according to the formula: \[ \frac{\text{infarct perimeter (epicardial + endocardial)}}{\text{total perimeter (epicardial + endocardial)}} \times 100 \].

Statistics

Values are expressed as mean ± SEM. For two group comparisons, Student's t-test was used to test for statistical significance. To analyze data containing more than two groups, we used ANOVA with the Tukey HSD post-hoc test. Both tests were performed using JMP 10.0 (SAS).

B. Supplemental References


C. Online Tables

Online Table I. Antibodies used in this study

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### Online Table II. Primers Used in This Study

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**ABI Taqman assays**

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*m=mouse; h=human
Online Figure I. Lin et al.

Representative images of sarcomere morphology of cardiomyocytes isolated from adult control and YAP\textsuperscript{Gof} hearts. Filamentous actin was stained with phalloidin. Cardiac myosin heavy chain antibody recognized both α and β isoforms. No difference in sarcomere organization was observed between treatment groups. Bar=50 μm
Online Figure II. Lin et al.

Cardiomyocyte cell cycle activity induced by expression of activated YAP. After Dox-induced expression of activated YAP from weeks of life 4 to 8, hearts were analyzed for cell cycle markers.

A. The fraction of cardiomyocytes in S-phase was determined by measuring EdU uptake. At least 5000 CMs were sampled per heart. n=6. Bar = 25 µm.

B. The fraction of cardiomyocytes in M-phase was determined by immunostaining for phosphohistone H3 (pH3). YAP increased the fraction of CMs in M-phase. At least 8000 CMs were sampled per heart. n=6. Bar = 25 µm.
Online Figure III. Lin et al.
Tamoxifen titration to achieve infrequent cardiomyocyte labeling by Myh6-MerCreMer.
A-B. The indicated dose of tamoxifen was administered to Myh6-MCM::CAG-fsRFP mice. We measured the frequency of labeled cardiomyocytes (B).
C. Tamoxifen-induced activation of RFP or YFP from the Rosa26<sup>fsConfetti</sup> reporter. Bar, 40 µm.
Online Figure IV. Lin et al.
AAV construction and validation.
A. AAV vectors used to generate AAV9:hYAP and AAV9:Luci contained the AAV inverted terminal repeats (ITRs), the chicken TNNT2 (cTNT) promoter, and a polyadenylation (pA) signal. 3Flag-hYAP[S127A] or luciferase were cloned into this AAV backbone to generate pAAV.cTNT.3Flag-hYAP (AAV9:hYAP) or pAAV.cTNT.Luciferase (AAV9:Luci), respectively.
B-C. AAV9:hYAP or AAV9:Luci viruses were injected subcutaneously into 3 day old mice. 5 days later, hearts were analyzed by western blotting. Mice that received no AAV9 injection served as control (Ctrl). Expression of 3Flag-hYAP (B, indicated by arrowhead) and Luciferase (C) was confirmed with western blot.
D. AAV9:hYAP expressed Flag-tagged YAP in the heart. Bar = 50 µm.
E. AAV9:Luci expressed luciferase in the heart. Bar = 50 µm.
Online Figure V. Lin et al.
Direct intramyocardial injection of AAV9-hYAP did not cause liver tumors.

A. Gross morphology of livers from sham control, AAV9-Luci and AAV9-hYAP.
B. AAV9-hYAP did not alter liver weight to body weight ratio (LiW/BW). No significant difference was found between groups. Sham, n=3; AAV9-Luci, n=3; AAV9-hYAP, n=6.
C. HE staining of liver sections did not reveal any hepatic tumors. Bar = 50 µm.
Online Figure VI, Lin et al.

**YAP-TEAD interaction is required for beneficial activities of YAP after MI.** Interaction of overexpressed YAP was abolished by mutation of serine 94 to alanine. Mice were treated with AAV9:YAP, AAV9:YAPS94A, or AAV9:Luci at the time of coronary artery ligation. Hearts were examined 1 month after MI.

**A.** Fraction shortening measured by echocardiography.

**B.** Heart weight to body weight ratio.

**C.** Total YAP mRNA level measured by qRT-PCR.

**D-E.** Cardiomyocyte proliferation assessed by EdU incorporation rate. EdU was administered in the fourth week after MI. White arrow heads indicate EdU positive non-cardiomyocytes. White arrow indicates EdU positive cardiomyocyte. Bar=50 µm.

*P<0.05. n=4