

The cytoprotective impact of yes-associated protein 1 after ischemia-reperfusion injury in AC16 human cardiomyocytes

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Impact statement

Genetically engineering the cells of the heart after myocardial infarction to display a more regenerative phenotype is a promising therapy for heart failure patients. Here, we support a regenerative role for yes-associated protein 1, the main effector protein of the Hippo signaling pathway, in AC16 human cardiomyocytes as a potential therapeutic gene target for cardiac repair after myocardial infarction.

Abstract

The Hippo-signaling pathway is a mechanism implicated in cardiomyocyte cytoprotection and regeneration after a myocardial infarction. Yes-associated protein 1, the main effector protein of this pathway, acts as a co-transcriptional activator to promote cardiomyocyte proliferation and survival. However, the biological mechanisms by which yes-associated protein 1 protects the heart post-MI are currently unknown. Here, we propose that yes-associated protein 1 plays a critical role in cardiomyocyte cytoprotection after simulated ischemia-reperfusion injury. AC16 human cardiomyocytes were infected with lentiviral plasmids containing normal human yes-associated protein 1 and a constitutively active form of

YAP, YAP1S127A. Cells were exposed to ischemia-reperfusion injury using a hypoxic chamber. Hippo-signaling characterization after ischemia-reperfusion injury was performed via Western blotting and reverse transcriptase polymerase chain reaction. Cell viability, apoptosis, and cellular hypertrophy were assessed as a measure of cytoprotection. The GSK3 β inhibitor CHIR99021 was used to investigate cross-talk between Hippo and Wnt-signaling and their role in cytoprotection after ischemia-reperfusion-injury. Ischemia-reperfusion injury resulted in significant decreased expression of the non-phosphorylated Hippo signaling kinases MST1 and LATS1, along with decreased expression of YAP/TAZ. Overexpression of yes-associated protein 1 improved cellular viability, while reducing hypertrophy and apoptosis via the ATM/ATR DNA damage response pathway. Activation of β -catenin in YAP-infected cardiomyocytes synergistically reduced cellular hypertrophy after ischemia-reperfusion-injury. Our findings indicate that yes-associated protein 1 is cytoprotective in AC16 human cardiomyocytes after ischemia-reperfusion injury, which may be mediated by co-activation of the canonical Wnt/ β -catenin pathway. Thus, activation of yes-associated protein 1 may be a novel therapeutic to repair the infarcted myocardium.

Keywords: Human, myocardial infarction, cardiomyocyte, Hippo, YAP1, hypertrophy, reactive oxygen species, cytoprotection, Wnt

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Introduction

Acute myocardial infarction (MI) is the most prevalent cause of heart failure, afflicting millions around the globe and a contributing to one of the largest financial burdens on the health care system.¹ It is caused by the occlusion of blood flow in the coronary arteries, resulting in the death of billions of cardiomyocytes, fibroblasts, and endothelial cells.² Following MI, the remaining cardiomyocytes attempt to compensate for the increased workload of the left ventricle.³ Although adaptive at first, this process

becomes detrimental overtime through left ventricular remodeling and subsequent hypertrophy. The remaining cardiomyocytes cannot keep up with the increased metabolic demands of the heart, inevitably leading to heart failure. Unfortunately, treatment options for heart failure patients are limited to invasive surgeries such as heart transplantation and the implantation of a left ventricular assist device.⁴ In order to innovate new treatment options, it is critical to understand the molecular pathophysiology of heart failure and the cardiomyocyte response to MI.

Until recently, cardiomyocytes were thought to have largely limited proliferative ability due to their terminal differentiation.⁵ The cardiomyocytes of neonatal mouse hearts display remarkable regenerative ability^{6–9}; however, this ability is quickly lost seven days after birth.¹⁰ After this transitional stage, the adult cardiomyocytes display limited proliferative ability after injury, a mechanism proposed to also occur in humans.^{11,12} Therefore, engineering the existing cardiomyocytes to display a fetal-like phenotype after MI may be a promising therapeutic for cardiac repair after MI.

Evolutionarily conserved from *drosophila*, the mammalian Hippo pathway functions in regulating organ size and cell proliferation.^{13–15} First, cytoplasmic mammalian Ste20-like kinase 1 (MST1) binds to its adaptor protein salvador (SAV).¹⁶ This complex phosphorylates the large tumor suppressor kinase 1/2 (LATS1/2),¹⁷ leading to binding with its cofactor Mps one binder kinase activator 1 (MOB1).¹⁸ This second complex then phosphorylates the co-transcriptional activators yes-associated protein 1 (YAP1) and tafazzin (TAZ), leading to YAP1's degradation via ubiquitination or inactivation via 14–3–3 binding.^{19,20} Inactivation of the upstream Hippo kinases results in the accumulation of unphosphorylated YAP1, promoting its nuclear entry,²¹ where it binds to several transcription factors including transcriptional enhancer factor 1 (TEAD1), forkhead box O (FoxO), T-cell factor/lymphoid enhancer factor (TCF/LEF), T-box transcription factor 5 (TBX5), and β -catenin to activate proliferative, autophagic, and anti-apoptotic genes.^{22–24} Wntless (Wnt)-signaling is well implicated during myocardial development and repair; however, there is limited research that directly studies the interaction between YAP1 and β -catenin after MI,²⁵ warranting further investigation into this interaction and the regeneration potential of these two transcription factors.

Interestingly, heart failure patients exhibit significant decreases in YAP1/phosphorylated YAP1 protein expression ratio in failed left ventricles,²⁶ indicating a vital role for YAP1 in the response to MI. In addition, several studies have attempted to manipulate members of the Hippo signaling pathway to elucidate its effects within the context of cardiac regeneration and have been reviewed extensively.²⁴ Depletion of LATS1/2 in murine models leads to myocardial expansion, while transgenic LATS1/2 mice display reduced ventricular size and increased apoptosis after pressure overload.^{17,27} Moreover, overexpression of YAP1 in a murine MI model resulted in significant regeneration of the myocardium and improved cardiac function.²⁸ Together, these findings suggest that inactivation of the Hippo-signaling cascade, or overexpressing YAP1, in the adult cardiomyocyte may result in regeneration of the cardiac tissue. However, a better understanding of these molecular mechanisms after MI is imperative in bringing these therapies to the clinic. Here, we investigate the molecular mechanisms of YAP1 overexpression in AC16 human cardiomyocytes after an *in vitro* model of ischemia-reperfusion (IR) injury. Using Western blotting and reverse transcriptase polymerase chain reaction (RT-PCR), we investigated the change in expression of Hippo-signaling proteins after IR injury. We utilized a lentiviral-based

technique to overexpress YAP1 in AC16 human cardiomyocytes and assessed its effects on cell viability, apoptosis, oxidative stress, and hypertrophy. Lastly, to investigate the cross-talk between Hippo and Wnt signaling, we activated both β -catenin and YAP1 in AC16 human cardiomyocytes and assessed their effects on cellular hypertrophy after IR injury.

Materials and methods

Cell culture

The AC16 human left ventricular cardiomyocytes (Millipore Sigma) were used for this study. AC16 cells stained positively for several cardiac markers including atrial natriuretic peptide, brain natriuretic peptide, α -catenin, myosin heavy chain 7, troponin I, connexin 43, and GATA4.²⁹ Cells were cultured in Dulbecco's Modified Eagle's Medium/Nutrient F-12 Ham (DMEM/F-12) (ThermoFisher Scientific) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin. Cells at passages three to five were used throughout the study.

Generation of YAP1-lentiviral vectors

Two lentiviral vectors promoting the overexpression of YAP1 in AC16 human cardiomyocytes were cloned into entry vectors containing a green fluorescence protein (GFP) gene, under the mouse phosphoglycerate kinase 1 promoter, and a puromycin resistance gene, under the CMV promoter. Gateway recombination was used to generate a flag-tagged YAP1 plasmid (YAP) and a flag-tagged YAP-mutant that is resistant to phosphorylation at the 127 serine position (YAP^{S127A}), thus promoting its nuclear entry.³⁰ Plasmid sequences were confirmed via polymerase chain reaction (PCR) (Supplemental Table 1, Supplemental Figure 1(a)) and genomic sequencing, which was performed at the McGill University and Génome Québec Innovation Centre, Montréal, Canada (data not shown). 293T competent cells were gifted by Dr. Trembley (McGill University, Montreal, Canada) and were transfected with the recombinant YAP and YAP^{S127A} plasmids. Two days following transfection, the viral supernatant was collected and used to infect the AC16 cardiomyocytes. Four days after infection, GFP-positive cells were selected for via fluorescence-activated cell sorting (FACS) (Supplemental Figure 1(b)) and cultured in 1–10 μ g/mL puromycin (Sigma). GFP expression was visualized on fixed cells using the Zeiss LSM 780 confocal microscope (Supplemental Figure 1(c)).

IR injury model

On the day of experimentation, the FBS-supplemented DME/F-12 media was replaced with serum-free DME/F-12 media and the cells were placed in a hypoxic chamber ($\sim 0\%$ O₂) (Mitsubishi Gas Company) using a single anaerobic sachet (Mitsubishi Gas Company). Reperfusion injury was performed by transferring the cells to normoxic conditions (20% O₂), and replacing the media with FBS-supplemented DME/F-12 media.

Detection of reactive oxygen species

Detection of H₂O₂ and superoxide radicals was performed using a reactive oxygen species (ROS) detection kit (Abcam). After IR injury, cells were stained with fluorescently-labeled antibodies for H₂O₂ (green) and superoxide radicals (orange). Detection of ROS was performed via fluorescence signal detection using TECAN Infinite® M200 Microplate reader (Tecan Group Ltd, Männedorf, 125 Switzerland). Experiments were performed using six to eight replicates on three different experimental days.

Immunoblotting

Total cell lysates were obtained using RIPA buffer (150 mM sodium chloride, 1.0% NP-40 or Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS (sodium dodecyl sulfate), 50 mM Tris, pH 8.0) supplemented with protease inhibitor (Sigma). Protein concentrations were determined via the DC protein quantification assay (BioRad) as per the manufacturer's instructions. Proteins were separated and transferred to nitrocellulose membranes (BioRad). The following antibodies were used from Cell Signaling Technologies at a 1:1000 dilution: rabbit anti-human MST1, rabbit anti-human LATS1, rabbit anti-human YAP/TAZ, rabbit anti-human pYAP, rabbit anti-human ATM, rabbit anti-human ATR, rabbit anti-human Chk1, rabbit anti-human Chk2, rabbit anti-human BRCA1, and rabbit anti-human β -actin. Secondary goat anti-rabbit-IgG-HRP (Cell Signaling Technologies) was used at 1:3000 dilution for all Western blots. All experiments were performed in triplicates.

Immunocytochemistry

Cardiomyocytes were seeded into chamber slides (BD Falcon). After IR injury, cells were fixed with 4% paraformaldehyde (Electron Microscopy Sciences) and permeated with 0.5% Triton X-100 (Electron Microscopy Sciences). The following antibodies were used from Cell Signaling Technologies: rabbit anti-human YAP/TAZ (1:500), rabbit anti-human pYAP (1:500), rabbit anti-human pp53 (1:500). Alexa Fluor 594 Dye-conjugated secondary antibody (Invitrogen) was used as the secondary antibody. Slides were mounted using DAPI (Vectashield; Vector Laboratories Inc.) and visualized using a Zeiss LSM 780 confocal microscope. For nuclear intensity quantification, images were randomly acquired at 40 \times magnification under a fixed exposure time. At least $n=200$ was used for all quantification of immunostaining data. CellProfiler imaging software was utilized for analyses. All experiments were performed using 8–10 replicated on three different experimental days.

Crystal violet cell viability staining

Crystal violet (CV) assay was utilized to determine the appropriate durations of hypoxia and reperfusion injury that resulted in significant cellular stress. Cardiomyocytes were seeded in 96-well plates. After IR injury, the media was removed and replaced with 100 μ L of 0.05% CV staining reagent (Sigma). The plates were washed three to five

times with running water and allowed to dry. Methanol was used to dissolve the CV stain. The plate was incubated on a rotator at room temperature for 30 min. All CV experiments were conducted using 8–10 replicates. Absorbance signal was detected using TECAN Infinite® M200 Microplate reader (Tecan Group Ltd, Männedorf, 125 Switzerland).

TUNEL assay

TUNEL assay (Abcam) was performed to assess cardiomyocyte apoptosis according to the manufacturer's instructions. Briefly, cardiomyocytes were seeded in 6-well plates at 70–80% confluency. After IR injury, the cell was fixed and labeled with DNA labeling solution (Abcam) and anti-BrdU-red antibody (Abcam). Apoptosis was measured via positive staining for BrdU (Ex/Em = 488/576) using the FACSCanto II Flow Cytometer (Becton Dickinson Biosciences). All experiments were performed in triplicates.

Assessment of cellular hypertrophy

Anti-actin (Life Technologies) was used to visually assess cell size according to the manufacturer's instructions in chamber slides (BD Falcon). Briefly, cells were fixed and incubated with anti-actin solution for 30 min. Following incubation, slides were mounted using DAPI (Vector Laboratories Inc.) and visualized using a Zeiss LSM 780 confocal microscope. Images were randomly acquired at 20 \times magnification under a fixed exposure time. ImageJ imaging software was used to quantify the cell area. At least $n=100$ was used for all quantification of immunostaining data. Cellular area is expressed relative to control cardiomyocytes in fully supplemented medium.

Detection of metabolic activity

Alamar blue assay (Fischer) was used to detect the metabolic activity of cardiomyocytes according to the manufacturer's instructions. Briefly, cells were seeded into 96-well plates at 60–70% confluency. After IR injury, cells were incubated in the Alamar Blue reagent for 4 h. Detection of metabolic activity was performed via absorbance detection using TECAN Infinite® M200 Microplate reader (Tecan Group Ltd, Männedorf, 125 Switzerland). All experiments were performed using 8–10 replicated on three different experimental days. Reduction percentage was calculated using the following formula

$$\text{Percentage reduction} = \frac{[(117.216) \times A_{570} - (80.586) \times A_{600}]}{[(155.677) \times A'_{600} - (14.652) \times A'_{570}] \times 100\%} \text{ where}$$

117.216: molar extinction coefficient of Alamar Blue in the oxidized form at 600 nm

80.586: molar extinction coefficient of Alamar Blue in the oxidized form at 570 nm

14.652: molar extinction coefficient of Alamar Blue in the reduced form at 600 nm

155.677: molar extinction coefficient of Alamar Blue in the reduced form at 570 nm

A_{570} : absorbance of test wells at 570 nm
 A_{600} : absorbance of test wells at 600 nm
 A'_{600} : absorbance of negative control wells at 600 nm
 A'_{570} : absorbance of negative control wells at 570 nm

RT-PCR. RNeasy Mini Kit (Qiagen) combining protocol: Total RNAs (1.5 μ g) were used for first strand cDNAs synthesis using SuperScript III First-Strand Synthesis System (Invitrogen). qPCRs were performed using QuantiFast[®] SYBR Green PCR kit (Qiagen) on LightCycler 1.5 (Roche). The qPCR data were analyzed using PrimePCR Analysis (Bio-RAD), and relative mRNA expressions of interested genes were normalized using housekeeping gene GAPDH. Sequences of gene-specific primers are listed in Supplemental Table 2.

Statistical analysis

Student *t* tests were used to determine significance between the protein expression of Hippo molecules before and after IR injury. One-way ANOVA tests were used to determine significance between YAP and YAP^{S127A} cardiomyocytes and control cells after IR injury for all other assays. This was followed by *post hoc* Tukey's multiple comparisons test to determine statistical differences between all groups. Results were analyzed at a significance level of $P < 0.05$. All graphs were generated using GraphPad Prism 8 software.

Results

Hippo-signaling is dysregulated after IR injury

We utilized a CV staining assay to determine the appropriate durations of hypoxia and reperfusion injury that resulted in significant cellular stress. Through our optimizations, 24 h of ischemia and 5 h of reperfusion caused significant damage, generation of ROS, and antioxidant enzyme response, resembling the phenotype of cardiomyocytes after MI (data not shown). Western blotting displayed significant reductions in protein expression of unphosphorylated MST1 ($P < 0.05$), LATS1 ($P < 0.01$), and YAP/TAZ ($P < 0.01$), with enhancement of pYAP expression

(Figure 1(a) and (b)). RT-PCR analysis revealed a significant increase in LATS1 ($P < 0.05$) gene expression, with no change in MST1 or YAP1 (Figure 1(c)). These results indicate that Hippo-signaling is dysregulated after IR injury.

Generation of YAP1-overexpressing AC16 cardiomyocytes

Western blotting and immunostaining were used to detect the expression of total and nuclear YAP1 expression in lentiviral generated YAP and YAP^{S127A}-overexpressing cardiomyocytes. A vehicle plasmid lacking the YAP1 sequence was also utilized as an infection control for all subsequent experiments. YAP and YAP^{S127A} cardiomyocytes displayed significant increased total YAP1 protein expression (Supplemental Figure 2(a) and (b), $P < 0.01$ and $P < 0.0001$, respectively) according to Dunett's *post hoc* multiple comparisons test. YAP/TAZ immunostaining suggested significantly greater nuclear YAP1 localization in YAP and YAP^{S127A} cells ($P < 0.01$, Supplemental Figure 2 (c) and (d)) according to Dunett's *post hoc* multiple comparisons test. These results confirm successful infection of the YAP1-lentiviral plasmids and also suggest increased expression of active YAP1 under normal conditions.

Infected AC16 cardiomyocytes have increased YAP1 total protein expression and activation after IR injury

One-way ANOVA revealed significant differences in YAP1 protein expression in infected and non-infected cardiomyocytes before and after IR injury (Figure 2(a) and (b), $P < 0.0001$). Tukey's *post hoc* multiple comparison's test revealed that after IR injury, the reductions seen in YAP1 protein expression were attenuated completely and increased by approximately 4-fold in both YAP and YAP^{S127A} cardiomyocytes ($P < 0.0001$). YAP^{S127A} cardiomyocytes also displayed significantly greater FLAG protein expression compared to YAP cardiomyocytes (Supplemental Figure 1(d) and (e), $P < 0.0001$), indicating that the YAP^{S127A} proteins are more resistant to degradation. We also performed YAP/TAZ immunostaining on infected and non-infected cardiomyocytes before and after IR injury. There was a statistically significant

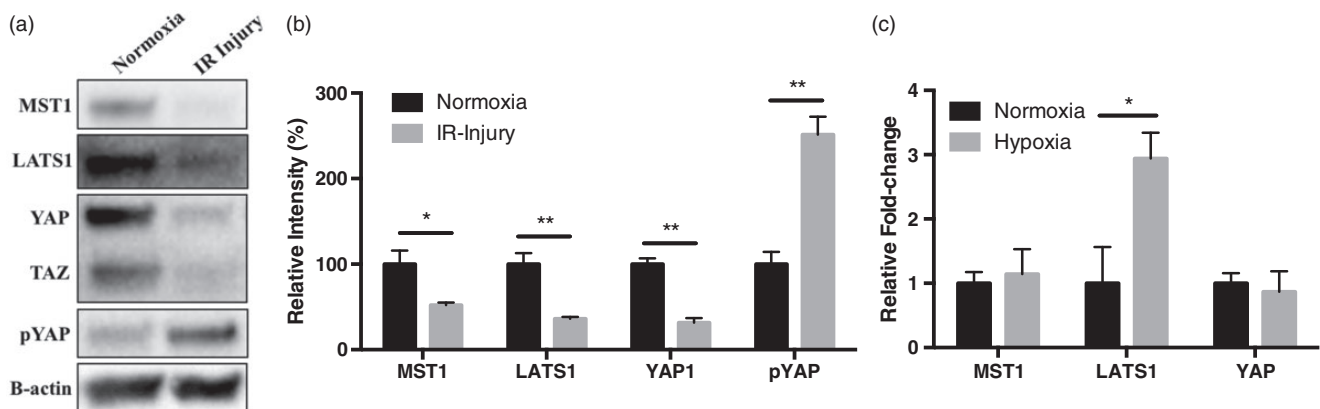


Figure 1. Hippo-signaling is dysregulated after IR injury. (a) Western blot of MST1, LATS1, YAP/TAZ, and pYAP protein expression after IR injury. (b) Densitometry of Hippo-signaling molecules after IR injury normalized to cardiomyocytes under normoxic conditions. (c) Relative fold-change of Hippo-signaling molecules after IR injury normalized to cardiomyocytes under normoxic conditions. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.0001$.

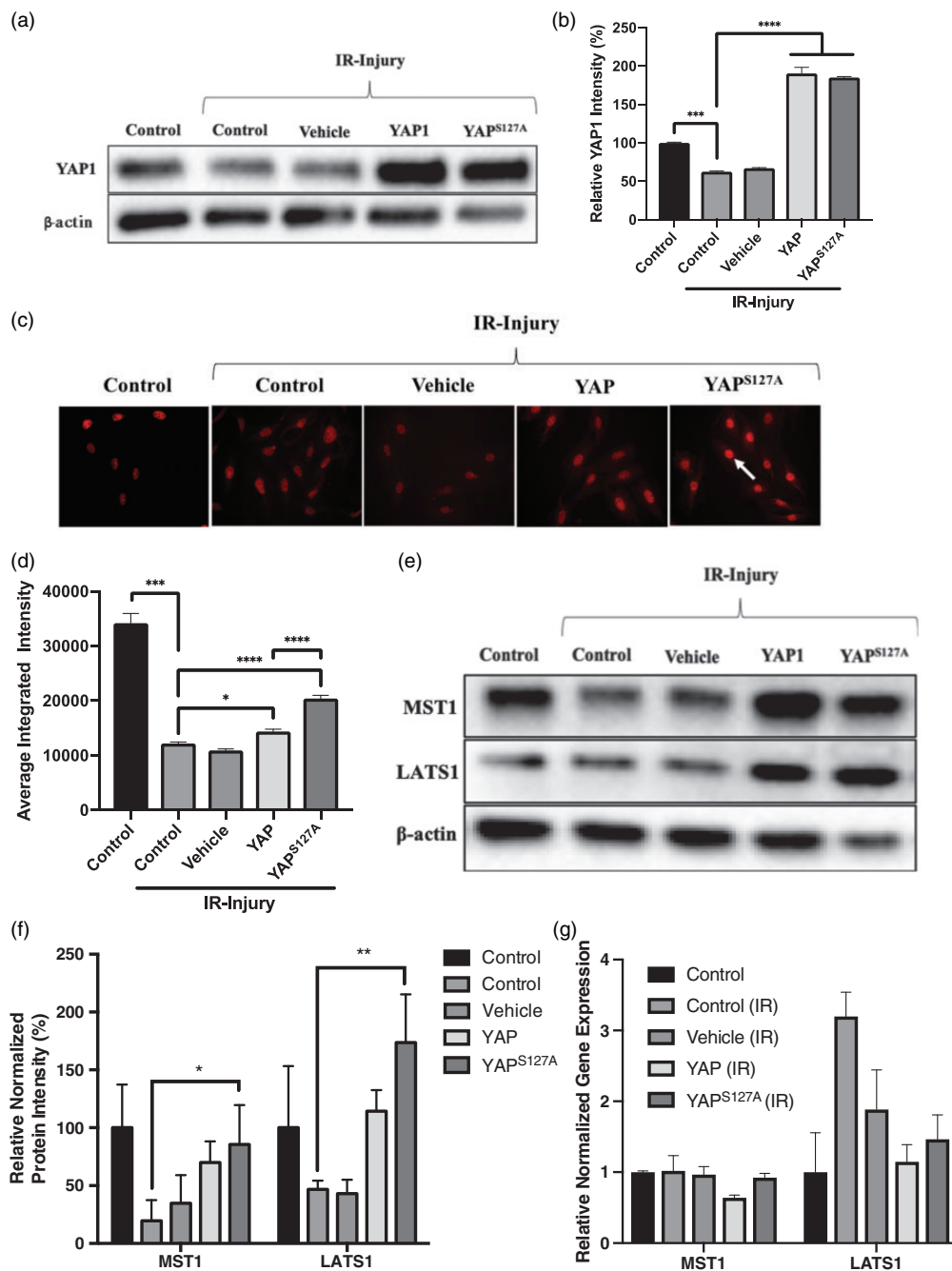


Figure 2. YAP and YAP^{S127A} cardiomyocytes increased total YAP1 protein expression and nuclear localization after IR injury. (a) Representative Western blot of YAP1 protein expression in non-infected and infected cardiomyocytes after IR injury. (b) Densitometry of YAP1 protein expression in infected cardiomyocytes normalized to non-infected cardiomyocyte after IR injury. (c) Representative images for YAP1 immunostaining of non-infected and infected cardiomyocytes after IR injury. (d) Average integrated intensity of nuclear YAP1 in non-infected and infected cardiomyocytes after IR injury. (e) Representative Western blot of MST1 and LATS1 protein expression in non-infected and infected cardiomyocytes after IR injury. (f) Relative fold-change in mRNA expression of MST1 and LATS1. * $P < 0.05$, *** $P < 0.001$, **** $P < 0.0001$. (A color version of this figure is available in the online journal.)

difference between all groups as determined by one-way ANOVA (Figure 2(c) and (d), $P < 0.0001$). Tukey's multiple comparisons test suggested significant nuclear localization of YAP1 in YAP and YAP^{S127A} cardiomyocytes ($P < 0.05$ and $P < 0.0001$, respectively), with even greater activation of YAP1 in the YAP^{S127A} cardiomyocytes in comparison to the YAP cardiomyocytes ($P < 0.0001$). In addition, YAP^{S127A} cardiomyocytes displayed significant increases in MST1 and LATS1 protein expression after IR-injury (Figure 2(e)

and (f)). RT-PCR analysis of Hippo genes revealed no significant differences in MST1 gene expression after IR injury; however, a trend towards significance was seen for LATS1 gene expression as determined by one-way ANOVA (Figure 2(g)). These data suggest successful overexpression and activation of YAP1 after IR injury in the infected cardiomyocytes and that phosphorylation of the S127 site on YAP may play a critical role in YAP1's nuclear exclusion.

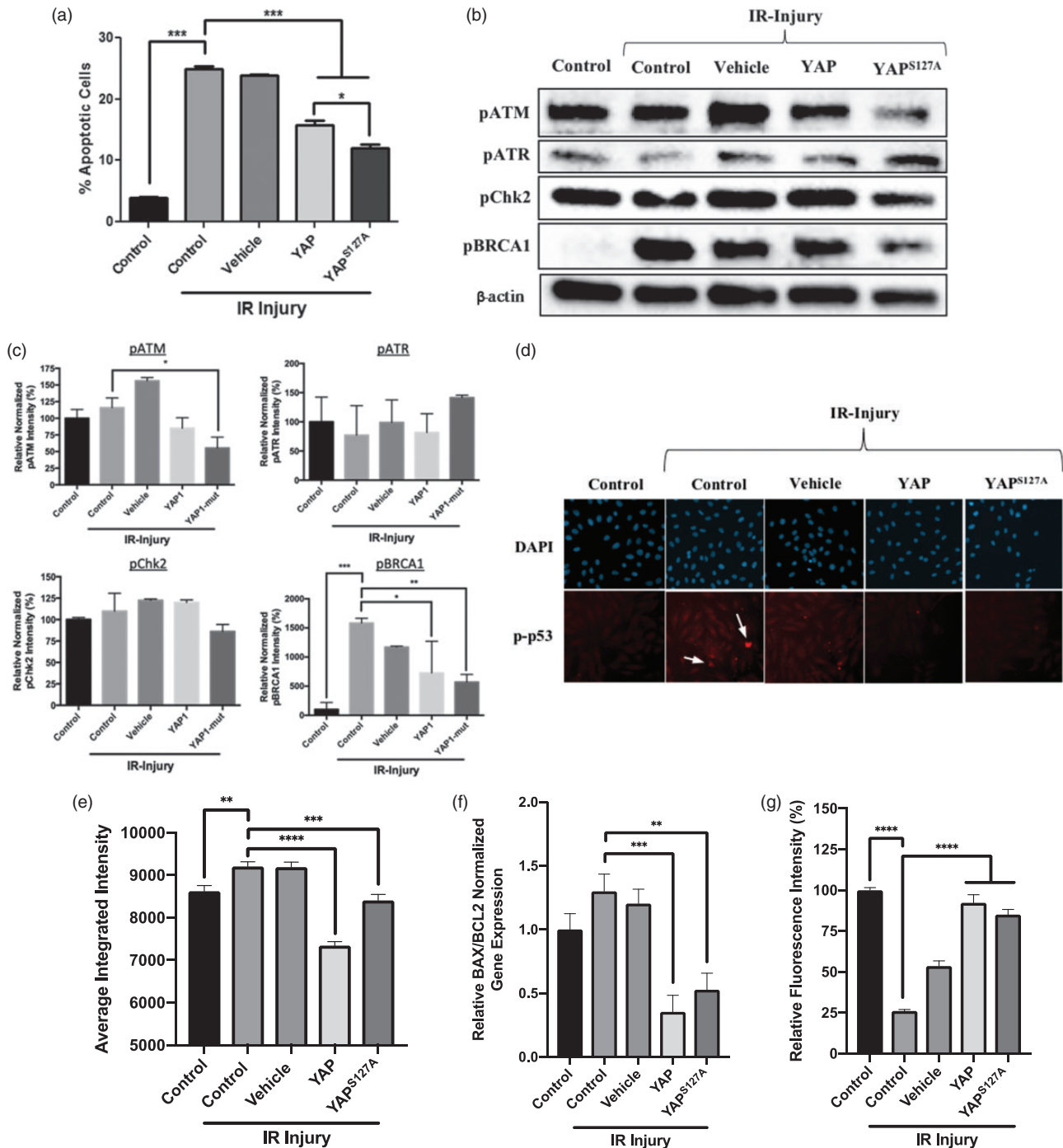


Figure 3. YAP1 protects cardiomyocytes from apoptosis after IR injury. (a) Quantification of BrdU⁺ cells via TUNEL flow cytometry. (b) Western blot of ATM/ATR DNA damage response elements in YAP and YAP^{S127A} cardiomyocytes after IR injury. (c) Densitometry of DNA damage protein expression in infected cardiomyocytes normalized to non-infected cardiomyocyte. (d) Representative images of p53 immunofluorescence in infected cardiomyocytes after IR injury. (e) Densitometry of p53 protein expression in infected cardiomyocytes normalized to non-infected cardiomyocyte. (f) Relative fold-change in mRNA expression of BAX/BCL2 ratio. (g) Relative fluorescence intensities of Alamar Blue metabolic activity assay. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. (A color version of this figure is available in the online journal.)

YAP1 protects AC16 cardiomyocytes from apoptosis after IR injury

After IR injury, cardiomyocytes displayed significant cell death as indicated by TUNEL assay flow cytometry (Figure 3(a), $P < 0.0001$). One-way ANOVA analysis showed a significant difference between all group means ($P < 0.0001$). Tukey's *post hoc* multiple comparisons test

indicated that YAP and YAP^{S127A} cells significantly attenuated apoptosis compared to non-infected cells ($P < 0.0001$). YAP^{S127A} displayed even greater reductions in apoptosis compared to YAP cardiomyocytes ($P < 0.05$). Furthermore, YAP^{S127A} cardiomyocytes displayed reductions in pATM and pBRCA1 protein expression after IR injury (Figure 3(b) and (c)). We also assessed for the

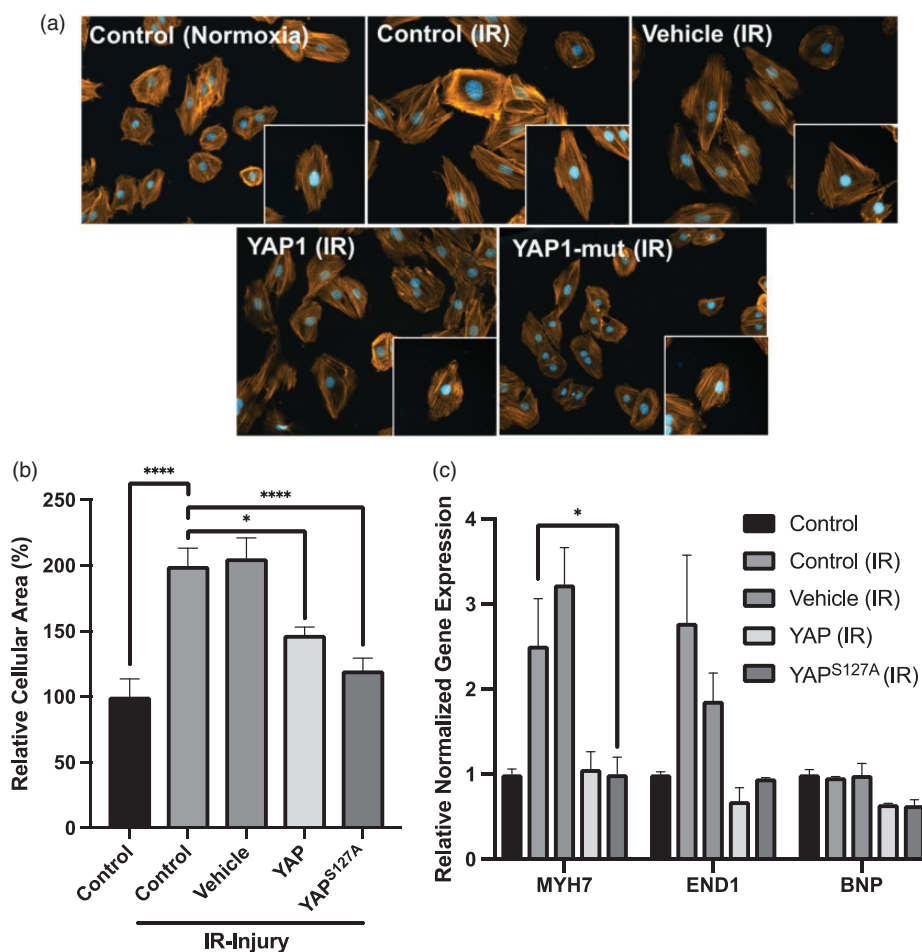


Figure 4. YAP overexpression attenuated the hypertrophic response from IR injury. (a) Representative images of actin immunostaining. (b) Quantification of cardiomyocyte cellular area. (c) Relative fold-change in mRNA expression of MYH7, END1 and BNP. * $P < 0.05$, **** $P < 0.0001$. (A color version of this figure is available in the online journal.)

presence of phosphorylated p53, a marker of apoptosis after IR injury. There was a statistically significant difference between all groups as determined by one-way ANOVA (Figure 3(d) and (e), $P < 0.0001$). Tukey's *post hoc* multiple comparisons test revealed a significant increase in phosphorylated p53 after IR injury ($P < 0.01$). In addition, YAP and YAP1^{S127A} cardiomyocytes were seen to reduce the gene expression ratio of BCL2-associated X/B-cell lymphoma 2 (BAX/BCL2), a marker of apoptosis (Figure 3(f), ($P < 0.001$ and $P < 0.01$, respectively), as determined by Tukey's *post hoc* multiple comparisons test after one-way ANOVA. These improvements in cell viability were also supplemented with improved metabolic activity of both YAP and YAP1^{S127A} cardiomyocytes after IR injury (Figure 3(g), $P < 0.0001$). Together, these results indicate that YAP1 protects cardiomyocytes from cell death during IR injury.

YAP1 reduces IR-induced cellular hypertrophy

Similar to the mechanism that occurs after MI, cardiomyocytes displayed significant cellular hypertrophy depicted by actin immunostaining after IR injury (Figure 4(a) and (b), $P < 0.001$). One-way ANOVA revealed

significant differences in cellular hypertrophy after IR injury in the infected cardiomyocyte groups ($P < 0.0001$). Tukey's *post hoc* multiple comparisons test indicated significant reductions in cellular hypertrophy were seen in the YAP and YAP1^{S127A} cardiomyocytes compared to non-infected cells after IR injury ($P < 0.05$, and $P < 0.0001$, respectively). Furthermore, YAP and YAP1^{S127A} cardiomyocytes were found to reduce the expression of several hypertrophy markers. YAP1^{S127A} reduced myosin heavy chain 7 (MYH7) expression significantly (Figure 4(c), $P < 0.05$). One-way ANOVA revealed significant changes in brain natriuretic peptide (BNP) gene expression (Figure 4(c), $P < 0.05$), with no significant changes seen between group means. Lastly, expression of endothelin 1 (END1) was found to be trending towards significance according to one-way ANOVA analysis (Figure 4(c), $P = 0.055$). Thus, YAP1 plays an important role in the reduction of cellular hypertrophy induced by IR injury.

YAP1 attenuates the generation of ROS after IR injury

IR injury was shown to induce significant generation of superoxide radicals in cardiomyocytes (Figure 5(a), $P < 0.0001$). One-way ANOVA analysis showed a

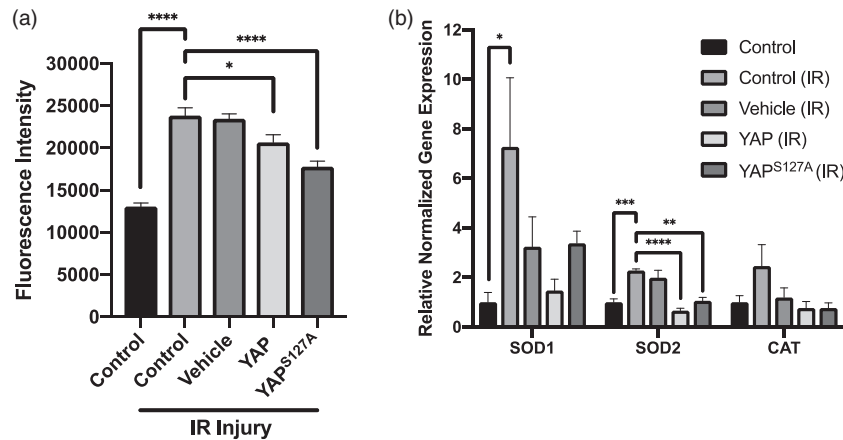


Figure 5. YAP1 reduces the generation of cellular ROS after IR injury. (a) Fluorescence intensity of superoxide radicals in YAP and YAP^{S127A} cardiomyocytes after IR injury. (b) Change in gene expression of SOD1, SOD2 and catalase in YAP and YAP^{S127A} cardiomyocytes after IR injury. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

significant difference between all group means ($P < 0.0001$). Tukey's *post hoc* multiple comparisons test indicated that both YAP and YAP^{S127A} cardiomyocytes were able to attenuate the generation of superoxide radicals after IR injury ($P < 0.05$ and $P < 0.0001$, respectively). The reductions in ROS were also shown through significant attenuation of the oxidative stress genes superoxide dismutase 2 (SOD2) for both YAP and YAP^{S127A} cardiomyocytes (Figure 5(b), $P < 0.0001$ and $P < 0.001$, respectively). Trends towards significant reductions were also seen for SOD1 and catalase genes (Figure 5(b)). These data suggest that YAP1 protects cardiomyocytes from ROS-induced damage after IR injury.

Cross-talk between Hippo and Wnt-signaling after IR injury

We used the GSK3 β inhibitor CHIR99021 to activate canonical Wnt-signaling in cardiomyocytes. Cardiomyocytes were treated with 5 μ M CHIR99021 during IR injury, which resulted in reduced phosphorylated-GSK3 β protein expression (Supplemental Figure 3(a) and (b), $P < 0.0001$). Cardiomyocytes treated with 5 μ M of CHIR99021 significantly increased the nuclear translocation of β -catenin (Supplemental Figure 3(c) and (d), $P < 0.0001$), indicative of transcriptional activation. We then determined the transcriptional activation of YAP1 in infected cardiomyocytes treated with CHIR99021. One-way ANOVA analysis showed a significant difference between all group means ($P < 0.0001$). Tukey's *post hoc* multiple comparisons test indicated that YAP^{S127A} cardiomyocytes treated with 5 μ M CHIR99021 displayed even greater nuclear translocation of YAP1 (Figure 6(a) and (b)) compared to control cells ($P < 0.0001$) and YAP cardiomyocytes ($P < 0.01$) after IR injury. This amplified transcriptional activity of YAP1 was found to completely attenuate the hypertrophic response of cardiomyocytes to IR injury, indicating a synergistic effect of YAP1 and β -catenin in reducing cellular hypertrophy (Figure 6(c) and (d)).

Discussion

The Hippo-signaling pathway has been gaining in interest as a novel mechanism to repair the myocardium after MI.^{24,31–33} In this study, we found that several of the key Hippo-signaling molecules were dysregulated after IR injury in AC16 human cardiomyocytes, indicating a key role of Hippo-signaling during the IR injury response. Cardiomyocytes overexpressing YAP1 improved attenuated apoptosis, cellular hypertrophy, and ROS generation after IR injury. Constitutive activation of both YAP1 and β -catenin resulted in synergistic attenuation of the hypertrophic response to IR injury. Together, these results indicate that YAP1 protects AC16 cardiomyocytes from IR injury that may be mediated by canonical Wnt/ β -catenin signaling.

There is no single stimulus that activates Hippo signaling. Rather, Hippo regulation occurs through a complex process involving multiple stimuli such as changes in cell polarity, junctional proteins, ROS generation, mechanical stress, and activation of G-protein coupled receptors.²⁴ The replacement of the infarcted tissue by cardiac myofibroblasts causes the detachment of cells from the ECM³⁴ and results in the activation of Rho GTPase, cytoskeletal reorganization, LATS activation, and YAP phosphorylation.³⁵ Our study found significant reductions in the inactive forms of MST1 and LATS1 kinases and YAP1 protein expression after IR injury. We also found that the response IR-injury upregulated LATS1 gene expression, with no change in MST1 or YAP1, indicating that IR injury may produce post-translational modifications in MST1 and YAP1. Similarly, Western blots in failed human left ventricle samples suggested significant decreases in YAP1 protein expression and significant increases in pYAP and pLATS protein expression.²⁶ Thus, our results coincide with others suggesting that Hippo-signaling is dysregulated after IR injury, but may also play a role in the transcriptional regulation of Hippo genes.

The ischemic environment from a MI causes significant apoptosis in cardiomyocytes.^{34,36} Due to the terminally

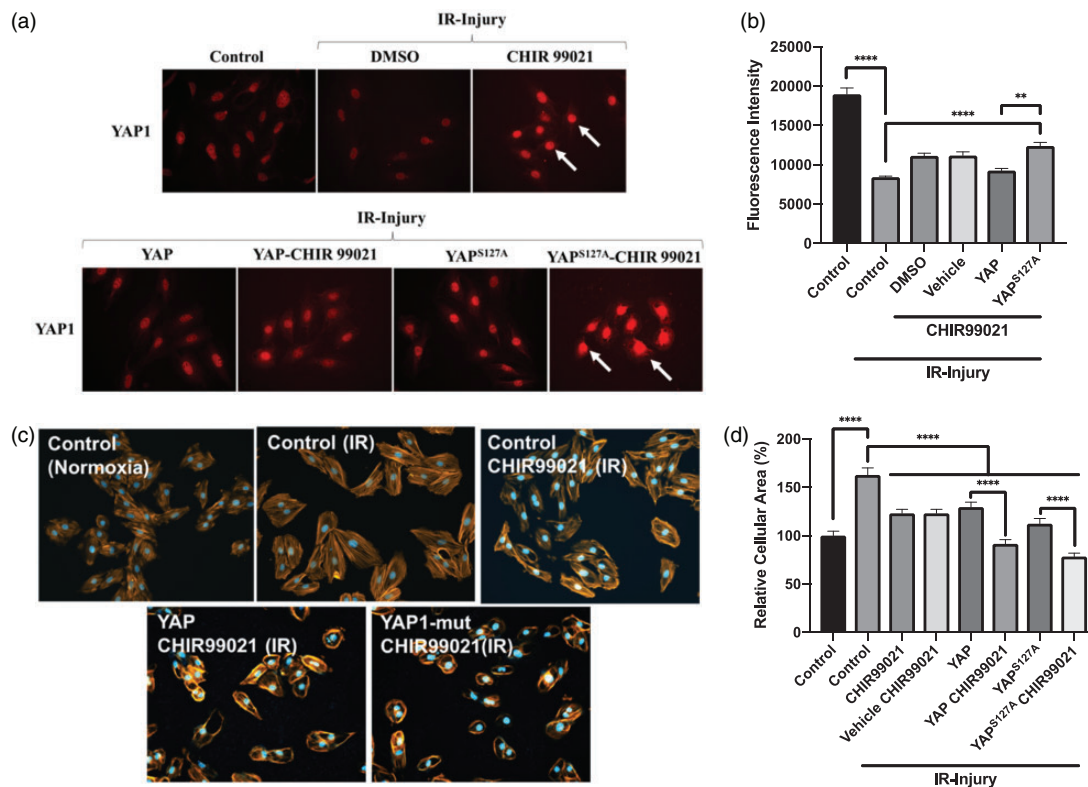


Figure 6. YAP1 and β -catenin work synergistically to attenuate the hypertrophic response of cardiomyocytes to IR injury. (a) Representative images of YAP1 immunostaining. (b) Average integrated intensity of nuclear YAP1 after treatment with 5 μ M CHIR99021. $^{**}P < 0.01$ and $^{****}P < 0.0001$ relative to control cardiomyocytes after IR injury, $\#P < 0.05$ relative to non-infected cardiomyocytes treatment with 5 μ M CHIR99021. (c) Representative images of actin immunostaining. (d) Quantification of cellular area. $^{**}P < 0.01$, $^{****}P < 0.0001$. (A color version of this figure is available in the online journal.)

differentiated nature of the adult cardiomyocyte, they are unable to proliferate and recover the damaged tissue, inevitably leading to heart failure.³⁷ It is thought that YAP1 activation may be able to attenuate the apoptotic response to MI³⁸; however, the mechanism by which this occurs is not well understood. YAP1 is suggested to play an anti-apoptotic role in podocytes,³⁹ endothelial cells,⁴⁰ and some cancer cells.^{41,42} Additionally, YAP1 knockouts in rat cardiomyocytes were found to significantly increase apoptosis.^{43,44} Here, we suggest that YAP1 reduced apoptosis in AC16 human cardiomyocytes through depression of the ATM/ATR DNA damage cascade, suggesting a novel pathway for YAP1 in reducing apoptosis. One of the most well-studied YAP1 partners within the context of proliferation and anti-apoptosis is TEAD, a transcription factor for YAP1-induced mitogenic activity and cell cycle re-entry.²⁸ Binding of YAP1 to TEAD has been shown to directly upregulate cell cycle genes minichromosome maintenance complex component 3 (MCM3), MCM6, cyclin-dependent kinase 1, and DNA polymerase alpha 1.⁴⁵ Thus, YAP1 may be used to reduce apoptosis and promote proliferation in cardiomyocytes after MI to prevent further deterioration of the cardiac tissue.

After MI, there is a surge in ROS generation that is thought to play an important role in the pathophysiology towards heart failure.⁴⁶ Shao *et al.*²² were the first to uncover a functional interaction between YAP1 and FoxO1, an important mediator of the anti-oxidant stress response.⁴⁷

Inhibition of Hippo signaling in a mouse MI model attenuated the generation of ROS through the direct interaction between YAP1-FoxO1 and the promoters of MnSOD and catalase antioxidant genes.²² ChIP sequencing and subsequent PCR analyses revealed that YAP1 was present during the binding of FoxO1 to catalase and *MnSOD* promoters,²² while hippo activation suppressed antioxidant gene expression. Our study suggests that AC16 human cardiomyocytes have an intrinsic antioxidant gene response to IR injury, and that activation of YAP is able to reduce ROS generation. In addition, there are several reports that indicate the rise in ROS after MI is in part responsible for cardiomyocyte hypertrophy and heart failure.⁴⁸ Treating neonatal rat cardiomyocytes with H₂O₂ activates PI3K-mediated cellular hypertrophy.^{49,50} Furthermore, adult rat cardiomyocytes treated with END1, a hypertrophic activator, resulted in significant ROS generation and ERK activation, which appeared to stem from NADH/NADPH oxidase.⁵¹ Therefore, the reductions in ROS found in YAP and YAP^{S127A} cardiomyocytes after IR injury may play a role in the reducing cellular hypertrophy.

The association between Hippo and Wnt-signaling is not well understood, yet both play an important role in the development of the myocardium and repair after injury.^{52,53} Studies that have investigated this cross-talk suggest that YAP1 and β -catenin activate similar cell cycle genes including Cyclin-dependent kinase 1, Cyclin A2, Cyclin B1, cell-division cycle protein 20, (sex determining region Y)-box 2,

Snail Family Transcriptional Repressor 2, and Baculoviral IAP Repeat Containing 2.^{54,55} In the present study, we found a novel functional role for β -catenin in reducing cellular hypertrophy after IR injury that may be mediated by YAP1. Together, these results indicate that β -catenin may have a similar function to YAP1, and that these proteins are highly regulated to maintain normal cellular morphology.

The current evidence regarding Hippo signaling for cardiac repair suggests that inhibition of this pathway may be beneficial to improve prognosis of patients suffering from heart failure. Of interest, the use of viral technology to infect cardiomyocytes in clinical trials is gaining awareness.^{28,56} There are currently more than 50 clinical trials investigating the use AAVs in inherited and acquired morbidities.⁵⁶ As mentioned previously, similar methodologies have been used to activate YAP1 in cardiomyocytes after MI using the AAV9.²⁸ Furthermore, Wnt-signaling has been well documented to play a significant role in several cardiovascular diseases.^{52,57} Developing therapeutics to target Wnt signaling in the heart may also be a novel method for cardiac regeneration. Better understanding of the molecular mechanisms that contribute to YAP1-induced cytoprotection will aid in bringing these therapies to the clinic.

One major strength of this study is that to our knowledge, it is the first to report the role of YAP1 on cytoprotection after IR injury in AC16 human cardiomyocytes. Second, we report that YAP1 reduces cell death in part through attenuation of ATM/ATR DNA damage response pathways, indicating a potentially new transcriptional target of YAP1. Lastly, we report a novel functional interaction between YAP1 and canonical Wnt/ β -catenin signaling within the context of IR injury, indicating that this interaction may play an important role in cytoprotection. Although novel in its approach, we utilized an *in vitro* model to study YAP1-related mechanisms and attempt to draw conclusions to complex 3D systems such as the human body. Furthermore, our *in vitro* model of ischemia-reperfusion does not fully mimic *in vivo* MI models, and therefore this system has its own set of limitations. Therefore, *in vivo* investigations will be necessary in order to corroborate our findings.

Targeting the Hippo-signaling cascade for myocardial regeneration is growing in interest. In the present study, we suggest that YAP1 plays an important role in cytoprotection after IR injury in AC16 human cardiomyocytes. In addition, we provide evidence for cross-talk between Hippo and Wnt signaling and significant overlap in their cytoprotective responses. Future investigations are encouraged to further characterize the interaction between Hippo and Wnt-signaling after IR injury and elucidate other downstream mechanisms upon YAP1 overexpression. Performing similar experimentations *in vivo* will help to deduce these mechanisms within complex models and perhaps identify other potential targets for cardiac regeneration.

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DECLARATION OF CONFLICTING INTERESTS

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