miR-206 Mediates YAP-Induced Cardiac Hypertrophy and Survival

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Rationale: In Drosophila, the Hippo signaling pathway negatively regulates organ size by suppressing cell proliferation and survival through the inhibition of Yorkie, a transcriptional cofactor. Yes-associated protein (YAP), the mammalian homolog of Yorkie, promotes cardiomyocyte growth and survival in postnatal hearts. However, the underlying mechanism responsible for the beneficial effect of YAP in cardiomyocytes remains unclear.

Objectives: We investigated whether miR-206, a microRNA known to promote hypertrophy in skeletal muscle, mediates the effect of YAP on promotion of survival and hypertrophy in cardiomyocytes.

Methods and Results: Microarray analysis indicated that YAP increased miR-206 expression in cardiomyocytes.

Increased miR-206 expression induced cardiac hypertrophy and inhibited cell death in cultured cardiomyocytes, similar to that of YAP. Downregulation of endogenous miR-206 in cardiomyocytes attenuated YAP-induced cardiac hypertrophy and survival, suggesting that miR-206 plays a critical role in mediating YAP function. Cardiac-specific overexpression of miR-206 in mice induced hypertrophy and protected the heart from ischemia/reperfusion injury, whereas suppression of miR-206 exacerbated ischemia/reperfusion injury and prevented pressure overload-induced cardiac hypertrophy. miR-206 negatively regulates Forkhead box protein P1 expression in cardiomyocytes and overexpression of Forkhead box protein P1 attenuated miR-206–induced cardiac hypertrophy and survival, suggesting that Forkhead box protein P1 is a functional target of miR-206.

Conclusions: YAP increases the abundance of miR-206, which in turn plays an essential role in mediating hypertrophy and survival by silencing Forkhead box protein P1 in cardiomyocytes. (Circ Res. 2015;117:891-904. DOI: 10.1161/CIRCRESAHA.115.306624.)

Key Words: apoptosis ■ heart ■ hypertrophy ■ microRNAs ■ signal transduction

The Drosophila Hippo pathway is an evolutionarily conserved signaling cascade and a key regulator of tissue growth and organ size. Activation of the core complex (consisting of the kinases Hippo and Warts and the adapter proteins Salvador and Mats) leads to phosphorylation and inhibition of Yorkie, the transcription cofactor and terminal effector of the Hippo pathway. Conversely, Hippo pathway loss of function, or direct phosphorylation by homeodomain-interacting protein kinase 2, can elicit Yorkie activation. Through association and activation of the transcription factor Scalloped, Yorkie regulates expression of cyclin E, diap1, and the microRNA (miRNA) bantam to promote proliferation and inhibit apoptosis. Hippo pathway components are highly conserved between flies and mammals, and mammalian homologs including Mst1/2 (Hippo), Lats1/2 (Warts), Yes-associated protein (YAP; Yorkie), and TEAD family transcription factors (Scalloped) exhibit similar, but not identical, regulation, and function outputs.

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Elucidation of Hippo signaling and the functional implications of Hippo modulation in the heart are highly relevant and of great interest. Our previous work demonstrated that transgenic mice with cardiac-specific overexpression of Mst1 develop dilated cardiomyopathy with increased cardiomyocyte apoptosis. Interestingly, cardiomyocyte size in these mice was smaller than nontransgenic controls despite dilation of the heart and increased wall stress. These findings suggested that activation of Hippo promotes cardiomyocyte apoptosis, whereas compensatory hypertrophy is suppressed by Mst1. We also observed that Lats2, a negative regulator...
of YAP, inhibits both hypertrophy and survival of cardiomyocytes during pressure overload stress. Furthermore, disruption of endogenous cardiac YAP led to impaired compensatory hypertrophy, proliferation, and survival of cardiomyocytes during chronic myocardial infarction (MI) and ischemia/reperfusion (I/R). However, overexpression of constitutively active YAP promoted mycardial regeneration by stimulating cardiomyocyte proliferation. Previous reports have demonstrated the importance of cross talk between Hippo and IGF-Wnt/β-catenin pathways for cardiomyocyte proliferation during heart development. These results suggest that the Hippo pathway plays an essential role in cardiomyocyte biology and that YAP is an important mediator of the actions of the Hippo pathway in the heart. However, the mechanisms underlying how Hippo/YAP modulates cardiomyocyte growth and survival remain largely undefined.

miRNAs regulate gene expression to affect cell growth, proliferation, and survival, and are important mediators of heart development, homeostasis, and disease. miR-206 belongs to the miR-1/miR-206 family of miRNAs. miR-206 is expressed in several known tissues/cell types including the brain, cancer cell lines, and brown adipocytes and mediates multiple functions, including tissue growth and differentiation, suppression of angiogenesis, as well as tumor suppression. In skeletal muscle, miR-206 positively regulates myogenesis, hypertrophy, and regeneration, and delays the progression of amyotrophic lateral sclerosis and Duchenne muscular dystrophy. These findings suggest that miR-206 is an important modulator of striated muscle growth. Importantly, miR-206 is also present in cardiomyocytes, but the cellular function of miR-206 in these cells is not well understood.

In this study, we explored the possibility that YAP regulates expression of miRNAs that modulate cardiomyocyte growth and survival. Through a microarray-based screening of miRNAs that showed altered expression in response to YAP, we found that the abundance of miR-206 was increased by YAP in cardiomyocytes. Despite some functional overlap between YAP and miR-206, a link between them has not been demonstrated. Therefore, the goals of this work were (1) to investigate the role of miR-206 in mediating the prohypertrophic and antiapoptotic actions of YAP in cardiomyocytes and (2) to identify the functional target of miR-206 in cardiomyocytes. Our results demonstrate that miR-206 mediates hypertrophy and cell survival stimulated by YAP through targeted degradation of the tumor suppressor, Forkhead box protein P1 (FoxP1).

Methods

An expanded Methods section is available in the online Data Supplement.

Adenoviral Vectors

Adenoviruses harboring genes of interest were made using the AdMax system (Microbix). Short hairpin RNA knockdown adenovirus for FoxP1 or miR-206 was made using the adenoviral shuttle vector pDC311 (Microbix), into which the U6 RNA polymerase III promoter and the polylinker region of Psilencer 1.0-U6 expression vector (Ambion) were subcloned. Sequence of sh-FoxP1: 5’-3’ GCCCAACACTG CAGAGGAAT TCAAGAGATTTCCTCTGC AGTGGTG GCC CAGGTCTC GAGCACACACT GCCTACATGC GCCACA CATCTTCTACCTACATCC A.

Transgenic Mice

Tg-miR-206 and Tg-206-SPONGE mice were generated on an FVB background, using the α-myosin heavy chain promoter to achieve cardiac-specific expression of around 300 bp of the genomic region of miR-206 or 2 repeats of GFP-tagged miR-206 antisense, respectively. All protocols concerning animal use were approved by the Institutional Animal Care and Use Committee at Rutgers, New Jersey Medical School.

miRNA Microarray

Total RNA was isolated from YAP- and LacZ-transduced neonatal rat cardiomyocytes (NRCMs). Ten micrograms of RNA were sent to L.C. Sciences for a miRNA microarray. Samples were enriched for small RNA, labeled with Cy3 and Cy5 fluorescent dyes and hybridized to a single Atactic μParaFlo microfluidics chip that held all 334 mature rodent miRNA probes identified to date, as well as perfectly matched and mismatched probes for quality control. Each miRNA probe is represented 9x on the microarray. Among the control probes, PUC2PM20B and PUC2MM20B are the perfect match and single-based match detection probes, respectively, of a 20-mer RNA-positive control sequence that is spiked into the RNA samples before labeling. One may assess assay stringency from the intensity ratio of PUC2PM20B to PUC2MM20B, which is normally larger than 30. After signal amplification, the background was subtracted and normalized using the LOWESS (locally weighted regression) method. For a transcript to be listed as detectable, it must meet the following criteria: signal intensity higher than 3× (background SD), spot coefficient of variation <0.5 (coefficient of variation=SD/signal intensity), and signals from at least 50% of the repeating probes above detection level.

Northern Blot

Total RNA, extracted using TRizol reagent (Invitrogen), was separated on a 1% agarose gel, transferred to an uncharged nylon membrane, Hybond-NX (Amersham Biosciences), and UV cross-linked. The membrane was prehybridized/hybridized with MiracleHyb Hybridization solution according to the instruction manual (Stratagene). DNA oligonucleotides, antisense sequences of mature miRNAs, were obtained from Integrated DNA Technologies. The probes were 5’-end labeled with ET adenosine 5’-triphosphate[γ-32P] (PerkinElmer) using a T4 Polynuclease Kinase kit and used for hybridization (1×106 cpm/mL).

Statistics

All values are expressed as mean±SEM. Statistical analyses were performed using ANOVA or t test with a P<0.05 considered significant.
Results

The Abundance of miR-206 Is Increased by YAP and Decreased by Mst1

To identify miRNAs involved in YAP-induced cardiac hypertrophy and survival, total RNA was extracted from NRCMs overexpressing wild-type YAP and enriched for small RNAs. We used microarrays containing rodent miRNAs to analyze the enriched extracts and selected miRNAs that showed the greatest increase in abundance, including miR-711, miR-483, and miR-206 (Online Table I). Of note, we found no significant similarity in the nucleotide sequences between these miRNAs and the established Yorkie target bantam (Online Figure I). Importantly, we found that overexpression of miR-206 significantly increased cardiomyocyte size (Online Figure II A), whereas neither miR-711 nor miR-483 overexpression significantly affected cell size (Online Figure II B). In addition, a link between miR-206 expression and skeletal muscle hypertrophy has been demonstrated previously.29 We therefore focused our efforts on investigating the possible role of miR-206 in mediating YAP-induced cardiac hypertrophy and survival for this study.

Both Northern blot and quantitative reverse transcription polymerase chain reaction (qRT-PCR) confirmed our microarray result that the abundance of miR-206 was significantly increased (+1.44 fold by Northern blot and +2.13-fold by qRT-PCR) in cardiomyocytes overexpressing wild-type YAP, in which the activity of YAP was confirmed by TEAD luciferase activation (Figure 1A and 1B; Online Figure II F). Interestingly, YAP did not alter miR-206 levels in C2C12 mouse myoblasts, which have a higher abundance of miR-206 than NRCMs at baseline (Online Figure IIC), suggesting that upregulation of miR-206 is cell type specific. Conversely, overexpression of Mst1, which induced inhibitory phosphorylation of YAP and reduced expression levels of the YAP target gene cardiac ankyrin repeat protein, decreased the abundance of miR-206 (Figure 1C; Online Figure IID). In addition, coexpression of an Mst1-resistant form of YAP (Serine-127-Alanine) abolished the repressive effect of Mst1 on miR-206 abundance (Online Figure IIIE), indicating that the mammalian Hippo pathway suppresses miR-206 expression in cardiomyocytes.

Given the reported similarity between miR-1 and miR-206,30 we sequenced the qPCR products detected by the miR-206 and miR-1 qPCR primers to ensure their intended mature miRNA sequences were measured (Online Figure IIIA). The specificity of the Taqman MicroRNA Assay was further verified in that qRT-PCR did not detect an increase in miR-206 in cardiomyocytes transduced with miR-1 adenovirus (Online Figure IIIB).

To investigate the mechanism by which YAP increases the abundance of miR-206, we focused on the role of enhancer box (E-box) elements in modulating miR-206 transcriptional activation. We constructed luciferase reporters containing either the wild-type miR-206 enhancer with 9 E-box sites or a mutant enhancer with mutations in the 3 most conserved E-box sites (E-box-3 m) (Figure 1D). YAP significantly activated the wild-type E-box, but not E-box-3 m enhancer-luciferase reporter, compared with control luciferase reporter (Figure 1E). However, Mst1 significantly decreased the E-box luciferase activity (Figure 1F). We also performed chromatin immunoprecipitation to test whether YAP associates with the miR-206 E-box. ChIP showed that YAP binds to the E-box located in the miR-206 promoter (Figure 1G). This suggests that the Hippo pathway inhibits miR-206 transcription through the E-box elements. Moreover, YAP did not stimulate the miR-1 enhancer-luciferase reporter, indicating that YAP selectively upregulates miR-206 and not miR-1 expression in cardiomyocytes (Online Figure IVA).

miR-206 Induces Cardiac Hypertrophy and Protects Myocytes From Death

Transduction of NRCMs with adenovirus harboring miR-206 (Ad-miR-206) induced upregulation of mature miR-206, as evaluated by Northern blot (Figure 2A). Adenoviral-mediated expression of either miR-206 or YAP for 48 hours induced cardiomyocyte hypertrophy, as evidenced by an increase in cell size and the total protein content normalized to DNA content (Figure 2B and 2C). Similarly, miR-206 and YAP both significantly increased mRNA expression of atrial natriuretic factor (ANF), a hypertrophic marker gene,31 as well as activation of the ANF-luciferase reporter gene in cardiomyocytes. ANF-luciferase activation was comparable with that elicited by phenylephrine, an α-1 adrenergic agonist known to stimulate cardiac hypertrophy32 and used here as a positive control to promote cardiac hypertrophy (Figure 2D and 2E). In contrast, miR-1 expression did not increase total protein normalized to DNA content in cardiomyocytes (Online Figure IVB). These results suggest that upregulation of miR-206 is sufficient to induce cardiomyocyte hypertrophy in vitro, whereas upregulation of miR-1 is not.

To examine the effect of miR-206 on cell death, cardiomyocytes were treated with chelerythrine, a known inducer of apoptosis.33 Adenoviral-mediated expression of miR-206 in cardiomyocytes inhibited chelerythrine-induced cell death as evaluated with the Cell Titer Blue viability assay, caspase-3 cleavage, and TUNEL-positive nuclei (Figure 2F; Online Figure IVC and IVD). These results indicate that miR-206 protects against cardiomyocyte death. However, miR-1 facilitated chelerythrine-induced apoptosis (Figure 2F; Online Figure IVC and IVD), evidence of further functional distinction between miR-206 and miR-1 in cardiomyocytes. Of note, neither miR-206 nor miR-1 expression affected proliferation of NRCMs (Online Figure IVE).

To test whether miR-206 is sufficient to induce cardiac hypertrophy in vivo, we generated 4 lines of transgenic (Tg) mice with cardiac-specific expression of the genomic region (≈300 bp) of miR-206, driven by the α-myosin heavy chain promoter (Tg-miR-206). Mature miR-206 was upregulated in Tg-miR-206 mouse myocardium compared with nontargeting (NTg) controls, as shown by Northern blot analysis (Figure 3A; Online Figure VA, left). Although we observed a modest increase in miR-1 levels by Northern blot, this was most probably because of detection of ectopic miR-206 by the miR-1 Northern probe, which, because of the nature of its design, has less selectivity compared with the qRT-PCR probes. In fact, we did not observe any increase in miR-1 expression levels using qRT-PCR analysis of Tg-miR-206 hearts compared with NTg (Online Figure VA, right). In
all lines, Tg-miR-206 mice developed baseline cardiac hypertrophy compared with NTg controls as evidenced by increased heart weight/body weight and left ventricle (LV) weight/body weight ratios, LV myocyte cross-sectional area, and ANF mRNA expression (Figure 3B-3F; Online Figure VB and VC). LV function was maintained in Tg-miR-206 mice as evidenced by normal LV ejection fraction and lung weight/body weight ratio (Online Figure VB and VD). The increase in LV weight/body weight can likely be attributed to individual cardiomyocyte hypertrophy because overexpression of miR-206 did not affect myocyte proliferation, as indicated by no change in the number of Ki67-positive nuclei or the estimated total myocyte number (Figure 3G; Online Figure VE and VF).

To confirm that cardiac hypertrophy is elicited specifically because of increased miR-206 expression, we crossed Tg-miR-206 mice with a separately generated transgenic mouse, Tg-206-SPONGE. These mice harbor 2 repeats of antisense miR-206 downstream of GFP driven by the α-myosin heavy chain promoter (Tg-206-SPONGE mouse). Expression of miR-206, as well as the extent of cardiac hypertrophy, was significantly reduced in the double-Tg mice compared with...
Taken together, these results suggest that miR-206 expression induces cardiac hypertrophy in vivo. Although we generated multiple lines of transgenic mice, none of the lines exhibited low levels of miR-206 overexpression. Thus, it is possible that the cardiac phenotype observed in Tg-miR-206 may not reflect physiological function of miR-206.

To address this issue in part, we also used a complementary loss-of-function approach to test the involvement of miR-206 in cardiac hypertrophy. Wild-type C57B/6J mice were administered LNA inhibitor designed to selectively inhibit miR-206, or control scrambled LNA, by tail vein injection. Specificity of the LNA inhibitor was confirmed by qRT-PCR analysis of miRNA expression 7 days after treatment (Online Figure VG). After 7 days, mice from both treatment groups were subjected to sham operation or transverse aortic constriction (TAC) to elicit hemodynamic overload. After 7 days of TAC, echocardiography was performed and mice were euthanized. Importantly, we found upregulation of myocardial miR-206 expression levels after 7 days TAC, consistent with its observed prohypertrophic effect (Online Figure VH). A significant increase in LV weight/tibia length...
in LNA control-treated mice after TAC was observed; however, no increase was observed in LNA-anti-206–treated mice (Figure 3J).

We also noted significant differences in chamber wall thickness between the control and anti-206 TAC groups (Online Table II). In addition, cardiomyocyte cross-sectional area and ANF expression were significantly increased in the LNA control-treated TAC group, and these responses were attenuated in the LNA-anti-206–treated mice (Figure 3K and 3L; Online Figure VI). These data demonstrate that inhibition of miR-206 impairs pressure overload-induced hypertrophy and indicates that miR-206 is an important endogenous promoter of heart growth in response to stress.

miR-206 Is an Important Mediator of YAP-Induced Cardiac Hypertrophy and Inhibition of Cardiac Cell Death

To further clarify the cell-autonomous effect of endogenous miR-206 in mediating YAP-induced cardiac hypertrophy, adenovirus harboring anti–miR-206 (Ad-anti–miR-206), comprising 2 repeats of a complementary sequence to LNA-Sc-CTRL LNA-anti-206

Figure 3. miR-206 promotes cardiac hypertrophy in vivo. A–G, Characterization of the lowest expressing line of Tg-miR-206 mice at 3 months of age. A, Tg-miR-206 (line 24) and nontransgenic control (NTg) mouse hearts were harvested for Northern blot analysis with an miR-206 probe, an miR-1 probe, or a 5S rRNA probe. B, Representative heart pictures taken in PBS under a microscope. C and D, Postmortem measurements of heart weight/body weight (HW/BW, mg/g) and left ventricular weight/body weight (LVW/BW, mg/g).

The level of atrial natriuretic factor (ANF) mRNA was evaluated by quantitative reverse transcription polymerase chain reaction (qRT-PCR) analyses. G, Ki67-positive nuclei/total cardiomyocyte nuclei was evaluated with anti–Ki67 and anti–Troponin T staining. H and I, Tg-miR-206 and Tg-miR-206/Tg-206-SPONGE bitransgenic mouse hearts were harvested for Northern blot analyses with an miR-206 probe and a 5S rRNA probe (H) and postmortem measurement of LVW/BW (mg/g; I).

J–L, Wild-type C57B6 male mice were treated with LNA scrambled control or LNA-anti-206 by tail vein injection 7 days before intervention. Groups were subjected to 1-week transverse aortic constriction (TAC) or sham operation. J, LV weight/tibia length (LVW/TL) was determined. K, Heart sections were stained with WGA and cardiomyocyte CSA was determined. L, mRNA was isolated from ventricles and subjected to qRT-PCR to detect ANF levels, n=4 to 6.
miR-206 under the control of a U6 promoter, was generated. Adenovirus harboring a scrambled sequence (Ad-scrambled) was used as a control. Transduction of cardiomyocytes with Ad-anti–miR-206 significantly (−50%) reduced expression of miR-206, but not miR-1 (Figure 4A), and attenuated YAP-induced cardiomyocyte hypertrophy, as evidenced by decreases in cell size, protein/DNA content, perinuclear ANF staining, and activation of the ANF-luciferase reporter (Figure 4B–4E; Online Figure VIA). We also used a miRIDIAN inhibitor to inhibit miR-206 in cardiomyocytes. The miRIDIAN miR-206 inhibitor, but not control miRIDIAN inhibitor, significantly inhibited miR-206 function, as shown by an increase of luciferase reporter containing miR-206 antisense sequence in the 3′ untranslated region (UTR), but not empty luciferase reporter (Online Figure VIB), and attenuated YAP-induced hypertrophy of cardiomyocytes, as evidenced by suppression of ANF-luciferase reporter and cardiomyocyte size (Online Figure VID and VIE). These results suggest that endogenous miR-206 is critical in mediating YAP-induced cardiomyocyte hypertrophy in vitro. Importantly, specific downregulation of miR-1 did not abolish YAP-induced cardiomyocyte hypertrophy, indicating
that miR-1 does not mediate hypertrophy elicited by YAP (Online Figure VIP and VIG).

We also tested the role of miR-206 in mediating cardiomyocyte survival elicited by YAP. miR-206 knockdown not only inhibited YAP-induced cardiomyocyte viability at baseline (Figure 4F) but also reversed the YAP-induced protection against chelerythrine-induced cell death, as shown by caspase-3 cleavage (Figure 4G). These results suggest that endogenous miR-206 is an important mediator of YAP-induced protection of cardiomyocytes in vitro.

**Endogenous miR-206 Is Protective During I/R Injury**

To examine the physiological function of miR-206 during stress, we first examined miR-206 expression in response to oxidative stress. H$_2$O$_2$ treatment reduced RNA and protein levels of miR-206 and YAP, respectively, in cardiomyocytes (Figure 5A and 5B; Online Figure VIIA). Similarly, miR-206 and YAP were downregulated in mouse hearts subjected to ischemia followed by reperfusion compared with sham-operated controls (Figure 5C and 5D; Online Figure VIIIB and VIIIC). In contrast, miR-1 was upregulated in response to ischemia followed by reperfusion (Online Figure VIIID). To evaluate the role of endogenous miR-206 in mediating cardiomyocyte survival in vivo, we used Tg-206-SPONGE mice, which have reduced miR-206, but not miR-1, expression compared with NTg controls (Figure 5E; Online Figure VIIIE and VIIIF). We found that I/R-induced cardiac injury was exacerbated in Tg-206-SPONGE compared with NTg mice, as indicated by a greater size of MI normalized by the area at risk (Figure 5F and 5G). In contrast, the size of MI normalized by the area at risk after I/R was significantly reduced in Tg-miR-206 compared with NTg hearts (Figure 5H and 5I). To test whether increased miR-206 expression can mitigate the chronic effects of I/R injury, including decreased cardiac function, we subjected NTg and Tg-miR-206 mice to I/R and followed them with serial echocardiography. Although there was no difference in basal heart function between Tg-miR-206 and NTg mice, we found that Tg-miR-206 mice had significantly better cardiac function, as determined by percent fractional shortening, post I/R compared with NTg controls (Online Figure VIIG). These results suggest that downregulation of endogenous miR-206 promotes myocardial I/R injury, whereas restoration of miR-206 levels with exogenous miR-206 protects against I/R injury and counteracts the progression to heart failure.
miR-206 Mediates Hypertrophy and Survival

miR-206 Induces Cardiac Hypertrophy Through Inhibition of FoxP1

A search of miR-206 targets using TargetScan (http://www.targetscan.org, Release 5.2) allowed us to identify FoxP1, a tumor suppressor protein, as a potential target and mediator of the prohypertrophic and prosurvival effects of miR-206 in cardiomyocytes. FoxP1 was ranked as seventh among 478 conserved targets of miR-206 by total context score predicted by TargetScan. Because our gain-of-function analyses suggested that miR-206 has growth promoting effects, we predicted that molecules known to inhibit cell growth responses might mediate the effect of miR-206. FoxP1 was ranked highest among the proteins known to have growth inhibitory function. In addition, FoxP1 has 4 predicted miR-206 binding sites in its 3′ UTR, 2 of which are conserved sites (Online Figure VIIIA) further strengthening our rationale to investigate FoxP1.

To demonstrate that FoxP1 is a target of miR-206, we first conducted luciferase assays using the FoxP1 3′ UTR. The 3′ UTR of the luciferase gene was replaced with either the WT FoxP1 3′ UTR or a mutated FoxP1 3′ UTR, in which the miR-206 binding sites were altered. Transduction of NRCMs with Ad-miR-206, but not Ad-scrambled, significantly inhibited the luciferase reporter containing the WT FoxP1 3′ UTR; however, the luciferase reporter containing the mutated FoxP1 3′ UTR was not affected (Figure 6A). Conversely, an miRIDIAN miR-206 inhibitor, but not control miRIDIAN inhibitor, significantly stimulated the luciferase reporter containing the WT FoxP1 3′ UTR but did not affect the control reporter containing the mutated FoxP1 3′ UTR (Online Figure VIC). Adenoviral-mediated expression of either YAP or miR-206 in cardiomyocytes inhibited FoxP1 expression, as determined by Western blotting (Figure 6B and 6C). Furthermore, YAP-induced downregulation of FoxP1 was attenuated in the presence of anti–miR-206, suggesting that YAP downregulates FoxP1 through miR-206 (Figure 6B). Adenoviral-mediated expression of miR-1 did not affect protein levels of FoxP1.

Figure 6. Yes-associated protein (YAP)-miR-206 signaling down regulates Forkhead box protein P1 (FoxP1) expression in cardiomyocytes. A, Cardiomyocytes were transfected with luciferase reporter constructs harboring either wild-type (untranslated region [UTR]) or mutated (UTRm) FoxP1 3′ UTR sequences and then transduced with Ad-scrambled or Ad-miR-206, n=3. B and C, Cardiomyocytes were transduced with Ad-LacZ, Ad-YAP, Ad-scrambled, or Ad-miR-206 for 48 hours. Cells were harvested for immunoblot analyses with anti-FoxP1 antibody and either anti-actin or anti-α-tubulin antibodies. The results of densitometric analyses are also shown, n=4. D and E, Tg-206-SPONGE, Tg-miR206/Tg-206-SPONGE, and nontransgenic control (NTg) mouse hearts were harvested for immunoblot analyses, using anti-FoxP1, anti-GFP, and anti-actin antibodies, n=4.
in cardiomyocytes (Online Figure VIIIB and VIIIC). FoxP1 was also downregulated in Tg-miR-206 hearts (Figure 6D), whereas it was upregulated in Tg-206-SPONGE mice, in which endogenous miR-206 is suppressed (Figure 6E). The upregulation of FoxP1 observed in Tg-206-SPONGE mice was normalized when Tg-206-SPONGE mice were crossed with Tg-miR-206 mice (Figure 6E). Taken together, these results suggest that FoxP1 is a specific target of miR-206 in the heart and the cardiomyocytes therein.

On the basis of our hypothesis, we predicted that FoxP1 inhibits cardiac hypertrophy. To test this, cultured cardiomyocytes were transduced with an adenovirus harboring shRNA targeting FoxP1 (Ad-sh-FoxP1). We confirmed that Ad-sh-FoxP1 efficiently downregulated both endogenous and overexpressed FoxP1 in cardiomyocytes (Figure 7A; Online Figure VIIID). Downregulation of FoxP1 significantly increased cell size at baseline (Figure 7B) and increased cell viability after H2O2 treatment (Online Figure VIIIG), indicating that endogenous FoxP1 negatively regulates cardiomyocyte hypertrophy and survival.

To elucidate the contribution of FoxP1 downregulation to the overall effect of miR-206 in cardiomyocytes, we generated an adenoviral vector harboring FoxP1 (Ad-FoxP1). The results show that overexpression of FoxP1 in Ad-FoxP1–transduced...
cardiomyocytes, and that miR-206– or YAP-induced down-regulation of FoxP1 was partially attenuated by overexpression of FoxP1 (Figure 7C and 7D; Online Figure VIIIIE and VIIIIF). Both miR-206– and YAP-induced cardiomyocyte hypertrophy, as evaluated by cell size, were significantly attenuated whenFoxP1 levels were normalized by adenoviral-mediatedFoxP1 expression (Figure 7C and 7D, bottom). These results indicate that YAP/miR-206 induce cardiomyocyte hypertrophy through downregulation of FoxP1. We then tested the involvement of FoxP1 in mediating the protective effect of YAP and miR-206. We found that when FoxP1 levels were normalized, the ability of miR-206 to inhibit chelerythrine-induced cardiomyocyte death was abrogated (Figure 7E and7F; Online Figure VIII). Interestingly, protection conferred by YAP was partially but not completely inhibited by FoxP1 (Figure 7G), suggesting that miR-206 promotes cell survival through downregulation of FoxP1, whereas YAP probably promotes survival through additional mechanisms.

Discussion
Considering the remarkable level of homology present between each component of the Hippo signaling pathway, it is reasonable to predict that YAP targets in mammalian cells might include miRNAs, similar to that observed in Drosophila (ie, bantam). To identify possible miRNAs that are functional targets of YAP, we conducted microarray analysis and detected several miRNAs whose expression was upregulated by YAP in cardiomyocytes. Here, we demonstrate that miR-206 acts as an important mediator downstream of YAP to promote hypertrophy and survival of cardiomyocytes.

Recent work has demonstrated that YAP upregulates miR-29, which in turn inhibits PTEN and activates the mTOR pathway. In cardiomyocytes, we found that YAP upregulated miR-29 1.9-fold (Online Table I) indicating that regulation of miR-29 by YAP may occur in multiple cell and tissue types. Although 1 study reported that miR-29c induces modest enlargement of NRCMs, our results show that overexpression of miR-29 did not induce hypertrophy in NRCMs (Online Figure IB). Previous work has shown that miR-29 is enriched in cardiac fibroblasts and inhibits cardiac fibrosis. In addition, miR-29 was found to negatively regulate cardiomyocyte proliferation during postnatal cardiac development demonstrating that miR-29 does play a role in cardiovascular biology. Thus, miR-29 is likely to mediate additional nongrowth-related functions of YAP in cardiomyocytes.

The enhancer region of miR-206 does not contain typical binding sites for the reported mammalian transcriptional partners of YAP, including TEAD, RUNX, ErbB4, p73, Tbx5, or Smad. Instead, we found that E-box elements are present in the miR-206 enhancer region and that 3 conserved E-box elements on the miR-206 enhancer are critical for basal, as well as YAP-induced, miR-206 expression in cardiomyocytes (Figure 1E). It has been shown that myogenic factors induce expression of muscle-specific miRNAs (including miR-1, miR-206, and miR-133) by binding to E-box regions in the promoter/enhancer. However, the identity of the E-box-binding transcription factor responsible for YAP-induced upregulation of miR-206 and its functional relationship to YAP remain to be demonstrated and warrant future investigation.

We have demonstrated that both YAP and miR-206 stimulate hypertrophy and survival of cardiomyocytes in vitro. In vivo, exogenous expression of either YAP or miR-206 protects cardiomyocytes against I/R-induced death and reduces heart injury. Furthermore, cardiac-specific heterozygous deletion of Yap1 promotes cardiac dysfunction but inhibits cardiac hypertrophy during chronic MI, suggesting that endogenous YAP plays an important role in promoting both survival and hypertrophy during cardiac remodeling in the adult mouse heart. In addition, we found that overexpression of miR-206 in the mouse heart induces hypertrophy without cardiac dysfunction, and that selective inhibition of miR-206 prevents 1-week TAC-induced hypertrophy without altering cardiac function. Taken altogether, we speculate that the YAP-miR-206 pathway may mediate physiological hypertrophy, and serves a compensatory function to help protect the heart against increased demand and stress. Interestingly, although phosphatidylinositol 3-kinase has been shown to promote physiological hypertrophy, we previously reported that YAP-induced cardiac hypertrophy was not affected by phosphatidylinositol 3-kinase inhibition, suggesting that the YAP-miR-206 pathway mediates physiological hypertrophy through PI3K-independent mechanisms. We have shown previously that YAP is activated in the peri-infarct area in the post-MI heart. Although YAP is activated by mechanical stress and agonists of G-protein-coupled receptors in other cell types, how YAP is activated in response to hypertrophic stimuli remains to be elucidated in cardiomyocytes.

Expression of miR-206 is relatively low at baseline and is further downregulated in response to oxidative stress in cardiomyocytes and I/R in the heart. This is consistent with our previous findings that both oxidative stress and I/R activate Mst1 and Lats2, which would in turn inhibit YAP. Because further downregulation of miR-206 exacerbated I/R injury (Figure 5F and 5G), whereas exogenous miR-206 expression reduced injury, endogenous miR-206 seems to have an essential role in promoting survival of cardiomyocytes during I/R. We also found that miR-206 expression was sufficient to counteract the decreased cardiac function observed in response to chronic I/R, further evidence of the cardioprotective effect of miR-206 in vivo.

We speculate that the effect of YAP on growth and survival of cardiomyocytes functions through multiple mechanisms in a context-dependent manner. YAP regulates proliferation of cardiomyocytes through YAP–TEAD interaction. We have shown recently that YAP acts as a nuclear cofactor of FoxO1 to protect the heart during myocardial I/R. Furthermore, YAP has been shown to regulate global biogenesis and processing of miRNA through an interaction with p72 (DDX17) and let-7-dependent downregulation of Dicer, respectively. Further investigation is required to elucidate the relative importance of these mechanisms in the regulation of growth and death of cardiomyocytes by YAP.

Our results indicate that FoxP1 is a physiological mediator of miR-206 function in cardiomyocytes. First, we found that its 3′ UTR is specifically targeted by miR-206. In NRCMs,
miR-206 expression downregulated FoxP1 protein and inhibited FoxP1-UTR luciferase activity. Our results suggest that miR-206 also targets FoxP1 in vivo. We observed downregulation of FoxP1 in Tg-miR-206 hearts, whereas FoxP1 expression was increased in Tg-206-SPONGE mouse hearts (Figure 6E). Although FoxP1 has been identified as a target of miR-1 in certain cell types,47 we clearly demonstrate that FoxP1 is specifically targeted by miR-206, and not by miR-1, in cardiomyocytes (Online Figure VIIC).

Second, we demonstrate that FoxP1 is an endogenous negative regulator of cardiac hypertrophy and survival. Indeed, downregulation of FoxP1 was sufficient to induce hypertrophy and to promote cell survival in cultured cardiomyocytes (Figure 7B; Online Figure VIIIIG). Furthermore, both the stimulation of hypertrophy and the suppression of apoptosis by miR-206 were attenuated when FoxP1 downregulation was reversed by adenoviral supplementation of FoxP1 in cardiomyocytes (Figure 7C, 7E, and 7F; Online Figure VIIIH). Taken together, our results suggest that downregulation of FoxP1 plays an important role in mediating the effects of miR-206 in cardiomyocytes.

FoxP1 systemic knockout mice die at E14.5 because of heart failure.48 These mice exhibit defects in valve formation caused by impaired apoptosis in the endocardial cushion mesenchyme, and have a thinner ventricular compact zone because of increased proliferation of the ventricular trabecular zone.48 Cardiac-specific FoxP1 knockout mice die within 24 hours of birth and have a thickened myocardium resulting from increased myocyte proliferation in a cell-autonomous fashion.49 These data suggest that FoxP1 promotes apoptosis and inhibits cardiomyocyte proliferation during cardiac development. FoxP1 also negatively regulates cardiac hypertrophy by interacting with and inhibiting Nfat3 transcriptional activity.50 It will be interesting to clarify the involvement of miR-206 in these cardiac events through FoxP1 targeting. In addition, FoxP1 is abundant in the nuclei of cardiomyocytes in failing human hearts.51 Because Mst1, a negative regulator of YAP, is activated in the failing heart,52 upregulation of FoxP1 may be mediated through downregulation of miR-206. The roles of miR-206 and FoxP1 in regulating myocardial cell death in the failing heart remain to be elucidated.

Some limitations in this study should be noted. First, the absolute level of miR-206 in the heart and the cardiomyocytes therein is low compared with skeletal muscle and the skeletal myocytes therein. Although we conducted multiple loss-of-function experiments, using anti-miRNA, SPONGE, LNA anti-miR, and miR-IDIAN inhibitor, the functional importance of endogenous miR-206 requires further scrutiny, for example, by using cardiac-specific miR-206 knockout mice. Second, increasing lines of evidence suggest that organs can take up miRNA from the circulation.53 Although the level of miR-206 in the circulation is low, it can be released from skeletal muscle after heavy workload, such as marathon running.54 Thus, we cannot formally exclude the possibility that exogenous, rather than endogenous, miR-206, such as that which originates from skeletal muscle, affects the function of the heart. Again, studies using skeletal muscle–specific miR-206 knockout mice would be helpful to address this issue.

In summary, we show here that miR-206 is upregulated by YAP and mediates YAP-induced hypertrophy and cardiomyocyte survival. The expression of miR-206 is regulated in response to I/R and oxidative stress. miR-206 promotes cardiomyocyte survival and cardiac hypertrophy in vivo, which in turn contributes to the regulation of myocardial injury and organ size. We also identify FoxP1 as a novel and critical downstream target of miR-206 that mediates its effects on cardiomyocyte survival and hypertrophy. To our knowledge, this is the first report demonstrating a functional miRNA target of YAP in cardiomyocytes that affects heart growth, death, and function.

Acknowledgments
We thank Daniela Zabolocki and Christopher D. Brady for critical reading of the article and Linda Reed for assistance with tail vein injections.

Sources of Funding
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Disclosures
None.

References


Novelty and Significance

What Is Known?

- Yes-associated protein (YAP) is a downstream effector of the Hippo signaling pathway, which controls organ size by modulating cell growth, proliferation, and apoptosis.
- YAP is a transcription cofactor, which can regulate multiple transcription factors, including TEAD, RUNX, TBX5, and FOXO.
- YAP promotes cardiomyocyte survival and proliferation in the postnatal heart, and cardiac regeneration and remodeling after myocardial infarction.

What New Information Does This Article Contribute?

- YAP upregulates miR-206, which in turn plays an important role in mediating the effect of YAP on cardiomyocyte survival and hypertrophy.
- miR-206 promotes cardiomyocyte survival during ischemia/reperfusion in the heart.
- miR-206 mediates cardiomyocyte hypertrophy and survival through inhibitory targeting of Forkhead box protein P1.

Increasing lines of evidence suggest that the Hippo pathway plays an important role in regulating the growth and death of cardiomyocytes by negatively regulating YAP, a transcription cofactor. The nuclear localization of YAP is negatively regulated by 2 upstream kinases, MST1 and LATS2, which constitute the core of the Hippo pathway. YAP that is localized in the nucleus positively regulates cardiomyocyte proliferation, hypertrophy, and survival. YAP is known to exert its function through TEAD and other transcription factors. However, the role of YAP in regulating miRNAs in the heart is unknown. We show that YAP transcriptionally upregulates miR-206, which in turn mediates the effect of YAP on cardiomyocyte hypertrophy and survival. Endogenous miR-206 also plays an essential role in mediating pressure overload-induced cardiac hypertrophy and cardiomyocyte survival during ischemia/reperfusion. One of the important targets of miR-206 is Forkhead box protein P1. Inhibition of Forkhead box protein P1 mediates the effect of miR-206 on cardiomyocyte hypertrophy and survival. Taken together, our results suggest that miR-206 is an important downstream effector of YAP that mediates both hypertrophy and cardiomyocyte survival at baseline and in response to stress. The YAP-miR-206 pathway seems to be a promising target for treatment of heart disease through modulation of myocardial growth and death mechanisms.
miR-206 Mediates YAP-Induced Cardiac Hypertrophy and Survival
Yanfei Yang, Dominic P. Del Re, Noritsugu Nakano, Sebastiano Sciarretta, Peiyong Zhai, Jiyeon Park, Danish Sayed, Akihiro Shirakabe, Shoji Matsushima, Yongkyu Park, Bin Tian, Maha Abdellatif and Junichi Sadoshima

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Materials and methods

Materials  Phenylephrine and propidium iodide were purchased from Sigma. miRIDIAN microRNA negative control and miRIDIAN miR-206 inhibitors were purchased from Thermo Scientific Dharmacon. The miRCURY LNA microRNA inhibitor targeting miR-206 and negative control were purchased from Exiqon. The LNA-anti-miR was used at a dose of 25 mg/kg and delivered by tail vein injection. The following antibodies were used: anti-α-tubulin (Sigma), anti-ANF (Santa Cruz), anti-α-sarcomeric actin (Sigma), anti-YAP (Cell Signaling), anti-phospho-YAP (Cell Signaling), anti-CARP (Santa Cruz), anti-GAPDH (Cell Signaling), anti-cleaved caspase-3 (Cell Signaling), anti-GFP (Cell Signaling) and anti-FoxP1 (Abcam).

Plasmids  YAP cDNA, FoxP1 cDNA and the genomic region of miR-206 were subcloned into pDC vectors with the CMV promoter for overexpression.

Adenoviral vectors  Adenoviruses harboring genes of interest were made using the AdMax system (Microbix). Short hairpin RNA knockdown adenovirus for FoxP1 or miR-206 was made using the adenoviral shuttle vector pDC316 (Microbix), into which the U6 RNA polymerase III promoter and the polylinker region of pSilencer 1.0U6 expression vector (Ambion) were subcloned.

Sequence of sh-FoxP1: 5’—3’ GCCCACACTGCAGAGGAAATTCAAGAGATTTCCTCTGCAGTGTGGGC TTTTTT;
Sequence of anti-miR-206: 5’—3’ CGCGTCTCGAGCCACACTGCAGAGGAAATTCAAGAGATTTCCTCTGCAGTGTGGGC TTTTTT.
We used adenovirus harboring scramble LacZ, miR-scramble, and shRNA-scramble as control virus. We confirmed that these control adenovirus vectors do not significantly affect hypertrophy or survival of CMs (Online Figure IX).

**Transgenic mice** miR-206 and anti-206-SPRONGE transgenic mice were generated on an FVB background, using the α-myosin heavy chain promoter to achieve cardiac-specific expression of around 300 bp of the genomic region of miR-206 or two repeats of GFP-tagged miR-206 antisense, respectively. All protocols concerning animal use were approved by the Institutional Animal Care and Use Committee at Rutgers, New Jersey Medical School.

**Primary culture of neonatal rat ventricular myocytes** Primary cultures of cardiac ventricular myocytes (NRCM) from 1-day-old Crl:(WI)BR Wistar rats (Charles River Laboratories) were prepared as previously described 1. In brief, ventricular myocytes were dispersed from the ventricles by digestion with collagenase type IV (Sigma), 0.1% trypsin (Life Technologies, Inc.) and 15 μg/ml DNase I (Sigma). Cell suspensions were applied to a discontinuous Percoll gradient. Culture media were changed to serum-free after about 24 hours. Myocytes were cultured under serum-free conditions for 36-48 hours before drug or virus treatments.

**miRNA microarray** Total RNA was isolated from YAP- and LacZ-transduced NRCMs. Ten micrograms of RNA were sent to L.C. Sciences for a miRNA microarray 2. Samples were enriched for small RNA, labeled with Cy3 and Cy5 fluorescent dyes and hybridized to a single Atactic μParaFlo microfluidics chip that held all 334 mature rodent miRNA probes identified to date, as well as perfectly matched and mismatched probes for quality control. Each miRNA
probe is represented 9X on the microarray. Among the control probes, PUC2PM-20B and PUC2MM-20B are the perfect match and single-based match detection probes, respectively, of a 20-mer RNA-positive control sequence that is spiked into the RNA samples before labeling. One may assess assay stringency from the intensity ratio of PUC2PM-20B to PUC2MM-20B, which is normally larger than 30. After signal amplification, the background was subtracted and normalized using the LOWESS (locally weighted regression) method. For a transcript to be listed as detectable, it must meet the following criteria: signal intensity higher than 3X (background SD), spot coefficient of variation<0.5 (coefficient of variation=SD/signal intensity) and signals from at least 50% of the repeating probes above detection level.

**Northern blot**  Total RNA, extracted using TRIzol reagent (Invitrogen), was separated on a 1% agarose gel, transferred to an uncharged nylon membrane, Hybond-NX (Amersham Biosciences), and UV cross-linked. The membrane was pre-hybridized/hybridized with MiracleHyb Hybridization solution according to the instruction manual (Stratagene). DNA oligonucleotides, anti-sense sequences of mature miRNAs, were obtained from Integrated DNA Technologies. The probes were 5’end- labeled with ET adenosine 5’-triphosphate[γ-32P] (PerkinElmer) using a T4 Polynucleotide Kinase kit (NEB) and used for hybridization (1X10^6cpm/ml).

**Transfection and luciferase assays**  Cells were transfected using FuGENE 6 transfection reagent (Roche) according to the manufacturer’s instructions. A plasmid containing the rat atrial natriuretic factor (ANF) promoter linked to firefly luciferase has been described. A TEAD luciferase reporter gene (8xGTIIC-luciferase) was a gift from Stefano Piccolo (Addgene plasmid
Forty-eight hours after transfection, cells were lysed with Passive Lysis Buffer (Promega), and the transcriptional activity was measured using the luciferase assay system (Promega) with an OPTOCOMP I luminometer (MGM instruments). For the 3’UTR luciferase assay, the FoxP1 3’UTR miR-206 target site (sequence with miR-206 binding sites) or a control site (sequence with mutated miR-206 binding sites) was cloned into the pMIR-REPORT vector (Ambion).

**Quantitative reverse transcription–polymerase chain reaction (qRT-PCR)** PCR primers for α-skeletal actin, ANF and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) have been described previously.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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</thead>
<tbody>
<tr>
<td>ANF s</td>
<td>ATGGGCTCCTTTCTCCATCAC</td>
</tr>
<tr>
<td>ANF as</td>
<td>ATCTTCGGTACCAGGAAGCTG</td>
</tr>
<tr>
<td>a-sk-Actin s</td>
<td>TATTCCTTCGTGACCACAGCTGAACGT</td>
</tr>
<tr>
<td>a-sk-Actin as</td>
<td>CGCGAACGCAGACGCGAGTGCGC</td>
</tr>
<tr>
<td>GAPDH s</td>
<td>TTCTTGTCAGTGCCAGCCTCGTC</td>
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<tr>
<td>GAPDH as</td>
<td>TAGGAACACGGAAGGCCATGCCAG</td>
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</tbody>
</table>

For miRNA qRT-PCR, we used the mirVana qRT-PCR miRNA Detection Kit and Primer Sets from Ambion as well as Taqman® miRNA assays from Applied Biosystems.

**Chromatin immunoprecipitation (ChIP) assay** ChIP assay was performed using the ChIP-IT Express Kit (Active Motif) according to the manufacturer’s instructions. Briefly, cultured cardiac myocytes were transduced with Ad-Yap (S127A) for 48 hours and then cells were fixed using a 1% concentration of formaldehyde for 10 minutes. Then, cells were washed with 1x PBS and
glycine was added to stop cross-linking reaction. Then, cells were scraped, nuclei were isolated and lysed, and sheared chromatin was isolated after sonication. Chromatin-protein complexes were then immunoprecipitated by incubating chromatin samples with anti-YAP antibody or control IgG antibody together with magnetic beads overnight. Beads were washed, chromatin was eluted and ChIP samples were analyzed by PCR. The primers used to amplify the mir-206 promoter region containing the 3 Ebox sequences are: sense 5’-TGCCAGTGTCGGTTCCCTCTC-3’; antisense 5’-CTTAGAGCTTGCCAAGGAGCTTC-3’.

**Cell viability assay** Viability of the cells was measured by CellTiter Blue assays (Promega). In brief, CMs (1X10^5 per 100μl) were seeded into 96-well culture dishes. After 24 hours, the medium was changed to a serum-free medium. CMs were transduced with the relevant adenoviruses for 48 hours, and then treated with 100 μM H2O2 for 48 hours or 5 μM chelerythrine for 1 hour. Viable cell numbers were measured by the CellTiter Blue assay according to the manual. Each experiment was performed in quadruplicate at least three times.

**Evaluation of apoptosis in cardiomyocytes** DNA fragmentation was detected in cultured cells using TUNEL as described previously 1. Nuclear density was determined by counting DAPI-stained nuclei in 20 different fields for each sample.

**Ischemia/reperfusion** The ischemia/reperfusion (I/R) procedure has been described previously4. In brief, ischemia was achieved by ligating the anterior descending branch of the LAD using an 8-0 nylon suture, with a silicon tubing (1 mm OD) placed on top of the LAD, 2 mm below the border between the left atrium and LV. Regional ischemia was confirmed by ECG change (ST
After occlusion for 45 minutes, the silicon tubing was removed to achieve reperfusion.

**Assessment of area at risk and infarct size** After 45 minutes of ischemia and 24 hours of reperfusion, mice were reanesthetized and intubated, and the chest was opened. After arresting the heart at the diastolic phase by KCl injection, the ascending aorta was cannulated and perfused with saline to wash out blood. The LAD was occluded with the same suture, which had been left at the site of the ligation. To demarcate the ischemic area at risk (AAR), Alcian blue dye (1%) was perfused into the aorta and coronary arteries. Hearts were excised, and LVs were sliced into six 1-mm-thick cross-sections. The heart sections were then incubated with a 1% triphenyltetrazolium chloride (TTC) solution at 37°C for 12 minutes. The infarct area (pale), the AAR (not blue) and the total LV area from both sides of each section were measured using ImageJ (ImageJ 1.42K), and the values obtained were averaged. The percentage of area of infarction and AAR of each section were multiplied by the weight of the section and then totaled from all sections. AAR/LV and infarct size/AAR were expressed as percentages.

**Echocardiography** Mice were anesthetized using 12 μl/g body weight of 2.5% tribromoethanol (Avertin, Sigma), and echocardiography was performed as described previously ¹, using a 13-MHz linear ultrasound transducer. Two-dimensional guided M-mode measurements of LV internal diameter were obtained from at least three beats and then averaged. LV end-diastolic dimension (LVEDD) was measured at the time of the apparent maximal LV diastolic dimension, and LV end-systolic dimension (LVESD) was measured at the time of the most anterior systolic excursion of the posterior wall. LVEF was calculated using the following formula: $LVEF(\%) = \frac{LVEDD - LVESD}{LVEDD} \times 100$. 

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¹ Authors: This is a citation. Please refer to the original research for the detailed methods.
100 X \( \frac{(LVEDD^3 - LVESD^3)}{LVEDD^3} \).

**Immunostaining.** Cells were fixed with PBS containing 4% paraformaldehyde, permeabilized in PBS containing 0.2% Triton X-100, blocked with PBS containing 3% BSA and incubated with the desired primary antibodies (dilution 1:400 for anti-troponin T antibody, 1:200 for anti-ANF antibody, 1:200 for anti-Ki67 antibody). Alexa Fluor 488 Dye- or Alexa Fluor 594 Dye-conjugated secondary antibody (Invitrogen) (dilution 1:400) was used for detecting indirect fluorescence. Ki67 positive myocytes were identified by calculating the percentage of Ki67 and Troponin T double positive cells in all the Troponin T positive cells.

**Histological analysis** Heart specimens were fixed with formalin, embedded in paraaffin, and sectioned at 6-μm thickness. Cardiomyocyte cross-sectional area was measured from images captured from wheat germ agglutinin-stained sections as described previously 5.

**Immunohistochemistry** Heart sections were stained with anti-Ki67 rabbit polyclonal antibody (Abcam), anti-troponin-T mouse monoclonal antibody (Neomarkers), Alexa-fluor 488 Dye-conjugated goat anti-rabbit IgG (Molecular Probes), Alexa-fluor 594 Dye-conjugated goat anti-mouse IgG (Molecular Probes) and Vectashield mounting medium with DAPI (Vector Laboratories). Analyses were performed using fluorescence microscopy (Zeiss).

**Statistics** All values are expressed as mean±SEM. Statistical analyses were performed using ANOVA or the t-test with a P<0.05 considered significant. The sample size needed to detect changes obtained through preliminary experiments was calculated with Power and Sample Size.
Analysis (http://biostat.mc.vanderbilt.edu/wiki/Main/PowerSampleSize), using 80% power and 5% significance level.
REFERENCES


Online Figure I: *bantam* and select miRNA stem loop/mature sequence alignment. Pictures of dme-bantam, mmu-mir-206, mmu-mir-711 and mmu-mir-483 stem loop structures were captured from miRBase (www.mirbase.org). Nucleotides in miR-206, mir-711 and mir-483 that are identical to those in *bantam* are highlighted in green (seed region) and red (outside of seed region).
A

**Relative Cell Size**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>YAP</th>
<th>miR-206</th>
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* P=0.0040 vs Control
# P=0.0036 vs Control
$ P=0.0043 vs Control

B

**Cell Size**

<table>
<thead>
<tr>
<th></th>
<th>Sh-sc</th>
<th>miR-29c</th>
<th>miR-711</th>
<th>miR-483</th>
</tr>
</thead>
</table>

![Graph](image2.png)

C

**miR-206/U6 (qRT-PCR)**

<table>
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<tr>
<td>YAP</td>
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P=0.0476
P=0.1575
P=0.5175

D

**Relative miR-206/5S rRNA (NB)**

<table>
<thead>
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<th>Mst1</th>
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P=0.0075

E

**miR206/U6 (qRT-PCR)**

<table>
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<th>LacZ</th>
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P=0.003
P=0.0468

F

**TEAD Luciferase**

<table>
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<th>YAP</th>
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P=0.0001
Online Figure II: The role of select miRNA in cardiomyocyte hypertrophy; YAP and Mst1 reguate the abundance of miR-206.

(A and B) Cardiomyocytes transduced with Ad-Control (Ad-short hairpin scrambled (sh-sc) or Ad-LacZ), Ad-YAP, Ad-miR-206, Ad-miR-29c, Ad-miR-711, Ad-miR-483 or treated with PE were harvested for relative cell surface area (cell size) measurement with live cell images, N=3 experiments. (C) Cardiomyocytes or C2C12 cells transduced with Ad-LacZ or Ad-YAP were harvested for qRT-PCR analyses with miR-1, miR-206 and U6 primers. N=3 experiments. (D) Cardiomyocytes transduced with Ad-LacZ or Ad-Mst1 were harvested for Northern blot analyses using a miR-206 probe and the results were quantified by densitometry. N=3 experiments. (E) NRCMs were transduced with control LacZ, Mst1 or Mst1 and mutant YAP (S127A), which is resistant to inhibitory phosphorylation, and qRT-PCR was performed 48 hours later to detect levels of miR-206 and U6. (F) NRCMs were transfected with GFP or wild-type YAP in combination with a TEAD-luciferase reporter construct and assayed 24 hours later. N=3 experiments. Values are mean ± SEM.
Online Figure III: miR-206 and miR-1 qRT-PCR primer specificity.
(A) qRT-PCR products obtained using either miR-206 or miR-1 primers were sequenced and aligned with miR-206 or miR-1 mature sequence. (B) Cardiomyocytes transduced with Ad-scrambled, Ad-miR-206 or Ad-miR-1 were harvested for qRT-PCR analyses with miR-206, miR-1 and 5S rRNA primers. N=3 experiments. Values are mean ± SEM.
Figure A: Bar graph showing relative luciferase activity for miR-206 and miR-1. The y-axis represents Luciferase Activity, and the x-axis represents Enhancer. The bars indicate LacZ, YAP, miR-206, and miR-1. The statistical significance is P=0.0006 for miR-206 and P=0.0002 for miR-1.

Figure B: Bar graph showing relative protein/DNA content for miR-206 and miR-1. The y-axis represents Relative Protein/DNA Content, and the x-axis represents Control, miR-206, and miR-1. The statistical significance is P=0.0002 for miR-206 and P=0.0110 for miR-1.

Figure C: Bar graph showing the relative cleaved-Casp3/Actin ratio. The y-axis represents Relative Cleaved-Casp3/Actin, and the x-axis represents Chelerythrine, miR-206, and miR-1. The statistical significance is P=0.0071 for miR-206 and P<0.0001 for miR-1.

Figure D: Bar graph showing the percentage of TUNEL-positive myocytes. The y-axis represents % TUNEL-positive myocytes, and the x-axis represents Vehicle and Chelerythrine. The statistical significance is * P=0.0151 vs. LacZ + Veh, # P=0.0001 vs. Sh-sc + Veh, § P=0.0004 vs. Sh-sc + Chele, and % P=0.0232 vs. Sh-sc + Chele.

Figure E: Images and bar graph showing the percentage of Ki-67-positive myocytes. The y-axis represents % Ki-67-positive myocytes, and the x-axis represents LacZ, Sh-sc, miR-206, and miR-1. The statistical significance is P=0.3103 for miR-206 and P=0.6319 for miR-1.
Online Figure IV: miR-206 and miR-1 have distinct effects upon hypertrophy and apoptosis in cardiomyocytes.

(A) Control or YAP-overexpressing cardiomyocytes co-transfected with luciferase constructs harboring the enhancer region of either miR-206 or miR-1 as the promoter were harvested for luciferase assay. N=4. (B) Cardiomyocytes transduced with Ad-Control, Ad-miR-206 or Ad-miR-1 were harvested for protein and DNA content measurement. N=4. (C and D) Control (LacZ or Sh-sc), miR-206 or miR-1-overexpressing cardiomyocytes treated with chelerythrine (Chele) were harvested for immunoblot analyses with anti-cleaved caspase 3 (Cleaved-Casp3) antibody and the results were quantified by densitometry (C), and TUNEL staining. The percentage of TUNEL-positive nuclei was calculated (D). N=4 experiments. (E) Cardiomyocytes transduced with Ad-Control, Ad-miR-206 or Ad-miR-1 were harvested for staining with anti-Ki67 (green), anti-Troponin T (red) antibodies and DAPI (blue). The percentage of Ki67-positive nuclei was calculated. N=3 experiments. Values are mean ± SEM.
Online Figure V: Tg-miR-206 mice develop cardiac hypertrophy with preserved function.

(A) Hearts of 8-week-old-Tg-miR-206 (line 2, 16, 24 and 36) and NTg (line 16 and 24) mice were harvested for Northern blot analysis using miR-206 probes (left), or subjected to qRT-PCR to detect miR-1 expression (line 24, right). (B) Postmortem measurements of heart weight/body weight (HW/BW, mg/g), left ventricular weight/body weight (LVW/BW, mg/g) and lung weight/body weight (LungW/BW, mg/g). (C) Representative pictures of wheat germ agglutinin (WGA) staining (left) and quantitative analysis of myocyte cross-sectional area (CSA) (right) are shown. Scale bar, 50 μm. (D) Echocardiographic analysis and ejection fraction was calculated and representative echocardiographic pictures are shown (line 24). The number of mice in each group is shown in parentheses.
Estimated cardiac myocyte number (millions)

miR-206/U6 (qRT-PCR)

P=0.7422
P=0.0075

miR-206/U6 (qRT-PCR)

P=0.039
P=0.0144

miR-206/U6 (qRT-PCR)
Online Figure V: Tg-miR-206 mice develop cardiac hypertrophy with preserved function (continued)

(E and F) Heart sections of 8-week-old Tg-miR-206 and NTg mice were harvested for staining with anti-Ki67 (green), anti-Troponin T (red) antibodies and DAPI (blue) (E), and H&E staining for total cardiac myocyte number estimation (Ref. 54) (F). N=4. (G) Wild-type male C57B/6J mice were administered scramble negative control (CTRL) or LNA-anti-miR-206 (25 mg/kg) by tail vein injection. 7 days later heart tissue was harvested and miRNA levels determined by qRT-PCR. (H) C57B/6J mice were administered scramble negative LNA control (CTRL) or LNA-anti-miR-206 (25 mg/kg) by tail vein injection. 7 days later, mice were subjected to TAC or sham operation. Heart tissue was harvested 7 days post-TAC for analysis. N=4-5. Values are mean ± SEM. (I) Heart sections were stained with WGA and CM CSA was determined. Representative images (left panel) and quantification (right panel) are shown. Scale bar, 50 μm.
Online Figure VI: miR-206 inhibitors attenuate YAP-induced cardiac hypertrophy.

(A) CMs were stained with anti-ANF antibody (red, upper) and DAPI (blue, lower). Representative ANF perinuclear staining is shown (upper). ANF-positive cells were quantified. N=3. (B-E) miRIDIAN microRNA negative control inhibitor (Mdn-Ctrl or CtrlM) or miRIDIAN miR-206 inhibitor (Mdn-206 or 206M) transfected cardiomyocyte were co-transfected with luciferase constructs harboring either an empty, miR-206 antisense (cp206) (B), FoxP1 3'UTR or a mutated FoxP1 3'UTR sequence (C) and harvested for luciferase assay, or transduced with Control (Ad-GFP or Ad-LacZ) or Ad-YAP and harvested for ANF luciferase assay (D) and cell size measurement (E). N=3-4 experiments. (F and G) LacZ or YAP-overexpressing cardiomyocytes co-transduced with or without Ad-anti-1 were harvested for Northern blotting using miR-1 and miR-206 probes (F), or protein and DNA content measurement (G). N=4-6 experiments. Values are mean ± SEM.
Online Figure VII: The abundance of YAP, miRNA-206 and miR-1 under stress conditions.
(A) Cardiomyocytes treated with H$_2$O$_2$ were harvested for Northern blot analysis using miR-206 and U6 probes and the results were quantified by densitometry. (B-D) Hearts of FVB mice at 3 months of age subjected to ischemia/reperfusion (I/R) were harvested for Northern blot analysis using miR-206 and miR-1 probes (B and D) or immunoblot analyses with anti-YAP antibody (C), and the results were quantified by densitometry. N=5 mice. (E and F) Hearts of Tg-206-SPONGE and NTg mice were harvested for Northern blot analysis using miR-206 and miR-1 probes, and the results were quantified by densitometry. N=3 mice. (G) Tg-miR-206 and NTg mice were subjected to 45 minutes of ischemia. Echocardiography was performed just prior to ischemia and at 1 and 2 weeks after ischemia. Percent fractional shortening (%FS) is shown. N=9 mice/group. Values are mean ± SEM.
### A

**miR-206 binding sites**

```
FoxP1 3'UTR
```

**miR-206 UGGAUGU AAGGAAGUGUGUGG**

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<th>Species</th>
<th>miR-206 Binding Sites</th>
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<td>Mmu</td>
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### B

**No virus**

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**Ad-Scramble**

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**P = 0.9083**

### C

**Scramble**

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**miR-206**

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**miR-1**

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### D

**Relative FOXP1/Actin**

- **LacZ**: P = 0.0004
- **FoxP1**: P = 0.0014

**P = 0.0008**

**Sh-sc**

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**Sh-FoxP1**

<table>
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<th>ACTIN</th>
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<tr>
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</table>

**P = 0.0014**
Online Figure VIII: miR-206 and miR-1 target specificity.
(A) Cartoon shows miR-206-binding sites (red indicates conserved sites) in FoxP1 3'UTR. A picture captured from TargetScan (www.targetscan.org) showing two conserved miR-206-binding sites in the 3’UTR of FoxP1. (B) Cardiomyocytes were untreated (no virus) or transduced with Ad-Scramble control and western blot performed to determine levels of FoxP1. Quantification of western results (right panel). (C) Cardiomyocytes transduced with Ad-short hairpin-scrambled (sh-sc), Ad-miR-206 or Ad-miR-1 were harvested for immunoblot analyses using an anti-FoxP1 antibody. (D) LacZ- or FoxP1-overexpressing cardiomyocytes co-transduced with Ad-short hairpin FoxP1 (sh-FoxP1) were harvested for immunoblot analyses with anti-FoxP1 antibody, and the results were quantified by densitometry. N=4 experiments. (E) Quantification of results shown in Figure 7C. (F) Quantification of results shown in Figure 7D. (G) H₂O₂-treated cardiomyocytes transduced with Ad-short hairpin scrambled (sh-sc) or Ad-short hairpin FoxP1 (sh-FoxP1) were harvested for viability assay using Cell Titer Blue. N=3 experiments. (H) Control or miR-206-overexpressing cardiomyocytes co-transduced with Ad-FoxP1 treated with chelerythrine were harvested for immunoblot analyses using anti-FoxP1 antibody, and the results were quantified by densitometry. N=4 experiments. Values are mean ± SEM.
Online Figure IX: The effect of control viral transduction on cardiomyocyte size and viability.

(A) Cardiomyocytes were either untreated (no virus) or transduced with Ad-LacZ, Ad-Scramble or Ad-short hairpin scrambled (Ad-sh-Scramble) adenovirus for 72 hours. Cells were then fixed, permeabilized and stained with α-actinin to selectively identify cardiomyocytes. Representative images are shown. Scale bar, 30 μm. (B) Cardiomyocytes were either untreated (no virus) or transduced with Ad-LacZ, Ad-Scramble or Ad-short hairpin scrambled (Ad-sh-Scramble) adenovirus for 72 hours. Viability was then determined using Cell Titer Blue assay. N=3 experiments. Values are mean ± SEM.
<table>
<thead>
<tr>
<th>miRNA</th>
<th>Ad-LacZ</th>
<th>Ad-YAP</th>
<th>Fold change (YAP/LacZ)</th>
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<tr>
<td>mmu-miR-711</td>
<td>1410.75</td>
<td>5121.44</td>
<td>3.63</td>
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<td>mmu-miR-483</td>
<td>722.61</td>
<td>2315.08</td>
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<td>mmu-miR-206</td>
<td>815.93</td>
<td>2219.02</td>
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<td>mmu-miR-29c</td>
<td>1041.12</td>
<td>1953.30</td>
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<td>mmu-miR-143</td>
<td>14,703.21</td>
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<td>mmu-miR-133a</td>
<td>29,462.95</td>
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<tr>
<td>mmu-miR-133b</td>
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<td>rno-miR-143</td>
<td>13,010.33</td>
<td>10,865.85</td>
<td>0.84</td>
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</tbody>
</table>

Table I: Cardiomyocyte miRNAs regulated by YAP as evaluated with miRNA microarray analysis.

Total RNA was extracted from Ad-LacZ or Ad-YAP transduced neonatal rat cardiomyocytes prepared from multiple dishes, enriched for small RNAs, and analyzed by microarray containing rodent microRNAs. miRNAs that were upregulated at least 2-fold by Ad-YAP and exceeded a raw value threshold of 400 are shown in the upper panel (green). miRNAs that have known homology to *Drosophila bantam* are shown in the lower panel (red).
Table II: Echocardiographic analysis of scramble control treated and anti-miR-206 treated wild-type mice subjected to pressure overload stress.

Wild-type male C57B/6 mice were administered scramble negative control (CTRL) or LNA-anti-miR-206 1 week prior to stress. Mice were then subjected to sham operation or TAC surgery. One week post-TAC all groups were evaluated by echocardiography. *, P=0.0003 versus CTRL + TAC. **, P=0.0049 versus CTRL + TAC. ***, P=0.0027 versus CTRL + TAC.

<table>
<thead>
<tr>
<th></th>
<th>SHAM</th>
<th>TAC</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>CTRL n=4</td>
<td>anti-206 n=4</td>
<td>CTRL n=6</td>
<td>anti-206 n=6</td>
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<tr>
<td>DSEP WT</td>
<td>0.74 ± 0.09</td>
<td>0.70 ± 0.02</td>
<td>0.97 ± 0.04</td>
<td>0.80 ± 0.04*</td>
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<tr>
<td>LVEDD</td>
<td>3.31 ± 0.05</td>
<td>3.21 ± 0.06</td>
<td>3.08 ± 0.10</td>
<td>3.56 ± 0.12</td>
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<td>DPW WT</td>
<td>0.89 ± 0.10</td>
<td>0.81 ± 0.04</td>
<td>1.00 ± 0.05</td>
<td>0.75 ± 0.03**</td>
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<tr>
<td>SSEP WT</td>
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<td>1.11 ± 0.05</td>
<td>1.39 ± 0.04</td>
<td>1.31 ± 0.05</td>
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<tr>
<td>LVESD</td>
<td>2.07 ± 0.04</td>
<td>1.98 ± 0.03</td>
<td>1.98 ± 0.07</td>
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<tr>
<td>SPW WT</td>
<td>1.32 ± 0.11</td>
<td>1.15 ± 0.06</td>
<td>1.42 ± 0.04</td>
<td>1.15 ± 0.07***</td>
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<tr>
<td>EF(%)</td>
<td>0.75 ± 0.02</td>
<td>0.77 ± 0.01</td>
<td>0.73 ± 0.03</td>
<td>0.77 ± 0.01</td>
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<tr>
<td>%FS</td>
<td>37.5 ± 1.7</td>
<td>38.5 ± 0.5</td>
<td>35.4 ± 2.1</td>
<td>38.8 ± 1.3</td>
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<td>BW(G)</td>
<td>28.0 ± 0.9</td>
<td>25.0 ± 0.7</td>
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