In Vivo Suppression of MicroRNA-24 Prevents the Transition Toward Decompensated Hypertrophy in Aortic-Constricted Mice

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Rationale: During the transition from compensated hypertrophy to heart failure, the signaling between L-type Ca2+ channels in the cell membrane/T-tubules and ryanodine receptors in the sarcoplasmic reticulum becomes defective, partially because of the decreased expression of a T-tubule–sarcoplasmic reticulum anchoring protein, junctophilin-2. MicroRNA (miR)-24, a junctophilin-2 suppressing miR, is upregulated in hypertrophied and failing cardiomyocytes.

Objective: To test whether miR-24 suppression can protect the structural and functional integrity of L-type Ca2+ channel–ryanodine receptor signaling in hypertrophied cardiomyocytes.

Methods and Results: In vivo silencing of miR-24 by a specific antagomir in an aorta-constricted mouse model effectively prevented the degradation of heart contraction, but not ventricular hypertrophy. Electrophysiology and confocal imaging studies showed that antagomir treatment prevented the decreases in L-type Ca2+ channel–ryanodine receptor signaling fidelity/efficiency and whole-cell Ca2+ transients. Further studies showed that antagomir treatment stabilized junctophilin-2 expression and protected the ultrastructure of T-tubule–sarcoplasmic reticulum junctions from disruption.

Conclusions: MiR-24 suppression prevented the transition from compensated hypertrophy to decompensated hypertrophy, providing a potential strategy for early treatment against heart failure. (Circ Res. 2013;112:601-605.)

Key Words: Ca2+ signaling • hypertrophic cardiomyopathy • hypertrophy • heart failure • myocardial contraction

Transition from compensated hypertrophy to decompensated hypertrophy represents a key step in the development of heart failure.1–5 One of the hallmarks of this transition is the decreased strength of cardiac contraction.5,6 In heart cells, the contraction is initiated by periodic transient increases in intracellular Ca2+. During each Ca2+ transient, the Ca2+ influx through L-type Ca2+ channels (LCCs) in the cell membrane and transverse tubules (TTs) triggers Ca2+ release from ryanodine receptors (RyRs) in the sarcoplasmic reticulum (SR).4–7 The structural integrity of the LCC–RyR signaling apparatus relies on a TT–SR linker protein, known as junctophilin-2 (JP2),8–10 which is downregulated in all tested animal models and human specimens of decompensated hypertrophy and heart failure.10–12 Recently, we found that microRNA (miR)-24, a miR that suppresses JP2 expression, is upregulated in hypertrophy/heart failure.15 Because overexpression of miR-24 suppresses both JP2 expression and excitation–contraction (E–C) coupling efficiency,15 we hypothesized that miR-24 upregulation is a key factor in the transition from compensated hypertrophy to heart failure.

In the present study, we tested this hypothesis by treating aorta-constricted mouse models of hypertrophy with a specific antagonist16 against miR-24. We found that in vivo silencing of miR-24 indeed protected the E–C coupling from structural and functional remodeling, preventing the transition from compensated hypertrophy to decompensated hypertrophy.

Methods

We created a chronic mouse model of pressure-overload hypertrophy by transverse aortic constriction (TAC) surgery, as described.17 In one

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Methods

We created a chronic mouse model of pressure-overload hypertrophy by transverse aortic constriction (TAC) surgery, as described.17 In one
of the TAC groups, we suppressed the expression of miR-24 by periodic injection (Online Figure I) of a chemically modified antisense oligonucleotide antagomir specific for miR-24. An oligonucleotide with mismatches to miR-24 was injected into another TAC group for negative control (NC). Single cardiomyocytes were isolated around 30 weeks after surgery for structural and functional analysis using electron microscopy,10 electrophysiology, 12 and confocal Ca2+ imaging,12 as described. The methods are detailed in the Online Data Supplemental Materials.

**Results**

**MiR-24 Suppression Prevented Decompensation but Not Hypertrophy**

Compared with that in the sham-operated group, the miR-24 level in isolated ventricular myocytes exhibited a ≈2.5-fold increase in the NC group, but not in the antagomir group (Figure 1A), indicating that the upregulation of miR-24 associated with TAC-induced hypertrophy was successfully suppressed by the antagomir treatment.

Echocardiographic measurements (Figure 1B) showed that left ventricle hypertrophy developed 4 weeks after TAC surgery in our models (Figure 1C). Around 15 weeks later, the fractional shortening became decreased (Figure 1D), indicating a transition from compensated to decompensated hypertrophy. Notably, although in vivo antagomir treatment did not interfere with the development of hypertrophy (Figure 1C), it did prevent the reduction of fractional shortening (Figure 1D), indicating that the transition toward decompensated hypertrophy was effectively prevented by miR-24 suppression.

**In Vivo MiR-24 Suppression Protected E–C Coupling in Cardiomyocytes**

To examine whether miR-24 suppression protected E–C coupling at the cellular level, we recorded the Ca2+ transient evoked by whole-cell LCC Ca2+ current (Figure 2A) under a condition (resting cardiomyocytes equilibrated in 2 mmol/L extracellular Ca2+), where the SR Ca2+ load was comparable among all groups (Online Figure II). In the NC group, TAC induced a significant reduction in Ca2+ transient amplitude without altering whole-cell LCC Ca2+ current density (Figure 2B), leading to a decreased gain of E–C coupling (Figure 2C) and reduced fraction of cell contraction (Figure 2D). In contrast, the Ca2+ transient amplitude (Figure 2B), the E–C coupling gain (Figure 2C), and the fractional shortening (Figure 2D) were well maintained after TAC in the antagomir group, indicating that miR-24 suppression protected the integrity of E–C coupling in hypertrophied cardiomyocytes.

Ca2+ transients are composed of numerous Ca2+ sparks evoked by LCC openings. Using unique loose-patch confocal imaging technology,7,12 we investigated the effect of the antagomir on LCC–RyR intermolecular Ca2+ signaling. To visualize single LCC activity, in the form of Ca2+ sparklets,7 we included in the pipette solution 20 mmol/L Ca2+ and 10 μmol/L FPL64176, an LCC agonist. Depolarization of on-cell patches evoked 2 distinct populations of local Ca2+ events (Figure 3A): steep, ryanodine-sensitive Ca2+ sparks from RyRs; and flat, ryanodine-resistant, but nifedipine-sensitive, Ca2+ sparklets from individual LCCs.7 With comparable Ca2+ release duration (time-to-peak), the amplitude of Ca2+ sparks was significantly lower in the NC group but not in the TAC antagomir group (Figure 3B), indicating that the TAC-induced decrease of local Ca2+ release flux was prevented by antagomir treatment. To quantify the fidelity of LCC–RyR coupling, we measured the percentage of the first detectable Ca2+ sparklets that successfully triggered Ca2+ sparks during the depolarization. The fidelity was decreased significantly in the NC group but unchanged in the antagomir group (Figure 3C, upper). Also, the percentage of depolarization pulses that

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Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>E–C</td>
<td>excitation–contraction</td>
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<tr>
<td>ICa</td>
<td>whole-cell Ca2+ current through L-type Ca2+ channels</td>
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<tr>
<td>JP2</td>
<td>junctophilin-2</td>
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<tr>
<td>LCC</td>
<td>L-type Ca2+ channel</td>
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<td>NC</td>
<td>negative control</td>
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<td>NFAT</td>
<td>nuclear factor of activated T-cells</td>
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<tr>
<td>RyR</td>
<td>ryanodine receptor</td>
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<tr>
<td>SR</td>
<td>sarcoplasmic reticulum</td>
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<td>TAC</td>
<td>transverse aortic constriction</td>
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<td>TT</td>
<td>transverse tubule</td>
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Figure 1. In vivo microRNA (miR)-24 silencing in mouse hypertrophy models. A, Real-time PCR assay of miR-24 expression in sham (n=4), negative control (NC; n=3), and antagomir (n=3) groups. B, Representative echocardiograms before and 25 weeks after transverse aortic constriction (TAC) surgery in NC and antagomir groups. C, Left ventricle posterior wall diastolic thickness (PWD, upper) and D, fractional shortening (FS, lower) measured by echocardiography. *P<0.05; **P<0.01 vs sham; #P<0.05; and ##P<0.01 vs NC.
failed to trigger a Ca\textsuperscript{2+} spark (miss index) was increased in the NC group but not in the antagonim group (Figure 3C, lower). We also quantified LCC–RyR coupling kinetics by the latency from the onset of a Ca\textsuperscript{2+} sparklet to the takeoff of a triggered Ca\textsuperscript{2+} spark (Figure 3D). Exponential fitting of the coupling latency (Figure 3E) showed that the time constant for LCC–RyR coupling was prolonged in the NC group but unchanged in the antagomir group (Figure 3F). These results indicated that miR-24 suppression effectively prevented the decreased efficiency and slowed kinetics of LCC–RyR signaling in failing heart cells.12,18

MiR-24 Suppression Prevented Structural Remodeling of E–C Coupling Apparatus

Next, we checked the ultrastructural basis of LCC–RyR communication using transmission electron microscopy. Stereological analysis (Online Figure III) showed that the volume density and the surface area of TTs apparently coupled to SRs were dramatically decreased in the NC group but not in the antagonim group (Figure 4A). The increase of bald TTs and decrease of junctional SRs were also suppressed by the antagomir. In failing heart cells, TT–SR junctions were displaced from the Z-line area, exhibiting increased junction-Z distance (Figure 4B and 4C).10 The increased junction-Z distance was not observed in the antagonim group (Figure 4C). The spatial span of individual TT–SR junctions is one of the determinants of LCC–RyR signaling efficiency.10 We found that the antagomir prevented the shrinkage of individual junction size (Figure 4D). These data indicated that the defects of TT–SR junctions in failing cardiomyocytes were prevented by miR-24 suppression.

JP2 is a structural protein maintaining the morphology of TT–SR junctions and efficiency of LCC–RyR signaling.8–10 We found that the levels of both JP2 mRNA and protein,
which were significantly decreased in the NC group, were unchanged in the antagonim group (Figure 4E).

Discussion

E–C coupling becomes defective during the chronic transition from compensated hypertrophy to heart failure.12,20 In the present study, we show that in vivo silencing of miR-24 in an aortic-constricted mouse model effectively protects cardiomyocytes from structural/functional disruption of E–C coupling and prevents the transition toward decompensated hypertrophy.

MiR-24 is expressed in cardiomyocytes and many other cell types, and regulates multiple target proteins.19–22 We have recently shown that overexpression of miR-24, as observed in heart failure/hypertrophy models, suppresses JP2 expression and leads to defective E–C coupling in cardiomyocytes.15 In the present study, we show that the JP2 downregulation is prevented by the miR-24 antagonim in TAC mice. As our bioinformatic analysis was not able to identify other miR-24 targets with known function related to E–C coupling, the stabilization of JP2 at least partially explains the protective effects of miR-24 suppression on TT–SR junctions and E–C coupling.

Besides E–C coupling, whether other histological/molecular hallmarks of decompensation, such as fibrosis, are altered by miR-24 modulation still needs further in-depth studies.

The pathogenesis of hypertrophy and heart failure involves a variety of intracellular signaling cascades, including the calcineurin–nuclear factor of activated T-cells (NFAT) pathway, the calmodulin-dependent protein kinase pathway, and pathways involving other protein kinases.23,24 The NFATc3 pathway controls the miR cluster miR-23a≈27a≈24-2, which is upregulated in hypertrophy.21,22,25 In this cluster, miR-23, but not miR-24 and miR-27, is found essential in the isoproterenol/aldosterone-induced cardiomyocyte hypertrophy.25 Agreeing with this report, our present study shows that miR-24 suppression in vivo does not prevent TAC-induced hypertrophy. Excitingly, miR-24 suppression does prevent the structural and functional degradation of E–C coupling, indicating that miR-24 upregulation is important in the transition from compensated hypertrophy to heart failure.

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Disclosures

None.
MicroRNA (miR)-24, a miR that suppresses JP2 expression, is upregulated in failing heart cells. During the transition from compensated hypertrophy to heart failure, cardiac E–C coupling becomes defective, partially because of the downregulation of TT–SR anchoring protein—JP2. Because miR-24, which suppresses JP2, is upregulated in failing cardiomyocytes, we tested whether suppression of miR-24 protects the integrity of E–C coupling. We found that in vivo silencing of miR-24 blocks the transition to decompensate hypertrophy, whereas allowing compensated hypertrophy to persist in mice subjected to TAC. Cellular studies showed that miR-24 antagonist treatment protects cardiomyocytes from structural and functional remodeling of E–C coupling apparatus. These findings suggest that miR-24 may be a potential target in the treatment of heart failure.

References

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Online Methods

TAC surgery
TAC surgery was performed on male C57BL/6 mice (8 weeks old) as described.1 All experimental protocols were approved by the Peking University Institutional Committee for Animal Care and Use. Briefly, mice were anesthetized with a ketamine-xylazine mixture (5:3, 1.32 mg/kg ip). A longitudinal cut was made in the proximal portion of the sternum. A 7-0 silk suture was tied around a 26-gauge needle and the aorta between the right innominate artery and left common carotid artery. After ligation, the needle was promptly removed. The sham procedure was identical except that the aorta was not ligated. To characterize the models, echocardiography was performed before and every two weeks after the surgery using a Vevo 770 ultrasound system (VisualSonics Inc., Toronto, ON, Canada) as reported.2 The left ventricular fractional shortening was calculated as FS = (LVDd - LVDs) / LVDd.

Oligonucleotide Administration and Echocardiographic measurements
A chemically-modified antisense oligonucleotide (antagomir)3 specific for miR-24 and a non-specific control oligonucleotide were synthesized by RiboBio Co., Ltd (Guangzhou, China). The sequence of the antagomir against microRNA-24 is: 5’-mC(s)mU(s)mGmUmUmCmCmUmGmCmUmGmAmAmCmUmGmAmG(s)mC(s)mC(s)mA(s)-Chol-3’, where m is a 2’-OMe-modified nucleotide, (s) is a phosphorothiate linkage, and Chol is a cholesterol group linked through a hydroxyprolinol linkage. After ~2 weeks of recovery from the TAC surgery, the mice were treated with the oligonucleotides (diluted in 0.2 ml saline) at 80 mg/kg body weight through tail vein injection for 3 consecutive days. The 3-day treatment was repeated every 6-8 weeks (Online Figure II) according to the manufacturer’s suggestion. Saline was injected into sham-operated mice and a group of TAC mice for control purposes.

MicroRNA and mRNA expression assays
Total RNA and total microRNAs were extracted from cardiac tissues and cell samples using Trizol reagent (Invitrogen) and a microRNA isolation kit (mirVana, Ambion), respectively, according to the manufacturer’s instructions. The first strand cDNA was first synthesized by microRNA-specific reverse-transcription primers (RiboBio Co., Ltd) (for miR-24 ) or oligodT15 (for JP2) using SuperScript III Reverse Transcriptase (Invitrogen Crop). 10 ng of cDNA was applied for real-time PCR amplification using Brilliant II SYBR Green QPCR master mix (Stratagene), and the fluorescent signals were monitored by an Mx3000p Real-Time PCR System (Stratagene). The thermo-cycling program was as follows: 95°C for 10 min, followed by 40 cycles at 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s, and finally an additional dissociation step to ensure the specificity of amplification. The primers for microRNA sample amplification were provided by RiboBio Co., Ltd, and the primers for mouse JP2 and GAPDH were the following: mouse JP2 (forward: 5’-AGG CCG GTG CCA AGA AGA AG-3’; reverse: 5’-CGA TGT TCA GCA AGA TCA CCA-3’); mouse GAPDH (forward: 5’-ATC AAG AAG GTG GTG AAG CA -3’; reverse: 5’-AAG GTG GAA GAG TGG GAG TTG -3’). The small nuclear RNA U6 was used as a control for microRNA samples and GAPDH was used as a control for JP2 mRNA quantification.
**MicroRNA target prediction**

Putative targets of miR-24 in mouse were predicted by the TargetScan software by searching for target sites within the 3' UTR of genes. Genes with at least one miR-24 target site which is conserved across mouse, rat and human were selected as miR-24 target candidates.

**Western blot**

Total proteins were extracted from isolated cells using lysis buffer containing 1% sodium deoxycholate, 10 mM Na₃P₂O₇, 1% Triton X-100, 10% glycerol, 150 mM NaCl, 5 mM EDTA-Na₂, 20 mM Tris (pH 7.4), 0.1% SDS, 50 mM NaF, 1 mM Na₃VO₄, 1 mM PMSF, and protease inhibitor cocktail (Roche). The sample lysate was separated on 10% SDS-PAGE and then transferred to PVDF membrane. The membrane was incubated with a self-made rabbit polyclonal antibody against JP2 (1 µg/ml), which specifically recognized the rat JP2 p434-p447 peptide (QEILENSESLLLEPR). A horseradish peroxidase-conjugated GAPDH antibody (KangChen Bio-tech Inc., China) was used to measure the GAPDH content as a loading control.

**Whole-cell and loose-seal patch clamp**

Myocytes were bathed in an extracellular solution containing (in mM) 137 NaCl, 4 KCl, 2 CaCl₂, 1 MgCl₂, 1.2 NaH₂PO₄, 10 glucose, 0.02 tetrodotoxin and 10 HEPES, pH 7.35 adjusted with NaOH. The pipette electrode was filled with a solution containing (in mM) 110 CsCl, 6 MgCl₂, 5 Na₂ATP, 15 TEA-Cl, 10 HEPES and 0.2 fluo-4 pentapotassium, pH 7.2 adjusted with CsOH. \(I_{ca}\) was activated at 10-s intervals using an EPC7 amplifier (List Medical Electronic, Germany). For loose-seal patch clamping, glass pipettes of 3-5 MΩ were filled with (in mM) 120 TEA-Cl, 20 CaCl₂, 10 HEPES, 0.01 tetrodotoxin and 10 µM FPL64176, pH 7.2 adjusted with TEA-OH. The membrane potential (\(V_m\)) was determined by proportionally dividing the test voltages between the pipette resistance and the seal resistance (15-20 MΩ). All experiments were performed at room temperature (25°C).

**Ca²⁺ imaging**

Intracellular Ca²⁺ dynamics were recorded using inverted confocal microscopes (LSM-510 or LSM-710, Carl Zeiss, Germany). Line-scan images were acquired at 3.84 ms/line for whole-cell recording and 0.47 ms/line for local Ca²⁺ detection. The Ca²⁺ concentration was either reported as the fluorescence normalized to the resting level (\(R = F/F_0\)), or calculated by \([Ca^{2+}] = k_d·R/(k_d/C_0+1−R)\), assuming a resting Ca²⁺ concentration \(C_0 = 100\) nM and a dissociation constant \(k_d = 1.1\) µM. The change in cell length was derived from edge-detection of the fluorescence.

**TEM and stereological measurement**

Cell samples were first fixed in 2.5% glutaraldehyde and 1% paraformaldehyde in 0.1 M PBS buffer (pH 7.4). To specifically stain membrane, the samples were post-fixed in a mixture of 0.8% potassium ferrocyanide and 2% osmium tetroxide in 0.1 M sodium cacodylate buffer for 30 min. After dehydration in a graded series of alcohol, the samples were embedded in Spurr resin and sectioned with a glass knife on a Leica Ultracut R cutter. Thin sections were stained with uranyl acetate and lead citrate, then observed and randomly imaged under an FEI Tecnai G² 20 Twin
system. For stereological measurement of the volume density and surface area of TTs and JSRs, we followed Mobley’s stereological method.\textsuperscript{6}

**Statistical analysis**

Results are expressed as mean ± SE. Statistical analysis was performed, where appropriate, using Student’s t-test, the Mann-Whitney rank sum test and two-way ANOVA with repeated measures. A value of $P < 0.05$ was considered significant.

**References**


Online Figures

**Online Figure I.** Design of *in vivo* experiment testing the effect of antagomir-24 on a TAC mouse model of hypertrophy and heart failure.

**Online Figure II.** Comparison of SR Ca\(^{2+}\) load among sham, NC and antagomir groups. **A,** Typical images showing that the SR Ca\(^{2+}\) load was measured as the amplitude (\(\Delta F/F_0\)) of 10 mM caffeine-induced Ca\(^{2+}\) transients. **B,** Statistical results of SR Ca\(^{2+}\) load from sham, NC and antagomir groups. Data from 59, 55 and 65 cells in 4 sham, 3 NC and 3 antagomir mice, respectively. Resting ventricular myocytes were equilibrated with 2 mM extracellular Ca\(^{2+}\). Under this condition, moderate differences in SERCA activity would not make differences in steady-state SR Ca\(^{2+}\) load.
Online Figure III. Stereological analysis of TT-SR junctions in human heart failure. The representative TEM images illustrate the stereological analysis of myocytes. The grid lines were spaced 0.167 μm apart. The closed and open circles denote examples of point counts for volume density and intersection counts for surface area per unit volume, respectively.