MiR-30-Regulated Autophagy Mediates Angiotensin II-Induced Myocardial Hypertrophy

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Abstract

Dysregulated autophagy may lead to the development of disease. Role of autophagy and the diagnostic potential of microRNAs that regulate the autophagy in cardiac hypertrophy have not been evaluated. A rat model of cardiac hypertrophy was established using transverse abdominal aortic constriction (operation group). Cardiomyocyte autophagy was enhanced in rats from the operation group, compared with those in the sham operation group. Moreover, the operation group showed up-regulation of beclin-1 (an autophagy-related gene), and down-regulation of miR-30 in cardiac tissue. The effects of inhibition and over-expression of the beclin-1 gene on the expression of hypertrophy-related genes and on autophagy were assessed. Angiotensin II-induced myocardial hypertrophy was found to be mediated by over-expression of the beclin-1 gene. A dual luciferase reporter assay confirmed that beclin-1 was a target gene of miR-30a. miR-30a induced alterations in beclin-1 gene expression and autophagy in cardiomyocytes. Treatment of cardiomyocytes with miR-30a mimic attenuated the Angiotensin II-induced up-regulation of hypertrophy-related genes and decreased in the cardiomyocyte surface area. Conversely, treatment with miR-30a inhibitor enhanced the up-regulation of hypertrophy-related genes and increased the surface area of cardiomyocytes induced by Angiotensin II. In addition, circulating miR-30 was elevated in patients with left ventricular hypertrophy, and circulating miR-30 was positively associated with left ventricular wall thickness. Collectively, these above-mentioned results suggest that Angiotensin II induces down-regulation of miR-30 in cardiomyocytes, which in turn promotes myocardial hypertrophy through excessive autophagy. Circulating miR-30 may be an important marker for the diagnosis of left ventricular hypertrophy.

Introduction

Myocardial hypertrophy induced by angiotensin II (Ang II) is an important cause of cardiac remodeling. Identification of the mechanisms through which Ang II induces myocardial hypertrophy would reveal novel targets for the development of new therapies for cardiac remodeling [1]. The development of myocardial hypertrophy is induced by the synthesis of cardiac contractile proteins, and this is increased by Ang II which stimulates anabolic pathways [2]. Homeostasis in cardiomyocytes is maintained by physiological autophagy, which not only removes misfolded or aggregated proteins, but also plays an important role in the clearance of damaged organelles, such as mitochondria and endoplasmic reticulum [3]. Autophagy may be activated by aberrant protein aggregation to help remove aggregates. However, excessive or deficient autophagy may lead to the development of disease. Cardiomyocyte physiological autophagy is an adaptive and self-protective mechanism that occurs during cardiac remodeling, but excessive autophagy may lead to cardiomyocyte death [4].

Beclin-1, a mammalian orthologue of yeast Atg6, was the first mammalian autophagy-related protein to be identified. Beclin-1 plays an important role in regulating vacuolar sorting protein 34 (Vps-34, a class III phosphatidylinositol-3 kinase), and advances the formation of beclin-1-Vps34-Vps15 core complexes, which induce autophagy [5–8]. Previous studies have shown that miR-30a could negatively regulate beclin-1 gene expression, resulting in decreased autophagic activity in cancer cells such as T98G, MDA-MB-468 and H1299 cells [9]. However, it is not known whether miR-30a can influence autophagy in cardiomyocytes through regulation of beclin-1 gene expression, and whether excessive autophagy mediates the actions of Ang II to cause myocardial hypertrophy. We hypothesize that myocardial hypertrophy induced by Ang II is mediated by excessive autophagy. The results of our study indicate that Ang II excessively up-regulates cardiomyocyte autophagy by decreasing miR-30 expression, and that this excessive autophagy promotes the development of myocardial hypertrophy. Consistent with this, the expression level of miR-30 in the plasma of peripheral blood was elevated in patients with left ventricular hypertrophy (LVH).

Materials and Methods

Animal Models, Echocardiography, and Tissue Staining

Wistar rats were divided randomly into 2 groups: Sham group (n = 6) and transverse abdominal aortic constriction (TAAC) group (n = 8). Rats in the Sham group only underwent exposure of the aorta. In the TAAC group, transverse abdominal aortic
constriction was performed between the abdominal aorta and anterior mesenteric artery, until the diameter of the aorta was 0.5 mm. 4 weeks after the operation, ventricular wall thickness and heart chamber size were measured using echocardiography (Philips ie 33, Netherlands). Blood samples were collected from the central veins, and the derived plasma was stored at -80°C. The heart was excised, cut along the ventricular septum, and heart chamber size were measured using echocardiography. The weight of the heart was determined. 0.5 mm. 4 weeks after the operation, ventricular wall thickness and heart chamber size were measured using echocardiography. The weight of the heart was determined.

The Construction of Plasmids
Coding sequence (CDS) fragments of beclin-1 were amplified from rat cDNA. Using HindIII and XbaI sites, the beclin-1 sequence was cloned into a pRc/CMV2 vector (Invitrogen) and identified by DNA sequencing. The over-expression vector, pRc/CMV2-beclin-1, was then constructed. The 3'-untranslated region (3'-UTR) of beclin-1 and a mutation sequence were amplified from cDNA by fusion PCR. The 3'-UTR and mutation sequence were inserted, respectively, into the HindIII and XbaI sites of a pGL3-control vector (Promega) and identified by DNA sequencing. The over-expression vector, pRc/CMV2-beclin-1, was then constructed. The 3'-UTR of beclin-1 (pGL3-Beclin-1 3'-UTR-Wild Type), and a mutant plasmid was created containing the mutation sequence (pGL3-Beclin-1 3'-UTR-Mutant). The pRL-TK vector (Promega) was used as an endogenous control.

Culture of Neonatal Ventricular Cardiomyocytes and Interventions Used
Rat ventricular myocytes were isolated by enzymatic digestion of 1- to 3-day-old neonatal rats, as described previously [10]. The intervention measures used were as follows: stimulation with

Table 1. primer and small RNA sequences.

| Designation | Sequences |
|-------------|-----------|
| 18s         | Forward: 5'-ACCCAGCTAGGAATAATGG-3', Reverse: 5'-TCCTCGATATTCCCAAA-3' |
| ANP         | Forward: 5'-GGGAGATTACGACTGAGGT-3', Reverse: 5'-CTACTAAGGTCTGCTGGG-3' |
| β-MHC       | Forward: 5'-CTCTGAAATACAGGGAATAA-3', Reverse: 5'-TACAGGTGTCATGCTGCC-3' |
| Beclin-1    | Forward: 5'-AAGATTGGAAGACAGCGAGCA-3', Reverse: 5'-GAGGACACCAAGCGAAAC-3' |
| U6          | Forward: 5'-GGCGCTACGATATTGC-3', Reverse: 5'-GAGGACACCAAGCGAAAC-3' |
| hsa-miR-30a | Forward: 5'-ACACTCCAGCTGGGTTGAACATCCTCGACTG-3', Reverse: 5'-CTCAACTGCTGTCAGTG-3' |
| cel-miR-39  | Forward: 5'-ACACTCCAGCTGGTACCGGTTAATACGCTG-3', Reverse: 5'-CTCAACTGCTGTCAGTG-3' |
| Negative control of beclin-1-specific siRNA | Sense: 5'-UUCUCGCAAGGUGUCAGGUTT-3', Antisense: 5'-ACGUGACAGUUCGGAAATTT-3' |
| Negative control of miR-30a mimic | Sense: 5'-UUCUCGCAAGGUGUCAGGUTT-3', Antisense: 5'-ACGUGACAGUUCGGAAATTT-3' |
| miR-30a mimic | Sense: 5'-UUCUCGCAAGGUGUCAGGUTT-3', Antisense: 5'-ACGUGACAGUUCGGAAATTT-3' |
| Negative control of miR-30a inhibitor | 5'-CAGUACUUUGUGUGUCAGA-3', 5'-UUUCGAGCGAGUUACAGA-3' |
| miR-30a inhibitor | 5'-CUUCCAGUGAGGAUGUAC-3' |

Table 2. clinical characteristics of patients with and without LVH.

|              | Control(n = 11) | LVH(n = 11) | P    |
|--------------|----------------|-------------|------|
| Age(years)   | 69.0±12.2      | 73.9±12.4   | 0.362|
| Male/female  | 4/7            | 6/5         | 0.670|
| Smoking      | 1(9.1%)        | 5(45.5%)    | 0.149|
| DM(n,%)      | 5(45.5%)       | 5(45.5%)    | 0.616|
| Hypertension | 5(45.5%)       | 11(100.0%)  | 0.214|
| ACS(n,%)     | 4(36.4%)       | 4(36.4%)    | 1.000|
| AF(n,%)      | 1(9.1%)        | 1(9.1%)     | 1.000|
| Fasting glucose(mmol/L) | 6.23±2.14 | 5.07±0.83 | 0.110|
| SBP(mmHg)   | 136.3±25.3     | 158.3±30.2  | 0.079|
| DBP(mmHg)   | 74.5±14.1      | 85.5±14.9   | 0.091|
| TC(mmol/L)   | 5.04±1.23      | 5.00±1.11   | 0.927|
| TG(mmol/L)   | 1.39±0.57      | 1.16±0.48   | 0.314|
| HDL(mmol/L)  | 1.12±0.27      | 1.26±0.38   | 0.320|
| LDL(mmol/L)  | 2.93±0.91      | 2.64±0.68   | 0.417|
| Cr(μmol/L)   | 129.6±120.5    | 105.1±72.4  | 0.949|
| CK-MB(U/L)   | 28.5±44.3      | 15.8±10.8   | 0.331|
| BNP(pg/L)    | 169.9±112.0    | 130.4±112.2 | 0.370|
| EF(%)        | 64.6±6.2       | 62.9±6.7    | 0.546|
| LV(cm)       | 4.418±0.506    | 4.318±0.536 | 0.658|
| IVSd(cm)     | 0.991±0.094    | 1.256±0.098 | 0.000|
| LVPWd(cm)    | 0.946±0.104    | 1.256±0.088 | 0.000|

DM = diabetes mellitus, ACS = acute coronary syndrome, AF = atrial fibrillation, SBP = systolic blood pressure, DBP = diastolic blood pressure, TC = total cholesterol, TG = total glyceride, HDL = high-density lipoprotein, LDL = low-density lipoprotein, Cr = creatinine, CK-MB = creatine kinase-MB, LV = left ventricular diameter.
1 μmol/L Ang II or 10 mmol/L 3-methyladenine (3-MA, an autophagy inhibitor; Sigma); transduction with lentivirus (Gene-Pharma, China), containing beclin-1-specific siRNA, control-siRNA, miR-30a mimic, miR-30a inhibitor, or control-miRNA (Sequences are shown in Table 1). 4×10⁵ of cardiomyocytes were transduced with 20 μl virus (10⁹TU/ml) and polybrene (at a final concentration of 5 μg/ml). The transfection of pRe/CMV2-beclin-1 was performed by using Lipofectamine™ LTX and PLUS Reagents according to the manufacturer’s instruction. Cells were stained with 50 μmol/L monodansylcadaverine (MDC, a specific marker for autophagic vacuoles; Sigma) in day 3 after transfection or treatment with drugs.

Dual Luciferase Reporter Assay

Neonatal rat cardiomyocytes were cultured in a 24-well plate. On the 5th day, cardiomyocytes were transduced with lentivirus containing miR-30a mimic and miR-30a inhibitor. 24h after the transduction, pGL3-Beclin-1 3’-UTR-Wild Type (200 ng) or pGL3-Beclin-1 3’-UTR-Mutant (200 ng) was co-transfected with pRL-TK vector (200 ng) into the cardiomyocytes using Lipofectamine™ LTX and PLUS Reagents. Cardiomyocytes were lysed and the relative luciferase activity was measured with the Dual-Luciferase Report Assay System (Promega) on a GLOMAX™96 microplate luminometer (Promega, USA).

Real-time RT-PCR

Total RNA was isolated using TRIzol Reagent (Invitrogen). Reverse transcription was performed at 50°C for 50 min, and 85°C for 5 min, using the SuperScript™ III First-Strand Synthesis SuperMix for qRT-PCR kit (Invitrogen). Real-time PCR was performed using the Platinum® SYBR® Green qPCR SuperMix-UDG kit (Invitrogen), with a 7500 Real-Time PCR System from Applied Biosystems. The relative expression of mRNA was calculated using the 2⁻ΔΔCt method, and normalized to the expression of 18S (Primer sequences are shown in Table 1).

Western Blotting

Protein was extracted from cardiac tissue and cardiomyocytes. Equal amounts of protein (50 μg) were separated by SDS-PAGE (150 V, 1 h) and transferred onto a polyvinylidene difluoride

Figure 1. Ventricular wall thickness was assessed using echocardiography in Wistar rats after TAAC surgery. (A) Representative ultrasound through left ventricular from rats after Sham operation and transverse abdominal aortic constriction (TAAC) for 4 weeks. (B) Quantitative analysis of echocardiography for the left ventricular posterior wall thickness at end-systole (LVPWs), interventricular septal thickness at end-systole (IVSs), left ventricular posterior wall thickness at end-diastole (LVPWd) and interventricular septal thickness at end-diastole (IVSd), and comparison was performed between TAAC (n = 8) and Sham group (n = 6). Data are presented as means ± SEM. *P<0.05 compared with Sham. doi:10.1371/journal.pone.0053950.g001
membrane (100 V, GAPDH and beclin-1 1.5 h, LC3 40 min). After blocking, the blots were probed with either anti-beclin-1 antibody, anti-LC3 antibody or anti-GAPDH antibody (Cell Signaling Technology) at 4°C overnight. After washing, the membrane was incubated with horseradish peroxidase (HRP)-conjugated AffiniPure rabbit anti-goat IgG(H+L) (EarthOx, USA) for 1 h. After washing again, immunological complexes were detected using the SuperSignal® West Pico Chemiluminescent Substrate (Thermo Scientific, USA).

Flow Cytometry

After incubation with MDC at 37°C in the dark for 1 h, the cardiomyocytes were digested with trypsin. The percentage of autophagic vacuoles was detected using MoFlo flow cytometry (355 nm excitation, 525 nm emission; Beckman Coulter, USA) and analyzed with Kaluza analysis software.

Electron Microscopy

Electron microscopy [11] was performed on a TECNAI 12 Spirit Twin transmission electron microscope, at a magnification of ×13500. Quantitative analysis of autophagosomes was carried out using 10 images from different fields, with the investigator blinded as to the origin of each image. Autophagosomes or autolysosomes were identified by the characteristic structure of a double- or multi-lamellar smooth membrane completely surrounding compressed mitochondria, or membrane-bound electron-dense material.

Confocal Microscopy

After removing the culture media, the samples were fixed with 3.7% formaldehyde at 37°C. The samples were permeabilized with 1% Triton X in PBST (Triton X: phosphate buffered saline = 1:100, volume ratio) for 15 minutes. The samples were then incubated with Alexa Fluor®555 Phalloidin (1:40, Invitrogen) for 20 minutes. The cells were stained with the chromatin dye, DAPI (300 nmol/L, Invitrogen), for 5 min. Images were obtained using a Zeiss LSM 710 confocal microscope (Carl Zeiss, Germany).

Detection of miRNA in Cardiac Tissue

The miRNA contents of cardiac tissue and cardiomyocytes were detected using the Hairpin-it™ miRNAs qPCR Quantitation Kit.
The optimal reverse transcription reaction system was: 40 U/mL RNaseOUT, 0.125 mM L, 10X RT Buffer 2 mM L, 10 mmol/L dNTPmix 0.75 mM L, 1 mM L, miRNA specific RT primer 1.2 mM L, 200 U/mL MMLV reverse transcriptase 0.1 mM L, total RNA 160 ng; DEPC-treated water was added to give a final reaction volume of 20 mL. The reaction conditions used were: 16°C for 30 min, 42°C for 30 min, and 85°C for 10 min. The optimal real-time PCR reaction system was: 2 x Real-time PCR Master Mix 10 mL, 5 mM L, miRNA specific primer set 0.36 mM L, 5 U/mL Taq DNA polymerase 0.2 mM L, 25 mM L, ROX reference dye 0.8 mM L, M-MLV Reverse Transcriptase 1 mL (Promega); 42°C for 60 min, and 85°C for 10 min. The cDNA obtained by reverse transcription was diluted (1:20) for real-time PCR. The reaction system used was as follows: miRNA specific forward primer 0.5 mM L (Invitrogen), miRNA general primer 0.5 mM L (Invitrogen), 2 x SYBR Green PCR Master Mix 10 mL (Toyobo, Japan), cDNA 5 mL; DEPC-treated water was added to give a final reaction volume of 20 mL. The real-time PCR reaction conditions used were: 95°C for 5 min, followed by 40 cycles of 95°C for 15 sec, 62°C for 15 sec, and 72°C for 32 sec. After adding TRizol, the samples were supplemented with cel-miR-39 as an endogenous control (GenePharma, China), as described previously [12] (Primer sequences are shown in Table 1).

Detection of miRNA in Plasma
A 3 mL volume of whole blood was collected into a tube containing EDTA, and the plasma was obtained by centrifugation. Total RNA was isolated from the plasma using 1.2 mL TRIzol LS Reagent (Invitrogen). 8 mL total RNA was dissolved in 2.5 mL DEPC-treated water, maintained at 85°C for 5 min. The optimal reverse transcription reaction system and reaction conditions used were: dissolved total RNA 10.5 mL, 10 mM dNTP 2 mM L (Promega), RNasin® Ribonuclease Inhibitor 0.5 mL (Promega), miR-30a reverse transcription primer 0.5 mL (LAND, Hongkong), cel-miR-39 reverse transcription primer 0.5 mL (LAND, Hongkong), 5 x buffer 5 mL, M-MLV Reverse Transcriptase 1 mL (Promega); 42°C for 60 min, and 85°C for 10 min. The cDNA obtained by reverse transcription was diluted (1:20) for real-time PCR. The reaction system used was as follows: miRNA specific forward primer 0.5 mM L (Invitrogen), miRNA general primer 0.5 mM L (Invitrogen), 2 x SYBR Green PCR Master Mix 10 mL (Toyobo, Japan), cDNA 5 mL; DEPC-treated water was added to give a final reaction volume of 20 mL. The real-time PCR reaction conditions used were: 95°C for 5 min, followed by 40 cycles of 95°C for 15 sec, 62°C for 15 sec, and 72°C for 32 sec. After adding TRizol, the samples were supplemented with cel-miR-39 as an endogenous control (GenePharma, China), as described previously [12] (Primer sequences are shown in Table 1).
Population Study

22 subjects were divided into 2 groups: LVH group (11 patients with LVH) and control group (11 subjects without LVH) (Table 2). Inclusion criteria for the LVH group included diagnosis by echocardiography: interventricular septal thickness at end-diastole (IVSd) and/or left ventricular posterior wall thickness at end-diastole (LVPWd) $\geq 1.2$ cm; ejection fraction (EF) $>40\%$. Patients with systolic heart failure, tumors, infection (acute phase), brain natriuretic peptide (BNP) $>400$ ng/L, Parkinson’s disease, idiopathic pulmonary fibrosis, shock or cirrhosis were excluded, as were patients treated recently with interferon or phenobarbitone. Whole blood was collected from the patients (fasted for 8 h), into a tube containing EDTA. The Institutional Ethics Committee of the Second Hospital Affiliated to Guangzhou Medical University approved the study, and all patients gave a written informed consent.

Statistical Analysis

All results for continuous variables were expressed as mean $\pm$ SEM, unless otherwise indicated. The Shapiro-Wilk test was used to establish whether the continuous data followed a normal distribution. For data that were normally distributed, Levene’s test, Mann-Whitney U Test or ANOVA were used, as appropriate. For categorical variables, a Fisher’s exact test was employed. Pearson correlation was used to measure the strength of the association between microRNA level and ventricular wall thickness. All tests were performed as 2-sided tests, and a significance level of $P<0.05$ was considered to indicate statistical significance. Statistical analyses were performed using PASW Statistics 18.0 software.
lower in rats from the TAAC group, compared with those in the Sham group (Figure 3D).

Myocardial Hypertrophy Induced by Excessive Activation of Autophagy in Cardiomyocytes

Compared with the controls, the expression of beclin-1, ANP (atrial natriuretic peptide) and β-MHC (beta-myosin heavy chain) genes was up-regulated in neonatal cardiomyocytes stimulated with Ang II. Compared with the Ang II-treated neonatal cardiomyocytes, the expression of beclin-1, ANP and β-MHC genes was down-regulated in cardiomyocytes treated with either 3-MA+Ang II, or beclin-1-specific siRNA+Ang II (Figure 4A and 4B). Hence, hypertrophy-related gene expression in these cardiomyocytes was attenuated by inhibition of excessive autophagy. The expression of myocardial hypertrophy-related genes increased by over-expression of the beclin-1 gene, as compared with cardiomyocytes transfected with the pRc/CMV2 vector (Figure 4C).

Figure 5. Expression of autophagy-related protein in cardiomyocytes varies with that of the beclin-1 gene. The relative expression of autophagy-related protein in cardiomyocytes was analyzed by Western blotting. GAPDH was used as an internal control. Data are presented as means ± SEM. (A) Evaluation of the influence of 3-MA on the expression of LC3II/LC3I and beclin-1 proteins. Con: control group, Ang II: treated with 1 μmol/L Angiotensin II, and Ang II+3-MA: treated with 1 μmol/L Angiotensin II and 10 mmol/L 3-MA. *P<0.05 compared with Con group; # P<0.05 compared with Ang II group. (B) Evaluation of the influence of beclin-1 specific siRNA on the expression of autophagy-related proteins. NC: treated with lentivirus containing negative control of beclin-1-specific siRNA, Ang II+ NC: treated with lentivirus containing negative control of beclin-1-specific siRNA and 1 μmol/L Angiotensin II, and Ang II+siRNA: treated with lentivirus containing beclin-1-specific siRNA and 1 μmol/L Angiotensin II. *P<0.05 compared with NC group; # P<0.05 compared with Ang II+NC group. (C) Evaluation of the influence of pRc/CMV2-beclin-1 vector on LC3II/LC3I and beclin-1 proteins. pRc/CMV2: transfected with pRc/CMV2 vector, and pRc/CMV2-beclin-1: transfected with pRc/CMV2-beclin-1. *P<0.05 compared with pRc/CMV2 group.

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Inhibition or over-expression of the beclin-1 gene in cardiomyocytes was associated with inhibition or up-regulation of autophagy, respectively. Over-expression of the beclin-1 gene was induced by Ang II or by transfection of a beclin-1 gene expression vector. After over-expression of the beclin-1 gene in cardiomyocytes, the expression of LC3II/LC3I and beclin-1 proteins significantly increased (Figure 5). The inhibition of beclin-1 gene expression was achieved using 3-MA or beclin-1-specific siRNA. After down-regulation of beclin-1 gene expression in cardiomyocytes, the expression of LC3II/LC3I and beclin-1 proteins significantly decreased (Figure 5A and 5B). The percentage and number of autophagic vacuoles were measured using flow cytometry of MDC-stained cardiomyocytes (Figure 6) or electron microscopy (Figure 7). The results of these experiments confirmed that alterations in cardiomyocyte beclin-1 gene expression can cause corresponding changes in autophagy.

miR-30-regulated Autophagy in Cardiomyocytes

Bioinformatic predictions suggested that the 3'UTR of the beclin-1 gene would have one binding site for miR-30a, and that the beclin-1 gene may be a target gene for miR-30a (Figure 6A). In cardiomyocytes transfected with pGL3-Beclin-1 3'-UTR-Wild Type, transduction with an miR-30a mimic decreased the relative luciferase activity by 45.4%, relative to cells treated with a negative control. In cardiomyocytes transfected with pGL3-Beclin-1 3'-UTR-Wild Type, transduction with an miR-30a inhibitor increased the relative luciferase activity 1.32-fold, compared with cells treated with a negative control. In cardiomyocytes transfected with pGL3-Beclin-1 3'-UTR-Mutant, there were no significant differences in the relative luciferase activities between cells treated with an miR-30a mimic or inhibitor and cells treated with a negative control (Figure 6C). In summary, these findings suggest that beclin-1 was a target gene of miR-30a. To determine the effect of miR-30a on the expression of beclin-1, the mRNA and protein expression levels of beclin-1 were measured in cardiomyocytes treated with either an miR-30a mimic or an miR-30a inhibitor. Compared with the negative control, the expression of beclin-1 mRNA decreased by 57.5% in cells treated with an miR-30a mimic, and increased by 2.25-fold in cells treated with an miR-30a inhibitor (Figure 6D). Compared with the negative control, beclin-1 protein levels decreased by 44.7% in cells treated with an miR-30a mimic, and increased by 1.87-fold in cells treated with an miR-30a inhibitor (Figure 6E).

miR-30a was found to not only induce changes in beclin-1 protein expression, but also alter the expression of other autophagy-related proteins. Compared to cardiomyocytes treated with a negative control, the expression of LC3II/LC3I protein decreased by 41.3% in cells treated with an miR-30a mimic, and increased 1.28-fold in cells treated with an miR-30a inhibitor (Figure 6F). The number of autophagic vacuoles in the visual field (×13500) significantly increased in cells treated with an miR-30a inhibitor, compared with control cells (0.52±0.224 vs 3.0±0.221, P<0.001). Compared with Ang II-stimulated cells, the number of autophagic vacuoles decreased in cardiomyocytes treated with...
Ang II + miR-30a mimic (2.8 ± 0.632 vs 1.0 ± 0.667, \( P = 0.000 \)) (Figure 7). Autophagic vacuole percentages were measured in MDC-stained cardiomyocytes using flow cytometry: these data confirmed that miR-30 functioned to regulate autophagy in cardiomyocytes (Figure 9).

**Downregulation of miR-30 Leads to Myocardial Hypertrophy**

The expression of miR-30a in Ang II-stimulated cardiomyocytes was only 32.9% of that in unstimulated cells (Figure 10A). To study the relationship between the down-regulation of miR-30a and the development of myocardial hypertrophy, the expression of \( ANP \) and \( \beta\-MHC \) was measured. In hypertrophic cardiomyocytes (hypertrophy induced by Ang II), treatment with an miR-30a mimic decreased the expression of \( ANP \) and \( \beta\-MHC \) by 48.3% and 46.5%, respectively, relative to the negative control. Conversely, in hypertrophic cardiomyocytes (induced by Ang II), treatment with an miR-30a inhibitor increased the expression of \( ANP \) and \( \beta\-MHC \) by 1.88- and 1.64-fold, respectively, relative to the negative control (Figure 10B). Morphological changes in these cardiomyocytes were observed using confocal microscopy (Figure 10C). The morphological observations indicated that the surface area of

![Figure 7. Autophagic vacuole number varies with beclin-1 gene expression.](image-url)
hypertrophic cardiomyocytes (induced by Ang II) was 2.95-fold that of untreated cells (Figure 10D). Furthermore, compared with hypertrophic cardiomyocytes treated with Ang II and negative control, the cell surface area decreased by 42.2% in cardiomyocytes treated with Ang II and miR-30a mimic, and increased 1.50-fold in cardiomyocytes treated with Ang II and miR-30a inhibitor (Figure 10E).

Circulating Levels of miR-30 Increased in Rats Following TAAC Surgery, and in Patients with LVH
The concentration of miR-30a in the plasma of rats from the TAAC group was 4.23-fold greater than that of rats from the Sham group ($P=0.000$, Figure 11). With the exception of IVSd and LVPWd, there were no significant differences in the clinical characteristics between patients with LVH and those without LVH (Table 1). Since 11 LVH patients suffered from primary hypertension, their LVH may be associated with abnormal blood pressure. The level of miR-30a expression was 2.07-fold higher in patients with LVH, compared with patients without LVH ($P=0.040$, Figure 12A). A receiver operating characteristic (ROC) curve showed that the areas under the curve (AUC) for the plasma level of miR-30a, for IVSd and for LVPWd were 0.760, 0.988 and 1.000, respectively. Use of plasma miR-30 levels for the diagnosis of LVH reached statistical significance ($P=0.039$, Figure 12B). Pearson correlation analysis indicated that the level of miR-30a expression in plasma was positively associated with IVSd and LVPWd (Figure 12C) ($R=0.466$ and $0.480$, $P=0.029$ and 0.024, respectively).

Discussion
Autophagy was originally defined as the process of sequestration of intracellular components and their subsequent degradation by lysosomal vacuoles [13]. This process is likely the main mechanism involved in the degradation of long-lived proteins and cytoplasmic organelles. In our study, we found that the expression of the beclin-1 gene and autophagy in rat heart were significantly increased in the TAAC group relative to the Sham group. Both autophagy and the expression of the beclin-1 gene increased in Ang II-stimulated neonatal cardiomyocytes, compared with control cells. Moreover, inhibition of the beclin-1 gene in cardiomyocytes was associated with reduced autophagic activity, whereas over-expression of beclin-1 resulted in enhanced autophagy. We were interested in determining whether over-expression of the beclin-1 gene and excessive autophagy are mechanisms that mediate Ang II-induced myocardial hypertrophy. We found that silencing of the beclin-1 gene by RNA interference caused decreased expression of genes related to myocardial hypertrophy, whereas over-expression of beclin-1 resulted in enhanced autophagy. Based on these observations, we propose that beclin-1 gene over-expression and excessive autophagy mediate Ang II-induced myocardial hypertrophy. When autophagy is excessively up-regulated in cardiomyocytes, redundant cytoplasmic organelles and spectrin are cleared by this autophagic process. Clearance of redundant cytoplasmic organelles, such as mitochondria, leads to disturbances in normal physiological function, while protein degradation via excessive autophagy results in a reactive acceleration of protein synthesis.
and the development of myocardial hypertrophy. Therefore, we speculate the existence of the following autophagy-associated pathway: Ang II → miR-30a → beclin-1 → excessive autophagy → myocardial hypertrophy.

MicroRNAs are endogenous (≈22-nucleotide) non-coding RNA molecules that regulate gene expression at the post-transcriptional level, by pairing with partially complementary sites in the 3′ UTR of the targeted mRNA, leading either to degradation or to translational repression. MicroRNAs control development, and are critically involved in many biological processes in health and disease, including cardiovascular diseases [14,15]. Bioinformatics software predicts that miR-30a may regulate the expression of beclin-1. The function of the miR-30 family is similar to that of other microRNAs. The miR-30 family is associated with the development of tumors and other diseases (of the nervous, genital, circulatory, alimentary, and respiratory systems), as well as adipogenesis, cellular senescence, drug metabolism and cell differentiation [16–31]. A study by Dusters et al. [10] found that miR-30 family members were significantly down-regulated in mouse hypertrophic hearts and in cardiac biopsies from patients with LVH. The pro-fibrotic protein, connective tissue growth factor (CTGF), was down-regulated by miR-30c, such that structural changes in the extracellular matrix of the myocardium were controlled by miR-30c. In another study, chronic alcohol intake in mice has been reported to lead to cardiac hypertrophy and down-regulation of miR-30a [32].

Our experiments revealed that expression of the miR-30 family was down-regulated in cardiomyocytes of rats from the TAAC group, and that miR-30a expression decreased in hypertrophic cardiomyocytes that had been treated with Ang II. It was thus of interest to investigate whether the down-regulation of miR-30 might mediate Ang II-induced myocardial hypertrophy. After treatment of cardiomyocytes with miR-30a mimic, the expression of genes related to myocardial hypertrophy decreased, and the hypertrophic cardiomyocytes showed improvements in their morphology. Furthermore, transduction of an miR-30a inhibitor into cardiomyocytes, to impair the function of miR-30a, caused an up-regulation of the expression of genes related to myocardial hypertrophy, with more severe morphological changes characteristic of hypertrophy. Therefore, we postulate that down-regulation of miR-30a expression mediates Ang II-induced myocardial hypertrophy, as follows: Ang II → miR-30a inhibitor → up-regulation of genes related to myocardial hypertrophy.

In additional experiments, we further demonstrated that beclin-1 is the target gene of miR-30a, and that miR-30a binds with the 3′UTR of beclin-1. The expression of beclin-1 was down-regulated in cardiomyocytes treated with an miR-30a mimic, and up-regulated in those treated with an miR-30a inhibitor. The results of our study also indicate that activation of autophagy was enhanced in Ang II-induced hypertrophic cardiomyocytes that had been treated with an miR-30a inhibitor, whereas autophagy was inhibited in Ang II-treated cells by over-expression of an miR-30a mimic. Consistent with our observations, the study of Zhu

Figure 9. MiR-30a regulates the percentage of autophagic vacuoles. The autophagic vacuoles was analyzed by calculating MDC-stained cardiomyocytes using flow cytometry. (A) Representative percentage of autophagic vacuoles measured in MDC-stained cardiomyocytes using flow cytometry. (B) Evaluation of the influence of miR-30a mimics on autophagic vacuoles. *P<0.05 compared with Ang II + NC group. (C) Evaluation of the influence of miR-30a inhibitors on autophagic vacuoles. *P<0.05 compared with NC group.

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et al. suggested that cardiac autophagy may be a maladaptive response to hemodynamic stress [5]. Taken together, these results provide strong evidence that autophagy mediates the development of myocardial hypertrophy in cardiomyocytes: a down-regulation of miR-30 induced by Ang II leads to excessive autophagy in cardiomyocytes, thereby promoting myocardial hypertrophy. A possible pathway by which Ang II may cause myocardial hypertrophy is as follows: Ang II \( \rightarrow \) miR-30a \( \rightarrow \) beclin-1 expression \( \rightarrow \) excessive autophagy \( \rightarrow \) myocardial hypertrophy. Since myocardial hypertrophy is not a key feature of all stages of cardiac remodeling, this pathway may not apply to all pathophysiologic processes that underlie cardiac remodeling.

Mitchell et al. [33], in 2008, were the first to report that miRNAs are present in human plasma in a remarkably stable state, protected from endogenous RNase activity. Cell-free miRNAs are relatively stable due to being packaged inside exosomes, which are 50–90 nm membrane-bound particles that are abundant in plasma. Circulating microRNAs in patients may prove to be a novel class of blood-based biomarker for the diagnosis of diseases. Since it is difficult to obtain samples of cardiac tissue from patients, detecting changes in microRNA expression in peripheral blood may prove to be a potentially more useful approach to acquiring information about the pathophysiologic processes underlying heart disease. Numerous studies have now reported the use of microRNA expression in peripheral

**Figure 10. Down-regulation of miR-30 leads to myocardial hypertrophy.** (A) Relative expression of miR-30a in Ang II-stimulated cardiomyocytes (treated with 1 \( \mu \)mol/L Angiotensin II) and that in unstimulated cells by real-time RT-PCR relative to U6. \( *P < 0.05 \) compared with Con group. (B) Evaluation of the influence of miR-30a on mRNA level of ANP and \( \beta \)-MHC in hypertrophic cardiomyocytes. \( *P < 0.05 \) compared with Ang II+NC group. (C) Morphological changes were observed using confocal microscopy in cardiomyocytes stained with Alexa Fluor®555 Phalloidin and DAPI. Summarized data are shown in (D and E). (D) Evaluation of the influence of Ang II on relative cell area (the cardiac muscle fiber surface area ratio) in cardiomyocytes. \( *P < 0.05 \) compared with Con group. (E) Evaluation of the influence of miR-30a on relative cell area in hypertrophic cardiomyocytes. \( *P < 0.05 \) compared with Ang II+NC group.

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blood for the diagnosis of cardiovascular diseases [34–40]. Since we found that miR-30a is involved in the development of myocardial hypertrophy, we were interested in measuring the changes in miR-30a expression in peripheral blood. The concentration of miR-30a was measured in the plasma of rats from the TAAC group, and patients with LVH. We reported, for the first time, that the plasma concentration of miR-30a increased in rats from the TAAC group, and in patients with LVH. Furthermore, the concentration of miR-30a in plasma was positively associated with IVSd and LVPWd, and use of the plasma level of miR-30a for diagnosis of LVH reached statistical significance. As it was not possible to obtain samples of myocardium from living patients, we were unable to examine the relationship between expression of miR-30a in plasma and its expression in samples of living myocardium. However, the results of our study indicate that miR-30a expression in the circulation has an opposite trend to its expression in cardiac tissue in the TAAC group. We speculate that miR-30a may be transformed from cardiac tissues to the peripheral circulation, and that this process contributes to the down-regulation of miR-30a expression in cardiac tissues of TAAC rats. Testing of this hypothesis deserves

Figure 11. circulating miR-30 expression in rats from the TAAC group. The circulating miR-30a in rats (miR-30a in Rattus norvegicus, rno-miR-30a) from TAAC group and Sham group was analyzed by real-time RT-PCR 4 weeks after the operation. cel-miR-39 was used an endogenous control. *P<0.05 compared with Sham group. doi:10.1371/journal.pone.0053950.g011

Figure 12. Relationship between the circulating miR-30 level and ventricular wall thickness. 22 subjects were divided into two groups: LVH group (n = 11) and control group (n = 11). (A) Comparison of the circulating miR-30a (miR-30a in Homo sapiens, has-miR-30a) between patients with LVH and patients without LVH by real-time RT-PCR relative to cel-miR-39. *P<0.05 compared with Con group. (B) Evaluation of the sensitivity and specificity of has-miR-30a on the diagnosis of LVH was performed by analyzing the ROC curve using MedCalc11.3 software. (C) The association between circulating has-miR-30a level and ventricular wall thickness was assessed by Pearson correlation analysis. R: Pearson correlation coefficient. doi:10.1371/journal.pone.0053950.g012
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Further study, as does investigation of the pathway by which miR-30a is transformed from cardiomyocytes to the blood. In conclusion, miR-30a may be a diagnostic marker for patients with LVH. We hypothesize that myocardial hypertrophy may be improved by supplying ectogenic miR-30a to patients with LVH. If this were shown to be the case, this would provide a novel strategy for the management of LVH and cardiac remodeling.