MicroRNA-350 induces pathological heart hypertrophy by repressing both p38 and JNK pathways

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1. Introduction

Epidemiological studies have shown that cardiac hypertrophy (CH) induced by sustained pressure-overload is a major risk factor for ischemic heart disease, arrhythmia, and sudden death [1,2]. Mitogen-activated protein kinases (MAPKs), including the extracellularly responsive kinases (ERKs), the stress-activated protein kinases (SAPKs) such as the c-Jun N-terminal kinases (JNKs), and the p38 MAPKs, have been found to play an important role in CH [3–5]. Although it has been shown that constitutive activation of the ERK 1/2 pathway significantly induces cardiac enlargement, the respective roles of the JNK and p38 pathways are controversial [3,4,6–10], suggesting that additional regulatory mechanisms remain to be identified.

MicroRNAs (miRNAs) are a class of short, noncoding RNAs of 22nt that serve as important regulators of gene expression [11]. They negatively regulate target gene expression through base paring to the 3′ untranslated region (UTR) of target mRNAs, which leads to translation repression and/or mRNA cleavage [11,12]. miRNAs have been shown to affect stem cell differentiation, organ development, apoptosis, signaling pathways, disease and cancer, and respond to biotic and abiotic environmental stresses [12,13]. Moreover, several studies have demonstrated that expression of some miRNAs displays unique tissue-specific, development-specific or etiology-specific patterns [11,14,15]. Recently, studies have identified expression patterns of microRNAs associated with cardiovascular diseases. For example, miRNA-21, miR-23a, miR-24, miR-133, miR-208/miR-195 and miR-199 are involved in CH [16–18], miRNA-1 in arrhythmia [19], miRNA-29 and — 21 in cardiac fibrosis [20,21], miR-210 and — 494 in ischemic heart disease [22,23] and miRNA-129 in heart failure [24]. However, it is not known how or
whether miRNAs control the SAPK pathway during the development of pathological CH and heart failure at later stages.

To address the role of JNK and p38 pathways in the response of the heart to stress and the molecular regulation of miRNA on the SAPK pathway in CH, we analyzed the expression profile of miRNAs in pressure-overload-induced CH. Our results show that miR-350 can be induced by mechanical stimuli, and convey the hypertrophic signal by repressing the translation of JNK1/2 and p38. MiR-350 may be a useful marker to indicate the transition from an adaptive response to maladaptive heart failure, and a promising target for treating CH.

2. Methods

2.1. Cell culture and transfection

Heart-derived H9c2 cardiomyoblasts were obtained from the American Tissue Culture Collection (ATCC). H9c2 cells were cultured with Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, 1 mM HEPES buffer, and 10% fetal bovine serum (Clontech) in humidified air (5% CO₂) at 37 °C. The day before transfection, the cells were trypsinized, diluted with fresh medium without antibiotics, and seeded into 6-well culture plates (approximately 10⁶ cells/well). shR-p38/JNK or miR or miR vectors were synthesized by Shanghai GenePharma (Shanghai, China). The sequences of shR-p38/JNK, miR-350 and mutant miR-350 (miR-350-m) are:

- shR-p38/JNK gene, TGA AGATTCTTG ATTTTCGTTCAAGAGACCAAAATCAAGAATCTTCA;
- Wild type miR-350 gene, GTGAAACTGTGATGCCGTTCGGAATTCAGAGATCCAAAGCCCTAC ACTTCCAC;
- Mutant miR-350 gene, GGGACAGTGTATGCTAAAGCCTATTCAAGAGATCCGTTAGCAGAT CACCTGCC.

The vector expressing GFP alone or mutant miR-350 was used as a control. Vectors were transfected using the Fugene transfection reagent (Roche, USA) according to the manufacturer’s instructions.

2.2. Microarray

Total RNA was extracted from rat cardiomyocytes that had undergone transverse aortic constriction (TAC) or sham operation, using TRIzol reagent according to the manufacturer’s protocol (Invitrogen). The miRCURY™ locked nucleic acid (LNA) arrays (Exiqon, Vedbaek, Denmark) consist of 1300 capture probes for rat and mouse and human. Twenty micrograms of RNA were sent to KangChen Bio-tech (Shanghai, China) for microRNA microarray analysis. Samples were enriched for small RNAs, after which each pair of sham and MAC samples was labeled with Cy3 and Cy5 fluorescent dyes and hybridized to a miRCURY™ chip that held all of the 1300 mature microRNA probes identified to date, as well as perfectly matched and mismatched probes for quality control. Data were normalized in two separate steps for each microarray as described previously [25].

2.3. Real-time TaqMan PCR analysis on the expression of miR-350

Total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. Reverse transcriptase reactions were performed with 1 μg of purified total RNA, 50 nM stem-loop RT primer (Applied Biosystems), 1 × RT Buffer, 0.25 mM of each dNTP, 5 U/μl M-MLV reverse transcriptase (Promega) and 0.25 U/μl RNase inhibitor (Promega). The reactions were incubated in a thermocycler for 30 min at 16 °C, 30 min at 30 °C, 10 min at 85 °C and then held at 4 °C. All reverse transcriptase reactions, including controls, were run in duplicate. Real-time PCR was performed using a standard TaqMan MicroRNA Assay protocol on a Roter-Gene 6000 (Corbett) Sequence Detection System [26]. The 20 μl PCR reactions included 1 μl RT product, 1 × Real-time PCR Master Mix including TaqMan probe, 0.15 μM miRNA specific primer set. The reactions were incubated at 95 °C for 3 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 40 s. All reactions were run in triplicate. The TaqMan C₅ value was converted into absolute copy numbers using a standard curve from a synthetic miRNA standard.

2.4. Antagomir experiments

Single-stranded RNAs were synthesized by GenePharma Co. Ltd. Chemically modified antagomir complementary to miR-350 was used to inhibit expression. The sequence used was 5′-GUGUAAAGUGUAAUG GCUUUGUGAA-3′ (miR-350-anti). A chemically modified mutant sequence 5′-GUAAAGUGUAGCCUGCUAGGA-3′ was used as a negative control (miR-350-NC). Underlined letters are mutated nucleotides. All of the bases were 2′-OMe modified. The transfection of antagomir or antagomir-NC was performed using Genofectin™ (Geno Biotech Beijuing, http://www.genobio-cn) [26].

2.5. Luciferase assays

pRL-TK vectors (Promega), with or without a target sequence such as MAPK11/14 or MAPK8/9, were cotransfected with miRNA-350 or mutant miRNA-350 vectors. Cells were harvested and lysed 24 h after transfection, and Renilla luciferase activities were measured consecutively using dual luciferase assays (Promega), according to the manufacturer’s instructions, using a Berthold Lumat 4097 Luminometer (Perkin Elmer Life Sciences, Carlsbad, CA).

2.6. Immunofluorescence

Cells grown in 24 well plates and subjected to various treatments were subsequently washed three times with cold PBS, and then fixed with 4% paraformaldehyde at room temperature for 30 min. After fixation, cells were washed three times with cold PBS and permeabilized with 0.5% Triton X-100 for 10 min at 4 °C. Non-specific binding of the fixed cells was blocked with PBS containing 2% bovine serum albumin at 37 °C for 30 min, followed by incubation with primary NFATc3 antibody (Santa Cruz, CA) overnight at 4 °C. After washing, the cells were incubated with Alexa Fluor 568-conjugated anti-goat antibody at 37 °C for 1 h; cells were stained with Alexa Fluor 568-conjugated antibody alone as a negative control. Actin filaments were visualized using rhodamine-labeled phalloidin. The cells were examined and photographed using a fluorescence microscope coupled to an image analysis system. Surface area was quantified by imaging to the boundary of individual cells under various conditions using computer assisted image analysis (Adobe Photoshop 5.0; Adobe, NIH Image J). Approximately 30–40 cells for each condition were counted and regarded as an independent experiment; three independent experiments were performed for each condition.

DAPI staining was used to visualize the nucleus. Cells grown in 24 well plates were divided into several groups for various treatments. After treatments, cells were fixed with 4% paraformaldehyde solution for 30 min at room temperature. After a rinse with PBS, the samples were incubated with 1 mg/ml 4-6-diamidino-2-phenylindole dihydrochloride (DAPI, Roche) for 30 min to enable the cell nucleus to be visualized (blue stains) by UV microscopy.

2.7. MTT assay

A total of 8 × 10³ cells were seeded into each well of a 96-well plate and allowed to adhere for 18 h. The cells were then transfected with various vectors. At day 1, 3, 5, and 7 after the transfection, cell viability was analyzed using a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT, Sigma) assay. The optical density was measured using a microculture plate reader (Bio-Rad 3550) at 540 nm. Absorbance values
were normalized to the values obtained for control cases to determine the percentage cell survival.

2.8. Flow cytometry

Cultured cells were treated with various vectors or regents. At day 2 after transfection, apoptotic cells were detected using Annexin V-PE and propidium iodide (PI) double labeling. The procedure was performed according to the manufacturer’s instructions. Flow cytometry (Becton Dickinson, USA) was used to assess the number of apoptotic cells.

2.9. Western blotting

Protein was prepared from cultured cells as previously described. Briefly, pre-stained molecular-weight markers and 50 μg cytosolic protein were separated on a 10% SDS-PAGE gel. Separated proteins were electrophoretically transferred to a 0.45-μm polyvinylidene difluoride membrane. The membrane was blocked overnight at 4 °C in PBS containing 5% nonfat milk, then probed with specific primary antibodies: phospho-MAPK14 (Cell Signaling Technology, Inc.), phospho-JNK1 (Cell Signaling Technology, Inc.), unphosphorylated NFATc3 (Santa Cruz Biotechnology, Inc.), beta-actin and GAPDH (Cell Signaling Technology). Horseradish peroxidase-labeled anti-rabbit IgG, diluted 1:3000 in PBS, were used as the secondary antibody. Specific protein bands were visualized using an enhanced chemiluminescence system (Amersham) according to the manufacturer’s instructions. Band densities from Western blots were quantified using Adobe Photoshop CS4 Extended version. The normalized intensity of each band is the ratio of control (actin)/background-corrected raw intensities of protein of interest.

2.10. Quantitative real-time PCR

Total RNA was extracted from snap-frozen heart using TRIzol reagent (Invitrogen). The yield and purity of the RNA were determined spectrophotometrically using the A260/A280 ratio. One microgram of RNA was reverse transcribed into cDNA using the SuperScript first-strand synthesis system (Applied Biosystems). cDNA (25 ng) was subjected to PCR amplification using the TaqMan RT-PCR Master Mix reagent (Applied Biosystems). TaqMan primers and probes for alpha-actin, beta-myosin heavy chain, atrial natriuretic peptide (ANP) and (BNP) and GAPDH were purchased from Applied Biosystems. The sequences of primers were listed in Table 1.

2.11. Transgenic mice

The linearized gene vectors (pGPU6/GFP/Neo) expressing miR-350 or miR-350-5p were injected into fertilized eggs of C57BL/6J mice using a TE2000-U micro-injection system. Thirty ICR mice were employed as sham pregnant receptors for the preparation of transgenic mice. All experimental procedures involving animals were approved by the Animal Welfare and Management Committee of Experimental Institute of Zoology, Chinese Academy of Medical Science (Approval number: GC-09-2078).

2.12. Statistical analysis

All values are expressed as mean ± SD. Student’s t-test was used for two-group comparisons. Comparisons of parameters among three or more groups were analyzed by 1-way ANOVA for single factor or 2-way ANOVA for two-factor variables with repeated measures, followed by Student’s t-test with Bonferroni’s correction for multiple-comparisons. Differences were considered statistically significant at a value of P < 0.05.

3. Results

3.1. TAC rats

To investigate whether and which miRNAs are involved in pathological late stage CH, we induced CH by TAC and compared miRNA expression profiles between different cases. Fig. 1A shows that TAC induced a remarkable enlargement in heart size compared with a sham operation (Fig. 1A and B, Table 2 and Suppl. Fig. 1). The heart-to-body weight ratio averaged 1.5-fold greater in the TAC cases than in control littersmates at week 7 post-surgery (Table 3). Histological analysis demonstrated CH wherein the diameter of the ventricular cardiomyocytes was significantly larger in TAC than in sham (Fig. 1C and Suppl. Fig. 2). In contrast to the well-organized, striated musculature of the normal ventricular wall, cardiomyocytes of TAC hearts were disorganized and obviously hypertrophic (Fig. 1C). In the early stage (4 weeks after treatment), TAC treatment showed a slight hypertrophic effect on LVPWTd and LVEDd without the features of heart failure present in the operated group (Table 2). By 7 weeks of age, however, all the animals treated with TAC showed a significant increase in LVPWTd, LVEDd and heart-to-body weight ratios. More importantly, these operated rats developed dilated cardiomyopathy with aging and exhibited heart failure in contrast to wild type (WT) and sham rats at later stages (Table 2).

3.2. Cardiac pressure overload induces the expression of miR-350

On the basis of the successful establishment of the TAC rat model with pathological CH, we employed miRNA chips to test the alteration of the expression of miRNAs in the cardiomyocytes of operated and sham-operated rats. Of the 1300 individual miRNAs represented on the miCURY™, 144 showed a decrease of more than 1.5-fold in the remote myocardium, whereas only one displayed an increase of more than 2-fold in expression (Fig. 1D and Suppl. Tables 1). The array data were confirmed by real-time PCR analysis using miRNA-specific probes. miR-350 expression within cardiomyocytes was at least eight times higher in TAC-treated than in sham-operated cases. However, miR-350 expression was not different between TAC-treated and sham-operated rats in the early stage (Fig. 1E). In contrast to the observation that 27 miRNAs showed increased expression in mouse hearts subjected to TAC intervention for 3 weeks [18,27], this highly upregulated miR-350 within cardiomyocytes was detected only in the late stage of pressure overload-induced hypertrophy (Fig. 1E), suggesting that the expression patterns of miRNAs in CH are different in the early and late stages.

3.3. miR-350 alone can inhibit both JNK and p38 pathways at the same time

To identify potential target genes of miR-350, we used our own bioinformatics tool [28] and miRanda to predict putative targets for miRNAs. Among the predicted target genes for miR-350 (Suppl. Table 2), six SAPK members - MAPK3/7, p38-α (MAPK14), -β (MAPK11) and MAPK8/9 (JNK1/2) - were identified. P38 and JNK were selected for further validation because they are phylogenetically conserved and contain more than...
one seed sequence matching miR-350 in their mRNA 3′ untranslated region (UTR) (Fig. 2A). Interestingly, MAPK4, p38 and JNK have been found to exert critical effects on pathological CH [3,10,29,30].

To determine whether miR-350 over-expression leads to the suppression of both p38 and JNK genes, we transfected a rat embryonic ventricular myocardial cell line (H9c2) with vectors expressing miR-350 and green fluorescence protein (GFP) transgenes. As shown in Fig. 2B, the levels of miR-350 were significantly higher following transfection with miR-350 vectors than with control vector, shR-p38/JNK vector, mutant miR-350 vector (miR-350 m), angiotensin II (Ang II) or fibroblast case. Subsequently, the luciferase activity assay was used to validate the efficacy of miRNA-350, using a luciferase reporter bearing different target sequences including MAPK11/14 (p38) or MAPK8/9 (JNK1/2) cloned into its 3′-UTR. Quantitative experiments revealed that miRNA-350 resulted in a significant decrease in luciferase expression (by 45–60%), while the mutant miRNA-350 caused no measurable reduction (Fig. 2C). This observation was further supported by the Western blotting data, which showed that increased levels of miR-350 caused a significant decrease in p38 and JNK1/2 phospho-proteins (Fig. 2E). In contrast, PCR analysis indicated that there were no detectable changes in the levels of phospho-p38 and phospho-JNK1/2 mRNAs, indicating that miR-350 modulates p38 and JNK expression by repressing translation (Fig. 2D). Conversely, when 100 nM Ang II was added to the medium, there was a significant increase in the levels of phospho-JNK1/2 and phospho-p38 MAPK and a decrease in unphosphorylated NFATc3; the cells from the control and miR-350 m cases showed no or slight changes at the mRNA and protein levels of both p38 and JNK (Fig. 2E). Further experiments have demonstrated that, in the cells treated with shR-JNK/p38, both JNK/p38 mRNAs and proteins were down-regulated. These data strongly support the suggestion that p38 and JNK are direct targets of miR-350 (Fig. 2E and F).

To clarify whether the increased expression of miR-350 is a cause or simply a consequence of CH, we first treated H9c2 cells with miR-350

Table 2
Effects of aortic banding on the left ventricular geometries and EF.

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Time</th>
<th>IVSTD (mm)</th>
<th>LVPWTD (mm)</th>
<th>LVEDD (mm)</th>
<th>EF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5</td>
<td>4 weeks</td>
<td>1.63 ± 0.21</td>
<td>1.73 ± 0.12</td>
<td>4.74 ± 0.13</td>
<td>90.72 ± 5.87%</td>
</tr>
<tr>
<td>Sham</td>
<td>5</td>
<td>4 weeks</td>
<td>1.87 ± 0.21</td>
<td>1.77 ± 0.17</td>
<td>4.50 ± 0.19</td>
<td>88.93 ± 4.57%</td>
</tr>
<tr>
<td>AB</td>
<td>5</td>
<td>7 weeks</td>
<td>1.73 ± 0.16</td>
<td>1.75 ± 0.13</td>
<td>4.83 ± 0.28</td>
<td>89.36 ± 5.11%</td>
</tr>
<tr>
<td>AB</td>
<td>10</td>
<td>4 weeks</td>
<td>2.34 ± 0.11*</td>
<td>1.84 ± 0.25</td>
<td>4.91 ± 0.48</td>
<td>88.34 ± 5.37%</td>
</tr>
<tr>
<td>AB</td>
<td>10</td>
<td>7 weeks</td>
<td>2.40 ± 0.03**</td>
<td>2.52 ± 0.24**</td>
<td>6.83 ± 0.28***</td>
<td>68.34 ± 4.27***</td>
</tr>
</tbody>
</table>

Values are means ± SEM. AB: aortic banding; LVEDD: Left ventricular end-diastolic dimension; LVPWTD: Left ventricular posterior wall end-diastolic thickness; IVSTD: Interventricular septal end-diastolic thickness; EF: ejection fraction.

* P < 0.05 and;
** P < 0.01 vs. sham.
The organization of the actin filament system was also examined. Data are mean±standard error (SE).

Table 3

<table>
<thead>
<tr>
<th>Group</th>
<th>Rat number</th>
<th>HW (g)</th>
<th>LVH (g)</th>
<th>HW/BW (g/kg)</th>
<th>LW/BW (g/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5</td>
<td>0.99±0.15</td>
<td>0.71±0.11</td>
<td>2.88±0.46</td>
<td>2.04±0.23</td>
</tr>
<tr>
<td>Sham</td>
<td>5</td>
<td>0.93±0.16</td>
<td>0.74±0.08</td>
<td>2.98±0.26</td>
<td>2.18±0.28</td>
</tr>
<tr>
<td>LVH</td>
<td>10</td>
<td>1.24±0.15</td>
<td>0.97±0.07</td>
<td>4.34±0.39</td>
<td>3.40±0.11</td>
</tr>
</tbody>
</table>

BW, body weight; LVH, left ventricular hypertrophied group; HW, heart weight; LVW, left ventricle weight; LW, left ventricular hypertrophied group. Data are mean±standard error (SE).

3.5. shR-JNK/p38 is able to imitate miR-350 in inducing hypertrophy

If an increase in miR-350 level is required for pathological hypertrophy of myocardial cells (MCs), then direct inhibition of its target genes, JNK and p38, should have similar effects on those cells. Therefore, we next studied whether over-expression of shR-JNK/p38 with the ability to knockdown both JNK1 and p38 mRNA targets in cultured H9c2 cells can mimic miR-350-induced CH. Quantitative RT-PCR analysis illustrated that shR-JNK/p38 greatly repressed endogenous p38 expression, compared with control and miR-350 m transfected cells (Fig. 2D). As expected, MCs treated with shR-JNK/p38 vectors were indeed induced to hypertrophy, as determined by the measurement of cell size (Fig. 3), whereas control vectors showed no effects on the enlargement of MCs. However, the alteration in the size of cardiomyocytes caused by shR-JNK/p38 was less than that observed in the miR-350-treated cells. Therefore, the over-expression of natural miR-350 may be more effective in inducing the enlargement of cardiomyocytes than artificial knockout of p38 and JNK1/2, suggesting that complete inhibition of the p38 and JNK pathways is essential for pathogenic hypertrophy of cardiomyocytes. Taken together, our findings provide direct evidence that miR-350 dictates the enlargement of cardiomyocytes via repression of the p38 and JNK pathways, suggesting that the activation of miR-350 may be significantly associated with massive cardiac enlargement.

3.6. miR-350 causes an increase in the level of unphosphorylated NFAT3 proteins and dramatic up-regulation of the hypertrophic markers

We next examined the translocation of unphosphorylated NFAT3 from the cytoplasm to the nucleus in response to administration of the miR-350 vector. As shown in Fig. 4, H9c2 cells were treated with miR-350 vectors (3 μg/ml) for 3 days, then assayed by immunofluorescence. The presence of the miR-350 vector induced a significant increase in the unphosphorylated NFAT3 proteins (Fig. 2E and F) and their translocation to the nucleus (Fig. 4A and B). We also examined the distribution of NFAT3 in both the cytoplasm and nucleus following treatment with different agents (Fig. 4B). We observed that the nuclear level of NFAT3 was dramatically increased following miR-350 vector (3 μg/ml) treatment, accompanied by a relative reduction in the cytosolic level of NFAT3. Similarly, shR-JNK/p38 induced the same response. In contrast, the presence of Ang II, mutant miR-350 vectors or miR-350 antagonist did not lead to a redistribution of NFAT3 from the cytoplasm to the nucleus (Fig. 4B). These results suggest that the miR-350 suppression of p38 and JNK leads to an increase in unphosphorylated NFAT3 and to its nuclear translocation.

To understand whether the expression of genes associated with myocardial pathological hypertrophy was induced by miR-350 vectors, cultured H9c2 cells were treated with various agents for 48 h, and then subjected to real-time PCR analysis. The results showed a dramatic up-regulation of the hypertrophic markers atrial natriuretic peptide (ANP), B-type natriuretic peptide (BNP), beta-myosin heavy chain (β-MHC), and skeletal alpha actin (SKA) in response to cardiac over-expression of miR-350 (Fig. 4C). These results suggest that the miR-350 suppression of p38 and JNK results in marked increases in the mRNA levels of the pathological hypertrophy markers ANP, BNP, β-MHC and SKA, implying that the induction/ regulation of their expression is mediated by NFAT3 (Fig. 4D).

3.7. Loss-of-function assay proves the hypertrophic roles of miR-350

The next question to be investigated was whether blocking miR-350 expression in cardiomyocytes could prevent hypertrophy. To test this idea, we performed in vitro studies in which an antagonist specific for miR-350 (miR-350-ant) or a mutant antagonist (miR-350-NC), and miR-350 vector were co-transfected into cells. When cells were treated with miR-350-ant, miR-350 activity was significantly repressed (Fig. 2B). As shown by PCR analysis and Western blotting (Fig. 2G and H), neither p38 and JNK genes nor proteins showed alternation. Quantitative analysis of cell size revealed that miR-350-ant treatment significantly attenuated the hypertrophy provoked by miR-350 (Fig. 3B and C). MTT analysis indicated that miR-350-ant effectively prevented the cell death mediated by miR-350 (Fig. 3D). Similarly, immunohistochemical staining illustrated that NFAT3 translocation could be blocked by this antagonist (Fig. 4B). However, treatment of H9c2 cells with both miR-350-NC and miR-350 vectors had no effect on the expression level of miR-350 compared with the control (Fig. 2B). In parallel, administration of antagonist-350, but not the antagonist-350 mutant, was associated with significantly increased levels of JNK and p38 protein in the cells (Fig. 2H). Furthermore, we observed that the relative area of cells was significantly reversed in the antagonist-350-treated cells (1396 ± 120 versus 1258 ± 86) compared to the control (P < 0.05; Fig. 3B and C). Apoptosis of H9c2 cells, NFAT3 translocation and expression of fetal genes did not differ significantly among the control, mutant and miR-350-ant groups (Figs. 3D and 4). Taken together, these results suggest that administration of inhibitory miR-350 molecules (i.e., miR-350-ant) may protect these cardiomyocytes from pathological hypertrophy [31,32].
Fig. 2. The ectopic expression of miR-350 can effectively inhibit the expression of endogenous p38 and JNK genes at the translational level. (A) Sequence alignment of the rat p38 and JNK 3′ UTRs and mature miR-350. Base-pairing between miR-350 seed sequences with MAPKs 3′ UTR is marked in red. (B) Differential expression of miR-350 in various cases. Cultured cardiocytes were treated with Ang II (100nM) or different vectors for 48 h. Small RNA was isolated and detected by real-time PCR. Data are averages of at least three independent determinations. Error bars indicate standard deviations. ⁎P < 0.001 and ⁎⁎P < 0.01; miRNA-350 compared with the control group and miR-350+anti, respectively. #P > 0.05; miRNA-350 compared with miR-350+NC. (C) The miRNA-350 or mutant miR-350 vector [2 μg] together with 2 μg pRL-TK reporter plasmids with or without a target sequence of the miRNA-350 were cotransfected into cardiocytes, and 24 h after transfection a dual luciferase assay was carried out. Relative levels of the expressed luciferase activity under various conditions were determined and normalized to their levels in the control. The data are the averages of at least three independent determinations. Error bars indicate standard deviations. ⁎P < 0.01; miRNA-350 compared with the control group. (D) Quantitative RT-PCR analysis of the expression levels of related mRNAs under various conditions was carried out 48 h after treatment. Data are averages of at least three independent determinations. Error bars indicate standard deviations. ⁎P < 0.005. (E) The levels of related proteins were analyzed by Western blotting. The Western blot was stripped and re-probed with β-actin antibody to determine total protein loading. (n = 3). (F) The quantitative analysis on the blotting was performed. Error bars indicate standard deviations. ⁎P < 0.05; shR-JNK/p38 compared with the control group. (G) PCR analysis of the expression levels of related mRNAs under three different conditions was carried out 48 h after treatment. (H) The levels of phospho-p38 and phospho-JNK proteins were analyzed by Western blotting. The graph is a representative of three experiments.
Fig. 3. The ectopic expression of miR-350 significantly induces the enlargement of cardiomyocytes and cell apoptosis. (A) Morphological changes in H9c2 myocardial cells in a time-dependent manner. (B) H9c2 myocardial cells were treated without or with various agents and stained by actin-immunofluorescence to reveal sarcomeres, and with Hoechst stain to reveal nuclei. Cells were transfected with mutant miR-350 vectors (miR-350 m) as a negative control or stimulated with Ang II (100 nM), a potent inducer of hypertrophy, as a positive control. (C) The relative cell size in response to various conditions at different time-points was analyzed. Data are means ± SD of 160 cell measurements from three independent experiments. *P < 0.01 and **P < 0.01; miRNA-350 and shR-JNK/p38 compared with the control group, respectively. #P > 0.01; miRNA-350 compared with miR-350+anti. (D) The effects of different agents on cell viability were examined by MTT assay. Data are means ± SD. *P < 0.05 or **P < 0.01 compared with control at day 5. #P > 0.05; miRNA-350 + anti or AngII compared with control, respectively. (E) H9c2 myocardial cells were transfected with miR-350, shR-p38 or miR-350 m. Cells were sampled at 60 h, and apoptosis was determined via Annexin V-PE and PI co-staining. Data are representative of three experiments. The corresponding quantitative analysis was shown in (F). *P < 0.05 and #P < 0.01; miRNA-350 and shR-JNK/p38 compared with the control group, respectively.
NFATc3 was not induced to translocate to the nucleus, as distinct from the effect of miR-350 and shR-p38 vectors. These results were observed in three independent experiments. (B) The results shown in (A) were quantified. Data shown are means ± SD of 120 cell measurements from three independent experiments. *P < 0.001 and **P < 0.001 compared with control. (C) Real-time PCR was performed on RNA samples extracted from H9c2 cells under different conditions. The expression levels of related mRNAs were normalized to that of GAPDH, then normalized to the levels in the control. Data are averages of at least three independent determinations. Error bars indicate standard deviations. Control values were fixed at 1. **P = 0.01 vs. control. (D) A schematic representation showing that miR-350 induces pathological hypertrophy via SAPK/NFAT-3 signaling pathways in myocardial cells. Stress provoked by TAC activates miR-350 expression, which represses p38 and JNK genes involved in cardiac hypertrophy, apoptosis, and the myocardial gene program. miR-350 directly targets p38 and JNK1/2, which causes decreased phosphorylation of NFATc3 and its nuclear translocation, resulting in the development of myocardial hypertrophy.

4. Discussion

In this study, our results have demonstrated that TAC in rats induces a novel miRNA, miR-350. It is the only highly expressed miRNA identified in the late stage of pressure overload-induced hypertrophy. Bioinformatics analysis revealed that miR-350 might interact with six key components of the SAPK pathway. They are MAPK3/7, MAPK11/14 (p38) and MARPK8/9 (JNK1/2), which all have seed sequences complementary to that of functional miR-350. Indeed, our data from Western blotting and luciferase assays provide strong evidence that to control the SAPK pathway with maximal efficiency, miR-350 may simultaneously inhibit MARPK8/9 and MAPK11/14. Moreover, gain- and loss-of-function studies in cultured cardiomyocytes have demonstrated the necessity and sufficiency of miR-350 alone to induce pathological CH, suggesting that miR-350 functions as a key mediator of pathological hypertrophy.

Expression of miRNAs might be differentially regulated under different conditions [33,34]. Recent studies have shown that some miRNAs such as miR-23a, miR-27a, miR-24-2, miR-195 and miR-21 are up-regulated in TAC-induced CH [18]. Their up-regulation may be necessary for the induction or suppression of early-stage or adaptive hypertrophy. However, our data indicated that miR-208 and miR-195 showed no notable alteration in late-stage CH, whereas miR-350 was found to increase significantly in response to late-stage TAC treatment (Suppl. Table 1). It is reasonable to propose that miR-350 plays a critical role in modulating the hypertrophic response and the succeeding transition towards heart failure caused by cell apoptosis. These studies suggest that, in response to environmental perturbations at different stages, cardiomyocytes alter the expression of miRNAs that participate in programs governing heart hypertrophic protein expression. These changes in miRNA expression profiles can either promote or avert progressive ventricular dysfunction and heart failure.

Pathological hypertrophy occurs in response to pathological stress signals. At the molecular level it is characterized by the activation of gene expression patterns associated with fetal development (Fig. 4C) [36,37]. Several studies have indicated that the MAPK signaling pathway is closely associated with CH. The MAPK pathway is generally sub-classified into three main branches consisting of p38 kinases, c-JunN-terminal kinases (JNKs), and extracellular signal-regulated kinases (ERKs) [38]. Transgenic mice over-expressing an activated MEK1 cDNA, which showed specific activation of only ERK1/2, were characterized by a prominent hypertrophic response [39]. It has been shown that angiotensin II (Ang II) is a major factor in the development of cardiomyocyte hypertrophy and a pivotal role for Ang II signals via ERK1/2 and NFATc4 has been identified. ERKs are particularly implicated in growth-associated responses [40]. Recently, Ang II has been found to up-regulate the expression of miR-21 associated with CH [27]. However, miR-350 showed no changes following stimulation with Ang II. In contrast, Ang II stimulated the phosphorylation of JNK and p38 but did not lead to the nuclear translocation of NFATc3 in cardiomyocytes (Figs. 2E and 4B). This suggests that the signaling pathways of miR-350 and Ang II are different. A combinatorial inhibition of JNK and p38 is particularly responsible for pathological responses.

Information reported in the literature has not yet given a clear view of the roles of specific MAPK pathways in CH. Rather, contradictory results have led to a perception that MAPKs are ambiguous characters in the heart, with both protective and detrimental effects [4,7,9,38]. Some studies have indicated that c-jun NH2-terminal kinases (JNKs) and p38 kinases function as specific transducers of the stress response.
For example, JNK activity is specifically up-regulated in response to pressure overload, while p38 activity is markedly induced in hearts subjected to volume overload [41]. Moreover, MAPK signaling pathways have been shown to cooperate with calcineurin such that unitary activation of calcineurin in myocytes leads to an up-regulation of ERK and JNK signaling, but down-regulation of p38 signaling [4,38]. Conversely, unitary activation of JNK or p38 in cardiac myocytes leads to a down-regulation in calcineurin effectiveness by directly antagonizing NFAT nuclear occupancy [42]. It is likely that both stress-activated protein kinase-signaling branches have somewhat overlapping functions in the heart aimed at counter-regulating cell behaviors such as apoptosis, fibrosis, survival, and growth. Considered together, these data imply that distinct combined effects on members of the MAPK signaling pathway, such that some are activated and some are inhibited, may be the cause of the conflicting results found in previous studies.

Our studies have revealed that inhibition of both JNK and p38 pathways by miR-350 and its mimic could result in hypertrophy of cardiomyocytes. This result is also consistent with the observation that dominant negative JNK1/2 transgenic mice demonstrated enhanced cardiac hypertrophic growth following pressure overload induced by aortic banding [6]. Furthermore, transgenic mice expressing activated MKK7 in the heart showed specific JNK activation but not CH [29,43]. A similar phenomenon was also observed with p38 signaling in the myocardium. Cardiac-specific transgenic mice (dominant negative mutants of p38a, MKK3, and MKK6) showed specific inhibition of p38 signaling that was associated with spontaneous CH [3]. On the other hand, JNK and p38 are capable of directly phosphorylating specific NFAT transcription factors in their N-terminal regulatory domains, resulting in net inhibition of nuclear occupancy [42]. For example, JNK factors can directly phosphorylate NFATc1, NFATc2 and NFATc3, but not NFATc4. Thus, suppressing the expression of both JNK and p38 by miR-350 can result in more effective inhibition of both the p38 MAPK and NFAT signaling branches. Moreover, MAPK signaling pathways may be of importance for the heart aimed at counter-regulating cell behaviors such as apoptosis, fibrosis, survival, and growth. Considered together, these data imply that distinct combined effects on members of the MAPK signaling pathway, such that some are activated and some are inhibited, may be the cause of the conflicting results found in previous studies.

Disclosures

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbadis.2012.09.004

References


