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Research paper

MicroRNA-29a-3p attenuates ET-1-induced hypertrophic responses in H9c2 cardiomyocytes

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ABSTRACT

Transcription factor nuclear factor of activated T cells c4 (NFATc4) is the best-characterized target for the development of cardiac hypertrophy. Aberrant microRNA-29 (miR-29) expression is involved in the development of cardiac fibrosis and congestive heart failure. However, whether miR-29 regulates hypertrophic processes is still not clear. In this study, we investigated the potential functions of miR-29a-3p in endothelin-1 (ET-1)-induced cardiomyocyte hypertrophy. We showed that miR-29a-3p was down-regulated in ET-1-treated H9c2 cardiomyocytes. Overexpression of miR-29a-3p significantly reduced ET-1-induced hypertrophic responses in H9c2 cardiomyocytes, which was accompanied by a decrease in NFATc4 expression. miR-29a-3p targeted directly to the 3′-UTR of NFATc4 mRNA and silenced NFATc4 expression. Our results indicate that miR-29a-3p inhibits ET-1-induced cardiomyocyte hypertrophy via inhibiting NFATc4 expression.

1. Introduction

In response to a number of sustained pathologic stimuli such as mechanical, hemodynamic and hormonal stimuli, the adult heart undergoes cardiac hypertrophy, which is accompanied by an increased cell size, sarcomeric reorganization and activation of a fetal gene program (Lorell and Carabello, 2000; Frey and Olson, 2003). There have been a number of studies in the exploration of molecular mechanism involved in the development of cardiac hypertrophy, but the exact regulatory mechanisms remain to be identified.

MicroRNAs (miRNAs) are a class of short, single-stranded RNAs that suppress protein expression through effects on the stability and/or translation of target mRNA. miRNAs are widely expressed in various tissues and organs and play an important role in temporal regulation during the development of organisms and control diverse aspects of disease (Bartel, 2009). An important role of miR-29 in cardiovascular disease is identified in recent years. miR-29 family, a fibroblast-enriched miRNA family, is composed of three mature members, miR-29a, miR-29b and miR-29c, differing only in two or three bases and sharing a common seed region sequence (AGCACCA) (Kriegel et al., 2012). miR-29 family members are predicted to target largely overlapping sets of genes, therefore, they have similar functions in many cases. For example, the three members all had strong antifibrotic effects on heart, lung, and other organs by targeting a broad collection of mRNAs encoding extracellular matrix proteins (He et al., 2013). van Rooij et al. (van Rooij et al., 2008) demonstrated that miR-29 family members were down-regulated in the border zone of the infarcted myocardium in mice, which was accompanied by an increase in the expression of extracellular matrix genes collagen-1A1, collagen-1A2, collagen-3A1 and fibrillin. miR-29a and miR-29c were demonstrated to induce cardiomyocyte apoptosis by targeting anti-apoptotic proteins and inhibition of miR-29a or -29c protected hearts against myocardial ischaemia–reperfusion injury by reducing myocardial infarct size and apoptosis (Ye et al., 2010). Moreover, members of the miR-29 family, miR-29a, -29b and -29c, were significantly down-regulated in mouse heart after aortic banding (Cheng et al., 2007), suggesting that miR-29 family may play an important role in cardiac hypertrophy. However, to date, no data is available on miR-29 in cardiac hypertrophy.

The Ca^{2+}-dependent signal transduction pathway is now recognized as an important regulatory system that controls cardiac hypertrophy (Heineke and Molkentin, 2006), in which nuclear factor of activated T cells (NFATs) occupy the central place. NFAT family has four structurally related members NFATc1, NFATc2, NFATc3, NFATc4 and a constitutively nuclear NFAT5 member, out of which, NFATc3 and NFATc4 are the best-characterized target for the development of cardiac hypertrophy

Abbreviations: ET-1, endothelin-1; microRNA, miRNA; NFAT, nuclear factor of activated T cell; ANF, atrial natriuretic factor; BNP, B-type natriuretic peptide; SK-α-actin, skeletal alpha actin; CAMKII, calcium/calmodulin-dependent protein kinase II; HDAC, histone deacetylase; MHC, myosin heavy chain; SRF, serum response factor; Gsk3β, glycogen synthase kinase-3β; ROS, reactive oxygen species; NCX1, sodium-calcium exchanger 1; PIP2, phosphatidylinositol 4,5-bisphosphate; IP3, inositol trisphosphate; DAG, diacylglycerol.

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(Rohini et al., 2010). In resting cells, NFATs are mainly located in the cytoplasm, in a highly phosphorylated form. Once activated by calcineurin, NFATs are dephosphorylated and translocated into the nucleus to regulate the expression of hypertrophic cardiac genes, such as skeletal alpha actin (SK-α-actin), atrial natriuretic factor (ANF) and B-type natriuretic peptide (BNP). Some previous studies have shown that overexpression of a constitutively activated NFATc4 in transgenic mice can induce cardiac hypertrophy (Molkentin et al., 1998). NFATc4 can be activated by endothelin-1 (ET-1) and up-regulates the BNP expression during phenylephrine stimulation, suggesting that NFATc4 activation is important for cardiac hypertrophy (Li et al., 2014; Le et al., 2012).

In this study, we show for the first time that miR-29a-3p is significantly down-regulated in ET-1-induced hypertrophy of H9c2 cardiomyocytes. miR-29a-3p mimics inhibit ET-1-stimulated expressions of hypertrophic cardiac genes partly by targeting NFATc4, suggesting that decreased miR-29a-3p and consequently increased NFATc4 expression contribute to ET-1-mediated cardiomyocyte hypertrophy.

2. Materials and methods

2.1. Cell culture, cell treatment and transfection

The rat cardiomyocyte-derived cell line H9c2 cells were obtained from American Type Culture Collection and cultured in high glucose Dulbecco's Modified Eagle's Medium (DMEM, GIBCO) supplemented with 10% fetal bovine serum (FBS, sijiqing).

ET-1 (Sigma-Aldrich) was dissolved in sterile ddH_2O, the working concentration is 100 nM. rno-miR-29a-3p mimic (miR29a-mimic), negative control for mimic (NC-mimic), anti-rno-miR-29a-3p (miR29a-inhibitor) and negative control for inhibitor (NC-inhibitor) were purchased from QIAGEN.

For transfection experiments, H9c2 cells were plated in DMEM medium without antibiotics when they were 40–50% confluent. Transfection was carried out with transfection reagent (Lipofectamine 2000 Reagent; Invitrogen) according to the instructions.

2.2. Measurement of cell surface area

Cell surface area was measured with a quantitative digital image analysis system (Image Pro-Plus, version 6.0) as described previously (Li et al., 2015). Fields of cells under each condition were randomly selected and 60 cells of each group were counted. The surface area was estimated by the ratio of control cells. Three independent experiments were performed under each condition.

2.3. Plasmid construction and site-directed mutagenesis

The pEGFP-C1 NFAT3 (NFATc4) expression plasmid was purchased from addgene (Plasmid 10961). The 3′-UTR of rat NFATc4 containing the predicted binding sites for miR-29a (+1 to +327) was amplified by PCR and then cloned it into the pmirGLO vector. The primers used in PCR reactions were as follows: forward primer-CCTGAGCTCACCAC ATGTAGTGGCCCCAGAAGTC, reverse primer-TAT TCTAGAGAGCCTGA GGCACCGGGCAGC. The mutant construct of NFATc4 3′-UTR was generated by introducing mutations into the putative binding sites by standard overlap PCR using mutagenic primers. The primers used in PCR reactions were as follows: forward primer-TAAGGTGTGGCCCCCAGG CCTGACTTTGCCT TGAAAGGGACTGAGGGTGTG, reverse primer- CACA CCCTCAGTCCCTTTCA AGGCAAAGTCAGGCCTGGGGGCCACACCTTA.

2.4. MiRNA quantification and quantitative real-time RT-PCR (qRT-PCR)

For miRNA analysis, H9c2 cells were lysed with Buffer MZ reagent and miRNA isolated using the miRcute miRNA Isolation kit (TIANGEN) according to the manufacturer's instructions. RNA was reverse transcribed using reverse transcription primers and M-MLV reverse transcriptase (Invitrogen) according to the manufacturer's instruction. The sequences of reverse transcription primers were as follows: rno-miR-29a-3p: GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACT GGATAC GACTAACCGA; U6: GTCGTATCCAGTGCAGGGTCCGAGGTATTCG CACT GGATACGACAAAATATG. For mRNA analysis, total RNA was extracted using Trizol reagent (Invitrogen), according to instructions. The RNA (2 μg of each sample) was reverse-transcribed into cDNA using random primers and M-MLV reverse transcriptase.

For the quantification of miRNA or mRNA, the samples were run in triplicates using Applied Biosystems StepOne™ Real-Time PCR System according to the manufacturer's instructions. GoTaq® qPCR Master Mix was obtained from Promega. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and U6 expression were used as internal controls. The qRT-PCR primer sequences were as follows: rno-miR-29a-3p: forward primer-GAGTGTTAGCACCATCTGAAA; reverse primer-GCAG GGTCCGAGGTATTC; U6: forward primer-GCGCGTCGTGAAGCGTTC; reverse primer-GTGCAGGGTCCGAGGT; ANF: forward primer-GAAGTC AACCCGTCTCA, reverse primer-ATCCTGTCAATCCTACCC; BNP: forward primer-TTCTGCTCCTGCTTTTCC, reverse primer-CTTTTGTAGGGCCT TGGT; α-actinin: forward primer-CGACCAGTGGGATAGATT, reverse primer-ATGCTGTGGACGATGAAC; SK-α-actin: forward primer-CCATTG AACACGGCATTA, reverse primer-CCACATACATAGCAGGCAC; NFATc4: forward primer- CCCTGAGTACAGCAACAA, reverse primer- CACAGGCA GGAACTTGA; GAPDH: forward primer-ATTCAACGGCACAGTCAAGG, reverse primer-GCAGAAGGGGCGGAGATGA.

2.5. Protein isolation and Western blotting

Nuclear protein was isolated with a Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime) according to the manufacturer's recommendations. Total protein was extracted from cells by lysis buffer and the protein concentration was measured with Coomassie brilliant blue method. Western blotting analysis was performed according to standard procedures. The membranes were incubated at 4 °C over night with different primary antibodies as indicated in figure legends, including NFATc4 (1:2000, LifeSpan BioScienses, LS-C289484), ANF (1:200, Santa Cruz, sc-20,158), α-actinin (1:250, abcam, ab9465), BNP (1:500, abcam, ab19645), Lamin B (1:500, Santa Cruz, sc-374,015) or β-actin (1:500, Santa Cruz, sc-47,778). The secondary antibody IRDye 680LT goat anti-rabbit IgG (H + L) (1:10,000, LI-COR, 926-68021) or IRDye 800CW goat anti-mouse IgG (H + L) (1:10,000, LI-COR, 926-32210) was incubated at room temperature for 60 min. The blots were scanned by a two-color infrared imaging system (Odyssey, LICOR) to quantify protein expression. Relative density of each band was analyzed with Image J software.

2.6. Luciferase assay

Luciferase activity assay was performed using the Dual-Luciferase® Reporter Assay System (Promega) according to the instructions. Briefly, the vector containing 3′-UTR of NFATc4 (0.4 μg) or mutant 3′-UTR were co-transfected with miR29a-mimic (5 nM), NC-mimic (5 nM), miR29a-inhibitor (50 nM) or NC-inhibitor (50 nM) using Lipofectamine 2000 for 24 h. Renilla luciferase was used to normalize the cell number and transfection efficiency. The transfected H9c2 cells were lysed in Passive Lysis Buffer. 20 μL of cell lysate was added into a 96-well enzyme label plate and reading was initiated by the injection of 100 μL of Luciferase Assay Reagent into the plate on a Synergy™ 4 (Bioteck).

2.7. Immunocytochemistry assay

The H9c2 cells after fixation and membrane permeabilization were blocked with normal goat serum for 40 min at room temperature. NFATc4 primary antibody (1:500, LifeSpan BioScienses, LS-C289484) was added and incubated in a humid chamber over night. After washing with PBS thrice, cells were incubated with appropriate secondary