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miRNA Expression in the Failing Human Heart: Functional Correlates

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Abstract

MicroRNAs (miRNAs) are small, noncoding ~22-nucleotide regulatory RNAs that are key regulators of gene expression programs. Their role in the context of the cardiovascular system has only recently begun to be explored; however, changes in the expression of miRNAs have been associated with cardiac development and with several pathophysiological states including myocardial hypertrophy and heart failure. We demonstrate that miRNA expression patterns are distinct in two types of heart failure: idiopathic dilated cardiomyopathy and ischemic cardiomyopathy. To pursue the observation that changes in expression levels of individual miRNAs are functionally relevant, microRNA mimics and inhibitors to miR-92, miR-100 and miR-133b were expressed in primary cultures of neonatal rat cardiac myocytes. These studies demonstrated that over expression of miR-100 is involved in the β -adrenergic receptor mediated repression of “adult” cardiac genes (i.e., α -myosin heavy chain, SERCA2a), and that over-expression of miR-133b prevents changes in gene expression patterns mediated by β -adrenergic receptor stimulation. In conclusion, some miRNA expression patterns appear to be unique to the etiology of cardiomyopathy and changes in the expression level of miR's 100 and 133b contribute to regulation of the fetal gene program. It is likely that this miR-directed reprogramming of key remodeling genes is involved in the establishment and progression of common human cardiomyopathies.

Keywords

microRNA; mRNA; gene regulation; cardiomyopathy; adrenergic receptor

1. Introduction

MicroRNAs (miRNAs) are small ~22 nucleotide noncoding RNAs that inhibit transcription or translation by interacting with the 3' untranslated region (3'UTR) of target mRNAs (1). miRNAs are important regulators of gene expression in both disease and development. Recently, several studies have demonstrated the importance of miRNAs in the regulation of cardiac differentiation and disease (2-5). In this context, several miRs including miR-21, 195, 133 and 208 appear to play an important role in the process of cardiac remodeling, putatively

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by regulating changes in gene expression that accompany pathological cardiac hypertrophy and contractile dysfunction (5-8).

β -adrenergic receptor (β -AR) signaling plays an important role in the progression of human heart failure, and in clinical trials, β -adrenergic receptor antagonists result in reverse remodeling and reduce morbidity and mortality (9,10). Since miRNAs can function by regulating the expression of cardiac genes, we sought to determine the relative overall abundance of miRNAs in failing versus nonfailing human heart, and further, to address the potential role(s) of a subset of differentially expressed miRNAs as they relate to β -adrenergic signaling in cardiac myocytes.

In this report we demonstrate that the expression levels of subsets of miRNAs are differentially regulated as a result of idiopathic (IDC) and ischemic cardiomyopathies (ISC) compared to control, nonfailing (NF) hearts. Moreover, in cell-based experiments, we show that down-regulation of miR-133b is sufficient to induce hypertrophic gene expression while over-expression of miR-133b attenuates aspects of β -AR mediated changes in gene expression. We also show that although miR-92 is down-regulated in human heart failure, it appears to play a minimal role in regulating hypertrophic gene expression. Finally we demonstrate that inhibition of miR-100, a miRNA up-regulated in the failing human heart, specifically prevents β -adrenergic mediated down-regulation of the adult gene component of the fetal gene program.

2. Materials and Methods

2.1 microRNA extraction

miRNA was extracted from 6 nonfailing, 5 idiopathic dilated cardiomyopathy, and 5 ischemic dilated cardiomyopathy patients. The cardiac function (ejection fraction) for each group of patients is summarized in Table 1. miRNA extraction was performed using the mirVana™ kit (Ambion) according to manufacturer's recommendation.

2.2 Array analysis

miRNA expression analysis was performed by LC Science, LLC (Houston, TX) using arrays based on the Sanger miRBase 9.0 database, (<http://www.sanger.ac.uk/Software/Rfam/mirna/>), capable of detecting 470 miRNAs.

2.3 miRNA RT-PCR

Reverse transcription of miRNAs was performed using the TaqMan™ MicroRNA Reverse Transcription Kit (Applied Biosystems) according to manufacturer's recommendations. Briefly, 5 ng of miRNA were combined with dNTPs, MultiScribe™ reverse transcriptase, and the primer specific for the target miRNA. The resulting cDNA was diluted 15-fold and used in PCR reactions. PCR was performed according to manufacturer's recommendations (Applied Biosystems). Briefly, cDNA was combined with the TaqMan™ assay specific for the target miRNA, and PCR reaction was done using the ABI7300.

2.4 Cell Culture

Neonatal rat cardiac ventricular myocytes (NRVMs) were cultured as described previously (11). Briefly, cells were isolated by trypsin digest of ventricles of 1 to 3 day-old rats. Twenty-four hours after plating, media was changed to serum-free media containing insulin, transferrin, bovine serum albumin, vitamin B12 and penicillin. All media solutions were buffered with Hepes (pH 7.5) to a final concentration of 20 mM. Protocol for animal work is in accordance with PHS Animal Welfare Assurance, ID A3269-01.

2.5 Transfection of mimic and inhibitors in neonate rat ventricular myocytes (NRVMs)

miRIDIAN™ mimic and inhibitors (Dharmacon) for miR-92, 100, and 133b, were transfected into NRVMs using Amaxa Nucleofector technology (Amaxa). Briefly, 2.4×10^6 cells were suspended in the appropriate Amaxa solution for cardiac myocytes and combined with 20 μ M of the mimic or inhibitor. Cells were electroporated using the cardiac myocyte program. This results in an approximate 95% transfection efficiency for short RNAs (data not shown). Media containing serum were added to the cells that were substituted with serum-free media 24 later. Cells were harvested 72 hours after transfection. A subset of cells was treated with β -adrenergic agonist isoproterenol (10^{-7} M)

2.6 RNA extraction from NRVMs and RT-PCR

Total RNA was extracted using TRIzol® (Invitrogen). cDNAs for poly(A) containing mRNAs was prepared using the iScript (Bio-Rad) essentially as described by the manufacture. Typically, 0.1 ng of cDNA, 12.5 nM of each primer and Syber Green PCR Master Mix (ABgene, ThermoFisher) were used in the RT-PCR reactions. Reactions were performed using the ABI7300 system. The primers used are described below. miRNA cDNAs were synthesized as described above, and RT-PCR reaction was performed according to Applied Biosystems' protocol.

2.7 Immunofluorescence and cell surface area quantification

Immunofluorescence was performed according to Harrison *et al* (12). Briefly, cells were washed with TBST and fixed with 10% formaldehyde for 20 minutes. Cells were again washed with TBST and incubate with 0.1% Triton-X for an additional 30 minutes. Cells were then blocked with 1% BSA in TBST for 1 hour followed by 1 hour incubation with 1:500 dilution of the anti-Flag antibody. Cells were then incubated with a 1:1000 dilution of Alexa 594 anti-mouse antibody and 2 μ g/ml Hoechst stain for 1 hour. Images were captured at a 40X magnification with a fluorescence microscope (Nikon E800) equipped with a digital camera (Zeiss AxioCam) and Zeiss AxioVision ver. 3.0.6.36 imaging software. Cell surface area of 30 cells from three different fields in each condition was quantified using the Image J software program (NIH).

3. Results and discussion

3.1 Differential expression of miRNAs in failing human hearts

To investigate differences in miRNA expression between NF, ISC and IDC hearts, miRNA was extracted from 16 subjects (n=6, 5, 5, respectively) and relative abundance of miRNAs comprising the Sanger 9.0 database were analyzed by microarray. Clinical characteristic of IDC and ISC patients are described in Table 1. As shown in Figure 1A, a number of miRNAs are differentially regulated in the failing heart, with subsets that are differentially regulated in both ISC and IDC and further subsets that are specific to each condition. Differential expression of several of the miRNAs observed in this study have been described in other recent studies (2,3,5,6,13). Based on the differential expression profiles for miRNAs expression profiles observed in both ISC and IDC patients, and based on results from other recent studies, above, a set of 6 miRNAs was selected for further analysis. These include miR-150 (5,13), miR-133a (2,5,6), miR-133b (2,14), miR-195 (3,5,13), miR-100 (3) and miR-92 (chosen because of dramatic repression in IDC and ISC samples). To confirm the results of the array experiments, the relative expression of these six miRNAs were examined by RT-PCR. In these studies, miRNA expression was normalized to that of either miR-24 or miR-143 (expression of both of these miRNA was found not change in our arrays). As shown in Figure 1B, in tissue samples from both ISC and IDC hearts, the up-regulation of miR-195 and miR-100 and down-regulation

of miR-92 and miR-133b detected in array data was confirmed by RT-PCR. For unknown reasons, down-regulation of miR-133a and miR-150 could not be confirmed by RT-PCR.

Because the sequences for miR-133a and miR-133b are highly similar, experiments were performed wherein either miR-133a or miR-133b inhibitors or mimics were over-expressed. Data obtained by RT-PCR provided confirmation that detection was specific to each miR. Thus, over-expression of miR-133a demonstrated increased expression of miR-133a with no change in the abundance of miR-133b. The results of the converse experiment yielded the same degree of specificity (Figure S1).

Given that the functional relevance of miR-195 up-regulation was described previously by van Rooij et al (5), further functional analysis was instead focused on the use of microRNA mimics and inhibitors specific to miR-92, miR-133b and miR-100.

3.2 Putative roles of miR-133b, miR-100 and miR-92 in the regulation of the hypertrophic gene program

In order to begin to understand the function of increased expression or down-regulation of each of the selected miR's, neonatal rat ventricular myocytes (NRVMs) were transfected with the appropriate miRIDIAN microRNA mimics and inhibitors. The role of the miRNAs in the hypertrophic process was determined by examining their ability to regulate expression of the Fetal Gene Program (FGP). In order to examine the role of each miRNA in preventing the hypertrophic gene expression profile, post transfection, cells were treated for 48 hours with the β -adrenergic receptor agonist, isoproterenol (ISO). As shown in Figure 2A, transfection of the cells with microRNA inhibitors or mimics resulted in dramatic down-regulation or up-regulation of target miRNAs. ISO-mediated induction of the fetal gene program was demonstrated by repression of expression of the adult genes, α -myosin heavy chain (α MyHC) and SERCA, and up-regulation of the fetal genes, b-type natriuretic peptide (BNP), atrial natriuretic factor (ANF), skeletal α -actin and β -myosin heavy chain (β MyHC). Data summarizing the effects in cells transfected with either a scrambled control inhibitor, a relevant mimic miRNA, followed by treatment with ISO are shown in Figures 2, B-D. As demonstrated in Figure 1, expression of miR-100 is increased in the failing heart. Up-regulation of miR-100 in NRVMs results in repression of the adult genes α MyHC and SERCA and increase ISO-mediated up-regulation of the fetal genes ANF and β MyHC (Figure 2B). Interestingly, down-regulation of miR-100 prevented ISO-mediated repression of α MyHC and SERCA but not the induction of the fetal isoforms (Figure 2B), suggesting that inhibition of miR-100 specifically regulates expression of genes involved in ISO-mediated repression of the adult isoforms.

miR-92 is down-regulated in heart failure, therefore, the expectation was that inhibition of miR-92 would result in induction of the fetal gene program, and that up-regulation of miR-92 would prevent induction of the fetal gene program. However, as shown in Figure 2C, inhibition or up-regulation of miR-92 has a minimal effect on the regulation of fetal or adult gene expression. These results suggest that down-regulation of miR-92 in heart failure might be involved in other pathways modulating the heart failure phenotype beyond the ones investigated in the current studies. It is interesting to note that down-regulation of miR-92 is not observed in any hypertrophic mouse model, suggesting again that miR-92 down-regulation may not be involved in the development of hypertrophy, but instead may be secondary to end-stage heart failure. Finally, miR-133b expression is down-regulated in heart failure. Inhibition of miR-133b resulted in a small but generalized up-regulation of the genes analyzed. However, up-regulation of miR-133b prevented ISO mediated down-regulation of α MyHC and SERCA, up-regulation of β MyHC, and reduced up-regulation of BNP. Over-expression of miR-133b also resulted in repression of skeletal α -actin and β MyHC with no changes in BNP and ANF expression. These results suggest that changes in miR-133b expression may have an important role in the regulation of the fetal gene program similar to the ones presented by Care et al (6),

a study demonstrating that up-regulation of miR-133 prevented or reduced α -adrenergic induction of hypertrophic gene program. These findings suggest that miR-133 family may be a global regulator of gene expression in cardiac disease. In fact, down-regulation of miR-133 in mouse hearts resulted in increased left ventricle/body weight, diastolic ventricle wall thickness, and septum thickness.

3.3 miR-133b and cellular hypertrophy

Since inhibition of miR-133b induced expression of the fetal isoforms BNP, skeletal α -actin and ANF, and over-expression of miR-133b blocked ISO-mediated activation of the fetal gene program, we tested the effect of over-expression or inhibition of miR-133b in cellular hypertrophy. NRVMs were transfected with mimic and inhibitors, as described above, and stained with an anti α -actinin antibody. Cell surface area was quantified using Image J program (NIH). As shown in Figure 3, down-regulation of miR-133b induced an increase in cell size while over-expression of miR-133b dramatically reduced ISO-mediated increases in cardiomyocyte cell size. Again, these results suggest that miR-133b may be a global regulator of cardiomyocyte hypertrophy.

4. Summary

miR-expression was examined in two models of human heart failure, IDC and ISC. These results herein demonstrate that subsets of miRNAs are differentially regulated in each of these disease state etiologies. More importantly, each etiology demonstrated dysregulation of unique sets of miRNAs, a finding generally supportive of the recent report of Ikeda et al (3). Of the miRNAs analyzed in greater depth in the current study, miR-92 and miR-100 function had not been previously determined to be relevant to hypertrophic gene expression. Here we showed that down-regulation of miR-92 has a detectable but relatively minimal effect in regulating the hypertrophic gene program. This result suggests that the role of miRNAs in heart disease is specific and not all miRNAs affect global aspects of the disease. The current results also suggest that miR-100 has a specific role in the gene regulation of the adult isoforms of cardiac genes. This is of particular interest given that, to date, it is not clear which specific factors promote down-regulation of α MyHC and SERCA. It will be important to identify candidate targets for miR-100. Finally, miR-133b, which is part of a highly conserved family consisting of miR-133a-1 and miR-133a-2, appears to have an important function in overall regulation of hypertrophic gene program. In this context, members of the Rho subfamily of small GTP-binding proteins are regulated by miR-133. It will be interesting to determine if preventing up-regulation of these proteins in cardiac myocytes is sufficient to prevent β -adrenergic mediated gene expression changes. In summary, differential regulation of miRNAs is an important mechanism regulating human heart muscle disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1A

Figure 1.

miRNA expression profiles in samples obtained from non-failing (NF), idiopathic cardiomyopathy (IDC) and ischemic (ISC) patients. (A) Relative expression of miRNAs is expressed at the log base 2 ratio of failing (F)/nonfailing (NF). NF vs. IDC (red), NF vs. ISC (blue). Only miRNAs with a p-value < 0.10 as determined by t-Test are shown. (B) Using miRNA specific ABI primers, the relative expression of a subset of miRNAs was confirmed by RT-PCR, as described in Methods.

Figure 2A

Figure 2.

Over-expression or down-regulation of a subset of miRNAs in NRVMs regulates β -AR changes in gene expression. (A) Changes in miR-92, miR-133b and miR-100 abundance upon transfection with microRNA mimics or microRNA inhibitors. miRNA abundance was determined by RT-PCR. (B-D) Changes in fetal gene expression in NRVMs transfected with microRNA mimic or microRNA inhibitors. Cells were treated with 10^{-7} M ISO (black bars) 24 hours after transfection and harvested 48 hours after treatment. Gene expression was measured by RT-PCR. Results were normalized to 18S rRNA expression levels and were compared to cells transfected with a control mimic or inhibitor, defined as 1 (line at 1). The graphs represent an average of 3-4 individual experiments.

Figure 3A

Figure 3.

Over-expression or down-regulation of miRNA-133b in NRVMs regulates cellular hypertrophy. Cells were transfected with miR-133b mimic or inhibitors and cell surface area was quantified. (A) Immunofluorescence with anti-actinin antibody. (B) Cell size was measured using the Image J software. A total of 30 cells from 3 different fields were measured.

Table 1
Cardiac performance (Ejection Fraction, %) in each group at the time of cardiac explantation, as determined by echocardiography.*

NF		IDC		ISC	
X	SEM	X	SEM	X	SEM
64	± 4.3	12	± 3.1	18.6	± 3.5

* At time of cardiac explantation, patients were on a number of pharmacological agents including: ACE inhibitors, diuretics, beta-blockers, digitalis, anticoagulants, insulin, beta-agonists, nitrates, etc.

RT-PCR Primers used to quantify miRNAs.

α MyHC F	CCTGTCCAGCAGAAAGAGC
α MyHC R	CAGGCAAAGTCAAGCATTCATATTTATTGTG
18S F	GCCGCTAGAGGTGAAATTCCTG
18S R	CTTTCGCTCTGGTCCGTCTT
BNP F	GGTGCTGCCCCAGATGATT
BNP R	CTGGAGACTGGCTAGGACTTC
SERCA F	GGCCAGATCGCGCTACA
SERCA R	GGGCAATTAGAGAGCAGGTTT
Sk α -actin F	CCACCTACAACAGCATCATGAAGT
Sk α -actin R	GACATGACGTTGTTGGCGTACA
β MyHC F	CGCTCAGTCATGGCGGAT
β MyHC R	GCCCCAATGCAGCCAT
ANF F	GCGAAGGTCAAGCTGCTT
ANF R	CTGGGCTCCAATCCTGTCAAT

Sequence of the primers used for the RT-PCR reaction. All primers are presented in a 5'-3' orientation.