Unique MicroRNA Profile in End-stage Heart Failure Indicates Alterations in Specific Cardiovascular Signaling Networks*

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It is well established that gene expression patterns are substantially altered in cardiac hypertrophy and heart failure, but the reasons for such differences are not clear. MicroRNAs (miRNAs) are short noncoding RNAs that provide a novel mechanism for gene regulation. The goal of this study was to comprehensively test for alterations in miRNA expression using human heart failure samples with an aim to build signaling pathway significantly perturbed neurohormonal and mechanical (hemodynamic) stimuli to the heart. The altered pathological signaling leads to remodeling of the heart with adaptive to mal-adaptive hypertrophy transitioning into diluted cardiomyopathy (DCM). DCM is the most common and well documented outcome of various deleterious stimuli the heart perceives (1). DCM is characterized clinically by left ventricular dilatation.

Heart failure has been classified as an epidemic of the 21st century and is now the major cause of morbidity in the elderly in the United States. End-stage heart failure is characterized by sequence-specific manner (3–5). MicroRNAs regulate gene expression by binding to mRNAs with the consequence of mRNA degradation or translational inhibition of targeted transcripts (3–5). Initial studies of miRNAs in animal models of hypertrophy and heart failure showed specific signature patterns suggesting that they could be used as valuable biomarkers for disease (6), but the high sequence conservation of miRNAs across the metazoan species suggests strong evolutionary pressure for potential regulatory roles in complex biologic processes (3, 7). Indeed, recent studies have documented the regulatory role of miRNAs in DCM and cardiac development in mice (8, 9). Studies by Srivastava and co-workers (10) using miRNA 1 knock-out mice have shown that miRNA 1 plays a

This article has been withdrawn by the authors. Evaluation by the Journal with image analysis software determined that in Fig. 5A, lanes 1–3 of the RB1 immunoblot were duplicated in lanes 4–6, lane 4 of the ERBB2 immunoblot was duplicated in lane 6, lane 5 of the STAT3 immunoblot was duplicated in lane 8, and lanes 1–3 of the actin immunoblot were flipped horizontally and reused in lanes 6–8. The authors state that RB1, ERBB2, STAT3, and actin in Fig. 5A were created from the phosphor-chemiluminescent digital imaging. The authors also state that they have replicate data supporting the conclusions of Fig. 5, A and B. In Fig. 6B, evaluation by the Journal of the original data determined that single cell background fluorescence was duplicated. The authors maintain that the concern is about a single background cell not a positive cell to show transfection efficiency and is inconsequential to proving transfection.

expression of the myriad proteins in heart failure and could be potential therapeutic targets.

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§ The abbreviations used are: DCM, dilated cardiomyopathy; miRNA, microRNA; TAC, transverse aortic constriction; IPA, Ingenuity Pathways Analysis; RT-PCR, real time PCR; HDGF, hepatoma-derived growth factor.

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critical role in cardiac development. Importantly, studies from Olson and co-workers (11) and Condorelli and co-workers (12) have shown in mice that miRNAs play a critical role in development and/or progression toward heart failure.

The targeting sequence of miRNA theoretically allows for binding of miRNAs to many gene products ultimately regulating the expression of target genes. Therefore, miRNAs could potentially play a significant role in global regulation of signaling networks during remodeling and transition to heart failure. Despite the potential role of miRNAs in targeting many genes, previous studies on miRNA targets in the cardiovascular system have been limited to analyzing specific gene products. Importantly, the appreciation that miRNAs could play a global role in regulating cardiac function and heart failure was recently elucidated by cardiac specific knock-out of dicer, an endonuclease critical for maturation of miRNAs (13). Absence of dicer in the heart leads to loss of mature miRNAs leading to significant DCM and heart failure suggesting a pivotal role for miRNA in globally regulating cardiac function. Considering the global regulatory role of miRNAs, previous cardiac miRNA studies are potentially limited as many of the targets could be concomitantly regulated in vivo. Furthermore, a single gene product may be a target for multiple miRNAs adding complexity in the regulatory process that cannot be appreciated by focusing on the effects of specific miRNA on specific genes.

This study was designed to elucidate the global regulation of the signaling networks by a unique miRNA pattern in human heart failure. Therefore, the goals of this study follow: (a) to measure the changes in expression of miRNAs using a microarray hybridization platform; (b) to measure the changes in expression of 288 human miRNAs using a microarray hybridization platform to validate by in silico analysis and by resolving the isolated samples on a denaturing formaldehyde gel; (c) to measure the changes in expression of miRNAs using a microarray hybridization platform; (d) to measure the changes in expression of miRNAs using a microarray hybridization platform; (e) to measure the changes in expression of miRNAs using a microarray hybridization platform.

EXPERIMENTAL PROCEDURES

Patient Samples—Tissue from the left ventricular free wall was obtained from explanted hearts of transplant recipients at the Cleveland Clinic with a diagnosis of DCM. The nonfailing control hearts were obtained from unmatched donors whose hearts were not suitable for transplantation despite normal ventricular structure and function as measured by echocardiography. The hearts were arrested and transported in ice-cold, oxygenated cardioplegic solution (14). Once in the laboratory the tissue was flash-frozen in liquid N₂ and stored at −80 °C. All protocols for tissue procurement and procedures carried out on the tissues were in performed in compliance with institutional guidelines for human research and approved by the Cleveland Clinic Institutional Review Board.

RNA Isolation—RNA was isolated as described previously (14). Briefly, 100 mg of left ventricular tissue was homogenized using TRizol reagent (Invitrogen), and the homogenized samples were incubated at room temperature for 5 min. Chloroform was added to the samples, vigorously mixed, and incubated at room temperature for 5 min. Following incubation, the samples were centrifuged at 12,000 × g for 15 min at 4 °C. RNA was precipitated from the aqueous phase by addition of isopro- pyl alcohol to a fresh tube containing the supernatant aqueous phase. The integrity of the RNA was tested by spectrophotometric analysis and by resolving the isolated samples on a denaturing formaldehyde gel.

Target Preparation and Array Hybridization—Target preparation and array hybridization were carried out as described previously (15). Briefly, 5 µg of total RNA was added to biotinylated oligonucleotide primer. Following incubation, the first strand was synthesized using Superscript II RNase H⁻ reverse transcriptase. After synthesis of the first strand, the reaction was incubated at 65 °C to denature the RNA/DNA hybrids and degrade RNA, and then labeled targets were then used for chip hybridization. The hybridization was carried out on the Mouse Comprehensive Cancer Centers Network (MCCCN) microarray platform. Biotin-labeled probes for mature miRNA corresponding to most precursor miRNAs were spotted in quadruplicate. Often, more than one probe set is present for a given potential targets of miRNAs in the global picture of the molecular network. Therefore could be used to evaluate canonical/functional pathways. We have used results of predicated targets recently released TargetScan 4.2 did not significantly alter the set pattern of target nodal molecules identified in this study. The need for analysis of patient samples to demonstrate inverse correlation between protein expression and miRNA level. By using this systems biology approach, multiple genes and pathways involved in the complex pathology of heart failure have been visualized simultaneously leading to identification of potential novel therapeutic targets.

Computational Analysis of miRNA Microarray Data and miRNA Target Prediction—Microarray images were analyzed by GenePix Pro. Average values of the replicate spots for each miRNA were background-subtracted, normalized, and subjected to further analysis. Global median normalization and Lowess normalization of the heart microarray data were carried out using BRB ArrayTools (15). The probes with >70% missing data were excluded from further analysis. Differentially expressed miRNAs between control and dilated cardiomyopathic samples were identified by using t test procedure within significance analysis of microarrays. Furthermore, Lowess normalization was carried out to analyze differentially expressed miRNAs in control versus diseased state. Following the identification of differentially expressing miRNAs, the predicted targets for these differentially expressed miRNAs were identified using TargetScan 3.1 and Pictar data bases (16–18). Use of the recently released TargetScan 4.2 did not significantly alter the nodal molecules identified in this study. The need for analysis using a variety of data bases was based on the need to encompass all the potential targets as they are built using slightly different algorithms. We have used results of predicated targets from TargetScan data base to carry out pathways and network analysis.

Network Analysis—A data set containing genes and the corresponding expression values was uploaded into the Ingenuity Pathways Analysis Network™ application. The dataset mole-
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Cell Culture, miRNA/Antagomir Sequences Used in Construct Generation, Transfection, and Northern Blot Analysis—Cell culture and transfection studies were carried out as described previously (1). Briefly, HEK 293 cells will be maintained in minimal essential medium supplemented with 10% fetal bovine serum and penicillin/streptomycin at 37 °C. Cells transfected at 60–70% confluence using FuGENE 6 (Roche Applied Science). Pre-miRNA 7 (5'-TTGATATGTTGGCCTAGTTCTGTGTGGAAGACTAGTGATTTTGTTGTTTTTATC-3'), Caspase 3 (5'-GTTCTGTGTGGAAGACTAGTGATTTTGTTGTTTTTATC-3'), and control sequences were used and detection carried out using enhanced chemiluminescence (ECL, Amersham Biosciences).

Cardiac Microarray Expression Data Base—To test for changes in expression in the target gene sets, microarray data from cardio-genomics data base for normal and idiopathic heart failure were used. The raw data were reprocessed using the GCRMA algorithm, which is a three-step function implemented in the GCRMA package (version 2.8.1) of the Bio-conductor open source library (version 2.5.0). Steps include correction of perfect match probe set expression signals for optical noise and nonspecific binding using probe sequence information, followed by quantile normalization to smooth individual probes intensities. Finally, expression values were summarized by the robust multichip model fit using median polish. The summarized probe set expression values were subsequently fit to a linear correlation analysis model.

Western Immunoblotting on Patient Samples—End-stage human heart failure samples (100 mg) were homogenized using Polytron homogenizer in 1.5 ml of lysis buffer (1% Nonidet P-40, 10% glycerol, 137 mM NaCl, 20 mM Tris-Cl (pH 7.4), 1 mM phenylmethylsulfonyl fluoride, 20 mM NaF, 1 mM sodium pyrophosphate, 1 mM sodium orthovanadate, and 2 μg/ml each of aprotinin and leupeptin) and centrifuged at 38,000 × g for 25 min. The supernatant myocardial lysates (180 μg) were resolved on SDS-polyacrylamide gel and immunoblotted with various primary antibodies, anti-RB1 (1:250, BD Biosciences), anti-ERBB2 (1:500, Santa Cruz Biotechnology), anti-STAT3 (1:1000, Santa Cruz Biotechnology), anti-NFκB (1:500, Santa Cruz Biotechnology), anti-HDAC4 (1:300, GenScript), anti-COL1A1 (1:250, BIORAD), anti-MMP2 (1:250, BIOMOL International), anti-MMP3 (1:1000, Abcam), anti-EZH2 (1:1000, Abcam), anti-β-actin (1:200, Santa Cruz Biotechnology), anti-Villin (1:200, Santa Cruz Biotechnology), anti-PCNA (1:500, Novus Biologicals), and anti-BCL2 (1:200). Appropriate secondary antibody (1:2000) was used and detection carried out using enhanced chemiluminescence technology (ECL, Amersham Biosciences).

Canonical Pathway and Biological Functions—Canonical Pathway and Biological Functions were analyzed using the Ingenuity Pathways Knowledge Base. Differentially regulated genes were mapped to the Ingenuity Pathways Knowledge Base using an internal software tool and compared to known biological functions in the Ingenuity Pathways Knowledge Base. The degree of association of a gene to a biological function was calculated using the right-tailed Fisher’s Exact Test and is represented as a negative log of this value determining the probability that the association between the nodes (i.e. p value) and is represented as a negative log of thisvalue. For example, a network of 35 molecules has a Fisher’s Exact Test p value of 1 × 10−8, the network score = −log(p value) = 6 (19).

Transverse Aortic Constriction (TAC)—The TAC was carried out on mice as described previously (20). Briefly, mice were anesthetized with a mixture of ketamine and xylazine. After endotracheal intubation, mice were connected to a rodent ventilator. Using microsurgical procedures, the chest cavity was entered in the second intercostal space, and the transverse aorta between the right (proximal) and left (distal) carotid arteries was isolated. TAC was performed by tying a 7-0 nylon suture ligature against a 27-gauge needle, the latter being promptly removed to induce pressure overload cardiac hypertrophy. After aortic constriction, the chest was closed; the pneumothorax was evacuated, and the mice were extubated and allowed to recover from the anesthesia. Sham-operated animals underwent the same operation without aortic constriction. After 0, 1, 4, 7, and 12 days of aortic constriction, mice were anesthetized, and hearts were rapidly excised. The individual chambers were separated and frozen in liquid N2 for miRNA and biochemical analysis.

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GATAAATAATCGACACAAATAACAGTCTGCCATA-TGGACACAGGCCATGCTCTACAG-3'), antagonir for
miRNA 7 (5'-AACCTACAACCGGATCAGACACCTT-
CTGATCATAAAACACAACAAATTATTTAGTTAC-
GGTGGTTTCTGTCAGACGCTATCCGCTGCTGCTG-
CGGAGATGTCC-3'), pre-miRNA 378 (5'-AGGCCTCTGAG-
TCCTCAGGCTGTGTTAATCTAGAATAGCTGCAG-
TGGAGCTGAAAGCT-3'), antimir for mirRNA 378 (5'-TCCCGAGGAGCTGAGTCCAGACACAAAG-
GATCPITATACGTGCAGCCTTAGCTAGCTCTGGG-
CCTTACGCTGCTCTAGCTCTCTCTCCGGAG-3'), pre-miRNA 214 (5'-GGCCCTGCTGCAAGAGTATAG-
GATCTTTATCGTGACCTGAACCTCAGTCTTCCGGA-
/H11032), antagomir for miRNA 214

**RESULTS**

**Patient Population**—To get a global expression pattern for the
miRNAs in end-stage heart failure, mRNA was isolated
from 70 patient samples (nonfailing (20) and end-stage heart
failure (50) with the diagnosis of DCM). The patient character-
istics are summarized in Table 1. All failing hearts had left
ventricular ejection fractions <15%, and all the nonfailing
had ejection fraction >61% (Table 1). Mean age for the
patients with DCM was 51 ± 2 years and for nonfailing was
54 ± 1 years. The age of the patients was 52.5 ± 3
years and was not race-independent. They all had rela-
tively normal function as measured by echo-
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**TABLE 1**

| Patient demographics |
|----------------------|
| The abbreviations used for nonfailing hearts are as follows: F, female; M, male; W, white; B, black; EF, left ventricular ejection fraction measured prior to explant; CVA, cerebrovascular accident; MVA, motor vehicle accident; GSW, gunshot wound. Drug therapy acute indicates treatment in the emergency room or intensive care unit prior to brain death: NE, norepinephrine (n = 9); DA, dopamine (n = 12); other, epinephrine, pitressin, phenylephrine, labetolol, lisinopril (n = 1 or 2). Drug therapy chronic, indicates drugs taken by patients prior to admission, as reported by family members (n = 6). The abbreviations used for failing hearts are as follows: F, female; M, male; W, white; B, black; EF, left ventricular ejection fraction measured prior to explant; DCM, dilated cardiomyopathy (pre-transplant diagnosis). Drug therapy lists those drugs taken by over 25% of patients in the group as follows: DIG, digoxin; DOB, dobutamine; AMIO, amiodarone; ACEI, angiotensin-converting enzyme inhibitor (usually lisinopril); BB, β-adrenergic blocker (metoprolol or carvedilol). |

| Age | Sex | Race | EF | Cause of death | Drug therapy |
|-----|-----|------|----|----------------|--------------|
| Nonfailing hearts (n = 20) | 51 ± 2 | 11F, 9 M | 19 W, 1 B | 61 ± 2 | 14 CVA, 2 MVA, 1 GSW, 1 trauma, 1 anoxia | Acute, NE, DA, other chronic “HTN meds” |
| Failing hearts (n = 50) | 54 ± 1 | 17 F, 33 M | 42 W, 8 B | 15 ± 1 | 50 DCM | DIG, DOB, AMIO ACEI, BB |

The abbreviations used for nonfailing hearts are as follows: F, female; M, male; W, white; B, black; EF, left ventricular ejection fraction measured prior to explant; CVA, cerebrovascular accident; MVA, motor vehicle accident; GSW, gunshot wound. Drug therapy acute indicates treatment in the emergency room or intensive care unit prior to brain death: NE, norepinephrine (n = 9); DA, dopamine (n = 12); other, epinephrine, pitressin, phenylephrine, labetolol, lisinopril (n = 1 or 2). Drug therapy chronic, indicates drugs taken by patients prior to admission, as reported by family members (n = 6). The abbreviations used for failing hearts are as follows: F, female; M, male; W, white; B, black; EF, left ventricular ejection fraction measured prior to explant; DCM, dilated cardiomyopathy (pre-transplant diagnosis). Drug therapy lists those drugs taken by over 25% of patients in the group as follows: DIG, digoxin; DOB, dobutamine; AMIO, amiodarone; ACEI, angiotensin-converting enzyme inhibitor (usually lisinopril); BB, β-adrenergic blocker (metoprolol or carvedilol).
heat map clearly demonstrates a distinctive pattern for specific miRNA expression associated with DCM.

To test whether the same set of miRNAs can be validated, we used a new independent set of patient samples (10 nonfailing and 20 DCM). To validate these miRNAs, RNA from a new set of samples was isolated and subjected to RT-PCR using specific primers for the set of miRNAs. As internal control we used both 18S and U6. Our studies found that U6 gave more consistent results compared with 18S, and therefore the miRNA data has been normalized to U6 values. RT-PCR from the new set of human patient samples showed that the differential expression of all the identified miRNAs could be validated except hsa-mir-145. To further confirm these results, we also carried out RT-PCR on the RNA from the samples that were first used for the miRNA microarray. Consistently, these studies also showed that differential expression of miRNAs could be validated with the exception of hsa-mir-145. The RT-PCR data from the two independent sets of human samples consistently showed the same results, and therefore it was appropriate to pool the data (20 nonfailing and 50 dilated cardiomyopathy human patient samples) from the two analyses (Fig. 3). The results from the pooled data show significant down-regulation of hsa-miRNA 1 (p < 0.00001), 29b (p < 0.00002), 7 (p < 0.00007), 378 (p < 0.001), and up-regulation of hsa-miRNA 214 (p < 0.0005), 342 (p < 0.003), 125b (p < 0.01), and 181b (p < 0.005) validating the microarray studies (Fig. 3). Critically, hsa-miRNA 145 showed a trend toward higher expression in DCM but did not reach a level of significance compared with nonfailing samples (Fig. 3g). Taken together, our data demonstrate that end-stage DCM has a specific miRNA signature that can be consistently revalidated in a large number of samples. Importantly, our studies have identified novel miRNAs, hsa-miRNA 7 and hsa-miRNA 378 which are significantly down-regulated in DCM, that may have critical roles in the pathophysiology of heart failure.

Altered miRNAs Are Significantly Associated with Specific Canonical and Functional Pathways—Since alterations in these miRNAs occur simultaneously and modulate their respective targets, the global effect would be a sum total of effects coordinated by individual miRNAs. A bioinformatic view of the global effects potentially mediated by the eight altered miRNAs on signaling in dilated cardiomyopathy is shown in supplemental Fig. 1. Analysis shows that a total of 1785 genes are predicted targets for the eight differentially expressed miRNAs in DCM. To evaluate potential functional consequences based on combinatorial effects of the predicted targets of the miRNAs, an unbiased computational approach (see “Experimental Procedures”) was taken using Ingenuity Pathway Knowledge base. Cardiovascular system development and function was the top canonical functional network with the highest level of significance (score 39). In comparison, significance score for nearest functional networks, cell development (score 35), cellular assembly (score 35), and cell death (score 33) was lower. Identification of this network function as potentially affected is entirely consistent with a role of the predicated targets of the altered miRNAs in cardiovascular disease. Cellular and molecular functions influenced by these network-eligible molecules, cell signaling (368 molecules, p < 5.91e-146), gene
expression (254 molecules, \( p < 1.67 \times 10^{-126} \)), and cell death (245 molecules, \( p < 4.55 \times 10^{-93} \)), are highly significant and consistent with the documented dysregulation of these events and pathways in DCM.

**Predicted Targets of Altered miRNAs Associate with Diverse Signaling Networks**—Of a total of 1785 predicted targets, 1716 could be mapped to signaling networks (see under “Experimental Procedures” for definition of network) in the IPATM, and 995 predicted targets were found to be network-eligible (see “Experimental Procedures” for definition of eligibility). The 995 network-eligible candidates mapped to 43 networks that are predicted to be involved in the cross-talk with the peripheral molecules bridging different networks (supplemental Fig. 1) resulting in the phenotype.

A representative network with NFκB, a known mediator in cardiac dysfunction (21) as a central node, is shown in Fig. 4 wherein the members that network with NFκB are targets for the miRNAs 1, 29b, 125b, 181b, 214, 342, and 378. As individual miRNA acts on each target, the net effect on the node would be the collective influence of all the members connected to the central node, NFκB (Fig. 4). Based on this consideration, we predict that the complete NFκB regulatory signaling network (Fig. 4) would be significantly down-regulated in DCM since molecules in this network are predicted targets for up-regulated miRNAs 125b, 181b, 214, and 243. Although Fig. 4 predicts one specific network with NFκB as a central node for various miRNA, each network does not affect a physiological process in isolation, and global regulation would involve integrative cross-talk among the networks to mediate the disease phenotype. To test for such a cross-talk between networks, we have used Ingenuity Pathways Analysis algorithm for overlaying and merging networking (supplemental Fig. 1). The analysis of networks involved in top molecular and cellular functions associated with DCM showed that out of the 75 networks, only 43 are predicted to be involved in the cross-talk with the peripheral molecules bridging different networks (supplemental Fig. 1). A representation of the merged networks (supplemental Fig. 2) predicts that the pathways are specific for DCM; 32 networks (supplemental Fig. 1) were not incorporated into the network merge, suggesting they are not involved in cross-talk. Taken together this iterative analysis indicates that a specific set of pathways are operational, which are associated with integrative connecting networks resulting in the manifestation of DCM phenotype.

We next analyzed whether nodes (see “Experimental Procedures”) of the various networks, which are interconnected and over-represented in DCM, are predicted targets for the differentially expressed miRNAs in end-stage heart failure. IPA-predicted nodal molecules on the various merged networks were analyzed for miRNA targets using target prediction algorithms (see “Experimental Procedures”). This analysis shows that a significant number of nodal molecules are predicted targets of miRNA that are altered in DCM (Table 2). Indeed, several of the...
predicted molecules are potential targets for two or three miRNAs, and some are targets for none. We analyzed whether these nodal molecules are altered in end-stage human dilated cardiomyopathy using the cardio-genomics expression data base (see “Experimental Procedures”). Many of the nodal molecules are significantly altered in end-stage heart failure (Table 3) consistent with prediction, thus relating the regulation of predicted targets by altered miRNAs in dilated cardiomyopathy. We have mapped the changes in the expression pattern of the nodal molecules to changes in miRNAs (Table 3, green and red represent down- and up-regulation, respectively). Indeed, the data show reverse complementary alterations in the levels of many of the predicted targets compared with our validated miRNA expression profile, e.g. MLL and STAT3 (Table 3). Interestingly, we also see parallel alterations for some nodal molecules and miRNAs, e.g. MMPs and TIMP2 (Table 3), and in some cases no effect on the nodal molecules, e.g. RB1 and EZH2 (Table 3), suggesting that the miRNA is a component of the complex regulation of signaling during pathophysiology of heart failure.

Analysis and Validation of the miRNA Targets and Network—
To directly evaluate whether nodal molecules are potential targets for altered miRNAs, we carried out Western immunoblotting studies in our collection of end-stage human heart failure samples. If the nodal molecules are targets for miRNAs, we would expect an inverse correlation between expression levels of nodal molecule to altered miRNA. Indeed Western analysis of some of the nodal molecules well known to have a role in heart failure shows an inverse co-relationship with the level of respective miRNAs (Fig. 5) and is very consistent with the data presented in Table 3. Western immunoblotting shows that ERBB2, HDAC4, COL1, MMP2, and TIMP2 are significantly up-regulated in human DCM (Fig. 5) and are inversely correlated to the down-regulation of their respective miRNAs. In
contrast, STAT3 and E2F3 are down-regulated, consistent with the observation of up-regulation of their respective miRNAs suggesting that expression of these molecules may be regulated by miRNAs. Interestingly, we did not observe changes in expression levels of RB1 or EZH2 (Fig. 5) despite being predicted targets for miRNAs consistent with our data in Table 3.

An important question arising from these studies is whether these miRNAs are the “cause” or an “effect” of heart failure. Since it is not possible to identify alterations in miRNA profile with initiation of cardiac dysfunction in humans, we have used a well known surgical mouse model of transverse aortic constriction (TAC) (20) to analyze alterations in eight miRNAs (identified in end-stage heart failure) with initiation of cardiac dysfunction. Mice (C57Bl/6) underwent TAC surgery, and the experiments were terminated on 0, 1–4, 7, and 12 days post-TAC. RNA isolated from the mouse hearts was subjected to RT-PCR to evaluate the alteration in the eight identified miRNAs. RT-PCR analysis showed no specific trends within 7 days of TAC except for miRNA 7, which consistently showed...
down-regulation and reached significance by 7 days (Fig. 6a). Importantly, 12 days post-TAC (a time point known from our previous studies to be characterized by initial cardiac dysfunction associated with cardiac hypertrophy (20)), significant alteration in a subset of four miRNAs (miRNA 7, 378, 214, and 181b) is observed (Fig. 6a) out of the eight altered miRNAs that in end-stage heart failure (Fig. 3). miRNA 7 was significantly down-regulated, and miRNAs 214 and 378 (out of eight miRNAs identified at end-stage heart failure) are very consistent with our findings in Table 3 depicting no change in human RB1 levels despite significant changes in miRNA levels. Taken together these data provide evidence that miRNAs may play an integral role in regulation of nodal molecules, including RB1, which is not a target of either miRNA 214 or 378. Although CLCF1 is a predicted target of miRNA 378, its expression pattern did not inversely correlate with initiation of cardiac stress resulting in modulation of NFκB, which is not a target of either miRNA 214 or 378. Although CLCF1 is a predicted target of miRNA 378, its expression pattern did not inversely correlate with miRNA expression profile indicating that it may be regulated by mechanism(s) independent of miRNA 378. Furthermore, none of the predicted targets for miRNA 181b were altered in the NFκB network.

To test whether NFκB is altered due to cross-talk in the signaling network, we transfected HEK cells with constructs expressing either miRNA 214 and 378 or miRNA 214 and antagonim of miRNA 378. Seventy two hours following transfection, cells were lysed, and Western immunoblotting was carried out. Immunoblotting showed an appreciable reduction of NFκB levels in cells expressing miRNA 214 along with antagonim for 378 compared with cells expressing miRNA 214 and 378 (Fig. 7a). To further identify which of the molecules feed into the alteration in NFκB, immunoblotting was carried out for HDGF, BCL2, and SLC2A, which are predicted targets for miRNA 214 and 378. Immunoblotting for HDGF and BCL2 did not show any appreciable changes in cells expressing miRNA 214 and 378 or miRNA 214 and antagonim 378 compared with the cells transfected with scrambled control (Fig. 7a). Interestingly, marked up-regulation of SLC2A was

To validate whether a combination of miRNAs/antagomirs will regulate the NFκB signaling network (depicted in Fig. 4), we assessed the predicted targets of the four altered miRNAs (Fig. 7a). Importantly, 12 days post-TAC (a time point known from our previous studies to be characterized by initial cardiac dysfunction associated with cardiac hypertrophy (20)), significant alteration in a subset of four miRNAs (miRNA 7, 378, 214, and 181b) is observed (Fig. 6a) out of the eight altered miRNAs that in end-stage heart failure (Fig. 3). miRNA 7 was significantly down-regulated, and miRNAs 214 and 378 (out of eight miRNAs identified at end-stage heart failure) are very consistent with our findings in Table 3 depicting no change in human RB1 levels despite significant changes in miRNA levels. Taken together these data provide evidence that miRNAs may play an integral role in regulation of nodal molecules, including RB1, which is not a target of either miRNA 214 or 378. Although CLCF1 is a predicted target of miRNA 378, its expression pattern did not inversely correlate with miRNA expression profile indicating that it may be regulated by mechanism(s) independent of miRNA 378. Furthermore, none of the predicted targets for miRNA 181b were altered in the NFκB network.

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observed in cells expressing miRNA 214 and antagomir 378 compared with cells expressing miRNA 214 and 378 (Fig. 7a) suggesting that NFκB may be regulated by input from SLC2A following perturbation of miRNA 378. To test whether such regulation exists in the NFκB network in hearts, we used 12-day-old TAC mice, which are characterized by up-regulation of miRNA 214 and down-regulation of miRNA 378. The ventricular lysates from 0-day (sham) and 12-day-old TAC were resolved on SDS-polyacrylamide gel and immunoblotted for NFκB. Marked reduction in NFκB was observed in the 12-day-old TAC mice compared with the control sham (Fig. 7b). There were no changes in BCL2 levels (Fig. 7b) but significant up-regulation in HDGF was observed following 12 days of TAC. The up-regulation of HDGF in TAC seems to be independent of miRNA 214 because HDGF is a predicted target for miRNA 214 and miRNA 214 is up-regulated post-TAC (Fig. 6a). Importantly, SLC2A is significantly up-regulated in TAC mice compared with the controls consistent with our results of down-regulation in miRNA 378 (Fig. 6a) following TAC. Taken together, these studies demonstrate that miRNAs do mediate regulation of signaling network, and more specifically the NFκB as shown in our studies may be modulated by single or multiple target proteins. In our studies, both in the heart as well as in transfected cells, miRNA 378 perturbation seems to modulate the nodal molecule, NFκB, by directly altering peripheral molecules that feed into the NFκB network as predicted by IPA network analysis.

**DISCUSSION**

The most important finding from our studies is identification of a unique miRNA fingerprint in human dilated cardiomyopathy empowering us to define the global molecular signaling...
these altered miRNAs enabled unbiased prediction of molecular cardiovascular disease bio-function in understanding heart failure biology within the Ingenuity™ knowledge compendium. The unique miRNA signature leads us to identify nodal molecules on the global signaling networks that are potential targets for miRNAs laying the foundation for us to propose for the first time the global regulatory role of miRNAs in modulating molecular networks. Furthermore, we have validated the expression pattern of nodal molecules through a meta-analysis of heart failure gene expression fingerprint from the publicly available cardio-genomics data base. Nodal molecules occupy key positions in the signaling networks, and alterations of these by combinatorial action of miRNAs could account for the observed global changes associated with cardiac dysfunction and failure. We have further used TAC mouse model of heart failure and transfected cells to demonstrate the regulation of NFκB nodal molecules and the inputs into this signaling network.

Our miRNA microarray and RT-PCR validation on independent sets of human cardiac samples consistently identified eight miRNAs differentially expressed in human dilated cardiomyopathy. Recently, along with identification of miRNAs relatively large set of human samples lead to identification of these by combinatorial action of miRNAs could account for the observed global changes associated with cardiac dysfunction and failure. We have further used TAC mouse model of heart failure and transfected cells to demonstrate the regulation of NFκB nodal molecules and the inputs into this signaling network.

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MicroRNA and Cardiovascular Signaling Networks

RB1. Importantly, analysis of some of the predicated targets of miRNA 7 like phospholipase Cβ, regulator of G-protein signaling, RAF1, and phosphodiesterase 4, which are well studied in heart failure (24–26), are all up-regulated in cardiac hypertrophy and heart failure (24–26) suggesting an important role for miRNA 7. Studies on neuronal development have shown that miRNA 7 targets basic helix-loop-helix and Brd box (27, 28) containing transcriptional repressor proteins that interpret Notch signaling. Whether such regulation exists in the cardiac system needs further elucidation. Similarly, transforming growth factor β, v-SRC, and platelet-derived growth factor receptor are some of the potential targets of miRNA 378. These molecules (24–26) are also dysregulated in cardiac hypertrophy and heart failure indicating potential regulation by miRNA 378. Recent studies in cancer have shown that expression of miRNA 378 enhances cell survival and promotes tumor growth and angiogenesis (29). What effect the down-regulation of these novel miRNA 7 and 378 will have in the pathology of heart failure is currently an active area of our study.

The miRNA-mediated regulation of gene expression within the myocardium is perhaps the most intriguing and novel mechanism in heart failure biology. Although several studies have begun focusing on miRNA in heart failure, genetic changes and miRNA-mediated critical transitions in progression of heart failure remain undefined. The miRNAs regulate gene expression primarily through repression, and the expressed miRNAs combinatorially delineate precise regulatory actions. Thus, combinatorial expression patterns in heart failure provide clear evidence showing that inhibition of NFκB, v-SRC, and platelet-derived growth factor receptor are some of the potential targets of miRNA 378. These studies indicate that miRNAs have a prominent role to play in altering global signaling networks and pathways in progression toward cardiac hypertrophy and failure. Interestingly, data for our TAC mice examined potential regulation of specific signaling networks by miRNA 214, we would have seen a marked inhibition in NFκB target genes. In the event that HDGF was regulated by miRNA 214, we cannot exclude the possibility that it may not be playing a role in the miRNA-mediated regulation of nodal molecule. Although HDGF may not be regulating NFκB by an miRNA-dependent mechanism, we cannot exclude the possibility that HDGF regulates NFκB independent of miRNA, which is not the focus of this study.

Despite the elucidation of several clinically relevant signal transduction pathways that can lead to heart disease progression, the means by which these pathways are coordinated with respect to the development of cardiac dysfunction remain obscure. Manifestation of the phenotype of dilated cardiomyopathy is the net result of traditional cross-talk between the molecules and the nontraditional regulation by miRNAs. Inclusion of all the predicted targets for these altered miRNAs showed that they represent 75 annotated networks out of which 44 networks could be merged to cross-talk leading to global regulation of signaling. The cross-talk between networks happens via peripheral interconnecting molecules that are represented in between the networks (supplemental Fig. 1).
of this kind provides a global understanding of signaling networks and sheds light on the atypical regulation of molecules by miRNAs resulting in a specific phenotype that cannot be explained by currently accepted signaling mechanisms. One of the major difficulties for functional studies of miRNAs is in determining their specific target genes as available algorithms predict hundreds of target genes for any single miRNA with a likely high fraction of false-positives. Despite these deficiencies, use of all the predicted targets for the differentially expressed miRNAs with the network analysis algorithm shows that these molecules regulate cardiovascular system and cell signaling.

Taken together our studies show that eight miRNAs are significantly altered in DCM compared with nonfailing controls. Importantly, we have identified two novel miRNAs that are down-regulated in end-stage heart failure. Our study in cell systems and mice indicates that miRNAs may play a pivotal role in altering global signaling networks during progression of cardiac pathology. Our analysis in this study provides the following: (a) a better understanding of the global regulation of signaling network pathways by miRNAs; (b) a reduced number of predicted targets, and (c) evidence that a single gene could be targeted by more than one miRNA, ultimately resulting in regulation that is a net effect of relative abundance of independent miRNAs. Importantly, our study lays a foundation for the concept that therapeutic interventions with miRNAs would have profound global effects on signaling networks in a nontraditional manner compared with the conventional mechanism of regulation.

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