MicroRNAs Play an Essential Role in the Development of Cardiac Hypertrophy

Danish Sayed, Chull Hong, Ieng-Yi Chen, Jacqueline Lypowy, Maha Abdellatif

Abstract—MicroRNAs are naturally existing, small, noncoding RNA molecules that downregulate posttranscriptional gene expression. Their expression pattern and function in the heart remain unknown. Here we report an array of microRNAs that are differentially and temporally regulated during cardiac hypertrophy. Significantly, the muscle-specific microRNA-1 (miR-1) was singularly downregulated as early as day 1 (0.56±0.036), persisting through day 7 (0.29±0.14), after aortic constriction–induced hypertrophy in a mouse model. Overexpression experiments showed that miR-1 inhibited its in silico–predicted, growth-related targets, including Ras GTPase–activating protein (RasGAP), cyclin-dependent kinase 9 (Cdk9), fibronectin, and Ras homolog enriched in brain (Rheb), in addition to protein synthesis and cell size. Thus, we propose that microRNAs play an essential regulatory role in the development of cardiac hypertrophy, wherein downregulation of miR-1 is necessary for the relief of growth-related target genes from its repressive influence and induction of hypertrophy. (Circ Res. 2007;100:0-0.)

Key Words: microRNA n cardiac hypertrophy n microarray n miR-1

With the discovery of microRNA, we are compelled to revise our understanding of the mechanisms regulating normal and pathological cellular functions. These fundamental regulators are noncoding RNA molecules (~21 nucleotides [nt]) that silence genes through posttranscriptional regulation. The first microRNA was discovered in 1993, when Lee et al found that lin-4, which downregulates the levels of lin-14 during the development of Caenorhabditis elegans, expresses a small noncoding 22-nt RNA.1 Not until 7 years later did Reinhart et al report the discovery of the second microRNA, let-7, which induces downregulation of lin-41 protein.2 This was shortly followed by identification of a multitude of human and fly microRNAs, some of which are highly conserved, such as let-7,3 thus reflecting their essentiality. Interestingly, it was found that in each of the heart, brain, and liver, there is a distinct tissue-specific microRNA that predominates, which led to the idea that microRNAs must be involved in tissue differentiation.4 There are collectively more than 4000 microRNA sequences existing in a wide range of species. These are annotated and catalogued in a searchable Web-based data registry by Welcome Trust Sanger Institute.5,6

Identification of the genomic locations of microRNAs revealed that they exist within introns or exons of protein-coding mRNA or noncoding RNA.7 Evidence suggests that microRNAs are expressed as an integral part of their host transcript.8 This transcript, or its processed products, is considered the primary microRNA and is cleaved in the nucleus by the enzyme Drosha, yielding a pre-microRNA, which is a short stem-loop structure ~70 nt.9,10 Pre-microRNA is then exported to the cytoplasm for further processing by Dicer into the mature microRNA sequence. This is where microRNA converges onto the same pathway that is known to process small interfering RNA.11 This includes incorporation of the single stranded microRNA into the RNA-induced silencing complex (RISC). Mature microRNA binds to the 3′ untranslated region of its target mRNA through exact complementarity, strictly, with its 5′-prime 7 to 8 nt, and partial complementarity with rest of the sequence.12 Using this criterion, computational predictions of microRNA targets reveal that a single microRNA has the potential to inhibit up to ~200 mRNAs. Depending on the overall degree of complementarity, microRNA will either inhibit translation or induce degradation of its target. The previous results showed that lin-4 microRNA downregulated lin-14 protein but not mRNA, which suggested that the partial complementarity between microRNA and its target inhibited protein translation,1 mechanism unknown.

Because of their major roles as oncogenes and tumor suppressors, a group of microRNAs have been dubbed “oncomirs.”13 In cancer, the discovery of microRNA is offering answers to previously unresolved questions. For example, after years of futile attempts to identify the gene(s) in the deleted 13q14 region that participate in the pathogenesis of chronic lymphocytic leukemia, Calin et al discovered that miR-15 and miR-16 are located within this sequence and...
that both are downregulated in \( \approx 68\% \) of cases.\(^{14}\) These microRNA molecules are now considered tumor suppressors, among their targets being Bcl2, the level of which reciprocally correlates with that of miR-15/16 in chronic lymphocytic leukemia.\(^{15}\) Calin et al have then gone on to show that 52.5% of microRNAs are located in fragile genomic sites that are frequently deleted in cancer.\(^{16}\) In contrast, overexpression of microRNA is also associated with cancer. For example, miR-155 is upregulated in B-cell lymphoma.\(^{17}\) Notably, tissue-specific overexpression of miR-155 in a transgenic mouse model proved it sufficient for induction of lymphoblastic leukemia.\(^{18}\) Overall, unique microRNA expression profiles have been detected in chronic lymphocytic leukemia,\(^{19}\) breast,\(^{20}\) and lung cancers,\(^{21}\) which could aid in the diagnosis and prognosis of cancer.\(^{21}\) Lu et al showed that classification of poorly differentiated tumors was possible with microRNA, but not mRNA, expression profiling.\(^{22}\) Thus, microRNAs provide us with a more accurate diagnostic tool.

miR-1 was identified as a muscle-specific microRNA.\(^4\) Its expression is detected as early as embryonic day (E) 8.5 in the mouse heart and increases with the progression of differentiation.\(^{23}\) When overexpressed with a \( \beta \)-myosin heavy chain (MHC) promoter in a transgenic mouse model, miR-1 inhibited myocyte proliferation and cardiac development. In Drosophila, a DmiR-1\(^{ko}\) mutant dies as a small second instar larvae, 2 to 7 days after hatching, from apparent paralysis.\(^{24}\) On the other hand, the first larvae stage is normal; it is only after feeding that paralysis and death are triggered. From this, it was concluded that miR-1 is not involved in muscle formation but in postmitotic muscle growth and function.

Although microRNA studies predominate in the cancer field, little is known about their expression patterns or role in other diseases, especially cardiovascular disorders. Pressure-overload cardiac hypertrophy is a multigene disease, which involves a global change in the gene expression profile, but the mechanism for this is unknown. In this study, we addressed whether an underlying change in the microRNA expression profile plays a hand in regulating the gene expression pattern characterizing cardiac hypertrophy. Our results point to a single muscle-specific microRNA molecule, miR-1, that is downregulated at the onset of pressure overload on the heart. An outcome that appears sufficient for inducing the gene expression changes underlying cardiac hypertrophy and initiating growth.

Figure 1. Progressive deregulation of microRNA expression during pressure-overload cardiac hypertrophy. Differentially expressed microRNAs \( (P<0.05) \) were analyzed by hierarchical clustering of the log2 value of each TAC/sham pair of microRNA microarray signal at 1, 7, and 14 days post-TAC, using Cluster v3.0 software. The results are displayed in a heatmap generated by Java TreeView version 1.0.13 software. Red indicates upregulation; green, downregulation; black, no change. The legend on the right indicates the microRNA represented in the corresponding row, all of which are Mus musculus specific. The bar code on the bottom right represents the color scale of the log2 values. Each column represents the data from a given time point indicated at the top of the heatmap.
Materials and Methods

Transverse Aortic Constriction
C57 BL/6 mice were anesthetized (IP) with a mixture of ketamine (0.066 mg/g), xylazine (0.013 mg/g), and acepromazine (0.002 mg/g). The animals were then ventilated via tracheal intubation connected to a rodent ventilator (Harvard Apparatus) with a tidal volume of 0.2 mL and a respiratory rate of 110 breaths per minute. The left chest was opened at the second intercostal space and the thymus glands were superficially reflected. The transverse thoracic aorta between the innominate artery and left common carotid artery was dissected and a 7-0 nylon suture tied around the aorta against a 28-gauge needle. A control group underwent a sham operation involving thoracotomy and aortic dissection without constricting the aorta.

MicroRNA Microarray
Total RNA was extracted form the hearts of the mice that have undergone transverse aortic constriction or sham operation, using TRIzol reagent according to the protocol of the manufacturer (Invitrogen). Ten micrograms of RNA were sent to L.C. Sciences for microRNA microarray. Samples were enriched for small RNA, after which each pair of sham and transverse aortic constriction (TAC) samples were labeled with Cys3 and Cys5 fluorescent dyes and hybridized to a single Atactic µParaFlo microfluidics chip that held all 334 mature rodent microRNA probes identified to date, as well as perfectly matched and mismatched probes for quality control. Each microRNA probe is represented 9\times on the microarray. Among the control probes, PUC2PM-20B and PUC2MM-20B are the perfect match and single-based match detection probes, respectively, of a 20-mer RNA positive control sequence that is spiked into the RNA samples before labeling. One may assess assay stringency from the intensity ratio of PUC2PM-20B and PUC2MM-20B, which is normally larger than 30. After signal amplification, the background was subtracted and normalized using LOWESS (locally weighted regression) method. For a transcript to be listed as detectable, it must meet the following criteria: signal intensity higher than 3× (background SD), spot coefficient of variation<0.5 (coefficient of variation=SD/signal intensity), and signals from at least 50% of the repeating probes above detection level.

The array output was received in Excel spreadsheets as lists of raw data and also as “simple detectable” data, which were the average of 9 signal values for each microRNA on the array. For each TAC/sham triplicate set at each time point analyzed, we calculate the significant differences (probability values) between TAC and sham for a given probe, using the t-test. With an FDR cut-off of 0.05, and |log2Ratio|>1, those microRNA signals were classified as differentially abundant.

The differences (probability values) between TAC and sham for a given microRNAs were obtained using the t-test. With an FDR cut-off of 0.05, and |log2Ratio|>1, those microRNA signals were classified as differentially abundant.

Culturing Cardiac Myocyte and Adenovirus Infection
Cardiac myocytes were prepared as previously described. Briefly, hearts were isolated from 1- to 2-day old Sprague–Dawley rats. After dissociation, the cells were subjected to Percoll gradient centrifugation, followed by differential preplating to enrich for cardiac myocytes and deplete nonmyocytes. Cells were then plated in DMEM/Ham F12 with 10% FCS, at a density of 0.5 to 1\times10^5 cells/cm². Twenty-four hours after plating, serum was removed and the cells were infected with recombinant adenoviruses at a multiplicity of infection (moi) of 10 to 20 particles per cell.

Construction of Adenoviruses
Recombinant adenoviruses were constructed, propagated, and titrated as previously described by Graham and Prevec. Briefly, recombinant adenoviruses were constructed, propagated, and titrated as previously described by Graham and Prevec.

Classification of MicroRNA

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Northern Blot
Total RNA (25 μg), extracted using TRIzol reagent according to the protocol of the manufacturer (Invitrogen), was separated on 1% agarose gel with 3% formaldehyde and 10% 10×4-morpholinepropanesulfonic acid. The RNA was transferred to an uncharged nylon membrane and UV cross-linked. The membrane was prehybridized at 42°C for 2 hours with 1 mL/cm² QuikHyb Hybridization solution (Stratagene). DNA oligonucleotides, complementary to the mature microRNAs, were obtained from Integrated DNA technologies. The probes were 5′-end labeled with Redivue adenose 5′-[γ-32P] triphosphate, triethylammonium salt (Amer sham Biosciences) using microRNA probe and marker kit (Ambion) and used for hybridization (1×10^6/cm²). The blot was hybridized overnight and then washed with 2× standard saline citrate/0.1% sodium dodecyl sulfate (SDS) and exposed to X-ray film for 24 hours at −80°C. Blots were stripped using 0.5% SDS for 1 hour at 60°C and reprobed after prehybridization.

MicroRNAs were classified into 3 subgroups: those that change preceding an increase in heart/body weight and persist through day 14 (1–14 days post-TAC), those that change correlative with the increase in heart/body weight (7–14 days post-TAC), and those that change during the latter stage of hypertrophy (14 days post-TAC).
pBH GloXΔE1.3Cre (Microbix), including the ΔE1 adenoviral genome, was cotransfected with the pDC shuttle vector containing the gene of interest, into 293 cells using Lipofectamine (Invitrogen). Through homologous recombination, the test genes integrate into the E1-deleted adenoviral genome. The viruses were propagated on 293 cells and purified using CsCl2 banding, followed by dialysis against 20 mmol/L Tris buffered saline with 2% glycerol. Titering was performed on 293 cells overlaid with DMEM plus 5% equine serum and 0.5% agarose.

**Cell Fractionation and Western Blotting**

Cells were fractionated into cytosol, membranes, nuclei, and cytoskeleton, using a Subcellular ProteoExtract kit (Calbiochem), according to the protocol of the manufacturer. The protein (5 to 10 µg) was analyzed on a 4% to 20% gradient SDS-PAGE (Criterion gels, Bio-Rad).

The antibodies used include: anti-Rheb (ProSci Incorporated), anti-fibronectin (BD Transduction), anti-Cdk9 (Santa Cruz Biotechnology), anti-RasGAP and anti–phospho-ribosomal protein S6 (S235) (Upstate Biotechnology), anti–α-sarcomeric actin (Biomedia Corp), anti-MHC (Hybridoma Bank, University of Iowa).

**Monitoring Proteins Synthesis**

Protein was monitored by the incorporation of [3H]-leucine into total cellular protein and normalized to the DNA content, as previously described.27

**Immunocytochemistry**

Cells were fixed in 3% paraformaldehyde plus 0.3% Triton X-100 in PBS at 25°C for 5 minutes followed by 3% parafomaldehyde in PBS at 25°C for 20 minutes. They were then incubated with anti-MHC (MF-20) at 1:100 in Tris-buffered saline with 1% BSA. After an overnight incubation, they were washed and the secondary antibody Alexa-598 plus phalloidin–Alex488 (Molecular Probes) were added to the cells. After washing, the slides were mounted using Prolong Gold Antifade with 4′,6-diamidino-2-phenylindole (DAPI) (Molecular Probes).

**Measurement of Imaged Cell Surface Area**

Cells were imaged at ×40 magnification. The relative surface area of an image a cell was calculated from the number of pixels outlined using “lasso” tool in Adobe Photoshop. Twenty cells were scored for each condition.

**Statistics**

Significant differences (probability values) between the experimental groups were calculated using 1-way, unpaired Student’s t test. All experiments were repeated 3 times and presented as average±SE of the mean.

**Results**

To identify microRNAs that are differentially expressed during cardiac hypertrophy, we used comprehensive, rodent-specific microRNA microarray analysis of RNA from the hearts of mice subjected to TAC or a sham operation. Hearts were isolated from mice 1, 7, and 14 day post-TAC or sham operation (n=3, each). Total RNA was extracted, enriched for small RNA, and analyzed by microarrays of mature rodent microRNA. TAC and sham samples were paired and analyzed on single arrays using dual labeling. From the microarray normalized values, the significant differences (probability values) between TAC and sham for a given detectable microRNA signal at each time point was calculated. Those with P<0.05 were analyzed using hierarchical clustering of the log2 value of each TAC/sham pair of signals and dis-

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**Figure 2.** Differentially regulated microRNA levels in TAC- and sham-operated hearts. The raw microRNA microarray signals (1 to 65 535) were averaged and those with a probability value of ≤0.05 of TAC (green, downregulated; red, upregulated microRNA) vs sham (black bars) were graphed, error bars representing ±SEM. The graphs represent data from 1 day post-TAC (a), 7 days post-TAC (b), and 14 days post-TAC (c).
played in a heatmap (Figure 1). The figure reflects the extent of temporal changes in microRNA levels during the progression of TAC-induced hypertrophy. Those changes equally represent microRNAs that are upregulated (red), as well as downregulated (green). Also, from examining the heatmap, one can classify microRNAs into 3 subgroups (Table): (1) microRNA with expression levels that change preceding an increase in heart/body weight and persist (1 to 14 days post-TAC); (2) microRNA changes that correlate with the increase in heart/body weight (7 to 14 days post-TAC); and (3) microRNA changes that are only observed during the later stage of hypertrophy (14 days post-TAC).

Figure 2 shows both the relative signal intensity of the microRNA microarray readout (1 to 65,535), which reveals the abundance of each microRNA in the heart. As early as 24 hours post-TAC, with no change in heart/body weight, there were significant (P<0.05), albeit small, changes in the expression of 5 microRNAs (Figure 2a). Notably, microRNA-1 (miR-1) is a skeletal and cardiac muscle-specific microRNA necessary for postmitotic muscle growth and differentiation.23,24 As expected, its signal intensity was the highest on the microarray. Most interestingly, miR-1 was downregulated at 1 day post-TAC (0.74±0.19). Of the 5 detected at 1 day, the decrease in miR-1 persisted and was accentuated in 7 days post-TAC hearts (0.62±0.05; Figure 2b). The hearts at that time showed 23±6% (P<0.01, sham versus TAC) increase in heart/body weight. Overall, at that time point, 11 microRNAs were downregulated and 16 upregulated, 22 of which persisted up to day 14 post-TAC. Of those mir-199a, -199a*, -199b, -21, -214 showed the greatest changes (>2-fold upregulation). Total, after 14 days, 26 microRNAs were downregulated and 25 were upregulated (Figure 2c). At this time, the heart/body weights were increased by 33±5% (P<0.01, sham versus TAC), ~1 week preceding heart failure in this model. Interestingly, miR-1 appeared to be returning back to normal levels (0.83±0.04). In contrast, miR-133a/b also a muscle-specific microRNA, was highly expressed in the heart but remained unchanged up to 14 days after TAC. We also noted that miR-206, which only differs from miR-1 by 3 of 21 nt, was undetectable on any of the microarrays, thus, reflecting the high specificity of these results.

To confirm the microarray data for miR-1 and miR-21, we performed Northern blot analysis on total RNA extracted from the hearts of 1-, 7-, and 14-day TAC or sham operated mice that was used for the microarray analysis (Figure 3a). After quantitation of the signal intensity and normalization to U6, we found that the results correlated well with the microarray data, with even greater differences between the TAC and sham signals (Figure 3b). In agreement, miR-1 was also 55% lower in neonatal myocytes cultured in the presence of growth-promoting 10% FBS versus growth-inhibited conditions (Figure 3c). Because miR-1 emerges as 1 of the earliest microRNA to be regulated and its downregulation persists through the initial phase of cardiac growth, we questioned whether it plays a role in the induction of hypertrophy. To address this, we constructed an adenoviral vector harboring the premature stem-loop sequence of mouse miR-1-2 (Figure 4). MicroRNA stem-loop is usually contained within a larger primary transcript, ranging from a few hundred to thousands of base pairs, that is processed by Drosha. Therefore, it was unclear...
whether it was best to express this construct from a polymerase II (cytomegalovirus [CMV])- or a polymerase III (U6)-dependent promoter; therefore, both were engineered. The latter has better defined start and stop sites that would allow the expression of the stem-loop with little flanking vector sequences. On the other hand, the CMV promoter would encompass any 5' and 3' untranslated vector sequences, including ∼200 bp of SV40 polyA signal sequence provided by the cloning plasmid for construction of the adenovirus. To determine the expression of miR-1 from these viral constructs, we delivered them to cultured neonatal cardiac myocytes and after 24 hours, analyzed them by Northern blotting using probes for miR-1 (top panel) and U6 (bottom panel). Overexpression from the CMV-miR-1 virus results in accumulation of the primary, premature, and mature miR-1 RNA, as indicated on the left.

Figure 4. Recombinant adenovirus constructs expressing miR-1. a. The stem-loop sequence of miR-1-2 that was cloned into recombinant adenoviruses under the control of CMV (CMV-miR-1) or U6 (U6-miR-1) promoters. The sequence in red is that of the mature miR-1. b. Cardiac myocytes were infected with ∼10 moi of adenoviruses expressing CMV-miR-1 or U6-miR-1. After 24 hours, total RNA was extracted and analyzed by Northern blotting using probes for miR-1 (top panel) and U6 (bottom panel). Overexpression from the CMV-miR-1 virus results in accumulation of the primary, premature, and mature miR-1 RNA, as indicated on the left.

Fully processed into mature sequences, the former, though, resulting in a more robust expression.

As shown above, miR-1 is decreased in the early stages of hypertrophy. Thus, the prediction is that miR-1 represses genes that are necessary for induction of cardiac hypertrophy. Sanger Institute, in silico–predicted targets of miR-1 include Ras GTPase–activating protein (RasGAP) \( P = 0.0002 \), cyclin-dependent kinase 9 (Cdk9) \( P = 0.0001 \), Ras homolog enriched in brain (Rheb) \( P = 0.006 \), and fibronectin \( P = 0.00013 \). Our previous reports have shown that both RasGAP and Cdk9 are upregulated and necessary for the development of cardiac hypertrophy.28,29 Rheb is an upstream activator of the mammalian target of rapamycin (mTOR)/S6 kinase pathway involved in protein synthesis and cell growth.30,31 Thus, an increase in those proteins, facilitated by reduction of miR-1, may be expected to support hypertrophy and vice versa.

To test this hypothesis, we overexpressed miR-1 in serum-starved neonatal cultured myocytes, before stimulation with serum-enriched medium, which is a robust hypertrophic stimulus. The assumption was that constitutive overexpression of exogenous miR-1 will counteract the reduction of endogenous miR-1 that is associated with serum-induced hypertrophy. Our results show that CMV-miR-1 effectively inhibited serum-induced RasGAP, Cdk9, fibronectin, and Rheb and partially phosphorylation of the downstream effector ribosomal S6 (Figure 5a) in a dose-dependent manner. In addition, the increase in sarcomeric \( \alpha \)-actin and MHC (localized to the cytoskeletal fractions) that is associated with hypertrophic growth was also inhibited, whereas cytosolic actin monomer remained unchanged. At a moi of 10, the miR-1 virus inhibited serum-stimulated expression of all aforementioned genes by >90%, except for cytosolic and membranous \( pS6 \), which was reduced by ∼40%, thus, reflecting the intactness of other upstream pathways regulating S6. In contrast, this dose of miR-1 did not perturb basal levels. At a moi of 15, though, basal levels were greatly reduced to below detectable levels in some cases. It should be mentioned here that markers of apoptosis, including phospho-H2AX and -H2B, were undetectable at the miR-1 doses used in this experiment (data not shown). RasGAP, histone H2A, and MHC expression patterns validate cytosolic and membranous, nuclear, and cytoskeletal fractions, respectively. Thus, the results suggested that miR-1 inhibits hypertrophic growth. This was verified by monitoring protein synthesis in cultured myocytes using \([3H]\)-leucine (Figure 5b). Myocytes treated with serum-enriched medium exhibited a 16% increase in \([3H]\)-leucine incorporation normalized to DNA content versus myocytes cultured in serum-free medium, over a period of 24 hours. In the presence of excess miR-1, basal, as well as serum-stimulated \([3H]\)-leucine incorporation was inhibited. This is plausibly attributable to the inhibition of basal and serum-stimulated levels of growth-related proteins, such as RasGAP, Cdk9, and Rheb, seen in Figure 5a. This was accompanied by 91.5% and 95% inhibition of cell spreading induced by FBS or endothelin \( P < 0.001 \), FBS or ET-1 versus FBS or ET-1 in the presence of miR-1, respectively, as well as inhibition of the increase in the

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Figure 5. Overexpressed miR-1 inhibits hypertrophy. a, Cultured neonatal myocytes in serum-free medium were infected with 10 or 15 moi of adenoviruses expressing CMV-miR-1, where indicated. A control virus expressing a luciferase-specific short hairpin RNA (shRNA) was used to normalize virus doses to 15 moi. After 48 hours, the cells were treated with 10% FBS where indicated (with a plus symbol). After an additional 24 hours, the cells were harvested and fractionated into cytosol (Cyto), membrane (Mem), nuclear (Nuc), and cytoskeletal (CytoSk) components. Equal amounts of each fraction were analyzed by Western blotting. Each blot was sequentially treated with the antibodies against the proteins listed on the left. Those include RasGAP, Cdk9, Rheb, fibronectin (FN), phospho-ribosomal S6 (pS6), sarcomeric actin (s.actin), and MHC. Data are representative of 4 experiments with similar results. b, Cultured neonatal myocytes in serum-free medium were infected with ~10 moi of adenoviruses expressing CMV-miR-1 or a control expressing a luciferase-specific stem-loop. After 48 hours, the cells were treated with 10% FBS or remained in serum-free medium, with the addition of 0.25 μCi/mL [H]-leucine. After an additional 24 hours, total protein and DNA were extracted and the [H]-leucine content was measured by a scintillation counter and normalized to the DNA content. The numbers were graphed, with the y-axis representing counts per minute (cpm) of [H]-leucine per micrograms of DNA. Bars represent basal levels of incorporated [H]-leucine (open bars) or 10% FBS-stimulated [H]-leucine (closed bars)±SEM (n=5). *P<0.01, control 10% FBS or miR-1 basal vs control basal; **P<0.001, miR-1 10% FBS vs control 10% FBS. c, Cultured neonatal myocytes plated on uncoated glass chamber slides, in serum-free medium, were infected with ~10 moi of adenoviruses expressing CMV-miR-1 (bottom panels) or a control expressing a luciferase-specific stem-loop (top panels). After 48 hours, the cells were treated with 10% FBS (middle panels), 0.1 μmol/L endothelin-1 (ET-1) (right panels), or remained in serum-free medium (left panels). After an additional 24 hours, the cells were fixed and stained with anti-MHC (red) to identify myocytes and atrial natriuretic factor (green), a marker of hypertrophy. The cells were imaged using an inverted fluorescence microscope with a ×40 objective.
hypertrophic marker atrial natriuretic factor in myocytes cultured on uncoated glass surface (Figure 5c).

Discussion

The molecular mechanism underlying cardiac hypertrophy involves a change in the gene expression profile that recapitulates the neonatal growth phenotype. This mostly includes the upregulation of translation-, transcription-, and survival-related genes. Because a single microRNA has the potential to target and inhibit up to 200 genes at a time, we hypothesized that they play a role in defining the expression profile that characterizes hypertrophy. Indeed our results reveal an array of more than 50 microRNAs with expression that progressively changes during development of pressure-overload cardiac hypertrophy. We have identified miR-1 as among the earliest microRNAs downregulated during hypertrophy. It has highly relevant in silico targets that included RasGAP, Cdk9, Rheb, and fibronectin, which we confirmed to be valid in vivo targets as well. miR-1 was previously identified as a muscle-specific microRNA. It has been shown that some microRNA targets regulate its expression in the mouse heart as it increases gradually in parallel with myocyte differentiation and where its levels are highest in the adult versus embryonic or neonatal hearts. In other words, relatively lower levels of miR-1 are associated with the neonatal hypertrophic growth of the heart. Accordingly, we propose that miR-1 is downregulated on induction of hypertrophy, plausibly through a serum response factor–dependent mechanism. This downregulation is required for the release of its growth-related targets, including RasGAP, Cdk9, Rheb, and fibronectin, from its inhibitory influence.

Another interesting microRNA includes miR-21, which is upregulated by day 7 post-TAC, gradually increasing to reach ≥8× greater than basal by day 14. Distinctively, miR-21 has been reported to be upregulated in a multitude of human cancers, in which it appears to play an antiapoptotic role. Some of its relevant, Sanger Institute, in silico–predicted targets include Fas ligand and transforming growth factor-β receptor, both of which await to be confirmed in vivo. Cardiac work overload is a stress condition that induces a degree of apoptosis, which correlates with the extent and duration of the imposed load. During the early stages, the heart counteracts this burden by compensatory mechanisms that involve cellular hypertrophy and activation of antiapoptotic pathways. We predict that upregulation miR-21 in the heart provides a powerful antiapoptotic mechanism, because of its capacity to simultaneously regulate multiple proapoptotic genes.

Functional studies of mammalian microRNAs have been lagging, possibly because of the slow development of the appropriate tools. Antisense, nonhydrolyzable microRNAs have recently been used as effective inhibitors of microRNAs. On the other hand, in vivo knockout is hampered by the fact that microRNAs may have more than 1 copy that may be differentially expressed and some contained within the mRNA of other genes. Studies in cardiac myocytes are further complicated by their poor capacity for transfection (3% to 10%). For that purpose, we emphasize here the successful use of recombinant adenovirus to transfer microRNA to cardiac myocytes with high efficiency by using the stem-loop pre-microRNA under the control of the CMV promoter. This also shows that the pre-microRNA is the minimum sequencing required for expression of mature microRNA and is independent of the context, circumventing the need for genomic cloning.

In brief, the deregulation of microRNA is proving to be an underlying cause of cellular dysfunction and disease. This is especially true in many forms of cancer, where these regulatory molecules have provided better prognostic and diagnostic tools, as well as new therapeutic targets. Our data suggest that microRNAs play an essential role during cardiac hypertrophy with a potential for similarly providing us with new diagnostic, prognostic and therapeutic targets for cardiovascular disorders.

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Disclosures

None.

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