Cardiac Disease Status Dictates Functional mRNA Targeting Profiles of Individual MicroRNAs

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Background—MicroRNAs are key players in cardiac stress responses, but the mRNAs, whose abundance and translational potential are primarily affected by changes in cardiac microRNAs, are not well defined. Stimulus-induced, large-scale alterations in the cardiac transcriptome, together with consideration of the law of mass action, further suggest that the mRNAs most substantively targeted by individual microRNAs will vary between unstressed and stressed conditions. To test the hypothesis that microRNA target profiles differ in health and disease, we traced the fate of empirically determined miR-133a and miR-378 targets in mouse hearts undergoing pressure overload hypertrophy.

Methods and Results—Ago2 immunoprecipitation with RNA sequencing (RNA-induced silencing complex sequencing) was used for unbiased definition of microRNA–dependent and microRNA–independent alterations occurring among ≈13,000 mRNAs in response to transverse aortic constriction (TAC). Of 37 direct targets of miR-133a defined in unstressed hearts (fold change ≥25%, false discovery rate <0.02), only 4 (11%) continued to be targeted by miR-133a during TAC, whereas for miR-378 direct targets, 3 of 32 targets (9%) were maintained during TAC. Similarly, only 16% (for miR-133a) and 53% (for miR-378) of hundreds of indirectly affected mRNAs underwent comparable regulation, demonstrating that the effect of TAC on microRNA direct target selection resulted in widespread alterations of signaling function. Numerous microRNA–mediated regulatory events occurring exclusively during pressure overload revealed signaling networks that may be responsive to the endogenous decreases in miR-133a during TAC.

Conclusions—Pressure overload–mediated changes in overall cardiac RNA content alter microRNA targeting profiles, reinforcing the need to define microRNA targets in tissue-, cell-, and status-specific contexts. (Circ Cardiovasc Genet. 2015;8:774-784. DOI: 10.1161/CIRCGENETICS.115.001237.)

Key Words: Ago2 protein ■ microRNAs ■ regulation, gene expression ■ RNA, messenger ■ RNA-induced silencing complex

MicroRNAs are short noncoding RNAs that serve to limit the translation of specific mRNAs, often but not always observed in conjunction with mRNA transcript degradation.1–2 MicroRNAs bound to Argonaute proteins recruit other protein components of RNA-induced silencing complexes (RISCs) to specific sites on target mRNAs. One key determinant of the strength of the mRNA–RISC interaction is the extent of nucleotide hybridization in the region of the initially exposed microRNA nt 2 to 7 seed region.3 Additional important parameters include the nucleation free energy of the microRNA–mRNA interaction,4 the accessibility of putative mRNA target regions, and conformational constraints placed on microRNAs by Argonaute binding.5 Furthermore, as for other biomolecular interactions, the amount of RISC-targeted mRNA depends not only on binding affinities but also on the concentrations of both the mRNA and of appropriately microRNA–programmed RISCs (ie, the law of mass action). Such data are critical for determining the final extent of interactions between individual microRNAs and mRNAs in biological contexts, given the degenerate nature of microRNA–mRNA interactions and the likelihood that numerous mRNAs will compete for binding to the same microRNA.

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The contribution of microRNAs to pathological cardiac remodeling induced by pressure overload has been studied by many groups, with key roles suggested for miR-1,6–8 miR-34a,9 miR-155,10 miR-199b,11 miR-208a,12 and miR-21/12213 among others. Despite potent effects of microRNA inhibition or elevation on hypertrophic outcomes, relatively few experimentally validated mRNA targets of these microRNAs have been uncovered. In addition, mRNA targets of microRNAs are frequently defined from in vitro models or unstressed hearts and assumed to continue to represent substantive targets of these microRNAs in the disease state. Considering that stressors provoke widespread changes in the cardiac cohort of microRNAs and mRNAs,14,15 thus altering the concentrations of the possible reactants in microRNA–RISC associations, we hypothesized that the mRNAs most substantially targeted by a
given microRNA may differ between unstressed and stressed hearts. The significance of this hypothesis is that, if supported, proper understanding of microRNA–dependent contributions to signaling pathways and planned microRNA–based interventions in pathological circumstances would need to be based primarily on RNA interaction data obtained from hearts exhibiting the same pathology rather than from other models.

To test these hypotheses, we took advantage of existing mice with cardiac-specific overexpression of 2 abundant microRNAs of importance to the heart, miR-133a and miR-378. We have previously developed methods (RISC sequencing) to define mRNAs that undergo increased targetting by RISCs in response to cardiac microRNA overexpression in vivo. Although the endogenous levels of these 2 microRNAs are decreased during transverse aortic constriction (TAC),19–24 persistent microRNA overexpression in both nondiseased and diseased states rendered comparisons of targeting profiles before and after TAC possible. By tracking the behavior of separate cohorts of microRNA–dependent mRNAs before and after TAC, we demonstrate here that the precise RISC-mediated mRNA suppression events that take place in response to either transgene are substantially altered under TAC conditions.

Methods

Mouse microRNAs are described according to the nomenclature used by miRBase 21, released in June 2014 (http://www.mirbase.org/).25,26 miR-133a refers to mmu-miR-133a-3p and miR-378 refers to mmu-miR-378a-3p unless otherwise mentioned.

Mice overexpressing pre-miR-133a and pre-miR-378 under the control of the Mvh6 promoter (αMHC–miR-133a and αMHC–miR-378 mice) were generated as previously described.21,27 All procedures used male mice, 8 to 12 weeks of age, and were performed in accordance with the policies of the Animal Studies Committee at Washington University School of Medicine. For miR-133a studies, RNA-sequencing libraries were prepared from 3 nontransgenic littermates and 7 mice of each genotype subjected to TAC. For miR-378 studies, libraries were prepared from 3 nontransgenic and 3 αMHC–miR-378 mice subjected to sham surgery, and 7 mice of each genotype subjected to TAC.

Unless otherwise specified, regulated RNAs were defined using a threshold of 25% (increased or decreased) at a false discovery rate of 0.02.

All mRNA-seq and RISC-seq data from this study have been deposited in the NCBI GEO under accession GSE65141 (for the miR-133a study cohort) and GSE61734 (for the miR-378 study cohort).

Detailed protocols and procedures for all experiments (Expanded Methods) are in the Data Supplement.

Results

RISC-Bound RNA and Global mRNA Alterations in Response to Pressure Overload

Previous studies from our group have documented dysregulation of microRNAs and mRNAs and consequent effects on microRNA suppression of mRNAs during murine cardiac hypertrophy induced by pressure overload.14 Our earlier investigation focused especially on TAC regulation of (1) mRNAs that were already substantially suppressed (enriched in RISCs compared with global mRNA) under sham conditions and (2) which exhibited converse regulation in the RISC-bound and global mRNA fractions. However, accumulating studies demonstrate that alterations in mRNA–RISC association manifest as changes in mRNA translation (ribosome initiation and elongation and ultimately protein content) that are temporally dissociated from changes in mRNA transcript levels.2–4 Thus, we analyzed data from a new cohort of mice subjected to 1 week of pressure overload and added a further category (3); alterations in mRNA RISC abundance without alterations in global mRNAs, considered as transcript level–independent changes in mRNA translational competence (Expanded Methods in the Data Supplement). Although the above definition captures a wider set of RISC-dependent mRNAs than that presented in prior studies,4,14,16,17 protein functional subcategories of transcription factors, kinases, and phosphatases continued to be over-represented in microRNA–dependent mRNAs defined in this manner in comparison with microRNA–independent mRNAs (Table I in the Data Supplement), similar to previous findings.14

The RISC-enrichment ratio (or RISC score) describes the amount of an individual mRNA measured in sequencing libraries prepared from Ago2 immunoprecipitates (Figure 1A) compared with the amount of the same mRNA measured in libraries prepared from total cellular mRNA (Figure 1B). The mRNAs affected by TAC in a microRNA–dependent manner were not exclusively mRNAs with high RISC scores in sham-operated mice nor were they exclusively mRNAs that tended not to be microRNA–targeted under sham conditions (Figure 1B, Table II in the Data Supplement). Together with the 911 mRNAs directly altered in a microRNA–dependent manner by TAC, an additional 1766 mRNAs were upregulated and 1948 were downregulated without RISC changes (Table III in the Data Supplement), suggesting that at least some direct mRNA targets of microRNAs feed back on nuclear signaling and promoter-driven mRNA transcription. Several previously described targets of TAC-regulated microRNAs from the literature were evaluated for changes in RISC RNA and total mRNA abundance (Figure 2). Strikingly, many of these mRNAs did not undergo alterations during TAC that would have been predicted from changes in their upstream microRNAs alone, revealing the importance of additional factors in determining final mRNA abundances and translational outcome.

To assess whether a majority of microRNA–dependent regulatory events had been captured by examining hearts after 1 week of TAC and to examine whether these events were maintained, further assays were performed at a 2-week time point. Pressure overload induces an initial concentric hypertrophy, which then progresses toward chamber dilation; echocardiographic data from the hearts used for RNA assays demonstrated a decreased r/r ratio at 1 week but not at 2 weeks (Figure 1 in the Data Supplement). Comparison of both RISC level and total mRNA level alterations at these time points demonstrated much less pronounced microRNA–dependent regulation after 2 weeks of TAC (Figure 3A and 3B), although typical changes in hypertrophic marker mRNAs selected from those altered during the fetal-adult transition were observed over the same time frame (Figure 3C). These data indicate that the acute response to pressure overload is not sustained, but rather that changes to the transcriptional and translational program occur later in TAC.
Cardiomyocyte and Nonmyocyte Contribution to TAC-Regulated mRNAs

In preparation for studies intended to monitor the behavior of cardiomyocyte mRNAs targeted by microRNAs during TAC, we first established which regulatory events were most likely taking place in cardiomyocytes. We took advantage of isolated adult cardiomyocyte and nonmyocyte microRNA- and mRNA-sequencing profiles obtained during previous studies\textsuperscript{27,41} and classified regulated RNAs from the 1- and 2-week TAC studies according to enrichment in myocyte or nonmyocyte fractions (Expanded Methods in the Data Supplement). Most mRNAs (and microRNAs) regulated by TAC fell into cardiomyocyte-enriched or cell type nonenriched categories (Figure 4; Figure II and Tables IV and V in the Data Supplement). Notably, we found that downregulation was much more prominent among cardiomyocyte-enriched mRNAs, whereas upregulation occurred much more often for nonmyocyte-enriched mRNAs. This observation was made both at 1- and 2-week time points (Figure 4). Indeed, immunostaining and fluorescence-activated cell sorter studies have shown that the proportion of fibroblasts to cardiomyocytes markedly increases in early pressure overload hypertrophy, such that these cells are present in a 1:1 ratio at 1 and 2 weeks after TAC compared with the wild-type 1:2 fibroblast:cardiomyocyte ratio.\textsuperscript{42} Thus, we propose that at least some of the observed downregulation of cardiomyocyte-enriched RNAs and upregulation of nonmyocyte-enriched RNAs, when measuring global RNAs extracted from intact hearts, reflect an alteration in the cardiomyocyte:fibroblast mass ratio. These data also indicate that caution must be used when assigning likely in vivo mRNA targets of regulated microRNAs.

Figure 1. Effect of pressure overload on RNA-induced silencing complex (RISC)–bound and global mRNAs. A (Top), Representative Ago2 immunoprecipitation from mouse heart. Center, Global microRNA vs RISC-bound (Ago2-immunoprecipitated) microRNA abundances for 300 detected cardiac microRNAs\textsuperscript{14}. Bottom, Global mRNA vs RISC-bound (Ago2-immunoprecipitated) mRNA abundances for 11158 detected cardiac mRNAs in nontransgenic, unstressed mice. B, Histogram of log\textsubscript{2} transformed RISC ratio positions of mRNAs suppressed or derepressed in a microRNA–dependent manner after 1-wk transverse aortic constriction (TAC; false discovery rate <0.02). FPKM indicates fragments per exon of transcript per million mapped reads (mRNAs); and RpM, reads per million mapped reads (microRNAs).

Figure 2. Effect of pressure overload on previously described mRNA targets of regulated microRNAs. RNA-induced silencing complex (RISC)–bound and global mRNA levels from 1-wk transverse aortic constriction (TAC) hearts are shown in a standardized heatmap, for microRNA–mRNA regulatory pairs described in previous reports. Red=increased mRNA abundance in RISC-bound or global fractions; blue=decreased RNA abundance. MicroRNAs observed to be regulated at 1-wk TAC in our studies\textsuperscript{14} are shown in bold, and mRNAs that exhibit predicted behavior are also shown in bold. *mRNA exhibits behavior in TAC consistent with previous report(s) but proposed regulatory microRNA does not change in the same way (eg, miR-24-3p, Jph2). #mRNA exhibits transcript abundance consistent with previous reports in TAC but does not seem to be dependent on proposed regulatory microRNA (Grb2 and Mapk1). mRNA targets of microRNAs are drawn from the following studies: miR-1a-3p\textsuperscript{6,7,29}; miR-21-3p\textsuperscript{30,31}; miR-24-3p\textsuperscript{32,33}; miR-25-3p\textsuperscript{34}; miR-34-5p family\textsuperscript{9,35}; miR-101a-3p\textsuperscript{36}; miR-133a-3p\textsuperscript{16,20,22}; miR-199b-5p\textsuperscript{11}; miR-208a-3p\textsuperscript{37,38}; miR-212/132-3p\textsuperscript{13}; miR-378-3p\textsuperscript{23,24,39}; and miR-499-5p.\textsuperscript{38}
Identification of Empirical mRNA Targets of MicroRNAs Resulting From MicroRNA Overexpression

miR-133a and miR-378 are highly abundant cardiomyocyte-enriched microRNAs (Figure III in the Data Supplement) whose expression, when measured from intact heart RNA preparations, is persistently decreased in response to pressure overload (Figure 5A). We overexpressed each of these microRNAs in cardiomyocytes (Figure 5B) so that mRNA targets of the microRNA transgenes could be identified under unstressed conditions and then could be further evaluated to judge whether these mRNAs were still regulated in a microRNA–dependent manner during TAC. Notably, a much higher degree of pre-miR overexpression was observed than that of the mature, processed microRNA, suggesting a bottleneck in downstream Dicer processing (Figure 5B). Using the same fold change and statistical selection criteria as for the TAC studies in Figure 1, and also taking care to filter the data to include only cardiomyocyte-enriched (or non-cell type–enriched mRNAs), 183 mRNAs displayed increased (microRNA–dependent) RISC association in response to αMHC–miR-133a (Table VIA in the Data Supplement). As might be expected from altered direct RISC targeting of several mRNAs, a consequence of miR-133a overexpression was RISC-independent (ie, indirect) regulation of many further mRNAs (Table VIB in the Data Supplement; 142 upregulated and 267 downregulated, excluding 86 regulated mRNAs enriched in the nonmyocyte fraction). The RNA-sequencing data on mRNA abundance changes in response to miR-133a compared favorably with previous microarray measurements of regulated transcripts in αMHC–miR-133a hearts21 (Figure IV in the Data Supplement).

We observed similar increases and decreases in global mRNA abundances in αMHC–miR-378 hearts to those...
previously reported 27 (309 upregulated and 361 downregulated, excluding 147 regulated mRNAs enriched in the nonmyocyte fraction), but relatively few mRNAs whose direct association with the RISC was increased in response to the transgene (only 7 mRNAs at false discovery rate <0.02; Table VII in the Data Supplement). At a less stringent statistical cutoff (false discovery rate <0.1), 38 mRNAs had increased RISC associations (Table VII in the Data Supplement). Although we have previously validated that mRNAs without significant global abundance changes, but with significant increases in RISC association, are indeed subject to translational suppression,17 we demonstrated that RISC-increased Gapdh is decreased at the protein level and that RISC-increased Rapgef4 is subject to miR-378 suppression in a luciferase reporter assay (Figure V in the Data Supplement).

Consistent with the notion that microRNA overexpression reprograms the RISC (and that the number of Ago2 molecules available for microRNA binding may represent a limiting factor), numerous mRNAs exhibited decreased rather than increased RISC abundance in the presence of either microRNA transgene (Table VII in the Data Supplement). Although we have previously validated that mRNAs without significant global abundance changes, but with significant increases in RISC association, are indeed subject to translational suppression, we demonstrated that RISC-increased Gapdh is decreased at the protein level and that RISC-increased Rapgef4 is subject to miR-378 suppression in a luciferase reporter assay (Figure V in the Data Supplement).

Pressure Overload Competes With MicroRNA Overexpression for Regulation of Numerous mRNAs

To be able to test the hypothesis that pressure overload–induced changes in RISC association profiles and mRNA abundances would alter functional microRNA targets, we needed to be able to track a set of microRNA–regulated mRNAs in the environment of TAC. Any independent effect of TAC (in nontransgenic mice) on any of the same mRNA targets identified in αMHC-miR hearts would confound further analyses. In addition, we were careful not to select tracking mRNAs which appeared to be enriched in cardiac nonmyocytes, as these are less likely to be authentic direct microRNA targets of cardiomyocyte-overexpressed microRNAs. Of the starting 183 mRNAs directly targeted by miR-133a in unstressed hearts, 146 underwent regulation at the RISC and global mRNA level in response to TAC alone, leaving a set of 37 mRNAs that could be used to monitor the effect of TAC on miR-133a–dependent RISC association. These served as the miR-133a tracking set (Table VIC in the Data Supplement). Similarly, we established a set of 32 tracking mRNAs directly targeted by miR-378 overexpression that were unaffected by TAC alone (Table VIIIC in the Data Supplement).

Figure 4. Cardiomyocyte and nonmyocyte distribution of transverse aortic constriction (TAC)–regulated mRNAs. A, Pie charts of global mRNAs regulated at false discovery rate (FDR) <0.02 after 1-wk TAC. Slices in each pie show cardiomyocyte (CM)–enriched (red), non-CM–enriched (green), and noncell type–enriched mRNAs (gray). B, As in A, but for 2-wk TAC.

Figure 5. Endogenous miR-133a and miR-378 decrease in transverse aortic constriction (TAC) and transgene overexpression. A, Reverse transcription coupled to quantitative polymerase chain reaction measurement of miR-133a and miR-378 during sustained TAC (normalized to U6 expression). White bars, sham; black bars, TAC. B, Reverse transcription coupled to quantitative polymerase chain reaction measurement of microRNA precursors and mature microRNAs during 1-wk TAC (for miR-133a) and 2-wk TAC (for miR-378). White bars, nontransgenic; black bars, transgenic. *P<0.05 relative to sham (unpaired t test); †P<0.05 relative to sham (Mann–Whitney). Numbers in bar plots designate biological replicates. Different mouse hearts were used in nontransgenic sham and TAC conditions shown in A to those used in B.
For αMHC-miR-378 mice and their nontransgenic controls, all RNA measurements were performed after 2 weeks of TAC (rather than the 1 week used for miR-133a studies) because parallel echocardiographic measurement suggested that this represented a key time point at which major functional differences were observed (vide infra).

We considered 2 broad classes of outcome with regard to RISC association of mRNAs in response to microRNA overexpression and TAC. First, RISC programming in αMHC-miR hearts, and separate RISC programming and mRNA abundance changes caused by TAC-mediated changes in microRNA and mRNA transcription, could comprise largely independent, additive actions. Second, competition between microRNA transgene–programmed RISCs and TAC for overlapping sets of mRNAs could lead to (1) synergistic effects from both stimuli and (2) events mediated by 1 stimulus that are canceled out by the other. Only 4 of the 37 mRNAs in the miR-133a–dependent tracking set (11%) continued to exhibit increased RISC association in a comparison of TAC miR-133a–transgenic with sham nontransgenic hearts (Figure 6A and 6B; Table VIIC in the Data Supplement), suggesting that TAC competes with miR-133a overexpression for regulation of many mRNAs, in accordance with the second model, rather than behaving in an independent or additive manner. A similar proportion of the tracking mRNAs (3 of the 32) in αMHC–miR-378 hearts maintained increased RISC associations during TAC (Figure 6E and 6F; Table VIEEE in the Data Supplement) further demonstrating competition between TAC effects and microRNA overexpression.

As only a small proportion of microRNA transgene–regulated, RISC-associated mRNAs showed similar changes under sham and TAC conditions, we reasoned that most of the mRNAs exhibiting indirect, downstream regulation because of the microRNA transgenes would also be unlikely to undergo similar regulation under sham and TAC conditions. As for the set of 37 directly RISC-regulated 37 mRNAs used to track miR-133a targeting during TAC, we defined 190 mRNAs that were indirectly regulated (either upward or downward) by miR-133a under sham conditions without exhibiting similar regulation because of TAC alone. However, only 31 of these (16%) were similarly regulated by the combined stimuli of miR-133a overexpression and TAC (Figure 6C and 6D; Table VIIID in the Data Supplement). In similar fashion, 491 mRNAs were identified (at false discovery rate <0.02) and were indirectly regulated by miR-378 overexpression but were not affected by TAC alone; 262 of these (53%) maintained similar regulation when TAC was performed on αMHC–miR-378 mice (Figure 6G and 6H; Table VIIIE in the Data Supplement).

We did observe that although miR-133a and miR-378 precursors and their mature microRNA products continued to be overexpressed compared with wild-type levels during TAC, there was an apparent decline in transgene expression levels in RNA extracted from intact heart (Figure 5B). First, as we noted when evaluating hypertrophic marker RNAs during TAC (Figure 3C), pressure overload causes a significant decrease in whole-heart αMHC/Myh6 levels after 2 weeks, and it is reasonable to expect that the transgene promoter would follow suit. Second, as referred to previously, fluorescence-activated cell sorter studies have demonstrated that nonmyocytes (especially fibroblasts) proliferate during the initial phase of TAC and contribute more substantially to total cardiac mass (and thus RNA) as a result. Thus, it is likely that at least some, if not a substantial fraction, of the apparent decline in Myh6-directed microRNA transgene activity measured from whole-heart preparations during TAC is a result of increased nonmyocyte numbers and their contribution to total RNA.

Taken together, these data show that miR-133a and miR-378 regulation of direct, RISC-associated targets and of indirect, secondarily affected mRNAs observed under unstressed conditions is largely overwhelmed by TAC-dependent transcriptional reprogramming, thus supporting the hypothesis that TAC-induced changes in mRNA abundances and mRNA RISC associations influence the biological function of microRNAs. Nonetheless, the above analyses did not investigate whether miR-133a or miR-378 overexpression could provoke changes in distinct subsets of RISC-associated and RISC-independent mRNAs in TAC.

For the purpose of visualization, the analytical data are represented in Figure 6. Transverse aortic constriction (TAC) effect on microRNA targets identified from RNA-inducing silencing complex (RISC) sequencing in unstressed hearts. A–D, Venn analyses of direct (left) and indirect mRNA targets (right) of miR-133a in unstressed hearts (yellow circles) compared with mRNAs regulated in a RISC-dependent or RISC-independent manner in TAC αMHC–miR-133a hearts (red circles). E–H, As in (A–D), but in αMHC–miR-378 hearts and with blue circles denoting miR-378 targets in unstressed hearts. mRNAs designated as tracking mRNAs (not affected by TAC alone in A, C, E, and G) are evaluated for similar regulation during TAC in the presence of the microRNA transgene (B, D, F, and H).
that were not observed to be so affected under unstressed conditions. To evaluate this further possibility and to seek to better understand the mechanisms underlying the previously observed salutary effects of miR-133a overexpression during TAC,21,22 we performed a pairwise comparison of RISC-associated and RISC-independent mRNA regulation during TAC in both αMHC–miR-133a mice and nontransgenic controls.

**Distinct Effects of miR-133a Overexpression on Pressure Overload mRNA Regulation**

We recapitulated the previously reported decline in terminal deoxyuridine triphosphate nick-end labeling-positive, apoptotic myocytes in αMHC–miR-133a hearts after 1 week of TAC and the lack of effect of the miR-133a transgene on the extent of hypertrophy21 (Figure VI in the Data Supplement). Independent studies from another group using an inducible αMHC–miR-133a mouse line have also replicated the effect on fibrosis and implicated miR-133a suppression of members of the β1-adrenergic receptor signaling cascade from in vitro studies and biotin pulldown from heart homogenates.23 However, in our in vivo RISC-sequencing data under unstressed conditions did not uncover further evidence of direct miR-133a targeting of members of this cascade (Table VI in the Data Supplement).

Numerous mRNA regulatory events observed during TAC were restricted to nontransgenic or to αMHC–miR-133a hearts. Only 38% (371 of 970) of direct microRNA targets and 32% of RISC-independent targets (1202 of 3807) were shared by TAC in either genotype (Figure VIIA and VIIIB in the Data Supplement). Thus, the interaction of TAC and miR-133a overexpression is nonlinear: TAC is able to override effects of miR-133a overexpression observed in unstressed hearts, whereas the miR-133a transgene alters the transcriptome typically induced by TAC, restricting some regulatory events while synergizing with TAC to produce new ones. To focus on differences in apoptotic signaling between αMHC–miR-133a TAC and nontransgenic TAC, we performed further analyses of only those genes with Gene Ontology biological process annotations44 including the keyword apoptosis, using these as input to construct a knowledge-based signaling network44 (Figure VIII in the Data Supplement). Both nontransgenic and αMHC–miR-133a TAC gave rise to multiple interacting pro-apoptotic and antiapoptotic signals. Although it was beyond the scope of these studies to elucidate which among these multiple altered signaling events most strongly favor cardiomyocyte survival during αMHC–miR-133a TAC, these data demonstrate differential regulation of multiple apoptotic effectors and protectors in response to elevated miR-133a. In combination with the observed phenotype of αMHC–miR-133a TAC hearts, these data suggest that the decrease in miR-133a observed during TAC in wild-type animals may rebalance these multiple pathways such that cell death is favored.

**Distinct Effects of miR-378 Overexpression on Pressure Overload mRNA Regulation**

In similar fashion to αMHC–miR-133a hearts, numerous mRNA regulatory events observed during TAC were restricted to nontransgenic or to αMHC–miR-378 hearts. Only 5% (7 of 146) of direct microRNA targets and 15% of RISC-independent targets (220 of 1427) were shared by TAC in either genotype (Figure VIIIC and VIID in the Data Supplement). However, in contrast to the miR-133a studies, the phenotype of αMHC–miR-378 mice (other than the effect of the transgene on the production of other microRNAs53) has not been previously described, either in unstressed or stressed conditions. Before these experiments, we had hypothesized that genetically increased levels of cardiomyocyte miR-378, in the context of pressure overload, may serve to limit myocyte hypertrophy, myocyte death, and replacement fibrosis, similar to adeno-associated virus serotype 9-mediated transduction of miR-378 to the heart.24 There were no changes in myocyte cross-sectional area or length or in cardiac chamber volume, in unstressed αMHC–miR-378 hearts (Figure 7A). Slight decreases in echocardiographic fractional shortening and in dobutamine-stimulated contractility measured during cardiac catheterization were evident (Figure 7B). At the global RNA abundance level, the molecular basis of the reduced contractility did not seem to involve downregulation of Adrb1 (β1-adrenergic receptor), G protein or adenylyl cyclase enzyme mRNAs but may depend, at least in part, on decreases in other key components of the contractile machinery, including calsequestrin 2 (Casq2), tropomyosin 1 (Tpm1), cardiac α-actin (Actc1), and cardiac myosin light chain 2 (Myl2; Figure 7C).

In contrast to the outcomes of adeno-associated virus serotype 9–miR-378 and anti–miR-378 studies,24,39 αMHC–miR-378 hearts exhibited a rapid decline in functional shortening and did not seem to undergo initial wall thickening (decreased r/h ratio) in response to pressure overload, unlike their wild-type controls (Figure 7D and 7F). Similar increases were observed in myocyte cross-sectional area, but myocyte length was increased only in transgenic mice subjected to pressure overload (Figure 7E). An increased r/h ratio measured via echocardiography was observed in parallel to the increase in sectioned myocyte length (Figure 7F). The extent to which further molecular, mechanistic dissection of the TAC phenotype of αMHC–miR-378 mice may be worthwhile is mitigated by the fact that αMHC–miR-378 hearts exhibit ≈12-fold miR-378 overexpression during TAC (Figure 5), in contrast to adeno-associated virus serotype 9–miR-378–treated hearts in which miR-378 expression during TAC is essentially normalized in comparison with pre-TAC conditions.24 Nonetheless, superposition of TAC in 2 different strains of microRNA–overexpressing hearts demonstrated that TAC altered microRNA–mRNA targeting relationships, regardless of whether the combined effect of TAC and microRNA overexpression was modest (miR-133a) or deleterious (miR-378).

**Discussion**

The primary goal of these studies was to determine whether microRNA–RISC associations mediated by individual microRNAs are the same under 2 different physiological conditions; a basal, unstressed state, and pressure overload hypertrophy. Although in silico or in vitro protocols can partially estimate the likelihood that a given microRNA–RISC binding
event is able to occur, they typically do not easily allow the stoichiometries of mRNAs and of microRNAs/RISCs to be factored in. Consequentially, it is difficult to estimate the proportion of an individual microRNA that might be bound to each of several potential mRNA targets and which of several microRNAs might constitute the most potent
regulator of an individual mRNA; factors which are likely to be highly sensitive to cell type and status. Here, we have used RISC sequencing of intact hearts to monitor changes in mRNA targeting by Ago2 during cardiac stress, in combination with microRNA-expressing transgenes to be able to accurately track microRNA–bound mRNAs. Overexpression of miR-133a and miR-378 provided a means of tracking microRNA–targeted mRNAs in unstressed and stressed hearts so that the primary goal of these studies could be achieved. Our findings demonstrate that some microRNA–targeted mRNAs in unstressed conditions no longer undergo RISC-mediated suppression during TAC, whereas other mRNAs not subject to RISC-mediated suppression in unstressed conditions become so during TAC. Thus, the signals influenced by particular microRNAs depend on precise tissue status, including the manner in which the remainder of the transcriptome is expressed, a parameter which will vary in health or disease. In consequence, we propose that rational design of microRNA–based interventions in cardiac disease needs to be based not principally on mRNA suppression events observed in vitro or in unstressed hearts, but needs to take into account whether the same events continue to occur in diseased hearts and whether inhibiting events that only occur in diseased hearts may provide new opportunities.

The use of microRNA overexpression combined with RISC sequencing in vivo may identify a greater range of cardiac targets of miR-133a and miR-378 in health or disease than is readily possible with in vitro or in silico approaches. Nonetheless, as we have previously discussed, RISC sequencing of microRNA transgenic hearts may not identify those targets of a microRNA that are already comprehensively suppressed in wild-type hearts, and microRNA transgenes that provoke disease alone or exacerbate disease responses severely complicate the analysis of endogenous microRNA targets in these contexts. In addition, the use of transgenes that encode microRNA stem-loop precursors means that both the more abundant guide microRNA strand and the less abundant passenger microRNA strand are overexpressed, each of which could have an independent set of mRNA targets. We have shown previously that a range of other microRNA species are dysregulated in cMHC–miR-378 hearts and could be responsible for some of the effects observed on the mRNA transcriptome. Thus, the direct applicability of our transgenic miR-133a and miR-378 targeting profiles for accurate definition of the mRNAs targeted by miR-133a and miR-378 in wild-type hearts is limited by how faithfully these targeting profiles reflect the wild-type context. More accurate determination of authentic miR-133a and miR-378 direct and indirect mRNA targets in healthy and stressed hearts, using microRNA–RISC interaction studies in vivo, may need to involve comparative studies in hearts lacking some but not all miR-133a alleles, in hearts lacking miR-378, and in hearts with temporal control of microRNA transgenes or shorter term microRNA manipulations made possible by viri or anti-miRs.

It could be argued that the fastest path to achieving useful therapies based on microRNA actions might be to work primarily to reverse disease-mediated microRNA changes with the expectation that the complex signals they regulate will regain equilibrium (eg, reversal of dysregulated miR-34a, miR-199b, miR-212/132, and miR-378 in hypertrophic hearts). Indeed, several such preclinical studies have already reported success in ameliorating consequences of pressure overload. Nonetheless, a more refined approach of altering particular microRNA–mRNA interactions (eg, with decoy target-protection oligonucleotides designed to disrupt specific binding events) made possible by detailed studies of the kind outlined here, may prove to be highly valuable as an alternative to affecting the entire targeting portfolio of a given microRNA. This presents a need for more specific determination of microRNA–binding sites and suppressive actions on mRNAs in vivo. Crosslinking before Ago2 immunoprecipitation, in combination with rigorous analysis of crosslinking-induced sequence mutations, can provide a comprehensive view of the binding of individual microRNAs to individual mRNAs, but has not yet been demonstrated using intact hearts nor has its sensitivity in differential expression studies been evaluated. Nonetheless, this or related approaches capable of interrogating endogenous in vivo interactions would avoid many of the complications arising from genetic or reagent-based microRNA overexpression and knockout. Continued methodological development and training of informatic helper algorithms with contextually appropriate empirical data should lead to a greater understanding of the actions of individual microRNAs, whether stress-regulated or of stable abundance, in health and disease. In turn, this will lead to optimized rationales for choosing individual microRNAs for therapeutic manipulation.

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Disclosures

None.

References

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Cardiac Stress Alters MicroRNA Target Profiles


**CLINICAL PERSPECTIVE**

Although microRNAs are known to possess important functions in cardiac stress responses, the mRNAs that are primarily affected by changes in cardiac microRNAs are not well defined. Because RNA-binding relationships depend on compatible nucleotide sequences, as well as complex stoichiometries, stress-dependent large-scale alterations in the cardiac transcriptome during disease suggest that the mRNAs most substantively targeted by individual microRNAs will vary between unstressed and stressed conditions. To test the hypothesis that microRNA target profiles differ in health and disease, we traced the fate of empirically determined miR-133a and miR-378 targets in mouse hearts undergoing pressure overload hypertrophy, using next-generation RNA sequencing techniques to define cohorts of mRNAs regulated in a microRNA–dependent or microRNA–independent manner. We found that only a small proportion of mRNAs targeted by either miR-133a or miR-378 in unstressed conditions continued to be targeted by these same micro RNAs in pressure overload hypertrophy. Thus, pressure overload alters individual microRNA targeting profiles, concomitant with widespread changes in other cardiac RNAs. This reinforces the need to define microRNA targets in tissue-, cell-, and status-specific contexts for a proper understanding of microRNA–dependent contributions to signaling pathways. Furthermore, it suggests that planned microRNA–based interventions in pathological circumstances may need to be based primarily on RNA interaction data obtained from hearts exhibiting similar pathology to permit an optimal choice of individual microRNAs for therapeutic manipulation.
Cardiac Disease Status Dictates Functional mRNA Targeting Profiles of Individual MicroRNAs

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Supplemental Material

Expanded Methods

*Generation, characterization and use of αMHC-miR-133a and αMHC-miR-378 transgenic mice.* Transgenic mice were created via cloning of a genomic DNA fragment flanking the mmu-miR-133a-1 or mmu-miR-378a microRNA stem-loop precursor regions into the αMHC/Myh6 cardiac transgenic promoter construct 1, as previously described 2, 3. Male mice between the ages of 8-12 weeks were used for these studies. Pressure-overload hypertrophy was induced via transverse aortic constriction (TAC) performed at Washington University’s Mouse Cardiovascular Phenotyping Core (Dr. Carla Weinheimer, director). For miR-133a studies, 3 nontransgenic littermate controls and 7 αMHC-miR-133a mice were subjected to sham surgery; 5 nontransgenic and 4 αMHC-miR-133a mice were subjected to TAC. For miR-378 studies, 3 nontransgenic littermate controls and 3 αMHC-miR-378 mice were subjected to sham surgery while 7 mice of each genotype were subjected to TAC. Mice were housed and surgeries were performed according to procedures approved by the Washington University Animal Studies Committee.

*Tissue harvest and RNA preparation.* Hearts were placed in ice-cold saline, rinsed, and sectioned into 5-6 coronal slices. One was immediately fixed in 10% neutral buffered formalin for later histological analysis, while right ventricle fragments were removed from the others prior to flash-freezing in liquid nitrogen. Total RNA was prepared from flash-frozen sections (or from saline-rinsed cell monolayers; see below) using Trizol (Invitrogen) as per the manufacturer’s directions, with the exception that isopropanol precipitation was carried out for 30 min at room temperature to improve small RNA yield. Ago2 immunoprecipitation prior to RNA isolation for RISC-sequencing was carried out as detailed below.

*Mouse adult cardiomyocyte isolation and gene expression analysis.* Cardiomyocyte and fibroblast fractions were separately isolated from the hearts of three 8 week-old, wild-type FVB/N mice, as previously described 4. Following 3 rounds of gravity filtration and washing with ice-cold PBS, myocytes were immediately dissolved in Trizol (Invitrogen) and total RNA was prepared. In order for sufficient nonmyocytes (fibroblasts) to be available for gene expression assays, cells were plated in tissue culture dishes in DMEM /10% fetal calf serum / antibiotics, grown at 37°C / 5% CO₂, and passaged once. Cell monolayers were harvested directly into Trizol. Previously, Applied Biosystems TaqMan microRNA and mRNA qPCR assays were performed on these total RNA preparations 3. All samples were subsequently assayed by microRNA-sequencing and mRNA-sequencing (described below), and both raw sequencing data and per-gene tabulated data have been deposited in NCBI GEO GSE58453. We defined a ‘cardiomyocyte enrichment ratio’ as [0.6*(FPKM in myocytes)]/[0.4*(FPKM in nonmyocytes)] to model the approximate 60%:40% ratio of cardiomyocyte to nonmyocyte mass 5. Cardiomyocyte-enriched mRNAs were selected at an enrichment ratio ≥2, nonmyocyte-enriched mRNAs were selecte at an enrichment ratio ≤0.5, and those with intermediate values were defined as nonenriched for either cell fraction. For a small number of mRNAs, detection was not possible in isolated cardiomyocyte or nonmyocyte fractions; these are classified as ‘unknown enrichment’ in data tables.
**MicroRNA annotation using miRBase 21.** Previous nomenclature for microRNAs often described the minor product (passenger strand) of a microRNA stem-loop structure as a miR* form. In this manuscript, we have annotated mouse microRNAs according to the nomenclature used by miRBase 21, released in June 2014 ([http://www.mirbase.org/](http://www.mirbase.org/))\(^6\,7\). MicroRNAs are designated as -5p or -3p forms according to their site of origin in the microRNA stem-loop precursor. While information on whether a microRNA form is ‘major’ or ‘minor’ is sometimes available from deep-sequencing data accumulated in the miRBase database from a variety of tissues, we designate ‘major’ and ‘minor’ forms in mouse hearts according to previously obtained deep-sequencing data \(^3\). For convenience, ‘miR-133a’ refers to mmu-miR-133a-3p and ‘miR-378’ refers to mmu-miR-378a-3p unless otherwise mentioned.

**MicroRNA-Seq for microRNA expression analysis.** Libraries were prepared with TruSeq Small RNA Sample Prep Kits (Illumina) following the manufacturer’s protocols, as previously described \(^8\). Briefly, small RNAs from 1 μg total cardiac RNA were sequentially ligated with 3’ and 5’ adapters, followed by reverse transcription to produce single stranded cDNAs, which were then amplified by PCR with primers including indexing capabilities to distinguish individual libraries after flowcell processing. The amplified libraries were size-selected/gel-purified and quantified. Twelve libraries were pooled in equimolar amounts and diluted to 14 pmol/L for cluster formation on a single flow cell lane, followed by single-end sequencing (50 nt reads, not including the index determination) on an Illumina HiSeq 2000 sequencer. Alignment and quantitation of microRNA sequencing reads was performed using sRNAbench ([http://bioinfo5.ugr.es/sRNAbench/sRNAbench.php](http://bioinfo5.ugr.es/sRNAbench/sRNAbench.php))\(^9\).

**mRNA-Seq for mRNA expression analysis.** mRNA-sequencing was performed essentially as described \(^8\,10\,11\,12\), using Illumina HiSeq 2000 sequencers and library indexing. Using prior criteria that a meaningfully expressed transcript should be present at a level equivalent to at least 1 mRNA copy/cell (3 FPKM; fragment [reads] per kilobase of exon per million mapped reads) \(^8\,10\,11\,12\), or as an alternate, that a detectable transcript must map to at least 1 millionth of the total mapped reads in an individual library, we identified approximately 13,000 coding mRNAs in mouse hearts. Concordance of these cardiac transcriptomes with those from previous RNA-Seq studies was high \(^8\,10\,11\,12\).

We and others have extensively compared RNA-sequencing analyses to microRNA and mRNA microarrays, finding that RNA-sequencing analyses generally offer superior dynamic range and accuracy (Supporting Information of \(^8\)). In addition, we have validated differential expression results from sequencing analyses with RT-qPCR techniques in several prior studies \(^8\,10\,13\).

**RISC-Seq for microRNA-dependent mRNA expression analysis.** Ago2 was immunoprecipitated from coronal sections of the same hearts used for mRNA-sequencing, followed by total RNA isolation from the Ago2 immunoprecipitate, as described in \(^10\) and the Supplemental Material of \(^8\).

**Alignment and quantitation of mRNA-Seq and RISC-Seq reads.** Single-end, 50 nt reads were obtained from Illumina HiSeq 2000 sequencers. Following separation of libraries according to 3’ indexes, both mRNA-Seq and RISC-Seq reads were aligned against the mouse transcriptome genes.gtf file defined from UCSC mm10 and present in the Illumina iGenomes package April 2014, using Tophat 2.0.10 ([http://ccb.jhu.edu/software/tophat/index.shtml](http://ccb.jhu.edu/software/tophat/index.shtml))\(^14\,15\) to generate bam files and HTSeq ([http://www-huber.embl.de/users/anders/HTSeq/doc/overview.html](http://www-huber.embl.de/users/anders/HTSeq/doc/overview.html)) to allocate
reads to gene locations. Tophat version 2 allows preparation of a pre-defined transcriptome (i.e. according to those genes defined in the genes.gtf list) to which reads are mapped; since ribosomal RNAs are not present in this list and these represent the major non-mRNA type in Ago2 immunoprecipitates, this allows for read mapping only to defined mRNAs in both RISC and global mRNA fractions. HTSeq read data were used as input for statistical calculations, including fold-change and false discovery rate determination, by the DESeq package \(^\text{16}\) (see below). Cufflinks (version 2) (http://cufflinks.cbcb.umd.edu/) \(^\text{14}\) was used to obtain FPKM values from bam files for presentation and inter-study comparison purposes.

For mRNA-seq, mean raw reads per heart were \((2.08 \pm 0.11) \times 10^7\), mean alignment to the transcriptome was \((61.4 \pm 0.8)\%\), and mean aligned reads per heart were \((1.26 \pm 0.06) \times 10^7\).

For RISC-seq, mean raw reads per heart were \((2.67 \pm 0.19) \times 10^7\), mean alignment to the transcriptome was \((31.5 \pm 1.4)\%\) (since a greater proportion of the raw reads comprised non-mRNA), and mean aligned reads per heart were \((0.77 \pm 0.04) \times 10^7\).

Detectable mRNAs were defined as those with at least 6 aligned reads (~1 millionth of the read depth of the lower RISC-seq libraries) in at least 50% of the samples. A total of 13,328 mRNAs were analyzed in downstream procedures.

All mRNA-Seq and RISC-Seq data from this study have been deposited in the NCBI GEO under accession GSE65141 (miR-133a cohort) and GSE61734 (miR-378 cohort). Relative abundance of microRNAs in intact hearts are under accession GSE55791, while microRNA abundances in cardiomyocyte and fibroblast fractions are under accession GSE58453.

**Calculation of differential gene expression using the DESeq package.** DESeq \(^\text{16}\) (version 1) was used to normalize read depth across multiple sequencing libraries, to calculate fold-changes, and to derive individual pairwise comparison p-values and false discovery rates (FDRs). While DESeq takes the non-normalized, absolute number of aligned reads as input, we reported RISC RNA and global mRNA abundance as FPKM, although the underlying fold-change and p-value / FDR comparisons used DESeq’s internal methods of library normalization. DESeq is part of the Bioconductor open-source software suite for the R statistical environment (http://bioconductor.org/packages/release/bioc/html/DESeq.html).

**Differential expression cutoff thresholds and other statistics.** Unless otherwise defined, regulated RNAs were defined using a threshold of 25% (increased or decreased) at an FDR of 0.02. Partek Genomics Suite 6.6 (Partek, St. Louis, MO) was used to derive principal components analysis plots and unsupervised hierarchical clustering heatmaps. Unpaired, 2-tailed t-tests (for parametric data) or Mann-Whitney U tests (for nonparametric data), or ANOVA tests were used for comparison of physiological parameters between mice and significance was taken at \(P<0.05\). Assessment of parametric distribution prior to ANOVA was made with a D’Agostino-Pearson omnibus normality test, and Bartlett’s test was used to assess homoscedasticity (GraphPad Prism 6 software).

**Classification of individual mRNAs according to dependence of regulation on microRNAs**

Considering numerous recent studies demonstrating translational suppression without detectable mRNA transcript degradation \(^\text{17-21}\) we have widened the scope of our RISC analyses here, in
comparison to previous studies\textsuperscript{8,10,11}, to classify regulated mRNAs into one of several possible categories of microRNA dependency. These are:

1) reciprocal regulation in RISC and global mRNA fractions, as used in prior studies\textsuperscript{8,10};

2) regulation only in the RISC fraction, implying an effect on translation without altering transcript levels;

3) regulation only at the global mRNA level, showing lack of direct targeting by microRNAs; and

Direct microRNA targets (microRNA-dependent mRNAs) are obtained from categories 1 and 2, while indirect targets (microRNA-independent mRNAs) are obtained from category 3.

Informatic suites. MetaCore \textsuperscript{22} (\url{http://thomsonreuters.com/metacore}) was used for pathway map and process network classification, while DAVID \textsuperscript{23} was used for Gene Ontology annotation.

\textit{RT-qPCR assays}. TaqMan RT-qPCR assays for microRNAs and mRNAs were performed as previously described\textsuperscript{8} using the following probes:

\begin{itemize}
  \item TaqMan miR-133a-3p: mmu-miR-133a, #002246
  \item TaqMan miR-378a-3p: mmu-miR-378, #002243
  \item TaqMan U6 snRNA: RNU6, #001973
\end{itemize}

For quantitation of pre-miRs, oligo(dT) was used to reverse transcribe 1 µg of total RNA using SuperScript III (Invitrogen) and 5% of the resulting product was used as input in qPCR with SybrGreenER supermix (Invitrogen). Primers for pre-miR-133a were: forward 5’-GAGCTGGTAAAATGGAACAA-3’, reverse 5’-ACAGCTGGTTGAAGGGGAC-3’, 61 bp product. Primers for pre-miR-378 were: forward 5’-AGGGCTCCTGACTCCAGGT-3’, reverse 5’-GTGACTCCACTCAGGCTTCT-3’, 78 bp product. Reference RNAs to normalize pre-miR data were Gapdh (TaqMan Mm99999915_g1) and Actb (TaqMan Mm01205647_g1).

\textit{Fluorescent lectin staining, cross-sectional area and length determinations}. Formalin-fixed, paraffin-embedded cardiac tissue was sectioned at 4 µm onto coated glass slides. Following deparaffinization in mixed xylenes, graded ethanol washes and three 5 min washes in phosphate-buffered saline (PBS), slides were incubated with 100 µg/mL Alexa Fluor \textsuperscript{488}-coupled wheat germ agglutinin (Molecular Probes) in PBS containing 1 mM CaCl\textsubscript{2} for 60 min, room temperature, in dark conditions. Following 3 further washes in PBS, slides were mounted in VectaShield + DAPI (Vector Laboratories) and imaged for AF\textsuperscript{488} and DAPI fluorescence.

The area enclosed by AF\textsuperscript{488}-fluorescent borders was obtained from an average of 500 non-elongated (rounded) cells per heart, with identifiable nuclei, and defined as myocyte cross-sectional area. Similarly, tissue sectioned in parallel to the long axis of myocytes was used to determine myocyte length, using elongated cells with recognizable nuclei and with clearly defined borders to demarcate their long axes. An average of 100 cells per heart with sharp border definition were used for length measurements.

\textit{Immunoblot analysis of cardiac proteins}. Proteins from cardiac homogenates (prepared in the presence of protease inhibitors) were separated using 10\% SDS-PAGE and transferred to
Immobilon-FL low-fluorescence PVDF membranes (Millipore). Heart sections were homogenized in ice-cold 50 mmol/L Tris, 5 mmol/L EDTA and 5 mmol/L EGTA, pH 7.6, containing Complete protease inhibitors (Roche), and all subsequent procedures were performed at 4°C. Debris was removed at 200g, 5 min, followed by nuclear removal at 1000g, 10 min. NP-40 (Sigma IGEPAL CA-630) was added at 0.2% (vol/vol) to the supernatant. After 15 min rocking, insoluble material was removed at 10000g, 10 min. A Bradford stain-based colorimetric dye assay (Bio-Rad) was used to determine protein concentration. For GAPDH and alpha-tubulin detection, 10 μg of NP-40-solubilized cardiac protein was separated on precast 4-20% SDS-PAGE minigels (Bio-Rad). Electrophoretic transfer to PVDF was achieved in 25 mmol/L Tris, 192 mmol/L glycine and 20% (vol/vol) methanol.

Primary antibody against Ago2 was the same used for immunoprecipitation; Wako Pure mouse monoclonal anti-Ago2 (at 0.5 μg/mL), catalog #018-222021, clone 2D4. Primary antibody against GAPDH was Abcam #8245 mouse anti-GAPDH, 1:5000, and primary antibody against alpha-tubulin was Sigma T6704 mouse anti-alpha-tubulin, 1:10000. All primary antibodies were diluted in LiCor Odyssey Blocking Buffer with 0.1% (vol/vol) Tween-20 and incubated with membranes overnight at 4°C. Bound primary antibodies were detected with LiCor IRDye-coupled goat anti-mouse (680LT) at 1:20000 in Odyssey Blocking Buffer with 0.1% (vol/vol) Tween-20 and 0.01% SDS. Signals were visualized and quantitated with a LiCor Odyssey digital imaging system and associated software.24

In vitro luciferase reporter assays for miR-378-mRNA interactions. pCMV-MIR and pCMV-MIR-378 microRNA expression constructs were from OriGene Technologies (Rockville, MD); catalog numbers PCMVMIR and SC401025, respectively. HEK293T cells were transiently transfected in 96-well plates (6 replicates per transfection condition) with 180 ng pCMV-MIR construct and 30 ng luciferase reporter construct together with 1.2 μL Fugene HD per well, and both firefly and Renilla luciferase activity were measured after 48 h using the Dual-GLO Luciferase Assay reagents (Promega) as per the manufacturer’s directions. The 3xGrb2 construct in pmiRGLO (Promega), containing 3 repeats of a Grb2-derived miR-378-binding sequence was a kind gift from Madhu Gupta, University of Illinois, Chicago. The mouse Rapgef4 cDNA region was PCR-amplified and cloned into psiCheck2 (Promega) between XhoI and NotI sites (italicized) using the following primers: forward 5’-agcctaCTCGAGacatctgaagtgccagagt-3’, reverse 5’-atgtatGCGGCCGCatctttgtcaagttatatat-3’.
Supplemental Figure 1. Echocardiographic assessment of TAC progression. A) Representative M-mode echocardiograms taken prior to and after TAC. B) Calculated echocardiographic parameters. C) Gravimetric heart weight relative to body weight. FVB/N nontransgenic mice, n=4-6 per group, mean ± s.e.m. * p<0.05 relative to preTAC or unstressed, unpaired t-test.
Supplemental Figure 2. Cardiomyocyte and nonmyocyte distribution of microRNAs regulated after 1 wk TAC. Cardiomyocytes and nonmyocytes were obtained from FVB/N nontransgenic hearts and subjected to small RNA-sequencing; this procedure and the definition of enrichment by cell type are described in Expanded Methods. RpM, reads per million reads mapped to microRNAs. Red bars, microRNAs upregulated in TAC vs sham whole-heart RNA preparations, FDR<0.02; blue bars, downregulated microRNAs.
Supplemental Figure 3. Cardiomyocyte and nonmyocyte distribution of abundant cardiac microRNAs. MicroRNA abundances defined by miR-sequencing of intact adult FVB/N mouse hearts (pie chart) and of isolated cardiomyocytes and nonmyocytes / fibroblasts (heatmap). RpM, reads per million reads mapped to microRNAs.
Supplemental Figure 4. Similarity of mRNA-sequencing and microarray data for αMHC-miR-133a-affected mRNA transcripts. A, 217 significantly regulated (p<0.05) mRNAs from microarray (x-axis) vs regulation obtained from RNA-sequencing in the present study (y-axis). As expected, RNA-sequencing reveals a wider dynamic range of mRNA regulation than that observed from microarray measurements. B, 413 significantly regulated mRNAs from RNA-sequencing (fold-change 25%, FDR<0.02) (x-axis) plotted against regulation observed from microarrays. Here, the more limited dynamic range of microarray determination tends to reduce the gradient of a linear correlation line.
Supplemental Figure 5

Supplemental Figure 5. Validation of translational suppression predicted from RISC-sequencing. A) Gapdh and Rapgef4 were two mRNAs predicted to be translationally suppressed from data indicating increased RISC abundance without global mRNA abundance changes in unstressed αMHC-miR-378 hearts. * FDR<0.02 compared to nontransgenic hearts. B) Immunoblot data demonstrating decreased Gapdh relative to aggregate alpha-tubulin (i.e. the protein products of Tuba1a, Tuba1b, Tuba1c, Tuba4a and Tuba8) in αMHC-miR-378 vs nontransgenic hearts (n=3 each; graph shows quantitative data, mean ± s.e.m., * p<0.05 relative to nontransgenic). C) miR-378-directed suppression (luciferase reporter assay) of a cloned fragment of Rapgef4 in HEK293T cells (Expanded Methods); comparison to a previously published 3xGrb2 construct is shown. * p<0.05 relative to pCMVmiR (empty microRNA precursor plasmid vector) transfection, white bars; pCMVmiR-378, black bars.
Supplemental Figure 6

A) Reduced TUNEL-positive myocytes were observed in αMHC-miR-133a hearts compared to nontransgenic hearts after 1 wk TAC, as originally reported 2.

B) In similar fashion, echocardiographic parameters were neither worsened nor improved in αMHC-miR-133a hearts at this timepoint, and the extent of gross hypertrophy (measured as gravimetric heart weight) did not differ 2.
Supplemental Figure 7. MicroRNA transgene effects on TAC mRNA targets identified from RISC-sequencing. Venn analyses of direct (A) and indirect mRNA targets (B) of 1 wk TAC in nontransgenic hearts (orange circles), compared to mRNAs regulated in a RISC-dependent or RISC-independent manner in TAC αMHC-miR-133a hearts (red circles). C-D) As for A-B), but in αMHC-miR-378 hearts and with green circles denoting targets of 2 wk TAC in nontransgenic hearts.
Supplemental Figure 8. Involvement of miR-133a-regulated mRNAs in apoptotic signaling pathways. Venn analyses of A) all RISC-dependent mRNA alterations and B) changes in global mRNA abundance during 1 week TAC in nontransgenic and αMHC-miR-133a hearts. All comparisons are relative to sham nontransgenic hearts. Genotype-unique ‘apoptosis’ genes in a MetaCore direct interaction network with increased (marked with red dots) or decreased (marked with blue) translational potential are shown for C) nontransgenic TAC and D) αMHC-miR-133a TAC. Boxes designate particular mRNAs of interest.
### Additional Tables

#### Supplemental Table 1

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Columns have the following meaning:
- **Protein class**: a broadly defined protein function
- **Actual**: number of network objects from the activated dataset(s) for a given protein class
- **n**: number of network objects in the activated dataset(s)
- **R**: number of network objects of a given protein class in the complete database or background list
- **N**: total number of network objects in the complete database or background list
- **Expected**: mean value for hypergeometric distribution (n*R/N)
**Ratio**
connectivity ratio (Actual/Expected)

**z-score**
z-score ((Actual-Expected)/sqrt(variance))

**p-value**
probability to have the given value of Actual or higher (or lower for negative z-score)

**In data set**
fraction of network objects with a selected function in the activated dataset

**In protein function**
fraction of network with a selected function in the activated dataset among network objects with this function in the complete database or background list

**Protein function in database**
fraction of network objects with a selected function in the complete database or background list

**Supplemental Table 1.** Functional categorization of mRNAs regulated by TAC in a microRNA-dependent manner. Direct and indirect mRNA targets (microRNA-dependent and –independent) identified using RISC-seq procedures were classified by MetaCore software 22 into protein functional groups. Although the definition of microRNA-dependent mRNAs was expanded compared to that previously used, the data recapitulate those of our earlier study 8.
**Supplemental Table 2.** microRNA-dependent mRNA regulation during 1 week of TAC in nontransgenic mice. Direct mRNA targets of microRNAs identified using RISC-seq procedures (Expanded Methods) are listed. Cardiomyocyte enrichment ratios for individual mRNAs are calculated as described in Expanded Methods. fc, fold-change; FDR, false-discovery rate; FPKM, fragments per kb of exon per million mapped (aligned) reads. Category designations are UN = RISC up, no global mRNA change; DN = RISC down, no global mRNA change; UD = RISC up, global mRNA down; DU = RISC down, global mRNA up.

**Supplemental Table 2** is supplied as an Excel (.xls) file.

**Supplemental Table 3.** microRNA-independent mRNA regulation during 1 week of TAC in nontransgenic mice. mRNAs not directly targeted by microRNAs identified using RISC-seq procedures (Expanded Methods) are listed. Cardiomyocyte enrichment ratios for individual mRNAs are calculated as described in Expanded Methods. fc, fold-change; FDR, false-discovery rate; FPKM, fragments per kb of exon per million mapped (aligned) reads. Category designations are NU = no RISC change, global RNA up; ND = no RISC change, global mRNA down.

**Supplemental Table 3** is supplied as an Excel (.xls) file.

**Supplemental Table 4.** Cardiomyocyte and nonmyocyte distribution of TAC-regulated mRNAs. mRNAs were classified as cardiomyocyte-enriched, noncardiomyocyte-enriched or nonenriched at an enrichment ratio ≥2, ≤0.5 or 0.5-2.0, respectively (Expanded Methods). mRNA fold-change and FDR (<0.02) are shown for each of 1 and 2 wk TAC vs sham.

**Supplemental Table 4** is supplied as an Excel (.xls) file.

**Supplemental Table 5.** Cardiomyocyte and nonmyocyte distribution of TAC-regulated microRNAs. MicroRNAs were classified cardiomyocyte-enriched, noncardiomyocyte-enriched or nonenriched at an enrichment ratio ≥2, ≤0.5 or 0.5-2.0, respectively (Expanded Methods). MicroRNA fold-change and FDR (<0.02) are shown for 1 wk TAC and are reprocessed from NCBI GEO GSE56891.

**Supplemental Table 5** is supplied as an Excel (.xls) file.

**Supplemental Table 6. A) Direct mRNA targets of miR-133a under unstressed conditions.** Direct mRNA targets of the miR-133a transgene in unstressed hearts, identified using RISC-seq procedures (Expanded Methods), are listed (FDR<0.02). Cardiomyocyte enrichment ratios for individual mRNAs are calculated as described in Expanded Methods. fc, fold-change; FDR, false-discovery rate; FPKM, fragments per kb of exon per million mapped (aligned) reads. TargetScan 6.2 predictions were taken from the 2012 mouse conserved and nonconserved site database; an entry in this column denotes the type of miR-mRNA site match. miRDB predictions
were obtained from the 2014 database release; entries in this column denote the mRNA RefSeq transcript predicted to bind to miR-133a. B) mRNAs indirectly regulated by miR-133a overexpression under unstressed conditions. As for A) but without TargetScan or miRDB annotations; category designations are NU = no RISC change, global RNA up; ND = no RISC change, global mRNA down. C) ‘Tracking’ set of direct miR-133a targets and fate in response to TAC. D) ‘Tracking’ set of indirect miR-133a targets and fate in response to TAC.

Supplemental Table 6 is supplied as an Excel (.xls) file.

Supplemental Table 7. A) Direct mRNA targets of miR-378 under unstressed conditions. Direct mRNA targets of the miR-378 transgene in unstressed hearts, identified using RISC-seq procedures (Supplemental Methods), are listed (FDR<0.02). Cardiomyocyte enrichment ratios for individual mRNAs are calculated as described in Expanded Methods. fc, fold-change; FDR, false-discovery rate; FPKM, fragments per kb of exon per million mapped (aligned) reads. TargetScan 6.2 predictions were taken from the 2012 mouse conserved and nonconserved site database; an entry in this column denotes the type of miR-mRNA site match. miRDB predictions were obtained from the 2014 database release; however no matches were found between transcripts predicted to bind to miR-378 and empirical data. B) mRNAs indirectly regulated by miR-378 overexpression under unstressed conditions. As for A) but without TargetScan or miRDB annotations; category designations are NU = no RISC change, global RNA up; ND = no RISC change, global mRNA down. C) ‘Tracking’ set of direct miR-378 targets and fate in response to TAC. D) ‘Tracking’ set of indirect miR-378 targets and fate in response to TAC.

Supplemental Table 7 is supplied as an Excel (.xls) file.

Supplemental Table 8. RISC-derepression of mRNAs in response to microRNA overexpression in cardiomyocytes. mRNAs whose abundance in the RISC fraction decreased, with converse change or no change in the global mRNA fraction (FDR<0.02), in response to the αMHC-miR-133a transgene (A) or the αMHC-miR-378 transgene (B).

Supplemental Table 8 is supplied as an Excel (.xls) file.
Additional References


