Regulation of Cardiac MicroRNAs by Cardiac MicroRNAs

Scot J. Matkovich, Yuanxin Hu, Gerald W. Dorn II

Rationale: MicroRNAs modestly suppress their direct mRNA targets, and these direct effects are amplified by modulation of gene transcription pathways. Consequently, indirect mRNA modulatory effects of microRNAs to increase or decrease mRNAs greatly outnumber direct target suppressions. Because microRNAs are products of transcription, the potential exists for microRNAs that regulate transcription to regulate other microRNAs.

Objective: Determine whether cardiac-expressed microRNAs regulate expression of other cardiac microRNAs, and measure the impact of microRNA-mediated microRNA regulation on indirect regulation of nontarget mRNAs.

Methods and Results: Transgenic expression of pre-microRNAs was used to generate mouse hearts expressing 6- to 16-fold normal levels of microRNA (miR)-143, miR-378, and miR-499. Genome-wide mRNA and microRNA signatures were established using deep sequencing; expression profiles provoked by each microRNA were defined. miR-143 suppressed its direct cardiac mRNA target hexokinase 2, but exhibited little indirect target regulation and did not regulate other cardiac microRNAs. Both miR-378 and miR-499 indirectly regulated hundreds of cardiac mRNAs and 15 to 30 cardiac microRNAs. MicroRNA overexpression did not alter normal processing of either transgenic or endogenous cardiac microRNAs, and microRNA-mediated regulation of other microRNAs encoded within parent genes occurred in tandem with parent mRNAs. MicroRNA regulation by miR-378 and miR-499 was stimulus specific, and contributed to observed mRNA downregulation.

Conclusions: MicroRNAs that modulate cardiac transcription can indirectly regulate other microRNAs. Transcriptional modulation by microRNAs, and microRNA-mediated microRNA regulation, help explain how small direct effects of microRNAs are amplified to generate striking phenotypes. (Circ Res. 2013;113:62-71.)

Key Words: deep sequencing ■ genetics ■ microRNAs ■ transgenic animals

MicroRNAs are important regulators of cardiac homeostasis and stress responses. By destabilizing messenger RNAs that encode proteins within cell metabolism, growth, calcium signaling, and programmed death pathways, microRNAs exert nodal control over critical biological processes. Individual microRNA–mRNA interactions typically only fractionally reduce levels of the target mRNA, leading to the notion that microRNAs are fine tuners of cell functions. However, striking phenotypes can be induced when some microRNA levels are artificially manipulated and, especially, when all forms of a given microRNA are genetically ablated. The disparity between modest individual effects of a microRNA on its direct mRNA targets and the dramatic end-organ changes that can be provoked by microRNA gain- or loss-of-function studies have been partially explained by targeting of transcriptional regulators by stress-regulated cardiac microRNAs. We designated the orchestration of nontarget mRNA levels through regulation of transcription pathways as epitranscriptional regulation.

Like all RNAs, microRNAs are products of transcription. It is, therefore, almost inescapable that microRNAs that regulate gene transcription also regulate the expression of some other microRNAs. An example of this type of microRNA-mediated microRNA regulation is the cross-regulation that occurs between members of the microRNA originating from myosin heavy chain gene (myomiR) family of microRNAs that are encoded within myosin heavy chain genes. Despite a strong logical foundation and the example of myomiRs, other microRNAs have been almost completely overlooked as microRNA targets. Yet, microRNA-mediated microRNA regulation is being detected when properly assayed. Thus, there is a critical need to determine whether cardiac microRNA–mediated microRNA regulation is unique to myomiRs. If such regulation is common, then these events illuminate a mechanism by which specific microRNAs can recruit to RNA-induced silencing complexes (RISCs) and suppress miRNAs that are not their direct targets, that is, indirect mRNA targeting.

Here, we investigated the potential for 3 structurally and functionally distinct cardiac-expressed microRNAs to direct microRNA-mediated cardiac microRNA regulation. We applied quantitative, unbiased, and genome-wide analytic techniques to novel mouse models in which microRNA (miR)-143, miR-378, and miR-499 were expressed at levels...
We observe that these microRNAs differ in their individual capacities to regulate expression of other cardiac-expressed microRNAs (cardiomiRs) in proportion to their modulation of gene transcription pathways. These results uncover a largely overlooked consequence of natural, artificial, or therapeutic microRNA regulation, microRNA-mediated microRNA regulation, that helps to explain unpredictable microRNA functions and that may contribute to off-target effects of synthetic molecules designed to inhibit microRNA function (antagomiR/anti-miRs).

### Methods

#### Mouse Models and Deep Sequencing Assays

Mice with Myh6-driven expression of pre-miR-143 and pre-miR-378 were created as previously described for miR-499. The late-developing cardiomyopathy phenotype of the miR-499 transgenic mice used herein has been reported (TG 15), but its RNA expression signature was not characterized. Procedural and analytic techniques for deep mRNA and microRNA sequencing of mouse hearts have been previously described. Genome-wide significance for multiple mRNA and microRNA comparisons was established as ±25% regulation at a false discovery rate of 0.05.

#### MicroRNA Annotation Using miRBase 19

Previous nomenclature for microRNAs often described the minor product (passenger strand) of a microRNA stem-loop structure as a miR form. In this article, we have annotated mouse microRNAs according to the nomenclature used by miRBase 19, released in August 2012 (http://www.mirbase.org/). miR base pairs; pre-miR-378: 926 base pairs) were amplified from mouse genomic DNA and expressed using the Myh6 promoter (Figure 2A). This system drives cardiac miR expression shortly after birth, which avoids potential confounding developmental effects of embryonic microRNA expression. miR-499 was expressed using the same system. The cardiac phenotypes of different miR-499 cardiac transgenic mouse lines have been described, but current studies used a line (TG 15) in which the transcriptional and posttranscriptional characteristics were not previously determined. Each of the 3 transgenic microRNAs was expressed at levels ranging from 6 to 16 times their respective endogenous levels in nontransgenic littersmates (Figure 2B), which is within the range observed in cases of human heart failure. All transgenic mice were born at expected frequencies. miR-143 and miR-378 transgenic hearts were functionally normal by echocardiographic examination at 8 weeks of age, and were studied at that age (Online Figure I). Because miR-499 induces cardiac hypertrophy and failure after 8 weeks, and hypertrophy/failure can primarily alter microRNA and mRNA expression, we studied functionally normal hearts from 4-week-old miR-499 transgenic mice and age-matched littermate controls. Comparing the results of miR-499 mice at 4 weeks and miR-378 and miR-143 mice at 8 weeks was uncomplicated, because the levels of each microRNA used for transgenic overexpression are stable between those ages (Figure 5A).

#### Transcriptional Redirection by Cardiac-Expressed miR-143, miR-378, and miR-499

We interrogated the effects of miR-143, -378, and -499 on cardiac mRNA levels using deep RNA-sequencing. A total of 1903 cardiac mRNAs were regulated by any expressed microRNA. Volcano plots depicting regulated mRNAs in each of the microRNA transgenic mouse lines are shown in Figure 3A. It is evident that miR-143 overexpression had little effect on cardiac mRNA levels at our prespecified levels of significance (25%-fold change; false discovery rate<0.05). By contrast, miR-378 and miR-499 each regulated large numbers of cardiac mRNAs (Online Table for these 3 cardiomiRs have not been determined in adult heart myocyte and nonmyocyte cell populations, we isolated cardiomyocytes and nonmyocytes from hearts of normal adult mice. As shown in Figure 1B, all 3 microRNAs are measurable in cardiac myocyte RNA. miR-143 was slightly more abundant, and miR-499 was not detectable, in the nonmyocyte myocardial fraction.

To quantify these 3 cardiomiRs in context, we performed deep sequencing of microRNAs from wild-type, 8-week mouse hearts. The 100 most abundant cardiomiRs are shown in Figure 1D and data on a further 200 cardiomiRs are shown in Online Figure III. Similarly to previous determinations using a separate cohort of mice, miR-143 was 1 of the 2 most abundant cardiomiRs, miR-378 was the 4th most abundant, and miR-499 was the 47th most abundant cardiomiR.

### Results

#### Characteristics of miR-143, miR-378, and miR-499 in Mouse Hearts

We interrogated 3 abundant cardiomiRs previously reported to be increased in heart disease: miRs-143, -378(a), and -499. The regulatory and structural diversity (Figure 1A) of these microRNAs suggest different actions (reviewed by Condorelli et al). The muscle-specific myomiR miR-499 is upregulated in transcriptional and post-translational regulation of pathological hypertrophy. Ubiquitous miR-378 is upregulated in human heart failure and decreased or unchanged in murine cardiac hypertrophy, and has been implicated in cardiac regulation of systemic metabolism. miR-143 is upregulated in human heart failure, but not in murine pressure-overload hypertrophy. miR-143 is highly expressed in vascular smooth muscle and in cardiomyocytes of the developing heart. Because the relative expression levels of mature microRNAs
A heat map of cardiac mRNA levels in miR-143 mice (Figure 3B) confirms that the transcript profile is virtually indistinguishable from that of nontransgenic littermates, with only 17 mRNAs significantly regulated (1 of these is the previously determined miR-143 target, hexokinase 225–28; Online Table I). By contrast, unsupervised hierarchical clustering of miR-378 and miR-499 transgenic mice and their respective nontransgenic controls reveals distinct mRNA signatures induced by miR-378 (954 regulated mRNAs) and miR-499 (1636 regulated mRNAs; Figure 3C and Online Table I). Thus, these 3 microRNAs display varying abilities to regulate cardiac mRNA levels. The observation that ≈50%

Figure 1. Characteristics of 3 cardiac-expressed microRNAs. A, Sequence alignment of guide strands for microRNA (miR)-143, -378, and -499. Box denotes seed sequences. B, MicroRNA real-time reverse transcription polymerase chain reaction (RT-qPCR) from cardiomyocytes, denoted as C (solid lines, amplification curves; black bars, summary graphs) and nonmyocytes, denoted as N (dashed lines, representative amplification curves; white bars, summary graphs). C, mRNA RT-qPCR from cardiomyocytes and nonmyocytes (represented as for B). B and C, fluorescence intensity data are all on the same scale, and bar graphs show the difference in Ct between target and control RNAs (U6 snRNA for microRNAs, Gapdh for mRNAs); n=3 biological replicates, mean±SEM. D, Contextual abundance of cardiac-expressed microRNAs (cardiomiRs) guide (black) and passenger (white) strands in 8 weeks mouse hearts; the most abundant 100 cardiomiRs are shown (Rpm [Reads per Million], log₁₀ scale). Black bars designate guide strands (major forms), white bars designate passenger strands (minor forms); further discussed in Figure 5. Abundances of the remaining cardiomiRs are shown in Online Figure III. Arrows indicate positions of guide strands for miR-143 to -378, and -499.

Figure 2. Forced expression of microRNA (miR)-143, -378, and -499 and effects on parent and guide strand abundance. A, Schematic diagram of constructs for miR-143 and miR-378(a) cardiac transgenic mice. Black and white bars in stem-loop structures indicate positions of guide and passenger strands, respectively. B, Overexpression of miR-143, -378, and -499 in their respective transgenic lines. Top is absolute quantity in reads per million (Rpm) microRNA reads. Bottom shows relative fold-increase in expression of transgenic (black) compared with respective littermate nontransgenic controls (white). Number of hearts used for each determination is shown in lower. All are significant at false discovery rate <1E-20. cMHC indicates α-myosin heavy chain.
of all miR-378-regulated mRNAs and 40% of miR-499-regulated mRNAs were increased (rather than destabilized and decreased) reveals epitranscriptional effects of these 2 cardiac microRNAs (Online Table I).7,11,19

MicroRNA Regulation of Cardiac microRNAs
To further assess gene expression control by miR-143, miR-378, and miR-499, we performed a systems analysis of the transcriptional signatures they invoke in mouse hearts. Principal components analysis of transcriptomes from each transgenic line validated the conclusion that miR-378 and miR-499 cardiac mRNA signatures are not only different from controls, but are remarkably distinct from each other (Figure 4A). By contrast, the miR-143 mRNA signature clustered together with the nontransgenic transcriptomes (Figure 4A). Thus, cardiomyocyte miR-143 overexpression does not meaningfully impact overall cardiac gene expression, whereas miR-378 and -499 do, but with different consequences.

MicroRNAs are encoded in the genome either as independent nonprotein-coding genes (∼56% of all mouse miRs) or within miRtrons, that is, microRNA-containing introns of protein-coding genes.29 Regardless, microRNA expression is directed by cis elements that bind transcription factors. Therefore, microRNAs that directly or indirectly modulate transcriptional activity have the potential to regulate the expression of genes encoding other microRNAs. Accordingly, both miR-378 and miR-499 should regulate other microRNAs because they have the capacity to modulate pathways that direct cardiac gene expression. By comparison, miR-143 should have minimal effects on other cardiac microRNAs. We tested this notion by deep sequencing cardiac microRNAs in miR-143, miR-378, and miR-499 transgenic mice. Principal components analysis of the cardiac microRNA expression signatures (absent the 3 overexpressed microRNAs) shows a pattern of clustering that is strikingly similar to the respective mRNA profiles (compare Figure
Unsupervised hierarchical clustering of 300 cardiomiRs according to expression level revealed individual differences in cardiomiR expression profiles invoked by miR-378 and miR-499 (Figure 4C; Online Table II). The same analysis for miR-143 transgenic mice was indistinguishable from nontransgenic mouse hearts (Online Figure II and Table II). Using our prespecified thresholds for genome-wide significance in microRNA regulation (25%-fold change at false discovery rate<0.05), miR-378 regulated 31 cardiomiRs (18 up, 31 down), and miR-499 regulated 17 cardiomiRs (11 up, 6 down), with only miRs-34a, 221, and 222 regulated by both (Figure 4D). These findings indicate that microRNA-mediated microRNA regulation in hearts is a specific response.

**MicroRNA Processing Is Unaffected by Overexpression, but Passenger Strands Are Rendered More Likely to Have Biological Effects**

MicroRNAs are transcribed, processed, and exported from the nucleus as hairpin premicroRNAs, and then further processed into a duplex encoding 2 microRNA strands with different sequences. In most instances, the mature dominant miR (guide strand) is incorporated into RISCs where it targets mRNAs with compatible sequences; the other (passenger) strand is rapidly degraded and consequently is far less abundant. Some microRNA -5p and -3p strands are both inserted into RISCs where they each target their respective complementary mRNAs (reviewed in 32). A recent report described retention of both miR-378 strands and, therefore, dual functionality in heart and other tissues.19

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**Figure 4. Transcriptome-wide mRNA and microRNA effects of microRNA (miR)-143, -378, and -499.**

A, Principal components analysis of ≈9500 cardiac-expressed mRNAs (cardiomisRs) in microRNA transgenic (TG) hearts and nontransgenic (ntg) controls. B, Principal components analysis of 300 cardiomisRs in microRNA transgenic (TG) hearts and nontransgenic (ntg) controls. In each data set, the overexpressed microRNA was excluded from analysis. C, Unsupervised hierarchical clustering of expression levels for the 300 cardiomisRs in miR-378 and miR-499 transgenic hearts, compared with littermate controls. D, Exploded heat maps depicting unsupervised clustering according to expression levels of microRNAs regulated at genome-wide significance in miR-378 and miR-499 transgenic hearts, compared with littermate controls. All microRNAs shown represent guide (major) strands of the respective microRNAs, with the exception of miRs-214 and -455, for which both guide and passenger strands were regulated and sufficiently abundant for inclusion within the set of 300 cardiomisRs. C and D, Blue indicates downregulation, whereas red indicates upregulation relative to mean level for each microRNA.
We determined levels of miR-143-3p, -378-3p, and -499-5p (the microRNA guide strands) and passenger strands in normal 4-week-old and 8-week-old hearts from our deep sequencing data. The 5p/3p ratio of each of these cardiomiRs was constant; the dominant guide strands are expressed at levels ≈1 order of magnitude greater than passenger strands (Figure 5A). An expanded analysis comprising the 300 most abundant cardiomiRs revealed that the pattern of a highly expressed guide strand and a less abundant passenger strand applies across all the cardiomiRs (Online Figure III). Remarkably, 85 of the 300 most abundant cardiomiRs are passenger strands, and 12 passenger strands are expressed at levels that place them within the 100 most abundant cardiomiRs (Figure 1D), suggesting contextual functionality for at least some cardiomiR passenger strands.

As with normal microRNA transcription, transgenic overexpression of microRNA precursors produces both guide and passenger microRNAs (see Figure 2A). Thus, transgenic passenger microRNA strands have the potential to be expressed, incorporated within RISCs, and to suppress mRNAs having complementary sequences within their 3' untranslated regions. We tested whether forced microRNA expression perturbs normal elimination of passenger microRNA strands by quantifying miR-143, -378, and -499 passenger and guide strand levels in our transgenic mouse hearts. The respective minor strand microRNAs were preferentially eliminated, maintaining normal ratios of guide and passenger strand even with overexpression (Figure 5B). Furthermore, the strong correlation of major-to-minor microRNA ratio for microRNAs regulated by miR-378 or miR-499 (Figure 5C) shows that these 2 cardiomiRs do not significantly perturb normal processing or stability of the cardiomiRs they regulate and support a mechanism that involves regulation of transcription. Nevertheless, the absolute levels of transgenically overexpressed miR-143, -378, and -499 passenger strands move them to the 102nd, 23rd, and 91st most abundant cardiomiRs in their respective transgenic hearts (Figure 5D). Although overexpression of the already highly abundant miR-143 and -378 guide strands barely changed their ranking among cardiomiRs, the somewhat less abundant miR-499 guide strand moved from the 51st to the 11th most abundant (Figure 5D). The law of mass action dictates that the proportional effect of overexpressed minor microRNAs will be small relative to the respective coexpressed major microRNAs, but transgenic minor microRNAs may nevertheless have measurable effects. We, therefore, identified passenger strand effects according to sequence complementarity and in comparison with biological data (Online Table III).

**Higher Order Consequences of MicroRNA-Mediated CardiomiR Regulation**

We identified 13 miR-378– or miR-499–regulated microRNAs that are encoded within introns of known parent genes...
(Figure 6A). Considering that a transcriptional mechanism of microRNA regulation should exert similar modulatory influences on the regulated cardiomiR and its parent mRNA, we compared the relative change induced by miR-378 and miR-499 on parent gene mRNA with that of the respective daughter cardiomiR. In almost every instance, the parent mRNA and daughter microRNA are coregulated (Figure 6A). Because the parent mRNA and daughter microRNA are products of the same transcriptional event, their coregulation supports transcriptional regulation.

To determine the extent to which microRNA-regulated microRNAs contribute to the altered transcriptomic profiles observed in miR-378 and miR-499 transgenic hearts, the FastamiRs algorithm was used at high stringency (see Online Methods) to predict binding between the upregulated microRNAs (including the transgenic microRNAs) and downregulated mRNAs. A total of 468 mRNAs were downregulated in miR-378 transgenic hearts compared with controls (Figure 6B, left and center). Seventy-four different mRNAs (16% of all downregulated mRNAs) are targeted by miR-378-3p or miR-378-5p, whereas a further 216 different mRNAs (46%) are targets of the other 3 upregulated microRNAs (Figure 6B, right; Online Table III). In miR-499 transgenic hearts (Figure 6C), 969 mRNAs were downregulated, with 376 of these being predicted targets of upregulated microRNAs. Seventy-six different mRNAs (7.8%) are targets of miR-499-5p or miR-499-3p, whereas 298 different mRNAs (31%) are targeted by the other 12 microRNAs (Figure 6C, right; Online Table III). Thus, secondary mRNA regulation by microRNA-regulated microRNAs accounts for ≈75% of the microRNA-dependent transcript downregulation in these transgenic models.

**Discussion**

Here, we describe 2 microRNAs that regulate different sets of other cardiac microRNAs. MicroRNA regulation by other microRNAs has been almost completely ignored when studying mechanisms of microRNA effects. Indeed, the 1 example of microRNA-mediated microRNA regulation within the family of myomiRs seems, since its description in 2007,8,9 to have been treated as a unique case rather than as an indication of a broader and more general function of microRNAs. Certainly microRNA-mediated microRNA regulation has not previously been assigned mechanistic importance outside of the special case of the myomiR family. The current studies show that, together with direct mRNA suppression,1,32 indirect regulation of mRNA transcription,7 and regulation of post-translational protein modification,11 microRNA-mediated microRNA regulation can be a major consequence for some microRNAs.

**Figure 6.** Regulated microRNA–mRNA interactomes in microRNA (miR)-378 and miR-499 hearts. **A**, Parent gene and encoded microRNA levels are regulated similarly by microRNAs. Fold-changes of parent gene (white bars) and encoded microRNA (black bars) are shown for miR-378 and miR-499-regulated microRNAs. *Significant regulation of parent mRNA at genome-wide significance for multiple comparisons. B and C, Heat maps of microRNA and mRNA regulation in miR-378 (B) and miR-499 (C) transgenic hearts and secondary consequences of regulated microRNAs on cardiac transcript levels. **Left,** mRNAs downregulated in transgenic hearts by the primary microRNA. **Center,** microRNAs secondarily upregulated in transgenic hearts. **Right,** mRNAs predicted by FastamiRs to be targeted by each secondarily regulated microRNA (Online Table III). Colors represent abundances of microRNAs and mRNAs.
These findings provide further insight into how microRNAs with modest direct effects can provoke dramatic end-organ phenotypes.6

In designing experiments to assess microRNA-mediated microRNA regulation in the in vivo mouse heart, we chose to study 3 highly expressed and disease-regulated cardiac microRNAs. MiR-143 is one of the most abundant mouse cardiomiRs and is highly expressed in vascular smooth muscle, but virtually nothing is known about its actions in cardiac myocytes. Based on our previous studies examining microRNA expression and regulation in early cardiac hypertrophy, we classified miR-143 as one of the housekeeping microRNAs because it is constitutively expressed at high levels, it regulates essential cellular functions (in other tissues), and was not regulated during early cardiac hypertrophy.7 Abnormal miR-143 activity has been linked to several cancers via destabilization of hexokinase 2.25-27 In our studies, forced expression of miR-143 suppressed its direct target hexokinase 2 by ≈30%, but had virtually no effect on other cardiac mRNAs. Consistent with absence of epitranscriptional activity, cardiomyocyte-specific miR-143 overexpression did not regulate the expression of other cardiomiRs. Of course, miR-143 might have epitranscriptional effects on mRNAs and other microRNAs in other cell types.28,33,34

In contrast to miR-143, miR-378 and miR-499 each regulated hundreds of cardiac-expressed mRNAs and a dozen or so cardiac microRNAs. These 2 stress-modulated cardiomiRs direct biological pathways, broadly orchestrating either metabolic remodeling (miR-378)39 or hypertrophic remodeling (miR-499).36 Indeed, we included miR-499 as the most likely direct candidate to prove our hypothesis that microRNAs directing transcriptional signaling would also regulate expression of other microRNAs.31 Although both miR-378 and miR-499 were capable of regulating other cardiomiRs, the pattern of their microRNA regulation showed almost no overlap. As illustrated by their respective principal components analyses, the different microRNA expression signatures induced by these 2 microRNAs mirrored their different transcriptional signatures. For example, 2 microRNAs that contribute to the molecular signature of doxorubicin toxicity, miR-34c and miR-208b,35 are induced by miR-499 but not by miR-378. As previously reported by the Olson laboratory,6 we detected regulation of miRs-208a and 208b by miR-499, and greatly expanded the number of miR-499-modulated cardiomiRs beyond interactions within this specific microRNA family.

In the absence of evidence for differential processing, and with data showing parallel regulation of microRNA and parent mRNA, our findings reveal microRNA-mediated microRNA regulation to be an epitranscriptional event.7 Based on these findings, we would anticipate that other microRNAs with modulatory effects on cardiac gene expression, such as miR-1 and miR-22,36 also have significant effects mediated via their regulation of other cardiac microRNAs. An important consequence of uncovering microRNA-mediated microRNA regulation is the recognition that RISC-enriched transcripts in microRNA-programmed hearts/tissues/cells represent direct mRNA targets of both the primary (transgenic) microRNA and any secondarily increased microRNAs.7,11,13,37 This does not represent a flaw in the approach of measuring RISC-associated microRNAs to define microRNA targets, but does suggest that concomitant knowledge of microRNA expression signatures is essential to properly interpreting RISC-Seq data. Thus, because the indirect effects of microRNAs include regulation of both mRNA transcription and microRNA transcription, we believe that formal assessment of microRNA-mediated microRNA regulation is essential to understand microRNA effects and phenotypes.

Our observation that some microRNAs regulate other microRNAs adds another layer of complexity to the mechanisms by which these simple molecules direct biological processes. Indeed, some authorities in the field have expressed concern that the actions of microRNAs are so complex that they can never be fully understood. Although correct from a purely reductionist perspective, we would argue that a linear view of molecular cause and effect ignores the obvious nonlinearity of microRNA signaling. MicroRNAs directly suppress a few mRNAs within target biological pathways. Positive or negative modulation of those pathways induces secondary effects that reverberate throughout the system, amplifying some responses and inhibiting others. miR-499, for example, directly targets phosphatases that post-translationally regulate transcriptional pathways,36 thereby broadly regulating cardiac gene expression. Indeed, transcriptional pathways seem to be preferentially targeted by cardiomiRs induced during hemodynamic stress.7 Now, we must include microRNA-mediated microRNA regulation as another higher order event that contributes to the ultimate effects of a given microRNA (Figure 7A). Based on the current results, we can apply this schema to real-world problems of how cardiac genes and microRNAs are epitranscriptionally regulated by miR-378 and miR-499: we bioinformatically identified putative transcription factor binding sites in the promoters of microRNA-regulated microRNAs and parent genes (Figure 4D; Online Tables IV and V) using Multi-genome Analysis of Positions and Patterns of Elements of Regulation,38 and delineated transactivating factors targeted by the respective microRNAs. Thus, miR-99a seems to be indirectly downregulated by miR-378 via its direct targeting of v-maf musculoaponeurotic fibrosarcoma oncogene homolog and retinoic acid receptor-related orphan receptor A. In turn, miR-99a directly targets 31 cardiac-expressed mRNAs (according to TargetScan predictions), accounting for their indirect regulation by miR-378 (Figure 7B). Further examples are in Online Table V. Given that the majority of microRNA effects are not the direct result of mRNA suppression, we suggest that terms such as off-target and nonspecific be abandoned in favor of indirect or higher order to reflect our observations that these effects are normal and real, but simply not predicted by available analytics.

The complexity and nonlinearity of microRNA effects does not preclude comprehension of their actions. Rather, like a Mandelbrot set in fractal geometry, where visual complexity results from simple mathematical rules,39 understanding simply requires a different approach than standard reductionism. Biological systems are sufficiently complex that they cannot be adequately described in terms of individual functioning units. But if the rules that govern the behavior of these units are understood, then the system-wide responses are predictable. It is the complexity of microRNA actions that
makes them therapeutically attractive, because parallel effects on multiple targets bypass the usual resiliency of biological systems to interventions that target a single factor. In this context, microRNA-mediated microRNA regulation is one of the consequences of microRNA-directed therapeutics to consider as they move toward clinical applications.

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Disclosures
None.

References
12. Matkovich SJ, Van Booven DJ, Youker KA, Torre-Amione G, Diwan A, Eschenbacher WH, Dorn LE, Watson MA, Margulies KB, Dorn GW 2nd. Reciprocal regulation of myocardial microRNAs and messenger RNA in...


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**Novelty and Significance**

**What Is Known?**

- MicroRNAs capture their direct mRNA targets via complementary nucleotide sequences and recruit them into RNA-induced silencing complexes to be rendered translationally incompetent.
- The overall effect of some microRNAs on the mRNA signature is driven by regulation of indirect mRNA targets that are not directly bound, rather than direct target suppression.
- The mechanisms for indirect mRNA regulation by microRNAs are unclear, but are thought to contribute substantially to observed phenotypes.

**What New Information Does This Article Contribute?**

- In mouse hearts, 2 microRNAs with numerous indirect mRNA targets, but not a microRNA with little indirect target activity, regulated expression of numerous other cardiac myocyte microRNAs.
- For microRNA (miR)-378 and miR-499, ≈30% to 50% of indirect cardiac mRNA target regulation is attributable to higher order effects from secondarily regulated microRNAs.
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Supplemental Material

Detailed Methods

*Generation and characterization of microRNA transgenic mice.* miR-143 and miR-378 transgenic mice were created via cloning genomic DNA fragments flanking microRNA stem-loop precursor regions (lengths as shown in Figure 2a of the main text) into the Sall/HindIII cloning site of the αMHC/Myh6 cardiac transgenic promoter construct. Mice were housed according to procedures approved by the Washington University Institutional Animal Care and Use Committee. miR-143 and -378 overexpression in hearts were initially measured using Applied Biosystems TaqMan microRNA qPCR assays with U6 snRNA as reference RNA (data not shown); later microRNA-Seq analysis (see below), as documented in the manuscript, found similar overexpression levels.

*Mouse adult cardiomyocyte isolation and gene expression analysis.* Cardiomyocyte and nonmyocyte fractions were separately isolated from the hearts of three 8 week-old, wild-type FVB/N mice, as previously described. 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 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. Applied Biosystems TaqMan microRNA and mRNA qPCR assays were performed as previously described, with the following mRNA expression probes:

- **Actc1:** Mm01333821_m1
- **Col1a1:** Mm00801666_g1
- **Gapdh:** Mm99999915_g1
- **Myh6:** Mm00440359_m1
- **Tpm1:** Mm00600378_m1

*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. Briefly, small RNAs from 1 μg total mouse heart 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 quantification of microRNA sequencing reads was performed by following the E-miR pipeline as described with minor modifications to the EmiR-Bowtie.pl Perl script to allow the Bowtie aligner to correctly map those microRNAs that arise from multiple genomic loci.

*microRNA annotation using miRBase 19.* 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 19, released in August 2012 (http://www.mirbase.org) with minor modifications to the EmiR-Bowtie.pl Perl script to allow the Bowtie aligner to correctly map those microRNAs that arise from multiple genomic loci.

MicroRNA forms 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 have designated ‘major’ and ‘minor’ forms in mouse hearts according to the deep-sequencing data that we obtained for this paper.
mRNA-Seq for mRNA expression analysis. mRNA-sequencing was performed essentially as described 2,7-9, using Illumina HiSeq 2000 sequencers and library indexing rather than bar-coding. 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) 2,7-9, 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 9,500 coding mRNAs in mouse hearts. Concordance of these cardiac transcriptomes with those from previous RNA-Seq studies was high 2,7-9.

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 2). In addition, we have validated differential expression results from sequencing analyses with RT-qPCR techniques in several prior studies 2,7,10.

Calculation of differential gene expression using the DESeq package. In comparison to previous analyses using Partek Genomics Suite to compare FPKM values of individual mRNAs 1,6-8, we used DESeq 11 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). This statistical approach, using a negative binomial distribution in comparison to the normal (Gaussian) distribution used by Partek on log-transformed data, is designed to be more robust in contexts where relatively few genes are highly abundant and there is a majority of less abundant genes. While DESeq takes the non-normalized, absolute number of aligned reads as input, we have reported microRNA and mRNA abundance in the main text and remainder of the Online Information as Reads per Million aligned to microRNA species (RPM) for microRNAs, and FPKM for mRNAs, although the underlying fold-change and p-value / FDR comparisons used DESeq’s internal methods of library normalization. We compared the DESeq method to Partek methods using FPKM and found similar direction and magnitude of changes in individual mRNAs (data not shown). However, DESeq tended to be more conservative in its assignment of p-values and FDRs.

Cardiac microRNAs and mRNAs regulated in microRNA-transgenic mice were defined using a threshold of 25% (increased or decreased) and a FDR of 0.05. Partek Genomics Suite 6.6 (Partek, St. Louis, MO) was used to derive principal components analysis plots, fold-change vs p-value volcano plots, and unsupervised hierarchical clustering heatmaps.

Explanation of principal component analysis plots: (used in Figures 4a and 4b as exploratory data analysis tools). Principal Components Analysis (PCA) is an exploratory multivariate statistical technique for simplifying complex data sets 12-14. The number of principal components is less than or equal to the number of original variables. This transformation is defined in such a way that the first principal component has the largest possible variance (that is, accounts for as much of the variability in the data as possible), and each succeeding component in turn has the highest variance possible under the constraint that it be orthogonal to (i.e., uncorrelated with) the preceding components. Principal components analysis has been used in a wide range of biomedical problems, including the analysis of microarray data in search of outlier genes 15 as well as the analysis of other types of expression 16,17. We and others use this data analysis method and graphical presentation for our RNA-sequencing work (see Figure 4 of 7 and the Supplemental Methods of 2) because it offers a compact way of viewing a great deal of (multidimensional) data in a fairly intuitive fashion. The above description is adapted from 18.

Informatic analyses. To compare the numbers of likely mRNA targets of primarily and secondarily regulated microRNAs (Figure 6, main text) we used our previously described FastamiRs algorithm 2 (http://epigenomics.wustl.edu/edwardsLab/index.php/downloads), since it permits prediction of targets for microRNA passenger or minor strands that are not present in the current TargetScan database.
Overexpressed or upregulated microRNAs were used as input to search the set of downregulated mRNAs (defined using the genome-wide significance criteria above); separate processing was carried out for miR-378 and miR-499 TG mice. Putative target mRNAs were stringently defined as those with a perfect match to the microRNA nucleotide 2-7 ‘seed’ region (G:U base pairing was excluded from consideration on the basis of recent work demonstrating that G:U pairing is poorly tolerated in the seed region for fly Ago2\textsuperscript{19}), a stretch of at least 7 contiguously paired nucleotides (which may include the ‘seed’), and with miR binding in the 3’UTR rather than in coding sequences or the 5’UTR.

miR-378- and miR-499-regulated transcription factor binding analysis for promoter regions of regulated miRs, Figure 7: we first used the RIKEN transcription factor database (http://genome.gsc.riken.jp/TFdb, accessed 11/5/12) to identify 1678 transcription factors or accessory proteins, and found 869 of these to be expressed in hearts (using the same abundance criteria we used in our other RNA-seq data throughout the paper). We then obtained predictions for miR-378 or miR-499 binding from the latest version of TargetScan (v6.2), resulting in 15 and 21 potentially microRNA-regulated entities, respectively (Online Table IV). Finally, the transcription factor-binding prediction database MAPPER\textsuperscript{20} (http://bio.chip.org/mapper) was used to identify putative binding sites 10 kb upstream of the start of the pre-miR, or for those cases in which the pre-miR was present in the intron of a host gene, 10 kb upstream of the transcription start site (Online Table V).

‘Defined miR-378 targets’ in Figure 7 (light red box) are derived from Online Table III, and are those mRNAs predicted by FastamiRs to be downregulated by miR-378-3p and by no other upregulated microRNA. ‘Indirect miR-378 targets’ in Figure 7 are those mRNAs which were upregulated in miR-378 transgenic hearts (Online Table I). miR-99a targets were those mRNAs predicted by TargetScan v6.2, and known to be expressed in the heart from our deep sequencing data. The categories of ‘muscle growth and development’, ‘muscle contraction’ and ‘metabolism’ were obtained from MetaCore (http://thomsonreuters.com/products_services/science/systems-biology/) / GeneOntology analysis of the defined and indirect miR-378 target gene lists, and the miR-99a TargetScan gene list.
Online Figure I.  *Echocardiographic examination of miR-143 and miR-378 transgenic hearts at 8 weeks of age, relative to littermate controls.* Representative M-mode echocardiograms on microRNA transgenic hearts and age- and sex-matched littermate controls.
Online Figure II. Transcriptome-wide microRNA effects of miR-143, -378, and -499. Unsupervised hierarchical clustering of expression levels for the 300 cardiomiRs in miR-143, -378 and -499 transgenic hearts, compared to littermate controls. Blue indicates downregulation while red indicates upregulation relative to mean level for each microRNA.
Online Figure III. *microRNA deep sequencing in nontransgenic mouse hearts.* Contextual abundance of cardiomiR guide (black) and passenger (white) strands in 8 wk mouse hearts. A, most abundant 100 cardiomiRs (log$_{10}$ scale); B and C are second and third tiers of 100 cardiomiRs (linear scales). Arrows indicate positions of guide and passenger strands for miR-143 -378, and -499.
Supplemental Tables

**Online Table I** is supplied as an Excel workbook (.xls)

**Online Table I. mRNAs regulated in miR-143, -378 and -499 transgenic hearts.** Regulated mRNAs were defined as those changed by at least 25% at FDR < 0.05, compared to littermate nontransgenics. The mean FPKM (abundance) of each mRNA in nontransgenic hearts is shown, together with fold-change and FDR in transgenic hearts calculated by DESeq. mRNAs are sorted in order of fold-change, from the most downregulated to the most upregulated. Separate Excel tabs are provided for each of the miR-143, -378 and -499 transgenic heart comparisons.

**Online Table II** is supplied as an Excel workbook (.xls)

**Online Table II. Regulation amongst 300 cardiomiRs in miR-143, -378 and -499 transgenic hearts.** Similarly to the mRNA analysis in Online Table I, regulated microRNAs were defined as those changed by at least 25% at FDR < 0.05, compared to littermate nontransgenics. MicroRNA expression is given as DESeq-adjusted ‘baseMeans’, representing the number of sequencing reads obtained for each microRNA, adjusted for sequencing depth (see Detailed Methods). Aligned read counts for microRNA libraries averaged 7.1 x 10^6; thus, any microRNA with less than 7 reads (~1 millionth of the total) was eliminated from further analysis. MicroRNAs are sorted in order of fold-change, from the most downregulated to the most upregulated. Separate Excel tabs are provided for each of the miR-143, -378 and -499 transgenic heart comparisons.

**Online Table III** is supplied as an Excel workbook (.xls)

**Online Table III. Downregulated mRNAs in miR-378 and -499 transgenic hearts, predicted to be targets of primarily and secondarily upregulated microRNAs.** The FastamiRs algorithm^2^ was used with stringent parameters (see Detailed Methods) to predict targeting of downregulated mRNAs by upregulated microRNAs.
<table>
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Online Table IV. Cardiac-expressed transcription factors predicted to be regulated by miR-378-3p or miR-499-5p according to TargetScan v6.2. Transcription factor genes were obtained from the RIKEN transcription factor database, selected for cardiac expression, and filtered for TargetScan-predicted regulation by miRs-378 or -499 (see Detailed Methods).

Online Table V is supplied as an Excel workbook (.xls)

Online Table V: Putative transcription factor binding sites in microRNA promoter regions. Hits predicted by MAPPER 20 in microRNA promoter regions (see Detailed Methods).
Supplemental References


