Loss of cardiac microRNA-mediated regulation leads to dilated cardiomyopathy and heart failure

The MIT Faculty has made this article openly available. Please share how this access benefits you. Your story matters.

<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>As Published</td>
<td><a href="http://dx.doi.org/10.1161/CIRCRESAHA.109.200451">http://dx.doi.org/10.1161/CIRCRESAHA.109.200451</a></td>
</tr>
<tr>
<td>Publisher</td>
<td>Ovid Technologies (Wolters Kluwer) -American Heart Association</td>
</tr>
<tr>
<td>Version</td>
<td>Author's final manuscript</td>
</tr>
<tr>
<td>Accessed</td>
<td>Fri Jan 25 07:43:53 EST 2019</td>
</tr>
<tr>
<td>Citable Link</td>
<td><a href="http://hdl.handle.net/1721.1/73885">http://hdl.handle.net/1721.1/73885</a></td>
</tr>
<tr>
<td>Terms of Use</td>
<td>Creative Commons Attribution-Noncommercial-Share Alike 3.0</td>
</tr>
<tr>
<td>Detailed Terms</td>
<td><a href="http://creativecommons.org/licenses/by-nc-sa/3.0/">http://creativecommons.org/licenses/by-nc-sa/3.0/</a></td>
</tr>
</tbody>
</table>
Loss of cardiac microRNA-mediated regulation leads to dilated cardiomyopathy and heart failure

Prakash K. Rao1, Yumiko Toyama2, H. Rosaria Chiang1, Sumeet Gupta1, Michael Bauer3, Rostislav Medvid4, Ferenc Reinhardt1, Ronglih Liao3, Monty Krieger2, Rudolf Jaenisch1,2, Harvey F. Lodish1,2,5, and Robert Blelloch4,5

1Whitehead Institute for Biomedical Research, Cambridge, MA 02142
2Dept of Biology, MIT, Cambridge, MA 02142
3Division of Cardiology, Brigham and Women’s Hospital, Harvard Medical School, Boston, Massachusetts 02115
4Eli and Edythe Broad Center for Regeneration Medicine and Stem Cell Biology, Department of Urology, University of California, San Francisco, California 94143, USA.

Abstract

RATIONALE—Heart failure is a deadly and devastating disease that places immense costs on an aging society. In order to develop therapies aimed at rescuing the failing heart, it is important to understand the molecular mechanisms underlying cardiomyocyte structure and function.

OBJECTIVE—microRNAs are important regulators of gene expression and we sought to define the global contributions made by microRNAs toward maintaining cardiomyocyte integrity.

METHODS AND RESULTS—First, we performed deep sequencing analysis to catalog the miRNA population in the adult heart. Secondly, we genetically deleted, in cardiac myocytes, an essential component of the machinery that is required to generate miRNAs. Deep sequencing of miRNAs from the heart revealed the enrichment of a small number of microRNAs with one, miR-1, accounting for 40% of all microRNAs. Cardiomyocyte-specific deletion of dgcr8, a gene required for microRNA biogenesis, revealed a fully penetrant phenotype that begins with left ventricular malfunction progressing to a dilated cardiomyopathy and premature lethality.

CONCLUSIONS—These observations reveal a critical role for microRNAs in maintaining cardiac function in mature cardiomyocytes and raise the possibility that only a handful of microRNAs maybe ultimately be responsible for the dramatic cardiac phenotype seen in the absence of dgcr8.

Introduction

A subset of endogenous non-coding small RNAs, known as microRNAs (miRNAs or miRs) are ~22 nucleotides long and modulate gene expression by targeting mRNAs for post-transcriptional repression. There are nearly 500 and 800 microRNAs in mice and humans respectively (http://microrna.sanger.ac.uk). In animals, repression is achieved through imperfect base-pairing between the microRNA and its target mRNA. Although there are certain
rare instances in which microRNAs have been reported to upregulate target gene expression 
3,4, repression is the most well-documented direct effect. The target mRNA is rendered labile 
through mechanisms involving deadenylation/decapping, translational repression or both. 
Target specificity is largely governed by the highly conserved seed region (nucleotides 2-8) of 
the miRNA5. Various target prediction programs have relied on this fact and an estimated 30% 
of the mRNAs are susceptible to miRNA-mediated regulation 6. Although this number is likely 
an overestimate, as it does not take into account the requirement for coexpression of miRNAs 
and mRNAs, it puts into perspective the enormous regulatory potential possessed by 
microRNAs. Not surprisingly, a number of studies have revealed the importance of the 
microRNA pathway as a whole while others have pinpointed specific roles for individual 
microRNAs in various tissues7-18.

Although mature microRNAs are only ~22 nucleotides in length, they are generated from 
longer precursors whose length distribution is similar to that of a mRNA. Indeed, the primary 
transcripts (pri-miRNAs) are transcribed by RNA Polymerase II, capped, polyadenylated and 
regulated by transcription factors like protein-coding mRNAs 19,21. Unlike mRNAs, miRNAs — 
because of their stem-loop structure— are cleaved within the nucleus by a Drosha/Dgcr8 
containing complex into ~60-80 bp precursor miRNAs (pre-miRNAs)22-24. The precursor 
miRNAs are transported out of the nucleus by Exportin-525 and subsequently processed by a 
cytoplasmic RNaseIII--Dicer29—which also resides in a multiprotein complex. Since the PAZ 
domain of Dicer recognizes the two-nucleotide 3′OH overhang generated by Drosha/Dgcr8, it 
is believed that the nuclear Drosha/Dgcr8 cleavage is required for Dicer-mediated cytoplasmic 
cleavage of pre-miRNAs. Exceptions to this general dependence on Drosha/Dgcr8 occurs in 
miRtrons and endogenous shRNAs 27-29 and in these rare cases, other nucleases generate the 
necessary ends for subsequent Dicer recognition and cleavage. Importantly, spatial segregation 
of Drosha/Dgcr8 and Dicer substrates allows for the two cleavage events to occur in a sequential 
manner.

We sought to uncover the regulatory potential of miRNAs in the heart by using two 
complementary approaches. First we catalog the known miRNA population of murine adult 
heart using deep (Solexa/Illumina) sequencing of a small RNA library. Secondly, we disrupt 
miRNA regulation by deleting dgcr8 and hence canonical microRNA biogenesis. We chose 
to focus on mature muscle tissue in order to establish the importance of microRNA function 
in the maintenance (as opposed to the development) of cardiac tissue. Mice lacking dgcr8 in 
muscle tissue die prematurely with signs of heart failure and dilated cardiomyopathy. 
Identification of the depleted microRNAs in dgcr8 deficient hearts led to the refined list of 
miRNA targets that may collectively play an important role in the development of the 
pathological state. Thus, the importance of the microRNA regulation in maintaining 
cardiomyocyte function is revealed by the fatal outcome associated with lack of dgcr8 in 
cardiomyocytes.

**Materials and Methods summary**

Details are included in the online supplement. Briefly a library of small RNAs were generated 
and sequenced using the Illumina platform 30. For the generation of conditional dgcr8 
knockout, floxed dgcr8 mice was crossed with Muscle Creatine Kinase (MCK)-Cre mice 31; 
mutant mice were genotyped using tail DNA by a PCR-based approach. Age and sex-matched 
mutant (2lox/2lox; Cre positive) and control (2lox/+; Cre positive) mice were analyzed 
pathologically; physiological studies were performed using telemetry and echocardiography. 
Molecular analyses were carried out using total RNA isolated from the heart; Northern blots 
were used to detect depletion of miR-1, miR-133 and miR-208. Array-based methods were 
employed to assess global loss of microRNAs.
Results

Deep sequencing of microRNAs from heart tissue

High throughput deep sequencing produces quantitative data with an extensive dynamic range, thereby enabling detailed insight into the relative levels of different microRNA in a particular tissue. Therefore, to gain such insight into the microRNA profile of the adult heart, we isolated small RNAs (16-24 nucleotides) from 6-8 week old male and female hearts, built tagged cDNA libraries and sequenced the libraries on a Illumina Genome analyzer producing over seven million reads from each sample. As has been reported previously, miR-1 and miR-133a were highly abundant (Fig 1A)—however the relative abundance of miR-1 reads was quite striking. miR-1 accounted for nearly 40% of all known microRNA reads. Also noteworthy is the fact that other microRNAs including miR-29a, miR-26a, let-7 family members, were more abundant than miR-133a. MicroRNAs from non-cardiomyocytes (miR-29a and miR-29c from fibroblasts 32, miR-126 from endothelial cells9,33) also contributed to the library as expected since the different cell types in the heart were not separated. Within the cardiac-specific miR-208 subsets, 50-100 times more reads were obtained for miR-208a (encoded within an intron of Myh6) when compared to miR-208b (encoded within an intron of Myh7), consistent with the relative overexpression of Myh6 compared to Myh7 in adult mice. miR-22 was highly expressed and showed gender-based differences in expression levels. While sexually dimorphic gene expression patterns in somatic tissues 34 has been established34; follow-up experiments will need to be carried out to confirm sex-based differences in miRNA expression in the heart. Reads from the miR-378 hairpin were also high; miR-378/378* (miR-378* is the same as miR-422b) is encoded within an intron of the PGC1b gene. Since PGC1a and PGC1b regulate mitochondrial biogenesis and the heart is a mitochondria-rich organ, the high expression levels of miR-378/miR-378* probably reflects the high endogenous levels of PGC1b transcription. Since the ability of a miRNA to repress target gene expression is largely dependent on the 5′ end of the miRNA, multiple miRNAs with identical 5′ ends are expected to function in a similar manner. This seed identity is the basis by which microRNAs are grouped into families. Therefore, we tabulated all the microRNA reads within individual families (as defined by TargetScan 4.1; www.targetscan.org) (Fig 1B). By this analysis, the miR-1/206 family still emerged as the most dominant microRNA family (the reads from miR-206 were insignificant). Since a considerable number of reads were obtained individually from members of the let7/miR-98 and miR-30a-5p family, these families were respectively, the second and third most abundant microRNA families in the heart.

Muscle-specific Dgcr8 knockout

The importance of the microRNA pathway during development has been largely inferred from studies in which Dicer has been deleted 18,35-39. As dicer has roles outside of the canonical miRNA pathway, we sought to block microRNA maturation (and therefore microRNA-mediated regulation) using another component of the microRNA biogenesis pathway, namely Dgcr8. Dgcr8 deletion in embryonic stem cells has revealed that it is essential for microRNA biogenesis and implicate microRNAs in regulating efficient ES-cell differentiation 40. Using MCK-Cre mice31 and a conditional floxed allele of dger8, we generated mice with a muscle-specific deletion of the dger8 gene. Endogenous MCK expression reportedly peaks around birth and declines to 40% of peak levels by day ten 31. This Cre line was deliberately chosen to match our interest in specifically disrupting microRNA biogenesis in mature, differentiated muscle as this allowed us to determine the importance of the microRNA pathway in muscle homeostasis. Genotyping analysis showed that although mutant (2lox/2lox; Cre positive) mice were slightly underrepresented at the time of genotyping, most mutant mice survived to at least 12 days after birth. We did not observe any pathology on 4-chamber sections (H & E stained) at two weeks of age. At three weeks of age, we detected fibrosis in the ventricular wall in all mice examined, and loss of ventricular function (as revealed by trans-thoracic
echocardiography—see below). Subsequently, all mutant mice died before two months of age and the median survival was 31 days (Figs. 2A & 2B). At end stage, the hearts of mutant mice showed marked decreases in the thickness of the left and right ventricular walls. Therefore the development of the pathology is quite rapid and highly penetrant. This demonstrates the stringent requirement for a threshold level of microRNAs below which heart function rapidly deteriorates.

In order to determine the extent of microRNA depletion in the heart, we performed Northern Blot and quantitative RT-PCR analyses to quantify cardiomyocyte-specific microRNAs (Fig. 2C) with RNA derived from the heart tissue of mutant and control (2lox/+; Cre positive) mice. At the time of sacrifice (when mutant mice were moribund), Northern Blot analysis showed that three cardiac-enriched mature microRNAs (miR-1, miR-133a, and miR-208) were dramatically depleted, but not completely absent, in mutant heart tissue. Quantification of the Northern blots revealed that depending on the microRNA, the mature forms were depleted 10 to 60 fold (Fig. 2C, bottom). Their precursor miRNAs (the ~ 60 base pair product of Drosha/Dgcr8 cleavage) were detectable in the control lanes and absent in the mutant lanes (Fig. 2D; shown for miR-208). The complete loss of the short-lived ~60 bp precursor, but not mature miRNA favors the argument that the residual amount of mature miRNA detected is due to its long half-life, rather than an incomplete excision of dger8 in these tissues.

The hearts of the mutant animals exhibited a variety of abnormalities that suggest cardiac dysfunction was responsible for their premature death. Preliminary ECG analysis of revealed dramatic drops in the heart rate of mutant mice along with an increased PQ interval and QRS width (all at end stage) indicative of a cardiac conduction defect (Online Fig S4). Histopathological analysis revealed that the hearts obtained from end-stage mutant mice were considerably enlarged with notable thinning of the ventricular walls (Fig. 3A & 3B; note end-stage mutant hearts). Fibrosis was also evident (Fig. 3C), an early and consistent pathological finding as it is observed in all mice at about three weeks of age (at which time there was no histopathologically obvious defect in the thickness of the ventricular wall; Fig. 3B and 3C). Quantitative RT-PCR analyses of cardiomyocyte-specific microRNAs was also carried out at end stage and at two weeks after birth. Precipitous decreases in miR-1, miR-133a and miR-208 levels was detected in two week old mice (Fig. 3D) and this preceded any pathophysiological changes that we observed.

In order to assess left ventricular function, we performed echocardiography. We conducted these studies at two timepoints: three weeks and four weeks after birth as histopathological analysis showed a dramatic progression between these two timepoints from mild fibrosis with otherwise no overt ventricular/wall defects (at three weeks) to extensive dilation (at four weeks). Accordingly, measurement of fractional shortening (FS) revealed that the mutant mice had dramatically reduced ventricular function at four weeks (Online Fig. S6); although wall thickness was not significantly different. This finding was not surprising considering the clear histopathological defects at this timepoint. The expectation at three weeks (Fig. 4B) was more ambiguous since we noted fibrosis at this point but did not see an obvious defect in wall thickness or ventricular volume in tissue sections. However, echocardiography at three weeks after birth, revealed that ventricular function (as assessed by FS readings) was decreased in mutant mice and the trend toward increased ventricular volume was already evident (see numbers for EDD at three weeks in the table in Fig. 4B).

Given the defects in ventricular function, one plausible explanation is that the myofibrillar apparatus was disorganized to the extent that contraction was ineffective. Such disarray has been noted in mice bearing a cardiac-specific loss of function allele of dicer. Ultrastructural analysis (Online Fig. S5) revealed mild myofibrillar disarray mostly related to misalignment of the contractile apparatus. In order to determine whether pathology-associated cardiac
markers are expressed and fetal genes are activated, we performed real-time RT-PCR analysis. Nppa and Nppb were expressed at higher levels in mutant heart (Fig. 5). Myh7, a fetal myosin whose re-expression in adulthood is associated with heart failure, was also expressed at higher levels in the mutant hearts. Myh6, the normal adult cardiac myosin, within which miR-208 is encoded, was expressed at similar levels in control and mutant hearts; thus the decrease seen in mature miR-208 is not attributable to differences in the regulation of the host gene. These molecular assays complement the pathological and echocardiographic observations and are consistent with a diagnosis of DCM.

Next, we isolated RNA from mutant and control hearts to examine the expression of marker genes expressed in striated muscle (Fig. 5). Cardiac markers were uniformly low in the mutant heart. In contrast, fast skeletal muscle markers were uniformly upregulated. One of the three slow skeletal markers (Tnni1) was also upregulated, while two (Tnnt1 and Tnnc1) were not; intriguingly Tnni1 also has a miR-133 binding site in its 3′ UTR and part of its upregulation may be attributable to the loss of miR-133. The upregulation of skeletal muscle genes has been previously noted in other miR knockout mice. As misexpression of skeletal muscle isoforms in the heart can lead to impaired cardiac function, at least part of the observed pathology may be attributed to increased expression of fast skeletal muscle transcripts at the expense of cardiac genes.

An array-based profiling approach was carried out to compare relative levels of mature microRNAs in RNA derived from the hearts of mutant and control mice. MicroRNAs that are less abundant in the mutant heart when compared to the control heart are likely to be those enriched in cardiomyocytes (as dgcr8 is knocked out only in cardiomyocytes). Hence this analysis allows us to indirectly detect cardiomyocyte-enriched microRNAs. As expected, we detected precipitous declines in the levels of cardiomyocyte-specific miR-1, miR-133, miR-208 and miR-499 in the mutant hearts (Online Fig S7, compare to Fig 2C). Others that were decreased by greater than 2 fold, and therefore likely to be enriched within the cardiomyocytes include miR-378/miR-378* (aka miR-422b), miR-22, miR-486, miR-30e*, miR-149, miR-709, miR-345 and members of the miR-30a-5p family (Online Fig. S7).

To uncover the scope of regulation that is disrupted by the loss of the microRNA pathway, we carried out an in silico analysis. We chose ten microRNAs that were downregulated the most and used Targetscan to obtain a target list of mRNAs with conserved miRNA binding sites. Next we extracted a published dataset that had compiled the list of genes that are expressed in the human heart. The intersection of these two lists (Online Suppl Table 1) provided us with a list of genes whose expression could be upregulated in the hearts of mutant mice. This analysis suggests that approximately 14% (1140/7896) of the genes expressed in the heart could be potentially upregulated due to the loss of these ten microRNAs that we determined to be cardiomyocyte-enriched. Included amongst this list of targets are genes that are involved in GPCR signaling (endothelin receptors), calcium signaling (Calcineurin subunits), smooth muscle contraction (Mylk) and calcification (Runx2). Thus it is likely that the complex phenotype is at least in part due to the misexpression of a subset of these genes.

**DISCUSSION**

**miR-1 and miR-133a**

While many studies utilizing inter-tissue comparisons can attest to the abundance of miR-1 within muscle tissue, our study, by focusing on the intra-tissue abundance has revealed a wide disparity between the levels of miR-1 and all the other microRNAs. This is especially significant when we compare miR-1 to miR-133 which are co-regulated (albeit differentially spliced) microRNAs. These results suggest that mechanisms other than transcription (e.g., processing or stability) can dramatically alter steady state levels of mature...
miRNAs. Recent evidence from other labs have shown such post-transcriptional regulation for let-7 and miR-214548. A second testament to the relative abundance of miR-1 is evident when comparing the levels of mature miR-1 and miR-208a. Since miR-208a is resident within a cardiac myosin, its levels should be representative of a highly transcribed miRNA. The fact that miR-1 levels far exceed that of miR-208a provides further indirect evidence for post-transcriptional mechanisms governing microRNA stability. Lastly, as we are sampling a multitude of cell types in the heart, it is possible that within cardiomyocytes, the percentage of miR-1 in relation to other microRNAs is even higher and further studies, using purified cardiomyocytes will be needed to verify this possibility. The very high levels of miR-1 suggests it plays a central role in sustaining heart muscle function; indeed previous analysis of a miR-1-2 knockout mouse 8 confirmed the importance of miR-1 dosage in maintaining proper cardiac function. The homozygous loss of miR-1-2, one of two mir-1 loci, causes multiple defects in heart function8. We await the generation of appropriate conditional knockout mice lacking both miR-1-1 & miR-1-2 mice to ascertain its singular importance in adult mouse myocardium. However, recent findings evaluating the mir-133a knockout mice clearly define its importance in cardiac development. Taken together, miR-1 and miR-133 appear to be attractive candidates for rescuing the phenotype associated with Dgcr8 loss.

Our sequencing revealed that the miRBase annotation for miR-133a is offset by one nucleotide at the 5′ end. The mature miRNA (which is the one that has the most common 5′ end and is read most often) is UUGGUCCCUUCAACCACUGU; the miRBase annotated miR-133a is UUUGGUCCCUUCAACCACUG. Given the importance of the 5′ end in determining target repression, this also changes the putative targets that may be repressed by miR-133a. We did obtain significant number of counts for the miR-133 species annotated on miRbase; however based on our criteria for miRNA classification, we denote the species with one less U as the mature miRNA. Other independent sequencing data (R.C, unpublished data) confirm this 5′ heterogeneity of mature miR-133a.

We also performed microarrays to determine the fold change in microRNA levels in mutant versus wild-type hearts. This strategy is particularly powerful as it enables the specific identification of microRNAs that are present in the cells expressing the cre transgene. Of note, the ranking of genes that were most dramatically reduced in the mutant hearts as determined by microRNA microarrays did not directly match with the relative amounts of individual microRNAs uncovered in the sequencing data. This difference is likely the result of a number reasons. First, the sequencing data represents the miRNAs in all the cell types of the heart, rather the cells expressing the transgene. Second, the absolute level of any microRNA will not necessarily correlate with the fold decrease following Dgcr8 loss as different miRNAs in the cardiomyocytes will almost certainly have different half-lives. Third, we isolated RNA from the hearts of slightly different aged mice for sequencing (6-8 week) versus the microarrays (4-5 weeks). Hence age-related differences may partly explain the differences between the array and the sequencing data sets.

**Phenotype of muscle-specific Dgcr8 KO mice**

We have utilized a loss of function allele of dger8 to uncover the importance of the microRNA pathway in cardiac integrity. The phenotypic outcome is similar to the cardiac-specific dicer deficient mice 18 and this similarity in phenotypes has also been shown in mice bearing conditional alleles of dger8 and dicer in the skin49. However, dger8 deficient mice have an advantage over dicer deficient as the former can potentially be rescued by a Dicer-substrate shRNA designed to produce mature miRNAs. Hence it will be possible to define, in a fairly straightforward manner, the “minimal microRNA” requirements for different cell types derived from these mice. This approach has been successfully employed to reveal microRNAs
important in the cell cycle regulation of murine ES cells\textsuperscript{50} and such an approach should be feasible in other cell types too.

The results from the muscle-specific dgcr8 knockout mice demonstrate the essential role of microRNA regulation in cardiac function. Although we have not identified the root cause of DCM and HF in our mice, our data clearly demonstrates a role for the microRNA pathway in proper functioning of the heart. Changes in ventricular diameters were further visible in trans-thoracic echocardiography. This resulted in a significant decrease in mutant ventricular function as assessed by fractional shortening at four weeks of age compared to control litters. Furthermore trans-thoracic echocardiography revealed functional deterioration was already present at three weeks of age with mutant mice showing markedly reduced fractional shortening. This functional deterioration preceded dilation seen at 4 weeks of age (Fig. 4). Heart rates and wall thickness were not significantly different in mutant mice (both at three weeks and at four weeks) supporting our histological observations that ruled out a hypertrophic phase prior to dilation. Ventricular walls from mutant mice did however, exhibit a thinning trend that was detectable by echocardiography at four weeks (Fig. 4B) and was very obvious histologically in end stage mice (Fig. 3B). Changes in microRNA levels have been noted to be secondary consequences of a stressed heart\textsuperscript{51}. We demonstrate that cardiomyocyte-specific microRNA levels are depleted prior to the occurrence of pathophysiological changes (Fig. 3D). These data are consistent with the microRNA loss being causative and representing a primary event in the emergence of the phenotype we have described.

In comparison to the other single-microRNA knockouts\textsuperscript{8,10,52}, the dgcr8 knockout exhibits a much more severe and penetrant phenotype. This is to be expected as a number of microRNAs are affected. Using a candidate gene approach and incorporating results from previously published work with the miR-208 -/- mice\textsuperscript{10}, we interrogated and detected the upregulation of several fast skeletal muscle genes in the heart. As cardiac muscle is more akin to a “slow” muscle the aberrant activation of fast skeletal genes could be pathological. Even though myofibrillar proteins are homologous, each striated muscle tissue has evolved to meet its particular needs and previous studies have demonstrated that cardiac-specific overexpression of skeletal muscle specific protein can cause loss of cardiac function\textsuperscript{41}. Clearly, a widespread increase in skeletal gene expression, suggested by our candidate gene analysis (Fig 5), could contribute to the loss of cardiac function. Another aspect of pathological remodeling is the re-establishment of a fetal gene program in failing cardiomyocytes. Clearly this is also a consequence of Dgcr8 loss as exemplified by an increase in Myh7, a fetal myosin.

**Comparison with other microRNA deficient mice**

In comparing our phenotype to the heart-specific Dicer deficient mice (using the alpha-MHC promoter driven Cre)\textsuperscript{13} we note the following important differences. We always detect fibrosis (Fig. 3C) and see marked increases in MYH7 expression (Fig. 5) in mutant mice. These could be due to differences in the timing of Cre-mediated excision, implying that loss of microRNA regulation at different times lead to different phenotypic outcomes. We also note that the recent report describing the knockout of dicer in the adult myocardium\textsuperscript{18} shows a broadly similar, but not an identical phenotype, to the dgcr8 KO mice described herein. Importantly, we did not detect any hypertrophy during routine pathological staining and the pathology of our mutant mice is more consistent with dilated cardiomyopathy that eventually leads to a phenotype that resembles human heart failure. Since the timing of the cre-mediated excision can cause different phenotypic outcomes\textsuperscript{18}, direct comparison of the two dicer KO studies with this dgcr8 KO (utilizing different promoters driving Cre) is complicated; nonetheless all these studies point to the clear importance for the microRNA pathway in cardiac function. A recent report from the Olson group reported the phenotype of a complete knockout of miR-133a\textsuperscript{52}. Pathologically, the dgcr8 muscle KO mice are very similar to the fraction of 133a-1\textsuperscript{ko};
133aa-2αKO (133a dKO) mice that survive to 2 or 4 months of age with fibrosis and ventricular wall thinning as common features. Similar to the 133a dKO mice, we did not detect any gross hypertrophy prior to the advent of dilation; however, in contrast to the 133a dKO mice, we detect only mild myofibrillar disarray in our ultrastructural analysis. In both the 133a dKO mice and the cardiac-specific dicer KO mice, deletion of the cognate genomic region occurs early and hence may have a more profound effect on the arrangement of myofibrils. When dicer8 expression (and therefore microRNA-mediated regulation) is perturbed after the establishment of the myofibrillar array, the requirement for an intact microRNA pathway may be less stringent (as is the case here). Nonetheless, overall pathological similarity suggests that one reason for the myopathy seen in the dger8 mice could be due to its lack of mature miR-133a.

Fast skeletal gene expression has also been noted in the hearts of miR-208-/- mice and this particular phenotypic characteristic could be a consequence of low levels of miR-208. However, miR-208-/- mice do not lose cardiac function and additional microRNAs have to be implicated in describing the complete phenotype associated with loss of Dgcr8 in the heart.

Cardiac heterochrony as a possible mechanism underlying the dramatic phenotype

An equally feasible and alternative (albeit speculative) explanation for the sustained expression of fetal gene markers in adulthood is that the lack of the microRNA pathway leads to an arrest in the development of the heart such that late embryonic/prenatal gene expression patterns are maintained in the adult. Indeed, such heterochronic events in Ca. elegans were instrumental in the identification of the founding members of microRNAs53-55 and recent reports have confirmed that this heterochronic pathway is conserved56-58. Two candidate genes that we have examined are normally repressed in adult tissues but continue to be expressed when Dgcr8 is absent: Myh7 (a fetal myosin) and Tnni (a slow skeletal muscle-specific troponin-complex subunit). Under normal physiological conditions, Tnni levels are downregulated in the heart following birth59, but it continues to be expressed at relatively higher levels in the Dgcr8 KO heart. Importantly, Tnni, as opposed to Myh7, is NOT expressed at higher levels in a pathological state59 and, therefore, its overexpression cannot be attributed to the cardiac reprogramming that occurs secondary to a failing heart. This observation suggests that the lack of the dger8 gene can cause a heterochronic phenotype (which in turn is incompatible with adult heart function). In addition, our in silico analysis (Online Suppl Table 1) suggests that a large number of genes that are expressed in the heart are susceptible to microRNA-mediated regulation. Comparison of temporal mRNA expression profiles in mutant mice and wild type mice will aid in the identification of the primary targets of the microRNA pathway as well as to provide evidence for the existence of cardiac heterochrony in the mutant mice.

In summary, we have, through complementary approaches, ascertained the importance of the microRNA pathway in maintaining cardiomyocyte function. We have identified high abundance microRNAs in the heart by performing intra-tissue comparisons and the preeminent position of miR-1 within muscle tissue has been quantitatively established. In addition, the requirement of the microRNA pathway in cardiac muscle maintenance has been unequivocally established (as its absence is lethal). Based on the results described, we suggest two distinct, but related, mechanisms to explain the drastic loss of cardiac function. The first one implicates fast skeletal muscle gene expression as a plausible causative factor in loss of cardiac function. Second, the loss microRNA function maybe causing cardiac heterochrony, which ultimately leads to heart failure. In addition, our data from the deep sequencing suggests that loss of a few microRNAs-including miR-1 and miR-133a may ultimately be responsible for the dramatic loss of function seen in Dgcr8 deficient cardiomyocytes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.
Acknowledgments

We thank Ron Kahn (Joslin Diabetes Center) for the MCK-Cre line and members of the Lodish and Bartel lab for their insightful comments. We are indebted to Carsten Russ and the Broad Sequencing Platform for carrying out Illumina/Solexa sequencing runs at the Broad Institute.

FUNDING SOURCES This work was supported by the following grants: PKR (Muscular Dystrophy Association-38823, RB [NIH K08 NS48118-01, NIH RO1 NS057221, Stem Cell Research Foundation and the Pew Scholars Program in the Biomedical Sciences]); HFL (NIH-R01 DK068348-04 and a SPARC grant from the Broad Institute); MK (NIH-HL52212); HFL & MK (NIH/NHLBI P01 - HL066105); RJ (NIH RO1-CA087869, NIH R37-CA084198, and NIH RO1-HD0445022); RL (NIH RO1s, HL071775, HL088533, HL090884, and HL093148); MB (Max Kade Foundation, Austria); HRC (NIH RO1 GM067031 to David Bartel).

Non-standard Abbreviations and Acronyms

- Dgcr8: DiGeorge syndrome critical region 8
- PAZ: Piwi Argonaut and Zwille
- shRNA: short hairpin RNA
- PGC1α: PPARg coactivator-1α
- PGC1β: PPARg coactivator-1β
- Myh6: Myosin Heavy Chain 6
- Myh7: Myosin Heavy Chain 7
- RT-PCR: Reverse Transcription-Polymerase Chain Reaction
- DCM: Dilated Cardiomyopathy
- 3’ UTR: 3’ Untranslated Region
- KO: knock-out

REFERENCES


Figure 1. microRNA abundance in the murine adult heart  
(A) The top 20 known microRNAs (in terms of normalized read number) from the male (dark bars) and the female (clear bars) heart small libraries were converted to percentage terms and plotted. Since the rank order differs slightly between the male and female libraries, the total number of microRNAs plotted is greater than 20. Note the abundance of miR-1 reads relative to other known microRNAs. (B) microRNAs belonging to the same family (as defined by Targetscan; www.targetscan.org) were summed and plotted together. This analyses reveals that aside from miR-1, the let-7 and miR-30 families are amongst the ones that are highly abundant in the heart.
Fig. 2. Lethality and microRNA expression in muscle-specific dgcr8 KO mice

(A) Actual numbers of Cre positive mice (and the expected numbers based on Punnett square analysis for two independent loci) obtained from matings between 2lox/+; Cre positive mice are shown. Expected numbers are based on the assumption that Cre transgene is heterozygous although this is not known. (B) Postnatal lethality of muscle-specific dgcr8 KO mice. Survival curves for Cre positive mice are shown and reveal the lethality when dgcr8 is excised in muscle tissue. Moribund, “hunched over” mice that had to be sacrificed due to animal care committee specifications were considered dead for survival analysis. Survival curves were plotted using a built-in module in Prism software. (C) miR-1, miR-133 and miR-208 expression was determined using Northern Blots from total RNA derived from heart tissue. Tissues from three mutant (2lox/2lox; Cre positive) and three sex and age-matched control (2lox/+; Cre positive) siblings were used for total RNA isolation; ages were 29 days (males), 29 days and 38 days (females). The same blots were reprobed for U6 (bottom part of each set) to normalize for differences in loading. Quantified miR/U6 ratios are plotted below for miR-1, miR-133 and miR-208 and the indicated p values were obtained using two-sample (unequal variance) one-tailed t-tests. (D) A larger region of the miR-208 Northern blot is shown to reveal the absence of pre-miR-208 in total RNA derived from the mutant heart.
Fig. 3.

(A). Intact, excised hearts from 30 day old mutant (2lox/2lox; Cre positive; left) and control (2lox/+; Cre positive; right) female sibling mice. (B). Representative long axis sections at different stages (as indicated) from mutant and control sex-matched sibling mice were stained with H & E (for end stage: left ventricle=LV and right ventricle=RV) or Masson’s Trichrome (for d14/15 & d21/22). Bar = 500 uM. The stage at which the mice were euthanized to reveal end-stage pathology was variable and defined by the health status of the mice and is d34 in this panel. (C). High magnification (20X) view of Masson’s Trichrome stained sections from 3 week and end-stage (d43 in this panel) mutant and control female sibling mice (interstitial blue staining (bright green arrows) is indicative of fibrotic collagen deposits; Bars = 50 uM. (D). RT-PCR assay to detect mature miR-1, miR-133a and miR-208 levels showing that the decline is already evident at two weeks after birth and continues to decrease by the time the mice are moribund (“end stage”). The ratio of mutant to control is shown on the y-axis and the pairs chosen for evaluation were age and sex matched.
Fig. 4. Trans-Thoracic Echocardiography. (A) Representative short axis B and M mode images for both mutant (left) and control (right) mice at three weeks of age showing dilation in the mutant mice. (B) Summary of echocardiographic data 3 weeks and 4 weeks after birth showing progressive dilation and reduction in ventricular function (see numbers for EDD and FS respectively) between three and four weeks in mutant mice. WT: Wall thickness, EDD: End-diastolic diameter, ESD: End-systolic diameter, FS: Fractional shortening, HR: Heart rate. *: p<0.05 vs. 2lox/+; Cre pos; † p<0.05 vs. 3 weeks.
Fig. 5.
Gene expression patterns in *dgcr8* KO heart. Total RNA was obtained from end stage (mutant) and sex and age-matched control mice. A minimum of seven pairs was used for the analysis of relative expression levels of the indicated genes. Box and whisker plots are represented for each gene. Expression is depicted as a ratio of mutant over control, (with each being first normalized to GAPDH to account for differences in total amount of RNA used). Lack of a difference should manifest itself as having a ratio of 1.0 (dotted line). While it is appreciated that some genes may fall into two or more categories during the embryonic and postnatal development, for convenience, they are grouped into a single category (as indicated) that is representative of adult gene expression.