

A Family of microRNAs Encoded by Myosin Genes Governs Myosin Expression and Muscle Performance

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SUMMARY

Myosin is the primary regulator of muscle strength and contractility. Here we show that three myosin genes, *Myh6*, *Myh7*, and *Myh7b*, encode related intronic microRNAs (miRNAs), which, in turn, control muscle myosin content, myofiber identity, and muscle performance. Within the adult heart, the *Myh6* gene, encoding a fast myosin, coexpresses miR-208a, which regulates the expression of two slow myosins and their intronic miRNAs, *Myh7/miR-208b* and *Myh7b/miR-499*, respectively. miR-208b and miR-499 play redundant roles in the specification of muscle fiber identity by activating slow and repressing fast myofiber gene programs. The actions of these miRNAs are mediated in part by a collection of transcriptional repressors of slow myofiber genes. These findings reveal that myosin genes not only encode the major contractile proteins of muscle, but act more broadly to influence muscle function by encoding a network of intronic miRNAs that control muscle gene expression and performance.

INTRODUCTION

The speed and force of both myocardial and skeletal muscle contraction are largely dependent on the intrinsic contractile properties of cardiac and skeletal myocytes. Myosin heavy chain (MHC) is the major contractile protein of cardiac and skeletal muscle cells and the primary determinant of the efficiency of muscle contraction. Cardiac and skeletal muscles modulate the expression of myosin genes in response to hormonal signaling and workload to meet physiological demands (Baldwin and Haddad, 2001).

Cardiac contractility depends on the expression of two MHC genes, α - and β -MHC (also known as *Myh6* and *Myh7*, respectively), which are linked in a head-to-tail orientation and are regulated in an antithetical manner (Weiss and Leinwand, 1996). The ratio of α - to β -MHC expression is species specific and varies in response to developmental, physiological, and pathological signaling. In rodents, β -MHC, a slow ATPase, is highly expressed

in the embryonic heart, whereas α -MHC, a fast ATPase, is expressed at relatively low levels in the prenatal heart and is up-regulated during the early postnatal period (Morkin, 2000). Mechanical stress and hypothyroidism induce a shift from α - toward β -MHC composition in the adult heart (Fatkin et al., 2000; Gupta, 2007; Kiriazis and Kranias, 2000; Krenz and Robbins, 2004; Vanderheyden et al., 2008), which correlates with a decline in mechanical performance and contractile efficiency (Lowe et al., 1997; Miyata et al., 2000; Stelzer et al., 2007). Because relatively minor changes in the ratio of these two myosin isoforms can have profound effects on cardiac contractility in human and rodent hearts (Herron and McDonald, 2002; Korte et al., 2005; Rundell et al., 2005; Schiaffino and Reggiani, 1996), and because increased expression of β -MHC in the myocardium decreases power output and contributes to depressed systolic function in end-stage heart failure, the mechanisms that regulate MHC gene switching have been the focus of intense interest.

Skeletal myofibers are also characterized by the expression of particular myosin isoforms and other contractile proteins that determine the efficiency of contraction (Bassel-Duby and Olson, 2006). In slow-twitch, type I fibers, which display oxidative metabolism and high endurance, β -MHC is the predominant myosin, whereas fast-twitch, type II myofibers express fast myosins and are primarily glycolytic and susceptible to fatigue. Thyroid hormone (T3) signaling represses β -MHC expression and type I myofiber formation, and promotes the fast myofiber phenotype. However, despite intense study, the exact regulatory mechanism behind myofiber switching in response to T3 signaling and other cues has remained elusive.

Recently, we reported that an intron of the α -MHC gene encodes a microRNA (miRNA), miR-208, that is required for up-regulation of slow β -MHC in the adult heart in response to stress and hypothyroidism (van Rooij et al., 2007). Given that miR-208 and its host myosin, α -MHC, are only expressed in the heart, these findings raised interesting questions as to whether other miRNAs might control myosin switching and contractile protein gene programs in fast versus slow skeletal muscle.

miRNAs inhibit mRNA translation or promote mRNA degradation by annealing to complementary sequences in the 3' untranslated regions (UTRs) of target mRNAs (Bartel, 2004). Individual miRNAs have numerous targets, and individual mRNAs are

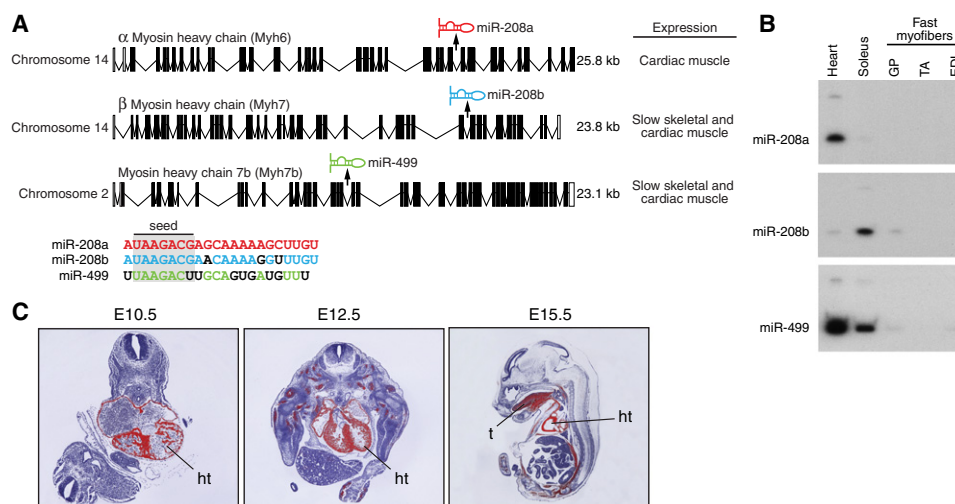


Figure 1. Distinct Expression Patterns of MyomiRs

(A) Schematic representation of the genomic location of the miRNAs present in the different myosin genes. miR-208a and miR-208b share a comparable seed region (nt 2–8) but differ at three bases in their 3' end (indicated in black). The seed region of miR-499 overlaps at 6 nt with miR-208a but differs significantly in the 3' region.

(B) Northern analysis on adult cardiac tissue shows that miR-208a expression correlates with the cardiac-specific expression of α -MHC, while miR-208b parallels the predominant slow skeletal expression of β -MHC. miR-499 is coexpressed with Myh7b, a slow myosin that is mainly expressed in the heart and soleus, a slow skeletal muscle, but not in fast myofibers. Label indicates the mature miRNA.

(C) In situ hybridization shows that *Myh7b* is expressed specifically in the heart at E10.5 and in somites as early as E12.5 during mouse embryogenesis. Silver grains are pseudocolored red. ht, heart; t, tongue.

commonly targeted by multiple miRNAs, providing combinatorial complexity and broad regulatory potential to miRNA:mRNA interactions. miRNAs often target multiple mRNAs with shared functions and in so doing can exert robust control over complex cellular processes through modulation of multiple interrelated targets (Mourelatos et al., 2002; Stefani and Slack, 2008). The T3 receptor associated protein-1 (Thrap1), which functions as a positive and negative regulator of T3 signaling, is one target of miR-208 that appears to mediate the functions of this miRNA in the heart (van Rooij et al., 2007; Callis et al., 2009).

In the present study, we show that miR-208 is essential for the expression not only of β -MHC in the heart, but also of a closely related myosin isoform, *Myh7b* (McGuigan et al., 2004). Remarkably, both of these genes encode slow myosins and contain intronic miRNAs (miR-208b and miR-499, respectively) (Berezikov et al., 2006; Landgraf et al., 2007) that are expressed in both cardiac and slow skeletal muscle. Through gain- and loss-of-function experiments in mice, we show that these myosin-encoded miRNAs act within a network to control myosin expression and skeletal myofiber phenotypes through the repression of a collection of transcriptional repressors of slow myofiber genes. Thus, myosin genes not only encode the major contractile proteins of muscle, but act more broadly to control muscle gene expression and performance through a network of intronic miRNAs.

RESULTS

A Family of miRNAs Encoded by Myosin Genes

miR-208 is encoded by intron 27 of the mouse α -MHC gene, which is expressed specifically in the heart (Figures 1A and

1B). Because of the important role of miR-208 in regulating cardiac gene expression in response to stress (van Rooij et al., 2007; Callis et al., 2009), we scanned other myosin genes for possible intronic miRNAs. Indeed, intron 31 of the mouse β -MHC gene is predicted to encode a closely related miRNA, miR-208b, which has an identical seed sequence to miR-208 and differs at only three nucleotides in the 3' region (Figure 1A). miR-208b is coexpressed with β -MHC, showing highest expression in soleus muscle, which is composed predominantly of slow type I myofibers, and lower expression in adult heart (Figure 1B). A third member of this miRNA family, miR-499, is encoded by intron 19 of the mouse *Myh7b* gene (McGuigan et al., 2004), a little-studied myosin gene that shares extensive homology with β -MHC (Figure 1A). *Myh7b* and miR-499 are highly expressed in the heart and are enriched in the soleus, compared with gastrocnemius/plantaris (G/P), tibialis anterior (TA), or extensor digitorum longus (EDL) muscles, which contain predominantly fast myofibers (Figure 1B). Similar to β -MHC, *Myh7b* is expressed in the embryonic heart and skeletal muscle, as revealed by in situ hybridization (Figure 1C). We refer to this family of miRNAs as MyomiRs, because of their location within and co-expression with their corresponding myosin genes. Henceforth, we refer to miR-208 as miR-208a to distinguish it from miR-208b.

T3 Regulation of *Myh7b*/miR-499 and β -MHC/miR-208b

Though *Myh7b*/miR-499 and β -MHC/miR-208b expression marks slow, type I myofibers in skeletal muscle, these genes display distinct regulation in the adult mouse heart, with β -MHC/miR-208b being expressed at a low level and induced to a high level in response to stress, and *Myh7b*/miR-499 being constitutively expressed at a relatively high level. In the adult

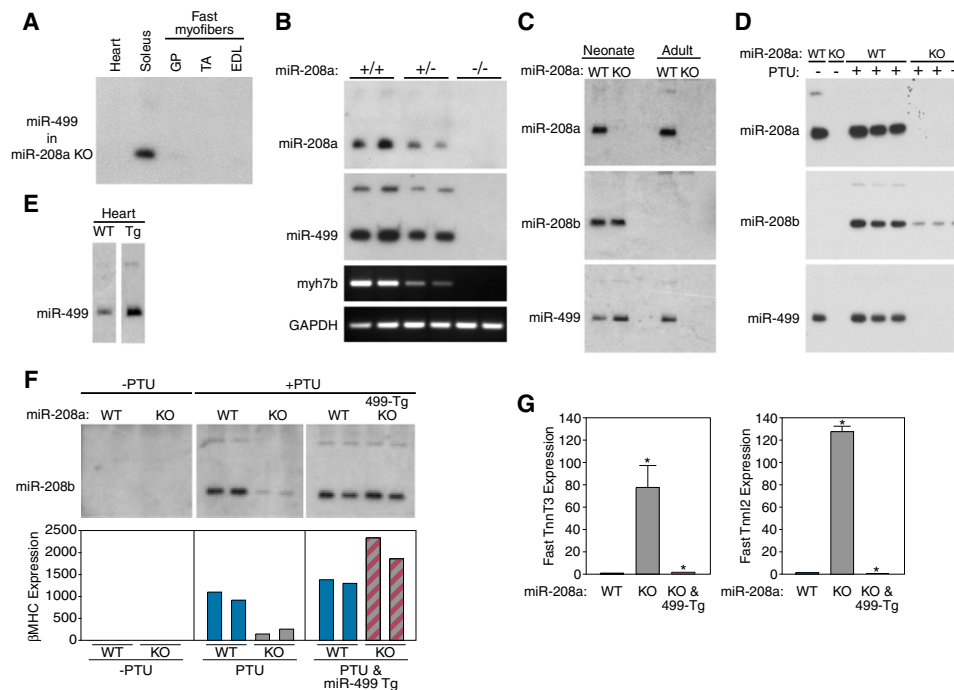


Figure 2. miR-208a Regulates Myh7b/miR-499

(A) Northern blot analysis of heart and skeletal muscle tissue of *miR-208a*^{-/-} animals shows that miR-499 expression is specifically extinguished in the heart while the expression in soleus remains unaffected. Compare with lower panel of Figure 1B.

(B) Northern blot analysis of heart tissue shows that miR-208a regulates the expression of Myh7b/miR-499 in a stoichiometric manner. In *miR-208a*^{+/-} animals, miR-499 and Myh7b expression is reduced by 50%, while miR-499 and Myh7b expression are eliminated in *miR-208a*^{-/-} animals. Myh7b and GAPDH were detected by RT-PCR. GAPDH serves as a loading control.

(C) Northern blot analysis on cardiac samples of wild-type and *miR-208a*^{-/-} (KO) neonate and adult mice. miR-208b and miR-499 are expressed normally in neonatal heart of *miR-208a*^{-/-} mice, whereas neither miRNA is expressed in adult *miR-208a*^{-/-} heart.

(D) Mice of the indicated miR-208a genotype were treated with PTU, as indicated, and miRNA expression was detected by northern blot. PTU treatment increases miR-208b in wild-type animals, and this effect is significantly blunted in *miR-208a*^{-/-} animals.

(E) Expression of miR-499 in hearts from wild-type and MCK-miR-499 transgenic mice detected by northern blot.

(F) Reintroducing miR-499 in the heart with an MCK-miR-499 transgene in the *miR-208a*^{-/-} background restores expression of miR-208b and β-MHC in response to PTU treatment. Upper panels show northern blots of hearts from duplicate animals under each condition. Lower panels show β-MHC expression as detected by real-time RT-PCR.

(G) Fast skeletal muscle troponins (TnnT3 and TnnI2) are upregulated in hearts of *miR-208a*^{-/-} animals. Introduction of the MCK-miR-499 transgene into *miR-208a*^{-/-} mice represses troponin expression as in wild-type. Results represent the average values obtained from two animals as detected by real-time PCR.

heart, T3 signaling induces α-MHC transcription and represses β-MHC expression (Morkin, 2000; Schuyler and Yarbrough, 1990). Inhibition of T3 biosynthesis with propylthiouracil (PTU) derepresses the expression of β-MHC. To determine whether miR-208b and miR-499 are coregulated with their corresponding myosin genes, we performed side-by-side comparison of myosin and miRNA expression in response to PTU (see Figure S1 available online). Like β-MHC, miR-208b was upregulated in the hypothyroid state induced by PTU, which could be reversed by T3 supplementation. Myh7b and miR-499 were also upregulated by PTU, but the magnitude of upregulation was blunted compared with β-MHC/miR-208b because the basal level of Myh7b/miR-499 expression was higher in the heart (Figure S1). Overall, the expression of the MyomiRs paralleled the expression of their host myosin genes during development and in response to T3 signaling, consistent with the conclusion that the MyomiRs are processed out of intronic sequences of their host myosin mRNAs.

miR-208a Is Required for Expression of Myh7b/miR-499

In light of the profound influence of miR-208a on cardiac gene expression (van Rooij et al., 2007), we investigated whether this miRNA might regulate the expression of subordinate miRNAs in the heart by comparing the miRNA expression profiles in hearts from wild-type and *miR-208a*^{-/-} mice by microarray analysis (Figure S2). The absence of miR-208a expression in *miR-208a*^{-/-} hearts served as an internal control for the microarrays. Remarkably, we found that miR-499 expression was extinguished in hearts of *miR-208a*^{-/-} mice, whereas it was unaffected in the soleus, consistent with the cardiac-specific expression of miR-208a (Figure 2A). The expression of miR-499 is exquisitely sensitive to the level of miR-208a expression, such that the expression of both miRNAs was reduced by ~50% in hearts from *miR-208a*^{+/-} mice (Figure 2B). The expression of miR-499 paralleled that of Myh7b, indicating a regulatory role for miR-208a in the control of Myh7b transcription (Figure 2B).

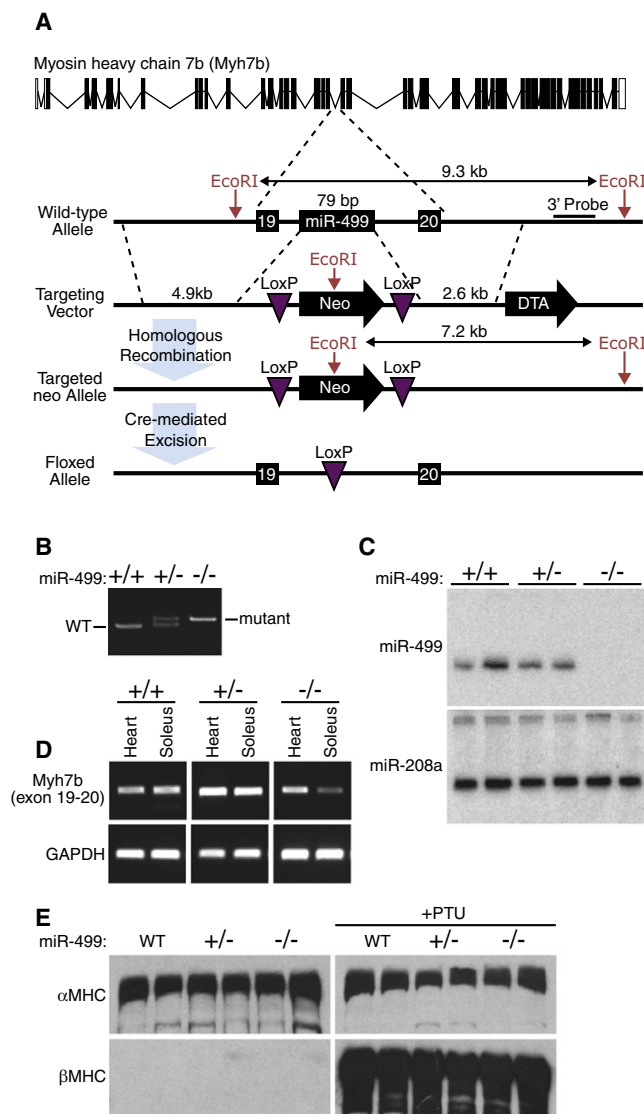


Figure 3. Generation of miR-499 Null Mice

(A) Strategy to generate miR-499 mutant mice by homologous recombination. The pre-miRNA sequence was replaced with a neomycin resistance cassette flanked by loxP sites. The neomycin cassette was removed in the mouse germline by breeding heterozygous mice to transgenic mice harboring the CAG-Cre transgene.

(B) Detection of the miR-499 mutation by PCR. Primers flanking the loxP site in intron 19 of the *Myh7b* gene generate PCR products as indicated. The mutant band in the PCR reaction is larger than the wild-type band due to replacement of the miRNA with a larger loxP site.

(C) Detection of miR-499 and miR-208a transcripts by northern analysis of hearts from wild-type, heterozygous, and miR-499 mutant mice.

(D) Detection of *Myh7b* expression by RT-PCR of RNA from heart and soleus of mice of the indicated genotypes using primers flanking intron 19. Deletion of miR-499 does not disrupt cardiac expression of *Myh7b*, while there is a decrease in expression in soleus.

(E) Western analysis of α -MHC and β -MHC protein levels in hearts of neonatal mice of the indicated genotypes in the absence (left) and presence (right) of PTU. Two mice of each genotype were analyzed. Unlike miR-208a, miR-499 is not required for upregulation of β -MHC in response to PTU.

Although miR-208a is required for upregulation of β -MHC in the adult heart in response to stress and hypothyroidism (van Rooij et al., 2007; Callis et al., 2009), expression of β -MHC and miR-208b during the neonatal period is independent of miR-208a (Figure 2C). Similarly, like the expression of its host gene, β -MHC, miR-208b expression is absent in the adult heart (Figure 2C). Based on the finding that miR-499 and *Myh7b* are expressed at comparable levels in wild-type and *miR-208a*^{-/-} hearts of neonates while miR-208b is still present, and that *Myh7b*/miR-499 expression is absent in adult *miR-208a*^{-/-} heart, we conclude that both miR-208a and miR-208b are capable of activating *Myh7b*/miR-499 expression (Figure 2C). While PTU upregulates miR-208b in wild-type hearts, this effect is nearly absent in *miR-208a*^{-/-} hearts (Figure 2D), and the slight induction of miR-208b seen in *miR-208a*^{-/-} animals treated with PTU is apparently insufficient to activate miR-499 expression. Thus, the expression of miR-208b and miR-499 correlates with the expression patterns of their host myosin genes even in the absence of miR-208a. The hearts of adult *miR-208a*^{-/-} mice are essentially analogous to those of MyomiR nulls, since neither miR-208b nor miR-499 are expressed at significant levels.

miR-499 Can Replace the Cardiac Functions of miR-208a

miR-208a is required for both the upregulation of β -MHC in the adult heart and the expression of *Myh7b*/miR-499. We investigated whether miR-499 could replace the functions of miR-208a by generating transgenic mice that expressed miR-499 under control of MCK regulatory elements, which direct expression in the heart and in skeletal muscle. Multiple transgenic lines were obtained that expressed miR-499 in the heart at levels approximately 3-fold above the endogenous level (Figure 2E). As shown in Figure 2F, transgenic expression of miR-499 in the heart of *miR-208a*^{-/-} mice was sufficient to restore the expression of β -MHC and miR-208b to normal levels in the presence of PTU. Genetic deletion of miR-208a also results in the expression of fast skeletal muscle genes, such as the fast troponins TnnT3 and TnnI2, in the heart (van Rooij et al., 2007), and transgenic expression of miR-499 in the absence of miR-208a repressed cardiac expression of fast skeletal muscle genes (Figure 2G). The finding that miR-499 alone is sufficient to replace the functions of miR-208a in the heart suggests that miR-499 is a downstream mediator of miR-208a actions.

Generation of miR-499 and miR-208b Null Mice

To further explore the functions of miR-499 and miR-208b in vivo, we generated mutant mice with germline deletions of the pre-miR regions of the *Myh7b* and β -MHC introns, respectively, encoding these miRNAs. LoxP sites were introduced into the corresponding introns at both ends of the miRNA-coding regions (Figures 3A and 4A). Breeding of these mice to mice expressing a CAG-Cre transgene, which is expressed ubiquitously, allowed deletion of the corresponding miRNA and its complete absence in homozygous mutant animals as shown by PCR using primers flanking the loxP site (Figures 3B and 4B). *miR-499*^{-/-} and *miR-208b*^{-/-} mice were obtained at Mendelian ratios from heterozygous intercrosses and displayed no overt abnormalities (Tables S1 and S2; data not shown).

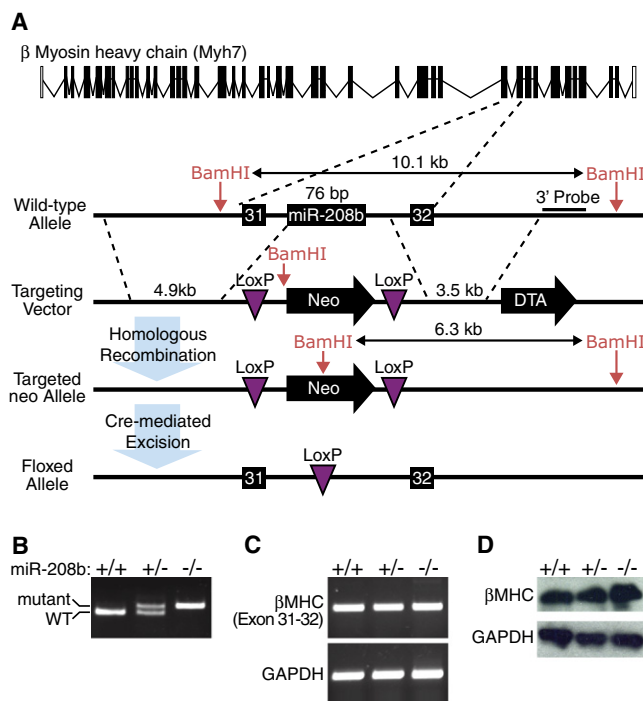


Figure 4. Generation of miR-208b Null Mice

(A) Strategy to generate miR-208b mutant mice by homologous recombination. The pre-miRNA sequence was replaced with a neomycin resistance cassette flanked by loxP sites. The neomycin cassette was removed in the mouse germline by breeding heterozygous mice to transgenic mice harboring the CAG-Cre transgene.

(B) Detection of the miR-208b mutation by PCR. Primers flanking the loxP site in intron 31 of the β -MHC gene generate PCR products as indicated.

(C) Detection of β -MHC expression by RT-PCR of RNA from hearts of mice of the indicated genotypes using primers flanking intron 31. Deletion of miR-208b does not disrupt expression of β -MHC.

(D) Detection of β -MHC by western blot analysis of heart from P0 mice of the indicated genotypes, showing that β -MHC expression is not altered by deletion of miR-208b. GAPDH was detected as a loading control.

Northern blot analysis on adult heart tissue showed that miR-208a expression was unperturbed by the deletion of miR-499 (Figure 3C). We confirmed that *Myh7b* mRNA expression was unaltered in hearts of *miR-499*^{-/-} mice, while there was a decrease in the soleus as detected by RT-PCR using primers in exons flanking the deletion in intron 19 (Figure 3D). Western blot analysis for both α -MHC and β -MHC showed no difference between wild-type or *miR-499*^{-/-} mice, and a comparable upregulation in β -MHC expression in wild-type and *miR-499*^{-/-} mice in response to PTU (Figure 3E). These data, combined with the finding that overexpression of miR-499 in *miR-208a*^{-/-} mice can restore β -MHC expression and repress fast skeletal muscle genes in the heart, indicate that both miR-208a and miR-499 must be absent for the blockade of β -MHC expression in response to stress and hypothyroidism.

Deletion of miR-208b also did not alter β -MHC expression as determined by both RT-PCR using primers in exons flanking the deletion in intron 31 (Figure 4C) and western blot analysis on neonatal cardiac tissue of *miR-208b*^{-/-} mice (Figure 4D). Thus, miR-208a appears to function as the most “upstream” and

dominant regulatory miRNA to control expression of both slow myosins, β -MHC and Myh7b, and their corresponding intronic miRNAs in the heart, whereas miR-499 and miR-208b are not individually required for expression of either myosin or other members of the MyomiR trio.

Control of Skeletal Muscle Fiber Type by miR-499 and miR-208b

The cardiac specificity of miR-208a led us to investigate the potential involvement of miR-208b and miR-499 in the control of skeletal muscle fiber types. Deletion of either miR-208b or miR-499 did not alter the expression of the other miRNA in the soleus (data not shown), and fiber type analysis showed little or no difference in the number of type I myofibers in either of these mutant mice compared to wild-type (Figure S3).

Wondering whether the homology of miR-208b and miR-499 and their coexpression in soleus might allow redundant functions that were masked in mice with either single gene deletion, we generated *miR-208b*^{-/-}; *miR-499*^{-/-} double knockout (dKO) mice. These mice were obtained at predicted Mendelian ratios from transheterozygous intercrosses and displayed no overt abnormalities (Table S3). However, staining of histological sections for myofiber type with metachromatic ATPase stain and immunohistochemistry against β -MHC showed a substantial loss of type I myofibers in the soleus of dKO mice (Figures 5A and 5B). The loss of slow myofibers in dKO mice was also evidenced by the reduced expression of slow β -MHC at the protein and mRNA levels (Figures 5C and 5E), and by a concomitant increase in the expression of fast type IIx/d and type IIb myosin isoforms (Figures 5C and S3).

Conversely, forced expression of miR-499 under control of MCK regulatory elements was sufficient to induce a complete conversion of all fast myofibers in soleus to a slow, type I phenotype (Figures 5A–5C). Analysis of gene expression in the soleus by real-time PCR also demonstrated an induction of the slow fiber gene program in response to miR-499 overexpression (Figure 5F). In the TA and EDL, which contain predominantly fast myofibers, we observed a pronounced induction of slow myofiber gene expression in MCK-miR-499 transgenic mice (Figure 5F). Metachromatic ATPase staining of TA and EDL muscles from MCK-miR-499 transgenic animals also revealed conversion to a slower myofiber type (Figure S3). Separation of myosin isoforms by gel electrophoresis indicated a switch from fast type IIb fibers to slower type IIx/d and type IIa fibers in the EDL and TA of transgenic mice (Figure 5C). This shift in fiber type was confirmed by real-time PCR for fiber-type-specific myosins (Figure S3). In contrast, fast myofiber genes were repressed in both soleus and EDL muscles from MCK-miR-499 transgenic mice (Figure 5E). Even more remarkably, when mice were subjected to a regimen of forced treadmill running, the miR-499 transgenic animals ran more than 50% longer than wild-type littermates, indicative of enhanced endurance resulting from the reprogramming of fast myofibers to a slower fiber type (Figure 5F). We conclude that miR-208b and miR-499 redundantly program skeletal myofibers to a slow phenotype at the expense of fast myofibers.

MyomiR Targets and Mechanism

To begin to define the mRNA targets of MyomiRs that mediate their influence on myosin expression and myofiber phenotypes,

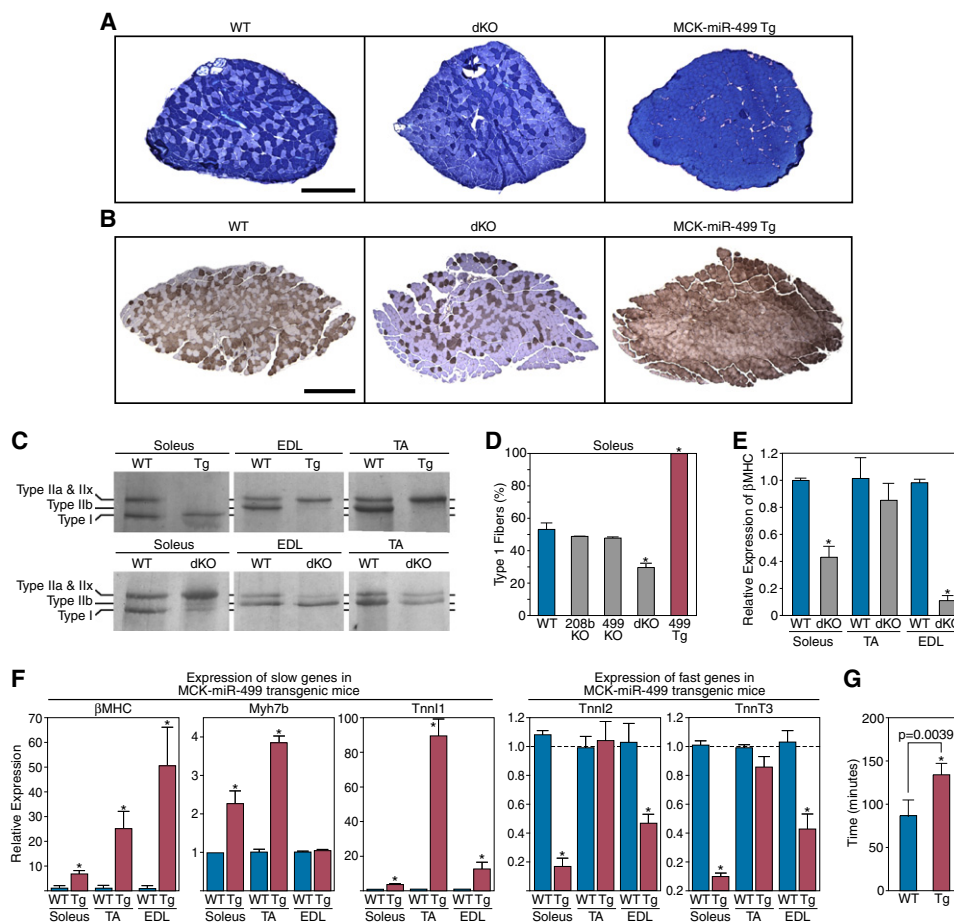


Figure 5. Control of Skeletal Muscle Fiber Type by miR-208b and miR-499

(A) Detection of type I myofibers in soleus of wild-type, *miR-208b*^{-/-}; *miR-499*^{-/-} dKO, and MCK-miR-499 transgenic mice by metachromatic ATPase staining. Scale bar, 500 μ m.

(B) Immunohistochemistry for β -MHC to identify type I myofibers in the soleus of wild-type, *miR-208b*^{-/-}; *miR-499*^{-/-} dKO, and MCK-miR-499 transgenic mice. Scale bar, 500 μ m.

(C) Identification of myosin isoform content of soleus, EDL, and TA muscles from wild-type, *miR-208b*^{-/-}; *miR-499*^{-/-} dKO, and MCK-miR-499 transgenic mice by gel electrophoresis.

(D) The percentage of type I myofibers within soleus muscles of wild-type, MyomiR mutant, and MCK-miR-499 transgenic mice was determined as shown in (B).

(E) β -MHC mRNA levels in skeletal muscle of *miR-208b*^{-/-}; *miR-499*^{-/-} dKO adult mice were detected by real-time PCR. Loss of MyomiRs in skeletal muscle results in repression of β -MHC.

(F) Expression of slow and fast myofiber genes in soleus, TA, and EDL muscles of MCK-miR-499 transgenic mice compared to their level of expression in wild-type muscles (set at a value of 1), as detected by real-time PCR. Transgenic overexpression of miR-499 is sufficient to upregulate slow myofiber genes and repress fast myofiber genes in soleus and EDL.

(G) Wild-type ($n = 4$) and MCK-miR-499 transgenic ($n = 5$) mice were subjected to a regimen of forced running to exhaustion on a treadmill. The time to run to exhaustion of each mouse is shown. $p = 0.0039$.

we used targeting algorithms (TargetScan) to search for predicted targets that were evolutionarily conserved and encoded transcriptional regulators implicated in myofiber gene expression. In addition to the thyroid hormone receptor coregulator Thrap1, shown previously to be a target for repression by miR-208a in the heart (van Rooij et al., 2007; Callis et al., 2009), we identified several transcriptional repressors among the predicted targets of the MyomiRs (Figure S4). These include Sox6, Pur β , and Sp3, each of which has been reported to repress β -MHC expression (Adolph et al., 1993; Azakie et al., 2006; Gupta et al., 2003; Hagiwara et al., 2005, 2007; Ji et al., 2007; Tsika et al., 2004; von Hofsten et al., 2008); and HP-1 β , a corepressor

of MEF2 (Zhang et al., 2002), which activates slow fiber gene expression (Wu et al., 2000).

Remarkably, the 3' UTR of Sox6 mRNA contains four evolutionarily conserved target sites for miR-499, one of which is also a predicted site for miR-208 (Figure S4). The 3' UTRs of Pur β and HP-1 β each contain single conserved predicted target sequences for miR-499 and miR-208, while Sp3 contains a conserved miR-208 targeting sequence. We cloned the 3' UTRs of each of these predicted targets downstream of a luciferase reporter and assayed for repression by miR-208 and miR-499 in transfected COS cells. Expression of the MyomiRs repressed wild-type 3' UTR-luciferase reporter constructs, but

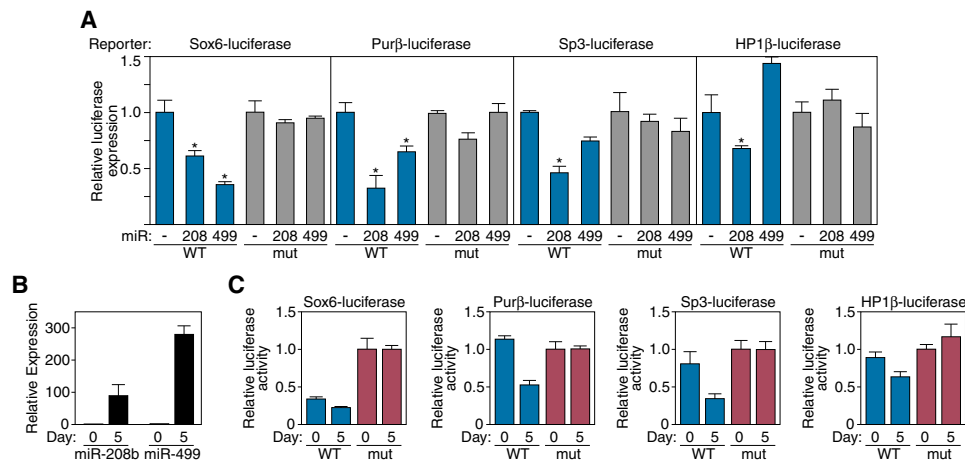


Figure 6. MyomiRs Directly Repress Predicted Target Genes

(A) Luciferase studies of predicted MyomiR target transcripts. Wild-type and mutant Sox6, Purβ, Sp3, and HP-1β 3' UTRs were cloned into a luciferase reporter plasmid. These constructs were cotransfected into COS cells with expression vectors for miR-208a or miR-499. Luminescence values were assayed 48 hr post transfection and were normalized to β-galactosidase activity.

(B) Expression of MyomiRs increases following C2C12 myoblast differentiation. Relative levels of miR-208b and miR-499 expression were detected by real-time PCR at days 0 and 5 of C2C12 differentiation.

(C) Wild-type, but not mutant luciferase, reporter constructs are repressed by MyomiRs in C2C12 cells.

had little effect on reporters containing 2 nucleotide mutations in the conserved targeting sequence (Figure 6A). Similarly, wild-type luciferase reporter constructs were repressed in C2C12 cells as MyomiR expression increased over the course of differentiation, while mutated constructs displayed no change in activity (Figures 6B and 6C). Consistent with previous results, the 3' UTR of Thrap1 displayed repression by both miR-208a and miR-499 (data not shown).

To further validate the above repressors as potential targets of MyomiRs, we compared the expression of the corresponding proteins in cardiac extracts from wild-type and *miR-208a*^{-/-} mice (Figure 7A). Consistent with a repressive effect of miR-208a on their expression, Purβ, Sp3, and HP-1β proteins were elevated in expression in hearts from mutant mice, while their mRNA levels were unchanged (data not shown). Additionally, Sox6 mRNA was upregulated in *miR-208a*^{-/-} hearts, indicative of a destabilizing effect of miR-208a on Sox6 mRNA. Transgenic expression of miR-499 was sufficient to reduce Sox6 mRNA expression in *miR-208a*^{-/-} hearts to wild-type levels (Figure 7B). In addition, Sox6 mRNA levels were reduced in skeletal muscles of MCK-miR-499 transgenic mice (Figure 7D). Such downregulation of Sox6 suggests that Sox6 mediates, at least in part, the increase in slow and decrease in fast myofiber gene expression in MCK-miR-499 transgenic mice, as shown in Figure 5F.

Transgenic Expression of Sox6 and Purβ Phenocopies MyomiR Gene Deletions

To further validate Sox6 and Purβ as potential mediators of MyomiR function, we generated MCK-Sox6 and MCK-Purβ transgenic mice. MCK-Sox6 mice appeared normal at birth; however, they demonstrated retarded growth at postnatal day 8 and ultimately died by 28 days of age. Analysis of multiple F0 transgenic animals revealed that simply elevating the expression of Sox6 by 3-fold in the neonatal heart was sufficient to extinguish expression of Myh7b and miR-499 (Figure 7C), and that

overexpression in skeletal muscle strongly repressed β-MHC and slow Tnni1 transcript levels (Figure 7E). Immunohistochemistry for β-MHC on skeletal muscle sections revealed a complete loss of type I fibers in MCK-Sox6 mice (Figure 7F). MCK-Purβ transgenic mice demonstrated that a 1.4-fold increase in Purβ expression was sufficient to repress β-MHC expression in skeletal muscle at the mRNA level, but had little effect on the overall number of type I fibers (Figure 7G and data not shown). Thus, overexpression of Sox6 at similar levels to those in the *miR-208a*^{-/-} heart, in addition to relatively modest elevation in expression of Sox6 and Purβ to levels comparable to those in MyomiR dKO mice (Figures 7E and 7G), was sufficient to evoke myofiber phenotypes similar to those of *miR-208a*^{-/-} and MyomiR dKO mice. These results provide strong support for the conclusion that these transcriptional repressors mediate the actions of MyomiRs on slow myofiber gene expression.

In contrast, these targets did not activate the expression of fast Tnni2 or Tnni3 (Figures 7E and 7G), as seen in *miR-208b*^{-/-}; *miR-499*^{-/-} dKO mice. The latter findings suggest that Sox6 and Purβ mediate the actions of miR-499 and miR-208 on the slow myofiber gene program, and that other targets of these MyomiRs are required for the activation of fast myofiber genes.

DISCUSSION

The results of this study reveal a network of miRNAs within myosin genes that regulates myosin expression, fiber type gene expression, and muscle performance. The functions of myosin genes thus extend far beyond the mere expression of myosin proteins to the control of myriad functions of striated muscles. A model to account for our results is shown in Figure 8.

Functions of MyomiRs in the Heart

As schematized in Figure 8, α-MHC and miR-208a sit atop a hierarchy of regulatory steps leading to myofiber diversification,

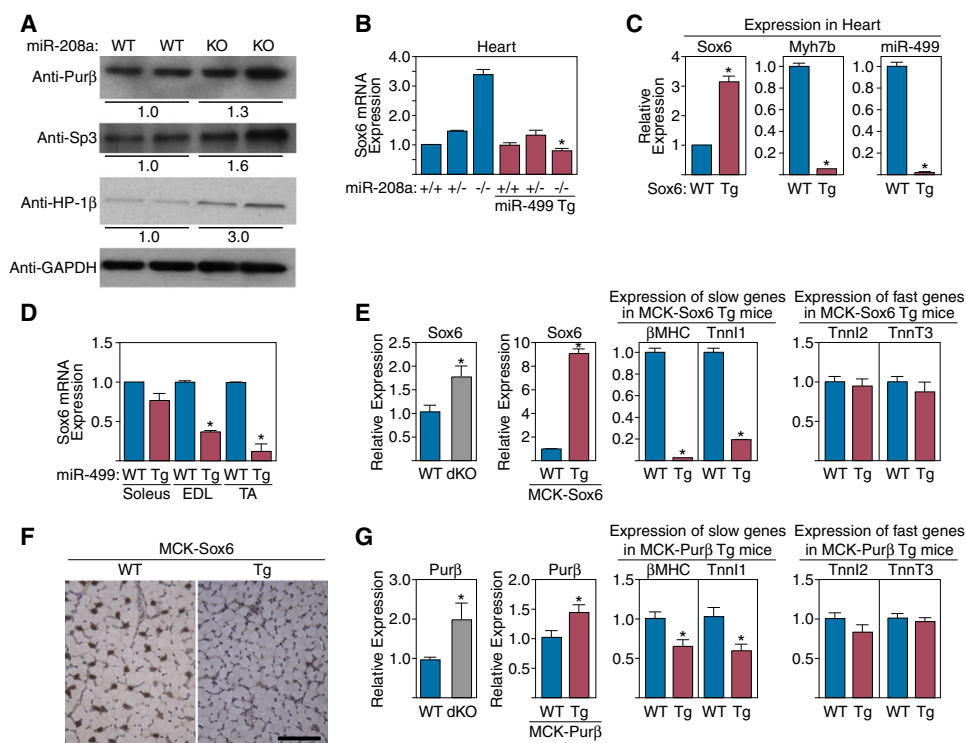


Figure 7. Target Proteins that Mediate the Actions of MyomiRs

(A) Western blot analysis of miR-208/499 target expression in adult wild-type (WT) and *miR-208a*^{-/-} (KO) mouse heart lysate. Relative expression was calculated from comparison of band intensity following normalization to GAPDH loading control.

(B) Sox6 mRNA levels are elevated in the *miR-208a*^{-/-} heart as measured by real-time PCR. Transgenic expression of miR-499 abolished the increase in Sox6 mRNA in hearts lacking miR-208a.

(C) Myh7b and miR-499 are repressed in the hearts of MCK-Sox6 transgenic mice. Transcript levels were measured in the hearts of P8 transgenic mice and wild-type controls (WT) by real-time PCR.

(D) Real-time PCR indicates that Sox6 mRNA levels are depressed in skeletal muscle of MCK-miR-499 mice.

(E) Sox6 mRNA transcript is overexpressed in MyomiR dKO skeletal muscle. MCK-Sox6 transgenic mice exhibit repression of slow skeletal muscle genes and unaltered levels of fast skeletal troponins in skeletal muscle. Expression was measured by real-time PCR in total hind limb muscle of MCK-Sox6 transgenic mice and wild-type (WT) controls.

(F) Immunohistochemistry for type I myosin (β-MHC) in the hind limb of MCK-Sox6 transgenic mice indicates an absence of type I fibers in the EDL of transgenic mice as compared with wild-type (WT) littermate controls. Scale bar, 100 μm.

(G) Purβ mRNA transcript is overexpressed in MyomiR dKO skeletal muscle. MCK-Purβ transgenic mice demonstrate repression of slow skeletal muscle genes and unaltered fast skeletal gene expression. Expression was measured by real-time PCR in total hind limb muscle of MCK-Purβ transgenic mice and wild-type (WT) controls.

stress-responsiveness, and thyroid hormone sensitivity of the heart. In the mouse heart, miR-208a, encoded by α -MHC, is required for upregulation of β-MHC and miR-208b in response to stress and hypothyroidism. miR-208a also regulates the expression of *Myh7b* and its intronic miRNA, miR-499; however, this regulatory step is distinct from the regulation of β-MHC/*miR-208b* in that *Myh7b/miR-499* are highly expressed in the adult heart in the absence of stress, whereas β-MHC/*miR-208b* require stress or hypothyroidism for expression. In addition to the requisite role of miR-208a in the expression of β-MHC and *Myh7b* in the adult heart, miR-208a is also required for repression of fast muscle genes in the heart.

Within the adult mouse heart, α -MHC is the predominant myosin, accounting for more than 90% of myosin expression (Lompre et al., 1984), and miR-208a is the dominant miRNA among the trio of MyomiRs. Although miRNAs are typically envisioned to function as fine-tuners of gene expression, miR-208a

operates in a precise, stoichiometric manner to control the expression of *Myh7b/miR-499* such that *Myh7b/miR-499* expression is reduced by ~50% in hearts of *miR-208a*^{+/-} mice and is extinguished in hearts of *miR-208a*^{-/-} mice. Forced expression of miR-499 in *miR-208a*^{-/-} hearts restores expression of β-MHC and represses ectopic expression of fast muscle genes, consistent with the conclusion that miR-499 functions downstream of miR-208a. Notably, transgenic expression of miR-499 in the hearts of *miR-208a*^{-/-} mice also reactivates the expression of *Myh7b*, indicative of a positive autoregulatory loop (Figure 8).

In large animals, β-MHC is the predominant myosin in the adult heart, so we presume that miR-208b, which shares an identical seed sequence with miR-208a, fulfills the functions of miR-208a in large animals. Whether miR-208b is required for pathological remodeling of hearts with predominantly β-MHC expression (Lompre et al., 1991), as is miR-208a in mice (van Rooij et al., 2007), is an important issue with respect to the

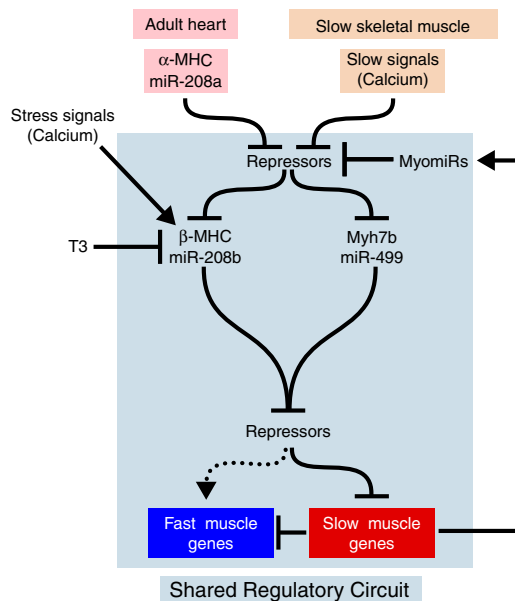


Figure 8. Control of Myosin and Fast Versus Slow Muscle Gene Expression by the MyomiR Network

In the adult heart, miR-208a encoded by the α -MHC gene is required for expression of β -MHC and *Myh7b*, which encode miR-208b and miR-499, respectively. Activation of *Myh7b* by miR-208a is constitutive, whereas activation of β -MHC also requires stress signals or absence of thyroid hormone. In slow skeletal muscle, *Myh7b* and β -MHC are regulated independently of miR-208a. miR-208a, miR-208b, and miR-499 repress the expression of a common set of transcriptional repressors that repress slow myosin and the slow myofiber gene program at the expense of fast muscle gene expression. Activation of the slow myofiber gene program also creates a positive feedback loop via the expression of miR-208b and miR-499, which further reinforce slow muscle gene program. Regulatory interactions within the blue box are shared by cardiac and slow skeletal muscle.

potential therapeutic manipulation of MyomiRs in the context of human heart disease.

There are several other noteworthy conclusions to be drawn about the regulation and functions of MyomiRs in the heart based on our findings with MyomiR mutant mice. First, the cardiac regulatory circuitry among the MyomiRs and their host myosins appears to be operative specifically in the adult, but not in the fetal, heart. We base this conclusion on the finding that β -MHC and *Myh7b* are expressed normally in neonatal hearts of *miR-208a*^{-/-} mice, whereas these myosin genes are silenced in heart of adult mutant mice. Thus, there must be a regulatory switch that distinguishes neonatal and adult cardiac gene programs with respect to their sensitivity to MyomiR functions. Second, none of the MyomiRs are essential for function of the adult heart, as evidenced by the phenotype of *miR-208a*^{-/-} mice, which are essentially MyomiR nulls, since the absence of miR-208a abolishes the expression of miR-208b and miR-499. Thus, the MyomiRs appear to function primarily to adapt adult cardiac gene expression to physiological and pathological signaling rather than to control cardiac homeostasis.

Functions of MyomiRs in Skeletal Muscle

Unlike α -MHC and miR-208a, which are expressed only in the heart, β -MHC/miR-208b and *Myh7b*/miR-499 are also

expressed in slow skeletal muscle. The latter two myosins and their intronic miRNAs appear to function in a shared regulatory circuit in cardiac and skeletal muscle; however, the upstream inputs into this myosin/miRNA circuit differ in cardiac and skeletal muscle in multiple ways (Figure 8). For example, the expression of miR-208b and miR-499 in slow skeletal muscle does not require an upstream MyomiR, since both of these miRNAs are expressed normally in the absence of the other, as shown from our analysis of miR-208b and miR-499 null mice. The activation of slow myofiber genes in skeletal muscle depends on calcium signaling through calcineurin and various kinases (Bassel-Duby and Olson, 2006; Wu et al., 2000), leading us to propose that β -MHC and *Myh7b* are activated independently through such signals, resulting in the production of their encoded MyomiRs, which then reinforce the slow skeletal muscle gene program through the actions of their targets. Importantly, this model infers the existence of a positive feedback loop whereby the targets of miR-208b and miR-499 in slow skeletal muscle enhance their own expression via their targets, which act on their host myosins. Such a mechanism also enables MyomiRs to function as binary regulators of slow versus fast muscle gene programs—activating slow and repressing fast—through their downstream targets. Moreover, we have observed that an increase of MyomiR levels in the soleus, TA, and EDL elicits a distinct shift toward the slow phenotype in each muscle. In addition to upregulating β -MHC, transgenic expression of miR-499 primarily drives these muscles to shift one myosin isoform slower in the sequential transition toward a slow myofiber phenotype (Pette and Staron, 1997). We hypothesize that divergent gene expression profiles, as well as different frequencies of muscle use between the soleus, TA, and EDL, may be responsible for the distinct changes we observe in response to gain of MyomiR function.

A Collection of Transcriptional Repressors Downstream of MyomiRs

It has been demonstrated that miRNAs function by finely modulating the expression of their downstream target genes at the protein level (Baek et al., 2008). Consistent with this idea, our results demonstrate that the MyomiRs target a cohort of transcriptional repressors to mediate their actions on striated muscle gene expression and function. Among their targets, Sox6, Pur β , and Sp3 have previously been shown to repress the expression of slow skeletal muscle genes, but the mechanisms that control the expression of these repressors have not been investigated. In Sox6 mutant mice, slow-fiber-type-specific gene expression, including the expression of β -MHC, is increased, whereas fast myofiber genes are repressed (Hagiwara et al., 2005, 2007; von Hofsten et al., 2008). It has been proposed that the repressive influence of Sox6 on β -MHC expression is mediated through direct binding of Sox6 to the promoter of the gene (Hagiwara et al., 2007). Pur β , a single-stranded DNA binding protein, cooperates with Sp3 to repress β -MHC expression (Ji et al., 2007). Thus, by dampening the expression of Sox6, Pur β , and Sp3, the MyomiRs enable the expression of β -MHC and other genes under the inhibitory influence of the repressors. Conversely, in the absence of MyomiRs, the expression of these repressors is enhanced, resulting in the inhibition of β -MHC and other downstream targets. Our finding that transgenic overexpression of Sox6 or Pur β represses slow genes, but does not activate fast

genes, suggests that another of the targets of MyomiRs likely mediates fast muscle gene expression downstream of MyomiRs or could require the combined actions of multiple targets.

In addition to the above targets, HP-1 β , a transcriptional repressor shown previously to function as a class II HDAC corepressor, is also a target for repression by the MyomiRs. Class II HDACs function as calcium-sensitive repressors of the MEF2 transcription factor, which is necessary and sufficient for activation of slow myofiber gene expression (Potthoff et al., 2007). The repressive influence of MyomiRs on HP-1 β expression would therefore be expected to diminish the repressive influence of class II HDACs on MEF2, promoting the expression of slow muscle genes. Thrap1, another validated target of the MyomiRs, functions as a modulator of thyroid hormone signaling and likely plays a key role in regulating the response of β -MHC and myofiber gene programs to hypothyroidism.

Interestingly, our data indicate that the MyomiR family functions redundantly in vivo, while luciferase reporter assays suggest that these microRNAs may exhibit differential effects on target gene repression. These differences in our reporter assays may be attributed to dissimilarity in sequence outside of the seed region of miR-208 and miR-499, which may have effects on the miRNA:target interactions that dictate repression, or possibly to differences in miRNA-associated machinery in vitro and in vivo.

Implications for Gene Regulatory Networks

Sequence analysis of MHC genes indicates that *Myh7b* is the most ancient of this trio of myosins and that gene duplications gave rise to α - and β -myosins. The MyomiRs are conserved from fish to human genomes, but do not exist in invertebrates, suggesting that the ancestral *Myh7b* gene contained the ancestral MyomiR in an intron, and when that myosin gene duplicated, so too did its intronic MyomiR. It is intriguing, in this regard, that the existence of MyomiRs correlates with myofiber diversification and the acquisition of hormonal sensitivity and stress responsiveness. These findings argue strongly for selection pressure to maintain not only the conserved sequence of MyomiRs, and presumably of their targets, but also the integration of this family of miRNAs into myosin genes, so as to ensure their regulatory relationship. The incorporation of MyomiRs into the introns of myosin genes that they regulate also provides an efficient means of ensuring the coregulation of the miRNA and the gene programs under its control, rather than creating separate sets of *cis*-regulatory elements to control expression of the miRNA and the myosin gene.

The functions and regulation of the MyomiR network serve as a paradigm for phenotypic control. This elaborate regulatory network provides communication between genes and reveals an unanticipated breadth of functions of myosin genes, previously recognized simply for their roles as protein-coding genes. We speculate that protein-coding genes with central roles in the functionality of other tissues will also be found to encode families of miRNAs that dictate the functions of those tissues, especially in settings of disease. Perhaps most provocative is the realization that miRNA functions can be perturbed without deleterious consequences on tissue function, but with dramatic effects on signal-dependent tissue remodeling responses. As such, miRNAs represent potentially powerful disease modifiers and therapeutic targets.

EXPERIMENTAL PROCEDURES

Northern Blot Analysis

Total RNA was isolated from mouse and human cardiac tissue samples by using Trizol reagent (GIBCO/BRL). Northern blots to detect miRNAs were performed as described previously (van Rooij et al., 2006). U6 was detected as a loading control using the following primers in RT-PCR reactions: U6 forward: 5-GTGCTCGCTTCGGCAGC-3, U6 reverse: 5-AAATATGGAACGC TTCACGAATTTGCG-3.

RNA In Situ Hybridization

Tissues used for histology were incubated in Krebs-Henselheit solution, fixed in 4% paraformaldehyde, sectioned, and processed for in situ hybridization by standard techniques (Shelton et al., 2000). ³⁵S-labeled RNA probes were generated using Maxiscript kit (Amersham). Signals were pseudocolored in red using Adobe Photoshop.

miRNA Microarray Analysis

Microarray assay was performed using a service provider (LC Sciences, Houston). The assay started from 10 μ g total RNA from cardiac tissue of both wild-type and *miR-208*^{-/-} mice, which was size fractionated, and the small RNAs (<300 nt) isolated were 3'-extended with a poly(A) tail using poly(A) polymerase. An oligonucleotide tag was then ligated to the poly(A) tail for later fluorescent dye staining. Hybridization was performed overnight on a μ Paraflo microfluidic chip on which each detection probe consisted of a chemically modified nucleotide coding segment complementary to target miRNA (from miRBase, <http://microrna.sanger.ac.uk/sequences/>). After RNA hybridization, tag-conjugating Cy5 dyes were circulated through the microfluidic chip for dye staining. Fluorescence images were collected using a laser scanner (GenePix 4000B, Molecular Device) and digitized using Array-Pro image analysis software (Media Cybernetics). Data were analyzed by first subtracting the background and then normalizing the signals using a Locally-weighted Regression (LOWESS filter). The ratio of the two sets of detected signals (log2 transformed, balanced) and p values of the t test were calculated; differentially detected signals were those with p < 0.01.

RT-PCR and Real-Time Analysis

RT-PCR with random hexamer primers (Invitrogen) was performed on RNA samples, after which the expression of a subset of genes was analyzed by either PCR or quantitative real-time PCR using Taqman probes purchased from ABI.

Generation of Transgenic Mice

A mouse genomic fragment flanking miR-499 was subcloned into a muscle-specific expression plasmid containing the MCK enhancer and human GH poly(A)⁺ signal (Sternberg et al., 1988). Genomic DNA was isolated from mouse tail biopsies and analyzed by PCR using primers specific for the human GH poly(A)⁺ signal.

Western Blot Analysis

Myosin was extracted from cardiac tissue as described (Bar and Pette, 1988). MHC isoforms were separated by SDS-PAGE and western blotting was performed with mouse monoclonal α -MHC (BA-G5) (ATCC, Rockville, MD) and mouse monoclonal anti-myosin (slow, skeletal M8421) (Sigma, MO), which is highly specific for β -MHC. Total heart lysate was prepared by homogenizing tissue in RIPA buffer, was resolved by SDS-PAGE, and was analyzed to detect HP-1 β , Pur β , and Sp3 using mouse monoclonal anti-HP-1 β (Cell Signaling #2613) at a dilution of 1:1000, anti-Pur β (a gift from Robert J. Kelm Jr. at University of Vermont College of Medicine; Kelm et al., 1999) at a dilution 1:1000, and anti-Sp3 (Abcam ab18334) at a dilution of 1:200. Anti-mouse IgG conjugated to horseradish peroxidase (Biorad) at a dilution of 1:5000 was used as a secondary antibody, followed by detection with ECL detection kit (Amersham).

Generation of miR-499 and miR-208b Mutant Mice

To generate the miR-499 targeting vector, a 4.9 kb region (5' arm) extending upstream of the miR-499 coding region was ligated into the pGKneoF2L2 Dta targeting plasmid upstream of the loxP sites and the Frt-flanked neomycin

cassette. A 2.6 kb fragment (3' arm) was ligated into the vector between the neomycin resistance and Dta negative selection cassettes. Targeted ES cells carrying the disrupted allele were identified by Southern blot analysis with 5' and 3' probes. Three miR-499 targeted ES clones were identified and used for blastocyst injection. A comparable approach was taken to generate the miR-208b targeting vector, with a 4.9 kb region extending upstream of the miR-208b coding region functioning as the 5' arm and a 3.5 kb fragment functioning as the 3' arm. The resulting chimeric mice were bred to C57BL/6 to obtain germline transmission of the mutant allele. PCR primer sequences are available upon request.

Fiber-Type Staining

Soleus, GP, TA, and EDL muscles were isolated at 8 weeks of age and were embedded in a 3:1 ratio of Tissue Freezing Medium to gum tragacanth. Samples were flash frozen and sectioned on a cryostat-microtome. Metachromatic ATPase staining was performed as previously described (Ogilvie and Feedback, 1990).

Forced Running

Forced treadmill running of 6-week-old wild-type and MCK-miR-499 transgenic mice was performed as follows. Animals were trained on the treadmill (Colombus Instruments, 10% incline) for 5 min at 7 m/min for 2 consecutive days. The following day mice were run at 8 m/min for 30 min, then 9 m/min for 15 min, followed by 10 m/min for 15 min. Finally, speed was incrementally increased by 1 m/min every 10 min until the mouse exhibited exhaustion. The end point was reached when a mouse received repeated electrical stimuli for greater than 5 s.

MHC Electrophoresis

Myosin was isolated from skeletal muscle and run on glycerol-SDS-PAGE gels as previously described (Talmadge and Roy, 1993). Gels were stained with a silver nitrate staining kit (Biorad).

Cell Culture, Transfection, and Luciferase Assays

A 300–500 bp genomic fragment encompassing the miR-208a or miR-499 coding region was amplified by PCR and ligated into pCMV6. Full-length 3' UTRs of Sox6, Sp3, HP-1 β , and Pur β were cloned into the pMiR-report vector (Ambion). Cell culture, transfection, and luciferase studies were performed as previously described (van Rooij et al., 2007). Mutations in the 3' UTRs alter the 2nd and 3rd nucleotides of the targeting sequence, and were generated using QuickChange Lightning kit (Stratagene).

Immunohistochemistry

Tissue samples were fixed in 4% paraformaldehyde and embedded in paraffin for sectioning. Deparaffinized sections were permeabilized with 0.04% Pro-nase E, then blocked in 0.5% bovine serum albumin/5% normal goat serum in PBS. NOQ7.5.4D (1:16,000) was used for primary detection of type I myosin, and HRP-conjugated secondary antibody (Sigma A8924) followed by DAB chromagen reaction was used for detection. Samples were then counterstained with hematoxylin.

Analysis of Transgenic Animals

Two or three F1 transgenic lines of MCK-miR-499 and MCK-Pur β animals, and three F0 transgenic MCK-Sox6 animals, were analyzed in this study.

Statistical Analysis

All graphs represent mean values \pm SD. * p < 0.05 as calculated by unpaired t test.

Animal Care

All animal procedures were previously approved by the Institutional Animal Care and Use Committee at University of Texas Southwestern Medical Center.

SUPPLEMENTAL DATA

Supplemental data for this article include four figures and three tables and can be found with this article online at [http://www.cell.com/developmental-cell/supplemental/S1534-5807\(09\)00435-3](http://www.cell.com/developmental-cell/supplemental/S1534-5807(09)00435-3).

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