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Evidence of MyomiR network regulation of β-myosin heavy chain gene expression during skeletal muscle atrophy

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McCarthy JJ, Esser KA, Peterson CA, Dupont-Versteegden EE. Evidence of MyomiR network regulation of β-myosin heavy chain gene expression during skeletal muscle atrophy. *Physiol Genomics* 39: 219–226, 2009. First published August 18, 2009; doi:10.1152/physiolgenomics.00042.2009.—There is a growing recognition that noncoding RNAs (ncRNA) play an important role in the regulation of gene expression. A class of small (19–22 nt) ncRNAs, known as microRNAs (miRs), have received a great deal of attention lately because of their ability to repress gene expression through a unique posttranscriptional 3′-untranslated region (UTR) mechanism. The objectives of the current study were to identify miRs expressed in the rat soleus muscle and determine if their expression was changed in response to hindlimb suspension. Comprehensive profiling revealed 151 miRs were expressed in the soleus muscle and expression of 18 miRs were significantly ($P < 0.01$) changed after 2 and/or 7 days of hindlimb suspension. The significant decrease (16%) in expression of muscle-specific miR-499 in response to hindlimb suspension was confirmed by RT-PCR and suggested activation of the recently proposed miR encoded by myosin gene (MyomiR) network during atrophy. Further analysis of soleus muscle subjected to hindlimb suspension for 28 days provided evidence consistent with MyomiR network repression of β-myosin heavy chain gene (β-MHC) expression. The significant downregulation of network components miR-499 and miR-208b by 40 and 60%, respectively, was associated with increased expression of Sox6 (2.2-fold) and Purβ (23%), predicted target genes of miR-499 and known repressors of β-MHC expression. A Sox6 3′-UTR reporter gene confirmed Sox6 is a target gene of miR-499. These results further expand the role of miRs in adult skeletal muscle and are consistent with a model in which the MyomiR network regulates slow myosin expression during muscle atrophy.

microRNA; microarray; hindlimb suspension; fiber type

SKELETAL MUSCLE ATROPHY IS associated with a number of different physiological and pathological conditions such as disuse, aging, congestive heart failure, and cancer (22). Muscle atrophy is often accompanied by a phenotypic transformation that is characterized by a slow- to fast-twitch shift in fiber-type (2). The transition to a fast-twitch phenotype during muscle atrophy is in part brought about by an alteration in the expression of myosin heavy chain (MHC) isoforms. In particular, the expression of the slow-twitch MHC isoform, commonly referred to as β-MHC, is downregulated concomitant with upregulation of fast-twitch MHC isoforms, most notably MHC type IIb and IIx (30).

Along with slow myosin light chain 2 and slow troponin I, the regulation of β-MHC gene expression has been used as a model system to elucidate the molecular mechanisms underlying the fiber-type shift that occurs with alterations in skeletal muscle contractile activity (5, 11, 25). Transgenic and biochemical analyses of the β-MHC promoter revealed that different transcription factors are involved in the activation and repression of the β-MHC gene during hypertrophy and atrophy, respectively, and that these factors bind to distinct regulatory regions within the β-MHC promoter (19, 20, 26, 32). During muscle atrophy, Sp3 in combination with Purα and Purβ were reported to repress β-MHC gene expression (19, 32). The increased expression of these negative regulators of β-MHC gene expression during muscle atrophy were paralleled by a loss in TEF-1 (transcription enhancer factor 1) and myogenin expression, known transcriptional activators (13).

In addition to the negative regulators described above, Hagiwara and colleagues (15) provided evidence that the transcription factor Sox6 (SRY-box containing gene 6) suppresses the slow-twitch phenotype. In skeletal muscle of a Sox6 mutant, which is unable to bind DNA as the result of a radiation-induced premature termination codon, there was upregulation of slow-twitch isoform genes and a concomitant downregulation of fast-twitch isoform genes. In particular, the β-MHC gene was upregulated almost 25-fold in skeletal muscle of the Sox6 mutant, suggesting Sox6 is a repressor of β-MHC expression (15). In a follow-up study, this same group demonstrated Sox6 was capable of repressing β-MHC expression and identified a negative regulatory *cis*-element within the proximal promoter of β-MHC gene bound by Sox6 (16).

Recent evidence suggests that, in addition to transcription factors, a new class of small, highly conserved, noncoding RNAs may have a role in the regulation of fiber type (23). These microRNAs (miR) have been shown to silence gene expression by binding to the 3′-untranslated region (UTR) of target mRNAs and inhibit initiation of translation and/or promote transcript cleavage (18). Muscle-specific miRs (miR-1, -133, 206, 208, 208b, and -499) have been identified and shown to be involved in a range of processes including myogenesis (proliferation, differentiation, and fiber type specification), regeneration, hypertrophy, and muscular dystrophy (4, 6, 10, 12, 21, 23, 24, 27, 28, 34, 37). Interestingly, miR-208a, -208b, and -499 are each encoded by an intron of a different myosin heavy chain gene, Myh6 (α-MHC), Myh7 (β-MHC), and Myh7b, respectively. Van Rooij and colleagues (33) have proposed the existence of a miR encoded by myosin gene (MyomiR) network composed of these three miRs that is involved in the regulation of MHC expression and potentially fiber type. One branch of the network has been shown to be necessary for the upregulation of the β-MHC gene during cardiac hypertrophy and hypothyroidism (34). Specifically, miR-208a, which is derived from intron 29 of the α-MHC gene, was required for

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β-MHC gene activation by repressing expression of Thrap1, a thyroid hormone receptor cofactor and known repressor of β-MHC expression (34).

More recently, van Rooij and coworkers (35) provided evidence for the MyomiR network in skeletal muscle by reporting overexpression of miR-499 was sufficient to promote a fast-to-slow-twitch fiber type transition, thought to be in part mediated by the downregulation of Sox6. Furthermore, an additional link in the network was uncovered when it was demonstrated that miR-208a or miR-208b can drive expression of miR-499 by activation of its host gene Myh7b by an unknown mechanism (35). An emerging theme is miRs within the network regulate MHC expression indirectly by targeting transcription factors that themselves directly regulate MHC gene expression.

Given the growing importance of miRs in regulating gene expression in striated muscle, the objectives of the present study were to identify those miRs expressed in the rat soleus muscle and determine if their expression was altered with muscle atrophy induced by hindlimb suspension (HS). Based on our previous studies examining the expression of the muscle-specific miRs during skeletal muscle hypertrophy and muscular dystrophy, we hypothesized that expression of one or more of the muscle-specific miRs would be altered during muscle atrophy (23, 24). The results from the profiling experiment revealed no change in the expression levels of the canonical muscle-specific miRs (miR-1, -133, and -206) following 2 and 7 days of HS but did detect reduced expression in the newest member of the family, miR-499. Evidence is provided consistent with a mechanism in which miR-499 indirectly regulates β-MHC expression via posttranscriptional regulation of Sox6 expression, a known repressor of β-MHC expression.

METHODS

Animal care. All animal procedures were conducted in accordance with institutional guidelines for the care and use of laboratory animals as approved by the Animal Care and Use Committee of the University of Arkansas for Medical Sciences. The tissue used in the current study was collected from Sprague-Dawley rats (male, 6 mo of age) subjected to 2, 7, and 28 days of HS as previously described by Dupont-Versteegden et al. (9). After 2 days of HS, there was a 17% loss in soleus muscle fiber cross-sectional area but no significant change in muscle weight when normalized to body weight. However, following 7 days of HS, a 32% decrease in soleus muscle fiber cross-sectional area was observed that was accompanied by a 11% loss in normalized muscle weight (9). For the present study, microRNA expression profiling was determined in three groups: control (C), HS for 2 days (HS-2), and HS for 7 days (HS-7).

RNA isolation. Total RNA was isolated from rat soleus muscle using ToTALLY RNA (Ambion, Austin, TX) according to manufacturer's directions. RNA samples were treated with TURBO DNase (Ambion) to remove genomic DNA contamination and RNA integrity assessed using the Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA); the average RIN (RNA integrity number) value for all samples was 8.4 ± 0.5 (scale 1–10), indicating high-quality RNA with minimal degradation.

MiR expression profiling. Three microarray chips were analyzed for each of the three groups (nine chips in total) representing one animal per chip. Total RNA samples were not pooled in an effort to minimize any influence possible outliers might have on miR expression. Total RNA (12 μg/sample) was sent to LC Sciences (Houston, TX) for miR expression profiling using their proprietary mParaFlo microfluidic chip containing 234 rat microRNA probes (Sanger miRBase 9.0) plus an additional 96 probes derived from predicted rat miRs that were conserved in the mouse and mapped to transcribed loci (3, 31). See supplemental data Table S1[1] for sequence information of the predicted rat miRs. Data analysis of the microarray results was performed by LC Science. Expression values were normalized by removing system related variation (sample amount variations, different labeling dyes, and signal gain differences of scanners) using a locally weighted regression (cyclic LOWESS). Data adjustment included data filtering, $\log_2$ transformation, gene centering ,and normalization. The data filtering removed miRs with normalized intensity values below a threshold value (twice the maximum background signal) across all samples. The $\log_2$ transformation converted intensity values into $\log_2$ scale. Gene centering and normalization transformed the $\log_2$ values using the mean and the standard deviation of individual genes across all samples using the following formula: Value = [(Value) − Mean (Gene)]/[Standard deviation (Gene)]. ANOVA analysis was performed to identify significant differences across groups. Student's *t*-tests were performed between C and HS-2 or HS-7 groups when each group contained at least two samples. MicroRNAs with P values <0.05 were selected for cluster analysis using a hierarchical method performed with average linkage and Euclidean distance metric. The complete microarray data set has been submitted to NCBI GEO with accession number GSE17776.

Taqman RT-PCR. To validate the microarray results the relative expression level of miR-23b, miR-107, miR-126*, miR-221, miR-206, and miR-499 were determined using Taqman MicroRNA Assay (Foster City, CA) according to the manufacturer's directions. The relative expression level for each miR was calculated using the comparative C_T method ($\Delta\Delta C_T$). To account for possible differences in the amount of starting RNA, miR expression was normalized to small nucleolar RNA U87.

RT-PCR analysis. First-strand cDNA synthesis from total RNA was performed with oligo$(dT)_{12–18}$ primer using SuperScript II RT (Invitrogen, Carlsbad, CA) according to manufacturer's directions. The PCR reaction (20 μl) consisted of 2 μl of 50-fold dilution of the cDNA, 1X *Power* SYBR Green Master Mix, and 0.2 μM of each primer. The primers for each gene of interest were designed using Biology Workbench 3.2 PrimerTm program (http://seqtool.sdsc.edu/CGI/BW.cgi) and spanned a single intron with a T_m of ~60°C. Primer sequences and product size for each gene are as follows: *Myh7*; forward (F) = 5′-CCC AGGTCAACAAGCTGC-3′, reverse (R) = 5′-GGGTTGGGTAGCACAAGATCT-3′, 84 bp; Sox6, F = 5′-GCGCTCACGAAGGCAG-3′, R = 5′-GCCATAGTAATAGCACCAGGA TACA-3′, 107 bp; Purβ, F = 5′-CCAGGAGCGACAGAGGG-3′, R = 5′-CCTCATCCACTT CCTCACCC-3′, 89 bp; cyclophilin A, F = 5′-GGACCAAACACAAATGGTTCC-3′, R = 5′-GGCTTCCACAATGC TCATG-3′, 114 bp. The UCSC Genome Browser *in-silico* PCR tool (http://genome.ucsc.edu) as well as melt curve analysis confirmed that each primer set amplified only a single product. To account for any starting difference in the amount of total RNA, gene expression levels were normalized to cyclophilin A expression, which did not change with HS.

Transfection experiments. To validate the prediction that Sox6 is a target gene of miR-499 a Sox6 3′-UTR luciferase reporter gene was constructed. The rat Sox6 3′-UTR was amplified from rat skeletal muscle cDNA and cloned into the *Xba*I site downstream of the luciferase gene in the pGL3-promoter vector (Promega, Madison, WI). The single miR-499 site within the cloned rat Sox6 3′-UTR was mutated by altering the first two nucleotides of the miR-499 seed sequence using Gene Editor System (Promega). Orientation and sequence of the wild-type and mutated rat Sox6 3′-UTR were confirmed by sequencing. NIH/3T3 cells (5×10^4) were plated in a

[1] The online version of this article contains supplemental material.

12-well plate and transfected 24 h later with Sox6 3′-UTR reporter gene (100 ng/well), with or without precursor miR (10 nM final concentration, Ambion) and pRL-CMV vector (5 ng) for normalization using Lipofectamine 2000 according to the manufacturer's directions (Invitrogen). Twenty-four hours posttransfection, cells were lysed and luciferase activities measured using the Dual-Luciferase Assay according the manufacturer's directions (Promega).

Statistical analysis. Data are reported as means ± SE with $n = 8$ per group except where indicated otherwise. Taqman RT-PCR results were analyzed by ANOVA followed by post hoc Tukey's test to identify significant differences between control and HS groups ($P < 0.05$). For experiments involving two groups (HS-28 analysis and transfections), a Student's *t*-test was used to determine if a significant difference existed between control and the experimental group. Significant difference ($P < 0.05$) was denoted by an asterisk.

RESULTS

miR expression profile of rat soleus muscle. A comprehensive microarray analysis was performed to determine the miR expression profile of the soleus muscle in normal adult male rats. We profiled a total of 330 miRs, which represented 234 rat miRs as listed by miRBase 9.0 and 96 predicted miRs as reported by Berezikov et al. and Takada et al. (3, 31). The 96 predicted miRs were chosen based on their conservation in the mouse and, when possible, mapped to a protein coding gene; designation and sequence information for each of the predicted miRs used in the array is provided as supplemental data (Supplemental Table S1). Of the 330 probe sets present on the array, 162 (49.1%) were detected in the rat soleus muscle including 11 predicted miRs (Supplemental Fig. S1). A search of miRBase 12.0 revealed that of the predicted miRs expressed in the rat soleus muscle, only MM_234 shared identity to the recently discovered murine miR-762. The low level of expression (mean intensity value was 96 with a range of 7 to 341) of the predicted miRs, including MM_234, suggested they are probably of minor biological significance in the soleus muscle, though further experiments are necessary to explore this possibility, as well as whether or not MM_234 is indeed the rat homolog of miR-762. As expected, the muscle-specific miRs (miR-1, -133, -206, and -499) were some of the most abundant miRs in the soleus muscle with miR-1 and miR-206 expression being almost twofold greater than any other detected miR (Supplemental Table S2).

Altered miR expression in response to hindlimb suspension. To identify HS-responsive miRs we compared the miR expression profiles of control soleus muscle to that of soleus muscle subjected to 2 or 7 days of HS. As shown in Fig. 1, the expression level of 18 miRs was found to be significantly different ($P < 0.05$) from control after 2 and/or 7 days of HS. These differentially expressed miRs were clustered into four distinct groups: *cluster 1*, downregulated after 2 and 7 days of HS (miR-20a, -221, -222, and -499); *cluster 2*, downregulated only after 2 days of HS (miR-23b, -27b, -333, and -505); *cluster 3*, upregulated only after 7 days of HS (miR-126*, -148b, -338, -183, -489, and -7*); and *cluster 4*, upregulated after 2 and 7 days of HS (MM_264, miR-189, -377, and -98). Of these differentially expressed miRs, only four (miR-23b, -27b, -98, and -499) were expressed at a level (>500, range 5,200–26,650) considered to be of biological significance (Supplemental Table S2) (8). We focused our downstream analysis on miR-499 because it is the newest member of the muscle-specific miR family and has been proposed to be part of a MyomiR network involved in the regulation of skeletal muscle fiber type (33).

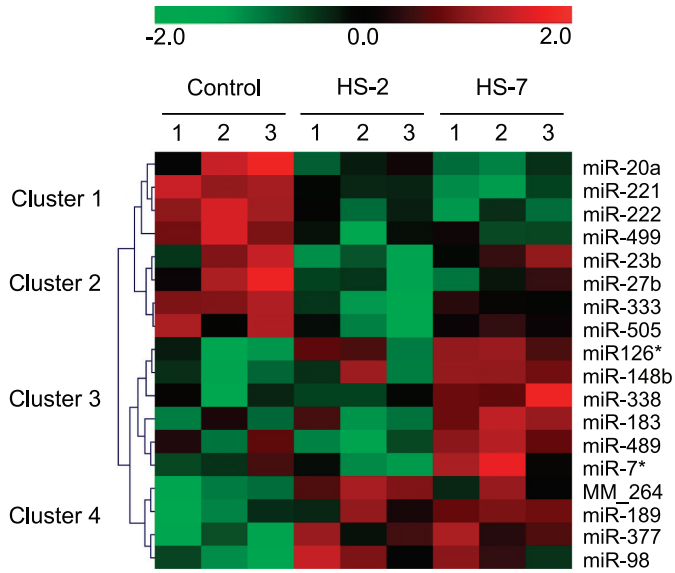

Fig. 1. Differentially expressed microRNAs (miRs) in rat soleus muscle following hindlimb suspension. Heat map displays the relative change in miR expression following 2 and 7 days of hindlimb suspension as detected by microarray. Columns (left to right) *1–3*, control (Control); *4–6*, hindlimb suspension for 2 days (HS-2), *7–9*, hindlimb suspension for 7 days (HS-7). Differentially expressed miRs ($P < 0.05$) were grouped into 4 clusters based on expression pattern: *1*) downregulated in HS-2 and HS-7 group relative to control, *2*) downregulated in HS-2 group relative to control, *3*) upregulated in HS-7 group relative control, and *4*) upregulated in HS-2 and HS-7 groups relative to control.

To confirm the profiling results showing the downregulation of miR-499 expression in soleus muscle undergoing atrophy, Taqman RT-PCR was performed. In addition to miR-499, the expression of miR-23b, -107, -126*, -206, and -221 were determined using Taqman analysis: miR-23b because it was the most abundant HS-responsive miR and miR-206 because its expression was previously shown to be altered with hypertrophy and muscular dystrophy (23, 24). The Taqman results confirmed a significant 16% reduction in miR-499 expression after 7 days of HS but, in contrast to the array result, did not detect a difference in expression following 2 days of HS (Fig. 2). In addition to miR-499, the expression of miR-107 and miR-221 was confirmed to be downregulated by ~40% in the HS-7 group. The expression levels of miR-23b, miR-126*, and miR-206 were, however, found to be unchanged from control level after 2 or 7 days of HS, though the decrease in miR-23b expression after 7 days of HS was statistically significant at $P = 0.054$ ($n = 8$).

Informatic analysis of predicted target genes of miR-499. MicroRNA-499 is encoded by intron 19 of the myosin heavy chain 7b gene (Myh7b) and is highly conserved, being present in the genome of the rat, human, mouse, frog, zebrafish, dog, chimp, bull, and chicken (http://www.targetscan.org/). In an effort to gain insight into the potential function of miR-499 in response to HS we evaluated the predicted target genes of miR-499, as identified by TargetScan 4.2 algorithm (http://www.targetscan.org/) and subjected this list to functional annotation by DAVID (http://david.abcc.ncifcrf.gov/). Annotation of 195 highly conserved target genes revealed enrichment

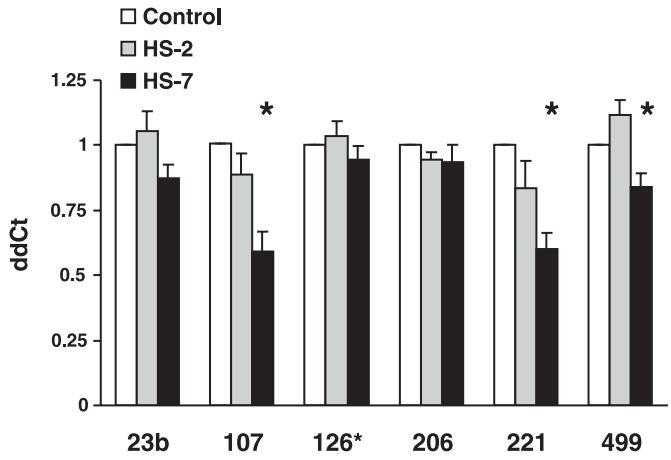

Fig. 2. Decreased expression of miR-499 following hindlimb suspension. Taqman RT-PCR was used to validate microarray results of differentially expressed miRs. Expression of miR-107, -221, and -499 were confirmed to be significantly different from control after 7 days of HS (HS-7). In contrast to microarray results, miR-23b and miR-126* expression was not significantly different from control after 2 or 7 days of hindlimb suspension. Relative to control, expression of miR-206 was unchanged in HS-2 and HS-7 groups, confirming the microarray results. miR expression levels were calculated by the $\Delta\Delta C_T$ (ddCt) method using small nucleolar RNA U87 for normalization. Values are presented as means ± SE ($n = 8$); *significant difference from control ($P < 0.05$).

of genes involved with RNA metabolism, regulation of transcription, organ development, phosphate metabolism, and cell migration. Table 1 highlights miR-499 target genes that have established roles in regulating fiber type (*Sox6*, *Purβ*, *Thrap1*, *CalnA,* and *CalnB*), muscle size (*myostatin*), and miR biogenesis and function (*Lin28b*, *Ago4,* and *Dgcr1*).

Of particular interest was the prediction that miR-499 targets Sox6 and Purβ, both known to repress expression of the Myh7 gene, the slow-twitch/cardiac isoform of the myosin heavy chain gene, commonly referred to as the β-myosin heavy chain (β-MHC) (16, 19). Of the 26 miRs predicted to target rat Sox6, miR-499 is ranked as number one in terms of 3′-UTR site efficacy with a context score of −0.83 (Table 1). This miR-499 site efficacy ranking is conserved in human and mouse with context scores of −1.59 and −0.90, respectively. The context score represents a summary of determinants that contribute to the ability of a miR binding site to mediate repression of gene expression (Table 1) (14). The more negative the context score, the greater the predicted efficacy of an miR binding site. In testing the validity of their model, Grimson and coworkers (14) reported context scores of −0.30 or greater were capable of repressing in vitro reporter gene expression. The possibility miR-499 controls β-MHC expression by regulating Sox6 expression was recently proposed by the Olson group to be part of a MyomiR regulatory network (33).

Table 1. *Predicted target genes of miR-499*

Gene Symbol	Description	Context Score
Sox6	SRY-box containing gene 6	−0.83
Purb	purine-rich element binding protein-β	−0.11
Thrap1	thyroid hormone receptor associated prot. 1	−0.19
CalnA	calcineurin, α-subunit	−0.34
CalnB	calcineurin, β-subunit	−0.17
Myostatin	growth differentiation factor 8 (myostatin)	−0.48
Lin28b	lin-28 homolog B (*C. elegans*)	−0.53
Ago4	agronaute 4	−0.27
Dgcr8	DiGeorge syndrome critical region gene 8	−0.31

miR, microRNA.

Analysis of MyomiR network during hindlimb suspension. As an initial effort to determine whether or not the MyomiR network was functioning during HS, the expression of components of the network (miR-499, miR-208b, β-MHC and Sox6) were measured by RT-PCR. The transcription factor Purβ was also included in the analysis because it has been shown to repress β-MHC expression in response to HS and is a predicted target gene of miR-499 (Table 1) (14, 19). On the other hand, miR-208a, which is a component of the network, was not considered for further analysis because it was not detected by the microarray analysis (Supplemental Table S2); the failure to detect miR-208a expression was not surprising because it is encoded by an intron of the α-MHC gene that is not expressed in skeletal muscle.

The expression of β-MHC was measured in the rat soleus muscle after 2, 7, and 28 days of HS. There was no change in β-MHC mRNA amount after 2 and 7 days of HS (data not shown) but was significantly reduced by 28% following 28 days of HS compared with control (Fig. 3). If this loss in β-MHC mRNA expression was, in part, due to regulation by the MyomiR network then one expectation would be a change in the expression of network components such as miR-499 and miR-208b.

As shown in Fig. 4, the expression of miR-499 significantly decreased by 40% in the soleus muscle of HS-28 group compared with control, a further reduction from the 16% observed in the HS-7 group. This change in miR-499 expression was paralleled by a significant 60% decrease in miR-208b expression in the HS-28 group relative to control (Fig. 4). These data are consistent with regulation of β-MHC expression by the MyomiR network and, furthermore, are in agreement with the previous finding that miR-208b controls miR-499 expression (35).

To date, the transcription factors which fall under MyomiR network regulation are known negative regulators of myosin

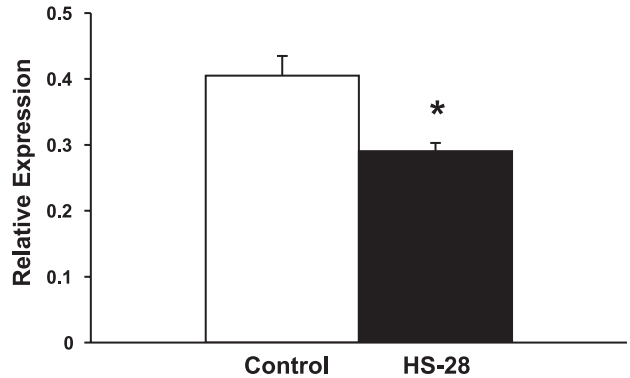

Fig. 3. Decreased expression of β-myosin heavy chain (MHC) following hindlimb suspension. The expression of β-MHC (Myh7) in the rat soleus muscle was measured by RT-PCR following hindlimb suspension for 2, 7, and 28 days (HS-28). There was a significant ($P = 0.031$) decrease of 28% in β-MHC expression in the HS-28 group relative to control but no change in β-MHC expression after 2 or 7 days of hindlimb unloading (data not shown). β-MHC expression was normalized to cyclophilin A expression. The relative expression values are presented as means ± SE ($n = 3$); *significance ($P < 0.05$).

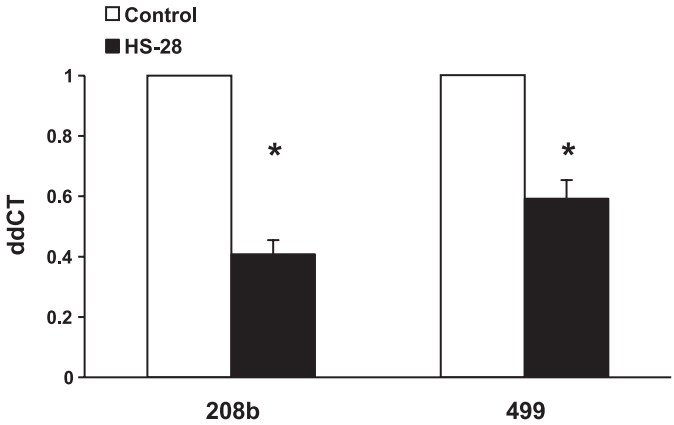

Fig. 4. Decreased expression of miR-208b and miR-499 following 28 days of hindlimb suspension. Taqman RT-PCR was used to measure the expression of miR-208b and miR-499 in the rat soleus muscle of control and hindlimb suspension (HS-28, hindlimb suspension for 28 days) groups. In the HS-28 group, miR-208b expression significantly ($P = 0.024$) decreased by 60% relative to the control group. Expression of miR-499 showed a significant ($P = 0.039$) decrease of 40% in the HS-28 compared with control. MicroRNA expression levels were calculated by the $\Delta\Delta C_T$ (ddCt) method using small nucleolar RNA U87 for normalization. Values are presented as means ± SE ($n = 3$); *significant difference from control ($P < 0.05$).

expression such as Sox6 and Purβ. According to the model, a decrease in miR-499 expression should lead to a de-repression of target gene expression, specifically Sox6 and Purβ. To test this prediction of the model, the transcript level of Sox6 and Purβ was measured by RT-PCR. As shown in Fig. 5, there was a modest, but significant, increase of 23% in Purβ expression in the soleus of HS-28 group compared with the control group. The change in Purβ expression in the HS-28 soleus was accompanied by a much greater increase in Sox6 expression of ~2.2-fold relative to control (Fig. 5). Interestingly, the magnitude of change in the expression of Purβ and Sox6 was inversely related to their miR-499 site context scores of −0.11 and −0.83, respectively (Table 1). These results together with the observed changes in β-MHC and miR-499 expression

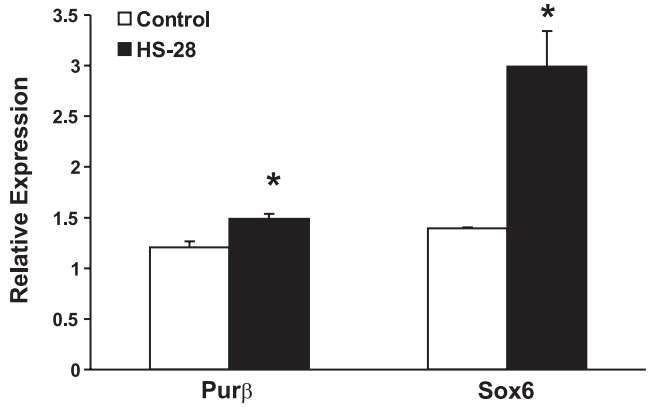

Fig. 5. Increase expression of Purβ and Sox6 following hindlimb suspension. The expression of Purβ and Sox6 in the rat soleus muscle was measured by RT-PCR in control and hindlimb suspension (HS-28, hindlimb suspension for 28 days) groups. Relative to the control group, the expression of Purβ significantly increased by 23%, whereas Sox6 expression increased ~2.2-fold in the HS-28 group. Gene expression was normalized to cyclophilin A expression with the relative expression values presented as means ± SE ($n = 3$); *significant difference from control ($P < 0.05$).

provide evidence supporting involvement of the MyomiR network in the downregulation of β-MHC during skeletal muscle atrophy.

Validation of Sox6 as a target gene of miR-499. To determine whether or not Sox6 is in fact a target gene of miR-499, a Sox6 3′-UTR luciferase reporter gene was constructed. Unlike the 6.3 kb and 5.6 kb human (NM_017508) and mouse (NM_011445) Sox6 3′-UTR, respectively, the rat (NM_001024751) Sox6 3′-UTR is 225 bp and harbors a single miR-499 binding site that is conserved in mouse and human (see Fig. 6*A*). Overexpression of miR-499 caused a 32% decrease in luciferase activity of the Sox6 3′-UTR reporter gene but not the parent vector lacking the Sox6 3′-UTR (Fig. 6*B*, Empty + 499 vs. Sox6 + 499). In contrast, overexpression of miR-206, which has no binding site in the rat Sox6 3′-UTR, resulted in no significant change in luciferase activity (Fig. 6*B*, Sox6 + 206). Mutation of the miR-499 seed region in the Sox6 3′-UTR (Fig. 6*A*) made the reporter gene unresponsive to miR-499 overexpression (Fig. 6*B*, Sox6m + 499). Collectively, these results validate the prediction that Sox6 is a target gene of miR-499 and, furthermore, provide a mechanistic link between the decrease in miR-499 expression and the upregulation of Sox6 expression during muscle atrophy.

DISCUSSION

The primary objectives of this study were to identify those miRs expressed in the rat soleus muscle and determine

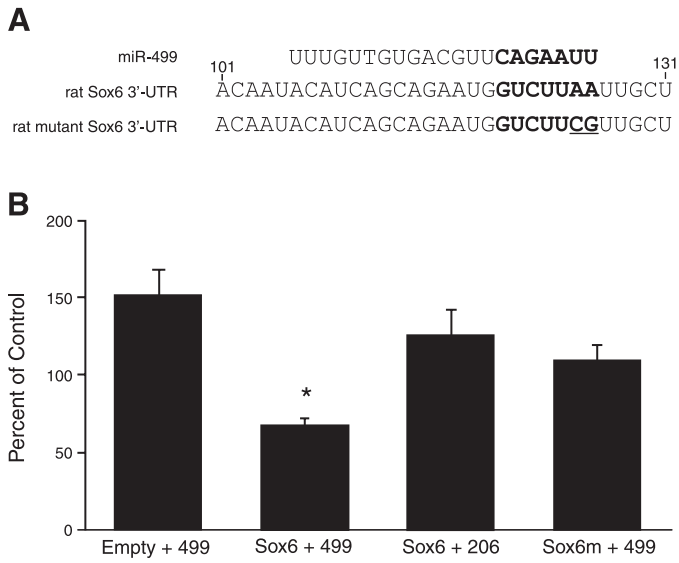

Fig. 6. Validation of Sox6 as target gene of miR-499. *A*: sequence alignment of miR-499 and the region of the rat Sox6 3′-UTR that contains the predicted miR-499 binding site (http://www.targetscan.org/). The seed sequence within miR-499 is in boldface as well as the corresponding region of homology within the rat Sox6 3′-untranslated region (UTR). The mutated seed nucleotides of the rat mutant Sox6 3′-UTR reporter gene are underlined. *B*: luciferase activity of a reporter gene containing the rat Sox6 3′-UTR was significantly decreased with overexpression of miR-499 (Sox6 + 499) but not in a reporter gene lacking the Sox6 3′-UTR (empty + 499). Luciferase activity of the Sox6 reporter gene or a mutant version (Sox6m) was not significantly changed by overexpression of miR-206 (Sox6 + 206) or miR-499 (Sox6m + 499), respectively, demonstrating the specificity of the interaction between miR-499 and Sox6 3′-UTR. Results are presented as percent of control (luciferase activity with no miR overexpression) representing 6 independent samples ($n = 6$) each performed in triplicate; *significant difference ($P < 0.05$) from control.

if their expression was significantly altered during muscle atrophy. Expression profiling by microarray revealed 65% of all known rat miRs (miRBase 9.0) were detectable in the rat soleus muscle and expression of 18 miRs was significantly changed after HS. The majority (88/151) of the detected miRs, however, were expressed at a low level (< 500) with the 25 most abundant miRs constituting 73% of all miR expression. In particular, the muscle-specific miRs (miR-1, -133, -206, and -499) accounted for almost 25% of miR expression. The most prevalent miRs likely have a substantial role in regulating gene expression in the soleus muscle, including muscle-specific miRs as well as the other highly expressed miRs such as miR-23, -26, and -29 (Supplemental Table S1).

We previously reported decreased expression of muscle-specific miR-1 and miR-133a during skeletal muscle hypertrophy induced by functional overload (23). Based on these findings, it was hypothesized that expression of one or more of the known muscle-specific miRs would be altered in response to HS. In support of our hypothesis, muscle-specific miR-499 expression was significantly reduced by 16 and 40% following 7 and 28 days of HS, respectively. The observation that expression of miR-1 and miR-133a was unchanged with HS suggests that the role of the muscle-specific miRs during periods of muscle plasticity is more complex than just a simple up- or downregulation of the same set of target genes. In agreement with our findings, Allen and colleagues (1) also detected no change in miR-1 or miR-133a expression in gastrocnemius muscle of mice exposed to ~12 days of spaceflight. In contrast to our results, however, they did report a significant 50% decrease in miR-206 expression with spaceflight (1). The reason for the discrepancy between the two studies with respect to miR-206 expression is not known but could be related to difference in species (rat vs. mouse), muscle (soleus vs. gastrocnemius) and/or duration (2 or 7 days vs. ~12 days). Regardless of the differences in the experimental design of the current study and Allen et al. (1), collectively the results suggest miRs have a role in skeletal muscle unloading.

The most significant finding of this study was the downregulation of miR-499 and miR-208b with HS-induced skeletal muscle atrophy. During the course of this study, the concept of a MyomiR network was put forward and proposed to be a master regulator of muscle fiber-type (33). The decreased expression of network components miR-499 and miR-208b was paralleled by an increase in the expression of two predicted target genes of miR-499, Sox6 and Purβ. The upregulation of Sox6 and Purβ, two known repressor of slow myosin expression, was in turn associated with a 28% decrease in β-MHC. In sum, these findings are completely consistent with the proposed MyomiR network and its regulation of β-MHC expression during skeletal muscle atrophy. Furthermore, given that changes in β-MHC gene expression have historically served as a marker of a fiber-type transition in skeletal muscle, the results from this study have broader implications by suggesting the MyomiR network may also be involved in regulating fiber-type conversion in other models of muscle plasticity.

Hints of a MyomiR network first appeared in a study by van Rooij and coworkers (34) in which they showed inactivation of miR-208a blunted induction of the β-MHC gene in response to a hypertrophic stimulus or hyperthyroidism in the heart. The notion that a miR encoded by a myosin (miR-208a is encoded by an intron of the α-MHC gene) would regulate the expression of a second myosin gene, in this case the β-MHC, was an exciting revelation and suggested other such relationships might exist. This possibility was realized when, in a subsequent review article, the Olson group sketched out a rough blueprint of the MyomiR network based on bioinformatic analysis (33). Figure 7 represents a synthesis of the latest information regarding the MyomiR network. MicroRNA-208a, miR-208b, and miR-499 are each encoded by an intron (29, 31, and 19,

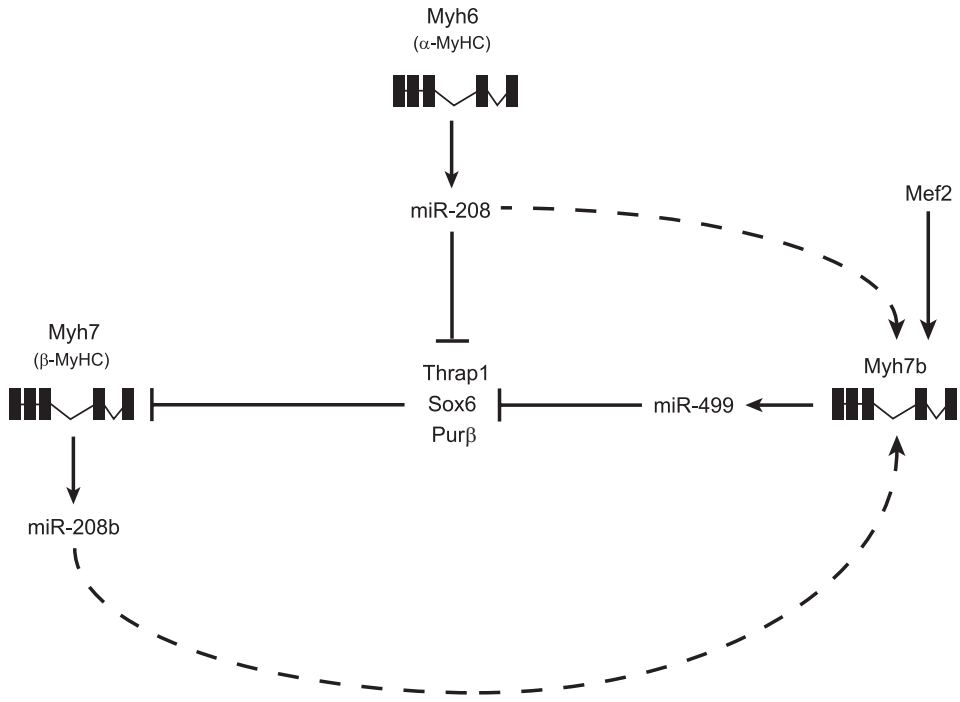

Fig. 7. MyomiR network. A relational presentation of the MyomiR network as proposed by the Olson laboratory. miR-208a, -208b, and -499 are each encoded by an intron (29, 31, and 19, respectively) within myosin heavy chain (Myh) gene 6, 7, and 7b, respectively. Myh6 and Myh7 are commonly known as α-myosin heavy chain (α-MHC) and β-MHC, respectively. miR-208a and -499 target genes (Sox6, Thrap1, and Purβ) have each been shown to repress β-MHC expression. Evidence indicates that miR-208a and miR-208b regulate, by an unknown mechanism (dashed line), expression of Myh7b, the host gene of miR-499. Overexpression of myocyte enhancer factor 2 (Mef2) transcription factor has been reported to activate expression of miR-499. In addition to regulating β-MHC expression in the heart during hypertrophy and hypothyroidism, emerging evidence indicates the MyomiR network may also be involved in regulating fiber type in skeletal muscle (33, 35).

respectively) within myosin heavy chain (*Myh*) gene 6 (α-MHC), 7 (β-MHC), and 7b, respectively. MicroRNA-208a and -499 target genes Sox6, Purβ, and Thrap1 have been independently shown to repress β-MHC gene expression (16, 19, 34). MicroRNA-208a and miR-208b, as well as the Mef2 transcription factor, can increase miR-499 expression via activation of its host gene Myh7b (35). In addition to regulating β-MHC expression in the heart during hypertrophy and hypothyroidism, the MyomiR network may also be involved in regulating fiber-type in skeletal muscle (29, 33, 35).

Promoter analysis using transgenic mice has revealed that separate regulatory regions are necessary for the induction or repression of the β-MHC gene during hypertrophy or atrophy, respectively (26). Additional studies have shown that Sp3 in cooperation with Purα and Purβ function to repress β-MHC expression during skeletal muscle atrophy (19, 32). These same authors also reported an increase in the abundance of Purα and Purβ protein of the rat soleus muscle following hindlimb suspension (19). Though Ji and coworkers did not quantify the change in Purβ protein content with muscle unloading it appeared to be much greater than the 23% increase in transcript level measured in this study (Fig. 5). The potential disparity between Purβ protein and mRNA levels is consistent with microRNA posttranscriptional regulation, miR-499 in particular. Thus, the previous findings demonstrating Purβ regulation of β-MHC expression under atrophic conditions can be incorporated in the MyomiR network with miR-499 functioning upstream of Purβ.

The notion that the increased expression of Sox6 is involved in the downregulation of β-MHC expression during muscle atrophy is supported by previous studies that have provided convincing evidence that Sox6 is a repressor of β-MHC expression. The regulatory link between miR-499 and Sox6 expression, however, remained speculative. The results from the transfection experiments validate the prediction that Sox6 is a target of miR-499 and suggest the decrease in miR-499 expression resulted in a de-repression of Sox6 expression. The transfection data are supported by a recent report by Hosoda and coworkers (17), which also confirmed by transfection and overexpression studies that Sox6 is a target gene of miR-499. Thus, the 40% decrease in miR-499 expression with muscle atrophy is consistent with de-repression of Sox6 expression, which consequently leads to repression of β-MHC expression.

In addition to regulating phenotype, the control of muscle mass may also be subject to miR regulation. The myostatin gene is predicted to be targeted by miR-499 and miR-23b with context scores of −0.48 and −0.33, respectively (Table 1). The possibility that myostatin expression can be regulated by a miR is supported by the finding that a polymorphism in the myostatin 3′-UTR, which created a miR-206 binding site, resulted in decreased myostatin expression and increased muscle mass (7). More recently, it was reported overexpression of miR-23a prevented glucocorticoid-induced muscle atrophy through posttranscriptional regulation of MAFbx expression (36). Future studies will determine if myostatin expression is regulated by miR-499 and/or miR-23b during muscle atrophy. If confirmed, miR-499 may represent a central node in the coordination of muscle fiber type and mass.

In conclusion, the results of the current study revealed abundant miR expression in the rat soleus muscle and that under atrophic conditions, expression of muscle-specific miR-499 and miR-208b were significantly reduced, suggesting activation of the MyomiR network. Future studies will test the novel hypothesis that miR-499 represents a regulatory node within the MyomiR network that coordinates changes in skeletal muscle fiber-type and mass.

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