Distinctive patterns of microRNA expression in extraocular muscles

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Submitted 7 October 2009; accepted in final form 2 February 2010

Zeiger U, Khurana TS. Distinctive patterns of microRNA expression in extraocular muscles. Physiol Genomics 41: 289–296, 2010. First published February 9, 2010; doi:10.1152/physiolgenomics.00169.2009.—The extraocular muscles (EOMs) are a unique group of muscles that are anatomically and physiologically distinct from other muscles. We and others have shown that EOMs have a unique transcriptome and proteome. Here we investigated the expression pattern of microRNAs (miRNAs), as they may play a role in generating the unique EOM allotype. We isolated RNA and screened LC Sciences miRNA microarrays covering the sequences of miRBase 10.0 to define the microRNAome of normal mouse EOM and tibialis anterior (TA) limb muscle. Seventy-four miRNAs were found to be differentially regulated (P value < 0.05) of which 31 (14 upregulated, 17 downregulated) were differentially regulated at signal strength > 500. Muscle-specific miRNAs miR-206 and miR-499 were upregulated and miR-1, miR-133a, and miR-133b were downregulated in EOM. Quantitative PCR (qPCR) analysis was used to validate the differential expression. Bioinformatic tools were used to identify potential miRNA-mRNA-protein interactions and integrate data with previous transcriptome and proteomic profiling data. Luciferase assays using cotransfection of precursor miRNAs with reporter constructs containing the 3′-untranslated region of predicted target genes were used to validate targeting by identified miRNAs. The definition of the EOM microRNAome complements existing transcriptome and proteome data about the molecular makeup of EOM and provides further insight into regulation of muscle genes. These data will also help to further explain the unique EOM muscle allotype and its differential sensitivity to diseases such as Duchenne muscular dystrophy and may assist in development of therapeutic strategies.

miR-206; miR-499; muscle-specific microRNAs; microarray profiling

EXTRAOCULAR MUSCLES (EOMs) are superfascial muscles that are engaged in the wide dynamic range of eye movements such as saccadic, vergence, and pursuit movements as well as optokinetic and vestibulococular reflexes (56). EOMs are highly specialized and adapted muscles, which is reflected in fundamental differences compared with other skeletal muscles in terms of mechanical properties, innervation, and development (9, 56). This is exemplified by coexpression of at least six different myosin heavy chains (Myh) including an EOM-specific Myh and continuous expression of embryonic Myh in EOMs (11, 56, 69). In contrast to other fast muscles, EOMs are engaged in the wide dynamic range of eye movements such as saccadic, vergence, and pursuit movements as well as optokinetic and vestibulococular reflexes (11, 56). This is exemplified by coexpression of at least six terms of mechanical properties, innervation, and development.

Material and methods

Animals. Adult normal (C57BL/10ScSn) mice aged 10–13 wk were obtained from Jackson Laboratory (Bar Harbor, ME). Tissues were dissected and stored in RNAlater (Ambion, Austin, TX) at −20°C until RNA extraction. All animal experiments were performed according to U.S. laws and were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania School of Medicine.

miRNAs are a class of small endogenous, 18- to 24-nucleotide-long, noncoding RNAs that bind to the 3′-untranslated regions (UTRs) of mRNAs, resulting in suppression of translation or degradation of the mRNA and reduced protein levels (6). In most cases miRNAs fine-tune mRNA expression levels rather than acting as on-off switches (17, 62). miRNAs are highly conserved among species, and it is believed that ~30% of all mRNAs are regulated by miRNAs (8). Many miRNAs are expressed in a tissue-specific manner (5, 38, 39) and are involved in the regulation of various cell functions ranging from developmental processes to differential expression in response to disease, such as cancer and primary muscular disorders (1, 2, 16). Important roles in myogenic differentiation, cardiogenesis, and muscle growth have been implicated for the muscle-specific miRNAs miR-1, miR-133a, miR-133b, and miR-206 (65). miR-206 is almost exclusively present in skeletal muscle (32, 37, 44), while miR-1 is crucial for the function of heart tissue (74).

To obtain insights into regulatory pathways that result in the molecular makeup of EOM we used miRNA microarray chips to define the unique microRNAome of normal mouse EOM and tibialis anterior (TA) limb muscle. We used a variety of molecular methods to validate the profiling. Quantitative PCR (qPCR) analysis was used to validate the differential expression of identified miRNAs. Bioinformatic tools were used to identify potential miRNA-mRNA-protein interactions. Luciferase reporter assays using precursor miRNA (pre-miRNA) cotransfections with constructs containing the 3′-UTRs of genes predicted to interact with identified miRNAs were used to validate the targeting by identified miRNAs.

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RNA extraction and microarrays. Total RNA was isolated from EOM and TA of normal mice (n = 7) with a mirVana miRNA Isolation Kit (Ambion), and RNA quality control was performed with Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA). EOM and TA total RNA samples (n = 4) were sent to LC Sciences (http://www.lcsciences.com; Houston, TX) for miRNA microarray screening. Three EOM and TA samples were retained for independent validation. Briefly, RNA from EOM and TA was labeled with Cy3 or Cy5, with dye swap between EOM and TA samples to eliminate dye bias, followed by hybridization to four chips with μParaflo microfluidic chip technology (LC Sciences), which contained probe sequences based on Sanger miRBase database release 10.0 (http://www.mirbase.org/) representing 568 mouse miRNAs. For quality control, spike-in positive and negative controls were used and each probe set was repeated six times on the chip. Data were analyzed by LC Sciences with in-house developed computer programs. The heat map was generated with TIGR MeV (Institute of Genomic Research; http://www.jcvi.org/cms/research/platforms/informatics/). Normalization across the four chips was carried out with a LOWESS (locally weighted regression) method (7) on the background-subtracted data. A probe set was listed as detectable when signal Table S4) (18–21, 30, 57).1

... the values from the microarrays (Fig. 3). Furthermore, 16 of the 31 miRNAs showed an expression difference of under twofold (1 log2-fold).

Validation of miRNA expression by TaqMan qPCR assay. The expression of selected miRNAs in our profile was validated by TaqMan-based real-time qPCR assay specific for mature miRNAs. Independent RNA samples that were prepared in parallel with the samples for the microarray were used. The expression levels of all tested miRNAs (miR-499, miR-206, miR-128a, miR-125a-3p, miR-133a, miR-133a*, and miR-196a) determined by TaqMan qPCR correlated well with the values from the microarrays (Fig. 3).

Distinct patterns of tissue-specific miRNA expression in EOM. The muscle-specific miRNAs were detected at high levels (Supplemental Table S1) and were differentially expressed in EOM versus TA. While miR-206 was expressed at ~17-fold higher levels in EOM, miR-1, miR-133a, and miR-133b were expressed at ~2.5-fold lower levels (Fig. 2). miR-1 showed the highest signal strength of all detected miRNAs in EOM and TA samples (Supplemental Table S1), consistent with its highly conserved role in muscle. Of the newest members of the muscle-specific miRNAs, miR-499, miR-208, and miR-208b, which are coexpressed with slow myosin heavy chain transcripts Myh7b, Myh7, and Myh6, respectively (65),
miR-499 was detected (and found to be upregulated) in EOM. miR-208 and miR-208b were either not detected or detected only in trace amounts, respectively (Supplemental Table S1). Interestingly, we found Myh7b to be upregulated (5.47 ± 0.87-fold) in EOM relative to TA, consistent with the recent findings of Rossi et al. (60).

A number of miRNAs known to be enriched in other tissues were found to be differentially expressed in EOM, including the brain-enriched miR-128a (upregulated), miR-125b-5p (upregulated), and miR-125a-5p (upregulated) and the heart-enriched miR-30c, miR-30a, miR-30d, and miR-143 (downregulated). Several miRNAs present in the profile have been proposed to play a role in transforming growth factor (TGF)-β signaling (miR-23b, miR-24, miR-27b), in differentiation of muscle (miR-27b, miR-24, miR-143), in neurons (miR-128a), in muscle metabolism (miR-23, miR-24, miR-125b), during angiogenesis (miR-27b, miR-16), and in regulation of developmental genes (miR-196a and miR-10b).

miRNA-mRNA interaction predictions and validation. To assign potential roles to the miRNAs differentially expressed in EOM, we used two approaches to predict targets or miRNAs with online target prediction tools. First, we chose to predict targets for the validated miRNAs, miR-499, miR-206, miR-128a, miR-125a-5p, and miR-196a, because they showed high fold changes (miR-499, miR-206, and miR-196a), because they showed high fold changes (miR-499, miR-206, and miR-196a) or had a known role in muscle (miR-499 and miR-206) and, in the case of miR-128a and miR-125a-5p, because of their known expression in brain and yet to be described role in muscle. Our approach was to use a combination of the following bioinformatic tools: TargetScan5.1 (40), DianaMicroT 3.0 (43), PicTar (35), miRDB (67, 68) and miRBase Targets V5 (22, 23). The predicted target gene lists were sorted according to the number of tools by which the genes were predicted. Moreover, only those genes that were predicted by two or more tools were listed, assuming that the more tools predicting any given gene to be a target, the more likely the miRNA-mRNA interaction would be relevant. When predicting targets for the validated miRNAs mentioned above, we found between 122 (for miR-499) and 503 (for miR-128a) potentially targeted genes for each of the miRNAs tested (Supplemental Table S2). Since miRNAs negatively regulated mRNA expression, where in many cases the mRNA and protein are downregulated while the miRNA is upregulated or vice versa (concordant matches), we cross-compared the target prediction lists with the gene lists of differentially expressed genes from previous transcriptome and proteome analysis (18-21, 30, 57) to increase the chance of finding mRNA-miRNA pairs. After matching the predicted gene lists with previous transcriptome and proteome data (rat and human), we found between 3 and 19 matching pairs per gene list and miRNA (Supplemental Table S4) containing
between 1 and 9 concordant matches. One of the top predicted genes identified with this approach was Mapk14, whose mRNA previously was found to be downregulated in EOM in several microarray studies (19, 30). We confirmed by qPCR that Mapk14 was -6.98(SE ±0.028)-fold downregulated in EOM. Mapk14 was predicted by four tools (TargetScan5.1, DianaMicroT 3.0, PicTar, and miRDB) to be targeted by miR-128a and by two tools (TargetScan5.1 and PicTar) to be targeted by miR-125a-5p (Supplemental Table S4). Both miRNAs were upregulated in EOM (2.51 and 2.03-fold, respectively; Supplemental Table S1). To test these predictions we tested the ability of miR-128a and miR-125a-5p to negatively regulate Mapk14 expression, using a Mapk14 3′-UTR luciferase reporter assay. Expression of miR-128a did not show significant downregulation of luciferase activity; however, miR-125a-5p significantly repressed expression of the Mapk14 3′-UTR luciferase reporter (Fig. 4, A–D).

In a second approach, we predicted miRNAs for one gene of interest in EOM. Mybpc2 is a fast muscle sarcomeric protein that is paradoxically known to be expressed at extremely low levels in EOM, despite their being capable of extremely rapid contractions (18, 30). By qPCR Mybpc2 transcripts were -12.96(SE ±0.019)-fold downregulated in EOM. We found 20 miRNAs to potentially target Mybpc2 (Supplemental Table S3). We tested the prediction that Mybpc2 could be targeted by miR-128a (Fig. 4, E and F) and found a significant repressive effect demonstrating interaction in the reporter system.

**DISCUSSION**

In this study, we used LC Sciences miRNA microarrays to identify the miRNAome of mouse EOM compared with limb muscle. The miRNAs differentially expressed in the unique EOMs include miRNAs thought to be brain- and heart enriched as well as differential expression of the canonical skeletal muscle-specific miRNAs (miR-206, miR-1, miR-133a, and miR-133b). Verification of expression changes was undertaken by qPCR and 3′-UTR luciferase reporter assays for Mapk14 and Mybpc2 by miR-125a-5p and miR-128a, respectively. The use of multiple independent assays to validate expression levels and functional consequences of individual miRNAs should allow a high degree of confidence for this study. Taken together, our results support the hypothesis that the miRNAome or overall pattern of miRNA expression of mouse EOMs is fundamentally different from that of limb muscle.

Microarray profiling reveals differentially expressed miRNAs in EOM. Using microarray profiling, we found 74 miRNAs that were differentially expressed in EOM and TA. Since the action of a miRNA seems to depend to a great extent on its abundance, the presence of competing miRNAs, and the levels of the target mRNA itself (8, 14), we used an additional cutoff (hybridization signals higher than 500) to prioritize 31 of the 74 miRNAs for further analysis. While we cannot rule out the possibility that miRNAs of low abundance have a subtle but pivotal effect on the expression of a target (e.g., in combination with other miRNAs), we concentrated on the miRNAs of high abundance in this study. In addition, 16 of 31 miRNAs showed
fold changes less than twofold between EOM and TA, suggesting subtle differences in posttranscriptional regulation by these miRNAs (62).

**Muscle-specific miRNAs in EOM.** We found several noteworthy patterns of miRNA expression in EOM among the muscle-specific miRNAs. The muscle-specific miRNAs have mainly been implicated in modulating muscle growth and differentiation. For instance, miR-206 was shown to promote muscle differentiation by downregulation of polymerase A1 (32). miR-1 also promotes muscle differentiation by targeting histone deacetylase 4, which enhances myocyte enhancer factor 2 (MEF2) promyogenic activity (10, 58). On the other hand, the closely related miR-133a and miR-133b promote myoblast proliferation by negatively regulating serum response factor (10). In addition, miR-27b can negatively regulate Pax3 in differentiating muscle and is seen at highly expressed levels in activated satellite cells (13). The skeletal muscle-specific miR-206 is highly expressed in regenerating muscle (44, 73). The higher expression of miR-206 and miR-27b coupled with the downregulation of miR-1, miR-133a, and miR-133b in EOM suggests that these miRNAs maintain a fine and specific balance between differentiation and proliferation in EOM, which is distinct from that of TA. Moreover, the slightly elevated miR-27b levels could be related to the high numbers of satellite cells in EOM. Indeed, in recent years it has become clear that EOM have higher regenerative capacity and fiber turnover in part due to higher satellite and stem cell content (48, 49, 53). Thus expression of these miRNAs may be mechanistically associated with the continuous cellular turnover that is taking place and their high regenerative potential.

miR-206, which is specific for skeletal muscle, and miR-499 were shown to be highly expressed in slow muscles (44, 46, 47). In our study we found that both miR-206 and miR-499 were highly upregulated in EOM and may emanate from the slow fibers within the EOM (Fig. 2). miR-499 is coexpressed with myosin heavy chain 7B (Myh7b) and is thought to play a major role in coordinating myosin heavy chain expression in the heart and slow muscle (65, 66). Interestingly, during the time this study was under review increased expression of miR-499 and Myh7b expression in EOM relative to TA muscle was described (60), and we were able to confirm this by qPCR. The presence of high levels of miR-206 and miR-499 in EOM is consistent with the known expression of different Myh and protein isoforms that are normally exclusively found in slow muscle fibers or heart tissue, such as sarcoplasmic Ca\(^{2+}\)-ATPase 2, Myh6, and cardiac actin (26, 29, 33, 69). The differential expression of muscle-specific miRNAs in different muscles supports the suggestion that miRNAs may play a significant role in defining a specific muscle type, as postulated by McCarthy and Esser (45).

**Bioinformatic predictions reveal miRNA interaction targets in EOM.** To identify potential miRNA-mRNA-protein interactions we used a combination of bioinformatic tools (61). While a number of miRNA target identification algorithms have been developed, only a handful of miRNA-target pairs have been validated to date. This is in part due to the characteristics of miRNAs to bind with partial complementarity to their targets. Usually, only within the seed region of the miRNA, which is a stretch of up to 8 nucleotides at the 5'-end of the miRNA, is full complementarity achieved (14). Therefore, it is thought that a single miRNA can regulate numerous mRNAs and miRNAs can be regulated by multiple miRNAs, complicating the identification of true interactions (8, 41). To find potential miRNA-target pairs the available target prediction algorithms commonly apply criteria such as complementarity of the seed region, conservation of the miRNA and the target site, as well as thermodynamic properties and other criteria (23, 24, 35, 40, 43, 67, 68). However, predictions by the available tools show varying levels of overlap and specificity; hence we also utilized reporter assays to validate the predictions. Prediction algorithms suggested that miR-128a targets Mybpc2 in vitro. Mybpc-C is involved in regulating contractile velocity of muscle (36), and Mybpc2 is the isoform normally found in fast muscles, but is highly downregulated in the rapidly contracting EOMs (18, 19, 30). The reasons for downregulation remain elusive and have been attributed to the uniqueness of the EOM phenotype (34). In addition, we found that miR-125a-5p but not miR-128a could efficiently target the 3'-UTR of the Mapk14 transcript. Mapk14 is important for myogenesis and the regulation of chromatin remodeling (42). Furthermore, Mapk14 was found to be downregulated in previous profiles (19, 30, 57), and we confirmed this by qPCR, which suggests that Mapk14 transcript levels might be controlled in part by miR-125a-5p in EOM. These results suggest that miRNAs known previously as brain specific may also regulate transcript levels in EOM.

**miRNA-mediated regulation of signaling, metabolism, regeneration, and angiogenesis in EOM.** The EOMs have distinct signaling, metabolism, regeneration, and vasculature compared with TA (3, 4, 19, 48–50, 52, 53, 57, 71). A number of miRNAs present in the EOM profile have been implicated in regulating
genes involved in these processes. The miRNA cluster miR-23b, miR-27b, and miR-24, for example, have been shown to target Smad proteins and are part of the TGF-β signaling cascade (59). Interestingly, miR-24 expression itself is regulated by TGF-β (63), thus generating mechanisms for precise local control in the EOMs. miR-23b, miR-24, and miR-125b have been implicated in targeting genes of energy metabolism (70). Many metabolic enzymes are differentially expressed in EOM (3, 4, 19, 50, 57), and the EOMs are unique in sharing features of fast and slow muscles in terms of their glucose-dependent oxidative metabolism and rapid rate of contraction. miR-27b is thought to be involved in angiogenesis, along with the downregulated miR-16 (which targets VEGF) (25), miR-378 (implicated in neovascularization) (64), and miR-143, which has been shown to play a role in
balancing differentiation and proliferation in arterial smooth muscle (12). Together these miRNAs could play a role in the organization of the extended network of vasculature in EOM (9, 19, 52, 71). Members of the miR-30 family appear to play roles in multiple tissues and pathologies and are expressed at higher levels in heart (15, 38) and might therefore contribute to fine-tuning the expression of heart isoforms of genes in EOM. Finally, some of the most downregulated miRNAs were miR-10b and miR-196a, which are both known to be coexpressed with Hox genes and hence may play a role in the continuous expression of developmental genes in EOM (72).

In conclusion, our identification of miRNA expression patterns in EOM strongly suggests that a network of miRNAs containing muscle-specific, brain- and heart-enriched miRNAs helps to define the EOM muscle group by regulating posttranscriptional expression of genes involved in structure, signaling, metabolism, angiogenesis, myogenesis, and regeneration in EOM. The miRNAome data described here identify an additional level of regulation for determination of the unique EOM allototype and provide the potential to identify new miRNA-target interactions in EOM and other muscles. The current availability of miRNAome, transcriptome, and proteome data sets for the EOM opens the possibility of applying bioinformatic strategies to integrate the information content in the future. Understanding miRNA expression and interactions in a tissue such as EOM, which exhibits differential susceptibility to disease, may help in understanding basic and pathophysiological processes in muscle.

ACKNOWLEDGMENTS

We thank Dr. John Tobias for bioinformatic support and Dr. Zissimos Mourelatos for helpful advice.

GRANTS

This research was supported by National Eye Institute Grant EY-013862 to T. S. Khurana via the American Recovery and Reinvestment Act of 2009.

DISCLOSURES

The authors have no conflicting financial interests.

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