Published in final edited form as:

Biochim Biophys Acta. 2008 November ; 1779(11): 682–691. doi:10.1016/j.bbagr.2008.03.001.

MicroRNA-206: the skeletal muscle-specific myomiR

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Abstract

MicroRNAs (miRNAs) are a class of non-coding RNAs involved in post-transcriptional gene silencing. A small number of striated muscle-specific miRNAs have been identified and shown to have an important role in myogenesis, embryonic muscle growth and cardiac function and hypertrophy. One of these myomiRs (myo = muscle + miR = miRNA), miR-206, is unique in that it is only expressed in skeletal muscle. The purpose of this review is to discuss what is currently known about miR-206 and its function in myogenesis as well as propose potential new roles for miR-206 in skeletal muscle biology. The review is also intended to serve as a comprehensive resource for miR-206 with the hope of encouraging further research on the role of miR-206 in skeletal muscle.

Keywords

microRNA; skeletal muscle; satellite cell; fiber-type; circadian

Introduction

The discovery that microRNA (miRNA) let-7 is expressed in a wide range of animals was the first evidence suggesting miRNAs might be a broad biological phenomenon and not just a novel regulatory mechanism restricted to the nematode *Caenorhabditis elegans* [1]. The finding that let-7 is phylogenetically conserved in bilateral animals built upon the pioneering work of the Ambros and Ruvkun laboratories and their identification of the first “short temporal RNA” lin-4 and its post-transcriptional regulation of the heterochronic gene lin-14 via a 3'-UTR mechanism [2–6]. For a first-hand, behind the scenes account of the journey that lead to the discovery of miRNAs, see [7,8]. A year after the let-7 discovery, three papers were published together that identified, by cloning and bioinformatics, ~30–50 new miRNAs in worm, fly and human [9–11]. These studies also hinted at the possibility that some miRNAs may be expressed in a tissue-specific manner. For example, the mature form of miRNA-1 (miR-1) was found to be expressed only in the human heart but not in the brain, kidney, liver, lung or HeLa cells [9,10]. The concept that some miRNAs might be tissue-specific was further supported by the finding that miR-1, -122a and -124a are expressed only in the muscle, liver and brain, respectively [12]. Shortly thereafter, Sempere and colleagues confirmed and expanded on these results by identifying 30 miRNAs that are enriched or specifically expressed in a particular tissue [13].

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The study of Sempere et al (2004) provided the first description of the canonical myomiRs miR-1, -133a and -206 by showing that their expression is highly enriched in both human and mouse heart and skeletal muscle [13]. Subsequent microarray studies confirmed their muscle-specificity and that expression of miR-206 is primarily restricted to skeletal muscle (see Table 1) [14–17]. Recently, the designation as a tissue-specific miRNA was formally defined as a mature miRNA that is expressed at >20-fold higher level in a specific tissue as compared to the mean expression in all other tissues; tissue-enriched was defined as <20-fold higher expression in the enriched tissue compared to other tissues [18]. Expression profiling revealed that heart and skeletal muscle, along with brain and pancreas, have the most tissue-specific miRNAs [18]. In addition to the original myomiRs, potentially new myomiRs have been identified in human and zebrafish (see Table 1) [18,19].

From the time of their initial identification, there has been great interest in determining the function of myomiRs in striated muscle. The demonstration that over-expression of miR-1 in HeLa cells caused a shift toward a myogenic gene profile suggested myomiRs may have a fundamental role in promoting a muscle cell identity [20]. The importance of myomiRs in muscle development was first shown by Sokol and Ambros (2005) who reported that deletion of miR-1 in flies caused premature death from a failure of skeletal muscle to properly grow during the second instar [21]. This initial observation was more recently extended to the mouse in which myomiRs miR-1-2 and miR-208 were knocked out; miR-1-2 was found to be necessary for heart morphogenesis, proper cardiac conduction and cell cycle progression whereas miR-208 was involved in the stress-dependent regulation of β -myosin heavy chain expression [22,23].

The majority of the research on myomiRs has focused on the function of miR-1 and miR-133a in cardiac development and disease [22–30] or during skeletal muscle differentiation using an in vitro model system [31–33]. Though less studied, miR-206 is unique among the myomiR family in that it is specifically expressed in skeletal muscle, being absent or expressed at relatively low levels in other tissues. Although our current understanding of miR-206 function in skeletal muscle biology is quite limited, some intriguing possibilities can be proposed based on predicted target genes. This review will discuss what is known about the role of miR-206 in skeletal muscle development and other potential functions in myogenesis and in adult skeletal muscle.

MiRNA-206 is a member of the muscle-specific miR-1 family of myomiRs. Phylogenetic analysis revealed miR-1 is an ancient miRNA, being one of only 20 miRNAs present in the last common ancestor of protostomes and deuterostomes [34,35]. The miR-1 family currently consists of six members clustered into three bicistronic pairs that most likely arose from an initial local gene duplication, giving rise to the original paralogous gene cluster (miR-1 and miR-133), followed by two “non-local” genomic duplications resulting in the new clusters being located on different chromosomes [35]. The identity of the primary miRNA transcript and location of the precursor and mature miRNAs within each transcript for each gene cluster is shown in Fig. 1A. The miR-1 family can be divided into two groups based on the sequence conservation of the seed region; either miR-1/206 or miR-133a/b (see Fig. 1B). As a result of the original local gene duplication, each bicistronic cluster consists of one member from the miR1/206 group and one from the miR-133a/b group.

Muscle-specificity

The miR-206/133b gene cluster is the most recent addition to the miR-1 family as it is only present in the vertebrate lineage whereas miR-1 and miR-133 are both conserved in the fly. Unlike other myomiR family members, miR-206 has been consistently found, by Northern blot, microarray, RNase protection assay and RT-PCR, to be highly expressed in skeletal

muscle and rarely detectable in the heart (see Table S1) [13–17,36]. The skeletal muscle-specific expression of miR-206 was first clearly demonstrated by microarray analysis and later confirmed by Northern blot (see Fig. 2) [14,37]. No consistent pattern of expression for miR-206 has been shown in other non-muscle tissue (see Table S1). In contrast to the aforementioned studies, rat miR-206 was recently shown by microarray analysis to be spleen-enriched; this initial observation remains to be confirmed by RT-PCR or Northern blot [38]. MiRNA expression profiling of adult skeletal muscle from human, mouse and rat has typically found miR-206 to be one of the most abundant miRNAs expressed in skeletal muscle [13,14,16,17,36,39–41] though exceptions have been reported [15,42,43] (see Table S2). The reason for this discrepancy in the relative expression level of miR-206 is not known but could be related to the species (zebrafish) [42], the method used for profiling (cloning) [43] or possibly the muscle group as miR-206 expression has been reported to be higher in muscle composed primarily of slow-twitch/oxidative fibers i.e. soleus versus plantaris [44].

Myogenesis

During embryonic development in the mouse, miR-206 is first detected at a very low level as early as 9.5 dpc and then, beginning around 11.5–12.5 dpc, miR-206 expression begins to significantly increase as determined by both Northern blot and cloning frequency [43]. Post-natal expression of miR-206 appears to peak at three days after birth and then begins to decline [45]. Similarly, in the zebrafish, miR-206 expression is low until between 12–16 hr post-fertilization at which point its expression increases over five-fold and continues to steadily increase out to 96 hr (latest time pointed examined) (see Table S3). This pattern of miR-206 expression is consistent with a global analysis of miRNA expression which found that miRNAs are generally expressed, for a given tissue, at higher levels in the embryo than in the adult [46]. Spatially, *in situ* hybridization studies have consistently observed miR-206 expression in the mouse, fish, frog and chick to be exclusively restricted to somites [42,47–49]. Unfortunately, to date, no studies have been published examining the expression pattern of miR-206 in adult skeletal muscle. The developmentally regulated pattern of miR-206 expression is similar to other miRNAs and is partly the basis for the hypothesis that miRNAs play an important role in cell differentiation and in the maintenance of cell identity [50].

The developmental regulation of miR-206 expression is recapitulated *in vitro* using the myogenic C2C12 cell line; upon initiation of differentiation there is a steady induction of miR-206 expression as well as the other myomiRs miR-1 and miR-133a [31,37,45,51]. Employing a *Myod1/Myf5* null fibroblast cell line that constitutively expresses an estradiol responsive MYOD1 protein, Rosenberg et al (2006) provided multiple lines of evidence demonstrating that MYOD1 directly regulates transcription of the primary miRNA-206 transcript AK132542 and that in the absence of MYOD1, AK132542 is not expressed [52]. These same authors used chromatin immunoprecipitation (ChIP) analysis to further show MYOD1 binds to a conserved E-box located just upstream of miRNA-206, supporting earlier ChIP-chip analysis that indicated both MYOD1 and MYOGENIN bind to sequences upstream of miRNA-206 [51,52]. In the rat myogenic L6 cell line, miR-206 was found to be expressed throughout the cytoplasm and co-localized with 28S rRNA within the granular component of the nucleolus; the functional significance of the miR-206 nucleolar localization remains to be determined [53].

To identify cis-elements that may be involved in regulating the skeletal muscle-specific expression of miR-206, one kilobase of sequence upstream of miRNA-206 was retrieved using CisView (<http://lgsun.grc.nia.nih.gov/geneindex/mm6/cisview.html>). CisView analysis identified two cis-regulatory modules based on a high density of conserved cis-elements (Fig. 4). The proximal cis-regulatory module (CM01030952) spans 89 bp and is located immediately upstream of miRNA-206. Most notably this module contains highly conserved TATA-box, c-

myc E-box and AML/Runx binding sites. The more distal regulatory module (CM01030951) is located approximately 800 bp upstream from miRNA-206 and is 189 bp in length. This upstream module harbors three predicted Myod1 binding sites, one of which is highly conserved. The complete set of transcription factor binding sites for both regulatory modules is provided as supplemental data (Table S6). Preliminary promoter analysis has indicated the distal upstream regulatory region is sufficient for induction during myogenic differentiation of C2C12 cells and confers Myod1 responsiveness (McCarthy, J.J. and Esser, K.A., unpublished data). These preliminary data are in good agreement with the findings of Rao and colleagues (2006) that identified a Myod1 binding site ~0.8 kb upstream of the miRNA-206 locus [51].

Established Function

Experiments designed to uncover the function of miR-206 in vivo have not been reported, however, in vitro studies using the C2C12 cell line have begun to elucidate the role of miR-206 during myogenesis. To date, four reports have used gain and loss of function experiments to rigorously test whether or not a predicted gene is an actual target of miR-206 [37,45,52,54]; the findings of each study are summarized below.

DNA polymerase alpha 1

Kim and coworkers (2006) expressed miR-206 in C2C12 myoblasts at a level comparable to that found in myotubes and then screened by microarray for genes that were predicted to be a target of miR-206 and were down-regulated [37]. The screen yielded a number of genes that fit this criteria of which the authors were able to experimentally confirm that *Pola1*, the largest subunit of DNA polymerase α , Rac1 interacting protein (formally known as *B-ind1*, butyrate induced transcript 1), *Cx43* (connexin-43) and *Mmd* (monocyte-to-macrophage differentiation-associated protein) were direct targets of miR-206 as determined by luciferase reporter assay [37]. These authors focused on *Pola1* and provided evidence showing that miR-206 inhibits DNA synthesis by repressing expression of *Pola1* which, subsequently, resulted in decreased cell proliferation and the induction of cell quiescence. The major conclusion of the study was miR-206 promoted myogenesis by inducing the transition from cell proliferation to differentiation by insuring the timely down-regulation of *Pola1* expression.

Connexin 43

Anderson and colleagues (2006) provided expression data and reporter gene analysis consistent with the prediction that connexin 43 (*Cx43*) is post-transcriptionally regulated by miR-206 during fusion of C2C12 myoblasts into myotubes [45]. *Cx43* is known to be a major component of gap-junctions and has been shown to be important for muscle regeneration and in vitro differentiation [55]. It is thought that the down-regulation of *Cx43* during perinatal muscle development is necessary for the proper formation of the mature neuromuscular junction (NMJ). The notion that miR-206 might have a role in NMJ formation via regulation of *Cx43* is circumstantially supported by the finding that the non-coding transcript 7H4 is synaptically enriched; 7H4 was first described in 1994 and later identified as the primary miRNA for miR-206 [56,57]. Interestingly, the 7H4 transcript, in addition to be synaptically enriched, was also found to be muscle-specific and up-regulated upon denervation.

Follistatin-like 1 and Utrophin

In an effort to understand how MYOD1 activation leads to repression of follistatin-like 1 (*Ftl1*) expression, Rosenberg and colleagues (2006) uncovered a regulatory mechanism that involved miR-206 [52]. Employing an in vitro model, these authors showed that activation of MYOD1 increased expression of the primary miRNA-206 transcript AK132542 which in turn led to the down-regulation of *Ftl1*, a predicted target of miR-206. MiRNA-206 was experimentally shown to be a direct transcriptional target of MYOD1 and that *Fstl1*, was in

turn, directly regulated via its 3'-UTR by miR-206. The function of *Fstl1* is not well understood but based on its pattern of expression it is thought to have a role in early myogenesis, possibly by antagonizing myogenic regulatory factors such as Myod1 [58]. In addition to *Fstl1*, utrophin (*Utrn*) was also verified as a target gene of miR-206. Understanding the regulation of *Utrn* gene expression is an important area of research in the muscular dystrophy field because *Utrn* can rescue the dystrophic phenotype by serving as a surrogate for a defective dystrophin gene [59]. The possibility that miR-206 may be involved in repressing *Utrn* expression opens up a novel therapeutic strategy for modulating *Utrn* expression during muscular dystrophy.

Estrogen receptor alpha

A miRNA screen of estrogen receptor alpha (ER α) positive and negative breast cancer cells found that miR-206 expression was elevated in ER α negative cells [60]. A follow-up study revealed the existence of a negative feedback loop in which miR-206 and ER α repress each other's expression [54]. Tavazoie et al., (2008), however, found a loss of miR-206 expression was associated with increased metastatic potential in human breast cancer cells and had no clear correlation to ER status [61]. Despite these contradictory findings, which may be related to the different cell types used in each study, under normal conditions ER α may function to modulate miR-206 levels in skeletal muscle. Studies in ovariectomized rats have found that changes in estrogen levels are associated with alterations in function, growth and myosin heavy chain expression in skeletal muscle [62,63]. It would be of interest to know if the responses of skeletal muscle to changes in estrogen level are in part mediated by miR-206 regulation of gene expression.

Predicted Functions of miR-206

One limitation to our understanding of miR-206 function is the small number of miR-206 target genes that have been experimentally verified. For this review, I have generated a unique list of predicted miR-206 target genes compiled from TargetScan (www.targetscan.org), PicTar (pictar.bio.nyu.edu) and Miranda (microrna.sanger.ac.uk) databases (see Table S4). The list of predicted miR-206 target genes was annotated using SymAtlas (symatlas.gnf.org/SymAtlas) and then grouped according to biological process (level 5) by DAVID (david.abcc.ncifcrf.gov/home.jsp). The results of this analysis are provided as supplemental data (Table S5) and reveal that genes associated with transcription, protein modification, transport, cytoskeleton organization and RNA metabolism are significantly over-represented. The most significantly enriched biological process was transcription, representing almost 22% of all the genes analyzed; a finding consistent with previous studies that have found miRNAs predominantly target regulators of transcription [64,65]. A closer inspection of the "regulators of transcription" list offers up some potential functions of miR-206 in skeletal muscle.

Increased skeletal muscle complexity

Among the established myomiRs, miR-206 is unique in that it is the only one specifically expressed in skeletal muscle and is the only myomiR to be exclusively expressed in vertebrates. These observations beg the question: What is different between skeletal muscle of vertebrates and invertebrates? In trying to answer this question it is important to consider the dramatic expansion in the number of vertebrate miRNAs relative to invertebrates; according to miRBase 10.0 (August 2007) there are currently 533 identified human miRNAs compared to 135 miRNAs in the worm. It has been noted that the increase in the number of miRNAs across metazoan evolution coincides with the increase in animal complexity (i.e., the number of cell types and organs) and has been proposed that miRNA regulation of gene expression may have contributed to the higher complexity of vertebrates [34,35,66]. Of the many differences that exist between vertebrate (primate, rodent and fish) and invertebrate (fly and worm) skeletal muscle, known and unknown, two major differences come to mind that can be considered as

an increase in the complexity of vertebrate skeletal muscle: fly and worm skeletal muscle do not have satellite cells, the presumptive stem cell of adult skeletal muscle, nor do they possess different fiber types such as slow-twitch and fast twitch fibers as found in vertebrate skeletal muscle [67,68]. The reason for the increase in the complexity of vertebrate skeletal muscle is not definitively known but more than likely reflects the unique functional demands of the vertebrate subphylum. Though miR-206 is not proposed to be solely responsible for the emergence of satellite cells or fiber type diversity in vertebrate skeletal muscle, it is postulated that miR-206 has an important role in regulating the expression of genes involved in satellite cell specification during development and fiber type transitions in adult muscle.

Satellite cell specification

Satellite cells are the presumptive stem cell of adult skeletal muscle responsible for post-natal muscle growth and muscle regeneration from injury [69]. Satellite cells are derived from myogenic progenitor cells of the dorsal region of the somite, the dermomyotome, and require the paired box transcription factors *Pax3* and *Pax7* for their specification [70–74]. Furthermore, the migration of these progenitor cells to the developing limb buds requires *Pax3* activation of *c-Met* gene expression [75–77]. *Pax3* expression is subject to post-transcriptional regulation and its timely down-regulation is necessary for terminal myogenic differentiation [78]. Both *Pax3* and *c-Met* are predicted target genes of miR-206. In particular, two highly conserved miR-206 binding sites have been identified in the 3'-UTR of *Pax3* and are calculated to have the highest efficacy of any miRNA predicted to target *Pax3*. In addition to *Pax3* and *c-Met*, *Six4*, *Meox2* and *Dmrt2*, transcription factors also known to have a role in *Pax3* regulation of early myogenesis, are predicted targets of miR-206 [79–82]. Though it is highly speculative as to whether or not miR-206 has a role in satellite cell specification, it does seem reasonable, given the number of miR-206 target genes associated with myogenesis, and the localized expression of miR-206 to the somite, that at a minimum, regulation of gene expression by miR-206 will be important for early myogenesis (see Fig. 4) [42,48]. The importance of miR-206 during myogenesis is circumstantially supported by the finding that skeletal muscle-specific Dicer-1 knockout mice have a significant reduction in muscle mass due to hypoplasia [83].

Regulation of fiber type transition

Vertebrate skeletal muscle is composed of different fiber types which possess unique contractile and metabolic properties. The fiber type composition of a given muscle is primarily determined during peri- and post-natal development but can be altered in adulthood in response to changes in contractile activity [84,85]. The plasticity of adult skeletal muscle fiber type is known to be regulated by calcium-dependent signaling pathways [86]. MiRNA-206 is predicted to regulate the expression of downstream effectors (*Mef2a* and *Hdac4*) of one branch of the calcium-dependent signaling pathway as well as a modulator the calcineurin/NFAT branch (*Dscr111/Rcan2*). Additionally, miR-206 is predicted to regulate the expression of *Sox6*, *Purβ* and *Sp3* – all known transcriptional repressors of the slow myosin heavy chain gene [87,88]. In two different models in which slow myosin expression is upregulated, expression of miRNA-206 was increased; a pattern consistent with a model in which miR-206 down-regulates the expression of the aforementioned repressors of slow myosin transcription [44, 89]. The idea that miRNAs may have a role in regulating skeletal muscle fiber type is supported by the finding that loss miR-214 expression leads to a reduction in slow fiber type [90]. Furthermore, Nishi and coworkers (2007) reported that over-expression of miR-206 in neonatal rat cardiomyocytes decreased the α/β myosin heavy chain ratio through regulation of retinoid X receptor alpha (RXR α) [91].

Polymorphisms

There is a growing recognition that single nucleotide polymorphisms (SNPs) which alter miRNA regulation of gene expression may be a source of phenotypic variation [92]. One of the most dramatic demonstrations of how a SNP involving a miRNA can result in an alteration in phenotype came from a study trying to identify the genetic basis for the increased muscle of mass of the Texel sheep [93]. Clop and colleagues (2006) identified a SNP within the myostatin 3'-UTR that created a functional miR-206 binding site which resulted in decreased myostatin protein expression [93]. As with the myostatin knockout mouse, the loss of myostatin expression in the sheep caused a significant increase in muscle mass [93,94]. In addition to providing a clear example of how potent miR-206 regulation can be in affecting skeletal muscle phenotype, these findings provided the first example of the degree to which a SNP involving miRNA can generate phenotypic variation [93]. Unexpectedly, a SNP (rs17578796) located within 100 bp of miR-206 was one of two miRNA SNPs identified as having a significant allelic association with schizophrenia [95]. It is difficult to reconcile these findings with the muscle-specific expression of miR-206 but, as the authors suggested, the association may be caused by a linkage disequilibrium between the miRNA SNP and some other unknown at-risk alleles [95]. A search of the human genome for polymorphisms within miRNAs themselves as well as in miRNA binding sites identified SNPs associated with miR-206 binding sites of *Slc16a9*, *Sdc4*, *Polr2k* and *Klhdc5* genes [96]. A query of the PolymiRTS database (<http://compbio.utm.edu/miRSNP/>) identified a SNP (SNPid: rs5031032) that destroys a predicted miR-206 binding site within the 3'-UTR of human insulin-like growth factor 1 (*IGF-1*) gene [97]. Given IGF-1's demonstrated ability to induce muscle hypertrophy, it would be of great interest to know if the rs5031032 polymorphism is associated with increased muscle mass in humans [98]. Unfortunately, as intriguing as these SNPs may be, there currently is insufficient population allele frequency data to assess their functional significance. In addition to PolymiRTS, a second, searchable miRNA SNP database called Patrocles is available (http://www.patrocles.org/Patrocles_targets.htm) [99]. A list of human and mouse genes with SNPs that alter miR-206 binding sites, as identified by PolymiRTS and Patrocles databases, is provided as supplemental data (Table S7 and Table S8).

Splicing

The muscle-specific miR-133a has been shown to regulate alternative splicing during myogenic differentiation by repressing expression of the neural homolog of polypyrimidine tract-binding protein (nPTB) [33]. Though nPTB is a predicted target of miR-206, current evidence suggests miR-206 plays a minor role in comparison to miR-133a in regulating the expression of nPTB during differentiation [33]. Although miR-206 does not appear to regulate splicing per se, alternative splicing may impact whether or not a predicted target gene is subject to miR-206 regulation. Similar to SNPs, alternative splicing can eliminate a potential miRNA binding site located within a spliced 3'-UTR sequence. Computational analysis identified predicted miRNA binding sites which could be affected by alternative splicing events that change the 3'-UTR sequence [100]. A list of miR-206 target genes that could potentially be affected by splicing is provided as supplemental data (Table S9). Of particular interest was identification of *IGF-1* as a gene in which alternative splicing could eliminate a miR-206 binding site (Table S9). It would be of interest to know if such a splice variant of *IGF-1* is present in skeletal muscle and, if so, does its expression change with contractile activity and age.

Aging

The original miRNA lin-4, and its target gene lin-14, has been shown to influence life span in *C. elegans* through a DAF-16 dependent mechanism [101]. A decrease in lin-14 expression by lin-4 over-expression caused an increase in life span whereas inhibition of lin-4, and thus an

increase in *lin-14* expression, reduced life span by accelerating the rate of aging [101]. In a separate study on aging in *C. elegans*, the muscle-specific miR-1, along with *lin-4* and *let-7*, showed a steady decline in expression as the worm aged [102]. The authors noted this progressive loss of miR-1 expression with age was qualitatively similar to the loss in body-wall muscle integrity observed with age in *C. elegans* and suggested miR-1 may have a role in this process [103]. Given that miR-206 is the vertebrate-specific homolog of miR-1, it is reasonable to speculate that its expression may also decline with age in vertebrate skeletal muscle and be involved in sarcopenia, the age-associated loss of muscle mass. Conversely, findings from a recent study found an increase in the expression of the myomiRs with muscle hypertrophy [44].

Muscle hypertrophy

MiRNA-206 is one of the most abundant miRNAs in adult skeletal muscle (Table S2). The function of miR-206 in adult skeletal muscle remains to be determined but given the importance of the muscle-specific miRNAs in muscle development, it is reasonable to propose an important role for myomiRs in skeletal muscle plasticity in the adult animal. We determined the expression of precursor and mature forms of the established myomiRs in the soleus and plantaris muscles and in the plantaris during the initial stage of muscle hypertrophy [44]. In control muscle, there was a direct correspondence in precursor and mature miRNA abundance for each of the myomiRs in both the soleus and plantaris muscles [44]. Interestingly, expression of miR-206 was 7-fold higher in the soleus muscle in comparison to the plantaris muscle suggesting miR-206 may have some role in setting fiber type [44]. During skeletal muscle hypertrophy induced by synergist ablation, transcript level of precursor miRNA-206 was elevated 18.3-fold whereas expression of miR-206 did not significantly change. The reason for this post-transcriptional regulation of miR-206 during muscle hypertrophy is not known but the authors suggested it might be the result of competitive inhibition of Drosha by ribosomal RNA (rRNA) [44]. Whatever the reason for the discordant expression of pri-miRNA-206 and miR-206 during hypertrophy, it would be of interest to determine if at a later time point, when the fast-to-slow fiber type transition is known to occur, if there is an increase miR-206 expression comparable to pri-miRNA-206 levels. If this scenario was found to be the true, it would provide further evidence to support the idea that miR-206 is involved in regulating fiber type. Determining the role of miR-206 in skeletal muscle hypertrophy and the associated phenotypic transition represent a fruitful area of research for future investigation.

Muscular dystrophy

The muscular dystrophies are a broad class of inherited myopathies distinguished by debilitating muscle wasting that often results in death as a consequence of respiratory complications [104]. Expression profiling of the established myomiRs revealed a 4.5-fold increase in the expression of miR-206 in the diaphragm of *mdx* mouse and not hindlimb muscle; the *mdx* mouse is an animal model of Duchenne muscular dystrophy (DMD) [89]. This finding is significant because the diaphragm of the *mdx* mouse, unlike muscles of the hindlimb, exhibits the pathological characteristics of DMD thus suggesting miR-206 may have a role in the dystrophic process [105]. McCarthy and coworkers (2007) proposed that increased miR-206 expression may contribute to the chronic pathology observed in the *mdx* diaphragm by repressing expression of genes that otherwise would serve a compensatory function, limiting the severity of the disease as observed in the hindlimb musculature [89].

Utrophin (*Utn*) is a verified target gene of miR-206 that can compensate for dystrophin and is known to be subject to a 3'-UTR-dependent post-transcriptional mechanism perfectly amenable to miRNA regulation [52,59,106,107]. In agreement with previous reports, McCarthy et al (2007) found *Utn* was post-transcriptionally regulated; *Utn* transcript level increased 69% whereas utrophin protein abundance increased 3.7-fold relative to control

[89]. The relationship between *Utrn* transcript and protein levels, however, is opposite of what would be expected if *Utrn* expression was regulated by miR-206. What could account for the discrepancy in the findings of Rosenberg et al (2007) and McCarthy et al (2007) with respect to miRNA regulation of *Utrn* expression [52,89]? One possible explanation is the effect of the different experimental conditions under which *Utrn* expression was assessed. The dystrophic cellular environment is characterized by a metabolic crisis that leads to a heightened susceptibility to oxidative stress [108,109]. The cellular stress associated with muscular dystrophy may inhibit miRNA repression of translation as has been shown in human hepatocarcinoma cells subjected to starvation (amino acid depletion) or oxidative stress (exposure to 0.1mM sodium arsenite) [110]. It may be that under dystrophic conditions, miR-206 is not able to effectively repress translation of *Utrn* mRNA.

The importance of miR-206 regulation of gene expression in muscular dystrophy needs to be carefully considered in the light of a recent study by Eisenberg and colleagues [41]. Expression profiling of 10 different human dystrophies, including DMD, did not find miR-206 differentially expressed in dystrophic muscle in comparison to control muscle [41]. One possible explanation for this discrepancy is that different muscles were used in surveying miRNA expression; Eisenberg et al (2007) collected muscle samples from limb muscle (bicep and quadricep) whereas McCarthy et al (2007) found a difference in miR-206 only in the diaphragm and not the limb muscles. Alternatively, the muscle samples from the two studies were more than likely collected at different time points in the progression of the disease which is an important factor given that DMD involves cycles of degeneration-regeneration. Though no myomiRs were found to be differentially expressed in any of the dystrophies surveyed, the author did identify a set of miRNAs (miR-146b, -221, -155, -214 and -222) that were consistently dysregulated in all but one of the dystrophies, suggesting miRNAs may indeed have a role in pathology associated with the disease [41].

Circadian rhythms

The first identified miRNA, *lin-4*, was shown to negatively regulate expression of the heterochronic gene *lin-14* [4]. Heterochronic genes act as timers during *C. elegans* development to insure the proper temporal specification of cell fate in somatic tissue. A RNAi screen of circadian gene homologs in *C. elegans* revealed that three of these homologs functioned as heterochronic genes, suggesting the intriguing possibility that circadian rhythms may be subject to regulation by miRNAs [111]. The novel idea that miRNAs may control circadian rhythms was confirmed by the demonstration that miR-132 and miR-219-1 regulate the circadian clock of the suprachiasmatic nucleus (SCN) [112]. MiRNA-219-1 was shown to be a direct transcriptional target of the CLOCK:BMAL1 heterodimer, i.e. a clock-controlled gene and is involved in setting the period length of the circadian cycle. On the other hand, the circadian expression of miR-132 was regulated by the light-inducible transcription factor CREB and shown to be involved in photic entrainment of the molecular clock. The additional identification of 12 circadianly expressed miRNAs in the retina suggests there may be a significant number of miRNAs whose expression oscillates over the course of 24 hours [113]; whether such proposed circadian miRNAs modulate the timing of the circadian clock, as do miR-219-1 and miR-132, remains to be determined.

A predicted target of miR-206 is the *Clock* gene, a critical component of the core circadian clock. The *Myod1* gene, which is known to regulate miR-206 expression, was recently identified as a constituent of the skeletal muscle circadian transcriptome in addition to being a clock-controlled gene [52,114]. Based on these observations, a model is proposed involving a Clock-Myod1-miR-206 feedback loop controlling the timing of the circadian cycle in skeletal muscle (see Fig. 5). The model proposes the CLOCK:BMAL1 heterodimer drives circadian expression of *Myod1* and, in turn, *Myod1* activates miR-206 expression. MiRNA-206

subsequently represses expression of *Clock*. Inhibition of *Clock* protein expression by morpholino oligonucleotide showed *Clock* expression is necessary to maintain normal circadian rhythmicity through regulation of *Period 2* levels [115]. Preliminary results indicate that over-expression of miR-206 in C2C12 cells can reduce *Clock* protein abundance, without altering *Clock* mRNA level, as well as decrease expression of a reporter gene containing the 3'-UTR of *Clock* (McCarthy, J.J. and Esser, K.A., unpublished data). This model is appealing because it provides a plausible mechanism through which tissue-specific factors such as *Myod1* and miR-206 can convey unique tissue requirements to the circadian clock. The exciting possibility that miR-206 regulates the circadian clock in skeletal muscle awaits experimental validation.

Concluding remarks

MiRNA-206 is unique among the muscle-specific miRNAs in that it is exclusively and highly expressed in skeletal muscle. Regulation of gene expression by miR-206 has a profound effect on skeletal muscle phenotype as shown by the work of Clop et al.,[93]. The characterization of miR-206 expression has provided a clear picture of the timing and localization of miR-206 during embryogenesis. Functional studies have validated target genes and demonstrated a role for miR-206 in myogenesis. In adult skeletal muscle, the function of miR-206 is unknown but circumstantial evidence strongly suggests a potential role in hypertrophy, fiber type switching and the pathophysiology of muscular dystrophy. Based on predicted target genes, additional roles for miR-206 in satellite cell specification, sarcopenia and circadian rhythms have been proposed. The key question that remains to be addressed is why is miR-206 expressed only in vertebrate skeletal muscle? Using this basic question to guide future research is anticipated to uncover a fundamental role for miR-206 in skeletal muscle biology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

I would like to thank Drs. Esser, Andrade and Shroeder for their careful reading of the manuscript. A special thanks to Dr. Sempere for providing raw data and insightful comments on the manuscript. This publication was made possible by grants from NIAMS/NIH to JJM (AR053641) and KAE (AR45617).

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Figure 1. Muscle-specific miRNAs

A, the location of mouse precursor and mature miRNA within the primary miRNA transcript for each of the muscle-specific gene clusters: miR-206 and miR-133b are processed from the non-coding RNA AK132542A; miR-1-1 and miR-133a-2 are derived from intron three of the predicted mRNA XM_989958; miR-1-2 and miR-133a-1 are encoded in the 12th intron of the mindbomb 1 transcript on the reverse strand. B, Sequence alignment of each muscle-specific miR. The “seed” region is boxed for the miR-1/206 and the miR-133a/b groups to emphasize their respective conservation. Each gene cluster consists of a member from each of these subgroups.

A.

B.

Figure 2. Skeletal muscle-specific expression of miR-206

A, MicroRNA expression profiling of various human tissues identify the myomiRs (miR-1, -133a and -206) and show miR-206 expression is restricted to skeletal muscle. Image is reproduced from Baskerville and Bartel (2005) with permission granted from Cold Spring Harbor Laboratory Press [14].

B. RNase protection assay of different human tissues clearly shows that miR-206 is skeletal muscle specific, confirming microarray analysis presented in A; Co, colon; Kd, kidney; Ht, heart; Lv, liver; Bs, breast; Lu, lung; Si, small intestine; Sm, skeletal muscle; Ut, uterus; At, atrium; Sp, spleen; Te, testis; Ov, ovary; Bm, bone marrow; Br, brain; Cv, cervix; Pt, prostate

tumor; Pe, prostate epithelium. The image is reproduced from Kim et al (2006) with permission from Rockefeller University Press [37].

Figure 3. Expression of miR-206 is restricted to somites in mouse, chick and frog embryos

A, miR-206 expression in somite of chick embryo (stage HH17) with small arrows at axial level indicating expression in lateral myotome; B–E, different magnifications of transverse sections through trunk of chick embryo (stage HH17) reveal miR-206 expression in myotome; F, mir-206 expression in chick embryo at stage HH21; G, somite-specific expression of miR-206 mouse embryo at E10; H, in contrast to miR-206, miR-1 is expressed in the heart and somites in the mouse embryo at E10; I–K, expression of miR-206 in *Xenopus laevis* tadpole (stage 35) is restricted to somites with arrow in (K) indicating lack of expression in heart, (J) transverse section through trunk show somite staining and (K) is higher magnification of (I); fl; forelimb, ht, heart; hl, hind limb. Image is reproduced from Sweetman et al (2006) with permission from John Wiley & Sons, Inc. [48].

A.

B.

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CCCTTCTCTCTCTAGCTTGCTTCTCTATTTCCTGACAGAGAATTCTA
TGCCAAGAAGTTCAAACCCCTTAATTTCCCATCCAGGGTGATCAGTCAG
ACCCGTGAATGGCTCaaacagctgCCAGTGTCATTTCATCTCTACAGCCCA
GGCCTCACAGATTGTGACGcaggtgATTGGCCACGAGCAGCAGGGGTGA
ACAAAAAAGATCCCTTCCACAATGAACAATTGTGTGAATGCTCATCT
ggcagctgCAGCCTGTCAGCATATTCCAAGCAACCCAAATAGGAAGTACA
ATAGCGCTGGGCCAGCCTCTAAGCTGAGCAGTTCTCCACATGTTACCCC
TGTTTAAATGTTCTATTAGTTCATAATATTAAGGAGGAAGAGGGAGGG
GGGATGGGGAGGGGGAGGAAGAGGAGGAGGAGGAAGGAGAAGGAGGA
GGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAAGGAGAAGGAGA
AGGAGAAGGAGAAGGAGAAGGAGAAGGAGAAGGAGAAGGAGAAGGAGA
AAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGA
GAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGA
AGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGA
AAGAAGCAATTTCAAATCCACCCTGCAACCTGCACCCTGGTAATTATAGT
GATAACACAGGTTGCCTGGCCATACTACGCTGAAATCTCTAGTGGCCTT
TGGGGGAGCCTTGGGGTCTTGTATCTCAGACTGAAGTGATAGGGGAAA
TTTGTGCCAACAAGCTCTGCCTGTAGGCCAAGATGGCACCTTACTCCA
GGATAGTTTCTGGGAGTGTAGAATGGATGAAGTCAGGTCCCAGAGATTCT
TCACACTTCTCACTTGTGCAAGGAAGAAAGCAGCTTTTCTTCTGCGTGA
CAAGTGCTCGGAGGACACAGCTTCCCCAGGCCACATGCTTctttatatc
CTCATAGATATCTCAGCACTATGGAATGTAAGGAAGTGTGTGGTTTGGC
AAGTGCTTCTTCAAGGCCGATGGCTCCACCCAGAGAATATGC
    
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— CRM-2

— CRM1

Figure 4. Promoter of miRNA-206 gene

A, a screen capture from CisView schematically displays characterization of upstream sequences of miRNA-206 gene. Within this presumptive promoter sequence (~ 1kb) two highly conserved cis-regulatory modules (CRM) are identified that are separated by a stretch of low complexity repeats.

B, nucleotide sequence of miRNA-206 promoter with the two regulatory modules, CRM-1 and CRM-2, as identified by CisView boxed. Highlighted (red) is the TATA-box in CRM-1 and the three Myod1 E-boxes located in CRM-2. Using an in vitro model, preliminary data revealed CRM-2 is sufficient for miRNA-206 up-regulation during myogenic differentiation.

Figure 5. MiRNA regulation of circadian rhythms in skeletal muscle

A diagram of a proposed model in which miR-206 is involved in a negative feedback loop that regulates Clock expression. Evidence to support the model are as follows; Expression profiling identified Myod1 as a clock-controlled gene with a circadian pattern of expression [114]. In turn, Myod1 has been shown to drive expression of primary miRNA-206 transcript AK132542 leading to increased expression of miR-206 [52]. MiRNA-206 is predicted to target Clock gene by three different databases (TargetScan, Miranda and PicTar). The model provides a novel mechanism by which skeletal muscle-specific factors (Myod1 and miR-206) can regulate circadian rhythms in a tissue-specific manner.

Table 1**MyomiRs: muscle-specific miRNAs**

Established myomiRs	Tissue expression	Reference
miR-1	cardiac & skeletal muscle	13
miR-133a	cardiac & skeletal muscle	
miR-206	skeletal muscle	
miR-208	cardiac muscle	22
Tentative myomiRs		
miR-95	skeletal muscle	18
miR-128a [†]	skeletal muscle & brain	
miR-302a [†]	cardiac muscle	
miR-302c [†]	cardiac muscle	
miR-302d	cardiac muscle	
miR-367 [†]	cardiac muscle	
miR-499	cardiac & skeletal muscle	19

Note. Tentative myomiR designation is based on a single study (18,19).

[†] Low level of expression in cardiac muscle but not detected in any other tissue as reported by (18).