Genome-wide profiling of the microRNA-mRNA regulatory network in skeletal muscle with aging

Ji Young Kim¹, Young-Kyu Park², Kwang-Pyo Lee¹, Seung-Min Lee¹, Tae-Wook Kang², Hee-Jin Kim²³, So Hee Dho¹, Seon-Young Kim²³, and Ki-Sun Kwon¹³

¹Aging Research Institute, Korea Research Institute of Bioscience & BioTechnology, 125 Gwahak-ro, Yuseong-gu, Daejeon, Korea
²Medical Genomics Research Center, Korea Research Institute of Bioscience & BioTechnology, 125 Gwahak-ro, Yuseong-gu, Daejeon, Korea
³Department of Functional Genomics, University of Science and Technology, Daejeon, Korea

Key words: Skeletal muscle, aging, microRNA profiling, imprinted Dlk1-Dio3, microRNA cluster, novel microRNA

Received: 4/8/14; Accepted: 7/11/14; Published: 7/12/14

Correspondence to: Ki-Sun Kwon, PhD; Seon-Young Kim, PhD E-mail: kwnks@kribb.re.kr; kimsy@kribb.re.kr

Copyright: © Kim et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited

Abstract: Skeletal muscle degenerates progressively, losing mass (sarcopenia) over time, which leads to reduced physical ability and often results in secondary diseases such as diabetes and obesity. The regulation of gene expression by microRNAs is a key event in muscle development and disease. To understand genome-wide changes in microRNAs and mRNAs during muscle aging, we sequenced microRNAs and mRNAs from mouse gastrocnemius muscles at two different ages (6 and 24 months). Thirty-four microRNAs (15 up-regulated and 19 down-regulated) were differentially expressed with age, including the microRNAs miR-206 and -434, which were differentially expressed in aged muscle in previous studies. Interestingly, eight microRNAs in a microRNA cluster at the imprinted Dlk1-Dio3 locus on chromosome 12 were coordinately down-regulated. In addition, sixteen novel microRNAs were identified. Integrative analysis of microRNA and mRNA expression revealed that microRNAs may contribute to muscle aging through the positive regulation of transcription, metabolic processes, and kinase activity. Many of the age-related microRNAs have been implicated in human muscular diseases. We suggest that genome-wide microRNA profiling will expand our knowledge of microRNA function in the muscle aging process.

INTRODUCTION

The progressive loss of skeletal muscle mass, strength, and quality, also known as sarcopenia, is a common characteristic of aging [1]. Decreased muscle mass and function accompany reduced physical activity and energy expenditure in the elderly. Consequently, sarcopenia results in muscle weakness, leading to disability and metabolic disorders, including insulin resistance, type 2 diabetes, and hypertension. Although, multiple factors contributing to sarcopenia are characterized by using transcriptome and proteome approaches [2], little is known about the molecular mechanisms of aging-related changes in skeletal muscle.

MicroRNAs (miRNAs) are endogenous, noncoding, short (20-22 nucleotides) RNAs that are involved in biological processes such as metabolism, development, cancer, and aging [3]. A long primary miRNA is initially transcribed by RNA polymerase II and processed in the nucleus by the endonuclease Drosha into precursor miRNA (pre-miRNA). The pre-miRNA is then exported into the cytoplasm and further cleaved by Dicer to generate mature miRNAs. MiRNAs recognize 3’ untranslated regions, they are guided by RNA inducing silencing complex (RISC), and they silence the target’s expression by either translational inhibition or degradation. The binding of miRNA to a target gene depends on the seed sequence (2-8
Recently, several works have demonstrated that various miRNAs are differentially expressed during aging and participate in the aging processes in tissue- and cell-type specific manners [5, 6]. For example, in the liver, age-regulated miRNAs such as miR-34, -93, and -214 target a gene important for oxidative stress defense and decrease its activity with aging [7]. In the brain, nearly 39% of age-regulated miRNAs are predicted to target components that play a critical role in oxidative phosphorylation [8]. A few groups have reported the profiling of miRNA expression in skeletal muscle with aging using microarray analysis [9, 10]. Let-7 family members were elevated in aged human skeletal muscle, contributing to reduced cellular proliferation and regenerative capacity in aged human muscle [9, 11]. Aging altered the expression of 57 miRNAs in mouse quadriceps muscles and it was suggested that age-regulated miRNAs decreased proliferation and favored the terminal differentiation of myogenic precursor [10]. Furthermore, resistance exercise, caloric restriction, or nutrient-related hormones such as the adipokine leptin changed the expression of age-regulated miRNAs, suggesting that these outputs may reverse muscle atrophy or induce changes to a younger phenotype [12, 13]. In addition to the normal muscle aging process, miRNAs participate in muscle pathologies such as muscular dystrophy and rhabdomyosarcoma. A total of 185 known miRNAs are deregulated in 10 major muscular disorders in humans and, of these deregulated miRNAs, five miRNAs, miR-146b, -221, -222, -155, and -214, were consistently regulated in almost all samples [14]. In rhabdomyosarcoma, which is a type of cancer originating from skeletal muscle progenitors, miR-1/206 and -29 directly targeted c-Met and YY1, respectively, suggesting a potential tumor suppressor role [15, 16].

Here, we profiled both miRNA and mRNA expression in young and aged mouse gastrocnemius muscles to investigate the potential role of miRNAs in skeletal muscle aging through next-generation sequencing (NGS) technology, which permits the precise identification of mature and novel miRNAs [17]. We found 34 age-related miRNAs in aged muscle and found that 57% of the down-regulated miRNAs are enriched as a cluster in the Dlk1-Dio3 imprinted genomic region on mouse distal chromosome 12. In combination with mRNA expression, we found that the genes targeted by age-related miRNAs in skeletal muscle appear to contribute to muscle aging by regulating transcription.

RESULTS

miRNA profiling using next-generation sequencing (NGS) in skeletal muscle

To study aging in skeletal muscle, we compared the muscle mass from young mice (6-month-old) to that of aged mice (24-month-old). Aged mice showed a significant loss of muscle mass in both the tibialis anterior (TA) and the gastrocnemius muscle (Fig. S1A). We focused particularly on the gastrocnemius muscle, which is a large skeletal muscle that gradually declines in mass through the age of 32 months (Fig. S1B).

To understand the molecular mechanisms of muscle aging, we analyzed the genome-wide miRNA expression of young and aged skeletal muscles by small-RNA sequencing. As shown in figure 1A, most sequences were 20–23 nucleotides in length with a peak at 22 nucleotides, representing mature miRNAs (96.9%). The remaining small RNAs (3.1%) included snRNA, scRNA, srpRNA, rRNA, tRNA, and piRNA (Fig. 1B). Interestingly, piRNA, mainly identified in germ cells, was up-regulated with aging (Fig. 1C, inset graph). The piwi-piRNA mechanism has a role in maintaining genome integrity and telomere protection in Drosophila [18], which might be a novel modulator of muscle aging and longevity. To see the abundance of mature miRNAs in skeletal muscle, we identified the top 10 miRNAs in the sequence reads. In skeletal muscle, the 10 most highly expressed mature miRNAs comprised nearly 75% of the total miRNAs, showing a relatively even distribution in abundance (Fig. 1D). Many of the top 10 miRNAs were previously reported for their roles in muscle. For example, two well-known miRNAs, miR-133 and miR-486, modulate skeletal muscle differentiation and repressing the proliferation of muscle cells [19]. MiR-143 regulates smooth muscle fate by promoting differentiation and repressing the proliferation of smooth muscle cells [20]. Recently, miR-22 was identified as a cardiac- and skeletal muscle-enriched miRNA that is up-regulated during myocyte differentiation and cardiomyocyte hypertrophy [21]. MiR-30a promotes muscle differentiation by inhibiting Snai1/2 in primary myoblast [22]. Finally, myogenic factor MyoD negatively regulates myogenic repressor MyoR via miR-378 during myoblast differentiation [23]. However, for the most abundant miR-10b, with over 6,000,000 reads per million (RPM), there were no previous reports concerning its role in either aging or muscle development.
Differentially expressed miRNAs in aged skeletal muscle

High throughput small-RNA sequencing detected 603 and 703 mature miRNAs having at least 5 reads from young and aged skeletal muscles, respectively. Among them, 20 miRNAs were up-regulated and 19 miRNAs were down-regulated in aged muscle (>1.5-fold difference, t-test, \( P < 0.05 \)) (Fig. S2). The selected miRNAs included several miRNAs that were previously reported to be involved in muscle aging. For example, miR-206 was up-regulated in aged human skeletal muscle, while miR-434 was down-regulated in aged mouse skeletal muscle [10, 12]. MiR-34, which is up-regulated with aging and modulates neurodegeneration in Drosophila and in aged heart by regulating cardiac aging [24], was also increased in aged skeletal muscle.
To validate the age-related miRNA identified by sequencing, we analyzed 11 representative miRNAs by TaqMan quantitative real-time PCR (qRT-PCR) on gastrocnemius muscles from young (6-month-old) or aged (24-month-old) mice. The qRT-PCR results for down-regulated miRNAs were consistent with the results of the sequencing (Fig. 2B). All five up-regulated miRNAs from sequencing data was consistently increased in qRT-PCR, though a modest up-regulation was observed in two miRNAs (Fig. 2A). To exclude ambiguity, we chose two cut-offs, >2 for up-regulation and >1.5 for down-regulation, and we selected 15 up-regulated and 19 down-regulated miRNAs (Tables 1 and 2).

**Down-regulation of miRNA cluster in aged skeletal muscle**

There is some evidence that miRNAs act as a group or pack in the aging process [25]. Recently, it was reported that age-regulated miRNAs identified in long-lived individuals were enriched on four chromosomes, 7, 9, 17, and X [26]. In addition, 11 miRNA clusters have been shown to be affected in the aged heart [27]. To see whether the expression of miRNAs was affected individually or collectively during muscle aging, we examined the genomic locations of age-regulated miRNAs in skeletal muscle. Notably, 8 of the 14 down-regulated miRNAs (57%) were located in the imprinted Dlk1-Dio3 genomic region on distal mouse chromosome 12 (see details in Discussion). Among them, seven miRNAs were distributed broadly across Rtl1 and the small nucleolar RNA Rian within the Dlk1-Dio3 region (Fig. S3). In contrast, the up-regulated miRNAs were randomly distributed throughout the genome.

**Novel miRNAs identified in skeletal muscle**

We used the miRDeep2 algorithm to identify novel miRNAs from our sequencing data [28]. Initially, miRDeep2 predicted 93 novel miRNAs. We then evaluated the candidates according to two criteria: 1) No mapping to sno/miRNA or non-coding RNAs according to the UCSC annotation. 2) Expression of at least two reads either in young or aged muscles. By these two criteria, the 93 candidates were narrowed down to 16 novel miRNAs (Table S1). The 16 novel miRNA precursor sequences folded well into a hairpin structure characteristic of bona fide miRNA (Fig. 3A). Six novel miRNAs shared homology with known miRNAs in their seed sequence (6-8 nucleotide long) as determined by the miRDeep2 algorithm, indicating that they may be new family members of those miRNAs (Fig. 3B). Interestingly, the mature sequences of two
novel miRNAs (chr1_524 and chr13_816) were nearly identical to miR-143-3p, which regulates the fate of smooth muscle by promoting the differentiation and repressing the proliferation of smooth muscle cells [20]. Another novel miRNA, chr11_3738, shared homology with miR-185, which participates in 22q11DS and schizophrenia by dysregulating sarco/endoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA2) [29]. While 2 of the novel miRNAs (chr7_8267 and chr13_816) exhibited high expression (>1,000 read counts), the remaining 14 novel miRNAs exhibited relatively low expression (<300 read counts). All of the novel miRNAs identified in skeletal muscle were distributed randomly in the genome.

Table 1. Up-regulated miRNAs in aged muscle. MiRNAs with >1.5-fold expression changes between young (6-month-old) and aged (24-month-old) muscles are listed from top to bottom according to fold changes and values of differences given in log2. MiRNAs in bold indicate TaqMan qRT-PCR validation.

| miRNA           | Fold Change | P-value |
|-----------------|-------------|---------|
| mmu-miR-185-3p  | 5.1         | 0.0012  |
| mmu-miR-615-5p  | 4.5         | 0.0128  |
| mmu-miR-196b-3p | 4.4         | 0.0179  |
| mmu-miR-215-5p  | 4.3         | 0.0126  |
| mmu-miR-3084-3p | 4.2         | 0.0446  |
| mmu-miR-6406    | 4.2         | 0.0193  |
| mmu-miR-223-5p  | 3.9         | 0.0091  |
| mmu-miR-483-3p  | 3.8         | 0.0077  |
| mmu-miR-369-5p  | 3.6         | 0.0138  |
| mmu-miR-7075-3p | 3.3         | 0.0253  |
| mmu-miR-7083-5p | 2.9         | 0.0469  |
| mmu-miR-206-3p  | 1.7         | 0.0216  |
| mmu-miR-34c-5p  | 1.5         | 0.0110  |
| mmu-miR-34a-5p  | 1.3         | 0.0275  |
| mmu-miR-146a-5p | 1.3         | 0.0325  |
| mmu-miR-29b-3p  | 1.0         | 0.0218  |
| mmu-miR-677-5p  | 0.9         | 0.0056  |
| mmu-miR-92b-3p  | 0.8         | 0.0417  |
| mmu-miR-155-5p  | 0.8         | 0.0392  |
| mmu-miR-203-3p  | 0.8         | 0.0399  |
RNA sequencing analysis in skeletal muscle

We performed RNA sequencing (RNA-Seq) to identify genes that were differentially expressed between young and old muscles. Overall, 123 genes were up-regulated, while only 13 genes were down-regulated, showing a dramatic increase in gene expression with muscle aging (>2-fold difference, t-test, P<0.05) (Fig. 4A). Young and aged samples were clearly separated into two distinct clusters by hierarchical clustering. Interestingly, 5 of the 123 up-regulated genes (4%) were over-expressed more than 7-fold. In contrast, all 13 down-regulated genes changed moderately. Several genes identified in RNA-Seq were previously reported to be associated with age-

Table 2. Down-regulated miRNAs in aged muscle

| miRNA               | Fold Change | P-value |
|---------------------|-------------|---------|
| mmu-miR-337-5p      | -5.2        | 0.0149  |
| mmu-miR-3544-3p     | -5.1        | 0.0147  |
| mmu-miR-540-5p      | -4.9        | 0.0200  |
| **mmu-miR-337-3p**  | -3.0        | 0.0324  |
| **mmu-miR-3544-5p** | -3.0        | 0.0308  |
| **mmu-miR-434-3p**  | -2.1        | 0.0001  |
| mmu-miR-3071-5p     | -2.0        | 0.0004  |
| mmu-miR-136-3p      | -2.0        | 0.0004  |
| mmu-miR-3071-3p     | -1.6        | 0.0000  |
| **mmu-miR-136-5p**  | -1.6        | 0.0000  |
| mmu-miR-143-5p      | -1.2        | 0.0004  |
| mmu-miR-190a-5p     | -1.0        | 0.0139  |
| mmu-miR-872-3p      | -0.9        | 0.0152  |
| mmu-miR-193a-3p     | -0.9        | 0.0164  |
| mmu-miR-144-3p      | -0.8        | 0.0298  |
| **mmu-miR-127-3p**  | -0.7        | 0.0002  |
| **mmu-miR-434-5p**  | -0.6        | 0.0082  |
| **mmu-miR-148a-3p** | -0.6        | 0.0130  |
| mmu-miR-411-5p      | -0.6        | 0.0091  |
related diseases. Early growth response 1 (Egr1) is a transcriptional regulator that is also known as a major regulator of cell senescence [30]. Another identified gene, apolipoprotein D (Apod), has a role in the aging brain and in Alzheimer’s dementia [31].

Next, we performed gene ontology (GO) analysis to understand the functional significance of the genes with changed expression. For the up-regulated genes, biological processes related to the immune response were highly over-represented: e.g., ISG15-protein conjugation, phagocytosis, engulfment, and complement activation (Fig. 4B, white bar). Other significantly enriched biological processes included the positive regulation of the transforming growth factor (TGF) beta signaling pathway and the negative regulation of the insulin pathway, apoptosis, and fat cell differentiation. Among these examples, the TGF beta pathway is a well-known regulator of skeletal muscle aging that represses myogenic differentiation and muscle regeneration [11]. In the ‘Molecular Functions’ category, up-regulated genes were significantly enriched for GO terms related to various enzyme activities such as kinase, peptidase, and endopeptidase. IgE binding, which is a molecular function related to the immune system and matrix binding, was also represented. The down-regulated genes were enriched in terms such as metabolic process, response to nutrient levels, platelet-derived growth factor binding, and SMAD binding (black bar).

![Figure 3. Sixteen novel miRNAs identified in skeletal muscle.](image-url)

(A) Secondary structure of the hairpin according to 16 novel miRNAs detected in skeletal muscle. Red denotes a mature sequence. Yellow denotes a loop and blue denotes a star sequence. (B) Conservation of six novel miRNAs to known miRNAs. Asterisk indicates conserved sequence.
Putative target genes regulated by age-related miRNAs and their roles in biological processes

Next, we tried to understand the biological significance of age-regulated miRNAs by identifying their target mRNAs. We used the TargetScan program (version 6.2) to predict the biological targets of each miRNA and then selected genes that showed a negative correlation between mRNA (1.5-fold difference) and miRNA expression. A total of 94 mRNA-miRNA interactions were identified, including 37 up-regulated and 57 down-regulated genes targeted by 14 age-related miRNAs (Table 3 and Fig. 5). By gene ontology analysis, the genes targeted by down-regulated miRNAs represented two clusters with enrichment scores ≥1.3 (Table S2). Of note, we found that the most enriched term in ‘biological process’ category was the regulation of transcription, in which Egr2, Klf4, Klf6, and Rybp was involved. Consistent with our RNA-Seq data, we confirmed that mRNA levels of all four genes were increased in aged muscle by qRT-PCR (Fig. 6A). Among them, Rybp, Ring1 and YY1-binding protein, is known to negatively regulate skeletal myogenesis in injury-induced muscle regeneration [32]. We confirmed that Rybp was also increased at the protein level in aged muscle (Fig. 6B). These results suggested that Rybp and its putative regulatory miRNA, miR-136, could contribute to muscle aging process.

Figure 4. Transcriptional signature of aged skeletal muscle and its selected gene ontology terms of the mRNA transcriptome. (A) Unsupervised hierarchical clustering of the 136 differentially regulated mRNAs with aging; 123 genes were up-regulated, and 13 genes were down-regulated. Each column presents the mRNA expression in young (n = 5) and aged (n = 5) muscle. The intensity represents the magnitude of the difference. Red and green denote high and low expression, respectively. (B) Bar plot for the -log10 of the P-value of the selected GO terms. The up- and down-regulated genes (2-fold change) were subjected to gene ontology analysis using DAVID. The enriched biological processes (upper) and molecular functions (lower) were plotted. White bar, enriched GO terms regulated by up-regulated genes; black bar, enriched GO terms regulated by down-regulated genes.
Disease enrichment analysis of age-related miRNAs

MiRNAs are implicated in various diseases [3]. To examine whether the 34 age-related miRNAs identified in skeletal muscle are implicated in human disease, we used a human miRNA-associated disease database (HMDD), which provides information on changes in miRNA regulation in various human diseases [33]. As a result, human homologues corresponding to the 11 up-regulated and 8 down-regulated miRNAs were related to 58 and 40 human diseases, respectively (data not shown). Interestingly, 8 of the 19 miRNAs were related to human muscular diseases (Tables S3 and S4 and Fig. 7A). Notably, the changes in the miRNA expression level in those diseases were correlated with up-regulation rather than down-regulation. For example, the human homologues for five miRNAs that were up-regulated in aged muscle were also up-regulated in muscular diseases, except for miR-29b in DMD and RMS. In contrast, only one miRNA, hsa-miR-193, was down-regulated in DMD. In addition, 61 miRNAs related to 10 different groups of human muscle disorders are annotated in HMDD. We found that 8 miRNAs were significantly enriched in 8 muscular diseases, such as Duchenne muscular dystrophy (DMD), facioscapulohumeral muscular dystrophy (FSHD), limb-girdle muscular dystrophies types 2A (LGMD2A) (Fig. 7B). This analysis thus represents a meaningful relationship between the age-regulated miRNAs identified in mouse skeletal muscle and human muscular diseases.

| Down-regulated miRNAs | Up-regulated target genes |
|-----------------------|---------------------------|
| mmu-mir-148a          | ARL6IP1, ARPP19, ATP2A2, CCNA2, CSF1, EGR2, ERLIN1, ERRFI1, FIGF, GADD45A, GMFB, ITGA5, KLF4, KLF6, LIMD2, MAFB, NFYA, PDIA3, PHIP, PPP1R10, PPP1R12A, PTPN14, RA14, RB1NL, SERPINE1, SIK1, SLC2A1, TMEM127, TMSB10, TMSB4X |
| mmu-mir-411           | APOLD1, SPRY4 |
| mmu-mir-136           | RYBP, ARL10, GLIPR2, UGGT1 |

| Up-regulated miRNAs | Down-regulated target genes |
|---------------------|-----------------------------|
| mmu-mir-34a/c       | DAB2IP, DMWD, EVI5L, FAM107A, MAZ, SPEG, TFRC, TTC19 |
| mmu-mir-92b         | COL1A2, DAB2IP, G3BP2, HOXC11, LBX1, NFIX, PKDCC, PRKAB2 |
| mmu-mir-132         | ACTR3B, AMD1, GPD2, HBEGF, KBTBD13, KCNJ12, PRRT2, SREBF1, TNN2 |
| mmu-mir-146a        | IRAK1, TNN2 |
| mmu-mir-152         | EML2, GPCPD1, NFIX, RPH3AL, SH3KBP1, TFRC, TRAK1, UCP3 |
| mmu-mir-155         | DUSP7, G3BP2 |
| mmu-mir-185         | DAB2IP, FAM134C, SYNM, TMEM233 |
| mmu-mir-203         | APBB2, CACNG7, FKB5, GADAP1, HBEGF, KCNC1, SIX5, TMEM182 |
| mmu-mir-206         | DMPK, G3BP2, GPD2, KCTD13, MKL1, SLC16A3, SPEG |
| mmu-mir-215         | KLHL23 |

Table 3. De-regulated miRNAs and their target genes from RNA-Seq. mRNAs are predicted as targets of up- or down-regulated miRNAs. TargetScan-predicted miRNA-mRNA pairs showing negative correlation in expression profile were selected.
**Figure 5. Network of miRNA-mRNA interaction.** The network displays the predicted interactions between age-related miRNAs and mRNAs from the sequencing and was generated using Cytoscape (version 3.0, www.cytoscape.org/). The up-regulated miRNAs are shown in yellow, down-regulated miRNAs in red. The miRNAs are depicted by circles and the mRNAs as squares. Their interaction is represented by one edge.

**Figure 6. Up-regulation of genes involved in the transcription regulation in aged muscle.** (A) Relative expressions of 4 genes, Egr2, Klf4, Klf6, and Rybp, were determined by qRT-PCR ($n = 3$ for each group). The data were normalized to the amount of GAPDH mRNA. Error bars indicate SEM (Student’s $t$-test, * $P < 0.05$). (B) Western blot and quantification of Rybp in young (white bar) and aged (black bar) gastrocnemius muscle ($n = 3$ for each group). GAPDH was used as loading control. * $P < 0.05$
**DISCUSSION**

Significant advances have been made regarding the roles of miRNAs in aging processes through massive analyses of miRNAs that are differentially expressed with aging in various tissues. Here, we systematically profiled miRNA and mRNA gene expression from young and aged skeletal muscle. A total of 34 age-related miRNAs were identified. Of the down-regulated miRNAs, miR-148a-3p and -127-3p belonged to the 50 most abundant miRNAs in skeletal muscle (Fig. S4, Asterisk). MiR-127 was identified as a cardiac valve-enriched miRNA [34]; however, there were no previous reports on its role in skeletal muscle development. MiR-148a, one of the down-regulated miRNAs, was identified as a myogenic miRNA that promotes myogenic differentiation through the ROCK1 gene. MiR-155, one of the up-regulated miRNAs, represses skeletal muscle differentiation by repressing MEF2A [35]. Thus, in accordance with their expression and role in myogenesis, miR-148a and miR-155 might be involved in the aging process through muscle differentiation or regeneration.

Several groups have reported genome-wide profiling of miRNA expression in skeletal muscle with aging. The expression of 57 miRNAs in mouse quadriceps muscles was altered by aging using a miRNA array [10]. In another study, 18 age-associated miRNAs were identified in aged human skeletal muscles by miRNA array [9]. Recently, age-associated miRNA alterations in the skeletal muscle were reported in rhesus monkeys based on small RNA sequencing [13]. The differentially expressed miRNAs in previous studies appeared consistently in mouse skeletal muscle from our study; for example, up-regulated miRNAs such as miR-29b, -34a, -206, -215, and -223 in aged muscle were also elevated, and the down-regulated miRNA miR-434 was decreased in aged mouse muscle; thus, our data reproduce previous findings.

The processing of pre-miRNA produces a miRNA duplex consisting of an miRNA (guide) and miRNA* (passenger) strand [36]. Although the miRNA* strand is generally considered to have no functional role due to its low abundance, some miRNA* have been detected with high abundance during large-scale small RNA sequencing. Furthermore, some miRNA* strands were shown to be incorporated into RISC-complex and to show inhibitory activity, suggesting the impact of miRNA* species on regulatory networks [37]. Of note, in our dataset, five miRNAs, miR-136, -337, -434, -3071, -3544, exhibited a high dominance in both the guide and passenger strands. These five miRNA/miRNA* were preferentially down-regulated with aging (Table 2). It remains unknown whether the down-regulated miRNA* strands, miR-136-3p, -337-3p,
the larger one between two groups; the smaller one between Dlk1-Dio3 clusters within the region are organized in changed in the same direction in muscle. The miRNA Dio3 belonged to the miRNA cluster derived from the Dlk1-regulated miRNAs (57%) identified in this work in the human genome [40]. Notably, 8 of the 14 down-contains 54 miRNAs, the largest miRNA cluster found skeletal muscle were derived from the smaller cluster, (maternally expressed non-coding RNA genes (paternally expressed genes chromosome 14 (mouse chromosome 12), contains the genomic region, which is located on human Gtl2/Rian muscle development [41]. The expression of ovine Dlk1, Gtl2, Rtl1, and Dio3 and the maternally expressed non-coding RNA genes Meg3 (Gtl2) and Meg8 (Rian) as well as the antisense Rtl1 (anti-Rtl1) [39]. Furthermore, the Dlk1-Dio3 region contains 54 miRNAs, the largest miRNA cluster found in the human genome [40]. Notably, 8 of the 14 down-regulated miRNAs (57%) identified in this work belonged to the miRNA cluster derived from the Dlk1-Dio3 region on mouse distal chromosome 12 and changed in the same direction in muscle. The miRNA clusters within the Dlk1-Dio3 region are organized in two groups; the smaller one between Gtl2 and Rian and the larger one between Rian and Mirg (also known as the miR-379-410 cluster); seven miRNAs found in skeletal muscle were derived from the smaller cluster, Gtl2/Rian (Fig. S3).

The miRNA clusters and genes within the Dlk1-Dio3 region are differentially regulated in various diseases such as cancer and schizophrenia as well as skeletal muscle development [41]. The expression of ovine Dlk1, Gtl2, Rtl1, and Meg8 is enriched in skeletal muscle, and overexpression of Dlk1 and Rtl1 is strongly correlated to muscle hypertrophy in Callipyge sheep [42]. The Gtl2-Dio3 miRNA cluster-mediated regulation of WNT signaling by MEF2A is required for skeletal muscle regeneration [43]. Of the eight miRNAs located in the imprinted Dlk1-Dio3 region, four mirRNAs, miR-127, -136, -434, and -3071, are located within anti-Rtl1, which contains seven miRNAs (Fig. S3, based on miRbase). In this respect, these observations raise the questions of (1) if the miRNA cluster within the Dlk1-Dio3 region is involved in muscle aging by participating in muscle regeneration or muscle atrophy; and (2) whether silencing of the miRNA cluster within the Dlk1-Dio3 region with aging occurs in other tissues. By the integrated analysis of small RNA-Seq and miRNA-Seq data, we identified 94 miRNAs targeted by 14 age-regulated miRNAs using the TargetScan algorithm. Interestingly, GO functional clustering analyses revealed that genes targeted by down-regulated miRNAs, such as the Ccna2, Egr2, Klf4, Klf6, Mafb, Nfya, and Rybp genes, are mainly involved in the regulation of transcription, whereas genes targeted by up-regulated miRNAs are not enriched in any of the GO categories. Recently, it was reported that Ring1 and YY1-binding protein (Rybp) is down-regulated by miR-29 during myogenesis and acts as a negative regulator of skeletal myogenesis in injury-induced muscle regeneration [32]. These results suggested that Rybp and its putative regulatory miRNA, miR-136, could contribute to muscle aging process. Future investigations into precise regulatory mechanisms for Rybp and miR-136 are needed.

In summary, we have identified 34 age-related miRNAs in the skeletal muscle, and of those 34, over 50% of down-regulated miRNAs are enriched as a cluster in the Dlk1-Dio3 imprinted genomic region. By analyzing miRNA-mRNA interactions, we have identified that the miRNA contributes to muscle aging primarily through the regulation of transcription. Therefore, our results provide a clue regarding the role of miRNA in skeletal muscle aging.

METHODS

Sample collection. Six young (6-month-old) and six aged (24-month-old) male C57BL/6 mice were purchased from the Laboratory Animal Resource Center (Korea Research Institute of Bioscience and Biotechnology). The body weight of the mice was recorded before sacrifice for the muscle dissection into four types of muscles, soleus (SOL), extensor digitorum longus (EDL), tibialis anterior (TA), and gastrocnemius (n = 12). Each muscle mass was recorded, and the gastrocnemius muscle was immediately selected for further experiments.

MiRNA sequencing and Data analysis. Small RNA-enriched total RNA from the gastrocnemius muscle (n = 12) was extracted using the mirVana miRNA isolation kit (Ambion, Austin, TX) according to the manufacturer’s protocol, and miRNA libraries were prepared following the Illumina library preparation protocol (Illumina Inc., San Diego, CA, USA). Each library was indexed with the Illumina adaptor (6-base barcode). The small RNA library was size fractionated on a 6% TBE urea polyacrylamide gel, and the 140-160 base pair fraction was excised from the gel. The purified miRNA library was quantified on the Agilent DNA 1000 chip. All indexed samples were pooled and sequenced in one lane of Illumina GAIIx (36 bp reads). Before mapping the raw small RNA sequencing reads on the reference genome, the base qualities of the reads were evaluated by FastQC (http://www.bioinformatics.
sequences, the read size distribution was analyzed by an in-house PERL script. After trimming the adapter (babraham.ac.uk/projects/fastqc), and the attached R (http://www.r-project.org.) script. Adapter-trimmed small RNA reads were then mapped on the reference genome mm10 downloaded from the UCSC Genome Browser (http://genome.ucsc.edu) by the BWA (http://bio-bwa.sourceforge.net.) software with the default options [44]. To assign the RNA classes for the mapped reads, we used the genomic position information of small RNAs and repeats in the annotation files from the UCSC Genome Browser and fRNAdb (http://www.ncrna.org/frnadb.) for miRNAs, tRNAs, rRNAs, and other non-coding RNAs [45]. By comparing the mapping position and annotation information, all mapped reads were classified into the corresponding RNA types, and the relative abundance of expression of each RNA type was evaluated based on the assigned read counts. To determine the portion of the main miRNA expression in the total small RNA sequencing data, we selected the top 10 expressed miRNAs from the pooled read set of 6 young mouse samples and evaluated the relative portion of the reads for those 10 most abundant miRNAs and other small portioned reads in the pooled young and aged mouse read sets.

For the known miRNAs (miRBase ver. 20), we calculated fragments per kilobase of transcript per million fragments mapped (FPKM) for each precursor and mature miRNA from the mapped reads using a custom Python script. For novel miRNA discovery, we used the mirDeep2 software (version 2.0.5) with the default parameters. MiRBase was used to filter known miRNAs, and Rattus norvegicus was used as a related species.

**RNA sequencing and data analysis.** Total RNA was extracted from young and aged gastrocnemius muscle (n = 5 for each group) using the RNasy Fibrous Tissue Mini Kit according to the manufacturer’s instructions (Qiagen). The quality and integrity of the RNA were confirmed by agarose gel electrophoresis and ethidium bromide staining, followed by visual examination under ultraviolet light. The sequencing library was prepared using the TruSeq RNA Sample Preparation kit v2 (Illumina, CA, USA) according to the manufacturer’s protocols. In brief, mRNA was purified from the total RNA using poly-T oligo-attached magnetic beads, fragmented, and converted into cDNA. Adapters were then ligated, and the fragments were amplified by PCR. The sequencing was performed in paired end reads (2×76 bp) using a Genome Analyzer IIx (Illumina).

Reference genome sequence data from Mus musculus were obtained from the University of California Santa Cruz Genome Browser Gateway (assembly ID: mm10). The reference genome index was built using the Bowtie2-build component of Bowtie2 (ver. 2.0) and SAMtools (ver. 0.1.18). Tophat2 (version 2.0.8) was applied to tissue samples to map the reads to the reference genome. Gene expression was measured as FPKM for each gene using the Refseq gene (mm10) model downloaded from the UCSC Browser gateway. Each FPKM was log2-transformed for further analysis.

Mature miRNA expression analyses. TaqMan MicroRNA Assays were performed according to the manufacturer’s recommended protocols (Applied Biosystems). Briefly, each reverse transcriptase (RT) reaction contained 10 ng of total RNA isolated using the mirVana miRNA isolation kit, 50 nM stem-loop RT primer, 0.25 mM each dNTP, 3.33 units/μl MultiScribe RT enzyme, 0.25 units/μl RNase inhibitor, and 1 × RT buffer (all purchased from Applied Biosystems). The reactions were incubated for 30 min at 16°C, 30 min at 42°C, and 5 min at 85°C, and then held at 4°C. Each quantitative PCR (qPCR) reaction included 1.33 μl of RT product, 1 μl of the 20 × primers and probe mix of the TaqMan MicroRNA Assay, 0.5 μl of H-Taq DNA polymerase (SolGent, Daejun, Korea), and 10 μl of homemade buffer mixture [40 mM Tris-HCl (pH8.4), 100 mM KCl, 6 mM MgCl2, 0.5 mM each dNTP, and 10% DMSO] instead of the 2 × TaqMan Universal PCR Master Mix in a 20-μl reaction volume. The reactions were incubated in a 96-well plate on a Bio-Rad CFX96 system at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The threshold cycle (Ct) is defined as the fractional cycle number at which the fluorescence passes the fixed threshold. U6 snRNA served as an endogenous control for normalization.

Gene functional analysis. To characterize the biological processes affected by age-related mRNAs and miRNA, we used Gene Ontology (GO) and functional annotation clustering to classify the similar annotations into a cluster of the Database for Annotation, Visualization and Integrated Discovery (DAVID).

Prediction of miRNA-mRNA interactions and network construction. We used the TargetScan (version 6.2, http://www.targetscan.org) database to predict potential target mRNAs for each miRNA. Based on the predicted miRNA-mRNA relationships, we selected miRNA-mRNA pairs showing opposite expression changes. The log₂-fold change over 0.5 was used as a cutoff. The network between miRNAs and mRNAs was created using Cytoscape (version 3.0, www.cytoscape.org/).
Quantitative real time RT-PCR. Total RNA was isolated using Qiazol reagent (Qiagen, Valencia, CA) followed by quantitative real time PCR using the StepOnePlus real-time PCR system (Applied Biosystems). Real-time PCR reactions were performed using specific primers at 95 °C for 3 min, followed by 40 cycles of 95 °C for 20 s, 60 °C for 20 s, and 72 °C for 20 s. The GAPDH housekeeping gene served as an endogenous control for normalization. The following primers were used: Eg2 forward 5'-TCAGTGTTTATGACCAAGC-3', Eg2 reverse 5'-TTGTCTGTGGAATGCTACGTTG-3', Klf4 forward 5'-GTGCCCGACTAACCGTG-3', Klf4 reverse 5'-GCACCAAGCTGCTACACATTG-3', Rybp forward 5'-AGGCCAAAAAGACAAGCGAAA-3', Rybp reverse 5'-TTCCTGGAAGATGCTACACATTG-3', GAPDH forward 5'-GTTTCTGCTCGGACTCCTGAT-3', GAPDH reverse 5'-GGCTGCTGGCTGCTGCTGCTGCT-3', Klf6 forward 5'-GTCGTTGAACTCCTCGGTCT-3', Klf6 reverse 5'-GTGCCCCGACTAACCGTTG-3', Klf4 forward 5'-GTTTCTGCTCGGACTCCTGAT-3', Klf4 reverse 5'-GGCTGCTGGCTGCTGCTGCTGCT-3', Rybp forward 5'-AGGCCAAAAAGACAAGCGAAA-3', Rybp reverse 5'-TTCCTGGAAGATGCTACACATTG-3', CC-3'. Rybp forward 5'-CGAGGTTTATGACCAAGC-3', Rybp reverse 5'-TTGTCTGTGGAATGCTACGTTG-3', Klf4 forward 5'-GTGCCCGACTAACCGTG-3', Klf4 reverse 5'-GCACCAAGCTGCTACACATTG-3', GAPDH forward 5'-GTTTCTGCTCGGACTCCTGAT-3', GAPDH reverse 5'-GGCTGCTGGCTGCTGCTGCTGCT-3', Klf6 forward 5'-GTCGTTGAACTCCTCGGTCT-3', Klf6 reverse 5'-GTGCCCCGACTAACCGTTG-3', Klf4 forward 5'-GTTTCTGCTCGGACTCCTGAT-3', Klf4 reverse 5'-GGCTGCTGGCTGCTGCTGCTGCT-3', Rybp forward 5'-AGGCCAAAAAGACAAGCGAAA-3', Rybp reverse 5'-TTCCTGGAAGATGCTACACATTG-3', CC-3'. Rybp forward 5'-CGAGGTTTATGACCAAGC-3', Rybp reverse 5'-TTGTCTGTGGAATGCTACGTTG-3', Klf4 forward 5'-GTGCCCGACTAACCGTG-3', Klf4 reverse 5'-GCACCAAGCTGCTACACATTG-3', GAPDH forward 5'-GTTTCTGCTCGGACTCCTGAT-3', GAPDH reverse 5'-GGCTGCTGGCTGCTGCTGCTGCT-3', Klf6 forward 5'-GTCGTTGAACTCCTCGGTCT-3', Klf6 reverse 5'-GTGCCCCGACTAACCGTTG-3', Klf4 forward 5'-GTTTCTGCTCGGACTCCTGAT-3', Klf4 reverse 5'-GGCTGCTGGCTGCTGCTGCTGCT-3', Rybp forward 5'-AGGCCAAAAAGACAAGCGAAA-3', Rybp reverse 5'-TTCCTGGAAGATGCTACACATTG-3', CC-3'.

Western blot analysis. The following antibodies were used in this study according to standard protocol: anti-GAPDH (Santa cruz) and anti-Rybp (Millipore).

Statistical analysis. The data were analyzed with Microsoft Excel using Student’s t-test, and probability values of less than 0.05 were considered significant. The data are presented as the mean, and the error bars indicate the standard error of the mean (SEM).

Data deposition. The miRNA sequencing data are available at the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) and are accessible through GEO Series accession number GSE55164. The RNA sequencing data are accessible through GEO accession number GSE55163.

ACKNOWLEDGEMENTS

This work was supported by the Bio & Medical Technology Development Program (20110030133, and 2013M3A9B6076413, KSK; 2012M3A9B4027954, SYK) of the National Research Foundation (NRF) funded by the Korean government (MSIP) and KIRIBB Research Initiative Program.

Conflict of interest statement

The authors have no conflict of interest to declare.

REFERENCES

1. Doherty TJ. Invited review: Aging and sarcopenia. Journal of applied physiology. 2003; 95:1717-1727.
2. Hwang CY, Kim K, Choi JY, Bahn YJ, Lee SM, Kim YK, Lee C and Kwon KS. Quantitative proteome analysis of age-related changes in mouse gastrocnemius muscle using mTRAQ. Proteomics. 2014; 14:121-132.
3. Sayed D and Abdellatif M. MicroRNAs in development and disease. Physiological reviews. 2011; 91:827-887.
4. Mourelatos Z, Destie J, Paushkin S, Sharma A, Charroux B, Abel L, Rappapoler J, Mann M and Dreyfuss G. miRNPs: a novel class of ribonucleoproteins containing numerous microRNAs. Genes & development. 2002; 16:720-728.
5. Smith-Vikos T and Slack FJ. MicroRNAs and their roles in aging. Journal of cell science. 2012; 125:7-17.
6. Grillari J and Grillari-Voglauer R. Novel modulators of senescence, aging, and longevity: Small non-coding RNAs enter the stage. Experimental gerontology. 2010; 45:302-311.
7. Li N, Muthusamy S, Liang R, Sarojini H and Wang E. Increased expression of miR-34a and miR-93 in rat liver during aging, and their impact on the expression of Mgs1 and Sirt1. Mechanisms of ageing and development. 2011; 132:75-85.
8. Li N, Bates DJ, An J, Terry DA and Wang E. Up-regulation of key microRNAs, and inverse down-regulation of their predicted oxidative phosphorylation target genes, during aging in mouse brain. Neurobiology of aging. 2011; 32:944-955.
9. Drummond MJ, McCarty JJ, Sinha M, Spratt HM, Volpi E, Esser KA and Rasmussen BB. Aging and microRNA expression in human skeletal muscle: A microarray and bioinformatics analysis. Physiological genomics. 2011; 43:595-603.
10. Hamrick MW, Herberg S, Aronlind P, He HZ, Shiver A, Qi RQ, Zhou L, Isaels CM and Mi QS. The adipokine leptin increases skeletal muscle mass and significantly alters skeletal muscle miRNA expression profile in aged mice. Biochemical and biophysical research communications. 2010; 400:379-383.
11. Carlson ME, Suetta C, Conboy MJ, Aagaard P, Mackay A, Kjaer M and Conboy I. Molecular aging and rejuvenation of human muscle stem cells. EMBO molecular medicine. 2009; 1:381-391.
12. Drummond MJ, McCarthy JJ, Fry CS, Esser KA and Rasmussen BB. Aging differentially affects human skeletal muscle microRNA expression at rest and after an anabolic stimulus of resistance exercise and essential amino acids. American journal of physiology Endocrinology and metabolism. 2008; 295:E1333-1340.
13. Mercken EM, Majounie E, Ding J, Guo R, Kim J, Bernier M, Mattison J, Cookson MR, Gorospe M, de Cabo R and Abdelmohsen K. Age-associated miRNA alterations in skeletal muscle from rhesus monkeys reversed by caloric restriction. Aging. 2013; 5:692-703.
14. Eisenberg I, Eran A, Nishino I, Moggio M, Lamperti C, Amato AA, Lidov HG, Kang PB, North KN, Mitran-Rosenbaum S, Flanagan KM, Neely LA, Whitney D, et al. Distinctive patterns of microRNA expression in primary muscular disorders. Proceedings of the National Academy of Sciences of the United States of America. 2007; 104:17016-17021.
15. Wang H, Garzon R, Sun H, Ladner KJ, Singh R, Dahlman J, Cheng A, Hall BM, Qualman SJ, Chandler DS, Croce CM and Guttridge DC. NF-kappabB-YY1-miR-29 regulatory circuitry in skeletal myogenesis and rhabdomyosarcoma. Cancer cell. 2008; 14:369-381.
16. Yan D, Dong Xda E, Chen X, Wang L, Lu C, Wang J, Qu J and Tu L. MicroRNA-1/206 targets c-Met and inhibits rhabdomyosarcoma development. The Journal of biological chemistry. 2009; 284:29596-29604.
17. Morozova O and Marra MA. Applications of next-generation sequencing technologies in functional genomics. Genomics. 2008; 92:255-264.

18. Khurana JS, Xu J, Weng Z and Theurkauf WE. Distinct functions for the Drosophila piRNA pathway in genome maintenance and telomere protection. PLoS genetics. 2010; 6:e1001246.

19. Luo W, Nie Q and Zhang X. MicroRNAs involved in skeletal muscle differentiation. Journal of genetics and genomics = Yi chuan xue bao. 2013; 40:107-116.

20. Cordes KR, Sheehy NT, White MP, Berry EC, Morton SU, Muth AN, Lee TH, Miano JM, Ivey KN and Srivastava D. miR-145 and miR-143 regulate smooth muscle cell fate and plasticity. Nature. 2009; 460:705-710.

21. Huang ZP, Chen J, Seok HY, Zhang Z, Kataoka M, Hu X and Wang DZ. MicroRNA-22 regulates cardiac hypertrophy and remodeling in response to stress. Circulation research. 2013; 112:1234-1243.

22. Soleimani VD, Yin H, Jahani-Asl A, Ming H, Kockx CE, van Ijcken WF, Grosveld F and Rudnicki MA. Snail regulates MyoD binding-site occupancy to direct enhancer switching and differentiation-specific transcription in myogenesis. Molecular cell. 2012; 47:457-468.

23. Gagan J, Dey BK, Layer R, Yan Z and Dutta A. MicroRNA-378 targets the myogenic repressor MyoR during myoblast differentiation. The Journal of biological chemistry. 2011; 286:19431-19438.

24. Boon RA, Iekushi K, Lechner S, Seeger T, Fischer A, Heydt S, Kaluza D, Treguer K, Carmona G, Bonauer A, Horrovoets AJ, Didier N, Girmasion Z, et al. MicroRNA-34a regulates cardiac aging and function. Nature. 2013; 495:107-110.

25. Lanceta J, Prough RA, Liang R and Wang E. MicroRNA group disorganization in aging. Experimental gerontology. 2010; 45:269-278.

26. ElSharawy A, Keller A, Flachsbart F, Wendenschlag A, Jacobs G, Kefer N, Brefort T, Leidinger P, Backes C, Meese E, Schreiber S, Rosenstiel P, Franke A, et al. Genome-wide miRNA signatures of human longevity. Aging cell. 2012; 11:607-616.

27. Zhang X, Azhar G and Wei JY. The expression of microRNA and microRNA clusters in the aging heart. PLoS one. 2012; 7:e34688.

28. Friedlander MR, Mackowiak SD, Li N, Chen W and Rajewsky N. miRDeep2 accurately identifies known and hundreds of novel microRNA genes in seven animal clades. Nucleic acids research. 2012; 40:37-52.

29. Earls LR, Fricke RG, Yu J, Berry RB, Baldwin LT and Zakharenko SS. Age-dependent microRNA control of synaptic plasticity in 22q11 deletion syndrome and schizophrenia. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2012; 32:14132-14144.

30. Krones-Herzig A, Adamson E and Mercola D. Early growth response 1 protein, an upstream gatekeeper of the p53 tumor suppressor, controls replicative senescence. Proceedings of the National Academy of Sciences of the United States of America. 2003; 100:3233-3238.

31. Kalman J, McConathy W, Araoz C, Kasa P and Lacko AG. Apolipoprotein D in the aging brain and in Alzheimer’s dementia. Neurological research. 2000; 22:330-336.

32. Zhou L, Wang L, Lu L, Jiang P, Sun H and Wang H. A novel target of microRNA-29, Ring1 and YY1-binding protein (Rybp), negatively regulates skeletal myogenesis. The Journal of biological chemistry. 2012; 287:25255-25265.

33. Lu M, Zhang Q, Deng M, Miao J, Guo Y, Gao W and Cui Q. An analysis of human microRNA and disease associations. PLoS one. 2008; 3:e3420.

34. Vacchi-Suzzi C, Hahne F, Scheubel P, Marcellin M, Dubost V, Westphal M, Boeglen C, Buchmann-Moller S, Cheung MS, Cordier A, De Benedetto C, Deurinck M, Frei M, et al. Heart structure-specific transcriptomic atlas reveals conserved microRNA-mRNA interactions. PLoS one. 2013; 8:e52442.

35. Seok HY, Tatsuguchi M, Callis TE, He A, Pu WT and Wang DZ. miR-155 inhibits expression of the MEF2A protein to repress skeletal muscle differentiation. The Journal of biological chemistry. 2011; 286:35339-35346.

36. Ameres SL and Zamore PD. Diversifying microRNA sequence and function. Nature reviews Molecular cell biology. 2013; 14:475-488.

37. Kuchenbauer F, Mah SM, Heuser M, McPherson A, Ruschmann J, Rouhi A, Berg T, Bullinger L, Argiropoulos B, Morin RD, Lair D, Starczynowski DT, Karsan A, et al. Comprehensive analysis of mammalian miRNA* species and their role in myeloid cells. Blood. 2011; 118:3350-3358.

38. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell. 2004; 116:281-297.

39. Schmidt JV, Matteson PG, Jones BK, Guan XJ and Tilghman SM. The Dlk1 and Gtl2 genes are linked and reciprocally imprinted. Genes & development. 2000; 14:1997-2002.

40. Glazov EA, McWilliam S, Barris WC and Dalrymple BP. Origin, evolution, and biological role of miRNA cluster in DLK-DIO3 genomic region in placental mammals. Molecular biology and evolution. 2008; 25:939-948.

41. Benetatos T, Hatzimichael E, Londin E, Vartholomatos G, Loher P, Rigoutsos I and Briasoulis E. The microRNAs within the DLK1-DIO3 genomic region: involvement in disease pathogenesis. Cellular and molecular life sciences : CMLS. 2013; 70:795-814.

42. Fleming-Waddell JN, Olbricht GR, Taxis TM, White JD, Vuocolo T, Craig BA, Teliam RL, Neary MK, Cockett NE and Bidwell CA. Effect of DLK1 and RTL1 but not MEG3 or MEG8 on muscle gene expression in Callipyge lambs. PLoS one. 2009; 4:e7399.

43. Snyder CM, Rice AL, Estrella NL, Feldh A, Kandarian SC and Naya FJ. MEF2A regulates the Gtl2-Dio3 microRNA mega-cluster to modulate WNT signaling in skeletal muscle regeneration. Development. 2013; 140:31-42.

44. Li H and Durbin R. Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics. 2009; 25:1754-1760.

45. Kin T, Yamada K, Terai G, Okida H, Yoshinari Y, Ono Y, Kojima A, Kimura Y, Komori T and Asai K. fRNAdb: a platform for mining/annotating functional RNA candidates from non-coding RNA sequences. Nucleic acids research. 2007; 35(Database issue):D145-148.
**SUPPLEMENTAL DATA**

**Figure S1. Muscle mass changes in different anatomical regions with age.** (A) The TA and gastrocnemius muscle showed a significant loss of weight in aged mice (24-month-old) compared to young mice (6-month-old) ($n = 12$ for each group). Muscle weight was normalized to animal body weight. Soleus, SOL. Extensor digitorum longus, EDL. Tibialis anterior, TA. (B) The mass of the gastrocnemius muscle gradually decreased through 32 months of age.

**Figure S2. Total read count for 39 differentially expressed miRNAs with aging in skeletal muscle.** Inset graph shows a magnified value for low read counts. Data are presented as the mean ± SEM. White bar; young muscle, black bar; aged muscle.
Figure S3. Eight down-regulated miRNAs located in Dlk1-Dio3 genomic regions. Genomic browser image around Rtl1 and Rian. Top, positions of eight down-regulated miRNAs are shown with the chromosomal location marked at the top. Bottom, seven miRNAs are distributed near the Rtl1 as indicated in the magnified view.

Figure S4. Fifty most abundant miRNAs identified in skeletal muscle. Data are presented as the normalized total read counts.
Table S1. 16 novel miRNAs identified in skeletal muscle. The following information is presented from left to right: provisional miRNA ID, genomic coordinate and strand (+ or -), mature sequence, miRNA length, genomic location, and mature/star read counts.

| Provisional ID | Genomic location | Mature sequence     | Length | Location   | Mature read | Star read |
|----------------|------------------|---------------------|--------|------------|-------------|-----------|
| chr7_8267      | chr7:16471899..16471933:+ | ACCGGUGCUUGAGGCUU  | 18     | Intronic   | 924         | 0         |
| chr13_816      | chr13:84058941..84058983:+ | UGAGAUGAAACACUGUAGCA | 20     | Exonic     | 22          | 0         |
| chr2_11277     | chr2:16657672..166576783:-   | CCGGGUGCUUAGGCACU   | 18     | Intronic   | 38          | 0         |
| chr2_5897      | chr2:16523441..165234461:-   | GGCUGCUGCUGCUGCUCCG | 18     | Intronic   | 26          | 0         |
| chr7_7384      | chr7:121040347..121040416:- | UGAGUUGGAAGACACUCUGCAU | 22     | Intronic   | 20          | 0         |
| chr11_2952     | chr11:87448818..87448900:+   | GGGAGGGAACGCAGUCUCUGAGU   | 23     | Intronic   | 44          | 0         |
| chr10_1092     | chr10:130557384..130557462:   | GAGAGGAAACACUGUAGUCU | 21     | Intergenic | 19          | 0         |
| chr12_1908     | chr12:33262824..33262884:+   | UCAGAAAACACCUGACCUCGUCC | 21     | Intronic   | 23          | 1         |
| chr3_5770      | chr3:147057717..147057785:-   | CACCAAGUGAGGAGCCUGGC | 19     | Intergenic | 11          | 0         |
| chr4_5989      | chr4:155858806..155858863:+   | UUCAAAAACUCUCUGUCGUCG   | 20     | Exonic     | 8           | 0         |
| chr16_524      | chr1:133827269..133827312:- | UGAGAUGGAAGCCUCUGAGG   | 19     | Intergenic | 6           | 0         |
| chr19_14188    | chr19:5840778..5840840:-     | CGGGGUGAUCGGAUGGCCC     | 19     | Intergenic | 2           | 0         |
| chr8_7686      | chr8:21095625..21095687:-    | GAUAAAUGGAUCAAGACAGCAA | 21     | Intronic   | 13          | 0         |
| chr11_3878     | chr11:12063334..12063383:-    | CCGGGCGUGGGCGGCGGCGG   | 18     | Intronic   | 1           | 0         |
| chr18_4213     | chr18:34759521..34759596:+   | CCAGUGAGUCUCUGAGGCCG   | 21     | Intronic   | 16          | 0         |
| chr2_4292      | chr2:28495933..28495984:+    | AUCUCUCUGGGGGCCUCCA    | 18     | Intergenic | 57          | 0         |
Table S2. Functional annotation clustering of enriched GO terms stimulated by down-regulated miRNAs in aged muscle. The up-regulated genes targeted by down-regulated miRNAs were subjected to gene ontology analysis with DAVID functional annotation clustering. The genes targeted by down-regulated miRNAs represented two clusters with enrichment scores ≥1.3. ( )*; an enrichment score of 0.05 is equivalent to an enrichment score of 1.3 in the minus log scale [1].

| Annotation cluster | Category                  | Term                                                                 | Count | P-value       |
|--------------------|---------------------------|----------------------------------------------------------------------|-------|---------------|
| Cluster 1 (1.5)*   | GO cellular component     | GO:0044432–endoplasmic reticulum part                               | 4     | 0.006473      |
|                    |                           | GO:0005783–endoplasmic reticulum                                     | 5     | 0.048089      |
| Cluster 2 (1.3)*   | GO biological process     | GO:0045941–positive regulation of transcription                      | 6     | 0.001521      |
|                    |                           | GO:0010628–positive regulation of gene expression                    | 6     | 0.001714      |
|                    |                           | GO:0045935–positive regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process | 6     | 0.002081      |
|                    |                           | GO:0051173–positive regulation of nitrogen compound metabolic process | 6     | 0.002382      |
|                    |                           | GO:0010557–positive regulation of macromolecule biosynthetic process  | 6     | 0.002462      |
|                    |                           | GO:0031328–positive regulation of cellular biosynthetic process       | 6     | 0.002938      |
|                    |                           | GO:0009891–positive regulation of biosynthetic process                | 6     | 0.003054      |
|                    |                           | GO:0045944–positive regulation of transcription from RNA polymerase II promoter | 5     | 0.003867      |
|                    |                           | GO:0006357–regulation of transcription from RNA polymerase II promoter | 6     | 0.004703      |
|                    |                           | GO:0010604–positive regulation of macromolecule metabolic process     | 6     | 0.005279      |
|                    |                           | GO:0045893–positive regulation of transcription, DNA-dependent        | 5     | 0.006577      |
|                    |                           | GO:0051254–positive regulation of RNA metabolic process               | 5     | 0.006744      |
| GO molecular function | GO:0030528–transcription regulator activity                           | 7     | 0.011563      |
|                    |                           | GO:0003700–transcription factor activity                             | 5     | 0.036412      |
Table S3. Human homologues for 3 down-regulated miRNAs are related to muscular disease. This table shows three human homologues for down-regulated miRNAs identified in aged skeletal muscle and their previously reported expression in each muscular disease. The three miRNAs are related to 6 muscular diseases.

| Muscular disease                                      | miRNAs   | Expression   |
|-------------------------------------------------------|-----------|--------------|
| Dermatomyositis (DM)                                  | hsa-miR-148a | up-regulated |
| Duchenne muscular dystrophy (DMD)                     | hsa-miR-148a | up-regulated |
| Miyoshi myopathy (MM)                                 | hsa-miR-148a | up-regulated |
| Limb-girdle muscular dystrophies types 2A (LGMD2A)    | hsa-miR-148a | up-regulated |
| Nemaline myopathy (NM)                                | hsa-miR-127  | up-regulated |
| Polymyositis (PM)                                     | hsa-miR-127  | up-regulated |

Table S4. Human homologues for 5 up-regulated miRNAs are related to muscular disease. This table shows five human homologues for up-regulated miRNAs identified in aged skeletal muscle and their previously reported expression in each muscular disease. The five miRNAs are related to 10 muscular diseases.

| Muscular disease                                      | miRNAs   | Expression   |
|-------------------------------------------------------|-----------|--------------|
| Becker muscular dystrophy (BMD)                       | hsa-miR-146b | up-regulated |
| Dermatomyositis (DM)                                  | hsa-miR-223  | up-regulated |
| Duchenne muscular dystrophy (DMD)                     | hsa-miR-369-5p | up-regulated |
| Facioscapulohumeral muscular dystrophy (FSHD)         | hsa-miR-369-5p | up-regulated |
| Inclusion body myositis (IBM)                         | hsa-miR-223  | up-regulated |
| Limb-girdle muscular dystrophies types 2A (LGMD2A)    | hsa-miR-223  | up-regulated |
| Miyoshi myopathy (MM)                                 | hsa-miR-223  | up-regulated |
| Nemaline myopathy (NM)                                | hsa-miR-223  | up-regulated |
| Polymyositis (PM)                                     | hsa-miR-34a  | up-regulated |
| Rhabdomyosarcoma (RMS)                                | hsa-miR-29b-2 | down-regulated |
REFERENCES

1. Huang da W, Sherman BT, Tan Q, Collins JR, Alvord WG, Roayaei J, Stephens R, Baseler MW, Lane HC and Lempicki RA. The DAVID Gene Functional Classification Tool: a novel biological module-centric algorithm to functionally analyze large gene lists. Genome biology. 2007; 8:R183.