MicroRNA-494 regulates mitochondrial biogenesis in skeletal muscle through mitochondrial transcription factor A and Forkhead box j3

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Yamamoto H, Morino K, Nishio Y, Ugi S, Yoshizaki T, Kashwagi A, Maegawa H. MicroRNA-494 regulates mitochondrial biogenesis in skeletal muscle through mitochondrial transcription factor A and Forkhead box j3. Am J Physiol Endocrinol Metab 303: E1419–E1427, 2012. First published October 9, 2012; doi:10.1152/ajpendo.00097.2012.—MicroRNAs (miRNAs) are important posttranscriptional regulators of various biological pathways. In this study, we focused on the role of miRNAs during mitochondrial biogenesis in skeletal muscle. The expression of miR-494 was markedly decreased in murine myoblast C2C12 cells during myogenic differentiation, accompanied by an increase in mtDNA. Furthermore, the expression of predicted target genes for miR-494, including mitochondrial transcription factor A (mtTFA) and Forkhead box j3 (Foxj3), was posttranscriptionally increased during myogenic differentiation. Knockdown of miR-494 resulted in increased mitochondrial content and upregulation of mtTFA and Foxj3 at the protein level. A 3′-untranslated region reporter assay revealed that miR-494 knockdown directly upregulated the luciferase activity of mtTFA and Foxj3. All of these observations were reversed by overexpression of miR-494. Furthermore, the miR-494 content significantly decreased after endurance exercise in C57BL/6j mice, accompanied by an increase in expression of mtTFA and Foxj3 proteins. These results suggest that miR-494 regulates mitochondrial biogenesis by downregulating mtTFA and Foxj3 during myocyte differentiation and skeletal muscle adaptation to physical exercise.

MicroRNAs (miRNAs) are small noncoding RNAs that posttranscriptionally regulate the expression of target genes. miRNAs suppress mRNA translation and/or stimulate mRNA degradation and thus influence protein expression levels (5). They are widely expressed in plants and animals, and their expression is tissue specific and dependent upon a cell’s developmental stage; thus, miRNAs play an important role in repressing gene expression at specific stages during various biological processes (9, 20, 29, 34). To date, >1,000 miRNAs have been identified within the human genome, and a single miRNA may inhibit many target genes; thus, ~60% of genes are thought to be regulated by miRNAs (6, 20, 13).

miRNAs play an active role in various processes within skeletal muscle. Chen and colleagues suggested that muscle-specific miRNAs (miR-1 and miR-133a) promote myocyte differentiation and proliferation, respectively, in the C2C12 mouse myoblast cell line (7). Wong and Tellam indicated that miR-26a contributes to myoblast differentiation by regulating the enhancer of zeste homolog 2 gene (35). In addition, numerous studies report that miRNAs regulate many genes during various biological processes within skeletal muscle (2, 8, 15, 25, 37). We therefore hypothesized that miRNAs may contribute to mitochondrial biogenesis within skeletal muscle.

To investigate our hypothesis, we analyzed miRNA expression in the C2C12 cell line during myogenesis, since these cells are thought to display large-scale alterations in mitochondrial content during myogenesis. The results showed that miR-494 expression significantly decreased during skeletal muscle differentiation. Furthermore, we found that miR-494 regulated the expression of mitochondrial transcription factor A (mtTFA) and Forkhead box j3 (Foxj3), both of which play important roles in mitochondrial biogenesis in C2C12 myocytes and in mice (1, 28).

EXPERIMENTAL PROCEDURES

Cell culture. C2C12 myoblasts were obtained from the American Type Culture Collection (ATCC; Manassas, VA, http://www.atcc.org/) and grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (vol/vol) FBS, 100 U/ml of penicillin, and 100 mg/ml of streptomycin in a humidified atmosphere of 95% air and 5% CO₂ at 37°C. At confluence, myoblasts were induced to differentiate in DMEM with 2% FBS, 100 U/ml of penicillin, and 100 mg/ml of streptomycin. Differentiation medium (DM) was replaced every 48 h.

Cell transfection. The miR-494 knockdown study was performed using antisense inhibitors and a scrambled sequence negative control (anti-miR miRNA inhibitor nos. AM17100 and AM17110; Life Technologies) and grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (vol/vol) FBS, 100 U/ml of penicillin, and 100 mg/ml of streptomycin. Differentiation medium (DM) was replaced every 48 h.

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MITOCHONDRIA ARE THE MAJOR ATP-synthesizing organelles and provide the cell with chemical energy. The number of mitochondria in a cell varies widely according to tissue type, energy requirements, and various other conditions (36). Skeletal muscles require a large amount of energy, and the number and function of mitochondria within skeletal muscle cells are related to their individual metabolizing capacity; thus, any dysfunction may result in metabolic disease. As an example, recent reports have suggested a relationship between mitochondrial dysfunction and diabetes (24). The function and number of mitochondria in skeletal muscle cells can change in response to stimulation. For example, endurance exercise increases the number of mitochondria in skeletal muscle (12, 32). Mechanisms of mitochondrial biogenesis have been the subject of much research, and some transcription factors, such as peroxisome proliferator-activated receptor-γ coactivator-1α (PGC1-α) and nuclear respiratory factor 1, are thought to play a primary role in mitochondrial biogenesis within skeletal muscle (11, 16, 31, 36); however, the mechanisms involved have not been identified.

microarray; mitochondrial deoxyribonucleic acid; exercise

100% confluence, C2C12 myoblasts were transfected using the siPORT NeoFX reagent (Life Technologies) according to the manufacturer’s instructions. Cells were harvested 24 h after transfection. Although it
is technically impossible to confirm a reduction in miRNA expression in an inhibitor experiment (4), transfection efficiency was confirmed using a previously reported combination of an miRNA inhibitor and its target gene (7) as a positive control (data not shown). The miR-494 overexpression study was performed using miR expression vector and control vector (miRNASelect pEGPmiR494 and pEGPnull expression vector; Cell Biolabs, San Diego, CA, http://www.cellbiolabs.com). At 100% confluence, C2C12 myoblasts were transfected using the FuGENE HD transfection reagent (Roche Applied Science, Mannheim, Germany, https://www.roche-applied-science.com). Cells were harvested 48 h after transfection.

Nucleic acid and protein isolation. Total DNA was isolated using a QIAamp DNA mini kit (QIAGEN, Mississauga, ON, Canada, http://www.qiagen.com) according to the manufacturer’s instructions. RNA and protein were isolated using a miRNAva PARIS kit (Life Technologies) according to the manufacturer’s instructions. Small RNAs were concentrated for quantification of mature miRNA.

Quantitative PCR for mitochondrial DNA content. DNA primers were designed to detect cytochrome oxidase 2 (COX2) and uncoupling protein 2 (UCP2) for mitochondrial DNA (mtDNA) and nucleic DNA, respectively (COX2 forward: 5’-TTTTCAGGCTTCACCCCTAGTGA-3’, COX2 reverse: 5’-GAAAGATGTGTTGTTTTACTCCTA-3’, UCP2 forward: 5’-GGGACAGCAGGCCATTAGA-3’, UCP2 reverse: 5’-GGTTCTCCATTACCATCCAAC-3’). The ratio of COX2 to UCP2 within the samples was used to calculate the mtDNA content.

miRNA microarray. The miRNA expression profiles of C2C12 myoblasts (80% confluent) and myotubes (on day 4 after switching to DM) were compared using the NCode Multi-Species miRNA microarray V2 (Life Technologies) according to the manufacturer’s instructions. The scanned array images were annotated and analyzed using GenePix software. The annotated data were normalized using the Latin Squares algorithm within the NCode Profiler data analysis software. The microarray contains optimized probes targeting all known mature miRNAs in the Sanger miRBase sequence database. Release 9.0 for human, mouse, rat, Drosophila, Caenorhabditis elegans, and zebra fish (spotted in triplicate). All microarray data in this study were obtained in accordance with the Minimum Information About a Microarray Experiment (MIAME) guidelines and have been deposited in the National Center for Biotechnology Information (NCBI) GEO database (accession no.: GSE29286).

miRNA quantification. miRNA expression was analyzed by RT-qPCR using TaqMan miRNA assays according to the manufacturer’s instructions (Life Technologies). U6 small RNA was used as an endogenous control for miRNA expression analysis.

miRNA quantification. cDNA was prepared using the PrimeScript 2 first-strand cDNA synthesis kit (Takara Bio, Otsu, Japan, http://www.takara-bio.co.jp). TaqMan RT-qPCR was performed using commercial primers and probe sets from Life Technologies. 18S rRNA was used as an endogenous control.

Western blotting. Proteins were separated by SDS-PAGE and transferred to polyvinylidene fluoride membranes. Immunoblotting was performed using antibodies against mtTFA (LS-C30495; Life-Span Biosciences, Seattle, WA, http://www.lsbio.com) diluted 1:1,000, Foxj3 (MAK1041AF; Protein Express, Chiba, Japan, http://www.proteinexpress.co.jp) diluted 1:2,000, and pan-actin (sc-1616; Santa Cruz Biotechnology, Santa Cruz, CA, http://www.scbt.com) diluted 1:3,000. Membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (GE healthcare Japan, Tokyo, Japan, http://www.gehealthcare.com) and visualized using a chemiluminescence detection reagent (PerkinElmer, Waltham, MA, http://www.perkinelmer.com). The intensity of the protein bands was quantified using Scion image analysis software.

3′-Untranslated region reporter assays. The following primers were used to amplify the 3′-untranslated region (UTR) of mouse mtTFA into the pMirTarget vector (OriGene, Rockville, MD, http://www.origene.com) (forward primer: AGCCGTACGGAGGTGT- CATTGGGATAGGCC, reverse primer: CTGGTCAGTCACTCGTGCA). The forward primer has a Mlu1 site (underlined) to facilitate ligation into the vector. The pMirTarget vector, including the 3′-UTR region of Foxj3, was purchased from OriGene. The mutant miTFA and Foxj3 3′-UTR reporters were created by mutating the seed regions of the predicted miR-494 site (AUGUUUC to CUGUCUCC or GUCUCUG, respectively) using the QuickChange Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, http://www.home.agilent.com). Constructs were cotransfected with anti-miR inhibitors (anti-miR-494 or scrambled sequence) to facilitate ligation into the vector. The pMirTarget vector, including the 3′-UTR region of Foxj3, was purchased from OriGene. The mutant miTFA and Foxj3 3′-UTR reporters were created by mutating the seed regions of the predicted miR-494 site (AUGUUUC to CUGUCUCC or GUCUCUG, respectively) using the QuickChange Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, http://www.home.agilent.com). Constructs were cotransfected with anti-miR inhibitors (anti-miR-494 or scrambled sequence).

Table 1. MicroRNA alterations during C2C12 myocyte differentiation

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Fold Change</th>
<th>P Value</th>
<th>Target Genes</th>
<th>Ref. No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-206</td>
<td>14.2</td>
<td>7.44E-06</td>
<td>HDAC4</td>
<td>6</td>
</tr>
<tr>
<td>miR-133b</td>
<td>10.2</td>
<td>4.61E-04</td>
<td>SRF</td>
<td>6</td>
</tr>
<tr>
<td>miR-444</td>
<td>7.8</td>
<td>4.46E-03</td>
<td>SRF</td>
<td>6</td>
</tr>
<tr>
<td>miR-494</td>
<td>-1.5</td>
<td>1.70E-02</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

miRNA, microRNA. *Positive fold changes represent the fold increase in the differentiated myotubes over the undifferentiated myoblasts. Negative fold changes represent the fold decrease in the myotubes below that of the myoblasts. Fold changes will always be absolute values equal to or >1. #The target genes involved in mitochondria biogenesis reported by previous papers. HDAC4, histone deacetylase 4; SRF, serum response factor.
negative control) or miRNA expression vectors (pEGPmiR494 or pEGPnull vector) into the C2C12 myoblasts at the following concentrations: 0.3 μg/ml of reporter gene (pMirTarget) or 0.1 μg/ml of control vector (pRL-SV40) using the FuGENE HD transfection reagent (Roche Applied Science). Luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, http://www.promega.co.jp).

Prediction of putative target genes for miRNAs altered during myogenesis. miRNA targets were predicted using two web-based programs: miRanda (17; http://www.microrna.org/microrna/home.do) and Targetscan (19; http://targetscan.org/mmu_50). Only target genes predicted by both programs were considered further.

Animals and experimental design. The present study was approved by the Animal Care and Use Committee of Shiga University of Medical Science. Nine-week-old male C57BL/6J mice were obtained from Charles River Japan and maintained on a chow diet with ad libitum access to water. They were randomly assigned (n = 6/group) into a chronic swimming exercise group (exercise) or nonexercise control group (control). They were exercised according to the method of Goto et al. (14) with some modifications. Briefly, mice were

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**Fig. 2.** Prediction of putative target genes for miR-494. Predicted miR-494 target site in the 3′-untranslated region (UTR) of mouse mitochondrial transcription factor A (mtTFA) and Forkhead box j3 (Foxj3). The seed region is enclosed with a square box. *M. musculus, Mus musculus (mmu); R. norvegicus, Rattus norvegicus; H. sapiens, Homo sapiens.*

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**Fig. 3.** miRNA-494 expression and that of its predicted target genes during C2C12 myocyte differentiation. A: expression levels of microRNAs (miRNAs) during C2C12 differentiation were analyzed by RT-qPCR. Myoblasts (80 or 100% confluent, grown in GM) and myotubes [2 days (d) or 4 days after switching to DM] were analyzed (n = 4). B: Western blotting. C: RT-qPCR analysis of mtTFA and Foxj3 expression during C2C12 differentiation (*P < 0.05 and **P < 0.01; n = 4).
accustomed to swimming for 5 and 10 min, with a 5-min rest interval. They then swam in seven 15-min bouts (105 min) separated by 5-min rest periods. All mice swim in a water-filled barrel with an average surface area of 210 cm²/mouse. The water temperature was maintained at 35°C. Mice performed the above program daily for 7 days (exercise). Approximately 2 h after the last bout of exercise, mice were anesthetized with sevoflurane, and the gastrocnemius muscles (exercise) were dissected out. The tissues were immediately frozen in liquid nitrogen and stored at −80°C.

Statistical analysis. Results are expressed as means ± SD. Significance was assessed using the Student’s t-test and one-way ANOVA. Differences between the groups were determined using Tukey’s multiple-range test. A P value of <0.05 was considered significant.

RESULTS

mtDNA content increases during skeletal muscle differentiation. mtDNA content was analyzed during C2C12 differentiation from myoblasts (80–100% confluent) to myotubes (2 and 4 days after switching to DM). As shown in Fig. 1A, the expression of myogenin mRNA significantly increased from day 2 to day 4 (after switching to DM), indicating that the cells were well differentiated. mtDNA content increased during differentiation by ~500% in the 80% confluent cells (Fig. 1B). Thus, comparative miRNA analysis during skeletal muscle differentiation is thought to be an effective method for identification of miRNAs that contribute to mitochondrial biogenesis.

miR-494 expression decreases during skeletal muscle differentiation. miRNA expression within myoblasts and myotubes was compared using a commercial miRNA microarray. Microarray analysis revealed that four miRNAs were significantly altered during skeletal muscle differentiation. The expression levels of three miRNAs increased (miR-130b 14.2-fold, P < 0.01; miR-133a 10.2-fold, P < 0.01; miR-133b 7.8-fold, P < 0.01) and one miRNA decreased (miR-494 1.5-fold, P = 0.017) (Table 1). Upregulation of miR-206, miR-133a, and miR-133b has been reported previously (32). miR-494 was selected for further analysis because there are no reports regarding the role of this miRNA in skeletal muscle. Computer programs predicted some genes, including mtTFA and Foxj3 [both of which are thought to be positive regulators of mitochondrial biogenesis (1, 28)], as potential target genes for miR-494 (Fig. 2). The putative binding sites are conserved in various species. The results of the TaqMan RT-qPCR assays showed that miR-494 expression decreased during skeletal muscle differentiation (Fig. 3A), which was in agreement with the microarray results. Because the major changes of miR-494 expression occurred between 100% confluence (day 0) and day 2, we checked the...
miR-494 expression levels from day 0 to day 2 in C2C12 cells and found that expression of miR-494 was decreased 12 h after initiation of myogenesis (data not shown). The results for miR-206 and miR-133b observed in the microarray analysis were confirmed by RT-qPCR (Fig. 3A).

Expression of mtTFA and Foxj3 increases during skeletal muscle differentiation. First, the expression of mtTFA and Foxj3 during C2C12 cell differentiation was analyzed. Western blot analysis showed that the expression of both proteins increased markedly during skeletal muscle differentiation and was accompanied by a decrease in miR-494 expression (Fig. 3B). In contrast, mtTFA mRNA levels decreased (Fig. 3C). The expression of Foxj3 mRNA moderately increased during skeletal muscle differentiation (Fig. 3C), in agreement with a report by Alexander et al. (1). The apparent dissociation of mRNA expression and protein expression suggests that their expression during skeletal muscle differentiation could be posttranslationally modified, and this may be regulated by miRNAs.

miR-494 negatively regulates mtTFA and Foxj3 expression and mitochondrial biogenesis in C2C12 myoblasts. miR-494 knockdown analysis in C2C12 myoblasts was achieved using antisense inhibitors of miR-494 and a scrambled sequence.
negative control. mtTFA and Foxj3 expression was then analyzed at the protein and mRNA level. The expression levels of mtTFA and Foxj3 proteins significantly increased ($P < 0.01$) after miR-494 suppression, but their mRNA levels were not changed (Fig. 4, A and B). We then investigated the effects on mtDNA copy number and myocyte enhancer factor 2c (Me2c), which is downstream of mtTFA and Foxj3. Both the mtDNA copy number and mRNA expression of Me2c significantly increased after miR-494 knockdown ($P < 0.01$, Fig. 4, B and C). The opposite of these findings was observed in response to miR-494 overexpression. miR-494 expression levels were ~10 times higher in the overexpression study (Fig. 5A), and the expression levels of mtTFA and Foxj3 proteins significantly decreased without any change in mRNA expression ($P < 0.01$, Fig. 5, B and C). The mtDNA copy number and mRNA expression of Me2c also decreased ($P < 0.01$, Fig. 5, C and D). These results suggest that miR-494 contributes to mitochondrial biogenesis by regulating the expression of mtTFA and Foxj3.

To examine the direct effects of miR-494 on its target genes, we conducted a luciferase assay using a reporter vector with the 3'UTR region inserted at the end of the luciferase gene. miR-494 knockdown significantly stimulated luciferase activity by the 3'UTR regions of mtTFA and Foxj3, whereas the control or mutant vector did not (Fig. 4D); the reverse of these findings was observed in response to miR-494 overexpression (Fig. 5E). These results strongly suggest that miR-494 directly regulates these genes by interaction with putative binding sites.

Endurance exercise decreases miR-494 expression in skeletal muscle. To investigate whether miR-494 contributes to mitochondrial biogenesis in vivo, C57BL/6J mice were subjected to endurance exercise tests, and skeletal muscle was analyzed. The results were then compared with those obtained from a “resting” control. As many researchers have also reported, mRNA expression of PGC1-α significantly increased in the exercise group ($P < 0.01$, Fig. 6D), and the mtDNA copy number also significantly increased in the exercise group ($P < 0.01$, Fig. 6A). In addition, miR-494 expression was significantly reduced in the exercise group ($P < 0.01$, Fig. 6B) along with a posttranscriptional increase in target gene expression (Fig. 6, C and D). These results suggest that miR-494 plays an important role in mitochondrial biogenesis in vivo by regulating the expression of its target genes.

DISCUSSION

To identify the miRNAs that contribute to mitochondrial biogenesis, we undertook microarray analysis of skeletal muscle during differentiation combined with the use of miRNA target prediction programs. We found that the expression of miR-494, which is predicted to regulate mtTFA and Foxj3 (key transcription factors of mitochondrial biogenesis), decreased during skeletal muscle differentiation (Fig. 3A). This finding is unique, since most previous reports show increased mRNA expression during skeletal muscle differentiation (7, 35). mtTFA is a major transcription factor that stimulates transcription in mitochondria (28). Foxj3 is...
a newly identified transcription factor that upregulates Mef2c (1). Mef2c belongs to the Mef2 protein family of transcription factors and regulates the formation of slow-twitch type 1 fibers, which contain abundant mitochondria (27). We found that the expression levels of these target genes were posttranscriptionally increased during skeletal muscle differentiation, accompanied by a decrease in miR-494 expression (Fig. 3, B and C). We also found that miR-494 knockdown led to an increase in target gene expression and mitochondrial copy number in C2C12 myocytes (Fig. 4A, B, and C). The reverse of these findings was observed in miR-494 overexpression studies (Fig. 5, B, C, and D). Furthermore, morphology of the cells and myogenin expression were not altered in response to miR-494 knockdown and overexpression (data not shown), suggesting that miR-494 directly regulates mtTFA and Foxj3 expression but not myogenesis per se. In addition, 3′-UTR reporter assays revealed that miR-494 directly regulates mtTFA and Foxj3 (Figs. 4D and 5E). Taken together, these findings suggest that miR-494 plays a critical role in mitochondrial biogenesis by regulating mtTFA and Foxj3 expression.

miR-494 expression also decreased after chronic endurance exercise. Moreover, the expression of its target genes was significantly increased at the protein level by chronic endurance exercise. To the best of our knowledge, this is the first report showing that Foxj3 levels increase in skeletal muscle after endurance exercise. The mRNA expression of Foxj3 was not changed by chronic endurance exercise, suggesting that Foxj3 protein expression is regulated by miRNAs. In our experiment, the mRNA expression of mtTFA was not changed, but the protein expression was increased after 2 h of endurance exercise. Park et al. reported that mtTFA mRNA expression was not changed after 2 h of exhaustive exercise, but its expression was increased after 12 h (26). These results are in agreement with our results and suggest that mtTFA expression is regulated at both the transcriptional and posttranscriptional levels in skeletal muscle after physical stimulation. It is thought that posttranscriptional regulation responds to exercise earlier than transcriptional regulation. Thus, we speculated that miR-494 may also regulate mitochondrial biogenesis via mtTFA and Foxj3 in vivo.

Little is known about the regulation of miR-494 expression. It has been reported that the expression level of miR-494 is transiently decreased in murine hearts on ischemia-reperfusion induced injury (33). It has also been reported that transforming growth factor-β upregulates miR-494 in myeloid-derived suppressor cells (23). In our study, it is interesting to note that the expression of miR-494 is regulated during both skeletal muscle differentiation and endurance exercise. Generally, miRNA is transcribed from the original gene or an intron of its host gene (30). miR-494 might be regulated by its original promoter because no genes reside close to the miR-494 region in the mouse genome. Further study is necessary to clarify the mechanisms underlying the regulation of miR-494 expression.

Recently, Aoi et al. showed that miR-696 was downregulated by physical activity and contributes to mitochondrial biogenesis by regulating PGC1-α expression (4). PGC1-α is a transcription coactivator that regulates mitochondrial biogenesis, and its overexpression in skeletal muscle or myocytes results in a large increase in mitochondrial numbers (18, 22, 36). After activation via phosphorylation, PGC1-α is translocated to the nucleus. Activated PGC1-α then docks with and coactivates transcription factors that regulate the expression of the nuclear genes encoding mitochondrial proteins that induce mtTFA, which regulates mitochondrial genome transcription (21). It is known that PGC1-α expression is regulated by Mef2 in a transcriptional manner (10). In the present study, Mef2c expression was thought to be indirectly regulated by miR-494 through Foxj3 (Figs. 4B and 5C). Thus, it was thought that miR-494-regulated Foxj3-Mef2c signaling stimulates mitochondrial biogenesis via PGC1-α signaling. In addition, we have observed dissociation of mtTFA mRNA expression and protein expression after endurance exercise tests (Fig. 6, C and D), suggesting that miR-494 also regulates mitochondrial biogenesis in a PGC-1-independent manner.

One potential limitation of this study is that the effects of miR-494 knockdown or overexpression were relatively small. However, because the mitochondrial content of skeletal muscle is critical for energy metabolism, it is not surprising that mitochondrial biogenesis is tightly controlled by multiple mechanisms. The dissociation between mRNA and protein expression of mtTFA and Foxj3 may be explained not only by miRNA but also by posttranslational regulatory mechanisms such as the ubiquitin-proteasome pathway. It has been reported that mtTFA is also controlled by this system (3), and it could be contributing to the observed dissociation in our study. Another limitation is that we do not have direct evidence of causal effects of miR-494 on mtTFA and Foxj3 in vivo. We speculate this causal relationship exists because there were negative correlations between the expression level of miR-494 and protein expression levels of mtTFA and Foxj3 (data not shown). Furthermore, we found the expression of miR-494 is high in extensor digitorum longus muscle, modest in tibialis anterior muscle, and low in gastrocnemius muscle, which negatively correlates with mitochondrial content. However, miR-494 in soleus muscle (high mitochondria content) is also high (data not shown). This is potentially because the

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**Fig. 7.** miR-494 contributes to mitochondrial biogenesis by regulating the expression of mtTFA and Foxj3 during skeletal muscle differentiation and endurance exercise. NRF-1, nuclear respiratory factor 1.
REFERENCES

H.Y. and K.M. prepared figures; H.Y. and K.M. drafted manuscript; H.Y. and K.M., Y.N., S.U., and T.Y. performed experiments; H.Y., K.M., Y.N., S.U., and T.Y. conceived and design of research; H.Y. and K.M. performed experiments; H.Y., K.M., Y.N., S.U., and T.Y. contributed to mitochondrial biogenesis through mtTFA and Foxj3-Mef2c signaling (Fig. 7). It is speculated that mir-494 might be a potent regulator of these transcription factors and connect muscle differentiation and mitochondrial biogenesis in skeletal muscle. The results of this study provide a new mechanism of mitochondrial biogenesis in skeletal muscle regulated by miRNAs. These findings will increase our understanding of mitochondrial biogenesis in skeletal muscle.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


REFERENCES