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miR-324-5p Inhibits C2C12 cell Differentiation and Promotes Intramuscular Lipid Deposition through lncDUM and PM20D1

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Skeletal muscle is an important metabolic organ of the body, and impaired skeletal muscle differentiation can result in a wide range of metabolic diseases. It has been shown that microRNAs (miRNAs) play an important role in skeletal muscle differentiation. The aim of this study was to investigate the role of mmu-miR-324-5p in the differentiation of C2C12 myoblasts and lipid droplet deposition in myotubes for future targeted therapies. We found that mmu-miR-324-5p was highly expressed in mouse skeletal muscle. Overexpression of miR-324-5p significantly inhibited C2C12 myoblast differentiation while promoting oleate-induced lipid accumulation and β-oxidation in C2C12 myoblasts. Conversely, inhibition of mmu-miR-324-5p promoted C2C12 myoblast differentiation and inhibited lipid deposition in myotubes. Mechanistically, mmu-miR-324-5p negatively regulated the expression of long non-coding Dum (lncDum) and peptidase M20 domain containing 1 (Pm20d1) in C2C12 myoblasts. Reduced lncDum expression was associated with a significant decrease in the expression of myogenesis-related genes. Knockdown of mmu-miR-324-5p increased the levels of lncDum and myogenesis-related gene expression. Following oleate-induced lipid deposition in C2C12 myoblasts, overexpression of mmu-miR-324-5p decreased the expression of Pm20d1 while increasing the expression of mitochondrial β-oxidation and long-chain fatty acid synthesis-related genes. In conclusion, we provide evidence that miR-324-5p inhibits C2C12 myoblast differentiation and promotes intramuscular lipid deposition by targeting lncDum and Pm20d1, respectively.

INTRODUCTION

In the human body, skeletal muscle comprises approximately 40% of total body weight and contains 50%–75% of all human proteins. It is also an important organ for maintaining the body's homeostasis.[1] Skeletal muscle cell differentiation is a complex biological process, during which skeletal muscle cells begin to express myogenic factors, such as myogenic factor 5 (MYF5), myoblast determination protein (MYOD), and myogenin (MYOG).[2] At the terminally differentiated stage, skeletal muscle cells form a multinuclear structure and begin to express myosins such as myosin heavy chains (MyHCs).[3] Mouse C2C12 cells can be induced to differentiate into myoblasts under *in vitro* culture conditions, and this cell line is widely used to study myogenic differentiation in skeletal muscle cells.

Obesity is caused by an excessive accumulation of fat,[4] which can also occur in skeletal muscle and has not been appreciated for a long time.[5] Ectopic skeletal muscle lipid deposition can result in the formation of intramuscular fat and the development of metabolic diseases such as insulin resistance and diabetes.[6,7] There are two types of intramuscular fat, namely, acellular lipid droplets in muscle cells (muscle fibers), called intramyocellular triacylglycerols (IMTGs), and adipocytes distributed in the muscle interstitium or surrounding muscle fascicles, known as intramuscular adipose tissue (IMAT).[5] Studies have shown that mutations in Pax7 lead to muscle being replaced by fibers and adipose tissue during muscle damage repair, while knockout of MYOD and MYF5 induces adipose tissue infiltration into skeletal muscle. In addition, in Duchenne muscular dystrophy (DMD) patients, intramuscular fat can comprise as much as 50% of total muscle mass.[8] Studies have also shown that free fatty acids can promote the accumulation of IMTGs. For example, oleic acid (OA) and, to a lesser extent, palmitic acid (PA) can promote the increased accumulation of triglycerides in human myotubes.[9,10] In a previous study, we also found that oleic acid can effectively promote the accumulation of lipid droplets in the myotubes of C2C12 myoblasts.[11]

MicroRNAs (miRNAs) are short, non-coding RNAs that posttranscriptionally exert their regulatory functions by targeting the 3′ UTR of mRNAs.[12] miRNAs play an important role in muscle development, as evidenced by the conditional deletion of Dicer in muscle

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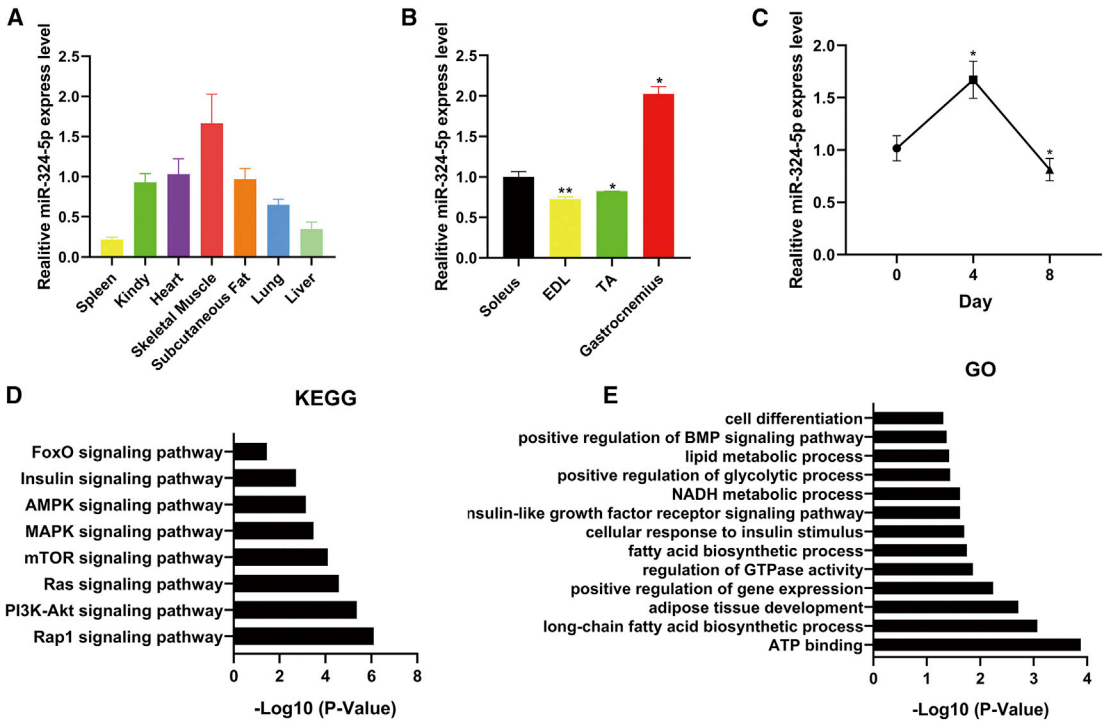

Figure 1. miR-324-5p Is Associated with Myogenic Differentiation and Lipid Synthesis

(A) The miR-324-5p expression level in different tissues of C57BL/6 mice as determined by quantitative real-time PCR. (B) Quantitative real-time PCR analysis of miR-324-5p expression in different skeletal muscles isolated from C57BL/6 mice at 3 months of age. (C) Quantitative real-time PCR detection of miR-324-5p expression during C2C12 cell differentiation. (D) Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of miR-324-5p target genes. (E) Gene Ontology (GO) term analysis of miR-324-5p target genes. The data are expressed as mean ± SD; n = 5. *p < 0.05, **p < 0.01. EDL, extensor digitorum longus; TA, tibialis anterior.

that causes a decrease in skeletal muscle mass and abnormal fiber morphology.[13] There are many skeletal muscle-specific miRNAs, among which miR-1/206 and miR-133 are the most studied, and are known to affect myoblast proliferation and differentiation.[14,15] However, there are also numerous ubiquitous miRNAs involved in regulating skeletal muscle cells, such as miR-22,[16] miR-19,[17] and miR-29c,[18] which indicates that both muscle-specific and ubiquitously expressed miRNAs have critical roles in myoblasts.

miR-324-5p is highly conserved among species, and its expression has been associated with several diseases. Studies have shown that miR-324-5p can inhibit gallbladder carcinoma cell metastasis by downregulating the expression level of transforming growth factor β2 (*TGFB2*),[19] as well as suppress the migration and induce the apoptosis of gastric cancer cells by inhibiting *TSPAN8*.[20] Another study found that miR-324-5p can promote the osteogenic differentiation of human mesenchymal stem cells (MSCs) and murine C3H10T1/2 cells by regulating the Hedgehog signaling pathway.[21] Mesenchymal stem cells have the potential for myogenic differentiation,[22] indicating that miR-324-5p has a potential role in myogenic differentiation. However, the role of miR-324-5p in skeletal muscle, as well as the associated mechanisms, remain unclear. In this study, we both overexpressed and inhibited mmu-miR-324-5p in C2C12 myoblasts and found that miR-324-5p has a suppressive effect on C2C12 myoblast differentiation, and this effect was achieved by targeting long non-coding RNA Dum (lncDum). We also found that, following oleate-induced lipid deposition in myotubes of C2C12 myoblasts, miR-324-5p promoted lipid deposition in myotubes by inhibiting the expression of peptidase M20 domain containing 1 (Pm20d1).

RESULTS

miR-324-5p Is Associated with Myogenic Differentiation and Lipid Synthesis

miRNAs play an important role in skeletal muscle differentiation. Differential analysis of the C2C12 myoblast differentiation microarray dataset GEO: GSE52410 showed that miR-324-5p expression was significantly reduced during the differentiation process (Figure S1A). Next, we compared the expression level of miR-324-5p in different mouse tissues and found that miR-324-5p was highly expressed in skeletal muscle compared with other tissues (Figure 1A). We also evaluated the expression level of miR-324-5p in different skeletal muscles of mice and found that miR-324-5p expression was highest in the gastrocnemius and lowest in the extensor digitorum longus (EDL) (Figure 1B). This suggested that miR-324-5p may play a significant role in mouse skeletal muscle. To further confirm that miR-324-5p is indeed involved in regulating C2C12 myoblast differentiation, we next evaluated the expression level of miR-324-5p at different C2C12 myoblast differentiation stages. The

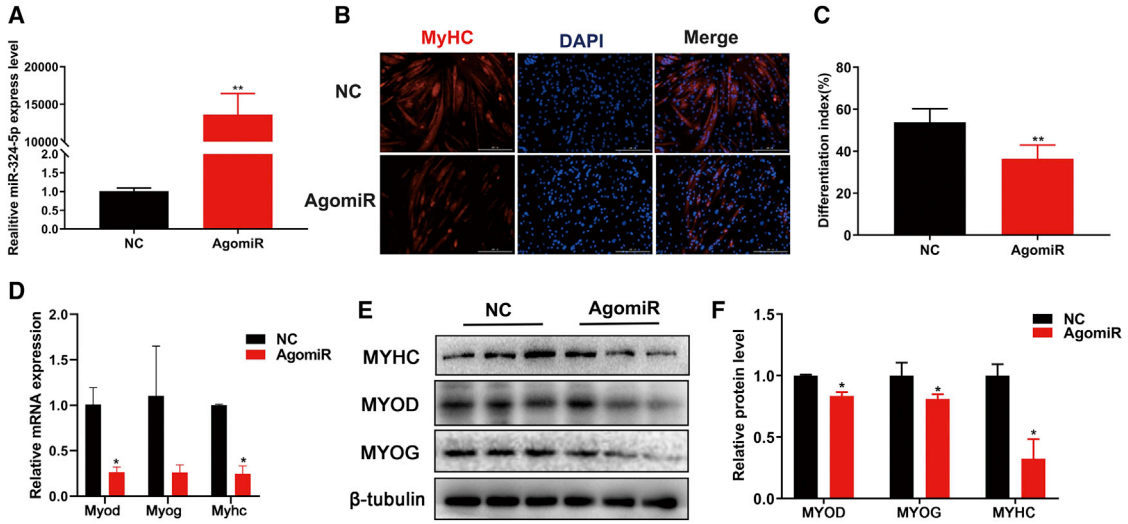

Figure 2. Overexpression of miR-324-5p Inhibits the Differentiation of C2C12 Myoblasts

(A) The efficiency of miR-324-5p overexpression after agomiR-324-5p transfection compared with that of the negative control (NC) on day 6 of differentiation. (B) Immunofluorescence staining of muscle myosin heavy chain (MyHC) in C2C12 myoblasts. Scale bars, 300 μm. (C) The fusion index was assessed as the ratio of MyHC-positive cells to the total number of nuclei. (D) The RNA level of myogenesis-related genes, including *Myod*, *Myog*, and *Myhc*, detected by quantitative real-time PCR after overexpression of miR-324-5p. (E) Western blot analysis of myogenesis-related proteins after overexpression of miR-324-5p. (F) Quantification of protein levels. The data are expressed as mean ± SD; n = 5. *p < 0.05, **p < 0.01.

results showed that miR-324-5p expression was significantly reduced at day 8 of differentiation, which was consistent with the microarray data. Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) analysis on miR-324-5p target genes further indicated that miR-324-5p is involved in the regulation of numerous signaling pathways (Figure 1C), most of which are involved in the regulation of myogenic differentiation or repair of muscle damage.[23] In addition, GO enrichment analysis indicated that miR-324-5p has roles in biological processes such as fatty acid and ATP synthesis (Figure 1D). These results implied that miR-324-5p has important effects on both skeletal muscle cell differentiation and adipogenesis.

Overexpression of miR-324-5p Inhibits Myogenic Differentiation of C2C12 Cells

To clarify the function of miR-324-5p in myogenic differentiation, C2C12 myoblasts were transfected with agomiR-324-5p or negative control (NC) at 80% confluence and then induced to differentiation. The results showed that agomiR-324-5p transfection led to a significant increase in the expression level of miR-324-5p (Figure 2A). We also performed MyHC immunofluorescence staining on C2C12 myoblasts 6 days after differentiation and found that miR-324-5p-overexpressing myoblasts presented fewer myotubes (Figure 2B) and a reduced fusion rate compared with the NC (Figure 2C). Additionally, overexpression of miR-324-5p markedly suppressed the mRNA expression levels of myogenesis-related genes, including *Myod*, *Myog*, and *Myhc* (Figure 2D), which was consistent with the protein level (Figures 2E and 2F). The above results indicated that overexpression of miR-324-5p inhibits C2C12 myoblast differentiation by suppressing the expression of myogenesis-related genes.

Inhibition of miR-324-5p Promotes C2C12 Myoblast Differentiation

To further investigate the effect of miR-324-5p on C2C12 myoblast differentiation, we transfected C2C12 myoblasts with antagomiR-324-5p or negative control (NA). At day 6 after differentiation, we observed that transfection of antagomiR-324-5p significantly inhibited the expression level of miR-324-5p (Figure 3A). MyHC immunofluorescence staining of differentiated cells demonstrated that AntagomiR-324-5p increased the number of myotubes (Figure 3B) and fusion rate when compared with the NA (Figure 3C). The quantitative real-time PCR results further showed that AntagomiR-324-5p significantly increased the expression levels of myogenesis-related genes such as *Myod*, *Myog*, and *Myhc* (Figure 3D), and the protein levels were also consistent with the mRNA expression data (Figures 3E and 3F). The above data implied that inhibition of miR-324-5p promotes the differentiation of C2C12 myoblasts.

miR-324-5p Inhibits C2C12 Myoblast Differentiation by Targeting lncDum

lncRNAs play an important role in myoblast differentiation,[24] and we identified lncDum as a candidate target gene of miR-324-5p through the online prediction website starBase (Figure 4A). To verify if lncDum is a target of miR-324-5p, we modulated the expression of miR-324-5p by transfection with agomiR-324-5p or antagomiR-324-5p. Overexpression of miR-324-5p strongly repressed lncDum expression (Figure 4B), whereas inhibition of miR-324-5p significantly increased the expression of lncDum (Figure 4C). To determine whether miR-324-5p can directly target

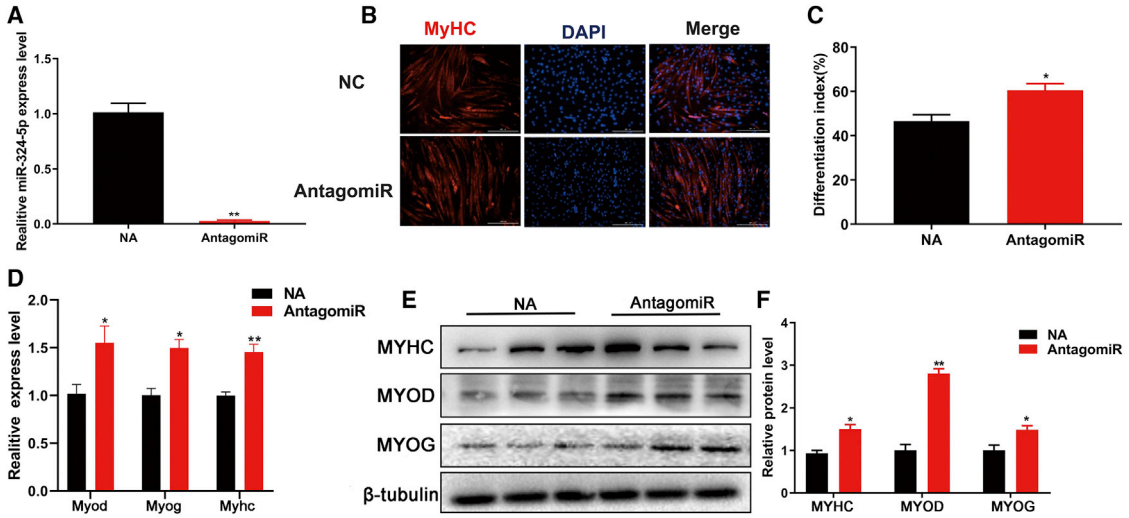

Figure 3. Inhibition of miR-324-5p Promotes C2C12 Myoblast Differentiation

(A) The efficiency of miR-324-5p inhibition after antagomiR-324-5p transfection compared with that of the negative control (NA) on day 6 of differentiation. (B) Immunofluorescence staining of MyHC in C2C12 myoblasts. Scale bars, 300 μm. (C) The fusion index was assessed as the ratio of MyHC-positive cells to the total number of nuclei. (D) Quantitative real-time PCR was used to detect the expression of myogenesis-related genes, including *Myod*, *Myog*, and *Myhc*, after inhibition of miR-324-5p. (E) Western blot analysis of myogenesis-related proteins after inhibition of miR-324-5p. (F) Quantification of protein levels. The data are expressed as mean ± SD; n = 5. *p < 0.05, **p < 0.01.

lncDum, we cloned the lncDum segment containing either the wild-type (WT) or mutant predicted miR-324-5p binding sequence into luciferase reporter plasmids. Dual-luciferase reporter assays showed that overexpression of miR-324-5p reduced the luciferase activity of plasmids containing the WT, but not the mutant, miR-324-5p binding sequence (Figure 4D). This demonstrated that miR-324-5p directly interacts with the predicted target site in lncDum.

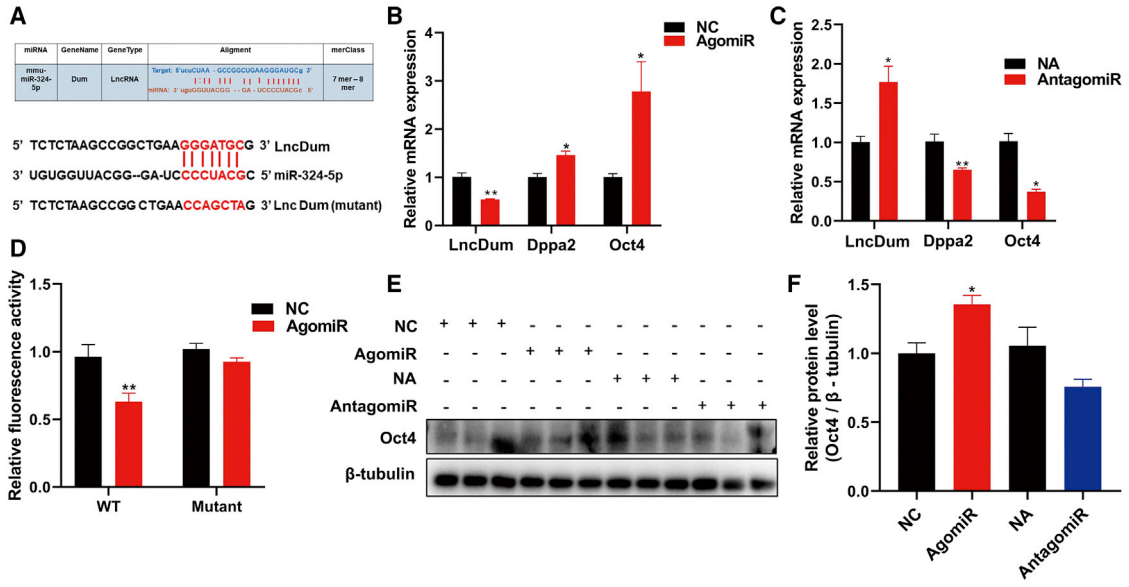

Figure 4. miR-324-5p Inhibits C2C12 Myoblast Differentiation by Targeting lncDum

(A) Upper panel: details of the alignment of miR-324-5p to the binding site of lncDum. Lower panel: red-marked nucleotides are WT, and mutant (Mut) lncDum binding sites to generate WT and Mut constructs are shown. (B) Quantitative real-time PCR analysis of the mRNA levels of lncDum, *Dppa2*, and *Oct4* after treat with AgomiR-324-5p. (C) The mRNA expression level of lncDum, *Dppa2*, and *Oct4* after inhibited miR324-5p. (D) AgomiR-324-5p or NC were co-transfected with psiCHECK2-lncDUM or psiCHECK2-lncDUM-Mut vectors into HEK293T cells. After 48 h, *Renilla* luciferase activity was quantified and normalized to that of firefly luciferase (n = 6). (E) Western blot analysis of OCT4 with overexpression or inhibition of miR-324-5p. (F) Quantification of protein levels. The data are expressed as mean ± SD; n = 5. *p < 0.05, **p < 0.01.

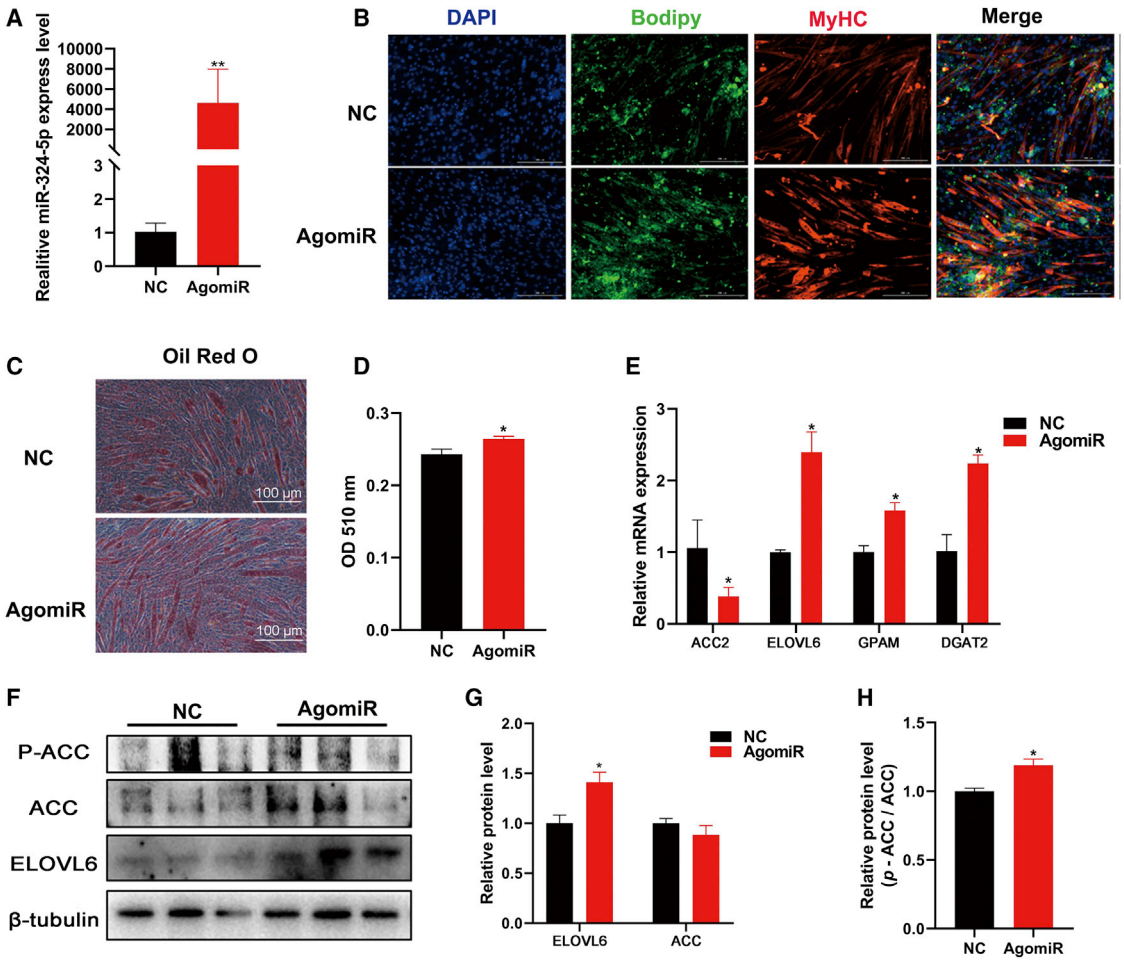

Figure 5. Overexpression of miR-324-5p Promotes Oleate-Induced Lipid Deposition in Myotubes

(A) The efficiency of miR-324-5p overexpression after agomiR-324-5p transfection compared with that of the NC. (B) BODIPY and immunofluorescence staining of lipid and muscle MyHC in C2C12 myoblasts. Scale bars, 300 μm. (C) Oil red O staining for lipids in C2C12 myoblasts. (D) Oil red O-stained cells were extracted with isopropyl alcohol for assessment of intracellular triglyceride concentrations. (E) The expression levels of fatty acid synthesis-related genes, including *Acc2*, *Elovl6*, *Gpam*, and *Dgat2*, were determined by quantitative real-time PCR after overexpression of miR-324-5p. (F) Western blot analysis of fatty acid synthesis-related proteins after overexpression of miR-324-5p. (G) Quantification of protein levels. (H) Quantification of p-ACC protein levels. The data are expressed as mean ± SD; n = 5; *p < 0.05, **p < 0.01.

Because lncDum can regulate myoblast differentiation through *cis*-regulation of *Dppa2* and *Oct4* expression,[25] we investigated whether miR-324-5p can regulate myogenic differentiation through lncDum. The results showed that overexpression of miR-324-5p promoted significantly the expression levels of *Dppa2* and *Oct4* (Figure 4B), whereas miR-324-5p inhibition markedly suppressed the expression levels of these two genes (Figure 4C); the result for OCT4 protein level was consistent with the quantitative real-time PCR data (Figures 4E and 4F). Moreover, the expression levels of *Dppa2* and *Oct4* were negatively correlated with that of lncDum. Collectively, these results indicated that the effect of miR-324-5p on myogenic differentiation perhaps achieved through targeting lncDum.

Overexpression of miR-324-5p Promotes Oleate-Induced Lipid Deposition in Myotubes

GO enrichment analysis of miR-324-5p target genes revealed that miR-324-5p was associated with biological processes such as long-chain fatty acid synthesis (Figure 1E), suggesting that miR-324-5p might exert a regulatory effect on fatty acid synthesis in the myotubes of C2C12 myoblasts. We have previously reported that oleate significantly promotes lipid deposition in the myotubes of myoblasts.[11] In this study, C2C12 myoblasts were induced to differentiate for 6 days; when myotubes were clearly visible, we transfected the myoblasts with agomiR-324-5p or NC. After 24 h, lipid deposition in myotubes was induced by the addition of 500 μmol/L oleate. We found that the expression level of miR-324-5p was increased near 4,000-fold under

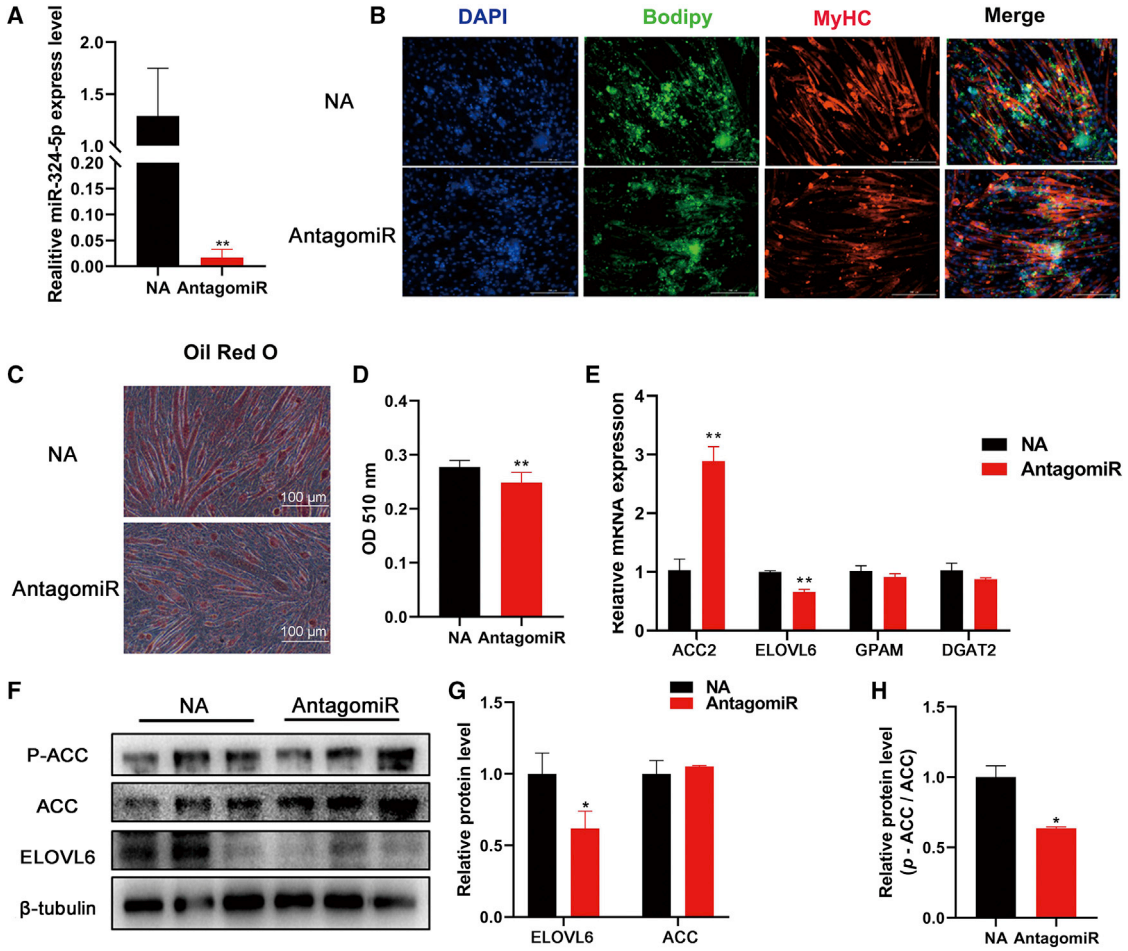

Figure 6. Inhibition of miR-324-5p Reduces Oleate-Induced Lipid Deposition in C2C12 Myoblasts

(A) The efficiency of miR-324-5p inhibition after antagomiR-324-5p transfection compared with that of the NA. (B) BODIPY and immunofluorescence staining of lipids and muscle MyHC in C2C12 myoblasts. Scale bars, 300 μm. (C) Oil red O staining for lipids in C2C12 myoblasts. (D) Oil red O-stained cells were extracted with isopropyl alcohol for assessment of intracellular triglyceride concentrations. (E) The expression levels of fatty acid synthesis-related genes, including *Acc2*, *Elovl6*, *Gpam*, and *Dgat2*, were determined by quantitative real-time PCR after inhibition of miR-324-5p. (F) Western blot analysis of fatty acid synthesis-related proteins ELOVL6 and ACC after inhibition of miR-324-5p. (G) Quantification of protein levels. (H) Quantification of p-ACC protein levels. The data are expressed as mean ± SD; n = 5. *$p < 0.05$, **$p < 0.01$.

these conditions (Figure 5A). BODIPY and oil red O staining demonstrated that, with overexpression of miR-324-5p, the amount of lipid was increased compared with the NC, as determined by the naked eye; however, the number of myotubes remained unchanged (Figures 5B and 5C). Further examination of intracellular triglyceride levels showed that the contents of triglyceride was significantly increased with overexpression of miR-324-5p (Figure 5D). We next analyzed the mRNA levels of genes related to long-chain fatty acid synthesis, including *Elovl6*, *Gpam*, and *Dgat2*. We found that overexpression of miR-324-5p significantly upregulated the expression of these genes, as well as the ELOVL6 protein level (Figures 5E–5G). The mRNA expression of the *Acc2* was significantly decreased, whereas the protein level of ACC remained unchanged; however, the level of phosphorylated ACC (p-ACC) was significantly increased (Figures 5E–5H). ACC2 catalyzes the production of malonyl-coenzyme A (CoA), which can inhibit the β-oxidation of mitochondrial fatty acids through inhibition of CPT1.[26] Collectively, these observations indicate that miR-324-5p promotes oleate-induced lipid deposition in myotubes by increasing the expression of fatty acid synthesis-related genes.

Inhibition of miR-324-5p Reduces Oleate-Induced Lipid Deposition in C2C12 Myoblasts

To further confirm the role of miR-324-5p on oleate-induced lipid deposition in C2C12 myoblasts, we transfected C2C12 myoblasts with antagomiR-324-5p or the NC. Transfection with antgomiR-324-5p significantly reduced the expression of miR-324-5p when compared with the NC (Figure 6A). BODIPY and oil red O staining results further showed that inhibition of miR-324-5p suppressed intracellular lipid deposition in myotubes (Figures 6B and 6C) and

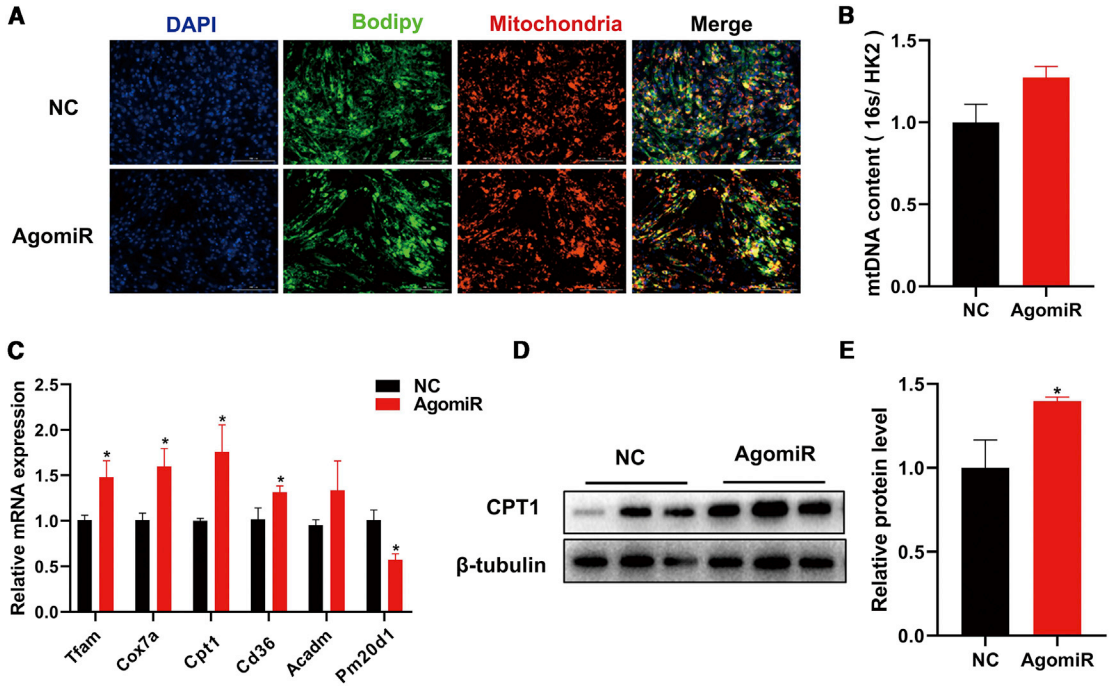

Figure 7. Overexpression of miR-324-5p Increases Oleate-Induced Mitochondrial β-Oxidation

(A) BODIPY and mitochondrial staining for oleate-induced C2C12 myoblasts after overexpression of miR-324-5p. Scale bars, 300 μm. (B) Mitochondrial DNA (mtDNA) copy number in C2C12 myoblasts transfected with agomiR-324-5p; total cellular DNA was extracted and quantitative PCR was performed. Relative mtDNA levels were calculated based on the ratio of mtDNA (16S RNA) to the nuclear gene hexokinase 2 (*HK2*). (C) Quantitative real-time PCR was used to measure the expression levels of mitochondrial metabolism-related genes (*Tfam*, *Cox7a*, and *Cpt1*) and fatty acid oxidation-related genes (*CD36* and *Acadm*) after overexpression of miR-324-5p. (D) Western blot analysis of CPT1 after overexpression of miR-324-5p. (E) Quantification of protein levels. The data are expressed as mean ± SD; n = 5. *$p < 0.05$, **$p < 0.01$.

significantly reduced intracellular triglyceride concentrations (Figure 6D). Quantitative real-time PCR and western blot results showed that knockdown of miR-324-5p significantly suppressed the expression levels of genes (Figure 6E) and proteins associated with fatty acid synthesis (Figures 6F–6H). However, inhibition of miR-324-5p markedly increased the expression level of *Acc2* and decreased the level of p-ACC (Figures 6E, 6F, and 6H). Collectively, these data demonstrated that antagomir-mediated inhibition of miR-324-5p suppresses oleate-induced lipid deposition in C2C12 myoblasts.

Overexpression of miR-324-5p Increases Oleate-Induced Mitochondrial β-Oxidation

Because we found that miR-324-5p modulated the expression of *Acc2*, a gene associated with β-oxidation of fatty acids, we then investigated the effect of miR-324-5p on oleate-induced mitochondrial fatty acid β-oxidation. For this, we overexpressed miR-324-5p and induced lipid deposition by oleate treatment. Lipid and mitochondrial staining showed that overexpression of miR-324-5p increased the number of mitochondria (Figure 7A). We also analyzed the mitochondrial DNA (mtDNA) copy number and found that the number of mtDNA copies was significantly increased with overexpression of miR-324-5p (Figure 7B). Furthermore, we observed a significant increase in the expression levels of genes associated with both mitochondrial metabolism (*Tfam*, *Cox7a*, and *Cpt1*) and fatty acid oxidation (*Cd36*), and the expression level of *Acadm* was rising as well, but the difference was not significant (Figure 7C), while the CPT1 protein expression level was also substantially increased (Figures 7D and 7E). Pm20d1 can inhibit mitochondrial fatty acid β-oxidation, and studies have shown that *Pm20d1* is a miR-324-5p target gene. In this study, we found that overexpression of miR-324-5p markedly inhibited the mRNA expression level of *Pm20d1* (Figure 7C). Taken together, these data suggested that miR-324-5p can promote oleate-induced mitochondrial fatty acid β-oxidation.

Inhibition of miR-324-5p Suppresses Oleate-Induced β-Oxidation of Mitochondrial Fatty Acids

In contrast with the overexpression results, inhibiting miR-324-5p expression using antagomiR-324-5p led to a reduction in the number of mitochondria, while the mtDNA copy number was significantly decreased (Figures 8A and 8B). In addition, inhibition of miR-324-5p significantly inhibited the expression level of mitochondrial metabolism- and fatty acid oxidation-related genes (Figure 8C), and the protein expression of CPT1 was also significantly reduced (Figures 8D and 8E). Moreover, inhibiting miR-324-5p promoted a remarkable increase in the expression level of *Pm20d1* (Figure 8C). These results indicated that miR-324-5p promotes β-oxidation of mitochondrial fatty acids perhaps through inhibition of *Pm20d1* expression.

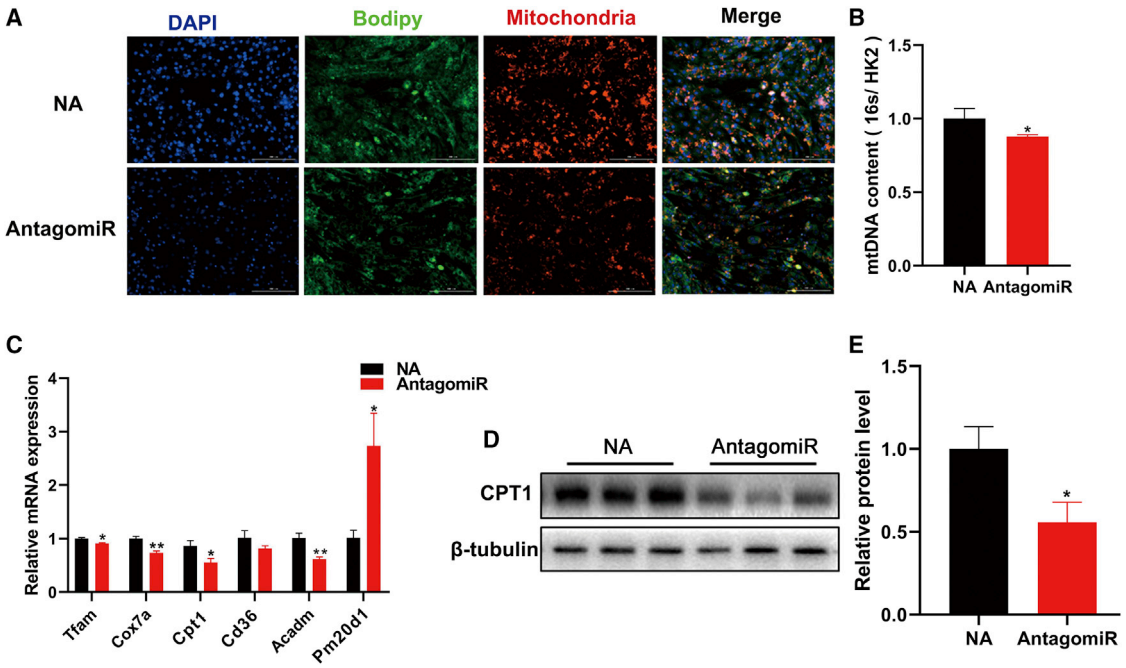

Figure 8. Inhibition of miR-324-5p Suppressed Oleate-Induced β-Oxidation of Mitochondrial Fatty Acids

(A) BODIPY and mitochondrial staining of oleate-treated C2C12 myoblasts after inhibition of miR-324-5p. Scale bars, 300 μm. (B) mtDNA copy number in C2C12 myoblasts transfected with antagomiR-324-5p; total cellular DNA was extracted and quantitative PCR was performed. Relative mtDNA levels were calculated based on the ratio of mtDNA (16S RNA) to the nuclear gene *HK2*. (C) Quantitative real-time PCR was used to measure the expression levels of mitochondrial metabolism-related genes (*Tfam*, *Cox7a*, and *Cpt1*) and fatty acid oxidation-related genes (*CD36* and *Acadm*) after inhibition of miR-324-5p. (D) Western blot analysis of CPT1 after inhibition of miR-324-5p. (E) Quantification of protein levels. The data are expressed as mean ± SD; n = 5. *$p < 0.05$, **$p < 0.01$.

DISCUSSION

Recent studies have found that numerous non-coding RNAs are involved in the regulation of skeletal muscle development, and knockout experiments have shown that miRNAs have important regulatory effects in this process.[13] Long non-coding RNAs, which are highly expressed but show poor sequence conservation, are key regulators of gene expression. They have different modes of action and play an important role in muscle development[27] and can also act as miRNA sponges, reducing their regulatory effect on mRNAs.[28] Several studies have reported that lncGpr19, lncH19, and lncTPT1-AS1 can absorb miR-324-5p, thereby influencing the migration of cancer cells.[29–31] In this study, we found that miR-324-5p targets lncDum and inhibits the differentiation of C2C12 myoblasts. lncDum can promote the differentiation of skeletal muscle satellite cells by inhibiting the expression of *Dppa2*.[25] We overexpressed miR-324-5p and found that the expression level of lncDum was significantly reduced, while that of *Dppa2* and *Oct4* was significantly increased; however, C2C12 differentiation was inhibited. These results indicated that miR-324-5p has a significant effect on C2C12 myoblast differentiation. The regulatory effect of miR-324-5p on C2C12 myoblast differentiation is indirectly regulated through *Oct4*, which is consistent with previous studies.[32] *Dppa2* can regulate the expression of *Oct4*, while OCT4 can inhibit the differentiation of C2C12 myoblasts. In this study, miR-324-5p had higher expression levels in mouse skeletal muscle and early differentiated C2C12 myoblasts, while overexpression of miR-324-5p inhibited C2C12 myogenic differentiation. Studies have shown that miR-324-5p can participate in the regulation of cancer cell proliferation;[33,34] therefore, we speculate that miR-324-5p may also be involved in the proliferation of C2C12 cells, but further research is still needed.

Skeletal muscle is an important organ for energy metabolism in the body.[35] Emerging evidences have indicated that lipid overflow to non-adipose tissues results in muscular fat storage, which is related to insulin resistance.[36] In addition, skeletal muscle fat deposition is also a characteristic of impaired muscle satellite cell function.[37] However, the mechanisms underlying skeletal muscle fat deposition are poorly understood. We analyzed the biological function of miR-324-5p and found that it has a role in fatty acid synthesis, which suggested that miR-324-5p might involved in C2C12 myoblast lipid deposition.

Because oleate can significantly induce lipid deposition in skeletal muscle cells,[11] we investigated the effect of miR-324-5p on oleic acid-induced C2C12 myoblast lipid deposition by overexpressing or inhibiting miR-324-5p. Our results showed that miR-324-5p can significantly promote intracellular lipid deposition in C2C12 myoblasts. Research has shown that miR-324-5p can inhibit lipid metabolism and promote fat deposition by targeting PM20D1,[38] a secreted

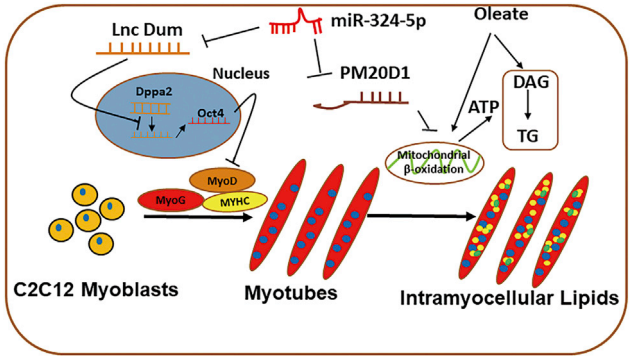

Figure 9. Summary Diagram Showing That miR-324-5p Inhibits C2C12 Myoblast Differentiation and Promotes Oleate-Induced Lipid Deposition in C2C12 Myoblasts

DAG, diacylglycerol; TG, triglyceride; MyoG, myogenin; MyoD, myoblast determination protein; Dppa2, developmental pluripotency associated 2.

enzyme that is enriched in UCP1^{+}, but not UCP1^{-}, adipocytes, and it catalyzes the condensation of fatty acids and specific amino acids to form *N*-acyl amino acids. *N*-acyl amino acid is an endogenous uncoupler of mitochondrial respiration that promotes mitochondrial oxidative metabolism without the production of ATP.[39] miR-324-5p has also been reported to promote apoptosis and oxidative stress by targeting *Mtfr1*,[29] indicating that miR-324-5p is tightly associated with mitochondrial function. As lipid deposition requires mitochondrial-provided ATP,[40] we hypothesize that miR-324-5p promotes oleate-induced lipid deposition in C2C12 myoblasts by improving mitochondrial function. The results of miR-324-5p overexpression and inhibition indicated that miR-324-5p is closely related to mitochondrial metabolism and fatty acid β-oxidation. Overexpression of miR-324-5p markedly increased mitochondrial metabolism and β-oxidation of fatty acids; however, miR-324-5p significantly reduced the expression level of *Pm20d1*. This indicated that miR-324-5p can promote lipid deposition by inhibiting mitochondrial uncoupling and increasing mitochondrial ATP output.

In conclusion, we found that miR-324-5p may inhibit the differentiation of C2C12 myoblasts by targeting lncDum. We also found that miR-324-5p may promote the β-oxidation of mitochondrial fatty acids, perhaps by targeting Pm20d1, thereby promoting oleate-induced lipid deposition in C2C12 myoblasts (Figure 9). Collectively, our data indicated that miR-324-5p inhibits C2C12 myoblast differentiation and promotes intramuscular lipid deposition by lncDUM and PM20D1, respectively.

MATERIALS AND METHODS

Ethics Statement

The protocols used in this study were approved by the Animal Research Ethics Committee of Northwest A&F University (NWAFU-314020038). The animal experiments were performed according to *Guide for the Care and Use of Laboratory Animals of China*.

Experimental Animals

The C57BL/6 male mice, weaned from 4 weeks of age, were purchased from the Medical Laboratory Animal Center of Xi'an Jiaotong University (Xi'an, China; approval XJTULAC-2013-024). Tissues were obtained from 3-month-old mice.

Bioinformatics Screening and miR-324-5p Target Gene Prediction

The C2C12 myoblast differentiation-related miRNA microarray dataset GEO: GSE52410 was initially downloaded from the NCBI GEO portal (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE52410). We systematically analyzed the differentially expressed genes (DEGs) at different time points (0, 2, and 8 days) of C2C12 myoblast differentiation using the R package limma. |Log fold change (FC)| >2 and a p value <0.05 were set as the threshold to screen out the DEGs in C2C12 myoblasts. Heatmap software was applied to generate a heatmap of DEGs. miR-324-5p target gene prediction was performed using starBase software (http://starbase.sysu.edu.cn/agoClipRNA.php?source=lncRNA).

Cell Culture

Mouse C2C12 myoblasts and human HEK293T cells were obtained from ATCC and cultured in DMEM supplemented with 10% fetal bovine serum (Zeta Life, Menlo Park, CA, USA) and 100 IU/mL penicillin-streptomycin at 5% CO_2 and 37°C. For the myogenic differentiation experiment, myoblasts were induced with differentiation medium (DMEM supplemented with 2% horse serum) when cells had reached the contact inhibition stage. Culture medium was changed every 2 days. After 6 days of C2C12 differentiation, when the myotubes were clearly visible, the cells were incubated for 24 h with DMEM containing 0.5% fatty acid-free bovine serum albumin (BSA) and 500 μmol/L oleate (Aladdin, Shanghai, China) for the induction of lipid deposition in myotubes.

Transfection of agomiRNA-324-5p and antagomiRNA-324-5p

C2C12 myoblasts were seeded in 12-well or 6-well plates and transfected with agomiR-324-5p or NC and antagomiR-324-5p or NA (Ribobio, Guangzhou, China) at 100 nmol/L using X-tremeGENE HP DNA transfection reagent (Roche, Mannheim, Germany). The culture medium was changed to fresh medium after 12 h.

Luciferase Reporter Assays

Luciferase reporter plasmids (psiCHECK2) containing the WT or mutant 3′ UTR of lncDum were manufactured by General Biosystems (Tongyong, Anhui, China). HEK293T cells were seeded in a 48-well plate, and Lipofectamine 2000 was used to co-transfect HEK293T cells with the reporter constructs containing either the WT or mutant 3′ UTR, with or without agomiR-324-5p. Luciferase activity in cell lysates was measured using the Dual-Glo luciferase assay system (Promega, Madison, WI, USA).

Quantitative Real-Time PCR

Total RNA was extracted with TRIzol reagent (Takara, Otsu, Japan). mRNA was reverse-transcribed using a reverse transcription kit

(Takara), and the cDNA was analyzed using SYBR Green PCR mix (Vazyme, Nanjing, China) in the Applied Biosystems StepOnePlus system (Thermo Fisher Scientific, USA). Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method. Gene expression was normalized to β-actin. The *U6* small RNA was the internal reference when examining the level of miR-324-5p. The sequences of the primers used are shown in Table S1.

Western Blot Analysis

Total protein was extracted using radioimmunoprecipitation assay (RIPA) buffer (Beyotime, Shanghai, China). A total of 20 μg of protein was separated by SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Boston, MA, USA). Membranes were then incubated overnight with primary antibodies as follows: anti-MYOD (Santa Cruz, Dallas, TX, USA), anti-MYOG (Novus Biologicals, CO, USA), anti-MyHC (Abcam, Cambridge, UK), anti-OCT4 (Santa Cruz), anti-ELOVL6 (Cell Signaling Technology), anti-ACC (Cell Signaling Technology), anti-p-ACC (Cell Signaling Technology), and anti-CPT1 (Santa Cruz Biotechnology). The next day, the membranes were washed three times with Tris-buffered saline containing Tween 20 (TBST). Then, secondary antibody was added and the samples were incubated for 1 h at 4°C with shaking. Secondary antibody was β-actin (Sungene Biotech, Tianjin, China). Protein bands were detected using ChemiDOC XRS+ and the Image Lab system (Bio-Rad, USA)

Immunofluorescence Staining

Cells were fixed in 4% paraformaldehyde for 30 min at room temperature, washed three times with PBS, and permeabilized with 0.5% Triton X-100 for 10 min. After blocking with 2% BSA in PBS for 2 h at room temperature, the cells were washed three times with PBS, and then incubated overnight at 4°C with anti-MyHC antibody. The next day, the cells were washed three times with PBS, 10 min each wash, and subsequently incubated with secondary antibody (Alexa Fluor 594-conjugated mouse immunoglobulin G [IgG] [1:200], #SA00006-3; Proteintech, Rosemont, IL, USA) for 1.5 h at room temperature, and then washed with PBS. Finally, the cells were counterstained with DAPI (Sigma-Aldrich, St. Louis, MO, USA) and viewed on a Nikon TE2000-U fluorescence microscope (Nikon, Tokyo, Japan).

BODIPY and DAPI Staining

Cells were washed three times with PBS and fixed in 4% paraformaldehyde for 30 min. After washing three times with PBS, BODIPY staining was performed for 30 min. Cells were then washed three times (5 min each wash) with PBS, with shaking. Finally, cells were stained with DAPI for 10 min, washed three times, and then imaged under a fluorescence microscope (Nikon).

Oil Red O Staining

After oleate-mediated induction of C2C12 myoblast differentiation for 24 h, the cells were washed twice with PBS, fixed in 4% paraformaldehyde for 30 min, and stained with oil red O for 30 min. The cells were visualized and imaged using a light microscope (Nikon).

Mitochondrial Staining

MitoTracker Red CMXRos (Solarbio, Beijing, China) was added to the cell culture medium, followed by incubation at 37°C for 12–30 min. The culture medium was then discarded and the cells washed three times with PBS. Images were obtained under a fluorescence microscope.

Statistical Analysis

All data were expressed as mean ± SD, and statistical analysis was performed using GraphPad Prism 7.0. (GraphPad, La Jolla, CA, USA). The data were analyzed by the Student's t test. $p < 0.05$ was considered significant, and $p < 0.01$ was considered highly significant ($^{*}p < 0.05$ and $^{**}p < 0.01$).

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.omtn.2020.09.037.

AUTHOR CONTRIBUTIONS

Y.L., J.W., and X.S. designed the research and analyzed the data. Y.L., J.W., X.Z., H.C., X.Z., K.H., X.L., and G.Y. conducted the experiments and collected the data. J.W., Y.L., and X.S. wrote the paper. All authors approved the final version of the manuscript.

CONFLICTS OF INTEREST

The authors declare no competing interests.

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