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MicroRNA-499-5p regulates skeletal myofiber specification via NFATc1/MEF2C pathway and Thrap1/MEF2C axis

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Abstract

Aims: This study aimed to investigate the role of microRNA-499-5p (miR-499-5p) in the regulation of skeletal myofiber specification and its underlying mechanisms.

Main methods: Mouse C2C12 cells were used in this study. Cyclosporin A and siRNA targeting Thrap1 (si-Thrap1) were used to inhibit NFATc1/MEF2C pathway and knockdown Thrap1, respectively. The expressions of miR-499-5p and genes were evaluated by real-time quantitative PCR and western blot analysis.

Key findings: Overexpression of miR-499-5p promoted oxidative fiber gene expression and repressed glycolytic fiber gene expression, affecting several factors associated with fiber specification including NFATc1/MEF2C pathway, PGC-1α, FoxO1 and Wnt5a. Inhibition of NFATc1/MEF2C pathway partly reduced the effect of miR-499-5p overexpression on muscle fiber gene expression. MiR-499-5p targeted *Thrap1* in proliferating and differentiating C2C12 cells. Knockdown of Thrap1 showed a parallel function with miR-499-5p overexpression on muscle fiber gene expression and NFATc1/MEF2C pathway, accompanied by an increase of miR-499-5p level. The effects of miR-499-5p inhibitor on muscle fiber type specific gene expression and NFATc1/MEF2C pathway were effectively reversed by *Thrap1* knockdown.

Significance: MiR-499-5p regulated skeletal myofiber specification and affected several factors associated with fiber specification. MiR-499-5p regulated muscle gene expression partly through NFATc1/MEF2C pathway. We also showed a clue that miR-499-5p regulates skeletal muscle fiber specification in C2C12 cells through targeting *Thrap1*, thereby, promoting NFATc1/MEF2C

pathway and then triggering a series of oxidative muscle fiber gene expression.

Keywords: MiR-499-5p; skeletal muscle; fiber specification; NFATc1/MEF2C pathway; Thrap1

1. Introduction

Skeletal muscle is composed of a mixture of different muscle fiber types, occupying approximately 30–42% of human body weight and exhibiting the contractile or stretchable characteristics. The major contractile protein of skeletal muscle cells is myosin heavy chain (MyHC) [1]. The expression of diverse type of MyHC isoform and muscle fiber type composition in skeletal muscle determine the ability of muscle contraction [2]. Skeletal myofibers are characterized by the expression of particular MyHC isoforms and classified into type I (slow-twitch) and type II (fast-twitch) [2, 3]. Type I (MyHC I) fibers are red, mitochondria-rich and high endurance, depending primarily on mitochondrial oxidative metabolism for ATP production [3, 4]. In contrast, type II (MyHC II) fibers are white, low endurance and contain fewer mitochondria, largely relying on glucose as an energy substrate. Type II fibers are subclassified as type IIa, IIx/d, or IIb based on the type of MyHC isoform expressed [3, 4].

Genetic factors, hormonal signaling and workload largely control the expression of skeletal muscle myosin genes and fiber type composition to meet physiological demands [2, 5]. However, the type of skeletal muscle fiber is directly determined by a wide range of genetic factors. MicroRNAs (miRNAs) are a group of small noncoding RNAs about 22 nucleotides long and involve in the post-transcriptional regulation of gene expression. MiRNAs have been shown to play critical roles in many aspects of muscle function, including satellite cell activity, muscle

development, contractility, energy production and diseases, which occur in fiber types and muscle mass in response to phenotypic changes [6, 7]. A number of miRNAs seem to be expressed in a muscle-specific manner, which regulate the vast majority of muscle activities, especially muscle fiber specification and MyHC expression. MiR-499-5p, highly expressed in cardiac and skeletal muscle and encoded by MyHC7b which is a member of the sarcomeric MyHC family [8], has been identified to be a key regulator of muscle fiber type switching and biomarker of muscle diseases [8-11]. Previous studies reported that miR-499-5p drove a mitochondrial oxidative metabolism program and played an important role in mitochondrial dynamics [6, 12]. The seed region of miR-499-5p overlaps at 6 nt with miR-208b, which was reported to have important roles in muscle performance, and they seem to present a parallel function and play redundant roles in the specification of muscle fiber identity [8]. Van Rooij et al. have proposed that miR-499-5p promoted slow muscle fiber formation and a fast-to-slow-twitch fiber type transition through activating slow and inhibiting fast myofiber gene programs [8]. Several transcriptional repressors including Purβ and Sox6, which have been determined to inhibit β-MyHC expression, were identified as miR-499-5p target genes [8, 10, 13].

In this study, we aimed to investigate the role of miR-499-5p in the regulation of skeletal myofiber specification and its underlying mechanisms. Our findings showed a clue that miR-499-5p regulates skeletal muscle fiber type gene expression in C2C12 cells through targeting *Thrap1*, thereby, promoting NFATc1/MEF2C pathway and then triggering a series of oxidative muscle fiber gene expression. This clue presents a new mechanism that miR-499-5p regulates muscle fiber gene

expression and fiber type specification.

2. Materials and Methods

2.1. Cell culture

C2C12 cells were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were cultured at 37 °C in a 5% CO_2 atmosphere in Dulbecco modified Eagle medium (DMEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen) and antibiotics (100 mg/L streptomycin and 100 U/mL penicillin) (ATCC). After cells reached about 80% confluence, myogenic differentiation was induced by replacing 10% FBS with 2% horse serum (ATCC). Medium was renewed daily during differentiation.

2.2. Cell transfection and treatment

C2C12 cells were transfected with miRNA-499-5p mimics (499M), mimics negative control (MNC), miR-499-5p inhibitor (499I), inhibitor negative control (INC), siRNA negative control (siNC) or siRNA targeting Thrap1 (si-Thrap1) (GenePharma, Shanghai, China) using Lipofectamine 3000 (Invitrogen) according to the manual of the manufacture. The si-Thrap1 was designed and synthesized by GenePharm (Shanghai, China). The sense strand of the Thrap1 siRNA was 5′-CCUCAUCCUUUCAGAAUCATT-3′ and the antisense strand was 5′-UGAUUCUGAAAGGAUGAGGTT-3′. The sense strand of the negative control siRNA was 5′-UUCUCCGAACGUGUCACGUTT-3′ and the antisense strand was 5′-ACGUGACACGUUCGGAGAATT-3′. We treated C2C12 cells with 5 μM cyclosporin A (CsA) (Amresco, USA) resolved in DMSO to inhibit NFAT signaling.

2.3. Real-time quantitative PCR

The mRNA level of *Thrap1* was quantified using real-time quantitative PCR (RT-qPCR) analysis. Briefly, Total RNA was isolated from C2C12 cells using RNAiso Plus reagent (TaKaRa,

Dalian, China). cDNA was synthesized using a PrimeScript® RT reagent Kit with gDNA Eraser (TaKaRa) following the manufacturer's recommendations. RT-qPCR was performed in triplicate using SYBR select Master Mix (Applied Biosystems, Foster, CA, USA) on a 7900HT Real-time PCR system (384-cell standard block) (Applied Biosystems). β-actin was used as an internal control. For detection of the miRNA-499-5p level, the cDNA template was prepared using TaqMan® miRNA Reverse Transcription Kit (Applied Biosystems) and TaqMan® MicroRNA Assay kit (Applied Biosystems) was used to examine the expression of mature miR-499-5p. U6 snRNA (Applied Biosystems) was used as an internal control.

2.4. Western blot analysis

Protein samples were extracted using lysis buffer (Beyotime, Shanghai, China) according to the manufacturer's recommendations. Western blot analysis was performed as before [14]. Briefly, proteins (25 μg/lane) were resolved by electrophoresis on 10% SDS-polyacrylamide gels and then transferred to a nitrocellulose membrane. After blocking with Tris-buffered saline Tween-20 (TBST; 0.14 mol/L NaCl, 0.02 mol/L Tris base, and 0.05% Tween) containing 5% BSA for 1 h at room temperature, the membrane was incubated with diverse primary antibodies overnight at 4 °C, followed by the corresponding secondary antibody (Cell Signaling Technology, Danvers, MA, USA). The primary antibodies used were: anti-β-actin (Santa Cruz, Cat. No. sc-1616); anti-MyHC I (DSHB, Cat. No. BA-F8); anti-MyHC IIa (DSHB, Cat. No. SC-71); anti-MyHC IIx (Sigma, Cat. SAB2104768); anti-MyHC IIb (DSHB, Cat. No. 10F5); anti-Tnni1 (Santa Cruz, Cat. No. SC-393330); anti-Tnni2 (Santa Cruz, Cat. No. SC-374549); anti-Myoglobin (Santa Cruz, Cat. No. SC-25607); anti-NFATc1 (Cell Signaling Technology, Cat. No. 8032); anti-MEF2C (Cell Signaling Technology, Cat. No. 5030); anti-MCIP1.4 (Santa Cruz, Cat. No. sc-377507); anti-PGC-1α (Cell Signaling Technology, Cat. No. 2178); anti-FoxO1 (Cell Signaling Technology, Cat. No. 2880);

anti-phospho-FoxO1 (P-FoxO1) (Cell Signaling Technology, Cat. No. 9461); anti-Wnt5a (Cell Signaling Technology, Cat. No. 2530); anti-AMPK (Cell Signaling Technology, Cat. No. 5831); anti-phospho-AMPK (P-AMPK) (Cell Signaling Technology, Cat. No. 2535) and anti-Thrap1 (Santa Cruz, Cat. No. SC5369). The primary antibodies from Cell Signaling Technology, DSHB, Sigma, and Santa Cruz were used at 1:1000, 1:500, 1:500 and 1:100 dilution, respectively. The secondary antibody was used at 1:5000 dilution. The membrane was visualized using a ClarityTM Western ECL Substrate (Bio-Rad, Hercules, CA, USA) and a ChemiDoc XRS Imager System (Bio-Rad). β-actin protein was used as a control for equal protein loading.

2.5. *Statistical analysis*

Data expressed as mean ± SE (standard error) were analyzed using SAS 8.2 software (SAS Inst. Inc., NC). Student's t test was performed to assess the statistical significance between groups. In all analyses, $p < 0.05$ was considered to be statistical significance.

3. Result

3.1. *MiR-499-5p regulates muscle fiber type specific gene expression in C2C12 myotubes*

To confirm the role of miR-499-5p in skeletal muscle fiber type specific gene expression, we performed both overexpression and loss-of-function studies for miR-499-5p in C2C12 myotubes. MiR-499-5p mimics (499M) were used to overexpress miR-499-5p and miR-499-5p inhibitor (499I) was used to knock down. The cells transfected with miRNA mimics negative control (MNC) or miRNA inhibitor negative control (INC) were used as control. The protein level of individual MyHC isoforms (type I, MyHC-I/β-MyHC; type II, MyHC-IIa, MyHC-IIx/d, and MyHC-IIb) as well as myoglobin and troponin I (Tnni) were evaluated in C2C12 cells transfected 499M or 499I. MyHC I, myoglobin and Tnni1 are typical markers of slow fiber genes, while MyHC II and Tnni2 are fast type genes. As shown in Fig. 1A, the expression levels of miR-499-

5p in C2C12 cells transfected with 499M and 499I were significantly higher and lower, respectively, than that of controls. Overexpression of miR-499-5p led to increases in MyHC I (Fig. 1B), MyHC IIa (Fig. 1D), as well as myoglobin (Fig. 1F) and Tnni1 (Fig. 1E), and decreases in MyHC IIx (Fig. 1D), MyHC IIb (Fig. 1B) and Tnni2 (Fig. 1E). Consistently, inhibition of miR-499-5p resulted in decreases of MyHC I (Fig. 1C), MyHC IIa (Fig. 1D), as well as myoglobin (Fig. 1F) and Tnni1 (Fig. 1E), and increases in MyHC IIx (Fig. 1D), MyHC IIb (Fig. 1C) and Tnni2 (Fig. 1E).

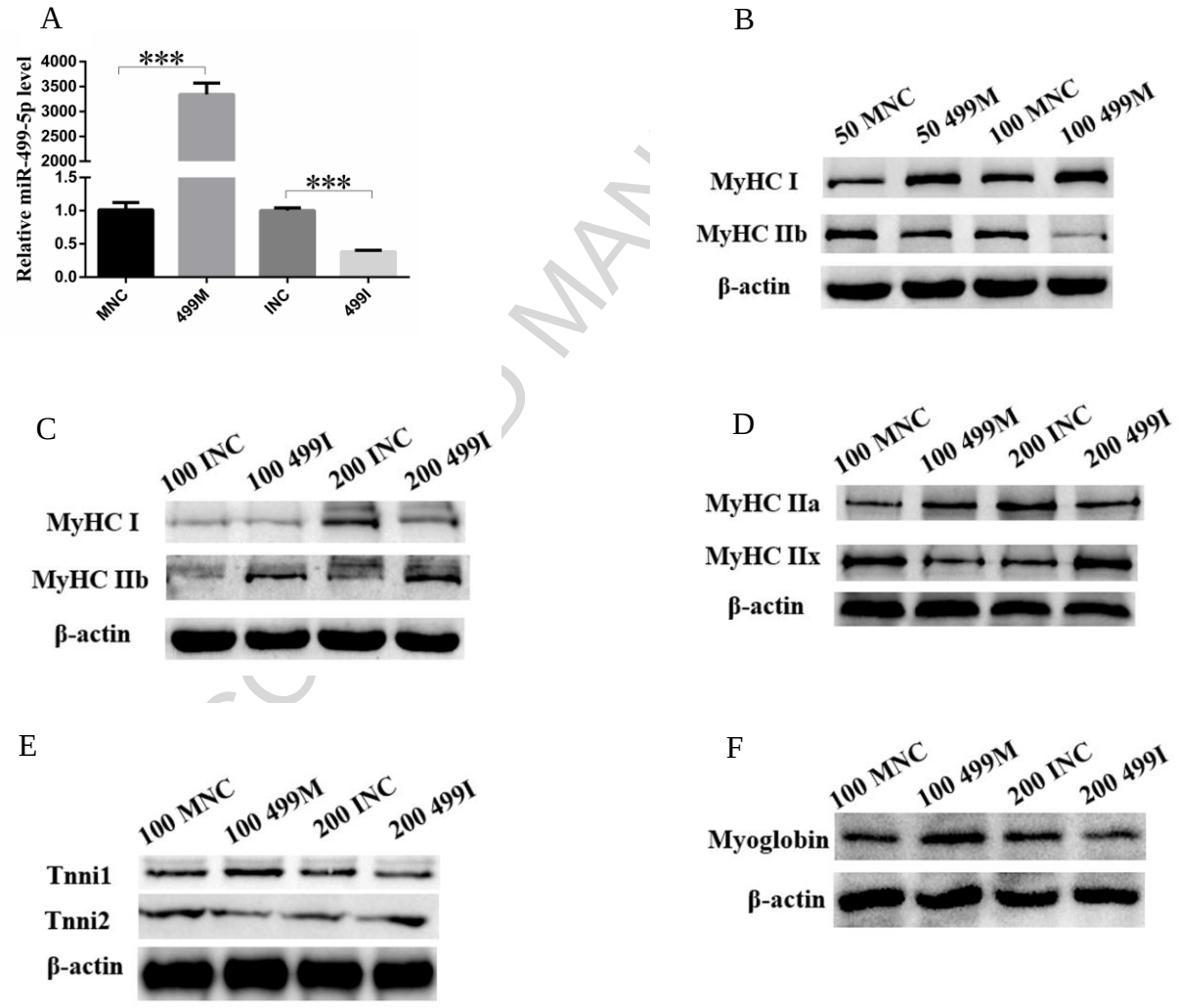


Figure 1. MiR-499-5p regulates muscle fiber gene expression. (A) To measure the transfection efficiency, the expression of miR-499-5p was determined by RT-qPCR normalized to U6 small nuclear

RNA. Cells were transfected with 100 nM of 499M, 100 nM of MNC, 200 nM of 499I or 200 nM of INC. (B) and (C) Effect of miR-499-5p on MyHC I and IIb expression. Cells were transfected with 50 nM, 100 nM of 499M and 100 nM, 200 nM of 499I, respectively. (D), (E) and (F) Effect of miR-499-5p on the expression of MyHC IIa, IIx and Tnni1, Tnni2 as well as myoglobin. Cells were transfected with 100 nM of 499M and 200 nM of 499I. C2C12 cells were transfected with 499M or 499I when they reached about 80% confluence and induced to differentiate for 3 days before analysis. β-actin served as the loading control. Data were presented as means ± SE (n=3). ***$p < 0.001$ as compared with control.

3.2. Effect of miR-499-5p on muscle fiber specification associated factors

To explore the underlying mechanism by which miR-499-5p regulates muscle fiber type specific gene expression, we performed western blotting to analyze the protein expression of certain signaling factors that have been reported to relate to muscle fiber specification. NFATc1 (nuclear factor of activated T cells, cytoplasmic 1, dephosphorylated in the nuclear), a known nuclear factor that promotes slow fiber type specific gene expression and potentially represses fast fiber type gene expression, is a cofactor of MEF2 (myocyte enhancer factor-2) isoforms and can interact with MEF2 depending on the promoter context in slow fiber gene expression [2, 15]. MEF2C transcription factor is selectively active in slow oxidative fibers and has been shown to promote the formation of oxidative muscle fiber [16, 17]. Modulatory calcineurin interacting protein 1 exon 4 isoform (MCIP1.4), a direct downstream target of the NFAT signaling pathway [18, 19], was reported to increase the number of slow muscle fibers and identified as oxidative muscle fiber genes [2, 20]. In this study, we found C2C12 myotubes transfected with 499M showed higher protein level of NFATc1, MEF2C and MCIP1.4 compared to cells transfected with MNC, whereas inhibition of miR-499-5p repressed NFATc1, MEF2C and MCIP1.4 protein expression (Fig. 2A). Peroxisome proliferator activated receptor-γ coactivator-1α (PGC-1α), a

nuclear receptor cofactor that activates expression of type I fiber-specific genes and drives the formation of type I fibers [21, 22], was shown to be upregulated in cells overexpressing miR-499-5p while it was reduced by inhibiting miR-499-5p (Fig. 2B). FoxO1, a member of the forkhead transcription factor forkhead box protein O (FoxO) family, was established as a negative regulator for type I fiber gene expression [16, 23]. Although FoxO1 protein expression was not changed by miR-499-5p overexpression, the P-FoxO1 level was pronounced increased (Fig. 2C), indicating miR-499-5p may inhibit the FoxO1 activity through phosphorylation. We also found the protein expression of Wnt5a, which has the potential to repress the fiber I formation [24, 25], was reduced and increased by miR-499-5p overexpression and inhibition, respectively (Fig. 2D). Moreover, another muscle fiber specification associated factor [2, 26], AMP activated protein kinase (AMPK) and its phosphorylated modality (P-AMPK), were not affected by miR-499-5p overexpression or inhibition.

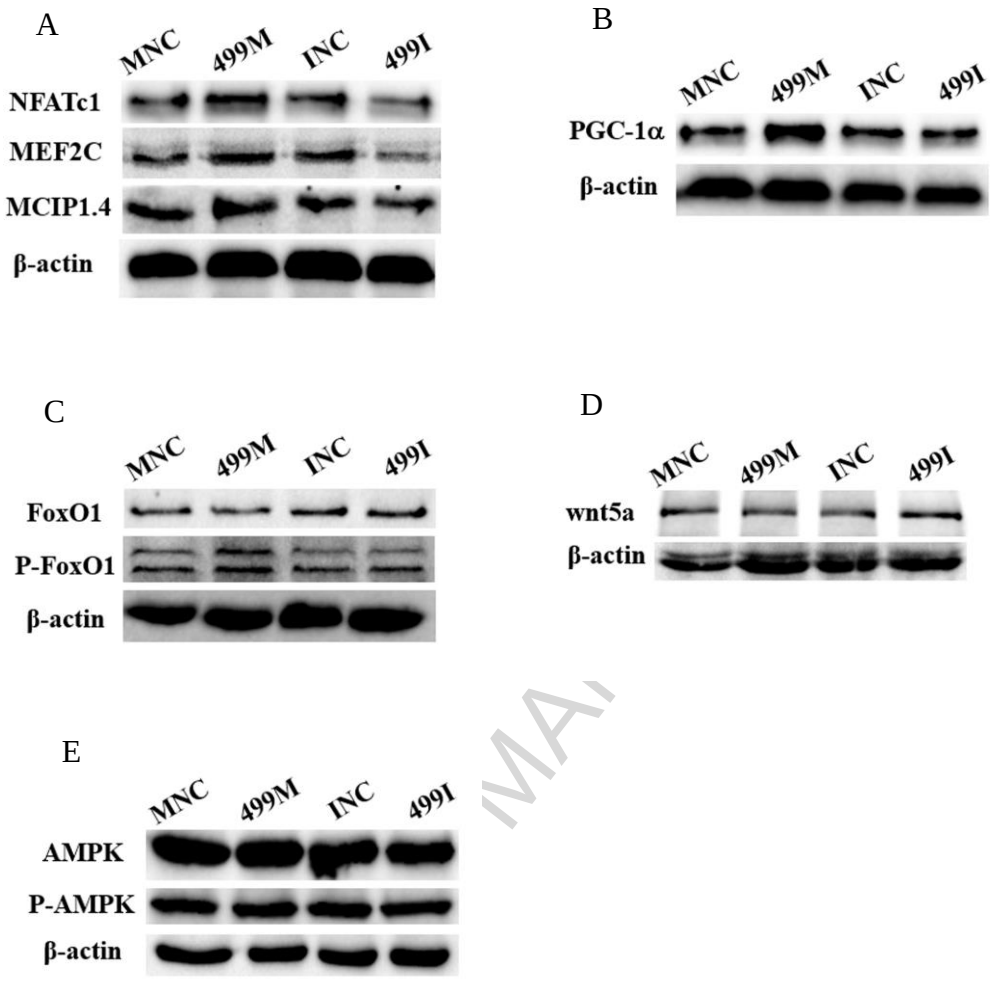


Figure 2. Effect of miR-499-5p on muscle fiber specification associated factors. (A) Effect of miR-499-5p on NFATc1/MEF2C pathway. (B-E) Effect of miR-499-5p on PGC-1α (B), FoxO1 and P-FoxO1 (C), Wnt5a (D), AMPK and P-AMPK (E). Cells were transfected with 100 nM of 499M and 200 nM of 499I, when they reached about 80% confluence and induced to differentiate for 3 days before analysis. β-actin served as the loading control.

3.3. MiR-499-5p mediates muscle fiber type specific gene expression through NFATc1/MEF2C pathway

The data (Fig. 2A) showed miR-499-5p affected NFATc1/MEF2C pathway in C2C12 myotubes. To confirm whether miR-499-5p regulates the expression of muscle fiber genes via NFATc1/MEF2C pathway, cells were treated with the calcineurin inhibitor cyclosporin A (CsA),

which is a well-known inhibitor of calcineurin and inhibits NFAT activity by blocking its dephosphorylation [27, 28], for 1 hour before transfection with 499M for 3 days in differentiation medium. As shown in Fig. 3A, the expression level of miR-499-5p in C2C12 myotubes transfected with 499M was significantly overexpressed than that of the control. CsA treatment remarkably reduced NFATc1 and MEF2C protein expression, and downregulated the miR-499-5p-induced increase of NFATc1 and MEF2C protein level (Fig. 3B). Consistent with the effect of CsA on miR-499-5p-induced promotion of NFATc1 and MEF2C protein level, CsA significantly eliminated the increase of MyHC I and MyHC IIa expression induced by miR-499-5p overexpression (Fig. 3C). In addition, CsA also partly rescued miR-499-5p overexpression-induced decrease of MyHC IIx and MyHC IIb expression (Fig. 3C). Moreover, the promotion and repression of miR-499-5p overexpression on Tnni1 and Tnni2, respectively, were consistently abolished by CsA treatment (Fig. 3D). Taken together, these results showed that miR-499-5p-mediated muscle fiber type specific gene expression is, at least partly, dependent on NFATc1/MEF2C pathway.

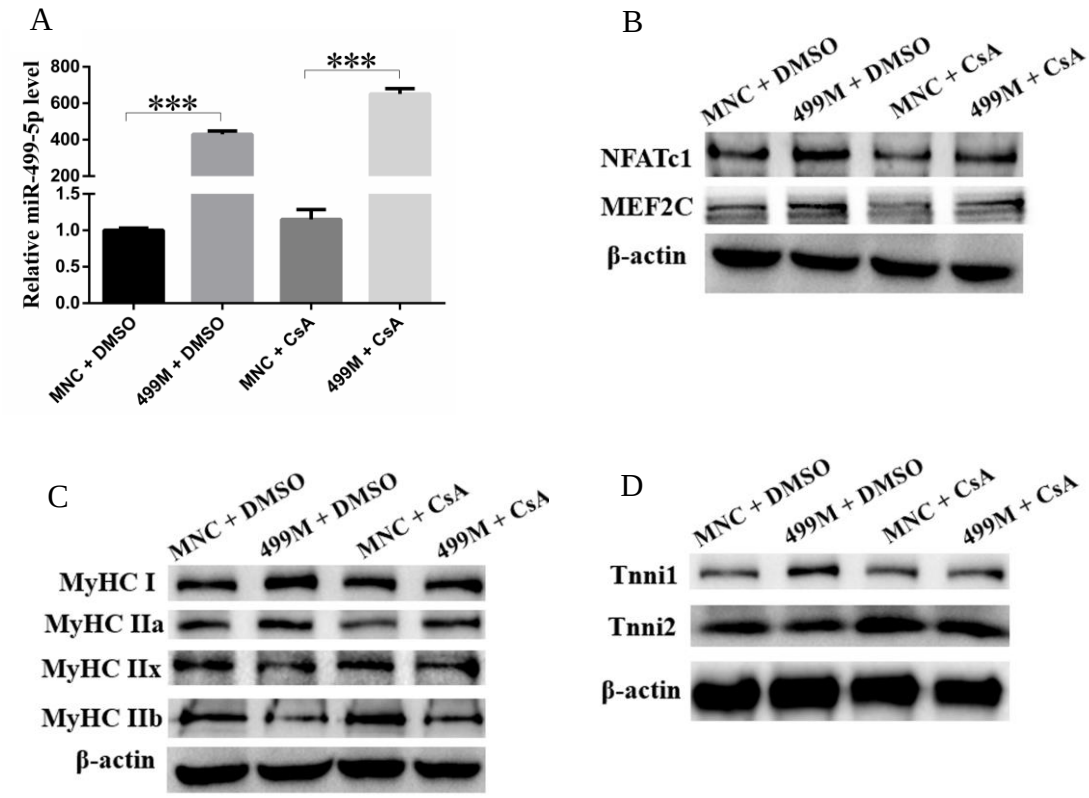


Figure 3. MiR-499-5p regulates muscle fiber type specific gene expression through NFATc1/MEF2C pathway. (A) The expression level of miR-499-5p in C2C12 cells transfected with 499M. MiR-499-5p was determined by RT-qPCR normalized to U6 small nuclear RNA. (B) CsA suppressed miR-499-5p overexpression-induced upregulation of NFATc/MEF2C pathway. (C) and (D) CsA partly reduced the effect of miR-499-5p on MyHCs (C) and Tnni (D) expression. C2C12 cells were treated with 5 μM CsA for 1 hour before transfection with 100 nM of 499M, when they reached about 80% confluence and induced to differentiate for 3 days before analysis. β-actin served as the loading control. Data were presented as means ± SE (n=3). ***$p < 0.001$ as compared with control.

3.4. Thrap1 is a target gene of miR-499-5p in proliferating and differentiating C2C12 cells

Thyroid hormone receptor associated protein-1 (Thrap1), also known as TRAP240 or Med13, a component of the TR-associated TRAP complex, modulates activity of the TR (thyroid hormone receptor) and has been shown to repress β-MHC gene expression in heart [8, 29-31]. Previous

researches have reported that miR-499-5p targeted the 3' UTR of mouse *Thrap1* [8, 29, 32]. To conform whether miR-499-5p targets *Thrap1* in proliferating and differentiating C2C12 cells, we performed overexpression and inhibition of miR-499-5p to evaluate Thrap1 mRNA and protein level. First, when myoblasts were about 50 - 60% confluence in proliferation medium, 499M and 499I were transfected for 1 day before analysis. As shown in Fig. 4A, cells transfected with 499M and 499I showed significantly higher and lower miR-499-5p expression compared to the controls, respectively. Transfection with 499M remarkably reduced both Thrap1 mRNA (Fig. 4B) and protein expression (Fig. 4C) in proliferating myoblasts, however, 499I pronouncedly upregulated Thrap1 mRNA (Fig. 4B) and protein level (Fig. 4D). Moreover, in order to evaluate whether miR-499-5p targets Thrap1 in C2C12 myoblast differentiation, we performed overexpression and inhibition of miR-499-5p for 3 days during myoblast differentiation. Consistent with the result from proliferating myoblasts, Thrap1 mRNA (Fig. 4E) and protein level (Fig. 4F) were significantly decreased and increased by miR-499-5p overexpression and inhibition, respectively, in differentiating myoblasts. Taken together, these data showed that *Thrap1* is a direct target gene of miR-499-5p not only in proliferating but also in differentiating C2C12 myoblasts.

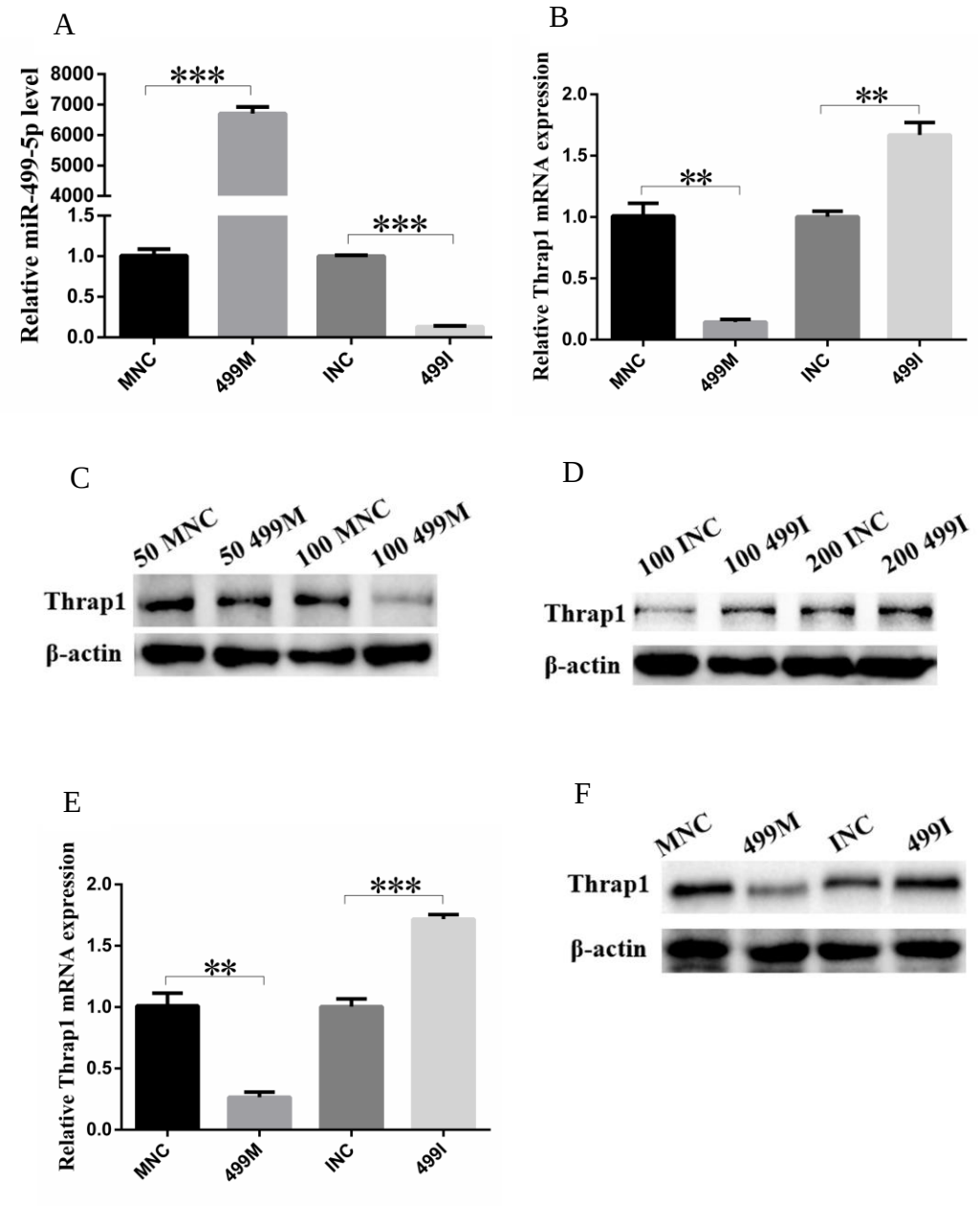


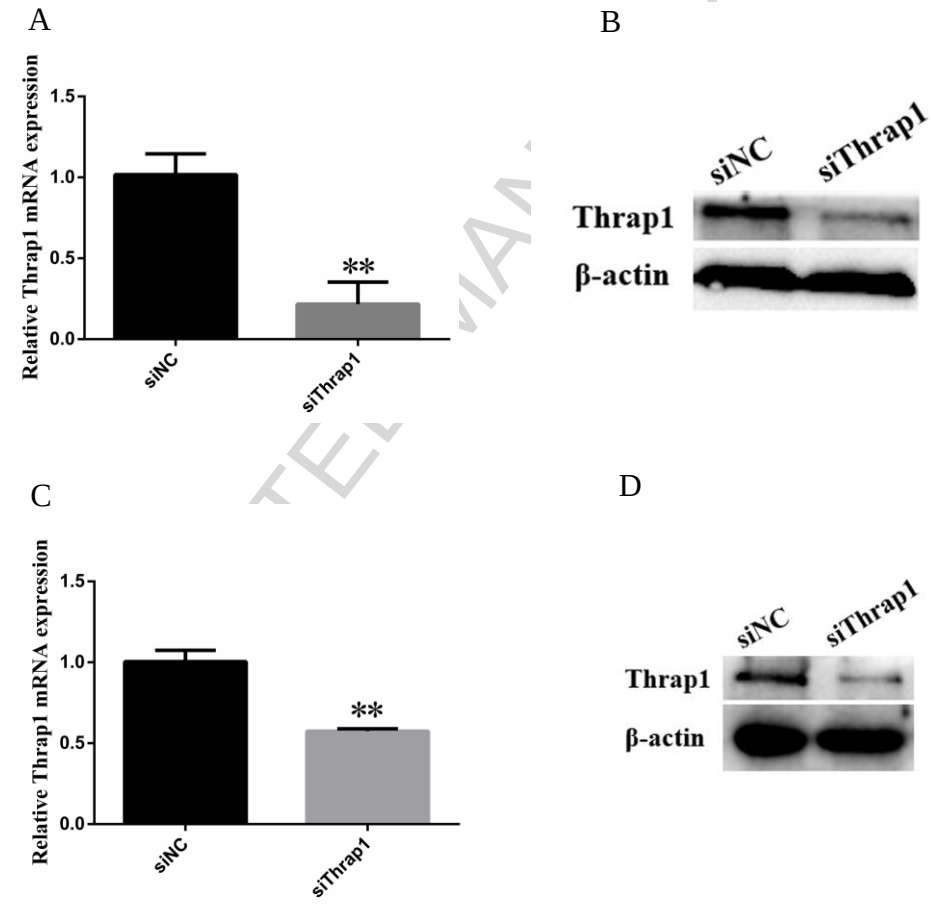
Figure 4. MiR-499-5p targets Thrap1 in proliferating and differentiating C2C12 cells. (A) The expression level of miR-499-5p in proliferating C2C12 cells transfected with 100 nM of 499M and 200 nM of 499I. (B) The mRNA level of *Thrap1* in proliferating C2C12 cells transfected with 100 nM of 499M and 200 nM of 499I. (C) and (D) The protein level of Thrap1 in proliferating C2C12 cells

transfected with 50 nM, 100 nM of 499M (C) and 100 nM, 200 nM of 499I (D). In figure A-D, when myoblasts were about 50 - 60% confluence in proliferation medium, 499M and 499I were transfected for 1 day before analysis. (E) and (F) The mRNA and protein level of Thrap1 in differentiating C2C12 cells transfected with 100 nM of 499M and 200 nM of 499I. In figure E and F, C2C12 cells were transfected with 499M and 499I, when they reached about 80% confluence and induced to differentiate for 3 days before analysis. MiR-499-5p and mRNA level of *Thrap1* were determined by RT-qPCR normalized to U6 small nuclear RNA and *β-actin*, respectively. Data were presented as means ± SE (n=3). $^{**}p < 0.01$, and $^{***}p < 0.001$ as compared with control.

3.5. Knockdown of Thrap1 by siRNA promotes oxidative fiber gene and represses glycolytic fiber gene expression, accompanied by an increase of miR-499-5p level

To evaluate the role of Thrap1 in fiber type specification, specific siRNA for Thrap1 (si-Thrap1) was transfected into C2C12 cells. Firstly, to determine whether the si-Thrap1 sequence could target Thrap1 specifically in both proliferating and differentiating C2C12 myoblast, we successfully screened the most effective si-Thrap1 and performed transfection with si-Thrap1 into proliferating and differentiating C2C12 cells, respectively. RT-qPCR and western blotting analyses showed that the mRNA and protein expression of Thrap1 were pronounced reduced not only in proliferating (Fig. 5A, B) but also in differentiating (Fig. 5C, D) C2C12 cells by si-Thrap1 transfection. Then, the expression of muscle fiber type specific genes were analyzed by western blotting. Consistent with the results from miR-499-5p overexpression, the results showed that the protein expressions of MyHC I and MyHC IIa in cells transfected with si-Thrap1 were higher than

that of control, whereas MyHC IIb protein was reduced, and there was no change in MyHC IIx expression (Fig. 5E). Moreover, knockdown of Thrap1 repressed Tnni2 protein level and had no effect on Tnni1 protein (Fig. 5F) but promoted another slow fiber marker myoglobin expression (Fig. 5G). In addition, knockdown of Thrap1 increased the level of miR-499-5p (Fig. 5H), suggesting that Thrap1 took part in regulating skeletal muscle fiber gene expression and miR-499-5p level.



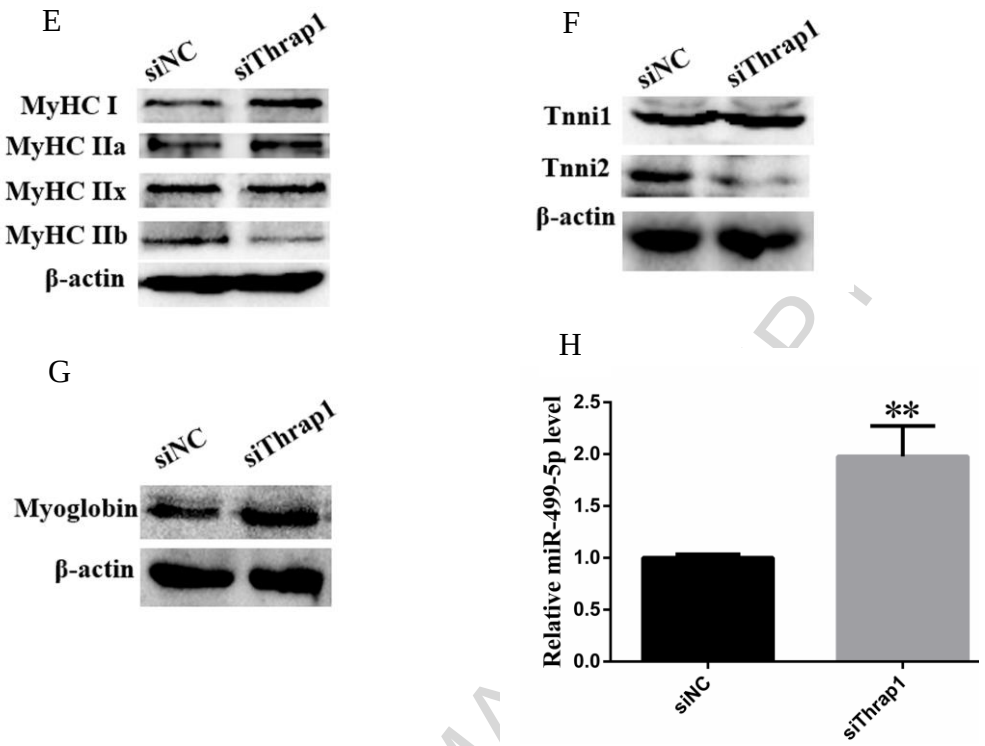
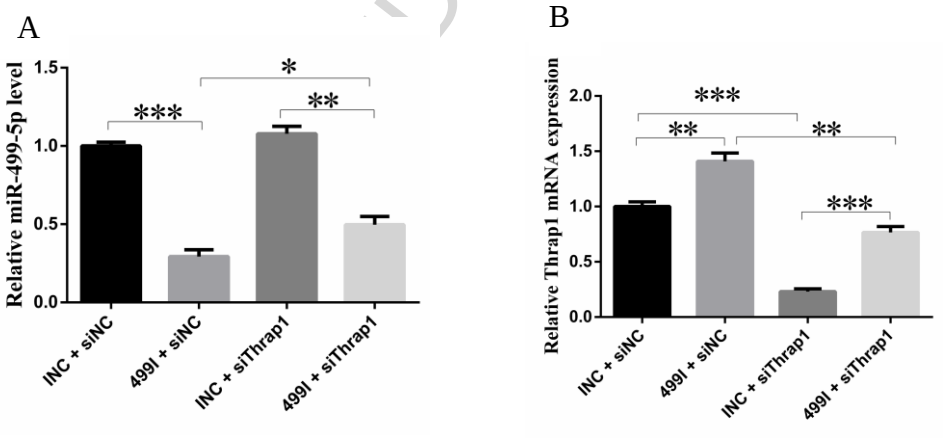


Figure 5. Si-Thrap1 promotes oxidative fiber gene and represses glycolytic fiber gene expression. (A) and (B) The designed sequence of si-Thrap1 inhibited the mRNA (A) and protein (B) level of Thrap1 in proliferating C2C12 cells. (C) and (D) The designed sequence of si-Thrap1 inhibited the mRNA (C) and protein (D) level of Thrap1 in differentiating C2C12 cells. (E-G) Effect of si-Thrap1 on protein expression of MyHCs (E), Tnni (F) and myoglobin (G). (H) Knockdown of Thrap1 increased the level of miR-499-5p. C2C12 cells were transfected with 200 nM of si-Thrap1 and the transfection was performed as described in Figure 4. The mRNA level of *Thrap1* were determined by RT-qPCR normalized to *β-actin*. Data were presented as means ± SE (n=3). $^{**}p < 0.01$ as compared with control.

3.6. Si-Thrap1 decreases the effect of miR-499-5p inhibitor on muscle fiber type specific gene expression

We examined whether knockdown of Thrap1 could inhibit the effect of miR-499-5p inhibitor

on muscle fiber type specific gene expression. MiR-499-5p inhibitor and si-Thrap1 were co-transfected into C2C12 cells. Cells transfected with 499I showed significant decrease of miR-499-5p level. RT-qPCR and western blotting analysis conformed that transfection of 499I led to downregulation of miR-499-5p (Fig. 6A) and upregulation of Thrap1 (Fig. 6B and C). Moreover, downregulation of Thrap1 expression by si-Thrap1 could be rescued by transfection with 499I (Fig. 6B and C). Western blotting analysis showed transfection with 499I pronouncedly repressed MyHC I and MyHC IIa and promoted MyHC IIx and MyHC IIb expression, whereas co-transfection with si-Thrap1 reduced the effect of miR-499-5p inhibitor on MyHCs expression (Fig. 6D). In addition, the effects of miR-499-5p inhibitor on Tnni were also inhibited by si-Thrap1 co-transfection (Fig. 6E). Taken together, these data suggested that miR-499-5p may regulate muscle fiber gene expression at least partly through Thrap1.



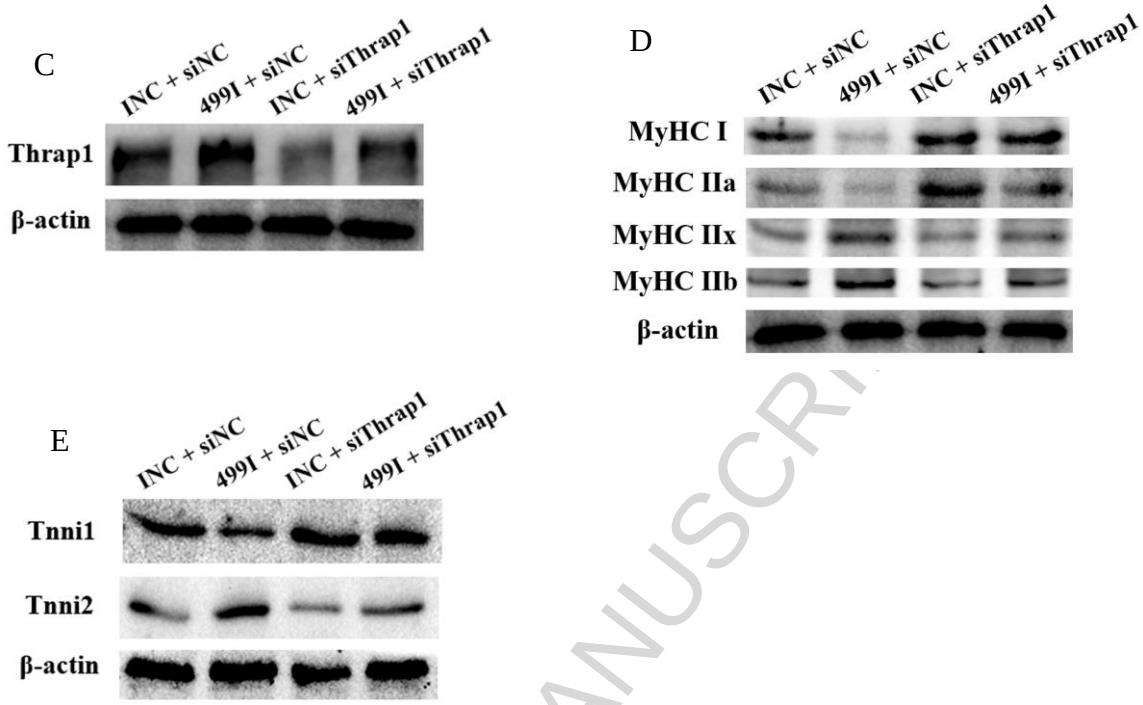


Figure 6. Si-Thrap1 decreases the effect of miR-499-5p inhibitor on muscle fiber type specific gene expression. (A) The expression level of miR-499-5p. (B) and (C) The decrease of mRNA (B) and protein (C) level of Thrap1 expression by si-Thrap1 could be partly rescued by transfection with miR-499-5p inhibitor. (D) and (E) The effect of miR-499-5p inhibitor on MyHCs (D) and Tnni (E) were partly suppressed by si-Thrap1. C2C12 cells were transfected with 200 nM of 499I and 200 nM of si-Thrap1 when they reached about 80% confluence and induced to differentiate for 3 days before analysis. Data were presented as means ± SE (n=3). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ as compared with control.

3.7. MiR-499-5p may regulate NFATc1 and MEF2C expression via Thrap1

In consideration of the effect of miR-499-5p on NFATc1/MEF2C pathway in C2C12 myotubes in this study and the inhibitory effect of Thrap1 on MEF2 in heart studied by Paulin et

al. [33] and in skeletal muscle studied by Amoasii et al. [15], we hypothesized that, as a target gene of miR-499-5p, Thrap1 may provide an important link to miR-499-5p regulating the NFATc1 and MEF2C expression and skeletal muscle fiber gene expression. To substantiate this, western blotting analysis was performed and showed that the expression level of NFATc1 and MEF2C in cells transfected with si-Thrap1 were significantly higher than that of control (Fig.7A). To conform whether miR-499-5p regulated NFATc1 and MEF2C through Thrap1, miR-499-5p inhibitor and si-Thrap1 were co-transfected into C2C12 cells. The results showed that attenuations of NFATc1 and MEF2C expression level induced by miR-499-5p inhibition were effectively reversed by si-Thrap1 (Fig.7B). These data suggested that miR-499-5p may regulate NFATc1 and MEF2C expression through Thrap1.

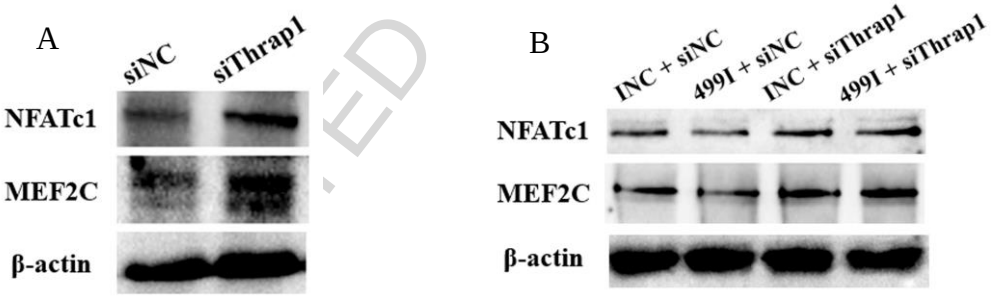


Figure 7. MiR-499-5p may regulate NFATc1 and MEF2C expression via Thrap1. (A) Si-Thrap1 repressed the expression of NFATc1 and MEF2C. (B) The attenuations of NFATc1 and MEF2C expression level induced by miR-499-5p inhibition were effectively reversed by si-Thrap1. The cell transfection was performed as described in Figure 6.

4. Discussion

Delineation of the mechanisms involved in the coordinate regulation of muscle fiber

composition and structural programs during muscle development has implications for new therapeutic approaches for many muscle diseases, including muscular dystrophy and metabolic disorders. MiR-499-5p has been identified as a positive regulator of slow muscle fiber formation and a negative regulator of fast muscle fiber formation *in vivo* [8-10], through targeting several transcriptional repressors that have been demonstrated to inhibit β-MyHC expression, including Purβ and Sox6 [8, 10, 13]. Recent reports have identified that miR-499-5p targets the 3'UTR of *Thrap1* [8, 29, 32] and Thrap1 suppresses MEF2 to regulate glucose uptake and metabolism in skeletal muscle [15] and right ventricular function in heart [33]. Herein, we show a clue that miR-499-5p regulates skeletal muscle fiber type gene expression through NFATc1/MEF2C pathway and Thrap1-MEF2C axis in C2C12 cells. Specifically, miR-499-5p targets *Thrap1* in differentiated C2C12 cells, increasing the NFATc1 and MEF2C protein expression, thereby, triggering a series of oxidative muscle fiber gene expression (Fig. 8). This miR-499-5p/Thrap1/MEF2C clue presents a new mechanism that miR-499-5p regulates muscle fiber type specification and switching.

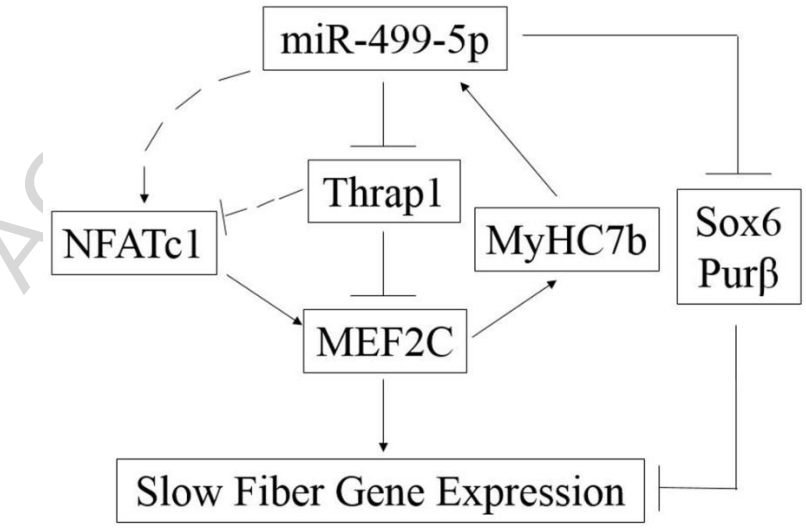


Figure 8. A proposed model of the mechanisms by which miR-499-5p regulates skeletal muscle fiber specification. In skeletal myocytes, miR-499-5p targets Purβ and Sox6 to control the muscle fiber gene expression. In this study, we found miR-499-5p targets Thrap1 in myogenesis, which suppresses the expression of MEF2C. MEF2C can active the transcriptional activation of slow fiber genes and increase MyHC7b expression, the host gene of miR-499-5p, thereby promoting miR-499-5p level. In addition, miR-499-5p and Thrap1 may control MEF2C through NFATc1 signaling. Taken together, miR-499-5p may regulate muscle fiber gene expression via NFATc1/MEF2C pathway and Thrap1-MEF2C axis. This clue composed of miR-499-5p/Thrap1/MEF2C may be a part of regulatory network involved in the regulation of skeletal muscle fiber type.

Skeletal muscle is composed of different types of muscle fibers that express distinct sets of metabolic enzymes and structural proteins. It is normally divided into four different types based on four different specific MyHC isoforms [3]. In this study, we found miR-499-5p promoted the oxidative muscle fiber gene expression and inhibited glycolic muscle fiber gene expression in C2C12 cells, accompanied by influencing several factors associated with muscle fiber specification, including NFATc1/MEF2C pathway, PGC-1α, FoxO1 and Wnt5a, each of which was reported to involve in different type of muscle fiber formation and transition. Specifically, miR-499-5p promoted the expression of NFATc1/MEF2C pathway and PGC-1α, which functioned as positive regulators of oxidative muscle fiber formation [2, 21, 28], and suppressed Wnt5a which has the potential to repress the type I fiber formation [24, 25]. Although FoxO1, a negative regulator for type I fiber gene expression [16, 23], was not changed by miR-499-5p overexpression,

the P-FoxO1 that disabled the inhibitory function to slow gene was pronouncedly increased, suggesting miR-499-5p may inhibit the FoxO1 activity through phosphorylation. MiR-499-5p did not change the AMPK or its phosphorylated modality level in C2C12 cells, which has been reported as a key regulator of myocyte energy metabolism and muscle mitochondrial oxidative metabolism and has been demonstrated to involve in regulation of exercise-induced fiber type transformation and mitochondrial biogenesis in skeletal muscle [34-36]. This data was not consistent with the report of Liu et al. that phosphorylated AMPK level was significantly increased in gastrocnemius muscle of miR-499-5p transgenic mice [6]. That may be due to difference between experiment model *in vivo* and *in vitro*. Taken together, our data suggested that miR-499-5p may regulate muscle fiber type specification through a series of muscle fiber specification associated factors.

NFATc1, a known nuclear factor that promotes slow fiber type-specific gene expression and potentially represses fast fiber type gene expression, is a cofactor of MEF2 isoforms and can interact with MEF2 depending on the promoter context in slow fiber gene expression [2]. MEF2C transcription factor is selectively active in slow oxidative fibers and has been shown to promote the formation of oxidative muscle fiber [16, 17]. Our previous study reported that miR-139-5p suppressed MyHC I and MyHC IIa expression via inhibition of NFATc1/MEF2C pathway in C2C12 cells [37]. In the present study, we found that overexpression of miR-499-5p activated NFATc1/MEF2C pathway and miR-499-5p may regulate MyHC isoforms expression through NFATc1/MEF2C pathway by treatment with CsA in C2C12 cells, which can inhibit NFATc1

activity by blocking its dephosphorylation [27, 28].

Previous studies reported that 3' UTR of mouse *Thrap1* is a target of miR-499-5p [8, 29, 32]. Thrap1 is a component of the TR-associated TRAP complex and modulates activity of the TR and has been shown to repress β-MHC gene expression in cardiac muscle [8, 29]. Here, we found miR-499-5p targeted Thrap1 both in proliferating and in differentiating C2C12 cells, suggesting that Thrap1 may be involved in miR-499-5p regulation of skeletal myogenesis and diverse fiber formation. Amoasii and the cooperators reported that Thrap1 has an important role in systemic glucose homeostasis in skeletal muscle [15]. Thrap1 suppressed the expression of genes involved in glucose uptake and metabolism in skeletal muscle via inhibiting MEF2 [15]. Taken together, we hypothesized miR-499-5p may regulate skeletal myofiber specification through Thrap1-MEF2C axis in C2C12 cells. In fact, our data showed that overexpression of miR-499-5p promoted oxidative fiber genes including MyHC I, MyHC IIa, Tnni1 and myoglobin expression, whereas it suppressed glycolytic fiber genes including MyHC IIx, MyHC IIb and Tnni2, accompanied with an increase of NFATc1/MEF2C signaling and a decrease of Thrap1 expression. We also found that knockdown of Thrap1 by siRNA promoted oxidative fiber gene and repressed glycolytic fiber gene expression, accompanied by upregulation of NFATc1/MEF2C pathway and an increase of miR-499-5p level, exhibiting a parallel result with miR-499-5p overexpression. Notably, MEF2 has been reported to promote expression of MyHC7b, the host gene of miR-499-5p, thereby resulting in an increase of miR-499-5p [38, 39]. The finding that knockdown of Thrap1 upregulated the level of miR-499-5p may be due to si-Thrap1-induced an increase of MEF2C expression, then

promoting the miR-499-5p level. Moreover, attenuations of NFATc1 and MEF2C expression induced by inhibition of miR-499-5p were effectively reversed by knockdown of Thrap1, indicating that miR-499-5p may regulate NFATc1 and MEF2C expression at least partly through targeting Thrap1. Taken together, a clue that miR-499-5p regulates skeletal muscle fiber type gene expression through Thrap1 and MEF2C axis in C2C12 cells was discovered.

5. Conclusion

In summary, we found that overexpression of miR-499-5p promoted oxidative fiber specification and suppressed glycolytic fiber specification, accompanied with upregulation of NFATc1/MEF2C pathway and downregulation of Thrap1 expression. The factors associated with fiber type specification were affected by miR-499-5p overexpression and inhibition. We also found that miR-499-5p might regulate skeletal myofiber specification partly through NFATc1/MEF2C pathway and Thrap1-MEF2C axis in C2C12 cells. We showed a clue that miR-499-5p regulates skeletal muscle fiber type gene expression in C2C12 cells through targeting *Thrap1*, thereby, promoting NFATc1/MEF2C pathway and then triggering a series of oxidative muscle fiber gene expression. This clue (Fig. 8) consisting of miR-499-5p/Thrap1/MEF2C may be a part of regulatory network involved in the regulation of skeletal muscle fiber type.

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Conflicts of Interest

The authors declare no conflict of interest.

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