MiR-142a-3p: A novel ACh receptor transcriptional regulator in association with peripheral nerve injury

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Long-term denervation leads to the disintegration of nicotinic acetylcholine receptor (nAChR) located at the endplate structure, which translates to deficits in functional activation despite nerve repair. Because of a lack of effective measures to protect AChR expression, we explored the effect of alterations in muscular miR-142a-3p on nAChR. In this study, we constructed a model of miR-142a-3p knockdown by transfecting a miR-142a-3p inhibitor short hairpin RNA (shRNA) into C2C12 myotubes, and we injected this miR-142a-3p inhibitor shRNA into the tibialis anterior (TA) muscle in uninjured mice and in denervated mice by transecting the sciatic nerve. Our results showed that miR-142a-3p knockdown led to an increased number and area of AChR clusters in myotubes in vitro and larger neuromuscular endplates in adult mice. Furthermore, miR-142a-3p knockdown delayed the disintegration of motor endplates after denervation. Last, upon miR-142a-3p knockdown in uninjured and denervated mice, we observed an increase in the mRNA levels of five AChR subunits as well as mRNAs of genes implicated in AChR transcription and AChR clustering. Together, these results suggest that miR-142a-3p may be a potential target for therapeutic intervention to prevent motor endplate degradation following peripheral nerve injury.

INTRODUCTION

Motor endplates (MEPs) are the anatomical and functional link that mediates the cross-talk between motor neurons and skeletal muscle fibers.1 As neuromuscular junctions mature, MEPs become perforated and complex, resembling pretzels with arrays or branches that are innervated by one axon per neuromuscular junction.2 Structural and functional MEP defects have been implicated in a number of neuromuscular diseases,3 of which the most frequent is peripheral nerve injury. Peripheral nerve injury is a growing topic due to the failure of regenerating nerves to establish a proper muscle-nerve interface because of MEP disintegration.4 These changes in MEPs may be due to acetylcholine receptor (AChR) dispersion after skeletal muscle denervation, which results in the decline of mature pretzel-shaped MEPS. A previous study showed that AChR aggregation induced by matrix metalloproteinase 3 (MMP3) deletion contributed to preventing MEP degradation and improving subsequent reinnervation.9 As the core protein at the MEP, AChR was analyzed in previous studies mainly on the basis of the general principles of AChR clustering, maturation, and stability.10 Although the regulation of acetylcholine receptor transcription is only beginning to be understood, it is a promising intervention for preventing MEP degradation and thereby contributing to dying-back motor neuropathy, similar to the fused in sarcoma (Fus) and peroxisome proliferator-activated receptor gamma coactivator 1-α (Pgc-1α) transcriptional regulation.11-13

In our previous study, we constructed mouse models of sciatic nerve injury and analyzed the whole transcriptome involved in denervated gastrocnemius muscle. We performed a careful bioinformatics analysis of sequencing datasets and found that miR-142a-3p was consistently highly upregulated within 2 months after denervation.14 To date, extensive studies have demonstrated that miR-142 is a major regulator of cell fate decisions in the hematopoietic system15 and plays pleiotropic roles in embryonic development,16 cancer,17-19 viral infection,20 inflammation and immune tolerance.21,22 Recent evidence has shown that miR-142a-3p can influence mitochondrial morphology and reduce lipid use,23 which indicates that miR-142a-3p might play important roles in skeletal muscle. This evidence suggests that miR-142a-3p may have a major effect on nicotinic AChR (nAChR) defects following peripheral nerve injury.

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Here, we constructed a model of miR-142a-3p knockdown by transfected a miR-142a-3p inhibitor short hairpin RNA (shRNA) into C2C12 myotubes and injected this miR-142a-3p inhibitor shRNA into the tibialis anterior (TA) muscle to investigate the effect of muscular miR-142a-3p on AChR expression and AChR cluster formation in vitro and in vivo, and we explored whether miR-142a-3p knockdown could delay MEP degradation following denervation.

RESULTS
miRNAs are differentially expressed following denervation injury
We performed a careful bioinformatics analysis of microRNA (miRNA) sequencing datasets of denervated gastrocnemius muscles in mice to look for differentially expressed miRNAs, which were obtained from our published literature.14 Gastrocnemius samples of denervation injury were obtained at 0 (control group), 1, 2, 4, and 8 weeks after injury. Differential miRNA expression between the control groups and denervated groups was presented, with a log2 fold change (FC) > 2 after injury at 0 (control group), 1, 2, 4, and 8 weeks (Figure S1). After peripheral nerve injury, compared with 0 weeks, there was more than a 3-fold increase in miRNA expression levels from 1 to 8 weeks in the denervation injury group. In addition, Pgc-1α and Sorbs2 were significantly downregulated in denervated muscle (Figure S2). Both genes were predicted as target genes of miR-142a-3p on the basis of the target gene prediction software miRanda (http://cbio.mskcc.org/miRNA2003/miranda.html) and were also proven to regulate the transcription of a broad neuromuscular junction (NMJ) gene program and regulate AChR cluster formation.13,24

MiR-142a-3p knockdown activates AChR clustering in C2C12 myotubes
To explore the effect of miR-142a-3p knockdown on AChR clustering in C2C12 myotubes, C2C12 cells were transfected with either miR-142a-3p inhibitor shRNA or negative control (NC) shRNA using a lentiviral vector (Figure 1A). The knockdown efficiency is presented in Figure 1B. After differentiation for 7 days, NC-transfected myotubes showed only sporadic clustering of AChR on the cell membrane. However, miR-142a-3p inhibitor-transfected myotubes showed great increases in the number and area of AChR clusters (Figures 1C and 1D).

MiR-142a-3p knockdown alters MEP morphology in the skeletal muscle of uninjured mice
To construct a model of miR-142a-3p knockdown, healthy male Thy1-YFP-16 mice (weighing 22–25 g and aged 6–8 weeks) were randomly assigned to two experimental groups as follows: one group was injected with miR-142a-3p inhibitor short hairpin RNA into the tibialis anterior muscle, and the other group was injected with negative control inhibitor short hairpin RNA into the tibialis anterior muscle. For the uninjured groups, TA muscle tissue samples were harvested on day 7 after injection.

We assessed whether miR-142a-3p knockdown in skeletal muscle induced morphological alterations in MEPs in vivo. Three-dimensional (3D) reconstruction showed that MEPs in the TA muscle were distributed in their original positions, as they appeared in the control group (shown in Figure 2A). MiR-142a-3p expression is shown in Figure 2B. Although the number of MEPs was not significantly changed, the volume of a single AChR-concentrated MEP was larger in the miR-142a-3p-knockdown mice than in the control mice (see Figures 2C and 2D). Confocal microscopy was used to visualize NMJs, and a representative confocal stack image is shown in Figure 2E. Consistent with the results above, in the TA, the endplate area was increased in miR-142a-3p-knockdown muscles by ~21.5% compared with that in NC-treated muscles (Figure 2F). However, endplate innervation with clear colocalization did not differ between the miR-142a-3p-knockdown group and the control group (Figure S3A).

MEPs always present a certain percentage of abnormalities at the postsynaptic level. To quantify this change, we used a previously described scheme to characterize endplate morphology.25 Endplates were categorized as pretzel (mature with weblike pattern including multiple perforations) and fragmentation (immature and smaller size lacking perforations).26,27 A representation of the morphology of AChRs encountered in muscle is shown in Figure S3B. The percentage of fragmentation was reduced in the miR-142a-3p-knockdown mice (see Figure 2G). In addition, we assessed the size of muscle fibers by measuring muscle mass and cross-sectional area, and no difference was found in these parameters between control and miR-142a-3p-knockdown mice (see Figure 2H; Figures S3C and S3D).

We next investigated whether miR-142a-3p expression in muscle could affect synaptic folds at the ultrastructural level using transmission electron microscopy (TEM), which are located at the interface between the motor neuron and the muscle and to a large extent governed the area of MEPS. Interestingly, the average length of these synaptic folds was increased in miR-142a-3p-knockdown muscles compared with NC-treated muscles (1014 ± 19.2 vs. 768.5 ± 19.3 nm, p < 0.001) (see Figure 2I; Figure S3E).

To further explore the functional relationship in uninjured mice, rectangular pulses (0.9 mA, wave width 0.1 ms, and frequency 50 Hz) were delivered to induce muscular contraction, which was used to judge the responses of the TA to electrical stimulation of the sciatic nerve. However, we did not observe a significant difference (p < 0.05) in muscle strength between the miR-142a-3p-knockdown animals and control animals (Figures S3F and S3G).

Denervation-induced changes in skeletal muscle are delayed by miR-142a-3p knockdown
We assessed whether MEPS were maintained in vivo in the skeletal muscle of miR-142a-3p-knockdown mice following sciatic nerve transection (Figure 3A). MiR-142a-3p expression is shown in Figure 3B. Three-dimensional reconstruction of MEPS in the TA muscle revealed a greater number and larger volume of MEPS in the knockdown group than in the control group (volume: 6,501 ± 41.7 vs.
8,761.4 ± 93.1 μm³ [2 weeks], 4,364.9 ± 71.7 vs. 6,787.8 ± 323.6 μm³ [4 weeks], 3,255.5 ± 3.1 vs. 7,730.6 ± 578.3 μm³ [8 weeks]; number: 3,208.3 ± 73.9% vs. 3,018.3 ± 3.6% [2 weeks], 2,522.3 ± 0.7% vs. 2,762.3 ± 74.2% [4 weeks], 1,886 ± 42.5% vs. 2,542 ± 18.6% [8 weeks]) (Figures 3C and 3D). Endplates from control animals at several time points following denervation underwent progressive decreases in area and the percentage of pretzel-shaped MEPs (shown in Figure 3E). However, the decline in area was delayed significantly in miR-142a-3p-knockdown mice across the same time interval (Figure 3F). (area: 82.0% ± 2.5% vs. 107% ± 3.6% [2 weeks], 38% ± 0.7% vs. 93.9% ± 9.3% [4 weeks], 30.9% ± 3.5% vs. 99.8% ± 4.4% [8 weeks]). Following denervation, pretzel-shaped MEPs were also maintained in miR-142a-3p-knockdown animals (Figure 3G). A post hoc Bonferroni correction confirmed that the differences were significant at 3 time points.

Measurements of muscle mass and cross-sectional area revealed that atrophy occurred at slower rates following denervation in miR-142a-3p-knockdown mice than in control mice at 2, 4, and 8 weeks postinjury (see Figure 3H, quantified in Figures 3I and
Thus, miR-142a-3p knockdown delayed the rate of muscle atrophy.

**MiR-142a-3p knockdown promotes AChR gene transcription**

The endplate size and number in miR-142a-3p-knockdown mice increased, which prompted us to investigate a possible role of miR-142a-3p in the regulation of Chrn gene expression. First, in uninjured mice, miR-142a-3p knockdown increased the mRNA and protein expression of each of the five Chrn genes in TA (Figures 4A–4C). In addition, some proteins are critical for concentrating AChRs to promote clustering, which anchors AChRs to the cortical cytoskeleton by interacting with cytoskeletal proteins or scaffold proteins, including Rapsyn, laminin α4 (Lama4), and laminin β2 (Lamb2).

The mRNA expression of the three genes (Rapsyn, Lama4, and Lamb2) was also elevated compared with that in the control group (Figure 4D).

To confirm the target gene of miR-142a-3p, luciferase assays were conducted, and a schematic diagram of the reporter containing either the wild-type 3′ UTR or the mutant 3′ UTR of Pgc-1α, Sorbs2, and mir-142a-3p is shown in Figure 4E. Treatment with the miR-142a-3p mimics significantly decreased the luciferase activity of the wild-type reporter of Pgc-1α in HEK-293 cells compared with treatment with the NC mimics (Figure 4F). Consistent with luciferase assays, in uninjured mice, miR-142a-3p knockdown upregulated the relative mRNA and protein expression of Pgc-1α in TA (Figures 4G–4I).
However, Sorbs2, another important gene for regulating AChR clustering, was not directly targeted by miR-142a-3p, although the relative mRNA expression was upregulated in TA (Figure 4J). Importantly, the transcript levels of GA-binding protein A (Gabpa), which is the DNA-binding subunit and has been proven to bind to N-box elements to promote NMJ gene transcription by directly binding Pgc-1α, were also elevated (Figure 4K).13

Consistent with the results from uninjured mice, miR-142a-3p knockdown was still able to increase the mRNA expression of AChR-related genes in injured mice (Figures S4A–S4C).

**DISCUSSION**

The AChR is a transmembrane ligand-gated ion channel composed of five protein subunits whose clustering and maintenance at the postsynaptic endplate is a hallmark of the mammalian NMJ.28 The nAChR has been implicated as a potential molecular target for therapeutic intervention in a variety of neuromuscular diseases; for example, active AChR prevents the atrophy of denervated skeletal muscles and favors reinnervation.29 In this study, we found that miR-142a-3p knockdown could strongly upregulate the expression of five AChR subunits and simultaneously greatly relieve denervation-induced changes in skeletal muscle.

MiR-142a-3p knockdown activated AChR clustering in C2C12 myotubes in vitro. We first transfected C2C12 cells with a miR-142a-3p inhibitor shRNA and found that the miR-142a-3p inhibitor shRNA and subsequent miR-142a-3p knockdown increased the number and average area of AChR clusters in myotubes in vitro. The receptors that were able to bind α-bungarotoxin (α-BTX) appeared as typical

Figure 3. MiR-142a-3p-knockdown mice show resistance to AChR area and morphological degradation following denervation
(A) Spatial conformation of AChR stained by α-BTX in MEPs following denervation. Scale bars represent 500 μm. (B) Expression of miR-142a-3p in the TA muscle at 2, 4, and 8 weeks after transection by qRT-PCR analysis. U6 was used as a loading control. For the NC inhibitor group, n = 6; for the miR-142a-3p inhibitor group at 2 weeks, n = 9; for both groups at 4 weeks and 8 weeks, n = 9. (C and D) Total number of AChR-concentrated fragments in MEPs and the volume of a single AChR-concentrated fragment in MEPs at 2, 4, and 8 weeks after denervation. For both groups at 2 weeks, 4 weeks, and 8 weeks, n = 3. (E) Representative confocal stack images of the MEP fragments are shown for control and miR-142a-3p-knockdown mice at 2, 4, and 8 weeks after denervation. The blue triangles represent pretzel-shaped MEPs (scale bars, 100 μm). (F and G) Quantification of the area and the pretzel-shaped percentage of MEP fragments was determined on confocal images stacked from at least 100 NMJs. The AChR area decreased to a lesser degree following denervation in miR-142a-3p-knockdown mice than in control animals. MiR-142a-3p-knockdown mice contained a larger percentage of pretzel-shaped receptors at 3 time points of denervation. For both groups at 2 weeks, n = 5; for the NC inhibitor group, n = 3; for the miR-142a-3p inhibitor group at 4 weeks, n = 4; for the NC inhibitor group, n = 3; for the miR-142a-3p inhibitor group at 8 weeks, n = 7. (H) Representative H&E images of TA muscle cross sections at 3 time points following denervation are shown. Scale bars, 50 μm. (I) Quantification of the muscle mass following denervation. For both groups at 2, 4, and 8 weeks, n = 6. (J) Cross-sectional area decreased more slowly following denervation in miR-142a-3p-knockdown mice than in control mice at 2, 4, and 8 weeks postinjury. For the NC inhibitor group, n = 3; for the miR-142a-3p inhibitor group at 2 weeks, n = 5; for both groups at 4 weeks, n = 4; for both groups at 8 weeks, n = 5. One-way analysis of variance with Bonferroni post hoc comparison was performed among multiple time points. *p < 0.05, **p < 0.01, and ***p < 0.001. Data are shown as mean ± SEM.
oval plaques, as previously reported, indicating proper morphology. The mature MEPs in vivo were pretzel shaped (mature with weblike patterns, including multiple perforations). However, the AChR clusters in myotubes in vitro lacked complex internal structures, which might be due to the absence of nerves, which is supported by the plaque-like shape of clusters that form in aneural muscles.10 Despite being immature, the number and area of these spots indicated the clustering ability of AChR. For example, a previous study showed that the average area of AChR clusters could increase up to 59% by applying electrical stimulation in vitro.30 In our study, compared with the control group, the average area of AChR clusters in the miR-142a-3p-knockdown group was doubled, which indicates a strong interaction to activate AChR clustering.

Treatment with the miR-142a-3p inhibitor shRNA improved MEP morphology in uninjured mice in vivo. Treatment with the miR-142a-3p inhibitor shRNA increased the MEP area in the muscle of miR-142a-3p-knockdown uninjured mice by 21.5%, similar to the stimulus of resistance training, which has been reported to increase the endplate area by 16% without alterations in muscle fiber size.11 In addition, MEPs always present a certain percentage of abnormalities at the postsynaptic level in vivo. For example, the pretzel shape can be irregular or fragmented, and the percentage increases with age. Caloric restriction and exercise have been shown to decrease the percentage of fragmentation in aged mice to reverse these age-related synaptic changes.32 Thus, the improvement in MEP morphology induced by the miR-142a-3p inhibitor shRNA may indicate a better muscle condition. In addition, the number and distribution of MEPs were not significantly altered in vivo, which indicated that the newly synthesized AChR activated by miR-142a-3p knockdown entered the original AChR microclusters and then formed larger clusters. At the ultrastructural level, the increase in synaptic fold length indicates a greater structural postsynaptic surface, which can harbor more AChRs and show a larger size, triggered by muscle miR-142a-3p knockdown. However, there was no significant difference (p < 0.05) in muscle contractility between the miR-142a-3p-knockdown animals and control animals. Perhaps the intervention time was too short to promote the remodeling of the NMJ, including presynaptic modulation. Alternatively, miR-142a-3p in skeletal muscle does not influence the release of synaptic vesicles in presynaptic

Figure 4. MiR-142a-3p inhibitor promotes AChR gene transcription

(A) The relative mRNA expression of five Chrm genes in uninjured mice by qRT-PCR. (B and C) Western blot analysis of the five AChR subunit proteins. (D) Relative mRNA expression of Rapshyn, laminin α4 (Lama4), and laminin β2 (Lamb2) in uninjured mice as determined using qRT-PCR. (E) Schematic representation of the sequences of the wild-type (WT) 3’ UTR or the mutant 3’ UTR of Pgc-1α and Sorbs2 containing the WT miR-142a-3p-binding site. (F) Luciferase assays were performed to demonstrate the direct interaction between miR-142a-3p and two targets. The points represent wells. (G–I) Western blot analysis of the five AChR subunit proteins. (J) Relative expression of Sorbs2 in uninjured mice by qRT-PCR. (K) The relative mRNA expression of Gabpa in uninjured mice by qRT-PCR. U6 and GAPDH were used as loading controls. Two-tailed unpaired t test; *p < 0.05, **p < 0.01, and ***p < 0.001; data are shown as mean ± SEM.
nerve terminals in vivo. Similarly, in a previous study, the volume of 
MEPs increased in Mmp3 null mutant mice, but electrophysiological 
recordings revealed no change in quantal content or miniature end-
plate potential (MEPP) frequency. We will perform further relevant 
experiments.

MiR-142a-3p knockdown greatly relieved the degradation of denerv-
ated muscle in vivo, including endplate morphology and muscle mass. Our previous study showed that AChRs in MEPS stained 
with α-BTX were disintegrated and fragmented after denervation in vivo, especially with a significant decrease in the mean volume of 
a single MEP. The area and volume of MEPS in miR-142a-3p-knock-
down mice remained broadly unchanged even after 8 weeks of denervation. Previous interventions that tried to delay the disintegration of 
denervated MEPS presented a positive effect on muscle function and 
health, such as the implantation of a flexible microelectrode array (MEA), a calcitonin gene-related peptide (CGRP) treatment in vivo and Mmp3 deletion. In addition, we observed that treatment with the miR-142a-3p inhibitor shRNA led to strong mainte-
nance of the percentage of pretzel-shaped MEPS for long-term denerva-
tion in vivo. Generally, following denervation, pretzel-shaped MEPS were diminished dramatically, which was indicative of endplate 
destabilization. The results above indicate that the MEPS of muscles injected with the miR-142a-3p inhibitor shRNA were more resistant to disassembly. Moreover, the rate of muscle atrophy was delayed 
following denervation in miR-142a-3p-knockdown mice. Given that neural repair following long-term denervation leads to improved 
functional endpoints when MEP stability is preserved, miR-142a-3p 
represents an important therapeutic target to relieve the degradation of 
denervated muscle.

MiR-142a-3p knockdown enhanced the synthesis of five AChR sub- 
units in uninjured and denervated mice in vivo. The genes encoding 
muscle nAChR include Chrnα1, Chrnβ1, Chrnγ, Chrnδ, and Chrnε, and 
arity mutations in these genes have been shown to impair neurotransmission at the nerve endplate. Previous studies focused on 
the suppression of AChR degradation but not the stimulation of 
protein synthesis, such as the CGRP treatment described above. In 
a recent study, simultaneous reduced expression of the five Chrn 
genes in Fus-amytrophic lateral sclerosis (ALS) model mice resulted 
in reduced endplate surface area and a loss of function. Interestingly, in our study above, the five Chrn genes were upregulated simul-
taneously, and MEP volume and area were increased. Thus, we specu-
late that simultaneous preservation of the five Chrn genes may result 
in an increase in MEP volume and area and better functional mainte-
nance.

To further determine how miR-142a-3p regulates AChR gene tran-
scription, we constructed a luciferase reporter to demonstrate that 
Pgc-1α was directly targeted by miR-142a-3p in vitro. However, 
Sorbs2, another important gene for regulating AChR clustering, 
was not directly targeted by miR-142a-3p. Regarding the link between 
Pgc-1α and AChRs, a previous study found that when activated, Pgc-
1α bound host cell factor (HCF) and the ets-related GA-binding pro-
tein transcription factor complex (Gabp α/β). PGC-1α enhanced 
the binding of Gabp to the N-box (the DNA-binding site for Gabp) 
by phosphorylation, an important sequence motif in the promoters 
of many NMJ synaptic genes. Thus, Pgc-1α elevated the expression of 
a broad neuromuscular junction gene program, including AChR 
α1, AChR δ, AChR ε and Rapsyn. In addition, Pgc-1α was also proven 
to protect skeletal muscle from atrophy. The expression of Gabpa, 
which has been demonstrated to mediate the transcriptional response 
of Chrn genes, was also elevated by miR-142a-3p knockdown. Notably, a previous study showed that Pgc-1α can regulate only three subunits, AChR α1, AChR δ, and AChR ε, by binding to and coacti-

The roles of Rapsyn and Laminins were reported as key genes in 
AChR concentration. Rapsyn binds tightly to AChR to the subsynap-
cytic cytoskeleton to form a high-density network. In the laminin protein 
family, the loss of laminin α4 (Lama4) and laminin β2 (Lamb2) was shown to result in the disruption of the pretzel-shaped end-
plates. The knockdown of miR-142a-3p, however, affects the 
expression of Sorbs2, which is also required for AChR cluster forma-
tion. Upregulation of the expression of these genes above favors the 
maintenance of the pretzel shape. A schematic diagram of miR-142a-
3p regulating AChR-related gene transcription is shown in Figure 5.

There are several limitations in this study. We did not perform miR-
142a-3p knockdown in the tibialis anterior muscle of Pgc-1α muscle-
specific knockout mice, which demonstrated that AChR transcription 
is partly due to the regulation of Pgc-1α. In addition, some potential 
regulatory mechanism of miR-142a-3p should be further explored. 
For example, how miR-142a-3p regulates the gene expression of 
Sorbs2 is not clear. In addition, further studies may be required to 
explore the long-term effects of miR-142a-3p on NMJs in uninjured 
mice. Perhaps transgenic animals of miR-142a-3p knockdown could 
help elucidate the contribution of miR-142a-3p to adaptation of the 
NMJ.

In summary, we found an important target that regulates a broad 
range of AChR transcription and thus led to an improvement in 
MEP morphology in many aspects in uninjured and denervated 
mice. To the best of our knowledge, this is the first time that a 
muscular microRNA has been directly implicated in AChR mainte-
nance and stability. Furthermore, these findings also indicate a 
possible contribution of muscle miR-142a-3p to the treatment of 
other neuromuscular diseases with prominent NMJ pathology, 
including myasthenic syndromes, ALS and spinal muscular atrophy.

METHODS

Dataset analysis
The data analysis presented refers to previously published data. 
Differentially expressed (DE) genes with corrected p values < 0.05 
were considered statistically significant. Differentially expressed
miRNAs whose log₂ fold change was >2 after injury at each time point are displayed using Venn diagrams in Figure S1. Heatmaps were generated using MATLAB software. Then, the intersections of the co-localized or coexpressed target mRNAs with miR-142a-3p, on the basis of the target gene prediction software miRanda (http://cbio.mskcc.org/miRNA2003/miranda.html), were displayed in a Venn diagram (Figure S2). Seventy-one targets of miR-142a-3p were downregulated at all time points. A heatmap showing 14 downregulated mRNAs (log₂ FC < -2) was generated using MATLAB software.

Plasmid transfections
miRNA inhibitors and NC inhibitors were designed and synthesized by GeneChem (Shanghai, China) and were prepared as lentiviruses to transfect C2C12 cells and adenoviruses to transfect TA muscle cells. The C2C12 myoblast cell line was procured from American Type Culture Collection (ATCC).

To obtain miR-142a-3p-knockdown myotubes, C2C12 myoblast cells were transfected with miRNA inhibitor and NC inhibitor plasmids in accordance with the manufacturer’s instructions. C2C12 myoblast cells were seeded into a 24-well plate containing the appropriate growth medium at 1 × 10⁵ to 5 × 10⁵ cells per well, and the cells were transfected when the cell density reached 30%–50%. The cells were cultured in a culture plate in a 5% CO₂ incubator at 37°C for 72 h and observed with a fluorescence microscope. When the transfection rate was above 80%, the medium was discarded, and the cells were rinsed gently 3 times with Hank’s balanced salt solution (HBSS) and then cultivated with differentiation medium (DMEM high-glucose medium supplemented with 2% horse serum and 1% penicillin/streptomycin). The solution was changed every 2 days for 7 days, and the process of myotube formation was observed with an inverted microscope. During myotube differentiation, the same number of C2C12 myoblasts were converted to thick and spindle shapes, which was regarded as mature differentiation.

AAV is a common family of viral vectors with hundreds of capsids that are designed for different gene therapy applications. Among these, AAV serotype 9 (AAV9) is particularly attractive for gene therapy because it can lead to robust and body-wide muscle transduction in animal models. For in vivo gene knockdown studies, 1.0 × 10¹¹ IU/mL adenovirus particles 9 composed of miR-142a-3p inhibitor (AAV9-miR-142a-3p inhibitor) or NC inhibitor (AAV9-NC inhibitor) shRNA were injected into the mouse right TA muscle. The injection was performed by multiple-point injection at four different points in the upper and lower muscle with 5 μL per injection. In each animal, we injected 20 μL virus into the right TA muscle.

Animals
Healthy male Thy1-YFP-16 mice (weighing 22–25 g and aged 6–8 weeks) were obtained from The Jackson Laboratory (Bar Harbor, ME) and kept in the Laboratory Animal Centre of Peking University (Beijing, China). The mice were assigned under a randomized allocation to two experimental groups as follows: one group was injected with miR-142a-3p inhibitor short hairpin RNA into the tibialis anterior muscle, and the other group was injected with negative control inhibitor short hairpin RNA into the tibialis anterior muscle.

For the uninjured groups, TA muscle tissue samples were harvested on day 7 after injection. The wet weight of the TA muscle was used...
to assess muscle mass. There were thirty mice for the uninjured groups and all the mice survived in this study. None of the mice showed toe self-biting or ulcers in the operated limbs. Six mice were used to obtain 3D images of MEPs in intact TA. Six mice were used for muscle strength measurement. To reduce the number of animals used in experiments, the TA muscles of eighteen mice were used for qPCR measurements and imaging studies at the same time. The detailed numbers used for qPCR measurements and imaging studies were specified in the legend.

For the injured groups, there were twenty-four mice for each time point at 2, 4, and 8 weeks after denervation. Six mice were used to obtain 3D images of MEPs in intact TA. The remaining mice were used for qPCR measurements and imaging studies.

This study was carried out in accordance with the principles of the Basel Declaration and recommendations of Chinese guidelines for the care and use of laboratory animals. All experiments complied with the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines and were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publication 8023, revised 1978). The protocol was approved by the Ethics Committee of the Peking University People’s Hospital (permit 2020PHE089).

Animal models: Denervation
Male Thy1-YFP-16 mice were anesthetized with isoflurane, and then the sciatic nerve and its main branches in the right limbs were exposed. The sciatic nerve was transected 5 mm proximal to the bifurcation with a 3-mm-long nerve defect. The proximal and distal stumps were ligated with 5-0 nylon sutures separately and stitched to the adjacent muscles in reverse to prevent neural growth. For the injured groups, tissue samples were harvested at 2, 4, and 8 weeks after transection. The wet weight of the TA muscle was used to assess muscular atrophy.

Immunofluorescence
AChR aggregates were visualized by incubating myotubes with a solution containing 1.0 μg/mL α-BTX-Alexa Fluor 647 in DMEM for 1 h at 37°C with 7.5% CO2. Cells were rinsed twice with warmed PBS and fixed immediately in a 4% paraformaldehyde (PFA)-PBS solution. After 30 min at room temperature in fixative, the cells were rinsed with PBS, and the cell nuclei were stained using DAPI for 10 min.

NMJ morphology in uninjured and denervated mice
Alexa Fluor 647-conjugated α-BTX (α-BTX 647) (Invitrogen, Carlsbad, CA) was injected via the tail vein to label MEPs. After injecting fluorescent α-BTX (0.3 μg/g) via the tail vein with a 2 h conjugation time, the TA muscle was dissected. The TA muscle was removed and fixed in 4% PFA in PBS. After 3 times the next day. Thereafter, the TA muscle was dehydrated at 4°C in 20% sucrose solution, dehydrated for 1 day in the dark and dehydrated in 30% sucrose solution for 2 days. The TA muscle tissues were embedded in OCT.

For the quantification of endplate morphology and innervation status, tissues were sliced into 50 μm sections using a vibratome (VT1000 S; Leica, Wetzlar, Germany) and imaged at 25× magnification on a Zeiss LSM700 confocal microscope. Fiji software was used to obtain maximum intensity projections of a limited number of confocal sections to generate images of a select number of individual, nonoverlapping endplates. We analyzed more than 100 endplates per muscle. The surface area of each of these endplates was determined using the “freehand selection” and “analyze particles” tools in Fiji, and the average area of MEPs was calculated. The morphology of each of these endplates was determined and described as pretzel-shaped and fragmented. The pretzel was mature with a weblike pattern, including multiple perforations, and the fragments were immature and smaller in size, lacking perforations. To quantify innervation status, composite images of the red and green channels were generated. Endplates that displayed at least three green-positive dots were scored as innervated, and the percentage of innervated endplates per muscle was calculated and used for statistical analysis.

For the quantification of endplate volume and the total number of endplates, the dissected and postfixed TA muscles were processed using a typical clearing method called 3DISCO, as described in our previous study. First, tetrahydrofuran (Sinopharm Chemical Reagent, Shanghai, China) and dibenzyl ether (Sigma-Aldrich, St. Louis, MO) were preprocessed with basic activated aluminum oxide (Sigma-Aldrich, St. Louis, MO) to remove the residual peroxides. Then, the fixed muscle samples were incubated with 50%, 70%, 80%, and 100% tetrahydrofuran for dehydration, each for 2–3 h (30 min for muscle slices) in glass vials while gently shaking. After that, the samples were placed into dibenzyl ether until they became completely transparent. Then, an UltraMicroscope I (LaVision BioTec, Bielefeld, Germany) equipped with a ×2/0.5 objective (dry, working distance 20 mm) was used to image the cleared intact muscles. This instrument could create a thin light sheet to illuminate the cleared biological samples while imaging perpendicular to the light sheet. For AChR labeled with α-BTX, 647 nm was applied as the excitation wavelength. The z step size was set to 5 μm. After setting the imaging parameters appropriately, images of the samples were acquired for subsequent processing and analysis. For both groups (uninjured mice and denervated mice), n = 3.

Electron microscopy
The TA muscles were dissected and postfixed in 2.5% glutaraldehyde and 2.5% PFA in PBS at 4°C overnight. Samples were embedded in Epon 812. Ultrathin sections were cut at 70 nm, contrasted with uranyl acetate and lead citrate and examined at 120 kV using an electron microscope (JEM 1400plus; JEOL). Synaptic fold lengths were determined for individual NMJs using ImageJ software (National Institutes of Health, Bethesda, MD), and lengths were measured cursively from the edge of the synapse to the end of each fold.
Muscle cross-sectional area

TA muscles from 0, 2, 4, and 8 weeks following denervation were fixed in 4% formalin and paraffin embedded. Muscles were sliced into transverse sections (20 μm) using a cryostat, and muscle sections were stained with H&E. One hundred fifty fibers per muscle were then analyzed for cross-sectional area using ImageJ software. For the NC inhibitor group, n = 3; for the miR-142a-3p inhibitor group at 2 weeks, n = 5; for both groups at 4 weeks, n = 4; and for both groups at 8 weeks, n = 5.

Luciferase assay

Plasmid transfections for luciferase assays in 293T cells were performed with 0.1 μg wild-type or mutant 3′ UTR luciferase reporter of Pgc-1α and Sorbs2 and then transfected with 0.8 μg miR-142a-3p mimics plasmid in a 24-well plate using Nucleofector transfection reagent (PAA, Colbe, Germany) as described by the manufacturer. The 293T cells were divided into (1) WT-3′ UTR-PGC-1α + miR-142a-3p mimics, (2) MUT-3′ UTR-Pgc-1α + miR-142a-3p mimics, (3) WT-3′ UTR-Sorbs2 + miR-142a-3p mimics, and (4) MUT-3′ UTR-Sorbs2 + miR-142a-3p mimics. Luciferase activity was measured 48 h posttransfection using the Dual Luciferase Reporter Assay System (Promega) as recommended by the manufacturer.

mRNA qRT-PCR

Total RNA was extracted from C2C12 myotubes and TA muscles with TRIzol. Template DNA was removed by treatment with DnaseI. RNA purity was checked using a NanoPhotometer spectrophotometer (IMPLEN). Only when the OD260/OD280 of RNA was between 1.8 and 2.0 was the RNA used for subsequent reverse transcription (RT) using a 5X All-In-One RT MasterMix kit (G492; abm). Stem-loop RT primers were used for RT of miRNAs. All qRT-PCR data were analyzed using the Livak method, where ΔΔCt values were calculated and reported as relative quantification (RQ) values, which were calculated using the 2-ΔΔCt method. Primers for RT-PCR are listed in Table S1.

Western blot

C2C12 myotubes and TA muscle tissues were homogenized in RIPA buffer containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and Protease Inhibitor Cocktail (Roche). Lysates were centrifuged for 20 min at 12,000 g wild-type or mutant 3′ UTR luciferase reporter of Pgc-1α and Sorbs2 and then transfected with 0.8 μg miR-142a-3p mimics plasmid in a 24-well plate using Nucleofector transfection reagent (PAA, Colbe, Germany) as described by the manufacturer. The 293T cells were divided into (1) WT-3′ UTR-PGC-1α + miR-142a-3p mimics, (2) MUT-3′ UTR-Pgc-1α + miR-142a-3p mimics, (3) WT-3′ UTR-Sorbs2 + miR-142a-3p mimics, and (4) MUT-3′ UTR-Sorbs2 + miR-142a-3p mimics. Luciferase activity was measured 48 h posttransfection using the Dual Luciferase Reporter Assay System (Promega) as recommended by the manufacturer.

mRNA qRT-PCR

Total RNA was extracted from C2C12 myotubes and TA muscles with TRizol. Template DNA was removed by treatment with DnaseI. RNA purity was checked using a NanoPhotometer spectrophotometer (IMPLEN). Only when the OD260/OD280 of RNA was between 1.8 and 2.0 was the RNA used for subsequent reverse transcription (RT) using a 5X All-In-One RT MasterMix kit (G492; abm). Stem-loop RT primers were used for RT of miRNAs. All qRT-PCR data were analyzed using the Livak method, where ΔΔCt values were calculated and reported as relative quantification (RQ) values, which were calculated using the 2-ΔΔCt method. Primers for RT-PCR are listed in Table S1.

Western blot

C2C12 myotubes and TA muscle tissues were homogenized in RIPA buffer containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and Protease Inhibitor Cocktail (Roche). Lysates were centrifuged for 20 min at 12,000 g (4°C). Supernatants were transferred to a separate tube, and the bicinchoninic acid (BCA) assay (Beyotime) was used for protein-level quantification. Proteins were separated using SDS-PAGE (Beyotime), transferred to polyvinylidene difluoride (PVDF) membranes (Millipore), and blocked for 1 h with 5% nonfat dry milk in Tris-buffered saline (TBS) at room temperature. Primary antibodies against the following targets were incubated overnight at 4°C: rabbit polyclonal to PGC1 alpha-N-terminal (1:1,000; Abcam), rabbit anti-CHRNA1 (α1) (1:1,000; Proteintech), rabbit anti-CHRNB1 (β1) (1:1,000; Proteintech), mouse anti-CHRNBD (β) (1:5,000; Novus Biologicals), mouse anti-AChRε (1:1,000; Santa Cruz Biotechnology), and rabbit anti-CHRNA3 (γ) (1:1,000; MyBioSource). After 3 washes, the blots were incubated with the appropriate secondary antibodies (Abcam) at room temperature for 1 h. Enhanced chemiluminescence (ECL) detection reagent and X-ray film were used for protein detection.

Muscle strength measurement

Mice were deeply anesthetized with isoflurane for surgical procedures and then placed on the operating table in the right lateral position. After preoperative skin preparation and disinfection with 0.5% iodo- phor in the right lower limb, an L-shaped incision was made at the anterolateral site to expose the sciatic nerve and the tibialis anterior muscle. The PCLAB-UE biomedical signal acquisition and processing system (MicroStar Science and Technology Development, Beijing, China) was applied for muscle strength measurement with its supporting PZ-1 tension sensor (50 g measuring range). “Continuous contraction” and “0–50 g” were chosen as the measurement type and range. The image recording parameters were set as 25.00 mV/div and 2,000 s/div. First, a zero setting was performed with 50 g of balancing weight. With the immobilization of the tibial plateau, the tibialis anterior tendon was dissociated with knitting sutures and connected parallel to the tension sensor. Then, the MedlecSynergy electrophysiological device was used as the stimulation producer, and the stimulating signal was set as square waves with 0.9 mA, a wave width of 0.1 ms, and a frequency of 50 Hz. The sciatic nerve was given continuous stimuli to record the waveforms of muscular contraction. Finally, we analyzed every waveform of muscular contraction and measured its maximal muscle strength to calculate the difference between the two groups.

Statistical analysis

Data are presented as mean ± standard error of the mean (SEM). One-way analysis of variance with Bonferroni post hoc comparison was performed unless otherwise indicated. Statistical significance is reported as p < 0.05.

DATA AVAILABILITY STATEMENT

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omtn.2022.10.005.

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AUTHOR CONTRIBUTIONS

Z.Q. performed most of the experiments in C2C12 cells and in miR-142a-3p-knockdown mice with the help of S.W. A.X. performed and analyzed the three-dimensional reconstruction of MEPs in the TA muscle. L.Z. performed electron microscopy. X.G. analyzed the three-dimensional reconstruction of MEPs in the TA muscle. C.H. wrote the manuscript. X.Y. initiated, conceived and...
supervised the project. All authors contributed to the experimental design and interpretation and commented on the manuscript.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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