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Original Article

Exercise preconditioning prevents immobilization-induced skeletal muscle atrophy by activating Prmt1-p38/ATF2-Sesn1 signaling axis in C57BL/6J mice

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ABSTRACT

Purpose: This study aimed to explore the effects of a 10-week combined exercise regimen on immobilization-induced muscle atrophy and elucidate the possible function of Protein arginine methyltransferase 1 (Prmt1) in this process.

Methods: 8-week-old male C57BL/6J mice were carried out combined exercise for 10 weeks. One week before the end of the intervention, mice underwent cast immobilization. Additionally, to investigate the potential mechanism in exercise-induced protection of skeletal muscle, mice in the exercise preconditioning group were administered TC-E−5003(an inhibitor of Prmt1 enzymatic activity). Exercise performance, muscle mass, and the cross-sectional area (CSA) of muscle fibers were analyzed. Besides, Prmt1 and Sestrin1 (Sesn1) were either overexpressed or inhibited in C2C12 myotubes to elucidate the underlying mechanism.

Results: Exercise preconditioning not only significantly improved muscle mass and motor ability in immobilized mice but also inhibited excessive activation of degradation pathways and enhanced protein synthesis. Importantly, Prmt1 mediated the protective effects of exercise preconditioning on muscle atrophy. Mechanistically, Prmt1 regulated the p38 mitogen-activated protein kinase (p38)/activating transcription factor 2 (ATF2) pathway, which modulates Sesn1 expression. Sesn1 acts as a downstream of Prmt1 and ATF2, contributing to the myoblast differentiation and skeletal muscle regeneration through AMP-Activated protein kinase α2 (AMPKα2)/transcriptional co-activator PPAR-γ co-activator-1 α (PGC-1α) signaling pathway.

Conclusions: Taken together, our results highlighted the effectiveness of exercise preconditioning in preventing muscle atrophy via the Prmt1-Sesn1 pathway.

1. Introduction

Skeletal muscle comprises approximately 40% of body weight in humans and serves as a reservoir for various myokines, which are vital in maintaining metabolic homeostasis.[1] Imbalance between protein synthesis and degradation resulting from multiple factors, including neuromuscular diseases, postoperative immobilization, and aging, leads to muscle atrophy, featured by the remarkable reduction of muscle mass and strength.[2] Studies have confirmed that activation of the ubiquitin-proteasome system (UPS) and impaired muscle regeneration promote muscle wasting.[3,4] Muscle RING finger 1 (MuRF1) and muscle atrophy F-box (MAFbx)/atrogin-1 are identified as two muscle-specific E3 ubiquitin ligases, which are upregulated in atrophic muscle.[5] Currently, exercise training is the frontline treatment for sarcopenia, with no drug currently approved for this indication.[6] Exercise training maintains metabolic homeostasis and induces potential beneficial effects on skeletal muscle, and this protective phenotype is termed "exercise preconditioning".[7] However, the molecular mechanisms underlying the protective effects of exercise preconditioning against immobilization-induced atrophy remain incompletely elucidated.

Prmts are a class of widely distributed catalytic enzymes responsible for catalyzing protein arginine methylation modifications in eukaryotes.[8] Prmt1, the most predominant subtype and crucial for muscle regeneration in skeletal muscle, regulates most arginine methylation among nine subtypes of Prmts.[9–12] In addition, muscle-specific Prmt1 knockout leads to muscle atrophy by accelerating protein degradation.[13] Notably, acute exercise can increase the expression and enzymatic activity of Prmt1 in

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Abbreviations

aDMA	asymmetric Di-Methyl Arginine Motif
Akt	protein kinase B
AMPKα2	AMP-Activated protein kinase α2
AP-1	activating protein 1
ATF2	activating transcription factor 2
Atrogin-1	muscle atrophy F-box
C	control
ChIp	Chromatin immunoprecipitation
Co-IP	CO-Immunoprecipitation
CSA	cross-sectional area
E	combined exercise
EDL	extensor digitorum longus
E + Im	a combination of combined exercise and immobilization
E+5003	a combination of combined exercise and TC-E5003 administration
E+5003+Im	a combination of E+5003 and immobilization
FoxO3	Forkhead box O3
GAS	gastrocnemius
H&E	hematoxylin and eosin
IGF-1	insulin-like growth factor 1
Im	immobilization
MEF2	myocyte enhancer factor 2
mTOR	mammalian target of rapamycin
MuRF-1	muscle RING-finger-1
MVCC	maximal voluntary carrying capacity
MyoD	myogenic differentiation antigen
Myf5	myogenic factor 5
MyoG	myogenin
PGC-1α	transcriptional co-activator PPAR-γ co-activator-1 α
Prmt1	protein arginine methyltransferase 1
PVDF	polyvinylidene fluoride
p38 (MAPK)	p38 mitogen-activated protein kinase (MAPK)
QUA	quadriceps femoris
Sesn1	Sestrin1
SOL	soleus
TA	tibialis anterior
UPS	ubiquitin-proteasome system

muscle,[14,15] yet its role in long-term exercise adaptation and disuse atrophy prevention remains unexplored. Concurrently, Sestrins, a family of stress-responsive proteins, has recently emerged as a critical regulator of mitochondrial quality and cellular energy metabolism by modulating AMPK and PGC-1α.[16,17] In addition, Sesn1 and Sesn2 play an important role in attenuating skeletal muscle atrophy and are mediated by the metabolic benefits of physical exercise.[18,19] Although Sesn1 is highly expressed in skeletal muscle compared to Sesn2 and Sesn3,[20] it remains unclear whether it is involved in the protective effects of muscle atrophy by exercise preconditioning. A previous study reported the presence of potential ATF2 binding sequences in the Sesn1 promoter region in human cancer cell lines.[21] Interestingly, Prmt1 has been reported to increase ATF2 expression by activating p38 in human leukemia cancer cell lines,[22] but this pathway has not been validated in skeletal muscle. Therefore, it is necessary to explore whether Prmt1 regulate the expression of Sesn1 via p38-ATF2 during exercise training and Prmt1 mediated exercise preconditioning to prevent muscle atrophy is dependent on Sesn1.

Based on these findings, we hypothesize that Prmt1 may regulate Sesn1 expression by activating p38/ATF2 in skeletal muscle, and Prmt1-p38/ATF2-Sesn1 pathway mediates the beneficial effects of exercise preconditioning on muscle atrophy.

2. Materials and methods

2.1. Ethical approval

Animals were housed at 22 °C-24 °C, 40%–70% humidity under 12 h dark and 12 h light cycle. Food and water were provided *ad libitum*. All protocols and animal studies were approved by the Tianjin Medical University Animal Care and Use Committee under the guidelines of the Chinese Academy of Sciences (approval number: SYXK-2019-0004).

2.2. Animal handling and experimental design

8-week-old male C57BL/6J mice were purchased from Sibeifu (Beijing, China) Biotechnology Co., Ltd. The mice were randomly divided into three weight-matched groups: a sedentary control group (C, $n = 16$), a combined exercise group (E, $n = 8$), and a combination of exercise and TC-E−5003 intervention group (E+5003, $n = 8$).

For aerobic exercise, mice underwent treadmill running for 60 min/day at 12 m/min. The running speed at 12 m/min corresponds to the intensity of 75% $\dot{V}O_2$max.[23] Training was terminated when the mice showed signs of exhaustion, such as staying in the shock zone of the treadmill continuously and failing to keep up with the running speed despite repeated stimuli. Resistance exercise was performed according to previous study.[24] Briefly, mice were subjected to ladder-climbing exercise and followed by attaching a load of 75% body weight to the base of the tail and incrementally adding 3 g of additional load each week. The training was conducted 3 sets of 5 repetitions and 2-min rest intervals during each set. The whole exercise protocol alternated between aerobic and resistance exercise three times each per week, for a total of six training days, and lasted for 10 weeks.

TC-E−5003 (MedChemExpress, HY-107574) is a selective inhibitor of Prmt1 enzymatic activity which does not affect the protein expression level of Prmt1. To investigate the role of Prmt1 in combined exercise-induced improvement of skeletal muscle function, mice were intraperitoneally injected with saline for control or TC-E−5003 (2 mg/kg per day), 5 days per week, for 10 weeks.[25]

After 10-week combined exercise preconditioning, half of the mice in C group (Im, $n = 8$) and the mice in E (E + Im, $n = 8$) and E+5003 (E+5003+Im, $n = 8$) groups were underwent cast immobilization for 1 week to induce disuse muscle atrophy, with both bilateral hindlimbs placed in knee extension and plantar flexion positions and wrapped in a 12-15 mm-wide cast (Fig. S1). All cast-immobilized mice were individually housed to prevent cast damage caused by mutual gnawing. Body weight and food intake were monitored daily during the immobilization. Following 1 week of immobilization, skeletal muscle function assessments were performed in all groups of mice. At last, the mice were euthanized by cervical dislocation after being anesthetized with 2.5% avertin (0.16 ml per 10 g).

2.3. Grip strength test

The YLS-13A grip strength tester (Jinan Yiyan Technology Development Co., Ltd, Shandong, China) was used for measuring grip strength. In brief, the tail of the mouse was gently pulled back at a constant speed after it grasped the grid. The peak force was recorded at the time the mouse released its paws from the grid in each measurement. Five measurements were taken at 1 min intervals, with the average value normalized by the body weight.

2.4. Inverted-grid test

Mouse was placed in the center of a wire mesh, and the grid was

gently inverted to evaluate the limb fatigability. The time before the mouse dropped from the wire mesh was recorded. Each test contains three measurements, allowing for sufficient rest between tests.

2.5. Rotarod test

The YLS-31A rotarod (Jinan Yiyan Technology Development Co., Ltd, Shandong, China) was applied to evaluate motor coordination and balance of mice. Briefly, the mouse was placed on a stationary rod to acclimate for 1 min, then the rod accelerated from 5 to 50 rpm in 8 min. Each mouse was measured three times, with 1 min rest between trials. The latency to fall was averaged as the final result.

2.6. Maximum voluntary climbing capacity test

The maximal voluntary carrying capacity (MVCC) test was performed to assess skeletal muscle function in mice. Briefly, the load of 75% of body weight was attached to the tail and the weight was gradually increased until the mouse could not overcome the resistance to complete three consecutive climbs. The weight before failure was recorded as the maximum load weight, which calibrated to body weight as maximum load capacity. Schematic diagrams of exercise performance tests could be found in Fig. S1.

2.7. Hematoxylin & eosin staining

For H&E staining, gastrocnemius (GAS) was fixed in 4% paraformaldehyde, embedded with OCT and frozen in liquid nitrogen. The frozen GAS was sectioned at 10 μm thickness with a Microtome Cryostat (CM1860UV, Leica). After fixation in 4% paraformaldehyde for 15 min, cryosections were stained for hematoxylin/eosin (H/E). Images were acquired from light microscopy (AF 6000, Leica). The cross-section area (CSA) of muscle fibers was analyzed using Image J software. The average CSA was calculated from 8 mice in each group, three randomly selected fields of view were analyzed in each section for each mouse.

2.8. Cell culture

C2C12 myoblasts obtained from ATCC were cultured in DMEM media (10% FBS, 1% penicillin/streptomycin) at 37 °C in a 5% CO_2 atmosphere. Upon reaching 80% confluency, C2C12 myoblasts were differentiated to myotubes in DMEM containing 2% horse serum for 5 days. Afterward, the differentiated myotubes were infected by adenoviruses (Ad-Prmt1, Ad-Sesn1) using a multiplicity of infection (MOI) of 100 and harvested 48 h after administration. Adenoviral vectors overexpressing Prmt1 and Sesn1 were produced in pAd/CMV/V5-DEST (Invitrogen) using Gateway technology. siRNA reagents (GenePharma, Shanghai, China) for Prmt1 (si-Prmt1), Sesn1 (si-Sesn1) and ATF2 (si-ATF2) were used according to the manufactures' instructions.

2.9. C2C12 cell hematoxylin & eosin staining

The specific steps are as follows: (1) After collecting the 6-well plate, perform operations on ice, remove the culture medium, wash twice with PBS, then fix with 95% ethanol for 20 min, followed by two washes with PBS; (2) Add 200 μL of hematoxylin staining solution to each well for 3 min, then rinse with tap water three times, each time for 1–2 min; (3) Add 200 μL of eosin staining solution to each well for 1–3 min, followed by rinsing with tap water, then adding 200 μL of PBS and photographing under a microscope.

2.10. Western blot

Total homogenates from tissue/cell were obtained in freshly prepared NP-40 lysis buffer supplemented with protease and phosphatase inhibitors. Equal amounts of protein were loaded and separated on SDS-PAGE gels (sodium dodecyl sulfate polyacrylamide gel electrophoresis) and then transferred to PVDF (polyvinylidene fluoride) membrane. The membranes were blocked in 5% skim milk for 2 h, and probed overnight at 4 °C with specific antibodies. The antibodies are listed below: aDMA (Anti-Asymmetric Di-Methyl Arginine Motif) (1:1 500, Rabbit, Cell Signal Tech, USA), Akt (protein kinase B) (1:2 000, Mouse, Proteintech, USA), pAkt-Ser473 (1:2 000, Rabbit, Cell Signal Tech, USA), AMPKα2 (1:2 000, Rabbit, Cell Signal Tech, USA), pAMPK-Thr172 (1:2 000, Rabbit, Cell Signal Tech, USA), ATF2 (1:2 000, Rabbit, Proteintech, USA), Atrogin-1 (FBXO32) (1:15 000, Mouse, Proteintech, USA), FoxO3a (Forkhead box O3) (1:1 000, Mouse, Proteintech, USA), pFoxO3a-Ser315 (1:2 000, Rabbit, Proteintech, USA), GAPDH (1:5 000, Rabbit, Utibody, CN), IGF-1 (insulin-like growth factor 1) (1:2 000, Mouse, Proteintech, USA), LaminB (1:2 000, Rabbit, Abcam, USA), MEF2 (myocyte enhancer factor 2) (1:2 000, Rabbit, Proteintech, USA), Myf5 (myogenic factor 5) (1:2 000, Rabbit, Abclonal, CN), MyoD (myogenic differentiation antigen) (1:2 000, Rabbit, Proteintech, USA), MyoG (myogenin) (1:2 000, Rabbit, Proteintech, USA). mTOR (mammalian target of rapamycin) (1:2 000, Rabbit, Cell Signal Tech, USA), p38 (1:2 000, Rabbit, WANLEIBIO, CN), p-p38-Thr180/Tyr182 (1:2 000, Rabbit, WANLEIBIO, CN), PGC-1α (1:1 000, Rabbit, Abcam, USA), Prmt1 (1:2 000, Rabbit, Cell Signal Tech, USA), Raptor (1:1 000, Rabbit, Cell Signal Tech, USA), Sesn1 (1:1 000, Rabbit, HUABIO, CN), Sesn1 (1:1 000, Rabbit, Abcam, USA), TRIM63 (MuRF1) (1:2 000, Rabbit, Proteintech, USA). Western blot was analyzed with a scanner and digitalized using image J (Quantity One, Hercules, CA, USA). All results are representative of three biological repeats.

2.11. Real-time PCR

Total RNA was extracted from GAS muscle by Trizol reagent (Invitrogen) and 1 μg of total RNA was reverse transcripted to complementary DNA using First-Strand cDNA Synthesis SuperMix kit (Transgen, Beijing, China). Real-time PCR was then performed using PerfectStartTM Green qPCR SuperMix (Transgen, Beijing, China) with Roche LightCycler 480 system (Roche, Basel, Switzerland). Relative mRNA amounts were normalized to GAPDH using the $2^{-\Delta\Delta CT}$ method. Primer sequences are listed in Supplementary Table 1.

2.12. Co-immunoprecipitation

Primary antibodies (Prmt1, ATF2, p38, PGC-1α, Sesn1, AMPKα2, MyoD) were cross-linked to Dynabeads® Protein A (Invitrogen, 10008D) according to the manufacturer protocol. Tissue lysates were precleared with IgG Dynabeads Protein A for 20 min before incubation with antibody–linked Dynabeads overnight at 4 °C. The immunoprecipitated complexes were washed five times with washing buffer. Proteins were eluted by boiling in loading buffer and then processed for Western blot analysis.

2.13. Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) is widely used to identify interaction between genomic DNA and proteins in eukaryotic cells. Briefly, approximately 50 mg muscle tissue was cross-linked with pre-warmed 1.5% formaldehyde for 20 min at 37 °C. Crude chromatin extracts were sonicated to small fragments about 180 bp– 900 bp by using micrococcal nuclease. ChIP grade antibodies to Prmt1 (1:100, Proteintech, USA) were used to perform immunoprecipitation. The chromatin– antibody complexes were captured on Dynabeads protein G (Invitrogen, USA), which was used to enrich the chromatin-antibody complexes. The complexes from the Dynabeads 3 were washed 3 times with elution buffer and then cross-linked in proteinase K at 65 °C for 4 h. Precipitated DNA was purified using phenol-chloroform extraction and re-suspended in ddH_2O. DNA from input and IP group was subjected to qPCR with specific primers. Primer designed for detecting Prmt1-associated promoters of Sesn1 was listed in Supplementary Table 1.

2.14. Statistical analysis

All data are presented as means ± standard error of the mean (*SEM*). We performed the unpaired Student's two-tailed *t*-test for comparison of the two experimental groups and two-way ANOVA for multiple comparisons followed by Tukey's post hoc test. Data analysis was conducted by GraphPad Prism, version 8. Statistical significance was considered at $p < 0.05$.

3. Results

3.1. 10-Week combined exercise preconditioning prevented 1-week hindlimb immobilization-induced muscle atrophy in mice

10-week combined exercise significantly blunted the body weight gain compared to the C group from the 4th to the 10th week, yet administration of TC-E−5003 increased body weight as opposed to E group from the 6th to the 10th week, suggesting that Prmt1 may modulate body weight regulation during exercise training (Fig. 1A–B). In addition, there were no significant differences in the food intake of each experimental group (Fig. 1C). The food intakes were also monitored during the immobilization period, a decrease in food intake was noted only during the first two days of immobilization, with no subsequent decrease observed (Fig. 1D). It has been previously shown that the expression of aDMA in skeletal muscle positively correlates with Prmt1 enzymatic activity,[14] and decreased aDMA level in the E+5003+Im group (Fig. 1E–F) confirmed the inhibitive effects on prmt1 of 10-week administration of TC-E−5003. We then found that the skeletal muscle dysfunction induced by immobilization supported by the decreasing grip strength (Fig. 1G), suspension time (Fig. 1H), the time of latency to fall (Fig. 1I), and MVCC (Fig. 1J) was improved by exercise preconditioning. Besides, the hindlimb muscle mass decreased in Im group, and increased in E + Im group (Fig. 1K). Subsequently, the GAS, soleus (SOL), quadriceps femoris (QUA), tibialis anterior (TA), and extensor digitorum longus (EDL) were dissected and weighed. Muscle atrophy was significantly observed in GAS, SOL, and QUA muscle mass. Combined exercise increased GAS, SOL, and EDL muscle mass as compared to the Im group. No changes in TA muscle weight were observed (Fig. 1L–P). The changes mentioned above might be implicated in the immobilization protocol performed in this study, which mainly induced the posterior calf muscle group atrophy, particularly the GAS and SOL muscles. Additionally, in line with the trend of GAS mass, we found that the CSA of GAS muscle fibers also significantly decreased in Im group, but increased in Im + E group (Fig. 2A–B). Conversely, TC-E−5003 administration negated the protective effects of combined exercise against muscle atrophy including exercise performance, muscle mass, and CSA (Fig. 1G–J, Fig. 2A–B). To further investigate the degree of muscle atrophy in GAS, genes and proteins involving protein synthesis and degradation were detected by qPCR and WB. Exercise preconditioning could significantly down-regulate skeletal muscle trophy genes MuRF1 and Atrogin-1 mRNA levels, which were increased induced by immobilization. However, we observed increased MuRF1 mRNA expression in the E+5003+Im group (Fig. 2C). Moreover, our results showed that Prmt1 and Sesn1 mRNA expression was significantly decreased after immobilization, increased in E + Im group, and decreased again after TC-E−5003 administration (Fig. 2D). WB experiments showed that exercise preconditioning mitigated the immobilization-induced decreases of Prmt1, Sesn1 and the ratio of p-FoxO3a/FoxO3a, and reduced FoxO3a target gene expression, including Atrogin-1 and MuRF1. Alternatively, exercise preconditioning increased the expression of mTOR, Raptor, and AKT activity (phosphorylation of Ser473) although their levels increased in Im group. As expected, exercise preconditioning upregulated the immobilization-induced decreases of IGF-1. Nevertheless, inhibiting Prmt1 by TC-E−5003 led to distinct degrees of inhibition on those changes resulted from exercise training (Fig. 2E–N) Taken together, exercise preconditioning improved the imbalance of protein synthesis-degradation homeostasis caused by immobilization, at least in part, by increasing Prmt1 and Sesn1 expression.

3.2. Exercise preconditioning increased Sesn1 expression by activating Prmt1-p38/ATF signaling in mice

In order to examine whether Prmt1 regulated Sesn1 transcription through forming transcription co-activator with transcription activating factor ATF2 during the exercise preconditioning, ChIP assay was conducted to investigate the binding level of Prmt1 to the underlying ATF2 binding sequence on Sesn1 gene promoter. We observed a significantly decreased binding level of Prmt1 with Sesn1 promoter region in Im group, but an increased binding level of them in E + Im group, and the level of Prmt1 at the promoter region of Sesn1 decreased again after TC-E−5003 treatment (Fig. 3A). Further, there were significant decreases in the expression levels of p-p38, p38 and ATF2 in immobilized mice, and increases in E + Im group, however, the effects of exercise on the levels of these genes were attenuated by TC-E−5003. Their binding relationship was also verified by Co-IP assay. Our results revealed a decline in Prmt1 binding to p38 in Im group, and an increase in E + Im group. Meanwhile, immobilization suppressed ATF2 binding to Prmt1, which was promoted by exercise. Lastly, p38 was immunoprecipitated; p38 binding to Prmt1 was strongly reduced in Im group, but increased in E + Im group. Although the binding of p38 and ATF2 was facilitated in Im group, exercise still upregulated their binding level in a greater degree. Interestingly, these effects of exercise were disrupted by TC-E−5003 (Fig. 3E–J). In summary, these results suggested that Prmt1 increased the expression of Sesn1 in skeletal muscle via activating Sesn1 transcription by forming transcription co-activator with ATF2 during exercise preconditioning.

3.3. Prmt1-Sesn1 coordinated the differentiation of C2C12 myoblasts into mature myotubes

Considering the role of Sesn1 in ameliorating muscle atrophy and Prmt1 in C2C12 myoblasts differentiation, we speculated that Prmt1 and Sesn1 may be involved in the differentiation of C2C12 myoblasts into myotubes. C2C12 myoblasts with different interventions were differentiated to 7 days, and the results of HE staining showed that Sesn1 deficiency suppressed C2C12 myoblasts differentiation to mature myotubes, but overexpression of Sesn1 promoted C2C12 differentiation (Fig. 4A–B). Examination of molecules involved in regulating protein degradation showed that Sesn1 deficiency increased the protein expression of FoxO3a, Atrogin-1, and MuRF1 in C2C12 myotubes, however, these changes were reversed after Sesn1 overexpression (Fig. 4C–D). Similar results were observed in C2C12 following inhibition or overexpression of Prmt1. Furthermore, Prmt1 overexpression diminished Prmt1 deficiency-induced decrease of p38 phosphorylation, ATF2, and Sesn1 protein expression (Fig. 4E–N). Taken together, these results indicated that overexpression of Prmt1 could activate p38/ATF2-Sesn1 pathway in C2C12, and Prmt1-Sesn1 coordinated the differentiation of C2C12 myoblasts into mature myotubes by inhibiting protein degradation pathway.

3.4. ATF2 mediated the regulation of Prmt1 on Sesn1 and Sesn1 acted as a downstream effector of Prmt1

The above results in vivo and in vitro confirmed the regulation of Prmt1-p38/ATF2 pathway on Sesn1, to fully understand the role of transcription factor ATF2 in forming the Prmt1-ATF2 co-activator and regulating Sesn1 expression, ATF2 was silenced in C2C12 treated with Prmt1 adenoviruses. Significant increases of ATF2 and Sesn1 levels were observed after Prmt1 overexpression, but ATF2 silencing disrupted the effects of Prmt1 on upregulating these genes (Fig. 4O–R). Therefore, ATF2 mediated the regulation of Prmt1 on Sesn1. Although Prmt1 upregulated Sesn1 via p38/ATF2 and Prmt1-Sesn1 coordinated the differentiation of C2C12 myoblasts into mature myotubes, it is still unclear

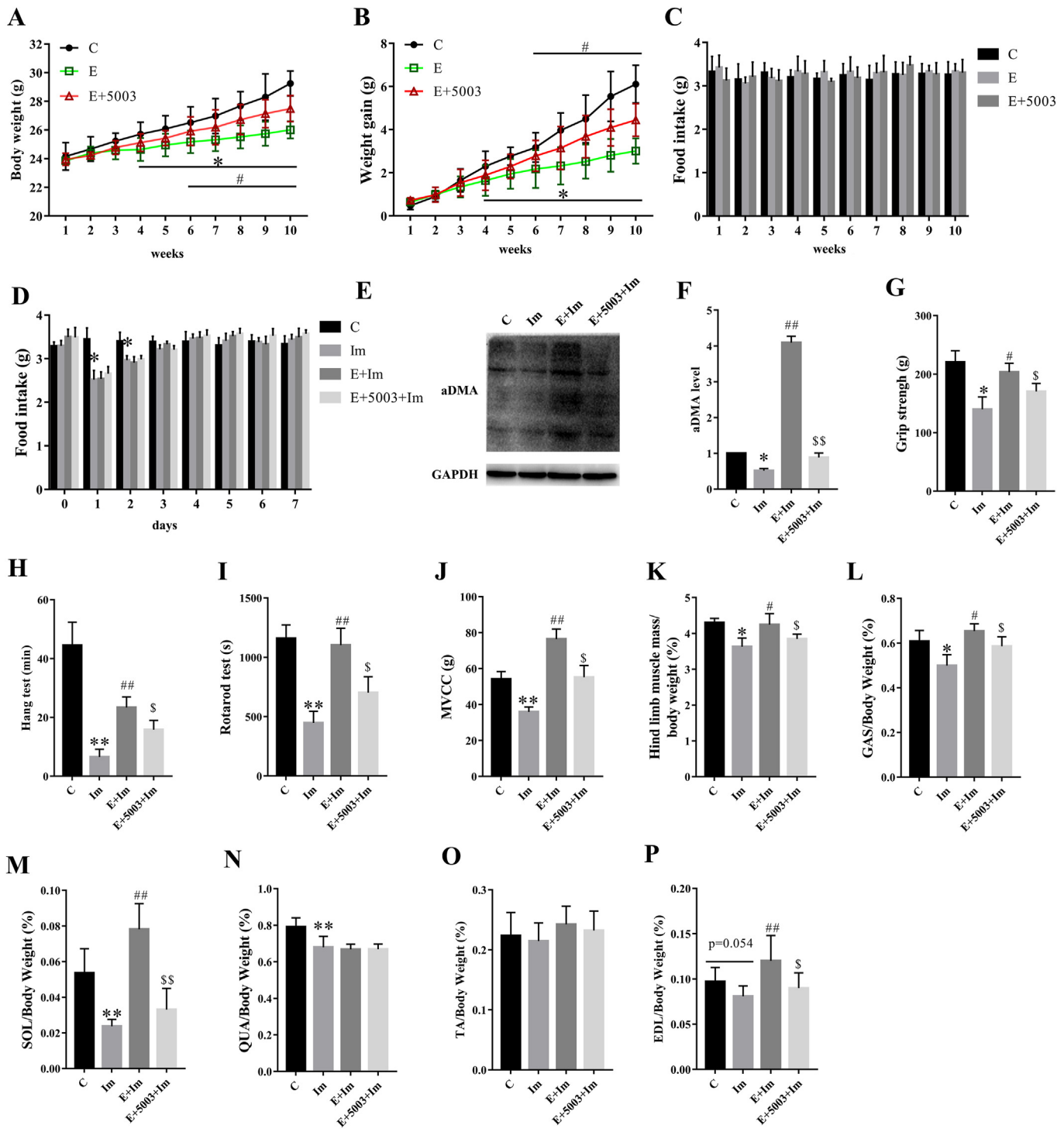

Fig. 1. 10-week combined exercise preconditioning prevented 1-week hindlimb immobilization-induced muscle atrophy in mice. (A–C) Body weight, gross weight gain and food intake data during 10-week exercise preconditioning. *$p < 0.05$ vs. C; #$p < 0.05$ vs. E. Two-way ANOVA was used and data are shown as means ± standard error of the mean (*SEM*) (C, $n = 16$, E, $n = 8$, E+5003, $n = 8$). (D) Food intake data during 1-week immobilization. *$p < 0.05$ vs. C. Two-way ANOVA was used and data are shown as means ± *SEM*. (E–F) aDMA level indicated for Prmt1 enzymatic activity after 10-week TC-E−5003 administration via subcutaneous injection at a dose of 2 mg/kg body weight, once daily, 5 days a week. (G–J) Skeletal muscle functions tests, G, grip strength, H, suspension time of hang test, I, time of the latency to fall in rotarod test, J, maximum voluntary climbing capacity (MVCC) test. (K–P) Total hindlimb mass and specific muscle mass of various parts of hindlimb, GAS, gastrocnemius, SOL, soleus, QUA, quadriceps femoris, lateralis, TA, tibialis anterior, EDL, extensor digitorum longus. H-S, *$p < 0.05$ vs. C; **$p < 0.01$ vs. C; #$p < 0.05$ vs. Im; ##$p < 0.01$ vs. Im; \$$p < 0.05$ vs. E + Im; \$\$$p < 0.01$ vs. E + Im. Two-way ANOVA was used and data are shown as means ± *SEM* (C, $n = 8$, Im, $n = 8$, E + Im, $n = 8$, E+5003+Im, $n = 8$).

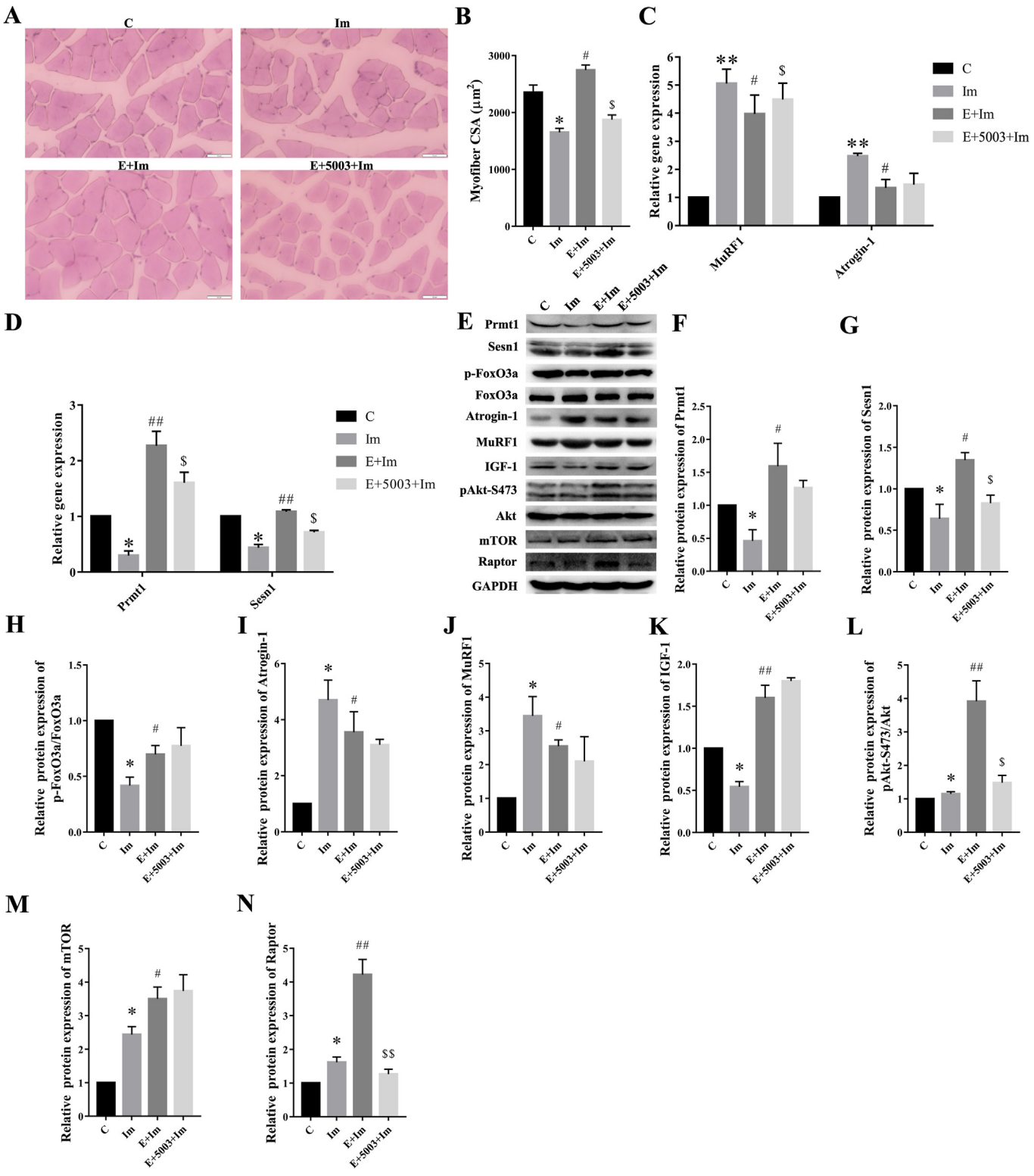

Fig. 2. 10-week combined exercise preconditioning ameliorated 1-week hindlimb immobilization-induced imbalance between synthesis and degradation of protein in skeletal muscle. (A) Representative images of hematoxylin and eosin staining (H&E) of GAS muscle cross-sections. Scale bar = 50 μm. (B) The average CSA of GAS muscle was quantified. (C–D) Real-time PCR results of TIRM63 (MuRF1), FBXO32 (Atrogin-1), Prmt1, and Sesn1 in GAS muscle. (E–N) Western blot results of Prmt1, Sesn1, p-FoxO3a, FoxO3a, Atrogin-1, MuRF1, IGF-1, pAkt-S473, Akt, mTOR, Raptor in GAS muscle. B-N, *$p < 0.05$ vs. C; **$p < 0.01$ vs. C; #$p < 0.05$ vs. Im; ##$p < 0.01$ vs. Im; $$p < 0.05$ vs. E + Im; $$$p < 0.01$ vs. E + Im. Two-way ANOVA was used and data are shown as means ± standard error of the mean (C, $n = 8$, Im, $n = 8$, E + Im, $n = 8$, E+5003+Im, $n = 8$).

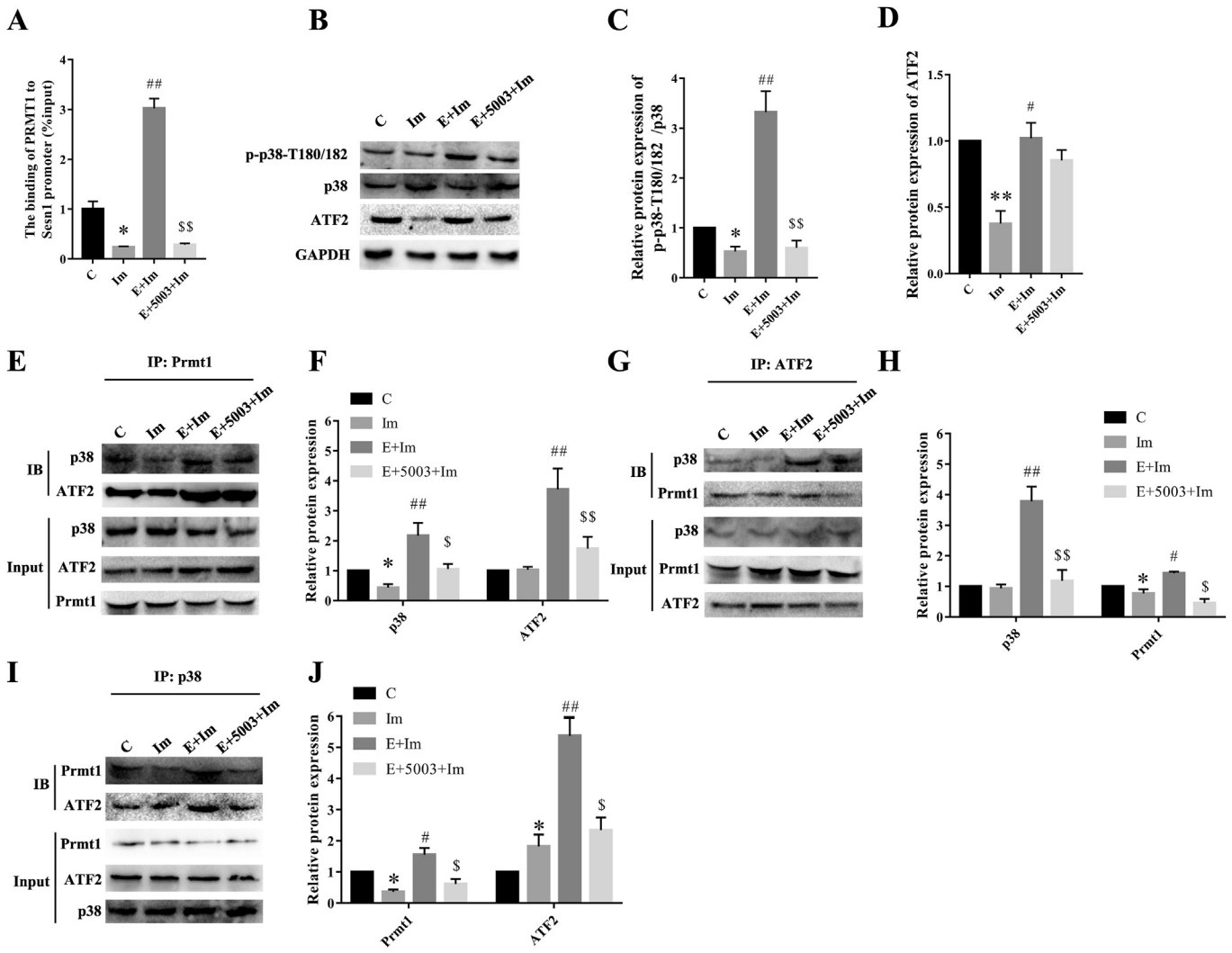

Fig. 3. Exercise preconditioning increased Sestrin1 (Sesn1) expression by activating protein arginine methyltransferase 1 (Prmt1)-p38/activating transcription factor 2 (ATF2) signaling in mice. (A) ChIP analysis of Prmt1 binding to the ATF2 sequence of mouse Sesn1 promoter in GAS muscle. (B–D) Western blot results of p-p38-T180/182, p38, and ATF2 in GAS muscle. (E–F) Co-IP results, IP: Prmt1, GAS muscle was used. (G–H) Co-IP results, IP: ATF2, GAS muscle was used. (I–J) Co-IP results, IP: p38, GAS muscle was used. A-J, *$p < 0.05$ vs. C; **$p < 0.01$ vs. C; #$p < 0.05$ vs. Im; ##$p < 0.01$ vs. Im; \$$p < 0.05$ vs. E + Im; \$\$$p < 0.01$ vs. E + Im. Two-way ANOVA was used and data are shown as means ± standard error of the mean (C, $n = 8$, Im, $n = 8$, E + Im, $n = 8$, E+5003+Im, $n = 8$).

whether Sesn1 acted as a downstream effector of Prmt1. To examine the role of Sesn1 in Prmt1 inhibiting protein degradation, C2C12 myotubes were treated with either si-Sesn1 or Ad-Sesn1 after Prmt1 overexpression. As shown in Fig. 4S–W, silencing Sesn1 disrupted the inhibitory effects of Prmt1 on the expression of FoxO3a, Atrogin-1, and MuRF1, which were diminished after Ad-Sesn1 treatment. Overall, these data indicated that Sesn1 mediated the effects of Prmt1 on inhibiting protein degradation.

3.5. *Exercise preconditioning activated AMPKα2-PGC-1α through Prmt1-Sesn1 in immobilized mice*

AMPKα2-PGC-1α was also activated by exercise through p38/ATF2, and multiple studies suggest that the expression of PGC-1α was modulated by Prmt1,[26,27] but it is still unclear if Sesn1 participated in it as well. The AMPKα2-PGC-1α signaling pathway was investigated as shown in Fig. 5A–C, 1-week immobilization decreased the AMPKα2 activity and PGC-1α level, which were reversed by exercise preconditioning. After inhibiting Prmt1 activity, the effects of exercise on activating AMPKα2-PGC-1α were disrupted, indicating that Prmt1 played an important role in modulating AMPKα2-PGC-1α during exercise training. Co-IP results in Fig. 5D–E revealed that immobilization prevented Prmt1 from binding to AMPKα2 and PGC-1α, but exercise increased their binding degree. Additionally, we also observed a decreased binding of PGC-1α with AMPKα2 and Prmt1 in the immobilized mice but an increased binding of them in E + Im group. However, these changes were reversed by TC-E−5003 administration (Fig. 5F–G), suggesting exercise preconditioning activated PGC-1α via enhancing Prmt1 from binding AMPKα2. In order to investigate the role of Sesn1 in activating PGC-1α in the mice of different groups, the interaction between Sesn1 and AMPKα2-PGC-1α was also examined. After Sesn1 was immunoprecipitated (Fig. 5H–I), the binding of AMPKα2 and PGC-1α to Sesn1 was also diminished by immobilization, and promoted in E + Im group. As expected, TC-E−5003 treatment weakened their binding compared to E + Im group. Additionally, the Co-IP results showed similar changes by immunoprecipitating PGC-1α (Fig. 5J–K) and AMPKα2 (Fig. 5L–M) in each group, implying that Sesn1 increased PGC-1α expression by activating AMPKα2 and this progress was also limited to the Prmt1 activity. In conclusion, these results suggested that 10-week combined exercise preconditioning activated AMPKα2-PGC-1α through Prmt1-Sesn1 in mice.

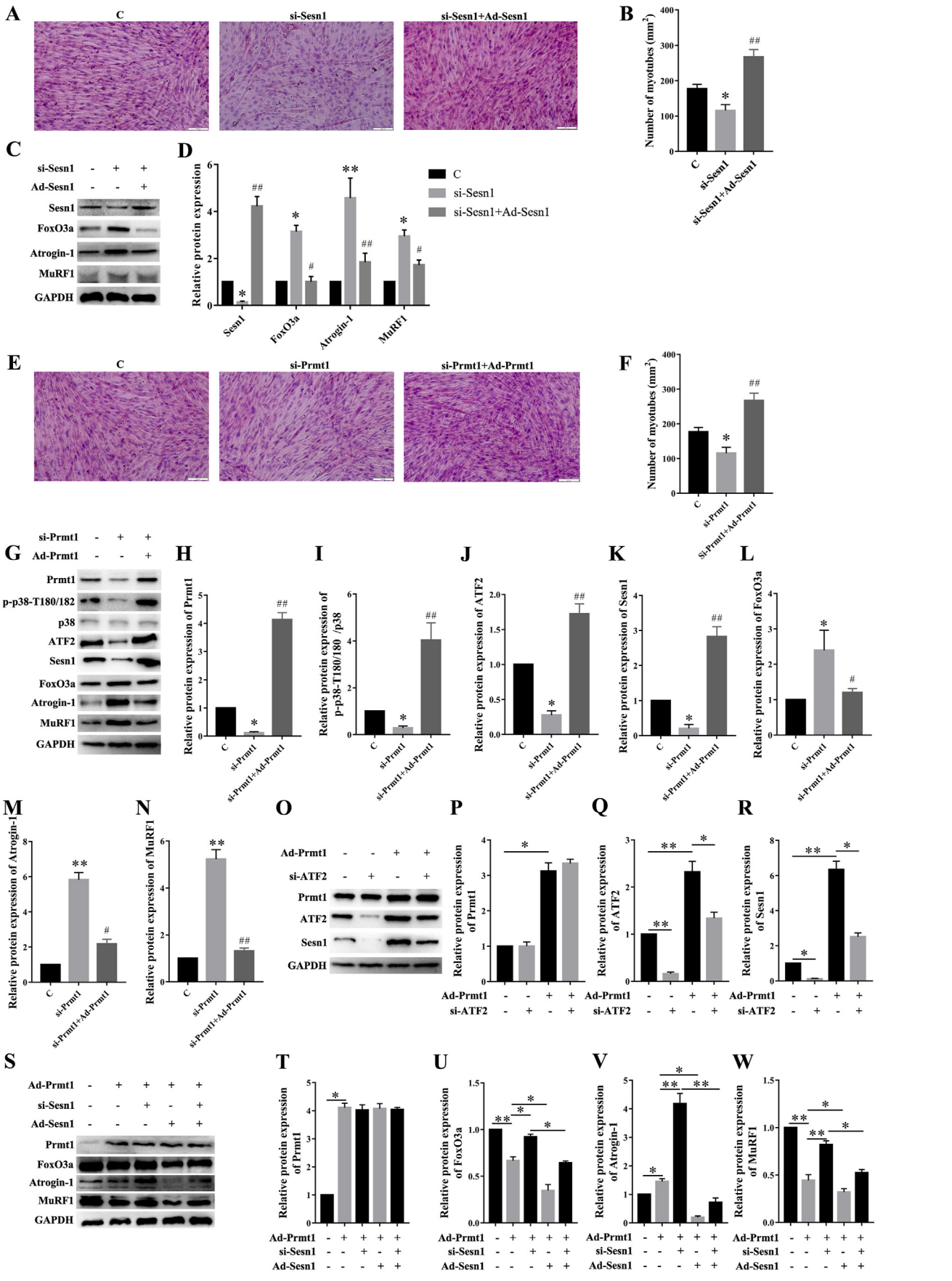

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Fig. 4. Protein arginine methyltransferase 1 (Prmt1)-Sestrin1 (Sesn1) coordinated the differentiation of C2C12 myoblasts into mature myotubes. (A–B) H&E staining of C2C12 myotubes at the 7th day of differentiation with si-Sesn, and Ad-Sesn1 treatment. Scale bar = 100 μm. (C–D) Western blot results of Sesn1, FoxO3a, Atrogin-1 and MuRF1 in C2C12. A-D, C, control, si-Sesn1, si-RNA of Sesn1, Ad-Sesn1, Adenoviruses overexpressing Sesn1, $*p < 0.05$ vs. C; $**p < 0.01$ vs. C; $\#p < 0.05$ vs. si-Sesn1; $\#\#p < 0.01$ vs. Si-Sesn1. Two-way ANOVA was used and data are shown as means ± standard error of the mean (*SEM*) ($n = 6$ in each group). (E–F) H&E staining of C2C12 myotubes at the 7th day of differentiation with si-Prmt1, and Ad-Prmt1. Scale bar = 100 μm. (G–N) Western blot results of Prmt1, p-p38-T180/182, p38, ATF2, Sesn1, FoxO3a, Atrogin-1 and MuRF1 in C2C12. E-N, C, control, si-Prmt1, si-RNA of Prmt1, Ad-Prmt1, Adenoviruses overexpressing Prmt1, $*p < 0.05$ vs. C; $**p < 0.01$ vs. C; $\#p < 0.05$ vs. si-Prmt1; $\#\#p < 0.01$ vs. si-Prmt1. Two-way ANOVA was used and data are shown as means ± *SEM* ($n = 3$ in each group). (O–R) Western blot results of Prmt1, ATF2 and Sesn1 in C2C12 myoblasts, $*p < 0.05$; $**p < 0.01$, unpaired Student's *t*-test was used and data are shown as means ± *SEM* ($n = 6$ in each group). (S–W) Western blot results of Prmt1, FoxO3a, Atrogin-1 and MuRF1 in C2C12 myoblasts, $*p < 0.05$; $**p < 0.01$, unpaired Student's *t*-test was used and data are shown as means ± *SEM* ($n = 6$ in each group).

3.6. Exercise preconditioning prevented muscle atrophy through Prmt1-Sesn1-PGC-1α mediated skeletal muscle regeneration

Previous study suggested that there was mutual regulation between PGC-1α and MyoD.[28] Under conditions of skeletal muscle injury or aging, the expression of MyoD was upregulated which in turn increased MyoG level to trigger muscle regeneration, subsequently they coordinated the activation of another two key factors including Myf5 and MEF2. In addition, Prmt1 enhanced MyoD activity and promoted MyoD mediated MyoG activation during muscle regeneration.[28,29] Considering our previous results found that Prmt1-Sesn1 coordinated the differentiation of C2C12 myoblasts, we inferred that PGC-1α may play a key role in muscle regeneration. As expected, the expression levels of the myogenic marker genes including MyoD, Myf5, MEF2, and MyoG were significantly downregulated in the Im group, increased in E + Im group, and decreased again after TC-E−5003 administration (Fig. 6A–B). Then we further verified the relationship between Prmt1 and MyoD. As shown in Fig. 6C–D and Fig. 5D–E, exercise preconditioning markedly reversed the diminished binding of Prmt1 to MyoD and PGC-1α induced by immobilization, which was also blunted by the inhibition of Prmt1 activity, suggesting that Prmt1 also enhanced the expression of MyoD and PGC-1α by protein–protein interaction during the exercise training. To determine whether Sesn1 was involved in PGC-1α-induced muscle regeneration, C2C12 myoblasts were transfected with Ad-Sesn1, and harvested at different time points during differentiation. The results showed that Sesn1 overexpression increased the number of myotubes at various days of differentiation (Fig. 6E-F), consisting with our previous result that Sesn1 promoted C2C12 cell differentiation. In addition, the expression levels of Sesn1, Prmt1, PGC-1α, and the genes involved in muscle regeneration composed of MyoD/MEF2/MyoG increased gradually with the prolongation of C2C12 cell differentiation time (Fig. 6G–H), although with no significant changes in Myf5. In view of the role of Sesn1 and PGC-1α in C2C12 differentiation, we speculated that nuclear translocation of PGC-1α was promoted by Sesn1 during C2C12 myoblasts differentiation. As shown in Fig. 6I–J, the expression of PGC-1α increased in the nucleus after Sesn1 overexpression during C2C12 differentiation. Moreover, Sesn1 also increased the level of PGC-1α in cytoplasm (Fig. 6K–L). In conclusion, these results suggested that Sesn1 overexpression promoted the translocation of PGC-1α into nucleus, subsequently activating muscle regeneration by upregulating the expression of MyoD, MyoG, and MEF2.

4. Discussion

Disuse muscle atrophy resulting from reduced physical activity because of soreness or disabilities caused by different type of diseases was common in multiple types of muscle atrophy.[30] Exercise training has been proven to be the most feasible and effective treatment for muscle atrophy.[31]

Considering the distinctive features of resistance exercise, which promotes skeletal muscle anabolism while simultaneously inhibiting catabolism, as well as the notable benefits of aerobic exercise in improving skeletal muscle mitochondrial activity and energy metabolism,[32,33] combined exercise was performed in mice before immobilization in this study. We found that exercise preconditioning could prevent immobilization-induced muscle mass and exercise capacity reduction. Moreover, exercise inhibited muscle protein breakdown via downregulating Atrogin-1, MuRF1, and FoxO3a. In addition, muscle protein synthesis was promoted by exercise, supporting by the higher expression level of IGF-1, pAkt-S473, mTOR, and Raptor compared with Im group. Study has demonstrated that IGF-1 can activate the phosphoinositide 3-kinase (PI3K)/Akt/mTOR signaling pathway, thereby promoting skeletal muscle protein synthesis.[34] Unexpectedly, Akt activity and the expression levels of mTOR and Raptor were all increased after immobilization. Considering 1-week immobilization serves as a short-term intervention, it is reasonable to elicit some adaptive responses in skeletal muscle, manifesting as the elevations in the expression of some key genes involved in protein synthesis.

Although the phenotype study suggests the myoprotective function of Prmt1 in muscle atrophy,[13] no study has examined whether prmt1 is involved in exercise preconditioning to prevent disuse muscle atrophy. In the present study, we found that the expression of Prmt1 decreased in the Im group, but increased in the E + Im group, and Prmt1 inhibition diminished the benefits of exercise training in preventing muscle atrophy and dysfunction. Therefore, this is the first study to demonstrate that Prmt1 is up-regulated by long-term exercise training in the skeletal muscle of C57BL/6J mice, and it mediates the protective effects of exercise preconditioning against immobilization-induced muscle atrophy. Additionally, our in vitro finding demonstrated that Prmt1 overexpression was able to mimic the protective effects of exercise training for decreasing the expression of atrogenes and facilitate the formation of mature myotubes. In summary, Prmt1 plays an important role in exercise preconditioning to prevent skeletal muscle atrophy and serves as a potential biomarker and novel therapeutic target to counteract sarcopenia.

Sesn1 is essential for the maintenance of skeletal muscle function and a decreased expression of Sesn1 has been observed in multiple models of muscle atrophy.[18,32] Consistently, immobilization significantly reduced Sesn1 protein expression. Besides, our results showed that Sesn1 overexpression promoted the differentiation of C2C12 myoblasts into mature myotubes and significantly reversed the expression of atrogenes. No changes in glucose homeostasis and running capacity were detected in $Sesn1^{-/-}$ mice compared to the WT counterparts during exercise training,[19] suggesting that Sesn1 is involved in the muscle response to exercise, we proposed that Sesn1 expression may be upregulated by exercise training, which has been controversial in recent years.[35,36] Surprisingly, our findings firstly revealed that a 10-week combined exercise significantly increased the expression of Sesn1 in the E + Im group compared with the Im group. In our view, such inconsistencies were cause by the application of different types, durations, intensities, and frequency of exercise training. Given the results described above, the mechanism by which exercise preconditioning protected muscle atrophy against immobilization may be associated with combined exercise training elevating the expression of Sesn1.

Despite catalyzing the formation of monomethyl and asymmetric dimethylarginine in protein substrates,[8] Prmt1 also regulates cellular gene transcription by binding to various transcriptional regulatory factors, including ATFs, to form transcriptional co-activators, which bind to the promoter regions of relevant genes. Although there is no definitive evidence to prove that ATF2 could regulate the transcription of Sesn1, potential ATF2 binding sequences have been found in the promoter

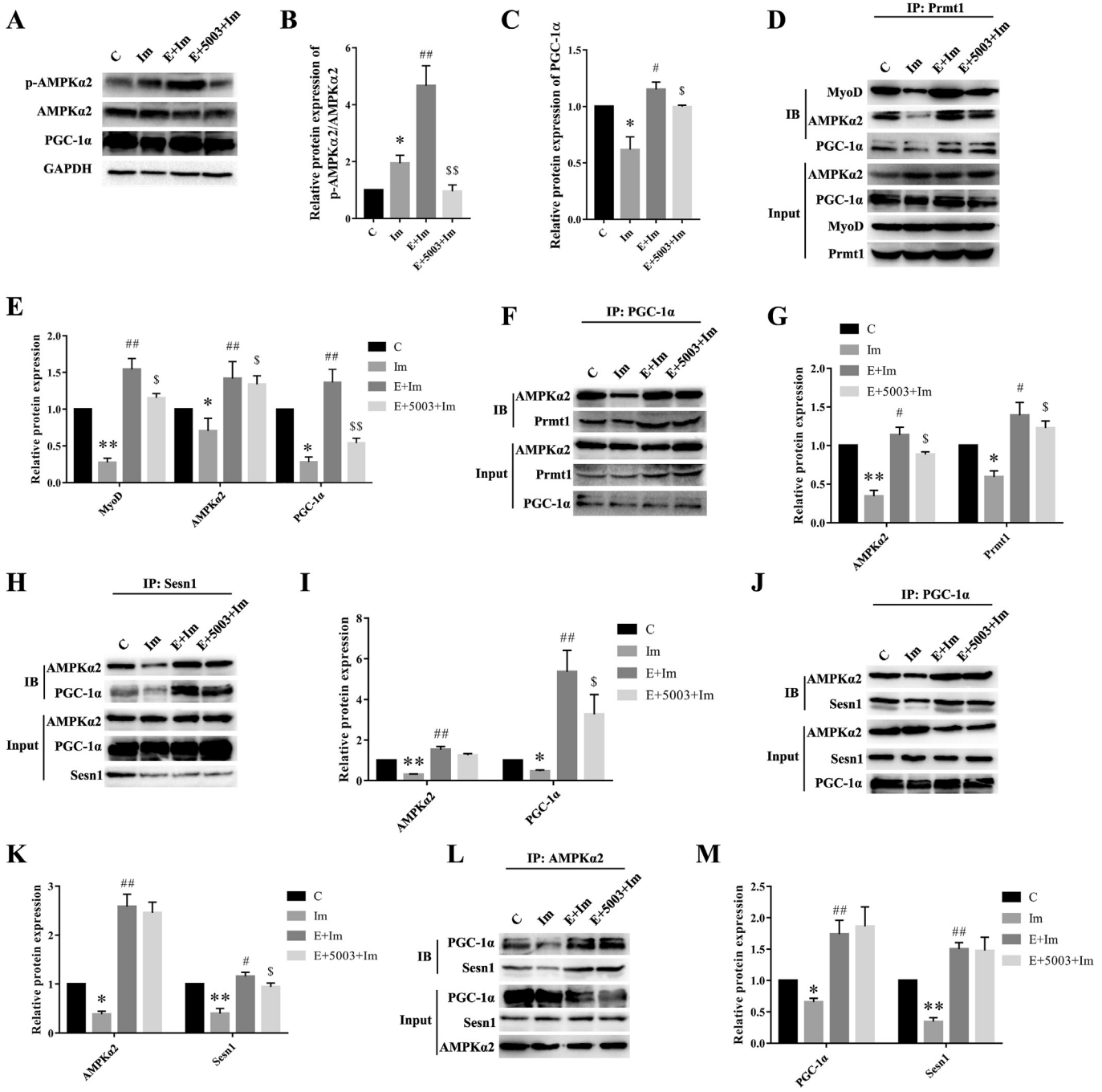
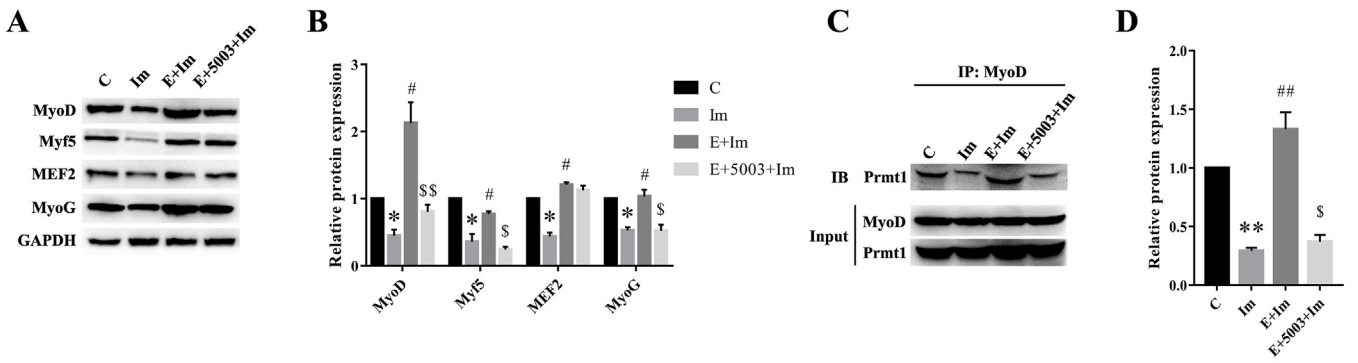
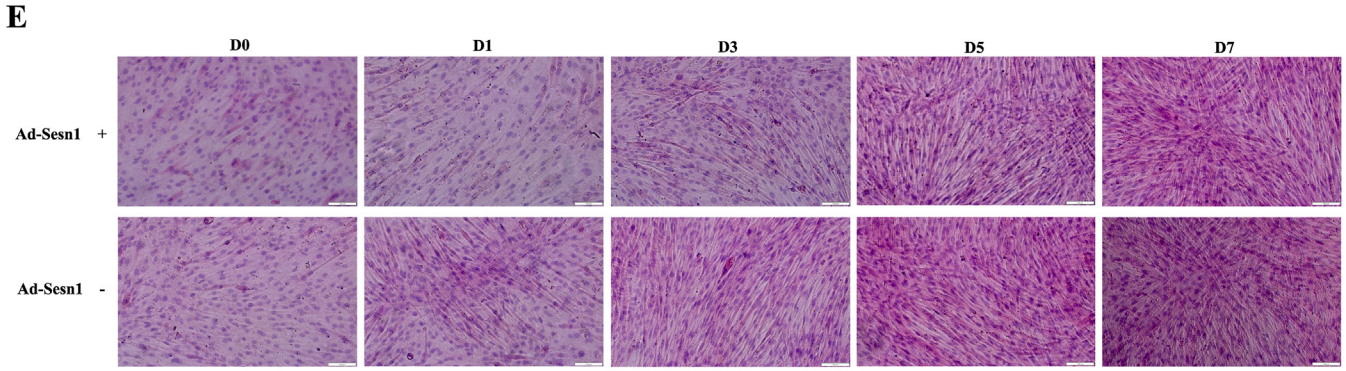
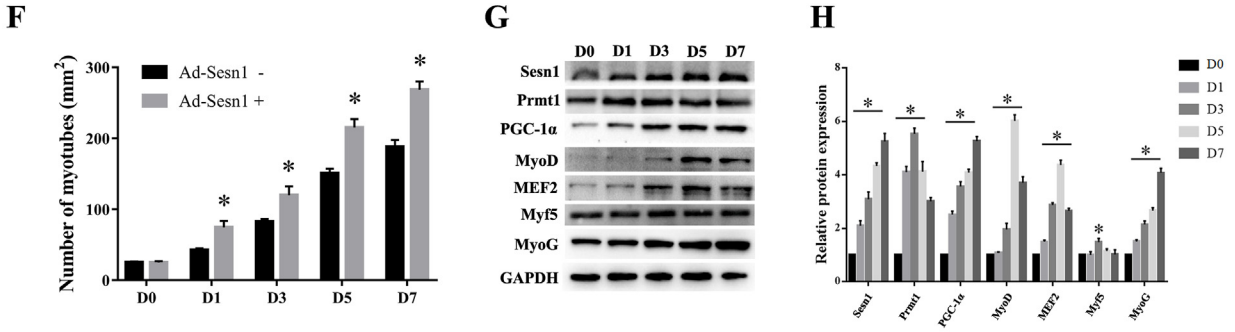


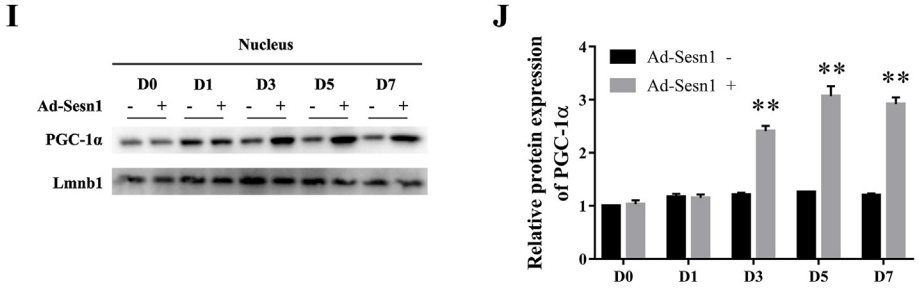
Fig. 5. Exercise preconditioning activated AMP-Activated protein kinase α2 **(AMPKα2)-transcriptional co-activator PPAR-γ co-activator-1 α (PGC-1α) through protein arginine methyltransferase 1 (Prmt1)-Sestrin1 (Sesn1) in immobilized mice.** (A–C) Western blot results of p-AMPKα2, AMPKα2, and PGC-1α in GAS muscle. (D–E) Co-IP results, IP: Prmt1, GAS muscle was used. (F–G) Co-IP results, IP: PGC-1α, GAS muscle was used. (H–I) Co-IP results, IP: Sesn1, GAS muscle was used. (J–K) Co-IP results, IP: PGC-1α, GAS muscle was used. (L–M) Co-IP results, IP: AMPKα2, GAS muscle was used. A-M, $^{*}p < 0.05$ vs. C; $^{**}p < 0.01$ vs. C; $\#p < 0.05$ vs. Im; $\#\#p < 0.01$ vs. Im; $\$p < 0.05$ vs. E + Im; $\$\$p < 0.01$ vs. E + Im. Two-way ANOVA was used and data are shown as means ± standard error of the mean (C, $n = 8$, Im, $n = 8$, E + Im, $n = 8$, E+5003+Im, $n = 8$).

region of Sesn1 in various human cancer cell lines.[21] Based on previous research and our bioinformatics analysis, Prmt1 might interact with ATF2 at the protein level (String database), and two ATF2-binding sequences on the promoter of Sesn1 were predicted (Supplementary Table 2). Besides this, Prmt1 could increase the expression of ATF2 by activating p38 in human leukemia cancer cell,[22] but whether such observation is available in skeletal muscle remains unclear. In our study, we found that the expression of Sesn1 was decreased in the E+5003+Im group, which confirmed Sesn1 is regulated by Prmt1. Subsequently, we further demonstrated that there were interactions between Prmt1, p38, ATF2, and Sesn1, and their binding levels raised in the E + Im group, but decreased after TC-E−5003 treatment. Meanwhile, overexpression of Prmt1 increased the levels of ATF2 and Sesn1 in C2C12 myotubes. After ATF2 silencing, the effect of Ad-Prmt1 on promoting Sesn1 expression was greatly blunted. Expectedly, the effect of Ad-Prmt1 on suppressing the expression of atrogenes was also diminished after Sesn1 silencing. Taken together, these results suggest a novel mechanism of Prmt1 in exercise preconditioning preventing muscle atrophy by binding to ATF2









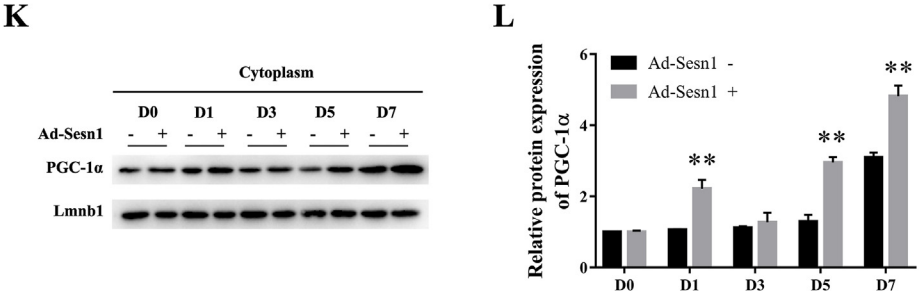


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Fig. 6. Exercise preconditioning prevented muscle atrophy through protein arginine methyltransferase 1 (Prmt1)-Sestrin1 (Sesn1)-transcriptional co-activator PPAR-γ co-activator-1 α (PGC-1α)-mediated skeletal muscle regeneration.
(A–B) Western blot results of MyoD, Myf5, MEF2 and MyoG in GAS muscle. (C–D) Co-IP results, IP: MyoD, GAS muscle was used. A-B, $^{*}p < 0.05$ vs. C; $^{**}p < 0.01$ vs. C; $^{\#\#}p < 0.01$ vs. Im; $\$p < 0.05$ vs. E + Im; $\$\$p < 0.01$ vs. E + Im. Two-way ANOVA was used, and data are shown as means ± standard error of the mean (*SEM*) (C, $n = 8$, Im, $n = 8$, E + Im, $n = 8$, E+5003+Im, $n = 8$). (E–F) H&E staining of myotubes at each differentiation time points of C2C12 myoblasts overexpressing Sesn1. Scale bar = 100 μm $^{*}p < 0.05$ vs. Ad-Sesn1-, unpaired Student's t-test was used and data are shown as means ± *SEM* ($n = 3$ in each group). (G–H) Western blot results of Sesn1, Prmt1, PGC-1α, MyoD, MEF2, Myf5, and MyoG in C2C12 myoblasts at each time points of differentiation. $^{*}p < 0.05$ vs. D0, unpaired Student's *t*-test was used and data are shown as means ± *SEM* ($n = 6$ in each group). (I–J) Western blot results of PGC-1α in nucleus of C2C12 myoblasts overexpressing Sesn1 at each time points of differentiation. $^{**}p < 0.01$ vs. Ad-Sesn1, unpaired Student's *t*-test was used and data are shown as means ± *SEM* ($n = 6$ in each group). (K–L) Western blot results of PGC-1α in cytoplasm of C2C12 myoblasts overexpressing Sesn1 at each time points of differentiation. $^{**}p < 0.01$ vs. Ad-Sesn1-, unpaired Student's *t*-test was used and data are shown as means ± *SEM* ($n = 6$ in each group).

to form transcriptional co-activator, which binds to the promoter regions of Sesn1, thus controlling the expression of it.

PGC-1α is a key regulator of neuronal and muscular plasticity, and it is well known that the AMPK-PGC-1α signaling pathway mediates the beneficial effects of exercise on skeletal muscle metabolism.[37] Exercise training enhances mitochondria homeostasis including mitochondrial biogenesis, and improving mitophagy through upregulation of PGC-1α expression.[38,39] Previous studies have clearly shown that upregulation of PGC-1α expression inhibits the FoxO3-MuRF1/Atrogin-1 axis, thereby suppressing the excessive activation of protein degradation signaling pathway.[40] Besides, activation of the p38-MAPK signaling pathway promotes the binding of ATF2 and MEF2 to the promoter of the PGC-1α gene, thereby increasing its protein expression.[41] Additionally, MyoD, which participates in muscle cell differentiation and muscle regeneration, not only acts as an upstream regulator of PGC-1α expression but is also regulated by PGC-1α itself.[42] Elevated expression of MyoD further promotes the expression of MyoG, which in turn leads to an upregulation of subsequent Myf5 expression. Consequently, the coordinated actions of MyoD/MyoG/Myf5 initiate the differentiation of muscle cells and muscle regeneration.

Prmt1 plays a pivotal role in promoting skeletal muscle cell differentiation and regeneration by activating MyoD and subsequently enhancing MyoG transcription.[43] Prmt1 can directly promote the activation of PGC-1α through methylation.[44] In this study, immobilization decreased the expression of PGC-1α and genes involved in muscle regeneration, which was accompanied by a reduction in the phosphorylation of AMPKα2. We also established that exercise preconditioning enhanced the interaction between Prmt1, Sesn1, AMPKα2, and PGC-1α. Besides, we found that the expression of PGC-1α was regulated by Prmt1 and Sesn1 through in vitro and in vivo experiments. During differentiation of myoblasts to myotubes, Sesn1 increased the expression of PGC-1α and promoted its nuclear translocation.

In conclusion, this study investigated the effects of combined exercise preconditioning on immobilization-induced skeletal muscle atrophy, revealing the importance of Prmt1 in maintaining muscle mass and function during exercise. We also unraveled the mechanism by which Sesn1, was induced by exercise training through Prmt1-p38/ATF2 axis, namely by activating PGC-1α to diminish protein degradation and promote skeletal muscle regeneration during exercise training.

CRediT authorship contribution statement

Xuege Yang: Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Yuchen Zou:** Investigation, Formal analysis, Data curation, Writing – review & editing, Supervision, Funding acquisition. **Haoyu Wang:** Investigation, Data curation. **Yanmei Niu:** Supervision, Project administration, Funding acquisition, Formal analysis, Conceptualization. **Li Fu:** Writing – review & editing, Funding acquisition.

Ethical approval statement for animal use

The mice were housed in a temperature-controlled environment of 22 °C-24 °C, and 40%–70% humidity, with 12 h light/dark cycles and received food and water *ad libitum*. All animal experiments were performed in accordance with Tianjin Medical University Animals Care and Use Committee and carried out following the Chinese Academy of Sciences guidelines (approval number: SYXK-2019-0004).

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests. Li Fu is an Editorial Board Member for Sports Medicine and Health Science and was not involved in the editorial review or the decision to publish this article. Li Fu reports the funding for this study was provided by National Natural Science Foundation of China.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.smhs.2025.04.001.

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