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# Effect of resistance training on satellite cells in old mice – a transcriptome study

## IMPLICATIONS FOR SARCOPENIA

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### Aims

The decrease in the number of satellite cells (SCs), contributing to myofibre formation and reconstitution, and their proliferative capacity, leads to muscle loss, a condition known as sarcopenia. Resistance training can prevent muscle loss; however, the underlying mechanisms of resistance training effects on SCs are not well understood. We therefore conducted a comprehensive transcriptome analysis of SCs in a mouse model.

### Methods

We compared the differentially expressed genes of SCs in young mice (eight weeks old), middle-aged (48-week-old) mice with resistance training intervention (MID+ T), and mice without exercise (MID) using next-generation sequencing and bioinformatics.

### Results

After the bioinformatic analysis, the PI3K-Akt signalling pathway and the regulation of actin cytoskeleton in particular were highlighted among the top ten pathways with the most differentially expressed genes involved in the young/MID and MID+ T/MID groups. The expression of *Gng5*, *Atf2*, and *Rtor* in the PI3K-Akt signalling pathway was higher in the young and MID+ T groups compared with the MID group. Similarly, *Limk1*, *Arhgef12*, and *Araf* in the regulation of the actin cytoskeleton pathway had a similar bias. Moreover, the protein expression profiles of *Atf2, Rptor*, and *Ccnd3* in each group were paralleled with the results of NGS.

### Conclusion

Our results revealed that age-induced muscle loss might result from age-influenced genes that contribute to muscle development in SCs. After resistance training, age-impaired genes were reactivated, and age-induced genes were depressed. The change fold in these genes in the young/MID mice resembled those in the MID + T/MID group, suggesting that resistance training can rejuvenate the self-renewing ability of SCs by recovering age-influenced genes to prevent sarcopenia.

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**Keywords:** Resistance training, Satellite cells, Age-induced sarcopenia

### Article focus

- To explore the underlying mechanisms of resistance exercise on satellite cells (SCs) in middle-aged mice by transcriptome analysis.

### Key messages

- In the forelimb grip strength test, resistance training prevented the age-induced muscle strength loss.
- Resistance training reversed the age-induced shifting proportion of muscle fibre.
- After resistance training, the age-impaired genes were reactivated, and the age-induced genes were depressed. The fold change of these genes in the young/MID group resembled those in the MID + T/MID group.
- Resistance training can rejuvenate the ability of SCs to self-renew by recovering

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the age-influenced genes, thereby preventing sarcopenia.

## Strengths and limitations

- The study found that resistance training prevented age-induced muscle loss by stimulating age-impaired genes and depressing age-induced genes, beneficial for muscle formation, thereby preventing sarcopenia.
- We did not figure out which pathway linked the resistance training to age-influenced genes in the SCs.

## Introduction

The ageing of the population has become a serious worldwide public health problem, increasing the emphasis on geriatric medicine. With age, numerous organ systems begin to functionally decline. In humans, age-related loss of muscle mass and decreased function can occur as early as 50 years,[1] a condition termed 'sarcopenia', which primarily occurs in the legs,[2,3] impairing physical activity and increasing the risk of falls,[4,5] leading to frailty, reduced capacity for independent living, and subsequent high healthcare costs.

Satellite cells (SCs), as known as muscle stem cells, are located between the myofibre plasma and basement membranes. The self-renewing ability of SCs not only maintains their population but also provides numerous myogenic cells for new myofibre formation and reconstitution of the muscle contractile apparatus.[6] In general, SCs are quiescent and stable under normal conditions. Quiescent SCs will activate the self-renewing process for the routine homeostasis of muscle, and will activate sporadic myofibre regeneration in response to muscle injury.[7] It has been reported that SC content decreases in the muscles of older adults and animals when compared with younger ones.[8-10] As age progresses, the number of SCs will decrease, and their proliferative capacity will diminish. These processes will cause a decline in myoblast proliferation and weaken myogenesis, leading to impaired muscle reconstitution following an injury.[11-13] A decrease in the number of SCs is therefore responsible for sarcopenia.

A number of studies have revealed the beneficial effect of resistance training on skeletal muscle in older adults.[14-17] A recent study showed that extra resistance exercise boosts the contribution of SCs to myofibre;[18] however, the underlying mechanisms of resistance exercise that affect SCs are not well understood. To explore these mechanisms, we conducted a comprehensive transcriptome analysis of SCs in middle-aged (MID) mice (48 weeks old), with or without resistance training, using next-generation sequencing (NGS), and analyzed the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment of the differentially expressed genes. Young (eight-week-old) mice were included in the comparison as a normal control. We hypothesized that resistance training would affect the expression of age-influenced genes in SCs.

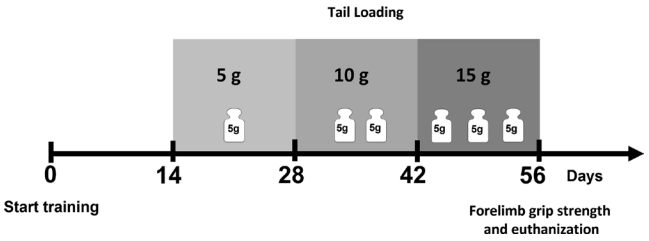


Fig. 1

Timeline of the weight loading and resistance training.

## Methods

**Animals and experiment design.** We employed two cohorts of young and middle-aged male BALB/c mice purchased from a commercial supplier (six mice per group).[19-21] The middle-aged mice were randomly divided into a progressive resistance training group (MID+ T) and a non-exercise group (MID). Mice in the MID+ T group underwent progressive resistance training on a treadmill with tail loading for eight weeks. The resistance training protocol has been described previously,[22,23] with subsequent modifications. Before training, the MID+ T mice were acclimated to the treadmill during week 1: on day 1, a five-minute run; on day 2, a ten-minute run; and on day 3, a 15-minute run followed by full-intensity exercise (10° incline; velocity, 10 m/min; duration, 30 mins/day). The frequency of resistance training was six days per week. After acclimatization, the MID+ T mice ran without weight-loading at full intensity for two weeks. The tail-load weight was then increased by 5 g every two weeks until a maximum weight of 15 g was reached (Figure 1). In the end, we collected blood from the mice and then euthanized them using carbon dioxide asphyxiation. All experimental procedures were approved by the Institutional Animal Care and Use Committee and complied with the Guidelines for the Care and Use of Laboratory Animals available through the National Academy of Sciences (IACUC number: 2017103101). All assessments in groups were done by the same experienced investigator (JSH), who was blinded to the animal allocation. We have adhered to the ARRIVE guidelines and supplied an ARRIVE checklist to show this (Supplementary Material).

**Forelimb grip strength test.** We measured mouse forelimb grip strength using a grip strength metre (Model: 47200; Ugo Basile, Italy) in accordance with a modified forelimb grip strength test.[24] While the mice grasped the vertically fixed T-bar with their forelimbs, we slowly pulled back on their tail. The force measurement was recorded until the mice released the T-bar; this was recorded for six repetitions for each mouse. The normalized grip strength

was obtained by dividing the mean grip strength by each mouse's body weight (n = 6).

**Muscle-related serum biomarker.** We measured the levels of muscle-related serum biomarkers, including secreted protein acidic and rich in cysteine (SPARC),[25] myostatin (growth/differentiation factor-8),[26] and musclin[27] (also named osteocrin) using customized MILLIPLEX multiplex assays and Luminex (MilliporeSigma, USA) following the manufacturer's instructions (n = 6).

**Muscle cryosections and immunofluorescence assay.** After euthanizing the mice, we froze the rectus femoris muscle from one hindlimb in liquid nitrogen-cooled isopentane for muscle cryosection, as previously described.[28] We transferred transverse cryosections (8 µm) to positively charged glass slides and stored them at –80°C. The transverse cryosections were fixed in ice-cold acetone, blocked with 5% bovine serum albumin, and incubated with anti-Pax7 (DHSB, USA), and anti-Laminin (ab11575, Abcam, UK) for staining satellite cell and basement membrane of muscle fibre, with anti-MYH7 (clone: BA-F8, DHSB) and anti-MYH2 (clone: SC-71, DHSB) antibodies for staining muscle fibre. To label the nuclear DNA, we stained the cells with 0.1 µg/ml of Hoechst 33,342 solution (Sigma-Aldrich, USA) for 30 minutes. The slide images were visualized with the Olympus BX51 microscope system (Olympus, Japan) (n = 6), or Leica TCS SP5 confocal microscope (Leica Microsystems, Germany) (n = 6).

**Primary satellite cell isolation.** After dissociating the hindlimb skeletal muscles including the gastrocnemius, tibialis anterior, soleus, triceps, and quadriceps from each group, we homogenized the muscles using a gentleMACS Dissociator (Miltenyi Biotec, Germany) and then purified the single cells using a MACS Satellite Cell Isolation Kit, mouse (130-104-268, Miltenyi Biotec) according to the manufacturer's instructions. The purified primary skeletal SCs were transferred into a 10 cm petri dish containing 20% horse serum Dulbecco's Modified Eagle Medium (ThermoFisher Scientific, USA) and then left for seven days. To verify the seven-day cultured SCs, we transferred the SCs onto the coverslips and performed immunofluorescence assay (IFA) with Pax7 (DHSB) and CD34 (orb348961, Biorbyt, UK) antibody, SC markers, (Supplementary Figure a). The characterized SCs were subjected to total RNA extraction for next-generation sequencing analysis.

**RNA extraction and next-generation sequencing by Illumina HiSeq.** The experimental process of transcriptomic sequencing includes quality assessment, sequencing library construction, purification, quality assessment, and quantification, followed by flow cell cluster formation and sequencing (Supplementary Figure b). We extracted total RNA from each sample using TRIzol Reagent (Thermo Fisher Scientific)/RNeasy Mini Kit (Qiagen, Germany) and quantified and qualified the total RNA using the Agilent 2100 Bioanalyzer (Agilent Technologies, USA), NanoDrop (Thermo Fisher Scientific), and 1% agarose gel. We employed 1 µg of total RNA with an RNA integrity number value above 7 for the following library preparation, and constructed the next-generation sequencing library preparations according to the manufacturer's protocol (NEBNext Ultra Directional RNA Library Prep Kit for Illumina, USA). We depleted the rRNA from total RNA using a Ribo-Zero Gold Kit (Illumina). The ribosomal-depleted RNA was then fragmented and reverse-transcribed. We synthesized first-strand cDNA using ProtoScript II Reverse Transcriptase with random primers and actinomycin D. The second-strand cDNA was synthesized using a Second Strand Synthesis Enzyme Mix (which includes dACG-TP/2´-Deoxyuridine, 5´-Triphosphate (dUTP)). The purified double-stranded complementary DNA by AxyPrep Mag PCR Clean-up (Axygen) was then treated with End Prep Enzyme Mix to repair both ends and add a dA-tailing in one reaction, followed by a T-A ligation to add adaptors to both ends. Size selection of adaptor-ligated DNA was then performed using AxyPrep Mag PCR Clean-up (Axygen, USA), and fragments of approximately 360 bp (with the approximate insert size of 300 bp) were recovered. The dUTP-marked second strand was digested with Uracil-Specific Excision Reagent (USER) enzyme (New England Biolabs, USA). We then amplified each sample by polymerase chain reaction (PCR) for 11 cycles using P5 and P7 primers, with both primers carrying sequences that can anneal with flow cell to perform bridge PCR and a P7 primer carrying a six-base index allowing for multiplexing. The PCR products were cleaned up using AxyPrep Mag PCR Clean-up (Axygen), validated using an Agilent 2100 Bioanalyzer (Agilent, USA), and quantified by Qubit 2.0 Fluorometer (Thermo Fisher Scientific). We then multiplexed the libraries with various indices and loaded them onto an Illumina HiSeq instrument according to the manufacturer's instructions (Illumina). Sequencing was performed using a 2 × 150 paired-end configuration; image analysis and base calling were conducted by the HiSeq Control Software (HCS)+ OLB + GAPipeline-1.6 (Illumina) on the HiSeq instrument. The sequences were processed and analyzed by GENEWIZ. The raw sequence data were deposited at the BioProject website (BioProject accession: PRJNA693114).

**Quality control.** The Illumina index adapters and low-quality bases of sequences were removed using Trimmomatic (v0.30) to obtain a high-quality clean data set.

**Expression analysis.** The transcript sequences were extracted from the known reference genome sequence and gff annotation file. The trimmed reads were then mapped to this transcript set, and the results were subjected to an estimation of gene/transcript expression levels using RNA-Seq by Expectation-Maximization (RSEM) pipeline (RSEM, v1.2.6).

**Differential expression analysis.** We performed differential expression analyses using the DESeq2 Bioconductor package, a model based on negative binomial distribution. After adjusting by the Benjamini and Hochberg's approach for multiple testing correction, the adjusted p-value (FDR) threshold was set to 0.05 to detect differential expressed values.

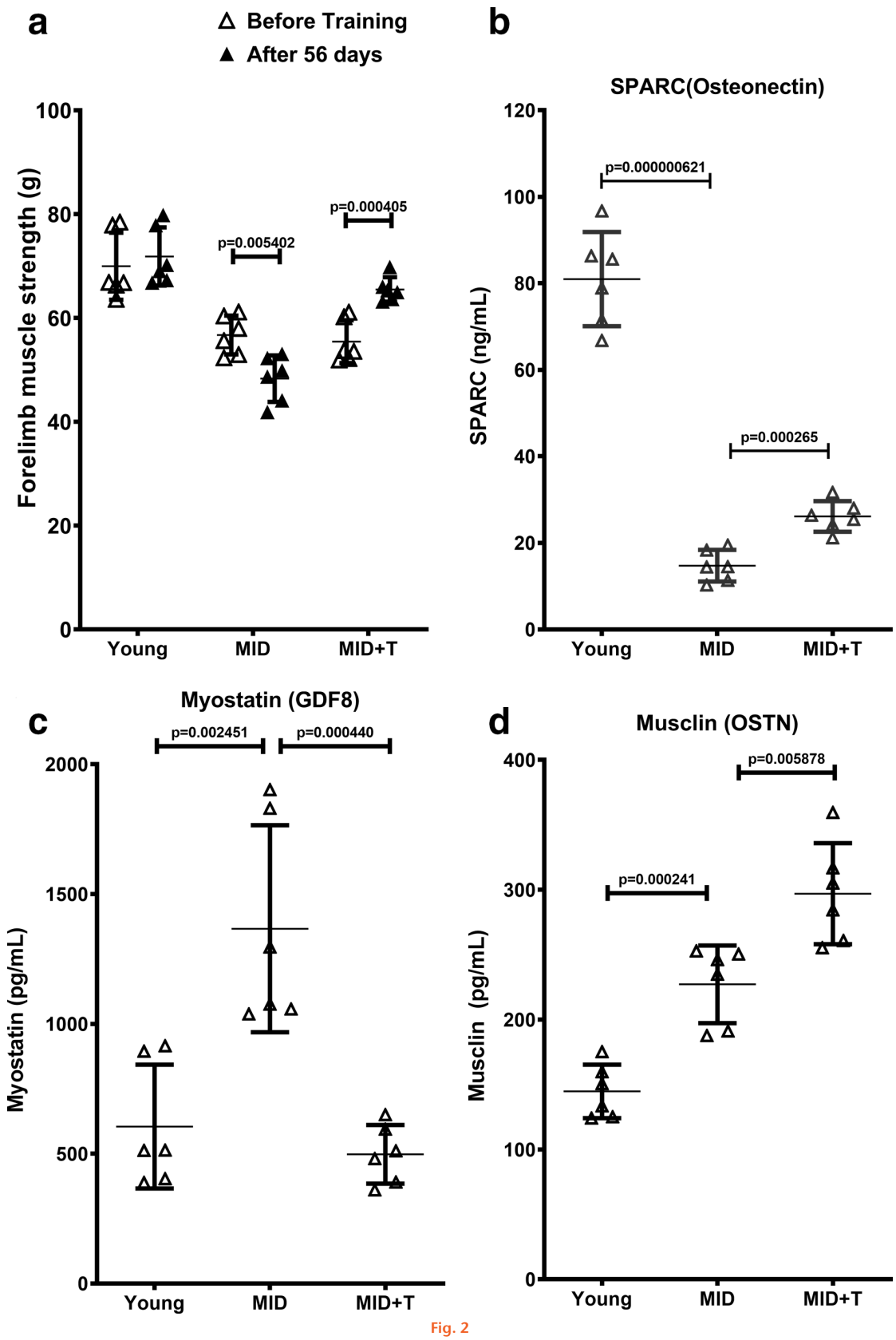


Fig. 2

Resistance training reversed age-induced muscle strength loss. a) The forelimb muscle strength assessment was performed on the young, MID, and MID + T groups before and after resistance training (n = 6). Meanwhile, the sarcopenia-related serum biomarkers, b) secreted protein acidic and cysteine rich (SPARC), c) myostatin, and d) musclin were measured by MILLIPLEX multiplex assays (Merck Millipore, USA) (n = 6). GDF, growth differentiation factor; OSTN, osteocrin.

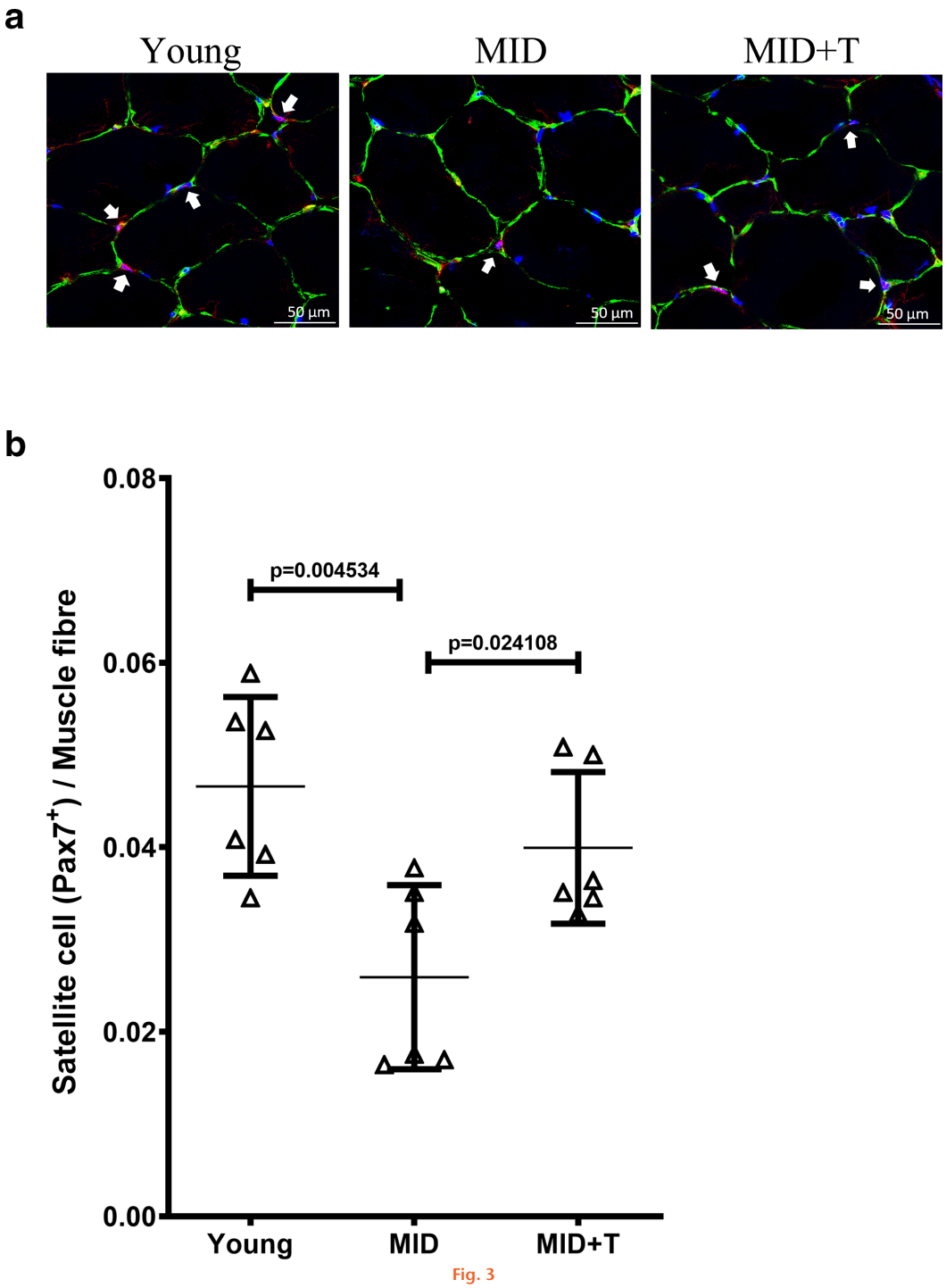


Fig. 3

The proportion of satellite cells (SCs) per muscle fibre type in each group. a) The muscle sections were isolated from the young, MID, and MID + T groups and stained with anti-Pax7 antibodies (Red), anti-Lamini antibodies (Green), and Hoechst 33,342 (Blue) by immunofluorescence assay. b) The graphic shows the SC quantification of the immunofluorescence image (n = 6). Arrowheads show Pax7-positive SCs.

**GO and KEGG enrichment analysis.** The list of differentially expressed genes with an absolute fold change greater than 2 were subjected to gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses using GO-TermFinder (MetaCPAN) and scripts written in-house, respectively.

**Western blot assay.** The seven-day cultured SCs were washed with cold phosphate-buffered saline (PBS) and lysed in RIPA buffer (20 to 188, EMD Millipore, USA) containing cOmplete protease inhibitor cocktail (EMD Millipore). Cell lysates were separated by SDS-PAGE followed by western blotting with the indicated antibodies (ATF2:

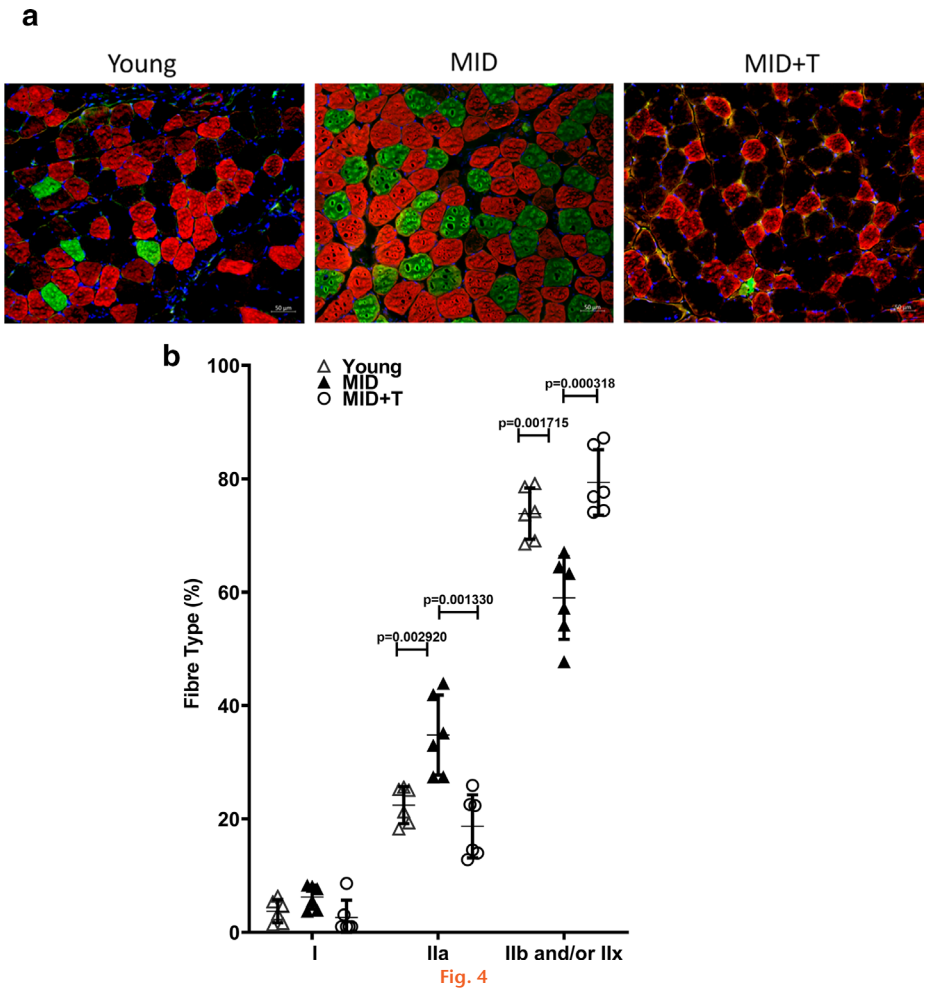


Fig. 4

The proportion of muscle fibre type was changed by resistance training. a) The muscle sections were isolated from the young, MID, and MID + T groups and stained with anti-MYH7 antibodies (type I: Green), anti-MYH2 antibodies (type IIa: Red), and Hoechst 33,342 (Blue) by immunofluorescence assay (200× magnification). b) The graphic shows the quantification of the immunofluorescence image (n = 6).

14834 to 1-AP, Raptor: 20984 to 1-AP, IKBKG: 18474 to 1-AP; α-Tubulin: 11224 to 1-AP; Proteintech, USA) (Cyclin D3:2936; Cell Signaling Technology, USA). Western blot analyses were performed as described previously.[29]

**Statistical analysis.** We employed a one-way analysis of variance (ANOVA) to determine the differences between the groups. A p-value < 0.05 was considered significant, and all analyses were performed using Prism (6.0; GraphPad, USA). The data are presented as mean and standard deviation.

## Results

**Forelimb grip strength assessment in each group.** First, we measured the forelimb grip strength of all the mice using a grip strength meter to assess the before- and after-effects of the 48-day resistance training. As shown in Figure 2a, the forelimb grip strength of the MID group was significantly decreased after 48 days (p = 0.005). In contrast, the forelimb grip strength of the MID+ T group was increased after 48 days (p < 0.001). However, the forelimb grip strength of the young group did not differ after 48 days. The results reveal that the resistance training programme did indeed enhance muscle strength in the mouse model.

**Detection of muscle-related serum biomarkers in each group.** Before euthanizing the mice, we collected peripheral blood to assess the level of muscle-related serum biomarkers such as secreted protein acidic and cysteine rich (SPARC), myostatin (growth differentiation factor-8), and musclin (also known as osteocrin) by multiplex assays. SPARC plays a crucial role in skeletal muscle development and regeneration,[25] and its expression is decreased in the skeletal muscle of older mice.[30] Myostatin is a negative regulator of skeletal muscle development and mass.[26] Musclin is an exercise-induced myokine, which we employed to assess the effect of resistance training.[27,31] The results revealed (Figures 2b to 2d) that SPARC levels were significantly higher in the young group than in the MID group (p < 0.001). In contrast, myostatin and musclin levels were lower in the young group than in the MID group (both p < 0.001). SPARC and musclin levels were higher in the MID+ T group than in the MID group (p <

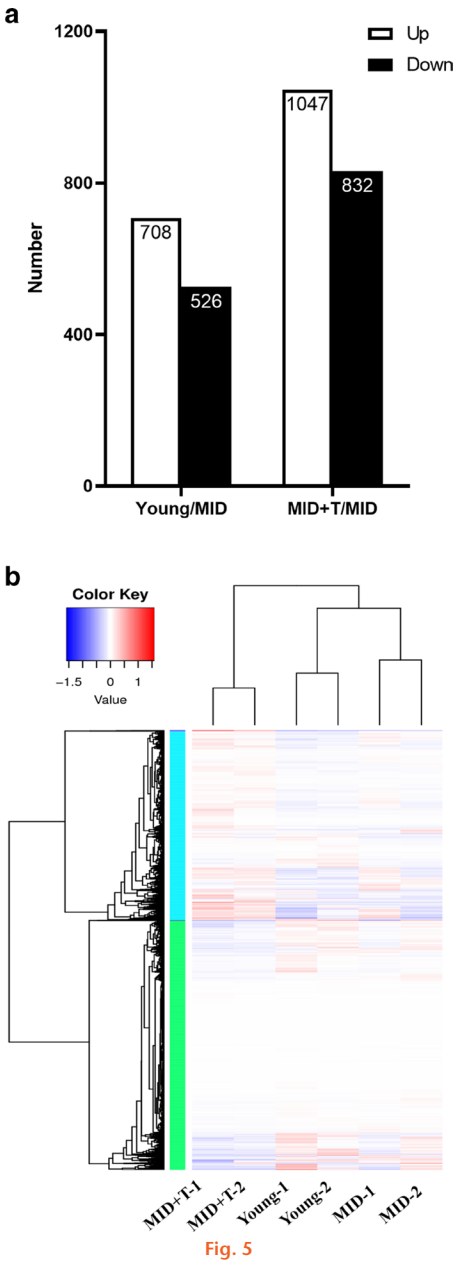


Fig. 5

The messenger RNA differential expression analysis. a) The graph presents the number of upregulated and downregulated differentially expressed genes in the young/MID and MID+ T/MID groups. b) Cluster analysis of the differentially expressed genes from each sample was performed, and log10 (fragments per kilo base per million reads + 1) values were employed for clustering. Highly expressed genes are in red, and weakly expressed genes are in blue.

0.001; p = 0.006); however, myostatin levels were lower in the MID+ T group than in the MID group (p < 0.001).

**The proportion of SC number and muscle fibre type in each group.** After euthanizing the mice, we excised the rectus femoris muscles and subjected them to an immunofluorescence examination. Sections of muscle samples in each group are shown in Figures 3 and 4. First, we identified the SCs and muscle fibre basement membrane with the specific Pax7 and Laminin antibodies, respectively, and quantified the proportion of SCs per muscle fibre in each group (Figure 3a). As shown in Figure 3b, the proportion of SCs per muscle fibre in the young and MID + T groups was significantly higher than in the MID group (p = 0.004; p = 0.024). Additionally, by immunostaining the specific type I muscle fibre (slow-twitch) and type IIa muscle fibre (fast-twitch oxidative) antibodies, we identified and quantified the different types of muscle fibre (unstained: type IIb/IIx muscle fibre (fast-twitch glycolytic)) (Figure 4a). As shown in Figure 4b, the proportion of type IIa muscle fibre in the MID group was significantly higher than in the young (p = 0.003) and MID + T group (p = 0.001). In contrast, the proportion of type IIb/IIx muscle fibre in the MID group was statistically lower than in the other groups (Young:MID, p = 0.002; MID:MID + T, p < 0.001). However, there was no difference in the proportion of type I muscle fibre between the groups.

**Next-generation sequencing and western blot.** Next, we isolated the SCs of skeletal muscle from each group and verified the stemness of seven-day cultured SCs by IFA with Pax7 and CD34 antibodies (Supplementary Figure a). The IFA results showed that the stemness was maintained in the seven-day cultured SCs. The seven-day cultured SCs were extracted from the total RNA for transcriptomic profiling using next-generation sequencing. The results from the DESeq2 analysis were further analyzed to determine the genes with significant differentially expressed genes according to the criteria of a fold change > 2. We then summarized the number of significantly upregulated and downregulated genes between the groups in Figure 5a. There were 708 upregulated and 526 downregulated differentially expressed genes when we compared the young group to the MID group (young/MID). When we compared the MID+ T group to the MID group (MID+ T/MID), there were 1,047 upregulated and 832 downregulated differentially expressed genes. We then performed a clustering analysis to group differentially expressed genes with similar functions by data analysis and classification based on similarity. The fragments per kilo base per million reads (FPKM) value of the various genes under differing experimental conditions was taken as the expression level and used for the hierarchical clustering. As shown in Figure 5b, the differentially expressed gene patterns of the young group were more similar to those of the MID group than those of the MID+ T group.

To explore the biological processes potentially associated with age or stimulated by resistance training, we analyzed the differentially expressed genes of the young/MID and MID+ T/MID groups by GO. We observed that the differentially expressed genes of the young/MID mice were annotated with 17 terms under the biological process category, eight terms under the molecular function category, and 14 terms under the cellular components category (Figure 6).

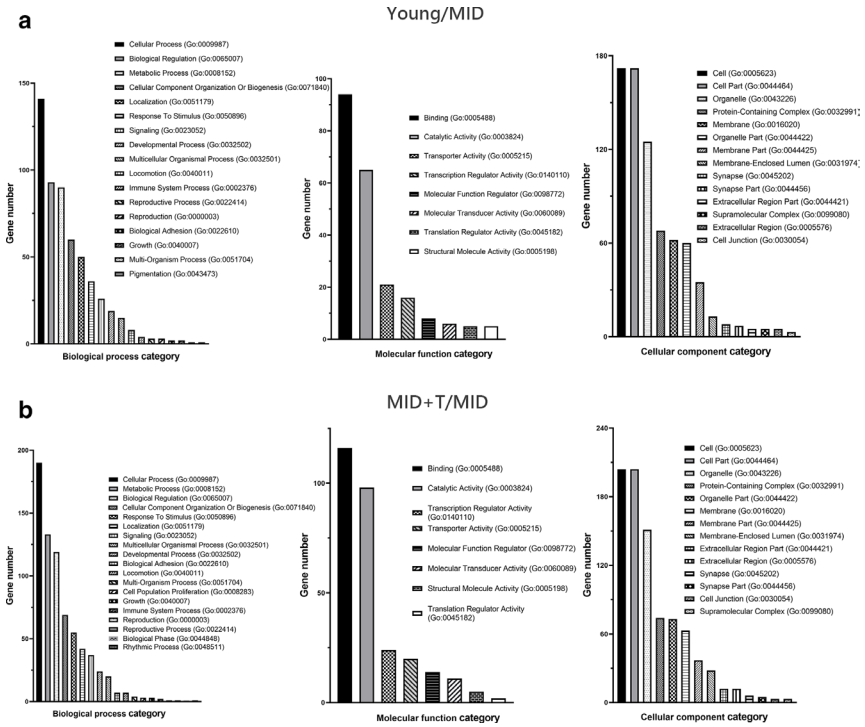


Fig. 6

The number of differentially expressed genes in each gene ontology term is shown in the histogram with the specification of the relevant biological process, molecular function, and cellular component. The differentially expressed genes were obtained from the a) young/MID and b) MID+ T/MID groups.

**Table I.** The top ten pathways of differentially expressed genes from the young and MID groups in the Kyoto Encyclopedia of Genes and Genomes analysis.

| KEGG pathway | Gene count | Gene symbol |
|---|---|---|
| Metabolic pathways (mmu01100) | 20 | *Atp5g1, Atp6v1h, Pip5k1a, Nme7, Cyp4f13, Ndufa5, Inpp5k, Eno3, Pi4ka, Enoph1, Ppt2, Mboat2, Hyal1, Uros, Ndufb7, Agpat4, Upp1, Kyat1, Atp5h, Alg1* |
| RNA transport (mmu03013) | 7 | *Sap18, Eif4g3, Nup37, Sumo2, Cyfip1, Eif4g2, Ndc1* |
| Viral carcinogenesis (mmu05203) | 7 | *Ikbkg, H2-T22, Hdac6, Atf2, Ccnd3, Kat2b, Rbpj* |
| Endocytosis (mmu04144) | 7 | *Dnm2, Snf8, H2-T22, Dnm3, Pip5k1a, Asap1, Psd3* |
| PI3K-Akt signalling pathway (mmu04151) | 7 | *Gng5, Ikbkg, Rptor, Pkn2, Atf2, Ccnd3, Ppp2r2d* |
| Regulation of actin cytoskeleton (mmu04810) | 6 | *Limk1, Arhgef12, Pip5k1a, Cyfip1, Araf, Arhgef7* |
| HTLV-I infection (mmu05166) | 6 | *Il15ra, Ikbkg, H2-T22, Atf2, Ccnd3, Kat2b* |
| Axon guidance (mmu04360) | 5 | *Limk1, Arhgef12, Rhod, Ephb1, Srgap2* |
| Oxidative phosphorylation (mmu00190) | 5 | *Atp5g1, Atp6v1h, Ndufb7, Ndufa5, Atp5h* |
| Adrenergic signalling in cardiomyocytes (mmu04261) | 5 | *Rapgef3, Cacna2d2, Atf2, Cacna2d1, Ppp2r2d* |

HLTV, human T-lymphotropic virus; KEGG, Kyoto Encyclopedia of Genes and Genomes.

The differentially expressed genes of the MID+ T/MID mice were annotated with 19 terms under the biological process category, eight terms under the molecular function category, and 14 terms under the cellular components category. The KEGG enrichment analysis further highlighted the top ten pathways in which most of the differentially expressed genes of the young/MID and MID+ T/MID groups were involved (Tables I and II). Additionally, there were six pathways (metabolic pathways, viral carcinogenesis, endocytosis, PI3K-Akt signalling pathway, regulation of actin cytoskeleton, and human T-lymphotropic virus (HTLV)-I infection) highlighted in both the young/MID and MID+ T/MID groups. Among these, the PI3K-Akt pathway directly promotes myogenic differentiation by inducing the transcriptional factor myocyte enhancer factor-2 (MEF2).[32] Actin is the principal component of filaments, organized into sarcomeres that function as the fundamental unit of contraction for skeletal and cardiac muscle.[33] We further listed differentially expressed genes that had the same regulation pattern in the young/MID and MID+ T/MID groups as the PI3K-Akt signalling and regulation of the actin cytoskeleton pathway. As shown in Tables III and IV, G Protein Subunit γ 5 (*Gng5*), Activating Transcription Factor 2 (*Atf2*), and Regulatory Associated Protein Of MTOR Complex 1 (*Rptor*) in the PI3K-Akt signalling pathway had higher expression in the young and MID+ T groups compared with the MID group, while Inhibitor Of

**Table II.** The top ten pathways of differentially expressed genes from the MID+ T and MID groups in the Kyoto Encyclopedia of Genes and Genomes analysis.

| KEGG pathway | Gene count | Involved genes |
|---|---|---|
| Metabolic pathways (mmu01100) | 28 | *Mvk, Sgsh, Pip5k1c, Atp6v1h, Akr1b3, Pafah1b3, Nadsyn1, Chka, Ndufb10, Azin2, Atp6v0a1, Mtmr2, Mboat2, Ndufab1, Ntpcr, Akr1b10, Dctd, Cyp4f13, Nme7, Fut8, Inpp5k, Cers4, Cept1, Alg3, Pfas, Coasy, Mthfs, Kyat1* |
| Pathways in cancer (mmu05200) | 15 | *Nfkb1, Ctbp2, Ikbkg, Mecom, Arhgef12, Fzd6, Flt3l, Araf, Myc, Arhgef1, Tpr, Itga3, Gng5, Rxrg, Lama2* |
| PI3K-Akt signalling pathway (mmu04151) | 12 | *Itga3, Nfkb1, Gng5, Ikbkg, Rptor, Rps6kb1, Ywhaz, Atf2, Ccnd3, Lama2, Myc, Spp1* |
| Transcriptional misregulation in cancer (mmu05202) | 9 | *Nfkb1, Uty, Cdk14, Tfe3, Rxrg, Fut8, Ewsr1, Aff1, Myc* |
| Proteoglycans in cancer (mmu05205) | 9 | *Drosha, Rps6kb1, Arhgef12, Ank2, Fzd6, Araf, Src, Myc, Arhgef1* |
| Regulation of actin cytoskeleton (mmu04810) | 9 | *Itga3, Limk1, Pip5k1c, Diaph2, Arhgef12, Cyfip2, Araf, Src, Arhgef1* |
| Endocytosis (mmu04144) | 9 | *H2-T22, Pip5k1c, Asap1, Iqsec1, Ccdc53, Psd3, Src, Iqsec3, Capzb* |
| HTLV-I infection (mmu05166) | 9 | *Il15ra, Nfkb1, Chek1, Ikbkg, H2-T22, Atf2, Ccnd3, Fzd6, Myc* |
| Hippo signalling pathway (mmu04390) | 8 | *Wwtr1, Mob1a, Ywhaz, Ccnd3, Fzd6, Serpine1, Myc, Dlg3* |
| Viral carcinogenesis (mmu05203) | 8 | *Nfkb1, Chek1, Ikbkg, H2-T22, Ywhaz, Atf2, Ccnd3, Src* |

HTLV, human T-lymphotropic virus; KEGG, Kyoto Encyclopedia of Genes and Genomes.

**Table III.** The fold change of differentially expressed genes in the PI3K-Akt signalling pathway in the young/MID and MID+ T/MID groups.

| Gene ID | Full name | Young/MID group fold change | MID + T/MID group fold change |
|---|---|---|---|
| *Gng5* | G Protein Subunit γ 5 | 3.08 | 3.22 |
| *Atf2* | Activating Transcription Factor 2 | 4.66 | 4.97 |
| *Rptor* | Regulatory Associated Protein Of MTOR Complex 1 | 5.58 | 3.33 |
| *Ikbkg* | Inhibitor Of Nuclear Factor Kappa B Kinase Regulatory Subunit γ | -3.99 | -4.00 |
| *Ccnd3* | Cyclin D3 | -4.30 | -6.27 |

**Table IV.** The fold change of differentially expressed genes in the regulation of actin cytoskeleton in the young/MID and MID+ T/MID groups.

| Gene ID | Full name | Young/MID group fold change | MID+ T/MID group fold change |
|---|---|---|---|
| *Limk1* | LIM Domain Kinase 1 | 3.08 | 3.22 |
| *Arhgef12* | Rho Guanine Nucleotide Exchange Factor 12 | 4.66 | 4.97 |
| *Araf* | A-Raf Proto-Oncogene, Serine/Threonine Kinase | 5.58 | 3.33 |

Nuclear Factor Kappa B Kinase Regulatory Subunit γ (*Ikbkg*) and Cyclin D3 (*Ccnd3*) had a lower expression. Similarly, LIM Domain Kinase 1 (*Limk1*), Rho Guanine Nucleotide Exchange Factor 12 (*Arhgef12*), and A-Raf Proto-Oncogene, Serine/Threonine Kinase (*Araf*) in the regulation of the actin cytoskeleton pathway had higher expression in the young and MID+ T groups than in the MID group.

Based on the results of next-generation sequencing, we assessed the protein level of *Atf2,* regulatory associated protein of MTOR complex 1 (*Rptor*), *Ikbkg*, and Cyclin D3 (*Ccnd3*) in the SCs from young, MID, and MID+ T group by western blot assay. The results showed that the ATF2 and Rptor protein level in the MID group was lower than in the young and MID+ T groups (Figure 7). By contrast, the Cyclin D3 protein level in the MID group was higher than in other groups. The IKBKG protein level was not different among groups.

## Discussion

Exercise is a well-known preventer of age-related muscle loss.[34] To the best of our knowledge, however, the effects of physical exercise on SCs have not been well defined. In the present study, we performed resistance training in a mouse model and showed that this activity significantly reverses the age-related decrease in forelimb muscle strength and serum SPARC (Figures 2a and 2b), which is responsible for muscle development. The results showed that resistance training also visibly restrained the age-related levels of serum myostatin, an inhibitor of muscle development (Figure 2c), echoing those of a previous study that showed that exercise can reduce myostatin levels.[35] Meanwhile, the age-induced decrease of the proportion of SCs per muscle fibre was restored after resistance training (Figure 3), echoing previous studies.[18,36]

Numerous studies have indicated that exercise-induced muscle fibre shifting is present only between type II fast-twitch muscle fibre.[37-39] Our results also revealed that the shifting proportion of muscle fibre by resistance training was apparently between type IIa and IIb/IIx muscle fibre (Figure 4). These results indicate that the resistance training mouse model was indeed valid.

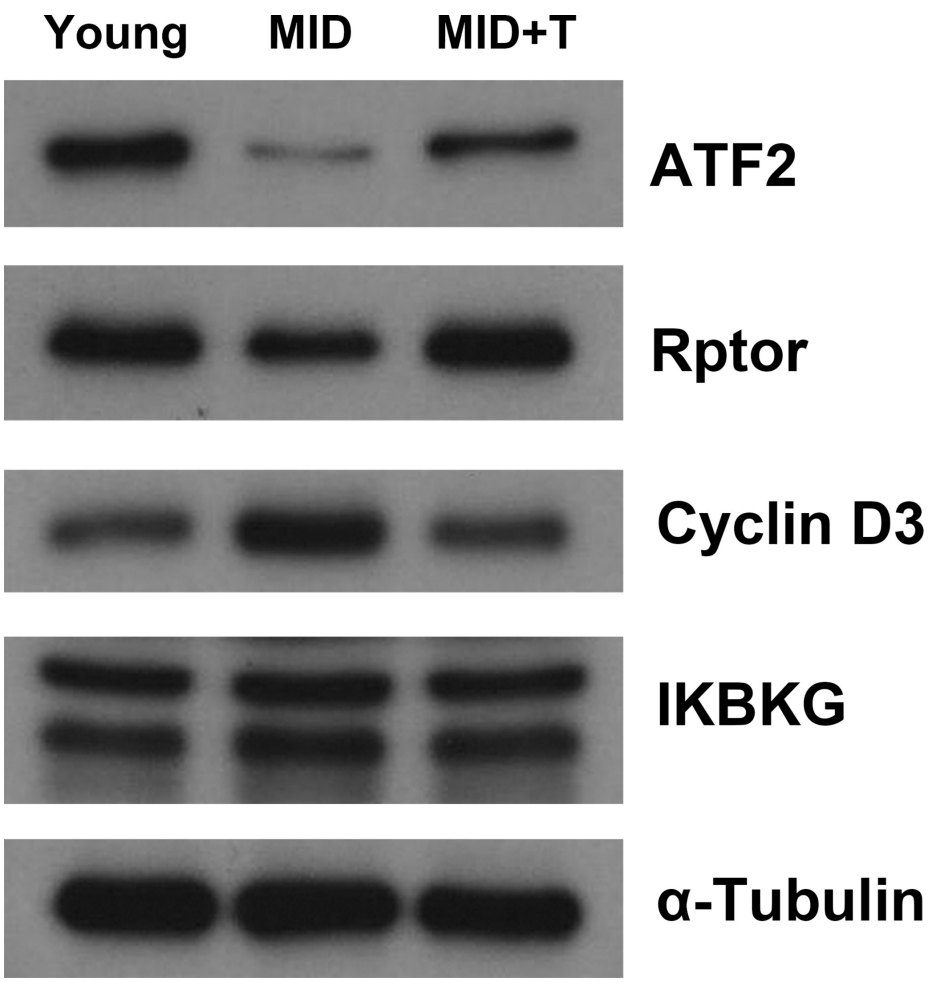


Fig. 7

The protein expression level of activating transcription factor 2 (*Atf2*), Regulatory Associated Protein Of MTOR Complex 1 (*Rptor*), inhibitor of nuclear factor kappa B kinase regulatory subunit γ (*Ikbkg*), and Cyclin D3 (*Ccnd3*) genes in satellite cells (SCs) from young, MID, and MID+ T groups by western blot assay. The seven-day cultured SCs from each group were lysed and the lysate was subjected to western blot assay with the indicated antibodies. α-Tubulin served as an internal control for amounts of protein loaded on the gel.

We then employed next-generation sequencing for genome-wide screening and compared the transcriptomes of SCs in the young, MID, and MID+ T groups. In the KEGG pathway enrichment analysis (Tables I and II), we found that six pathways (metabolic pathways, viral carcinogenesis, endocytosis, PI3K-Akt signalling pathway, regulation of actin cytoskeleton, and HTLV-I infection) were highlighted in the young/MID and MID+ T/MID groups, suggesting that resistance training and age might have similar epigenetic mechanisms in SCs. In the PI3K-Akt signalling pathway, the expression pattern of *Gng5, Atf2, Rtor, Ikbkg,* and *Ccnd3* were the same in the young/MID and the MID+ T/MID groups. Likewise, *Limk1, Arhgef12,* and *Araf* in the regulation of actin cytoskeleton pathway also had the same trend in both the young/MID and MID+ T/MID groups. Interestingly, the fold change of these differentially expressed genes was highly similar between the young/MID and MID+ T/MID groups. We believed that resistance training might reactivate the age-influenced genes to assist SCs in regenerating muscle fibre to prevent muscle loss. Moreover, the protein expression profiles of *Atf2, Rptor*, and *Ccnd3* (Figure 7) in each group were paralleled with the result of NGS (Table III).

Musclin is a secretory peptide in the muscle and bone, and contributes to myocyte and osteoblast differentiation, respectively.[27,40] However, our data revealed an age-dependent increase in circulating musclin (Figure 2d), a similar result observed in a previous study,[41] the reasons for which are still unclear and require further studies to validate. Nevertheless, previous studies have concluded that musclin is an exercise-responsive myokine,[31,42] and is enriched in fast glycolytic muscle.[43] The results echo our data that serum musclin levels (Figure 2d) and the portion of type IIb/IIx muscle fibre were increased (fast-twitch glycolytic) (Figure 4) after resistance training.

Heterotrimeric G proteins are composed of α, β, and γ subunits, and activated by G protein-coupled receptors to regulate numerous cellular responses. G protein subunit γ 5 (*Gng5*) has a higher expression in the skeletal muscle of younger women than in older women,[44] and

is expressed during the development of skeletal muscle in the Hainan black goat.[45] It has been suggested that *Gng5* might play a role in skeletal muscle development, however its mechanism is not entirely clear. *Rptor* deletion results in progressive muscle dystrophy,[46] and is necessary for mechanical load-induced hypertrophy.[47] In our results, we found that *Gng5* and *Rptor* expression in the SCs was impaired by age, and that the impaired genes were reversed by resistance training (Table III). There were similar results for *Limk1, Arhgef12,* and *Araf* in the regulation of the actin cytoskeleton (Table IV). It has been suggested that resistance training can reactivate the age-impaired genes that contribute to muscle development. Interestingly, *Ccnd3* expression plays a critical role in myoblast differentiation;[48] however, our results revealed that *Ccnd3* expression in the SCs was upregulated in the MID group, which had lower muscle strength compared with the young group (Table III). A previous study also found that *Ccnd3* expression in the myoblast progenitors of aged (24 months old) DBA/2JNIA mice was higher than that of adult (eight months old) DBA/2JNIA mice.[49] Similarly, upregulated *Ccnd3* expression in the old mice (when compared with the young mice) also occurred in liver cells.[50,51] Therefore, the role of *Ccnd3* in muscle development requires further studies for validation. Nevertheless, most differentially expressed genes in the SCs influenced by age (including upregulation and downregulation) were reversed by resistance training, suggesting that this could rejuvenate the self-renewal ability of SCs to prevent sarcopenia.

The actin and myosin filament are components of the cylindrical myofibril, which is a basic contractile unit of muscle cells. In the sliding filament theory, the attachment-detachment cycle of actin and myosin contributes to muscle contraction. *Limk1* is involved in actin polymerization,[52] required for muscle contraction.[53] *Arhgef12*[54] and DA-Raf, an isoform protein of A-Raf,[55] have been reported to serve as a positive regulator of myogenic differentiation. The age-decreased expression of these genes might result in muscle loss, leading to sarcopenia. However, the age-impaired gene expression was reversed by RT.

Interestingly, three cancer-related pathways (pathways in cancer, transcriptional misregulation in cancer, and proteoglycans in cancer) were enriched in the MID + T/MID group (Table II). Several genes in the PI3K-Akt signalling pathway and/or regulation of actin cytoskeleton were also involved in the three pathways such as *Itga3, Gng5*, *Lama2*, and *Src* genes. The functions of *Itga3, Gng5*, *Lama2*, and *Src* are associated with cell migration and/or metastasis during tumorigenesis.[56-59] Their expression levels were significantly increased in the MID + T/MID group (Table II), indicating an alternative function in SCs after resistance training. However, our transcriptome data revealed that the expression level of a proto-oncogene, *Myc*, was significantly decreased in the MID + T/MID group and we did not find any obvious tumour formation in each group mouse, especially in the MID + T group. Furthermore, numerous studies have indicated that physical activity and exercise are beneficial for reducing cancer risks.[60-62] It was suggested that the upregulated cancer-associated genes may have alternative functions in SCs than cancer cells after resistance training.

Previous studies have shown that exercise can increase the number of stem cells.[63,64] A recent transcriptomic study also showed that the mice exercised on a rotating wheel can induce stem cell rejuvenation through the reactivation of Cyclin D1.[65] Interestingly, inhibition of Src-class kinases repressed the proliferation of the muscle cell line accompanied by a reduced level of Cyclin D1.[66] It was speculated that the upregulated genes involved in the cancer pathway, such as *SrC*, may function as promoting SC proliferation after resistance training.

The present study had some limitations. First, the muscle mass is an important morphological parameter that could be detected by MRI, micro-CT, or dual-energy x-ray absorptiometry (DXA). In the current study, we had assessed the muscle mass by DXA, but mice are so small that precise detection is difficult in DXA. Instead, we presented the grip strength, serum markers, and muscle fibre type as the effect of resistance training. Second, the pathways linking the resistance training to age-influenced genes in the SCs demanded further investigations. Nevertheless, the comprehensive analysis of the present study still provided informative data regarding the transcriptomic changes in response to resistance training.

In conclusion, we performed next-generation sequencing to screen the transcriptome of SCs in young, MID, and MID + T mice. After the comparison and bioinformatics analysis, we found that most differentially expressed genes in the SCs influenced by age (including upregulation and downregulation) were reversed by resistance training. In particular, the differentially expressed genes in the muscle development pathway, the PI3K-Akt signalling pathway, and the regulation of actin cytoskeleton pathway showed similar results. To sum up, age-induced muscle loss might result from the age-influenced genes that contribute to muscle development in SCs. After resistance training, the age-impaired genes were reactivated, and the age-induced genes were depressed. The fold change of these genes in the young/MID group resembled those in the MID + T/MID group, suggesting that RT can rejuvenate the ability of SCs by recovering the age-influenced genes, thereby preventing sarcopenia.

## Supplementary material

The satellite cells staining after seven-day culture, and experimental workflow of transcriptome sequencing; ARRIVE checklist.

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- W-H. Hsu: Conceptualization.
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