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ORIGINAL ARTICLE

Mechanism of thyroid hormone signaling in skeletal muscle of aging mice

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Abstract

Background and aim Skeletal muscle (SM) has been shown as a target of thyroid hormones (THs). However, the status of TH signaling in aged SM remains unclear. This study aimed to explore the mechanism of TH signaling in SM of aging mice.

Methods Thirty C57BL/6J male mice were divided into 6-, 15- and 22-month (6, 15 and 22M) groups according to different age. Physical parameters were evaluated by analytical balance, grip strength test and histological analysis. Thyroid function was detected by enzyme-linked immunosorbent assay. TH signaling was compared among the three groups by real-time PCR and western blotting analysis.

Results p16, p21, and p53 mRNA levels in SM increased in age-dependent manner. The muscle weight and strength decreased in 22M group compared to 6 and 15M groups. Concentrations of thyroid hormones, including free triiodothyronine (FT3), free thyroxine (FT4) and thyroid-stimulating hormone (TSH) in 22 M mice were not shown significant difference compared to 6M or 15M mice, although FT3 showed slightly decrease and TSH appeared a mild increase accompanying with age. mRNA levels of TH transporters, including MCT8 and MCT10, as well as iodothyronine deiodinase type 2 (DIO2) and type 3 (DIO3), were higher in 22M, while TH receptor α (TRα) mRNA and protein expression was lower in 22M, compared to the other groups. Type-I myosin heavy chain (MyHC I), MyHC IIx, and MyHC IIa were upregulated and Type-IIb MyHC (MyHC IIb) was downregulated in SM with advancing age.

Conclusions TH signaling in SM changes with aging.

Keywords Aging · Skeletal muscle · Thyroid hormone signaling · Mice

Introduction

Aging is accompanied by numerous time-associated changes in cellular structure and function [1]. The typical characteristics of geriatric skeletal muscle are attenuated muscle mass, strength, and function. Such decline in muscle system is defined as a senescence-related disease sarcopenia [2], which causes limited physical activity and mobility or even disability in elderly people [3, 4].

SM plays a significant role in maintaining basal posture, movement and energy metabolism. According to twitch speeds and metabolic properties, SM fibers are classified into fast- and slow-twitch fibers. Fast-twitch fibers are characterized by the expression of myosin heavy chain (MyHC) II, which includes MyHC IIa, MyHC IIb, and MyHC IIx subtypes, while slow-twitch fibers are characterized by the expression of MyHC I [5, 6]. Muscle fibers could transform to other types of fibers in different condition, such as aging, innervation and exercise training [7, 8], but such transformation of SM in aging mice is still unclear.

SM had been confirmed as a target organ of thyroid hormones (THs) and TH signaling. TH signaling plays an essential role in contractile function, myogenesis, metabolism, and regeneration of SM [9]. The effects of TH signaling mainly depend on regulatory factors, such as THs transmembrane transporters, MCT8, and MCT10,

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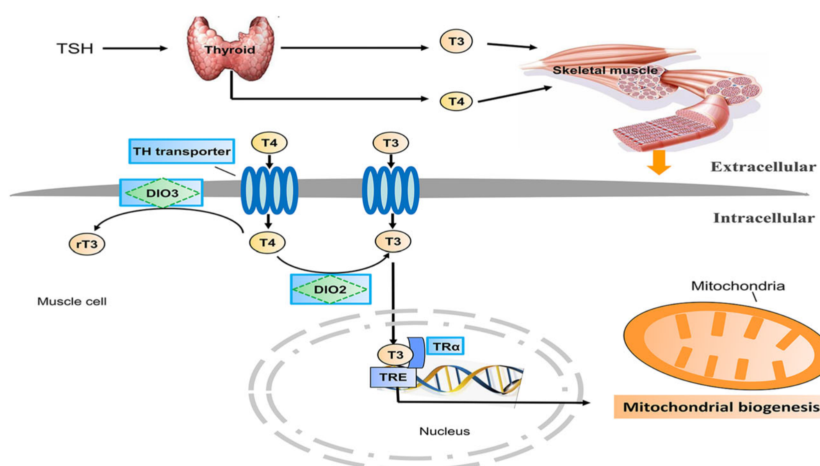

Fig. 1 Diagrams of TH signaling mechanism. TRIAD: THs transmembrane transport, intracellular deiodination and TRs-mediated biological effect. TRE: thyroid hormone response elements

the iodothyronine deiodinase type 2 (DIO2) and type 3 (DIO3), and nuclear TH receptors (TRs), which are described as the thyroidal signaling triad (TRIAD, Fig. 1). It is known that triiodothyronine (T3), an active type of TH that is mainly converted from thyroxine (T4), binds TRs and modulates target genes to promote metabolic pathways [10]. However, the mechanism of TH signaling, including TRIAD in SM during aging is uncertain. This study aimed to explore the mechanism of TH signaling in aged SM by using an aging mouse model.

Materials and methods

Animals

Thirty C57BL/6J male mice of different age were obtained from the Model Animal Center of Nanjing University and maintained at 12 h light/12 h dark cycle at 22 ± 1 °C with free access to water and food. Young (6 months old, 6M; $n = 10$), middle-aged (15 months old, 15M; $n = 10$), and old (22 months old 22M; $n = 10$) mice were included and their weight was recorded weekly. After all animals were euthanized, gastrocnemius (Ga) muscle tissues were excised from the lower limb, and fixed in 4% paraformaldehyde or stored at −80 °C. All animal protocols were approved by the Institutional Animal Care and Use Committee.

Histology

Ga muscle tissues were fixed in 4% paraformaldehyde, embedded in paraffin, and then cut into sections (8 μm thin). Next, the sections were stained with hematoxylin-eosin (HE) and observed under a microscopy. The myocyte cross-sectional area (MCSA) was analyzed.

Grip strength test

The limb grip strength was analyzed by a grip strength meter (Columbus Instruments, USA). The tested mouse was first allowed to grasp a grid with four paws, and then the tail of mouse was pulled backwards away from the grid until it released the grid. The pulling speed should be gentle and slow enough to let it resist against the pulling force. The peak force was recorded once the mouse moved away from the grid. Each mouse was tested at least three times. The results were measured in grams (g), and finally analyzed by average griping strength (Grip) and normalized grip force (Grip/Body weight).

Real-time PCR (RT-PCR)

Total RNA was extracted from tissues with RNAiso Plus (Takara, Japan) and cDNA was synthesized with PrimeScript RT Master Mix Kit (Takara). RT-PCR was performed with TB-Green Kit (Takara) on StepOnePlus system (USA). The primer sequences for β-actin, p16, p21, p53, MCT8, MCT10, TRα, MyHC I, MyHC IIa, MyHC IIb, MyHC IIx, DIO2, and DIO3 are shown in Table 1, with β-actin as internal control. Relative mRNA level was analyzed by $2^{-\Delta\Delta CT}$ method.

Enzyme-linked immunosorbent assay (ELISA)

Serum samples were isolated from mice and stored at −80 °C. Serum levels of thyroid hormones, FT3, FT4, and TSH were measured by ELISA (MEIMIAN, China). The reference normal ranges of THs and TSH in mice were as below: 18.36 ± 4.67 pmol/L (FT3), 16.83 ± 3.90 pmol/L (FT4), and 3.826 ± 1.107 mIU/L (TSH), respectively.

Table 1 Primers used for RT-PCR

Primer	Primer sequence (5′ → 3′)
β-actin	Forward: GACAACTTTGGCATCGTGGA Reverse: ATGCAGGGATGATGTTCTGG
p16	Forward: GCTCAACTACGGTGCAGATTC Reverse: GCACGATGTCTTGATGTCCC
p21	Forward: CGAGAACGGTGGAACTTTGAC Reverse: CCAGGGCTCAGGTAGACCTT
P53	Forward: GCGTAAACGCTTCGAGATGTT Reverse: TTTTTATGGCGGGAAGTAGACTG
MCT8	Forward: GTGCTGAGTACCTTCATGTTTG Reverse: ACACGCATGTTGAAGTACTTTC
MCT10	Forward: GGCCGCATTGCTGACTATTT Reverse: CAATGGGCGCCATGATAGA
TRα	Forward: GGTCACCAGATGGAAAGCGAA Reverse: CCTTGTCCCCACACACGA
MyHC I	Forward: ACTGTCAACACTAAGAGGGTCA Reverse: TTGGATGATTTGATCTTCCAGGG
MyHC IIa	Forward: AAGTGACTGTGAAAACAGAAGCA Reverse: GCAGCCATTTGTAAGGGTTGAC
MyHC IIb	Forward: CTTTGCTTACGTCAGTCAAGGT Reverse: AGCGCCTGTGAGCTTGTAAA
MyHC IIx	Forward: CTCTTCCCGCTTTGGTAAGTT Reverse: CAGGAGCATTTCGATTAGATCCG
DIO2	Forward: CTTCCTCCTAGATGCCTACAAAC Reverse: GGCATAATTGTTACCTGATTCAGG
DIO3	Forward: AGCGCAGCGAGAGTACTACA ACAA Reverse: ACATGATGGTGCCACTCTGGATGA

Western blotting

Total protein was isolated from tissues using the protein extraction kit (Beyotime, China). Western blot analysis was performed using standard protocol with primary antibodies for GAPDH (Cell Signaling Technology, USA) and TRα (Abcam, UK). Quantification of protein bands was performed with Image-J software (USA).

Statistics

Statistical analysis was performed with GraphPad Prism 7.0 software (USA). Results were shown as mean ± standard error of mean (SEM) for at least triplicate experiments and analyzed by one-way ANOVA. $P < 0.05$ indicated significance.

Results

Altered physical parameters of SM during aging

As shown in Fig. 2, senescence-associated markers including p16, p21, and p53 mRNA levels increased with advancing age (Fig. 2a–c). Compared to 6M group, body weight (BW) significantly increased in 15 and 22M groups ($P < 0.01$) (Fig. 2d) and the SM weight of Ga significantly increased in 15M group ($P < 0.05$), but slightly declined in 22M group ($P > 0.05$) (Fig. 2e). However, the Ga muscle weight normalized by BW (Ga/BW) decreased in age-related manner, although the difference was not significant among three groups ($P > 0.05$) (Fig. 2f). Furthermore, four limb grip strength significantly attenuated in 22M group compared to 6 and 15M groups ($P < 0.01$) (Fig. 2k). Similarly, the ratio of grip strength (Grip) to BW (Grip/BW) reduced in 22 and 15M groups compared to 6M group, and showed significant difference between 22 and 15M groups ($P < 0.01$) (Fig. 2l). Representative images of SM section are shown in Fig. 2g–i. We found that MCSA was lower in 15 and 22M groups than in 6M group, while the difference was not significant ($P > 0.05$) (Fig. 2j).

Serum concentrations of thyroid hormones in mice

Serum levels of FT3 in three groups showed slight decrease with age, yet no significant difference was found in 22M compared to 15 and 6M groups ($P > 0.05$) (Fig. 3a). There was no significant difference in FT4 among the three groups ($P > 0.05$) (Fig. 3b). Serum levels of TSH in 22M group were higher than in the other two groups, although the difference was not significant ($P > 0.05$) (Fig. 3c).

Altered expression of TH transporters in skeletal muscle

Real-time PCR analysis of two major TH transporters MCT8 and MCT10 showed that MCT8 mRNA expression level was higher in 15 and 22M groups compared to 6M group, while the difference was not significant ($P > 0.05$) (Fig. 4a). However, MCT10 mRNA expression level was significantly higher in both 15 and 22M groups compared to 6M group ($P < 0.01$) (Fig. 4b).

Altered expression of deiodinases in skeletal muscle

Next, we detected two types of deiodinases DIO2 and DIO3. DIO2 mRNA expression significantly increased in 22M group compared to 6 and 15M groups ($P < 0.01$). However, there was no significant difference between 6 and 15M mice ($P > 0.05$) (Fig. 5a). The expression of DIO3 mRNA increased with advancing age, and was significantly higher in 15 and 22M groups compared to 6M group ($P < 0.05$) (Fig. 5b).

Altered expression of TRα in skeletal muscle

Compared to 6 and 15M groups, TRα mRNA level significantly decreased in 22M group ($P < 0.05$), while showed

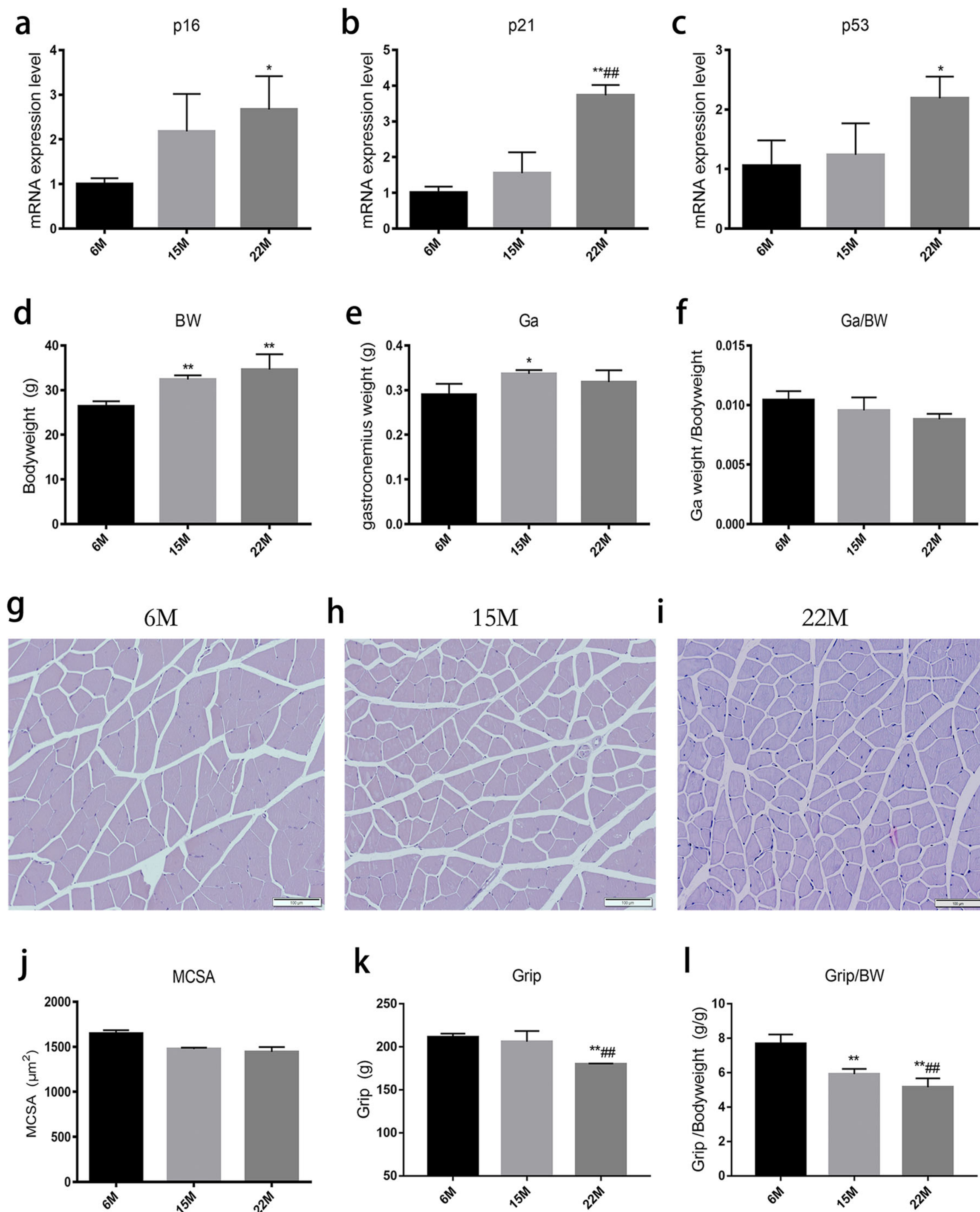

Fig. 2 Changes in senescence-associated genes expression, BW, muscle weight, MCSA and grip strength during aging. **a**–**c** RT-PCR analysis of p16, p21, and p53 mRNA expression of three groups. **d** BW of three groups; **e**, **f** Ga muscle weight and normalized Ga muscle weight in different-aged mice; **g**–**i** representative HE staining of Ga muscle sections; **j** quantitative analysis of MCSA level of three groups; **k**, **l** four limb grip strength and normalized grip strength of three groups. Scale bar, 100 μm. Data were mean ± SEM. *$P < 0.05$ vs. 6M group, **$P < 0.01$ vs. 6M group; ##$P < 0.01$ vs. 15M group

no significant difference between 6 and 15M groups ($P > 0.05$) (Fig. 6a). Consistently, TRα protein level significantly decreased in aged mice group compared to the young and middle-aged groups ($P < 0.05$) (Fig. 6b, c).

Change in skeletal muscle phenotypes during aging

To investigate whether senescence affected muscle fiber types, we examined the expression of four major MyHC in

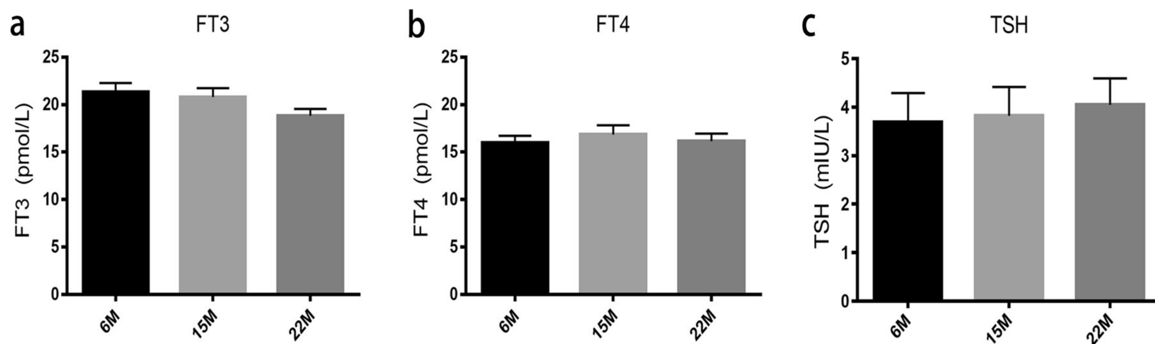

Fig. 3 Serum THs levels in mice of different age. Circulating FT3 **a**, FT4 **b** and TSH **c** levels of three groups. Data were mean ± SEM

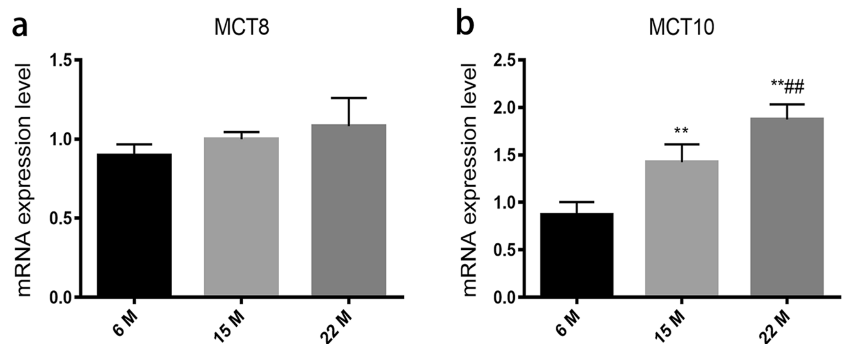

Fig. 4 MCT8 and MCT10 expression in mice at different ages. RT-PCR analysis of relative MCT8 **a** and MCT10 **b** mRNA levels. Data were mean ± SEM. $^{**}P < 0.01$ vs. 6M group; $^{\#\#}P < 0.01$ vs. 15M group

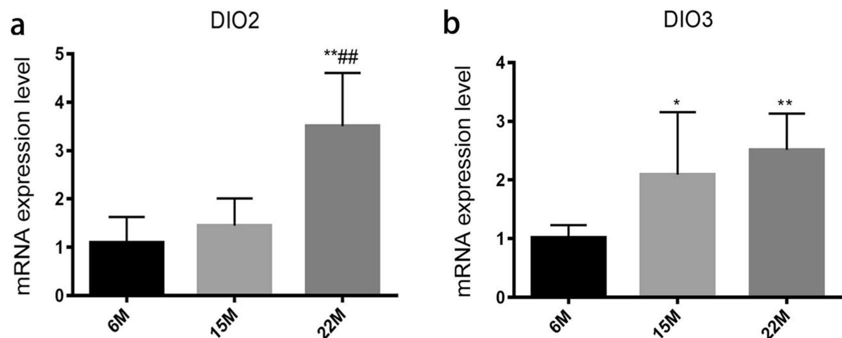

Fig. 5 Deiodinases expression in different-aged mice. RT-PCR analysis of relative mRNA levels of DIO2 **a** and DIO3 **b**. Data were mean ± SEM. $^{*}P <$ 0.05 vs. 6M group, $^{**}P < 0.01$ vs. 6M group; $^{\#\#}P < 0.01$ vs. 15M group

mice at different ages. For slow-twitch fibers, MyHC I mRNA level significantly increased in 22M group compared to 6 and 15M groups, respectively ($P < 0.01$) (Fig.7a). For fast-twitch fibers, MyHC IIa and MyHC IIx exhibited an age-dependent increase in 15 and 22M groups compared to 6M group (Fig. 7b, c), while MyHC IIb mRNA level was significantly lower in 22M group than in 6 and 15M groups ($P < 0.01$) (Fig. 7d).

Discussion

In present study, we demonstrated that expression levels of three senescence-related genes p16, p21, and p53 were significantly higher in aging mice. In addition, histological analysis of lower limb skeletal muscle showed that old mice had increased BW, decreased muscle mass and MCSA level, and their muscle strength attenuated with advanced age. These findings were consistent with those of previous studies [11]. Therefore, we postulated that progressive reduction in mass and strength of SM, as well as typical manifestation of sarcopenia, would occur during aging.

The association between TH signaling and SM has been extensively investigated. TH signaling plays an essential role in regulating physiological processes, such as development, growth, thermogenesis and metabolism [12]. SM is a crucial target of THs and is affected by alteration in TH signaling, such as T3 concentration, deiodinase activity, the

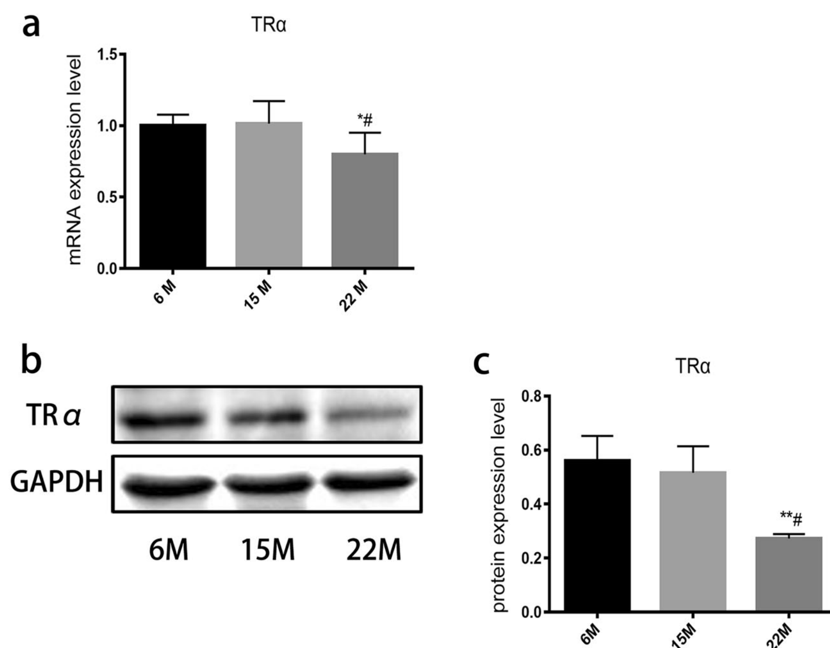

Fig. 6 TRα expression in different-aged mice. RT-PCR analysis of relative TRα mRNA level **a**; Representative blots **b**; Quantification of TRα protein level **c**. Data were mean ± SEM. $^{*}P < 0.05$ vs. 6M group, $^{**}P < 0.01$ vs. 6M group; $^{\#}P < 0.05$ vs. 15M group

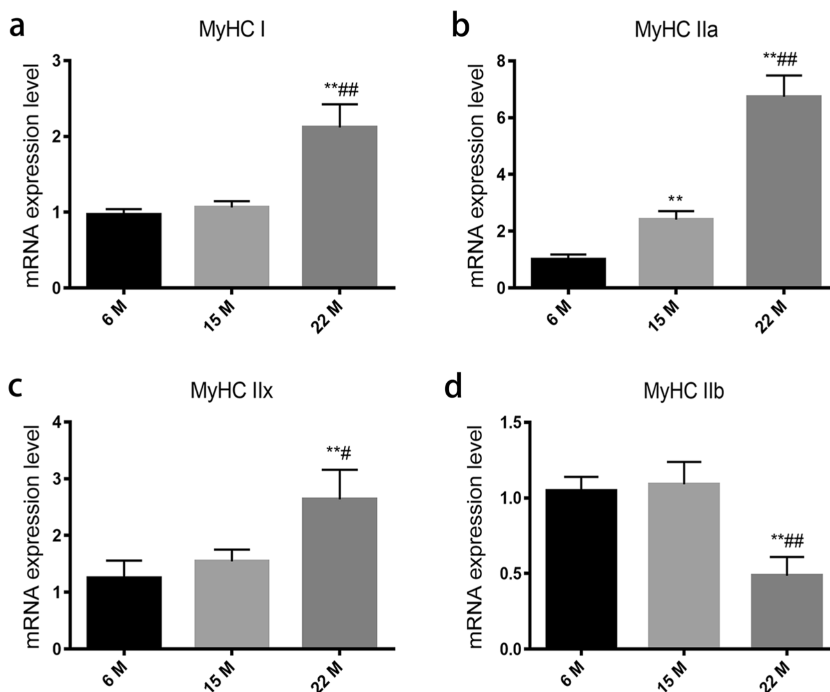

Fig. 7 SM phenotypes in mice at different ages. RT-PCR analysis of mRNA levels of MyHC I **a**, MyHC IIa **b**, MyHC IIx **c** and MyHC IIb **d**. Data were mean ± SEM. $^{**}P < 0.01$ vs. 6M group; $^{\#}P < 0.05$ vs. 15M group, $^{\#\#}P < 0.01$ vs. 15M group

density and function of TH transporters, and thyroidal receptors in SM, but the underlying mechanisms remain uncertain.

In this study, we found that serum level of FT3, the more active form of THs, decreased with aging, while TSH level showed slight increase with aging without significant difference among the three groups, consistent with our previous report that subjects with sarcopenia had lower level of FT3 [13]. In addition, another investigation of us focused on 7122 community population detected the phenomena of gradual increase of TSH with aging, but no significant difference was found compared the young to the middle-aged or older-aged people [14]. These results may be due to attenuated synthesis and secretion of thyroid or consistent attenuation of hypothalamus-pituitary-thyroid axis with aging [15].

During process of THs activation, two T-type TH transmembrane transporters MCT8 and MCT10 are widely expressed in SM of rodents and human [16, 17]. In this study, the expression of MCT8 mRNA showed age-related

increased in mouse SM tissues. It was reported that MCT8-defficient mice had influenced on TH secretion and led to low serum T4 level [18]. In addition, we found that MCT10 mRNA level significantly increased with advanced age and the highest expression occurred in 22M mice group. Compared to MCT8, MCT10 could uptake both T3 and T4, although the ability of transforming T4 was less effective [16]. Our findings suggested that THs may be insufficient in aged muscle fiber, leading to compensatory increase of MCT8 and MCT10 to maintain the concentration of THs in SM microenvironment.

Previous studies demonstrated that THs concentration in skeletal muscle was determined by the balance between DIO2 and DIO3 rather than by serum THs [19, 20]. In this study, we found that both DIO2 and DIO3 mRNA levels significantly increased in aged mice compared to young mice. In addition, the expression of DIO3 but not DIO2 significantly increased in middle-aged mice. DIO2 converts T4 to T3, which possesses stronger biological activity of thyroid hormone. By contrast, DIO3 inactivates T3 through two types, one is promoting T3 degrade to diiodothyronine (T2), and the other is converting T4 reverse to T3 (rT3) [21]. Our results suggested that accompanying with aging, the activity of DIO3 in SM showed more intensive than that of DIO2, in another words, the converting rate of inactive T3 (T2 or rT3) was more prominent compared to T3 production in senescent SM.

As an active form of THs, T3 exerts effects by binding to various nuclear TRs and interacting with specific TH response elements (THEs). TRs ($TR\alpha_{1-2}$ and $TR\beta_{1-3}$) belong to nuclear hormone superfamily, encoding of TRα and TRβ, respectively [22, 23]. TRα is the major isoform widely distributed in SM and plays a significant role in SM differentiation, proliferation, and homeostasis [7]. However, circulating FT3 only occupies about 50% TRα and the other 50% of TRα remain unoccupied in many tissues [24, 25]. In this study, both TRα mRNA and protein showed decreased expression in age-related manner. Previous study reported that TRα-mediated regulation of muscle gene expression is the main mode to active THs, and mutations of TRα may cause TH resistance syndrome [26]. Considering the above-mentioned evidence of TH transporters and deiodinases, we speculated that aging related attenuation of TH signaling may be occurred in defection of T3 binding to TRα, or further dysfunction of TRα.

TH signaling regulates diverse myogenesis-related gene expression in SM. By analyzing SM phenotypes with aging, we found that MyHC I, MyHC IIa, MyHC IIx mRNA levels significantly increased, while MyHC IIb mRNA level significantly decreased during aging. Recent study found that the deletion of TRα increased the expression of MyHC I and the deletion of TRβ only affected MyHC I of fast-twitch fiber [27]. Furthermore, circulating T3 could promote SM fiber transition from slow- to fast-twitch fibers [8]. Our findings speculated that the transition from fast-twitch MyHC IIb to slow-twitch MyHC I occurred with advancing age, since SM fibers could be transformed under stimulation of TH signaling, neuronal innervation and myosin change [28]. In addition, we found that MyHC IIb, a kind of participant in glycolytic mechanism and lowering mitochondrial density, was significantly downregulated with advancing age. These results suggest that TH signaling may be related to mitochondrial metabolism in SM, but need confirmed by further studies.

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Author contributions Y.D.: experiments design and revise. W.X.: performing the experiments. L.W. and Y.S.: data analysis and interpretation. M.S., S.L. and J.Y.: critical review of this paper. X.W. and G.D.: editing this paper. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval Animal studies were in agreement with series of ethical standards, including the institutional and/or national research committee, 1964 Helsinki declaration and its later amendments, and the National and Institutional Guidelines for Animal Welfare.

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