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Age-related reductions in expression of serum response factor and myocardin-related transcription factor A in mouse skeletal muscles

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ABSTRACT

The molecular signaling pathways linking the atrophy of skeletal muscle during aging have not been identified. Using reverse transcription (RT)-PCR, Western blotting, and immunofluorescence microscopy, we investigated whether the amounts of RhoA, RhoGDI, SRF, MRTF-A, and MyoD in the triceps brachii and quadriceps muscles change with aging in mice. Young adult (3 mo) and aged (24 mo) C57BL/6J mice were used. Senescent mice possessed many fibers with central nuclei in the quadriceps muscle. Western blotting using a homogenate of whole muscle or the cytosolic fraction clearly showed that the amount of SRF protein was significantly decreased in the aged skeletal muscles. Immunofluorescence labeling indicated more SRF-positive muscle fibers in young mice. Both young and old mice possessed SRF immunoreactivity in some satellite cells expressing Pax7. MRTF-A and STARS mRNA levels significantly declined with aging in the triceps brachii and quadriceps muscles. The amount of MRTF-A protein was markedly reduced in the nuclear fraction of aged muscle of mice. The amounts of RhoA and RhoGDI in the crude homogenate or the cytosolic and membrane fractions were greater in the aged muscle. Senescent mice possessed significantly higher levels of MyoD protein in the cytosol and nucleus. Decreased SRF and MRTF expression may induce the atrophy of skeletal muscle with aging.

1. Introduction

Sarcopenia is a term that refers to a loss of skeletal muscle mass associated with a decrease in muscle strength and increased fatigability. Sarcopenia is the common denominator of the aging process, responsible for a general and substantial decline in physical performance, which leads ultimately to physical disability. Muscle loss during aging may partly depend on how a muscle repairs itself after damage [1]. This includes overt injury but also the daily minor damage that may not be perceived via pain or alterations in function. The capacity of skeletal muscle to regenerate relies on satellite cells, a population of myogenic precursor cells located between the basal lamina and the plasma membrane of mature myofibers. As the process of aging occurs, the number of satellite cells is reduced in rat skeletal muscle [2] and regeneration is less successful [3]. Furthermore, the repeated participation of satellite cells in muscle regeneration throughout life results in a significant reduction in their cumulative mitogenic potential [4]. Finally, because of a decrease in compliance of myofibers, aging increases sensitivity to contraction-induced injury and muscle injury [5,6]. Altogether, these factors suggest that the age-related loss of skeletal muscle mass and function may partly result from cumulative repeated episodes of incomplete repair [3].

In the initial phase of muscle regeneration, satellite cells are activated and proliferate in response to stimulation by growth factors and cytokines such as insulin-like growth factor-I (IGF-I), hepatocyte growth factor (HGF), interleukin-6, and leukemia inhibitory factor (LIF) [7]. After the initial phase, adult myoblasts differentiate into myotubes, and then fuse with the damaged fibers or form new fibers [7]. Myogenic regulatory factors (MRFs), myocyte enhancer factor (MEF2), and serum response factor (SRF) tightly regulate the muscle differentiation process [8–10]. Most previous studies dealt with the adaptive changes in MRFs with aging. All these studies showed [11–13] an aging-induced increase in MRF mRNA but not protein levels in skeletal muscles of experimental animals. In contrast, no study conducted to date has examined the aging effect of the amounts of MEF2 and SRF, the other factors that promote muscle regeneration during the phase of differentiation.

SRF, the MADS box transcription factor, enhances the hypertrophic process of muscle fibers after functional overload [14,15] as well as muscle differentiation and MyoD gene expression [8] *in vitro*. For example, we showed that, in mechanically overloaded muscles of rats, SRF protein is co-localized with MyoD and myogenin in myoblast-like cells during the active differentiation phase [15]. In contrast, hindlimb unweighting leads to a rapid atrophy along with a marked reduction in

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levels of SRF and the SRF-linking molecules (RhoA, FAK, paxillin, etc) [16]. Maintenance of SRF expression with aging may thus be critical to ensure a proper response of skeletal muscle to damaging insults and to prevent the atrophy of muscle fiber.

It is proposed that the RhoA–FAK–SRF pathway is of potential importance in transducing mechanical signals from the cell membrane into the nucleus in skeletal muscle fibers [17,18]. In contrast, RhoGDI would inhibit the functional role of RhoA, even if RhoA protein was abundant in these tissues. C_2C_{12} cells overexpressing RhoGDI do not cause differentiation into myotubes [19].

Like MEF2 and other MADS box transcription factors, SRF activates transcription by associating with a variety of signal-responsive and cell type-restricted cofactors [20]. Myocardin, an especially powerful SRF coactivator expressed specifically in cardiac and smooth muscle cells, has been shown to be necessary and sufficient for cardiac and smooth muscle gene expression [21]. The myocardin-related transcription factors (MRTFs) MRTF-A (also called MAL/MKL1/BSAC) and MRTF-B (also called MKL2) also are expressed in skeletal, cardiac, and smooth muscle cells, as well as other cell types [22–24]. Although a recent study showed that the complex of SRF and MRTF enhanced the transcription of α-actin in cultured cells [25], their potential contributions to the growth, atrophy, or regeneration of skeletal muscle *in vivo* have not yet been investigated.

In the present study, we examined whether the mRNA and/or protein levels of RhoA, RhoGDI, SRF, MRTF-A, and MyoD in the skeletal muscle change with aging.

2. Materials and methods

2.1. Experimental animals

Young adult (3 mo; $n=10$) and aged (24 mo; $n=10$) C57BL/6J mice were used in this study. The animals were housed in a temperature (22±2 °C) and humidity (60±5%)-controlled room regulated to provide alternating 12-h periods of light and darkness and were allowed to feed (commercial chow) and drink *ad libitum*. Using excess pentobarbital, mice were killed and the quadriceps and triceps brachii muscles were dissected. The muscles were quickly weighed, frozen in liquid nitrogen, and then stored at −80 °C. This experimental procedure was approved by the Committee for Animal Research of the Kyoto Prefectural University of Medicine.

2.2. Primary antibodies

The antibodies employed in the present study were as follow: affinity-purified mouse monoclonal antibody to RhoA (dilution 1:300, Santa Cruz Biotechnology Inc., Santa Cruz, CA), SRF (1:100, GeneTex Inc., San Antonio, TX), MyoD (1:1000, Pharmingen, Becton, Dickinson and Company, San Diego, CA), cadherin (1:2000, Abcam Ltd, UK), HSP70 (1:2400, Abcam), dystrophin (1:50, Sigma-Aldrich Corp., St. Louis, MO) and RhoGDI (1:3000, Pharmingen), and affinity-purified rabbit polyclonal antibody to SRF (1:60–1:300, Santa Cruz), MRTF-A (1:400, Santa Cruz), Histone 2B (1:600, Chemicon International Inc., CA) and Pax7 (1:100, Genway Biotech, Inc., San Diego, CA).

2.3. RNA isolation and RT-PCR

Total RNA was isolated from skeletal muscle using Sepasol-RNA II Super (Nacalai Tesque, Inc., Kyoto, Japan) according to the manufacturer's directions. First-strand cDNA was produced using 1 μg of RNA with a 1st Strand cDNA Synthesis Kit for RT-PCR (AMV; Takara Bio Inc., Otsu, Japan). Complementary DNA was then used as a template for PCR carried out in a 15-μl reaction mixture containing 1 μm of each primer, 40 μM $MgCl_2$, Ex Taq Buffer, and 0.15 μl of Ex Taq polymerase (Takara). The specific primers used are summarized in Table 1. The oligonucleotides for each PCR primer were purchased from Sigma-Genosys (Hokkaido, Japan). The reaction consisted of 30 s of denaturing at 96 °C, 30 s of annealing at 51 °C (STARS), 54 °C (MRTF-A and cyclophillin), 58 °C (Rock1), 57 °C (18S rRNA) and 61 °C (β1 integrin), and 60 s of elongation at 72 °C. The PCR conditions for the analysis of the expression of each gene were designed to avoid saturation and to enable a semiquantitative determination. The amplicons were resolved by electrophoresis on 1.5% agarose gels and visualized by ethidium bromide staining. Each sample was run in duplicate and was normalized against the data for 18S rRNA or cyclophillin, averaged, and statistically compared.

2.4. Tissue preparation, gel electrophoresis, and immunoblotting

Each tissue was homogenized in 10–20 vol of 50 mM Tris–HCl pH 7.4, 5 mM EDTA, 10 μg/ml phenylmethylsulfonylfluoride, 0.5 μg/ml leupeptin, 0.2 μg/ml aprotinin, 0.2% NP-40, 0.1% Triton X-100, and 1 mM Na_3VO_4 in a polytron (DIAX 900, Heidolph-Instruments, Schwabach, Germany) for 30 s. The homogenized tissues were then centrifuged at 15,000 *g* for 25 min at 4 °C, and the protein concentration of the supernatant was colorimetrically determined (Bio-Rad protein determination kit, Bio-Rad Laboratories, Richmond, CA). Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (8–12.5% acrylamide) and Western blotting were next performed as previously described [26]. Proteins separated by SDS-PAGE were electrophoretically transferred onto nitrocellulose membranes (Hybond-ECL Western, Amersham, Arlington Heights, IL). The blots were then incubated with blocking buffer composed of 0.1% Tween-20 and 1% gelatin in 10 mM Tris-buffered saline (TBS, 10 mM Tris, 135 mM NaCl, 1 mM KCl, 0.02% NaN_3, pH 7.4) for 10 min. The blots were next incubated with primary antibodies for 15 h, and with goat anti-mouse or donkey anti-rabbit IgG-conjugated AP (1:10000, Promega Co., Madison, WI) for l h and were visualized with Western blue stabilized substrate for alkaline phosphatase (Promega). A densitometric analysis of each blot was performed with a computerized image processing system (NIH image 1.63).

Table 1
Primers used in PCR

Gene		Sequence (5′-3′)	Position (5′-3′)	Size	Cycles
MRTF-A	Forward	CACATTCTGGAAGAGACCTCGG	583–1049	467	37
	Reverse	ACGATGCTTCCATTGGCTAGG			
18S rRNA	Forward	CTCTTTCGAGGCCCTGTAATTGG	538–1015	478	33
	Reverse	CATTCTTGGCAAATGCTTTCGC			
β1 integrin	Forward	GGCACACTGTCTGGAAACTC	1251–1650	400	36
	Reverse	ACCTGCAGGCTCCACACTCA			
Rock1	Forward	ATTCCAAGATGACCGGTATC	901–1307	407	38
	Reverse	AAGGTGTGTCACCTACGAGC			
Cyclophillin	Forward	CACAGAATTATTCCAGGATTC	200–590	391	31
	Reverse	CTGAGCTACAGAAGGAATGGT			
STARS	Forward	ATTGACAGAATCCTTCTTAGT	421–811	391	35
	Reverse	TGGACATGGCTAGGTCATAGT			

The primers were derived from the following Genebank accession numbers: MRTF-A: AF532597; 18S rRNA: X00686; β1 integrin: NM_010578; Rock1: NM_009071; cyclophillin: X52803; STARS; AF504061.

2.5. Subcellular fractionation

To compare the amount of RhoA, RhoGDI, SRF, and MyoD protein between young and aged quadriceps muscles in the cytosol, nucleus, and membrane, rapidly frozen muscle tissue (25–50 mg) was fractionated into cytosol, membrane, nucleus, and cytoskeleton using a Subcellular ProteoExtract proteome kit from Calbiochem (EMD Biosciences, La Jolla, CA) according to the manufacturer's directions. We identified pure subcellular fractions using antibodies for heat shock protein 70 (HSP70), Histone 2B, and cadherin, each of which was selectively detected in cytosol, nucleus, and membrane fractions, respectively.

2.6. Immunofluorescence

Mouse quadriceps muscle was isolated and frozen in isopentane. Serial 7-μm transverse sections made with a cryostat (Sakura Coldtome, CM-502, Japan) were mounted on silanized slides (Dako Japan, Tokyo). Cryosections were fixed with methanol (3 min) and incubated in blocking solution (10% normal horse serum in PBS) for 20 min at room temperature. Sections were incubated with monoclonal anti-SRF and polyclonal anti-Pax7 antibodies at 4 °C overnight. After being washed in PBS, sections were incubated with anti-mouse FITC-conjugated (1:100 final dilution; Rockland Immunochemicals, Inc., USA) and anti-rabbit Rhodamine-conjugated (1:100; Chemicon) secondary antibodies. The muscle sections were mounted using a slowfade antifade kit with DAPI (Molecular Probes, Eugene, OR). Images were acquired on an Olympus BX50 inverted microscope with a fluorescent attachment (Olympus) and Photonic Science CCD camera (Olympus DP70).

2.7. Morphometric analysis

The cryosections were stained with hematoxylin–eosin (H&E) to observe the histological differences in quadriceps muscle between young adult and aged mice. The percentage of centrally nucleated fibers was determined on at least 480 fibers (489~693) per muscle, using microphotographs taken at three different positions in the muscle. Direct tracings of each muscle fiber were carried out from cryosections processed for H&E staining. The cross-sectional area was determined with at least 200 fibers at different positions of the entire muscle and an image analysis computer program (NIH Image 1.63).

2.8. Statistical analysis

All values were expressed as means±SEM. A one-way analysis of variance (ANOVA) was used to evaluate the significance of differences in morphometric parameters and in the mRNA and protein levels of SRF-linking molecules in muscle. A Scheffés post hoc

Table 2
Muscle characteristics

	Quadriceps		Triceps brachii	
	3-month-old	24-month-old	3-month-old	24-month-old
Wet weight (mg)	185.3±8.2	135.3±5.5**	105.1±4.5	83.3±3.6**
Muscle wet weight/body weight (mg/g)	6.90±0.32	4.25±0.16**	3.91±0.16	2.61±0.08**

Values are means±SEM; $n=10$/group; ** Significantly different from 3-month-old mice ($P<0.01$).

test was conducted if the ANOVA indicated a significant difference. $P<0.05$ was considered to be statistically significant.

3. Results

The body weight of the aged mice (31.9±0.6 g) was 18.6% higher than that of the young adult mice (26.9±0.3 g). The extent of aging-associated sarcopenia was similar in each of the skeletal muscles from aged mice compared with the young adults as shown by the ~27% reduction in muscle wet weight with age (Table 2). The degree of muscle loss was even more striking when muscle mass was normalized to body weight (mg/g body wt), which was 33.2~38.4% lower in aged mice than young adult mice.

Histological characteristics of the quadriceps muscle of 3-month (A) and 24-month-old (B) mice are shown in Fig. 1. Each cryosection was stained with hematoxylin & eosin. As previously reported [27] the muscle of aged mice contained several atrophic, centrally nucleated fibers (Fig. 1B). The percentage (19.1±1.5%) of muscle fibers with central nuclei was significantly increased in the aged quadriceps muscle (Fig. 1C). Compared with young mice, the mean fiber cross-sectional area was significantly lower in the quadriceps muscle of aged mice (Fig. 1D). Comparing the distributing pattern of the muscle fiber area, aged mice possessed small (atrophic?) muscle fibers more frequently than young mice (Fig. 1E).

As shown in Fig. 2, immunoblotting with antibodies against SRF and β-actin revealed prominent bands consistent with a molecular size of 56–60 and 30 kDa, respectively (Fig. 2A). Western blots showed that SRF protein was abundantly expressed in skeletal muscles of young mice compared with those of aged mice. A densitometric analysis ($n=6$) showed the amount of SRF in quadriceps and in triceps

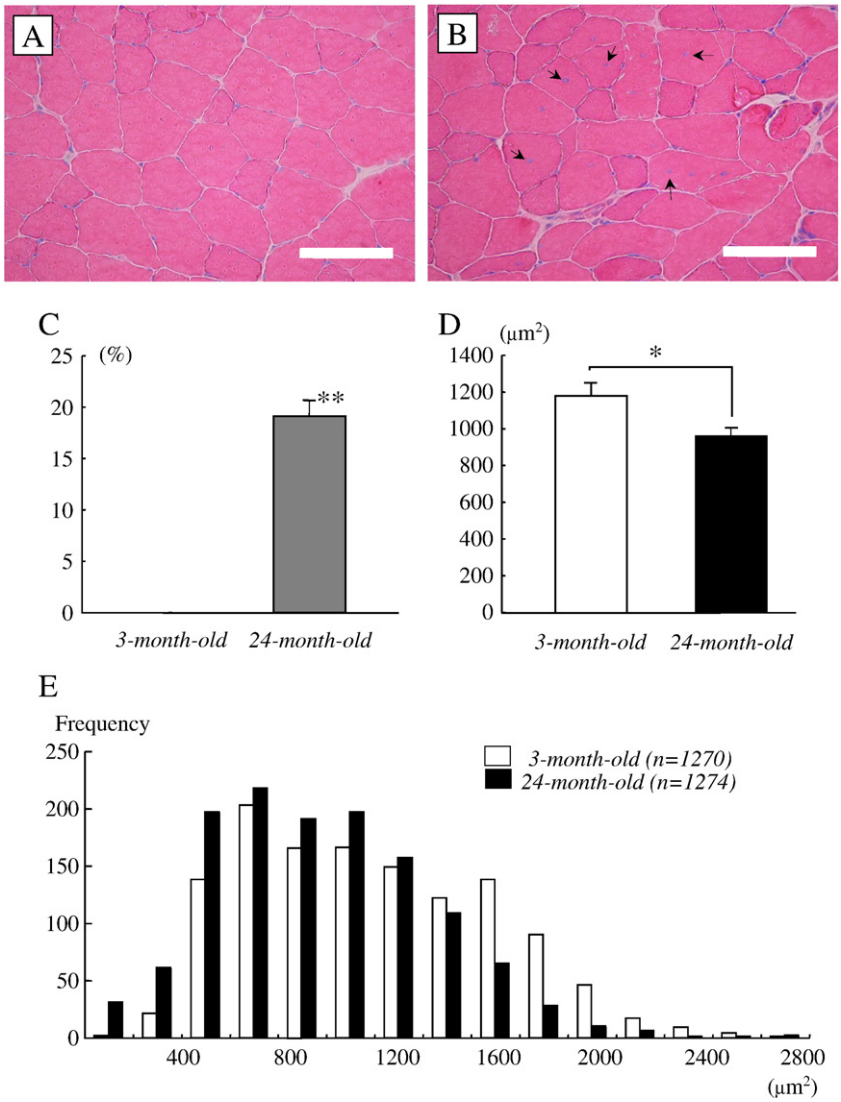

Fig. 1. Histological characteristics of the quadriceps muscle of 3-month-old (A) and 24-month-old (B) mice. Each cryosection was stained with hematoxylin & eosin. The muscle of aged mice had several atrophic and centrally nuclear fibers (B). The percentage of muscle fibers with central nuclei was significantly increased in the aged quadriceps muscle (C). The mean fiber cross-sectional area was significantly lower in the quadriceps muscle of aged mice than young mice (D). Aged mice possessed small (atrophic?) muscle fibers more frequently than young mice (E). Values are means±SEM ($n=6$/group). Bar=50 μm.

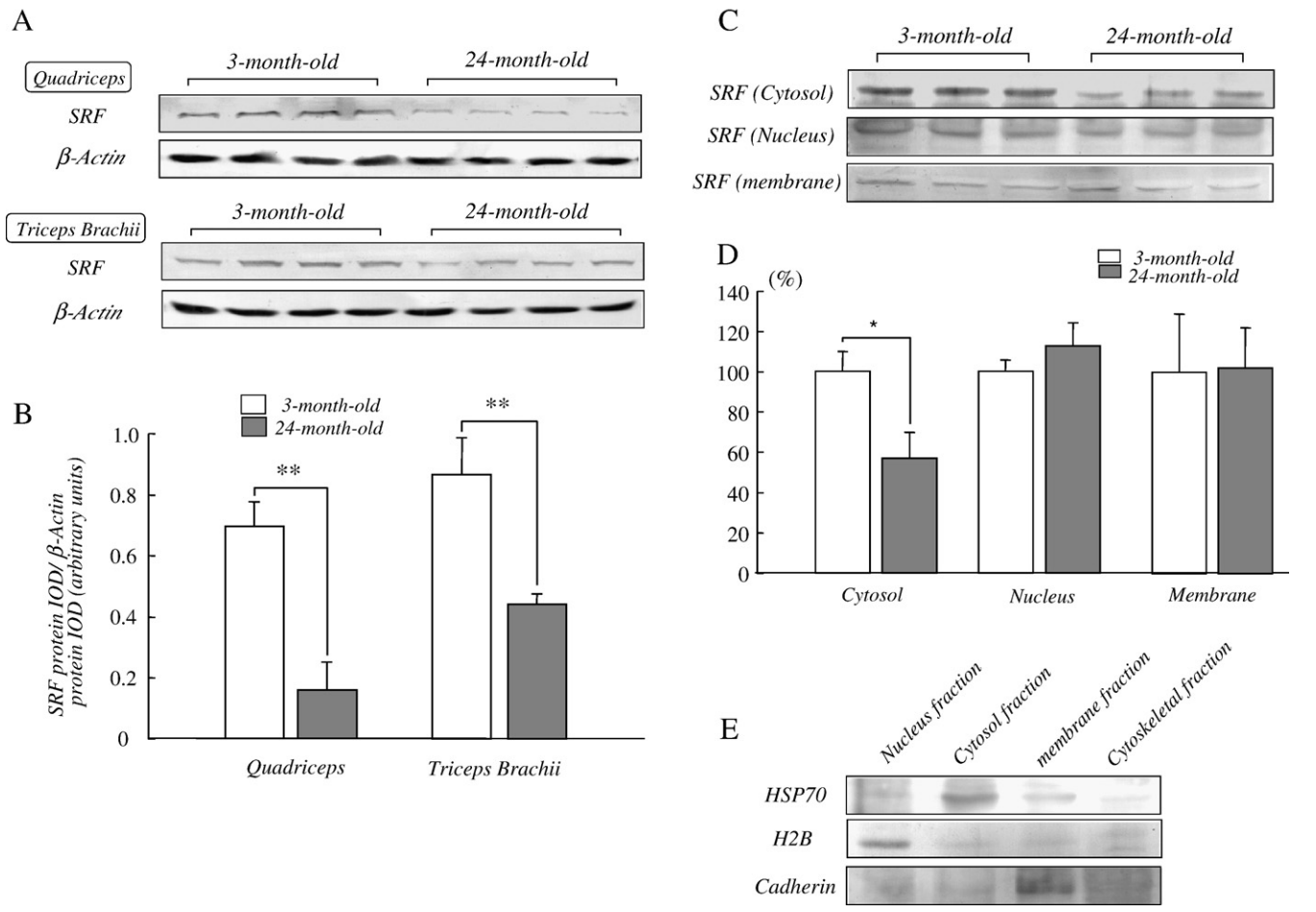

Fig. 2. Western blot analysis showed that SRF protein was more abundantly expressed in both the quadriceps and triceps brachii muscles of young mice compared with those of aged mice (A). No significant effect of aging on the amount of β-actin was observed in either muscle. Densitometric analysis (n=6) of aged mice showed the amount of SRF in skeletal muscles to be 85 and 50% lower than that in young mice. The integrated optical density (IOD) of SRF protein was normalized to the IOD of β-actin protein (arbitrary units). Values are means ± SEM (n=6/group). The amount of SRF in the cytosolic fraction was greater in the skeletal muscle of young mice (C and D). No significant difference in the amount of SRF in the nuclear and membrane fractions was observed in the quadriceps muscle between 3-month and 24-month-old mice (C and D). The values are expressed as percentages of the immunostaining intensity of SRF protein in 3-month-old mice, which was taken as 100% (D). Control experiments with subcellular pure fractions using each selective marker indicated a selective immunoblots of HSP70 in cytosol, H2B in the nucleus, and cadherin in the membrane fraction (E).

brachii muscles to be 85 and 50% lower, respectively, in aged than young mice (Fig. 2B). We did not observe a significant effect of aging on the amount of β-actin in these muscles (Fig. 2A). We performed Western blotting using homogenate after cell fractionation, such as the cytosol, nucleus, membrane and cytoskeleton of the muscle fiber, in order to understand the difference in the cellular distribution of SRF between young and aged muscles of the mice. The amount of SRF in the cytosol but not nucleus and membrane was greater in the young quadriceps muscle than the aged muscle (Fig. 2C). We conducted control experiments with subcellular pure fractions using each selective marker (HSP70, H2B, and cadherin). We observed selective immunoblots of HSP70 in cytosol, H2B in the nucleus, and cadherin in the membrane fraction (Fig. 2E).

To more clearly understand the cellular localization of SRF protein in the young and aged quadriceps muscles of mice, we performed immunofluorescence staining of single cryosections. SRF and Pax7 immunoreactivity was visualized using FITC- or Rhodamine-conjugated antibodies. In young quadriceps muscle, immunolabeling of SRF was detected in the cytosol and membrane of several muscle fibers (Fig. 3A). Apparent SRF immunoreactivity was also observed in membrane but not cytosol of aged muscle fibers (Fig. 3B). Both young and aged mice similarly possessed apparent SRF immunoreactivity in some satellite cells expressing Pax7 (Figs. 3C–F). In the quadriceps muscle of aged mice, Pax7-positive satellite cells did not possess the immunoreactivity of SRF without the primary antibody to SRF (Figs. 3G, H, and I). The control experiment indicated immunoreactivity for Pax7 outside (satellite cells) of the plasma membrane labeled with dystrophin (Figs. 3J, K, and L).

MRTF-A, which is expressed in skeletal muscles, seems to be a signal-responsive transcription factor, which moves to the nucleus and activates SRF target genes [25]. RT-PCR analysis showed that MRTF-A mRNA of a suitable size (467 bp) was present. Both muscles of young mice abundantly possessed MRTF-A mRNA compared with the muscles of aged mice (Fig. 4A). The amount of 18S rRNA in these muscles did not significantly differ between young and aged mice (Fig. 4A). The level of MRTF-A mRNA was 70% and 50% lower in the quadriceps and triceps brachii of 24-month-old mice, respectively (Fig. 4B). The quadriceps of senescent mice possessed similar amounts of MRTF-A protein in the cytosol and membrane (Figs. 4C and D). In contrast, we detected a significantly lower level of MRTF-A in the nuclear fraction (75.8% lower than the control) in the skeletal muscle of aged mice.

RhoA is a candidate for the upstream modulator of SRF activity and transcription. However, it is possible that RhoGDI protein inhibits the functional role of RhoA in the muscle [19]. Thus, we investigated whether levels of these proteins change in skeletal muscles of mice with age. Compared with young mice, aged mice possessed more RhoA and RhoGDI protein in their muscles (Figs. 5A and C). Densitometric

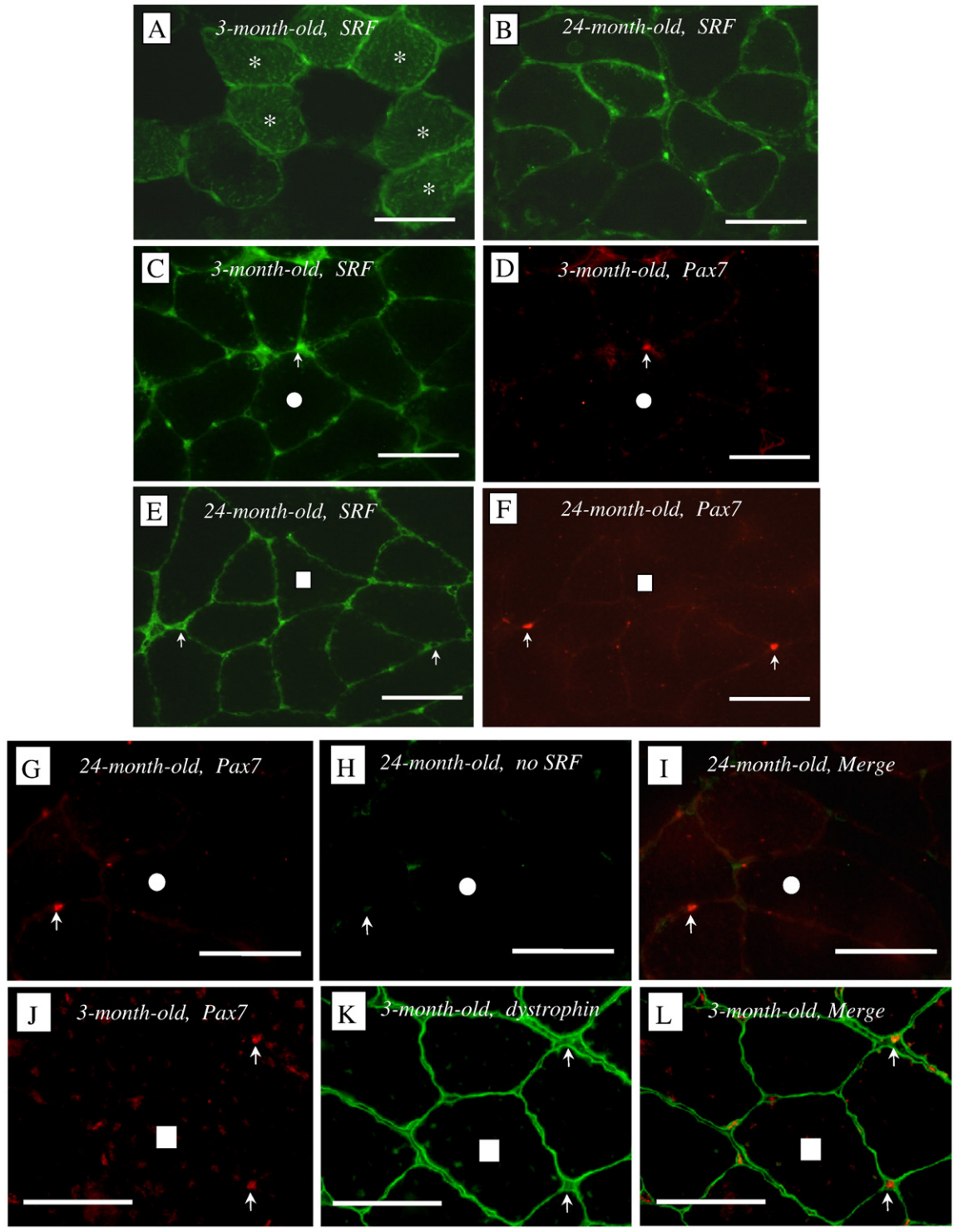

Fig. 3. Serial cryosections of the quadriceps muscle of 3-month and 24-month-old mice. SRF and Pax7 immunoreactivity was visualized using FITC- or Rhodamine-conjugated antibodies. In young quadriceps muscle, immunofluorescence labeling showed that SRF was present in the cytosol and membrane of several muscle fibers (A). Apparent SRF immunoreactivity was also observed in membrane but not cytosol of aged muscle fibers (B). Both young (C and D) and aged (E and F) mice similarly possessed apparent SRF immunoreactivity in some satellite cells expressing Pax7. In the quadriceps muscle of aged mice, Pax7-positive satellite cells did not possess the immunoreactivity of SRF without the primary antibody to SRF (G, H and I). The control experiment indicated immunoreactivity for Pax7 outside (satellite cells) of the plasma membrane labeled with dystrophin (J, K, and L). The filled circles and squares indicate the same fibers in different immunoimages. White arrows denote the same satellite cells. The asterisks (*) denote the SRF-positive fibers. Scale bar = 50 μm.

analysis ($n=6$) showed the amount of RhoA and RhoGDI to be 60–120% and 50–70%, respectively, greater in aged mice than young mice (Figs. 5B and D). The quadriceps of senescent mice possessed more RhoA protein in the cytosol and membrane than did those of young mice (Figs. 5E and F). In contrast, we did not detect nuclear RhoA protein in the skeletal muscle of either mouse. More RhoGDI protein was expressed in the aged quadriceps in the nucleus (44.8% over the control level), cytosol (15.6%), and membrane (52.7%), than in the corresponding fractions in young mice (Figs. 5E and F), although the difference was not significant in the nuclear fraction ($P=0.059$). The

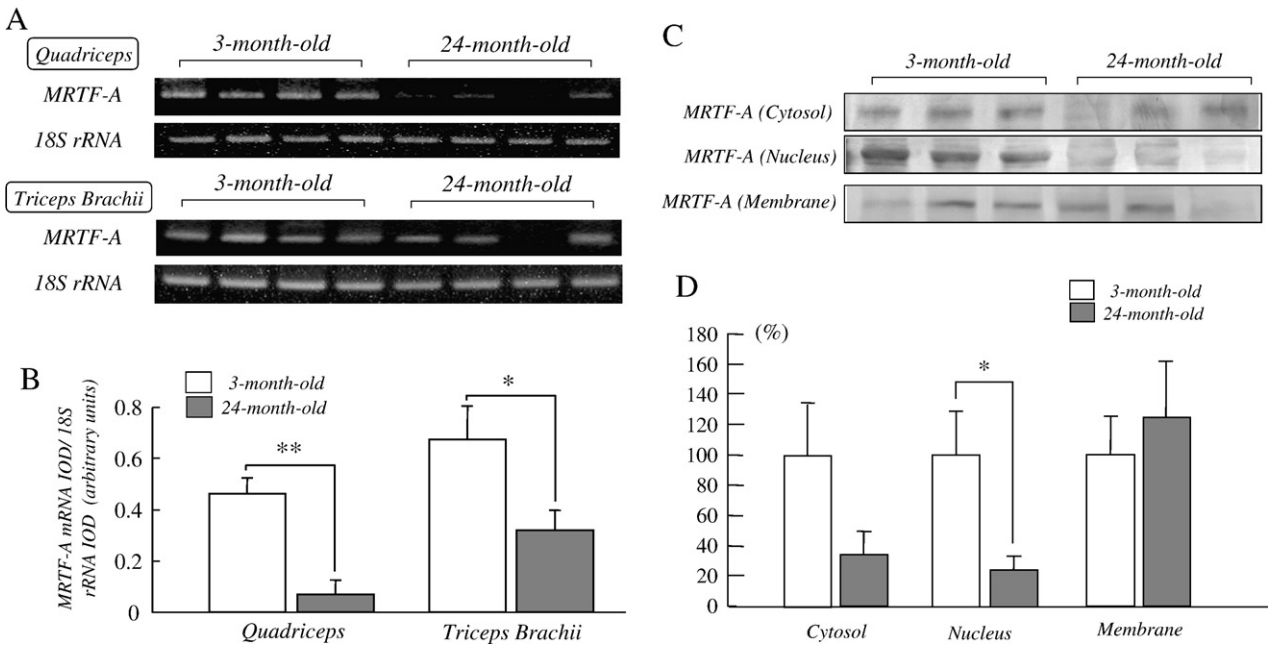

Fig. 4. A: RT-PCR analysis showing that MRTF-A mRNA of a suitable size (467 bp) was present. Both muscles of young mice abundantly possessed MRTF-A mRNA compared with those of aged mice (A). The lanes show ethidium bromide-stained PCR product bands generated from individual muscle cDNA samples where 18S rRNA served as a loading control. The amount of 18S rRNA did not significantly differ in these muscles between young and aged mice. Densitometric analysis showed a significant decrease in MRTF-A mRNA levels in aged muscle (B). The amount of MRTF-A mRNA was 70% and 50% smaller in the quadriceps and triceps brachii of 24-month-old mice, respectively (B). The integrated optical density (IOD) of MRTFs was normalized against the IOD of 18S rRNA (arbitrary units). The values are means ± SEM (n = 6/group). The quadriceps of senescent mice possessed similar amounts of MRTF-A protein in the cytosol and membrane (C and D). In contrast, we detected a significantly lower level of MRTF-A in the nuclear fraction (75.8% lower than the control) in the skeletal muscle of aged mice (C and D).

amount of RhoA/RhoGDI was significantly increased in the cytosol but not membrane in aged mice.

In myogenesis, RhoA signaling via SRF appears to regulate MyoD expression at the transcriptional level [8]. We compared the expression pattern of MyoD protein in the muscle between young and senescent mice. MyoD was more abundantly expressed in the aged mice in the crude homogenate from the quadriceps and triceps brachii muscles or the nucleus and cytosol in quadriceps muscle (Figs. 6A–C).

The disruption of signaling between RhoA and SRF in aged muscles may be attributable to the changes of cytoskeletal dependent molecules such as β1 integrin, STARS, and α-actin and/or independent ones such as Rock kinase, PKN, and MuRF2. The level of STARS mRNA was 60% and 40% lower in the quadriceps and triceps brachii of 24-month-old mice, respectively (Figs. 7A and B). In contrast, we observed no significant differences in the mRNA levels of β1 integrin and Rock1 in either muscle between 3-month-old and 24-month-old mice (Figs. 7C and D).

4. Discussion

Three main conclusions were drawn from the present study. First, there was a marked reduction in the amount of SRF protein in the quadriceps and triceps brachii muscles of mice with aging. Second, aged mice showed an increase in RhoA and the inhibitor RhoGDI in both muscles. Third, mRNA levels of the SRF-linking molecules MRTF-A and STARS were also decreased in skeletal muscle of senile mice.

It is proposed that the RhoA–SRF pathway is of potential importance in transducing mechanical signals from the cell membrane into the nucleus of skeletal muscle fibers [17,18] To our knowledge, this is the first study to investigate SRF signaling in aged animals. In both forelimb and hindlimb muscles of mice, we observed a marked reduction in the amount of SRF protein using three different methods (Western blotting with a crude or fractionated homogenate and immunofluorescence labeling). One interpretation for the loss of SRF protein would be a decrease in satellite cells during aging, because our previous study demonstrated a slight expression of SRF protein in quiescent satellite cells [15]. Indeed, many satellite cells of young and aged mice possessed SRF, although both biochemical and immunohistochemical results in our study clearly showed a more marked reduction of SRF in aged mice in the cytosol of mature muscle fibers. It is widely accepted that the number of satellite cells declines with aging [2,28] and the regeneration of aged muscle is less successful due to a significant reduction in the cumulative mitogenic potential of satellite cells throughout life [4]. More recently, Brack et al. [29] proposed an intriguing hypothesis, that a decline in satellite cell function and/or number during aging leads to a loss of nuclei from large fibers and an associated increase in domain size that triggers cytoplasmic atrophy through the cell-size-regulating machinery.

A decrease in the SRF protein level would downregulate the gene expression of several structural proteins such as α-actin [30], SM-22a, myosin light chain 1A [31], beta myosin heavy chain, and desmin. In addition, we demonstrated a decrease in the expression of MRTF-A in aged skeletal muscles of mice. The functional disruption of MRTF-A using a dominant-negative form induced marked atrophy, extensive fibrosis, and centrally located nuclei, indicative of muscle damage and regeneration, due to a reduction in mRNA levels of α-actin, muscle-type CK, and MyoD [32].

In addition, the mutation of SRF delineated the translocational action of MRTF-A induced *in vitro* by striated muscle activators of Rho signaling (STARS), a muscle-specific actin-binding protein [25]. Moreover, we observed a marked reduction in STARS mRNA in the quadriceps and triceps brachii of 24-month-old mice. Therefore, it is possible that the smaller amounts of SRF, MRTF-A and STARS lead to atrophy and the degeneration of muscle fibers during aging.

Our RT-PCR and Western blot analysis using skeletal muscles of aged mice clearly showed no significant change in the expression pattern of β1 integrin, α-actin (data not shown), and Rock1 compared with those of young mice. In skeletal muscle cells, nuclear translocation of MuRF2, a muscle-specific ubiquitin ligase, in response to mechanical inactivity causes a reduction of nuclear SRF [33]. A few findings were obtained

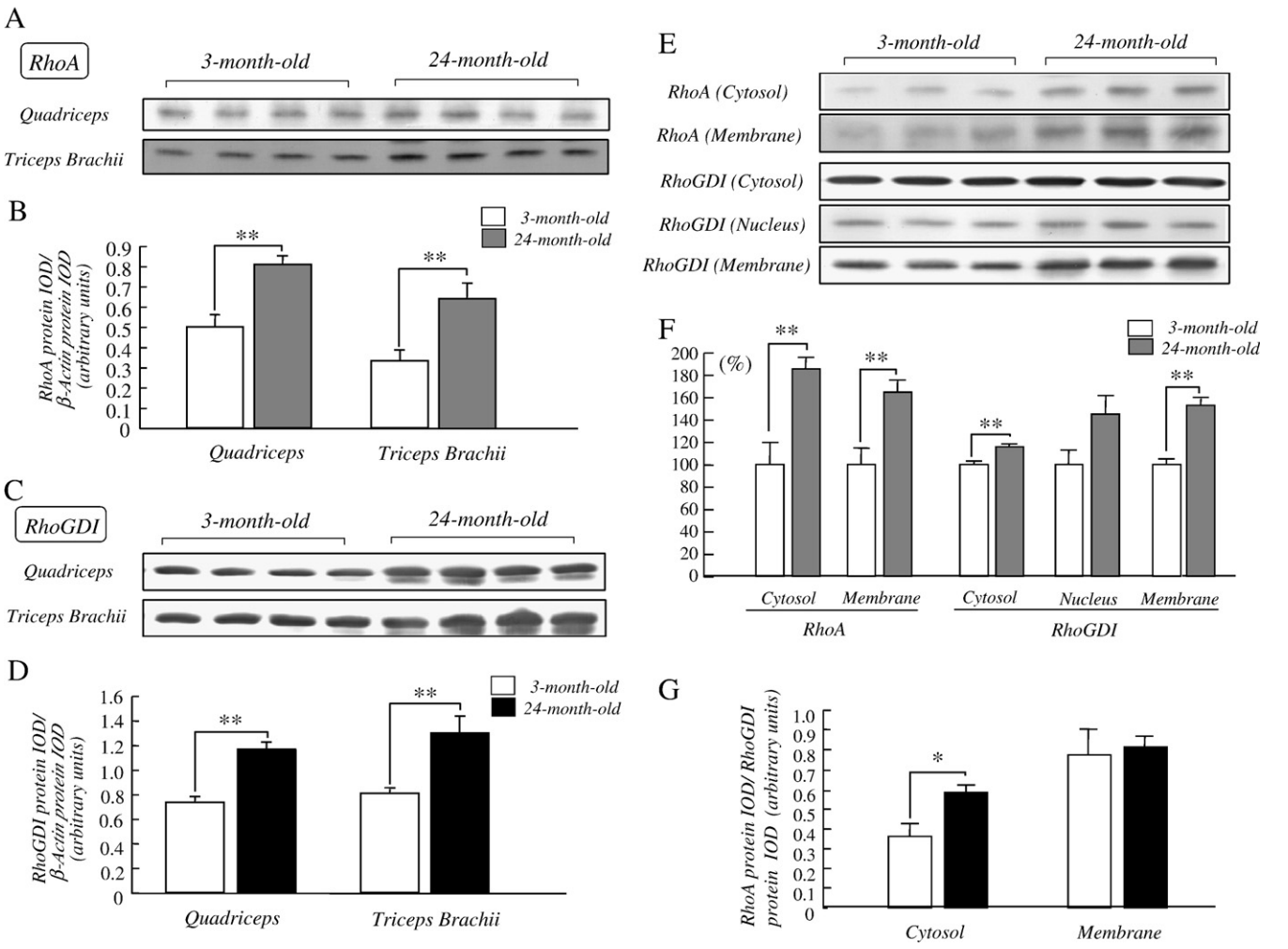

Fig. 5. Compared with 3-month-old mice, aged mice showed markedly increased levels of RhoA protein in quadriceps and triceps brachii muscles (A, B). The amount of RhoGDI protein was also increased in both muscles of 24-month-old mice (C and D). The amount of RhoA and RhoGDI in the cytosol and membrane was significantly greater in the skeletal muscle of aged mice (E and F). In contrast, senescent mice did not possess more RhoGDI protein in the nucleus than did young mice. The amount of RhoA/RhoGDI was significantly increased in the cytosol but not membrane in aged mice (G).

using skeletal muscle of rats concerning the adaptive changes with age in a similar ring-finger protein, MuRF1, although these two studies were very controversial [34,35]. If MuRF2 protein is accumulated in quadriceps and biceps brachii muscle fibers of aged mice in our study, it may contribute to a reduction of SRF in these regions.

Various models of inactivity have shown a reduction in the expression of RhoA in skeletal muscle. For example, McClung et al. [36] demonstrated that suspending the hindlimb for 3 days resulted in a 46% decrease in the amount of RhoA protein in rat plantaris muscle. Surprisingly, the amount of RhoA actually increased in aged muscles. In contrast, the amount of RhoGDI also increased in the cytosol, membrane, and nucleus. RhoA GTPase and the other Rho family proteins become active GTP-bound forms. RhoGDI inhibits the dissociation of GDP and constructs an inactive form of Rho family proteins. C_2C_{12} cells overexpressing RhoGDI do not cause differentiation into myotubes due to marked reductions in myogenin, MRF4, and MEF2C [19]. The RhoA protein existing in aged muscles may activate SRF and/or other downstream molecules by inhibiting RhoGDI protein in the same tissues. It remains to be elucidated whether the ratio of RhoGDI bound to RhoA increases in aged muscle.

The MyoD promoter region contains several putative CArG boxes that diverge more or less from the consensus CArG sequence CC(A/T)6GG. In myogenesis, RhoA–SRF signaling appears to regulate MyoD expression [8]. Additionally, we previously observed the co-localization of SRF and MyoD, in the nucleus and cytosol of myoblast-like cells, respectively, in mechanically hypertrophied muscle [15]. However, different to the changes in SRF, MyoD protein was more abundant in the aged forelimb and hindlimb muscles of mice. Our data differ from the results of Dedkov et al. [37] and Alway et al. [11]. Dedkov et al. [37] reported that the level of MyoD-specific protein in the 43- to 45-kDa band remained relatively unchanged during aging in adult WI/HicksCar rats. In contrast, Alway et al. [11] suggested that in two fast-twitch and soleus muscles of 37-month-old Fisher 344×Brown Norway F1 hybrid rats, the amount of MyoD protein was reduced to 33–66% and unaffected, respectively. Although these studies employed a crude homogenate of skeletal muscle for Western blotting, we also recognized an increase in the cytosol and nucleus but not the membrane in the senile mice using the homogenate obtained by cell fractionation as well as crude homogenate. We assume the significant discrepancy in the results reported in these papers to be related to at least three factors: (1) the use of various types of muscles, (2) significant variation in the age at which the mice or rats from different strains become senescent, (3) and Western blotting using different samples of muscle tissue (crude or fractionated).

An important question that was not addressed in the current study is what cell types are expressing MyoD in aged muscle. Although it has been demonstrated the existence of MyoD protein in myonucleus of skeletal muscle fibers of normal rats [38], our Western blot analysis

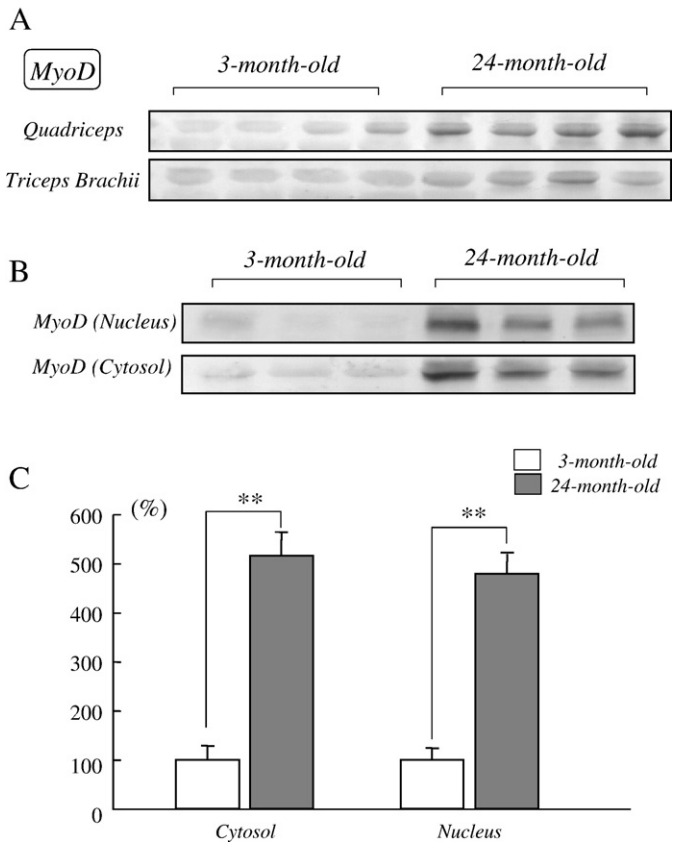

Fig. 6. MyoD was more abundantly expressed in the aged mice in the crude homogenate (A) of the quadriceps and triceps brachii muscles or the nuclear and cytosolic fractions (B) of quadriceps muscle. The amount of MyoD protein was 380–400% greater in the quadriceps and triceps brachii of 24-month-old mice (C).

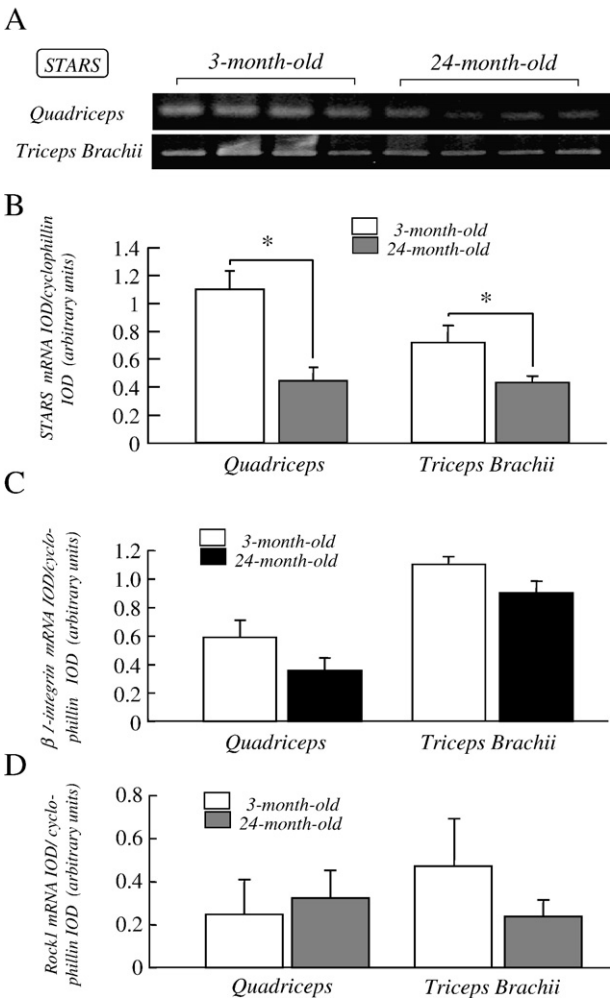

Fig. 7. The level of STARS mRNA was 60% and 40% lower in the quadriceps and triceps brachii of 24-month-old mice, respectively (A and B). We observed no significant differences in the mRNA levels of β1 integrin and Rock1 in either muscle between 3-month-old and 24-month-old mice (C and D). The amount of cyclophillin mRNA serving as a loading control did not significantly differ in these muscles between young and aged mice.

using fractionated homogenates showed abundant MyoD protein in the cytosol fraction as well as nuclear one in several muscles of aged mice. This finding might be attributed to myotube-like cells (about 10%) possessing developmental myosin heavy chain in aged muscle [39]. In fact, MyoD localization is recognized to not only satellite cells and myoblasts, but to some myotubes of the centrally located nuclei [40]. Further study will be required to investigate the precise localization of MyoD protein in aged muscles.

In summary, the present study showed that the expression of SRF, MRTF-A, and STARS decreased in the muscle of aged mice. We suggest that RhoA–SRF signaling was ameliorated in the muscle with aging. The precise functional role of this pathway in aged skeletal muscle *in vivo* should be studied further.

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