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学位論文

Passive repetitive stretching is associated with greater muscle mass and cross-sectional area in sarcopenic muscle

（他動的反復ストレッチによるサルコペニア筋の筋量および筋断面積の増加）

2021 年 9 月

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List of Publications

1. Wang, Y., Ikeda, S. & Ikoma, K. Passive repetitive stretching is associated with greater muscle mass and cross-sectional area in the sarcopenic muscle. Scientific Reports, under review.

2. Wang, Y., Ikeda, S. & Ikoma, K. Efficacy of passive repetitive stretching of skeletal muscle on myofiber hypertrophy and genetic suppression on MAFbx, MuRF1, and myostatin. Journal of Muscle Research and Cell Motility, under review.

List of Presentations

1. Wang, Y., Koganemaru, S., Tobisawa, Y., Tanaka, S., Ninomiya, S., Iguchi H., Mima T., Ikeda S. & Ikoma K. Modulation of cerebella function by paired associative stimulation in patients with spinocerebellar degeneration. The 48th Annual Meeting of Japanese Society of Clinical Neurophysiology, November 8-9, 2018, Tokyo, Japan (Poster).

2. Wang, Y., Koganemaru, S., Tobisawa, Y., Tanaka, S., Ninomiya, S., Iguchi H., Mima T., Ikeda S. & Ikoma K. Improvement of cerebellar and motor functions after repeated sessions of cerebellar paired associative stimulation using TMS in patients with spinocerebellar degeneration. The13th International Society of Physical and Rehabilitation Medicine World Congress, June 9-13, 2019, Kobe, Japan (Poster).

Summary

Background and Purpose:

Age-associated loss of muscle mass and function, known as sarcopenia, is characterized by a progressive decline in muscle fiber number and size, a shift in fiber-type composition, and modification of adverse metabolic parameters. It is imperative to identify effective countermeasures that induce hypertrophy of senescent muscle and combat progression of sarcopenia.The regulation of skeletal muscle mass and fiber size is largely determined by the dynamic turnover between protein synthesis and degradation. Muscle hypertrophy occurs when the overall rate of protein synthesis exceeds the rate of protein degradation. Insulin-like growth factor 1/Akt/mammalian target of the rapamycin (IGF1/Akt/mTOR) signaling primarily controls muscle protein synthesis. Akt stimulates protein synthesis by activating mTOR and inhibits protein degradation through Forkhead box O (FoxO)-mediated ubiquitin-ligases including Muscle Atrophy F-box (MAFbx)/atrogin-1, and Muscle Ring Finger 1 (MuRF1) downregulation. Myostatin has been extensively studied as another potential mediator of sarcopenia owing to its potent negative effects on cell growth and intracellular catabolic and anabolic signaling pathways. In addition, skeletal muscle hypertrophy can be induced by the activation of muscle satellite cells. When activated, a surge of Myogenic regulatory factors (MRFs), including Myoblast determination protein 1 (MyoD), Myogenic factor 5 (Myf5), and myogenin, is required owing to the role in driving the differentiation of myoblasts to mature myotubes. Evidence has shown that stretching applied to cultured skeletal muscle cells provides mechanical stimuli and triggers cellular biomarkers essential for muscle growth. However, recent studies in vivo investigated the effect of static and low-frequency stretching using immobilization approaches on skeletal muscles with very limited hypertrophied outcomes. It has been observed that induction of myogenesis is more effective in fast, repetitive stretching than static stretching. However, the mechanisms underlying muscle hypertrophy resulting from passive repetitive stretching remain poorly understood. Moreover, aging processes alter mechano-transduction, indicating a perturbed load-induced plasticity for aged muscle hypertrophy. Thus, the purpose of this study was to (1) characterize the efficacy of clinically feasible protocols of daily passive repetitive stretching on healthy skeletal muscle mass, myofiber morphology, and then decipher the underlying mechanisms of action in terms of (a) signaling molecules involved in muscle protein synthesis and degradation, and (b) satellite cell content and MRFs expression. (2) further

elucidate that whether or not passive repetitive stretching exerts hypertrophic effect against sarcopenic atrophy.

Materials and Methods:

This study mainly consisted of two experiments: The *Experiment 1* using 6-weeks-old Institute of Cancer Research (ICR) mice and the *Experiment 2* using 35-weeks-old Senescence-Accelerated Mouse Prone-8 (SAM-P8) mice. In order to isolate active isometric contractile activity during stretching duration, I anesthetized the mice with isoflurane solution. I stretched the right gastrocnemius muscles of the animals 15 times/min for 15 min daily, 5 days a week for 2 weeks. Contralateral unstretched muscles were used as controls. Twenty-four hours after the final stretching session, the gastrocnemius muscles of both legs of the mice were removed under deep anesthesia. The mean muscle fiber cross-sectional area (CSA) and the number of myonuclei were determined by the hematoxylin and eosin (H&E) staining. Pax7+ cells were detected by immunohistochemistry (IHC) staining. The messenger ribonucleic acid (mRNA) expression of MRFs and signaling molecules involved in muscle protein synthesis and degradation were detected by real time polymerase chain reaction (PCR). The phosphorylation level of Akt, ribosomal protein S6 kinase beta-1 (p70S6K) and eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) were measured with western blotting.

Results:

In *Experiment 1*, I found that the muscle weight and muscle fiber CSA of the stretched side increased as compared with unstretched side. The mRNA expression of p70S6K and myogenin increased while 4E-BP1, MAFbx, MuRF1 and myostatin expression decreased on the stretched side as compared with the unstretched side. Passive repetitive stretching increased the phosphorylation level of p70S6K. I found there was no significant difference in the number of Pax7+ cells and myonuclei per fiber between the stretched and unstretched sides. In *Experiment 2*, I found that the muscle weight and fiber CSA of the stretched side was greater compared with that of the unstretched side. Passive repetitive stretching increased the mRNA expression level of Akt, p70S6K, 4E-BP1, Myf5, myogenin, MuRF1.The phosphorylation level of p70S6K was significantly increased in stretched muscles as compared with unstretched ones. The Pax7+ cells and myonuclei content doesn't differ between stretched and unstretched muscle.

Discussion:

Akt signaling enhances protein synthesis primarily via activation of mTORC1 (mammalian target of the rapamycin complex 1). The activation of mTORC1 mediates the phosphorylation of S6 kinase and the inhibition of 4E-BP1. In the *Experiment 1*, I found that the mRNA expression of p70S6K significantly increased while 4E-BP1 expression decreased in the stretched side compared to the unstretched contralateral limb. I provided further evidence that in vivo repetitive stretching strongly increased the phosphorylation level of p70S6K in both *Experiment 1* and *Experiment 2*. Overexpression of MuRF1 and MAFbx results in muscle atrophy. In *Expeiment 1*, I observed a decreased expression of MuRF1 and MAFbx after two weeks of passive repetitive stretching. Inhibition of MAFbx and MuRF1 in stretched muscles would help to maintain muscle mass and fiber size. In *Experiment 2*, however, I found that MuRF1 mRNA expression was elevated, indicating the involvement of the cellular degradation pathway in aged skeletal muscle adaptation to passive stretching. Another potential contributor to the increase in muscle mass and myofiber CSA is the significant decrease in myostatin expression as observed in *Experiment 1*. Myostatin inhibits muscle hypertrophy via negative regulation of myogenesis and protein synthesis; therefore, decreased levels of myostatin permit increased protein synthesis and muscle growth. Myostatin expression was not affected by stretching in *Experiment 2*. I also assumed that the hypertrophic or suppressed atrophic observations in passive repetitive stretching may result from satellite cells activation. However, there was no significant difference in the number of Pax7+ cells between the stretched and unstretched muscles in both ICR and SAM-P8 mice. Finally, the number of nuclei per fiber was measured in each muscle section to determine whether or not stretching result in satellite cell activation and incorporation of new nuclei. As a result, the stretched and unstretched muscles did not differ in both ICR and SAM-P8 mice. These findings suggest the hypertrophic or suppressed atrophic observation in stretched muscles are mainly attributable to the protein turnover rather than triggering satellite cell activation and fusion.

Conclusion:

Passive repetitive stretching for 2 weeks induces muscle hypertrophy via regulation of Akt/mTOR and MuRF1/MAFbx pathway as well as Myostatin in healthy ICR mice. In addition, passive repetitive stretching proved to be effective in preserving muscle mass and fiber area in sarcopenic muscle in SAM-P8 mice.

List of Abbreviations

4E-BP1	Eukaryotic translation initiation factor 4E-binding protein 1
CSA	Cross-sectional area
FoxO	Forkhead box O
H&E	The hematoxylin and eosin
ICR	Institute of Cancer Research
IGF1/Akt/mTOR	Insulin-like growth factor 1/Akt/mammalian target of the rapamycin
IHC	Immunohistochemistry
MAFbx/atrogin-1	Muscle Atrophy F-box /atrogin-1
mTORC1	Mammalian target of the rapamycin complex 1
MuRF1	Muscle Ring Finger 1
mRNA	Messenger ribonucleic acid
MRFs	Myogenic regulatory factors
MyoD	Myoblast determination protein 1
Myf5	Myogenic factor 5
Pax7	Paired box protein Pax-7
PCR	Polymerase chain reaction
p70S6K	Ribosomal protein S6 kinase beta-1
SAM-P8	Senescence-accelerated mouse prone-8

Introduction

1. Age related muscle atrophy (Sarcopenia)

Skeletal muscle is one of the most abundant tissues human body, accounting for approximately 40%-45% of total body mass. Any dysfunction or deterioration of skeletal muscle can have serious impacts on human health and quality of life (QOL) due to its vital roles in locomotion, respiration, and energy metabolism. The term sarcopenia derives from the Greek roots sarx, for flesh, and penia, for loss, and it is first described in the 1980s as an age-related decline in the loss of muscle mass affecting mobility, nutritional status, and independence (Rosenberg, 1997). In 2010, the European Working Group on Sarcopenia in Older People (EWGSOP) defined sarcopenia as a syndrome characterized by progressive and generalized loss of skeletal muscle mass and strength (Cruz-Jentoft et al., 2010). In 2016, sarcopenia was recognized as an independent condition with an International Classification of Diseases-10 code (Anker et al., 2016). In EWGSOP 2018, the low muscle strength, which was the most reliable measure of muscle function, was considered as a primary parameter of sarcopenia (Cruz-Jentoft et al., 2019a). Asia is the most populated continent with fast aging population, thus facing increasing challenges of sarcopenia. In 2019, Asian Working Group for Sarcopenia (AWGS) updated diagnostic algorithm based on Asian data, defining sarcopenia as low skeletal muscle mass with low muscle strength or low physical performance (Chen et al., 2020). AWGS 2019 also added a term "possible sarcopenia" defined as the presence of reduced of muscle strength and/or reduced physical performance, to promote early intervention in seniors at risk of or with sarcopenia (Chen et al., 2020).

Sarcopenia is an age-related reduction in both muscle mass and quality. The prevalence of sarcopenia ranges from 15% at 65 years to 50% at 80 years in humans, with normal ageing associated with a 1–2% muscle loss beyond the age of 50 years (Kim and Choi, 2013). Cellular changes in sarcopenic muscle include reduction in both size and number of skeletal muscle fibers (Cruz-Jentoft and Sayer, 2019b). Human evidence showed that a ~30% reduction in muscle cross-sectional area and a ~40% decline in muscle strength are observed at 70 years (Porter et al., 1995). From a histological point of view, sarcopenia predominantly affects type II muscle fiber, whereas type I fibers are less affected (Doherty, 2003). Accompanied by physical frailty, functional disability, falls, hospitalization, and mortality, sarcopenia has become a major geriatric disorder owing to the increasing life expectancy and rapidly expanding ageing population (Mankhong et al., 2020). The etiology of sarcopenia is complex and not fully understood. Multiple factors appear to be involved in the development

of sarcopenia, such as cellular senescence, oxidative stress, mitochondrial dysfunction, fat accumulation, low-grade inflammation, inadequate nutrition, hormonal changes as well as a reduction in the number and regenerating capacity of satellite cells (Mankhong et al., 2020). Despite the growing recognition of clinical importance, sarcopenia is poorly managed by routine treatment. It is imperative to identify effective countermeasures that induce hypertrophy of senescent muscle and combat progression of sarcopenia.

2. Protein homeostasis (synthesis and degradation)

Disrupted protein homeostasis is well established as an initial process that regulates age-related muscle mass reduction (Mankhong et al., 2020). The regulation of skeletal muscle mass and fiber size is largely determined by the dynamic turnover between protein synthesis and degradation (Bowen et al., 2015). Muscle hypertrophy occurs when the overall rate of protein synthesis exceeds the rate of protein degradation while muscle atrophy, occurs when protein degradation rates exceed protein synthesis (Schiaffino et al., 2013). Ageing appears to disturb the balance between muscle protein anabolic and catabolic pathways, leading to overall loss of skeletal muscle (Cruz-Jentoft and Sayer, 2019b). Hence, strategies that enhance protein synthesis may prevent sarcopenic progression.

The IGF1/Akt/mTOR pathway primarily controls muscle protein synthesis (Schiaffino et al., 2013). Akt stimulates protein synthesis by activating mTOR and inhibits protein degradation through negative regulation of FoxO-mediated ubiquitin ligases (Mankhong et al., 2020). The major muscle-specific ubiquitin ligases include MAFbx/atrogin-1 and MuRF1, which are associated with myonuclear apoptosis and reduced fiber size (Bodine and Baehr, 2014). Decreases in MuRF1 and MAFbx in skeletal msucle would support better maintenance of fiber size with inhibition of the ubiquitin–proteosome pathway (Schiaffino et al., 2013).

Myostatin, a member of the TGF-β superfamily, has been extensively studied as another potential mediator of sarcopenia owing to its potent negative effects on cell growth and intracellular catabolic and anabolic signaling pathways (Rodriguez et al., 2014). Myostatin negatively regulates skeletal muscle growth, primarily by acting via activin type II receptors (ActRII), resulting in the activation of Smad signaling (Rodriguez et al., 2014). Smad signaling suppresses Akt signaling and its downstream effectors such as mTOR and FoxO, thus inhibiting protein synthesis (Rodriguez et al., 2014; Trendelenburg et al. 2009).

Collectively, therefore, these findings suggest that the promotion of protein synthesis through IGF-1/Akt/mTOR signaling and suppression of protein degradation via inhibition of MAFbx/ MuRF1 and myostatin, are considered playing a pivotal strategy for sarcopenia prevention.

3.Satellite cells

The regulation of muscle mass and fiber size essentially reflects protein turnover, i.e. the balance between protein synthesis and degradation within the muscle fibers. However, skeletal muscle fibers are multinucleated structures, thus the growth of skeletal muscle also encompasses myonuclear accretion, that is, the addition of new myonuclei due to the fusion of satellite cells (Schiaffino et al., 2013). Skeletal muscle stem cells, also called satellite cells, have been first identified more than half a century ago by Alexander Mauro. Their name pertains to their location, residing under the basal lamina of the myofiber; this position, between the myofiber and the surrounding extracellular matrix (ECM) (Mauro, 1961). Satellite cells play an important role in afterbirth growth and regeneration of skeletal muscle. In mature muscles, satellite cells are generally quiescent but become activated in response to stimuli or under muscle regeneration (Schmidt et al., 2019). When activated, a surge of MRFs, including MyoD, Myf5, and myogenin expression, is required owing to the role of MRFs in driving the differentiation of myoblasts to mature myotubes (Perry and Rudnick, 2000; Schmidt et al., 2019). Markers of early myogenesis, MyoD and Myf5, contribute to determination, while the marker of late myogenesis, myogenin, plays a downstream role in muscle cell differentiation to form muscle fibers (Perry and Rudnick, 2000; Schmidt et al., 2019). Accumulating evidence suggests that satellite cell numbers and responsiveness are altered during aging, which may be associated with sarcopenia (Sousa-Victor and Muñoz-Cánoves 2016). The age-related dysregulation of satellite cell appears to be fiber-type specific, as satellite cells associated with fast twitch type II fiber are more susceptible to impairment (Verdijk et al., 2007). Some studies suggested that the restoration of satellite cells would have therapeutic effects for sarcopenia (Verdijk et al., 2009; Shefer et al., 2010). It has been demonstrated that the depletion of satellite cells in adult mice does not directly accelerate sarcopenic muscle atrophy (Fry et al., 2015). Although satellite cells are indispensable for muscle regeneration, whether they play a key role in the maintenance of skeletal muscle mass and myofiber size throughout life is still controversial.

4. Passive stretching

Mechanical stimuli play an important role in the regulation of muscle mass and function. Skeletal muscle undergoes adaptations in gene expression and protein accretion via mechanotransductive mechanisms in response to mechanical signals (Burkholder 2007). Passive stretching, a type of mechanical stimulus, is widely used in rehabilitation medicine to prevent muscle shortening, maintain the range of joints, and muscle flexibility (Marshall, 2011; Williams, 1990). It is performed with minimal risk of injury at relatively light intensity as compared with resistant exercise and is used to prevent muscle disuse or enhance recovery from exposure to prolonged inactivity (Mirzoev et al., 2019; Sasa et al., 2004).

Evidence has shown that stretching applied to cultured skeletal muscle cells provides mechanical stimuli and triggers cellular biomarkers essential for muscle growth (Tatsumi, 2010). However, recent studies in vivo have investigated the effect of continuous static and low-frequency stretching using immobilization approaches on skeletal muscles with very limited hypertrophied outcomes. As such, divergences in the stretching protocols applied also affect muscular hypertrophic adaptations. According to previous reports, intermittent stretching for 60 s with 30 s of rest between sets for 10 sets/day did not affect myofiber CSA after 15 days (Peviani et al., 2018). And in specific circumstances such as aging, this may lead to a decrease in myofiber CSA (Zotz et al. 2016). Intermittent stretching for 3.2 min with 10 min of rest between sets for 4 sets/day for 7 days was reportedly insufficient for preventing muscle fiber atrophy due to denervation (Russo et al. 2010). In contrast, repetitive stretching performed every 5 s for 15 min/day for 2 weeks successfully prevented denervation-induced atrophy (Agata et al. 2009). Therefore, I hypothesized that rapid, repetitive sessions of passive stretching may exert greater hypertrophic effects.

The mechanisms underlying muscle hypertrophy resulting from passive repetitive stretching remain poorly understood. Prior reports highlighted the advantages of daily repetitive stretching in regulating the myogenic regulatory pathway and suppressing denervation-induced atrophy through the Akt/mTOR signaling cascade (Agata et al., 2009; Kamikawa et al., 2013). It has been observed that induction of myogenesis is more effective in fast, repetitive stretching than continuous static stretching (Kamikawa et al., 2013). Based on previous studies, I found that stretch parameters such as frequency and duration have been identified as important factors that potentially affect the skeletal muscle adaptive process, as the gene expressions involved in muscle growth and atrophy are responsive to the number of stretch sessions (Peviani et al., 2007; Russo et al., 2010; Soares et al., 2007). The effects of

passive stretching on MuRF1, MAFbx, and myostatin expression have been reported to be decreased, unchanged and even increased with different stretch protocols (Gomes et al., 2006; Faturi et al., 2016; Peviani et al., 2007; Russo et al., 2010; Soares et al., 2007).

Although previous studies have shown the effect of long-duration stretching in cultured quiescent satellite cells and hindlimb suspension models (Tatsumi et al., 2001; 2006), indicating a potential activation and fusion of satellite cells during stretching. To date, no studies have directly evaluated whether satellite cells play a role in muscle hypertrophy in response to daily passive repetitive stretching with short-duration in vivo.

In addition, aging processes alter mechano-transduction, which is the ability for skeletal muscle cells perceive and respond to mechanical inputs, indicating a perturbed load-induced plasticity for aged muscle hypertrophy (Bajpai et al., 2021). The age-related alterations to the complex signaling pathway including IGF1/Akt/mTOR, and FOXO-MAFbx/MuRF1, myostain-Smad, as well as other interlinked pathways in sarcopenic muscle are not yet clear. To date, no studies have demonstrated the effect of daily passive repetitive stretching on senescent skeletal muscles and decipher how these signaling pathways act in the muscular adaptation to stretching.

5. Purpose of this study

This study is an extension of other researches that investigate the effect of passive stretching has on skeletal muscle. First, I characterize the efficacy of clinically feasible protocols of daily passive repetitive stretching on healthy skeletal muscle mass, myofiber morphology, and then decipher the regulatory elements of action in terms of (a) gene expression and the phosphorylation status of signaling molecules involved in muscle protein synthesis and degradation, and (b) satellite cells, myonuclei content and MRFs expression as compared to unstretched contralateral limb in *Expeiment 1*. The *Experiment 1* also included a supplemented experiment investigating the effect of continuous static stretching on skeletal muscle mass and fiber size. Then I investigated whether or not passive repetitive stretching can be used as a practical therapeutic approach preventing against sarcopenic progression in senescent muscles in *Expeiment 2*.

Methods

1. Materials

The source and methods for preparing the experimental materials used in this study are as follows.

1.1 Animals

Male ICR mice (CLEA Japan, Tokyo, Japan)

Male SAMP8 mice (Japan SLC, Hamamatsu, Japan)

1.2 Equipment

Inhalation anesthesia equipment NARCOBIT-E-II (Natsume Seisakusho, Tokyo, Japan)

Hitachi centrifuge himac CT15RE (Hitachi Koki, Osaka, Japan)

Step One Plus Real Time PCR System (Applied Biosystems, Waltham, MA, USA)

NanoDrop 2000 Spectrophotometers (Thermo Fisher Scientific, Waltham, MA, USA)

Cryostat HM550 (Thermo Fisher Scientific, Waltham, MA, USA)

PALM MicroBeam IV (ZEISS, Oberkochen, Germany)

Hand homogenizer DREMEL® 3000 (DREMEL Europe, Breda, The Netherlands)

Multiple refrigerated centrifuge TOMY EX-126 (Tomy, Tokyo, Japan)

Image Quant LAS 4000(GE Healthcare, Chicago, IL, USA)

Ultra-low temperature freezer CLN-30U (NIHON FREEZER, Tokyo, Japan)

1.3 Kits

ImmunoCruz® rabbit ABC Staining System (Santa Cruz Biotechnology, Dallas, TX, USA)

Power SYBR Green RNA-to-CT™ 1-Step Kit (Applied Biosystems, Waltham, MA, USA)

EzRIPA Lysis kit (Atto, Tokyo, Japan)

Q Blot kit C (Atto, Tokyo, Japan)

Western Blotting Rabbit IgG DAB Chromogenic Reagent Kit (Boster Biological Technology, Pleasanton, CA, USA)

TaKaRa BCA Protein Assay Kit (Takara Bio, Shiga, Japan)

1.4 Histology and immunohistochemistry-related materials

Tissue-Tek® O.C.T. compound (Sakura Finetek, Torrance, CA, USA)

Tissue-Tek® Cryomold 1 (Sakura Finetek, Torrance, CA, USA)

NEO Micro cover glass (Matsunami Glass, Osaka, Japan)

Mounting reagent NEW M·X (Tokyo Garasu Kikai, Tokyo, Japan).

Mayer's hemalum solution (Merck KGaA, Darmstadt, Germany)

Tissue-Tek® Eosin (Sakura Finetek, Tokyo, Japan)

Ethanol 99.5 (KANTO CHEMICAL, Tokyo, Japan)

Hydrogen Peroxide 30% (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan)

1.5 Antibodies

MYH4 (Cloud-Clone Corp, Katy, TX, USA)

MYH2 (Cloud-Clone Corp, Katy, TX, USA)

MYH7 (Cloud-Clone Corp, Katy, TX, USA)

Pax7 (Cloud-Clone Corp, Katy, TX, USA).

AKT1/AKT2/AKT3 (CSB-PA008132, CUSABIO, Houston, TX, USA)

Phospho-AKT1/AKT2/AKT3 (S473) (CSB-PA000466, CUSABIO, Houston, TX, USA)

P70S6K (CSB-PA003686, CUSABIO, Houston, TX, USA)

Phospho-P70S6K (Thr389) (A0533, Assay Biotechnology Company, Sunnyvale, CA, USA)

4EBP1 (CSB-PA007994, CUSABIO, Houston, TX, USA)

Phospho-4EBP1 (Thr37/46) (#2855, Cell Signaling technology, Danvers, MA, USA)

GAPDH (CSB-PA004666 CUSABIO, Houston, TX, USA)

IgG-HRP (sc-2357; Santa Cruz Biotechnology, Dallas, TX, USA)

1.6 RT-PCR-related materials

Trizol Reagent (Thermo Fisher Scientific, Waltham, MA, USA)

Chloroform (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan)

2-Peopanol (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan)

Ethanol 99.5 (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan)

Primer (Hokkaido System Science, Sapporo, Japan)

MicroAmp® Fast 96-well Reaction Plate (0.1 mL) (Applied Biosystems, Waltham, MA, USA)

Optical Adhesive Covers (Applied Biosystems, Waltham, MA, USA)

Microplate Mixer MX-M (DLAB Scientific, Ontario, CA, USA)

Vortex Mixer MX-F (DLAB Scientific, Ontario, CA, USA)

Milli-Dry (MILLIPORE, Temecula, CA, USA)

1.7 Western blot-related materials

EzApply (Atto, Tokyo, Japan)

EzRun (Atto, Tokyo, Japan)

EzTween (Atto, Tokyo, Japan)

EzStandard PrestainBlue (Atto, Tokyo, Japan)

EzWestLumi plus (Atto, Tokyo, Japan)

C-PAGEL HR (Atto, Tokyo, Japan)

Compact PAGE AE7300 (Atto, Tokyo, Japan)

Power supply (M&S Instruments, Osaka, Japan)

Semi-dry transfer BE-300 (Bio Craft, Tokyo, Japan)

Microplate 96 well (300μl) (AS ONE, Osaka, Japan)

1.8 Software

ImageJ software (imagej.nih.gov/ij/)

Graph Pad Prism 8 (GraphPad Software, La Jolla, CA, USA)

SPSS Statistics 26 (IBM Corp., Armonk, NY, USA).

Microsoft PowerPoint for Mac version 16.49 (Microsoft, Redmond, WA, USA).

Microsoft Excel for Mac version 16.49 (Microsoft, Redmond, WA, USA).

1.9 Others

Pipetman P2 (M&S Instruments, Osaka, Japan)

Pipetman P10 (M&S Instruments, Osaka, Japan)

Pipetman P20 (M&S Instruments, Osaka, Japan)

Pipetman P100 (M&S Instruments, Osaka, Japan)

Pipetman P200 (M&S Instruments, Osaka, Japan)

Pipetman P1000 (M&S Instruments, Osaka, Japan)

Isoflurane solution (FUJIFILM Wako Pure Chemical Corporation, Japan)

PARAFILM® M (Sigma-Aldrich, Saint Louis, MO, USA)

Normal saline (Otsuka Pharmaceutical Factory, Naruto, Japan)

RNase Quiet (Nacalai tesque, Kyoto, Japan)

Water for Molecular Biology (Merck, Tokyo, Japan)

Preparation of 10 × Phosphate Buffered Saline (PBS) buffer (1000 mL):

Na2HPO4 • 2H2O (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan)	17.8 g
KH2PO4 (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan)	2.4 g
NaCl (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan)	80g
KCl (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan)	2 g
Distilled water	Adjust final volume to 1 L

Preparation of 10 × running buffer for SDS-PAGE (1000mL):

EzRun powder	1 bag
Distilled water	Dissolve powder to a final volume of 1 L

Preparation of 1× gel washing buffer:

5× Gel Wash Buffer (Atto, Tokyo, Japan)	10mL
Distilled water	40ml

Preparation of 1× Tris Buffered Saline (TBS) buffer (1000mL):

10 ×TBS Stock Solution (Atto, Tokyo, Japan)	100 mL
Distilled water	900mL

Preparation of 1× Tris Buffered Saline with Tween 20 (TBS-T) buffer:

1×TBS buffer	1000mL
EzTween	10mL

Preparation of 1× blocking buffer:

Protein dry powder (Boster Biological Technology, Pleasanton, CA, USA)	2g
1× TBS buffer	100mL

2. Experimental procedures

The experimental procedures were approved by the Animal Care and Use Committee of the Hokkaido University. The study protocol was carried out in accordance with the Fundamental Guidelines for Proper Conduct of Animal Experiments and Related Activities in Academic Research Institutions, under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology, Japan, and the NIH Guide for the Care and Use of Laboratory Animals. The study was carried out in compliance with the ARRIVE guidelines.

2.1 Animal breeding

Male ICR mice (30-40g in weight) and Male SAMP8 mice (25-40g in weight) were housed in plastic cages in a temperature-controlled room (24 °C) with a 12-hour light-dark cycle and given free access to water and standard food until the time of the study. The *Experiment 1* using ICR mice was started at 6 weeks old (n=16); The *Experiment 2* using SAM-P8 mice was started at 35-week-old (n=8).

2.2 Stretching protocol

Repetitive stretching:

The repetitive stretching protocol was based on a previously validated method (Agata et al., 2009; Kamikawa et al., 2013). After weighted, I anesthetized the animals with isoflurane solution using inhalation anesthesia equipment to isolate active isometric contractile activity during stretching intervention. The right gastrocnemius muscles were stretched by manual ankle dorsiflexion of the right ankle joint to 30°dorsiflexion with the knee at the extended position (RS). I performed the stretching repeatedly with a timer to stabilize at the frequency of 15 times/minutes for 15 min daily (as one single stretching session). Stretching was performed 5 days (5 sessions) a week for 2 weeks in *Experiment 1* and *Experiment 2*. Contralateral unstretched muscles were used as controls (US).

Continuous static stretching:

The continuous static stretching protocol was based on a previously validated method (Russo, 2010). The stretching was performed by holding the right ankle of the animal in full dorsiflexion, fixed with tape, and maintained in this position for 15 minutes daily (CS). Stretching was performed 5 days (5 sessions) a week for 2 weeks in supplemented experiment included in *Experiment 1*.

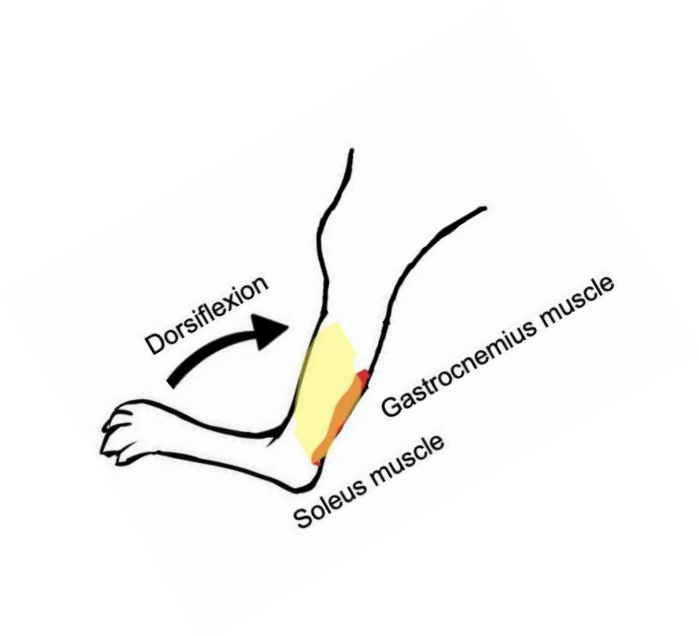


Figure 1. Muscle passive repetitive stretching by ankle dorsiflexion under knee extended position. The images were drawn with Microsoft PowerPoint for Mac version 16.49.

2.3 Tissue collection and histology

Twenty-four hours after the final stretching session, the gastrocnemius muscles of both legs of the mice were removed under deep anesthesia. The muscles were weighed before divided into two blocks. Samples for RNA extraction and western blot analysis were immediately preserved in liquid nitrogen and stored at −80°C. Samples intended for histology were collected in Tissue-Tek® O.C.T. compound for frozen section analysis.

Transverse cross-section (8 μm) frozen sections of the gastrocnemius muscles were cut using Cryostat HM550. I performed H&E staining as follows. (1) The sections were hydrated in distilled water for 30 seconds. (2) Adequate Hematoxylin was applied until completely cover tissue section and incubated for 5 minutes. (3) The slides were rinsed in two changes of distilled water to remove excess stain. (4) Adequate Eosin solution was applied until completely cover tissue section to excess and incubate for 2-3 minutes. (5) After rinsed in two changes of distilled water, the slides were dipped in 90% ethanol for 30 seconds, two changes of 95% ethanol for 1 minute, three changes of 99.5% ethanol for 3minutes, and three changes of xylene for 5 minutes. (6) The sections were mounted with coverslips using mounting reagent.

Tissue slides were observed and photographed using PALM MicroBeam IV at the magnification of 100×. The cross-sectional area of the myofibers and the number of myonuclei was measured from 200 myofibers per muscle sample using ImageJ software. Mean cross-sectional area of the myofibers, frequency distribution and number of the myonuclei per fiber were analyzed by using Microsoft Excel software.

2.4 Immunohistochemistry

2.4.1 Fiber typing

Myosin heavy chain (MYH) isoform content is the basis for classification of myofibers as slow twitch type 1(MYH7), or fast twitch type 2A (MYH2), 2B (MYH4) (Qaisar et al., 2016). I determined fiber types by immunohistochemistry staining using the ImmunoCruz® rabbit ABC Staining System according to the manufacturer's guidelines. 8 μm frozen muscle sections were preincubated for 5 minutes in H_2O_2 and washed twice for 5 minutes in PBS before being blocked for 1 hour at room temperature. The sections were subsequently incubated overnight at 4°C with primary antibodies against primary antibodies for MYH7 (1:500), MYH2 (1:500), MYH4 (1:500). On the next day, the sections were washed three

times for 5 minutes in PBS and incubated with biotinylated secondary antibodies for 1.5 hour. After washing with PBS, the sections were incubated for 30 minutes with AB enzyme reagent. The sections were then washed and incubated in three drops of peroxidase substrate for 5 minutes. Finally, the sections were washed with deionized H_2O for 5 minutes, dipped in 90% ethanol for 30 seconds, two changes of 95% ethanol for 1 minutes, three changes of 99.5% ethanol for 3minutes, and three changes of xylene for 5 minutes, and mounted with coverslips using mounting reagent. Tissue slides were observed and photographed using PALM MicroBeam IV. The CSA of each muscle fiber type and fiber-type distribution were measured from 200 myofibers per muscle sample using ImageJ and analyzed using Microsoft Excel software.

2.4.2 Satellite cells

Pax7 expression has been recognized as a marker of satellite cells (Bazgir et al., 2017). Pax7+ cells were identified by immunohistochemistry staining with same procedures of fiber typing. Briefly, 8 μm frozen muscle sections were preincubated for 5 minutes in H_2O_2 and washed twice for 5 minutes in PBS before being blocked for 1 hour at room temperature. The sections were subsequently incubated overnight at 4°C with primary antibodies against Pax7 (1:100). On the next day, the sections were washed three times for 5 minutes in PBS and incubated with biotinylated secondary antibodies for 1.5 hour. After washing with PBS, the sections were incubated for 30 minutes with AB enzyme reagent. The sections were then washed and incubated in three drops of peroxidase substrate for 5 minutes. Finally, the sections were washed with deionized H_2O for 5 minutes, dipped in 90/95/99.5% ethanol and xylene, and mounted with coverslips using mounting reagent. Tissue slides were observed and photographed using PALM MicroBeam IV. The number of Pax7+ cells were measured from 200 fibers per muscle sample using ImageJ and the number of Pax7+ cells per muscle fiber was analyzed using Microsoft Excel software.

2.5 Ribonucleic acid (RNA) isolation

The skeletal muscle tissue was mashed using a hammer and immediately preserved in liquid nitrogen. 1 mL of TRIzol Reagent was added into a 1.5mL collection tube, followed by the addition of about 50–100 mg of muscle tissue. I homogenized the muscle tissue in TRIzol Reagent with a hand homogenizer for 2 minutes. After that, 0.2 mL of chloroform was added

for lysis, then securely cap the tube and incubated for 3 minutes. Next, the samples were centrifuged for 15 minutes at 12,000 × g at 4°C and separated into a lower red phenol-chloroform, and interphase, and a colorless upper aqueous phase. The aqueous phase was transferred by pipetting to a fresh tube and 0.5 ml of 2-propanol was added. The samples were allowed to stand for 10 minutes at room temperature and centrifuged at 12,000 × g for 10 minutes at 4 °C, then the RNA precipitate formed a pellet on the side and bottom of the tube. Subsequently, the supernatant was removed and RNA pellet was washed by adding 1 mL of 75% ethanol. The sample was vortexed for 15 seconds and then centrifuged at 7,500 × g for 5 minutes at 4 °C. After the removal of ethanol, the RNA pellet was dried for 5minutes by using an air-drying. At last, the RNA pellet was dissolved in 100 μL of RNase-free water and the concentration was measured with NanoDrop 2000 Spectrophotometer.

2.6 Reverse-transcription polymerase chain reaction (RT-PCR)

One-step real-time reverse transcription polymerase chain reaction was performed using the Power SYBR Green RNA-to-CT™ 1-Step Kit. PCR was carried out in a total reaction volume of 20 μL containing 9 μL of RNA template, 10 μL of t Power SYBR® Green RT-PCR Mix (2×), 0.16 μL of RT Enzyme Mix (125×) , 0.44 μL of RNase-free H_2O and 0.4 μL of corresponding primer mixture in a 96-well plate (0.1 mL). PCR conditions, running on a Step One Plus Real Time PCR System with the following steps: 48°C for 30 minutes, 95°C for 10 minutes, and 40 cycles at 95°C for 15 seconds with an annealing and extension step at 60°C for 1 minutes. A melting curve was created to validate the specificity of the amplification products. The relative expression of target genes was determined using the relative standard curve method. The primers used were listed in Table 1. The GAPDH gene was used as a housekeeping gene.

Table 1. Sequences of real-time PCR primers used

Genes	Forward primer	Reverse primer
Pax7	5′-AAATCCGGGACCGGCTGCTGAA-3′	5′-AGACGGTTCCCTTTGTCGCCCA-3′
MyoD	5′-CCCCGGCGGCAGAATGGCTACG-3′	5′-GGTCTGGGTTCCCTGTTCTGTGT-3′
Myf5	5′-GAGGGAACAGGTGGAGAACTATTA-3′	5′-CGCTGGTCGCTGGAGAG-3′
myogenin	5′-ACTCCCTTACGTCCATCGTG-3′	5′-CAGGACAGCCCCACTTAAAA-3′
MHCe	5′-GAGCAGCTGGCGCTGAA-3	5′-TCTGATCCGTGTCTCCAGTTTCT-3′
Myostatin	5′-CTGTAACCTTCCCAGGACCA-3′	5′-TCTTTTGGGTGCGATAATCC-3′
p70S6K	5′-TGAGTCAAGCCTTGGTCGAG-3′	5′-AAGAGTCGAGAGAGACGCCC-3′
4E-BP1	5′-CGGAAGATAAGCGGGCAG-3′	5′-CAGTGTCTGCCTGGTATGAG-3′
MAFbx	5′-CTCTGCTGTGAGTGCCACAT-3′	5′-CAATGAGCCTGGGTACCACT-3′
MuRF1	5′-TGGAAACGCTATGGAGAACC-3′	5′-AACGACCTCCAGACATGGAC-3′
GAPDH	5′-TGACGTGCCGCCTGGAGAAA-3	5′-AGTGTAGCCCAAGATGCCCTTCAG-3

2.7 Western blot assay

2.7.1 Total protein extraction

The skeletal muscle tissue (30 mg) was homogenized in 1 mL of RIPA lysis buffer: 20mM HEPES, 150mM NaCl, 1.0%IGEPAL® CA-630, 0.1%SDS, 0.5% Sodium deoxycholate/pH7.5 supplemented 1 μl of protease inhibitor and 1 μl of phosphatase inhibitor. The lysates were incubated for 15 minutes on ice and then centrifugated for 15 minutes at 14,000 × g. After that, the supernatant was collected in a 1.5mL tube.

2.7.2 Protein quantification

The protein content of the supernatants was quantified by using TaKaRa Bicinchoninic Acid (BCA) Protein Assay Kit. Before measurement, 10 mL of working solution was prepared by mixing BCA Reagent A and BCA Reagent B at a 100:1 ratio. Dilutions of the bovine serum

albumin (BSA) standard solution as shown below. PBS buffer was used to dilute the BSA standard solution and the sample.

Table 2. Dilutions of the BSA standard solution

2mg/ml BSA standard (µl)	Diluent (µl)	Final concentration of BSA (µg/ml)
120	0	2000
90	30	1500
60	60	1000
45	75	750
30	90	500
15	105	250
10	150	125
0	120	0

10 µl of each dilution of the BSA standard solution was dispensed to 96-wells reaction plate. 200 µl of the working solution was added and mixed immediately and incubated for 30 minutes in a 37°C water bath. After that, the plate was returned to room temperature and cooled. The absorbance at 562 nm was measured using NanoDrop 2000 Spectrophotometers and the standard curve was generated by using Microsoft Excel software. The absorbance at 562 nm of each samples were measured and protein concentration was determined by using the standard curve as a reference.

2.7.3 Electrophoresis, blotting, and detection

The samples were then diluted in RIPA buffer at a concentration of 2 mg/mL, mixed with DTT-added EzApply solution at a ratio of 1:1 and boiled for 5 minutes. After that, 7µl of samples and 5µl of EzStandard PrestainBlue were applied to the electrophoresis gel for each preparation and separated by SDS-PAGE. The electrophoresed gel was rinsed with gel wash

buffer and placed gently on the bottom stack with PVDF membrane, covered with top stack. Air bubbles were removed by using a blotting roller. The transfer was started at 24V transferred to PVDF membrane in 5 minutes. After confirming the migration of the band from the gel to the membrane, the membrane was cut apart according to the molecular weight. Subsequently, the membranes were blocked for 1.5 hour at room temperature with the blocking buffer and washed 10 minutes with TBS-T. The membrane was incubated for 2 hour at room temperature with primary antibodies (diluted in TBS-T) against p-p70S6K (Thr 389) (1:1000) and p70S6K (1:1000), p-4E-BP1 (Thr37/46) (1:1000) and 4E-BP-1 (1:1000), p-AKT (S473) (1:1000), AKT (1:1000),GAPDH (1:1000). The membrane was wash 3 time for 10 minutes each with TBS-T. After that, the membranes were incubated for 1.5 hour at room temperature with diluted secondary antibody (1:2000). The membranes were then washed again in wash buffer 3 times for 5 minutes each. The protein bands were detected using a chemiluminescence kit (*Experiment 1*) and DAB chromogenic regent kit (*Experiment 2*). The protein bands were quantified using Image Quant LAS 4000 and ImageJ software and normalized to GAPDH expression.

2.8 Statistics

The Student's t-test or Wilcoxon signed-rank test were used as appropriate after ascertaining normality of distribution via the Shapiro-Wilk test. A p-value < 0.05 was considered to be statistically significant. Statistical analyses were performed using GraphPad Prism and SPSS Statistics. Data are expressed as mean ± standard error of the mean (SEM).

3. Graphical representation

The overall flow of this study is shown in Figure 2.

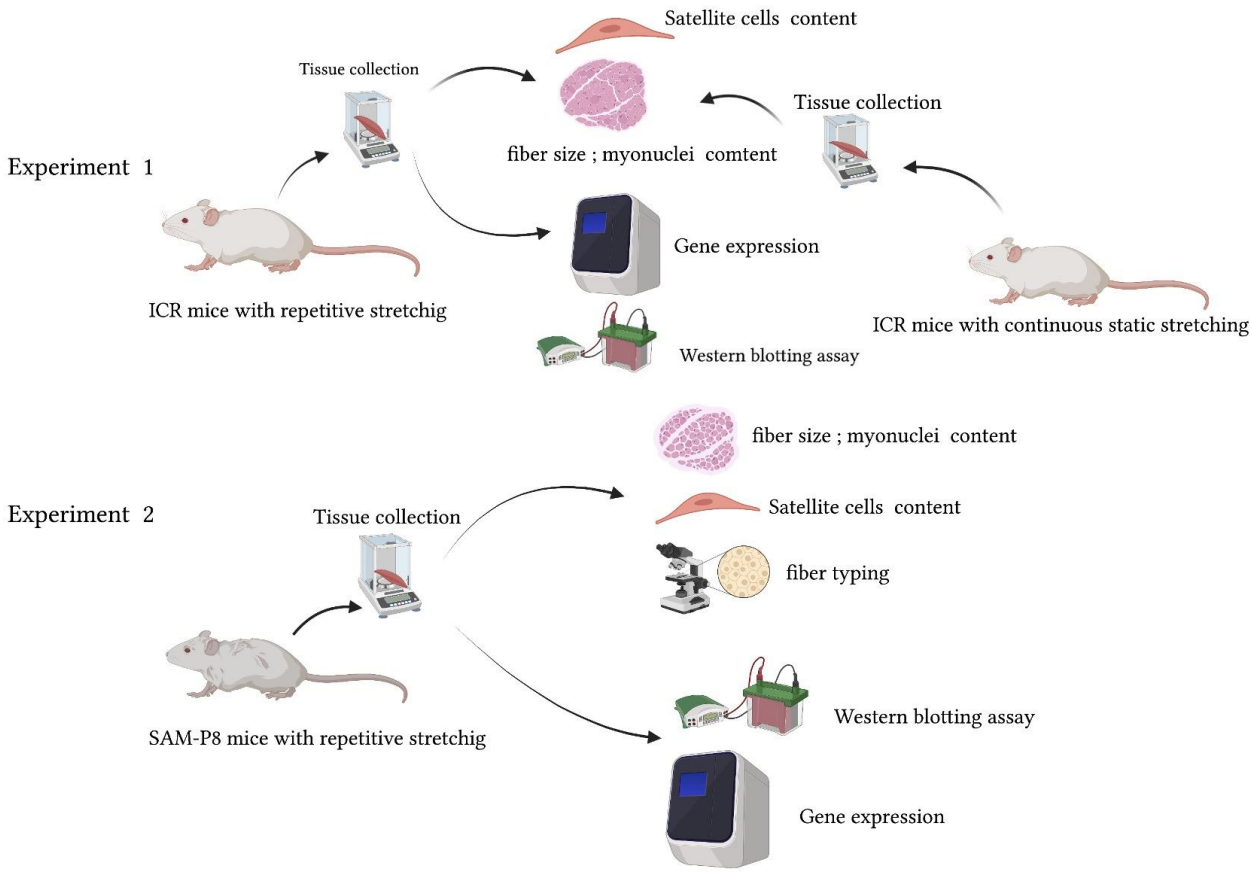


Figure 2. The overall flow chart of the study created with BioRender.com.

Results

1.Experiment 1

1.1 Muscle mass and myofiber cross-sectional area

There was no significant difference in body weight between the pre- and post-stretching animals (Figure 3A). I observed about 11.8% increase in muscle mass in stretched gastrocnemius muscle as compared with unstretched side (Figure 3B). In addition to the increase in muscle mass, histological analysis using H&E staining showed that the mean gastrocnemius muscle fiber CSA of the stretched side was 11.7% larger than that of the unstretched side ($P < 0.05$; Figure 3C, 3D). By the way, the frequency distribution of the muscle fiber areas revealed that stretched gastrocnemius muscle shifted toward larger myofibers compared to the unstretched side (Figure 3E).

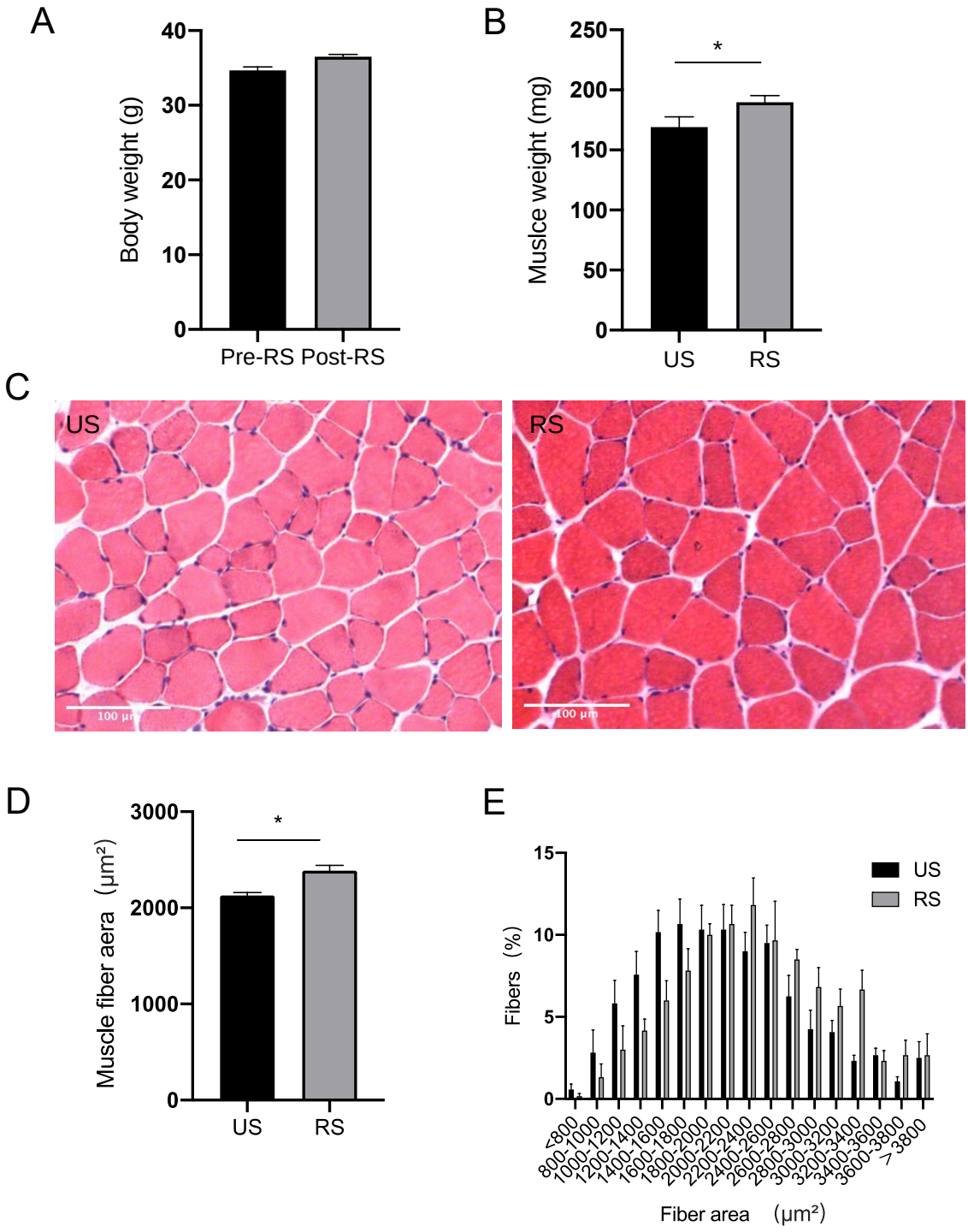


Figure 3. Body weight, muscle weight and fiber analysis (A) Body weight. (B) Mean weight of gastrocnemius muscle. (C) H&E staining of the gastrocnemius muscle. The bar indicates 100 μm. Magnification: 100×. (D) Mean cross-sectional fiber area of gastrocnemius muscle. (E) Frequency distribution of cross-sectional gastrocnemius muscle fiber area. US: unstretched. RS: repetitively stretched. Values are expressed as mean ± SEM. *$p < 0.05$, n = 8/group.

1.2 Satellite cells and myonuclei content

Pax7 expression has been recognized as a marker of satellite cells. I quantified the number of Pax7+ cells to analyze the population of satellite cells after 2 weeks of passive repetitive stretching by immunohistochemistry staining. However, there was no significant difference in the number of Pax7+ cells between the stretched and unstretched sides in ICR mice (Figure 4A, 4B). Total number of nuclei per fiber in each muscle section was measured by to determine whether stretch did in fact result in the incorporation of new nuclei. As a consequence, I didn't observe any diffidence in the number of myonuclei per fiber between stretched and unstretched muscles (Figure 4C)

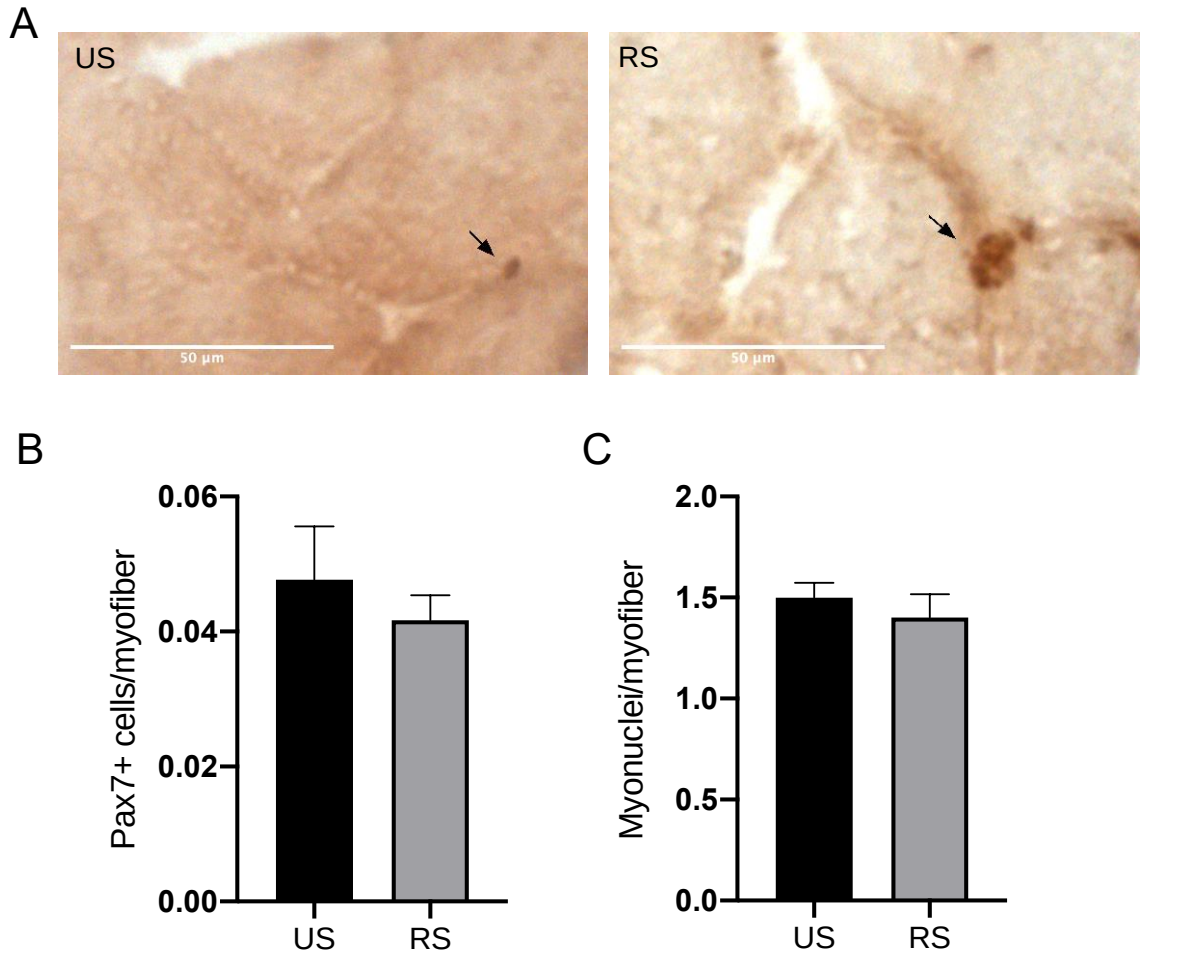


Figure 4. Immunohistochemical analysis of Pax7+ cells (A) Representative images showing Pax7+ myonuclei (Arrowheads). The bar indicates 50 μm. Magnification: 400×. (B) Quantification of the number of satellite cells (Pax7+ myonuclei/fiber) in gastrocnemius muscle. (C) Quantification of the number of myonuclei per fiber in gastrocnemius muscle US: unstretched. RS: repetitively stretched. Values are expressed as mean ± SEM. *$p < 0.05$, n = 6/group.

1.3 Gene expression

4E-BP1 and p70S6K are two downstream targets of mTORC1. On the stretched side, the mRNA expression of p70S6K increased by 4.7-fold ($P < 0.01$; Figure 5A), while 4E-BP1 expression decreased by 0.64-fold compared to the unstretched side ($P < 0.05$; Figure 5A). The mRNA expressions of MAFbx and MuRF1 were observed decreased by 0.56- and 0.59-fold on the stretched side, respectively, compared with the unstretched side ($P < 0.05$; Figure 5A). Myostatin expression in the stretched side was decreased by 0.61-fold when compared to the unstretched side ($P < 0.05$; Figure 5A). While there was no difference in Pax7 expression between the stretched and unstretched sides, MyoD expression decreased by 0.4-fold in the stretched side ($P < 0.05$; Figure 5B). The expression of Myf5 and MHCe remained unchanged, while myogenin increased by 8.4-fold on the stretched side compared to the unstretched side ($P < 0.01$; Figure 5B).

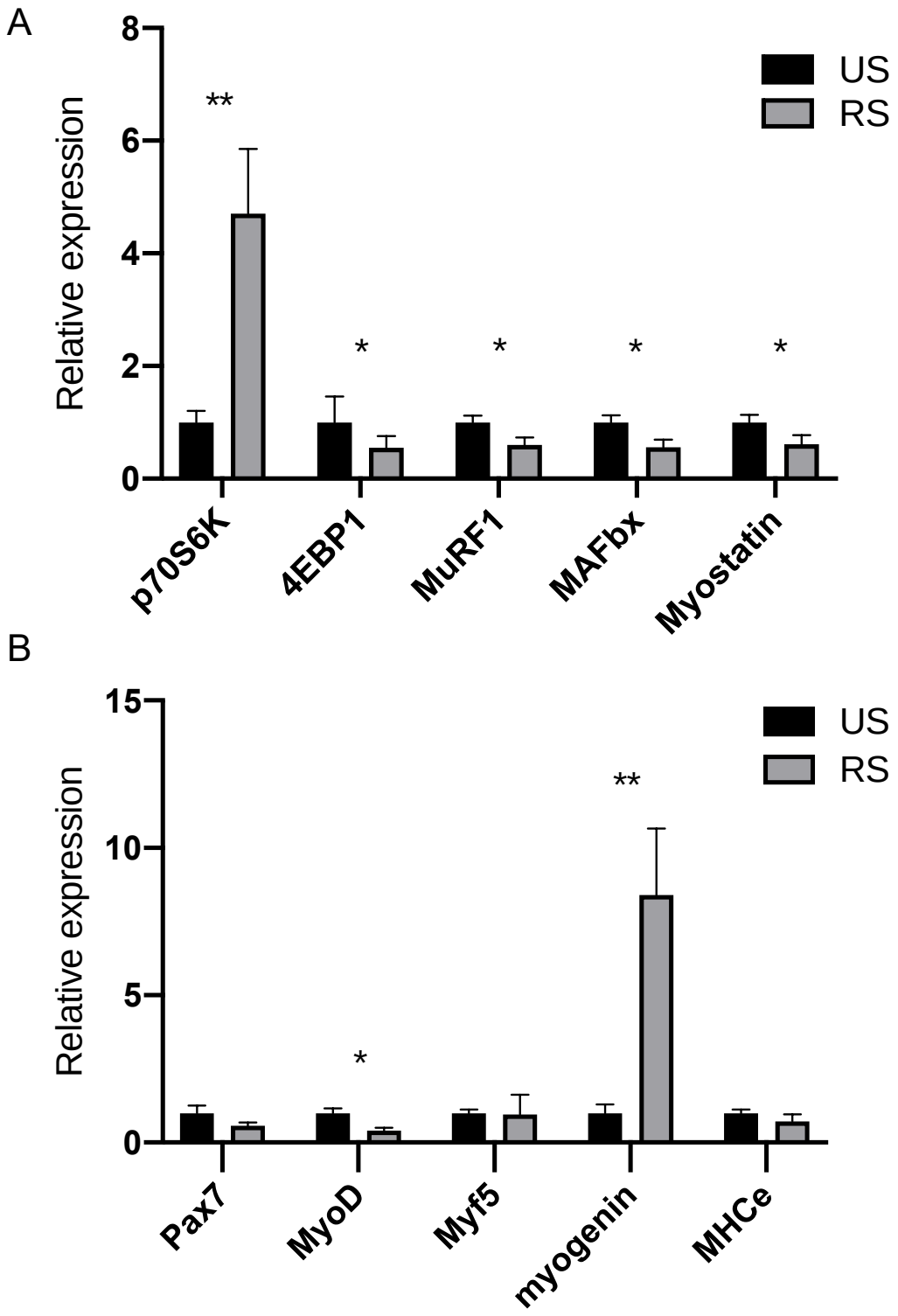


Figure 5. Real-time PCR gene expression of p70S6K, 4E-BP1, MAFbx and MuRF1, Pax7, MyoD, Myf5, myogenin and MHCe. US: unstretched. RS: repetitively stretched. Values are expressed as mean ± SEM. *$p < 0.05$, **$p < 0.01$, n = 6/group.

1.4 Phosphorylation status of Akt, p70S6K, 4E-BP1

The level of Akt phosphorylation in the stretched muscles did not differ significantly from that in the unstretched muscles (Figure 6A). The phosphorylation status of p70S6K increased by 54.5% on the stretched side compared to that on the unstretched side ($P<0.05$; Figure 6B). The phosphorylation level of 4E-BP1 remained unchanged compared to that in the unstretched muscles (Figure 6C).

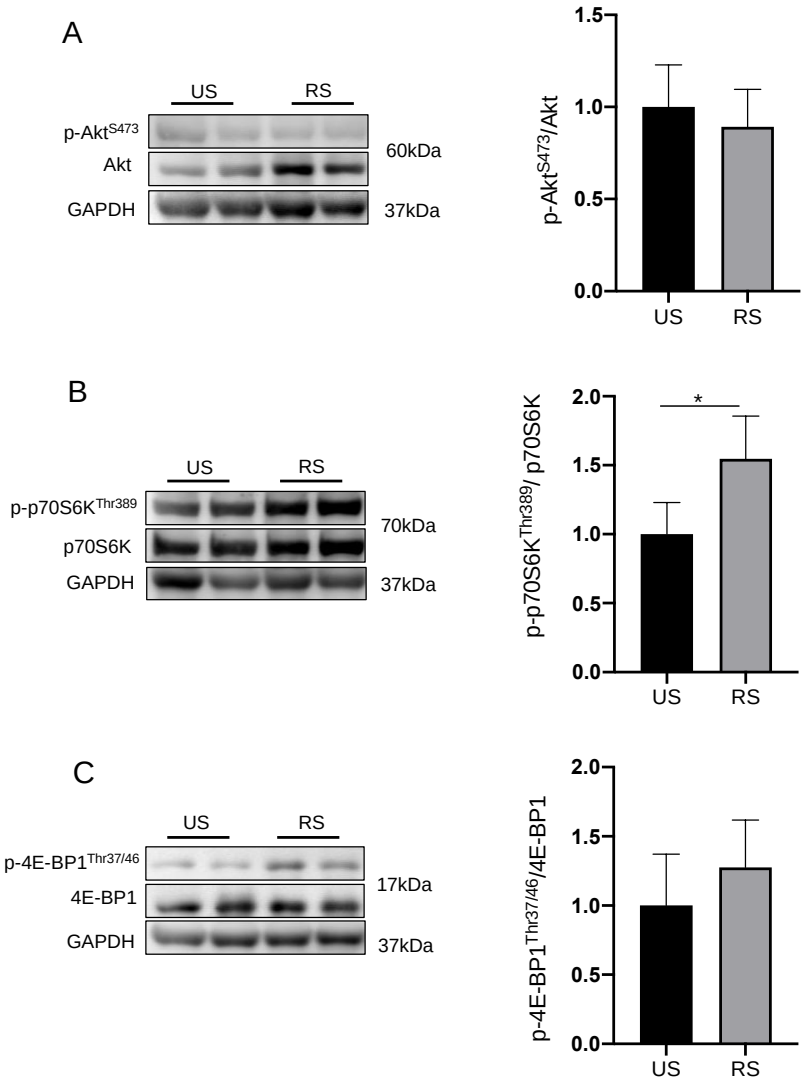


Figure 6. The phosphorylation levels of Akt, p70S6K, and 4E-BP1 measured with western blot analysis. Representative western blot images and quantification of phosphorylation levels of (A) Akt, (B) p70S6K, and (C) 4E-BP1. US: unstretched. RS: stretched. Values are expressed as mean ± SEM. *$p < 0.05$, n =6 /group.

※ Supplemented experiment

Muscle mass and myofiber cross-sectional area

There was no significant difference in muscle mass and fiber size between stretched and unstretched gastrocnemius muscle (Figure 7).

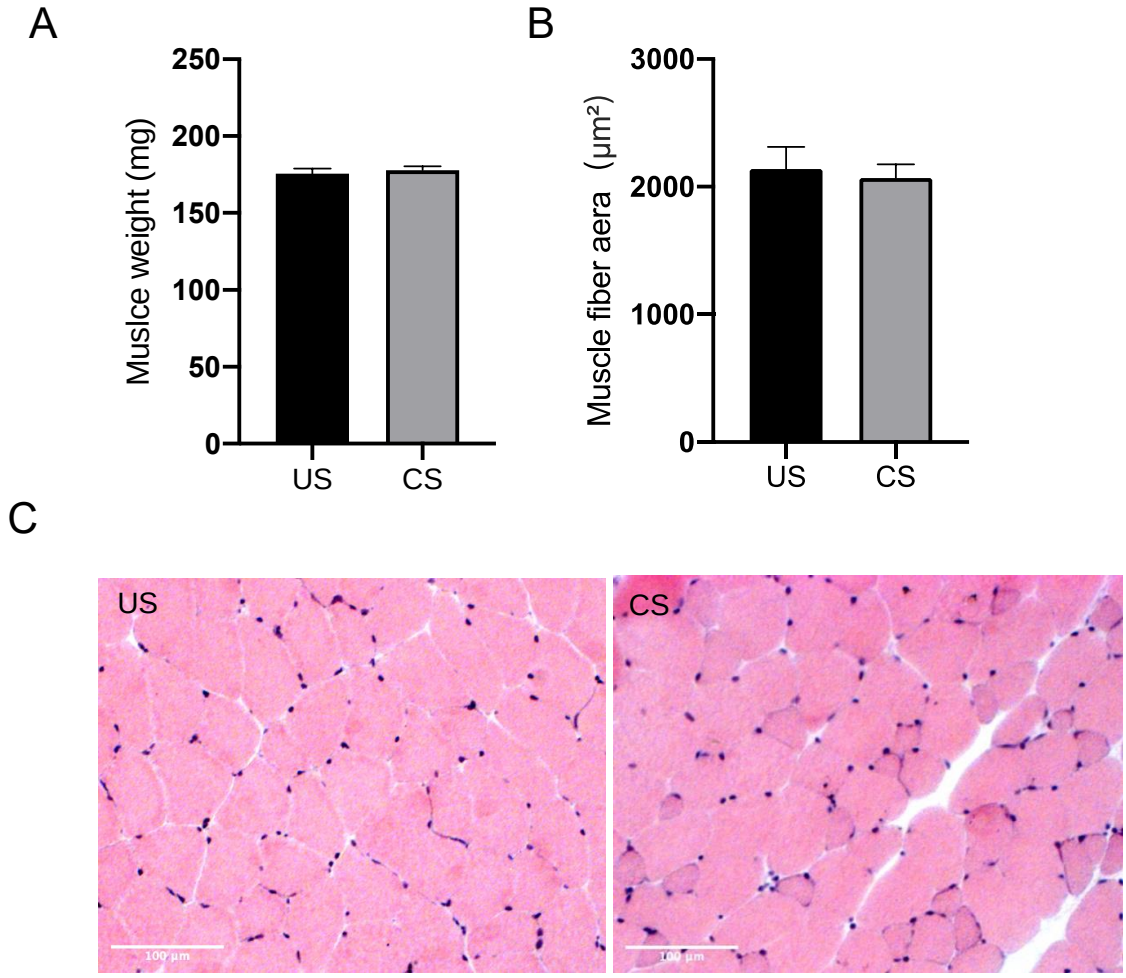


Figure 7. Muscle weight and fiber analysis (A) Mean weight of gastrocnemius muscle. (B) Mean cross-sectional fiber area of gastrocnemius muscle. (C) H&E staining of the gastrocnemius muscle. The bar indicates 100 μm. Magnification: 100×. US: unstretched. CS: continuously stretched. Values are expressed as mean ± SEM. *$p < 0.05$, n =8 /group.

2. Experiment 2

2.1 Muscle mass and myofiber cross-sectional area

There was no significant difference in body weight between the pre -and post-stretching animals (Figure 8A). I found the gastrocnemius muscle weight of the stretched side was ~10.7% greater as compared with that of the unstretched side ($P<0.05$; Figure 8B). The mean gastrocnemius muscle fiber cross-sectional area of the stretched side was ~19.9% larger than that of the unstretched side ($P <0.05$; Figure 8C, 8D). The frequency distribution of the muscle fiber area revealed that the stretched gastrocnemius muscle had a shift toward larger myofibers as compared with the unstretched side (Figure 8E).

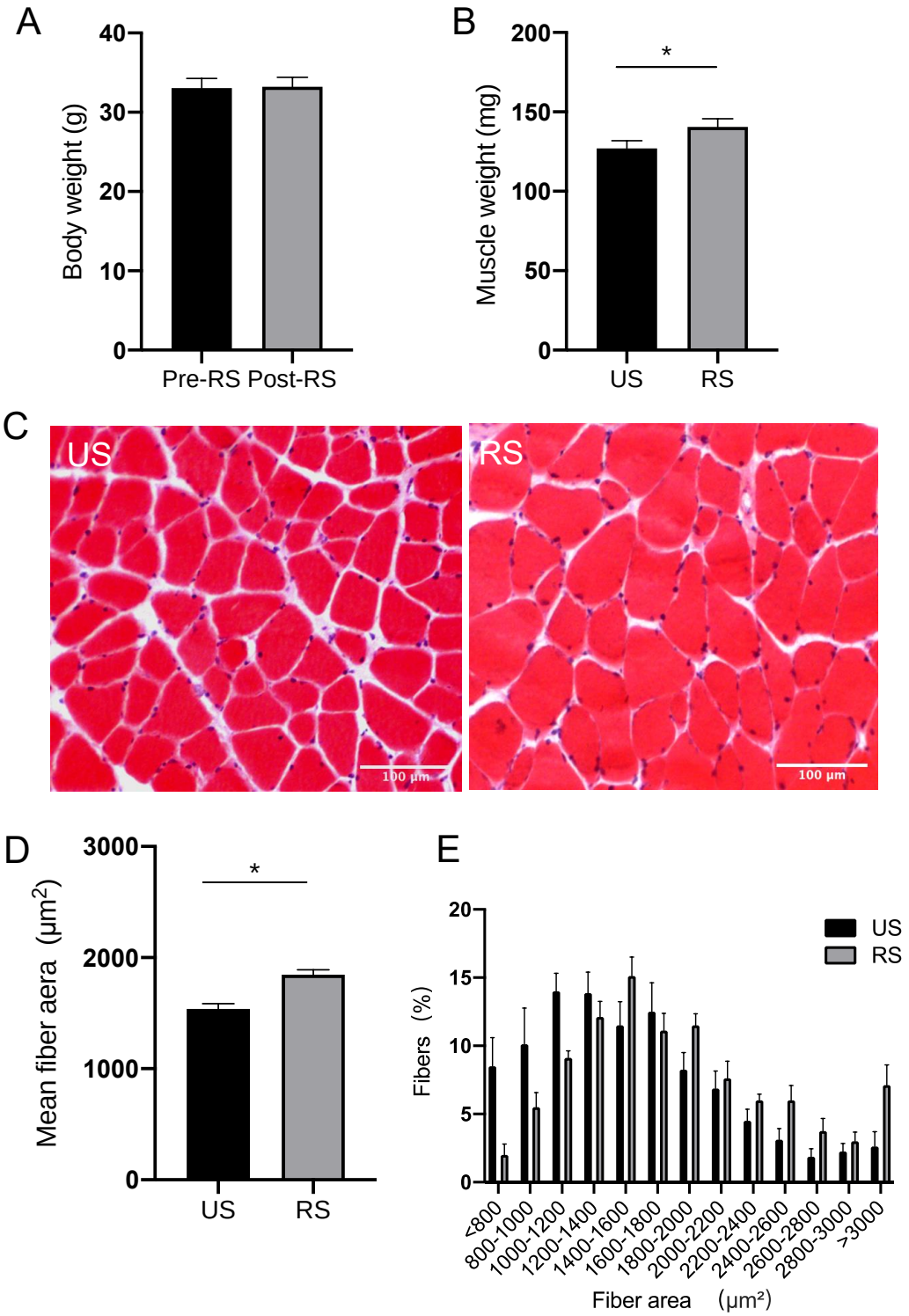


Figure 8. Body weight, muscle weight and fiber analysis (A) Body weight. (B) Mean weight of gastrocnemius muscle. (C) H&E staining of the gastrocnemius muscle. The bar indicates 100 μm. Magnification: 100×. (D) Mean cross-sectional fiber area of gastrocnemius muscle. (E) Frequency distribution of cross-sectional gastrocnemius muscle fiber area. US: unstretched. RS: repetitively stretched. Values are expressed as mean ± SEM. *$p < 0.05$, n = 8/group.

2.2 Muscle fiber type

No clear differences in the distribution of fiber types were observed when the fiber-type composition was examined with IHC staining (Figure 9A, 9B). Type 2A fibers were the most common in the gastrocnemius muscle, with lower proportions of type 2B and type 1 fibers (Figure 9B). This suggests that fiber-type switching is not regulated by passive repetitive stretching for a short duration within 2 weeks. In addition, I found that type 2A fibers were mostly responsible for the difference in cross-sectional area, which was ~27.4% greater in stretched side compared to unstretched side ($P < 0.05$; Figure 9C).

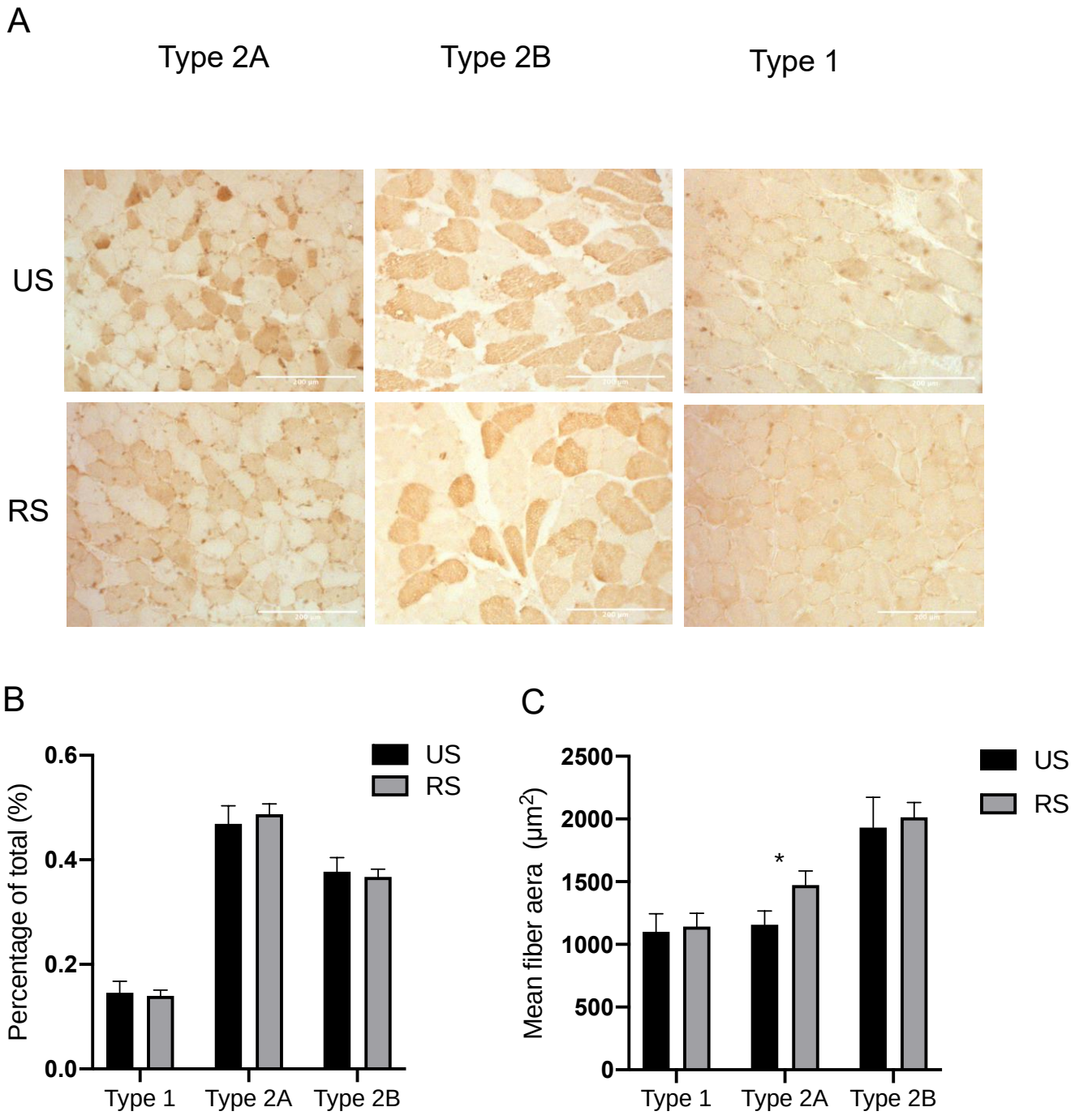


Figure 9. Immunohistochemical analysis of muscle fiber type (A) Representative images are shown for muscle fiber type 2A, type 2B, type 1, visualized with DAB (brown). The bar indicates 200 μm. Magnification: 100×. (B) Distribution of fiber types in gastrocnemius muscle. (C) Mean cross-sectional fiber area of type 2A, type 2B and type US: unstretched. RS: repetitively stretched. Values are expressed as mean ± SEM. *$p < 0.05$, n = 8/group.

2.3 Myonuclei and satellite cells content

I found the Pax7+ cells were rare, and there was no significant difference in the number of Pax7+ cells between the stretched and unstretched sides (Figure 10A, 10B). The number of myonuclei per fiber determined by H&E staining showed no difference between the stretched and unstretched muscles (Figure 10C).

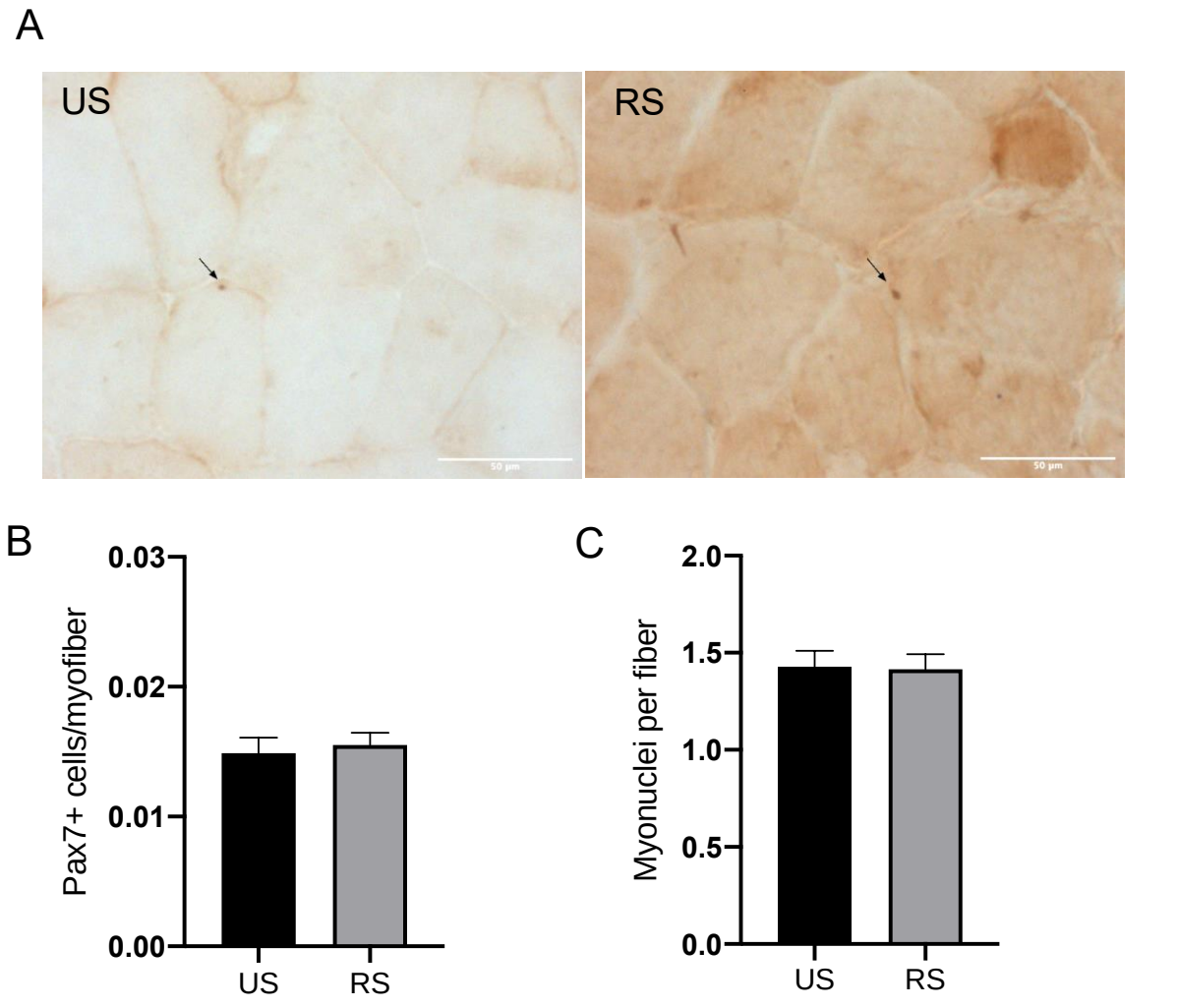


Figure 10. Immunohistochemical analysis of Pax7+ cells (A) Representative images showing Pax7+ myonuclei (Arrowheads). The bar indicates 50 μm. Magnification: 400×. (B) Quantification of the number of satellite cells (Pax7+ myonuclei/fiber) in gastrocnemius muscle. (C) Quantification of the number of myonuclei (myonuclei/myofiber) in gastrocnemius muscle. US: unstretched. RS: repetitively stretched. Values are expressed as mean ± SEM. *$p < 0.05$, n = 8/group.

2.4 mRNA expression of Akt, p70S6K, 4E-BP1, MAFbx and MuRF1, MRFs and myostatin.

The messenger RNA expression levels of Akt/mTOR pathway signal molecules, Akt, p70S6K, and 4E-BP1 are affected by passive repetitive stretching (Figure 11). The mRNA expression of Akt increased by 2.5-fold ($P < 0.05$; Figure 11A) on the stretched side as compared with the unstretched side. 4E-BP1 and p70S6K are two downstream targets of mTORC1. The mRNA expression of p70S6K increased by 4.9-fold ($P<0.05$; Figure 11B) and 4E-BP1 increased by 5.7-fold ($P<0.05$; Figure 11C) in the stretched side as compared with the unstretched side. I then measured the expression of major muscle-specific E3 ubiquitin ligases, including MAFbx and MuRF1. The MAFbx mRNA expression was not altered (Figure 11D), whereas the MuRF1 mRNA expression increased by 2.1-fold ($P < 0.05$; Figure 11E) on the stretched side as compared with the unstretched side. Myostatin mRNA expression did not differ between the stretched and unstretched sides (Figure 11F).

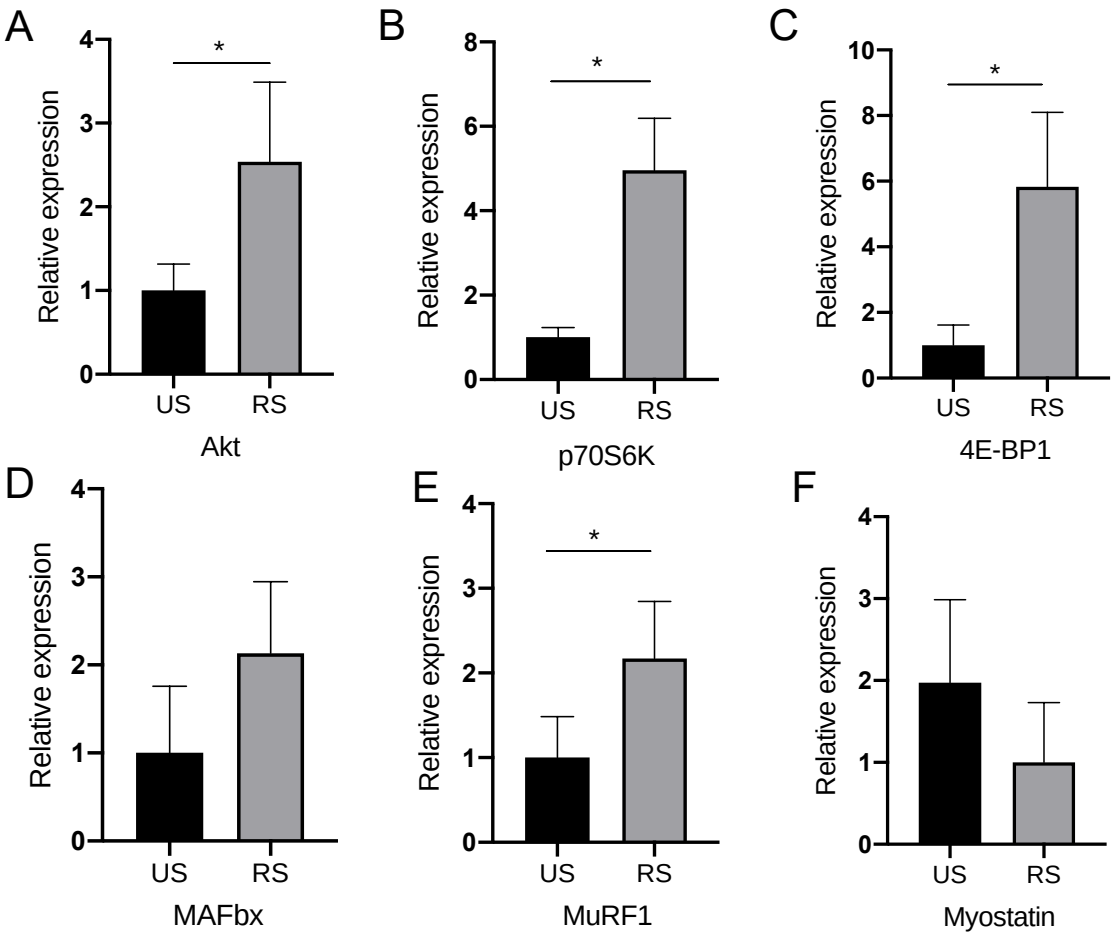


Figure 11. Real-time PCR gene expression of Akt, p70S6K, 4E-BP1, and muscle-specific E3 ubiquitin ligases MAFbx and MuRF1. US: unstretched. RS: repetitively stretched. Values are expressed as mean ± SEM. *p < 0.05, n = 8/group.

The expressions of MRFs, including MyoD, Myf5, and myogenin mRNA, are shown in Figure 12. The MyoD mRNA expression remained unchanged (Figure 12A). Myf5 increased by 3.8-fold (P<0.05; Figure 12B) and myogenin increased by 2.7-fold (P<0.05; Figure 12C) on the stretched side as compared with the unstretched side.

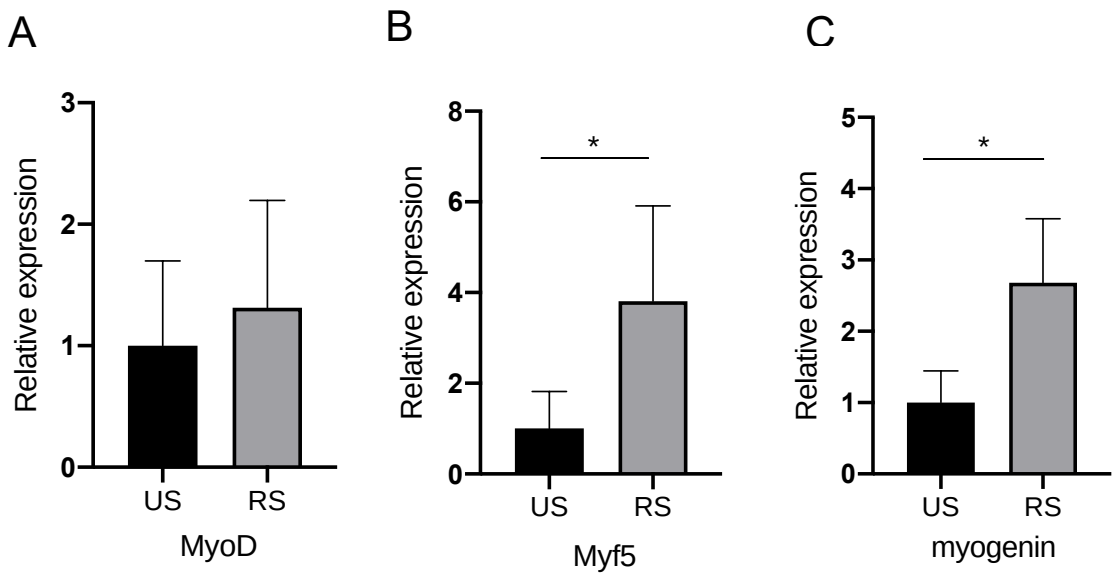


Figure 12. Real-time PCR gene expression of Myogenic regulatory factors. US: unstretched. RS: repetitively stretched. Values are expressed as mean ± SEM. *p < 0.05, n = 8/group.

2.5 Phosphorylation status of Akt, p70S6K, 4E-BP1

I found that the level of Akt phosphorylation in the stretched muscles did not differ significantly from the unstretched side (Figure 13A). The phosphorylation status of the key mTORC1 activity marker, p70S6K, increased significantly by 22% on the stretched side compared to the unstretched side (P <0.05; Figure 13B). The phosphorylation level of another mTORC1 downstream target, 4E-BP1, remained unchanged compared to the unstretched muscles (Figure 13C).

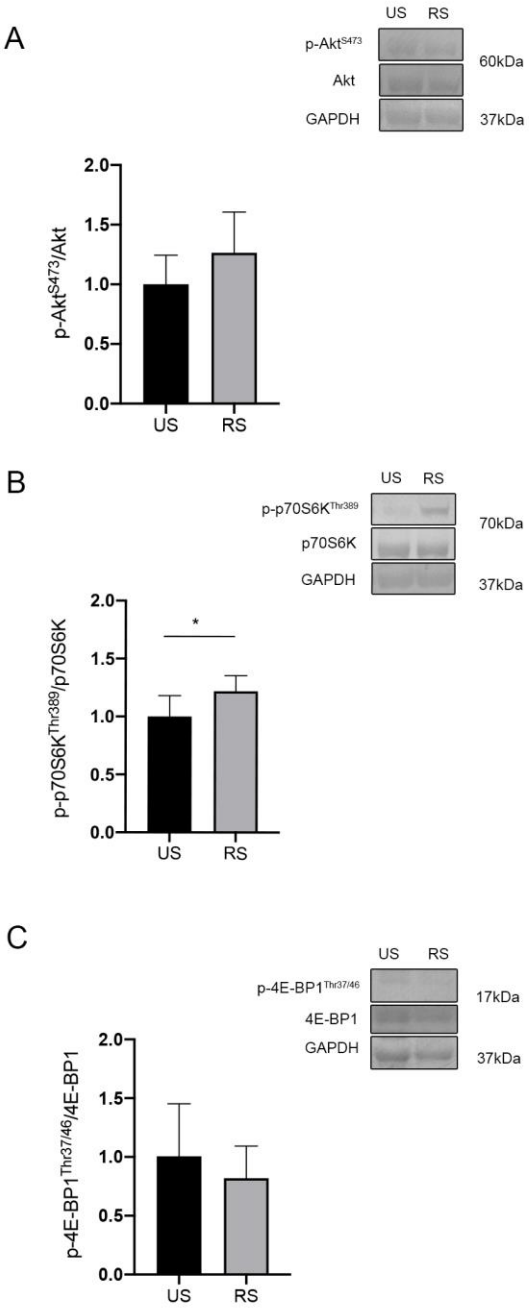


Figure 13. The phosphorylation levels of Akt, p70S6K, and 4E-BP1 measured with Western blotting. Representative western blot images and quantification of phosphorylation levels of (A) Akt, (B) p70S6K, and (C) 4E-BP1. US: unstretched. RS: repetitively stretched. Values are expressed as mean ± SEM. *$p < 0.05$, n = 7/group.

Discussion

Herein, I investigated the effects of daily passive repetitive stretching for two weeks has on skeletal muscle mass and myofiber morphology in both healthy and sarcopenic mice. I also compared the changes in gene expression and phosphorylation status of signaling molecules involved in protein synthesis and degradation, as well as satellite cell content and MRFs expression to further elucidate the underlying mechanisms of action. In the *Experiment 1*, I found that passive repetitive stretching (1) significantly increased muscle mass and myofiber CSA while continuous static stretching didn't 2) promoted genetic activation of AKT/mTOR and suppression of MuRF1/MAFbx signaling pathway, and 3) inhibited myostatin expression while increasing myogenin expression. In the *Experiment 2*, I found that passive repetitive stretching (1) attenuated sarcopenic muscle loss in aged mice and were associated with a greater muscle fiber cross-sectional area, particularly in the type 2A fiber. 2) promoted a positive protein turnover via Akt/mTOR signaling pathways, however, accompanied by an elevated expression level of MuRF1, which is involved in protein degradation, in the senescent skeletal muscles of SAM-P8 mice. My thesis was, daily passive repetitive stretching would prove to be effective in inducing muscle hypertrophy and can be used as a practical therapeutic approach preventing against sarcopenic progression in senescent muscles.

The conceptual basis for stretch-induced skeletal muscle hypertrophy dates back to in vitro studies (Goldberg et al., 1975; Tatsumi, 2010). Evidence showed that repetitive stretching applied to cultured skeletal muscle cells provided mechanical stimuli and triggered cellular biomarkers essential for muscle growth. In vivo studies in animals then showed robust muscle hypertrophy after several weeks of stretching intervention. For example, Goldspink (1977) reported that rat soleus and extensor digitorum longus muscles immobilized in lengthened positions increased in size whereas atrophied in shortened position, indicating that passive stretch at long muscle length stimulated protein synthesis and induced the growth of muscles. Antonio and Gonyea (1993) observed huge muscle growth in quails following a period of progressive stretch overload of wing muscles, performing by adding a cuff weight filled with lead pellets secured around the wing of each bird. Unlike the previous studies that used chronic stretching protocols, a study by Coutinho et al. (2004) found that stretching the immobilized soleus muscles by typing for 40 minutes every 3 days for 3 weeks prevented the muscle shortening and reduced the magnitude of muscle atrophy as compared with an immobilized group. Muscles submitted to stretching significantly increased the length serial

sarcomere number, and fiber area compared with the contralateral muscles (Coutinho et al., 2004). A recent study by Hotta et al. (2018) also reported an increase in muscle weights after 4 weeks of stretching by placing an ankle dorsiflexion splint for 30 min day, 5 days week, for 4 weeks. However, much of the research in this area using long-duration immobilization method as stretching, which may not commonly used in routine rehabilitation practice due to a risk of articular contracture. Furthermore, it is difficult to isolate the effects of stretching from isometric contractile activity as the animals were awake during muscle stretching duration. In another study, contractions were eliminated by animal anesthesia during stretching, while muscle weight and fiber area remained unaltered (Baewer et al., 2004). A number of subsequent studies endeavoured to investigate the effect of short-duration passive stretching on skeletal muscle muscles under animal anesthesia conditions with clinically feasible protocols. However, the results obtained in these studies supporting stretch-induced muscle hypertrophy are equivocal. Peviani et al. (2018) reported that intermittent stretching for 60 s with 30 s of rest between sets for 10 sets/day did not affect myofiber CSA after 15 days. And in specific circumstances such as aging, this may lead to a decrease in myofiber CSA (Zotz et al., 2016). Intermittent stretching for 3.2 min with 10 min of rest between sets for 4 sets/day for 7 days was reportedly insufficient for preventing muscle fiber atrophy due to denervation (Russo et al., 2010). In contrast, repetitive stretching performed every 5 s for 15 min/day for 2 weeks successfully prevented denervation-induced atrophy (Agata et al., 2009). Therefore, I hypothesized that rapid, repetitive sessions of passive stretching may exert greater hypertrophic effects than continuous static stretching.

I confirmed my hypotheses by observing an increase in muscle mass and myofiber size in repetitively stretched muscles of healthy ICR mice in *Experiment* 1, whereas continuously stretched muscles showed no positive effects. Passive repetitive stretching with a short duration for only 15 minutes daily for two weeks did induce muscle hypertrophy whereas continuous static stretching didn't. However, it remains unclear whether it is still effective when performed to sarcopenic muscle. Although prior studies have demonstrated that senescent muscles preserved the capacity to undergo hypertrophy, the ability to perceive and respond to mechanical inputs and translate them into biochemical signals, which is called "mechano-transduction," was reportedly blunted during aging (Bajpai et al., 2021). The impact of aging on the cellular mechano-transduction process is rooted in multiple factors such as modifications in cell cytoskeleton structures, alterations in mechanosensitive signaling, and the extracellular matrix environment (Bajpai et al., 2021). To clarify this, I

performed *Experiment 2* using 35-weeks-old SAM-P8 mice, which exhibits characteristics of accelerated muscle aging, and is frequently used as a model in sarcopenia research (Mankhong et al., 2020). Indeed, I observed that muscle mass and myofiber size of stretched side were greater as compared with that of unstretched side. Our results here extend those of previous observations by demonstrating that clinically feasible protocol of passive repetitive stretching is effective in preserving or improving muscle mass and fiber area in the senescent muscles.

It has been indicated that sarcopenia predominantly affects type 2 muscle fibers, whereas type 1 fibers are less affected. In SAM-P8 mice aging from 6 to 10 months, the proportion of fiber type 2A became greater from 53% to 66%, whereas that of muscle fiber type 2B decreased from 42% to 30%, as reported by Guo et al (2015). In addition, the type 2A fiber area increased from 6 to 8 months, followed by a sharp reduction up to 10 months (Guo et al., 2015). The remarkable amelioration of the type 2A fiber area with passive repetitive stretching in the present study is notable considering that the preferential atrophy of these high-power generation muscle fibers is a hallmark of sarcopenia progression. As a countermeasure, passive repetitive stretching with a short duration may mitigate or delay the characteristic age-related muscle loss. I also tried to find out that whether stretching induced any changes in fiber type transition by IHC staining. However, it appears that muscle fiber-type composition seemed not to be susceptible to regulation within two weeks of stretching. Fiber-type plasticity within skeletal muscle is regulated by a sophisticated signaling network with two major pathways, calcineurin signaling and AMP-activated protein kinase (AMPK) signaling with a major mediator, PGC-1α (Talbot and Maves, 2016). It suggested that the hypertrophic effect of passive repetitive stretching occurred without modifying the fiber type composition within a short duration of two weeks.

The enlargement of individual muscle fibers, that is, hypertrophy, occurs when the overall rates of protein synthesis exceed the rates of protein degradation. Therefore, to decipher the mechanism of action behind the hypertrophic effect of passive repetitive stretching, I first verified whether the Akt/mTOR pathway, which predominantly controls muscle protein synthesis, was involved in muscular adaptation. Akt is an upstream regulator of mTOR, and it is widely recognized that signaling by mTOR is a core module of the pathway through which mechanical stimuli regulate protein synthesis and muscle growth (Hornberger et al., 2004). The regulation is primarily mediated by two downstream targets of the mTORC1: translational suppressor 4E-BP1 and ribosomal protein p70S6K (Bodine et al., 2001). The

activation of mTOR1 increase p70S6K activity and mediates the phosphorylation of S6 kinase (S6K) to induce protein synthesis (Jefferies et al., 1997). 4E-BP1 generally inhibits eIF4E activity, while mTOR activity separates 4E-BP1 from eIF4E to form eIF4F, a complex of eIF4E and eIF4G, and initiates protein synthesis (Beugnet et al., 2003). Skeletal muscle stretching activates these signaling molecules with elevated phosphorylation levels, including Akt, p70S6K, and 4E-BP1, in vitro (Baar et al., 2000; Sakamoto et al., 2003). Enhanced Akt, p70S6K, and 4E-BP1 phosphorylation were observed in denervated mice soleus muscles when subjected to repetitive stretching in vivo (Agata et al., 2009). In the *Experiment 1,* I found that the mRNA expression of p70S6K significantly increased while 4E-BP1 expression decreased in the stretched side compared to the unstretched contralateral limb. Passive repetitive stretching increased the phosphorylation level of p70S6K. Several studies have demonstrated that the responsiveness of Akt/mTOR signaling is diminished in overload-induced muscle growth during aging, suggesting limited plasticity for aged muscle hypertrophy (Thomson and Gordon, 2006). In *Experiment 2*, I provided further evidence that in vivo repetitive stretching strongly increases the gene expression of Akt, p70S6K, and 4E-BP1 and the phosphorylation level of p70S6K in senescent skeletal muscles. The stretch-activated Akt/mTOR signaling pathway involved in skeletal muscle protein synthesis is intact in aged mice. The phosphorylation level of Akt and 4E-BP1 remained unchanged, compared to the unstretched muscles in both *Experiment 1* and *Experiment 2*. Previous studies have shown that the phosphorylation status of the key signaling proteins implicated in the regulation of protein synthesis exerted time-course changes in response to a period of acute stretching (Mirzoev, 2019; Agata;2009). Thus, a time course study of the acute effect of a single bout of passive repetitive stretching on phosphorylation would be useful to further elucidate protein synthesis regulation induced by stretching of the senescent skeletal muscles.

Moreover, Akt normally blocks the upregulation of several ubiquitin-proteasome genes related to protein degradation in skeletal muscles by negatively regulating FoxO transcription factors (Schiaffino et al., 2013). In skeletal muscles, the major muscle-specific ubiquitin ligases include MAFbx/atrogin-1 and MuRF1, which are associated with myonuclear apoptosis and muscle atrophy (Bodine and Baehr, 2014). Overexpression of MuRF1 and MAFbx results in muscle atrophy. However, the expression of MuRF1 and MAFbx in response to passive stretching has been inconsistent. Peviani et al. (2007) found that stretching for 60 s with 30 s of rest between sets for 10 sets per day for 7 days increased MAFbx expression in the soleus muscles of rats. Russo et al. (2010) reported that stretching

for 3.2 min with 10 min of rest between sets for 4 sets/day for 7 days could reduce the accumulation of MAFbx and MuRF1 in denervated rat skeletal muscle. Soares et al. (2007) showed that the time course changes of MAFbx and MuRF1 decreased drastically after 24-h stretching and then recovered after 48 and 96-h of stretching in immobilized muscles. These divergent findings suggest that proteasome activity is potentially influenced by stretching protocols or responds differently under physiological and pathological conditions. In *Expeiment 1*, I observed a decreased expression of MuRF1 and MAFbx after two weeks of passive repetitive stretching. MAFbx mediates protein synthesis by targeting MyoD and eIF3-f, whereas MuRF1 controls protein degradation (Bodine and Baehr, 2014). Thus, inhibition of MAFbx and MuRF1 in stretched muscles would help to maintain muscle mass and fiber size. The alternation of MAFbx and MuRF1 expression in aged skeletal muscle has been reported to be inconsistent. Several studies found that the expression levels of MAFbx and MuRF1 increased in skeletal muscles with aging, which may contribute to sarcopenia (Altun et al., 2010; Clavel et al., 2006). However, unaltered and even decreased expression levels of MAFbx and MuRF1 have been shown in other studies (Whitman et al., 2005; Edström et al., 2006). In *Experiment 2*, I found that MuRF1 mRNA expression was elevated, indicating the involvement of the cellular degradation pathway in aged skeletal muscle adaptation to passive stretching. In combination with the greater muscle mass and increased muscle fiber size, this may suggest that stretch-induced hypertrophy or suppressed atrophy of senescent muscles may result from relatively enhanced overall rates of protein synthesis that possess a superior position in protein homeostasis during the experimental period.

Another potential contributor to the increase in muscle mass and myofiber CSA is the significant decrease in myostatin expression as observed in *Experiment 1*. Myostatin is a member of the TGF-β superfamily and has been extensively studied as a target for stimulating muscle growth due to its potent negative effects on muscle growth. Myostatin binds to activin type II receptors and activates Smad signaling, which subsequently interacts with Akt signaling and suppresses its downstream targets to regulate muscle growth (Trendelenburg et al., 2009). Likewise, differential expression patterns of myostatin in stretching have been observed in previous studies. Peviani et al. (2007) showed that short sets of stretching increased myostatin expression. Aoki et al. (2009) reported time course alterations of myostatin expression that increased at 12–24 h, followed by a decrease at 96 h in continuously stretched muscles. Faturi et al. (2016) examined muscle atrophy following denervation and reported that intermittent stretching does not prevent atrophy but increases

fibrosis with an elevated level of myostatin expression. In the present study, I found that repetitive stretching for 2 weeks significantly decreased myostatin expression in ICR mice. Myostatin inhibits muscle hypertrophy via negative regulation of myogenesis and protein synthesis; therefore, decreased levels of myostatin permit increased protein synthesis and muscle growth. Alterations in myostatin expression and signaling activity in the context of aging are not completely understood. I could speculate that passive stretching is a potential intervention to counter, at least in part, sarcopenia via myostatin inhibition. However, myostatin expression was not affected by stretching in *Experiment 2*. A further time-course study may help to define the myostatin expression alternation in response to passive repetitive stretching of senescent skeletal muscles.

As stated previously, the growth of skeletal muscle encompasses both protein turnover within individual myofibers and myonuclear accretion, the addition of new myonuclei due to the fusion of satellite cells (Schiaffino et al., 2013). When satellite cells activated, a surge of MRFs, including MyoD, Myf5, and myogenin expression, is required owing to the role of MRFs in driving the differentiation of myoblasts to mature myotubes. Previous studies have shown the stretch-induced activation of skeletal muscle satellite cells in vitro and in vivo (Tatsumi et al. 2001; 2006). Elevated expression levels of MRFs have also been observed after short-term passive repetitive stretching (Kamikawa et al., 2013). Based on these findings, I assumed that the hypertrophic or suppressed atrophic observations in passive repetitive stretching may result from a combination of both two mechanisms. To elucidate whether or not passive repetitive stretching triggered an active regenerative process that may contribute to healthy or senescent skeletal muscle mass and fiber size, I first sought to detect Pax7+ cells by immunohistochemical analysis. However, there was no significant difference in the number of Pax7+ cells between the stretched and unstretched muscles in both ICR and SAM-P8 mice. In *Expeiment 1*, gene expression analysis by RT-PCR showed no differences in Pax7 expression. Myogenin mRNA expression was largely upregulated whereas MyoD expression was reduced in stretched muscles compared to unstretched ones. In *Experiment 2*, the mRNA expression of Pax7 was undetected, which indicates a contraction of the satellite cells content and impaired cell function associated with aging. The expression of MyoD was unchanged, whereas the Myf5 and myogenin mRNA expressions were upregulated in the stretched side when compared with the unstretched side. It has been reported that MyoD mRNA expression increases early in response to stimulation and then tends to decrease (McKay et al., 2008). Thus, a further examination of time course changes may help to better

define how passive repetitive stretching regulates MyoD expression. At last, the total number of nuclei per fiber was measured in each muscle section to determine whether stretch did in fact result in satellite cell activation and the incorporation of new nuclei. As a consequence, I didn't observe any diffidence in the number of myonuclei per fiber between stretched and unstretched muscles in both ICR and SAM-P8 mice. Concomitant expressions of MyoD and PAX7 are well recognized as a satellite cells activation index (Cornelison and Wold, 1997; Relaix et al., 2006). I am not convinced of the possibility of stretch-induced myogenesis without an increase in MyoD and Pax7 expression levels and new nuclei addition. It seems that satellite cells proliferated and contribute to hypertrophy normally when muscle damage occurs (Schiaffino et al., 2013). I speculate that muscle damage is greatly avoided by stretching muscles under deep anesthesia within the natural range of motion and a short duration. Furthermore, it has been indicated that overload-induced muscle hypertrophy requires the involvement of satellite cells in growing mice, whereas it is not necessary for hypertrophic growth in mature adult mice (Murach et al., 2017). These findings indicate that stretch-induced hypertrophy mainly attributes to the regulation of signaling pathways involved in muscle protein rather than triggering satellite cell activation and fusion.

The present study had some limitations. Considering that SAMP8 mice muscle was not collected before stretching intervention as controls, it remains unclear that how sarcopenic morphological changes progressed during stretching intervention. Therefore, I cannot draw a robust conclusion regarding the extent of the effect of passive repetitive stretching on the muscle mass and fiber area: suppressing the progression of atrophy or inducing hypertrophy. Although pax7+ cells and MRFs related to muscle satellite cells were assessed after the final section of stretching, it is better detected at an early time point in the protocol because of potential time course changes. The time course of the acute effect of a single bout of passive repetitive stretching on the phosphorylation status of the key signaling molecules would be useful to further elucidate protein synthesis regulation induced by stretching of the senescent skeletal muscles.

Conclusion

In conclusion, I propose that passive repetitive stretching for a duration of two weeks contributes to the improvement of muscle mass and myofiber size and is proved to be effective in preserving and improving muscle mass and fiber area in sarcopenic muscle. However, the mechanism(s) responsible for stretch-induced muscle hypertrophy and suppressed atrophy in two experiments are different. The hypertrophic observations in healthy muscles are attributed to comprehensive promotion of Akt/mTOR pathway, which is involved in muscle protein synthesis, and the suppression of MuRF1/MAFbx pathway, which is involved in protein degradation, as well as myostatin; Whereas passive repetitive stretching, induces hypertrophy of senescent muscles mainly via regulation of the Akt/mTOR signaling pathway, with unchanged myostatin expression, accompanied by an elevated expression level of MuRF1. This study also highlights the contribution of protein turnover rather than satellite cells activation in the stretch-induced muscle growth in adult and aged mice. These results support the performance of passive repetitive stretching as an effective measure to induce skeletal muscle hypertrophy, for sarcopenia prevention and the maintenance of skeletal muscle mass and function in patients exposed to prolonged inactivity, unconscious or paralyzed.

Future studies are required to determine the time course of the acute effect of passive repetitive stretching on the expression of key factors implicated in the regulation of protein synthesis (For example, the phosphorylation status of Akt/mTOR signaling pathway, the expression of myostatin) and also myogenesis pathway to further elucidate the mechanisms and optimize the stretching protocols. In addition, I speculate that the combination of passive repetitive stretching together with nutritional supplement, for example β-hydroxy-β-methylbutyrate-free acid (HMB-FA) would provide an additive effect on muscle hypertrophy when paired with stretching on certain variables. In fact, our laboratory has begun to record the food intake and body weight in ICR mice, considering a possible dietary supplementation with HMB-FA. Furthermore, I intend to clinically apply the findings obtained by animal experiments and develop a more effective muscle strengthening strategy to fight against illnesses related to muscle atrophy as well as enhance hypertrophy.

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Disclosure of Conflict of Interest

The author declares no conflict of interest.

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