Age-dependent increase in angiopoietin-like protein 2 accelerates skeletal muscle loss in mice

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Running title: ANGPTL2 accelerates skeletal muscle atrophy

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ABSTRACT

Skeletal muscle atrophy, or sarcopenia, is commonly observed in older individuals and in those with chronic disease and is associated with decreased quality of life. There is recent medical and broad concern that sarcopenia is rapidly increasing worldwide as populations age. At present, strength training is the only effective intervention for preventing sarcopenia development, but it is not known how this exercise regimen counteracts this condition. Here, we report that expression of the inflammatory mediator angiopoietin-like protein 2 (ANGPTL2) increases in skeletal muscle of aging mice. Moreover, in addition to exhibiting increased inflammation and accumulation of reactive oxygen species...
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(ROS), denervated atrophic skeletal muscles in a mouse model of denervation-induced muscle atrophy had increased ANGPTL2 expression. Interestingly, mice with a skeletal myocyte specific \textit{Angptl2} knockout had attenuated inflammation and ROS accumulation in denervated skeletal muscle, accompanied by increased satellite cell activity and inhibition of muscular atrophy compared with mice harboring wild-type \textit{Angptl2}. Moreover, consistent with these phenotypes, wild-type mice undergoing exercise training displayed decreased ANGPTL2 expression in skeletal muscle. In conclusion, ANGPTL2 upregulation in skeletal myocytes accelerates muscle atrophy, and exercise-induced attenuation of ANGPTL2 expression in those tissues may partially explain how exercise training prevents sarcopenia.

Sarcopenia is defined as age-related loss of skeletal muscle mass and strength, a condition that worsens subjects' quality of life (1). Sarcopenia is now a medical and social concern because the aging population is increasing worldwide. Sarcopenia also develops in patients with chronic disease, such as diabetes, cardiovascular disease and cancer, and these pathologies accelerate clinical mortality (2). Therefore, clarification of molecular mechanisms underlying sarcopenia development is important to devise effective therapeutic and/or preventive approaches to treat this condition.

Several lines of evidence support the idea that in skeletal muscle chronic inflammation and reactive oxygen species (ROS) accumulation due to redox imbalance contribute to sarcopenia development (3-8). Chronic inflammation in aging skeletal muscle is positively correlated with sarcopenia development in humans and mice (3,4). The pro-inflammatory cytokines interleukin-6 (IL-6) and interleukin-1β (IL-1β) both decrease skeletal muscle mass by causing inflammation and subsequently facilitating muscle proteolysis, ROS accumulation, and growth hormone resistance (9-11). Moreover, excess ROS accumulation causes oxidative damage to skeletal muscles, resulting in myofibers loss (5-8). Both inflammation and ROS accumulation inactivate “satellite cells”, the precursors of skeletal muscle cells (12,13), thereby accelerating sarcopenia development (14). Currently, strength training exercise, which increases the volume of skeletal muscle myofibers, is the only effective way to prevent sarcopenia development. Recent reports suggest that exercise reduces inflammation (15) and ameliorates ROS
ANGPTL2 accelerates skeletal muscle atrophy accumulation by increasing antioxidant activity (16) and also enhances satellite cell activation (17) in animal models of aging. However, molecular mechanisms underlying these activities remain unclear.

Previous studies reveal that expression and secretion of angiopoietin-like 2 (ANGPTL2) significantly increase in cells stressed by pathophysiological stimuli, such as hypoxia and pressure-overload (18,19). ANGPTL2 expression also increases in cells undergoing senescence (20,21), suggesting that ANGPTL2 is a senescence-associated secretory phenotype (SASP) factor. Moreover, excess ANGPTL2 signaling is pro-inflammatory in pathological states and contributes to development of aging-associated diseases such as obesity, diabetes, atherosclerotic disease, chronic kidney disease, and some cancers (22,23).

Although ANGPTL2 hyperactivation is associated with age-related diseases, ANGPTL2 function in sarcopenia development remains unknown. Here, we investigated the roles of ANGPTL2 in sarcopenia development using aging mice and denervation-induced muscle atrophy mouse model. We report that ANGPTL2 expression increases in skeletal myocytes of aging mice and that running exercise decreases that expression, suggesting that excess ANGPTL2 signaling in aged skeletal muscular myofibers accelerates sarcopenia development. Moreover, ANGPTL2 deficiency in skeletal myocytes attenuated loss of skeletal muscle by reducing muscular inflammation and ROS accumulation and increasing satellite cell activity. To the best of our knowledge, this is the first report showing that ANGPTL2 signaling may accelerate sarcopenia pathologies.

RESULTS
ANGPTL2 expression increases in skeletal myocytes with age

Since ANGPTL2 expression and its circulating levels are positively correlated with aging (19,23,24), we asked whether ANGPTL2 expression increases in aging skeletal muscles. To do so, we first evaluated age-related changes in skeletal muscles of adult (8-month-old) compared to aging (18-month-old) mice. We observed significantly increased expression of pro-inflammatory and cellular senescence genes in skeletal muscle of aging relative to adult mice (Figure 1a). Skeletal muscle mass of aging mice was significantly decreased compared with that seen in adult mice (Figure 1b). In animal tissues, 4-HNE (4-hydroxynonenal) is a lipid peroxidation product whose formation is closely related to
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oxidative stress (25). Because of its electrophilic nature, 4-HNE reacts with nucleophilic amino acid residues to form 4-HNE-adducted proteins, which serves as a marker of oxidative stress. We also confirmed increased 4-HNE-adducted protein levels and decreased catalase expression in skeletal muscle of aging relative to adult mice; however, we observed no change in expression of superoxide dismutases (Sods) or glutathione peroxidase 1 (Gpx1) (Figures 1c and d). Moreover, expression of genes associated with satellite cell activation, such as Cd34, Pax3, and Pax7, in skeletal muscles was significantly decreased in aging relative to adult mice (Figures 1e, g and Supplementary Figure 1a, b).

We next evaluated Angptl2 expression in aging and adult mice. Angptl2 mRNA and protein levels significantly increased in skeletal muscle tissues of aging relative to adult mice (Figures 1f and g). Skeletal muscle tissue is composed not only skeletal myocytes but of various stromal cells, such as endothelial cell, macrophages and infiltrated blood cells. Thus, to determine which cell type in skeletal muscle predominantly expresses ANGPTL2, we prepared both skeletal myocytes (SMC)- and stromal cells-enriched fractions from skeletal muscle of adult and aging mice (Supplementary Figure 2). In adult mice, ANGPTL2 protein levels were comparable in both fractions, whereas, in aging mice, ANGPTL2 protein expression was markedly increased in SMC fraction relative to stromal cells fraction (Figure 1h). These results suggest that ANGPTL2 is predominantly increased in skeletal myocytes of aging mice.

ANGPTL2 suppression in skeletal myocytes decreases inflammation and ROS levels and improves cellular senescence phenotypes

To determine the relationship between ANGPTL2 expression in skeletal myocytes and age-related changes in skeletal muscle, we generated skeletal myocyte-specific Angptl2 knockout (Angptl2^Flox/Flox; MCK-Cre) mice. We first confirmed that both Angptl2 mRNA and protein expression was decreased by half in Angptl2^Flox/Flox; MCK-Cre versus Angptl2^Flox/Flox mice (Figure 2a) and that expression was specifically decreased in skeletal myocytes (Figure 2b). Skeletal muscle masses were comparable between Angptl2^Flox/Flox; MCK-Cre and Angptl2^Flox/Flox mice (Figure 4a), suggesting that ANGPTL2 deficiency in skeletal myocytes does not alter myogenesis or skeletal muscle homeostasis.

On the other hand, expression of pro-inflammatory and cellular senescence
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genes in skeletal muscle significantly decreased in \textit{Angptl2}^{\text{Flx/Flx}}; MCK-Cre relative to \textit{Angptl2}^{\text{Flx/Flx}} mice (Figure 2c). In addition, although we observed slightly decreased ROS level in some low-molecular-weight proteins, there was no significant difference in global ROS levels in young skeletal muscle of either genotype (Figure 2d). However, catalase activity in skeletal muscle of \textit{Angptl2}^{\text{Flx/Flx}}; MCK-Cre mice was significantly higher than that seen in \textit{Angptl2}^{\text{Flx/Flx}} mice (Figure 2e).

To further assess the effect of ANGPTL2 suppression on myocytes, we conducted loss-of-function studies by transfecting differentiated mouse C2C12 myoblasts with Angptl2 siRNA or control siRNA. ANGPTL2 protein levels were significantly decreased in Angptl2-knockdown cells and in their culture medium compared to control cells (Figure 2f and Supplementary Figure 3a). Expression levels of \textit{p21}, \textit{p57}, and \textit{II-6} and senescence-associated β-gal activity were all significantly decreased in Angptl2 knockdown relative to control C2C12 cells (Figures 2g and h). Moreover, intracellular ROS levels in Angptl2 knockdown cells were significantly lower than those in control cells, while catalase expression and activity were significantly increased in knockdown relative to control cells (Figures 2i and j). These results suggest that ANGPTL2 suppression in skeletal myocytes enhances ROS clearance capacity by up-regulating catalase, decreasing inflammation and cellular senescence phenotypes.

\textbf{ANGPTL2 deficiency in myocytes increases skeletal muscle satellite cell activity}

Because inflammation and ROS antagonize development of skeletal muscle satellite cells (26), we evaluated satellite cell activity in skeletal muscle of \textit{Angptl2}^{\text{Flx/Flx}}; MCK-Cre and \textit{Angptl2}^{\text{Flx/Flx}} mice. Expression of genes associated with satellite cell activation in \textit{Angptl2}^{\text{Flx/Flx}}; MCK-Cre mice was significantly increased compared to that seen in \textit{Angptl2}^{\text{Flx/Flx}} mice (Figure 3a). Moreover, immunofluorescence staining revealed that the number of quiescent satellite cells (marked by CD34+/PAX7+ expression) did not change, however the number of activated satellite cells (as marked by CD34-/PAX7+ expression) increased in skeletal muscle of \textit{Angptl2}^{\text{Flx/Flx}}; MCK-Cre relative to \textit{Angptl2}^{\text{Flx/Flx}} mice (Figure 3b and Supplementary Figure 3c). Immunoblotting analysis also revealed relatively increased PAX7 protein levels in skeletal muscle of \textit{Angptl2}^{\text{Flx/Flx}}; MCK-Cre mice (Figure 3c).

Overall, these results suggest that ANGPTL2
suppression in skeletal myocytes promotes satellite cell activation without any impairing of their self-renewal.

**ANGPTL2 deficiency in myocytes prevents skeletal muscle atrophy**

Since aging-associated inflammation, ROS accumulation, and satellite cell inactivation all reportedly contribute to sarcopenia (7,27,28), we asked whether ANGPTL2 deficiency in skeletal myocytes would protect skeletal muscles from atrophy. Given that spinal motor neuron loss and reduced numbers of motor units characterize aging and directly induce sarcopenia (29), we established a denervation-induced muscle atrophy mouse model to mimic sarcopenia. As anticipated, we observed a significant decrease in skeletal muscle mass relative to wild-type by 7 days after denervation (Supplementary Figure 4).

We also observed relatively increased ANGPTL2 protein expression in skeletal muscle post-denervation (Figure 4b). To assess the pathophysiological significance of this increase, we performed denervation in Angptl2^Flox/Flox; MCK-Cre mice. By 14 days after denervation, the reduction in skeletal muscle mass seen in wild-type Angptl2 mice was significantly suppressed in Angptl2^Flox/Flox; MCK-Cre mice (Figures 4c and d). Decreased cellular volume seen on a wild-type Angptl2 background was also significantly suppressed in Angptl2^Flox/Flox; MCK-Cre mice (Figures 4e and f), suggesting overall that ANGPTL2 deficiency in skeletal myocytes prevents development of muscle atrophy.

Angptl2^Flox/Flox; MCK-Cre mice also showed decreased expression of pro-inflammatory genes in denervated skeletal muscle compared to Angptl2^Flox/Flox mice (Figure 5a). By 14 days after denervation, ROS accumulation and decreased catalase expression seen in denervated skeletal muscle of mice harboring wild-type Angptl2 were significantly suppressed in Angptl2^Flox/Flox; MCK-Cre mice (Figures 5b and c). Expression levels of genes associated with satellite cell activation and PAX7 protein in skeletal muscle were also significantly increased in Angptl2^Flox/Flox; MCK-Cre mice compared to mice harboring wild-type Angptl2 (Figures 5d and e). These results suggest that ANGPTL2 deficiency in skeletal myocytes blocks muscle atrophy by activating satellite cells and decreasing inflammation and ROS accumulation.

**Exercise training suppresses ANGPTL2 expression in skeletal muscle**

Since exercise training reportedly
prevents sarcopenia development (30), we asked whether exercise altered Angptl2 expression in skeletal muscle. To do so, we exposed wild-type mice to exercise training involving running on a treadmill (see Experimental procedures). We firstly confirmed that after exercise training, expression of pro-inflammatory or cellular senescence genes in skeletal muscles significantly decreased and that of genes associated with satellite cell activation significantly increased (Figure 6a and b), as previously reported (15-17). Moreover, expression of Angptl2 mRNA and protein in skeletal muscle was significantly decreased after exercise training (Figure 6c and d).

To further evaluate the effect of exercise training on Angptl2 expression in skeletal myocytes, we used two cellular exercise models in which differentiated C2C12 cells are either subjected to mechanical stretch or treated with AICAR (AMP-activated kinase activator) (31,32), both of which lead to cellular changes associated with exercise training. Angptl2 expression levels in differentiated C2C12 cells significantly decreased after mechanical stretch (Figure 6e), while AICAR treatment of C2C12 cells also significantly decreased expression of Angptl2 mRNA and protein (Figure 6f).

**DISCUSSION**

This study reveals that ANGPTL2 expression in skeletal myocytes increases with age and in a denervation-induced muscle atrophy mouse model. Interestingly, Angptl2-deficient mice in the atrophy model showed relatively decreased inflammation and ROS accumulation and increased satellite cell activity. In sarcopenia development, both inflammation and excess ROS accumulation damage skeletal myocytes, and inactivate satellite cells, accelerating muscle mass loss (14). Taken together, these findings suggest that ANGPTL2 contributes to sarcopenia development by accelerating inflammation and ROS accumulation in skeletal muscle and subsequently inactivating satellite cells.

Large amounts of ROS released from senescent skeletal myocytes may enhance oxidative damage to skeletal muscle (33). Excessive ROS accumulation with age is reportedly due to decreased activity and production of antioxidant enzymes (34). Here, using young mice, we observed that global ROS levels did not significantly change in Angptl2Flox/Flox; MCK-Cre mice compared to Angptl2Flox/Flox mice, but slight decreased ROS levels was observed in some low-molecular-weight proteins (Figure 2d). However, others have reported that global ROS level induced by denervation
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significantly decrease in Angptl2\textsuperscript{Flox/Flox}, MCK-Cre mice related to Angptl2\textsuperscript{Flox/Flox} mice (35). Consistent with these results, we found that Angptl2 knockdown in differentiated C2C12 cells resulted in low ROS levels (Figures 2i). Accordingly, catalase levels increased in both normal and denervated Angptl2\textsuperscript{Flox/Flox}; MCK-Cre mice as seen in Angptl2-knockdown differentiated C2C12 cells (Figures 2e, 5c and 2j). Overall, these results indicate that ANGPTL2 induced ROS accumulation may require catalase down-regulation.

Peroxisome proliferator-activated receptors (PPARs) reportedly play important roles in activating transcription of genes encoding antioxidant enzymes (36). Recent studies report that PPAR\(\alpha\) activates catalase expression and that PPAR\(\alpha\) expression decreases with age in tissues like skin and heart (37,38). Moreover, our recent study showed that in cardiomyocytes, excess ANGPTL2 signaling down-regulates PPAR\(\alpha\) expression, while ANGPTL2 suppression up-regulates it (19). Taken together with findings reported here, we propose that excess ANGPTL2 signaling in aging skeletal muscle may enhance ROS accumulation by decreasing PPAR\(\alpha\)-mediated catalase expression.

Here, we also report that Angptl2 loss in skeletal muscle decreases expression of genes associated with inflammation and cellular senescence. We previously reported that ANGPTL2 promotes chronic tissue inflammation by activating the integrin \(\alpha 5\beta 1\)/nuclear factor-\(\kappa B\) (NF-\(\kappa B\)) pathway and through p38 MAPK activity (22,39,40). Therefore, we hypothesize that increased pro-inflammatory gene expression in skeletal muscle downstream of ANGPTL2 could be due to activation of these two pro-inflammatory pathways. Since inflammation and ROS induce cellular senescence (4,5), ANGPTL2 may accelerate muscle cell senescence by promoting both. As noted above, ANGPTL2 is a SASP factor and its expression increases in senescent cells (20,21). Overall, upregulated ANGPTL2 signaling in skeletal muscle cell may underlie inflammation, ROS accumulation, and cellular senescence.

In aging muscle, satellite cell self-renewal activity is reduced and the number of quiescent satellite cells decreases (41). Moreover, differentiation of activated satellite cells and myotube formation are impaired in aging muscles (42). Interestingly, in young mice, ANGPTL2 suppression in skeletal myocytes increases the number of activated satellite cells without affecting quiescent satellite cell population, suggesting...
that ANGPTL2 deficiency in skeletal myocytes does not impair satellite cell self-renewal capacity. We also showed that decreased expression of several genes associated with both satellite cell activation and increased inflammation/ROS accumulation in a denervation-induced muscle atrophy mouse model is ameliorated by \textit{Angptl2} knockout. Inflammation and ROS block activity of satellite cell precursors of skeletal muscle cells (14), suggesting that at least, ANGPTL2 antagonizes satellite cell development via these mechanisms. Further studies are necessary to investigate whether ANGPTL2 directly modulates satellite cell function or whether suppression of ANGPTL2 production from skeletal myocytes could prevent or ameliorate sarcopenia development.

Activated satellite cells reportedly proliferate, differentiate into myocyte, and fuse in muscle fibers, increasing muscle mass (43). Here, we showed that exercise-induced ANGPTL2 suppression decreases inflammation and ROS accumulation and facilitates satellite cell activation. Taken together, these findings suggest that satellite cells activated by exercise-induced ANGPTL2 suppression in skeletal myocytes could generate muscle fibers and that their presence could prevent loss of muscle mass.

Exercise training, including running and walking, counteracts development of heart failure and sarcopenia in humans (15-17). We recently observed decreased ANGPTL2 expression in mouse heart tissues after exercise training through exercise-induced, cardiac miR-222-mediated ANGPTL2 suppression (19). We also found that inactivation of heart-derived autocrine/paracrine ANGPTL2 signaling protected mice from heart failure development (19). Interestingly, the present study shows that exercise training also suppresses ANGPTL2 expression in skeletal muscle and that ANGPTL2 suppression antagonizes muscle fiber loss induced by denervation. It is noteworthy that exercise-induced ANGPTL2 suppression is a potential mechanism underlying exercise-associated prevention of sarcopenia development. Further studies are needed to determine how exercise training decreases ANGPTL2 expression in skeletal muscle on a mechanistic level.

We previously reported that circulating levels of ANGPTL2 increase with age in the general Japanese population and in mice (23). Interestingly, we found that in mice, age-dependent increases in serum ANGPTL2 concentration are suppressed by skeletal myocyte-specific ANGPTL2 deletion.
ANGPTL2 accelerates skeletal muscle atrophy (Supplementary Figure 3b), suggesting that increased ANGPTL2 production from myocytes contributes in part to age-dependent increases in circulating levels of this factor.

In summary, this is the first study to report that increased ANGPTL2 produced from skeletal muscle may be a novel factor involved in promoting loss of muscular volume, enhancing inflammation and ROS accumulation, and suppressing satellite cell activity (Figure 7, left panel). We also propose that exercise-induced ANGPTL2 suppression could reduce inflammation and ROS accumulation, facilitating satellite cell activation required to maintain muscle mass (Figure 7, right panel).

**EXPERIMENTAL PROCEDURES**

**Animal study**

Male or female C57BL/6NJcl and female Angptl2 conditional knockout (Angptl2^{Flox/Flox}; MCK-Cre and Angptl2^{Flox/Flox}) mice were used in this study. MCK-Cre mice were purchased from The Jackson Laboratory. Angptl2^{Flox/Flox} mice were described previously (19). Mice were fed a normal diet and water *ad libitum*, bred in a mouse house with automatically controlled lighting (12 h on, 12 h off), and maintained at a stable temperature of 23°C. All experimental procedures were approved by the Kumamoto University Ethics Review Committee for Animal Experimentation.

**Mouse denervation-induced muscle atrophy model**

Twelve-week-old female Angptl2^{Flox/Flox}; MCK-Cre mice were anesthetized by intraperitoneal injection with sodium pentobarbital. The sciatic nerve of the left hindlimb was excised approximately 0.5-1 cm, while the sciatic nerve of the contralateral hindlimb was sham-operated by exposure without excision. At 3, 7, and 14 days after denervation, mice were sacrificed for analysis.

**Exercise training**

Exercise was performed as described previously (19). Briefly, 12-week-old male mice were exposed to a treadmill chamber with free movement for environmental adaptation for 30 min before the exercise. Mice were then trained using the following protocol: 5 meters / min for 5 min, 10 miters / min for 5 min, 15 meters / min for 5 min, and 20 meters / min for 60 min. These mice were sacrificed immediately or 3 hrs after exercise. A different group of endurance-trained mice were trained for 2 weeks (5 days / week) and were sacrificed 3 hrs after the last run.
Isolation of skeletal muscle myofibers and non-myofibers

Isolation followed a previously described protocol (44). Briefly, as shown in Supplementary Figure 2a, gastrocnemius muscle was dissected and incubated in collagenase solution (L-15 medium (1601301, Gibco™ Life Technologies, Carlsbad, CA, USA) containing 500U/ml collagenase type I (CLS1, Worthington Biochemical Corporation, Lakewood, NJ, USA)) at 37°C for 2h. Muscle was transferred to pre-warmed PBS and then flushed with PBS using a pipette under a microscope until myofibers were isolated. The entire solution was then collected and passed in turn through 100 µm and 40 µm mesh filters. The filtrate was collected into a new falcon tube and centrifuged 3000rpm for 5min to collect non-myofibers. The 100 µm mesh filter was flushed with pre-warmed PBS from the underside and the flushed fluid was transferred to a new falcon tube and centrifuged as above to collect myofibers. The supernatant was discarded and the pellet was subjected to RNA extraction or to western analysis.

C2C12 culture and AICAR treatment

C2C12 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; WAKO 044-29765, Tokyo, Japan) supplemented with 10% fetal calf serum in a humidified atmosphere with 5% CO2 at 37 °C. Cells reached to 60-80% confluence after being seeded for 24 h and then the medium was replaced by DMEM containing 2% horse serum for 96 h to induce differentiation. All in vitro studies reported here were conducted in differentiated C2C12 cells. Differentiated C2C12 cells for knockdown analysis were established using Mission siRNA Universal Negative Control (siControl, Sigma-Aldrich, St. Louis, MO, USA) or Mission siRNA targeting Angptl2 (siAngptl2: SASI_Mn01_00095185: 5'-GAGAGUACAUUUACCUCAATT-3'. Sigma-Aldrich, St. Louis, MO, USA). Cells were transfected using Lipofectamine™ RNAiMAX Transfection Reagent (13778-150, Invitrogen, Carlsbad, CA, USA).

For AICAR treatment, differentiated C2C12 cells were incubated with 1mM AICAR (A9978, Sigma, Aldrich, St. Louis, MO, USA) for 1, 6, or 12 hours.

ANGPTL2 ELISA

A Human ANGPTL2 Assay Kit-IBL (27745; Immuno-Biological Laboratories, Gunma, Japan) was used to measure ANGPTL2 concentration in supernatants or
in mouse serum in accordance with the manufacturer's instructions. Briefly, supernatants were diluted 10-fold with EIA buffer and 100ul of the sample was placed into wells of a tissue culture plate. Samples were incubated 60min at 37°C, washed 4 times with washing buffer provided, and then treated with labeled antibody solution and incubated another 30min at 4°C. Samples were then washed 5 times and the treated with TMB solution for 30min at room temperature protected from light. After addition of stop solution, plates were then analyzed for absorbance at OD 450 nm using an iMark™ microplate reader (168-1130 JA, BIO-RAD, Hercules, CA, USA), and catalase activity was calculated using formulas supplied by the manufacturer (Abcam, Cambridge, UK).

**ROS detection**

C2C12 cellular ROS was detected using CM-H2DCFDA reagent (General Oxidative Stress Indicator) (C6827, Invitrogen, Carlsbad, CA, USA) based on the manufacturer's instructions. Briefly, cells were seeded in a 96-well microplate and incubated for 30 minutes at 37 °C with 10 µM CM-H2DCFDA and then washed twice with PBS. Cellular ROS levels were then detected at an Ex/Em of 485/515 nm using a Fluoroskan Ascent Microplate Luminometer (Thermo Fisher Scientific Inc., Carlsbad, CA, USA). Data was analyzed using Ascent software (Version 2.6).

Skeletal muscular ROS levels were assessed by examining the 4-HNE-adducted protein levels in skeletal muscle samples (see Western Blotting).

**SPiDER-βgal staining**

SPiDER-βgal reagent (SG02, DOJINDO, Kumamoto, Japan) was used for cellular βgal staining based on the manufacturer's instructions. Briefly, cells were seeded on 96-well microplates or chamber slides (SCS-002, Matsunami, Osaka, Japan) for
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differentiation. After Angptl2 knockdown, 1µmol/L SPiDER-βgal working solution was added and incubated for 15min at 37 °C protected from light. After 2 washes with PBS, cells were evaluated using a Fluoroskan Ascent Microplate Luminometer (Thermo Fisher Scientific Inc., Carlsbad, CA, USA), or images were obtained using a fluorescence microscope (model BZ-9000; Keyence, Osaka, Japan).

**Cellular mechanical stretch**

C2C12 cells were seeded on BIOFLEX® PLATE COLLAGEN I-BF 3001C (Flexcell® International Corporation, Burlington, NC, USA) plates. After differentiation, cells were subjected to 5%, 10% or 15% elongation with 1Hz sine wave cyclic stretch for 15min or 30min. Control cells were not subjected to stretch. Cells were then harvested and immediately analyzed.

**Histological staining**

For routine hematoxylin and eosin (HE) staining, skeletal muscle was dissected, fixed in 4% formalin for >12 hours at room temperature, and then embedded in paraffin and cut into 5µm thick sections. Images were obtained using a fluorescence microscope (model BZ-9000; Keyence, Osaka, Japan). The area of each cellular cross section was analyzed using BZ-X analyzer software (Keyence, Osaka, Japan).

For immunofluorescence, we used frozen section of skeletal muscle. Sections were fixed with cold acetone for 20min, and blocked with 5% goat serum for 20min at room temperature. The primary antibody was incubated at 4°C overnight and then second antibodies or Wheat Germ Agglutinin (WGA) were incubated at room temperature for 1h. Antibodies used in immunofluorescence were: anti-CD34 (1:50, 13-0341-85, RAM34, eBioscience, Carlsbad, CA, USA), anti-Pax7 (1:20, PA1-117, Invitrogen, Carlsbad, CA, USA), WGA (W11262, Invitrogen, Carlsbad, CA, USA). Image was obtained from confocal microscopy (FV1200 IX83, OLYMPUS, Tokyo, Japan) and quantified by ImageJ.

**RT-PCR**

Total RNA was isolated from C2C12 cells or skeletal muscle using an RNeasy mini kit (Qiagen, Valencia, CA, USA). mRNA was converted to cDNA using a Prime Script RT reagent kit (Takara Bio Inc., Shiga, Japan). PCR reactions were performed in a Thermal Cycler Dice Real-Time System TP870 (Takara Bio Inc., Shiga, Japan) using SYBR Premix EX Taq (Takara Bio Inc., Shiga, Japan) according to the manufacturer's
instructions. Relative gene expression was determined using the standard curve method, and fold-changes of targeted genes were normalized to 18S mRNA. Primer sequences used are shown in Supplementary Table 1.

**Western blotting**

Differentiated C2C12 cells or skeletal muscle tissues were homogenized in lysis buffer (10mM Tris–HCl, 1% Triton X-100, 50mM NaCl, 30mM sodium pyrophosphate, 50mM NaF, 5mM EDTA, 0.1mM Na3VO4, plus a protease inhibitor cocktail (Nacalai Tesque, Kyoto, Japan), pH 7.5). SDS–PAGE was performed on gradient gels (SuperSep™Ace, 5-20%, 17well, WAKO, Tokyo, Japan), and immunoblotting was performed as described (19). Antibodies for anti-Angptl2 (BAF1444; R&D SYSTEMS, Systems, Minneapolis, MN, USA), anti-Pax7 (PAX7497, Abcam, Cambridge, UK), anti-4-hydroxynonenal (anti-4-HNE) (MAB3249, R&D SYSTEMS, System, Minneapolis, MN, USA), anti-MYL (F-5, sc-365243; Santa Cruz Biotechnology, Dallas, Texas, USA) and anti-Hsc70 (sc-7298; Santa Cruz Biotechnology, Dallas, Texas, USA) were used. CBB staining was performed using Quick-CBB reagent (299-50101, WAKO, Tokyo, Japan). Briefly, after image acquisition, the PVDF membrane was washed with TBST and incubated 15min with stripping buffer (46430, Thermo Fisher Scientific Inc., Carlsbad, CA, USA), followed by 5 min wash for 3 times in TBST. Membranes were dipped into the Quick-CBB reagent mixture (solution A + solution B) for 20min, washed with deionized water 4-6 times, and imaged.

**Statistical analyses**

All data are represented as the mean ± standard deviation (S.D) or mean ± standard error of the mean (S.E.M). Statistical significance of two-group comparisons of variables was determined using Student's t-test, and multiple comparisons were assessed by one-way or two-way ANOVA. \( P < 0.05 \) was considered statistically significant.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest with the contents of this article.

AUTHOR CONTRIBUTIONS

J.Z., Z.T., and Y.O. conceived the study and designed experiments. J.Z., Z.T., P.X. and K.M. performed experiments and collected or analyzed data. T.Y. and K.Y. provided instructions and assisted with the cellular stretch experiment. J.Z., Z.T., T.K. and Y.O. wrote the paper. K.M., T.S., M.E., S.Z., H.F., M.S., H.H., J.M. and K.T. provided necessary assistance.

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**FOOTNOTES**
ANGPTL2 accelerates skeletal muscle atrophy

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FIGURE LEGENDS

Figure 1. ANGPTL2 levels increase in skeletal muscle of aging mice. (a) Relative transcript levels of cellular senescence-associated (p16, p19, p21, and p57) and pro-inflammatory (IL-1β and IL-6) genes in musculus gastrocnemius and musculus soleus of adult (8-month-old) and aging (18-month-old) female wild-type mice (n = 4 per group) (b) Absolute muscle mass and body weight as well as tibia length normalized muscle mass in adult and aging mice (n = 7-10 per group). (c) Representative western blot of 4-HNE (4-hydroxynonenal)-adducted proteins, a product of oxidative stress, in musculus soleus of adult and aging mice. Asterisks indicate increased levels of 4-HNE-adducted proteins in aging mice. (d-f) Relative expression of genes encoding antioxidant enzymes (catalase, Sod1, Sod2, and Gpx1) (d) or Cd34, Pax3, and Pax7 (e) or Angptl2 (f) transcripts in musculus gastrocnemius and musculus soleus of adult and aging mice (n = 4 per group). (g) Representative western blot and the quantifications of ANGPTL2 and PAX7 proteins in musculus gastrocnemius and musculus soleus of adult and aging mice. (h) Representative western blot of ANGPTL2 in skeletal myocytes (SMC)-rich or stromal cells-rich fractions isolated from musculus gastrocnemius of adult and aging mice. MYL (myosin light chain) serves as a skeletal myocyte marker. Relative mRNA expression was normalized to 18S mRNA (a, d, e and f). Hsc70 and CBB (Coomassie Brilliant Blue) staining serves as an internal loading control (c, g and h). Values in adult mice were set to 1 (a, d, e, f and g). All data are presented as means ± S.D (a, b, d, e and f) or means ± SEM (g); statistical significance was determined by Student’s t-test, *p < 0.05, **p < 0.01, †p < 0.001.

Figure 2. Angptl2 deficiency suppresses inflammation and cellular senescence and increases CATALASE activity in skeletal myocytes. a-e shows in vivo (skeletal muscles)
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analysis; f-j shows the in vitro (differentiated C2C12) analysis. (a) Relative mRNA expression (left) and representative western blot (middle) and ANGPTL2 quantification in skeletal muscle of Angptl2^Flx/Flx; MCK-Cre mice and Angptl2^Flx/Flx mice (n = 4 per group). (b) Relative Angptl2 mRNA expression in skeletal myocyte (SMC)-rich or stromal cells-rich fractions isolated from musculus gastrocnemius of Angptl2^Flx/Flx; MCK-Cre mice and Angptl2^Flx/Flx mice (n = 6-8 per group). (c) Relative expression of cellular senescence-associated or pro-inflammatory genes in musculus gastrocnemius and musculus soleus of Angptl2^Flx/Flx; MCK-Cre mice and Angptl2^Flx/Flx mice (n = 4 per group). (d) Representative western blot of 4-HNE-adducted proteins in musculus gastrocnemius of Angptl2^Flx/Flx; MCK-Cre and Angptl2^Flx/Flx mice. Asterisks indicate decreased levels of these proteins seen in Angptl2^Flx/Flx; MCK-Cre mice. (e) Relative CATALASE activity in musculus gastrocnemius of Angptl2^Flx/Flx; MCK-Cre and Angptl2^Flx/Flx mice (n = 6-8 per group). (f) Representative western blot and the quantification of ANGPTL2 expression in differentiated C2C12 cells transfected with siRNA control (siControl) or siRNA Angptl2 (siAngptl2) (n = 3 per group for ANGPTL2 quantification). (g) Relative transcript levels of cellular senescence-associated genes in differentiated C2C12 cells transfected with Angptl2 or control siRNA (UD = undetected) (n = 6 per group). (h) Representative images and relative fluorescence levels of SPIDER-βgal staining of differentiated C2C12 cells transfected with Angptl2 or control siRNA (Scale bar: 50µm) (n = 10 per group). (i) Relative fluorescence levels detected by CM-H2DCFDA (General Oxidative Stress Indicator) of differentiated C2C12 cells transfected with Angptl2 or control siRNA (n = 12-13 per group). (j) Relative catalase transcript levels (left) or activity (right) in differentiated C2C12 cells transfected with Angptl2 or control siRNA (n = 5-6 per group). Relative mRNA expression was normalized to 18S mRNA (a, b, c, g and j). Hsc70 and CBB (Coomassie Brilliant Blue) serve as internal loading controls (Figure 2a, d and f). Values in Angptl2^Flx/Flx mice were set to 1 (a-c, e-g and i-g). All data are presented as means ± S.D (a: left, b, c, e, g-j) or means± SEM (a: right and f); statistical significance was determined by Student’s t-test. *p < 0.05, **p < 0.01, †p < 0.001.

**Figure 3. Angptl2 deficiency in skeletal myocytes enhances satellite cell activity.** (a) Relative transcript levels of satellite cell activation-associated genes (Cd34, Pax3, Pax7, Myf5, MyoD, and Myogenin) and skeletal muscle myosin subtypes (Myh1, Myh2, Myh4, and Myh7) in
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musculus gastrocnemius and musculus soleus of Angptl2Flox/Flox; MCK-Cre or Angptl2Flox/Flox mice (n = 4 per group). (b) Representative immunostaining and quantifications of CD34 (green) and PAX7 (red) in musculus gastrocnemius of Angptl2Flox/Flox; MCK-Cre or Angptl2Flox/Flox mice. Myofibers are co-stained with DAPI (blue) and wheat germ agglutinin (WGA; grey). Yellow arrowheads in Merge indicate CD34- and PAX7-positive cells. Red arrowheads in Merge indicate PAX7-positive cells. (Scale bar: 50µm. Six different fields are quantified per mouse, n = 3 per group) (c) Representative western blot and PAX7 quantification in musculus gastrocnemius and musculus soleus of Angptl2Flox/Flox; MCK-Cre and Angptl2Flox/Flox mice. Hsc70 serves as the internal loading control. Values in Angptl2Flox/Flox mice were set to 1 (a and c). All data are presented as means ± S.D (a and b) or means ± SEM (c); statistical significance was determined by Student’s t-test, *p < 0.05, **p < 0.01.

Figure 4. Angptl2 deficiency in skeletal myocytes prevents skeletal muscle atrophy. (a) Absolute muscle mass and body weight normalized muscle mass in musculus gastrocnemius and musculus soleus of normal Angptl2Flox/Flox, MCK-Cre or Angptl2Flox/Flox male or female mice (n = 5 per group). (b) Representative western blot and ANGPTL2 quantification in musculus gastrocnemius and musculus soleus of wild-type mice after denervation surgery (n = 4 per group). Hsc70 serves as an internal loading control. (c) Representative images of lower limbs and samples of musculus gastrocnemius and musculus soleus from sham-operated or 2-week denervated Angptl2Flox/Flox; MCK-Cre or Angptl2Flox/Flox mice. Red arrow indicates atrophied muscle. (d) Muscle mass is shown as a percentage of the day 0 value of denervated musculus gastrocnemius and musculus soleus in Angptl2Flox/Flox, MCK-Cre or Angptl2Flox/Flox mice (n = 5 per group). (e-f) Representative HE staining images (left) and cross sections of myofibers (right) in musculus gastrocnemius (e) and musculus soleus (f) of sham-operated or 2-week denervated Angptl2Flox/Flox; MCK-Cre or Angptl2Flox/Flox mice (Scale bar: 50mm. N = 5, n = 1200-1700 per group for gastrocnemius, n = 800-1000 per group for soleus). Values in sham mice were set to 1 (b). All data are presented as means ± S.D (a, e and f) or means ± SEM (b and d); statistical significance was determined by Student’s t-test (a, d, e and f) or one-way ANOVA (b), *p < 0.05, †p < 0.001.

Figure 5. Angptl2-deficient skeletal myocytes show decreased inflammation and ROS
levels and increased satellite cell activity. (a, c, d) Relative expression of *I*1-1β, *I*-6 (a), *catalase* (c), and satellite cell activation-associated (d) mRNAs in denervated musculus gastrocnemius of *Angptl2*^Flx/Flx; MCK-Cre or *Angptl2*^Flx/Flx mice (n = 5 per group). Relative mRNA expression was normalized to 18S mRNA. (b) Representative western blot of 4-HNE-adducted proteins in denervated musculus gastrocnemius of *Angptl2*^Flx/Flx; MCK-Cre or *Angptl2*^Flx/Flx mice. Asterisks indicate decreased levels of these proteins seen in *Angptl2*^Flx/Flx, MCK-Cre mice. (e) Representative western blot and PAX7 quantification 14 days after denervation in musculus gastrocnemius of *Angptl2*^Flx/Flx; MCK-Cre or *Angptl2*^Flx/Flx mice. Hsc70 and CBB (Coomassie Brilliant Blue) serve as internal loading controls (b and e). Values at day 0 of denervation in both *Angptl2*^Flx/Flx, MCK-Cre and *Angptl2*^Flx/Flx mice (a, c and d) and in *Angptl2*^Flx/Flx mice (e) were set to 1. All data are presented as means ± SEM (a, c and d) or means ± SD (e); statistical significance was determined by Student’s t-test, *p* < 0.05, **p** < 0.01.

**Figure 6. Exercise training suppresses Angptl2 expression in skeletal muscles.** a-d show *in vivo* (skeletal muscles) analysis; e-f show *in vitro* (differentiated C2C12) analysis. (a-b) Relative transcript levels of markers of senescence and pro-inflammation (a), and satellite cells (b) in musculus gastrocnemius and musculus soleus of wild-type mice, 3 hours after an endurance run (n = 7-12 per group). (c-d) Relative *Angptl2* mRNA (c) and protein (d) expression in musculus gastrocnemius and musculus soleus of wild-type C57BL/6NJcl male mice after exercise training. (Sed: sedentary; AR: post-acute run; AR3h: post-acute run plus 3h; ER3h: post-endurance run plus 3h). (n = 6-8 per group for c; n = 3-4 per group for d). (e) Relative *Angptl2* mRNA expression in differentiated C2C12 cells exposed to mechanical stretch by 5%, 10% or 15% extension with 1Hz sine wave cyclic for 15 min or 30 min (n = 4-6 per group). (f) Relative *Angptl2* mRNA (left) and protein (right) expression in differentiated C2C12 cells treated with 1mM AICAR for indicated time points (1, 6 and 12 hours) (n = 6 per group). Relative mRNA expression was normalized to 18S mRNA (a, b, c, e and f). Hsc70 serves as the internal loading control (d and f). Values in sedentary mice (a-d), in the 0 min group (e) and in the veh. group (f) were set to 1. All data are presented as means ± S.D (a-c, e and f: left) or means ± SEM (d and f: right); statistical significance was determined by Student’s t-test (a and b) or one-way ANOVA (c, d, e and f), *p* < 0.05, **p** < 0.01, †p < 0.001.
Figure 7. Model of ANGPTL2 activity in skeletal muscle. (Left) ANGPTL2 expression in skeletal myocytes increases with age. Skeletal myocyte-derived ANGPTL2 promotes inflammation and facilitates ROS accumulation by decreasing catalase expression in skeletal muscle. Increased ANGPTL2 and associated inflammation and ROS accumulation impair satellite cell activity in skeletal muscle, leading to atrophy. (Right) Exercise training decreases ANGPTL2 expression in skeletal muscle as it suppresses inflammation and ROS accumulation. These activities facilitate satellite cell activation that contribute to maintenance of muscle mass. Thus, exercise training-induced ANGPTL2 down-regulation represents a potential mechanism underlying exercise-induced protection from muscle atrophy.
Figure 1. Zhao et al.
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Figure 6. Zhao et al.
Figure 7. Zhao et al.
Age-dependent increase in angiopoietin-like protein 2 accelerates skeletal muscle loss in mice
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