Skeletal Muscle

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Decreased number of satellite cells-derived myonuclei in both fast- and slow-twitch muscles in HeyL-KO mice during voluntary running exercise

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Abstract

Background Skeletal muscles possess unique abilities known as adaptation or plasticity. When exposed to external stimuli, such as mechanical loading, both myofiber size and myonuclear number increase. Muscle stem cells, also known as muscle satellite cells (MuSCs), play vital roles in these changes. HeyL, a direct target of Notch signaling, is crucial for efficient muscle hypertrophy because it ensures MuSC proliferation in surgically overloaded muscles by inhibiting the premature differentiation. However, it remains unclear whether HeyL is essential for MuSC expansion in physiologically exercised muscles. Additionally, the influence of myofiber type on the requirement for HeyL in MuSCs within exercised muscles remains unclear.

Methods We used a voluntary wheel running model and HeyL-knockout mice to investigate the impact of HeyL deficiency on MuSC-derived myonuclei, MuSC behavior, muscle weight, myofiber size, and myofiber type in the running mice.

Results The number of new MuSC-derived myonuclei was significantly lower in both slow-twitch soleus and fast-twitch plantaris muscles from exercised HeyL-knockout mice than in control mice. However, expect for the frequency of Type IIb myofiber in plantaris muscle, exercised HeyL-knockout mice exhibited similar responses to control mice regarding myofiber size and type.

Conclusions HeyL expression is crucial for MuSC expansion during physiological exercise in both slow and fast muscles. The frequency of Type IIb myofiber in plantaris muscle of HeyL-knockout mice was not significantly reduced compared to that of control mice. However, the absence of HeyL did not affect the increased size and frequency of Type IIa myofiber in plantaris muscles. In this model, no detectable changes in myofiber size or type were observed in the soleus muscles of either control or HeyL-knockout mice. These findings imply that the requirement for MuSCs

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in the wheel-running model is difficult to observe due to the relatively low degree of hypertrophy compared to surgically overloaded models.

Keywords Muscle satellite cells, Wheel running, Hypertrophy, HeyL

Background

Skeletal muscles have a distinctive ability to adapt to internal and external physiological changes, known as plasticity. Skeletal muscles may weaken and decrease in size due to various diseases or disuses, a phenomenon termed as muscle atrophy. Conversely, increased mechanical loading triggers the remodeling of skeletal muscles, resulting in enhanced strength and mass, a process known as muscle hypertrophy [1]. Muscle-resident stem cells called muscle satellite cells (MuSCs) [2] respond to increased mechanical loads, proliferate, and subsequently supply new myonuclei to myofibers [3]. This process is vital for load-dependent muscle hypertrophy in resistance-training models [4–6].

Surgical overload models (resistance training models) have been widely used to analyze the contribution of MuSCs to myofibers in loaded muscles. These models have contributed to revealing mechanisms underlying MuSC proliferation, differentiation, and fusion [5, 7–10]. Myomaker, a fusogenic protein, is essential for MuSCs to fuse with myofibers in surgically loaded and regenerated muscles [5]. The overloaded models also shed light on the critical roles of macrophages and mesenchymal progenitors (FAPs) in MuSC proliferation and differentiation [7, 10, 11]. Furthermore, our previous study using a surgical overloaded model demonstrated that sustained expression of hairy/enhancer-of-split related to the YRPW motif-like protein (HeyL) is necessary for MuSC proliferation by suppressing MyoD expression in proliferating MuSCs [12]. HeyL, along with Hey1 and Hey2, belongs to the Hey family, which are direct targets of the canonical Notch signaling pathway, similar to the Hes family genes [13]. *Hes1*, *Hey1*, and *HeyL* are canonical and essential effector genes for the Notch-dependent inhibition of myogenic differentiation, including MyoD expression [14–18]. Although *Hey1/HeyL* double mutant mice show marked defects in skeletal muscle development and MuSC numbers, neither *Hey1* nor *HeyL* single knockout mice exhibit substantial abnormalities [14]. However, *HeyL*-knockout mice display blunted muscle hypertrophy due to the premature differentiation of proliferating MuSCs in overloaded muscles [12, 19]. Whether similar mechanisms apply to MuSC behavior in slow-type muscles, as observed in fast-twitch muscles in our previous studies, and other physiological models, such as the voluntary wheel-running model, remain unclear.

Voluntary wheel running is a well-established model for endurance training. Plantar flexor muscles, including the soleus, plantaris, and gastrocnemius muscles, exhibit high responsiveness and adaptability to wheel running [20, 21]. In some cases, the addition of weight may be necessary to induce substantial muscle hypertrophy. However, Masschelein et al. observed an increased number of myonuclei contributed by MuSCs in mice running on a commercially available, non-weighted wheel [20]. In this study, using commercially available equipment, we aimed to investigate the role of HeyL in MuSC behavior in both slow and fast muscles of mice undergoing voluntary wheel running.

Methods

Mice

C57BL/6J mice, 8–12 weeks of age, were purchased from Charles River Laboratories (Yokohama, Kanagawa, Japan). Dr. Kokubo generated *HeyL*-knockout (KO) mice as previously described [14]. All mice were housed in a controlled environment, at a maintained temperature of 24±2 °C, humidity at 50% ± 10%, and subjected to a 12/12-h light/dark cycle. Sterilized standard chow (DC-8; Nihon Clean, Tokyo, Japan) and water were provided ad libitum.

Wheel running

The mice were housed individually in cages measuring 225 mm (width) × 345 mm (depth) × 210 mm (height). The cages were equipped with running wheels measuring 140 mm in diameter (MELQUEST, Toyama, Japan), and the mice were allowed to run voluntarily within the cages for a specified duration. The number of rotations was recorded using a CNT-10 (MELQUEST). Mice in the Non-exercise group were housed individually without access to the running wheels.

In vivo EdU labeling and detection

EdU (5- ethynyl −2′-deoxyuridine; Thermo Fisher Scientific, #A10044) was dissolved in sterilized PBS at 10 mg/mL and stored at -20 °C. Alzet mini-osmotic pumps (Model 2002, Durect, Cupertino, CA, http://www.durect.com/) [22] were used to continuously administer EdU during wheel running or home cage control for 14 days. The pumps were filled with 200 µL of EdU solved in PBS (10 mg/mL), thus giving a delivery rate of 0.5 µL/h (120 µg EdU/day). EdU^+ nuclei were detected using Click-iT™ EdU Cell Proliferation Kit for Imaging, Alexa Fluor™ 647 dye (Thermo Fisher Scientific, #C10340). To identify EdU^+ myonuclei, dystrophin staining (Abcam, #AB15277) was performed. Fluorescence was recorded using a BZ-X700 fluorescence microscope (Keyence;

Osaka, Japan), and cell size was quantified using Hybrid Cell Count software (Keyence).

Muscle fixation

The isolated soleus and plantaris muscles were carefully positioned on the cork using kneaded Tragacanth Gum (Wako Pure Chemicals Industries, Osaka, Japan). Subsequently, the muscles were rapidly frozen by immersion in isopentane (Wako Pure Chemical Industries), which was pre-cooled with liquid nitrogen for 1 min. The muscles were then transferred onto dry ice and allowed to remain for 1 h to facilitate isopentane vaporization. Finally, the frozen muscles were stored in sealed containers at -80 °C.

Immunohistochemistry

Transverse cryosections (6 μm thick) were cut using a cryostat (Leica CM1860; Nussloch, Germany). After drying at room temperature for 30 min, the sections were fixed with 4% paraformaldehyde (PFA) for 10 min and washed with PBS in a coupling jar. For fiber type stainings, the sections were fixed with −20°C acetone for 10 min. After blocking with 5% skim milk/phosphate-buffered saline (PBS), the sections were reacted with primary antibodies, anti-Laminin α2 (Enzo, RRID: AB_2051764) and Dystrophin (Abcam, RRID: AB_301813) overnight at 4°C. For myofiber type analyses, SC-71 (Mouse Myosin heavy chain Type-IIa, RRID: AB 2147165), BF-F3 (Mouse Myosin heavy chain Type-IIb, RRID: AB_2266724), and BA-D5 (Mouse Myosin heavy chain Type-I, RRID: AB_2235587) were purchased from Developmental Studies Hybridoma Bank (Iowa, IA, USA). The next day, the sections were washed three times with PBS and incubated with secondary antibodies. Signals were photographed using a BZ-X700 fluorescence microscope (Keyence). For all analyses, sections were obtained from the middle third of the plantaris or soleus muscles from control or KO mice. For the quantification of EdU+myonuclei or $Pax7^+$ cells, the average number of EdU^+ myonuclei or $Pax7^+$ cells from two sections was used.

Measurement of CSA

Cross sections of soleus and plantaris muscles were stained with anti-Laminin α2 antibody, and fluorescence images of entire cross sections were recorded using BZ-X700 fluorescence microscope. For the analyses of Type-I and Type-IIa in soleus muscles and Type-IIb in plantaris muscles, three to four fields per sample were randomly selected to calculate the cross-sectional area. For the analyses of Type-IIa in plantaris muscles, all fibers were analyzed to calculate the cross-sectional area.

Single myofiber staining

Following a previously established protocol [23], single myofibers were isolated from the plantaris muscles of wild-type and *HeyL*-KO mice. The isolated myofibers were immediately fixed in 4% PFA/PBS solution and washed with PBS containing 0.1% Triton-X. To permeabilize the myofibers, they were treated with a solution containing 20 mM HEPES, 300 mM sucrose, 50 mM NaCl, 3 mM $MgCl_2$, and 0.5% Triton X-100. After washing with PBS, the myofibers were treated with PBS containing 5% FCS and 0.01% Triton-X and reacted with anti-Pax7 (DSHB), MyoD (Abcam, RRID: AB_2890928), and Ki67 (Thermo Fisher Scientific, RRID: AB_10854564) antibodies overnight at 4°C. The following day, the myofibers were washed and incubated with secondary antibodies for 1 h. After washing and encapsulation, the stained myofibers were observed under a BZ-X700 fluorescence microscope (Keyence).

Statistics

Values were expressed as means±standard deviation (S.D.). A two-sided unpaired Student's t-test was used to conduct statistical comparisons between two groups. For comparisons involving more than two groups, one-way analysis of variance (ANOVA) followed by the Tukey–Kramer test was employed. All statistical analyses were performed using the Prism 8 software. A p-value less than 0.05 was considered statistically. Additionally, Prism 10 software was used to generate all graphs.

Results

Decreased number of myonuclei derived from muscle satellite cells in exercised *HeyL*-KO mice

Voluntary wheel running is an exercise in which rodents engage in spontaneous running, driven by internal motivation, effectively loading their plantar flexor muscles. HeyL expression is specific to MuSCs in skeletal muscle [14, 24]. Because Hey1 compensates the function of HeyL, *HeyL*-KO did not exhibit a significant difference in MuSC number and EdU-uptake [12, 14, 15, 25]. However, *HeyL*-KO exhibited the decrease in MuSC proliferation in the surgically overloaded plantaris muscle [12]. To explore the role of HeyL in MuSC behavior in slow- and fast-twitch muscles of wheel-running mice, we analyzed two plantar flexor muscles, the soleus and plantaris muscles, which are representative slow- and fast-twitch muscles, respectively. Consistent with our previous study, we confirmed that MuSC number in the soleus or plantaris muscles of non-exercised *HeyL*-KO (KO: $HeyL^{-/-}$) was comparable to that observed in non-exercised littermate controls (wild-type [WT]: $HeyL^{+/+}$)(Fig. 1A). Then, *HeyL*-KO and littermate control mice were allowed free access to the wheel equipment for voluntary running (Fig. 1B). Two weeks after exercise, the total rotation number of

HeyL-KO mice was comparable to that of control mice (Fig. 1C), indicating that the physical activity of *HeyL*-KO mice was not influenced by the loss of *HeyL*.

Using this model, we counted the number of MuSC-derived myonuclei in soleus and plantaris muscles by labeling MuSCs with EdU during exercise (Fig. 1B). The number of newly formed EdU-positive myonuclei in both the soleus and plantaris muscles was significantly reduced in *HeyL*-KO mice compared with controls (Fig. 1D and E). The number of EdU-positive MuSCs were also reduced in both the soleus and plantaris muscles from *HeyL*-KO mice (Fig. 1F and G). These findings demonstrate that HeyL plays a critical role in MuSCs in exercised muscles subjected to exercise, similar to observations in surgically overloaded muscles [12]. Furthermore, these results illustrated that HeyL is essential for MuSCs in both fast- and slow-twitch myofibers.

Increased number of MyoD-positive proliferating MuSCs in *HeyL*-KO mice

We previously reported that MuSCs proliferate without significant MyoD expression in surgically overloaded muscles [12, 26]. HeyL is involved in the suppression of MyoD, as the loss of HeyL leads to increased MyoD expression in proliferating MuSCs, eventually resulting in a reduced number of new MuSC-derived myonuclei and biased myogenic differentiation, rather than proliferation [12]. Therefore, the same mechanism was observed for the reduced number of EdU^+ myonuclei in running-exercise *HeyL*-KO mice (Fig. 1E). To verify this, we isolated single myofibers from the plantaris muscles of exercised control and *HeyL*-KO mice and stained them with anti-MyoD and Pax7 antibodies (Fig. 2A). Because the extent of exercise affected the results, we analyzed mice that exercised more than 50,000 wheel-rotations for 7 days (Fig. 2B). As expected, the number of $MyoD^+$ MuSCs was significantly higher in *HeyL*-KO mice than in control mice (Fig. 2C–E). Further, MuSC number on single myofiber from *HeyL*-KO mice was significantly lower than that of control mice (Fig. 2F). While, the frequency of $Ki67^+$ activated/proliferating cells was not significantly different between control and *HeyL*-KO mice (Fig. 2G). These findings suggest that HeyL does not directly influence the proliferation of MuSCs; rather, it indirectly affects MuSC expansion in exercised muscles by inhibiting MyoD-dependent premature differentiation. Our previous study demonstrated that untrained/sedentary *HeyL*-KO mice exhibited a modestly higher percentage of MyoD-positive MuSCs compared to wild-type (WT) mice (2.4% vs. 12.4%) [14]. In exercised mice, the frequency of MyoD-positive cells increased dramatically, reaching approximately 60% in $Ki67^+$ MuSCs. This finding suggests that HeyL is necessary to suppress muscle differentiation during the exercise-induced proliferation of MuSCs, a phenomenon also observed in overloaded muscle [12].

Increase in muscle weight by voluntary wheel running in *HeyL*-KO mice

To investigate the impact of HeyL deficiency on muscle hypertrophy, male *HeyL*-KO and littermate control (WT) mice were randomly assigned to two groups. One group had free access to the wheel equipment and ran voluntarily for 4 weeks, while the other remained in cages without the wheel equipment (Fig. 3A). Consistent with the results observed in female mice after two weeks of exercise (Fig. 1C), the total number of rotations over four weeks in male *HeyL*-KO mice was comparable to that in control mice (Fig. 3B). After euthanasia, muscle weights of these exercised (+Ex) or non-exercised (-Ex) mice were analyzed. In control mice, the plantaris and soleus muscle weights per body weight significantly increased after 4 weeks of voluntary running compared to those in the (-Ex) group (Fig. 3C). Although there was no significant difference in the plantaris muscle between (-Ex) and (+Ex) *HeyL*-KO mice, the soleus muscle weight per body weight was significantly increased in *HeyL*-KO mice, similar to the observations in WT mice (Fig. 3C). Mechanical loading in the EDL muscles did not increase with this voluntary wheel-running exercise [20], therefore, EDL muscle weights remained unchanged by exercise in both *HeyL*-KO and control mice (Fig. 3C).

Size or type of myofiber in exercised *HeyL*-KO mice

Wheel running exercise induces a transition in myofiber types from fast-to slow-twitch fibers [27]. Therefore, we analyzed the myofiber type composition along with myofiber size (CSA: cross-sectional area) of the plantaris and soleus muscles of control and KO mice in Fig. 3 (Fig. 4A). In the plantaris muscles, the CSA of Type IIa increased or tended to increase in both control and KO mice after the running exercise, whereas the CSA of Type IIb remained unchanged by exercise (Fig. 4B). Concerning myofiber-type composition, the proportion of Type IIa increased, whereas that of Type IIb decreased in WT mice (Fig. 4C), which was consistent with published data [27]. In KO mice, the proportion of Type IIa was increased, but that of Type IIb was not significantly decreased (Fig. 4C). In the soleus muscles, no significant differences were observed in myofiber type and size between the control and KO mice (Fig. 4D and E). These results suggest that the absence of HeyL did not dramatically impact myofiber sizes and types of plantaris and soleus muscles in exercised mice.

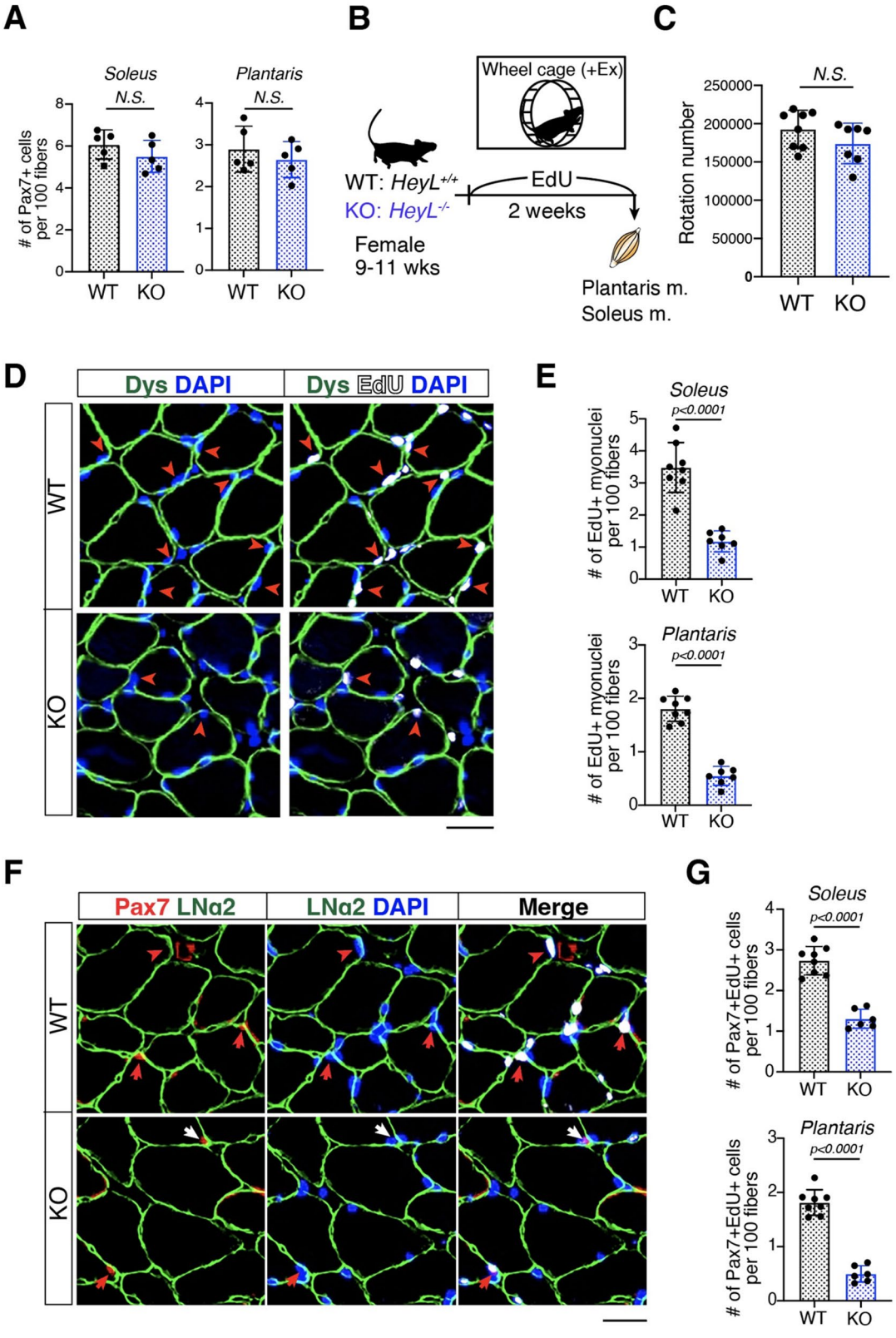

Fig. 1 (See legend on next page.)

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Fig. 1 Reduced number of myonuclei derived from muscle satellite cells in exercised *HeyL*-KO mice. (**A**) Number of $Pax7^+$ cells per 100 myofibers in sections of soleus or plantaris muscles from male non-exercised WT ($HeyL^{+/+}$, $n=5$) or KO ($HeyL^{-/-}$, $n=5$). (**B**) Experimental design for analyzing the effect of a 2-week wheel running exercise on the number of MuSC-derived myonuclei. Littermate control (WT: $HeyL^{+/+}$) or *HeyL*-KO (KO: $HeyL^{-/-}$) mice were randomly separated into two groups; home cage (-Ex: non-exercise group) or wheel running cage (+Ex: exercise group). EdU was administrated through osmotic pumps during 2-week running. (**C**) Number of wheel rotations during 2 weeks in WT ($n=8$) or KO ($n=7$) mice. (**D**) Immunostaining of dystrophin (green) and EdU (white) in soleus muscle 2 weeks after wheel running. Arrowheads indicate EdU^+ myonuclei. Scale bar, 25 μm. (**E**) Number of EdU^+ myonuclei per 100 myofibers in sections of soleus or plantaris muscle from exercised WT ($n=8$) or KO ($n=7$) mice. (**F**) Immunostaining of Pax7 (red), Laminin α2 (LNα2, green), and EdU (white) in soleus muscle 2 weeks after wheel running. Arrows indicate $Pax7^+EdU^+$ cells. Red arrows or arrowheads indicate $Pax7^+EdU^+$ cells or EdU^+ myonuclei, respectively. White arrow indicates $Pax7^+EdU^-$ cell. Scale bar, 25 μm. (**G**) Number of $Pax7^+EdU^+$ cells per 100 myofibers in sections of soleus or plantaris muscle from exercised WT ($n=8$) or KO ($n=6$) mice

Discussion

In this study, we found that proliferating MuSCs in exercising mice required HeyL to suppress MyoD expression, similar to observations in surgically overloaded *HeyL*-KO mice [12]. Surgically overloaded models have been employed for resistance training, whereas wheel-running has been utilized for endurance training. Each training type elicited a distinct response. Endurance training diminishes the activity of glycolytic enzymes but enhances oxidative enzyme activity and mitochondrial density, leading to a shift from fast-type to slow-type myofibers [28]. In contrast, resistance training decreases mitochondrial density and has a minimal effect on enzymatic activity [28]. Therefore, endurance training facilitates aerobic processes, whereas resistance training increases anaerobic responses to muscle strength. Accordingly, the present study demonstrated that the requirement of HeyL for MuSC proliferation in exercised muscles is similar in endurance and resistance training, despite inducing distinct biological responses in myofibers.

MuSCs have properties distinct from those of slow- and fast-twitch myofibers [29]. Motohashi et al. reported that MuSCs derived from the soleus exhibited higher self-renewal and lower differentiation abilities than those derived from the EDL [29]. The present study demonstrated reduced myonuclear accretion in both fast- and slow-twitch muscles in *HeyL*-KO mice, suggesting that the requirement for HeyL and the proliferation mechanism of MuSCs are likely to be common in both muscle types, fast- and slow-switch muscles. Notably, de Lima et al. demonstrated that HeyL suppresses myomaker expression during muscle development [30]. Given that the myomaker is essential for myogenesis in both slow and fast myofibers, it is not surprising that HeyL is also critical for MuSC proliferation in both types of exercised myofibers. In contrast to Myomaker-mutant mice, *HeyL*-KO mice did not exhibit any developmental defects, suggesting the presence of a compensatory pathway that mitigates the loss of HeyL during development. The precise mechanism remains unidentified; however, Hey1, which plays a redundant role in maintaining quiescence in MuSCs, is a potential candidate involved in developmental processes. Among the Hes and Hey family genes, *HeyL* transcripts are highly expressed in MuSCs and are the most sensitive to induction by Notch ligands in mice [14]. Hes1 and Hey1 are predominantly expressed and induced by Notch ligands [31]. Although HeyL primarily functions in loaded murine MuSCs, other Hes and Hey genes may play similar roles in loaded human MuSCs.

The requirement for MuSCs and MuSC-derived myonuclei has been highly debated. In surgically overloaded muscles, blunted muscle hypertrophy has been observed in mice with defective MuSC proliferation and fusion, at least in long-term designed experiments [6]. Our group also demonstrated low efficiency of muscle hypertrophy in *HeyL*-null mice [12]. The present data demonstrated that the requirement of HeyL for the increased number of MuSC-derived myonuclei in our wheel-running model. However, we could not observe the effect associated with a reduction in newly formed myonuclei in this model. Compared to the surgically overloaded models, the increased ratios of muscle weight and myofiber size in running mice were very mild. The increase in the number of MuSC-derived myonuclei in running mice was lower than that in surgically overloaded mice. In addition, owing to the decreased motivation of mice, the running distance was decreased in the wheel-running exercise [20], suggesting that loading to the muscle is also decreased during long-term exercise. Considering the blunted muscle hypertrophy in MuSC-depleted or fusion-defective mice using other running models [32, 33], our model seems insufficient to observe the effect of the reduced number of MuSC-derived myonuclei on the efficiency of muscle hypertrophy. Thus, there may be a threshold for the rate of hypertrophy required to detect the effects of additional MuSC-derived myonuclei.

Conclusions

HeyL is required for MuSC expansion and increases the myonuclei number by suppressing MyoD expression in a wheel-running model similar to the surgical overload model.

Moreover, our current findings illustrate that HeyL is essential for MuSC proliferation in both fast- and slow-twitch muscles. However, the absence of HeyL did not affect myofiber size or type in the wheel-running model.

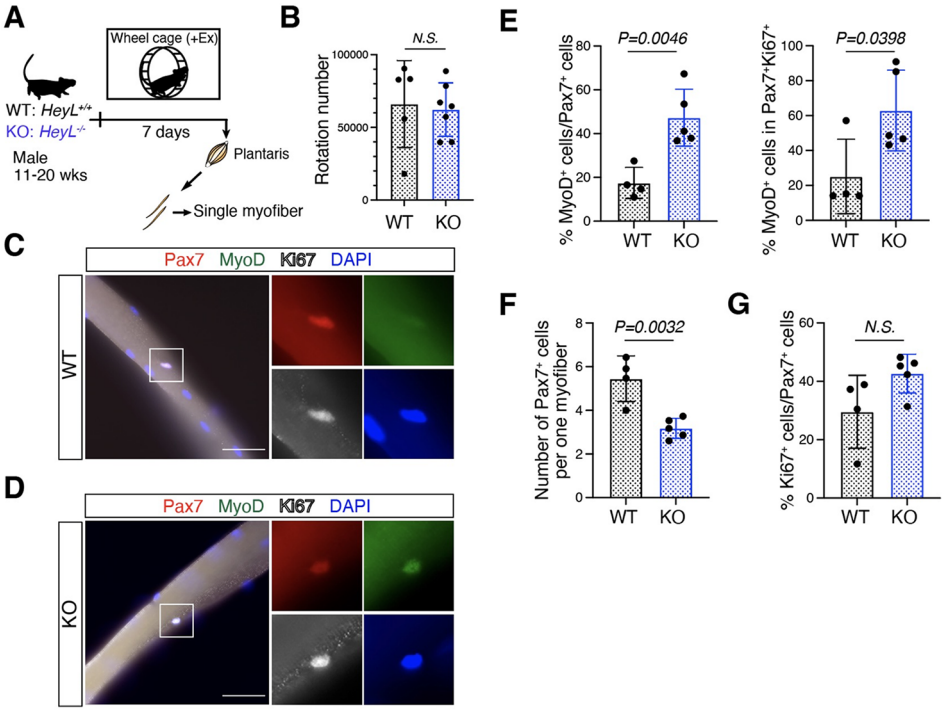

Fig. 2 Increased MyoD expression in proliferating *HeyL*-KO MuSCs. (**A**) Experimental scheme for analyzing the effect of *HeyL*-KO on MyoD expression in MuSCs on isolated myofibers from plantaris muscles 7 days after voluntary wheel running. (**B**) Number of wheel rotations during 7 days in WT ($n=5$) or KO ($n=7$) mice. (**C** and **D**) Immunostaining of Pax7 (red), MyoD (green), and Ki67 (white) in MuSCs on isolated myofibers at 7 days after wheel running from WT (**C**) or KO (**D**) mice. Nuclei were counterstained with DAPI. Scale bar, 100 μm. (**E**) Percentage of $MyoD^+$ cells in $Pax7^+$ cells (left) or $Pax7^+Ki67^+$ (right) cells on isolated myofibers from exercised WT ($n=4$) or KO ($n=5$) mice. (**F**) Number of $Pax7^+$ cells on isolated myofibers from exercised WT ($n=4$) or KO ($n=5$) mice. (**G**) Percentage of $Ki67^+$ cells in $Pax7^+$ cells on isolated myofibers from exercised WT ($n=4$) or KO ($n=5$) mice

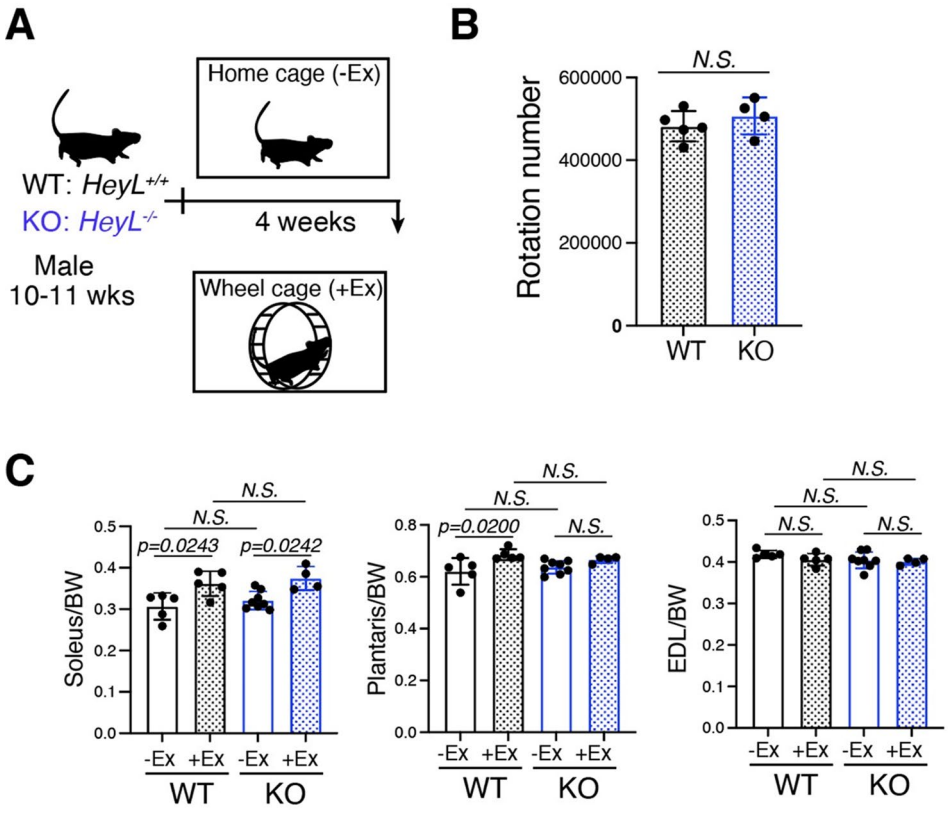

Fig. 3 Muscle response to exercise in *HeyL*-KO mice. (**A**) Experimental scheme for analyzing the effect of 4-week wheel running on muscle weight and myofiber types. Littermate male control (WT: $HeyL^{+/+}$) or *HeyL*-KO (KO: $HeyL^{-/-}$) mice were randomly separated into two groups; home cage (-Ex: non-exercise group) or wheel running cage (+ Ex: exercise group). (**B**) Number of wheel rotations during 4 weeks in WT ($n = 5$) or KO ($n = 4$) mice.(**C**) Normalized muscle weight [muscle weight/body weight (BW)] from WT or KO mice. N.S.: not significant

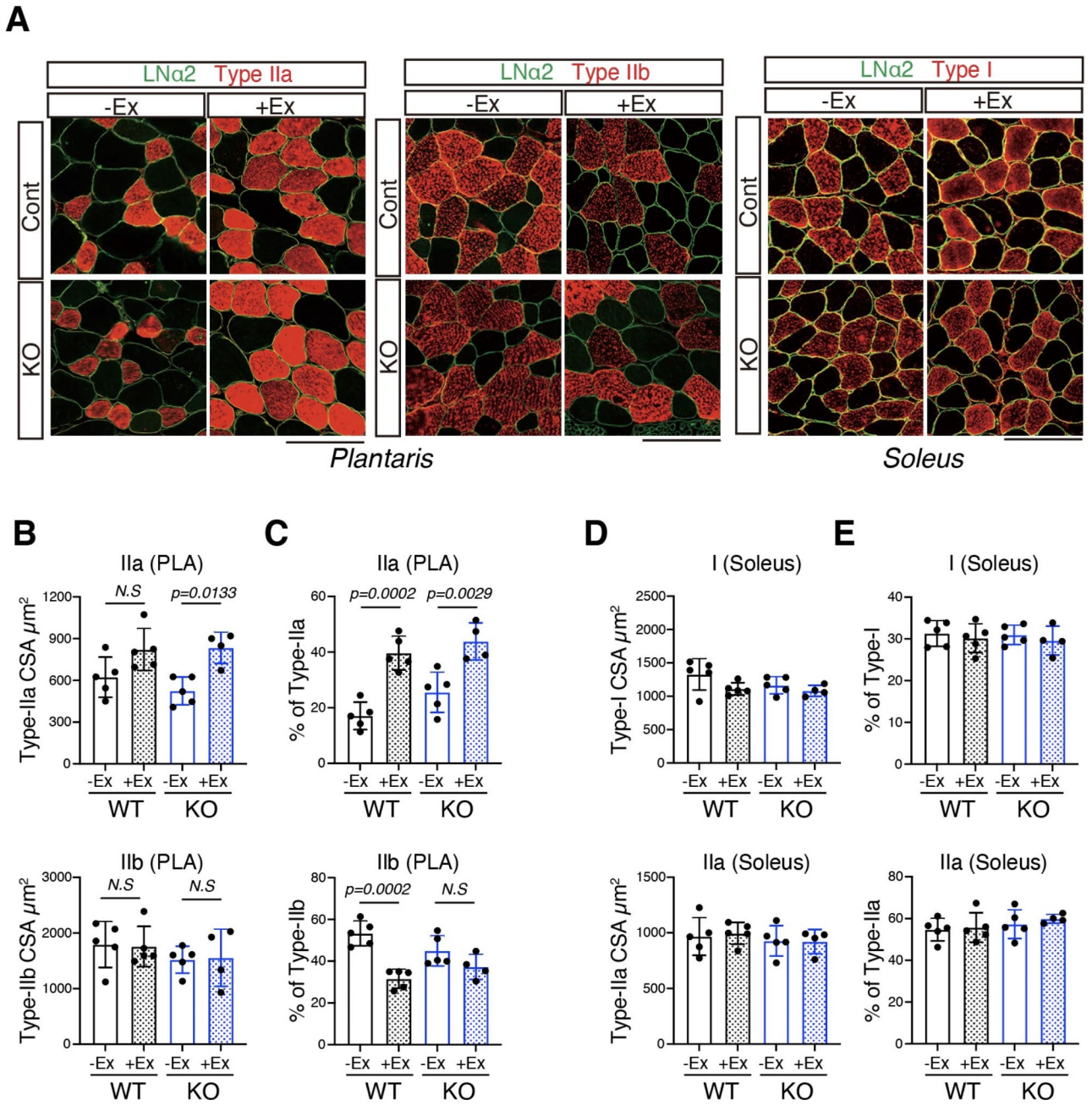

Fig. 4 Myofiber size and types in running *HeyL*-KO mice. (**A**) Representative images of staining for Type-IIa (plantaris), Type-IIb (plantaris), and Type-I (soleus) with anti-laminin staining (green) in male WT or KO mice. Scale bar: 100 μm. (**B**) Cross sectional area (CSA) of Type-IIa or -IIb myofibers in plantaris muscle (PLA) from male WT (-Ex; $n=5$, +Ex; $n=5$) or KO (-Ex; $n=5$, +Ex; $n=4$) mice. (**C**) The frequency of Type-IIa or -IIb myofibers in plantaris (PLA) muscle from male WT (-Ex; $n=5$, +Ex; $n=5$) or KO (-Ex; $n=5$, +Ex; $n=4$) mice. (**D**) CSA of Type-I or -IIa myofibers in soleus muscle from male WT (-Ex; $n=5$, +Ex; $n=5$) or KO (-Ex; $n=5$, +Ex; $n=4$) mice. (**E**) The frequency of Type-I or -IIa myofibers in soleus muscle from male WT (-Ex; $n=5$, +Ex; $n=5$) or KO (-Ex; $n=5$, +Ex; $n=4$) mice

Abbreviations

CSA	cross-sectional area
EdU	5-ethynyl-2′-deoxyuridine
Ex	exercise
FAP	Fibro/adipogenic progenitor
HeyL	hairy/enhancer-of-split related to YRPW motif-like
KO	knockout
MuSC	muscle satellite cell
MyoD	myogenic differentiation 1
Pax7	paired box 7
PFA	paraformaldehyde
WT	wild-type

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Author contributions
KI, MK, LZ, and KK. performed the experiments and analyzed the data. HK provided the critical materials (HeyL-KO mice) for this study. TK and SF designed wheel-running experiments. SF designed the experiments, interpreted data, assembled input data, and wrote the manuscript. AK supported KI, and KK performed the experiments and wrote the manuscript. All authors discussed the results and implications, and commented on the manuscript. All the authors have read and approved the final version of the manuscript.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval
All animal experiments were performed in accordance with guidelines approved by the Experimental Animal Care and Use Committee of Osaka University (approval number: 30 – 15, R02-1-10).

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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