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ORIGINAL ARTICLE

Insulin-like growth factor-I is required to maintain muscle volume in adult mice

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Abstract

Insulin-like growth factor-I (IGF-I) is a peptide with diverse functions, among them regulation of embryonic development and bone homeostasis. Serum IGF-I levels decline in the elderly; however, IGF-I function in adults has not been clearly defined. Here, we show that IGF-I is required to maintain muscle mass in adults. We crossed *Igf-I* flox'd and *Mx1* Cre mice to yield *Mx1 Cre/Igf-I*$^{flox/flox}$ (IGF-I cKO) mice, and deleted *Igf-I* in adult mice by polyIpolyC injection. We demonstrate that, although serum IGF-I levels significantly decreased after polyIpolyC injection relative to (*Igf-I*$^{flox/flox}$) controls, serum glucose levels were unchanged. However, muscle mass decreased significantly after IGF-I down-regulation, while bone mass remained the same. In IGF-I cKO muscle, expression of anabolic factors such as *Eif4e* and *p70S6K* significantly decreased, while expression of catabolic factors *MuRF1* and *Atrogin-1* was normal and down-regulated, respectively, suggesting that observed muscle mass reduction was due to perturbed muscle metabolism. Our data demonstrate a specific role for IGF-I in maintaining muscle homeostasis in adults.

Keywords Insulin-like growth factor-I · Aging · Muscle wasting · Adult

Introduction

Insulin-like growth factor-I (IGF-I) is a small peptide mainly produced by liver and expressed in various organs [1]. IGF-I is first produced as 158 amino acid pro-peptide and then cleaved to 70 amino acids to become an active peptide. IGF-I is structurally similar to insulin and IGF-II, but insulin and IGF-II show high affinity for the insulin receptor and IGF-II receptor, respectively, while IGF-I binds to IGF-I receptor (IGFIR) with high affinity to transduce signals. IGF-I signaling is also regulated by IGF-binding proteins (IGFBPs) such as IGFBP3 [2].

IGF-I-deficient mice were reportedly exhibited growth retardation, but were otherwise normal, suggesting that IGF-I is required for the early development [3]. IGF-II expression is high in various tissues embryonically but is down-regulated after birth. IGF-II-deficient mice were demonstrated to show growth retardation phenotypes similar to IGF-I-deficient mice [4]. Insulin is encoded by two genes *Ins1* and *Ins2*. Either insulin-deficient or *Ins1*/*Ins2* doubly deficient mice show growth retardation but no sign of glycosuria at birth, but soon develop diabetes mellitus with

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ketoacidosis and die within 48 h [5]. These results suggest that IGF-I and IGF-II are required for embryonic development, while insulin regulates both embryonic development and glucose metabolism.

In adult mice, IGF-I expression is reportedly required to regulate bone and muscle homeostasis [6]. Bone homeostasis is regulated by a balance between osteoclastic bone resorption and osteoblastic bone formation, and these activities are coupled with each other [7]. IGF-I resides in bone matrix, and bone resorption by osteoclasts reportedly promotes IGF-I release from matrix, activating mesenchymal stem cell migration to produce osteoblastic new bone [8]. Osteoblast-specific deletion of the *Igf1r* gene mediated by either *Osterix* Cre or *ColIa2.3* Cre reportedly results in reduced bone mass [8, 9]. Moreover, conditional *Igf-I* deletion in *type1alpha2 collagen* Cre mice alters bone formation and promotes postnatal lethality [10]. Meanwhile, osteocyte-specific *Igf-I* deletion seen in *DMP1* Cre mice accelerates bony union of the fracture gap [11].

The IGF-I/IGFIR axis is also crucial for muscle homeostasis [12]. IGF-I signaling activates anabolic signals and inhibits catabolic signals. IGF-I stimulates MAPK and AKT signals to activate protein synthesis via Eif4e and p70S6K [13]. IGF-I also inhibits muscle atrophy by inactivating expression of MuRF1 and Atrogin-1, both of which are E3 ligases targeting muscle-specific proteins such as MyoD [14, 15]. Smad2 and Smad3 reportedly accumulate in atrophic immobilized muscle and are required for immobilization-induced muscle atrophy [16], while IGF-I inhibits Smad2 and Smad3 protein accumulation via AKT and ERK pathways [16]. Furthermore, IGF-I overexpression promotes increased muscle volume in mice [17].

In humans, low IGF-I levels are associated with various diseases such as stroke [18]. Moreover, relatively high IGF-I levels have been shown to promote tumorigenesis [19]. Serum IGF-I levels are known to decline in the elderly, and the impact of that decrease remains unknown. Here, we established a low IGF-I model in adult mice and demonstrated that lowering circulating IGF-I levels decreased muscle mass. Our data may shed light on the physiological role of IGF-I in regulating muscle homeostasis in adults.

Materials and methods

Mice

IGF-I flox mice (The Jackson Laboratory, Bar Harbor, ME) were crossed with *Mx1 Cre* transgenic mice to yield *Mx1 Cre/Igf-I*$^{flox/flox}$ mice (IGF-I cKO). *Igf-I*$^{flox/flox}$ mice served as controls. Wild-type (WT) mice were purchased from The Jackson Laboratory.

Animals were maintained under specific pathogen-free conditions in animal facilities certified by the Keio University Animal Care Committee. All mice were kept under a 12-h light/dark cycle and were fed standard diets. Animal protocols were also approved by the committee. All animal experiments were carried out in accordance with the Guidelines of the Keio University animal care committee.

polyIpolyC treatment

Eight-week-old female IGF-I cKO and control mice were injected with 250 μg of polyIpolyC (P0913, Sigma-Aldrich Co., St Louis, MO) intraperitoneally for 3 days, and once 4 weeks later. Mice were sacrificed 10 weeks after the first polyIpolyC injection, when mice were 18 weeks old.

Grip power

Tails of mouse that holding a metal grid with forearm paws were pulled backwards horizontally until the mice could no longer hold the grid, and grip power was measured as the pulling power for releasing that wire 10 times in each mouse by MK380Si (Muromachi Kikai Co., Tokyo, Japan).

Serum IGF-I measurement

Serum samples were collected from 8-week-old IGF-I cKO and control mice before and after 10 weeks of polyIpolyC treatment. Serum IGF-I levels were measured by ELISA (MG100, R&D Systems, Minneapolis, MN) using a multiple plate analyzer (POWERSCAN HT, DS Pharma Biomedical, Osaka, Japan).

Histology

Excised muscle was embedded in paraffin, cut into 4-μm sections and stained with hematoxylin and eosin. Fiber cross-sectional area (CSA) was measured in gastrocnemius and quadriceps muscle sections using BioRevo (Keyence, Osaka, Japan). To calculate the CSA, 33–86 and 75–108 myofibers per mouse were examined in control and IGF-I cKO mice, respectively.

Real-time PCR analysis

Total RNA was isolated from gastrocnemius muscle using TRI Reagent (TR118, Molecular Research Center, Inc., Cincinnati, OH) and an RNeasy Mini Kit (74106, QIAGEN, Hamburg, Germany). cDNA was synthesized by reverse transcription with an Advantage® RT-for-PCR Kit (Takara Bio Inc., Otsu, Shiga, Japan). Real-time PCR was performed using SYBR Premix ExTaq II (Takara Bio Inc.) with a DICE

thermal cycler (Takara Bio Inc.). β-actin expression was analyzed as an internal control.

Primer sequences for real-time PCR were as follows:

β-actin forward: 5′-TGAGAGGGAAATCGTGCGTGAC-3′
β-actin reverse: 5′-AAGAAGGAAGGCTGGAAAAGAG-3′
Atrogin-1 forward: 5′-GAGACCATTCTACACTGGCAGCA-3′
Atrogin-1 reverse: 5′-GTCACTCAGCCTCTGCATGATGT-3′
MuRF1 forward: 5′-ACCTGCTGGTGGAAAACATCATT-3′
MuRF1 reverse: 5′-AGGAGCAAGTAGGCACCTCACAC-3′
Eif4e forward: 5′-CAAGCAAACCTTCGATTGATCTCT-3′
EIf4e reverse: 5′-ATAGGCTCAATCCCGTCCTTAAAA-3′
p70S6K forward: 5′-TGGGGCATTTACATCAAAAAGG-3′
p70S6K reverse: 5′-AATGTGTGCGTGACTGTTCCAT-3′.

Western blot analysis

Lysates were obtained from frozen minced gastrocnemius and quadriceps muscle using RIPA buffer [1% Tween 20, 0.1% SDS, 150 mM NaCl, 10 mM Tris–HCl (pH 7.4), 0.25 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM Na3VO4, and 5 mM NaF (Sigma)]. 30 µg of proteins were loaded onto 12.5% SDS–PAGE (e-PAGEL, ATTO Corporation, Tokyo, Japan), and were transferred to PolyVinylidene DiFluoride (PVDF) membranes (Immobilon, Merck KGaA, Darmstadt, Germany). The membranes were blocked with a buffer-containing 10 mM Tris–HCl (pH 7.4), 150 mM NaCl, 0.1% Tween 20, and 5% skim milk or bovine serum albumin, and were incubated with each primary antibody overnight at 4 °C. Then, membranes were incubated by the appropriate secondary antibodies, and the immune complexes were visualized using ECL Western Blotting Analysis System (GE Healthcare, Tokyo, Japan).

Primary antibodies used to detect proteins were: anti-phospho-Smad2 (3101, Cell Signaling Technology, Inc., Beverly, MA), anti-phospho-Smad3 (9520, Cell Signaling), anti-Smad2/3 (3102, Cell Signaling), anti-phospho Akt (4051, Cell Signaling), anti-Akt (9272, Cell Signaling), anti-phospho Erk1/2 (9106, Cell Signaling), anti-Erk1/2 (9102, Cell Signaling), and anti-Actin (A2066, Sigma-Aldrich). Secondary antibodies were: Goat anti-Mouse IgG (G21040, Thermo Fisher Scientific, Waltham, Massachusetts, USA) and Goat anti-Rabbit IgG (G21234, Thermo Fisher Scientific). Image J v. 1.51 (the National Institutes of Health, Bethesda, MD) was used to quantify each band.

Bone mineral density

Bone mineral density (BMD) was analyzed by dual-energy X-ray absorptiometry (DEXA) measurements in 20 equal cross sections of tibiae using a DCS-600R densitometer (Aloka Co. Ltd., Tokyo, Japan).

Blood glucose levels

Blood glucose levels were measured before polyIpolyC treatment and at sacrifice using a Glutest Neo Super meter (Sanwa Kagaku Kenkyusho Co. Ltd., Nagoya, Japan).

Statistical Analysis

Data are shown as means ± SD. Statistical significance was assessed using Student's *t* test. A probability of less than 5% was considered statistically significant (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ns, not significant).

Results

Establishment of a serum IGF-I reduction model in adult mice

To evaluate outcomes associated with IGF-I decline in adults, we established a mouse model in which IGF-I levels are down-regulated in adults following *Igf-I* deletion globally. *Mx1 Cre* and *Igf-I*$^{flox/flox}$ mice were crossed to yield *Mx1 Cre*/*Igf-I*$^{flox/flox}$ mice (cKO), and *Igf-I*$^{flox/flox}$ mice served as controls. PolyIpolyC was injected at 8 weeks of age into both genotypes to induce Cre for *Igf-I* deletion (Fig. 1a). Although serum IGF-I levels were equivalent in cKO and control mice at 8 weeks of age before polyIpolyC injection, levels significantly decreased in cKO mice 1 week after polyIpolyC injection and differences remained significant up to 10 weeks after injection (Fig. 1b; Fig. S1). Given IGF-I's structural similarity to insulin, we evaluated potential changes in glucose metabolism [20]. However, blood glucose levels were equivalent before and 10 weeks after polyIpolyC injection in cKO and control mice (Fig. S2).

Decreased IGF-I levels promote muscle loss in mice

We next analyzed phenotypes 10 weeks after polyIpolyC injection when mice were 18 weeks of age (Fig. 2). Body weight did not differ between cKO and control mice prior to polyIpolyC injection but was significantly lower in cKO

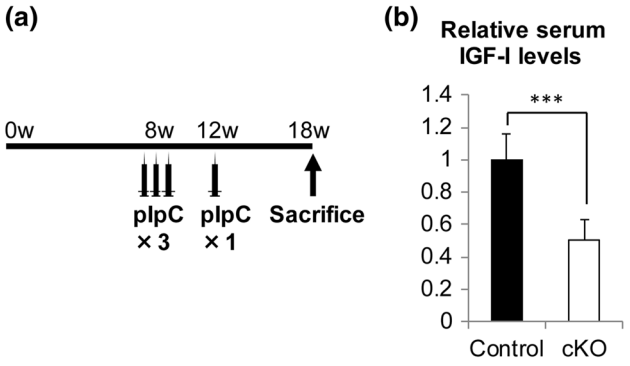

Fig. 1 Establishment and characterization of low IGF-I models. **a** Experimental protocol. 8-week-old IGF-I cKO (cKO) and control mice were injected with polyIpolyC for 3 consecutive days intraperitoneally, followed by additional polyIpolyC administration at 12 weeks (w) of age. Ten weeks after the first injection sera were collected and muscles were analyzed. **b** Sera were collected from cKO and control mice at 18 weeks of age, and serum IGF-I levels were determined by ELISA. Data are mean relative serum IGF-I levels ± SD (control $n=5$, cKO $n=5$; $^{***}P<0.001$). Statistical analysis was done by Student's t test. Data are representative of two independent experiments

than control mice 10 weeks after injection (Fig. 2a). At that time point, gastrocnemius and quadriceps weights were significantly lower in polyIpolyC-injected cKO compared with control mice (Fig. 2b), while these weights were not different when muscle weight was normalized to body weight (Fig. 2c). The cross-sectional areas (CSA) of gastrocnemius and quadriceps muscle were significantly smaller in cKO than in control mice (Fig. 2d, e). Similar muscle weight loss was seen in the slow-twitch soleus muscle after IGF-I reduction as well as in fast-twitch muscles, such as the gastrocnemius and quadriceps (Fig. S3a–e). Moreover, grip power was significantly weaker in IGF-I cKO than in control mice (Fig. 2f). Reduction in body weight or in weight of gastrocnemius, quadriceps, or soleus muscle in polyIpolyC-injected cKO mice was confirmed by comparison with normal mice (Fig. S4). Although IGF-I reportedly regulates bone mass, bone mineral density as analyzed by DEXA in tibia was comparable between cKO and control mice 10 weeks after polyIpolyC injection (Fig. S5).

Muscle metabolism is perturbed by IGF-I reduction

Muscle volume is regulated by anabolic and catabolic signals [12]. IGF-I signals reportedly induce anabolic factors such as Eif4e and p70S6K with concomitant inhibition of catabolic factors such as Atrogin-1 and MuRF1 [21–23]. *Eif4e* and *p70S6K* mRNA expression was significantly inhibited in cKO relative to control mouse gastrocnemius muscle 10 weeks after polyIpolyC injection (Fig. 3a, b). In contrast, *MuRF1* expression was unchanged and *Atrogin-1* was significantly lower in cKO compared to controls (Fig. 3c, d), suggesting that both anabolic and catabolic signals were inhibited and muscle acquired a low turnover state following IGF-I reduction.

Anabolic IGF-I signals are transduced via Akt and Erk [13]. Indeed, both Akt and Erk expression was inhibited by IGF-I reduction in cKO mice in gastrocnemius or quadriceps muscle tissue (Fig. 4a, c; Fig. S6). Catabolic signals underlying muscle atrophy are transduced via Smad2/3 activation and blocked by IGF-I [24]. However, both Smad2 and Smad3 protein accumulation and phosphorylation were unchanged in muscle tissues from cKO mice following IGF-I reduction (Fig. 4b, d; Fig. S6), supporting idea that muscle turnover decreases during following IGF-I cKO.

Discussion

In aging societies, maintaining musculoskeletal health in the elderly is crucial to preserve daily living activities [25]. Both muscle and bone volume decrease with age [26]; however, underlying mechanisms remain to be elucidated. In humans, IGF-I levels reportedly increase after birth, plateau around the middle teens, and then decline with age [27]. Analysis of knockout mouse phenotypes indicates that IGF-I and IGFIR are required for the embryonic development [3]; however, IGF-I function in adults has not been fully characterized possibly due to lack of appropriate adult models. Here, we show that IGF-I is required to maintain muscle homeostasis and prevent muscle loss in adults (Fig. 5). We report that weight of gastrocnemius, quadriceps, or soleus muscle decreased following IGF-I reduction (Fig. 2b, c, S3), but these values did not differ when muscle weight was normalized to body weight. These observations suggest that reduced body weight seen in cKO mice exhibiting decreased serum IGF-I levels was due to the loss of muscle volume. IGF-I continuously activates catabolic signals via Akt and Erk, stimulating Eif4e and S6K expression to prevent muscle atrophy (Fig. 5a). We found that decreased serum IGF-I levels block these anabolic signals, leading to muscle atrophy (Fig. 5b). Our data also suggest that catabolic signals are unchanged by IGF-I reduction (Fig. 5b), and muscles assume a low turnover state. Bone homeostasis is cooperatively regulated by the activities of bone-resorbing osteoclasts and bone-forming osteoblasts [28], and inhibition of either cell type reportedly promotes low turnover conditions associated with decreased bone mass [29]. Thus, low turnover status due to inhibition of either anabolic or catabolic signals likely promotes muscle loss. Both decreased muscle volume and serum IGF-I levels have been demonstrated in the elderly [30]. Various factors likely contribute to age-dependent muscle weight loss, such as physical inactivity [31]. Our data in a mouse model recapitulating this loss suggest that a

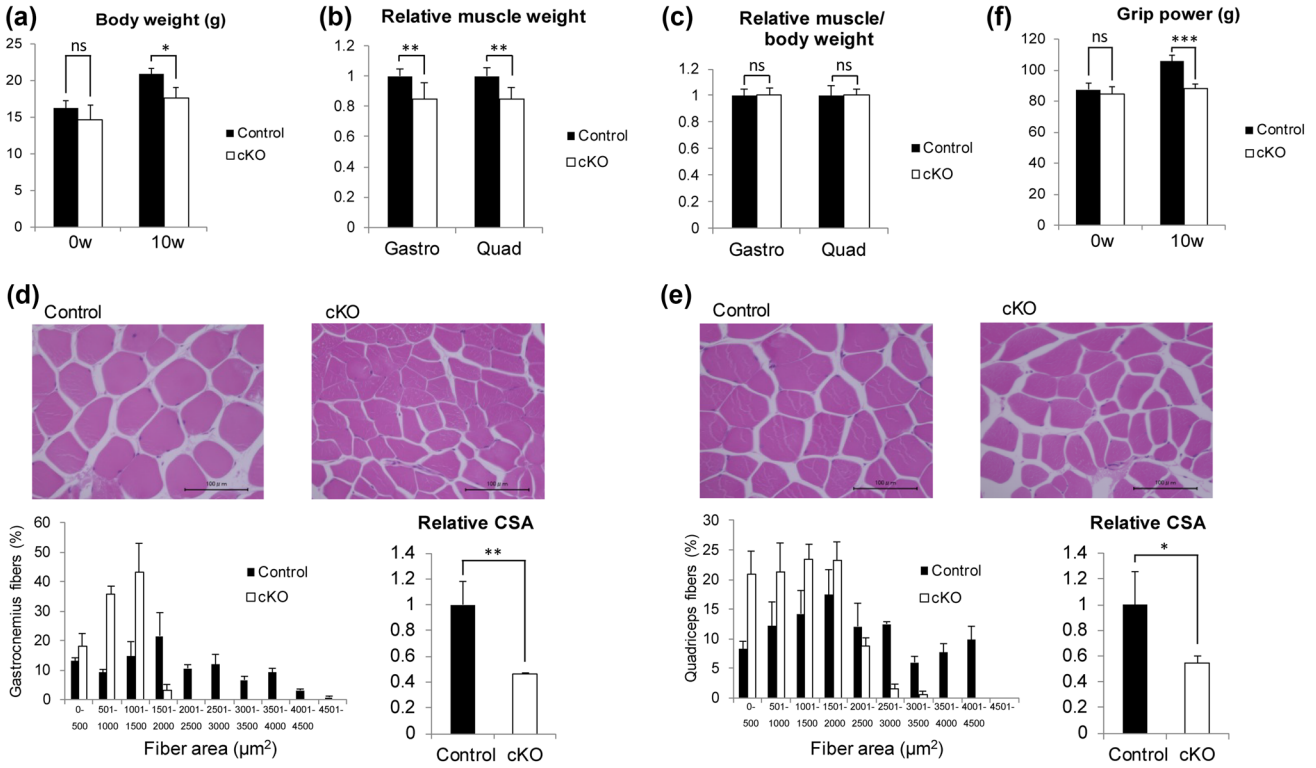

Fig. 2 IGF-I cKO mice show skeletal muscle atrophy. polyIpolyC was injected into 8-week-old IGF-I cKO or control mice. Ten weeks later, body weight (**a**), gastrocnemius or quadriceps muscle weight (**b**), gastrocnemius or quadriceps muscle weight/body weight (**c**), cross-sectional area (CSA) of gastrocnemius (**d**), and quadriceps (**e**) were analyzed in IGF-I cKO and control mice. Body weight (**a**) and grip power (**f**) was also analyzed before polyIpolyC injection (0w). Data represent mean body weight (**a**), gastrocnemius or quadriceps muscle weight (**b**), gastrocnemius or quadriceps weight relative to body weight (**c**), grip power (**f**) ± SD (**a** control $n=4$, cKO $n=3$; **b** control $n=8$, cKO $n=6$; **c** control $n=8$, cKO $n=6$; **f** control $n=5$, cKO $n=5$). Hematoxylin and eosin staining of gastrocnemius or quadriceps muscle of IGF-I cKO and control mice is shown (**d**, **e** upper panels. Scale bar 100 μm). Frequency distribution of fiber area of gastrocnemius or quadriceps muscle in IGF-I cKO and control mice (**d**, **e** lower left panels. *x* axis, fiber area; *y* axis, % of cross-sectional area (CSA) of muscle fiber; data are mean % of CSA ± SD; control $n=4$, cKO $n=3$). Mean relative CSA ± SD (control $n=4$, cKO $n=3$) are also shown (**d**, **e** lower right panels). Statistical analysis was done by Student's *t* test (*$P<0.05$; **$P<0.01$; *ns* not significant). Representative data of two independent experiments are shown (**a–c**)

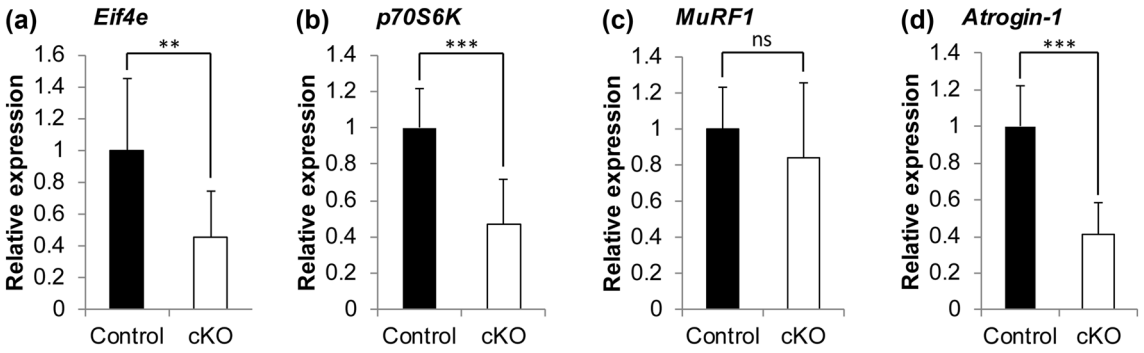

Fig. 3 Expression of anabolic and catabolic markers in skeletal muscle following IGF-I reduction. Expression of *Eif4e* (**a**), *p70S6K* (**b**), *MuRF1* (**c**), or *Atrogin-1* (**d**) mRNA relative to *β-actin* was assessed in gastrocnemius muscle of IGF-I cKO and control mice 10 weeks after the first polyIpolyC injection (when mice were 18 weeks old) by real-time PCR. Data are mean indicated gene expression relative to *β*-actin ± SD (control $n=8$, cKO $n=6$, **$P<0.01$; ***$P<0.001$; ns, not significant). Data are representative of two independent experiments

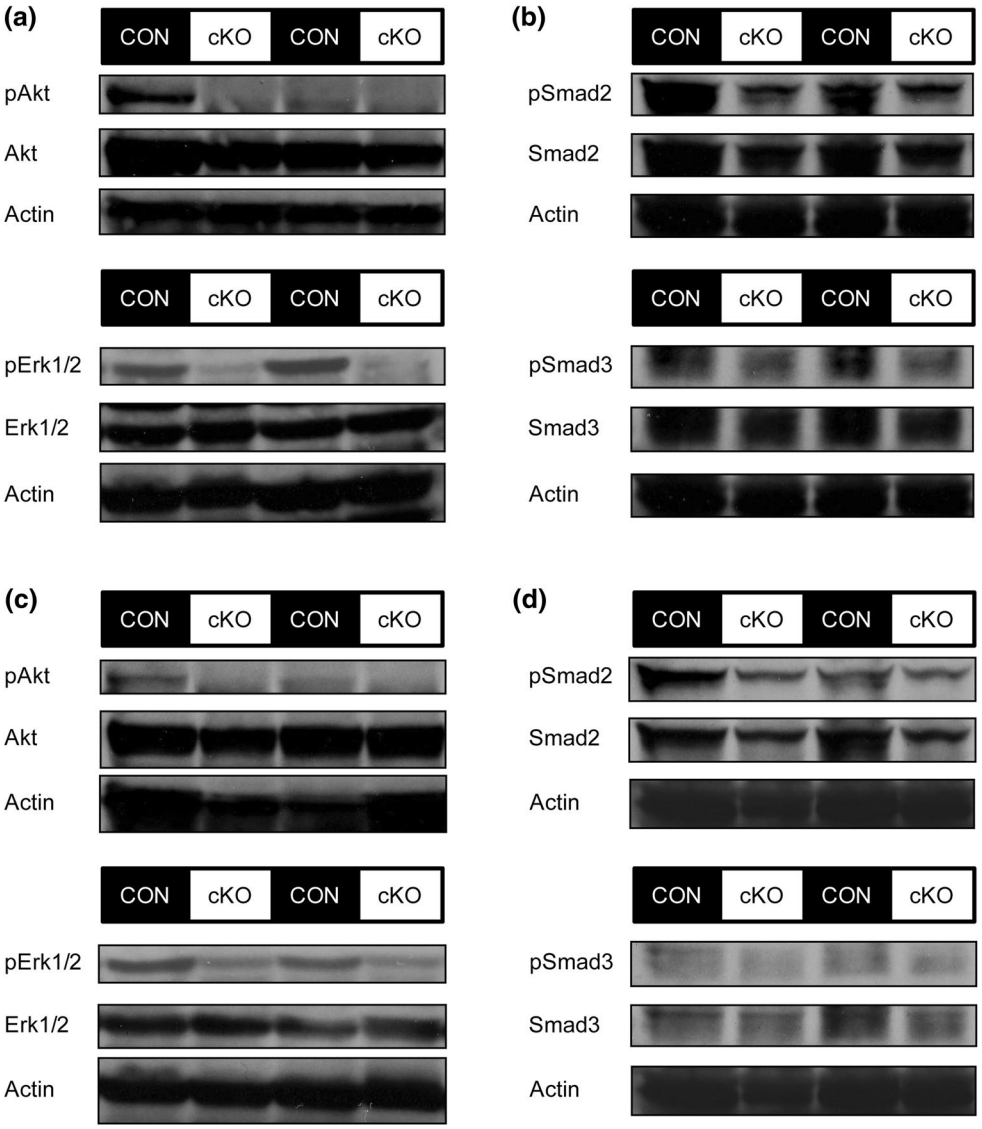

Fig. 4 Changes in the expression of anabolic and catabolic signaling factors in skeletal muscle following IGF-I suppression. Ten weeks after poly-IpolyC injection, expression of catabolic (**a**, **c** pAkt, Akt, pErk, and Erk) and anabolic (**b**, **d** pSmad2, Smad2, pSmad3, and Smad3) proteins in gastrocnemius (**a**, **b**) and quadriceps (**c**, **d**) muscle of IGF-I cKO (cKO) and control (CON) mice was identified by Western blot. Two independent data sets are shown

decrease in serum IGF-I levels also underlies age-dependent muscle loss.

Various factors regulate muscle volume [32]. Among them, either Myostatin-deficient mice or animals with *Myostatin* mutations exhibit elevated muscle mass with increased muscle power [33–35]. Administration of anti-Myostatin antibody to adult mice elevates muscle weight and cross-sectional area [36, 37]. Indeed, several neutralizing antibodies against human Myostatin exist [38, 39], although they are currently not available for use in human subjects. Moreover, Myostatin inhibition in mice reportedly is not effective in elevating muscle mass in adults [16], suggesting that Myostatin likely regulates muscle volume during development.

Other than aging, muscle atrophy is induced by either immobilization or unloading [40, 41]. Recently, we demonstrated that accumulation of Smad2 and Smad3 protein in muscle occurred following immobilization and was required for immobilization-induced muscle atrophy [16]. In vitro, serum starvation promotes *MuRF1* and *Atrogin-1* expression in C2C12 myogenic cells via Smad2 and Smad3 protein accumulation, and *MuRF1* and *Atrogin-1* expression or Smad2 and Smad3 protein accumulation is blocked by IGF-I [16]. Unloading is known to promote muscle atrophy by inducing Cbl-b, an E3 ligase, and Cbl-b inhibits IGF-I signaling by degrading IRS-1, a signal transducer for IGF-I [42]. Thus, IGF-I likely prevents muscle loss by several mechanisms.

IGF-I also functions in bone. Recently, muscle and bone were shown to regulate each other in an interactive manner [43]. Several myokines produced by muscle, such as Fam5c, osteoglycin, Irisin, and Myostatin, were identified to act on bones [44]. In contrast, bone-derived factors such as TGF-β reportedly inhibit muscle homeostasis [45]. IGF-I is secreted

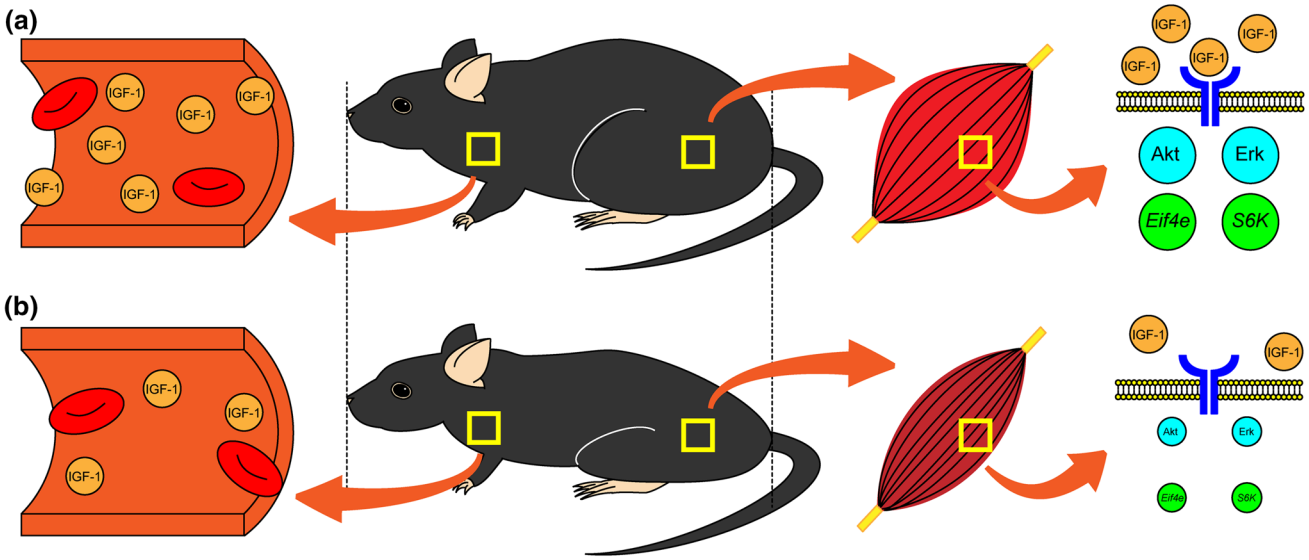

Fig. 5 Schematic model of IGF-I reduction-induced muscle atrophy. **a** In IGF-I sufficient conditions, muscle homeostasis is maintained by IGF-I-induced continuous activation of Akt and Erk, which, in turn, activates the expression of *Eif4e* and *p70S6K*, both anabolic signals that prevent muscle atrophy. **b** In low IGF-I conditions, IGF-I-induced catabolic signals decrease, leading to muscle atrophy

from bones during bone resorption and promotes subsequent bone formation [8], but it also inhibits muscle atrophy. Ibandronate, a bisphosphonate, inhibits bone resorption but also prevents immobilization-induced muscle atrophy [46]. IGF-I production is reportedly regulated by vitamin D [47, 48], and vitamin D plays crucial roles in regulating bone homeostasis [49]. Nonetheless, IGF-I plays positive roles in both bone and muscle. In our study, muscle weight but not bone mineral density decreased in IGF-I cKO compared with control mice. At present, the reason for this apparent contradiction is unclear. However, since our model showed an ~ 50% reduction in serum IGF-I levels in cKO compared to control mice, these differences may be due to differences in sensitivity to serum IGF-I levels among various organs.

Our study has some limitations. In a mouse model, serum IGF-I levels likely decrease more rapidly than occurs in elderly people [27], given a mouse's decreased life span. IGF-I plays diverse roles in various tissues other than muscle and bone; among them, IGF-I activates Schwann cell function and differentiation [50, 51]. Although IGF-I also functions in glucose metabolism, in this study, we show that down-regulation of serum IGF-I levels did not promote elevated blood glucose. At present, mechanisms underlying differences in sensitivity to serum IGF-I levels among skeletal muscle, glucose, and bone metabolism were not clear. Taken together, our study demonstrates that IGF-I may represent a potential therapeutic factor to maintain muscle volume in the elderly.

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Compliance with ethical standards

Conflict of interest The authors declare no competing financial and non-financial interests.

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