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Construction of Plasmid DNA Expressing Two Isoforms of Insulin-Like Growth Factor-1 and Its Effects on Skeletal Muscle Injury Models

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Insulin-like growth factor-1 (IGF-1) plays a significant role in the development of various organs, and several studies have suggested that IGF-1 isoforms, IGF-1 Ea and IGF-1 Ec, are expressed in skeletal muscle to control its growth. In this study, we designed a novel nucleotide sequence, IGF-1-X10, consisting of IGF-1 exons and introns to simultaneously express both IGF-1 Ea and IGF-1 Ec. When transfected into human cells, the expression of both isoforms was observed at the transcript and protein levels. In an animal study, intramuscular injection of plasmid DNA comprising IGF-1-X10 induced the expression of IGF-1 Ea and IGF-1 Ec, leading to the production of functional IGF-1 protein. Finally, the efficacy of this plasmid DNA was tested in a cardiotoxin (CTX)-mediated muscle injury model and age-related muscle atrophy model. We found that IGF-1-X10 increased the muscle mass and controlled several key factors involved in the muscle atrophy program in both models. Taken together, these data suggest that IGF-1-X10 may be utilized in the form of gene therapy for the treatment of various muscle diseases related to IGF-1 deficiency.

Keywords: IGF-1, Ea, Ec, plasmid DNA, skeletal muscle injury, muscle satellite cell

INTRODUCTION

INSULIN-LIKE GROWTH factor-1 (IGF-1) is well characterized for its roles in promoting the proliferation and differentiation of various cell types, eventually leading to organ development.[1–3] This growth factor also plays a critical role in controlling physiological conditions such as maintaining metabolic homeostasis, protecting nerve tissues, and inducing blood vessel formation.[2,4,5] IGF-1 activates several receptor tyrosine kinases such as IGF-1R, IGF-2R, insulin receptor (IR), and the IR/IGF-1R complex, leading to the activation of various downstream signaling cascades. With the highest binding affinity, IGF-1 binds to IGF-1R and controls the activity of signaling pathways such as the Ras/MAPK/ERK1/2 and PI3K/AKT pathways.[6]

The human IGF-1 gene consists of 6 exons and 5 introns, which theoretically induces the expression of six isoforms by the combination of two signal peptides at the N-terminal and three E-peptides at the C-terminal through alternative splicing.[3,7,8] It has been reported that the signal peptides from exon 1 and exon 2 determine Class I and Class II IGF-1, respectively. The Class I isoform plays a role in controlling the autocrine and paracrine effects of IGF-1, whereas the Class II isoform is a circulating form of IGF-1 involved in the endocrine system. The combination of exon 5 and exon 6 results in the formation of three different E-peptides, Ea (exon 6), Eb (exon 5), and Ec (parts of exon 5 and 6); however, the exact role of these E-peptides is still unclear.

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In skeletal muscle, it has been shown that the expression of IGF-1 isoforms is differentially controlled during myotube differentiation.[9,10] At the early stage of satellite cell activation, IGF-1 Ec rapidly increases and gradually decreases during the phase of myoblast proliferation and myotube differentiation. At this stage of differentiation, the level of IGF-1 Ea gradually increases, and the level is maintained during the course of myotube formation.[10] In addition to the expression kinetics, it has been reported that IGF-1 Ea activates AMPK and SIRT-1 to promote mitochondrial biogenesis and reduce the inflammation, whereas IGF-1 Ec induces the formation of autophagosome to maintain the protein homeostasis. These data imply the potential distinct role of IGF-1 isoforms in the skeletal muscle and these isoforms may be a useful target for the development of efficient therapeutic agents for the treatment of muscular diseases and utilizing both isoforms simultaneously can be a strategy to maximize the biological potential of IGF-1 gene.[11]

Based on the biological activities of IGF-1 isoforms, we designed a genomic-complementary DNA (cDNA) hybrid construct, IGF-1-X10, to induce the expression of both IGF-1 Ea and IGF-1 Ec simultaneously and investigated the effect of this construct in skeletal muscle injury models. Intramuscular injection of IGF-1-X10 induced the expression of IGF-1 Ea and IGF-1 Ec both *in vitro* and *in vivo* and properly activated IGF-1R and its downstream signaling pathways. Moreover, IGF-1-X10-mediated expression of IGF-1 isoforms ameliorated skeletal muscle injury by accelerating the activation of muscle satellite cells. These data suggest that inducing the expression of IGF-1 isoforms simultaneously by IGF-1-X10 may be a useful gene therapy strategy for the treatment of various diseases related to skeletal muscle.

MATERIALS AND METHODS

Animal studies

Eight-week-old male mice were purchased from Orient Bio, Inc. (Seoul, Korea) and 18-month-old male mice were purchased from Janvier Labs (Le Genest-Saint-Isle, France). All experimental procedures were reviewed and approved by the International Animal Care and Use Committee (IACUC) at Seoul National University or Helixmith Co., Ltd. Mice were housed at least 7 days before any treatments and were given *ad libitum* access to food and water under a light–dark cycle of 12 h at 24°C ± 2°C.

Cardiotoxin (CTX) injury was induced as previously described.[12] To induce skeletal muscle injury in mice, animals were first anesthetized with a gas mixture of 2–4% isoflurane and 1–2% oxygen. Then, 50 μL of 10 μM CTX (Latoxan) diluted in phosphate-buffered saline was injected intramuscularly into the tibialis anterior (TA) muscle using an insulin syringe. Plasmid DNA encoding the IGF-1 gene was also i.m. injected 3 days before CTX injury. TA muscles were then prepared, and the muscle mass was measured at appropriate time points.

Cell culture and reagents

The mouse myoblast cell line C2C12, the human embryonic kidney cell line HEK293T/17 and the human neuroblastoma cell line SH-SY5Y were purchased from American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (Gibco), antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin) and 2 mM GlutaMax (Gibco) at 37°C in a 5% CO_2 humidified incubator. Lipofectamine 3000 (Invitrogen) was mixed with 1 μg of plasmid DNA and transfected into C2C12 and HEK293T/17 cells according to the manufacturer's instructions.

Cloning of IGF-1 constructs

To construct a genomic-cDNA hybrid sequence from the IGF-1 gene, IGF-1 cDNA containing exons 1, 2, and 4 and the 5′ region of intron 4 was synthesized (425 bp). Ten variants in combination with the 3′ untranslated region (UTR) of intron 4, exon 5, a fragment of intron 5, and exon 6 were generated (X1–X10). All variants were further subcloned into a human cytomegalovirus (HCMV)-based expression cassette[13] and we tested whether these variants expressed two transcripts corresponding to IGF-1 Ea and IGF-1 Ec (data not shown). Three plasmid DNAs were additionally constructed for the control (Supplementary Fig. S1). IGF-1 Ea and IGF-1 Ec encode human IGF-1 Ea and Ec, respectively, and IGF-1 Mature encodes exons 3 and 4 of IGF-1, which is responsible for binding to the IGF-1 receptor.[3]

RNA extraction and real time-quantitative polymerase chain reaction

TA muscles from mice or cultured cells were prepared for the analysis of messenger RNA (mRNA) expression levels. Total RNA was isolated from homogenized TAs or cultured cells using TRIzol reagent (Invitrogen), chloroform, and isopropanol according to the protocol described in previous studies.[12,14] cDNA was synthesized using 1 μg of extracted RNA in the presence of avian myeloblastosis virus (AMV) reverse transcriptase (Takara Bio), dNTPs (Takara Bio), oligo-dT primers (Qiagen), and RNase inhibitors (Takara Bio). To measure gene expression levels, real time-quantitative polymerase chain reaction was performed with a Thermal Cycler Dice Real Time System TP800 (Takara Bio). Synthesized cDNA was amplified using TB Green Premix (Takara Bio) and normalized to the level of GAPDH. The primers used in this study are listed in Table 1.

Enzyme-linked immunosorbent assay

Human IGF-1 in cell culture supernatants and mouse TA muscles were measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits (SG100B; R&D Systems) according to the manufacturer's instructions. Total protein was extracted from cells in RIPA lysis buffer (Sigma-Aldrich) containing a phosphatase inhibitor (Sigma-Aldrich), and a protease inhibitor

Table 1. *RT-qPCR primers used in this study*

Gene	*Direction*	*Sequence*
Human IGF-1 Ea	Forward	CAAGCCTGCCAAGTCAGCTC
	Reverse	TAGTTCTTGTTTCCTGCACTCCCT
Human IGF-1 Ec	Forward	GATCTAAGGAGGCTGGAGATGTAT
	Reverse	TTGGTAGATGGGGGCTGATACT
Human GAPDH	Forward	CTGTTCGACAGTCAGCCGCATC
	Reverse	GCGCCCAATACGACCAAATCCG
Mouse GAPDH	Forward	CTGGAAAGCTGTGGCGTGAT
	Reverse	CCAGGCGGCACGTCAGATCC

IGF-1, insulin-like growth factor-1.

(Roche). The lysed cells and tissues were centrifuged at 4°C and 12,000 *g* for 15 min, and the supernatants were analyzed using human IGF-1 ELISA (DYC1770-5; R&D Systems) according to the manufacturer's instructions.

Western blot

Mechanistically homogenized TA muscles or cultured cells were prepared, and total proteins were extracted in RIPA lysis buffer (Sigma-Aldrich) containing a phosphatase inhibitor (Sigma-Aldrich), and a protease inhibitor (Roche). To precipitate human IGF-1 protein, immunoprecipitation kit (10007D; Thermo Fisher Scientific) and human IGF-1 antibody (MAB291-100; R&D Systems) were used according to the manufacturer's instructions. Equal amounts of protein were separated on a Bolt™ 4–∼12% Bis-Tris gel (Invitrogen), and transferred to a polyvinylidene difluoride (PVDF) membrane (GE Healthcare).

The membrane was blocked in 3% bovine serum albumin (BSA) in 0.1% TBST (Thermo Fisher Scientific) at room temperature for 1 h and probed overnight at 4°C with a primary antibody diluted in 3% BSA in 0.1% TBST. The membrane was then incubated with horseradish peroxidase (HRP)-conjugated secondary IgG (Abcam) diluted in 2% skim milk in 0.1% TBST at room temperature for 1 h. After development with ECL substrate (Thermo Fisher Scientific), the bands were visualized by a Solo 6S instrument (Vilber Lourmat), and quantified using ImageJ software. The antibodies used in this study are listed in Table 2.

Hematoxylin and eosin staining and morphometric analysis

TA muscles were fixed in 10% normalized buffered formalin (Sigma-Aldrich), followed by the dehydration with a gradient series of ethanol from 70% to 100%. Samples were then embedded in the paraffin block. A paraffin section (6 μm) was stained by hematoxylin and eosin (H&E), and the cross-sectional area (CSA) was determined by ImageJ software. More than 200 myofibers were analyzed in each group ($n=6$).

Statistical analysis

All values are represented as the mean ± standard error of the mean (SEM) or ±standard deviation from more than two independent experiments. Statistical significance was determined using unpaired Student's *t*-test, one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test or two-way ANOVA followed by Bonferroni's *post hoc* test in GraphPad Prism 9.0 software.

Table 2. *Antibodies used in this study*

Antibody	*Company*	*Catalog No.*
IGF-1	R&D Systems	MAB291-100
IGF-1	Abcam	Ab9572
p-AKT(T308)	Cell Signaling Technology	4056
p-AKT(S473)	Cell Signaling Technology	9271
AKT(Pan)	Cell Signaling Technology	4691
p-p70S6K(T389)	Cell Signaling Technology	8209
p70S6K	Cell Signaling Technology	2708
p-GSK3α/β	Cell Signaling Technology	9331
GSK3α/β	Cell Signaling Technology	5676
GAPDH	Cell Signaling Technology	2118
Ea-peptide	Abclon	Custom
Ec-peptide	Abclon	Custom
Pax7	Santa Cruz	SC-81648
MyoD	Santa Cruz	SC-32758
MyoG	Santa Cruz	12732
Myh3	Abcam	Ab124205
IGF-1Rβ	Cell Signaling Technology	9750
p-IGF-1Rβ (Y1131)	Cell Signaling Technology	3021
p-AMPK (T172)	Cell Signaling Technology	2535
AMPK	Cell Signaling Technology	5831

RESULTS

Construction of a genomic-cDNA hybrid sequence of IGF-1 expressing two isoforms

In the human *igf1* gene, six different isoforms may be theoretically expressed by alternative splicing, which combines two distinct signal peptide sequences at the N-terminal (Class I and Class II) and three types of E-peptides at the C-terminal (Ea, Eb, and Ec) (Fig. 1A).[3] It has also been reported that Class I IGF-1 Ea and Class I IGF-1 Ec exhibited significant therapeutic effects in several disease models.[8] Based on these reports, we hypothesized that inducing the expression of both IGF-1 isoforms may be a strategy to maximize the biological function of IGF-1 gene and the preliminary result suggested that the expression of IGF-1 Ea and IGF-1 Ec simultaneously showed enhanced therapeutic effects in skeletal muscle atrophy model, compared with the groups solely injected with IGF-1 Ea or IGF-1 Ec expression vector (data not shown).

Among the various candidate constructs (described in Materials and Methods section Cloning of IGF-1 constructs), we found that IGF-1-X6 and its derivative IGF-1-X10 sequence induced the expression of both IGF-1 isoforms (data not shown). IGF-1-X10 is a sequence consisting of IGF-1 exons 1, 3, and 4, followed by the fragment of intron 4, Ec peptide (exons 5 and 6), the fragment of intron 5, and Ea peptide (exon 6), and this construct was chosen for the further experiments as it

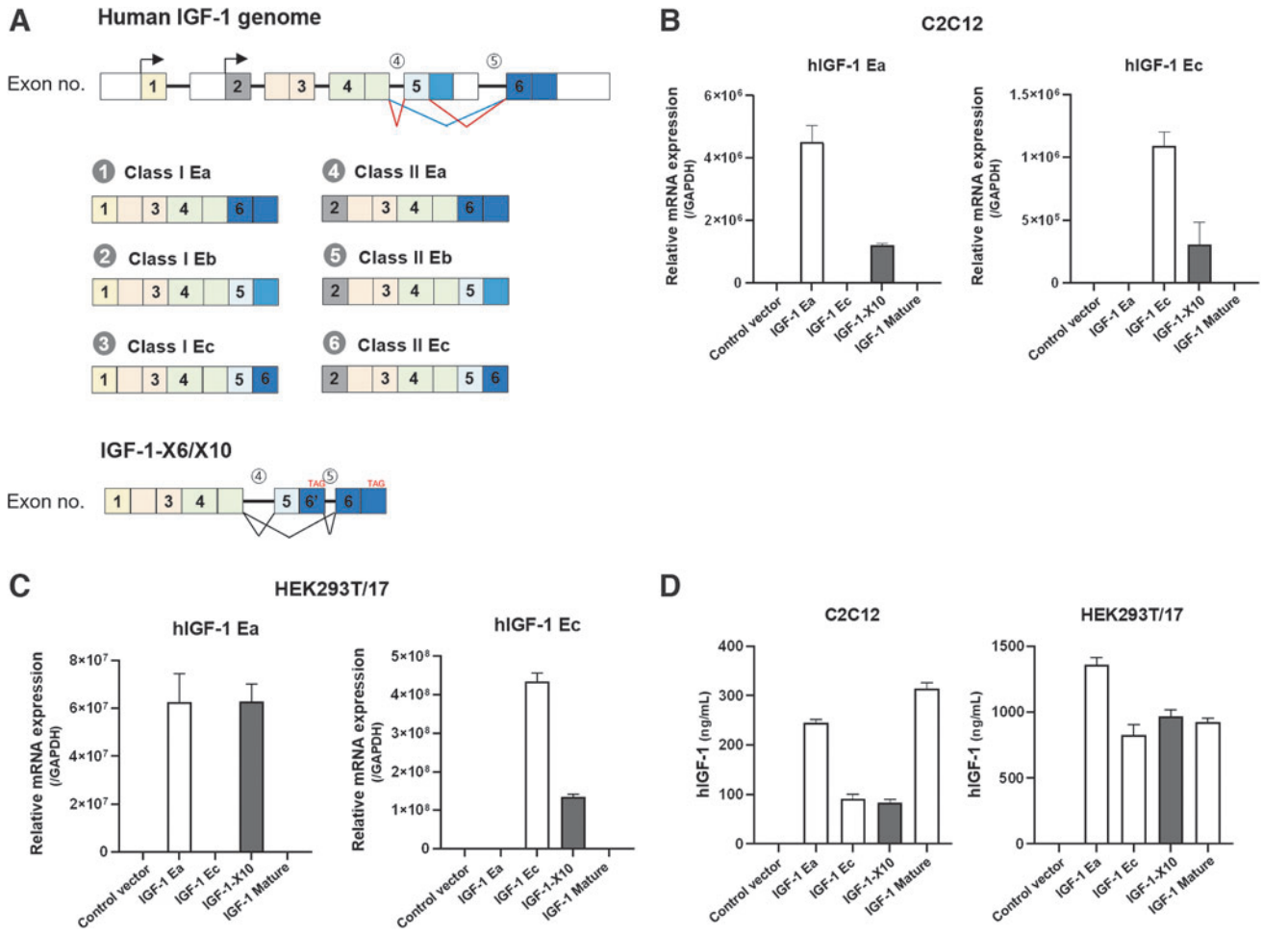


Figure 1. Construction of the genomic-cDNA hybrid sequence of the human IGF-1 gene and the expression of IGF-1 isoforms. **(A)** Schematic illustration of the human IGF-1 genome and IGF-1-X10 construct. Among the six exons of the IGF-1 gene, exon 1 was selected as the signal peptide sequence (Class I). The IGF-1-X10 sequence was constructed by the combination of exons 1, 2, 4, 5, and 6 and introns 4 and 5 as described in the Results and Materials and Methods sections. **(B, C)** To determine the IGF-1 isoform transcripts *in vitro*, C2C12 and HEK293T/17 cells were transfected with plasmid DNAs expressing different human IGF-1 isoforms. Total RNA was prepared, and the levels of human IGF-1 isoforms were analyzed by RT-qPCR, using a specific primer to E-peptide region (Table 1). The RT-qPCR reactions to each IGF-1 isoforms were specific as the transcript level of IGF-1 Ea was negligible in IGF-1 Ec and IGF-1 Mature group and vice versa. GAPDH was used as a control and the values were normalized to the expression level of GAPDH. Plasmid DNAs expressing IGF-1 Ea, IGF-1 Ec, and IGF-1 Mature were used as controls. IGF-1 Mature contains exons 1, 3, and 4 without E peptides. **(D)** The expression levels of human IGF-1 proteins in culture supernatants of IGF-1 plasmid-transfected cells were analyzed by ELISA specific to IGF-1 exon 3/4. All data are represented as the mean ± SD. Two or more independent experiments were performed, and the representative results are shown, $n=3$ per group. cDNA, complementary DNA; ELISA, enzyme-linked immunosorbent assay; IGF-1, insulin-like growth factor-1; RT-qPCR, real time-quantitative polymerase chain reaction; SD, standard deviation.

contains shorter version of the intron 4 and intron 5 fragments (Fig. 1A and Supplementary Fig. S1). The IGF-1-X10-mediated expression of both isoforms was further confirmed in two different cell lines using primers specific to each IGF-1 isoform (Fig. 1B, C).

We further tested the protein level of IGF-1 produced by IGF-1-X10 in the culture supernatants. As shown in Fig. 1D, a significant amount of IGF-1 protein was detected in all of the experimental group, including IGF-1-X10-transfected cells. The isoform pattern of IGF-1 protein was also confirmed by Western blotting using antibodies specific to the exon 3 and 4 regions of IGF-1 (Supplementary Fig. S2). Taken together, these data suggested that plasmid DNA containing the genomic-cDNA hybrid sequence of the *igf1* gene (IGF-1-X10) induced the expression of IGF-1 Ea and IGF-1 Ec simultaneously *in vitro*.

Intramuscular injection of IGF-1-X10 induces the expression of IGF-1 protein *in vivo*

Based on these data from *in vitro* experiments, we further tested whether this plasmid DNA induces the expression of IGF-1 isoforms *in vivo*.[15] To investigate the time-dependent expression kinetics of IGF-1-X10, IGF-1-X10 encoding plasmid DNA was i.m. injected into mouse TA muscle and the level of human IGF-1 protein was analyzed. IGF-1 protein from IGF-1-X10 gradually increased and peaked at day 7. This expression then decreased and reached the basal level below the lower

limit of quantitation value of ELISA (0.1 ng/mL) at day 14 (Fig. 2A). This kinetic pattern was similar to the results from other publications using plasmid DNA.[16,17]

Next, we determined the dose-dependent expression of IGF-1-X10. Animals administered with 25 μg/head IGF-1-X10 showed no detectable level of IGF-1 protein. When 50 μg/head was administered, IGF-1 protein was induced and further increased in the 100 μg/head-injected group. Mice injected with 200 μg/head showed slightly increased level; however, this increase was not statistically significant compared with the 100 μg/head group. Based on this dose response of IGF-1-X10, 100 μg/head was chosen for further animal studies. Consistent with the data from the *in vitro* experiment, IGF-1-X10 significantly induced the expression of both IGF-1 Ea and IGF-1 Ec transcripts, compared with the untreated group (Fig. 2C). Taken together, IGF-1-X10 induces the expression of IGF-1 Ea and IGF-1 Ec both *in vitro* and *in vivo* possibly by the mechanism illustrated in Fig. 2D.

IGF-1-X10 showed therapeutic effects in a muscle injury model

IGF-1 isoforms play a critical role in the regeneration of injured skeletal muscle by promoting satellite cell activation and proliferation, leading to the acceleration of myotube formation.[9,10] To investigate whether i.m. injection of IGF-1-X10 shows therapeutic effects in injured muscle, we first used CTX-induced muscle injury, a representative model for studying muscle injury and regeneration in mice.[12] Before the *in vivo* investigation in murine system, we confirmed that the IGF-1-X10-induced human IGF-1 proteins activated both human and murine signaling pathways related to the IGF-1R (Supplementary Figs. S3 and S4).[6] We also confirmed that i.m. injection of this plasmid DNA did not induce the hypertrophic effects in normal skeletal muscle (Fig. 3A).[18]

To test the effects of IGF-1-X10 in the CTX-induced muscle injury model, plasmid DNAs were i.m. injected into the TA muscle 3 days before CTX injury. Because the

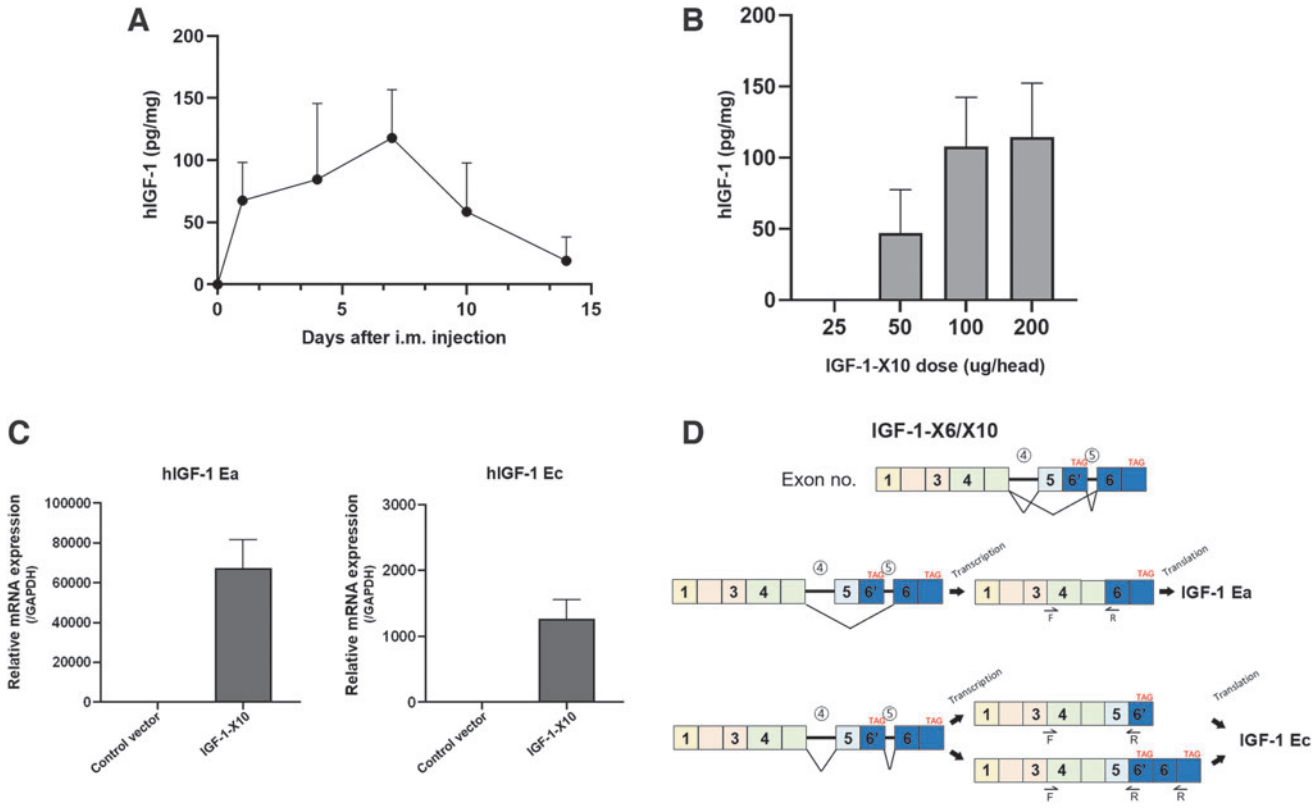


Figure 2. The expression of human IGF-1 proteins in mice. **(A)** IGF-1-X10 encoding plasmid DNA was i.m. injected into the TA muscle at a dosage of 100 μg/head. The skeletal muscles were prepared at the indicated time points. Total proteins were extracted, and the levels were quantified using a BCA assay. Samples were then subjected to ELISA specific to human IGF-1 protein. **(B)** To determine the dose dependency of protein expression, IGF-1-expressing plasmid DNA was injected intramuscularly at doses ranging from 25 to 200 μg/head. Seven days after administration, total proteins were extracted from skeletal muscle, followed by human IGF-1-specific ELISA. **(C)** IGF-1 isoform pattern in IGF-1-X10 injected muscle. IGF-1-X10 was i.m. injected, and total RNA was isolated 7 days after administration. After cDNA synthesis, the transcript levels of IGF-1 Ea and IGF-1 Ec were analyzed using the specific primers described in Table 1. The level of GAPDH was determined as a control. **(D)** Possible mechanism of IGF-1 isoform expression. IGF-1-X10 may induce the expression of IGF-1 Ea and IGF-1 Ec by alternative splicing between exon 4 and exon 6 (for Ea) and exon 4 and eon 5/6 (for Ec), respectively. IGF-1 Ea can be expressed by IGF-1-X10 through the alternative splicing between the donor site at the end of exon 4 and the acceptor site at the end of intron 5. IGF-1 Ec appears to be expressed by the splicing between the end of exon 4 and the end of the intron 4. The removal of intron 5 during the expression of IGF-1 Ec may be occurred, but the amino acid sequence after the translation is identical due to the stop codon presented in the end of Ec peptide (exon 6′). TA, tibialis anterior.

IGF-1-X10 significantly induced the gene expression starting from day 3 (Fig. 2A), this injection protocol has been chosen to synchronize the kinetic of the plasmid DNA-mediated gene expression and the initiation of muscle injury progression.[12] Upon CTX injury, the mass of skeletal muscle decreased by 14% compared with the control group at day 7 (Fig. 3B). In animals injected with IGF-1-X10, CTX-mediated muscle damage was significantly ameliorated, as indicated by the increased level of muscle mass at day 7 (Fig. 3B). When mice were injected with the IGF-1 Mature construct, an increasing trend was observed; however, this difference was not statistically significant.

To further investigate the mechanism of action of IGF-1-X10, we analyzed several key factors involved in the satellite cell activation such as Pax7, MyoD, and MyoG as the activation of this cell is a key event during the skeletal muscle regeneration.[19] In the CTX-injured group, the protein levels of these factors were increased at day 1, indicating the activation of muscle satellite cells as previously described.[19] When animals were injected with IGF-1-X10, the protein levels of Pax7 and MyoD was further upregulated and that of MyoG showed down-regulated pattern, compared with CTX-injured group (Fig. 3C, D). The protein levels in IGF-1 Mature-treated group were comparable with those in CTX-injured animals. At 3 days after CTX injury, the protein levels of Pax7 and MyoD were further increased (Fig. 3E, F). When animals were injected with IGF-1-X10, these levels were decreased to basal levels compared with the control vector-injected group (Fig. 3E, F).

We further analyzed the level of Myh3, a marker for regenerating myofibers.[20] As shown in Fig. 3E and F, the CTX-injected group showed decreased levels of Myh3 compared with the uninjured animals. However, i.m. administration of IGF-1-X10 increased the protein level of Myh3, suggesting the promotion of muscle regeneration process. Taken together, these data collectively suggested that IGF-1-X10 may show therapeutic effects in a skeletal muscle injury model by controlling the activity of muscle satellite cells, leading to the acceleration of the regeneration process.

Therapeutic effects of IGF-1-X10 in an age-related muscle atrophy model

To further assess the therapeutic effect of IGF-1-X10 in a disease condition, we tested this construct in an age-related muscle atrophy model.[9] As shown in Fig. 4A, the ratio between the TA muscle mass and the body weight was decreased in 18-month-old mice, when compared with that from the 8-week-old animals indicating the age-related sarcopenia condition. When IGF-1-X10 was i.m. administered to aged mice, the skeletal muscle mass was significantly ameliorated (Fig. 4A). We further analyzed the CSA of the muscle fiber at the histological level.

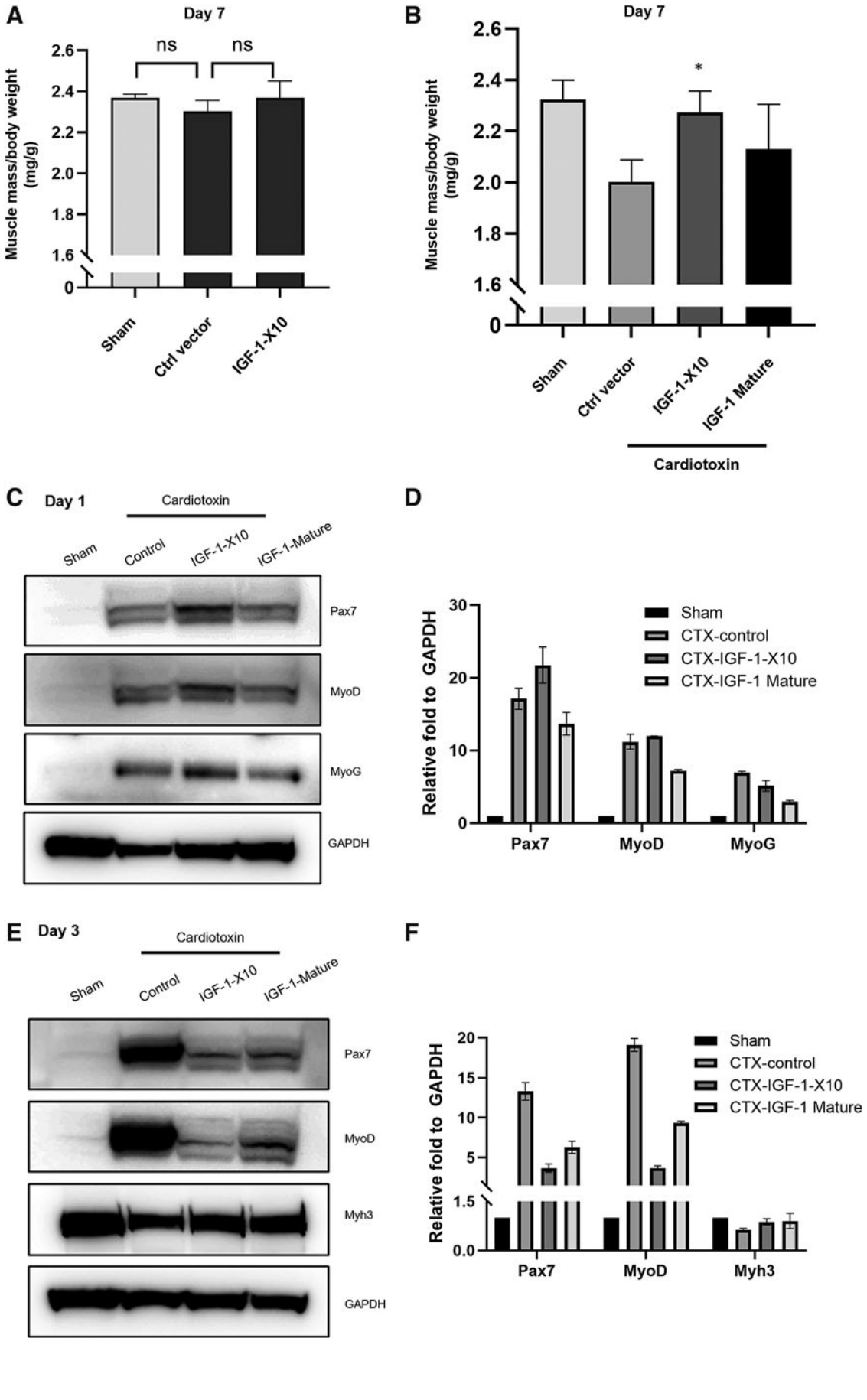
The proportion of the muscle fiber with 0–1,200 μm^2 diameter was increased and that of fiber with larger than 3,200 μm^2 diameter was decreased in the 18-month-old skeletal muscle, when compared with 8-week-old sample (Fig. 4B). In IGF-1-X10 treated group, the distribution pattern of CSA diameter was significantly shifted. The proportion of 0–400 μm^2 diameter was decreased and that of 800–2,000 μm^2 diameter fiber was increased (Fig. 4B). These data suggested that IGF-1-X10 showed therapeutic effects in sarcopenic animals by ameliorating the muscle atrophy and changing the pattern of skeletal muscle CSA, implying the possible improvements of muscle function.[21–23]

The protein levels of key factors involved in the skeletal muscle atrophy were further investigated. The level of Pax7 showed increasing trend in IGF-1-X10-treated animals but that of MyoD and MyoG was comparable between the experimental groups (Fig. 4C, D). The protein level of Myh3 was significantly upregulated in IGF-1-X10-injected group. In addition, the activity of AMPK protein, a key enzyme involved in the skeletal muscle homeostasis, was further investigated as this kinase was previously reported to be activated by IGF-1 isoforms to show therapeutic effects in sarcopenic mice.[9] As shown in Fig. 4C and D, the level of phosphorylated AMPK protein was increased in the IGF-1-X10-injected skeletal muscles. Taken together, these data suggested that i.m. injection of IGF-1-X10 construct showed therapeutic effects in sarcopenia model by ameliorating the atrophic condition of aged skeletal muscle.

DISCUSSION

In this study, we demonstrate that the genomic-cDNA hybrid sequence of the *igf1* gene can express two types of human IGF-1, IGF-1 Ea and IGF-1 Ec, both *in vitro* and *in vivo*, and that IGF-1 expression activated IGF-1R and its

Figure 3. Effects of IGF-1-X10 in the CTX-induced skeletal muscle injury model. **(A)** Effect of IGF-1-X10 on normal skeletal muscle. To test whether supplementation with the IGF-1 gene induces muscle hypertrophy, IGF-1-X10 plasmid DNA was injected into the TA muscle and the muscle mass was measured after 7 days. **(B)** Effect of IGF-1-X10 in CTX muscle injury model. To test the efficacy of IGF-1-X10 encoding plasmid DNA, 100 μg/head was i.m. injected to TA muscle 3 days before CTX injury. CTX (10 μM) was administered to skeletal muscle, and the damaged skeletal muscle was prepared 7 days after injury. The mass of the TA muscle was measured and normalized to body weight. The mock control vector and the plasmid DNA expressing mature IGF-1 protein (IGF-1 Mature) were used as controls. Data are presented as the mean ± SEM. $*p < 0.05$ (one-way ANOVA or unpaired *t*-test), $n = 6$ per group. **(C–F)** Effect of IGF-1-X10 on the markers related to skeletal muscle regeneration. Plasmid DNA was i.m. injected 3 days before CTX administration. Damaged skeletal muscles were prepared at the indicated time points, and total protein was extracted, followed by Western blot analysis using specific antibodies against Pax7, MyoD, MyoG, and Myh3. GAPDH was used as loading control **(C, E)**. The intensities of the bands were quantified using ImageJ software **(D, F)**. ANOVA, analysis of variance; CTX, cardiotoxin; SEM, standard error of the mean.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Day 7
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Muscle mass/body weight (mg/g)
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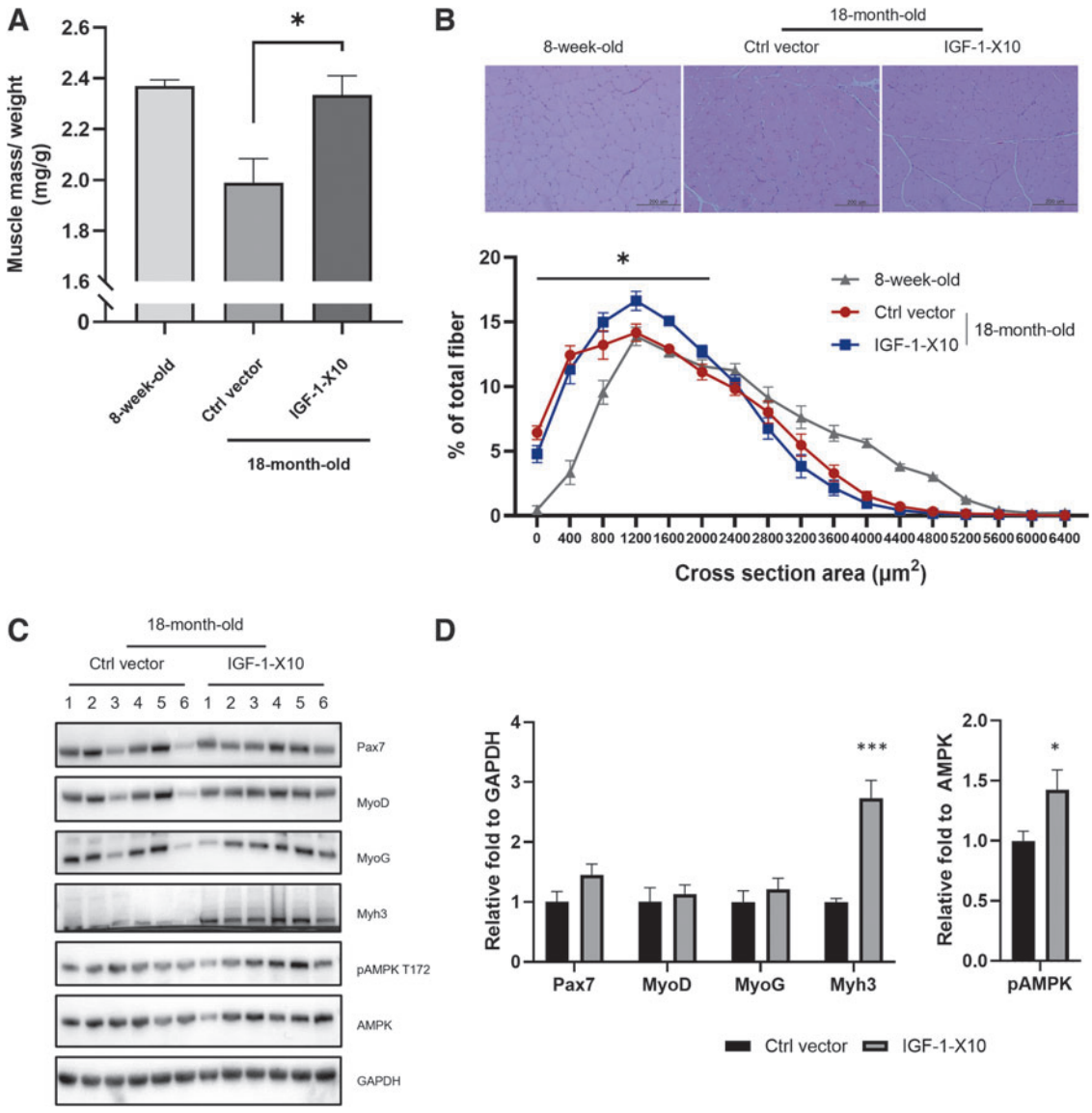

Figure 4. Effects of IGF-1-X10 in the sarcopenia model. **(A)** Effect of IGF-1-X10 on aged skeletal muscle. To test the therapeutic effects of IGF-1-X10, 100 μg/head of IGF-1-X10 encoding plasmid DNA was i.m. injected to the TA muscle and the muscle mass was analyzed 7 days after the injection. The mass of the skeletal muscle was normalized to the body weight. **(B)** Analysis of CSA in TA muscle. Skeletal muscles were fixed, and sectioned slides were subjected to H&E staining as described in Materials and Methods section. The CSA was measured in each sample using ImageJ software ($n=6$) and the representative images are shown. **(C, D)** The protein level of key factors involved in the muscle atrophy was analyzed by Western blot using specific antibodies against Pax7, MyoD, MyoG, Myh3, and pAMPK. GAPDH was used for internal control and AMPK was used to normalize pAMPK **(C)**. The intensities of the bands were quantified using ImageJ software **(D)**. Data are presented as the mean ± SEM. *$p<0.05$, ***$p<0.0001$ (one-way ANOVA, two-way ANOVA, or unpaired t-test), $n=6$ per group. CSA, cross-sectional area; H&E, hematoxylin and eosin.

downstream signaling cascades. Moreover, the transient expression of IGF-1 isoforms by i.m. injection of plasmid DNA can promote the regeneration of injured or aged skeletal muscle. It has been reported that IGF-1 and its isoforms play important roles in the differentiation and development of skeletal muscle and show therapeutic effects in multiple muscle-related disease models.[8,11]

Consistent with these reports, our results strongly suggest the involvement of IGF-1 in the skeletal muscle regeneration. The level of Myh3 protein was induced in the IGF-1-X10 injected animals and more importantly, the proportion of larger fiber diameter was increased (Figs. 3 and 4). We also observed that the activity of AMPK was significantly induced in the IGF-1-X10 treated group. AMPK has been reported to exhibit various protective effects such as anti-inflammatory and antioxidative stress in skeletal muscle, and IGF-1-X10 may show these protective effects in aged skeletal muscle by activating AMPK.[24,25] Further investigations are currently underway to elucidate the AMPK-dependent bioactivities of IGF-1.

We observed that the simultaneous injection of both IGF-1 Ea and IGF-1 Ec showed enhanced therapeutic

effects, compared with the group solely treated with IGF-1 Ea or IGF-1 Ec (data not shown). In this study, we demonstrated that the factors related to the satellite cell activation showed increased trend in IGF-1-X10-treated group, which may be mainly controlled by the IGF-1 Ec.[3] It has been reported that IGF-1 isoforms have distinct biological role(s) in aged skeletal muscle[9,25] IGF-1 Ea has been reported to increase the quantity of mitochondria and induce the antioxidant activities and IGF-1 Ec has been shown to upregulate the autophagy activity and contribute to the maintenance of neuromuscular junction integrity. Therefore, IGF-1-X10 may show pleiotropic effects in aged condition by inducing the expression of both IGF-1 Ea and IGF-1 Ec. The detailed mechanism of action investigation of IGF-1-X10 is currently underway.

As shown in Fig. 4, we demonstrated that the i.m. injection of IGF-1-X10 showed therapeutic effects in the aged skeletal muscle. Although the skeletal muscle mass in the IGF-1-X10-treated group was comparable with that in the control group, the pattern of muscle fiber distribution was different. These data indicate that there may be smaller fibers or larger fibers in the IGF-1-X10-administered group. The shift in the distribution pattern (0–400 to 800–2,000 μm^2) also strongly suggests the potential IGF-1-mediated proliferation of skeletal muscle as previously reported.[11,26] Further investigations are warranted to address the detailed mechanism of action of IGF-1-X10-induced muscle growth associated with proliferation, autophagy, or inflammatory responses, and to assess the functional recovery of skeletal muscle including the regeneration of neuromuscular junction.[9]

To our knowledge, this is the first article to demonstrate a novel sequence capable of expressing both IGF-1 isoforms simultaneously. Given the differential roles of these isoforms, it will be meaningful to induce the expression of the two isoforms simultaneously to maximize the therapeutic effect of the IGF-1 gene.[3,7] IGF-1-X10 is a minimized version of human *igf1* gene as this sequence contains exons (1, 3, 4, 5, and 6) and shorter version of introns (4 and 5), arranged identically to IGF-1 genome. IGF-1-X10 construct induced the expression of three isoforms, IGF-1 Ea, IGF-1 Eb, and IGF-1 Mature (exon 3 and exon 4), and the future development of this construct for the clinical use will be more convenient, efficient, and timesaving than that of two constructs each expressing IGF-1 Ea and IGF-1 Ec. Considering the pleiotropic effects of IGF-1 gene on the various tissue, and the growing utility of gene therapy vectors such as adeno-associated virus, it will be meaningful to develop efficient gene therapy programs based on the IGF-1-X10 sequence.

CONCLUSIONS

Taken together, we demonstrated a novel genomic-cDNA hybrid sequence of IGF-1, IGF-1-X10, expressing two isoforms simultaneously both *in vitro* and *in vivo*. This construct showed a therapeutic effect in muscle disease models by accelerating the skeletal muscle regeneration. Given the various role(s) of IGF-1 isoforms, IGF-1-X10 may be a useful agent for the efficient treatment of various skeletal muscle diseases.

AUTHORS' CONTRIBUTIONS

Data curation, formal analysis, investigation, methodology, and writing—original draft by J.L. Conceptualization, data curation, formal analysis, investigation, methodology, and supervision by K.-R.K. Conceptualization, formal analysis, and investigation by N.L. Formal analysis, investigation, and methodology by S.K. Funding acquisition, project administration, writing—review and editing by S.-s.Y. Supervision, funding acquisition, and project administration by S.K. Conceptualization, data curation, formal analysis, investigation, methodology, supervision, validation, writing—original draft, and writing—review and editing by J.L.

AUTHOR DISCLOSURE

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SUPPLEMENTARY MATERIAL

Supplementary Figure S1
Supplementary Figure S2
Supplementary Figure S3
Supplementary Figure S4

REFERENCES

1. Laron Z. Insulin-like growth factor 1 (IGF-1): A growth hormone. Mol Pathol 2001;54(5):311–316; doi: 10.1136/mp.54.5.311.

2. Yakar S, Adamo ML. Insulin-like growth factor 1 physiology. Endocrinol Metab Clin North Am 2012; 41(2):231–247; doi: 10.1016/j.ecl.2012.04.008.

3. Oberbauer AM. The regulation of IGF-1 gene transcription and splicing during development and aging. Front Endocrinol (Lausanne) 2013;4:39; doi: 10.3389/fendo.2013.00039.

4. Zheng W-H, Kar S, Doré S, et al. Insulin-like growth factor-1 (IGF-1): A neuroprotective trophic factor acting via the Akt kinase pathway. In: Advances in Research on Neurodegeneration. Springer Vienna: Vienna; 2000; pp. 261–272; doi: 10.1007/978-3-7091-6301-6_17.

5. Delafontaine P, Song Y-H, Li Y. Expression, regulation, and function of IGF-1, IGF-1R, and IGF-1

binding proteins in blood vessels. Arterioscler Thromb Vasc Biol 2004;24(3):435–444; doi: 10.1161/01.ATV.0000105902.89459.09.

6. Hakuno F, Takahashi S-I. 40 Years of IGF1: IGF1 receptor signaling pathways. J Mol Endocrinol 2018;61(1):T69–T86; doi: 10.1530/JME-17-0311.

7. Philippou A, Maridaki M, Pneumaticos S, et al. The complexity of the IGF1 gene splicing, post-translational modification and bioactivity. Mol Med 2014;20(1):202–214; doi: 10.2119/molmed.2014.00011.

8. Philippou A, Barton ER. Optimizing IGF-I for skeletal muscle therapeutics. Growth Horm IGF Res 2014;24(5):157–163; doi: 10.1016/j.ghir.2014.06.003.

9. Ascenzi F, Barberi L, Dobrowolny G, et al. Effects of IGF-1 isoforms on muscle growth and sarcopenia. Aging Cell 2019;18(3):e12954; doi: 10.1111/acel.12954.

10. Hill M, Wernig A, Goldspink G. Muscle satellite (stem) cell activation during local tissue injury and repair. J Anat 2003;203(1):89–99; doi: 10.1046/j.1469-7580.2003.00195.x.

11. Song Y-H, Song JL, Delafontaine P, et al. The therapeutic potential of IGF-I in skeletal muscle repair. Trends Endocrinol Metab 2013;24(6):310–319; doi: 10.1016/j.tem.2013.03.004.

12. Choi W, Lee J, Lee J, et al. Hepatocyte growth factor regulates macrophage transition to the M2 phenotype and promotes murine skeletal muscle regeneration. Front Physiol 2019;10:914; doi: 10.3389/fphys.2019.00914.

13. Lee Y, Park EJ, Yu SS, et al. Improved expression of vascular endothelial growth factor by naked DNA in mouse skeletal muscles: Implication for gene therapy of ischemic diseases. Biochem Biophys Res Commun 2000;272(1):230–235; doi: 10.1006/bbrc.2000.2758.

14. Choi W, Lee J, Lee J, et al. Hepatocyte growth factor regulates the MiR-206-HDAC4 cascade to control neurogenic muscle atrophy following surgical denervation in mice. Mol Ther Nucleic Acids 2018;12:568–577; doi: 10.1016/j.omtn.2018.06.013.

15. Wolff JA, Malone RW, Williams P, et al. Direct gene transfer into mouse muscle in vivo. Science (1979) 1990;247(4949):1465–1468; doi: 10.1126/science.1690918.

16. Pyun W-B, Hahn W, Kim D-S, et al. Naked DNA expressing two isoforms of hepatocyte growth factor induces collateral artery augmentation in a rabbit model of limb ischemia. Gene Ther 2010;17(12):1442–1452; doi: 10.1038/gt.2010.101.

17. Nho B, Lee J, Lee J, et al. Effective control of neuropathic pain by transient expression of hepatocyte growth factor in a mouse chronic constriction injury model. FASEB J 2018;32(9):5119–5131; doi: 10.1096/fj.201800476R.

18. Musarò A, McCullagh K, Paul A, et al. Localized Igf-1 transgene expression sustains hypertrophy and regeneration in senescent skeletal muscle. Nat Genet 2001;27(2):195–200; doi: 10.1038/84839.

19. Sousa-Victor P, García-Prat L, Muñoz-Cánoves P. Control of satellite cell function in muscle regeneration and its disruption in ageing. Nat Rev Mol Cell Biol 2022;23(3):204–226; doi: 10.1038/s41580-021-00421-2.

20. Guiraud S, Edwards B, Squire SE, et al. Embryonic myosin is a regeneration marker to monitor utrophin-based therapies for DMD. Hum Mol Genet 2019;28(2):307–319; doi: 10.1093/hmg/ddy353.

21. Maughan RJ, Watson JS, Weir J. Strength and cross-sectional area of human skeletal muscle. J Physiol 1983;338(1):37–49; doi: 10.1113/jphysiol.1983.sp014658.

22. Pant I, Chaturvedi S, Bala K, et al. Muscle histopathology in today's era of molecular genetics: Role and limitations. Ann Indian Acad Neurol 2015;18(4):398–402; doi: 10.4103/0972-2327.165455.

23. Babcock LW, Hanna AD, Agha NH, et al. MyoSight—Semi-automated image analysis of skeletal muscle cross sections. Skelet Muscle 2020;10(1):33; doi: 10.1186/s13395-020-00250-5.

24. Steinberg GR, Carling D. AMP-activated protein kinase: The current landscape for drug development. Nat Rev Drug Discov 2019;18(7):527–551; doi: 10.1038/s41573-019-0019-2.

25. Musarò A, Scicchitano BM. Counteracting sarcopenia: The role of IGF-1 isoforms. Aging 2019;11(11):3410–3411; doi: 10.18632/aging.102027.

26. Yu M, Wang H, Xu Y, et al. Insulin-like growth factor-1 (IGF-1) promotes myoblast proliferation and skeletal muscle growth of embryonic chickens via the PI3K/Akt signalling pathway. Cell Biol Int 2015;39(8):910–922; doi: 10.1002/cbin.10466.

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