



Absence of estrogen receptors delays myoregeneration and leads to intermuscular adipogenesis in a low estrogen status: Morphological comparisons in estrogen receptor alpha and beta knock out mice

Rattanatrai CHAIYASING^{1,2)}, Takuro ISHIKAWA¹⁾, Katsuhiko WARITA^{1,3)} and Yoshinao Z. HOSAKA^{1,3)*}

¹⁾Laboratory of Basic Veterinary Science, United Graduate School of Veterinary Science, Yamaguchi University, Yamaguchi 753-8515, Japan

²⁾Faculty of Veterinary Sciences, Office of Academic Affairs, Maha Sarakham University, Maha Sarakham 44000, Thailand

³⁾Department of Veterinary Anatomy, Faculty of Agriculture, Tottori University, Tottori 680-8553, Japan

ABSTRACT. This study aimed to investigate the function of estrogen receptors (ERs) in myoregeneration and intermuscular adipogenesis. Ovariectomized (OVX) ER α knockout (KO) mice and ER β KO mice were used to assess the effect of estrogen on the myoregenerative process. Tibialis anterior muscle was collected on days 7, 10, and 14 after cardiotoxin injection to assess myotube morphology and adipogenesis area. Regenerated myotubes from OVX-ER β KO mice were consistently smaller in diameter than those from OVX-ER α KO and OVX-wild-type mice, whereas the adipogenesis area of OVX-ER β KO mice was consistently greater than that of the other types. Therefore, ER β may be an influential factor in promoting myoregeneration and adipogenesis inhibition compared to ER α .

KEY WORDS: adipogenesis, estrogen, estrogen receptors, myoregeneration

J. Vet. Med. Sci.

83(7): 1022–1030, 2021

doi: 10.1292/jvms.20-0696

Received: 7 December 2020

Accepted: 22 April 2021

Advanced Epub:

10 May 2021

Estrogen deficiency in women after menopause or ovariectomy affects not only reproductive dysfunction but also non-reproductive functions such as sarcopenia, obesity and predisposition to develop frailty [17, 25, 28, 31, 33]. So far, there have been several reports that low estrogen status inhibits regeneration of damaged muscle tissue [5, 22, 29]. Skeletal muscles express estrogen receptor α (ER α) and ER β ; however, the functional difference between the two types of ERs remains unclear, especially in the morphology of muscle regeneration. Collins *et al.* [5] reported that estrogen and muscle satellite cell expression of ER α is necessary to prevent apoptosis of satellite cells. On the other hand, Seko *et al.* [36] insisted that ER β knockout (KO) mice exhibited impaired muscle regeneration following acute muscle injury, probably due to reduced proliferation and increased apoptosis of satellite cells. Thus, there are several reports on the involvement of both ERs in muscle tissue regeneration, but it was not concluded which ER action has a strong effect on myoregeneration [2, 9]. Moreover, estrogen has been reported to affect the regulation of adipocyte accumulation in regenerating muscle [6, 12, 29, 32]. When the estrogen concentration decreased, adipogenesis was accelerated in skeletal muscle tissue [3, 15, 18, 21]. However, the effect of ERs in the process of adipogenesis is poorly understood.

In this study, we chemically induced muscle damage in ER KO mice with low estrogen status and morphologically evaluated the effect of estrogen and ERs on myoregeneration and adipose tissue formation.

MATERIALS AND METHODS

Animals

Two subtypes of ERs KO (ER α KO and ER β KO) and wild-type (WT; C57BL/6) mice were used in this study. ER α KO mice were obtained by mating mice of a mixed C57BL/6 background that were heterozygous for ER α gene disruption, as described

*Correspondence to: Hosaka, Y. Z.: y-hosa@tottori-u.ac.jp

(Supplementary material: refer to PMC <https://www.ncbi.nlm.nih.gov/pmc/journals/2350/>)

©2021 The Japanese Society of Veterinary Science



This is an open-access article distributed under the terms of the Creative Commons Attribution Non-Commercial No Derivatives (by-nc-nd) License. (CC-BY-NC-ND 4.0: <https://creativecommons.org/licenses/by-nc-nd/4.0/>)

previously [10]. *Ers2^{tm1Unc/J}*, ER β gene disruption mice were purchased from The Jackson Laboratory (Bar Harbor, MA, USA), and ER β KO was obtained by mating mice of a mixed C57BL/6 background that were heterozygous [7, 23]. After 28 days of age, genotypes of both subtypes of ERs KO mice were determined by multiplex PCR. Female WT, ER α KO, and ER β KO mice were fed a commercial diet (CLEA Rodent Diet CE-2; CLEA, Tokyo, Japan) and tap water *ad libitum*. Animals were kept in an environment of a temperature of $23 \pm 1.0^\circ\text{C}$ under 12 hr light/12 hr dark by artificial illumination (lights on from 7 am to 7 pm). The animal experiments were approved by the Animal Research Committee of Tottori University, Japan (approval number: 18-T-37, 32-040).

Animal operation and muscle injury procedure

All mice (aged 8 weeks old) underwent ovariectomy (OVX) under anesthesia by intraperitoneal administration of medetomidine (Kyoritsu Seiyaku, Tokyo, Japan), midazolam (Maruishi, Osaka, Japan), and butorphanol (Meiji Seika, Tokyo, Japan) [20]. OVX mice were divided into 3 groups (n=3): OVX-WT (control), OVX-ER α KO, and OVX-ER β KO group. Four weeks after OVX (aged 12 weeks), 50 μl of 10 μM cardiotoxin (CTX) (Latoxan, Portes lès Valence, France) was injected into the right tibialis anterior (TA) muscles [13, 14, 27]. The contralateral left TA muscle was left intact and served as the non-injured control. The injured TA muscles were collected on after euthanasia on days 7, 10, and 14 (D7, D10, and D14, respectively) after CTX injection. In addition, the non-injured control (left) TA muscles were also collected at D7. All mice were sacrificed by inhalation of an overdose of isoflurane (MSD Animal Health, Osaka, Japan).

Measurement of estrogen level in serum

To measure the estradiol (E2) concentration in serum, blood was collected from the tail immediately before OVX (8 weeks old) and 4 weeks after OVX treatment (12 weeks old). Separated serum from the blood was stored at -30°C until use. The E2 concentration was measured with ELISA (Estradiol ELISA Kit; Cayman Chemical, Ann Arbor, MI, USA) and we confirmed the mice used in the experiment were in a low estrogen state.

Histological analysis

The collected muscle tissue was immediately immersed in 10% neutral buffered formalin and fixed at room temperature for 16 hr. Then, a series of procedures were performed to embed the tissue in paraffin. Hematoxylin and eosin (HE)-stained and Masson's trichrome-stained paraffin sections (6 μm thickness) were examined using an inverted light microscope (IX71, Olympus, Tokyo, Japan). Digital images were obtained and used to evaluate morphological changes in muscle fibers, regenerated myotube diameter, adipocyte accumulation and connective tissue deposition.

To evaluate myogenesis, the regenerated muscle region was defined as follows: a region of newly formed myotubes with a central nucleus, residual region of necrotic muscle fibers, and a region of regenerated myotubes of various sizes with a central nucleus. In the myoregenerated region, we randomly selected approximately 150 myotubes (including the center region of the section) per specimen (at 200 \times magnification), and the minimum diameter of muscle fiber was measured as the axis diameter of the fibers [27, 29]. Adipogenesis area (%) was defined as the area of adipocytes distributed between and among individual regenerated myotubes with central nuclei at all time points post injection or non-injured muscle fibers. For the adipogenesis area (%) calculation, 4 images of non-overlapping areas at 200 \times magnification were chosen after manual outlining of the intermuscular adipocyte area and dividing by the total area of the image using the image analysis software (ImageJ; v1.46r, National Institutes of Health, Bethesda, MD, USA) [29, 34].

Muscle sections were stained with Masson's trichrome stain to detect changes in tissue composition after CTX injury and quantify the areas of muscle (red), adipocytes (transparent) and connective tissue (blue). The stained sections were observed at a magnification of 200 \times with a light microscopy, 4 images of each section, 3 mice at each time point were randomly selected and analyzed with Image J software (National Institutes of Health). After adjusting the threshold, each area was displayed as a percentage of the total area [27].

Assessment of skeletal muscle recovery

The recovery ratio (%) of OVX mice to that of non-injured control was calculated by the average diameter of newly formed myotubes with a central nucleus of OVX mice in each group and divided by the average diameter of muscle fibers of non-injured control at each time point. Recovery ratio (%) of OVX-ER KO mice to compare with OVX-WT mice was calculated by the average diameter of muscle fibers or newly formed myotubes with a central nucleus in each group was divided by the average diameter of OVX-WT mice at each time point. The myoregeneration rate ($\mu\text{m}/\text{day}$) of OVX mice was calculated by the inclination is changed in average diameter of regenerated muscle fibers in each group was divided by the duration period of day after injury.

Statistical analysis

Data were expressed as average \pm standard deviation (SD). Obtained data were analyzed with StatView software, version 5.0 (SAS Institute, Cary, NC, USA) using Student's *t*-test, two-tailed to compare the E2 concentration in serum. One-way ANOVA followed by Bonferroni's *post-hoc* test was used to compare the histometrical data. Significance level was set as $P < 0.05$.

RESULTS

Before OVX treatment of the ER α KO group, E2 concentration in serum was particularly higher than that in the other groups. The E2 concentration in the serum of all groups (three types of OVX mice) 4 weeks after OVX treatment was significantly lower than that before treatment. For this reason, the mice used in the muscle regeneration experiment are in a “low estrogen state” (Fig. 1).

On D7 after CTX treatment, the muscle fibers with a central nucleus appeared, and the start of muscle regeneration was confirmed. Moreover, adipocytes appeared between the regenerated muscle fibers in all mice. That is, muscle regeneration and adipocyte differentiation were observed on the same specimen on D7 (Supplementary Fig. 1). Cell infiltration in connective tissue and regenerated muscle fibers appeared by D7 in all OVX treated mice. However, OVX-ER β KO mice showed residual necrotic muscle fibers on D7. At D10, OVX-ER β KO mice showed cell infiltration between the regenerated muscle fibers. Moreover, muscle fibers of all three types of OVX mice on D14 appeared to be well regenerated when compared with non-injured muscle fibers (Fig. 2A).

When comparing the diameters of muscle fibers of the non-injured control, there was a significant difference between each group, and the diameters of non-injured control of OVX-ER α KO mice and -ER β KO mice were significantly smaller than those of non-injured control of OVX-WT. Moreover, when comparing the ER KO mice, OVX-ER β KO mice showed a significantly lower value than that of OVX-ER α KO mice (Fig. 2B). Throughout the 14 days of the experimental period after CTX treatment, the diameter of newly formed myotubes increased over time in all groups. The average diameter of the myotube at D14 was OVX-WT mice: $40.2 \pm 2.83 \mu\text{m}$, OVX-ER α KO mice: $35.4 \pm 2.69 \mu\text{m}$ and OVX-ER β KO mice: $30.2 \pm 2.81 \mu\text{m}$, with a recovery ratio from $65.4 \pm 0.37\%$ to $71.5 \pm 0.92\%$ of the average diameter of non-injured control in each group.

The ratio of the average diameter of muscle fibers in each group to non-injured control at each time point is shown in Table 1. The value of OVX-ER β KO group was significantly lower than those of OVX-WT and OVX-ER α KO group, respectively, but all values tended to increase. On the other hand, Table 2 shows the ratio (%) of the average diameter of muscle fibers in each group to the average diameter of OVX-WT mice at each time point. From D7 to D14, the value of OVX-ER α KO mice changed from $83.5 \pm 1.39\%$ to $88.0 \pm 0.50\%$, and the value of OVX-ER β KO mice changed from $63.6 \pm 1.47\%$ to $75.2 \pm 0.42\%$. In both groups, their values increased with the passage of days. However, the value of OVX-ER β KO mice group was significantly lower than OVX-ER α KO mice. Table 3 shows the rate of increase in muscle fiber diameter during the 7 days (first half period: D7 to D10, second half period: D10 to D14) after injury, that is, repair speed of regenerated muscle fibers. At the same duration period, there was no significant difference between groups, but OVX-ER β KO mice was higher than the other groups on first half period and OVX-ER α KO mice was higher than the other groups on second half period. Of the three groups, the OVX-ER β KO mice indicated the highest value through the period (D7 to D14).

Intermuscular adipocytes appeared among the regenerated myotubes in OVX mice at D7. Adipocyte differentiation was observed within the regenerated muscle in OVX mice at D14 (Fig. 3A). The adipogenesis area (%) in OVX-ER β KO mice was the largest compared to other types at all time points. At D14, the adipogenesis area in OVX-ER β KO mice was about 1.49 and 1.95 times higher than that in OVX-ER α KO and OVX-WT mice, respectively (Fig. 3B).

Masson's trichrome staining revealed the ratio of muscle, fat and connective tissue. Connective tissue was originally present between muscle fibers, but in D7, the ratio (%) of connective tissue in all three types of OVX mice increased sharply compared to non-injured control. As the days went by, the ratio of connective tissue decreased with D10 and D14, and on the contrary, the ratio of fat increased. Interestingly, the ratio of muscle in each group remained almost constant regardless of the time after injury (Fig. 4).

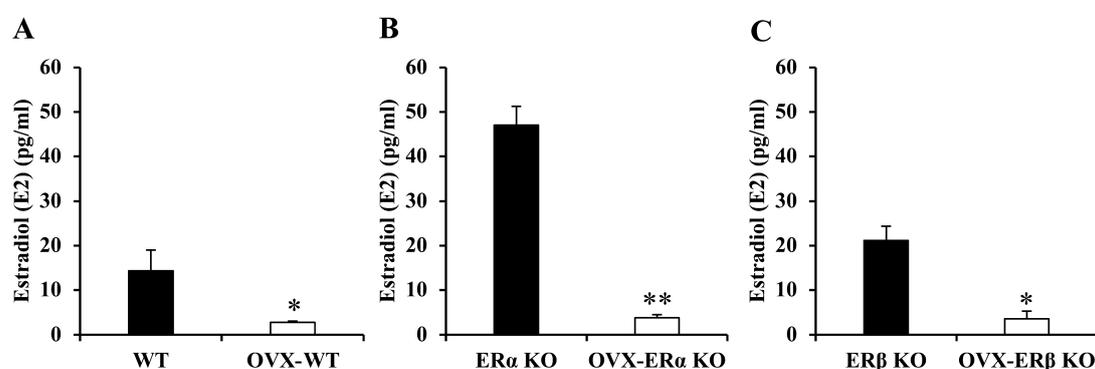


Fig. 1. The estradiol (E2) concentration in serum before and after ovariectomy (OVX) treatment of all three types of mice. E2 concentration in wild-type (WT), estrogen receptor α knockout (ER α KO) and ER β KO mice (A, B, C). Data were shown as average \pm standard deviation (SD), $n=3$ /groups, * indicates significant difference between group before and after OVX treatment, $P<0.05$, ** indicates significant difference between groups before and after OVX treatment, $P<0.01$, Student's t -test, two-tailed.

Table 1. Ratio (%) of the average diameter of muscle fibers in each group to the average diameter of non-injured control at each time point

Type of mice	Time post injury		
	D7	D10	D14
OVX-WT	53.4 ± 0.08	62.9 ± 1.57	71.5 ± 0.92
OVX-ER α KO	49.1 ± 0.82**	58.8 ± 1.16*	69.1 ± 0.39**
OVX-ER β KO	41.5 ± 0.96** [‡]	55.0 ± 1.04** [†]	65.4 ± 0.37** [‡]

Average ratio percentage values of each group were compared using the Bonferroni/Dunn *post-hoc* tests. Average ± standard deviation; n=3. * $P < 0.05$, ** $P < 0.01$ vs. OVX-WT, respectively, [†] $P < 0.05$, [‡] $P < 0.01$ vs. OVX-ER α KO, respectively. D, day; OVX, ovariectomized; WT, wild-type; ER, estrogen receptor; KO, knockout.

Table 2. Ratio (%) of the average diameter of muscle fibers in each group to the average diameter of wild-type mice at each time point

Type of mice	Non-injured	Time post injury		
		OVX		
		D7	D10	D14
ER α KO	90.9 ± 1.06	83.5 ± 1.39	85.0 ± 1.68	88.0 ± 0.50
ER β KO	82.1 ± 1.09**	63.6 ± 1.47**	71.7 ± 1.35**	75.2 ± 0.42**

Data were shown as average ratio percentage ± standard deviation. ** indicates significant difference from ER α KO mice, $P < 0.01$. OVX, ovariectomized; D, day; ER, estrogen receptor; KO, knockout.

Table 3. Repair speed ($\mu\text{m}/\text{day}$) of regenerated muscle fibers in each group at several duration periods of days after injury

Type of mice	Duration period		
	D7 to D10	D10 to D14	D7 to D14
OVX-WT	1.79 ± 0.29	1.20 ± 0.13	1.45 ± 0.07
OVX-ER α KO	1.66 ± 0.20	1.32 ± 0.05	1.47 ± 0.03
OVX-ER β KO	2.08 ± 0.16	1.21 ± 0.04	1.58 ± 0.02

Data were shown as average/day ± standard deviation. D, day; OVX, ovariectomized; WT, wild-type; ER, estrogen receptor; KO, knockout.

DISCUSSION

In this study, we measured the diameter of regenerated muscle fibers in ER KO mice after injury in low estrogen status. The diameter of muscle fibers in ER KO mice was significantly smaller than that in WT mice. To maintain muscle fiber formation, the following steps should proceed in an orderly manner: 1) proliferation of satellite cells, 2) differentiation into myoblasts, 3) fusion myoblasts, and 4) elongation of myotubes [11, 19, 35]. However, in ER KO (both subtypes) mice, the muscle fiber diameter was significantly low. Satellite cells are stem cells of muscle fibers, and their initial cell growth is important for muscle regeneration smoothly. Several researchers mentioned that estrogen and ERs are necessary for satellite cell survival and proliferation [5, 22, 24, 36, 43]. If satellite cell survival and proliferation were inhibited due to low estrogen status, this experiment may imply that the low number of satellite cells at early stages reflects the delay of myoregeneration.

The muscle fiber diameter was significantly smaller in ER β KO mice than in ER α KO mice. Based on these results, it can be inferred that the action of estrogen *via* ER β has a stronger effect on the maintenance of muscle tissue homeostasis than ER α . ER β is thought to regulate not only satellite cell growth, but also prevent apoptosis in the differentiation steps of muscle fiber formation [36]. However, ER α is thought to be involved in the maintenance of the number of satellite cells in the proliferation process [5]. Our results indicated that the recovery ratio of ER β KO mice was lower than that of ER α KO mice, indicating that the loss of ER β could not accelerate muscle fiber formation in several steps. On the other hand, ER α KO mice showed better regeneration of muscle fiber formation than ER β KO mice. Taken together, ER β is likely to play a more important role in regulating myoregeneration than ER α in muscle tissue.

A difference was observed in the muscle regeneration speed between ER α KO mice and ER β KO mice. That is, the muscle regeneration speed of ER β KO mice tended to be high in the first half period (D7 to D10) and that of ER α KO tended to be high in the second half period (D10 to D14). It is considered that this difference in myoregeneration rate depending in the timing after injury may reflect the functional regulation by each ER at each stage of myoregeneration process such as proliferation and differentiation of satellite cells, and myotube formation. The detail of functional regulation by ER need to be further examined.

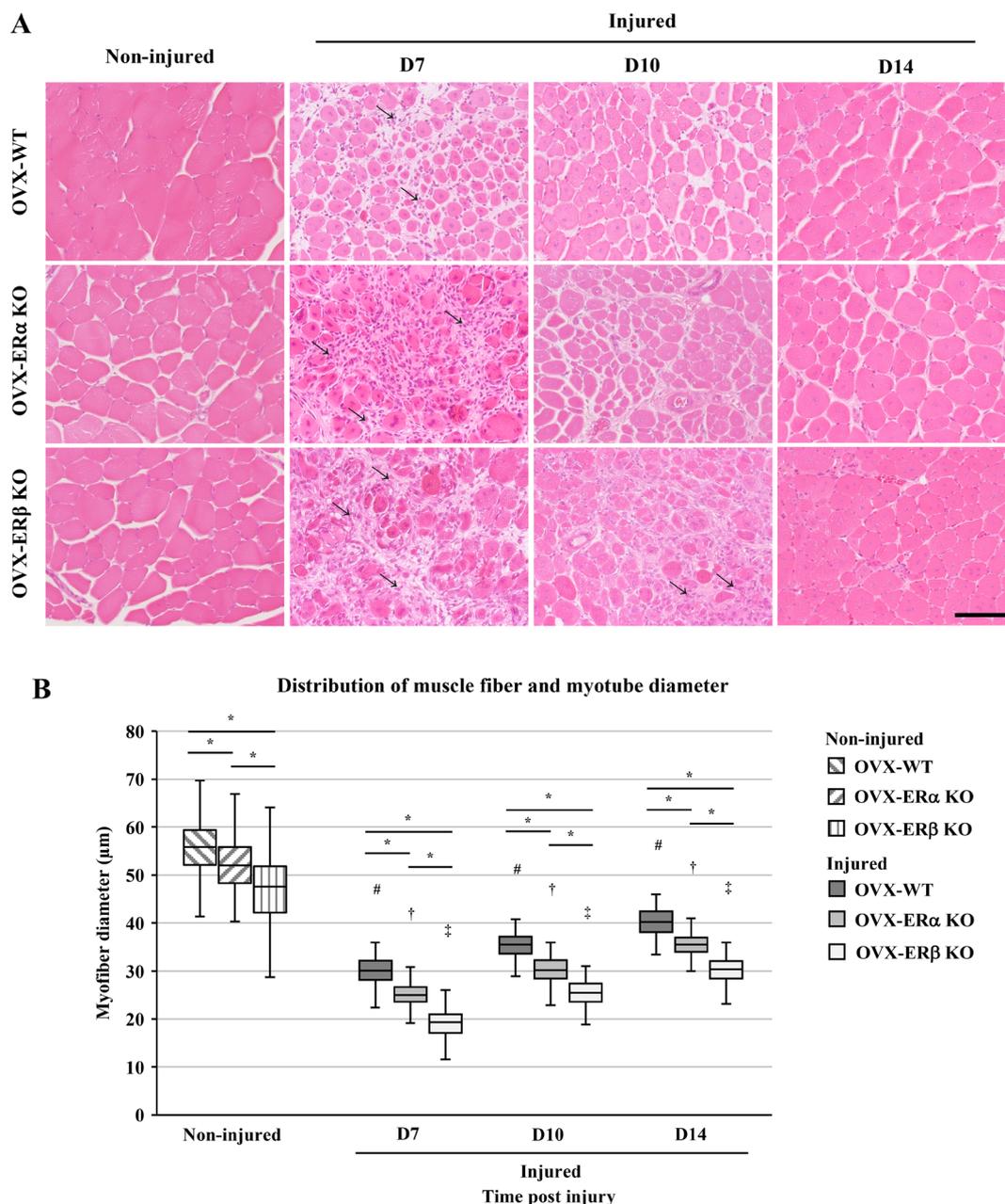


Fig. 2. Regeneration of muscle in ovariectomy (OVX) treated wild-type (WT), estrogen receptor α knockout (ER α KO) and ER β KO mice by cardiotoxin (CTX) injection. (A) Sections of the tibialis anterior (TA) muscle of non-injured control of OVX-WT mice, OVX-ER α KO and OVX-ER β KO. Sections of the TA muscle injected with CTX at day 7, 10 and 14 (D7, D10 and D14) post injection. Arrows indicate cell infiltration among regenerated muscle fibers. (B) Distribution of non-injured muscle fiber diameter and newly formed muscle fiber diameter at D7, D10 and D14 post CTX injection. Data are expressed as average \pm standard deviation (SD), # indicates significant differences from non-injured control in OVX-WT mice, † indicates significant differences from non-injured control in OVX-ER α KO mice, ‡ indicates significant differences from non-injured control in OVX-ER β KO mice, $P < 0.01$, * indicates significant difference between groups at time post injury, $P < 0.01$. Scale bar=100 μ m.

All types of OVX mice after CTX injury were significantly increased in the adipogenesis area (%) at D7, D10, and D14 after injury compared with non-injured control. OVX-ER β KO mice showed a larger adipogenesis area (%) than OVX-ER α and OVX-WT mice. Delay of myoregeneration and induction of intermuscular adipogenesis in CTX-injured OVX-ER KO mice may alter the muscle and fat metabolism pathways and alter gene expression in muscle tissue. For example, myostatin, a member of the transforming growth factor- β superfamily, is a protein synthesized and released by myocytes that act as muscle cells to suppress myogenesis, such as muscle cell growth and differentiation [8, 30]. In a previous study, myostatin inhibited intermuscular preadipocyte differentiation *in vitro* [26, 39]. However, in a low estrogen state, muscle regeneration itself was suppressed,

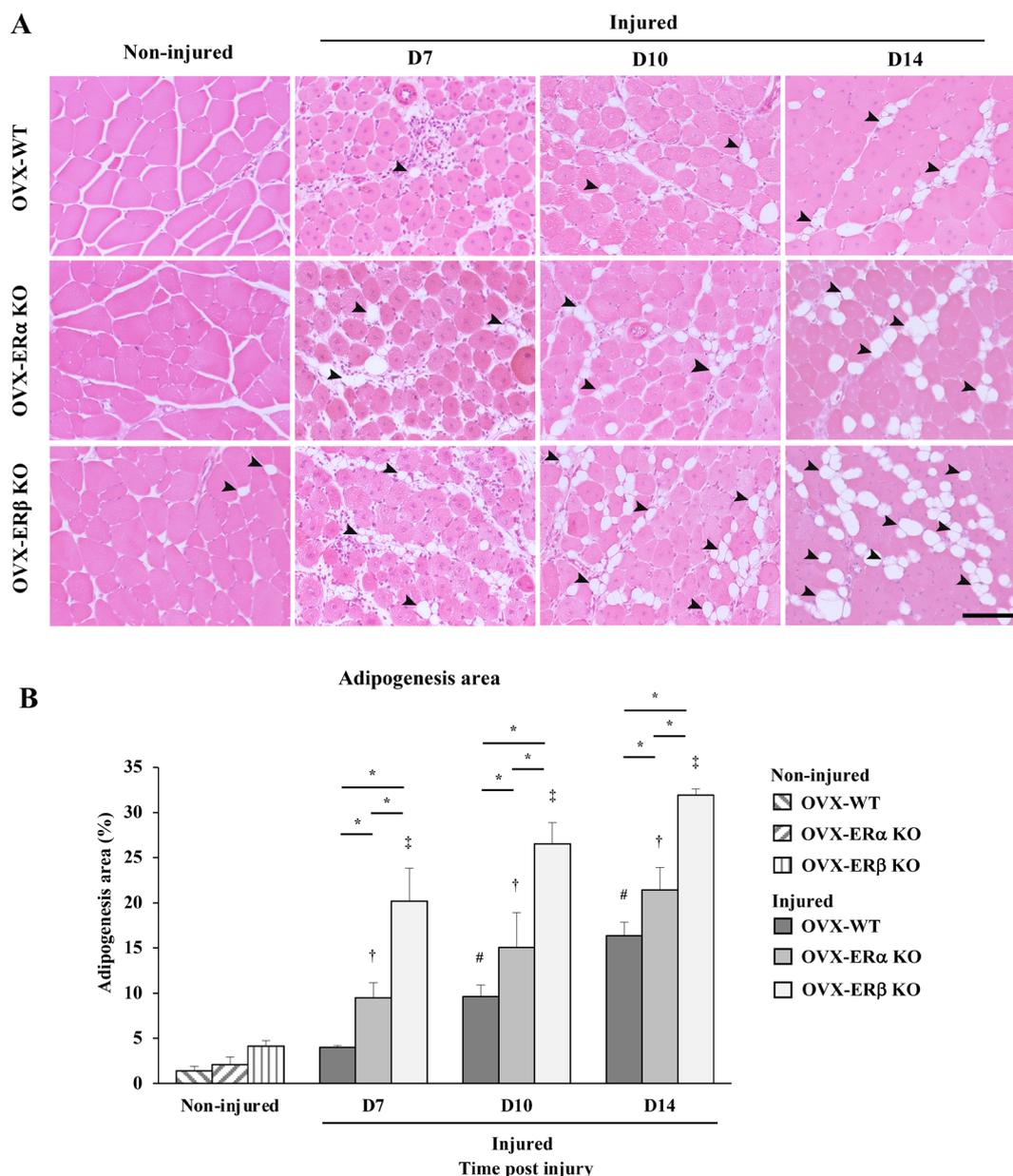


Fig. 3. Intermuscular adipogenesis in ovariectomy (OVX) treated wild-type (WT), estrogen receptor α knockout (ER α KO) and ER β KO mice after cardiotoxin (CTX) injection. (A) Sections of the non-injured tibialis anterior (TA) muscle of OVX-WT mice, OVX-ER α KO mice and OVX-ER β KO mice. Sections of the TA muscle injected with CTX in OVX-WT mice, OVX-ER α KO mice and OVX-ER β KO mice at day 7, 10 and 14 (D7, D10 and D14) post injection. Arrow heads indicate adipocytes among non-injured muscle fibers and regenerated muscle fibers. (B) Adipogenesis area (%) of all three types of non-injured OVX mice and OVX mice injected CTX at D7, D10 and D14 post injection. Data are expressed as average \pm standard deviation (SD), # indicates significant differences from non-injured control in OVX-WT mice, † indicates significant differences from non-injured in OVX-ER α KO mice, ‡ indicates significant differences from non-injured control in OVX-ER β KO mice, $P < 0.05$, * indicates significant difference between groups at time post injury, $P < 0.05$. Scale bar=100 μ m.

resulting in a decrease in muscle mass and production and expression of myostatin, which may have promoted the proliferation and differentiation of adipocytes. In contrast, it has been reported that adipocyte stimulated interleukin-6 expression in muscle cells could suppress muscle cell differentiation [37]. Platelet-derived growth factor receptor α (PDGFR α)-positive mesenchymal progenitor cells are thought to be the origin of intermuscular adipocytes [42]. In our experiments, adipocytes increased between muscle fibers after tissue damage, but the area of adipocytes formed varied significantly between ER α KO mice and ER β KO mice. It is easy to imagine that the number and proliferation of PDGFR α -positive mesenchymal progenitor cells have a great influence on the proliferation of adipocytes after muscle tissue damage, but to our best knowledge, there are no reports of ER expression in PDGFR α -positive mesenchymal progenitor cells. By elucidating the relationship between ER and PDGFR α -positive mesenchymal progenitor cells, we will be able to get closer to elucidating the mechanism of ectopic adipocyte accumulation after postmenopausal

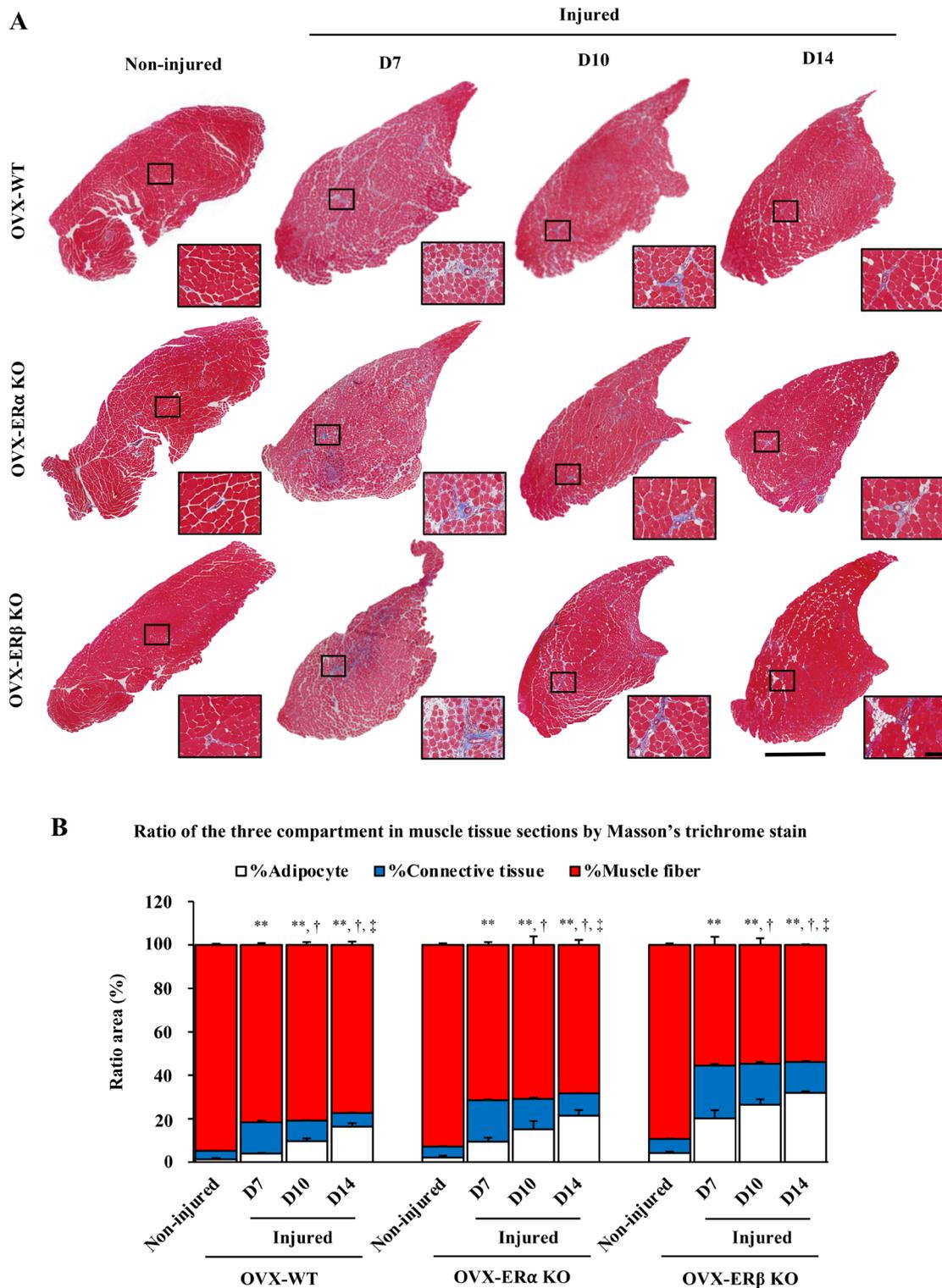


Fig. 4. Masson's trichrome stain. (A) Sections of tibialis anterior (TA) muscle injected with cardiotoxin (CTX) and without CTX (non-injured as control) in ovariectomized-wild-type (OVX-WT) mice, OVX- estrogen receptor α knockout (OVX-ER α KO) mice and OVX-ER β KO mice all time post CTX injection at day 7, 10 and 14 (D7, D10 and D14). Square areas within low magnification of whole section images are magnified in the right bottom panels. (B) Ratio of the three compartments in muscle tissue sections by Masson's trichrome stain. Muscle fiber area (red), adipocyte area (white), and connective tissue area (blue) (%) of all three types of non-injured OVX mice and OVX mice injected CTX at several time points post injection at day 7, 10 and 14. Data are expressed as average \pm standard deviation (SD), ** indicates significant differences from non-injured control of connective tissue area (%), † indicates significant differences from D7 of connective tissue area (%), ‡ indicates significant differences from D10 of connective tissue area (%), $P < 0.01$, Scale bar=1 mm (low magnification), 100 μ m (high magnification).

muscle injury.

Initially, this study aimed to clarify whether estrogen and ER affect muscle regeneration. The results of the comparison in ER KO mice revealed that ER function in myoregeneration is more important for ER β than ER α . Also, it has been reported that estrogen and ER are important factors in the proliferation and differentiation of satellite cells [5, 22, 36, 43]. However, it is interesting that even in OVX-ER KO mice, in which the low estrogen status and the absence of ER, muscle regeneration was delayed. This phenomenon may indicate that there are other mechanisms besides estrogen-ER that maintain the progression of myoregeneration. Insulin-like growth factor-1 (IGF-1), which is known to be a major factor in the increase in skeletal muscle mass [1], and IGF-1 and its receptor (IGF-1R) appear to be linked to estrogen and ER. Temporary interruption of estrogen supply weakened ER signaling and dramatically increased IGF-1 compared to normal conditions [16]. In addition, administration of estrogen to OVX animals suppressed IGF-1 production [41]. There are other cases where IGF-1 signaling is regulated by ER pathway. For example, in lung cancer cells, estrogen has been shown to elevate the IGF-1 signaling pathway *via* ER β [40]. In addition, there is an interaction between IGF-1R and ER α , which act synergistically to promote the proliferation of nucleus pulposus cells [4]. However, there are still many uncertainties about the relationship between estrogen and IGF-1, and their receptors in muscle tissue. Focusing on these factors, it is necessary to elucidate the detailed mechanism of myogenesis and myoregeneration. Of course, it is quite possible that factors other than those mentioned above are involved in the formation of muscle, fat and connective tissue after muscle injury. It is also reasonable to assume that the many number of infiltrated cells observed in the connective tissue between muscle fibers after muscle injury perform a variety of functions in a complex manner. Therefore, further research is needed to determine the biological significance of these cells and phenomena.

In this experiment, adipose accumulation was observed in the skeletal muscle tissue of OVX mice after CTX injury (Figs. 3 and 4). Moreover, muscle fiber diameter and cross-sectional area of TA muscle did not recover until the CTX pre-injury level (Figs. 2 and 4, Table 1). In sarcopenia, accumulation of ectopic adipose tissue is observed in skeletal muscle, as well as atrophy of muscle fibers and deterioration of muscle strength and physical function [17, 38]. Comparing the CTX-injured and sarcopenic muscle, there are many morphologically similar parts. We have not investigated the function of CTX-injured mice, but it can be easily inferred that the muscle mass and strength of TA muscle of these mice are reduced. The mice used in this experiment may be a good model candidate for pathophysiological studies of sarcopenia.

In conclusion, our data may indicate that low estrogen affects myoregeneration *via* the ER. In addition, its action was more remarkable *via* ER β rather than by ER α , and it was morphologically shown that removal of these ERs delayed muscle regeneration and promoted adipose tissue formation. Targeting estrogen and ER may provide clues for maintaining muscle tissue homeostasis.

POTENTIAL CONFLICTS OF INTEREST. The authors have nothing to disclose.

ACKNOWLEDGMENTS. The Authors thank Dr. Pierre Chambon, Institute for Genetics and Cellular and Molecular Biology, France, for providing ER α KO mice. This work was supported by JSPS Grant-in-Aid for Scientific Research (B) Grant Number JP17H03934.

REFERENCES

1. Adams, G. R. and McCue, S. A. 1998. Localized infusion of IGF-I results in skeletal muscle hypertrophy in rats. *J. Appl. Physiol.* (1985) **84**: 1716–1722. [Medline] [CrossRef]
2. Brown, M., Ning, J., Ferreira, J. A., Bogener, J. L. and Lubahn, D. B. 2009. Estrogen receptor- α and - β and aromatase knockout effects on lower limb muscle mass and contractile function in female mice. *Am. J. Physiol. Endocrinol. Metab.* **296**: E854–E861. [Medline] [CrossRef]
3. Campbell, S. E. and Febbraio, M. A. 2001. Effect of ovarian hormones on mitochondrial enzyme activity in the fat oxidation pathway of skeletal muscle. *Am. J. Physiol. Endocrinol. Metab.* **281**: E803–E808. [Medline] [CrossRef]
4. Chen, R. S., Zhang, X. B., Zhu, X. T. and Wang, C. S. 2020. The crosstalk between IGF-1R and ER- α in the proliferation and anti-inflammation of nucleus pulposus cells. *Eur. Rev. Med. Pharmacol. Sci.* **24**: 5886–5894. [Medline]
5. Collins, B. C., Arpke, R. W., Larson, A. A., Baumann, C. W., Xie, N., Cabelka, C. A., Nash, N. L., Juppi, H. K., Laakkonen, E. K., Sipilä, S., Kovanen, V., Spangenburg, E. E., Kyba, M. and Lowe, D. A. 2019. Estrogen regulates the satellite cell compartment in females. *Cell Rep.* **28**: 368–381.e6. [Medline] [CrossRef]
6. Contreras-Shannon, V., Ochoa, O., Reyes-Reyna, S. M., Sun, D., Michalek, J. E., Kuziel, W. A., McManus, L. M. and Shireman, P. K. 2007. Fat accumulation with altered inflammation and regeneration in skeletal muscle of CCR2-/- mice following ischemic injury. *Am. J. Physiol. Cell Physiol.* **292**: C953–C967. [Medline] [CrossRef]
7. Curtis Hewitt, S., Couse, J. F. and Korach, K. S. 2000. Estrogen receptor transcription and transactivation: Estrogen receptor knockout mice: what their phenotypes reveal about mechanisms of estrogen action. *Breast Cancer Res.* **2**: 345–352. [Medline] [CrossRef]
8. Deng, B., Zhang, F., Wen, J., Ye, S., Wang, L., Yang, Y., Gong, P. and Jiang, S. 2017. The function of myostatin in the regulation of fat mass in mammals. *Nutr. Metab. (Lond.)* **14**: 29. [Medline] [CrossRef]
9. Diel, P. 2014. The role of the estrogen receptor in skeletal muscle mass homeostasis and regeneration. *Acta Physiol. (Oxf.)* **212**: 14–16. [Medline] [CrossRef]
10. Dupont, S., Krust, A., Gansmuller, A., Dierich, A., Chambon, P. and Mark, M. 2000. Effect of single and compound knockouts of estrogen receptors α (ER α) and β (ER β) on mouse reproductive phenotypes. *Development* **127**: 4277–4291. [Medline] [CrossRef]
11. Forcina, L., Cosentino, M. and Musarò, A. 2020. Mechanisms regulating muscle regeneration: insights into the interrelated and time-dependent phases of tissue healing. *Cells* **9**: 1297. [Medline] [CrossRef]
12. Girousse, A., Gil-Ortega, M., Bourlier, V., Bergeaud, C., Sastourné-Arrey, Q., Moro, C., Barreau, C., Guissard, C., Vion, J., Arnaud, E., Pradère, J. P., Juin, N., Casteilla, L. and Sengenès, C. 2019. The release of adipose stromal cells from subcutaneous adipose tissue regulates ectopic

- intramuscular adipocyte deposition. *Cell Rep.* **27**: 323–333.e5. [Medline] [CrossRef]
13. Hardy, D., Besnard, A., Latil, M., Jouvion, G., Briand, D., Thépenier, C., Pascal, Q., Guguin, A., Gayraud-Morel, B., Cavaillon, J. M., Tajbakhsh, S., Rocheteau, P. and Chrétien, F. 2016. Comparative study of injury models for studying muscle regeneration in mice. *PLoS One* **11**: e0147198. [Medline] [CrossRef]
 14. Harris, J. B. 2003. Myotoxic phospholipases A2 and the regeneration of skeletal muscles. *Toxicon* **42**: 933–945. [Medline] [CrossRef]
 15. Hausman, G. J., Basu, U., Du, M., Fernyhough-Culver, M. and Dodson, M. V. 2014. Intermuscular and intramuscular adipose tissues: Bad vs. good adipose tissues. *Adipocyte* **3**: 242–255. [Medline] [CrossRef]
 16. Iida, M., Tsuboi, K., Niwa, T., Ishida, T. and Hayashi, S. I. 2019. Compensatory role of insulin-like growth factor 1 receptor in estrogen receptor signaling pathway and possible therapeutic target for hormone therapy-resistant breast cancer. *Breast Cancer* **26**: 272–281. [Medline] [CrossRef]
 17. Ikeda, K., Horie-Inoue, K. and Inoue, S. 2019. Functions of estrogen and estrogen receptor signaling on skeletal muscle. *J. Steroid Biochem. Mol. Biol.* **191**: 105375. [Medline] [CrossRef]
 18. Jackson, K. C., Wohlers, L. M., Lovering, R. M., Schuh, R. A., Maher, A. C., Bonen, A., Koves, T. R., Ilkayeva, O., Thomson, D. M., Muoio, D. M. and Spangenburg, E. E. 2013. Ectopic lipid deposition and the metabolic profile of skeletal muscle in ovariectomized mice. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **304**: R206–R217. [Medline] [CrossRef]
 19. Karalaki, M., Fili, S., Philippou, A. and Koutsilieris, M. 2009. Muscle regeneration: cellular and molecular events. *In Vivo* **23**: 779–796. [Medline]
 20. Kawai, S., Takagi, Y., Kaneko, S. and Kurosawa, T. 2011. Effect of three types of mixed anesthetic agents alternate to ketamine in mice. *Exp. Anim.* **60**: 481–487. [Medline] [CrossRef]
 21. Kiens, B. 2006. Skeletal muscle lipid metabolism in exercise and insulin resistance. *Physiol. Rev.* **86**: 205–243. [Medline] [CrossRef]
 22. Kitajima, Y. and Ono, Y. 2016. Estrogens maintain skeletal muscle and satellite cell functions. *J. Endocrinol.* **229**: 267–275. [Medline] [CrossRef]
 23. Kregel, J. H., Hodgins, J. B., Couse, J. F., Enmark, E., Warner, M., Mahler, J. F., Sar, M., Korach, K. S., Gustafsson, J. Å. and Smithies, O. 1998. Generation and reproductive phenotypes of mice lacking estrogen receptor β . *Proc. Natl. Acad. Sci. USA* **95**: 15677–15682. [Medline] [CrossRef]
 24. LaBarge, S., McDonald, M., Smith-Powell, L., Auwerx, J. and Huss, J. M. 2014. Estrogen-related receptor- α (ERR α) deficiency in skeletal muscle impairs regeneration in response to injury. *FASEB J.* **28**: 1082–1097. [Medline] [CrossRef]
 25. La Colla, A., Pronsato, L., Milanese, L. and Vasconsuelo, A. 2015. 17 β -Estradiol and testosterone in sarcopenia: Role of satellite cells. *Ageing Res. Rev.* **24** Pt B: 166–177. [Medline] [CrossRef]
 26. Li, F., Yang, H., Duan, Y. and Yin, Y. 2011. Myostatin regulates preadipocyte differentiation and lipid metabolism of adipocyte via ERK1/2. *Cell Biol. Int.* **35**: 1141–1146. [Medline] [CrossRef]
 27. Mahdy, M. A. A., Lei, H. Y., Wakamatsu, J., Hosaka, Y. Z. and Nishimura, T. 2015. Comparative study of muscle regeneration following cardiotoxin and glycerol injury. *Ann. Anat.* **202**: 18–27. [Medline] [CrossRef]
 28. Maher, A. C., Akhtar, M. and Tarnopolsky, M. A. 2010. Men supplemented with 17 β -estradiol have increased β -oxidation capacity in skeletal muscle. *Physiol. Genomics* **42**: 342–347. [Medline] [CrossRef]
 29. McHale, M. J., Sarwar, Z. U., Cardenas, D. P., Porter, L., Salinas, A. S., Michalek, J. E., McManus, L. M. and Shireman, P. K. 2012. Increased fat deposition in injured skeletal muscle is regulated by sex-specific hormones. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **302**: R331–R339. [Medline] [CrossRef]
 30. McPherron, A. C. and Lee, S. J. 1997. Double muscling in cattle due to mutations in the myostatin gene. *Proc. Natl. Acad. Sci. USA* **94**: 12457–12461. [Medline] [CrossRef]
 31. Muramatsu, M. and Inoue, S. 2000. Estrogen receptors: how do they control reproductive and nonreproductive functions? *Biochem. Biophys. Res. Commun.* **270**: 1–10. [Medline] [CrossRef]
 32. Nagai, S., Ikeda, K., Horie-Inoue, K., Takeda, S. and Inoue, S. 2018. Estrogen signaling increases nuclear receptor subfamily 4 group A member 1 expression and energy production in skeletal muscle cells. *Endocr. J.* **65**: 1209–1218. [Medline] [CrossRef]
 33. Nilsson, S. and Gustafsson, J. Å. 2011. Estrogen receptors: therapies targeted to receptor subtypes. *Clin. Pharmacol. Ther.* **89**: 44–55. [Medline] [CrossRef]
 34. Ochoa, O., Sun, D., Reyes-Reyna, S. M., Waite, L. L., Michalek, J. E., McManus, L. M. and Shireman, P. K. 2007. Delayed angiogenesis and VEGF production in CCR2 $^{-/-}$ mice during impaired skeletal muscle regeneration. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **293**: R651–R661. [Medline] [CrossRef]
 35. Relaix, F. and Zammit, P. S. 2012. Satellite cells are essential for skeletal muscle regeneration: the cell on the edge returns centre stage. *Development* **139**: 2845–2856. [Medline] [CrossRef]
 36. Seko, D., Fujita, R., Kitajima, Y., Nakamura, K., Imai, Y. and Ono, Y. 2020. Estrogen receptor β controls muscle growth and regeneration in young female mice. *Stem Cell Reports* **15**: 577–586. [Medline] [CrossRef]
 37. Seo, K., Suzuki, T., Kobayashi, K. and Nishimura, T. 2019. Adipocytes suppress differentiation of muscle cells in a co-culture system. *Anim. Sci. J.* **90**: 423–434. [Medline] [CrossRef]
 38. Srikanthan, P., Hevener, A. L. and Karlamangla, A. S. 2010. Sarcopenia exacerbates obesity-associated insulin resistance and dysglycemia: findings from the National Health and Nutrition Examination Survey III. *PLoS One* **5**: e10805. [Medline] [CrossRef]
 39. Sun, W. X., Dodson, M. V., Jiang, Z. H., Yu, S. G., Chu, W. W. and Chen, J. 2016. Myostatin inhibits porcine intramuscular preadipocyte differentiation in vitro. *Domest. Anim. Endocrinol.* **55**: 25–31. [Medline] [CrossRef]
 40. Tang, H., Liao, Y., Chen, G., Xu, L., Zhang, C., Ju, S. and Zhou, S. 2012. Estrogen upregulates the IGF-1 signaling pathway in lung cancer through estrogen receptor- β . *Med. Oncol.* **29**: 2640–2648. [Medline] [CrossRef]
 41. Tsai, W. J., McCormick, K. M., Brazeau, D. A. and Brazeau, G. A. 2007. Estrogen effects on skeletal muscle insulin-like growth factor 1 and myostatin in ovariectomized rats. *Exp. Biol. Med. (Maywood)* **232**: 1314–1325. [Medline] [CrossRef]
 42. Uezumi, A., Fukada, S., Yamamoto, N., Takeda, S. and Tsuchida, K. 2010. Mesenchymal progenitors distinct from satellite cells contribute to ectopic fat cell formation in skeletal muscle. *Nat. Cell Biol.* **12**: 143–152. [Medline] [CrossRef]
 43. Velders, M., Schleipen, B., Fritzemeier, K. H., Zierau, O. and Diel, P. 2012. Selective estrogen receptor- β activation stimulates skeletal muscle growth and regeneration. *FASEB J.* **26**: 1909–1920. [Medline] [CrossRef]