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FOXO1 delays skeletal muscle regeneration and suppresses myoblast proliferation

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Unloading stress, such as bed rest, inhibits the regenerative potential of skeletal muscles; however, the underlying mechanisms remain largely unknown. FOXO1 expression, which induces the upregulated expression of the cell cycle inhibitors p57 and Gadd45α, is known to be increased in the skeletal muscle under unloading conditions. However, there is no report addressing FOXO1-induced inhibition of myoblast proliferation. Therefore, we induced muscle injury by cardiotoxin in transgenic mice overexpressing FOXO1 in the skeletal muscle (FOXO1-Tg mice) and observed regeneration delay in skeletal muscle mass and cross-sectional area in FOXO1-Tg mice. Increased p57 and Gadd45α mRNA levels, and decreased proliferation capacity were observed in C2C12 myoblasts expressing a tamoxifen-inducible active form of FOXO1. These results suggest that decreased proliferation capacity of myoblasts by FOXO1 disrupts skeletal muscle regeneration under FOXO1-increased conditions, such as unloading.

Key words: FOXO1; cell proliferation; skeletal muscle

Skeletal muscle is the largest organ in the human body comprising approximately 40% of the normal body weight; therefore, it is important to maintain skeletal muscle function for normal metabolism and mobility. Although damaged skeletal muscle has the ability to regenerate through proliferation of satellite cells (muscle stem cells, progenitor of myoblasts), several studies have indicated that unloading stress, as evidenced by bed rest and tail suspension in mice, inhibits the regenerative potential of the skeletal muscle.[1,2] Therefore, the regenerative capacity of skeletal muscle is considered to be decreased under unloading conditions.

Forkhead box protein O1 (FOXO1) is a member of the forkhead-type family of transcription factors and regulates various physiological phenomena, including cell proliferation, differentiation, survival, and metabolism.[3] It is well known that FOXO1 expression levels increase in skeletal muscle under low nutrient, unloading, and certain pathological conditions,[4,5] and transgenic mice specifically overexpressing FOXO1 in skeletal muscle show remarkable muscle atrophy.[6]

It is reported that FOXO1 induces the upregulation of the cell cycle inhibitors p57[7] and Gadd45α,[8] which bind to cyclin-dependent kinase and inhibit transition of the cell cycle at the G1/S and G2/M phases, respectively. However, the ability of FOXO1 to inhibit proliferation of myoblasts remains unknown.

Although unloading stress inhibits the regenerative potential of skeletal muscles,[2] the underlying mechanisms remain unclear. We speculated that the suppression of cell proliferation in the skeletal muscle by FOXO1, whose expression is increased, is involved in this process. Therefore, in this study, we used transgenic mice specifically overexpressing FOXO1 in skeletal muscle (FOXO1-Tg mice)[6] to determine whether muscle regeneration is delayed by FOXO1 overexpression. Furthermore, we used C2C12 myoblasts expressing a FOXO1-estrogen receptor fusion protein, which translocates to the nucleus and is activated by the addition of tamoxifen (TAM),[8] to examine whether FOXO1 increases p57 or Gadd45α expression or suppresses myoblast proliferation.

Materials and methods

Transgenic (Tg) mice. FOXO1-Tg mice were generated previously[6] and were maintained at a constant temperature of 24 °C under a constant 12-h light/dark cycle. Care of the mice was conducted in accordance with the guidelines of our institution.

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Abbreviations: FOXO1, forkhead box protein O1; Tg, transgenic; WT, wildtype; Gadd45α, growth arrest and DNA damage 45α; Pax7, paired box protein 7; EDL, extensor digitorum longus; DAPI, 4,6-diamidino-2-phenylindole; CTX, cardiotoxin; HE, hematoxylin–eosin; TAM, tamoxifen; ER, estrogen receptor; EdU, 5-ethynyl-2′-deoxyuridine.

Isolation of skeletal muscle single fibers. Ten-weeks-old C57BL/6 mice were sacrificed by cervical dislocation. The extensor digitorum longus (EDL) muscle was isolated and digested in collagenase type 1, as previously described.[9] Isolated fibers were used for immunostaining.

Immunostaining of muscle fibers. The immunostaining protocol was performed as previously described.[10] Briefly, 30–40 isolated muscle fibers were collected in 1.5-ml tubes, then fixed with 4% paraformaldehyde, and washed with phosphate-buffered saline (PBS) containing 0.025% Tween20. Subsequently, the cells were permeabilized and concomitantly blocked with 0.3% Triton X-100 in PBS containing 10% goat serum. Mouse anti-Pax7 (sc-81648) and rabbit anti-FOXO1 (H-128) primary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Secondary antibodies were obtained from Life Technologies (Paisley, UK). Nuclei were counterstained with mounting medium containing 4,6-diamidino-2-phenylindole (DAPI).

Muscle injury and histology. Muscle injury was induced by injections of cardiotoxin (CTX) into the gastrocnemius muscles. The experiments were performed twice on separate days. In experiment 1, ten-weeks-old WT ($n = 3$) and FOXO1-Tg ($n = 3$) mice were used. In experiment 2, 19–25-weeks-old WT ($n = 4$) and 19–22-weeks-old FOXO1-Tg ($n = 4$) mice were used. Briefly, mice were anesthetized using somnopentyl (Kyoritsu Seiyaku Corporation, Tokyo, Japan), and then, 100 of 10 μM CTX was injected into both the gastrocnemius muscles at 5–6 points. Muscles were harvested at 0 (untreated), 7, and 14 days post-injection; their weights were measured, and the muscles were fixed in 10% formaldehyde neutral buffer to assess the completion of regeneration and repair. Relative muscle weights were calculated using combined data from experiments 1 and 2 ($n = 7$ for both WT and FOXO1-Tg mice). In experiment 1, hematoxylin–eosin (HE) staining was consigned to Applied Medical Research Laboratory (Osaka, Japan). Cryosections were viewed on an Olympus IX81 inverted fluorescence microscope, and digital images were acquired with an Olympus DP72 microscope digital camera using CellSens imaging software. Images were optimized and assembled into figures using ImageJ software (Image Processing and Analysis in Java; http://imagej.nih.gov/ij/).

Cell culture. We used C2C12 myoblasts, as previously described,[8] that express fusion proteins containing a constitutively active form of human FOXO1 [FOXO1(3A), where three Akt phosphorylation sites (Thr24, Ser256, and Ser319) are replaced by alanine residues] in frame with a modified TAM-specific version of the murine estrogen receptor ligand-binding domain: C2C12 (FOXO1-3A-ER). C2C12 myoblasts were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 1% penicillin–streptomycin in 5% CO_2 at 37 °C. Whenever applicable, TAM was added to the medium.

Study of myoblast proliferation. In experiments measuring C2C12 (FOXO1-3A-ER) cell proliferation, the Click-iT Plus EdU Alexa Fluor 488 Flow Cytometry Assay Kit (Invitrogen, Life Technologies, Paisley, UK) was used as per the manufacturer's instructions. Briefly, 1×10^4 myoblasts/ml in 12-well plates were cultured with TAM for 24 h to activate the FOXO1-ER fusion protein. Then, 10-mM EdU stock solution was added to the culture up to a 10 μM final concentration and subsequently cultured for an additional 24 h. Immunostained myofibers and plated myoblasts were viewed on a inverted laboratory microscope with LED Illumination Leica DM IL LED. Digital images were acquired with a Leica DFC3000 G system using Leica LAS Image Overlay software (ver. 4.6), optimized, and assembled into figures using ImageJ software.

Quantitative RT-PCR. Total RNA was prepared using TRIzol reagent (Life Technologies Japan, Tokyo, Japan). cDNA was synthesized from 0.5 μg of total RNA using the ReverTra Ace qPCR RT Master Mix (TOYOBO, Tokyo, Japan). Gene expression levels were measured as previously described.[11] The following mouse-specific primer pairs were used: 36B4 Fw, 5′-GGCCCTGCACTCTCGCTTTC-3′; 36B4 Rv, 5′-TGCCAGGACGCGCTTGT-3′; p57 Fw, 5′-GACGATGGAAGAACTCTGGG-3′; p57 Rv, 5′-AGCGTACTCCTTGCACATGG3′; Gadd45α Fw, 5′-CGTAGACCCCGATAACGTGGTA-3′; Gadd45α Rv, 5′-CGGATGAGGGTGAAATGGAT-3′.

Statistical analysis. Statistical analysis was performed using the Student's *t*-test. Data are expressed as mean ± SE. A probability (p) value of < 0.05 was considered statistically significant.

Results and discussion

FOXO1 transgene expression in skeletal muscle of FOXO1-Tg mice

We used previously generated FOXO1-Tg mice.[6] Skeletal muscle single fibers were isolated from the EDL muscle of FOXO1-Tg and WT mice. When isolated fibers were co-immunostained using an antibody against FOXO1 and satellite cell marker protein Pax7, strong FOXO1 signals were observed from the FOXO1-Tg mice. In addition, Pax7-positive cells, namely satellite cells, were observed on fibers of both WT and Tg-mice (Fig. 1). The FOXO1 transgene is driven by the α-actin promoter, which is known to be expressed in satellite cells.[12] Though we did not observe the co-localization of FOXO1 or Pax7 in satellite cells because the FOXO1 signal was unexpectedly strong, we considered that FOXO1 was likely overexpressed in the satellite cells of the FOXO1-Tg mice.

Overexpressed FOXO1 inhibits muscle regeneration

To examine how skeletal muscle regeneration is affected by FOXO1, we compared the extent of muscle regeneration by injecting CTX into the gastrocnemius muscles of WT and FOXO1-Tg mice.

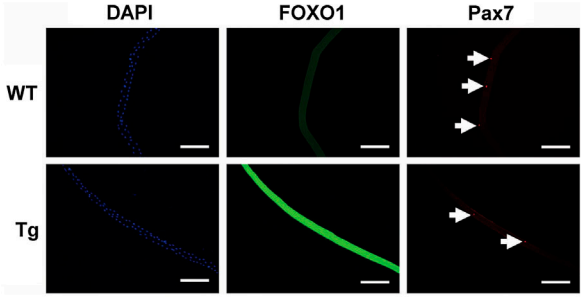

Fig. 1. FOXO1 transgene expression in FOXO1-Tg mice.

Notes: Representative fields of approximately 30–40 myofibers isolated from WT and FOXO1-Tg mice that were collected in microtubes and immediately fixed, immunostained, and counterstained are shown. Myonuclei, blue; FOXO1, green; and Pax7, red. White arrows indicate Pax7-positive cells (satellite cells). Exposure times were unified between colors. Scale bar = 100 μm.

The gastrocnemius muscles were isolated on days 0 (untreated, control), 7, and 14 post-injection, weighed, and cryosectioned for HE staining. The results showed that the muscle weight of WT mice recovered at 7 days, but that of FOXO1-Tg did not recover at 14 days (WT 7 days vs. Tg 7 days, $p = 0.106$, and WT 14 days vs. Tg 14 days, $p < 0.05$; Fig. 2(A)). The average muscle weights were graphed in relative values, taking the weight on day 0 as 100. The data on the absolute muscle weights are shown in the Supplemental Table 1. The muscle weight of the FOXO1-Tg mice was approximately 30 mg less than that of the WT mice on day 0; subsequently, the difference increased up to 52 mg at 14 days post-injection (Supplemental Table 1). By the histochemical analysis of HE staining on day 0, a smaller myofiber diameter and more cell nuclei were observed in the FOXO1-Tg mice than those observed in the WT mice (Fig. 2(B)). Furthermore, HE staining revealed more severe muscle damage in FOXO1-Tg mice than in WT mice at 7 days, as indicated by a greater influx of likely inflammatory cells. At 14 days, there were many myofibers in WT mice that were positive for centronuclear, as a regeneration marker. On the other hand, myofibers were thinner, and condensed nuclear areas were observed in the FOXO1-Tg mice, which suggests incomplete regeneration of injured muscles (Fig. 2(B)). We measured and graphed the cross-sectional area (CSA) per myofiber of the images shown in Fig. 2(B). CSA of both mice decreased at 7 days post-injection. At 14 days, CSA of fibers in WT mice recovered to higher levels than at

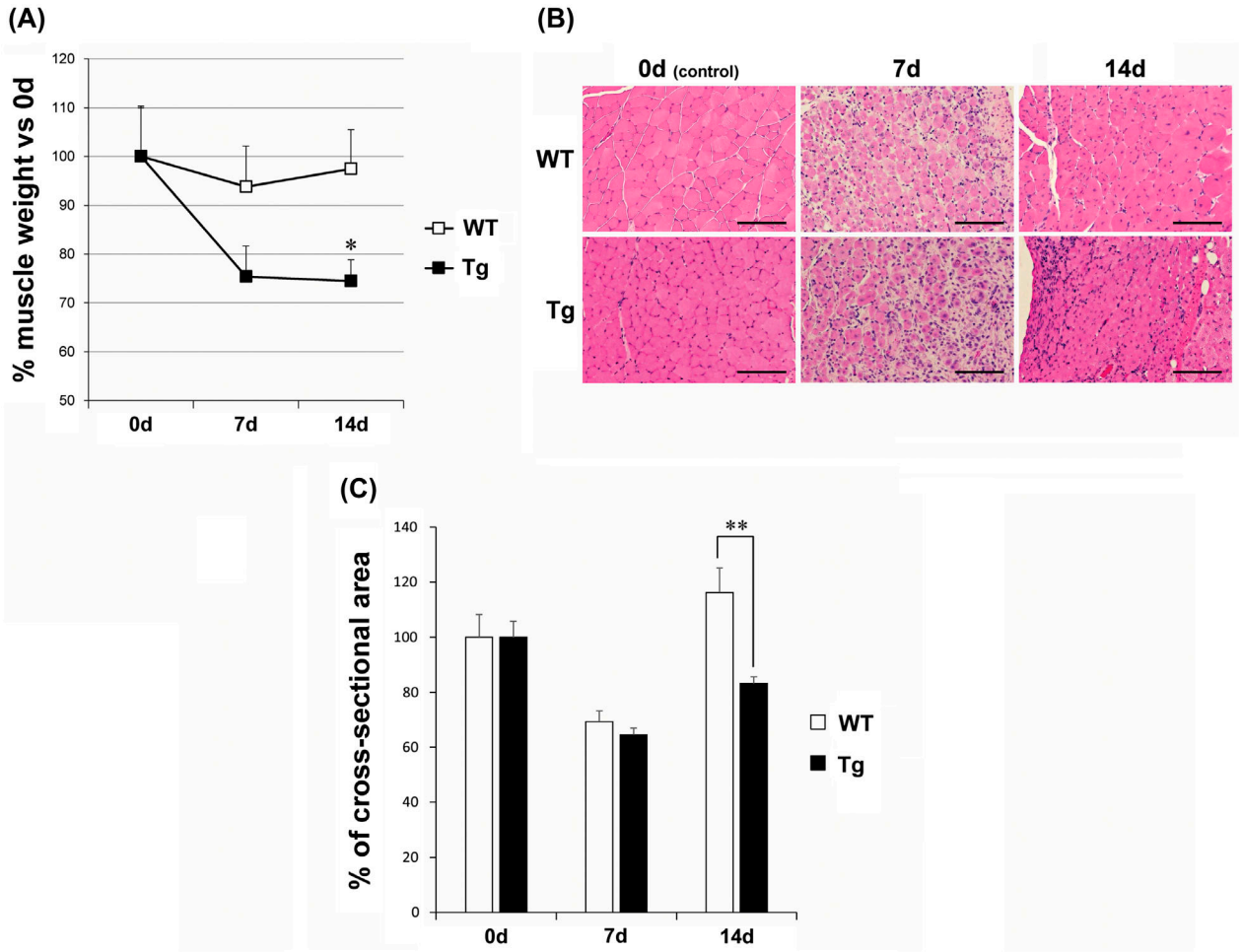

Fig. 2. Muscle regeneration declines in FOXO1-Tg mice.

Notes: CTX was injected into both gastrocnemius muscles of WT and FOXO1-Tg mice. The gastrocnemius muscles were isolated on days 0 (untreated), 7, and 14 post-injection. (A) Average muscle weights were graphed in relative values to day 0 as 100. WT: $n = 7$, FOXO1-Tg: $n = 7$ (B) HE staining of the cross-section of injured muscles (scale bar = 100 μm). (C) Cross-sectional area (CSA) per fiber. Average CSA was graphed in relative values to day 0 as 100. WT: $n = 3$, FOXO1-Tg: $n = 3$ The graphs include an average of approximately 300 fibers. *$p < 0.05$, **$p < 0.01$.

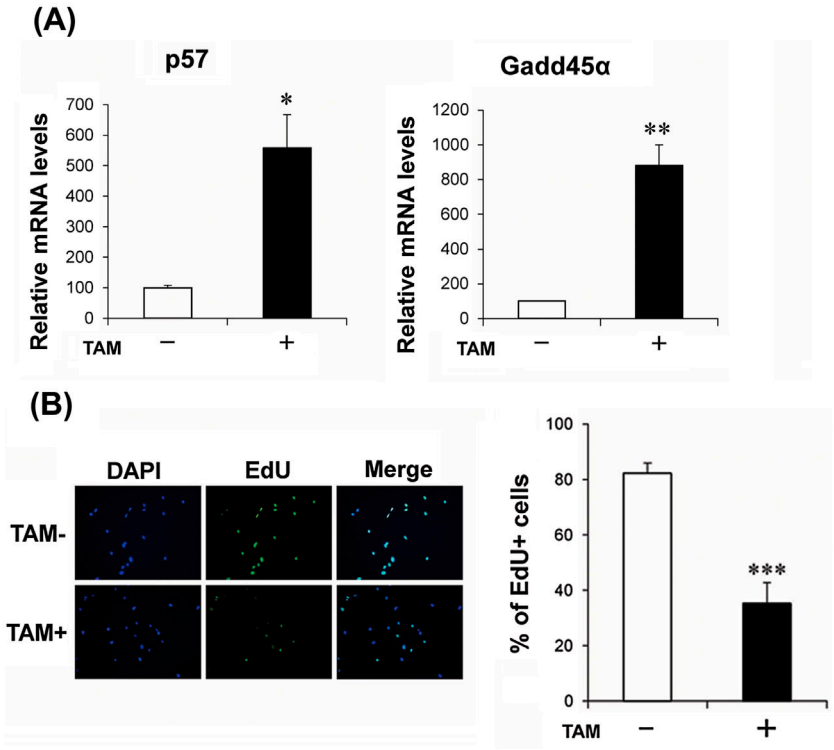

Fig. 3. Proliferation capacity was decreased in C2C12 by FOXO1 activation.

Notes: C2C12 (FOXO1–3A-ER) myoblasts were cultured in 12-well plates at 1×10^4 cells/ml, then incubated for 24 h with 1 μM TAM. (A) qRT-PCR was performed after an additional 24-h culture. Graph shows relative mRNA levels. (B) Consequently, 10 μM EdU was added, and the culture was incubated for 24 h. Nuclear and EdU in C2C12 myoblasts were stained and enclosed. >800 DAPI+ cells were counted in ten random fields from three individual wells. Representative fields and average numbers of EdU+ cells are shown. Nuclei, blue, and EdU, green. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with TAM (−).

0 day, but not that of FOXO1-Tg mice (Fig. 2(C)). These results suggest that FOXO1 overexpression delays skeletal muscle regeneration.

FOXO1 induces cell cycle inhibitors and decreases C2C12 myoblasts proliferation

It is reported that FOXO1 induces the expression of cell cycle inhibitors.[7,8] Therefore, to determine whether FOXO1 suppresses myoblast proliferation, we activated FOXO1 in C2C12 myoblasts and measured proliferation capacity and gene expression. When treated with TAM, FOXO1 localized to the nucleus in activated C2C12 myoblasts. As shown in Fig. 3(A), p57 and Gadd45α mRNA levels were increased in TAM-treated C2C12 cells seeded at the same density and cultured in growth medium containing TAM or vehicle for 24 h. Next, C2C12 cells were treated with 5-ethynyl-2′-deoxyuridine (EdU) for 24 h, and then fixed and stained for comparison of proliferation capacity by counting the number of EdU-positive cells. As a result, proliferation capacity was decreased in the TAM-treated group (Fig. 3(B)). These observations suggest that FOXO1 decreases C2C12 myoblast proliferation through the upregulation of p57 and Gadd45α. Conditional deletion of FOXO3, a member of the FOXO family, in satellite cells reportedly increases cell proliferation.[13] Thus, FOXO1 overexpression may decrease primary myoblast proliferation; however, to date, no reports have been published.

From the above, these results suggest that FOXO1 suppresses cell proliferation in skeletal muscle. Therefore, it is conceivable that formation of new muscle tissue is disrupted under conditions of upregulated FOXO1 expression. On the other hand, FOXO1 is reported to suppress myoblast differentiation.[14,15] Notably, suppression of myoblast differentiation may also be involved in disturbances in skeletal muscle regeneration. Therefore, a decline in skeletal muscle regeneration capacity during unloading, especially under bedridden or inactive conditions, may be associated by upregulation of FOXO1 expression.

Authors contribution

A.Y., Y.H., and Y.K. conception and design of research; A.Y, Y.H., Y.H., and Y.K. performed experiments; A.Y., Y.H., and Y.O. analyzed data; A.Y, Y.H., Y.O., and Y.K. interpreted results of experiments; A.Y., Y.H., and Y.K. drafted manuscript; All authors read and approved the final manuscript.

Disclosure statement

No potential conflict of interest was reported by the authors.

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Supplemental materials

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