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## TGF $\beta$ 1 signaling protects chondrocytes against oxidative stress via FOXO1–autophagy axis

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### Abstract

**Objective:** The forkhead box O1 (FOXO1) transcription factor is a key regulator of autophagy. In chondrocytes, reduced FOXO1 expression with aging causes osteoarthritis due to dysfunction of autophagy, but the mechanisms underlying regulation of FOXO1 expression and the reduction in expression with aging remain unclear. We investigated the mechanism by which transforming growth factor  $\beta$ 1 (TGF $\beta$ 1) signaling regulates the FOXO1–autophagy axis.

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#### Contributions

IK, YA, MKL, and YN contributed to the conception and design of the study. IK, HT, TS, MT, MK, and TU contributed to the acquisition of data. IK and YA analyzed data and drafted the manuscript. All authors have approved the final version of the manuscript.

#### Conflict of interest

The authors declare that they have no conflict of interest.

**Methods:** Expression of FOXO1 was measured in chondrocytes after TGFβ1 treatment. Immunohistochemistry was performed to estimate the levels of activin receptor-like kinase 5 (ALK5) and FOXO1 in the knee joints of young, middle-aged and old mice. The effects of the ALK5 inhibitor and SMAD3 or SMAD2 knockdown on FOXO1 expression were evaluated. The role of TGFβ1 in autophagy after hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) treatment was analyzed. The protective effect of TGFβ1 against H<sub>2</sub>O<sub>2</sub> treatment was assessed by cell viability assay and TUNEL assay.

**Results:** TGFβ1 promoted the expression of *FOXO1* mRNA and protein. Both ALK5 and FOXO1 expression decreased with aging. ALK5 inhibition and SMAD3 knockdown suppressed induction of FOXO1 expression by TGFβ1, whereas SMAD2 knockdown increased it. TGFβ1 promoted the expression of microtubule-associated proteins 1A/1B light chain 3B (LC3)-I protein via the SMAD3–FOXO1 pathway. Furthermore, under H<sub>2</sub>O<sub>2</sub> treatment, TGFβ1 promoted expression of LC3-II. TGFβ1 pretreatment suppressed cell death of chondrocytes following H<sub>2</sub>O<sub>2</sub> treatment, but this protective effect was abolished by FOXO1 knockdown.

**Conclusions:** TGFβ1 protects chondrocytes against oxidative stress via the FOXO1–autophagy axis, and a reduction in ALK5 expression might cause reduced FOXO1 expression with aging.

### Keywords

Transforming growth factor β1; Activin receptor-like kinase 5; Forkhead box O1; Autophagy; Oxidative stress

## Introduction

Aging is a major risk factor for osteoarthritis (OA), and aged chondrocytes exhibit multiple senescent phenotypes<sup>1 2 3 4</sup>. In particular, dysfunctional autophagy in aged chondrocytes leads to impaired maintenance of cellular homeostasis under oxidative stress<sup>5 6</sup>. Autophagy prevents oxidative stress–induced cell death by degradation and recycling damaged organelles and proteins<sup>7 8 9</sup>. Reactivation of autophagy is a promising therapeutic strategy for OA<sup>10 11 12 13 14 15</sup>. Therefore, it is necessary to further elucidate the mechanism underlying dysfunctional autophagy in OA progression.

Forkhead box O (FOXO) transcription factors play pivotal roles in autophagy-related gene expression and are intimately involved in aging<sup>16 17 18 19 20 21</sup>. In human chondrocytes, expression of FOXO1 and FOXO3 decreases with aging, and FOXO1 downregulation is associated with reduced expression of LC3 and BECLIN1<sup>22 23</sup>. Furthermore, cartilage-specific *Foxo1*-knockout mice develop OA at a young age, concomitant with reduced expression of autophagy-related genes<sup>24</sup>.

The mechanisms underlying regulation of FOXO1 expression and its reduced expression in aged chondrocytes remain unclear. Transforming growth factor β1 (TGFβ1) is a positive regulator of FOXO1 protein expression in human chondrocytes<sup>22</sup>. In addition, in chondrogenic progenitor cells undergoing chondrogenic differentiation, TGFβ1 promotes the expression and activity of FOXO1 via SMAD3 signaling<sup>25</sup>. Intriguingly, expression of activin receptor-like kinase 5 (ALK5), a receptor for TGFβ1, also decreases in aged chondrocytes, resulting in OA<sup>6 26 27 28</sup>.

Hence, we hypothesized that TGF $\beta$ 1 promotes the expression of FOXO1 through the ALK5-SMAD3 pathway in chondrocytes, and that the reduction in the level of ALK5 might contribute to the reduction in FOXO1 expression with aging. In this study, we investigated the mechanism by which TGF $\beta$ 1 signaling promotes FOXO1 expression in chondrocytes and assessed the role of TGF $\beta$ 1 in autophagy.

## Method

### Cell isolation from human articular cartilage

Human articular cartilage was obtained from 30 donors with OA undergoing knee replacement surgery and providing informed consent with the approval of the Ethics Committee of Kyushu University Hospital. Cartilage was incubated with collagenase (2 mg/ml) in Dulbecco's Modified Eagle Medium (DMEM)/Ham's F12 medium (Gibco, Langley, OK, USA) with 10% fetal bovine serum (FBS) (Gibco) for 12 hours. After digestion, chondrocytes were isolated and cultured in 10-cm dishes in DMEM/Ham's F12 medium with 10% FBS. The chondrocytes were used in experiments after they reached sub-confluence.

### Chemical inhibitor treatment

RepSox (Merck Millipore, Billerica, MA, USA) (1  $\mu$ M) was used to inhibit the activity of ALK5<sup>29</sup>. The inhibitor was dissolved in dimethyl sulfoxide (DMSO), and control groups were incubated with an equivalent amount of DMSO lacking inhibitor.

Chloroquine (CQ) (MBL, Aichi, Japan) (25  $\mu$ M) was used to assess autophagy flux.

### siRNA transfection

Human chondrocytes were seeded in 12-well plates at a density of  $1 \times 10^5$  cells/well. After the cells reached sub-confluence, they were transfected with small interfering RNAs (siRNAs) (5 nM) against *FOXO1* (siFOXO1), *SMAD3* (siSMAD3) or *SMAD2* (siSMAD2) (Santa Cruz Biotechnology, Dallas, TX, USA) in Opti-MEM medium (Gibco) for 6 hours using Lipofectamine RNAiMAX (Thermo Fisher Scientific, Waltham, MA, USA). Subsequently, culture medium was changed to DMEM/Ham's F12 with 10% FBS. The control group was transfected with Control siRNA (siCtrl) (Santa Cruz Biotechnology). After 2 days, the cells were used in experiments. The transfection efficiency was evaluated by qRT-PCR and western blotting.

### RNA extraction and quantitative real-time RT-PCR (qRT-PCR)

Total RNA was extracted from human chondrocytes using the TRIzol reagent (Thermo Fisher Scientific). Total RNA (100 ng) from each sample was reverse-transcribed to complementary DNA using the PrimeScript RT reagent kit (Takara Bio, Kusatsu, Japan). qRT-PCR was performed on a Light Cycler 2.0 System (F. Hoffmann-La Roche AG, Basel, Switzerland) using TB Green Premix EX TaqII (Takara Bio). The sequences of the forward and reverse primers were shown in Supplementary Table 1. Data were normalized against the corresponding levels of *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) or *ribosomal protein S18* (*RPS18*), house keeping genes.

## Western blotting

Whole-cell lysates were extracted from human chondrocytes using the Cell Lytic M (Sigma-Aldrich, Saint Louis, MO, USA) with protease inhibitor (cOMplete™ Mini; Sigma-Aldrich) and phosphatase inhibitor (PhosSTOP; Roche Diagnostics, Mannheim, Germany). Nuclear and cytoplasmic extracts were isolated using nuclear and cytoplasmic extraction reagents (Thermo Fisher Scientific). Cell lysates were electrophoresed in 4–12% gradient polyacrylamide gels (Thermo Fisher Scientific), and the resolved proteins were transferred to nitrocellulose membranes (Amersham Biosciences, Arlington Heights, IL, USA). Membranes were blocked with blocking buffer (Takara Bio), washed in Tris-buffered saline with Tween (TBST), and incubated with primary antibodies against FOXO1 (#2880; Cell Signaling Technology, Danvers, MA, USA), pFOXO1 (#9461; Cell Signaling Technology), SMAD2/3 (#8685; Cell Signaling Technology), SMAD3 (#9523; Cell Signaling Technology), SMAD2 (#5339; Cell Signaling Technology), pSMAD3 (#9520; Cell Signaling Technology), pSMAD2 (#3108; Cell Signaling Technology), LC3 (#3868; Cell Signaling Technology), GAPDH (#5174; Cell Signaling Technology) diluted 1:1000, and LAMIN A/C (H-110; Santa Cruz Biotechnology) diluted 1:200 in Can Get Signal Immunoreaction Enhancer Solution 1 (TOYOBO, Osaka, Japan). After washing in TBST, secondary anti-rabbit IgG antibodies (#7074; Cell Signaling Technology) diluted 1:1000 in Can Get Signal Immunoreaction Enhancer Solution 2 (TOYOBO, Osaka, Japan) were added. Immunoreactivity was detected with ECL Prime (Amersham Biosciences) and photographed on an Ez Capture MG (ATTO, Tokyo, Japan). Band densities were calculated using CS Analyzer 3.0 (ATTO).

## Mice

C57BL/6J male mice were used in the experiments. Mice were housed in a specific pathogen-free condition with freedom to access to food and water. Young, middle-aged and old knee joints were collected from the hind legs of 3-, 13- and 20-month-old mice, respectively (n=5 per group). All animal experiments were approved by the Animal Experiment Committee of Kyushu University. Experiments were performed under the rules of our institution.

## Immunohistochemistry

Knee joints from mice were fixed with 4% paraformaldehyde (PFA) (Wako Pure Chemical Industries) for 2 days, decalcified with K-CX (FALMA, Tokyo, Japan) for 2 days, and embedded in paraffin. Sections were cut into 4 µm-thick sections, deparaffinized, and rehydrated; antigen retrieval was performed by incubation overnight with ethylenediaminetetraacetic acid (EDTA) (1 mM) at pH 8.0. Endogenous peroxidase activity was blocked by incubation with 3% hydrogen peroxidase (H<sub>2</sub>O<sub>2</sub>) in methanol for 30 minutes. After blocking with normal horse serum (Vectastain Universal Elite ABC kit; Vector Laboratories, Burlingame, CA, USA) for 30 minutes, sections were incubated with antibodies against ALK5 (sc-398; Santa Cruz Biotechnology), FOXO1 (#2880; Cell Signaling Technology) and normal rabbit IgG (AB-105-C; R&D Systems, Minneapolis, MN, USA) for 1 hour. Sections were incubated with biotinylated secondary antibodies for 30 minutes, followed by incubation with streptavidin-peroxidase complex (Vectastain

Universal Elite ABC kit) for 30 minutes. Antibody complexes were visualized using the diaminobenzidine substrate system (Wako Pure Chemical Industries), and counterstained with hematoxylin. The percentage of cells positive for ALK5 and FOXO1 was determined using the BZ-II Analyzer software (Keyence, Osaka, Japan).

### Immunofluorescence

Sections were blocked with 5% goat serum (Wako Pure Chemical Industries) and 0.3% Triton X-100 (Sigma-Aldrich) for 1 hour, and then incubated with antibodies against ALK5 (sc-398; Santa Cruz Biotechnology) and FOXO1 (#2880; Cell Signaling Technology) for 1 hour. Subsequently, the sections were washed three times with PBS and incubated with Alexa Fluor 488 (A11008; Thermo Fisher Scientific) and 568 (A11004; Thermo Fisher Scientific) for 1 hour. SlowFade diamond antifade mountant with DAPI (Thermo Fisher Scientific) was used for nuclear staining. Immunostaining was visualized by fluorescence microscopy (BZ-X700; Keyence).

### Immunocytochemistry

Human chondrocytes were seeded in 8-well chamber slides at a density of  $2 \times 10^4$  cells/well. The cells were fixed with 4% PFA for 10 min at room temperature for antibodies against FOXO1 (#14952; Cell Signaling Technology), SMAD2/3 (#8685; Cell Signaling Technology), SMAD3 (#9523; Cell Signaling Technology), SMAD2 (#5339; Cell Signaling Technology) or ice-cold 100% methanol for 15 min at  $-20^\circ\text{C}$  for antibody against LC3 (#3868; Cell Signaling Technology), and then blocked with 5% goat serum and 0.3% Triton X-100 for 1 hour. Subsequently, the cells were incubated with each primary antibody for 1 hour. The cells were washed three times with PBS and incubated with Alexa Fluor 488 (A11008; Thermo Fisher Scientific) and 568 (A11004; Thermo Fisher Scientific) for 1 hour.

### MTT assay

Human chondrocytes were seeded in 96-well plates at a density of  $0.6 \times 10^4$  cells/well and transfected with siCtrl or siFOXO1. After incubation for 3 hours with recombinant human TGF $\beta$ 1 (R&D Systems, Minneapolis, MN, USA), H<sub>2</sub>O<sub>2</sub> was added to each well to a final concentration of 0, 100, or 250  $\mu\text{M}$ . After 48 hours, 25  $\mu\text{l}$  of MTT (5mg/ml) was added to each well. Three hours later, supernatant was aspirated, and 200  $\mu\text{l}$  of MTT solvent (4 mM HCl in isopropanol) was added. Optical densities were measured at 595 nm using an iMark microplate reader (Bio-Rad Laboratories, Hercules, CA, USA). Data are expressed as the percent absorbance of cells in each group relative to cells transfected with siCtrl, and not treated with TGF $\beta$ 1 and H<sub>2</sub>O<sub>2</sub>.

### ATP assay

Human chondrocytes were seeded in 96-well plates at a density of  $0.6 \times 10^4$  cells/well and transfected with siCtrl or siFOXO1. After incubation for 3 hours with TGF $\beta$ 1, H<sub>2</sub>O<sub>2</sub> was added to each well to a final concentration of 0, 100, or 250  $\mu\text{M}$ . After 48 hours, ATP production was evaluated using CellTiter-Glo (Promega). Data are expressed as the percent luminescence of cells in each group relative to cells transfected with siCtrl, and not treated with TGF $\beta$ 1 and H<sub>2</sub>O<sub>2</sub>.

## TUNEL assay

TUNEL assay was performed using the in situ Apoptosis Detection kit (Takara Bio). Human chondrocytes were seeded in 8-well chamber slides at a density of  $4 \times 10^4$  cells/well and transfected with siCtrl or siFOXO1. After incubation for 3 hours with TGF $\beta$ 1, H<sub>2</sub>O<sub>2</sub> (0 or 250  $\mu$ M) was added to each well. After 48 hours, the cells were fixed with 4% PFA for 15 min at room temperature, and then incubated with terminal deoxynucleotidyl transferase buffer for 1 hour at 37°C. The percentage of apoptotic cells was determined using the BZ-II Analyzer software (Keyence).

## Statistical analysis

All experiments were repeated with at least five samples from independent donors. Data are presented as dot plots with means  $\pm$  S.D. In each experiment, the effect size and the power were calculated. For *in vivo* experiment, a sample size  $n=5$  per group provided 82% power, given an  $\alpha$  level of 0.05, to detect the difference in means of the percentage of ALK5 positive cells between  $48.3 \pm 9.5\%$  in 13-month-old group and  $31.1 \pm 6.9\%$  in 20-month-old group. Similarly, all experiments provided  $>80\%$  power. Wilcoxon's rank-sum test was used for two-group comparisons. After normality was confirmed by the Shapiro-Wilk test, multiple comparisons were evaluated by one-way repeated measures ANOVA with the Tukey-Kramer post hoc test. All data analyses were performed using the JMP 14 statistical software (SAS Institute, Cary, NC, USA).  $P < 0.05$  was considered statistically significant.

## Results

### TGF $\beta$ 1 promotes FOXO1 expression in human chondrocytes

First, we investigated whether TGF $\beta$ 1 treatment promotes the expression of *FOXO1*, *FOXO3*, and *FOXO4* in human chondrocytes. Among the three genes, only *FOXO1* expression was promoted by TGF $\beta$ 1 treatment for 24 hours (5.22-fold increase). Conversely, *FOXO3* expression was reduced after TGF $\beta$ 1 treatment (1.82-fold decrease). *FOXO4* expression was unchanged (Fig. 1A). The reproducibility of the result in Fig. 1A was confirmed by *RPS18*, another housekeeping gene (Fig. 1B). Western blotting also revealed that expression of total FOXO1 protein significantly increased after TGF $\beta$ 1 treatment for more than 8 hours (Fig. 1C). The activity of FOXO1 is negatively regulated by phosphorylation at Ser-256<sup>30</sup>. For example, FOXO1 is phosphorylated by nutrient load (Fig. S1). By contrast, the level of phosphorylated FOXO1 (pFOXO1) was not changed after incubation with TGF $\beta$ 1 (Fig. 1C). In addition, we performed immunocytochemistry to confirm the localization of FOXO1. After incubation with TGF $\beta$ 1 for 24 hours, the level of FOXO1 protein significantly increased in the nucleus (Fig. 1D).

### ALK5 and FOXO1 levels decrease in aged mouse joints

To assess whether both ALK5 and FOXO1 expression decrease with aging, we investigated the levels of ALK5 and FOXO1 in young (3 months old, 3M), middle-aged (13 months old, 13M), and old (20 months old, 20M) mouse knee joints. ALK5 was strongly expressed in chondrocytes of young joints at 3M, whereas its expression was significantly reduced with aging (Fig. 2A and B). Similarly, levels of FOXO decreased with aging (Fig. 2A and B). In

13M mouse cartilage, the distribution pattern of FOXO1 expression was similar to ALK5 expression (Fig. 2C).

### **Inhibition of ALK5 suppresses FOXO1 expression induced by TGF $\beta$ 1**

To clarify the effect of ALK5 inhibition on the induction of FOXO1 expression by TGF $\beta$ 1 *in vitro*, we incubated human chondrocytes with RepSox, a specific inhibitor of ALK5. Inhibition of ALK5 significantly suppressed the expression of *FOXO1* after TGF $\beta$ 1 treatment (Fig. 3A). Western blotting revealed that induction of FOXO1 protein by TGF $\beta$ 1 was suppressed by RepSox; however, the level of pFOXO1 was not affected (Fig. 3B). FOXO1 protein both in the cytoplasm and nucleus significantly increased upon incubation with TGF $\beta$ 1. The induction of FOXO1 in the cytoplasm and nucleus by TGF $\beta$ 1 was completely suppressed by RepSox (Fig. 3C). Inhibition of ALK5 suppressed the phosphorylation of SMAD2 and SMAD3 after TGF $\beta$ 1 treatment for 30 min (Fig. 3D). Consistently, nuclear translocation of SMAD2 and SMAD3 was induced after TGF $\beta$ 1 treatment, but inhibited by RepSox (Fig. 3E). In immunocytochemistry, the localization of both SMAD2/3 and FOXO1 protein to the nucleus following TGF $\beta$ 1 treatment were suppressed by RepSox (Fig. 3F).

### **FOXO1 expression via TGF $\beta$ 1 is mediated by SMAD3 and repressed by SMAD2**

To verify the discrete effect of SMAD3 and SMAD2 on the expression of FOXO1 by TGF $\beta$ 1, we transfected human chondrocytes with siSMAD3 or siSMAD2. Gene and protein expression of SMAD3 significantly decreased after TGF $\beta$ 1 treatment for 24 hours, and were further suppressed by siSMAD3 (Fig. 4A and C). SMAD3 knockdown decreased expression of FOXO1 at mRNA and protein level after TGF $\beta$ 1 treatment (Fig. 4B and C). In immunocytochemistry, SMAD3 knockdown in the nucleus with siSMAD3 suppressed the induction of FOXO1 protein by TGF $\beta$ 1 (Fig. 4D). These findings were consistent with the effect of ALK5 inhibition by RepSox.

On the other hand, TGF $\beta$ 1 treatment did not affect the gene and protein expression of SMAD2 (Fig. 4E and G). In contrast to SMAD3, SMAD2 knockdown increased expression of FOXO1 at mRNA and protein level after TGF $\beta$ 1 treatment (Fig. 4F and G). In immunocytochemistry, SMAD2 knockdown in the nucleus with siSMAD2 increased the induction of FOXO1 protein by TGF $\beta$ 1 (Fig. 4H).

### **TGF $\beta$ 1 promotes autophagy under oxidative stress through the SMAD3-FOXO1 pathway**

We next investigated whether TGF $\beta$ 1 can activate autophagy in human chondrocytes. First, we evaluated the effect of TGF $\beta$ 1 on the expression of autophagy-related genes. Expression of *autophagy related 3 (ATG3)* and *microtubule associated protein 1 light chain 3 beta (MAP1LC3B)* increased after incubation with TGF $\beta$ 1 (Fig. 5A). *MAP1LC3B* encodes LC3, which has two forms: LC3-I and LC3-II. When autophagy is activated, LC3-I is converted to LC3-II, a phosphatidylethanolamine (PE)-conjugated form<sup>31</sup>. In western blotting, TGF $\beta$ 1 increased the expression of LC3-I, but had no effect on LC3-II (Fig. 5B). To further evaluate autophagy flux after TGF $\beta$ 1 treatment, chondrocytes were incubated with chloroquine (CQ) for 2 hours after TGF $\beta$ 1 pretreatment for 22 hours. In this condition, TGF $\beta$ 1 also increased the expression of LC3-I, but did not affect LC3-II (Fig. 5C). Next, we assessed the effect

of TGF $\beta$ 1 on the activity of autophagy under oxidative stress. H<sub>2</sub>O<sub>2</sub> treatment increased the expression of LC3-II relative to cells not exposed H<sub>2</sub>O<sub>2</sub> (Fig. 5B and D). Furthermore, expression of LC3-II was higher in chondrocytes pretreated with TGF $\beta$ 1 than in cells not pretreated with TGF $\beta$ 1 as well as LC3-I (Fig. 5D). In chondrocytes incubated with H<sub>2</sub>O<sub>2</sub> and CQ, TGF $\beta$ 1 pretreatment also increased the expression of LC3-I and LC3-II (Fig. 5E). By immunocytochemistry, we assessed the level of LC3 puncta (dots) after incubation with H<sub>2</sub>O<sub>2</sub>. The number of LC3 dots increased following exposure to H<sub>2</sub>O<sub>2</sub>, and TGF $\beta$ 1 pretreatment increased the number of LC3 dots under oxidative stress relative to cells not pretreated with TGF $\beta$ 1 (Fig. 5F). To confirm that the effect of TGF $\beta$ 1 on the regulation of LC3 protein expression was mediated by the SMAD3–FOXO1 pathway, we transfected chondrocytes with siSMAD3 or siFOXO1. FOXO1 knockdown decreased the induction of LC3-I protein by TGF $\beta$ 1 (Fig. 5G). SMAD3 knockdown also decreased the induction of FOXO1 and LC-3I protein by TGF $\beta$ 1 (Fig. 5G). Furthermore, following exposure to H<sub>2</sub>O<sub>2</sub>, knockdown of either FOXO1 or SMAD3 suppressed the induction of LC3-I and LC3-II by TGF $\beta$ 1 pretreatment (Fig. 5H).

### TGF $\beta$ 1 protects chondrocytes against oxidative stress–induced cell death via FOXO1

To investigate the protective effect of TGF $\beta$ 1 against cell death induced by oxidative stress via FOXO1, we assessed the cell viability after exposure to H<sub>2</sub>O<sub>2</sub>. After pretreatment with or without TGF $\beta$ 1 in chondrocytes transfected with siCtrl or siFOXO1, the cells were exposed to H<sub>2</sub>O<sub>2</sub>. In MTT assay, exposure to H<sub>2</sub>O<sub>2</sub> decreased cell viability in a dose-dependent manner relative to cells not exposed to H<sub>2</sub>O<sub>2</sub>, and in chondrocytes transfected with siCtrl, TGF $\beta$ 1 pretreatment suppressed the decrease in cell viability following exposure to H<sub>2</sub>O<sub>2</sub> (Fig. 6A). On the other hand, in chondrocytes transfected with siFOXO1, the protective effect of TGF $\beta$ 1 against exposure to H<sub>2</sub>O<sub>2</sub> was abolished (Fig. 6A). ATP assay revealed that ATP production was decreased by FOXO1 knockdown (Fig. 6B). TGF $\beta$ 1 pretreatment suppressed the decrease of ATP production following exposure to H<sub>2</sub>O<sub>2</sub>, whereas in cells transfected with siFOXO1, the decrease of ATP production was not suppressed by TGF $\beta$ 1 pretreatment (Fig. 6B). Consistently, in TUNEL assay, TGF $\beta$ 1 suppressed cell apoptosis against exposure to H<sub>2</sub>O<sub>2</sub> via FOXO1 (Fig. 6C).

## Discussion

In this study, we clarified the mechanism by which TGF $\beta$ 1 signaling promotes FOXO1 expression in chondrocytes. It is well known that the activity of FOXO proteins is strictly regulated by post-translational modifications such as phosphorylation and acetylation<sup>32 33</sup>. In particular, insulin and insulin-like growth factor I (IGF-I) are the main negative regulators of FOXO proteins, and promote phosphorylation of FOXO protein through the phosphatidylinositol 3-kinase (PI3K)/AKT pathway<sup>34</sup>. Phosphorylated FOXO proteins are exported from the nucleus to the cytoplasm, followed by cytoplasmic retention mediated by binding to 14-3-3 proteins or ubiquitination-mediated degradation<sup>35 36</sup>. However, a previous report revealed that in chondrocytes, IGF-I did not affect either the expression or the phosphorylation of FOXO proteins<sup>22</sup>. In addition, that report demonstrated that TGF $\beta$ 1 promoted expression of the FOXO1 protein. These previous findings motivated

us to investigate the mechanistic details of FOXO1 regulation by TGF $\beta$ 1 signaling in chondrocytes.

We first showed that only *FOXO1* mRNA expression was promoted by TGF $\beta$ 1 treatment, whereas *FOXO3* and *FOXO4* were not promoted. In addition, we showed that TGF $\beta$ 1 promoted the expression of the FOXO1 protein without affecting phosphorylation at Ser-256, a key site regulated by the PI3K/AKT pathway<sup>30</sup>. Considering the result that TGF $\beta$ 1 promoted the expression of cytoplasmic FOXO1 protein, our findings indicate that TGF $\beta$ 1 promotes the expression of FOXO1 at a transcriptional level, rather than via post-translational modifications. Little is known about the regulation of *FOXO* at the transcriptional level, although a few studies have described the effects of transcription factors such as E2F1 and Forkhead box C1 on the expression of *FOXO* genes<sup>37 38</sup>. Furthermore, although previous studies described the regulation of FOXOs through TGF $\beta$ 1 signaling in some cell types, they reported that TGF $\beta$ 1 promoted the expression of FOXO proteins by inhibiting cytoplasmic translocation and did not refer to the expression of *FOXO* genes<sup>39 40</sup>. Our findings demonstrate the potential of TGF $\beta$ 1 to promote the expression of *FOXO1* genes in chondrocytes. In addition, we revealed that TGF $\beta$ 1 promotes the localization of FOXO1 to the nucleus. This indicates that TGF $\beta$ 1 promotes not only the expression of FOXO1, but also activation of FOXO1 by nuclear translocation.

Next, we confirmed the decreases in ALK5 and FOXO1 levels in aged chondrocytes *in vivo*. In addition, we showed that inhibition of ALK5 suppressed the induction of FOXO1 expression by TGF $\beta$ 1 *in vitro*. In the canonical pathway, after binding with TGF $\beta$ 1, ALK5 phosphorylates SMAD2 and SMAD3, which form a complex with SMAD4. The complex translocates to the nuclei and regulates their target genes<sup>41</sup>. In our results, although the nuclear translocations of SMAD3 and SMAD2 were induced via the TGF $\beta$ 1-ALK5 pathway, the inverse impact of siRNA between SMAD3 and SMAD2 on the expression of FOXO1 was observed. SMAD3 knockdown inhibited the induction of FOXO1 expression by TGF $\beta$ 1, whereas SMAD2 knockdown promoted TGF $\beta$ 1-induced FOXO1 expression. Recent studies showed that SMAD3 and SMAD2 have distinct roles. In dendritic cells, the TGF $\beta$ 1-SMAD3 pathway positively regulates TGF $\beta$ 1 autoinduction, whereas SMAD2 negatively regulates TGF $\beta$ 1 expression<sup>42</sup>. In pancreatic ductal adenocarcinoma cells, SMAD3 mediates the inhibition of cell growth and migratory response by TGF $\beta$ 1, whereas SMAD2 induces cell growth and migration<sup>43</sup>. The regulatory mechanism of FOXO1 expression by TGF $\beta$ 1 in chondrocytes is similar to the results of these reports. Our findings suggest that TGF $\beta$ 1 promotes the expression of FOXO1 through the ALK5-SMAD3 pathway, whereas SMAD2 negatively regulate FOXO1 expression (Fig. 7A), and a decrease in ALK5 expression suppresses the activity of SMAD3 signaling, resulting in the reduction of FOXO1 expression in aged chondrocytes (Fig. 7B). Further studies will provide more information on the regulatory mechanism of FOXO1 expression by TGF $\beta$ 1 signaling. For example, the observation of mice younger than 13 months old will determine the detailed time point at which the expression of ALK5 and FOXO1 begins to decline.

TGF $\beta$ 1-ALK5-SMAD3 signaling is an important pathway for the maintenance of the extracellular matrix in articular cartilage<sup>44 45 46 47</sup>. On the other hand, little has been reported about the role of TGF $\beta$ 1 signaling in autophagy in chondrocytes, although TGF $\beta$ 1

has been reported to promote the activity of autophagy in other cell types<sup>48 49</sup>. In this study, we demonstrated that TGF $\beta$ 1 signaling activates autophagy via FOXO1. We first showed that expression of *MAP1LC3B* increased after TGF $\beta$ 1 treatment. Consistent with this, overexpression of FOXO1 in chondrocytes promotes the expression of *MAP1LC3B*<sup>24</sup>. Next, we examined the expression of LC3 proteins in order to assess autophagic flux. When cells are exposed to stressful conditions, autophagy is activated<sup>7 8</sup>. When this happens, LC3-I is conjugated to phosphatidylethanolamine and forms LC3-II, which is involved in formation of the autophagosome membrane. Therefore, elevated expression of LC3-II indicates activation of autophagic flux<sup>31</sup>. Importantly, TGF $\beta$ 1 treatment promoted only LC3-I expression, whereas the level of LC3-II did not change. This observation indicates TGF $\beta$ 1 treatment is not stressful for chondrocytes, and TGF $\beta$ 1 alone does not induce autophagic flux. In addition, we investigated LC3-II expression under oxidative stress because autophagic flux is induced by oxidative stress<sup>50</sup>. TGF $\beta$ 1 promoted oxidative stress-induced expression of LC3-II. This result was consistent with the observation in the cells incubated with CQ. On the other hand, SMAD3 and FOXO1 knockdown suppressed induction of LC3-I expression by TGF $\beta$ 1 and subsequently decreased oxidative stress-induced expression of LC3-II. These findings suggest that TGF $\beta$ 1 plays an essential role in the expression of LC3-I by promoting the expression of *MAP1LC3B* via the SMAD3-FOXO1 pathway, and that under oxidative stress, TGF $\beta$ 1 signaling makes autophagy more effective (Fig. 7A). Conversely, reduced TGF $\beta$ 1 signaling causes a reduction in FOXO1 expression, which in turn decreases expression of LC3-I. This results in dysfunction of autophagy under oxidative stress because autophagosomes are not formed effectively due to insufficient expression of LC3-II (Fig. 7B). To clarify further detailed regulatory mechanism of autophagy by TGF $\beta$ 1 signaling, we have to do some additional experiments in the future, for instance, the observation of autophagosomes by electron microscopy.

FOXO1 is important for protecting cells against oxidative stress<sup>19</sup>. Because TGF $\beta$ 1 promotes autophagy under oxidative stress via FOXO1, we speculated that TGF $\beta$ 1 signaling could protect chondrocytes against oxidative stress. Indeed, TGF $\beta$ 1 suppressed cell death following oxidative stress in a FOXO1-dependent manner. This is the first evidence that TGF $\beta$ 1 signaling protects chondrocytes against oxidative stress.

In summary, our findings indicate that TGF $\beta$ 1 promotes the expression of FOXO1 via the ALK5-SMAD3 pathway, maintains the expression of LC3-I, and protects chondrocytes against oxidative stress-induced cell death by activating autophagy (Fig. 7A). Conversely, in aged chondrocytes, the reduction in ALK5 expression may cause a reduction in the level of FOXO1 and subsequent defects in autophagy. Consequently, because damaged organelles and proteins are not effectively degraded and recycled, chondrocytes cannot maintain homeostasis and undergo cell death (Fig. 7B). Our findings reveal a novel function of TGF $\beta$ 1 signaling in the maintenance of cartilage homeostasis under oxidative stress, mediated by the FOXO1-autophagy axis, and emphasize the important role of TGF $\beta$ 1 signaling in preventing OA.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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## Funding sources

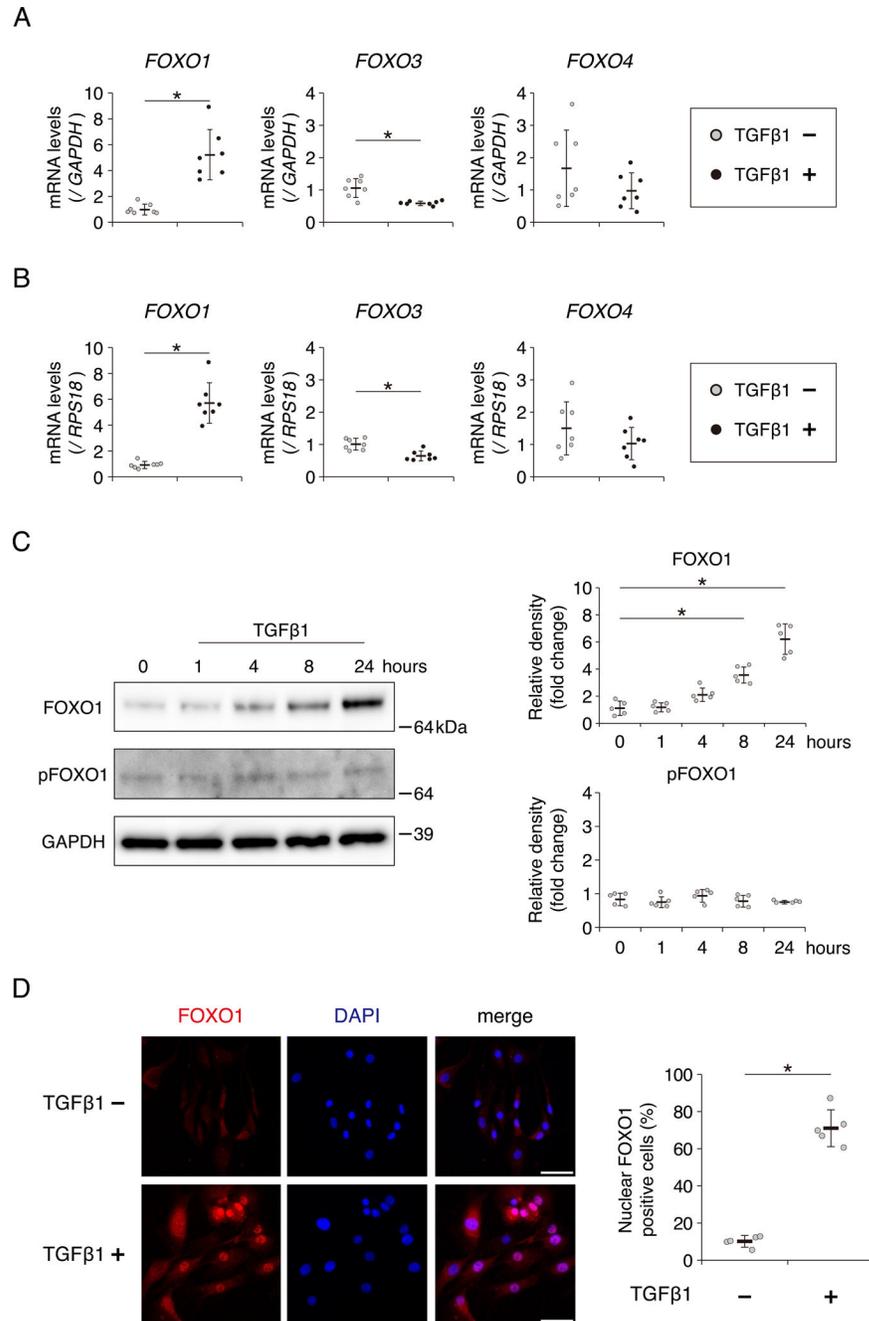
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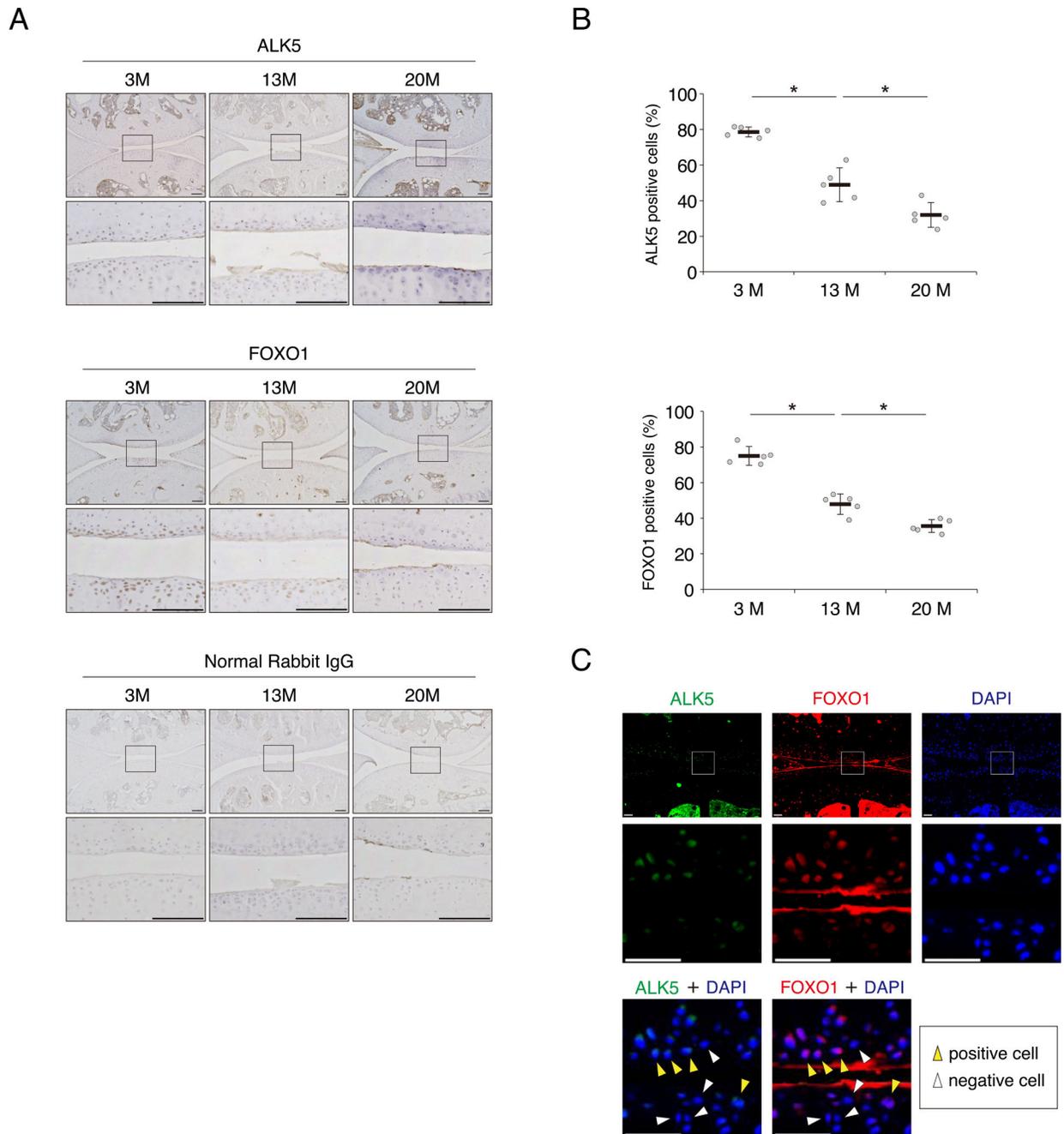
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**Fig. 1. TGFβ1 promotes FOXO1 expression in human chondrocytes.**

Relative mRNA levels of *FOXO1*, *FOXO3*, and *FOXO4* after incubation with TGFβ1 (10 ng/ml) for 24 hours, as determined by qRT-PCR using the primer of *GAPDH* (A) or *RPS18* (B), house keeping genes. Gene expression is shown relative to the level in cells incubated without TGFβ1; n = 7 independent experiments. (C) Levels of total FOXO1 and phosphorylated FOXO1 (pFOXO1) at Ser-256 after incubation with TGFβ1 (10 ng/ml) for 1, 4, 8, or 24 hours, as determined by western blotting. GAPDH was used as a loading control. Graphs show levels of total FOXO1 and pFOXO1, relative to the corresponding levels in cells incubated without TGFβ1; n = 5 independent experiments. (D)

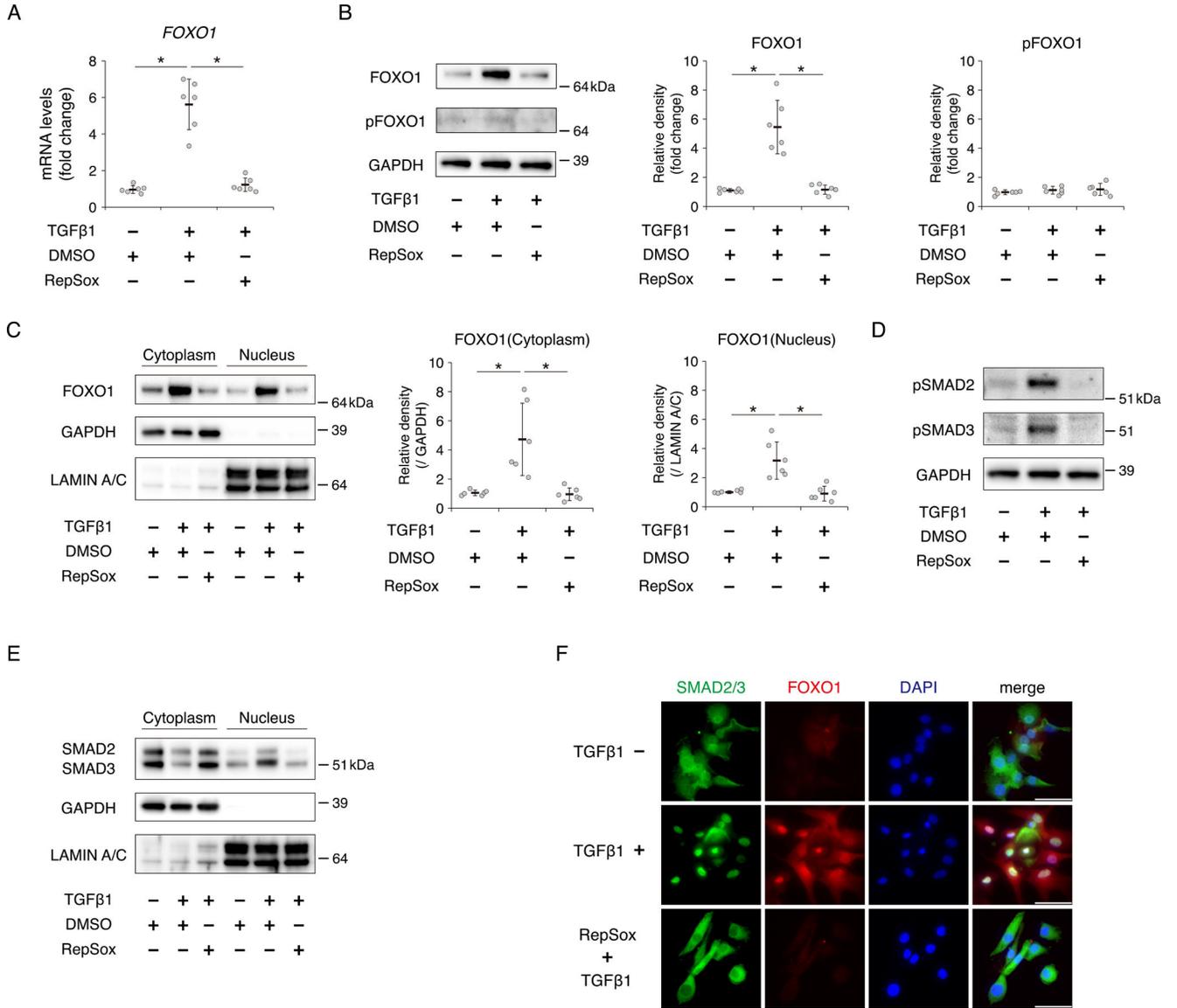
FOXO1 localization after incubation with TGF $\beta$ 1 (10 ng/ml) for 24 hours, visualized by immunocytochemistry using anti-FOXO1 antibody (red staining). Nuclei were detected with DAPI (blue staining). Bars, 50  $\mu$ m. Graph shows the percentage of nuclear FOXO1 positive cells; n = 5 independent experiments. Data are presented as dots showing individual values and as means  $\pm$  S.D. Statistical analysis in (A), (B) and (D) was performed using Wilcoxon's rank-sum test. Statistical analysis in (C) was performed using one-way repeated measures ANOVA with the Tukey-Kramer post hoc test. \* $P < 0.05$ .



**Fig. 2. Expression of ALK5 and FOXO1 decrease with aging in mouse cartilage.**

Knee joints were collected from the hind legs of 3-month-old (3M) young mice, 13-month-old (13M) middle-aged mice or 20-month-old (20M) old mice. **(A)** Representative images of ALK5 and FOXO1, detected by immunohistochemistry. Sections stained with normal rabbit IgG are shown as negative controls. Bottom images in each group show higher-magnification views corresponding to the boxed areas in the top images. Bars, 100  $\mu$ m. **(B)** Quantification of ALK5 and FOXO1 positive cells; n = 5 mice per group. **(C)** Co-localization of ALK5 and FOXO1 in 13M mouse knee joint were visualized by immunofluorescence. ALK5 is shown in green, and FOXO1 is shown in red. Nuclei were

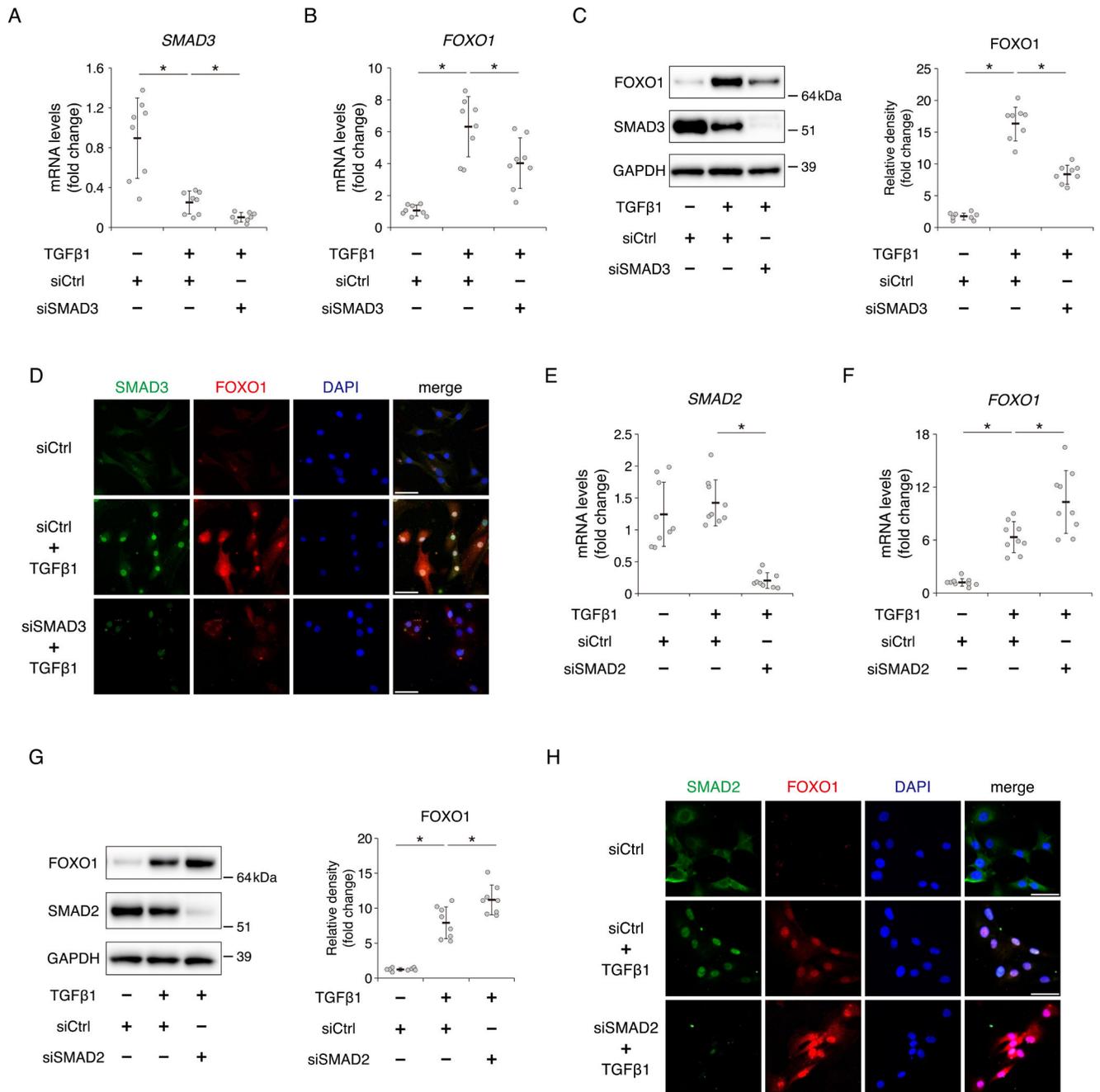
detected with DAPI (blue staining). Middle and bottom images show higher-magnification views corresponding to the boxed areas in the top images. Yellow arrows indicate both ALK5 and FOXO1 positive cells, and white arrows indicate both ALK5 and FOXO1 negative cells. Bars, 100  $\mu\text{m}$ . Data are presented as dots showing individual values and as means  $\pm$  S.D. Statistical analysis was performed using one-way repeated measures ANOVA with the Tukey–Kramer post hoc test. \* $P < 0.05$ .



**Fig. 3. ALK5 inhibition suppresses induction of FOXO1 expression by TGFβ1 in human chondrocytes.**

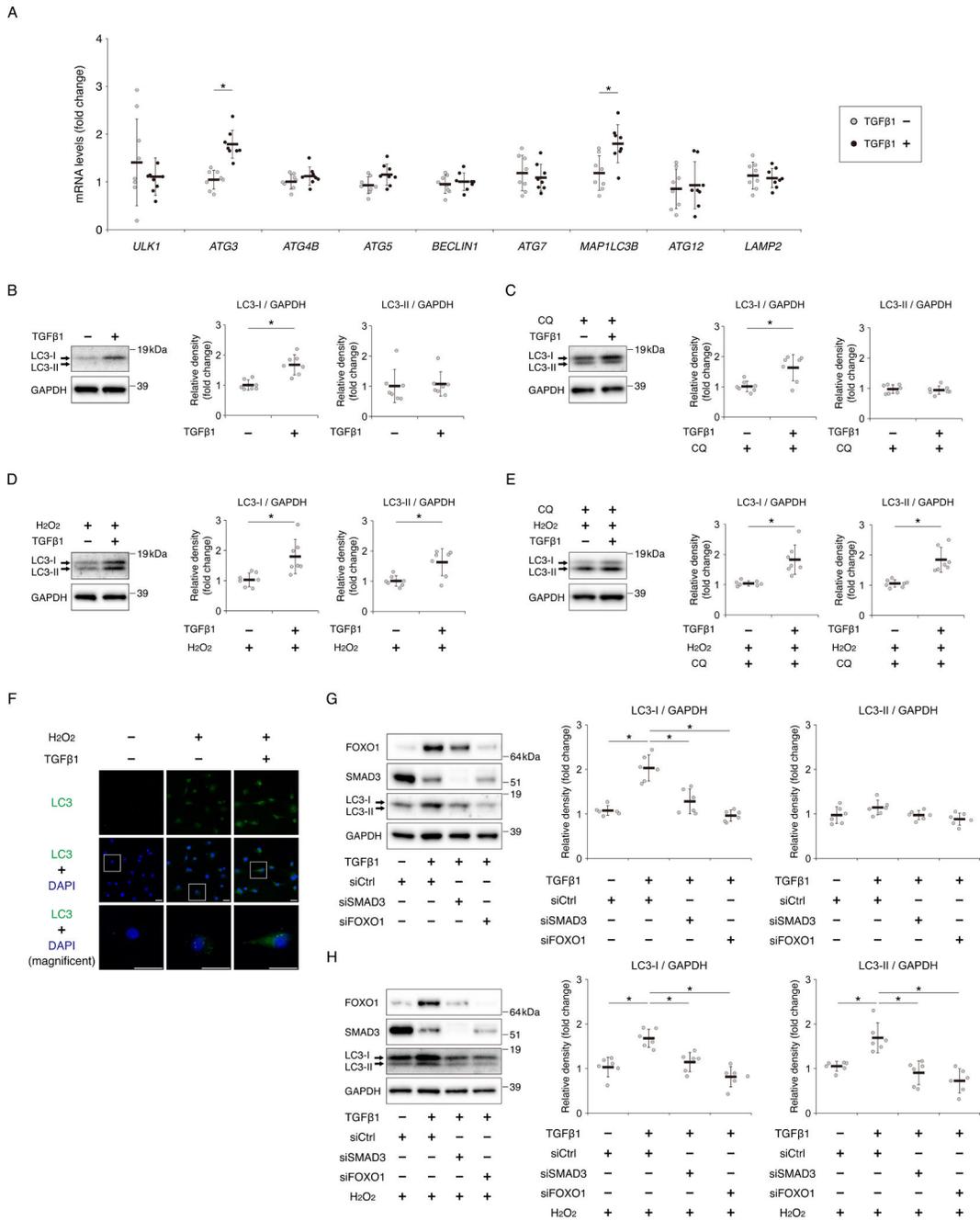
Chondrocytes were incubated with or without TGFβ1 (10 ng/ml) for 24 hours after pretreatment with or without RepSox (1 μM) for 3 hours. (A) Relative mRNA levels of *FOXO1* were measured by qRT-PCR. Gene expression at each stage is expressed relative to the level in cells incubated without TGFβ1 and RepSox; n = 6 independent experiments. (B) Levels of total FOXO1 and pFOXO1 were analyzed by western blotting. GAPDH was used as a loading control. Graphs show total FOXO1 protein level and pFOXO1 protein level, relative to the corresponding levels in cells incubated without TGFβ1 and RepSox; n = 6 independent experiments. (C) Levels of cytoplasmic FOXO1 and nuclear FOXO1 were analyzed by western blotting. GAPDH was used as a loading control for cytoplasmic extracts. LAMIN A/C was used as a loading control for nuclear extracts. Graphs show cytoplasmic FOXO1 protein level and nuclear FOXO1 protein level, relative to the corresponding levels in cells incubated without TGFβ1 and RepSox; n = 6

independent experiments. **(D)** Levels of phosphorylated SMAD2 (pSMAD2) at Ser-465/467 and phosphorylated SMAD3 (pSMAD3) at Ser-423/425 in chondrocytes incubated with or without TGF $\beta$ 1 (10 ng/ml) for 30 min after pretreatment with or without RepSox (1  $\mu$ M) for 3 hours, as determined by western blotting. **(E)** Levels of cytoplasmic SMAD2/3 and nuclear SMAD2/3 in chondrocytes incubated with or without TGF $\beta$ 1 (10 ng/ml) for 30 min after pretreatment with or without RepSox (1  $\mu$ M) for 3 hours, as determined by western blotting. **(F)** SMAD2/3 and FOXO1 localizations in chondrocytes incubated with or without TGF $\beta$ 1 (10 ng/ml) for 24 hours after pretreatment with or without RepSox (1  $\mu$ M) for 3 hours were visualized by immunocytochemistry. SMAD2/3 is shown in green, and FOXO1 is shown in red. Nuclei were detected with DAPI (blue staining). Bars, 50  $\mu$ m. Data are presented as dots showing individual values and as means  $\pm$  S.D. Statistical analysis was performed using one-way repeated measures ANOVA with the Tukey–Kramer post hoc test. \* $P$  < 0.05.



**Fig. 4. FOXO1 expression via TGFβ1 is mediated by SMAD3 and repressed by SMAD2.** Chondrocytes were incubated with or without TGFβ1 (10 ng/ml) for 24 hours after transfection with siCtrl or siSMAD3. Relative *SMAD3* (A) and *FOXO1* (B) mRNA levels were measured by qRT-PCR. Gene expression at each stage is expressed relative to the level in cells transfected with siCtrl and incubated without TGFβ1; n = 8 independent experiments. (C) Levels of total FOXO1 and SMAD3 were analyzed by western blotting. GAPDH was used as a loading control. Graphs show total levels of FOXO1, relative to the corresponding levels in cells transfected with siCtrl and incubated without TGFβ1; n = 8 independent experiments. (D) SMAD3 and FOXO1 proteins were visualized by

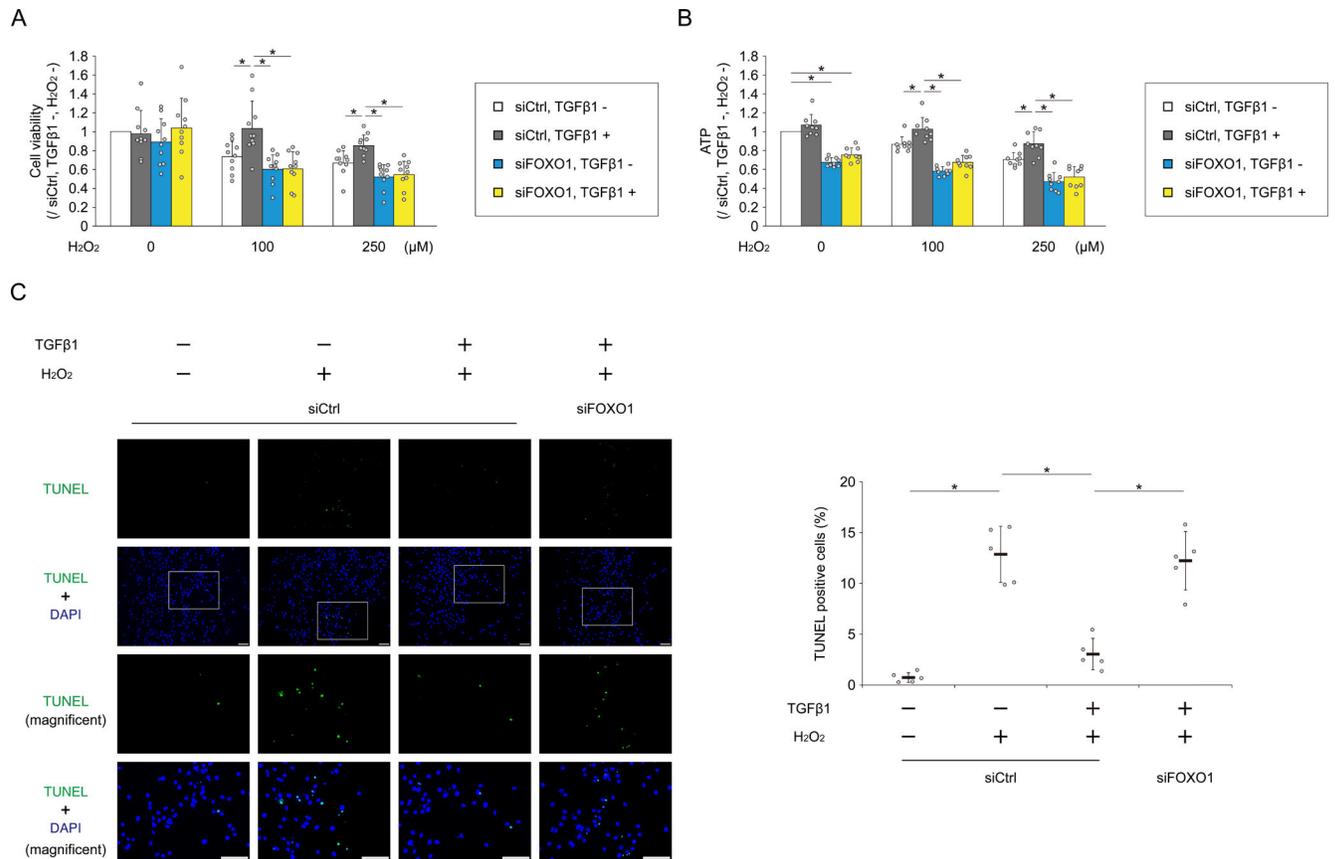
immunocytochemistry. SMAD3 is shown in green, and FOXO1 is shown in red. Nuclei were detected with DAPI (blue staining). Bars, 50  $\mu$ m. Chondrocytes were incubated with or without TGF $\beta$ 1 (10 ng/ml) for 24 hours after transfection with siCtrl or siSMAD2. Relative *SMAD2* (E) and *FOXO1* (F) mRNA levels were measured by qRT-PCR. Gene expression at each stage is expressed relative to the level in cells transfected with siCtrl and incubated without TGF $\beta$ 1; n = 9 independent experiments. (G) Levels of total FOXO1 and SMAD2 were analyzed by western blotting. Graphs show total levels of FOXO1, relative to the corresponding levels in cells transfected with siCtrl and incubated without TGF $\beta$ 1; n = 8 independent experiments. (H) SMAD2 and FOXO1 proteins were visualized by immunocytochemistry. SMAD2 is shown in green, and FOXO1 is shown in red. Bars, 50  $\mu$ m. Data are presented as dots showing individual values and as means  $\pm$  S.D. Statistical analysis was performed using one-way repeated measures ANOVA with the Tukey–Kramer post hoc test. \* $P < 0.05$ .



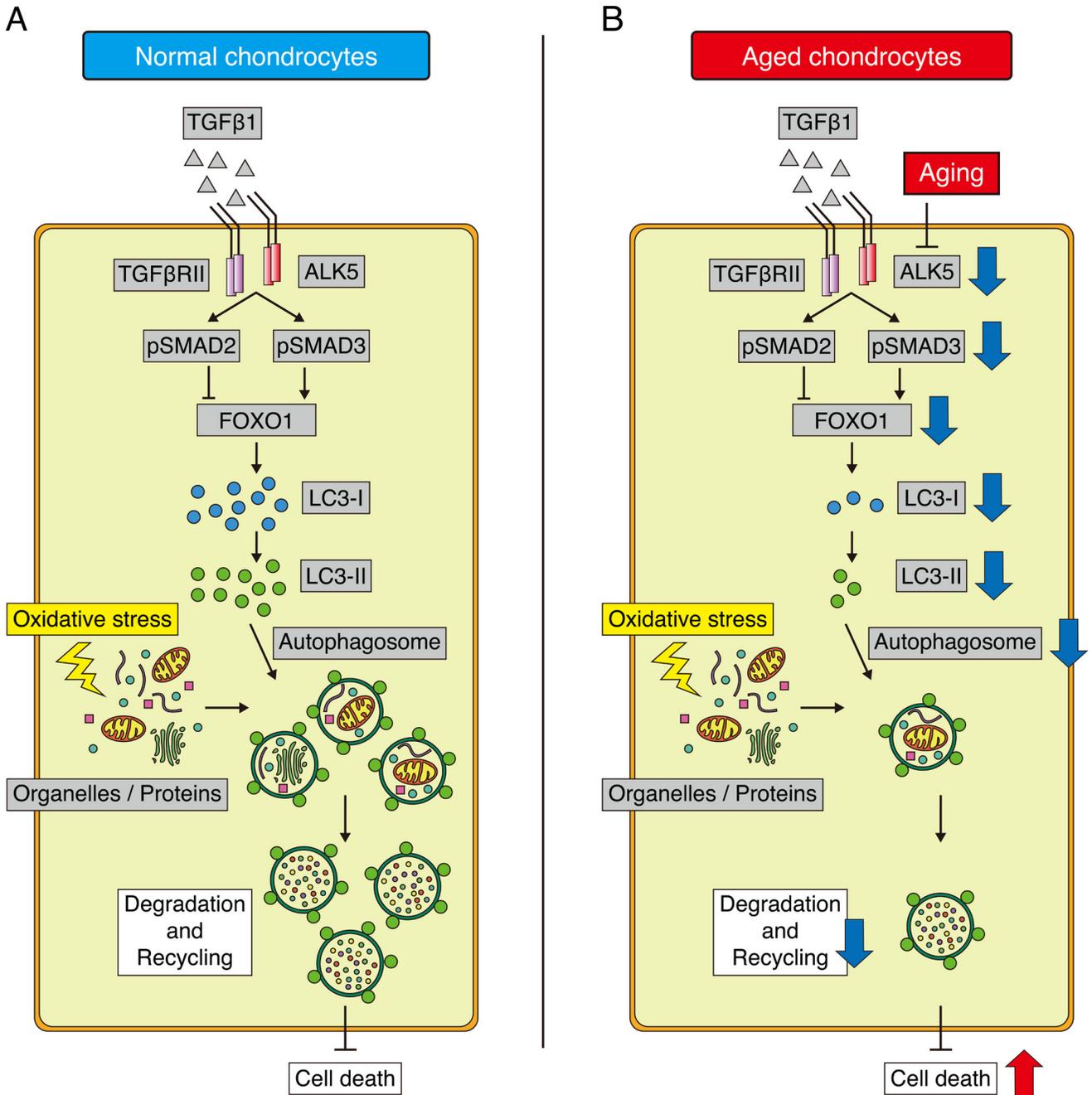
**Fig. 5. TGFβ1 promotes autophagic activity under oxidative stress through the SMAD3–FOXO1 pathway.**

(A) Relative mRNA levels of *ULK1*, *ATG3*, *ATG4B*, *ATG5*, *BECLIN1*, *ATG7*, *MAP1LC3B*, *ATG12*, and *LAMP2* in human chondrocytes after incubation with TGFβ1 (10 ng/ml) for 24 hours, as determined by qRT-PCR. Gene expression is shown relative to the level in cells incubated without TGFβ1; n = 8 independent experiments. (B) Levels of LC3-I and LC3-II after incubation with TGFβ1 (10 ng/ml) for 24 hours, as determined by western blotting. GAPDH was used as a loading control. Graphs show levels of LC3-I and LC3-II, relative to the corresponding levels in cells incubated without TGFβ1; n = 7

independent experiments. **(C)** Levels of LC3-I and LC3-II in chondrocytes incubated with chloroquine (CQ) (25  $\mu$ M) for 2 hours after pretreatment with or without TGF $\beta$ 1(10 ng/ml) for 22 hours, as determined by western blotting. Graphs show levels of LC3-I and LC3-II, relative to the corresponding levels in cells incubated without TGF $\beta$ 1; n = 7 independent experiments. **(D)** Levels of LC3-I and LC3-II in chondrocytes exposed to H<sub>2</sub>O<sub>2</sub> (500  $\mu$ M) for 2 hours after pretreatment with or without TGF $\beta$ 1(10 ng/ml) for 22 hours, as determined by western blotting. Graphs show levels of LC3-I and LC3-II, relative to the corresponding levels in cells pretreated without TGF $\beta$ 1; n = 7 independent experiments. **(E)** Levels of LC3-I and LC3-II in chondrocytes incubated with CQ (25  $\mu$ M) and H<sub>2</sub>O<sub>2</sub> (500  $\mu$ M) for 2 hours after pretreatment with or without TGF $\beta$ 1(10 ng/ml) for 22 hours, as determined by western blotting. Graphs show levels of LC3-I and LC3-II, relative to the corresponding levels in cells incubated without TGF $\beta$ 1; n = 7 independent experiments. **(F)** LC3 proteins in chondrocytes incubated with or without H<sub>2</sub>O<sub>2</sub> (500  $\mu$ M) for 2 hours after pretreatment with or without TGF $\beta$ 1 (10 ng/ml) for 22 hours were visualized by immunocytochemistry. LC3 is shown as green dots. Nuclei were detected with DAPI (blue staining). Bottom images show higher-magnification views corresponding to the boxed areas in the middle images. Bars, 30  $\mu$ m. **(G)** Levels of FOXO1, SMAD3, LC3-I, and LC3-II, as determined by western blotting, in chondrocytes transfected with siCtrl, siSMAD3, or siFOXO1 after incubation with or without TGF $\beta$ 1 (10 ng/ml) for 24 hours. Graphs show total levels of LC3-I and LC3-II, relative to the corresponding levels in cells transfected with siCtrl and incubated without TGF $\beta$ 1; n = 6 independent experiments. **(H)** Levels of FOXO1, SMAD3, LC3-I, and LC3-II, as determined by western blotting, in chondrocytes transfected with siCtrl, siSMAD3, or siFOXO1 following exposure to H<sub>2</sub>O<sub>2</sub> (500  $\mu$ M) for 2 hours after pretreatment with or without TGF $\beta$ 1 (10 ng/ml) for 22 hours. Graphs show total levels of LC3-I and LC3-II, relative to the corresponding levels in cells transfected with siCtrl and incubated without TGF $\beta$ 1; n = 6 independent experiments. Data are presented as dots showing individual values and as means  $\pm$  S.D. Statistical analysis in (A), (B), (C), (D) and (E) was performed using Wilcoxon's rank-sum test. Statistical analysis in (G) and (H) was performed using one-way repeated measures ANOVA with the Tukey-Kramer post hoc test. \* $P$  < 0.05.



**Fig. 6. TGFβ1 protects chondrocytes against oxidative stress–induced cell death via FOXO1.** Human chondrocytes were transfected with siCtrl or siFOXO1. After incubation with or without TGFβ1 for 3 hours, H<sub>2</sub>O<sub>2</sub> was added to each well to a final concentration of 0, 100, or 250 μM for 48 hours. **(A)** Cell viability was analyzed by MTT assay; n = 10 independent experiments. **(B)** ATP production was analyzed using the CellTiter-Glo assay; n = 9 independent experiments. Data are expressed as the percent absorbance (A) or luminescence (B) of cells in each group relative to cells transfected with siCtrl, and not treated with TGFβ1 and H<sub>2</sub>O<sub>2</sub>. Human chondrocytes were transfected with siCtrl or siFOXO1. After incubation with or without TGFβ1 for 3 hours, H<sub>2</sub>O<sub>2</sub> (0 or 250 μM) was added to each well. After 48 hours, cell apoptosis was analyzed by TUNEL assay **(C)**. Higher-magnification views are corresponding to the white boxed areas in each group. Graph shows the percentage of TUNEL positive cells; n = 5 independent experiments. Data are presented as dots showing individual values and as means ± S.D. Statistical analysis was performed using one-way repeated measures ANOVA with the Tukey–Kramer post hoc test. \**P* < 0.05.



**Fig. 7. Schematic model of the regulation of the FOXO1–autophagy axis by TGFβ1 signaling.** (A) In young normal chondrocytes, TGFβ1 promotes FOXO1 expression via the ALK5-SMAD3 pathway, whereas SMAD2 negatively regulate the expression of FOXO1. SMAD3-FOXO1 axis maintains the expression of LC3-I. Organelles and proteins damaged by oxidative stress are degraded and recycled by autophagosomes, and chondrocytes are protected against cell death. (B) In aged chondrocytes, the reduction in ALK5 expression suppresses SMAD3 activity and FOXO1 expression. Under these conditions, organelles and proteins damaged by oxidative stress are not degraded effectively because of a dysfunction

of autophagy caused by reductions in LC3-I and LC3-II expression, resulting in induction of cell death.

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