OSTEOARTHRITIS

FoxO transcription factors modulate autophagy and proteoglycan 4 in cartilage homeostasis and osteoarthritis

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Aging is a main risk factor for osteoarthritis (OA). FoxO transcription factors protect against cellular and organismal aging, and FoxO expression in cartilage is reduced with aging and in OA. To investigate the role of FoxO in cartilage, Col2Cre-FoxO1, 3, and 4 single knockout (KO) and triple KO mice (Col2Cre-TKO) were analyzed. Articular cartilage in Col2Cre-TKO and Col2Cre-FoxO1 KO mice was thicker than in control mice at 1 or 2 months of age. This was associated with increased proliferation of chondrocytes of Col2Cre-TKO mice in vivo and in vitro. OA-like changes developed in cartilage, synovium, and subchondral bone between 4 and 6 months of age in Col2Cre-TKO and Col2Cre-FoxO1 KO mice. Col2Cre-FoxO3 and FoxO4 KO mice showed no cartilage abnormalities until 18 months of age when Col2Cre-FoxO3 KO mice had more severe OA than control mice. Autophagy and antioxidant defense genes were reduced in Col2Cre-TKO mice. Deletion of FoxO1/3/4 in mature mice using Aggrecan(Acan)-CreERT2 (AcanCreERT-TKO) also led to spontaneous cartilage degradation and increased OA severity in a surgical model or treadmill running. The superficial zone of knee articular cartilage of Col2Cre-TKO and AcanCreERT-TKO mice exhibited reduced cell density and markedly decreased *Prg4*. In vitro, ectopic FoxO1 expression increased *Prg4* and synergized with transforming growth factor–β stimulation. In OA chondrocytes, overexpression of FoxO1 reduced inflammatory mediators and cartilage-degrading enzymes, increased protective genes, and antagonized interleukin-1β effects. Our observations suggest that FoxO play a key role in postnatal cartilage development, maturation, and homeostasis and protect against OA-associated cartilage damage.

INTRODUCTION

Articular cartilage is an integral component of the musculoskeletal system that absorbs compressive and shear forces during joint movement (*1*). Aging- or trauma-related damage to cartilage is a principal event in the pathogenesis of osteoarthritis (OA), the most prevalent joint disease (*2*). Tissue- and cellular-level changes in cartilage with aging have been characterized (*3*), but the mechanisms responsible for cellular homeostasis and the reasons for their failure in aging remain to be discovered. Recent findings suggest that cartilage aging and the development of structural changes are related to the failure of cellular homeostasis mechanisms, such as autophagy and oxidative stress responses (*4*).

The FoxO proteins are an evolutionarily conserved family of transcription factors with important functions in development, aging, and longevity (*5*). In mammals, the FoxO family is composed of four members (FoxO1, FoxO3, FoxO4, and FoxO6) with distinct and overlapping functions (*6*). FoxO1, FoxO3, and FoxO4 are ubiquitously expressed, whereas FoxO6 expression is largely restricted to the brain (*7*). The triple deletion of FoxO1, 3, and 4 leads to more severe phenotypes than deletion of an individual FoxO (*8*, *9*). However, each FoxO regulates gene expression in a tissue-specific pattern (*10*). In bone, FoxO1 modulates osteoblast differentiation by interacting with other signaling pathways, including Runx2 (*11*) and Wnt/β-catenin (*12*, *13*). FoxO1 also mediates the effects of 1α,25-dihydroxyvitamin D_3 on glucose metabolism and bone development (*14*).

In humans, FoxO3 single-nucleotide polymorphisms are associated with exceptional longevity (*15*). FoxO is thought to regulate life span through control of cellular homeostasis and maintenance of stem/progenitor cells populations during aging (*7*, *16*). FoxO expression and activity are induced under oxidative stress conditions (*16*), and FoxO transcriptionally induce expression of several antioxidant enzymes such as catalase and manganese superoxide dismutase (*17*). FoxO proteins also regulate two major intracellular clearance mechanisms, autophagy and the ubiquitin-proteasome system, to eliminate damaged and aggregated proteins (*16*).

Dysregulation of FoxO expression or activity contributes to the pathogenesis of age-related diseases in several different tissues, including bone (*18*) and muscle (*19*). We reported earlier that the expression of autophagy genes, which are regulated by FoxO, were reduced in OA (*20*) and that autophagy activation by rapamycin reduced the severity of OA in mice (*21*). Subsequently, we found that the expression of FoxO is reduced in aging and OA-affected human and mouse cartilage (*22*). Here, we analyzed the impact of cartilage-specific developmental deletion of FoxO1, FoxO3, and FoxO4, and of developmental and postnatal deletion of all three FoxO, on joint development, postnatal maturation, maintenance, and OA pathogenesis.

RESULTS

FoxO1 controls postnatal skeletal size

To generate mice with cartilage-specific developmental deletion of single FoxO1, FoxO3, and FoxO4 or of all three FoxO isoforms, $FoxO1^{lox/lox}$, $FoxO3^{lox/lox}$, and $FoxO4^{lox/lox}$ single transgenic mice and $FoxO1^{lox/lox}$;$FoxO3^{lox/lox}$;$FoxO4^{lox/lox}$ triple transgenic mice (*8*) were crossed with the Col2a1-Cre/+ mice (*23*). Real-time polymerase chain reaction (PCR) analysis showed about 90% reduction in FoxO mRNA in the cartilage of FoxO single knockout (KO) mice (Col2Cre-FoxO1/3/4 KO mice) and in FoxO triple KO (Col2Cre-TKO) mice (fig. S1A). FoxO1/3/4 proteins in cartilage were markedly reduced in Col2Cre-TKO mice (fig. S1B).

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Skeletal preparations of newborn mice [postnatal day 1 (P1)] showed no differences in length between control and FoxO KO mice (fig. S1C). However, Col2Cre-TKO and Col2Cre-FoxO1 KO mice showed a significantly increased total body (TKO, $P = 0.011$; FoxO1 KO, $P = 0.008$) and tail length (TKO, $P = 0.002$; FoxO1 KO, $P < 0.001$) at 1 month of age compared with control mice (Fig. 1A). The proximal tibial growth plate showed increased height of the proliferative zone in Col2Cre-TKO mice at P1, P7, and 1 month and the proliferative and hypertrophic zones in Col2Cre-FoxO1 KO mice at P7 and 1 month (Fig. 1, B to D, and fig. S1D). There was an irregular columnar arrangement of the hypertrophic chondrocytes and acellular areas in the hypertrophic zone (Fig. 1B). There were no differences in body weight between the FoxO KO mice and Cre-negative littermate controls at 1 month of age (fig. S1E).

FoxO deletion results in thickened articular cartilage

During postnatal growth and maturation, Col2Cre-TKO and Col2Cre-FoxO1 KO mice, but not Col2Cre-FoxO3 KO or Col2Cre-FoxO4 KO mice, developed significantly thicker cartilage on the femoral condyle and tibial plateau (TKO 1 month, $P < 0.001$; TKO 2 months, $P < 0.001$; FoxO1 KO 2 months, $P = 0.02$) than control mice (Fig. 1, E and F) at 1 or 2 months of age. This was mainly the result of increased thickness of the noncalcified region above the tidemark (Fig. 1G). Cell density in the noncalcified area was significantly reduced in Col2Cre-TKO and Col2Cre-FoxO1 KO mice ($P < 0.001$ each) (Fig. 1H). Articular chondrocytes were also larger in Col2Cre-TKO mice than in controls (Fig. 1I).

Because increased cartilage thickness may be the direct result of an overall increase in cell number, we tested whether FoxO controls chondrocyte proliferation. In vivo 5-bromo-2′-deoxyuridine (BrdU) labeling revealed more proliferating cells in the articular cartilage of 1-month-old Col2Cre-TKO mice than control mice (Fig. 2A). In addition, cultured immature mouse articular chondrocytes (IMACs) from Col2Cre-TKO mice also showed increased BrdU incorporation (Fig. 2B), increased cell metabolic activity (Fig. 2C), and increased numbers of S-phase cells (Fig. 2D). Gene expression analysis showed reduced expression of the cell cycle inhibitors *Cdkn1b* and *Ccng2* in cartilage from 1-month-old Col2Cre-TKO mice (Fig. 2E). Reductions in *Cdkn1a* in cartilage from

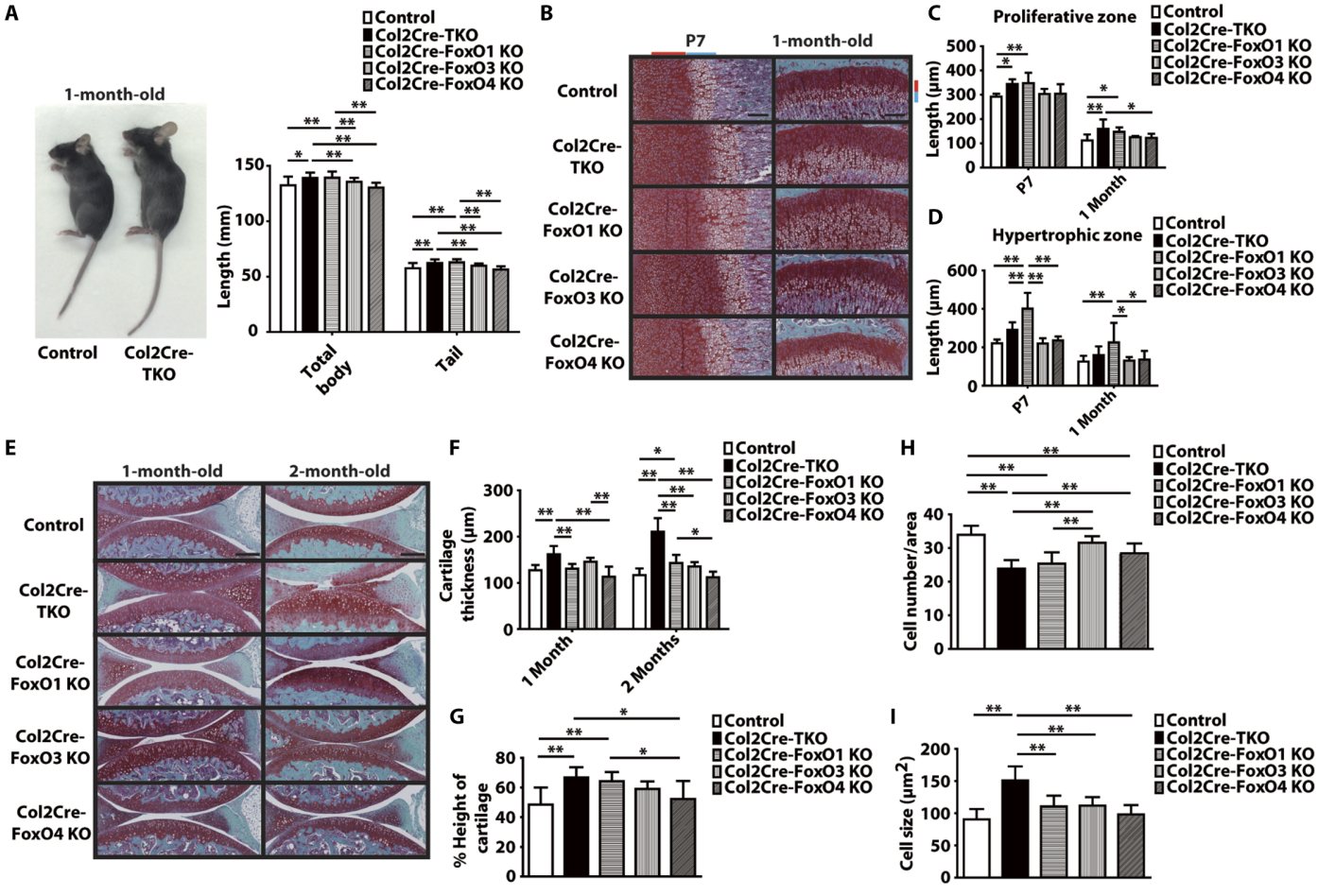

Fig. 1. Body size, growth plate, and articular cartilage in Col2Cre-FoxO KO mice. (**A**) Photograph and quantification of body length and tail length in 1-month-old mice. KO, knockout; TKO, triple KO. (**B**) Histological sections of proximal tibial growth plate from postnatal day 7 (P7) and 1-month-old mice. Scale bar, 200 μm. Red bar indicates proliferative zone and blue bar indicates hypertrophic zone. (**C**) Quantification of lengths of proliferative zone and (**D**) hypertrophic zone. (**E**) Histological sections of articular cartilage from 1- and 2-month-old mice. Scale bar, 200 μm. (**F**) Articular cartilage thickness measured as the distance between the articular surface and the subchondral bone interface across three points in each medial tibial plateau of the knee joint in mice at 1 and 2 months. (**G**) Ratios of height of cartilage above tidemark measured at 2 months. (**H**) Cell density above the tidemark and (**I**) cell size in the cartilage of tibial plateau measured at 2 months. Numbers of mice are listed in table S1. Data are means ± SD. *$P < 0.05$, **$P < 0.01$ [one-way analysis of variance (ANOVA) and Bonferroni's post test].

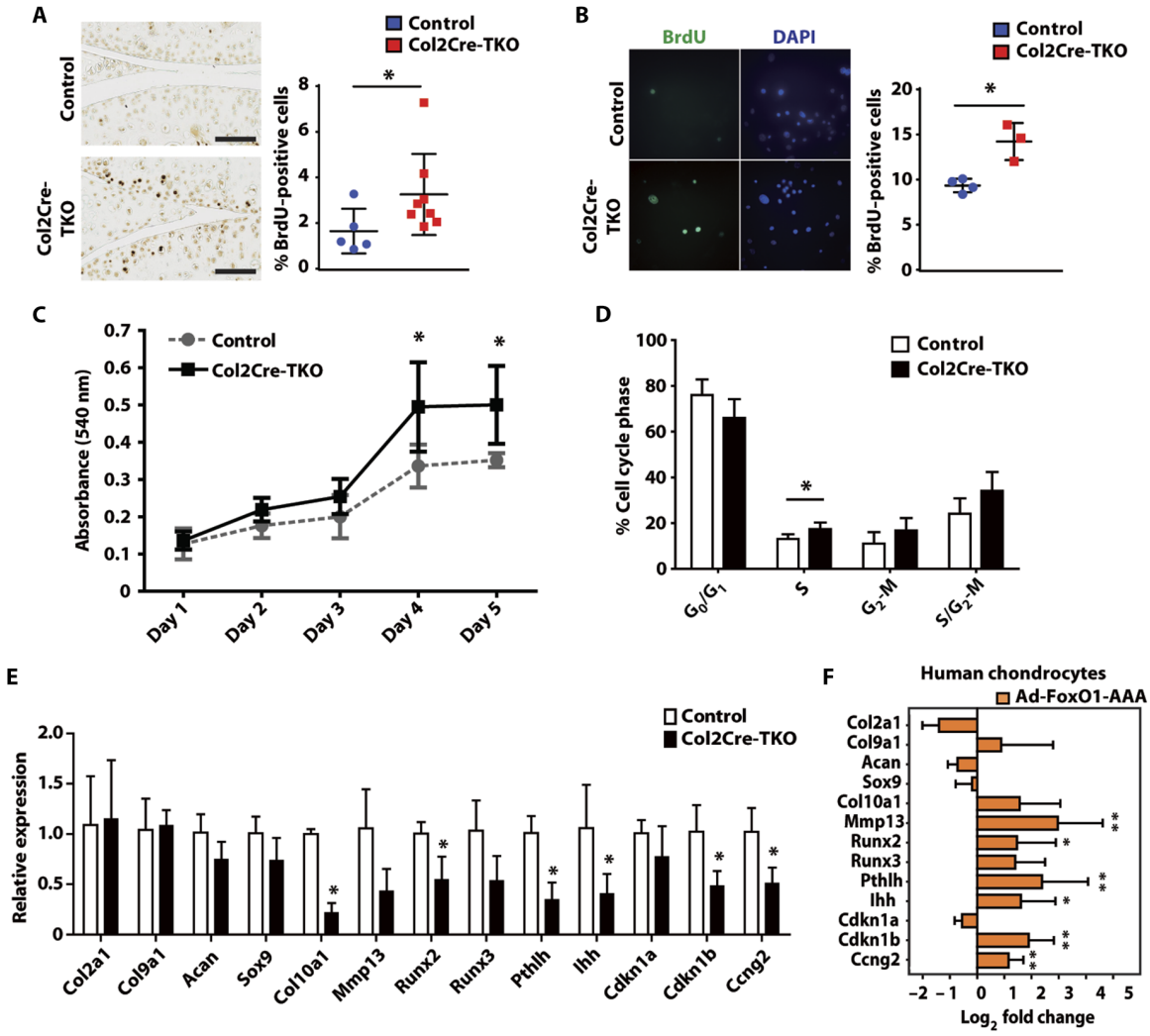

Fig. 2. FoxO effect on cell proliferation and gene expression in chondrocytes. (**A**) Immunohistochemistry and quantification of 5-bromo-2′-deoxyuridine (BrdU) in control (n = 5) and Col2Cre-TKO mouse (n = 8) femur and tibia sections. Scale bars, 100 μm. (**B**) Fluorescence images and quantification of BrdU-positive cells in immature mouse articular chondrocytes (IMACs) from P6 control (n = 4) and Col2Cre-TKO mice (n = 3) in culture (×40 magnification). DAPI, 4′,6-diamidino-2-phenylindole. (**C**) Quantification of proliferation (MTT assay) in IMACs from P6 control (n = 4) and Col2Cre-TKO mice (n = 5). (**D**) Flow cytometry cell cycle analysis of IMACs from P6 control and Col2Cre-TKO mice. The percentages of cells in G_1, S, and G_2-M phase are indicated (n = 5 each). (**E**) Real-time polymerase chain reaction (PCR) analysis for chondrogenic markers and cell cycle genes using RNA from knee joint cartilage from 1-month-old Col2Cre-TKO mice and control mice (n = 4 each). Expression values are relative to *Gapdh*. (**F**) Real-time PCR analysis for chondrogenic markers and cell cycle genes in adenoviral FoxO1-AAA–transduced human chondrocytes (n = 6). Values are relative to *Actb*, and expression values are normalized to Ad-GFP (green fluorescent protein)–transfected cells. Data are means ± SD. *P < 0.05, **P < 0.01 (Mann-Whitney test).

Col2Cre-TKO mice, *Cdkn1b* from Col2Cre-TKO, Col2Cre-FoxO1, and Col2Cre-FoxO3 KO mice, and *Ccng2* from Col2Cre-TKO and Col2Cre-FoxO3 KO mice were seen at 2 months of age (fig. S2A). FoxO1 overexpression in cultured human chondrocytes increased expression of *Cdkn1b* and *Ccng2* mRNA (Fig. 2F). Collectively, these data suggest that FoxO controls chondrocyte proliferation in vivo and in vitro.

Next, we analyzed extracellular matrix genes. *Col2a1* in cartilage was significantly increased only in Col2Cre-FoxO1 (1 month; P = 0.001; 2 months, P = 0.006) but not in Col2Cre-FoxO3 or Col2Cre-FoxO4 KO mice (fig. S2B), indicating specific functions of FoxO isoforms. Col10a1 was significantly reduced in Col2Cre-TKO mice (Fig. 2E). In periods of rapid skeletal growth, abnormal chondrocyte differentiation may alter articular cartilage dynamics and result in increased cartilage thickness (*24*). Analysis of chondrocyte differentiation-related genes in the cartilage from 1-month-old Col2Cre-TKO mice showed reduced expression of genes associated with hypertrophic chondrocyte differentiation such as *Mmp13* (P = 0.057), *Runx2*, *Runx3* (P = 0.057), *Pthlh*, and *Ihh* (Fig. 2E). Deletion of single FoxO1, 3, or 4 led to variable changes in genes related to chondrocyte hypertrophy (fig. S2C). Overexpression of a constitutively active FoxO1 mutant, in which three phosphorylation sites were mutated to alanine (FoxO1-AAA), in human chondrocytes resulted in increased expression of *Mmp13*, *Runx2*, *Pthlh*, and *Ihh* (Fig. 2F).

FoxO deletion results in spontaneous degradation of articular cartilage

The articular cartilage surfaces of 1-month-old FoxO KO mice were intact, and there were no apparent degenerative changes; however,

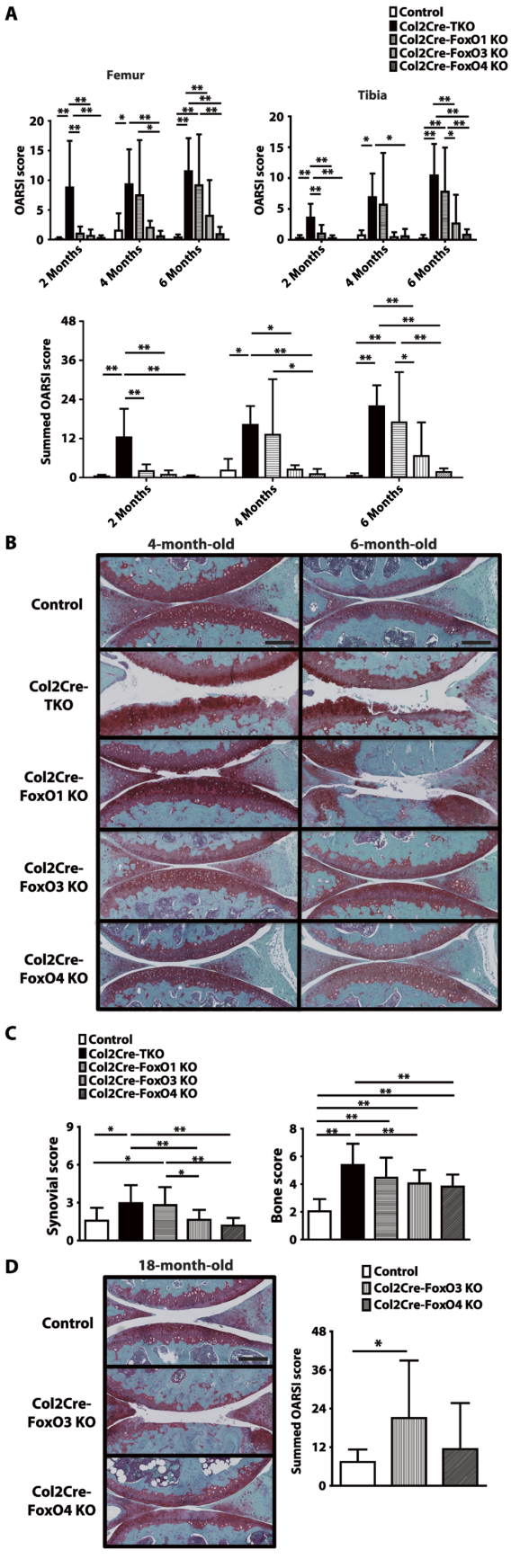

Fig. 3. Spontaneous development of OA-like changes in joint tissues of Col2Cre-FoxO KO mice. (**A**) Summed Osteoarthritis Research Society International (OARSI) scores for the medial femoral condyle and tibial plateau from 2-, 4-, and 6-month-old mice. (**B**) Histological sections of knee joints from 4- and 6-month-old mice. Scale bar, 200 μm. (**C**) Synovium and bone scores obtained from the same sections from 6-month-old mice. (**D**) Histological sections of knee joints and summed OARSI scores for the medial femoral condyle and tibial plateau from 18-month-old control, Col2Cre-FoxO3 KO, and Col2Cre-FoxO4 KO mice. Numbers of mice are listed in table S1. Data are means ± SD. *P < 0.05, **P < 0.01 (one-way ANOVA and Bonferroni's post test).

Col2Cre-TKO mice showed cartilage surface defects by 2 months of age, resulting in increased OA scores (Figs. 1 and 3A). Between 4 and 6 months of age, full-thickness cartilage lesions developed in Col2Cre-TKO mice (Fig. 3, A and B). In a similar fashion, Col2Cre-FoxO1 KO mice developed severe cartilage lesions at 6 months, whereas Col2Cre-FoxO3 and FoxO4 KO mice showed no histological cartilage abnormalities up to 6 months of age (Fig. 3, A and B). In the growth plate, there was malalignment of cells in 4-month-old Col2Cre-TKO mice, and cell numbers were significantly reduced in 6-month-old Col2Cre-TKO and Col2Cre-FoxO1 KO mice (fig. S3, A and B) (TKO, P < 0.001; FoxO1 KO, P < 0.001). In addition, other joint tissues were affected in Col2Cre-TKO and Col2Cre-FoxO1 KO mice. Synovial inflammation and bone changes were more severe in Col2Cre-TKO and Col2Cre-FoxO1 KO mice than in control mice at 6 months of age (Fig. 3C). Bone scores were also higher in the Col2Cre-FoxO3 KO and Col2Cre-FoxO4 KO mice (Fig. 3C). Furthermore, Col2Cre-FoxO3 KO showed significantly more severe cartilage lesions at 18 months (P = 0.043) than control mice (Fig. 3D).

FoxO are chondrocyte survival factors in postnatal articular cartilage

To further identify mechanisms involved in cartilage degradation, we examined the cartilage of 2-month-old mice because severe lesions had not yet developed at this time. Terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick end labeling (TUNEL) staining revealed a marked increase in the number of apoptotic chondrocytes in the articular cartilage of Col2Cre-TKO mice (Fig. 4A). This result suggests that FoxO are important regulators of cell survival in articular cartilage. In keeping with this, decreased cell viability of IMACs from Col2Cre-TKO mice was seen under H_2O_2 stimulation (Fig. 4B).

Because FoxO are known transcriptional regulators of cellular stress responses, we next examined the expression of key genes involved in homeostatic responses. FoxO deletion resulted in decreased expression of genes involved in antioxidant defenses (*Sesn1*, *Sesn3*, and *Gpx3*), autophagy (*Lc3b*, *Atg4b*, *Becn1*, *Gabarapl1*, and *Bnip3*), redox regulation (*Cat* and *Txnip*), and adaptation to energy stress (*Prkaa2*) (Fig. 4C). Consistent with these in vivo findings, overexpression of the constitutively active FoxO1-AAA mutant in human normal chondrocytes from normal articular cartilage and IMACs from C57BL6/J wild-type mice increased the expression of *Lc3b*, *Becn1*, and *Gabarapl1* (Fig. 4D). Collectively, these data suggest that FoxO are essential regulators of cell viability in articular cartilage by coordinating key cellular stress responses.

FoxO1 regulates *Prg4* expression and is essential for maintaining cartilage superficial zone integrity

The earliest histological feature indicative of cartilage damage in the Col2Cre-TKO mice was an irregular articular surface at 1 month (Fig. 5A). Cell density in the superficial zone in the tibial cartilage was

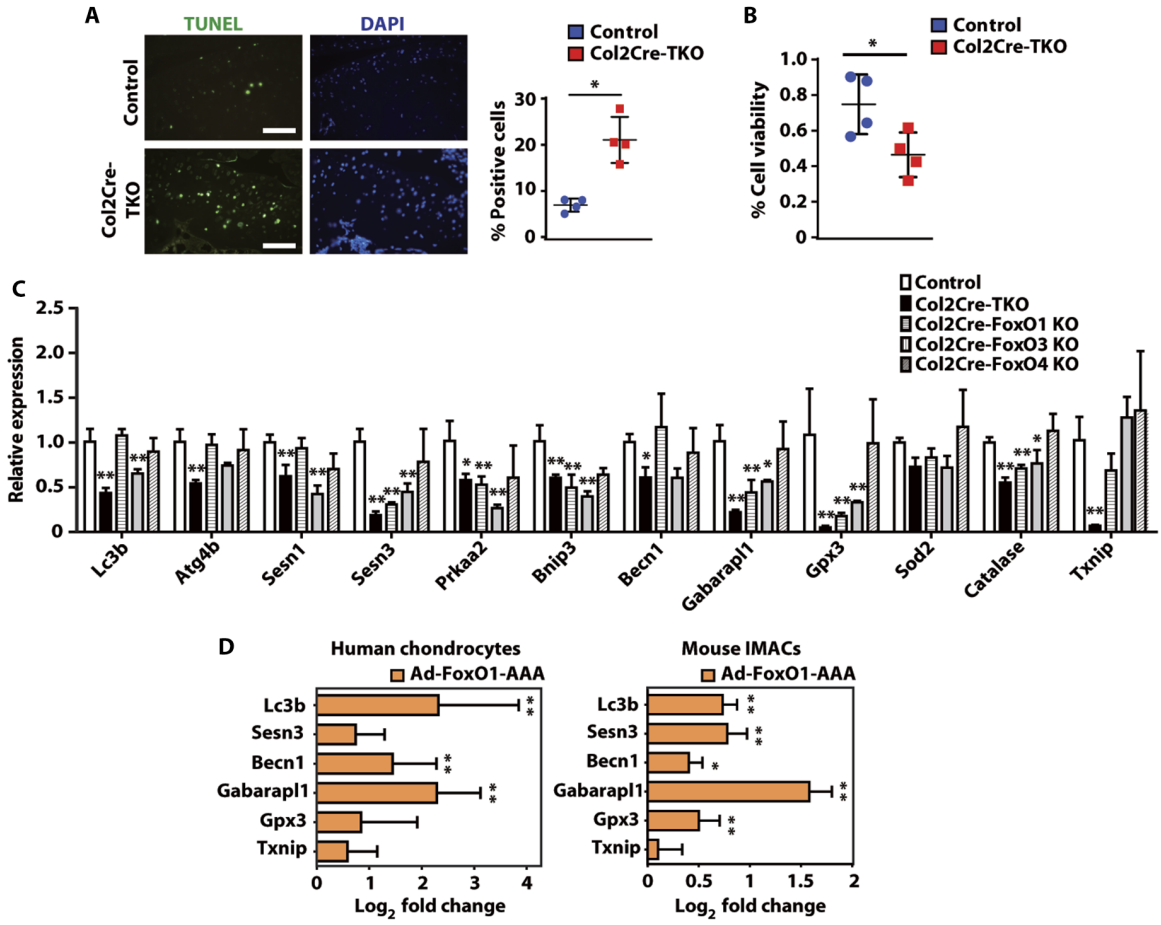

Fig. 4. Cell viability and cellular homeostasis gene expression. (**A**) TUNEL (terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick end labeling) staining (scale bars, 100 μm) and quantitative analysis of the TUNEL-positive cell counts per field in the central weight-bearing region of the medial tibial plateau in 2-month-old mice. Cell viability under 500 μM H_2O_2 was measured by resazurin assay (n = 4 each). Results are shown as % viability compared to cells not treated with H_2O_2. (**C**) Real-time PCR analysis using RNA from knee joint cartilage from 2-month-old Col2Cre-TKO, Col2Cre-FoxO1 KO, and control mice (control, n = 4; Col2Cre-TKO, n = 4; Col2Cre-FoxO1 KO, n = 4; Col2Cre-FoxO3 KO, n = 3; Col2Cre-FoxO4 KO, n = 3). Expression values were normalized to *Gapdh* as control. (**D**) Real-time PCR analysis from adenoviral FoxO1-AAA–transduced healthy human chondrocytes (n = 4) and C57BL6/J wild-type mouse IMACs (n = 6). The values are relative to *Actb*, and expression values are normalized to Ad-GFP–transfected cells. Data are means ± SD. *P < 0.05, **P < 0.01 (Mann-Whitney test for two groups and one-way ANOVA and Bonferroni's post test for multiple groups).

significantly reduced (P < 0.001) with a >50% depletion of the elongated superficial zone cells (Fig. 5A). Picrosirius red staining, which allows visualization of collagen fibers, showed abnormal collagen organization with an overall reduction of the stained area in the superficial zone of Col2Cre-TKO mice (Fig. 5B). Superficial zone chondrocyte markers, including superficial zone protein (SZP) (also known as lubricin, encoded by the *Prg4* gene) and vascular cell adhesion molecule 1 (VCAM1), were also lower in Col2Cre-TKO mice (Fig. 5C). Reduction of *Prg4* expression was also confirmed by PCR on cartilage RNA from P1 mice, 1-month-old Col2Cre-TKO mice, and 2-month-old Col2Cre-TKO and Col2Cre-FoxO1 KO mice, but not in Col2Cre-FoxO3 KO or Col2Cre-FoxO4 KO mice (Fig. 5D), suggesting that FoxO1 is a transcriptional regulator of *Prg4* in cartilage.

FoxO regulate cartilage homeostasis and OA pathogenesis in skeletally mature mice

Because FoxO conditional deletion using the Col2Cre promoter resulted in defects in cartilage maturation, we used the Aggrecan-CreERT2 (AcanCreERT) knock-in mice (*25*) to directly analyze the role of FoxO in the maintenance of mature articular cartilage and OA pathogenesis. $FoxO1^{lox/lox}$;$FoxO3^{lox/lox}$;$FoxO4^{lox/lox}$ triple transgenic mice were crossed with AcanCreERT mice to generate FoxO triple KO mice (AcanCreERT-TKO). FoxO deletion was confirmed by real-time PCR (fig. S4). The postnatal FoxO deletion resulted in decreased mRNA expression of *Prg4*, *Cdkn1b*, *Sesn3*, *Gabarapl1*, *Gpx3*, and *Txnip*, whereas the expression of *Col2a1* and *Col9a1* were increased (Fig. 6A). In the same manner as Col2Cre-TKO mice, 4-month-old AcanCreERT-TKO showed thicker cartilage (Fig. 6B) and reduced cell density in the cartilage superficial zone (Fig. 6C), which was associated with reduced SZP expression in the cartilage on tibial plateau (Fig. 6D). The AcanCreERT-TKO mice developed mild cartilage lesions 2 months after tamoxifen administration (Fig. 6E), and this progressed to full-thickness cartilage defects 5 months after tamoxifen administration (Fig. 6F). Synovial inflammation and subchondral bone changes were also more severe in AcanCreERT-TKO mice than in control mice (fig. S5). In addition, the severity of OA induced by surgical destabilization of the medial

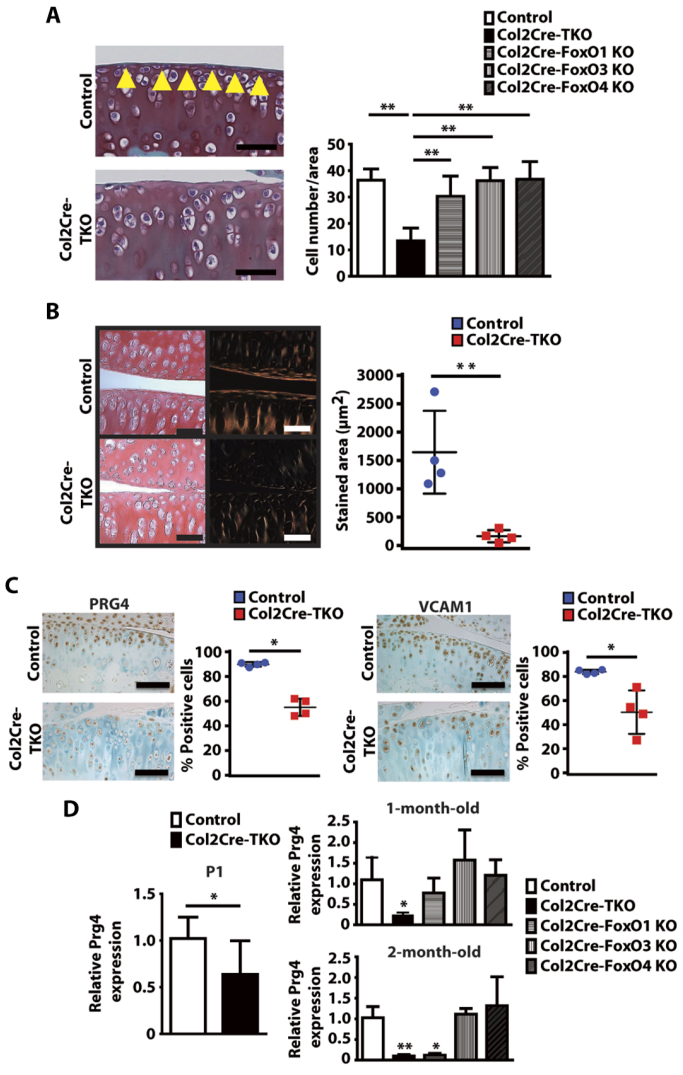

Fig. 5. Cartilage superficial zone changes in Col2Cre-FoxO KO mice. (**A**) Images and quantification of superficial zone cells in the cartilage on the tibial plateau per superficial zone area (10 μm from the surface) measured in 1-month-old mice. Scale bar, 50 μm. Yellow arrows indicate superficial zone cells in healthy cartilage. Numbers of mice are listed in table S1. (**B**) Images of Picrosirius red–stained knee joint sections from 1-month-old mice under standard light (left) or polarized light (right). Intensity of stained area in superficial zone of cartilage on tibial plateau (10 μm from the surface) was measured by ImageJ software (n = 4 each). Scale bar, 100 μm. (**C**) Immunohistochemistry and quantification for superficial zone cell markers superficial zone protein (SZP) and vascular cell adhesion molecule 1 (VCAM1) in the cartilage on the tibial plateau from 1-month-old mice (n = 4 each). Scale bar, 100 μm. (**D**) Real-time PCR analysis for *Prg4* using RNA isolated from cartilage from P1, 1-month-old, and 2-month-old mice (P1: n = 7 each; 1-month-old: control, n = 4; Col2Cre-TKO, n = 4; Col2Cre-FoxO1 KO, n = 3; Col2Cre-FoxO3 KO, n = 3; and Col2Cre-FoxO4 KO, n = 3; 2-month-old: control, n = 4; Col2Cre-TKO, n = 4; Col2Cre-FoxO1 KO, n = 4; Col2Cre-FoxO3 KO, n = 3; and Col2Cre-FoxO4 KO, n = 3). The expression values are relative to *Gapdh*, and each value is compared to control. Data are means ± SD. *P < 0.05, **P < 0.01 (Mann-Whitney test for two groups and one-way ANOVA and Bonferroni's post test for multiple groups).

meniscus (DMM) and treadmill running was also significantly higher in AcanCreERT-TKO mice (P = 0.027 and P = 0.001, respectively) than in control mice (Fig. 6, G and H), indicating a critical role of FoxO in articular cartilage homeostasis during aging and excess mechanical load.

FoxO1 activates *Prg4* expression and synergizes with transforming growth factor–β

To investigate the molecular mechanisms by which FoxO1 regulates PRG4 expression, we overexpressed FoxO1-AAA in IMACs and ATDC5 chondrogenic cells using adenovirus or plasmid and observed increased *Prg4* mRNA expression (Fig. 7A). In a three-dimensional pellet culture of adipose tissue–derived mesenchymal stem cells (MSCs) isolated from FoxO triple floxed mice, transduction of cells with adenovirus expressing Cre recombinase also reduced *Prg4* mRNA during chondrogenic differentiation (Fig. 7B). Because transforming growth factor–β (TGFβ) is a well-known inducer of chondrogenesis and *Prg4* expression (*26*, *27*), we further investigated the relationship between TGFβ and FoxO. TGFβ1 treatment increased *Prg4* expression in control mouse chondrocytes. Although FoxO-depleted IMACs showed an increase in *Prg4* expression in response to TGFβ, the *Prg4* levels were significantly lower than in TGFβ-stimulated control cells (Fig. 7C). In addition, exogenous TGFβ1 did not promote increased secretion of PRG4 protein in IMACs of Col2Cre-TKO mice (Fig. 7D). In ATDC5 cells, FoxO1 overexpression synergistically increased *Prg4* expression in the presence of TGFβ1 (Fig. 7E). This effect was dependent on FoxO1 ability to bind DNA, because overexpression of the DNA binding mutant FoxO1H215RAAA showed no effect on *Prg4* expression by itself or in combination with TGFβ1 (Fig. 7F).

FoxO1 normalizes gene expression in human OA chondrocytes and antagonizes the effect of interleukin-1β

In human OA chondrocytes, inflammatory genes (*Il6*, *Nos2*, and *Ptgs2*) and catabolic genes (*Mmp3* and *Mmp13*) were increased (Fig. 8A), whereas *FoxO1* and autophagic genes including *Map1lc3b*, *Sesn3*, *Prkaa2*, and *Becn1* were decreased as compared to chondrocytes from healthy human knee joints (Fig. 8B). Adenoviral overexpression of FoxO1-AAA in OA chondrocytes increased autophagic genes (*Map1lc3b*, *Sesn3*, and *Prkaa2*) (Fig. 8C). Stimulation of the chondrocytes with interleukin-1β (IL-1β), a prototypic inducer of OA-related genes (*28*), increased the expression of *Il6*, *Nos2*, *Ptgs2*, *Adamts4*, and *Adamts5*. FoxO1 transduction suppressed all IL-1β–induced genes (Fig. 8D).

DISCUSSION

Our previous findings that FoxO1, FoxO3, and FoxO4 mRNA and protein expression are reduced in aging and OA-affected cartilage in humans and mice (*22*) motivated the present study to analyze consequences of chondrocyte-specific FoxO deletion. First, we used the Col2a1-Cre mouse strain with Cre-mediated recombination in Col2a1-expressing cells, especially in chondrocytes (*29*), to analyze mice during postnatal growth and maturation from P1 until 18 months of age. We saw phenotypic changes in the epiphyseal growth plate, articular cartilage, and other joint tissues in Col2Cre-TKO mice and similarly in Col2Cre-FoxO1 KO mice. In addition, deletion of FoxO3 led to more severe and earlier onset of age-related OA-like changes at 18 months.

The role of FoxO in endochondral ossification was recently studied using the same gene-targeting strategy to create Col2Cre-TKO mice (*30*). Neonatal mice showed elongation of the hypertrophic zone of the growth plate and had increased overall body

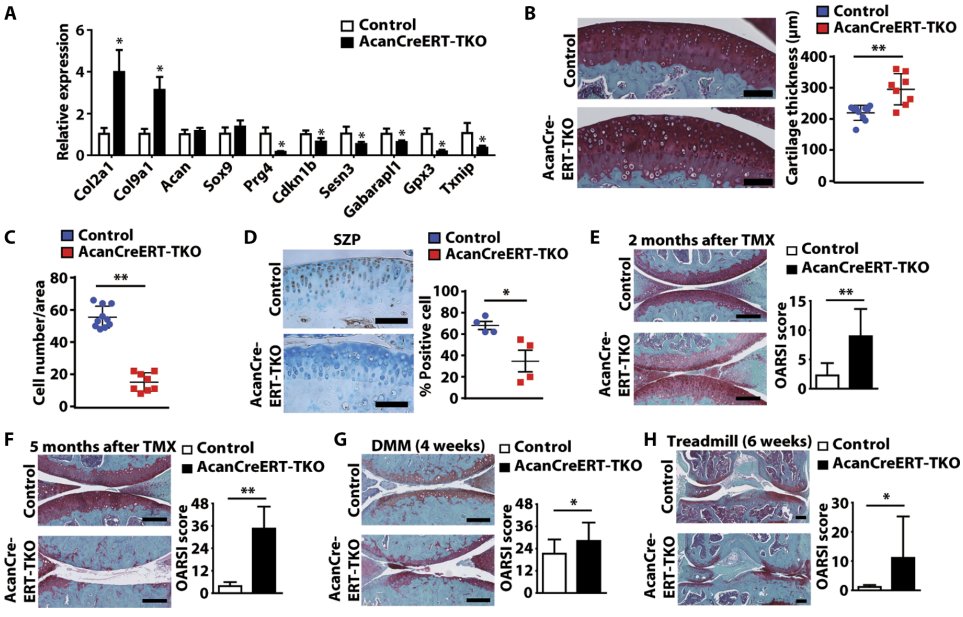

Fig. 6. Phenotype of postnatal deletion of FoxO in cartilage using Aggrecan-CreERT knock-in mice. (**A**) Real-time PCR analysis using RNA from knee joint cartilage in control and Aggrecan-CreERT2 TKO (AcanCreERT-TKO) mice 2 weeks after tamoxifen (TMX) injection ($n = 4$ each). The expression values are relative to *Gapdh*. (**B**) Images and quantification of the thickness of articular cartilage measured as the distance between the articular surface and the subchondral bone interface (scale bar, 100 μm) across three points in each medial tibial plateau of the knee joint in mice at 2 months after tamoxifen injection (control, $n = 10$; AcanCreERT-TKO, $n = 8$). (**C**) Quantification of the numbers of superficial zone cells of cartilage on the tibial plateau per superficial zone area (10 μm from the surface) measured in mice 2 months after tamoxifen injection (control, $n = 10$; AcanCreERT-TKO, $n = 8$). (**D**) Immunohistochemistry for SZP and quantification of percent SZP-positive cells in the cartilage on the tibial plateau of mice 2 months after tamoxifen injection ($n = 4$ each). Scale bar, 100 μm. (**E**) Histological sections and OARSI scores of knee joints from mice 2 and (**F**) 5 months after tamoxifen injection, (**G**) 4 weeks after surgical osteoarthritis (OA) model (scale bar, 200 μm), and (**H**) 6 weeks after treadmill-induced OA model (coronal section; scale bar, 200 μm). DMM, destabilization of the medial meniscus. OARSI scores were obtained by summing the scores for the medial femoral condyle and tibial plateau. Numbers of mice are listed in table S1. Data are means ± SD. *$P < 0.05$, **$P < 0.01$ (Mann-Whitney test).

and tail length at 8 weeks of age (*30*). The present study revealed that FoxO1 appears to be the FoxO isoform that is largely responsible for the growth plate abnormalities.

The epiphyseal cartilage in the KO mice appeared normal at birth, and there were no apparent changes in tissue volume, cell density, size, or organization. However, the articular cartilage was significantly thicker in Col2Cre-TKO mice at 1 month and Col2Cre-FoxO1 KO mice at 2 months. The cartilage of these mutant mice also exhibited increased cell proliferation and abnormal expression of chondrocyte differentiation markers. These findings indicate that FoxO deletion leads to increased cartilage thickness by regulating chondrocyte proliferation and differentiation.

Articular cartilage lesions developed spontaneously in Col2Cre-TKO and Col2Cre-FoxO1 KO mice between 2 and 6 months of age. Because these degradative changes could be at least in part due to abnormal cartilage growth and maturation during the postnatal period, we deleted FoxO1/3/4 in skeletally mature mice using the Aggrecan-CreERT2 knock-in mice (*25*). These mice started to show OA-like changes within 2 months after FoxO deletion and full-thickness cartilage defects after 5 months. Surgical destabilization of the knee joint in mice (*31*) is associated with mechanisms and features similar to posttraumatic OA in humans (*32*). We also used a treadmill running model with a protocol, which leads to mild cartilage damage in wild-type mice (*33*). In both models, the mice with postnatal FoxO deletion showed more severe cartilage damage, suggesting that FoxO have protective functions in the response of cartilage to joint trauma and mechanical overload.

The earliest morphological changes in the FoxO KO mice were irregularities in the superficial zone and a depletion of the elongated superficial zone cells (*34*). We observed reduced expression of *Prg4* in FoxO KO mice, which is consistent with a previous study in T lymphocytes (*35*). The *Prg4* gene (*36*) encodes a mucin-like, O-linked glycosylated protein, termed lubricin (*37*) or SZP (*38*). It is produced by cells in the articular cartilage surface (*38*), meniscus (*39*), and synovium (*40*) and present in the extracellular matrix of the superficial zone (*41*) and in synovial fluid (*42*). Lubricin functions as a boundary lubricant in articular cartilage to decrease friction and wear, and the accumulation of lubricin at the surface of cartilage is thought to be important for joint homeostasis (*34*). Mice lacking lubricin have increased baseline coefficient of friction values and are not protected against further increases in friction values caused by loading (*43*). *Prg4*-deficient mice also develop superficial and upper mid zone chondrocyte apoptosis and cell loss (*44*, *45*). Human cartilage aging is characterized by decreased mechanical function in the superficial zone, with reduced tensile integrity and surface wear, reduced cellularity, and a decrease in matrix glycosaminoglycan content (*46*). The findings observed in FoxO KO mice are thus consistent with changes that were seen in lubricin-deficient mice (*44*, *45*) and during early stages of human joint aging (*46*).

The present findings support a direct role of FoxO1 in up-regulating the expression of *Prg4*. Transfection of a constitutively active form but not of a DNA binding deficient form of FoxO1 increased *Prg4* expression. TGFβ is a main stimulus of *Prg4* (*26*, *27*), and the chondrocyte response to TGFβ decreases with aging (*47*). Because the present results show that FoxO1 synergizes with TGFβ1 to increase *Prg4* gene expression, the aging-related loss of FoxO is a likely factor in the reduced *Prg4* expression and potentially in other compromised TGFβ-induced cellular responses.

Although the FoxO deficiency–related reduction in *Prg4* expression appears to be an early and important event in initiating structural changes in the superficial zone, other chondrocyte functions are abnormal in FoxO-deficient mice and are likely to contribute to rapid and severe cartilage destruction. In addition to reduced *Prg4* expression, we observed lower expression of important cellular homeostasis genes in FoxO-deficient mice. Genes involved in antioxidant

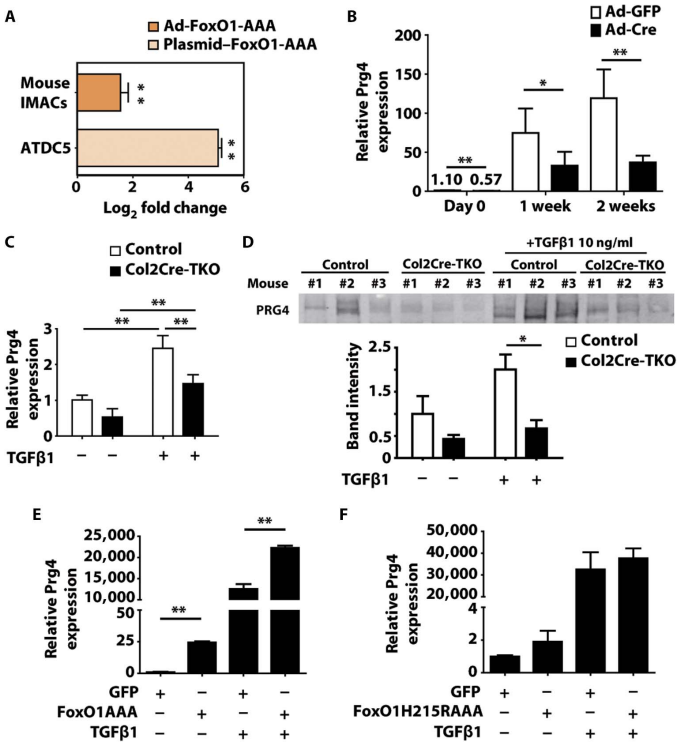

Fig. 7. FoxO and *Prg4* expression in mouse articular chondrocytes and chondrogenic cells. (**A**) Real-time PCR analysis for *Prg4* in adenoviral FoxO1-AAA– or Ad-GFP–transduced IMACs (n = 6) and plasmid FoxO1-AAA– or plasmid GFP control–transfected ATDC5 cells (n = 3). The values are relative to *Actb*, and expression values are normalized to Ad-GFP– or plasmid GFP–transduced cells. (**B**) Real-time PCR for *Prg4* expression in cultured adipose tissue–derived mesenchymal stem cells from FoxO triple floxed mice with or without adenovirus-Cre infection (n = 6 each). Day 0 was defined as the start date 72 hours after transfection. Values are shown relative to those from day 0 cells not infected with adenovirus. (**C**) Real-time PCR analysis of *Prg4* in IMACs from control and Col2Cre-TKO mice treated with TGFβ1 (transforming growth factor–β; 10 ng/ml) or vehicle for 6 hours (control, n = 5; Col2Cre-TKO, n = 4). The expression values are relative to *Gapdh*. (**D**) Western blotting for secreted PRG4 protein. IMACs from control and Col2Cre-TKO mice (n = 3 each) were treated with TGFβ1 (10 ng/ml) or vehicle for 24 hours. Secreted PRG4 protein in the culture supernatants was measured by Western blotting. Equal amounts of concentrated supernatants from identical cell numbers were loaded on the gels. Band intensity was quantified by ImageJ. (**E**) Real-time PCR analysis of *Prg4* expression in ATDC5 cells transfected with GFP or FoxO1-AAA and treated with TGFβ1 or vehicle for 24 hours. The expression values are relative to *Gapdh*. (**F**) Real-time PCR analysis of Prg4 in ATDC5 cells transfected with GFP, FoxO1H2015RAAA, and treated with TGFβ1 (10 ng/ml) or vehicle for 24 hours. The expression values are relative to *Gapdh*. All experiments were performed in duplicate. Data are means ± SD. *P* values were calculated with Mann-Whitney test (A to D) and Student's *t* test (E and F). **P* < 0.05, ***P* < 0.01.

defenses (*Sesn3* and *Gpx3*), autophagy (*Map1lc3b*, *Atg4b*, *Becn1*, *Gabarapl1*, and *Bnip3*), redox regulation (*Txnip*), and adaptation to energy stress (*Prkaa2*) were reduced in cartilage of FoxO KO mice. Notably, expression of several of these genes including autophagy proteins (*20*), *Txnip* (*48*), sestrins (*49*), and *Prkaa2* (*50*) are reduced in aging and OA cartilage, raising the possibility that these gene expression changes are due to the reduced expression of FoxO that we reported earlier to occur spontaneously in aging and OA in mice and humans (*22*).

To test this and examine the potential therapeutic benefit of targeting FoxO in OA, we cultured human OA chondrocytes, which show abnormal gene expression patterns. Overexpression of FoxO1 in human OA chondrocytes increased autophagy genes, reduced inflammatory mediators and cartilage-degrading enzymes, and antagonized the effect of IL-1β stimulation. These results indicate that reduced FoxO expression in human OA chondrocytes is at least in part responsible for abnormal expression of homeostasis genes and mediators of OA pathogenesis.

The present results suggesting FoxO as a therapeutic target in OA are limited to studies using FoxO-deficient mice and human cells. Further studies are needed to demonstrate in vivo that overexpression of FoxO using genetic approaches in mice or using pharmacological approaches in larger animal models can protect against OA.

OA is a disease that affects all joint tissues, and FoxO KO mice showed changes not only in cartilage but also in synovium and subchondral bone. It should be noted that the Col2a1-Cre driver used in the present study targets not only articular and growth plate chondrocytes but also other cells in joint tissues, including cells in synovium (*51*). We observed OA-like changes in synovium and subchondral bone in 6-month-old Col2Cre-TKO and Col2Cre-FoxO1 KO mice. Down-regulation of FoxO1 is involved in synoviocyte survival and synovial hyperplasia in rheumatoid arthritis (*52*). Consistent with this FoxO function, synovial hyperplasia was the main manifestation of FoxO deficiency observed in the present study. FoxO are also key regulators of bone formation and remodeling (*53*). However, the subchondral bone changes observed in the mice with chondrocyte-specific deletion of FoxO are likely secondary to initial changes in the articular cartilage. Mouse models of OA are characterized by a close association of changes within the osteochondral unit (*54*).

The results from the present study revealed overlapping and distinct functions of FoxO isoforms. A likely explanation for the mild changes observed in FoxO4-deficient mice is that this isoform is expressed in chondrocytes at much lower expression than FoxO1 or FoxO3 (*22*). The most severe phenotype during postnatal articular cartilage development was seen in the FoxO TKO mice. FoxO1 KO mice had similar changes as the TKO mice, but all changes in the TKO mice were consistently more severe than in the FoxO1 KO mice, suggesting that FoxO3 also has some, although lesser, effects on these processes. In this regard, FoxO3 KO mice also had changes in cell cycle regulators. The main difference between FoxO1 and FoxO3 KO mice was that only FoxO1 KO mice exhibited deficient Prg4 expression, and this is a likely explanation for why cartilage in these mice rapidly degenerated. The main reason for the aging-related OA development in the FoxO3 KO mice appears to be the reduction in protective genes (autophagy and antioxidants). These differences in FoxO function are in part related to different sets of interacting proteins (*55*).

In summary, these studies identify FoxO as essential transcription factors regulating postnatal articular cartilage growth and homeostasis. The role of FoxO in cartilage growth is mainly mediated by FoxO1 and is related to effects on chondrocyte proliferation, survival, and regulation of chondrocyte differentiation. The role of FoxO in maintaining postnatal articular cartilage integrity is mediated by their role in activating cellular defense mechanisms and in regulating the expression of PRG4, an essential protein in cartilage lubrication and superficial zone protection. These findings support the pathogenic significance of the reduction of FoxO in aging and OA-affected cartilage and suggest that

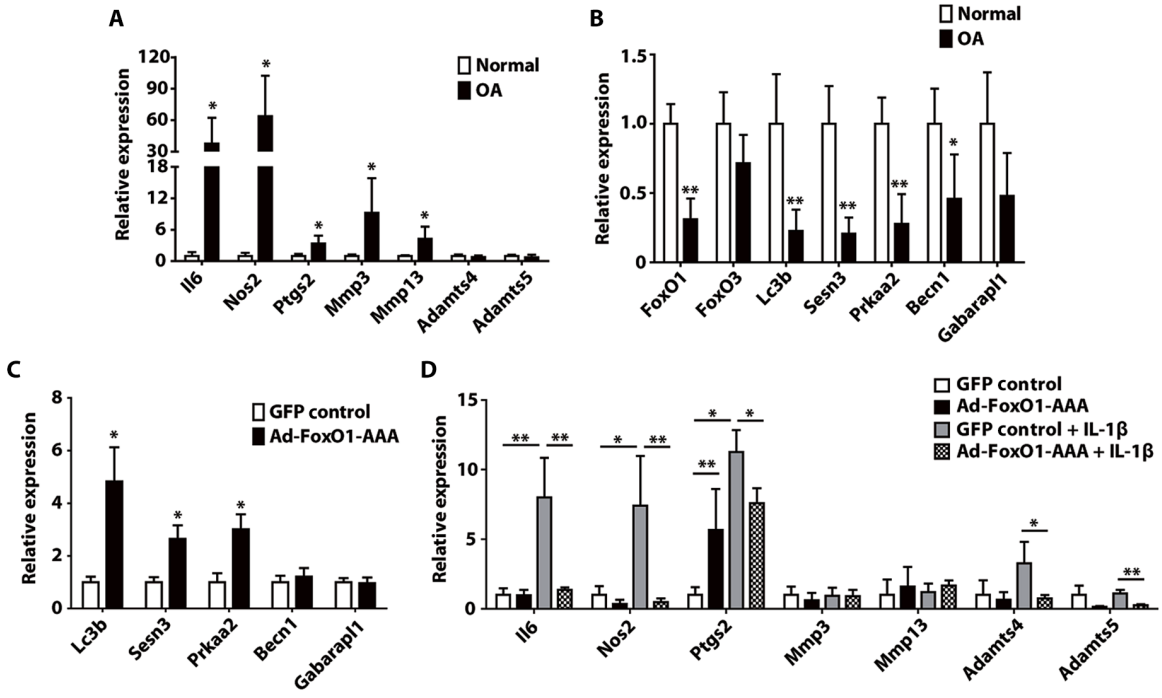

Fig. 8. Overexpression of FoxO1 in human OA chondrocytes and effects on IL-1β stimulation. (**A**) Real-time PCR analysis for inflammatory and cartilage catabolic genes and (**B**) FoxO expression and autophagic genes using RNA from healthy human (n = 4) and OA chondrocytes (n = 4) with basal medium. (**C**) Real-time PCR analysis of autophagic genes in OA chondrocytes (n = 3) transfected with Ad-GFP or FoxO1-AAA. (**D**) Real-time PCR analysis for inflammatory and cartilage catabolic genes in Ad-GFP control or FoxO1-AAA–transduced human OA chondrocytes (n = 3) with or without interleukin-1β (IL-1β). The expression values in all experiments are relative to *B2M*. Data are means ± SD. *P* values were calculated with Mann-Whitney test (A and B) and Student's *t* test (C and D). $^*P < 0.05$, $^{**}P < 0.01$.

maintaining or restoring FoxO expression can prevent OA onset and delay disease progression.

MATERIALS AND METHODS

Study design

The primary goals of the study were to investigate whether developmental or postnatal cartilage-specific deletion of FoxO1, FoxO3, and FoxO4 and of all three FoxO affects cartilage development and degeneration and to investigate whether FoxO have a protective role for mature articular cartilage. In all experiments, mice were randomly assigned to experiments. All animal studies were performed with approval by the Scripps Institutional Animal Care and Use Committee. Human tissues were obtained with approval by the Scripps Human Subjects Committee. Quantification of histopathological changes in the joint tissues was performed by at least two independent observers blinded to the experimental conditions. Sample sizes were determined on the basis of previous studies using wild-type and mutant mice to detect similar changes in the joint tissues (*22*, *56*).

Mice

Col2a1-Cre/+ transgenic mice (*23*) and Aggrecan-CreERT2 knock-in mice (*25*) on a C57BL6/J background were obtained from The Jackson Laboratory (JAX #003554). $FoxO1^{lox/lox}$, $FoxO3^{lox/lox}$, and $FoxO4^{lox/lox}$ were obtained from R. DePinho (*8*). Tamoxifen (Sigma-Aldrich) was injected intraperitoneally at a dose of 1.5 mg/10 g body weight for five consecutive days to 4-month-old mice. Genotyping was performed by PCR using tail DNA. Littermates homozygous for the floxed FoxO not expressing Cre recombinase were used as controls of Col2Cre-FoxO KO mice, and littermates with tamoxifen injections were used as controls for AcanCreERT-TKO mice. Numbers of mice are listed in table S1.

Histological analyses

Mouse knee joints were fixed in 10% zinc-buffered formalin for 2 days and decalcified in TBD-2 for 24 hours. Sections were stained with Safranin O–fast green and Picrosirius red staining. Intensity of the picrosirius red–stained area in the superficial zone was measured by ImageJ software 1.51j8 (National Institutes of Health). Histological scoring of OA-like changes on the medial femoral condyle and tibial plateau was performed using the Osteoarthritis Research Society International (OARSI) scoring system (score, 0 to 24) (*57*). Synovial changes were evaluated using Krenn's synovitis scoring system (score, 0 to 9) (*58*). To assess the subchondral bone, we used a scoring system (score, 0 to 8) that evaluates trabecular bone structure (mostly cystic, 2; partially cystic, 1), lamellar structure (destroyed, 2; partially destroyed, 1), angiogenesis (number of vessels ≥3, 2; 1 to 2, 1), and ectopic ossification/articular cartilage absorption in the tibia (present, 2).

Skeletal preparation

Whole skeletons of control and FoxO KO mice on P1 were skinned and fixed in 95% ethanol. Alcian blue staining was performed, followed by placement into potassium hydroxide, and Alizarin red staining was performed as described (*59*).

Immunohistochemistry

Knee joint sections were deparaffinized, washed, and blocked with 10% goat serum. Primary antibodies against SZP (1:300; Abcam) or

VCAM1 (1:100; Abcam) were applied and incubated overnight at 4°C, followed by ImmPRESS regents (Vector Laboratories). The signal was developed with diaminobenzidine (Sigma-Aldrich) and counterstained with methyl green. The number of SZP- or VCAM1-positive cells was quantified.

Chondrocyte proliferation, cell cycle, and apoptosis assays

BrdU (Sigma-Aldrich) was injected to 1-month-old mice with 100 mg/kg intraperitoneally daily for five consecutive days. Immunohistochemical detection of BrdU-positive cells was performed using BrdU antibody (1:1000; Proteintech). IMACs were incubated with 10 μM BrdU (Sigma-Aldrich) for 2 hours, then stained with anti-BrdU (1:200) primary antibody. To assess apoptotosis, we performed TUNEL staining using In Situ Cell Death Detection Kit (Roche Applied Science). In MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, IMACs were incubated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), and cells were analyzed on days 1 to 5. MTT (5 mg/ml) was added. After adding dimethyl sulfoxide, absorbance values were measured at 540 nm. For cell cycle analysis, fluorescence-activated cell sorting analysis was performed using FxCycle PI/RNase Staining Solution (Molecular Probes). Cells were resuspended and analyzed on a BD LSR II flow cytometer (BD Biosciences). The percentages of cells in G_1, S, and G_2-M phase were determined.

IMACs and human chondrocytes

Mouse chondrocytes were isolated as described (*60*). IMACs were stimulated with TGFβ1 (10 ng/ml) (PeproTech) for 6 or 24 hours. Human normal chondrocytes were isolated from macroscopically normal knee joints that were harvested at autopsy from six donors (mean ± SD age, 45.8 ± 9.5 years old; range, 34 to 58 years old), as described previously (*61*). Human OA chondrocytes were harvested from four donors undergoing total knee arthroplasty (mean ± SD age, 71.8 ± 7.8 years old; range, 64 to 81 years old). First or second passage cells were used. Human OA chondrocytes were incubated with IL-1β (10 ng/ml) (PeproTech) for 6 hours.

Chondrocyte viability

IMACs were incubated with 500 μM H_2O_2 for 24 hours when resazurin solution (500 μM in phosphate-buffered saline) was added and incubated for 2 hours. Fluorescence was measured using excitation and emission wavelengths of 530 and 590 nm. Cell viability was calculated as the percent absorbance of H_2O_2-treated cells compared to the cells not treated with H_2O_2.

RNA isolation and real-time PCR

Articular cartilage was collected from both sides of the femoral condyle and tibial plateau (knee joint) from 2-month-old mice and mice with tamoxifen-induced FoxO deletion and from the knee joint and femoral head of P1 and 1-month-old mice (tissues from three mice were pooled and considered one sample; $n = 1$). Total RNA was extracted from mouse cartilage or cultured cells using TRIzol (Invitrogen), followed by Direct-zol RNA MiniPrep kits (Zymo Research). Real-time PCR was performed on a LightCycler 480 instrument (Roche Diagnostics) using TaqMan probes listed in table S2.

MSC isolation and chondrogenic differentiation assay

Adipose tissue–derived MSCs were isolated from subcutaneous adipose tissue of control mice (*62*). To delete FoxO, we infected the cells with Adeno-CMV-Cre (Vector Biolabs) at 25 MOI (multiplicity of infection). Cells were then cultured in pellets in DMEM with 10% FCS, rhTGFβ3 (10 ng/ml) (PeproTech), 1× insulin-transferrin-selenium, with sodium pyruvate (11 μg/ml) (Invitrogen), 100 nM dexamethasone (Sigma-Aldrich), L-ascorbic acid 2-phosphate (50 μg/ml) (Sigma-Aldrich), L-proline (40 μg/ml) (Sigma-Aldrich), and penicillin/streptomycin to induce chondrogenic differentiation, and samples were collected after 1 and 2 weeks.

FoxO1 overexpression and suppression

Recombinant adenoviral vector encoding constitutively active FoxO1-AAA (*63*) was constructed using pAd/CMV/V5-DEST Gateway vector with pcDNA3–FLAG–FoxO1-AAA (Ad-FoxO1-AAA; Addgene plasmid #13508). Human normal chondrocytes, OA chondrocytes, and IMACs were infected with Ad-FoxO1-AAA or control Ad-eGFP (enhanced green fluorescent protein; Addgene) by using Lipofectamine 3000 reagent (Invitrogen) at 5 MOI (IMACs) and 20 MOI (human normal and OA chondrocytes). Cells were collected after 48 hours. ATDC5 murine chondrocytic cells were transfected with plasmids encoding constitutively active FoxO1 (FoxO1-AAA; Addgene plasmid #13508) (*63*), DNA binding FoxO1 domain mutant (FoxO1H215RAA; Addgene plasmid #13510) or GFP control using Lipofectamine 3000. ATDC5 cells were stimulated with TGFβ1 (10 ng/ml) (PeproTech) for 24 hours.

Western blotting

Western blotting for FoxO was performed as described (*22*). Secreted PRG4 protein in the culture supernatants was detected by anti-Lubricin (Abcam) antibody.

Surgical and treadmill-induced OA models

An experimental OA model was created in mice 2 weeks after tamoxifen injection (age, 4.5 months). A surgical OA model was created by DMM (*31*). In the treadmill-induced OA model, mice were placed on the treadmill (Columbus Instruments Exer 3/6 Treadmill) at 10° incline for 45 min at a speed of 15 m/min including 2 min of warming up (*33*). Four weeks after DMM surgery and 6 weeks after treadmill exercise, knee joints were collected.

Statistical analyses

All data were expressed as means ± SD. Results were analyzed using Prism version 7 (GraphPad Software Inc.). Student's *t* test and Mann-Whitney test were used to establish statistical significance between two groups. One-way analysis of variance (ANOVA) was used to compare multiple groups, with subsequent pairwise (group) comparisons assessed via Bonferroni's procedure, at an experiment-wise error level of 0.05. *P* values less than 0.05 were considered statistically significant. Individual subject-level data are reported in table S3.

SUPPLEMENTARY MATERIALS

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Fig. S1. Skeletal and growth plate changes in Col2Cre-FoxO KO mice.
Fig. S2. Real-time PCR of cartilage from 1- and 2-month-old Col2Cre KO mice.
Fig. S3. Histological features in the growth plate in 4- and 6-month-old Col2Cre KO mice.
Fig. S4. Real-time PCR of cartilage from AcanCreERT-TKO mice.
Fig. S5. Synovial and bone histological scores in AcanCreERT-TKO mice.
Table S1. Numbers of mice used for each experiment.
Table S2. TaqMan probes used for real-time PCR analysis.
Table S3. Subjective-level data.

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