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FoxO transcription factors influence cartilage maturation, homeostasis and osteoarthritis pathogenesis by modulating autophagy and proteoglycan 4

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Abstract

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 FoxO in cartilage function, single and triple FoxO1/3/4 floxed mice were crossed with Col2-Cre mice to generate Col2Cre-FoxO1, 3, and 4 single knockout (KO) and triple KO mice (Col2Cre-TKO). Articular cartilage in Col2Cre-TKO and Col2Cre-FoxO1 KO mice was significantly thicker than in control mice at 1 or 2 months. 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 when Col2Cre-FoxO3 KO mice had more severe spontaneous OA. Autophagy and anti-oxidant defense genes were reduced in Col2Cre-TKO mice. Deletion of triple FoxO1/3/4 in mature mice using Aggrecan(Acan)-CreERT2 (AcanCreERT-TKO) also led to spontaneous cartilage degradation and increased severity of OA induced by surgical knee instability or treadmill running. The superficial zone 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 TGFβ stimulation. In OA chondrocytes, overexpression of FoxO1 reduced inflammatory mediators, cartilage-degrading enzymes, increased protective genes and antagonized IL-1β effects. Our observations suggest that FoxOs play a key role in postnatal cartilage development, maturation and homeostasis and protects against OA-associated cartilage damage.

One Sentence Summary:

FoxOs play a key role in postnatal cartilage development, maturation, homeostasis, and osteoarthritis pathogenesis.

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Introduction

Articular cartilage is an integral component of the musculoskeletal system and its main function is to absorb 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). While tissue level and cellular changes in cartilage aging have been characterized (3), mechanisms that are responsible for cellular homeostasis and reasons for their failure in aging remain to be discovered. Recent findings support the concept 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 comprised 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 individual FoxO (8, 9). However, each FoxO regulates gene expression in a tissue specific pattern (10). In bone, FoxO1 is more abundant than FoxO3 and FoxO4 (11) and modulates osteoblast differentiation by interacting with the Runx2 promoter (12).

In humans, FoxO3 single nucleotide polymorphisms are associated with exceptional longevity (13). The role of FoxO in regulating lifespan is thought to be through control of cellular homeostasis and maintenance of stem/progenitor cells populations during aging (7, 14). FoxO expression and activity are induced under oxidative stress conditions (14) and FoxO transcriptionally induce expression of several antioxidant enzymes such as catalase and manganese superoxide dismutase (15). FoxO proteins also regulate two major intracellular clearance mechanisms, autophagy and the ubiquitin-proteasome system, to eliminate damaged and aggregated proteins (14).

Dysregulation of FoxO expression or activity contributes to the pathogenesis of age-related diseases in several different tissues, including bone (11) and muscle (16). We reported earlier that the expression of autophagy genes which are regulated by FoxO were reduced in OA (17) and that autophagy activation by rapamycin reduced the severity of OA (18). Subsequently, we found that the expression of FoxO is reduced in aging and OA-affected human and mouse cartilage (19). The objective of this study was to analyze the impact of cartilage specific developmental deletion of FoxO1, FoxO3, FoxO4 and of all three FoxOs, and of postnatal deletion of all three FoxOs, 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 (20). Real-time PCR analysis showed an approximately 90% reduction in FoxO mRNA in 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 significantly reduced in Col2Cre-TKO mice (Fig. S1B).

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 and tail length at 1 month of age (Fig. 1A). The tibial growth plate at P7 and 1 month showed increased height of the proliferative zone in Col2Cre-TKO mice and the proliferative and hypertrophic zones in Col2Cre-FoxO1 KO mice (Fig. 1B-D). 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 femoral and tibial cartilage than control mice (Fig. 1E, F). This was mainly the result of increased thickness of the non-calcified region above the tidemark (Fig. 1G). Cell density in the non-calcified area was significantly reduced in Col2Cre-TKO and Col2Cre-FoxO1 KO mice (Fig. 1H). Articular chondrocytes were also larger in Col2Cre-TKO mice than in controls (Fig. 1I).

Since increased cartilage thickness may be the direct result of an overall increase in cell number, we tested whether FoxO control chondrocyte proliferation. *In vivo* BrdU (5-bromo-2'-deoxyuridine) 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), 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). The reduction of *Cdkn1a* in cartilage from Col2Cre-TKO, *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 levels of *Cdkn1b* and *Ccng2* mRNA (Fig. 2F). Collectively, these data suggest that FoxO control chondrocyte proliferation *in vivo* and *in vitro*.

Next, we analyzed extracellular matrix genes. *Col2a1* in cartilage was significantly increased only in Col2Cre-FoxO1 but not in Col2Cre-FoxO3 or Col2Cre-FoxO4 KO mice (Fig. S2B), indicating specific functions of FoxO isoforms.

In periods of rapid skeletal growth, abnormal chondrocyte differentiation may alter of articular cartilage dynamics and result in increased cartilage thickness (21). Analysis of

chondrocyte differentiation related genes in cartilage from 1-month-old Col2Cre-TKO and Col2Cre-FoxO1 KO 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). Overexpression of a constitutively active FoxO1 mutant (FoxO1-AAA) in human chondrocytes resulted in increased expression of *Mmp13, Runx2, Pthlh* and *Ihh* (Fig. 2F).

Spontaneous degradation of articular cartilage in Col2Cre-FoxO KO mice.

The articular cartilage surfaces of 1-month-old FoxO KO mice were intact and there were no apparent degenerative changes (Fig. 1). However, Col2Cre-TKO mice showed cartilage surface defects by 2 months of age, resulting in increased OA scores (Fig. 1E, Fig. 3A, B). Between 4 and 6 months, full thickness cartilage lesions developed in Col2Cre-TKO mice (Fig. 3A, 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. 3A, 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. S3A, B). 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 (Fig. 3C). Bone scores were also significantly higher in the Col2Cre-FoxO3 KO and Col2Cre-FoxO4 KO mice (Fig. 3C). Further, Col2Cre-FoxO3 KO showed significantly more severe cartilage lesions at 18 months than control mice (Fig. 3D).

FoxOs 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 since severe lesions had not yet developed at this time. 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 FoxOs 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).

Since FoxOs 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 (*Map1lc3b, Atg4b, Becn1, Gabarapl1, Bnip3*), redox regulation (*Cat, Txnip*) and adaptation to energy stress (*Prkaa2*) (Fig. 4C). Consistent with these in vivo findings, FoxO1-AAA overexpression in human normal chondrocytes and IMACs from C57BL6/J wild-type mice increased the expression of *Map1lc3b, Sesn3, Becn1, Gabarapl1,* and *Gpx3* (Fig. 4D). Collectively, these data suggest that FoxOs 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 was significantly reduced 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 (VCAM-1), were also lower in Col2Cre-TKO mice (Fig. 5C). Reduction of *Prg4* expression was also confirmed by PCR on cartilage RNA from P1, 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.

FoxOs regulate cartilage homeostasis and OA pathogenesis in skeletally mature mice.

Since FoxO conditional deletion using the Col2Cre promoter resulted in defects in cartilage maturation, we used the Aggrecan-CreERT2 (AcanCreERT) knockin mice (22) 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). Four-month-old AcanCreERT-TKO developed mild cartilage lesions two months after tamoxifen injection (Fig. 6E). Five months after tamoxifen administration, mutant mice exhibited full thickness cartilage defects (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 meniscus (DMM) and treadmill running were also significantly higher in AcanCreERT-TKO mice than in control mice (Fig. 6G, H), indicating a critical role of FoxO in articular cartilage homeostasis during aging and excess mechanical load.

FoxO1 activates *Prg4* expression and synergizes with TGFβ.

We sought to investigate the molecular mechanisms whereby FoxO1 regulates PRG4 expression. In IMACs and ATDC5 chondrogenic cells, adenoviral or plasmid-mediated overexpression of FoxO1-AAA significantly increased *Prg4* mRNA levels (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 levels during chondrogenic differentiation (Fig. 7B). Since TGFβ is a well-known inducer of chondrogenesis and *Prg4* expression (23, 24), we further investigated the relationship between TGFβ and FoxO. TGFβ1 treatment increased *Prg4* expression in control mouse chondrocytes but this effect was blunted in FoxO depleted chondrocytes (Fig. 7C). In addition, secreted PRG4 protein in the medium from IMACs of Col2Cre-TKO mice was not increased with TGFβ1 (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, since overexpression of the DNA binding mutant FoxO1H215RAAA showed no effect on *Prg4* expression (Fig. 7F).

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

In human OA chondrocytes, inflammatory genes (*Il6, Nos2, Ptgs2*) and catabolic genes (*Mmp3, Mmp13*) were increased (Fig. 8A) while *FoxO1* and autophagic genes including *Map1lc3b, Sesn3, Prkaa2*, and *Becn1* were decreased as compared to chondrocytes from normal knee joints (Fig. 8B). Adenoviral overexpression of FoxO1-AAA in OA chondrocytes significantly increased autophagic genes (*Map1lc3b, Sesn3, Prkaa2*) (Fig. 8C). Stimulation of the chondrocytes with IL-1β, a prototypic inducer of OA-related genes (25) 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 (19) 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 (26). We analyzed mice during postnatal growth and maturation from P1 until 18 months of age. The most profound phenotypic changes in the epiphyseal growth plate, articular cartilage and other joint tissues were observed in Col2Cre-TKO mice and to a very similar extent 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 (27). Neonatal mice showed elongation of the hypertrophic zone of the growth plate and had increased overall body and tail length at eight weeks of age (27). 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. As 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 knockin mice (22). 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

(28) is associated with mechanisms and features similar to posttraumatic OA in humans (29). We also used a treadmill running model with a protocol, which leads to mild cartilage damage in wild-type mice (30). In both models, the mice with postnatal FoxO deletion showed more severe cartilage damage, suggesting that FoxOs 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 (31). We observed reduced expression of *Prg4* in FoxO KO mice, which is consistent with a previous study in T-lymphocytes (32). The *Prg4* gene (33) encodes a mucin-like, O-linked glycosylated protein, termed lubricin (34) or SZP (35). It is produced by cells in the articular cartilage surface (35), meniscus (36), and synovium (37) and present in the extracellular matrix of the superficial zone (38) and in synovial fluid (39). Lubricin functions as a boundary lubricant in articular cartilage to decrease friction and wear and the accumulation of lubricin at the surface of cartilage are thought to be important for joint homeostasis (31). Mice lacking lubricin have increased baseline coefficient of friction values and are not protected against further increases caused by loading (40). *Prg4* deficient mice also develop superficial and upper mid zone chondrocyte apoptosis and cell loss (41, 42). 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 (43). The findings observed in FoxO KO mice are thus consistent with changes that were seen in lubricin deficient mice and during early stages of human joint aging.

The present findings support a direct role of FoxO1 in upregulating 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* (23, 24) and the chondrocyte response to TGFβ decreases with aging (44). As the present results show that FoxO1 synergizes with TGFβ1 to increase *Prg4* gene expression, the aging-related loss FoxO is a likely factor in the reduced *Prg4* expression and potentially in other compromised TGFβ induced cellular responses.

While 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 levels of important cellular homeostasis genes in FoxO deficient mice. Genes involved in antioxidant defenses (*Sesn3* and *Gpx3*), autophagy (*Map1lc3b, Atg4b, Becn1, Gabarapl1, 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 (17), *Txnip* (45), sestrins (46), *Prkaa2* (47) are reduced in aging and OA cartilage, raising the possibility that this is due to the reduced expression of FoxO.

To test this and examine the potential therapeutic benefit of targeting FoxO in OA, we cultured OA chondrocytes, which show abnormal gene expression patterns. Overexpression of FoxO1 in 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 OA chondrocytes is at least in part responsible for abnormal expression of homeostasis genes and mediators of OA pathogenesis.

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 cells in other joint tissues, including cells in synovium (48). We observed OA-like changes in synovium and subchondral bone in 6-month-old Col2Cre-TKO and Col2Cre-FoxO1 KO mice. Downregulation of FoxO1 is involved in synoviocyte survival and synovial hyperplasia in rheumatoid arthritis (49). Consistent with this FoxO function, synovial hyperplasia was the main manifestation of FoxO deficiency observed in the present study. FoxOs are also key regulators of bone formation and remodeling (50). However, the subchondral bone changes observed in the mice with chondrocyte specific deletion of FoxO are probably secondary to initial changes in the articular cartilage. Mouse models of OA are characterized by a close association of changes within the osteochondral unit (51).

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 levels than FoxO1 or FoxO3 (19). 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 deficient Prg4 expression and this is a likely explanation 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, antioxidants). These differences in FoxO function are in part related to different sets of interacting proteins (52).

In summary, these studies identify FoxOs 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 FoxOs 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 maintaining or restoring FoxO expression can prevent OA onset and delay disease progression.

Materials and Methods

Mice.

All animal studies were performed with approval by the Scripps Institutional Animal Care and Use Committee. Col2a1-Cre/+ transgenic mice (20) and Aggrecan-CreERT2 knockin mice (22) on a C57BL6/J background were obtained from The Jackson Laboratory (JAX#003554, Bar Harbor, ME, USA). FoxO1$^{lox/lox}$, FoxO3$^{lox/lox}$ and FoxO4$^{lox/lox}$ were obtained from Dr. R. DePinho (8). Tamoxifen (Sigma-Aldrich, St. Louis, MO, USA) was injected intraperitoneally at a dose of 1.5 mg/10g body weight for 5 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 as controls for AcanCreERT-TKO mice. Numbers of mice were listed in supplementary materials.

Histological analyses.

Mouse knee joints were fixed in 10% zinc buffered formalin for 2 days, decalcified in TBD-2 for 24 hours. Sections were stained with Safranin-O–fast green and picrosirius red staining. 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 0–24) (53). Synovial changes were evaluated using Krenn's synovitis scoring system (score 0–9) (54). To assess the subchondral bone, we used a scoring system (score 0–8) that evaluates trabecular bone structure (mostly cystic: 2, partially cystic: 1), lamellar structure (destroyed: 2, partially destroyed: 1), angiogenesis (number of vessels ≥3: 2, 1–2: 1) and ectopic ossification/articular cartilage absorption in the tibia (present: 2).

Immunohistochemistry.

Knee joint sections were deparaffinized, washed and blocked with 10% goat serum. Primary antibodies against SZP (1:300, Abcam, Cambridge, MA, USA) or VCAM1 (1:100, Abcam) were applied and incubated overnight at 4ºC, followed by ImmPRESS regents (Vector Laboratories, Burlingame, CA, USA). The signal was developed with diaminobenzidine (DAB, 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, Rosemont, IL, USA). IMACs were incubated with 10 μM BrdU (Sigma-Aldrich) for 2 hours then stained with anti-BrdU (1:200) primary antibody. To assess apoptotosis, TUNEL staining was performed using *In Situ* Cell Death Detection Kit (Roche Applied Science, Indianapolis, IN, USA). In MTT assay, IMACs were incubated in DMEM supplemented with 10% FCS, and cells were analyzed on days 1–5. MTT (5 mg/ml) was added. After adding DMSO, absorbance values were measured at 540 nm. For cell cycle analysis, fluorescence-activated cell sorting (FACS) analysis was performed using FxCycle PI/RNase Staining Solution (Molecular Probes,

Eugene, OR, USA). Cells were resuspended and analyzed on a BD LSR II Flow Cytometer (BD Biosciences, San Diego, CA). The percentages of cells in G1, S and G2/M phase were determined.

Immature mouse articular chondrocytes (IMACs) and human chondrocytes.

Mouse chondrocytes were isolated as described (55). IMACs were stimulated with 10 ng/ml TGFβ1 (PeproTech, Rocky Hill, NJ, USA) for 6 hours. Human normal chondrocytes were isolated from macroscopically normal knee joints that were harvested at autopsy from 6 donors (mean ± SD age 45.8 ± 9.5 years old, range 34–58 years old), as described previously (56). OA chondrocytes were harvested from 4 donors undergoing total knee arthroplasty (mean ± SD age = 71.8 ± 7.8 years old, range 64–81 years old). First or second passage cells were used. OA chondrocytes were incubated with 10 ng/ml IL-1β (PeproTech) for 6 hours.

Chondrocyte viability.

IMACs were incubated with 500 μM H_2O_2 for 24 hours when resazurin solution (500 μM in PBS) 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 from the knee joint and femoral head of 1-month-old mice (tissue from 3 mice were pooled). Total RNA was extracted from mouse cartilage or cultured cells using TRIzol (Invitrogen, Carlsbad, CA, USA), followed by Zymo Direct-zol RNA MiniPrep kits (Zymo Research, Irvine, CA, USA). Real-time PCR was performed on a Light Cycler 480 instrument (Roche Diagnostics) using TaqMan probes listed in Table S1.

Mesenchymal stem cell isolation and chondrogenic differentiation assay.

Adipose tissue-derived MSCs were isolated from subcutaneous adipose tissue of control mice (57). To delete FoxOs, cells were infected with Adeno-CMV-Cre (Vector BioLabs, Malvern, PA, USA) at 25 MOI (multiplicity of infection). Cells were then cultured in pellets in DMEM with 10% FCS, 10 ng/mL rhTGFβ3 (PeproTech), 1x ITS with 11 μg/ml sodium pyruvate (Invitrogen), 100 nM dexamethasone (Sigma-Aldrich), 50μg/ml L-ascorbic acid 2-phosphate (Sigma-Aldrich), 40 μg/ml L-proline (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 (58) was constructed using pAd/CMV/V5-DEST Gateway vector with pcDNA3-FLAG-FoxO1-AAA (Addgene Plasmid #13508, Cambridge, MA, USA). Human normal chondrocytes, OA chondrocytes, and IMACs were infected with Ad-FoxO1-AAA or control Ad-eGFP (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 (FoxO1AAA, Addgene plasmid #13508) (58), DNA binding FoxO1 domain mutant (FoxO1H215RAA, Addgene plasmid # 13510) or GFP control using Lipofectamine 3000. ATDC5 cells were stimulated with 10 ng/ml TGFβ1 (PeproTech) for 24 hours.

Western Blotting.

Western blotting for FoxO was performed as described (19).

Surgical and treadmill induced OA models.

Experimental OA model was created in mice 2 weeks after tamoxifen injection (age 4.5 months). Surgical OA model was created by destabilizing the medial meniscus (DMM) (28). In the treadmill-induced OA model, mice were placed on the treadmill (Columbus Instruments Exer 3/6 Treadmill, Columbus, OH, USA) at 10 degrees incline for 45 minutes at a speed of 15 m/min including 2 minutes warming up (30). Four weeks after DMM surgery and 6 weeks after treadmill exercise, knee joints were collected.

Statistical analyses.

All data were expressed as the mean ± standard deviation (SD). Results were analyzed using Prism version 5.2 (GraphPad Software, Inc., La Jolla, CA). 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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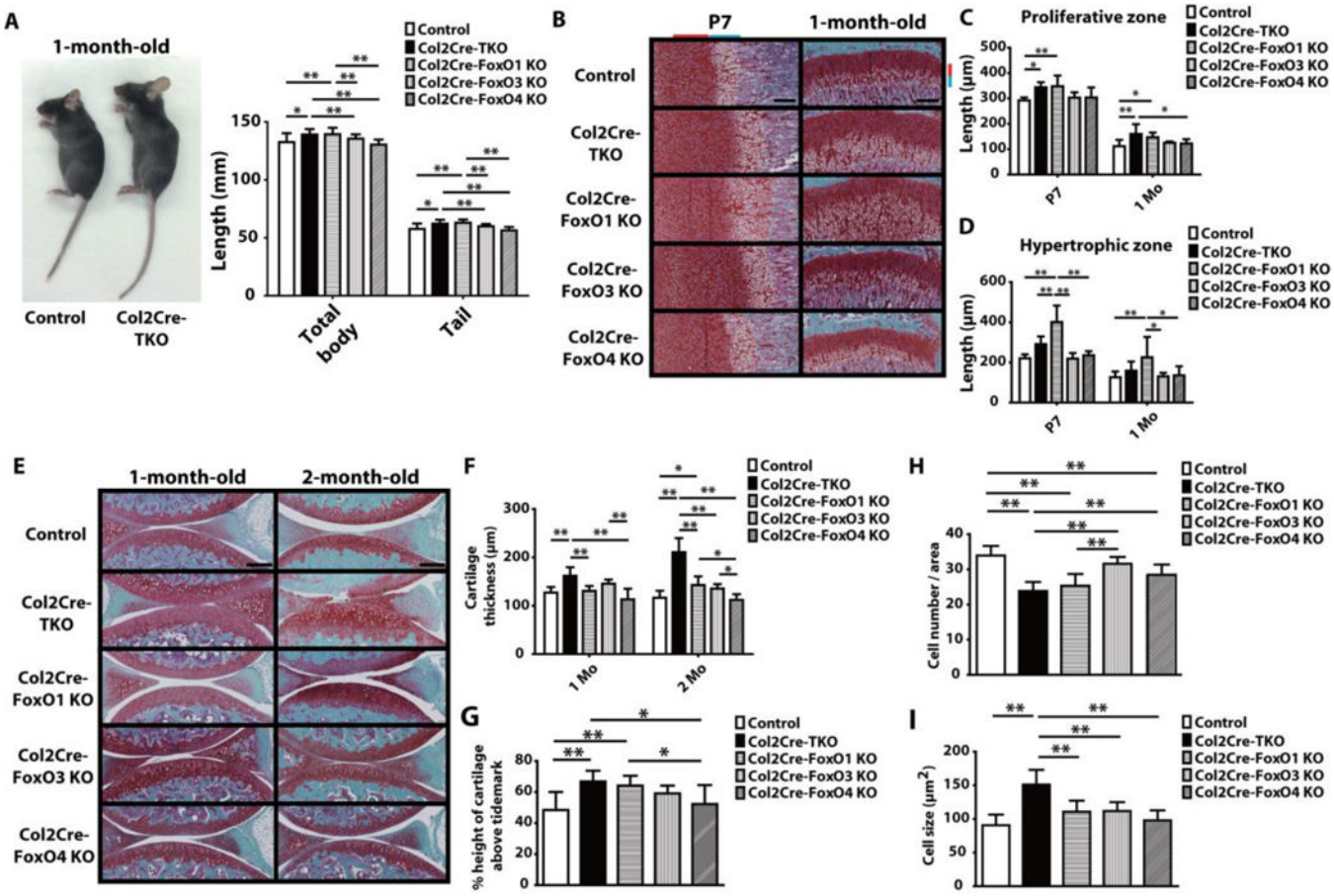

Figure 1. Body size, growth plate and articular cartilage in Col2Cre-FoxO KO mice.

(A) Body length and tail length in 1-month-old mice. **(B)** Histological sections of growth plate from 1- and 2-month-old mice (scale bar = 200μm). Red bar indicates proliferative zone and blue bar indicates hypertrophic zone. **(C)** Lengths of proliferative zone and **(D)** hypertrophic zone were measured. **(E)** Histological sections of articular cartilage from 1- and 2-month-old mice (scale bar = 200μm). **(F)** The thickness of articular cartilage was measured as the distance between the articular surface and the subchondral bone interface across 3 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 were measured at 2 months. **(H)** Cell density was measured at 2 months. **(I)** Cell size was measured at 2 months. Numbers of mice were listed in supplementary materials. Data are mean ± SD. *p<0.05, **p<0.01 (one-way ANOVA and Bonferroni's posttest).

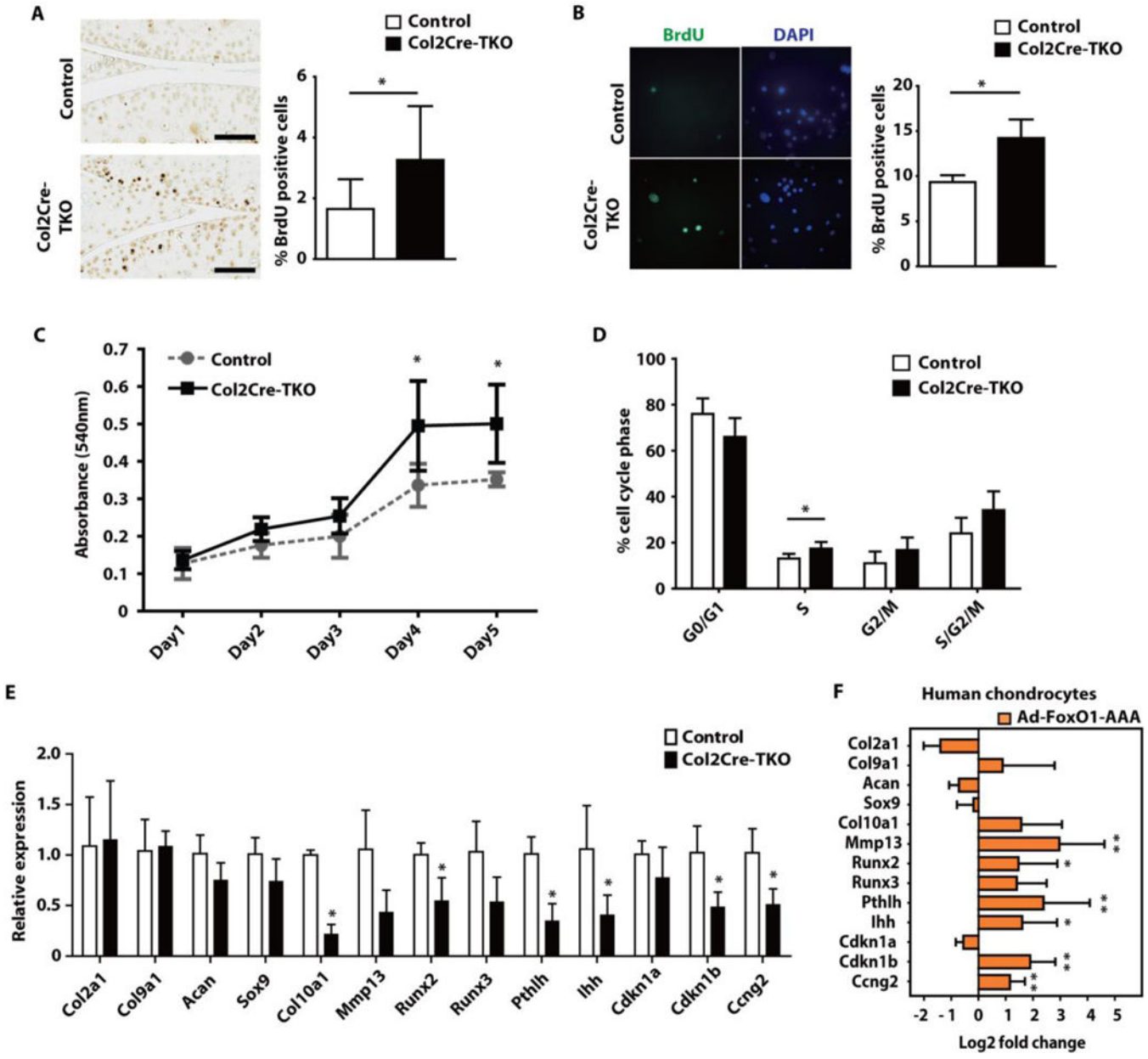

Figure 2. FoxOs effect on cell proliferation and gene expression in chondrocytes.

(A) Immunohistochemistry for BrdU was detected using DAB (scale bars = 100 μm) in control (n = 5) and Col2Cre-TKO mice (n = 8). Positive cells were counted at femur and tibia. **(B)** IMACs from P6 control and Col2Cre-TKO mice were incubated DMEM containing 10% fetal calf serum with 10uM BrdU for 2 hours. Signal was detected with Alexa fluor 488. BrdU positive cell were counted in multiple areas under 40X magnification. **(C)** IMACs from P6 control and Col2Cre-TKO mice were incubated in DMEM containing 10% fetal calf serum for 5 days. MTT assay was performed at each day (n = 4, each). **(D)** Cell cycle analysis was performed using flow cytometry on IMACs from P6 control and Col2Cre-TKO mice. The percentages of cells in G1, S and G2/M phase are indicated (n = 5, each). **(E)** Real-time PCR analysis for chondrogenic markers and cell cycle genes using

RNA from knee joint cartilage from 1-month-old mice Col2Cre-TKO and control mice (n = 4, each). The expression values were relative to *Gapdh*. **(F)** Real-time PCR analysis for chondrogenic markers and cell cycle genes in adenoviral FoxO1-AAA transduced human chondrocytes (n = 6). The values were relative to *Actb* and expression values were normalized to Ad-GFP transfected cells. Each value was compared to Ad-GFP transfected cells. Data are mean ± SD. *p<0.05, **p<0.01 (Mann-Whitney test).

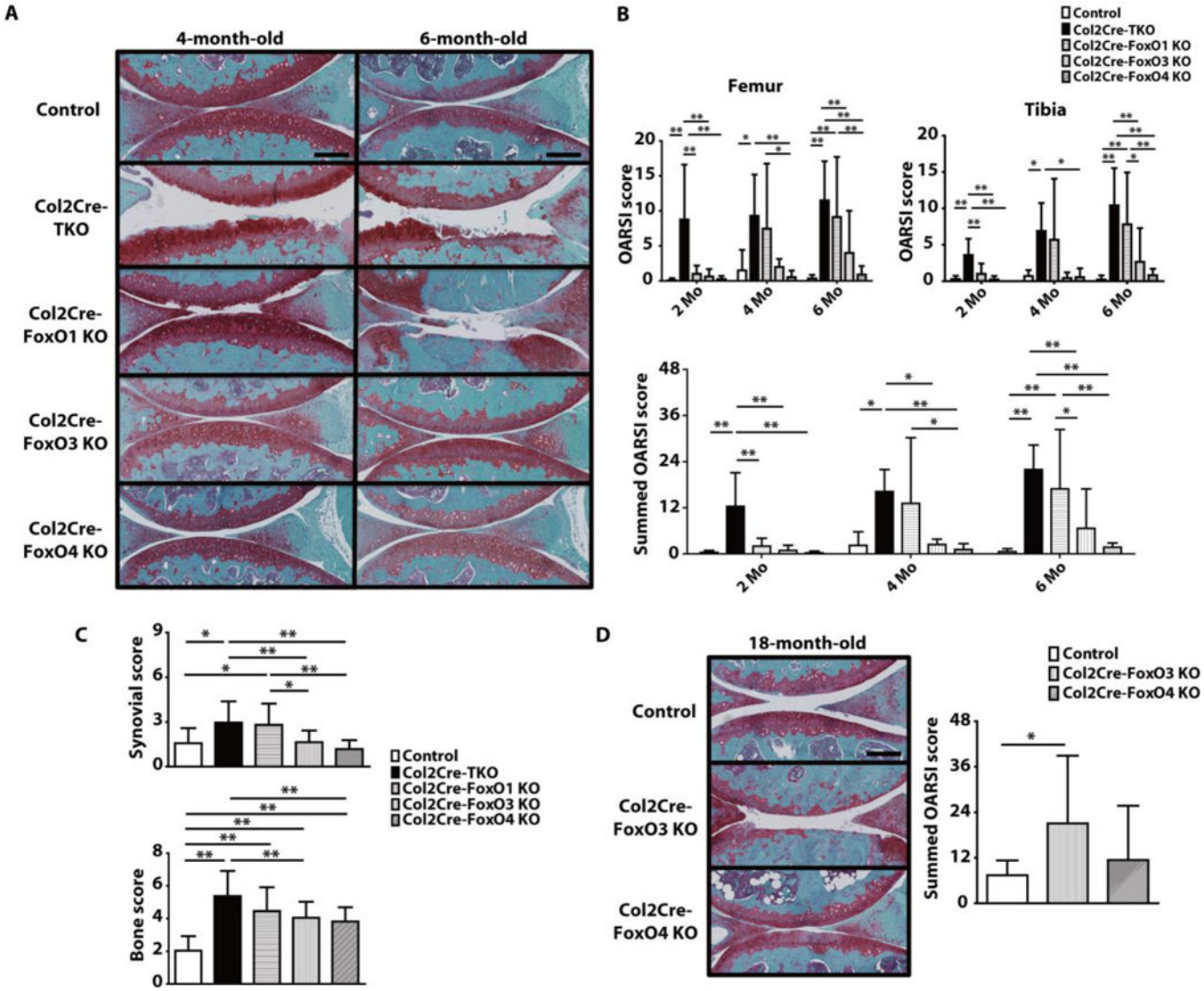

Figure 3. Spontaneous development of OA-like changes in joint tissues of Col2Cre-FoxO KO mice.

(A) Histological sections of knee joints from 4- and 6-month-old mice (scale bar = 200μm). **(B)** OARSI scores were obtained by summing the scores for the medial femoral condyle and tibial plateau. **(C)** The synovium and bone scores were obtained from the same sections from 6-month-old mice. Data are mean ± SD. *p<0.05, **p<0.01 (one-way ANOVA and Bonferroni's posttest).

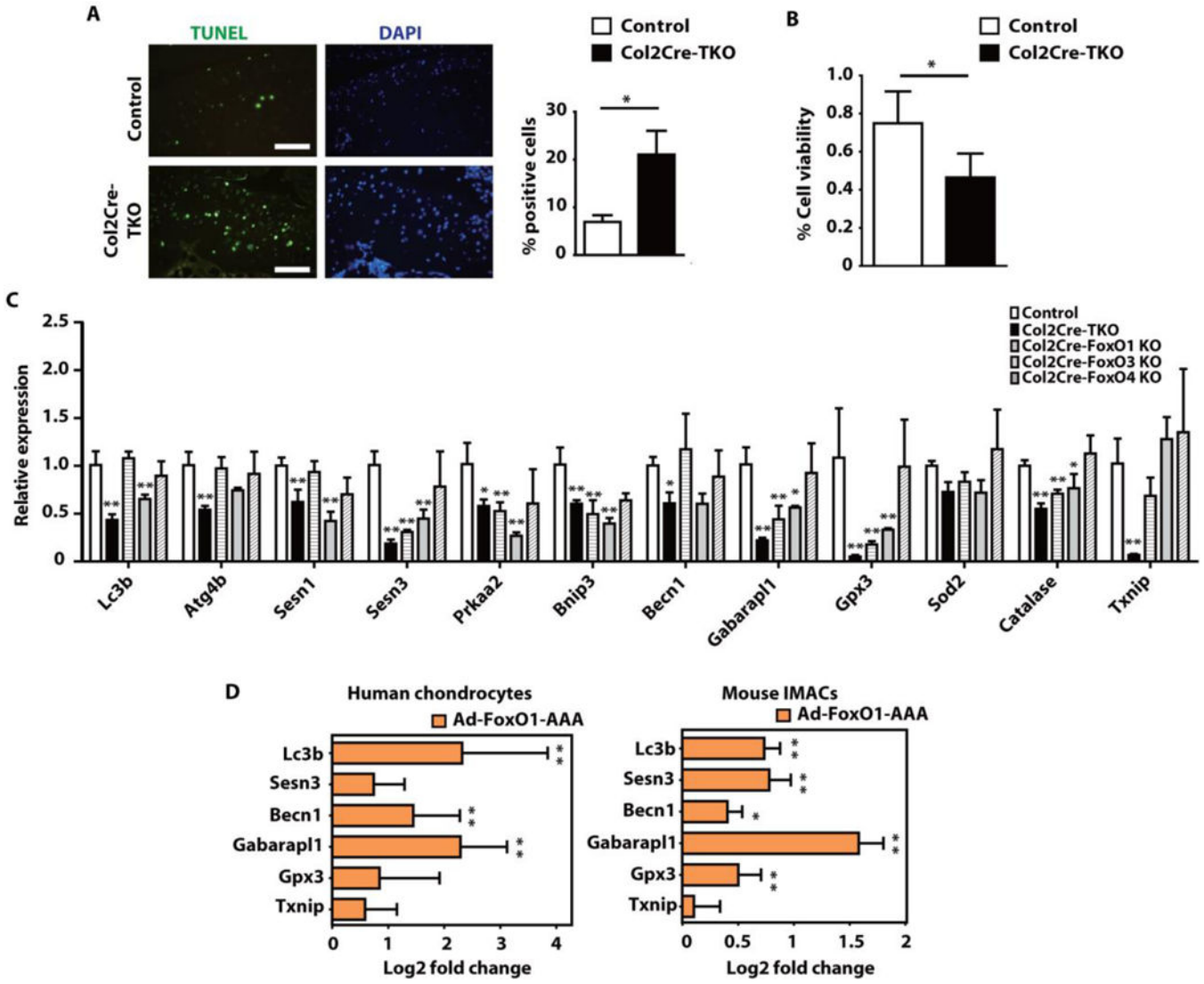

Figure 4. Cell viability and cellular homeostasis genes.

(A) TUNEL 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. **(B)** 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). The expression values were relative to *Gapdh*. Each value was compared to control. **(D)** Real-time PCR analysis from adenoviral FoxO1-AAA transduced human normal chondrocytes (n = 4) and mouse IMACs (n = 6). The values were relative to *Actb* and expression values were normalized to Ad-GFP transfected cells. Each value was compared to Ad-GFP transfected cells. Data are mean ± SD. *p<0.05, **p<0.01 (Mann-Whitney test for 2 groups and one-way ANOVA and Bonferroni's posttest for mutiple groups).

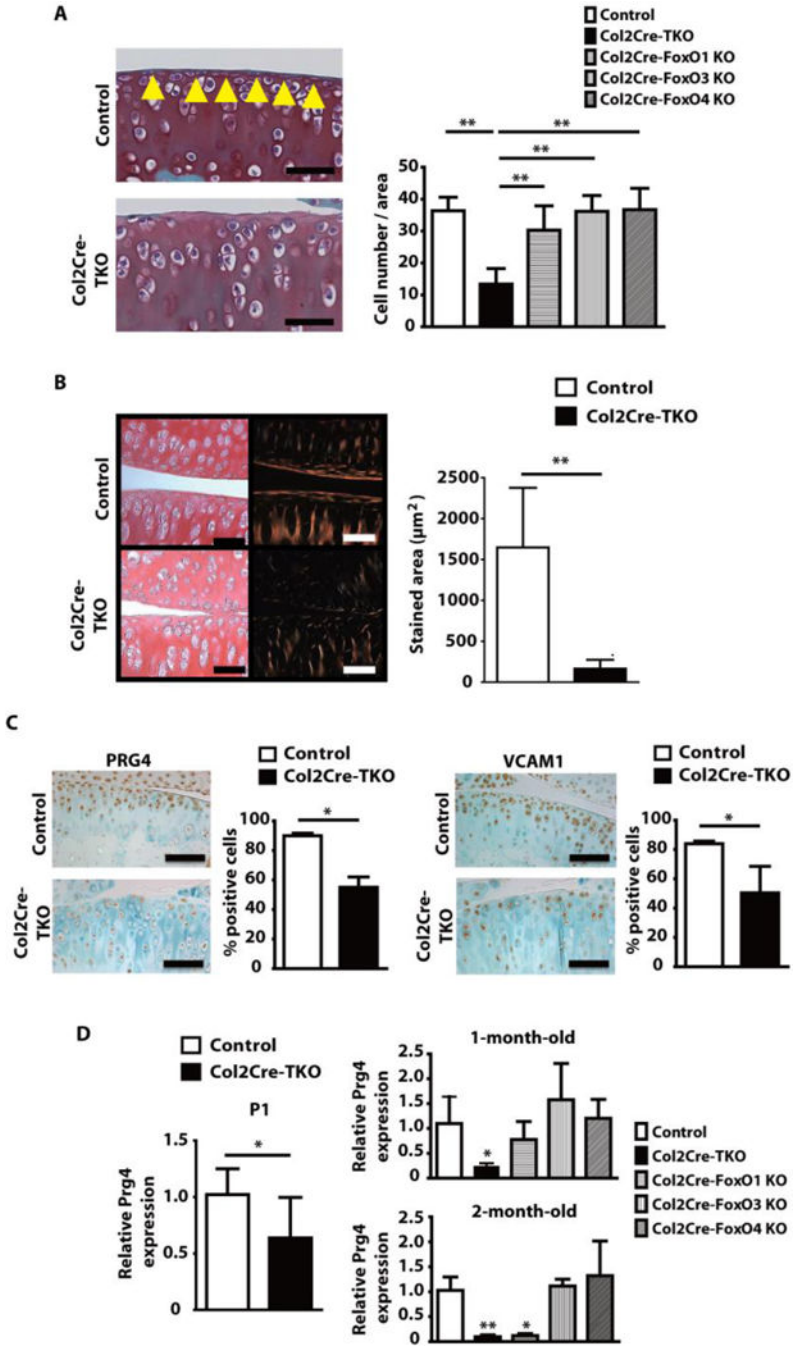

Figure 5. Cartilage superficial zone changes in Col2Cre-FoxO KO mice.

(A) Total numbers of superficial zone cell per superficial zone area (10μm from the surface) were measured in 1-month-old mice (scale bar = 50μm). Yellow arrows indicate superficial zone cells in normal cartilage. **(B)** Picrosirius red staining images were obtained under standard light (Left panel) or polarized light (right panel, scale bar = 100μm). Stained area was calculated using ImageJ software at 1 month (n = 4, each). **(C)** Immunohistochemistry for superficial zone cell markers SZP and VCAM1 in 1-month-old mice (n = 4, each, scale bar = 100μm). The number of SZP percent positive cells was quantified in the tibial

cartilage. **(D)** Real-time PCR analysis for *Prg4* using RNA isolated from cartilage from P1, and 1, and 2-month-old mice (n = 4, each). The expression values were relative to *Gapdh*. Each value was compared to control. Data are mean ± SD. *p<0.05, **p<0.01 (Mann-Whitney test for 2 groups and one-way ANOVA and Bonferroni's posttest for mutiple groups).

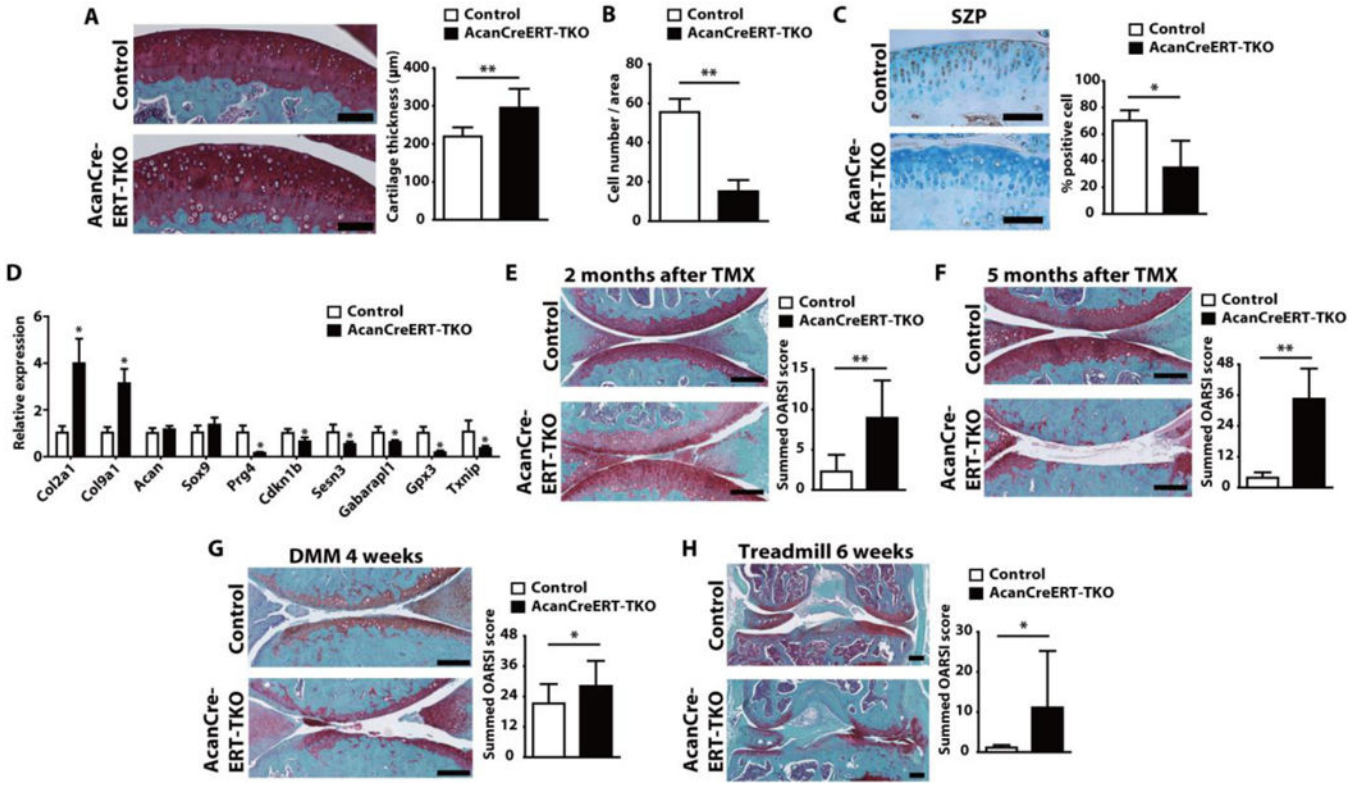

Figure 6. Phenotype of postnatal deletion of FoxOs in cartilage using Aggrecan-CreERT knockin mice.

(A) The thickness of articular cartilage was measured as the distance between the articular surface and the subchondral bone interface (scale bar = 100μm) across 3 points in each medial tibial plateau of the knee joint in mice at 2 months after tamoxifen injection. **(B)** Total numbers of superficial zone cell per superficial zone area (10μm from the surface) were measured in mice at 2 months after tamoxifen injection. **(C)** Immunohistochemistry for SZP and the number of SZP percent positive cells was quantified in the tibial cartilage of mice at 2 months after tamoxifen injection (n = 4, each, scale bar = 100μm). **(D)** Real-time PCR analysis using RNA from knee joint cartilage in control and AcanCreERT-TKO mice of 2 weeks after tamoxifen injection (n = 4, each). The expression values were relative to *Gapdh*. **(E)** Histological sections of knee joints from mice 2- and **(F)** 5 months after tamoxifen injection, **(G)** 4 weeks after surgical OA model (scale bar = 200μm), and **(H)** 6 weeks after treadmill induced OA model (Coronal section, scale bar = 200μm). OARSI scores were obtained by summing the scores for the medial femoral condyle and tibial plateau. Data are mean ± SD. *p<0.05, **p<0.01 (Mann-Whitney test).

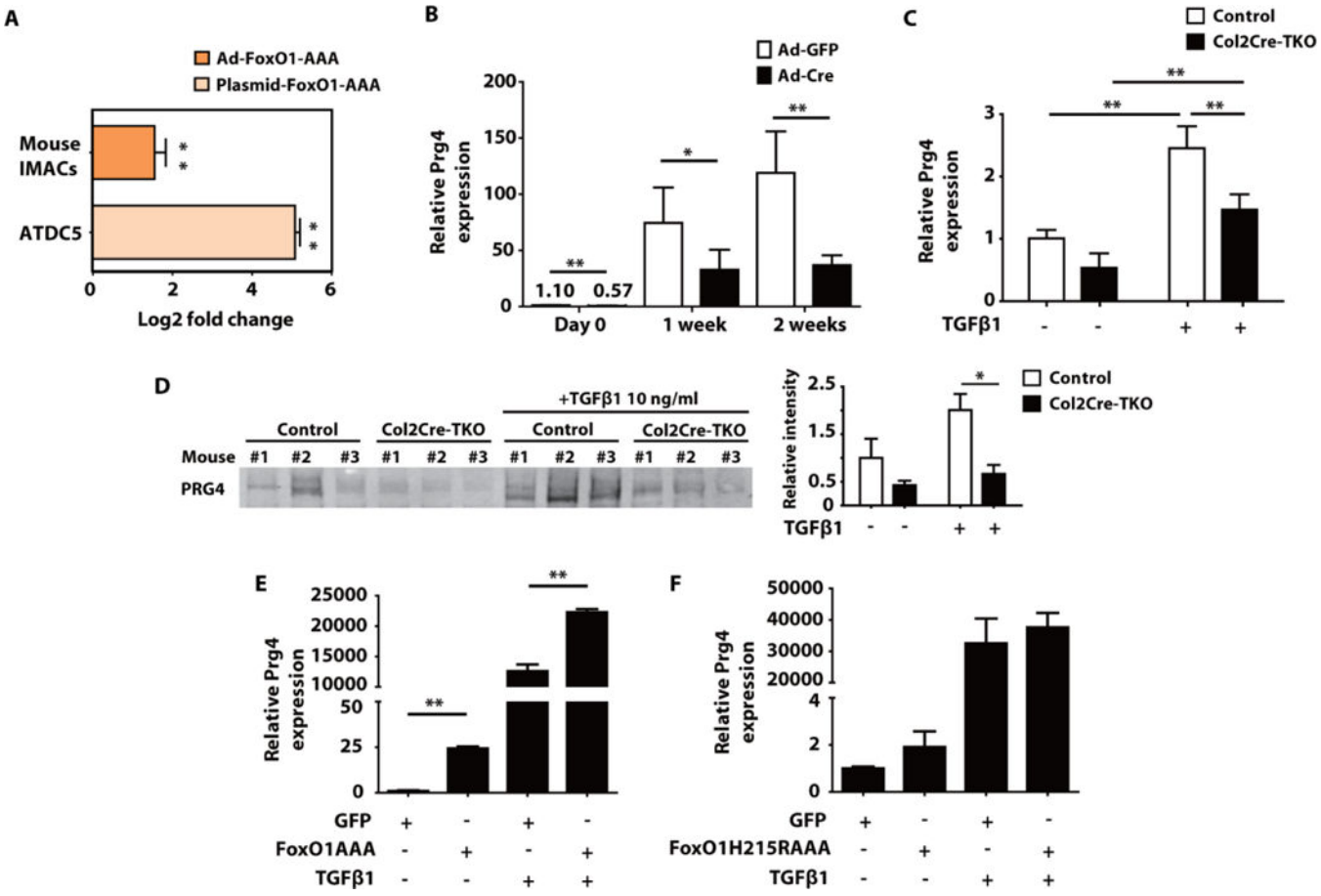

Figure 7. FoxO and *Prg4* expression.

(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 were relative to *Actb* and expression values were normalized and compared to Ad-GFP or plasmid GFP transduced cells. **(B)** Adipose tissue derived MSCs from FoxO triple floxed mice with or without adenovirus-Cre infection (n = 6, each) were cultured as pellets in chondrogenic medium. Real-time PCR for *Prg4* was performed at day 0, and at 1 and 2 weeks. Day 0 was defined as 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 10ng/ml TGFβ1 or vehicle for 6 hours (Control n = 5, Col2Cre-TKO n = 4). The expression values were relative to *Gapdh*. **(D)** Western blotting for secreted PRG4 protein from IMACs of control and Col2Cre-TKO mice (n = 3 each) with 10ng/ml TGFβ1 or vehicle for 24 hours. Quantitative analysis of band intensity was measured. **(E)** Real-time PCR analysis of *Prg4* expression in ATDC5 cells transfected with GFP or FoxO1AAA and treated with 10ng/ml TGFβ1 or vehicle for 24 hours. The expression values were relative to *Gapdh*. **(F)** Real-time PCR analysis of Prg4 in ATDC5 cells transfected with GFP, FoxO1H2015RAAA and treated with 10ng/ml TGFβ1 or vehicle for 24 hours. The expression values were relative to *Gapdh*. All experiments were performed in duplicate. *p<0.05, **p<0.01. Data are mean ± SD. P

values were calculated with Mann-Whitney test (A-D) and Student t-test (E). *p<0.05, **p<0.01.

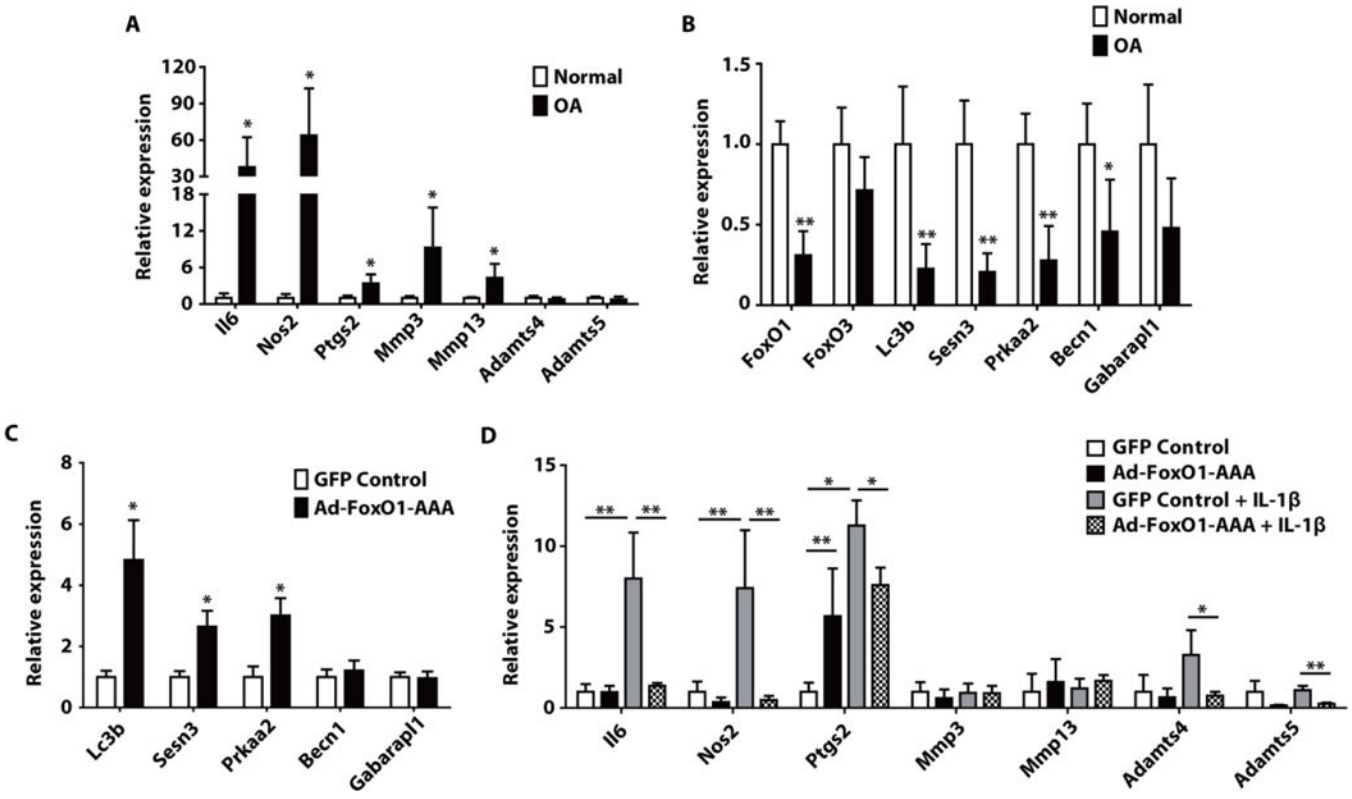

Figure 8. Overexpression of FoxO1 in OA chondrocytes and effects on IL-1β stimulation

(A) Real-time PCR analysis for FoxOs expression, autophagic genes and **(B)** inflammatory and cartilage catabolic genes using RNA from in human normal (n = 4) and OA chondrocytes (n = 4) with basal medium. **(C)** Real-time PCR analysis of autophagic genes in OA chondrocytes (n = 4) transfected with adenoviral GFP or FoxO1-AAA **(D)** Real-time PCR analysis for inflammatory and cartilage catabolic genes in adenoviral GFP control or FoxO1-AAA transduced OA chondrocytes (n = 4) with or without IL-1β. The expression values in all experiments were relative to *B2M*. Data are mean ± SD. *p<0.05, **p<0.01 (Mann-Whitney test for 2 groups and one-way ANOVA and Bonferroni's posttest for mutiple groups).