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Dysregulated FOXO transcription factors in articular cartilage in aging and osteoarthritis

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SUMMARY

Objective: Aging is a major risk factor for osteoarthritis (OA). Forkhead-box class O (FoxO) transcription factors regulate mechanisms of cellular aging, including protein quality control, autophagy and defenses against oxidative stress. The objective of this study was to analyze FoxO transcription factors in normal, aging and OA cartilage.

Design: Knee joints from humans ages 23–90 and from mice at the age of 4–24 months and following surgically induced OA were analyzed for expression of FoxO proteins. Regulation of FoxO protein expression and activation was analyzed in cultured chondrocytes.

Results: Human cartilage expressed FOXO1 and FOXO3 but not FOXO4 proteins. FOXO1 and FOXO3 were more strongly expressed the superficial and mid zone as compared to the deep zone and were mainly localized in nuclei. During human joint aging, expression of FOXO1 and FOXO3 was markedly reduced in the superficial zone of cartilage regions exposed to maximal weight bearing. In OA cartilage, chondrocyte clusters showed strong FOXO phosphorylation and cytoplasmic localization. Similar patterns of FOXO expression in normal joints and changes in aging and OA were observed in mouse models. In cultured chondrocytes, IL-1β and TNF-α suppressed FOXO1, while TGF-β and PDGF increased FOXO1 and FOXO3 expression. FOXO1 and FOXO3 phosphorylation was increased by IL-1β, PDGF, bFGF, IGF-1, and the oxidant t-BHP.

Conclusions: Normal articular cartilage has a tissue specific signature of FoxO expression and activation and this is profoundly altered in aging and OA in humans and mice. Changes in FoxO expression and activation may be involved in cartilage aging and OA.

Introduction

The primary risk factor for osteoarthritis (OA) development is aging, but the mechanisms by which aging contributes to OA susceptibility and progression remain to be investigated[1].

The aging process, where oxygen radical production is increased and oxidant defense mechanisms are compromised[2–5], is accompanied by a progressive accumulation of damaged molecules and organelles, leading to the decreased ability of cells to function normally[6]. Therefore, protein quality control mechanisms are essential in maintaining normal biosynthetic cell functions and their failure plays an important role in the pathogenesis of age-related disease[7,8]. One of the prominent pathways involved in turnover of cellular constituents is autophagy. Compromised autophagy associated with a reduction and loss of ULK1, Beclin1, and LC3 expression was observed in human OA and age-related and surgically induced OA in mice[9].

One of the major signaling pathways that regulate cellular aging and stress resistance is the Insulin/IGF-1 signaling pathway. The protein kinase Akt is an important upstream signaling component in this pathway that regulates diverse cellular functions related to longevity, cellular senescence, and metabolism. Among of the most evolutionarily conserved targets of Akt are the forkhead-box class O (FOXO) transcription factors[10,11]. The mammalian FOXO family consists of three members, FOXO1, FOXO3, and FOXO4[12,13]. Activity of the three FOXO molecules is controlled by phosphorylation,

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which modulates their cellular localization. Akt directly phosphorylates FOXO1, FOXO3, and FOXO4 at three conserved sites, resulting in nuclear export and subsequent degradation[14].

FOXOs regulate not only genes governing oxidative defense such as MnSOD, catalase, and the DNA repair enzyme GADD45[15,16] but also regulate protein degradation mediated by the ubiquitin–proteasome system[17] and the autophagic/lysosomal pathway[18]. Abnormal expression and activation of FOXOs is involved in the pathogenesis of age-related diseases affecting bone[19], muscle[20], and CNS[21].

Importantly, expression and activation of FOXO transcriptional factors are highly context and cell-lineage specific[19–21]. The objectives of this study were to analyze protein expression and activation of FOXO transcription factors in normal cartilage and to determine changes in aging and OA.

Methods

Human knee joints

Human knee joints from individuals ages 23–90 were obtained at autopsy under approval by the Scripps Human Subjects Committee. The entire femoral condyles of young normal knee joints were harvested from six donors (age 23–48 years, mean ± SD = 36.0 ± 9.6, OA grade I, Mankin score = 0) having no history of joint disease. Aged normal knee joints were also obtained at autopsy from four donors having no history of joint diseases or overt OA (age 68 to 76, mean ± SD = 72.5 ± 3.6, OA grade I–II, Mankin score = 1–3). Human OA joints were obtained from four donors (age from 64 to 90, mean ± SD = 81.5 ± 10.3, OA grade III–IV, Mankin score = 7–8). Articular surfaces were graded macroscopically according to a modified Outerbridge scale[22]. Osteochondral slabs (5 mm thickness) were harvested from the central part of medial femoral condyle for histomorphologic analysis. Subsequently, the slabs were cut into six tissue blocks from the anterior to the posterior condyle [Fig. 1(B)]. Each block was fixed in 10% zinc-buffered formalin for 2 days, decalcified in TBD-2 for 7 days, followed by paraffin embedding. Serial sections (4 μm each) were cut, stained with Safranin O-fast green, and graded according to Mankin scoring system[23].

Cell and protein isolation from human cartilage

Human chondrocytes were isolated and cultured as described previously[24]. For protein isolation directly from cartilage tissue, the cartilage strips obtained from patients undergoing total knee arthroplasty were separated based on the extent of degradation and frozen in liquid nitrogen. Normal cartilage samples were collected from young donors having no history of joint disease. The frozen tissues were crushed and homogenized. Samples were incubated in TRIzol (Invitrogen) at room temperature. After addition of chloroform, samples were vortexed vigorously and centrifuged for 15 min at 12,000× g at 4°C. The interphase and organic phase was collected, followed by addition of 100% ethanol. After centrifugation, the supernatant was collected for protein isolation. Proteins were precipitated by the addition of isopropanol and diluted in 6M Urea, 2% SDS.

Human chondrocyte cultures

The isolated chondrocytes were plated at high density in DMEM with 10% CS and antibiotics and allowed to attach to the culture flasks. The cells were incubated at 37°C in a humidified gas mixture containing 5% of CO_2 balanced with air. The chondrocytes were used in the experiments at confluence (2–3 weeks in primary culture).

Human chondrocytes were seeded in six-well plates at a density of 4.0×10^5 cells/well. After 1 day, the cells were washed and incubated in DMEM with 0.5% CS for 24 h. Cytokines, growth factors, tert-Butyl hydroperoxide (t-BHP), and CS were added at the following final concentrations: IL-1β (1 ng/ml), TNF-α (10 ng/ml), IL-6 (10 ng/ml), TGF-β1 (10 ng/ml), BMP-7 (100 ng/ml), bFGF2 (25 ng/ml), PDGF-AA (25 ng/ml), IGF-1 (100 ng/ml), t-BHP (25 μM and 250 μM), and 10% CS. Cells were harvested after 30 min, 60 min, 1 day, 2 days, and 5 days of incubation.

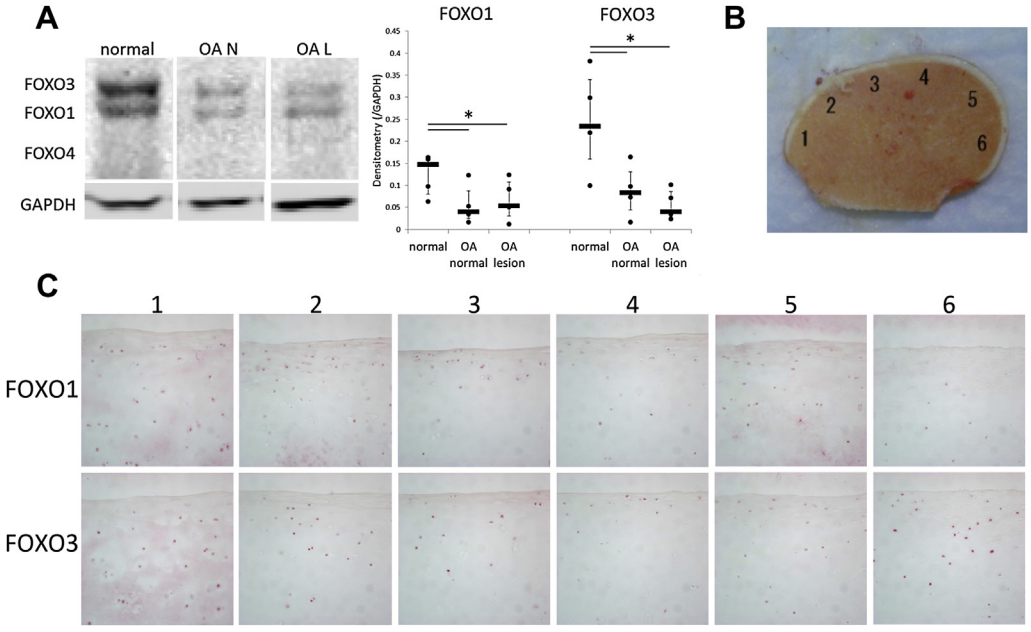

Fig. 1. FOXO protein expression in human articular cartilage. A. Protein extracts from articular cartilage were analyzed by western blotting using antibodies to FOXOs as indicated. Graph shows the results of a total of five normal and five OA donors. B. Photograph of osteochondral slab from the central region of the medial femoral condyle showing regions that were used to cut sections from the most proximal (1) to the most distal (6) location. C. Immunohistochemistry was performed for FOXO1 and FOXO3. Numbers of sections correspond to the regions in panel B. Magnification 40×.

Mouse knee joints

All animal experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee (IACUC) at The Scripps Research Institute. In the spontaneous aging-related OA model, C57BL/6J mice were kept under normal conditions and knee joints were collected at 4, 12 and 24 months of age. The surgical OA model was induced in 4 months old C57BL/6J mice by transection of the medial meniscotibial ligament and the medial collateral ligament (MMTL + MCL) as described[25] and animals were euthanized 10 weeks later.

Knee joints from both murine models were resected from both hind legs, fixed in 10% zinc-buffered formalin for 2 days, decalcified in TBD-2 for 24 h. Serial sections (4 μm each) were cut, and expression of FoxO proteins was analyzed by immunohistochemistry.

Immunohistochemistry

For antigen unmasking, the tissue sections were incubated with 2.5 mg/ml of hyaluronidase for 60 min at 37°C. After washing with phosphate buffered saline (PBS), sections were blocked with 10% goat serum for 1 h at room temperature. This condition was obtained after optimization and provided specific positive staining without non-specific signals. Anti-FOXO1A (1:50 dilution), Anti-FOXO1A (phospho S256) (1:100 dilution), Anti-FOXO3A (1:100 dilution), Anti-FOXO3A (phospho S253) (1:100 dilution), and negative control rabbit IgG (1 μg/ml) were applied with 0.1% Tween 20 and incubated overnight at 4°C. All primary antibodies were purchased from Abcam. Incubation with secondary antibody, substrate and hematoxylin were performed as described[9] [Fig. 1(C)].

Quantification and localization of positive cells in human cartilage

FOXO localization in each cartilage zone was assessed systematically by counting positive cells in three pictures under 40× magnification starting from the cartilage surface to the deep zone[9]. The identification of each zone [Fig. 2(A)] was based on previously reported characteristics that comprise cell shape, morphology, orientation, and pericellular matrix deposition[26]. The percentages of positive cells for total FOXO and phosphorylated FOXO were

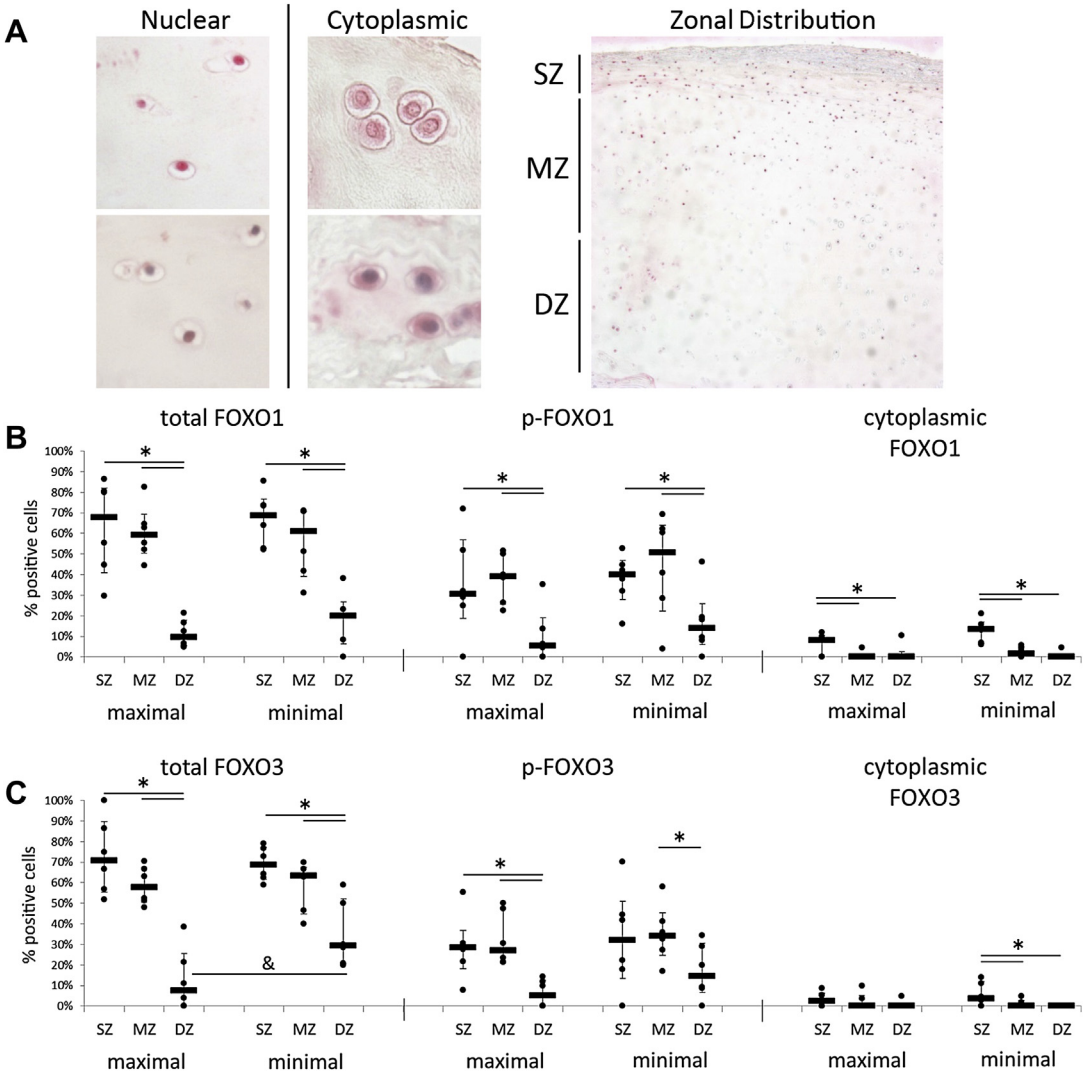

Fig. 2. Zonal distribution of total and phospho-FOXO in human articular cartilage. Cartilage sections from maximal and minimal weight bearing regions were stained with antibodies to total FOXO and phospho-FOXO. Representative images for nuclear and cytoplasmic localization of FOXO1 and zonal distribution are shown in panel A. Images for cellular localization are 100×, for zonal distribution is 10× magnification. Results are shown as % positive cells for FOXO1 in panel B and for FOXO3 in panel C. Values are the median and quartile. Data are representative of six normal donors.* = $P < 0.05$ vs each zone; & = $P < 0.05$ vs maximal weight bearing.

determined independent on cellular localization. Cells were counted as positive for cytoplasmic p-FOXO when staining was predominantly cytoplasmic positive staining on sections that were counter-stained with hematoxylin [Fig. 2(A)]. The frequency of positive cells was expressed as a percentage relative to the total number of cells counted in each zone.

Quantification of FoxO immunoreactivity in mouse knee joints

Cartilage cellularity in C57BL/6J mice was quantified by counting the chondrocytes in a microscopic field[27]. Three pictures were taken under 40× magnification, representing the center of the femoral condyle that is not covered by the meniscus as well as the anterior and posterior femoral condyles covered by the meniscus. Then, the total numbers of cells and FoxO positive cells were counted in each section.

Quantitative western blotting

Quantitative western blotting was performed with the LiCor immunofluorescence detection system (Licor, Lincoln, NE). Primary antibodies from Cell Signaling were used: Anti-FOXO1A (1:1000 dilution), Anti-FOXO1A (phospho S256) (1:1000 dilution), Anti-FOXO3A (1:1000 dilution), Anti-FOXO3A (phospho S253) (1:1000 dilution), Anti-Akt (phospho S473) (1:1000 dilution), and GAPDH (1:5000) in 1/2× Odyssey buffer in PBS with 0.1% Tween 20. After washing in TBST, secondary antibodies goat anti-rabbit – IRDye 800 (1:5000 dilution) for FOXOs and goat anti mouse – IRDye 680 (1:10,000 dilution) for GAPDH, diluted in 1/2× Odyssey buffer in PBS with 0.1% Tween 20 and 0.01% SDS, were applied. Blots were washed in PBS and then water before acquisition on the LiCor Odyssey. In-lane background was removed (Median: Top/Bottom) before analysis with the Odyssey software version 3.0 (LiCor). Integrated intensity values (K counts) for each protein of interest were normalized to those of GAPDH.

Statistical analysis

Statistically significant differences between three groups were determined with Kruskal Wallis H-test and Friedman test. When a significant differences were found among three groups, Mann–Whitney *U* test and Wilcoxon signed-rank test were used to analyze the specific sample pairs for significant differences. The results are reported as median and quartile 25%–75%. *P* values less than 0.05 were considered significant.

Results

FOXO protein expression in young normal human cartilage

Articular cartilage from normal human knee joints expressed predominantly FOXO1 and FOXO3 proteins but not FOXO4 as detected by western blotting [Fig. 1(A)]. Both non-fibrillated and fibrillated OA cartilage showed significant FOXO1 and FOXO3 reduction compared to normal cartilage [Fig. 1(A)]. Immunohistochemistry was used to determine the distribution and phosphorylation of FOXO1 and FOXO3. Locations in the knee joint differ in regard to exposure of articular cartilage to weight bearing and susceptibility to OA. To determine regional changes in FOXO protein expression, adjacent cartilage sections representing the entire femoral condyle ($n = 6$ per condyle) were analyzed [Fig. 1(B)]. The most proximal and the most distal sections (#1 or 2 and # 5 or 6) are exposed to minimal, while the central sections (#3 or 4) are exposed to maximal weight bearing [Fig. 1(C)]. We also assessed differences in FOXO among the superficial, mid and deep zone [Fig. 2(A)]. FOXO1 and FOXO3 proteins ($n = 6$ donors each) were more highly expressed in the superficial and middle than the deep zone [Fig. 2(B), (C)]. When determining nuclear/cytoplasmic localization with FOXO by counter-staining of hematoxylin, FOXO1 and FOXO3 were found mainly in the nuclei [Fig. 2(B), (C)]. Comparison of areas of cartilage exposed to minimal vs maximal weight bearing showed that FOXO3 protein in the deep zone was more strongly expressed in areas exposed to minimal weight bearing.

Phosphorylated FOXO1 and FOXO3 ($n = 6$ donors each) were detected at higher levels in the superficial and middle zones as compared to the deep zone. The levels of total FOXO proteins and phosphorylated FOXOs were significantly greater in the nucleus as compared to cytoplasm [Fig. 2(B), (C)].

In summary, among the FOXO isoforms, FOXO1 and FOXO3 proteins are most strongly expressed in normal cartilage. Overall, their protein expression is higher in the superficial and middle zone as compared to deep zone. In regard to regional differences, only FOXO3 protein in the deep zone was more strongly expressed in areas exposed to minimal weight bearing. Nuclear localization of FOXO proteins indicates that most cells in the superficial and mid zone express activated FOXO1 and FOXO3 proteins.

Aging and OA-associated changes in FOXO protein expression in human cartilage

For the analysis of aging and OA cartilage, we selected representative maximal weight bearing regions [#3 or as shown in Fig. 1(B)]. OA cartilage had no superficial zone in maximal weight bearing areas.

In aged donors, FOXO1 and FOXO3 ($n = 4$ donors each) were significantly decreased in the superficial zone compared with young normal cartilage (Fig. 3). In contrast, FOXO1 and FOXO3 in OA cartilage ($n = 4$ donors each) were significantly increased in the middle zone compared with normal cartilage. In terms of nuclear/cytoplasmic localization, FOXO1 and FOXO3 in the middle zone of OA cartilage were stronger in the cytoplasm compared with normal cartilage [Fig. 3(B)]. Moreover, phosphorylated FOXO1 and FOXO3 were significantly stronger in the middle zone compared with normal cartilage. This increase was due to the cell clusters localized in the middle zone in OA cartilage (Fig. 3). These cell clusters showed strong protein expression and phosphorylation of FOXO1 and FOXO3. These results indicate an age-related reduction in FOXO1 and FOXO3 protein expression in the superficial zone and increased phosphorylation and cytoplasmic localization in the OA cluster chondrocytes.

FoxO protein expression in normal, aging and OA mouse cartilage

Two different types of OA models in mice were used, including aging-related OA and mechanical overload induced OA in order to mitigate their limitations and to correlate with aging and OA-related changes in human knee cartilage. Both mouse models showed cartilage degradation with only small variations in OA severity.

Normal joints from skeletally mature 4-month-old C57BL/6J mice showed high levels of FoxO1 and FoxO3 proteins in the superficial and upper middle zones. FoxO1 and FoxO3 were localized mainly in the nucleus. The signals for the phosphorylated FoxOs were less intense as compared to the total FoxOs in 4-month-old mice. At the ages of 12 months and 24 months, there was a significant aging-related reduction in FoxO1- and FoxO3-positive cells compared with 4-month-old mice, as well as a significant reduction in FoxO1 and FoxO3 in 24-month-old mice compared with 12-month-old mice [Fig. 4(B)]. In 12- and 24-month-old mice, the reduction of FoxO protein was more marked in the meniscus non-covered regions compared to the meniscus-covered regions [Fig. 4(A)].

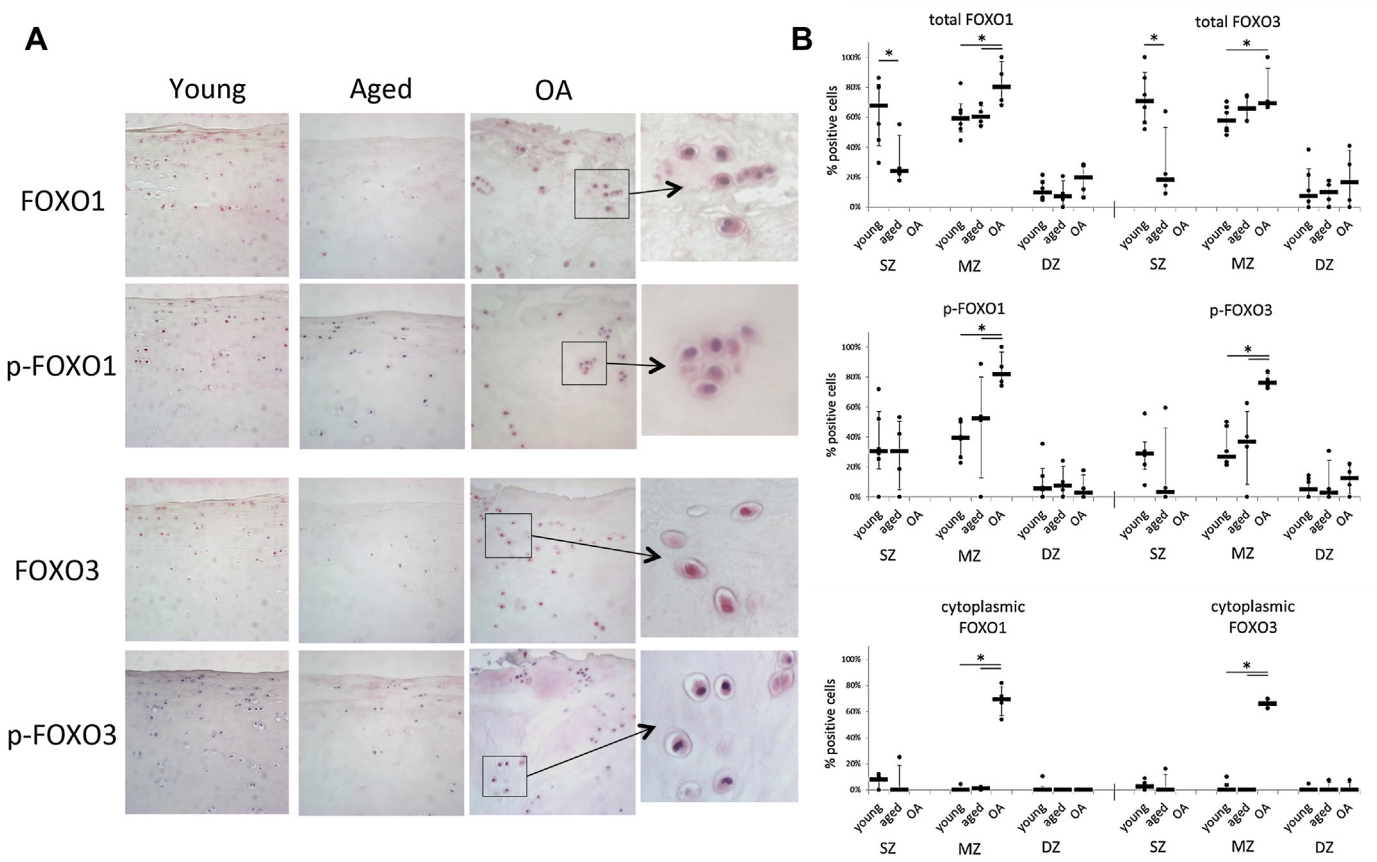

Fig. 3. Changes in FOXO protein expression in human cartilage from aging and OA-affected joints. A. Cartilage was collected from the femoral condyles of normal (weight bearing regions) and OA knee joints and analyzed by immunohistochemistry with antibodies as indicated. Images are 40×, insets on the right are 100× magnification. B. Quantification of total FOXO, phospho-FOXO in cartilage zones and cytoplasmic vs nuclear localization (shown as % cytoplasmic localization). Data are representative of six young, four aged and four OA donors.

Articular cartilage in joints with surgical OA showed a reduction of FoxO1-positive cells and FoxO3-positive cells compared with non-operated 4-month-old mice. The quantitative analysis of positive cells showed a significant reduction of FoxO1 and FoxO3 10 weeks after surgery. In contrast, the cluster-like chondrocyte aggregates in fibrillated lesions, showed strong expression of FoxO1 and FoxO3 protein and also of the phosphorylated forms of FoxO1 and FoxO3.

Regulation of FOXO protein expression and activation in cultured human chondrocytes

In cultured human chondrocytes, FOXO1 and FOXO3 proteins were basally expressed, whereas FOXO4 was expressed to a lesser extent. IL-1β and TNF-α significantly repressed FOXO1 at 2 and 5 days. FOXO3 protein expression was also reduced by IL-1β at 5 days, but to a lesser extent by TNF-α. On the other hand, stimulation with TGF-β1 significantly increased FOXO1 protein at 2 days. FOXO3 was also increased 2 days and 5 days after the addition of PDGF (Fig. 5). Shorter treatment periods (30 min–48 h) did not significantly affect expression of the FOXO proteins.

Phosphorylation of FOXO1, FOXO3, and FOXO4 increased in chondrocytes stimulated with IL-1β, TNF-α, bFGF, PDGF, t-BHP, and serum (Fig. 6). We used antibodies directed against phospho-serine 256 of FOXO1 (which cross-react with FOXO4) or antibodies against phospho-serine 253 of FOXO3. These conserved sites are phosphorylated by Akt, which was also activated in chondrocytes upon these stimuli, as judged from blots with the Akt anti-phospho-serine 473 antibody (Fig. 6).

Discussion

The present study is the first to elucidate the characteristics of the protein expression and activation of FOXO transcription factors in articular cartilage. Our results demonstrated that among three FOXOs, FOXO1 and FOXO3 proteins were highly expressed in normal human and mouse cartilage, suggesting that they play important roles in cartilage homeostasis. FOXO protein expression showed differences among cartilage zones with higher expression in superficial and middle zones compared to the deep zone. These findings are consistent with observations that MnSOD, one of the major FOXO target antioxidants, was abundantly expressed in the superficial but not in the deep zone of human cartilage[5].

In aged human cartilage, the protein expression of FOXO transcription factors was significantly decreased in the superficial zone of regions exposed to maximal weight bearing. Similarly, in mouse knee joints there was an age-related reduction of FOXO positive cells especially in the meniscus non-covered regions. In bone, FoxO1 expression also progressively decreased with aging, whereas FoxO3 and FoxO4 levels remained stable[19]. In muscle, the aging-related dramatic decrease of the nuclear FoxO1 and FoxO3 was associated with muscle atrophy[28]. In the present study, we observed increased FOXO positive cells in the middle zone of human OA cartilage and in fibrillated areas and osteophytes in mouse knees. Importantly, the cellular localization of FOXO in OA cartilage was predominantly in the cytoplasm in contrast to normal cartilage, indicating inactivation of FOXO transcription factors. The

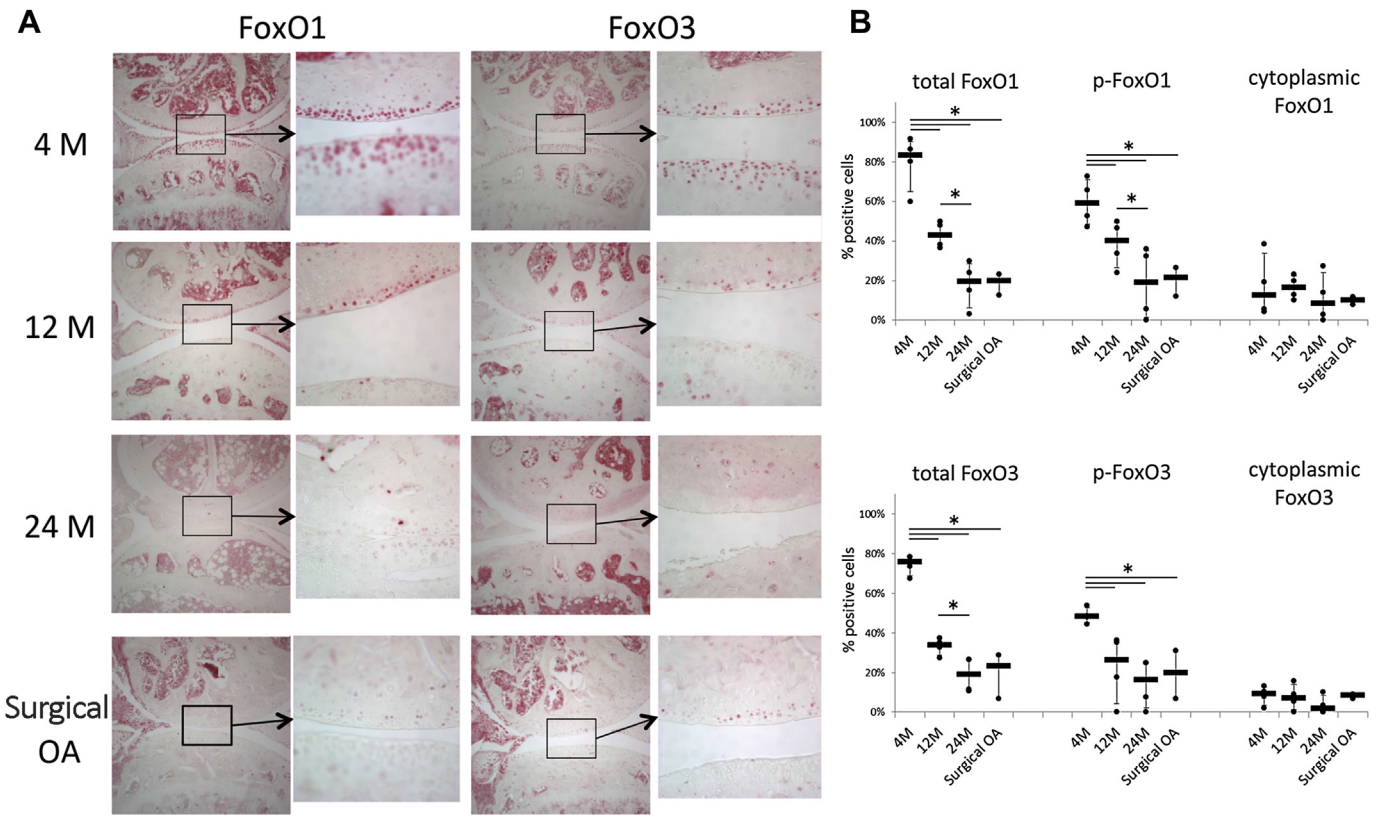

Fig. 4. FoxO protein expression in normal, aging and OA-affected mouse joints. Knee joints were collected from C57/Bl6 mice at the indicated ages or 10 weeks following induction of experimental OA by surgical knee destabilization. A. Representative images normal young, aging and experimental OA cartilage. Images are 10×, insets are 40× magnification. B. Quantification of total FoxO, phospho-FoxO and cytoplasmic localization. Data represent four mice per time point and three for surgical OA.

chondrocytes in these regions formed clusters containing cells that are abnormally activated and differentiated[29].

Cell density and composition in OA cartilage are different as compared to normal tissue. Cell density is reduced in some areas but in fibrillated areas there are chondrocyte clusters, which represent a different cell phenotype as compared to the normal zone-specific chondrocytes. In non-weight bearing areas of cartilage from OA joints where the superficial zone is still present and

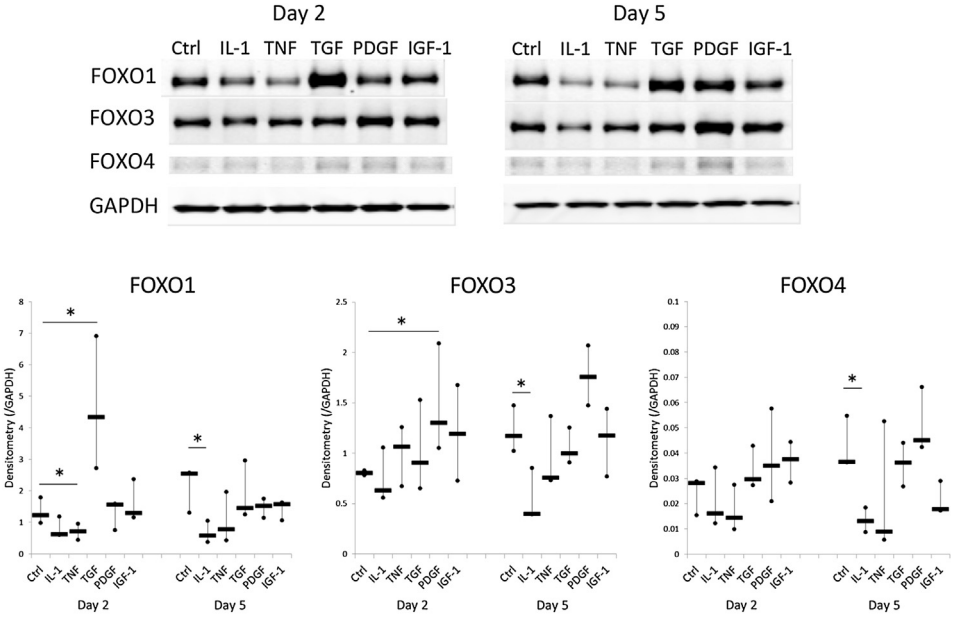

Fig. 5. Changes in FOXO protein expression in response to stimulation of cultured human chondrocytes. Cells were treated with the indicated stimuli for 2 or 5 days and cell lysates were analyzed by western blotting with the antibodies indicated. Representative western blot images are shown and graphs represent quantification of western blots from three different experiments using cells from different donors, each including all stimuli and time points.

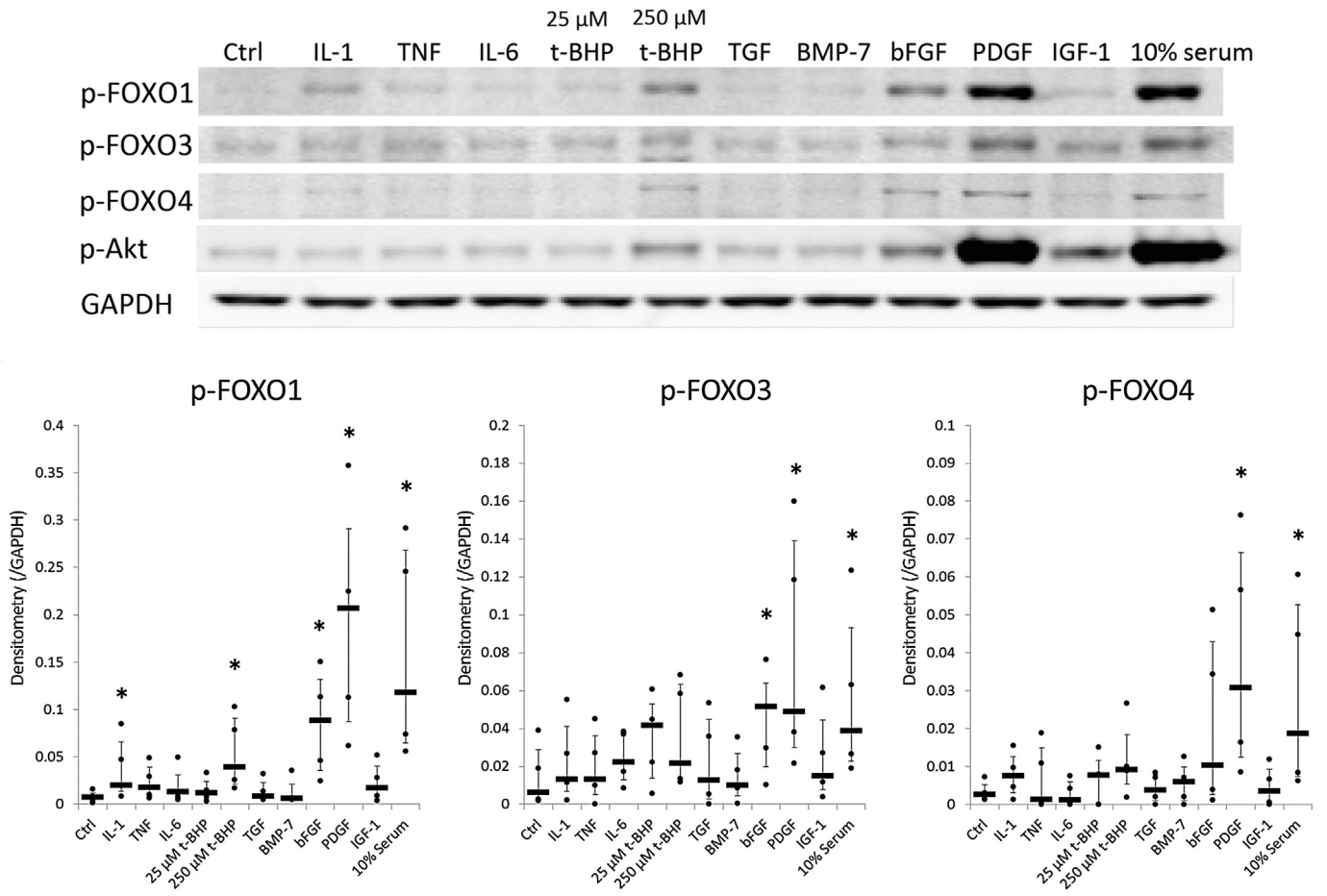

Fig. 6. Changes in FOXO phosphorylation in response to stimulation of cultured human chondrocytes. Cells were treated with the indicated stimuli for 60 min and cell lysates were analyzed by western blotting with the antibodies for phosphorylated FOXOs and Akt as indicated. Representative western blot images are shown and graphs represent quantification of western blots from five different experiments using cells from different donors, each including all stimuli.

there are no fibrillations, there is a reduction in the total number of cells, in the total number of FOXO positive cells and in the percentage of FOXO positive cells.

The activity of FOXOs is tightly controlled by a phosphorylation-dependent shuttling system that modulates their cellular localization[10] and this is predominantly controlled by the Insulin/IGF-1 pathway acting through PI3K and Akt-mediated phosphorylation of FOXO. Phosphorylation of FOXOs by Akt provokes their nuclear export. In this study, we observed significantly higher levels of phosphorylated FOXOs in the cytoplasm of OA cartilage compared to normal cartilage. High levels of IGF-1 and IGF-1 receptor have been found in human OA cartilage, especially in chondrocytes of the upper zone[30–32]. However, the superficial zone of normal cartilage had reduced IGF-1 receptor levels[30] and IGF-induced activation of Akt activation is reduced in OA chondrocytes[33]. These prior observations suggest a similar pattern that may be mechanistically related to the specific distribution of phosphorylated FOXOs seen in this study. Mechanical load is an alternative mechanism for dysregulated FOXOs in OA cartilage, as shear stress downregulated FOXO DNA-binding activity by Akt activation in muscle[28].

In cultured normal chondrocytes IL-1β and TNF-α reduced FOXOs protein expression, and increased phosphorylation of FOXOs. This observation is similar to synovial cells where FOXO1 and FOXO4 were phosphorylated following stimulation with IL-1β and TNF-α[34]. Therefore, proinflammatory cytokines appear to inhibit the activity of FOXOs. On the other hand, our results showed that TGF-β up-regulated FOXO1 protein expression but did not affect phosphorylation, whereas PDGF up-regulated FOXO3 and increased phosphorylation of all FOXOs. PDGF was reported to induce the phosphorylation and inactivation of FOXO protein in hepatic stellate cells[35], in fibroblasts[36,37], and in vascular smooth muscle cells[38]. PDGF, FGF, and IGF-I also repressed the expression of FOXO genes in human fibroblasts[36].

Increased intracellular ROS was reported to facilitate the localization of FOXO to the nucleus where it is transcriptionally active[39]. Upon treatment with H_2O_2, c-Jun-N-terminal kinase (JNK) phosphorylates FOXO at two threonine residues (Thr447 and Thr451 in FOXO4), which are different from the Akt phosphorylation sites, resulting in increased nuclear localization and activation of transcriptional activity[39–42]. In contrast, oxidative stress can increase Akt activity and subsequent FOXO phosphorylation, dependent on the cellular context. Our results in cultured chondrocytes stimulated with t-BHP showed FOXO phosphorylation (serine 256 of FOXO1 and serine 253 of FOXO3). The opposing forces of Akt vs JNK signaling are considered to determine whether FOXO will direct a transcriptional response regulating entry into quiescence or senescence[43]. To address functions of FOXO and phosphorylated FOXO in chondrocytes, further studies with manipulating FOXO expression are needed.

In summary, the present results suggest that attenuated FOXO protein expression or altered FOXO activation may represent a novel mechanism in the development of OA. In view of their central function in aging, FOXO proteins may provide a potential molecular target for the treatment of OA.

Author contributions

ML, YA, HA and YI conceived of the study, and participated in its design and coordination. YA, AH and MS carried out histology and immunohistochemistry experiments and performed quantitative analysis. All authors read and approved the final manuscript. Dr Lotz had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Ethics approval

This study was conducted with the approval of the Human Subjects Committee and the Institutional Animal Care and Use Committee at The Scripps Research Institute.

Role of funding source

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Conflict of interest

None.

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