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Autophagy Is a Protective Mechanism in Normal Cartilage, and Its Aging-Related Loss Is Linked With Cell Death and Osteoarthritis

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Objective. **Autophagy is a process for turnover of intracellular organelles and molecules that protects cells during stress responses. We undertook this study to evaluate the potential roles of Unc-51–like kinase 1 (ULK1), an inducer of autophagy, Beclin1, a regulator of autophagy, and microtubule-associated protein 1 light chain 3 (LC3), which executes autophagy, in the development of osteoarthritis (OA) and in cartilage cell death.**

Methods. **Expression of ULK1, Beclin1, and LC3 was analyzed in normal and OA human articular cartilage and in knee joints of mice with aging-related and surgically induced OA, using immunohistochemistry and Western blotting. Poly(ADP-ribose) polymerase (PARP) p85 expression was used to determine the correlation between cell death and autophagy.**

Results. **ULK1, Beclin1, and LC3 were constitutively expressed in normal human articular cartilage. ULK1, Beclin1, and LC3 protein expression was reduced in OA chondrocytes and cartilage, but these 3 proteins were strongly expressed in the OA cell clusters. In mouse knee joints, loss of glycosaminoglycans (GAGs) was observed at ages 9 months and 12 months and in the surgical OA model, 8 weeks after knee destabilization. Expression of ULK1, Beclin1, and LC3 decreased together with GAG loss, while PARP p85 expression was increased.**

Conclusion. **Autophagy may be a protective or homeostatic mechanism in normal cartilage. In contrast, human OA and aging-related and surgically induced OA in mice are associated with a reduction and loss of ULK1, Beclin1, and LC3 expression and a related increase in apoptosis. These results suggest that compromised autophagy represents a novel mechanism in the development of OA.**

Disruption of the articular cartilage surface, degradation of extracellular matrix (ECM), and reduced cartilage cellularity are major histologic features of osteoarthritis (OA), the most common aging-related joint pathology (1,2). Because chondrocytes maintain the dynamic equilibrium between production of the ECM and its enzymatic degradation, it is important to uncover the molecular mechanisms that control cell fate in cartilage (3). Many factors can be implicated in the development of OA, but one of the most important risk factors is aging (4). Aging is a process characterized by a progressive accumulation of damaged macromolecules and organelles in somatic cells during the postdevelopmental period, leading to the decreased ability of cells to function normally and survive (5). However, mechanisms leading to aging-related cartilage degeneration remain to be determined.

Macroautophagy is a major physiologic mechanism that targets altered and dysfunctional cytosolic macromolecules, membranes, and organelles for delivery to lysosomes for degradation and recycling (5–8). *Atg* genes control the autophagy process leading to the induction and nucleation of autophagic vesicles as well as to their expansion and fusion with lysosomes, allowing enzymatic degradation and recycling (9,10). Among the *Atg* genes, *Atg1, Atg6,* and *Atg8* (Unc-51–like kinase 1 [ULK1], Beclin1, and microtubule-associated protein 1

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light chain 3 [LC3], respectively, in mammals) are 3 major regulators of the autophagy pathway. ULK1 is a key intermediate in the transduction of proautophagic signals to autophagosome formation (11). Beclin1 forms a complex with type III phosphatidylinositol 3-kinase and Vps34 that allows nucleation of the autophagic vesicle (12). Finally, the formation and expansion of the autophagosome requires 2 protein conjugation systems that involve the Atg proteins LC3 and Atg12 (13).

LC3 is present in 2 forms, LC3-I in the cytoplasm and LC3-II bound to the autophagosome membrane. During autophagy, LC3-I is converted to LC3-II through lipidation by a ubiquitin-like system, resulting in the association of LC3-II with autophagy vesicles. The amount of LC3-II is correlated with the extent of autophagosome formation (14). Autophagy plays a fundamental role in cellular homeostasis and functions primarily to promote cellular and organism health. In certain physiologic and pathologic conditions, it can also lead to a form of cell death that is characterized by cytoplasmic vacuolation and is termed type II programmed cell death or cell death by autophagy (15–17). In most experimental models, suppression of autophagy genes leads to cell death, indicating a protective and survival-promoting function of autophagy (18–20).

In articular cartilage, which is characterized by a very low rate of cell turnover (21), this mechanism would appear to be essential to maintain cellular integrity, function, and survival. Furthermore, while autophagy changes in various models and tissues with aging (22), this has not been investigated in articular cartilage. In the present study, we demonstrate that autophagy is a constitutively active and apparently protective process for maintenance of the homeostatic state in normal cartilage. In contrast, human OA and aging-related and surgically induced OA in mice are associated with a reduction and loss of ULK1, Beclin1, and LC3 expression in articular cartilage. Furthermore, the reduction of these key regulators of autophagy is accompanied by an increase in apoptosis. These results suggest that compromised autophagy may contribute to the development of OA.

MATERIALS AND METHODS

Human cartilage procurement and processing. Normal human articular cartilage from 14 adult donors (mean ± SD age 22.4 ± 6.2 years; Mankin score 0, OA grade I) having no history of joint disease (inflammatory arthritis, OA, microcrystalline arthritis, or osteonecrosis) was harvested from femoral condyles and tibial plateaus at autopsy. Human cartilage was also obtained at autopsy from 8 older donors having no history of joint diseases or overt OA (mean ± SD age 69.5 ± 9.02 years; Mankin score 3, OA grade II) but with mild aging-related changes. OA human articular cartilage was obtained from 16 patients (mean ± SD age 74.7 ± 9.4 years; Mankin score 7–8, OA grade III–IV) undergoing knee replacement surgery. Human tissues were obtained under approval by the Scripps Human Subjects Committee. All tissue samples were graded macroscopically according to a modified Outerbridge scale (23), and Safranin O–stained sections of normal and OA cartilage were graded according to Mankin (24) (Mankin score 0 = normal cartilage; Mankin score 1–4 = mild OA cartilage; Mankin score ≥5 = OA cartilage [25]).

Once cartilage surfaces were rinsed with saline, scalpels were used to cut parallel sections 5 mm apart, vertically from the cartilage surface onto the subchondral bone. These cartilage strips were then resected from the bone. Human chondrocytes were isolated and cultured as described previously (26). The cartilage tissue was incubated with trypsin (0.5 mg/ml; Sigma-Aldrich, St. Louis, MO) at 37°C for 10 minutes. After the trypsin solution was removed, the tissue slices were treated for 12–16 hours with type IV clostridial collagenase (2 mg/ml; Sigma-Aldrich) in Dulbecco's modified Eagle's medium (DMEM; Mediatech, Manassas, VA) with 5% fetal calf serum.

Primary culture of chondrocytes. The isolated chondrocytes were recovered and plated at high density in high-glucose DMEM supplemented with 10% calf serum, L-glutamine, and antibiotics and allowed to attach to the culture flasks. The cells were incubated at 37°C in a humidified gas mixture containing 5% CO_2 balanced with air. The chondrocytes were used in the experiments at confluency (2–3 weeks in primary culture).

Experimental OA models in mice. All animal experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee at The Scripps Research Institute. In the spontaneous aging-related OA model, C57BL/6J mice were kept under normal conditions, and knee joints were compared at ages 2, 9, and 12 months. The surgical OA model was induced in 2-month-old C57BL/6J mice by transection of the medial meniscotibial ligament and the medial collateral ligament, and animals were killed 8 weeks later. Knee joints from both OA models were resected from both hind legs, fixed in 10% zinc-buffered formalin (Z-Fix; Anatech, Battle Creek, MI) for 2 days, decalcified in Shandon TBD-2 decalcifer (Fisher Scientific, Pittsburgh, PA) for 24 hours, and embedded in paraffin. Serial sections (4 μm each) were cut, stained with Safranin O–fast green, and examined for histopathologic changes. ULK1, Beclin1, LC3, and poly(ADP-ribose) polymerase (PARP) p85 expression was analyzed by immunohistochemistry in the 2 different OA animal models.

Quantification and localization of positive cells in human cartilage. ULK1, Beclin1, and LC3 localization in each cartilage zone was assessed systematically by counting positive cells in 50 × 50–μm grids starting from the cartilage surface to the deep zone. This was repeated a minimum of 3 times for each section. The identification of each zone was based on previously reported characteristics that comprise cell shape, morphology, orientation, and pericellular matrix deposition (27). The depth of each zone was recorded for each section for comparative analysis of the frequency of positive cells in each zone. The frequency of positive cells was expressed as a percentage relative to the total number of cells counted in each zone.

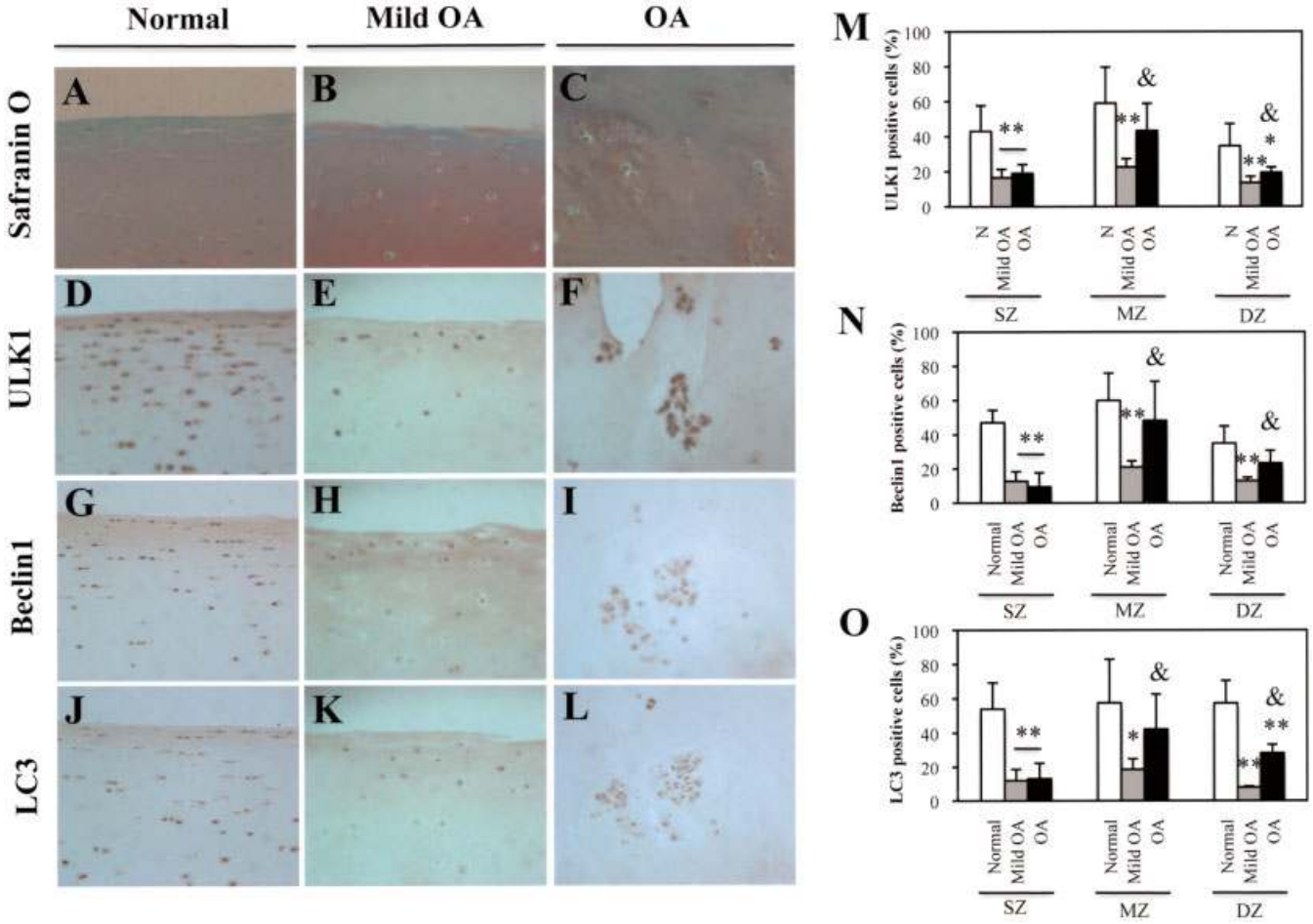

Figure 1. Unc-51–like kinase 1 (ULK1), Beclin1, and microtubule-associated protein 1 light chain 3 (LC3) expression is reduced in human osteoarthritic (OA) cartilage. **A–C,** Safranin O–stained sections representative of cartilage samples from 6 normal donors, 4 donors with mild OA, and 6 donors with OA. **D–L,** Immunohistochemical analysis with anti-ULK1, anti-Beclin1, and anti-LC3. **M–O,** Quantification of ULK1-, Beclin1-, and LC3-positive cells in normal (N), mild OA, and OA cartilage. In mild OA, the percentage of ULK1-, Beclin1-, and LC3-positive cells was significantly reduced in the superficial zone (SZ), middle zone (MZ), and deep zone (DZ) compared with that in normal cartilage. In OA cartilage, ULK1, Beclin1, and LC3 expression was significantly reduced in the superficial zone compared with that in normal cartilage. In the deep zone, only ULK1 and LC3 expression was significantly reduced. Furthermore, ULK1, Beclin1, and LC3 expression was significantly increased in OA cartilage in the middle and deep zones compared with that in mild OA. Values are the mean and SD. $* = P < 0.05$ and $** = P < 0.01$ versus normal cartilage; $\& = P < 0.05$ versus mild OA. (Original magnification × 40 in **A–L**.) Color figure can be viewed in the online issue, which is available at http://www.arthritisrheum.org.

Quantification of positive chondrocytes in mouse models. Cartilage cellularity in C57BL/6J wild-type (WT) mice was quantified by counting the chondrocytes in a microscopic field (28). In cartilage from mice in the spontaneous aging-related OA model (2-, 9-, and 12-month-old mice) and from mice with surgically induced OA (shortly after sham surgery and 8 weeks after surgery), 3 pictures were taken under 40× magnification, showing the center of the femoral condyle that is not covered by the menisci as well as the medial and lateral femoral condyles. Then, the total number of ULK1-, Beclin1-, and LC3-positive cells was counted in each section and expressed as the percentage relative to the 2-month-old mice (control) and sham-operated mice (control) in aging-related OA and surgically induced OA, respectively.

Western blotting. Cells were washed in ice-cold phosphate buffered saline (PBS), pH 7.5, and lysed in 0.2*M* Tris HCl, pH 6.8, containing 2% sodium dodecyl sulfate (SDS), 20% glycerol, 1 mg/ml protease inhibitor cocktail (Sigma-Aldrich), and 1 m*M* phenylmethylsulfonyl fluoride (Sigma-Aldrich). Whole cell lysates were boiled for 5 minutes. Cartilage was cut into 1-mm–thin slices, and 200–1,000 mg of frozen cartilage was pulverized in a liquid nitrogen–cooled freezer mill for 2 cycles of 1.5 minutes at the rate of maximum impact frequency. Dry weight of normal and OA cartilage was measured, and the same amount of protein was resuspended in SDS gel loading buffer (50 m*M* Tris, pH 6.8, 10% glycerol, 4% SDS, 10% 2-mercaptoethanol, and 0.001% bromphenol blue) and mixed for 2 hours at room temperature. Centrifugation at

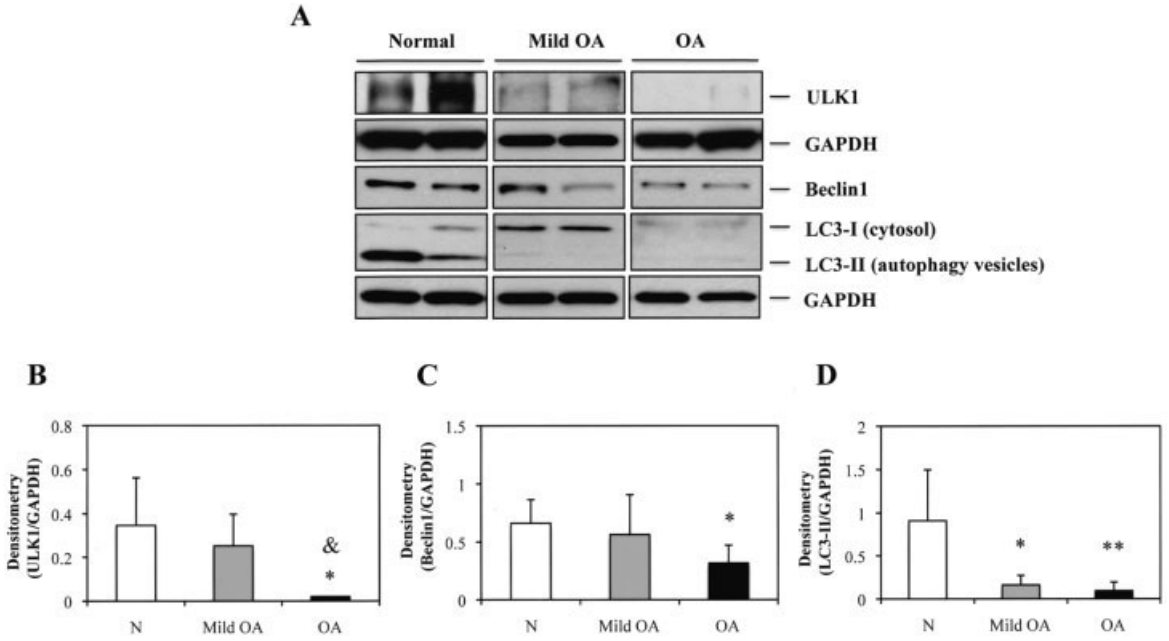

Figure 2. Unc-51–like kinase1 (ULK1), Beclin1, and microtubule-associated protein 1 light chain 3 (LC3) expression is decreased in human osteoarthritic (OA) chondrocytes. **A,** Total protein from normal (N), mild OA, and OA chondrocytes was analyzed by Western blotting, using anti-ULK1, anti-Beclin1, anti-LC3, and GAPDH as described in Materials and Methods. The images shown are representative of normal donors, donors with mild OA, and donors with OA (n = 4 each) for ULK1 and of 6 normal donors, 4 donors with mild OA, and 6 donors with OA for Beclin1 and LC3. **B–D,** Densitometric analysis showed significant (17-fold, 2.1-fold, and 9-fold) decreases in expression of ULK1, Beclin1, and LC3-II protein, respectively, in OA chondrocytes compared with normal chondrocytes. ULK1 protein expression was also significantly reduced in OA chondrocytes compared with those from donors with mild OA. Values are the mean and SD. $* = P < 0.05$ and $** = P < 0.01$ versus normal cartilage; $\& = P < 0.05$ versus mild OA.

14,000 revolutions per minute was performed for 30 minutes, and then supernatants were harvested and heated at 80°C for 10 minutes.

The protein concentrations were determined using a bicinchoninic acid reagent assay (Pierce, Rockford, IL). The concentrated samples were then adjusted to equal volumes before resolution on 12% Tris–glycine gels (Invitrogen, Carlsbad, CA). Protein was transferred to nitrocellulose membranes (Invitrogen), blocked with 5% dry milk in Tris buffered saline–Tween (TBST), and blotted with rabbit polyclonal antibody specific for ULK1 and Beclin1 (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit polyclonal antibody for LC3 (Abgent, San Diego, CA), and GAPDH (Ambion, Austin, TX) for 1 hour. The membranes were then incubated with horseradish peroxidase (HRP)–conjugated anti-mouse IgG (Cell Signaling Technology, Beverly, MA) for 1 hour. Afterward, the membranes were washed 3 times with TBST and developed using enhanced chemiluminescence substrate (Pierce).

Immunohistochemistry. Paraffin-embedded samples were first deparaffinized in the xylene substitute Pro-Par Clearant (Anatech) and rehydrated in graded ethanol and water. For antigen unmasking, sections in 10 m*M* sodium citrate buffer (pH 6.0) were heated in a microwave oven and kept at 80–85°C for 1.5 minutes. Slides were cooled for 20 minutes at room temperature after antigen unmasking. After washing with PBS, sections were blocked with 5% serum for 30 minutes at room temperature. The following steps were performed. Antibodies to ULK1 (1:100 dilution), Beclin1 (1:100 dilution), and LC3 (1:100 dilution) as well as negative control IgG (1 μg/ml) were applied and incubated overnight at 4°C. After washing with PBS, sections were treated with 3% H_2O_2 for 10 minutes, washed with PBS, incubated with biotinylated goat anti-rabbit secondary antibody for 30 minutes at room temperature, and then incubated for 30 minutes using the Vectastain ABC-AP kit (Vector, Burlingame, CA). Slides were washed, and sections were incubated with 3,3′-diaminobenzidine (DAB) substrate for 3–10 minutes.

Detection of PARP cleavage. Polyclonal antibody specific for the p85 fragment of PARP (Promega, Madison, WI) was used. Sections were microwaved in PBS–citrate buffer, pH 6.0, then digested with hyaluronidase, washed, and blocked for 30 minutes in PBS containing 0.1% Tween 20 and 10% normal serum. Primary antibody was applied at a 1:200 dilution and incubated overnight at 4°C. The following day, the sections were washed, blocked with 3% H_2O_2 for 5 minutes, washed again, and incubated with diluted secondary antibody (HRP-conjugated anti-rabbit IgG) for 1 hour. The slides were incubated for 30 minutes with peroxidase complex using the peroxidase-based Elite ABC system (Vector) and then incubated for 4–10 minutes in DAB substrate, followed by mounting as described above.

Statistical analysis. Statistically significant differences between 2 groups were determined with *t*-tests. The results are reported as the mean ± SD. *P* values less than 0.05 were considered significant.

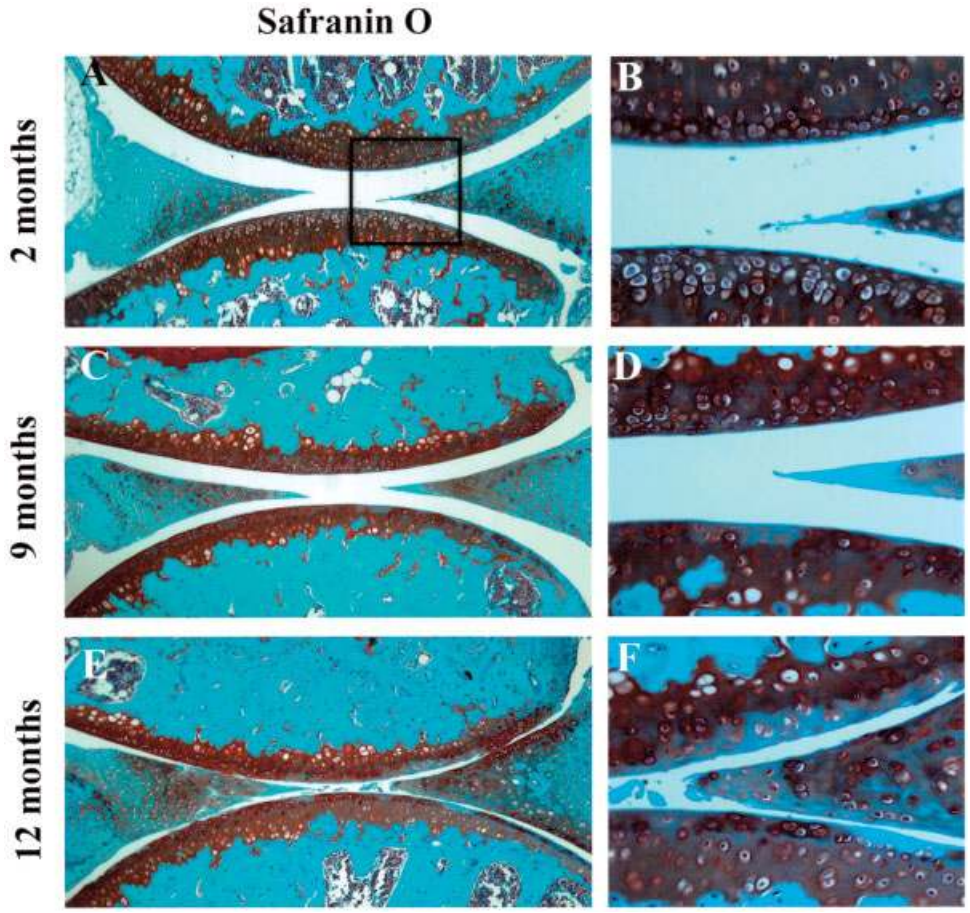

Figure 3. Aging-related loss of glycosaminoglycans in mouse joints. Knee joints from C57BL/6J mice at ages 2 months (n = 4), 9 months (n = 4), and 12 months (n = 4) were analyzed using Safranin O staining. Areas in **A** (boxed), **C,** and **E** are shown at higher magnification in **B, D,** and **F,** respectively. (Original magnification × 10 in **A, C,** and **E**; × 40 in **B, D,** and **F**.) Color figure can be viewed in the online issue, which is available at http://www.arthritisrheum.org.

RESULTS

ULK1, Beclin1, and LC3 expression in human articular cartilage. To study the role of autophagy in the development of OA, we evaluated the expression of ULK1, an inducer of autophagy, Beclin1, a regulator of autophagy, and LC3, which executes autophagy, in human normal (Mankin score 0, OA grade I), mild OA (Mankin score 3, OA grade II), and OA (Mankin score 7–8, OA grade III–IV) articular cartilage. Cartilage samples were evaluated by staining with Safranin O (Figures 1A–C), and ULK1, Beclin1, and LC3 expression was analyzed by immunohistochemistry (Figures 1D–L).

Quantitative analysis of the distribution of positive cells (Figures 1M–O) showed that in normal cartilage, ULK1 (n = 5 donors) and Beclin1 and LC3 (n = 6 donors each) were highly expressed in the superficial, middle, and deep zones (Figures 1D, G, and J). In mild OA, expression of ULK1, Beclin1, and LC3 (n = 4 donors each) was significantly decreased in the superficial zone ($P < 0.01$), middle zone ($P < 0.01$ and $P < 0.05$), and deep zone ($P < 0.01$) compared with that in normal cartilage. In OA cartilage, expression of ULK1 (n = 5 donors) and Beclin1 and LC3 (n = 6 donors each) was reduced in all zones compared with that in normal cartilage. This reduction was significant in the superficial zone for ULK1, Beclin1, and LC3 ($P < 0.01$) and in the deep zone for ULK1 and LC3 ($P < 0.05$ and $P < 0.01$, respectively). In contrast, expression of ULK1, Beclin1, and LC3 in OA cartilage

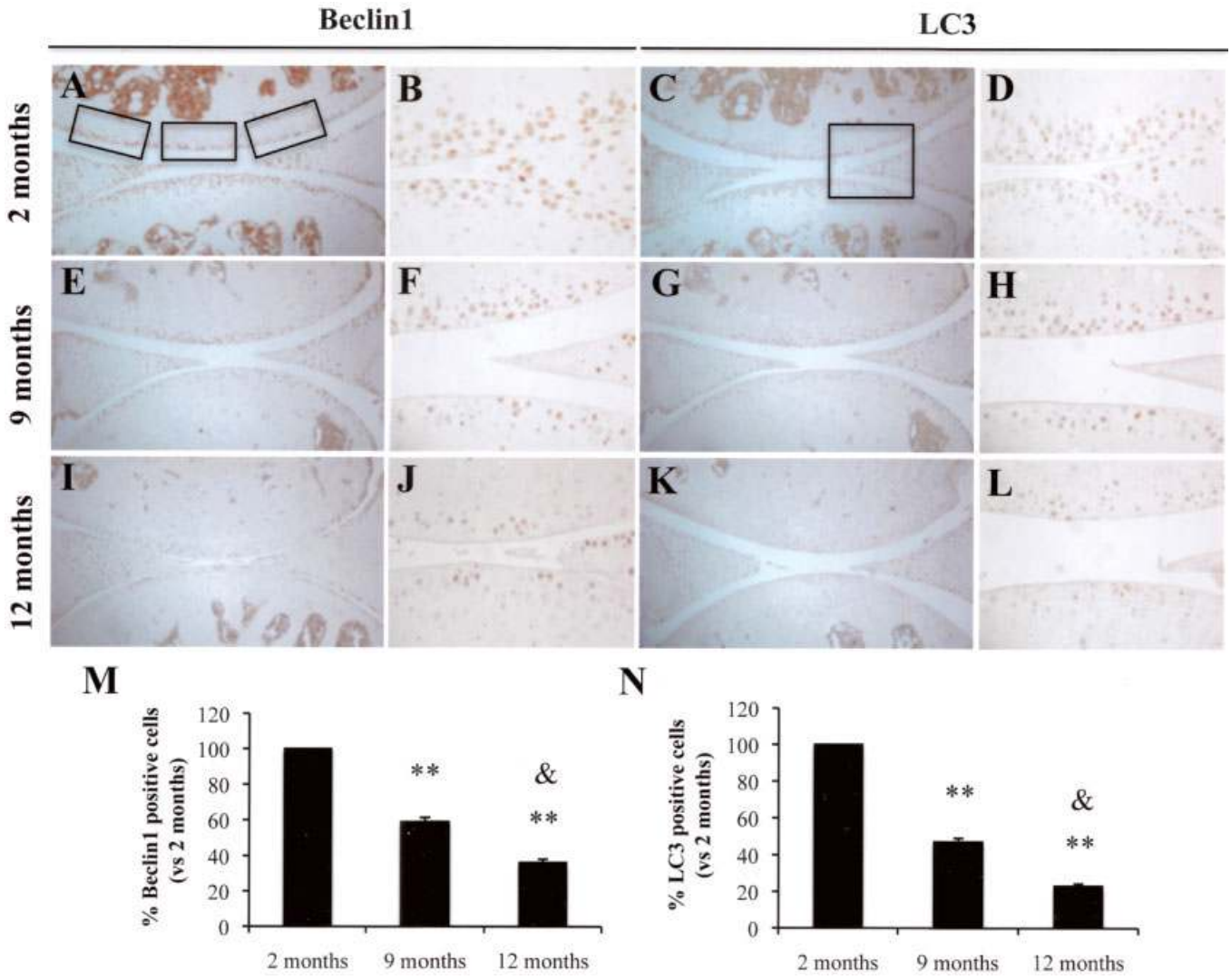

Figure 4. Aging-related reduction in Beclin1 and microtubule-associated protein 1 light chain 3 (LC3) expression in mouse joints. **A–L,** Immunohistochemical analysis for Beclin1 and LC3 in knee joints from 2-, 9-, and 12-month-old C57BL/6J mice. **M** and **N,** Quantification of Beclin1- and LC3-positive cells at ages 2, 9, and 12 months. Percentages of Beclin1- and LC3-positive cells were significantly decreased at ages 9 months and 12 months compared with percentages at age 2 months. Furthermore, percentages of Beclin1- and LC3-positive cells were significantly reduced at age 12 months compared with percentages at age 9 months. Values are the mean and SD (n = 4 per group). ** = $P < 0.01$ versus age 2 months; & = $P < 0.05$ versus age 9 months. The 3 boxed areas in **A** represent areas used for quantification of the positive calls in **M** and **N.** The boxed area in **C** represents the areas in **A, C, E, G, I,** and **K** shown at higher magnification in **B, D, F, H, J,** and **L,** respectively. (Original magnification × 10 in **A, C, E, G, I,** and **K**; × 40 in **B, D, F, H, J,** and **L**.)

was significantly increased in the middle and deep zones (both $P < 0.05$) compared with that in mild OA. This increase was due to the cell clusters localized in the middle and deep zones in OA cartilage. These cell clusters showed strong expression of ULK1, Beclin1, and LC3 (Figures 1F, I, and L). These results indicate that ULK1, Beclin1, and LC3 are expressed in normal cartilage, with overall reduced expression in mild OA and OA cartilage and high expression in the OA cluster chondrocytes.

ULK1, Beclin1, and LC3 protein expression in human cultured articular chondrocytes. To confirm the results obtained in cartilage, we evaluated ULK1, Beclin1, and LC3 protein expression by Western blotting in chondrocytes from normal, mild OA, and OA donors (Figure 2A). ULK1, Beclin1, and LC3-II, an indicator of

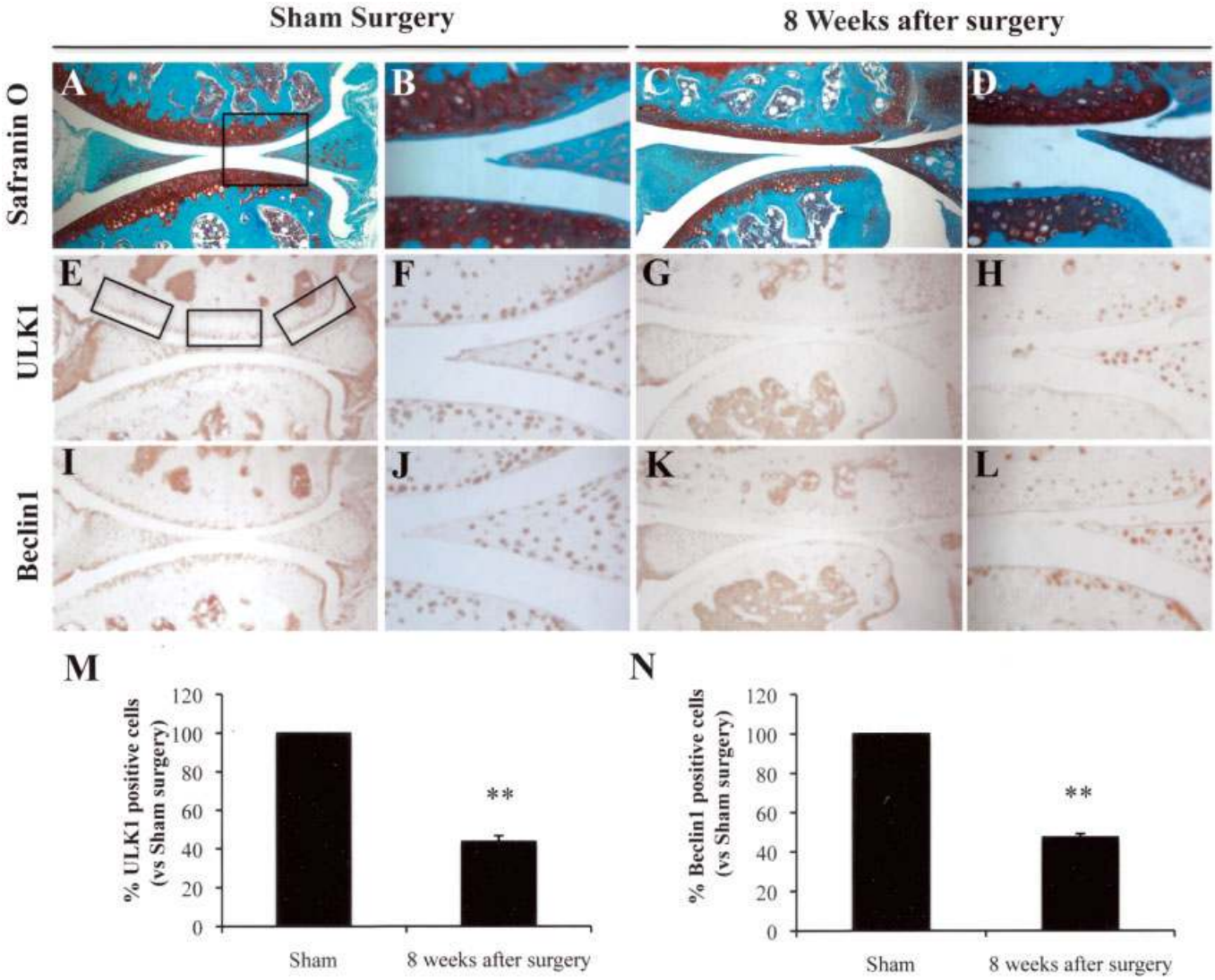

Figure 5. Reduction in Unc-51–like kinase 1 (ULK1) and Beclin1 expression in knee joints from mice with surgically induced osteoarthritis (OA). **A–L,** Knee joints from 2-month-old C57BL/6J mice that underwent sham surgery (n = 4) and from mice 8 weeks after surgical OA induced by transection of the medial meniscotibial ligament and the medial collateral ligament (n = 4), analyzed using Safranin O staining for expression of ULK1 and Beclin1. **M** and **N,** Quantification of ULK1- and Beclin1-positive cells. Knee joints 8 weeks after surgery showed OA pathology, indicated by loss of glycosaminoglycans, and a signifciant decrease of ULK1 and Beclin1 expression. Values are the mean and SD. ** = $P < 0.01$. The 3 boxed areas in **E** represent areas used for quantification of the positive cells in **M** and **N**. The boxed area in **A** represents the areas in **A, C, E, G, I,** and **K** shown at higher magnification in **B, D, F, H, J,** and **L,** respectively. (Original magnification × 10 in **A, C, E, G, I,** and **K**; × 40 in **B, D, F, H, J,** and **L**.) Color figure can be viewed in the online issue, which is available at http://www.arthritisrheum.org.

autophagosome formation, were expressed in normal chondrocytes, and their expression was decreased in mild OA and OA chondrocytes. Densitometric analysis showed significant 17-fold, 2.1-fold, and 9-fold decreases in expression of ULK1, Beclin1, and LC3-II, respectively, in OA chondrocytes compared with normal chondrocytes ($P < 0.05$ and $P < 0.01$) (Figures 2B–D). In addition, ULK1 protein expression was significantly reduced in OA chondrocytes compared with chondrocytes from donors with mild OA ($P < 0.05$) (Figure 2B).

These results suggest that the autophagy inducer ULK1, the autophagy regulator Beclin1, and the autophagy marker LC3 show similar expression patterns in normal cartilage and chondrocytes and similar abnormal expression in OA. Importantly, the expression of LC3-II,

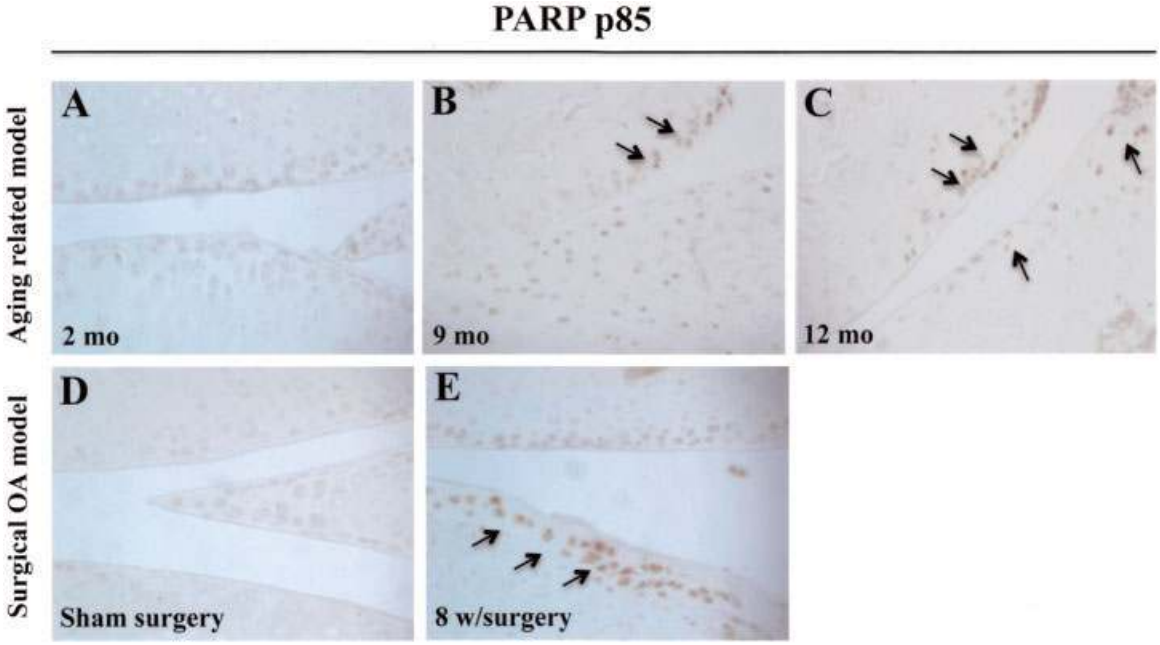

Figure 6. Increased cell death in aging-related and surgically induced osteoarthritis (OA) in mouse joints. **A–C**, Knee joints from wild-type 2-, 9-, and 12-month-old C57BL/6J mice (n = 3 per age group) were used to evaluate poly(ADP-ribose) polymerase (PARP) p85 expression by immunohistochemistry. **D** and **E,** Knee joints from 2-month-old C57BL/6J mice that underwent sham surgery and from mice 8 weeks after surgical induction of OA by transection of the medial meniscotibial ligament and the medial collateral ligament (n = 3 per group) were evaluated for PARP p85, expression using immunohistochemistry. **Arrows** indicate PARP p85–positive cells. (Original magnification × 40.) Color figure can be viewed in the online issue, which is available at http://www.arthritisrheum.org.

which indicates active autophagy in normal tissue, was reduced in OA.

Aging-related cartilage degradation and reduction in Beclin1 and LC3 expression in mouse articular cartilage. Articular cartilage from 2-month-old C57BL/6J WT mice showed homogeneous Safranin O staining in all zones (represented by normal glycosaminoglycan [GAG] content), intact surface, and high cell density (Figures 3A and B). At age 9 months, C57BL/6J WT mice developed mild OA-like changes as previously reported (29), with a modest reduction in Safranin O staining (Figures 3C and D). At age 12 months, C57BL/6J WT mice developed more obvious OA changes with moderate reduction of Safranin O staining and reduction in cartilage thickness and cellularity (Figures 3E and F). Immunohistochemical analysis of knee joints from C57BL/6J mice ages 9 months and 12 months showed an aging-related significant reduction in Beclin1- and LC3-positive cells ($P < 0.01$) compared with 2-month-old mice, as well as a significant reduction in Beclin1 and LC3 in 12-month-old mice compared with 9-month-old mice ($P < 0.05$) (Figures 4M and N). At 2 months, Beclin1 and LC3 were highly expressed in the superficial and upper middle zones (Figures 4A–D); however, this expression was reduced at 9 months (Figures 4E–H) and 12 months (Figures 4I–L), when OA-like changes had developed.

Reduction in ULK1 and Beclin1 expression in mouse joints with surgically induced OA. OA was induced in the knee joints of 2-month-old C57BL/6J mice by transection of the medial meniscotibial ligament and the medial collateral ligament. Knee joints 8 weeks after surgery showed OA pathology, as indicated by loss of GAGs (Figures 5C and D). Immunohistochemical analysis of knee joints at that time point showed a reduction of ULK1-positive cells (Figures 5G and H) and Beclin1-positive cells (Figures 5K and L) compared with control knees that underwent sham surgery (Figures 5E, F, I, and J). The quantitative analysis of positive cells showed a significant reduction of ULK1 and Beclin1 8 weeks after surgery ($P < 0.01$) (Figures 5M and N).

Increased cell death in knee joints from mice with aging-related and surgically induced OA. To further investigate the relationship between autophagy and apoptotic cell death, immunohistochemistry was per-

formed for the apoptosis marker PARP p85 on the same samples that were analyzed in the preceding experiments for the autophagy markers. In knee joints from 9- and 12-month-old C57BL/6J mice, significantly more chondrocytes were positive for PARP p85 than in knee joints from 2-month-old mice (Figures 6A–C). In knee joints from mice with surgically induced OA, an increased number of PARP p85–positive cells was observed 8 weeks after surgery compared with that in control knee joints (Figures 6D and E). Together, these findings demonstrate that human aging and OA cartilage and chondrocytes as well as aging-related and surgically induced OA mouse models are associated with a reduction in autophagy (ULK1, Beclin1, and LC3 expression) along with an increase of apoptosis (PARP p85 expression).

DISCUSSION

The relationship between aging and OA is clinically and epidemiologically evident, and recent findings provide insight into mechanisms that lead to aging-related changes in cells and ECM (30). Articular cartilage appears to be highly susceptible to the accumulation of aging-related changes, in part due to the relatively low turnover of ECM and cells. It has been estimated that the half-life of type II collagen is ~117 years (31), and various measurements in mature cartilage revealed only a very small fraction of proliferating cells (32). Thus, cartilage matrix and cells are prone to accumulating changes related to trauma or to mechanical or oxidant stress over time (33). At the cellular level, a large number of aging-related changes have been documented. These include an overall reduction in cartilage cellularity, reduced antioxidant defenses, altered responses to growth factors and cytokines, and changes in gene and protein expression patterns (21). Physiologic mechanisms that allow for repair of cellular damage are thus critical for the maintenance of chondrocyte survival and function. Best characterized in this regard are mechanisms for nuclear and mitochondrial DNA repair. In contrast, mechanisms capable of replacing damaged proteins and organelles in chondrocytes are essentially unknown.

Autophagy has gained interest in the past decade due to its role in the pathogenesis of various diseases and in regulation of the aging process. Autophagy has been characterized as a highly regulated cellular mechanism with both beneficial and pathogenic effects (34). Normal cellular development and growth require a well-regulated balance between protein synthesis and degradation. Eukaryotic cells have 2 major mechanisms for degradation, the proteasome and autophagy pathways. Autophagy is involved in the bulk degradation of long-lived cytosolic proteins and organelles, whereas the ubiquitin proteasome system degrades specific short-lived proteins (35). Recent studies provide compelling evidence that, at least in model organisms, autophagy protects against diverse pathologies, such as neurodegeneration, heart diseases, infections, cancer, and aging (8). Genetic studies in mice support the importance of autophagy in physiologic and pathologic events. In fact, loss of autophagy genes leads to neurodegeneration, cardiomyopathies, and abnormalities in skeletal development and is associated with the accumulation of cytoplasmic protein aggregates (18,36–39).

The mechanisms by which autophagy is lost with aging are mainly related to the failure of the lysosomal hydrolases, resulting in an increase of toxic protein products and slow clearance of autophagosomes in the aging tissues (40). In addition, other reports described alterations in the response of macroautophagy to hormonal changes with aging. In particular, the effects of oxidative stress on the insulin receptor signaling pathway seem to play a critical role in decreased autophagy in aged organisms (41). The signaling network involving longevity factors sirtuin (silent mating type information regulation 2 homolog) 1 (SIRT1), mechanistic target of rapamycin (mTOR), FoxO3, NF-κB, and p53 regulate autophagy and might have a role in the aging process. NF-κB and mTOR are repressors of the autophagy pathway under input signals of stress and inflammation, while SIRT1, a stress resistance and longevity factor, and FoxO3, a major regulator of cellular metabolism, proliferation, and stress resistance, enhance autophagy (42).

The present study demonstrates that ULK1, Beclin1, and LC3 are expressed in normal human and murine articular cartilage, suggesting activation of autophagy, and the presence of LC3-II directly indicates autophagosome formation (14). However, in OA cartilage and chondrocytes, the expression of these autophagy markers was significantly decreased. Importantly, the reduction in LC3-II implies defective or reduced autophagy in OA. These observations are consistent with the notion that basal autophagic activity decreases with age, thus contributing to the accumulation of damaged macromolecules and susceptibility to aging-related diseases (43). Abnormal protein aggregation and formation of characteristic pathologic structures are central features of such diseases. Interestingly, cellular responses to protein misfolding and aggregation are

intimately related to mechanisms of pathogenesis and are potential targets of new therapies (44).

The present results also show that autophagy was decreased in the surgically induced OA model in mice. Since this was observed in relatively young mice (ages 2–4 months), it is apparently not a consequence of aging-related mechanisms. We found similar results in porcine cartilage explants following exposure to mechanical injury (Caramés B: unpublished observations). It will be of interest to determine mechanisms that are responsible for the suppression or loss of autophagy in the surgical OA model and after mechanical injury. However, both the surgical OA model and mechanically injured cartilage feature increased cell death, suggesting that loss of autophagy may contribute to cell death.

Little is known about the role of autophagy in articular cartilage. Morphologic changes similar to autophagic cell death have been described (45). More information is available in autophagy studies of the epiphyseal growth plate. Autophagy regulates maturation and promotes survival of terminally differentiated chondrocytes under conditions of stress and hypoxia (46–48). In fact, silencing the transcription factor hypoxia-inducible factor 1 (HIF-1) in chondrocytes decreases the expression of Beclin1, suggesting that this factor may have a role as a positive autophagy regulator and that it could play a protective role against cell death (48). In contrast, suppression of HIF-2 in OA and aging cartilage was associated with increased HIF-1 expression and autophagy induction (49).

The protective role of autophagy in endochondral ossification was further supported by observations that its inactivation leads to severe skeletal abnormalities, due in part to cell death (39). In addition, autophagy is essential for early embryonic development, supported by observations that Beclin1 disruption causes early embryonic lethality, autophagy deficiency, and apoptosis (50). In order to investigate the relationship between autophagy and apoptotic cell death as well as the mechanism of chondrocyte loss and cartilage degradation in aging and OA, immunohistochemistry was performed in the present study for the apoptosis marker PARP p85. We found that autophagy was decreased in the aging-related and surgically induced OA mouse models, while apoptotic cell death was increased. This correlation requires further analysis to determine whether a direct causal relationship exists between the 2 processes in cartilage.

In summary, this study is the first to demonstrate that autophagy may be a protective or homeostatic mechanism in normal cartilage. In contrast, human OA and spontaneous aging-related and surgically induced OA in mice are associated with a reduction and loss of ULK1, Beclin1, and LC3 expression and a related increase in apoptosis. These results suggest that compromised autophagy may represent a novel mechanism in the development of OA.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. 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.

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