Aging-related loss of the chromatin protein HMGB2 in articular cartilage is linked to reduced cellularity and osteoarthritis

Noboru Taniguchi[a], Beatriz Caramés[a], Lorenza Ronfani[b], Ulrich Ulmer[a], Setsuro Komiya[c], Marco E. Bianchi[b], and Martin Lotz[a,1]

[a]Division of Arthritis Research, The Scripps Research Institute, La Jolla, CA 92037; [b]Department of Biological and Technological Research, San Raffaele University, 20132 Milan, Italy; and [c]Department of Orthopaedic Surgery, Kagoshima University Graduate School of Medical and Dental Sciences, Kagoshima 890-8520, Japan

Edited by Dennis A. Carson, University of California at San Diego, La Jolla, CA, and approved November 21, 2008 (received for review June 23, 2008)

Osteoarthritis (OA) is the most common joint disease and typically begins with an aging-related disruption of the articular cartilage surface. Mechanisms leading to the aging-related cartilage surface degeneration remain to be determined. Here, we demonstrate that nonhistone chromatin protein high-mobility group box (HMGB) protein 2 is uniquely expressed in the superficial zone (SZ) of human articular cartilage. In human and murine cartilage, there is an aging-related loss of HMGB2 expression, ultimately leading to its complete absence. Mice genetically deficient in HMGB2 (*Hmgb2*$^{-/-}$) show earlier onset of and more severe OA. This is associated with a profound reduction in cartilage cellularity attributable to increased cell death. These cellular changes precede glycosaminoglycan depletion and progressive cartilage erosions. Chondrocytes from *Hmgb2*$^{-/-}$ mice are more susceptible to apoptosis induction *in vitro*. In conclusion, HMGB2 is a transcriptional regulator specifically expressed in the SZ of human articular cartilage and supports chondrocyte survival. Aging is associated with a loss of HMGB2 expression and reduced cellularity, and this contributes to the development of OA.

HMGB | chondrocytes | apoptosis | superficial zone

Osteoarthritis (OA) represents the most common musculoskeletal disorder, and the number of affected individuals is predicted to increase as a result of population aging and an increase in life expectancy (1). Pharmacologic interventions that alter the progressive loss of articular cartilage are currently unavailable (2). Secondary forms of OA can develop in individuals with specific risk factors, such as joint trauma, malalignment, or metabolic disorders (3). Primary OA, the most common form, is not associated with specific risk factors, but its prevalence increases with age (4).

The earliest manifestations of the OA process include changes in the superficial zone (SZ) of articular cartilage, which evolve into the progressive remodeling and degradation of the cartilage extracellular matrix, and other joint structures are also affected at later stages in the disease process (5). Mechanical stress has been implicated in initiating the superficial lesions (6); the SZ is more susceptible to cell death induced by mechanical stress (7), but molecular mechanisms remain to be elucidated. The SZ of articular cartilage is unique in cell morphology and extracellular matrix composition. The SZ is important in many respects because it forms a fluid-tissue interface of articular cartilage in the synovial cavity. The SZ spans the first 10–20% of full-thickness articular cartilage and is composed of densely packed collagen II fibrils maintained by elongated and flattened cells that lie in a parallel orientation to the cartilage surface (8). Unique to SZ is the production of superficial zone protein (SZP), also called proteoglycan-4 (PRG4) or lubricin, which is thought to contribute to the low-friction properties of articular cartilage (9, 10). The SZ possesses 3.5-fold more cells compared with the radial zone (11), and a decrease in cell number, along with surface fibrillation, is the earliest indicator of OA development (12). Although these observations detail certain unique attributes of the SZ, molecular mechanisms that determine differentiation, maintenance, and aging-related changes of SZ cells are unknown.

The high-mobility group box (HMGB) protein family includes the ubiquitous HMGB1, the tissue-specific HMGB2, and the embryo-specific HMGB3 (13). HMGB1 is an abundant component of all mammalian nuclei and acts as an architectural factor that bends DNA and promotes protein assembly on specific DNA targets (14). HMGB1 also has an extracellular role as a chemoattractant (15) as well as a proinflammatory cytokine (16). We reported that HMGB1 is expressed in growth plates and regulates endochondral ossification (17).

Contrary to the ubiquitous expression of HMGB1, HMGB2 is restricted mainly to the lymphoid organs and testis in adult mice, although it is widely expressed during embryogenesis (18). Functionally, HMGB1 and HMGB2 are interchangeable in certain in vitro models. They both bind to Hox proteins (19), steroid hormone receptors (20), and Rag1 recombinase (21) and enhance the transcription and recombination activities of their partner proteins when transiently transfected in mammalian cells (14). However, despite their sequence and structural and biochemical similarities, functions of HMGB1 and HMGB2 are not identical. *Hmgb1*$^{-/-}$ but not *Hmgb2*$^{-/-}$ mice die shortly after birth because of hypoglycemia (22). Male *Hmgb2*$^{-/-}$ but not *Hmgb1*$^{-/-}$ mice have reduced fertility, which correlates with Sertoli and germ cell apoptosis in seminiferous tubules (18).

Here, we identify HMGB2 as a transcriptional regulator that is unique to the SZ of human articular cartilage. Spontaneous aging-related and genetic deficiencies of HMGB2 are related to chondrocyte cell death and the development of OA.

Results

HMG Gene Expression in Normal and OA Cartilage. DNA array studies were performed on normal and OA human articular cartilage, and data were analyzed for differences in the expression of HMG genes [see supporting information (SI) *Text*]. The results showed significantly reduced expression of HMGB1 and HMGB2 and increased expression of HMGA1 and HMGN1 in

Author contributions: N.T. and M.L. designed the study; N.T., B.C., and U.U. performed research; L.R. and M.E.B. generated *Hmgb2*-deficient mice; N.T. and M.L. analyzed data; and N.T., S.K., M.E.B., and M.L. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

[1]To whom correspondence should be addressed at: Division of Arthritis Research, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037. E-mail: mlotz@scripps.edu.

This article contains supporting information online at www.pnas.org/cgi/content/full/0806062106/DCSupplemental.

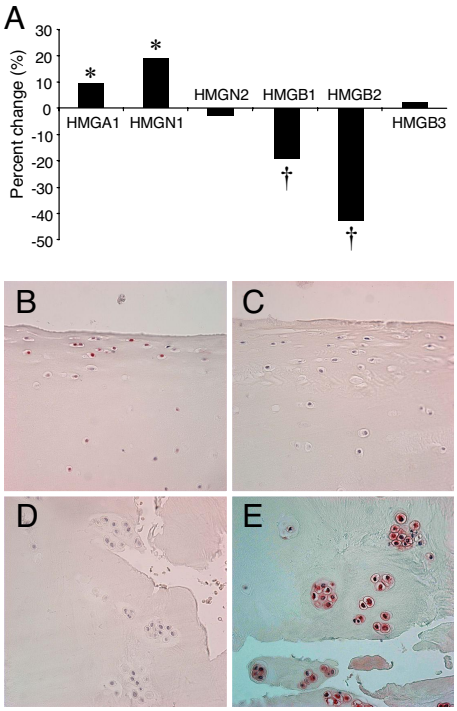

Fig. 1. HMGB2 expression in human articular cartilage. (*A*) Microarray analysis of HMG family genes in normal and OA cartilage. RNA was isolated from 9 normal donors (26.0 ± 8.6 years; OA grade I) and 13 donors with OA (77.3 ± 8.8 years; OA grades III–IV). Total RNA was applied to U95A Affymetrix DNA arrays in duplicate. Statistically significant differences between normal and OA groups are indicated (*, $P < 0.05$; †, $P < 0.01$). *y* axis: % change in gene expression in OA cartilage compared with normal cartilage. (*B–E*) Immunohistochemical analysis of human knee articular cartilage sections. Normal tissue, donor aged 27 years (*B*); mild OA tissue, donor aged 85 years (*C*); and OA tissue, donor aged 71 years (*D* and *E*) stained with anti-HMGB2 antibody (*B–D*) or anti-HMGB1 antibody (*E*). Images shown are representative of normal (19.0 ± 5.1 years, Mankin score = 0, $n = 6$), mild OA (68.8 ± 8.6 years; Mankin score, 3.0 ± 0.6; $n = 6$), and OA (69.0 ± 9.8 years; Mankin score, 7.1 ± 0.7; $n = 6$) donors. (Magnification: ×400.)

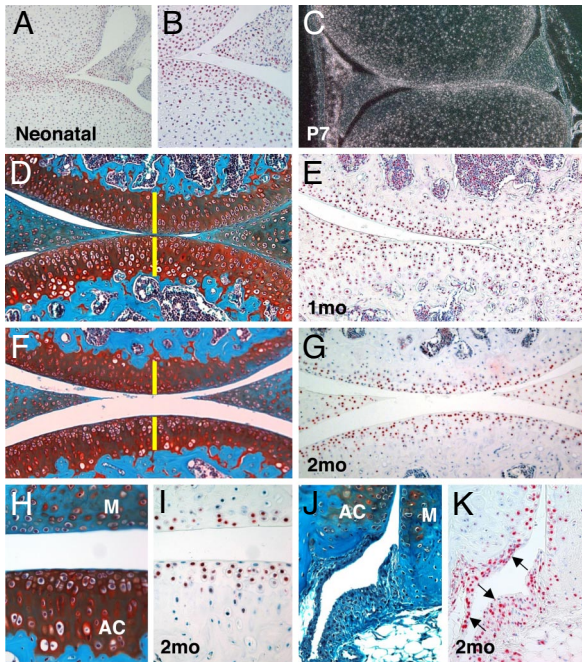

Fig. 2. HMGB2 expression in normal young mouse knee joints. Immunostaining for HMGB2 in knee joints from neonatal C57BL/6J mice (*A* and *B*) and in situ hybridization for HMGB2 at 7 days (P7) after birth (*C*). (*D* and *F*) Between 1 and 2 months of age, there is a reduction in the thickness of the articular cartilage as seen on safranin O stains (bars). HMGB2 is expressed throughout articular cartilage at 1 month (*E*) and becomes more restricted to the superficial and upper middle zones at 2 months (*G*). (*H–K*) High-magnification pictures of cartilage of 2-month-old mice. HMGB2 is also expressed in the superficial layer of cells in menisci (*I*) and in synovial lining (*K*, arrows) from 2-month-old mice. (*H* and *J*) Safranin O staining of adjacent sections. AC, articular cartilage; M, meniscus. (Magnification: *A*, *C–G*, ×100; *B*, *H–K*, ×400.)

OA, whereas the expression of the other family members was similar in the 2 types of tissues (Fig. 1*A*).

HMGB2 Expression in Human Articular Cartilage and Mouse Joints. Patterns of HMGB2 expression in human articular cartilage were characterized by immunohistochemistry. In normal cartilage (Mankin grade 0) from young donors (age: 19.0 ± 5.1 years), HMGB2 expression was unique to the top 1–2 cell layers in the SZ (Fig. 1*B*), where 64.5% of the cells were HMGB2-positive (Table S1). In contrast, expression of HMGB2 was much lower in human cartilage from old donors (age: 68.8 ± 8.6 years) with mild OA (Fig. 1*C*). OA cartilage sections from old donors (age: 69.0 ± 9.8 years) that featured the characteristic fibrillations and chondrocyte clusters were completely negative for HMGB2 (Fig. 1*D*). This observation was remarkable, because the cells in the OA clusters were positive for many markers, such as Notch1 (23), Runx2, and matrix metalloproteinase (MMP)-13 (24). In comparison, strong staining in the clusters was shown for HMGB1 (Fig. 1*E*). Control IgG was negative in adjacent sections (Fig. S1).

Immunostaining of knee joints from neonatal C57BL/6J mice revealed that HMGB2 was expressed in cells in all zones of the articular cartilage (Fig. 2 *A* and *B*). This expression was confirmed by in situ hybridization (Fig. 2*C*). At 1 month of age, HMGB2 was also localized in all zones of articular cartilage (Fig. 2*E*). Between 1 and 2 months of age, there was a reduction in the thickness of the articular cartilage (Fig. 2 *D* and *F*); at 2 months, HMGB2 expression became more restricted to the superficial and upper middle zones (Fig. 2 *G* and *I*). HMGB2 was also expressed in cells in the superficial layer of menisci (Fig. 2*I*) and in the synovial lining (Fig. 2*K*). HMGB2 staining was not detectable in sections of *Hmgb2*$^{-/-}$ mice (Fig. S1).

Aging-Related Reduction in HMGB2 Expression in Mouse Articular Cartilage. Immunohistochemical analysis of knee joints from C57BL/6J mice aged 6, 9, and 12 months showed an aging-related reduction in the number of HMGB2-positive cells. At 6 months, HMGB2 was detected in the top 3–4 cell layers (Fig. 3*B* and *C*); however, this expression was reduced at 9 months (Fig. 3 *E* and *F*) and almost completely absent at 12 months (Fig. 3 *H* and *I*). To determine whether the reduction of HMGB2-positive cells in articular cartilage was associated with loss of chondrocytes, SZ cellularity was quantified (25). The results showed that the number of SZ chondrocytes was significantly reduced at 9 and 12 months (Fig. 3*J*). There was a similar reduction in HMGB2-expressing cells at 6 months, and this became more profound at 9 and 12 months (Fig. 3*K*). A reduction in HMGB2-expressing cells was also observed in menisci, although it was less profound.

There was no apparent aging-related reduction of HMGB2 expression in bone marrow (Fig. 3 *C*, *F*, and *I*) or synovium (Fig. S2), suggesting that the loss of HMGB2 in articular cartilage does not appear to be a general transcriptional suppression of *Hmgb2* expression in the joints.

Aging-Related Joint Pathology in *Hmgb2*$^{-/-}$ Mice. *Hmgb2*$^{-/-}$ mice are on C57BL/6J background (18). Skeletal development in *Hmgb2*$^{-/-}$ mice was normal (Fig. S3). Articular cartilage from 4- and 6-month-old C57BL/6J WT mice showed homogeneous safra-

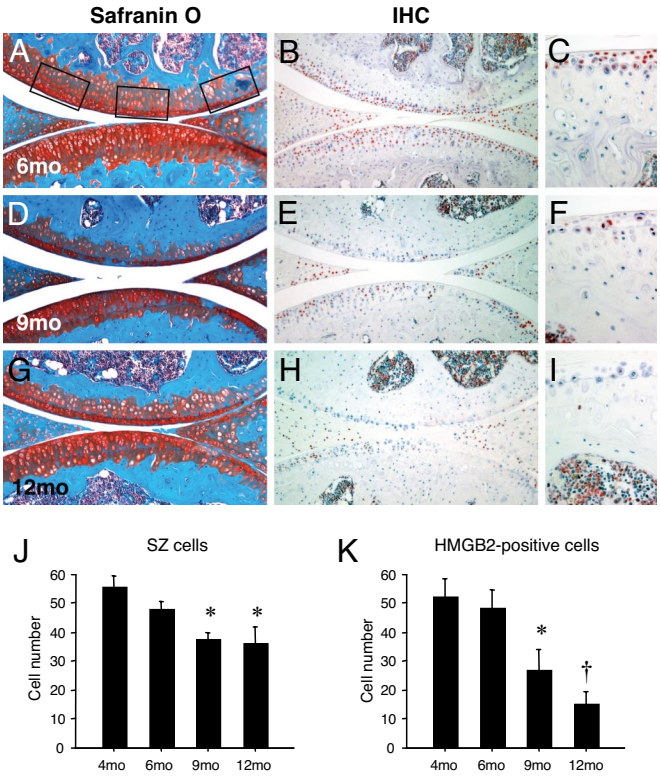

Fig. 3. Aging-related reduction in HMGB2 expression in mouse joints. Knee joints from 6-, 9-, and 12-month-old C57BL/6J mice were analyzed by safranin O staining (*A*, *D*, *G*) or for HMGB2 expression by immunohistochemistry (IHC; *B*, *E*, *H*). HMGB2-positive cells are significantly decreased at 9 months (*E*) and almost completely absent in articular cartilage at 12 months (*H*). (*C*, *F*, *I*) High-magnification pictures of the central area of the femoral condyle at 6, 9, and 12 months. HMGB2-positive cells are found in the bone marrow without apparent age-related differences. (*J*) Quantification of SZ cells in cartilage in 4-, 6-, 9-, and 12-month-old mice. Cells were counted on safranin O-stained sections. Cell counting was performed in 3 microscopic fields as shown in *A*, and summed scores are shown. (*K*) Quantification of HMGB2-positive cells in the adjacent sections. Knee joints from C57BL/6J WT mice aged 4 months ($n = 7$), 6 months ($n = 7$), 9 months ($n = 9$), and 12 months ($n = 7$) were examined. Statistically significant differences are indicated (*, $P < 0.05$; †, $P < 0.01$). (Magnification: *A*, *B*, *D*, *E*, *G*, and *H*, ×100; *C*, *F*, and *I*, ×400.)

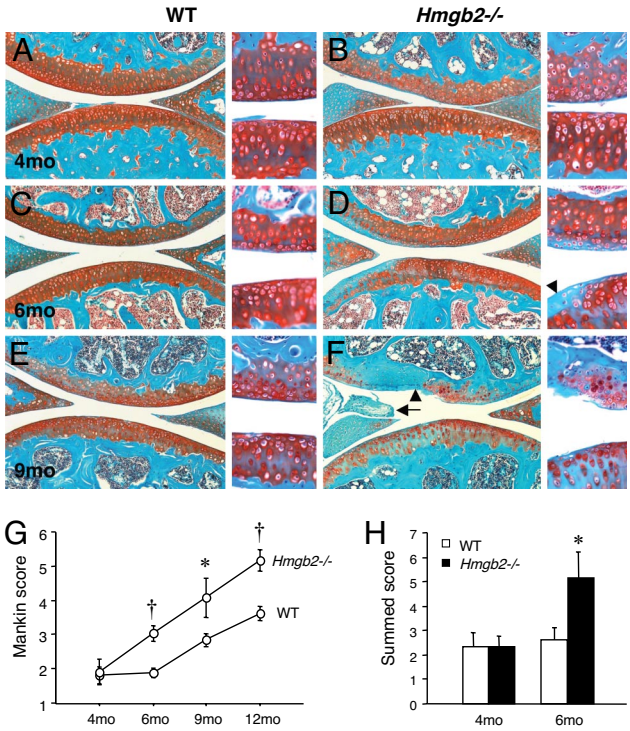

Fig. 4. Aging-related joint pathology in *Hmgb2*$^{-/-}$ mice. Safranin O staining of knee joints from 4-, 6-, and 9-month-old WT mice (*A*, *C*, *E*) and *Hmgb2*$^{-/-}$ mice (*B*, *D*, *F*) with high-magnification pictures (×400) of the articular cartilage. (*D*) Joints from 6-month-old *Hmgb2*$^{-/-}$ mice exhibit reduced safranin O staining in the medial femoral condyles and tibial plateaus. There is also a profound reduction in cellularity (arrowhead). (*F*) *Hmgb2*$^{-/-}$ mice at 9 months of age show structural cartilage defects (arrowhead); this is associated with synovial hyperplasia (arrow). (Magnification: *A*–*F*, ×100.) Quantification of histopathological changes in C57BL/6J WT and *Hmgb2*$^{-/-}$ mice. (*G*) Pathological changes in knee joints were quantified by a modified Mankin scoring system. C57BL/6J WT mice develop mild OA changes at 9 months, whereas in *Hmgb2*$^{-/-}$ mice on the same background, this appears at 6 months. (*H*) Modified semiquantitative scoring system for WT and *Hmgb2*$^{-/-}$ mice at 4 and 6 months. The disruption of the cartilage surface in *Hmgb2*$^{-/-}$ mice occurs at 6 months. Statistically significant differences in scores between WT and *Hmgb2*$^{-/-}$ mice at each time point are indicated (*, $P < 0.05$; †, $P < 0.01$).

nin O staining in all zones, intact surface, and high cell density (Fig. 4*A* and *C*). In contrast, joints from 6-month-old *Hmgb2*$^{-/-}$ mice showed reduced safranin O staining in the medial femoral condyles and tibial plateaus (Fig. 4*D*). The interterritorial staining was completely absent, and territorial staining was only present in some middle and deep zone cells. There was also a profound reduction in cellularity (Fig. 4*D*, *Right*). Areas of the SZ were acellular, and the cell density in the middle and deep zones was significantly reduced. By 9 months of age, the *Hmgb2*$^{-/-}$ mice showed structural cartilage defects and this was associated with synovial hyperplasia (Fig. 4*F*). WT mice had a modest reduction in safranin O staining, but this was restricted to the femoral condyle, without cartilage destruction (Fig. 4*E*). In coronal views of the knee joints, safranin O loss and synovial hyperplasia were prominent in the medial femoral condyle of *Hmgb2*$^{-/-}$ mice. Some knee joints from *Hmgb2*$^{-/-}$ mice showed osteophyte formation in the patellar ligament (Fig. S4).

The pathological changes in the knee joints were quantified by the modified Mankin scoring system (Fig. 4*G*) (26). C57BL/6J WT mice developed mild osteoarthritic changes at 9 months as previously reported (27). In contrast, *Hmgb2*$^{-/-}$ mice on the same background already had OA-like changes at 6 months, and Mankin scores were significantly different between WT and *Hmgb2*$^{-/-}$ mice at 6, 9, and 12 months. Because OA typically begins with an aging-related disruption of the articular cartilage surface (5), we assessed the severity of OA-like changes in the cartilage surface of WT and *Hmgb2*$^{-/-}$ mice (28, 29). The result showed that the cartilage surface in *Hmgb2*$^{-/-}$ mice was already significantly affected at 6 months of age (Fig. 4*H*).

Apoptosis and Matrix Metalloproteinase Expression. To investigate the mechanisms of chondrocyte loss and cartilage degradation, immunohistochemistry was performed for the apoptosis marker poly (ADP-ribose) polymerase (PARP)-p85 and for MMP-3 and MMP-13. In articular cartilage from 6-month-old *Hmgb2*$^{-/-}$ mice, a large number of chondrocytes in the SZ and upper middle zone were positive for PARP-p85, particularly in the non-meniscus-covered areas of the articular cartilage (Fig. 5*F*). In WT knee joints, only a few chondrocytes were positive, mainly in the area adjacent to the lateral meniscus (Fig. 5*E*). At 9 months, PARP-p85 staining was almost absent in the *Hmgb2*$^{-/-}$ mice (Fig. 5*H*), whereas some chondrocytes were positive for PARP-p85 in WT mice (Fig. 5*G*, arrows), although they were not confined to the superficial layer. The corresponding safranin O stain showed that articular cartilage was depleted of glycosaminoglycans and cell density was profoundly

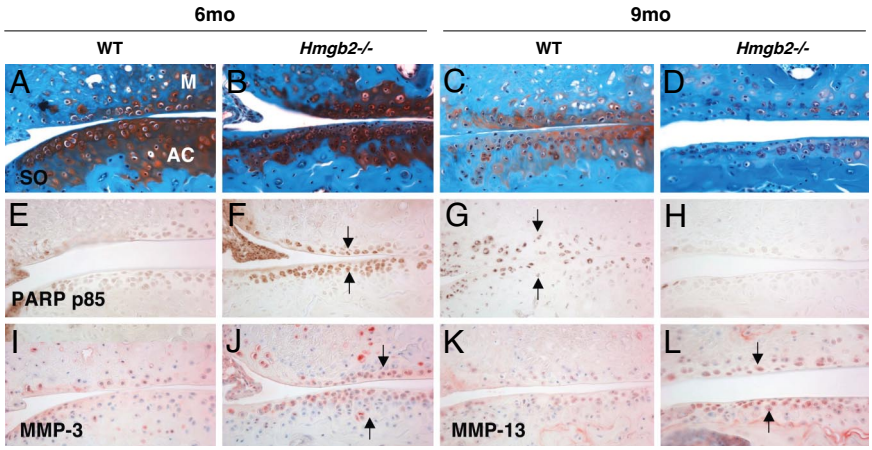

Fig. 5. Apoptosis and MMP expression. Adjacent sections of knee joints from 6- and 9-month-old WT and *Hmgb2*$^{-/-}$ mice were stained with safranin O and by immunohistochemistry for PARP-p85, MMP-3, and MMP-13. (*B*) Articular cartilage from 6-month-old *Hmgb2*$^{-/-}$ mice shows diffuse safranin O reduction with a large number of PARP-p85 chondrocytes (*F*, arrows), particularly in the nonmeniscus-covered areas of cartilage surface. In WT knee joints from 6-month-old mice, safranin O staining is intact (*A*); only a few chondrocytes are positive for PARP p85 (*E*). (*G* and *H*) At 9 months, PARP-p85 staining is almost absent in the *Hmgb2*$^{-/-}$ mice, whereas some chondrocytes are positive for PARP-p85 in WT mice (*G*, arrows). (*C* and *D*) Corresponding safranin O stain shows that articular cartilage is depleted of glycosaminoglycans and cell density is profoundly reduced in *Hmgb2*$^{-/-}$ mice compared with WT mice. (*I* and *J*) MMP-3 is expressed by a higher number of chondrocytes from 6-month-old *Hmgb2*$^{-/-}$ mice (*J*, arrows) compared with WT mice. (*K* and *L*) MMP-13 expression is not detected at 6 months (data not shown), but at 9 months, staining intensity is greater in *Hmgb2*$^{-/-}$ mice (*L*, arrows) than in WT mice. Representative pictures from PARP-p85 staining ($n = 4$ each) and MMP-3 and MMP-13 staining ($n = 2$–4 each) are shown. AC, articular cartilage; M, meniscus. (Magnification: *A*–*L*, ×400.)

reduced at this time point (Fig. 5*D*). MMP-3 was expressed by a higher number of chondrocytes from 6-month-old *Hmgb2*$^{-/-}$ mice compared with WT mice (Fig. 5 *I* and *J*). At 9 months, the intensity of MMP-3 expression in *Hmgb2*$^{-/-}$ mice was lower than at 6 months (not shown). MMP-13 expression was not detected at 6 months (not shown), but at 9 months, staining intensity was greater in *Hmgb2*$^{-/-}$ mice compared with WT mice (Fig. 5 *K* and *L*).

These findings suggest a sequence of events in *Hmgb2*$^{-/-}$ mice with occurrence of apoptosis in articular cartilage at 6 months that is associated with MMP-3 expression, followed by decreased cellularity and high MMP-13 expression at 9 months.

***Hmgb2* Deficiency and PRG4/SZP Expression.** PRG4/SZP has a similar distribution as HMGB2 and is involved in cartilage homeostasis and joint lubrication (10). Furthermore, *Prg4*-deficient mice have structural and cellular abnormalities in the cartilage surface (10). We thus investigated the relation between HMGB2 and PRG4/SZP in articular cartilage. PRG4/SZP and HMGB2 expression in murine articular cartilage from 2-month-old mice was examined by immunofluorescence assay. The results indicated that in most (67.9%) SZ chondrocytes, HMGB2 and PRG4/SZP were colocalized (Fig. 6*A*). We also compared PRG4/SZP expression between WT and *Hmgb2*$^{-/-}$ mice by in situ hybridization. At 2 months, PRG4/SZP expression was found in the cartilage surface and synovium and there was no apparent difference between WT and *Hmgb2*$^{-/-}$ mice (Fig. 6*B*, *Top*). At 6 months, WT mice had the expected expression pattern; however, *Hmgb2*$^{-/-}$ mice showed a substantial or, in some cartilage regions, complete loss of PRG4/SZP expression (Fig. 6*B*, *Bottom*). PRG4/SZP expression was not reduced in the synovium of *Hmgb2*$^{-/-}$ mice.

The Role of HMGB2 in Articular Chondrocyte Survival. Based on the observed reduction in cartilage cellularity and increased apoptosis

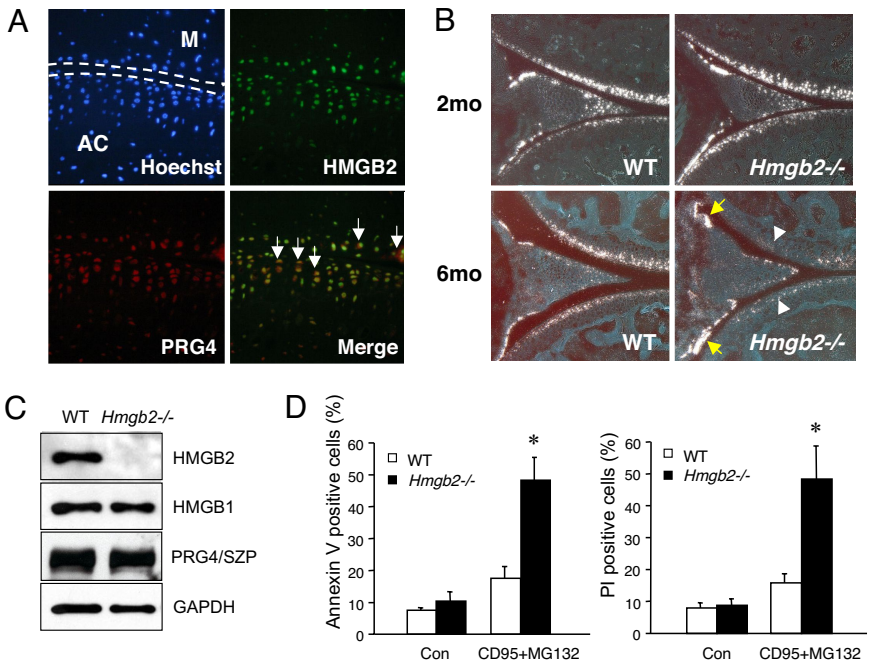

Fig. 6. Localization of HMGB2 and PRG4/SZP in murine cartilage. (*A*) Double staining by immunofluorescence shows colocalization of HMGB2 and PRG4/SZP in articular cartilage and meniscus at 2 months. PRG4/SZP is localized in the cytosol, which is distinct from HMGB2 expression in the nuclei (arrows). Nuclei were labeled by using Hoechst 33258 dye. AC, articular cartilage; M, meniscus. (Magnification: ×400.) (*B*) PRG4/SZP expression in knee joints as visualized by in situ hybridization. At 2 months, PRG4/SZP-positive cells are found in the SZ and synovium in both WT and *Hmgb2*$^{-/-}$ strains without apparent difference. At 6 months, PRG4/SZP-positive cells are significantly reduced in the cartilage in *Hmgb2*$^{-/-}$ mice (arrowheads), whereas PRG4/SZP expression in synovium is higher in *Hmgb2*$^{-/-}$ mice (arrows) than in WT mice. (Magnification: ×100.) (*C*) PRG4/SZP and HMGB2 expression in cultured mouse chondrocytes. Chondrocytes were isolated from articular cartilage of 5-day-old WT and *Hmgb2*$^{-/-}$ mice and cultured for 3 days until analysis. Western blotting shows similar PRG4/SZP and HMGB1 expression in WT and *Hmgb2*$^{-/-}$ chondrocytes. HMGB2 expression is absent in chondrocytes from *Hmgb2*$^{-/-}$ mice. (*D*) Apoptosis assays for articular chondrocytes from WT and *Hmgb2*$^{-/-}$ mice. After culture in control media (Con) or treatment with CD95 antibody (1 μg/mL) and proteasome inhibitor MG132 (20 μM) for 12–14 h, chondrocytes were labeled with annexin V or propidium iodide (PI) and analyzed by flow cytometry. Three independent experiments with chondrocytes from three different littermate mice were performed. Statistically significant differences between *Hmgb2*$^{-/-}$ and WT chondrocytes are indicated (*, $P < 0.05$).

in *Hmgb2*$^{-/-}$ mice, we examined the role of HMGB2 in articular chondrocyte survival. We isolated articular chondrocytes from 5-day-old C57BL/6J WT mice (30) and examined HMGB2 and PRG4/SZP expression. Western blotting showed that chondrocytes isolated from WT and *Hmgb2*$^{-/-}$ mice had similar expression of HMGB1 and PRG4/SZP; HMGB2 was not detected in *Hmgb2*$^{-/-}$ chondrocytes (Fig. 6*C*). Immunofluorescence assay showed that HMGB2 and PRG4/SZP were positive in 80% and 83% of the chondrocytes, respectively, and colocalized in 80% of the cells (not shown). These findings imply that isolated chondrocytes from immature (5-day-old) joints resemble SZ cells that are positive for both HMGB2 and PRG4/SZP (Fig. 6*A*). Using chondrocytes in primary culture from WT and *Hmgb2*$^{-/-}$ mice, we examined apoptotic cells by annexin V and propidium iodide staining. Under control conditions, the number of annexin V- or propidium iodide-positive cells was similar between the groups (Fig. 6*D*). In chondrocytes stimulated with antibody to CD95/Fas, there was a low level of apoptosis, but this was augmented by the proteasome inhibitor MG132 (31). In response to this apoptosis challenge, the number of annexin V- and propidium iodide-positive cells was significantly higher in *Hmgb2*$^{-/-}$ than in WT chondrocytes (Fig. 6*D*). These findings suggest that HMGB2 regulates cell survival of SZ chondrocytes and that loss of this function represents a mechanism of early-onset OA in *Hmgb2*$^{-/-}$ mice and in aging in WT mice and humans.

Discussion

Aging is the major risk factor for OA, the most prevalent joint disease (2). Among the earliest lesions during OA development is disruption of the SZ of articular cartilage (5, 6), but underlying mechanisms remain to be elucidated.

The present results demonstrate that HMGB2 expression is uniquely restricted to cells in the SZ in normal mature human articular cartilage. This is a demonstration of a transcriptional regulator that has selectivity for a particular zone of cartilage. Further studies on HMGB2 have the potential to generate insight into molecular mechanisms that control the unique differentiation status of SZ chondrocytes. This information will not only be relevant to OA but to cartilage tissue engineering. HMGB2 is also expressed in the superficial or lining cell populations of the menisci and synovium. Recent studies indicate that these regions harbor adult stem cells (23, 32, 33). HMGB2 is highly expressed in human bone marrow-derived mesenchymal stem cells (N.T. and M.L.; unpublished data), raising the possibility that the cell population in the SZ that expresses HMGB2 is an immature mesenchymal stem cell-like chondrocyte subset.

We reported previously that extracellular HMGB1 released by differentiating cartilage acts as a chemoattractant for osteoblasts, osteoclasts, and endothelial cells at the primary ossification center (17). HMGB1 protein was localized in the nuclei of prehypertrophic chondrocytes and the cytosol of hypertrophic chondrocytes. In contrast, HMGB2 was found only in the nucleus of prehypertrophic chondrocytes (Fig. S3), suggesting that HMGB1 and HMGB2 exert temporally and spatially different functions in skeletal development. This is consistent with our observations that *Hmgb1*$^{-/-}$ mice have defects in skeletal development (17), whereas *Hmgb2*$^{-/-}$-deficient mice have no apparent skeletal defects.

Joint aging in mice is associated with a reduction in HMGB2 expression in articular cartilage. A link between aging and HMGB2 has been observed in earlier studies. Phosphorylation as well as ADP-ribosylation of HMGB2 in the liver of old rats decreased drastically more than in young rats after spermine or sodium butyrate treatment, although HMGB1 remained unchanged (34, 35). The same group also reported that dexamethasone stimulated the methylation of HMGB2 and inhibited that of other HMGs in young rats, whereas it stimulated all major HMG proteins except for HMGB2 in old age (36). Regarding HMGB2 expression during aging, Ly *et al.* (37) measured mRNA levels in actively dividing fibroblasts isolated from young, middle-aged, and old-aged humans and humans with progeria. Interestingly, *Hmgb2* was among 9 genes that were down-regulated in both groups of old age and progeria among the 6,000 human genes that were examined. Because HMGB2 participates in chromosomal processing and assembly (14), the loss of HMGB2 may cause chromosomal pathological changes that result in misregulation of genes involved in tissue homeostasis and the aging process. The loss of HMGB2 in articular cartilage does not appear to be a general transcriptional suppression of *Hmgb2* expression, because it was still detected in certain parts of the synovium and bone marrow.

PRG4/SZP has a similar distribution compared with HMGB2 and is involved in cartilage and joint homeostasis (9, 10). In young *Hmgb2*$^{-/-}$ mice, there was no apparent reduction in PRG4/SZP expression. However, with advancing age, *Hmgb2*$^{-/-}$ mice showed a substantial or, in the nonmeniscus-covered weight-bearing regions of the knee joint, complete loss of PRG4/SZP expression. Joint aging in mice is associated with a reduction in HMGB2 expression, and this correlates with a reduction in expression of PRG4/SZP. Mice lacking *Prg4* have normal joint development; however, as the mice age, abnormal protein deposits on the cartilage surface occur and underlying SZ chondrocytes disappear (10). We observed a correlated reduction and loss of HMGB2 and PRG4/SZP expression in the SZ of articular cartilage but not in the synovium, suggesting that HMGB2 plays a unique role in cartilage SZ cells.

Several lines of evidence indicate that a major function of HMGB2 is to support SZ chondrocyte survival. The aging-related loss of HMGB2 expression in the cartilage surface was associated with a reduction in cellularity, and the aging-related reduction in cellularity was more profound in knee and temporomandibular joints from *Hmgb2*$^{-/-}$ mice (Fig. S5). This correlated with increased apoptosis in *Hmgb2*$^{-/-}$ mice. Our in vitro studies on chondrocytes from *Hmgb2*$^{-/-}$ mice demonstrate an increased susceptibility to apoptosis, as induced by antibody to the CD95/Fas receptor, and the SZ is more susceptible to cell death induced by mechanical stress (7). These findings suggest that chromatin factor HMGB2 may facilitate survival of SZ chondrocytes. The pathway may involve interactions of HMGB2 with other regulators of cell survival that are specific to the SZ. A role for HMGB proteins in protecting against cell death has also been suggested in polyglutamine diseases (38), Sertoli cells (18), and certain cancer cells (39).

Our results suggest that *Hmgb2*$^{-/-}$ mice represent a relevant model of OA. These mice not only develop early-onset OA, like many of the previously reported mutant strains of mice, but more importantly, feature a specific phenotype in the SZ of articular cartilage. This closely resembles human OA, which develops its first manifestations in the SZ (5). The earliest lesions we detected in articular cartilage of *Hmgb2*$^{-/-}$ mice were a reduction in cellularity and loss of glycosaminoglycans. This was also associated with increased expression of MMP-3 and MMP-13. Our mechanistic model proposes that HMGB2 regulates chondrocyte survival exclusively in the SZ, where HMGB2 is highly expressed. Aging-related loss of HMGB2 results in loss of SZ cells and deficient production of PRG4/SZP. This, in combination with increased expression of matrix-degrading enzymes, accelerates cartilage degradation.

In conclusion, we found that HMGB2 is specifically expressed in the SZ of mature human articular cartilage. HMGB2 is a critical factor for survival of SZ cells. The spontaneous aging-associated loss of HMGB2 is linked to the loss of PRG4/SZP and compromises the integrity of the cartilage surface. This represents a mechanistic model of one of the earliest events in OA pathogenesis. Preventing or restoring loss of HMGB2-expressing SZ chondrocytes has potential as a therapeutic approach toward chondroprotection.

Materials and Methods

Human Articular Cartilage. Human knee joints were obtained (with approval of the Scripps Human Subjects Committee) from tissue banks and graded according

to a modified Outerbridge scale (40); grades III–IV are considered as OA. Full-thickness normal and OA cartilage was used for DNA array analysis. Safranin O-stained sections of normal and osteoarthritic cartilage were graded according to Mankin (41): Mankin score 0, normal cartilage; Mankin score 1–4, mild osteoarthritic cartilage; and Mankin score ≥5, osteoarthritic cartilage (42).

Mice. *Hmgb2*$^{-/-}$ mutant mice used in this study were described previously (18). The *Hmgb2* coding sequence was replaced by homologous recombination in embryonic stem cells with the ble-LacZ coding sequence starting from the ATG transcription start site in exon 2. *Hmgb2* transcripts were completely absent in tissues from the mutant mice. Age- and gender-matched WT mice of the same parental C57BL/6J lineage were included in each experiment as controls. Experiments were performed with mice aged 0–12 months. All animal experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee at The Scripps Research Institute. Knee joints from C57BL/6J WT mice and *Hmgb2*$^{-/-}$ mice aged 4 months ($n = 7$ WT, $n = 8$ *Hmgb2*$^{-/-}$), 6 months ($n = 7$ each), 9 months ($n = 9$ WT, $n = 7$ *Hmgb2*$^{-/-}$), and 12 months ($n = 7$ each) were collected and processed as described (25), stained with safranin O-fast green, and examined for histopathological changes. Whole-mount Alcian blue and alizarin red S staining of skeletons was performed as described previously (17).

Grading of Histopathological Changes. Pathological changes in the knee joints were evaluated by using a modified Mankin scoring system (26). All quadrants of the joint (medial tibial plateau, medial femoral condyle, lateral tibial plateau, and lateral femoral condyle) were scored separately and averaged. The same slides were also graded by using a modification of a semiquantitative scoring system (28, 29). All quadrants of the joint were scored separately and expressed as the summed histological score, which represented the additive scores for each quadrant of the joint on each histological section through the joint. This method of analysis enabled assessment of the severity of lesions as well as reflecting the surface area of cartilage affected with OA lesions (28).

Immunohistochemistry and in Situ Hybridization. Immunohistochemistry was performed with rabbit anti-HMGB2 antibody (BD PharMingen) or chicken anti-HMGB1 antibody (Shino-Test), goat anti-MMP-3 antibody (Santa Cruz Biotechnology), and rabbit anti-MMP-13 antibody (Chemicon) as described (17, 25). Polyclonal antibody specific for the p85 fragment of PARP (Promega) was used to detect PARP cleavage (25). RNA in situ hybridization was performed as described (17). The probes for HMGB2 and PRG4/SZP were provided by Marco Bianchi (San Raffaele University, Milan) (18) and Matthew Warman (Harvard Medical School, Boston) (10), respectively.

Quantification of Cartilage Cellularity. Cartilage cellularity in C57BL/6J WT mice was quantified by counting the chondrocytes in a microscopic field (25). In normal cartilage from 4-, 6-, 9-, and 12-month-old mice, 3 pictures were taken under magnification ×400, representing 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 SZ chondrocytes and HMGB2-positive cells was counted in each section. The elongated and flattened cells located in the first 20% of full-thickness articular cartilage were counted as SZ cells (8).

Apoptosis Induction and Analysis in Vitro. Murine articular chondrocytes were prepared from hip and knee cartilage of 5-day-old C57BL/6J and *Hmgb2*$^{-/-}$ mice as described (30). Before reaching confluency, the cells were plated in 6-well plates at 5×10^5 cells per well and cultured in DMEM/F12 with 0.5% FBS for 24 h. Then, medium was replaced and the chondrocytes were stimulated with NA/LE hamster anti-mouse CD95 antibody (PharMingen) and proteasome inhibitor MG132 (Sigma) for 12–14 h. The cells were labeled with FITC-labeled annexin V (PharMingen) or propidium iodide (Sigma) as described (31, 43) and measured on a BD FACSCalibur (Becton Dickinson). For each condition, 15,000 events were collected.

Statistical Analysis. Results are expressed as mean ± SD. Statistical comparison between genotypes or treatment groups was performed with a 2-tailed Student's *t* test. Histologic scores were analyzed by using the Mann–Whitney rank sum test. *P* values less than 0.05 were considered significant.

ACKNOWLEDGMENTS. We thank Lilo Creighton, Jean Valbracht, and Jackie Quach for technical support. This study was supported by National Institutes of Health Grants AG007996, AG033409, and the Sam and Rose Stein Endowment Fund (to M.L.) and grants from the Arthritis National Research Foundation and Japan Orthopaedics and Traumatology Foundation, Inc., No. 179 (to N.T.).

1. Felson DT, *et al.* (1987) The prevalence of knee osteoarthritis in the elderly. The Framingham Osteoarthritis Study. *Arthritis Rheum* 30:914–918.
2. Jordan KM, *et al.* (2003) EULAR recommendations 2003: An evidence based approach to the management of knee osteoarthritis: Report of a Task Force of the Standing Committee for International Clinical Studies Including Therapeutic Trials (ESCISIT). *Ann Rheum Dis* 62:1145–1155.
3. Sharma L, Kapoor D (2007) *Osteoarthritis*, ed Moskowitz R (Wolters Kluwer, Philadelphia).
4. Bierma-Zeinstra SM, Koes BW (2007) Risk factors and prognostic factors of hip and knee osteoarthritis. *Nature Clinical Practice Rheumatology* 3:78–85.
5. Poole AR, Guilak F, Abramson S (2007) *Osteoarthritis*, ed Moskowitz R (Wolters Kluwer, Philadelphia).
6. Temple MM, *et al.* (2007) Age- and site-associated biomechanical weakening of human articular cartilage of the femoral condyle. *Osteoarthritis Cartilage* 15:1042–1052.
7. Otsuki S, *et al.* (2008) The effect of glycosaminoglycan loss on chondrocyte viability: A study on porcine cartilage explants. *Arthritis Rheum* 58:1076–1085.
8. Youn I, *et al.* (2006) Zonal variations in the three-dimensional morphology of the chondron measured in situ using confocal microscopy. *Osteoarthritis Cartilage* 14:889–897.
9. Flannery CR, *et al.* (1999) Articular cartilage superficial zone protein (SZP) is homologous to megakaryocyte stimulating factor precursor and is a multifunctional proteoglycan with potential growth-promoting, cytoprotective, and lubricating properties in cartilage metabolism. *Biochem Biophys Res Commun* 254:535–541.
10. Rhee DK, *et al.* (2005) The secreted glycoprotein lubricin protects cartilage surfaces and inhibits synovial cell overgrowth. *J Clin Invest* 115:622–631.
11. Hunziker EB, Quinn TM, Hauselmann HJ (2002) Quantitative structural organization of normal adult human articular cartilage. *Osteoarthritis Cartilage* 10:564–572.
12. Meachim G, Collins DH (1962) Cell counts of normal and osteoarthritic articular cartilage in relation to the uptake of sulphate (35SO4) in vitro. *Ann Rheum Dis* 21:45–50.
13. Muller S, Ronfani L, Bianchi ME (2004) Regulated expression and subcellular localization of HMGB1, a chromatin protein with a cytokine function. *J Intern Med* 255:332–343.
14. Agresti A, Bianchi ME (2003) HMGB proteins and gene expression. *Curr Opin Genet Dev* 13:170–178.
15. Palumbo R, *et al.* (2004) Extracellular HMGB1, a signal of tissue damage, induces mesoangioblast migration and proliferation. *J Cell Biol* 164:441–449.
16. Taniguchi N, *et al.* (2003) High mobility group box chromosomal protein 1 plays a role in the pathogenesis of rheumatoid arthritis as a novel cytokine. *Arthritis Rheum* 48:971–981.
17. Taniguchi N, *et al.* (2007) Stage-specific secretion of HMGB1 in cartilage regulates endochondral ossification. *Mol Cell Biol* 27:5650–5663.
18. Ronfani L, *et al.* (2001) Reduced fertility and spermatogenesis defects in mice lacking chromosomal protein Hmgb2. *Development* 128:1265–1273.
19. Zappavigna V, *et al.* (1996) HMG1 interacts with HOX proteins and enhances their DNA binding and transcriptional activation. *EMBO J* 15:4981–4991.
20. Boonyaratanakornkit V, *et al.* (1998) High-mobility group chromatin proteins 1 and 2 functionally interact with steroid hormone receptors to enhance their DNA binding in vitro and transcriptional activity in mammalian cells. *Mol Cell Biol* 18:4471–4487.
21. Aidinis V, *et al.* (1999) The RAG1 homeodomain recruits HMG1 and HMG2 to facilitate recombination signal sequence binding and to enhance the intrinsic DNA-bending activity of RAG1-RAG2. *Mol Cell Biol* 19:6532–6542.
22. Calogero S, *et al.* (1999) The lack of chromosomal protein Hmg1 does not disrupt cell growth but causes lethal hypoglycaemia in newborn mice. *Nat Genet* 22:276–280.
23. Hiraoka K, Grogan S, Olee T, Lotz M (2006) Mesenchymal progenitor cells in adult human articular cartilage. *Biorheology* 43:447–454.
24. Wang X, *et al.* (2004) Regulation of MMP-13 expression by RUNX2 and FGF2 in osteoarthritic cartilage. *Osteoarthritis Cartilage* 12:963–973.
25. Zemmyo M, *et al.* (2003) Accelerated, aging-dependent development of osteoarthritis in alpha1 integrin-deficient mice. *Arthritis Rheum* 48:2873–2880.
26. Xu L, *et al.* (2003) Osteoarthritis-like changes and decreased mechanical function of articular cartilage in the joints of mice with the chondrodysplasia gene (cho). *Arthritis Rheum* 48:2509–2518.
27. Stanescu R, Knyszynski A, Muriel MP, Stanescu V (1993) Early lesions of the articular surface in a strain of mice with very high incidence of spontaneous osteoarthritic-like lesions. *J Rheumatol* 20:102–110.
28. Glasson SS, *et al.* (2005) Deletion of active ADAMTS5 prevents cartilage degradation in a murine model of osteoarthritis. *Nature* 434:644–648.
29. Glasson SS, *et al.* (2004) Characterization of and osteoarthritis susceptibility in ADAMTS-4-knockout mice. *Arthritis Rheum* 50:2547–2558.
30. Salvat C, *et al.* (2005) Immature murine articular chondrocytes in primary culture: A new tool for investigating cartilage. *Osteoarthritis Cartilage* 13:243–249.
31. Kuhn K, Lotz M (2001) Regulation of CD95 (Fas/APO-1)-induced apoptosis in human chondrocytes. *Arthritis Rheum* 44:1644–1653.
32. Alsalameh S, Amin R, Gemba T, Lotz M (2004) Identification of mesenchymal progenitor cells in normal and osteoarthritic human articular cartilage. *Arthritis Rheum* 50:1522–1532.
33. Dowthwaite GP, *et al.* (2004) The surface of articular cartilage contains a progenitor cell population. *J Cell Sci* 117:889–897.
34. Prasad S, Thakur MK (1990) Effects of spermine and sodium butyrate on the in vitro phosphorylation of HMG nonhistone proteins of the liver of young and old rats. *Arch Gerontol Geriatr* 10:231–238.
35. Thakur MK, Prasad S (1990) ADP-ribosylation of HMG proteins and its modulation by different effectors in the liver of aging rats. *Mech Ageing Dev* 53:91–100.
36. Prasad S, Thakur MK (1991) Differential methylation of HMG proteins by dexamethasone in the liver of aging rats. *Aging (Milano)* 3:333–335.
37. Ly DH, Lockhart DJ, Lerner RA, Schultz PG (2000) Mitotic misregulation and human aging. *Science* 287:2486–2492.
38. Qi ML, *et al.* (2007) Proteome analysis of soluble nuclear proteins reveals that HMGB1/2 suppress genotoxic stress in polyglutamine diseases. *Nat Cell Biol* 9:402–414.
39. Brezniceanu ML, *et al.* (2003) HMGB1 inhibits cell death in yeast and mammalian cells and is abundantly expressed in human breast carcinoma. *FASEB J* 17:1295–1297.
40. Yamada K, *et al.* (2002) Subchondral bone of the human knee joint in aging and osteoarthritis. *Osteoarthritis Cartilage* 10:360–369.
41. Mankin HJ, Dorfman H, Lippiello L, Zarins A (1971) Biochemical and metabolic abnormalities in articular cartilage from osteo-arthritic human hips. II. Correlation of morphology with biochemical and metabolic data. *J Bone Jt Surg Am Vol* 53:523–537.
42. Pfander D, *et al.* (1999) Hepatocyte growth factor in human osteoarthritic cartilage. *Osteoarthritis Cartilage* 7:548–559.
43. Carames B, *et al.* (2008) Differential effects of tumor necrosis factor-alpha and interleukin-1beta on cell death in human articular chondrocytes. *Osteoarthritis Cartilage* 16:715–722.