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Restoration of the Extracellular Matrix in Human Osteoarthritic Articular Cartilage by Overexpression of the Transcription Factor *SOX9*

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Objective. **Human osteoarthritis (OA) is characterized by a pathologic shift in articular cartilage homeostasis toward the progressive loss of extracellular matrix (ECM). The purpose of this study was to investigate the ability of rAAV-mediated *SOX9* overexpression to restore major ECM components in human OA articular cartilage.**

Methods. **We monitored the synthesis and content of proteoglycans and type II collagen in 3-dimensional cultures of human normal and OA articular chondrocytes and in explant cultures of human normal and OA articular cartilage following direct application of a recombinant adeno-associated virus (rAAV) *SOX9* vector in vitro and in situ. We also analyzed the effects of this treatment on cell proliferation in these systems.**

Results. **Following *SOX9* gene transfer, expression levels of proteoglycans and type II collagen increased over time in normal and OA articular chondrocytes in vitro. In situ, overexpression of *SOX9* in normal and OA articular cartilage stimulated proteoglycan and type II collagen synthesis in a dose-dependent manner. These effects were not associated with changes in chondrocyte proliferation. Notably, expression of the 2 principal matrix components could be restored in OA articular cartilage to levels similar to those in normal cartilage.**

Conclusion. **These data support the concept of using direct, rAAV-mediated transfer of chondrogenic genes to articular cartilage for the treatment of OA in humans.**

Osteoarthritis (OA) is a progressive disease that affects diarthrodial joints and is mainly characterized by a gradual deterioration of the articular cartilage. A disturbed balance in cartilage metabolism is thought to play an important role in the pathogenesis of OA and to be a key factor in determining its progression. Over the course of OA, the cartilage loses major components of its extracellular matrix (ECM), such as proteoglycans and type II collagen (1). Proinflammatory cytokines, such as interleukin 1 (IL-1) and tumor necrosis factor α, produced locally by the inflamed synovium likely contribute to the pathophysiology of OA (2). Articular chondrocytes are the focus of OA because of pathologic changes in their gene expression pattern (3), the loss of their capacity to synthesize cartilage-specific matrix molecules, and their increased production of matrix-degrading enzymes (4).

Despite various therapeutic options, including systemic nonsteroidal antiinflammatory drugs, local corticosteroids, physical therapy, regular exercise, use of orthopedic appliances, or with the advent of disease- and structure-modifying drugs, the management of OA remains an unresolved problem, especially for patients who are too young to undergo endoprosthetic total joint replacement. The difficulty in treating OA is largely due to its slow and irreversible progression and to the limited intrinsic ability of the cartilage to reequilibrate its natural components. Application of therapeutic genes to OA cartilage may offer potent alternatives for reestab-

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lishing the structural integrity of the damaged cartilage architecture.

Most of the current approaches to restoring the physiologic balance in injured articular cartilage are based on the delivery of factors that modulate the metabolic functions of chondrocytes, such as agents that counteract the processes of matrix degradation or ones that enhance the synthesis of matrix components. Protective effects of an IL-1 receptor antagonist (IL-1Ra) sequence against IL-1–induced cartilage breakdown have been documented in experimental models ex vivo (5,6) and in vivo (7,8). The transfer of the gene for a heat-shock protein (Hsp70) was also shown to afford protection against cellular injuries in chondrocytes (9). Alternatively, the delivery of sequences encoding for growth and enzymatic factors can potentially stimulate cartilage anabolism in vitro and in situ, such as insulin-like growth factor 1 (IGF-1) (10), fibroblast growth factor 2 (FGF-2) (11), bone morphogenetic protein 7 (BMP-7) (12), transforming growth factor β (TGFβ) (13), and glucuronosyltransferase I (14). However, application of external stimuli to damaged articular cartilage has not yet proved sufficient in fully reestablishing an original cartilage surface, and little is known about the effects on human OA cartilage. Other avenues of research may thus have value in the identification of supplementary treatments for OA.

Strategies to correct the altered gene expression patterns in OA chondrocytes may prove beneficial in readjusting the disturbed cartilage homeostasis. Transcription factors are key regulators of cartilage metabolism since they stimulate chondrogenesis in physiologic and pathologic conditions. Among them, SOX9, a member of the sex-determining region Y–type high mobility group box family of DNA binding proteins, plays critical roles in the regulation of skeletal and cartilage formation (15) and chondrocyte differentiation (16). *SOX9* is expressed during embryonic development in a pattern that closely parallels that of cartilage matrix synthesis (17) and exerts its properties by activating the gene for type II collagen and other cartilage-specific genes (17–20). Notably, the levels of *SOX9* expression decline in OA cartilage (21,22). Modulation of the chondrocyte phenotype in OA cartilage by genetically modifying the levels of intracellular *SOX9* expression might be advantageous in shifting the disrupted balance toward the synthesis of ECM components and contribute to the reproduction of an original articular cartilage surface.

A prerequisite for the development of an applicable gene treatment against OA is the ability of the gene vehicle to mediate the efficient and sustained expression of a candidate agent in order to counterbalance the progression of the disease. Recombinant adeno-associated virus (rAAV) vectors are particularly well suited for this purpose because they can transfer genes into human OA chondrocytes in vitro and in situ with high efficiencies and for extended periods of time (23–25). The rAAV vectors are derived from a replication-defective human parvovirus that is nonpathogenic in humans. The rAAV can transduce both dividing and nondividing cells, such as chondrocytes, and they drive transgene expression from highly stable episomes, which can persist for months to years (26). In addition, rAAV exhibit a reduced immunogenicity due to the complete removal of the viral protein coding sequences in the recombinant genome. These features are in marked contrast with the properties of other classes of vectors (27), such as retroviral vectors, which necessitate the division of the target cells (28), or adenoviral vectors, which generally mediate only short-term transgene expression (29).

In the present study, we tested the hypothesis that *SOX9* overexpression via rAAV promotes the synthesis of proteoglycans and type II collagen in human OA chondrocytes in vitro and in cartilage explants in situ. We also evaluated whether application of the *SOX9* vector restores the cartilage matrix in human OA cartilage as compared with normal cartilage.

MATERIALS AND METHODS

Reagents. All reagents were obtained from Sigma (Munich, Germany), except for dimethylmethylene blue dye, which was obtained from Serva (Heidelberg, Germany). Anti–β-galactosidase (anti–β-gal; GAL-13) and anti-FLAG (BioM2) antibodies were from Sigma, anti-SOX9 antibody (C-20) was from Santa Cruz Biotechnology (Heidelberg, Germany), and anti–type II collagen antibody (AF-5710) was from Acris (Hiddenhausen, Germany). Type II collagen contents were measured with an Arthrogen-CIA native type II collagen capture enzyme-linked immunosorbent assay (ELISA) kit (Chondrex, Redmond, WA).

Cartilage and cells. Normal articular cartilage was obtained from unaffected knee joints that were removed during tumor surgery in patients ages 67–72 years. OA was excluded by analysis of Safranin O–stained sections according to the Mankin scale (Mankin score 1–2) (30). OA cartilage (Mankin score 7–9) was obtained from joints undergoing total knee arthroplasty in patients ages 65–78 years. All patients provided informed consent prior to inclusion in the study. Explant cultures and chondrocytes were prepared as previously described (11,24).

Plasmids and rAAV vectors. Plasmid pACP is an AAV-2–derived vector plasmid from the genomic clone pSSV9, and pAd8 contains the AAV-2 replication and encapsidation functions (31). The rAAV-LacZ plasmid is an

AAV-2–based vector plasmid carrying the *lacZ* gene that encodes β-gal under the control of the cytomegalovirus immediate-early (CMV-IE) promoter (24,25,32). A human *SOX9* (h*SOX9*) complementary DNA (cDNA; 1.7 kb) obtained from G. Scherer (Institute for Human Genetics and Anthropology, Albert-Ludwig University, Freiburg, Germany) (33) was modified to incorporate a FLAG tag on its 5′ end. The h*SOX9* fragment was first extracted from pcDNA3-SOX9 (33) by *Xho* I and *Xba* I and recloned in pGEM-11Zf(+) (Promega, Mannheim, Germany). An h*SOX9* fragment was extracted from the resulting plasmid by *Sal* I and *Hind* III and cloned in pACP. The 5′ end of the resulting h*SOX9* sequence was removed by *Pst* I and *Hind* III and replaced by a polymerase chain reaction (PCR) fragment containing the 5′ end of h*SOX9*, where a FLAG tag sequence was incorporated by PCR using the following primers: 5′ F-SOX9 primer AAAAATCTAGA(*Xba* I)AAAAGACCCAAGCTGGCTAGCCACCATGGACTACAAAGACGATGACGACAAGAATCTCCTGGACCCCTTCATGAAGATGACCG and 3′ F-SOX9 primer AAAAACTGCAG(*Pst* I)CGCCTTGAAGATGGCGTT. The resulting plasmid vector is rAAV-FLAG-hSOX9, where the presence of the FLAG-hSOX9 fragment was confirmed by sequencing. The rAAV vectors were packaged using adenovirus 5 to provide helper functions in combination with pAd8 (24,25,32). Vector preparations were purified by dialysis (24,25,32) and titered by real-time PCR (24,25,32), averaging 10^{10} functional units/ml.

Gene transfer. Chondrocytes (10^6) were transduced with rAAV (300 μl) for 2 days and encapsulated in alginate spheres that were maintained in culture for 26 days to monitor cell number and viability (25). Explant cultures were transduced by direct application of rAAV to the surface of the samples (24) and maintained in culture for 10 days.

Analysis of transgene expression. The activity of β-gal was detected by X-Gal staining to determine the transduction efficiencies (24,25,32,34). Transgene expression was also monitored by indirect immunostaining (25). Transduced explant cultures were processed for Western blot analysis using the same amount of proteins (14 μg) (35). Expression was revealed with the use of specific antibodies, horseradish peroxidase–labeled secondary antibodies (Vector, Grünberg, Germany), and the ECL Advance Western blotting detection kit (Amersham Biosciences, Freiburg, Germany).

Histologic and immunohistochemical analyses. Spheres and explant cultures were processed, and paraffin-embedded sections (5 μm) were stained with Safranin O to detect proteoglycans and with hematoxylin and eosin (H&E) to detect cells (11,24,25). Type II collagen was detected with a specific antibody, a biotinylated secondary antibody (Vector), and the avidin−biotin−peroxidase method (Vector) using diaminobenzidine as the chromogen. Samples were examined under light microscopy (Olympus BX45 microscope; Hamburg, Germany).

Morphometric analyses. Safranin O staining intensities and cell densities were measured at 3 standardized sites using SIS AnalySIS (Olympus), Adobe Photoshop (Adobe Systems, Unterschleissheim, Germany), and Scion Image (Scion, Frederick, MD) software packages. The percentage of Safranin O staining intensity was calculated as the ratio of positively stained tissue surface to the total surface of the site evaluated.

Determination of the type II collagen, proteoglycan, and DNA contents. Solubilized spheres and explant cultures were digested (25,36) and the type II collagen contents were determined by ELISA. The proteoglycan contents were detected by binding to dimethylmethylene blue dye (25). The DNA contents were monitored using Hoechst 33258 dye (25). Measurements were performed with a GENios spectrophotometer/fluorometer (Tecan, Crailsheim, Germany).

Statistical analysis. Each condition was performed in triplicate in 3 independent experiments with the spheres and in triplicate in 2 independent experiments with the explant cultures. Data were obtained by 2 individuals (MC and TT) who were blinded with regard to the treatment groups. Values are expressed as the mean ± SD. The *t*-test and Mann-Whitney rank sum test were used where appropriate. *P* values less than 0.05 were considered statistically significant.

RESULTS

Expression of a *SOX9* gene cassette in human articular chondrocytes by rAAV transduction in vitro. The candidate rAAV-FLAG-hSOX9 and control rAAV-LacZ vectors were first applied to primary cultures of isolated normal and OA articular chondrocytes. The rAAV-FLAG-hSOX9 vector carries a human *SOX9* cDNA tagged by a FLAG sequence to discriminate between the endogenous *SOX9* (18,22) and the transgene. Chondrocytes were next encapsulated in alginate spheres and maintained in culture for 26 days. On the day of encapsulation, β-gal activity, restricted to the control chondrocytes, was already detected and remained present for at least 26 days (results not shown). Transduction efficiencies were between 76% and 80%, consistent with previous data using rAAV (24,25). In contrast, the FLAG tag was detected only in the rAAV-FLAG-hSOX9–treated chondrocytes (Figure 1). In contrast, *SOX9* expression was observed in normal and OA chondrocytes transduced by either vector (results not shown), which is consistent with the endogenous *SOX9* expression in chondrocytes (18).

Stimulation of type II collagen and proteoglycan synthesis in human articular chondrocytes by *SOX9* overexpression. On the day of encapsulation, type II collagen immunostaining was present in the rAAV-FLAG-hSOX9–transduced and rAAV-LacZ–transduced chondrocytes (results not shown), extending beyond the cell-associated matrix (25). After 26 days, the immunoreactivity was stronger in the treated chondrocytes (Figure 2). As expected (37), the staining was less intense in OA chondrocytes than in normal chondrocytes, but it was stronger in rAAV-FLAG-hSOX–treated OA chondrocytes than in rAAV-LacZ–treated control normal chondrocytes.

Chondrocytes transduced with rAAV-FLAG-hSOX9 and those transduced with rAAV-LacZ had similar type II collagen contents on the day of encapsulation ($P = 0.705$ for normal and $P = 0.168$ for OA chondrocytes) (Table 1). After 26 days, the contents increased significantly in the rAAV-FLAG-hSOX9–treated chondrocytes (3.93-fold in normal [$P < 0.001$] and 3.30-fold in OA [$P = 0.029$] chondrocytes), whereas the contents did not vary in the rAAV-LacZ–treated control chondrocytes ($P = 0.686$ for normal and $P = 0.886$ for OA chondrocytes). OA chondrocytes always contained less type II collagen than did their normal counterparts. Remarkably, the contents were significantly higher in the treated OA chondrocytes than in the control normal chondrocytes (2.20-fold; $P < 0.001$).

There was no difference in the proteoglycan contents of the rAAV-FLAG-hSOX9–transduced and rAAV-LacZ–transduced chondrocytes on the day of encapsulation ($P = 0.940$ for normal and $P = 0.844$ for OA chondrocytes) (Table 1). After 26 days, the amounts of proteoglycans significantly increased in the treated chondrocytes (1.73-fold in normal [$P = 0.029$] and 1.70-fold in OA [$P < 0.001$] chondrocytes), whereas they remained unchanged in the control chondrocytes ($P = 0.939$ for normal and $P = 0.912$ for OA chondrocytes). OA chondrocytes always contained less proteoglycans than the normal counterparts, as has previously been reported (38). Notably, the contents were significantly higher in treated OA chondrocytes than in control normal chondrocytes (1.62-fold; $P < 0.001$).

In contrast to the data for the ECM, the number of cells in rAAV-FLAG-hSOX9–transduced and rAAV-LacZ–transduced spheres were not different at any time point of the analysis ($P = 0.775$ on day 1 and $P = 1.000$ on day 26 in normal chondrocytes; $P = 0.851$ on day 1 and $P = 1.000$ on day 26 in OA chondrocytes) (Table 1). Over time, a decrease in the cell numbers (for treated spheres, 2.33-fold [$P < 0.001$] in normal and 2.20-fold [$P = 0.029$] in OA chondrocytes; for control spheres, 2.37-fold [$P < 0.001$] in normal and 2.12-fold [$P = 0.029$] in OA chondrocytes) and cell viability (from an overall mean ± SD of 82 ± 3% on day 1 to 35 ± 2% on day 26)

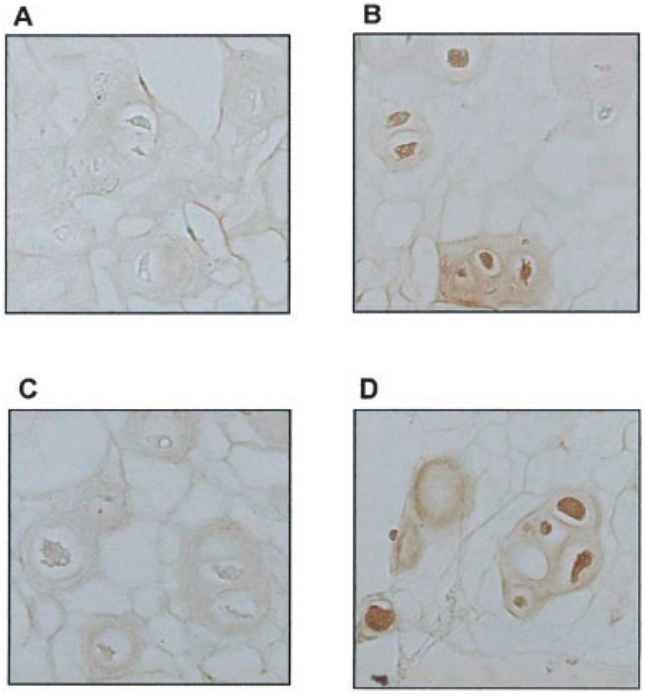

Figure 1. Detection of transgene expression in transduced chondrocytes. Normal (**A** and **B**) and osteoarthritic (**C** and **D**) chondrocytes were transduced with rAAV-LacZ (**A** and **C**) or rAAV-FLAG-hSOX9 (**B** and **D**), and transduced spheres (n = 9 per condition) were processed after 26 days to detect the FLAG tag (1:200 dilution). (Original magnification ×100.) Color figure can be viewed in the online issue, which is available at http://www.arthritisrheum.org

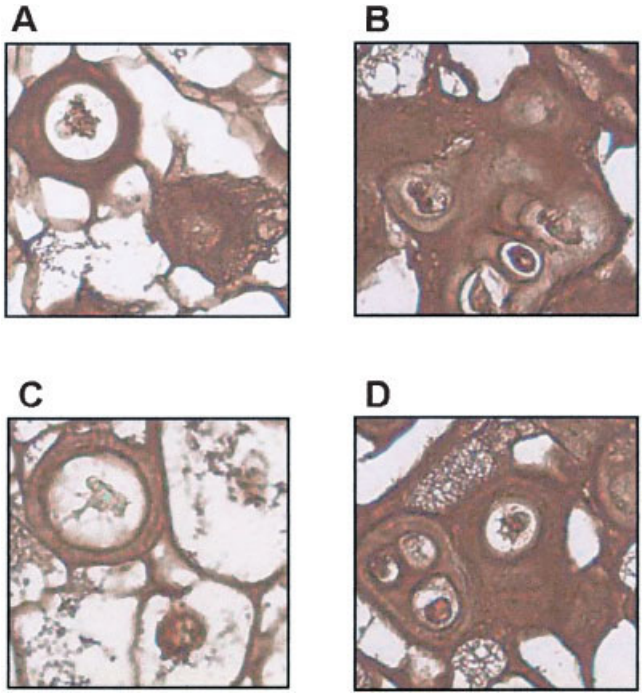

Figure 2. Immunohistochemical detection of type II collagen in transduced chondrocytes. Normal (**A** and **B**) and osteoarthritic (**C** and **D**) chondrocytes were transduced with rAAV-LacZ (**A** and **C**) or rAAV-FLAG-hSOX9 (**B** and **D**), and transduced spheres (n = 9 per condition) were processed after 26 days to detect type II collagen (1:30 dilution). (Original magnification × 200.) Color figure can be viewed in the online issue, which is available at http://www.arthritisrheum.org

Table 1. Type II collagen, proteoglycan, cell, and DNA contents in transduced chondrocytes

	Normal chondrocytes, mean ± SD		Osteoarthritic chondrocytes, mean ± SD	
	rAAV-LacZ	rAAV-FLAG-hSOX9	rAAV-LacZ	rAAV-FLAG-hSOX9
Type II collagen, ng/10^4 cells				
Day 1	0.15 ± 0.01	0.15 ± 0.01	0.09 ± 0.01	0.10 ± 0.01
Day 26	0.15 ± 0.01	0.59 ± 0.02*	0.10 ± 0.01	0.33 ± 0.02*
Proteoglycans, μg/10^4 cells				
Day 1	4.41 ± 0.09	4.41 ± 0.09	4.00 ± 0.02	4.01 ± 0.01
Day 26	4.20 ± 0.01	7.62 ± 0.02*	3.78 ± 0.01	6.82 ± 0.02*
Cells, $\times 10^4$/sphere				
Day 1	0.64 ± 0.02	0.63 ± 0.03	0.53 ± 0.07	0.55 ± 0.10
Day 26	0.27 ± 0.04	0.27 ± 0.05	0.25 ± 0.05	0.25 ± 0.04
DNA, μg/10^4 cells				
Day 1	0.31 ± 0.01	0.31 ± 0.01	0.28 ± 0.01	0.28 ± 0.01
Day 26	0.21 ± 0.01	0.21 ± 0.01	0.20 ± 0.01	0.20 ± 0.01

* Significant treatment effect compared with rAAV-LacZ.

was noted in both types of spheres. Consistent with this finding, there was no difference in the cell densities on H&E-stained histology sections of tissues treated with the spheres at all time points analyzed, and a decrease in the densities was seen over time (data not shown). The DNA contents were similar at all time points analyzed ($P = 1.000$ on days 1 and 26 in normal and $P = 0.705$ on day 1 and $P = 0.670$ on day 26 in OA chondrocytes) (Table 1), and they decreased over time in both types of spheres (1.48-fold [$P < 0.001$] in normal and 1.40-fold [$P < 0.001$] in OA chondrocytes).

Expression of the *SOX9* gene cassette in human articular cartilage by direct rAAV application in situ. In order to evaluate the effects of *SOX9* overexpression on chondrocyte metabolism in a native environment, the vectors were directly applied to normal and OA cartilage explant cultures. As early as 5 days after vector application, β-gal activity, restricted to the control cartilage, was detected both in the superficial and middle zones and remained present for at least 10 days (results not shown). Transduction efficiencies were dose-dependent, increasing from a mean ± SD of 45.3 ± 0.5% with 20 μl of vector to 58.0 ± 0.8% with 50 μl of vector in normal cartilage (1.28-fold; $P < 0.001$) and from 44.5 ± 0.6% to 57.5 ± 0.6% with 20 μl and 50 μl of vector in OA cartilage (1.29-fold; $P = 0.029$). In contrast, the FLAG tag was detected only in rAAV-FLAG-hSOX9–transduced treated cartilage (results not shown). *SOX9* expression, however, was observed in normal and OA explant cultures where either vector was applied (Figure 3), which is consistent with the previously reported results of *SOX9* expression in cartilage (22). However, marked differences were noted depending on the vector applied and the type of cartilage evaluated.

The percentage of cells immunoreactive for the

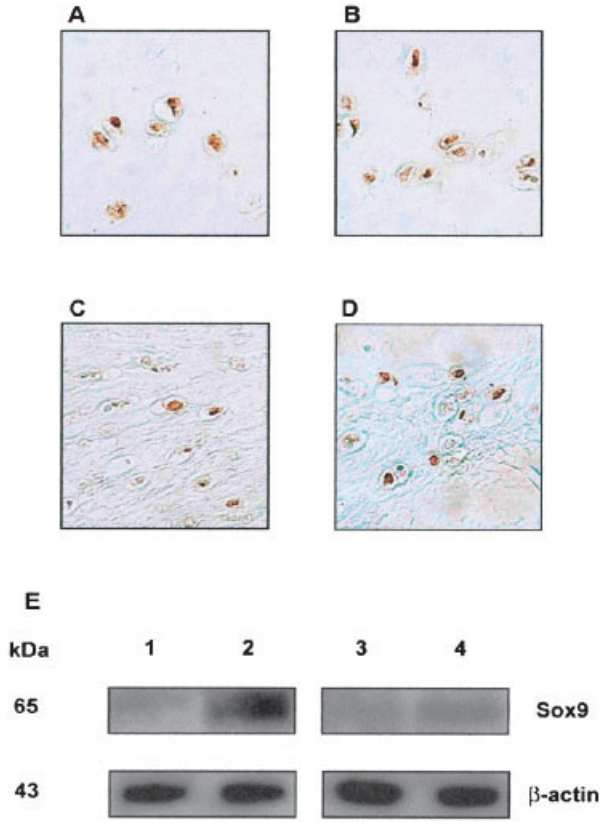

Figure 3. Detection of transgene expression in transduced cartilage. Normal (**A** and **B**) and osteoarthritic (**C** and **D**) cartilage explant cultures were transduced with 50 μl of rAAV-LacZ (**A** and **C**) or rAAV-FLAG-hSOX9 (**B** and **D**) and processed after 10 days (n = 6 per condition) to detect SOX9 (1:300 dilution). Shown are the middle zones of the cartilage samples. (Original magnification × 100.) *SOX9* expression in cartilage was also analyzed by Western blotting (**E**) of rAAV-LacZ–transduced normal cartilage (lane 1), rAAV-FLAG-hSOX9–transduced normal cartilage (lane 2), rAAV-LacZ–transduced osteoarthritic cartilage (lane 3), and rAAV-FLAG-hSOX9–transduced osteoarthritic cartilage (lane 4). Molecular weight markers are shown at the left. Color figure can be viewed in the online issue, which is available at http://www.arthritisrheum.org

endogenous SOX9 was significantly lower in control OA cartilage than in normal cartilage (mean ± SD 44.8 ± 0.5% versus 66.6 ± 0.5%; 1.49-fold increase [$P = 0.029$]), confirming the previously reported decrease in *SOX9* expression in OA cartilage (21,22). Application of rAAV-FLAG-hSOX9 resulted in a significant increase in the percentages of cells immunoreactive for SOX9 as compared with control cartilage due to overexpression of the transgene (from 66.6 ± 0.5% to 81.7 ± 0.5%; 1.23-fold [$P < 0.001$] in control and from 44.8 ± 0.8% to 65.6 ± 1.7%; 1.46-fold [$P < 0.001$] in OA cartilage). Importantly, expression of the transgene in OA cartilage restored the number of SOX9-positive chondrocytes to levels seen in the control normal cartilage (65.6 ± 1.7% and 66.6 ± 0.5%; $P = 1.000$).

Western blot analysis of protein extracts from normal cartilage transduced by either rAAV demonstrated a single primary SOX9-immunoreactive band of ~65 kd (Figure 3E) that was ~5 times more intense in the treated cartilage than in the control cartilage due to overexpression of the transgene. Similar results were obtained in OA cartilage, where the band was ~3 times more intense in the treated cartilage. Notably, the intensity of the immunoreactive band in the treated OA cartilage was similar to that seen in the control normal cartilage.

Stimulation of type II collagen synthesis in human articular cartilage by *SOX9* overexpression. Consistent with previous findings (39), immunoreactivity to type II collagen was uniformly distributed in control normal cartilage, whereas typical pericellular deposits of type II collagen, representative of an active synthesis, were noted in the OA counterpart. Following treatment with rAAV-FLAG-hSOX9, areas of synthesis became evident in the superficial and middle zones of normal cartilage, most particularly when the higher dose of vector was applied (Figure 4). When this vector was administered to OA cartilage, the deposits observed in the control counterpart became even more frequent.

A significant, dose-dependent increase in the type II collagen content was noted after treatment with rAAV-FLAG-hSOX9 (Table 2). In normal cartilage, the content increased 1.76-fold ($P < 0.001$) with 20 μl of vector and increased 2.54-fold ($P < 0.001$) with 50 μl as compared with control treatment (a 1.65-fold dose-dependent increase; $P < 0.001$). In OA cartilage, the content increased 1.72-fold ($P < 0.001$) with 20 μl of vector and 1.95-fold ($P < 0.001$) with 50 μl (a 1.32-fold dose-dependent increase; $P < 0.001$). OA cartilage always contained less type II collagen than did normal cartilage, as has been described previously (40,41). Most notably, the content was significantly higher in treated OA cartilage than in control normal cartilage (1.48-fold [$P < 0.001$] with 20 μl and 1.71-fold [$P < 0.001$] with 50 μl of vector), probably due to restored *SOX9* expression levels (see Figure 3).

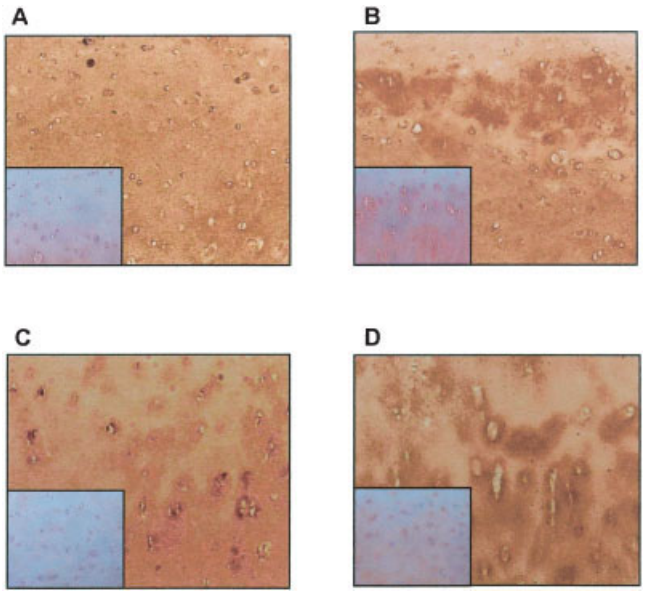

Figure 4. Immunohistochemical detection of type II collagen and Safranin O and hematoxylin and eosin (H&E) staining in transduced cartilage. Normal (**A** and **B**) and osteoarthritic (**C** and **D**) cartilage explant cultures were transduced with 50 μl of rAAV-LacZ (**A** and **C**) or rAAV-FLAG-hSOX9 (**B** and **D**) and processed after 10 days (n = 6 per condition) to detect type II collagen (1:50 dilution) and for staining with Safranin O and H&E (**insets**). Shown are the middle zones of the cartilage samples. (Original magnification × 40.)

Stimulation of proteoglycan synthesis in human articular cartilage by *SOX9* overexpression. The intensity of Safranin O staining was significantly higher in the superficial and middle zones of the cartilage to which rAAV-FLAG-hSOX9 was applied (see insets in Figure 4). In normal cartilage, the percentage increased 1.16-fold (from a mean ± SD of 47.5 ± 0.6% to 55.0 ± 0.8%; $P < 0.001$) with 20 μl of vector and increased 1.51-fold (from 47.3 ± 1.0% to 71.3 ± 1.0%; $P < 0.001$) with 50 μl of vector as compared with control treatment (a 1.30-fold dose-dependent increase; $P < 0.001$). In OA cartilage, the percentage increased 1.13-fold (from 31.3 ± 1.0% to 35.5 ± 0.6%; $P < 0.001$) with 20 μl of vector and increased 1.51-fold (from 31.3 ± 1.0% to 47.3 ± 1.0%; $P < 0.001$) with 50 μl (a 1.33-fold dose-dependent increase; $P < 0.001$). The staining intensity was always less in OA cartilage than in normal cartilage, as previously reported (42). Importantly, the

Table 2. Type II collagen, proteoglycan, and DNA content in transduced cartilage

	Normal cartilage, mean ± SD				Osteoarthritic cartilage, mean ± SD			
	rAAV-LacZ		rAAV-FLAG-hSOX9		rAAV-LacZ		rAAV-FLAG-hSOX9	
	20 μl	50 μl	20 μl	50 μl	20 μl	50 μl	20 μl	50 μl
Type II collagen, ng/mg dry weight	0.021 ± 0.001	0.024 ± 0.001	0.037 ± 0.001*	0.061 ± 0.001*	0.018 ± 0.001	0.021 ± 0.001	0.031 ± 0.001*	0.041 ± 0.001*
Proteoglycans, μg/mg dry weight	2.67 ± 0.01	2.67 ± 0.01	2.82 ± 0.01*	3.20 ± 0.01*	2.46 ± 0.01	2.48 ± 0.01	2.58 ± 0.01*	2.67 ± 0.01*
DNA, μg/mg dry weight	1.31 ± 0.01	1.31 ± 0.01	1.31 ± 0.01	1.31 ± 0.01	1.25 ± 0.01	1.26 ± 0.01	1.26 ± 0.01	1.25 ± 0.01

* Significant treatment effect compared with rAAV-LacZ.

staining intensity in the treated OA cartilage using the higher dose of vector was similar to that in the control normal cartilage ($P = 0.670$).

Treatment with rAAV-FLAG-hSOX9 induced a significant, dose-dependent increase in the proteoglycan content of the cartilage (Table 2). In normal cartilage, the content increased 1.06-fold ($P < 0.001$) with 20 μl of vector and increased 1.20-fold ($P < 0.001$) with 50 μl of vector as compared with control treatment (a 1.13-fold dose-dependent increase; $P < 0.001$). In OA cartilage, the values increased 1.05-fold ($P < 0.001$) with 20 μl and 1.08-fold ($P < 0.001$) with 50 μl (a 1.03-fold dose-dependent increase; $P < 0.001$) of vector. OA cartilage always contained less proteoglycans than did normal cartilage, which is consistent with previous findings (41). Most important, the content in the OA cartilage treated with the higher dose of vector was similar to that in the control normal cartilage ($P = 0.278$), probably due to restored levels of *SOX9* expression (see Figure 3).

Lack of effect of *SOX9* overexpression on chondrocyte proliferation in human articular cartilage. There was no difference in the cell densities of the rAAV-FLAG-hSOX9–transduced and the rAAV-LacZ–transduced cartilage samples (mean ± SD 288 ± 5 versus 287 ± 7 cells/mm^2 with 20 μl [$P = 0.869$] and 266 ± 1 versus 267 ± 2 cells/mm^2 with 50 μl [$P = 0.390$] of vector in normal cartilage; 286 ± 5 versus 286 ± 6 cells/mm^2 with 20 μl [$P = 0.903$] and 286 ± 6 versus 286 ± 5 cells/mm^2 with 50 μl [$P = 1.000$] of vector in OA cartilage) (Figure 4, insets). Increasing the dose of the candidate vector had no effect on the cell densities ($P = 0.912$ in normal and $P = 0.954$ in OA cartilage). Application of rAAV-FLAG-hSOX9 did not affect the DNA content in normal ($P = 0.670$ with 20 μl and $P = 1.000$ with 50 μl of vector) or OA ($P = 0.168$ with 20 μl and $P = 0.168$ with 50 μl of vector) cartilage (Table 2). Increasing the dose of the vector had no effect on the content ($P = 0.670$ in normal and $P = 0.168$ in OA cartilage).

DISCUSSION

Application of candidate therapeutic genes that restore the ECM in OA articular cartilage is an attractive approach to balancing the progression of the disease. In this regard, the effects of external stimuli provided by the expression of potent chondroprotective and chondroregenerative factors, such as IL-1Ra or IGF-1, have been widely evaluated (6,10,11), but complete reproduction of an original articular cartilage surface has not been achieved thus far with the use of these agents. Additional treatments might be required to expand the processes of regeneration in OA cartilage, such as those based on the use of factors that regulate chondrogenesis. Transcription factors are good candidates because they have the potential to correct the phenotype of OA chondrocytes and might thus contribute to the restoration of cartilage homeostasis.

The identification of a suitable gene vehicle is particularly important for the treatment of a slow, progressive disease such as OA, in which the effects of an agent may be required over prolonged periods of time. Vectors based on AAV might be advantageous to achieving this goal because they have the unique ability to mediate both efficient and stable transgene expression throughout the entire depth of cartilage (23,24), in marked contrast with the properties of other classes of vectors (27). In the present study, we evaluated the ability of rAAV-mediated *SOX9* overexpression to restore the cartilage matrix in human OA cartilage as compared with normal cartilage.

Our data indicate that sustained expression of a *SOX9* gene cassette significantly increased the proteoglycan and type II collagen content in 3-dimensional

cultures of human normal and OA chondrocytes, consistent with the effects of *SOX9* upon the expression of cartilage matrix components (17–19,43). Notably, the amount of proteoglycans and type II collagen noted in the treated OA chondrocytes was significantly higher than that in the control normal chondrocytes. Application of the rAAV-FLAG-hSOX9 vector did not promote cell proliferation in these systems, which is consistent with the properties of the transcription factor (26). These data demonstrate the ability of a therapeutic candidate to stably restore matrix synthesis in human OA chondrocytes. Delivery of a *SOX9* gene cassette via adenoviral and retroviral vectors has been shown to enhance the type II collagen and glycosaminoglycan content in OA chondrocytes (43,44), but the effects reported were incomplete in the absence of anabolic supplements and were noted only over a short period of time, in contrast with our findings using rAAV. This might be the result of the persistence of the rAAV transgenes in their targets (26), in contrast with adenoviral or retroviral transgenes that are either less stable or require cell division prior to expression.

Direct application of rAAV-FLAG-hSOX9 to human normal and OA cartilage in situ mediated high levels of transgene expression that was distributed throughout the thickness of the explant cultures, probably due to the ability of these small vectors to penetrate the dense ECM of the cartilage (23,24). The manipulation of vectors such as rAAV might thus be desirable for introducing candidate genes directly into the targets as compared with other classes of vectors still used in ex vivo gene transfer protocols (5,12,45). While no effects on cell proliferation were seen, expression of the *SOX9* gene cassette promoted a significant, dose-dependent increase in the proteoglycan and type II collagen content in OA cartilage to levels higher than or similar to those in the untreated normal cartilage and to depths relevant for clinical applications (24).

To the best of our knowledge, this is the first evidence of the ability of SOX9, or of any other therapeutic candidate, to compensate for the loss of ECM components in human OA cartilage. Interestingly, in the present system, matrix synthesis was enhanced in the treated OA cartilage as compared with the control normal cartilage. It remains to be seen whether altered (different from normal) levels of proteoglycans and type II collagen caused by *SOX9* overexpression influence the structure of articular cartilage over time. Kypriotou et al (46) reported that concentrations of SOX9 that were too high might inhibit type II collagen expression in chondrocytes by disturbing the cellular balance between transcription factors, depending on the stage of cell differentiation. Regulation of *SOX9* expression in these cells will be critical to the development of an appropriate gene treatment for OA that does not alter the integrity of the cartilage. Instead of using the strong CMV-IE transcription element, transgene expression might be controlled by regulatable (tetracycline-sensitive) or tissue-specific (*SOX9*, type II collagen, cartilage oligomeric matrix protein) promoters.

Kypriotou et al (46) also suggested that *SOX9* alone might not be sufficient to reorient the chondrocyte phenotype. Accordingly, Ikeda et al (16) showed that codelivery of the genes for *SOX5* and *SOX6* with *SOX9* (the SOX trio) was more efficient for inducing a chondrocyte phenotype in human mesenchymal stem cells in vitro as compared with *SOX9* treatment alone, although effects of the SOX trio on OA cartilage have not yet been reported. Indeed, regeneration of an original cartilage surface was not afforded by administration of the *SOX9* vector in the present study. Renewal of a native phenotype in OA chondrocytes might thus require the action of more than one intracellular therapeutic agent. This may be achieved by applying combinations of rAAV vectors at the same time (47). Restoration of a normal architecture in OA cartilage may certainly benefit from coapplication of factors that stimulate the metabolic and proliferative responses of the chondrocytes, such as IGF-1 (10,48,49), FGF-2 (11,48), TGFβ (49), BMP-7 (12), IL-1Ra (5), and Hsp70 (9). Nevertheless, recovery from cartilage degradation using direct application of gene treatments will be practicable only if some cartilage surface and resident chondrocytes are maintained, as in the early stages of OA, while methods based on the transplantation of genetically modified cells or progenitors might be desirable for more advanced cases of the disease.

In summary, the results of this study indicate that direct, rAAV-mediated overexpression of *SOX9* within human OA cartilage restores the synthesis of proteoglycans and type II collagen, 2 key ECM components of the cartilage. Additional studies in established experimental models (7,8) will be required in order to evaluate the effects of *SOX9* overexpression in OA cartilage in vivo. The present findings provide motivation to further develop this therapeutic gene-transfer approach to the treatment of OA in humans.

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AUTHOR CONTRIBUTIONS

Dr. Cucchiarini 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.

Study design. Drs. Cucchiarini and Madry.
Acquisition of data. Dr. Cucchiarini, Ms Thurn, and Ms Weimer.
Analysis and interpretation of data. Dr. Cucchiarini, Ms Thurn, Ms Weimer, and Drs. Kohn, Terwilliger, and Madry.
Manuscript preparation. Drs. Cucchiarini, Terwilliger, and Madry.
Statistical analysis. Dr. Cucchiarini.

REFERENCES

1. Poole AR. An introduction to the pathophysiology of osteoarthritis. Front Biosci 1999;4:D662–70.
2. Pelletier JP, DiBattista JA, Roughley P, McCollum R, Martel-Pelletier J. Cytokines and inflammation in cartilage degradation. Rheum Dis Clin North Am 1993;19:545–68.
3. Aigner T, Vornehm SI, Zeiler G, Dudhia J, von der Mark K, Bayliss MT. Suppression of cartilage matrix gene expression in upper zone chondrocytes of osteoarthritic cartilage. Arthritis Rheum 1997;40:562–9.
4. Smith RL. Degradative enzymes in osteoarthritis. Front Biosci 1999;4:D704–12.
5. Baragi VM, Renkiewicz RR, Jordan H, Bonadio J, Hartman JW, Roessler BJ. Transplantation of transduced chondrocytes protects articular cartilage from interleukin 1-induced extracellular matrix degradation. J Clin Invest 1995;96:2454–60.
6. Nixon AJ, Haupt JL, Frisbie DD, Morisset SS, McIlwraith CW, Robbins PD, et al. Gene-mediated restoration of cartilage matrix by combination insulin-like growth factor-I/interleukin-1 receptor antagonist therapy. Gene Ther 2005;12:177–86.
7. Pelletier JP, Caron JP, Evans C, Robbins PD, Georgescu HI, Jovanovic D, et al. In vivo suppression of early experimental osteoarthritis by interleukin-1 receptor antagonist using gene therapy. Arthritis Rheum 1997;40:1012–9.
8. Frisbie DD, Ghivizzani CS, Robbins PD, Evans CH, McIlwraith CW. Treatment of experimental equine osteoarthritis by in vivo delivery of the equine interleukin-1 receptor antagonist gene. Gene Ther 2002;9:12–20.
9. Grossin L, Cournil-Henrionnet C, Pinzano A, Gaborit N, Dumas D, Etienne S, et al. Gene transfer with HSP 70 in rat chondrocytes confers cytoprotection in vitro and during experimental osteoarthritis. FASEB J 2006;20:65–75.
10. Madry H, Zurakowski D, Trippel SB. Overexpression of human insulin-like growth factor-I promotes new tissue formation in an ex vivo model of articular chondrocyte transplantation. Gene Ther 2001;8:1443–9.
11. Madry H, Emkey G, Zurakowski D, Trippel SB. Overexpression of human fibroblast growth factor 2 stimulates cell proliferation in an ex vivo model of articular chondrocyte transplantation. J Gene Med 2004;6:238–45.
12. Hidaka C, Quitoriano M, Warren RF, Crystal RG. Enhanced matrix synthesis and in vitro formation of cartilage-like tissue by genetically modified chondrocytes expressing BMP-7. J Orthop Res 2001;19:751–8.
13. Ulrich-Vinther M, Stengaard C, Schwarz EM, Goldring MB, Soballe K. Adeno-associated vector mediated gene transfer of transforming growth factor-β1 to normal and osteoarthritic human chondrocytes stimulates cartilage anabolism. Eur Cell Mater 2005; 10:40–59.
14. Venkatesan N, Barre L, Benani A, Netter P, Magdalou J, Fournel-Gigleux S, et al. Stimulation of proteoglycan synthesis by glucuronosyltransferase-I gene delivery: a strategy to promote cartilage repair. Proc Natl Acad Sci U S A 2004;101:18087–92.
15. Bi W, Deng JM, Zhang Z, Behringer RR, de Crombrugghe B. Sox9 is required for cartilage formation. Nat Genet 1999;22:85–9.
16. Ikeda T, Kamekura S, Mabuchi A, Kou I, Seki S, Takato T, et al. The combination of SOX5, SOX6, and SOX9 (the SOX trio) provides signals sufficient for induction of permanent cartilage. Arthritis Rheum 2004;50:3561–73.
17. Bell DM, Leung KK, Wheatley SC, Ng LJ, Zhou S, Ling KW, et al. SOX9 directly regulates the type-II collagen gene. Nat Genet 1997;16:174–8.
18. Lefebvre V, Huang W, Harley VR, Goodfellow PN, de Crombrugghe B. SOX9 is a potent activator of the chondrocyte-specific enhancer of the proα1(II) collagen gene. Mol Cell Biol 1997;17: 2336–46.
19. Ng LJ, Wheatley S, Muscat GE, Conway-Campbell J, Bowles J, Wright E, et al. SOX9 binds DNA, activates transcription, and coexpresses with type II collagen during chondrogenesis in the mouse. Dev Biol 1997;183:108–21.
20. Xie WF, Zhang X, Sakano S, Lefebvre V, Sandell LJ. Transactivation of the mouse cartilage-derived retinoic acid-sensitive protein gene by Sox9. J Bone Miner Res 1999;14:757–63.
21. Salminen H, Vuorio E, Saamanen AM. Expression of Sox9 and type IIA procollagen during attempted repair of articular cartilage damage in a transgenic mouse model of osteoarthritis. Arthritis Rheum 2001;44:947–55.
22. Aigner T, Gebhard PM, Schmid E, Bau B, Harley V, Poschl E. SOX9 expression does not correlate with type II collagen expression in adult articular chondrocytes. Matrix Biol 2003;22:363–72.
23. Arai Y, Kubo T, Fushiki S, Mazda O, Nakai H, Iwaki Y, et al. Gene delivery to human chondrocytes by an adeno associated virus vector. J Rheumatol 2000;27:979–82.
24. Madry H, Cucchiarini M, Terwilliger EF, Trippel SB. Recombinant adeno-associated virus vectors efficiently and persistently transduce chondrocytes in normal and osteoarthritic human articular cartilage. Hum Gene Ther 2003;14:393–402.
25. Cucchiarini M, Madry H, Ma C, Thurn T, Zurakowski D, Menger MD, et al. Improved tissue repair in articular cartilage defects in vivo by rAAV-mediated overexpression of human fibroblast growth factor 2. Mol Ther 2005;12:229–38.
26. Xiao X, Li J, Samulski RJ. Efficient long-term gene transfer into muscle tissue of immunocompetent mice by adeno-associated virus vector. J Virol 1996;70:8098–108.
27. Cucchiarini M, Madry H. Gene therapy for cartilage defects. J Gene Med 2005;7:1495–509.
28. Mason JM, Grande DA, Barcia M, Grant R, Pergolizzi RG, Breitbart AS. Expression of human bone morphogenic protein 7 in primary rabbit periosteal cells: potential utility in gene therapy for osteochondral repair. Gene Ther 1998;5:1098–104.
29. Mi Z, Ghivizzani SC, Lechman ER, Jaffurs D, Glorioso JC, Evans CH, et al. Adenovirus-mediated gene transfer of insulin-like growth factor 1 stimulates proteoglycan synthesis in rabbit joints. Arthritis Rheum 2000;43:2563–70.
30. Mankin HJ, Dorfman H, Lippiello L, Zarins A. Biochemical and metabolic abnormalities in articular cartilage from osteo-arthritic human hips. II. Correlation of morphology with biochemical and metabolic data. J Bone Joint Surg Am 1971;53:523–37.
31. Samulski RJ, Chang LS, Shenk T. Helper-free stocks of recombinant adeno-associated viruses: normal integration does not require viral gene expression. J Virol 1989;63:3822–8.
32. Cucchiarini M, Ren XL, Perides G, Terwilliger EF. Selective gene

expression in brain microglia mediated via adeno-associated virus type 2 and type 5 vectors. Gene Ther 2003;10:657–67.
33. Sudbeck P, Schmitz ML, Baeuerle PA, Scherer G. Sex reversal by loss of the C-terminal transactivation domain of human SOX9. Nat Genet 1996;13:230–2.
34. Madry H, Trippel SB. Efficient lipid-mediated gene transfer to articular chondrocytes. Gene Ther 2000;7:286–91.
35. Merrihew C, Soeder S, Rueger DC, Kuettner KE, Chubinskaya S. Modulation of endogenous osteogenic protein-1 (OP-1) by interleukin-1 in adult human articular cartilage. J Bone Joint Surg Am 2003;85-A Suppl 3:67–74.
36. Madry H, Cucchiarini M, Stein U, Remberger K, Menger MD, Kohn D, et al. Sustained transgene expression in cartilage defects in vivo after transplantation of articular chondrocytes modified by lipid-mediated gene transfer in a gel suspension delivery system. J Gene Med 2003;5:502–9.
37. Wang J, Verdonk P, Elewaut D, Veys Em, Verbruggen G. Homeostasis of the extracellular matrix of normal and osteoarthritic human articular cartilage chondrocytes in vitro. Osteoarthritis Cartilage 2003;11:801–9.
38. Loeser RF, Pacione CA, Chubinskaya S. The combination of insulin-like growth factor 1 and osteogenic protein 1 promotes increased survival of and matrix synthesis by normal and osteoarthritic human articular chondrocytes. Arthritis Rheum 2003;48: 2188–96.
39. Von der Mark K, Kirsch T, Nerlich A, Kuss A, Weseloh G, Gluckert K, et al. Type X collagen synthesis in human osteoarthritic cartilage: indication of chondrocyte hypertrophy. Arthritis Rheum 1992;35:806–11.
40. Hollander AP, Heathfield TF, Webber C, Iwata Y, Bourne R, Rorabeck C, et al. Increased damage to type II collagen in osteoarthritic articular cartilage detected by a new immunoassay. J Clin Invest 1994;93:1722–32.
41. Squires GR, Okouneff S, Ionescu M, Poole AR. The pathobiology of focal lesion development in aging human articular cartilage and molecular matrix changes characteristic of osteoarthritis. Arthritis Rheum 2003;48:1261–70.
42. Sharif M, Whitehouse A, Sharman P, Perry M, Adams M. Increased apoptosis in human osteoarthritic cartilage corresponds to reduced cell density and expression of caspase-3. Arthritis Rheum 2004;50:507–15.
43. Tew SR, Li Y, Pothacharoen P, Tweats LM, Hawkins RE, Hardingham TE. Retroviral transduction with SOX9 enhances re-expression of the chondrocyte phenotype in passaged osteoarthritic human articular chondrocytes. Osteoarthritis Cartilage 2005;13:80–9.
44. Li Y, Tew SR, Russell AM, Gonzalez KR, Hardingham TE, Hawkins AR. Transduction of passaged human articular chondrocytes with adenoviral, retroviral, and lentiviral vectors and the effects of enhanced expression of SOX9. Tissue Eng 2004;10: 575–84.
45. Doherty PJ, Zhang H, Tremblay L, Manolopoulos V, Marshall KW. Resurfacing of articular cartilage explants with genetically-modified human chondrocytes in vitro. Osteoarthritis Cartilage 1998;6:153–9.
46. Kypriotou M, Fossard-Demoor M, Chadjichristos C, Ghayor C, de Crombrugghe B, Pujol JP, et al. SOX9 exerts a bifunctional effect on type II collagen gene (COL2A1) expression in chondrocytes depending on the differentiation state. DNA Cell Biol 2003;22: 119–29.
47. Rendahl KG, Leff SE, Otten GR, Spratt SK, Bohl D, Van Roey M, et al. Regulation of gene expression in vivo following transduction by two separate rAAV vectors. Nat Biotechnol 1998;16:757–61.
48. Trippel SB, Wroblewski J, Makower AM, Whelan MC, Schoenfeld D, Doctrow SR. Regulation of growth-plate chondrocytes by insulin-like growth-factor I and basic fibroblast growth factor. J Bone Joint Surg Am 1993;75:177–89.
49. Nixon AJ, Lillich JT, Burton-Wurster N, Lust G, Mohammed HO. Differentiated cellular function in fetal chondrocytes cultured with insulin-like growth factor-I and transforming growth factor-β. J Orthop Res 1998;16:531–41.