Molecular Medicine

Benefits of Recombinant Adeno-Associated Virus (rAAV)-Mediated Insulinlike Growth Factor I (IGF-I) Overexpression for the Long-Term Reconstruction of Human Osteoarthritic Cartilage by Modulation of the IGF-I Axis

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Administration of therapeutic genes to human osteoarthritic (OA) cartilage is a potential approach to generate effective, durable treatments against this slow, progressive disorder. Here, we tested the ability of recombinant adeno-associated virus (rAAV)-mediated overexpression of human insulinlike growth factor (hIGF)-I to reproduce an original surface in human OA cartilage in light of the pleiotropic activities of the factor. We examined the proliferative, survival and anabolic effects of the rAAV-hIGF-I treatment in primary human normal and OA chondrocytes *in vitro* and in explant cultures *in situ* compared with control (reporter) vector delivery. Efficient, prolonged IGF-I secretion via rAAV stimulated the biological activities of OA chondrocytes in all the systems evaluated over extended periods of time, especially *in situ*, where it allowed for the long-term reconstruction of OA cartilage (at least for 90 d). Remarkably, production of high, stable amounts of IGF-I in OA cartilage using rAAV advantageously modulated the expression of central effectors of the IGF-I axis by downregulating IGF-I inhibitors (IGF binding protein (IGFBP)-3 and IGFBP4) while upregulating key potentiators (IGFBP5, the IGF-I receptor and downstream mitogen-activated protein kinase/extracellular signal–regulated kinase 1/2 (MAPK/ERK-1/2) and phosphatidylinisitol-3/Akt (PI3K/Akt) signal transduction pathways), probably explaining the enhanced responsiveness of OA cartilage to IGF-I treatment. These findings show the benefits of directly providing an IGF-I sequence to articular cartilage via rAAV for the future treatment of human osteoarthritis.

INTRODUCTION

Osteoarthritis (OA) is a progressive disease of the entire joint that affects millions of people worldwide. OA is mainly characterized by a gradual, irreversible degeneration of the articular cartilage with a loss of its major matrix components (proteoglycans, type II collagen) (1), with concomitant changes in the subchondral bone and synovium. Although several pharmacological treatment options and surgical interventions are available to manage the progression of OA, regeneration of the articular cartilage remains an unresolved problem, in particular for patients who are too young to undergo partial or total joint replacement.

Disturbances in cartilage homeostasis are believed to play determining roles in the pathogenesis and progression of OA. Proinflammatory cytokines (interleukin [IL]-1 and tumor necrosis factor [TNF]-α) and adipokines (leptin, adiponectin and resistin) locally produced by the inflamed synovium, infrapatellar fat pad, osteophytes or the chondrocytes themselves may all contribute to the pathophysiology of OA (2–5). The putative implication of articular chondrocytes during OA progression has received particular attention because of pathological changes in their gene expression patterns, their incapacity to restore natural levels of extracellular matrix components and an ability to produce increased

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amounts of matrix-degrading enzymes. Interestingly, several investigations have focused on these cells, the unique cellular components in adult articular cartilage, as targets to develop new therapeutic interventions.

Delivery of candidate genes to articular chondrocytes is an attractive strategy that has the potential to allow for a durable reestablishment of the structural integrity in OA cartilage. Gene transfer approaches might be better suited to treat a slow and irreversible disorder such as OA over time instead of systems on the basis of the application of recombinant factors with relatively short pharmacological half-lives. Current approaches that aim at re-equilibrating the metabolic balance in OA cartilage are on the basis of the transfer of sequences coding for agents that either counteract the processes of matrix degradation or enhance the synthesis of matrix components. Protective effects against cartilage breakdown have been documented in experimental models of OA using sequences coding for inhibitors of inflammatory pathways (an IL-1 receptor antagonist [IL-1Ra], soluble TNF receptor [sTNFR], tissue inhibitor of metalloproteinases [TIMP-1]) (6–14) or factors such as IL-10 (12), heat shock protein 70 (15), glutamine:fructose-6-phosphate amidotransferase (16), thrombospondin-1 (17), kallistatin (18) and inhibitors of nuclear factor κB (19). Even though application of these stimuli was capable of containing cartilage degradation, it was not sufficient to compensate for the loss of matrix elements and cells and to reestablish an original cartilage surface. Growth, transcription and enzymatic factors are potent candidates to achieve these goals because of their anabolic or mitogenic properties such as fibroblast growth factor 2 (FGF-2) (20,21), bone morphogenetic proteins 2 and 4 (BMP-2 and BMP-4) (22,23), transforming growth factor β (TGF-β) (22,24,25), transcription factor sex-determining region Y–type high mobility group box 9 (SOX9) (20,26,27), glucuronosyltransferase-I (28), bcl-2 (29) and telomerase (30). A critical

agent that may have the strongest value to readjust the disturbed homeostasis in OA cartilage is insulinlike growth factor (IGF)-I, since it has the ability to influence concomitantly metabolic and proliferative processes, affording protection against extracellular matrix degradation in horse and rabbit articular cartilage explant cultures experimentally treated with proinflammatory cytokines (13,14,22).

Importantly for the treatment of OA, the development of effective gene treatments will necessitate that the gene vehicle allows for high and sustained levels of expression of the candidate sequence due to the slow and irreversible progression of this disorder. In contrast with vectors derived from adenoviruses (6,10,13,14,16–19,21,22,25) and retroviruses (7,11,12,23,26,30) or with nonviral compounds (8,15,28,29), systems based on the replication-defective, nonpathogenic human adeno-associated virus (AAV) may provide better tools for OA, since recombinant AAV (rAAV) can deliver genes in nondividing cells such as chondrocytes both *in vitro* and *in situ* in their dense extracellular matrix at high efficiencies and for extended periods of time (20,24,27,31). Also, removal of the viral protein coding sequences in rAAV make them less immunogenic than adenoviral vectors characterized by short-term transgene expression levels (32).

In the present study, we tested the hypothesis that efficient and prolonged IGF-I overexpression can be achieved via rAAV in primary human normal and OA chondrocytes in monolayer and three-dimensional hydrogel cultures *in vitro* and, most importantly, within their extracellular matrix in articular cartilage explants *in situ*, leading simultaneously to enhanced levels of cell proliferation and survival and of matrix synthesis vis-à-vis control (reporter gene vector) treatment. We also analyzed the extent by which the candidate treatment with the rAAV–human IGF–I (rAAV-hIGF-I) vector was capable of durably restoring the structure of human OA cartilage vis-à-vis normal cartilage. Finally, we investigated the influence of rAAV-mediated IGF-I

production upon the expression of key effectors of the IGF-I axis, specifically the IGF-binding proteins 3, 4 and 5 (IGFBP-3 to IGFBP-5) as well as the IGF-I receptor (IGF-IR) and downstream mitogen-activated protein kinase/extracellular signal–regulated kinase 1/2 (MAPK/ERK-1/2) and phosphatidylinisitol-3/Akt (PI3K/Akt) signal transduction pathways (33–39), reported as being inhibitors (IGFBP3 and IGFBP4) (40-42) or potentiators (IGFBP5 and IGF-IR) (42,43) of the IGF-I actions during OA pathogenesis.

MATERIALS AND METHODS

Reagents

Reagents were from Sigma (Munich, Germany) unless indicated. The dimethylmethylene blue dye was from Serva (Heidelberg, Germany). The anti–β-gal (GAL-13) and anti-bromodeoxyuridine (BrdU) (BU-33) antibodies were from Sigma; the anti–IGF-I antibody (AF-291-NA) was from R&D Systems (Wiesbaden-Nordenstadt, Germany); the anti–type I collagen (AF-5610) and anti–type II collagen (AF-5710) antibodies were from Acris (Hiddenhausen, Germany); the anti–IGF-I receptor (IGF-IR) antibody (Ab-4) was from Oncogene Research (Merck, Darmstadt, Germany); the anti–IGF binding proteins 3, 4 and 5 (IGFBP3, IGFBP4 and IGFBP5) (YY07, C-20 and E87, respectively) and anti-SOX9 (C-20) antibodies were from Santa Cruz Biotechnology (Heidelberg, Germany); and the anti–phospho-ERK-1/2 (D13.14.4E), anti–phospho-Akt (D9E) and anti–phospho-Elk-1 (2B1) antibodies were from Cell Signaling Technology (New England Biolabs, Frankfurt am Main, Germany). Biotinylated secondary antibodies and ABC reagent were from Vector Laboratories (Alexis Deutschland, Grünberg, Germany). Production of IGF-I, IGFBP3, IGFBP4 and IGFBP5 was measured with Quantikine IGF-I and Quantikine IGFBP3 enzyme-linked immunosorbent assays (ELISAs) (DG100 and DGB300, respectively) and the DuoSet IGFBP-4 and IGFBP5 ELISAs (DY804 and DY875, respectively) (all from R&D
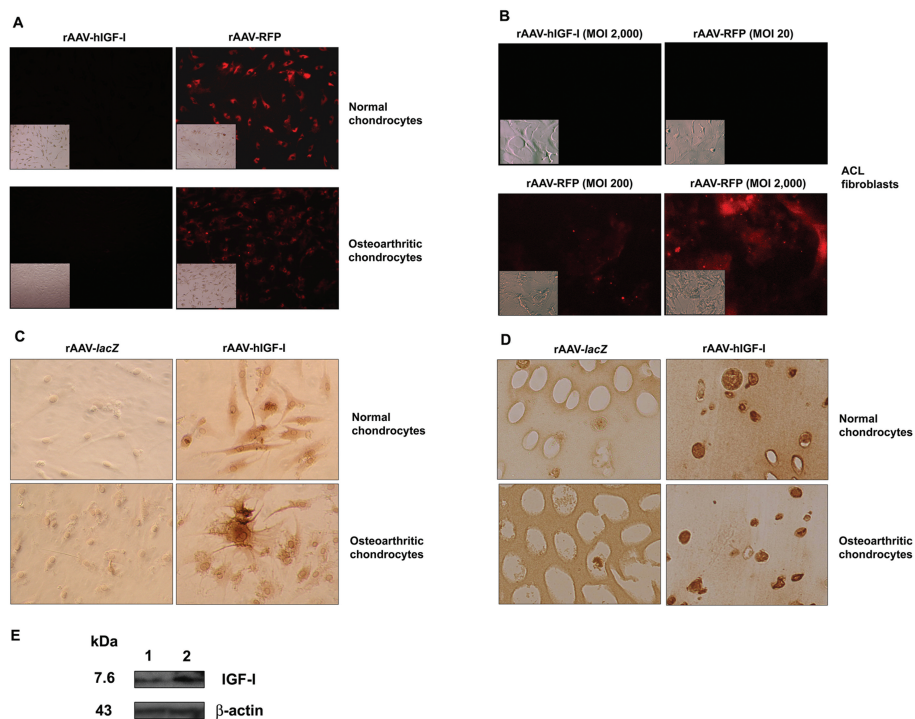
Figure 1. Detection of transgene expression in monolayer and alginate cultures of rAAV-transduced human articular chondrocytes and human ACL fibroblasts. Primary human normal and OA chondrocytes were transduced with either rAAV-hIGF-I or a control vector (rAAV-RFP or rAAV-*lacZ*) for maintenance in monolayer (A, C, E: MOI = 20) or alginate culture (D: MOI = 6). The vectors were also applied to monolayer cultures of primary human ACL fibroblasts using increasing vector doses (B: MOI = 20, 200 or 2,000). Transgene expression was detected by live fluorescence (A: magnification 10×; B: magnification 20×; insets: same fields under transmitted light; d 20 after transduction), anti–IGF-I immunocyto- and immunohistochemistry (C: d 20 after transduction; D: d 26 after transduction; both: magnification 20×) and Western blotting analysis (E: lane 1, rAAV-*lacZ*–transduced OA chondrocytes; lane 2, rAAV-hIGF-I–transduced OA chondrocytes; d 5 after transduction).

Plasmids and rAAV Vectors

The constructs and pACP were derived from pSSV9, an AAV-2 genomic clone (47,48). pAd8 contains the AAV-2 replication and encapsidation functions (48). rAAV-*lacZ* carries the *lacZ* gene for β-galactosidase (β-gal) under the control of the cytomegalovirus immediate-early (CMV-IE) promoter (20,27,31,45,49,50). rAAV–red fluorescent protein (rAAV-RFP) carries a 776-bp *Discosoma* sp. RFP cDNA fragment (45,49,50). A hIGF-I cDNA (51) was generated by polymerase chain reaction (PCR) using the primers 5′-I-A (A_5ctgcag[*Pst* I]G_{17}CTTCA GAAGC A) and 3′-I-A (A_5aagctt[*Hind* III]TGCGG TGGCA TGTCA CTCTT CAC) with pCMVhIGF-I (52) as a template for amplification. The resulting hIGF-I sequence (536 bp) was cloned in pACP to generate rAAV-hIGF-I, where the hIGF-I fragment was confirmed by sequencing. rAAVs were packaged as conventional (not self-complementary) vectors in the 293 cell line, an adenovirus-transformed human embryonic kidney cell line, using adenovirus 5 and pAd8 for helper functions. The preparations were purified by dialysis and titered by real-time PCR (20,27,31, 45,49,50), averaging 10^{10} transgene copies/mL (ratio virus particles to functional vectors = 500/1) (49).

rAAV Gene Transfer

Chondrocytes (passages 2–3, 10 d of culture) in monolayer culture (4×10^4 cells) were transduced with rAAV (multiplicity of infection [MOI] = 20) and kept in Dulbecco's modified Eagle's medium, 100 U/mL penicillin G, 100 μL/mL streptomycin and 10% fetal bovine serum (growth medium) in a humidified atmosphere of air with 5% CO_2 at 37°C for up to 20 d (45). Chondrocytes (10^6) were also transduced with rAAV (MOI = 6) for 2 d and encapsulated in alginate spheres in growth medium for up to 26 d (27,31,45). For comparison, human ACL fibroblasts in monolayer culture (4×10^4 cells) were transduced with increasing doses of rAAV (MOI = 20, 200 or 2,000) and kept in growth medium in a humidified at-

Systems). The type I and type II collagen ELISAs (Arthrogen-CIA Capture ELISAs) were from Chondrex (Redmond, WA, USA), the cell proliferation reagent WST-1 was from Roche Applied Science (Mannheim, Germany), and apoptosis was determined using the Cell Death Detection ELISA^{PLUS} (Roche Applied Science) and ApopTag® Plus Peroxidase *In Situ* Apoptosis Detection Kit (Chemicon-Millipore, Schwalbach/Ts., Germany).

Cartilage and Cells

Human normal articular cartilage was obtained from unaffected areas in knee joints removed during tumor surgery (nine patients, 68–72 years of age). OA was excluded on safranin O–stained

sections according to the Mankin scale (Mankin score 1–2) (44). Human OA articular cartilage was obtained from joints undergoing total knee arthroplasty (14 patients, 67–77 years of age) (Mankin score 7–9). The study was approved by the Ethics Committee of the Saarland Physicians Council. Cartilage explant cultures (6.2-mm diameter, 2-mm thick) and articular chondrocytes were prepared as previously described (20,27,31,45). Human anterior cruciate ligament (ACL) was obtained in patients undergoing total knee arthroplasty (three patients, 70–76 years of age), and primary human ACL fibroblasts were prepared as previously described (46).

mosphere of air with 5% CO_2 at 37°C for up to 20 d.

Cartilage explant cultures were transduced by direct application of rAAV (4×10^8 functional vectors) to the surface of the samples downwards during 1–2 min of contact (27,31,45). Growth medium was then added to the cultures without removal of the vector solution to allow for further in-depth penetration of the viral particles. The explants were then maintained in growth medium for up to 90 d with regular medium change every 2–3 d, starting on d 2 after vector administration.

Transgene Expression

RFP was detected by live fluorescence using a fluorescent microscope with a 568-nm filter (Olympus CKX41, Hamburg, Germany) (20,27,31,45,50). To assess IGF-I secretion, samples were washed twice and placed for 24 h in serum-free medium. Supernatants were collected and centrifuged, and IGF-I production was measured by ELISA on a GENios spectrophotometer/fluorometer (Tecan, Crailsheim, Germany). Expression was also monitored by immunocytochemical and immunohistochemical analyses with specific primary antibodies, biotinylated secondary antibodies and the ABC method using diaminobenzidine (DAB) as the chromogen (20,27,31,50). To control for secondary immunoglobulins, samples were processed with omission of primary antibodies. Samples were examined under light microscopy (Olympus BX 45). Western blotting analyses were performed using 15-µg proteins prepared from transduced cells. Signals were revealed with specific antibodies, horseradish peroxidase–labeled secondary antibodies (Vector Laboratories) and the ECL Advance Western blotting detection kit (Amersham Biosciences, Freiburg, Germany) (20,27).

Histological, Immunocytochemical and Immunohistochemical Analyses

Monolayer, alginate and explant cultures were harvested and fixed in 10% buffered formalin. Spheres and explants were dehydrated in graded alcohols and embedded in paraffin. Paraffin-embedded sections (5 µm) were stained with safranin O (proteoglycans) and hematoxylin and eosin (H&E) (cells) (20,27,31,45). Expression of type I and type II collagen, IGF-IR, IGFBP3, IGFBP4, IGFBP5, phospho-ERK-1/2, phospho-Akt, phospho-Elk-1 and SOX9 was detected using specific antibodies, biotinylated secondary antibodies, the ABC method and DAB, as described above.

Cell Proliferation

Proliferative activities were assessed by immunolabeling after BrdU incorporation (50). BrdU was introduced at a final concentration of 3 µg/mL in the culture medium 24 h after rAAV transduction. Samples were immunochemically processed as described above to monitor proliferation rates with a specific anti-BrdU antibody and a biotinylated secondary antibody and by revelation using the ABC method and DAB. Proliferation was also assessed using the cell proliferation reagent WST-1, with optical density (OD) being proportional to the cell numbers (50).

Apoptosis Assays

In vitro, the specific enrichment of cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes) released from monolayer cultures was estimated using the Cell Death Detection ELISA[PLUS] according to the manufacturer's protocol. *In situ*, nuclear DNA fragmentation consistent with apoptosis was determined by the terminal deoxynucleotidyl transferase-mediated dUTP nickend labeling (TUNEL) method (20).

Morphometric Analyses

The cell numbers and viability were monitored by trypan blue exclusion and by counting cells on H&E-stained histological sections (20,27,31,45,49). The transduction efficiencies; the percentage of cells positive for BrdU uptake; apoptotic events; IGF-IR, IGFBP3, IGFBP4, IGFBP5, phospho-ERK-1/2, phospho-Akt, phospho-Elk-1 and SOX9 immunoreactivity; the cell densities; and the intensities of safranin O staining and of type I and type II collagen immunostaining were measured at three standardized sites or by using 10 serial histological and immunohistochemical sections for each parameter, test and replicate condition. Analysis programs included SIS AnalySIS (Olympus), Adobe Photoshop (Adobe Systems, Unterschleissheim, Germany) and Scion Image (Scion Corporation, Frederick, MD,

Table 1. IGF-I production in rAAV-transduced chondrocytes *in vitro* and *in situ*.

		Normal chondrocytes		OA chondrocytes	
	d	rAAV-*lacZ*	rAAV-hIGF-I	rAAV-*lacZ*	rAAV-hIGF-I
Monolayer cultures ($ng/10^7$ viable cells/24 h)					
	5	3.40 (0.08)	29.30 (0.81)[a]	2.60 (0.05)	18.87 (0.92)[a]
	10	0.08 (0.01)	27.03 (0.70)[a]	0.06 (0.01)	18.89 (0.68)[a]
	20	n.d.	21.84 (0.53)[a]	n.d.	16.68 (0.64)[a]
Alginate cultures ($ng/10^7$ viable cells/24 h)					
	5	2.70 (0.03)	25.02 (0.22)[a]	3.75 (0.02)	24.73 (0.09)[a]
	26	n.d.	24.60 (0.16)[a]	n.d.	22.79 (0.13)[a]
Explant cultures (pg/mg dry weight/24 h)					
	5	0.42 (0.06)	16.97 (1.22)[a]	0.27 (0.03)	14.14 (1.02)[a]
	12	0.12 (0.04)	13.61 (1.16)[a]	0.10 (0.05)	12.71 (1.01)[a]
	30	0.09 (0.02)	26.24 (2.31)[a]	0.16 (0.03)	29.23 (1.58)[a]
	60	0.14 (0.03)	26.82 (1.98)[a]	0.11 (0.02)	28.91 (2.06)[a]
	90	0.11 (0.03)	32.41 (1.84)[a]	0.09 (0.03)	37.20 (2.12)[a]

Data are means (SD). n.d., Not detectable.
[a]Statistically significant vis-à-vis control treatment.

USA) (20,27,31,45,49,50). The safranin O staining intensities were in percentages as the ratios of positively stained tissue surface to the total surface of the site evaluated. The type I and type II collagen immunostaining intensities were in pixels per standardized area (intensity units).

Biochemical Assays

Solubilized spheres and explant cultures were digested to monitor the DNA contents, proteoglycan production and type I and type II collagen contents by Hoechst 33258 assay, binding to the dimethylmethylene blue (DMMB) dye and ELISA, respectively (20,27,31,50). Data were normalized to total cellular proteins using a protein assay (Pierce Thermo Scientific, Fisher Scientific, Schwerte, Germany). To assess IGFBP secretion, samples were washed twice and placed for 24 h in serum-free medium. Supernatants were collected and centrifuged, and production of IGFBP3, IGFBP4 and IGFBP5 was measured by ELISA. All measurements were performed with a GENios spectrophotometer/fluorometer.

Statistical Analysis

Each condition was performed in triplicate in three independent experiments with monolayer, alginate and explant cultures. Data were obtained by three individuals blinded with respect to the groups. The *t* test and Mann-Whitney rank sum test were used where appropriate. *P* values <0.05 were considered statistically significant.

RESULTS

Overexpression of IGF-I via rAAV in Human Normal and OA Chondrocytes *In Vitro*

The candidate (rAAV-hIGF-I) and control (rAAV-RFP or rAAV-*lacZ*) vectors were first applied to primary human normal and OA chondrocytes in monolayer culture to define transgene expression *in vitro*. A strong fluorescent signal restricted to cells transduced with rAAV-RFP was already detected on d 5 (not shown) and for at least 20 d compared

Table 2. Biochemical analyses in rAAV-transduced alginate-chondrocyte spheres.

	d	Normal chondrocytes		OA chondrocytes	
		rAAV-*lacZ*	rAAV-hIGF-I	rAAV-*lacZ*	rAAV-hIGF-I
Cells (10^4/sphere)					
	5	1.40 (0.02)	1.70 (0.03)[a]	1.30 (0.07)	1.80 (0.10)[a]
	26	0.04 (0.02)[b]	1.55 (0.05)[a]	0.04 (0.05)[b]	1.45 (0.04)[a]
Cell densities (cells/mm^2)					
	5	122 (5)	178 (9)[a]	119 (4)	165 (3)[a]
	26	16 (1)[b]	149 (7)[a]	14 (2)[b]	137 (6)[a]
DNA (µg/10^4 cells)					
	5	0.36 (0.03)	3.07 (0.14)[a]	0.32 (0.02)	3.18 (0.16)[a]
	26	0.12 (0.01)[b]	2.88 (0.12)[a]	0.10 (0.01)[b]	2.73 (0.11)[a]
Proteoglycans (µg/10^4 cells)					
	5	3.91 (0.16)	4.70 (0.25)[a]	3.36 (0.14)	4.42 (0.21)[a]
	26	3.07 (0.12)[b]	10.91 (1.04)[a,b]	2.97 (0.11)[b]	9.37 (1.01)[a,b]
Type II collagen (ng/10^4 cells)					
	5	0.14 (0.03)	0.33 (0.04)[a]	0.09 (0.01)	0.28 (0.04)[a]
	26	0.12 (0.02)[b]	0.79 (0.05)[a,b]	0.08 (0.01)[b]	0.54 (0.05)[a,b]
Type II collagen staining (%)					
	5	18 (3)	37 (5)[a]	15 (5)	29 (4)[a]
	26	15 (3)[b]	70 (4)[a,b]	12 (3)[b]	65 (3)[a,b]

Data are means (SD).
Statistically significant vis-à-vis [a]control treatment and [b]earlier time point.

with rAAV-hIGF-I (Figure 1A), with transduction efficiencies ranging between 80% and 95% when applying an MOI of 20. For comparison, a similar signal was noted in rAAV-RFP–transduced primary human fibroblasts only when an MOI of at least 2,000 was provided to the cells by d 20 (transduction efficiencies of 60–71%) (Figure 1B). Conversely, strong IGF-I immunoreactivity was noted in cells where rAAV-hIGF-I was provided vis-à-vis control treatment (Figure 1C). Western blotting analyses revealed an IGF-I immunoreactive band of approximately 7.6 kDa that was about four-fold more intense in rAAV-hIGF-I–treated OA cells than in control cells (Figure 1E). Similar results were noted in normal chondrocytes. Significant, prolonged synthesis of IGF-I was achieved with rAAV-hIGF-I compared with control conditions (at least an 8.6-fold increase in normal cells and a 7.3-fold increase in OA cells; *P* ≤ 0.001) (Table 1), with well-maintained levels in both types of cells over time (*P* ≥ 0.200 between d 5 and d 20) (see Table 1).

Transduced cells were next encapsulated in alginate spheres to characterize transgene expression in a three-dimensional environment supportive of the chondrocyte phenotype. β-Gal activity, restricted to control cells, was already detected on the day of encapsulation and for at least 26 d (not shown). Significant, prolonged IGF-I expression and synthesis was seen when rAAV-hIGF-I was provided compared with control treatment (at least a 9.3-fold increase in normal cells and a 6.6-fold increase in OA cells; *P* ≤ 0.001) (Figure 1D and Table 1), with well-maintained levels over time (*P* ≥ 0.165 between d 5 and d 26) (Table 2). Transduction efficiencies were again between 80% and 92%.

Effects of IGF-I Overexpression on the Proliferative and Synthetic Activities of Human Normal and OA Chondrocytes *In Vitro*

rAAV-hIGF-I was next administered to chondrocytes in monolayer and alginate cultures to examine the effects of IGF-I
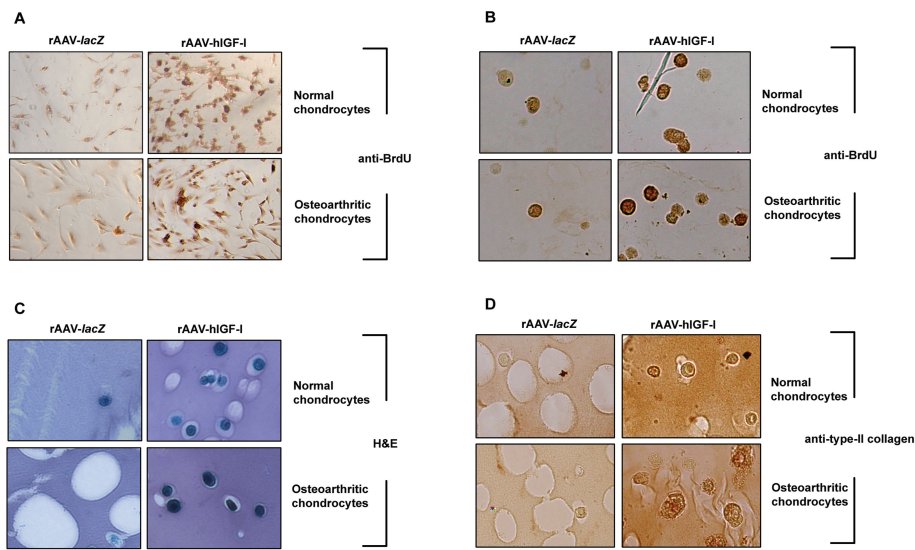
Figure 2. Analysis of cell proliferation and extracellular matrix synthesis in rAAV-transduced human articular chondrocytes in monolayer and alginate cultures. Primary human normal and OA chondrocytes were transduced with either rAAV-*lacZ* or rAAV-hIGF-I, as described in Figure 1, and cell encapsulation was performed where indicated, as also denoted in Figure 1. Monolayer cultures were processed for the evaluations on d 20 after transduction and alginate cultures on d 26. (A, B) BrdU immunodetection in monolayer (A: magnification 10×) and alginate (B: magnification 20×) cultures of transduced chondrocytes after BrdU incorporation. (C) H&E staining of histological sections from transduced alginate cultures (magnification 20×). (D) Immunodetection of type II collagen on sections from transduced alginate cultures (magnification 20×).

overexpression on cell proliferation and survival *in vitro* over time compared with rAAV-*lacZ* treatment. Immunodetection of BrdU incorporation in monolayer cultures revealed significantly increased levels of proliferation mediated by rAAV-hIGF-I vis-à-vis rAAV-*lacZ* (75–80% versus <10% of BrdU$^+$ normal or OA cells on d 20 [that is, up to eightfold difference]; $P \leq 0.001$) (Figure 2A). These results were corroborated by a WST-1 assay performed at the same time point (0.628 versus 0.312 OD^{450nm} in normal cells or 1.175 versus 0.716 OD^{450nm} in OA cells using rAAV-hIGF-I versus rAAV-*lacZ* [that is, a 2- or 1.6-fold increase]; $P \leq 0.001$). Also, the apoptotic events significantly decreased in the presence of rAAV-hIGF-I on d 20 compared with rAAV-*lacZ* (normal cells: a 1.15 versus 1.50 factor [that is, a 1.3-fold decrease]; OA cells: a 0.89 versus 1.62 factor [that is, a 1.8-fold decrease]; $P \leq 0.001$). The levels of proliferation in en-

capsulated cells were also significantly enhanced by treatment with rAAV-hIGF-I compared with the controls (70–82% versus <12% of BrdU$^+$ normal or OA cells on d 26 [that is, up to seven-fold difference]; $P \leq 0.001$) (Figure 2B), a result confirmed when estimating the viable cell numbers in rAAV-hIGF-I–transduced spheres vis-à-vis control spheres (up to 38.8-fold difference; $P \leq 0.001$) (see Table 2), the cell densities on histological sections (up to 9.8-fold difference; $P \leq 0.001$) (Figure 2C and Table 2), and the DNA contents (up to 27.3-fold difference; $P \leq 0.001$) (see Table 2). Interestingly, a significant decrease in the viable cell numbers, cell densities and DNA contents was noted over time in both types of control spheres (up to 35-fold; $P \leq 0.001$), whereas these parameters were well maintained in the rAAV-hIGF-I–treated spheres ($P \geq 0.598$), probably due to the steady expression levels of the IGF-I transgene in cells (see Table 1).

rAAV-hIGF-I was next provided in the alginate system to determine the effects of IGF-I on the synthesis of extracellular matrix components in three-dimensional culture conditions *in vitro* over time vis-à-vis rAAV-*lacZ*. The proteoglycan and type II collagen contents were significantly enhanced in spheres from both types of cells using rAAV-hIGF-I versus rAAV-*lacZ* (up to 6.8-fold difference; $P \leq 0.001$) (see Table 2). The results related to the type II collagen were corroborated by an analysis of the staining intensities for this marker on sections from spheres (up to 5.4-fold difference; $P \leq 0.001$) (Figure 2D and Table 2). Notably, a significant decrease in the amounts of proteoglycans and type II collagen and in the intensities of type II collagen immunoreactivity was observed over time in both types of control spheres (up to 1.3-fold; $P \leq 0.001$), whereas these parameters significantly increased in the rAAV-hIGF-I–treated spheres (up to 2.4-fold; $P \leq 0.001$), probably because of the steady expression levels of the IGF-I transgene in cells (see Table 1). Little or no expression of type I collagen was detected in any of the treatment groups when the samples were processed either by immunohistochemistry or by ELISA to monitor the type I collagen contents.

Expression of the IGF-I Transgene in Human Normal and OA Articular Cartilage After Direct rAAV Application *In Situ*

The candidate rAAV-hIGF-I vector was then directly applied to articular cartilage explant cultures to monitor IGF-I expression over time in a native environment compared with rAAV-*lacZ* treatment. Immunoreactivity to the growth factor was preferentially detected when rAAV-hIGF-I was provided to the explants (normal and OA cartilage), both in the superficial and middle zones, as early as 5 d after vector application (not shown) and for at least 90 d (Figure 3A). About 72–81% of the cells expressed the transgene compared with the controls (below 4%; up to 20.3-fold difference; $P \leq 0.001$). Significant, prolonged IGF-I syn-

thesis was achieved in rAAV-hIGF-I–transduced explants compared with control treatment (at least a 294.6-fold increase in normal explants and a 413.3-fold increase in OA explants; $P \leq 0.001$) (see Table 1), with increasing levels detected in both types of rAAV-hIGF-I–treated cultures over time (1.9- and 2.6-fold increase between d 5 and d 90 in normal and OA explants, respectively; $P \leq 0.001$) (see Table 1).

Effects of IGF-I Overexpression on the Proliferative, Synthetic and Restorative Activities in Human Normal and OA Articular Cartilage *In Situ*

rAAV-hIGF-I was next provided to cartilage explant cultures to evaluate the effects of IGF-I overexpression on the proliferative, survival and metabolic activities of normal and OA chondrocytes in a native environment compared with rAAV-*lacZ* treatment. An histomorphometric analysis revealed significant increases in the cell densities of the explants transduced with rAAV-hIGF-I vis-à-vis rAAV-*lacZ* for at least 90 d, the longest time point examined, both in the superficial and middle zones of the cartilage (a 2.8-fold increase in either normal or OA cartilage; $P \leq 0.001$) (Figure 3B and Table 3). These results were confirmed when evaluating the percentage of BrdU$^+$ cells (28- and 19.3-fold increases in normal and OA cartilage, respectively; $P \leq 0.001$) (Figure 3C and Table 3) and the DNA contents (a 1.9-fold increase in either normal or OA cartilage; $P \leq 0.001$) (see Table 3), overall reflecting the long-term stimulatory effects of the IGF-I treatment on cell proliferation *in situ*, probably due to the steady expression of the factor (see Table 1). Of note, these parameters were higher in rAAV-hIGF-I–treated OA cartilage than in control normal cartilage ($P \leq 0.001$). A TUNEL analysis further showed that application of the IGF-I vector promoted a significant decrease in the percentage of apoptotic cells in OA cartilage vis-à-vis rAAV-*lacZ* treatment (9.8-fold; $P \leq 0.001$), bringing back the levels to those observed in control normal cartilage (al-
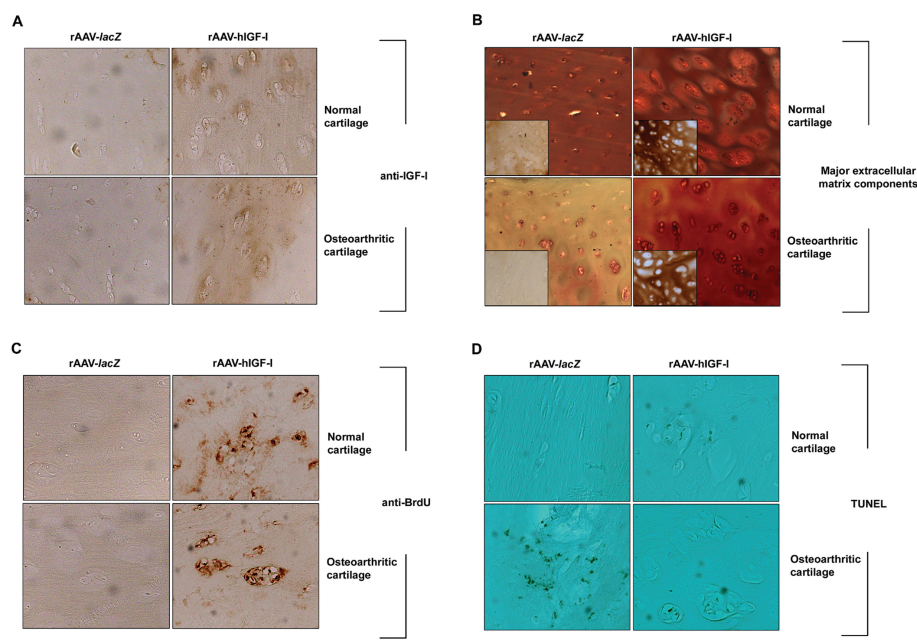

Figure 3. Immunohistochemical analyses in rAAV-transduced human articular cartilage. Primary human normal and OA cartilage explant cultures were transduced with either rAAV-hIGF-I or the control rAAV-*lacZ* vector (4×10^8 each functional vector) and processed after 90 d to detect transgene expression by anti–IGF-I immunohistochemistry (A: magnification 20×), the expression of major extracellular matrix components (B: safranin O at magnification 10× and type II collagen in the insets at magnification 10×), the rates of cell proliferation after BrdU incorporation and immunodetection (C: magnification 20×) and apoptotic events by TUNEL assay (D: magnification 4×). All are views of the middle zone.

most undetectable levels) (Figure 3D and Table 3).

Remarkably, transduction with rAAV-hIGF-I compared with control conditions led to significant increases in safranin O staining intensity for at least 90 d, both in the superficial and middle zones of the cartilage (2- and 8.5-fold increases in normal and OA cartilage, respectively; $P \leq 0.001$) (Figure 3B and Table 3), in the proteoglycan contents (3.7- and 8.7-fold increases, respectively; $P \leq 0.001$) (see Table 3), in type II collagen immunostaining intensity (2.3- and 4.3-fold increases, respectively; $P \leq 0.001$) (insets of Figure 3B and Table 3) and in the type II collagen contents (2.8- and 4.7-fold increases, respectively; $P \leq 0.001$) (see Table 3). Interestingly, expression of these components was higher in rAAV-hIGF-I–treated OA cartilage than in control normal cartilage ($P \leq 0.001$). In good agreement with the findings *in vitro*, little

or no type I collagen expression was detected in any of the treatment groups when the samples were processed either by immunohistochemistry (superficial and middle zones) or by ELISA to monitor the type I collagen contents (53).

Regulation of Central Effectors of the IGF-I Axis by rAAV-Mediated IGF-I Overexpression

rAAV-hIGF-I was next provided to human normal and OA articular cartilage explant cultures to determine the possible effects of rAAV-mediated IGF-I production on the expression of components of the IGF-I axis known to play key roles in the pathogenesis of OA, precisely the IGF-binding proteins 3, 4 and 5 (IGFBP-3 to IGFBP-5) as well as IGF-IR and downstream MAPK/ERK-1/2 and PI3K/Akt signal transduction pathways. Remarkably, treatment with the rAAV IGF-I vector promoted a significant de-

Table 3. Histomorphometric and biochemical analyses in rAAV-transduced cartilage (d 90).

	Normal cartilage		OA cartilage	
	rAAV-*lacZ*	rAAV-hIGF-I	rAAV-*lacZ*	rAAV-hIGF-I
Cell densities (cells/mm^2)	192 (4)	539 (6)[a]	178 (4)	498 (5)[a]
BrdU labeling (%)	3 (1)	84 (3)[a]	4 (2)	77 (4)[a]
DNA (μg/mg total protein)	12.5 (1.2)	23.4 (1.4)[a]	9.3 (0.8)	17.3 (0.7)[a]
Apoptotic cells (%)	4 (1)	3 (1)	59 (3)	6 (2)[a]
Matrix staining (%)	48 (4)	98 (3)[a]	11 (2)	94 (2)[a]
Proteoglycans (μg/mg total protein)	16.3 (1.4)	59.6 (1.5)[a]	6.2 (1.1)	53.9 (1.2)[a]
Type II collagen staining (intensity units)	38 (3)	87 (2)[a]	19 (4)	82 (3)[a]
Type II collagen (ng/mg total protein)	6.4 (0.5)	17.8 (1.2)[a]	3.1 (0.4)	14.5 (1.3)[a]
IGFBP3 (%)	17 (1)	1 (1)[a]	64 (2)	1 (1)[a]
IGFBP3 (ng/mg total protein)	0.23 (0.03)	0.03 (0.01)[a]	0.55 (0.04)	0.02 (0.01)[a]
IGFBP4 (%)	21 (2)	1 (1)[a]	67 (2)	1 (1)[a]
IGFBP4 (ng/mg total protein)	0.35 (0.02)	0.01 (0.01)[a]	0.58 (0.04)	0.04 (0.02)[a]
IGFBP5 (%)	23 (2)	77 (3)[a]	71 (2)	84 (2)[a]
IGFBP5 (ng/mg total protein)	0.26 (0.02)	0.73 (0.03)[a]	0.69 (0.02)	1.07 (0.04)[a]
IGF-IR (%)	22 (3)	49 (2)[a]	52 (4)	86 (3)[a]
Phospho-ERK-1/2 (%)	6 (2)	67 (3)[a]	57 (2)	87 (3)[a]
Phospho-Akt (%)	2 (1)	58 (2)[a]	58 (3)	78 (3)[a]
Phospho-Elk-1 (%)	4 (2)	75 (3)[a]	54 (3)	88 (2)[a]
SOX9 (%)	65 (2)	76 (3)[a]	40 (2)	58 (3)[a]

Data are means (SD).
[a]Statistically significant vis-à-vis control treatment.

crease in IGFBP3 and IGFBP4 expression, reaching almost undetectable levels both in normal and OA cartilage vis-à-vis control vector application after 90 d, the longest time point evaluated (up to 35- and 67-fold in normal and OA cartilage, respectively; $P \le 0.001$) (Figures 4A, B; Table 3). In good agreement with previous observations (54–58), expression of these markers was higher in control OA versus control normal cartilage.

Strikingly, and in marked contrast with the observations for IGFBP3 and IGFBP4, application of rAAV-hIGF-I significantly increased IGFBP5 and IGF-IR expression both in normal and OA cartilage vis-à-vis control vector application after 90 d (up to 3.3- and to 1.7-fold in normal and OA cartilage, respectively; $P \le 0.001$) (Figures 4C, D; Table 3). This effect mediated by IGF-I overexpression was more substantial in normal cartilage than in OA cartilage, since the expression levels of these markers were lower

in control normal versus control OA cartilage, consistent with previous findings (54,56,59). Remarkably, these effects of IGF-I via rAAV gene transfer in normal and OA cartilage were associated with significant increases in the expression levels of downstream components of the IGF-IR signal transduction pathways, that is, MAPK/ERK-1/2 (Figure 5A and Table 3) and PI3K/Akt (Figure 5B and Table 3) compared with control conditions (up to 28.9- and 1.5-fold increase in normal and OA cartilage, respectively; $P \le 0.001$) and with an activation of the specific transcription factors Ets-like gene-1 (Elk-1) (Figure 5C and Table 3) and SOX9 (Figure 5D and Table 3) (up to 18.8- and 1.6-fold increase in normal and OA cartilage, respectively; $P \le 0.001$).

DISCUSSION

Application of sequence coding for factors that might successfully and durably influence the metabolic and pro-

liferative pathways in OA articular cartilage is an attractive approach to readjust the disturbed balance in this disorder and to counteract its long-term progression. Among the candidates tested for their reparative properties, IGF-I was described for its ability to modulate cell proliferation and extracellular matrix synthesis in horse and rabbit experimental models of cytokine-induced cartilage matrix degradation *in situ* (13,14,22). Yet, to the best of our knowledge, the effects of IGF-I gene transfer upon the long-term remodeling of human OA cartilage have not been evaluated to date. Notably, in these previous reports, delivery of IGF-I was performed using adenoviral vectors, a class of vectors that promote high but only very transient levels of gene expression, an important issue for the treatment of a disorder with a slow and irreversible progression such as OA (32). Vectors on the basis of the nonpathogenic adeno-associated virus might be thus better suited to treat this disease, since rAAVs are capable of transducing OA cells with high efficiency and for persistent periods of time and also when the cells are still surrounded by extracellular matrix components *in situ* (20,24,27,31). In the present study, we therefore examined the effects of rAAV-mediated overexpression of IGF-I on the long-term reconstruction of human OA cartilage and on the expression of the major components of its own axis.

The data first indicate that highly effective, significant and prolonged expression of IGF-I could be achieved via rAAV in primary human normal and OA chondrocytes in monolayer and alginate cultures *in vitro* (maintenance for up to 20 d and 26 d, respectively) and most remarkably in cartilage explants *in situ* (regular increase until d 90). Transduction efficiencies reached up to 95%, even when using relatively low MOI and vector doses (MOI = 6–20 *in vitro*; 4×10^8 functional recombinant particles *in situ*), probably because of the high permissivity of the cells to rAAV transduction and to their ability to rapidly process the viral particles compared
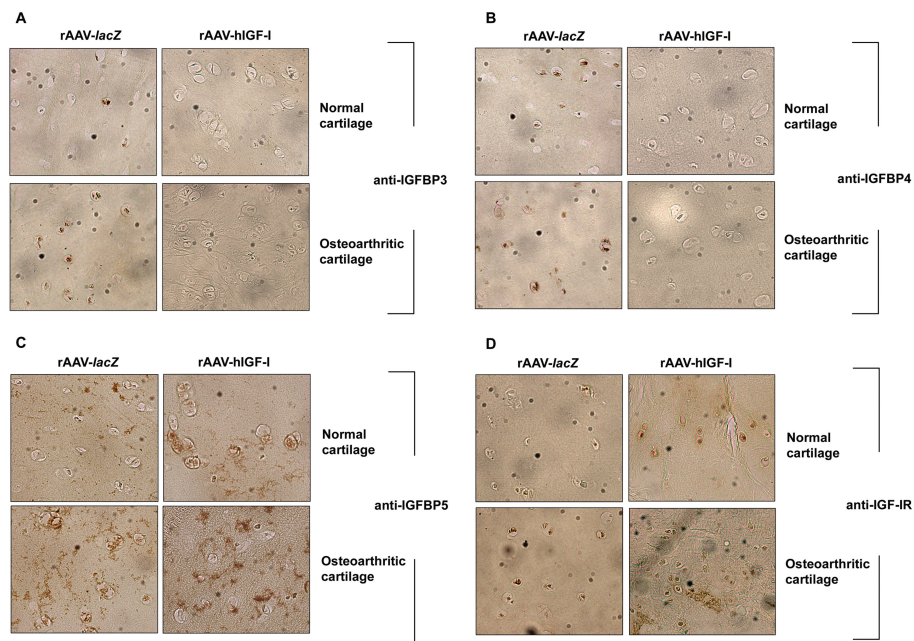
Figure 4. Expression of IGFBPs and IGF-IR in rAAV-transduced human articular cartilage. Primary human normal and OA cartilage explant cultures were transduced with either rAAV-hIGF-I or the control rAAV-*lacZ* vector, as described in Figure 3, and processed after 90 d to detect the expression of IGFBP3 (A), IGFBP4 (B), IGFBP5 (C) and IGF-IR (D). Magnification 20×. All are views of the middle zone.

with fibroblasts, which require much higher amounts of rAAV (MOI of at least 2,000) to allow for an efficient viral trafficking to the nucleus and to detectable transgene expression, in good agreement with previous findings (20,27,31,45, 60–62). Interestingly, transgene expression was seen throughout the thickness of the cartilage, probably because of the ability of the small rAAV particles to penetrate the dense cartilage matrix and in agreement with previous findings using this class of vector (20,27,45). In monolayer cultures, high concentrations of IGF-I were produced in transduced normal and OA cells (at least 18.87 ± 0.92 ng/10^7 cells/24 h on d 5), remaining steady until d 20 (above 16.68 ± 0.64 ng/10^7 cells/24 h; $P \geq 0.200$). The levels produced early on via rAAVs were lower than those achieved at similar time points with nonviral vectors (up to 83 ng/10^7 cells/24 h with 2 μg DNA [that is, 2.8- to 4.4-fold higher than here using a relatively low MOI of 20]) (52,63) or with plasmid vectors carrying AAV

sequences (up to 900 ng/10^7 cells/24 h with 2 μg DNA [that is, 30.7- to 47.7-fold more than with our recombinant particles]) (64). Nevertheless, such levels decreased rapidly (230 ng/10^7 cells/24 h on d 6) (64) and were probably undetectable by d 20, as is commonly observed with nonviral compounds (65). A rapid decline in IGF-I expression was also reported when using adenoviral vectors (22,66,67). On d 3, IGF-I production levels were of 200–600 ng/10^7 cells/24 h at high adenoviral vector doses (MOI = 100–500 [that is, 5- to 25-fold higher than here]), which relates well with the concentrations achieved here with an MOI of 20 (that is, 6.8- to 31.8-fold difference), followed by a dramatic decrease on d 20 (67 ng/10^7 cells/24 h at an MOI of 500 [that is, 25-fold more adenoviral vector than here, for a difference of only 3.1- to 4.1-fold in concentration]) (67). Next, we measured long-term IGF-I secretion in encapsulated cells at vector doses that were even lower than those applied in the monolayer cultures (from 25.02 ±

0.22 to 24.60 ± 0.16 and from 24.73 ± 0.09 to 22.79 ± 0.13 ng/10^7 cells/24 h between d 5 and d 26 in normal and OA cells, respectively, at an MOI of 6; $P \geq 0.165$). This result was also reported when using nonviral vectors, but again with high amounts of genetic material (28.4 ng/10^7 cells/24 h on d 36 with 2 μg DNA) (68). Finally (and for the first time to the best of our knowledge), we demonstrate that rAAV allows for a significant ($P \leq 0.001$ compared with each respective control) and increasing production of IGF-I in human normal and OA cartilage over a sustained period of time that might be relevant for successful treatment OA (from 16.97 ± 1.22 to 32.41 ± 1.84 and from 14.14 ± 1.02 to 37.20 ± 2.12 pg/mg dry weight/24 h between d 5 and d 90, respectively; $P \leq 0.001$).

Most remarkably, the levels of IGF-I achieved here via rAAV stimulated the proliferative, survival and biosynthetic activities of human normal and OA chondrocytes *in vitro* and *in situ* vis-à-vis control treatment, even at prolonged time points, with well-maintained cell proliferation and enhanced matrix synthesis *in vitro* over time, consistent with the properties of the growth factor (64,69–71). The levels of activation attained in normal and OA cells *in vitro* and *in situ* after rAAV-hIGF-I treatment vis-à-vis control treatment were superior to those reported when using nonviral (52,64) or adenoviral vectors (13,14,22, 66,67). Whereas increases of up to 2.9-fold (proliferation) and 3.2-fold (anabolism) were evidenced with these other classes of vectors, we noted enhanced responses of up to 38.8- and 8.7-fold, respectively, with rAAV compared with the controls, probably due to the elevated, well-maintained (*in vitro*) or increasing (*in situ*) levels of IGF-I expression that may reflect the persistence of the rAAV transgenes in their targets (32). Interestingly, application of the candidate rAAV-hIGF-I vector had no detectable effect on the expression of type I collagen, an observation in good agreement with previous findings when applying an
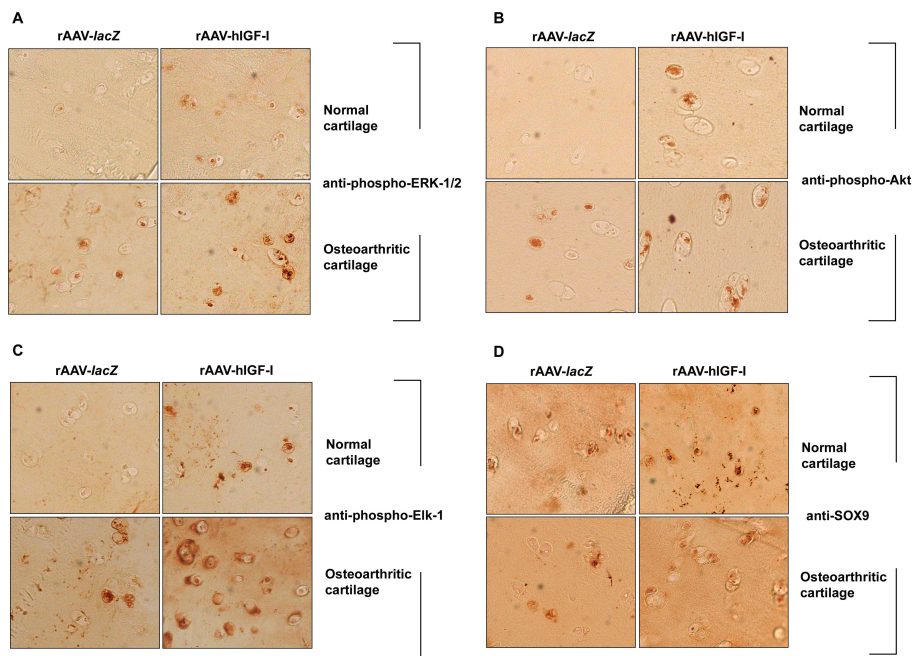
Figure 5. Expression of phospho-ERK-1/2, phospho-Akt, phospho-Elk-1 and SOX9 in rAAV-transduced human articular cartilage. Primary human normal and OA cartilage explant cultures were transduced with either rAAV-hIGF-I or the control rAAV-*lacZ* vector, as described in Figure 3, and processed after 90 d to detect the expression of phospho-ERK-1/2 (A), phospho-Akt (B), phospho-Elk-1 (C) and SOX9 (D). Magnification 20×. All are views of the middle zone.

IGF-I gene sequence to chondrocytes via adenoviral gene transfer (22,67). Quite strikingly, OA chondrocytes were particularly well responsive to the treatment by rAAV IGF-I here, in marked contrast with their otherwise established hyporesponsiveness to the growth factor (59,72). Of note, we also observed that IGF-I overexpression in OA cartilage *in situ* was capable of significantly decreasing the expression of IGFBP3 and IGFBP4, two inhibitors of the IGF-I actions (40–42), while increasing the levels of two of its potentiators (IGFBP5 and its own receptor [IGF-IR]) (42,43), an effect noted at prolonged time points and probably the result of the steady levels of IGF-I production from the rAAV transgene sequence. In this regard, the effects of IGF-I on these components of the IGF-I axis via rAAV in OA cartilage are in good agreement with previous findings when providing IGF-I as a recombinant molecule to articular chondrocytes *in vitro* (73–76). Also noteworthy, the ele-

vated, sustained levels of IGF-I produced in OA cartilage via rAAV might have been capable of counteracting the well-known effects of pathogenic adipokines on the destruction of articular cartilage (3,4). Of further note, the activating effects of IGF-I via rAAV on the expression of its receptor in normal and OA cartilage were associated with significant increases in the expression of effectors of the IGF-IR signal transduction pathways (MAPK/ERK-1/2 and PI3K/Akt) and downstream transcription factors (Elk-1, SOX9) described for their impact on cell proliferation and survival and on the synthesis of extracellular cartilage matrix components (proteoglycans, type II collagen) (38,39,77–79). These results are in good agreement with previous findings using recombinant IGF-I (35–37).

Importantly, we also report that overexpression of IGF-I in human OA cartilage led to higher levels of proliferation and biosynthesis than noted in control normal cartilage. It remains to be seen

whether elevated levels of activities will influence the cartilage structure over time. Smith *et al.* (22) and Brower-Toland *et al.* (67) reported that production of IGF-I via adenoviral vectors led to decreased amounts of matrix synthesis in chondrocytes at high vector doses (MOI = 200–500). Equally important, sustained overexpression of IGF-I via gene transfer may have undesirable effects on bone formation by stimulating the replication and metabolic activities of osteoblasts that might be targeted after growth factor release in an *in vivo* setting (72,80,81). Thus, regulation of IGF-I expression may be critical when developing an appropriate treatment for OA that does not further alter the cartilage and bone integrity. Instead of CMV-IE, expression might be controlled by regulated (tetracycline-sensitive), cartilage tissue-specific (SOX9, type II collagen) or disease-induced transcription elements (82,83).

Despite significant effects of IGF-I, application of the candidate rAAV vector did not fully restore the native architecture in OA cartilage, and additional treatments might be necessary to refine the processes of regeneration. Combined delivery with other factors, such as those that prevent cartilage degradation (IL-1Ra, sTNFR, IL-10) (12–14,21) or correct the OA phenotype in the cells (SOX9) (20,26,27), might be useful to further attenuate pathological events in OA. Again, rAAVs are strong tools, since they can be used for cotransduction strategies in OA chondrocytes and cartilage (20). Yet, recovery from cartilage degeneration by direct gene transfer will be practicable only with some remaining cartilage surface and cells (early OA), whereas transplantation approaches will be desirable for the advanced cases of the disease.

CONCLUSION

In summary, the present results demonstrate the ability of rAAV-mediated IGF-I overexpression to stimulate cellular activities in human OA chondrocytes *in vitro* and *in situ* over extended periods of time, making rAAV an advantageous vec-

tor to develop long-term effective therapies against human OA. Additional future studies will be required to test the effects of the construct *in vivo*, taking care of selecting an experimental model of OA that is the most adequate to reproduce the clinical situation among the many systems available to date (8,12,21,84) and of first reproducing the current findings using human cells by translation to such animal cells. The present findings provide motivation to further elaborate the current approach for human OA.

DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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