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ARTICLE

Improved Tissue Repair in Articular Cartilage Defects *in Vivo* by rAAV-Mediated Overexpression of Human Fibroblast Growth Factor 2

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Therapeutic gene transfer into articular cartilage is a potential means to stimulate reparative activities in tissue lesions. We previously demonstrated that direct application of recombinant adeno-associated virus (rAAV) vectors to articular chondrocytes in their native matrix *in situ* as well as sites of tissue damage allowed for efficient and sustained reporter gene expression. Here we test the hypothesis that rAAV-mediated overexpression of fibroblast growth factor 2 (FGF-2), one candidate for enhancing the repair of cartilage lesions, would lead to the production of a biologically active factor that would facilitate the healing of articular cartilage defects. *In vitro*, FGF-2 production from an rAAV-delivered transgene was sufficient to stimulate chondrocyte proliferation over a prolonged period of time. *In vivo*, application of the therapeutic vector significantly improved the overall repair, filling, architecture, and cell morphology of osteochondral defects in rabbit knee joints. Differences in matrix synthesis were also observed, although not to the point of statistical significance. This process may further benefit from cosupplementation with other factors. These results provide a basis for rAAV application to sites of articular cartilage damage to deliver agents that promote tissue repair.

Key Words: articular cartilage defects, chondrocytes, tissue repair, gene therapy, AAV, FGF-2

INTRODUCTION

The management of articular cartilage lesions, such as in joint trauma and osteoarthritis, remains a major unresolved problem due to the very limited intrinsic ability of articular cartilage to heal [1]. Diverse therapeutic options are currently employed to improve the quality of articular cartilage repair tissue, but restoration of a tissue similar to the native cartilage has not been achieved to date [1]. The introduction of gene candidates into articular cartilage defects in localized areas may represent a potent alternative approach to enhance tissue healing. Several studies have shown that reparative signals may be provided using nonviral [2,3] or viral vectors, including agents based on retroviruses [4] and adenoviruses [5,6]. Nevertheless, neither efficient nor stable transduction of the highly differentiated chondrocytes, in particular within their native matrix, has been achieved with most of these gene vehicles [7]. This is particularly important for the treatment of cartilage damage in conditions such as osteoarthritis, when the effects of a gene agent may be required over a relatively long period of time.

Recently, viral vectors derived from adeno-associated virus (AAV) have been successfully applied as an alternative gene delivery system to allow direct gene transfer into articular cartilage [8,9]. AAV is a replication-defective human parvovirus that is nonpathogenic. Most recombinant AAV (rAAV) generated to date have been derived from serotype 2 of the virus (AAV-2), although other AAV have been cloned and partially characterized. No serotype that displays a specific tropism for chondrocytes, or the bone marrow-derived mesenchymal stem cells (MSC) from which they may be derived, has been described. In generating rAAV vectors, all of the viral protein coding sequences can be deleted. Their diminished immunogenicity compared with adenoviruses make rAAV a particularly attractive gene transfer system

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for *in vivo* applications [10]. rAAV vectors also effectively transduce nondividing cells, unlike agents such as retroviruses [11]. This is essential for gene transfer into articular cartilage *in vivo,* since adult articular chondrocytes do not divide, or do so to only a limited extent [12]. Most rAAV transgenes persist as highly stable episomes that can be maintained and transcribed for months to years [13]. Consequently, sustained rAAV-mediated transgene expression can be achieved and has been documented for over 1.5 years in mouse skeletal muscle [14]. Using rAAV based on AAV-2 carrying reporter genes, we previously provided evidence that transgene expression could be achieved with high efficiency in isolated normal and osteoarthritic articular chondrocytes, both within their native matrix *in situ* to depths relevant for clinical applications and *in vivo* by direct vector administration [9,15]. Sustained transgene expression was demonstrated in these systems and may be sufficient to promote articular cartilage repair *in vivo* by overexpressing therapeutic genes [9]. The efficiency levels attainable with rAAV also minimize the need for the selectable markers and cell selection required when using retroviral vectors.

Fibroblast growth factor 2 (FGF-2) is a member of the multifunctional fibroblast growth factor family and a strong candidate factor for articular cartilage repair. Mitogenic properties have been ascribed to FGF-2 *in vitro* for articular and growth plate chondrocytes [16,17]. Enhancement of tissue repair has also been observed following the application of recombinant FGF-2 protein into articular cartilage defects *in vivo* [18]. Based upon our earlier success employing vectors derived from AAV-2, we tested the hypotheses that rAAV are capable of delivering a functional FGF-2 gene cassette to isolated articular chondrocytes and to sites of articular cartilage damage *in vivo*. We specifically examined the effects of rAAV-delivered FGF-2 on cell proliferation and matrix synthesis in chondrocytes *in vitro* and on the improvement of tissue repair in osteochondral defects in the knee joints of rabbits.

RESULTS AND DISCUSSION

rAAV-Mediated Expression of FGF-2 in Chondrocytes

Our construction and use of an AAV-2-derived *lac*Z vector, rAAV-*lac*Z, have been previously described [9,19]. The human FGF-2 sequence was substituted in this plasmid in place of *lac*Z, and both vectors were packaged as described under Materials and Methods. Primary rabbit articular cartilage chondrocytes were then transduced with either rAAV-hFGF-2 (hFGF-2, human basic fibroblast growth factor) or rAAV-*lac*Z in monolayer cultures. Two days after the addition of the vectors, the cells were encapsulated in alginate and maintained in three-dimensional cultures (alginate–chondrocyte constructs) in order to ascertain whether the FGF-2 transgene was expressed and the gene product released in a biologically active form. Immunohistochemical analysis performed on sections of alginate–chondrocyte constructs revealed that FGF-2 expression could be detected in a high proportion of cells forming the rAAV-hFGF-2-transduced (treated) constructs as well as in areas surrounding the cells (n = 6) (not shown), but not in the rAAV-*lac*Z-transduced (control) constructs (n = 6). Conversely, *lac*Z expression was seen only in cells forming the control constructs by immunohistochemistry (n = 6), a result confirmed by X-Gal staining. Transduction efficiencies were between 75 and 80%, which is consistent with previous data using rAAV [9]. Western blotting analysis of protein extracts from rabbit articular chondrocytes transduced with either rAAV demonstrated a single primary FGF-2 immunoreactive band of approximately 18 kDa (Fig. 1A) that was about fourfold more intense in cells transduced with rAAV-hFGF-2 than in controls exposed to rAAV-*lac*Z. The size of this product was in good agreement with a report by Luan *et al.* in chick chondrocytes [20]. This result shows that chon-

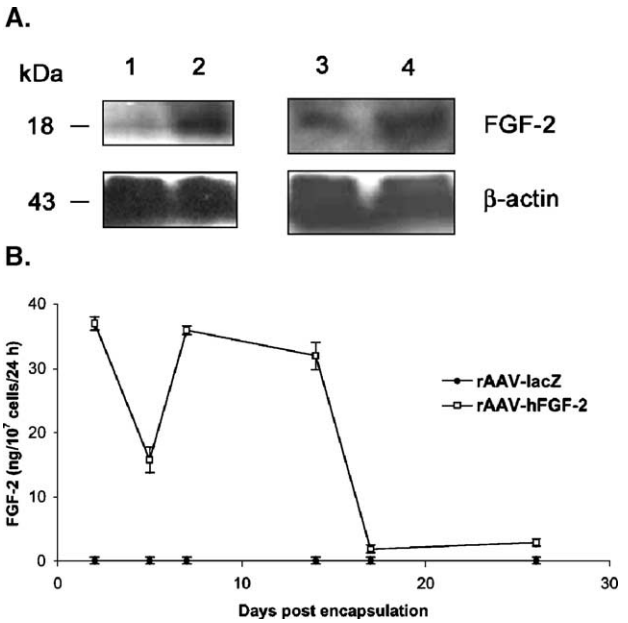

FIG. 1. Analysis of FGF-2 expression *in vitro*. (A) Western blotting of lysates from rabbit articular chondrocytes and rabbit bone marrow clots transduced by rAAV. Lane 1, extracts from rAAV-*lac*Z-transduced chondrocytes (10 μg); lane 2, extracts from rAAV-hFGF-2-transduced chondrocytes (10 μg); lane 3, extracts from rAAV-*lac*Z-transduced bone marrow clots (60 μg); lane 4, extracts from rAAV-hFGF-2-transduced bone marrow clots (60 μg). (B) Time course analysis of FGF-2 production in transduced alginate–chondrocyte constructs. Cells were transduced by rAAV-*lac*Z or rAAV-hFGF-2 and encapsulated in alginate 2 days after vector application. rAAV-*lac*Z- and rAAV-hFGF-2-transduced constructs were prepared and maintained in culture for 26 days. Conditioned medium was collected at the denoted time points after encapsulation (n = 9 per time point and condition) and FGF-2 production was measured by ELISA (±SD) with a detection limit of 3 pg/ml.

drocytes engineered with rAAV-hFGF-2 were induced to produce higher levels of the form of FGF-2 normally synthesized, rather than a novel isoform. For comparison, the FGF-2 isoforms were also examined in cultures of rabbit bone marrow clot cells [21]. An analysis of protein extracts from transduced clots again revealed the presence of a single FGF-2 immunoreactive band of about 18 kDa, which was several-fold more intense in the rAAV-hFGF-2-treated clots (Fig. 1A).

Secretion of FGF-2 in supernatants collected from transduced constructs was monitored by ELISA. Prior to encapsulation (2 days after transduction), production of FGF-2 in the monolayer cultures was 12.00 ± 0.71 ng/10^7 cells/24 h in rAAV-hFGF-2-transduced chondrocytes, while levels were below the limit of detection in the control chondrocytes. After encapsulation, the secretion of FGF-2 from the treated constructs was noted as early as day 2 post encapsulation (37.00 ± 0.70 ng/10^7 cells/24 h) (Fig. 1B). The early onset of FGF-2 expression in the chondrocytes *in vitro* is consistent with the relatively high permissivity of these cells to these vectors [9]. A second peak of secretion was observed at day 7. Elevated concentrations of FGF-2 were present until day 14, followed by lower but still detectable levels until day 26, the longest time period examined. In marked contrast, time course measurements of FGF-2 secretion from the control constructs revealed that the levels of FGF-2 remained below the threshold of detection of the assay at each time point of the analysis. Sustained transgene expression has been documented in encapsulated chondrocytes carrying the *Photinus pyralis* luciferase [22], *lac*Z [23], and GFP (green fluorescent protein) marker genes [24]. Notably, the proteins encoded by these genes are all expressed intracellularly, whereas FGF-2 is a peptide secreted in the extracellular compartment. As a heparin-binding growth factor that does not possess a conventional secretion signal [25], FGF-2 remains mostly cell-associated, presumably through interactions with matrix proteoglycans after export across the plasma membrane [26,27], without a loss of biological activity [28]. Because FGF-2 also has an affinity for alginate, as it is an acidic polysaccharide similar to glycosaminoglycan [29], levels of FGF-2 measured in culture supernatants are likely underestimates, particularly later in the time course, as the density of the extracellular matrix continues to increase. To confirm that the protein signal noted resulted from intracellularly synthesized FGF-2 rather than from a soluble fraction carried in the vector preparation, we measured the amounts of FGF-2 present in the supernatants of monolayer cultures transduced by rAAV-*lac*Z in the presence or absence of a high dose (20 ng) of recombinant FGF-2 ($n = 6$ per condition). A transient signal was observed for 2 days after the addition of the recombinant peptide (16 ng/10^7 cells/24 h) but not beyond this time, out to 12 days, the longest time period evaluated.

Biological Effects of rAAV-Mediated FGF-2 Production on Chondrocytes *in Vitro*

On the day of encapsulation, after 8 h in culture, the constructs harboring the rAAV-*lac*Z-transduced cells (2.89 ± 0.01 mm in diameter) averaged $0.79 \pm 0.03 \times 10^4$ viable cells/construct. By contrast, treated constructs (3.01 ± 0.01 mm in diameter; $P < 0.001$) contained $1.34 \pm 0.14 \times 10^4$ viable cells/construct ($P < 0.001$). The higher cell numbers noted initially in the treated constructs likely resulted from the proliferative activity of FGF-2 during the 2-day posttransduction period prior to encapsulation, as suggested by the ELISA results (Fig. 1B). At the end of the evaluation period (26 days), the number of cells in the treated constructs averaged $1.76 \pm 0.20 \times 10^4$ viable cells/construct for a diameter of 3.21 ± 0.01 mm, showing good maintenance of the cells in the constructs ($P < 0.001$), as well as an increase in their total volume ($P < 0.001$). In contrast, cell numbers in the control constructs ultimately declined to $0.13 \pm 0.01 \times 10^4$ viable cells/construct ($P < 0.001$), with a corresponding decrease in the volume of the constructs (2.77 ± 0.01 mm in diameter; $P < 0.001$). Consistent with this, viability in the control constructs was only 31% at the end of the evaluation period, much lower than in the treated constructs (86%) and a dramatic decline from the initial viability of 80% when they were established. The differences in outcome were therefore a combination of increased viability, as well as an increased index of cell division produced by FGF-2. Histological analysis of sections of constructs showed that the number of cells stained by hematoxylin and eosin (H&E) was more elevated (about 3-fold) in the treated constructs (Fig. 2C) compared to the control constructs (Fig. 2A). Type-II collagen staining was evident on sections prepared from both the control (Fig. 2B) and treated constructs (Fig. 2D) and extended well beyond the cell-associated matrix, in agreement with reports of collagen production in this culture system [23]. The total amount of proteoglycan (PG) produced by the constructs after 26 days in culture was not significantly different between the treated (4.58 ± 0.21 μg/10^4 cells) and the control constructs (4.13 ± 0.63 μg/10^4 cells) ($P = 0.233$). In contrast, the DNA content of the treated constructs (1.45 ± 0.04 μg/10^4 cells) was significantly higher (6.9-fold) than in the control constructs (0.21 ± 0.02 μg/10^4 cells) ($P < 0.001$). These results were consistent with the established mitogenic activity of FGF-2 [16].

rAAV-Mediated Transfer and Expression of FGF-2 *in Vivo*

Encouraged by the findings in the alginate–chondrocyte constructs, the vectors were next tested in an animal model. Each vector (10 μl) was directly applied to osteochondral defects created in the patellar groove of knee joints in rabbits [15], a situation analogous to the

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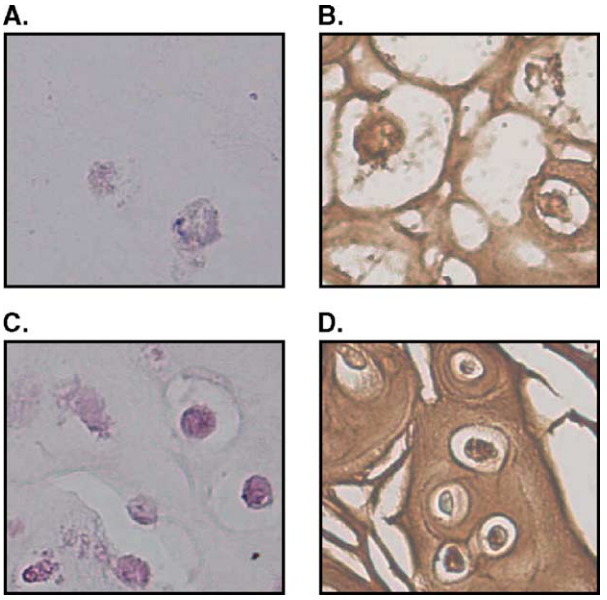

FIG. 2. Histological sections of transduced alginate–chondrocyte constructs. rAAV-*lacZ*- (A and B) and rAAV-hFGF-2-transduced constructs (C and D) were histologically processed at day 26 after encapsulation ($n = 6$ per condition) and analyzed for HE staining (A and C) and for immunohistological detection of type-II collagen with a mouse anti-type-II antibody (1:100), using a biotinylated goat anti-mouse antibody (1:200). Revelation was performed by the ABC method using DAB as the chromogen. Samples were examined under light microscopy. Original magnification, ×20.

common clinical circumstance in which defects penetrate the subchondral bone [1]. Macroscopic examination of knees retrieved at day 10 after vector administration showed that both rAAV-hFGF-2- and rAAV-*lac*Z-treated defects were filled to the level of the articular surface with repair tissue that was whiter and softer than the surrounding host cartilage. After 20 days, this initial repair tissue was well integrated with the surrounding cartilage in both types of defects. The color of the new tissue closely resembled that of the host cartilage, but the margins of the defects were still visible. Four months after vector application in both types of defects, the color of the defects was similar to that of the surrounding cartilage and the margins of the defects were difficult to discern. rAAV application *in vivo* was well tolerated, with no signs of synovitis, adhesions, or adverse reactions, and no macroscopically descriptive differences between joints that received rAAV-*lac*Z or rAAV-hFGF-2 at any time point. Immunohistochemical analysis of tissue sections using specific antibodies to screen for CD3- (T-lymphocytes), CD11b- (activated macrophages), or HLA-DRα- (class II MHC antigens) positive cells [30] revealed no immune cell infiltration of the defects in knees exposed to either rAAV at any time point during the period of observation. The absence of immune system provocation over the period of observation is an additional mitigating factor favoring the use this class of vector in joints, in contrast to the use of more immunogenic agents such as adenoviruses [10].

*lac*Z expression was analyzed by X-Gal staining and by indirect immunohistochemistry to detect β-gal activity. A strong signal was observed in all the defects to which rAAV-*lac*Z had been applied after 10 days (Figs. 3A and C), in contrast to findings in knees treated with rAAV-hFGF-2 (Figs. 3B and D). After 20 days, the staining was milder, as observed by macroscopic examination, but β-gal reactivity could still be seen in the cells filling the defects by histological analysis of serial sections (Fig. 3E). At 4 months, areas of transgene expression in cells within the repair tissue were still noted by immunohistochemistry (Fig. 3G).

On histological transverse sections of rAAV-hFGF-2-treated knees, FGF-2 expression was detected as early as day 10 after vector administration (Fig. 4B), in contrast to control samples (Fig. 4A). Staining was persistent at day 20 (Fig. 4D), and, although reduced, the specific signal was still present 4 months after application (Fig. 4F). These results show that direct application of the FGF-2 gene sequence via rAAV allowed for sustained overexpression in osteochondral articular cartilage defects, extending our previous findings that showed *in vivo* reporter gene expression for up to 20 days [9,15]. The use of this vector should therefore prove advantageous over agents mediating short-term transgene expression [10]. FGF-2 expression was detected in the cells forming the repair tissue through their full thickness, as previously observed with rAAV bearing reporter genes [9].

This ability to transfer genes in depth within articular cartilage lesions makes rAAV particularly attractive for this type of application [9,31] and indicates that the cells transduced by rAAV include bone marrow-derived MSC that migrate into the site of injury. MSC are considered the principal cells that repopulate such full-thickness defects [31], undergoing chondrocytic differentiation upon stimulation by FGF-2 and other growth factors [32,33]. Although MSC migration is rapid [31], this observation further indicates that rAAV persists for several days after delivery into the defect. Consistent with this, Chamberlain *et al.*, among others, have reported that MSC are permissive to rAAV transduction *in vitro* [34]. Transgene expression was observed not only within the site of regeneration, but in chondrocytes residing in the surrounding intact articular cartilage, primarily localized within the internal zones adjacent to the defects [35]. It is likely that long-term FGF-2 production by these cells, as well as by transduced MSC repopulating the defects themselves, both contribute to the enhanced level of cartilage regeneration induced by the gene treatment. The *in vitro* experiments are also consistent with this conclusion and extend our appreciation of the effects of therapeutic rAAV upon metabolic changes in these cells.

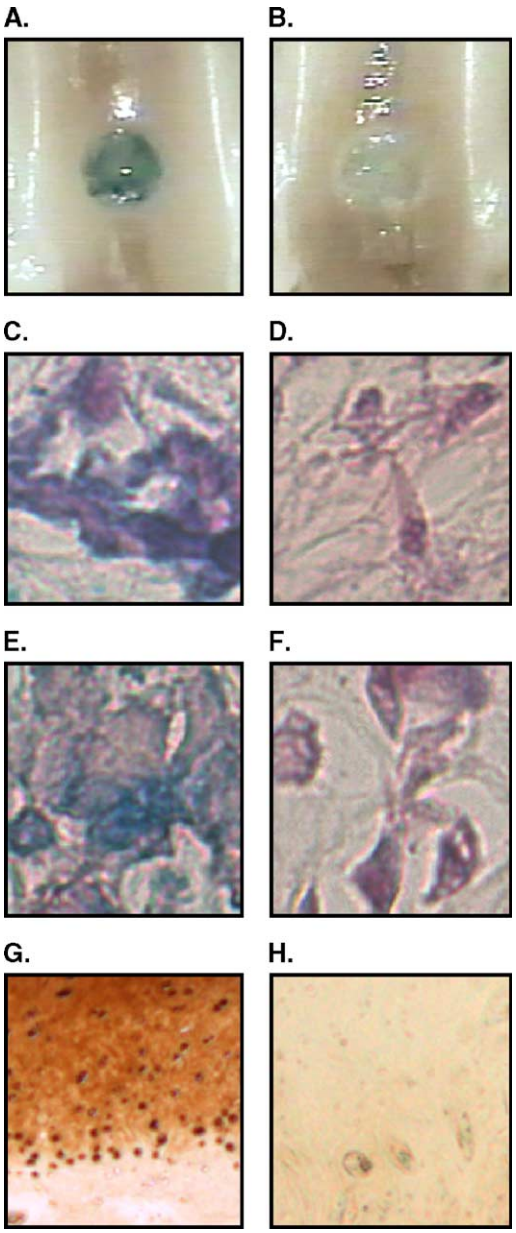

FIG. 3. Representative analysis of *lac*Z expression in osteochondral cartilage defects *in vivo*. Transgene expression was detected by X-Gal staining in knees retrieved 10 days (A–D; C and D, original magnification, ×100) or 20 days (E and F, original magnification, ×100) after vector application and by immunohistochemistry using a mouse anti-β-gal antibody (1:50) in knees retrieved 4 months postadministration (G and H, original magnification, ×20), as described in the legend to Fig. 2 and under Materials and Methods. (A, C, E, and G) Application of rAAV-*lac*Z (10 μl); (B, D, F, and H) application of rAAV-hFGF-2 (10 μl).

Mild staining was also apparent in a few parts of the synovium of joints to which rAAV-hFGF-2 was applied, at all three time points, as well as in muscle cells of the quadriceps muscle adjacent to the patella, and in the infrapatellar fat pad, although the levels of expression in these sites were always less elevated than those noted within the defects. This observation probably reflects an intraarticular distribution of rAAV after closure of the arthrotomy, with resulting synovial transduction [9,15]. More extensive synovial gene transfer has been reported using vectors other than rAAV [7].

FGF-2 expression was not detectable in the subchondral bone marrow, or in the more distant marrow (central cavity of the femora) at any time point by immunohistochemistry. Analysis of FGF-2 concentrations in the synovial fluid and blood by ELISA also showed no differences between control and rAAV-hFGF-2 treatment groups, nor between these groups and samples from rabbits in which no osteochondral defects were created, at any time point. The observation of minimal transgene expression in nontarget tissues of the knee joint cavity and the absence of contamination at the periphery are consistent with the procedure employed to inject our vectors in the defects, i.e., by direct application in opened

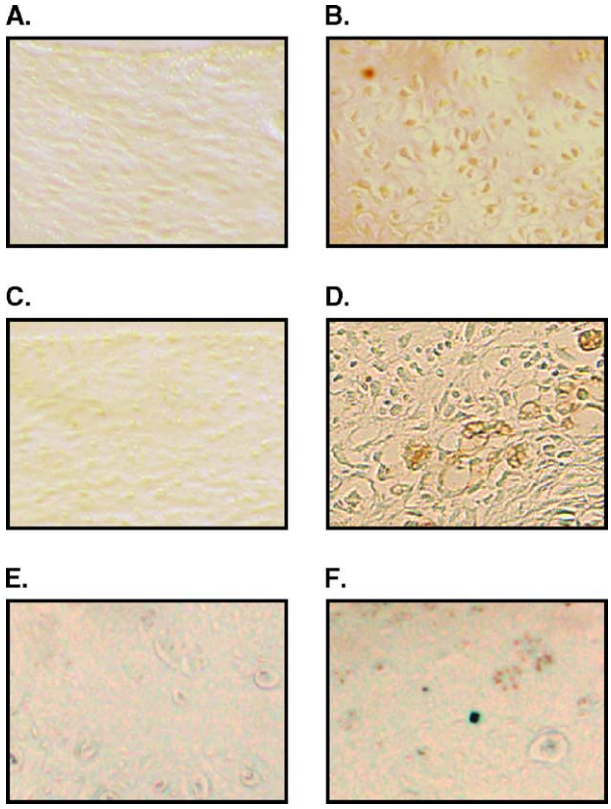

FIG. 4. Representative analysis of FGF-2 expression in osteochondral cartilage defects *in vivo*. Transgene expression was detected by immunohistochemistry in sections from knees retrieved 10 days (A and B), 20 days (C and D), and 4 months (E and F) after vector application using a mouse anti-FGF-2 antibody (1:100), as described in the legend to Fig. 2 and under Materials and Methods. (A, C, and E) Application of rAAV-*lac*Z (10 μl); (B, D, and F) application of rAAV-hFGF-2 (10 μl). Original magnification, ×20.

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knees [9]. This expression pattern contrasts with the more overt diffusion of vectors that can occur with simple intraarticular injection [35].

Effects of rAAV-Mediated Production of FGF-2 *in Vivo*

No immunoreactivity to type-II collagen was detectable in any of the defects at day 10 after vector addition (not shown), but was apparent at day 20 (Figs. 5A and C). After 4 months, type-II collagen staining in the defects treated with rAAV-hFGF-2 (Fig. 5D) was more intense than that observed in the defects that received rAAV-*lac*Z (Fig. 5B) and was also more regular and consistent with that noted in the surrounding articular cartilage. In contrast, type-I collagen immunoreactivity was reduced over time, in particular when the defects were treated with rAAV-hFGF-2 (Fig. 5H).

On histological sections stained by safranin O, limited amounts of extracellular matrix were observed 10 days after application of the vectors (not shown). At this time point, the defects were filled with repair tissue composed of spindle-shaped cells with elongated nuclei. By day 20, matrix staining was more intense in the defects that received treatment with rAAV-hFGF-2 (Fig. 6D). The presence of round cells exhibiting the morphology of

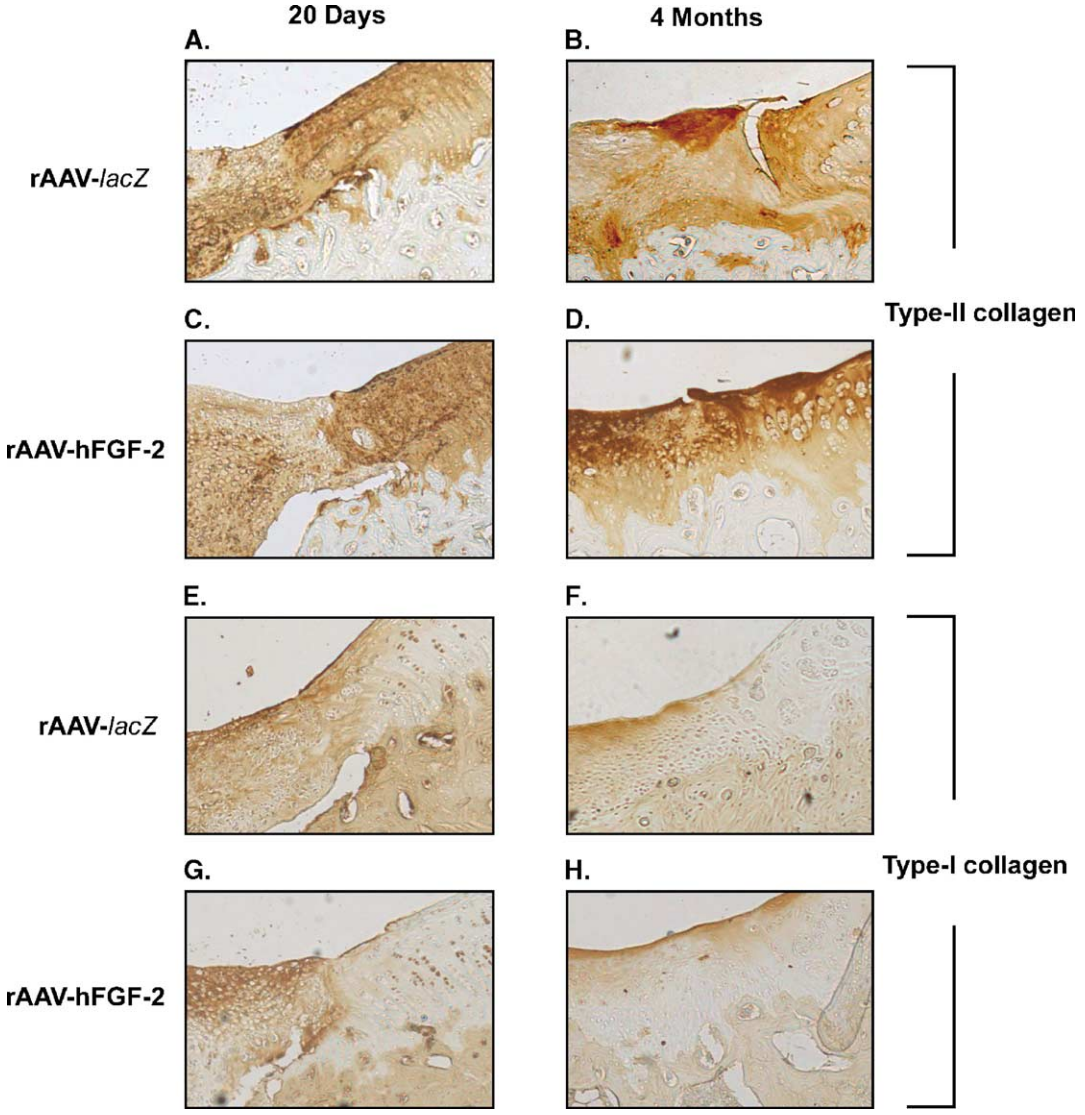

FIG. 5. Analysis of type-II and type-I collagen expression in osteochondral cartilage defects *in vivo*. Immunostaining was performed in sections from knees retrieved 20 days (A, C, E, and G) and 4 months (B, D, F, and H) after vector application using a mouse anti-type-II collagen antibody (1:50) (A–D) and a mouse anti-type-I collagen antibody (1:100) (E–H), as described in the legend to Fig. 2 and under Materials and Methods. (A, B, E, and F) Application of rAAV-*lac*Z (10 μl); (C, D, G, and H) application of rAAV-hFGF-2 (10 μl). Original magnification, ×4.

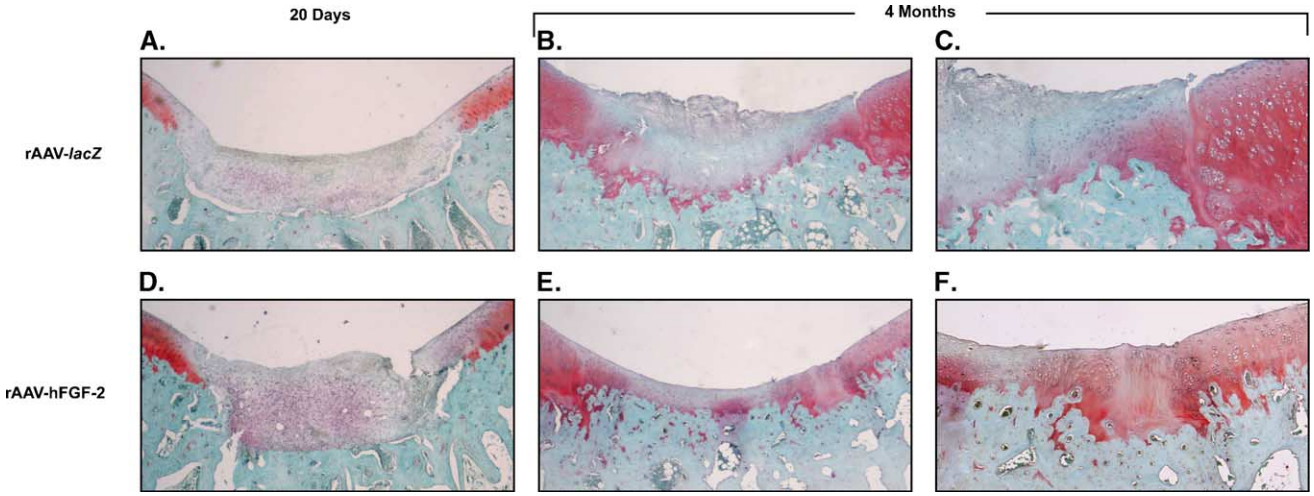

FIG. 6. Histological sections of osteochondral cartilage defects. Safranin O staining was performed on sections from knees retrieved 20 days (A and D, original magnification, ×2) and 4 months after vector application (B and E, original magnification, ×2; C and F, original magnification ×4). (A–C) Application of rAAV-*lacZ* (10 μl); (D–F) application of rAAV-hFGF-2 (10 μl).

chondrocytes was also most evident in defects to which rAAV-hFGF-2 had been applied. Four months after vector administration, enhanced tissue healing and organization were observed in the defects that received treatment with rAAV-hFGF-2 (Figs. 6E and 6F) compared to the controls (Figs. 6B and 6C). The bone front under the defects also appeared shifted upward in those receiving the rAAV-hFGF-2 vector.

Using a grading system developed for the quantitative assessment of articular cartilage defect repair [36], significant improvement of individual histological parameter scores was observed in defects receiving rAAV-hFGF-2 after 4 months for the filling and architecture of the defects ($P < 0.05$ and $P < 0.01$, respectively) as well as cell morphology ($P < 0.001$) (Table 1). The therapeutic treatment resulted in the appearance of many round cells with the typical phenotype and columnar organization of chondrocytes within the new cartilage. By contrast, very few cells with this appearance were seen within control defects, even after 4 months. Other individual parameter scores, such as matrix synthesis, were improved but did not reach statistical significance at this time. The total score of the histological grading was also significantly improved for defects receiving the rAAV-hFGF-2 treatment ($P < 0.01$). These observations are in good agreement with the reported ability of FGF-2 to modulate tissue healing, cell differentiation, and proliferation *in vivo*, when applied as a recombinant factor [18], and to stimulate chondrocyte mitotic activity but not matrix synthesis in a model of genetically modified chondrocytes transplanted *ex vivo* [37]. However, using rAAV to deliver FGF-2 improves the transfer of this therapeutic

TABLE 1: Effects of FGF-2 gene transfer and expression on histological grading of the repair tissue 4 months after rAAV application

Category	rAAV-*lacZ* mean (95% CI)	rAAV-hFGF-2 mean (95% CI)	F-test	*P* value[†]
Filling of defect	1.22 (0.82–1.64)	0.40 (0.02–0.78)	5.75	<0.05[†]
Integration	1.73 (1.36–2.10)	1.27 (0.91–1.64)	1.95	0.08
Matrix staining	2.45 (1.80–3.11)	1.88 (1.22–2.53)	1.40	0.19
Cell morphology	2.98 (2.40–3.57)	1.34 (0.75–1.92)	19.49	<0.001[†]
Architecture of defect	2.73 (1.88–3.58)	1.05 (0.30–1.80)	9.78	<0.01[†]
Architecture of surface	2.63 (2.07–3.19)	1.94 (1.38–2.50)	1.94	0.08
Subchondral bone	2.15 (1.49–2.82)	1.36 (0.70–2.03)	1.87	0.09
Tidemark	2.43 (1.71–3.15)	1.87 (1.15–2.60)	1.46	0.25
Average total score	18.5 (15.5–21.2)	11.0 (8.2–14.0)	15.65	<0.01[†]

Each category and total score are based on the average of two independent evaluators. Points for each category and total score were compared between the rAAV-hFGF-2 and rAAV-*lacZ* groups using a mixed general linear model with repeated-measures analysis of variance (knees tested within the same animals; CI, confidence interval). A cumulative score of 0 indicates complete healing; a total score of 31 indicates no healing. Means indicate the estimated scores in points for each category.

[†] Significant treatment effect.

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agent into joint cartilage in terms of delivery efficiency and duration, as well as reduces the risk of inflammatory immune responses compared with other vectors.

The ability of rAAV to transfer bioactive therapeutic gene sequences to enhance the healing process of articular cartilage lesions, particularly into defects that necessitate cellular repopulation, is highly promising from the perspective of therapeutic development. In the present model system, the regeneration of native articular cartilage at the site of the defect was not complete at the end of the evaluation period. A similar finding has been noted when using FGF-2 as a recombinant protein [18]. The shift apparent in the bone front beneath cartilage lesions exposed to the rAAV-hFGF-2 vector is intriguing in light of the effects of FGF-2 reported upon osteogenesis and osteoblast function and may have implications for the long-term durability of the repair tissue. The delivery of more than one therapeutic agent capable of augmenting articular cartilage repair or modulating the differentiation of MSC progenitors may be necessary to fully reproduce the normal articular tissue [38]. To achieve this, combinations of rAAV vectors can be delivered together [39]. Sequential challenges with the same or different vectors can also be performed under conditions under which immune interference can be avoided [40]. In this way, the inclusion of candidate factors that promote matrix synthesis, such as insulin-like growth factor-I [16,41] and transforming growth factor-β [41,42], can also be evaluated. The regulation of transgene expression levels and duration can also be provided where desirable [43], as some factors may inhibit tissue repair over time at high doses [17,41,44]. For example, the application of recombinant FGF-2 has been reported to lead to the downregulation of cell surface FGF-2 receptors [18] and desensitization. In summary, the results of this study demonstrate that therapeutic rAAV can enhance articular cartilage repair by direct application to sites of cartilage damage. The findings provide motivation for further research into the optimization of beneficial gene transfer approaches to treat articular cartilage diseases.

MATERIALS AND METHODS

Antibodies, kits, and chemicals. Collagenase type I (activity, 232 U/mg) was purchased at Biochrom (Berlin, Germany). Alginate, papain, chondroitin sulfate, and Hoechst 33258 were from Sigma (Munich, Germany). The dimethylmethylene blue (DMMB) dye was obtained from Serva (Heidelberg, Germany). The recombinant FGF-2 peptide was purchased at R&D Systems GmbH (234-FSE; Wiesbaden, Germany). The monoclonal mouse anti-type-I and anti-type-II collagen antibodies (Medicorp AF-5610 and AF-5710) were purchased at Acris Antibodies GmbH (Hiddenhausen, Germany). The monoclonal mouse anti-β-gal antibody (GAL-13) was from Sigma. The monoclonal mouse anti-human FGF-2 antibody (Ab-3) was obtained from Oncogene Research Products (Darmstadt, Germany). Quantitative measurements of FGF-2 production were performed using the human FGF basic Quantikine ELISA (DFB50; R&D Systems GmbH) with a detection limit of 3 pg/ml.

Cells. Rabbit chondrocytes were prepared and maintained in culture as previously described [22]. All assays were performed with chondrocytes at passage 2, 10–14 days after isolation. The 293 line, an adenovirus-transformed human embryonic kidney cell line, was maintained in Eagle's minimal essential medium containing 10% FBS and antibiotics.

Plasmids, rAAV vector packaging, and titration. rAAV-*lac*Z is an AAV-2 vector plasmid containing the *lac*Z reporter gene under the control of the CMV-IE promoter and the simian virus 40 small t antigen intron/polyadenylation signal [9,19] and was employed as a control and to verify the efficiency of gene transfer and expression of rAAV in the targets. rAAV-hFGF-2 carries a 480-bp human basic fibroblast growth factor (hFGF-2) cDNA fragment [45] that was cloned in rAAV-*lac*Z in place of the *lac*Z gene. rAAV were packaged using adenovirus 5 to provide helper functions, in combination with the *trans*-acting AAV factors supplied by pAd8, as previously described [9,19,46]. Purified vector preparations were obtained by dialysis, a method successfully employed for gene transfer approaches *in vivo* [9,19]. Titers of the vector preparations screened by real-time PCR [9,19] were on the order of 10^{10} functional units/ml.

Cell transduction and encapsulation in alginate. Chondrocytes (10^6 cells) were transduced with the vectors (300 μl) as previously described [9]. Encapsulation of transduced cells in alginate was then carried out as previously described [22,23]. The cultured alginate–chondrocyte constructs were assessed for diameter, cell number, and viability at days 0, 2, 5, 7, 14, 17, and 26 postencapsulation [22]. Single constructs were solubilized and the released chondrocytes were counted and their viability assessed using a Neubauer chamber and trypan blue exclusion staining based on four counts per sample.

Gene transfer to articular cartilage defects in vivo. All animal procedures were approved by the Saarland University Animal Committee according to German guidelines and have been described [9,15]. Eleven female chinchilla bastard rabbits (mean weight 2.6 ± 0.4 kg; Charles River, Sulzfeld, Germany) (two animals for the time point of 10 days; two animals for 20 days; seven animals for 4 months) were employed for the study. The animals were determined to be in their late juvenile stage by histological analysis of their growth plate, which contained few layers of chondrocytes. A cylindrical osteochondral cartilage defect was created in the middle of each patellar groove (n = 22 defects) with a manual cannulated burr (3.2 mm in diameter). Care was taken not to perforate the subchondral plate. Defects were washed with saline and blotted dry, and 10 μl of rAAV was applied. Each animal received rAAV-*lac*Z treatment on one knee and rAAV-hFGF-2 treatment on the contralateral knee. Control and experimental treatments were evenly distributed between the right and left knees. One rabbit was removed from the protocol because of death following a gastrointestinal infection 4 months postoperation. At 10 days (n = 2), 20 days (n = 2), and 4 months (n = 6) postoperation, the animals were euthanized and the knee joints were exposed and examined grossly for synovitis, contractures, adhesions, or other adverse reactions. The appearance of the repair tissue (color, integrity, contour) and articular surfaces was noted. The distal femora with adjacent synovium were removed and subjected to transgene expression and histological analyses.

Histological evaluations and immunohistochemical analyses. Alginate–chondrocyte constructs and retrieved knees were histologically processed as previously described [22,23]. Paraffin-embedded sections (5 μm) were stained with safranin O to detect proteoglycans and with H&E to detect cells according to routine protocols [22]. Serial histological sections of distal femora were taken at 200-μm intervals. All sections were taken within approximately 1.2 mm from the center of the defects (n = 6–12 per defect). All articular cartilage sections were graded blind by two individuals independently using a standard articular cartilage repair scoring system that rates nine different parameters (a cumulative score of 0 indicates complete regeneration; a total score of 31 indicates an empty defect, i.e., no healing) [36]. Each section was scored, and all scores for each treatment group were combined to determine the mean score for each group. A total of 109 sections were scored.

Immunohistochemical detection of type-I and type-II collagen expression was performed on paraffin-embedded sections by indirect

immunostaining using specific primary antibodies and a biotinylated goat anti-mouse antibody (Vector Laboratories, Alexis Deutschland GmbH, Grünberg, Germany), according to routine protocols. Revelation was performed with the ABC method (Vector Laboratories) using diaminobenzidine (DAB) as the chromogen. To control for secondary immunoglobulins, sections were processed with the omission of the primary antibody. Samples were examined by light microscopy using an Olympus microscope (BX 45; Hamburg, Germany).

Analyses of transgene expression. Detection of β-gal activity was performed by X-Gal staining using a standard method [9,19]. Expression of the transgenes was also determined by immunohistochemistry using specific antibodies. The presence of specific immunostaining was examined within the repair tissue and in the intact surrounding articular cartilage, as well as in the synovium, quadriceps muscle adjacent to the patella, infrapatellar pad, subchondral bone marrow, and bone marrow in the central cavity of the femora.

To monitor FGF-2 secretion, transduced samples were washed twice and placed for 24 h in serum-free medium. Supernatants were next collected at the denoted time points and centrifuged to remove cell debris. FGF-2 production was measured by ELISA in these samples, as well as in the synovial fluid and blood (ear vein puncture) from rAAV-treated animals and from rabbits in which no osteochondral defects were created [37].

Western blotting analyses. Rabbit bone marrow clots were prepared as previously described [21]. Under sterile surgical conditions, approximately 1 ml of bone marrow was aspirated from each femur and aliquots of 500 μl were rapidly mixed with 100 μl rAAV-*lac*Z or rAAV-hFGF-2. The mixtures were allowed to coagulate and the clots were then placed in individual wells of 24-well plates. Transduction of primary cultures of rabbit articular chondrocytes (0.4×10^6) was performed in parallel using 100 μl rAAV. 20 days later, the transduced articular chondrocytes and clots were processed according to standard protocols to detect the expression of FGF-2 and β-actin by Western blotting using specific antibodies [20]. Revelation was performed with horseradish peroxidase-labeled secondary antibodies (Vector Laboratories) using the ECL Advance Western blotting detection kit (Amersham Biosciences Europe GmbH, Freiburg, Germany).

Measurements of DNA and matrix component contents in alginate–chondrocyte constructs. Constructs were solubilized and samples were digested in papain solution [22,47]. The PG concentrations were measured by binding to the DMMB dye [47]. The DNA content was determined with a fluorimetric assay using Hoechst 33258 [47,48]. Measurements were performed using a GENios spectrophotometer/fluorometer (Tecan Deutschland GmbH, Crailsheim, Germany).

Statistical analysis. Each test condition *in vitro* was performed in triplicate in three independent experiments for each time point and with 12 defects for the time point of 4 months for the *in vivo* experiments. Data are expressed as the means ± standard deviation (SD) of separate experiments. The *t* test and the Mann–Whitney rank sum test were employed for the *in vitro* experiments when appropriate. To evaluate the *in vivo* experiments, points for each category and total score were compared between the two groups using a mixed general linear model with repeated-measures analysis of variance (knees tested within the same animals). Data are expressed as the means ± 95% confidence interval. Any *P* value of less than 0.05 was considered statistically significant.

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