Expedited gene delivery for osteochondral defect repair in a rabbit knee model: A one-year investigation

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Abstract

Objective: To evaluate a single-step, gene-based procedure for repairing osteochondral lesions.

Design: Osteochondral lesions were created in the patellar groove of skeletally mature rabbits. Autologous bone marrow aspirates were mixed with adenovirus vectors carrying cDNA encoding green fluorescent protein (Ad.GFP) or transforming growth factor-β_1 (Ad.TGF-β_1) and allowed to clot. The clotted marrow was press-fit into the defects. Animals receiving Ad.GFP were euthanized at 2 weeks and intra-articular expression of GFP examined by fluorescence microscopy. Animals receiving Ad.TGF-β_1 were euthanized at 3 months and 12 months; repair was compared to empty defects using histology and immunohistochemistry. Complementary *in vitro* experiments assessed transgene expression and chondrogenesis in marrow clots and fibrin gels. In a subsequent pilot study, repair at 3 months using a fibrin gel to encapsulate Ad.TGF-β_1 was evaluated.

Results: At 2 weeks, GFP expression was seen at variable levels within the cartilaginous lesion. At 3 months, there was no statistically significant improvement ($p > 0.05$) in healing of lesions receiving Ad.TGF-β_1 and variability was high. At 12 months, there were still no significant difference ($p > 0.05$) between the empty defects and those receiving Ad.TGF-β_1 in the overall, cartilage, and bone scores. Variability was still high. In vitro experiments suggested that variability reflected variable transduction efficiency and chondrogenic activity of the marrow clots; using fibrin gels instead of marrow may address this issue but more research is needed.

Conclusions: This approach to improving the repair of osteochondral lesions needs further refinement to reduce variability and provide a more robust outcome.

1. Introduction

Trauma to articular cartilage is found in 60% of knee arthroscopies [1]. A majority of lesions include injuries to both the cartilage and subchondral bone (osteochondral defects) [1]. These defects do not heal spontaneously and, if left untreated, can cause pain and dysfunction leading to post-traumatic osteoarthritis. Cartilage has no innate ability to repair, because the tissue is avascular, aneural, alymphatic, and does not contain stem or progenitor cells [2]. This has led to cartilage repair surgery being the current clinical standard of care for patients with chondral lesions; surgeons perform approximately 500,000 cartilage repair procedures annually in the US [3].

Autologous chondrocyte implantation (ACI) and microfracture are two popular cell-based therapies used by surgeons to treat chondral lesions. ACI has shown great promise as a cartilage repair procedure. However, this is a two-step procedure which involves harvesting cartilage from the periphery of the joint, expanding the isolated chondrocytes in culture, and transplanting these cells back into the defect to regenerate tissue [4]. This increases the cost of the procedure, and patients incur a prolonged post-operative rehabilitation period during which full weight bearing is delayed [5,6]. In contrast, microfracture is a one-step, point of care procedure which involves exposing and penetrating the subchondral plate underneath the cartilage defect to allow bone marrow to enter and form a fibrin clot within the lesion [7]. The coagulated bone marrow contains mesenchymal stromal cells (MSCs)

with the potential to differentiate into chondrocytes which initiate the repair process [7]. Microfracture has demonstrated promising short-to-medium term relief for many patients allowing them back to previous levels of activity [8]. However, according to multiple long-term reports both ACI and microfracture have high failure rates and patients are likely to develop repair tissue resembling a fibrocartilaginous scar instead of native articular cartilage [9–14]. Fibrocartilage has inferior biomechanical properties and does not endure the joint environment. Its deterioration causes pain, dysfunction, and eventual failure of the repair, requiring salvage surgery.

The present study is based on the hypothesis that the long-term clinical outcome of microfracture and related marrow stimulation techniques would be improved if the MSCs differentiated fully into articular chondrocytes instead of fibrochondrocytes. Morphogens, such as TGF-β_1, hold promise in this respect but they are difficult to deliver to osteochondral lesions in a sustained fashion. Gene transfer offers a technology for overcoming this barrier, and we have shown that MSCs transduced with adenovirus vectors encoding TGF-β_1 undergo efficient chondrogenesis [15,16]. Based upon these considerations, the research described in this paper investigates a gene therapy approach for osteochondral defect repair.

We have developed a technology in which autologous bone marrow coagulates incorporating adenoviral vectors are used for gene transfer to osteochondral defects [17]. The MSCs within the coagulate are transduced by the adenovirus, and the fibrin scaffold retains additional vector for transducing MSCs as they enter the lesion. The marrow clot also has excellent handling properties and conforms to the dimensions of the structure within which it clots. Sieker et al. [18] have published promising results using the marrow clot technology in conjunction with adenovirus encoding bone morphogenetic protein 2 (BMP-2) and Indian hedgehog (IHH) transgenes. While successful in the short term, defects receiving BMP-2 progressively formed bone, while the cartilage in those receiving Indian hedgehog was immature and the subchondral bone absent.

The primary objective of the study is to investigate whether delivering adenoviral vectors carrying TGF-β_1 cDNA (Ad.TGF-β_1) using autologous bone marrow coagulates can improve the long-term (12-months) repair of osteochondral defects in the rabbit knee. We also evaluate a small number of rabbits at an early (3-month) time point. We hypothesize that rabbit knee's receiving autologous bone marrow clots with Ad.TGF-β_1 would demonstrate significantly better long-term osteochondral defect repair than empty defects.

2. Methods

2.1. Study overview

We first created three different groups using the knees of New Zealand white rabbits. These groups included: 1) an empty osteochondral defects group (control), (2) autologous bone marrow clots (BMCs) with adenovirus carrying the enhanced green fluorescent protein (GFP) cDNA (BMC + Ad.GFP) group, and (3) autologous bone marrow clots with Ad.TGF-β_1 (BMC + Ad.TGF-β_1) group. Two-weeks after surgery, the BMC + Ad.GFP group (n = 3 rabbits; n = 6 defects total) were sacrificed to verify transgene expression within the

healing tissue. We then compared healing between the Empty Defect (control) and BMC + Ad.TGF-β_1 (treatment) groups 3 months (n = 2 rabbits/group; n = 4 defects/group) and 12 months (n = 3 rabbits/group; n = 6 defects/group) after surgical intervention. Following this, we cultured BMCs with and without Ad.TGF-β_1 for 15 days to evaluate *in vitro* chondrogenesis of the clots. Lastly, we performed pilot *in vitro* (n = 4 hydrogels/group) and in vivo (n = 2 rabbits/group; n = 4 defects total) studies using fibrin hydrogels to deliver the cDNA of Ad.TGF-β_1 (fibrin + Ad.TGF-β_1) as an alternative to BMCs. For the pilot in vivo study, we evaluated healing at 3-months, and three osteochondral defects (n = 3) received fibrin + Ad.TGF-β_1 hydrogels and one osteochondral defect (n = 1) received an empty fibrin only hydrogel as a control. Studies involving animals were completed in accordance with Mayo Clinic's Institutional Animal Care and Use Committee.

2.2. Adenovirus vector preparation

First generation adenovirus vectors, serotype 5, carrying TGF-β_1 (Ad.TGF-β_1) or GFP (Ad.GFP) cDNA under the transcriptional control of the CMV promoter were produced in 293 cells as previously described [19].

Cell lysates were purified by density gradient ultracentrifugation and dialyzed against 10 mM Tris-HCl, pH 7.8, 150 mM NaCl, 10 mM $MgCl_2$ and 4% sucrose. Aliquots were stored at −80°. Titer was estimated as 4×10^{12} viral particles (vp) ml^{-1} by OD 260.

2.3. Osteochondral defect surgery and bone marrow clot harvesting, implantation, and culturing

A total of 13 (n = 26 total knees) skeletally mature male New Zealand white rabbits (average weight: 3.54 ± 0.29 kg; average age:9.1 ± 0.6 months) were injected with ketamine (35 mg/kg) and xylazine (5 mg/kg) intra-muscularly and given buprenorphine (0.18 mg/kg) for pre-emptive analgesia. Both hindlimbs and the lumbar region were shaved, and the skin was sterilized. Rabbits were then intubated with a size 2.5–3.5 endotracheal tube and maintained on oxygen and 2–2.5% isoflurane during surgery.

If autologous bone marrow aspirates were required, rabbits were placed in the prone position in a sterile field exposing the lumbar region and a 1 cm incision was made exposing the posterior iliac crest after soft tissue dissection. A bone marrow biopsy needle was used to penetrate the iliac crest and aspirate 2 mL of marrow. This was distributed in 250 μl aliquots among wells in a 96-well plate. After adding 25 μl saline or adenovirus suspension (10 [9] vp; Ad.TGF-β_1 or Ad.GFP) the mixture was homogenized with the pipette and then allowed to clot for 20 min, by which time the clot was firm enough to enable handling (Fig. 1). After the skin was closed, rabbits were turned to a supine position in order to create bilateral osteochondral defects within the patellar groove. After disinfection and draping, a 3-cm medial anterior parapatellar incision was made, and the knee joint accessed by opening the joint capsule medial to the patella. The knee was extended and the patella dislocated laterally giving access to the patellar groove. The knee was then flexed and a full-thickness osteochondral defect (3.2 mm diameter × 5–8 mm deep) created within the patellar groove with continuous irrigation. After the defect was created, the surgeon checked to verify that the defect did not perforate the metaphysis or extend into the medullary canal.

If the rabbits were receiving autologous marrow clots, they were press-fit into the defects, the patella relocated, and the joint capsule and skin closed using 3–0 and 4–0 resorbable sutures, respectively. If the rabbits were within the empty defect group, the osteochondral defects were left empty, and the joint capsule and skin were closed as formerly described. A summary of the surgical procedure is shown in Fig. 1.

Rabbits receiving BMC + Ad.GFP were euthanized after two weeks. Synovium, adjacent cartilage, and tissue from within the osteochondral defect was harvested and transgene expression verified by fluorescence microscopy (Olympus IX83, Olympus Corporation, Tokyo, Japan). Groups of rabbits receiving Ad.TFG-β_1 were euthanized after 3 months and 12 months. The distal femurs were stained with safranin orange-fast green and repair of the osteochondral defects assessed in a blinded fashion by four individuals using a modified O'Driscoll score. Sections at 12 months were further stained for type I and type II collagen by immunohistochemistry. Detailed methods are described below.

For *in vitro* experiments, BMCs were transferred to Ultra Low-Attachment 24-well plates (Corning, Corning, NY) and cultured in 1 mL incomplete chondrogenic medium (DMEM-HG, 10% FBS, 100 nm dexamethasone, and 1% penicillin/streptomycin) for 28 days with change of medium every 2 or 3 days. The TGF-β_1 concentration in the conditioned medium was measured by ELISA (R&D Systems, Minneapolis, MN). At day 28 samples were processed for histology using safranin orange and fast green staining.

2.4. Pilot in vitro and in vivo studies using fibrin hydrogels

Fibrin hydrogels were formed in 96-well plates using Tisseel fibrin glue kit (Baxter Healthcare Corporation, Deerfield, IL). The following ingredients were added in order: 125 μl PBS, 25 μl Ad.GFP or Ad.TGF-β_1 (10 [9] vp) or PBS, 50 μl thrombin, 150 μl fibrinogen. After 15–20 min at room temperature the polymerized gels were sliced into 4 individual discs and placed in wells on a 96-well plate. To each well were added 250,000 human bone marrow MSCs that were monolayer expanded (P3–P5, Lonza Ltd., Basel, Switzerland) and incubation continued at 37 °C, 5% CO_2 for 2 days. At this time media were changed to incomplete chondrogenic medium and incubated for a further 16 days, with medium change every 2 days. The TGF-β_1 concentrations of the media were measured by ELISA and GFP expression observed by fluorescence microscopy.

We then used the fibrin hydrogels with Ad.TGF-β_1 in a pilot in vivo study. Osteochondral defects were again created within the patellar groove of New Zealand white rabbits (n = 2 rabbits; n = 4 knees total) in the same manner as described above. The hydrogels were formed within the operating room during the knee surgery using the same protocol that was described for the *in vitro* studies but did not include any MSCs. Three osteochondral defects received the fibrin + Ad.TGF-β_1 construct, and one received fibrin alone. After 3 months, the femurs were harvested, and the samples were processed for histology using safranin orange - fast green and collagen type 2 staining.

2.5. Histology

Rabbit condyles were fixed in 10% neutral buffered formalin for 15 days and decalcified in unbuffered 10% EDTA pH 7.4 (Mol-decalcifier, Milestone Medical, Kalamazoo, MI)

with constant stirring at 37° using a decalcifying microwave apparatus (KOS Histostation. Milestone Medical). Decalcification was evaluated using a digital x-ray cabinet (MX-20, Faxitron Bioptics, Tucson, AZ). Fixed and decalcified specimens were dehydrated through graded alcohols, embedded in paraffin; 5 μm sections were cut using an automatic microtome (HM 355 S, Thermo Scientific Kalamazoo, MI) and mounted onto positively charged slides (Superfrost Plus Microscope Slides, Fisher Scientific, Pittsburgh, PA). Safranin orange – fast green staining was performed according to standard protocols.

2.6. Dual immunohistochemistry

Expression of collagen type I and collagen type II was examined by double staining. Briefly, formalin-fixed tissue sections (5 μm-thick) were deparaffinized in xylene and rehydrated in an ethanol/water gradient series, then rinsed in phosphate-buffered saline/0.05% Tween-20 (PBS-T) (Sigma; St. Louis, MO). Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 10 min, followed by treatment with 0.1% hyaluronidase with 0.1% pronase in tris-buffered saline (all from Sigma) in a water bath for 30 min at 37 °C to unmask collagen antigens. Slide sections were washed 3 times in PBS-T for 5 min each. Endogenous biotin activity was blocked by incubating the slides with the avidin and biotin solutions (SP-2001, Vector Laboratories), then washed 3 times in PBS-T for 5 min each. Nonspecific binding was blocked with animal-free blocker (SP-5035; Vector Laboratories, Burlingame, California) for 30 min at room temperature). Slides were incubated with goat polyclonal anti-type I collagen antibody (dilution 1:250), (Cat No. 1310–01, Southern Biotech, Birmingham, AL) for 1 h at room temperature (RT). Then, the slides were washed 3 times in PBS-T for 5 min each, followed by incubation with biotinylated rabbit anti-goat IgG antibody (PK-6105; Vector laboratories) for 30 min at RT, washed, and incubated with the avidin-biotin-peroxidase complex solution (PK-6105; Vector laboratories) for a further 30 min at RT.

Collagen type I staining was developed with DAB (3,3′-diaminobenzidine) (SK-4103, Vector Laboratories), a horseradish peroxidase (HRP) substrate that yields a brown product. DAB-slides slides were washed; and subsequently incubated with the second primary antibody, a mouse monoclonal to collagen II (dilution 1:200) (Cat No. 11–116B3, Developmental Studies Hybridoma bank, University of Iowa) in a humidified chamber at 4 °C overnight. The next day, slides were washed 3 times in PBS-T for 5 min each and incubated with the biotinylated horse anti-mouse IgG antibody (AK-5002, Vector laboratories) for 30 min, washed, and then treated with avidin-biotin-alkaline phosphatase complex solution (AK-5002, Vector laboratories) for 30 min at RT. Collagen type II staining was developed with red alkaline phosphatase substrate (SK-5105, Vector Laboratories) that yields a magenta precipitate. Nuclei were counterstained with hematoxylin (H-3404–100, Vector Laboratories), then quickly dehydrated in a graded ethyl alcohol series, cleared with xylene, and mounted with xylene-based mounting medium. To verify specificity of the immunolabeling, isotype controls consisting of goat IgG polyclonal (1:250) and mouse IgG1 kappa (1:200) isotype were included in a set of sections.

2.7. Statistical analysis

Histological scoring of sections closest to the middle of the defect were done by 4 blinded investigators (CVN, CHE, RDLV, MC) using the modified O'Driscoll scoring scale. The investigators' scores were averaged for each defect so that the mean results of the four scorers will be used for comparisons. A Mann-Whitney U, non-parametric test was performed to determine statistical significance ($p < 0.05$) of the 3-month ($n = 2$ rabbits/ group; $n = 4$ defects/group) and 12-month histological scores ($n = 3$ rabbits/group; $n = 6$ defects/group).

3. Results

3.1. Gene delivery using bone marrow clots for in vivo osteochondral defect repair

After two-weeks, the BMC + Ad.GFP group ($n = 3$ rabbits; $n = 6$ knees) was euthanized and both knees were harvested from each rabbit (Fig. 2A). The tissue within the defect was detached and loosened using a sterile blade along the edges of the defect and then removed using sterile forceps and placed into a well of an ultra low-binding, 24-well plate filled with culture media. Fluorescence microscopy was used to detect transgene expression within the tissue. We found GFP expression within the harvested tissue from all 6 knees confirming gene delivery with transgene expression for at least two weeks after surgery. However, the GFP expression was present in variable degrees (Fig. 2B), with negligible expression in 3 samples. There was no GFP activity in the cartilage surrounding the defect, and there was negligible expression in the synovium (Fig. 2C).

Clots containing Ad.TGF-β_1 were implanted into osteochondral defects in the patellar grooves of rabbit knees, with euthanasia at 3 and 12 months after surgery. Sections through defects in the left and right knees for each rabbit, stained with safranin orange – fast green at 3- and 12-months are shown in Fig. 3A and Fig. 4A, respectively. Using a modified O'Driscoll score we found that, despite considerable variability within groups, defects receiving BMC + Ad.TGF-β_1 were not significantly different than empty controls at 3 months (Fig. 3B). These included the overall score ($p = 0.88$), the cartilage component of the score ($p > 0.9$), and the osseous component of the score ($p = 0.45$).

There was still no significant difference between the groups at 12-months in the overall scores ($p = 0.73$), cartilage scores (0.43), and bone scores ($p = 0.18$) (Fig. 4B). However, in all groups there was a decline in all scores from 3- to 12-months.

We also performed dual immunohistochemical staining for collagen type I and type II within the repair tissue of the same groups at 12 months (Fig. 4C). We found staining for collagen-type II within the repair tissue of the BMC + Ad.TGF-β_1 and empty defect groups, but the staining was still highly variable within each group. A few defects within the empty defect group demonstrated strong collagen-type II staining compared to the BMC + Ad.TGF-β_1 defects. However, there was also presence of collagen-type I staining within the repair tissue of both groups.

3.2. Chondrogenic differentiation of bone marrow clots with Ad.TGF-β_1

Because of the high variability seen in the healing of the Ad.TGF-β_1 group, we performed a follow-up experiment in which marrow clots were formed with or without Ad.TGF-β_1 (n = 3 per group) and maintained in culture for 28 days with incomplete chondrogenic medium. During this period, the secretion of TGF-β_1 was measured by ELISA of conditioned media. After 28 days, clots were prepared for histology with safranin orange-fast green staining. Unmodified, control clots secreted little TGF-β_1 and did not show any evidence of chondrogenesis (Fig. 5A, C). Only one of the three clots containing Ad.TGF-β_1 secreted markedly elevated TGF-β_1 and had regions of chondrogenesis under histological examination (Fig. 5B and C).

3.3. Pilot studies using fibrin hydrogels to deliver chondrogenic genes

Much of the variability shown in Figs. 2–6 may reflect variation in the quality of the bone marrow aspirated from the iliac crests of the rabbits. As a more uniform and reliable alternative to bone marrow, we investigated the use of commercially available fibrin.

Human MSCs were co-cultured with a fibrin hydrogel incorporating adenoviral vectors encoding GFP (Ad.GFP). As shown in Fig. 6A, we observed MSCs on the surface of the scaffold with transduction and expression of GFP after only 2 days of co-culture. When the fibrin hydrogels contained Ad.TGF-β_1, expression of TGF-β_1 was very high, exceeding 180 ng/mL at its maximum on day 6. Control groups produced a basal level of approximately 4 ng/mL TGF-β_1 (Fig. 6B).

With the encouraging *in vitro* data shown in Fig. 6, a small pilot study explored the use of the fibrin + Ad.TGF-β_1 scaffold to repair osteochondral defects. Three osteochondral defects received the fibrin + Ad.TGF-β_1 construct, and one received fibrin alone. After 3-months, the femurs were harvested and processed for histology and immunohistochemistry for collagen type II.

Sections through the middle of the repair tissue were stained with safranin orange-fast green are shown in Fig. 7A. The control defect which received the empty fibrin scaffold demonstrated very poor healing. Defects which received the fibrin +Ad.TGF-β_1 scaffolds had repair tissue that was rich in proteoglycan and resembled articular cartilage; with one exception, was continuous with the surrounding native tissue.

We also performed immunohistochemistry for collagen-type II for these knees (Fig. 7B). We found strong staining for collagen type II in defects receiving the fibrin +Ad.TGF-β_1 scaffold whereas defects receiving the control fibrin scaffold had less staining for type II collagen.

4. Discussion

In this study, we compared the long-term healing of osteochondral defects within knees which received no treatment (empty defects) and ones that were treated with BMC + Ad.TGF-β_1. The BMC + Ad.GFP verified at 2-weeks there was transgene expression with the repair tissue. Early, 3-month comparisons in a small number of rabbits between the two

groups showed no differences in repair tissue. However, we found no significant differences in healing at 12-months between the two groups based on the modified O'Driscoll score. This was one of the first studies to investigate the long-term efficacy of gene delivery on osteochondral defect repair in an animal model. In the context of cartilage repair, previous research has confirmed that bone marrow clots with embedded adenovirus vectors can deliver transgenes to osteochondral defects, leading to transgene expression within the lesion [17]. Consistent with this, Sieker et al. [18] used bone marrow coagulates with adenoviral vectors encoding BMP-2 and IHH in a rabbit osteochondral defect model. Expression of BMP-2 gave encouraging early repair but led to the formation of osteophytes and elevated subchondral bone after 13 weeks [18]. IHH, in contrast, generated an abundance of immature, highly cellular cartilage that did not remodel into mature articular cartilage during the 13 week experiment [18]. Ivkovic et al. [20] also used BMC to deliver TGF-β1 cDNA in a sheep chondral defect model. Although areas of the defect showed *de novo* cartilage formation by 6 months complete repair was not achieved, possibly because there was no connection to the underlying bone marrow.

One critical factor that osteochondral repair strategies to promote in vivo tissue repair and regeneration must overcome is the inflammatory joint environment. Our group and other researchers have shown that even low-to-moderate levels of circulating inflammatory cytokines such as interleukin-1 and tumor necrosis factor-α inhibit the differentiation of MSCs to articular chondrocytes [21–23]. We did not control for these factors in our experiments which may have influenced the osteochondral defect repair in both groups. We also observed that the healing of osteochondral defects in the patellar groove of the rabbit knee proved highly variable, both in empty defects and in defects receiving bone marrow clots incorporating Ad.TGF-β_1. There are several sources of variability. Because the surgeries were conducted under closely controlled conditions, variability in the healing of empty defects presumably reflects biological variability between individual New Zealand white rabbits which, although inbred, are not syngeneic. A seminal publication from Shapiro et al. [24] investigated the natural healing of 364 individual knee osteochondral defects from 122 New Zealand White rabbits at various early (1 day) and late (48 weeks) time points. These authors have also noted large variation in the natural healing of empty osteochondral defects created in the same manner as we performed in this study [24]. Such intrinsic biological variability would have been compounded in defects receiving Ad.TGF-β_1 by the heterogeneous nature of bone marrow aliquots. This is best illustrated in Fig. 5, showing that only 1 in 3 marrow clots incorporating Ad.TGF-β_1 secreted TGF-β_1 and contained areas of chondrogenesis. This may be attributable to increasing dilution of the marrow by blood as aspiration continues. It appears that the variability in the quality of different marrow aspirates can be obviated by the use of a fibrin gel where, under *in vitro* conditions, the production of TGF-β_1 by human MSCs was high and uniform. Although a fibrin scaffold lacks the chondroprogenitor cells of marrow, the number of such cells in a 250 μl aliquot of marrow is likely to be small and an acceptable compromise in using the fibrin scaffolds to ensure greater uniformity in gene delivery. It is important to note that this difference in number of cells may explain the difference in expression values of TGF-β_1 between the fresh clots (Fig. 5) and fibrin hydrogels with Ad.TGF-β_1 (Fig. 6). Moreover, by eliminating the need to harvest marrow, the use of fibrin eliminates one

procedure. The small exploratory experiment using fibrin gels in conjunction with Ad.TGF-β_1 provided encouraging results that demonstrated less variability and better cartilage repair than using the marrow clots. These merits further development of this technology. In future experiments using fibrin gels to deliver chondrogenic genes, we will conduct experiments which evaluates gene expression and the overexpression of TGF-β_1 at early and time points. The alternative approach of administering vector directly to the marrow as it enters the lesion from the underlying marrow is also showing promising results in animal models [25,26].

This study has limitations that should be considered. The BMC + Ad.GFP group was sacrificed at two-weeks after treatment to verify transgene expression within the defects and not in the synovium. This did not allow us to use this group as a control for healing at later time points. In addition, this study was limited because we did not include a BMC only group for our in vivo studies to distinguish between chondrogenic effects of the marrow clot and Ad.TGF-β_1. However, we were limited by a budget, and we omitted this group but note that unmodified BMCs cultured *in vitro* did not undergo chondrogenesis, whereas those including, whereas those including Ad.TGF-β that expressed the transgene did so. Also, expressing TGF-β within the osseous region of the defect is not an obvious way to stimulate bone formation. With our main focus being on cartilage regeneration, we speculated that the intrinsic ability of bone to heal would enable eventual restoration of the subchondral plate. Should the TGF-β have formed cartilage in this region, this would occur by endochondral ossification. Indeed, we saw evidence of the osseous region within the BMC + Ad.TGF-β_1 group undergoing endochondral ossification 3-months after surgical intervention (Fig. 3A, right panel). Additionally, the overexpression of TGF-β within these defects is also known to promote the homing and recruitment of progenitor cells but we did not notice high levels of cellularity in our treatment defects.

The pilot, in vivo experiments using fibrin hydrogels had a limited number of samples and considering the high variability of healing in the previous experiments, definite conclusions cannot be made from this small study. Within these constraints, however, the fibrin hydrogels allowed for more uniform gene delivery which we suspected was an issue with the marrow clots. While a much larger study needs to be conducted to draw any conclusions regarding the use of fibrin + Ad.TGF-β_1 for osteochondral defect repair, the data presented here may help explain the lack of differences in repair between the empty defect and BMC + Ad.TGF-β_1 groups.

Gene transfer offers to enhance the regenerative behavior of musculoskeletal tissues [27], including cartilage [28] while single-step procedures that can be delivered at point-of-care will facilitate clinical translation and utilization [29]. As noted above, the clinical outcomes following cartilage repair surgery are inferior and many patients develop tissue resembling fibrocartilage which eventually breaks down. Gene deliver can augment these procedures to deliver specific morphogens to tissue and cells for a sustained period of time which is a major limitation of traditional intra-articular injections. However, more basic science research is warranted for its effective clinical translation.

We found that delivering BMC + Ad.TGF-β_1 does not significantly improve long-term osteochondral defect repair in a rabbit model which does not support our initial hypothesis. The overall O'Driscoll score and that of the cartilage component fell between 3 and 12 months for both the empty defect and BMC + Ad.TGF-β_1. This is reminiscent of the previously mentioned study by Shapiro et al. [24] who noted a similar trend in the spontaneous healing of osteochondral defects in skeletally mature rabbits. Thus, although transfer of TGF-β_1 slightly improved early healing, it could not overcome the subsequent delayed decline noted in this model. The use of a fibrin hydrogel may allow for more uniform gene delivery that initially improves osteochondral defect repair, but further study is needed.

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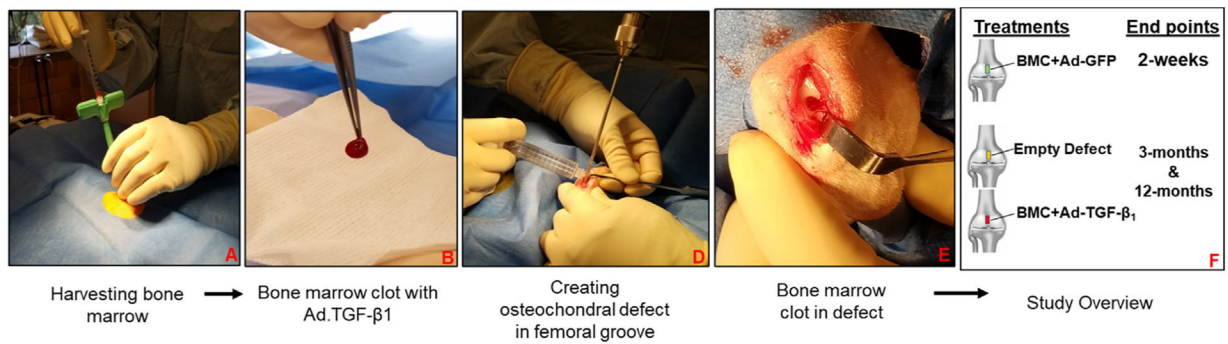
Fig. 1.

Summary of surgical procedures and study overview. Bone marrow was harvested from the iliac crest of the New Zealand white rabbits (A) and 250 μl aliquots of marrow were dispensed into a 96-well plate; 25 μl of Ad.GFP or Ad.TGF-β1 were added and the mixture titurated. After approximately 15–20 min, the bone marrow aliquots coagulated and formed into a gelatinous plug, which could be picked up with sterile forceps (B). An osteochondral defect (3.2 mm wide and 5–8 mm deep) was created in the patellar groove (D) and the bone marrow clot (BMC) was press-fit into the defect (E). The following groups were created: BMC + Ad.GFP, Empty Defect, and BMC + Ad-TGF-β1 (F). BMC + Ad.GFP was sacrificed 2 weeks to check for transgene expression (F). Healing was compared between the Empty Defect and BMC + Ad-TGF-β1 at 3- and 12-months (F).
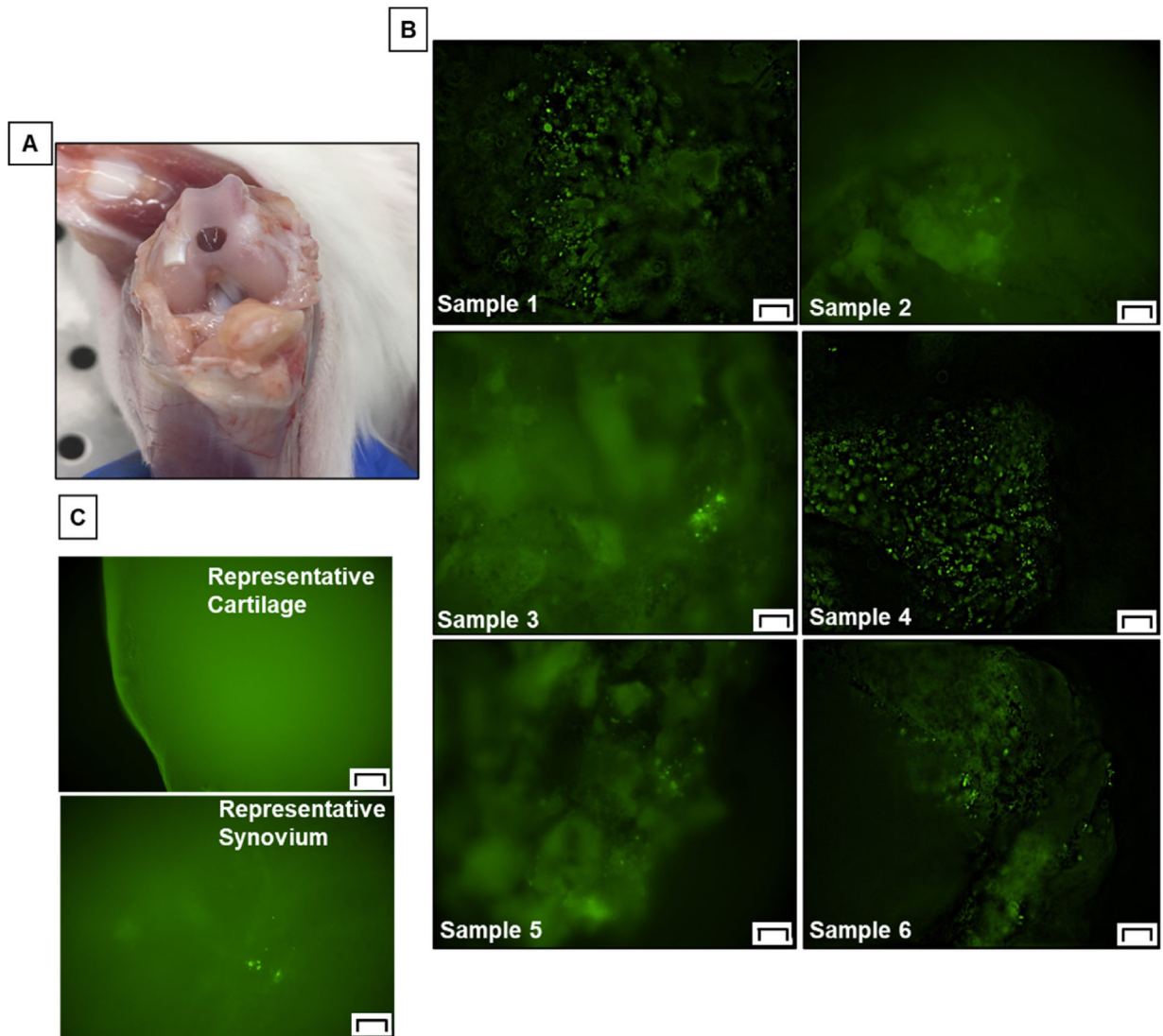
Fig. 2.

In vivo transgene expression. Rabbits in the BMC + Ad.GFP group were sacrificed 2 weeks after surgery and tissue within osteochondral defects (n = 3 rabbits; n = 6 defects) harvested, GFP expression was confirmed by fluorescence microscopy (A, B). Scale bars represent 100 μm in length. Representative images of the femoral cartilage surrounding the defect and synovium are shown (C). We observed no GFP expression in cartilage and negligible GFP expression in the synovium. Scale bars represent 100 μm in length.
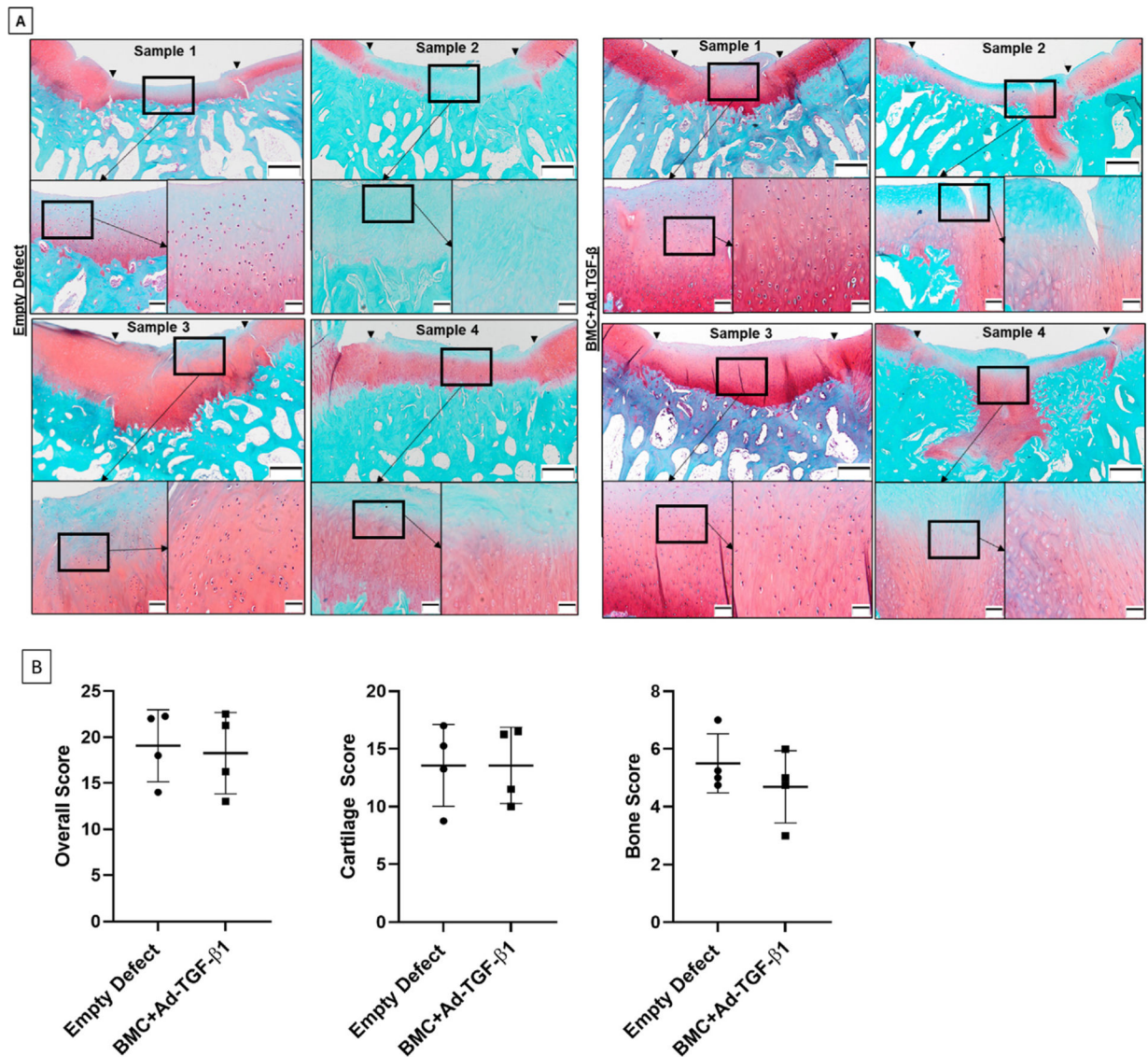
Fig. 3.
Osteochondral defect repair in empty defect and BMC + Ad.TGF-β1 groups at 3 months. Safranin orange - fast green staining of osteochondral defects was used to assess healing in each group (A) (n = 2 rabbits/group; n = 4 defects/group. Scale bars in images from lower to higher magnification represent 500 μm, 100 μm, and 50 μm in length, respectively. Using the modified O'Driscoll score, knees were scored by four blinded raters who found statistically significant differences in overall scores, cartilage scores and bone scores between the two groups (B). A Mann-Whitney U, non-parametric test was performed to determine statistical significance (p < 0.05). There were no significant differences between the groups.
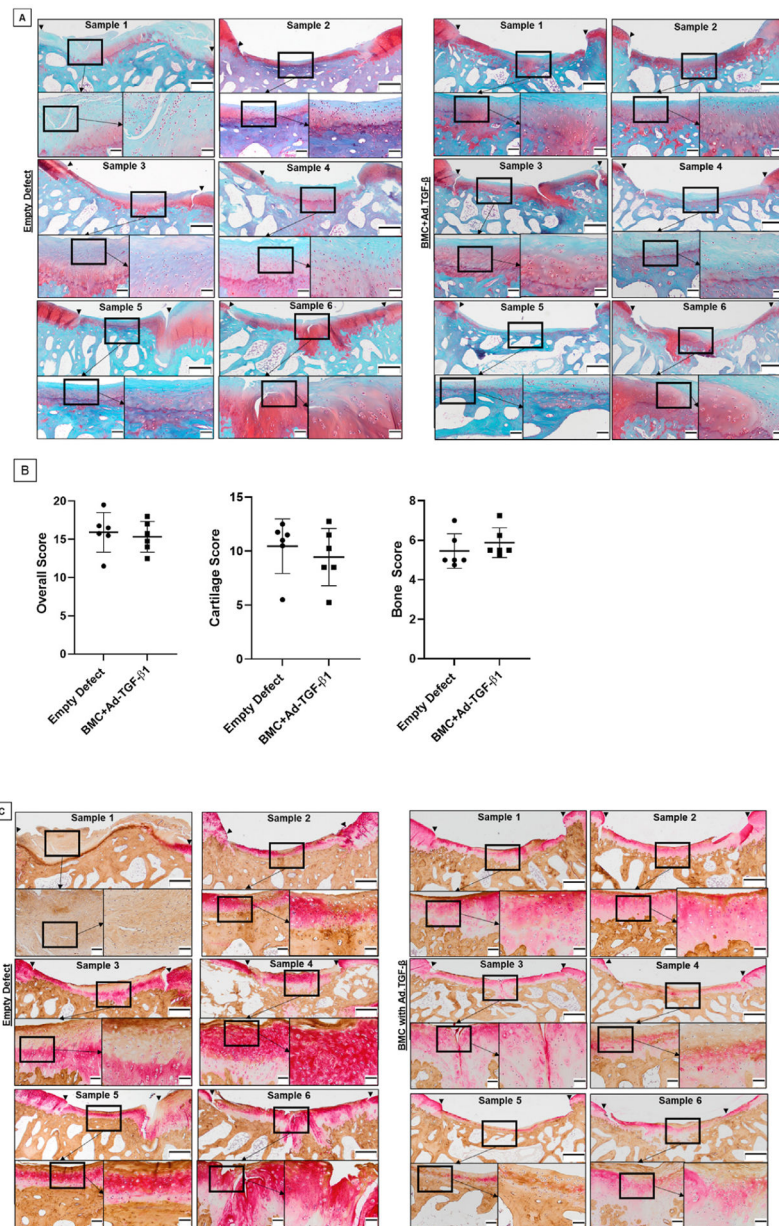
Fig. 4.

Osteochondral defect repair in the empty defect and BMC + Ad.TGF-β_1 groups at 12 months. Safranin orange - fast green staining of osteochondral defects was used to assess healing in each group (A) (n = 3 rabbits/group; n = 6 defects/group). Using the modified O'Driscoll score, knees were scored by four blinded raters who found no statistically significant differences in overall scores or cartilage scores, but there a significant difference in bone scores between treatment groups (B). A Mann-Whitney U, non-parametric test was performed to determine statistical significance (p < 0.05). There were no significant differences between the groups. Dual immunohistochemistry staining for collagen type I (brown) and type II (red/pink) of the osteochondral repair tissue from the empty defect and BMC + Ad.TGF-β1 (C) groups one year after surgery was also performed. Panels in (A) and

(C) have scale bars in images from lower to higher magnification which represent 500 μm, 100 μm, and 50 μm in length, respectively.
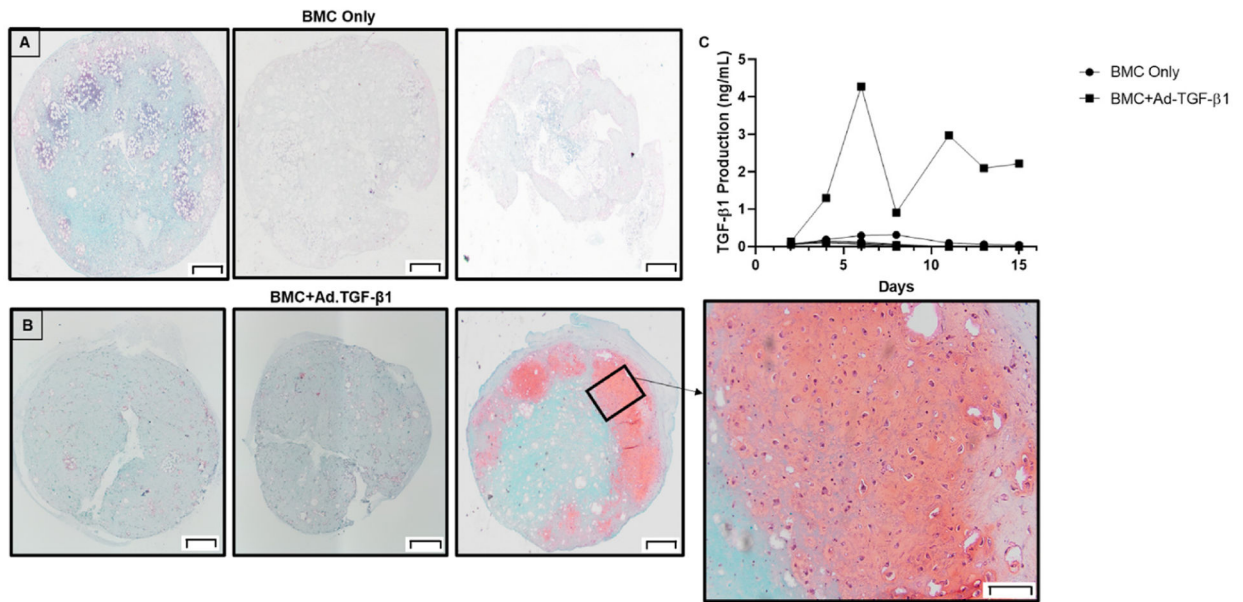
Fig. 5.

In vitro chondrogenesis of bone marrow coagulates (BMC) with or without Ad.TGF-β1 incorporation after 15 days. Safranin-orange staining at day 15 of the BMC only group (A) (n = 3 clots) found that none of the clots underwent chondrogenesis while only one of the three clots containing BMC + Ad.TGF (B) (n = 3) had evidence of chondrogenesis showing the presence of chondrocytes surrounded by a rich proteoglycan extracellular matrix. The supernatant from each clot was assayed for TGF-β1 (C); the single clot that underwent chondrogenesis was also the only clot producing significant amounts of TGF-β1. Panels (A) and (B) have scale bars which represent 500 μm in length. The higher magnification panel on the bottom right has a scale bar which represents 100 μm in length.
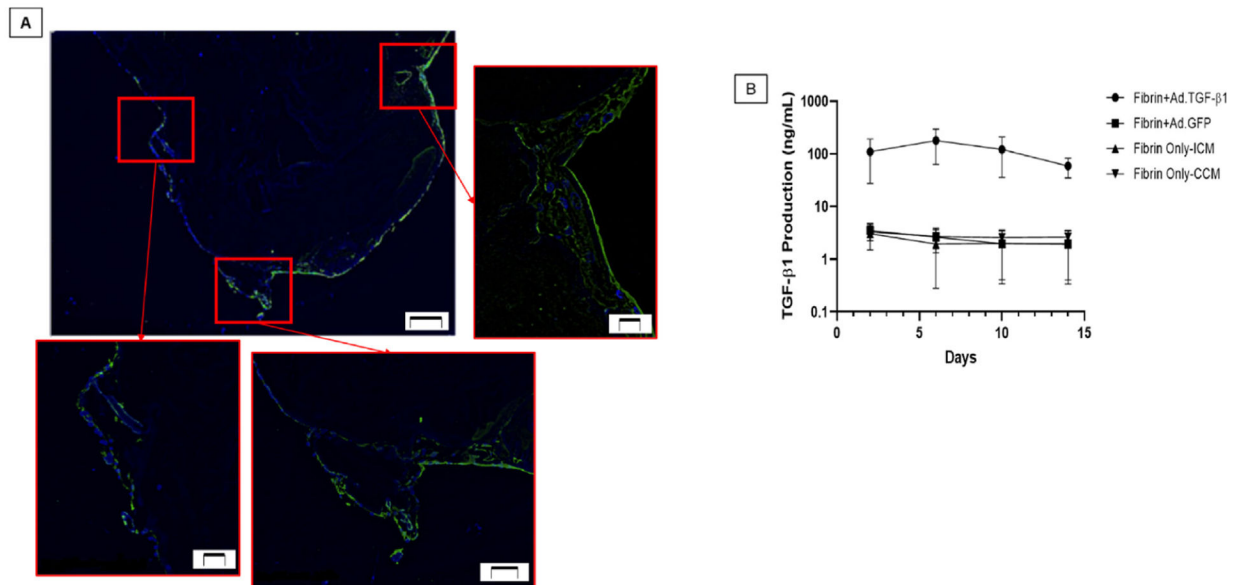
Fig. 6.
Gene transfer to human MSCs in culture using fibrin scaffolds. MSCs were co-cultured with a fibrin hydrogel incorporating Ad.GFP (n = 4). By 2 days of co-culture the MSCs were migrating into the scaffold, becoming transduced with Ad.GFP, and expressing GFP (A). The lower magnification image in panel (A) has a scale bar which represents 500 μm in length, while the higher magnification images from left to right (red boarder panels) have scale bars that represent 50 μm, 100 μm, and 50 μm in length, respectively. We also cultured MSCs with fibrin scaffolds containing Ad.TGF-β1 (n = 4) and found that the expression of TGF-β1 to be much higher than the control scaffolds (n = 4) lacking Ad.TGF-β1 (B).
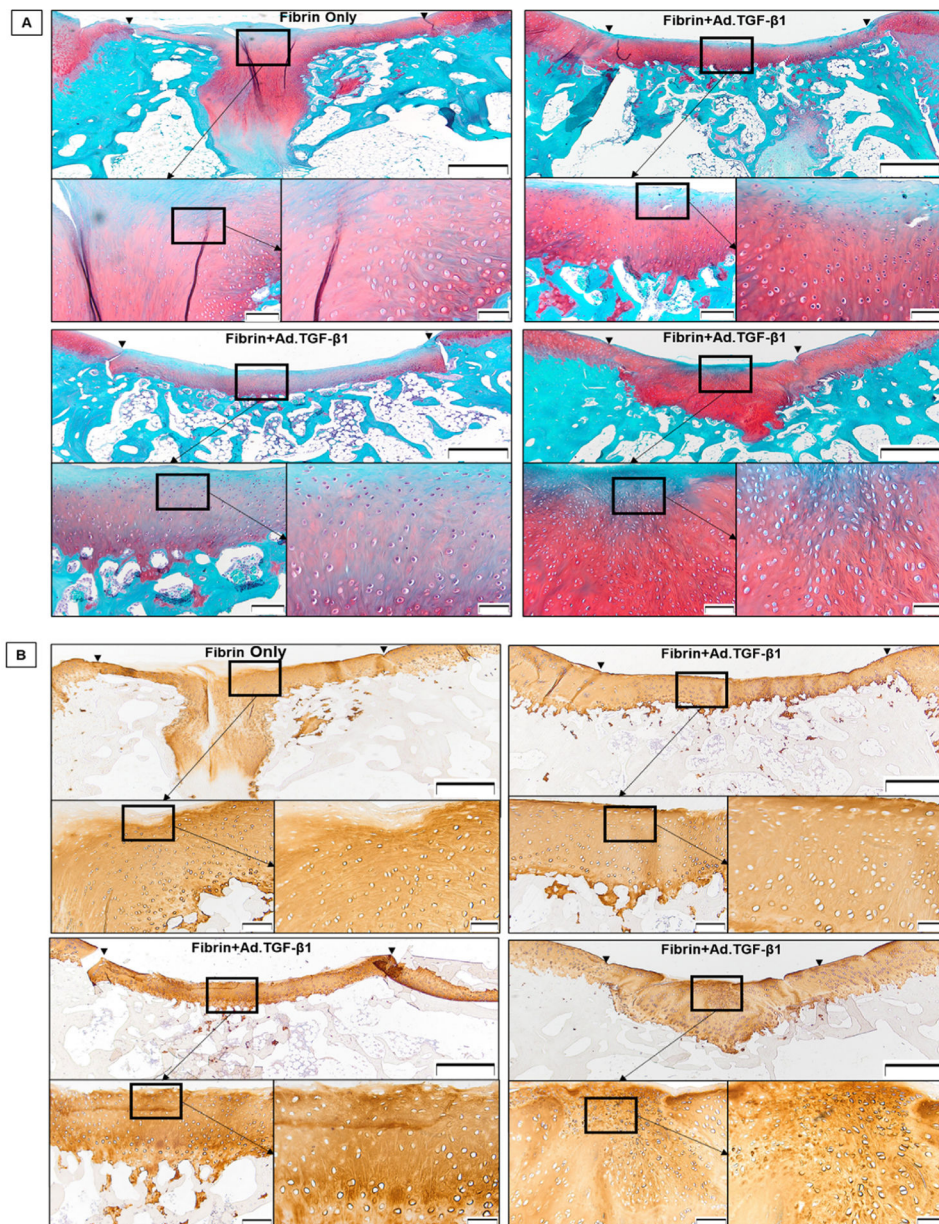
Fig. 7.

Gene delivery using fibrin scaffolds for in vivo osteochondral defect repair. Safranin orange - fast green staining of histological sections of the osteochondral defects demonstrate differential healing response between defects containing empty scaffolds (n = 1 defects) and those containing Ad.TGF-β1 (n = 3 defects) (A). Collagen type II staining of the same sections revealed collagen type II in the repair cartilage tissue of the knees which received the fibrin scaffolds with Ad.TGF-β1 compared to the empty scaffold defect which had collagen type II staining within the osseus region (B). Panels in (A) and (B) have scale bars in images from lower to higher magnification which represent 500 μm, 100 μm, and 50 μm in length, respectively.