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1 **TITLE:** Expedited gene delivery for osteochondral defect repair in a rabbit knee model: a one-
2 year investigation

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27 **RUNNING TITLE:** Gene delivery for osteochondral repair

28 **ABSTRACT**

29 **Objective:** To evaluate a single-step, gene-based procedure for repairing osteochondral
30 lesions.
31 **Design:** Osteochondral lesions were created in the patellar groove of skeletally mature rabbits.
32 Autologous bone marrow aspirates were mixed with adenovirus vectors carrying cDNA
33 encoding green fluorescent protein (Ad.GFP) or transforming growth factor-β_1 (Ad.TGF-β_1) and
34 allowed to clot. The clotted marrow was press-fit into the defects. Animals receiving Ad.GFP
35 were euthanized at 2 weeks and intra-articular expression of GFP examined by fluorescence
36 microscopy. Animals receiving Ad.TGF-β_1 were euthanized at 3 months and 12 months; repair
37 was compared to empty defects using histology and immunohistochemistry. Complementary in
38 vitro experiments assessed transgene expression and chondrogenesis in marrow clots and
39 fibrin gels. In a subsequent pilot study, repair at 3 months using a fibrin gel to encapsulate
40 Ad.TGF-β_1 was evaluated.
41 **Results:** At 2 weeks, GFP expression was seen at variable levels within the cartilaginous
42 lesion. At 3 months, there was a statistically significant improvement in healing of lesions
43 receiving Ad.TGF-β_1, although variability was high. At 12 months, there was no difference
44 between the empty defects and those receiving Ad.TGF-β_1 in overall score and cartilage score,
45 but the bone healing score remained higher. Variability was again high. In vitro experiments
46 suggested that variability reflected variable transduction efficiency and chondrogenic activity of
47 the marrow clots; using fibrin gels instead of marrow provided more uniformity in healing.
48 **Conclusions:** This approach to improving the repair of osteochondral lesions holds promise but
49 needs further refinement to reduce variability and provide a more robust outcome.
50 **Key Words:** Gene delivery, cartilage repair, osteochondral defects, knee, rabbit, long-term

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52 **INTRODUCTION**

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

61 Autologous chondrocyte implantation (ACI) and microfracture are two popular cell-based
62 therapies used by surgeons to treat chondral lesions. ACI has shown great promise as a
63 cartilage repair procedure. However, this is a two-step procedure which involves harvesting
64 cartilage from the periphery of the joint, expanding the isolated chondrocytes in culture, and
65 transplanting these cells back into the defect to regenerate tissue.[4] This increases the cost of
66 the procedure, and patients incur a prolonged post-operative rehabilitation period during which
67 full weight bearing is delayed.[5] [6] In contrast, microfracture is a one-step, point of care procedure
68 which involves exposing and penetrating the subchondral plate underneath the cartilage defect
69 to allow bone marrow to enter and form a fibrin clot within the lesion.[7] The coagulated bone
70 marrow contains mesenchymal stromal cells (MSCs) with the potential to differentiate into
71 chondrocytes which initiate the repair process.[7] Microfracture has demonstrated promising
72 short-to-medium term relief for many patients allowing them back to previous levels of activity.[8]
73 However, according to multiple long-term reports both ACI and microfracture have high failure
74 rates and patients are likely to develop repair tissue resembling a fibrocartilaginous scar instead
75 of native articular cartilage.[9-11] [12-14] Fibrocartilage has inferior biomechanical properties and does
76 not endure the joint environment. Its deterioration causes pain, dysfunction, and eventual failure
77 of the repair, requiring salvage surgery.

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

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

96 In the present study, we investigated whether delivering adenoviral vectors carrying
97 TGF-β_1 cDNA (Ad.TGF-β_1) using autologous bone marrow coagulates can improve the repair of
98 osteochondral defects in the rabbit knee at early (3 months) and late (12 months) time points.
99
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101

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102 **METHODS**

103 *Study Design*

104 *In vivo experiments*

105 Osteochondral defects were surgically created in the knees of skeletally mature New
106 Zealand White Rabbits. Depending on the experiment, the defects were left untreated (empty
107 defects, controls) or filled with clotted bone marrow, clotted bone marrow containing Ad.TGF-β_1
108 (BMC+ Ad.TGF-β_1) or adenovirus carrying the enhanced green fluorescent protein (GFP) cDNA
109 (BMC+Ad.GFP), fibrin hydrogel or fibrin hydrogel containing Ad.TFG-β_1.

110 Rabbits receiving Ad.GFP were euthanized after two weeks. Synovium, adjacent
111 cartilage, and tissue from within the osteochondral defect was harvested and transgene
112 expression verified by fluorescence microscopy. Groups of rabbits receiving Ad.TFG-β_1 were
113 euthanized after 3 months and 12 months. The distal femurs were stained with safranin orange-
114 fast green and repair of the osteochondral defects assessed in a blinded fashion by four
115 individuals using a modified O'Driscoll score. Sections at 12 months were further stained for
116 type I and type II collagen by immunohistochemistry.

117 *In vitro experiments*

118 Bone marrow was aspirated from the iliac crests of rabbits and aliquots distributed
119 among wells in a 96-well plate. Saline or Ad.TGF-β_1 were added and mixed with the marrow,
120 which was then allowed to clot. The marrow clots were cultured in well plates for 15 days.
121 Conditioned media were assayed for TGF-β_1 expression by ELISA. After 28 days the clots were
122 examined by histology for evidence of chondrogenesis.

123 Fibrin gels were formed in 96-well plates. Saline, Ad.TGF-β_1 or Ad.GFP were
124 incorporated into the gel as it formed. Gels were then co-cultured with human MSCs in growth

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125 medium. Two days later media were changed to either complete chondrogenic medium or
126 incomplete chondrogenic medium. Transgene expression was assessed by immunofluorescent
127 identification of GFP+ cells and by ELISA measurement of TGF- β_1 in conditioned media.

128 *Adenovirus vector preparation*

129 First generation adenovirus vectors, serotype 5, carrying TGF-β_1 (Ad.TGF-β_1) or GFP
130 (Ad.GFP) cDNA under the transcriptional control of the CMV promoter were produced in 293
131 cells as previously described.[19] Cell lysates were purified by density gradient ultracentrifugation
132 and dialyzed against 10 mM Tris-HCl, pH 7.8, 150 mM NaCl, 10 mM $MgCl_2$ and 4% sucrose.
133 Aliquots were stored at -80°. Titer was estimated as 4 x 10^{12} viral particles (vp) ml^{-1} by OD 260.

134 *Marrow clots*

135 Bone marrow (2 ml) was aspirated from the iliac crest of an anesthetized rabbit using a
136 16-gauge needle. This was distributed in 250 µl aliquots among wells in a 96-well plate. After
137 adding 25 µl saline or adenovirus suspension (10^9 vp) the marrow was titrated and then allowed
138 to clot for 20 min, by which time the clot was firm enough to enable handling (figure 1).

139 Implantation of bone marrow clots (BMC) into osteochondral defects is described in the
140 section on rabbit surgery. For *in vitro* experiments, BMCs were transferred to Ultra Low-
141 Attachment 24-well plates (Corning, Corning, NY) and cultured in 1 ml incomplete chondrogenic
142 medium (DMEM-HG, 10% FBS, 100 nm dexamethasone, and 1% penicillin/streptomycin) for 28
143 days with change of medium every 2 or 3 days. The TGF-β_1 concentration in the conditioned
144 medium was measured by ELISA (R&D Systems, Minneapolis, MN). At day 28 samples were
145 processed for histology using safranin orange and fast green staining.

146

147

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148 *Fibrin hydrogels*

149 Fibrin hydrogels were formed in 96-well plates using Tisseel fibrin glue kit (Baxter
150 Healthcare Corporation, Deerfield, IL). The following ingredients were added in order: 125 µl
151 PBS, 25 µl Ad.GFP or Ad.TGF- β_1 (10^9 vp) or PBS, 50 µl thrombin, 150 µl fibrinogen. After 15-
152 20 min at room temperature the polymerized gels were sliced into 4 individual discs and placed
153 in wells on a 96-well plate. To each well were added 250,000 human MSCs (P3-P5, Lonza Ltd.,
154 Basel, Switzerland) and incubation continued at 37°C, 5% CO_2 for 2 days. At this time media
155 were changed to incomplete chondrogenic medium and incubated for a further 16 days, with
156 medium change every 2 days. The TGF-β_1 concentrations of the media were measured by
157 ELISA and GFP expression observed by fluorescence microscopy. Samples were processed for
158 histology using safranin orange - fast green staining.

159 *Osteochondral defect surgery*

160 A total of 15 (n=30 total knees) skeletally mature male New Zealand white rabbits
161 (average weight: 3.54±0.29 kg; average age:9.1±0.6 months) were injected with ketamine (35
162 mg/kg) and xylazine (5 mg/kg) intra-muscularly and given buprenorphine (0.18 mg/kg) for pre-
163 emptive analgesia. Both hindlimbs and the lumbar region were shaved and the skin sterilized.
164 Rabbits were then intubated with a size 2.5 – 3.5 endotracheal tube and maintained on oxygen
165 and 2-2.5% isoflurane during surgery.

166 If bone marrow aspirates were required, rabbits were placed in the prone position in a
167 sterile field exposing the lumbar region and a 1 cm incision was made exposing the posterior
168 iliac crest after soft tissue dissection. The iliac crest was penetrated with a bone marrow biopsy
169 needle and the bone was used to aspirate marrow was aspirated from the iliac crest. After the
170 skin closure was closed, rabbits were turned to a supine position in order to create bilateral
171 osteochondral defects within the patellar groove. After disinfection and draping, a 3-cm medial

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172 anterior parapatellar incision was made, and the knee joint accessed by opening the joint
173 capsule medial to the patella. The knee was extended and the patella dislocated laterally giving
174 access to the patellar groove. The knee was then flexed and a full-thickness osteochondral
175 defect (3.2 mm diameter x 5-8 mm deep) created within the patellar groove with continuous
176 irrigation. After the defect was created, the surgeon checked to verify that the defect did not
177 perforate the metaphysis or extend into the medullary canal. Where necessary, marrow clots or
178 fibrin hydrogels were press-fit into the defects, the patella relocated, and the joint capsule and
179 skin closed using 3-0 and 4-0 resorbable sutures, respectively. A summary of the surgical
180 procedure is shown in figure 1.

181 *Histology*

182 Rabbit condyles were fixed in 10% neutral buffered formalin for 15 days and decalcified
183 in unbuffered 10% EDTA pH 7.4 (Mol-decalcifier, Milestone Medical, Kalamazoo, MI) with
184 constant stirring at 37° using a decalcifying microwave apparatus (KOS Histostation. Milestone
185 Medical). Decalcification was evaluated using a digital x-ray cabinet (MX-20, Faxitron Bioptics,
186 Tucson, AZ). Fixed and decalcified specimens were dehydrated through graded alcohols,
187 embedded in paraffin; 5 µm sections were cut using an automatic microtome (HM 355S,
188 Thermo Scientific Kalamazoo, MI) and mounted onto positively charged slides (Superfrost Plus
189 Microscope Slides, Fisher Scientific, Pittsburgh, PA). Safranin orange – fast green staining was
190 performed according to standard protocols.

191 Histological scoring of the sections by 4 blinded investigators (CVN, CHE, RDLV, MC)
192 using the modified O'Driscoll scoring scale. An unpaired, student's t-test was performed to
193 determine statistical significance ($p<0.05$).

194

195

196

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197 *Dual Immunohistochemistry*

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

214 Collagen type I staining was developed with DAB (3,3'-diaminobenzidine) (SK-4103,
215 Vector Laboratories), a horseradish peroxidase (HRP) substrate that yields a brown product.
216 DAB-slides slides were washed; and subsequently incubated with the second primary antibody,
217 a mouse monoclonal to collagen II (dilution 1:200) (Cat No. 11-116B3, Developmental Studies
218 Hybridoma bank, University of Iowa) in a humidified chamber at 4 °C overnight. The next day,
219 slides were washed 3 times in PBS-T for 5 min each and incubated with the biotinylated horse
220 anti-mouse IgG antibody (AK-5002, Vector laboratories) for 30 min, washed, and then treated
221 with avidin-biotin-alkaline phosphatase complex solution (AK-5002, Vector laboratories) for 30
222 min at RT. Collagen type II staining was developed with red alkaline phosphatase substrate

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223 (SK-5105, Vector Laboratories) that yields a magenta precipitate. Nuclei were counterstained
224 with hematoxylin (H-3404-100, Vector Laboratories), then quickly dehydrated in a graded ethyl
225 alcohol series, cleared with xylene, and mounted with xylene-based mounting medium. To verify
226 specificity of the immunolabeling, isotype controls consisting of goat IgG polyclonal (1:250) and
227 mouse IgG1 kappa (1:200) isotype were included in a set of sections.
228

229 **RESULTS**

230 *Gene delivery using bone marrow clots for in vivo osteochondral defect repair*

231 After two-weeks, the BMC+Ad.GFP group (n=3 rabbits; n=6 knees) was euthanized and
232 both knees were harvested from each rabbit (figure 2A). The tissue within the defect was
233 removed using sterile forceps and placed into a well of an ultra low-binding, 24-well plate filled
234 with culture media. Fluorescence microscopy was used to detect transgene expression within
235 the tissue. We found GFP expression within the harvested tissue from all 6 knees, although to
236 variable degrees (figure 2B), confirming gene delivery with transgene expression for at least two
237 weeks after surgery. There was no GFP activity in the cartilage surrounding the defect, and
238 there was negligible expression in the synovium (not shown).

239 Clots containing Ad.TGF-β_1 were implanted into osteochondral defects in the patellar
240 grooves of rabbit knees, with euthanasia at 3 and 12 months after surgery. Both femurs were
241 harvested from each rabbit and processed for histological and immunohistochemistry analysis.
242 Sections through defects in the left and right knees for each rabbit, stained with safranin orange
243 – fast green at 3- and 12-months are shown in figure 3A and figure 4A, respectively. Using a
244 modified O'Driscoll score we found that, despite considerable variability within groups, defects
245 receiving BMC+Ad.TGF-β_1 had scores that were statistically higher than empty controls at 3
246 months (figure 3B). These included the overall score, the cartilage component of the score and
247 the osseous component of the score.

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248 However, the differences for overall and cartilage scores disappeared by 12-months
249 after surgery (figure 4B). In all groups there was a decline in overall and cartilage scores by 12-
250 months ($p<0.01$) such that intergroup differences were no longer apparent. Nevertheless, the
251 bone score continued to improve in the group receiving BMC+Ad.TGF-β_1 thereby enhancing the
252 statistical significance of the difference (figure 4B).

253 We also performed dual immunohistochemical staining for collagen type I and type II
254 within the repair tissue of the same groups at 12 months (figure 4C). We found uniform strong
255 staining for collagen-type II within the repair tissue of the BMC+Ad.TGF-β_1 group, whereas the
256 empty defects stained only weakly for type II collagen.

257

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

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

267

268 *Chondrogenic differentiation of MSCs using fibrin scaffolds incorporating Ad.TGF-β_1*

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

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272 Human MSCs were co-cultured with a fibrin hydrogel incorporating adenoviral vectors
273 encoding GFP (Ad.GFP). As shown in figure 6A, we observed MSC migration into the scaffold
274 with transduction and expression of GFP after only 2 days of co-culture. When the fibrin
275 hydrogels contained Ad.TGF-$\beta_{1,}$ expression of TGF-β_1 was very high, exceeding 180 ng/mL at
276 its maximum on day 6. Control groups produced a basal level of approximately 4 ng/mL TGF-β_1
277 (figure 6B).

278

279 *Gene delivery using fibrin scaffolds for in vivo osteochondral defect repair*

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

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

290 We also performed immunohistochemistry for collagen-type II for these knees (Figure
291 7B). We found strong staining for collagen type II in defects receiving the fibrin+Ad.TGF-β_1
292 scaffold whereas defects receiving the control fibrin scaffold had less staining for type II
293 collagen.

294

295

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296 **DISCUSSION**

297 Gene transfer offers to enhance the regenerative behavior of musculoskeletal tissues,[20]
298 including cartilage[21] while single-step procedures that can be delivered at point-of-care will
299 facilitate clinical translation and utilization.[22] In the context of cartilage repair, previous research
300 has confirmed that bone marrow clots with embedded adenovirus vectors can deliver
301 transgenes to osteochondral defects, leading to transgene expression within the lesion.[17]
302 Consistent with this, Sieker et al.[18] used bone marrow coagulates with adenoviral vectors
303 encoding BMP-2 and IHH in a rabbit osteochondral defect model. Expression of BMP-2 gave
304 encouraging early repair but led to the formation of osteophytes and elevated subchondral bone
305 after 13 weeks.[18] IHH, in contrast, generated an abundance of immature, highly cellular
306 cartilage that did not remodel into mature articular cartilage during the 13 week experiment.[18]
307 Ivkovic et al.[23] also used BMC to deliver TGF-β1 cDNA in a sheep chondral defect model.
308 Although areas of the defect showed *de novo* cartilage formation by 6 months complete repair
309 was not achieved, possibly because there was no connection to the underlying bone marrow.

310 In the present experiments, healing of osteochondral defects in the patellar groove of the
311 rabbit knee proved highly variable, both in empty defects and in defects receiving bone marrow
312 clots incorporating Ad.TGF-β_1. There are several sources of variability. Because the surgeries
313 were conducted under closely controlled conditions, variability in the healing of empty defects
314 presumably reflects biological variability between individual New Zealand white rabbits which,
315 although inbred, are not syngeneic. Such intrinsic biological variability would have been
316 compounded in defects receiving Ad.TGF-β_1 by the heterogeneous nature of bone marrow
317 aliquots. This is best illustrated in figure 6, showing that only 1 in 3 marrow clots incorporating
318 Ad.TGF-β_1 secreted TGF-β_1 and contained areas of chondrogenesis. This may be attributable
319 to increasing dilution of the marrow by blood as aspiration continues. Nevertheless it is

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320 encouraging that, despite this background variability, a statistically significant improvement in
321 healing was evident at 3 months.

322 It appears that the variability in the quality of different marrow aspirates can be obviated
323 by the use of a fibrin gel where, under in vitro conditions, the production of TGF-β_1 by human
324 MSCs was high and uniform. Although a fibrin scaffold lacks the chondroprogenitor cells of
325 marrow, the number of such cells in a 250 μl aliquot of marrow is likely to be small and an
326 acceptable compromise in using the fibrin scaffolds to ensure greater uniformity in gene
327 delivery. Moreover, by eliminating the need to harvest marrow, the use of fibrin eliminates one
328 procedure. The small exploratory experiment using fibrin gels in conjunction with Ad.TGF-
329 β_1 provided encouraging results that merit further development of this technology. The
330 alternative approach of administering vector directly to the marrow as it enters the lesion from
331 the underlying marrow is also showing promising results in animal models.[24, 25]

332 The overall O'Driscoll score and that of the cartilage component fell between 3 and 12
333 months. This is reminiscent of the study by Shapiro et al[26] who noted a similar trend in the
334 spontaneous healing of osteochondral defects in young rabbits. Thus, although transfer of TGF-
335 β_1 accelerated and improved early healing, it could not overcome the subsequent delayed
336 decline noted in this model. Addressing this problem will be a matter for future research.

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340 ***CONTRIBUTIONS***

341 All authors have made substantial contributions to the study and approved the final submitted
342 manuscript. Nagelli (nagelli.christopher@mayo.edu) and Evans (evans.christopher@mayo.edu)
343 take full responsibility for the integrity of the research.

344 Nagelli: Collection and assembly of data, analysis and interpretation of the data, drafting of the
345 article, final approval of the article

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346 De la Vega: Collection and assembly of data, analysis and interpretation of the data, technical
347 support, editing of manuscript

348 Coenen: Collection and assembly of data, analysis and interpretation of the data, technical
349 support, editing of manuscript

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354 Trovar: Collection and assembly of data, technical support

355 Müller: Collection and assembly of data, analysis and interpretation of the data, technical
356 support, editing of manuscript

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359

360 ***ROLE OF FUNDING SOURCE***

361

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366 ***CONFLICT OF INTEREST***

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368 There are no conflicts of interests for the authors for this study.

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438

439 *Figure 1*: Summary of surgical procedures. Bone marrow was harvested from the iliac crest of
440 the New Zealand white rabbits (A) and 250 µL aliquots of marrow were dispensed into a 96-well
441 plate (B); 25 µL of Ad.TGF-β1 were added and the mixture titurated. After approximately 15-20
442 min, the bone marrow aliquots coagulated and formed into a gelatinous plug, which could be
443 picked up with sterile forceps (B). An osteochondral defect (3.2 mm wide and 5-8 mm deep)
444 was created in the patellar groove (C) and the bone marrow clot was press-fit into the defect
445 (D).
446
447 *Figure 2: In vivo* transgene expression. Rabbits in the BMC+Ad.GFP group were sacrificed 2
448 weeks after surgery and tissue within osteochondral defects (n=3 rabbits; n=6 knees) harvested,
449 GFP expression was confirmed by fluorescence microscopy (A, B).
450
451 *Figure 3*: Osteochondral defect repair in empty defect and BMC+Ad.TGF-β1 groups at 3
452 months. Safranin orange - fast green staining of osteochondral defects was used to assess
453 healing in each group (A) (n=4 rabbits). Using the modified O'Driscoll score, knees were scored
454 by four blinded raters who found statistically significant differences in overall scores, cartilage
455 scores and bone scores between the two groups (B).
456
457 *Figure 4:* Osteochondral defect repair in the empty defect and BMC+Ad.TGF-β_1 groups at 12
458 months. Safranin orange - fast green staining of osteochondral defects was used to assess
459 healing in each group (A) (n=6 rabbits; n=6 knees/group). Using the modified O'Driscoll score,
460 knees were scored by four blinded raters who found no statistically significant differences in
461 overall scores or cartilage scores, but there a significant difference in bone scores between
462 treatment groups (B). Dual immunohistochemistry staining for collagen type I and type II of the
463 osteochondral repair tissue from the empty defect and BMC+Ad.TGF-β1 (B) groups one year
464 after surgery was also performed.
465
466 *Figure 5*: In vitro chondrogenesis of bone marrow coagulates (BMC) with or without Ad.TGF-β1
467 incorporation after 15 days. Safranin-orange staining at day 15 of the BMC only (A) found that
468 none of the clots underwent chondrogenesis while only one of the three clots containing
469 BMC+Ad.TGF (B) had evidence of chondrogenesis showing the presence of chondrocytes
470 surrounded by a rich proteoglycan extracellular matrix. The supernatant from each clot was
471 assayed for TGF-β1 (C); the single clot that underwent chondrogenesis was also the only clot
472 producing significant amounts of TGF-β1.
473
474 *Figure 6*: Gene transfer to human MSCs in culture using fibrin scaffolds. MSCs were co-cultured
475 with a fibrin hydrogel incorporating Ad.GFP. By 2 days of co-culture the MSCs were migrating
476 into the scaffold,becoming transduced with Ad.GFP, and expressing GFP (A). We also cultured
477 MSCs with fibrin scaffolds containing Ad.TGF-β1 and found that the expression of TGF-β1 to be
478 much higher than the control scaffolds lacking Ad.TGF-β1 (B).
479
480 *Figure 7*: Gene delivery using fibrin scaffolds for in vivo osteochondral defect repair. Safranin
481 orange - fast green staining of histological sections of the osteochondral defects demonstrate
482 differential healing response between defects containing empty scaffolds and those containing

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483 Ad.TGF-β1 (A). Collagen type II staining of the same sections revealed abundant collagen type
484 II in the repair tissue of the knees which received the fibrin scaffolds with Ad.TGF-β1 (B).
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534 **Figure 1**
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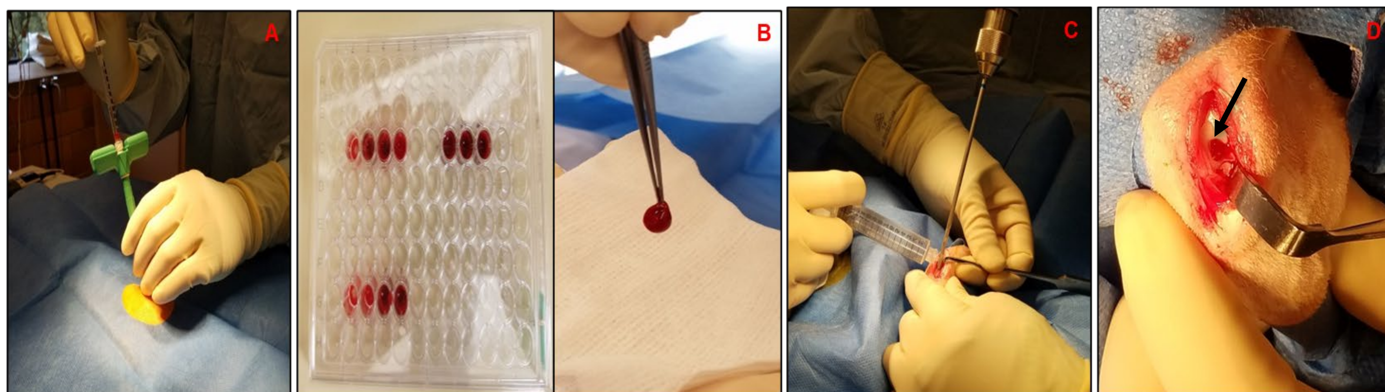

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572 **Figure 2**
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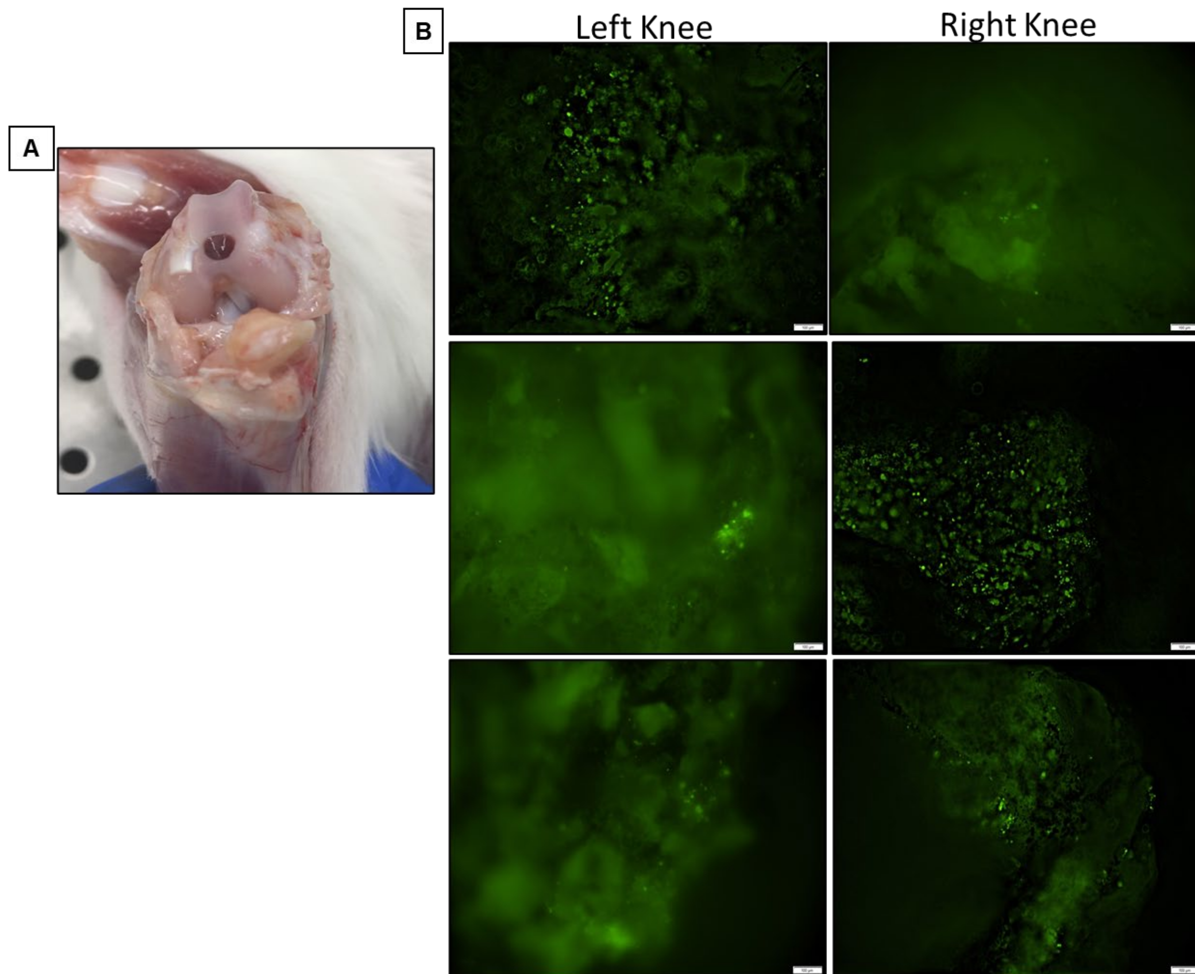

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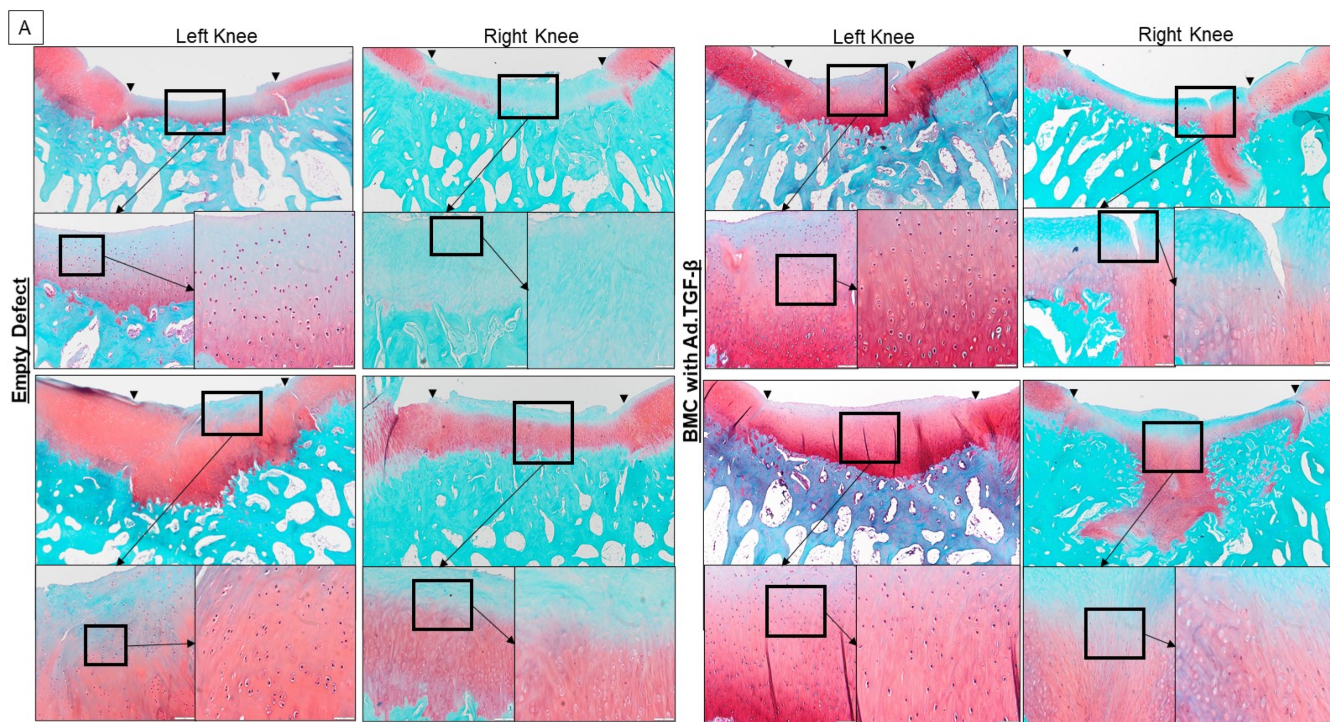
576 **Figure 3**



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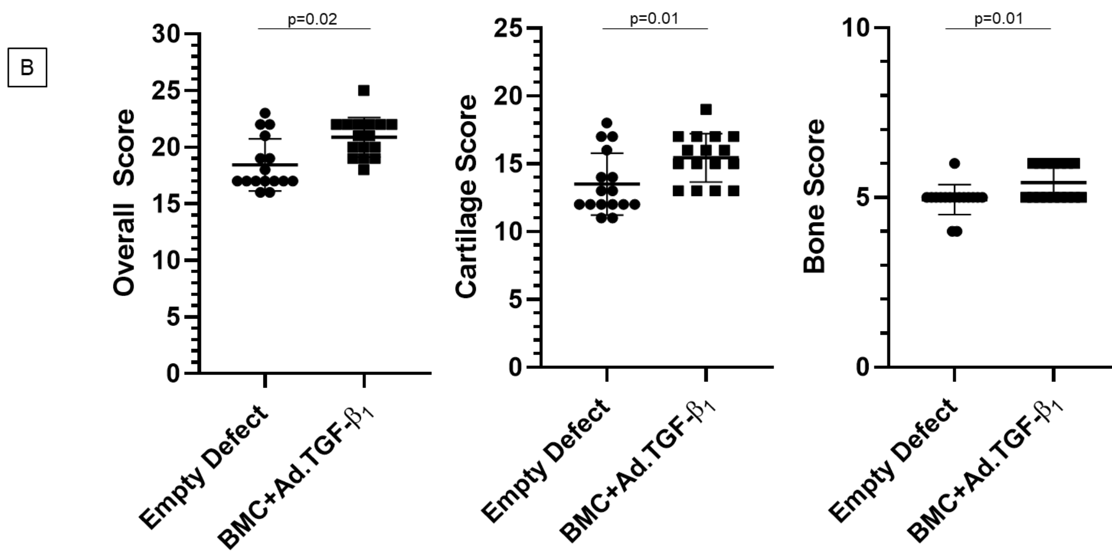

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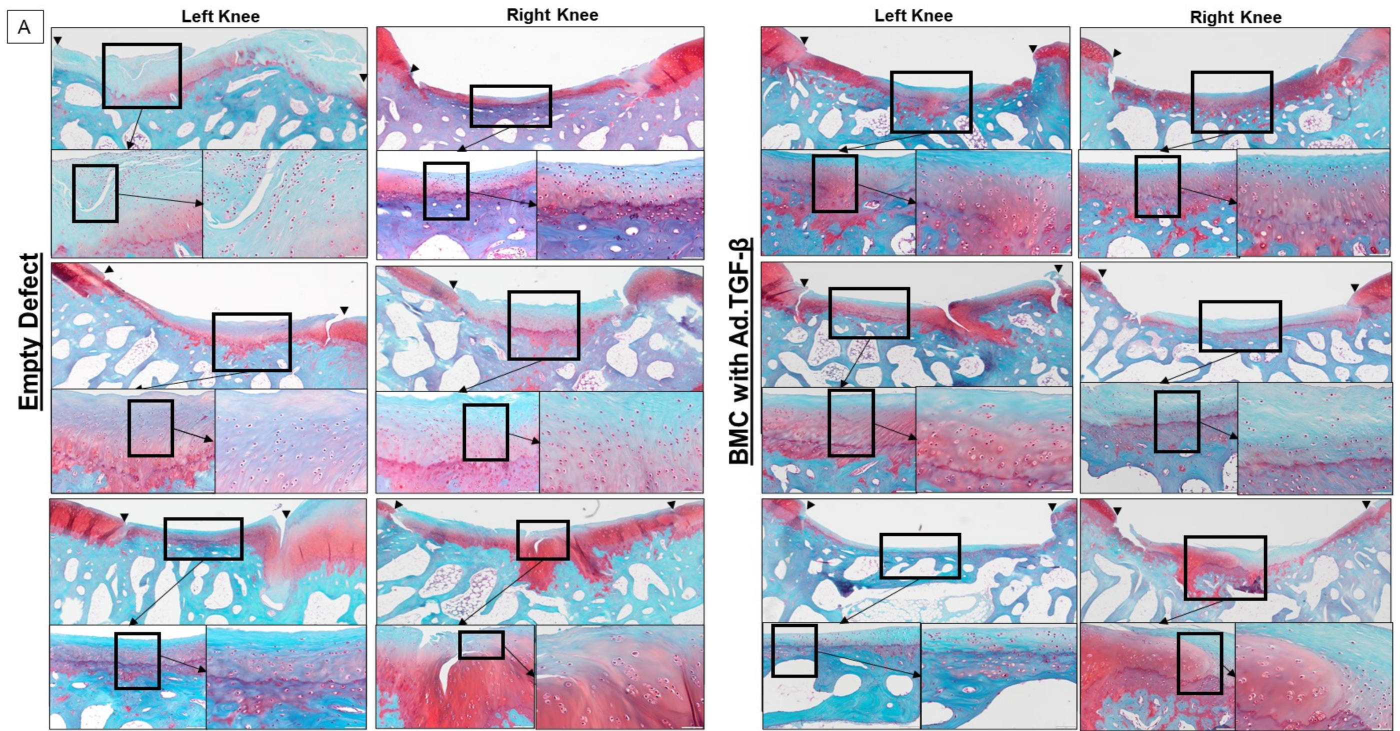
605 **Figure 4**



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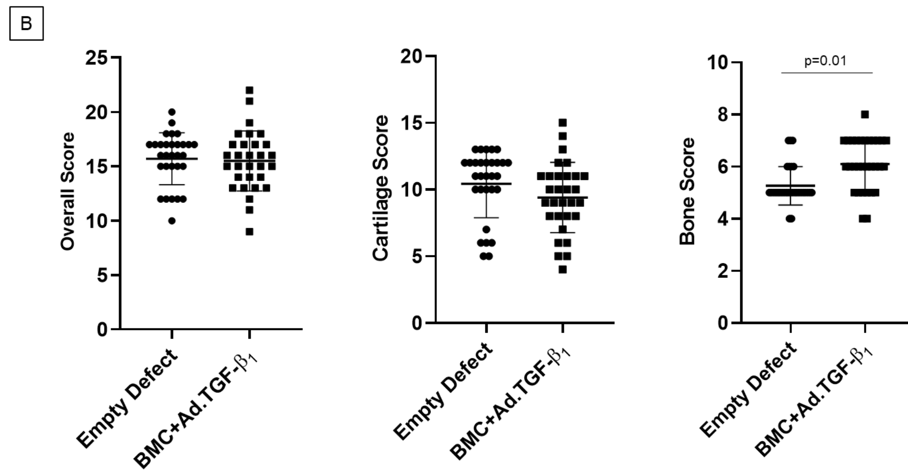

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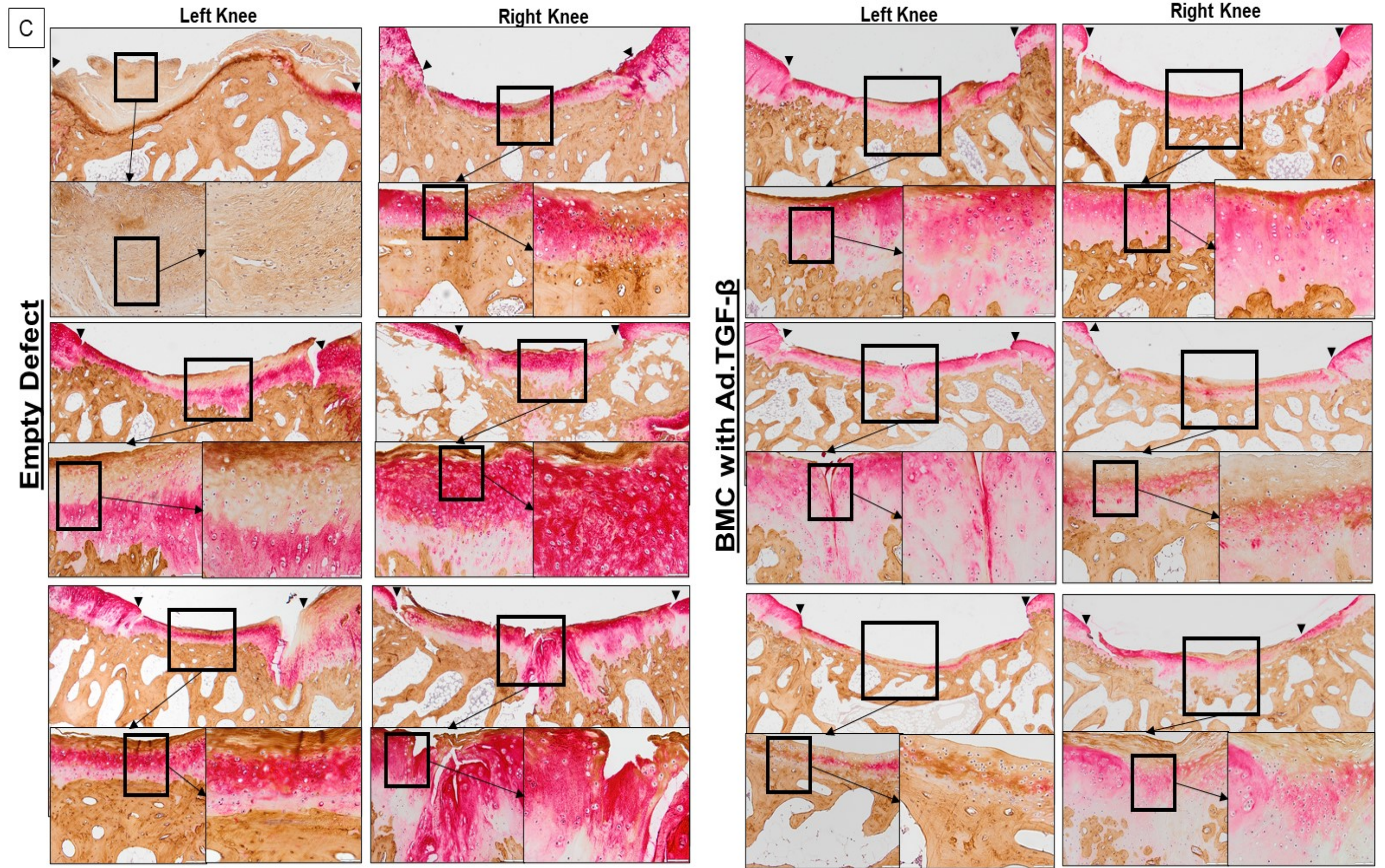

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639 **Figure 5**
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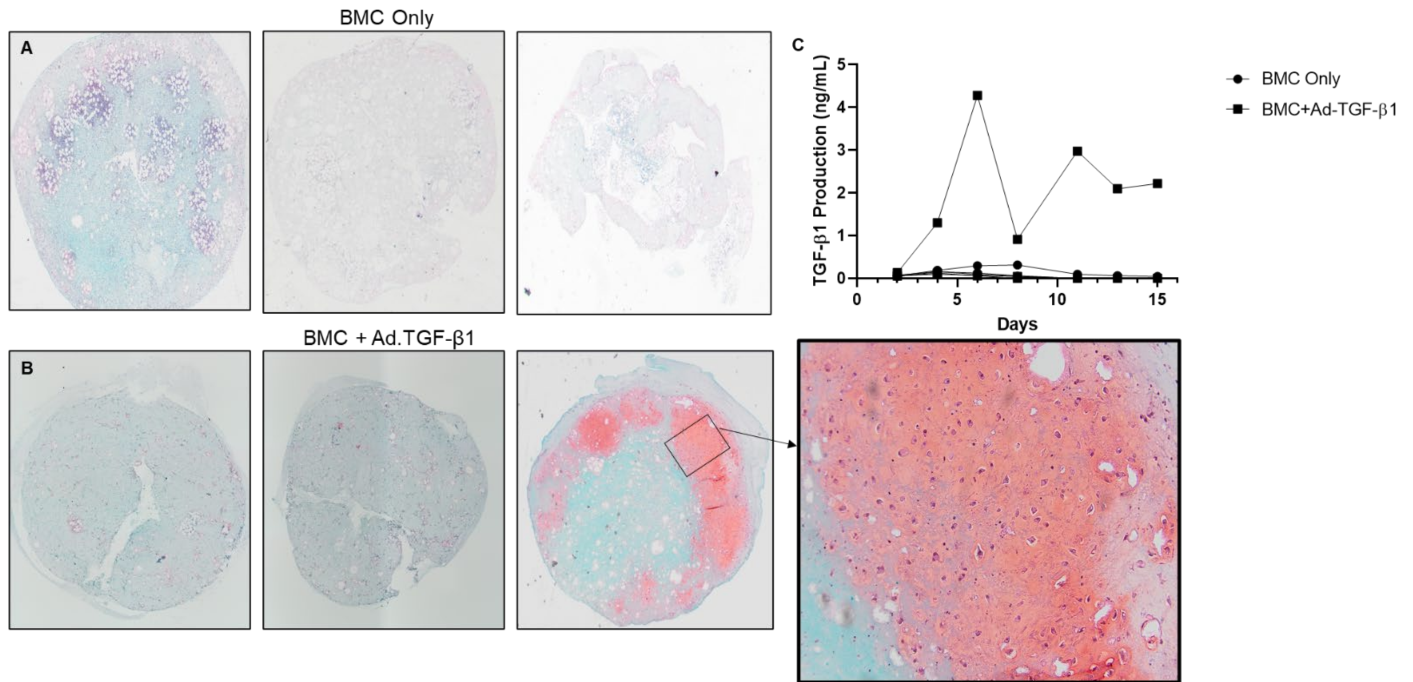

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669 **Figure 6**
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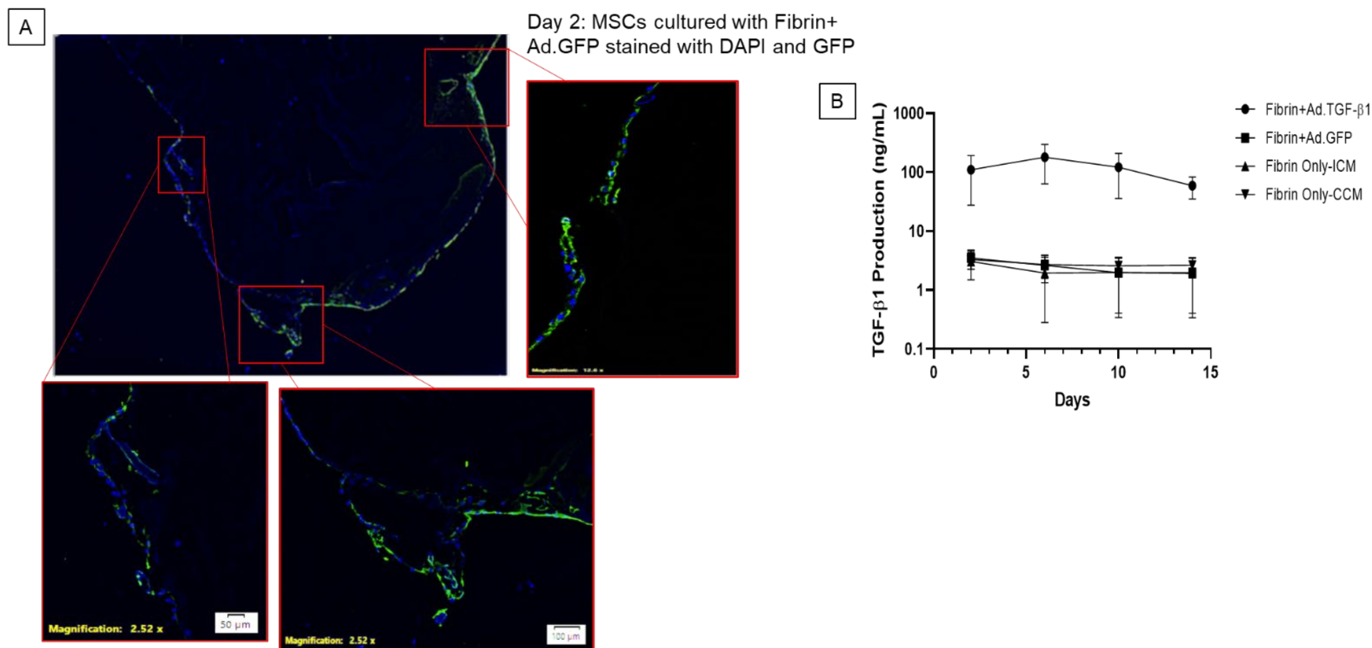

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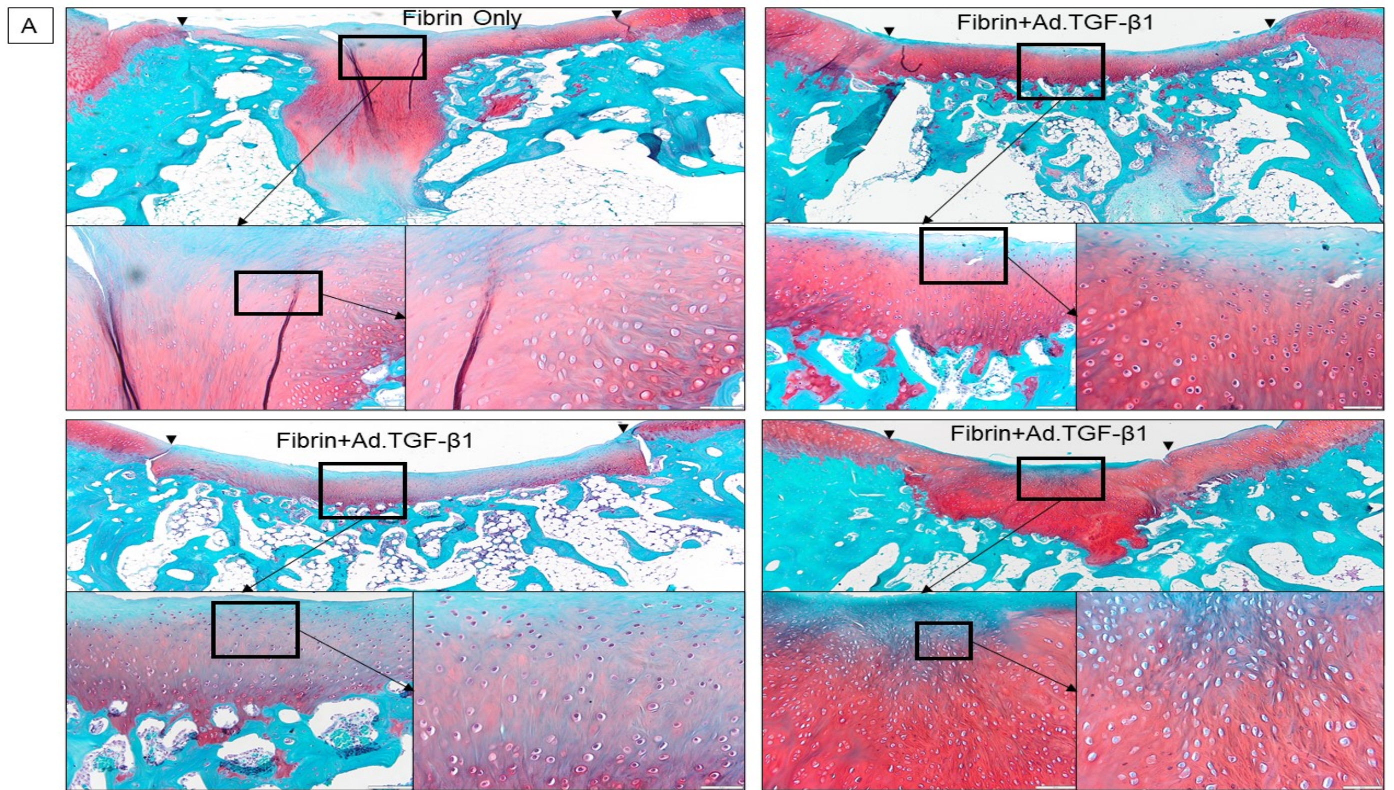
701 **Figure 7**



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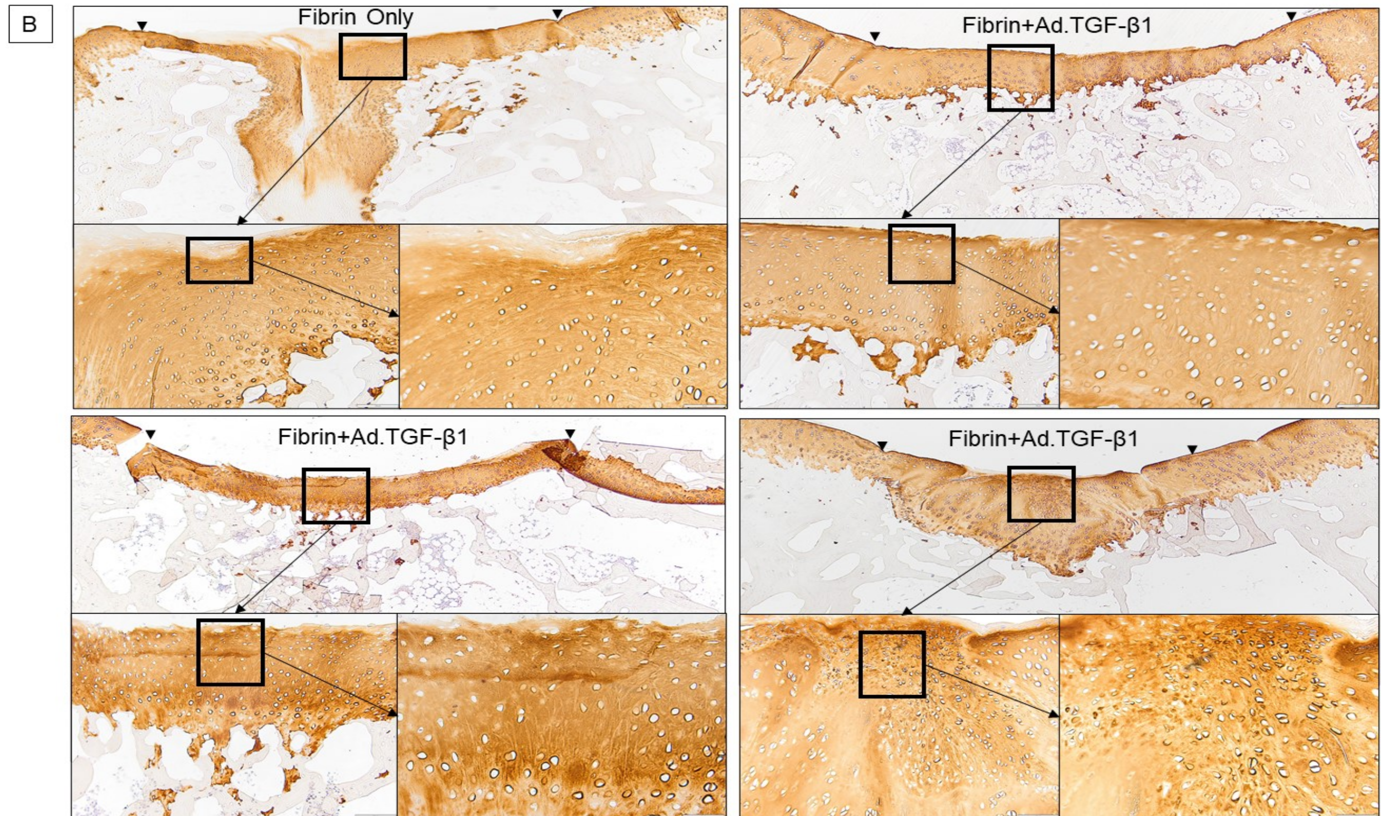

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