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Original Article

ALGINATE HYDROGEL-GUIDED RAAV-MEDIATED *FGF-2* AND *TGF-β* DELIVERY AND OVEREXPRESSION STIMULATES THE BIOLOGICAL ACTIVITIES OF HUMAN MENISCAL FIBROCHONDROCYTES FOR MENISCUS REPAIR

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Abstract

Meniscus lesions are unsolved problems that impede the functions of stability, load bearing, and shock absorption in the knee joint and for which there is no satisfying therapeutic option to date. Gene therapy using clinically adapted recombinant adeno-associated virus (rAAV) vectors is a powerful tool to enhance meniscus repair, especially when applied in combination with tissue engineering strategies that may allow to counteract a possible neutralization and/or dissemination of the rAAV particles in the recipient. Here, we examined the ability of an alginate (AlgPH155) hydrogel to formulate and deliver rAAV vectors carrying the genes for the reparative fibroblast growth factor (*FGF-2*) and transforming growth factor beta (*TGF-β*) as a means to trigger the biological activities in human meniscal fibrochondrocytes. The results show that effective rAAV-mediated *FGF-2* and *TGF-β* overexpression via alginate (AlgPH155) hydrogel-guided vector administration equally led to enhanced levels of cell proliferation and of specific matrix deposition (proteoglycans, type-I/-III collagen) in the cells over an extended period of time (21 days, the latest time point evaluated) relative to the control treatments (hydrogel without vector or with a vector carrying the reporter *lacZ* gene). These effects were associated with an increase in the expression of the contractile alpha smooth muscle actin (α-SMA) marker, a determinant of the meniscus response injury, and with decreased levels of pro-inflammatory markers (interleukin 1 beta—IL1β, tumor necrosis factor alpha—TNF-α). These findings show the potential of alginate hydrogel-guided rAAV-mediated gene therapy as a new, off-the-shelf therapeutic system for meniscus repair.

Keywords: Human meniscal fibrochondrocytes, recombinant adeno-associated virus, fibroblast growth factor 2, transforming growth factor beta, alginate hydrogel, reparative activities.

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Introduction

Meniscal tears are common, unsolved problems in sports medicine (Englund and Lohmander, 2005; Langhans *et al.*, 2023; Makris *et al.*, 2011), impairing the critical functions of this highly specialized fibrocartilaginous structure in knee joint stability, load bearing, and shock absorption (Langhans *et al.*, 2023; Makris *et al.*, 2011). Although tears occurring in the peripheral (vascularized) region of the meniscus may be sutured, those located in the central (avascular) portion do not properly heal, potentially leading to knee osteoarthritis (Englund and Lohmander, 2005; Langhans *et al.*, 2023; Roos *et al.*, 1998; Salata *et al.*, 2010), and their reconstruction is unsatisfying while the long-term benefits of allografts are not conclusive (Ahmed *et al.*, 2022; Kunze *et al.*, 2023; Langhans *et al.*, 2023; Makris *et al.*, 2011; Ozeki *et al.*, 2021; Wang *et al.*, 2021; Wirth *et al.*, 2002), showing the pressing need for new therapeutic options. The meniscus is composed of resident meniscal fibrochondrocytes that produce an extracellular matrix (ECM) predominantly containing type-I collagen, with also smaller amounts of proteoglycans (Arnoczky, 1999; Makris *et al.*, 2011; McDevitt and Webber, 1990). Such features are substantially altered in meniscal tears and disease, with impaired levels of cellularity and of ECM compounds (Herwig *et al.*, 1984; Hough and Webber, 1990; Meister *et al.*, 2004; Mesiha *et al.*, 2007).

Gene therapy offers strong tools to manage such pathological changes in torn menisci via the application

of gene sequences coding for reparative factors that may durably enhance meniscal cell proliferation and ECM deposition relative to short-lived recombinant molecules (Evans *et al.*, 2005; Evans and Huard, 2015; Evans and Robbins, 1999; Huard *et al.*, 2003; Lamsam *et al.*, 1998; Madry *et al.*, 2011; Martinek *et al.*, 2000; Shen *et al.*, 2005). Recombinant adeno-associated virus (rAAV) vectors are particularly well adapted to achieve these goals as these constructs are derived from a replication-defective, non-pathogenic human parvovirus (Atchison *et al.*, 1965) capable of stably targeting primary meniscal fibrochondrocytes at very high efficiencies (up to 100 % for at least 4 weeks *in vitro*) (Arrigoni *et al.*, 2021; Cucchiarini *et al.*, 2009; Madry *et al.*, 2004) even when the cells are embedded in their ECM (up to 75 % for 2 weeks *in situ*) (Cucchiarini *et al.*, 2009). Such effects are probably due to the small size (20 nm) (Atchison *et al.*, 1965) and long maintenance of rAAVs as stable episomal forms in their targets (months to years) (Xiao *et al.*, 1996) that may avoid the risk of insertional mutagenesis inherent to integrative retro-/lentivial vectors (Goto *et al.*, 2000) and in contrast to nonviral and adenoviral vectors with short-term functionality (Evans *et al.*, 2005; Evans and Huard, 2015; Evans and Robbins, 1999; Huard *et al.*, 2003; Lamsam *et al.*, 1998; Lee *et al.*, 2014; Madry *et al.*, 2011; Martinek *et al.*, 2000; Shen *et al.*, 2005; Steinert *et al.*, 2007), making currently rAAV the most suited gene transfer vehicles for clinical applications (Cucchiarini, 2016; Evans *et al.*, 2009; Evans *et al.*, 2013; Evans *et al.*, 2018; Evans *et al.*, 2006; Mease *et al.*, 2010; Vrouwe *et al.*, 2022). Nevertheless, a number of obstacles still impede the optimal use of rAAV *in vivo*, in particular the presence of neutralizing antibodies against the AAV capsid proteins in the human population (Abdul *et al.*, 2023; Cottard *et al.*, 2004; Mingozzi *et al.*, 2013) and the possible dissemination of the vectors to unwanted, nontarget sites (Cucchiarini, 2016). A potent approach to circumvent these hurdles is to take advantage of tissue engineering procedures as a means to deliver rAAV via biocompatible materials adapted for meniscus repair (Bilgen *et al.*, 2018; Cucchiarini, 2016; Cucchiarini *et al.*, 2016; Grogan *et al.*, 2020; Huard *et al.*, 2003; Kluyskens *et al.*, 2022; Kwon *et al.*, 2019; Makris *et al.*, 2011; Rey-Rico *et al.*, 2017), based on the pioneering work from Brunger and colleagues who used a scaffold-mediated lentiviral transduction of *TGF-β* for functional cartilage tissue engineering (Brunger *et al.*, 2014). Hydrogels provide optimal systems to achieve this goal due to their high water contents that make them consistent with the microenvironment of the meniscus and to their ability to deliver drugs and gene vectors in target tissues in a spatiotemporal manner (De Laporte and Shea, 2007; Jo and Tabata, 2015; Lombardo *et al.*, 2021; Rey-Rico *et al.*, 2017; Seidlits *et al.*, 2013).

The aim of this study was to explore the value of using an alginate hydrogel formulating rAAV vectors (Diaz-Rodriguez *et al.*, 2015) as a new, improved gene therapy platform for meniscus repair. Among the various factors reported for their therapeutic benefits in this tissue (connective tissue growth factor—CTGF; insulin-like growth factor I—IGF-I; hepatocyte growth factor—HGF) (Hidaka *et al.*, 2002; Romanazzo *et al.*, 2018; Zhang *et al.*, 2015; Zhang *et al.*, 2009a; Zhang *et al.*, 2009b), we examined the ability of the fibroblast growth factor (FGF-2) (Lee *et al.*, 2014) and of the transforming growth factor beta (TGF-β) (Goto *et al.*, 2000; Steinert *et al.*, 2007) to trigger the reparative activities of human meniscal fibrochondrocytes upon rAAV-mediated gene transfer guided via alginate (AlgPH155) hydrogel administration based on our previous findings showing the potential of these two gene vectors when applied directly to these cells (Cucchiarini *et al.*, 2009; Cucchiarini *et al.*, 2015).

Materials and Methods

Study Design

Human meniscal fibrochondrocytes were prepared from human adult menisci of knee joints from patients undergoing total knee arthroplasty, placed in monolayer culture, and directly incubated with rAAV (candidate *FGF-2* or *TGF-β versus* control *lacZ* or no vector)/alginate hydrogels for up to 21 days prior to performing the evaluations that included biochemical, immunohistological, and real-time RT-PCR analyses (Fig. 1).

Reagents

Reagents were from Sigma (Munich, Germany) otherwise indicated. Collagenase type-I (C1-22; 232 U/mg) was from Biochrom (Berlin, Germany). Sodium alginate (GRINDSTED AlgPH155, molecular weight = 140 kDa, mannuronic to glucuronic (M:G) ratio = 1:5, viscosity = 350-550 mPass) was obtained from Danisco (Copenhagen, Denmark). The dimethylmethylene blue dye (DMMB) was from Serva (20335.01; Darmstadt, Germany). The AAVanced Concentration Reagent was from System Bioscience (Heidelberg, Germany). The anti-FGF-2 (C-18), anti-TGF-β (V), anti-type-I collagen (COL-1), and anti-type-III collagen (C15) antibodies were purchased at Santa Cruz Biotechnology (Heidelberg, Germany), the anti-type-II collagen (II-II6B3) antibody at DSHB Iowa (Iowa City, IA, USA), and the anti-type-X collagen (C7974) and anti-α-SMA (1A4) antibodies at Sigma. Biotinylated secondary antibodies and the ABC reagent with diaminobenzidine (DAB, D5637) were obtained at Vector Laboratories (Alexis Deutschland GmbH, Grünberg, Germany). The FGF-2 ELISA (human FGF basic Quantikine ELISA, DFB50), the TGF-β ELISA (human TGF-β Quantikine ELISA, DB100B), the IL-1β ELISA (human IL-1β Quantikine ELISA, DLB50), and the TNF-α ELISA (human TNF-α Quantikine ELISA, DTA00D) were from R&D Systems (Wiesbaden-Nordenstadt, Germany). The Cell Proliferation Reagent WST-1 was purchased at Roche Applied Science (Mannheim, Germany). The type-I collagen

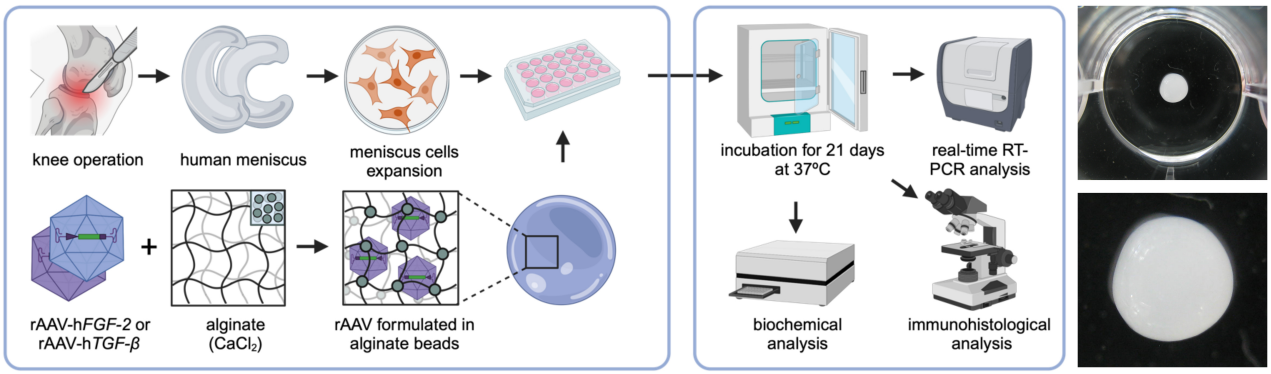

Fig. 1. Study design. Human meniscal fibrochondrocytes were prepared from human adult menisci of knee joints from patients undergoing total knee arthroplasty and placed in monolayer culture as described in the Materials and Methods. The rAAV-h*FGF-2* and rAAV-h*TGF-β* vectors were independently incubated in alginate to form rAAV (FGF-2 or TGF-β)/alginate hydrogels (macroscopic views) and added to the human meniscal fibrochondrocyte monolayer cultures for up to 21 days as described in the Materials and Methods. The obtained cultures were processed to perform biochemical, immunohistological, and real-time RT-PCR analyses as described in the Materials and Methods (created with BioRender.com).

ELISA (abx585048) was from Abbexa (Cambridge, UK) and the type-III collagen ELISA (EK403315-BM) from BioCat (Heidelberg, Germany).

Cell Culture

Human meniscal fibrochondrocytes were prepared from normal human adult menisci of knee joints from patients undergoing total knee arthroplasty (n = 3, ages 55-75 years) as previously described (Cucchiarini *et al.*, 2009; Cucchiarini *et al.*, 2015; Rey-Rico *et al.*, 2016) and with approval by the Ethics Committee of the Saarland Physicians Council (*Ärztekammer des Saarlandes*, *Ethik-Kommission*, no. 67/12). All patients provided informed consent before inclusion in the study, with all procedures in accordance with the Helsinki Declaration. Menisci with tears or visible degenerative changes on gross examination were excluded. Briefly, the menisci were washed, diced in 2 × 2 mm samples, and placed DMEM, 100 U/mL penicillin G, 100 µL/mL streptomycin (basal medium) with 10 % fetal bovine serum (FBS) (growth medium) containing 0.01 % (w/v) collagenase for 16 h at 37 °C in a humidified atmosphere with 5 % CO_2 (Cucchiarini *et al.*, 2009; Cucchiarini *et al.*, 2015; Rey-Rico *et al.*, 2016). Isolated cells were filtered through a 100 µm mesh to remove any undigested matrix, washed 2× with phosphate-buffered saline (PBS), and placed in 75-cm^2 culture flasks with growth medium at 37 °C with 5 % CO_2 with medium change every 2-3 days (Cucchiarini *et al.*, 2009; Cucchiarini *et al.*, 2015; Rey-Rico *et al.*, 2016). The cells were replated at the indicated densities for the experiments (passage ≤ 2) (Cucchiarini *et al.*, 2009; Cucchiarini *et al.*, 2015; Rey-Rico *et al.*, 2016).

Plasmids and rAAV Vectors

All constructs derive from pSSV9, an AAV-2 genomic clone (Samulski *et al.*, 1987; Samulski *et al.*, 1989). rAAV-*lacZ* carries the *E. coli* β-galactosidase (*lacZ*) reporter gene, rAAV-h*FGF-2* a human basic fibroblast growth factor (*FGF-2*) sequence (0.48-kb), and rAAV-h*TGF-β* a human transforming growth factor beta 1 (*TGF-β*) sequence (1.2-kb), all controlled by the CMV-IE promoter/enhancer (Cucchiarini *et al.*, 2009; Cucchiarini *et al.*, 2015; Diaz-Rodriguez *et al.*, 2015; Madry *et al.*, 2004; Venkatesan *et al.*, 2022). All vectors were packaged as conventional (not self-complementary) vectors with a helper-free, two-plasmid transfection system using the 293 packaging cell line (an adenovirus-transformed human embryonic cell line), the pXX2 packaging plasmid, and the pXX6 Adenovirus helper plasmid (Cucchiarini *et al.*, 2015; Diaz-Rodriguez *et al.*, 2015; Venkatesan *et al.*, 2022). Purification of the vectors using the AAVanced Concentration Reagent and their titration by real-time PCR were performed according to the manufacturer's recommendations (Cucchiarini *et al.*, 2009; Cucchiarini *et al.*, 2015; Diaz-Rodriguez *et al.*, 2015; Madry *et al.*, 2004; Venkatesan *et al.*, 2022) and using routine protocols (Cucchiarini *et al.*, 2009; Cucchiarini *et al.*, 2015; Diaz-Rodriguez *et al.*, 2015; Madry *et al.*, 2004; Venkatesan *et al.*, 2022), with ~ 10^{10} transgene copies/mL (~ 1/500 functional recombinant particles).

Incorporation of rAAV Vectors in Alginate Hydrogel

A 1.5 % alginate (AlgPH155) solution was prepared in PBS (Diaz-Rodriguez *et al.*, 2015; Rey-Rico *et al.*, 2016), mixed 1:1 (v/v) with the different rAAV preparations (80 µL alginate/80 µL vector in 10 % sucrose, i.e., 1.6 × 10^6 transgene copies) or with 10 % sucrose (80 µL alginate/80 µL 10 % sucrose, i.e., alginate hydrogel without vector), and dropped at room temperature in calcium chloride (102 mM) using a syringe with a needle of 27 G in 24-well plates for alginate crosslinking with calcium (3-8 sec) (Diaz-Rodriguez

et al., 2015; Rey-Rico *et al.*, 2016). Studies showing the effective and durable (21 days) rAAV vector controlled release from the alginate (AlgPH155) hydrogels have been already previously performed and reported (Diaz-Rodriguez *et al.*, 2015).

Gene Transfer via the rAAV/alginate Hydrogels

Monolayer cultures of human meniscal fibrochondrocytes (10^4 cells/well in 48-well plates) were kept in growth medium for 24 h at 37 °C with 5 % CO_2 and the rAAV/alginate hydrogels were directly added to the cultures (multiplicity of infection, i.e., MOI = 160) that were kept in growth medium for up to 21 days with medium change every 3-4 days (Cucchiarini *et al.*, 2009; Cucchiarini *et al.*, 2015; Diaz-Rodriguez *et al.*, 2015). Controls included hydrogels without rAAV vectors. Direct application of the vectors in their free form was not performed here as it was already reported in previous work (Cucchiarini *et al.*, 2009; Cucchiarini *et al.*, 2015).

Detection of Transgene Expression

Transgene (*FGF-2*, *TGF-*β) expression was monitored by specific, respective ELISAs as previously described (Cucchiarini *et al.*, 2009; Cucchiarini *et al.*, 2015). Briefly, monolayer cultures of human meniscal fibrochondrocytes were washed 2× and kept for 24 h in basal medium prior to collecting culture medium supernatants at the denoted time points that were centrifuged to remove debris. Measurements were performed using a GENios spectrophotomer/fluorometer (Tecan, Crailsheim, Germany) (Cucchiarini *et al.*, 2009; Cucchiarini *et al.*, 2015).

Expression of FGF-2 and TGF-β was also examined by immunocytochemistry on fixed cultures (4 % formalin) using specific, respective primary antibodies (anti-FGF-2 and anti-TGF-β: 1:50), biotinylated secondary antibodies (1:200), and the ABC method with DAB as the chromogen (Cucchiarini *et al.*, 2009; Cucchiarini *et al.*, 2015). Cultures were also processed with omission of the primary antibodies to control for secondary immunoglobulins (Cucchiarini *et al.*, 2009; Cucchiarini *et al.*, 2015). Cultures were then examined under light microscopy (Olympus BX 45; Hamburg, Germany) (Cucchiarini *et al.*, 2009; Cucchiarini *et al.*, 2015).

Immunocytochemical Analyses

Expression of type-I, -III, -II, and -X collagen and of α-SMA was examined by immunocytochemistry on fixed cultures (4 % formalin) using specific, respective primary antibodies (anti-type-II collagen: undiluted; anti-type-III collagen: 1:50; anti-type-I and -X collagen: 1:200; anti-α-SMA: 1:400), biotinylated secondary antibodies (1:200), and the ABC method with DAB as the chromogen (Cucchiarini *et al.*, 2009; Cucchiarini *et al.*, 2015; Diaz-Rodriguez *et al.*, 2015; Rey-Rico *et al.*, 2016; Venkatesan *et al.*, 2022). Cultures were also processed with omission of the primary antibodies to control for secondary immunoglobulins (Cucchiarini *et al.*, 2009; Cucchiarini *et al.*, 2015; Diaz-Rodriguez *et al.*, 2015; Rey-Rico *et al.*, 2016; Venkatesan *et al.*, 2022). Cultures were then examined under light microscopy (Olympus BX 45) (Cucchiarini *et al.*, 2009; Cucchiarini *et al.*, 2015; Diaz-Rodriguez *et al.*, 2015; Rey-Rico *et al.*, 2016; Venkatesan *et al.*, 2022).

Histomorphometry

The percentage of FGF-2-, TGF-β-, type-I collagen-, type-III collagen-, type-II collagen-, type-X collagen-, and α-SMA-positive (FGF-2^+, TGF-β^+, type-I collagen$^+$, type-III collagen$^+$, type-II collagen$^+$, type-X collagen$^+$, and α-SMA$^+$, respectively) cells (cells positively stained by immunocytochemical analyses for FGF-2, TGF-β, type-I collagen, type-III collagen, type-II collagen, type-X collagen, and α-SMA, respectively, to the total number of cells) (Cucchiarini *et al.*, 2009; Cucchiarini *et al.*, 2015; Venkatesan *et al.*, 2022) were measured at three standardized and randomized sites in the cultures using the CellSens program 1.12 (Olympus) and Adobe Photoshop Adobe Systems software CS6 (Adobe Systems, Unterschleissheim, Germany) (Venkatesan *et al.*, 2022).

Biochemical Analyses

The levels of IL-1β and TNF-α production were measured in the supernatants of the cultures by respective ELISAs as previously described (Cordaro *et al.*, 2020). The indices of cell proliferation were directly estimated in the cultures using the Cell Proliferation Reagent WST-1, with optical densities ($OD^{450\ nm}$) proportional to the cell numbers as previously described (Diaz-Rodriguez *et al.*, 2015; Steinert *et al.*, 2009; Venkatesan *et al.*, 2022). The cells were then harvested, digested overnight in 200 µL of 125 µg/mL papain (Sigma), and collected to assess the DNA contents by Hoechst 33258 assay (20 µL), the proteoglycan contents by binding to DMMB dye (20 µL), and the type-I and -III collagen contents by respective ELISA (100 µL of a 1:5 dilution) as previously described (Cucchiarini *et al.*, 2009; Cucchiarini *et al.*, 2015; Venkatesan *et al.*, 2022). Measurements were performed using a GENios spectrophotomer/fluorometer (Cucchiarini *et al.*, 2009; Cucchiarini *et al.*, 2015; Diaz-Rodriguez *et al.*, 2015; Venkatesan *et al.*, 2022).

Total RNA Extraction and Real-time RT-PCR Analyses

Total cellular RNA was prepared using the RNeasy Protect Mini Kit with on-column RNase-free DNase treatment (Qiagen, Hilden, Germany) (Venkatesan *et al.*, 2022) and RNA was eluted in 40 µL RNase-free water for reverse transcription with 8.2 µL of eluate and the 1st Strand cDNA Synthesis kit for RT-PCR (AMV) (Roche Applied Science) (Venkatesan *et al.*, 2022). RT-PCR amplification was executed with 2 µL of cDNA product using Brilliant SYBR Green QPCR Master Mix (Stratagene, Agilent

Technologies, Waldbronn, Germany) on an Mx3000P QPCR system (Stratagene) (Venkatesan *et al.*, 2022) with the following conditions: (10 min at 95 °C), cycles of amplification (30 sec denaturation at 95 °C, 1 min annealing at 60 °C, 30 sec extension at 72 °C), denaturation (1 min at 95 °C), and final incubation (30 sec at 55 °C) (Venkatesan *et al.*, 2022). The primers (Applied Biosystems, Inchinnan, UK) used were: type-I collagen (COL1A1; meniscus marker; forward 5′-ACGTCCTGGTGAAGTTGGTC-3′; reverse 5′-ACCAGGGAAGCCTCTCTCTC-3′), type-III collagen (COL3A1; meniscus marker; forward 5′-CACAAGGAGTCTGCATGTCT-3′; reverse 5′-GTTCACCAGGCTCACCAGCA-3′), alpha smooth muscle actin (α-SMA; contractile marker; forward 5′-GAACATGTAGTCCTTCCTGGAG-3′; reverse 5′-TAACTGTAGTCCTTCCTGGAG-3′), type-II collagen (COL2A1; chondrogenic marker; forward 5′-GGACTTTTCTCCCCTCTCT-3′; reverse 5′-GACCCGAAGGTCTTACAGGA-3′), type-X collagen (COL10A1; marker of hypertrophy; forward 5′-CCCTCTTGTTAGTGCCAACC-3′; reverse 5′-AGATTCCAGTCCTTGGGTCA-3′), interleukin 1 beta (IL-1β; pro-inflammatory marker; forward 5′- CCGTGCCTACGAACATGTC-3′; reverse 5′-CACACAGAAGCTCATCGGAG-3′), tumor necrosis factor alpha (TNF-α; pro-inflammatory marker; forward 5′-AGAACCCCCTGGAGATAACC-3′; reverse 5′-AAGTGCAGCAGGCAGAAGAG-3′), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; housekeeping gene and internal control; forward, 5′-GAAGGTGAAGGTCGGAGTC-3′; reverse, 5′-GAAGATGGTGATGGGATTTC-3′) (all 150 nM final concentration) (Venkatesan *et al.*, 2022). Controls included reactions using water and non-reverse-transcribed mRNA while product specificity was verified by melting curve analysis and agarose gel electrophoresis. The threshold cycle (Ct) value for each sequence was acquired for each amplification with the MxPro QPCR software (Stratagene) and values were normalized to GAPDH expression using the $2^{-\Delta\Delta Ct}$ method (Venkatesan *et al.*, 2022).

Statistical Analysis

Data are expressed as mean ± standard deviation of separate experiments. Each condition was performed in duplicate in three independent experiments with all patients′ samples. Data were obtained by two individuals blinded with respect to the treatment groups. The *t*-test was employed with $p \leq 0.05$ considered statistically significant.

Results

Effective rAAV-mediated FGF-2 and TGF-β Overexpression in Human Meniscal Fibrochondrocytes upon Alginate Hydrogel-guided Vector Delivery

The candidate rAAV-h*FGF-2*/alginate (FGF-2/AlgPH155) and rAAV-h*TGF-β*/alginate (TGF-β/AlgPH155) hydrogels were first tested for their respective ability to promote *FGF-2* and *TGF-β* overexpression in human meniscal fibrochondrocytes relative to the control conditions that included the reporter rAAV-*lacZ*/alginate (*lacZ*/AlgPH155) hydrogel and hydrogels without rAAV vectors (-/AlgPH155).

Effective overexpression of *FGF-2* via rAAV delivered using an alginate hydrogel (FGF-2/AlgPH155) was significantly achieved in human meniscal fibrochondrocytes relative to the control conditions (-/AlgPH155, *lacZ*/AlgPH155) as noted by immunocytochemical detection of FGF-2 (~ 85 % of FGF-2$^+$ cells with FGF-2/AlgPH155 *versus* < 10 % in the controls on day 21, i.e., an ~ 8.5-fold difference, always $p \leq 0.001$) and by specific (FGF-2) ELISA (up to 8.9-fold increase with FGF-2/AlgPH155 *versus* the controls on day 21, always $p \leq 0.001$) (Fig. 2**A**), probably due to the ability of AlgPH155 to support an effective, controlled release of these vectors (Diaz-Rodriguez *et al.*, 2015). Similarly, effective overexpression of *TGF-β* via rAAV delivered using an alginate hydrogel (TGF-β/AlgPH155) was significantly achieved in human meniscal fibrochondrocytes relative to the control conditions (-/AlgPH155, *lacZ*/AlgPH155) as seen by immunocytochemical detection of TGF-β (~ 78 % of TGF-β$^+$ cells with TGF-β/AlgPH155 *versus* < 10 % in the controls on day 21, i.e., an ~ 7.8-fold difference, always $p \leq 0.001$) and by specific (TGF-β) ELISA (up to 1.3-fold increase with TGF-β/AlgPH155 *versus* the controls on day 21, always $p \leq 0.001$) (Fig. 2**B**), again probably due to the ability of AlgPH155 to support an effective, controlled release of these vectors (Diaz-Rodriguez *et al.*, 2015). No significant differences were noted between the two control -/AlgPH155 and *lacZ*/AlgPH155 conditions for any of these parameters (always $p \geq 0.050$) (Fig. 2).

Effects of rAAV-mediated FGF-2 and TGF-β Overexpression on The Biological Activities of Human Meniscal Fibrochondrocytes upon Alginate Hydrogel-guided Vector Delivery

The candidate FGF-2/AlgPH155 and TGF-β/AlgPH155 hydrogels were next tested for their respective ability to stimulate the biological activities (cell proliferation, matrix deposition, inflammation) in human meniscal fibrochondrocytes relative to the control conditions including the reporter *lacZ*/AlgPH155 hydrogel and hydrogels without rAAV vectors (-/AlgPH155).

Application of the FGF-2/AlgPH155 and TGF-β/AlgPH155 hydrogels significantly enhanced the indices of cell proliferation in human meniscal fibrochondrocytes compared with the control conditions as noted using the Cell Proliferation Reagent WST-1 (up to 1.2-fold difference with FGF-2/AlgPH155 and TGF-β/AlgPH155, respectively, *versus* -/AlgPH155 and *lacZ*/AlgPH155 on day 21, $p \leq 0.006$) (Table 1). Overall, these findings were corroborated by the results of an estimation of the DNA contents using the Hoechst 33258 assay in cells treated

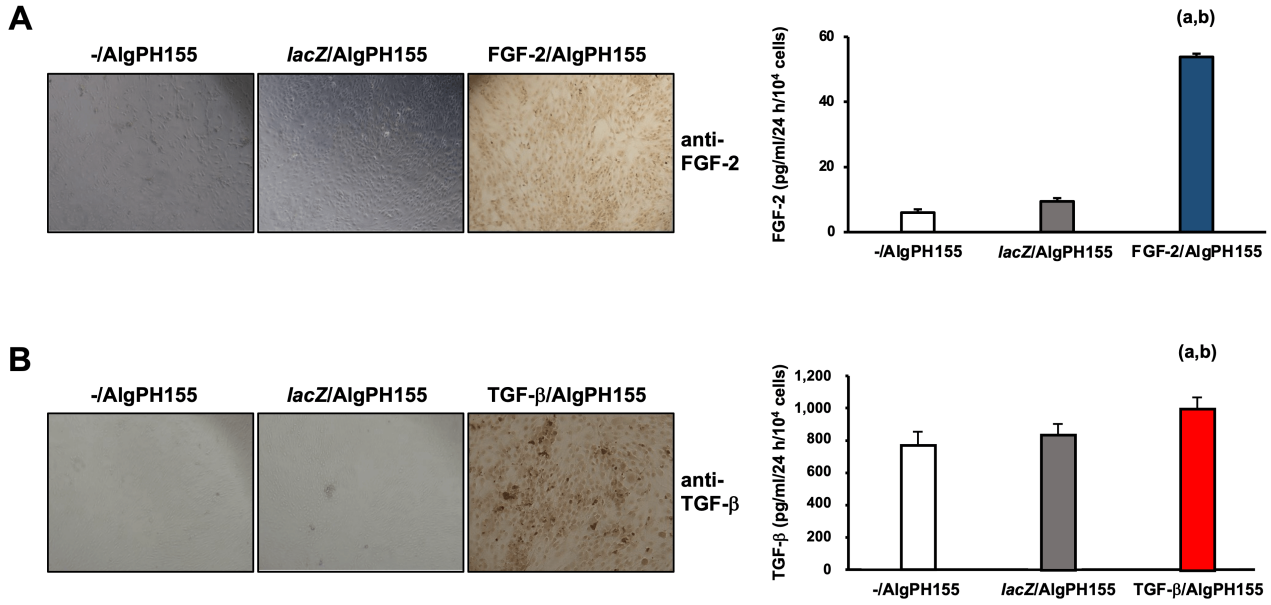

Fig. 2. Detection of transgene (*FGF-2*, *TGF-β*) expression in human meniscal fibrochondrocytes treated with the rAAV/alginate hydrogels. Cells in monolayer cultures (10^4 cells/well in 48-well plates) were directly incubated with the independently generated rAAV (FGF-2 or TGF-*β*)/alginate hydrogels (80 µL alginate/80 µL rAAV, i.e., 1.6×10^6 transgene copies and MOI = 160) and kept in culture as described in the Materials and Methods to monitor FGF-2 (**A**) and TGF-*β* (**B**) expression on day 21 by immunocytochemistry and specific ELISA as described in the Materials and Methods. Statistically significant relative to [a]-/AlgPH155 and [b]*lacZ*/AlgPH155. Abbreviations: -/AlgPH155, hydrogel without rAAV vector; *lacZ*/AlgPH155, rAAV-*lacZ*/alginate hydrogel; FGF-2/AlgPH155, rAAV-h*FGF-2*/alginate hydrogel; TGF-*β*/AlgPH155, rAAV-h*TGF-β*/alginate hydrogel.

with the FGF-2/AlgPH155 and TGF-*β*/AlgPH155 hydrogels (up to 1.1-fold difference with FGF-2/AlgPH155 and TGF-*β*/AlgPH155, respectively, *versus* -/AlgPH155 and *lacZ*/AlgPH155 on day 21, $p \leq 0.033$) (Table 1). No significant differences were noted between the two control -/AlgPH155 and *lacZ*/AlgPH155 conditions or between the two candidate FGF-2/AlgPH155 and TGF-*β*/AlgPH155 conditions for any of these parameters (always $p \geq 0.050$) (Table 1).

Application of the FGF-2/AlgPH155 and TGF-*β*/AlgPH155 hydrogels further led to significantly enhanced levels of matrix deposition in human meniscal fibrochondrocytes compared with the control conditions, including the proteoglycan contents using binding to DMMB (up to 1.9- and 2.2-fold difference with FGF-2/AlgPH155 and TGF-*β*/AlgPH155, respectively, *versus* -/AlgPH155 and *lacZ*/AlgPH155 on day 21, $p \leq 0.049$), the proteoglycan contents normalized to the DNA contents (up to 1.8- and 2-fold difference with FGF-2/AlgPH155 and TGF-*β*/AlgPH155, respectively, *versus* -/AlgPH155 and *lacZ*/AlgPH155 on day 21, $p \leq 0.036$), the type-I collagen contents by specific ELISA (up to 1.3-fold difference with FGF-2/AlgPH155 and TGF-*β*/AlgPH155, respectively, *versus* -/AlgPH155 and *lacZ*/AlgPH155 on day 21, $p \leq 0.048$), and the type-III collagen contents by specific ELISA (up to 1.9- and 1.6-fold difference with FGF-2/AlgPH155 and TGF-*β*/AlgPH155, respectively, *versus* -/AlgPH155 and *lacZ*/AlgPH155 on day 21, $p \leq 0.045$) (Table 1). Overall, these findings were corroborated by the results of an immunocytochemical analysis revealing significantly higher type-I and -III collagen deposition with FGF-2/AlgPH155 and TGF-*β*/AlgPH155 compared with the control conditions (~ 71 % and ~ 68 % of type-I collagen$^+$ cells with FGF-2/AlgPH155 and TGF-*β*/AlgPH155, respectively, *versus* < 6 % in -/AlgPH155 and *lacZ*/AlgPH155 on day 21, i.e., an ~ 11.8- and ~ 11.3-fold difference, respectively, always $p \leq 0.001$; ~ 76 % and ~ 66 % of type-III collagen$^+$ cells with FGF-2/AlgPH155 and TGF-*β*/AlgPH155, respectively, *versus* < 7 % in -/AlgPH155 and *lacZ*/AlgPH155 on day 21, i.e., an ~ 10.9- and ~ 9.4-fold difference, respectively, always $p \leq 0.001$) (Fig. 3). No significant differences were noted between the two control -/AlgPH155 and *lacZ*/AlgPH155 conditions or between the two candidate FGF-2/AlgPH155 and TGF-*β*/AlgPH155 conditions for any of these parameters (always $p \geq 0.050$) (Table 1 and Fig. 3).

These observations were further supported by the results of a real-time RT-PCR analysis showing significantly increased COL1A1 and COL3A1 expression compared with the control conditions (COL1A1: up to 9.4- and 7.1-fold difference with FGF-2/AlgPH155 and TGF-*β*/AlgPH155, respectively, *versus* -/AlgPH155 and *lacZ*/AlgPH155 on day 21, always $p \leq 0.001$; COL3A1: up to 3.2- and 2.2-fold difference with FGF-2/AlgPH155

Table 1. Biological activities in human meniscal fibrochondrocytes treated with the rAAV/alginate hydrogels (day 21).

Assay	-/AlgPH155	*lacZ*/AlgPH155	FGF-2/AlgPH155	TGF-β/AlgPH155
WST-1 ($OD^{450\ nm}/10^4$ cells)	1.17 ± 0.01	1.27 ± 0.03	1.43 ± 0.09[a,b]	1.39 ± 0.05[a,b]
DNA (ng/10^4 cells)	18.89 ± 0.03	19.51 ± 0.15	20.20 ± 0.05[a,b]	21.25 ± 0.18[a,b]
Proteoglycans (µg/10^4 cells)	0.79 ± 0.05	0.86 ± 0.03	1.54 ± 0.12[a,b]	1.75 ± 0.10[a,b]
Proteoglycans/DNA (µg/ng)	0.042 ± 0.002	0.044 ± 0.001	0.076 ± 0.006[a,b]	0.082 ± 0.004[a,b]
Type-I collagen (pg/mL/24 h/10^4 cells)	466.50 ± 14.85	483.50 ± 22.63	607.75 ± 1.77[a,b]	604.25 ± 3.18[a,b]
Type-III collagen (pg/mL/24 h/10^4 cells)	4,650.00 ± 494.97	5,075.00 ± 459.62	8,750.00 ± 282.84	7,500.00 ± 70.71[a,b]
IL-1β (pg/mL/24 h/10^4 cells)	3.25 ± 0.03	2.92 ± 0.29	2.20 ± 0.09	2.45 ± 0.04
TNF-α (pg/mL/24 h/10^4 cells)	106.53 ± 0.14	97.64 ± 5.54	75.69 ± 3.86	86.72 ± 0.39

Abbreviations: -/AlgPH155, alginate hydrogel without rAAV vector; *lacZ*/AlgPH155, alginate hydrogel carrying the rAAV-*lacZ* vector; FGF-2/AlgPH155, alginate hydrogel carrying the rAAV-h*FGF-2* vector; TGF-β/AlgPH155, alginate hydrogel carrying the rAAV-h*TGF-*β vector. Data are expressed as mean ± standard deviation of separate experiments. Statistically significant relative to [a]-/AlgPH155 and [b]*lacZ*/AlgPH155.

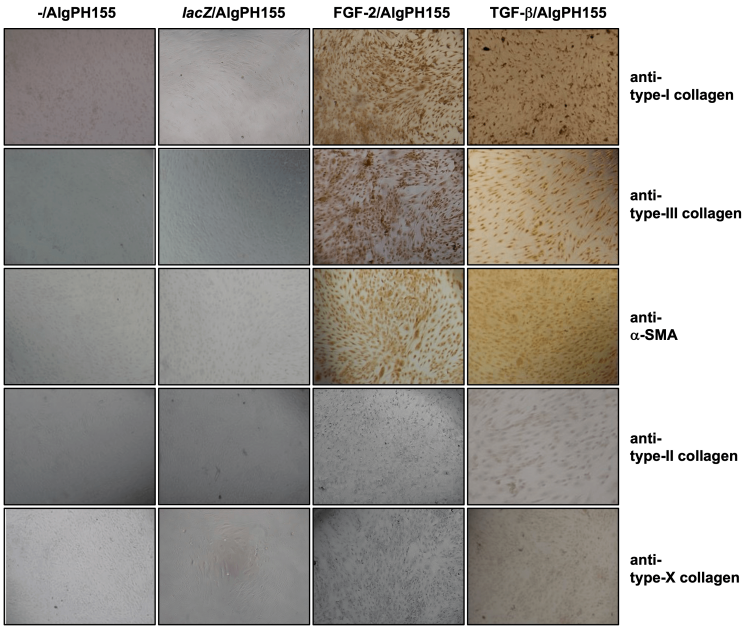

Fig. 3. Detection of matrix deposition and markers in human meniscal fibrochondrocytes treated with the rAAV/alginate hydrogels. Cells in monolayer cultures were directly incubated with the independently generated rAAV (FGF-2 or TGF-β)/alginate hydrogels as described in Fig. 2 and kept in culture as described in the Materials and Methods to monitor the deposition of type-I, -III, -II, and -X collagen and the expression of α-SMA on day 21 by immunocytochemistry with specific primary antibodies, biotinylated secondary antibodies, and the ABC method with DAB as the chromogen as described in the Materials and Methods (magnification 4×; all representative data). Abbreviations: -/AlgPH155, hydrogel without rAAV vector; *lacZ*/AlgPH155, rAAV-*lacZ*/alginate hydrogel; FGF-2/AlgPH155, rAAV-h*FGF-2*/alginate hydrogel; TGF-β/AlgPH155, rAAV-h*TGF-*β/alginate hydrogel.

and TGF-β/AlgPH155, respectively, *versus* -/AlgPH155 and *lacZ*/AlgPH155 on day 21, always $p \leq 0.001$) (Fig. 4). No significant differences were noted between the two control -/AlgPH155 and *lacZ*/AlgPH155 conditions or between the two candidate FGF-2/AlgPH155 and TGF-β/AlgPH155 conditions for any of these parameters (always $p \geq 0.050$) (Fig. 4).

These effects were associated with increased levels of specific α-SMA expression as seen on an immunocytochemical analysis (~ 85 % and ~ 80 % of α-SMA$^+$ cells with FGF-2/AlgPH155 and TGF-β/AlgPH155, respectively, *versus* < 8 % in -/AlgPH155 and *lacZ*/AlgPH155 on day 21, i.e., an ~ 10.6- and ~ 10-fold difference, respectively, always $p \leq 0.001$) (Fig. 3), a result again confirmed by a real-time RT-PCR analysis (up to 3.1- and 2.8-fold increase with FGF-2/AlgPH155 and TGF-β/AlgPH155, respectively, *versus* -/AlgPH155 on day 21, always $p \leq 0.001$) (Fig. 4). No significant differences were noted

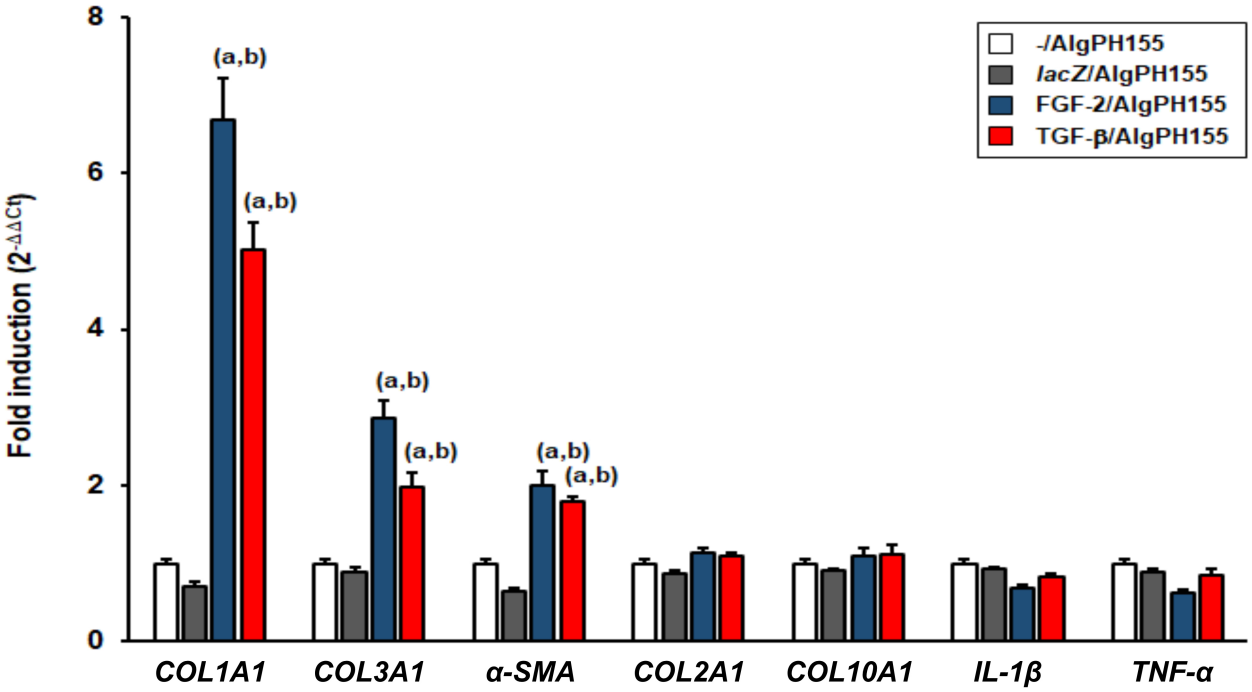

Fig. 4. Gene expression profiles in human meniscal fibrochondrocytes treated with the rAAV/alginate hydrogels. Cells in monolayer cultures were directly incubated with the independently generated rAAV (FGF-2 or TGF-β)/alginate hydrogels as described in Figs. 2,3 and kept in culture to monitor the following gene expression profiles by real-time RT-PCR on day 21 as described in the Materials and Methods: COL1A1, COL3A1, α-SMA, COL2A1, COL10A1, IL-1β, and TNF-α, with GAPDH serving as a housekeeping gene and internal control. Ct values were obtained for each target and for GAPDH (control for normalization) and fold inductions relative to the -/AlgPH155 condition were measured using the $2^{-\Delta\Delta Ct}$ method as described in the Materials and Methods. Statistically significant relative to [a]-/AlgPH155 and [b]*lacZ*/AlgPH155. Abbreviations: -/AlgPH155, hydrogel without rAAV vector; *lacZ*/AlgPH155, rAAV-*lacZ*/alginate hydrogel; FGF-2/AlgPH155, rAAV-h*FGF-2*/alginate hydrogel; TGF-β/AlgPH155, rAAV-h*TGF-*β/alginate hydrogel.

between the two control -/AlgPH155 and *lacZ*/AlgPH155 conditions or between the two candidate FGF-2/AlgPH155 and TGF-β/AlgPH155 conditions for any of these parameters (always $p \geq 0.050$) (Figs. 3,4).

In contrast, there was no significant difference in the modest levels of type-II and -X collagen deposition between the FGF-2/AlgPH155 and TGF-β/AlgPH155 *versus* the control conditions as noted by the results of an immunocytochemical analysis (~ 8 % and ~ 9 % of type-II collagen$^+$ cells with FGF-2/AlgPH155 and TGF-β/AlgPH155, respectively, *versus* < 7 % in -/AlgPH155 and *lacZ*/AlgPH155 on day 21, $p \geq 0.138$; ~ 9 % and ~ 10 % of type-X collagen$^+$ cells with FGF-2/AlgPH155 and TGF-β/AlgPH155, respectively, *versus* < 9 % in -/AlgPH155 and *lacZ*/AlgPH155 on day 21, $p \geq 0.126$) (Fig. 3), as also observed via real-time RT-PCR analysis (always $p \geq 0.05$ for COL2A1 and COL10A1 expression when comparing FGF-2/AlgPH155 and TGF-β/AlgPH155 with -/AlgPH155 and *lacZ*/AlgPH155 on day 21) (Fig. 4). No significant differences were noted between the two control -/AlgPH155 and *lacZ*/AlgPH155 conditions or between the two candidate FGF-2/AlgPH155 and TGF-β/AlgPH155 conditions for any of these parameters (always $p \geq 0.050$) (Figs. 3,4).

Interestingly, application of the FGF-2/AlgPH155 and TGF-β/AlgPH155 hydrogels decreased the expression of inflammatory IL-1β and TNF-α mediators compared with the control conditions as assessed by respective ELISA (IL-1β: up to 1.5- and 1.3-fold difference with FGF-2/AlgPH155 and TGF-β/AlgPH155, respectively, *versus* -/AlgPH155 and *lacZ*/AlgPH155 on day 21; TNF-α: up to 1.4- and 1.2-fold difference with FGF-2/AlgPH155 and TGF-β/AlgPH155, respectively, *versus* -/AlgPH155 and *lacZ*/AlgPH155 on day 21) (Table 1) and by real-time RT-PCR analysis (IL-1β: up to 1.5- and 1.2-fold difference with FGF-2/AlgPH155 and TGF-β/AlgPH155, respectively, *versus* -/AlgPH155 and *lacZ*/AlgPH155 on day 21; TNF-α: up to 1.6- and 1.2-fold difference with FGF-2/AlgPH155 and TGF-β/AlgPH155, respectively, *versus* -/AlgPH155 and *lacZ*/AlgPH155 on day 21), although statistical significance was not reached (always $p \geq 0.050$) (Table 1 and Fig. 4). No significant differences were noted between the two control -/AlgPH155 and *lacZ*/AlgPH155 conditions or between the two candidate FGF-2/AlgPH155 and TGF-β/AlgPH155 conditions for any of these parameters (always $p \geq 0.050$) (Table 1 and Fig. 4).

Discussion

Scaffold-guided gene therapy (Cucchiarini, 2016; Cucchiarini *et al.*, 2016; De Laporte and Shea, 2007; Jo and Tabata, 2015; Rey-Rico *et al.*, 2017; Seidlits *et al.*, 2013) is an innovative, powerful strategy for meniscus repair, especially when applied to deliver clinically adapted rAAV gene transfer vectors (Cucchiarini, 2016; Cucchiarini *et al.*, 2016; Evans *et al.*, 2009; Evans *et al.*, 2018; Evans *et al.*, 2006; Rey-Rico *et al.*, 2017). The goal of this study was to explore the possibility of triggering the reparative activities of primary human meniscal fibrochondrocytes by the overexpression of two potent growth factors (FGF-2, TGF-β) produced from rAAV gene transfer vectors delivered in the cells via an alginate (AlgPH155) hydrogel.

The data first show that the alginate (AlgPH155) hydrogel was capable of significantly and durably promoting the overexpression of the two candidate (*FGF-2*, *TGF-*β) genes in human meniscal fibrochondrocytes upon rAAV-mediated delivery relative to control treatments (hydrogel without vector or with a reporter rAAV-*lacZ* vector) (up to 8.9-fold difference for at least 21 days, the longest time point evaluated), probably due to the ability of this hydrogel to support the effective, controlled release of this class of vectors (Diaz-Rodriguez *et al.*, 2015). Overall, the levels of transgene (*FGF-2*, *TGF-*β) expression reached here in human meniscal fibrochondrocytes from rAAV via hydrogel-guided vector delivery were higher and more prolonged than those achieved when directly applying the same vectors in their free form to the cells in the same culture conditions and at similar vector doses (~ 55 pg FGF-2/mL/24 h/10^4 cells for 21 days with FGF-2/AlgPH155 here *versus* ~ 60 pg FGF-2/mL/24 h/10^4 cells for no longer than 10 days with free rAAV-hFGF-2; ~ 1,000 pg TGF-β/mL/24 h/10^4 cells for 21 days with TGF-β/AlgPH155 here *versus* only ~ 210 pg TGF-β/mL/24 h/10^4 cells for 21 days with free rAAV-hTGF-β) (Cucchiarini *et al.*, 2009; Cucchiarini *et al.*, 2015), again probably resulting from the vector controlled release capabilities of this hydrogel (Diaz-Rodriguez *et al.*, 2015).

The results next demonstrate that the effective, durable overexpression of the two candidate (*FGF-2*, *TGF-*β) genes from rAAV via alginate hydrogel-guided vector delivery significantly and durably enhanced the levels of cell proliferation and of specific matrix (proteoglycan, type-I/-III collagen) deposition in human meniscal fibrochondrocytes relative to control treatments (hydrogel without vector or with a reporter rAAV-*lacZ* vector) (up to 1.2-fold difference for cell proliferation and up to 11.8-fold difference for matrix deposition, for at least 21 days, the longest time point evaluated), probably again due to the vector controlled release ability of this hydrogel (Diaz-Rodriguez *et al.*, 2015), while no effects were noted on type-II/-X collagen expression. These observations are in good agreement with the properties of these growth factors applied either as recombinant agents (Adesida *et al.*, 2006; Collier and Ghosh, 1995; Webber *et al.*, 1988) or via less adapted vectors (nonviral, adenoviral, retroviral gene vehicles) (Goto *et al.*, 2000; Lee *et al.*, 2014; Steinert *et al.*, 2007) and with our findings when directly applying the same vectors in their free form to such cells in similar conditions (Cucchiarini *et al.*, 2009; Cucchiarini *et al.*, 2015). Interestingly, compared with control treatments (hydrogel without vector or with a reporter rAAV-*lacZ* vector), the effects of FGF-2/AlgPH155 and TGF-β/AlgPH155 were associated with an increased expression of contractile α-SMA, a determinant of the meniscus response to injury (Lin *et al.*, 2002) (up to 10.6-fold difference for at least 21 days, the longest time point evaluated) and with a decreased inflammatory response (IL-1β, TNF-α) in the specific conditions applied here (up to 1.6-fold difference for at least 21 days, the longest time point evaluated), concordant with previous observations (Cucchiarini *et al.*, 2009; Cucchiarini *et al.*, 2015; Kuo *et al.*, 2019; Rabie *et al.*, 2023; Zaleskas *et al.*, 2001). Interestingly, the FGF-2/AlgPH155 and TGF-β/AlgPH155 systems were equally potent, in good agreement with previous findings when applying low, optimally safe doses of recombinant FGF-2 and TGF-β factors to these cells (Pangborn and Athanasiou, 2005) as achieved here.

Conclusions

The current study provides the proof of principle of the potential of therapeutic (*FGF-2*, *TGF-*β) rAAV gene vectors delivered via an alginate (AlgPH155) hydrogel as a convenient, off-the-shelf system to advantageously trigger the reparative activities in human meniscal fibrochondrocytes to enhance the processes of meniscus repair in future translational applications. Of note, this strategy was used to durably heal cartilage defects in minipigs over one year with a functional rAAV-h*IGF-I*/AlgPH55 hydrogel system (Maihöfer *et al.*, 2021) from which rAAV is being released in a sustained, controlled manner by diffusion without degradation (Diaz-Rodriguez *et al.*, 2015; Madrigal *et al.*, 2019). To optimize therapeutic outcomes, it will be crucial to define the ideal vector dose provided in the hydrogel for applications *in vivo* by performing an extensive, comparative analysis in future work. This scaffold-guided gene therapy approach may provide less invasive options compared with the use of genetically modified cells encapsulated in an alginate hydrogel to achieve this goal (Collier and Ghosh, 1995; Cucchiarini *et al.*, 2009; Lee *et al.*, 2014; Rey-Rico *et al.*, 2016; Zhang *et al.*, 2009a). Work is ongoing to test the benefits of the present strategy to stimulate the reparative activities of human meniscal fibrochondrocytes in a three-dimensional (3D) environment (Liang *et al.*, 2020) known to be adapted for the effective transduction and overexpression of rAAV vectors (3D pellet cultures) (Cucchiarini *et al.*, 2011). A study is currently being performed to monitor the effective repair of meniscal lesions in experimental models *ex vivo* (Cucchiarini *et al.*, 2009;

Cucchiarini *et al.*, 2015; Rey-Rico *et al.*, 2016) and in relevant animal models *in vivo* (Goto *et al.*, 1999; Madry *et al.*, 2004; Zhang *et al.*, 2009a) with this approach and to test its value over a direct administration of the rAAV vectors in their free form. Such an approach may further be adapted for a safe delivery of multiple rAAV vectors without interference (Tao *et al.*, 2016) to synergize and extend the benefits reported here with FGF-2 and TGF-β as noted with recombinant factors, with also a potential to mechanistically increase the integration of the material at repair sites (Ionescu *et al.*, 2012). Other therapeutic gene treatments may be also envisaged like CTGF that induces the deposition of a fibrochondrocyte matrix (Romanazzo *et al.*, 2018), provided that rAAV supports its effective overexpression and activities in the cells. Taken together, these results provide motivation to apply alginate hydrogel-guided gene therapy to treat human meniscal lesions.

List of Abbreviations

rAAV, recombinant adeno-associated virus; AlgPH155, alginate; FGF-2, basic fibroblast growth factor; TGF-β, transforming growth factor beta; α-SMA, alpha smooth muscle actin; IL-1β, interleukin 1 beta; TNF-α, tumor necrosis factor alpha; ECM, extracellular matrix; CTGF, connective tissue growth factor; IGF-I, insulin-like growth factor I; HGF, hepatocyte growth factor; *lacZ*, β-galactosidase; DMMB, dimethylmethylene blue; DAB, diaminobenzidine; FBS, fetal bovine serum; MOI, multiplicity of infection; -/AlgPH155, hydrogel without rAAV vector; *lacZ*/AlgPH155, rAAV-*lacZ*/alginate hydrogel; FGF-2/AlgPH155, rAAV-hFGF-2/alginate hydrogel; TGF-β/AlgPH155, rAAV-hTGF-β/alginate hydrogel.

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Author Contributions

JKV and MC designed the research study. JKV and WL performed the research. JKV, WL, HM, and MC analyzed the data. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

With approval by the Ethics Committee of the Saarland Physicians Council (*Ärztekammer des Saarlandes*, *Ethik-Kommission*, no. 67/12). All patients provided informed consent before inclusion in the study, with all procedures in accordance with the Helsinki Declaration.

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Conflict of Interest

The authors have no conflicts of interest to declare.

References

Abdul TY, Hawse GP, Smith J, Sellon JL, Abdel MP, Wells JW, Coenen MJ, Evans CH, De La Vega RE (2023) Prevalence of AAV2.5 neutralizing antibodies in synovial fluid and serum of patients with osteoarthritis. Gene Therapy 30: 587-591. DOI: 10.1038/s41434-022-00326-5.

Adesida AB, Grady LM, Khan WS, Hardingham TE (2006) The matrix-forming phenotype of cultured human meniscus cells is enhanced after culture with fibroblast growth factor 2 and is further stimulated by hypoxia. Arthritis Research & Therapy 8: R61. DOI: 10.1186/ar1929.

Ahmed AF, Rinaldi J, Noorzad AS, Zikria BA (2022) Return to Sports Following Meniscal Allograft Transplantation Is Possible but Remains Questionable: A Systematic Review. Arthroscopy: the Journal of Arthroscopic & Related Surgery: Official Publication of the Arthroscopy Association of North America and the International Arthroscopy Association 38: 1351-1361. DOI: 10.1016/j.arthro.2021.11.003.

Arnoczky SP (1999) Building a meniscus. Biologic considerations. Clinical Orthopaedics and Related Research S244-S253.

Arrigoni P, Ruprecht JC, Chasse DAD, Glass KA, Andress B, Guilak F, Weinberg JB, McNulty AL (2021) Optimization of Meniscus Cell Transduction Using Lentivirus and Adeno-Associated Virus for Gene Editing and Tissue Engineering Applications. Cartilage 13: 1602S-1607S. DOI: 10.1177/1947603519880321.

ATCHISON RW, CASTO BC, HAMMON WM (1965) ADENOVIRUS-ASSOCIATED DEFECTIVE VIRUS PARTICLES. Science (New York, N.Y.) 149: 754-756. DOI: 10.1126/science.149.3685.754.

Bilgen B, Jayasuriya CT, Owens BD (2018) Current Concepts in Meniscus Tissue Engineering and Repair. Advanced Healthcare Materials 7: e1701407. DOI: 10.1002/adhm.201701407.

Brunger JM, Huynh NPT, Guenther CM, Perez-Pinera P, Moutos FT, Sanchez-Adams J, Gersbach CA, Guilak F (2014) Scaffold-mediated lentiviral transduction for func-

tional tissue engineering of cartilage. Proceedings of the National Academy of Sciences of the United States of America 111: E798-806. DOI: 10.1073/pnas.1321744111.

Collier S, Ghosh P (1995) Effects of transforming growth factor beta on proteoglycan synthesis by cell and explant cultures derived from the knee joint meniscus. Osteoarthritis and Cartilage 3: 127-138. DOI: 10.1016/s1063-4584(05)80045-7.

Cordaro A, Zagami R, Malanga M, Venkatesan JK, Alvarez-Lorenzo C, Cucchiarini M, Piperno A, Mazzaglia A (2020) Cyclodextrin Cationic Polymer-Based Nanoassemblies to Manage Inflammation by Intra-Articular Delivery Strategies. Nanomaterials (Basel, Switzerland) 10: 1712. DOI: 10.3390/nano10091712.

Cottard V, Valvason C, Falgarone G, Lutomski D, Boissier MC, Bessis N (2004) Immune response against gene therapy vectors: influence of synovial fluid on adeno-associated virus mediated gene transfer to chondrocytes. Journal of Clinical Immunology 24: 162-169. DOI: 10.1023/B:JOCI.0000019781.64421.5c.

Cucchiarini M, Schetting S, Terwilliger EF, Kohn D, Madry H (2009) rAAV-mediated overexpression of FGF-2 promotes cell proliferation, survival, and alpha-SMA expression in human meniscal lesions. Gene Therapy 16: 1363-1372. DOI: 10.1038/gt.2009.91.

Cucchiarini M (2016) Human gene therapy: novel approaches to improve the current gene delivery systems. Discovery Medicine 21: 495-506.

Cucchiarini M, McNulty AL, Mauck RL, Setton LA, Guilak F, Madry H (2016) Advances in combining gene therapy with cell and tissue engineering-based approaches to enhance healing of the meniscus. Osteoarthritis and Cartilage 24: 1330-1339. DOI: 10.1016/j.joca.2016.03.018.

Cucchiarini M, Schmidt K, Frisch J, Kohn D, Madry H (2015) Overexpression of TGF-β via rAAV-Mediated Gene Transfer Promotes the Healing of Human Meniscal Lesions Ex Vivo on Explanted Menisci. The American Journal of Sports Medicine 43: 1197-1205. DOI: 10.1177/0363546514567063.

Cucchiarini M, Ekici M, Schetting S, Kohn D, Madry H (2011) Metabolic activities and chondrogenic differentiation of human mesenchymal stem cells following recombinant adeno-associated virus-mediated gene transfer and overexpression of fibroblast growth factor 2. Tissue Engineering. Part a 17: 1921-1933. DOI: 10.1089/ten.TEA.2011.0018.

De Laporte L, Shea LD (2007) Matrices and scaffolds for DNA delivery in tissue engineering. Advanced Drug Delivery Reviews 59: 292-307. DOI: 10.1016/j.addr.2007.03.017.

Díaz-Rodríguez P, Rey-Rico A, Madry H, Landin M, Cucchiarini M (2015) Effective genetic modification and differentiation of hMSCs upon controlled release of rAAV vectors using alginate/poloxamer composite systems. International Journal of Pharmaceutics 496: 614-626. DOI: 10.1016/j.ijpharm.2015.11.008.

Englund M, Lohmander LS (2005) Patellofemoral osteoarthritis coexistent with tibiofemoral osteoarthritis in a meniscectomy population. Annals of the Rheumatic Diseases 64: 1721-1726. DOI: 10.1136/ard.2005.035568.

Evans CH, Ghivizzani SC, Herndon JH, Robbins PD (2005) Gene therapy for the treatment of musculoskeletal diseases. The Journal of the American Academy of Orthopaedic Surgeons 13: 230-242. DOI: 10.5435/00124635-200507000-00003.

Evans CH, Ghivizzani SC, Robbins PD (2009) Progress and Prospects: genetic treatments for disorders of bones and joints. Gene Therapy 16: 944-952. DOI: 10.1038/gt.2009.73.

Evans CH, Ghivizzani SC, Robbins PD (2013) Arthritis gene therapy and its tortuous path into the clinic. Translational Research: the Journal of Laboratory and Clinical Medicine 161: 205-216. DOI: 10.1016/j.trsl.2013.01.002.

Evans CH, Ghivizzani SC, Robbins PD (2018) Gene Delivery to Joints by Intra-Articular Injection. Human Gene Therapy 29: 2-14. DOI: 10.1089/hum.2017.181.

Evans CH, Gouze E, Gouze JN, Robbins PD, Ghivizzani SC (2006) Gene therapeutic approaches-transfer in vivo. Advanced Drug Delivery Reviews 58: 243-258. DOI: 10.1016/j.addr.2006.01.009.

Evans CH, Huard J (2015) Gene therapy approaches to regenerating the musculoskeletal system. Nature Reviews. Rheumatology 11: 234-242. DOI: 10.1038/nrrheum.2015.28.

Evans CH, Robbins PD (1999) Genetically augmented tissue engineering of the musculoskeletal system. Clinical Orthopaedics and Related Research S410-8. DOI: 10.1097/00003086-199910001-00040.

Goto H, Shuler FD, Lamsam C, Moller HD, Niyibizi C, Fu FH, Robbins PD, Evans CH (1999) Transfer of lacZ marker gene to the meniscus. The Journal of Bone and Joint Surgery. American Volume 81: 918-925. DOI: 10.2106/00004623-199907000-00003.

Goto H, Shuler FD, Niyibizi C, Fu FH, Robbins PD, Evans CH (2000) Gene therapy for meniscal injury: enhanced synthesis of proteoglycan and collagen by meniscal cells transduced with a TGFbeta(1)gene. Osteoarthritis and Cartilage 8: 266-271. DOI: 10.1053/joca.1999.0300.

Grogan SP, Baek J, D'Lima DD (2020) Meniscal tissue repair with nanofibers: future perspectives. Nanomedicine (London, England) 15: 2517-2538. DOI: 10.2217/nnm-2020-0183.

Herwig J, Egner E, Buddecke E (1984) Chemical changes of human knee joint menisci in various stages of degeneration. Annals of the Rheumatic Diseases 43: 635-640. DOI: 10.1136/ard.43.4.635.

Hidaka C, Ibarra C, Hannafin JA, Torzilli PA, Quitoriano M, Jen SS, Warren RF, Crystal RG (2002) Formation of vascularized meniscal tissue by combining gene therapy with tissue engineering. Tissue Engineering 8: 93-105.

DOI: 10.1089/107632702753503090.

Hough AJ Jr, Webber RJ (1990) Pathology of the meniscus. Clinical Orthopaedics and Related Research 32-40.

Huard J, Li Y, Peng H, Fu FH (2003) Gene therapy and tissue engineering for sports medicine. The Journal of Gene Medicine 5: 93-108. DOI: 10.1002/jgm.344.

Ionescu LC, Lee GC, Huang KL, Mauck RL (2012) Growth factor supplementation improves native and engineered meniscus repair in vitro. Acta Biomaterialia 8: 3687-3694. DOI: 10.1016/j.actbio.2012.06.005.

Jo JI, Tabata Y (2015) How controlled release technology can aid gene delivery. Expert Opinion on Drug Delivery 12: 1689-1701. DOI: 10.1517/17425247.2015.1048221.

Kluyskens L, Debieux P, Wong KL, Krych AJ, Saris DBF (2022) Biomaterials for meniscus and cartilage in knee surgery: state of the art. Journal of ISAKOS: Joint Disorders & Orthopaedic Sports Medicine 7: 67-77. DOI: 10.1136/jisakos-2020-000600.

Kunze KN, Davie RA, Ramkumar PN, Chahla J, Nwachukwu BU, Williams RJ, 3rd (2023) Risk Factors for Graft Failure After Meniscal Allograft Transplantation: A Systematic Review and Meta-analysis. Orthopaedic Journal of Sports Medicine 11: 23259671231160296. DOI: 10.1177/23259671231160296.

Kuo SJ, Liu SC, Huang YL, Tsai CH, Fong YC, Hsu HC, Tang CH (2019) TGF-β1 enhances FOXO3 expression in human synovial fibroblasts by inhibiting miR-92a through AMPK and p38 pathways. Aging 11: 4075-4089. DOI: 10.18632/aging.102038.

Kwon H, Brown WE, Lee CA, Wang D, Paschos N, Hu JC, Athanasiou KA (2019) Surgical and tissue engineering strategies for articular cartilage and meniscus repair. Nature Reviews. Rheumatology 15: 550-570. DOI: 10.1038/s41584-019-0255-1.

Lamsam C, Fu FH, Robbins PD, Evans CH (1998) Gene therapy in sports medicine. Sports Medicine (Auckland, N.Z.) 25: 73-77. DOI: 10.2165/00007256-199825020-00001.

Langhans MT, Lamba A, Saris DBF, Smith P, Krych AJ (2023) Meniscal Extrusion: Diagnosis, Etiology, and Treatment Options. Current Reviews in Musculoskeletal Medicine 16: 316-327. DOI: 10.1007/s12178-023-09840-4.

Lee HP, Rey-Rico A, Cucchiarini M, Madry H (2014) Nonviral gene transfer into human meniscal cells. Part II: effect of three-dimensional environment and overexpression of human fibroblast growth factor 2. International Orthopaedics 38: 1931-1936. DOI: 10.1007/s00264-014-2405-z.

Liang Y, Szojka ARA, Idrees E, Kunze M, Mulet-Sierra A, Adesida AB (2020) Re-Differentiation of Human Meniscus Fibrochondrocytes Differs in Three-Dimensional Cell Aggregates and Decellularized Human Meniscus Matrix Scaffolds. Annals of Biomedical Engineering 48: 968-979. DOI: 10.1007/s10439-019-02272-7.

Lin BY, Richmond JC, Spector M (2002) Contractile actin expression in torn human menisci. Wound Repair and Regeneration 10: 259-266. DOI: 10.1046/j.1524-475x.2002.10410.x.

Lombardo MDM, Mangiavini L, Peretti GM (2021) Biomaterials and Meniscal Lesions: Current Concepts and Future Perspective. Pharmaceutics 13: 1886. DOI: 10.3390/pharmaceutics13111886.

Madrigal JL, Shams S, Stilhano RS, Silva EA (2019) Characterizing the encapsulation and release of lentivectors and adeno-associated vectors from degradable alginate hydrogels. Biomaterials Science 7: 645-656. DOI: 10.1039/c8bm01218k.

Madry H, Cucchiarini M, Kaul G, Kohn D, Terwilliger EF, Trippel SB (2004) Menisci are efficiently transduced by recombinant adeno-associated virus vectors in vitro and in vivo. The American Journal of Sports Medicine 32: 1860-1865. DOI: 10.1177/0363546504265189.

Madry H, Orth P, Cucchiarini M (2011) Gene Therapy for Cartilage Repair. Cartilage 2: 201-225. DOI: 10.1177/1947603510392914.

Maihöfer J, Madry H, Rey-Rico A, Venkatesan JK, Goebel L, Schmitt G, Speicher-Mentges S, Cai X, Meng W, Zurakowski D, Menger MD, Laschke MW, Cucchiarini M (2021) Hydrogel-Guided, rAAV-Mediated IGF-I Overexpression Enables Long-Term Cartilage Repair and Protection against Perifocal Osteoarthritis in a Large-Animal Full-Thickness Chondral Defect Model at One Year In Vivo. Advanced Materials (Deerfield Beach, Fla.) 33: e2008451. DOI: 10.1002/adma.202008451.

Makris EA, Hadidi P, Athanasiou KA (2011) The knee meniscus: structure-function, pathophysiology, current repair techniques, and prospects for regeneration. Biomaterials 32: 7411-7431. DOI: 10.1016/j.biomaterials.2011.06.037.

Martinek V, Fu FH, Huard J (2000) Gene therapy and tissue engineering in sports medicine. The Physician and Sportsmedicine 28: 34-51. DOI: 10.3810/psm.2000.02.691.

McDevitt CA, Webber RJ (1990) The ultrastructure and biochemistry of meniscal cartilage. Clinical Orthopaedics and Related Research 8-18.

Mease PJ, Wei N, Fudman EJ, Kivitz AJ, Schechtman J, Trapp RG, Hobbs KF, Greenwald M, Hou A, Bookbinder SA, Graham GE, Wiesenhutter CW, Willis L, Ruderman EM, Forstot JZ, Maricic MJ, Dao KH, Pritchard CH, Fiske DN, Burch FX, Prupas HM, Anklesaria P, Heald AE (2010) Safety, tolerability, and clinical outcomes after intraarticular injection of a recombinant adeno-associated vector containing a tumor necrosis factor antagonist gene: results of a phase 1/2 Study. The Journal of Rheumatology 37: 692-703. DOI: 10.3899/jrheum.090817.

Meister K, Indelicato PA, Spanier S, Franklin J, Batts J

(2004) Histology of the torn meniscus: a comparison of histologic differences in meniscal tissue between tears in anterior cruciate ligament-intact and anterior cruciate ligament-deficient knees. The American Journal of Sports Medicine 32: 1479-1483. DOI: 10.1177/0363546503262182.

Mesiha M, Zurakowski D, Soriano J, Nielson JH, Zarins B, Murray MM (2007) Pathologic characteristics of the torn human meniscus. The American Journal of Sports Medicine 35: 103-112. DOI: 10.1177/0363546506293700.

Mingozzi F, Chen Y, Edmonson SC, Zhou S, Thurlings RM, Tak PP, High KA, Vervoordeldonk MJ (2013) Prevalence and pharmacological modulation of humoral immunity to AAV vectors in gene transfer to synovial tissue. Gene Therapy 20: 417-424. DOI: 10.1038/gt.2012.55.

Ozeki N, Seil R, Krych AJ, Koga H (2021) Surgical treatment of complex meniscus tear and disease: state of the art. Journal of ISAKOS: Joint Disorders & Orthopaedic Sports Medicine 6: 35-45. DOI: 10.1136/jisakos-2019-000380.

Pangborn CA, Athanasiou KA (2005) Growth factors and fibrochondrocytes in scaffolds. Journal of Orthopaedic Research: Official Publication of the Orthopaedic Research Society 23: 1184-1190. DOI: 10.1016/j.orthres.2005.01.019.

Rabie MA, Sayed RH, Venkatesan JK, Madry H, Cucchiarini M, El Sayed NS (2023) Intra-articular injection of rAAV-hFGF-2 ameliorates monosodium iodoacetate-induced osteoarthritis in rats via inhibiting TLR-4 signaling and activating TIMP-1. Toxicology and Applied Pharmacology 459: 116361. DOI: 10.1016/j.taap.2022.116361.

Rey-Rico A, Cucchiarini M, Madry H (2017) Hydrogels for precision meniscus tissue engineering: a comprehensive review. Connective Tissue Research 58: 317-328. DOI: 10.1080/03008207.2016.1276576.

Rey-Rico A, Klich A, Cucchiarini M, Madry H (2016) Biomedical-grade, high mannuronic acid content (BioMVM) alginate enhances the proteoglycan production of primary human meniscal fibrochondrocytes in a 3-D microenvironment. Scientific Reports 6: 28170. DOI: 10.1038/srep28170.

Romanazzo S, Vedicherla S, Moran C, Kelly DJ (2018) Meniscus ECM-functionalised hydrogels containing infrapatellar fat pad-derived stem cells for bioprinting of regionally defined meniscal tissue. Journal of Tissue Engineering and Regenerative Medicine 12: e1826-e1835. DOI: 10.1002/term.2602.

Roos H, Laurén M, Adalberth T, Roos EM, Jonsson K, Lohmander LS (1998) Knee osteoarthritis after meniscectomy: prevalence of radiographic changes after twenty-one years, compared with matched controls. Arthritis and Rheumatism 41: 687-693. DOI: 10.1002/1529-0131(199804)41:4<687::AID-ART16>3.0.CO;2-2.

Salata MJ, Gibbs AE, Sekiya JK (2010) A systematic review of clinical outcomes in patients undergoing meniscectomy. The American Journal of Sports Medicine 38: 1907-1916. DOI: 10.1177/0363546510370196.

Samulski RJ, Chang LS, Shenk T (1987) A recombinant plasmid from which an infectious adeno-associated virus genome can be excised in vitro and its use to study viral replication. Journal of Virology 61: 3096-3101. DOI: 10.1128/JVI.61.10.3096-3101.1987.

Samulski RJ, Chang LS, Shenk T (1989) Helper-free stocks of recombinant adeno-associated viruses: normal integration does not require viral gene expression. Journal of Virology 63: 3822-3828. DOI: 10.1128/JVI.63.9.3822-3828.1989.

Seidlits SK, Gower RM, Shepard JA, Shea LD (2013) Hydrogels for lentiviral gene delivery. Expert Opinion on Drug Delivery 10: 499-509. DOI: 10.1517/17425247.2013.764864.

Shen W, Li Y, Huard J (2005) Musculoskeletal gene therapy and its potential use in the treatment of complicated musculoskeletal infection. Infectious Disease Clinics of North America 19: 1007-1022. DOI: 10.1016/j.idc.2005.07.005.

Steinert AF, Palmer GD, Capito R, Hofstaetter JG, Pilapil C, Ghivizzani SC, Spector M, Evans CH (2007) Genetically enhanced engineering of meniscus tissue using ex vivo delivery of transforming growth factor-beta 1 complementary deoxyribonucleic acid. Tissue Engineering 13: 2227-2237. DOI: 10.1089/ten.2006.0270.

Steinert AF, Proffen B, Kunz M, Hendrich C, Ghivizzani SC, Nöth U, Rethwilm A, Eulert J, Evans CH (2009) Hypertrophy is induced during the in vitro chondrogenic differentiation of human mesenchymal stem cells by bone morphogenetic protein-2 and bone morphogenetic protein-4 gene transfer. Arthritis Research & Therapy 11: R148. DOI: 10.1186/ar2822.

Tao K, Rey-Rico A, Frisch J, Venkatesan JK, Schmitt G, Madry H, Lin J, Cucchiarini M (2016) rAAV-mediated combined gene transfer and overexpression of TGF-β and SOX9 remodels human osteoarthritic articular cartilage. Journal of Orthopaedic Research: Official Publication of the Orthopaedic Research Society 34: 2181-2190. DOI: 10.1002/jor.23228.

Venkatesan JK, Schmitt G, Speicher-Mentges S, Orth P, Madry H, Cucchiarini M (2022) Effects of Recombinant Adeno-Associated Virus-Mediated Overexpression of Bone Morphogenetic Protein 3 on the Chondrogenic Fate of Human Bone Marrow-Derived Mesenchymal Stromal Cells. Human Gene Therapy 33: 950-958. DOI: 10.1089/hum.2022.004.

Vrouwe JPM, Meulenberg JJM, Klarenbeek NB, Navas-Cañete A, Reijnierse M, Ruiterkamp G, Bevaart L, Lamers RJ, Kloppenburg M, Nelissen RGHH, Huizinga TWJ, Burggraaf J, Kamerling IMC (2022) Administration of an adeno-associated viral vector expressing interferon-β in patients with inflammatory hand arthritis, results of a phase I/II study. Osteoarthritis and Cartilage 30: 52-60.

DOI: 10.1016/j.joca.2021.09.013.

Wang D, Gonzalez-Leon E, Rodeo SA, Athanasiou KA (2021) Clinical Replacement Strategies for Meniscus Tissue Deficiency. Cartilage 13: 262S-270S. DOI: 10.1177/19476035211060512.

Webber RJ, Zitaglio T, Hough AJ Jr (1988) Serum-free culture of rabbit meniscal fibrochondrocytes: proliferative response. Journal of Orthopaedic Research: Official Publication of the Orthopaedic Research Society 6: 13-23. DOI: 10.1002/jor.1100060103.

Wirth CJ, Peters G, Milachowski KA, Weismeier KG, Kohn D (2002) Long-term results of meniscal allograft transplantation. The American Journal of Sports Medicine 30: 174-181. DOI: 10.1177/03635465020300020501.

Xiao X, Li J, Samulski RJ (1996) Efficient long-term gene transfer into muscle tissue of immunocompetent mice by adeno-associated virus vector. Journal of Virology 70: 8098-8108. DOI: 10.1128/JVI.70.11.8098-8108.1996.

Zaleskas JM, Kinner B, Freyman TM, Yannas IV, Gibson LJ, Spector M (2001) Growth factor regulation of smooth muscle actin expression and contraction of human articular chondrocytes and meniscal cells in a collagen-GAG matrix. Experimental Cell Research 270: 21-31. DOI: 10.1006/excr.2001.5325.

Zhang H, Leng P, He T, Wang Y (2015) Comparison of hIGF-1 gene transfection to the hBMSCs and human meniscal fibrochondrocytes. Medical Science Monitor: International Medical Journal of Experimental and Clinical Research 21: 681-688. DOI: 10.12659/MSM.891410.

Zhang H, Leng P, Zhang J (2009a) Enhanced meniscal repair by overexpression of hIGF-1 in a full-thickness model. Clinical Orthopaedics and Related Research 467: 3165-3174. DOI: 10.1007/s11999-009-0921-8.

Zhang HN, Leng P, Wang YZ, Zhang J (2009b) Treating human meniscal fibrochondrocytes with hIGF-1 gene by liposome. Clinical Orthopaedics and Related Research 467: 3175-3182. DOI: 10.1007/s11999-009-0870-2.

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