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Gene transfer to the patellar tendon

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Abstract Growth factors have the potential to enhance native repair responses in ligamentous lesions. However, methods for applying these cytokines to sites of injury for extended periods are lacking. We suggest that local transfer of genes which encode the relevant healing factors merits investigation as a potential solution to this problem. In the present study, the retroviral vectors MFG lacZ and BAG lacZ neo^r and adenovirus LacZ were evaluated for their ability to deliver genes to cells of ligamentous origin. The posterior and anterior cruciate ligaments, medial collateral ligament, semitendinosus tendon and patellar tendon were harvested from New Zealand white rabbits. Cells grown from these tissues were then investigated for their susceptibility to genetic alteration by these vectors in vitro. Based upon their ability to convert cells in culture to a lacZ(+) phenotype, adenovirus was the most effective vector in short-term experiments. However, expression was transient. Although retrovirus gave lower initial transduction efficiencies, the percentage of transduced cells could be increased by the use of the selectable marker gene neo^r. In an in vivo marker study, we injected adenovirus into the rabbit patellar tendon. Transduced cells could be observed preferentially in the subsynovial layer at a declining frequency over a 6-week period. The allogeneic transplantation of in vitro retrovirally transduced fibroblasts into the patellar tendon resulted in a greater number of transduced cells. Although the number of lacZ(+) cells declined with time, positive cells were still present 6 weeks after transplantation. Furthermore, the transplanted cells, unlike cells transduced in situ with adenovirus, migrated from the injection site and integrated into the crimp of the tendon.

Key words Viral transduction · Growth factors · Knee ligament · Fibroblast · LacZ

Introduction

In recent years, research has provided significant insight into the biomechanics, ultrastructure, and biochemistry of the knee ligaments. The biomechanical principles of anterior (ACL) and posterior (PCL) cruciate ligament reconstruction have been well studied, and techniques of ligament replacement based on this knowledge have provided reproducibly good midterm results. The biology of ligament healing, however, is inadequately studied and has contributed little to clinical practice.

Experimental examinations of the reconstructed ACL and PCL have shown that grafts remain structurally and mechanically inferior to the normal ligament. The grafts undergo morphologically distinct phases of wound healing, but do not achieve a normal stage of maturation [5, 16]. Also, recent data suggest that even if joint stability is

achieved after ACL reconstruction, this fails to reduce the incidence of osteoarthritis [14].

Since further refinement of the surgical techniques has not resulted in a better clinical outcome, additional approaches are required for future improvement in the treatment of ligamentous injuries. Recent results suggest that certain growth factors [8, 17, 23] such as insulin-like growth factor-I (IGF-I) and transforming growth factor β (TGF-β) aid natural repair processes. Hence, there is growing interest in the use of mediators which enhance one or more of the stages of tissue repair, including cell migration, cell division, collagen synthesis, and vascular ingrowth. Utilization of such factors demands local application to avoid potential detrimental effects of more general administration. Unfortunately, at this time there are no convenient methods by which prolonged local application could be achieved.

One potential solution to the problem of growth factor delivery is through gene therapy, by transferring exogenous genes to a target cell [2, 10–13]. Therapeutic benefit is achieved through the transgene product that is expressed by the genetically modified cell. Since naked DNA is not taken up and expressed well by most cells, this approach depends on vectors that facilitate gene transfer and expression. As viruses do this very efficiently, most currently available vectors are viral. Elements of the viral genome are removed to prevent viral replication and replaced by the gene(s) of interest.

In the present study, we evaluated retrovirus and adenovirus for their ability to transfer the *lacZ* marker gene in vitro to ligamentous cells of rabbit origin. Finally, gene delivery to rabbit patellar tendon in living animals was done using vivo and ex vivo techniques.

Materials and methods

In vitro study

Cell culture

Three-month-old New Zealand white rabbits, weighing approximately 2.5 kg, were killed by i.v. injection of pentothal. ACL, PCL, medial collateral ligament (MCL), patellar tendon and semitendinosus tendon were harvested from the knee joint under aseptic conditions. Attached fat and synovium were sharply dissected from the tissues. Ligaments and tendons were minced into pieces of about 1 mm in diameter and placed in 100-mm tissue culture dishes (Corning, N.Y.) containing Ham's F12 medium (Gibco, Grand Island, N.Y.) supplemented with 10% fetal bovine serum (Gibco) and 100 U/ml penicillin and 10 μg/ml streptomycin (Gibco). Cells were cultured at 37°C in an atmosphere of 5% CO_2 and 95% air. The medium was changed every 10 days until cells had migrated from the tissue fragments, divided, and reached full confluence. Cultures were then trypsinized (trypsin 0.25 w/v %, Gibco) and subcultured in 25-cm^2 polystyrene tissue culture flasks (Corning) at a 1:2 to 1:4 split ratio.

Table 1 Viral and nonviral vectors evaluated in this study. Retroviral vectors are derived from the Moloney murine leukemia virus (MoMLV). In the MFG derivative, the gene of interest is inserted in place of the viral "env" gene, and its expression is driven by the endogenous viral promoter within the 5′LTR. Genes: *lacZ* β-galactosidase, *neo*^{r} neomycin resistance; promoter: *LTR* long terminal repeat, *SV40* simian virus 40, *HCMV* human cytomegalovirus

Name	Vector	Gene	Promoter	Integration into the host genome
Ad lacZ	Adenovirus	*lacZ*	HCMV	No
MFG lacZ	Retrovirus	*lacZ*	LTR	Yes
BAG lacZ neo^r	Retrovirus	*lacZ*	LTR	Yes
		neo^r	SV40	

Virally mediated gene transfer

Since it has been shown that retroviruses only infect actively replicating cells, cell cultures were used at approximately 70% confluence for infection with the retroviruses BAG lacZ neo^r [20] and MFG lacZ (Table 1). As adenovirus infects both dividing and non-dividing cells, cell cultures were infected with the cells at full confluence. All viral supernatants had titers of 10^6 Pfus/ml medium and contained 8 μg/ml Polybrene (Sigma), a polycationic infection facilitator [6, 9, 25]. One milliliter of the viral supernatant was layered onto a monolayer of cells in a 25-cm^2 tissue culture flask and incubated at 37°C with gentle agitation of the viral supernatant every 20 min. After 3 h incubation, 3 l Ham's F-12 medium were added, and the cultures returned to the incubator.

Assays for gene expression

The marker gene *lacZ* encodes β-galactosidase, whose activity within lacZ(+) cells can be detected by a histochemical stain. To evaluate the efficiency of transfer and expression of the *lacZ* gene, cells were stained 3 days after infection by adding a substrate β-galactosidase: X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyrosanide; Sigma). Results after X-gal staining are expressed semiquantitatively; the efficiency of transduction was determined by the percentage of blue cells (Table 2).

The marker gene *neo*^{r} encodes neomycin phosphotransferase. Infected cells which express this transgene are resistant to the toxic effects of G-418, a synthetic neomycin analogue. Cell cultures transduced with this gene were trypsinized at a 1:2 split ratio 3 days after infection and transferred to a medium containing 0.5 mg/ml G-418. The medium was changed every 4 days with the addition of fresh G-418 (Table 2).

Table 2 Assays of gene expression. The vectors used in this study carried one or more of the listed genes. Shown are the gene products and the assays used to detect transgene expression

Gene	Gene product	Assay of gene expression
lacZ	β-Galactosidase	Staining with X-gal, a chromogenic substrate for β-galactosidase
neo^r	Neomycin phosphotransferase	Selection using G-418 (a synthetic neomycin analogue)

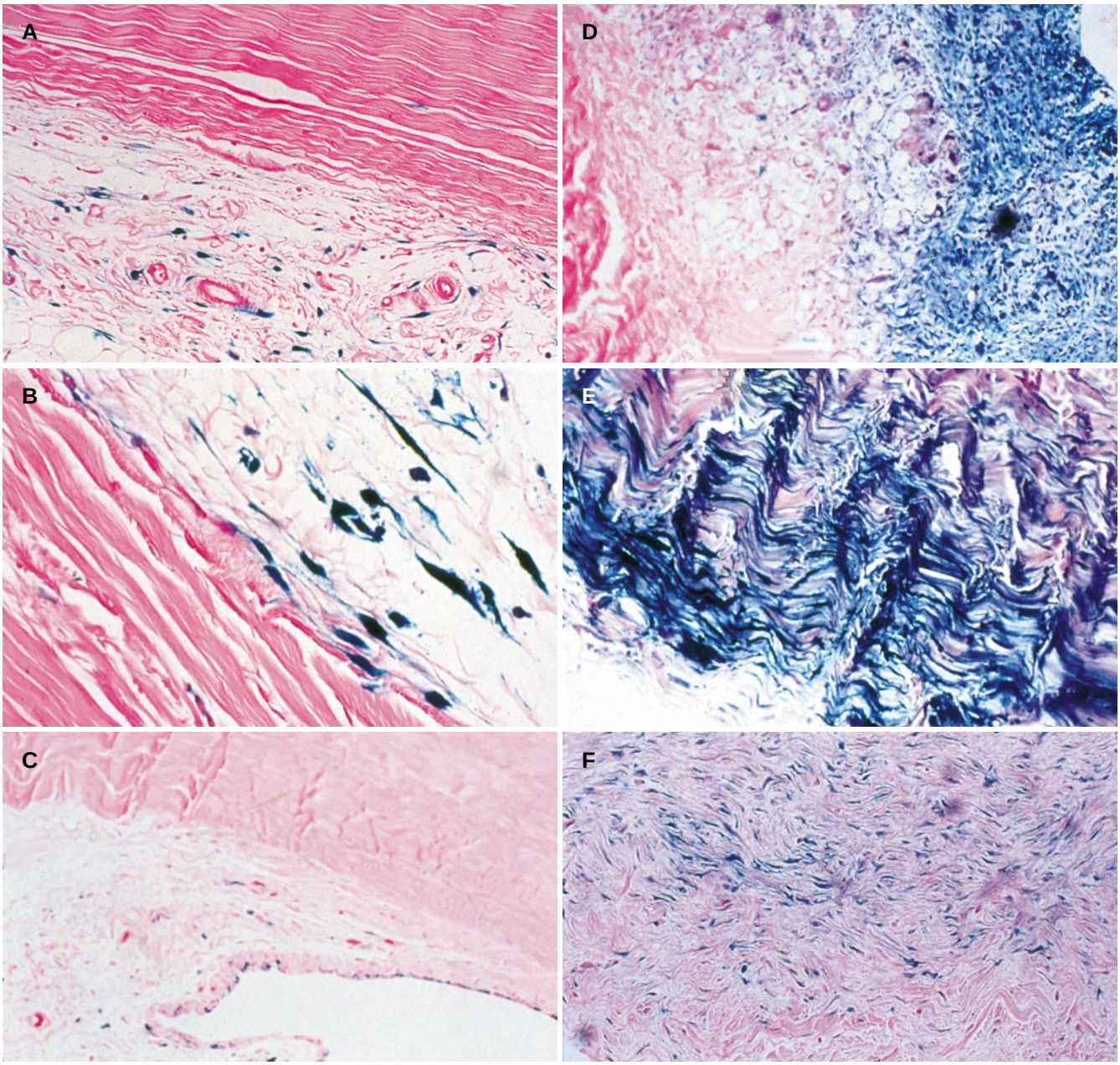

Fig. 1A–F *lacZ* gene expression in patellar tendon following in vivo and ex vivo delivery. X-gal and eosin staining, 1 week after injection of adenovirus lacZ (5×10^6 particles/250 µl saline solution) into the patellar tendon. Fibroblasts expressing β-galactosidase are found in the periphery of vessels in the synovial layer (**A, B**). Six weeks after injection of adenovirus lacZ into the patellar tendon, expression is still found in the synovial layer; however, the number of cells expressing the *lacZ* gene has decreased significantly (**C**). One week after allogeneic transplantation of 5×10^6 patellar tendon fibroblasts suspended in 250 µl saline solution into the patellar tendon, allogeneic fibroblasts expressing the *lacZ* gene are found in high numbers in the synovial layer (**D**) and in the tendon (**E**). Cells regain their fibroblastic shape and are aligned parallel to the crimp pattern. Six weeks after transplantation, the number of fibroblasts expressing the *lacZ* gene has decreased. Transduced fibroblasts are found remote from the site of injection (**F**)

In vivo study

There are two principal techniques of gene transfer to the body. In the direct, in vivo method, the vector is directly administered to the tissue in situ. In the indirect, ex vivo approach, tissue is removed, and the cells are genetically modified in vitro, and then returned to the body.

Based on the results of our in vitro study, we concluded that adenovirus lacZ was most suitable for an in vivo approach and BAG lacZ neo^r most suitable for the ex vivo approach. The patellar tendon was chosen as an appropriate target tissue, since the tendon is palpable and accessible to percutaneous injection.

Nine 3-month-old New Zealand white rabbits were allocated to the different treatment regimens. Group I consisted of four rabbits who received an injection of adenovirus lacZ (5×10^6 particles/250 µl normal saline) in the right patellar tendon. The left tendon

served as a control and was injected with 250 µl normal saline. Group II consisted of five rabbits who received an allogeneic cell transplantation of fibroblasts which had been infected with the BAG virus and selected in G-418. A total of 5×10^6 *neo*^{r}, lacZ(+) patellar tendon fibroblasts suspended in 250 µl saline solution were injected into the right patellar tendon. The left patellar tendon was injected with an equal number of native fibroblasts. After the procedure, unrestricted cage activity was allowed. Rabbits were killed after 1, 2, and 6 weeks. The patellar tendon was harvested with the adjacent synovial tissue and the fat pad. The tendon was sectioned longitudinally and stained with X-gal for 24 h. Specimens were then paraffin embedded, sectioned into 5-µm slices, and eosin counterstained for histological examination.

Results

In vitro study

Adenovirus

Cell cultures were infected with adenovirus lacZ with a multiplicity of infection of approximately 10, and then maintained in culture for a further 3 days. By X-gal staining, the overall efficiency of transduction was found to be between 90% and 100%. No change in expression was observed when cells were maintained for 7 days after infection, and no cytotoxic effects were evident.

Retrovirus

Cells were infected with MFG-lacZ and maintained in culture for a further 3 days. The percentage of lacZ(+) cells ranged from 10% to 60% at both 3 and 7 days postinfection. Infection with a retrovirus vector expressing both *lacZ* and *neo*^{r} (BAG) resulted in over 90% of the cells lacZ(+) after G-418 selection. There were no significant differences between the cell types in their susceptibility to selection in G-418.

In vivo study

Macroscopic evaluation

At the time of killing, all knee joints appeared macroscopically normal. Using a medial parapatellar approach, the joint was opened. There was no effusion or synovitis, and the articular cartilage, menisci, and ligaments were normal. The patellar tendon was then dissected and freed of adjacent fat.

Rabbits of group I (adenovirus, in vivo approach) had tendons of normal appearance without hypervascularity 1, 2, and 6 weeks after injection. Tendons of group II (ex vivo approach) were generally enlarged 1 week after transplantation of allogeneic patellar tendon fibroblasts. The surrounding soft-tissue layers and the retropatellar fat pad were adherent to the tendon. These changes resolved after 2 weeks and were not observed at week 6.

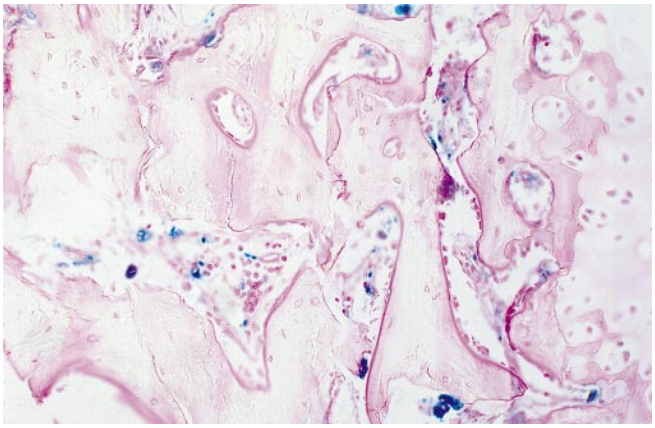

Fig. 2 *lacZ* gene expression in bone following injection of adenovirus. X-gal and eosin staining, 1 week after injection of adenovirus into the patellar tendon. Accidental injection into the patellar tendon insertion site transduced bone cells

Histologic evaluation

In vivo approach. After harvest, the specimens were processed for X-gal and eosin staining. One and 2 weeks after injection of adenovirus lacZ into the tendon, blue cells could be observed mainly in the subsynovial layer near the site of injection where a significant number of lacZ(+) cells were found surrounding vessels (Fig. 1A,B). Only a few blue cells were seen in the tendon itself. Those cells did not migrate, but remained at the site of injection. Accidental injection of adenovirus LacZ into the patellar insertion site of the tendon revealed that this technique also transduced bone cells (Fig. 2). Six weeks after injection, transduced cells were still found in the synovial layer, but expression had decreased significantly (Fig. 1C). No hypervascularity, cellular infiltration, or other evidence of an inflammatory response was observed. The control-injected side was negative for X-gal staining (not shown).

Ex vivo approach. One week after injection of BAG-infected and *neo*^{r}-selected fibroblasts into the patellar tendon, transduced cells could be found in the synovial layer (Fig. 1D) and throughout the entire tendon. Formation of allogeneic cell clusters was observed at the sites of injection. Cells in the tendinous portion expressing the *lacZ* gene were spindle-shaped and aligned parallel to the crimp pattern of the collagen fibers (Fig. 1E).

Although a precise quantitation of transduced cells was not feasible, the number of lacZ(+) cells was clearly higher than in the in vivo approach. In several histologic sections, fibroblasts were found remote from the site of injection. By week 2, there was a reduction in the number of lacZ(+) cells, but lacZ(+) cells were still found in the ligament and synovial layer. A few areas displayed disorganized fibrous tissue surrounded by lacZ(+) cells, suggesting a fibrotic response (not shown). The control-in-

jected side did not display blue cells. At week 6, expression of the *lacZ* gene had decreased significantly. LacZ(+) cells were found mainly in the tendon itself (Fig. 1F).

Discussion

Numerous in vitro and in vivo studies suggest that growth factors enhance the native repair response in wound healing. These factors have been shown to exhibit stimulatory functions, thereby increasing cell division, migration, and synthesis of collagenous and noncollagenous proteins [4, 15, 19, 22, 26].

Application of these cytokines in experimental wound healing models raises the question about the time-dependent decrease of concentration at the site of injury. Experiments to determine the stability of growth factors applied at sites of wounding generally report a rapid loss of the factor [7]. Therefore, systems that allow a continuous release of growth factors are being developed. These significantly increase the concentration of growth factors, but it is still unclear whether they will achieve clinical success.

As the direct application of growth factors either as free molecules or as molecules associated with slow release devices may not provide sufficiently high local concentrations of the factors for sufficient periods of time, we investigated a novel approach to growth factor delivery. Since gene transfer to synoviocytes has proven to be a feasible and efficacious way to express transgenes in joints [2, 3] we hypothesize that fibroblasts from ligaments, which have been engineered to secrete cytokines of interest, can be used to produce sustained, local concentrations of these cytokines after ligament replacement.

In this initial study, adenovirus and retrovirus were evaluated for their ability to transfer marker genes to the target cells in vitro and in vivo. In these experiments, we were able to demonstrate that all cells harvested from rabbit ligaments were susceptible to various degrees to gene transfer mediated by viruses. However, there was a considerable variation between these vectors in terms of their efficiency. Adenovirus was highly effective in all cell types tested. Gene expression following adenoviral transduction was lost on passaging; gene transfer and expression following retroviral infection were stable.

Retroviruses are the best developed vectors. They are RNA viruses that contain reverse transcriptase which copies the viral RNA genome into DNA, which is then stably integrated into the host genome at random sites. A major limitation of retroviruses is their inability to infect nondividing cells [18]. For practical use, this has certain implications since gene transfer is possible only in tissues whose cells divide or can be made to divide in vitro. The packaging capability of retroviruses is limited to about 8 kb, but this should not pose a problem for delivering growth factors, most of which are small molecules.

Adenovirus is an alternative vector with a number of features that makes it attractive as a vector for delivery of growth factors to healing tissues. This DNA virus can be generated at high titers and infects both dividing and nondividing cells with high efficiency. However, a potential problem is the host immune response against the vector itself, since cells infected with the present generation of adenovirus continue to express antigenic viral proteins [21, 28]. Based on these findings, we concluded that adenovirus was best suited to in vivo gene delivery and retrovirus best suited to ex vivo gene delivery. Although the in vivo approach is straightforward, there are limitations. To ensure adequate expression of the transgene product, there has to be a sufficient density of target cells. The ex vivo technique is more complex, but provides a greater margin of safety. Genetically modified cells can be subjected to any necessary test in vitro and viral DNA is not directly administered to the body. Additionally, gene transfer in vitro allows selection of cells that express the transgene at high concentrations by using a selectable gene, such as *neo*^{r}.

Using adenovirus in a direct in vivo approach to transfer a marker gene to the patellar tendon, blue cells could be found preferentially in the synovial layer surrounding vessels; only a few cells were observed in the hypocellular tendon itself. Expression of the transgene decreased during a 6-week period after transduction, but was still present at 6 weeks after injection.

Although gene expression was limited to the synovial sheath, genes expressed here could still serve as source of cytokines for cells in the tendon itself, as indicated by a number of studies. For example, Skyhar et al. [24] demonstrated by injection of $Na_2{}^{35}SO_4$ in saline solution into the rabbit knee joint that the ACL uses diffusion as its primary nutritional pathway.

Whiteside and Sweeney [27] confirmed these results in the PCL of dogs. Using the hydrogen washout technique, they demonstrated that the PCL depends primarily on an intact synovial layer for its nutrition.

We transplanted retrovirally transduced, lacZ(+), *neo*^{r}-selected, allogeneic patellar tendon fibroblasts into the patellar tendon after they had been in culture for 6 weeks. Cells remained viable in the synovial layer and in the tendon itself and expressed the transgene for at least 6 weeks. The number of transduced cells found at the time of killing was significantly higher compared with the in vivo approach. Cells in the tendon regained a spindle-shaped appearance and, unlike cells transduced in vivo with adenovirus, were aligned along the collagen fibers parallel to the crimp pattern. In addition, cells were found in the tendon remote from the sites of injection. Those cells were integrated into the matrix, indicating that viable cells keep their ability to migrate and adapt to biomechanical stress. By week 2, a few fibrotic areas were found, suggesting an inflammatory response to the injection of cells. Later, these fibrotic responses resolved and were not observed at week 6.

With this study, we have demonstrated the feasibility of both in vivo and ex vivo gene transfer to ligamentous fibroblasts from the rabbit knee with respect to transgene expression in situ for a period of 6 weeks. Whether this holds true for ligament replacement and ligament repair has to be investigated in further experimental studies, since expression of the transgene may be limited by cell division and necrosis. A graft for ligament replacement undergoes cell necrosis [1, 5] and is repopulated by fibroblasts of synovial origin [1]. At this moment, we can only hypothesize whether early stimulation with growth factors using either an ex vivo or in vivo approach can initiate a repair response before the onset of graft necrosis.

Through transfer of a therapeutic growth factor into these fibroblasts, expression of the transgene product may facilitate better healing of injured ligaments. Although future studies are needed to evaluate (1) which cytokine(s)/growth factors best improve healing of injured ligaments and menisci, (2) the long-term viability of the transplanted genetically modified fibroblasts, and (3) the duration of transgene expression, gene therapy holds great promise as a technique with which to optimize healing of injured tissues of the joint.

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