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Lipid-Mediated Gene Transfer for Cartilage Tissue Engineering

Henning Madry

Laboratory for Experimental Orthopaedics, Department of Orthopaedic Surgery, Saarland University Medical Center, Saarland University, 66421 Homburg, Germany, hmad@hotmail.com

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1. INTRODUCTION TO GENE TRANSFER

Recombinant DNA technology has provided tools to introduce exogenous DNA into cultured mammalian cells. In cartilage tissue engineering, these methods can be used to study the effect of single genes on the regulation of chondrogenesis or to enhance structural and functional properties of engineered cartilage [Madry et al., 2002]. The techniques of transferring DNA molecules to cells can be divided into nonviral methods (including chemical, mechanical, and electrical techniques) and viral methods. The first critical step is the delivery of a gene of interest to the chondrocyte. For the purposes of cartilage tissue engineering, this can be achieved by two different strategies. First, the DNA may be introduced into component chondrocytes before seeding them into a scaffold. The second approach would be to transfer the DNA at a later time point directly into tissue-engineered cartilaginous constructs. This step requires the transfection of chondrocytes within their native matrix in situ and can be performed efficiently by using recombinant adeno-associated virus (rAAV) vectors [Madry et al., 2003b]. In general, an adequate delivery system requires (1) a high efficiency of transmission, (2) a desired level and length of gene expression, and (3) biological safety during the course of the transgene expression.

Recombinant genes have been efficiently introduced into isolated articular chondrocytes by virus-based carriers [Madry et al., 2003b; Baragi et al., 1995; Doherty et al., 1998; Ikeda et al., 2000; Smith et al., 2000; Brower-Toland et al., 2001] and nonviral synthetic methods. Lipid-based vectors in particular offer many advantages [Madry et al., 2002; Kim et al., 1996; Madry and Trippel, 2000]. They are relatively easy to prepare and to handle, do not restrict the length of the DNA molecule of interest that is to be transferred, and very rarely elicit an immune response [Fogler et al., 1987]. The lipid-based gene transfer protocol described in this chapter has been optimized for overexpressing recombinant DNA in primary articular chondrocytes.

1.1. Principle of Lipid-Mediated Gene Transfer

Liposomes and other lipid-mediated gene shuttle systems are important tools to transfect primary cells because of their very high transfer efficiencies. Nonviral,

cationic lipid-mediated gene transfer was first described by Felgner [Felgner et al., 1987]. Cationic liposomes are composed of an amphiphilic cationic lipid and a neutral phospholipid, the helper lipid [Felgner and Ringold, 1989; Felgner et al., 1995]. When brought into aqueous solution, these lipids form liposomes as hexagonal phases [Zhou F and Huang, 1994; Safinya, 2001]. After addition of plasmid DNA, the liposomes spontaneously assemble into liposome-DNA complexes. The cellular events that facilitate the entry of the transferred DNA into the nucleus are not completely known as yet [Escriou et al., 2001; Neves et al., 1999; Seisenberger et al., 2001]. The interaction of the lipid-DNA complexes with the lipid bilayer occurs either by nonspecific adsorption or by endocytosis. Once the DNA has passed the cellular membrane, it is trapped in endosomes. The destabilization of the endosomal membrane is thought to be facilitated by the helper lipid [Farhood et al., 1992], enabling the DNA to escape degradation [Zhou X and Huang, 1994; Crystal, 1995]. The DNA is thereafter released into the cytoplasm, where it enters the nucleus through the nuclear pores by an unidentified mechanism [Seisenberger et al., 2001].

Cationic liposomes that are currently used to transfect a variety of cell lines and primary cells are the monocationic cholesterol derivative DC-Chol [Gao and Huang, 1991], the polycationic lipid DMRIE [San et al., 1993], DOTMA (part of Lipofectin®) [Felgner et al., 1987], and DOSPA (part of Lipofectamine®) [Felgner et al., 1995]. Transfection of chondrocytes of avian origin has been successful with cationic liposomes like Lipofectin [Long and Linsenmayer, 1995] or Lipofectamine [Pallante et al., 1996]. However, transfection by these methods with bovine and human articular chondrocytes has not been forthcoming. Recently, nonliposomal lipid-mediated methods have been described to offer high transfection efficiencies in primary chondrocytes. Among them, FuGENE 6, a nonliposomal mixture of lipids and other components, has been particularly effective [Madry et al., 2001, 2002, 2003a; Madry and Trippel, 2000; Lefebvre et al., 1998; Dinser et al., 2001]. The protocol outlined below will focus on transfection with FuGENE 6.

1.2. Advantages and Shortcomings

Lipid-based transfection offers many advantages over other transfection systems to introduce genes of interest into primary articular chondrocytes. The high transfection efficiency avoids the need for cell selection that might result in undesired phenotypic drift, as observed after prolonged monolayer culture of articular chondrocytes [Benya et al., 1978]. The very low toxicity avoids the need to change the cell culture medium after transfection. In addition, lipid-based transfections can be carried out in the presence of serum.

The main shortcoming of lipid-based transfection is its relatively high cost, especially when large-scale transfections are required.

1.3. Critical Factors for Efficient Transfection

Several factors greatly influence the success of lipid-based transfection with FuGENE 6. The starting point is to identify the optimal quantity of DNA per cell. For chondrocytes cultured in a 24-well plate, 1 μg of DNA per well is commonly

used. The ratio of FuGENE 6 (μl) to DNA (μg) must always be greater than 1. In our own investigations, ratios of 2–5 (v/w) FuGENE 6 to DNA have been successful. Another important consideration is the pericellular matrix that surrounds the chondrocytes, thereby acting as a physical barrier. It is thought to inhibit the uptake of the lipid-DNA complexes into the cell by limiting their interaction with the cell membrane. When this matrix is partially degraded by incubation with hyaluronidase, transfection efficiencies are significantly enhanced [Madry and Trippel, 2000]. Other factors for a successful transfection include the growth state of the cells [Corsaro and Pearson, 1981] and an optimal cell density at the time of transfection. It is therefore important to maintain a standard seeding protocol.

The protocol described in Section 3 has proven successful for the authors in transfecting neonatal bovine and human articular chondrocytes. It may serve as a starting point for further improvements, as transfection conditions vary among species, as well as with the anatomic location and the age of the donor. Although a list of reagents is included with the protocols, similar results may be obtained with reagents from different manufacturers. When chondrocytes from a different species (e.g., avian chondrocytes) are used, transfections with the commercially available Lipofectin or Lipofectamine may be considered. Alternatively, cationic liposomes may be synthesized from their individual components [Ravid and Freshney, 1998].

2. PREPARATION OF REAGENTS AND MEDIA

2.1. Expression Plasmid Vectors

Plasmid vectors that may be manipulated to contain a gene of interest are commercially available. These vectors contain an antibiotic resistance gene to allow for the selection of positive transformants. To express large quantities of DNA in vitro, a strong (or tissue specific) promoter and a stable polyadenylation sequence are desirable. The addition of a consensus Kozak sequence has been demonstrated to further increase translation. The efficiency of gene transfer is greatly affected by the purity of the plasmid DNA. Purification by cesium chloride centrifugation has been traditionally used to prepare DNA for transfections [Sambrook et al., 1989]. Alternatives based on commercially available column systems (e.g., Endofree Plasmid Maxi Kit, Qiagen) are less time consuming and less labor intensive and result in ultrapure DNA. Their additional advantage is the removal of endotoxins, in order to reduce the interference of bacterial endotoxins with the transfection processes [Cotten et al., 1994]. After purification, the DNA is usually resuspended in sterile Tris-EDTA (TE) buffer at a final concentration of 0.5–2.0 μg/μl. The vectors used in these protocols are as follows:

pCMVβgal

To express the *Escherichia coli* (*E. coli*) β-galactosidase (*lacZ*) marker gene, any vector containing the *lacZ* gene under the control of a strong promoter (e.g., the cytomegalovirus immediate-early (CMV-IE) promoter and enhancer) can be used [MacGregor and Caskey, 1989]. These vectors are also commercially available (e.g., pCMVβgal, Invitrogen, Carlsbad, CA). Expression of β-galactosidase

can be monitored by staining the cells in situ or by applying a colorimetric assay to quantitatively determine the enzyme activity.

pcDNA3.1(−)

If expression of a therapeutic gene is required, pcDNA3.1/Zeo(+) (Invitrogen) containing the human CMV-IE promoter/enhancer and the bovine growth hormone polyadenylation signal may be used. This vector contains a multiple-cloning site.

2.2. Transfection Reagent

FuGENE 6 is a sterile-filtered mixture of different nonliposomal lipids dissolved in 80% ethanol. Any direct contact with plastic surfaces must be avoided because this interaction may greatly decrease transfection efficiency. We recommend the use of polypropylene tubes with a round bottom to prepare the transfection complexes as these have the lowest risk of binding, but direct contact with the FuGENE 6 reagent should still be avoided. Sometimes a precipitate may form in cold FuGENE 6 reagent that disappears once the reagent is warmed up to room temperature.

2.3. Tissue Culture Equipment and Preparation of Reagents

The use of regular tissue culture-treated plasticware for the cell cultures gives the best results. Special coatings (e.g., collagens) do not improve cell attachment. Transfection optimization experiments can be performed in 24-well plates. To transfect articular chondrocytes for tissue engineering, we found 10-cm cell culture dishes useful.

2.3.1. Basal Medium

Dulbecco's modified Eagle medium (DMEM). DMEM, correctly formulated, requires a gas phase of 10% CO_2.

Penicillin G	50 U/ml
Streptomycin	50 µl/ml
Ascorbic acid	50 µg/ml

2.3.2. Growth Medium

Basal medium
Fetal bovine serum (FBS) 10% (v/v)

Heat inactivate the FBS by incubation (in a 500-ml bottle) in a water bath for 1 h at 56 °C. Aliquot in 15-ml conical polypropylene centrifuge tubes (use 12 ml FBS per tube). Store at −20 °C. Thaw immediately before use.

2.3.3. Collagenase

Collagenase 0.08% (m/v)
In basal medium

Vortex briefly to dissolve at room temperature. Sterilize the solution through a 0.22-µm filter. Use immediately.

2.3.4. Hyaluronidase

Hyaluronidase 200 U/ml
In UPW

Vortex to dissolve at room temperature. Sterilize the solution through a 0.22-μm filter. Add 20 μl of this stock solution to 1 ml of growth medium, resulting in a 4 U/ml final concentration. Use immediately, do not store.

2.3.5. Tris-EDTA (TE) Buffer

Tris HCl 10 mM
EDTA 1 mM
In distilled deionized water

Mix to dissolve with a stir bar at room temperature for 15–30 min. Adjust to pH 8.0. Sterilize the solution by autoclaving.

2.3.6. Fixative for X-Gal Staining

Paraformaldehyde (pH 7.0) 2% (m/v)
Glutaraldehyde 0.2% (v/v)
In phosphate-buffered saline without Ca^{2+} and Mg^{2+} (PBSA)

2.3.7. X-Gal Stock Solutions

Solution A

Potassium ferricyanide crystalline 5 mM
Potassium ferrocyanide trihydrate 5 mM
Magnesium chloride 2 mM
In PBSA

Store at 4 °C. Protect from light. Warm at 37 °C before use.

Solution B

X-Gal 40 mg/ml
In dimethyl sulfoxide (DMSO)

Store at −20 °C in polypropylene tubes. Protect from light. Warm at 37 °C before use. Dilute solution B (X-Gal stock) 1:40 in solution A in a polypropylene tube. Combination of cold solutions may result in precipitation of X-Gal. Vortex, use immediately. Do not store.

3. PROTOCOLS

3.1. Tissue Harvest, Cell Isolation, and Primary Culture

Protocol 5.1. Primary Culture of Bovine Cartilage

Reagents and Materials

Sterile or aseptically prepared

- ❑ Radiocarpal joints of 1- to 2-week-old calves
- ❑ Basal medium (See Section 2.3.1)
- ❑ Growth medium (See Section 2.3.2)
- ❑ Collagenase (See Section 2.3.3)
- ❑ PBSA
- ❑ Scalpel
- ❑ Anatomic forceps
- ❑ Flat-end spatula
- ❑ Cell strainer with 100-μm nylon mesh
- ❑ Spinner flask (100 ml) with adjustable hanging bar
- ❑ Cell culture dishes, 10 cm, tissue culture treated
- ❑ Pipette (10 ml)

Nonsterile

- ❑ Hemocytometer
- ❑ Trypan Blue
- ❑ Centrifuge

Protocol

(a) Using bovine articular cartilage from radiocarpal joints of 1- to 2-week-old calves:
  i) Open the joint in a sterile fashion.
  ii) Harvest articular cartilage by removing cartilage chips from the articular surface with a sterile scalpel. Exclude the underlying vascularized cartilage.
  iii) Transfer the cartilage chips to a 10-ml dish filled with 10 ml PBSA.
  iv) Remove the PBSA.

(b) Wash the harvested cartilage 3× with PBSA.

(c) Dice the articular cartilage into 2-mm cubes with a scalpel.

(d) Remove the PBSA and measure the wet weight of the cartilage.

(e) Transfer cartilage chips with the spatula to a 100-ml spinner bottle.

(f) Add 100 ml basal medium containing 0.08% collagenase (use 100 ml enzyme solution for 1 g wet weight cartilage).

(g) Incubate at 37 °C in a humidified atmosphere with 10% CO_2 for 16 h (rotation speed: 50 rpm).

(h) Resuspend cells, using a 10-ml pipette to break down cell aggregates.

(i) Filter the solution containing isolated chondrocytes through a 100-μm cell strainer to remove undigested matrix.

(j) Wash once in 40 ml basal medium in a 50-ml Falcon tube. Centrifuge at 2000 *g* for 5 min.
(k) Determine the cell number by hemocytometer and viability by Trypan Blue exclusion. Do not use cells if their viability is below 90%.
(l) Place isolated chondrocytes in monolayer culture in 10-cm cell culture dishes at a density of 6.0×10^6 cells per dish in 10 ml growth medium. This cell number should be sufficient to obtain reliable results and will not overgrow the dish in 3 days. However, if cells are already near confluence after 1 day, decrease the initial seeding density, as a high cell density reduces transfection efficiency significantly.
(m) After 24 h check appearance of the cells. Not all cells may have settled on the plastic surface. Change the growth medium.
(n) Perform transfection experiments with primary cell cultures 1–2 days after seeding when cells reach 50–70% confluence.

3.2. Gene Transfer

Protocol 5.2. Gene Transfer to Bovine Cartilage Cells

Reagents and Materials

Sterile

- ❑ Basal medium (See Section 2.3.1)
- ❑ Growth medium (See Section 2.3.2)
- ❑ Opti-MEM
- ❑ PBSA
- ❑ Trypsin-EDTA (TE) buffer (See Section 2.3.5)
- ❑ Hyaluronidase (See Section 2.3.4)
- ❑ FuGENE 6
- ❑ Polypropylene tubes, round bottom, with cap
- ❑ Pipette tips, 10–1000 μl, aerosol resistant

Nonsterile

- ❑ Hemocytometer
- ❑ Trypan blue

Protocol

(a) When the cells are in exponential growth and at 50–70% confluence, add 200 μl hyaluronidase solution (4 U/ml) to the cells. It is important to prepare fresh hyaluronidase solution immediately before use.
(b) After 12 h, warm all remaining solutions to room temperature.
(c) For each 10-cm cell culture dish, prepare and label two polypropylene tubes. Add 177 μl Opti-MEM to the first polypropylene tube.
(d) Very gently add, in a dropwise fashion, 87 μl undiluted FuGENE 6 reagent directly into the Opti-MEM in this polypropylene tube with sterile, aerosol-resistant pipette tips. Gently tap the bottom of the tube. Be careful to avoid

direct contact of FuGENE 6 with the plastic surface of the tube, as a decrease in transfection efficiency may occur. Never add FuGENE 6 reagent to an empty tube.

(e) In the second polypropylene tube, add 29 μg plasmid DNA into a suitable volume of TE buffer to achieve a final concentration of 0.5–2.0 μg/μl to the bottom of the tube. Use sterile, aerosol-resistant pipette tips.

(f) Incubate the solutions in each of the two tubes for 5 min at room temperature.

(g) **Very carefully, dropwise, and slowly** add the diluted FuGENE 6 reagent from the first polypropylene tube directly onto the DNA drop in the second tube. Be careful to avoid direct contact of FuGENE 6 with the plastic surface of the tube. Gently tap the bottom of the tube to mix the two components. Close the tube with the cap. You may notice a precipitate as the lipid-DNA complexes form. It is very important to prepare this cocktail fresh, approximately 15–45 min before adding it to the cells.

(h) Incubate for 15–45 min at room temperature.

(i) In the meantime, remove the growth medium of the chondrocytes and add 10 ml fresh growth medium to the cells. A washing step is not necessary.

(j) Prepare fresh hyaluronidase solution.

(k) Approximately 15–45 min after combining the FuGENE 6 with the DNA, add the FuGENE 6-DNA complexes slowly and dropwise directly to the medium in the 10-cm cell culture dish. Swirl carefully to distribute the FuGENE 6-DNA complexes.

(l) Immediately after adding the FuGENE 6-DNA complexes, add 200 μl hyaluronidase solution (4 U/ml) directly to the medium in the 10-cm cell culture dish. It is important to prepare this solution fresh immediately before use. Swirl carefully.

(m) Check the appearance of the cells under the microscope. You may note the transfection complexes.

(n) Incubate the chondrocytes at 37 °C in a humidified atmosphere with 10% CO_2 for 6 h. As FuGENE 6-DNA complexes have not been reported to be harmful to the cells, it is usually not necessary to monitor the cells for signs of toxicity.

(o) After 6 h, add 5 ml growth medium to the cell culture dish containing 10 ml of medium and hyaluronidase.

(p) After 24 h, remove all medium.

(q) Wash the cell layer once with 5 ml PBSA.

(r) Trypsinize the cells.

i) Apply 2 ml trypsin-EDTA solution.

ii) Return the cells to the incubator for 15–45 min.

iii) After dissociation from the culture vessel, wash the cells once in 40 ml basal medium in a 50-ml Falcon tube. Centrifuge at 2000 *g* for 5 min.

iv) Determine the cell number by hemocytometer and cell viability by Trypan Blue exclusion.

v) Proceed to Protocol 5.3 to determine transfection efficiency.

3.3. Cell Seeding in Scaffolds

Functional substitutes for native articular cartilage can be created in vitro from chondrogenic cells attached to polymer scaffolds in bioreactors. During this process, chondrocytes deposit extracellular matrix consisting of proteoglycans and type II collagen in parallel with the scaffold degradation [Vunjak-Novakovic et al., 1998, 1999; Freed et al., 1998]. For a detailed protocol on seeding scaffolds, See Chapter 6.

3.4. Reporter Gene Expression

Marker genes like the *E. coli* β-galactosidase (*lacZ*) gene, the chloramphenicol-acetyl transferase (CAT) or the *Photinus pyralis* luciferase gene are often used to detect transgene expression. A direct estimation of the efficiency of transfection is possible with *lacZ*. Transfection efficiency is expressed as the percentage of positive transfected cells. The sensitive CAT or luciferase assays are very useful to compare different transfection systems or to optimize transfection conditions. They do not allow a direct determination of transfection efficiency.

After a successful gene transfer, the exogenous bacterial enzyme β-galactosidase, which breaks β-galactoside into its component sugars, is produced inside the cell. It can be detected easily in the cytoplasm by a colorimetric reaction. The substrate 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal) is cleaved and an insoluble indigo blue reaction product is generated in the presence of potassium ferricyanide and potassium ferrocyanide (Fig. 5.1). Thus all cells carrying the *lacZ* gene can be identified by their deep blue color. Some cells (e.g., synoviocytes) possess endogenous β-galactosidase-like enzymatic activity. The optimal pH for endogenous β-galactosidase is between 3.5 and 5.5, whereas that for the *E. coli* enzyme is 7.3 [Hatton and Lin, 1992]. Possible interference can therefore be minimized by adjusting the pH of the staining solution to 8.4 and by limiting the incubation to 2–4 h. The X-Gal staining solution should be prepared fresh every time. Alternatively, commercial β-galactosidase staining kits can be used, at somewhat higher total cost.

Protocol 5.3. Detection of Reporter Gene Expression in Transfected Cartilage

Reagents and Materials

Sterile

- ❑ Tissue culture-treated plates, 24 well
- ❑ PBSA

Nonsterile

- ❑ Fixative for X-Gal staining (See Section 2.3.6)
- ❑ X-Gal stock solutions, solutions A and B (See Section 2.3.7)

Protocol

(a) At 48 hours after transfection in a 24-well plate, remove medium, wash the cell layers twice in 10 ml PBSA, and fix the cells for 5 min in fixative.

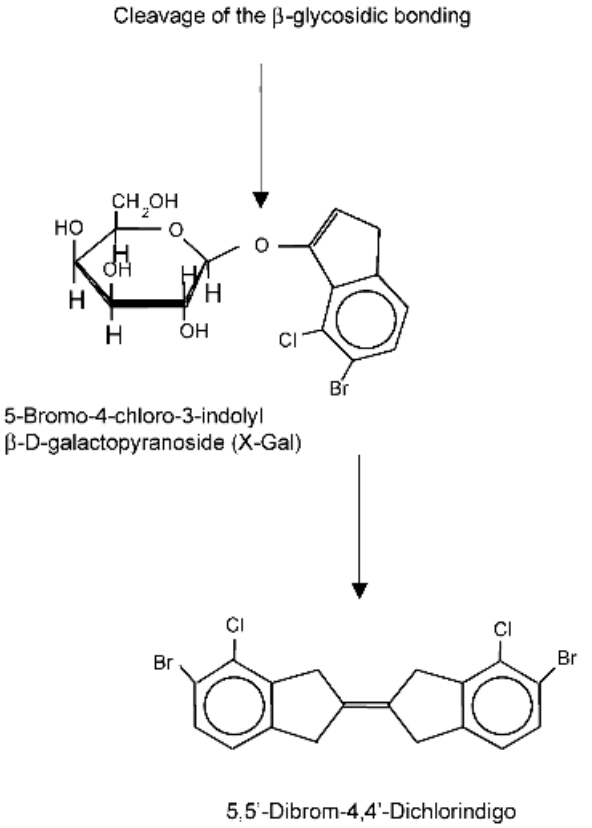

Figure 5.1. Cleavage of X-Gal by β-galactosidase. The colorless substrate 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal) is cleaved by the bacterial enzyme β-galactosidase in galactose and in an indoxyl derivate. The indoxyl derivate later oxidizes to the blue dibrome-dichlor derivate that is insoluble in water. It can be easily identified as it stains the cytoplasm of the transfected cell blue.

(b) Rinse cells 3× with 10 ml PBSA; be careful not do dislodge the cell layer.
(c) Add 0.5 ml X-Gal staining solution to each well.
(d) Incubate for 2–4 h at 37 °C in a humidified incubator.
(e) Remove cells from the incubator after each 1 h to check for transgene expression. As there is no false-positive staining in chondrocytes when the solutions are properly prepared and its pH is adjusted, one may also stain overnight. The stain should develop after 1–2 h.
(f) Once the stain has developed, remove the X-Gal staining solution and add 10 ml PBSA.
(g) Express transfection efficiency as the percentage of positive to total cells by counting cells in 10–20 sequential microscope fields at 40× magnification along the horizontal and vertical diameters of each well of the 24-well plate. When transfection efficiencies are low (e.g., $<$1.0%), all stained cells per well may be counted.

Table 5.1. Structural and functional parameters of engineered cartilage after 4 weeks of bioreactor cultivation.

Construct parameters measured after 4 weeks of bioreactor cultivation	Constructs		
	Lac Z	IGF-I	Nontransfected
Construct structure			
Wet weight (mg/construct)	26.5 ± 2.1 ($n = 10$, $P < 0.001$)	67.7 ± 10.8 ($n = 10$)	29.0 ± 8.5 ($n = 10$, $P < 0.001$)
Dry weight (mg/construct)	2.2 ± 0.4 ($n = 10$, $P < 0.001$)	5.8 ± 1.1 ($n = 10$)	2.8 ± 0.4 ($n = 10$, $P < 0.001$)
Diameter (mm)	5.6 ± 0.1 ($n = 10$, $P < 0.001$)	6.5 ± 0.2 ($n = 10$)	5.3 ± 0.2 ($n = 10$, $P < 0.001$)
Water (% wet weight)	92.5 ± 1.0 ($n = 6$, $P > 0.05$)	91.3 ± 0.9 ($n = 5$)	88.7 ± 3.5 ($n = 6$, $P > 0.05$)
DNA (μg/construct)	48.8 ± 7.0 ($n = 6$, $P = 0.001$)	74.3 ± 11.5 ($n = 5$)	38.4 ± 7.0 ($n = 6$, $P = 0.001$)
Glycosaminoglycans (μg/construct)	96.3 ± 15.3 ($n = 6$, $P = 0.001$)	1068.6 ± 239.6 ($n = 5$)	133.3 ± 51.1 ($n = 6$, $P < 0.001$)
Glycosaminoglycans (μg/μg DNA)	2.0 ± 0.2 ($n = 6$, $P < 0.001$)	14.3 ± 1.2 ($n = 5$)	3.4 ± 0.9 ($n = 6$, $P < 0.001$)
Collagen (mg/construct)	0.6 ± 0.1 ($n = 6$, $P < 0.001$)	1.3 ± 0.2 ($n = 2$)	0.5 ± 0.1 ($n = 6$, $P < 0.001$)
Collagen (μg/μg DNA)	11.6 ± 1.3 ($n = 6$, $P < 0.001$)	17.0 ± 0.9 ($n = 5$)	14.1 ± 1.9 ($n = 6$, $P = 0.015$)
Construct function			
[^{35}S]sulfate incorporation (cpm/μg DNA/16 h)	2.3 ± 0.6 ($n = 6$, $P < 0.001$)	9.4 ± 1.0 ($n = 5$)	3.8 ± 0.9 ($n = 6$, $P < 0.001$)
[^{3}H]proline incorporation (cpm/μg DNA/16 h)	38.9 ± 8.3 ($n = 6$, $P > 0.05$)	45.4 ± 6.1 ($n = 5$)	43.6 ± 7.1 ($n = 6$, $P > 0.05$)
Glucose in medium (mg/cm^3)	3.22 ± 0.07 ($n = 2$, $P > 0.05$)	2.21 ± 0.52 ($n = 2$)	3.11 ± 0.20 ($n = 2$, $P > 0.05$)
Lactate in medium (mg/cm^3)	0.998 ± 0.07 ($n = 2$, $P > 0.05$)	1.80 ± 0.49 ($n = 2$)	1.04 ± 0.12 ($n = 2$, $P > 0.05$)
Equilibrium modulus (kPa)	35 ± 33 ($n = 3$, $P < 0.05$)	126 ± 52 ($n = 4$)	30 ± 4 ($n = 3$, $P < 0.03$)

Parentheses indicate the number of samples per group followed by the P value for comparing the respective control group with IGF-I constructs. Data represent average $\pm$ standard deviation.

4. APPLICATIONS

One focus of interest of our laboratory is the healing of articular cartilage defects by chondrocytes overexpressing therapeutic genes. We have reported previously that FuGENE 6-mediated transfection in the presence of hyaluronidase produced high transfection efficiencies in normal bovine and human articular chondrocytes [Madry and Trippel, 2000]. When articular chondrocytes were transfected with an expression plasmid vector based on pcDNA3.1/Zeo(+) containing the cDNA for human insulin-like growth factor-I (IGF-I), a polypeptide that is anabolic and mitogenic for cartilage, biologically relevant amounts of IGF-I protein were synthesized [Madry et al., 2001]. We have used Protocol 5.2 to modify bovine articular chondrocytes that were then used to engineer cartilaginous constructs. After seeding onto biodegradable polyglycolic acid

scaffolds and culture in bioreactors, the structural and functional properties of tissue engineered cartilage were significantly enhanced when a human IGF-I cDNA was transfected (Table 5.1). Transgene expression was maintained over 28 days of in vitro cultivation followed by an additional 10 days either in vitro or in vivo [Madry et al., 2001]. In these experiments, the human IGF-I gene served as a model to demonstrate the potential benefits of gene transfer for tissue engineering. Genetically modified cartilaginous constructs overexpressing a growth or transcription factor gene may be used as a tissue substitute that simultaneously provides a stimulus for repair.

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SOURCES OF MATERIALS

Item	*Supplier*
Ascorbic acid	Sigma
Bovine testicular hyaluronidase	Sigma
5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal)	Roche
Cell culture dishes, standard tissue culture treated (100×20 mm)	Falcon (BD Biosciences)
Cell strainer with 100-μm nylon mesh	Falcon (BD Biosciences)
Collagenase (type I)	Worthington
DMEM (high glucose)	Invitrogen
Endofree Maxi Kit	Qiagen
FuGENE 6	Roche
Magnesium chloride	Fisher Scientific
N,*N*-dimethylethylformamide	Sigma
Opti-MEM	Invitrogen
PBSA	Invitrogen
pcDNA3.1/Zeo(+)	Invitrogen
pCMVβgal	Invitrogen
Penicillin	Invitrogen
Pipette tips, aerosol resistant	Fisher Scientific
Plasticware	Falcon (BD Biosciences)
Potassium ferricyanide, $K_3Fe(CN)_6$	Fisher Scientific
Potassium ferrocyanide trihydrate, $K_4Fe_3(CN)_6 \cdot 3H_2O$	Fisher Scientific
Round bottom test tubes with cap	Falcon (BD Biosciences)
Spatula, flat end	Fisher Scientific
Spinner flask, 100 ml, with adjustable hanging bar	Bellco Glass
Streptomycin	Invitrogen
Trypsin-EDTA (0.25% trypsin in 1.0 mM EDTA)	Invitrogen

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