Engineering Endostatin-Producing Cartilaginous Constructs for Cartilage Repair Using Nonviral Transfection of Chondrocyte-Seeded and Mesenchymal-Stem-Cell-Seeded Collagen Scaffolds

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Although there is widespread recognition of the importance of angiogenesis in tissue repair, there is little work on the inhibition of angiogenesis in the context of tissue engineering of naturally avascular tissues, like articular cartilage. The objective was to engineer a collagen-scaffold-based cartilaginous construct overexpressing a potent antiangiogenic factor, endostatin, using nonviral transfection. Endostatin-plasmid-supplemented collagen scaffolds were seeded with mesenchymal stem cells and chondrocytes and cultured for 20–22 days. The effects of the following variables on endostatin expression and chondrogenesis were examined: collagen scaffold material, method of nonviral vector incorporation, plasmid load, culture medium, and oxygen tension. An increase and peak of endostatin protein was observed during the first week of culture, followed by a decrease to low levels, suggesting that overexpression of endostatin could be sustained for several days using the nonviral vector. The amount of endostatin produced was tunable with the external factors. Chondrogenesis was observed in the engineered constructs cultured in chondrogenic medium at the 3-week time point, demonstrating that endostatin did not inhibit the chondrogenic potential of mesenchymal stem cells or the general viability of the cells. The ability to engineer endostatin-expressing cartilaginous constructs will be of value for future work exercising regulatory control of angiogenesis in cartilage repair.

Introduction

UNLIKE MOST TISSUES, articular cartilage, a specialized form of hyaline cartilage, is normally resistant to vascularization and contains no blood vessels. If articular cartilage is damaged, the tissue exhibits only limited healing potential. Damage may be accompanied by the invasion of blood vessels from the underlying subchondral bone into the cartilage; osteoarthritic cartilage has been shown to lose its resistance to vascular invasion compared to normal articular cartilage.¹ Moreover, angiogenesis in articular cartilage is believed to be a key contributor to the symptoms and pathology associated with osteoarthritis.² The active resistance of normal articular cartilage to neovascularization and the associations of angiogenesis with pathological conditions in

cartilage suggest that angiogenesis may interfere with cartilage regeneration and may play a critical role in cartilage degeneration. Early investigations examining the use of various antiangiogenic factors, such as Flt-1, endostatin, and suramin, in articular cartilage tissue engineering include the use of soluble Flt-1 to block vascular endothelial growth factor in osteochondral defects,³ the use of nonviral gene therapy to engineer cells to overexpress endostatin,⁴ and a proof-of-principle study demonstrating that by inhibiting angiogenesis with suramin, an environment promoting the exclusive production of cartilage could be created in the space between the tibia and the periosteum.⁵

The objective of this study was to engineer *in vitro* a collagen-scaffold-based cartilaginous construct overexpressing a potent antiangiogenic factor, endostatin, using

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nonviral transfection and small amounts of plasmid to address safety issues. The construct would serve as an implant for defects in the articular surface of joints, providing an immature cartilage for subsequent maturation *in vivo*, and endostatin-expressing cells to prevent vascular invasion. Endostatin is a 20-kDa proteolytic fragment located at the COOH-terminal of type XVIII collagen that has been shown to strongly inhibit endothelial cell proliferation and migration and prevent the formation of blood vessels.⁶ It is synthesized by chondrocytes *in vitro* and *in vivo*⁷ and has been found in articular cartilage *in vivo*.⁸

Our supposition was that the following select variables may affect the endostatin expression profile and select measures of chondrogenesis, in this study cell morphology and glycosaminoglycan (GAG) and type II collagen content: collagen scaffold material (type I/III vs. II); method of nonviral vector, lipoplex, incorporation; plasmid load, with an emphasis on small amounts for safety; culture medium (chondrogenic medium [CM] vs. nonchondrogenic medium [NCM]); and oxygen tension, recognizing that articular cartilage levels are low, estimated to range from 1% to 7%.⁹ These studies were conducted using both caprine chondrocyte-seeded and marrow-stromal-cell (also referred to as mesenchymal stem cell [MSC])-seeded constructs, recognizing the advantage with respect to procurement of the latter. Previous studies have investigated the utility of type I/III and type II collagen scaffolds for the regeneration of articular cartilage.¹⁰ Of particular interest is the use of the collagen scaffolds for nonviral gene delivery^{11,12}; however, prior studies have not yet investigated the differences between type I/III and II collagen as delivery vehicles for nonviral vectors.

Materials and Methods

Plasmid propagation and isolation

The endostatin plasmid (pEndo) vector *pCEP-Pu AC7* and the propagation and isolation protocol have been previously described.⁴ Briefly, plasmid was obtained by heat shock transformation of *Escherichia coli* DH5 α competent cells (Invitrogen, Carlsbad, CA) and isolation using a Mega QIAfilter™ Plasmid kit (Qiagen, Valencia, CA).

Cell isolation and two-dimensional monolayer expansion

MSCs were isolated from heparinized bone marrow aspirates from the iliac crests of adult Spanish goats. Adherent cells were expanded in monolayer using a standard MSC expansion medium consisting of low-glucose Dulbecco's modified Eagle's medium (DMEM-LG), containing 10% (v/v) fetal bovine serum (FBS) and 1% penicillin/streptomycin (PS) (Invitrogen), and supplemented with 10 ng/mL fibroblast growth factor-2 (R&D Systems, Minneapolis, MN). The cells were incubated in one of two conditions: (1) a humidified chamber at 37°C, 5% CO₂, and atmospheric (standard) O₂ (~21%), or (2) a humidified chamber at 37°C, 5% CO₂, and 5% (low) O₂. MSCs were grown through two subcultures to obtain passage 2 (P2) cells.

Chondrocytes were isolated from articular cartilage shavings obtained from nonarticulating regions of the trochlear ridges and trochlear notch of the left knee of an adult Spanish goat. Cells were expanded in monolayer culture using a standard chondrocyte expansion medium consisting of high-glucose DMEM (DMEM-HG), containing 1% (v/v) nonessential amino acids, 1% HEPES buffer, 1% PS/L-glutamine, and 10% FBS (Invitrogen), and supplemented with 1 ng/mL transforming growth factor- β 1, 5 ng/mL fibroblast growth factor-2, and 10 ng/mL platelet-derived growth factor- β (R&D Systems). The cells were incubated at in a humidified chamber at 37°C, 5% CO₂, and 21% O₂ and grown through two subcultures to obtain P2 cells.

Scaffold fabrication

Two scaffold types were prepared. One type was made using 0.5% (w/v) porcine type I/III collagen (Geistlich Biomaterials, Wolhusen, Switzerland), and a second type using 1% porcine type II collagen (Geistlich Biomaterials). Porous sheets (~1.5 mm thick) were fabricated by freeze-drying (VirTis, Gardiner, NY) as previously described.⁴ The sheets were then sterilized and lightly crosslinked by dehydrothermal treatment using a vacuum >30 inHg at a temperature of 105°C. Disks (8 mm in diameter and 1.5 mm in thickness) were cut from the porous sheets using a dermal biopsy punch.

TABLE 1. SUMMARY OF EXPERIMENTAL CONDITIONS AND EVALUATION METHODS

Exp. no	Cell type	Collagen type	Lipoplex incorporation ^a	pEndo (μ g)	Culture medium	Oxygen tension ^b	Cells (millions) ^c	Evaluation method (n)
1	MSC	I/III	Half X-L	4	NCM	21%–21%	2	ELISA (2–3)
2	MSC	I/III	None X-L	20	CM	21%–21%	2	Histochemical (1)
			Half X-L					ELISA (6)
			All X-L					Histochemical (2)
3	MSC	I/III	All X-L	20	CM	21%–21% (MSCs, Chon)	4	ELISA (3)
	Chon	II				21%–5% (Chon)		Histochemical (2–3)
						5%–5% (MSC)		Biochemical (2–3)

^aX-L, crosslinked.

^bOxygen percentage in monolayer expansion-oxygen percentage in culture of the cell-seeded collagen construct.

^cNumber of cells seeded into the scaffold.

Chon, chondrocyte; CM, chondrogenic medium; ELISA, enzyme-linked immunosorbent assay; MSC, mesenchymal stem cell; NCM, nonchondrogenic medium.

Experimental design

Caprine MSCs and chondrocytes were subcultured twice (P2) and transfected with lipoplexes prepared with human endostatin plasmid (Table 1).

1. In the first experiment, MSCs expanded at 21% O₂ were seeded into endostatin-lipoplex-supplemented type I/III collagen (CI) scaffolds and cultured in NCM or CM for 22 days at 21% O₂. The CM was composed of DMEM-HG, 1% (v/v) nonessential amino acids, 1% HEPES buffer, 1% PS/L-glutamine, 1.25 mg/mL of bovine serum albumin (Invitrogen), and 1×ITS + 1 (Sigma Chemical Co., St. Louis, MO), and supplemented with 10 ng/mL transforming growth factor-β1, 100 nM dexamethasone (Sigma Chemical Co.), and 0.1 mM L-ascorbic acid 2-phosphate. The NCM consisted of DMEM-LG containing 10% FBS and 1% PS. The variables included the plasmid amount and the culture medium used for three-dimensional (3D) scaffold culture.
2. In the second experiment, MSCs expanded at 21% O₂ were seeded into endostatin-lipoplex-supplemented type I/III collagen scaffolds and cultured for 22 days at 21% O₂ in CM. The variable was the method of carbodiimide crosslinking.
3. In the third experiment, MSCs and chondrocytes from the same goat were seeded into endostatin-lipoplex-supplemented type I/III or type II (CII) collagen scaffolds. The cultures were maintained for 20 days in CM. Four different oxygen groups were used: (1) MSCs expanded in two-dimensional (2D) monolayer at 21% (standard) O₂ and cultured in 3D scaffolds at 21% (21%–21%), (2) chondrocytes expanded in 2D at 21% and cultured in 3D at 21% (21%–21%), (3) chondrocytes expanded in 2D at 21% and cultured in 3D at 5% (low) (21%–5%), and (4) MSCs expanded in 2D at 5% and cultured in 3D at 5% (5%–5%). The variables included the scaffold material, cell type, and oxygen tension.

The principal outcome variable was the amount of endostatin released by the cells, recovered in the medium.

Preparation of collagen scaffolds incorporating GenePORTER 2/endostatin plasmid complexes (lipoplexes)

Endostatin plasmid was encapsulated in a lipid-mediated transfection reagent, GenePORTER™ 2 (GP2; Gene Therapy Systems, Inc., San Diego, CA) following the manufacturer's instructions.

In the first experiment, a ratio of μL of GP2:μg of pEndo of 3.5 was used. Our previous work showed that there was no significant effect of GP2:pEndo ratio (3.5, 7, and 10) on endostatin levels.⁴ Lipoplexes were prepared using 4 and 20 μg of pEndo and incorporated into type I/III collagen scaffolds using two steps. Half of the lipoplex solution was pipetted on one side of the scaffold and incubated for 10 min. A 1 mL aliquot of an aqueous carbodiimide solution consisting of 0.6 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride and 0.6 mM N-hydroxysuccinimide (Sigma Chemical Co.) was added to the scaffold, followed by incubation for 30 min. The same carbodiimide solution concentration was used for all subsequent experiments as well.

Excess carbodiimide was removed by soaking the scaffolds in phosphate-buffered saline (PBS) for 1 h. Then, the other half of the lipoplex solution was added to the second side of the scaffold and incubated for 10 min. Scaffolds with no lipoplex served as controls.

In the second experiment, 20 μg of pEndo and a GP2:pEndo ratio of 3.5 were used. Three different methods were used for incorporation and crosslinking of the endostatin lipoplexes to type I/III collagen scaffolds. For the group in which none of the lipoplex was crosslinked to the scaffold, the scaffolds were incubated in carbodiimide for 30 min and soaked in PBS for 1 h to remove excess carbodiimide. Then, half of the lipoplex solution was added to each side, with an incubation period of 10 min for each side. For the group in which half of the lipoplex solution was crosslinked to the scaffold, half of the lipoplex solution was added to one side, the scaffolds were incubated for 10 min, carbodiimide was added for an incubation period of 30 min, the scaffolds were soaked in PBS for 1 h to remove excess carbodiimide, and the other half of the lipoplex solution was added to the second side for 10 min. For the group in which all of the lipoplex was crosslinked, half of the lipoplex solution was added to each side, with an incubation period of 10 min for each side, followed by incubation in carbodiimide for 30 min and PBS for 1 h. Control scaffolds with no lipoplex supplementation were also prepared.

In the third experiment, 20 μg of pEndo and a GP2:pEndo ratio of 2.5 were used. Half of the lipoplex solution was added to each side with 20 min incubations, followed by carbodiimide incubation for 30 min. Scaffolds were rinsed and maintained in PBS until cell seeding. Both type I/III and type II collagen scaffolds were supplemented with lipoplexes. Scaffolds with no lipoplex served as controls.

Transfection and culture of cells in 3D collagen scaffolds

Collagen scaffolds were placed in agarose-coated wells for cell seeding. P2 cells were trypsinized and resuspended in DMEM-LG. In the first two experiments, 1 million MSCs were pipetted onto each side of the scaffold, with an incubation period of 10 min at 21% O₂ for each side, for a total of 2 million cells seeded per scaffold. For the third experiment, 2 million MSCs or chondrocytes were pipetted onto one side of the scaffold and incubated at 5% or 21% O₂, as appropriate, for 10 min, followed by the addition of 2 million cells on the second side for 30 min, for a total of 4 million cells seeded per scaffold. All constructs were then incubated in 0.5 mL of DMEM-LG at 5% or 21% O₂, as appropriate, for 2.5–4 h to allow for transfection. Following transfection, all constructs were cultured at 5% or 21% O₂, as appropriate, in CM for the remainder of the experiment, with the exception of control scaffolds in the first experiment cultured in NCM. Non-cell-seeded scaffolds were also cultured as controls.

Every 1–3 days, expended medium was collected and frozen at –20°C until analysis, and fresh medium was added. Cultures were terminated after 20–22 days for histological examination and biochemical analysis.

Endostatin detection in the medium

Endostatin protein in the culture medium was measured using a sandwich enzyme-linked immunosorbent assay

(ELISA) kit for human endostatin protein (R&D Systems) following the manufacturer's instructions.

Analysis of DNA and GAG content

Constructs were lyophilized and enzymatically digested overnight using proteinase K (Roche Diagnostics, Indianapolis, IN). Determination of the DNA content was carried out using the Picogreen dye assay kit (Molecular Probes, Inc., Eugene, OR) according to the manufacturer's instructions. Previous work in our laboratory found that the average DNA content was 5.6 pg DNA/cell for goat MSCs and 7.7 pg DNA/cell for goat chondrocytes. The sulfated GAG content was determined by the dimethylmethylene blue dye assay, with a standard curve obtained using chondroitin-6-sulfate from shark cartilage (Sigma Chemical Co.).

Histological and immunohistochemical evaluation

Constructs allocated for histology were fixed in 10% formalin or 4% paraformaldehyde for at least 3 h, processed and embedded in paraffin, and sectioned by microtomy. The sections were mounted on glass slides and stained with hematoxylin and eosin, safranin-O, and Masson's trichrome using standard histological techniques.

Endostatin and type II collagen distribution were examined immunohistochemically using an antitype II collagen mouse monoclonal antibody (CIIC1, 1:150 dilution; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA) and an antiendostatin rabbit polyclonal antibody (1:40 dilution; Millipore, Billerica, MA). The immunohistochemical staining was performed using the Dako Autostainer (DakoCytomation, Carpinteria, CA) and the peroxidase-aminoethyl carbazole-based Envision + kit (DakoCytomation) following the manufacturer's recommendations.

Statistical analysis

Data are presented as the mean $\pm$ standard error of the mean. Analysis of variance (ANOVA) and Fisher's protected least squares difference (PLSD) *post hoc* testing were performed using StatView software (SAS Institute Inc, Cary, NC). Statistical significance was set at $p < 0.05$.

Results

The effects of plasmid load and culture medium (first experiment), method of nonviral vector incorporation (second experiment), and collagen scaffold material and oxygen tension (third experiment) on endostatin expression and chondrogenesis of MSC- and chondrocyte-seeded constructs were examined. Our experience with the seeding method indicated that $\sim 65\%$ of the cells were retained in the scaffold 1 day postseeding.

Endostatin released into the medium by MSC-seeded type I/III constructs cultured in CM and NCM

Endostatin detection in the medium. The MSC-seeded type I/III collagen control groups, which received no lipoplex and were not transfected, displayed no notable en-

dostatin in the medium (data not shown). Endostatin in the medium of MSC-seeded, lipoplex-supplemented type I/III constructs increased to a peak in the first week of culture and then decreased to low levels (Fig. 1a). The kinetics of expression was similar for constructs supplemented with 4 and 20 μ g of plasmid. While the 6-day accumulated endostatin release of the 20 μ g pEndo group in CM (90 ± 20 ng/mL) was around twofold higher than in NCM, the results were not statistically significant ($p = 0.181$, power = 0.249), likely owing to variation. For the 3-day collection period ending at day 6, the endostatin level of constructs with 20 μ g of plasmid in CM reached 45 ± 10 ng/mL, the highest level seen in the experiment (Fig. 1a).

Three-factor ANOVA for the cumulative endostatin data from the first 6 days (including the controls with no plasmid) revealed significant effects of plasmid load ($p < 0.0001$, power = 1.0) and collection period ($p = 0.045$, power = 0.596) on endostatin level, but failed to find a significant effect of medium type ($p = 0.181$, power = 0.249). Fisher's PLSD *post hoc* testing demonstrated a significant difference in endostatin level between the group treated with 20 μ g pEndo and the other two groups.

Histological and immunohistochemical evaluation of the constructs. Immunohistochemical evaluation of the cell-seeded constructs collected 6 days postseeding demonstrated positive staining for endostatin for a few cells in the lipoplex-supplemented scaffolds, cultured in CM and NCM (Fig. 2a), suggesting that most of the endostatin protein was not retained in the engineered constructs. Samples cultured in CM, collected at the termination of the 22-day culture period, revealed many cells that were rounded and resided in lacunae, typical characteristics of chondrocyte morphology, for scaffolds cultured in CM (Fig. 2b). Cells could be seen dispersed throughout the construct entrapped in newly synthesized extracellular matrix, with very little original scaffold material remaining (Fig. 2b, c). The fibrous appearance of the matrix was consistent with fibrocartilage. There were only a few small areas demonstrating a hyaline matrix. MSC-seeded constructs, both lipoplex-supplemented and controls, cultured in CM showed intense positive staining with safranin-O (Fig. 2d), indicating the presence of sulfated GAGs, and stained positively for type II collagen (Fig. 2e). Constructs cultured in NCM did not display cells with a chondrocytic morphology and did not stain for GAG or type II collagen (data not shown).

Transfection of MSCs following crosslinking modification of the type I/III collagen scaffold for retaining the lipoplex

Endostatin detection in the medium. Substantial overexpression of endostatin was observed in the MSC-seeded, lipoplex-supplemented constructs compared to non-supplemented controls (Fig. 1b). Endostatin levels of the lipoplex-supplemented constructs were not noticeably different than the control non-lipoplex-supplemented constructs on days 20 and 22 (data not shown). Endostatin synthesized by cell-seeded constructs in which none or half of the lipoplexes were crosslinked to the scaffolds increased to a peak by the 2-day collection period ending at day 4,

FIG. 1. Endostatin in the medium of cell-seeded, lipoplex-supplemented collagen constructs and control constructs. (a) Mesenchymal-stem-cell (MSC)-seeded type I/III constructs cultured in chondrogenic medium (CM) and nonchondrogenic medium (NCM) ($n = 3$ for first 6 days of lipoplex-supplemented scaffolds; $n = 2$ for all others). (b) Modification of the scaffold via crosslinking none, half, or all of the lipoplexes ($n = 6$), in MSC-seeded type I/III constructs cultured in CM. (c) MSCs cultured at standard and low oxygen in type I/III and type II collagen scaffolds in CM ($n = 3$). (d) Chondrocytes cultured at standard and low oxygen in type I/III and type II collagen scaffolds in CM ($n = 3$).

FIG. 2. Micrographs of histological sections of chondrocyte- and MSC-seeded constructs cultured in CM and NCM at standard oxygen. (a) Endostatin immunohistochemistry of MSC-seeded CI scaffold supplemented with 20 μ g pEndo after 6 days of culture in NCM (red chromogen [arrows] indicates positive stain). Scale bar, 50 μ m, 40 $\times$ magnification. (b) Hematoxylin and eosin stain of the interior of MSC-seeded CI scaffold supplemented with 4 μ g pEndo after 22 days of culture in CM. Scale bar, 50 μ m, 40 $\times$ magnification. Inset shows the section at lower magnification, scale bar, 100 μ m, 10 $\times$ magnification. (c) Hematoxylin and eosin stain of the surface of MSC-seeded CI scaffold supplemented with 4 μ g pEndo after 22 days of culture in CM. Scale bar, 100 μ m, 10 $\times$ magnification. (d) Safranin-O staining of glycosaminoglycan (GAG) for MSC-seeded CI scaffold supplemented with 4 μ g pEndo after 22 days of culture in CM (red chromogen indicates sulfated GAGs). Scale bar, 200 μ m, 10 $\times$ magnification. (e) Type II collagen immunohistochemistry of MSC-seeded CI scaffold supplemented with 4 μ g pEndo after 22 days of culture in CM (red indicates positive stain). Scale bar, 200 μ m, 10 $\times$ magnification. (f) Safranin-O staining of GAG for chondrocyte-seeded CI scaffold supplemented with 20 μ g pEndo after 20 days of culture in CM (red chromogen indicates sulfated GAGs). Scale bar, 50 μ m, 40 $\times$ magnification. Inset shows the section at lower magnification, scale bar, 500 μ m, 4 $\times$ magnification. (g) Type II collagen immunohistochemistry of chondrocyte-seeded CI scaffold supplemented with 20 μ g pEndo after 20 days of culture in CM (brownish-red chromogen indicates positive stain). Scale bar, 500 μ m, 4 $\times$ magnification. (h) Masson's trichrome staining of chondrocyte-seeded CI scaffold supplemented with 20 μ g pEndo after 20 days of culture in CM (blue chromogen indicates collagen, red chromogen indicates cytoplasm, and black chromogen indicates nuclei). Scale bar, 500 μ m, 4 $\times$ magnification. Color images available online at www.liebertonline.com/ten.

whereas the peak for the MSC-seeded constructs in which all of the lipoplexes were crosslinked occurred on the 2-day collection period ending on day 6 (Fig. 1b). The peak amounts per collection period for the 3 lipoplex-supplemented groups were comparable, around 13 ng/mL (Fig. 1b). A gradual decrease in endostatin was seen in the following 2 weeks, but sustained expression was observed for nearly 3 weeks (Fig. 1b).

The average 6-day accumulated endostatin in the half crosslinked (20 μ g) group (30 ± 5 ng/mL; Fig. 1b) was lower than that recorded in the first experiment (90 ± 20 ng/mL; Fig. 1a).

Two-factor ANOVA for the data from the three lipoplex-supplemented groups revealed a significant effect of collection period ($p \leq 0.0001$, power = 1) on endostatin level. The effect of crosslinking method ($p = 0.50$, power = 0.162) on

endostatin level did not achieve statistical significance. However, for the endostatin protein peak day data of the three lipoplex-supplemented groups, one-factor ANOVA revealed a significant effect of crosslinking method on peak day ($p < 0.0001$, power = 1). Fisher's PLSD *post hoc* testing demonstrated a significant difference in peak day between the group in which all the lipoplexes were crosslinked to the scaffold and the other two groups.

*Endostatin released into the medium
by MSC- and chondrocyte-seeded type I/III
and type II collagen scaffolds cultured
at 5% and 21% oxygen*

Endostatin detection in the medium. Endostatin in the medium of cell-seeded, lipoplex-supplemented scaffolds increased to a peak during the first week, followed by a gradual decrease (Fig. 1c, d). Notable endostatin levels could be measured in several of the groups even after 17 days (Fig. 1c, d). The highest endostatin level in the experiment was observed to be 3.8 ± 0.3 ng/mL for 21%–21% MSCs cultured on type II collagen scaffolds for the 3-day collection period ending on day 3 (Fig. 1c). MSC-seeded constructs resulted in higher endostatin levels than chondrocyte-seeded constructs during the initial week after transfection, and constructs cultured in 3D scaffolds at standard oxygen showed more endostatin compared with those cultured at low oxygen (Fig. 1c, d). For constructs cultured at low oxygen, after the first 3 days, endostatin expression levels for MSCs were higher in the type II collagen versus the type I collagen scaffolds (Fig. 1c). For standard oxygen, there was no notable effect of scaffold type on expression (Fig. 1c, d).

Including all data, five-factor ANOVA demonstrated significant effects of 3D oxygen tension, scaffold material, cell type, plasmid load (0 vs. 20 μ g), and collection period on endostatin level ($p < 0.0001$, power = 1). Analyzing data for the two cell types separately, four-factor ANOVA revealed significant effects of oxygen tension, scaffold material, plasmid load, and collection period ($p < 0.0001$, power = 1) on endostatin level of the 21%–21% and the 21%–5% MSCs. For the data for the 21%–21% and the 21%–5% chondrocytes, four-factor ANOVA demonstrated significant effects of oxygen tension ($p = 0.002$, power = 0.913), plasmid load ($p < 0.0001$, power = 1), and collection period ($p = 0.0002$, power = 0.991) on endostatin level. The effect of scaffold material ($p = 0.57$, power = 0.086) on endostatin level did not achieve statistical significance.

Biochemical analysis of DNA and GAG content. Biochemical analyses of cell-seeded samples terminated after 20 days of culture found that the GAG density (GAG content per volume) of the constructs ranged from <10% to about 150% of that in native cartilage (Fig. 3a, b); the value for the normal GAG density that was used is $15.8 \mu\text{g}/\text{mm}^3$.¹³ No notable GAG (<2%) was detected in non-cell-seeded controls (data not shown). Higher percentages of GAG were observed in chondrocyte-seeded constructs, compared to the respective MSC-seeded scaffolds, and the GAG density was higher in the type I/III collagen constructs compared to the type II collagen constructs (Fig. 3a, b). Biochemical findings also indicated that constructs cultured at standard oxygen resulted in higher percentages of GAG compared to those at low oxygen (Fig. 3a,

b). It was apparent in the chondrocyte-seeded constructs that there was a higher GAG density in scaffolds with endostatin-expressing cells compared to the scaffolds with no lipoplex (Fig. 3b).

Including all data for the cell-seeded constructs, four-factor ANOVA demonstrated significant effects of 3D oxygen tension ($p < 0.0001$, power = 1), scaffold material ($p < 0.0001$, power = 1), cell type ($p < 0.0001$, power = 1), and plasmid load ($p = 0.0290$, power = 0.597) on the percentage of GAG. Analyzing data for the 21%–21% and the 5%–5% MSCs, three-factor ANOVA revealed significant effects of oxygen tension ($p < 0.0001$, power = 1) and scaffold material ($p < 0.0001$, power = 1), but not plasmid load ($p = 0.9278$, power = 0.051), on the percentage of GAG. For the data for the 21%–21% and the 21%–5% chondrocytes, three-factor ANOVA again demonstrated significant effects of oxygen tension ($p < 0.0001$, power = 1) and scaffold material ($p < 0.0001$, power = 1) on the percentage of GAG. Plasmid load was also found to have a significant effect ($p = 0.011$, power = 0.778).

At the end of the 20-day culture period, the number of MSCs and chondrocytes in the cell-seeded scaffolds ranged from about 0.5 to 2.75 million cells per scaffold (Fig. 3c, d), compared to the 4 million cells seeded. More cells were found in the scaffolds cultured at standard oxygen than in those cultured at low oxygen (Fig. 3c, d). A larger number of MSCs than chondrocytes were seen in the 21%–21% group at the termination of the culture period (Fig. 3c, d). Including all data for the cell-seeded constructs, four-factor ANOVA demonstrated significant effects of 3D oxygen tension ($p < 0.0001$, power = 1) and scaffold material ($p = 0.0042$, power = 0.866) on cell number, but failed to find a significant effect of plasmid load ($p = 0.1097$, power = 0.342) or cell type ($p = 0.8984$, power = 0.052). Including the data for the 21%–21% and the 5%–5% MSCs, three-factor ANOVA revealed a significant effect of oxygen tension ($p = 0.0001$, power = 1) on cell number but failed to find significant effect of scaffold material ($p = 0.055$, power = 0.485) or plasmid load ($p = 0.131$, power = 0.308). For the 21%–21% and the 21%–5% chondrocytes, three-factor ANOVA demonstrated significant effects of oxygen tension ($p < 0.0001$, power = 1) and scaffold material ($p = 0.011$, power = 0.775) on cell number and found no significant effect of plasmid load ($p = 0.845$, power = 0.054).

Histological and immunohistochemical evaluation of the constructs. Immunohistochemical analysis of the constructs cultured for 20 days revealed the presence of newly synthesized extracellular matrix staining strongly positive for sulfated GAG (Fig. 2f) and type II collagen (Fig. 2g), generally around the periphery of the constructs. Few areas of newly synthesized matrix were observed in the centers of the constructs. Cells were distributed throughout the constructs, and much of the original collagen scaffold could still be seen (Fig. 2f–h). Some of the cells in the GAG-rich and type-II-collagen-rich matrix appeared rounded in lacunae; many had a fibroblast-like appearance (without lacunae) (Fig. 2f). The fibrous appearance of the matrix was consistent with fibrocartilage and fibrous tissue. The safranin-O staining results of the 20-day samples correlated with the biochemical findings, in that increasing amounts of positive staining for sulfated GAG were seen in constructs with

FIG. 3. Biochemical analyses of MSC- and chondrocyte-seeded constructs cultured at standard and low oxygen ($n = 3$ for all samples except CI/21%-21% MSCs, where $n = 2$). (a) Percentage GAG content per volume compared to native articular cartilage in the MSC-seeded samples after 20 days of culture. (b) Percentage GAG content per volume compared to native articular cartilage in the chondrocyte-seeded samples after 20 days of culture. (c) Cell numbers in the MSC-seeded samples after 20 days of culture, estimated from the DNA content. (d) Cell numbers in the chondrocyte-seeded samples after 20 days of culture, estimated from the DNA content.

increasing percentages of GAG. The amount and distribution of positive staining for type II collagen correlated with that of the GAG staining. Masson's trichrome staining indicated the presence of collagen around the periphery of the constructs, in the same areas where positive staining for type II collagen was observed in the newly synthesized matrix (Fig. 2g, h). Many of the struts of the collagen scaffold stained red (Fig. 2h), likely due to a difference in tension of the collagen struts compared to that found in the collagen in the matrix.¹⁴

Discussion

In this study, we engineered endostatin-expressing cartilaginous constructs *in vitro* using a small amount of pEndo in lipoplex-supplemented scaffolds. An increase and peak in endostatin protein in the expended medium during the first week of culture was seen in each of the three experiments, followed by a decrease to low levels, suggesting that overexpression of endostatin could be sustained for at least sev-

eral days using the nonviral vector. Similar kinetics of endostatin expression were found in the three experiments despite differences in the GP2:pEndo ratio and number of seeded cells.

Of note is that the endostatin levels released by the constructs were comparable to physiological values found in human serum, ~ 30 ng/mL,¹⁵ and to levels shown to inhibit endothelial cell migration *in vitro*, reported to be 0.1–10 ng/mL.¹⁶ The smallest maximum endostatin level per collection period in this study was 4 ng/mL, and the smallest maximum total amount of endostatin released over the duration of the experiment was 16 ng, both observed in experiment 3. The largest observed maximum endostatin level per collection period in this study and maximum total amount of endostatin released over the duration of the experiment occurred in experiment 1, with values of 45 ng/mL and 95 ng, respectively. It is not yet clear why select groups repeated in the experiments under the same experimental conditions yielded different expression levels. Although the cells were from the same goat donors, unrecognized differences in the thawing and growth of the cells for the various experiments may have affected their response to the lipoplex. Another possible reason could be the incidental loss of activity of one or more of the reagents.

The average amount of endostatin produced per cell cannot be commented on because it is unclear how many cells were present at the time of synthesis. The percentage of cells that were transfected was not determined in this study and requires further work. One potential method for determining the percentage of cells that are actually transfected would be the use of a plasmid encoding for both endostatin and green fluorescent protein (GFP); however, this plasmid was not available. A prior experiment in our lab using a GFP plasmid alone in the same lipoplex system incorporated into collagen scaffolds imaged GFP expression in chondrocytes grown in the scaffolds, but did not quantify transfection efficiency.¹²

Another finding of this study in experiment 3 was that chondrocyte-seeded, lipoplex-supplemented constructs were observed to have higher percentages of GAG per volume compared to chondrocyte-seeded, non-lipoplex-supplemented controls, which achieved statistical significance. This suggests that endostatin may stimulate chondrocyte production of sulfated GAGs, and thus supports previous observations that *in vitro* exposure to recombinant human endostatin protein promoted the anabolic activity of articular chondrocytes.⁷ However, in our study the percentage increase ranged only from 2% to 32%. Our contemporaneous negative control for endostatin expression and chondrogenesis were the cells alone. A control scaffold incorporating a lipoplex containing an irrelevant DNA was not included in the study, because our own prior study⁴ demonstrated that increasing the lipid in the lipoplex had no significant effect on endostatin expression. There was no reason to expect that the lipid alone would induce endostatin expression or increase GAG production. Our own data and the absence of prior studies demonstrating that lipid alone would affect endostatin levels and GAG synthesis make it unlikely that the absence of a lipid control is a confounding factor in the interpretation of the effects of various factors on endostatin expression and chondrogenesis.

To our knowledge, no studies have yet reported on the effects of endostatin on MSCs. Of note, this study found that endostatin transfection using lipoplex-supplemented scaffolds did not appear to inhibit the chondrogenic potential of MSCs, in that MSC-seeded constructs were seen to have cells that expressed chondrocyte morphology and contained areas rich in type II collagen and sulfated GAGs at 3 weeks, and notable GAG percentages were measured.

Endostatin overexpression was affected by a number of variables. In experiment 1, plasmid load was found to affect endostatin levels, with higher amounts of plasmid resulting in higher amounts of endostatin protein. In experiment 3, MSC- and chondrocyte-seeded constructs were found to behave differently. MSC-seeded constructs were generally observed to have more endostatin in the expended medium in the initial week following transfection compared to chondrocytes. Oxygen tension was found to play an important role in experiment 3, significantly affecting endostatin expression. In this experiment, 5% oxygen resulted in lower endostatin levels compared to 21% oxygen. How oxygen tension affects endostatin production is unclear—some studies have found that low oxygen induced endostatin production,¹⁷ whereas other studies have observed that low oxygen downregulated endostatin.¹⁸ Similarly, the mechanism by which these variables are affecting endostatin overexpression, whether they are affecting gene transfection efficiency in general or whether they are specific to endostatin production, is not known. These issues remain to be addressed in future studies.

While the timing of endostatin release necessary for articular cartilage repair applications is not yet known, the ability to manipulate the endostatin expression profile, such as through the use of different carbodiimide crosslinking procedures for lipoplex retention in experiment 2, will be of value.

Chondrogenesis was observed in the engineered constructs cultured in CM at the 3-week time point and was found to be affected by a number of variables. In experiment 3, chondrocyte-seeded scaffolds demonstrated greater degrees of chondrogenesis than MSC-seeded scaffolds after the same amount of time in culture, likely due to MSCs requiring time to first differentiate down the chondrogenic lineage. The correlation between the GAG per cell cannot be commented on because it is unclear how many cells were present when the GAG was synthesized, and the amount of GAG that was lost to the medium cannot be accounted for.

A higher GAG percentage and more positive staining of newly synthesized matrix for type II collagen and GAG were seen when using type I/III collagen scaffolds compared to type II collagen in experiment 3. It is possible that the weight percentage of collagen used when fabricating the scaffolds (1% type II collagen vs. 0.5% type I/III collagen), and not only the collagen type, may account for the observed differences in GAG percentages.

Oxygen tension was also found to have a significant effect on chondrogenesis and cell number in experiment 3. Both MSC- and chondrocyte-seeded constructs cultured at 21% oxygen demonstrated more chondrogenesis than those cultured at 5%. Additionally, more cells were observed in the standard oxygen constructs after 20 days of culture than in the low oxygen constructs, even though the same number of cells was initially seeded onto each scaffold, suggesting that low oxygen may result in decreased cell proliferation or viability.

Given that 5% oxygen tension is closer to normoxia for cartilage *in vivo*, these findings could have implications for endostatin expression and chondrogenesis of cells transfected by the endostatin-lipoplex-supplemented scaffolds in an *in vivo* setting. However, it is important to note that the *in vivo* environment has many other soluble and mechanical factors that were not simulated in our *in vitro* setting. Here, only the effects of oxygen tension during 3D culture of the constructs were examined. Future work is needed to address the role of oxygen tension during 2D monolayer expansion of the cells.

Carbodiimide was used as a crosslinking agent in this study. Both the lipoplexes and the collagen protein contain amine and carboxyl groups in their molecular structure, and there exists the potential for formation of peptide bonds with the aid of carbodiimide crosslinking. Previous work in our lab¹² showed that scaffolds that were treated with carbodiimide after supplementing with plasmid did not exhibit passive bolus release of plasmid like scaffolds treated with carbodiimide before plasmid supplementation did, but work has not yet been performed to confirm covalent attachment of lipoplexes to collagen scaffolds.

As mentioned in other studies, the extent of lipoplex cytotoxicity is unclear¹⁹; however, no significant effect of lipoplex was seen on cell number after 3 weeks in culture, suggesting that there are no long-term negative effects of lipoplexes on cell viability.

In this study, we engineered endostatin-producing constructs using nonviral transfection of MSC- and chondrocyte-seeded, pEndo-lipoplex-supplemented collagen constructs and showed that cells retained their chondrogenic capacity. The endostatin expression profile and degree of chondrogenesis were altered through the manipulation of select variables, including oxygen tension, scaffold material, plasmid load, and method of lipoplex incorporation. The quantity and duration of endostatin protein necessary for cartilage repair is unknown, and more studies are needed in this area. On the basis of the current results, the optimal group of experimental conditions appears to be MSCs seeded in 0.5% type I/III collagen scaffolds, supplemented with 20 µg of pEndo half crosslinked to the scaffold, and cultured at 21% oxygen, balancing considerations of high levels of overexpression and sustained production over an extended period of time. Other potential strategies to increase the duration of sustained production include modifications to the nonviral vector. Given the associations of angiogenesis and pathological conditions in normally avascular cartilage, a pEndo-supplemented scaffold provides a promising off-the-shelf product that could be used to treat patients, possibly in conjunction with other repair options, to inhibit angiogenesis, improve healing of a focal defect, and prevent further cartilage degeneration. The ability to engineer endostatin-expressing cartilaginous constructs will be of value for future *in vitro* and *in vivo* work exercising regulatory control of angiogenesis in cartilage repair processes.

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Disclosure Statement

No competing financial interests exist.

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