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Tissue-Engineered Cartilage Constructs Using Composite Hyaluronic Acid/Collagen I Hydrogels and Designed Poly(Propylene Fumarate) Scaffolds

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ABSTRACT

Our approach to cartilage tissue-engineering scaffolds combines image-based design and solid free-form (SFF) fabrication to create load-bearing constructs with user-defined parameters. In this study, 3-dimensional scaffolds with cubic and ellipsoidal pore architecture were fabricated using poly(propylene fumarate) (PPF). To increase seeding efficiency and cellular retention, hydrogels were used to deliver cells into the scaffolds. The first objective of this study was to evaluate the concentrations of composite hyaluronic acid (HyA) and collagen I hydrogels best able to stimulate proteoglycan synthesis in porcine chondrocytes *in vitro* and *in vivo*. The second objective was to evaluate the differences in extracellular matrix production due to pore geometry and scaffold design. For the *in vitro* assessment, chondrocytes were encapsulated in collagen I hydrogels with varying concentrations of HyA. Hydrogels were cultured for 1 and 2 weeks, and then the sulfated glycosaminoglycan (sGAG) content was quantified using a dimethyl-methylene blue assay. The concentration of HyA best able to increase ECM synthesis was 5% HyA/collagen I, or 0.23 mg/mL HyA. The results from the *in vitro* experiment were used as culture parameters for the *in vivo* analysis. Composite 5% HyA/collagen I or collagen I–only hydrogels were used to seed chondrocytes into SFF-fabricated scaffolds made of PPF with designed cubic or ellipsoidal pore geometry. The scaffolds were implanted subcutaneously in immunocompromised mice for 4 weeks. Histomorphometric analyses of sections stained with Safranin O were used to quantify the amount of ECM deposited by cells in the scaffolds. Scaffolds seeded with 5% HyA/collagen hydrogels had significantly greater areas of positive Safranin O staining (approximately 60%, compared with 30% for scaffolds with collagen I hydrogels only), indicating that greater numbers of chondrocytes retained their metabolic activity in the ectopic environment. These scaffolds also had greater stain intensities (corresponding to greater amounts of sGAG in the ECM) than their counterparts seeded with collagen I hydrogels alone. Significant differences in matrix production were not found between the scaffold pore designs. Overall, these results indicate that a combination of composite HyA hydrogels and designed SFF scaffolds could provide a functional tissue-engineered construct for cartilage repair with enhanced tissue regeneration in a load-bearing scaffold.

INTRODUCTION

ARTICULAR CARTILAGE LESIONS have poor regenerative capacity. Tissue engineering provides new methods of combining cells, matrices, and biological factors, potentially facilitating the regeneration of cartilage tissue after injury. Hydrogels and porous scaffolds implanted at defect sites increase the retention of cells and provide a 3-dimensional matrix within which the cells can differentiate. Growth factors and other biologics can also be delivered

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using these matrices, to further improve the characteristics of the repair tissue. Although many studies have shown that scaffolds can support chondrogenesis, the regeneration of viable, fully functional cartilage capable of supporting loads is yet to be achieved.

Collagen is an ideal substrate for cartilage tissue engineering because it can be formed *in situ*, is easily manipulated, and can serve as a natural, biologic delivery vehicle for cells and growth factors. Collagen hydrogels have been explored in numerous applications, including regeneration of vocal chords[1] and repair of spinal cord conduits[2] and cartilage defects.[3,4] However, collagen I fibrils have been shown to destabilize the phenotype of chondrocytes, acquiring fibroblast-like morphologies and producing collagen I and collagen X instead of collagens II, IX, and XI.[5,6] Chondrocytes cultured in collagen I gels hence exhibit less proliferation and biosynthetic activity.[7,8]

The addition of hyaluronic acid (HyA) to collagen I hydrogels may help stabilize chondrocyte phenotype and increase proteoglycan synthesis, presenting a possible solution to mitigate the effect of collagen I fibrils. HyA acid is a ubiquitous proteoglycan found in many tissues of the body. HyA is required for maintenance of the pericellular matrix and lubrication of the joint surface and is partially responsible for the characteristic ability of cartilage to withstand compression due to its organization and retention of aggrecan and sulfated glycosaminoglycans (sGAGs) within the extracellular matrix. Chondrocytes bind to HyA through cell surface receptors, which initiate signals that modulate cell proliferation, migration, and differentiation.[9,10] The benefits of HyA in facilitating chondrogenesis have been shown in several studies. Bovine chondrocytes cultured in gelatin sponges with exogenous high-molecular-weight HyA increase cell retention and proteoglycan synthesis.[11] Chondrocytes embedded in collagen I hydrogels cultured with exogenous HyA increase cell proliferation and chondroitin sulfate synthesis at 0.1 and 1 mg/mL concentrations.[12] Furthermore, chondrocytes cultured in composite HyA/alginate beads also have higher sGAG deposition and greater synthesis of deoxyribonucleic acid (DNA) and hydroxyproline.[13]

Hydrogels, however, inherently have weak mechanical properties and are incapable of withstanding forces normally exerted at clinically relevant defect sites. Scaffolds used to provide structural support may also direct cell growth and tissue regeneration when parameters such as scaffold pore size, porosity, and surface area are taken into consideration. Image-based design[14] and solid free-form (SFF) fabrication methods enable the creation of custom scaffolds with user-defined characteristics that have complex internal architectures and external geometries that are capable of mimicking the irregular surfaces of a defect.[15] In this study, scaffolds with cubic or ellipsoidal pore architecture were used to compare differences in tissue growth due to changes in the ratio of surface area to volume. A large surface area encourages cell attachment and growth, whereas a large volume allows greater seeding density to facilitate tissue development.[16] The ellipsoid pores should encourage cell-substrate interactions and stimulate ECM production by increasing cell density in the scaffold pores.

For this study, porous poly(propylene fumarate) (PPF) scaffolds with the designed architectures described above were created using indirect SFF methods.[17] PPF is a biodegradable, biocompatible polymer that has been used previously for orthopedic applications.[18,19] PPF is an unsaturated linear polyester that can be cross-linked through its fumarate double bonds using N-vinyl pyrrolidone (NVP). The cross-linking reaction is propagated using free-radical polymerization using benzoyl peroxide and N, N dimethyl-*p*-toluidine. The result is an injectable polymer that is moldable to fill irregularly shaped defects.[20] When used in combination with SFF fabrication methods, 3-dimensional PPF matrices with defined pores and pore interconnections can be fabricated that are capable of supporting load. These scaffolds will provide a mechanically protective environment for the hydrogels and allow nutrient diffusion to support the developing tissue.

In this study, we evaluated the effects of HyA on chondrocyte behavior using a combination of composite HyA/collagen I hydrogels and designed porous PPF scaffolds. Our hypothesis was that addition of low concentrations of high-molecular-weight HyA to collagen I hydrogels would have a beneficial effect on matrix production and phenotype stability. In an *in vitro* phase of this study, we evaluated the metabolism and proteoglycan synthesis of chondrocytes embedded in composite HyA/collagen I hydrogels to determine HyA concentrations with the greatest benefit (Fig. 1). In a separate *in vivo* phase, composite hydrogels were used in combination with designed scaffolds and implanted in mice to evaluate the effects of the hydrogels and of scaffold pore geometry on cell function and ECM production. The cubic and ellipsoidal pore geometry was investigated to determine whether the differences in surface area:volume ratio and the permeability of the 2 scaffold designs would affect the metabolic activity of the cells.

MATERIALS AND METHODS

Cell isolation

Primary porcine chondrocytes were isolated from the metacarpophalangeal joints of domestic pigs. Cartilage slices were extracted aseptically within 4 h of slaughter. Chondrocytes were isolated from the tissue using a solution of 1 mg/mL collagenase II (Sigma, St. Louis, MO) in Dulbecco's modified Eagle medium (DMEM) containing 500 U/mL penicillin and 500 μg/mL streptomycin (P/S), 100 U/mL kanamycin, and 1:500 amphotericin B (Fungizone, Gibco, Carlsbad, CA). The tissue was digested for 6 h at 37°C with gentle agitation. The cell suspension was filtered through a nylon sieve (70-μm pore size) and centrifuged at 700×g for 8 min. The pelleted cells were resus-

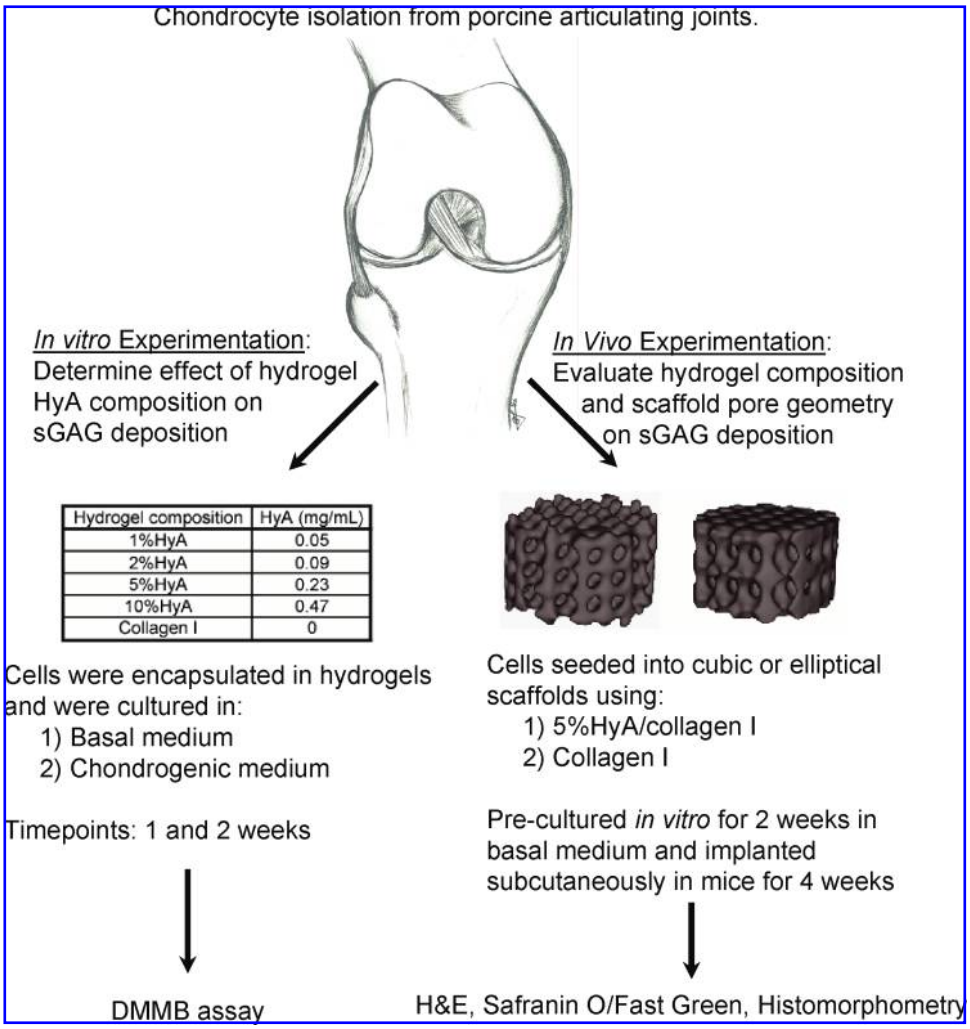

FIG. 1. Schematic of experimental design used for this study. Two research stages (*in vitro* and *in vivo* experimentation) were used to evaluate the effect of hyaluronic acid (HyA) on chondrocyte proteoglycan synthesis. *In vitro* experiments were designed to determine HyA compositions best able to stimulate sulfated glycosaminoglycan (sGAG) deposition in basal or chondrogenic medium. The results of the *in vitro* experimentation were used as culture parameters for *in vivo* experiments. The hydrogels were evaluated in solid free-form designed scaffolds with cubic- or ellipsoidal-pored scaffolds to determine whether pore geometry had an effect on sGAG deposition. DMMB, dimethyl-methylene blue; H&E, hematoxylin and eosin.

pended in basal culture medium (DMEM, 10% fetal bovine serum (FBS), 1% P/S, Gibco) supplemented with 50 μg/mL 2-phospho-L-ascorbic acid (Sigma) and plated onto tissue culture flasks. Cells were allowed to adhere for 24 h and were then trypsinized and used for experimentation.

In vitro *experimentation*

In vitro *cell culture*. Cells were encapsulated in collagen I or composite HyA/collagen I gels and suspended in basal or chondrogenic media to determine the combination most favorable for chondrogenesis. After trypsinization, cells were resuspended at 3.5×10^6 cells/mL in 2 mL of collagen I (stock concentration: 4.66 mg/mL; BD Bioscience Discovery Labs, San Jose, CA) with 200 μL of culture medium. The pH of the collagen/cell suspension was increased with the addition of 20 μL of 0.5 M sodium hydroxide with 220 mg/mL sodium bicarbonate. The mixture was pipetted in 70-μL aliquots into 96-well tissue culture plates. The resulting collagen I hydrogels were 2.2 mm in height and 6.35 mm in diameter. To create the composite HyA/collagen I gels, HyA (stock concentration: 2.7 mg/mL in 0.8 M sodium chloride (NaCl), molecular weight 3×10^6 Da; Hyalogic LLC, Edwardsville, KS) was added to the collagen I cell suspension to produce 1%, 2%, 5%, and 10% (w/w) HyA/collagen I gels. The HyA concentrations for the corresponding weight percentage gels were 0.05, 0.09, 0.23, and 0.47 mg/mL, respectively. The pH was raised to physiologic levels using sodium hydroxide as described above. (The amount of collagen I used to resuspend the cells in composite hydrogels was adjusted so that the final volumes of the HyA/collagen cell suspension

was similar to collagen I only hydrogels.) All hydrogels were placed in an incubator at 37°C and allowed to solidify for 30 min. Basal medium or chondrogenic medium (basal medium supplemented with 50 μg/mL 2-phospho-L-ascorbic acid, 0.4 mM proline (Sigma), 5 μg/mL insulin (Gibco), and 0.1 mM non-essential amino acids (Gibco)) was then added to the tissue culture wells. Chondrocytes were cultured for up to 2 weeks under gentle agitation on an orbital shaker and with the media replaced every other day. Hydrogels was assessed at 1 week and 2 weeks for GAG content, cell viability, and cell number. Three replicates of each sample were used for each analysis.

Dimethyl-methylene blue assay for GAG quantification. Cultured hydrogels were rinsed with Hank's Balanced Salt Solution (HBSS, Gibco) and then digested with 75 μg/mL proteinase K in 100 mM sodium acetate buffer at 65°C for 2 h. After the gels were completely digested, the temperature was increased to 95°C for 10 min to inactivate the enzyme. The digested samples were centrifuged for 10 min at 14,000 rpm and the supernatant kept. Dimethyl-methylene blue (DMMB) assay was conducted as described by Farndale.[21] Briefly, 16 mg of DMMB dye was dissolved in 1 L of water with 3.04 g glycine, 2.37 g NaCl, and 95 mL 0.1 M HCl. Five μL of supernatant from each sample was mixed with 200 μL of DMMB solution, and 525 nm absorbance was read in a spectrophotometer. Chondroitin sulphate from shark cartilage (Sigma) was used to create a calibration curve to correlate the measured absorbance to known amounts of sGAG. Data from the DMMB analyses were assessed using the Student t-test with $\alpha = 0.05$. The data of the groups were compared using analysis of variance (ANOVA) using hydrogel composition and culture medium as the factors for the Tukey *post hoc* test.

Cell viability and count. Cell viability and count were determined using the Cell Titer 96 AQ_{ueous} One non-radioactive cell proliferation assay (Promega, Madison, WI). Cell viability was determined by reading the 490-nm absorbance of a formazan product bioreduced from a tetrazolium compound. Cell count was also determined using this assay via a linear cell standard correlating the amount of formazan product formed in a given amount of time and number of cells present. A hemacytometer and Coulter counter was used to verify the cell count from digested hydrogels and validate the cell standard. The amount of DNA present was calculated from the cell number, assuming 7.7 pg of DNA per cell.

In vivo *experimentation*

Scaffold design and fabrication. Image-based design techniques[14] were used to create cylindrical scaffolds with defined internal pore architectures. The external dimensions of all designed scaffolds were 3 mm in height and 5 mm in diameter. Unit cells for cubic and ellipsoidal pore designs were [0.7 mm, 0.7 mm, 0.7 mm] and [0.7 mm, 0.7 mm, 0.9 mm], respectively (Fig. 2A1, B1). The strut size of the cubic scaffolds was designed to be 350 μm thick, which corresponds to pore space dimensions of 350 μm in length, width, and height. The designed porosity of the scaffold was 50%. Pore dimensions for ellipsoidal scaffolds were designed to be 700 μm in diameter and 900 μm in height. The interconnections between pores for the ellipsoidal scaffolds were measured to be 260 μm for the top and bottom and 300 μm for the sides of the ellipsoid. The designed porosity for the ellipsoid scaffold was 52%. The unit cells were then used to create the interconnected pore architecture of the scaffolds shown in Figure 2 (A2, B2).

Wax molds were printed from design files using a 3-dimensional rapid prototyping system (Patternmaster, Solidscape, Inc., Merrimack, NH). The wax molds were cast into PPF using indirect SFF fabrication techniques described previously.[22] Briefly, 1 g of PPF (molecular weight 1500 Da) was dissolved in 1 mL of NVP. After homogenization, 0.02 g of benzoyl peroxide was added to the PPF/NVP mixture. Then, 2 μL of N, N-dimethyl-*p*-toluidine was added to induce cross-linking immediately before casting.[23] A custom polytetrafluoroethylene (PTFE) mold (diameter = 6 mm) was used to force the polymer solution through the pore spaces of the wax molds. The PTFE mold was placed in a nitrogen chamber at room temperature for 24 h until the polymer cured. The wax was then removed with acetone, and the remaining PPF scaffolds were rinsed in distilled deionized water and sterilized in 70% ethanol.

Scaffold design verification and permeability calculations. Scaffolds cast in PPF from the wax molds were scanned using micro-computed tomography (MS-130, GE Medical Systems, Toronto, Canada) to determine the accuracy of the fabrication process. The scaffolds were scanned in air at a resolution of 16 μm.

A finite difference technique was used to calculate the effective permeability tensor, K, defined as:

$$K_{ij} = \frac{1}{\mu}\langle v_i^j \rangle \qquad (m^3/N\cdot \sec)$$

where μ is the fluid viscosity and ν is the velocity field. Using standard homogenization theory, the effective permeability is derived from combining Darcy's Law (describing fluid flow at macroscopic level) and the Navier-Stokes equation, which assumes that the scaffold is a homogeneous medium with a defined characteristic resistance to the fluid surrounding it.[24]

In vitro *pre-culture.* In preparation for cell seeding, the PPF scaffolds were rinsed twice with sterile HBSS and once with culture medium. The PPF scaffolds were then placed in a custom-made PTFE mold (diameter = 5.1 mm). Cells were seeded into the scaffold pores using a collagen I or 5% HyA/collagen I hydrogel similar to those described

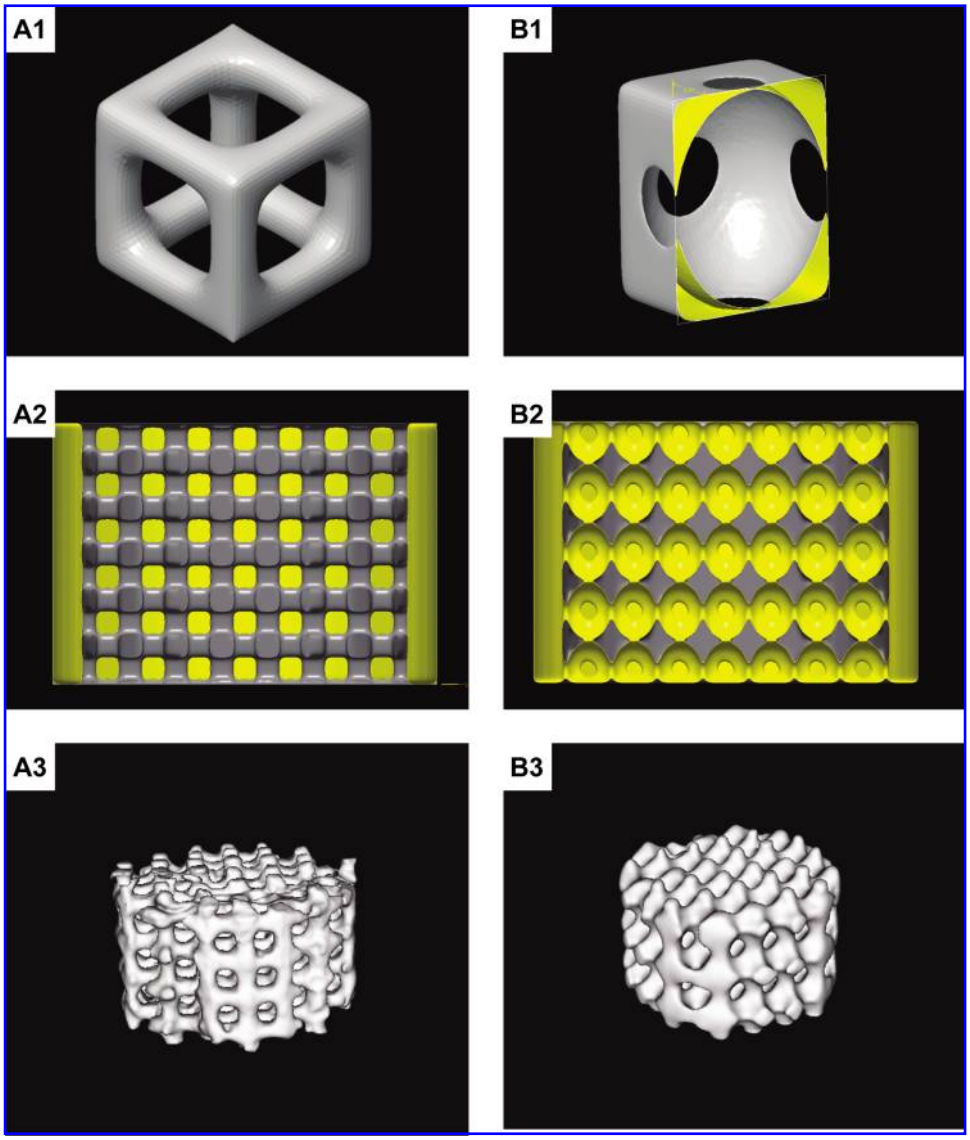

FIG. 2. Images of scaffolds designed with cubic (**A**) or ellipsoidal (**B**) interconnected pore architecture. The external scaffold dimensions are 5 mm in diameter and 3 mm in height. Image-based design files showing the unit cell used for each design. The cubic unit cell (A1) is [0.7, 0.7, 0.7 mm], with designed pore space dimensions: 350×350×350 mm. The ellipsoidal unit cell (B1) is [0.7, 0.7, 0.9 mm], with a designed diameter of 700 μm and a height of 900 μm. Interconnections between ellipsoids are between 260 (top and bottom) and 300 μm (sides). (A2, B2) View of the xz plane of the designed scaffolds. (A3, B3) Reconstructed micro-computed tomography scans of fabricated poly(propylene fumarate) scaffolds. Color images available online at www.liebertpub.com/ten.

above. For these hydrogels, 40×10^6 cells/mL were resuspended in a solution containing ~3.8 mg/mL collagen I or 5% (w/w) HyA/collagen I after trypsinization. The pH of the solution was increased, and the cell/hydrogel suspension was pipetted into the scaffold pores (~60 μL) immediately after sodium hydroxide addition. The gels were allowed to solidify within the scaffolds in a humidified chamber at 37°C for 30 min. The PPF scaffolds were then removed from the PTFE mold and placed in a 24-well tissue culture plate. Basal culture medium (DMEM, 10% FBS, 1% P/S) was added to the wells, and then the plate was placed in the incubator and gently agitated on an orbital shaker. The chondrocyte-seeded scaffolds were pre-cultured for 2 weeks, with the culture medium changed every other day during this period.

In vivo *implantation.* After pre-culture, 16 PPF scaffolds (4 of each combination: cubic or ellipsoidal pore architecture seeded with collagen I or 5% HyA/collagen I) were implanted subcutaneously in 5- to 8-week-old immunocompromised mice (N:NIH-bg-nu-xid; Charles River, Wilmington, MA). The animals were anesthetized with intramuscular injections of ketamine (50 μg/g) and xylazine (5 μg/g) in saline. Four dorsal subcutaneous pockets were

created by blunt dissection, and 1 PPF scaffold from each experimental group was placed in each pouch and in a different pouch location in each mouse. The implantation sites were closed with surgical wound clips. The animals were housed in groups for 4 weeks with free access to food and water.

Evaluation of implants. The mice were killed after 4 weeks. The scaffolds were harvested and fixed in Z-FIX (Anatech, Battle Creek, MI) overnight. The scaffolds were then dehydrated and processed for histology. The specimens were embedded in paraffin and sectioned at 7 μm. Sections were affixed to slides and stained with hematoxylin and eosin (H&E) or Safranin O counterstained with fast green FCF. Briefly, hydrated slides were immersed for 30 s in Harris hematoxylin then cleared in running tap water, followed by 3 min in 0.02% fast green FCF, 10 s in 1% acetic acid, and 6 min in 0.1% Safranin O. The slides were then dehydrated and mounted with Permount (Sigma).

Immunohistochemistry was used to detect collagen I and collagen II. Paraffin sections were deparaffinized and rehydrated. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide v/v in methanol. The slides were rinsed once in distilled water and twice in 0.5 M Tris, 0.15 M NaCl, pH 7.6 (Tris-buffered saline (TBS)) buffer for 5 min. The slides were then placed in 50 mU/mL sheep hyaluronidase (Sigma) with 1% bovine serum albumin (BSA, Sigma) solution for 30 min at 37°C to retrieve the antigen. The slides were rinsed twice in TBS buffer for 5 min, and then 10% (v/v) normal goat serum was used to block non-specific background staining (1 h at room temperature). All incubations were performed using a humidified chamber. The goat serum was gently shaken off the slides, and the primary antibody, polyclonal rabbit antisera raised against human type I collagen or type II collagen (US Biological, Swampscott, MA) diluted in TBS with 1% BSA, was placed on the sections for 1 h at room temperature. The slides were rinsed 3 times in TBS for 5 min to wash away excess primary antibodies. The primary antibody was detected using a biotinylated-goat anti-rabbit secondary antibody and Vectastain ABC reagent according to the manufacturer's instructions (Vectastain ABC kit for rabbit immunoglobulin G, Vector Laboratories, Burlingame, CA). Peroxidase activity was visualized using diaminobenzidine (DAB substrate kit, Vector Laboratories) for 5 to 7 min. The reaction was stopped by rinsing the sections with water. The sections were counterstained using 0.02% fast green FCF, dehydrated, and mounted using Permount. Paraffin slides of a mouse joint were used as positive controls and to determine the optimal concentrations for the primary antibody. For negative controls, the primary antibodies were not added.

Histomorphometry and image processing. Histological sections taken from 2 separate planes (100- to 500-μm difference) of each scaffold were used for analyses. Low-magnification (2× objective) images of whole cross-sections of tissue samples were acquired using a CCD camera (RT Color 2.2.1, Diagnostic Instruments, Sterling Heights, MI) mounted on an inverted microscope (E600, Eclipse, Nikon, Melville, NY). RGB images were acquired using Spot, an image-acquisition program (Diagnostic Instruments, Sterling Heights, MI). The illumination of each picture was controlled so that the exposure for each image was the same; thus the brightness, contrast, and saturation of the images were captured using the same parameters.

Image processing was performed on the RGB images in Matlab (The Mathworks, Natick, MA) to determine the ratio of positive Safranin O staining to the available pore space and the average stain intensity of the section. The prior calculation was correlated to the number of chondrocytic cells that have not dedifferentiated, whereas the latter was correlated to the amount of sGAG present in the ECM.[25,26] Matlab code was written to convert the RGB image (Fig. 3A) to hue saturation value (HSV) and threshold out the scaffold and background so that only the areas of positive Safranin O staining remained (Fig. 3B). The pixel area was calculated for these extracted areas to determine the area of positive Safranin O staining for the section. The background was subtracted from the picture and was thresholded again to remove the scaffold, but this time, pore space that did not stain for Safranin O was selected (Fig. 3C). The 2 extracted areas were combined to calculate the total pore space that was available for that particular section (Fig. 3D). Using these 2 areas, the percentage of positive Safranin O staining for the section is calculated. The second set of histomorphometric data assessed was the overall intensity of the stain. The extracted areas of positive Safranin O staining were used for this calculation. The intensity (I) was calculated using the equation $I = (R + G + B)/3$.[27] A value between 0 and 1 corresponding to the overall density of stain was assigned for each pixel. The image was divided into 50×50 pixel blocks, and the average intensity of each block was calculated (Fig. 3E). The values from each 50×50 pixel block were used to determine the average intensity of the entire section. The histomorphometric data was statistically analyzed using ANOVA to determine whether hydrogels containing HyA/collagen I promoted more chondrogenesis than hydrogels with collagen I alone. All statistical tests were performed using SPSS (SPSS Inc., Chicago, IL).

RESULTS AND CONCLUSIONS

In vitro *comparison of hydrogels*

After up to 2 weeks in culture with basal or chondrogenic medium, 97% to 99% of all cells in collagen I-only hydrogels and HyA/collagen I composite hydrogels remained viable. Additionally, the number of viable cells after 2 weeks in culture was greater than after 1 week in culture. The number of cells increased with increasing concentra-

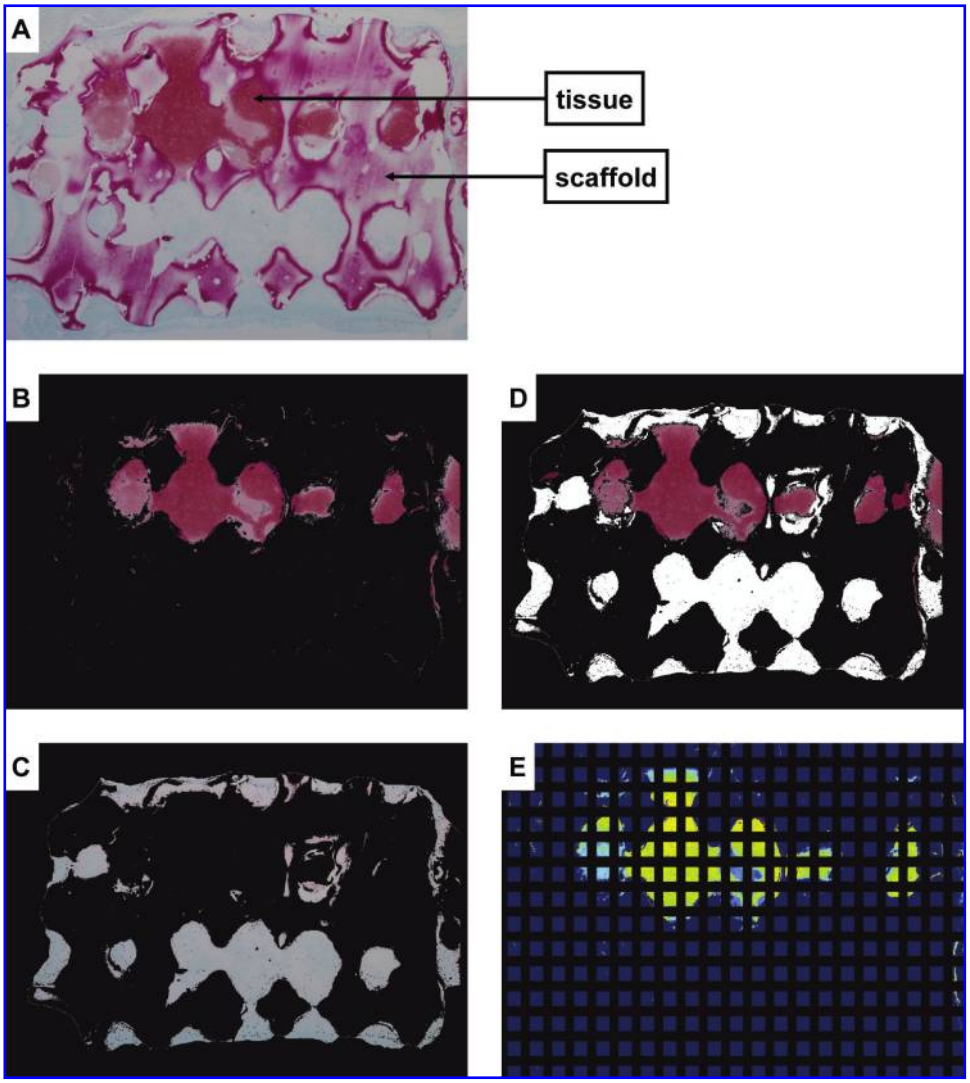

FIG. 3. Example of the histomorphometrical analysis used to quantify the amount of Safranin O staining. (**A**) RGB image acquired from microscope with 2× objective. (**B**) Areas of positive Safranin O staining extracted from RGB image. (**C**) Remaining areas of non-positive staining extracted from RGB image. (**D**) Combined image of positive (**B**) and non-positive staining (**C**) representing the total scaffold pore space. The ratio of positive Safranin O to total pore space is expressed as a percentage. For this particular specimen, the ratio was 36%. (**E**) Calculation of stain intensity (I) using the equation, $I = (R + G + B)/3$. The average intensity of each block is correlated to the amount of sulfated glycosaminoglycan present in the block. Color images available online at www.liebertpub.com/ten.

tions of HyA and was also greater in hydrogels cultured in chondrogenic medium than in basal medium. These results verify that all combinations of medium and hydrogel studied are capable of supporting chondrocyte activity.

The addition of HyA to collagen I hydrogels generally resulted in greater sGAG deposition than with hydrogels with collagen I alone (controls), irrespective of the culture medium or the culture duration (Fig. 4A). After 1 week in basal culture medium, 1%, 2%, and 5% HyA/collagen hydrogels had significantly greater ($p < 0.05$) sGAG/DNA ratios than collagen I-only hydrogels; a 10% HyA/collagen hydrogel had significantly less sGAG/DNA than the control. Similarly, after 1 week in chondrogenic culture medium, 2%, 5%, and 10% HyA/collagen hydrogels had greater sGAG/DNA ratios than collagen I-only hydrogels (only the 5% result was statistically significant); a 1% composite hydrogel had slightly, but not significantly, less sGAG/DNA than the control. Composite hydrogels cultured in chondrogenic medium had higher sGAG/DNA on average than those cultured in basal medium although only significantly for the 10% hydrogel. Collagen I-only hydrogels also had significantly higher sGAG/DNA in chondrogenic medium.

After 2 weeks in culture, all composite hydrogels displayed greater sGAG deposition than controls (Fig. 4B). In the basal medium, 1%, 2%, 5%, and 10% HyA/collagen hydrogels had significantly greater ($p < 0.05$) sGAG/DNA ratios than collagen I-only hydrogels. In the chondrogenic culture, only the 5% HyA/collagen hydrogels had

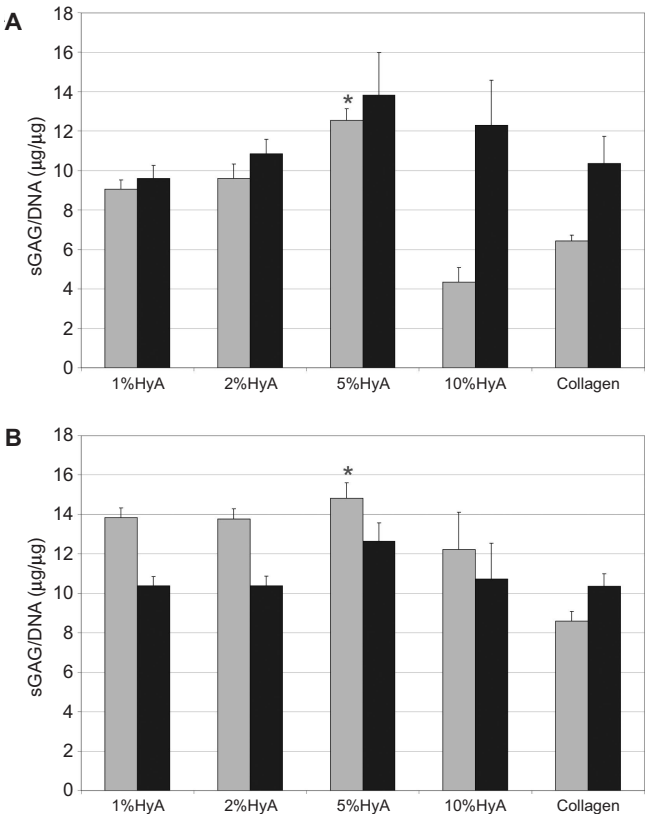

FIG. 4. Results from dimethyl-methylene blue analysis of composite hyaluronic acid (HyA)/collagen and collagen I-only hydrogels cultured in basal medium (gray bars) and chondrogenic (black bars) medium. One-week data (**A**) and 2-week data (**B**) are presented. Statistical analyses comparing groups showed significant differences (indicated with *) between 5% HyA hydrogels cultured in basal medium and all other conditions ($p < 0.05$). The sulfated glycosaminoglycan/deoxyribonucleic acid (sGAG/DNA) ratios of 5% HyA hydrogels cultured in chondrogenic medium were greater than all other conditions, but only significantly greater than 1% and 2% HyA and control hydrogels.

significantly greater sGAG/DNA ratios than collagen I-only hydrogels. In contrast to the 1-week results, composite hydrogels cultured for 2 weeks in chondrogenic medium had significantly lower sGAG/DNA on average than those cultured in basal medium. However, sGAG/DNA for collagen I-only hydrogels remained significantly higher in the chondrogenic medium. Comparison of the 1-week and 2-week results showed that sGAG/DNA increased significantly for all hydrogels (composite and controls) cultured in basal medium and conversely decreased, although not significantly, for hydrogels in the chondrogenic medium.

Of all combinations of hydrogel and medium, the highest amount of matrix formation, as quantified according to the amount of sGAG normalized by the amount of DNA, was obtained with a 5% composite hydrogel cultured in basal medium. This combination was thus chosen to seed chondrocytes into PPF scaffolds that subsequently were implanted into mice in *in vivo* experiments.

Fabricated scaffold properties

The micro-computed tomography scans of representative fabricated PPF scaffolds are shown in Figure 2 (A3, B3). The measured features of the fabricated scaffolds were within 5% of the designed specifications. The porosities of the fabricated scaffolds were 50% and 49% for the cubic and ellipsoidal scaffolds, respectively. The effective permeability tensor, K, for the [x-axis, y-axis, z-axis] was calculated for each scaffold design. For the cubic scaffold, the calculated K was [2.16×10^{-7}, 2.16×10^{-7}, 2.16×10^{-7} m^3/N*sec]. For the ellipsoid scaffold, the effective permeability was [0.36×10^{-7}, 0.36×10^{-7}, 0.29×10^{-7} m^3/N*sec]. The surface area:volume ratios were also calculated for each unit cell using Solidview software (Solid Concepts, Valencia, CA). The cubic pore had a surface area of 2.7 mm^2 and a volume of 0.12 mm^3. The surface area:volume ratio was therefore 23 mm^{-1}. For the ellipsoid unit cell, the calculated values were 4.4 mm^2 for the surface area and 0.17 mm^3 for the volume. This corresponds to a surface area:volume ratio of 26 mm^{-1}. For reference, the analogous spheroid unit cell with [0.7, 0.7, 0.7 mm] dimensions would have a surface area:volume ratio of 28 mm^{-1}.

In vivo *comparison of hydrogels*

The PPF scaffolds were seeded with chondrocytes using 5% HyA/collagen or collagen I hydrogels and cultured for 2 weeks *in vitro*. All scaffolds were implanted, and the mice survived surgery without complication. All mice lived 4 weeks without signs of abnormality. All scaffolds were extracted, found intact, and processed for histological analysis.

Cell phenotype verification. Microscopic examination of the tissue/scaffold sections showed widespread positive Safranin O staining of the pore spaces, confirming the presence of sGAG and viable chondrocytes. Chondrocytes filled the pore spaces of the scaffolds and were surrounded by dense ECM, which stained positive with Safranin O. The general morphology of the cells were round and chondrocytic in appearance, with established lacunae, even at the center of the scaffolds (Fig. 5A4, B4).

Within these same sections, some areas counterstained with fast green, indicating the presence of non-cartilaginous matrix. Spindle-shaped fibroblast-like cells and endothelial cells were found in these areas, which were primarily at the edges of the scaffolds, and in scaffold pores where cells were embedded in collagen I. Positive fast green staining was more prevalent in scaffolds containing collagen I-only hydrogels and less so in scaffolds containing HyA hydrogels.

Immunohistochemistry verified the presence of collagen II and collagen I in the ECM. The prior confirms the presence of chondrocytes and the latter the presence of fibroblasts and dedifferentiated chondrocytes. Areas of detected collagen II correlated well with the areas of positive Safranin O staining, confirming the presence of cartilage-like

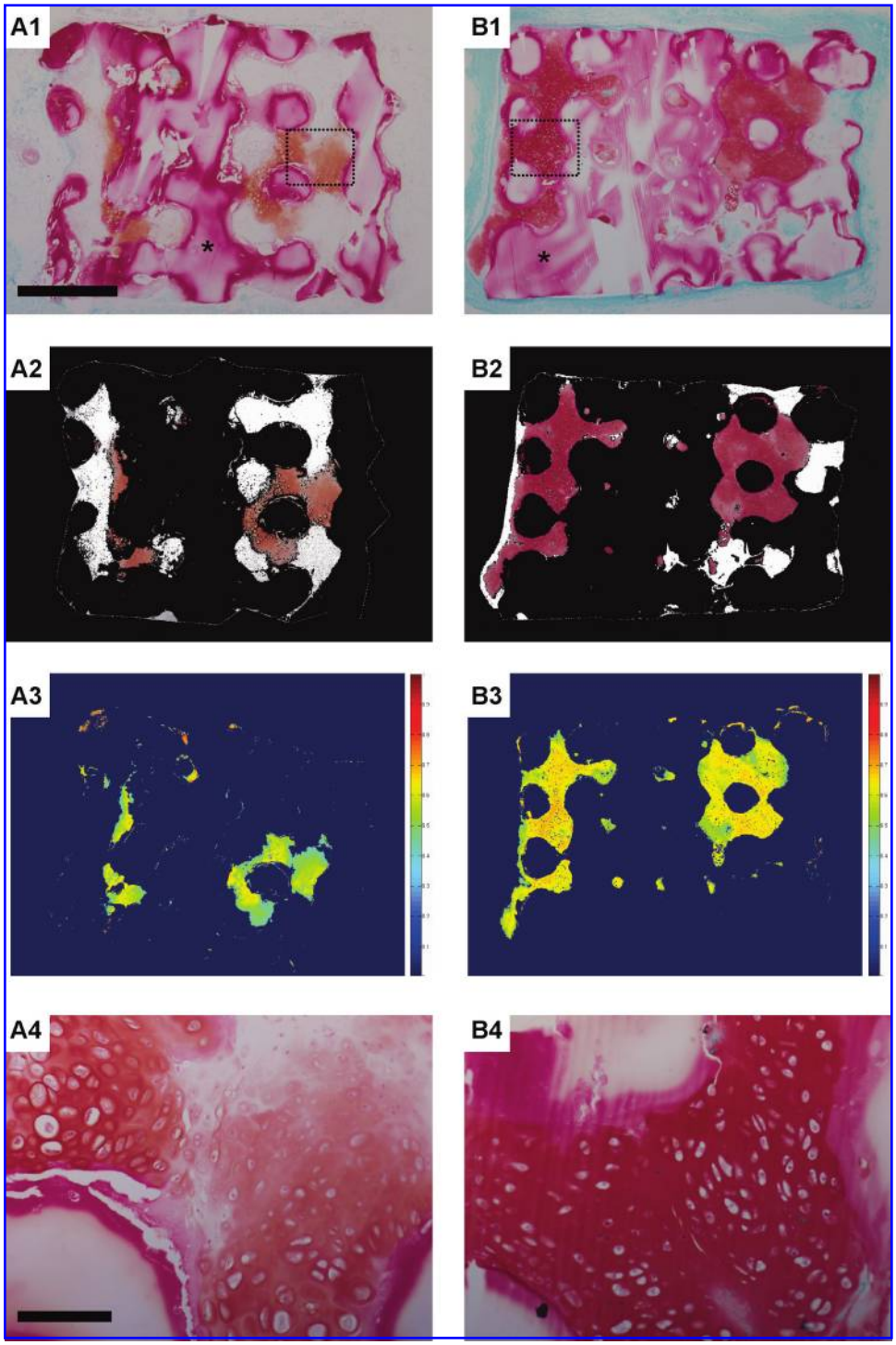

FIG. 5. Representative micrographs of scaffolds seeded with collagen (**A**) and 5% hyaluronic acid (HyA)/collagen (**B**) hydrogels. Sections are stained with Safranin O/fast green FCF. Residual poly(propylene fumarate)is visible in the sections (stars) and stained dark pink because the negatively charged polymer attracts the cationic Safranin O dye. The bar represents 1 mm for images A1 and B1. Results from histomorphometric analyses performed on sections A1 and B1 are shown in A2, A3 and B2, B3, respectively. A2 is a representative section from scaffolds with collagen I hydrogel showing approximately 30% positive Safranin O staining, and B2 is a representative section from scaffolds with 5% HyA/collagen hydrogel that has approximately 60% positive Safranin O staining. The calculated intensities of the Safranin O staining for the sections are shown in A3 and B3. The average intensity or fixed charge density of the sections is 14 and 25 per mm^2 for A3 and B3, respectively. Higher magnifications of the areas within the dashed boxes show round chondrocytic cells surrounded by positively stained extracellular matrix. The bar represents 125 μm for A4 and B4. Color images available online at www.liebertpub.com/ten.

matrix (Fig. 6). Collagen I was detected in areas of non-cartilaginous connective tissue and in some regions of weak Safranin O staining, such as the edges of the scaffolds and in pores with fibroblastic cells or where vascularization was observed (Fig. 7).

Histomorphometric analysis. Positive staining was found in all specimens, but more-robust and -intense staining was seen in scaffolds with composite 5% HyA/collagen hydrogels than with collagen I-only hydrogels (Fig. 5). Histomorphometrical analysis of the Safranin O stained slides confirmed these observations (Fig. 8A). The percentage of pore area stained positive with Safranin O for scaffolds seeded with composite 5% HyA hydrogels was $63 \pm 6\%$ for cubic geometries ($n = 4$) and $59 \pm 2\%$ ($n = 4$) for ellipsoidal geometries. The percentage of pore area stained positive with Safranin O for scaffolds seeded with collagen I-only hydrogels was $29 \pm 4\%$ for cubic-pore geometries ($n = 4$) and $32 \pm 5\%$ ($n = 4$) for ellipsoidal-pore geometries. Statistical analysis showed that the chondrocytes cultured in composite 5% HyA/collagen I hydrogels had significantly greater areas of positive Safranin O staining. These results indicate that more chondrocytes are present in the HyA hydrogel than the collagen I hydrogel and lead to the conclusion that HyA promotes proteoglycan synthesis and has a chondroprotective effect on chondrocytes, increasing phenotype stability at an ectopic site.

The average intensity of the Safranin O staining was also higher in the HyA scaffolds than the collagen I scaffolds (Fig. 8B). The average intensities per mm^2 of pore space for scaffolds with 5% HyA hydrogels were 25 ± 2 for cubic-pore geometries ($n = 4$) and 23 ± 3 ($n = 4$) for ellipsoidal-pore geometries. The average intensities per mm^2 for the scaffolds with collagen I hydrogels alone were 14 ± 3 for cubic-pore geometries ($n = 4$) and 14 ± 2 ($n = 4$) for ellipsoidal-pore geometries. The intensity of the Safranin O stain correlates directly to the amount of sGAG present in the matrix.[25] The results showed significantly greater amounts of sGAG in the ECM of the scaffolds with 5% HyA hydrogels.

Both scaffold pore designs were capable of supporting cartilage tissue development, although a statistical difference was not found between designed cubic and ellipsoidal scaffolds in the area of positive Safranin O staining or the intensity of the stain.

DISCUSSION

The aim of this study was to evaluate the effects of composite HyA/collagen I hydrogels on chondrocytes *in vitro* and *in vivo*. Most clinical applications of HyA are administered as interarticular injections, with which the residence time of the HyA is limited to days.[28] The incorporation of

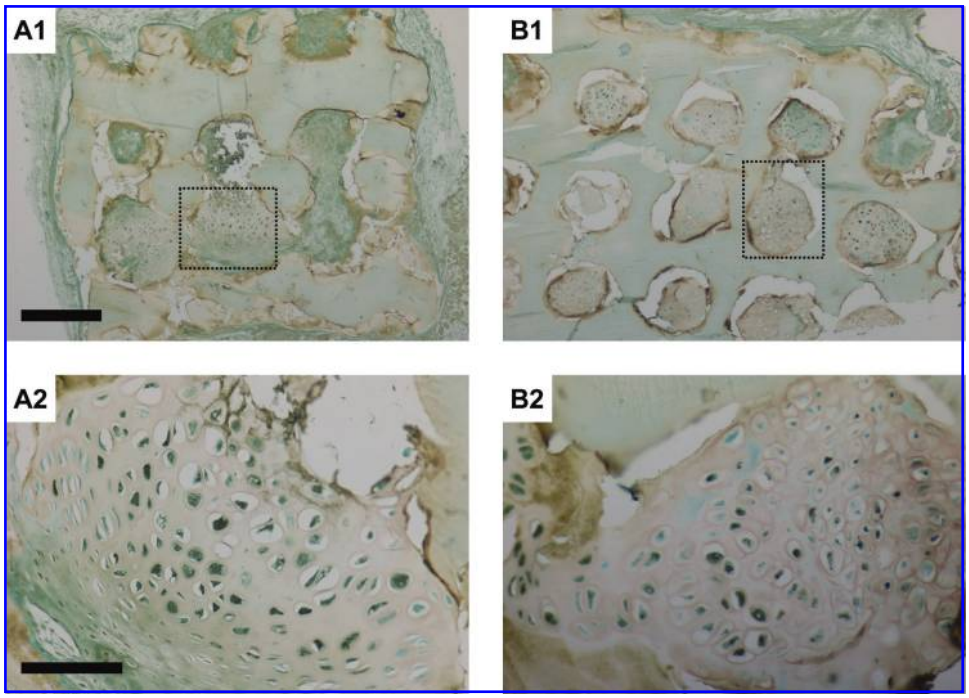

FIG. 6. Representative micrographs of immunohistochemistry detecting collagen II of scaffolds seeded with collagen I (**A**) and 5% hyaluronic acid (HyA)/collagen I (**B**) hydrogels. Positive staining for collagen II was found in all scaffolds, but greater areas of positive staining were seen in scaffolds seeded with 5% HyA/collagen I hydrogels. The areas of detected collagen II correlated well with areas of positive Safranin O staining. The bar represents 0.5 mm for images A1 and B1. Higher magnification of areas within dashed boxes shows chondrocytic cellular morphology. The bar represents 125 μm for images A2 and B2. Color images available online at www.liebertpub.com/ten.

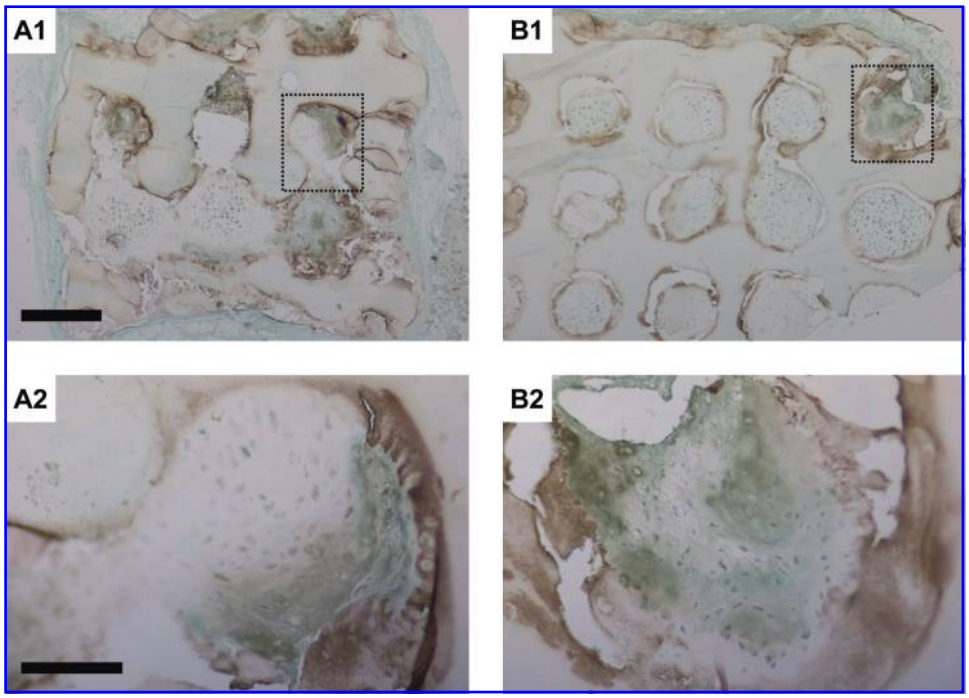

FIG. 7. Representative micrographs of immunohistochemistry detecting collagen I in scaffolds seeded with collagen I (**A**) and 5% hyaluronic acid (HyA)/collagen I (**B**) hydrogels. Positive staining for collagen I was found in some pore spaces, mainly at the edges of scaffolds, and was more prominent in scaffolds seeded with collagen I alone. The bar represents 0.5 mm for images A1 and B1. Higher magnifications of areas within dashed boxes are shown in A2 and B2. The bar represents 125 μm for A2 and B2. Color images available online at www.liebertpub.com/ten.

HyA into collagen I hydrogels may increase the lifetime *in vivo* and extend the timeframe of its chondroprotective properties.

At low concentrations, HyA modulates cell proliferation and matrix synthesis. Our results are consistent with previous studies, in which increases in sGAG production and cell proliferation were seen with the addition of HyA concentrations between 0.1 and 0.5 mg/mL.[11,29] At high concentrations, individual HyA domains overlap and may form entanglements through self-association and steric interactions.[30,31] These steric interactions and hydrophobic patches may deter proteins and other large molecules with large hydrodynamic sizes from diffusing through the composite HyA hydrogels. However, our results show that, at certain concentrations of HyA (5% or 0.23 mg/mL), it may be possible to overcome this effect. At a favorable concentration of HyA, a larger number of cells is able to bind to HyA that is in the environment to promote cell movement, proliferation, aggregation, and degradation of HyA and mediate the formation of pericellular matrices.[32] Beyond this concentration, the aggregation effect is inhibited because of the saturation of cell receptors for HyA.[33]

The culture medium is also a contributing factor in cellular metabolism and matrix synthesis. Our results show that the chondrogenic medium significantly increased sGAG/DNA at both timepoints for hydrogels containing collagen I and the 10% HyA hydrogel at 1 week, although mainly small differences were seen between the other composite HyA hydrogels cultured in basal and chondrogenic medium. This difference may also be attributed to the steric inhibitions and entanglements of the HyA molecules preventing the proteins in the chondrogenic medium from diffusing into the hydrogels. This indicates that the interactions of HyA chains may be a candidate for influencing nutrient diffusion. Furthermore, contrary to our expectations, a proportionate increase in sGAG production did not accompany the increase in cell proliferation seen with cells cultured in chondrogenic medium. This may explain the decrease of the sGAG/DNA ratio from week 1 to week 2, although, further analysis of the gene expression and assessment of the collagen production of these cells may help clarify these results.

Overall, our results show that the addition of HyA to collagen I hydrogels creates an environment that may be more conducive for chondrocyte proliferation and greater ECM production. Chondrocytes are able to bind to HyA through surface receptors such as CD44 and receptor for hyaluronic acid mediated motility.[34,35] The binding of chondrocytes to HyA may not only increase cellular retention within the hydrogels, but also reduce morphological changes due to interactions with collagen I fibrils.[5] The presence of HyA may also increase the number of attachment sites for chondroitin sulphate, keratan sulphate, and other sGAGs. This increase in sGAG retention within the hydrogel can be seen with the increase of sGAG measured

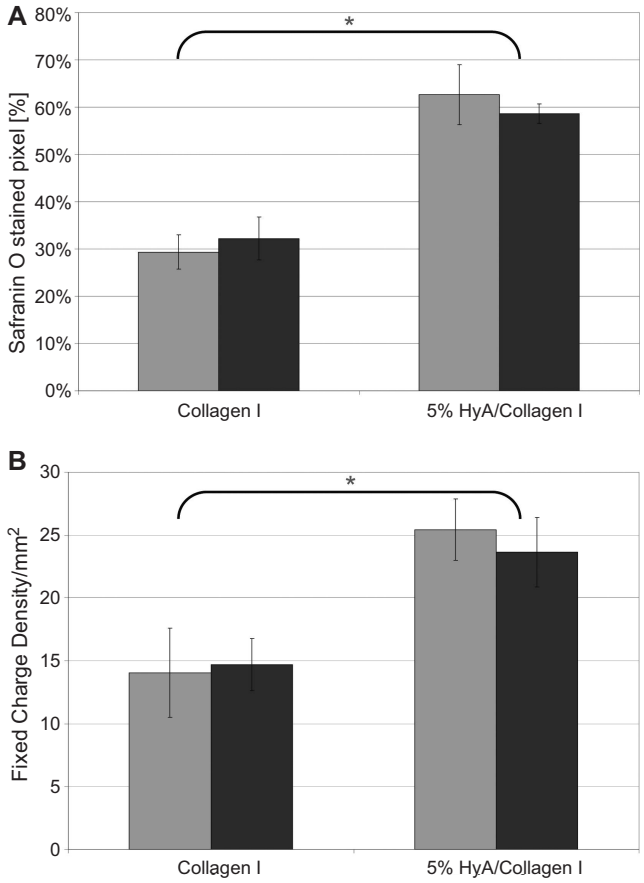

FIG. 8. Results from histomorphometric analyses of Safranin O stained sections. Data from scaffolds with cubic pores are shown in gray bars, and data from scaffolds with ellipsoidal pores are shown in black. Significantly greater areas and densities were found in scaffolds seeded with 5% hyalinuric acid (HyA) hydrogels. (**A**) The percentage of Safranin O staining is equivalent to the area of positive Safranin O staining normalized by the available pore space. (**B**) The fixed-charge density is the average stain intensity of the section, which is directly correlated to the amount of sulfated glycosaminoglycan in the extracellular matrix. *Significant difference ($p < 0.05$) from scaffolds with collagen I-only hydrogels.

in the composite gels with the DMMB assay. The increase in sGAG is also seen *in vivo*, where the area of positive Safranin O staining and the intensity of the stain was significantly higher than in the collagen I hydrogels. Furthermore, the increase of sGAG binding to HyA may enhance the compressive properties of the hydrogel by recreating the negative charge repulsions present in native cartilage.

In previous studies, various hydrogels, cross-linked ethylic and benzylic esters of hyaluronan (Hyaff)[36,37] and polyglycolic acid[37,38] were able to support chondrocyte attachment and ECM formation. However, the mechanical properties of these constructs must be assessed to determine whether these scaffolds are able to support adequate loads. The primary function of the PPF scaffold in this study was to support the developing repair tissue and protect it from excessive external forces. Our results show that PPF is able to support chondrocyte growth and matrix deposition. PPF is a biodegradable polymer that will allow the regenerating tissue to slowly replace the degrading biomaterial. It is biocompatible with minimal immunological reactions.[23] In the future, the mechanical properties of the designed PPF scaffolds alone and with cells will need to be evaluated. Ideally, the scaffold properties should match those of native cartilage. The mechanical properties of these scaffolds can be easily adjusted by altering the ratio of PPF to NVP, which changes the amount of cross-linking.[39] Thus, the properties of the scaffold and polymer degradation time can be calibrated to match tissue regeneration.

An advantage of using SFF scaffolds over non-woven meshes and scaffolds fabricated using particulate leaching is that the user can define the pore architectures, pore sizes, and overall scaffold porosity. We designed scaffolds with pores that were cubic or ellipsoid in shape. Although the results did not show a significant difference between these scaffold designs, it is promising that, even with lower effective permeabilities, the tissue that developed in the ellipsoid pore was fairly robust and was comparable with the tissue found in the cubic-pored scaffolds. The permeability in this study may not be a large factor in ECM production because the chondrocytes are able to alter their aerobic pathways in favor of anaerobic metabolism easily. Chondrocytes are well adapted to conditions with low permeabilities and low oxygen tensions similar to those found *in vivo* in the joint. Although we did not see a difference between the ECM formation due to the scaffold designs, some studies have shown that lower oxygen tensions help support greater matrix production and lower dedifferentiation.[40] Furthermore, we believe that an increase in the surface area: volume ratio due to pore geometry should increase cellular attachment and growth and increase the amount of ECM that is deposited in the scaffold pore space. Perhaps a spheroidal-shaped pore or a different cell type will help to further elucidate the differences between scaffold pore geometries. It is possible that the cellular encapsulation in the hydrogels prevented attachment to the polymer surface; therefore, the cellular density and composition of the hydrogels were the major factors in determining ECM deposition and cell proliferation.

In conclusion, a combination of composite HyA hydrogels and a custom-designed SFF scaffold may provide a promising functional construct for cartilage repair. The beneficial effects of HyA make it a good substrate for tissue-engineering applications, but there are limits to this effect depending on the concentration of HyA. At high concentrations, the effects of steric inhibition, self-association, and chain entanglement become important factors influencing nutrient diffusion and cellular metabolism. The ability to design scaffold properties such as porosity, permeability, and shape using SFF will be important in the creation of a functional construct that is capable of load bearing and allows tissue regeneration.

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