Silk-Fibrin/Hyaluronic Acid Composite Gels for Nucleus Pulposus Tissue Regeneration

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Scaffold designs are critical for *in vitro* culture of tissue-engineered cartilage in three-dimensional environments to enhance cellular differentiation for tissue engineering and regenerative medicine. In the present study we demonstrated silk and fibrin/hyaluronic acid (HA) composite gels as scaffolds for nucleus pulposus (NP) cartilage formation, providing both biochemical support for NP outcomes as well as fostering the retention of size of the scaffold during culture due to the combined features of the two proteins. Passage two (P2) human chondrocytes cultured in 10% serum were encapsulated within silk-fibrin/HA gels. Five study groups with fibrin/HA gel culture (F/H) along with varying silk concentrations (2% silk gel only, fibrin/HA gel culture with 1% silk [F/H+1S], 1.5% silk [F/H+1.5S], and 2% silk [F/H+2S]) were cultured in serum-free chondrogenic defined media (CDM) for 4 weeks. Histological examination with alcian blue showed a defined chondrogenic area at 1 week in all groups that widened homogenously until 4 weeks. In particular, chondrogenic differentiation observed in the F/H+1.5S had no reduction in size throughout the culture period. The results of biochemical and molecular biological evaluations supported observations made during histological examination. Mechanical strength measurements showed that the silk mixed gels provided stronger mechanical properties for NP tissue than fibrin/HA composite gels in CDM. This effect could potentially be useful in the study of *in vitro* NP tissue engineering as well as for clinical implications for NP tissue regeneration.

Introduction

LOWER BACK PAIN (LBP) is a predominant cause of disability, with significant ensuing socioeconomic consequences.¹ LBP is generally associated with degeneration of the intervertebral disc (IVD).² The IVD is a unique and complex connective tissue that plays an important role in spinal weight transmission. It consists of two developmentally and morphologically separate regions: a central hydrated gel-like zone (the nucleus pulposus [NP]) and a ligamentous peripheral tissue (the annulus fibrosus [AF]).³ Collagens and proteoglycans (PGs) are the primary macromolecules that make up the extracellular matrix (ECM) of the IVD.⁴ Degenerative disc disease, in particular, is believed to originate in the NP of the disc, because this is the region of tissue that shows the most dramatic transformation with age. Thus, most tissue engineering studies are directed toward treatment of the NP.⁵

The principal function of a scaffold for tissue engineering is to emulate the ECM in the body, thus creating an environment that is conducive to cell adhesion, proliferation, gene expression, and synthesis of functional new tissue.⁶

While a wide variety of biomaterials have been used in IVD tissue engineering, strategies typically involved incorporation of isolated cells into scaffold materials to form three-dimensional (3D) constructs.⁷ Specifically, after selection of appropriate hydrogel scaffold materials, their physical properties, mass transport properties, and biological interaction requirements are extensively scrutinized for each application. Although a hydrogel system itself lacks mechanical strength, they allow diffusion of nutrients and metabolites to and from the encapsulated cells and surrounding tissue.⁸ In addition, chondrocyte activities are maintained in a proper 3D environment within a hydrogel system as they are constantly exposed to cell-matrix interactions. This cell-matrix interaction is essential for chondrocyte proliferation, differentiation, and survival.⁹ Therefore, many researchers have tried to find an optimal gel scaffold, based on the characteristics of the target cells and tissues.

Various hydrogels such as collagen,^{10,11} alginate,¹² chitosan,¹³ fibrin,^{14,15} and hyaluronic acid (HA)^{16,17} have been tested for their effectiveness as scaffolds for NP tissue. These materials have frequently been used in tissue engineering applications, being either components of or having

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macro-molecular properties similar to the natural ECM and have advantages over synthetics, with excellent physiological characteristics such as selective cell adhesion, cellular properties similar to natural tissues, and biodegradability. However, naturally derived materials offer limited versatility in designing an exogenous/ECM with specific properties for clinical applications (e.g., porosity and mechanical strength). Therefore, the concept of hybrid materials incorporating cell-recognizing sites of naturally derived biomaterials is attractive for improving the properties of synthetic materials. These hybrid materials allow for a favorable combination of properties of their constituent components, including varying mechanical and degradation properties, reproducible large-scale production, and good process ability, as well as specific biological activity of naturally derived materials.⁸ In particular, scaffolds for disc regeneration must exhibit the necessary mechanical properties as well as provide physical support. Due to the large number of loading cycles experienced in the spine, it is important that the scaffold be able to withstand significant physical loading.⁹ The NP is known to support approximately 70% of the compressive axial load exerted on the spine because of its PG-rich ECM and its interaction with water.¹⁸

In our previous studies, we focused on fibrin/HA composite gel as a chondrocyte delivery vehicle, because of its current prevalent use in tissue engineering applications, and showed the possibility of the gel for artificial cartilage engineering using chondrocytes.^{19,20} However, the fibrin/HA composite gel showed fast degradation. In cartilage tissue engineering, it is important that the rate of ECM synthesis and degradation be matched and kept in an equilibrium condition.²¹ Taking the mechanical strength into consideration, cartilage chips were added to fibrin glue to create a scaffold with increased mechanical strength and to prevent shrinkage after culturing for extended periods.²² To overcome similar challenges in achieving superior mechanical strength, we added silk to the fibrin/HA gel in this study. Silk has been used in the tissue engineering field because of its excellent mechanical properties, biocompatibility, controllable degradation rates, and inducible formation of crystalline β -sheet structure networks.^{23–26} In addition, sonication-induced silk gels can be readily formed,²⁷ with cell encapsulation and superior mechanical strength to several other materials (fibrin, alginate, gelatin, and agarose).

We hypothesized that the modification of fibrin/HA composites with silk would provide appropriate mechanical strength with support for cartilage formation. In this study, we studied whether silk-fibrin/HA would provide a favorable environment for chondrogenesis compared to fibrin/HA gel alone.

Materials and Methods

Chondrocyte culture

Human chondrocytes were purchased from Lonza Walkersville, Inc. (Walkersville, MD; CC-2550), the cell pellet was washed twice with phosphate-buffered saline (PBS), and resuspended in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Gibco BRL), 100 U/mL penicillin G (Gibco BRL), and 100 μ g/mL streptomycin (Gibco BRL). Both cell number and viability were determined using trypan blue exclusion test. The cells were then plated at a

density of 1.5×10^5 cells/cm² and cultured at 37°C in a 5% CO₂ incubator. The culture medium was changed every other day. The chondrocytes were passaged twice before the experiments.

Preparation of silk fibroin solution

Silk fibroin (SF) solutions were prepared according to procedures described previously.^{28,29} Briefly, cocoons of *Bombyx mori* were boiled for 20 min in an aqueous solution of 0.02 M Na₂CO₃, and then rinsed thoroughly with distilled water to extract the glue-like sericin proteins. The extracted SF was dissolved in 9.3 M LiBr solution at 60°C for 4 h, yielding a 20 w/v% solution. This solution was dialyzed in distilled water using a Slide-a-Lyzer dialysis cassette (MWCO 3500; Thermo Fisher Scientific, Inc., Rockford, IL) for 2 days. The final concentration of SF aqueous solution was ~8 w/v%, which was determined by weighing the remaining solid after drying. Silk solutions with lower concentrations were prepared by diluting the 8% solution with water.

Preparation of silk composite gel with fibrin/HA gel

To prepare fibrin/HA composite gel, the chondrocytes were pelleted by centrifugation, and then resuspended in a solution containing both fibrinogen (10 mg/mL; Green Cross, Young In, Korea) and 1300 kDa HA (10 mg/mL with 6000 cps viscosity; Lifecore, Chasca, MN).²⁰ A chondrocyte suspension of 5×10^6 cells/mL was then homogeneously mixed with aprotinin (Green Cross), 60 U/mL thrombin (1000 U/mg protein; Sigma, St. Louis, MO), a fibrin stabilizing Factor XIII, and 50 mM CaCl₂. The fibrin/HA mixture (250 μ L) was dropped into an empty Petri dish to form a gel.

To perform sonication-induced silk gelation, we followed our previous method.^{27,30} Five milliliters of each silk solution (2%, 3%, and 4%) in a 15 mL Falcon tube was sonicated with a Branson 450 Sonifier (Branson Ultrasonics Co., Danbury, CT), which consisted of the Model 450 power supply, converter, externally threaded disruptor horn, and 1/8" (3.175 mm) diameter-tapered microtip. Sonication time was 30 s at 35% amplitude setting. Two hundred fifty microliters of sonicated silk solutions was dropped into an empty Petri dish after sonication to form a gel and incubated at 37°C for 30 min.

For silk composite gels with fibrin/HA, 125 μ L of different wt% sonicated silk solutions was mixed with 125 μ L of 2% fibrinogen/HA solution. The mixture was then placed in a Petri dish in the same manner as above. Each drop was transferred to a six-well culture plate and cultured in chondrogenic defined media, consisting of high-glucose DMEM (Gibco BRL) supplemented with 1% antibiotic-antimycotic (Gibco BRL), ITS supplement (1.0 mg/mL insulin from bovine pancreas, 0.55 mg/mL human transferrin, and 0.5 mg/mL sodium selenite), 50 μ g/mL ascorbic acid, 100 nM dexamethasone, 40 μ g/mL proline, 1.25 mg/mL bovine serum albumin (BSA), and 100 μ g/mL sodium pyruvate. All products were purchased from Sigma. The gels were divided into 5 experimental groups: 2% silk gel, fibrin/HA gel (F/H), with 1% silk (F/H+1S), with 1.5% silk (F/H+1.5S), and with 2% silk (F/H+2S).

Fourier transform infrared spectroscopy analysis

Fourier transform infrared spectroscopy (FTIR) analysis was performed with a JASCO FTIR 6200 Spectrometer

(JASCO, Tokyo, Japan), equipped with a deuterated triglycine sulfate detector and a multiple-reflection, horizontal MIRacle ATR attachment (using a Ge crystal). For each measurement, 32 scans were coded with resolution 4 cm^{-1} , with the wave number ranging from 400 to 4000 cm^{-1} . Fourier self-deconvolution (FSD) of the infrared spectra covering the amide I region ($1595\text{--}1705\text{ cm}^{-1}$) was performed by Opus 5.0 software. Deconvolution was performed using Lorentzian line shape with a half-bandwidth of 25 cm^{-1} and a noise reduction factor of 0.3. FSD spectra were curve-fitted to measure the relative areas of the amide I region components.

Volume measurement

For measuring volume of composite gels, custom image analysis software was used (Cell Therapy Center, Ajou University, Korea).³¹ The sequence of image processing algorithms was divided into five stages, that is, shape extraction, and measurement of surface area and volume: (1) image acquisition operations to convert images into digital form (256 by 256); (2) preprocessing operations to obtain an improved image with the same dimensions as the original image; (3) image segment operations to partition a digital image into disjoint and nonoverlapping regions (calculation of area); (4) object measurement operations to measure the characteristics of objects, such as size, shape, and height; and (5) measurement of surface area and volume. One method was developed to measure the surface area and volume of tissue-engineered constructs, which is the derived method. The method was based on the three principal dimension, length (l), width (w), and thickness (t), and the surface area and volume were derived mathematically; the 3D image of the target material is partitioned into a number of sections, followed by summation of each section to obtain a real volume.

Biomechanical strength analysis

The retrieved specimens ($n=4$) were subjected to unconstrained compression tests using an Instron 3366 testing frame (Grove City, PA) equipped with a 10 N capacity load cell. Tests were conducted at room temperature in PBS (wet state). A displacement control mode was used, with a crosshead displacement rate of $5\text{ mm}\cdot\text{min}^{-1}$. The compressive stress and strain were graphed for the measured sample dimensions: cross-sectional area based on the sample punch size and sample height (measured automatically @ 0.02 N tare load). The yield strength as well as the compressive modulus and standard deviation were determined after testing. The elastic modulus was calculated based on a least-squares' fitting between 0% and 10% strain (based on the new sample height measured automatically). The compressive strength was determined using an offset-yield approach. A line was drawn parallel to the modulus line, but offset by 0.5% of the sample gauge length. The corresponding stress value at which the offset line crossed the stress-strain curve was defined as the compressive yield strength of the scaffold.

Live/dead cell viability assay in gels

Viable cells within gels were screened using a live/dead kit (Molecular Probes, Eugene, OR). Following the manufacturer's instructions, *in vitro* sample was treated with 2 mM ethidium homodimer-1, PBS, and 4 mM calcein AM for 40 min.

After washing in sterilized PBS, the sample-embedded slide was observed with a Leica TCS SP2 AOBs microscopy with Leica confocal software. The region of interest was selected from z-plane images to include either the surface or the internal pores, beginning with a bottom section at least 1 mm above the surface of the scaffolds. Depth projection micrographs were obtained from 20 horizontal sections imaged at a depth distance of 50 mm from each other. Live cells appeared green, and dead cells appeared red. Viability of cells was measured by dividing the number of viable cells (green cells) with that of total cells (green cells+red cells), and determined using the ImageJ program.

Histology and immunohistochemistry

Following macroscopic observation, tissues were fixed with 4% formalin for 24 h. These were then embedded in paraffin and sectioned in $4\text{ }\mu\text{m}$ thickness. Serial sections were stained with hematoxylin and eosin, safranin-O, and alcian blue for sulfated proteoglycan. Immunohistochemistry was also carried out to screen for expression of type II collagen as the major ECM protein of IVD. The sections were washed sequentially in 70% ethanol and PBS followed by 3% H_2O_2 in PBS. Finally, 0.15% Triton X-100 was added. Once blocked with 1% BSA solution, monoclonal antibody raised against human type II collagen was added (1:200; Chemicon, Temecula, CA) and incubated for 1 h, followed by addition of biotinylated secondary antibody. The protein was then detected using a horseradish peroxidase-conjugated avidin system (Vector Laboratories, Burlingame, CA). The immunostained sections were counterstained with Mayer's hematoxylin (Sigma) before microscopic examination with a Leica DMIL light microscope (Wetzlar, Germany).

Biochemical assays

The recovered samples ($n=4$) for DNA and glycosaminoglycans (GAGs) were digested for 16 h with papain solution ($125\text{ }\mu\text{g/mL}$ of papain, 5 mM L-cystein, 100 mM Na_2HPO_4 , and 5 mM EDTA, pH 6.2) at 60°C . DNA content was measured using the PicoGreen DNA Assay according to the manufacturers protocol (Molecular Probes). After centrifugation $25\text{ }\mu\text{L}$ aliquot of supernatant was taken from each sample and placed into 96-well plates with each well containing $75\text{ }\mu\text{L}$ of $1\times\text{TE}$ buffer. A standard curve was generated using lambda phage DNA in 0, 2.5, 5, and $10\text{ }\mu\text{g/mL}$ concentrations. One hundred microliters of a 1:200 dilution of Quant-iT PicoGreen reagent was added to each well and read using a fluorometer with an excitation wavelength of 480 nm and an emission wavelength of 528 nm. Total GAG content was analyzed using a 1,9-dimethylmethylene blue (DMB) assay.³² Individual samples were mixed with the DMB solution, and the absorbance was measured at 525 nm. Total GAG of each sample was extrapolated using a standard plot of shark chondroitin sulfate (Sigma) in the range of 0– $100\text{ }\mu\text{g/mL}$.

The tissue-engineered constructs were digested with pepsin solution (1 mg/mL of pepsin, pH 3.0) at 4°C for 48 h to determine total collagen content. Total collagen was measured as previously reported.²⁰ A dye solution (pH 3.5) was prepared with Sirius red dissolved in picric acid-saturated solution (1.3%; Sigma) to a final concentration of 1 mg/mL . The digested samples were dried at 37°C in 96-well plates for 24 h and then reacted with the dye solution for 1 h on a shaker.

The samples were then washed five times with 0.01 N HCl and the dye-sample complex in each well was resolved in 0.1 N NaOH and absorbance read at 550 nm (Versa MAX; Molecular Devices, Sunnyvale, CA). Total collagen in each sample was extrapolated using a standard plot of bovine collagen (Sigma) in the range of 0–500 µg/mL.

Real-time PCR analysis

Cultured gels ($n=4$ per group) were transferred into 2 mL plastic tubes and 1.0 mL of Trizol was added. Scaffolds were chopped with micro scissors on ice. The tubes were centrifuged at 12,000 g for 10 min, and the supernatant was transferred to a new tube. Chloroform (200 µL) was added to the solution and incubated for 5 min at room temperature. Tubes were centrifuged at 12,000 g for 15 min and the upper aqueous phase was transferred to a new tube. One volume of 70% ethanol (v/v) was added and applied to an RNeasymini spin column (Qiagen, Hilden, Germany). The RNA was washed and eluted according to the manufacturer's protocol. The RNA samples were reverse transcribed into cDNA using oligo (dT)-selection according to the manufacturer's protocol (High Capacity cDNA Archive Kit; Applied Biosystems, Foster City, CA). Collagen type II α 1 (*Coll α 1*), *aggrecan*, and *sox-9* levels were quantified using the Mx3000 Quantitative Real Time PCR system (Stratagene, La Jolla, CA). All data analysis employed the Mx3500 software (Stratagene) based on fluorescence intensity values after normalization with an internal reference dye and baseline correction. Differences in gene expression were analyzed using the comparative Ct method (C_t [delta][delta] C_t comparison). C_t values for samples were normalized to an endogenous housekeeping gene. PCR conditions were 2 min at 50°C, 10 min at 95°C, and then 50 cycles at 95°C for 15 s, and 1 min at 60°C. The data were normalized to the expression of the housekeeping

gene, glyceraldehyde-3-phosphate-dehydrogenase (*GAPDH*) within the linear range of amplification and differences.²⁹ The *GAPDH* probe was labeled at the 5' end with fluorescent dye VIC and with the quencher dye TAMRA at the 3' end. Primer sequences for the human *GAPDH* gene were as follows: forward primer 5'-ATG GGG AAG GTG AAG GTC G-3', reverse primer 5'-TAA AAG CCC TGG TGA CC-3', and probe 5'-CGC CCA ATA CGA CCA AAT CCG TTG AC-3'. Primer sequences for the human collagen type II α (*Coll α 1*) gene were as follows: forward primer 5'-CAG CCG CTT CAC CTA CAG C-3', reverse primer 5'-TTT TGT ATT CAA TCA CTG TCT TGC C-3', and probe 5'-CCG GTG TGA CTC GTG CAG CCA TC-3'. Primer sequences for the human *aggrecan* gene were as follows: forward primer 5'-CAG CCG CTT CAC CTA CAG C-3', reverse primer 5'-TTT TGT ATT CAA TCA CTG TCT TGC C-3', and probe 5'-CCG GTG TGA CTC GTG CAG CCA TC-3'. Primer sequences for the human *sox-9* gene were as follows: forward primer 5'-CAG CCG CTT CAC CTA CAG C-3', reverse primer 5'-TTT TGT ATT CAA TCA CTG TCT TGC C-3', and probe 5'-CCG GTG TGA CTC GTG CAG CCA TC-3'. Probes were purchased from Assay on Demand (Applied Biosciences).

Statistical analysis

Statistical differences in biochemical and mechanical quantitative analyses were determined using Mann-Whitney U test (Independent t -test; SPSS). Statistical significance was assigned as $*p<0.05$, $**p<0.01$, and $***p<0.001$, respectively.

Results

Gross observation

The size change in F/H+1S gels was not significant at 1 week compared to day 1; however, the overall size of this

FIG. 1. Degradation test of silk-mixed fibrin/HA composite gels. (a) Gross observation; (b) volume change. Statistically significant differences ($*p<0.05$, $**p<0.01$, and $***p<0.001$). Gross images of the gels were observed at 1 day, 1 week, 2 weeks, and 4 weeks after culture. The gross image reveals that fibrin/HA composite gels were reduced in size more rapidly than silk-mixed fibrin/HA composite gels. HA, hyaluronic acid.

FIG. 2. Fourier transform infrared spectroscopy (FTIR) spectra of composite gels. (a) F/H, (b) F/H+1S, (c) F/H+1.5S, (d) F/H+2S, and (e) silk 2%. FTIR spectra of silk fibroin scaffolds showed characteristic peaks of silk II at 1701 and 1623 cm^{-1} (amide I). F/H, fibrin/HA gel culture. Color images available online at www.liebertonline.com/tea

group decreased with time. The F/H group showed significant reduction (about 85%) in size after 4 weeks, whereas specimens of F/H+1.5S and 2S gels did not have any major difference in their sizes over time (Fig. 1b). The 2% silk gel group also did not show any loss in size during the time of study (Fig. 1a). The silk only gel was easily broken during

handling of the samples, suggesting a stiffness that was too high for the needs of the present study.

FTIR analysis

To check β -sheets in the composite gel, change in the amide I curve in various gels was analyzed using FTIR. All silk and F/H composite gel groups showed β -sheet crystal structures (1697 cm^{-1} , 1627 cm^{-1} , and 1528 cm^{-1}), whereas the F/H gel alone exhibited a mostly amorphous structure (Fig. 2).

Analysis of mechanical strength

Gels were tested for their mechanical properties. The silk gel mixed with F/H showed a higher modulus and yield strength than the F/H gel alone. These differences were statistically significant and gradually increased with increases in silk concentration. However, there was no significant difference between F/H+1.5S and +2S (Fig. 3a, b). On the other hand, the 2% silk gel was brittle, whereas the silk gel with F/H exhibited more elasticity (Fig. 3c).

Cell morphology and viability

Cultured chondrocytes retained morphology and remained attached on the gel matrix. Cell aggregation appeared in the F/H gels (Fig. 4a). However, F/H+1S and 1.5S gels showed even cell distributions (Fig. 4b, c). Proliferating cells in the lower silk concentration gels (F/H+1S and 1.5S gels) showed more organized cytoskeleton than cells in F/H+2S and 2% silk. In addition, cell populations were lower

FIG. 3. Mechanical measurement of composite gels. (a) Modulus, kPa; (b) yield strength, kPa; (c) elasticity by manual bending test. Silk gel mixed with F/H showed a higher modulus and yield strength than F/H gel alone. The 2% silk gel was brittle, whereas silk gels with F/H exhibited more elasticity. Data are shown as mean $\pm$ standard deviation from $n=4$, statistically significant differences ($*p<0.05$, $**p<0.01$, and $***p<0.001$). Color images available online at www.liebertonline.com/tea

FIG. 4. Live/dead assay. (a) F/H, (b) F/H+1S, (c) F/H+1.5S, (d) F/H+2S, and (e) silk 2%. Scale bars = 300 μ m. The live cells were stained in green by Live/Dead assay. Cell aggregation appeared in the F/H gels, whereas F/H+1S and 1.5S gels showed even cell distributions. Proliferated cells in F/H+1S and 1.5S gels had longer cytoskeleton than cells in F/H+2S and 2% silk. In addition, the fibrin/HA gel showed decreased cell populations with higher silk concentrations. Color images available online at www.liebertonline.com/tea

in pure silk gels and the fibrin/HA gel showed decreased cell populations with higher silk concentrations, particularly in F/H+2S gels (Fig. 4d, e). The percentage of viable cells (green color) were approximately 90% of the entire cell population (green color + red color) in all gels based on Live/Dead assay (Fig. 4).

Histology and immunohistochemistry analysis

For identification of cartilage-specific ECM molecules, thin sections of each specimen were stained with safranin-O and alcian blue at 4 weeks. Cells in all composite gels were homogeneously distributed within the matrix. However, safranin-O and alcian blue staining showed different accumulation of sulfate proteoglycan. Although the 2% silk

gel and F/H+2S gels showed less accumulated ECM (Fig. 5d, e, i, g), F/H+1S and 1.5S gels had increased proteoglycan (Fig. 5a–c, f–h). Although expression of type II collagen was detected in F/H, F/H+1S, and 1.5S gels at 4 weeks, F/H+2S and 2% silk gels showed less staining for the collagen (Fig. 5k–o).

Chemical analysis for chondrogenesis

When the total amount of GAGs and collagen was measured during *in vitro* culture, F/H, F/H+1S, and 1.5S silk composite gels had significantly higher levels than the 2% silk and F/H+2S. F/H+1.5S showed the highest level of total GAG synthesis and DNA-normalized GAG content after 4 weeks (Fig. 6a, b). The average ECM concentration per

FIG. 5. Histology of GAGs and collagen staining showing chondrogenic extracellular matrix deposition in individual composite gels in chondrogenic medium. (a–e) Alcian blue staining for deposition of cartilage-like matrix in the gels, blue color (f–j) Immunohistochemistry for type II collagen, brown color (k–o), F/H (a, f, k), F/H+1S (b, g, l), F/H+1.5S (c, h, m), F/H+2S (d, i, n), and silk 2% (e, j, o). Scale bars: 3 mm (a–e), 100 μ m (f–o). GAGs, glycosaminoglycans. Color images available online at www.liebertonline.com/tea

FIG. 6. Measurement of chemical content of the composite gels. (a) GAGs content, (b) GAGs/DNA, (c) GAGs/gel, (d) total collagen content, (e) collagens/DNA, and (f) collagens/gel. The amount of GAGs and collagens in each gel was measured at the time points indicated by 1,9-dimethylmethylene blue (DMB) assay and Hride tullberg-reinert method in chondrogenic medium at 1 day, 1 week, 2 weeks, and 4 weeks after culture. The data were presented as normalized values by the total DNA ($\mu\text{g}/\mu\text{g}$) and dry weight of gel ($\mu\text{g}/\text{mg}$). Data are shown as mean $\pm$ standard deviation from $n=4$, statistically significant differences ($*p<0.05$, $**p<0.01$, and $***p<0.001$, respectively).

gel ($\mu\text{g}/\text{mg}$ dry weight) in the F/H and F/H+1S gels was 19.8 ± 5.1 and 16.7 ± 2.3 , respectively, for GAGs at 4 weeks. However, the 2% silk and F/H+2S gels did not show a significant increase with time (Fig. 6c). Total collagen content in both groups (F/H+1S and 1.5S) gradually increased with time and showed higher levels than the fibrin/HA gels at 4 weeks (Fig. 6d). The groups with silk added did not show differences after DNA normalization at 4 weeks, whereas F/H+2S showed the highest level of collagen content at 2 weeks (Fig. 6e). Although the collagen values per gel of F/H+1S and 1.5S groups did not reach those of the F/H gels at 4 weeks, they showed a statistically significant difference compared to the 2% silk and F/H+2S gels (Fig. 6f). This result is consistent with the collagen staining by immunohistochemistry. Total DNA content within the silk constructs was determined to quantify cell proliferation. The cell numbers within the 2% silk and F/H+2S gels were lower than in the other groups after 4 weeks of culture (Supplementary Fig. S1; Supplementary Data are available online at www.liebertonline.com/tea).

Chondrogenic gene expression

To further support the results from histological observations, the expression of chondrogenic markers such as *Col III α 1*, *Sox-9*, and *aggrecan* was analyzed by real-time PCR. Transcript levels were normalized to *GAPDH* within the linear range of amplification and differences. Three genes in the F/H, F/H+1S, and 1.5S gels showed significantly higher values than those in the 2% silk and F/H+2S gels. At 4 weeks, *Col III α 1* gene expression in the F/H 1S and 1.5S gels exhibited 3.5 and 2.3 times higher value than F/H+2S gel, respectively (Fig. 7a). Transcript level of *Sox-9* in the F/H+1.5S gel was 7.6-fold higher than that in the F/H+2S silk composite gels (Fig. 7b). *Aggrecan* gene expression in the F/H+1S and 1.5S gels also showed 2.7 and 2.3 times higher levels than in the F/H+2S gels, respectively (Fig. 7c).

Discussion

Tissue engineering requires an artificial 3D ECM for tissue regeneration to facilitate cell proliferation and differentiation,

FIG. 7. Transcript levels related to chondrogenic differentiation in the composite gels. (a) *Col III α 1*, (b) *Sox-9*, and (c) *aggrecan*. mRNA levels were measured in each gel at the time points indicated by RT-PCR analysis. The mRNA level of glyceraldehyde-3-phosphate-dehydrogenase was measured as an internal control. Data are shown as mean $\pm$ standard deviation from $n=4$; statistically significant differences ($*p<0.05$, $**p<0.01$, and $***p<0.001$).

resulting in tissue regeneration.³³ IVD tissue engineering aims to restore the function of the NP tissue by repairing or replacing degenerated tissue. In repairing the IVD, the aim is to induce tissue regeneration *in situ* via biological manipulation, whereas replacing the IVD requires developing a functional tissue unit *in vitro* and implanting it into the body. The physiological properties of the disc are linked to the composition of its ECM. Thus, tissue engineering methods tend to focus on techniques directed toward replenishing the ECM components of the disc to restore disc function.³⁴

To date, most studies of cell transplantation for the treatment of degenerative disc disease have used autologous disc cells.^{6,35} However, diseased IVD with pathological characterizations such as degraded disc matrix and degenerative NP cells are not present in healthy IVD organ cultures.³⁶ In a clinical setting, a healthy disc would have to be damaged to harvest autologous disc cells to be implanted, unless the disc cells were harvested from the degenerative disc to be treated. In addition, progress on tissue engineering of the IVD has been slow because of the complexities involved in cultivating disc cells, particularly NP cells.³⁷ There is a need for increasing NP cell viability because of their low cell yields from harvested IVDs and low proliferative capacity during cultivation. Chondrocytes in contrast are widely available and can be harvested from nonspinal sites with relatively low morbidity and risks. There is also substantial clinical experience with autologous articular chondrocyte harvest, expansion, and then implantation to treat articular chondral defects in the knee and other joints.^{38,39} For this reason, previous experiments have been conducted using chondrocytes as an alternative cell source. Implanted elastic cartilage chondrocytes could survive and synthesize a cartilage-specific matrix in the IVD of rabbits.⁴⁰ Bovine autologous articular chondrocytes transduced with bioactive proteins (bone morphogenetic proteins [BMP]-2 or -7) were delivered to stimulate IVD healing and proved effective in promoting NP collagen and proteoglycan production.⁴¹ In addition, there are many similarities between hyaline cartilage and the NP. Within the NP, rounded chondrocyte-like cells are embedded in a random network of type II collagen and aggrecan, supplemented with other proteoglycans, including versican.^{42,43} The mature NP of the IVD becomes similar to hyaline cartilage, expressing markers of chondrocytes such as *collagen type II*, *collagen type IX*, *aggrecan*, and *SOX-9*.^{43,44}

Although bone marrow stromal cells and other multipotential cells might also be considered for this application, and have the advantage of being harvested with relative ease and low morbidity, the use of these more primitive cells requires considerable *ex vivo* manipulation to induce the cells into a more chondrocyte-like phenotype before transplantation.^{45,46} Hyaline-like cartilage produced after implantation as far may represent the best substitute for degenerated NP. Therefore, we believe that an alternate source of chondrocytic cells, such as non-weight-bearing articular cartilage, would be preferable and could provide a large number of metabolically active cells. However, further studies are needed to demonstrate that chondrocytes would be an appropriate cell source for NP regeneration. In this study, to achieve more NP-like tissue we also used transforming growth factor beta (TGF)- β 1. Because of the low cellular activity and nutrition-deprived environment in degenerated IVDs, it may be necessary to employ signals, such as those

provided by growth factors (GFs), for tissue engineering strategies to be more effective. Most IVD tissue engineering studies have examined growth factors that are members of the TGF- β super family, such as TGF- β 1 and BMPs. Treatment with TGF- β 1 stimulated new ECM synthesis in old or degenerated discs.⁴⁷ In addition, TGF- β 1 was found to be the optimal GF for stimulating GAG synthesis with both NP and AF cells.⁴⁸ TGF- β 1 also increased proteoglycan synthesis and cell proliferation when cultured without FBS.⁴⁹

A well-chosen scaffold may be important for effective regenerative cell therapy. The 3D structure of the scaffold provides good cell adherence, effectively guides tissue regeneration, and promotes cell growth and migration. A 3D structure is believed to be required to induce chondrogenic phenotype *in vitro*.^{7,33} The IVD is a highly compressed structure, sensitive to physical stress and may be subject to degeneration with even the slightest damage. Thus, injectable materials are preferable for IVD engineering, allowing needle-guided graft delivery to minimize trauma of the disc to be regenerated.⁵⁰ Injected materials can interdigitate with irregular surgical defects and may physically bond to the adjacent tissue. In addition, they allow for incorporation and uniform dispersion of cells and/or therapeutic agents.⁹ Therefore, several gel types have been used as injectable biomaterials for NP cartilage tissue engineering. Both NP and AF cells maintained their characteristic phenotype within alginate gels.⁵¹ Alginate gels may be useful for neocartilage formation, but it is unlikely that this substrate will be used *in vivo* because of its mechanical fragility.^{7,52} Collagen has been used by several researchers. For example, collagen/cell implants that preserved IVD structure compared with nontreatment have been reported.^{40,53} In addition, this research group showed well-maintained human NP cells in atelocollagen scaffolds.¹¹ However, atelopeptide collagen has been prepared from animals. Although it is quality-controlled as a medical device to prevent disease transmission, future risks for disease transmission remain a major concern.³³

In the present study, we utilized fibrin, HA, and silk as a triple-composite gel for NP tissue regeneration. It has been common practice that two or more biomaterials are combined to utilize the benefits of both. Matrix engineering by adding various components has been successful in producing composite materials, such as fibrin-alginate,⁵⁴ HA-alginate,⁵⁵ and collagen-fibrin.⁵⁶ The results indicated the feasibility of the combined matrix gels as supports for cell proliferation without losing their chondrogenic potential. However, here we used fibrin as an alternative natural scaffold candidate because it can be made from autologous blood and already has a good record of biocompatibility as a wound adhesive.⁷ HA is a nonsulfated GAG that constitutes a large proportion of cartilaginous ECM. In addition, in the central NP region of the IVD, the *aggrecan* molecules accumulate on a HA to form structures called aggregates.⁵⁷ Therefore, several studies have been conducted using fibrin and/or HA for cartilage and disc regeneration. A 2 wt% HA gel showed intense and uniform type II collagen staining in constructs with greatest neocartilage production.⁵⁸ In another study, HA induced chondrocyte proliferation in a dose-dependent manner and stimulated a large increase in chondroitin sulfate synthesis.⁵⁹ Disc height increased with the injection of a cell-seeded HA scaffold.⁶⁰ Moreover, a

fibrin/HA matrix for porcine NP cell culture has been reported. The proteoglycan synthesis in relation to DNA content was significantly higher in the fibrin/HA matrix.⁶¹ We also have previously suggested fibrin/HA composite gels as a scaffold matrix for chondrogenesis *in vitro* and *in vivo*: transplantation of chondrocytes in the fibrin/HA composite gel showed promise in repairing full-thickness cartilage defects of articular cartilage.¹⁹ This gel has also been tested as a scaffold matrix for chondrogenic differentiation of mesenchymal stem cells (MSCs) *in vitro* and, compared to other chondrogenic methods, the results showed good chondrogenesis of MSCs without the use of growth factors.⁶² This system thus represents a natural, biodegradable, and biocompatible matrix for chondrocytes and MSCs for *in vivo* regeneration. However, the main problem of natural scaffolds (fibrin and HA) is the large amount of shrinkage *in vivo*.

To improve gel mechanical properties (strength and shrinkage) we selected SF gels (from silkworm cocoons) as it is a naturally occurring degradable material that has unique mechanical properties, excellent biocompatibility, and processability. It has been identified as a suitable scaffold material for skeletal tissue engineering.¹⁹ The present results of inducing chondrogenesis seem to provide a favorable environment for NP cartilage tissue formation to synthesize cartilage ECM. In particular, silk-mixed fibrin/HA composite gel provided appropriate mechanical properties and uniform cell distribution in suspension (Figs. 3 and 4). The system also could be prepared simply and controllably. In addition, silk gels showed good elasticity when mixed with F/H gel (Fig. 3c).

A specific cell environment is a key determinant of cartilage formation. The importance of tissue environmental factors in the induction of cell differentiation has recently been emphasized. In particular, degradation rate is essential in many small and large molecule release applications and in functional tissue regeneration applications. The desired kinetics for scaffold degradation depends on the tissue engineering application.⁸ Although fibrin/HA gel showed potential as a cell delivery vehicle for chondrogenesis, the weak mechanical properties and rapid degradation remain problematic for some applications. To this end, a longer term stable fibrin gel has been prepared by controlling Ca^{2+} and fibrinogen concentrations and pH.⁶³ We also tried to find effective methods to inhibit degradation of the fibrin/HA scaffold [various fibrinolysis inhibition factors (epsilon aminocaproic acid, Aprotinin, and leukocyte protease) were added into gel]; however, little progress was made for fibrin/HA composite gels as a scaffold, and we failed to make a significant improvements.⁶² Silk gels have been investigated as alternative materials to provide mechanical support. In this study, we employed three study groups (F/H+1% silk, F/H+1.5% silk, and F/H+2% silk) to decrease volume shrinkage as well as provide stronger gel stability than the F/H gel alone during chondrogenesis. In particular, mechanical strength and volume maintenance in the F/H +2% silk composite gel provided superior features to the F/H and F/H+1% silk composite gels (Figs. 1 and 3). This may be due to β -sheet formation of the silk gel. Hydrogels have three basic degradation mechanisms: hydrolysis, enzymatic cleavage, and dissolution. In particular, fibrin/HA composite gels can be degraded by enzymatic action, but can also undergo controlled hydrolysis after partial oxidation.⁸

Therefore, the hydrophobic β -sheets formed by the silk gel decreased gel shrinkage during cell culture, as expected based on our prior studies with silk degradation mechanisms.⁶⁴ Mechanical strength may also benefit from β -sheet formation in silk the structure.²⁵ The present results of histology, immunohistochemistry, and chemical analysis showed that chondrogenesis of the lower concentrations of silk-added F/H (F/H+1% and 1.5% silk) composite gels were superior to the F/H +2% composite gel. Notably, although F/H+1% silk composite gel showed some volume shrinkage, *type II collagen*, *Sox-9*, and *aggrecan* expression appeared to be improved when compare to the F/H +2% silk composite gel (Fig. 7). The benefits of the addition of silk in maintaining mechanical integrity and slowing degradation rates for chondrogenesis, as found here, must be factored against limitations in cell proliferation, as we have previously reported,²⁷ if the silk content is too high and thus gel stiffness is too high.

It should be noted that while the present study was focused on the topic of NP regeneration, the strategies, cells, and approaches could also be applicable to other chondrogenesis needs, such as for articular cartilage repair. We have previously studied the use of porous sponge scaffolds of silk for chondrogenesis,^{23,65} and the present work extends these earlier observations into injectable hydrogel formats, where a broad range of potential utility can be considered particularly with this ability to regulate rates of degradation of the gels.

Conclusions

Advances in the field of tissue engineering are associated with the performance of biomaterials that help guide tissue formation or regeneration. Design principles in biomaterials have typically been influenced by the function of ECM.

The present study showed that sonicated silk gels could influence the properties of natural composite gels (mechanical strength was improved and shrinkage was prevented) for cartilage formation. Further, we demonstrated that the addition of silk to fibrin/HA gels decreased degradation of the gels while maintaining NP-like chondrogenic cell growth, making this hybrid gel system an attractive candidate for future NP tissue engineering studies.

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Disclosure Statement

No competing financial interests exist.

References

1. Ehrlich, G.E. Low back pain. *Bull World Health Organ* **81**, 671, 2003.
2. Kelsey, J.L., and White, A.A., 3rd. Epidemiology and impact of low-back pain. *Spine (Phila Pa 1976)* **5**, 133, 1980.
3. Gan, J.C., Ducheyne, P., Vresilovic, E.J., Swaim, W., and Shapiro, I.M. Intervertebral disc tissue engineering I: characterization of the nucleus pulposus. *Clin Orthop Relat Res* (411), 305, 2003.

4. Kitahara, H. Histochemical study of the human intervertebral disc. *Nippon Seikeigeka Gakkai Zasshi* **53**, 817, 1979.
5. Risbud, M.V., Izzo, M.W., Adams, C.S., Arnold, W.W., Hillibrand, A.S., Vresilovic, E.J., Vaccaro, A.R., Albert, T.J., and Shapiro, I.M. An organ culture system for the study of the nucleus pulposus: description of the system and evaluation of the cells. *Spine (Phila Pa 1976)* **28**, 2652, 2003.
6. Gruber, H.E., and Hanley, E.N., Jr. Biologic strategies for the therapy of intervertebral disc degeneration. *Expert Opin Biol Ther* **3**, 1209, 2003.
7. Hubbell, J.A. Materials as morphogenetic guides in tissue engineering. *Curr Opin Biotechnol* **14**, 551, 2003.
8. Drury, J.L., and Mooney, D.J. Hydrogels for tissue engineering: scaffold design variables and applications. *Biomaterials* **24**, 4337, 2003.
9. Boyd, L.M., and Carter, A.J. Injectable biomaterials and vertebral endplate treatment for repair and regeneration of the intervertebral disc. *Eur Spine J* **15 Suppl 3**, S414, 2006.
10. Sakai, D., Mochida, J., Yamamoto, Y., Nomura, T., Okuma, M., Nishimura, K., Nakai, T., Ando, K., and Hotta, T. Transplantation of mesenchymal stem cells embedded in Atelocollagen gel to the intervertebral disc: a potential therapeutic model for disc degeneration. *Biomaterials* **24**, 3531, 2003.
11. Sakai, D., Mochida, J., Iwashina, T., Watanabe, T., Suyama, K., Ando, K., and Hotta, T. Atelocollagen for culture of human nucleus pulposus cells forming nucleus pulposus-like tissue *in vitro*: influence on the proliferation and proteoglycan production of HNPSV-1 cells. *Biomaterials* **27**, 346, 2006.
12. Mizuno, H., Roy, A.K., Zaporozhan, V., Vacanti, C.A., Ueda, M., and Bonassar, L.J. Biomechanical and biochemical characterization of composite tissue-engineered intervertebral discs. *Biomaterials* **27**, 362, 2006.
13. Mwale, F., Iordanova, M., Demers, C.N., Steffen, T., Roughley, P., and Antoniou, J. Biological evaluation of chitosan salts cross-linked to genipin as a cell scaffold for disk tissue engineering. *Tissue Eng* **11**, 130, 2005.
14. Sha'ban, M., Yoon, S.J., Ko, Y.K., Ha, H.J., Kim, S.H., So, J.W., Idrus, R.B., and Khang, G. Fibrin promotes proliferation and matrix production of intervertebral disc cells cultured in three-dimensional poly(lactic-co-glycolic acid) scaffold. *J Biomater Sci Polym Ed* **19**, 1219, 2008.
15. Gruber, H.E., Leslie, J., Ingram, J., Norton, H.J., and Hanley, E.N. Cell-based tissue engineering for the intervertebral disc: *in vitro* studies of human disc cell gene expression and matrix production within selected cell carriers. *Spine J* **4**, 44, 2004.
16. Cloyd, J.M., Malhotra, N.R., Weng, L., Chen, W., Mauck, R.L., and Elliott, D.M. Material properties in unconfined compression of human nucleus pulposus, injectable hyaluronic acid-based hydrogels and tissue engineering scaffolds. *Eur Spine J* **16**, 1892, 2007.
17. Kogan, G., Soltes, L., Stern, R., and Gemeiner, P. Hyaluronic acid: a natural biopolymer with a broad range of biomedical and industrial applications. *Biotechnol Lett* **29**, 17, 2007.
18. Broberg, K.B. On the mechanical behaviour of intervertebral discs. *Spine (Phila Pa 1976)* **8**, 151, 1983.
19. Park, S.H., Cui, J.H., Park, S.R., and Min, B.H. Potential of fortified fibrin/hyaluronic acid composite gel as a cell delivery vehicle for chondrocytes. *Artificial Organs* **33**, 439, 2009.
20. Park, S.H., Park, S.R., Chung, S.I., Pai, K.S., and Min, B.H. Tissue-engineered cartilage using fibrin/hyaluronan composite gel and its *in vivo* implantation. *Artif Organs* **29**, 838, 2005.
21. Chou, C.H., Cheng, W.T., Kuo, T.F., Sun, J.S., Lin, F.H., and Tsai, J.C. Fibrin glue mixed with gelatin/hyaluronic acid/chondroitin-6-sulfate tri-copolymer for articular cartilage tissue engineering: the results of real-time polymerase chain reaction. *J Biomed Mater Res A* **82**, 757, 2007.
22. Peretti, G.M., Randolph, M.A., Zaporozhan, V., Bonassar, L.J., Xu, J.W., Fellers, J.C., and Yaremchuk, M.J. A biomechanical analysis of an engineered cell-scaffold implant for cartilage repair. *Ann Plast Surg* **46**, 533, 2001.
23. Seda Tigli, R., Ghosh, S., Laha, M.M., Shevde, N.K., Daheron, L., Gimble, J., Gumusderelioglu, M., and Kaplan, D.L. Comparative chondrogenesis of human cell sources in 3D scaffolds. *J Tissue Eng Regen Med* **3**, 348, 2009.
24. Kim, H.J., Kim, U.J., Kim, H.S., Li, C., Wada, M., Leisk, G.G., and Kaplan, D.L. Bone tissue engineering with pre-mineralized silk scaffolds. *Bone* **42**, 1226, 2008.
25. Altman, G.H., Diaz, F., Jakuba, C., Calabro, T., Horan, R.L., Chen, J., Lu, H., Richmond, J., and Kaplan, D.L. Silk-based biomaterials. *Biomaterials* **24**, 401, 2003.
26. Altman, G.H., Horan, R.L., Lu, H.H., Moreau, J., Martin, I., Richmond, J.C., and Kaplan, D.L. Silk matrix for tissue engineered anterior cruciate ligaments. *Biomaterials* **23**, 4131, 2002.
27. Wang, X., Kluge, J.A., Leisk, G.G., and Kaplan, D.L. Sonication-induced gelation of silk fibroin for cell encapsulation. *Biomaterials* **29**, 1054, 2008.
28. Kim, U.J., Park, J., Kim, H.J., Wada, M., and Kaplan, D.L. Three-dimensional aqueous-derived biomaterial scaffolds from silk fibroin. *Biomaterials* **26**, 2775, 2005.
29. Kim, H.J., Kim, U.J., Vunjak-Novakovic, G., Min, B.H., and Kaplan, D.L. Influence of macroporous protein scaffolds on bone tissue engineering from bone marrow stem cells. *Biomaterials* **26**, 4442, 2005.
30. Matsumoto, A., Chen, J., Collette, A.L., Kim, U.J., Altman, G.H., Cebe, P., and Kaplan, D.L. Mechanisms of silk fibroin sol-gel transitions. *J Phys Chem B* **110**, 21630, 2006.
31. Choi, K.H., Yoo, B.S., Park, S.R., Choi, B.H., and Min, B.H. Novel imaging analysis system to measure the spatial dimension of engineered tissue construct. *Artif Organs* **34**, 158, 2009.
32. Whitley, C.B., Ridnour, M.D., Draper, K.A., Dutton, C.M., and Neglia, J.P. Diagnostic test for mucopolysaccharidosis. I. Direct method for quantifying excessive urinary glycosaminoglycan excretion. *Clin Chem* **35**, 374, 1989.
33. Rosso, F., Marino, G., Giordano, A., Barbarisi, M., Parmeggiani, D., and Barbarisi, A. Smart materials as scaffolds for tissue engineering. *J Cell Physiol* **203**, 465, 2005.
34. O'Halloran, D.M., and Pandit, A.S. Tissue-engineering approach to regenerating the intervertebral disc. *Tissue Eng* **13**, 1927, 2007.
35. Paesold, G., Nerlich, A.G., and Boos, N. Biological treatment strategies for disc degeneration: potentials and shortcomings. *Eur Spine J* **16**, 447, 2007.
36. Chen, W.H., Liu, H.Y., Lo, W.C., Wu, S.C., Chi, C.H., Chang, H.Y., Hsiao, S.H., Wu, C.H., Chiu, W.T., Chen, B.J., and Deng, W.P. Intervertebral disc regeneration in an *ex vivo* culture system using mesenchymal stem cells and platelet-rich plasma. *Biomaterials* **30**, 5523, 2009.
37. Gruber, H.E., Stasky, A.A., and Hanley, E.N., Jr. Characterization and phenotypic stability of human disc cells *in vitro*. *Matrix Biol* **16**, 285, 1997.
38. Podskubka, A., Povysil, C., Kubes, R., Sprindrich, J., and Sedlacek, R. [Treatment of deep cartilage defects of the knee with autologous chondrocyte transplantation on a hya-

- luronic Acid ester scaffolds (Hyalograft C)]. *Acta Chir Orthop Traumatol Cech* **73**, 251, 2006.
39. Robert, H., and Bahuaud, J. Autologous chondrocyte implantation. A review of techniques and preliminary results. *Rev Rhum Engl Ed* **66**, 724, 1999.
 40. Gorensek, M., Jaksimovic, C., Kregar-Velikonja, N., Gorensek, M., Knezevic, M., Jeras, M., Pavlovic, V., and Cor, A. Nucleus pulposus repair with cultured autologous elastic cartilage derived chondrocytes. *Cell Mol Biol Lett* **9**, 363, 2004.
 41. Zhang, Y., Li, Z., Thonar, E.J., An, H.S., He, T.C., Pietryla, D., and Phillips, F.M. Transduced bovine articular chondrocytes affect the metabolism of cocultured nucleus pulposus cells *in vitro*: implications for chondrocyte transplantation into the intervertebral disc. *Spine (Phila Pa 1976)* **30**, 2601, 2005.
 42. Humzah, M.D., and Soames, R.W. Human intervertebral disc: structure and function. *Anat Rec* **220**, 337, 1988.
 43. Sive, J.L., Baird, P., Jeziorski, M., Watkins, A., Hoyland, J.A., and Freemont, A.J. Expression of chondrocyte markers by cells of normal and degenerate intervertebral discs. *Mol Pathol* **55**, 91, 2002.
 44. Roberts, S., Menage, J., Duan, V., Wotton, S., and Ayad, S. Volvo Award in basic sciences. Collagen types around the cells of the intervertebral disc and cartilage end plate: an immunolocalization study. *Spine (Phila Pa 1976)* **16**, 1030, 1991.
 45. Tapp, H., Hanley, E.N., Jr., Patt, J.C., and Gruber, H.E. Adipose-derived stem cells: characterization and current application in orthopaedic tissue repair. *Exp Biol Med* (Maywood) **234**, 1, 2009.
 46. Hiyama, A., Mochida, J., and Sakai, D. Stem cell applications in intervertebral disc repair. *Cell Mol Biol (Noisy-le-grand)* **54**, 24, 2008.
 47. Gruber, H.E., Fisher, E.C., Jr., Desai, B., Stasky, A.A., Hoelscher, G., and Hanley, E.N., Jr. Human intervertebral disc cells from the annulus: three-dimensional culture in agarose or alginate and responsiveness to TGF- β 1. *Exp Cell Res* **235**, 13, 1997.
 48. Alini, M., Li, W., Markovic, P., Aebi, M., Spiro, R.C., and Roughley, P.J. The potential and limitations of a cell-seeded collagen/hyaluronan scaffold to engineer an intervertebral disc-like matrix. *Spine (Phila Pa 1976)* **28**, 446, 2003.
 49. Thompson, J.P., Oegema, T.R., Jr., and Bradford, D.S. Stimulation of mature canine intervertebral disc by growth factors. *Spine (Phila Pa 1976)* **16**, 253, 1991.
 50. Leung, V.Y., Chan, D., and Cheung, K.M. Regeneration of intervertebral disc by mesenchymal stem cells: potentials, limitations, and future direction. *Eur Spine J* **15 Suppl 3**, S406, 2006.
 51. Chiba, K., Andersson, G.B., Masuda, K., and Thonar, E.J. Metabolism of the extracellular matrix formed by intervertebral disc cells cultured in alginate. *Spine (Phila Pa 1976)* **22**, 2885, 1997.
 52. Raghunath, J., Rollo, J., Sales, K.M., Butler, P.E., and Seifalian, A.M. Biomaterials and scaffold design: key to tissue-engineering cartilage. *Biotechnol Appl Biochem* **46**, 73, 2007.
 53. Sakai, D., Mochida, J., Iwashina, T., Hiyama, A., Omi, H., Imai, M., Nakai, T., Ando, K., and Hotta, T. Regenerative effects of transplanting mesenchymal stem cells embedded in atelocollagen to the degenerated intervertebral disc. *Biomaterials* **27**, 335, 2006.
 54. Perka, C., Arnold, U., Spitzer, R.S., and Lindenhayn, K. The use of fibrin beads for tissue engineering and subsequential transplantation. *Tissue Eng* **7**, 359, 2001.
 55. Yoon, D.M., Curtis, S., Reddi, A.H., and Fisher, J.P. Addition of hyaluronic acid to alginate embedded chondrocytes interferes with IGF-1 signaling *in vitro* and *in vivo*. *Tissue Eng Part A* **15**, 3449, 2009.
 56. Perka, C., Schultz, O., Lindenhayn, K., Spitzer, R.S., Muschik, M., Sittlinger, M., and Burmester, G.R. Joint cartilage repair with transplantation of embryonic chondrocytes embedded in collagen-fibrin matrices. *Clin Exp Rheumatol* **18**, 13, 2000.
 57. Buckwalter, J.A., Smith, K.C., Kazarian, L.E., Rosenberg, L.C., and Ungar, R. Articular cartilage and intervertebral disc proteoglycans differ in structure: an electron microscopic study. *J Orthop Res* **7**, 146, 1989.
 58. Chung, C., Mesa, J., Miller, G.J., Randolph, M.A., Gill, T.J., and Burdick, J.A. Effects of auricular chondrocyte expansion on neocartilage formation in photocrosslinked hyaluronic acid networks. *Tissue Eng* **12**, 2665, 2006.
 59. Kawasaki, K., Ochi, M., Uchio, Y., Adachi, N., and Matsusaki, M. Hyaluronic acid enhances proliferation and chondroitin sulfate synthesis in cultured chondrocytes embedded in collagen gels. *J Cell Physiol* **179**, 142, 1999.
 60. Crevensten, G., Walsh, A.J., Ananthakrishnan, D., Page, P., Wahba, G.M., Lotz, J.C., and Berven, S. Intervertebral disc cell therapy for regeneration: mesenchymal stem cell implantation in rat intervertebral discs. *Ann Biomed Eng* **32**, 430, 2004.
 61. Stern, S., Lindenhayn, K., Schultz, O., and Perka, C. Cultivation of porcine cells from the nucleus pulposus in a fibrin/hyaluronic acid matrix. *Acta Orthop Scand* **71**, 496, 2000.
 62. Park, S.H., Park, S.R., and Min, B.H. Reinforcement fibrin-hyaluronic acid composite gel for tissue engineering cartilage genesis. *Key Eng Mat* **342-343**, 253, 2007.
 63. Eylich, D., Brandl, F., Appel, B., Wiese, H., Maier, G., Wenzel, M., Staudenmaier, R., Goepferich, A., and Blunk, T. Long-term stable fibrin gels for cartilage engineering. *Biomaterials* **28**, 55, 2007.
 64. Numata, K., Cebe, P., and Kaplan, D.L. Mechanism of enzymatic degradation of beta-sheet crystals. *Biomaterials* **31**, 2926, 2010.
 65. Wang, Y., Kim, U.J., Blasioli, D.J., Kim, H.J., and Kaplan, D.L. *In vitro* cartilage tissue engineering with 3D porous aqueous-derived silk scaffolds and mesenchymal stem cells. *Biomaterials* **26**, 7082, 2005.

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