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Cell-Seeded Adhesive Biomaterial for Repair of Annulus Fibrosus Defects in Intervertebral Discs

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Defects in the annulus fibrosus (AF) of intervertebral discs allow nucleus pulposus tissue to herniate causing painful disability. Microdiscectomy procedures remove herniated tissue fragments, but unrepaired defects remain allowing reherniation or progressive degeneration. Cell therapies show promise to enhance repair, but methods are undeveloped and carriers are required to prevent cell leakage. To address this challenge, this study developed and evaluated genipin-crosslinked fibrin (FibGen) as an adhesive cell carrier optimized for AF repair that can deliver cells, match AF material properties, and have low risk of extrusion during loading. Part 1 determined that feasibility of bovine AF cells encapsulated in high concentration FibGen (F140G6: 140 mg/mL fibrinogen; 6 mg/mL genipin) for 7 weeks could maintain high viability, but had little proliferation or matrix deposition. Part 2 screened tissue mechanics and *in situ* failure testing of nine FibGen formulations (fibrin: 35–140 mg/mL; genipin: 1–6 mg/mL). F140G6 formulation matched AF shear and compressive properties and significantly improved failure strength *in situ*. Formulations with reduced genipin also exhibited satisfactory material properties and failure behaviors warranting further biological screening. Part 3 screened AF cells encapsulated in four FibGen formulations for 1 week and found that reduced genipin concentrations increased cell viability and glycosaminoglycan production. F70G1 (70 mg/mL fibrinogen; 1 mg/mL genipin) demonstrated balanced biological and biomechanical performance warranting further testing. We conclude that FibGen has potential to serve as an adhesive cell carrier to repair AF defects with formulations that can be tuned to enhance biomechanical and biological performance; future studies are required to develop strategies to enhance matrix production.

Keywords: annulus fibrosus repair, intervertebral disc herniation, tissue engineering, adhesive biomaterial, fibrin, genipin

Introduction

BACK PAIN IS a chronic condition that significantly impacts quality of life and affects up to 1 billion people worldwide.^{1,2} Back pain severity and disability are strongly correlated with herniation, severe intervertebral disc (IVD) degeneration, or the presence of prominent modic changes.^{3–6} Conservative care for IVD disorders includes pain medication, spinal injections, and physical therapy, yet these treatments often have limited efficacy.^{7,8} Microdiscectomy, in which herniated tissue is removed, is the most common procedure performed after conservative care has failed for mechanical back pain and leg pain with herniation with strong symptomatic improvement compared to conservative treatment.^{9–11} However these procedures do not repair annulus fibrosus (AF)

defects created by herniation and subsequent microdiscectomy, and 10–30% of patients experience reherniation, degeneration, and/or recurrent pain at the operative level.^{12,13} We believe that biomaterials that match IVD material properties and seal AF defects may help inhibit back pain by preventing reherniation and restoring IVD biomechanical behaviors to the healthy condition.

Clinical trials utilizing cellular therapies for reducing pain from IVD degeneration show promise and it is speculated that efficacy is due to the new cells modulating inflammation or promoting repair.^{14,15} However, implantation of cells is challenging due to poor cell survival, cell leakage, and potential for unwanted off-target effects such as osteophyte formation.^{16–20} The optimal cell type for AF repair has not been established yet, although some clinical improvement has been observed for

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human clinical trials utilizing bone marrow-derived mesenchymal stem cells (MSCs),^{21,22} juvenile articular chondrocytes,²³ and IVD cells.^{24,25} The largest clinical trials thus far have used autologous IVD cells. IVD cell injections reduced symptoms of low back pain and prevented IVD height loss and suggested that development of carriers that might imbue additional potential or scaffolds that enhance placement as a basis for treatment should remain the next considerations.²⁴

Several biomaterials for IVD repair have been developed.^{26–28} Using a cell carrier biomaterial may address the challenges for IVD cell delivery by improving cell survival, preventing cell leakage, and providing a supportive scaffold for cellular growth and activity. An ideal cell carrier for AF repair would be injectable and allow easy translation to current surgical procedures, would seal irregularly shaped IVD defects to prevent or slow chemical degeneration, would restore IVD height and biomechanical behaviors, would withstand the challenging physiological mechanical loading environment of the IVD without reherniation, and would degrade slowly to enable healing to occur.^{27,29,30}

There is an unmet need to develop a biomaterial that can fulfill mechanical demands required for AF repair while enabling cell proliferation and extracellular matrix (ECM) production. Genipin-crosslinked fibrin (FibGen) fulfills many of the required mechanical roles of a cell carrier for AF repair since it is injectable, fills irregularly shaped defects, rapidly gels for easy clinical translation, can be mechanically tuned to match native AF shear properties, and partially restores IVD biomechanical behaviors following injury with little herniation risk.^{31–34} While high genipin concentrations can prevent cell growth or induce cell death,^{35–37} there is some evidence that fibrin with low genipin concentrations can promote cell survival and matrix production.³⁴ The objectives of this

study were to develop FibGen as a cell-seeded AF repair sealant and to determine optimal fibrin and genipin concentrations that meet mechanical design requirements for AF repair while also promoting cell survival and extracellular matrix production.

Materials and Methods

Study design

The three-part study design included the following. Part 1 determined the feasibility of using FibGen with high fibrin and genipin concentrations as a cell seeded hydrogel by performing a 49-day three-dimensional (3D) cell culture study that assessed bovine AF cell viability and proliferation, glycosaminoglycan (GAG) production, and hydrogel biomechanical properties (Fig. 1). Part 2 screened nine distinct FibGen acellular hydrogel formulations to identify the lowest fibrin and genipin concentrations that would match AF tissue material properties in unconfined compression and shear. Four formulations were chosen for additional *in situ* failure testing to identify the failure strength when hydrogel failure occurred. These acellular formulations were analyzed immediately following gelation. Part 3 screened biological performance of bovine AF cells encapsulated in four formulations of FibGen in a 7-day 3D cell culture experiment with assessments of cell viability, proliferation, Collagen I production, and GAG content.

Hydrogel fabrication

FibGen formulations were mixed using a dual barrel syringe with mixing tip (4:1 syringe; Pacific Dental, Walnut, CA). Fibrinogen dissolved in phosphate buffered saline (PBS) and mixed thoroughly with low glucose Dulbecco's

FIG. 1. Schematic of methods and outcome measurements. This was a three part study. Part 1 validated feasibility of using high concentration FibGen for cell seeding. Part 2 involved mechanical screening with material testing on nine Fibgen formulations and motion segment failure testing on four FibGen formulations to identify formulations capable of providing balanced mechanical and biological performance. Part 3 involved biological screening to identify which of four FibGen formulations were most amenable to cell seeding. FibGen, genipin-crosslinked fibrin. Color images available online at www.liebertpub.com/tea

TABLE 1. FibGen FORMULATION NOMENCLATURE

Final genipin concentration	Final fibrinogen concentration		
	140 mg/mL	70 mg/mL	35 mg/mL
6 mg/mL	F140G6 ^a	F70G6	F35G6
2 mg/mL	F140G2	F70G2	F35G2
1 mg/mL	F140G1 ^a	F70G1 ^a	F35G1 ^a

^aIndicates formulation selected for advanced screening. FibGen, genipin-crosslinked fibrin.

modified Eagle's medium (DMEM; Fisher Scientific, PA) with or without cells was pipetted into the large syringe barrel, and thrombin, genipin (dissolved in DMSO), and serum-free DMEM was pipetted into the small barrel. After mixing, FibGen was injected into a 5×5 mm cylindrical mold and cured for 4 h at 37°C. Formulations of fibrinogen and genipin denote final concentrations of each component (Table 1). FibGen concentrations were selected to include 140 mg/mL of fibrinogen and 6 mg/mL of genipin, which were previously optimized and analyzed for mechanical performance,^{32,35} as well as a wide range of FibGen concentrations with reduced fibrin and genipin concentration to screen for improved biological and mechanical performance for cell delivery. The formulations presented in this study are not clinically approved for use in humans.

Cell isolation and expansion

Bovine AF cells were obtained by primary isolation from skeletally mature bovine IVDs ($n=4$) obtained from a local abattoir (Green Village Packing Co., Green Village, NJ). AF cells were seeded at 4.4×10^3 cells/cm² and expanded in high glucose DMEM (Fisher Scientific), containing 10% FBS (Gemini Bio-Products, CA), 1% penicillin/streptomycin (Life Technologies, New York), and 0.2% ascorbic acid (Fisher Scientific) at 37°C, 21% O₂, 5% CO₂ in a humidified incubator.

Culture conditions

Cells between passage 3 and 5 were trypsinized with a 0.25% porcine trypsin solution (HyClone Laboratories, Logan, Utah), resuspended in serum-free DMEM, and mixed into fibrinogen by thorough pipetting. *Part 1*: AF cells from several different bovines were seeded in hydrogels (F140G6) ($n=4$ biological donors and 3 technical replicates/biological). Biological replication was used to screen biological performance in these gels. Constructs were cultured in high-glucose DMEM (supplemented with 10% FBS and 1% pen/strep) for 7, 28, or 49 days under normoxia (21% O₂) at 37°C and 5% CO₂ (Napco Series 8000 WJ; Thermo Scientific, PA). *Part 3*: AF cells ($n=1$ biological donor) were seeded in the four candidate hydrogel formulations ($n=4$ technical replicates/formulation). Technical replicates were used to screen hydrogel formulation with reduced biological variability. Constructs were cultured in low-glucose DMEM (Fisher Scientific) under hypoxia (5% O₂) at 37°C and 5% CO₂, to better approximate the low glucose environment expected in IVDs *in situ*.

Cell viability and proliferation

Cell viability and proliferation were assessed using confocal microscopy on calcein AM/DAPI dual-stained FibGen gels that were cut in half to visualize the middle of the gel. Gels were stained for 30 min in low-glucose DMEM containing 5 U/mL of calcein AM dye (Fisher Scientific), washed thrice with PBS, stained for 20 min in low-glucose DMEM containing 5 U/mL of DAPI (Fisher Scientific), and washed thrice in PBS and then stored in low-glucose DMEM until imaging. Gels were imaged at 4 μm intervals, ~120 μm in depth, at 10× magnification or 20× magnification with 2× zoom for an effective magnification of 40×. Any cell stained with calcein was considered live, and cells with DAPI staining alone (no calcein) were considered dead. Live and dead cells were counted using a custom CellProfiler script (www.cellprofiler.org), which was confirmed with manual counts. Cells were counted in three separate areas of the 10× image per flattened stack and then averaged. Multiple stacks from gels that were seeded on the same day were averaged.

Cell proliferation was also assessed quantitatively in Part 3 by measuring the amount of DNA (QuantiFluor, Promega, WI) present at day 1 and 7 on digested FibGen samples and normalizing the amount of DNA to the weight of each gel. FibGen was digested for 2 days in a papain digestion solution (0.3 mg/mL papain, 3 mg/mL EDTA, 0.12 mg/mL L-cysteine, 8.2 mg/mL Sodium acetate; Sigma-Aldrich, Saint Louis, MO) at 65°C. Cell proliferation levels within FibGen were also assessed in a semiquantitative manner by means of immunohistochemistry (IHC; see Immunohistochemistry section). Manual cell counting of immunostained samples was completed as follows: cells without any chromogen presence were considered immunonegative, cells with chromogen present in the cytoplasm but not nucleus were considered immunonegative, and cells with chromogen present in the nucleus and cytoplasm or nucleus alone were considered immunopositive.

Immunohistochemistry

Chromogenic IHC was performed to assess Collagen I (COL1) production and cellular proliferation (Ki67) after 1 week of culture (Part 3 of this study). Day 7 cell-laden FibGen samples were fixed in aqueous buffered zinc formalin fixative (Anatech Ltd., Battle Creek, MI). After fixation and processing, samples were embedded in paraffin wax using Leica HistoCore Arcadia H and C. Blocks were then sectioned using a Leica RM 2165 Rotary Microtome to produce 5 μm thick sections mounted on Millennia 1000 Adhesion Slides (Cat. No. M1000W; StatLab Medical Products, McKinney, TX). Slides were heated in a dry incubator at 60°C overnight before deparaffinizing and immunostaining.

Slides were deparaffinized by consecutive rinsing in petroleum ether solution (Cat. No. E139-4; Fisher Chemical, Fair Lawn, NJ) and ethylene glycol monomethyl ether solution (Cat. No. E180-4; Fisher Chemical). Enzymatic antigen retrieval was then performed by applying Histo/Zyme pH 7.2 Antigen Retriever (Cat. No. H3292; Sigma-Aldrich) to sections for 3 min at room temperature. Once completed, samples were rinsed thrice with sterile ultrapure water (ddH₂O), and Dako Serum-Free Protein Block (Cat. No. X0909; Dako North America, Carpinteria, CA) was applied

to sections for 30 min at room temperature. After half an hour of blocking, excess blocking solution was shaken off, and primary antibody was applied to sections at the appropriate dilutions. To assess collagen I elaboration, rabbit polyclonal anti-collagen 1 (ab34710; Abcam, Cambridge, MA) was used at a dilution of 1:150 (6.66 $\mu\text{g/mL}$) in Dako Antibody Diluent with Background Reducing Components (Cat. No. S3022; Dako North America). To assess cell proliferation, rabbit polyclonal anti-Ki67 (Cat. No. ab15580; Abcam) was used at a dilution of 1:3000 (0.3 $\mu\text{g/mL}$) in antibody diluent solution. Samples were incubated with primary antibody overnight at 4°C in a humidity chamber.

Once primary antibody incubation was complete, samples were dipped in 1 $\times$ PBS-Tween[®] 20 (0.01 M NaPO₄, 0.15 M NaCl, 0.05% Tween 20) (Cat. No. 28352; Pierce Biotechnology, Rockford, IL) thrice and consecutively washed with ddH₂O thrice. Secondary antibody (ImmPRESS[™] HRP Goat Anti-Rabbit IgG) (Cat. No. MP-7451; Vector Laboratories, Burlingame, CA) was then applied to sections for 30 min at room temperature. After the completion of secondary antibody incubation, samples were dipped in 1 $\times$ PBS-Tween 20 thrice and consecutively washed with ddH₂O thrice. After washing, slides were incubated in 10% hydrogen peroxide solution (Cat. No. H325-500; Fisher Chemical) for 10 min at room temperature. Slides were then rinsed thrice with ddH₂O, and chromogen (ImmPACT DAB Peroxidase Substrate) (Cat. No. SK-4105; Vector Laboratories) was applied for 90 s at room temperature. After chromogen incubation, slides were dipped in 1 $\times$ PBS-Tween 20 thrice and consecutively washed with ddH₂O thrice.

Samples were then counterstained with Toluidine Blue O solution (Cat. No. 198161; Sigma-Aldrich) for 2 min at room temperature. Once counterstaining was completed, slides were rinsed thrice with ddH₂O and dehydrated by consecutive rinsing with ethylene glycol monomethyl ether solution and xylene (Cat. No. X3P; Fisher Chemical). After dehydration, Eukitt Mounting Medium (Cat. No. 15320; Electron Microscopy Sciences, Hatfield, PA) and Fisherfinest[®] Premium Cover Glass (Cat. No. 12-544-14; Fisher Scientific, Fair Lawn, NJ) were applied to slides. All samples were imaged using bright-field microscopy on the Zeiss Axio Imager Z1 (Carl Zeiss Microscopy GmbH, Jena, Germany).

To assess the validity of the immunostaining patterns, antigen/positive tissue controls and reagent controls were incorporated. For anti-Collagen 1, the mouse IVD was included alongside experimental samples (outer AF structure). For anti-Ki67, the mouse small intestine was included alongside experimental samples (Crypts of Lieberkühn). Reagent control conditions for experimental and positive tissue control samples involved a secondary antibody-only application to ensure that antigen detection was strictly due to the primary antibody.

Matrix production

Visual assessment of pericellular matrix production was performed using scanning electron microscopy (SEM). FibGen gels ($n=3$) were fixed in 3% glutaraldehyde followed by 1% Osmium Tetroxide and then dehydrated in ethyl alcohol and embedded in EMbed 812 for TEM (H760 TEM; Hitachi High Technologies, Northridge, CA) or dried and gold sputtered for SEM (5 kV, S4300 SEM; Hitachi High Technologies).

Quantitative assessment of GAG content produced by cells over time was performed using Blyscan Assay (UK Biocolor, Carrickfergus, UK) on FibGen digests according to the manufacturer's protocol.

A semiquantitative analysis was completed on the Collagen 1 immunostained samples to determine if there were any significant differences in Collagen 1 production when screening FibGen formulations. Manual cell counting of immunostained samples was completed as follows: cells without any chromogen presence in the pericellular space were considered immunonegative, and cells with chromogen present in the pericellular space were considered immunopositive.

Biomechanics and formulation screening

Biomechanical and degradation properties of FibGen F140G6 hydrogels from Part 1 were assessed by measuring dry weight at each time point and normalizing to day 1 values of dry weight. Young's compressive modulus was calculated from unconfined compression testing at a 0.2% strain/s rate using the initial 20% strain (ELF 3200; TA Instruments, New Castle, DE). Complex shear modulus ($|G^*|$, 0.5 Hz) was measured using a frequency sweep (0.05 to 5 Hz at 1% strain) on a parallel plate rheometer on 5 mm diameter and 5 mm height gels (AR2000ex; TA Instruments). Sample size $n=4$ was used for biomechanical measurements in Part 1 and Part 2 studies.

In situ failure testing

Bovine caudal IVD motion segments were isolated, and ligaments and tendons were removed to expose the AF. All IVDs were frozen at -20°C until use. The motion segments ($n=9$ /formulation group) underwent two parallel cuts (Iso-Met 1000; Buehler, Lake Bluff, IL) 3 mm away from each end plate and then thawed at 37°C for 2 h. A 4 mm biopsy punch was used to create an injury on the left posterolateral side of the IVD, and the nucleus pulposus (NP) was disrupted with a curette for 1 min before 90–120 mg of NP tissue was removed with a rongeur. Repaired groups were filled with F140G6, F140G1, or F70G1 FibGen formulation shortly after injury. All groups were submerged in PBS overnight at 37°C before mechanical testing. IVD height and diameter were measured, and specimens were placed in the mechanical testing system (Bionix 370; MTS Systems Corporation, Eden Prairie, MN) with the posterolateral aspect of the IVD aligned with the 5° incline of the stage. The specimen underwent a 25 N compressive preload and was then compressed at a rate of 2 mm/min until failure, defined as end plate cracking or greater than 2 mm protrusion of NP tissue or FibGen from the injury. Videos were recorded for each test run to confirm failure strength measurements by identifying that material extrusion occurred during dips in the stress-displacement curves. Failure strength was calculated from the force exerted at failure divided by the cross-sectional area of the IVD, and subsidence to failure was defined as the displacement to failure. The upper range of physiological stress values experienced by human lumbar IVDs was calculated from estimated compressive forces applied during strenuous exercise^{38,39} divided by lumbar IVD cross-sectional area,^{40,41} mirroring the calculation for experimental failure strength.

FIG. 2. AF cells retained high viability in FibGen F140G6. AF cells retained high viability in FibGen. (A) Confocal microscopy images of Calcein/DAPI stained gels indicate that cells maintained high viability (B) with no difference over time ($p > 0.69$) through 49 days, but no significant change ($p = 0.78$) in the number of cells (C) was observed. Scale bar = 50 μm . AF, annulus fibrosus. Color images available online at www.liebertpub.com/tea

Statistics

Statistical analysis was performed using GraphPad Prism software, and results are represented as mean $\pm$ standard deviation. One-way analysis of variance (ANOVA) with Tukey's *post hoc* test with significance of $p < 0.05$ was used. Two-way ANOVA was used in Part 2 to analyze the interaction of fibrin and genipin concentrations on compressive and shear moduli.

Results

Feasibility of FibGen as a cell carrier

Bovine AF cells seeded within FibGen had high viability above 85% with no significant change through day 49 ($p > 0.69$), but notably had no increase in cell number ($p = 0.78$) (Fig. 2). These seeded bovine AF cells produced extracellular matrix that was localized to pericellular regions within FibGen as observed by SEM (Fig. 3). However GAG production was not significantly increased as measured by Blyscan ($p = 0.74$). Dry weight and compressive modulus of both cell seeded and acellular gels remained stable for up to 49 days with no effect of cell seeding ($p > 0.17$) (Fig. 4).

FibGen formulation screening

Compression and shear testing demonstrated that the biomechanical properties of FibGen could be adjusted by varying the concentrations of fibrin and genipin (Table 1 and Fig. 5). Two-way ANOVA detected significant effects of fibrin concentration ($p < 0.0001$), genipin concentration ($p = 0.0009$), and significant interactions ($p = 0.016$). At 140 mg/mL of fibrin, genipin concentration had a significant effect on compressive and shear moduli when going from F140G6 to F140G2 ($p = 0.027$) or F140G1 ($p = 0.0002$), but no differences were observed between F140G2 and F140G1. Modulus values for F70 and F35 groups were insensitive to decreasing genipin concentrations. For compressive

modulus, decreasing fibrin concentration from 140 to 70 mg/mL had no effect for a given genipin concentration, but using 35 mg/mL of fibrin decreased the compressive modulus when using 6 or 2 mg/mL of genipin ($p < 0.0033$). For shear modulus, decreasing fibrin concentration at a given genipin concentration significantly reduced modulus values ($p < 0.0029$) (Fig. 5B).

FIG. 3. AF cells deposited small amounts of matrix. (A) SEM images of a bovine AF cell encapsulated in FibGen at day 1 compared to day 49 suggested minimal matrix production around the cell. (B) Blyscan assay to measure GAG indicated no significant increase ($p = 0.74$) over 49 days. Scale bar = 10 μm . GAG, glycosaminoglycan; SEM, scanning electron microscopy. Color images available online at www.liebertpub.com/tea

FIG. 4. Biomaterial properties of FibGen were unaffected by cells. Cells seeded in FibGen had no effect on gel degradation or biomechanics. **(A)** No significant change in dry weight over time ($p=0.17$). **(B)** No significant change in compressive modulus ($p=0.46$).

In situ failure testing

The four candidate FibGen formulations were easily injected into large IVD defects. Greater attention was required when handling high macromer and crosslinking formulations to ensure that no bubbles formed within the hydrogels. All intact samples failed through end plate fracture which was identified as a drop in the stress-displacement curve with no visual sign of herniation (Fig. 6A). Injured and repair samples failed through herniation of NP tissue which was confirmed visually and with a corresponding abrupt drop in the stress-displacement curve (Fig. 6B). Failure strength (Fig. 6C) and subsidence to failure (Fig. 6D) for all injured and FibGen repaired samples were significantly lower than intact samples ($p<0.0001$). F140G6 significantly improved those parameters compared to the injured group ($p=0.0343$).

Biological screening of FibGen formulations

Bovine AF cells were seeded in four FibGen formulations for 7 days and were assessed for viability, proliferation, Collagen 1 production, and GAG content. Viability increased

as FibGen concentration decreased, with viability of cells in F35G1 significantly greater than viability within F140G6 ($p=0.014$) (Fig. 7A, C). Cell counts were greater in lower concentration FibGen compared to F140G6, with F70G1 having significantly more cells than F140G6 at day 1 ($p=0.014$) (Fig. 7D). However, no groups displayed an increase of cells within this time frame. DNA quantification displayed similar trends where F70G1 and F35G1 had significantly greater DNA than F140G6 at day 1 ($p<0.0469$). DNA in F35G1 was also significantly greater than F140G1 at day 1 ($p=0.0047$) (Fig. 7E). Day 7 DNA quantification data agreed with semi-quantitative Ki67 IHC data, as no significant differences were found in Ki67 immunopositivity across all screened FibGen formulations ($p>0.3$) (Fig. 7B). Semiquantitative analysis of Collagen 1 IHC (Fig. 8A) showed a significant increase in Collagen 1 production as fibrin concentration decreased from 140 to 70 mg/mL (F140G6 vs. F70G1, $p=0.003$; F140G1 vs. F70G1, $p=0.0004$) and 35 mg/mL (F140G6 vs. F35G1, $p=0.0004$; F140G1 vs. F35G1, $p<0.0001$), but not when genipin concentration decreased (F140G6 vs. F140G1, $p=0.275$) (Fig. 8B). GAG content

FIG. 5. Material properties of nine FibGen formulations with varying fibrin and genipin concentrations. Young's compressive modulus **(A)** was significantly different between F140 and F35, as well as F70 and F35, at genipin concentrations G6 and G2 ($p<0.0033$). Complex shear modulus **(B)** was significantly different among F140, F70, and F35 at each genipin concentration ($p<0.0029$). Two-way ANOVA had an overall significant effect due to fibrinogen concentration ($p<0.0001$), genipin concentration ($p=0.0009$), and interaction between the two ($p=0.016$), for both compressive and shear modulus values. Horizontal gray band represents range of human physiological values. ANOVA, analysis of variance.

FIG. 6. Bovine IVD motion segments repaired with F140G6 had decreased herniation risk. Representative stress displacement curves of Intact and F140G6 samples, which show the two modes of failure: End plate fracture (**A**) and NP herniation (**B**). Failure strength (**C**) and subsidence to failure (**D**) of bovine IVD motion segments were significantly reduced for the Injured and all FibGen repair groups ($p < 0.0001$). The F140G6 repair significantly reduced herniation risk by increasing failure strength and subsidence to failure compared to the Injured group ($p = 0.034$). * = $p < 0.05$ compared to Intact group, $bar = p < 0.05$, horizontal gray bar = range of human physiological values. IVD, intervertebral disc. Color images available online at www.liebertpub.com/tea

was significantly increased for F70G1 ($p = 0.04$) compared to F140G6 (Fig. 8C).

Discussion

This study developed FibGen hydrogel as a cell carrier of bovine AF cells and optimized multiple formulations with mechanical and biological tests. The first feasibility test demonstrated that AF cells maintained high viability and displayed signs of limited matrix production in F140G6. Biomechanical screening tests demonstrated F140G6 matched AF shear and compressive properties while significantly reducing herniation risk. FibGen formulations with lower fibrin and genipin concentrations had increased cell viability and GAG production but inferior mechanical performance. F70G1 was a formulation that balanced acceptable mechanical and biological performance.

Part 1 demonstrated that FibGen maintained live AF cells for 49 days, but produced little matrix beyond some pericellular matrix observed on SEM. Cytocompatibility of FibGen has previously been demonstrated with two-dimensional cell culture, *in vivo* subcutaneous implantation, and organ

culture studies,^{32,35} yet had not been evaluated with encapsulated cells. Bovine AF cells seeded within FibGen remained highly viable through 49 days; however, cell counts did not increase which suggests that no cell proliferation occurred. We considered any calcein stained cell to be live in this study but noted that 20–40% of the cells were dual stained for calcein and DAPI. We believe that dual staining occurred when live cells were damaged from exposure to vital stains and imaging procedures. However, it is also possible that genipin crosslinking damaged the AF cells. Signs of matrix production surrounding a cell could be seen in the day 49 SEM images, but GAG quantification with Blyscan assay indicated no significant changes over time. AF cells mainly produce collagens and it is possible that the matrix observed in SEM images consisted mainly of collagen, which was not measured in this part of the study, and it was apparent that extracellular matrix production was limited.

Biomechanical performance of F140G6 FibGen gels was excellent and showed no significant changes in compressive modulus over time or between acellular and cell-seeded hydrogels. The lack of substantial matrix production in F140G6

FIG. 7. FibGen with lower concentrations had higher viability at day 7 and cell proliferation at day 1 than the F140G6 formulation. **(A)** Confocal images of Calcein/DAPI stained FibGen for four formulations at day 1 and 7. Scale bar = 50 μ m. **(C)** Semiquantification of day 7 confocal images in **(A)** indicated a significant increase in viability for F35G1 than F140G6 ($p=0.014$). **(B)** Ki67 immunostaining of the four selected FibGen formulations featured no significant differences in immunopositivity ($p>0.3$). **(D)** Cell count normalized by area, with a significant difference between F140G6 and F70G1 at day 1 ($p=0.014$). **(E)** DNA quantification normalized by mass of gel with significant differences between F140 groups and F70 and F35 groups at day 1 ($p<0.047$). Color images available online at www.liebertpub.com/tea

may have been due to the high concentration of macromolecules surrounding the cell, as seen in the day 1 SEM images, which limited cell motility, proliferation, and matrix production for the duration of the 49-day experiment. Genipin crosslinking provided a stable and stiff hydrogel, which might account for the limited biological cell activity once embedded. It is notable that the mechanics of this FibGen formulation were stable with time also confirming that little degradation occurred.

The lack of proliferation or significant extracellular matrix production in F140G6 gels motivated the reformulation of FibGen to increase biological performance. Part 2 screened multiple FibGen formulations with biomaterial testing and *in situ* failure testing to identify formulations with reduced fibrin and/or genipin concentrations that retained effective mechanical performance. Studies of other cell-seeded hydrogels have shown similar observations that decreasing macromer concentration and crosslinking density of the hydrogel can increase matrix production.^{42,43} Although the shear and compressive moduli values for the FibGen formulations with lower concentrations fell below physiological values for AF tissues, it is hoped that increased

extracellular matrix production by cells within these gels may enhance the mechanics over time. The compressive and shear moduli of FibGen gels were less affected by genipin concentration than fibrin concentration. As a result, three additional FibGen formulations at 1 mg/mL genipin concentration with three different fibrin concentrations were selected for continued evaluation with biological screening to enable comparisons of distinct fibrin and genipin concentration effects on mechanical and biological performance. This decision was further supported by knowledge that genipin has dose-dependent cytotoxicity,³⁷ so that decreasing the amount of genipin within FibGen was considered an important variable for biological screening.

A key mechanical design requirement for safety of an AF repair is its ability to reduce or prevent herniation of the NP once injected *in situ*. Cyclic biomechanical studies at physiological loads have been conducted using acellular F140G6, and results indicated restoration of many biomechanical properties with little herniation risk.^{32,33} This study investigated the ultimate failure strength of FibGen using bovine motion segments injured with a 4 mm biopsy punch and compressed at 5° posterolateral flexion to controllably

FIG. 8. Assessment of ECM content indicated that lower FibGen concentrations supported more ECM elaboration after 1 week of culture. **(A)** IHC for Collagen Type 1 across four candidate gel formulations. Green and red arrows indicate representative immunopositive and immunonegative cells, respectively. **(B)** Semiquantitative analysis of COL1 immunopositivity, with a significant difference between low and high FibGen concentrations ($p \leq 0.003$). **(C)** F70G1 gels had increased GAG content at day 7 compared to day 1 ($p = 0.040$). ECM, extracellular matrix; IHC, immunohistochemistry. Color images available online at www.liebertpub.com/tea

provoke herniation in a worst-case scenario.⁴⁴ *In vivo* compressive axial loads in humans are estimated at 230–1300N during daily activities and up to 3700N while lifting 180N,^{38,39} which can induce intradiscal pressures of ~ 2.3 MPa.⁴⁵ The failure strength and subsidence to failure of acellular F140G6 were significantly greater than injured samples and exhibited failure strength values greater than the physiological axial compressive stresses experienced by human lumbar IVDs during moderate physical activity. The three reduced-concentration FibGen formulations had failure strengths that were not significantly greater than injured values, but the mean values for F140G1 and F70G1 were in the upper range of physiological values. If cells seeded within these lower concentration hydrogels are able to produce matrix, then it is likely that biomechanical performance and failure strength would also improve during a healing process when the patient's activities would be more limited. As a result, we believe that some short-term compromise of biomechanical performance is acceptable if there is good evidence of improved cellular performance.

Part 3 assessed whether the lower concentrations of fibrin and genipin affected cell viability, proliferation, and Collagen I production and GAG content in a 7-day cell culture study. Cell viability increased as FibGen concentration decreased, with F35G1 significantly greater than F140G6. The nonsignificant increase from F140G6 to F140G1 may indicate reduced cytotoxicity at lower genipin concentrations. The trend of increasing viability with decreasing fibrin concentration may be attributed to a lower hydrogel density, and it is known that reducing fibrin concentration can enhance cellular proliferation and chemotaxis.^{46,47} Cell counts, DNA quantification, and Ki67 IHC indicated no suggestion of cell proliferation over the 7-day culture period, and it is

notable that the concentrations of fibrin used in our studies on AF repair are substantially greater than fibrin-based biomaterials for most other purposes.⁴⁶ There were more cells/mm² in the 1 mg/mL genipin concentration formulations than F140G6, which suggests some genipin cytotoxicity during gel creation.

This three-part study has some limitations requiring further investigation before translation to humans. IVD cells and MSCs are both commonly used for IVD repair.¹⁴ This study selected bovine AF cells for seeding since they are readily available, are terminally differentiated, and have lower variability than human AF cells. Although bovine AF cells were convenient for formulation screening and optimization, translation for human applications will require investigations with human cells. Harvesting autologous AF cells is challenging due to the additional surgery needed to acquire native AF tissue, which has limited availability and cellularity. MSCs, adipose-derived stem cells, or induced pluripotent cells may be more easily translatable from autologous cell sources and also warrant future investigation.^{14,48–51} The limited matrix elaboration and spherical AF cell morphology highlight a need to better understand cell-material interactions in this material, perhaps with the addition of cell adhesion molecules. Biological performance of cells is also likely to be improved with delivery of growth factors or cytokines (e.g., TGF- β , GDF-5), as has been used in IVD tissue engineering.^{52,53} Consequently, future studies optimizing cell source, cell adhesion molecules, and growth factor supplementation are required before additional long-term culture with greater numbers of biological replicates and eventual *in vivo* studies.

Multiple AF repair biomaterials are available with demonstrated capacity to fill voids from AF defects and restore

IVD volume, and in some cases they can also restore biomechanical properties of the motion segment and produce extracellular matrix.^{26,54–59} Biological performance of some AF repair biomaterials surpasses the cell-seeded FibGen described in the present study. However, many of these biomaterials with strong biological performance have failure behaviors that have not been evaluated and are expected to be poor due to relatively low moduli values. We apply a screening methodology in this study that most strongly focused on biomechanical performance and reherniation risk²⁹ to prioritize safety and reduce need for a potential surgical revision procedure. Cell delivery with AF biomaterials or NP replacement biomaterials with greater biological performance and inferior biomechanical performance can be considered^{27,28,30} particularly if augmented with an AF closure device that helps to reduce reherniation risk.⁶⁰ Repair biomaterials or patches that mimic the AF fibrillar structure can also better match tensile properties of the AF,^{61,62} yet adhesion and integration with native tissues remain a challenge. The appeal of enhancing cell-seeded FibGen performance is that it is an adhesive void-filling biomaterial that can be delivered in a simple single injection procedure and has low herniation risk.

We conclude that FibGen is a tunable AF sealant with potential for cell delivery to promote AF repair after microdiscectomy procedures or during intradiscal injection procedures. This study screened nine FibGen formulations to arrive at two formulations deserving additional investigation. F140G6 had excellent initial and long-term mechanical performance. While cells can survive within this formulation, there was little matrix production, so that this material may be best suited as an acellular adhesive AF repair biomaterial. F70G1 had balanced performance with acceptable material properties and failure behaviors and early promise for GAG production. Future studies are required to evaluate if growth factor supplementation can enhance matrix production of AF cells or other cell types seeded in FibGen. Long-term organ culture or *in vivo* experiments are also required to investigate if cell-seeded FibGen hydrogels have sufficient mechanical and biological performance to achieve initial biomechanical stabilization while enabling long-term healing and AF repair.

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Disclosure Statement

No competing financial interests exist. The material described is covered under US patents (US CIP Application 13/907,442 filed May 31, 2013. US Publication No. US-2013-0345321 A1 and US Divisional Application 14/483,284 filed September 11, 2014. US Publication No. 2015-0004138 A1). No licensing agreement exists.

References

1. Balagué, F., Mannion, A.F., Pellisé, F., and Cedraschi, C. Non-specific low back pain. *Lancet* **379**, 482, 2012.
2. Hoy, D., March, L., Brooks, P., *et al.* The global burden of low back pain: estimates from the Global Burden of Disease 2010 study. *Ann Rheum Dis* **73**, 968, 2014.
3. Adams, M.A., and Dolan, P. Intervertebral disc degeneration: evidence for two distinct phenotypes. *J Anat* **221**, 497, 2012.
4. Livshits, G., Popham, M., Malkin, I., *et al.* Lumbar disc degeneration and genetic factors are the main risk factors for low back pain in women: the UK Twin Spine Study. *Ann Rheum Dis* **70**, 1740, 2011.
5. Mok, F.P.S., Samartzis, D., Karppinen, J., Fong, D.Y.T., Luk, K.D.K., and Cheung, K.M.C. Modic changes of the lumbar spine: prevalence, risk factors, and association with disc degeneration and low back pain in a large-scale population-based cohort. *Spine J* **16**, 32, 2016.
6. Samartzis, D., Karppinen, J., Mok, F., Fong, D.Y.T., Luk, K.D.K., and Cheung, K.M.C. A population-based study of juvenile disc degeneration and its association with overweight and obesity, low back pain, and diminished functional status. *J Bone Joint Surg Am* **93**, 662, 2011.
7. Lu, Y., Guzman, J.Z., Purmessur, D., *et al.* Nonoperative management of discogenic back pain: a systematic review. *Spine* **39**, 1314, 2014.
8. Koes, B.W., van Tulder, M., Lin, C.-W.C., Macedo, L.G., McAuley, J., and Maher, C. An updated overview of clinical guidelines for the management of non-specific low back pain in primary care. *Eur Spine J* **19**, 2075, 2010.
9. Abdu, R.W., Abdu, W.A., Pearson, A.M., Zhao, W., Lurie, J.D., and Weinstein, J.N. Reoperation for recurrent intervertebral disc herniation in the spine patient outcomes research trial: analysis of rate, risk factors, and outcome. *Spine* **42**, 1106, 2017.
10. Lurie, J.D., Tosteson, T.D., Tosteson, A.N.A., *et al.* Surgical versus nonoperative treatment for lumbar disc herniation: eight-year results for the spine patient outcomes research trial. *Spine* **39**, 3, 2014.
11. Weinstein, J.N., Lurie, J.D., Tosteson, T.D., *et al.* Surgical vs nonoperative treatment for lumbar disk herniation: the Spine Patient Outcomes Research Trial (SPORT) observational cohort. *JAMA* **296**, 2451, 2006.
12. Carragee, E.J., Han, M.Y., Suen, P.W., and Kim, D. Clinical outcomes after lumbar discectomy for sciatica: the effects of fragment type and anular competence. *J Bone Joint Surg Am* **85-A**, 102, 2003.
13. McGirt, M.J., Eustacchio, S., Varga, P., *et al.* A prospective cohort study of close interval computed tomography and magnetic resonance imaging after primary lumbar discectomy: factors associated with recurrent disc herniation and disc height loss. *Spine* **34**, 2044, 2009.
14. Sakai, D., and Andersson, G.B.J. Stem cell therapy for intervertebral disc regeneration: obstacles and solutions. *Nat Rev Rheumatol* **11**, 243, 2015.
15. Benneker, L.M., Andersson, G., Iatridis, J.C., *et al.* Cell therapy for intervertebral disc repair: advancing cell therapy from bench to clinics. *Eur Cell Mater* **27**, 5, 2014.
16. Vadalà, G., Sowa, G., Hubert, M., Gilbertson, L.G., Denaro, V., and Kang, J.D. Mesenchymal stem cells injection in degenerated intervertebral disc: cell leakage may induce osteophyte formation. *J Tissue Eng Regen Med* **6**, 348, 2012.
17. Li, Y.Y., Diao, H.J., Chik, T.K., *et al.* Delivering mesenchymal stem cells in collagen microsphere carriers to rabbit degenerative disc: reduced risk of osteophyte formation. *Tissue Eng Part A* **20**, 1379, 2014.

18. Bertram, H., Kroeber, M., Wang, H., *et al.* Matrix-assisted cell transfer for intervertebral disc cell therapy. *Biochem Biophys Res Commun* **331**, 1185, 2005.
19. Tong, W., Lu, Z., Qin, L., *et al.* Cell therapy for the degenerating intervertebral disc. *Transl Res* **181**, 49, 2017.
20. Kandel, R., Roberts, S., and Urban, J.P.G. Tissue engineering and the intervertebral disc: the challenges. *Eur Spine J* **17 Suppl 4**, 480, 2008.
21. Orozco, L., Soler, R., Morera, C., Alberca, M., Sánchez, A., and García-Sancho, J. Intervertebral disc repair by autologous mesenchymal bone marrow cells: a pilot study. *Transplantation* **92**, 822, 2011.
22. Yoshikawa, T., Ueda, Y., Miyazaki, K., Koizumi, M., and Takakura, Y. Disc regeneration therapy using marrow mesenchymal cell transplantation: a report of two case studies. *Spine* **35**, E475, 2010.
23. Coric, D., Pettine, K., Sumich, A., and Boltes, M.O. Prospective study of disc repair with allogeneic chondrocytes presented at the 2012 Joint Spine Section Meeting. *J Neurosurg Spine* **18**, 85, 2013.
24. Meisel, H.J., Ganey, T., Hutton, W.C., Libera, J., Minkus, Y., and Alasevic, O. Clinical experience in cell-based therapeutics: intervention and outcome. *Eur Spine J* **15 Suppl 3**, S397, 2006.
25. Meisel, H.J., Siodla, V., Ganey, T., Minkus, Y., Hutton, W.C., and Alasevic, O.J. Clinical experience in cell-based therapeutics: disc chondrocyte transplantation A treatment for degenerated or damaged intervertebral disc. *Biomol Eng*, **24**, 5, 2007.
26. Bowles, R.D., and Setton, L.A. Biomaterials for intervertebral disc regeneration and repair. *Biomaterials* **129**, 54, 2017.
27. Iatridis, J.C., Nicoll, S.B., Michalek, A.J., Walter, B.A., and Gupta, M.S. Role of biomechanics in intervertebral disc degeneration and regenerative therapies: what needs repairing in the disc and what are promising biomaterials for its repair? *Spine J* **13**, 243, 2013.
28. Moriguchi, Y., Alimi, M., Khair, T., *et al.* Biological treatment approaches for degenerative disk disease: a literature review of in vivo animal and clinical data. *Global Spine J* **6**, 497, 2016.
29. Long, R.G., Torre, O.M., Hom, W.W., Assael, D.J., and Iatridis, J.C. Design requirements for annulus fibrosus repair: review of forces, displacements, and material properties of the intervertebral disk and a summary of candidate hydrogels for repair. *J Biomech Eng* **138**, 021007, 2016.
30. Guterl, C.C., See, E.Y., Blanquer, S.B.G., *et al.* Challenges and strategies in the repair of ruptured annulus fibrosus. *Eur Cell Mater* **25**, 1, 2013.
31. Schek, R.M., Michalek, A.J., and Iatridis, J.C. Genipin-crosslinked fibrin hydrogels as a potential adhesive to augment intervertebral disc annulus repair. *Eur Cell Mater* **21**, 373, 2011.
32. Likhitpanichkul, M., Dreischarf, M., Illien-Junger, S., *et al.* Fibrin-genipin adhesive hydrogel for annulus fibrosus repair: performance evaluation with large animal organ culture, in situ biomechanics, and in vivo degradation tests. *Eur Cell Mater* **28**, 25, 2014.
33. Long, R.G., Bürki, A., Zysset, P., *et al.* Mechanical restoration and failure analyses of a hydrogel and scaffold composite strategy for annulus fibrosus repair. *Acta Biomater* **30**, 116, 2016.
34. Dare, E.V., Griffith, M., Poitras, P., *et al.* Fibrin sealants from fresh or fresh/frozen plasma as scaffolds for in vitro articular cartilage regeneration. *Tissue Eng Part A* **15**, 2285, 2009.
35. Guterl, C.C., Torre, O.M., Purmessur, D., *et al.* Characterization of mechanics and cytocompatibility of fibrin-genipin annulus fibrosus sealant with the addition of cell adhesion molecules. *Tissue Eng Part A* **20**, 2536, 2014.
36. Koo, H.-J., Lim, K.-H., Jung, H.-J., and Park, E.-H. Anti-inflammatory evaluation of gardenia extract, geniposide and genipin. *J Ethnopharmacol* **103**, 496, 2006.
37. Wang, C., Lau, T.T., Loh, W.L., Su, K., and Wang, D.-A. Cytocompatibility study of a natural biomaterial crosslinker—genipin with therapeutic model cells. *J Biomed Mater Res Part B Appl Biomater* **97**, 58, 2011.
38. Han, J.S., Goel, V.K., Ahn, J.Y., *et al.* Loads in the spinal structures during lifting: development of a three-dimensional comprehensive biomechanical model. *Eur Spine J* **4**, 153, 1995.
39. Dreischarf, M., Shirazi-Adl, A., Arjmand, N., Rohlmann, A., and Schmidt, H. Estimation of loads on human lumbar spine: a review of in vivo and computational model studies. *J Biomech* **49**, 833, 2016.
40. Pooni, J.S., Hukins, D.W., Harris, P.F., Hilton, R.C., and Davies, K.E. Comparison of the structure of human intervertebral discs in the cervical, thoracic and lumbar regions of the spine. *Surg Radiol Anat* **8**, 175, 1986.
41. Natarajan, R.N., and Andersson, G.B. The influence of lumbar disc height and cross-sectional area on the mechanical response of the disc to physiologic loading. *Spine* **24**, 1873, 1999.
42. Gupta, M.S., and Nicoll, S.B. Functional nucleus pulposus-like matrix assembly by human mesenchymal stromal cells is directed by macromer concentration in photocrosslinked carboxymethylcellulose hydrogels. *Cell Tissue Res* **358**, 527, 2014.
43. Lin, H.A., Gupta, M.S., Varma, D.M., Gilchrist, M.L., and Nicoll, S.B. Lower crosslinking density enhances functional nucleus pulposus-like matrix elaboration by human mesenchymal stem cells in carboxymethylcellulose hydrogels. *J Biomed Mater Res A* **104**, 165, 2016.
44. Vergroesen, P.-P.A., Bochynska, A.I., Emanuel, K.S., *et al.* A biodegradable glue for annulus closure: evaluation of strength and endurance. *Spine* **40**, 622, 2015.
45. Wilke, H.J., Neef, P., Caimi, M., Hoogland, T., and Claes, L.E. New in vivo measurements of pressures in the intervertebral disc in daily life. *Spine* **24**, 755, 1999.
46. Brown, A.C., and Barker, T.H. Fibrin-based biomaterials: modulation of macroscopic properties through rational design at the molecular level. *Acta Biomater* **10**, 1502, 2014.
47. Hanson, A.J., and Quinn, M.T. Effect of fibrin sealant composition on human neutrophil chemotaxis. *J Biomed Mater Res* **61**, 474, 2002.
48. Pirvu, T., Blanquer, S.B.G., Benneker, L.M., *et al.* A combined biomaterial and cellular approach for annulus fibrosus rupture repair. *Biomaterials* **42**, 11, 2015.
49. Liu, Y., Fu, S., Rahaman, M.N., Mao, J.J., and Bal, B.S. Native nucleus pulposus tissue matrix promotes notochordal differentiation of human induced pluripotent stem cells with potential for treating intervertebral disc degeneration. *J Biomed Mater Res A* **103**, 1053, 2015.
50. Chen, J., Lee, E.J., Jing, L., Christoforou, N., Leong, K.W., and Setton, L.A. Differentiation of mouse induced pluripotent stem cells (iPSCs) into nucleus pulposus-like cells in vitro. *PLoS One* **8**, e75548, 2013.

51. Richardson, S.M., Kalamegam, G., Pushparaj, P.N., *et al.* Mesenchymal stem cells in regenerative medicine: focus on articular cartilage and intervertebral disc regeneration. *Methods* **99**, 69, 2016.
52. Stoyanov, J.V., Gantenbein-Ritter, B., Bertolo, A., *et al.* Role of hypoxia and growth and differentiation factor-5 on differentiation of human mesenchymal stem cells towards intervertebral nucleus pulposus-like cells. *Eur Cell Mater* **21**, 533, 2011.
53. Nakai, T., Sakai, D., Nakamura, Y., *et al.* CD146 defines commitment of cultured annulus fibrosus cells to express a contractile phenotype. *J Orthop Res* **34**, 1361, 2016.
54. Borde, B., Grunert, P., Härtl, R., and Bonassar, L.J. Injectable, high-density collagen gels for annulus fibrosus repair: an in vitro rat tail model. *J Biomed Mater Res A* **103**, 2571, 2015.
55. Grunert, P., Borde, B.H., Towne, S.B., *et al.* Riboflavin crosslinked high-density collagen gel for the repair of annular defects in intervertebral discs: an in vivo study. *Acta Biomater* **26**, 215, 2015.
56. Sloan, S.R., Galesso, D., Secchieri, C., Berlin, C., Hartl, R., and Bonassar, L.J. Initial investigation of individual and combined annulus fibrosus and nucleus pulposus repair ex vivo. *Acta Biomater* **59**, 192, 2017.
57. Guillaume, O., Daly, A., Lennon, K., Gansau, J., Buckley, S.F., and Buckley, C.T. Shape-memory porous alginate scaffolds for regeneration of the annulus fibrosus: effect of TGF- β 3 supplementation and oxygen culture conditions. *Acta Biomater* **10**, 1985, 2014.
58. Naqvi, S.M., and Buckley, C.T. Differential response of encapsulated nucleus pulposus and bone marrow stem cells in isolation and coculture in alginate and chitosan hydrogels. *Tissue Eng Part A* **21**, 288, 2015.
59. Chang, G., Kim, H.J., Vunjak-Novakovic, G., Kaplan, D.L., and Kandel, R. Enhancing annulus fibrosus tissue formation in porous silk scaffolds. *J Biomed Mater Res A* **92**, 43, 2010.
60. Wilke, H.-J., Ressel, L., Heuer, F., Graf, N., and Rath, S. Can prevention of a reherniation be investigated? Establishment of a herniation model and experiments with an annular closure device. *Spine* **38**, E587, 2013.
61. Nerurkar, N.L., Baker, B.M., Sen, S., Wible, E.E., Elliott, D.M., and Mauck, R.L. Nanofibrous biologic laminates replicate the form and function of the annulus fibrosus. *Nat Mater* **8**, 986, 2009.
62. Borem, R., Madeline, A., Walters, J., Mayo, H., Gill, S., and Mercuri, J. Angle-ply biomaterial scaffold for annulus fibrosus repair replicates native tissue mechanical properties, restores spinal kinematics, and supports cell viability. *Acta Biomater* **58**, 254, 2017.

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