European Cells and Materials Vol. 21 2011 (pages 373-383) DOI: 10.22203/eCM.v021a28 ISSN 1473-2262

GENIPIN-CROSSLINKED FIBRIN HYDROGELS AS A POTENTIAL ADHESIVE TO AUGMENT INTERVERTEBRAL DISC ANNULUS REPAIR

R.M. Schek[§], A.J. Michalek[§] and J.C. Iatridis*

School of Engineering, University of Vermont, Burlington, VT, USA

[§]These authors contributed equally to this manuscript.

Abstract

Treatment of damaged intervertebral discs is a significant clinical problem and, despite advances in the repair and replacement of the nucleus pulposus, there are few effective strategies to restore defects in the annulus fibrosus. An annular repair material should meet three specifications: have a modulus similar to the native annulus tissue, support the growth of disc cells, and maintain adhesion to tissue under physiological strain levels. We hypothesized that a genipin crosslinked fibrin gel could meet these requirements. Our mechanical results showed that genipin crosslinked fibrin gels could be created with a modulus in the range of native annular tissue. We also demonstrated that this material is compatible with the *in vitro* growth of human disc cells, when genipin:fibrin ratios were 0.25:1 or less, although cell proliferation was slower and cell morphology more rounded than for fibrin alone. Finally, lap tests were performed to evaluate adhesion between fibrin gels and pieces of annular tissue. Specimens created without genipin had poor handling properties and readily delaminated, while genipin crosslinked fibrin gels remained adhered to the tissue pieces at strains exceeding physiological levels and failed at 15-30%. This study demonstrated that genipin crosslinked fibrin gels show promise as a gap-filling adhesive biomaterial with tunable material properties, yet the slow cell proliferation suggests this biomaterial may be best suited as a sealant for small annulus fibrosus defects or as an adhesive to augment large annulus repairs. Future studies will evaluate degradation rate, fatigue behaviors, and long-term biocompatibility.

Keywords: Fibrin, genipin, hydrogel, crosslinker, annular repair, biomaterial, sealant, adhesive, intervertebral disc.

*Address for correspondence:
James C. Iatridis
Leni and Peter W. May Department of Orthopaedics
Mount Sinai School of Medicine
One Gustave L. Levy Place, Box 1188
New York, NY 10029-6574, USA
Telephone Number: 212-241-1517
E-mail: james.iatridis@mssm.edu

Introduction

Low back pain is a serious public health problem in the US affecting more that 25% of adults (Deyo *et al.*, 2006). For cases in which this pain stems from the intervertebral disc (IVD), current surgical treatments include spinal fusion, total disc replacement, and discectomy. Unfortunately, these procedures do not repair the disc or restore its original function and may further limit mobility or otherwise alter the biomechanics of the spine which can lead to adjacent segment degeneration (Lee, 1988; Schlegel *et al.*, 1996). Newer treatments for damaged IVDs include strategies to replace and regenerate the nucleus pulposus (NP), the central, gelatinous region of the IVD (Di Martino *et al.*, 2005; Sebastine and Williams, 2007; Hegewald *et al.*, 2008). These techniques are likely to have limited success without adequate repair of the annulus fibrosus (AF), the outer ring of the IVD. The AF is necessarily damaged during surgery to remove or repair the NP, and to a lesser extent from discography procedures (Carragee *et al.*, 2009), and yet a functional, intact AF is key to preventing re-herniation of the NP and retention of any NP replacement device (Alini *et al.*, 2002; Wilke *et al.*, 2006). Thus, it is possible that NP replacement approaches could be augmented by incorporating AF repair. A successful method to repair the AF could also be useful for repairing small needle punctures, such as those that occur for discography or delivery of therapeutics.

Methods for repairing damaged AF are currently limited largely to sutures and modified sutures, which do not compensate for the loss of AF tissue or restore the lost biomechanical properties (Bron *et al.*, 2009a). An appealing alternative is the development of a tissue engineered material to repair the gap in the AF and contain the NP or its replacement. If such a material were degradable, it would ultimately be replaced with host tissue; to fill a large defect in the AF, the material may be seeded with progenitor cells, such as mesenchymal stem cells. An ideal material would need to meet the following three requirements: match the mechanical properties of the AF tissue, allow the growth of disc cells, and adhere to the surrounding tissues under physiological levels of strain. A number of materials have been investigated for this purpose including hydrogels, bioglass, collagen, silk and degradable polymers such as polycaprolactone and polyglycolic acid (Sato *et al.*, 2003; Mizuno *et al.*, 2004; Nerurkar *et al.*, 2007; Chang *et al.*, 2007; Shao and Hunter, 2007; Helen and Gough, 2008; Wan *et al.*, 2008). While many of these materials show promise, none have satisfactorily addressed the need for fixing the implanted material within the annular defects. To ensure a biomaterial remains in place and encourage the

formation of new tissue, it is critical that any material be able to strongly adhere and be fully integrated with the native annulus tissue.

We hypothesize that a crosslinked hydrogel can meet the requirements for annular tissue repair. Hydrogels are useful in a wide range of tissue engineering applications due to their high water content, biocompatibility, ease of cell seeding, and mechanical properties that mimic soft tissue (Lavik and Langer, 2004; Ahmed *et al.*, 2008). Delivery of a hydrogel is also straightforward as it can be injected in liquid form and sets *in situ* over the course of several minutes. This property allows its use in irregularly shaped defects. Additionally, a hydrogel could be used in conjunction with other porous biomaterials to improve structure and expand the possibilities for cell seeding. The major limitation of a hydrogel may be their somewhat poor mechanical properties. An appropriately chosen crosslinker, however, can increase the stiffness of the gel such that it closely matches the native tissue. Just as importantly, the crosslinker serves to chemically connect the proteins in the hydrogel with the native proteins in the disc, thus "bridging" the disrupted collagen fibers.

For this work, we have chosen to employ a crosslinked fibrin gel. The components of fibrin, fibrinogen and thrombin, can be purified from human plasma, and fibrin glue has a long history of clinical use in Food and Drug Administration (FDA) approved products such as Tisseel®, Evicel™, and Crosseal™. Additionally, fibrin has shown to be an excellent scaffold for cell delivery and tissue ingrowth in a number of tissue engineering applications (for review, see Ahmed *et al*., 2008). Of specific relevance to connective tissues such as the AF, fibrin has been used to engineer muscle (Hecker *et al.*, 2005; Huang *et al.*, 2005; Nieponice *et al.*, 2007; Rowe *et al.*, 2007), skin (Hojo *et al.*, 2003; Balestrini and Billiar, 2006;), cartilage (Passaretti *et al.*, 2001; Connelly *et al.*, 2004; Johnson *et al.*, 2004; Mesa *et al.*, 2006; Peretti *et al.*, 2006; Eyrich *et al.*, 2007), and connective tissue (Chong *et al.*, 2007; Hankemeier *et al.*, 2007). Recently, fibrin has been shown to be an effective carrier to deliver stem cells to denucelated discs (Allon *et al.*, 2010) and to improve the structure and function of surgically damaged IVDs (Buser *et al.*, 2011).

To crosslink the fibrin gel, we have chosen to utilize genipin. Genipin ($C_{11}H_{14}O_5$, FW 226.23) is a plant-derived crosslinking agent traditionally used in herbal medicine and as a food dye. While crosslinkers have the capacity to kill cells, genipin is an attractive choice for crosslinking due its relatively low cytotoxicity (Bedran-Russo *et al.*, 2007; Tsai *et al.*, 2000); specifically, genipin has been shown to be 10,000 times less cytotoxic than glutaraldehyde to 3T3 fibroblasts (Sung *et al.*, 1999). Additionally, genipin has anti-inflammatory properties when used as a crosslinker *in vivo* (Liang *et al.*, 2003; Koo *et al.*, 2006;) and may even protect neuronal cells from stress-induced cytotoxicity (Yamazaki *et al.*, 2009). Genipin is known to crosslink proteins by binding amine groups on adjacent proteins (Touyama *et al.*, 1994; Yao *et al.*, 2004) and has previously been used to crosslink a variety of materials including collagenous tissues (Huang *et al.*, 1998; Sung *et al.*, 2003), chitosan (Mi *et al.*, 2002; Mwale *et al.*, 2005), gelatin (Chen *et al.*, 2005), and fibrinogen electrospun scaffolds (Sell *et al.*, 2008). Recently, it has also been used to crosslink fibrin hydrogels for articular cartilage engineering (Dare *et al.*, 2009). Genipin crosslinked fibrin gels were shown to be compatible with chondrocytes *in vitro* and to inhibit the inflammatory response when implanted in rats (Dare *et al.*, 2009).

In this work, we proposed to determine the suitability of a genipin crosslinked fibrin hydrogel for repair of the annulus fibrosus. Particularly, we sought to determine whether such a hydrogel could meet the three requirements of matching the AF tissue properties, supporting cell growth, and strongly adhering to native tissue. To investigate this first requirement, we measured the modulus of gels with varied formulation and compared them to samples of human AF tissue. We then performed *in vitro* experiments in which human disc cells were cultured on gels and their survival measured. Finally, to evaluate adhesion, we created gel samples that were allowed to crosslink in contact with AF tissue and measured the strain at which this adhesion failed. The current study is focused on developing an adhesive biomaterial with potential to be used as a sealant (rather than full cell seeded scaffold) for small AF defects or as an adhesive to augment repair of large AF repairs with other biomaterials. The material was optimized to match native tissue material properties, while maintaining suitable adhesion to native tissue and low cytotoxicity.

Materials and Methods

Gel fabrication

Fibrinogen isolated from bovine plasma (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in phosphate buffered saline (PBS) at concentrations of 200, 250, or 300 mg/mL; above 300 mg/mL the fibrinogen could not be readily dissolved. Thrombin isolated from bovine plasma (Sigma-Aldrich) was dissolved in PBS at a concentration of 100 U/mL. Genipin (Wako, Richmond, VA, USA) was dissolved in dimethyl sulfoxide (Fisher Scientific, Hampton, NH, USA) at a concentration of 400 mg/mL. The fibrinogen was pipetted into molds as specified below; thrombin and genipin were mixed together and then added to the fibrinogen in quantities specified below. Genipin quantities were determined such that desired genipin:fibrin weight ratios could be obtained (i.e., to create a 0.25:1 gel for cell culture testing, 600 μL of 200 mg/mL fibrinogen was mixed with 75 μL of genipin). The gels were allowed to rest for 24 h to completely crosslink prior to mechanical testing or cell seeding. Pilot studies (not shown) demonstrated that a genipin:fibrin concentration ratio of 1:1 and 2:1 resulted in nearly zero cell survival. Therefore, we have tested ratios of 0.25:1, 0.5:1, and 0:1 as a control. The ratio of 0:1 was fibrin gel alone. A pilot study, which used a spectrophotometer to determine the relative number of crosslinks in genipin-containing gels, indicated that the use of larger amounts of genipin did not lead to the formation of additional crosslinks beyond a genipin:fibrin ratio of approximately 10:1 (Sung *et al.*, 1999; Starcher, 2001; Chang *et al.*, 2003). Therefore, we assumed that at the sub-saturating genipin:fibrin ratios used, the vast majority

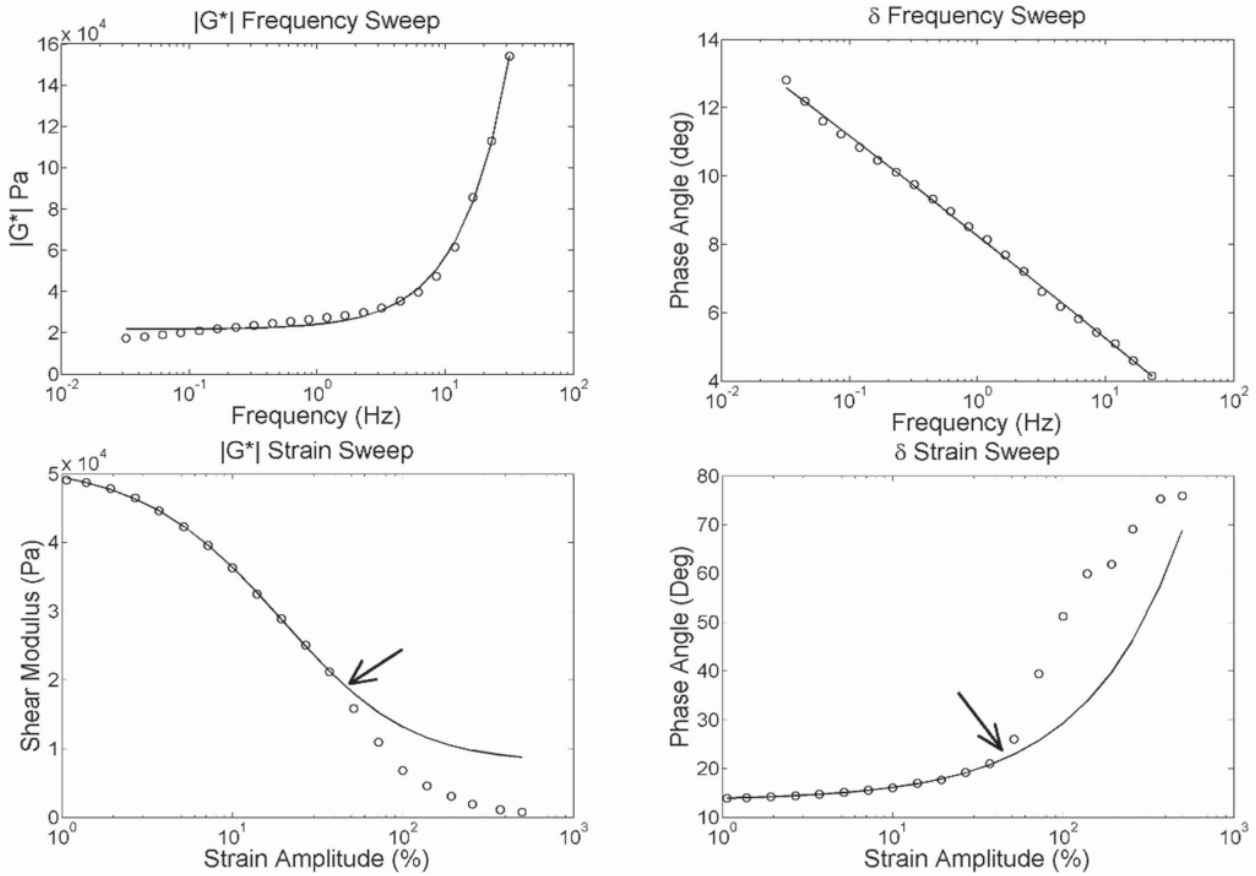

Fig. 1. Representative model fits, -, to experimental data, o. Black arrows indicate slippage between the specimen and the rheometer platens. Measurements after this point were not analyzed.

of the genipin in the gel would have formed crosslinks and be bound to amine groups on the fibrin (Touyama *et al.*, 1994; Yao *et al.*, 2004).

Rheological testing

Samples for rheometer testing were created using custom made Teflon molds with cylindrical wells 5 mm in diameter and 5 mm deep. Into each well, 50 µL of the fibrinogen was pipetted, followed by 5 µL of thrombin mixed with sufficient genipin to create the desired ratio of genipin:fibrin. The resulting gel specimens were approximately 2.5 mm thick.

Gel dynamic stiffness tests were carried out using a rheometer (TA Instruments, www.tainstruments.com) equipped with flat platens covered with 100 grit sand paper (Gator Grit; www.gatorfinishing.com) and a humidified chamber. The test protocol was adapted from Bron *et al.* (Bron *et al.*, 2009b) and consisted of a 20 min equilibration at 0.1N of axial compression followed by a dynamic frequency sweep at 10% strain from 0.032 to 32 Hz. The protocol concluded with a strain sweep at 0.5Hz from 1 to 500% strain. Dynamic modulus magnitude, $|G^*|$, and phase angle, δ, were calculated at each point of the frequency and strain sweeps. Parameters from both sweeps demonstrated power law dependence on frequency and strain, and were thus characterized by fitting the following functions using a least squares routine in Matlab (Mathworks Inc., Natick, MA, USA) (Fig. 1).

$$|G^*|(\omega) = a + b\omega^{\alpha_{|G^*|}}, \delta(\omega) = c + d\omega^{\alpha_\delta} \quad (1)$$

$$|G^*|(\gamma) = j + k(\gamma + \Gamma)^{\beta_{|G^*|}}, \quad \delta(\gamma) = m + n\gamma^{\beta_\delta} \quad (2)$$

Five parameters from the model fit were used to compare $|G^*|$ and δ across groups, and with human AF tissue. For the frequency sweep, dynamic modulus was characterized by its value at 1Hz, $|G^*|_{1Hz}$ which is equivalent to parameters $a+b$, and its dependence on frequency was characterized by power law exponent t, $\alpha_{|G^*|}$. Phase angle was likewise characterized by its value at 1 Hz, δ_{1Hz} which is equivalent to parameters $c+d$, and its power law exponent, α_δ. For the strain sweep, accurate description of the experimental data also required a strain offset term. Therefore, dynamic modulus during the strain sweep was characterized by strain offset, Γ, modulus at the offset strain, $|G^*|_{\Gamma 0}$, and strain dependence exponent $\beta_{|G^*|}$. Phase angle during the strain offset was accurately described with 2 parameters, its value at 1% strain, $\delta_{1\%} = m+n$, and strain dependence exponent for δ, β_δ. The dependence of these nine parameters on fibrin and genipin concentrations was assessed using a two way ANOVA with factors fibrin concentration (200, 250 and 300 mg/mL), and relative genipin concentration (i.e., as given by genipin:fibrin ratio of 0:1, 0.25:1 and 0.5:1).

In order to assess the suitability of the genipin crosslinked fibrin gels for tissue repair, the rheological

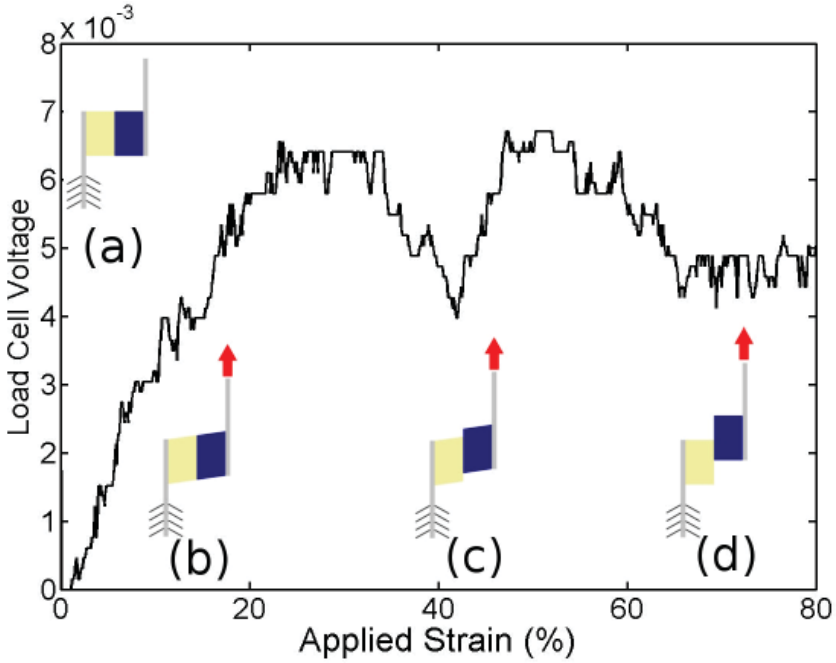

Fig. 2. Representative plot of load cell voltage *vs*. applied strain for a bi-layered lap test. Schematics show bi-layered specimen initially **(a)**, under shear **(b)**, at initial failure **(c)**, and completely failed **(d)**.

testing protocol was also performed on thirteen human AF specimens with an average age of 53±16 years. The average Thompson grade of these discs was 3, indicating a tissue with mild to moderate degeneration. Model fit parameters were compared between the gel groups and the native tissue using unpaired *t*-tests with the significant *p* value adjusted to <0.005 to account for multiple comparisons.

Lap testing

Gel-tissue adhesion was characterized by a modified lap test carried out using a custom built axial testing machine using a linear actuator (www.physikinstrumente.com). The upper and lower faces of the bi-layered specimen were adhered to brass platens using cyanoacrylate glue (Loctite 410 Gel). In order to ensure that the test was measuring adhesion and not boundary friction, platen spacing was adjusted to minimize axial force on the specimen. Specimen thickness was measured with calipers in order to calculate shear displacements required for given strains. One platen was then displaced in order to shear the specimen at a constant rate of 1 %/s to 100%. Pilot studies showed that this magnitude was sufficient to ensure specimen failure. Specimen failure strain was assessed by identifying peak stress on the resulting load versus strain plot (Fig. 2). Mode of failure was determined visually for each specimen and recorded.

Cell culture

Human AF cells were obtained from tissue removed by a surgeon performing discectomy procedures with IRB approval and patient consent. Disc tissue from the outer AF only was obtained from patients with an average age of 45 ±14 years; discs were graded as either moderate or severely degenerate. The harvested AF tissue was first rinsed in a wash solution of 1.5% fungizone (Gibco/Invitrogen-Life Technologies, www.invitrogen.com) and 3% penicillin-streptomycin (Gibco) in phosphate buffered saline (Gibco) and then digested in 50 mL of Dulbecco's Modified Eagle Medium (DMEM, Gibco) containing 0.2% pronase (Sigma-Aldrich), 1% penicillin-streptomycin, and 0.5% fungizone for 1 h at 37 ºC. After 1 h, 0.2% collagenase (Sigma-Aldrich) was added to the digest solution and the digest was continued for an additional 4 h. The digest was then filtered through a 70 µm nylon filter, centrifuged, and cells resuspended and plated into flasks. Cells were fed every 3-4 d with growth medium (DMEM supplemented with 10% fetal bovine serum (Gibco), 1% penicillin-streptomycin, 0.5% fungizone, and 50 µg/ mL ascorbic acid (Sigma-Aldrich)). Cells used for viability experiments were P3-P4.

Gels were created in the wells of 6-well tissue culture plates. Into each well, 600 µL of 200, 250, or 300 mg/mL fibrinogen was pipetted. Thrombin (60 µL/well) and genipin were added to obtain genipin: fibrin of 0:1, 0.25:1, or 0.5:1 (n=3). After 24 h, the gels were rinsed with PBS and 30,000 human disc cells were plated in each well. Cells were fed with growth medium every two days and allowed to grow on the gels for 1, 3, or 7 d and then rinsed with PBS. To assess the number of live cells adhered on the surface of the gels, they were incubated with a solution of 4mM calcein in PBS (Invitrogen) for 30 min at 37 ºC. Calcein is transported only through the membrane of live cells and is commonly utilized to assess number of live cells (Sun *et al.*, 2010). Calcein positive cells were imaged on an Olympus (Tokyo, Japan) upright BX microscope equipped with a Chroma GFP filter cube and a Leica

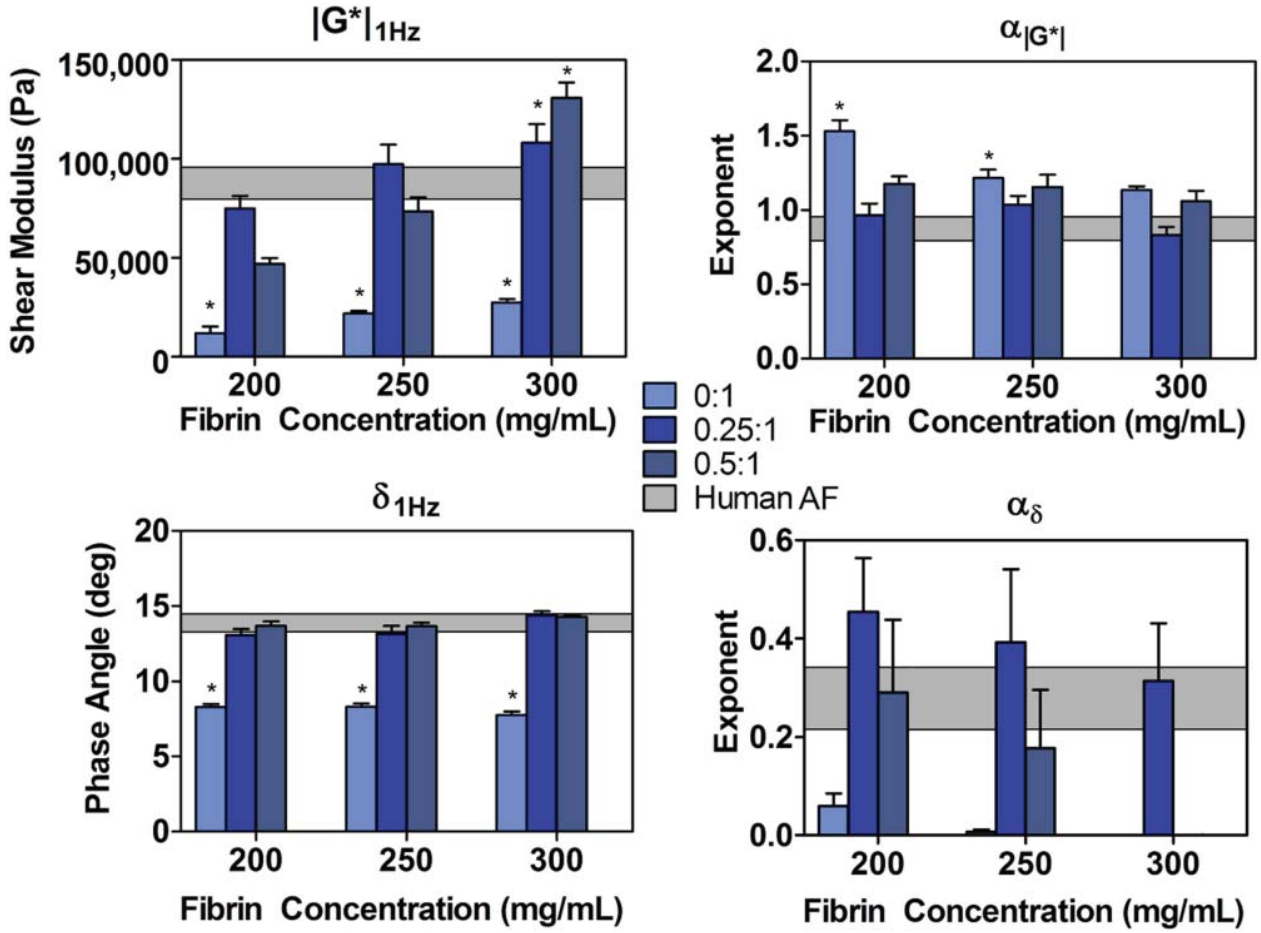

Fig. 3. Average frequency sweep model fit parameters ± SEM for gels with varying fibrin concentrations and genipin to fibrin ratios. * indicates significantly different from human AF (p<0.005).

R3 camera. Photographs were processed using ImageJ (National Institutes of Health, Bethesda, MD, USA); they were thresholded and the total number of cells was counted. From each experimental well, five fields of view were captured and counted. For each gel formulation at each time point, a total of 15 values were averaged and standard error calculated. The differences in the number of calcein positive cells were compared using paired t tests with p< 0.05 significant. To determine whether cell number had increased over the duration of the culture period, the number of live cells on day 1 was compared to the number of live cells on day 7.

Results

Gel fabrication

Gels formed readily in both the Teflon molds and cell culture dishes. Gels set in less than 5 min and evidence of crosslinking (appearance of dark blue color) was apparent within 2 h. Following the 24 h curing, crosslinked gels were very firm to the touch and easily removable from the Teflon mold. Gels fabricated without genipin were more difficult to remove from the mold and more easily torn during handling.

Stiffness results from rheological testing

Rheological testing indicated that material properties of this genipin crosslinked fibrin gel follow power law relationships (c.o.d. = 0.84±0.28) with strain amplitude and frequency, the parameters of which are tuneable by varying fibrin concentration and genipin:fibrin ratio. The two way ANOVA showed that nearly all parameters were significantly affected by fibrin concentration, genipin:fibrin ratio, and their interaction (p-values are summarized in Table 1, and parameter values are in Figs. 3 and 4), although some exceptions were found particularly for the strain sensitivity of several material parameters.

Gels made with 250 mg/mL of fibrin and either 0.25:1 or 0.5:1 genipin:fibrin appear to be the most suitable for mimicking the shear behavior of native tissue, as these samples were not significantly different from human AF in any of the nine calculated parameters. The frequency sweep showed that genipin crosslinked fibrin gels generally demonstrate greater strain rate stiffening (increase in $|G^*|$ with increasing frequency) than the native tissue, with significance achieved either without genipin or with 0.5:1 genipin:fibrin at lower fibrin concentrations. Strain rate viscosity effects (change in δ with increasing frequency) were not significantly different from native in any of the gel groups. The strain dependency exponents, $\beta_{|G^*|}$ and β_{δ}, were not significantly different from native AF for most gel

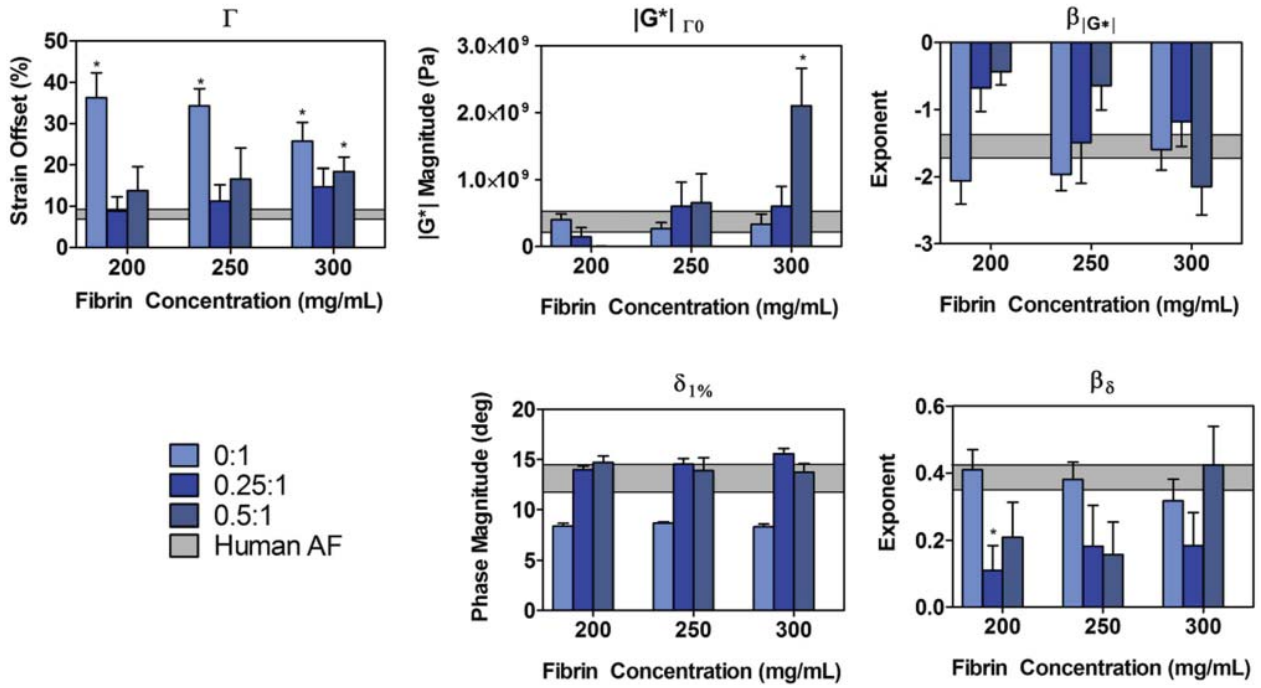

Fig. 4. Average strain sweep model fit parameters ± SEM for gels with varying fibrin concentrations and genipin:fibrin ratios. * indicates not significantly different from human AF (p<0.005).

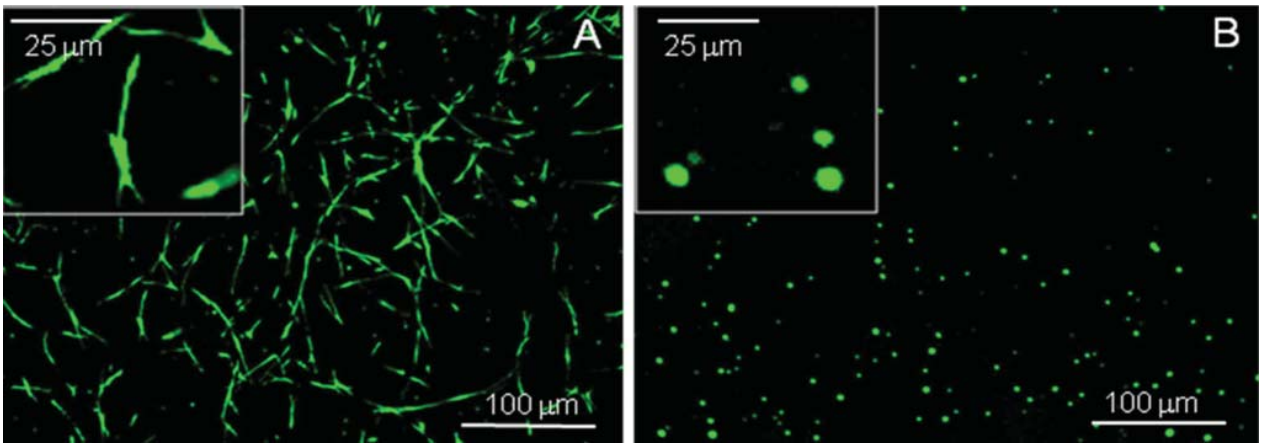

Fig. 5. Images of calcein stained cells after 7 d grown on 200 mg/mL fibrin gel containing no genipin **(A)** or with a genipin to fibrin ratio of 0.25:1 **(B)**. Inset of greater magnification to highlight cytomorphology.

Table 1. Two way ANOVA showing effects of fibrin and relative genipin concentrations on frequency and strain

	Frequency				**Strain**				
	Modulus		**Phase**	**Angle**	**Modulus**			**Phase**	**Angle**
	$G^*\|_{1Hz}$	$\alpha_{\|G^*\|}$	δ_{1Hz}	α_{δ}	Γ	$\|G^*\|_{\Gamma 0}$	$\beta_{G^*\|}$	$\delta_{1\%}$	β_{δ}
Fibrin	<0.001	<0.001	0.080	<0.001	0.015	0.709462	<0.001	0.436	0.050
Relative Genipin	<0.001	<0.001	0.008	<0.001	0.567	0.467944	<0.001	0.045	<0.001
Interaction	0.074	<0.001	0.541	0.018	0.995	0.546036	<0.001	0.352	0.088

groups. Both magnitude parameters, as calculated by both the frequency and strain sweeps, were significantly lower in the gels without genipin at all fibrin concentrations.

Adhesion results from lap testing

The modified lap test resulted in an average failure strain ±SD of 26 ±7%. Detachment at the specimen-platen interface was not observed in any specimen. The mode of specimen failure was equally distributed between slippage of the gel-tissue interface and fracture of the gel itself.

Cell viability

Disc cells assumed a spindle morphology when grown on fibrin gel alone (i.e., a ratio of 0:1) that is typical when grown on tissue culture plastic (Fig. 5). When grown on the genipin containing gels, the disc cells assumed a round shape and did not exhibit cell processes extending across the surface of the gel. This difference was observed for all fibrinogen concentrations and genipin:fibrin ratios; the presence of genipin at these levels was sufficient to alter the morphology.

The number of live cells was quantified and averaged across 15 fields of view for each condition. Graphs of the cell number over time show similar results for gels with fibrin concentrations of 200 or 250 mg/mL (Fig. 6). Between day 1 and day 7, the increase in cell number was statistically significant ($p<0.05$) for all gels containing no genipin. On the crosslinked gels, we observed the cell number to be similar between days 1 and 3; however, multiplication between days 3 and 7 was more rapid and similar in speed to cells on the gels containing no genipin. For both 200 and 250 mg/mL gels, higher cells numbers were seen for genipin: fibrin ratios of 0.25:1 than for 0.5:1. Cell cultured on 300 mg/mL gels multiplied more slowly that those grown on 200 or 250 mg/mL, even in the absence of genipin. Statistical analysis showed that the increase in cell number between day 1 and day 7 was significant for all the gels with a genipin:fibrin ratio of 0.25:1. However, for gels with a genipin: fibrin ratios of 0.5:1, only the 200 mg/mL gel showed a significant increase in cell number.

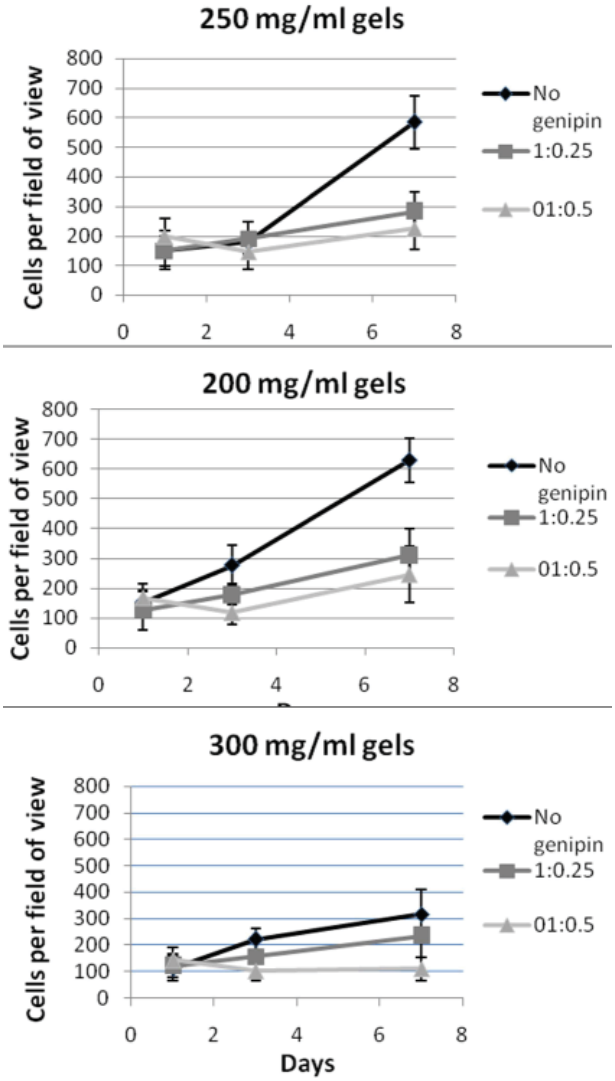

Fig. 6: Graphs demonstrating the multiplication of cells on the crosslinked fibrin gels over time; cell number is per field of view.

Discussion

In this work, we have been guided by the principle that an annular repair material should meet three specifications: have a modulus that matches the native annulus tissue, support the growth of disc cells, and maintain adhesion to tissue under physiological strain levels. We hypothesized that a genipin crosslinked fibrin gel could meet these requirements. Our mechanical results showed that genipin crosslinked fibrin gels could be created with a modulus in an appropriate range, and that genipin was required to achieve a modulus matching that of native AF tissue across a wide range of strain rates and amplitudes. By altering fibrin concentration and the genipin:fibrin ratio, the modulus of the gel is tuneable to specific applications. We also demonstrated that this material is compatible with the *in vitro* growth of human disc cells, when genipin:fibrin ratios were 0.25:1 or less. Lastly, the results of our lap tests demonstrated that genipin crosslinked fibrin gels remained adhered to pieces of annular tissue at physiological strains of 5-10% (Krismer *et al.*, 1996; Costi *et al.*, 2007; O'Connell *et al.*, 2010) and failed at higher strains of 15-30%.

Cell viability testing demonstrated that the presence of genipin in the gels results in rounded cell morphology and slows cell proliferation. An elongated morphology and the presence of long cell processes are key features of AF cells in their native matrix (Errington *et al.*, 1998). Because these processes are important for proper AF cell function (Errington *et al.*, 1998; Bruehlmann *et al.*, 2002), the ideal biomaterial should likewise facilitate this cell morphology. These results show that AF cells can survive and proliferate at a retarded but statistically significant rate on gels with a genipin to fibrin ratios of 0.25:1 or less, yet the cell morphology was rounded. Further evaluation is required to evaluate if normal AF cell morphology can be achieved in the presence of this fibrin-genipin hydrogel in a 3D culture system that more closely represents *in situ* repair, or with lower genipin concentrations. Future studies

are also warranted to evaluate the compatibility of this material for cells types where a rounded morphology is normal, such as for chondrocytes or stem cell condensation.

An optimal material must simultaneously balance mechanical properties and adhesion, which are improved by the addition of genipin, with biological properties, which are improved by lowering genipin concentrations. In this work, our goal was to map out that design space by probing the limits of these two variables. Because these experiments determined levels at which cells could survive, but not thrive, we know now the maximum concentration and that lower levels will be preferable for cell survival and growth. Interactions between AF cells and their matrix are complex; in addition to the chemical make-up of the matrix, its stiffness, alignment, and surface chemistry, as well as time in culture, all affect cell attachment and morphology (Shao and Hunter, 2007; Yang *et al.*, 2009; Attia *et al.*, 2011; Koepsell *et al.*, 2011; Zhang *et al.*, 2011). Thus, it is possible that the cell behavior seen in this work may derive from surface properties or culture time, rather than being solely a result of the genipin to fibrin ratio. The abnormal AF cell behavior could also be caused by the unique surface structure of the crosslinked gel. The presence of the genipin crosslinks may affect how cells adhere and spread upon the surface. However, other investigations have shown that genipin crosslinked scaffolds do support cellular growth (Mekhail *et al.*, 2010), and we therefore hypothesize that the behavior of AF cells in the presence of genipin crosslinked fibrin gels will be improved in a more three dimensional culture environment, and/or with different concentrations. Future work will investigate properties of gels with lower genipin concentrations and the effect on cell viability and phenotype as well as the capacity of this crosslinked hydrogel to be used to augment adhesion of other biomaterials that may be better able to promote AF cell morphology and proliferation *in situ*.

Another interesting phenomenon noted in the cell culture study was the tendency, in the gels containing genipin, for little cell proliferation in the first 48 h, following by a slow increase in cell number. While we did not specifically investigate a mechanism, two possibilities likely exist for this trend. Firstly, if any free genipin in the media was hindering cell growth, it would have been largely removed by the media change. Secondly, a number of cells may have died during seeding and a few days were required for cell number to rebound to initial seeding density. Only after this happened, could an increase in cell number become apparent.

In this work, we primarily reported genipin concentration as the ratio of genipin to fibrin. Genipin crosslinks proteins (including fibrin) by binding amine groups on adjacent proteins; thus degree of crosslinking is dependent on the percentage of amine groups that are bound to a genipin molecule (Touyama *et al.*, 1994; Yao *et al.*, 2004). Unlike absolute genipin concentration, the ratio of genipin to fibrin gives us a relative measure of the extent of fibrin crosslinking. Additionally, our cell culture testing showed that rates of cell survival in gels depended upon genipin to fibrin ratios rather than absolute genipin concentration. For instance, a similar number of cells survived in the 200 mg fibrin/mL gel with a genipin to fibrin ratio of 0.25:1 as in the 300 mg fibrin/mL with a ratio of 0.25:1 gels. This was despite the fact that the absolute genipin concentration was 1.5 times greater in the latter gel. We hypothesize that cell toxicity largely results from genipin which remains unbound to fibrin. Accordingly, gels with higher ratios of genipin to fibrin had larger amounts of free genipin, which resulted in fewer numbers of viable cells in these constructs. Overall, this indicates that the ratio of genipin to fibrin is a more relevant variable than the absolute genipin concentration.

The results of rheological testing demonstrated power law relationships with both strain rate and amplitude for the genipin crosslinked hydrogel and for human AF tissue. Assessing these behaviors across a broad range of loading conditions is essential for determining an ideal material formulation for repairing the AF. The similarities of material constants of the hydrogel (of certain formulations) with human AF tissue demonstrates strong mechanical compatibility over a large range of frequencies and strain amplitudes. The shear modulus of the hydrogel depends on both the fibrin concentration and the ratio of genipin to fibrin. Increasing the fibrin concentration leads to an apparently linear increase in the modulus of the gels. Increasing the ratio of fibrin to genipin, however, does not lead to such a regular increase in modulus. Specifically, we found that, for a given fibrin concentration, modulus increases steeply from 0:1 to 0.25:1 genipin to fibrin; further increases in modulus were seen only when this ratio was substantially increased to 4:1 (data not shown because cells did not remain viable at this high genipin to fibrin ratio). Further exploration of mechanical behavior at lower genipin ratios may show that we can maintain mechanical properties while improving cell survival. Adjustments in fibrin concentration and/or the ratio of genipin to fibrin allow us multiple formulation options to create a gel with the desired modulus.

Genipin crosslinked fibrin gels have met basic design requirements for acute annular repair as outlined above, although future work must focus on longer term behavior before these gels can be considered for clinical use. Most importantly, we must understand how the material might perform under long-term implantation conditions including characterizing both its fatigue life and its biodegradation profile. Additional bench testing should also include evaluating the gels performance within an intact vertebral motion segment. Genipin and fibrin's history of use along with the cell viability data presented here suggest that this material has potential clinical utility. However, these data do not establish true biocompatibility or the specific effects of the gel on disc cell phenotype, which both need to be fully understood. Future cell culture studies focusing on gene expression will require the use of AF cells at the lowest possible passage since phenotypic changes have been observed when disc cells are cultured for extended periods (Chou *et al.*, 2006). These *in vitro* tests cannot fully predict the mechanical or biological performance of these gels *in vivo* and so future evaluation in an animal injury model is needed.

The biomechanical compatibility, "tunable" material properties, and strong adherence to native tissues demonstrate high utility of this biomaterial for AF repair

as well as potential repair of other tissues. The "tunable" nature of the mechanical properties indicates that we can maintain a desired modulus by simultaneously adjusting the fibrinogen content and the ratio of genipin to fibrin so that we can optimize the formulation of AF repair or potentially for other tissues with similar design requirements. The gel's formulation can also be altered as we learn more about its degradation and fatigue behavior over time. For example, the gel could be augmented to improve its performance with enzymes to slow degradation. The relatively rapid set time of the gels also enables refinement of delivery and application techniques. For example, the genipin crosslinked fibrin gel may be used as a sealant to repair punctures with injection techniques. The strong adherence of genipin crosslinked fibrin gel to human AF tissues further indicates the gel may be used for larger AF defects in combination with recently developed fibrous materials, creating a composite scaffold with excellent stiffness and adhesive properties (Mizuno *et al.*, 2004; Chang *et al.*, 2007; Wan *et al.*, 2007; Nerurkar *et al.*, 2009). Additionally, the positive results of recent studies utilizing fibrin to repair IVDs (Allon *et al.*, 2010; Buser *et al.*, 2011) suggest that there could be significant applications for an enhanced, stiffer fibrin-based material in the future. We conclude that genipin crosslinked fibrin gels are effective gap filling biomaterials with tunable material properties and strong adhesion to native tissues with substantial promise to be used as a sealant for small AF defects or as an adhesive to augment other biomaterials for larger AF repairs, although additional testing to evaluate safety and biocompatibility *in situ* are required.

Acknowledgements

This project was supported by the National Institutes of Health (R01AR051146). We gratefully acknowledge the assistance procuring surgical tissue samples from Dr. Robert Monsey, and the technical assistance on shear testing from Ms. Kristin Funabashi. A preliminary patent was submitted for this biomaterial.

References

Ahmed TA, Dare EV, Hincke M (2008) Fibrin: A versatile scaffold for tissue engineering applications. Tissue Eng Part B Rev, in press.

Alini M, Roughley PJ, Antoniou J, Stoll T, Aebi M (2002) A biological approach to treating disc degeneration: Not for today, but maybe for tomorrow. Eur Spine J **11** Suppl 2: S215-220.

Allon AA, Aurouer N, Yoo BB, Liebenberg EC, Buser Z, Lotz JC (2010) Structured coculture of stem cells and disc cells prevent disc degeneration in a rat model. Spine J **10**: 1089-1097.

Attia M, Santerre JP, Kandel RA (2011) The response of annulus fibrosus cell to fibronectin-coated nanofibrous polyurethane-anionic dihydroxyoligomer scaffolds. Biomaterials **32**: 450-460.

Balestrini JL, Billiar KL (2006) Equibiaxial cyclic stretch stimulates fibroblasts to rapidly remodel fibrin. J Biomech **39**: 2983-2990.

Bedran-Russo AK, Pereira PN, Duarte WR, Drummond JL, Yamauchi M (2007) Application of crosslinkers to dentin collagen enhances the ultimate tensile strength. J Biomed Mater Res B Appl Biomater **80**: 268-272.

Bron JL, Helder MN, Meisel HJ, Van Royen BJ, Smit TH (2009a) Repair, regenerative and supportive therapies of the annulus fibrosus: Achievements and challenges. Eur Spine J **18**: 301-313.

Bron JL, Koenderink GH, Everts V, Smit TH (2009b) Rheological characterization of the nucleus pulposus and dense collagen scaffolds intended for functional replacement. J Orthop Res **27**: 620-626.

Bruehlmann SB, Rattner JB, Matyas JR, Duncan NA (2002) Regional variations in the cellular matrix of the annulus fibrosus of the intervertebral disc. J Anat **201**: 159-171.

Buser Z, Kuelling F, Liu J, Liebenberg E, Thorne KJ, Coughlin D, Lotz JC (2011) Biological and biomechanical effects of fibrin injection into porcine intervertebraldiscs. Spine (Phila Pa 1976), in press.

Carragee EJ, Don AS, Hurwitz EL, Cuellar JM, Carrino JA, Herzog R (2009) 2009 ISSLS prize winner: Does discography cause accelerated progression of degeneration changes in the lumbar disc: A ten-year matched cohort study. Spine (Phila Pa 1976) **34**: 2338-2345.

Chang G, Kim HJ, Kaplan D, Vunjak-Novakovic G, Kandel RA (2007) Porous Silk Scaffolds Can Be Used for Tissue Engineering Annulus Fibrosus. Eur Spine J **16**: 1848-1857.

Chang WH, Chang Y, Lai PH, Sung HW (2003) A genipin-crosslinked gelatin membrane as wound-dressing material: *In vitro* and *in vivo* studies. J Biomater Sci Polym Ed **14**: 481-495.

Chen YS, Chang JY, Cheng CY, Tsai FJ, Yao CH, Liu BS (2005) An *in vivo* evaluation of a biodegradable genipin-cross-linked gelatin peripheral nerve guide conduit material. Biomaterials 2**6**: 3911-3918.

Chong AK, Ang AD, Goh JC, Hui JH, Lim AY, Lee EH, Lim BH (2007) Bone marrow-derived mesenchymal stem cells influence early tendon-healing in a rabbit Achilles tendon model. J Bone Joint Surg Am **89**: 74-81.

Chou AI, Bansal A, Miller GJ, Nicoll SB (2006) The effect of serial monolayer passaging on the collagen expression profile of outer and inner anulus fibrosus cells. Spine (Phila Pa 1976) **31**: 1875-1881.

Connelly JT, Vanderploeg EJ, Levenston ME (2004) The influence of cyclic tension amplitude on chondrocyte matrix synthesis: Experimental and finite element analyses. Biorheology **41**: 377-387.

Costi JJ, Stokes IA, Gardner-Morse M, Laible JP, Scoffone HM, Iatridis JC (2007) Direct measurement of intervertebral disc maximum shear strain in six degrees of freedom: Motions that place disc tissue at risk of injury. J Biomech **40**: 2457-2466.

Dare EV, Griffith M, Poitras P, Kaupp JA, Waldman SD, Carlsson DJ, Dervin G, Mayoux C, Hincke MT (2009) Genipin cross-linked fibrin hydrogels for *in vitro* human

articular cartilage tissue-engineered regeneration. Cells Tissues Organs **190**: 313-325.

Deyo RA, Mirza SK, Martin BI (2006) Back pain prevalence and visit rates: Estimates from U.S. National Surveys, 2002. Spine (Phila Pa 1976) **31**: 2724-2727.

Di Martino A, Vaccaro AR, Lee JY, Denaro V, Lim MR (2005) Nucleus pulposus replacement: Basic science and indications for clinical use. Spine (Phila Pa 1976) **30**: S16-22.

Errington RJ, Puustjarvi K, White IR, Roberts S, Urban JP (1998) Characterisation of cytoplasm-filled processes in cells of the intervertebral disc. J Anat **192**: 369-378.

Eyrich D, Brandl F, Appel B, Wiese H, Maier G, Wenzel M, Staudenmaier R, Goepferich A, Blunk T (2007) Long-term stable fibrin gels for cartilage engineering. Biomaterials **28**: 55-65.

Hankemeier S, van Griensven M, Ezechieli M, Barkhausen T, Austin M, Jagodzinski M, Meller R, Bosch U, Krettek C, Zeichen J (2007) Tissue engineering of tendons and ligaments by human bone marrow stromal cells in a liquid fibrin matrix in immunodeficient rats: Results of a histologic study. Arch Orthop Trauma Surg **127**: 815-821.

Hecker L, Baar K, Dennis RG, Bitar KN (2005) Development of a three-dimensional physiological model of the internal anal sphincter bioengineered *in vitro* from isolated smooth muscle cells. Am J Physiol Gastrointest Liver Physiol **289**: G188-196.

Hegewald AA, Ringe J, Sittinger M, Thome C (2008) Regenerative treatment strategies in spinal surgery. Front Biosci **13**: 1507-1525.

Helen W, Gough JE (2008) Cell viability, proliferation and extracellular matrix production of human annulus fibrosus cells cultured within PDLLA/bioglass composite foam scaffolds *in vitro*. Acta Biomater **4**: 230-243.

Hojo M, Inokuchi S, Kidokoro M, Fukuyama N, Tanaka E, Tsuji C, Miyasaka M, Tanino R, Nakazawa H (2003) Induction of vascular endothelial growth factor by fibrin as a dermal substrate for cultured skin substitute. Plast Reconstr Surg **111**: 1638-1645.

Huang LL, Sung HW, Tsai CC, Huang DM (1998) Biocompatibility study of a biological tissue fixed with a naturally occurring crosslinking reagent. J Biomed Mater Res **42**: 568-576.

Huang YC, Dennis RG, Larkin L, Baar K (2005) Rapid formation of functional muscle in vitro using fibrin gels. J Appl Physiol **98**: 706-713.

Johnson TS, Xu JW, Zaporojan VV, Mesa JM, Weinand C, Randolph MA, Bonassar LJ, Winograd JM, Yaremchuk MJ (2004) Integrative repair of cartilage with articular and nonarticular chondrocytes. Tissue Eng **10**: 1308-1315.

Koepsell L, Zhang L, Neufeld D, Fong H, Deng Y (2011) Electrospun nanofibrous polycaprolactone scaffolds for tissue engineering of annulus fibrosus. Macromol Biosci **11**: 391-399.

Koo HJ, Lim KH, Jung HJ, Park EH (2006) Anti-inflammatory evaluation of Gardenia extract, geniposide and genipin. J Ethnopharmacol **103**: 496-500.

Krismer M, Haid C, Rabl W (1996) The contribution of anulus fibers to torque resistance. Spine (Phila Pa 1976) **21**: 2551-2557.

Lavik E, Langer R (2004) Tissue engineering: Current state and perspectives. Appl Microbiol Biotechnol **65**: 1-8.

Lee CK (1988) Accelerated degeneration of the segment adjacent to a lumbar fusion. Spine (Phila Pa 1976) **13**: 375-377.

Liang HC, Chang WH, Lin KJ, Sung HW (2003) Genipin-crosslinked gelatin microspheres as a drug carrier for intramuscular administration: *In vitro* and *in vivo* studies. J Biomed Mater Res A **65**: 271-282.

Mekhail M, Wong KK, Padavan DT, Wu Y, O'Gorman DB, Wan W (2010) Genipin-cross-linked electrospun collagen fibers. J Biomater Sci Polym Ed., in press.

Mesa JM, Zaporojan V, Weinand C, Johnson TS, Bonassar L, Randolph MA, Yaremchuk MJ, Butler PE (2006) Tissue engineering cartilage with aged articular chondrocytes *in vivo*. Plast Reconstr Surg **118**: 41-49.

Mi FL, Tan YC, Liang HF, Sung HW (2002) *In vivo* biocompatibility and degradability of a novel injectable-chitosan-based implant. Biomaterials **23**: 181-191.

Mizuno H, Roy AK, Vacanti CA, Kojima K, Ueda M, Bonassar LJ (2004) Tissue-engineered composites of anulus fibrosus and nucleus pulposus for intervertebral disc replacement. Spine (Phila Pa 1976) **29**: 1290-1298.

Mwale F, Iordanova M, Demers CN, Steffen T, Roughley P, Antoniou J (2005) Biological evaluation of chitosan salts cross-linked to genipin as a cell scaffold for disk tissue engineering. Tissue Eng **11**: 130-140.

Nerurkar NL, Elliott DM, Mauck RL (2007) Mechanics of oriented electrospun nanofibrous scaffolds for annulus fibrosus tissue engineering. J Orthop Res **25**: 1018-1028.

Nerurkar NL, Baker BM, Sen S, Wible EE, Elliott DM, Mauck RL (2009) Nanofibrous biologic laminates replicate the form and function of the annulus fibrosus. Nat Mater **8**: 986-992.

Nieponice A, Maul TM, Cumer JM, Soletti L, Vorp DA (2007) Mechanical stimulation induces morphological and phenotypic changes in bone marrow-derived progenitor cells within a three-dimensional fibrin matrix. J Biomed Mater Res A **81**: 523-530.

O'Connell GD, Vresilovic EJ, Elliott DM (2010) Human intervertebral disc internal strain in compression: The effect of disc region, loading position, and degeneration. J Orthop Res., in press.

Passaretti D, Silverman RP, Huang W, Kirchhoff CH, Ashiku S, Randolph MA, Yaremchuk MJ (2001) Cultured chondrocytes produce injectable tissue-engineered cartilage in hydrogel polymer. Tissue Eng **7**: 805-815.

Peretti GM, Xu JW, Bonassar LJ, Kirchhoff CH, Yaremchuk MJ, Randolph MA (2006) Review of injectable cartilage engineering using fibrin gel in mice and swine models. Tissue Eng **12:** 1151-1168.

Rowe SL, Lee S, Stegemann JP (2007) Influence of thrombin concentration on the mechanical and morphological properties of cell-seeded fibrin hydrogels. Acta Biomater **3**: 59-67.

Sato M, Asazuma T, Ishihara M, Kikuchi T, Masuoka K, Ichimura S, Kikuchi M, Kurita A, Fujikawa K (2003) An atelocollagen honeycomb-shaped scaffold with a membrane seal (ACHMS-scaffold) for the culture of annulus fibrosus cells from an intervertebral disc. J Biomed Mater Res A **64**: 248-256.

Schlegel JD, Smith JA, Schleusener RL (1996) Lumbar motion segment pathology adjacent to thoracolumbar, lumbar, and lumbosacral fusions. Spine (Phila Pa 1976) **21**: 970-981.

Sebastine IM, Williams DJ (2007) Current developments in tissue engineering of nucleus pulposus for the treatment of intervertebral disc degeneration. Conf Proc IEEE Eng Med Biol Soc **2007**: 6401-6406.

Sell SA, Francis MP, Garg K, McClure MJ, Simpson DG, Bowlin GL (2008) Cross-linking methods of electrospun fibrinogen scaffolds for tissue engineering applications. Biomed Mater **3**: 45001.

Shao X, Hunter CJ (2007) Developing an alginate/chitosan hybrid fiber scaffold for annulus fibrosus cells. J Biomed Mater Res A **82**: 701-710.

Starcher B (2001) A ninhydrin-based assay to quantitate the total protein content of tissue samples. Anal Biochem **292**: 125-129.

Sun J, Zheng Q, Wu Y, Liu Y, Guo X, Wu W (2010) Biocompatibility of KLD-12 peptide hydrogel as a scaffold in tissue engineering of intervertebral discs in rabbits. J Huazhong Univ Sci Technolog Med Sci **30**: 173-177.

Sung HW, Huang RN, Huang LL, Tsai CC (1999) *In vitro* evaluation of cytotoxicity of a naturally occurring cross-linking reagent for biological tissue fixation. J Biomater Sci Polym Ed **10**: 63-78.

Sung HW, Chang WH, Ma CY, Lee MH (2003) Crosslinking of biological tissues using genipin and/or carbodiimide. J Biomed Mater Res A **64**: 427-438.

Touyama R, Inoue K, Takeda Y, Yatsuzuka M, Ikumoto T, Moritome N, Shingu T, Yokoi T, Intuye H (1994) Studies on the blue pigments produced fromgenipin and methylamine II: The formation mechanisms of brownish-red intermediates leading to the blue pigment formation. Chem Pham Bull **42**: 1571-1578.

Tsai CC, Huang RN, Sung HW, Liang HC (2000) *In vitro* evaluation of the genotoxicity of a naturally occurring crosslinking agent (genipin) for biologic tissue fixation. J Biomed Mater Res **52**: 58-65.

Wan Y, Feng G, Shen FH, Balian G, Laurencin CT, Li X (2007) Novel biodegradable poly(1,8-Octanediol Malate) for annulus fibrosus regeneration. Macromol Biosci **7**: 1217-1224.

Wan Y, Feng G, Shen FH, Laurencin CT, Li X (2008) Biphasic scaffold for annulus fibrosus tissue regeneration. Biomaterials **29**: 643-652.

Wilke HJ, Heuer F, Neidlinger-Wilke C, Claes L (2006) Is a collagen scaffold for a tissue engineered nucleus replacement capable of restoring disc height and stability in an animal model? Eur Spine J **15** Suppl 3: S433-438.

Yamazaki M, Chiba K, Yoshikawa C (2009) Genipin suppresses A23187-induced cytotoxicity in Neuro2a cells. Biol Pharm Bull **32**: 1043-1046.

Yang L, Kandel RA, Chang G, Santerre JP (2009) Polar surface chemistry of nanofibrous polyurethane scaffold affects annulus fibrosus cell attachment and early matrix accumulation. J Biomed Mater Res A **91**: 1089-1099.

Yao CH, Liu BS, Chang CJ, Hsu SH, Chen YS (2004) Preparation of networks of gelatin and genipin as degradable biomaterials. Mater Chem and Phys **83**: 204-208.

Zhang YH, Zhao CQ, Jiang LS, Dai LY (2011) Substrate stiffness regulates apoptosis and the mRNA expression of extracellular matrix regulatory genes in the rat annular cells. Matrix Biol **30**: 135-144.

Editor's Note: All questions/comments by the reviewers were answered by text changes; hence, there is no "Discussion with Reviewers" section.