Published in final edited form as:

Biomaterials. 2011 January ; 32(2): 461–468. doi:10.1016/j.biomaterials.2010.09.015.

HOMOLOGOUS STRUCTURE-FUNCTION RELATIONSHIPS BETWEEN NATIVE FIBROCARILAGE AND TISSUE ENGINEERED FROM MSC-SEEDED NANOFIBROUS SCAFFOLDS

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Abstract

Understanding the interplay of composition, organization and mechanical function in load-bearing tissues is a prerequisite in the successful engineering of replacement tissues for diseased ones. Mesenchymal stem cells (MSCs) seeded on electrospun scaffolds have been successfully used to generate organized tissues that mimic fibrocartilages such as the knee meniscus and the annulus fibrosus of the intervertebral disc. While matrix deposition has been observed in parallel with improved mechanical properties, how composition, organization, and mechanical function are related is not known. Moreover, how this relationship compares to that of native fibrocartilage is unclear. Therefore, in the present work, functional fibrocartilage constructs were formed from MSC-seeded nanofibrous scaffolds, and the roles of collagen and glycosaminoglycan (GAG) in compressive and tensile properties were determined. MSCs deposited abundant collagen and GAG over 120 days of culture, and these extracellular molecules were organized in such a way that they performed similar mechanical functions to their native roles: collagen dominated the tensile response while GAG was important for compressive properties. GAG removal resulted in significant stiffening in tension. A similar stiffening response was observed when GAG was removed from native inner annulus fibrosus, suggesting an interaction between collagen fibers and their surrounding extracellular matrix that is shared by both engineered and native fibrocartilages. These findings strongly support the use of electrospun scaffolds and MSCs for fibrocartilage tissue engineering, and provide insight on the structure-function relations of both engineered and native biomaterials.

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INTRODUCTION

The overarching goal of tissue engineering is to develop strategies by which cells (generally in combination with biocompatible materials) can be appropriately instructed to form new tissues to replace damaged or diseased ones. This task is quite challenging for load-bearing soft tissues of the musculoskeletal system, where the combination of composition and microstructural organization gives rise to anisotropic and nonlinear mechanical behaviors that are necessary for tissue function. Despite this challenge, recent advances in the engineering of orthopaedic soft tissues such as meniscus [1,2], tendon and ligament [3–6], cartilage [7,8], and the annulus fibrosus of the intervertebral disc [9–12] have shown promise in the generation of functional replacement tissues. Outcome measures in such studies are often focused on compositional analyses to measure the accumulation of collagen and glycosaminoglycan (GAG), organizational analyses to determine how these constituents are assembled, and ultimately, mechanical testing to determine how these tissues perform functionally with respect to their native counterparts. While many testing modalities are available and germane to native tissue function, two of the most commonly employed methods include uniaxial tension [13–15] and unconfined compression [16–18].

A number of load-bearing tissues exemplify the interrelation between composition, structure, and function. For instance, tissues subject to large tensile stresses, such as ligaments and tendons, contain a large amount of collagen, while tissues subject primarily to compressive loading, such as articular cartilage and the nucleus pulposus of the intervertebral disc, contain large amounts of proteoglycan containing charged GAG chains [19]. Fibrocartilage tissues are subjected to both tension and compression and therefore contain relatively large amounts of both collagen and GAG, and include such tissues as the annulus fibrosus of the intervertebral disc and the knee meniscus.

To arrive at function from composition, it is necessary to consider structural organization. Fibrocartilages like the annulus fibrosus and knee meniscus are highly ordered structures, densely packed with collagen fibers that are locally aligned. Therefore, engineering successful replacements for these tissues necessitates replication of structural organization in order to ultimately achieve similar mechanical function. We have recently shown that electrospinning permits the fabrication of ordered nanofibrous assemblies that direct cell alignment and extracellular matrix deposition [20]. These scaffolds have been employed to engineer the annulus fibrosus across multiple length scales, from a single lamella [14,21] to angle-ply laminates [9] and most recently, whole disc composites containing an angle-ply annulus fibrosus surrounding a central nucleus pulposus [17]. In each case, mesenchymal stem cells seeded on nanofibrous scaffolds deposited GAG and collagen. Concomitant with extracellular matrix deposition, mechanical properties improved, and in the case of engineered angle-ply laminates, matched the uniaxial tensile modulus of the native annulus fibrosus after 10 weeks of *in vitro* culture [9]. While, clearly, matrix deposition and mechanical properties are related in these engineered tissues, the nature of this relationship, and how it compares to native tissue, is unknown. Therefore, the objective of the present study was to determine the functional role of GAG and collagen in engineered fibrocartilage formed from aligned electrospun nanofibrous poly(ϵ -caprolactone) (PCL) scaffolds seeded with mesenchymal stem cells (MSCs), and to determine whether these constituents are integrated in a manner consistent with their function in native fibrocartilage. While digestion studies have demonstrated the importance of GAG for the compressive properties of annulus fibrosus [22], their precise functional role in tension is not known. In the present study, the inner annulus fibrosus, which contains large amounts of both GAG and collagen, was investigated as a model for native fibrocartilage, and therefore, a second objective of the present work was to understand the role of GAG in the tensile behavior of inner annulus fibrosus.

MATERIALS AND METHODS

Scaffold fabrication

Nanofibrous PCL scaffolds were electrospun as described previously [1,9,14]. Eight grams of poly(ϵ -caprolactone) (PCL, Sigma Aldrich, batch # 00702CE) was dissolved at 37°C overnight in 56 mL of equal parts tetrahydrofuran and N, N-dimethylformamide. The PCL solution was ejected via syringe pump at 2.5 mL/h through a spinneret charged to +13 kV, generating a nanofibrous jet that was collected on a grounded mandrel rotating at 10 m/s at a distance of 20 cm from the spinneret tip. The spinneret was fanned axially along the mandrel to ensure uniform fiber deposition. Aluminum shields on either side of the spinneret were charged to +9 kV in order to focus the jet toward the mandrel [23]. Fibers were collected onto the mandrel for 6 to 8 hours, resulting in an aligned nanofibrous mesh of approximately 1 mm thickness. Rectangular scaffolds (5 mm $\times$ 30 mm) were excised from the mesh with the long axis parallel to the prevailing fiber direction.

Isolation of bovine mesenchymal stem cells (MSCs) and seeding on nanofibrous scaffolds

MSCs were isolated from bone marrow obtained from juvenile (3 – 6 month old) bovine knee joints as described previously [9,20] and expanded to passage 2 in basal medium containing 10% fetal bovine serum and 1% penicillin streptomycin and fungizone in DMEM. Scaffolds were hydrated by sequential washes in 100%, 70%, 50% and 30% ethanol and finally phosphate buffered saline (PBS). Before seeding, scaffolds were incubated overnight in 20 μ g/mL fibronectin at 25°C. MSCs were trypsinized, washed, and concentrated to 1×10^7 cells/mL in basal medium. 50 μ m of cell solution were applied to each side of the scaffold, allowing one hour at 37°C between applications. After an additional two hour incubation, samples were transferred to chemically defined media (DMEM, 0.1 μ M dexamethasone, 40 μ g/mL L-Proline, 100 μ g/mL Sodium Pyruvate, 1% Insulin, Transferrin, Selenium/Premix, and 1% penicillin, streptomycin and fungizone supplemented with 10 ng/mL Transforming Growth Factor β 3) [24]. Media was replaced twice weekly for 120 days of *in vitro* culture. This culture duration was chosen to ensure formation of a mature tissue with abundant extracellular matrix, so that the relative functional roles of collagen and GAG could be assessed.

Enzymatic digestion of mature engineered fibrocartilage constructs

Enzymatic digestions were performed in order to assess the roles of GAG and collagen in engineered fibrocartilage mechanics. All digestions were performed in PBS at 37°C for 36 hours. Collagen depletion was achieved by digestion with collagenase (COL-ASE, 10 U/mL) and GAG depletion was achieved by digestion with chondroitinase ABC (ChABC, 0.125 U/mL). A control group was subject to the digestion protocol, but without the addition of enzyme (PBS). A fourth group was tested directly from culture (Fresh) in order to determine whether the digestion protocol alone (PBS) had any effects on the mechanics or composition of the native and engineered fibrocartilages. Additional acellular scaffolds maintained in culture under identical conditions throughout the 120 culture period were also tested in order to establish baseline mechanical properties in the absence extracellular matrix deposition (SCAF). All digestion protocols were developed in preliminary studies to confirm effective removal of GAG and collagen. Because in preliminary experiments protease inhibitors were not necessary to maintain engineered construct biochemistry in PBS, none were included here; in native tissue experiments (below), however, protease inhibitors were used.

Mechanical, histologic, and biochemical analyses

Samples were either subject to uniaxial tensile testing followed by biochemical analyses ($n = 5$), unconfined compression testing ($n = 3$), or histologic examination ($n = 2$). Cross-sectional area was measured using a custom laser device that provided noncontact measurements of thickness and width [25]. Samples were lightly airbrushed with black enamel paint to generate texture for strain analysis (below). Testing in uniaxial tension was performed using an Instron 5542. All samples were placed into customized serrated grips and maintained in a PBS bath during the testing protocol: 5 minute preload of 0.1 N, followed by 15 cycles of preconditioning to 0.1% at 0.5%/sec, and finally a quasi-static constant elongation until failure at a rate of 0.1% strain/sec [26]. During the test, strain control was performed via crosshead displacement and images of the sample midsubstance were collected per 0.5% strain increments. Images were analyzed by texture correlation to determine the local surface deformations, and two-dimensional components of Lagrangian strain were calculated (Vic-2D, Correlated Solutions Inc.). Modulus was calculated by a linear regression to either the toe or linear region of the stress-strain response, and similarly Poisson's ratio was computed from the negative slope of longitudinal (applied) vs. transverse strain within the linear range of the stress-strain curve. After tensile testing, water content was determined by measuring the change in weight with overnight lyophilization. Dried samples were digested with papain, and biochemical analyses were conducted to determine total GAG and collagen content via 1,9-dimethylmethylene blue (DMMB) dye binding assay and measurement of ortho-hydroxyproline (OHP) following acid hydrolysis, respectively [24].

Compression testing was performed on 4 mm diameter samples cored from three separate constructs. Unconfined compression was performed by first applying a 2 gram creep preload for five minutes, followed by a ramp to 10% axial strain applied at 0.05% per second and then relaxation for 1000 seconds [15].

For histologic analyses, samples from each group ($n = 2$) were embedded in OCT freezing medium and flash frozen in liquid nitrogen. Embedded samples were cryo-sectioned in the plane perpendicular to the fiber direction (i.e. in cross-section) and stained to visualize GAG (Alcian Blue) or collagen (Picrosirius Red) as described previously [24].

Native inner annulus fibrosus preparation and mechanical testing

Intervertebral discs were excised from adult bovine caudal spines. Inner annulus fibrosus samples were harvested from each disc. The bovine caudal disc has previously been established as a valid model for disc research due to its functional and geometric similarities with the human disc [27,28]. Samples were taken circumferentially from the discs and sectioned to 1.5 mm thickness by freezing-stage microtome. Samples were further cut to uniform dimensions of $14 \times 3 \times 1.5 \text{ mm}^3$ (length $\times$ width $\times$ thickness) as described previously [26], and wrapped with PBS-soaked gauze for storage at -20°C until testing.

To parallel the above experiments on engineered fibrocartilage, native inner annulus fibrosus specimens were subject to GAG depletion by chondroitinase digestion (ChABC, $n = 6$ each) or were used in one of two control conditions: those tested after being freshly thawed (Fresh, $n = 7$) and those subject to the digestion protocol but lacking chondroitinase (PBS, $n = 6$). The digestion protocol consisted of 6 hours incubation with gentle agitation at 37°C in PBS with protease inhibitors (10mM N-ethylmaleimide, 5mM benzoic acid, 1mM phenylmethane-sulfonylfluoride) either with (ChABC) or without (PBS) the addition of chondroitinase ABC (1 U/ml). To control swelling, samples were hydrated in PBS for 30 minutes prior to tensile testing. Uniaxial tensile testing was performed as described above for engineered fibrocartilage. Effects of enzymatic digestion on the compressive properties

of native annulus fibrosus have been investigated elsewhere [22] and were therefore omitted from the present work. GAG content for each group was measured as above by DMMB assay. Although originally measured with respect to wet weight, data were converted to % dry weight by measuring water content for Fresh, PBS, and ChABC samples (n=3). This was done to compare with literature and engineered tissue data; no differences in water content were observed between the three groups, and all dry weight comparisons were preserved when compared on a wet weight basis.

RESULTS

Selective removal of extracellular matrix components in mature engineered fibrocartilage

Following 120 days of *in vitro* culture on aligned, electrospun PCL scaffolds, MSCs deposited a GAG- and collagen-rich extracellular matrix throughout the thickness of the scaffold (Fig. 1A, 2A). This deposition equated to approximately 15% and 25% dry weight of GAG and collagen, respectively (Figure 3A, B), demonstrating that MSCs produce a robust fibrocartilage tissue with abundant extracellular matrix. Effects of chondroitinase and collagenase digestions were examined by histology to confirm removal of GAG and collagen, respectively. Performing the digestion protocol in the absence of any degradative enzyme had no effect on GAG content or distribution (Fig. 1B). Similarly, no differences in collagen were observed between the freshly sectioned samples and those subject to the PBS incubation without degradative enzymes (Fig. 2A, B). Treatment with chondroitinase resulted in a complete depletion of GAG from the construct (Fig. 1C). Similarly, collagenase treatment resulted in a complete removal of GAG as well (Fig. 1D). These findings were confirmed quantitatively by biochemical analyses (Fig. 3A). Chondroitinase treatment had no effect on collagen content (Fig. 2C). However, treatment with collagenase resulted in a complete removal of both collagen and GAG from the construct (Fig. 2D). These findings were also confirmed quantitatively by biochemical analyses; a slight elevation in collagen content was observed for the PBS-incubated constructs compared to Fresh (Fig. 3B). Finally, water content was high (approximately 80%) in all groups, with only a slight reduction observed with chondroitinase treatment compared to PBS control (Fig. 3C).

Role of GAG and collagen in the mechanical properties of engineered fibrocartilage

Compression testing of acellular scaffolds revealed approximately 1 kPa peak stress followed by minimal stress relaxation (< 10%, Fig. 4A–C). Engineered constructs demonstrated significantly higher peak stress and percent stress relaxation (~ 8 kPa and 80%, respectively) when compared to acellular scaffolds, indicating that extracellular matrix deposition altered the mechanical behavior (Fig. 4A–C). Despite these differences, however, equilibrium compressive modulus of Fresh samples remained unchanged with extracellular matrix deposition compared to the acellular scaffold, while PBS-incubation of cell-seeded controls elevated the modulus with respect to Fresh samples (Fig. 4D). Removal of GAG significantly altered the initial response with a dramatic reduction in peak stress, returning to acellular scaffold levels; the relaxation behavior was unaltered relative to cell-seeded controls (Fig. 4A–C). Removal of GAG alone with chondroitinase caused a reduction in equilibrium modulus below scaffold levels (Fig. 4D), and removal of collagen and GAG with collagenase treatment resulted in a return of peak stress and percent stress relaxation to the levels of acellular scaffolds (Fig. 4B, C).

Tensile testing revealed a significant reduction in toe region modulus and a significant increase in linear region modulus for the mature (Fresh) cell-seeded constructs compared to acellular scaffold (Fig. 5A, B). No significant difference was observed between the Fresh and the PBS-incubated controls, indicating that the digestion protocol alone had no effect on tensile properties. Surprisingly, removing only the GAG via chondroitinase treatment

resulted in a significant increase (>10 MPa) in the linear region tensile modulus compared to PBS and Fresh samples. However, removal of GAG and collagen with collagenase resulted in a return of toe and linear region moduli to acellular scaffold levels (Fig. 5A). Linear region Poisson's ratio was significantly reduced with matrix deposition in the cell-seeded controls (Fresh and PBS, Fig. 5C). Chondroitinase digestion resulted in a significant increase in Poisson's ratio compared to all other groups.

Role of GAG in the tensile mechanics of native inner annulus fibrosus

While the importance of GAG and collagen for compressive and tensile properties of native fibrocartilage, respectively, have been widely investigated, what role if any GAGs play in defining tensile behavior are not well understood. To determine whether chondroitinase-induced changes in tensile behavior of engineered fibrocartilage mimic the response of native fibrocartilage to GAG depletion, the effect of chondroitinase treatment on the tensile behavior of native inner annulus fibrosus was next determined. A significant reduction in GAG was observed with chondroitinase treatment of the inner annulus fibrosus, while there was no GAG loss in the PBS group (Fig. 6A). No changes in toe-region modulus were observed with chondroitinase treatment or PBS incubation (Fig. 6B). However, chondroitinase treatment resulted in a significant increase in linear region modulus relative to PBS and Fresh controls (Fig. 6C), paralleling the findings for engineered fibrocartilage (Fig. 5B).

DISCUSSION

In this study, MSCs were seeded onto aligned, electrospun nanofibrous scaffolds to generate mature engineered fibrocartilage constructs that mirror many key functional aspects of native fibrocartilage. MSCs deposited and retained the two major fibrocartilage extracellular matrix components, GAG and collagen; moreover, these extracellular matrix molecules were organized in such a way that their mechanical functions were conserved with respect to native load-bearing soft tissues of the musculoskeletal system. Constructs achieved extracellular matrix contents similar to native fibrocartilage: 15 % dry weight GAG compared to 25 % dry weight in the inner annulus fibrosus and 25 % dry weight collagen compared to 25 – 40 % for the inner annulus fibrosus.

Consistent with conventional thinking for native tissues, GAG played an important role in the compressive response of engineered fibrocartilage (Fig. 4). GAG removal with chondroitinase or with collagenase (which also removed collagen) resulted in a compressive peak stress equal to acellular scaffold values, indicating that GAG – and not collagen or other extracellular matrix molecules – dictates the immediate, or short-term, compressive response of these engineered tissues (Fig. 4A). This is consistent with experiments on the intervertebral disc, as well as cartilage and meniscus, where loss of GAG is associated with a dramatic decline in compressive properties [22,29,30]. Stress relaxation was minimal for the acellular scaffold. As scaffold porosity is quite high (80–90%), it is likely that water was exuded during the loading phase of the stress relaxation, such that fluid pressurization was minimal and equilibrium reached at or soon after peak stress. Moreover, in the absence of GAG or other molecules to establish osmotic pressure, there is no osmotic restriction to fluid exudation. After extracellular matrix deposition in Fresh and PBS-incubated samples, however, stress relaxation of over 80% was observed. This suggests that fluid pressurization occurs during loading, and is subsequently dissipated as fluid slowly leaves the tissue under constant load. This biphasic response is consistent with the behavior of many load-bearing soft tissues. Because GAG removal did not affect the transient response, but GAG and collagen removal with collagenase did, it is expected that collagen, or its interaction with GAG, is primarily responsible for determining the hydraulic permeability and therefore the transient response of the engineered tissue under compression. This is consistent with

studies of native cartilage mechanics that indicate that collagen defines the hydraulic permeability of the tissue [31]. These results suggest that MSCs seeded on electrospun, aligned nanofibrous PCL scaffolds generate a tissue that resists compressive loads by mechanisms similar to load-bearing soft tissues of the musculoskeletal system.

Concomitant with extracellular matrix deposition and altered compressive mechanics, tensile properties were also significantly altered in engineered fibrocartilage relative to acellular scaffolds. Matrix deposition resulted in a reduction in the toe-region modulus, which was incrementally restored to acellular scaffold levels by the removal of GAG, and GAG and collagen (Fig. 5A). Why mature, cell-seeded constructs were more compliant than acellular scaffold at small deformations is unclear, however it is possible that an increasing contrast in stiffness between the fibrous phase and the surrounding extrafibrillar matrix phase (due to anisotropic tissue deposition) may favor fiber rotation (soft response) at small strains and consequently fiber stretch (stiff response) at large strains. This is consistent with the observed stiffening in the linear region of the engineered fibrocartilage constructs compared to acellular scaffold. The linear region modulus increased significantly with matrix deposition, and this increase was eliminated upon removal of collagen via collagenase treatment (Fig. 5B). This finding is also in keeping with the well-established notion that collagen resists tensile stresses in load-bearing soft tissues.

Perhaps most interesting, however, was that removal of GAG, a molecule conventionally thought to have little to no role in tensile properties, resulted in a large (> 10 MPa) increase in the linear region modulus compared to the untreated mature construct (Fig. 5B). Remarkably, this pronounced stiffening effect was achieved without altering the collagen network known to dictate tensile properties. Additionally, because GAG depletion did not significantly alter cross-sectional area (not shown) relative to Fresh and PBS-incubated controls, this increase in modulus indicates an increase in load-bearing capacity, and not a shrinking or reduction of cross-section.

It is possible that GAG depletion may increase the tensile properties by altering recruitment under load. If a population of fibers, dispersed about some prevailing direction of alignment, are enmeshed within an isotropic GAG-rich extrafibrillar matrix, application of a uniaxial load would result in re-organization of the fibers, rotating them toward the direction of loading [26,32,33]. In the process, rotating fibers must compress their surrounding matrix as the fibers collapse inward toward the loading direction, and consequently water is exuded from the material as it contracts laterally. However, if the GAG-rich extrafibrillar matrix is perturbed, for instance via chondroitinase treatment as in the present study, fibers would be able to reorient with less impediment from the surrounding matrix. Therefore, it is possible that GAG depletion results in enhanced fiber recruitment, as the matrix provides less resistance to fiber re-organization after GAG removal. The significant increase in Poisson's ratio with chondroitinase treatment supports this mechanism, as enhanced fiber rotation would increase lateral contractions under fixed longitudinal stretch. In other words, for a given extension along the loading direction, an increase in the Poisson's ratio indicates an increase in fiber rotation toward the loading direction, consistent with the proposed mechanism of stiffening.

Because it was unclear whether the observed tensile stiffening following GAG depletion was an aberrant behavior of engineered neo-tissues, or whether this behavior is also present in native fibrocartilage, we next focused on understanding how GAG depletion affects the tensile response of native fibrocartilage, and we selected the inner annulus fibrosus of the intervertebral disc as a representative tissue. The annulus fibrosus has a radially heterogeneous distribution of GAG. Therefore the inner annulus, where GAG content is similar to that of engineered constructs, was chosen for analysis. Despite similar

extracellular matrix composition, the tensile modulus of inner annulus fibrosus was considerably lower than engineered fibrocartilage (approximately 3 MPa for native vs. 35 MPa for engineered). This is due to the difference in tensile testing orientation and tissue architecture: engineered fibrocartilages were tested along their direction of collagen alignment, while the native inner annulus was tested along the disc circumferential direction, oblique to the local directions of collagen alignment. Nonetheless, native inner annulus demonstrated more than a two-fold increase in linear modulus upon GAG depletion, similar to engineered constructs. PBS incubation of inner annulus samples did not alter GAG content or tensile modulus, indicating that stiffening was a direct consequence of GAG depletion. Therefore, engineered and native fibrocartilage both stiffen in response to GAG depletion. This further support the notion that engineered fibrocartilages formed from MSC-seeded nanofibrous scaffolds obey the same composition-function laws as native tissue.

These findings present new insight into the interplay between GAG and collagen in load-bearing soft tissues, indicating that for fibrocartilage tissues, the association of GAG with compression and collagen with tension does not fully define their mechanical roles. Instead, a complex interaction permits the shielding of collagen by its surrounding GAG-rich matrix, whereby fiber reorganization requires fluid exudation as the rotating fibers compress their surrounding matrix. While this interaction has been proposed recently for cartilage [34], this is the first evidence for such an interaction within the annulus fibrosus and other fibrocartilages.

The role of GAG in tensile mechanics has been widely investigated for a number of other fiber-reinforced soft tissues that span a wide range of endogenous GAG contents, from tendon and ligament (0.1% – 1% dry weight) to articular cartilage (approximately 25% dry weight, Fig. 7). While their role in resisting compressive forces in these tissues well established, there is no clear consensus regarding what role, if any, GAGs play in the response to tensile forces across different tissues. In tissues with relatively low GAG content, there are conflicting reports on the effects of GAG removal on tensile properties [35–37]. Unlike studies in ligament and tendon, where very little GAG is present, investigations in cartilage demonstrate that indeed GAG depletion does result in stiffening of the tensile response in the linear region of the stress-strain curve (Fig. 7). This has been demonstrated in native cartilage [38,39] as well as engineered cartilage constructs [40]. Finally, recent theoretical work has demonstrated that by modeling cartilage as a disperse population of fibers within an osmotically swollen matrix, it is possible to capture this stiffening response of cartilage upon reduction in fixed charge density (akin to chondroitinase-induced GAG depletion) [34]. The authors reasoned that tensile stiffening proceeds from a reduction in GAG-related osmotic swelling of the matrix, which under normal conditions may restrict fiber reorganization under uniaxial load. This interpretation is consistent with the experimental findings of the present study (Fig. 7).

The present work may also have important implications for MSCs as a promising cell source for fibrocartilage tissue engineering. While there has been a substantial amount of work done to understand the appropriate environmental conditions and stimuli for MSC differentiation along chondrogenic, adipogenic, and osteogenic lineages [41], much less has been done to understand what factors drive MSC differentiation toward a fibrous or fibrochondrogenic phenotype. In the present study, MSCs elaborated a functional fibrocartilagenous extracellular matrix. While genetic markers that signify differentiation were not investigated, an important downstream output measure, tissue-level mechanics, indicated that MSCs on aligned electrospun scaffolds in the presence of TGF- β 3 give rise to a fibrocartilage tissue in which mechanical function of its constituents is conserved with respect to native load-bearing soft tissues of the musculoskeletal system.

CONCLUSIONS

The present study demonstrates that mesenchymal stem cells on aligned electrospun scaffolds not only produce and accumulate the correct extracellular molecules – GAG and collagen – but that they assemble these molecules in such a way that their mechanical functions are conserved with respect to load-bearing soft tissues of the musculoskeletal system. This is a strong indication that aligned electrospun scaffolds direct the formation of functional tissue analogs, and that this approach can be successfully used to engineer fibrocartilage constructs to replace injured or degenerated load-bearing tissues. Further, the present work demonstrates the important role of GAGs in the tensile behavior of the annulus fibrosus, where GAG depletion was found to significantly stiffen the tissue. A complete understanding the multi-faceted interrelation of structure, composition, and mechanical function will be an invaluable platform for the development of treatments for degenerative diseases of load bearing soft tissues of the musculoskeletal system.

Acknowledgments

The authors gratefully acknowledge Sounok Sen and Emily Wible for their assistance with mechanical testing and histologic analyses, respectively. This work was funded by NIH grants AR 056624 and EB 02425. Additional support was provided by the Penn Center for Musculoskeletal Disorders (AR 050950).

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Fig. 1. Alcian Blue staining of MSC-seeded electrospun PCL scaffolds after 120 days of culture, when sectioned without prior incubation (Fresh, A), when subjected to the digestion protocol but without enzymatic digestion (PBS, B), after treatment with chondroitinase (ChABC, C), or after treatment with collagenase (COL-ASE, D). Scale = 250 μ m.

Fig. 2. Picosirius Red staining of MSC-seeded electrospun PCL scaffolds after 120 days of culture, when sectioned without prior incubation (Fresh, A), when subjected to the digestion protocol but without enzymatic digestion (PBS, B), after treatment with chondroitinase (ChABC, C), or after treatment with collagenase (COL-ASE, D). Scale = 250 μ m.

Fig. 3.

GAG (A), collagen (B), and water content (C) of acellular scaffolds (SCAF) and matured tissue constructs after no treatment (Fresh), PBS incubation (PBS), chondroitinase treatment (ChABC) and collagenase treatment (COL-ASE). DW = dry weight. * and # = $p < 0.05$ compared to PBS and SCAF, respectively.

Fig. 4. Representative stress relaxation curves (A), peak stress (B), percent stress relaxation (C), and equilibrium compressive modulus (D) measured by unconfined compression foracellular scaffolds (SCAF) and matured tissue constructs after no treatment (Fresh), PBS incubation (PBS), chondroitinase treatment (ChABC) and collagenase treatment (COL-ASE). * = $p < 0.05$ compared to PBS; # = $p < 0.05$ compared to SCAF.

Fig. 5.

Toe region modulus (A), linear region modulus (B), and linear region Poisson's ratio (C) measured by uniaxial tensile testing for acellular scaffolds (SCAF) and matured tissue constructs after no treatment (Fresh), PBS incubation (PBS), chondroitinase treatment (ChABC) and collagenase treatment (COL-ASE). * = $p < 0.05$ compared to PBS; # = $p < 0.05$ compared to SCAF.

Fig. 6. GAG content (A) and toe (B) and linear (C) region moduli measured by uniaxial tensile testing for native inner annulus fibrosus tested without prior incubation (Fresh), with incubation in PBS (PBS), or after treatment with chondroitinase (ChABC). * = $p < 0.05$ compared to PBS; # = $p < 0.05$ compared to SCAF.

Fig. 7.

The effect of GAG depletion on tensile properties has been studied in a number of tissues ranging from low GAG contents (tendon and ligament) to high GAG contents (cartilage). In the present study, inner annulus fibrosus and engineered fibrocartilage constructs possessed a moderate to high GAG content, and GAG depletion in these tissues induced tensile stiffening, which is observed in cartilage, but not tendon or ligament. References where GAG depletion-induced stiffening was observed are provided in red, while those in which little or no change was observed are in black. DW = dry weight.