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Superficial Zone Extracellular Matrix Extracts Enhance Boundary Lubrication of Self-Assembled Articular Cartilage

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Abstract

Objective. Previous work has shown that increasing the production of boundary lubricant, superficial zone protein (SZP), did not reduce the friction coefficient of self-assembled articular cartilage constructs and was possibly due to poor retention of the lubricant. The aim of this investigation was to reduce the friction coefficient of self-assembled articular cartilage constructs through enhancing SZP retention by the exogenous addition of extracellular matrix (ECM) extracted from the superficial zone of native articular cartilage. *Design*. Superficial zone cartilage was shaved from juvenile bovine femoral condyles using a dermatome, minced finely with razor blades, extracted with 4 M guanidine-hydrochloride, buffer exchanged with culture medium, and added directly to the culture medium of self-assembled articular cartilage constructs at low (10 μg/mL) and high (100 μg/mL) concentrations for 4 weeks. Biochemical and biomechanical properties were determined at the conclusion of 4 weeks culture. *Results*. ECM treatment increased compressive and tensile stiffness of self-assembled articular cartilage constructs and decreased the friction coefficient. Glycosaminoglycan content decreased and collagen content increased significantly in self-assembled constructs by the ECM treatment. *Conclusions*. Friction coefficients of self-assembled articular cartilage constructs were reduced by adding extracted superficial zone ECM into the culture medium of self-assembled articular cartilage constructs.

Keywords

chondrocytes, articular cartilage, extracellular matrix, cartilage lubrication, SZP

Introduction

Superficial zone protein (SZP) (345 kDa), lubricin (227 kDa), and PRG4 (460 kDa) are all encoded by the *prg4* gene and are used interchangeably in the literature as the critical boundary lubricant for articular cartilage.[1,2] Historically, SZP has been used in our laboratory due to the availability of the antibody that recognizes the 345 kDa gene product. Therefore, to avoid confusion, SZP will be used in this article thereon to refer to the cartilage lubricant. SZP was identified in the articular cartilage, specifically in the superficial zone, and in synoviocytes and synovium of the diarthrodial joint.[3] From explant cultures of superficial zone cartilage, SZP was found to be mainly secreted into the culture medium and not retained in the matrix. Immunochemical localization studies revealed that although SZP is not incorporated into the matrix, it does accumulate in the superficial zone of articular cartilage and at the interface between articular joint tissues such as the synovial lining, tendons, and ligaments.[4] It is believed that SZP binds onto cartilage surfaces as a thin monolayer film where it confers low friction and minimizes wear of the tissue.[3,5]

It is hypothesized that putative binding macromolecules for SZP are available at cartilage surfaces.[6] The articular surface consists of an acellular layer known as the lamina splendens.[7] Scanning electron microscopy revealed the surface as a tangentially arranged layer of collagen fibrils covered by a single layer of electron dense, amorphous material[4] that has yet to be identified. It has been suggested that in some instances this area contains type I collagen.[8] Type IX collagen has also been reported to be found on the surfaces of articular cartilage collagen fibrils covalently bound to type II collagen or type XI collagen forming structures

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referred to as the collagen II:IX:XI heteropolymer.[9] Some major proteins found in the cartilage surface include the collagen-binding small proteoglycans fibromodulin, decorin, biglycan, and the glycoprotein fibronectin.[4]

To identify critical SZP binding molecules, cell culture plates have been coated with different extracellular matrix (ECM) proteins and incubated with varying concentrations of SZP.[10] Fibronectin, hyaluronan, and bovine serum albumin (BSA) coated wells were shown to bind with SZP in a dose-dependent manner. Heparin has also been suggested to be involved in cartilage matrix binding to SZP. SZP isoforms, lacking exons 4 and 5 encoding heparin binding sites, demonstrated reduced binding to ECM-coated and heparin-coated 96-well plates.[11] Although these molecules have shown the ability to bind and interact with SZP, no definitive data have been presented yet as to the underlying mechanism.

Previous studies have shown that increasing production of SZP in tissue engineered cartilage had no effect on its frictional properties.[12] This paradoxical result was hypothesized to be due to the poor retention of SZP in the matrix of the engineered cartilage. Therefore, the aim of this study was to reduce the friction coefficient of self-assembled cartilage by enhancing SZP retention in the engineered tissues through exogenous addition of ECM components. Since the putative SZP binding macromolecules of the superficial zone articular cartilage are unknown, the complete superficial zone ECM was extracted from bovine femoral condyles and added directly into the culture media of self-assembled cartilage.

Materials and Methods

Extracellular Matrix Preparation

Superficial zone articular cartilage ECM was extracted from the femoral condyles of juvenile bovine stifle joints (Research 87, Boylston, MA) using 4 M guanidine-hydrochloride (guanidine-HCl) in 50 mM Tris–HCl buffer, pH 7.4. Superficial zone articular cartilage was shaved using a dermatome and collected into a petri dish containing wash media. Wash media consisted of Dulbecco's Modified Eagle Medium (DMEM), containing 4.5 mg/mL glucose and GlutaMAX (Gibco, Grand Island, NY), and supplemented with 1% penicillin/streptomycin and 0.5% Fungizone. Shaved cartilage was washed 3 times with wash media and finely minced into ~1 mm pieces using 2 single-edged razor blades held side-by-side. Approximately 0.25 g of minced tissue was then transferred to one 14 mL polypropylene round-bottom tube (BD Biosciences) and homogenized in 2 mL of 4 M guanidine-HCl. After homogenization, 5.5 mL of 4 M guanidine-HCl was added to the homogenized tissue (30 mL extraction solution per gram of tissue) and incubated overnight at 4°C. Tubes were centrifuged to remove insoluble components and supernatant was concentrated 10-fold with the Amicon Ultra-Centrifugal Filter, with a molecular weight cutoff of 10 kDa (Millipore, Billerica, MA). The guanidine solution was then buffer exchanged with culture medium consisting of DMEM, containing 4.5 mg/mL glucose and GlutaMAX (Gibco), and supplemented with 1% ITS+ Premix (BD Biosciences, Franklin Lakes, NJ), 100 nM dexamethasone (Sigma, St. Louis, MO), 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA), 0.5% Fungizone (Invitrogen), 50 µg/mL ascorbate-2-phosphate, 40 µg/mL L-proline, and 100 µg/mL sodium pyruvate, until the final concentration of guanidine-HCl was reduced to less than 6 µM. This final solution was then sterile filtered through a 0.2 µm pore size Nalgene Rapid-Flow filter unit and the total protein concentration (31.7 mg/mL) was quantified using the BCA protein assay kit (Pierce ThermoScientific, Waltham, MA). The extracted, soluble ECM stock solution was stored at 4°C.

Extracellular Matrix Treatment

The stock solution of ECM was diluted in culture medium to yield a high (100 µg/mL) and low (10 µg/mL) concentration, which was directly added to the constructs. Control and ECM groups were treated with 10 ng/mL of TGF-β1 throughout the entire culture duration (4 weeks). For the first 7 days, a full media change was performed daily with the treatments. After day 7, constructs were unconfined and full media changes were performed every 2 to 3 days.

Chondrocyte Isolation

Articular cartilage from the femoral condyle and patellofemoral groove was harvested from juvenile bovine stifle joints. Cartilage slices were finely minced into ~1 mm pieces using 2 single-edge razor blades held side-by-side, washed 3 times in the wash media, and digested for 16 hours in digestion media (DM) containing 0.2% Collagenase II (Worthington, Lakewood, NJ) in wash medium and 3% fetal bovine serum (FBS, Gibco). The released chondrocytes were filtered through a cell strainer (70 µm, BD Falcon, Franklin Lakes, NJ), washed in wash medium and the cell number determined using a hemocytometer.

Self-Assembly of Engineered Cartilage Constructs

Nonadherent, agarose wells were prepared by placing sterile, stainless steel molds consisting of 5 mm diameter × 10 mm long cylindrical prongs into a 48-well plate containing 1 mL/well of molten 2% agarose (Fisher Scientific, Waltham, MA) in phosphate-buffered saline (PBS). After the agarose solidified at room temperature, the mold was removed and the agarose was perfused with culture medium. Into each well, 4 million cells were seeded in 100 µL of serum-free culture media, followed by an additional 400 µL

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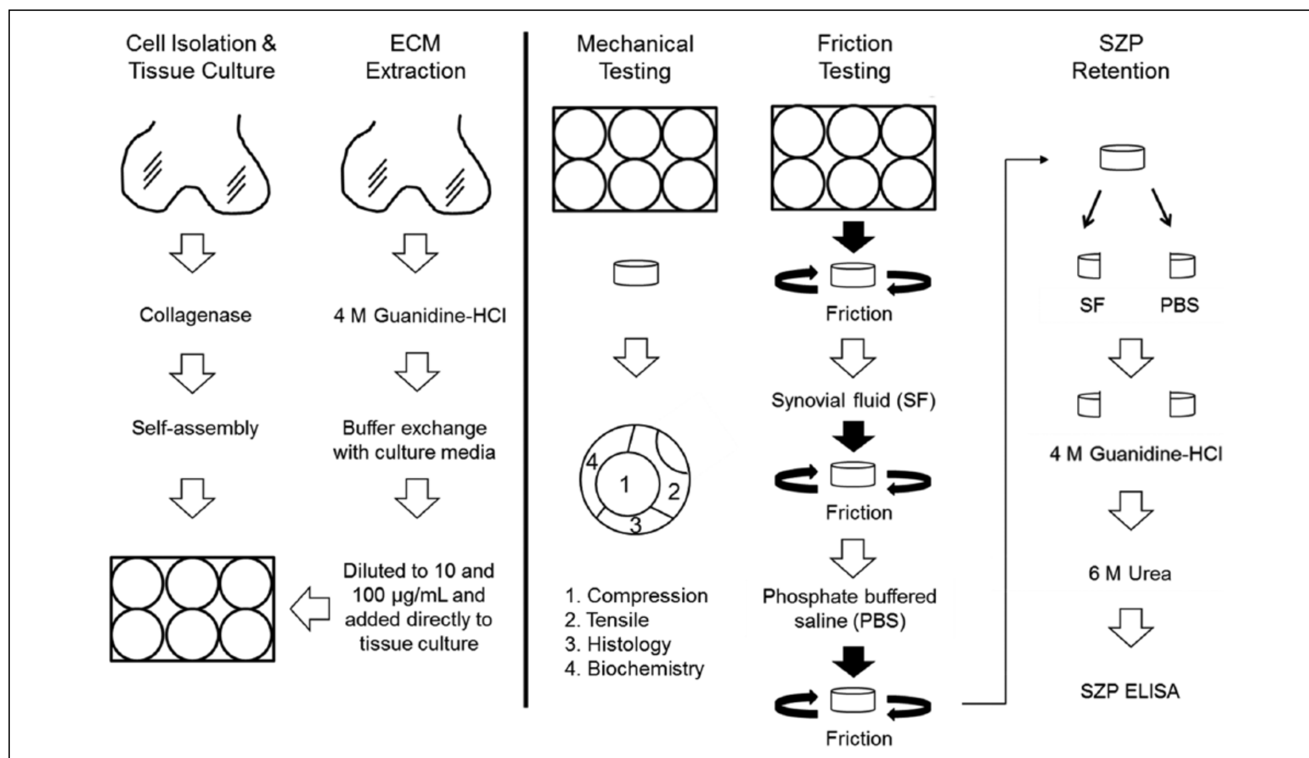

Figure 1. Schematic drawing of experimental procedure. Cartilage from the femoral condyle was harvested and digested in collagenase solution. Cells were isolated and self-assembled in tissue culture plates. Extracellular matrix (ECM) from the femoral condyle was extracted using 4 M guanidine-HCl, buffer exchanged with culture medium, diluted to the desired concentrations in culture medium, and then added directly to culture medium of self-assembled cartilage. Self-assembled cartilage cultured for 4 weeks was then taken directly out of culture and either used for mechanical or friction testing. For mechanical testing, one construct was divided, as shown, for compression, tension, histology, and biochemistry analyses. For friction testing, another construct was taken directly out of culture and tested for friction, immersed in synovial fluid for 30 seconds, tested for friction, immersed in phosphate buffered saline (PBS) for 30 seconds, tested for friction, and then saved for determination of superficial zone protein (SZP) retention. Self-assembled constructs were split in half; one immersed in synovial fluid and the other in PBS for 30 seconds. Retained SZP was then extracted from the tissue and measured using ELISA.

of culture media 4 hours later. On the seventh day of culture, constructs were unconfined and transferred to nonadherent tissue culture plates coated with a thin layer of agarose. Full media changes were performed daily during the first week of culture. After unconfinement, full media changes were performed every 2 to 3 days until the end of culture duration (4 weeks). All engineered tissues were cultured at 37°C in a humid atmosphere containing 5% carbon dioxide. After 4 weeks of culture, constructs were processed for gross morphology, histology, quantitative biochemistry, and mechanical testing. A schematic drawing of the experimental design is presented in **Figure 1**.

Gross Morphology, Histology, and Immunohistochemistry

The dimensions of the engineered constructs were determined using ImageJ (National Institutes of Health, Bethesda, MD). For histology, constructs were fixed in Bouin's solution (Sigma) for 24 hours, followed by paraffin embedding and sectioning (4 µm thick). Sections were stained with toluidine blue to demonstrate metachromasia due to proteoglycans. For immunohistochemistry (IHC) of SZP, slides were deparaffinized using xylene and rehydrated with graded ethanol, quenched of peroxidase activity with hydrogen peroxide, and blocked with 1% BSA. SZP immunostaining was performed according to prior methods[13,14] using monoclonal antibody S6.79 (1:1,000 dilution) as the primary antibody. To expose collagen epitopes, samples were pretreated with Proteinase K (Sigma). Collagen type II immunostaining utilized mouse monoclonal antibodies (Pierce) at 1:200 dilution. The signal detection for all immunostaining was performed using a mouse IgG secondary antibody with an avidin-biotin-peroxidase kit (Vector Laboratories, Cambridgeshire, UK) following the manufacturer's directions. As a comparison, 5 mm diameter explants were obtained from bovine femoral condyles, cut to a thickness of ~400 µm (surface zone), and processed for histology and IHC.[14]

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Biochemical Composition Analysis

Constructs were frozen overnight and lyophilized for 48 hours, after which the dry weights were obtained. They were then digested in 125 μg/mL papain (Sigma) in phosphate buffer (pH 6.5) containing 2 mM N-acetyl-L-cysteine (Sigma) and 2 mM EDTA for 18 hours at 60°C. Glycosaminoglycan (GAG) content was quantified by a 1,9-dimethylmethylene blue binding assay using a commercially available kit according to the manufacturer's directions (Blyscan GAG Assay, Biocolor, Westbury, NY). Total collagen was quantified after hydrolyzing samples with 2 N NaOH for 20 minutes at 120°C using a chloramine-T hydroxyproline assay with trans-4-hydroxyl-L-proline (Sigma) as a standard, assuming a 12.5% hydroxyproline content in collagen.[15]

Compressive and Tensile Testing

The compressive aggregate modulus was determined using a creep indentation apparatus.[16] A 2.5 mm diameter punch was taken from the center of each construct (**Fig. 1**), attached to a flat stainless steel surface with a thin layer of cyanoacrylate glue, and allowed to equilibrate for 20 minutes in PBS. The sample was then placed into the creep indentation apparatus, which automatically loaded and unloaded the specimen while recording the tissue's creep and recovery behavior. A tare load of 0.2 g, followed by a test load of 0.7 g, was applied to all samples with a 0.8-mm-diameter, flat-ended, porous, rigid tip. All loads were applied until equilibrium was reached. To calculate the specimen's aggregate modulus, a semianalytical, seminumeric, linear, biphasic model was used.[17]

Tensile mechanical properties were determined using the Test Resources 840L.[18] Briefly, 2.5 mm punches were used to trim construct samples and generate a dog-bone shape (**Fig. 1**), which were glued to paper tabs to establish a gauge length of 1.3 mm. Samples were loaded at a constant rate of 1% strain per second. Young's modulus and ultimate tensile strength (UTS) were quantified from stress-strain curves generated from the load-displacement results.

Friction Testing

The friction coefficients of the constructs were determined using a pin-on-disk tribometer operated in the boundary lubrication regime in reciprocating sliding mode as described previously.[14] Briefly, a 5 mm punch was taken from the center of each construct, affixed to acrylic pins by cyanoacrylate glue, and then brought into contact with a polished glass disk while fully immersed in PBS. Prior to the initiation of each friction test, the sample was allowed to equilibrate unconfined under the applied load (0.1 MPa) for 2 minutes to minimize any fluid effects during testing. The test duration of each friction experiment was fixed at 5 minutes at a sliding speed of 0.5 mm/s. Data were collected (Daqview, IOtech, Cleveland, OH) and processed using a standard software package (Microsoft, Seattle, WA).

The friction coefficient of self-assembled constructs was measured by testing its friction immediately out of culture as described above. Constructs were then blotted dry with a Kimwipe, immersed in synovial fluid for 30 seconds, washed 3 times in PBS for 30 seconds each, and then placed on the tribometer to measure its friction coefficient. After 5 minutes of testing, the construct was again blotted dry, immersed in PBS for 30 seconds, washed 3 times again, and placed on the tribometer to measure friction. Constructs were then cut in half, immersing one half of the tissue in PBS and the other half in synovial fluid for 30 seconds. Tissues were washed again 3 times in PBS, homogenized in 4 M guanidine-HCl, and then frozen at −20°C (**Fig. 1**).

SZP Detection and Quantification

Tissue extracts in 4 M guanidine-HCl were buffer exchanged with 6 M urea in 50 mM Tris (pH 7.4) using the Amicon Ultra-Centrifugal Filter. Extracts were then used to determine SZP retention in the matrix and quantified by enzyme-linked immunosorbent assay (ELISA) using SZP purified from bovine synovial fluid as a standard. Each well of 96-well MaxiSorp plates (Nalge Nunc International, Rochester, NY) was coated with 1 μg/mL peanut lectin (EY Laboratories, San Mateo, CA) in 50 mM sodium carbonate buffer (pH 9.5). The wells were then blocked with 1% BSA in the same buffer. Aliquots of extracted SZP in 6 M urea, monoclonal antibody S6.79 (1:5,000 dilution), and horseradish peroxidase-conjugated goat anti-mouse IgG (1:3,000) were sequentially incubated for 1 hour each in the wells. Finally, the SuperSignal ELISA Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific) was applied and the chemiluminescence of each well was measured. Wells were washed with PBS containing 0.05% Tween 20 (Sigma) between all steps. SZP levels were calculated using a SZP standard that was purified by affinity chromatography on a peanut lectin column; the SZP standard was verified by immunoblot analysis and quantified using a Micro BCA Protein Assay Kit (Pierce). SZP levels were normalized against total protein using the Micro BCA Protein Assay Kit.

Statistics

Biochemical and biomechanical testing of self-assembled cartilage consisted of a sample size of $n = 6$. A sample size of $n = 6$ was used for SZP ELISA and $n = 3$ for histology and IHC. Both one- and two-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test were performed (JMP10) to determine if treatments and lubricant baths were significant factors. Groups not connected by the

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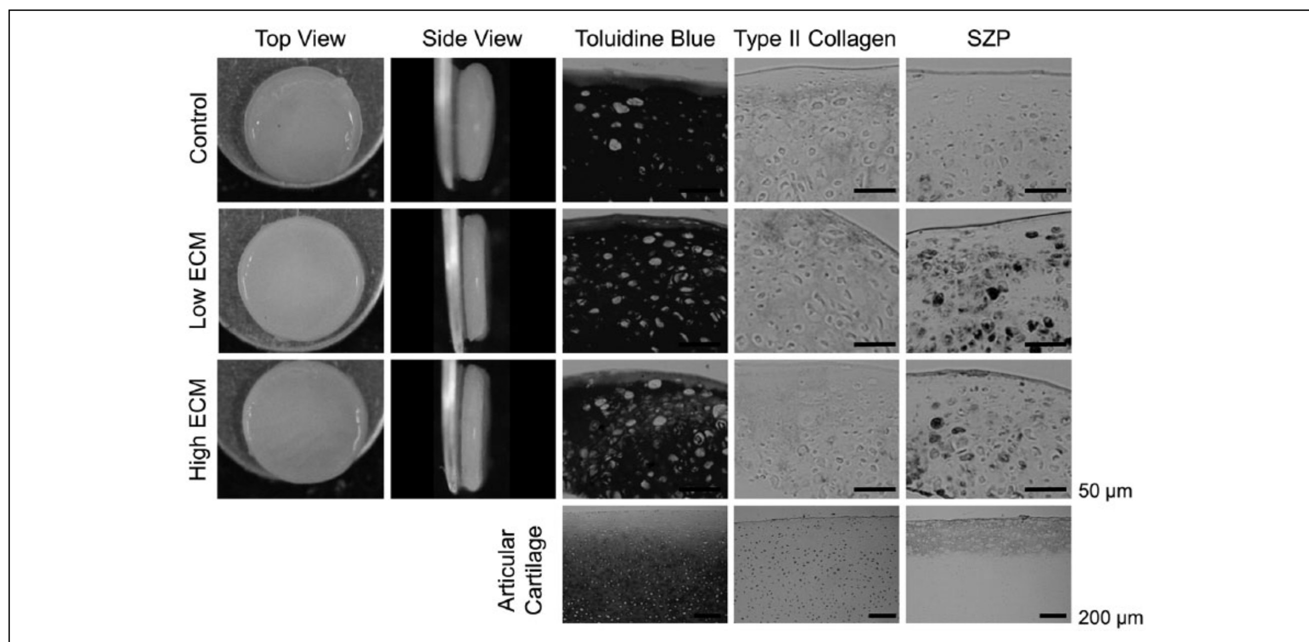

Figure 2. Gross morphology, histology, and immunohistochemistry of self-assembled constructs and native articular cartilage. The gross morphology from a top and side view of constructs after t = 4 weeks of culture is shown. Histology sections of constructs were stained with toluidine blue to detect sulfated glycosaminoglycans (GAG). Immunohistochemistry was performed on sections for type II collagen and superficial zone protein (SZP) (constructs scale bar: 50 µm). Native articular cartilage was also stained with toluidine blue, type II collagen, and SZP (native cartilage scale bar: 200 µm). ECM = extracellular matrix.

Table 1. Physical Properties of Constructs After 4 Weeks of Culture[a].

Treatment Group	Wet Weight (mg)	Hydration (%)	Diameter (mm)	Thickness (mm)
Control	13.7 ± 1.1^{A}	77 ± 2^{A}	4.2 ± 0.1^{B}	1.0 ± 0.1^{A}
Low ECM	12.3 ± 0.3^{B}	78 ± 1^{A}	4.5 ± 0.1^{A}	0.8 ± 0.1^{B}
High ECM	11.8 ± 0.5^{B}	78 ± 3^{A}	4.5 ± 0.1^{A}	0.7 ± 0.1^{B}

[a]The diameter, thickness, wet weight, and hydration of constructs were measured. Extracellular matrix (ECM) treatment decreased wet weight and thickness but increased construct diameter. Hydration was not affected. A one-way ANOVA followed by Tukey's *post hoc* test was applied. Values not connected by the same letter are significantly different. Values are presented as mean ± standard deviation.

same letter are significantly different. A Student's paired t test (Microsoft Excel) was performed on the effects of lubricant bath on SZP retention with $P < 0.05$ considered significant.

Results

Gross Morphology, Histology, and Immunohistochemistry

Grossly, all constructs appeared flat and thin (**Fig. 2**). Compared to controls, ECM treatment produced constructs that were thinner, larger in diameter, and had lower wet weights (**Table 1**). Hydration was similar among all groups (**Table 1**). Toluidine blue demonstrated metachromasia due to proteoglycans in the constructs (**Fig. 2**). However, metachromasia intensity was slightly decreased in constructs treated with low and high ECM. Type II collagen was found in all groups while SZP immunolocalization was increased in constructs treated with low and high ECM (**Fig. 2**).

Biochemical and Biomechanical Properties

The percentage of GAG per dry weight (dw) of control, low ECM, and high ECM constructs were 39 ± 1%, 36 ± 1%, and 33 ± 4%, respectively (**Fig. 3A**). Collagen per dry weight of control, low ECM, and high ECM constructs were 8 ± 2%, 13 ± 3%, and 14 ± 4%, respectively (**Fig. 3B**). ECM treatment significantly decreased levels of GAG while increasing levels of collagen.

Although ECM treatment decreased the levels of GAG, it increased their aggregate modulus (**Fig. 3C**). The aggregate modulus of control, low ECM, and high ECM groups were 89 ± 25, 124 ± 18, and 114 ± 15 kPa, respectively.

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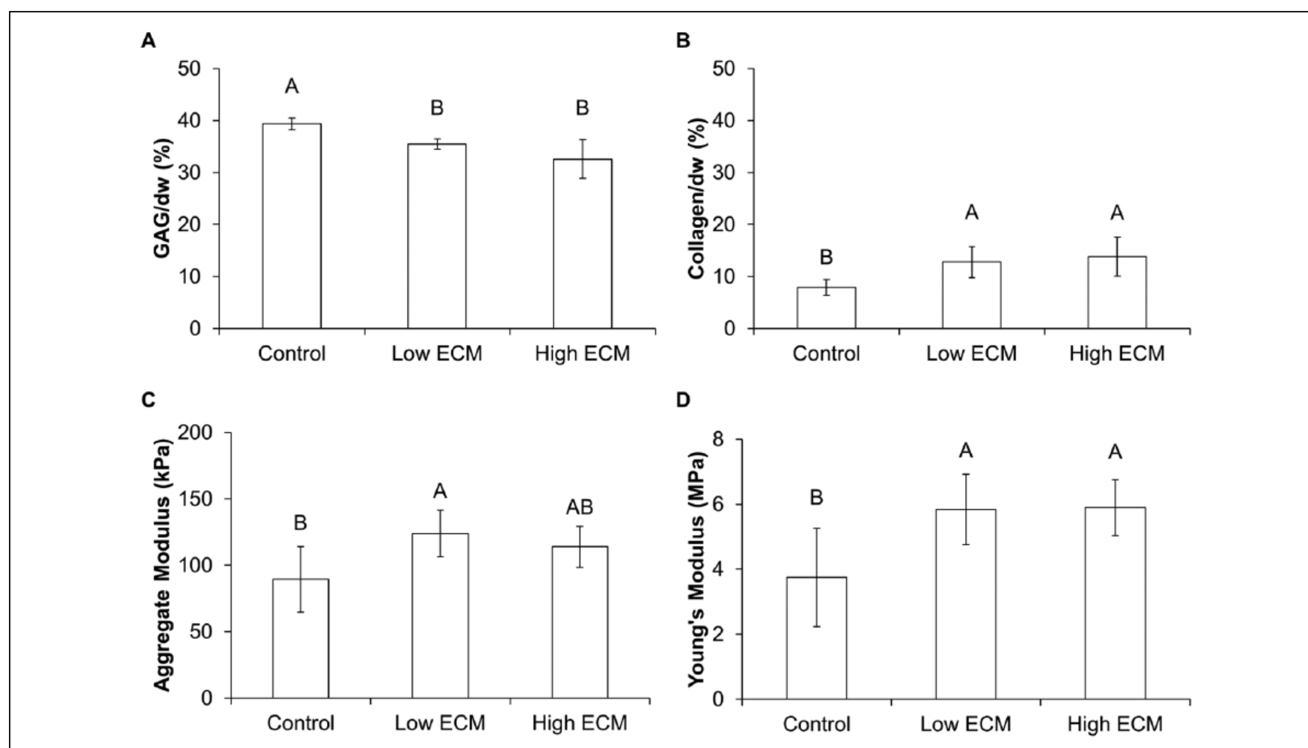

Figure 3. Biochemical and biomechanical properties of constructs after 4 weeks of culture. Glycosaminoglycans (GAG) (**A**) and collagen content (**B**) of each construct was assayed using the dimethylmethylene blue and hydroxyproline assays, respectively. All values are normalized by construct dry weight (dw). GAG content was significantly lower in extracellular matrix (ECM) treated groups while collagen content was higher in ECM-treated constructs compared to nontreated control. ECM-treated constructs exhibited a higher aggregate modulus (**C**), but only the low ECM treatment was significant. The Young's modulus (**D**) increased significantly for constructs treated with ECM. Values are presented as mean ± standard deviation.

Only the low ECM treatment group was significantly different from the control. Following the increased trend in collagen, ECM treatments also increased the tensile stiffness of engineered cartilage, as determined by the Young's modulus (**Fig. 3D**). The Young's modulus of control, low ECM, and high ECM groups were 3.7 ± 1.5, 5.8 ± 1.1, and 5.9 ± 0.9 MPa, respectively. There was an increasing trend in UTS when treated with low and high concentrations of ECM, but none of the groups were significantly different. Control, low ECM, and high ECM groups had an UTS of 1.7 ± 0.8, 2.3 ± 0.7, and 2.4 ± 0.8 MPa, respectively.

SZP and Friction Characteristics

SZP production from the engineered constructs was measured via SZP accumulation in the media during weeks 1, 2, and 4 of culture using quantitative ELISA (**Fig. 4A**). ECM treatment had no effect on SZP production. Control, low ECM, and high ECM groups secreted 7 ± 2, 7 ± 3, and 8 ± 2 μg/mL of SZP (week 1), 19 ± 5, 20 ± 4, and 23 ± 7 μg/mL (week 2), and 9 ± 2, 11 ± 3, and 8 ± 2 μg/mL (week 4), respectively. SZP production was significantly higher during the second week of culture. By week 4, SZP synthesis returned to similar levels as week 1.

ECM-treated constructs exhibited a significantly lower coefficient of friction than constructs without ECM treatment (**Fig. 4B**). Synovial fluid and PBS baths did not affect the observed friction coefficients. Friction coefficients of control, low ECM, and high ECM groups during the last minute of a 5-minute friction test were 0.10 ± 0.02, 0.03 ± 0.02, and 0.02 ± 0.01, respectively. After incubation for 30 seconds in synovial fluid, friction coefficients of control, low ECM, and high ECM were 0.11 ± 0.03, 0.03 ± 0.01, and 0.02 ± 0.01, respectively. After PBS incubation, friction coefficients of control, low ECM, and high ECM were 0.13 ± 0.03, 0.03 ± 0.01, and 0.03 ± 0.01, respectively.

The concentration of SZP in the matrix was normalized against total protein content and control groups had a higher SZP content compared to either ECM treatment groups (**Fig. 4C**). There was no difference in SZP matrix concentration between low and high ECM groups. Incubation in synovial fluid significantly increased SZP concentration in the matrix for ECM treatment groups but not for control group.

Discussion

Based on a previous investigation demonstrating that increased production of SZP in self-assembled articular cartilage did not

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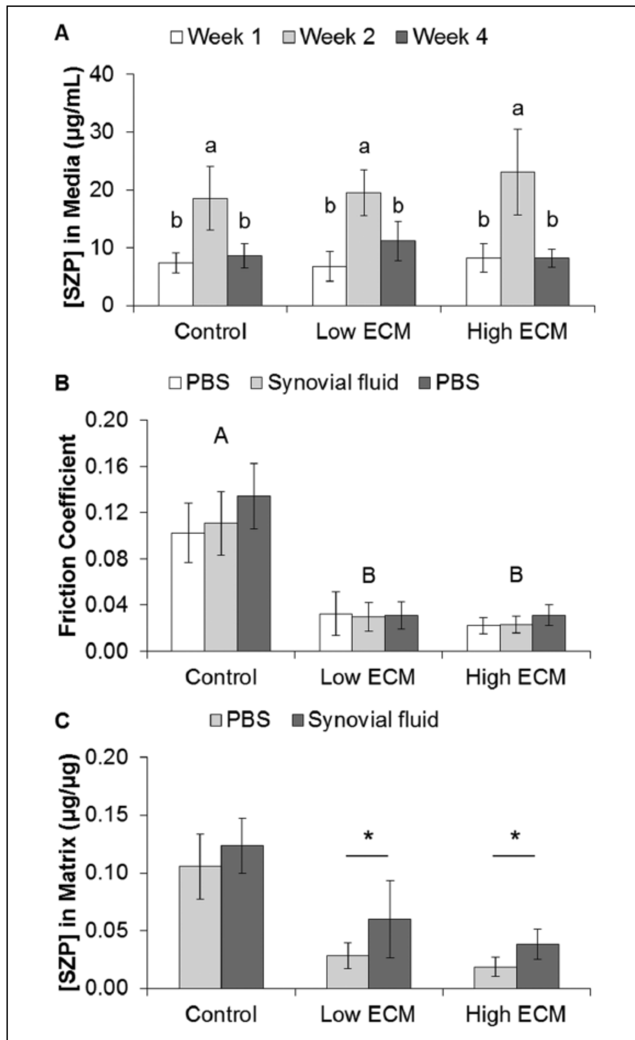

Figure 4. Superficial zone protein (SZP) synthesis and friction characteristics. (**A**) SZP accumulation in the media during weeks 1, 2, and 4 of culture were determined by ELISA. A two-way ANOVA was performed with treatment and time as the factors. No difference in friction coefficient was observed with treatment, but significant differences with time were observed. Tukey's *post hoc* test determined greater SZP accumulation in week 2 of culture compared to weeks 1 and 4. (**B**) Friction coefficients were significantly reduced with ECM treatment. (**C**) Immediately after friction testing, constructs were cut in half, incubated in either synovial fluid or phosphate-buffered saline (PBS), and SZP retention in the matrix was determined by ELISA, normalized relative to total protein. A Student's paired *t* test was performed against synovial fluid and PBS incubated samples. An asterisk (*) indicates significance with $P < 0.05$. Values are presented as mean ± standard deviation.

significantly reduce friction,[12] the aim of this study was to enhance SZP retention through the exogenous addition of superficial zone ECM extracts. It was hypothesized that SZP was not binding to the surface of self-assembled articular cartilage due to the lack of SZP-binding macromolecules and, thus, unable to reduce friction. Although SZP is mainly secreted into culture medium and surrounding synovial fluid *in vivo*,[3] accumulation of SZP on the surfaces of native tissue is detected by immunohistochemistry.[6,19] SZP is an important boundary lubricant in articular cartilage and must be present at the tissue surface in order to provide lubrication.[20,21]

SZP is localized to the articular surface and is not found in the middle or deep zone layers of cartilage.[3] SZP removal could be reversed on an intact cartilage surface, but not on cut or deep zone tissue.[6,22] This observation suggests that there are putative binding macromolecules found only at the surface of articular cartilage that can interact with SZP. Although several ECM macromolecules have been identified to bind with SZP *in vitro*, the exact mechanism of how SZP binds to cartilage surfaces *in vivo* remains to be elucidated. Previous work has shown that SZP is able to bind to cell culture plates coated with fibronectin, hyaluronan, and BSA in a dose-dependent manner.[10] Wells coated with heparin and superficial zone ECM also bind to SZP.[11] Therefore, superficial zone ECM extracted from bovine femoral condyles, which should include all putative SZP binding macromolecules, were directly added to the culture medium at a low (10 µg/mL) and high (100 µg/mL) concentration.

Both ECM treatments improved the lubrication properties of self-assembled constructs, with a reduction in friction of approximately 4-fold compared to control constructs (**Fig. 4B**). However, while SZP IHC revealed high SZP retention in the matrix of ECM treated constructs (**Fig. 2**), the biochemical assays detected greater quantities of SZP in the matrix of controls than ECM-treated constructs (**Fig. 4C**). This discrepancy in the data cannot be readily explained. It is possible that ECM treatment may be presenting more SZP at the surface rather than the bulk of the tissue, but since the entire tissue was homogenized in 4 M guanidine-HCl, both the bulk and surface retention of SZP are being measured and no conclusions in SZP localization can be made. Incubation of ECM-treated constructs in synovial fluid also significantly increased SZP in the matrix but not for control constructs (**Fig. 4C**), suggesting that the surfaces of ECM-treated constructs are not completely saturated with SZP.

Self-assembled constructs were all treated with 10 ng/mL of TGF-β to increase SZP synthesis[23] and improve biomechanical properties.[24,25] Grossly, all constructs appeared flat and thin (**Fig. 2**). ECM-treated constructs were slightly thinner and larger in diameter than the control constructs (**Table 1**). This morphological change could be attributed to lower GAG content and higher collagen content in the ECM-treated constructs (**Fig. 3A** and **B**). Although GAG content decreased with ECM treatment (**Fig. 3A**), the compressive properties of the ECM-treated constructs improved (**Fig. 3C**). This phenomenon could be due to the resistance of collagen to compression.[26,27] The Young's modulus also increased with ECM treatment in accordance with increases

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in collagen content (**Fig. 3B**). The improved mechanical properties of constructs from the ECM treatment could be contributing to its frictional properties as well.

Articular cartilage *in vivo* experiences many types of dynamic mechanical loading. Mechanical stimuli, such as hydrostatic pressure, have been reported to improve the compressive and tensile properties of self-assembled tissues.[25] The increase in stiffness could potentially improve its frictional properties. Mechanical loading under certain instances such as shear,[14,28] continuous passive motion,[29] and compression[30] have also been demonstrated to stimulate SZP metabolism in cartilage explants. Therefore, mechanical loading could be applied to investigate its effects on the friction coefficient of self-assembled cartilage. However, stimulating SZP production may not have much effect on its friction coefficient. As demonstrated in a previous study, increasing the proportion of superficial zone chondrocytes in self-assembled cartilage increased SZP synthesis but did not concomitantly decrease its friction coefficient.[12] It was hypothesized that the extracellular matrix of self-assembled cartilage may be lacking in matrix macromolecules that facilitate SZP binding. On the other hand, mechanical loading such as hydrostatic pressure improves mechanical properties and increases matrix synthesis such as collagen[25] and may provide an alternative method to stimulate production of extracellular matrix macromolecules that bind SZP. However, the effects of mechanical stimuli on the friction coefficient of self-assembled cartilage need to be first investigated.

The tribology of engineered cartilage is an important area of research. There has been a lot of research on the friction and wear characteristics of hemiarthroplasty biomaterials that articulate against native cartilage in the joint.[31-34] Friction is the resistance of solid surfaces sliding against one another. Wear is defined as the removal of material from solid surfaces due to mechanical action induced by surface sliding. All smooth surfaces contain asperities, or surface roughness and irregularities, at the atomic scale. Surface asperities make contact with each other as surfaces slide across one another and can produce friction. Rougher surfaces may exhibit lower friction due to less asperity contacts, but can cause greater wear. Therefore, higher friction does not necessarily result in greater surface wear. Wear occurs as a result of asperity adhesions and abrasions during sliding. In native articular cartilage, fluid films and boundary lubricants prevent direct contact between the cartilage surface asperities. When thick fluid films are not present to separate opposing surfaces, boundary lubricants, such as SZP, adsorbed along the surfaces, minimize friction and wear by preventing adhesions and direct contact between the asperities of the articulating surfaces. When SZP is depleted, high incidences of adhesion and abrasion can occur resulting in increased wear. As surfaces wear down, the mechanical integrity of the implant or the articulating native cartilage will diminish along with its functionality. Similar to orthopedic biomaterials and prostheses, the longevity of a biological implant will depend heavily on its surface friction and wear properties. Therefore, it is important to consider both the friction and wear properties of engineered cartilage tissues.

Based on the accumulation of the current experimental data alone, it is likely that other factors besides SZP are contributing to its frictional properties. It is possible that the ECM is altering the surface roughness of the constructs. The morphological difference between diameters and thicknesses of the ECM-treated constructs compared to control constructs were easily observed. Since there was an effect on the bulk morphology of the constructs, it is possible that the added ECM also had an effect on construct surface morphology. ECM extracts could be also directly influencing friction. Free glycosaminoglycans, such as chondroitin sulfate or heparan sulfate, may present themselves at the surface and attract a layer of fluid film to provide lubrication. Although the exact mechanism for reducing the friction coefficient of self-assembled cartilage cannot be identified from this study alone, this investigation demonstrated that superficial zone ECM has the ability to enhance the friction properties of self-assembled articular cartilage constructs. Future experiments are needed to identify the components of the extracted ECM that contributed to the observed reductions in friction. The effects of ECM treatment on surface roughness and adhesion forces of self-assembled cartilage should also be investigated.

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Declaration of Conflicting Interests

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Ethical Approval

Ethical approval was not sought for the present study because neither living animals nor humans nor human tissues were used in the conduct of this research.

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