SPATIAL RESOLUTION OF CHONDROCYTE RESPONSE TO MECHANICAL SIGNALS FOR CARTILAGE TISSUE ENGINEERING

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ABSTRACT

Cartilage is a supportive structure able to transmit and distribute loads within the body, as a result of its composition of chondrocytes and extracellular matrix (ECM). Damage to cartilage associated with osteoarthritis and injury is difficult to repair because of the tissue's avascular nature. Growth and maintenance of cartilage tissue *in vivo* result from the synergistic effects of biochemical molecules and mechanical stimulation. Specifically, the chondrocyte functions as a biological sensor that detects and transduces extracellular mechanical signals (mechanotransduction). However, many of the fundamental mechanisms of chondrocyte mechanotransduction remain unclear. The mitogen-activated protein kinases (MAPKs) are a family of molecules involved in responding to extracellular stimuli and regulating subsequent intracellular activities, a series of events known as the MAPK signaling cascade. In this study, a bioreactor capable of dynamic compression was used to stimulate ECM gene transcription in chondrocyte-laden agarose disks (2.25 mm × 5 mm Ø, 30×10^6 cells/mL) cultured in chondrogenic medium with or without TGFβ-3 for one- and four-hour loading periods. After loading, disks were divided into inner core (2 mm Ø) and outer annulus regions and analyzed for ECM gene expression (aggrecan, type II collagen) by real-time PCR, and for active MAPK signaling molecules (ERK 1/2, SAPK/JNK, p38 MAPK) by Western blotting. In the future, this characterization of specific MAPK signaling cascades involved in chondrocyte mechanotransduction will be valuable in efforts to repair damaged cartilage tissue through functional tissue engineering solutions and in further understanding the role of the chondrocyte as a sensor.

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1. INTRODUCTION

Cartilage is a unique structure within the body that transmits and distributes physiological loads. In order to properly maintain these functions, cartilage tissue is dynamic and constantly remodels itself in response to mechanical changes in its environment. This load-bearing ability of cartilage results from a combination of specialized cells known as chondrocytes and a structural foundation of extracellular matrix (ECM) consisting of collagen, proteoglycans, and fluid. Specifically, one type, articular cartilage, is commonly found lining bone in joints [1]. Articular cartilage ECM contains predominantly type II collagen and proteoglycans composed of glycosaminoglycans with an aggrecan core protein [2].

The avascularity of cartilage causes the tissue to rely on diffusion for transport of nutrients and other cell materials and also limits the extent of native tissue repair possible [3]. This limited healing potential is especially a concern in the treatment of osteoarthritis, a common form of arthritis which is characterized by degradation of cartilage tissue. An estimated 21 million Americans suffer from stiffness, pain, and even loss of function associated with osteoarthritic joints [4].

As a result, functional tissue engineering solutions have since been sought to produce biological replacements for damaged native cartilage tissue. The field of tissue engineering combines the principles of multiple disciplines, including biochemistry and materials science, with the goal of restoring the lost or compromised functions of organs by producing new, healthy tissue. Tissue-engineered constructs are created through the combination of cells with biodegradable scaffolds, which are later implanted into the body [5]. While chondrocytes are the natural cell source for use in cartilage tissue engineering, recently mesenchymal stem cells (MSCs), a related cell type, have also seen application. MSCs are multipotential cells capable of differentiating along several lineages, such as chondrogenesis, adipogenesis, and osteogenesis. Although MSCs can be conditioned toward differentiation into chondrocytes, their functional properties have not been shown to be as robust as chondrocytes in studies involving their use in tissue-engineered constructs [6].

In cartilage tissue engineering, one key feature that constructs must exhibit in order to be functionally useful are mechanical properties similar to native cartilage. To achieve this, tissue-engineered constructs have been subjected to treatments such as culture in medium containing biochemical factors that promote chondrogenesis (differentiation into cartilage) and mechanical stimulation reminiscent of physiological conditions by loading regimes. In particular, this loading can be controlled through the design and use of bioreactors, devices capable of supporting biologically active environments [5].

Despite the work of many in cartilage tissue engineering, results thus far demonstrate one fundamental problem: the functional properties of tissue-engineered constructs remain far less than that of native cartilage, even with enhancement by chondrogenic factors and mechanical stimulation. The search therefore continues for methods by which the functionality of tissue-engineered constructs can be increased to more closely resemble the original tissue.

2. BACKGROUND

Normal cartilage tissue growth and development *in vivo* is governed by both dynamic mechanical stimulation and the presence of biochemical growth factors. Results from previous studies suggest that cartilage is a tissue strongly influenced by mechanical changes in its environment and requires physiological loading to maintain its proper functions and homeostasis [2]. Mechanical loading of cartilage explants by methods such as static and cyclic compression and shear with variations in duration and frequency *in vitro* has been shown to influence ECM gene expression and remodeling of cartilage [7]. In general, these studies have shown that dynamic loading increases biosynthesis of ECM components, proteoglycans and collagen [8].

Loading studies with cylindrical constructs have also shown that regional variation in biological response exists between the inner core and outer annulus regions of constructs. Distinct patterns of gene expression and ECM biosynthesis in the inner and outer regions result from the different mechanical forces experienced by each region. Specifically, the inner core region experiences high fluid pressures, while the outer annulus experiences high fluid fluxes [9]. Thus, the regional response of chondrocytes to dynamic loading is of interest for study.

Hydrogel-based systems have been widely used as models for the study of chondrocyte response to biochemical and mechanical stimulation, because of their characteristic high water content reminiscent of native cartilage and capacity for even dispersion of cells within the scaffold during preparation. Agarose, a polysaccharide derived from red algae, is one type of hydrogel that is commonly used for chondrocyte encapsulation. The ability of chondrocyte-laden agarose hydrogel constructs to be consistently and uniformly manufactured makes them simple and popular model systems for use in studies [5]. Finite element modeling of agarose constructs as a biphasic (fluid and solid) medium yields the same regional variation in mechanical forces discussed previously (Fig. 1) [10].

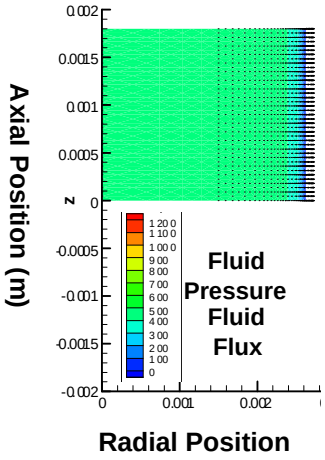

Figure 1 Finite element modeling of a hydrogel construct shows the mechanical forces experienced by a construct vary radially from its center. High fluid pressures predominate at and directly around the center of the construct, and high fluid fluxes are present at its outer edges. In the axial direction, fluid pressures within the inner core region and fluid fluxes are uniform.

While the pathways involved in mechanical loading are very complex, the biochemical mechanisms beginning with the application of a mechanical stimulus and leading to the response of individual cells remain largely unclear. In the cell, the mechanism by which extracellular, mechanical stimuli are interpreted and converted into biochemical signals is known as mechanotransduction. One well-studied family of biochemical signaling molecules, the mitogen-activated protein kinases (MAPKS), may be a critical component in chondrocyte mechanotransduction. Although their exact role is yet to be determined, these molecules are known to be involved in many cellular activities such as mitosis and gene expression, by sensing stimuli and initiating a cascade of events which lead to a biological response [11, 12].

Protein kinases such as MAPKs are capable of regulating biological events by phosphorylating other molecules. Currently, the MAPK family has been separated into five groups: extracellular signal-regulated kinases (ERKs) 1 and 2 (ERK 1/2), p38 MAPK and its isoforms (α, β, γ, δ), c-Jun amino-terminal kinases (JNKs) 1, 2, and 3, and the less-studied ERKs 3, 4 and ERK5. In general, the MAPK signaling cascade consists of three conserved kinases, MAPK, MAPK kinase (MAPKK), and MAPKK kinase (MAPKKK), which act sequentially in response to stimuli such as mitogens, cytokines, and stress (Fig. 2) [11].

Stimulus ⟶ MAPKKK ⟶ MAPKK ⟶ MAPK ⟶ Biological Response

Figure 2. The general sequence of events in the MAPK signaling cascade.

Previous studies involving static compression of cartilage explants have suggested that several members of the MAPK family, ERK1/2, p38 MAPK, and SAPK/JNK, have time-dependent roles in chondrocyte mechanotransduction [13]. ERK1/2 was found to be rapidly activated at 10 min, followed by a rapid decay in activity, with a final elevation in activated ERK1/2 levels for at least 24 hr. Active p38 MAPK levels were observed to be transiently elevated at 10 min, while activated SAPK/JNK levels were greatest at 1 hr, Alternatively, cartilage explants exhibited elevated phosphorylated ERK1/2 levels when subjected to 0.5 and 16 hr of dynamic loading relative to static compression [14].

In this study, chondrocytes encapsulated in agarose hydrogel constructs were cultured in media containing TGFβ-3, a chondrogenic factor, and subjected to short-term dynamic loading regimes and evaluated for regional changes in gene expression and MAPK activation. The purposes of this study are to evaluate regional differences in ECM gene expression and to identify members of the MAPK family that are activated in response to dynamic loading and culture containing TGFβ-3, as well as to determine a time course for these gene expression and MAPK signaling events.

3. EXPERIMENTAL METHODS

3.1 CHONDROCYTE AND MSC ISOLATION

Articular cartilage was harvested from the carpalmetacarpal joint of freshly slaughtered three to six-month old calves (Fresh Farms Beef, Rutland, VT, USA) and stored in serum-containing medium (SCM; high glucose Dulbecco's Modification of Eagle's Medium [hgDMEM], 10%

fetal bovine serum, 1x penicillin/streptomycin/fungizone [PSF]). Chondrocytes were isolated from the cartilage pieces after digestion in a solution of hgDMEM containing 2.5 mg/mL pronase (Calbiochem, San Diego, CA) for one hour, followed by a 0.5 mg/mL collagenase type II (Sigma Chemicals, St. Louis, MO) solution for four hours at 37°C with stirring. The resulting cartilage digest was poured through a 70 μm sterile filter to remove any undigested parts, and cells were collected by centrifugation at 300×*g* for 5 min. The resulting cell pellet was resuspended in fresh hgDMEM, counted, and diluted to a concentration of 60×10^6 cells/mL.

Bovine MSCs were harvested from the tibiae of freshly slaughtered three to six-month old calves (Fresh Farms Beef). Tibiae marrow pieces were mixed in an anti-coagulation solution (hgDMEM, 1x PSF, 300 U/mL heparin) and centrifuged at 300×*g* for 5 min after agitation. Resulting pellets were plated onto 30 cm plates and cultured in fresh SCM twice weekly. When cells reached confluence, sub-culturing was performed at a 1:3 expansion ratio in 60 cm plates. To harvest MSCs for studies, cells were washed with 1x PBS and incubated for 5 min at 37°C in 10 mL trypsin-EDTA. After cells detached from the plates, the reaction was terminated by adding 20 mL SCM, and the resulting solution was collected into a 50 mL tube and centrifuged for 5 min at 300×*g*. The cell pellet was resuspended in fresh hgDMEM, counted, and resuspended to a concentration of 10^6 cells/mL.

3.2 CHONDROCYTE ENCAPSULATION IN AGAROSE

The chondrocyte solution containing 60×10^6 cells/mL was mixed with an equal volume of 4% type VII agarose in PBS and casted into a 2.25 mm thick gel at a final concentration of 2% agarose at 30×10^6 cells/mL. The cell-seeded gel was cultured overnight in chemically defined medium (CDM-; 1x PSF, 0.1 mM dexamethasone, 50 mg/mL ascorbate 2-phosphate, 40 mg/mL L-proline, 100 mg/mL sodium pyruvate, 1x ITS+). Disks were punched from the gel the next day using a 5 mm Ø biopsy punch. For J1–3, the disks were dynamically loaded after two days of culture in CDM-. For J4, disks were pre-cultured for three weeks in CDM- prior to loading. After three weeks of culture in CDM-, chondrocytes within the agarose scaffold have begun to synthesize matrix and form cell-cell interactions (Fig. 3).

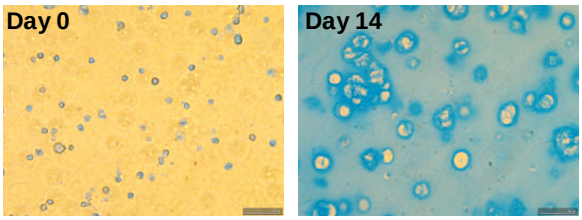

Figure 3 Alcian Blue staining of proteoglycans shows increased proteoglycan content in chondrocyte-laden agarose disks at day 14 of culture in CDM- relative to day 0.

3.3 DYNAMIC LOADING

In preparation for loading, 4% Type VII agarose gels were cast in 60 cm plates, and 6 mm Ø holes were punched into the plates using a biopsy punch to serve as placeholders for the disks. Samples were loaded onto these plates directly before loading with 7 mL of chemically defined medium containing TGF-β3 (CDM+) at a final concentration of 10 ng/mL. TGF-β3 is a growth

factor previously shown to enhance chondrogenesis (R&D Systems, Minneapolis, MN) [6]. Using a compressive loading bioreactor, dynamic (2% tare + 10% cyclic load at 1 Hz) and tare loading (2% tare) were applied to disks for one- or four-hour periods (Fig. 4*A*). For studies J1–3, both one- and four-hour loading durations were performed. For J4, disks were subjected only to the four-hour loading regime.

After loading, disks were harvested into a 2 mm Ø inner core and remaining outer annulus regions using a 2 mm biopsy punch for RNA and Western blotting (Fig. 4***B***). Subsequently, one sample in these studies represents 3-4 disks (inner or outer region) collected into one for RNA, and 3-5 disks for Western blotting. For RNA, samples for each loading condition were stored into one tube in 1 mL TRIZOL reagent (Invitrogen), while samples for Western blotting were flash-frozen in liquid nitrogen. Both RNA and Western blotting samples were stored at -80°C until further processing.

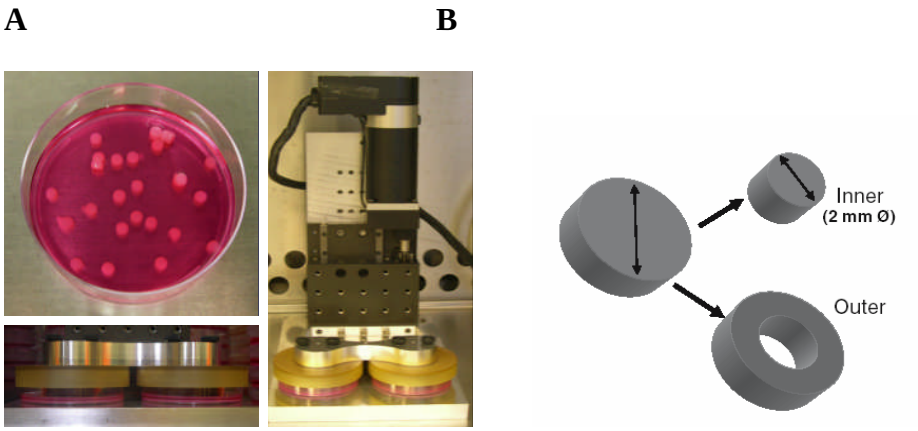

Figure 4 A compressive loading bioreactor was used to apply specific dynamic loading regimes on chondrocyte-laden agarose disks (2.25 mm × 5 mm Ø) placed into Petri dishes *(A)*. After loading, each disk was harvested into inner core (2 mm Ø) and outer annulus regions for further analysis *(B)*.

3.4 REAL-TIME PCR

Aggrecan and type II collagen gene expression were evaluated by real-time PCR. RNA was isolated from the TRIZOL-stored samples by a double chloroform extraction method. Samples were initially grinded by a motorized pestle and extracted by an initial 200 µL of chloroform. After centrifugation of vortexed samples at 4°C for 15 min at 12,000 rpm, the aqueous layer containing RNA was transferred to a new tube along with an additional 500 µL of TRIZOL reagent. Another 200 µΛ of chloroform was added to the sample, and the remainder of the RNA extraction was performed according to the manufacturer's instructions. The final purified RNA product was quantified using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Rockland, DE).

Complementary or cDNA was synthesized with random hexamers for 2000 ng of product using the First Strand cDNA Synthesis Kit (Invitrogen), according to manufacturer's protocol. 7 µL of RNA sample was added if the concentration was insufficient to produce 2000 ng of cDNA. The

resulting cDNA product was diluted to 50 or 100 μL in DEPC water prior to use in real-time PCR.

Real-time PCR was performing using an Applied Biosystems 7300 Real-time PCR machine. The real-time PCR reaction consisted of cDNA sample, SYBR Green, and forward and reverse primers specific for bovine aggrecan, type II collagen, and GAPDH. After the reactions were completed, the initial quantities of aggrecan and type II collagen gene transcripts were determined using the respective standard curves and normalized by corresponding GAPDH values to account for variations in starting cDNA concentrations. Data for dynamically loaded samples were further normalized by data for tare loaded samples of the same conditions.

3.5 WESTERN BLOTTING

To extract protein, frozen samples were lysed in 100 μL RIPA buffer (1x PBS, 1% nonidet P-40, 0.5% sodium deoxycholate) and sonicated twice on ice for five seconds each. In addition, chondrocyte-laden agarose disks were ground using a motorized pestle prior to sonication. Lysed samples were then centrifuged at 12,000 rpm for 10 min at 4°C, and the supernatant was collected. An additional 25 μL RIPA buffer was added to the collected supernatants, and the concentrations of protein within these samples were measured using the BCA Protein Assay Kit (Pierce).

Neat samples were diluted to a 20 μL volume in RIPA buffer, such that the concentration of all samples matched the sample with the lowest concentration of protein. Samples were then mixed with 5 μL of 5x ImmunoPure Lane Marker Non-Reducing Sample Buffer (Pierce) containing 100 mM β-mercaptoethanol, and heated at 99°C for six minutes to denature the protein.

Denatured protein samples were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on a 12% resolving and 5% stacking gel of 0.75 mm thickness for ~1 hr at 130 V. After SDS-PAGE, proteins from the gel were electrophoretically transferred to a polyvinylidene fluoride membrane for 1 hr at 100 V and 30 min at 60 V. After transfer, the gel was stained using Gel Code Blue (Pierce) to visualize protein banding patterns.

The transferred membrane was incubated at 25°C for 1 hr in 10 mL blocking buffer (5% w/v nonfat dry milk in 1x PBS/0.1% Tween-20) with gentle agitation and washed three times for 5 min each with 1x PBS/0.1% Tween-20 (PBS-T) to remove residual blocking buffer prior to incubation over night at 4°C in a primary antibody solution (10 μL primary antibody in 10 mL 5% bovine serum albumin/PBS-T) with gentle agitation. The membrane was washed three times for 5 min each with PBS-T the next day to remove any unattached primary antibody, before incubation for 1 hr at 25°C in a secondary antibody solution (5 μL secondary antibody in 10 mL blocking buffer) with gentle agitation. Finally, the membrane was washed three times for 5 min each with PBS-T. To develop the blot, the membrane was mixed with a 1:1 solution of SuperSignal West Pico Chemiluminescent Substrate (Pierce) and exposed to film using a Kodak RP-X-Omat automatic X-ray film processor.
For MSC1, harvested MSCs were collected at 10^6, 5×10^5, 2.5×10^5, 10^5, and 5×10^4 cells/pellet and pelleted at 10 min at 300×*g*. These pellets were immediately flash-frozen in liquid nitrogen

and stored at -80°C until they were probed for BiP/GRP78 (BD Biosciences), a constitutively expressed chaperone protein, by Western blotting.

For MSC2, cells were pelleted at a density of 2.5×10^5 cells/pellet for 10 min at 300×*g*. These pellets were cultured in either CDM- or CDM+ to induce chondrogenesis for 24 hr, with 12-14 pellets collected per sample at 0, 1, 3, 4, 8, and 24 hr for both media conditions [15]. Collected pellets were flash-frozen in liquid nitrogen and stored at -80°C. For these samples, 300 μL RIPA buffer was used to lyse the cells, since the total number of cells per sample was much higher than in MSC2. Finally, the lysed samples were probed for total ERK1/2 (Cell Signaling Technologies) by Western blotting.

For J1, protein extracted from the collected chondrocyte-laden agarose disks was probed for total p38 MAPK (Cell Signaling Technologies) by Western blotting.

4. RESULTS

4.1 AGGRECAN AND TYPE II COLLAGEN GENE EXPRESSION VARY REGIONALLY IN TWO-DAY CULTURED CHONDROCYTE-LADEN DISKS SUBJECTED TO ONE- AND FOUR-HOUR DYNAMIC LOADING IN CDM+ MEDIA

Real-time PCR was used to quantify aggrecan and type II collagen gene expression after one- and four-hour tare and dynamic loading regimes in CDM+ media (Fig. 5). All data for dynamically loaded samples were normalized by data for tare loaded samples with the same loading time and media conditions. A normalized value of 1 therefore corresponds to no difference in gene expression between the tare and dynamically loaded samples. For both genes, gene expression levels for inner region samples are ~1, while the outer region samples exhibited higher levels of gene expression (~1.5 – 2). Thus, spatial resolution between the inner and outer regions of chondrocyte-laden agarose disks is substantially different for investigation, because the two regions exhibit different levels of gene expression.

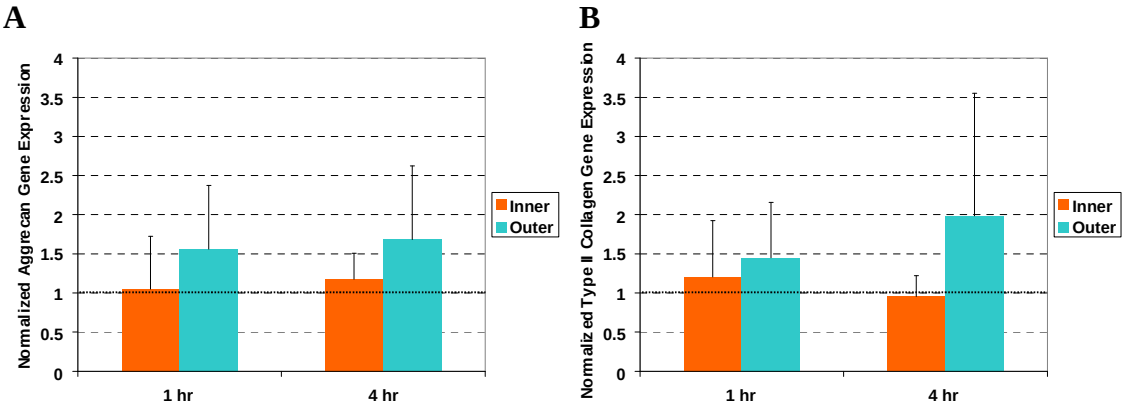

Figure 5 Aggrecan *(A)* and type II collagen *(B)* gene expression vary between the inner and outer regions of two-day cultured samples subjected to dynamic loading for one or four hours in CDM+ media (n = 3).

4.2 AGGRECAN AND TYPE II COLLAGEN GENE EXPRESSION IS SUSTAINED FOR TWO-DAY CULTURED CHONDROCYTE-LADEN DISKS AFTER ONE- AND FOUR-HOUR DYNAMIC LOADING IN CDM+ MEDIA

For both aggrecan and type II collagen, gene expression levels for inner region samples subjected to one- or four-hour dynamic loading times do not vary considerably (Fig. 5). Likewise, outer region samples do not exhibit appreciable differences in aggrecan and type II collagen gene expression levels between one- or four-hour loading times. This similarity suggests that the level of gene expression activity is sustained from one to four hours of loading, and more importantly, that activity within the samples does not substantially change between one and four hours of loading.

4.3 AGGRECAN GENE EXPRESSION IS ELEVATED IN THE OUTER REGION FOR THREE-WEEK CULTURED CHONDROCYTE-LADEN DISKS SUBJECTED TO FOUR-HOUR DYNAMIC LOADING IN CDM+ MEDIA RELATIVE TO TWO-DAY CULTURED DISKS

The differences observed in two-day cultured samples between aggrecan gene expression levels of the inner and outer regions were maintained in three-week cultured samples (Fig. 6). Samples after three weeks of culture exhibit elevated levels of aggrecan gene expression in the outer region (2.494) relative to samples after two days of culture (1.683). Type II collagen gene expression *(not shown)* was also assessed for three-week culture samples, but inner and outer region levels were not observed to be substantially different.

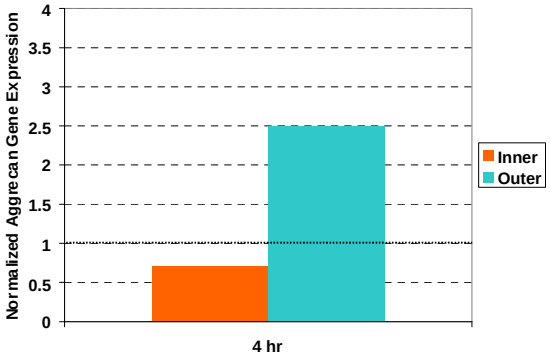

Figure 6 Aggrecan gene expression varies between the inner and outer regions of three-week cultured samples subjected to dynamic loading for one or four hours in CDM+ media.

4.4 PROTEIN EXTRACTION LEVELS AND TOTAL ERK1/2 DETECTION VARY WITH MSC PELLET DENSITY

In MSC1, the concentrations of protein extracted from MSC pellets of various cell densities were quantified (Fig. 7*A*). A linear positive correlation was found between pellet cell density and the concentration of protein extracted from the pellet, with the greatest concentration of protein extracted (3.679 μg/μL) from the sample of the highest cell density (1000×10^3 cells/pellet). Gel electrophoresis and staining of the samples further supported this relationship, as the intensity of staining was greatest for the sample of the highest cell density and lowest for the sample of the lowest cell density (50×10^3 cells/pellet) (Fig. 7*B* – SG). Lastly, BiP/GRP78 protein levels as

shown by band intensity were also found to be greater in samples of higher cell density (Fig. 7*B* – WB). Thus, protein levels can be quantified through a BCA assay and correlated with staining intensity on polyacrylamide gels and band intensity on Western blots.

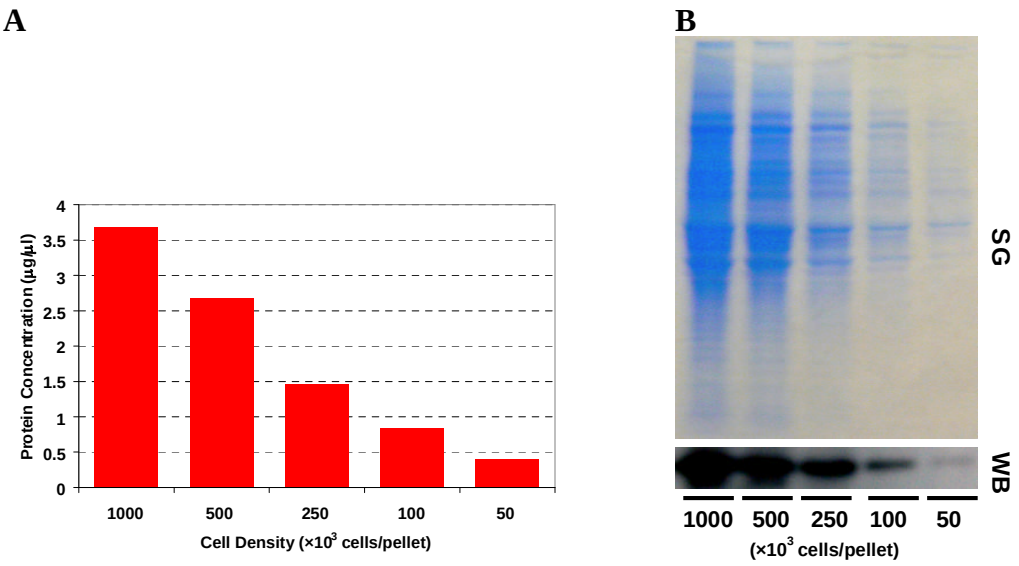

Figure 7 The concentration of protein extracted from MSC pellets exhibits a linear and positively correlated relationship with cell density *(A)*. Equal volumes of neat samples loaded onto a polyacrylamide gel show distinct variation in staining intensity, with the greatest staining associated with the sample of highest cell density *(B)*. Bands on the Western blot corresponding to BiP/GRP78 detection of the samples show the same relationship between chemiluminescence intensity and sample cell density.

4.5 PHOSPHORYLATED ERK1/2 PROTEIN LEVELS IN MSC PELLETS VARY OVER THREE-DAY CULTURE IN CHONDROGENIC MEDIA

The concentration of protein extracted from the MSC pellets collected at various time points during three days of culture in CDM- or CDM+ were all found to be at least 2.5 µg/µL (Fig. 8*A*). All samples were diluted to match the sample with the lowest concentration (2.606 µg/µL) for Western blotting, and gel staining verified that these samples have similar staining intensity and were loaded with approximately the same amount of protein in each well (Fig. 8*B* – SG**)**. Total ERK1/2 levels were observed to be high for all samples, although phosphorylated ERK1/2 levels vary with time and media conditions over the 24 hours of culture (Fig. 8*B* – WB). Specifically, phosphorylated ERK1/2 levels are highest at early and late times (1 hr; 8, 24 hrs) and lower between these times. The presence of TGFβ-3 in CDM+ media is known to stimulate chondrogenesis in MSCs, and the appearance of stronger band intensities for samples cultured in CDM+ suggests that TGFβ-3 is also related to ERK1/2 activation in MSCs. From these results, cell lysis methods to extract protein from MSC pellet tissue were verified, and the functionality of total and phosphorylated ERK1/2 antibodies was confirmed for subsequent use with Western blotting of chondrocyte-laden agarose disks.

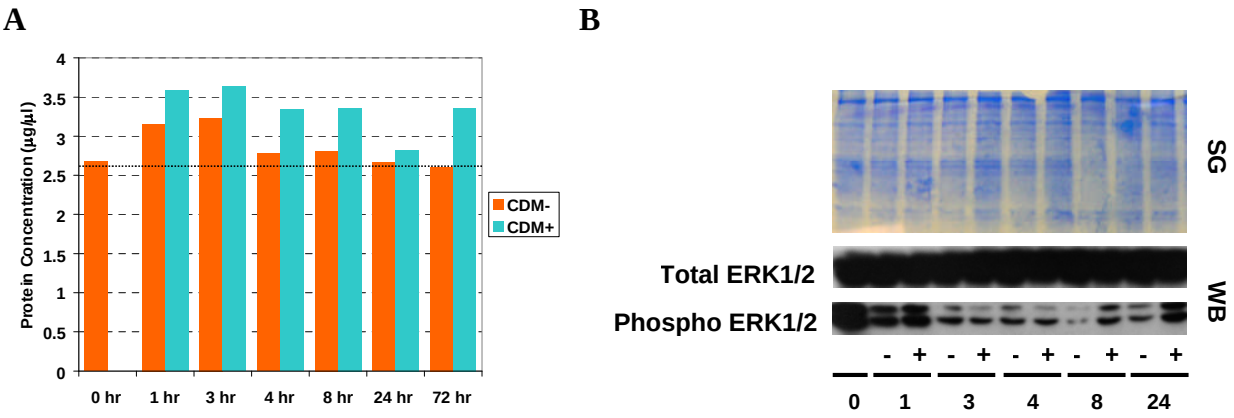

Figure 8 The concentrations of protein extracted from MSC pellets exceed 2.5 µg/µL for all samples, with the black dotted line designating the sample of lowest concentration *(A)*. All other samples were diluted to match this sample for Western blotting. Gel staining after SDS-PAGE confirms equal loading of protein sample in each well *(B)*. Total ERK1/2 protein levels are constant for all samples, while phosphorylated ERK1/2 protein levels vary on the Western blot.

4.6 PROTEIN EXTRACTION FROM INTACT CHONDROCYTE-LADEN AGAROSE DISKS AND SDS-PAGE OF EXTRACTED PROTEINS SUGGEST FUNCTIONALITY OF REVISED WESTERN BLOTTING METHODS

Intact two-day cultured chondrocyte-laden agarose disk seeded at 30×10^3 cells/mL were tested for protein extraction efficacy and verified by SDS-PAGE separation of proteins. The concentration of protein extracted from one sample of three intact disks was 2.422 µg/µL (Fig. 9*A*). Gel staining after SDS-PAGE verified the presence of protein extracted from the disks (Fig. 9*B*), although the banding pattern of the proteins was unique from that of MSC pellets (Fig. 8*B* – SG). The modified cell lysis protocol was therefore determined to be successful for extraction from agarose disks.

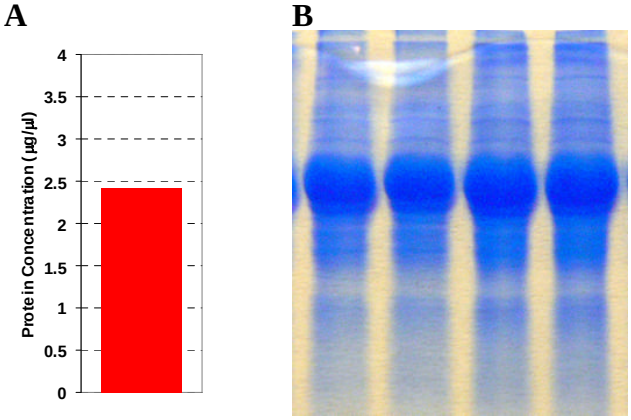

Figure 9 The concentration of protein extracted from three intact chondrocyte-laden agarose disks is 2.422 µg/µL *(A)*. Gel staining after SDS-PAGE shows a distinct banding pattern for protein extracted from chondrocytes in agarose disks from that of MSCs in pellets *(B)*.

4.7 TOTAL P38 MAPK PROTEIN WAS DETECTED IN CHONDROCYTES EXTRACTED FROM THE OUTER REGION OF AGAROSE DISKS

For Western blotting, all inner region samples were diluted to match the sample of lowest concentration (0.533 μg/μL), and likewise for outer region samples (1.245 μg/μL) (Fig. 10*A*). However, the diluted inner region samples contained insufficient amounts of protein for p38 MAPK antibody detection, and only bands for the outer region samples appeared after developing (Fig. 10*B*). Thus, MAPK proteins can be detected in chondrocyte-laden agarose disks, although samples of higher protein concentrations are necessary (> 0.533 μg/μL).

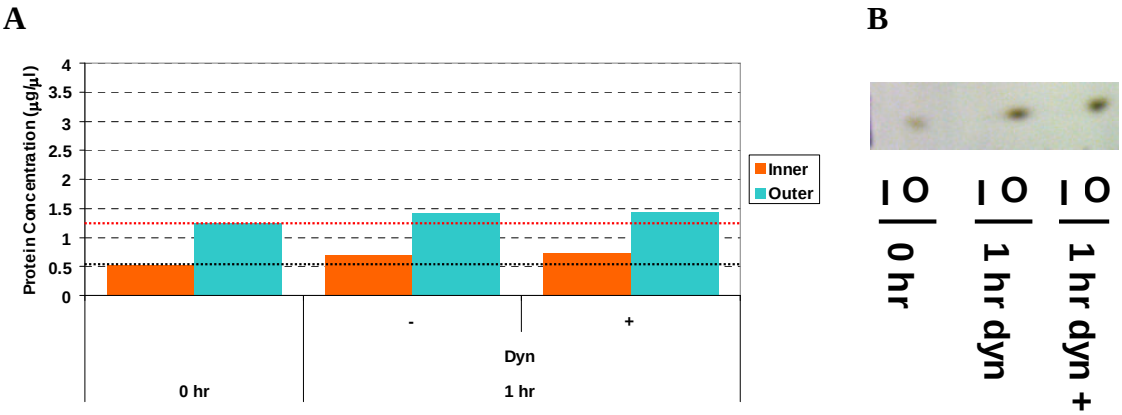

Figure 10 The concentration of protein extracted from the inner (~0.6 μg/μL) and outer regions (~1.4 μg/μL) of chondrocyte-laden agarose disks are much less than that of intact disks (*A*). The black and red dotted lines designate the inner and outer region samples of lowest concentration, respectively. Total p38 MAPK protein levels appear only for the outer region samples and are elevated in samples with dynamic loading relative to time zero *(B)*.

5. DISCUSSION AND CONCLUSIONS

One of the aims of this project was to evaluate regional differences in ECM gene expression in response to TGFβ-3 induction and dynamic loading. Real-time PCR results from three replicate studies show that two-day cultured chondrocyte-laden agarose disks exhibit varying levels of aggrecan and type II collagen gene expression between the inner and outer regions. For the inner region, gene expression levels for both aggrecan and type II collagen are ~1, suggesting that short-term dynamic loading (one and four hours) at 10% strain and 1Hz is insufficient for initiating changes in gene expression. In contrast, gene expression levels for both aggrecan and type II collagen are elevated for the outer region. Since the two regions differ in the type of mechanical forces experienced, the regional variation in gene expression is not unexpected. However, the differences in gene expression levels present between the inner and outer regions do validate one of the first aims of these studies.

Although gene expression levels differ between the two regions, they do not vary greatly for duration of loading time. Specifically, aggrecan gene expression levels are not vastly different after one hour of loading versus four hours (~1.5; Fig. 5*A*). While type II collagen levels show more variance between one and four hours of loading, this difference is not appreciable (Fig. 5*B*). This sustained response between one and four hours of loading suggests that the gene expression

activity exhibits greatest change within the first hour of loading and is steady during the latter three hours. These results give us a time frame to focus upon in future studies.

The standard deviation between values from replicate studies J1–3 is noticeably large, which are likely due to low yields of RNA extracted from the two regions of samples. The inner region was especially difficult to extract large quantities of RNA from because of its small volume. Even with three disks collected into one sample for processing, resulting RNA concentrations were often low, which may account for much of the variation in starting gene transcript quantities.

In J4, a preliminary study of three-week cultured chondrocyte-laden agarose disks, results from one replicate show aggrecan gene expression levels are elevated in the outer region (~2.5) in comparison to results for two-day cultured disks (~1.7; Fig. 6). The elevation in aggrecan gene expression may result from the presence of ECM synthesized by cells with additional time in CDM- culture prior to loading (Fig. 3). In contrast, type II collagen gene expression levels were not observed to be substantially different between the inner and outer region. This study must be replicated to evaluate any robust trends in gene expression and differences between two days and three weeks of pre-culture in CDM- prior to loading.

Western blotting techniques were tested on MSC pellets initially, in order to develop a working protocol for eventual use with inner and outer region samples of chondrocyte-laden agarose disks. The concentration of protein extracted (~2.6-3.7 μg/μL) from the two MSC pellet studies, MSC1 and MSC2, and used for blotting (~2.6 μg/μL) were found to be adequate for detection of BiP/GRP78 chaperone protein and total ERK1/2 proteins (Figs. 7, 8). From MSC1, a linear, positively correlation was observed between pellet cell density and the amount of protein extracted from the pellet (Fig. 7*A*), a relationship which extended to the band intensity of stained gels and probed blots (Fig. 7*B*). From MSC2, the functionality of the MAPK antibodies was verified to advance towards use on protein extracted from chondrocyte-laden agarose disks (Fig. 8*B*).

Next, Western blotting techniques were tested on intact chondrocyte-laden agarose disks. Difficulty arose in the initial steps of cell lysis and protein extraction for the agarose disks. MSC pellets are primarily composed of cells, and sonicating the samples twice on ice was sufficient to lyse cells for the release of proteins. In contrast, chondrocytes encapsulated in agarose must be extracted from the gel before they can be lysed and proteins are released. The original cell lysis protocol was thus modified by using a motorized pestle to grind the agarose before sonication, similar to the method described previously for RNA extraction. Despite this additional step to extract protein, the concentration of protein extracted from the disks (2.422 μg/μL) was lower than that of MSC pellets, although the volume of lysis buffer was less for the disks (100 μL) than for the pellets (300 μL). Nonetheless, SDS-PAGE and gel staining showed the steps of protein extraction, denaturation, and separation by electrophoresis were successful, prompting the final transition to performing Western blots with inner and outer region samples from dynamic loading studies, J1–4 (Fig. 9). Notably, the proteins extracted from the chondrocyte-laden disks did exhibit a distinct banding pattern than that of the MSC pellets, but this observation is not unexpected given the differences between the two cell types (Fig. 9*B*).

Inner and outer samples harvested from J1 were used for testing of Western blotting. The concentration of protein for both inner (~0.6 μg/μL) and outer (~1.4 μg/μL) samples were both much less than that of the MSC pellets used previously for Western blot testing (Fig. 10*A*). The concentration of protein extracted from one sample containing three agarose disks (2.25 × Ø 5.0 mm) seeded at 30×10^6 cells/mL and lysed in 100 μL was only 2.422 μg/μL (Fig. 9*A*). Thus, the concentration of protein extracted from one sample containing 3-4 inner cores (2.25 × Ø 2.0 mm) or outer annuli seeded at 30×10^6 cells/mL and lysed in 100 μL was not expected to be much greater.

For the first trial blot, all samples were diluted to match the sample of the lowest concentration, an inner region sample (0.533 μg/μL) (Fig. 10*A*). However, this concentration proved to be insufficient for antibody detection of phosphorylated ERK1/2 and phosphorylated p38 MAPK, resulting in blank sheets of film after developing *(not shown)*. Thus, before performing more blots, sample concentrations were increased to improve protein detection. Since the concentration of outer region samples is generally at least two times greater than that of the inner region, all outer region samples were diluted to match the outer region sample of the lowest concentration (1.245 μg/μL), and similarly for the inner region samples (0.533 μg/μL) (Fig. 10*A*). Probing for total p38 MAPK would test if the amount of protein loaded onto the gel for the inner or region samples was sufficient for detection, since total MAPK levels are greater than phosphorylated MAPK levels in samples (Fig. 10*B*).

Overall, protein was successfully extracted from chondrocyte-laden agarose disks and probed for MAPKs, although difficulty was encountered in insufficient concentrations of protein loaded on the gel, similar to the trouble encountered during RNA extraction for real-time PCR.

6. RECOMMENDATIONS

For J1–3, these studies should be repeated with additional samples harvested for real-time PCR and Western blotting analysis. Real-time PCR results and trends between different loading durations would likely be more robust with higher yields of extracted RNA. Likewise, the blotting process would be much more efficient with additional samples to increase the concentration of protein loaded onto gels for Western blotting. With sufficient protein extracted from samples, the active and total MAPK profiles can be completed for the dynamically loaded chondrocyte-laden agarose disks in these studies.

The time frame established in J1–3 should also be explored in future studies in order to evaluate the changes that occur in aggrecan and type II collagen gene expression within the first hour of loading. Correspondingly, phosphorylated MAPK activity is also of interest relative to these changes in gene expression.

In J4, the differences in gene expression activity between two days and three weeks of pre-culture prior to loading were considered, but additional replicate studies must be conducted to assess the accuracy of the observed elevation in aggrecan gene expression levels with additional time in culture and ECM biosynthesis. Similarly, the protocol for these studies can also be modified to test other durations of pre-culture time and their effects on gene expression and MAPK activity.

For Western blotting, the initial studies performed with MSC pellets should be repeated with chondrocytes to distinguish differences in banding patterns among MSCs, chondrocytes, and agarose-encapsulated chondrocytes. In addition, this test will also check for the presence of any contamination from residual agarose.

Finally, these studies in elucidating chondrocyte mechanotransduction can be translated to MSCs in the future, since these cells are an important alternative source in cartilage tissue engineering. With the knowledge of the biochemical mechanisms underlying chondrocyte and MSC response to mechanical signals, functional cartilage tissue engineering can be vastly improved for application to osteoarthritis and other diseases involving cartilage damage and degradation.

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