

Regulatory Effects of Mechanical Strain on the Chondrogenic Differentiation of MSCs in a Collagen-GAG Scaffold: Experimental and Computational Analysis*

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Abstract—The effective treatment of cartilage defects by tissue engineering requires an improved understanding of the effect of mechanical forces on cell differentiation within three-dimensional (3D) matrices. The objective of this study was to investigate the effects of mechanical constraint and cyclic tensile strain on the chondrogenic differentiation of mesenchymal stem cells (MSCs) in a 3D collagen type I-glycosaminoglycan (GAG) scaffold. A multi-station uniaxial stretching bioreactor was fabricated to facilitate application of cyclic strain to the constructs cultured in a chondrogenic medium. Mechanical constraint, created by uniaxial clamping, prevented the cell-mediated contraction of the scaffolds and resulted in a reduction in the rate of GAG synthesis as measured by [³⁵S] sulfate incorporation relative to unconstrained controls. However, the rate of GAG synthesis was increased following application of continuous 10% cyclic tensile loading at 1 Hz for 7 days. A poroelastic finite element analysis of the 3D scaffold computed a maximum fluid flow of 19 $\mu\text{m/s}$ and maximum principal strains of 8% under 10% stretch suggesting these magnitudes were sufficient to mechano-regulate the chondrogenic differentiation process.

Keywords—Chondrogenic differentiation, Mesenchymal stem cells, Collagen-GAG scaffold, Mechanoregulation, Tensile strain, Computational analysis.

INTRODUCTION

Mesenchymal stem cells (MSCs) are an undifferentiated cell phenotype that have the potential to differentiate into the specialized cells of the skeletal system. Although mechanical loading is known to influence skeletal tissue formation, the regulatory effects of

cyclic tensile strain on the chondrogenic differentiation of MSCs in a three-dimensional (3D) scaffold has yet to be reported. Some studies have shown that intermittent hydrostatic pressure^{2,39,49} and cyclic compressive loading^{1,10,21,22} can regulate the chondrogenic differentiation of MSCs. It has also been demonstrated that cyclic compressive loading alone was a sufficient stimulus to promote the chondrogenic differentiation of MSCs in agarose gels; that is, MSC differentiation along the chondrogenic lineage may be regulated by mechanical factors alone.^{21,22,36}

The material properties and pore architecture of scaffolds affect the morphology, phenotype, and synthesis of cells.³ Furthermore scaffold designs influence the mechanical-induced development of complex biophysical microenvironments (strain, fluid flow, and pressure gradients) acting on cells seeded in these materials. Collagen-GAG scaffolds have been shown to support the synthesis of a cartilaginous matrix using chondrocytes,^{40,53} and have also supported the chondrogenic and osteogenic differentiation of MSCs.¹⁸ The authors have previously demonstrated collagen II and GAG synthesis³⁸ in the MSC-seeded collagen-GAG scaffolds treated with TGF- β 1 thereby showing that it supports chondrogenic differentiation.

Cell-mediated contraction of collagen-based matrices has been observed in culture by various cell types and this has been demonstrated to benefit matrix synthesis.^{19,23,53,54} For example, Vickers *et al.*⁵⁴ found that scaffolds with increased stiffness prevented cell-mediated contraction and that this resulted in reduced matrix synthesis. A recent and promising approach for cartilage tissue repair involves using chondrocyte-seeded collagen I/III matrices in a technique known as matrix-induced autologous chondrocyte implantation (MACI).^{4,5,35,51,55} In such studies chondrocyte-seeded matrices are secured into defects with fibrin glue⁴ which

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would cause a scaffold to be constrained should it try to contract. However, it is not known what effect this constraint may have on cell synthesis in such constructs *in vivo*. MSCs are of interest in the repair of chondral defects³⁰ and they offer a potential alternative to chondrocytes and could therefore be used in the MACI procedure. However, little information is available regarding the effects of mechanical constraint and physiological loading on MSC-seeded collagen matrices, which forms the basis of our investigations.

Mechanoregulation models have been proposed that attempt to quantify the relationship between mechanical stimulation and the differentiation of MSCs, and the subsequent development of various connective tissues.^{11,13,48} Such models have used finite element (FE) analysis as a tool to better understand how mechanical stimuli correlate with tissue differentiation patterns found during biological processes such as fracture healing^{11,12,31} and osteochondral defect healing.²⁴ This method has also been implemented to quantify biophysical stimuli developed in tissue engineering constructs to better understand the microenvironment to which cells are exposed in bioreactors when subjected to various loading patterns.^{14,21,36}

In this study we hypothesize that mechanical constraint and cyclic tensile strain will modulate the chondrogenic differentiation of MSCs within a 3D environment of a collagen-GAG scaffold, in the presence of chondrogenic growth factors. A computational model will be developed to calculate the biophysical stimuli developed within the scaffold during loading. If it is demonstrated that mechanical constraint affects the chondrogenic differentiation of MSCs, and that cyclic tensile loading plays a role, then this information would provide further knowledge which may benefit future approaches to the engineering of constructs for cartilage tissue repair.

MATERIALS AND METHODS

Isolation and Cultivation of MSCs

MSCs were isolated from the tibia and femur of adult male Wistar rats (250–350 g) as detailed in Farrell *et al.*¹⁸ MSCs were expanded for 3 weeks in culture medium consisting of Dulbecco's modified Eagle's medium (DMEM, Sigma–Aldrich, Poole, UK) supplemented with 10% fetal bovine serum, 100 U/ml penicillin/streptomycin, 3 mM glutaMAX™, 1 mM L-glutamine, and 1% non-essential amino acids (all from GIBCO, supplied by Biosciences, Dublin, Ireland), to allow for proliferation. Medium was replaced every 3–4 days. MSCs were harvested from individual animals for each observation reported.

Phenotypic Characterization Using Immunocytochemistry and Flow Cytometry Analysis

MSCs were plated onto 2D plastic coverslips, were cultured for 2 days in culture medium, and fixed with 4% paraformaldehyde. Immunocytochemistry was carried out to probe for reported MSC surface proteins, CD105 and CD90. Non-specific binding was blocked with 20% fetal calf serum (FCS)/2% bovine serum albumin (BSA). Cells were incubated overnight at 4 °C with rabbit polyclonal CD105 (Santa Cruz Biotechnology, Santa Cruz, CA) and mouse monoclonal CD90 (Abcam, Cambridge, UK). Cells were incubated for 1 h with either biotinylated anti-rabbit or anti-mouse IgG secondary antibodies (Vector Labs, UK), followed by a 1 h incubation with ExtrAvidin-FITC (Sigma–Aldrich, Dublin, Ireland). Cells were mounted using Vectashield and examined by confocal microscopy.

For flow cytometry, cells were trypsinized after 3 weeks of expansion and incubated for 45 min at 4 °C with CD90-phycoerythrin (PE) and CD45-fluorescein isothiocyanate (FITC) monoclonal antibodies (BD Biosciences, Oxford, UK). Isotype-identical antibodies were used as controls. Samples were analyzed with 488 and 633 nm lasers (CyAn™ ADP, DakoCytomation Ltd.) and 10,000 events were collected for each sample.

Induction of Chondrogenesis in a Collagen-GAG Scaffold

In order to assess the behavior of MSCs in a 3D environment, a collagen type I-glycosaminoglycan scaffold (donated by Dr. Fergal O'Brien and Mr. Matthew Haugh, RCSI, Dublin and Integra LifeSciences) was employed which was fabricated using a lyophilization (freeze-drying) process, described previously.^{18,42–44} Briefly, the collagen-GAG scaffold was produced by mixing bovine tendon collagen I and shark chondroitin-6-sulfate in acetic acid which was frozen to –40 °C. The ice-crystals were sublimated leading to the formation of a homogenous highly interconnected architecture which was crosslinked in a vacuum oven at 105 °C, producing a scaffold with a pore size of 96 μm.

MSCs were rinsed with phosphate-buffered saline, detached with trypsin-EDTA, and centrifuged at 650g for 5 min at 20 °C. Two milliliters of prewarmed culture medium was added to the resulting pellet and a single cell suspension was obtained by aspirating through a 20-gauge needle. The cells were counted and diluted to achieve a cell density of 2×10^6 cells/ml for static 3D experiments.

To seed the collagen-GAG scaffolds, 2 mL of 2% agarose was placed into each well of 6-well plates to prevent cells from attaching to the plastic. Thirteen

millimeter diameter discs of dry scaffold were obtained using a stainless-steel punch, seeded with 200 μL of the cell suspension and incubated for 30 min at 37 °C. The volume of cell suspension was used to correlate to the number of cells seeded in the volume of scaffold used in the bioreactor. The scaffolds were turned over, seeded with another 200 μL of the cell suspension, and incubated for a further 30 min, after which, the wells were flooded with 2 mL of culture medium. After 2 days, the medium was changed and replaced either with culture medium or with chondrogenic-supplemented medium (10 ng/mL TGF- β 1, 100 nM dexamethasone, and 50 μM ascorbic acid (Sigma–Aldrich, Dublin, Ireland)). Constructs were cultured unconstrained (i.e., free-swelling condition where the scaffold could contract or swell in all directions) for 2 weeks after which they were labeled with [^{35}S] sulfate to assess the rate of GAG synthesis.

[^{35}S] Sulfate Radiolabelling and Cell Number Quantification

To determine the rate of GAG synthesis as an index of chondrogenic differentiation, constructs were labelled with 5 $\mu\text{Ci}/\text{mL}$ of [^{35}S] sulfate (GE Healthcare, UK) during the final 24 h of culture. Constructs were digested in 200 μL of papain for 6 h at 60 °C. Radiolabelled-GAG chains were precipitated from half the digest with 0.1% cetylpyridinium chloride and dissolved in 0.5 mL of 2 M NaCl with 15% ethanol at 37 °C. Samples were transferred into scintillation vials containing 4 mL of scintillation fluid (Perkin Elmer, supplied by Foss Ireland, Dublin, Ireland) and the counts per minute (CPM) were detected using a liquid scintillation counter (Tri-carb 2100, Packard). The remainder of the digests were frozen at -80 °C for quantification of total cell number per construct for normalization. Total cell number was quantified using the Hoechst 33258 dye-binding assay, as described by Kim *et al.*²⁶ The cell number for each

were mixed with 0.1 $\mu\text{g}/\text{mL}$ Hoechst to create standard curve, from which the total cell number per construct was determined. Fluorescence of all samples was determined on a fluorescent plate reader (Fluoroskan Ascent, Germany) at an excitation of 355 nm and emission of 460 nm.

Fabrication of Multi-station Bioreactor and Dynamic Culture Conditions

A custom-designed 5-station uniaxial stretching bioreactor was fabricated to apply cyclic tensile loading to 3D cell constructs (see Fig. 1). In the bioreactor, the strain was provided by a cam-follower assembly connected to a DC motor. A cam with a 2 mm offset was selected to apply 10% strain to 20 mm construct lengths. A dial gauge was used to determine the exact displacement provided by the cam in the bioreactor, and an error of 4.5% was determined. Since the collagen-GAG scaffolds were extremely flexible when hydrated, 30 mm \times 10 mm dry sections were attached to similar sized pieces of silicone at the extreme ends only (Fig. 1a). Constructs were clamped into static frames (Figs. 1b and 1c) leaving a grip-to-grip length of 20 mm. A total of 550 μL of 2×10^6 cells/mL cell density was seeded onto each scaffold. Constructs were incubated for 60 min to allow for cell attachment, then flooded with 8 mL of control medium. After 2 days, the medium was replaced with chondrogenic medium consisting of 10 ng/mL TGF- β 1, 100 nM dexamethasone, and 50 μM ascorbic acid. Half the medium was replaced every 3–4 days thereafter. Constructs were left under static conditions for 7 days to allow for cell migration. After the 7-day period one clamping frame was transferred into the bioreactor (Fig. 1d) and subjected to a continuous loading regime of 10% strain at 1 Hz for 7 days, and the other frame was maintained in static culture to serve as an unloaded control (i.e., constrained). Therefore, the culture conditions investigated were:

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| <ul style="list-style-type: none"> (i) Unconstrained without chondrogenic factors ($n = 10$) (ii) Unconstrained ($n = 10$) (iii) Constrained (i.e. clamped, $n = 10$) (iv) Constrained + stretched (10% ϵ, 1 Hz, $n = 10$) | } | + Chondrogenic growth factors |
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construct digest was measured in triplicate by mixing 10 μL aliquots of each sample with 200 μL of a working concentration of 0.1 $\mu\text{g}/\text{mL}$ Hoechst 33258 (Sigma–Aldrich, Dublin, Ireland). Aliquots of digested MSCs

Constructs were cultured in the presence of 5 $\mu\text{Ci}/\text{mL}$ of [^{35}S] sulfate for the final 24 h of culture and CPM were normalized to the total cell number per construct, as detailed above.

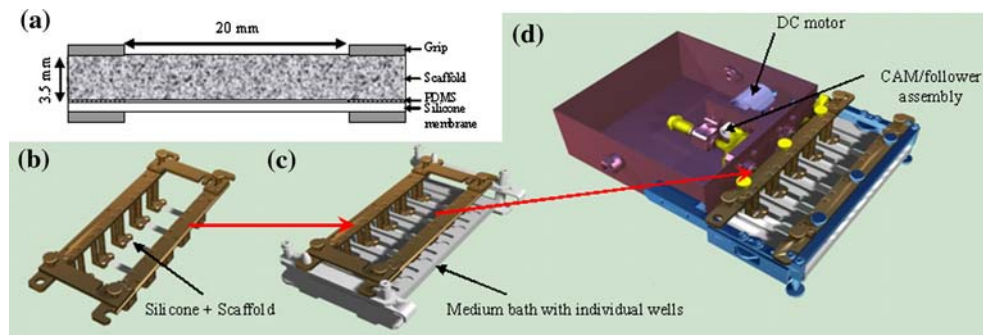


FIG. 1. Design images of (a) schematic of scaffold set-up for tensile stretching experiments where the scaffold is attached to a silicone membrane with liquid PDMS, (b) clamping frame containing collagen-GAG scaffolds, (c) clamping frame slotted into static culture frame with individual wells in the medium bath for each construct, and (d) 5-station uniaxial stretching rig, fabricated for use in the dynamic tensile loading experiments.

Quantification of Scaffold Contraction during Chondrogenesis

To quantify contraction, 13 mm diameter pieces of collagen-GAG scaffolds were seeded on each side with 200 μL of a 2×10^6 cells/mL cell density as described above, and cultured for 14 days in normal culture medium or in chondrogenic medium. Digital images were taken of the constructs during culture. These were used to determine the average diameter of the constructs by calculating the total cross sectional area using UT-HSCSA ImageTool (<http://ddsdx.uthscsa.edu/>). Contraction was determined by analyzing the change in diameter over 14 days of culture compared to the initial diameter at day 0 (seeding), minus the contraction of cell-free scaffolds over the same time period.^{32,53}

Mechanical Testing of the Collagen-GAG Scaffold

The tensile modulus and the Poisson's ratio of the scaffold, required for computational analysis, was determined experimentally by carrying out mechanical tests on the scaffolds, using a 5 N load cell on a Zwick Z005 materials testing machine. Briefly, sections of scaffold were fully hydrated and samples were strained to failure at a rate of 1% every 6 s.⁴⁷ A tensile modulus of 2.625 kPa was determined from the linear slope on

the stress-strain graph up to 10% strain ($n = 4$). This value correlates well with the value of 2 kPa reported by Harley *et al.*²⁰

Confined and unconfined compression tests were carried out to obtain values for the compressive and Young's modulus, respectively. Briefly, cylindrical samples were obtained using a dermal punch and stress relaxation tests performed on hydrated samples. Samples were compressed to 50% of their original thickness and allowed to undergo stress relaxation for 10 min. The equilibrium force value after stress relaxation was used to calculate the moduli, and these values were used to indirectly calculate a Poisson's ratio of 0.33 using an equation developed by Korhonen *et al.*²⁹ ($n = 4$).

Finite Element Analysis

A poroelastic finite element (FE) model of the collagen-GAG scaffold was developed using Abaqus 6.5-6. An illustration of the collagen-GAG scaffold clamped uniaxially between the grips in the clamping frame is shown in Fig. 2a. Due to symmetry it was only necessary to model a quarter of the scaffold. Severe deformation of the scaffold occurs upon clamping (see Fig. 2a). At the grip interface, there is a curvature on

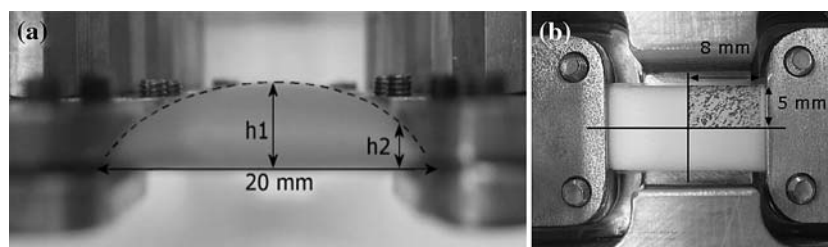


FIG. 2. Clamped collagen-GAG scaffold (a) side view showing deformation upon clamping (40% ϵ between positions h_1 and h_2) with dashed line showing the upper surface of the clamped scaffold and (b) top view showing the quarter region of the clamped scaffold modelled for finite element analysis.

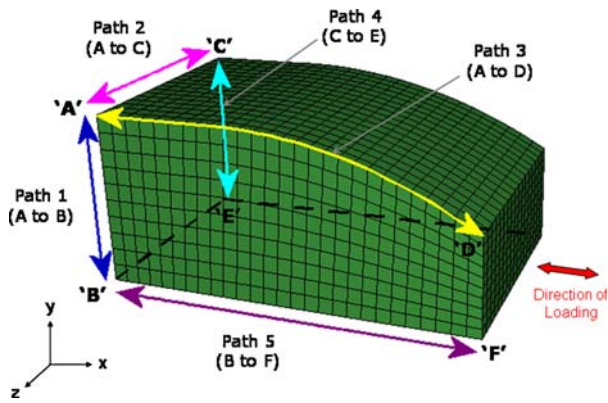


FIG. 3. Finite element model of the quarter model showing five paths along which the biophysical stimuli were analyzed.

the grips with accounts for 1 mm of the length of the scaffold. Along with the severe deformation of the scaffold at the grips, it is not expected that cells would be viable in the 2 mm close to the grip interface. Therefore, the modelled quarter geometry was created to replicate an 8 mm length from the central axis of the scaffold (see Figs. 2b and 3). The model included a curvature to replicate the deformation caused as a result of the applied compressive force during clamping. The physical clamping of the scaffold reduces the permeability across the length of the scaffold, and this was taken into account when applying material properties to the model. For the 8 mm length of scaffold modelled, it was determined that the scaffold was compressed by 40% from height (h_1) to height (h_2) (see Fig. 2a). Therefore, when applying the material properties to the model the permeability was reduced to correspond to no strain at the top of the model to 40% strain at maximum compression (using data from O'Brien *et al.*⁴² who determined experimental permeability values for the collagen-GAG scaffold under various amounts of compression). A linear fit to the values cited by O'Brien *et al.*⁴² was obtained and this linear fit was used to calculate permeability values for 2% compressive strain increments, and this was applied to parallel rows of elements. The other material properties applied are given in Table 1.

The scaffold was modelled using 4590 C3D8P pore fluid/stress 8-noded brick elements (Fig. 3). Appro-

priate symmetry boundary conditions were applied to the quarter model to restrain the central axes in the x and z directions. In the experiments, scaffolds were supported by silicone strips. Therefore, the nodes on the bottom face of the model were restrained in the y direction. The pore pressure was set to zero at the external nodes on the top and side faces of the scaffold. This means that fluid may flow in or out of the modelled region at these faces.

The experimental conditions were simulated by applying 10% strain (0.8 mm displacement) to the model at a frequency of 1 Hz in time steps of 0.1 s, and employed the non-linear geometry option to account for large deformations. Ten loading cycles were applied and fluid flow, pore pressure, and maximum principal strain were analyzed at a maximum tensile strain during the tenth loading cycle along the five pathways outlined in Fig. 3.

Statistics

Values are given as means \pm standard error of the mean (SEM). One-way analysis of variance (ANOVA) and the Newman-Keuls multiple comparisons post hoc test was used to detect significance between groups (GraphPad Software Inc., San Diego). Values of $p < 0.05$ were considered to be significant.

RESULTS

Experimental Results

Analysis of the cell population demonstrated that the cells were positive for both the CD90 and CD105 epitopes as assessed with immunocytochemistry (data not shown). FACS analysis for the expression of CD90 and the leukocyte common antigen, CD45, a hematopoietic cell surface marker found that $96.9 \pm 0.4\%$ ($n = 7$) of cells were CD90 positive, whereas only $2.65 \pm 0.8\%$ ($n = 7$) were CD45 positive. These results demonstrate that, although a homogenous population of MSCs was not achieved, a high percentage of the cells express MSC markers which is in accordance with other studies in literature.²⁷

Analysis of scaffold contraction found that the initial diameter of the circular plug of unconstrained cell-free scaffolds reduced by $20 \pm 1.7\%$ ($n = 4$) after 14 days in culture. The initial diameter of MSC-seeded unconstrained scaffolds after 14 days in culture reduced by 35.5% ($p < 0.01$, $n = 4$). The addition of chondrogenic growth factors to unconstrained MSC-seeded scaffolds further increased contraction to 46.9% ($n = 4$, $p < 0.01$); the time course of this contraction is shown in Fig. 4.

TABLE 1. Material properties applied to FE model.

Parameter	Value
Young's Modulus (kPa)	2.625
Poisson's ratio	0.33
Fluid compression modulus (MPa)	2300
Solid compression modulus (MPa)	6
Porosity	0.995

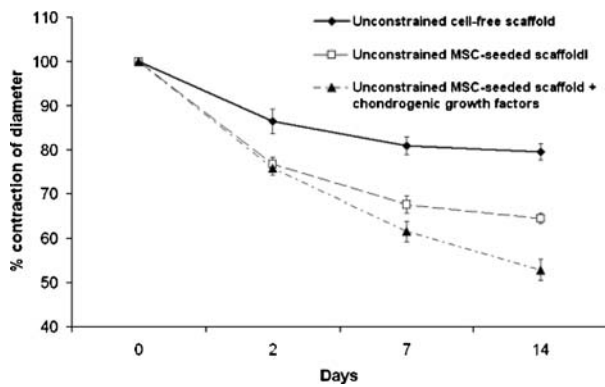


FIG. 4. Quantification of the collagen-GAG scaffold contraction in unconstrained cell-free, unconstrained MSC-seeded (i.e., no chondrogenic-factors) and unconstrained chondrogenic-treated scaffolds over 14 days of free-swelling culture. Unconstrained cell-free scaffolds contracted by $20 \pm 1.7\%$. MSC-seeded scaffolds contracted by a further $15.4 \pm 1.15\%$, while chondrogenic-treated MSC-seeded scaffolds contracted by $26.9 \pm 2.2\%$ compared to the cell-free group. Mean \pm SEM, $n = 4$.

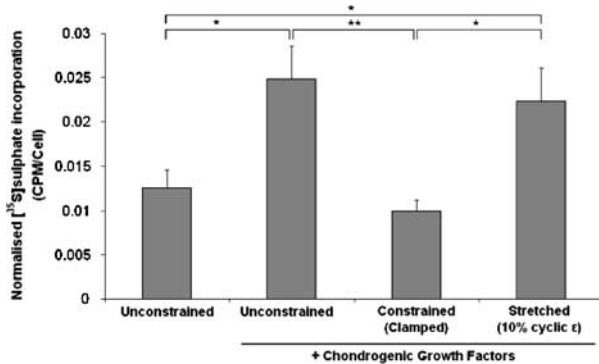


FIG. 5. The effect of mechanical constraint and cyclic loading on the chondrogenic differentiation of MSCs in a collagen-GAG scaffold. Unconstrained chondrogenic-treated samples had a higher rate of GAG synthesis compared to unconstrained controls (i.e., without chondrogenic growth factors). Mechanical constraint reduced the rate of GAG synthesis relative to unconstrained chondrogenic-treated constructs. However, application of 10% cyclic tensile stretch increased the rate of GAG synthesis compared to clamped controls, and restored the chondrogenic potential of the cells to levels similar to that observed in the free-swelling chondrogenic-treated controls. One-way ANOVA, Student–Newman–Keuls post hoc test, * $p < 0.05$, ** $p < 0.01$, $n = 10$.

The rate of GAG synthesis was significantly higher in unconstrained (i.e., free to contract) chondrogenic-treated scaffolds compared to unconstrained controls (i.e., without growth factors) (Fig. 5). This increase in GAG synthesis with growth factor treatment corresponded to an increase in cell-mediated scaffold contraction, as illustrated in Fig. 4. By constraining the scaffold (i.e., clamping) the rate of GAG synthesis was significantly reduced in the presence of chondrogenic factors relative to unconstrained chondrogenic-treated scaffolds ($p < 0.01$, see Fig. 5). Application of contin-

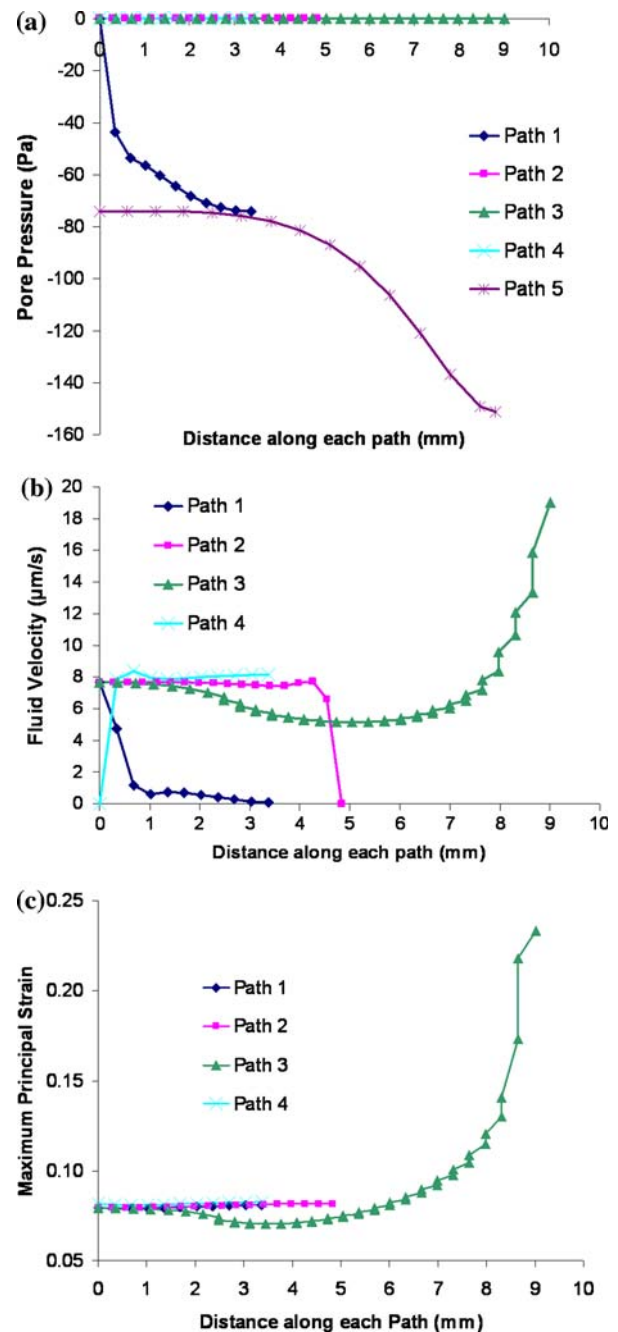


FIG. 6. Pressure (a), fluid flow (b), and max principal strain (c) along the paths as outlined in Fig. 3.

uous 10% cyclic stretch at 1 Hz for 7 days abrogated the constraint-induced reduction in the rate of GAG synthesis and restored it to levels observed in the unconstrained chondrogenic treated scaffolds ($p < 0.01$, Fig. 5). This result demonstrates that cyclic stretching under the specified parameters resulted in a significant increase in GAG synthesis relative to the constrained condition which indicates that mechanical stimulation influenced the differentiation process.

Biophysical Stimuli in the Scaffolds

A heterogeneous distribution of fluid flows, pressures, and matrix strains were developed within the scaffold under a cyclic stretch of 10%, as illustrated in Fig. 6. At a maximum tensile stretch of 10%, a negative pore pressure developed within the scaffold and reached a maximum of -151 Pa (see Fig. 6a). This negative pressure caused fluid to flow into the scaffold under the tensile stretch.

The fluid velocity was greatest at the surface (see Paths 2, 3, and 4) and reduced to zero through the depth (see Path 1). The lower permeability toward the grips resulted in a reduction in fluid flow from $7.7 \mu\text{m/s}$ to $5.2 \mu\text{m/s}$, before it increased to a maximum of $19 \mu\text{m/s}$ (see Path 3), as illustrated in Fig. 6b. This increase to $19 \mu\text{m/s}$ corresponded to the increase in the negative pore pressure from -74 Pa to -151 Pa along the base of the scaffold (see Path 5, Fig. 6a), due to the constraint. The average fluid velocity throughout the scaffold under 10% stretch was computed as $1.75 \mu\text{m/s}$.

Maximum principal strains along the four pathways (pathways shown in Fig. 3) under 10% stretch during the tenth cycle are presented in Fig. 6c. For pathways 1, 2, and 4, the maximum principal strain averaged at 8%. Along path 3 (i.e., along the curved edge at the top of the scaffold), the maximum principal strain ranged from 7.1% to 23.3% (Fig. 6c).

DISCUSSION

In this study we have demonstrated that mechanical constraint and cyclic strain in a 3D scaffold modulated the chondrogenic differentiation of MSCs, as assessed by the rate of GAG synthesis. Scaffold constraint by clamping impeded the cell-mediated contraction that would have occurred naturally, and resulted in a reduction in the rate of GAG synthesis compared to unconstrained chondrogenic-treated scaffolds. However, the application of 10% cyclic tensile stretch restored chondrogenic differentiation to similar levels observed in unconstrained chondrogenic-treated constructs. Analysis of the macroscopic biophysical stimuli within the scaffold during cyclic loading using a continuum poroelastic FE model quantified the magnitudes of stimuli that were developed in the scaffold which impacted cell synthesis, as demonstrated experimentally.

It is acknowledged that the cell population used in this study was not purely MSCs. Analysis of the cell population found that the cells were immunoreactive against CD90 and CD105 antibodies. Flow cytometry analysis demonstrated that the plastic adherence technique provided a population with 96% of cells

expressing the CD90 marker which is comparable to literature,²⁷ and illustrates that a large percentage of cells had the potential to undergo differentiation. A collagen I-GAG scaffold has been used in this study, however, collagen II matrices have been demonstrated to better support the chondrocyte phenotype than collagen I matrices.^{41,53} Nonetheless, collagen I is successfully used in matrices for MACI procedures.^{4,5,35,51,55} Additionally the collagen-GAG scaffold is fabricated from chondroitin-6-sulfate, and chondroitin sulfate has been demonstrated to provide a chondro-inductive environment for MSCs.⁵² A limitation of the work is that although 10% tensile stretch is applied to the whole construct, the actual microscopic strains experienced by the cells at a local level will be varied. This is due to the strut-pore architecture of the collagen-GAG scaffold to which MSCs can adhere to in numerous ways; for example, one strut or multiple strut attachments. Therefore it is likely that (i) there is a large variability in cellular strains and (ii) the strains experienced by the majority of cells are lower than the applied stretch of 10%. Investigations reported by Stops *et al.*⁵⁰ used FE analysis to show a variable distribution of microscopic cellular strains experienced by cells in the collagen-GAG scaffold when the scaffold is subjected a single macroscopic tensile strain.

A significant increase in cell-mediated scaffold contraction was observed in unconstrained chondrogenic-treated constructs compared to unconstrained controls over a period of 14 days. Scaffold contraction by the cells is attributed to cytomechanical forces, which have previously been demonstrated in cord blood stem cells undergoing osteogenic differentiation in collagen I/III scaffolds.²³ In 2006, Vickers *et al.*⁵⁴ demonstrated that cell-mediated contraction of collagen-GAG scaffolds by chondrocytes promoted cartilage formation. As chondrocytes require a 3D morphology to maintain their phenotype, we propose that the reduction in GAG synthesis found in MSC-seeded scaffolds treated with chondrogenic growth factors and exposed to uniaxial clamping was due to the inability of the cells to contract their local matrix to allow them to attain the rounded chondrocytic morphology. This hypothesis is supported by the findings of Galois *et al.*¹⁹ who found that chondrocyte-collagen I gels lost their chondrocytic phenotype when they were attached to the walls of a 24-well plate, compared to a free-floating condition.

The negative effect of mechanically constraining MSC-seeded scaffolds undergoing chondrogenic differentiation has possible implications for the MACI technique which is under investigation for the clinical treatment of articular cartilage defects.^{4,17,34,35} Although MACI uses chondrocytes and not MSCs,

there is a move toward the use of MSC-seeded 3D matrices for the repair of articular cartilage defects.³⁰ The MACI procedure involves seeding collagen membranes with autologous chondrocytes, and securing them into defects with fibrin glue.⁴ Therefore, the glue creates a ‘clamping’ effect and it has been demonstrated to last for up to 2 weeks *in vivo* prior to resorption.⁶ It has also been demonstrated in an *in vitro* MACI model that fibrin sealant enhanced the proliferation and migration of chondrocytes from collagen membranes,²⁸ which may potentially aid the integration of constructs to the surrounding native cartilage. Our results show that in an environment where cell-mediated contraction of the matrix is impeded due to mechanical constraint, the growth-factor induced increase in the rate of GAG synthesis is significantly reduced compared to unconstrained chondrogenic-treated constructs. Based on the findings of this study, the authors propose that if MSCs are to be used for such MACI procedures that constructs should be cultured prior to implantation so that cells can reshape their local environment to achieve a desired morphology and hence, maximum rate of matrix synthesis. A time limit on *in vitro* culturing would be necessary to ensure that engineered tissues would integrate appropriately with cartilage when implanted *in vivo*.⁴⁵

The mechanical loading regime of 10% stretch at 1 Hz chosen for this study is within the range of physiological strains and cyclic frequencies found in articular cartilage.³⁷ When constrained constructs were mechanically stimulated with continuous 10% cyclic tensile stretch at 1 Hz for 7 days, the rate of GAG synthesis was statistically increased compared to the constrained group, to levels similar to those achieved in unconstrained chondrogenic-treated constructs. This illustrates that mechanical loading influenced the chondrogenic differentiation of the MSCs. Although compressive loading alone has previously been demonstrated to induce the differentiation of MSCs along the chondrogenic lineage in agarose gels,^{21,22,36} this study provides new insight of the effects of mechanical loading in a tissue engineered scaffold of the kind under investigation for the clinical repair of damaged articular cartilage. Additionally, it demonstrates that although mechanical constraint has a negative effect on cell synthesis, that the application of dynamic loading can overcome this limitation and therefore implies that *in vitro* conditioning or post-operative joint loading would be beneficial to patients following MACI procedures.

The poroelastic computational analysis quantified the magnitudes of biophysical stimuli developed within the collagen-GAG scaffold during cyclic tensile loading (which resulted in an increase in the rate of GAG

synthesis as demonstrated experimentally). Fluid flows of 0–19 $\mu\text{m/s}$ and maximum principal strains up to 23% are comparable to stimuli that others have shown to regulate chondrocyte biosynthesis.^{7,16,33,46} These stimuli are also comparable to those computed to develop within agarose gels subjected to 10% compressive loading, in which MSCs were directed to differentiate along the chondrogenic lineage.²¹ In a study carried out by Buschmann *et al.*⁸ involving the dynamic compression of cartilage disks, it was found that GAG synthesis was localized to areas of high interstitial fluid velocity. It is expected that the maximum negative pore pressure of 150 Pa did not influence cell synthesis as physiological pressures in cartilage during walking are three orders of magnitude higher at approximately 5 MPa.¹⁵ Similarly, high pressures (ranging from 1 to 10 MPa) have been required to successfully regulate the chondrogenic differentiation of MSCs.^{2,39} Therefore, from the evidence presented, we propose that the magnitudes of strain and fluid flow generated within the scaffold were the main biophysical stimuli responsible for the observed mechanoregulation of MSC differentiation. These findings corroborate an existing mechanoregulation model where strain and fluid flow are proposed to be the main regulators of MSC differentiation,⁴⁸ which has been successfully implemented into computational models to predict fracture healing,³¹ osteochondral defect healing,²⁴ and optimal scaffold design for tissue engineering applications.^{9,25}

In conclusion, our results confirm the hypothesis that the chondrogenic differentiation of MSCs can be modulated by mechanical constraint and cyclic tensile stretch in a collagen-GAG scaffold. The mechanical constraint of constructs by uniaxial clamping resulted in a significant reduction in the rate of GAG synthesis. Application of cyclic tensile loading of 10% restored the rate of GAG synthesis to levels achieved in unconstrained chondrogenic-treated constructs. A poroelastic computational model demonstrated that strain and fluid flow were of sufficient magnitude to regulate the observed mechanoregulatory response. These results—particularly the finding that scaffold constraint impedes chondrogenic differentiation—have implications for the design of bioreactors and repair techniques in cartilage tissue engineering using MSCs.

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