The effect of simulated joint motion on gene expression and localization of superficial zone markers in tissue engineered cartilage constructs

INTRODUCTION
Enhancing the mechanical properties of tissue engineered cartilage has been the focus of several studies. Many concentrate on the application of mechanical stimuli, based on the premise that the mechanical environment regulates the development and maintenance of the tissue in vivo[1-2]. These studies have primarily focused at improving the bulk tissue properties, with little attention being paid to the layered structural hierarchy of cartilage. The superficial surface layer of articular cartilage is a highly organized structure that possesses exceptional wear-resistance while also providing a near frictionless articulation surface. Chondrocytes of this region produce superficial zone protein (SZP), a glycoprotein[3]unique to the superficial layer encoded by the proteoglycan 4 (PRG4) gene. Studies have illustrated the contribution of SZP to the frictional properties and joint health of articular cartilage, as well as the deleterious effects of tissues deficient in SZP[4]. It has been hypothesized that in order to engineer a successful superficial layer in tissue engineered constructs, simulated joint loading can be applied to stimulate and localize the expression of PRG4 and other superficial zone markers in a preferential manner. The purpose of this study was to measure the mRNA expression and in situ localization of superficial zone markers to determine the optimal stimuli required for the basis of forming a functional superficial layer in tissue engineered constructs.

MATERIALS AND METHODS
Chondrocyte Cultures: Primary articular chondrocytes were isolated from the calf (12-18 months) metacarpal-carpal articular cartilage by sequential enzymatic digestion. Cells were suspended in 20% FBS and encapsulated in 2% agarose. Aliquots of the cell/agarose suspension were placed into 24 well plates so a resultant culture thickness of 2mm was attained. The cultures were incubated at 37°C and 5% CO2 in Ham’s F-12 medium supplemented with 20% FBS. Cultures were maintained under static (no load) conditions for a period of 72 hours before subjected to simulated joint loading.

Mechanical Stimulation: Simulated joint loading was applied using a two-axis Mach-1™ material testing device (Biomomentum Inc.) in conjunction with custom-designed jig and loading pins. Spherical ended loading pins were compressed into, and reciprocated against the surface of the culture in order to generate combined dynamic compression, shearing and friction across the surface of the agarose constructs. Samples were stimulated under a compressive load of 1g, a reciprocating frequency of 1 or 2 Hz and a duration of 10,15, 30 or 60 minutes per day for three consecutive days. Samples were then snap-frozen in liquid N2 for RNA isolation.

Semi-quantitative and qRT-PCR: Total RNA was isolated from frozen constructs, dissected into unstimulated control and stimulated sections, using a Roche High Pure RNA isolation Kit with a slight modification to allow elution of agarose byproducts[5]. First strand cDNA was synthesized from random nonanucleotide primed total RNA using the Applied Biosystems High Capacity RNA to cDNA kit. Semi-quantitative PCR was performed using a Bio-Rad C1000 thermocycler with 18S (ribosomal RNA) used as the endogenous control and genes of interest were measured using custom oligonucleotide primers designed with NCBI Nucleotide sequences. Real time quantitative PCR was performed using the Roche LC480 Thermal Cycler with the same endogenous control and primers utilized via semi-quantitative PCR.

In-situ Hybridization: DIG labelled cRNA Probes were produced by in vitro transcription using T7 RNA polymerase. Paraffin embedded tissue sections were hybridized with the DIG labelled cRNA probes for 16 hours in at 40 °C. Labelled chondrocytes were then localized with an anti-DIG antibody and the sections were counterstained with methyl green. Bright field microscopy was utilized for imaging.

Statistics: All results were compared statistically amongst the loading conditions using a one-way ANOVA and the Fisher’s LSD post-hoc test and difference from unity using a one-sided T-test. Significance was assigned at p-values less than 0.05.

RESULTS
Initial semi-quantitative PCR, utilized as a coarse screening method to refine conditions, illustrated that shorter stimulation durations of 10 and 15 minutes at 1 Hz, enhanced expression of PRG4 and Collagen II in a significant manner (* p<0.05) (Figure 1). Similar increases in PRG4 and Collagen II expression were observed at 2 Hz, but in a non-significant manner (data not shown).

<table>
<thead>
<tr>
<th>Stimulation Duration</th>
<th>PRG4 (Mean ± SEM)</th>
<th>Collagen II (Mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Hz</td>
<td>2.09 ± 0.58</td>
<td>1.27 ± 0.16</td>
</tr>
<tr>
<td>2 Hz</td>
<td>0.94 ± 0.20</td>
<td>1.53 ± 0.31</td>
</tr>
</tbody>
</table>

DISCUSSION
This study illustrates the viability of applying simulated joint loading to produce positive change in gene expression of superficial zone markers (PRG4, Decorin, Biglycan) and localization of PRG4. The modest 2-fold increase in gene expression, as well as the distribution of PRG4 localization, is thought to be the result of the localized aspect of the stimulation applied. Simulated joint loading approximates the small moving contact area in normal joint contact, which limits the depth and breadth of the strain field produced, leading to a small amount of stimulated cells relative to the overall size of the construct. The localization of PRG4 expression appears to occur near the top 10% of the construct, and not directly at the construct surface. Further investigation of the traction forces at the tissue surface by FEA is underway to explain this phenomena as well as further in-situ work for other superficial zone markers.

REFERENCES