# Material-Directed Chondrogenic Differentiation under Mechanical Stimulation

LMC 4702 Research Thesis

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**Abstract**

The mechanical properties of scaffolds used to encapsulate stem cells are widely known to help direct the differentiation of those stem cells down different lineages. When studying chondrogenesis, the ability of the scaffold to withstand force is important as joints are expected to bear loads after implantation. The goal of the project is to study how differing perfusion and compression conditions influence human mesenchymal stem cell (hMSC) chondrogenesis. We hypothesize that sinusoidal dynamic compressive stimuli along with perfusion bioreactor culture of hydrogel-MSC constructs will further enhance zonal cartilage differentiation. Using a C9-x CartiGen perfusion bioreactor, cyclic compression was applied to PEG-based hydrogel constructs laden with hMSCs. The constructs were subjected to dynamic compression following one week of static preculture. At week 3, they were removed from the bioreactor and histological sections were stained for collagen and sulfated glycosaminoglycans. Gene expression of collagen I, II, X, Sox 9 and aggrecan were analyzed. It was shown that the material composition had more of an effect on the differentiation of hMSCs into chondrocytes than the addition of mechanical stimulation, but that chondrogenesis can be enhanced when both material properties and mechanical stimulation are varied. While perfusion bioreactor culture coupled with sinusoidal dynamic loading provides a more accurate model of the articular cartilage environment when compared to static culture due to loading at joints. Dynamic culture can provide insight into cell and material interactions during differentiation, leading to a more biomimetic neocartilage tissue construct.

## Introduction

Articular cartilage provides important cushioning and lubrication in joints in the body. It is a highly organized, low cell density avascular tissue made up of four spatially-variable layers exhibiting a gradient of mechanical and biochemical properties and markers as shown in Figure 1.

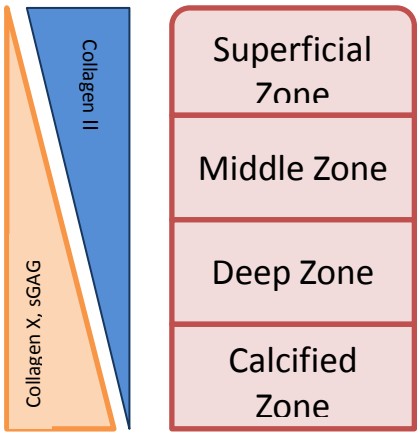


**Fig 1. Articular Cartilage Tissue Schematic.** The tissue is oriented into four distinct zones whose chemical and physical properties differ.

Due to the lack of vasculature and low cellularity, the tissue does not exhibit regenerative properties, and defects in the tissue can worsen over time if left untreated (Fox et al., 2009). Cartilage injuries are prominent in the US as well as osteoarthritis. Therefore, the need for tissue engineered cartilage implants is high (Temenoff & Mikos, 2000).

Current studies call for a biomaterial-based 3D scaffold to differentiate mesenchymal stem cells (MSCs) into chondrocytes and mimic the native tissue. Crosslinked polyethylene glycol (PEG)-based gels are attractive due to their ability to mimic the extracellular environment and mechanical properties of articular cartilage tissue (Nicodemus & Bryant, 2008). Studies have shown that scaffold composition has a strong effect on chondrogenesis in static culture (Nguyen et al., 2011). Our study uses PEG-based hydrogels with the addition of other biomolecules to create a biomimetic scaffold. While altering gel scaffold make-up is promising in static culture, the extracellular environment of articular cartilage is hardly static. The tissue is expected to withstand loads and implants are expected to do the same. Perfusion bioreactors can mimic the diffusion of nutrients into cells and the loading that articular cartilage must withstand.

Studies have shown that cyclic mechanical stimulation enhances chondrogenesis compared to a variety of conditions. This includes pellet culture and various hydrogel scaffold compositions. The media perfusion rate and duration, force amplitude and frequency conditions vary among studies. Steinmetz et al. used a 0% to 2.5% amplitude strain with a frequency of 1 Hz (1 h on, 23 off) for two weeks after a week of free swelling culture and found that it was critical for differentiation in a multi-layered spatially varying scaffold (Steinmetz et. al, 2015). Nicodemus and Bryant investigated the role of continuous versus intermittent loading on chondrogenesis and determined that intermittent loading up regulated important genes (Nicodemus & Bryant, 2010). Terraciano et al varied the amount of time that the gels were subjected to stimulation in a study. They stimulated the gels for 2.5 h/day and 4 h/day for 6, 14 and 21 days. They found that sulfated glycosaminoglycan (sGAG) production increased with time (Terraciano et al., 2007). Other studies have shown that mechanical stimulation inhibits chondrogenesis. Thorpe et al applied intermittent dynamic compression with a 10% amplitude strain with 0.01 N and a frequency of 0.5 Hz for 1 h on 23 h off for 5 days/ week. Their results showed no evidence of chondrogenesis enhancement (Thorpe et al., 2008).

Studies have also been moving away from single-layered scaffolds to multilayered scaffolds in order to mimic the spatial variation of the articular cartilage tissue. Nguyen et al used mouse-derived cells encapsulated in a tri-layered PEG based hydrogel scaffold and studied the differentiation of those cells into chondrocytes (Nguyen et al, 2011). They found optimal chondrogenesis in a multilayered scaffold, but only under static culture. Steinmetz et al combined both a spatially-varying scaffold with mechanical stimulation and found that both were critical for chondrogenesis of human MSCs (Steinmetz et al, 2015).

There is high variability in studies of mechanical stimulation and chondrogenesis due to differing material and culture conditions. The goal of our study is to explore the relationships between material and culture conditions for optimization of chondrogenesis of MSCs. We propose that through the variation of material compositions and dynamic culture conditions, more biomimetic articular cartilage tissue can be engineered.

Using a Cartigen C9-x perfusion bioreactor, sinusoidal dynamic compression was applied to PEG-based hydrogel scaffolds seeded with MSCs. PEGDA will be combined with hyaluronic acid methacrylate, chondroitin sulfate methacrylate, and matrix-metalloprotease peptide. We will be observing multiple scaffolds, including a multi-layered gel, over multiple mechanical culture conditions to study how these properties work together to influence chondrogenesis.

## Literature Review

Biomaterial based scaffold systems are desirable for tissue replacement due to their abilities to mimic tissue mechanical and biochemical properties while allowing cells to grow within. Many studies focus on the direction of the differentiation of mesenchymal stem cells (MSCs) due to their ability to differentiate down many different cell lines. One such area of research is in the regeneration of articular cartilage due to the heavy dependency on spatially-variable ECM components leading to a gradient of chemical and mechanical properties. Hydrogel-based scaffolds have shown to be promising to direct MSCs down a chondrogenic phenotype while also mimicking the natural tissue. An emerging area of research is observing how mechanical stimulation affects the differentiation of these cells encapsulated within hydrogel based scaffolds.

### *Biomaterials*

It is important to determine what biomaterial should be used for this experiment as that will be the main source of signals that the cells receive as well as the main contributor to the mechanical properties. Natural polymers have been studied such as collagen, which is already a key component in articular cartilage ECM, for the synthesis of scaffolds (Temenoff et al., 2000). Synthetic polymers have been studied as well as they can be mass produced and therefore do not rely on the body's natural production. Poly(ethylene glycol) diacrylate (PEGDA) gels are promising due to their inert behavior when interacting with cells and the ability to incorporate other biomolecules into a crosslinked scaffold (Nguyen et al., 2011, Nicodemus et al., 2008). Studies have shown that scaffold composition has a strong effect on chondrogenesis in static culture (Nguyen et al., 2011). Our study uses PEGDA based hydrogel scaffolds with the addition of other molecules such as hyaluronic acid methacrylate (HAMA) and chondroitin sulfate methacrylate (CSMA).

*Mechanical Stimulation*

Studies have observed the effect of mechanical stimulation on chondrogenesis. Nicodemus and Bryant used 15% strain at a frequency of 1 Hz of sinusoidal dynamic compression on a PEG based hydrogel seeded with chondrocytes (Nicodemus & Bryant, 2008). In later work, the group studied continuous vs. intermittent and delayed vs. immediate loading on the performance of chondrocytes in PEG hydrogels. They concluded that a combination of intermittent and immediate loading is favorable for the performance of the chondrocytes (Nicodemus & Bryant, 2010). While both of these studies show promising culture conditions, they use predifferentiated chondrocytes as opposed to MSCs. It is unknown how the performance of MSCs would be when compared to the chondrocytes and how these specific mechanical

loading regimes might affect the differentiation of the MSCs. Terraciano et al observed the effect of a 10% strain and 1 Hz dynamic compression regime on the chondrogenic differentiation of MSCs encapsulated in PEG gels. They altered the amount of time that the gels were under stimulation. They studied 1, 2, 2.5 or 4 hours/day for 1, 2 and 3 weeks. They found that the expression of chondrogenic markers on these cells increased under dynamic compression (Terraciano et al, 2007). Thorpe et al found that under their culture conditions, chondrogenic differentiation was downregulated. They applied intermittent dynamic compression with a 10% amplitude strain with 0.01 N and a frequency of 0.5 Hz for 1 h on 23 h off for 5 days/ week (Thorpe et al., 2008). Studies have shown, therefore, that higher frequencies and lower strains are more favorable for chondrogenesis of MSCs. Guo et al used alginate beads to encapsulate MSCs and subjected them to dynamic culture conditions. These beads were placed in a bioreactor that exposed them to dynamic flow. There was an additional group that was subjected to dynamic compression at a frequency of 0.5 Hz. The MSCs showed higher proliferation under the dynamic flow but no significant difference in the chondrogenic markers. They found higher regulation of chondrogenic markers in the compressed group (Guo et al., 2016). Steinmetz et al used a custom bioreactor to apply intermittent dynamic compression to hydrogels. They allowed the gels to culture in static culture for 1 week prior to loading in a sinusoidal manner within their bioreactor. They observed 0%-15% strain at a frequency of 0.3 Hz for one week with 0.5 h on, 1.5 h off for 16 h and 8 h off (Steinmetz et al., 2012). In later work, the group observed mechanical loading of a multilayered hydrogel and determined that mechanical loading is favorable in multilayered, spatially variable hydrogels for the differentiation of MSCs (Steinmetz et al., 2015).

As shown, there is a lot of variability in studies of mechanical stimulation and chondrogenesis due to differing material and culture conditions. The cell type, material composition and culture conditions must all work together for the optimization of articular cartilage regeneration. The goal of our study is to explore the relationships between material and culture conditions for optimization of chondrogenesis of MSCs.

## Methods and Materials

### *Cell expansion*

Mesenchymal stem cells purchased from Rooster Bio were expanded to passage 2. The cells were cultured in Rooster Bio expansion media for one week and allowed to proliferate with a media change on day 4.

### *Hydrogel synthesis*

A mold of polydimethylsiloxane (PDMS) containing 9 circular cutouts was fabricated and cut to the size of a glass slide. Each mold is designed to hold 200 microliters of prepolymer and is roughly the size of the bioreactor pistons, about 5 mm diameter. The gels were fabricated from varying weight percentages of polyethylene glycol diacrylate (PEGDA), hyaluronic acid methacrylate (HAMA), chondroitin sulfate methacrylate (CSMA) and matrix metalloproteinase-sensitive peptides (MMP-pep). The material compositions are as shown in table 1.

**Table 1. Material Composition of Hydrogels**

| Sample | PEGDA (%) | CSMA (%) | HAMA (%) | MMP-pep (%) |
|---|---|---|---|---|
| A | 20 | - | - | - |
| B | 19 | - | 1 | - |
| C | 18 | - | 2 | - |
| D | 18 | - | - | 2 |
| E | 15 | 5 | - | - |
| F | 14 | 5 | 1 | - |
| G | 10 | 10 | - | - |
| H | 10 | - | 2 | - |
| I | 9 | 10 | 1 | - |

Enough of each was weighed out for 2 mL of prepolymer. The solids were exposed to UV light for 5 minutes on each side in order to sterilize. These were dissolved overnight in 2mL of 0.05% lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP) in Dulbecco's modified eagle medium (DMEM) solution. The following day cells were resuspended in the prepolymer. The cell seeding was 20 million cells/mL prepolymer. 200 microliters of prepolymer was pipetted into each mold and crosslinked under UV light for 30 seconds. Figure 2 shows the gel synthesis process.

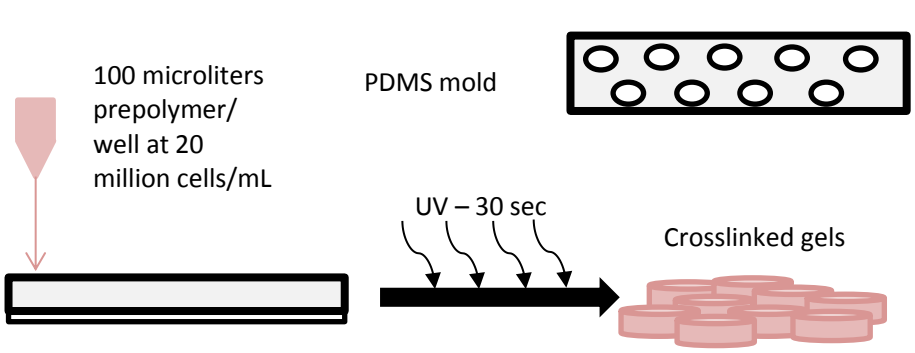


**Figure 2. Hydrogel polymerization**. Prepolymer and cell solution was pipetted into PDMS molds and exposed to UV light for 30 seconds, resulting in crosslinked three dimensional hydrogel constructs with encapsulated cells.

*Cell Culture*

The bioreactor being used is the C9-x CartiGen perfusion bioreactor, as seen in figure 3. It consists of a chamber with 9 circular wells for construct or cell loading. There are porous

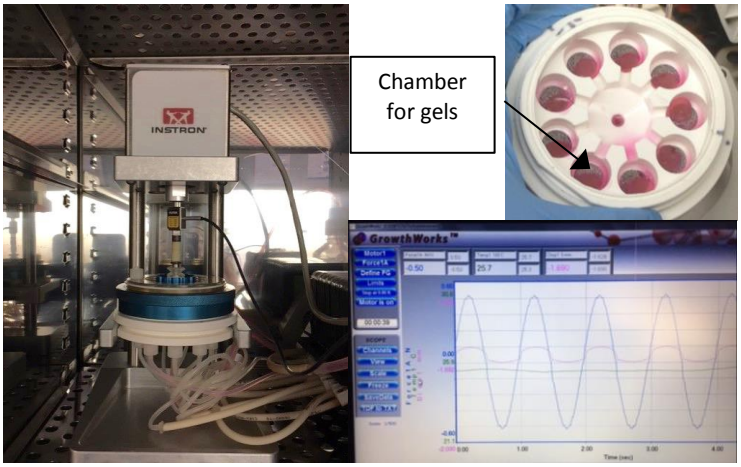


**Figure 3. C9-x Cartigen perfusion bioreactor from BISS.** Hydrogel constructs were placed in the chambers and subjected to sinusoidal dynamic compression. Media was perfused at a constant rate of 0.5mL/min.

platens at the bottom of each well to allow media perfusion into the constructs. Media is perfused through the system using a peristaltic pump system. It can be autoclaved after each use. The bioreactor has a mechanical stimulator that applies a compressive force to the constructs.

Once crosslinked, the gels were cultured statically and without perfusion in a 6 well plate covered with chondrogenic media, containing 1% penicillin/streptomyocin, 10nM Dexamethosone, 40 µg/mL l-proline, 5% ITS+, 50 µg/mL ascorbic acid –2- phosphate, and 10ng/mL TGF-β1 in DMEM solution, for one week. After 24 hours they were moved to a new plate with new media. Media was exchanged every 2 days. The gels were loaded into the C9-x CartiGen perfusion bioreactor after 1 week of static culture. The gels were subjected to a sinusoidal force with an amplitude of 0.5 N and a frequency of 1 Hz. The force was 4 hours on, 20 hours off for 14 days. Chondrogenic media was perfused at a rate of 0.5mL/min. Additional gels were statically cultured for the same time point to compare the effects of the loading.

*qRT-PCR*

6 gels were saved and 2 each were pooled to give 3 biological replicates. Gels were ground in 2mL Trizol using a homogenizer. They were precipitated with 0.2 mL chloroform for 5 minutes and centrifuged at 12 kg for 10 minutes. The less dense aqueous solution was saved

and precipitated again with IPA for 10 minutes and centrifuged at 10 kg for 10 minutes. The supernatant was removed and the pellet was rinsed with 70% EtOH made with RNAse-free water. They were centrifuged again at 10 kg for 5 minutes. The EtOH was removed and pellets were allowed to dry. They were then redissolved in 50 microliters of RNAse-free water. RNA purity was confirmed. cDNA was synthesized from the RNA using between 500-1000 ng of RNA per sample. This was done using the SuperScript III First-Strand Synthesis System for qRT-PCR using the oligo(dT)20 primer. PCR was performed using primers for collagen I, collagen II, collagen X, Sox 9, aggrecan and beta actin gene expression using the SYBR/Green Master Mix on a StepOnePlus machine.

*Histology*

3 gels were saved for histological analysis. The gels were rotated overnight in 4% paraformaldehyde at 4 degrees C. The formaldehyde was removed and replaced with 70% ethanol. The gels were rotated at 4 degrees C for 24 hours in the ethanol. The ethanol was removed and the gels were rotated at room temperature for one hour in each of the following solutions; 80% EtOH/H2O, 95% EtOH/H2O, 100% EtOH (repeated once), 50% CitriSolv/50%EtOH, 100%CitriSolv for two hours. They were then embedded in paraffin wax blocks, sectioned, and stained for Masson's Trichrome and Safranin-O.

*Statistical Analysis*

A one way ANOVA was performed on the results of the PCR gene expression to evaluate significance between groups, followed by Tukey's multiple comparisons test. All calculations were done in GraphPad Prism 7.03.

## Results and Discussion

*Histology*

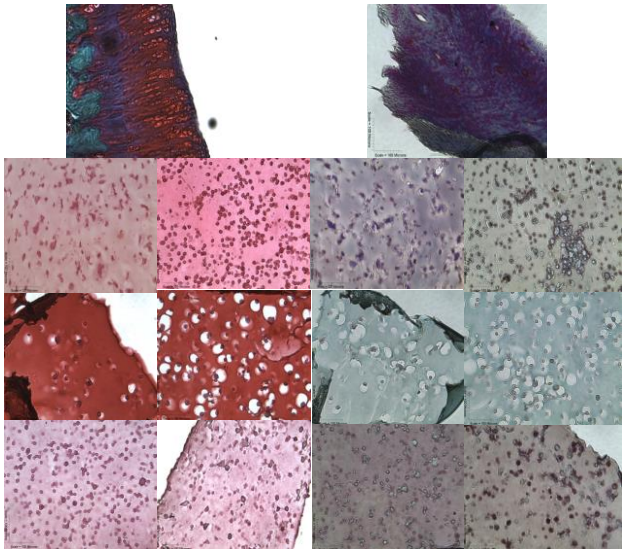

**Fig 4. Left: Safranin-O staining for sGAG concentration. Right: Masson's Trichrome staining for collagen.** Mouse cartilage is shown as a reference at the very top. The left columns of each are statically cultured, the right are the gels after mechanical stimulation. In order from top to bottom: 19PEGDA/1HAMA, 14PEGDA/5CSMA/1HAMA, 18PEGDA/2MMPpep

Masson's Trichrome and Safranin-O stains are shown in figure 4. The material conditions shown are B, F and D, chosen due to previous work from our group. Mouse cartilage is shown as a positive reference to determine the amount of staining. It is apparent that the gels do not stain as strongly as the mouse cartilage, but it is enough to see differences in staining between materials. Specifically, there is strong Masson's Trichrome staining in gel B when statically cultured, showing collagen deposition. It is important to note that Masson's Trichrome stains nonspecifically for collagens, therefore it is unknown what type of collagen is present in the hydrogel. In the Safranin-O stain, it is important to note that the hydrogel conditions that already had chondroitin sulfate present stain stronger, as the chondroitin sulfate stains positively for Safranin-O.

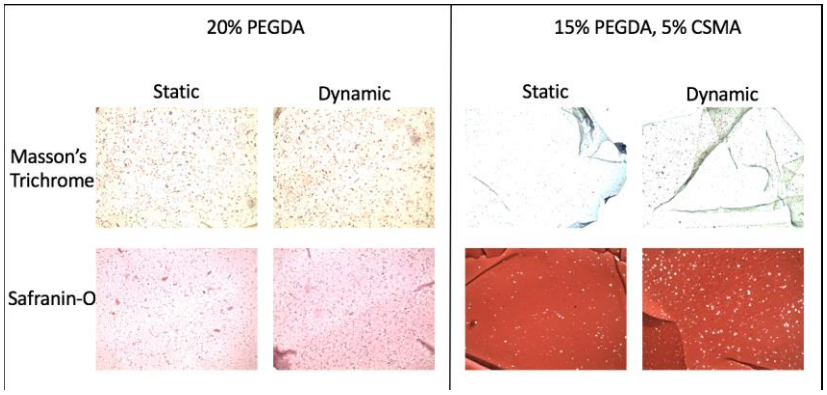


**Fig 5. Histological Staining** of gel A and E after static culture and dynamic culture for 3 days.

Figure 5 shows the

results of a second study, in order to determine if we can use the same stains to see differences in materials and dynamic culture conditions. This study uses different materials and a different loading regime. Specifically, the loading regime is as follows: Frequency of 1 Hz, 2.5% strain, 1 h on/23 h off for 3 days. As shown, there were no real differences in staining between static and dynamic conditions, but there are differences in the staining profiles between materials, although the increase in Safranin-O staining in the 15% PEGDA 5% CSMA gel can be attributed to the incorporation of a natural sGAG into the gel matrix. It is difficult to deduce if the staining profiles are due to the gel itself or if there is an increase in GAG production, therefore gene expression was analyzed in order to determine the effect of the materials and mechanical loading on the expression of specific chondrogenic genes.

*qRT-PCR*

Gene expression was analyzed using quantitative reverse transcription polymerase chain reaction (qRT-PCR).

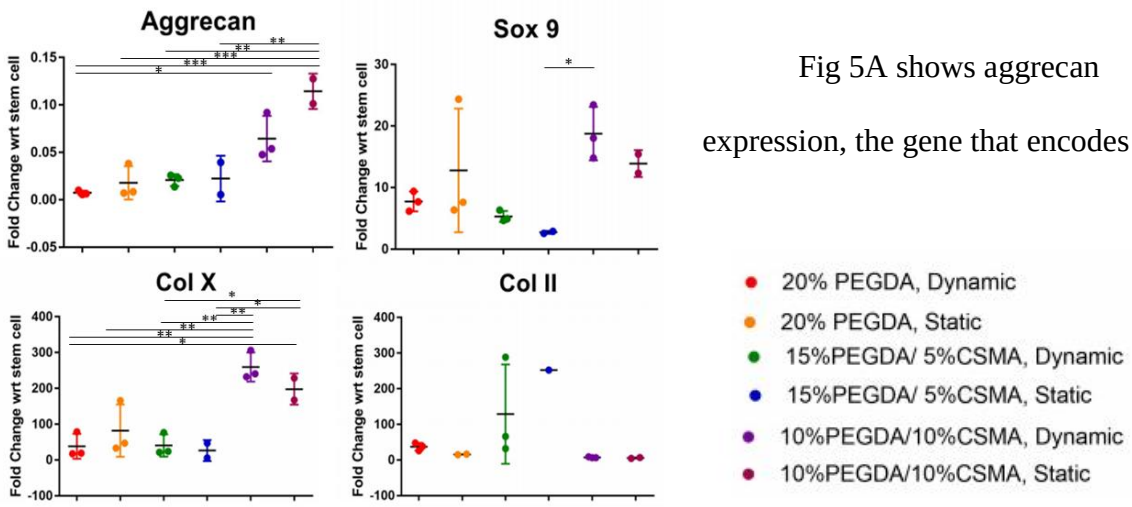


Fig 5A shows aggrecan expression, the gene that encodes for

**Figure 6. Gene Expression.** A - Aggrecan expression. B - Sox 9 Expression. C - Collagen X Expression. D - Collagen II Expression. Key is shown to the right. One way ANOVA with Tukey's multiple comparisons test was performed on each gene. * p<0.05, **p<0.005, ***p<0.001

sGAGs. Fig 5B shows Sox 9 expression, a transcription factor for collagen II. Fig 5C,D show collagen X and collagen II expression, which should have an approximate inverse relationship. It can be seen that with the aggrecan and sox 9 expression, there are differences between the 10%PEGDA/10%CSMA gels and the other materials, as well as slight differences between static and dynamic culture. This shows promise because the dynamically cultured gel is higher in sox 9 and lower in aggrecan, which could show the possibility of representing the superficial layer of the tissue. When looking at the same material but collagen X and collagen II, there are no real differences in collagen II, while collagen X is the highest in the 10%PEGDA/10%CSMA material condition. No significant difference was shown between statically and dynamically cultured samples of the same material composition. This shows that material composition had a stronger effect on cell differentiation than mechanical loading did.

**Conclusion**

Material composition and mechanical loading are factors that must work together in order to enhance the differentiation of human MSCs into neo-cartilage. Though previous work has shown differences between static and dynamically cultured cell scaffolds, the results of this study show that material composition causes greater differences than mechanical stimulation in the differentiation of MSCs. The relationship between material composition and mechanical loading must be studied further,  as these two factors must work together to influence the differentiation of human mesenchymal stem cells into chondrocytes. This study must be expanded past the three materials that were further explored to better understand the interplay between material properties and mechanical stimulation in the hopes of creating a functional, cell based cartilage mimicking tissue.

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