RECOVERY OF PASSAGED CELLS THROUGH THE RECAPITULATION OF THE PERICELLULAR MATRIX

By

Stefan Balko

Bachelor of Science in Mechanical Engineering, University of Manitoba, 20014

A thesis presented to Ryerson University in partial fulfillment of the requirements for the Degree of Master of Applied Science in the program of Biomedical Engineering

Toronto, Ontario, Canada, 2019

AUTHOR'S DECLARATION FOR ELECTRONIC SUBMISSION OF A THESIS

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I authorize Ryerson University to lend this thesis to other institutions or individuals for the purpose of scholarly research

I further authorize Ryerson University to reproduce this thesis by photocopying or by other means, in total or in part, at the request of other institutions or individuals for the purpose of scholarly research. I understand that my thesis may be made electronically available to the public.

Recovery of Passaged Cells Through the Recapitulation of the Pericellular Matrix, Stefan Balko, Master of Applied Science, Biomedical Engineering, Ryerson University, 2019

Abstract

In order to create successful cartilage tissue engineered constructs, an abundant supply of healthy cartilage cells (chondrocytes) are required. Since only a small amount can be excised from a patient, chondrocytes must be expanded and passaged *in* vitro to reach the desired population numbers. As chondrocytes are expanded *in vitro* they start to lose their chondrogenic phenotype and synthesize less cartilaginous extracellular matrix (ECM); including a pericellular matrix (PCM). A promising method used to upregulate the synthesis of ECM constituents is mechanical stimulation. The objective of this study was to recover the PCM of passaged chondrocytes and determine whether mechanical stimuli could restore their phenotype and function. Although the passaged chondrocytes did not show an increase in cartilaginous ECM accumulation over the long-term culture, they did show a positive response to mechanical stimulation, highlighting the importance of the PCM in maintaining chondrocyte phenotype and mechanosensitivity.

Acknowledgements

First off, I would like to thank my supervisor and mentor, Dr. Stephen Waldman. Without his help and constant guidance, I would not be where I am today and for that I am very thankful. My sincere thanks also goes out to the rest of the members of my thesis committee, Dr. Michael Olson and Dr. Warren Lee. Thank you for having the patience to read my work and coming up with insightful questions for me to think about.

I would also like to thank all my lab mates at St.Michaels for making these past few years a memorable experience. The discussions we had over the years were thought provoking and entertaining to say the least. Specifically I want to thank Warren Tang, Loraine Chiu and Arianna Soave for helping me with my work when I needed it most.

I would like to thank my family and friends. You were always there for me to lend a supporting hand and helped me get through the more stressful parts and for that I am forever thankful. Specifically I would like to thank my mother and father for helping support me financially and lovingly over these past few years. I would also like to thank my brother and sister for always finding a way to cheer me up, or make me laugh when the work got slow and drawn out. Lastly, and most importantly, I would like to thank my fiancé Ashley Dixon, for putting up with me for these last few years as I pursued my dreams. You are an amazing woman and I cannot put into words how much you have helped me.

Table of Contents

List of Tables

List Of Figures

1.0 Introduction

1.1 Research Problem

Cartilage tissue has an inept ability to repair and heal itself, mainly due to its lack of vascularity. This is an issue, especially when cartilage becomes unhealthy or compromised because of injury or disease. Osteoarthritis, a disease of cartilage tissue, is one of the leading causes of disability in adults in the United States, with more than 10% of the entire population suffering from osteoarthritis [1]. Although some medical treatments exist for Osteoarthritis, the majority focus on alleviating pain rather than solving the underlying issue. In order to fix and heal damaged cartilage tissue, engineering practices have been sought after to develop tissue engineered cartilage constructs with the aim of repairing the damaged tissue.

Although different methods exist to create tissue engineered constructs, the majority of them focus on using explanted chondrocytes as the primary cell source. A major issue is that there isn't an abundant supply of healthy autologous chondrocytes available. In addition to this, when a healthy supply is isolated, only a small amount can be excised without causing damage. This leads to the requirement of *in vitro* expansion in order to achieve a sufficient chondrocyte population to create tissue engineered constructs. In vitro expansion is a well explored area of study, and it is widely accepted that in vitro expansion of chondrocytes causes dedifferentiation and loss of the chondrogenic phenotype[2,3]. This is an issue as chondrocytes that shift from their chondrogenic phenotype will not behave properly after implantation and will ultimately fail. Methods of redifferentiating expanded cells needs to be explored in order to ensure the success of tissue engineered constructs.

A method which has been used to enhance cartilage tissue formation *in vitro* is the application of mechanical stimuli to the chondrocytes as they grow, similar to their *in vivo* environment. It has been shown that applying mechanical stimuli to cartilage constructs *in vitro* allow for better mechanical properties and uniformly organized tissue growth, similar to native cartilage tissu[4–10]. Although there have been other methods explored for redifferentiating chondrocytes, the use of mechanical stimuli for the purpose of redifferentiation has never been explored. Mechanical stimulation is very effective in enhancing cartilage tissue growth and shows great potential for recovering the loss of phenotype caused by passaging.

1.2 Research Objectives

The overall objective of this work was to explore a means by which passaged chondrocytes could be recovered to a chondrogenic phenotype similar to primary chondrocytes. Specifically, the primary goal was to ascertain whether or not passaged chondrocytes could be recovered through the application of mechanical stimuli to the chondrocytes, in an effort to recover the percellular matrix (PCM).

The first study explored the application of dynamic compression, vibration and stochastic resonance (combined dynamic compression with superimposed random vibration[11]) applied to chondrocytes seeded in agarose constructs in a short-term culture. The agarose constructs were stimulated for 20 minutes after which they were analyzed for DNA accumulation as well as collagen and proteoglycan synthesis. The objective was to understand how passaged cells react to mechanical stimulation, and to see whether we could elicit a positive anabolic response in the passaged chondrocytes.

The second study explored the effects of mechanical stimulation on passaged chondrocytes in a long-term culture. The constructs were stimulated for 20 min a day, 3 days a week for a total of 4 weeks. The purpose of this experiment was to determine the effects of the stimuli in a long term culture. Furthermore it was to investigate the architecture of the extra cellular matrix (ECM) and whether or not it would be similar to primary chondrocytes.

The third study explored recapitulating the pericellular matrix (PCM) in the passaged chondrocytes as it was discovered that passaged cells lose their ability to create a robust PCM on their own. Specific to mechanical stimulation and matrix synthesis, the PCM plays a crucial role in both and the desired outcome of this study was to allow for the chondrocytes to recreate their PCM after being seeded in alignate beads.

The fourth and final study explored a long term mechanical stimulation study with passaged chondrocytes with a recapitulated PCM. Three modes of stimuli were used (unstimulated control, dynamic compression or randomized vibration). The objective was to recapitulate the PCM in the passaged chondrocytes and to mechanically stimulate them. With the recovered PCM, the passaged cells should receive the proper signalling from the mechanical stimulation and in turn accumulate cartiligous ECM, in a similar manner to primary chondrocytes.

2.0 Background and Literature Review

2.1 Articular Cartilage

Articular cartilage is located at the distal ends of the bones in all articulating joints, providing a gliding surface for the bones to articulate on. It is composed of four major components: 1) collagens, 2) proteoglycans, 3) water and 4) chondrocytes [4,10,12–14]. Collagens are fibril proteins that provide tensile properties to the cartilage and make the tissue resistant to mechanical forces. Proteoglycans are negatively charged glycosaminoglycans which are covalently bonded to a protein core that attracts cations and water into the collagen network, causing swelling and further resistance to compressive forces. Water, which is the largest component of articular cartilage, contributes between 65-80% of the wet weight. Lastly, chondrocytes are the specialized cartilage cells distributed throughout the extracellular matrix (ECM) of the tissue, comprising roughly only 5% of the tissue volume. Although they play no direct role in resisting mechanical forces, they are responsible for tissue maintenance and control the synthesis and degradation of both collagen and proteoglycans.

Articular cartilage has four different zones, each with a unique composition and organization allowing for a complex structure which is able to not only act as a smooth gliding surface but also is able to withstand large loads while not compromising joint function. The 4 different zones are: i) superficial zone; ii) middle zone; iii) deep zone and; iv) calcified zone (Figure 2-1).

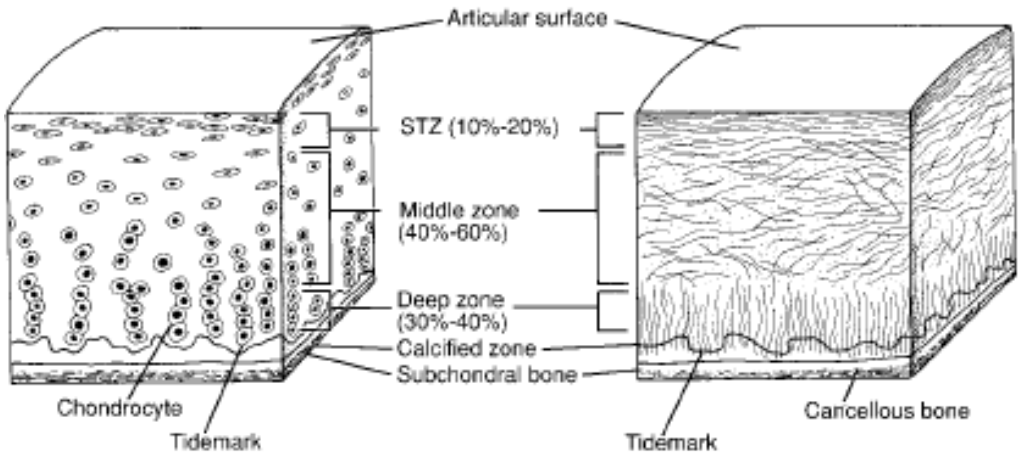

Figure 2-1: Architecture of articulating cartilage, with specified zones outlined[14]

The superficial zone protects against tangential shear forces and comprises approximately 10-20% of the overall volume of the tissue. The outer most region of the superficial zone is the articulating surface itself. In the superficial zone, there is a denser population of chondrocytes which take on a flatter, elongated shape. The collagen fibres in this zone are oriented parallel to the articulating surface. This layer is responsible for the majority of the tensile properties of cartilage, allowing it to resist shear, tensile, and compressive forces. In this zone, resident chondrocytes also synthesize superficial zone protein (also known as lubricin or proteoglycan 4), a specialized proteoglycan which accumulates at the surface and is secreted into the synovial fluid of the joint, acting as a boundary lubricant. The zone just below the superficial zone is the middle zone, which comprises about 40-60% of the overall volume. It is a transitional zone, providing a bridge between the superficial and deep zone. The collagen fibres in this zone are organized obliquely and are thicker than in the superficial zone. Proteoglycans are also present in this zone making it the first resistor to compressive forces. The chondrocytes in this layer are more sparsely distributed and have a spherical shape. The deep zone is the zone just below the middle zone and comprises approximately 30% of the total volume of tissue. The collagen fibers

in this zone are oriented perpendicular to the surface, having the largest diameter fibrils of all the zones. The deep zone also has the largest content of proteoglycans, and combined with the size and orientation of the collagen fibers, makes the deep zone the largest resistor to compressive forces. The chondrocytes are organized in columns oriented perpendicular to the surface. A notable feature of the deep zone is the tide mark, which separates the three previous zones from the calcified zone. The calcified zone is responsible for ensuring that the cartilage is adhered to the subchondral bone. In this zone, the population of chondrocytes is very limited. The unique orientation of chondrocytes in each zone leads to the specific organization and maintenance of the overall ECM of the tissue [4,10,13–15].

2.1.1 Chondrocytes

The only cells present, which are responsible for the repair, maintenance and formation of cartilage tissue, are chondrocytes. Chondrocytes originate from the mesenchymal stem cell lineage and constitute about 2% of the total volume of cartilage tissue [13,14,16]. They are spherical in shape and are responsible for secreting proteoglycans and the different collagens that make up the ECM. Chondrocytes are unique in that they do not form direct cell-to-cell contacts and rely on signals from their surrounding ECM, both mechanical and chemical in nature, to properly synthesize and accumulate the matrix constituents required for healthy cartilage tissue[13,14,16,17]. As chondrocytes develop, they essentially entrap themselves within their surrounding ECM, inhibiting mobility throughout the surrounding ECM. Combined with the fact that they have limited potential for replication, chondrocytes exhibit a difficultly in healing and repairing damaged cartilage tissue after injury[13]. The metabolic activity of chondrocytes can be altered by the local mechanical and biochemical environment, and therefore it is crucial that the correct environment is maintained for cartilage tissue homeostasis[13].

2.1.2 Extracellular Matrix of Articular Cartilage

As mentioned earlier, the majority of cartilage tissue is comprised of it's ECM. The ECM acts as the mesh network, within which chondrocytes are entrapped and enclosed. In terms of mechanical properties, the ECM is responsible for the majority of the structural integrity of cartilage tissue and is also responsible for transmitting signals between cells. The ECM of cartilage tissue is mainly composed of two constituents: i) collagens and ii) proteoglycans.

Collagens

Collagens are extended extracellular proteins composed of three polypeptide α-chains, each possessing a characteristic tripeptide sequence which forms a lefthanded helical structure[16]. The major collagen present in the ECM is collagen type II, representing 90-95% of all the collagen in the ECM. Other collagens which are present, but not as abundant, are collagen types I,IV,V,VI,IX and XI[13]. Collagen type II is a fibril collagen which forms the mesh structure of the cartilage matrix. It is responsible for anchoring the proteoglycans in place and accounts for a majority of the mechanical properties of cartilage tissue[13,16]. The mechanical properties of collagen type II stem from the intermolecular crosslinking that occurs. There is also evidence that collagen type XI has an interconnected role with collagen type II in that it helps maintain the structure and organizational stability of collagen type II [18,19].

Proteoglycans

As mentioned earlier, proteoglycans are glycosaminoglycans entrapped within the collagen mesh network and are responsible for attracting water and positively charged ions into the cartilage tissue. They comprise of 10-15% of the total volume of cartilage tissue and consist of one major protein core, with 1 or more glycosaminoglycan chains attached to it[13]. Healthy articular cartilage contains many proteoglycans, such as biglycan, decorin and fibromodulin but the most

abundantly found is aggrecan. Aggrecan is a bottlebrush proteoglycan which has as many as 100 chondroitin sulphate chains and as many as 30 keratan sulphate chains attached to its core. Aggrecan molecules have an affinity for binding towards hyaluronan, with the ability to create large aggregate macromolecules through the binding of "link proteins". Aggrecan is responsible for providing the osmotic properties of articular cartilage, from which the resistance to compressive loads as well as nutrient deliver and waste removal is achieved[13,16].

Although decorin, biglycan and fibromodulin are smaller than aggrecan, they may be present in similar molar quantities. Decorin and fibromodulin interact with collagen type II within the ECM as well as play a role in fibrillogenesis. Biglycan is mainly found in the PCM and has shown to interact with collagen type VI[13].

2.1.3 Pericellular Matrix of Articular Cartilage

Within the ECM, a region specific to the periphery of the chondrocytes is known as the Pericellular Matrix (PCM). The PCM is a specialized layer of the ECM and acts as the communication channel between chondrocytes and the surrounding ECM [20,21]. The cell and its enclosing PCM together are known as a Chondron [22]. The PCM has been shown to have a major role in biomechanical and biochemical signalling to both the cell and the surrounding ECM [23]. Similar to the ECM, the PCM is composed of collagens and proteoglycans that make up the core of its structure. Very recently, it has been discovered that healthy articular cartilage contains a primary cilium, an organelle which acts as a major sensory component of the chondron in response to mechanical forces[24–27].

Collagens

The most notable collagen that is present in the PCM is collagen type VI. Collagen type VI is a beaded filament structure which has 3 distinct polypeptide chains: α1, α2 and α3

respectively[23,28]. Type VI collagen interacts with many other ECM components, such as aggrecan, biglycan, decorin, hyaluronan, fibronectin, perlecan, and heparin through these polypeptide chains. Type VI also forms a mesh network that anchors the PCM to the chondrocyte itself. Because of the high affinity of collagen type VI for other ECM molecules and its direct anchorage to the chondrocyte cell, it is hypothesized that collagen type VI plays a crucial role in cell-ECM signaling and interactions[20,23,28–30]. The expression of type VI collagen is also crucial in maintaining the chondrocyte phenotype, showing diminished levels of collagen type-VI expression after chondrocyte dedifferentiation and exhibiting restored levels of expression after chondrocyte redifferentiation[20,31].

Although not as notable, another collagen type integral to the overall PCM structure is collagen type-IX. Collagen type-IX is a Fibril Assisted Collagen with Interrupted Triple Helices (FACIT) with three distinct triple helical domains. Localized in the PCM, collagen type IX bonds to both collagen type II and itself, limiting the diameter of the collagen type II fibrils[32,33]. Type IX collagen may also regulate the surface properties of the localized collagens, limiting their size and interaction with nearby fibrils, aiding in the stabilization of the microenvironment of the PCM[33].

The last major collagen present in the PCM is collagen type II. Although the PCM contains type-II collagen, it does not have the larger band and aggregate architecture more commonly found in collagen type II in the surrounding ECM. Again this is due to the interaction with the collagen type IX found in the PCM, as previously discussed [22,33–35]. Type II collagen acts as the major structural component of the ECM and binds to type VI collagen in the PCM, anchoring the PCM to the rest of the ECM.

Proteoglycans

Similar to the ECM, the PCM contains a vast array of different proteoglycans. In contrast to the ECM, the PCM contains a higher concentration of proteoglycans and glycoproteins[20,34]. Of the proteoglycans present, the most abundant is aggrecan.

A high concentration of aggrecan has been shown to exist in the PCM. In addition to this, a high concentration of hyluronan and link protein exists and it has been suggested that the localized confinement in the PCM enables the hyluronan-aggrecan-link protein complexes to form[34,36].

Perlecan is a proteoglycan which is present exclusively in the PCM. Perlecan is a large heparin sulphate proteoglycan that co-localizes with collagen type VI in the PCM and has been shown to regulate the mechanical properties of the PCM. Perlecan has also been shown to have a role in chondrocyte attachment to the surrounding matrix[29,34,37].

Other smaller proteoglycans and glycoproteins exist in the PCM, but to a smaller extent, such as, biglycan, decorin, fibronectin and heparin[20,34,35]. Biglycan and decorin help connect collagen type VI with matralins, which in turn connect and anchor the collagen type VI to collagen type II. Biglycan has also been shown to restrict fibrillogenesis, altering collagen type II fibres found in the PCM causing them to be smaller than those found in the surrounding ECM[20,29,34,35].

Primary Cilium

In chondrocytes, as well as many other cell types, there is an antenna like organelle called the primary cilium. Primary cilia consist of an intracellular basal body and a membrane-coated axoneme which projects from the cell surface into the extracellular microenvironment. The antenna like axoneme is made up of microtubules rich in acetylated α-tubulin. Primary cilium in

chondrocytes exist in a 9+0 configuration, as opposed to the 9+2 configuration of regular cilia responsible for motility in other cell types[24,26].

In chondrocytes, it has recently been suggested that the primary cilium plays a major role in mechanotransduction and mechanosensitivity. The primary cilium is thought to sense deformations in the ECM as they are structurally associated with collagen fibres, they express collagen anchoring receptors and integrins and are physically deflected by ECM interactions[24,27,38,39]. It has also been shown that activated intracellular Ca^{2+} via mechanical loading is only achieved with chondrocytes containing primary cilium[24,26,40]. It has also been shown that integrins located on the chondrocyte primary cilium surface, anchor mechanically functional collagen fibres to the ciliary membrane and, after bending occurs due to mechanical loading, transduces these signals via intracellular signalling pathways such as hedgehog, Wnt and PDGFαα[27,41,42].

Alternatively, the primary cilium has also been revealed to have a close relationship with ECM maintenance, having a structural relationship with the Gogi apparatus which in turn suggests it could be involved in the secretion of ECM macromolecules during tissue repair and turnover[38]. Specifically Col2aCre;Ift88$^{fl/fl}$ mice, which were shown to have depleted primary cilia, ended up developing abnormal articular cartilage and early signs of osteoarthritis [25,41].

2.1.4 Mechanical Properties of Articular Cartilage

From a materials standpoint, cartilage is a porous, viscoelastic material[43] and has three key phases: i) a solid phase which is predominately a strong collagen mesh with proteoglycans interwoven in; ii) a fluid phase, which is comprised of water; and iii) an ion phase, which has many dissolved electrolytes with positive and negative charges [10,44]. It is the combination of these three phases that allows articular cartilage to withstand large loads imposed on it by the

human body. The most notable theory on describing the stress-strain relationship is the biphasic theory, originally introduced by Mow et al.[44–48]. The biphasic theory states that three major internal forces act within cartilage tissue when it is under a mechanical load: i) the stress developed in the solid phase (collagen and proteoglycan woven network); ii) the pressure developed in the liquid phase; and iii) the drag force acting on each phase as they pass through one and other [44–46]. Soon after, the triphasic theory was introduced by Lai et al., which builds on the biphasic model, but also takes into account the Donnan osmotic pressure. The Donnan osmotic pressure is pressure created by the imbalance of the mobile counter-ions between the inner proteoglycan molecules and the outer solution [44,49]. The interaction of these different phases lead to a complex coupled mechanoelectrochemical environment in which chondrocytes are exposed to multiple stimuli. These stimuli include mechanical forces, fluid flow, hydrostatic pressure, an osmotic pressure gradient, electric current, and electric potential differences within the ECM itself[10].

Cartilage is also characterized as a viscoelastic material, exhibiting three crucial time dependant phenomena that relate to it: creep, hysteresis, and stress relaxation. Creep is the tendency for a material to permanently deform under constant stress. Hysteresis is the phenomenon in which previous loading influences the current behaviour of the tissue. Stress relaxation is the tendency for a material, under constant strain, to slowly decrease in stress until an equilibrium is reached [12]. These three time dependant phenomena stem from the complex nature of the mechnoelctrochemical environment found in articular cartilage. In regards to healthy cartilage found in humans, the aggregate modulus and Poisson's ratio is between 0.5 - 0.7 MPa and 0.07 – 0.1 respectively [10,50].

2.2 Cartilage Tissue Engineering

Since cartilage has an inability to repair and heal itself, a need has arisen to find solutions to the problem of deteriorating articular cartilage. Through engineering principles and techniques, tissue engineered constructs have been created *in vitro* to be implanted into the defect site to resurface the affected joint. In order to engineer viable tissue constructs, three general considerations are required: i) cell sourcing; ii) scaffold design and; iii) growth stimulus.

Although chondrocytes are the primary cells involved in cartilage tissue formation, there are different means by which one can obtain chondrocytes. Chondrocytes can be directly harvested from existing cartilaginous tissues in the body. Alternatively, stem cells from different sources can be differentiated into chondrocytes. Thus far clinically, chondrocytes have typically served as the primary cell source for articular cartilage repair, although they do have their limitations, such as dedifferentiation during *in vitro* expansion and limited availability of healthy autologous chondrocytes [2,3]. To avoid the issues related to chondrocyte sourcing, extensive research has been conducted into the use of stem cells, including: embryonic (ESCs), mesenchymal (MSCs), and adipose-derived (ASCs) stem cells, as cell sources. MSCs and ASCs are multipotent stem cells that can be easily isolated from many mesenchymal tissues and have the ability to undergo chondrogenesis given the correct physiochemical cues, making it a popular choice for cell sourcing and cellular therapy [51,52]. ESCs have the trait of unlimited proliferation and can essentially differentiate into any type of cell, making them extremely promising in tissue regeneration and cell sourcing. As research into the use of ESCs develops, there are ethical concerns as well as many unknown safety concerns that may limit their usability[10].

When designing tissue engineered constructs it is also important to take into consideration scaffold design. Scaffolds can provide a 3D structure to support cell growth and proliferation,

ECM deposition, and tissue regeneration. Scaffolds must be biocompatible to minimize the host response, be biodegradable to allow for replacement with newly grown tissue, have suitable porosity to allow cellular proliferation and interconnectivity, and possess the proper mechanical properties to support tissue growth under mechanical loads, all while encouraging the growth of newly formed tissue [53]. In order to meet all these requirements, the biomaterial chosen for the scaffold must be chosen carefully. Generally speaking, scaffolds are designed using a natural or synthetic based biomaterial. Natural biomaterials are a popular choice for scaffolds due to their biocompatibility. Specifically, carbohydrate-based hyaluronic acid, agarose, alginate, chitosan, protein based collagen or fibrin are currently used [53]. For synthetic biomaterials, polymers are used because of their ease of fabrication and the ability to tailor the surface and bulk properties. The most popular synthetic polymers for scaffold design are poly-lactic acid, poly-glycolic acid, and their copolymer poly-lactic-co-glycolic acid [2]. Alternatively, there are several scaffold-free techniques for tissue engineered constructs also, such as pellet culture, aggregate cultures, and self-assembling techniques [2]. Scaffold-free techniques benefit from being free of excess material that would otherwise be used to create the scaffold which would need to degrade to allow for new tissue formation to replace it. In addition to this, scaffold material may potentially induce other problems, such as stress shielding and toxicity that scaffold-free designs do not suffer from[10].

Lastly, when designing tissue engineered constructs the incorporation of growth stimuli are crucial in ensuring the tissue is formed properly and in a time-efficient manner. Growth factors have been studied extensively since the 1950's as a means to encourage tissue growth *in vitro*. Many different growth factors have been used and incorporated into tissue engineered constructs to elicit differentiation, proliferation and synthesis [2,54,55]. Alongside growth factors, there are

other biophysical stimulation methods which are extensively studied. The three most common are mechanical stimulation, electrical stimulation, and magnetic stimulation. The use of magnetic stimulation in the treatment of diseases has been of great interest for a long time and because of it there is a vast amount of literature covering the use of biomagnetism. Although the field of study is robust, much of it is met with skepticism [56]. Similar to magnetic stimulation, the use of electrical stimulation is a well-established clinical therapy readily available with promising uses in cartilage tissue engineering [57].

2.3 Mechanical Stimulation in Cartilage Tissue Engineering

Articular cartilage is constantly exposed to mechanical forces inside the human body which are essential to the growth of healthy human cartilage. Areas of the joint that are load-bearing, have cartilage which is thicker and mechanically stronger than those areas that are non-load-bearing [4,5,58]. Similar to bone, articular cartilage will reform itself in order to withstand the loads applied to it. Articular cartilage must be able to withstand the applied forces or it will start to deteriorate which can lead to severe joint pain and eventually osteoarthritis. There are a number of mechanisms that have been proposed to be involved in the transduction of mechanical forces to biochemical signals in chondrocytes. As cartilage deforms, certain effects occur within the tissue. These effects include: interstitial fluid flow, the generation of electrical potentials, increases in osmotic pressure, and decreases in pH levels[59]. Changes in pH have been linked to changes in proteoglycan and collagen metabolism during compression. Osmotic pressure has been shown to change the mechanical properties of chondrocytes, further altering the deformation rate of the tissue under a given load. The flow of ions in and out of the cell is also affected by deformation via stretch activated ion channels. Integrins also play a crucial role during mechanotransduction as they act as the primary bridge between the cell and its ECM.

Salter el al. have shown that mechanical stimulation results in an influx of Ca^{2+} via stretch activated channels and a multitude of signal transduction events through integrin receptors [6,58,60–65]. In addition to this, the cytoskeleton and the nucleus of the chondrocyte play a role in transducing mechanical forces into different signals to alter gene expression and secreted constituents of the ECM [66,67]. All of this leads to the fact that, although the exact mechanisms of mechanotransduction are unclear, what is clear is that mechanical loading has a direct influence on tissue formation. This has led engineers and scientists to study mechanical forces and mechanical stimulation to induce cartilaginous tissue growth *in vitro*[10].

With the desire to make constructs that better represent what is found in native healthy cartilage, the environment in which cartilage grows *in vivo* was assessed and the application of mechanical forces to the cartilage constructs *in vitro* was determined to be a viable method to create a healthier stronger construct [2,4–9]. Applying mechanical stimulation to cartilage constructs *in vitro* allows for better tissue growth, uniformly organized tissue constituents and better mechanical properties, similar to that of native cartilage. Studies have shown that in general, low to moderate magnitude loads applied at frequencies on the order of 1 Hz substantially enhance the expression and synthesis of matrix proteins [6]. Although mechanical forces applied to cartilage have been extensively studied, the means by which the cells sense these mechanical signals and affect change remains poorly understood [6]. In the human body, articular cartilage is exposed to stresses between 3 and 10 MPa and because of this, the focus has been on applying forces in this physiological range [10,68].

There are currently many different methods for applying mechanical stimulation to cartilage constructs, but each of the methods can generally be broken down into one of two following categories: static and dynamic loading. Static loading refers to a constant force being applied to

a tissue engineered construct over a given period of time whereas dynamic stimulation refers to the application of a force that changes over time. Usually in a cyclical pattern, the force will be applied at a certain frequency over a given time period. Dynamic mechanical stimulation has been shown to have the greatest effect on tissue engineering constructs thus far, showing the highest growth rates as well as stronger tissue constructs with better mechanical properties.

Although each individual stimulation method has it's own individual merits, the three methods which showed the best promise for the current work presented were vibration, dynamic compression and combined vibration and dynamic compression. Further reviews by Brown, Grad et al. and our lab describe the different methodologies currently used for mechanically stimulating cartilage constructs *in vitro*[7,10,69].

2.3.1 Dynamic Compression

Dynamic compression applied to tissue engineered constructs has been the focus for studies in compressive mechanical stimulation for quite some time. The most common method for applying a dynamic compressive load is to directly apply the load to the construct through a loading surface and alter the load via a sinusoidal wave form. Compressive loading falls under three categories: i) confined compression; ii) unconfined compression and; iii) indentation

The different parameters of interest in terms of applying the stimulation, which have also been the focus of optimization studies, have been frequency (or duty cycle), duration, and strain or force amplitude used. Typically, frequencies ranging from 0.0001 to 3 Hz, strains from 0.1 to 35%, loads from 0.1 to 24 MPa and durations lasting hours to weeks have been examined at various cycles and waveforms, attempting to find an optimal configuration while staying in the realm of physiological conditions [2,70–72].

Confined Compression

Confined compression refers to the construct being radially confined during the application of force. Special chambers are created to inhibit the transverse strain of constructs during force application, while allowing for uniaxial compression to still occur. Because of confinement, hydrostatic pressure plays a larger role in the force being applied, depending on the frequency of the compressive force. Soltz and Ateshian were able to show that as the frequency of dynamic load approaches 0.00044 Hz, the magnitude and phase of fluid pressurization matched that of the applied stress [73]. Studies in confined compression seem to further elucidate the crucial role of interstitial fluid pressurization in the load bearing capabilities of cartilage [71,73–75]. In terms of tissue growth, Davisson et al. showed a dramatic increase in sulfated glycosaminoglycan and protein synthesis for confined dynamic compression at 0.1 Hz with a 50% static compression offset, showing results that agree with the literature in terms of dynamic compression increasing matrix synthesis [71]. In general, confined compression requires a more rigorous compression apparatus as it requires a chamber designed to inhibit transverse strain and higher precision in construct shape consistency[10].

Unconfined Compression

Unconfined compression, in contrast to confined compression, refers to the construct being free to expand radially. The majority of research in dynamic compression falls into this category. As a construct is compressed, it expands radially, introducing a transverse strain in the construct in addition to the axial strain caused by the compression. The introduction of a secondary strain has the ability to aid in further tissue growth. In addition, the method is easier to apply as there are no constraints applied to the constructs being stimulated. Hydrostatic pressure still increases

inside the constructs being stimulated, but not exponentially as it does with a confined compression stimulation mode[10].

Numerous short-term and long-term studies have applied unconfined compression protocols to cartilage constructs using hydrogels or microporous scaffolds, differentiated, undifferentiated, or de-differentiated cells to stimulate cell differentiation, proliferation, biosynthetic activity and functional ECM development [76]. In regards to short-term studies, Wong et al. showed that 45 hours of unconfined cyclical compression increased protein synthesis by 50% above control values [77]. Sah et al. has shown that depending on the frequency, the biosynthetic response of cartilage would either decrease or increase. Frequencies between 0.0001 and 0.001 Hz and compressions of up to 4% total strain showed little effect but at a frequency of 0.01 Hz, compressions of 1.1 - 4.5% total strain caused a 40% increase and a 30% increase in collagen type-II and proteoglycan synthesis respectively [78]. Through the use of 3D scaffolds and dynamic compression stimulus, Démarteau et al. showed that cartilage construct response does not depend directly on the stage of cell differentiation [10,79].

The development of functional ECM can be further appreciated when looking at long-term studies that apply unconfined compressive loading. Through a 2-month long study, Mauck et al. showed that constructs loaded with intermittent loading (10% deformation, at a 1 Hz frequency, 1 hour on 1 hour off, 3 hours per day 3 days per week) had a 2-fold increase in mechanical properties relative to the control, even though the proteoglycan content was similar between the control and stimulated groups. This suggests that the organization and assembly of the ECM was regulated by the dynamic loading, allowing for better mechanical properties [80]. Mauck et al. have also reported a 3-fold increase in the equilibrium aggregate modulus in argarose-seeded constructs which were dynamically stimulated, as compared to their free-swelling controls. It

was also noted that a significant difference in stiffness occurred in the last week of growth as compared to the first 3 weeks, further eluding to the role of the ECM on material properties and response to stimulation [10,81].

The mechanisms by which the dynamic compression affects the growth of cartilage can be categorized as: i) cell deformation; ii) transport-related fluid flow and cell-protein interaction; iii) physicochemical; and iv) cell-matrix interactions. Most notably, it has been shown that constructs cultured for longer periods of time respond to compressive stimulation to a greater degree than constructs with shorter culture periods. This indicates that the cell-matrix interactions play crucial roles in the biosynthetic response to compression stimulation. It has also been shown that proteoglycan accumulation in cartilage constructs only occurs if the proteoglycan content prior to compression is sufficiently high, once again indicating that the ECM plays a significant role in supporting the biosynthesis of the cartilage construct [72,79,82]. With regard to the application of the force, Suh et al. reported that when cartilage constructs undergo dynamic compressive loading, the ECM goes through repeated compression-expansion cycles, causing an oscillating positive-negative hydrostatic pressure together with interstitial fluid flow, which in turn leads to tissue biosynthesis [10,83].

Although dynamic loading has shown many benefits for both short-term and long-term culture periods, there are still drawbacks that arise. One of the drawbacks is the desensitization of the constructs to the stimulation itself, over longer periods of time. Weber & Waldman have shown that a minimum amount of stimulation was required to elicit an anabolic response, but desensitization would quickly be reached with an increase in loading cycles [12]. In addition to this, although the properties can be altered, not all mechanical properties are affected in the same manner. Kelly et al. demonstrated that, although the dynamic loading increased the bulk

properties, the overall profile of construct properties in the axial direction were qualitatively the same as free swelling controls over the course of 42 days. That being the case, the Poisson's ratio did increase, hinting at an improved collagen network [10,84].

Indentation

Indentation stimulation is a special type of compressive stimulation where the size of the stimulating platen is smaller than the surface of the construct [85]. Indentation stimulation, therefore, results in a complex loading profile throughout the construct where directly under the indenter experiences direct compression while the surrounding area remains uncompressed. Through special analysis techniques that allow for correlation to spatial mapping (e.g. autoradiography), this type of stimulation allows for the comparison of directly stimulated, indirectly stimulated and unstimulated conditions. Parkkinen et al. noted differences in the amount of proteoglycan synthesis due to direct vs. indirect stimulation with different trends observed in each area under several loading magnitudes. Depth-dependent variations were also observed due to the different loading magnitudes[85].

The complex loading state created by indentation stimulation is difficult to define for constructs with irregular geometry and non-uniform material properties, thus it is not a common mode of stimulation. More often, indentation (or double indentation) is used for mechanical property testing rather than stimulation [86,87].

2.3.2 Vibration

Vibration stimulation of cells *in vitro* is a novel approach that many studies are examining. The two main categories are: high-frequency ultrasonic and lower frequency mechanical vibrations.

High-frequency Ultrasonic Vibration

The application of ultrasonic vibration requires a secondary medium to pass on the vibration to the cells themselves. Different methods of achieving this have been studied, with the most common approach having transducers transmitting the ultrasonic waves through a coupling medium to culture plates. Different forms of ultrasound have been investigated in terms of low and high intensity. Thakurta et al. have shown that ultrasound increases proliferation of chondrocytes, maintains the chondrocyte phenotype in scaffolds over 21 days and selectively enhances the gene expression of the chondrogenic growth factor TGF-β3 over TGF-β1[88]. Parvizi et al. were able to show an increase in proteoglycan synthesis and aggrecan mRNA expression through the use of a low intensity-pulsed ultrasound stimulation [89]. Similarly Noriega et al. showed an increase in collagen type-II and mRNA expression in chondrocytes seeded in 3D scaffolds stimulated with a 5 MHz ultrasonic wave applied for 51 seconds, twice a day [90]. Further studies from the same group also showed an up regulation of both the ROCK-I and Rho-A when the same application of ultrasound was applied. Both ROCK-I and Rho-A are genes known to regulate cytoskeleton formation in chondrocytes, more specifically the formation of actin stress fibres [10,91].

Lower-frequency Mechanical Vibrations

A common mechanism used for inducing low-frequency mechanical vibrations in cells is to have a stage on which a cell culture plate rests and to apply the mechanical vibration with a modular piezoelectric device through the stage itself. Jankovitch et al. showed that a 0.3 gram amplitude, 30 Hz vibration increased chondrogenic differentiation *in vitro* as well as upregulated SOX9 expression and downregulated MMP-13 activity [92]. Kaupp et al. obtained similar results, showing an increase in cell proliferation at 1 gram and 350 Hz. They also showed that over a

longer culture period (1 week), there was in increase in ECM accumulation that led to a decrease in the effectiveness of the stimulation [93].

Although vibrational therapies show a promising effect, it is still unsure as to their use in long-term applications to chondrocyte biosynthesis and tissue growth.

2.3.3 Combined Dynamic Compression and Vibration (Stochastic Resonance)

As of recent, studies have begun exploring the use of stimulating cartilage constructs with a combined dynamic compression and vibration stimulation protocol. This combined mode of loading was termed stochastic resonance. Stochastic resonance has been observed in many biological systems, from molecular level DNA transcription systems in gene expression, to whole body level devices developed for regular use to maintain blood pressure, blood oxygenation, and balance [12]. Originally, stochastic resonance was investigated as a method to inhibit the desensitization of chondrocytes to dynamic mechanical compression *in vitro*. Weber & Waldman showed improved cellular sensitivity to mechanical loading and increased matrix synthesis between 20-60% over short-term culture. Stochastic resonance also limited the load-induced desensitization by maintaining sensitivity under desensitized loading conditions [11].

Further work to examine the long-term effects of stochastic resonance on younger and older cell populations has also been done showing increased development of collagen type-VI in the territorial region of the ECM, connecting the cells with a network like pattern similar to what is seen in growth plate development and endochondral ossification [94]. Furthermore, the combined loading reduced the cell clustering typically seen in older and degenerative cartilage[10,11,95].

3.0 Materials and Methodology

3.1 Short-Term Mechanical Stimulation of Passaged Cells

3.1.1 Chondrocyte Isolation and Passaging

Articular cartilage was harvested from the lower metacarpophalangeal joint of cows (age range: 16-24 months) obtained from Millgrove Packers Ltd. Cartilage slices were dissected from the upper and lower condyles found within the joint capsule. Primary chondrocytes were isolated from harvested cartilage slices by sequential enzymatic digestion: 0.5% w/v protease (Sigma Aldrich) for 1 hour followed by 0.15% w/v collagenase A (Sigma Aldrich) for 18 hours at 37°C[96]. The enzymes were prepared in 20 mL of Hams F12 media supplemented with 25 mM HEPES. The cell digest mixture was then filtered through a 200-mesh filter to remove any excess fragments/undigested tissue, washed with Ham's F12 media, and then centrifuged at 700g for 7 minutes. The supernatant was removed, and the remaining cell pellet was resuspended in Ham's F12 media and centrifuged again. This step was repeated 4 times. Viable cells were counted using a hemocytometer and trypan blue dye exclusion method (Sigma Aldrich)[96]. Viable cells were then resuspended in Ham's F12 media supplemented with 20% v/v Fetal Bovine Serum (FBS Sigma Aldrich) at a concentration of 20 x 10^6 cells/mL and seeded in T-175 flasks at a density of 20,000 cells/mL in complete media (Ham's F12 supplemented with 10% v/v FBS, 1% v/v antibiotics/antimycotics, 0.2% v/v ascorbic acid and 20mM HEPES). Seeded flasks were incubated at 37°C and 5% CO_2 for further passaging (up to passage 4) with the media exchanged 3 times a week.

Upon obtaining confluence, cells in the flasks were passaged. The media was first aspirated followed by incubation in 4.5 mL of trypsin (Sigma Aldrich) at 37°C for 5 minutes. Flasks were then agitated to loosen up any remaining attached cells, followed by trypsin inactivation by

adding 10 mL of complete media. The cell suspension was then centrifuged and counted, as described previously, prior to use or re-seeding flasks for subsequent passaging.

3.1.2 Preparation of Cell-Seeded Agarose Constructs

To encapsulate the cells, a 4% w/v solution of type VII low melting agarose (Sigma Aldrich) was prepared in Ham's F12 media. 500µL of the agarose solution was mixed slowly together with equal parts of the remaining cell suspension in a 15mL conical tube to create a final cell-agarose mixture of 10 x 10^6 cells/mL in 2% agarose. The cell-agarose solution was then cast into a custom Teflon™ cylindrical moulds (3mm diameter x 3mm height). After gellation at room temperature, cell-seeded constructs were leveled using a scalpel and then removed and placed in petri dishes with complete media (as described previously). The cell-seeded agarose constructs were then allowed to incubate for 24 hours at 37 °C and 5% CO_2 prior to mechanical stimulation[97].

3.1.3 Application of Mechanical Stimuli

Cell-seeded agarose constructs created from different passages (P0, P1, P2, P3 and P4) were then mechanically stimulated using a custom-made device. Briefly, the custom stimulation device consisted of a plate with cylindrical loading platens which fitted on the top of a standard 24-well culture plate allowing for 6 cell-seeded agarose constructs to be stimulated at a time[97] (Figure 3-1). The device was attached to a Mach-1 Micromechanical Testing System (Biomomentum, Laval) equipped with a 1 kg load cell to apply direct mechanical compression. For the application of the vibrational loading (with or without additional compressive loading), a vibration actuator (LFA-10, Equipment Solutions Inc., CA, USA) was positioned under the custom-designed culture plate [97,98].

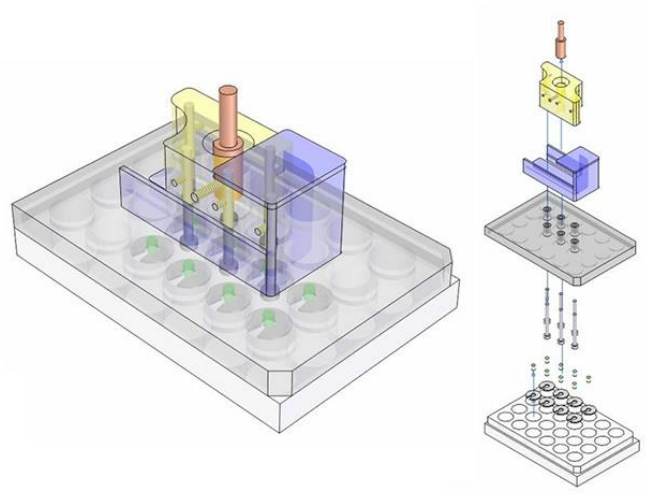

Figure 3-1: Schematic of custom-made mechanical stimulation plate for 24 well plate[97]

For this study four different stimulation methods were used: control (no loading), vibration only, dynamic compression only, and combined vibration-dynamic compression (termed: “stochastic resonance”). The constructs were placed into individual wells of a 24-well culture plate with 500µL of complete media containing 20% v/v FBS. For the vibration loading, a 1g vibration force was applied with a random waveform between 20 and 50 Hz. For the dynamic compression loading, a 5% total strain amplitude at 1 Hz was used. For the stochastic resonance loading, both the vibration and dynamic compression loading protocols were used simultaneously. All stimulations were conducted once, for 20 minutes in duration[11].

3.1.4. Quantification of Matrix Biosynthesis

After mechanical stimulation, matrix biosynthesis was measured using radioisotope incorporation. Immediately after mechanical stimulation, 5µCi of [^{3}H]-proline and [^{35}S]-sulfate,

respectively, was added to each well containing a construct. The plates were incubated at 37°C at 5% CO_2 for 24 hours. After incubation, the agarose-cartilage constructs were removed from the plates and washed three times with phosphate buffered saline (PBS) to remove any unincorporated isotope. After washing, the constructs were enzymatically digested in 1mL of 40 µg/ mL papain buffered with 20mM ammonium acetate, 1mM EDTA and 2mM DTT for 72 hours at 65°C. After digestion, 100 µL of sample was aliquoted for quantification of DNA using the fluorometric PicoGreen DNA assay[99]. Proteoglycan and collagen synthesis were then estimated through the quantification of the radioisotope incorporation using a LS6500 β-liquid scintillation counter (Beckman Coulter, Mississauga, ON, Canada) using 100µL of the digested sample. Proteoglycan and collagen synthesis was then normalized to DNA content. Each sample was then normalized to the no-load controls.

3.2 Long-Term Mechanical Stimulation of Passaged Cells

3.2.1 Chondrocyte Isolation and Passaging

Chondrocytes were isolated and passaged (up to P4) as described in Section 3.1.1.

3.2.2 Preparation of Cell-Seeded Agarose Constructs

Passaged chondrocytes (P0, P2 and P4) were seeded in agarose constructs as described in Section 3.1.2.

3.2.3 Application of Mechanical Stimuli

Mechanical stimulation was applied to the cell-seeded agarose constructs, as described in Section 3.1.3; however, stimulation was conducted over a 4 week period, with constructs stimulated every second day, with a 2 day break over weekends.

3.2.4 Mechanical Testing

After the 4 weeks of mechanical stimulation, cell-seeded agarose constructs were harvested for mechanical testing. Prior to mechanical testing, the diameter and height of the constructs were measured using digital calipers. Mechanical properties of the constructs were then determined using an unconfined compression stress relaxation protocol in conjunction with a Mach-1 Micromechanical Testing System (Biomomentum, Laval, Canada. Using a flat loading platen, constructs were first preloaded to 5mN preload to ensure contact with the platen. Sequential step strains of 2% were then applied to the construct, until 10% total strain was achieved, with the force recorded for each strain step until equilibrium was achieved (defined as <2mN/min change in force). The equilibrium stress (determined from the sample cross-sectional area and equilibrium force) was plotted against the equilibrium strain. The equilibrium modulus was determined from the slope of equilibrium stress-strain curve by linear regression[12,100–103] .

3.2.5 Determination of Accumulated Extracellular Matrix

After mechanical testing, samples were weighed (wet weight), lyophilized overnight, and then weighed again (dry weight). Samples were then digested in 1mL of 40 µg/ mL papain buffered with 20mM ammonium acetate, 1mM EDTA and 2mM DTT for 72 hours at 65°C. After digestion, samples were aliquoted for quantification of DNA, proteoglycan, and collagen content. DNA content was determined using the fluorometric PicoGreen DNA assay[99]. Proteoglycan content was determined using dimethyl methylene blue (DMMB) sulfated glycosaminoglycan binding assay (Sigma Aldrich)[104]. For collagen content, aliquots of digested sample (100 µL) were first hydrolyzed (6N HCl for 18 hours) followed by the determination of hydroxyproline content using the colorimetric chloramine-T/Ehrlich's reagent assay[105,106].

Collagen content was then determined assuming that 10% of the collagen mass was made up of hydroxyproline[106].

3.2.6 Histological and Immunohistochemical Evaluation

Representative samples, after mechanical stimulation, were assessed by histological and immunohistochemical evaluation. Harvested constructs were fixed in 4% paraformaldehyde for 24 hours, transferred to 70% EtOH, embedded in paraffin, and then sectioned (5µm sections). Sections were deparaffinised, rehydrated, and stained with either Safranin-O with fast green counterstain, or picrosirius red with haematoxylin counterstain for visualization of the proteoglycan and collagen distribution respectively. Histological slides were analyzed by light microscopy and images were acquired using a Nikon Eclipse TS100 system. The sections were also assessed for the localization of collagen type I, II and VI. Once again, after rehydration the sections underwent a heat-mediated antigen retrieval using a citrate-tween20 buffer at 95 °C for 20 min, followed by blocking with a 1% w/v BSA solution in PBS for 30 minutes at room temperature. After blocking the sections were incubated over night at 4 °C with either mouse monoclonal antibody against collagen I (ab90395 at 1:100 dilution; Abcam, Cambridge, MA), mouse monoclonal antibody against collagen II (II-II6B3 at 1:100 dilution; DSHB, Iowa City, IA) or rabbit polyclonal antibody against collagen VI (ab118955 at 2.5 lg mL21 , Abcam) for collagen type I, II and VI respectively. Sections were then washed with PBS and incubated with either Fluorescein Isothiocyanate labeled polyclonal sheep anti-rabbit IgG secondary antibody (ab97094 at a 1:200 dilution, Abcam) or Texas Red labeled polyclonal goat anti-mouse IgG secondary antibody (ab6787 at a 1:200 dilution; Abcam) for 2 hours at room temperature for collagen type VI and collagen type I and II respectively. Nuclear counterstaining was done using mounting media containing DAPI (Vector Laboratories, Burlington, ON). Sections were

examined immediately and images were taken using a ZOE florescent microscope (Bio-Rad ZOE Fluorescent cell imager). Non-specific staining was assessed by replacement of the primary antibody with non-immune 1% BSA[12,95].

3.3 Recovery of the Pericellular Matrix in Passaged Cells

3.3.1 Chondrocyte Isolation and Passaging

Chondrocytes were isolated and passaged (up to P4) as described in Section 3.1.1.

3.3.2 Preparation of Cells Seeded in Alginate Beads

Passaged chondrocytes (P0, P2 and P4) were seeded in alginate beads to determine the time required for the cells to recapitulate a pericellular matrix. First, a 3% w/v alginate solution was created by mixing alginic acid sodium salt from brown algae (Sigma Aldrich) with ddH_2O, buffered with 150mM NaCl, 20mM of HEPES and adjusted to a pH of 7.4. This solution was placed on a heating block, mixing and heating simultaneously until it was complete homogenous, and the alginate was completely dissolved. The alginate solution was cooled to room temperature and then mixed with the cell suspension (1:1 ratio) to create a final alginate-cell solution of 10 x 10^6 cells/mL in 1.5% alginate. A 110mM calcium chloride solution, buffered with 10mM HEPES, mixed with ddH_2O and adjusted to a pH of 7.4, was created to allow for the chelation of the alginate beads. A 200µL pipette was used to drop 30µL at a time of the cell-alginate mixture into the $CaCl_2$ solution which instantly chelated the alginate and formed alginate beads[107]. The beads remained in the $CaCl_2$ solution for 5 minutes to fully cure before they were removed and placed in 6-well culture plate containing complete media with 20% v/v FBS. The beads were incubated for 1, 2 and 4 days at 37°C and 5%CO_2, after which they were harvested for immunohistochemical evaluation.

3.3.3 Immunohistochemical Evaluation

Harvested cell-seeded alginate beads were fixed in 4% paraformaldehyde for 24 hours, transferred to 70% EtOH, embedded in paraffin, and then sectioned (5µm sections). The sections were stained for collagen type VI using the procedure described in Section 3.2.6.

3.4 Long-Term Mechanical Stimulation of Passaged Cells with a Recovered Pericellular Matrix

3.4.1 Chondrocyte Isolation and Passaging

As passage 4 (P4) chondrocytes were unable to recapitulate a pericellular matrix (Section 3.3), chondrocytes were isolated and passaged (up to P2) as described in Section 3.1.1.

3.4.2 Recovery of the Pericellular Matrix in Alginate Beads

After passaging to P2, the chondrocytes were isolated and encapsulated in alginate beads to allow for the recovery of the pericullular matrix, as previously described in Section 3.3.2.

3.4.3 Preparation of Cell-Seeded Agarose Constructs

After cells were recovered from the alginate beads, they were then seeded in agarose constructs as previously described in Section 3.1.2.

3.4.4 Application of Mechanical Stimuli

The chondrocyte-agarose constructs were then stimulated 3 times a week for 4 weeks. The methods of stimulation used were: unstimulated controls, compression and vibration, as previously described in Section 3.2.3.

3.4.5 Mechanical Testing

After 4 weeks of stimulation, the chondrocyte-agarose constructs were tested for mechanical properties, as previously described in Section 3.2.4.

3.4.6 Determination of Accumulated Extracellular Matrix

After mechanical testing, samples were weighed and analyzed for DNA, proteoglycan and collagen contents, as described in Section 3.2.5.

3.4.7 Histological and Immunohistochemical Evaluation

Representative samples, after mechanical stimulation, were assessed by histological and immunohistochemical evaluation, as described in Section 3.2.6.

3.5 Statistical Analyses

Data was collected and pooled together from repeated experiments, resulting in data sets of $n \geq 8$. All data was analyzed statistically using a one-way ANOVA and Dunnett's post hoc testing (version 17, SPSS Inc., Chicago, IL), for comparison to both the P0 controls and the controls of the respective passage number. Significance was associated with p-values less than 0.05 and trends were associated with p-values between 0.05 and 0.1[12].

4.0 Results

4.1 Effect of Short-Term Mechanical Stimulation on Passaged Chondrocytes Seeded in Agarose Constructs

For the short term mechanical stimulation study, the effects of mechanical stimulation on ECM synthesis was observed through the incorporation of the radioisotopes [^{3}H]-Proline and [^{35}S]-Sulphur for the measurement of collagen and proteoglycan respectively. Specifically, unstimulated controls were compared to vibration (1g randomized between 20 and 50 Hz), compression (5% total strain amplitude at 1 Hz) and stochastic resonance (combined vibration and compression).

When compared to their respective controls of each passage number, collagen synthesis varied with stimulation mode and passage number (Figure 4-1). Vibrational stimulation showed a 1.45-fold ($p = 0.033$) and 1.22-fold ($p = 0.013$) increase in synthesis as compared to unstimulated controls for the P1 and P2 cells, respectively. Stochastic resonance elicited a similar response, primarily in further passaged cells and showed a 1.56- fold ($p = 0.007$) and a 1.65-fold increase ($p = 0.003$) for the P3 and P4 cells, respectively. However, a trend was observed in P1 cells exposed to stochastic resonance ($p = 0.09$) which experienced a 1.46-fold increase in synthesis. Compressive stimulation alone, had no effect on collagen synthesis on any of the cells (P0 through P4).

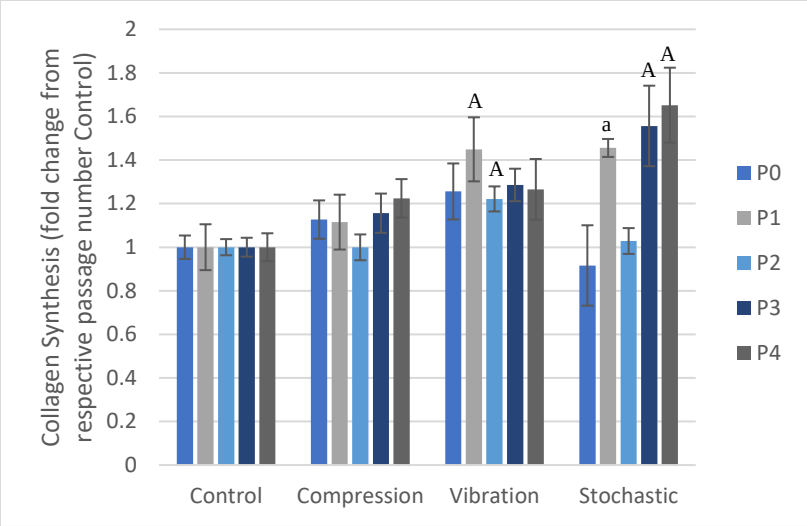

Figure 4-2: Effect of short term mechanical stimulation to collagen synthesis of passaged cells seeded in agarose constructs, normalized to DNA and to the respective passage number control. A – Statistically significant difference as compared to the respective passage number control ($p < 0.5$). a – Statistical trend as compared to the respective passage number control ($0.05 < p < 0.1$). Data represented as mean ± SEM; $n \geq 5$.

For proteoglycan synthesis (Figure 4-2), similar trends were observed. Vibrational stimulation elicited a 1.69-fold increase ($p = 0.011$) and a 1.32-fold increase ($p = 0.007$) in proteoglycan synthesis for the P1 and P2 cells, respectively. Alternatively, stochastic resonance had a greater effect in the P1, P3 and P4 cells, which experienced a 1.7-fold increase ($p = 0.029$), 1.79-fold increase ($p = 0.003$), and 1.84-fold increase ($p = 0.004$), respectively.

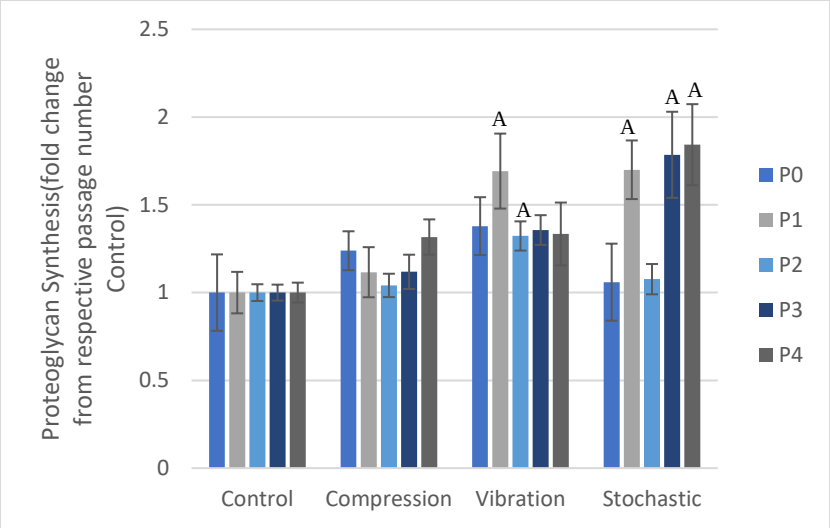

Figure 4-2: Effect of short term mechanical stimulation on proteoglycan synthesis of passaged cells seeded in agarose constructs, normalized to DNA and respective passage number control. A – Statistically significant difference as compared to respective passage number control ($p < 0.5$). Data represented as mean ± SEM; $n \geq 6$.

Matrix synthesis was also normalized to the primary (P0) cells to better understand the effects of the stimulation on the cells. Although there appeared to be a large (non-significant) increase in synthesis for the P2 cells for all stimulation modes, later passaged cells (P3 and P4) displayed decreases in synthesis (less than half; $p<0.001$) (Figures 4-3 and 4-4).

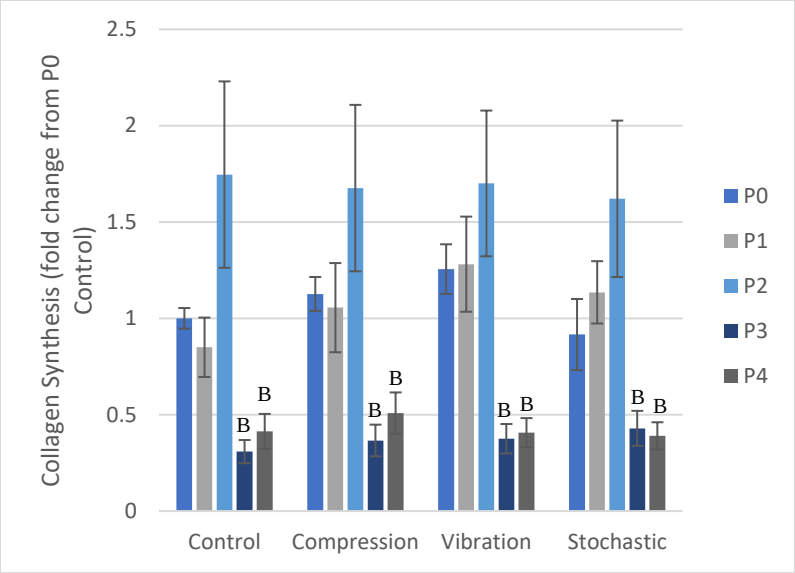

Figure 4-3: Effect of short term mechanical stimulation on collagen synthesis of passaged cells seeded in agarose constructs, normalized to DNA and P0 control respectively. Data represented as mean ± SEM; n ≥ 8. B – Statistically significant difference as compared to P0 control ($p < 0.05$).

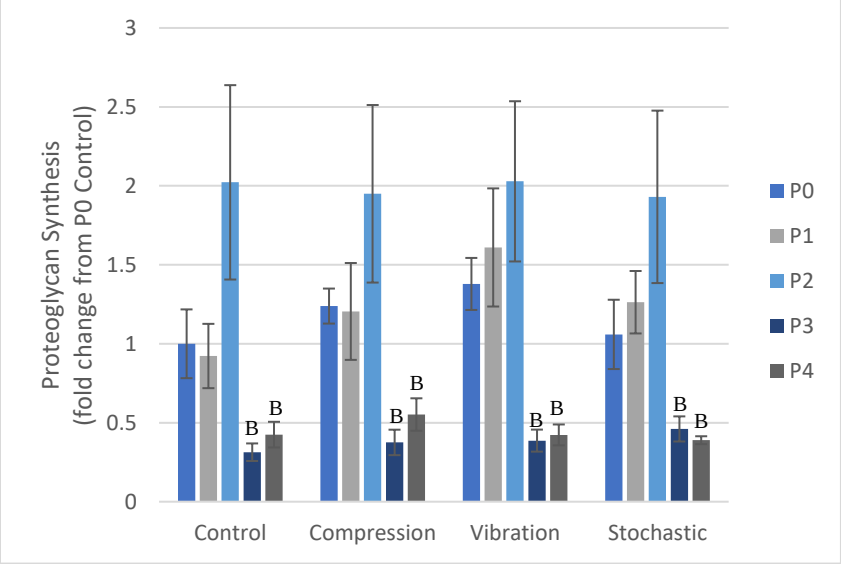

Figure 4-4: Effect of short term mechanical stimulation on proteoglycan synthesis of passaged cells seeded in agarose constructs, normalized to DNA and P0 control respectively. Data represented as mean ± SEM; n ≥ 7. B – Statistically significant difference as compared to P0 control ($p < 0.05$).

DNA content for each stimulation group and passage number was also compared to primary cells (Figure 4-5). Significant increases in DNA content was observed at all passage numbers which

also varied with stimulation mode. For the unstimulated controls, there was a significant increase in DNA content as the passage number increased (up to P3). The P3 cells showed the largest increase in DNA with a 4.3-fold increase as compared to P0 controls ($p < 0.001$). Interestingly, the stimulation had no observable effect on the DNA content of the P0 cells, regardless of the stimulation mode.

The stimulation had the greatest effect on DNA content in the further passaged cells (P3 and P4), showing a significant increase in DNA for all modes of stimulation. For the earlier passaged cells, the vibration mode caused a significant increase in DNA for both the P1 and P2 cells (P1 $p = 0.017$; P2 $p = 0.041$), where as the stochastic mode only had a significant effect on the P1 cells ($p = 0.014$). Both the unstimulated control and the compression mode of stimulus showed no significant increase in DNA for the P1 cells.

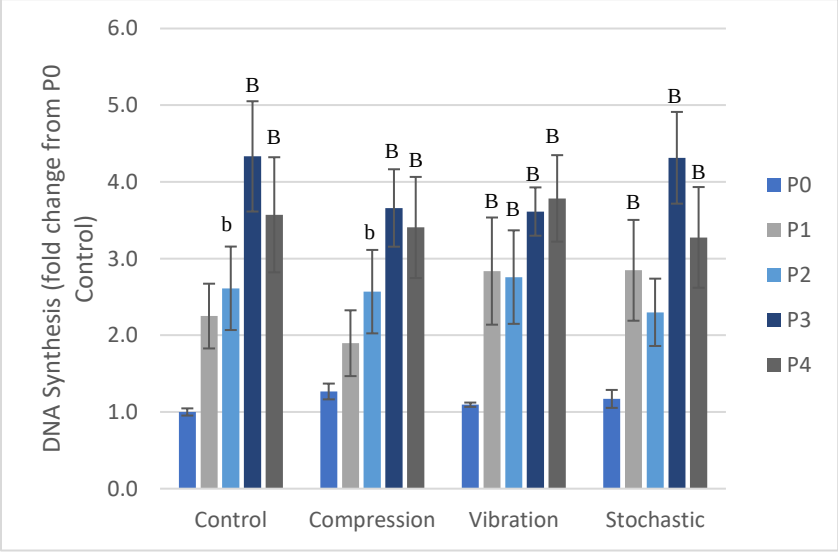

Figure 4-5: Effect of short-term mechanical stimulation on DNA content of passaged cells seeded in agarose constructs, as compared to Primary control. B – Statistically significant difference as compared to P0 control ($p < 0.05$). b – Statistical trend as compared to P0 control ($0.05 < p < 0.1$). Data represented as mean ± SEM; $n \geq 6$.

An observable difference was noted between the earlier passaged cells (P1 and P2) and the further passaged cells (P3 and P4) with respect to DNA and matrix synthesis as compared to the P0 controls. Each passage number within the groups responded in a similar manner to the different forms of stimulus. Based on this, it was assumed that the P1 cells would respond similar to the P2 cells, and the P3 cells would respond similarly to the P4 cells and this is what led to the exclusion of the P1 and P3 cells moving forward.

4.2 Effect of Long-Term Mechanical Stimulation on Passaged Chondrocytes Seeded in Agarose Contsructs

For the long-term stimulation study, the effects of mechanical stimulation on DNA, collagen and proteoglycan accumulation was observed. Primary (P0), P2 and P4 cells were split into four groups: unstimulated controls, compression, vibration and stochastic resonance, and were stimulated accordingly for 20 min a day, 3 days a week for 4 weeks. Constructs were analyzed for their biochemical (matrix accumulation and DNA content) and biomechanical (equilibrium modulus) properties. The constructs were also analyzed for their immunohistochemical and histological appearances.

Collagen accumulation, as compared to the respective passage number control, can be seen in Figure 4-6 below. No significant differences were observed between the P0 and P4 cells for all modes of stimulation, including the unstimulated controls. The stochastic mode of stimulus elicited the only observable effect in the P2 cells with a 1.55-fold increase in collagen accumulated as compared to controls ($p = 0.048$)

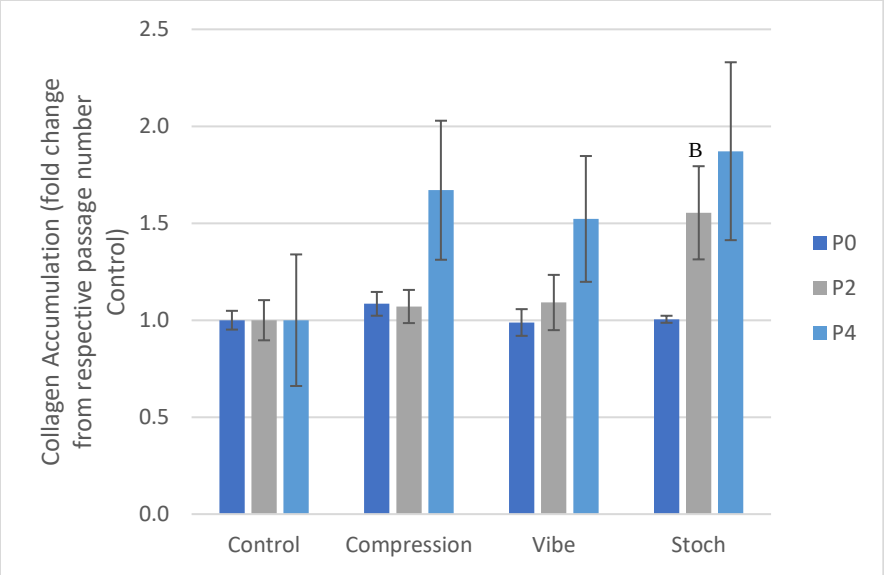

Figure 4-6: Effect of long-term mechanical stimulation to collagen accumulation of passaged cells seeded in agarose constructs, normalized to DNA and passage number control respectively. B – Statistically significant difference as compared to respective passage number control ($p < 0.05$). Data represented as mean ± SEM; $n \geq 6$.

The collagen accumulation was also compared to the P0 controls (Figure 4-7). For the unstimulated controls, P2 cells showed the only difference, with a large decrease of over 80% ($p = 0.086$) compared to the P0 controls. There was no observable difference in the collagen accumulated in P0 and P4 cells, compared to P0 controls for all modes of stimulation. Alternatively, the compression mode of stimulus showed a large decrease in collagen accumulation of the P2 cells ($p = 0.088$) compared to P0 controls.

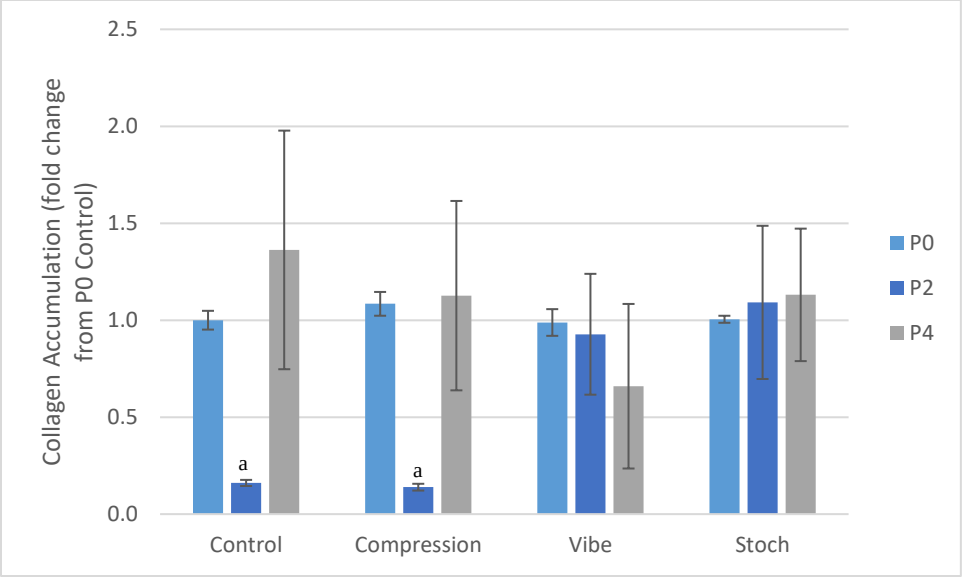

Figure 4-7: Effect of long-term mechanical stimulation on collagen accumulation of passaged cells seeded in agarose constructs, as compared to P0 control, normalized to DNA. Data represented as mean ± SEM; n≥6/group. a – Statistical trend as compared to P0 control ($0.05 < p < 0.1$).

Figure 4-8 below shows the effects of the mechanical stimulation on the accumulation of proteoglycan, as compared to the respective passage number control. There was no noted difference between the unstimulated controls. The P0 and P2 cells showed no significant difference in the proteoglycan accumulation, as compared to the respective controls, for all modes of stimulation. On the other hand, the vibration mode of stimulus caused a significant increase in accumulation for the P4 cells, with a 1.85-fold increase ($p = 0.03$) compared to controls.

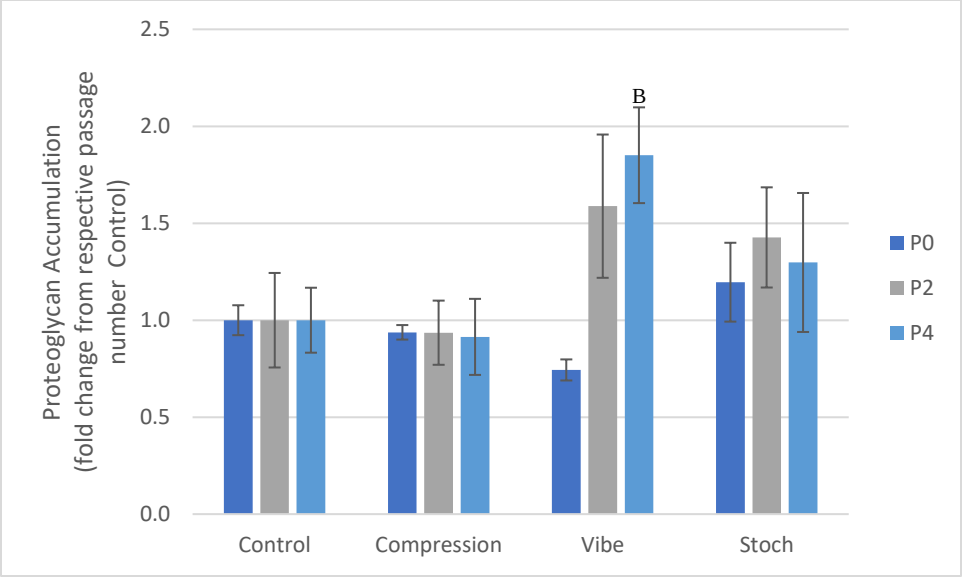

Figure 4-8: Effect of long-term mechanical stimulation on proteoglycan accumulation of passaged cells seeded in agarose constructs, normalized to DNA and passage number control respectively. B – Statistically significant difference as compared to respective passage number control ($p < 0.5$). Data represented as mean ± SEM; $n \geq 7$.

The accumulation of proteoglycan content was also normalized to the P0 controls, and can be seen in Figure 4-9 below. Both the P2 and P4 cells showed a significant difference in accumulation in the unstimulated controls, decreasing synthesis by 84% and 67% respectively ($p < 0.001$; $p = 0.013$) A significant decrease in proteoglycan accumulation was also observed for all forms of stimulation for the P2 and P4 cells, except for the P4 vibration. The largest decrease was seen in P4 stochastic resonance with a 90% decrease in synthesis ($p < 0.001$). There was no significant difference in accumulation for the P0 cells for all modes of stimulation.

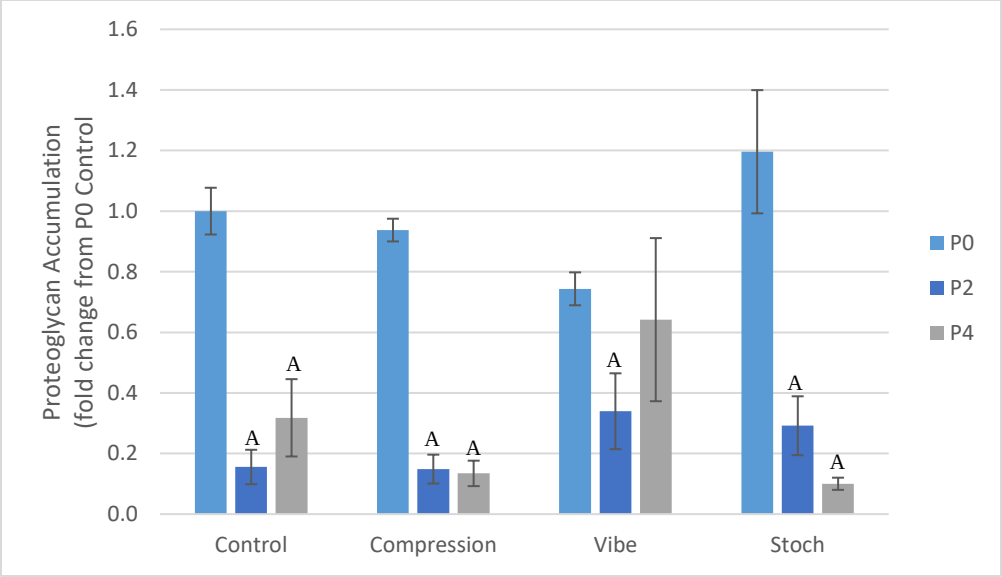

Figure 4-9: Effect of long-term mechanical stimulation on proteoglycan accumulation of passaged cells seeded in agarose constructs, as compared to P0 control, normalized to DNA. Data represented as mean ± SEM; n≥6/group. A – Statistically significant difference as compared to P0 control ($p < 0.05$).

The effects of the long-term stimulation on DNA accumulation was quantified and compared to P0 control, as can be seen in Figure 4-10 below. For the unstimulated controls, both the P2 and P4 cells showed a distinct decrease in DNA accumulated ($p < 0.001$). As for the stimulation groups, the only increase in accumulation was caused by the vibration mode of stimulus and only to the P0 cells ($p = 0.043$). All modes of stimulation caused a significant decrease in DNA accumulation for the P2 and P4 cells respectively ($p < 0.001$).

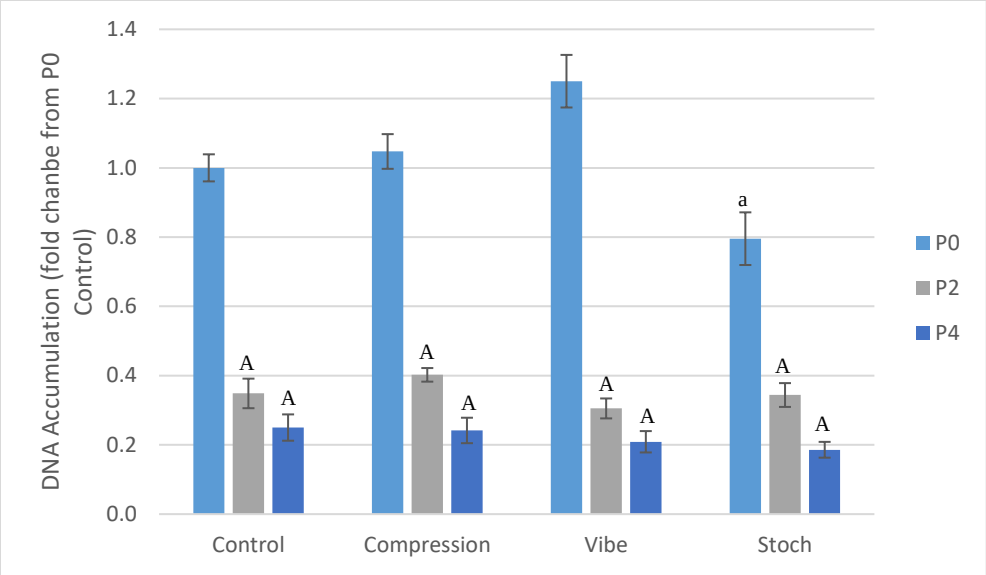

Figure 4-10: Effect of long-term mechanical stimulation on DNA accumulation of passaged cells seeded in agarose constructs, as compared to Primary control. Data represented as mean ± SEM; n=10/group. A – Statistically significant difference as compared to P0 control ($p < 0.05$). a – statistical trend as compared to P0 control ($0.05 < p < 0.01$).

The biomechanical properties (equilibrium modulus) of the cell-seeded constructs appeared to be affected by mechanical stimulation mode for the primary (P0) cells (Table 4-1). Compression and vibration stimulated constructs increased construct modulus by ~ 2-fold (*compression*: 1.94-fold, p=0.06; *vibration*: 2.09-fold, p=0.019) compared to the unstimulated controls. Alternatively, the equilibrium modulus was unaffected by stochastic resonance applied to the primary cell constructs. Similarly, the equilibrium modulus was also unaffected by any stimulation mode in the passaged cell constructs (P2 and P4).

Table 4-1: Equilibrium modulus for primary and passaged cells. Data presented as mean plus or minus the standard error mean (SEM); n=3/group.

	P0 (kPa)	**P2 (kPa)**	**P4 (kPa)**
Control	11.66 ± 3.14	18.58 ± 1.59	11.92 ± 1.89
Compression	**22.61 ± 1.73[a]**	17.67 ± 2.71	14.72 ± 1.80
Vibration	**24.27 ± 0.72[A]**	16.11 ± 1.07	17.87 ± 3.28
Stochastic	15.01 ± 2.30	14.64 ± 1.41	9.73 ± 0.74

A - Statistically significant difference as compared to respective control ($p < 0.05$). a - statistical trend as compared to respective control ($p < 0.1$).

Histological staining for collagen (picrosirius red; Figure 4-11) displayed similar results to that of the biochemical accumulation, with the P0 constructs seeded with primary cells accumulating the most collagen which, declined with increasing passage number (P2 and P4). The effect of stimulation mode was also apparent with vibrational and stochastic resonance stimulation modes showing the most staining within each passage number, and vibrational loading displaying the most collagen staining within the P2 cells.

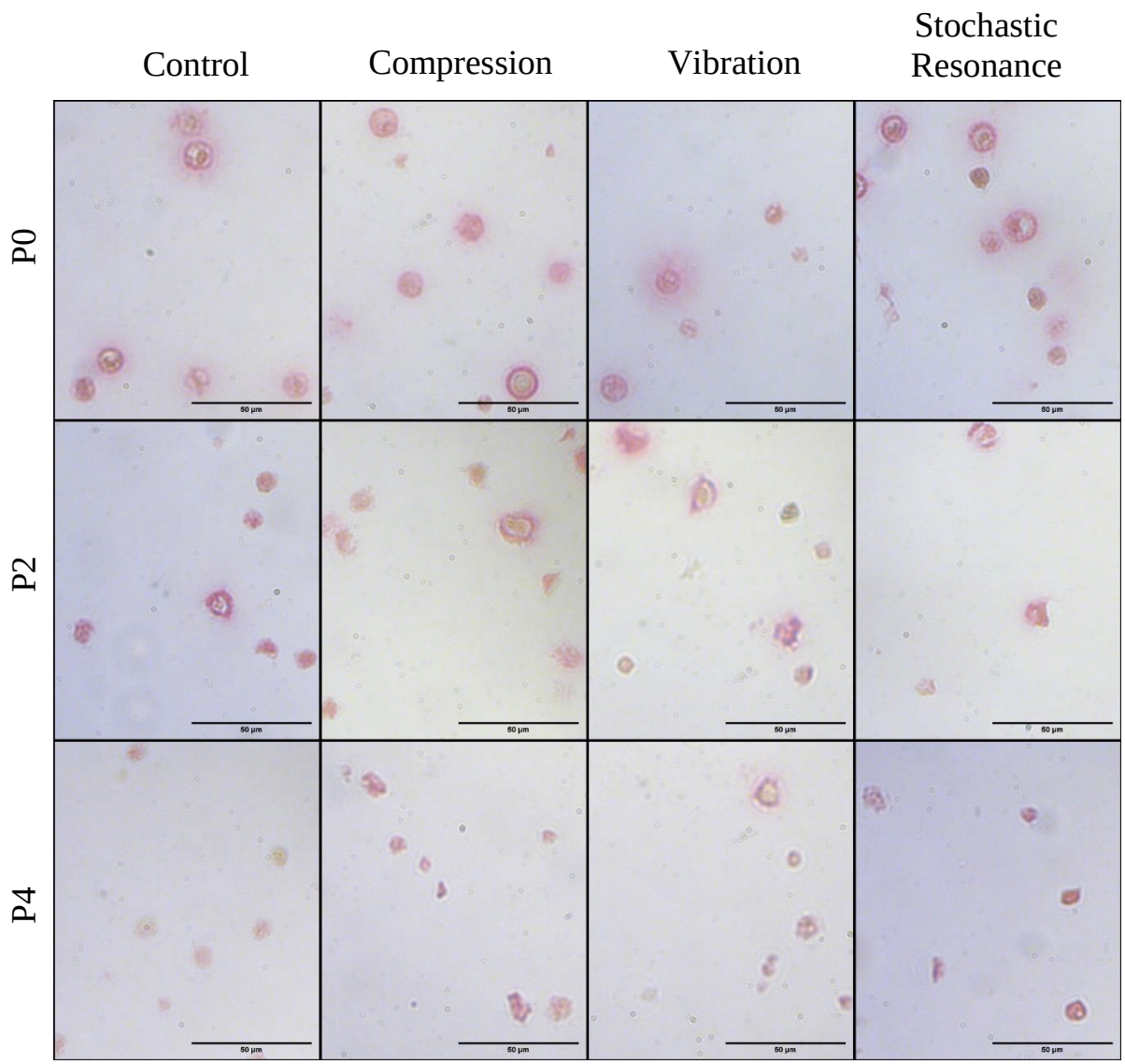

Figure 4-11: Histological staining of stimulated constructs with Picrosirius red staining for general Collagens. Scale bar: 50 µm.

In a similar fashion, the proteoglycan staining (safranin-O; Figure 4-12) also showed reduced staining as the passage numbers increased, with the staining becoming completely intercellular at P4. The vibration and compression stimulus modes seemed to show the most staining within the primary cells. This effect is seemingly lost in the P2 and P4 cells.

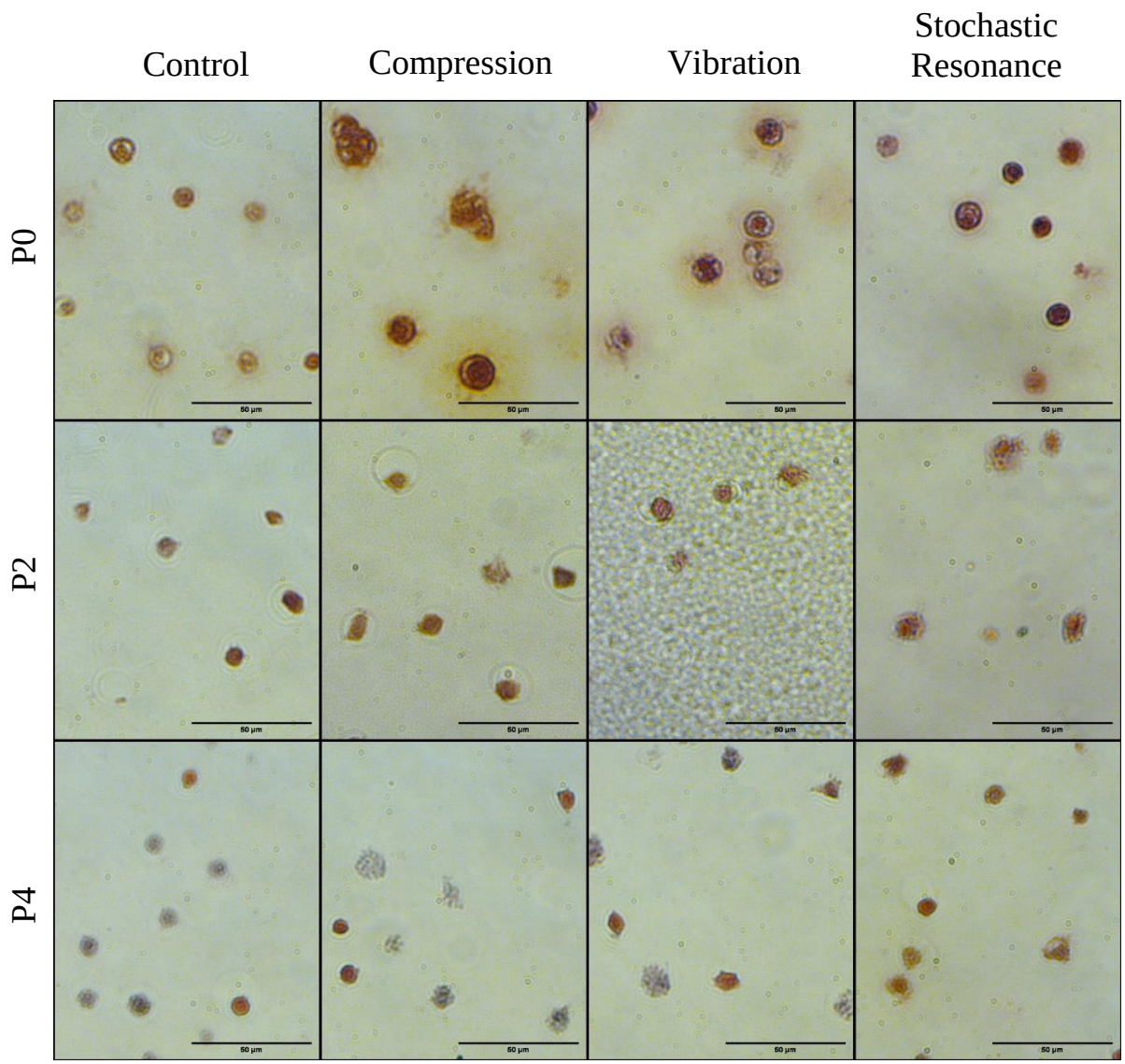

Figure 4-12: Histological staining of stimulated constructs with Safranin-O for proteoglycans. Scale bar: 50 µm.

Immunohistochemical (IHC) staining for collagen type I (Figure 4-13) appeared to be minimal throughout the primary cell constructs and increasing with the P2 and P4 cell constructs. The majority of the collagen type I staining appeared to be intercellular for the P0 constructs, and then shifted outwards to the pericellular region. With respect to stimulation mode, compression appeared to have the greatest effect on collagen type I staining for all cells (P0 through P4), whereas the other stimulation modes (vibration and stochastic resonance) did not have any observed effects on collagen type I deposition.

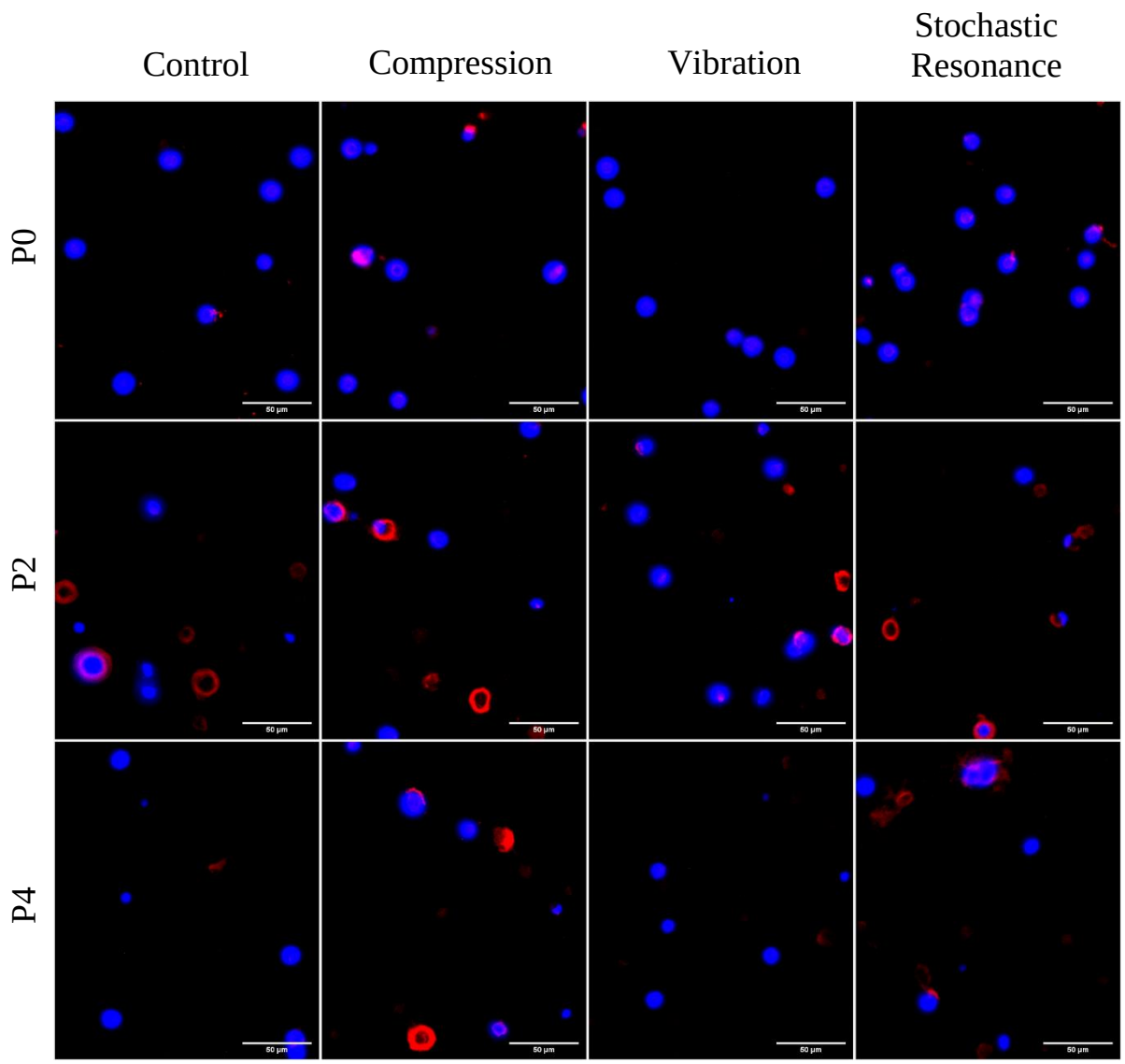

Figure 4-13: Collagen Type I immunoflorescent staining of stimulated constructs. Nuclei are counterstained with DAPI. Scale bar: 50 µm.

Collagen type II staining (Figure 4-14) was abundant in the P0 cells, and decreased rapidly with the P2 and P4 constructs, predominantly appearing in the territorial matrix between the cells. Regarding stimulation mode, the vibration and stochastic resonance modes showed the greatest effect on collagen type II staining for the P0 cells. There did not seem to be any effect on the staining of type II collagen for the passaged cells (P2 and P4).

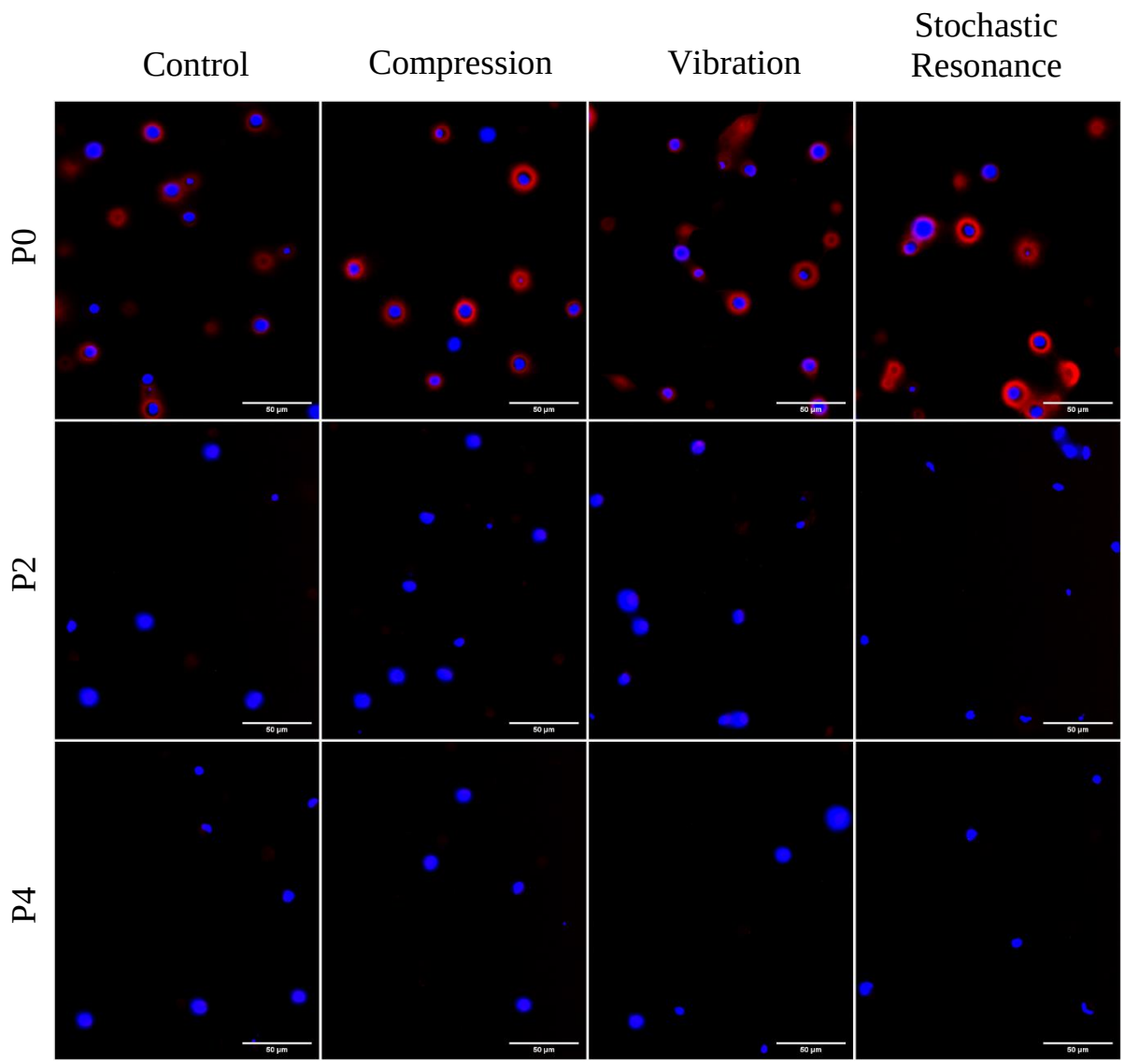

Figure 4-14: Collagen Type II immunoflorescent staining of stimulated constructs. Nuclei are counterstained with DAPI. Scale bar: 50 µm.

Collagen type VI was generally observed in the pericellular matrix (PCM) for all of the cell-seeded constricts (Figure 4-15). Upon passaging, the cells appeared to lose their ability to form a PCM, which was especially noted in the P4 cell constructs. Vibrational stimulation appeared to have the greatest effect on collagen type VI deposition in the primary cells with robust PCM staining and even showing web-like formations occurring in the territorial regions between the cells, as noted by the arrows. There did not appear to be an effect of stimulation mode on collagen type VI deposition in the passaged cell constructs (P2 and P4).

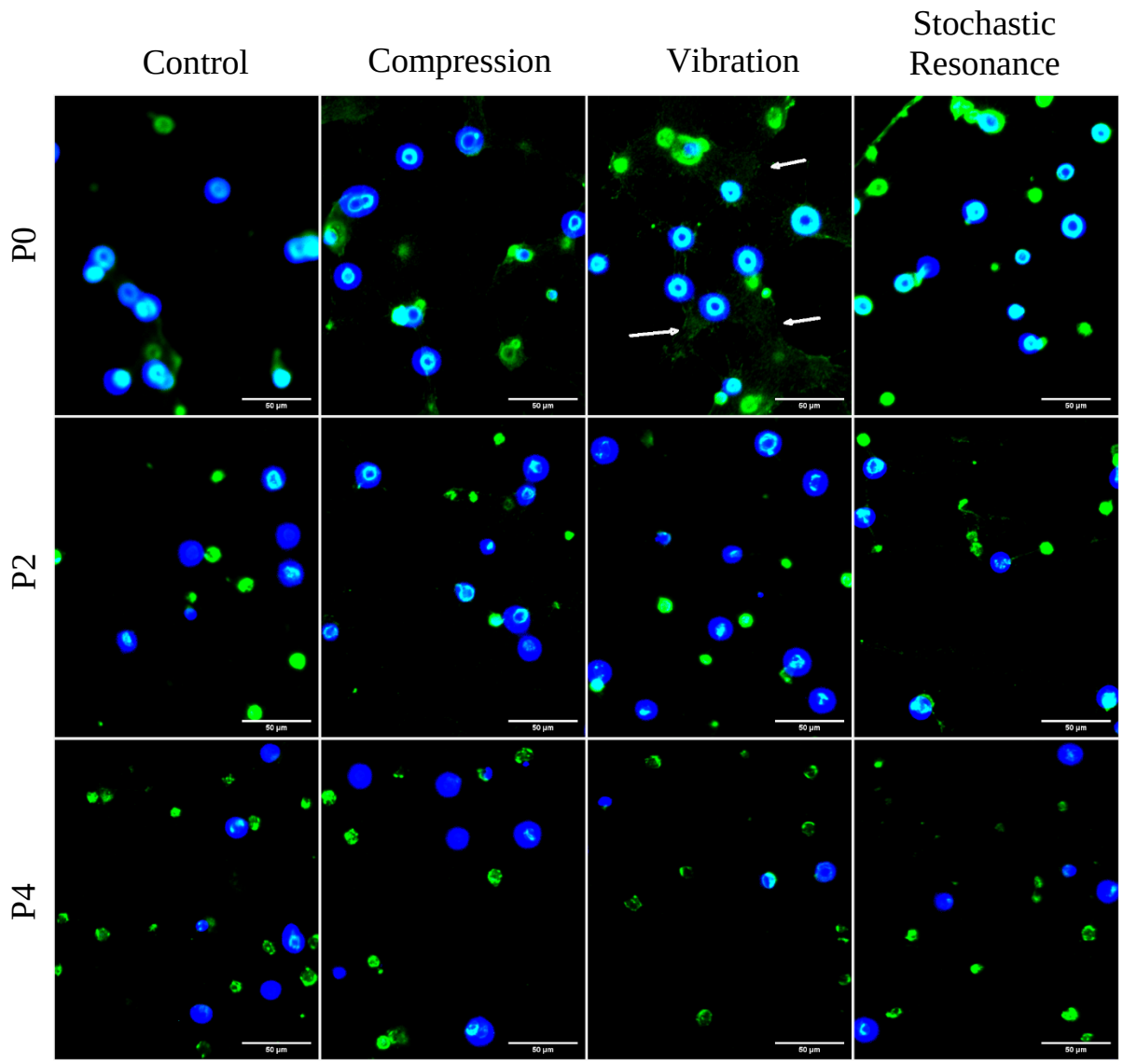

Figure 4-15: Collagen Type VI immunoflorescent staining of stimulated constructs. Nuclei are counterstained with DAPI. Scale bar: 50 µm.

Although stochastic resonance showed promise for the primary cells, for the passaged cells this method did not appear to have much effect with respect to matrix accumulation or histological appearance. For this reason, it was decided to exclude stochastic resonance as a mode of stimulation from any further long-term studies.

4.3 PCM Recovery

The PCM recovery experiment involved encapsulating passaged chondrocytes in alginate beads to allow for the PCM to fully develop. Encapsulated beads were fixed at 1, 2 and 4 days respectively and the PCM was evaluated using immunohistochemical staining of collagen type VI.

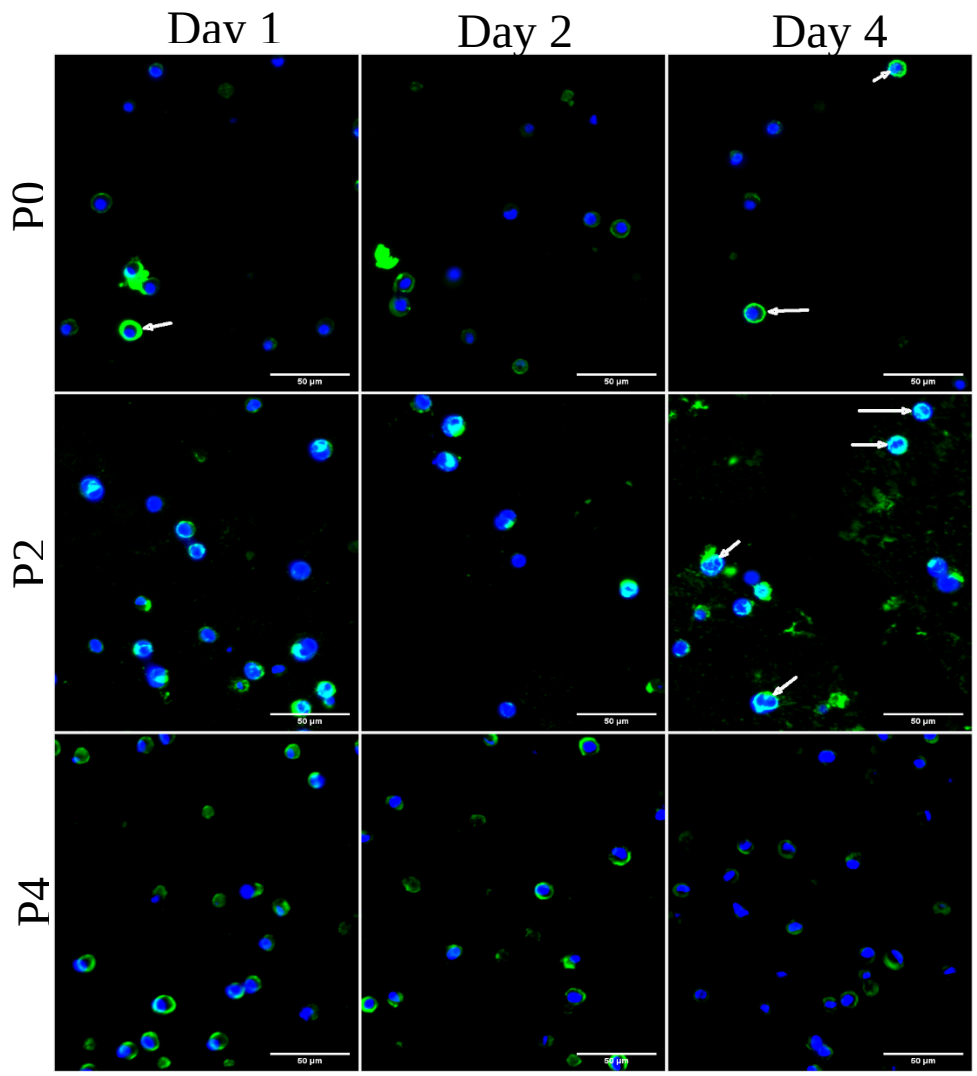

Figure 4-16: Collagen type VI immuno-florescent staining of alginate beads. Nuclei are counterstained with DAPI. PCM marked with arrows Scale bar: 50 µm.

The extent of collagen type VI staining (Figure 4-16) did not appear to change with incubation period for the P0 cells. For the P2 cells, the staining was similar between the day 1 and day 2 time periods but increased significantly by day 4, showing robust PCM staining as well as a small amount of collagen type VI staining in the territorial region. The P4 cells seem to decline in staining each day, showing almost no collagen type VI staining by day 4.

As it was apparent that a PCM was not able to be recovered in the P4 cells, it was decided to not include P4 cells in the following long-term study.

4.4 The Effect of Long-Term Mechanical Stimulation on Passaged Cells with a Recovered PCM

For this long-term stimulation study, the effects of mechanical stimulation on DNA, collagen and proteoglycan accumulation in cells with a recovered PCM were determined. Primary (P0), P2 non-bead and P2 bead cells were split into three groups: unstimulated controls, compression or vibration, and were stimulated accordingly for 20 min a day, 3 days a week for 4 weeks. Constructs were analyzed for their biochemical (matrix accumulation and DNA content) and biomechanical (equilibrium modulus) properties. The constructs were also analyzed for their immunohistochemical and histological appearances.

Collagen type VI was visible in constructs created immediately after alginate bead disassociation, showing a developed PCM in the cells (Figure 4-17).

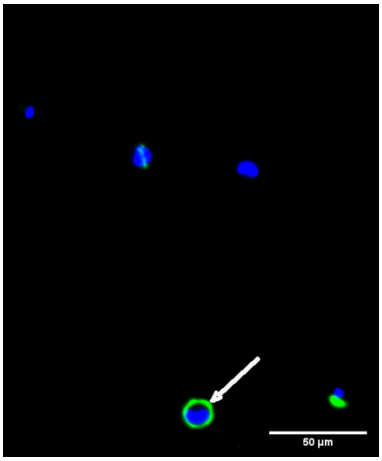

Figure 4-17: Collagen type VI immuno-florescent staining of constructs seeded containing passaged cells with recapitulated PCM, immediately after cells recovered from alginate beads. Nuclei are counterstained with DAPI. PCM marked with arrows Scale bar: 50 µm.

There was no significant difference between the unstimulated controls, with respect to collagen accumulation, when compared to their respective passage number controls (Figure 4-17). Mechanical stimulation also showed no significant effect on collagen accumulation for the P2 non-bead and P2 bead groups, respectively. Alternatively, mechanical stimulation elicited both positive and negative effects on the P0 cells, for vibration ($p = 0.007$) and compression ($p = 0.002$) modes, respectively.

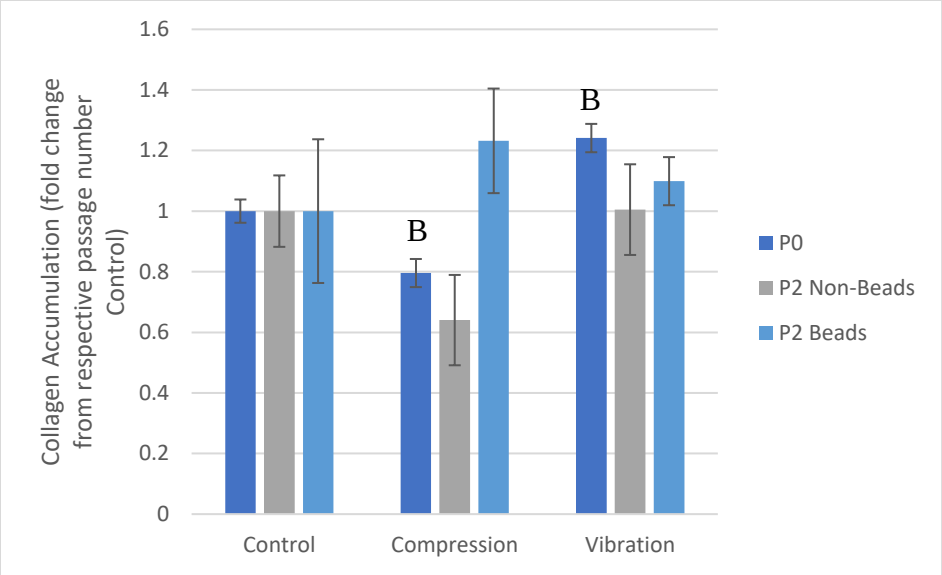

Figure 4-18: Effect of long-term mechanical stimulation on collagen accumulation of passaged cells seeded in agarose constructs with recovered PCM's, normalized to DNA and passage number control respectively. B – Statistically significant difference as compared to respective passage number control ($p < 0.05$). Data represented as mean ± SEM; $n \geq 6$.

When normalized to the P0 controls (Figure 4-19), there was also no significant difference in the collagen content accumulated for all groups (P0, P2 non-bead and P2 bead). With respect to mechanical stimulation, the compression mode of stimulus elicited a decrease in collagen accumulation for both the P0 and P2 non-bead cells ($p = 0.007$; $p = 0.05$). The P2 bead cells showed no effect on collagen accumulation for any mode of stimulation.

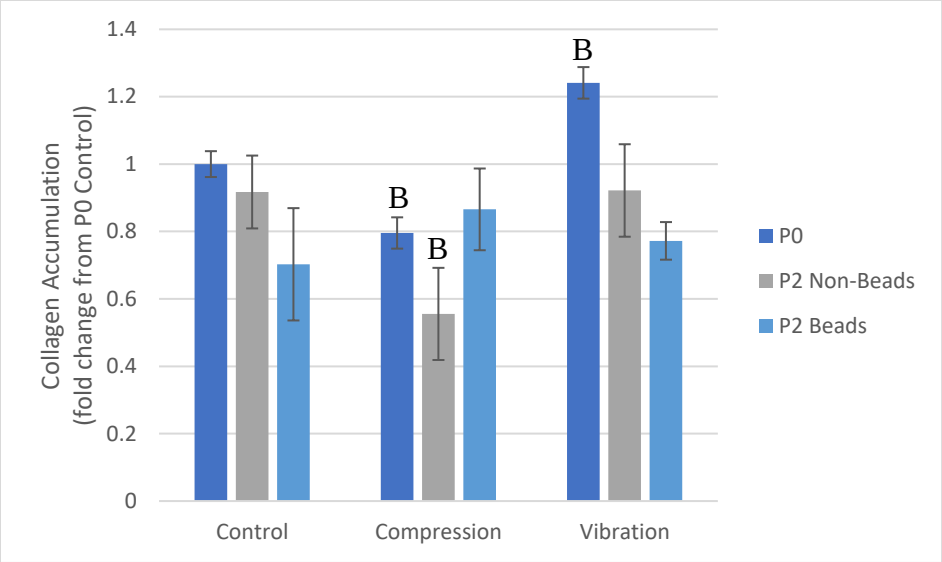

Figure 4-19: Effect of long-term mechanical stimulation on collagen accumulation of passaged cells seeded in agarose constructs with recovered PCM's, normalized to DNA and P0 control respectively. B – Statistically significant difference as compared to P0 control ($p < 0.05$). Data represented as mean ± SEM; $n \geq 6$.

With respect to proteoglycan accumulation as compared to the respective control groups (Figure 4-20), no significant changes were seen regardless of stimulation mode.

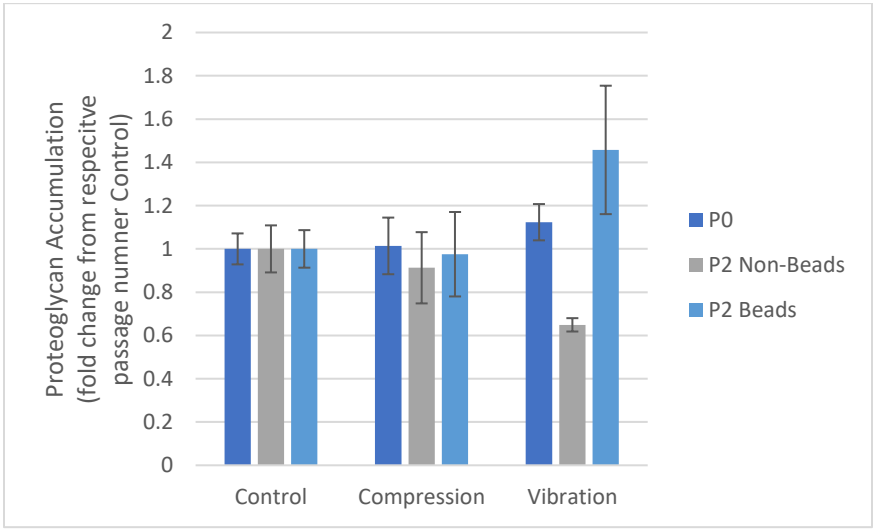

Figure 4-20: Effect of long-term mechanical stimulation on proteoglycan accumulation of passaged cells seeded in agarose constructs with recovered PCM's, normalized to DNA and passage number control respectively. B – Statistically significant difference as compared to respective passage number control ($p < 0.05$). Data represented as mean ± SEM; $n \geq 6$.

When compared to the P0 controls, the P2 bead unstimulated controls showed a significant 63% decrease in proteoglycan accumulation (Figure 4-21; $p < 0.001$). With respect to stimulation mode, once again only the P2 beads showed significant changes, with both compression and vibration eliciting a decreased response (*compression*: 64% decrease, $p < 0.001$; *vibration:* 46% decrease, $p < 0.001$), as compared to P0 controls.

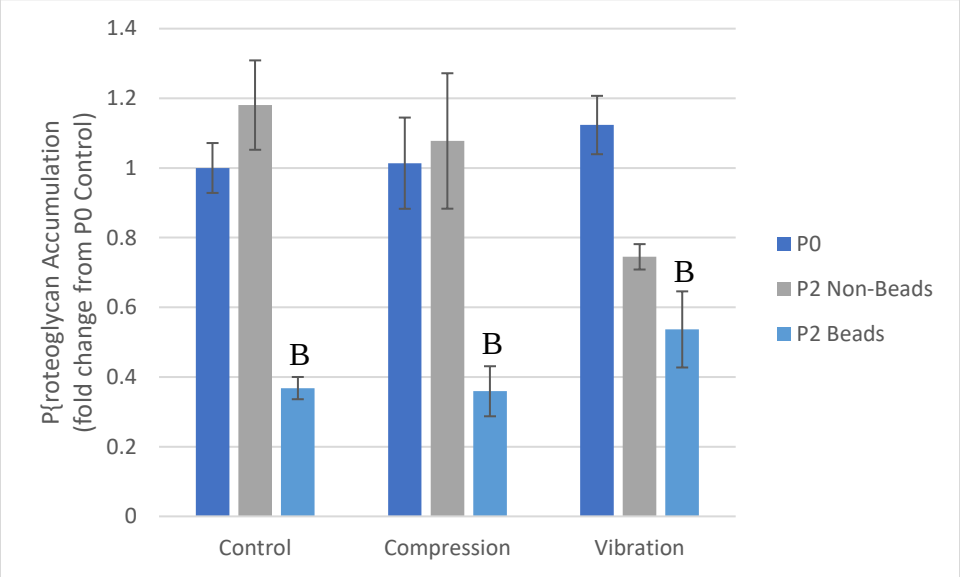

Figure 4-21: Effect of long-term mechanical stimulation on collagen accumulation of passaged cells seeded in agarose constructs with recovered PCM's, normalized to DNA and P0 control respectively. B – Statistically significant difference as compared to P0 control ($p < 0.05$). Data represented as mean ± SEM; $n \geq 6$.

DNA content (Figure 4-22), with respect to P0 control, showed a similar response as was seen in Section 4.3. The passaged cell groups showed a significant decrease of more than 60% in DNA accumulated for both the unstimulated controls and the different control groups respectively ($p < 0.001$). The P0 cells showed no significant difference.

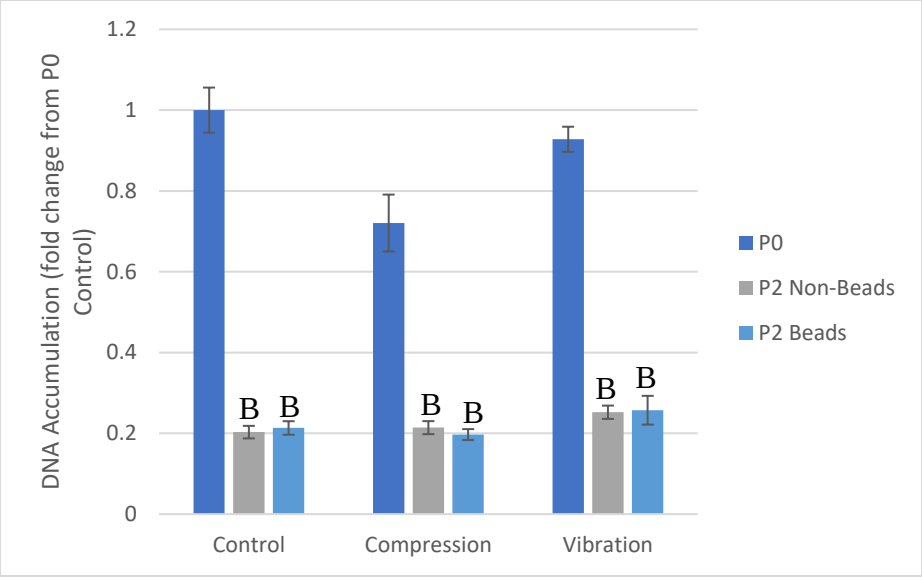

Figure 4-22: Effect of long-term mechanical stimulation on DNA accumulation of passaged cells seeded in agarose constructs with recovered PCM's, normalized to DNA and P0 control respectively. B – Statistically significant difference as compared to P0 control ($p < 0.05$). Data represented as mean ± SEM; $n \geq 6$.

The biomechanical properties (equilibrium modulus) of the cell-seeded constructs appeared to be affected by mechanical stimulation predominantly for the P2 Bead constructs as well as the P2 Non-Bead cells (Table 4-2). The unstimulated P2 Bead controls exhibited a decrease in modulus of roughly half (0.48-fold, $p = 0.011$). Similarly, compression showed a 0.58-fold decrease ($p = 0.005$) in modulus or the P2 Bead constructs as well as a 0.68-fold ($p = 0.089$) decrease in the P2 Non-Bead constructs, as compared to the P0 unstimulated controls. Vibration only elicited a response in the P2 Bead constructs, with a 0.68-fold decrease in modulus as compared to the P0 controls ($p = 0.06$). Alternatively, the equilibrium modulus was unaffected by any stimulation mode in the P0 constructs.

Table 4-2: Equilibrium modulus of chondrocytes with recapitulated PCM's, seeded in agarose constructs and stimulated over a 4 week period. Data represented as mean ± SEM; n≥3.

	P0 (kPa)	P2 Non-Bead (kPa)	P2 Bead (kPa)
Control	22.13±1.74	24.4±1.67	**12.85±2[A]**
Compression	28.03±2.43	**15.08±3.09[a]**	**10.4±2.55[A]**
Vibration	24.95±3.7	22.65±1.74	**15.1±2.2[a]**

A - Statistically significant difference as compared to respective control ($p < 0.05$). a - statistical trend as compared to respective control ($0.05 p < 0.1$).

The histological staining for proteoglycans (Safranin-O, Figure 4-22) displayed similar results, showing decreases in staining from the P0 cells and the P2 non-beads and P2 beads, respectively. There also did not appear to be any differences in staining between the different modes of stimulation for both the P2 non-beads and the P2 beads. However, for the P0 cells, the vibration stimulation showed the greatest amount of staining.

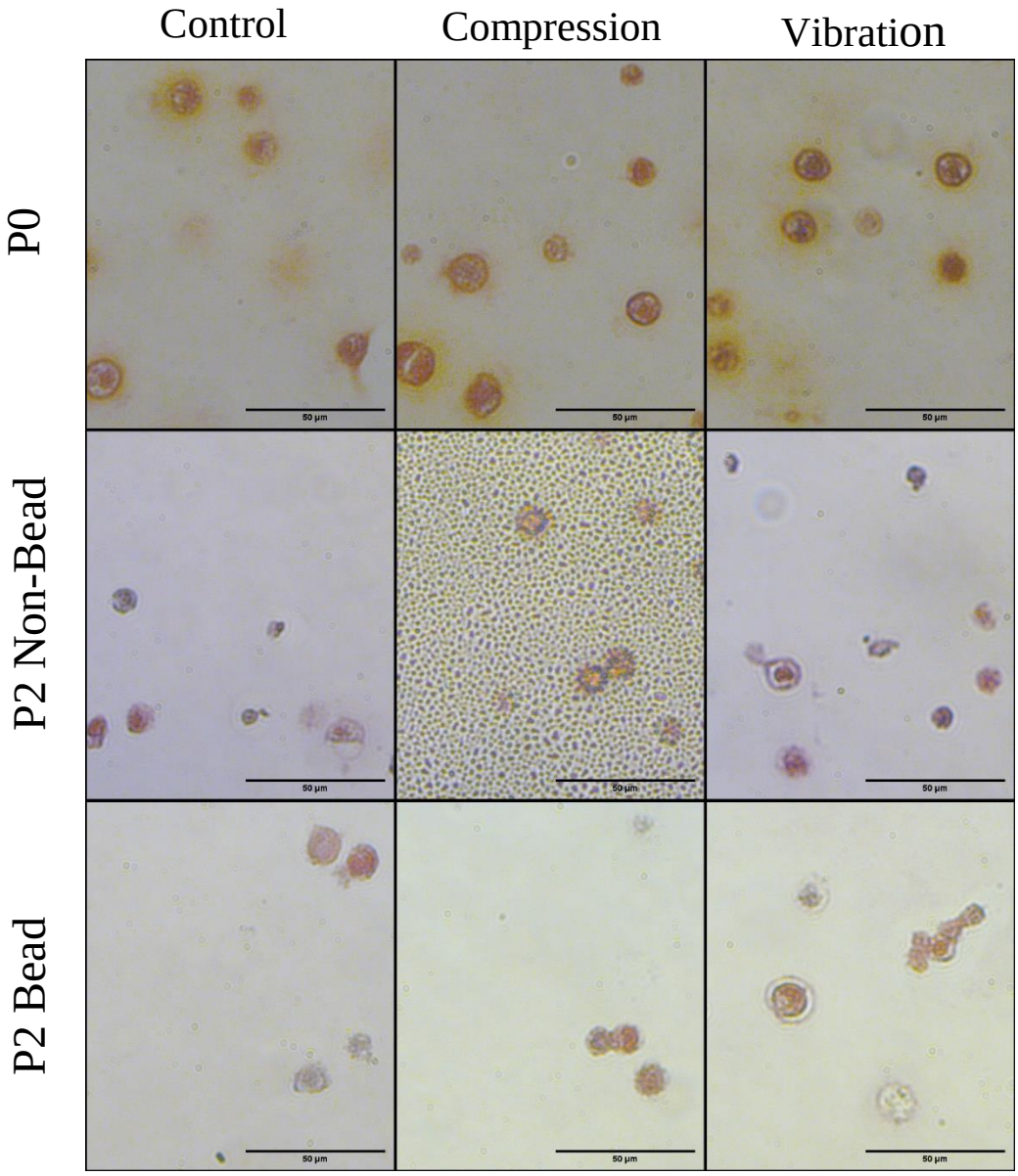

Figure 4-23: Histological staining of stimulated constructs, containing passaged cells with recapitulated PCM, with Safranin-O for proteoglycans. Scale bar: 50 µm.

The histological staining for collagens (picrosirius red, Figure 4-23) also displayed similar results, with the P0 cells showing the greatest amount of collagen staining, and both P2 groups showing decreased staining. Interestingly, the P2 non-beads showed better staining for both

vibration and control compared to the P2-beads. Compression appeared to elicit no response in regard to staining for the P2 non-beads and P2 beads, respectively. There also seemed to be no difference in the staining with respect to simulation mode for the P2 beads.

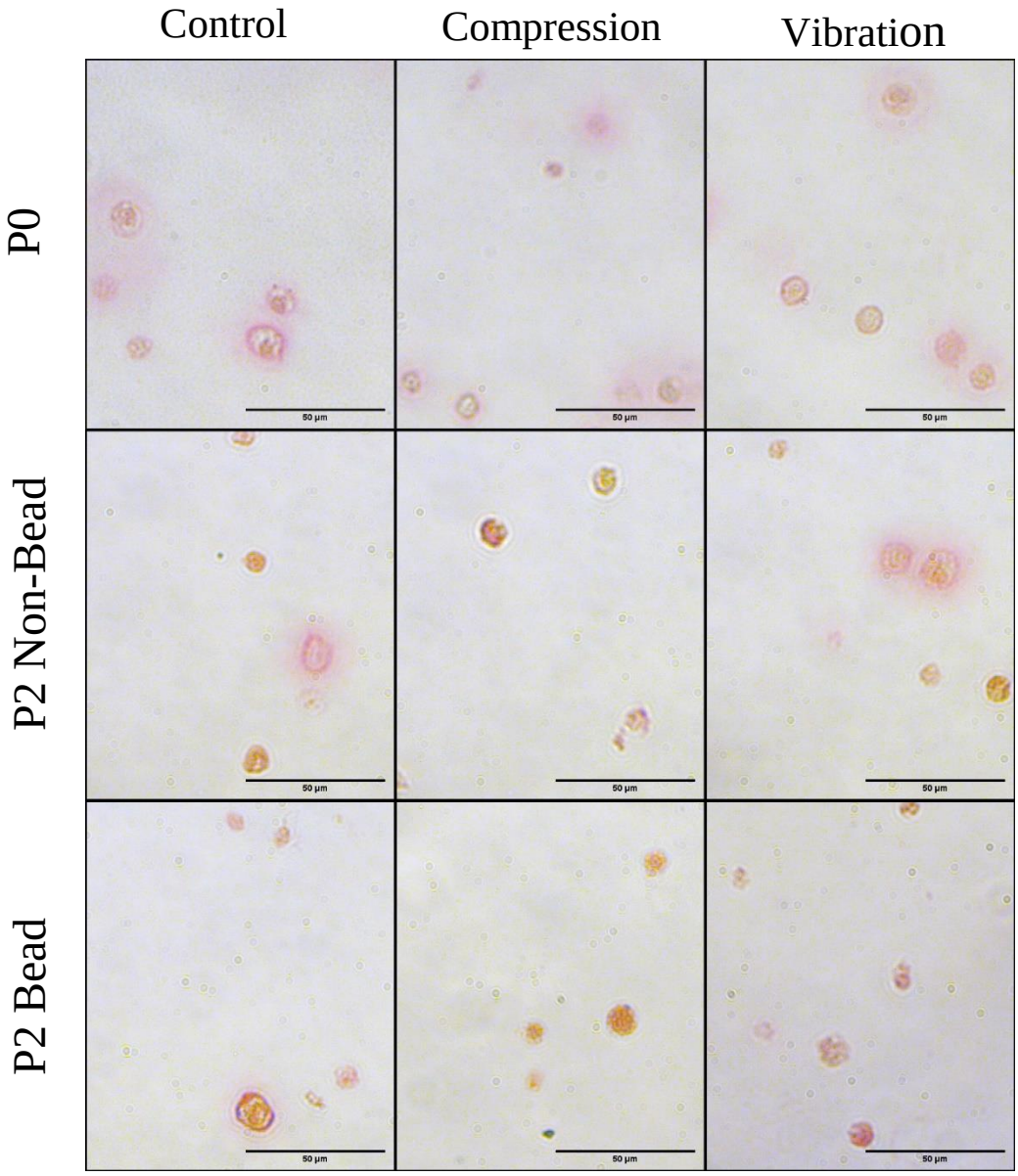

Figure 4-24: Histological staining of stimulated constructs, containing passaged cells with recapitulated PCM, with Picrosirius red staining for general Collagens. Scale bar: 50 µm.

There appeared to be no effect on collagen type I staining for the P0 cells regardless of stimulation group (Figure 4-24). On the other hand, the staining appeared to increase for the P2

non-beads and then even more so with the P2 beads. Compression seemed to cause the greatest effect on the staining of the P2 non-bead and P2 bead cells, respectively.

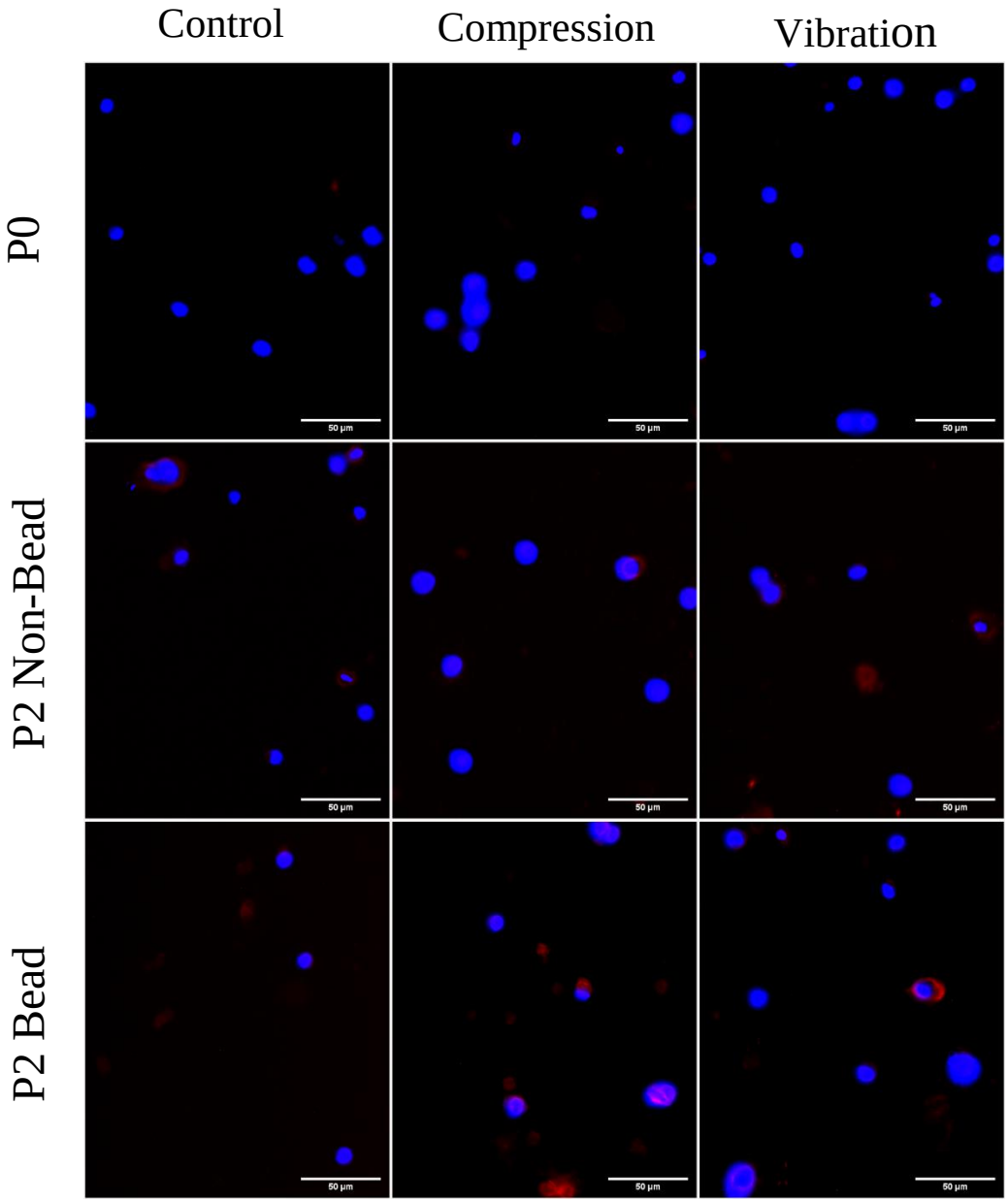

Figure 4-25: Collagen type I immunoflorescent staining of stimulated constructs containing passaged cells with recapitulated PCM. Nuclei are counterstained with DAPI. Scale bar: 50 µm.

In contrast to the collagen type I staining, staining for collagen type II (Figure 4-25) showed an abundant amount of staining in the P0 constructs, with less staining in both P2 groups. The P0

unstimulated control showed the greatest staining for the P0 group, followed by vibration and then compression. Both P2 non-bead and P2 bead groups showed significantly less staining for collagen type II, with no observable differences between the different stimulation groups.

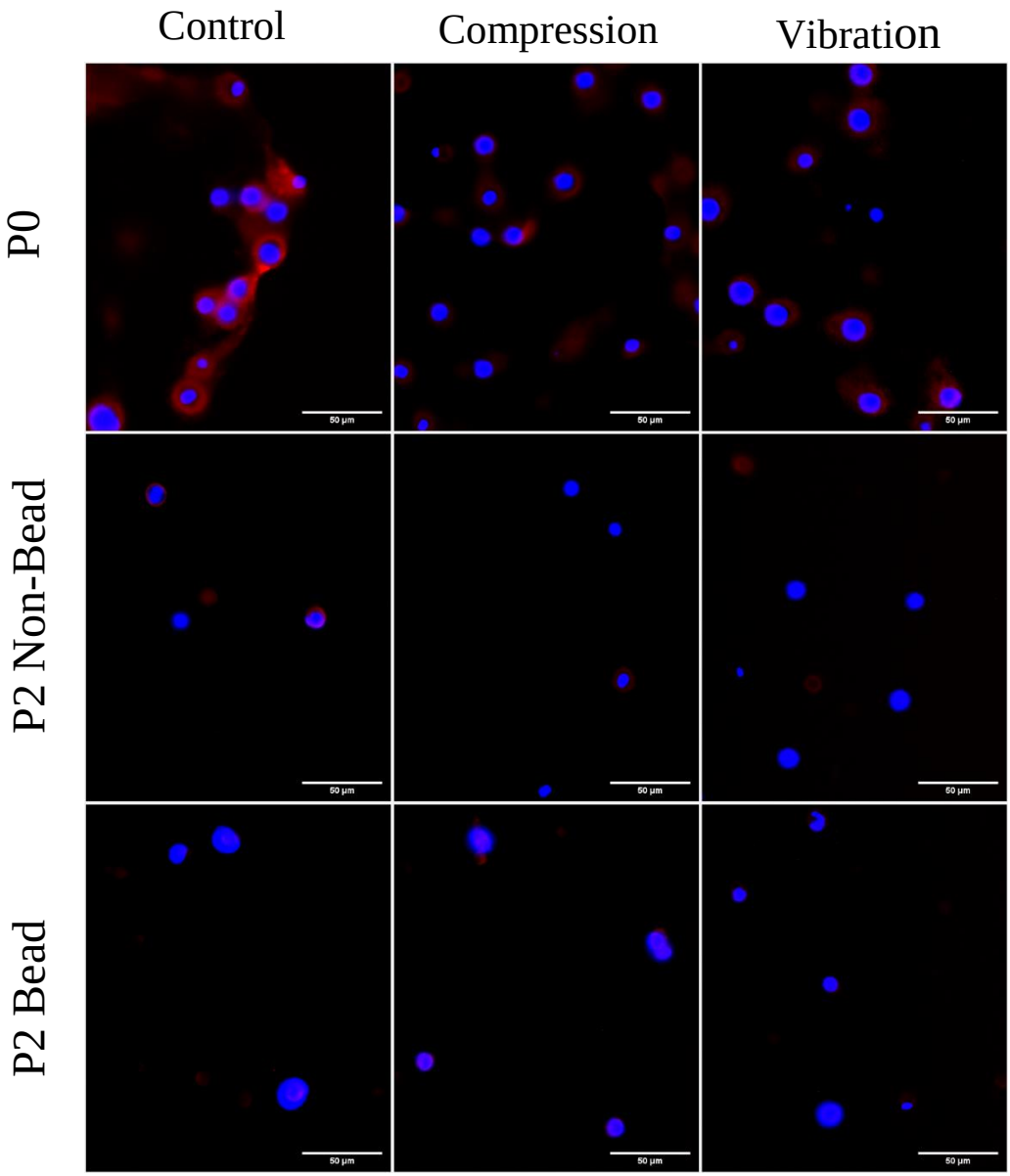

Figure 4-26: Collagen type II immunoflorescent staining of stimulated constructs containing passaged cells with recapitulated PCM. Nuclei are counterstained with DAPI. Scale bar: 50 µm.

A similar trend was observable in the collagen type VI staining (Figure 4-26). The P0 constructs exhibited a good dispersion of collagen type VI staining. The vibration mode of stimulus seemed to cause the greatest effect in the collagen type VI staining for the P0 constructs, showing not only pericellular staining but also staining extending into the territorial matrix, as noted by the arrows. For the P2 non-bead cells there was no observable difference on the collagen type VI staining between the different stimulation groups. Interestingly, the P2 bead group showed a noticeable increase in collagen type VI staining in response to the vibration mode of stimulus, as compared to control, showing a better developed PCM.

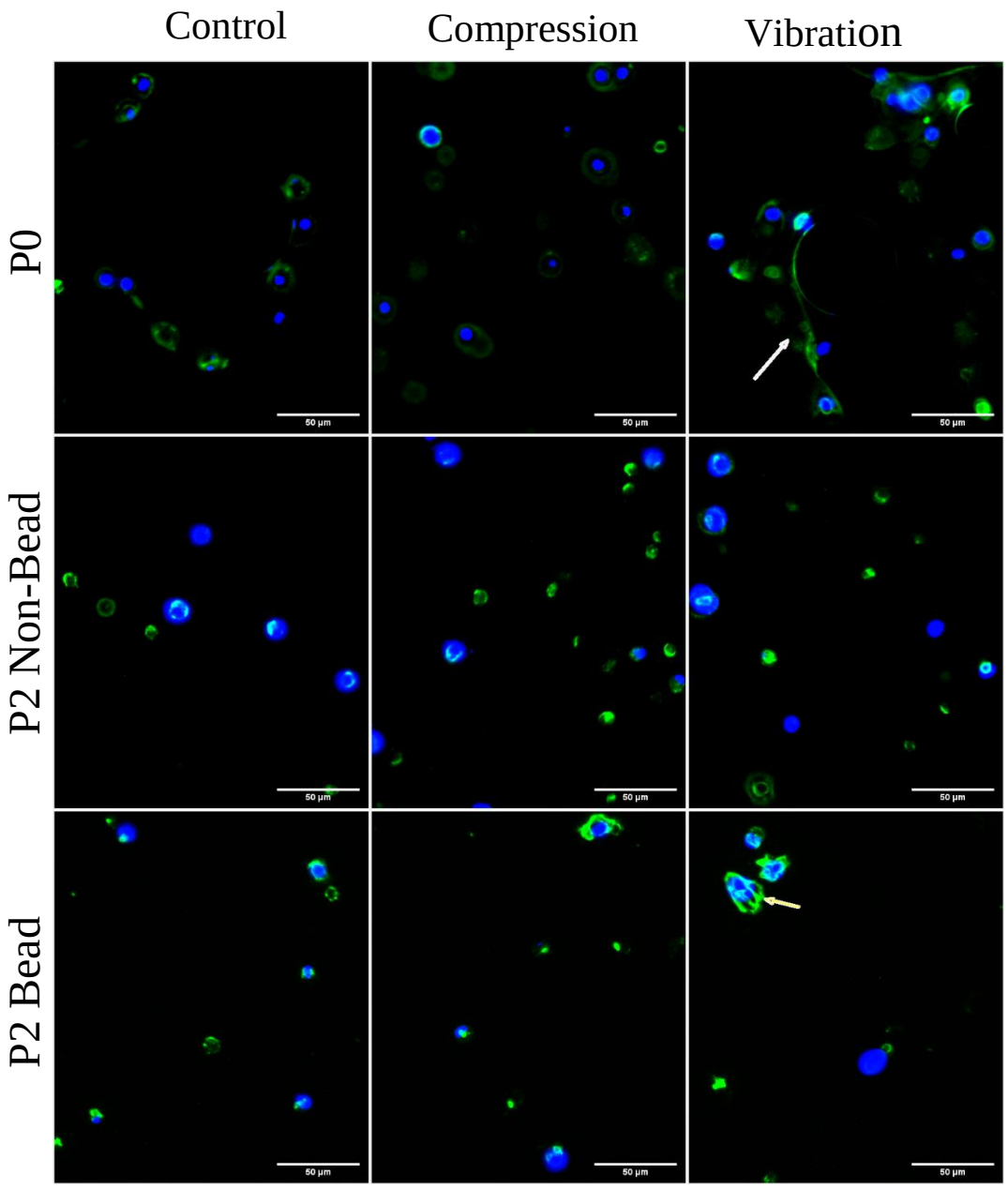

Figure 4-27: Collagen type VI immuno-florescent staining of constructs seeded containing passaged cells with recapitulated PCM. Nuclei are counterstained with DAPI. Areas of interest marked with arrows Scale bar: 50 µm.

5.0 General Discussion

5.1 Effect of Short-Term Mechanical Stimulation on Passaged Chondrocytes in Agarose Constructs

In the short-term mechanical stimulation study, isolated bovine chondrocytes were passaged from P0 (primary) to P4, seeded into agarose constructs, and subjected to mechanical stimuli. Four modes of mechanical stimulation were used: unstimulated (controls), dynamic compression, random vibration, and stochastic resonance (dynamic compression with superimposed random vibrations). Stimulated constructs were evaluated for changes in collagen and proteoglycan synthesis as well as DNA content.

With respect to passage number, the rate of DNA synthesis appeared to increase. It is well known that passaged chondrocytes, compared to primary chondrocytes, display increased proliferation. Specifically, in monolayer culture, as chondrocytes are passaged, they start to adopt a fibroblastic phenotype and proliferate at a faster rate than primary chondrocytes[108–111]. It is expected that these cells would continue to behave in a similar manner after seeding in agaose gels which would account for these results. Accompanying the increases in DNA synthesis with increasing passage number was a general decrease in proteoglycan and collagen synthesis, as compared to the P0 controls. This decrease in matrix synthesis may also be a result of phenotypic changes that occur during passaging. As mentioned, passaged chondrocytes tend to adopt a more fibroblastic phenotype leading to decreases in extracellular matrix gene expression [3,112–115].

Unexpectedly, all modes if mechanical stimulation had no significant effect on biosynthesis in primary cells. Many studies have shown beneficial effects on chondrocyte matrix synthesis in response to mechanical stimuli[72,93,95,98,116,117]. One explanation for the insignificant change could

be the fact that the primary cells were behaving more phenotypical and therefore were able to interpret and resist the physical effects of the stimulation. It has been shown that chondrocytes with a proper cartilage matrix can temper the effects of mechanical stimulation[112,113]. Another explanation could be that the mechanical stimulation modes were not optimized for enhancing and upregulating matrix synthesis in primary cells.

When comparing the effect of the mechanical stimulus on the passaged cells, both vibration and stochastic resonance had a positive effect on matrix synthesis. Interestingly, early passaged cells (P1 and P2) responded preferentially to vibrational loading, whereas later passaged cells (P3 and P4) preferentially responded to stochastic resonance. While mechanical vibrations have shown a variety of effects depending on duration and frequency, in general, vibrations elicit matrix biosynthesis in a short term setting [92,93,118,119]. The earlier passaged cells responding better to the vibration cold be attributed to the fact that the P1 and P2 cells still display a chondrogenic phenotype, which would allow them to respond positively to a vibration mode of stimulus.

In relation to the further passaged cells (P3 and P4), the vibration component of the stochastic resonance could be counteracting the negative aspects of the compression allowing the chondrocytes to receive the benefits of both modes combined. It has been shown that passaged chondrocytes under dynamic compression have an increase in the interstitial fluid flow that occurs, but also an increase in matrix proteins released into the media[120]. With further passaged cells, the cartilage matrix is sparser resulting in a greater effect of fluid flow and potential protein loss, but with the application of the vibration, the matrix proteins might be able to remain in the construct instead of being washed out. Previous work has explored the use of Stochastic resonance as a mode of stimulation for chondrocytes, showing an increase in matrix synthesis in the short term setting[11,95]. Interestingly, the further the cells are passaged, the greater the effect

stochastic resonance had on matrix synthesis, as compared to the other modes of stimulation with respect to the passage number control. This may be attributed to the fact that the combined vibration-compression mode of stimulus works better for chondrocytes that are further shifted away from the chondrocyte phenotype.

Although mechanical stimulation showed positive increases in matrix synthesis of passaged chondrocytes, this short-term study was limited to only determining the anabolic response. It is well known that mechanical stimuli can also elicit changes in chondrocyte catabolism[121,122] and thus the next series of experiments were focused on the long-term effect of mechanical stimulation of passaged chondrocytes.

5.2 Effect of Long-Term Mechanical Stimulation on Passaged Chondrocytes Seeded in Agarose Constructs

DNA accumulation decreased as passage number increased, as compared to control. This is opposite as to what was observed during the short-term stimulation study. This was an unexpected result as most studies note either an increase in DNA accumulation[80,123], or no observable change in DNA accumulation, when mechanical stimulation is applied during long-term culture[12,100,103,123,124]. This may be due to the notion that chondrocytes can switch from a proliferative state to one of matrix synthesis when the room for proliferation is limited[125] ; especially during high density culture such as this experiment. In addition, passaged cells not only start to shift in terms of phenotype but they also show a higher rate of apoptosis[126]. Thus, the lower DNA accumulation may have been a combination of the lower rate of proliferation rate along with higher rate of cell apoptosis, characteristic of passaged chondrocytes.

In regard to the mode of stimulation, random vibrations showed a significant increase in DNA accumulation for primary (P0) chondrocytes. This is similar to other studies that have shown that vibrational stimuli can upregulate DNA incorporation in high density cultures, although the exact mechanism(s) responsible are currently unknown[93].

As compared to primary (P0) chondrocytes, proteoglycan accumulation was drastically decreased for passaged cells, across all stimulation modes (except for P4 cells exposed to random vibrations). These results were similar to what was observed during the short-term stimulation study, except to a greater extent. As previously stated, passaged chondrocytes lose their ability to properly synthesize matrix constituents due to their altered phenotype. Mallein-Gerin et al. noted that dedifferentiating chondrocytes are associated with the loss of a chondrocyte phenotype as measured by increased collagen type I expression and decreased proteoglycan expression[3,127,128], which could account for these results. Guilak & Kisiday have also shown that mechanical stimulation can increase the expression of matrix degrading enzymes responsible for ECM maintenance and remodelling, such as MMP-3, MMP-13, ADAMTS4 and ADAMTS5 [117,129]. Thus, over a long-term culture, increases in matrix turnover could also account for the overall decrease in the proteoglycan accumulation in this study.

In contrast, when compared to their respective passage number, vibrational stimuli showed a significant increase in the proteoglycan accumulation of the P4 cells. Studies by Lui et al. have shown that mechanical vibrations applied over long term-culture can increase proteoglycan accumulation[130]. Mechanical vibrations have been shown to upregulate SOX9, the master transcriptional regulator of chondrogenesis[92,93]. Thus, it is possible that the vibrations elicited an increase in not only proteoglycan accumulation, but other matrix constituents which, in turn, would allow for the entrapment of the synthesized proteoglycans.

Collagen type VI expression was increased in primary chondrocytes (P0) subjected to mechanical vibrations. In these stimulated cells, a robust PCM as well as a web-like network of collagen type VI was observed in the territorial matrix. Collagen type VI plays an important role in linking the PCM to the ECM and acting as a transducer (or regulator) of mechanical loading[23,29,60,95,131–133]. In addition, the web-like network of collagen type 6 could potentially act as a link between cells, further allowing for signalling in response to mechanical stimuli[95]. These effects also led to changes in construct mechanical properties with vibrations eliciting a 2-fold increase in the equilibrium modulus.

Slight increases in collagen accumulation were also observed in the P4 cells when stimulated by random vibrations. This was similar to the proteoglycan accumulation results and may also be a result of increased SOX9 expression when exposed to vibrational stimuli[92,93]. However, in general, it appeared that stimulation had a varied effect on collagen accumulation in passaged chondrocytes. Interestingly, as the passage number increased, there appeared to be a decrease in accumulation of collagen. This was also accompanied by the lack of a pericellular matrix (PCM) with further passaged cells as noted by the decline in collagen type VI expression with increasing passage number. This could explain why further passaged chondrocytes were generally less responsive to mechanical stimuli. Without a proper PCM, the cells most likely were unable to transduce mechanical signals properly. For example, the primary cilium, a PCM organelle thought to be responsible for mechanotransduction[26,134] are progressively lost with passaging as noted by decrease in primary cilia length and prevelance[135,136]. Thus, ascertaining the importance of the PCM led to the notion that in order to properly recover passaged cells, they first need to be able to recapitulate a functional PCM.

5.3 Effect of Long-Term Mechanical Stimulation on Passaged Chondrocytes with a Recapitulated PCM Seeded in Agarose Constructs

Pre-culturing passaged chondrocytes in alginate beads led to a recapitulation of a PCM which appeared to remain after encapsulation in agarose. However, these cells (those with a recapitulated PCM), displayed no significant increases in ECM accumulation for any of the modes of stimulation investigated as compared to primary cells. That being said, it did appear that the P2 cells pre-cultured in alginate beads showed a positive response to mechanical stimuli when compared to their respective passaged cell controls. This response could be potentially explained by the presence of a PCM. The PCM stabilizes and shields chondrocytes from excessive forces, thereby allowing for a increase in biosynthetic response[20,29,31,137–139]. It should also be noted that the alginate recovery method used, although successful at recapitulating a PCM was not optimized as not all of the cells had a recovered a PCM. In order to further explore this area, the PCM recovery method should be refined to ensure all chondrocytes have a recovered PCM. In addition, further analysis of the recovered PCM should be conducted (for example, exploring the primary cilium) to determine its functionality compared to the PCM of primary cells.

In terms of collagen types, there were no observable differences between the passaged cells with or without a recovered PCM, both showing an increase in collagen type I and a decrease in collagen type II with passaging. This suggests that although the PCM was recovered, the cells were still unable to fully recover a chondrogenic phenotype.

Similar to the previous studies, vibrational stimuli had a great effect on the collagen type VI staining in primary cells. The stimulated cells also developed web-like structures in the territorial matrix between the cells. Interestingly, passaged cells with recovered PCM's also showed an

increase in collagen type 6 staining when exposed to mechanical vibrations, compared to both the unloaded controls and those subjected to compressive stimulation. This increase could be the passaged chondrocytes trying to respond and lay down collagen type VI in a similar manner to the primary cells, but to a lesser degree. This effect was not observed in the passaged cells without the recovered PCM, once again highlighting the role that the PCM plays in mechanotransduction.

6.0 Conclusions and Future Work

6.1 Conclusions

In conclusion, the effect of mechanical stimuli was explored on passaged chondrocytes. The major objective of the work presented was to explore the application of mechanical stimulation on passaged chondrocytes to determine its efficacy in recovering passaged chondrocytes to behave more like primary cells. It has been shown that as cells are passaged, they start to lose their chondrogenic phenotype and start to adopt a fibroblast-like phenotype[3,112,140]. When attempting to heal cartilage defects with tissue engineered constructs, a large population of chondrocytes is typically required. This would require the passaging of primary cells multiple times. In the work presented, 4 separate studies were conducted in order to determine the effects of different forms of mechanical stimuli on passaged chondrocytes.

6.1.1 Mechanical Stimulation on Passaged Chondrocytes

When mechanical stimulation was applied to passaged chondrocytes, in a short-term setting, an increase in DNA synthesis was observed for all modes of stimulation. There was also an upregulation of proteoglycan and collagen content for the later passaged cells (P3 and P4) when stochastic resonance was applied, as well as an upregulation for the earlier passaged cells (P1 and P2) when vibrations were applied. However, when long-term mechanical stimuli were applied, these increases in DNA and matrix accumulation were lost. This response was attributed to the dedifferentiation of the passaged cells away from their chondrogenic phenotype. What was also noted was that the passaged cells were unable to recreate and maintain a properly robust PCM, as observed by a decrease in collagen type VI expression. Ultimately, without a functional PCM, mechanical stimuli would not be properly transduced in the passaged cells and therefore a means to recapitulate the PCM was required.

6.1.2 PCM Recapitulation

Passaged chondrocytes pre-cultured in alginate beads showed an increase in collagen type VI expression when incubated for up to 4 days. Specifically, chondrocytes displayed a robust PCM structure for the P2 chondrocytes after 4 days of incubation. Comparatively, the P4 cells appeared to be unable to recapitulate a PCM, highlighting that the P4 chondrocytes may have been too far shifted in phenotype to recapitulate a PCM.

6.1.3 Mechanical Stimulation of Passaged Chondrocytes with a Recapitulated PCM

Passaged chondrocytes with a recapitulated PCM showed a positive response when mechanical stimuli were applied. In terms of matrix accumulation, although the magnitude was not as high as the P2 control cells (non-recapitulated PCM), they responded in a preferential manner to the stimulus. The PCM plays a crucial role in mechanotransduction of the chondrocyte and potentially the recapitulated PCM had an effect in shielding the chondrocytes from excessive loading. Although this method did not led to the creation of constructs with increased ECM accumulation, recapitulation of the PCM is a crucial step in recovering passaged chondrocytes as it allows the cells to properly transduce mechanical forces and thus crucial in developing proper cartilaginous tissue.

6.2 Future Work

6.2.1 Repeat Studies

The PCM recovery long-term stimulation study should be repeated to increase the sample sizes and associated power of the statistical methods used.

6.2.2 PCM Recovery Optimization

In addition to repeating the long-term study, the PCM recovery method should be further optimized. As none of the timepoints investigated (up to day 4) worked with the P4 cells, it might be that the P4 cells require longer incubation times. In addition, further optimization may be required to ensure that all the cells recover a robust PCM before encapsulation in agarose. The current methodology employed was focused on ensuring cell viability and not the frequency of PCM recovery. Thus, further exploring this method is warranted.

6.2.3 Mechanism(s)

Lastly, the mechanism(s) by which the PCM aids in the mechanotransduction of applied forces to the chondrocyte should be explored. Specifically, exploring the different elements of the PCM and how they function together would be beneficial. For example, the role of primary cilium in chondrocytes has only been recently discovered. Exploring the effects of cell passaging on the primary cilium and how to recover them would be valuable in further understanding how to recover dedifferentiated chondrocytes.

References

1. Murphy L, Helmick CG. The Impact of Osteoarthritis in the United States. *Orthop Nurs*. 2012;31(2):85-91. doi:10.1097/NOR.0b013e31824fcd42

2. Zhang L, Hu J, Athanasiou KA. The role of tissue engineering in articular cartilage repair and regeneration. *Crit Rev Biomed Eng*. 2009;37(1-2):1-57. doi:10.1126/scisignal.3104re1.Basal

3. Darling EM, Athanasiou KA. Rapid phenotypic changes in passaged articular chondrocyte subpopulations. *J Orthop Res*. 2005;23(2):425-432. doi:10.1016/j.orthres.2004.08.008

4. Hall AC, Horwitz ER, Wilkins RJ. The cellular physiology of articular cartilage. *Exp Physiol*. 1996;81(3):535-545. doi:10.1113/expphysiol.1996.sp003956

5. Stockwell RA. Cartilage failure in osteoarthritis: Relevance of normal structure and function. A review. *Clin Anat*. 1991;4(3):161-191. doi:10.1002/ca.980040303

6. Shieh AC, Athanasiou KA. Principles of cell mechanics for cartilage tissue engineering. *Ann Biomed Eng*. 2003;31(1):1-11. doi:10.1114/1.1535415

7. Brown TD. Techniques for mechanical stimulation of cells in vitro: a review. *J Biomech*. 2000;33(1):3-14. doi:10.1016/S0021-9290(99)00177-3

8. Natenstedt J, Kok AC, Dankelman J, Tuijthof GJ. What quantitative mechanical loading stimulates in vitro cultivation best? *J Exp Orthop*. 2015;2(1):15. doi:10.1186/s40634-015-0029-x

9. Ryan JA, Eisner EA, DuRaine G, You Z, Reddi AH. Mechanical compression of articular cartilage induces chondrocyte proliferation and inhibits proteoglycan synthesis by

activation of the ERK pathway: Implications for tissue engineering and regenerative medicine. *J Tissue Eng Regen Med*. 2009;3(2):107-116. doi:10.1002/term.146

10. Balko S, Weber JF, Waldman SD. Mechanical Stimulation Methods for Cartilage Tissue Engineering. In: *Orthopedic Biomaterials*. Cham: Springer International Publishing; 2018:123-147. doi:10.1007/978-3-319-89542-0_7

11. Weber JF, Waldman SD. Stochastic resonance is a method to improve the biosynthetic response of chondrocytes to mechanical stimulation. *J Orthop Res*. 2016;34(2):231-239. doi:10.1002/jor.23000

12. Weber JF. The sensitivity of articular chondrocytes to dynamic mechanical stimulation. 2015;(December).

13. Sophia Fox AJ, Bedi A, Rodeo SA. The basic science of articular cartilage: structure, composition, and function. *Sports Health*. 2009;1(6):461-468. doi:10.1177/1941738109350438

14. Buckwalter JA, Mow VC, Ratcliffe A. Restoration of Injured or Degenerated Articular Cartilage. *J Am Acad Orthop Surg*. 1994;2(4):192-201. doi:10.5435/00124635-199407000-00002

15. Brady MA, Waldman SD, Ethier CR. The Application of Multiple Biophysical Cues to Engineer Functional Neo-Cartilage for Treatment of Osteoarthritis (Part I: Cellular Response). *Tissue Eng Part B Rev*. 2015;21(1):1-19. doi:10.1089/ten.teb.2013.0757

16. Muir H. The chondrocyte, architect of cartilage. Biomechanics, structure, function and molecular biology of cartilage matrix macromolecules. *BioEssays*. 1995;17(12):1039-

1048. doi:10.1002/bies.950171208

17. O'Conor CJ, Case N, Guilak F. Mechanical regulation of chondrogenesis. *Stem Cell Res Ther*. 2013;4(4):1. doi:10.1186/scrt211

18. Eyre DR. The collagens of articular cartilage. *Semin Arthritis Rheum*. 1991;21(3 SUPPL. 2):2-11. doi:10.1016/0049-0172(91)90035-X

19. Eyre D. Collagen of articular cartilage. - PubMed - NCBI. *Arthritis Res Ther*. 2002;4(1):30-35. https://www.ncbi.nlm.nih.gov/pubmed/11879535.

20. Zhang Z. Chondrons and the Pericellular Matrix of Chondrocytes. *Tissue Eng Part B Rev*. 2015;21(3):267-277. doi:10.1089/ten.teb.2014.0286

21. KNUDSON CB, Nofal GA, Pamintuan L, Aguiar DJ. The chondrocyte pericellular matrix: a model for hyaluronan-mediated cell-matrix interactions. *Biochem Soc Trans*. 1999;27(2):142-147. http://eutils.ncbi.nlm.nih.gov/entrez/eutils/elink.fcgi?dbfrom=pubmed&id=10093723&retmode=ref&cmd=prlinks%0Apapers3://publication/uuid/CF9C5862-220D-4B1A-AE66-41CF2A3C9731.

22. C. Anthony Poole, Michael H. Flint and BWB. Chondrons in Cartilage: Ultrastructi ral Analj sis of the Pericellular Microenvironment in Adult Human Articular Cartilages. *J Orthop Res*. 1987;5(4):509-522. doi:10.1002/jor.1100050406

23. Guilak F, Alexopoulos LG, Upton ML, et al. The pericellular matrix as a transducer of biomechanical and biochemical signals in articular cartilage. *Ann N Y Acad Sci*. 2006;1068(1):498-512. doi:10.1196/annals.1346.011

24. Wann AKT, Zuo N, Haycraft CJ, et al. Primary cilia mediate mechanotransduction through control of ATP-induced Ca2+ signaling in compressed chondrocytes. *FASEB J*. 2012;26(4):1663-1671. doi:10.1096/fj.11-193649

25. Rich DR, Clark AL. Chondrocyte primary cilia shorten in response to osmotic challenge and are sites for endocytosis. *Osteoarthr Cartil*. 2012;20(8):923-930. doi:10.1016/j.joca.2012.04.017

26. Ruhlen R, Marberry K. The chondrocyte primary cilium. *Osteoarthr Cartil*. 2014;22(8):1071-1076. doi:10.1016/j.joca.2014.05.011

27. McGlashan SR, Jensen CG, Poole CA. Localization of extracellular matrix receptors on the chondrocyte primary cilium. *J Histochem Cytochem*. 2006;54(9):1005-1014. doi:10.1369/jhc.5A6866.2006

28. Miller RA, Waitman LR, Chen S, Rosenbloom ST. DEVELOPMENTAL AND OSTEOARTHRITIC CHANGES IN Col6a1 KNOCKOUT MICE: THE BIOMECHANICS OF COLLAGEN VI IN THE CARTILAGE PERICELLULAR MATRIX. 2006;38(6):469-485. doi:10.1038/ki.2009.479.Commonly

29. Wilusz RE, Sanchez-Adams J, Guilak F. The structure and function of the pericellular matrix of articular cartilage. *Matrix Biol*. 2014;39:25-32. doi:10.1016/j.matbio.2014.08.009

30. Pfaff, Martin; Aumailley, Monique; Specks, Ulrich; Knolle, Joachim; Zerwes, Hans G; Timpl R. Integrin and Arg-Gly-Asp Dependence of Cell Adhesion to the Native and Unfolded Triple Helix of Collagen Type VI. *Exp Cell Res*. 1993;206:167-176.

31. Twomey JD, Thakore PI, Hartman DA, Myers EGH, Hsieh AH. Roles of type VI collagen and decorin in human mesenchymal stem cell biophysics during chondrogenic differentiation. *Eur Cells Mater*. 2014;27:237-250. doi:10.22203/eCM.v027a17

32. Wotton SF, Duance VC, Fryer PR. Type IX collagen: A possible function in articular cartilage. *FEBS Lett*. 1988;234(1):79-82. doi:10.1016/0014-5793(88)81307-3

33. Poole CA, Gilbert RT, Herbage D, Hartmann DJ. Immunolocalization of type IX collagen in normal and spontaneously osteoarthritic canine tibial cartilage and isolated chondrons. *Osteoarthr Cartil*. 1997;5(3):191-204. doi:10.1016/S1063-4584(97)80014-3

34. Poole CA. Articular cartilage chondrons: Form, function and failure. *J Anat*. 1997;191(1):1-13. doi:10.1017/S0021878297002185

35. Lee GM, Loeser RF. Interactions of the chondrocyte with its pericellular matrix. *Cells Mater*. 1998;8:135-149. http://www.ecmjournal.org/journal/smi/pdf/cm98-11.pdf.

36. Robinson HC, Handleyc CJ. Identification of Distinct Metabolic Pools of Aggrecan and Their Relationship to Type VI Collagen in the Chondrons of Mature Bovine Articular Cartilage Explants, Connective Tissue Research, Informa Healthcare. 1998;17. http://informahealthcare.com.ezproxy.lib.ucalgary.ca/doi/abs/10.3109/03008209809002445.

37. Nirmala SundarRaj, Dana Fite1, Steven Ledbetter SC and JRH. Perlecan is a component of cartilage matrix and promotes chondrocyte attachment. 2006;2672:2663-2672. http://biochemj.org/lookup/doi/10.1042/bj2620823.

38. McGlashan SR, Knight MM, Chowdhury TT, et al. Mechanical loading modulates

chondrocyte primary cilia incidence and length. *Cell Biol Int*. 2010;34(5):441-446. doi:10.1042/CBI20090094

39. Malone AMD, Anderson CT, Tummala P, et al. Primary cilia mediate mechanosensing in bone cells by a calcium-independent mechanism. *Proc Natl Acad Sci*. 2007;104(33):13325-13330. doi:10.1073/pnas.0700636104

40. Praetorius HA, Spring KR. Bending the MDCK cell primary cilium increases intracellular calcium. *J Membr Biol*. 2001;184(1):71-79. doi:10.1007/s00232-001-0075-4

41. Chang CF, Ramaswamy G, Serra R. Depletion of primary cilia in articular chondrocytes results in reduced Gli3 repressor to activator ratio, increased Hedgehog signaling, and symptoms of early osteoarthritis. *Osteoarthr Cartil*. 2012;20(2):152-161. doi:10.1016/j.joca.2011.11.009

42. Lin L, Shen Q, Xue T, Duan X, Fu X, Yu C. Sonic Hedgehog Improves Redifferentiation of Dedifferentiated Chondrocytes for Articular Cartilage Repair. Xie J, ed. *PLoS One*. 2014;9(2):e88550. doi:10.1371/journal.pone.0088550

43. Trickey WR, Lee GM, Guilak F. Viscoelastic properties of chondrocytes from normal and osteoarthritic human cartilage. *J Orthop Res*. 2000;18(6):891-898. doi:10.1002/jor.1100180607

44. Lu XL, Mow VC. Biomechanics of articular cartilage and determination of material properties. *Med Sci Sports Exerc*. 2008;40(2):193-199. doi:10.1249/mss.0b013e31815cb1fc

45. Mow VC, Holmes MH, Michael Lai W. Fluid transport and mechanical properties of

articular cartilage: A review. *J Biomech*. 1984;17(5):377-394. doi:10.1016/0021-9290(84)90031-9

46. Mow VC, Kuei SC, Lai WM, Armstrong CG. Biphasic creep and stress relaxation of articular cartilage in compression? Theory and experiments. *J Biomech Eng*. 1980;102(1):73-84. doi:10.1115/1.3138202

47. Guilak F, Mow VC. The mechanical environment of the chondrocyte: a biphasic finite element model of cell-matrix interactions in articular cartilage. *J Biomech*. 2000;33(12):1663-1673. http://www.ncbi.nlm.nih.gov/pubmed/11006391.

48. Ateshian GA, Kim JJ, Grelsamer RP, Mow VC, Warden WH. Finite deformation biphasic material properties of bovine articular cartilage from confined compression experiments. 1997;30(97). doi:10.1016/S0021-9290(97)85606-0

49. Lai WM, Hou JS, Mow VC. A triphasic theory for the swelling and deformation behaviors of articular cartilage. *J Biomech Eng*. 1991;113(3):245-258. doi:10.1115/1.2894880

50. Athanasiou KA, Rosenwasser MP, Buckwalter JA, Malinin TI, Mow VC. Interspecies Comparisons of Insitu Intrinsic Mechanical-Properties of Distal Femoral Cartilage. *J Orthop Res*. 1991;9(3):330-340. doi:10.1002/jor.1100090304

51. Wakitani S, Goto T, Pineda SJ, et al. Mesenchymal cell-based repair of large, full-thickness defects of articular cartilage. *J Bone Joint Surg Am*. 1994;76(4):579-592. doi:10.1007/978-1-4471-5451-8

52. Yoo J, Barthel T, Nishimura K. The chondrogenic potential of human bone- marrow-derived mesenchymal progenitor cells. *J Bone Jt Surg*. 1998;80A(12):1745-1759.

doi:10.1002/jbm.a.35603.

53. Getgood A, Brooks R, Fortier L, N R. Articular cartilage tissue engineering. *J Bone Jt Surg*. 2009;91-B(5):565-576. doi:10.1302/0301-620X.91B5.21832

54. Takeichi M. The factor affecting the spreading of chondrocytes upon inorganic substrate. *J Cell Sci*. 1973;13(1):193-204.

55. Corvol MT, Malemud CJ, Sokoloff L. A pituitary growth-promoting factor for articular chondrocytes in monolayer culture. *Endocrinology*. 1972;90(1):262-271. doi:10.1210/endo-90-1-262

56. Haddad JB, Obolensky AG, Shinnick P. The biologic effects and the therapeutic mechanism of action of electric and electromagnetic field stimulation on bone and cartilage: new findings and a review of earlier work. *J Altern Complement Med*. 2007;13(5):485-490. doi:10.1089/acm.2007.5270

57. Snyder MJ, Wilensky JA, Fortin JD. Current applications of electrotherapeutics in collagen healing. *Pain Physician*. 2002;5(2):172-181. http://www.ncbi.nlm.nih.gov/pubmed/16902668.

58. Grodzinsky AJ, Levenston ME, Jin M, Frank EH. Cartilage Tissue Remodeling in Response to Mechanical Forces. *Annu Rev Biomed Eng*. 2000;2:691-713.

59. Mow VC, Wang CC, Hung CT. The extracellular matrix, interstitial fluid and ions as a mechanical signal transducer in articular cartilage. *Osteoarthr Cartil*. 1999;7(1):41-58. doi:10.1053/joca.1998.0161

60. Guilak F, Jones WR, Ting-Beall HP, Lee GM. The deformation behavior and mechanical

properties of chondrocytes in articular cartilage. *Osteoarthritis Cartilage*. 1999;7(1):59-70. doi:10.1053/joca.1998.0162

61. Gray ML, Pizzanelli AM, Grodzinsky AJ, Lee RC. Mechanical and physicochemical determinants of the chondrocyte biosynthetic response. *J Orthop Res*. 1988;6(6):777-792. doi:10.1002/jor.1100060602

62. Salter DM, Nuki G, Wright MO. Integrin – Interleukin-4 Mechanotransduction Pathways in Human Chondrocytes. 2001;(391):49-60.

63. Graff RD, Lazarowski ER, Banes AJ, Lee GM. ATP release by mechanically loaded chondrocytes in pellet culture. *Trans Orthop Res Soc*. 2000;25(7):1571-1579.

64. Mobasheri A, Carter SD, Martín-Vasallo P, Shakibaei M. Integrins and stretch activated ion channels; putative components of functional cell surface mechanoreceptors in articular chondrocytes. *Cell Biol Int*. 2002;26(1):1-18. doi:10.1006/cbir.2001.0826

65. Han SK, Wouters W, Clark A, Herzog W. Mechanically induced calcium signaling in chondrocytes in situ. *J Orthop Res*. 2012;30(3):475-481. doi:10.1002/jor.21536

66. Buschmann MD, Hunziker EB, Kim YJ, Grodzinsky a J. Altered aggrecan synthesis correlates with cell and nucleus structure in statically compressed cartilage. *J Cell Sci*. 1996;109 (Pt 2:499-508. http://www.ncbi.nlm.nih.gov/pubmed/8838673.

67. Guilak F. Pression-Induced Changes in Cyte Nucleu. *J Biomech*. 1995;28(12):1529-1541. doi:10.1016/0021-9290(95)00100-x

68. Elder BD, Athanasiou KA. Hydrostatic pressure in articular cartilage tissue engineering: from chondrocytes to tissue regeneration. *Tissue Eng Part B Rev*. 2009;15(1):43-53.

doi:10.1089/ten.teb.2008.0435

69. Grad S, Eglin D, Alini M, Stoddart MJ. Physical stimulation of chondrogenic cells in vitro: A review. *Clin Orthop Relat Res*. 2011;469(10):2764-2772. doi:10.1007/s11999-011-1819-9

70. Lin WY, Chang YH, Wang HY, et al. The study of the frequency effect of dynamic compressive loading on primary articular chondrocyte functions using a microcell culture system. *Biomed Res Int*. 2014;2014(1):1-11. doi:10.1155/2014/762570

71. Davisson T, Kunig S, Chen A, Sah R, Ratcliffe A. Static and dynamic compression modulate matric metabolism in tissue engineered cartilage. *J Orthop Res*. 2002;20:842-848.

72. Buschmann MD, Gluzband YA, Grodzinsky AJ, Hunziker EB. Mechanical compression modulates matrix biosynthesis in chondrocyte/agarose culture. *J Cell Sci*. 1995;108 (Pt 4:1497-1508. http://www.ncbi.nlm.nih.gov/pubmed/7615670.

73. Soltz M a, Ateshian G a. Interstitial fluid pressurization during confined compression cyclical loading of articular cartilage. *Ann Biomed Eng*. 2000;28:150-159. doi:10.1114/1.239

74. Heiner AD, Martin JA. Cartilage responses to a novel triaxial mechanostimulatory culture system. *J Biomech*. 2004;37(5):689-695. doi:10.1016/j.jbiomech.2003.09.014

75. Gu WY, Yao H, Huang CY, Cheung HS. New insight into deformation-dependent hydraulic permeability of gels and cartilage, and dynamic behavior of agarose gels in confined compression. *J Biomech*. 2003;36(4):593-598. doi:10.1016/S0021-

9290(02)00437-2

76. Lee C, Grad S, Wimmer M, Alini M. The Influence of Mechanical Stimuli on Articular Cartilage Tissue Engineering. *Top Tissue Eng*. 2006;2(2):1-32.

77. Wong M, Siegrist M, Cao X. Cyclic compression of articular cartilage explants is associated with progressive consolidation and altered expression pattern of extracellular matrix proteins. *Matrix Biol*. 1999;18(4):391-399. doi:10.1016/S0945-053X(99)00029-3

78. Sah RL, Kim YJ, Doong J-YH, Grodzinsky AJ, Plaas AH, Sandy JD. Biosynthetic response of cartilage explants to dynamic compression. *J Orthop Res*. 1989;(7):619-636. doi:10.1007/978-1-4471-5451-8_108

79. Démarteau O, Wendt D, Braccini A, et al. Dynamic compression of cartilage constructs engineered from expanded human articular chondrocytes. *Biochem Biophys Res Commun*. 2003;310(2):580-588. doi:10.1016/j.bbrc.2003.09.099

80. Mauck RL, Wang CCB, Oswald ES, Ateshian GA, Hung CT. The role of cell seeding density and nutrient supply for articular cartilage tissue engineering with deformational loading. *Osteoarthr Cartil*. 2003;11(12):879-890. doi:10.1016/j.joca.2003.08.006

81. Mauck RL, Soltz M a, Wang CC, et al. Functional tissue engineering of articular cartilage through dynamic loading of chondrocyte-seeded agarose gels. *J Biomech Eng*. 2000;122(3):252-260. doi:10.1115/1.429656

82. Babalola OM, Bonassar LJ. Parametric finite element analysis of physical stimuli resulting from mechanical stimulation of tissue engineered cartilage. *J Biomech Eng*. 2009;131(6):061014. doi:10.1115/1.3128672

83. Suh JK. Dynamic Unconfined Compression of Articular Cartilage Under a Cyclic Compression Load. *Biorheology*. 1996;33(4-5):289-304.

84. Kelly TAN, Ng KW, Wang CCB, Ateshian GA, Hung CT. Spatial and temporal development of chondrocyte-seeded agarose constructs in free-swelling and dynamically loaded cultures. *J Biomech*. 2006;39(8):1489-1497. doi:10.1016/j.jbiomech.2005.03.031

85. Parkkinen JJ, Lammi MJ, Helminen HJ, Tammi M. Local stimulation of proteoglycan synthesis in articular cartilage explants by dynamic compression in vitro. *J Orthop Res*. 1992;10(5):610-620. doi:10.1002/jor.1100100503

86. Hayes WCC, Keer LMM, Herrmann G, Mockros LFF. A mathematical analysis for indentation tests of articular cartilage. *J Biomech*. 1972;5(5):541-551. doi:10.1016/0021-9290(72)90010-3

87. Hori RY, Mockros LF. Indentation tests of human articular cartilage. *J Biomech*. 1976;9(4):259-268. doi:10.1016/0021-9290(76)90012-9

88. Guha Thakurta S, Kraft M, Viljoen HJ, Subramanian A. Enhanced depth-independent chondrocyte proliferation and phenotype maintenance in an ultrasound bioreactor and an assessment of ultrasound dampening in the scaffold. *Acta Biomater*. 2014;10(11):4798-4810. doi:10.1016/j.actbio.2014.07.013

89. Parvizi J, Wu CC, Lewallen DG, Greenleaf JF, Bolander ME. Low-intensity ultrasound stimulates proteoglycan synthesis in rat chondrocytes by increasing aggrecan gene expression. *J Orthop Res*. 1999;17(4):488-494. doi:10.1002/jor.1100170405

90. Noriega S, Mamedov T, Turner JA, Subramanian A. Intermittent Applications of

Continuous Ultrasound on the Viability, Proliferation, Morphology, and Matrix Production of Chondrocytes in 3D Matrices. *Tissue Eng*. 2007;13(3):611-618. doi:10.1089/ten.2006.0130

91. Noriega S, Hasanova G, Subramanian A. The effect of ultrasound stimulation on the cytoskeletal organization of chondrocytes seeded in three-dimensional matrices. *Cells Tissues Organs*. 2012;197(1):14-26. doi:10.1159/000339772

92. Gauthier BJ. The Effects of Mechanical Vibration on Human Chondrocytes In Vitro. 2016. http://epublications.marquette.edu/theses_open/369.

93. Kaupp J a, Waldman SD. Mechanical vibrations increase the proliferation of articular chondrocytes in high-density culture. *Proc Inst Mech Eng H*. 2008;222(5):695-703. doi:10.1243/09544119JEIM376

94. Cescon M, Gattazzo F, Chen P, Bonaldo P. Collagen VI at a glance. *J Cell Sci*. 2015;128(19).

95. Weber JF, Chiu LLY, Balko S, Waldman SD. Stochastic Resonance with Dynamic Compression Improves the Growth of Adult Chondrocytes in Agarose Gel Constructs. *Ann Biomed Eng*. 2018. doi:10.1007/s10439-018-02123-x

96. Kuettner KE, Pauli BU, Gall G, Memoli VA, Schenk RK. Synthesis of cartilage matrix by mammalian chondrocytes in vitro. I. Isolation, culture characteristics, and morphology. *J Cell Biol*. 1982;93(3):743-750. doi:10.1083/jcb.93.3.743

97. Kaupp JA, Weber JF, Waldman SD. Mechanical Stimulation of Chondrocyte-agarose Hydrogels. *J Vis Exp*. 2012;(68):1-5. doi:10.3791/4229

98. Fan JCY, Waldman SD. The effect of intermittent static biaxial tensile strains on tissue engineered cartilage. *Ann Biomed Eng*. 2010;38(4):1672-1682. doi:10.1007/s10439-010-9917-5

99. McGowan KB, Kurtis MS, Lottman LM, Watson D, Sah RL. Biochemical quantification of DNA in human articular and septal cartilage using PicoGreen® and Hoechst 33258. *Osteoarthr Cartil*. 2002;10(7):580-587. doi:10.1053/joca.2002.0794

100. Waldman SD, Spiteri CG, Grynpas MD, Pilliar RM, Kandel RA. Long-term intermittent shear deformation improves the quality of cartilaginous tissue formed in vitro. *J Orthop Res*. 2003;21(4):590-596. doi:10.1016/S0736-0266(03)00009-3

101. Waldman SD, Grynpas MD, Pilliar RM, Kandel RA. Characterization of cartilagenous tissue formed on calcium polyphosphate substrates in vitro. *J Biomed Mater Res*. 2002;62(3):323-330. doi:10.1002/jbm.10235

102. Waldman SD, Spiteri CG, Grynpas MD, Pilliar RM, Hong J, Kandel RA. Effect of biomechanical conditioning on cartilaginous tissue formation in vitro. *J Bone Jt Surg - Ser A*. 2003;85(SUPPL. 1):101-105. doi:10.2106/00004623-200300002-00013

103. Waldman SD, Spiteri CG, Grynpas MD, Pilliar RM, Kandel RA. Long-Term Intermittent Compressive Stimulation Improves the Composition and Mechanical Properties of Tissue-Engineered Cartilage. *Tissue Eng*. 2004;10(9-10):1323-1331. doi:10.1089/ten.2004.10.1323

104. Farndale RW, Buttle DJ, Barrett AJ. Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue. *BBA - Gen Subj*. 1986;883(2):173-177. doi:10.1016/0304-4165(86)90306-5

105. Lin YK, Kuan CY. Development of 4-hydroxyproline analysis kit and its application to collagen quantification. *Food Chem*. 2010;119(3):1271-1277. doi:10.1016/j.foodchem.2009.08.009

106. Rioche AOF, Bi TRYA. The Determination of Hydroxyproline in Tissue and Protein Samples Containing Small Proportions of Imino Acid. *Arch Biochem Biophys*. 1961;93:440-447.

107. De Ceuninck F, Lesur C, Pastoureau P, Caliez A, Sabatini M. Culture of Chondrocytes in Alginate Beads. *Cartil Osteoarthr*. 100:015-022. doi:10.1385/1-59259-810-2:015

108. Lee BYDA, Bentley G, Charles W. The control of cell division in articular chondrocytes. *Osteoarthr Cartil*. 1993;1:137-146.

109. Benya D, Nimni E. The Stability of the Collagen Phenotype during Stimulated and DNA Synthesis by Articular Cartilage Organ Cultures The behavior of chondrocytes in culture has been studied in a variety of situations . differentiated functions of cartilage-specific tion whe. 1979;192(2).

110. Lee DA, Reisler T, Bader DL. Expansion of chondrocytes for tissue engineering in alginate beads enhances chondrocytic phenotype compared to conventional monolayer techniques. *Acta Orthop Scand*. 2003;74(1):6-15. doi:10.1080/00016470310013581

111. Newman P, Watt FM. Influence of cytochalasin d-induced changes in cell shape on proteoglycan synthesis by cultured articular chondrocytes. *Exp Cell Res*. 1988;178(2):199-210. doi:10.1016/0014-4827(88)90391-6

112. Das RHJ, Jahr H, Verhaar JAN, van der Linden JC, van Osch GJVM, Weinans H. In vitro

expansion affects the response of chondrocytes to mechanical stimulation. *Osteoarthr Cartil*. 2008;16(3):385-391. doi:10.1016/j.joca.2007.07.014

113. Wright MO, Davies LW, Nuki G, Salter DM, Al MET. Mechanotransduction via Integrins and Interleukin-4 results in Altered Aggrecan adn Matrix Metalloproteinase-3 Gene Expression in Normal, but nit Osteoarthritic, Human Articular Chondrocytes. *Arthritis Rheum*. 2000;43(9):2091-2099.

114. Goessler UR, Bieback K, Bugert P, et al. In vitro analysis of integrin expression during chondrogenic differentiation of mesenchymal stem cells and chondrocytes upon dedifferentiation in cell culture. *Int J Mol Med*. 2006;17(2):301-307. doi:10.3892/ijmm.17.2.301

115. Parreno J, Nabavi Niaki M, Andrejevic K, Jiang A, Wu PH, Kandel RA. Interplay between cytoskeletal polymerization and the chondrogenic phenotype in chondrocytes passaged in monolayer culture. *J Anat*. 2017;230(2):234-248. doi:10.1111/joa.12554

116. Ando K, Imai S, Isoya E, et al. Effect of dynamic compressive loading and its combination with a growth factor on the chondrocytic phenotype of 3-dimensional scaffold-embedded chondrocytes. *Acta Orthop*. 2009;80(6):724-733. doi:10.3109/17453670903413111

117. Kisiday JD, Frisbie DD, McIlwraith CW, Grodzinsky AJ. Dynamic compression stimulates proteoglycan synthesis by mesenchymal stem cells in the absence of chondrogenic cytokines. *Tissue Eng Part A*. 2009;15(10):2817-2824. doi:10.1089/ten.TEA.2008.0357

118. Marycz K, Lewandowski D, Tomaszewski KA, Henry BM, Golec EB, Marędziak M.

Low-frequency, low-magnitude vibrations (LFLM) enhances chondrogenic differentiation potential of human adipose derived mesenchymal stromal stem cells (hASCs). *PeerJ*. 2016;4:e1637. doi:10.7717/peerj.1637

119. Liu J, Sekiya I, Asai K, Tada T, Kato T, Matsui N. Effects of mechanical vibration on DNA and proteoglycan syntheses in cultured articular chondrocytes. *Mod Rheumatol*. 2001;11(1):40-46. doi:10.1007/s101650170042

120. Lee CR, Grodzinsky AJ, Spector M. Biosynthetic response of passaged chondrocytes in a type II collagen scaffold to mechanical compression. *J Biomed Mater Res - Part A*. 2003;64(3):560-569. doi:10.1002/jbm.a.10443

121. Nicodemus GD, Bryant SJ. Mechanical loading regimes affect the anabolic and catabolic activities by chondrocytes encapsulated in PEG hydrogels. *Osteoarthr Cartil*. 2010;18(1):126-137. doi:10.1016/j.joca.2009.08.005

122. De Croos JNA, Dhaliwal SS, Grynpas MD, Pilliar RM, Kandel RA. Cyclic compressive mechanical stimulation induces sequential catabolic and anabolic gene changes in chondrocytes resulting in increased extracellular matrix accumulation. *Matrix Biol*. 2006;25(6):323-331. doi:10.1016/j.matbio.2006.03.005

123. Mauck RL, Yuan X, Tuan RS. Chondrogenic differentiation and functional maturation of bovine mesenchymal stem cells in long-term agarose culture. *Osteoarthr Cartil*. 2006;14(2):179-189. doi:10.1016/j.joca.2005.09.002

124. Wernike E, Li Z, Alini M, Grad S. Effect of reduced oxygen tension and long-term mechanical stimulation on chondrocyte-polymer constructs. *Cell Tissue Res*. 2008;331(2):473-483. doi:10.1007/s00441-007-0500-9

125. Abbott J. The Loss of Phenotyipic By Differentiated TraitsT H E LOSS OF P H E N O T Y P I C BY D I F F E R E N T I A T E D TRAITS IIL The Reversible Behavior of Chondrocytes in Primary Cultures. 1966:473-487.

126. Kang SW, Yoo SP, Kim BS. Effect of chondrocyte passage number on histological aspects of tissue-engineered cartilage. *Biomed Mater Eng*. 2007;17(February 2007):269-276. internal-pdf://kang_2007-0310635776/Kang_2007.pdf%0Ahttp://www.ncbi.nlm.nih.gov/pubmed/17851169.

127. Mallein-Gerin F, Garrone R van der RM. Proteoglycan and collagen synthesis are correlated with actin organization in dedifferentiating chondrocytes. *Eur J Cell Biol*. 1991;56(2):364-373.

128. Darling EM, Pritchett PE, Evans BA, Superfine R, Zauscher S, Guilak F. Mechanical properties and gene expression of chondrocytes on micropatterned substrates following dedifferentiation in monolayer. *Cell Mol Bioeng*. 2009;2(3):395-404. doi:10.1007/s12195-009-0077-3

129. Guilak F, Meyer BC, Ratcliffe A, Mow VC. The effects of matrix compression on proteoglycan metabolism in articular cartilage explants. *Osteoarthr Cartil*. 1994;2(2):91-101. doi:10.1016/S1063-4584(05)80059-7

130. Liu J, Sekiya I, Asai K, Tada T, Kato T, Matsui N. Biosynthetic response of cultured articular chondrocytes to mechanical vibration. *ResExpMed(Berl)*. 2001;200(0300-9130 (Print) LA-eng PT-Journal Article RN-0 (Proteoglycans) RN-0 (Sulfur Radioisotopes) RN-0 (Sulfuric Acids) RN-10028-17-8 (Tritium) RN-50-89-5 (Thymidine) RN-7664-93-9 (sulfuric acid) RN-9007-49-2 (DNA) SB-IM):183-193.

131. Knight MM, Lee DA, Bader DL. The influence of elaborated pericellular matrix on the deformation of isolated articular chondrocytes cultured in agarose. *Biochim Biophys Acta - Mol Cell Res*. 1998;1405(1-3):67-77. doi:10.1016/S0167-4889(98)00102-5

132. Graff RD, Kelley SS, Lee GM. Role of pericellular matrix in development of a mechanically functional neocartilage. *Biotechnol Bioeng*. 2003;82(4):457-464. doi:10.1002/bit.10593

133. Zelenski NA, Leddy HA, Sanchez-Adams J, et al. Type VI Collagen Regulates Pericellular Matrix Properties, Chondrocyte Swelling, and Mechanotransduction in Mouse Articular Cartilage. *Arthritis Rheumatol*. 2015;67(5):1286-1294. doi:10.1002/art.39034

134. Muhammad H, Rais Y, Miosge N, Ornan EM. The primary cilium as a dual sensor of mechanochemical signals in chondrocytes. *Cell Mol Life Sci*. 2012;69(13):2101-2107. doi:10.1007/s00018-011-0911-3

135. Thompson CL, Plant JCT, Wann AKT, et al. Chondrocyte expansion is associated with loss of primary cilia and disrupted hedgehog signalling. *Eur Cells Mater*. 2017;34:128-141. doi:10.22203/eCM.v034a09

136. Kim J, Lee JE, Heynen-Genel S, et al. Functional genomic screen for modulators of ciliogenesis and cilium length. *Nature*. 2010;464(7291):1048-1051. doi:10.1038/nature08895

137. DiMicco MA, Kisiday JD, Gong H, Grodzinsky AJ. Structure of pericellular matrix around agarose-embedded chondrocytes. *Osteoarthr Cartil*. 2007;15(10):1207-1216. doi:10.1016/j.joca.2007.03.023

138. Alexopoulos LG, Haider MA, Vail TP, Guilak F. Alterations in the Mechanical Properties of the Human Chondrocyte Pericellular Matrix With Osteoarthritis. *J Biomech Eng*. 2003;125(3):323. doi:10.1115/1.1579047

139. Alexopoulos LG, Setton LA, Guilak F. The biomechanical role of the chondrocyte pericellular matrix in articular cartilage. *Acta Biomater*. 2005;1(3):317-325. doi:10.1016/j.actbio.2005.02.001

140. Wiseman M, Bader DL, Reisler T, Lee DA. Passage in monolayer influences the response of chondrocytes to dynamic compression. *Biorheology*. 2004;41(3-4):283-298. http://www.ncbi.nlm.nih.gov/pubmed/15299261.