Calcium Regulates Cyclic Compression-Induced Early Changes In Chondrocytes During *In Vitro* Tissue Formation

by

Igal Raizman

A thesis submitted in conformity with the requirements
for the degree of Master of Science

Graduate Department Laboratory Medicine and Pathobiology
University of Toronto

© Copyright by Igal Raizman 2009

Calcium Regulates Cyclic Compression-Induced Early Changes In Chondrocytes During *In Vitro* Tissue Formation

Igal Raizman

Master of Science

Graduate Department of Laboratory Medicine and Pathobiology
University of Toronto

2009

Abstract

A single application of cyclic compression to bioengineered cartilage improves tissue formation through cell shape changes that are mediated by α5β1 integrin and membrane-type metalloprotease (MT1-MMP). To determine if this response is controlled by calcium, we investigated how calcium regulated cell shape changes, MT1-MMP and integrin activity in response to stimulation. Stimulation-induced changes in cell shape and MT1-MMP expression were abolished with chelation of extracellular calcium, and reinstated with its re-introduction. Spreading and retraction were inhibited by blocking the stretch-activated and L-Type voltage-gated channels, respectively; channel blocking also inhibited MT1-MMP upregulation. Channels' role was confirmed through treatment with calcium A23187 ionophore, which alleviated the effects of channel blocking. Calcium regulated the integrin-mediated signalling pathway, which was facilitated through the kinase Src. Both calcium- and integrin-mediated pathways converged on activating ERK in response to stimulation. Understanding the molecular mechanisms regulating chondrocyte mechanotransduction may lead to the development of improved bioengineered cartilage.

Acknowledgments

First and foremost I would like to thank my supervisor, Dr. Rita Kandel, whose teaching, guidance, and support enabled me to develop an understanding of the subject. Dr. Kandel provided invaluable assistance, encouragement and patience over the course of my degree. I would like to thank my committee members, Dr. Christopher McCulloch and Dr. Michelle Bendeck, for their helpful comments and suggestions.

I would also like to thank and acknowledge:

- Dr. Kandel's Lab, Mount Sinai Hospital for all their help, advice and support
- Dr. Amritha De Croos for his technical expertise and assistance
- Jean-Phillippe St. Pierre for his technical expertise and support
- Dr. Jian Wang for his assistance with mechanical stimulation
- Dr. Svitlana Prada for her preparation of CPP
- Menat Attia for her assistance with confocal microscopy
- Justin Parreno for his tissue culture expertise
- Douglas Holmyard & Robert Temkin (Advanced Bioimaging Centre, Mount Sinai) for their invaluable assistance with SEM imaging
- Dr. Marc Grynpas for chairing my thesis defence

Table of Contents

LIST OF FIGURES

LIST OF TABLES

CHAPTER ONE: BACKGROUND

1.1 Introduction

With the world population continually aging, cartilage-related pathologies are becoming more pronounced and their toll on the worlds' health care systems in ever increasing. It is estimated that over 36 million individuals, in the United States alone, are afflicted with some form of cartilage pathology arisen from arthritis or sports injury [Buckwalter & Mankin, 98b]. Given the debilitating nature of severe joint pain, and the limited ability of cartilage to self-repair, great effort has been dedicated to elucidating ways to repair native cartilage tissue. Unfortunately, due to the tissue's complex biomechanical properties, little progress has been made and most clinical treatments resort to prosthetic replacements of the damaged tissue. Consequently, the creation of bioengineered articular cartilage tissue has been suggested as a viable possible alternative to current clinical approaches.

1.1.2 Cartilage: Role & Function

The designation of cartilage is given to a specialized type of dense connective tissue that is composed of chondrocyte cells embedded in a rich matrix composed of mainly of proteoglycans, collagens and elastin fibres. Depending on the relative abundance of these components cartilage is further classified into three distinct types, notably: elastic cartilage, fibrocartilage, and articular (hyaline) cartilage [Temenoff & Mikos, 00b]. Briefly, elastic cartilage is characterized by a significant presence of elastin in the extracellular matrix (ECM) and is found in the ear and nose of individuals. Fibrocartilage, which is found at the ends of tendons and ligaments, possesses a higher constituency of collagens. Hyaline cartilage, in turn, possesses a white, glassy appearance and is most often found in the lining of articulating surfaces[Mow & Lai, 09].

1.1.3 Articular Cartilage

Articular cartilage, a specialized form of hyaline cartilage, is a unique connective tissue of the musculoskeletal system that forms a 3-4mm thick layer covering the articulating ends of diarthrodial (synovial) joints, which enables smooth articulation and consequently movement and activity (Figure 1) [Mow & Lai, 09]. Due to their role of providing flexion and extension, joints are repeatedly subject to complicated motions at varying speeds. Throughout such activities, joints are exposed to a wide range of forces, at times totaling up to several times the body weight [Mow & Lai, 09;Brower & Hsu, 69]. The presence of articular cartilage at the ends of diarthrodial joints serves a dual purpose: 1) even transfer of forces to neighbouring subchondral bone plates and, 2) along with the synovial fluid to provide a nearly frictionless surface for the joint-forming bones to move freely over one another during motion [Huber *et al.*, 00].

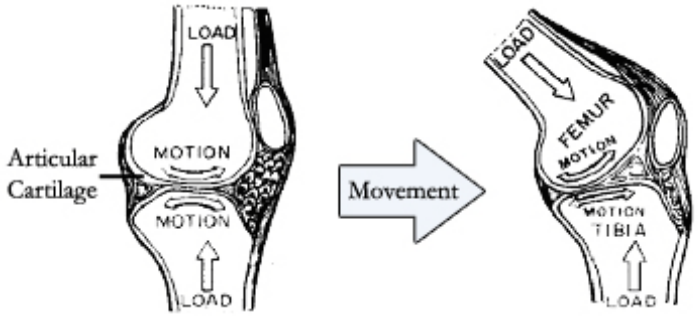


Figure 1: Articular cartilage tissue is a specialized connective tissue lining the ends of articulating joints and serves a dual function of: 1) providing for a nearly frictionless join movement, and 2) facilitating the transfer or mechanical loads during movement. Illustration adapted from Mow & Lai, 1979.

When cartilage is loaded (i.e. during walking or physical activity) an initial increase in pressure squeezes tissue fluid out of the solid extracellular matrix, causing the tissue's matrix to consolidate and the cells present to undergo compressive deformation. However, the increase in negative charge density due to proteins embedded in the extracellular matrix, along with the

relatively low fluid permeability of the matrix, assists in minimizing water escape. The resulting hydrostatic pressure (~95% of resistance), along with the solid extracellular matrix (~5% resistance), is then able to withstand the compressive forces experienced by the joint [Pearle *et al.*, 05;Wong & Carter, 03].

The ability of articular cartilage to withstand such life-long taxing mechanical loads without any significant erosion in healthy individuals, is made possible by the tissue's unique morphological and biomechanical properties [Grodzinsky *et al.*, 00;Mow & Lai, 09;Pearle, Warren & Rodeo, 05]. Normal and moderate joint loading serves to maintain cartilage health and function throughout the lifetime of an individual. It is well accepted that mechanical forces, and changes in loading, result in metabolic alterations that allow the tissue to respond to these changes [Eckstein *et al.*, 06]. Failure to properly respond to such changes results in tissue pathology and degeneration, of which osteoarthrosis is a prime example [Buckwalter, 95].

1.1.4 Composition & Architecture of Articular Cartilage

Similarly to other cartilages, articular cartilage is considered to be a permeable, visoelastic material consisting of three principal phases: a solid phase mainly composed of a collagen network and embedded proteoglycans; a fluid phase of water; and an ion phase containing electrolytes and dissolved gases [Huber, Trattnig & Lintner, 00]. A distinguishing characteristic of articular cartilage, as compared to most other tissues, is the lack of vascular, neural and lymphatic innervation. As a result, all nutrient and waste exchanges occur solely through diffusion to and from the synovial fluid. This fluid, which also serves to lubricate the joint, is a plasma ultrafiltrate containing electrolytes, small molecules and glucose [O'Hara *et al.*, 90].

1.1.5 Chondrocytes

Chondrocytes, the sole cellular component of articular cartilage, constitute up to 5% of the tissue's total volume, and are embedded in an extracellular matrix (ECM). The cells originate from mesenchymal stem cells (MSCs) which, in mature individuals, are localized to the bone marrow. Chondrocytes arise during embryogenesis when mesenchymal stem cells undergo differentiation and begin secreting cartilaginous matrix [Buckwalter & Mankin, 98a]. With time, chondrocytes destined to become bone acquire a hypertrophic phenotype and begin producing proteins that are required for matrix calcification. Other chondrocytes, on the periphery, continue to secrete collagen and other matrix molecules, and create the characteristic ECM of cartilage. The signals controlling in which direction the chondrocytes mature have not been fully elucidated. Mature chondrocytes are completely encased in matrix, and have a limited ability to proliferate *in vivo*. In addition, due to their role of maintaining the ECM through secretion of its constituents, they possess prominent endoplasmic reticulum and golgi apparati [Temenoff & Mikos, 00a]. Although chondrocytes within articular cartilage are said to be of a single cell type, minor but important phenotypic differences exist between chondrocytes depending on their relative position in the tissue. For instance, chondrocytes located at the articular surface are more flattened in morphology and are known to express proteins required for lubrication [Schumacher *et al.*, 94]. In contrast, chondrocytes residing closer to the subchondral bone are rounded and express proteins that are required for matrix calcification [Xu *et al.*, 94].

Owing to the low cell density, articular cartilage is said to be a *matrix-continuous* structure, indicating that each cell is fully surrounded by extracellular matrix molecules, a feature that prohibits both cell-to-cell contact and cellular migration through the ECM. Despite their low numbers, the chondrocytes play a crucial role in regulating tissue properties through the

synthesis, secretion, organization and degradation of the surrounding ECM [Kinner *et al.*, 05;Muir, 95]. The mechanical characteristics of the tissue depend on the properties and constituents of the ECM, primarily: 1) collagens, which provide tensile strength. 2) Proteoglycans, which are negatively charged and trapped within the collagen network and attract cations and water, thus allowing the tissue to withstand compression. 3) The interstitial fluid whose composition influences cellular responses to mechanical loading [Hall *et al.*, 96].

1.1.6 Extracellular Matrix

The extracellular matrix of articular cartilage, which may be considered as a fiber-reinforced gel, is primarily composed of water and macromolecules such as collagens, proteoglycans, and noncollagenous proteins. Human articular cartilage has a substantial fluid content (primarily water but also dissolved ions, gases and metabolites), which corresponds to 60-80% of the total wet weight of the tissue. The remaining 20-40% of the total tissue weight is attributed to a variety of extracellular matrix molecules, primarily: collagens (50-70%, dry weight), proteoglycans (15-30%, dry weight), and other non-collagenous proteins (15-20%, dry weight) (Figure 2). The precise composition of macromolecules, along with the fluid phase, is responsible for the inherent structure and mechanical properties of articular cartilage; an interaction between the fluid phase and the various matrix macromolecules provides the tissue's stiffness and its resilience to mechanical loads [Pearle, Warren & Rodeo, 05].

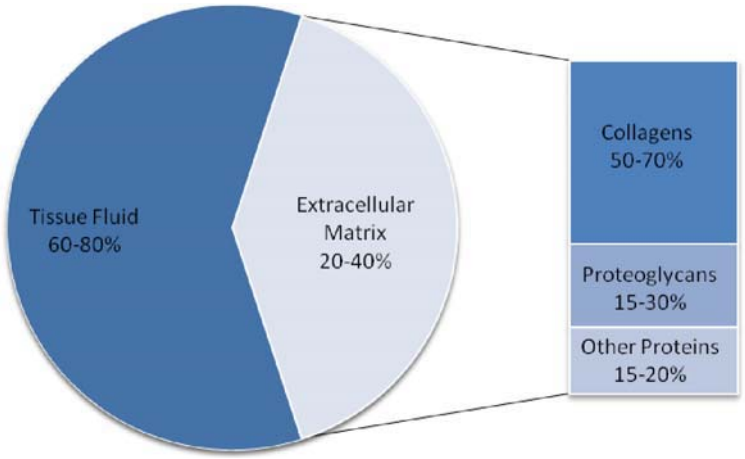


Figure 2: The composition of articular cartilage tissue by weight (chondrocytes excluded): The extracellular matrix is further divided into its constituent collagens, proteoglycan and other non-collagenous proteins.

Collagens, which constitute the major portion of dry weight of the ECM, are a family of proteins that consist of three polypeptide chains, called α chains, which have triple-helix configuration. To date, 43 distinct α chains have been identified and arrange to form at least 27 known proteins that are defined as collagens [Eyre, 04]. Depending on the particular type of collagen, the three constituent α chains may all be identical or different. However, regardless of the isoform, all α chains contain at least one domain composed of repeating –Glycine-X-Y sequences to facilitate the correct formation of the triple helix. Glycine, being the smallest amino acid, is required as larger amino acids would not fit into the restricted spaces in the centre of the triple-helix. Most collagens form supramolecular assemblies, such as fibrils and networks, and are divided into several subgroups based on structural and functional characteristics [Cremer *et al.*, 98]. While the most abundant and characteristic collagen of articular cartilage is Collagen II, other types are also found to a lesser extent: V, VI, IX, X and XI. The extensive cross-linking of the various collagen types is vital to the tissue's material strength [Eyre, 04;Poole *et al.*, 01]. A brief overview of the collagens found in articular cartilage tissue, and their respective roles, is

presented in the table below (Table I). Collagen type I, which is not normally found in the tissue, is often used as a marker for cellular dedifferentiation during culture [Schnabel *et al.*, 02].

Table I: Collagens are a key component of articular cartilage's extracellular matrix, and constitute the greater portion of the tissue's dry weight. They are a family of proteins that consist of three polypeptide chains bound in a triple-helix configuration. Listed below are the collagens, along with their respective functions, that are found in articular cartilage tissue.

Collagen	**Function**	**Reference**
Collagen I	Usually not present in healthy articular cartilage, and is used as a marker for cellular de-differentiation during culture	[Schnabel, Marlovits, Eckhoff, Fichtel, Gotzen, Vecsei & Schlegel, 02]
Collagen II	Forms the backbone of the cartilage heteropoymeric fibrils. Responsible for the tensile strength of tissue. Most prominent cartilage in healthy tissue.	[Cremer, Rosloniec & Kang, 98;Eyre, 04]
Collagen V	Participates in the formation of the fibrillar collagen network.	[Gelse *et al.*, 03]
Collagen VI	Ubiquitous matrix component of most tissues. Found predominately in the pericellular matrix (around chondrocytes). Interacts with a variety of ECM proteoglycans.	[Chang *et al.*, 97]
Collagen IX	Acts as a macromolecular bridge between collagen fibrils and between collagen fibrils and other ECM components. Regulates fibril size. Found predominately in the pericellular matrix (around chondrocytes).	[Poole, 97]
Collagen X	Secreted by chondrocytes in the zone of cartilage that is destined to be calcified, and in zones of secondary ossification. Provides support as the cartilage matrix is degraded during endochondral ossification	[Alini *et al.*, 94]

Proteoglycans, the second largest constituent of the ECM, are a specific class of glycoproteins that consist of glycoaminoglycan (GAG) chains covalently bound to the central core protein. The

most predominant proteoglycan found in articular cartilage is Aggrecan, which consists of a protein core and covalently bound keratan sulphate and chondroitin sulphate glycoaminoglycan chains. Aggrecan monomers bind to hyaluronan, an un-branched polysaccharide (Figure 3), to form large multi-molecular aggregates that are distributed within the collagen network (molecular weight in the millions). The interaction between the Aggrecan monomers and hyaluronan is facilitated and stabilized by a polypeptide termed Link Protein. Such distribution of proteoglycan aggregates serves several purposes: the intrinsic electrical charges of the proteoglycans provide a high fixed-charge, and the hydrophilic nature entraps water and thus allows the tissue to withstand compression.

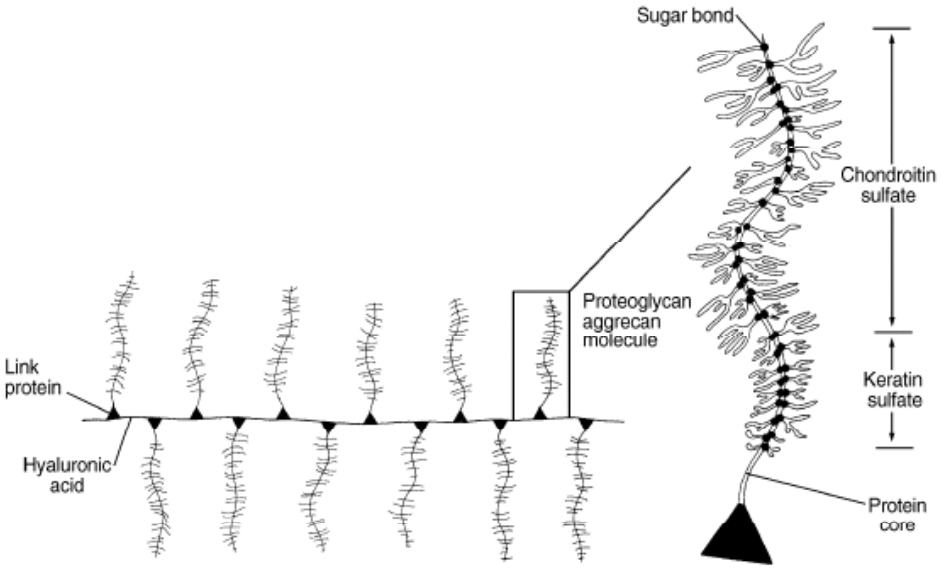


Figure 3: A diagram of the proteoglycan aggrecan, which consists of covalently bound keratan sulphate and chondroitin sulphate glycoaminoglycan (GAG) chains. Aggrecan monomers bind to hyaluronan, via link protein, to form large multi-molecular aggregates that are distributed within the collagen network of the articular cartilage. Aggrecan serves a primary role of providing the osmotic resistance necessary for cartilage to resist compressive loads. Diagram taken from (Pearle *et al.*, 2005)

Other smaller proteoglycans, belonging to a family of leucine-rich proteoglycans (SLRPs), are also found in the ECM, although to a lesser extent. Despite their relative low abundance in the ECM, knockout studies have indicated that they play a crucial role in providing matrix stability

(Knudson & Knudson, 2001;Roughley, 2006;Poole *et al.*, 2001). The proteoglycans, along with their respective roles are listed in the table below (Table II).

Table II: Proteoglycans, the second largest constituent of the ECM, are a specific class of glycoproteins that consist of glycoaminoglycan chains covalently bound to the central core protein. Listed below are they major proteoglycans found in articular cartilage tissue, along with their respective functions (where known). Table adapted from (Poole *et al.*, 2001)

Proteoglycan	Function
Aggrecan	Through binding to hyaluronan, via Link Protein, Aggrecan aggregates form a structural backbone that is resistant to compression and provides stiffness. It is also the majority proteoglycan by weight.
Perlecan	Located on the cell surface and facilitates cell-matrix interactions and adhesion.
Biglycan	Bind TGF-β. Found only in the pericellular matrix. Precise function has not been elucidated.
Decorin	Bind TGF-β. Found only in the interterritorial matrix. Regulates collagen fibril formation. Equimolar to aggrecan and is concentrated in pericellular sites.
Versican	Regulates the formation of collagen macrofibrils.

Lastly, other non-collagenous proteins constitute the remaining 15-20% of the ECM. Belonging to this group of proteins is Link Protein, which acts as a link between hyaluronan and Aggrecan, as well as several proteins involved in cell-matrix binding: Chondroadherin and CD44. Also present is Cartilage Oligomeric Protein that binds to Collagen Type II and is believed to be involved in macrofibril assembly.

1.1.7 Zonal architecture

Mature articular cartilage possesses a zonal architecture, and is often classified into four distinct zones in succeeding horizontal sections. From the articular surface and proceeding deeper to the subchondral bone, cartilage is separated into the superficial, middle (also referred to as transitional and tangential), deep (radial), and calcified zones (figure 4)[Pearle, Warren & Rodeo,

05]. The zones vary in their structure and composition, and it is suggested that each zone plays a discrete role in responding to mechanical loads. Additionally, though articular cartilage chondrocytes are categorized as possessing identical phenotypes, transient metabolic differences between chondrocytes of different sizes and shapes in different zonal locations have been observed in-vitro [Darling *et al.*, 04;Hauselmann *et al.*, 98;Hidaka *et al.*, 06;Schmidt *et al.*, 04].

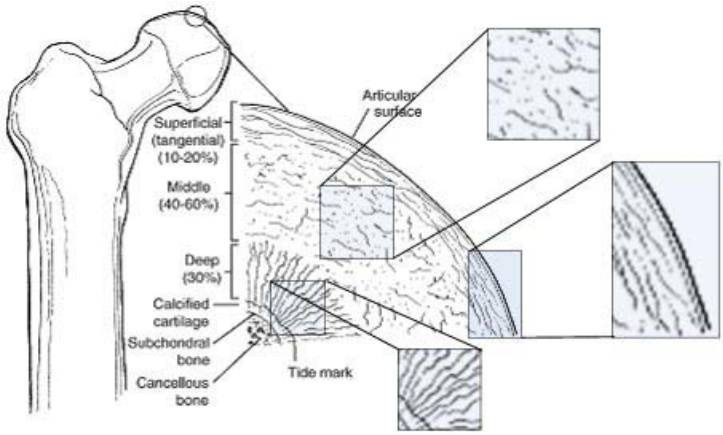


Figure 4: Mature articular cartilage possesses a zonal architecture, and can be classified into four distinct zones in succeeding horizontal sections. From the surface and proceeding deeper towards the subchondral bone are the: superficial, middle, deep and the calcified zones. The zones vary in their composition, fibril alignment, and the residing chondrocytes' phenotypes. It is suggested that each zone plays a discrete role in responding to mechanical loads. Illustration adapted from (Pearle *et al.*, 2005)

The superficial zone, which constitutes the upper-most 10-20% of cartilage, is the thinnest zone of articular cartilage and forms the gliding surface. In comparison to the other zones, the superficial zone has the highest content of water (~84%, wet weight), Type II collagen (~85%, dry weight) and the lowest content of proteoglycans (~15%, dry weight). The densely packed collagen fibres, collectively known as *lamina splendens*, are thin in diameter and are oriented parallel to the articular surface. The parallel arrangement determines the mechanical properties of the tissue, by providing tensile strength that aids in resisting shear stresses produced during movement. It has also been speculated that such an arrangement acts as a barrier, and prevents the passage of larger molecules (e.g. antibodies) from the synovial fluid into cartilage. The

resident chondrocytes possess a decreased cellular volume, and are flattened along the direction of shear stress (parallel to surface) [Huber, Trattnig & Lintner, 00]. Of the proteoglycans found in this zone, decorin and biglycan constitute the predominant ones [Poole *et al.*, 96]. An additional proteoglycan, Superficial Zone Protein (SZP), is uniquely expressed in this zone and is involved in the lubrication at the joint-cartilage interface [Schumacher, Block, Schmid, Aydelotte & Kuettner, 94]. Recent studies suggested that the superficial zone contains a subpopulation of chondrocytes with progenitor-like characteristics, and it has been speculated that the cells may have the capability to produce cartilage with healthy structural and biochemical properties [Dowthwaite *et al.*, 04].

Directly below the superficial zone is the intermediate (also known as transitional or middle) zone, which corresponds to the largest the zone in terms of thickness (40-60% of full thickness). Due to the layer's purpose of serving as a transition between the superficial and deep zones, the contents of water, collagen and proteoglycans (of which aggrecan is the most prominent) are intermediate and are in-between the contents of the superficial and deep zones [Pearle, Warren & Rodeo, 05]. The relative abundance of proteoglycans provides the middle zone with the appropriate mechanical properties that are required to withstand and absorb the compressive forces transmitted to the joint. The chondrocytes in this zone are spherical and are rich in synthetic organelles such as the Endoplasmic Reticulum and Golgi bodies. The collagen fibrils are larger compared to superficial zone and are arranged randomly with respect to the articular surface [Poole, Kojima, Yasuda, Mwale, Kobayashi & Laverty, 01]. A characteristic protein restricted to the middle zone is the Cartilage Intermediate Layer Protein (CILP), and while its exact function has not been fully elucidated evidence suggests its amount increases with age [Mori *et al.*, 06].

The deep (or radial) zone has the lowest water and collagen contents, however the collagen fibrils are of the largest diameter and proteoglycan content is maximal. The chondrocytes possess several properties that are usually attributed to hypertrophic-like chondrocytes, primarily: expression of alkaline phosphatase and collagen Type X. The cells are aligned in columns perpendicular to the articular surface (as well as to the subchondral plate). Similarly, the collagen fibres are oriented perpendicular to the joint surface and penetrate the underlying calcified cartilage which strengthens the bonding of cartilage to bone. Immediately below the deep zone is the tidemark which serves as an interface between the deep zone and the calcified layer. The tidemark has not been fully characterized; however it is known to contain crystals of calcium salts and hyaluronan. The tidemark is often considered to be the boundary between cartilage and bone [Huber, Trattnig & Lintner, 00].

The calcified layer facilitates the transmission of forces across the joints and serves to anchor the cartilage to the subchondral bone. Although during development the chondrocytes of the calcified zone are active, the cells become quiescent as the cartilage ages and mineralizes. Consequently, the calcified zone contains relatively few metabolically active cells that are entrapped within a calcified matrix. Despite the mineralization, however, the arrangement of collagen fibers in the calcified layer makes it less stiff than bone. The stiffness of the zone (elastic modulus = 0.32 GPa) is approximately an order of magnitude greater than non-mineralized cartilage, but an order of magnitude less than bone [Mente & Lewis, 94]. This apparent gradient in stiffness, from non-mineralized cartilage to bone, is essential for the proper functionality of cartilage as it allows the appropriate distribution of forces along the joint [Huber, Trattnig & Lintner, 00].

1.1.8 Pericellular Matrix

In addition to horizontal subdivisions into the respective zones, a circumferential differentiation of matrix components exists around individual chondrocytes. Each of the zones can be further divided into three distinct regions or matrices: the pericellular, territorial and interterritorial regions/matrices [Poole, 97]. While each of the regions plays a crucial role in the functionality of the tissue (territorial regions allows for chondrocyte attachment to the ECM, and the interterritorial region contributes to the mechanical properties of the tissue), the pericellular matrix (PCM) region is of particular interest.

Within the ECM individual chondrocytes are surrounded by a narrow region of matrix, termed the pericellular matrix, that is characterized by a higher concentration of proteoglycans, finer arrangement of collagen fibres, and the presence of Collagen Type VI [Poole, 97]. Together, the PCM and the surrounded chondrocyte have been grouped to a form a basic structural unit termed the "chondron". In each chondron, the chondrocyte is linked at its surface to a filamentous coating of carbohydrate-rich molecules and enclosed by a fibrillar pericellular capsule [Guilak *et al.*, 06]. Although the precise function of the pericellular matrix is not fully elucidated, considerable evidence suggests it is involved in the regulation of biochemical and biomechanical interactions between the cells and the surrounding ECM [Alexopoulos *et al.*, 05;Graff *et al.*, 03;Guilak *et al.*, 05]. It is well accepted that in healthy articular cartilage the residing chondrocytes must function synergistically with the ECM in order to absorb, redistribute and transmit physiological compressive and shearing forces to the subchondral bone. An interaction between the cell surface and ECM components significantly influences metabolism, gene expression and cellular responses to growth factors. Because chondrocytes are completely surrounded by the PCM any signals – of either mechanical or chemical nature – originating in

the ECM must pass through the pericellular environment before they are sensed by the chondrocytes. As such it has been hypothesized that the PCM may function as a transducer of biomechanical and biochemical signalling, selectively trapping, modifying, or retaining soluble mediators such as growth factors [Larson *et al.*, 02;Lee *et al.*, 00]. The lower permeability of the PCM, relative to the ECM, has been postulated to inhibit fluid flux near the cells by a factor of 30, thus having a considerable effect on convective transport and diffusion from and to the chondrocyte [Alexopoulos, Setton & Guilak, 05]. Similarly, it has been proposed that any direct interactions between cell surface receptors and the ECM must be facilitated by constituent macromolecules present within the PCM. In fact, it has been previously shown that retention of native PCM significantly influences the metabolic activity of chondrocytes *in vitro* [Larson, Kelley, Blackwood, Banes & Lee, 02].

It has also been suggested that the primary function of the PCM is to provide a protective effect for the chondrocytes during loading through an "adaptive water loss from the PCM proteoglycans" [Poole, 97]. Several investigators have supported the notion that a chondron acts as a compression resistant, fluid filled 'bladder' which is able to absorb mechanical loads, deform and recover completely when unloaded. Recent studies have shown that the observed mismatch between elastic moduli of the PCM and ECM results in a significant stress shielding effect for the chondrocytes [Alexopoulos, Setton & Guilak, 05].

While the PCM's composition is similar to that of the ECM, distinct differences in collagen and proteoglycan composition exist. Of the collagens found in articular cartilage, Collagen Type VI (see Table I) is found exclusively in the pericellular region and is used as a marker for chondrons [Poole, 97]. Supporting the idea of the PCM as a signal transducer is the ability of Type VI collagen to interact with a wide array of ECM components including biglycan, hyaluronan,

perlecan, fibromodulin as well as Type II collagen [Bidanset *et al.*, 92;Kielty *et al.*, 92]; in addition, it also serves as a site of chondrocyte attachment [Alexopoulos, Setton & Guilak, 05]. Similarly, type IX Collagen is also concentrated within the PCM, however, smaller quantities may also be found in the territorial matrix - none is found in the interterritorial regions [Poole, 97]. In terms of proteoglycans, the region has been shown to be rich in decorin.

1.2 Cartilage Pathology & Tissue Engineering

The loss of articular cartilage, typically through injury or disease, often results in limited mobility, pain, and debilitating effects in individuals. It is estimated that cartilage degeneration due to osteoarthritis and sports-related injuries, afflicts some 36 million individuals in North America [Temenoff & Mikos, 00a]. As the population ages, the number of individuals with joint damage is only expected to increase and burden both the health care system and the population.

1.2.2 Articular Cartilage Injury & Repair Obstacles

Articular cartilage injuries may be classified into three distinct categories: matrix disruptions, partial thickness defects, and full thickness defects. In Matrix disruptions a blunt trauma force results in damage to the ECM, but in many cases the resident chondrocytes are able to replenish ECM macromolecules through increased anabolic activity. In more severe injuries, such as partial thickness defects, disruption of cartilage tissue occurs but does not propagate to the subchondral bone. While a cellular response is initiated to repair the damage, for reasons yet unknown, the cellular response is terminated before the tissue is repaired. In the most severe type of injury, damage transverse the entire thickness of the tissue and penetrates the subchondral bone. In such cases, the defect is filled with a fibrin clot and classical wound healing ensues. However, the resultant tissue displays a fibrocartilage phenotype, and possesses inferior

mechanical properties that result in the eventual degradation of the tissue over a period of years [Temenoff & Mikos, 00a].

Interestingly enough, the 200-year-old observation that "cartilage once destroyed never heals" (as stated by Hunter in 1743[1]) remains appropriate even today, as adult articular cartilage possesses a very limited capacity for self-repair. The tissue's avascular nature, the limited proliferative and metabolic capacity of chondrocytes, along with the inability of chondrocytes to migrate through the ECM are all factors that preclude proper tissue healing [Hunziker, 99;Coutts *et al.*, 97].

While clinical cartilage restoration techniques are available, all possess limitations and are of debatable effectiveness. Briefly, in patients with smaller lesions, attempts are made to restore joint-surface cartilage through surgical implantation of autografts or through utilizations of techniques that encourage native tissue repair processes [O'Driscoll, 98]. In the former technique, cartilage defects are replaced with smaller plugs from a less weight-bearing region of the joint. While the procedure has been shown to decrease pain in some patients, it suffers from several limitations: 1) the resultant damage to the donor site, 2) the amount of cartilage available for transplantation, 3) discrepancy between native and transplanted tissue properties, and 4) the difficulty to match the topology of the grafts with the injured site. As such, the long-term success of this approach remains to be verified [Martin *et al.*, 07]. Other techniques aiming to enhance native tissue regenerative properties through pharmacological agents, electrical stimulation, or even transplantation of extra chondrocytes have not been completely successful and the effectiveness decreases with the patients' age and extent of damage [O'Driscoll, 98]. In cases where cartilage damage is severe, the optimal treatment constitutes the replacement of

[1] Hunter W (1743) Philos Trans 470:514

articulating joints with synthetic prosthesis (i.e. hip replacements). However, failure rates as high as 20% have been reported after seemingly successful implantations [Soderman *et al.*, 01].

As a result from the various limitations of currently available clinical approaches, tissue engineering has emerged as a novel therapeutic alternative for tissue regeneration. It is hoped that tissue engineering approaches would allow the properties (structural, biological, biomechanical) of grafted tissues to be specifically tailored to the affected joints [Beris *et al.*, 05].

1.2.3 Tissue Engineering Approaches

Because of current surgical limitations in the repair of complex cartilage lesions, tissue engineering holds great promise for the generation of functional tissue *in vitro* for subsequent implantation *in vivo*. In general, tissue engineering of articular cartilage can be achieved through three different strategies: utilizing culture dishes, scaffolds, or substrates.

The first approach, termed autologous chondrocyte implantation, utilizes the patient's own chondrocytes to encourage cartilage repair and regeneration. In this strategy a sample of the patient's cartilage is harvested and the resident chondrocytes are isolated and expanded, through growth in monolayer culture, *in vitro*. Following a period of expansion, cells are collected, transplanted back into the defect site, and confined there utilizing a periosteal flap [Gillogly *et al.*, 98]. Although there is evidence that the treatment is effective at alleviating pain, the resulting tissue is at least partially fibrocartilaginous and thus is subject to degradation over time [Breinan *et al.*, 97]. Moreover, while it is relatively easy to grow cells in a 2D monolayer, a limiting feature to this technique is the inability to shape the cells into a pre-defined, three-dimensional shape that is able to perfectly fit the defect site. Likewise, continued monolayer expansion is known to result in an irreversible loss of chondrocyte-phenotype and cellular dedifferentiation

which results in inferior extracellular matrix that is inadequate for articulating joints [Schnabel, Marlovits, Eckhoff, Fichtel, Gotzen, Vecsei & Schlegel, 02;Martin, Miot, Barbero, Jakob & Wendt, 07;Darling & Athanasiou, 05].

The second strategy involves the seeding of cells into porous materials (scaffolds) that may be untreated or coupled with chemicals, such as growth factors, to promote cell and/or matrix in-growth. Upon seeding with cartilage-forming cells the scaffold is grown in culture and subsequently implanted directly into the defect site [Martin, Miot, Barbero, Jakob & Wendt, 07]. Because the chondrocytes are cultured in a 3D structure, which may be created in a variety of geometries, they allow a better approach to fix irregularly-shaped defects. More importantly, however, growing chondrocytes in 3D has been shown to be conducive to the maintenance of cell phenotype and cell retention in the defect site [Stewart *et al.*, 00]. Scaffolds may be constructed utilizing either biodegradable or non-biodegradable components to further tailor to individual needs. Unfortunately, several studies have shown that the resulting tissue possesses inferior mechanical properties and low collagen content, as compared to native tissue. It has been hypothesized that the absence of weight bearing, or mechanical loading, during tissue formation is required for proper ECM gene expression and protein synthesis to occur. Additionally, because substrates cannot be anchored fully in the implanted tissue, they may be dislodged by shearing forces during locomotion [Temenoff & Mikos, 00a].

As all the previous strategies have limitations, recent approaches to cartilage tissue engineering have focused on developing biphasic articular cartilage constructs that include a bone-interfacing component. The biphasic constructs are composed of cartilage tissue formed on the top of a substrate that serves as the bone interface component. Similar to scaffolds, substrates are porous, which allows for bone in-growth and subsequent anchorage in the underlying bone. The

substrates may be either biodegradable or non-biodegradable and may be coupled to molecules modulating tissue growth. The substrate acts to both support cartilage formation and facilitates fixation after implantation. Moreover, as the cartilage tissue is already formed prior to implantation, lateral integration to the adjacent native cartilage is possible [Grynpas *et al.*, 02;Redman *et al.*, 05;Kuo *et al.*, 06].

To enable the construction of a biphasic construct for tissue implantation, a suitable bone substitute must be used. The ideal material should exhibit several properties in order to be considered adequate. Firstly, the material must not be cytotoxic and allow for the proper attachment and growth of seeded chondrocytes, as well as the formation of cartilage tissue. In addition, the substitute bone material should be osteoconductive, as to allow rapid integration with the host bone, and biodegradable (at a predetermined rate) in order to be eventually replaced by the in-growing host bone. As importantly, the substrate must be strong enough to fulfill required load-bearing functions before significant bone in-growth occurs, and the degradation products of the substrate must not induce an inflammatory reaction that may jeopardize the implantation [Coutts *et al.*, 01].

1.2. 4 Calcium Poly-Phosphate (CPP) and Biphasic Constructs

Our lab has previously developed and characterized a porous calcium polyphosphate (CPP) substrate that possesses mechanical properties approximating cancellous bone. Utilizing the CPP we are able to form biphasic constructs *in vitro*, consisting of cartilaginous tissue grown on top of the porous ceramic substrate (Figure 5) [Kandel *et al.*, 06]. Owing to the porous properties of CPP (37 vol.% porosity), chondrocytes are able to enter into the pores of the immediate subsurface and produce cartilaginous tissue that is anchored to the surface of the substrate [Pilliar *et al.*, 01]. Likewise the presence of interconnected empty pores in the substrate allows for bone

in-growth after implantation, and thus providing a secure fixation into the host bone. The substrate is not cytotoxic to the cells and the resulting tissue has been previously shown to resemble the native tissue and to contain Collagen Type II and various characteristic proteoglycans. CPP is biodegradable and the degradation products, calcium and phosphate, do not incite an inflammatory reaction; as the substrate degrades with time, it will ultimately be replaced with natural host bone [Grynpas, Pilliar, Kandel, Renlund, Filiaggi & Dumitriu, 02]. Unfortunately, similar to the cartilage formed in scaffolds, the cartilage tissue formed contained less ECM and possesses only a fraction of the mechanical properties of native tissue. Such mechanical deficiencies may affect the tissue's ability to resurface a large joint defect and to provide adequate load-bearing functionality to the defect site [Waldman *et al.*, 02].

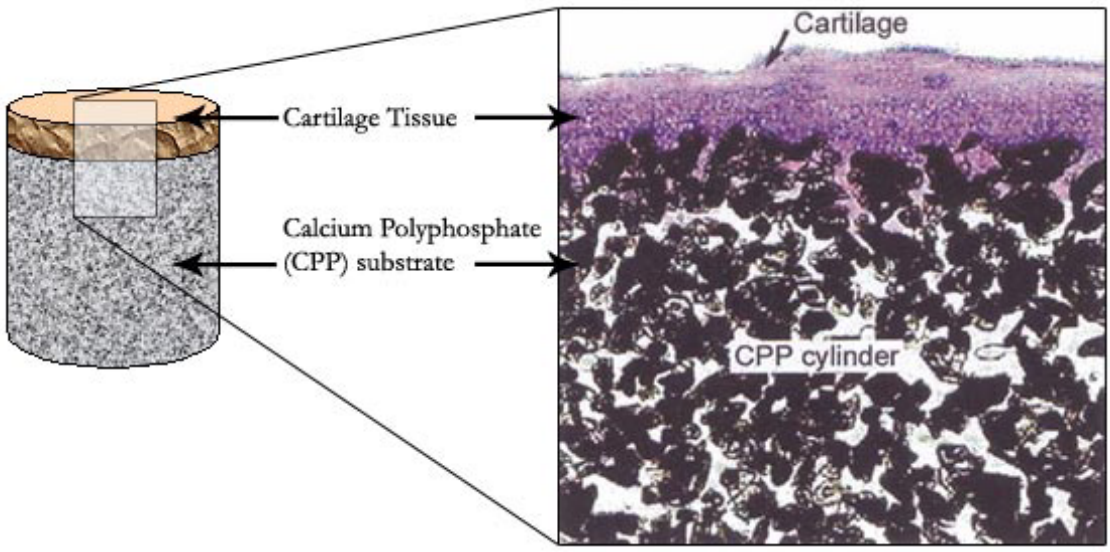


Figure 5: A diagrammatic representation (left) of a biphasic construct of in-vitro formed articular cartilage tissue grown on Calcium Polyphosphate (CPP) substrate. On the right is the corresponding histogram of a 4-week-old articular cartilage tissue as grown in our lab.

1.3 Mechanical stimulation

It has been known for years that the mechanical environment of a tissue regulates its growth, development and, when required, its remodelling [Grodzinsky, Levenston, Jin & Frank, 00;Kim *et al.*, 94;Beaupre *et al.*, 00]. On a cellular level mechanical forces have been shown to influence cellular function through regulation of gene and protein expression, induction of differentiation, apoptosis and/or maintenance of cellular homeostasis [Grodzinsky, Levenston, Jin & Frank, 00;Wang & Thampatty, 06]. Although the underlying mechanisms by which cells sense mechanical forces and convert them into biological responses are not fully understood, many cell types (i.e. Tendon and ACL fibroblasts, Cardiac Fibroblasts, smooth muscle cells, and chondrocytes) have been shown to respond to changes in their mechanical environment [Grodzinsky, Levenston, Jin & Frank, 00;Waldman *et al.*, 03;Yang *et al.*, 04;Hsieh *et al.*, 00;Lee *et al.*, 99]. While the underlying mechanotransduction signalling pathways have not been fully elucidated, several potential signalling events have been suggested to be the primary triggers which ultimately influence cell functionality. These extracellular events include cell deformation, increased hydrostatic pressure, compression of the charged extracellular matrix, changes in osmolarity and pH, as well as fluid flow [Zhang *et al.*, 06;Knight *et al.*, 06b;Knight *et al.*, 06a;Guilak, Alexopoulos, Upton, Youn, Choi, Cao, Setton & Haider, 06].

As chondrocytes are found in a load-bearing tissue, they are exposed to a wide variety of mechanical forces, including tension, compression and shear. It has been demonstrated that, under physiological conditions, the presence of mechanical stimulation is required for the development and maintenance of healthy articular cartilage tissue [Eckstein, Hudelmaier & Putz, 06]. In fact, it is now evident that articular cartilage undergoes atrophy under reduced loading conditions such as postoperative immobilization. On a cellular level, mechanical loading of

articular cartilage tissue results in cell deformation, hydrostatic pressure gradients, fluid flow, deformation of the charged extracellular matrix and the accompanying changes in osmolarity and pH [Grodzinsky, Levenston, Jin & Frank, 00;Huber, Trattnig & Lintner, 00]. These extracellular events are believed to influence the biosynthetic activity of chondrocytes through an array of intracellular events such as modulation of the cytoskeleton, nucleus deformation and alterations of intracellular concentrations of ions - namely Ca^{2+} [Wong & Carter, 03]. Experimentally, chondrocytes have been shown to be mechanosensitive *in-vitro*, and depending on the magnitude, duration (static vs. dynamic) and type of strain (i.e., osmotic, shear, compression), have altered their biosynthetic activity. In general, high frequencies and low-moderate strains are associated with increased biosynthetic activity, while static compression, low frequency or high strains have resulted in a decreased synthesis [Davisson *et al.*, 02;Mauck *et al.*, 00;Waldman, Spiteri, Grynpas, Pilliar, Hong & Kandel, 03;Waldman *et al.*, 04;Waldman *et al.*, 06].

Because of the vital role mechanical loading plays in the development and maintenance of healthy cartilage *in vivo*, it has been suggested that the application of mechanical stimulation may be beneficial to the *in-vitro* formation of cartilage tissue. Several studies have demonstrated that loading chondrocytes, in a controlled manner, results in improved tissue formation [De Croos *et al.*, 06;Waldman, Couto, Grynpas, Pilliar & Kandel, 06]. For instance, the application of fluid-induced shear forces to chondrocytes seeded in monolayer has been shown to increase proteoglycan synthesis. Similarly, application of mechanical forces to cartilage explants, isolated chondrocytes grown in scaffolds and/or the surfaces of substrates has resulted in the stimulated synthesis of cartilaginous ECM macromolecules [Shieh & Athanasiou, 03].

In our lab, we have previously shown that a single application of cyclic compression results in a greater accumulation of ECM macromolecules, and the consequent improvement in the tissue's

mechanical properties (Figure 6). Specifically, the application of uniaxial cyclic compressive forces of approximately 9.81 mN (equivalent to 1g) for 30 minutes at 1Hz have significantly enhanced ECM production (15-30%) and improved the mechanical properties of the tissue (both load-bearing capacity and stiffness) by twofold [Waldman, Couto, Grynpas, Pilliar & Kandel, 06].

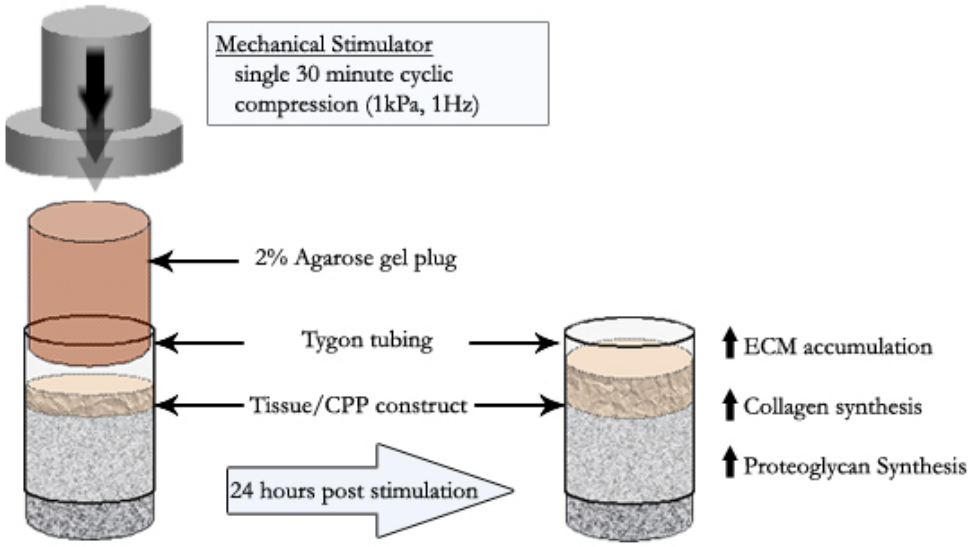


Figure 6: A diagrammatic representation of the application of confined compressive mechanical forces 30 min, 1gm, 1Hz) through a compliant agarose plug on *in vitro* formed cartilage tissue grown on Calcium Polyphosphate (CPP) substrate.

Studies aiming to elucidate the underlying mechanism(s) regulating the tissue's response to cyclic compression have demonstrated the existence of a biphasic response, composed of sequential catabolic and anabolic changes, in response to this mechanical stimulation. Briefly, it was shown that mechanical stimulation induces an ERK-mediated upregulation of Matrix Metalloproteiase (MMP)-3 and MMP-13 (2 hours post stimulation) through activation of the AP-1 consensus sequence [De Croos, Dhaliwal, Grynpas, Pilliar & Kandel, 06]. AP-1 consensus sequences are found in most MMPs, and transcriptionaly regulate their activity [Crawford & Matrisian, 96]. Following the upregulation of MMP-13 and MMP-3, which resulted in increased

tissue degradation, significant increases in type II collagen and aggrecan gene expression was observed, ultimately resulting in increased synthesis and accumulation of the tissue's ECM (24 hours post stimulation)[De Croos, Dhaliwal, Grynpas, Pilliar & Kandel, 06]. The upregulation of MMP-13, in turn, was shown to be dependent upon the activation of Membrane Type-1 MMP (MT1-MMP), which is known to be mechanosensitive in several cell types [Yamaguchi *et al.*, 02;Bradley *et al.*, 01] and is believed to be crucial to matrix remodelling [Holmbeck *et al.*, 99]. The activation of MT1-MMP, which was facilitated through ERK induced binding of early growth factor-1 (Egr-1) to the MT1-MMP promoter, is believed to cause degradation in the pericellular matrix and thus elicit the anabolic phase of the remodelling response [De Croos *et al.*, 07]. When the MT1-MMP upregulation was blocked, through decoy MT1-MMP oligonucleotide or through MT1-MMP siRNA, the increase in tissue ECM accumulation was not observed following mechanical stimulation (submitted).

Since several studies have suggested that MT1-MMP is important in regulating cell morphology (for instance, it regulates the appropriate cell shape and cytoarchitecture required for adipose differentiation and allows cells to adjust their cellular architecture in response to matrix stiffness [Beningo *et al.*, 04;Chun *et al.*, 06]) and owing to the role cell morphology plays in facilitating chondrocytes' response to mechanical stimuli [Campbell *et al.*, 07;Knight, Toyoda, Lee & Bader, 06b;Spiteri *et al.*, 08;Smith *et al.*, 95], the relationship between MT1-MMP and cell shape changes following mechanical stimulation was investigated. It was observed that following cyclic compression chondrocytes underwent a rapid, transient cell spreading and returned to pre-stimulated morphology within six hours. This spreading and retraction was associated with increased accumulation of newly synthesized proteoglycan and collagen. Chondrocyte spreading was shown to be dependent upon the $\alpha 5\beta 1$ integrin, as blocking it (or the $\beta 1$ subunit) abolished

spreading. However blocking the integrin only partially inhibited the compression-induced increase in matrix accumulation, suggesting other mechanosensitive components may be involved. It was further shown that cell retraction following mechanical stimulation is partially mediated by MT1-MMP, as silencing MT1-MMP inhibited both the cellular retraction and matrix accumulation (submitted).

While it is apparent that both the morphological changes (spreading/retraction) and the upregulation of MT1-MMP are correlated and crucial to facilitating the chondrocytes' response to cyclic compression, the underlying mechanisms of mechanotransduction are still not understood.

1.3.2 Mechanotransduction

Generally speaking, mechanotransduction refers to the cellular processes that are responsible for the conversion of mechanical stimuli into biochemical signals which allow cells to adapt and respond to surrounding environmental cues. These cues, in turn, are known to influence nearly every single cellular activity, including growth, differentiation, migration, apoptosis, gene expression and protein synthesis [Alenghat & Ingber, 02a]. While the type and the strength of the applied mechanical stimuli vary considerably in different tissues (i.e. vascular endothelial cells are experience shear forces totalling a few pascals, cartilage may experience compressive forces up to 20MPa) all mechanotransduction processes can be divided into four general phases[Millward-Sadler & Salter, 04b;Urban, 94]:

I. Mechanocoupling – forces at the tissue level are translated into local actions at the surfaces of the residing cells.

II. Coupling – forces at the cell surface (outside) are transduced into biochemical cells in the cytoplasm (inside)

III. Signal transmission – signal transduction takes place to the effector target (i.e. the alteration of cellular gene expression)

IV. ECM coupling – Changes in the effector cell line result in changes in the ECM and/or the altered ECM environment results in the modification of intracellular signalling.

Furthermore, the cellular responses to mechanical stimuli can be separated into rapid responses (seconds to minutes) or long-term responses (hours to days). Rapid responses arise as a result of the activation of various intracellular signalling pathways, which ultimately result in changes in the ECM composition or tissue functionality [Millward-Sadler & Salter, 04b].

Despite the crucial role mechanotransduction plays in cellular functions, cellular survival, and disease (i.e. deafness, muscular dystrophies, cancer, and developmental disorders), the

underlying mechanisms have not been fully elucidated yet [Jaalouk & Lammerding, 09]. A key feature, distinguishing mechanotransduction from other types of signal transduction, is the lack of a ligand-mediated activation of cell surface receptors; instead, activation occurs through mechanical stimuli induced distortions in the cellular membrane, surrounding ECM, or the cells' internal components [Alenghat & Ingber, 02b;Orr *et al.*, 06]. These generalized physical changes are sensed, and converted to a biochemical signal, by several classes of cellular components, namely integrins (which are localized in focal contacts between the cell and the ECM) and ion channels (which are located throughout the cellular membrane).

1.3.3 Integrins as Mechanosensors

Cell adhesion to the surrounding ECM plays multiple roles in assembling, maintaining and coordinating tissues in multicellular organisms [Huveneers & Danen, 09]. However, besides functioning as structural anchor points, cell-matrix adhesion sites play a crucial role in transmitting mechanical stimuli into cells through coupling to integrins and the actin cytoskeleton. The precise composition of the adhesion complex (its maturity) and the nature of the underlying ECM both regulate how external forces are sensed by the cells.

Adhesion Complexes:

One of the earliest abilities of cells to respond to mechanical forces requires the attachment of cells to the underlying ECM. Recently it has been suggested that cell-associated hyaluronan regulates the early stages of cell attachment, particularly in chondrocytes [Cohen *et al.*, 03;Zimmerman *et al.*, 02;Cohen *et al.*, 06]. Studies have shown that treatment of cells with hyaluronidase drastically reduces cell attachment, and that longer periods of time are required for these cells to firmly adhere to the substrate; specifically, it was determined that hyaluronan-

mediated attachment occurs within seconds after the cell's initial encounter with a surface [Zaidel-Bar *et al.*, 04;Zimmerman, Geiger & Addadi, 02].

The hyaluronan-medicated adhesion, which is transient, is replaced within a few minutes with integrin-containing contacts (integrins are discussed below). The earliest integrin-containing adhesion sites are termed focal complexes (FX) and are formed by the activation of small Rho-family GTPase Rac [Rottner *et al.*, 99]. These focal complexes are small, dot-like and provide early cell attachment in protruding areas such as the leading edge. The componential makeup of FXs is dynamically regulated in response to cell signals and the exogenous tension (i.e. matrix rigidity), and may vary in different cell types[Berrier & Yamada, 07]. Nonetheless, it is currently believed that the earliest molecules present at the FX are the $\alpha_V\beta3$ integrin, talin and paxilin. As the FXs mature (for example as a result of increased stress) additional molecular components may be added including, vinculin, α-actinin, focal adhesion kinase (FAK), Arp2/3 [Zaidel-Bar, Cohen, Addadi & Geiger, 04]. Focal complexes are highly dynamic and last for a few minutes before being replaced, dismantled or, through the activation of Rho, mature into focal adhesions (FA)[Rottner, Hall & Small, 99]. Focal adhesions are more pronounced in size and involve the additional recruitment of molecules such as zyxin and the concomitant assembly of an actin-bundle. This transitions appears to be actomyosin-driven and involved contractility of the membrane that results in force generation at the cell-matrix adhesion site [Zaidel-Bar, Cohen, Addadi & Geiger, 04;Berrier & Yamada, 07]. While the biological relevance of FA was initially uncertain, in recent years it was shown that FAs are found *in vivo* in areas of high fluid shear stress (i.e. blood vessels) and that they play a crucial role during development [Cukierman *et al.*, 01;Romer *et al.*, 06]. It is believed that local mechanical forces activate the growth and maturation of FAs [Riveline *et al.*, 01].

Despite their size FA remain highly dynamic with various components, in particular vinculin and α-actinin, interchanging between their FA-associated and cytoplasmic pools. In addition, FA can further mature into fibrillar adhesions (FBs) in which the α5β1 integrin associates with fibronectin fibrils [Zamir *et al.*, 99]. Visually, FBs appear as elongated fibrils or an array of dots and are localized to more central regions in cells. Unlike FAs, which contain high levels of paxillin and vinculin, FBs possess high levels of tensin and little phosphotyrosine[Zamir, Katz, Aota, Yamada, Geiger & Kam, 99]. It has been suggested that FBs play a role in matrix reorganization, as the pliability of the ECM was shown to influence FB assembly [Katz *et al.*, 00]. While the exact mechanism regulating the transformation of FAs into FBs has not been fully elucidated, it was demonstrated that c-Src-null cells fail to form FBs, indicating that c-Src may be involved in this process [Volberg *et al.*, 01].

Integrins:

The adhesion of the cells to the surrounding ECM is mediated, in part, by specialized transmembrane adhesion receptors termed integrins. This receptor family encompasses several non-covalently bound heterodimeric transmembrane proteins composed of an α (120-180 kDa) and β (90-110 kDa) subunits. Within each subunit is found a large extracellular domain, which interacts with various ECM components, a single transmembrane spanning domain, and a short cytoplasmic tail which interacts with various adapter molecules in the cytoplasm [Humphries, 00]. Currently 24 different mammalian receptors are known, which are composed of 18 different α and 8 different β subunits; the specificity of the integrin towards a particular ligand and its downstream cellular effect are regulated by the respective subunits (Table III) [Brakebusch & Fassler, 05;Takada *et al.*, 07]. Integrins are able to recognize a wide array of physiological ligands, including both soluble and surface-bound proteins. Binding of ligands attached to a rigid

surface results in the generation of force at the focal contacts, and directly influences the cell's contractile apparatus. Integrin binding to their respective ligands requires the presence of divalent cations [Arnaout *et al.*, 05].

Table 3: The currently known mammalisn integrins and their ligand-binding specificities, adapted from [49].

Integrin	Ligand	Integrin	Ligand
α1β1	Laminin, collagen	αLβ2	ICAM, ICAM-4
α2β1	Laminin, collagen, thrombospondin, E -	αMβ2	ICAM, iC3b, factor X, fibrinogen, ICAM-4, heparin
α3β1	Laminin, thrombospondin,	αXβ2	ICAM, iC3b, fibrinog en, ICAM -4,
α4β1	Thrombospondin, MAdCAM-1, VCAM -1,	αDβ2	ICAM, VCAM-1, fibrinogen, fibronectin, vitronectin, Cyr61, plasminogen
α5β1	Fibronectin, osteopontin, fibrillin, thrombospondin, ADAM, COMP, L1	αIIbβ3	Fibrinogen, thrombospondin,, fibronectin, vitronectin, vWF, Cyr61, ICAM-4, L1, CD40 ligand
α6β1	Laminin, thrombospondin, ADAM, Cyr61	αVβ3	Fibrinogen, vitronectin, vWF, thrombospondin, fibrillin, tenascin, PECAM-1, fibronectin, osteopontin, BSP, MFG-E8, ADAM-15, COMP,
α7β1	Laminin	α6β4	Laminin
α8β1	Tenascin, fibronectin, osteopontin, vitronectin,	αVβ5	Osteopontin, BSP, vitronectin, CCN3, LAP-TGF-β
α9β1	Tenascin, VCAM -1, osteopontin, uPAR, plasmin, angiostatin,	αVβ6	LAP-TGF-β, fibronectin, osteopontin, ADAM
α10β1	Laminin, collagen	α4β7	MAdCAM-1, VCAM -1, fibronectin, osteopontin
α11β1	Collagen	αEβ7	E-cadherin
αVβ1	LAP-TGF-β,	αVβ8	LAP-TGF-β

Many integrins are known to exist in different activation states (active, intermediate, and inactive) which correspond to their relative affinity states towards extracellular ligands. Utilizing these different affinity states integrins are utilized by cells for both outside-in and inside-out signalling, whereby cytoplasmic changes influence the integrins ability to bind/attach extracellular ligands (inside-out) which then is able to influence cellular functionality (outside-in) [Ingber, 03]. Integrins utilize their cytoplasmic domains, commonly referred to as the tail, to elicit various intracellular signalling responses through interaction with various adapter proteins and the actin cytoskeleton. Owing to the intrinsic lack of enzymatic activity, and lack of an actin binding domain, all of the integrins' functionality is facilitated through integrin associated adapter molecules [Arnaout, Mahalingam & Xiong, 05].

Owing to the rich extracellular matrix surrounding chondrocytes, and the known role of integrins to interact with ECM components, integrins have long been suggested as one mechanism by which chondrocytes sense and respond to mechanical forces. Previous studies have suggested that integrins mediate multiple aspects of chondrocyte mechanotransduction in response to compressive and shear forces [Spiteri, Young, Simmons, Kandel & Pilliar, 08;Millward-Sadler & Salter, 04a;Onodera *et al.*, 05]. In particular, chondrocytes have been shown to secrete interleukin-4 in response to mechanical compression through a signalling cascade activated by the α1β1 integrin; when chondrocytes were stimulated in the presence of a GRDDSP peptide, which competes for the integrin-matrix Ligand binding spots, the response was abolished [Millward-Sadler & Salter, 04a]. In another monolayer study, mechanical stimulation failed to result in increased proteoglycan synthesis in chondrocytes stimulated in the presence of blocking antibody for αVβ5 suggesting that this particular integrin also plays a role in mechanotransduction [Holledge *et al.*, 08]. Studies conducted in 3D culture further supported the

role of integrin in mediating chondrocytes' response to mechanical forces where, for instance, blocking the α5β1 integrin regulated cell morphology and influenced synthesis of collagens and proteoglycans in response to shear forces [Spiteri, Young, Simmons, Kandel & Pilliar, 08].

While the role integrins play in chondrocyte mechanotransduction has been illustrated through experiments utilizing blocking antibodies or peptide-competing ligands, the precise downstream events have not been fully elucidated. Upon interaction between an integrin and the respective matrix ligand a 2 pN talin-dependent adhesion point forms [Jiang *et al.*, 03]. This early adhesion signals for the activation of Rac and Cdc42 which ultimately regulate the nature and extent of the adhesion (FX, FC or FB)[Schwartz & Shattil, 00]. Upon the formation of the adhesion various adapter molecules may interact with the newly formed complex and, depending on the composition of adapter molecules, send various downstream signals. One molecule of interest is the focal adhesion kinase (FAK), which is one of the first molecules that are recruited to the site [Schwartz, 01]. Upon recruitment, FAK autophosphorylates to reveal a Src-homology-2 (SH2) binding domain which then enables it to interact with other kinases such as Src and Fyn [Schaller *et al.*, 94;Schlaepfer *et al.*, 97]. These molecules, either in cohort or independently, are able to phosphorylate other components of the complex such as paxilin and tensin, and thus elicit a diverse array of downstream signals [Vuori *et al.*, 96]. It is interesting to note that depending on the perceived mechanical force, different phosphorylation sites on FAK and Src are activated thus influencing the downstream cellular response; for instance it was shown that FAK-tyrosine397 is specifically phosphorylated upon binding of integrins to rigid surfaces whereas other surfaces regulate the phosphorylation of other sites [Ren *et al.*, 00]. Cellular responses to mechanical forces also depend on the specific ECM components to which integrins can bind. A study investigating the effects of biaxial stretch on cardiac fibroblasts noted that while JNK1

activation was seen in cells plated on fibronectin, vitronectin or laminin while ERK2 was only activated in cells plated on fibronectin [MacKenna *et al.*, 98]. Similarly, cyclic stretch of vascular smooth muscle resulted in increased apoptosis in cells grown on collagen I matrix, but not on other matrixes [Yano *et al.*, 96].

Of the multiple integrins known to be expressed in mammalian cells, of particular interest are those integrins which are known to be expressed in articular cartilage, namely: α1β1, α5β1 (the classical "fibronectin" receptor), α10β1 and the αVβ5 [Loeser, 00]. Of these, the β1 integrins have been the most studied. Upon activation, β1 integrin signalling results in the activation of the Ras-MAPK pathway [Shakibaei *et al.*, 99;Shakibaei & Merker, 99]. This signalling pathway plays a crucial role in maintaining cellular function (i.e. growth, differentiation, migration) in many cell types through a step-wise activation of a cascade of proteins. Upon activation, Ras, which is membrane anchored, phosphorylates and activates the Ser/Thr kinase Raf. Raf, in turn, phosphorylates mitogen activated protein kinase kinase (MAPKK) also known as Extracellular Regulated Kinase (ERK). Upon activation, ERK translocates from the cytosol to the nucleus and regulates the activity of transcription factors such as *jun*, *c-fos*, *c-myc* and AP-1 [MacKenna, Dolfi, Vuori & Ruoslahti, 98;Shakibaei & Merker, 99;Karin, 95]. These transcription factors, in turn, regulate the transcription of gene and activate numerous cellular responses.

1.3.4 Calcium Signalling

An additional mechanism by which cells are able to sense to respond to mechanical stimulation is through the utilization of calcium signalling. Calcium (Ca^{2+}) is a ubiquitous second messenger that is known to regulate a diverse range of cellular functions including gene transcription, metabolism, muscle contraction, proliferation and mechanotransduction. This diverse functionality of Ca^{2+} ions is attributed to the ability of cells to vary their Ca^{2+} signals in space,

time, amplitude as well through expression of selective intracellular components that are either Ca^{2+} dependent or responsive [Berridge *et al.*, 00].

The intracellular concentration of is Ca^{2+} regulated by two simultaneous processes, one of which one aims to introduce calcium into the cytoplasm ('on' reactions), while the other is employed to remove cytoplasmic calcium through the use of buffers, pumps, and ion exchangers ('off' reactions). It is generally believed that in resting cells, in which an equilibrium is established between the 'on' and 'off' mechanisms, the intracellular concentration of calcium ($[Ca^{2+}]_i$) is approximately 100nM. When cells are stimulated, through events such as depolarisation or mechanical deformation, the equilibrium shifts towards the 'on' reactions, causing the $[Ca^{2+}]_i$ to increase to levels that may surpass 1μM [Bootman *et al.*, 01].

In general, Ca^{2+} utilized in signalling is derived either from internal stores or from the extracellular medium. Internally, calcium is predominately stored in the endoplasmic reticulum (ER) or its muscle equivalent the sarcoplasmic reticulum (SR), although in some instances calcium may be sequestered in the mitochondria [Bootman, Collins, Peppiatt, Prothero, MacKenzie, de, Travers, Tovey, Seo, Berridge, Ciccolini & Lipp, 01;Collins *et al.*, 01]. The release of sequestered calcium is regulated both by calcium itself and by a group of secondary messengers which include: inositol-1,4,5-triphosphate (IP_3), cyclic ADP ribose (cADPR) and nicotinic acid adenine dinucleotide phosphate (NAADP) [Berridge *et al.*, 03]. Briefly, the activation of many cell-surface receptors by their ligands (i.e. growth factors, hormones) results in the activation of phospholipase C (PLC) which catalyzes the hydrolysis of phosphatidylinositol 4,5-biophosphate (PIP_2) to produce the IP_3 and diacylglycerol. Upon its production, IP_3 diffuses into the cytoplasm and, through activation of specific receptors on the ER/SR (IP_3Rs) causes the release of stored calcium ions. Similarly, cADPR induces the release

of ER-stored calcium through binding to Ryanodine Receptors (RyRs), which are structurally and functionally analogous to IP_3Rs [Bootman, Collins, Peppiatt, Prothero, MacKenzie, de, Travers, Tovey, Seo, Berridge, Ciccolini & Lipp, 01;Berridge, Bootman & Roderick, 03]. Recently it was shown that NAADP also possesses the ability to induce the release of stored calcium from ER/SR stores, although the receptor through which it is acting is not yet known [Genazzani & Galione, 97].

External calcium is utilized by cells through several specialized Ca^{2+} influx channels that are grouped together based on their activation mechanisms [Berridge, Lipp & Bootman, 00]. Voltage-Gated Calcium Channels (VGCC) are activated by the depolarization of the cellular membrane and help regulate various cellular processes including secretion, contraction and gene expression [Catterall *et al.*, 05]. Structurally, mammalian VGCCs are comprised of 5 distinct subunits (α_1, α_2, β, γ and δ) which exist in different isoforms and are differentially expressed in tissues throughout the body. The largest of subunits, the α_1 subunit (190-250 kDa), compromises the conduction pore (channel), the voltage sensor, gating apparatus, and contains several regulatory sites acted on by second messengers. The remaining four subunits in any given VGCC serve to regulate the channel's gating and impart distinctive conductance, current, and pharmacological properties to various channel subtypes [Berridge, Lipp & Bootman, 00;Catterall, Perez-Reyes, Snutch & Striessnig, 05]. The various subtypes are classified according to electrophysiological and pharmacological properties. Briefly, all channels are divided either into Low-Voltage Activated (LVA) or High-Voltage Activated (HVA) groups. LVA channels require a strong depolarization of the membrane (-70 mV), while the HVA channels require a smaller depolarization (-20 mV) [Lacinova, 05]. Subsequently, channels in each group are further divided according to their respective current conductance patterns (Table

4). The first characterized LVA channel possessed a large, slow decaying, current and was termed L-Type (L for large and long lasting). A pharmacological property shared amongst all L-type channels is their sensitivity to 1,4-DHPs, a wide class of drugs, with inhibitory action (nifedipine, nisoldipine, isradipine). Other HVA channels, which possess a smaller/shorter conductance and are insensitive to DHPs, were discovered and termed according to sensitivity to other selective inhibitors: N-type (N for neuronal; sensitive to ω-conotoxin GVIA), P/Q-type (P Purkinje cells; for sensitive to ω-Aga IVA) and R-type (R for rapid and resistant; resistant to the above named toxins). The single subtype that belongs to the HVA group is the T-type channel which is named for its tiny and transient conductance [Berridge, Lipp & Bootman, 00;Catterall, Perez-Reyes, Snutch & Striessnig, 05;Lacinova, 05].

Table 4: Classification of VGCC, adapted from [Lacinova, 05]

Channel Class	Type of Conductance	Subunit
HVA	L-Type (Long-, Large)	α_{1S} ($Ca_V1.1$)
		α_{1C} ($Ca_V1.2$)
		α_{1D} ($Ca_V1.3$)
		α_{1F} ($Ca_V1.4$)
	P/Q-Type (Purkinje Cells)	α_{1A} ($Ca_V2.1$)
	N-Type (Neuronal)	α_{1B} ($Ca_V2.2$)
	R-Type (Rapid)	α_{1E} ($Ca_V2.3$)
LVA	T-Type (Tiny, Transient)	α_{1G} ($Ca_V3.1$)
		α_{1H} ($Ca_V3.2$)
		α_{1I} ($Ca_V3.3$)

In contrast to the VGCC, extracellular calcium can enter cells through mechanically activated calcium channels that respond to cell deformation – their open probability increases with membrane stretch. Owing to their sensitivity to compression, shear stress as well as stretch these channels are often referred to as mechanotransducers as they convert direct physical forces into biological signals [Guibert *et al.*, 08]. Unlike the VGCCs, these stretch activated channels are less selective for the precise species of ions, and tend to allow for ions of similar size/charge to pass through the conduction pore. Of particular interest is the cationic stretch activated channel (SAC), which allows for calcium to pass through the conduction pore, albeit non selectively as both sodium and potassium have may pass as well [Wu & Davis, 01]. Previous research suggested two major roles that SACs may assume: (1) induction of sustained membrane depolarization via cation influx, thus activating VGCCs and inducing increase in $[Ca^{2+}]_i$, or (2) allow for local increases in cytosolic calcium and thus influence local signalling pathways [Wu & Davis, 01;Zou *et al.*, 02]. While the channel has not been fully characterized on a molecular level, and its mechanism of activation not full elucidated, three models have been proposed: (1) the "bilayer model" proposes that mechanical deformations in the lipid bilyaer cause the channel to activate [Maroto *et al.*, 05], (2) the "tether model", in turn, proposes that the channel is directly linked to extracellular or intracellur proteins that upon deformation generate and pass tension to the channel[Piao *et al.*, 03], (3) lastly, the "secondary signal model" suggests that a distance mechanical-sensitive protein generates a diffusible second messenger which causes the channel to open [Kalapesi *et al.*, 05]. Unlike voltage-gated channels, very few selective inhibitors of SACs are currently known. The trivalent lanthanide, gadolinium (Gd^{3+}), is the most widely used inhibitor, however its inhibitory – albeit not major - effect on other types of calcium channels have been documented [Catterall, Perez-Reyes, Snutch & Striessnig, 05;Wu & Davis,

01]. A single specific inhibitor (GsMTx-4) has been isolated from tarantula (*Grammostola spatulata*) venom [Suchyna *et al.*, 00].

Previous studies have implicated calcium signalling in chondrocyte mechanotransduction following fluid shear forces [D'Andrea *et al.*, 00], hydrostatic forces [Mizuno, 05], compressive forces [Roberts *et al.*, 01;Pingguan-Murphy *et al.*, 05b], and micropipette indentation [Kono *et al.*, 06]. However, the underlying mechanism(s) through which calcium facilitates the chondrocytes' response to mechanical stimuli have not been fully elucidated. Of the multitude of calcium channels, chondrocytes have been shown to predominately express the L-Type VGCC [Shao *et al.*, 05;Zuscik *et al.*, 97], and the cationic SAC (as shown through gadolinium inhibitor studies) [Guilak *et al.*, 99;Perkins *et al.*, 05]. Recent studies have shown that selective inhibitors of VGCCs can reduce the expression of MMP-3 and MMP-13 in osteoarthritic canine chondrocytes [Boileau *et al.*, 05], possibly suggesting that the upregulation of these MMPs following mechanical stimulation may be attributed, at least partially, to calcium signalling. Similarly, studies in human pulmonary artery endothelial cells have shown that calcium can regulate the binding activity of AP-1 (a regulator of MT1-MMP expression)[Fantozzi *et al.*, 03], while other studies have specifically implicated calcium currents through the L-Type VGCC in regulation of AP-1 binding activity [Premkumar *et al.*, 00]. Further evidence that calcium may be involved in facilitating the improvement of cartilage tissue following mechanical stimulation is seen in studies that indicated that intracellular calcium levels can influence aggrecan mRNA[Alford *et al.*, 03] and collagen type-X expression [Bonen & Schmid, 91], as well as modulate the ratio of collagens type I/type II in articular chondrocytes [Deshmukh & Sawyer, 77]. Other studies have implicated the SACs in regulation of aggrecan, sox9 and collagen types I,

II, IX, and XI gene expression, as treatment with Gd^{3+} influenced their expression [Perkins, Derfoul, Ast & Hall, 05].

Recently it was observed that several ion channels, including VGCCs, are co-localized in their expression with β1-integrins in mouse limb-bud chondrocytes [Mobasheri *et al.*, 02;Shakibaei & Mobasheri, 03]. Other studies, albeit in vascular smooth muscles cells, have shown that the α5β1 integrin regulates a tyrosine phosphorylation cascade involving Src and other focal adhesion proteins that control the function of the L-type VGCC [Wu *et al.*, 01b]. In fact, it is now known that both Src and Pyk2 (an additional focal adhesion associated protein) are able to bind to the II-III linker and C-terminal regions of the α_{1c} subunit on L-type VGCC and differentially regulate the channel's activity [Dubuis *et al.*, 06]. Consequently, it is quite possible that calcium is involved in mechanotransduction pathways, in chondrocytes, both independently through various ion channels as well as through synergistic action with co-localized integrins.

1.3.5 Cell Morphology

Regulation of cell morphology through adhesion and de-adhesion between the cells and their surrounding ECM is critical for numerous cellular functions, including migration, development, apoptosis and response to mechanical stimuli [Woods *et al.*, 07;Spiteri *et al.*, 06]. Rapid cell shape changes have been characterized in numerous tissues and are now known to play an essential role in the body. In the endothelium, for instance, the regulation of cell-cell and cell-ECM adhesions causes rapid changes in cell shape and vascular architecture in response to both normal and disease processes [Dudek & Garcia, 01]. Similarly, cell spreading has been observed in many other cell types including fibroblasts, platelets, B cells, smooth muscle cells, and chondrocytes [Spiteri, Pilliar & Kandel, 06;Thakar *et al.*, 09;Fleire *et al.*, 06;Cavalcanti-Adam *et al.*, 07]. In particular, in terms of mechanical stimuli, it was previously shown that the application of mechanical forces, both compressive and shear, can enhance the formation of stress fibres and through changes in the cellular actin cytoskeleton influence cell morphology [Millward-Sadler & Salter, 04a;Spiteri, Pilliar & Kandel, 06;Guilak, 95;Knight, Toyoda, Lee & Bader, 06b]. Thus, it is believed that cell spreading can serve as a mechanism through which cells sense, integrate and response to external mechanical forces.

In response to external stimuli which causes cell spreading, actin polymerization drives the assembly of early contacts between the cell and the ECM, ultimately leading to the formation of focal complexes and/or focal adhesions [Pollard & Borisy, 03]. These events are tightly regulated by the small GTPases Rac1 and Rho which regulate actin polymerization (spreading) and actomyosin contractility (retraction), respectively [Ballestrem *et al.*, 01;Wittmann *et al.*, 03]. These GTPases are in may be regulated by integrin-mediated adhesion which induces autophosphorylation of FAK at tyrosine 397 which activates FAK and creates additional binding

sites for Src. The activated FAK-Src complex then stimulates Rac1 activity through recruitment and phoshporylation of p130Cas and its associated adapter proteins (Dock190, ELMO1, Crk), which functions as a Guanine-Exchange Factor (GEF) and activates Rac1 resulting in membrane protrusions [Brugnera *et al.*, 02;Kiyokawa *et al.*, 98]. Thus, the exact localization and activation of integrins is able to regulate localized activity of GEFs and coordinate membrane protrusions [Huveneers & Danen, 09]. Interestingly, MT1-MMP has been shown to play a role in regulating integrin clustering which further implicates this MMP in regulating cell morphology and consequently matrix accumulation [Takino *et al.*, 06].

As spreading progresses, RhoA activity gradually increases in concert with formation of stress fibres and the maturation of focal contacts. This increased RhoA activity promotes myosin activity and thus results in the contractility (retraction) of the actin cytoskeleton [Huveneers & Danen, 09]. While the exact mechanism by which RhoA is activated has not been fully elucidated, one possible explanation is that tension caused during the spreading triggers Src-mediated activation specific GEFs (p115RhoGEF, Gef12, p190RhoGEF) that activate RhoA [Huveneers & Danen, 09;Danen *et al.*, 02]. In contrast, it has been shown that activation of FAK-Src signalling can result in increased phosphorylation of Endophilin A2 (a protein involved in endocytosis) at Tyr315 phosphorylation which inhibits its activity and thus prolongs the proteolytic activity of MT1-MMP and the degradation of the surrounding ECM [Wu *et al.*, 05]. The increased degradation of the ECM would then weaken the focal adhesion stability and cause the cell to retract – an event which may signal the cell to increase its metabolic activity.

Nonetheless, despite the heavy involvement of integrins and their associated proteins in cell spreading and retraction, it is now accepted that calcium may exert a potent effect on these processes. Studies have shown that under certain loading conditions chondrocytes exhibit a rapid

(~8 minutes) reorganization of their action cytoskeleton in a calcium-dependent manner [Erickson *et al.*, 03]. In an additional study, chondrocytes assumed a flattened fibroblast-like phenotype (marked by the appearance of stress fibers) upon treatment with Gd^{3+}; upon the removal of the inhibitor, the cells assumed their normal morphology suggesting SACs are also involved in regulation chondrocyte morphology [Perkins, Derfoul, Ast & Hall, 05].

1.4 Hypothesis

Owing to the close interaction of calcium channels, integrins and the cytoskeleton, we hypothesized the following: improved tissue formation following the application of cyclic compression occurs via a transient cell spreading, activation of MT1-MMP, and subsequent cell retraction which are all regulated by converging calcium and integrin signalling pathways.

Research Goals

1. Investigate the role calcium plays in chondrocyte cell spreading and retraction following mechanical stimulation.
    1.1. Determine whether the response is regulated by extracellular calcium and, if so, determine which calcium channels are implicated.
    1.2. Determine whether calcium is involved in regulating MT1-MMP expression following mechanical stimulation.
2. Investigate the involvement of integrins, and their respective adapter proteins, in the response to cyclic compression.
3. Determine whether calcium signalling pathways interact with the integrin signalling pathways in mediating the chondrocytes' response to mechanical stimulation.

CHAPTER TWO: PAPER MANUSCRIPT

Calcium regulates cyclic compression-induced early changes in chondrocytes during *in vitro* tissue formation.

Igal Raizman, MSc; Rita Kandel, MD

CIHR-BioEngineering of Skeletal Tissue Team, Mount Sinai Hospital, University of Toronto, Toronto, Canada M5G 1X5.

Running title: Calcium regulation of chondrocyte mechanotransduction

Keywords: Calcium, Chondrocytes, Tissue-engineering, Mechanotransduction

Manuscript submitted to *Cell Calcium*.

Abstract:

A single application of cyclic compression (30min) to bioengineered cartilage results in improved tissue formation through sequential catabolic and anabolic changes mediated via cell shape changes that are regulated by α5β1 integrin and membrane-type metalloprotease (MT1-MMP). To determine if calcium exerts a regulatory role over this process, we investigated how calcium regulated cell shape changes, MT1-MMP and integrin activity in response to mechanical stimulation. Stimulation-induced changes in cell shape and MT1-MMP expression were abolished with chelation of extracellular calcium, and reinstated with its re-introduction. Spreading was inhibited by blocking the stretch-activated channel (with gadolinium), while retraction was prevented by blocking the L-Type voltage-gated channel (with nifedipine); both compounds inhibited MT1-MMP upregulation. Channels' role was further confirmed with the use of either inhibitor in conjunction with the calcium A23187 ionophore, which restored cellular response. Calcium regulated the integrin-mediated signalling pathway, which was facilitated through the kinase Src. Both calcium- and integrin-mediated pathways converged on activating ERK in response to stimulation. While both integrins and calcium signalling mediate chondrocyte mechanotransduction, calcium appears to play the major regulatory role. Understanding the underlying molecular mechanisms involved in chondrocyte mechanotransduction may lead to the development of improved bioengineered cartilage and a better treatment for osteoarthritis.

2.1 Introduction

Articular cartilage covers the articulating ends of bones in synovial joints. Due to lack of vascular supply the tissue possesses a limited ability for self-repair, and with injury or disease it often fails to properly heal. Current attempts to address this issue have been only partially successful [Darling & Athanasiou, 03]. Consequently, repair of cartilage defects with *in vitro*-formed cartilage tissue is a promising alternative approach that would circumvent many limitations inherent in current treatment approaches. Unfortunately the biomechanical properties of bioengineered cartilage tissue are inferior to the native tissue, a factor which precludes its utilization clinically. In our lab, we have shown that the application of 30 minutes of compressive forces enhances collagen and proteoglycan accumulation by articular chondrocytes grown on the surface of a porous ceramic substrate [Waldman, Spiteri, Grynpas, Pilliar & Kandel, 04;Waldman, Couto, Grynpas, Pilliar & Kandel, 06]. This increase in matrix appears to be dependent on a biphasic remodelling process, which involves an initial catabolic phase (characterized by ERK activation and upregulation of MT1-MMP) followed by a subsequent anabolic response which increases the amount of extracellular matrix (ECM) synthesis and accumulation [De Croos, Dhaliwal, Grynpas, Pilliar & Kandel, 06]. Furthermore, it was observed that this stimulation-induced increase in matrix synthesis is dependent upon transient chondrocyte spreading and subsequent retraction, mediated through the α5β1 integrin and was dependent on the upregulation of membrane-type 1 metalloproteinase (MT1-MMP), which was observed to occur immediately following mechanical stimulation [De Croos, Jang, Dhaliwal, Grynpas, Pilliar & Kandel, 07] (submitted for publication). However, the underlying mechanism(s) by which chondrocytes sense mechanical forces and convert them to the previously delineated pathways are poorly understood. For example, it is not known whether ERK activation occurs prior to cell spreading.

As calcium fluxes have been shown to occur in mechanically stimulated chondrocytes [Alford, Yellowley, Jacobs & Donahue, 03;D'Andrea, Calabrese, Capozzi, Grandolfo, Tonon & Vittur, 00;Guilak, Zell, Erickson, Grande, Rubin, McLeod & Donahue, 99;Zhang, Wang & Chen, 06], and can occur within seconds of stimulation [Kono, Nishikori, Kataoka, Uchio, Ochi & Enomoto, 06;Pingguan-Murphy *et al.*, 05a], it has been proposed that the first signalling event in response to mechanical stimuli may involve calcium. Of the multitude of calcium channels described, chondrocytes have been shown to express the L-Type Voltage Gated Calcium Channel (VGCC) and the cationic stretch activated cationic channel (SAC) that are permissive to calcium ion fluxes [Perkins, Derfoul, Ast & Hall, 05;Shao, Alicknavitch & Farach-Carson, 05;Zuscik, Gunter, Puzas & Rosier, 97]. Studies in canine chondrocytes have shown that selective inhibitors of VGCCs are able to reduce the expression of MMP- 3 and -13 [Boileau, Martel-Pelletier, Brunet, Tardif, Schrier, Flory, El-Kattan, Boily & Pelletier, 05], while studies in human pulmonary artery endothelial cells have shown that calcium signalling regulates the binding activity of AP-1 (a known regulator of MT1-MMP expression) [Fantozzi, Zhang, Platoshyn, Remillard, Cowling & Yuan, 03]. Moreover, chondrocytes appear to reorganize their actin cytoskeleton in a calcium-dependent manner [Erickson, Northrup & Guilak, 03], and intracellular calcium levels have been shown to influence aggrecan, collagen types I, II, and X expression levels [Alford, Yellowley, Jacobs & Donahue, 03;Bonen & Schmid, 91;Deshmukh & Sawyer, 77]. Interestingly, ion channels co-localize with β1 integrins [Mobasheri, Carter, Martin-Vasallo & Shakibaei, 02;Shakibaei & Mobasheri, 03], and some studies have shown that cross-talk between the ion channels and integrins exist which can regulate cell function [Wu, Davis, Meininger, Wilson & Davis, 01b]. As such, calcium signalling could be involved in the

regulation of both the cell morphology changes, and the anabolic/catabolic response that occurs in our system.

The role of calcium in mediating enhanced cartilage tissue formation in response to cyclic compression has not been elucidated. The aim of this study, therefore, was to determine whether the initial chondrocyte response that leads to the cyclic compression-induced increase in matrix accumulation, namely cell spreading and MT1-MMP expression, are regulated by calcium. In addition, because of the putative role of α5β1 integrin in this process, we investigated whether the calcium signalling pathway is involved in regulating the integrin associated kinase Src. Understanding the mechanism by which mechanical compression results in increased matrix accumulation may further help us to improve the properties of *in vitro*-formed cartilage tissue.

2.2 Methods

Cell Culture

Chondrocytes were isolated by sequential enzymatic digestion from bovine (6 to 9 month old) metacarpal-phalangeal joints as described previously [Boyle *et al.*, 95]. Briefly, cartilage harvested from 2-3 joints was pooled together in order to obtain sufficient cells, and digested with 0.5% protease (Sigma Chemical Co., MI) for 1 hour at 37°C, followed by digestion with 0.1% collagenase (Roche Molecular Biochemicals, IN) overnight under standard culture conditions (37°C, 5% CO_2). Chondrocytes were seeded in Ham's F-12 supplemented with 5% fetal bovine serum (Sigma Chemical Co.) at a density of 160,000 cells/mm^2 onto the surfaces of calcium polyphosphate (CPP) substrates (2mm height x 4mm diameter). Cultures were grown at 37°C under standard cell culture conditions.

Mechanical Stimulation

Following 3 days of static culture, cells were subjected to uniaxial, confined cyclic compression (1kPa, 1 Hz, 30min) using a MACH-1 mechanical stimulation (Biosyntech, Montreal, CAN) as described previously [De Croos, Dhaliwal, Grynpas, Pilliar & Kandel, 06]. To avoid dislodging the small amount of tissue formed by this time, the cells were subjected to compressive forced through a compliant 2% agarose gel disk (3.5 mm diameter x 8 mm height) that was placed on top of the cells. The control constructs were maintained under identical culture conditions but did not receive any mechanical stimulation.

Calcium Studies

To investigate the role of calcium in mechanotransduction, constructs were preincubated in serum free Hams F-12 alone (as a control) or supplemented with EGTA (10μM), or EGTA

(10μM) combined with $CaCl_2$ (5μM), for 4 hours prior to mechanical stimulation. These samples were then subjected to mechanical stimulation under the same conditions. To determine which calcium permissive channels are involved, chondrocytes were preincubated for 4 hours in the absence or presence of the ion channel inhibitors, nifedipine and gadolinium (Sigma)which were dissolved in DMSO and then diluted with HAMs F-12 to form a 20μM solution (0.01% DMSO). The role of calcium fluxes was further confirmed by utilizing the A23187 ionophore (10μM) in conjunction with the inhibitors. To investigate the involvement of Src, constructs were preincubated in the presence of PP2 (4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine) or its negative control PP3 (4-Amino-7-phenylpyrazol[3,4-d]pyrimidine) (20 μM), purchased from Calbiochem, for 4 hours prior to mechanical stimulation .

Chondrocyte Morphology and Area

Following mechanical stimulation, tissue-CPP constructs were washed three times in cold phosphate buffered saline (PBS) and fixed in 2.5% glutaraldehyde for at least an hour. Subsequently, constructs were rinsed three additional times in PBS, dehydrated in graded ethanol (up to 100%), critical point dried (Bal-Tec, Liechtenstein; CPD 030), and sputter coated with gold (Denton Vacuum, NJ; Desk II). The cells at the surface of the CPP substrate were visualized by secondary electron imaging under scanning electron microscopy (SEM, FEI, OR; XL30). Resulting images were imported into Image J (http://rsb.info.nih.gov/ij/) and chondrocyte area was determined by tracing the cell outline and calculating the area using the Image J program, utilizing the scale bar in the images. Only cells that were in direct contact with the CPP substrate were measured. At least three constructs with a total of at least 100 cells were examined for each condition.

Western Blot Analysis

The tissue was removed from the CPP and homogenized with a motorized pestle on ice (100 µl) in RIPA Buffer (50 mM Tris-HCL pH 7.4, 150 mM NaCl, 1% Triton x-100, 1% Sodium deoxycholate, 0.1% SDS, 1mM EDTA) with protease inhibitors NaF (1mM) and phenylmethylsulfonyl fluoride (1mM) added immediately prior to use. Protein extracts were clarified by centrifugation for 10 min at 12,000*g* and protein content quantified utilizing a BCA protein assay kit (Pierce, Rockford, IL). Proteins from each sample (20 µg) were separated by sodium-dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (12% acrylamide,1.5 h at 150 V), electroblotted (1.5 h, 0.3 mA) onto nitrocellulose membranes (BioTrace NT, Pall Life Sciences, Pensacola, FL) then incubated with antibodies reactive with total ERK1/2 or phospho-ERK1/2 (1:2000; Cell Signaling, Beverly, MA) in tris-buffered saline, 0.1% Tween-20 (10 mM Tris, pH 7.5, 150 mM NaCl, 0.05% v/v Tween-20) containing 5.0% non-fat dry milk overnight at 4°C. Immunoreactivity was visualized using secondary antibody conjugated with horseradish peroxidase (1:2000, Cell Signaling) and enhanced chemiluminescence (ECL Plus; Amersham Biosciences).

Semi-quantification of gene expression by RT-PCR

To determine the expression of MT1-MMP following mechanical stimulation, total RNA was extracted from stimulated and unstimulated tissues using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's directions. Total RNA was reverse transcribed (RT) into cDNA using reverse transcriptase (Superscript II, Invitrogen) according to the manufacturer's instructions. Relative gene expression was examined by semi-quantitative RT-PCR using Taq

Polymerase (Invitrogen) and primers in reactions designed to amplify the sequence of interest as outlined previously [De Croos, Jang, Dhaliwal, Grynpas, Pilliar & Kandel, 07]. 18S rRNA was the housekeeping gene against which gene expression was normalized. PCR products were run on a 1.6%-agarose gel stained with ethidium bromide. Band intensity was semi-quantified by densitometry using Lab Works software (V4.0, Media Cybernectics).

Statistical Analysis

All experiments were performed in duplicate and all experiments repeated at least three times. The data were pooled and expressed as mean ± standard error of the mean (SEM). The results were evaluated using a one-way analysis of variance (ANOVA) with a bonferroni *post-hoc* correction. Statistical significance was assigned at $p<0.05$.

2.3 Results

Characterization of ERK phosphorylation and cell spreading following cyclic compression

ERK MAP Kinase activation and cell spreading are amongst the early changes induced in chondrocytes by mechanical stimulation. To examine which of them occurred first we examined the time course of ERK phosphorylation (Figure 1A) and cell shape (Figure 1C) at various times up to 30 minutes following the initiation of mechanical stimulation. Significant upregulation of ERK phosphorylation was observed by 5 minutes (5 fold increase), with the levels peaking by 15 (10 fold increase) minutes of stimulation. Albeit still significantly elevated compared to control constructs, a decline in phosphorylation was noted with 25 minutes of stimulation. Chondrocyte area peaked (two-fold increase) with 5 minutes of cyclic compression and, although slightly decreasing, remained well above control values at the subsequent time points. This suggested that changes in cell shape and activation of the MAP Kinase signalling were rapid. As maximal cell spreading occurred prior to maximal ERK activation, it suggested that cell spreading was the earlier change so only cell shape changes were quantified in the subsequent experiments.

Extracellular calcium regulates cell morphology

Given that the chondrocytes responded within 5 minutes to cyclic compression, and since calcium signalling is known to be involved in mechanotransduction and regulation of cell morphology, the role of extracellular calcium in regulating cell spreading was investigated. As shown in Figure 2A cells that were stimulated in the presence of EGTA-treated media failed to spread following mechanical stimulation (EGTA treated: 58.8 ± 1.81 μm^2; EGTA untreated: 86.5 ± 5.44 μm^2). This did not represent a delayed response as no spreading was seen as late as 6 hours after stimulation. As EGTA can chelate ions other than calcium, we confirmed that calcium was responsible for this effect by adding calcium back into the media after EGTA

treatment but prior to mechanical stimulation. The final amount of calcium chosen to be added to the media was determined through dose-response experiments which ensured all cation binding capacity of EGTA was saturated. Addition of calcium into the EGTA-treated media prior to mechanical stimulation allowed for cell spreading similar to that seen in constructs undergoing cyclic compression in untreated media as determined morphologically by SEM and by measuring cell size (Figure 2B).

Cell spreading and retraction are regulated by two distinct ion channels in chondrocytes

As extracellular calcium influenced chondrocyte response to cyclic compression, the role of two calcium transitory channels, the voltage-gated calcium channel and the cationic stretch-activated channels, in regulating these cell shape changes were investigated (Figure 3). Involvement of these channels would also provide additional support for the involvement of ion (calcium) fluxes regulating cell response to cylic compression. Mechanical stimulation in the presence of nifedipine, a selective inhibitor of the L-type VGCC, had no effect on cell spreading observed immediately following mechanical stimulation (0 hours); however while untreated cells retracted to their original cell size by 6 hours, those exposed to nifedipine remained significantly more spread relative to the unstimulated controls. In contrast, constructs stimulated in the presence of gadolinium, a selective inhibitor of the stretch activated cationic channel (Gd^{3+}), did not exhibit cyclic compression-induced cell spreading following the stimulation, suggesting that the L-Type VGCC is involved in cell retraction while the stretch activated channel is involved in regulating cell spreading.

To support the role of calcium and to exclude indirect effect of the inhibitors in the regulation of cell morphology, constructs were mechanically stimulated in the presence of

calcium ionophore A23187 and either nifedipine or gadolinium. Treatment with the A23187 ionophore partially reversed the inhibition of gadolinium, and mechanically stimulated chondrocytes spread immediately following mechanical stimulation, compared to unstimulated chondrocytes. Similarly mechanical stimulation in the presence of both nifedipine and the A23187 ionophore reversed the effects of nifedipine-inhibition as chondrocytes had partially retracted by six hours post mechanical stimulation as compared to vehicle treated constructs (Figure 3E).

Extracellular calcium regulated MT1-MMP expression

We had determined previously that MT1-MMP expression following cyclic compression plays a role in modulating cell shape changes observed following cyclic loading. Thus we examined whether blocking the calcium influx prevented changes in MT1-MMP mRNA expression following stimulation (Figure 4). As expected, a significant upregulation in MT1-MMP, approximately four-fold, gene expression was seen in constructs stimulated in the absence of any chemical pre-treatment. In contrast, no increase in MT1-MMP mRNA was seen in constructs that were mechanically stimulated in the presence of EGTA-treated media. Upregulation of MT1-MMP expression was reversed by addition of calcium to the EGTA-treated media, to levels similar to that seen in mechanically stimulated untreated control constructs. Unstimulated control constructs that were placed in EGTA-treated media showed upregulated MT1-MMP gene expression (approximately three-fold) in the absence of mechanical stimulation suggesting a role for calcium in regulation of basal levels of MT1-MMP in chondrocytes (Figure 4B).

The effect of blocking L-Type VGCC and SAC on the induction of MT1-MMP mRNA expression following cyclic compression was analyzed as well. As shown in Figure 5, both nifedipine and Gd^{3+} prevented compression-induced upregulation of MT1-MMP mRNA as compared to the corresponding untreated controls ($p<0.05$). Interestingly, similar to the observed in EGTA-treated constructs, both nifedipine and gadolinium induced upregulation of MT1-MMP in the absence of mechanical stimulation. Treatment of constructs with the A23187 ionophore reversed the inhibitory effects of both nifedipine and gadolinium resulting in upregulation of MT1-MMP gene expression following cyclic compression supporting the role of calcium in these processes (Figure 6B).

Involvement of integrin signalling in cell spreading and MT1-MMP expression

The α5β1 integrin has also been shown to regulate chondrocyte spreading (and MT1-MMP expression) following cyclic compression. Integrins translate the signal by activation of adapter proteins such as Src. To further evaluate the role of calcium in this process we examined whether calcium ionophore would reverse the inhibitory effects induced by blocking integrin activation through the use of the Src inhibitor, PP2. PP3 served as a negative control as was the media with the vehicle. As expected given the involvement of α5β1, treatment with PP2 prevented cell spreading and upregulation of MT1-MMP following cyclic expression. Cell spreading did occur in the controls (vehicle and PP3) so the cells were able to respond to mechanical stimulation (Figure 7). The ionophore A23187 was able to overcome the inhibitory effect of PP2 ($p<0.05$) as cell size increased with cyclic compression although not to the same level observed in control constructs (vehicle only). Increasing the ionophore concentration did not have any further effect on cell shape suggesting that it was unlikely that the partial effect was due to an insufficient amount of the drug.

Regulation of ERK MAP Kinase activation

To examine whether calcium also regulates ERK MAPK activation we examined the effect of calcium channel blockers on ERK phosphorylation (Figure 8). As expected cyclic compression resulted in increased ERK phosphorylation. Inhibition of either calcium channel (SAC or L-type VGCC) did not prevent ERK phosphorylation following stimulation, and actually increased the phosphorylation in Gd^{3+}-treated constructs, although variability in the responsiveness of the cells to these drugs was observed. Mechanical stimulation in the presence of PP2 did not prevent ERK phosphorylation, however when the cells were stimulated in the presence of both the Src inhibitor, PP2, and nifedipine, ERK phosphorylation was inhibited completely ($p < 0.05$).

2.4 Discussion

We have previously shown that the mechanical stimulation-induced improvement in tissue properties is facilitated through transient chondrocytes spreading and retraction that are regulated via α5β1 integrin, ERK MAPkinase activation and upregulation of MT1-MMP expression (submitted for publication). While calcium has been implicated in other studies examining chondrocyte mechanotransduction [Roberts, Knight, Lee & Bader, 01;Tanaka *et al.*, 05;Zhang, Wang & Chen, 06], its role in regulating the mechanism(s) leading to the improved cartilage formation as a result of mechanical stimulation have not been fully investigated. This study demonstrated that the transient cell spreading and retraction, as well as the associated upregulation of MT1-MMP, that follows a single application of cyclic compression are regulated via calcium as treatment of the media with EGTA, prior to stimulation abolished these changes and re-introduction of calcium was able to reverse this inhibitory effect. Cell spreading and cell retraction were regulated through two distinct calcium permissive ionic channels, the cationic stretch-activated channel (SAC) and the L-type voltage gated channel (VGCC), respectively. Despite the differential regulation of cell morphology, inhibition of either channel prevents upregulation of MT1-MMP gene expression following stimulation. Treatment with the calcium ionophore A23187 partially reversed the inhibitory effects of nifedipine and gadolinium on cell morphology, and completely reversed the effects of stimulation on MT1-MMP expression. Inhibition of the activation of integrin associated kinase Src abolished cell spreading and MT1-MMP upregulation, but treatment with the A23187 ionophore partially re-established this cellular response. The two pathways, calcium and integrin associated kinase Src converged on the activation of ERK MAP Kinase, as inhibition of both pathways are required to achieve down-regulation of ERK phosphorylation in response to mechanical stimulation.

The role of calcium in regulating these early events was demonstrated through the use of EGTA, which abolished these stimulation-induced changes, and the reintroduction of calcium into the media which returned the cellular response to mechanical stimulation. While EGTA can chelate a variety of cations (namely Ca^{2+}, Na^{2+}, K^{+}), it was the re-introduction of calcium into the media that returned cell response to normal levels, suggesting that the mechanism regulating this response is attributed to calcium and not other media soluble cations. The role of calcium influx was further confirmed through the use of the A23187 ionophore alone or in conjunction with nifedipine or gadolinium. A23187 ionophore has been previously shown to elevate intracellular calcium levels in our laboratory [Howarth *et al.*, 93]. It is interesting that ionophore treatment was only able to partially reverse the inhibitory effects of the nifedipine and gadolinium on cell morphology. The reasons for this incomplete reversal are not known, but one possible explanation may be that the localization of calcium, and not merely its presence, following mechanical stimulation is an important aspect of this response. Localized calcium currents have been previously documented to influence local signalling cascades and thus activate signalling pathways in specific locations in the cell [Janmey, 98;Zou, Lifshitz, Tuft, Fogarty & Singer, 02].

Cell spreading was shown to be regulated through the SAC, which is known to be activated through direct physical deformation of the cellular membrane [Wu & Davis, 01;Zou, Lifshitz, Tuft, Fogarty & Singer, 02]. Owing to previous studies implicating SAC as the initial step in mechanotransduction [Guilak, 95;Knight, Bomzon, Kimmel, Sharma, Lee & Bader, 06a], it is not unexpected that we found that this channel was involved in our system. While it is not possible to characterize the expression of the SAC in chondrocytes, as the channel has not been fully sequenced or characterized [Maroto, Raso, Wood, Kurosky, Martinac & Hamill, 05], its

presence in chondrocytes has been supported through the use of gadolinium [Guilak, Zell, Erickson, Grande, Rubin, McLeod & Donahue, 99;Mouw *et al.*, 07;Perkins, Derfoul, Ast & Hall, 05]. In contrast, we observed that the L-Type VGCC regulated chondrocyte retraction following mechanical stimulation. While the VGCC has been shown to be involved in mechanotransduction in other studies [Gomez *et al.*, 02;Guibert, Ducret & Savineau, 08;Lacinova, 05], its relationship to chondrocyte morphology was not explored by others. In preliminary studies we were able to determine that articular cartilage chondrocytes express VGCC mRNA as determined by RT-PCR (data not shown), similar to what was observed by Zuscik *et al.,* in growth plate chondrocytes [Zuscik, Gunter, Puzas & Rosier, 97]. The sequential role of the SAC and the L-type VGCC is in line with literature suggesting that one of the functional outcomes of SAC activation, at least in smooth muscle cells, is cell depolarization and subsequent activation of VGCCs [Zou, Lifshitz, Tuft, Fogarty & Singer, 02].

Chelation of extracellular calcium abolished the stimulation-induced upregulation of MT1-MMP suggesting that either calcium directly regulates MT1-MMP expression, or that MT1-MMP expression is dependent upon cell morphology changes. The former is supported by several studies showing that calcium can regulate the binding activity of AP-1 (a regulator of MT1-MMP expression) in human pulmonary artery endothelial cells [Fantozzi, Zhang, Platoshyn, Remillard, Cowling & Yuan, 03], and that L-type VGCC generated currents are implicated in direct regulation AP-1 binding activity [Premkumar, Mishra, Overholt, Simonson, Cherniack & Prabhakar, 00]. It is interesting to note that in inhibitor-treated, but mechanically unstimulated samples, an increase in MT1-MMP gene expression was observed. Since this change was observed without any morphological changes in cell shape it suggests that calcium signalling exerts a role regulating constitutive expression of MT1-MMP. Further supporting this

notion is the restoration of MT1-MMP gene expression with the ionophore treatment, perhaps further indicating the existence of a separate calcium signalling pathway that controls MT1-MMP expression. This is in keeping with previous work by Lohi *et al.* who showed that ionophore treatment can induce MMP expression, such as MMP-9 and MMP-2. Although they did not observe a change in MT1-MMP in their system [Lohi & Keski-Oja, 95], it is possible that this is a result of the different cell type (fibrosarcoma) used, as the tumour cells may have different calcium signalling mechanisms [Whitfield, 92].

Due to the previously documented role of α5β1 integrin in mediating cell shape and MT1-MMP changes, we examined the role of the kinases Src and FAK, which are known downstream modulators of integrin activation, in regulating these changes, and investigated the role of calcium in this process. The response to cyclic compression was facilitated through the Src, as its inhibition abolished both cell spreading and MT1-MMP upregulation. No changes in FAK phosphorylation were seen with mechanical stimulation (data not shown). This may be explained by noting that different types of mechanical forces can differentially regulate the phosphorylation of FAK and Src [Ren, Kiosses, Sieg, Otey, Schlaepfer & Schwartz, 00]. Alternatively, it is possible that the cell-matrix interactions formed during spreading are somewhere between focal adhesions and fibrillar adhesions and thus the constitution of associated kinases could predominantly rely on Src. Fibrillar adhesions are known to play a role in matrix reorganization [Katz, Zamir, Bershadsky, Kam, Yamada & Geiger, 00], and it is believed that their formation (through maturation) is dependent upon Src activity [Volberg, Romer, Zamir & Geiger, 01]. Similarly, since the α5β1 integrin can be localized with MT1-MMP in intercellular contacts [Galvez *et al.*, 02], and Src phosphorylation has been previously shown to regulate MT1-MMP activity [Wu, Gan, Yoo & Guan, 05] it is not surprising that

inhibition of Src can influence the mechanosensitive upregulation of MT1-MMP following stimulation.

The dual regulation of cell morphology and MT1-MMP is not unexpected as others have shown that both integrins and calcium channels are components of a cellular mechanosensing complex. VGCCs are known to co-localize with β1 integrins in mouse limb-bud chondrocytes [Mobasheri, Carter, Martin-Vasallo & Shakibaei, 02;Shakibaei & Mobasheri, 03], and studies using vascular smooth muscle cells showed that α5β1 integrin can regulate the function of the L-Type VGCC [Wu, Davis, Meininger, Wilson & Davis, 01b]. It is now known that Src can bind to both the II-III linker and C-terminal regions of the α_{1C} subunit of the L-Type VGCC and thus regulate its activity [Dubuis, Rockliffe, Hussain, Boyett, Wray & Gawler, 06]. Our data suggests that calcium plays a major regulatory role in mediating this response, possibly to the extent of lying upstream to α5β1 and regulating its activation. This idea is supported by the observation that blocking calcium signalling completely abolished cell spreading in response to mechanical stimulation, and that ionophore treatment was able to partially reverse the effects of Src inhibition. Moreover, the ability of calcium to influence basal levels MT1-MMP expression in unstimulated samples further implicates calcium in exerting the major regulatory role in mediating chondrocyte responses. The activation of ERK, however, may be suggestive of a parallel pathway where both signalling cascades converge to regulate ERK, which then exerts a regulatory role on AP-1 and MT1-MMP expression. To fully address this, it would be necessary to visualize calcium fluxes in response to mechanical stimulation in real-time and determine whether inhibition of Src activity influences these currents. Unfortunately, due to the technical limitations of mechanically stimulating our biphasic 3D constructs, it was not possible for us to monitor calcium fluxes in real-time.

Lastly, it is worth noting that the observation that significant changes in cell morphology occurred prior to the peak in phosphorylation of ERK suggests that physical changes in cell shape precede/initiate the downstream signalling cascades such as ERK MAP Kinase. Past studies in our lab indicated that MT1-MMP upregulation in response to cyclic compression is dependent on ERK activation [De Croos, Jang, Dhaliwal, Grynpas, Pilliar & Kandel, 07], and owing to MT1-MMP's role in facilitating cellular retraction (submitted for publication), it is possible that the activation of ERK may not be involved in cell spreading but rather the subsequent events. This notion is supported by studies which observed that physical deformations in the cellular membrane comprise the first step in mechanotransduction [Guilak, 95;Knight, Bomzon, Kimmel, Sharma, Lee & Bader, 06a].

Based on the results obtained, we would like to suggest a pathway through which mechanical forces result in improved formation of cartilage tissue in vitro (figure 9): cyclic compression causes cellular deformations which activate SAC, resulting in calcium influx. This influx results in cell spreading and activation of the L-Type VGCC and α5β1 integrin with its associated Src kinase. This results in the phosphorylation of ERK and subsequent upregulation of MT1-MMP. Simultaneously, Src signalling may result in increased phosphorylation of Endophilin A2 (a protein involved in MT1-MMP endocytosis), which inhibits endocytosis and prolongs the proteolytic activity of MT1-MMP [Wu, Gan, Yoo & Guan, 05].The increased degradation of the ECM would then weaken any cell-matrix adhesions causing the cells to retract to their original morphology, while the degradation products of the ECM will signal the cells to increase synthesis of novel matrix molecules (for example, fibronectin fragments have been shown previously to increase proteoglycan synthesis by chondrocytes [Homandberg *et al.*, 92]).

In summary, calcium currents through SAC and L-Type VGCC channels appear to regulate the transient cell spreading and retraction, respectively, that are observed following the application of cyclic compressive forces. In addition, calcium exerts a regulatory role on the integrin-mediated signalling pathways, that is facilitated through the kinase Src. Further understanding of the underlying molecular mechanisms involved in chondrocyte mechanotransduction will ultimately lead to the development of improved bioengineered cartilage and may lead to a better treatment for osteoarthritis and other cartilage pathologies.

Acknowledgements:

I.R. was supported by fellowship from CAN-arthritis Society and Ontario Student Scholarship OGS. The research was supported by a grant from the Canadian Institutes of Health Research (CIHR). We thank Mr. Harry Bojarski and Ryding-Regency Meat Packers for providing the tissues for these studies, Dr Svitlana Prada for manufacturing the CPP substrates and Dr. Jian Wang for technical assistance.

2.5 Figures & Legends

Figure 1: Characterization of chondrocyte response to cyclic compression. (A) A time course of a confined cyclic compression stimulation over 1, 3, 5, 15 and 25 minutes shows an increase in ERK phosphorylation as early as five minutes during mechanical stimulation, with maximal phosphorylation observed by 15 minutes. (B) Representative western blot indicating the phosphorylation of ERK (pERK) and the respective total ERK over the time course. (C) Representative SEM micrographs of chondrocytes as seen through scanning electron microscopy. (D) Chondrocyte cell spreading is observed as early as 5 minutes into a cyclic compression. * denotes statistically significant difference ($p<0.05$) as compared to unstimulated control constructs at the respective time points. Results are expressed as means ± SEM (n=3, in duplicates).

Figure 2: Chondrocyte cell spreading following cyclic compression is regulated by extracellular calcium. (A) Representative SEM micrographs (A1) unstimulated chondrocytes, (A2) stimulated chondrocytes after stimulation, (A3 and A5) unstimulated chondrocytes in EGTA-treated media at 0 and 6 hours, (A4, A6) stimulated chondrocytes in EGTA-treated media at 0 and 6 hours, (A7) unstimulated chondrocytes in EGTA-treated media supplemented with calcium at 0 hours, and (A8) stimulated chondrocytes in EGTA-treated media supplemented with calcium at 0 hours. (B) Quantification of chondrocyte area in control (C) and stimulated (S) constructs at 0 and 6 hours post mechanical stimulation in the presence of EGTA or vehicle. *denotes statistically significant difference ($p<0.05$) as compared to respective unstimulated samples (n=3, in duplicates).

Figure 3: Chondrocyte cell spreading and contraction following mechanical stimulation are controlled by two different ion channels. Chondrocytes were stimulated in the presence of Nifedipine (10 μM), Gadolinium (10 μM), or control vehicle (0.01% DMSO in HAM'S-F12) Chondrocytes visualized by SEM at 0 hours (A) or 6 hours (B) and area quantified morphometrically (C, D). Treatment with the A23187 ionophore partially reversed the effects on nifedipine and gadolinium (E). (A1) unstimulated, (A3) stimulated, (A2) unstimulated and gadolinium treated, (A4) stimulated and gadolinium treated, (A5) unstimulated and nifedipine treated, (A6) stimulated and nifedipine treated. (B1) unstimulated, (B3) stimulated, (B2) unstimulated and gadolinium treated, (B4) stimulated and gadolinium treated, (B5) unstimulated and nifedipine treated, (B6) stimulated and nifedipine treated. *denotes statistically significant difference ($p<0.05$) as compared to respective unstimulated samples.

Figure 4: Upregulation of MT1-MMP in response to mechanical stimulation in chondrocytes is dependent extracellular calcium. Cells were stimulated in the presence of 10 μM EGTA, 10 μM EGTA with the addition of 5 μM $CaCl_2$, or control vehicle (PBS in HAM'S F-12). (A) Chelation with EGTA abolished the up-regulation of MT1-MMP mRNA following mechanical stimulation. Re-introduction of calcium into the chelated media returned the expected increase MT1-MMP mRNA expression following mechanical stimulation. (C) Representative gel illustrating the effects of calcium chelation and re-introduction on MT1-MMP gene expression. * denotes statistically significant difference ($p<0.05$) as compared to unstimulated control constructs at the respective time points. Rsults are expressed as means ± SEM (n=3, in duplicates).

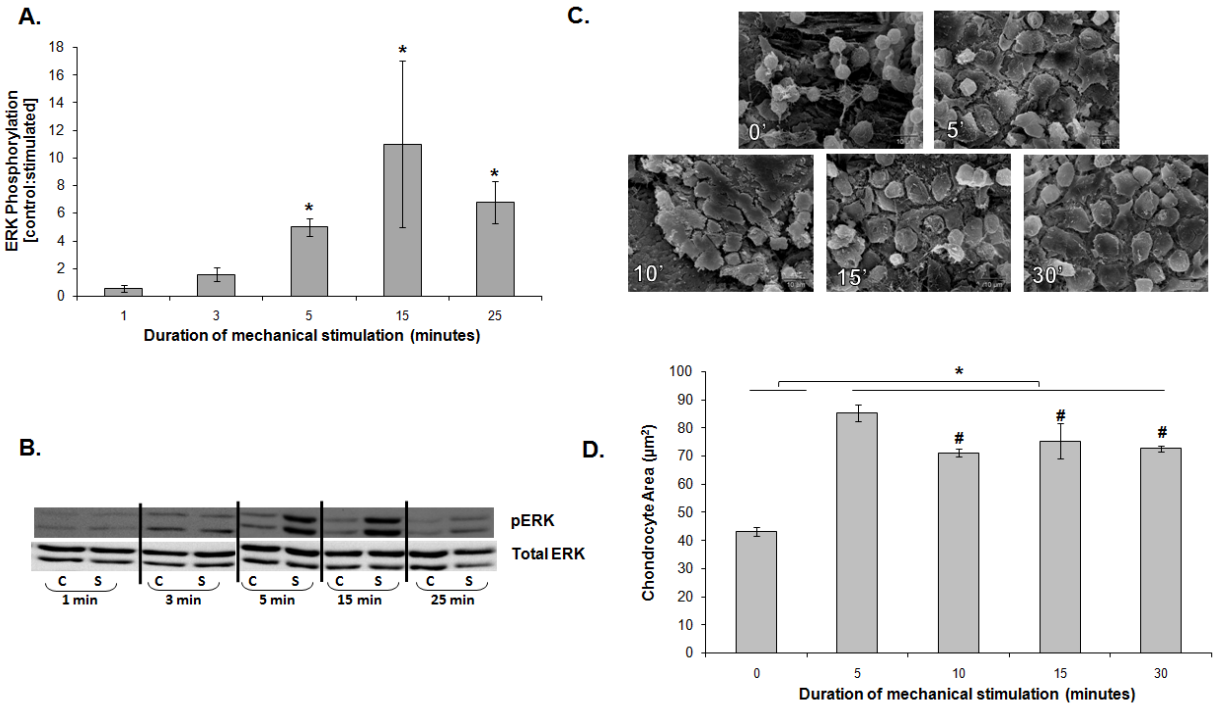
Figure 5: Upregulation of MT1-MMP in response to mechanical stimulation in chondrocytes is dependent upon calcium. Chondrocytes were stimulated in the presence of Nifedipine (10 μM), or Gadolinium (10 μM), or vehicle (0.01% DMSO in HAM'S-F12). (A) pre-treatment with Nifedipine and Gadolinium abolished the up-regulation of MT1-MMP mRNA, as seen in vehicle-treated constructs, following mechanical stimulation. (B) Pre-treatment of constructs with Nifedipine resulted in a significantly increased MT1-MMP mRNA expression in unstimulated constructs, suggesting calcium signalling is involved in regulating constitutive MT1-MMP levels in chondrocytes. (C) Representative gel illustrating the effects of Nifedipine and Gadolinium on MT1-MMP gene expression. * denotes statistically significant difference ($p<0.05$) as compared to unstimulated control constructs at the respective time points. Results are expressed as means ± SEM (n=3, in duplicates)

Figure 6: Treatment with calcium ionophore reversed the effects of nifedipine/golinium inhibition on MT1-MMP following cyclic compression. (A) Treatment with the A23187 ionophore also returned MT1-MMP expression to stimulated constructs which mimicked the changes seen in vehicle-treated constructs. (B) Representative gel illustrating the effects of A23187 ionophore treatment on MT1-MMP gene expression in nifedipine/gadolinium treated constructs. * denotes statistically significant difference ($p<0.05$) as compared to unstimulated control constructs at the respective time points. Results are expressed as means ± SEM (n=3, in duplicates).

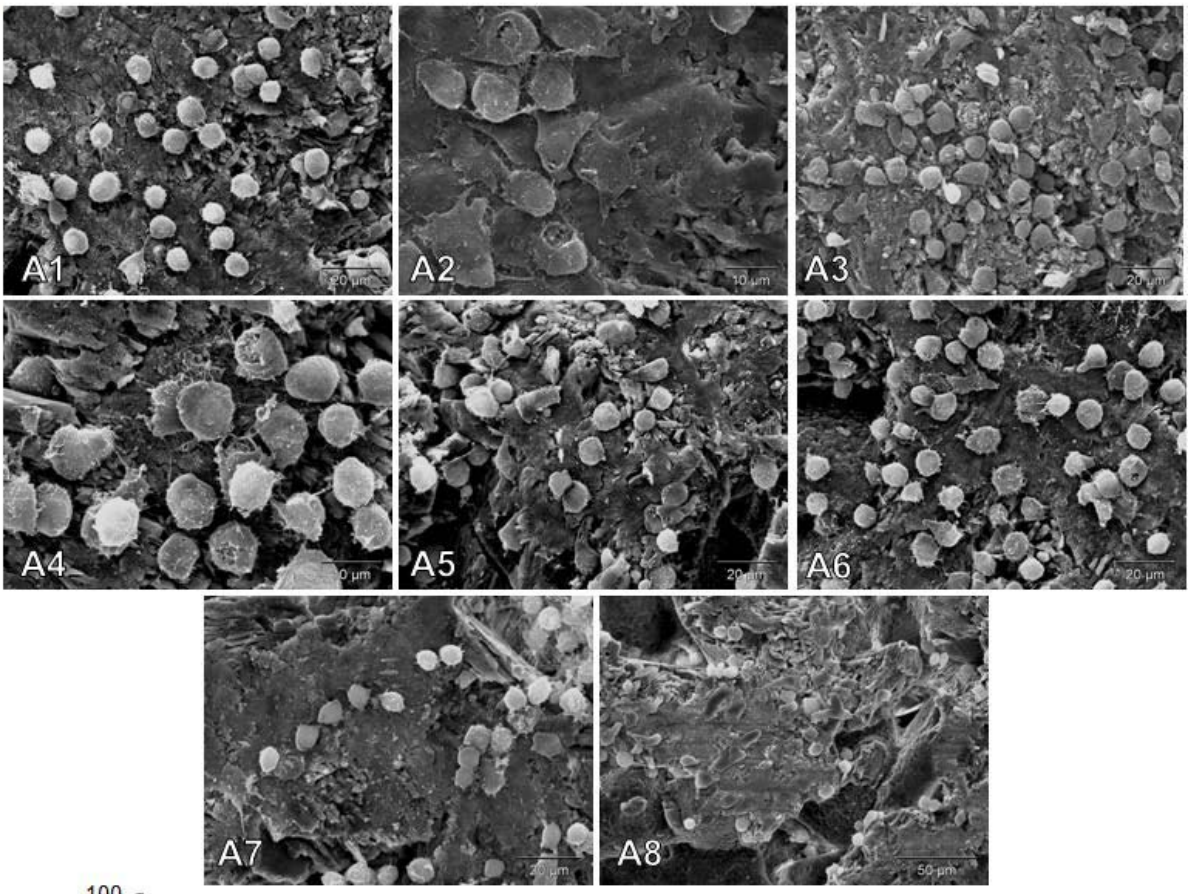
Figure 7: Treatment with the Src inhibitor PP2 inhibited cell spreading and upregulation of MT1-MMP following cyclic compression. (A) Treatment with PP2 abolished cell spreading following mechanical stimulation, which was observed in vehicle and PP3 (negative control of PP2) treated cells. (B) Treatment of cells with PP2 prior to mechanical stimulation also abolished the expected increase in MT1-MMP mRNA which was seen in vehicle and PP3 treated constructs. (C) Representative gel illustrating the effects of PP2 treatment on MT1-MMP gene expression in stimulated constructs. * denotes statistically significant difference ($p<0.05$) as compared to unstimulated control constructs at the respective time points. Results are expressed as means ± SEM (n=3, in duplicates).

Figure 8: Calcium signalling and integrin signalling regulate cell response through mutual regulation of ERK phosphorylation following mechanical stimulation. (A) Treatment of cells with both the Src inhibitor PP2 and the calcium ionophore partially reversed the effects of PP2 in terms of cell area; the partial effect was not attributed to insufficient intracellular calcium (dark bar). (B) Inhibition with gadolinium did not abolish ERK phosphorylation, neither did treatment with nifedipine and PP2 although variability was observed. Co-treatment with PP2 and nifedipine completely abolished ERK phosphorylation following mechanical stimulation. (C) Representative blots illustrating the effects of PP2, nifedipine and gadolinium treatment on ERK phosphorylation in stimulated constructs. * denotes statistically significant difference ($p<0.05$) as compared to unstimulated control constructs at the respective time points. Results are expressed as means ± SEM (n=3, in duplicate).

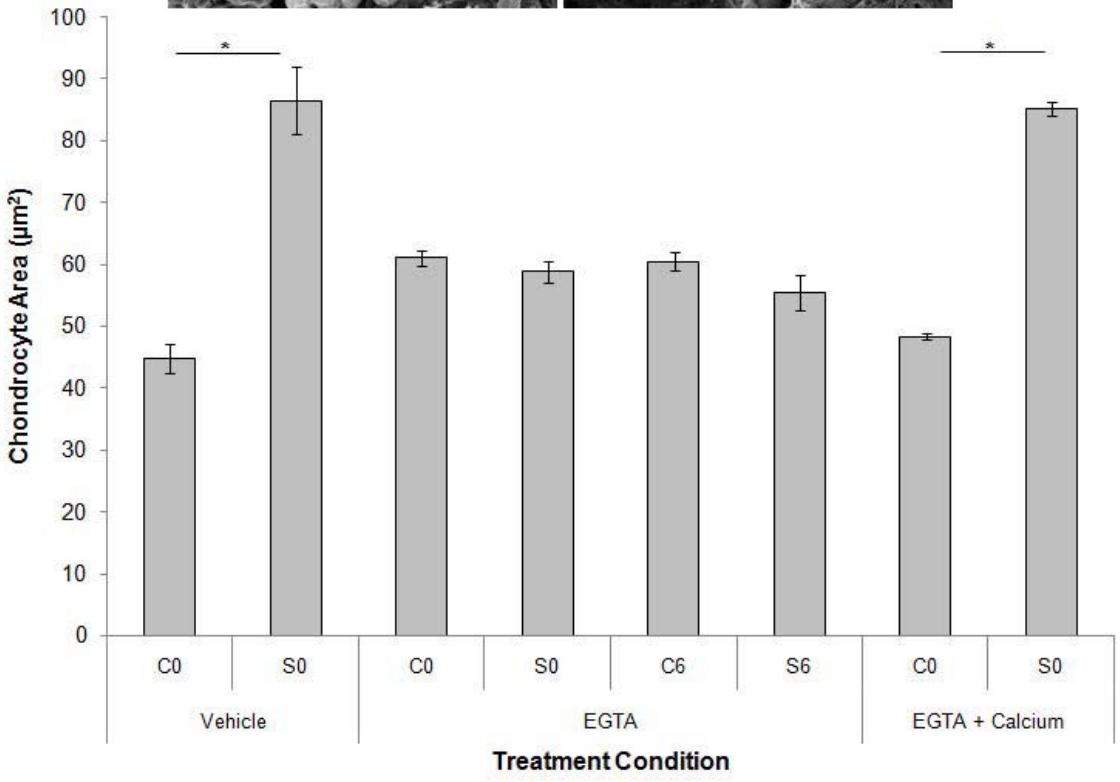
Figure 9: **Proposed mechanism by which chondrocytes respond to mechanical stimulation with increased synthesis and accumulation of extracellular matrix.**

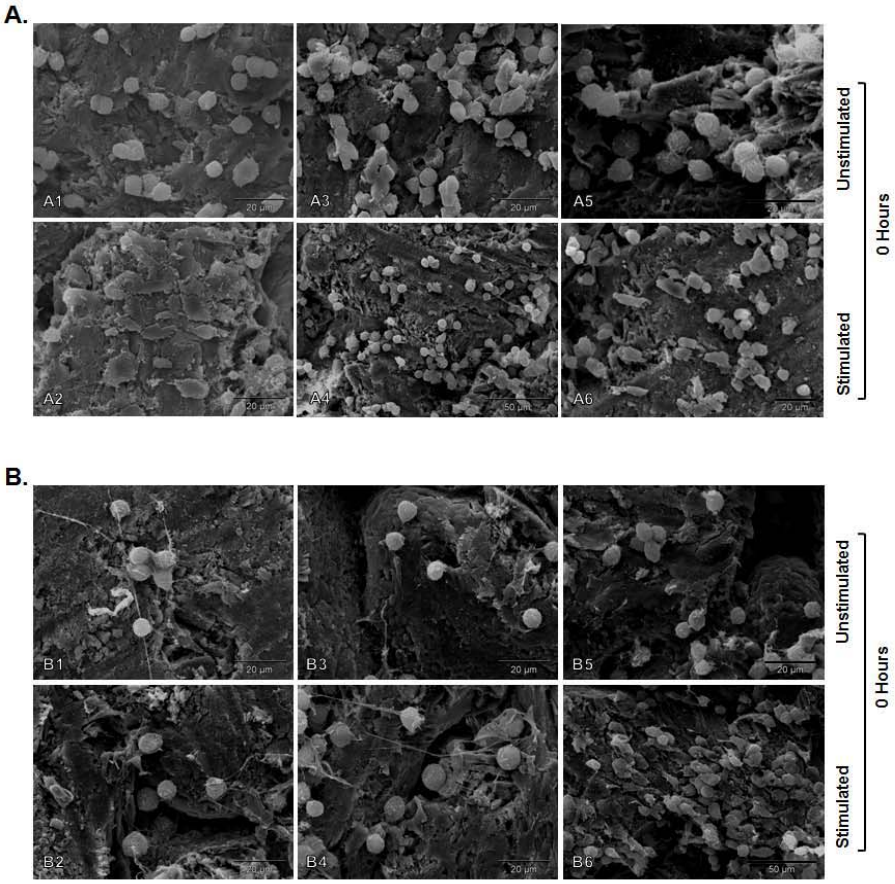

A.
ERK Phosphorylation [control:stimulated]
Duration of mechanical stimulation (minutes)
B.
pERK
Total ERK
C S
1 min
3 min
5 min
15 min
25 min
C.
0'
5'
10'
15'
30'
D.
Chondrocyte Area (μm²)
Duration of mechanical stimulation (minutes)

A.

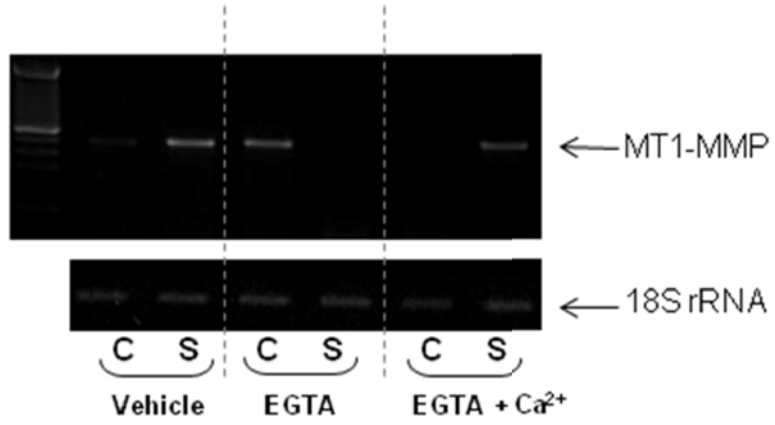

A1
A2
A3
A4
A5
A6
A7
A8

B.

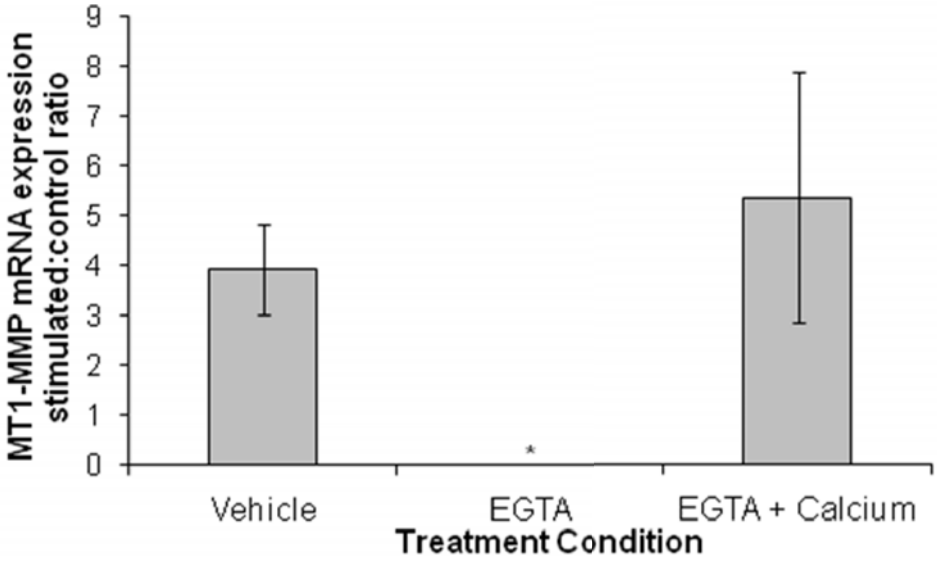

Chondrocyte Area (µm²)
100
90
80
70
60
50
40
30
20
10
0
*
*
C0
S0
C0
S0
C6
S6
C0
S0
Vehicle
EGTA
EGTA + Calcium
Treatment Condition

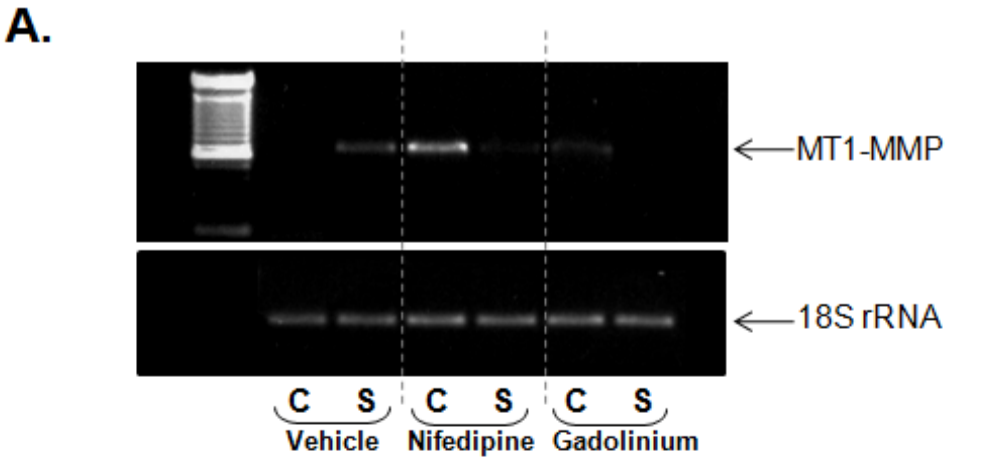

A.
A1
A3
A5
A2
A4
A6
Unstimulated
Stimulated
0 Hours
B.
B1
B3
B5
B2
B4
B6
Unstimulated
Stimulated
0 Hours
C.
Chondrocyte Area (um²)
Stimulated
Nifedipine Treated
0 hours
6 Hours
D.
Chondrocyte Area (um²)
Stimulated
Gadolinium Treated
0 hours
6 Hours
E.
Chondrocyte Area (µm2)
U
S
0 hours
6 hours
Nifedipine + Ionophore
Gadolinium + Ionophore
Vehicle
Treatment Condition

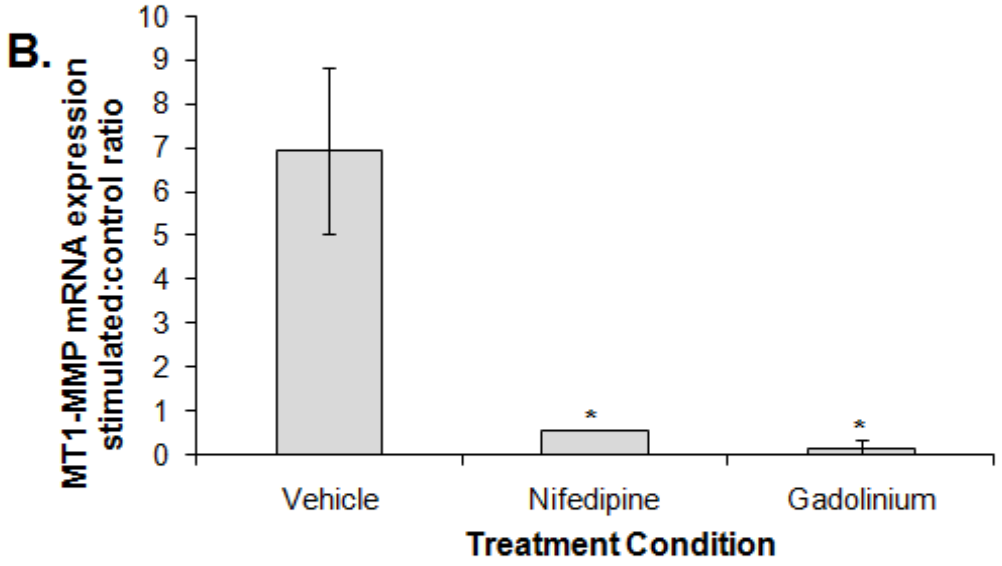
A.

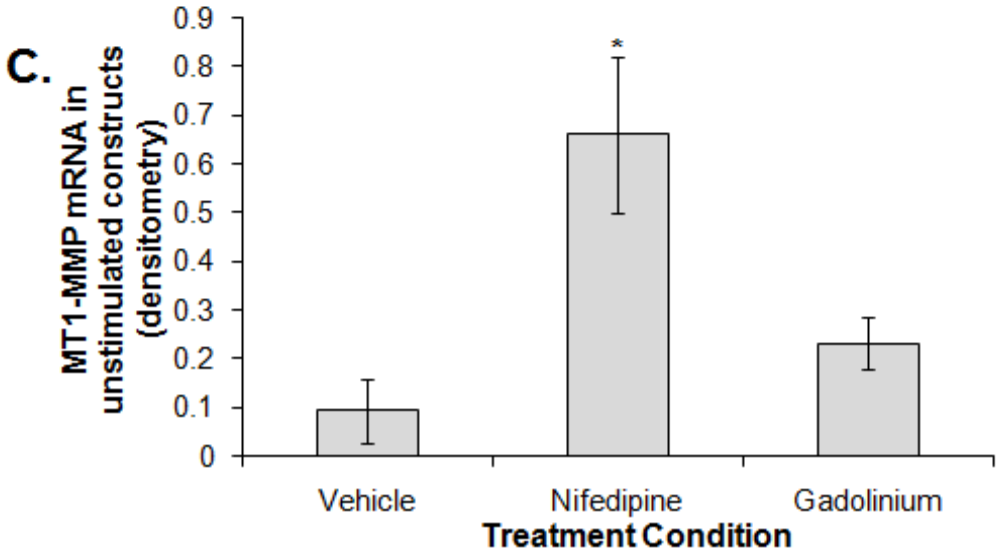

MT1-MMP
18S rRNA
C S C S C S
Vehicle
EGTA
EGTA + Ca2+

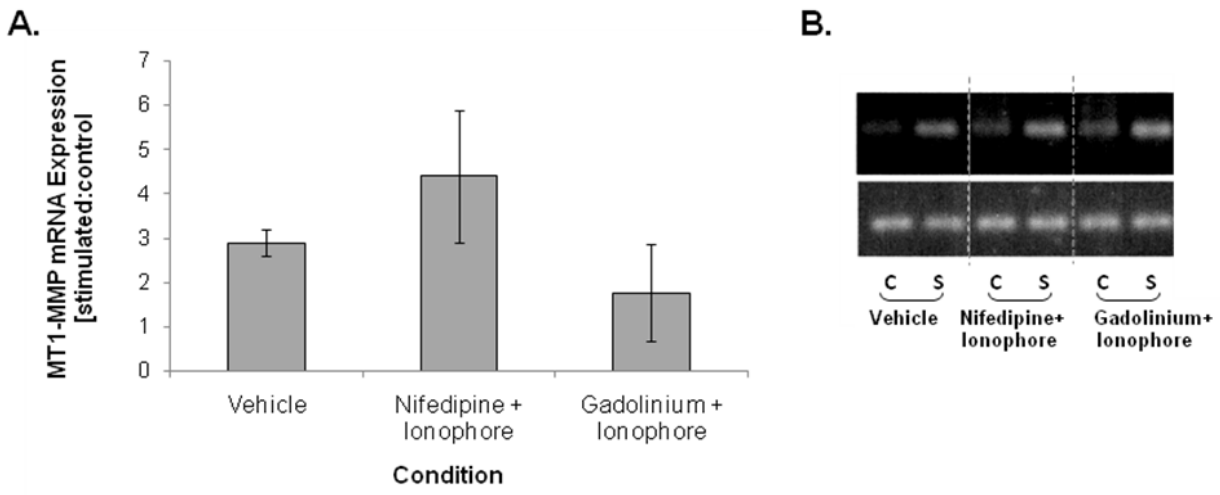
B.

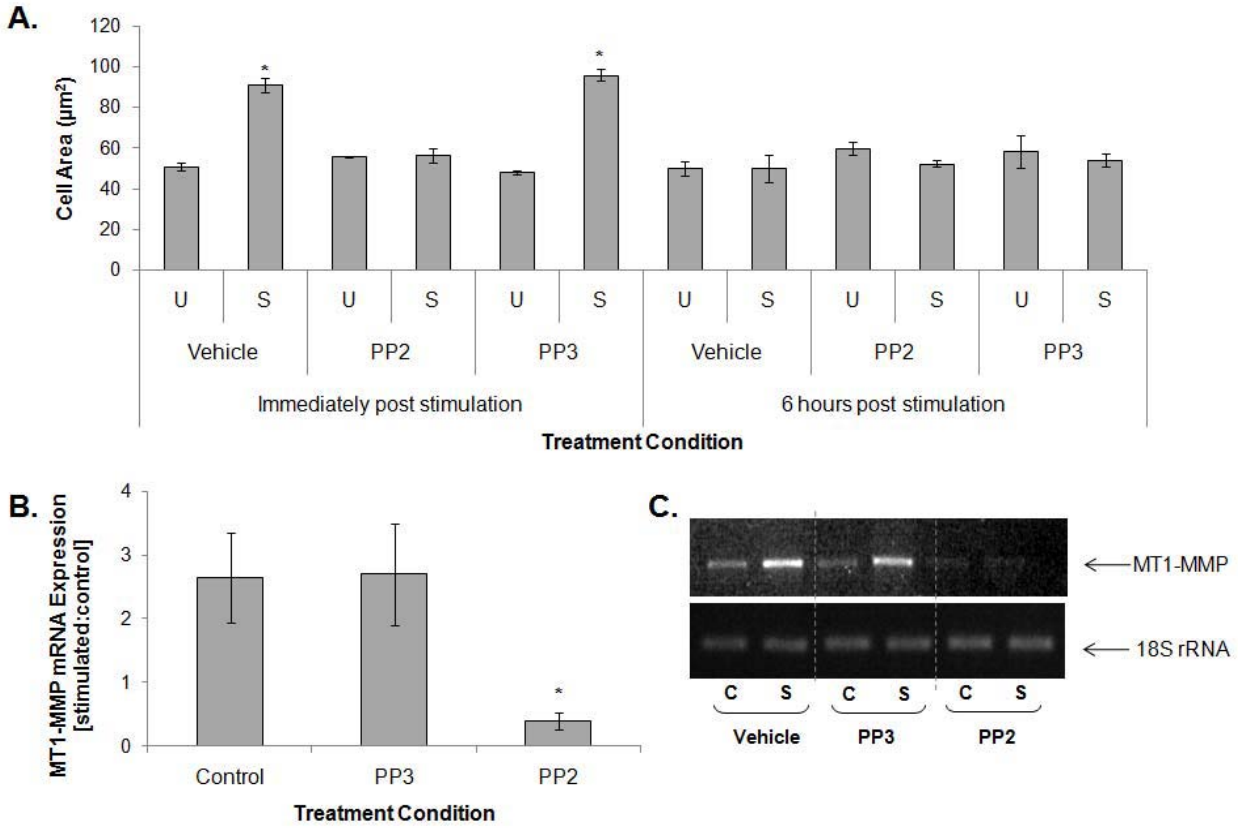

MT1-MMP mRNA expression
stimulated:control ratio
0
1
2
3
4
5
6
7
8
9
*
Vehicle
EGTA
EGTA + Calcium
Treatment Condition

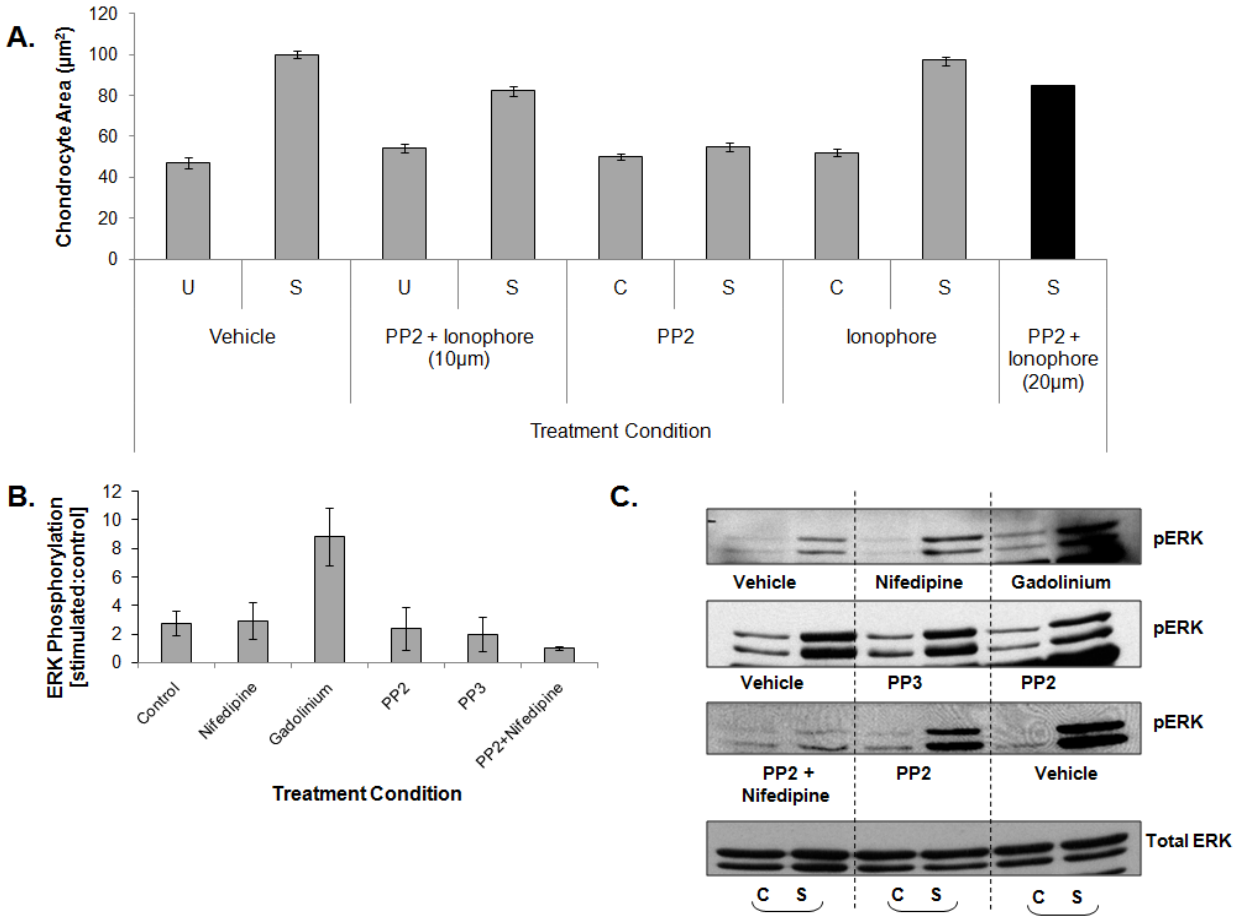

A.
MT1-MMP
18S rRNA
C S C S C S
Vehicle
Nifedipine
Gadolinium

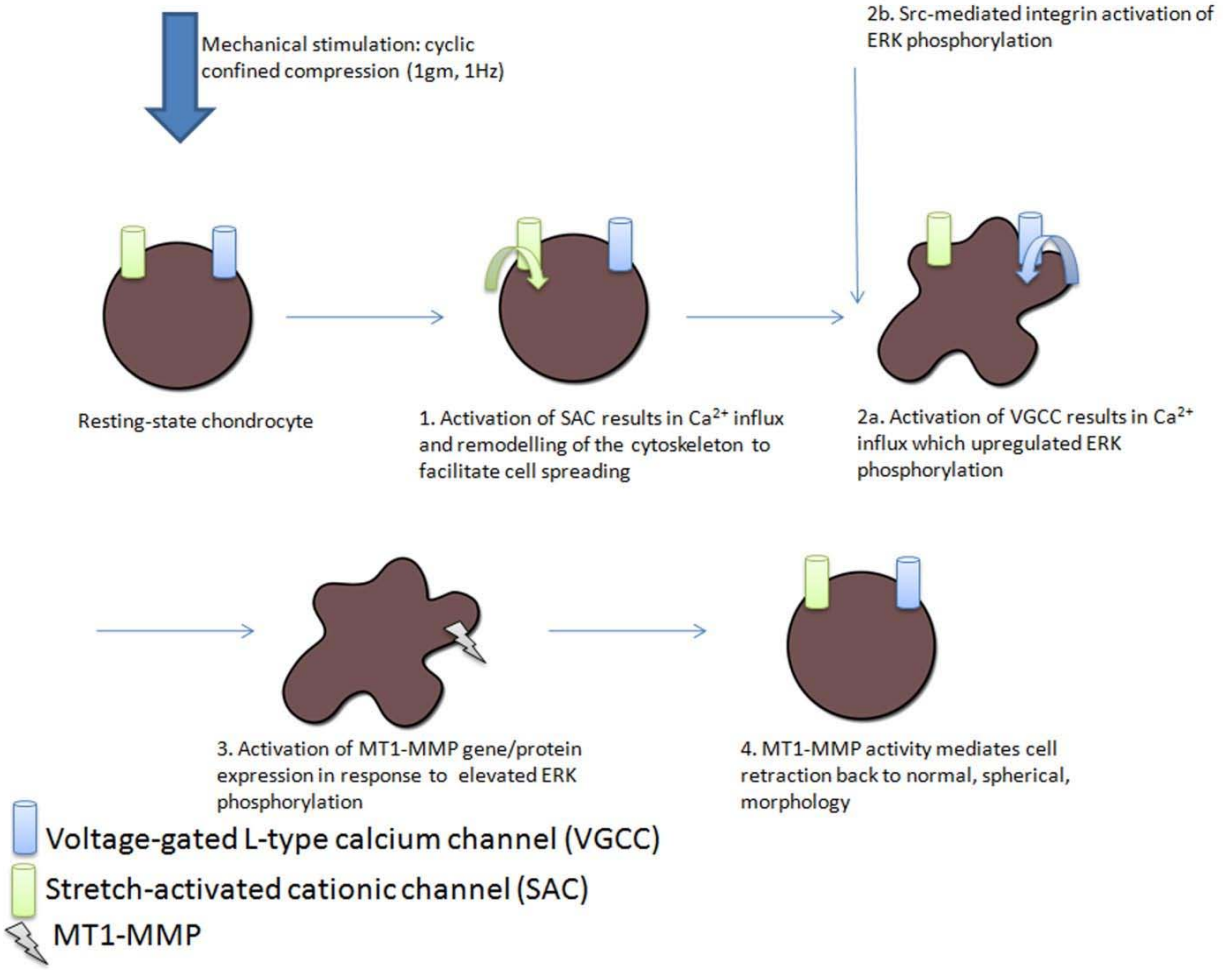

B.
MT1-MMP mRNA expression stimulated:control ratio
10
9
8
7
6
5
4
3
2
1
0
*
*
Vehicle
Nifedipine
Gadolinium
Treatment Condition


C.
MT1-MMP mRNA in unstimulated constructs (densitometry)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*
Vehicle
Nifedipine
Gadolinium
Treatment Condition


A.
MT1-MMP mRNA Expression [stimulated:control
7
6
5
4
3
2
1
0
Vehicle
Nifedipine + Ionophore
Gadolinium + Ionophore
Condition
B.
C S
C S
C S
Vehicle
Nifedipine+ Ionophore
Gadolinium+ Ionophore


A.
Cell Area (μm²)
120
100
80
60
40
20
0
*
*
U
S
Vehicle
PP2
PP3
Immediately post stimulation
6 hours post stimulation
Treatment Condition
B.
MT1-MMP mRNA Expression [stimulated:control]
4
3
2
1
0
*
Control
PP3
PP2
Treatment Condition
C.
MT1-MMP
18S rRNA
C
S
Vehicle
PP3
PP2


A.
Chondrocyte Area (µm²)
120
100
80
60
40
20
0
U
S
U
S
C
S
C
S
S
Vehicle
PP2 + Ionophore (10µm)
PP2
Ionophore
PP2 + Ionophore (20µm)
Treatment Condition
B.
ERK Phosphorylation [stimulated:control]
12
10
8
6
4
2
0
Control
Nifedipine
Gadolinium
PP2
PP3
PP2+Nifedipine
Treatment Condition
C.
pERK
Vehicle
Nifedipine
Gadolinium
pERK
Vehicle
PP3
PP2
pERK
PP2 + Nifedipine
PP2
Vehicle
Total ERK
C S
C S
C S


Mechanical stimulation: cyclic confined compression (1gm, 1Hz)
2b. Src-mediated integrin activation of ERK phosphorylation
Resting-state chondrocyte
1. Activation of SAC results in Ca^{2+} influx and remodelling of the cytoskeleton to facilitate cell spreading
2a. Activation of VGCC results in Ca^{2+} influx which upregulated ERK phosphorylation
3. Activation of MT1-MMP gene/protein expression in response to elevated ERK phosphorylation
4. MT1-MMP activity mediates cell retraction back to normal, spherical, morphology
Voltage-gated L-type calcium channel (VGCC)
Stretch-activated cationic channel (SAC)
MT1-MMP

CHAPTER THREE: DISCUSSION

Previously we had shown that a single application of cyclic compression of 30 minutes duration resulted in enhanced cartilage tissue formation in vitro. This change was shown to be dependent on transient changes in cell shape regulated via α5β1 integrin, ERK MAPkinase activation and upregulation of MT1-MMP expression. The current study demonstrated that the transient cell spreading and retraction, as well as the associated upregulation of MT1-MMP, that follows a single application of cyclic compression are regulated via calcium as treatment of the media with EGTA, a cation chelator, prior to stimulation abolished these changes and re-introduction of calcium was able to reverse this inhibitory effect. Cell spreading and retraction were regulated through two distinct calcium-permissive ionic channels, the cationic stretch-activated channel and the L-Type voltage gated channel, respectively, which also supports the involvement of calcium fluxes. Both channels were also shown to exert a regulatory role on the expression of MT1-MMP both in unstimulated and stimulated constructs. Calcium also appeared to be involved in regulating integrin activation as mechanical stimulation in the presence of A23187 ionophore and PP2, an inhibitor of integrin-associated kinase Src, returned cell spreading and MT1-MMP upregulation almost back to control levels. The two pathways appear to converge on the phosphorylation of ERK, as inhibition of both pathways was required to achieve a complete down regulation of ERK phosphorylation in response to mechanical stimulation.

Interestingly it was shown that significant changes in cell morphology (5 minutes into stimulation) occur prior to the peak in phosphorylation of ERK (15 minutes into stimulation) possibly suggesting that physical changes in cell shape precede/initiate the downstream signalling cascades such as ERK MAP Kinase. Past studies in our lab showed that when ERK

activation is inhibited MT1-MMP upregulation in response to cyclic compression does not occur [De Croos, Jang, Dhaliwal, Grynpas, Pilliar & Kandel, 07]. Since it is postulated that MT1-MMP facilitates cellular retraction, through destruction of cell-matrix contacts, it is possible to conclude that the activation of ERK may not be involved in cell spreading but rather subsequent events. This notion is supported by studies which observed that physical deformations in the cellular membrane comprise the first step in mechanotransduction [Guilak, 95;Knight, Bomzon, Kimmel, Sharma, Lee & Bader, 06a]. However, this is controversial as several studies in other cell types, such as Chinese hamster ovary cells and smooth muscle cells, have suggested that ERK upregulation occurs prior, and in fact drives, any cell shape changes associated with physical membrane perturbation or intergrin-activation [Levy *et al.*, 03;Samarakoon & Higgins, 02]. To fully delineate the sequence of events in our system, it would be necessary to fully inhibit or knockout ERK expression prior to mechanical stimulation and observe whether changes in cell morphology occur. However, owing to the extensive role ERK plays in multitude of cellular processes it may be hard to conclusively prove a direct influence of ERK on cell shape in response to cyclic compression. Thus, based on the data obtained in our study, we cannot determine definitively whether cell spreading drives ERK phosphorylation or that the two occur simultaneously.

The involvement of calcium in regulating cell response in our system was not unexpected as others have previously documented the role of calcium in chondrocyte mechanotransduction [Roberts, Knight, Lee & Bader, 01;Tanaka, Ohno, Honda, Tanimoto, Doi, Ohno-Nakahara, Tafolla, Kapila & Tanne, 05;Zhang, Wang & Chen, 06]. The role of calcium signalling in our system was demonstrated through the use of EGTA which was shown to abolish the stimulation-induced increase in cell spreading, and the reintroduction of calcium into the media which

returned the cellular response to mechanical stimulation (Figure 2). It is worthwhile noting that EGTA is able to chelate a variety of cations (namely Ca^{2+}, Na^{2+}, K^{+}), but as the re-introduction of calcium alone into the media was able to return cell response to normal levels, the mechanism regulating this response was attributed to calcium and not other media soluble cations. The precise amount of calcium that was re-introduced into the media (in the form of $CaCl_2$) was determined previously in the lab through dose-response experiments which reintroduced calcium into EGTA-chelated media at various concentrations until cellular response was identical to that observed in EGTA-untreated samples. It was important to fully saturate any EGTA present in the media to ensure sufficient free calcium was present. The lack of cellular shape changes in the absence of calcium may be a result of either prevention of calcium-dependent reorganization of the actin cytoskeleton or activation of other calcium-associated downstream signalling events. Calcium is required for substantial remodelling of the actin cytoskeleton, which is necessary for cell spreading, and is controlled through activation of calmodulin and other calcium-dependent actin binding proteins [Toyoda *et al.*, 03]. It is known that under certain loading conditions chondrocytes exhibit a rapid (~8 minutes) reorganization of their actin cytoskeleton in a calcium-dependent manner [Erickson, Northrup & Guilak, 03], and that calcium fluxes through the SAC are involved in regulating chondrocyte morphology [Perkins, Derfoul, Ast & Hall, 05]. Interestingly, however, a study by Lurrant *et al.*, suggested that chondrocytes in fact do not alter their actin cytoskeleton in response to mechanical stimulation, and instead modify their vimentin cytoskeleton [Durrant *et al.*, 99]. Past studies have implicated PKC, a calcium dependent enzyme, in regulating vimentin cytoskeleton reorganization in cell types such as astrocytes [Mangoura *et al.*, 95], and thus it is possible that a similar mechanism may exist in chondrocytes. PKC, which encompasses a family of 11 closely related isozymes (of which the PKCα, -β_1, -β_2,

and -γ forms are calcium-dependent) has also been previously shown to regulate the Ras/Raf/MAP Kinase pathway in a variety of systems [Kolch, 00;Marshall, 95] and thus is a plausible regulator of this system as well. We attempted to visualize the actin cytoskeleton following mechanical stimulation through the use of confocal microscopy, and although it was just a preliminary study (Appendix A, Figure 1) we observed that there was no significant alteration in the actin cytoskeleton with stimulation. This observation suggests that the morphological changes observed in our system may be modulated by the vimentin cytoskeleton as well. To further investigate this, the vimentin cytoskeleton should be observed through confocal microscopy as well.

Interestingly it was also observed that EGTA treatment of the medium resulted in a slightly (not significant) increase in chondrocyte cell area independent of mechanical stimulation. This observation can be possibly explained by either EGTA-induced changes in media osmolality or to calcium's regulatory function in maintaining cell shape and volume. While osmolality has been shown to be a significant regulator of chondrocyte function [Hopewell & Urban, 03], the authors utilized solutions with drastically lower osmolality (380 mOsmol) than that which could be induced by the EGTA chelation in our system, suggesting that the minor changes that might have occurred in our system were unlikely to be responsible for changes in cell shape. However measurements of the media osmolality would have to be done to determine the actual levels in our system. Alternatively, it could be possible to increase the osmolality in our system through the addition of ions such as Na^+, however this may interfere with some cellular processes which are activated by Na^+ channels. There is some evidence that sodium mechanosensitive channels are expressed in chondrocytes and may play a role in regulating cell response as well [Shakibaei & Mobasheri, 03]. Thus it is more plausible that the

changes in cell area are attributed to the regulatory role calcium exerts in regulating cell shape and volume. For instance, $[Ca^{2+}]_i$ has been shown to control Regulatory Volume Decreases (RVD) and Increases (RVI) in various cell types including osteocytes and chondrocytes [Miyauchi *et al.*, 00;Yellowley *et al.*, 02]. In a study by Kerrigan *et al.*, the authors showed that changes in $[Ca^{2+}]_i$ influences RVD in only a subpopulation of freshly isolated bovine chondrocytes [Kerrigan & Hall, 08], thus suggesting that the slight increase observed in cell area in our system can be attributed to interference with calcium regulation in the cells.

Having confirmed the role of extracellular calcium, we further characterized its role in mediating the response through the use of the inhibitors nifedipine and gadolinium (Gd^{3+}) to selectively block either the voltage-gated L-Type calcium channel (VGCC) or the stretch-activated channel (SAC), respectively (Figure 4). In the presence of Gd^{3+} cell spreading was entirely abolished in response to mechanical stimulation, suggesting that calcium flow through SACs is directly involved in influencing cytoskeletal remodelling leading to cell spreading. This particular channel has been previously shown to be mechanosensitive [Wu *et al.*, 01a;Mouw, Imler & Levenston, 07] and it is possible that those responses were, at least in part, attributed to cytoskeletal rearrangements that have been facilitated through calcium currents passing through the channel. The channel is known to be activated through direct physical deformation of the cellular membrane [Wu & Davis, 01;Zou, Lifshitz, Tuft, Fogarty & Singer, 02], which some argue is the initial step in mechanotransduction [Guilak, 95;Knight, Bomzon, Kimmel, Sharma, Lee & Bader, 06a], and thus it might expected that any subsequent processes (spreading, retraction, etc.) be regulated through this channel. Unfortunately it is currently impossible to characterize the expression of the SAC in chondrocytes as the channel has not been fully described [Maroto, Raso, Wood, Kurosky, Martinac & Hamill, 05], but its presence in

chondrocytes has been supported through other studies utilizing the inhibitor gadolinium [Guilak, Zell, Erickson, Grande, Rubin, McLeod & Donahue, 99;Mouw, Imler & Levenston, 07;Perkins, Derfoul, Ast & Hall, 05].

In contrast, cells stimulated in the presence of nifedipine exhibited a spread morphology indicating that either calcium currents through this particular channel are not involved in the early stages of cytoskeletal remodelling, or that the channel remains inactive throughout the stimulation itself and that it becomes active sequentially after the SACs. The latter explanation is, however, less likely as we have observed an effect and other have previously reported that VGCC influences some cellular responses to mechanical stimulation in chondrocytes , such as the secretion of PTHrP in response to cyclic strain[Tanaka, Ohno, Honda, Tanimoto, Doi, Ohno-Nakahara, Tafolla, Kapila & Tanne, 05]. Nonetheless, while the chondrocytes spread in the presence of nifedipine it was observed that their subsequent retraction, which normally occurs by six hours post-stimulation, was abolished. We have previously shown that the retraction phase of the response is mediated via the activity of the membrane-bound MT1-MMP (submitted for publication), which itself is regulated through ERK phosphorylation [De Croos, Jang, Dhaliwal, Grynpas, Pilliar & Kandel, 07]. Consequently it is possible that calcium currents through the VGCC are not directly involved in the remodelling of the cytoskeleton, but rather through activation of ERK which regulates the activity of MT1-MMP and thus indirectly influence retraction. This possibility is supported by previous literature indicating that calcium influx through VGCC is able influence ERK phosphorylation in several cell types [Mulvaney *et al.*, 99;Gomez, Pritchard & Herbert, 02]. Overall, however, the sequential role of the SAC and the L-type VGCC is in keeping with literature suggesting that one of the functional outcomes of SAC activation is cell depolarization and subsequent activation of VGCCs [Zou, Lifshitz, Tuft,

Fogarty & Singer, 02]. The expression of the channel by the chondrocytes in our system was confirmed through RT-PCR (Appendix A, Figure 2).

To further study the role of calcium in mechanotransduction, as well as confirm the effects of SAC and L-Type VGCC inhibition were due to cation fluxes, we utilized the A23187 ionophore to modulate calcium levels. The calcium ionophore A23187 is a mobile ion-carrier that forms stable complexes with divalent cations (such as Ca^{2+}) and allows their transport across the cellular membrane. Unlike other ionophores which form hydrophilic pores in the cellular membrane and thus allowing ions to pass, the A23187 ionophore binds divalent cations and through shielding of their charge from the surrounding environment facilitates their crossing into the cell. The addition of A23187 ionophore is utilized to increase intracellular calcium levels, and has been previously shown to elevate intracellular calcium levels in chondrocytes [Schwartz *et al.*, 91]. Treatment of cells with the A23187 ionophore during cyclic compression was able to partially reverse the effects of nifedipine and gadolinium on cell morphology. The reason for only the partial restoration of changes in cell area with the ionophore treatment is not known, but one possible explanation may be that the localization of calcium, and not merely its presence, following mechanical stimulation is an important aspect of this response. Localized calcium currents have been previously documented to influence local signalling cascades and thus affect localized signalling pathways [Janmey, 98;Zou, Lifshitz, Tuft, Fogarty & Singer, 02]. Alternatively, it is possible that exposure of chondrocytes to the ionophore activates additional, mechanical stimulation-unrelated, signalling pathways that influence processes which indirectly may regulate cell area. For instance, in our lab we have previously shown that treatment of bovine synovial fibroblasts with the ionophore has resulted in the production of a gelatinase [Howarth, Pritzker, Cruz & Kandel, 93] without the application of any mechanical forces.

Consequently, treatment of chondrocytes with A23187 ionophore may elicit numerous other cellular effects which could prevent a complete restoration of the response.

Owing to the previously established role of MT1-MMP in modulating cell and chondrocyte morphology (submitted for publication)[Chun, Hotary, Sabeh, Saltiel, Allen & Weiss, 06], we investigated whether the stimulation-induced upregulation MT1-MMP is also regulated via calcium. Chelation of extracellular calcium abolished the stimulation-induced upregulation of MT1-MMP suggesting either calcium directly exerts a control over MT1-MMP expression or that MT1-MMP expression is dependent upon cell morphology changes (Figure 3). The former is supported through studies that have shown that calcium can regulate the binding activity of AP-1 (a regulator of MT1-MMP expression) in human pulmunary artery endothelial cells [Fantozzi, Zhang, Platoshyn, Remillard, Cowling & Yuan, 03], and through studies that implicated L-type VGCC currents in direct regulation of AP-1 binding activity [Premkumar, Mishra, Overholt, Simonson, Cherniack & Prabhakar, 00]. Further supporting the idea that calcium directly regulates MT1-MMP expression was seen in tissue-constructs that were treated with EGTA resulting in a significant increase in MT1-MMP mRNA levels in the absence of mechanical stimulation. It is interesting to note that while this increase in MT1-MMP produced a temporary increase in the synthesis of collagen and proteoglycans, it did not result in improved tissue formation in the long term (Appendix A, Figure A3). One may hypothesize that without the synchronized changes in cell morphology, which perhaps result in the actual activation of MT1-MMP and the subsequent degradation of the pericellular matrix, the newly synthesized macromolecules are not incorporated into the tissue's matrix. The observed changes in MT1-MMP mRNA could not be replicated when chondrocytes were grown on monolayer, suggesting that the shape of cells, which is very different in monolayer compared to 3D culture, is important

in regulating MT1-MMP as well (Appendix A, Figure A4). The complete restoration of MT1-MMP gene expression in the absence of complete restoration of cell shape changes with ionophore treatment may also be indicative of a separate calcium signalling pathway that exerts control over MT1-MMP expression or, in contrast, that partial spreading and retraction are sufficient to elicit MT1-MMP gene expression. A study by Lohi *et al.* showed that ionophore treatment can induce MMP-9 and MMP-2. The absence if an effect on MT1-MMP expression in their system [Lohi & Keski-Oja, 95] may reflect the different cell type, fibrosarcoma, utilized. Fibrosarcoma cells which, being cancerous, are known to often have abnormal calcium signalling mechanisms (i.e. calcium signals to differentiate, undergo apoptosis, proliferate are ignored)[Whitfield, 92].

Previous studies in our lab suggested that cell morphology changes, MT1-MMP expression and the subsequent improvement in tissue matrix synthesis are regulated via the α5β1 integrin (submitted for publication), and owing to the closely documented relationship between calcium channels and integrins, it was important to investigate the role calcium plays in regulating integrin activity. In light of the above results, it was not clear how the calcium-related and integrin-associated signalling pathways interact to regulate cell shape changes and MT1-MMP expression. Since integrins lack any inherent enzymatic activity, signal transduction is accomplished via activation of various adapter proteins such as FAK and Src. Consequently, to determine the role calcium plays with respect to α5β1 integrin activity, the adapter proteins involved had to be elucidated. A timeline examining the phosphorylation of Focal Adhesion Kinase (FAK) conducted over 3, 5, 15 and 25 minutes of mechanical stimulation (Appendix A, Figure 5) indicated that no significant changes in the protein's phosphorylation state occurs, suggesting FAK is not involved in mediating this response. It should be pointed out, however,

that the antibody utilized to determine the phosphorylation of FAK by western blot analysis was a generic phospho-tyrosine antibody that targeted all cellular proteins with phosphorylated tyrosine residues, with the protein of interest (FAK) identified through incubation with an anti-FAK antibody. Consequently the lack of differences in FAK phosphorylation may be possibly attributed to non-selective binding of the anti-pTyr antibody to other proteins of similar molecular weight. Similarly, FAK contains multiple tyrosine residues that are differentially phosphorylated and differentially regulate cellular function; for instance the major phosphorylation site is tyrosine-397 which forms a Src Homology 2 (SH2) domain capable of interacting with Src-family kinases (i.e. Src, Fyn), while other sites include tyrosine-576 and -577 and -925 which may also interact with the kinase Grb2. Recently it was also demonstrated that phosphorylation at tyrosine-861 forms an additional SH2 binding domain [Calalb *et al.*, 96;Schaller, Hildebrand, Shannon, Fox, Vines & Parsons, 94;Schlaepfer, Broome & Hunter, 97;Yano, Geibel & Sumpio, 96]. As such, changes in phosphorylation in individual tyrosine residues may be masked with antibodies targeting total phospho-tyrosine.

A second kinase that is known to be heavily involved in mediating integrin signalling, Src, was likewise investigated in our system. However, due to a lack of a reliable antibody, its role was investigated through the use of the chemical inhibitor PP2 which selectively inhibits its activity. Based on the results (Figure 7) it appears that the response to cyclic compression is mainly facilitated through the kinase Src, as its inhibition abolished the subsequent cell spreading and MT1-MMP upregulation. Constructs incubated with the chemical PP3, a negative control for PP2, exhibited a response that was similar to that observed in vehicle treated samples. It is important to mention that while PP2 is utilized extensively in literature as a selective inhibitor of Src, it is able to inhibit several other tyrosine kinases, albeit not as effectively as indicated by the

following IC_{50} values: Lck 0.004 μM, Fyn 0.005 μM, ZAP-70 >100 μM, JAK2 >50 μM and EGF-R 0.49 μM (note: Lck and Fyn are members of the Src family)[Hanke *et al.*, 96]. Similarly, some authors have shown that the ability of PP2 to inhibit Src kinases may interfere with integrin-induced FAK phosphorylation [Salazar & Rozengurt, 01]. A study by Xia *et al.*, demonstrated that PP2 can effectively abolish the phosphorylation of Tyr-397 of FAK, the p85 subunit of PI3k, and Ser-473 of Akt which occur upon the activation of β1 ingetrins [Xia *et al.*, 04]. The same study indicated that PP3 had no effect on any of the investigated kinases. Consequently, while PP2 can be used to indicate the involvement of Src kinase in the signalling pathway, it is not, in itself, sufficient to reject the involvement of other kinases. The observation that no changes in FAK phosphorylation were seen with mechanical stimulation, in particular as cell spreading occurred, could be explained by noting that different types of mechanical forces differentially regulate the phosphorylation of FAK and Src [Ren, Kiosses, Sieg, Otey, Schlaepfer & Schwartz, 00]. Alternatively, it is possible that the cell-matrix interactions formed during this spreading are somewhere between Focal Adhesions and Fibrillar adhesions and thus the constitution of associated kinases could predominantly rely on Src. Fibrillar adhesions are known to play a role in matrix reorganization [Katz, Zamir, Bershadsky, Kam, Yamada & Geiger, 00], and it is believed that their formation (through maturation) is dependent upon Src activity [Volberg, Romer, Zamir & Geiger, 01]. Similarly, since the α5β1 integrin can be localized with MT1-MMP in intercellular contacts [Galvez, Matias-Roman, Yanez-Mo, Sanchez-Madrid & Arroyo, 02], and Src phosphorylation has been previously shown to regulate MT1-MMP activity [Wu, Gan, Yoo & Guan, 05] it is not surprising that inhibition of Src can influence the mechanosensitive upregulation of MT1-MMP following stimulation.

Analysis of the results indicated that both the α5β1 integrin and calcium channels exert a significant influence over chondrocytes' response to cyclic compression. It is probable that both integrins and calcium channels are components of a cellular mechanosensing complex. Supporting this notion is the observation that VGCCs are known to co-localize with β1 integrins in mouse limb-bud chondrocytes [Mobasheri, Carter, Martin-Vasallo & Shakibaei, 02;Shakibaei & Mobasheri, 03]. Another study, albeit in vascular smooth muscle cells, has shown that the α5β1 integrin has the ability to regulate the function of the L-Type VGCC [Wu, Davis, Meininger, Wilson & Davis, 01b], and it is now known that both Src and Pyk2 (integrin-associated proteins) are able to bind to the II-III linker and C-terminal regions of the α_{1C} subunit of the L-Type VGCC and this regulate its activity [Dubuis, Rockliffe, Hussain, Boyett, Wray & Gawler, 06]. Furthermore, in our system, treatment with the A23187 ionophore managed to partially offset the effects of Src inhibition by PP2 further implicating the convergence of these two pathways. These data suggest that calcium plays a major regulatory role in mediating this response, possibly to the extent of lying upstream to α5β1 and regulating its activation. This idea is supported by the observation that blocking calcium signalling completely abolished cell spreading in response to mechanical stimulation, and that ionophore treatment was able to partially reverse the effects of Src inhibition. Moreover, the ability of calcium to influence basal levels MT1-MMP expression in unstimulated samples further implicates calcium in exerting the major regulatory role in mediating chondrocytes' response. Nonetheless, to fully confirm this, it would be worthwhile to block calcium signalling (i.e. with nifedipine) and activate the α5β1 component through specific activating antibodies. This experiment will allow us to fully confirm whether calcium plays a major regulatory role exerting control over integrin activation. Alternatively, although unlikely based on our results, the results may indicate that the calcium

activation lies downstream of integrin activation. Such signalling pathways have been previously shown to exist in synapses, where activation of Src by integrins ultimately regulated the phosphorylation of the NMDA channel [Lin *et al.*, 03]. To fully determine which of the pathways is activated first it would be imperative to investigate and measure real-time calcium currents in response to mechanical stimulation. If the calcium current were to be characterized in this system, it would allow us to inhibit or knockout various cellular components and to determine their relative order in the pathway. For instance, if inhibiting α5β1 integrin would abolish calcium currents in response to compression that that would be a definitive indication that integrin activation transpires upstream of calcium signalling. The phosphorylation of ERK, however, is suggestive of a parallel pathway where both signalling cascades converge to regulate ERK, which then exerts a regulatory role on AP-1 and MT1-MMP expression.

In addition, aside from potentially establishing the mechanism by which chondrocytes sense and respond to the mechanical stimulation, this study further sheds some light on the manner by which mechanical stimulation results in increased matrix production. Previous studies in our lab have indicated that the minimum duration of mechanical stimulation that is able to produce an increase in matrix macromolecules was 30 minutes of cyclic compression [Waldman, Spiteri, Grynpas, Pilliar, Hong & Kandel, 03;Waldman, Couto, Grynpas, Pilliar & Kandel, 06]. However, the results obtained in this study indicate that all of the previously characterized crucial events, namely cell spreading and ERK phosphorylation, occur much earlier into the stimulation cycle. This observation raises the possibility that additional crucial events, unknown as of yet, that must transpire to induce increased accumulation of matrix molecules. For instance, the secretion of various signalling chemicals, such as ATP, PTHrP, and IL-1 in response to mechanical stimulation have been previously observed in chondrocytes, and

interestingly their release have been shown to influence or be influenced by either calcium or integrin related signalling [Millward-Sadler *et al.*, 99;Millward-Sadler *et al.*, 04;Tanaka, Ohno, Honda, Tanimoto, Doi, Ohno-Nakahara, Tafolla, Kapila & Tanne, 05;Elfervig *et al.*, 01]. It is possible that these molecules require a longer stimulation period before they can be released and accumulate in a sufficient concentration to act in an autocrine manner to regulate the cell response. A study by Ionescu *et al.,* revealed that PTHrP can modulate chondrocyte differentiation through regulation of AP-1 signalling [Ionescu *et al.*, 01]. Since AP-1 also regulated MT1-MMP expression it is plausible that PTHrP signalling is also involved in our system and further studies should be undertaken to determine if and when this signalling molecule is secreted in response to cyclic compression. Alternatively, since it is known that the stimulation-induced increase in matrix accumulation involves a catabolic phase characterized by degradation of the surrounding matrix [De Croos, Dhaliwal, Grynpas, Pilliar & Kandel, 06], it is possible that only 30 minutes of compression produces a sufficient amount of degradation to elicit this response. To this end, studies describing the kinetics of MT1-MMP should be undertaken in our system to determine how quickly and efficiently is MT1-MMP activated on the cell surface in response to mechanical stimulation.

Similarly, past studies in our lab determined that mechanical stimulation-induced increase in matrix synthesis of approximately 30% in terms of accumulation of collagens and proteoglycans [Waldman, Couto, Grynpas, Pilliar & Kandel, 06].It was not clear, however, why the observed increase was limited to 30%, and it was suspected that this can be attributed to the differential contribution of the zones composing articular cartilage. When the individual zonal response to cyclic compression was investigated it was found that their response still could not explain why the overall increase in the tissue is limited to that value [Raizman *et al.*, 09],

suggesting other factors are involved. This study provided another plausible explanation for this phenomenon, as observed in Figure A6 (Appendix A, Figure 6) where the distribution of cell areas following mechanical stimulation is shown. As seen in the figure, following mechanical stimulation only a certain fraction of the total cells undergo cell spreading in response to the applied compressive forces. In effect roughly 30% of the examined cells remained in their spherical morphology despite cyclic compression. Since only cells at the CPP interface was analyzed, and since cell spreading appears to increase with the depth of the tissue (Appendix A, Figure 7), it is quite possible that the total percentage of non-responsive cells in the entire 3D tissue is much higher (possibly as high as 70%). As such, it is possible that the 30% increase in matrix synthesis is attributed to the fact that only 30% of the cells in the entire 3D construct undergo cell spreading and retraction. Alternatively, owing to the inherent phenotypical differences that between chondrocytes of different populations, it is feasible that the observed response can be attributed to only a subset of the cell population responding to mechanical stimulation. A study by Shieh *et al.,* indicated that chondrocytes from the superficial zone are stiffer than the corresponding middle/deep zone chondrocytes, raising the possibility these two subpopulations may not undergo identical changes in cell morphology in response to mechanical stimulation [Shieh & Athanasiou, 06].

Although the study provided significant insight into chondrocyte mechanotransduction, it possessed several limitations. One limitation inherent in the utilized system is the inability to investigate individual cell area throughout the entire thickness of the tissue. For the purposes of consistency in measuring chondrocyte area only cells at the CPP-tissue interface were analyzed after peeling the tissue off. Examination of the entire tissue by SEM revealed a highly dense tissue packed with chondrocytes and ECM molecules which did not allow for accurate

measurement of cell area. To ensure that there was no selection bias introduced, we examined the under-surface of the detached tissue. Spread cells, similar to that attached to the substrate, were seen. As such it is difficult to extrapolate whether the results obtained apply to all chondrocytes or simply to a subset of chondrocytes which are located at the interface. Similarly, owing to a variety of factors both technical (such as porosity of the CPP produced at different times,) and biological variability in cells obtained from different calfs it is possible that a different amount of matrix was deposited by the cells prior to mechanical stimulation. While the difference could not be clearly seen by a visual inspection, any minor differences in ECM-amount and composition may influence the manner by which the cells respond to mechanical stimulation. For instance, we have previously suggested that the differential response of superficial-zone and deep-zone chondrocytes to mechanical stimulation can be attributed to the amount of ECM present around the cells at the time of the stimulation [Raizman, De Croos, St-Pierre, Pilliar & Kandel, 09]. In that study, we observed that the response of deep zone chondrocytes to cyclic compression, in terms of matrix synthesis, varied drastically in constructs stimulated 48 hour apart. The difference in the response was attributed to a significantly greater accumulation of ECM molecules in constructs that were stimulated at the later time (for further information refer to paper attached in Appendix B). This observation is in keeping with previous research that indicated that the composition of the ECM, and in particular of the PCM, influences the responsiveness of chondrocytes [Guilak, Alexopoulos, Haider, Ting-Beall & Setton, 05;Guilak, Alexopoulos, Upton, Youn, Choi, Cao, Setton & Haider, 06]. Moreover, owing to the differences in the relative abundance of chondrocytes from different zone, which is a result of the chondrocyte extraction process, the composition of the ECM may differ slightly from experiment to experiment. As such, the particular response we are seeing could be dependent on the amount

of matrix, and that mechanical stimulation conducted under different conditions (i.e. later time point) may result in the activation of other components of the signalling pathway.

In addition, it would be useful for the purposes of elucidating the mechanism regulating chondrocyte response to cyclic compression, to visualize real-time calcium fluxes. Numerous technologies and dyes exist for this purpose and are widely used in literature in single-cell and monolayer cultures. However, these methods are not applicable to our system, as the technical hurdles of mechanically stimulating a 3D tissue construct inside a mechanical stimulator prevent us from visualizing the cells in real-time. Nonetheless, as mentioned above, designing an apparatus that will allow the visualization of calcium fluxes in real-time would be tremendously useful in attempting to determine the relative order of integrin and calcium-channel activation in response to cyclic compression.

Some of the limitations mentioned above are a direct consequence of the 3D culture system utilized in our lab. Other studies which are conducted utilizing systems such as monolayer, pellet cultures, cartilage explants or even 3D cultures utilizing agarose or alginate allow for a greater analysis with a wider array of tools due to the easier technical aspects associated with the cultures (i.e. visualization of real-time calcium currents in monolayer). However, none of these systems are able to generate continuous layer of tissue to more closely mimic what is occurring in a natural *in vivo* environment where chondrocytes are surrounded by a specialized ECM matrix and are situated on top of a stiff material (bone). As such, despite the limited type of analysis which can be undertaken in our system, this system provides a far more realistic model, and more a physiologically accurate environment, to study and investigate chondrocyte response to compressive forces that lead to enhanced tissue formation.

Owing to these limitations, other experiments should be conducted in the future to further study the involved mechanisms. Firstly, it would be important to selectively knock-out the involved calcium channels and integrins (i.e. through the use of siRNA) to further confirm their involvement in this process. Similarly, it would be interesting to determine if other integrin-associated proteins may be involved in this response as well and, if so, what is their function; it is entirely possible that different adapter proteins may be called upon depending on the type of force chondrocytes sense.

Additional studies should be conducted to investigate which of the calcium-dependent pathways are activated by the calcium channels in response to mechanical stimulation. This will indicate whether calcium is involved in simply mediating the cytoskeletal rearrangement (which is calcium dependent) or directly influences gene translation through proteins such as PKC. Moreover, it would be important to investigate the possible involvement of other ionic channels such as the sodium and potassium channels, as these channels are also known to be involved in mechanotransduction in other cell types and can co-localized with integrins as well.

Overall, an in-depth understanding of the molecular mechanisms involved in chondrocyte mechanotransduction, in particular those pathways which are responsible for the synthesis and maintenance of the tissue's ECM, will contribute to our understanding of various cartilage-related pathologies such as osteoarthritis. Moreover, knowing the underlying mechanisms may lead to the development of new strategies for treatment of damaged cartilage tissue through the use of bioengineering approaches.

CHAPTER FOUR: REFERENCES

References

Alenghat FJ, Ingber DE. Mechanotransduction: all signals point to cytoskeleton, matrix, and integrins. *Sci.STKE.* 2002a; **2002**:E6.

Alenghat FJ, Ingber DE. Mechanotransduction: all signals point to cytoskeleton, matrix, and integrins. *Sci.STKE.* 2002b; **2002**:E6.

Alexopoulos LG, Setton LA, Guilak F. The biomechanical role of the chondrocyte pericellular matrix in articular cartilage. *Acta Biomater.* 2005; **1**:317-325.

Alford AI, Yellowley CE, Jacobs CR, Donahue HJ. Increases in cytosolic calcium, but not fluid flow, affect aggrecan mRNA levels in articular chondrocytes. *J.Cell Biochem.* 2003; **90**:938-944.

Alini M, Carey D, Hirata S, Grynpas MD, Pidoux I, Poole AR. Cellular and matrix changes before and at the time of calcification in the growth plate studied in vitro: arrest of type X collagen synthesis and net loss of collagen when calcification is initiated. *J.Bone Miner.Res.* 1994; **9**:1077-1087.

Arnaout MA, Mahalingam B, Xiong JP. Integrin structure, allostery, and bidirectional signaling. *Annu.Rev.Cell Dev.Biol.* 2005; **21**:381-410.

Ballestrem C, Hinz B, Imhof BA, Wehrle-Haller B. Marching at the front and dragging behind: differential alphaVbeta3-integrin turnover regulates focal adhesion behavior. *J.Cell Biol.* 2001; **155**:1319-1332.

Beaupre GS, Stevens SS, Carter DR. Mechanobiology in the development, maintenance, and degeneration of articular cartilage. *J.Rehabil.Res.Dev.* 2000; **37**:145-151.

Beningo KA, Dembo M, Wang YL. Responses of fibroblasts to anchorage of dorsal extracellular matrix receptors. *Proc.Natl.Acad.Sci.U.S.A* 2004; **101**:18024-18029.

Beris AE, Lykissas MG, Papageorgiou CD, Georgoulis AD. Advances in articular cartilage repair. *Injury* 2005; **36 Suppl 4**:S14-S23.

Berridge MJ, Bootman MD, Roderick HL. Calcium signalling: dynamics, homeostasis and remodelling. *Nat.Rev.Mol.Cell Biol.* 2003; **4**:517-529.

Berridge MJ, Lipp P, Bootman MD. The versatility and universality of calcium signalling. *Nat.Rev.Mol.Cell Biol.* 2000; **1**:11-21.

Berrier AL, Yamada KM. Cell-matrix adhesion. *J.Cell Physiol* 2007; **213**:565-573.

Bidanset DJ, Guidry C, Rosenberg LC, Choi HU, Timpl R, Hook M. Binding of the proteoglycan decorin to collagen type VI. *J.Biol.Chem.* 1992; **267**:5250-5256.

Boileau C, Martel-Pelletier J, Brunet J *et al*. Oral treatment with PD-0200347, an alpha2delta ligand, reduces the development of experimental osteoarthritis by inhibiting metalloproteinases and inducible nitric oxide synthase gene expression and synthesis in cartilage chondrocytes. *Arthritis Rheum.* 2005; **52**:488-500.

Bonen DK, Schmid TM. Elevated extracellular calcium concentrations induce type X collagen synthesis in chondrocyte cultures. *J.Cell Biol.* 1991; **115**:1171-1178.

Bootman MD, Collins TJ, Peppiatt CM *et al*. Calcium signalling--an overview. *Semin.Cell Dev.Biol.* 2001; **12**:3-10.

Boyle J, Luan B, Cruz TF, Kandel RA. Characterization of proteoglycan accumulation during formation of cartilagenous tissue in vitro. *Osteoarthritis.Cartilage.* 1995; **3**:117-125.

Bradley JM, Kelley MJ, Zhu X, Anderssohn AM, Alexander JP, Acott TS. Effects of mechanical stretching on trabecular matrix metalloproteinases. *Invest Ophthalmol.Vis.Sci.* 2001; **42**:1505-1513.

Brakebusch C, Fassler R. beta 1 integrin function in vivo: adhesion, migration and more. *Cancer Metastasis Rev.* 2005; **24**:403-411.

Breinan HA, Minas T, Hsu HP, Nehrer S, Sledge CB, Spector M. Effect of cultured autologous chondrocytes on repair of chondral defects in a canine model. *J.Bone Joint Surg.Am.* 1997; **79**:1439-1451.

Brower TD, Hsu WY. Normal articular cartilage. *Clin.Orthop.Relat Res.* 1969; **64**:9-17.

Brugnera E, Haney L, Grimsley C *et al*. Unconventional Rac-GEF activity is mediated through the Dock180-ELMO complex. *Nat.Cell Biol.* 2002; **4**:574-582.

Buckwalter JA. Osteoarthritis and articular cartilage use, disuse, and abuse: experimental studies. *J.Rheumatol.Suppl* 1995; **43**:13-15.

Buckwalter JA, Mankin HJ. Articular cartilage: tissue design and chondrocyte-matrix interactions. *Instr.Course Lect.* 1998a; **47**:477-486.

Buckwalter JA, Mankin HJ. Articular cartilage: degeneration and osteoarthritis, repair, regeneration, and transplantation. *Instr.Course Lect.* 1998b; **47**:487-504.

Calalb MB, Zhang X, Polte TR, Hanks SK. Focal adhesion kinase tyrosine-861 is a major site of phosphorylation by Src. *Biochem.Biophys.Res.Commun.* 1996; **228**:662-668.

Campbell JJ, Blain EJ, Chowdhury TT, Knight MM. Loading alters actin dynamics and up-regulates cofilin gene expression in chondrocytes. *Biochem.Biophys.Res.Commun.* 2007; **361**:329-334.

Catterall WA, Perez-Reyes E, Snutch TP, Striessnig J. International Union of Pharmacology. XLVIII. Nomenclature and structure-function relationships of voltage-gated calcium channels. *Pharmacol.Rev.* 2005; **57**:411-425.

Cavalcanti-Adam EA, Volberg T, Micoulet A, Kessler H, Geiger B, Spatz JP. Cell spreading and focal adhesion dynamics are regulated by spacing of integrin ligands. *Biophys.J.* 2007; **92**:2964-2974.

Chang J, Nakajima H, Poole CA. Structural colocalisation of type VI collagen and fibronectin in agarose cultured chondrocytes and isolated chondrons extracted from adult canine tibial cartilage. *J.Anat.* 1997; **190 (Pt 4)**:523-532.

Chun TH, Hotary KB, Sabeh F, Saltiel AR, Allen ED, Weiss SJ. A pericellular collagenase directs the 3-dimensional development of white adipose tissue. *Cell* 2006; **125**:577-591.

Cohen M, Kam Z, Addadi L, Geiger B. Dynamic study of the transition from hyaluronan- to integrin-mediated adhesion in chondrocytes. *EMBO J.* 2006; **25**:302-311.

Cohen M, Klein E, Geiger B, Addadi L. Organization and adhesive properties of the hyaluronan pericellular coat of chondrocytes and epithelial cells. *Biophys.J.* 2003; **85**:1996-2005.

Collins TJ, Lipp P, Berridge MJ, Bootman MD. Mitochondrial Ca(2+) uptake depends on the spatial and temporal profile of cytosolic Ca(2+) signals. *J.Biol.Chem.* 2001; **276**:26411-26420.

Coutts RD, Healey RM, Ostrander R, Sah RL, Goomer R, Amiel D. Matrices for cartilage repair. *Clin.Orthop.Relat Res.* 2001;S271-S279.

Coutts RD, Sah RL, Amiel D. Effects of growth factors on cartilage repair. *Instr.Course Lect.* 1997; **46**:487-494.

Crawford HC, Matrisian LM. Mechanisms controlling the transcription of matrix metalloproteinase genes in normal and neoplastic cells. *Enzyme Protein* 1996; **49**:20-37.

Cremer MA, Rosloniec EF, Kang AH. The cartilage collagens: a review of their structure, organization, and role in the pathogenesis of experimental arthritis in animals and in human rheumatic disease. *J.Mol.Med.* 1998; **76**:275-288.

Cukierman E, Pankov R, Stevens DR, Yamada KM. Taking cell-matrix adhesions to the third dimension. *Science* 2001; **294**:1708-1712.

D'Andrea P, Calabrese A, Capozzi I, Grandolfo M, Tonon R, Vittur F. Intercellular Ca2+ waves in mechanically stimulated articular chondrocytes. *Biorheology* 2000; **37**:75-83.

Danen EH, Sonneveld P, Brakebusch C, Fassler R, Sonnenberg A. The fibronectin-binding integrins alpha5beta1 and alphavbeta3 differentially modulate RhoA-GTP loading, organization of cell matrix adhesions, and fibronectin fibrillogenesis. *J.Cell Biol.* 2002; **159**:1071-1086.

Darling EM, Athanasiou KA. Rapid phenotypic changes in passaged articular chondrocyte subpopulations. *J.Orthop.Res.* 2005; **23**:425-432.

Darling EM, Athanasiou KA. Biomechanical strategies for articular cartilage regeneration. *Ann.Biomed.Eng* 2003; **31**:1114-1124.

Darling EM, Hu JC, Athanasiou KA. Zonal and topographical differences in articular cartilage gene expression. *J.Orthop.Res.* 2004; **22**:1182-1187.

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

De Croos JN, 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**:323-331.

De Croos JN, Jang B, Dhaliwal SS, Grynpas MD, Pilliar RM, Kandel RA. Membrane type-1 matrix metalloproteinase is induced following cyclic compression of in vitro grown bovine chondrocytes. *Osteoarthritis.Cartilage.* 2007; **15**:1301-1310.

Deshmukh K, Sawyer BD. Synthesis of collagen by chondrocytes in suspension culture: modulation by calcium, 3':5'-cyclic AMP, and prostaglandins. *Proc.Natl.Acad.Sci.U.S.A* 1977; **74**:3864-3868.

Dowthwaite GP, Bishop JC, Redman SN *et al*. The surface of articular cartilage contains a progenitor cell population. *J.Cell Sci.* 2004; **117**:889-897.

Dubuis E, Rockliffe N, Hussain M, Boyett M, Wray D, Gawler D. Evidence for multiple Src binding sites on the alpha1c L-type Ca2+ channel and their roles in activity regulation. *Cardiovasc.Res.* 2006; **69**:391-401.

Dudek SM, Garcia JG. Cytoskeletal regulation of pulmonary vascular permeability. *J.Appl.Physiol* 2001; **91**:1487-1500.

Durrant LA, Archer CW, Benjamin M, Ralphs JR. Organisation of the chondrocyte cytoskeleton and its response to changing mechanical conditions in organ culture. *J.Anat.* 1999; **194 (Pt 3)**:343-353.

Eckstein F, Hudelmaier M, Putz R. The effects of exercise on human articular cartilage. *J.Anat.* 2006; **208**:491-512.

Elfervig MK, Graff RD, Lee GM, Kelley SS, Sood A, Banes AJ. ATP induces Ca(2+) signaling in human chondrons cultured in three-dimensional agarose films. *Osteoarthritis.Cartilage.* 2001; **9**:518-526.

Erickson GR, Northrup DL, Guilak F. Hypo-osmotic stress induces calcium-dependent actin reorganization in articular chondrocytes. *Osteoarthritis.Cartilage.* 2003; **11**:187-197.

Eyre DR. Collagens and cartilage matrix homeostasis. *Clin.Orthop.Relat Res.* 2004;S118-S122.

Fantozzi I, Zhang S, Platoshyn O, Remillard CV, Cowling RT, Yuan JX. Hypoxia increases AP-1 binding activity by enhancing capacitative Ca2+ entry in human pulmonary artery endothelial cells. *Am.J.Physiol Lung Cell Mol.Physiol* 2003; **285**:L1233-L1245.

Fleire SJ, Goldman JP, Carrasco YR, Weber M, Bray D, Batista FD. B cell ligand discrimination through a spreading and contraction response. *Science* 2006; **312**:738-741.

Galvez BG, Matias-Roman S, Yanez-Mo M, Sanchez-Madrid F, Arroyo AG. ECM regulates MT1-MMP localization with beta1 or alphavbeta3 integrins at distinct cell compartments modulating its internalization and activity on human endothelial cells. *J.Cell Biol.* 2002; **159**:509-521.

Gelse K, Poschl E, Aigner T. Collagens--structure, function, and biosynthesis. *Adv.Drug Deliv.Rev.* 2003; **55**:1531-1546.

Genazzani AA, Galione A. A Ca2+ release mechanism gated by the novel pyridine nucleotide, NAADP. *Trends Pharmacol.Sci.* 1997; **18**:108-110.

Gillogly SD, Voight M, Blackburn T. Treatment of articular cartilage defects of the knee with autologous chondrocyte implantation. *J.Orthop.Sports Phys.Ther.* 1998; **28**:241-251.

Gomez E, Pritchard C, Herbert TP. cAMP-dependent protein kinase and Ca2+ influx through L-type voltage-gated calcium channels mediate Raf-independent activation of extracellular regulated kinase in response to glucagon-like peptide-1 in pancreatic beta-cells. *J.Biol.Chem.* 2002; **277**:48146-48151.

Graff RD, Kelley SS, Lee GM. Role of pericellular matrix in development of a mechanically functional neocartilage. *Biotechnol.Bioeng.* 2003; **82**:457-464.

Grodzinsky AJ, Levenston ME, Jin M, Frank EH. Cartilage tissue remodeling in response to mechanical forces. *Annu.Rev.Biomed.Eng* 2000; **2**:691-713.

Grynpas MD, Pilliar RM, Kandel RA, Renlund R, Filiaggi M, Dumitriu M. Porous calcium polyphosphate scaffolds for bone substitute applications in vivo studies. *Biomaterials* 2002; **23**:2063-2070.

Guibert C, Ducret T, Savineau JP. Voltage-independent calcium influx in smooth muscle. *Prog.Biophys.Mol.Biol.* 2008; **98**:10-23.

Guilak F. Compression-induced changes in the shape and volume of the chondrocyte nucleus. *J.Biomech.* 1995; **28**:1529-1541.

Guilak F, Alexopoulos LG, Haider MA, Ting-Beall HP, Setton LA. Zonal uniformity in mechanical properties of the chondrocyte pericellular matrix: micropipette aspiration of canine chondrons isolated by cartilage homogenization. *Ann.Biomed.Eng* 2005; **33**:1312-1318.

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**:498-512.

Guilak F, Zell RA, Erickson GR *et al*. Mechanically induced calcium waves in articular chondrocytes are inhibited by gadolinium and amiloride. *J.Orthop.Res.* 1999; **17**:421-429.

Hall AC, Horwitz ER, Wilkins RJ. The cellular physiology of articular cartilage. *Exp.Physiol* 1996; **81**:535-545.

Hanke JH, Gardner JP, Dow RL *et al*. Discovery of a novel, potent, and Src family-selective tyrosine kinase inhibitor. Study of Lck- and FynT-dependent T cell activation. *J.Biol.Chem.* 1996; **271**:695-701.

Hauselmann HJ, Stefanovic-Racic M, Michel BA, Evans CH. Differences in nitric oxide production by superficial and deep human articular chondrocytes: implications for proteoglycan turnover in inflammatory joint diseases. *J.Immunol.* 1998; **160**:1444-1448.

Hidaka C, Cheng C, Alexandre D, Bhargava M, Torzilli PA. Maturational differences in superficial and deep zone articular chondrocytes. *Cell Tissue Res.* 2006; **323**:127-135.

Holledge MM, Millward-Sadler SJ, Nuki G, Salter DM. Mechanical regulation of proteoglycan synthesis in normal and osteoarthritic human articular chondrocytes--roles for alpha5 and alphaVbeta5 integrins. *Biorheology* 2008; **45**:275-288.

Holmbeck K, Bianco P, Caterina J *et al*. MT1-MMP-deficient mice develop dwarfism, osteopenia, arthritis, and connective tissue disease due to inadequate collagen turnover. *Cell* 1999; **99**:81-92.

Homandberg GA, Meyers R, Xie DL. Fibronectin fragments cause chondrolysis of bovine articular cartilage slices in culture. *J.Biol.Chem.* 1992; **267**:3597-3604.

Hopewell B, Urban JP. Adaptation of articular chondrocytes to changes in osmolality. *Biorheology* 2003; **40**:73-77.

Howarth D, Pritzker KP, Cruz TF, Kandel RA. Calcium ionophore A23187 stimulates production of a 144 kDa gelatinase. *J.Rheumatol.* 1993; **20**:97-101.

Hsieh AH, Tsai CM, Ma QJ *et al*. Time-dependent increases in type-III collagen gene expression in medical collateral ligament fibroblasts under cyclic strains. *J.Orthop.Res.* 2000; **18**:220-227.

Huber M, Trattnig S, Lintner F. Anatomy, biochemistry, and physiology of articular cartilage. *Invest Radiol.* 2000; **35**:573-580.

Humphries MJ. Integrin structure. *Biochem.Soc.Trans.* 2000; **28**:311-339.

Hunziker EB. Articular cartilage repair: are the intrinsic biological constraints undermining this process insuperable? *Osteoarthritis.Cartilage.* 1999; **7**:15-28.

Huveneers S, Danen EH. Adhesion signaling - crosstalk between integrins, Src and Rho. *J.Cell Sci.* 2009; **122**:1059-1069.

Ingber DE. Mechanosensation through integrins: cells act locally but think globally. *Proc.Natl.Acad.Sci.U.S.A* 2003; **100**:1472-1474.

Ionescu AM, Schwarz EM, Vinson C *et al*. PTHrP modulates chondrocyte differentiation through AP-1 and CREB signaling. *J.Biol.Chem.* 2001; **276**:11639-11647.

Jaalouk DE, Lammerding J. Mechanotransduction gone awry. *Nat.Rev.Mol.Cell Biol.* 2009; **10**:63-73.

Janmey PA. The cytoskeleton and cell signaling: component localization and mechanical coupling. *Physiol Rev.* 1998; **78**:763-781.

Jiang G, Giannone G, Critchley DR, Fukumoto E, Sheetz MP. Two-piconewton slip bond between fibronectin and the cytoskeleton depends on talin. *Nature* 2003; **424**:334-337.

Kalapesi FB, Tan JC, Coroneo MT. Stretch-activated channels: a mini-review. Are stretch-activated channels an ocular barometer? *Clin.Experiment.Ophthalmol.* 2005; **33**:210-217.

Kandel RA, Grynpas M, Pilliar R *et al*. Repair of osteochondral defects with biphasic cartilage-calcium polyphosphate constructs in a sheep model. *Biomaterials* 2006; **27**:4120-4131.

Karin M. The regulation of AP-1 activity by mitogen-activated protein kinases. *J.Biol.Chem.* 1995; **270**:16483-16486.

Katz BZ, Zamir E, Bershadsky A, Kam Z, Yamada KM, Geiger B. Physical state of the extracellular matrix regulates the structure and molecular composition of cell-matrix adhesions. *Mol.Biol.Cell* 2000; **11**:1047-1060.

Kerrigan MJ, Hall AC. Control of chondrocyte regulatory volume decrease (RVD) by [Ca2+]i and cell shape. *Osteoarthritis.Cartilage.* 2008; **16**:312-322.

Kielty CM, Whittaker SP, Grant ME, Shuttleworth CA. Type VI collagen microfibrils: evidence for a structural association with hyaluronan. *J.Cell Biol.* 1992; **118**:979-990.

Kim YJ, Sah RL, Grodzinsky AJ, Plaas AH, Sandy JD. Mechanical regulation of cartilage biosynthetic behavior: physical stimuli. *Arch.Biochem.Biophys.* 1994; **311**:1-12.

Kinner B, Capito RM, Spector M. Regeneration of articular cartilage. *Adv.Biochem.Eng Biotechnol.* 2005; **94**:91-123.

Kiyokawa E, Hashimoto Y, Kobayashi S, Sugimura H, Kurata T, Matsuda M. Activation of Rac1 by a Crk SH3-binding protein, DOCK180. *Genes Dev.* 1998; **12**:3331-3336.

Knight MM, Bomzon Z, Kimmel E, Sharma AM, Lee DA, Bader DL. Chondrocyte deformation induces mitochondrial distortion and heterogeneous intracellular strain fields. *Biomech.Model.Mechanobiol.* 2006a; **5**:180-191.

Knight MM, Toyoda T, Lee DA, Bader DL. Mechanical compression and hydrostatic pressure induce reversible changes in actin cytoskeletal organisation in chondrocytes in agarose. *J.Biomech.* 2006b; **39**:1547-1551.

Kolch W. Meaningful relationships: the regulation of the Ras/Raf/MEK/ERK pathway by protein interactions. *Biochem.J.* 2000; **351 Pt 2**:289-305.

Kono T, Nishikori T, Kataoka H, Uchio Y, Ochi M, Enomoto K. Spontaneous oscillation and mechanically induced calcium waves in chondrocytes. *Cell Biochem.Funct.* 2006; **24**:103-111.

Kuo CK, Li WJ, Mauck RL, Tuan RS. Cartilage tissue engineering: its potential and uses. *Curr.Opin.Rheumatol.* 2006; **18**:64-73.

Lacinova L. Voltage-dependent calcium channels. *Gen.Physiol Biophys.* 2005; **24 Suppl 1**:1-78.

Larson CM, Kelley SS, Blackwood AD, Banes AJ, Lee GM. Retention of the native chondrocyte pericellular matrix results in significantly improved matrix production. *Matrix Biol.* 2002; **21**:349-359.

Lee AA, Delhaas T, McCulloch AD, Villarreal FJ. Differential responses of adult cardiac fibroblasts to in vitro biaxial strain patterns. *J.Mol.Cell Cardiol.* 1999; **31**:1833-1843.

Lee V, Cao L, Zhang Y, Kiani C, Adams ME, Yang BB. The roles of matrix molecules in mediating chondrocyte aggregation, attachment, and spreading. *J.Cell Biochem.* 2000; **79**:322-333.

Levy Y, Ronen D, Bershadsky AD, Zick Y. Sustained induction of ERK, protein kinase B, and p70 S6 kinase regulates cell spreading and formation of F-actin microspikes upon ligation of integrins by galectin-8, a mammalian lectin. *J.Biol.Chem.* 2003; **278**:14533-14542.

Lin B, Arai AC, Lynch G, Gall CM. Integrins regulate NMDA receptor-mediated synaptic currents. *J.Neurophysiol.* 2003; **89**:2874-2878.

Loeser RF. Chondrocyte integrin expression and function. *Biorheology* 2000; **37**:109-116.

Lohi J, Keski-Oja J. Calcium ionophores decrease pericellular gelatinolytic activity via inhibition of 92-kDa gelatinase expression and decrease of 72-kDa gelatinase activation. *J.Biol.Chem.* 1995; **270**:17602-17609.

MacKenna DA, Dolfi F, Vuori K, Ruoslahti E. Extracellular signal-regulated kinase and c-Jun NH2-terminal kinase activation by mechanical stretch is integrin-dependent and matrix-specific in rat cardiac fibroblasts. *J.Clin.Invest* 1998; **101**:301-310.

Mangoura D, Sogos V, Dawson G. Phorbol esters and PKC signaling regulate proliferation, vimentin cytoskeleton assembly and glutamine synthetase activity of chick embryo cerebrum astrocytes in culture. *Brain Res.Dev.Brain Res.* 1995; **87**:1-11.

Maroto R, Raso A, Wood TG, Kurosky A, Martinac B, Hamill OP. TRPC1 forms the stretch-activated cation channel in vertebrate cells. *Nat.Cell Biol.* 2005; **7**:179-185.

Marshall MS. Ras target proteins in eukaryotic cells. *FASEB J.* 1995; **9**:1311-1318.

Martin I, Miot S, Barbero A, Jakob M, Wendt D. Osteochondral tissue engineering. *J.Biomech.* 2007; **40**:750-765.

Mauck RL, Soltz MA, Wang CC *et al*. Functional tissue engineering of articular cartilage through dynamic loading of chondrocyte-seeded agarose gels. *J.Biomech.Eng* 2000; **122**:252-260.

Mente PL, Lewis JL. Elastic modulus of calcified cartilage is an order of magnitude less than that of subchondral bone. *J.Orthop.Res.* 1994; **12**:637-647.

Millward-Sadler SJ, Salter DM. Integrin-dependent signal cascades in chondrocyte mechanotransduction. *Ann.Biomed.Eng* 2004b; **32**:435-446.

Millward-Sadler SJ, Salter DM. Integrin-dependent signal cascades in chondrocyte mechanotransduction. *Ann.Biomed.Eng* 2004a; **32**:435-446.

Millward-Sadler SJ, Wright MO, Flatman PW, Salter DM. ATP in the mechanotransduction pathway of normal human chondrocytes. *Biorheology* 2004; **41**:567-575.

Millward-Sadler SJ, Wright MO, Lee H *et al*. Integrin-regulated secretion of interleukin 4: A novel pathway of mechanotransduction in human articular chondrocytes. *J.Cell Biol.* 1999; **145**:183-189.

Miyauchi A, Notoya K, Mikuni-Takagaki Y *et al*. Parathyroid hormone-activated volume-sensitive calcium influx pathways in mechanically loaded osteocytes. *J.Biol.Chem.* 2000; **275**:3335-3342.

Mizuno S. A novel method for assessing effects of hydrostatic fluid pressure on intracellular calcium: a study with bovine articular chondrocytes. *Am.J.Physiol Cell Physiol* 2005; **288**:C329-C337.

Mobasheri A, Carter SD, Martin-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**:1-18.

Mori M, Nakajima M, Mikami Y *et al*. Transcriptional regulation of the cartilage intermediate layer protein (CILP) gene. *Biochem.Biophys.Res.Commun.* 2006; **341**:121-127.

Mouw JK, Imler SM, Levenston ME. Ion-channel regulation of chondrocyte matrix synthesis in 3D culture under static and dynamic compression. *Biomech.Model.Mechanobiol.* 2007; **6**:33-41.

Mow, V. C. and Lai, M. F. Mechanics of Animal Joints. Annual Review of Fluid Mechanics 11, 247-288. 2009.
Ref Type: Generic

Muir H. The chondrocyte, architect of cartilage. Biomechanics, structure, function and molecular biology of cartilage matrix macromolecules. *Bioessays* 1995; **17**:1039-1048.

Mulvaney JM, Zhang T, Fewtrell C, Roberson MS. Calcium influx through L-type channels is required for selective activation of extracellular signal-regulated kinase by gonadotropin-releasing hormone. *J.Biol.Chem.* 1999; **274**:29796-29804.

O'Driscoll SW. The healing and regeneration of articular cartilage. *J.Bone Joint Surg.Am.* 1998; **80**:1795-1812.

O'Hara BP, Urban JP, Maroudas A. Influence of cyclic loading on the nutrition of articular cartilage. *Ann.Rheum.Dis.* 1990; **49**:536-539.

Onodera K, Takahashi I, Sasano Y *et al*. Stepwise mechanical stretching inhibits chondrogenesis through cell-matrix adhesion mediated by integrins in embryonic rat limb-bud mesenchymal cells. *Eur.J.Cell Biol.* 2005; **84**:45-58.

Orr AW, Helmke BP, Blackman BR, Schwartz MA. Mechanisms of mechanotransduction. *Dev.Cell* 2006; **10**:11-20.

Pearle AD, Warren RF, Rodeo SA. Basic science of articular cartilage and osteoarthritis. *Clin.Sports Med.* 2005; **24**:1-12.

Perkins GL, Derfoul A, Ast A, Hall DJ. An inhibitor of the stretch-activated cation receptor exerts a potent effect on chondrocyte phenotype. *Differentiation* 2005; **73**:199-211.

Piao L, Ho WK, Earm YE. Actin filaments regulate the stretch sensitivity of large-conductance, Ca2+-activated K+ channels in coronary artery smooth muscle cells. *Pflugers Arch.* 2003; **446**:523-528.

Pilliar RM, Filiaggi MJ, Wells JD, Grynpas MD, Kandel RA. Porous calcium polyphosphate scaffolds for bone substitute applications -- in vitro characterization. *Biomaterials* 2001; **22**:963-972.

Pingguan-Murphy B, Lee DA, Bader DL, Knight MM. Activation of chondrocytes calcium signalling by dynamic compression is independent of number of cycles. *Arch.Biochem.Biophys.* 2005b; **444**:45-51.

Pingguan-Murphy B, Lee DA, Bader DL, Knight MM. Activation of chondrocytes calcium signalling by dynamic compression is independent of number of cycles. *Arch.Biochem.Biophys.* 2005a; **444**:45-51.

Pollard TD, Borisy GG. Cellular motility driven by assembly and disassembly of actin filaments. *Cell* 2003; **112**:453-465.

Poole AR, Kojima T, Yasuda T, Mwale F, Kobayashi M, Laverty S. Composition and structure of articular cartilage: a template for tissue repair. *Clin.Orthop.Relat Res.* 2001;S26-S33.

Poole AR, Rosenberg LC, Reiner A, Ionescu M, Bogoch E, Roughley PJ. Contents and distributions of the proteoglycans decorin and biglycan in normal and osteoarthritic human articular cartilage. *J.Orthop.Res.* 1996; **14**:681-689.

Poole CA. Articular cartilage chondrons: form, function and failure. *J.Anat.* 1997; **191 (Pt 1)**:1-13.

Premkumar DR, Mishra RR, Overholt JL, Simonson MS, Cherniack NS, Prabhakar NR. L-type Ca(2+) channel activation regulates induction of c-fos transcription by hypoxia. *J.Appl.Physiol* 2000; **88**:1898-1906.

Raizman I, De Croos JN, St-Pierre JP, Pilliar RM, Kandel R. Articular Cartilage Subpopulations Respond Differently to Cyclic Compression in vitro. *Tissue Eng Part A* 2009.

Redman SN, Oldfield SF, Archer CW. Current strategies for articular cartilage repair. *Eur.Cell Mater.* 2005; **9**:23-32.

Ren XD, Kiosses WB, Sieg DJ, Otey CA, Schlaepfer DD, Schwartz MA. Focal adhesion kinase suppresses Rho activity to promote focal adhesion turnover. *J.Cell Sci.* 2000; **113 (Pt 20)**:3673-3678.

Riveline D, Zamir E, Balaban NQ *et al*. Focal contacts as mechanosensors: externally applied local mechanical force induces growth of focal contacts by an mDia1-dependent and ROCK-independent mechanism. *J.Cell Biol.* 2001; **153**:1175-1186.

Roberts SR, Knight MM, Lee DA, Bader DL. Mechanical compression influences intracellular Ca2+ signaling in chondrocytes seeded in agarose constructs. *J.Appl.Physiol* 2001; **90**:1385-1391.

Romer LH, Birukov KG, Garcia JG. Focal adhesions: paradigm for a signaling nexus. *Circ.Res.* 2006; **98**:606-616.

Rottner K, Hall A, Small JV. Interplay between Rac and Rho in the control of substrate contact dynamics. *Curr.Biol.* 1999; **9**:640-648.

Salazar EP, Rozengurt E. Src family kinases are required for integrin-mediated but not for G protein-coupled receptor stimulation of focal adhesion kinase autophosphorylation at Tyr-397. *J.Biol.Chem.* 2001; **276**:17788-17795.

Samarakoon R, Higgins PJ. MEK/ERK pathway mediates cell-shape-dependent plasminogen activator inhibitor type 1 gene expression upon drug-induced disruption of the microfilament and microtubule networks. *J.Cell Sci.* 2002; **115**:3093-3103.

Schaller MD, Hildebrand JD, Shannon JD, Fox JW, Vines RR, Parsons JT. Autophosphorylation of the focal adhesion kinase, pp125FAK, directs SH2-dependent binding of pp60src. *Mol.Cell Biol.* 1994; **14**:1680-1688.

Schlaepfer DD, Broome MA, Hunter T. Fibronectin-stimulated signaling from a focal adhesion kinase-c-Src complex: involvement of the Grb2, p130cas, and Nck adaptor proteins. *Mol.Cell Biol.* 1997; **17**:1702-1713.

Schmidt TA, Schumacher BL, Klein TJ, Voegtline MS, Sah RL. Synthesis of proteoglycan 4 by chondrocyte subpopulations in cartilage explants, monolayer cultures, and resurfaced cartilage cultures. *Arthritis Rheum.* 2004; **50**:2849-2857.

Schnabel M, Marlovits S, Eckhoff G *et al*. Dedifferentiation-associated changes in morphology and gene expression in primary human articular chondrocytes in cell culture. *Osteoarthritis.Cartilage.* 2002; **10**:62-70.

Schumacher BL, Block JA, Schmid TM, Aydelotte MB, Kuettner KE. A novel proteoglycan synthesized and secreted by chondrocytes of the superficial zone of articular cartilage. *Arch.Biochem.Biophys.* 1994; **311**:144-152.

Schwartz MA. Integrin signaling revisited. *Trends Cell Biol.* 2001; **11**:466-470.

Schwartz MA, Shattil SJ. Signaling networks linking integrins and rho family GTPases. *Trends Biochem.Sci.* 2000; **25**:388-391.

Schwartz Z, Langston GG, Swain LD, Boyan BD. Inhibition of 1,25-(OH)2D3- and 24,25-(OH)2D3-dependent stimulation of alkaline phosphatase activity by A23187 suggests a role for calcium in the mechanism of vitamin D regulation of chondrocyte cultures. *J.Bone Miner.Res.* 1991; **6**:709-718.

Shakibaei M, John T, De SP, Rahmanzadeh R, Merker HJ. Signal transduction by beta1 integrin receptors in human chondrocytes in vitro: collaboration with the insulin-like growth factor-I receptor. *Biochem.J.* 1999; **342 Pt 3**:615-623.

Shakibaei M, Merker HJ. Beta1-integrins in the cartilage matrix. *Cell Tissue Res.* 1999; **296**:565-573.

Shakibaei M, Mobasheri A. Beta1-integrins co-localize with Na, K-ATPase, epithelial sodium channels (ENaC) and voltage activated calcium channels (VACC) in mechanoreceptor complexes of mouse limb-bud chondrocytes. *Histol.Histopathol.* 2003; **18**:343-351.

Shao Y, Alicknavitch M, Farach-Carson MC. Expression of voltage sensitive calcium channel (VSCC) L-type Cav1.2 (alpha1C) and T-type Cav3.2 (alpha1H) subunits during mouse bone development. *Dev.Dyn.* 2005; **234**:54-62.

Shieh AC, Athanasiou KA. Biomechanics of single zonal chondrocytes. *J.Biomech.* 2006; **39**:1595-1602.

Shieh AC, Athanasiou KA. Principles of cell mechanics for cartilage tissue engineering. *Ann.Biomed.Eng* 2003; **31**:1-11.

Smith RL, Donlon BS, Gupta MK *et al*. Effects of fluid-induced shear on articular chondrocyte morphology and metabolism in vitro. *J.Orthop.Res.* 1995; **13**:824-831.

Soderman P, Malchau H, Herberts P. Outcome of total hip replacement: a comparison of different measurement methods. *Clin.Orthop.Relat Res.* 2001;163-172.

Spiteri CG, Pilliar RM, Kandel RA. Substrate porosity enhances chondrocyte attachment, spreading, and cartilage tissue formation in vitro. *J.Biomed.Mater.Res.A* 2006; **78**:676-683.

Spiteri CG, Young EW, Simmons CA, Kandel RA, Pilliar RM. Substrate architecture and fluid-induced shear stress during chondrocyte seeding: role of alpha5beta1 integrin. *Biomaterials* 2008; **29**:2477-2489.

Stewart MC, Saunders KM, Burton-Wurster N, Macleod JN. Phenotypic stability of articular chondrocytes in vitro: the effects of culture models, bone morphogenetic protein 2, and serum supplementation. *J.Bone Miner.Res.* 2000; **15**:166-174.

Suchyna TM, Johnson JH, Hamer K *et al*. Identification of a peptide toxin from Grammostola spatulata spider venom that blocks cation-selective stretch-activated channels. *J.Gen.Physiol* 2000; **115**:583-598.

Takada Y, Ye X, Simon S. The integrins. *Genome Biol.* 2007; **8**:215.

Takino T, Watanabe Y, Matsui M *et al*. Membrane-type 1 matrix metalloproteinase modulates focal adhesion stability and cell migration. *Exp.Cell Res.* 2006; **312**:1381-1389.

Tanaka N, Ohno S, Honda K *et al*. Cyclic mechanical strain regulates the PTHrP expression in cultured chondrocytes via activation of the Ca2+ channel. *J.Dent.Res.* 2005; **84**:64-68.

Temenoff JS, Mikos AG. Review: tissue engineering for regeneration of articular cartilage. *Biomaterials* 2000b; **21**:431-440.

Temenoff JS, Mikos AG. Review: tissue engineering for regeneration of articular cartilage. *Biomaterials* 2000a; **21**:431-440.

Thakar RG, Cheng Q, Patel S *et al*. Cell-shape regulation of smooth muscle cell proliferation. *Biophys.J.* 2009; **96**:3423-3432.

Toyoda T, Seedhom BB, Yao JQ, Kirkham J, Brookes S, Bonass WA. Hydrostatic pressure modulates proteoglycan metabolism in chondrocytes seeded in agarose. *Arthritis Rheum.* 2003; **48**:2865-2872.

Urban JP. The chondrocyte: a cell under pressure. *Br.J.Rheumatol.* 1994; **33**:901-908.

Volberg T, Romer L, Zamir E, Geiger B. pp60(c-src) and related tyrosine kinases: a role in the assembly and reorganization of matrix adhesions. *J.Cell Sci.* 2001; **114**:2279-2289.

Vuori K, Hirai H, Aizawa S, Ruoslahti E. Introduction of p130cas signaling complex formation upon integrin-mediated cell adhesion: a role for Src family kinases. *Mol.Cell Biol.* 1996; **16**:2606-2613.

Waldman SD, Couto DC, Grynpas MD, Pilliar RM, Kandel RA. A single application of cyclic loading can accelerate matrix deposition and enhance the properties of tissue-engineered cartilage. *Osteoarthritis.Cartilage.* 2006; **14**:323-330.

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**:323-330.

Waldman SD, Spiteri CG, Grynpas MD, Pilliar RM, Hong J, Kandel RA. Effect of biomechanical conditioning on cartilaginous tissue formation in vitro. *J.Bone Joint Surg.Am.* 2003; **85-A Suppl 2**:101-105.

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**:1323-1331.

Wang JH, Thampatty BP. An introductory review of cell mechanobiology. *Biomech.Model.Mechanobiol.* 2006; **5**:1-16.

Whitfield JF. Calcium signals and cancer. *Crit Rev.Oncog.* 1992; **3**:55-90.

Wittmann T, Bokoch GM, Waterman-Storer CM. Regulation of leading edge microtubule and actin dynamics downstream of Rac1. *J.Cell Biol.* 2003; **161**:845-851.

Wong M, Carter DR. Articular cartilage functional histomorphology and mechanobiology: a research perspective. *Bone* 2003; **33**:1-13.

Woods A, Wang G, Beier F. Regulation of chondrocyte differentiation by the actin cytoskeleton and adhesive interactions. *J.Cell Physiol* 2007; **213**:1-8.

Wu Q, Zhang Y, Chen Q. Indian hedgehog is an essential component of mechanotransduction complex to stimulate chondrocyte proliferation. *J.Biol.Chem.* 2001a; **276**:35290-35296.

Wu X, Davis GE, Meininger GA, Wilson E, Davis MJ. Regulation of the L-type calcium channel by alpha 5beta 1 integrin requires signaling between focal adhesion proteins. *J.Biol.Chem.* 2001b; **276**:30285-30292.

Wu X, Davis MJ. Characterization of stretch-activated cation current in coronary smooth muscle cells. *Am.J.Physiol Heart Circ.Physiol* 2001; **280**:H1751-H1761.

Wu X, Gan B, Yoo Y, Guan JL. FAK-mediated src phosphorylation of endophilin A2 inhibits endocytosis of MT1-MMP and promotes ECM degradation. *Dev.Cell* 2005; **9**:185-196.

Xia H, Nho RS, Kahm J, Kleidon J, Henke CA. Focal adhesion kinase is upstream of phosphatidylinositol 3-kinase/Akt in regulating fibroblast survival in response to contraction of type I collagen matrices via a beta 1 integrin viability signaling pathway. *J.Biol.Chem.* 2004; **279**:33024-33034.

Xu Y, Pritzker KP, Cruz TF. Characterization of chondrocyte alkaline phosphatase as a potential mediator in the dissolution of calcium pyrophosphate dihydrate crystals. *J.Rheumatol.* 1994; **21**:912-919.

Yamaguchi S, Yamaguchi M, Yatsuyanagi E *et al*. Cyclic strain stimulates early growth response gene product 1-mediated expression of membrane type 1 matrix metalloproteinase in endothelium. *Lab Invest* 2002; **82**:949-956.

Yang G, Crawford RC, Wang JH. Proliferation and collagen production of human patellar tendon fibroblasts in response to cyclic uniaxial stretching in serum-free conditions. *J.Biomech.* 2004; **37**:1543-1550.

Yano Y, Geibel J, Sumpio BE. Tyrosine phosphorylation of pp125FAK and paxillin in aortic endothelial cells induced by mechanical strain. *Am.J.Physiol* 1996; **271**:C635-C649.

Yellowley CE, Hancox JC, Donahue HJ. Effects of cell swelling on intracellular calcium and membrane currents in bovine articular chondrocytes. *J.Cell Biochem.* 2002; **86**:290-301.

Zaidel-Bar R, Cohen M, Addadi L, Geiger B. Hierarchical assembly of cell-matrix adhesion complexes. *Biochem.Soc.Trans.* 2004; **32**:416-420.

Zamir E, Katz BZ, Aota S, Yamada KM, Geiger B, Kam Z. Molecular diversity of cell-matrix adhesions. *J.Cell Sci.* 1999; **112 (Pt 11)**:1655-1669.

Zhang M, Wang JJ, Chen YJ. Effects of mechanical pressure on intracellular calcium release channel and cytoskeletal structure in rabbit mandibular condylar chondrocytes. *Life Sci.* 2006; **78**:2480-2487.

Zimmerman E, Geiger B, Addadi L. Initial stages of cell-matrix adhesion can be mediated and modulated by cell-surface hyaluronan. *Biophys.J.* 2002; **82**:1848-1857.

Zou H, Lifshitz LM, Tuft RA, Fogarty KE, Singer JJ. Visualization of Ca2+ entry through single stretch-activated cation channels. *Proc.Natl.Acad.Sci.U.S.A* 2002; **99**:6404-6409.

Zuscik MJ, Gunter TE, Puzas JE, Rosier RN. Characterization of voltage-sensitive calcium channels in growth plate chondrocytes. *Biochem.Biophys.Res.Commun.* 1997; **234**:432-438.

CHAPTER FIVE: APPENDIX A

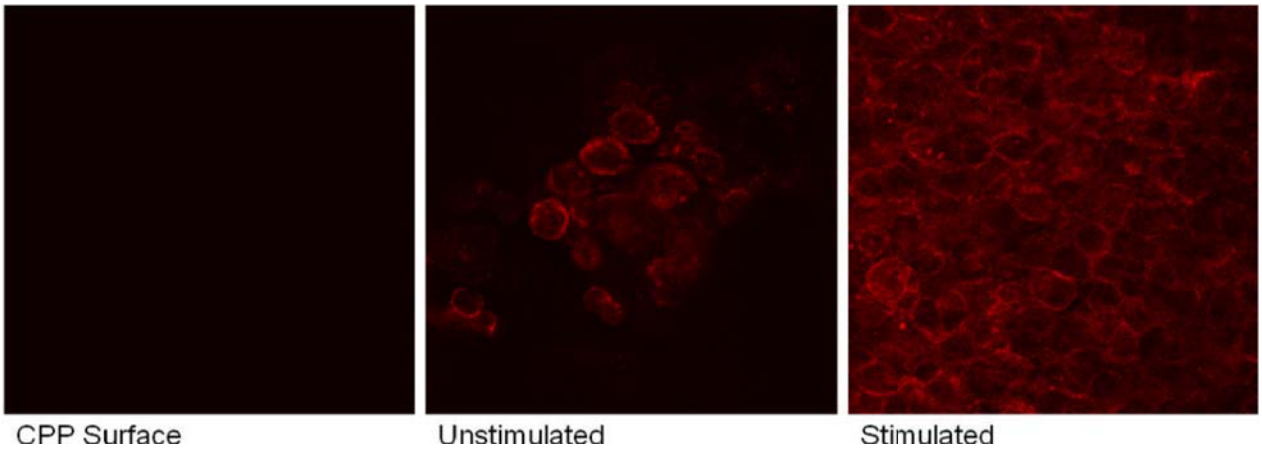
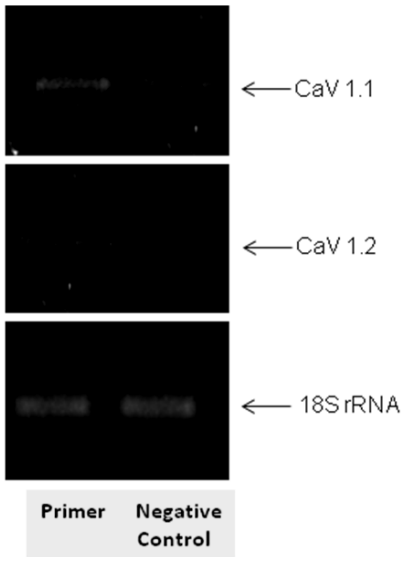


Figure A1: Chondrocyte Actin cytoskeleton as visualized by confocal microscopy. Control and stimulated tissue constructs were washed in PBS (3x) and fixed with 4% w/v paraformadehyde overnight at 4°C. Samples were then washed again (3x) with PBS followed by cell membrane permeabilization using 1% Triton X for 15 minutes at room temperature with gentle shaking. The actin cytoskeleton was stained using phalloidin-rhodamine (Alexa-fluor 586, Invitrogen) diluted 1:20 in 1% Triton X solution. Samples were mounted onto slides with a drop of Permafluor Mountant solution (anti-fade Immunon, Pittsburgh), sealed using a coverslip and stored in the dark at 4°C until analysis with a confocal microscope. Images were collected at a resolution of 512 x 512 units using a 40x objective (Leica TCS SP2, Leica Microsystems, Mannheim, Germany). Preliminary studies suggested that mechanical stimulation did not appear to alter the distribution of the actin cytoskeleton as compared to control unstimulated constructs.

A.



B.

Gene	Primer Sequence	Product Size
Cav 1.1	F: 3'- cagtgccaccgcattgtcaatgaca -5'	**491 bp**
	R: 3'- ctttcccttgaagagctggacccc -5'	
Cav 1.2	F: 3'- cgaagcttcttcatgatgaacatct -5'	**928 bp**
	R: 3'- gcggatccatgtagaagctgatgaa -5'	

Figure A2: Bovine articular cartilage chondrocytes express the L-Type VGCC. (A) The expression of the L-Type VGCC was confirmed in our system by RT-PCR, as the cells were shown to express the mRNA of the α subunit of the channel (B) The utilized primer sequences, along with the expected product size, for the receptor subunits. Primer sequences were taken from A. Benavides *et. al.*

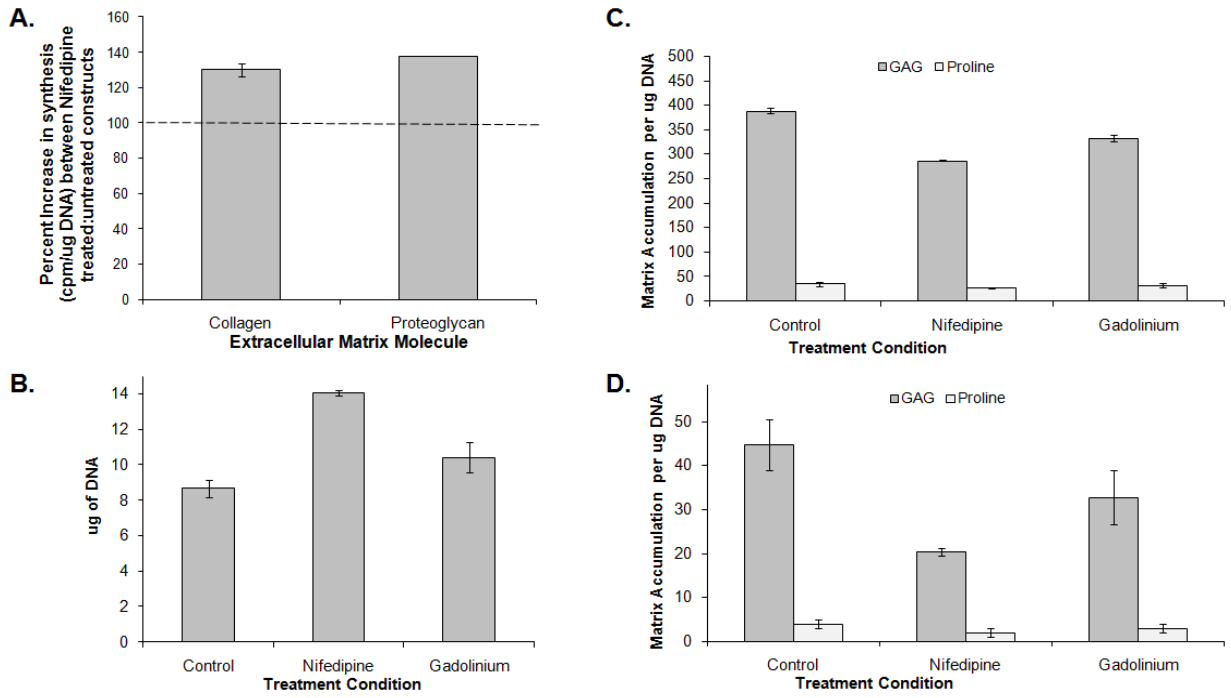


Figure A3: Tissue formation not affected with inhibitor treatments. Treatment with Nifedipine alone, without mechanical stimulation, induces increased synthesis of collagens and proteoglycans within 24 hours of treatment, however no improvement in tissue formation is observed by 3 weeks. In addition, Nifedipine treatment increases cell proliferation. Following the application mechanical stimulation, constructs were incubated with [^{35}S]-SO4 (2 µCi/construct) and [^{3}H]-proline (2 µCi/construct) for 24 hours under standard tissue culture conditions to measure proteoglycan and collagen synthesis respectively. Cultures were then washed in PBS and digested by papain (40 µg/ml; Sigma) in papain digestion buffer (40 mM ammonium acetate, 1mM EDTA, 2mM DTT) at 65ºC for 48 hours. The amount of newly synthesized proteoglycan and collagen retained in the tissue was quantified by measuring radioisotope incorporation using a β-scintillation counter and normalized to DNA content. The papain digested samples were assessed for DNA content using the Hoechst 33258 dye binding assay and fluorometry. (A) A 4-hour treatment with Nifedipine induces an approximately 25% increase in the synthesis of new matrix molecules in chondrocyte constructs. (B) It was observed that Nifedipine treatment also results in increased chondrocyte proliferation as compared to the vehicle- and Gadolinum-treated conditions. (C) A one-time 4-hour treatment by Nifedipine or Gadolinum did not result in improved tissue formation following a 3 week culture period under standard conditions. (D) Owing to the increase in DNA content in the Nifedipine-treated constructs there is an apparent decrease in proteoglycan and collagen content.

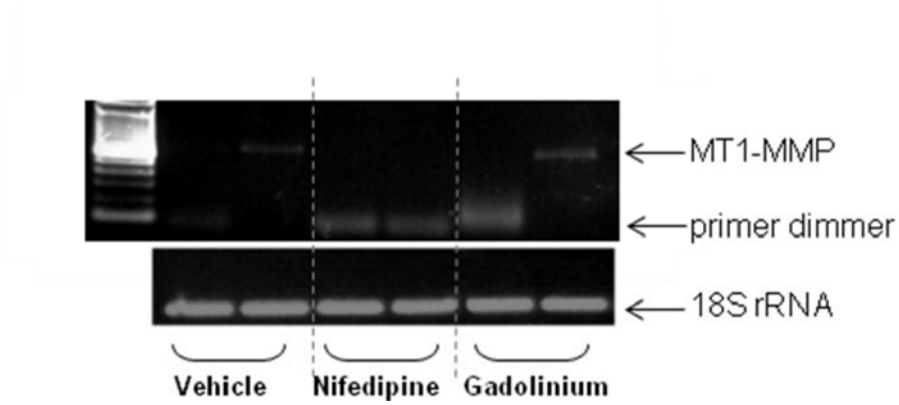


Figure A4: MT1-MMP expression in chondrocytes cultured on monolayer. MT1-MMP gene expression is dependent upon the chondrocytes' environment and the three dimensional architecture of the tissue. Likewise, the role of calcium in regulating MT1-MMP expression may be dependent on the culture system. Representative gel of MT1-MMP gene expression in chondrocytes grown in monolayer cultures. The chondrocytes exhibit indiscriminate expression of the mRNA in untreated, Nifedipine and Gadolinium treatments. This suggests that calcium regulation of MT1-MMP gene expression may be dependent upon the environment of the chondrocyte and is different in chondrocytes cultured in 3D. Chondrocytes were cultured on monolayer at 80-90% confluence prior to treatment with either inhibitors or vehicle media. Treatment times and concentrations were otherwise identical to those utilized previously.

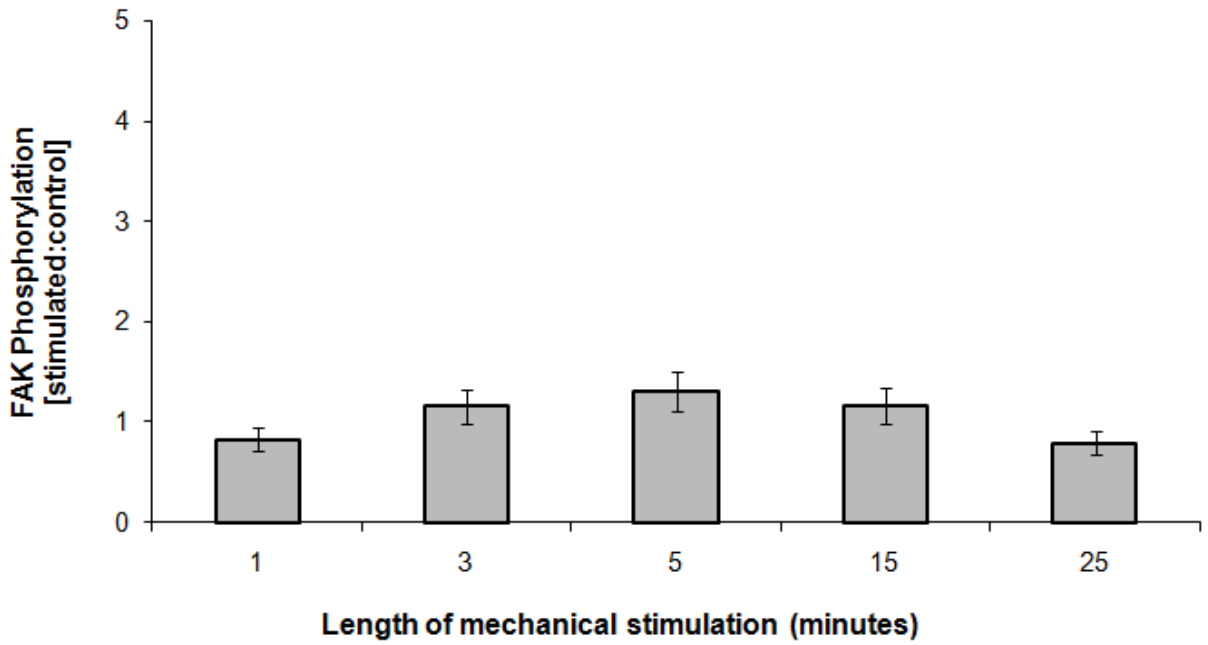


Figure A5: Phosphorylation of Focal Adhesion Kinase (FAK) in response to mechanical stimulation. Cells were mechanically loaded for various periods of time ranging from 1 to 25 minutes. Total cell protein was extracted and analyzed by western blot for pTyr and Total FAK, as per the previously outlined western blotting methods. No significant changes in FAK phosphorylation were observed. Three independent experiments were performed in duplicate (n=6), and the results are pooled and expressed as mean ± SEM.

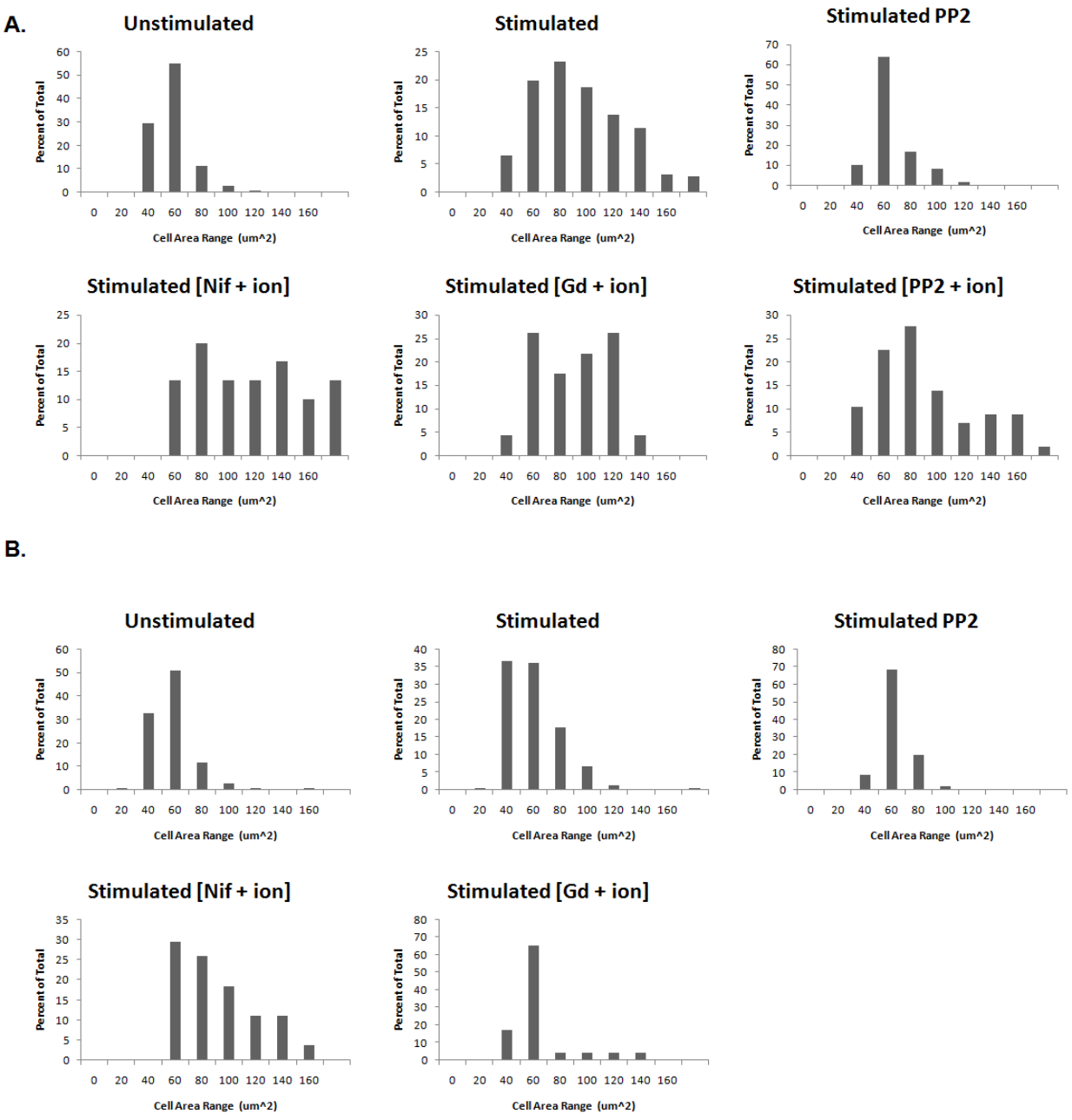


Figure A6: Cell Area Distributions Following Stimulation. Distribution of chondrocyte cell area, expressed as percentage of total, in the various conditions. Total cell areas in each condition were pooled together and separated, utilizing Excels frequency function, into bins in the range of 20um. The value of each bin was then quantified and expressed as a percentage of the total cell number. Cell area was quantified immediately following stimulation (A) and at six hours later (B) and the distribution was determined. Three independent experiments were performed in duplicate (n=6), and the results are pooled and expressed as mean ± SEM.

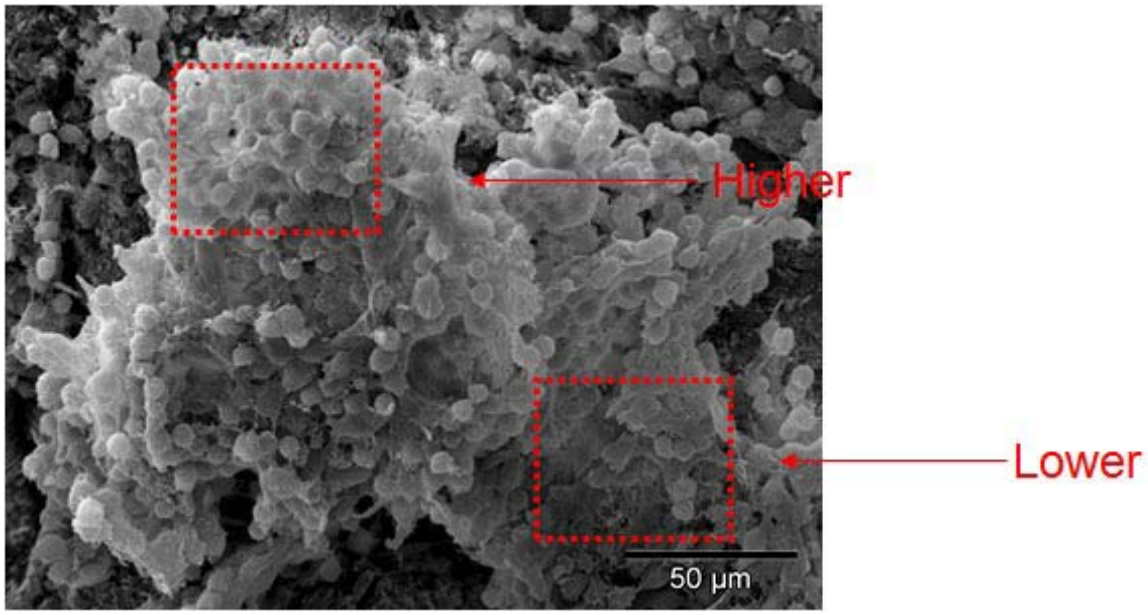


Figure A7: Cell spreading appears to be depth-dependent. Stimulated tissue constructs were cut at an angle prior to Scanning Electron Microscopy visualization, to attempt to visualize the cell morphology gradient, following mechanical stimulation. It appears that cells situated deeper within the tissue, closer to the CPP surface, undergo a more significant spreading as a result of mechanical stimulation.

CHAPTER SIX: APPENDIX B

Articular Cartilage Subpopulations Respond Differently to Cyclic Compression *in vitro*

*[1]I. Raizman, *[1]J.N. Amritha De Croos, [1,2]J-P. St-Pierre, [1,3]R. M. Pilliar and [1,2,4]R. A. Kandel

*both authors contributed equally to this work.

[1] CIHR BioEngineering of Skeletal Tissues Team, Mount Sinai Hospital, and University of Toronto.

[2] Institute of Biomaterials and Biomedical Engineering, University of Toronto,

[3] Faculty of Dentistry, University of Toronto, Toronto, Canada.

[4] Address correspondence to:

Rita Kandel, MD

Department of Pathology and Laboratory Medicine,

Mount Sinai Hospital 600 University Avenue, Suite 6-500,

Toronto, Ontario, Canada M5G 1X5.

Telephone: 416-586-8516

Fax: 416-586-8628

E-mail: rkandel@mtsinai.on.ca

Running title: Cartilage subpopulation response

Keywords: cyclic compression, subpopulation, chondrocytes, deep zone, superficial-mid zone.

Manuscript accepted: Journal of Tissue Engineering A

Introduction

Articular cartilage, a specialized tissue present at the ends of bones, allows for smooth articulation and transmission of applied forces to the subchondral bone. When damaged by disease or trauma, it has a limited self-repair potential due, in part, to its avascularity. Tissue engineering articular cartilage *ex vivo* which is then transplanted into a joint defect is a promising therapeutic approach. In previous studies we developed the methodology to form biphasic constructs consisting of an articular cartilage-like component and a bone-interfacing component [1]. When submitted to mechanical stimulation, either cyclic compression or shear, the amount of extracellular matrix (ECM) produced by chondrocytes in the *in vitro* formed cartilage significantly increased as did the mechanical properties [2, 3]. We have previously shown that the increased ECM synthesis induced by a single episode of cyclic compression of chondrocytes isolated from the full-thickness (FT) of bovine articular cartilage is a result of a remodelling process involving a catabolic response followed by an anabolic response [4]. This single application of cyclic compression increased both the synthesis of collagen and proteoglycan measured 24 hours post-stimulation and at four weeks post-stimulation [3, 4]. The initial response included upregulation of both type 1 membrane-bound (MT1) matrix metalloproteinase (MMP) MT1-MMP as well as MMP-13. Increased MT1-MMP gene expression was detected within 30 minutes of initiating cyclic compression and 2 hours later MMP-13 was elevated. Changes in protein levels occurred after the gene changes. By 24 hours both MT1-MMP and MMP-13 had returned to constitutive levels of expression [4, 5]. These transient changes appear to be critical to the increased matrix accumulation that occurs under these stimulatory conditions as shown through the use of pharmacological inhibitors and decoy oligodeoxynucleotides to suppress catabolic changes of MMP-13 and MT1-MMP [4, 5]. A biphasic construct in which there is more

cartilage tissue with greater mechanical properties has the advantage in that it more closely mimics native tissue and could potentially experience less problems following transplantation into the joint defect.

Articular cartilage, *in vivo*, can be divided into four distinct regions: A) superficial zone B) middle zone C) deep zone, and D) calcified layer which together are essential for proper cartilage functionality. Each zone is characterized by a specific matrix composition and organization. In keeping with this, bioengineered tissue from different cartilage subpopulations yields tissue with different properties [6-10]. Given the phenotypic differences observed, it is likely that cyclic compression may affect one zone of cartilage preferentially. If only a select subpopulation of chondrocytes can respond to mechanical loading under our conditions this may explain why we observed an approximately 30% increase in tissue formation. This possibility is supported by work showing that the pericellular matrix is important in influencing chondrocyte response to mechanical loading [11]. Indeed, one study has shown that subpopulations of chondrocytes grown on agarose beads have differential responses to compressive mechanical strain [12]. Furthermore, the cells in each of the zones experience different types of forces when loaded *in vivo* [13]. For instance, as a result of their location, deep zone (DZ) chondrocytes are subjected to shearing stresses upon loading [13]. In contrast, chondrocytes from the superficial and mid zones (SMZ) encounter more tensile and compressive forces respectively [13]. So the cell subpopulation used to engineer cartilage might be very critical to the development of hyaline cartilage with the ability to survive long term.

In the current study, the effect of cyclic compression on matrix synthesis and the critical early gene and protein changes that occur following mechanical stimulation were examined in DZ and SMZ chondrocyte subpopulations during tissue formation. This will allow us to

determine whether a particular subpopulation is responsible for the increased ECM synthesis and provide further insight into the contribution of MMP-13 and MT1-MMP to matrix remodelling. Understanding the response of different chondrocyte subpopulations to mechanical stimulation during tissue formation will facilitate our understanding of tissue growth and as well allow for further optimization of cartilage tissue development *in vitro*.

Materials and Methods

Cell Isolation

Chondrocytes were selectively harvested from the upper 70% (superficial-mid zone) and the bottom 30% (deep zone) of cartilage.from bovine metacarpal-phalangeal joints (6 to 9 month old calves). Two different subpopulations of chondrocytes representing the superficial-mid zone (SMZ) and the deep-zone (DZ) as well as full-thickness (FT) chondrocytes were isolated as previously described by sequential digestion with 0.5% proteinase (Sigma-Aldrich Chemical Co., St. Louis, MO) and 0.1% Collagenase A (Roche Applied Science, Indianapolis, IN) at 37^{o}C, 5% CO_2 [1, 14]. Cells were re-suspended in Ham's F-12 supplemented with 5% fetal bovine serum (Sigma-Aldrich) and seeded at a density of 160,000 cells/mm^2 onto the top surface of cylindrical discs of calcium polyphosphate (CPP) surrounded by Tygon tubing (4.3 mm diameter x 6 mm height; Thermoplastics Processor Inc., San Jose, CA). CPP substrates (4 mm diameter x 2 mm height) were prepared as previously described [15]. Cells were obtained from two to three animals and pooled together to obtain sufficient numbers to do an experiment.

Mechanical Stimulation

Following three days of static culture, the chondrocytes formed a thin layer of tissue on top of

and integrated with the surface of the CPP substrate. These biphasic constructs were then cultured in serum-free DMEM overnight before undergoing uniaxial, confined cyclic compression of 1 kPa (corresponding to a strain of 1.4%) using a mechanical stimulator (MACH-1, Biosyntech, Montreal, CAN) for 30 minutes at a frequency of 1Hz. Serum-free conditions were used to eliminate the effects of serum factors. The resultant tissue formation after mechanical stimulation in serum-free conditions used in De Croos et al., (2006) was similar to that seen in Waldman et al., (2005) performed in serum. Cyclic compression of the *in vitro* formed cartilage was applied through a compliant 2% agarose gel cylinder (3.5 mm diameter x 8 mm height; approximately 10±1 kPa compressive stiffness, n=6) (Sigma-Aldrich). The agarose plug-tissue layer construct was deformed under displacement control with the amplitude of cyclic deformation adjusted manually to apply sinusoidal loading to the construct [3]. Agarose cylinders were removed immediately after mechanical stimulation. Control samples were treated identically to stimulated samples, but did not receive any mechanical stimulation. Constructs remained in serum-free DMEM until the appropriate harvest time-point. Previous studies have shown that chondrocytes do not adhere to the agarose plug during loading [3].

Histological assessment of the different chondrocyte subpopulations

After 3 days of culture, the tissues formed on the top surface of the CPP were removed using a scalpel blade (no. 11) and embedded in 2% agar (Difco, Detroit, MI). Agar was used to facilitate handling of the tissue during subsequent processing. Samples were fixed in 10% neutral-buffered formalin and embedded in paraffin. Sections were cut, stained with hematoxylin and eosin (H&E) or toluidine blue, and examined by light microscopy.

Analysis of gene expression

Total RNA was extracted from the different chondrocyte subpopulations before and during various periods of culture using TRIzol Reagent (Invitrogen, Carlsbad, CA). Total RNA was then reverse-transcribed (RT) into cDNA using Superscript II (Invitrogen). The resulting cDNA was subjected to PCR in an Eppendorf Mastercycler PCR using primers and conditions previously described [4, 5]. PCR products were run on a 1.5% agarose gel and visualized by staining with ethidium bromide. Band intensity was semi-quantified by densitometry using Lab Works software (V4.0, Media Cybernectics). Relative gene expression was normalized to 18S rRNA.

Analysis of protein expression

After TRizol extraction and removal of the aqueous phase and RNA, proteins were obtained from the remaining interphase and organic phases respectively. Proteins were quantified utilizing a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL) and spectrophotometry at 562 nm absorbance (Thermo Electron MultiSkan Ex). Bovine serum albumin was used to generate a standard curve. Total proteins from each sample (20 μg) were mixed (1:1 v/v) with sample buffer containing 100 mM Tris-HCL (pH 6.8), 4% (w/v) SDS, 20% (v/v) glycerol, 0.2% (w/v) bromophenol blue, and 5% β-mercaptoethanol (Sigma-Aldrich) and separated by a 12% sodium-dodecyl-sulfate polyacrylamide gel (SDS-PAGE) (2 hours at 150V) and electroblotted onto nitrocellulose membranes (BioTrace NT, Pall Life Sciences, Pensacola, FL) for 1.5 hours at 0.3 mA. The membranes were washed in TBS-T (10mM Tris, pH 7.5, 150 mM NaCl, and 0.05% v/v Tween-20) and subsequently blocked for 1 hour at room temperature in 5% (w/v) solution of non-fat dry milk in TBS-T. Membranes were then probed with a primary

antibody reactive with either MMP-13 (Calbiochem, 1:1500) or MT1-MMP (Santa-Cruz Biotechnologies; 1:2000) overnight at 4°C. Membranes were then incubated with secondary antibodies for detection of MMP-13 (anti-mouse, 1:5000; Santa Cruz Biotechnologies) or MT1-MMP (anti-rabbit, 1:2000; Cell Signaling) for 1 hour at room temperature. Immunoreactivity was detected using enhanced chemiluminescence (ECL Plus) (GE Healthcare, Buckinghamshire, UK) and band intensity was semi-quantified by densitometry using Lab Works software (V4.0, Media Cybernectics). To compensate for loading variations, all densitometric values were corrected by normalizing to β-actin (Sigma; 1:5000).

Extracellular matrix accumulation by chondrocytes from the different subpopulations

Following the application of cyclic compressive loading, chondrocyte-seeded constructs (stimulated and unstimulated controls) were incubated with [^{35}S]-SO_4 (2 μCi/construct) and [^{3}H]-proline (2 μCi/construct) for 24 hours (37°C, 5% CO_2) to measure proteoglycan and collagen synthesis respectively. Cultures were then washed in PBS and digested by papain (40 μg/ml; Sigma-Aldrich) in papain digestion buffer (40 mM ammonium acetate, 1mM EDTA, 2mM DTT) at 65ºC for 48 hours. The amount of newly synthesized proteoglycan and collagen retained in the tissue was quantified by measuring radioisotope incorporation using a β-scintillation counter and normalized to DNA content. The papain digested samples were assessed for DNA content using the Hoechst 33258 dye binding assay and fluorometry as described previously (Waldman et al. 2002).

Extracellular matrix accumulation by deep zone chondrocytes at different time points

Accumulation of collagen and proteoglycan was quantified in DZ chondrocytes at 1, 3, and 5 days after initial seeding. For these studies, cultures were incubated in serum-free DMEM for four hours prior to mechanical stimulation as it was not possible to do a 24hr serum-free pre-incubation for the day 1 cultures. Cultures were then incubated with radioisotopes immediately following mechanical stimulation as described above and the amount of newly synthesized collagen and proteoglycan quantified.

Statistical Analysis

Each condition was performed in triplicate and all experiments were repeated at least 3 times. The data was pooled and expressed as mean ± SEM. Results were evaluated using a student's *t*-test or an ANOVA followed by Fisher's protected least significant difference test (*post-hoc*). Statistical significance was assigned at $p<0.05$.

Results

Chondrocyte population enrichment

To determine the efficacy of the harvesting of the chondrocyte subpopulations, isolated cells were analyzed for the expression of subpopulation-specific markers prior to seeding on CPP substrates. Cells isolated from DZ showed greater expression of MMP-13 and Collagen type X whereas expression of superficial zone protein (SZP) and cartilage intermediate layer protein (CILP) were preferentially expressed in the SMZ cells (Fig. 1). Expression of aggrecan and type II collagen (col2a1) was evident in all three subpopulations with a slightly greater expression in DZ cells (Fig. 1). Extracellular matrix (ECM) accumulation in unstimulated constructs also differed amongst the different subpopulation (Fig. 3). DZ cells accumulated four-fold more

collagen per cell than SMZ cells (SMZ= 409±66 cpm/μg of DNA, DZ= 2339±576 cpm/ug DNA; $p<0.01$). Similarly DZ chondrocytes accumulated significantly more proteoglycans than SMZ cells (SMZ= 570±82 cpm/μg of DNA, DZ =2856±528 cpm/μg of DNA) ($p<0.05$). This confirms that the method to obtain these subpopulations was appropriate for use in the subsequent studies.

Histological appearance of the tissue subjected to mechanical stimulation

To confirm that the different subpopulations formed tissue, the cultures after 3 days (at the time when they would be mechanically stimulated) were harvested, and the tissue removed from the top of the substrate, and examined histologically. Figure 2 shows that the different chondrocyte subpopulations can form tissue. Interestingly cells from the DZ appeared to form slightly thicker tissue than the cells from SMZ and some cells resembled hypertrophic chondrocytes with pericellular clearing on the toluidine blue stained tissues.

Effect of mechanical stimulation on different chondrocyte subpopulations

The effect of cyclic compression on accumulation of newly synthesized collagen and proteoglycans by the different subpopulations was examined. There was a significant increase in accumulation of proteoglycans after cyclic compression by SMZ cells (increase of 75%). In tissues formed by DZ chondrocytes, however, proteoglycan accumulation decreased by 40% (Fig. 3A). Mechanical stimulation increased the accumulation of collagen in the tissue formed by SMZ chondrocytes by approximately 65% compared to unstimulated control tissue while decreasing the accumulation of collagen in the tissue formed by DZ chondrocytes by approximately 40% ($p<0.01$, Fig. 3B). The application of cyclic compression did not significantly alter the DNA content of the *in vitro*-formed tissues (Table 1).

MT1-MMP gene and protein expression

As differences were observed in matrix accumulation by the different chondrocyte populations, the cells were examined for changes in MT1-MMP over time as this protease shows very early changes following cyclic compression in full thickness chondrocytes and has been implicated in the mechanism leading to the remodelling of the extracellular matrix resulting in improved matrix accumulation. The profile of MT1-MMP gene expression differed between the two different chondrocyte subpopulations (Fig. 4). A significant increase in MT1-MMP mRNA was observed immediately following mechanical stimulation in SMZ cells (2.5 fold increase compared to controls, data not shown, $p<0.05$). The response was transient and decreased to constitutive levels by 2 hours. The changes in MT1-MMP expression were similar to that observed in full thickness cells as described previously [4]. In contrast, DZ chondrocyte MT1-MMP gene expression did not increase until 24 hours following mechanical stimulation (2.5 fold increase compared to controls, $p<0.05$). The levels of expression at this time were significantly different than the expression in SMZ cells ($p<0.05$). The responses of the SMZ and DZ cells were opposite to each other.

MT1-MMP protein changes were also detected (Fig. 4C). Western blot analysis showed a significant increase (2.5 fold) in MT1-MMP protein expression in SMZ chondrocytes at two hours ($p<0.05$) which was different from the DZ cells ($p<0.05$). The increase was transient and by 12 hours had returned to constitutive levels in keeping with the gene changes. In contrast, significantly increased protein levels of MT1-MMP in the tissues formed by DZ cells were only detected at 24 hours, which was in keeping with the gene changes.

MMP-13 gene and protein expression

The gene expression profile of MMP-13, another matrix protease known to be involved in the early response to mechanical stimulation, was also examined (Fig. 5A). There were significant differences observed between the SMZ and DZ chondrocytes immediately following stimulation ($p<0.05$). The expression levels in SMZ were significantly elevated (stimulated: control ratio of 1.5, $p<0.05$) immediately following stimulation and remained increased throughout the 24 hour period. In contrast, in DZ chondrocytes there was a decrease in expression immediately following stimulation but the gene levels increased until 12 hours (5-fold increase compared to 0 hour, $p<0.05$). The increase was transient and by 24 hours MMP-13 had returned to constitutive levels of expression.

Following mechanical stimulation there was a significant decrease in MMP-13 protein expression in the SMZ chondrocytes (Fig. 5B) but protein expression returned to constitutive levels by 2 hours and showed no further changes. In contrast, no significant changes in the expression of MMP-13 were observed in DZ chondrocytes at the times examined. No significant changes were observed in aggrecan and collagen gene expression at the time-points investigated (data not shown).

Matrix accumulation by deep zone chondrocytes influences cell response to mechanical loading

To investigate why DZ chondrocytes responded differentially to mechanical stimulation, the role of the ECM in mechanotransduction was investigated owing to the differences in thickness between SMZ and DZ tissue by Day 3. This was done by subjecting DZ cultures to cyclic compression at Days 1, 3, and 5 to test how ECM accumulation would influence the

response of mechanical stimulation (Fig. 6). DZ cells stimulated at day 1 had a significant increase in the accumulation of both newly synthesized proteoglycans and collagens when compared to corresponding unstimulated control tissues ($p<0.05$). A significant decrease in accumulation was detected in cultures stimulated at days 3 and 5 ($p<0.05$). DZ cultures at Day 3 had significantly less accumulated proteoglycans compared to Day 5 DZ cultures (data not shown).

Discussion

In this study the responsiveness of superficial-mid zone (SMZ) or deep zone (DZ) chondrocytes to one application of cyclic compression during *in vitro* tissue formation was examined. The enrichment of the chondrocyte subpopulations was confirmed through RT-PCR, quantification of matrix synthesis, and by histology. Cells isolated from the DZ and seeded onto CPP substrates yielded a thicker tissue compared to the tissue formed by FT or SMZ cells within 3 days of culture. The FT and SMZ tissues appeared to be of similar thicknesses. This was not unexpected as SMZ makes up about 70% of the FT cartilage. As well as producing morphologically distinct tissues, these phenotypic changes translated into significant differences in matrix accumulation following mechanical stimulation. There were significant increases in proteoglycan and collagen accumulation within 24 hours ($p<0.05$) in tissues formed by SMZ cells, while tissues formed by DZ cells showed decreased accumulation of these macromolecules. As would be expected based on our previous studies, MT1-MMP showed an increase in gene and protein expression in the early time points following mechanical stimulation [4, 5]. The DZ cells, which did not respond positively to stimulation, did not develop these early changes. However the DZ cells were responsive to stimulation as gene and protein changes did

occur but at a later time point.

The inhibitory response of DZ cells to cyclic compression seems to be related to the amount and/or composition of the ECM that is generated because stimulation of DZ cells when there was little pericellular matrix, 24 hours after seeding, had a positive response to cyclic compression. Taken together these results indicate that the SMZ cells are responsible for the improvement in ECM accumulation that occurs in developing cartilage after cyclic compression. The degradative response by DZ cells may explain why we observed only a small, approximately 30%, increase in matrix accumulation in our previous studies. Although our data suggests that DZ cells degrades the ECM when exposed to this type of mechanical loading, it is not possible to predict how these cells will behave in the presence of SMZ cells as occurs when they are mixed together to bioengineer tissue as occurs following cell isolation from cartilage. It may be that the different subpopulations of articular cartilage communicate with each other, perhaps by synthesizing for example, soluble mediators and/or other ECM components, resulting in non-additive effects. We have shown previously that chondrocytes are capable of cross-talk in co-culture [16] and although the mechanism(s) has not been determined, it likely occurs via a soluble factor. ATP [17] or PTHrP [18] are possible factors that have been shown to mediate interactions between chondrocytes.

Our data also indicates that the pericellular matrix (PCM) plays an important role in regulating the chondrocytes' response to cyclic compression and may explain why DZ and SMZ cells respond differently to mechanical stimulation. We observed that with increasing matrix accumulation that mechanical stimulation went from having a positive effect on the first day to a negative effect by the third day of culture. Although the precise function of the PCM has not been fully elucidated, there is considerable evidence that suggests it is involved in the regulation

of biochemical and biomechanical interactions between the cells and the surrounding ECM [11, 19, 20]. It is well accepted that in articular cartilage the residing chondrocytes must function synergistically with the ECM in order to absorb, redistribute and transmit physiological compressive and shearing forces to the subchondral bone. As chondrocytes are completely surrounded by the PCM any signals from the ECM must pass through the PCM before they are sensed by the chondrocytes. As such it has been hypothesized that the PCM may function as a transducer of biomechanical forces and it can do so in a variety of ways. The PCM influences the fixed charge density experienced during cartilage loading, which can affect chondrocyte activity [21, 22]. Alternatively chondrocytes are known to produce and respond to factors such as ATP [23], FGF [24], and the BMP family [25]. As components of the ECM, such as decorin and biglycan, are differentially produced by DZ and SMZ cells, these could regulate cells directly or indirectly by sequestering different growth factors [26]. This interplay between ECM molecules and soluble mediators at the intracellular level adds further to the complexity underlying the chondrocytes' response to mechanical loading. .Another mechanism could involve binding of chondrocyte integrins to ECM molecules. This interaction results in the formation of adhesion plaques, which in turn, is a prerequisite for chondrocyte responsiveness to various growth factors [27]. Recent work has shown that the PCM functions to provide a homogenous cell-level strain amongst the cartilage subpopulations when exposed to variable local ECM strains [28]. Further study is required to delineate the exact mechanism(s) regulating chondrocyte function.

The timing of the MMP-13 gene and protein changes differ from those observed in our previous studies [4, 5]. There are three possible reasons for this. Firstly, the present study was done using enriched zonal populations and if there is cross-talk between the different zones this could not occur thus resulting in different responses. Secondly the studies were performed in a

different culture media, DMEM as opposed to Ham's F12, the former is a culture condition favoured by DZ cells [29]. Ham's F-12 has lower sulfate compared to DMEM which along with other differences in chemical composition may also play a role in cellular response. As well, DMEM has a higher Ca^{2+}concentration than Ham's F12 (200 mg/L and 33.20 mg/L respectively) and previous studies have shown that Ca^{2+} signalling is involved in the mechanotransduction events in response to fluid flow [30, 31] and hydrostatic pressure [32] in chondrocytes. The application of physiological levels of compressive strain on chondrocytes seeded within a three-dimensional agarose construct results in intracellular Ca^{2+} signalling that can be blocked with EGTA, a chelator of extracellular Ca^{2+} and significantly reduced with Gd^{3+}, which is known to block voltage-operated Ca^{2+} channels [33]. Another study reported that extracellular Ca^{2+} is crucial for the propagation of Ca^{2+} waves in cultured chondrocytes following mechanical stimulation [23]. Our results suggest that chondrocytes may respond differentially depending on the extracellular Ca^{2+} concentration which would be in keeping with the studies. However, as these media differ in other components it is possible that one of these other, as yet unidentified, molecules may be responsible. Thirdly, in the previous study MMP-13 protein levels in the media were evaluated; whereas the current study measure tissue levels of MMP-13. Nevertheless, even though there are differences in the timing of the MMP-13 alterations, MT1-MMP changes occur within the first two hours in SMZ tissues and these correlated with increased matrix accumulation similar to the changes we described previously in the tissues formed by cells obtained from full thickness cartilage. This also raises the possibility that MT1-MMP might be more important than MMP13 in regulating tissue growth in vitro.

In summary, cyclic compression has a differential effect on the individual cartilage subpopulations. SMZ responded positively to a single application of cyclic compression,

whereas DZ responded negatively. The DZ response can be attributed to the amount and/or type of matrix that accumulates as cyclic compression applied at an earlier time point in growth did increase matrix accumulation by DZ cells. Our results suggest that SMZ cells may be responsible for the improved tissue formation that results following cyclic compression. Forming cartilage with the appropriate amount of SMZ cells may be critical to successfully bioengineer articular cartilage, a particular problem if using osteoarthritic cartilage, which may lack a superficial zone and portions of the mid zone, as the source of cells.

Acknowledgments

This work was supported by CIHR and the Arthritis Society. We thank Mr. Harry Bojarski and Ryding-Regency Meat Packers for providing the tissues for these studies.

Figure Legends

Figure 1: Gene expression of chondrocyte subpopulation phenotype markers. Total RNA was extracted from superficial-mid zone (SMZ), deep zone (DZ) and full thickness (FT) chondrocytes immediately after isolation from cartilage. RT-PCR using primers specific for genes indicative of phenotype were used to assess the purity of each subpopulation. Analyses were performed in triplicate from three separate experiments (n=9). Representative gels from a single experiment are shown. Average densitometric values normalized to 18S rRNA are shown below each gene.

Figure 2: Histological appearance of tissues after three days of culture. Chondrocytes were isolated from A) superficial-mid zone, B) deep zone or C) full thickness cartilage, grown for three days, removed from the surface of the CPP substrates, and processed for histological examination. Sections were stained with either hematoxylin and eosin or toluidine blue. *indicates where substrate was located.

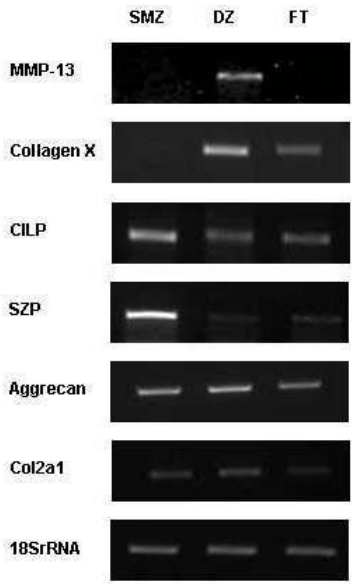
Figure 3: Effect of a single application of mechanical stimulation on accumulation of newly synthesized collagen and proteoglycan. Chondrocytes from either superficial-mid or deep zone were mechanically stimulated and then incubated with ^{3}H-proline and ^{35}S-SO_4 to label collagens and proteoglycans respectively; control constructs did not receive any mechanical stimulation. Twenty-four hours later, the amount of newly synthesized proteoglycan (A) and collagen (B) accumulated was determined by quantifying radioactivity in the tissue as described under the methods. Results were pooled and expressed as mean ± standard error of the mean (n=9). As indicated there is a significant increase from respective unstimulated constructs, *$p<0.02$, #$p<0.05$.

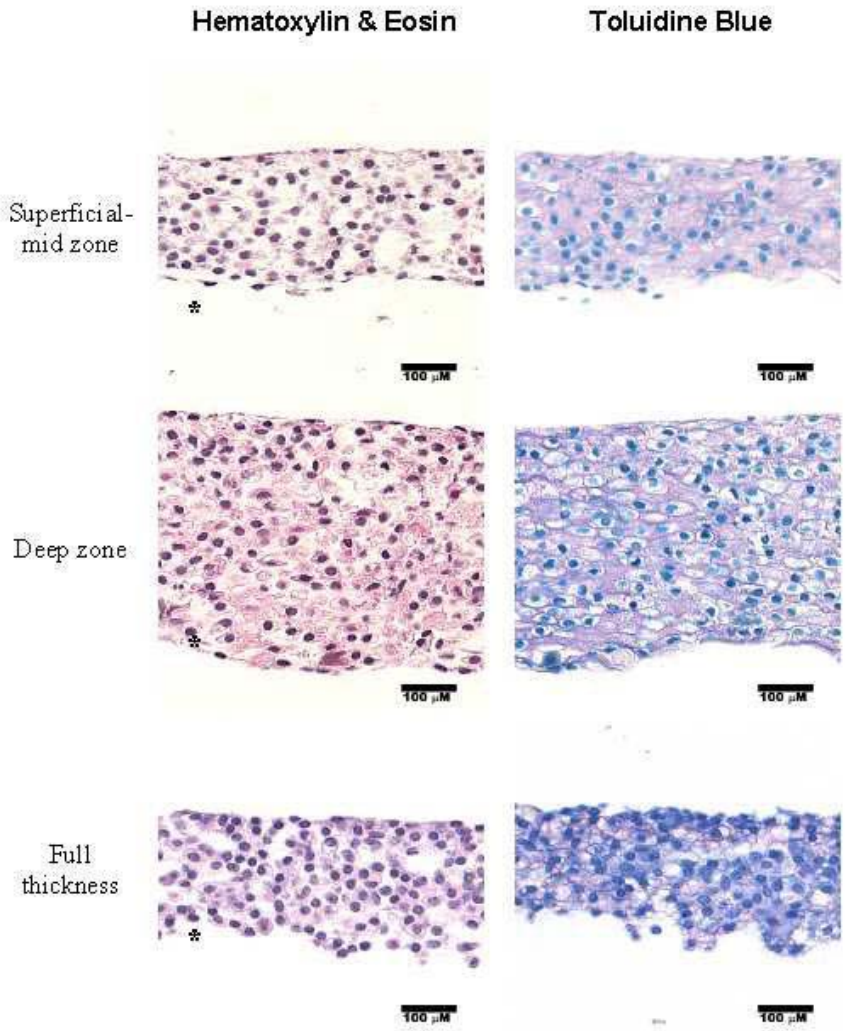
Figure 4: MT1-MMP gene and protein expression following a single application of cyclic compression. A) Representative gel showing PCR products over time in both superficial-mid zone or deep zone tissues. The levels of expression were semi-quantified by densitometry and the fold changes (B) between mechanically stimulated and control tissues formed by different chondrocyte populations over a 24 hour time period following mechanical stimulation were determined. Significant differences within each zone and over time, are indicated, where *$p=0.016$, **$p=0.006$, and #$p<0.05$ with respect to zero time-point. C) Western blot analysis and densitometry were used to determine fold changes in MT1-MMP proteins levels between stimulated and control unstimulated tissues formed by different chondrocyte subpopulations over time. Results were pooled and expressed as mean ± standard error of the mean (n=6).***,** #denotes significant differences where *$p<0.05$ with respect to the 2 hour time-point; #$p<0.05$ with respect to the 24-hour time point.

Figure 5: MMP-13 gene and protein expression following a single application of cyclic compression in chondrocytes from superficial-mid and deep zones. A) Representative gel showing PCR products. The levels of expression were semi-quantified by densitometry and the fold changes (B) between mechanically stimulated and control tissues formed by different chondrocyte populations over a 24 hour time period following mechanical stimulation were determined. Significant differences within each zone and over time, are indicated, where *$p=0.02$ with respect to zero time-point. #$p<0.05$ to corresponding unstimulated control. C) Western blot

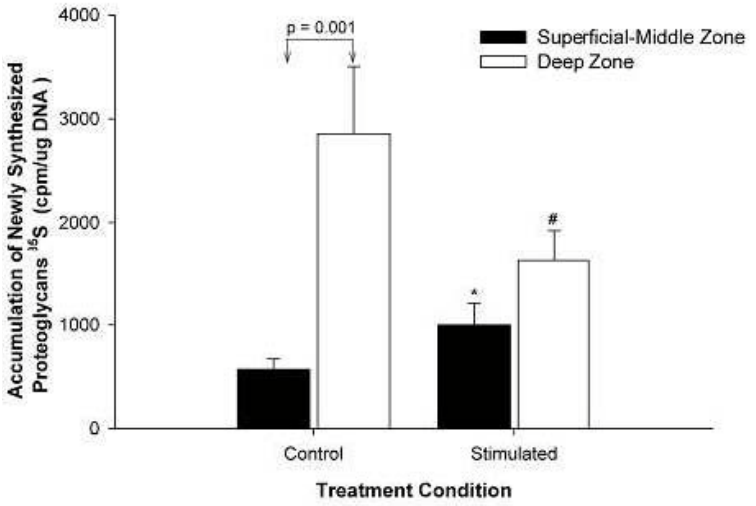
analysis and densitometry were used to determine fold changes in MMP-13 proteins levels between stimulated and control unstimulated tissues formed by different chondrocyte subpopulations overtime. Results were pooled and expressed as mean ± standard error of the mean (n=6) Results were pooled and expressed as mean ± standard error of the mean (n=6).*p=0.008 with respect to zero time-point.

Figure 6: Accumulation of newly synthesized collagen and proteoglycans by deep zone chondrocytes. Chondrocytes from the deep zone at either 1, 3 or 5 days following initiation of the cultures were mechanically stimulated and then incubated with ^{3}H-proline and ^{35}S-SO_4 to label collagens and proteoglycans respectively; control constructs did not receive any mechanical stimulation. Twenty-four hours later, the amount of newly synthesized proteoglycan (open bar) and collagen (filled bars) accumulated were determined by quantifying radioactivity in the tissue as described under the methods and normalized for DNA. Experiments were performed in duplicates and repeated three times (n=6). Results were pooled and expressed as mean ± standard error of the mean. *denotes significant increase from corresponding unstimulated control, $p<0.05$. [a]denotes significant decrease from corresponding unstimulated control, $p<0.05$.

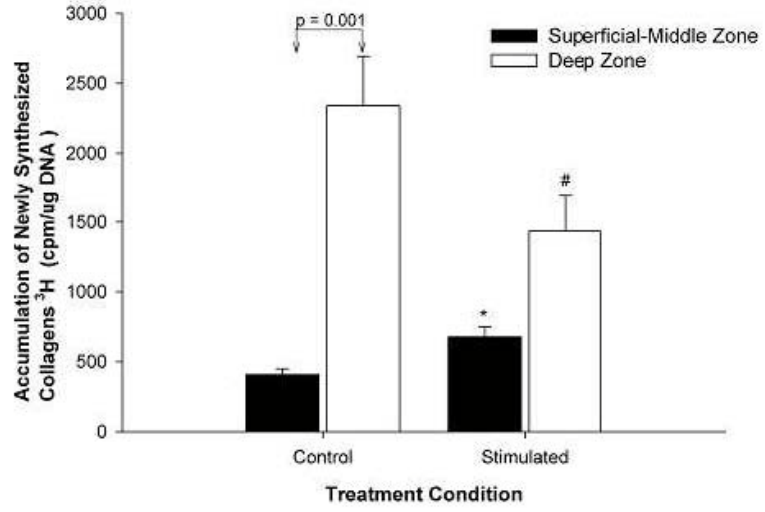

SMZ
DZ
FT
MMP-13
Collagen X
CILP
SZP
Aggrecan
Col2a1
18SrRNA

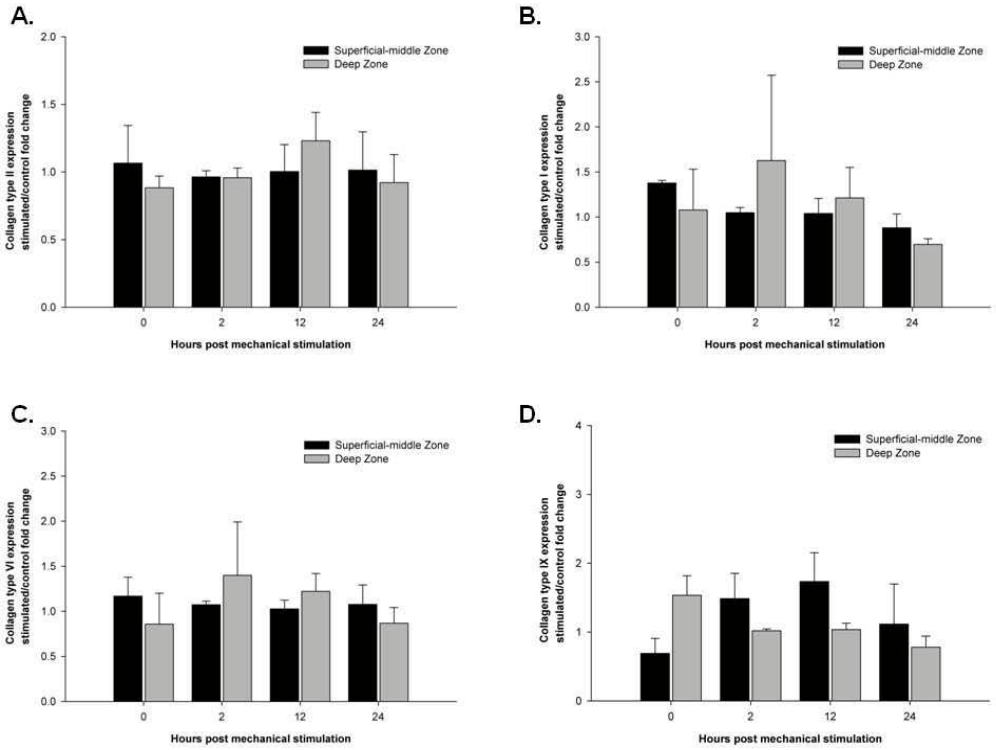

Hematoxylin & Eosin
Toluidine Blue
Superficial-mid zone
Deep zone
Full thickness
100 µM

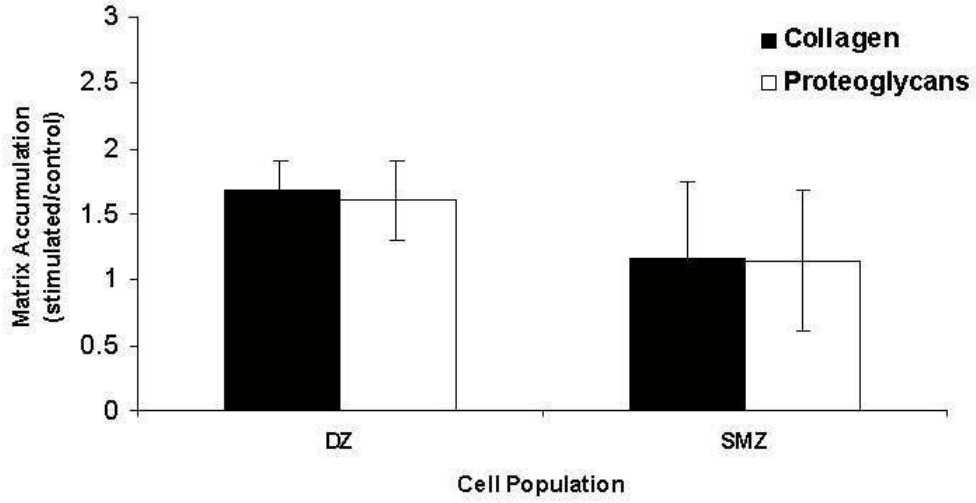
A)

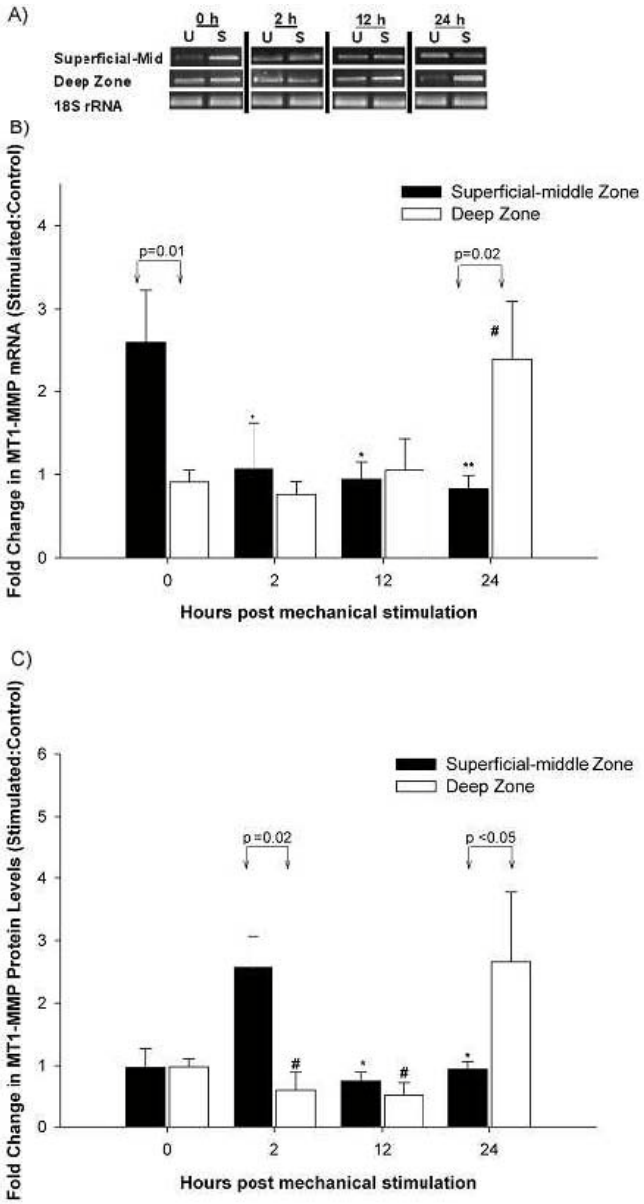

4000
3000
2000
1000
0
p = 0.001
Superficial-Middle Zone
Deep Zone
Accumulation of Newly Synthesized Proteoglycans ^{35}S (cpm/ug DNA)
*
#
Control
Stimulated
Treatment Condition

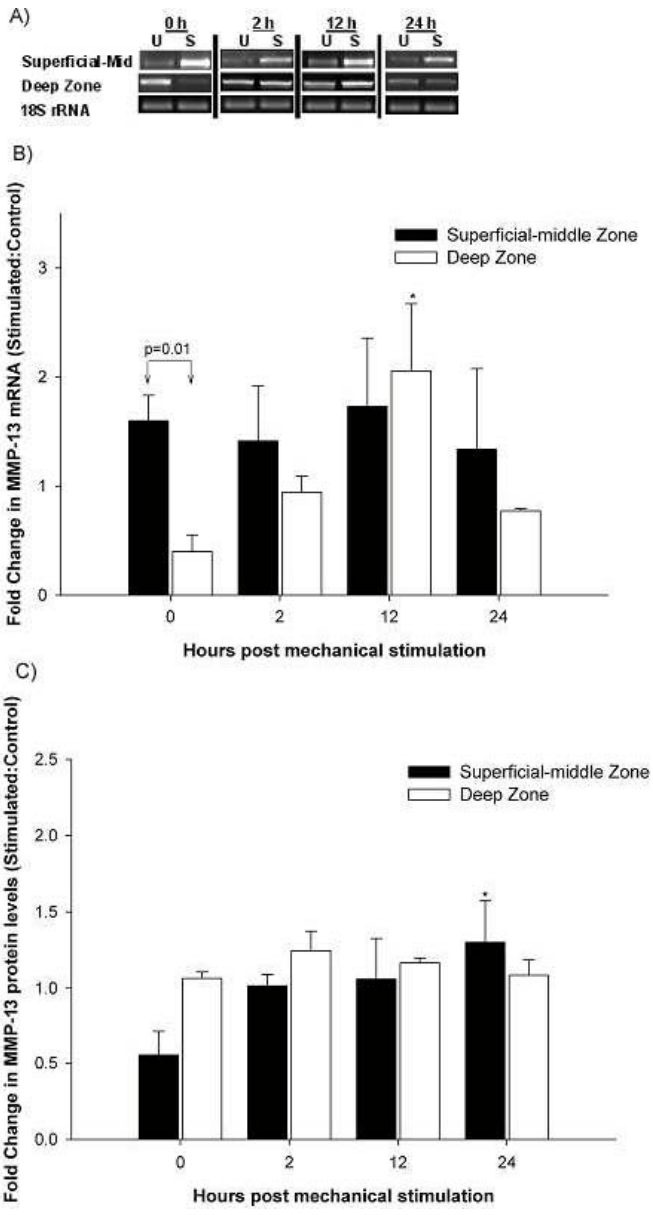
B)


3000
2500
2000
1500
1000
500
0
p = 0.001
Superficial-Middle Zone
Deep Zone
Accumulation of Newly Synthesized Collagens ^{3}H (cpm/ug DNA)
*
#
Control
Stimulated
Treatment Condition


A.
Collagen type II expression stimulated/control fold change
Superficial-middle Zone
Deep Zone
Hours post mechanical stimulation
B.
Collagen type I expression stimulated/control fold change
Superficial-middle Zone
Deep Zone
Hours post mechanical stimulation
C.
Collagen type VI expression stimulated/control fold change
Superficial-middle Zone
Deep Zone
Hours post mechanical stimulation
D.
Collagen type IX expression stimulated/control fold change
Superficial-middle Zone
Deep Zone
Hours post mechanical stimulation


3
2.5
2
1.5
1
0.5
0
Matrix Accumulation (stimulated/control)
Collagen
Proteoglycans
DZ
SMZ
Cell Population


A)
0 h
2 h
12 h
24 h
U S
U S
U S
U S
Superficial-Mid
Deep Zone
18S rRNA
B)
Fold Change in MT1-MMP mRNA (Stimulated:Control)
Superficial-middle Zone
Deep Zone
p=0.01
p=0.02
#
*
*
**
0
1
2
3
4
0
2
12
24
Hours post mechanical stimulation
C)
Fold Change in MT1-MMP Protein Levels (Stimulated:Control)
Superficial-middle Zone
Deep Zone
p =0.02
p <0.05
#
*
#
*
0
1
2
3
4
5
6
0
2
12
24
Hours post mechanical stimulation


A)
0 h
2 h
12 h
24 h
U S
U S
U S
U S
Superficial-Mid
Deep Zone
18S rRNA
B)
Fold Change in MMP-13 mRNA (Stimulated:Control)
Superficial-middle Zone
Deep Zone
p=0.01
*
0
1
2
3
0
2
12
24
Hours post mechanical stimulation
C)
Fold Change in MMP-13 protein levels (Stimulated:Control)
Superficial-middle Zone
Deep Zone
*
0.0
0.5
1.0
1.5
2.0
2.5
0
2
12
24
Hours post mechanical stimulation

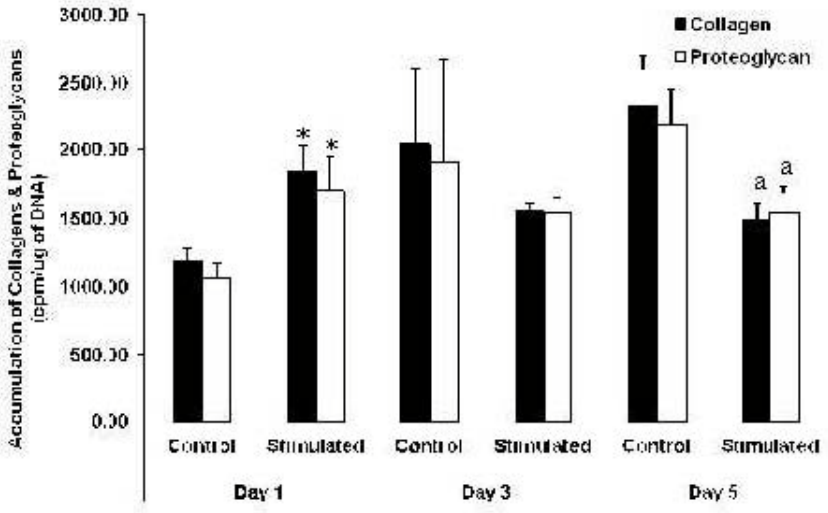

Accumulation of Collagens & Proteoglycans (cpm/ug of DNA)
3000.00
2500.00
2000.00
1500.00
1000.00
500.00
0.00
Collagen
Proteoglycan
Control
Stimulated
Control
Stimulated
Control
Stimulated
Day 1
Day 3
Day 5

Table 1. **DNA content of tissue formed by the chondrocyte subpopulations.** Chondrocytes were isolated from superficial-mid zone (SMZ), deep zone (DZ) or full thickness (FT) of cartilage and grown in culture for 3 days. The cells were mechanically stimulated as described under the Methods. DNA content was determined 24 hours after stimulation. The results from 3 experiments were pooled and expressed as mean ± standard error of the mean (n = 9). No significant difference in DNA content was observed between unstimulated (control) and stimulated tissues formed by the different chondrocyte populations.

Chondrocyte Population	Control tissue (μg of DNA/construct)	Stimulated tissues (μg of DNA/construct)
FT	9.04 ± 0.8	8.55 ± 1.4
SMZ	9.74 ± 2.0	7.35 ± 0.5
DZ	6.84 ± 1.4	8.48 ± 1.2

Reference List

1. Waldman SD, Grynpas MD, Pilliar RM, Kandel RA. Characterization of cartilagenous tissue formed on calcium polyphosphate substrates in vitro. J Biomed Mater Res. 2002;62:323-330.

2. 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;21:590-596.

3. Waldman SD, Couto DC, Grynpas MD, Pilliar RM, Kandel RA. A single application of cyclic loading can accelerate matrix deposition and enhance the properties of tissue-engineered cartilage. Osteoarthritis Cartilage. 2005.

4. De Croos JN, 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;25:323-331.

5. De Croos JN, Jang B, Dhaliwal SS, Grynpas MD, Pilliar RM, Kandel RA. Membrane type-1 matrix metalloproteinase is induced following cyclic compression of in vitro grown bovine chondrocytes. Osteoarthritis Cartilage. 2007.

6. Aydelotte MB, Greenhill RR, Kuettner KE. Differences between sub-populations of cultured bovine articular chondrocytes. II. Proteoglycan metabolism. Connect Tissue Res. 1988;18:223-234.

7. Aydelotte MB, Kuettner KE. Differences between sub-populations of cultured bovine articular chondrocytes. I. Morphology and cartilage matrix production. Connect Tissue Res. 1988;18:205-222.

8. Klein TJ, Schumacher BL, Schmidt TA, Li KW, Voegtline MS, Masuda K *et al.* Tissue engineering of stratified articular cartilage from chondrocyte subpopulations. Osteoarthritis Cartilage. 2003;11:595-602.

9. Siczkowski M, Watt FM. Subpopulations of chondrocytes from different zones of pig articular cartilage. Isolation, growth and proteoglycan synthesis in culture. J Cell Sci. 1990;97 (Pt 2):349-360.

10. Waldman SD, Grynpas MD, Pilliar RM, Kandel RA. The use of specific chondrocyte populations to modulate the properties of tissue-engineered cartilage. J Orthop Res. 2003;21:132-138.

11. Guilak F, Alexopoulos LG, Haider MA, Ting-Beall HP, Setton LA. Zonal uniformity in mechanical properties of the chondrocyte pericellular matrix: micropipette aspiration of canine chondrons isolated by cartilage homogenization. Ann Biomed Eng. 2005;33:1312-1318.

12. Lee DA, Noguchi T, Knight MM, O'Donnell L, Bentley G, Bader DL. Response of chondrocyte subpopulations cultured within unloaded and loaded agarose. J Orthop Res. 1998;16:726-733.

13. Poole AR, Kojima T, Yasuda T, Mwale F, Kobayashi M, Laverty S. Composition and structure of articular cartilage: a template for tissue repair. Clin Orthop Relat Res. 2001;S26-S33.

14. Kandel RA, Boyle J, Gibson G, Cruz T, Speagle M. In vitro formation of mineralized cartilagenous tissue by articular chondrocytes. In Vitro Cell Dev Biol Anim. 1997;33:174-181.

15. Pilliar RM, Filiaggi MJ, Wells JD, Grynpas MD, Kandel RA. Porous calcium polyphosphate scaffolds for bone substitute applications -- in vitro characterization. Biomaterials. 2001;22:963-972.

16. Gan L, Kandel RA. In vitro cartilage tissue formation by Co-culture of primary and passaged chondrocytes. Tissue Eng. 2007;13:831-842.

17. Kudirka JC, Panupinthu N, Tesseyman MA, Dixon SJ, Bernier SM. P2Y nucleotide receptor signaling through MAPK/ERK is regulated by extracellular matrix: involvement of beta3 integrins. J Cell Physiol. 2007;213:54-64.

18. Jiang J, Leong NL, Mung JC, Hidaka C, Lu HH. Interaction between zonal populations of articular chondrocytes suppresses chondrocyte mineralization and this process is mediated by PTHrP. Osteoarthritis Cartilage. 2008;16:70-82.

19. Alexopoulos LG, Setton LA, Guilak F. The biomechanical role of the chondrocyte pericellular matrix in articular cartilage. Acta Biomater. 2005;1:317-325.

20. Graff RD, Kelley SS, Lee GM. Role of pericellular matrix in development of a mechanically functional neocartilage. Biotechnol Bioeng. 2003;82:457-464.

21. Lai WM, Sun DD, Ateshian GA, Guo XE, Mow VC. Electrical signals for chondrocytes in cartilage. Biorheology. 2002;39:39-45.

22. Sanchez JC, Powell T, Staines HM, Wilkins RJ. Electrophysiological demonstration of Na+/Ca2+ exchange in bovine articular chondrocytes. Biorheology. 2006;43:83-94.

23. D'Andrea P, Vittur F. Propagation of intercellular Ca2+ waves in mechanically stimulated articular chondrocytes. FEBS Lett. 1997;400:58-64.

24. Powers CJ, McLeskey SW, Wellstein A. Fibroblast growth factors, their receptors and signaling. Endocr Relat Cancer. 2000;7:165-197.

25. Zhu Y, Oganesian A, Keene DR, Sandell LJ. Type IIA procollagen containing the cysteine-rich amino propeptide is deposited in the extracellular matrix of prechondrogenic tissue and binds to TGF-beta1 and BMP-2. J Cell Biol. 1999;144:1069-1080.

26. Hildebrand A, Romaris M, Rasmussen LM, Heinegard D, Twardzik DR, Border WA *et al.* Interaction of the small interstitial proteoglycans biglycan, decorin and fibromodulin with transforming growth factor beta. Biochem J. 1994;302 (Pt 2):527-534.

27. Clancy RM, Rediske J, Tang X, Nijher N, Frenkel S, Philips M *et al.* Outside-in signaling in the chondrocyte. Nitric oxide disrupts fibronectin-induced assembly of a subplasmalemmal actin/rho A/focal adhesion kinase signaling complex. J Clin Invest. 1997;100:1789-1796.

28. Choi JB, Youn I, Cao L, Leddy HA, Gilchrist CL, Setton LA *et al.* Zonal changes in the three-dimensional morphology of the chondron under compression: the relationship among cellular, pericellular, and extracellular deformation in articular cartilage. J Biomech. 2007;40:2596-2603.

29. Sun Y, Kandel R. Deep zone articular chondrocytes in vitro express genes that show specific changes with mineralization. J Bone Miner Res. 1999;14:1916-1925.

30. Yellowley CE, Jacobs CR, Donahue HJ. Mechanisms contributing to fluid-flow-induced Ca2+ mobilization in articular chondrocytes. J Cell Physiol. 1999;180:402-408.

31. Yellowley CE, Hancox JC, Donahue HJ. Effects of cell swelling on intracellular calcium and membrane currents in bovine articular chondrocytes. J Cell Biochem. 2002;86:290-301.

32. Hall AC. Differential effects of hydrostatic pressure on cation transport pathways of isolated articular chondrocytes. J Cell Physiol. 1999;178:197-204.

33. Roberts SR, Knight MM, Lee DA, Bader DL. Mechanical compression influences intracellular Ca2+ signaling in chondrocytes seeded in agarose constructs. J Appl Physiol. 2001;90:1385-1391.