Biomechanics of the Single Chondrocyte and its Developing Extracellular Matrix

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ABSTRACT

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Degradation of articular cartilage results in poor joint movement and afflicts millions of patients each year. Since this tissue is incapable of self-repair, developing new approaches to treat injured cartilage would be a tremendous boon to patient quality of life, as well as have important economic ramifications. To address this debilitating condition, this thesis investigated the biomechanical nature of single chondrocytes and studied their emergent biophysical environment.

Detailed insight into the role of intracellular structures on chondrocyte mechanical characteristics is a vital first step in understanding the etiology of cartilage degradation and identifying potential treatments. This thesis demonstrated that actin, intermediate filaments, and microtubules each play a unique function in cellular compressive stiffness, Poisson’s ratio and its strain dependence, as well as recovery behavior in response to a range of applied strains. The in situ stiffness of the nucleus was found to be minimally greater than that of the cytoplasm, countering current theories in chondrocyte biomechanics and identifying a potential new avenue for mechanotransduction. A videocapture method was also developed to examine the response of single chondrocytes to direct shear, whose results were further correlated with alterations in actin and focal adhesions.
This thesis then examined the effect of two key components of cartilage regenerative processes, phenotypic modulation and growth factors, on cellular mechanics. A 'mechanical range' was observed for single cells along a chondrogenic lineage and a subpopulation of differentiated stem cells was identified with similar characteristics as chondrocytes. Moreover, growth factors were found to induce changes in chondrocyte stiffness and volumetric properties.

The second major component of this thesis examined the developing biophysical milieu of chondrocytes. Through a novel 'self-assembly' tissue engineering approach, the evolving matrix composition and mechanical properties of cartilage neotissue were examined. Moreover, several notable similarities were identified between tissue maturation in self-assembled cartilage and known developmental processes for native tissue.

This thesis sheds light on how chondrocytes respond to physicochemical stimuli, the role of biophysical factors in the maintenance of the cellular phenotype, and the composition of the emergent chondrocyte environment. This work can greatly aid researchers toward developing effective treatments for deteriorated cartilage.
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INTRODUCTION

Articular cartilage is a highly functional tissue that covers the ends of long bones and serves to ensure proper joint movement. Due to its lack of vasculature, cartilage is unable to adequately repair itself during injury, leading to a debilitating condition known as osteoarthritis that afflicts millions of Americans each year. Assessment of the mechanical properties and behavior of single chondrocytes is an important first step in understanding tissue level responses and the etiology of osteoarthritis. Information on how chondrocytes respond to various forces, which are prevalent within their own microenvironment, will greatly aid researchers in developing functional tissue replacements for deteriorated cartilage. Correspondingly, studying the maturation of cartilage neotissue in vitro yields valuable information regarding the emergent biophysical environment of single chondrocytes and elucidates ideal intervention windows for biochemical or biomechanical stimulation. Thus, toward establishing effective treatments for cartilage degradation, the objectives of this thesis were to analyze the biomechanical characteristics of single chondrocytes and to study their developing pericellular and extracellular matrices.

These two separate objectives were achieved under the following governing hypotheses. First, biomechanical or biochemical stimuli may alter the mechanical characteristics of single chondrocytes. Second, chondrocyte biomechanical properties and behavior are modulated by intracellular structural components. Third, extracellular matrix maturation in our 'self-assembling' tissue engineering approach would mirror the known developmental process of articular cartilage. To test these hypotheses, the following three specific aims were developed:
1. **To study the contribution of intracellular structures to the biomechanical characteristics of single chondrocytes.** Consisting of three independent studies, this aim specifically investigated the role of the cytoskeleton, nucleus, and focal adhesions in the biomechanical properties and behavior of single chondrocytes. First, the influence of the primary three cytoskeletal elements on the morphology, compressibility, stiffness, and recovery behavior characteristics of single chondrocytes in response to a range of applied compressive strains was assessed. *It was hypothesized that each cytoskeletal component would contribute differentially to the compressive properties and behavior of single chondrocytes, based on known dissimilarities in their structure and spatial orientation within chondrocytes.* Next, the *in situ* mechanical properties of the nucleus and cytoplasm were determined through finite element modeling of compressive axial and lateral strains. Based on the previously observed nearly one-to-one correlation of cellular and nuclear strains, *it was hypothesized that nuclear stiffness is similar to that of the cytoplasm.* In the third study, a videocapture method was developed to examine how single cells responded to direct shear. *This study hypothesized that cells would exhibit shear strain-dependent morphological and biomechanical characteristics, which further correlate with changes in the focal adhesion or actin cytoskeleton organization.*

2. **To examine the effects of cartilage engineering processes on cellular mechanics.** This aim seeks to understand how two vital methodologies relevant to cartilage tissue engineering, chondrogenesis and growth factor stimulation, affect cellular biomechanics. In the first study, the viscoelastic and morphological
properties of chondrogenically differentiated embryonic stem cells were compared to those of single cells along a chondrogenic lineage. *It was hypothesized that a differentiated stem cell subpopulation could be identified which was similar to native chondrocytes in biomechanical characteristics.* In the second study, chondrocytes were incubated with either TGF-β1 or IGF-I, which are frequently employed to augment functional tissue properties. The mechanical properties and recovery behavior of single chondrocytes were subsequently measured over a range of applied compressive strains to assess the hypothesis that chondrocytes would exhibit strain-dependent and growth factor-dependent characteristics.

3. **To study matrix development of self-assembled engineered cartilage.** Using a novel self-assembly approach for engineering articular cartilage, the maturing biophysical environment of chondrocytes was examined. Changes in specific collagen and glycosaminoglycan (GAG) extracellular matrix components were characterized up to 8 weeks in culture and correlated with the developing compressive and tensile properties of the neotissue. This aim was performed in two phases: the first was to study mechanisms of cellular aggregation during early stages of neotissue development and to screen for time points to be used in the second phase, which then examined maturing structure-function relationships. *The hypotheses of this aim were that: (a) the temporal distribution of collagen types II and VI and chondroitin 4- and chondroitin 6- sulfate would be similar to that of typical articular cartilage development; and (b) changes in construct compressive*
and tensile mechanical properties would correlate with modulating levels of specific GAG and collagen types, respectively.

The following chapters explain the experiments and studies related to the specific aims of this thesis. Chapter 1 is a review of current cartilage research on the single cell level and seeks to motivate the research set forth in each of the aims. This chapter first discusses the structure-function relationships and developmental characteristics of articular cartilage, which can be used as benchmarks for future cartilage regeneration endeavors. The micromechanical environment of the chondrocyte was subsequently described in detail, with an emphasis on the mechanical models and experimental techniques utilized to examine its physical characteristics. This information was then related to changes in cellular behavior and its potential toward tissue engineering articular cartilage. It is of specific importance to examine the thresholds for mechanical perturbation which may precipitate regenerative or degenerative cellular changes and, thus, provide valuable information regarding the loading regimens facilitating tissue construct growth.

Chapters 2, 3 and 4 present work conducted toward the completion of Specific Aim 1. The overall objective of Chapter 2 was to examine the contribution of actin, intermediate filaments, and microtubules to the compressive biomechanical characteristics of single chondrocytes. Cells were incubated with cytoskeletal disrupting agents to target each of the aforementioned cytoskeletal components and then tested singly over a range of applied axial strains for changes in their stiffness, compressibility, and recovery behavior. Chapter 3 complements Chapter 2 by studying the in situ mechanical properties of the chondrocyte nucleus. Through finite element modeling,
cellular and nuclear strains were utilized to determine the optimal material properties for the nucleus and cytoplasm under compression, as well as elucidate the specific contribution of the nucleus to the overall biomechanics of the chondrocyte. In Chapter 4, a videocapture method for examining the biomechanical and morphological response of single chondrocytes to direct shear was developed. Through this novel approach, precise force and deformation measurements were obtained during the shear event and analyzed as functions of the initial cell to probe contact configuration. The intracellular organization of the actin cytoskeleton and focal adhesions under mechanical shear were further investigated toward understanding the response of single chondrocytes to this applied shear force.

Chapters 5 and 6 discuss the research conducted in Specific Aim 2. These chapters focus on the biomechanical changes of single chondrocytes during two processes germane to cartilage regeneration efforts: cellular differentiation and growth factor enhancement. Classical tissue engineering paradigms involve a modular approach that entails the differentiation of an alternative cell source, such as embryonic stem cells, and subsequent biochemical or biomechanical stimulation to promote robust extracellular matrix production. As such, Chapter 5 of this thesis examined the biomechanical characteristics of embryonic and mesenchymal stem cells, as well as native chondrocytes, to yield a cartilage-specific spectrum of mechanical properties for stem cell differentiation. Embryonic stem cells were also chondrogenically-differentiated and purified based on their ensuing densities, with the goal of identifying a subpopulation of differentiated cells with similar mechanical characteristics as native chondrocytes. Chapter 6 then investigated the effect of two cartilage-relevant growth factors, TGF-β1
and IGF-I, on the biomechanical properties and behavior of single chondrocytes, using similar end-point analyses as described in Chapter 2.

Chapter 7 describes the work performed in Specific Aim 3. Within this chapter, the development of self-assembled engineered cartilage neotissue was thoroughly examined, with an emphasis on the emergent chondrocyte microenvironment. While previous studies in our laboratory have focused on the end functionality of tissue constructs, it is of great interest to study the maturation of these constructs to identify intervention strategies employing exogenous stimulation or implantation time points for \textit{in vivo} work. A specific focus was placed on the spatiotemporal profile of various collagen and GAG types, as well as the process of cellular aggregation. The observed changes in extracellular matrix components were further related to the compressive and tensile mechanical properties of the neotissue to provide insight on the invaluable structure-function relationships during neotissue growth.
CHAPTER 1: Micromechanical properties of chondrocytes and chondrons: relevance to articular cartilage tissue engineering*

Abstract

Articular cartilage is a highly mechanical tissue, performing multiple functions to ensure proper joint movement. Degradation of this tissue may be due to improper loading conditions that lead to a debilitating condition known as osteoarthritis. Furthermore, it is believed that mechanical signals transmitted from the tissue to cellular levels are necessary for the production of essential extracellular matrix components responsible for cartilage viability. Examinations of the tissue on its most rudimentary level will elucidate mechanical regimens related to cartilage health and disease. A ‘fundamental unit approach’ has been employed to study the biomechanical properties of single cells with discrete pericellular and extracellular matrix layers. This approach enables researchers to develop definitive relationships between mechanical stimulation and changes in gene expression corresponding to regenerative or catabolic processes. The knowledge gained from these studies will shed light on the etiology of osteoarthritis and elucidate the mechanical loading regimens useful for promoting articular cartilage health. This chapter article discusses the micromechanical environment of the cartilage cell, the chondrocyte,

and the mechanical models and experimental techniques utilized to examine its physical characteristics. This information is then related to changes in cellular behavior and its potential toward tissue engineering articular cartilage.
Introduction

Articular cartilage is the load-bearing material lining diarthrodial joints. It is a specialized type of hyaline cartilage and a highly versatile tissue, serving multiple functions to ensure proper joint movement. Together with the synovial fluid, it provides a lubricating and wear resistant surface, facilitating nearly frictionless motion about the articulating joints of our body. In addition, articular cartilage works to resist and distribute high compressive loads from one subchondral bone to another. Due to its biomechanical nature, articular cartilage health is largely determined by a variety of mechanical factors. It is well known that mechanical forces can elicit particular changes in the viability of articular cartilage, from the cellular to tissue levels.1-13 Mechanical stimuli can induce modulations in cartilage tissue metabolism, in either a catabolic or anabolic manner. Therefore, the goal of much current research is to elucidate specific regimens of mechanical forces that will lead to the growth and strengthening of articular cartilage.

Under conditions of improper joint loading, the signaling pathways leading to normal gene regulation may not be stimulated correctly, and articular cartilage will degenerate. It has been shown that an abnormal mechanical environment within the tissue, created in cases of high joint impact or due to an accumulation of small repeated loading events, significantly affects cellular behavior and may result in pathological extracellular matrix (ECM) synthesis and apoptosis.14-20 These detrimental tissue changes lead to a condition known as osteoarthritis, which afflicts millions of Americans and significantly affects the United States economy.21
This chapter will begin by briefly discussing the physiological and structural properties of articular cartilage and the need to pursue tissue engineering techniques in order to treat patients with osteoarthritis. If the reader is interested, a more compressive discussion of articular cartilage physiology can be found in several excellent reviews.\textsuperscript{22-24} Due to the heterogeneous nature of the tissue, it is important to individually look at articular cartilage's most fundamental units in order to develop definitive relationships between applied stimuli and changes in cellular behavior. Therefore, to formulate effective treatment methodologies for osteoarthritis, it is critical to first study articular cartilage on its most basic level, the \textit{chondrocyte} cell, so that regulatory mechanisms within cartilage can be directly correlated with mechanical factors. Researchers may then move upwards toward the tissue level, building upon single cell research by including discrete ECM regions, to elucidate on all fronts the role of mechanical stimulation in cartilage health and disease. As such, this chapter will then primarily focus on the mechanical environment necessary to bring about a favorable response from the cell and the \textit{chondron}, the cell within its local microenvironment; and its tissue engineering implications.

The single cell, chondron, microexplant, and tissue construct are metabolic units of increasing complexity. This chapter utilizes the concept of a 'fundamental unit approach' to demonstrate how research conducted at each of these phases may be interrelated. The fundamental unit approach is a modular template that facilitates research and understanding of these phases and consists of \textit{modeling}, \textit{experimental validation}, and \textit{stimulating} the unit of interest. Studies regarding single chondrocytes and chondrons will be presented in this manner. Information gained from this research can be used in the
formulation of necessary treatment methodologies, such as tissue replacement strategies, for patients suffering from osteoarthritis.

Articular cartilage structure and tissue engineering

Heterogeneous tissue properties

Articular cartilage tissue is non-uniform in its composition, varying in terms of matrix components, matrix organization, cellular phenotypes and organization. It is largely composed of chondrocytes sparsely interdispersed within an intricate network of collagen fibrils, proteoglycans, lipids, and various ionic and non-ionic solutes. Due to the inherent heterogeneity of articular cartilage, chondrocytes in one location may sense and respond to forces differently than other cells. Therefore observations of the tissue on the bulk level may not accurately reflect the intrinsic phenotypic and morphological differences existing within various subpopulations of chondrocytes in cartilage. As will be further discussed in detail, chondrocytes perceive stimuli within their individual mechanical microenvironments. To begin with, an understanding of articular cartilage structural properties is necessary to appreciate the scope of the cellular microenvironment and grasp the heterogeneous nature of this tissue.

Articular cartilage is often considered as consisting of both a solid and fluid phase. The interaction and arrangement of these various tissue components and phases provide articular cartilage with its unique functional capabilities. The primary component of articular cartilage is interstitial water that accounts for 60-85% of its wet weight. Water and various electrolytes in the tissue are denoted as the fluid phase. Collagens,
particularly type II collagen, make up approximately 50% - 75% of the solid phase and are responsible for the tensile characteristics of the tissue.\textsuperscript{23} Proteoglycans and other glycoproteins compose a majority of the remaining solid phase,\textsuperscript{22} and contribute to the compressive and flow-dependant viscoelastic properties of articular cartilage.\textsuperscript{26} Proteoglycans are a highly specific type of glycoprotein, containing long, unbranched, negatively charged chains of glycosaminoglycans (GAGs) attached to a central link protein. Aggrecan is the most predominant proteoglycan in articular cartilage.\textsuperscript{22,23}

The spatial arrangement of collagens and proteoglycans has been shown to affect cellular metabolism.\textsuperscript{27} The thickness of the collagen fibers is determined by a fixed charge density created by the adjacent GAG chains.\textsuperscript{28} The interaction of electrostatic charges from the GAGs and cations from the fluid phase contribute to the regulation of interstitial water content through a swelling pressure following the Donnan osmotic pressure law.\textsuperscript{29} The swelling generated by the resulting outward osmotic pressure is fettered by the cross-linked network of collagen and aggregan.\textsuperscript{30} When articular cartilage is compressed, fluid exits the tissue and experiences a drag via osmotic pressure. This drag enables the tissue to deform viscoelastically and act as a damper and distributor of applied forces.

Articular cartilage consists of four distinct zones: superficial, middle, deep, and calcified layers. Each layer is known to vary in matrix composition, and cellular, metabolic and mechanical properties.\textsuperscript{12,31-39} Transitioning from the superficial layer to the deep layer, water content decreases and collagen fiber alignment changes from a tangential orientation to a radial direction along the tissue.\textsuperscript{22,23,40} The superficial layer is the outermost level of cartilage, encompassing the upper 10-20% of the tissue, and is
characterized by a high tensile strength due to the tangential alignment of collagen fibrils. Chondrocytes in this region are stiffest and produce a specific superficial zone protein that aids in providing articular cartilage with its lubricating surface and prevents undesirable cell adhesion in this region. The middle layer contains more rounded chondrocytes and the greatest proteoglycan levels compared to the other layers. The deep layer is the thickest region of articular cartilage, although it contains the lowest amounts of interstitial fluid and collagen. Despite their low abundance in the deep layer, collagen fibers in this region are the greatest in diameter and function as a connection between articular cartilage and the underlying subchondral bone. The high compressive mechanical properties of this region can also be attributed to the radial orientation of these fibers. Lining the underside of the deep layer is a tidemark, conspicuously separating the region from the calcified layer below. In this final layer, chondrocytes are mostly inert and sheathed within a calcium fortified milieu.

With the cell as the center, distinct matrix divisions also exist concentrically within articular cartilage. Extending radially from the cell, pericellular, territorial, and interterritorial matrices each play a role in chondrocyte maintenance and articular cartilage function. The ECM region consists of both the territorial and interterritorial matrices and is known to contain the vast majority of collagen and aggrecan proteins in cartilage tissue. The pericellular matrix (PCM) is most prominent in the middle and deep layers of the tissue and immediately surrounds the chondrocyte within a lacuna. The PCM, together with its enclosed cell, is defined as the chondron and is considered to be the smallest metabolic and functional unit of articular cartilage. Examinations of isolated chondrons have provided researchers with vast insight into the micromechanical
environment of the chondrocyte \textit{in vivo}. It has been found that the PCM is important in directly conveying biomechanical and biochemical stimuli on to the chondrocyte and will be discussed in greater detail later.

\textit{Articular cartilage development}

Articular cartilage is known to arise during an intricate process of joint development, first involving the formation of an interzone region through mesenchymal condensation at the future joint site. The interzone then separates into three cartilaginous tissue layers: two layers of perichondrium-like layers along the epiphyseal ends of the developing joint and one intermediate layer consisting of softer tissues. Articular cartilage begins to form with the chondrogenesis of mesenchymal progenitor cells located at the subsequent perichondrium regions. Growth at the epiphyseal ends will continue until the formation of the growth plates, which will become the primary source of self-renewing, proliferating chondrocytes.\textsuperscript{45}

An understanding of articular cartilage development is of great importance to researchers, as it will provide essential benchmarks for tissue growth \textit{in vitro}. The various collagen and GAG types, and their associated spatial arrangement, can be used as indicators for the ability of a myriad of tissue engineering approaches to recapitulate the different stages of the developmental process of articular cartilage\textsuperscript{46, 47} and the formation of functional replacements for diseased tissue. Moreover, the changing extracellular matrix composition during cartilage maturation will result in a different structural microenvironment surrounding individual chondrocytes,\textsuperscript{48} potential altering their local stress-strain environment and mechanical cues.
Role of cadherins

It has been reported that neural cadherin (N-cadherin), a calcium-dependent adhesion molecule, plays a substantial role in chondrogenesis and mesenchymal condensation. They are detected with a faint intensity at very early stages of joint development, with a sparse localization around the center of the presumptive limb. Then during the condensation phase for the mesenchymal cells, N-cadherin activity spikes to its highest level. The staining intensity for N-cadherins decreases around the central condensation region, as the cells spread apart and begin to differentiate into chondrocytes. At later stages of joint development, N-cadherin staining is only evident in the perichondrium and not in mature cartilage tissue.

A differential adhesion hypothesis has been proposed to explain the role of cadherins in cellular aggregation and tissue development. This theory postulates that tissues are viscoelastic liquids, whose basic structural elements are mutually adhesive cells which produce a measurable surface tension. Cadherin-cadherin interactions act to minimize the free energy of the population of cells by sorting cells with varying intracellular adhesiveness. In articular cartilage, the sorting of cells during the condensation phase of development may be a direct result of the relative cadherin activity amongst the mesenchymal cells.

Collagen maturation

Like many musculoskeletal tissues, articular cartilage undergoes significant changes within a developing embryo and fetus. Various collagen types are specifically and locally expressed, contributing to the physical characteristics of the nascent tissue.
The spatial arrangement of the different collagens further helps distinguish the various components of the emergent joint. Within the interzone, collagen types III and V predominate, with small amounts of type I collagen also present. A decrease in total collagen is also observed within the interzone as it develops, which decreases the region's tensile properties and enables the newly formed musculature to separate the epiphyseal ends and cause cavitation. Interestingly, collagen type II, which is a known marker for articular cartilage, is not found until later stages of development of the tissue, either in terms of mRNA expression or protein production. However, collagen type II is present in the underlying cartilaginous matrix past the developing epiphyseal region.

It has recently been observed that collagen type VI plays an essential role in the development of articular cartilage and tissue integrity. As previously mentioned, collagen type VI is localized within the PCM for mature tissue. However, this protein is present throughout matrix development and it has been suggested that it plays a key role in stabilizing other ECM proteins and tissue remodeling. Collagen type VI expression also spikes at chondrocyte differentiation, supporting the idea that the protein is essential to the formation of cartilage matrix. The steep rise in expression soon levels off at later stages of chondrogenesis, coinciding with an upregulation in collagen type II.

Soon after partuition, interterritorial collagen fibers can be unequivocally identified in the still developing tissue, pointing to the near complete formation of the ECM. Radial fibers from the central growth region now extend, in a parallel alignment, toward the tissue's surface. Moreover, these collagen fibers are tightly packed and appear to be continuous. Within the cellular microenvironment, a non-uniform fibrous capsule enclosed the cell, indicative of a PCM and mature chondrons. The fibers in this capsule
are noticeably thinner than those of running tangentially along the cartilage surface and radially toward the surface.\textsuperscript{59} A summary of the predominate collagen changes during articular cartilage development are delineated in Table 1.

**GAG maturation**

The various GAG types located on aggrecan also undergo noticeable changes during articular cartilage maturation. During embryogenesis, chondroitin-6-sulfate (CS-6) can be observed throughout the developing matrix, then from parturition onwards the staining intensity for the GAG increases uniformly.\textsuperscript{60} Conversely, chondroitin-4-sulfate (CS-4) is not very apparent in the developing ECM, yet is visible at parturition with a strong affinity to the pericellular region.\textsuperscript{60} The specific chondroitin sulfation patterns are also variable during cartilage growth post-parturition. While there are nearly equivalent amounts of CS-4 and CS-6 immediately post-parturition, CS-levels increase during tissue maturation as the percentage of CS-4 drops.\textsuperscript{61,62}

At birth, CS comprises nearly all of the GAG in articular cartilage; then decreases over time as KS chains are introduced onto aggrecan.\textsuperscript{60,62,63} KS chains can initially be identified pericellularly during prenatal development, but become evident throughout the ECM in mature tissue.\textsuperscript{60} Dermatan sulfate content also increases during cartilage development post-parturition\textsuperscript{64} and localizes predominately to the superficial zone.\textsuperscript{65} In addition, HA was found to increase in abundance with age in articular cartilage.\textsuperscript{63,66} The protein has also been shown to associate directly with collagen type VI,\textsuperscript{67,68} eluding to its role in matrix organization and development. A summary of the major GAG changes during cartilage development are summarized in Table 2.
Tissue engineering potential for articular cartilage

Though primarily serving a mechanical function, articular cartilage lacks the vasculature and lymphatic system to repair itself under conditions of wear and tear or traumatic injury.\textsuperscript{22, 23} Since articular cartilage is unable to naturally restore its original structure and functionality after damage, ample research has focused toward understanding the etiology of osteoarthritis and to tissue engineer fully functional neotissue as a replacement for diseased cartilage. In cases of severe articular cartilage pathology, tissue replacement may become necessary. In the laboratory, it is now possible to engineer cartilage of clinically relevant dimensions and properties,\textsuperscript{69-71} and this may have profound implications for orthopedics in the near future.

Due to the inherent biomechanical nature and function of articular cartilage, the majority of tissue engineer techniques employ various mechanical stimuli as promoters of ECM synthesis and tissue growth. While external loading regimens are applied onto the bulk tissue construct level, it is the chondrocyte itself which produces the proteins necessary for healthy and viable cartilage. Therefore, an understanding of chondrocyte mechanotransduction and their mechanical environment is paramount toward elucidating the ideal methodologies for stimulating cartilage tissue. As will be later discussed, forces and deformations applied onto the construct will precipitate down through the chondrocyte microenvironment and can effect gene expression. Therefore, when considering a tissue engineering approach to articular cartilage, it is critical to understand how mechanical signals are interpreted by the individual cell.

While unified by a realm of exogenous stimuli, such as mechanical factors, which modulate cartilage development and growth, current tissue engineering techniques can
themselves vary in their methods to coalesce isolated cells. Traditionally, tissue engineering techniques employ a degradable polymer scaffold upon which to seed a desired cell density. Since chondrocytes are already able to create relevant ECM elements, such as the collagen and GAG types found in cartilage, they immediately come to mind as the appropriate cell type to be used in tissue engineering articular cartilage. As the scaffold degrades, it is replaced by a natural matrix produced by the cells. Some common scaffold types include collagen, poly(DL-lactic acid) and poly(glycolic acid), and more recently poly(1,8-octanediol citrate), with promising results.

Despite the vast potential of scaffold-based tissue engineering approaches, they do include several notable drawbacks. Whenever dealing with degradable polymers, researchers must consider issues of biodegradability, degradation products’ toxicity, stress-shielding, and hindrance of cell-to-cell communication. To avoid such scaffold-related concerns, many scaffold-less approaches have been tested and utilized in the past few years. Autologous chondrocyte implantation is the most common technique of this sort and the leading method to treat osteoarthritic patients under the age of 55. In this procedure, the defected cartilage region is cleared of debris and covered by a periosteum flap. The surgeon then injects a high density of the patient’s own chondrocyte cells underneath flap where they may develop into healthy cartilage tissue. In contrast, scaffold-less procedures have also been employed to grow articular cartilage outside the body with the goal of then transplanting the neotissue into the defective region. Pellet culture and aggregate culture techniques have been implemented to grow articular cartilage constructs and tested under various mechanical conditions. Another scaffold-less approach, known as the self-assembling process, has recently been validated by Hu
and Athanasiou\textsuperscript{69} to produce cartilage tissue constructs with biomechanical and biochemical properties nearing those of native tissue. This novel approach, which involves seeding of primary chondrocytes at high-density over agarose wells, can be employed using various molds to create the desired shape for an articular cartilage implant.

Fundamental unit approach

Elucidating the mechanical properties of single cells and chondrons and of their behavior in response to mechanical stimuli will provide great insight into the most rudimentary level of articular cartilage. This is a necessary first step toward tissue engineering articular cartilage and understanding the regimens of mechanical stimuli needed to elicit favorable gene responses, in terms of ECM production and cellular proliferation, from within a tissue construct. Information on how chondrocytes respond to various forces within their own microenvironment will greatly aid researchers toward developing a functional tissue replacement for deteriorated cartilage in patients suffering from osteoarthritis.

The majority of previous studies investigating chondrocyte mechanotransduction have looked at the mechanical response of an entire population of cells in their ECM. Though these experiments have been essential in furthering our knowledge of mechanotransduction and of the influence of mechanical stimuli on cellular behavior, they are subject to significant limitations. Most notably, testing cells in a bulk manner does not take into consideration variables such as individual cell shape, position, orientation, or local ECM characteristics. Cells within the same population may
experience different stress-strain patterns under similar testing modalities. Therefore, the observed response may not accurately reflect a true response to the presumed applied force. Moreover, past research has strongly suggested that an individual chondrocyte responds to mechanical stimuli within its local environment, as opposed to the aggregate mechanical environment of the whole tissue.\textsuperscript{78,79}

To overcome such drawbacks, a fundamental unit approach can be utilized, wherein mechanical forces are applied to a single metabolic unit and the resulting changes in cellular behavior are recorded. By applying a well-defined force and recording the subsequent changes in the same unit, definitive relationships between mechanical stimuli and their intracellular effect can be developed. Therefore, the effects of the magnitude, duration, and frequency of a certain load regimen application can be examined unequivocally. This approach presents the additional advantage of uncovering the direct role of biomechanical forces as potential stimulators of tissue healing or degeneration via observation of particular changes in gene regulation related to regenerative, catabolic, or apoptotic cell behavior (Fig. 1). Forces precipitating the onset of osteoarthritis may be observed and studied to further our knowledge of this degenerative disease. Similarly, loading regimens inducing favorable gene responses can be easily identified and utilized in further work to develop articular cartilage neotissue. Furthermore, mechanical stimuli may also be examined in conjunction with additional exogenous factors, such as growth factors or oxygen tension levels. Combinations of these factors have exhibited a synergistic effect on chondrocyte metabolism and may prove to be quite useful in tissue engineering articular cartilage,\textsuperscript{80-84} yet are beyond the scope of this chapter.
While examinations on a single cell level present several distinct advantages in tissue engineering, it is not without its own caveats. The response of individual cells seeded on a dish can potentially be quite different to that of cells interdispersed within an extracellular matrix or polymer scaffold. In these situations, which are more related to the chondrocyte’s environment in vivo, mechanical stimuli are not directly applied onto the cell but, rather, are a result of a secondary response, such as gel compression or shear due to induced fluid flow. Therefore, the methods used to stimulate and mechanically test individual cells, which will be discussed in great detail later, can never fully reproduce the true mechanical environment within articular cartilage. It is further known that cells within articular cartilage are in constant communication with each other and these molecular signals can also effect chondrocyte behavior and gene response. However, it should be noted that single cell research is simply the first step in a modular approach to tissue engineer articular cartilage. The research performed with individual cells can provide great insight into the mechanical environment necessary to elicit favorable cellular responses, which can then be translated upwards toward the tissue level. Findings which elucidate loading parameters favorable to the individual cell can be utilized in subsequent phases, which will eventually involve cells embedded within a matrix. This knowledge may include the ideal type of loading regimen (hydrostatic pressure, direct compression, shear, etc.) and their corresponding force levels, applied onto the tissue construct, which will bring about the production of essential matrix components from the cells.

The fundamental unit approach to tissue engineer articular cartilage consists of four interrelated phases, single cells, single chondrons, microexplants, and tissue
constructs, each thoroughly examining the mechanical behavior via increasingly complex models and using a series of steps that are translatable from one phase to the next. Each phase contains three main steps. Researchers first need to develop mathematical models for the properties and characteristics of each unit. These models then need to be validated through direct experimental techniques. Finally, various combinations of the previously described exogenous factors can be applied to the single unit in order to determine ideal stimulation levels to produce a well-developed neotissue (Fig. 2). Moving stepwise through each phase, researchers could carry this knowledge and adapt it to account for the additional PCM and ECM regions to reach desired levels for gene expression. Advanced mechanical modeling will enable researchers to correlate the applied stresses or strains back down to the cellular level for each phase. Multi-scale models have recently been developed to account for the intrinsic matrix variation and cellular spatial arrangement within articular cartilage. These models can be utilized to predict the local mechanical environment of chondrocytes under various loading conditions. Moreover, models used to examine the properties of individual cells can be translated to the tissue level by maintaining the same underlying physical principles and assumptions governing cellular properties. With each added level of complexity, the mechanical model for chondrocytes will not change, enabling researchers to translate these properties to different spatial scales. Therefore, those mechanical loading regimens which elicit the upregulation of ECM genes can be applied and tailored to each subsequent phase in the approach, in order to work toward the greatest ECM production due to stimulation at the tissue level. The fundamental unit approach provides researchers the indispensable tool of
translating the effect of mechanical forces back down to the cellular level and using this indispensable knowledge to develop strong and healthy cartilage tissue in vitro.

Cellular microenvironment

To accurately model the mechanical characteristics of single cells and chondrons, an appreciation of the cellular microenvironment is necessary. This is an essential component of the first step of the fundamental unit approach and will enable researchers to develop appropriate mechanical models of increasing complexity describing each matrix layer surrounding the cell, as well as the cell itself. All components within the immediate surroundings of the chondrocyte can potentially act as force transducers onto the cell and affect cellular transcriptional changes. Mechanical forces applied onto cartilage tissue are conveyed down to the subcellular levels via linkages between the ECM, PCM, cytoskeleton, and nuclear lamina. The cellular microenvironment consists of the latter three connections, which serve as the principal mechanical components of the chondron.

PCM composition and function

The chondron, the chondrocyte together with its PCM, has been studied for years and much progress has been made to establish its structure. The PCM is known to contain collagen types II, VI, and IX, and significant concentrations of decorin, hyaluronan, and sulphated glycosaminoglycans. Of the aforementioned proteins, type VI collagen is considered a key molecular marker of chondron microanatomy and is
essential in maintaining the microenvironment of the cell. The glycoprotein fibronectin is also localized within the pericellular region in adult articular cartilage\textsuperscript{94} and has been shown to interact directly with type VI collagen to create a scaffold for the assembly of other essential PCM proteins.\textsuperscript{96}

The biochemical and biomechanical contributions of the PCM to the chondrocyte microenvironment have also been studied. In particular, it is known that the PCM surrounding the chondrocyte organizes and constructs collagen fibrils,\textsuperscript{44} regulates cellular osmolarity,\textsuperscript{97} and modulates growth factor interactions with the enclosed cell.\textsuperscript{98} The mechanical properties of the PCM are known to differ from the larger territorial and interterritorial matrices inside the cartilage tissue and is approximately 10-fold greater in stiffness than the enclosed chondrocyte.\textsuperscript{78, 99-102} In this manner, the PCM acts as a biomechanical buffer of stresses applied onto the chondrocyte. Considering these biochemical and biomechanical functional roles, it is no surprise that the PCM has received special attention in tissue engineering of articular cartilage and toward maintaining a healthy, well-developed ECM.\textsuperscript{47, 71, 103, 104}

\textit{Cytoskeleton}

The cytoskeleton provides a framework for cellular structure and plays a key role in the mechanical characteristics of the cell. It is primarily composed of three main structural proteins: microtubules, microfilaments, and intermediate filaments.\textsuperscript{105} Understanding the properties and behavioral changes of cytoskeletal components is important toward discerning the mechanobiology of individual chondrocytes.
Cytoskeletal components

Microtubules extend over the entire cytoplasm of the cell, forming a scaffold upon which other cytoskeletal elements can branch out.\textsuperscript{106} Due to their omnipresence, microtubules function to support cellular shape and the movement of organelles. They are also the largest fibers of the cytoskeleton and consist of hollow cylindrical tubes of the globular protein tubulin.\textsuperscript{105}

Intermediate filaments span the entirety of the cell and serve primarily to mechanically link the nuclear lamina with integrin receptors on the cell surface.\textsuperscript{107} They are considered the most stable of cytoskeletal elements, comprising of protofilaments or fibrous proteins. Intermediate filaments are also involved in providing additional support in maintaining cell shape and structure.\textsuperscript{105}

Microfilaments, which are the smallest of cytoskeletal elements and are composed of two threaded polymer chains of F-actin, are involved primarily in cell motility.\textsuperscript{105} These proteins are located cortically, with many focal adhesion points along the cell membrane. In addition, microfilaments are important in resisting cell deformation due to applied mechanical forces, particularly shear stresses.\textsuperscript{108}

Tensegrity model

The organization of the cytoskeleton has been described using a tensegrity model.\textsuperscript{109-112} This approach attempts to explain cellular structure and behavior by modeling the cytoskeleton as an interwoven mesh of discrete compressive and tensile elements in a three-dimensional configuration. Applying the tensegrity model to the cytoskeleton, the microtubules serve as struts which resist compression and can reorient
their position due to prestresses produced by the contractility of both microfilaments and intermediate filaments. The various mesh elements can be anchored to each other or the ECM. Applied stresses are distributed and transmitted through this lattice, depending on the preferred directionality of the mechanical coupling between elements.\textsuperscript{111}

\textbf{Response to mechanical forces}

It is well believed that the cytoskeleton plays a direct role in mechanotransduction within the cell.\textsuperscript{110, 112, 113} The structure and organization of cytoskeletal proteins has been shown to have a strong influence in force transmission onto the nucleus\textsuperscript{110} and the mechanical properties of the chondrocyte as a whole.\textsuperscript{114, 115} It has further been reported that the cytoskeleton remolds and thickens in response to applied mechanical loads,\textsuperscript{106, 116, 117} suggesting that these organizational or conformational changes may effect mechanical signal transduction within the cell, and thereby alter the regulation of essential ECM proteins.\textsuperscript{117}

\textit{Nucleus}

The mechanical properties of the chondrocyte’s nucleus are also of great interest. The chondrocyte nucleus has been described a viscoelastic structure,\textsuperscript{118} containing both solid and fluid elements. It is known to be considerably stiffer than the cell itself, potentially indicating a unique nuclear function to mediate mechanical forces with the enclosed genomic DNA. Moreover, it has been shown that the nucleus will respond, in terms of its physical dimensions and volumetric properties, to an induced strain applied
on the cartilage tissue level\textsuperscript{119} and that these changes correlate with the synthesis of certain ECM proteins.\textsuperscript{120}

The nucleus is supported by a filamentous meshwork known as the nuclear lamina, positioned underneath the nuclear envelope. This lamina provides a framework for nuclear structure and serves as an intermediary between the cytoskeletal network and chromatin.\textsuperscript{121} Hence, deformation of the nucleus is often seen as the most direct transducer of cellular mechanotransduction. Alterations in the structural characteristics of the nucleus can cause changes in chromosome alignment via the lamin network, affecting the accessibility of genomic sequences to various transcriptional factors or other molecular signals.

**Single cell approach**

While studies of cartilage mechanobiology can quantify stress and strain at the bulk tissue level, few studies have attempted to quantify the local mechanical environment around the cell. Doing so would help to identify levels of mechanical stimuli at the cellular level relevant to cartilage health and disease.\textsuperscript{122} By applying different mechanical models and making the necessary geometric and physical assumptions, researchers can describe with growing accuracy the mechanical characteristics of single cells. This first phase of the fundamental unit approach consists of mathematical modeling, validating these models through direct experimentation, and determining appropriate mechanical loading regimens to elicit favorable gene responses on the single cell level.
Cellular mathematical modeling

Three models, of increasing complexity, have been used in recent literature to determine the mechanical properties of individual chondrocytes: (1) punch model; (2) viscoelastic model; and (3) linear biphasic model. While all arriving at different constitutive relations between stress and strain within the continuum, all of the models have been developed using basic physical principles and can accurately predict the equilibrium deformation of a cell due to an applied force. The various models serve as a foundation for later mathematical models describing more complex situations such as with the chondron or tissue microexplants.

Punch model

The punch model is one of the simplest in single cell mechanics, treating the cell as a linearly elastic, homogeneous, isotropic, and incompressible half-space. Although the punch model does not account for strain- or time-dependant responses by the cell, it is useful in ascertaining the elastic modulus of stiffer, anchorage-dependant cells, namely chondrocytes. It has been applied in both the testing modalities of cell indentation and micropipette aspiration to determine the mechanical properties of single cells.

The solution to indentation experiments can be obtained by solving Cauchy’s equations of motion under conditions of axial symmetry. Neglecting inertial forces, the equations governing stress distributions reduce to:

\[
\frac{d\sigma_{rr}}{dr} + \frac{d\sigma_{\theta\theta}}{dr} + \frac{1}{r} (\sigma_{rr} - \sigma_{\theta\theta}) = 0
\]  

(1)
\[
\frac{d\sigma_r}{dr} + \frac{d\sigma_z}{dz} + \frac{\sigma_r}{r} = 0
\]  
(2)

Then by representing the stress components by a single function and applying the method of Hankel transforms, a relationship between applied force and Young's modulus can be derived for a given indenter displacement.\textsuperscript{123, 124} There are three common indenter shapes that can be used to prod the cell: conical, spherical, and flat-ended cylindrical punch. For the rigid conical punch, the solution for Young's modulus is:

\[
E = \frac{2P(1-\nu^2)}{\pi \varepsilon a}
\]  
(3)

where \(P\) is the applied force, \(\nu\) is the Poisson's ratio of the cell, \(\varepsilon\) is the indentation depth, and \(a\) is the indenter radius at the cell's surface.\textsuperscript{124} For a rigid spherical indenter, the Young's modulus is shown to be:

\[
E = \frac{3P(1-\nu^2)}{4R^{1/2}\varepsilon^{3/2}}
\]  
(4)

where \(R\) is the radius of the indenting sphere.\textsuperscript{124} The last case of a cylindrical end is the most common among indentation experiments. In this situation, the cell's Young's modulus is:

\[
E = \frac{P(1-\nu^2)}{2\alpha a}
\]  
(5)

The punch model solution under the experimental modality of micropipette aspiration has been previously described by Theret and associates\textsuperscript{125} and later compared to more complex continuum mechanics models by Haider and Guilak\textsuperscript{36} and Jones and associates.\textsuperscript{126, 127} This model describes the Young's modulus of the cell as:

\[
E = \frac{3a\Delta P}{2\pi L} \phi_p(\eta)
\]  
(6)
where $\Delta P$ is the applied external suction pressure, $a$ is the inner radius of the micropipette, $L$ is the distance the cell is aspirated in the micropipette, and $\varphi_p(\eta)$ is a function of the inner and outer radii of the micropipette.

**Viscoelastic model**

The viscoelastic model accurately depicts the behavioral response of chondrocyte cells to an applied stress or strain, by describing the cell as containing both fluid-like and solid-like structural elements. The most common viscoelastic model for the chondrocyte is the standard linear solid (SLS), where the cell is represented using a circuit analog of springs and dashpots. The springs are strain-dependant elements, whereas the dashpots are varying according to strain-rate. In the SLS model, the spring is in series with an element consisting of a spring and dashpot in parallel. Using this one-dimensional analog, the constitutive equation describing the stress and stress relationship is written as:

$$
(E_1 + E_2) \sigma + \eta \frac{d\sigma}{dt} = E_1 E_2 \varepsilon + E_1 \eta \frac{d\varepsilon}{dt}
$$

where $E_1$ and $E_2$ are the elastic constants for the two springs, $\eta$ is the coefficient of viscosity for the fluid element, and $\sigma$ and $\varepsilon$ represent the stress and strain within the system, respectively.\textsuperscript{128}

By including characteristics of both viscous fluids and elastic solids, this model can predict the cellular creep response to an applied constant stress and stress-relaxation response to a step strain. The SLS model has been used to describe chondrocyte behavior in many testing modalities, including cell indentation, unconfined compression, and micropipette aspiration. The indentation solution was first developed by Ting\textsuperscript{129} to
describe the behavior of a viscoelastic half space. A more recent creep indentation solution was developed by Cheng and associates\textsuperscript{130} and used by Koay and associates\textsuperscript{131} to test the material properties of single chondrocytes. The SLS model has also been applied to micropipette aspiration experiments using a solution originally developed by Sato and associates\textsuperscript{132} and to unconfined compression creep tests on single cells using a relationship described by Liepzig and Athanasiou.\textsuperscript{133} This model allows researchers to elucidate three mechanical parameters describing the individual chondrocyte: instantaneous modulus ($E_0$), relaxed modulus ($E_{\infty}$), and apparent viscosity ($\mu$) of the continuum.\textsuperscript{131}

**Linear biphasic model**

Developed in 1980 by Mow and associates,\textsuperscript{134} the biphasic model describes a material as having both a fluid and solid phase. Though initially difficult to conceive, this theoretical model treats each spatial point within the continuum as a mixture of both a liquid and a solid. To reduce relations of conservation of mass and momentum describing this continuum to workable equations, several assumptions need to be made. The solid matrix is assumed to be linearly elastic, isotropic, nondissipative, and incompressible and the fluid phase is inviscid and also incompressible. Additionally, the fluid is assumed to flow through the porous solid medium, creating a frictional drag force which accounts for the time-dependant behavior of the continuum. These assumptions enable the following constitutive relationships to be made for the solid matrix and fluid components:

\[ T^s = -\phi^s p I + \lambda_e I + 2\mu_e E \]  
\[ T^f = -\phi^f p I \]
where \( T^s \) and \( T^f \) are the stress tensors for the solid and fluid phases respectively, \( \varphi \) denotes the volume fraction for each phase, and \( p \) is the hydrostatic pressure within the medium. Further denoted for the solid matrix, \( E \) is the infinitesimal strain tensor and \( e \) is its volumetric change, and \( \lambda \) and \( \mu \) are the Lamé constants.\(^\text{135}\)

Applied to single cells, this model correctly depicts the interaction of both fluid and solid behavioral components inside the confines of the cell membrane. Solid elements, such as cytoskeletal proteins, the nucleus, and organelles create a porous continuum for the free flowing cytoplasmic fluid. The biphasic model has been used to extract the following three intrinsic mechanical properties using cell indentation,\(^\text{136}\) unconfined compression,\(^\text{133}\) and micropipette aspiration\(^\text{137}\) experimental techniques: the aggregate modulus (\( H_A \)), permeability (\( k \)) and Poisson’s ratio (\( \nu_s \)) of the material’s solid phase. In addition, more complex versions of the biphasic theory have recently been developed to predict cellular behavior within articular cartilage tissue.\(^\text{78,138}\)

**Experimental validation**

Several experimental modalities have been used on single chondrocytes in recent years. Applying the three mechanical models described in the first step of the single cell approach, researchers have obtained cellular characteristics through either stress or strain controlled experiments. Major advancements in high precision systems have made it possible to apply forces or deformations on a scale relevant to that of a single cell. While not an all-inclusive list, several of the more prominent single cell experimental techniques include: (1) atomic force microscopy; (2) cytoindentation; (3) cytodetachment; (4) unconfined compression; and (5) micropipette aspiration (Fig. 3).
The first four methods take advantage of the anchorage-dependent characteristics of chondrocytes to elucidate salient mechanical properties, while micropipette aspiration tests generally treat the cells as free-floating within a fluid medium. A summary of all available data on chondrocyte mechanical properties is shown in Table 3.

**Atomic force microscopy**

Atomic force microscopy (AFM) has been utilized to provide researchers with a high-resolution topographic description of variations along the cell surface. With accuracy on the order of picometers, AFM is a popular technique to obtain a detailed spatial map regarding local pressure points on the cell. As an example, Bader and associates\(^1\) used AFM to examine the non-linear force-displacement response of single chondrocytes at various positions on the cell surface. Their results showed that the center of the cell was most resistant to an applied indentation. Therefore, AFM can be quite advantageous in studying heterogeneous materials, such as chondrocytes and their associated matrix, where highly localized differences exist.

Current AFM techniques generally consist of a pyramidal-shaped probe, with a diameter on the order of nanometers, attached to cantilever beam indenting the cell. The minute contact area of the probe and the cell provides AFM its high local precision. A piezoelectric motor displaces the probe toward the cell and force levels upon indentation are measured using Hooke’s law \(F = kx\), based on the deflection \(x\) and stiffness \(k\) of the cantilever beam. Controlled feed-back loops are used to maintain either desired constant force or displacement levels. Force and displacement data, over time, can then be fitted to elastic, viscoelastic, or biphasic models to acquire specific material properties.
A study by Darling and associates\textsuperscript{36} examined the viscoelastic nature of porcine articular chondrocytes from both superficial and middle/deep zones using AFM. The authors derived a viscoelastic solution for the punch problem with a spherical indenter (see eq. 4). Applying a ramp velocity of 6.25 $\mu$m/s to reach a target force of 2.5 nN, held for 60 seconds at the center of the cell, the stress-relaxation experiments yielded relevant mechanical properties. It was found that superficial zone chondrocytes were stiffer than middle/deep cells in terms of instantaneous moduli (0.55 kPa vs. 0.29 kPa) and relaxed moduli (0.31 kPa vs. 0.09 kPa), and had a greater apparent viscosity (1.15 kPa-s vs. 0.61 kPa-s).

Ng and associates\textsuperscript{140} recently utilized AFM to investigate chondrocyte viscoelastic properties with its developing PCM. Individual chondrocytes, with and without their associated matrix, were placed in custom-made pyramidal wells in preparation for testing. This provides the significant advantage of testing individual cells or chondrons without requiring them to be adherent on a substrate. Accurate spatial maps were obtained and finite element and Hertzian modeling was used to obtain biomechanical properties. In addition, a hysteresis upon unloading the probe was also observed, confirming previous findings regarding the time-dependant behavior of chondrocyte cells.

Cytoindentation

One increasingly popular technique to study the mechanical properties of single cells is cytoindentation. This recently developed procedure investigates a cell’s ability to withstand controlled indentations by a flat-ended miniature borosilicate glass probe, 5 $\mu$m
in diameter, which is attached to a much larger cantilever beam. Cytoindentation utilizes similar cantilever principles as AFM to determine the applied force based upon the deflection of the beam. A force transducing system is then employed to measure the cell's reactive force, on the order a single nanonewton, for a given displacement of the probe. The earliest model of the cytoindenter was developed by Shin and Athanasiou\textsuperscript{136} to study the biomechanical properties of individual MG63 osteosarcoma cells. In this version of the device, the deflection of the cantilever was measured by a dual photodiode system, enhanced by light microscopy. This system monitors the displacement of the end of the cantilever beam as a function of the voltage difference between the two photodiodes and is designated as the displacement on the cell. A piezoelectric motor applied a controlled ramp displacement with 0.275 \( \mu \text{m} \) increments to a 2.0 \( \mu \text{m} \) final depth and a linear biphasic finite element model was used to determine cellular characteristics based upon recorded force measurements. The results showed that the cells had a permeability of \( 1.18 \times 10^{-10} \text{ m}^4 \text{N}^{-1} \text{s}^{-1} \), which is strikingly greater than that of articular cartilage.\textsuperscript{141} Additionally, the cells had an aggregate modulus of 2.05 kPa, shear modulus of 0.41 kPa, and Poisson's ratio of 0.37.

Recently, Koay and associates\textsuperscript{131} modified the cytoindenter set-up to enable a laser micrometer to record the displacement at the end of the cantilever. Using a closed-loop algorithm, the authors applied creep tests on single chondrocytes at a test load of 50 nN for either 15 or 20 seconds. Both punch and viscoelastic continuum models were used to determine the mechanical properties of the chondrocytes. The punch model (see eq. 5) yielded an average Young's modulus of 1.10 kPa. The viscoelastic model resulted in an
instantaneous modulus of 8 kPa, relaxed modulus of 1.01 kPa, and apparent viscosity of 1.5 kPa-s for the chondrocyte.

Cytodetachment

The cytodetacher measures the adhesive properties of cells attached to various substrata and also can be used to examine the response in cellular behavior to an applied shearing force. In this device, a piezoelectric translator drives a small diameter carbon filament, attached to a larger glass cantilever, parallel to the cell substratum. Similar to experimentation with the cytoindenter, cantilever beam theory is utilized to calculate the reactive force based upon the deflection of the beam recorded by dual photodiodes. The first study employing the cytodetacher examined the force necessary to detach articular chondrocytes after seeding for 2 hours on untreated glass, bovine serum albumin-coated, and fibronectin coated glass. It was observed that the quantified adhesiveness of the cells on fibronectin (72.6 nN) were significantly greater than both bovine serum albumin (20.3 nN) and untreated glass (38.9 nN) experimental groups. Follow up by studies Hoben and associates and by Huang and associates further modified the cytodetacher and examined the temporal characteristics of chondrocyte adhesive properties. It was observed that cellular adhesion strength increased significantly with seeding time and could be attributed to a spreading of the actin cytoskeleton. Quantifying cellular adhesion strength will prove to be quite useful when examining cell-matrix interfaces in more complex situations in subsequent phases of the fundamental unit approach.
Unconfined cytocompression

Additional simple modifications of the cytoindenter and cytodetacher have made it possible to examine the effects of unconfined compression on single chondrocytes. This testing modality has been extensively utilized to study both the material properties and mechanical behavior of chondrocytes. In order to adapt the mechanical models to extract properties via this experimental technique, a suitable geometry for the chondrocyte was identified to accurately depict the anchorage-dependant properties and structure of the cell. Using vertical scanning interferometry, Scott and associates determined that a disc-shape would be an appropriate model for the cell, and Leipzig and Athanasiou used this shape to develop unconfined creep compression solutions for elastic, standard linear solid, and linear biphasic mechanical models. Using the cytoindenter device, the original 5 μm diameter indenter was replaced with a wider 50.8 μm diameter tungsten probe to apply constant loads of 75 nN for 45 seconds onto the chondrocyte. It was observed that viscoelastic model best fit the creep behavior of the individual cell, most notably in the early response phase to the applied force. They determined the elastic modulus for chondrocyte to be 2.55 kPa. Curvefitting the data to the viscoelastic model resulted in an instantaneous modulus of 2.47 kPa, relaxed modulus of 1.48 kPa, and apparent viscosity of 1.92 kPa-s. Finally the biphasic fit yielded an aggregate modulus of 2.58 kPa, cell permeability of 2.57 x 10^{-12} m^4 N^{-1} s^{-1} and Poisson’s ratio of 0.069.

Shieh and Athanasiou expanded unconfined compression experiments to look at zonal variations and attachment times in chondrocyte viscoelastic mechanical properties. Similar to later findings by Darling and associates, it was determined that superficial
zone cells were significantly stiffer than those from the middle/deep region in terms of instantaneous moduli (1.59 kPa vs. 0.69 kPa), relaxed moduli (1.20 kPa vs. 0.49 kPa), and apparent viscosity (6.32 kPa-s vs. 0.18 kPa-s) after 18 hours of seeding. Similar trends were also observed in the experimental group given three hours to attach.

Shieh and associates\textsuperscript{145} also modified the original cytodetacher set-up to study the response of single chondrocytes to various levels of strain under unconfined compression. By placing seeded chondrocytes perpendicular to the advancing probe, the authors were able to apply specific deformations onto the individual cell for 30 seconds and to record the subsequent cellular recovery behavior through videocapture. Quite interestingly, the cells exhibited a change in their response around 25-30\% strain levels. This critical point in chondrocyte behavior may have vast implications toward determining threshold levels for eliciting particular biochemical responses from the cell in terms of tissue engineering articular cartilage. Moreover, this yield strain may be analogous to an intrinsic threshold where bulk tissue is damaged and chondrocytes no longer experience their normal \textit{in vivo} mechanical environment.

\textbf{Micropipette aspiration}

One of the earliest methods to measure the mechanical characteristics of individual cells is through micropipette aspiration. In this technique, a miniature pipette applies a negative suction pressure onto the surface of the cell, thereby deforming its membrane inward through the pipette. By recording this event, researchers are able to calculate salient mechanical properties of the cell based up on the amount of membrane deformation, the required suction pressure levels, and the geometric relations pertaining
to the shape of the pipette. Similar to the cytodetacher, this technique can also be used to quantify the mechanical adhesiveness of a cell attached to a particular substratum.

Micropipette aspiration has frequently been used to determine the mechanical properties of chondrocytes harvested from both normal and osteoarthritic articular cartilage. Jones and associates\textsuperscript{127} first used this approach to compare cellular mechanical characteristics, such as Young's modulus and volumetric properties, from both healthy and diseased tissue. They applied maximum pressures between 0.05 and 1 kPa through a micropipette with an inner diameter of approximately 5 \( \mu \)m. By modeling the cell as a homogenous elastic half-space (see eq. 6), they determined that no differences existed between the Young's modulus of normal and osteoarthritic chondrocytes (0.65 kPa vs. 0.67 kPa). However, significant differences were observed in cell volume changes immediately and 600 seconds after complete aspiration of the cells into the micropipette. Normal chondrocytes only exhibited an 11% volume change, while osteoarthritic cells lost 20% of their volume. A similar study by Trickey and associates\textsuperscript{146} applied the standard linear solid model to micropipette experiments on normal and osteoarthritic chondrocytes. Their results showed that cells from diseased cartilage tissue were stiffer and more viscous than healthy cells, with regards to instantaneous moduli (0.63 kPa vs. 0.41 kPa), relaxed moduli (0.33 kPa vs. 0.24 kPa), and apparent viscosity (5.8 kPa-s vs. 3.0 kPa-s). Trickey and associates\textsuperscript{137} recently applied a biphasic model to compare the recovery behavior of chondrocytes from the two sources. While there were no significant differences between poisson's ratio of normal and osteoarthritic cells (0.38 vs. 0.36), osteoarthritic chondrocytes did exhibit a greater characteristic recovery time upon release of the suction pressure.
Effects of mechanical stimuli on single cells

The last step in the single cell approach is to determine regimens of mechanical stimulation that elicit favorable gene responses. Using the knowledge attained through modeling and experimental validation, researchers can tailor the applied stimuli to the individual cell. Shieh and Athanasiou\textsuperscript{147} and Leipzig and Athanasiou\textsuperscript{148} have most recently used the technique of single cell reverse transcriptase polymerase chain reaction (scRT-PCR) to qualify gene expression changes due to direct mechanical stimulation. scRT-PCR has recently been validated\textsuperscript{149} as a highly sensitive and effective means to detect alterations in gene regulation within a single cell. In short, their experimental set-up involved using the previously described unconfined compression device to apply particular loading regimens upon adherent chondrocytes. Then via a custom designed micropipette aspirator, the same cell was removed from its substrate and placed into a lysis buffer in order to disrupt the cell membrane. Following protocols essentially similar to that of traditional RT-PCR, the RNA for each individual cell was isolated and reverse transcribed into cDNA. Specific primers and probes for DNA sequences of interest were optimized in conjunction with real-time PCR to measure relative gene expression levels.

Shieh and Athanasiou\textsuperscript{147} examined the effects of static, intermittent, and dynamic compression cycles on the gene expression levels for type II collagen, aggrecan, tissue inhibitor of metalloproteinase-1 (TIMP-1), and matrix metalloproteinase-1 (MMP-1) on individual chondrocytes. Both type II collagen and aggrecan genes are considered related to regenerative pathways for articular cartilage. In contrast, TIMP-1 and MMP-1 are catabolic genes, known to be involved in matrix degeneration. A housekeeping gene of GAPDH was used to identify any variability between samples. It was found that static
compression of 50 and 100 nN downregulated type II collagen and aggrecan, while
dynamic loading at the same force levels significantly alleviated these negative effects.
Leipzig and Athanasiou\textsuperscript{148} also looked at the effects of mechanical stimulation on
chondrocyte gene expression. Adding an additional complication, the authors examined
25nN, 50 nN, and 100 nN static loading in the presence of known growth factors, TGF-
\( \beta \)1 and IGF-1. It was observed that increased static loading significantly decreased type II
collagen and aggrecan levels, and increased TIMP-1 abundance. Furthermore, the
addition of soluble growth factors provided a form of mechanoprotection against the
detrimental effects of static loading. Taken together, these results clearly demonstrate that
it is possible to measure gene expression on the single cell level, providing credence to
future experiments on the chondron level. Moreover, these findings correlate very well
with tissue engineering studies involving large number of chondrocytes in dynamically
loaded matrices, with and without the addition of growth factors.\textsuperscript{80, 150, 151} Research on the
single cell level may therefore prove to be useful toward identifying the ideal frequency
of these dynamic loads applied onto the tissue construct, and their associated magnitudes.

\section*{Single chondron approach}

After studying the biomechanical properties and behavior of single cells, the next
logical progression within the fundamental approach is to look at single chondrons.
Examinations of the characteristics of the chondron include cell-matrix interactions and
the three-dimensional configuration of the chondrocyte embedded in articular cartilage
tissue. The information gained from experiments with single chondrons sheds light on
how the tissue functions and responds in its mechanical environment and contributes to
the overall goal of generating a tissue that more closely mimics the properties of native cartilage. By including the PCM in these experiments, researchers gain a physiologically relevant model of chondrocyte behavior and structure *in vitro*, while maintaining all the previously mentioned advantages as in the single cell approach. To date, little research has been completed to directly correlate the effect of mechanical stimuli and gene expression on single chondrons. However, the potential for this research is extensive, with significant strides already taken in chondron mathematical modeling and experimental techniques.

*Chondron mathematical modeling*

To accurately depict the mechanical effect of the PCM on chondrocyte behavior, mathematical models for the chondron need to include a cell-matrix interface to describe the bilayered nature of this continuum. There are currently two main models used in the mechanical testing of single chondrons: (1) layered half space model; and (2) multi-scale biphasic model. Modeling the interface of the cell and its immediate surroundings presents several significant advantages in articular cartilage micromechanics. First, researchers can accurately portray the role of the PCM as an immediate transducer of mechanical forces onto the enclosed chondrocyte. Second, peak stress and flow conditions around the cell may be predicted. Third, the micromechanical environment of the cell can be directly linked to cellular metabolic and structural changes to further understand chondron mechanobiology.
Layered half space model

The layered elastic half-space model is an extension of the previously described punch problem, including an elastic layer lining the elastic half space. The outer layer (corresponding to the PCM) is assumed to be infinite, isotropic, and homogeneous. Similarly, the enclosed region (corresponding to the chondrocyte) is treated as an isotropic, homogeneous, and semi-infinite half space. Each layer is assumed to be a linearly elastic solid, with different Lamé elastic constants, and both layers are in perfect contact with each other. Solutions have been developed for the stress distribution within a bilayered material undergoing static deformation by a flat-ended rigid circular indenter or by a given force application. These solutions can be potentially applied to the indentation problem of individual chondrons, although presenting practical experimental hurdles. Kumar and Hiremath have solved the punch problem for an annular shaped indenter and Alexopoulos and associates have extended this solution in order to determine the mechanical characteristics of single chondrons using micropipette aspiration.

Multi-scale biphasic model

The multi-scale biphasic model utilizes a finite element approach to elucidate the mechanical characteristics of the individual matrix layers extending radially outward from the chondrocyte. This approach considers cell-PCM and PCM-ECM interactions within articular cartilage and has been employed to determine the mechanical properties of the chondrocyte’s PCM. Bachrach and associates originally described the cell-matrix interface within soft biological tissues under confined compression conditions.
Later, Wu and Herzog\textsuperscript{156} developed a model to determine the effect of time and position under unconfined loading conditions in the local mechanical environment of the chondrocytes embedded within the articular cartilage matrix. A further advancement of this approach was the inclusion of distinct matrix layers surrounding the cell, particularly pertaining to the PCM and ECM, into a biphasic finite element model by Guilak and Mow.\textsuperscript{78} Each level, including the cell, is assumed to be a biphasic material with continuous boundary conditions at each interface. Most recently, a radial biphasic model has been developed by Haider\textsuperscript{157} to describe the transmission of mechanical forces throughout the chondron.

\textit{Experimental validation}

Due to practical limitations, the majority of single chondron mechanical experiments have utilized the technique of micropipette aspiration. This is in large part due to the non-adhesive properties of the PCM, thus making it difficult to apply a constant load without the chondron “slipping away.” Micropipette aspiration has been extensively used by Alexopoulos and associates\textsuperscript{100, 101} and Guilak and associates\textsuperscript{158} to elucidate the osteoarthritic and zonal differences in chondrocyte biomechanical characteristics and its associated PCM. Alexopoulos and associates\textsuperscript{101} developed an analytical solution for the layered half-space model for chondron micropipette aspiration. This was the first reported study to directly measure PCM mechanical properties of single chondrons. The authors compared their newly developed layered half-space model to elastic half-space and shell models for the PCM and chondron, respectively. Single chondrons were isolated mechanically from the superficial and middle/deep zones of both
healthy and osteoarthritic human articular cartilage tissue. The layered half-space model yielded Young’s moduli of 68.9 kPa and 39.1 kPa for normal and osteoarthritic chondrons, respectively, from the superficial zone, and 62.0 kPa and 43.9 kPa, respectively, from the middle/deep zone. Alexopoulos and associates later applied a biphasic model to similar single chondron micropipette aspiration experiments and found that osteoarthritic PCM was less stiff and more permeable than normal PCM. These results bolster the observation that PCM, in addition to ECM, degrades in terms of its mechanical integrity upon the onset of osteoarthritis. The layered half space model was again used by Guilak and associates to study the zonal variations in chondron mechanical properties within canine articular cartilage. Confirming previous observations, little differences existed in the Young’s Modulus of the PCM between superficial and middle/deep zones (24.0 kPa vs. 23.2 kPa).

Conclusions

The fundamental unit approach provides several advantages toward elucidating and effecting tissue engineering of articular cartilage. The ability to develop definitive relationships between mechanical stimulation and cellular behavior will enable researchers to define appropriate loading regimens that are favorable to the cell as it develops within a tissue engineered construct. Conversely, an understanding of the mechanical forces pertaining to degenerative processes, i.e., osteoarthritis, may allow researchers to devise intervention regimens and other possible treatment modalities. Advanced mechanical modeling enables researchers to correlate mechanical forces between layers and has progressed substantially in recent years. As previously described,
the punch model for single cells can be related to the layered half-space model for chondrons and the biphasic model can be adapted to account for discrete matrix layers. Mechanical models on the cellular level can be scaled up to the tissue level by maintaining the same governing principles of chondrocyte behavior and physical properties. Loading regimens can then be translated between phases until eventually determining an ideal regimen on the tissue construct level which will elicit the production of necessary ECM proteins.

Of further special interest is the possibility of using one of the previously described tissue engineering approaches, particularly on the scale of a single chondron, as a model for articular cartilage development. On this level, researchers can elucidate the role of the pericellular and territorial matrices in the formation of strong and healthy cartilage tissue. Research in this field may enable investigators to answer several pertinent questions regarding articular cartilage development:

(1) How does the expansion of the PCM affect the growth of other ECM regions?

(2) Do the thresholds for ideal cartilage mechanical stimulation change as the PCM creates a more robust mechanical buffer around the chondrocyte?

(3) How do these local matrix changes facilitate or modulate the passage of cytokines and growth factors among the cells in the construct?

While ample research has already been performed on single chondrocytes, there are several areas for future studies aside from the mechanical aspects described in this chapter. Additional combinations of various exogenous factors, including oxygen tension
levels and growth factors, may be used in conjunction with mechanical stimulation to optimize the biochemical response of individual cells. It would also be of great interest to examine the temporal effects of gene expression after mechanical stimulation. This knowledge could potentially provide researchers with a working time frame for eliciting particular cellular responses. For instance, after a certain time point, changes in gene expression may return to baseline values or remain permanently changed. Lastly, deformations on the cellular and nuclear level can be directly linked to changes in gene expression to further our understanding of chondrocyte mechanobiology.

Mechanical testing of single chondrons can be seen as a logical next step after single cell experiments. Many single chondrocyte experiments require seeding the cells onto a substrate, which does not account for their three-dimensional configuration \textit{in vivo} and may alter cytoskeletal structure and cellular organization. Studying single cells with their associated matrix allows researchers to observe changes in cellular behavior more indicative of an \textit{in vivo} response. However, as of yet, micropipette aspiration is the only technique used to directly measure the mechanical properties of single chondrons. Despite the ability of micropipette aspiration to quantify cellular and PCM properties, it cannot be used to apply compressive forces or controlled deformations on single chondrons. This can be seen as a significant drawback, as it prevents researchers from performing conventional stress relaxation experiments. Furthermore, the types of forces normally experienced by chondrocytes \textit{in vivo}, namely compressive, shear, or hydrostatic forces, can not be achieved using micropipette aspiration alone. This leaves the window wide open for new techniques to be developed, which may be used to directly stimulate individual chondrons and measure their gene expression response.
As previously described, Ng and associates\textsuperscript{140} recently fabricated microwells that are capable of holding chondrons in place during AFM experimentation, thereby providing a potentially significant experimental modality to be used on single chondrons. In addition, other techniques may employ a type of "matrix glue" to attach the chondrons onto a given substrate prior to mechanical testing. Coverslips coated with monoclonal antibodies specific to PCM proteins can be used to immobilize chondrons for direct compression experiments. Similarly, micropipette aspiration also may be employed to apply a small tare load to hold the chondrons in position while a probe compresses the sample on the other end. In short, the potentials for chondron mechanical testing and stimulation are vast, with profound implications in articular cartilage micromechanics. Future experiments will need to examine the possibilities of various new approaches and study the threshold levels necessary to elicit positive responses from single chondrons. Then following the fundamental unit approach, researchers can work upwards toward the overarching goal of tissue engineering articular cartilage.
Table 1: Overview of major collagen changes during cartilage development

<table>
<thead>
<tr>
<th>Matrix Component</th>
<th>Animal Model</th>
<th>Prenatal</th>
<th>Post-Parturition</th>
<th>Mature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen (general)</td>
<td>Human knee</td>
<td>• Present in interzone and joint cavitation(^6)</td>
<td>• ECM is filled by random network of small banded fibrils (~40 nm in diameter)(^9)</td>
<td>• Largest fibrils are ~100 nm thick, running vertically in deep zone(^9)</td>
</tr>
<tr>
<td></td>
<td>Rabbit tibia</td>
<td>• Present in articular surface layer of epiphysis, but no new mRNA production(^56)</td>
<td>• Not present in interzone or joint cavitation(^2)</td>
<td>• Not present in adult tissue and no mRNA production(^56)</td>
</tr>
<tr>
<td>Collagen Type I</td>
<td>Rabbit knee</td>
<td>• Detected in the presumptive cartilage along the epiphyses and the perichondrium(^160)</td>
<td>• Not present in cartilage lining the epiphysis, but mRNA production is observed by chondrocytes(^56)</td>
<td>• Detected throughout the developing ECM and the perichondrium(^160)</td>
</tr>
<tr>
<td></td>
<td>Marsupial knee</td>
<td>• Present in interzone and newly formed cartilaginous layers(^56)</td>
<td>• Detected in the interterritorial matrix of the cartilage along the epiphysis(^56)</td>
<td>• Present in the perichondrium(^160)</td>
</tr>
<tr>
<td>Collagen Type II</td>
<td>Rabbit knee</td>
<td>• Present in interzone and newly formed cartilaginous layers, localized pericellularly(^26)</td>
<td>• Strong pericellular presence in superficial layer(^56)</td>
<td>• Initially located within interterritorial matrix, then localizes to PCM(^15)</td>
</tr>
<tr>
<td></td>
<td>Marsupial knee</td>
<td>• Expression spikes at chondrocyte differentiation(^2)</td>
<td></td>
<td>• Present in the perichondrium(^160)</td>
</tr>
<tr>
<td>Collagen Type III</td>
<td>Rabbit knee</td>
<td>• Expression spikes at chondrocyte differentiation(^2)</td>
<td>• Intensity of stain decreases in regions associated with chondrocyte maturity(^57)</td>
<td>• Not detected in mature tissue(^160)</td>
</tr>
<tr>
<td></td>
<td>Marsupial knee</td>
<td>• Intensity of stain decreases in regions associated with chondrocyte maturity(^57)</td>
<td></td>
<td>• Observed in pericellular and territorial matrices thorough the tissue, above the tidemark(^56)</td>
</tr>
<tr>
<td>Collagen Type VI</td>
<td>Avian embryo tibia</td>
<td>• Expression spikes at chondrocyte differentiation(^2)</td>
<td>• Intensity of stain decreases in regions associated with chondrocyte maturity(^57)</td>
<td>• Observed pericellularly around the deep hypertrophic cells and with the growth plate(^160)</td>
</tr>
<tr>
<td></td>
<td>Fetal calf femur</td>
<td>• Expression spikes at chondrocyte differentiation(^2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Marsupial knee</td>
<td>• Expression spikes at chondrocyte differentiation(^2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Collagen Type X</td>
<td>Marsupial knee</td>
<td>• Expression spikes at chondrocyte differentiation(^2)</td>
<td>• Intensity of stain decreases in regions associated with chondrocyte maturity(^57)</td>
<td>• Observed pericellularly around the deep hypertrophic cells and with the growth plate(^160)</td>
</tr>
</tbody>
</table>
Table 2: Overview of major GAG changes during cartilage development

<table>
<thead>
<tr>
<th>Component</th>
<th>Animal Model</th>
<th>Prenatal</th>
<th>Post-Parturition</th>
<th>Mature</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>GAG (general)</strong></td>
<td>Equine</td>
<td>• ~17% by dry weight&lt;sup&gt;141&lt;/sup&gt;</td>
<td>• ~16% by dry weight&lt;sup&gt;141&lt;/sup&gt;</td>
<td>• Represented ~85% of total GAG&lt;sup&gt;63&lt;/sup&gt;</td>
</tr>
<tr>
<td>metacarpophalangeal joint</td>
<td>Human femur</td>
<td>• ~5% by wet weight&lt;sup&gt;141&lt;/sup&gt;</td>
<td>• ~8% by wet weight&lt;sup&gt;141&lt;/sup&gt;</td>
<td>• Ratio of CS-4 to CS-6 is 15% to 85%&lt;sup&gt;61&lt;/sup&gt;</td>
</tr>
<tr>
<td>Hyaluronan</td>
<td>Equine</td>
<td>• ~50% by dry weight&lt;sup&gt;63&lt;/sup&gt;</td>
<td>• ~15% by dry weight&lt;sup&gt;63&lt;/sup&gt;</td>
<td>• CS-6 predominant throughout the ECM&lt;sup&gt;61&lt;/sup&gt;</td>
</tr>
<tr>
<td>metacarpophalangeal joint</td>
<td>Human knee</td>
<td>• ~0.03% by wet weight&lt;sup&gt;141&lt;/sup&gt;</td>
<td>• ~0.06% by wet weight&lt;sup&gt;141&lt;/sup&gt;</td>
<td>• Intense labeling for CS-6, but not CS-4&lt;sup&gt;60&lt;/sup&gt;</td>
</tr>
<tr>
<td>Chondroitin Sulfate</td>
<td>Equine</td>
<td>• ~0.05% by wet weight&lt;sup&gt;66&lt;/sup&gt;</td>
<td>• Consists of &lt;1% of GAG&lt;sup&gt;63&lt;/sup&gt;</td>
<td>• CS-4 most pronounced in interterritorial matrix&lt;sup&gt;60&lt;/sup&gt;</td>
</tr>
<tr>
<td>metacarpophalangeal joint</td>
<td>Human femur</td>
<td>• Accounts for nearly all of the GAG at birth&lt;sup&gt;63&lt;/sup&gt;</td>
<td>• CS-4 located mainly in deeper layers&lt;sup&gt;61&lt;/sup&gt;</td>
<td>• Unchanged 4-:6- sulfation ratio&lt;sup&gt;62&lt;/sup&gt;</td>
</tr>
<tr>
<td>Marsupial knee</td>
<td>• Contains a greater amount of 4-sulfation than 6-sulfation&lt;sup&gt;62&lt;/sup&gt;</td>
<td>• Both CS-4 and CS-6 are observed throughout the ECM&lt;sup&gt;60&lt;/sup&gt;</td>
<td>• DS-PGII synthesis barely detectable relative to DS-PGII&lt;sup&gt;65&lt;/sup&gt;</td>
<td>• DS-PGII localized to superficial zone&lt;sup&gt;63&lt;/sup&gt;</td>
</tr>
<tr>
<td>Dermatan Sulfate</td>
<td>Human knee</td>
<td>• DS-PGII is ~0.15% by wet weight&lt;sup&gt;63&lt;/sup&gt;</td>
<td>• CS-4 located throughout articular and epiphyseal cartilages&lt;sup&gt;63&lt;/sup&gt;</td>
<td>• Gel electrophoresis shows an increase in KS levels over time&lt;sup&gt;63&lt;/sup&gt;</td>
</tr>
<tr>
<td>Bovine</td>
<td>• DS-PGII was located mainly in superficial and middle zones&lt;sup&gt;63&lt;/sup&gt;</td>
<td>• In deep zones, DS-PGII was found pericellularly&lt;sup&gt;63&lt;/sup&gt;</td>
<td>• DS-PGII was confined mainly to superficial and middle zones&lt;sup&gt;63&lt;/sup&gt;</td>
<td>• Comprises 12% of total GAG&lt;sup&gt;63&lt;/sup&gt;</td>
</tr>
<tr>
<td>metacarpophalangeal joint</td>
<td>Human femur</td>
<td>• Present in trace amounts&lt;sup&gt;63&lt;/sup&gt;</td>
<td>• In deep zones, DS-PGII was found pericellularly&lt;sup&gt;63&lt;/sup&gt;</td>
<td>• Detected throughout ECM, but with a stronger binding near the superficial zone&lt;sup&gt;60&lt;/sup&gt;</td>
</tr>
<tr>
<td>Keratan Sulfate</td>
<td>Human femur</td>
<td>• Weakly positive pericellular binding observed&lt;sup&gt;60&lt;/sup&gt;</td>
<td>• Present in low levels with a pericellular localization&lt;sup&gt;63&lt;/sup&gt;</td>
<td>• Gel electrophoresis shows an increase in KS levels over time&lt;sup&gt;63&lt;/sup&gt;</td>
</tr>
</tbody>
</table>
Table 3: Overview of published mechanical properties for the individual chondrocyte and its associated PCM

<table>
<thead>
<tr>
<th>Tissue source</th>
<th>Experimental technique</th>
<th>Mechanical model</th>
<th>Material properties</th>
</tr>
</thead>
<tbody>
<tr>
<td>Porcine femoral joints</td>
<td>AFM</td>
<td>Viscoelastic</td>
<td>$E_o = 0.55 \text{kPa}, E_w = 0.31 \text{kPa}, \mu = 1.15 \text{kPa-s}$ **</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$E_o = 0.29 \text{kPa}, E_w = 0.17 \text{kPa}, \mu = 0.41 \text{kPa-s}$</td>
</tr>
<tr>
<td>Bovine metatarsal joints</td>
<td>Micropipette aspiration</td>
<td>Viscoelastic</td>
<td>$E_o = 0.45 \text{kPa}, E_w = 0.14 \text{kPa}, \mu = 2.570 \text{kPa-s}$</td>
</tr>
<tr>
<td>Bovine metatarsal joints</td>
<td>Cytoindentation$^{131}$</td>
<td>Elastic</td>
<td>$E_Y = 1.10 \text{kPa}$</td>
</tr>
<tr>
<td>Bovine metatarsal joints</td>
<td>Unconfined compression$^{133}$</td>
<td>Viscoelastic</td>
<td>$E_o = 8.00 \text{kPa}, E_w = 1.09 \text{kPa}, \mu = 1.50 \text{kPa-s}$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$E_o = 2.47 \text{kPa}, E_w = 1.48 \text{kPa}, \mu = 1.92 \text{kPa-s}$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Biphasic</td>
<td>$H_A = 2.58 \text{kPa}, k = 2.37 \times 10^{-12} \text{mN}^{-1}\text{s}^{-1}, \nu_s = 0.069$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$E_o = 1.59 \text{kPa}, E_w = 1.20 \text{kPa}, \mu = 6.32 \text{kPa-s}$ **</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$E_o = 0.69 \text{kPa}, E_w = 0.49 \text{kPa}, \mu = 3.18 \text{kPa-s}$</td>
</tr>
<tr>
<td>Canine femoral articular cartilage</td>
<td>Micropipette aspiration</td>
<td>Layered half-space</td>
<td>$E_Y = 24.0 \text{kPa}$ $$ $$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$E_Y = 23.2 \text{kPa}$ $$ $$</td>
</tr>
<tr>
<td>Healthy human articular cartilage</td>
<td>Micropipette aspiration</td>
<td>Punch$^{123}$</td>
<td>$E_Y = 0.65 \text{kPa}$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$E_Y = 0.6 \text{kPa}$ $$ $$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Viscoelastic$^{146}$</td>
<td>$E_o = 0.41 \text{kPa}, E_w = 0.24 \text{kPa}, \mu = 3.0 \text{kPa-s}$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$\nu_s = 0.38$</td>
<td>$$ $$ $$ $$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Layered half-space$^{101}$</td>
<td>$E_Y = 68.9 \text{kPa}$ $$ $$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$E_Y = 62.0 \text{kPa}$ $$ $$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Multi-scale biphasic$^{100}$</td>
<td>$E_Y = 39.7 \text{kPa}, k = 4.71 \times 10^{-17} \text{mN}^{-1}\text{s}^{-1}$ $$ $$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$E_Y = 36.8 \text{kPa}, k = 3.69 \times 10^{-17} \text{mN}^{-1}\text{s}^{-1}$ $$ $$</td>
</tr>
<tr>
<td>Osteoarthritic human articular cartilage</td>
<td>Micropipette aspiration</td>
<td>Punch$^{127}$</td>
<td>$E_Y = 0.67 \text{kPa}$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$E_Y = 0.63 \text{kPa}, E_w = 0.33 \text{kPa}, \mu = 5.80 \text{kPa-s}$</td>
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<tr>
<td></td>
<td></td>
<td>Viscoelastic$^{146}$</td>
<td>$\nu_s = 0.38$ $$ $$</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>$E_Y = 39.1 \text{kPa}$ $$ $$</td>
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<tr>
<td></td>
<td></td>
<td>Layered half-space$^{101}$</td>
<td>$E_Y = 43.9 \text{kPa}$ $$ $$</td>
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<tr>
<td></td>
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<td></td>
<td>$E_Y = 20.8 \text{kPa}, k = 10.46 \times 10^{-17} \text{mN}^{-1}\text{s}^{-1}$ $$ $$</td>
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<tr>
<td></td>
<td></td>
<td>Multi-scale biphasic$^{100}$</td>
<td>$E_Y = 24.4 \text{kPa}, k = 9.91 \times 10^{-17} \text{mN}^{-1}\text{s}^{-1}$ $$ $$</td>
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** All material properties are for chondrocytes isolated from the middle/deep region of articular cartilage unless otherwise denoted.

++ Superficial zone characteristics

## PCM mechanical parameters
Figure 1: Response of single cells to mechanical forces

Mechanical stimuli inducing tissue degradation and cellular apoptosis may be used at models for osteoarthritis. In contrast, stimuli leading to ECM synthesis should be included in any tissue engineering approach for articular cartilage.
Figure 2: ‘Functional unit approach’

Research with a single cell will provide an excellent starting point for subsequent research of the chondron, microexplant, and cartilage tissue. Each phase consists of three steps which examine the biomechanical properties of individual units within articular cartilage. With each added level of complexity, researchers gain invaluable insight toward understanding the effects of mechanical forces on cellular behavior. This knowledge may be used to elucidate regimens of mechanical stimulation necessary toward tissue engineering articular cartilage.
Figure 3: Experimental modalities for mechanically testing single chondrocytes

Techniques of atomic force microscopy (A); cytoindentation (B), unconfined compression (C), and cytodetachment (D) utilize the anchorage-dependant characteristics of chondrocytes to obtain relevant mechanical properties. Each of these four techniques apply a controlled force or displacement onto the cell via a probe attached to a cantilever. The probe deflection ($x$) due to the cell’s reactive force is as shown. In contrast, the testing modality of micropipette aspiration (E) employs a micropipette to apply a controlled suction pressure ($\Delta P$) on the cell, and the resulting deformation is recorded.
CHAPTER 2: Contribution of the cytoskeleton to the compressive properties and recovery behavior of single cells*

Abstract

The cytoskeleton is known to play an important role in the biomechanical nature and structure of cells, however its particular function in compressive characteristics has not yet been fully examined. This study investigated the contribution of the main three cytoskeletal elements to the bulk compressive stiffness (as measured by the compressive modulus), volumetric or compressibility changes (as indicated by apparent Poisson’s ratio), and recovery behavior of individual chondrocytes. Prior to mechanical testing, cytochalasin D, acrylamide, or colchicine was used to disrupt actin microfilaments, intermediate filaments, or microtubules, respectively. Cells were subjected to a range of compressive strains and allowed to recover to equilibrium. Analysis of the video recording for each mechanical event yielded relevant compressive properties and recovery characteristics, related to the specific cytoskeletal disrupting agent and as a function of applied axial strain. Inhibition of actin microfilaments had the greatest effect (~50% decrease vs. control) on bulk compressive stiffness. Meanwhile, intermediate filaments and microtubules were each found to play an integral role in either the diminution (compressibility) or retention (incompressibility) of original cell volume during compression. In addition, microtubule disruption had the largest effect on the

* Chapter under review as: Ofek G, Wiltz DC, and Athanasiou KA. Contribution of the cytoskeleton to the compressive properties and recovery behavior of single cells. *Biophys J*, 2009.
"critical strain threshold" in cellular mechanical behavior (33% decrease vs. control), as well as the characteristic time for recovery (~100% increase vs. control). Elucidating the role of the cytoskeleton in the compressive biomechanical behavior of single cells is an important step toward understanding the basis of mechanotransduction and the etiology of cellular disease processes.
Introduction

Biomechanical factors play an important role in healthy cellular function, tissue regeneration efforts, and the etiopathogenesis of a myriad of disease types. Individual cells sense and respond to mechanical changes in their microenvironment through mechanotransduction, whereby mechanical signals are translated to a biologic change, as reviewed elsewhere. The cytoskeleton, composed primarily of actin microfilaments, intermediate filaments, and microtubules, is considered to be an important mediator of mechanical forces in individual cells, as well as serving to support cellular structure and function. Actin microfilaments, which are the smallest elements of the cytoskeleton consisting of two threaded polymer chains of F-actin and positioned cortically with numerous focal adhesion points along the cell membrane, facilitate cell migration and can form tightly woven bundles of ‘stress fibers’ in response to mechanical stimuli. Intermediate filaments, which are composed of fibrous proteins (i.e., vimentin for chondrocyte cells) and distributed throughout the cell, function to link the nucleus to the cell surface and exert tension within the cell. Microtubules, comprised of rigid hollow tubes of the globular protein tubulin spanning the entire cell, facilitate organelle movement and function as a scaffold or truss.

The mechanical interactions for each of the cytoskeletal components have been described using a tensegrity model. This approach considers the cytoskeleton as an interwoven mesh of discrete compressive (microtubule) and tensile (intermediate and actin filaments) elements in a three-dimensional configuration, which are stabilized through a constant state of tension. The prestresses generated by the contractility of actin and intermediate filaments are resisted by microtubule struts within the cell. It is
therefore necessary to study how each integral element in this structural lattice plays a role in the overall mechanical characteristics of a cell to aid theoretical models which may be predictive of a cell’s response to mechanical loading.¹⁰⁹

Many cell types, such as articular chondrocytes, experience high compressive loads during everyday activity.³⁵, ¹³⁸ As the structural basis for cells, the various cytoskeletal elements may each serve an important function in the ability of a cell to resist and recover from mechanical forces. Compression of single cells may result in changes in the organization and characteristics of individual cytoskeletal components.¹¹⁶, ¹⁶⁷ The cytoskeleton has also been shown to aid in the transmission of forces to the nucleus during compression,¹⁰⁷, ¹¹⁹ resulting in direct strain on the nucleus¹⁶⁸ and, thus, altering the biosynthetic regulation of single cells.¹²⁰ Though prior research has investigated the role of the cytoskeleton in resisting compression, these studies only examined forces applied  \textit{locally} onto the cell.¹⁶⁹, ¹⁷⁰ However, cells typically experience mechanical forces applied along their entirety.⁷⁸ Therefore, this study was motivated to examine unique relationships of each cytoskeletal element to the \textit{bulk} compressive biomechanics of single cells toward understanding the cytoskeleton’s role in mediating forces and reinforcing cellular structure during compression.

It is of further importance to examine the ranges of mechanical perturbation which precipitate anabolic or catabolic cellular pathways. Previous research from our group reported a “critical strain threshold” in the mechanical behavior of single chondrocytes¹⁴⁵ at around 30% applied axial strain, wherein the cells are no longer able to fully recover from the applied compressive load. At strains above this level, gene expression patterns also display catabolic characteristics.¹⁹, ¹⁶⁸ Thus, one of the aims of
this study is to understand the contribution of the cytoskeleton in modulating this strain threshold, which can be considered akin to a yield point in the stress-strain behavior of the single cell. Moreover, since alterations in cellular mechanical properties may be indicative of degenerative and metastatic changes, a detailed insight into the contribution of individual cytoskeletal components on the compressive characteristics of single cells may aid in elucidating the underlying mechanisms for cellular disease alterations and identifying potential treatments for restoring homeostatic conditions.

The overall objective of this study was to examine the contribution of actin, intermediate filaments, and microtubules to the compressive biomechanical characteristics of single cells. Using an anchorage-dependent cell model, individual chondrocytes were incubated with a cytoskeletal disrupting agent to target each of the aforementioned cytoskeletal components and then tested for their biomechanical characteristics over a range of applied axial strains. This was accomplished employing an unconfined compression approach to test the hypothesis that each cytoskeletal component would contribute differentially to the compressive properties and behavior of single chondrocytes, based on known dissimilarities in their structure and spatial orientation within chondrocytes. Specific emphasis was placed on the role of these cytoskeletal elements in the compressive modulus, apparent Poisson’s ratio, volumetric changes, and recovery behavior of the cells.
Methods

Cell isolation and seeding

Articular cartilage was harvested from adult bovine fetlock joints obtained from a local abattoir (Doreck and Sons Packing Company, Santa Fe, Texas). A total of 15 joints from nine different animals were used in this study. Single chondrocytes were isolated from the middle/deep region of the cartilage tissue as previously described. Post tissue digestion, chondrocytes were counted and resuspended at a concentration of 200,000 cells/ml in supplemented DMEM (0.1 mM NEAA, 100U/ml penicillin/streptomycin, 0.25 μg/ml fungizone), which also contained one of the following cytoskeletal disrupting agents: cytochalasin D (2 μM) for actin microfilaments, acrylamide (40 mM) for intermediate filaments, colchicine (10 μM) for microtubules, or none (control). The concentrations for the specific disrupting agents were chosen based upon prior literature demonstrating their efficacy with isolated chondrocytes. Chondrocytes were then seeded for 3 hrs on cut glass slides, which were placed inside a 6-well plate at 37°C and 10% CO₂. The 3 hr incubation period has been previously demonstrated by our group to be sufficient in achieving proper cell attachment for cytocompression testing.

Immunocytochemistry

Fluorescent staining was performed to observe the effectiveness of cytochalasin, acrylamide, and colchicine at disrupting actin microfilaments, intermediate filaments, and microtubules, respectively. Chondrocytes were seeded in a similar manner as for compressive testing with each cytoskeletal disrupting agent or control. After 3 hrs of
culture, cells were fixed with 4% paraformaldehyde, blocked with 10% FBS, and permeabilized with 0.1% Triton X-100. Cells were then incubated with AlexaFluor 647 Phalloidin (Invitrogen, Carlsbad, CA) for actin visualization, Paclitaxel (Molecular Probes, Eugene, OR) for microtubule detection, mouse anti-Vimentin primary antibody (Invitrogen, Carlsbad, CA) followed by a goat anti-mouse secondary antibody (AlexaFlour 488; Invitrogen, Carlsbad, CA) for intermediate filaments imaging, and Hoescht’s dye for nuclei staining. These slides were viewed with an Axioplan 2 microscope (Carl Zeiss, Oberkochen, Germany) and a CoolSNAP_HQ CCD camera (Photometrics, Tuscon, AZ). All images for the same fluorescent stain were acquired at the same exposure time and analyzed using Metamorph 4.15 (Universal Imaging Corp., Downingtown, PA).

Unconfined cytocompression and videocapture

A previously described cytocompression device\textsuperscript{145} was utilized to apply a range of compressive strains (~5% - 60%) onto single chondrocytes via a 50.8 µm diameter tungsten probe. Glass slides were transferred from the 6-well plate and positioned upright in a Petri dish containing supplemented DMEM with 30 mM HEPES Buffer Solution and either cytochalasin D (2 µM), acrylamide (40 mM), colchicine (10 µM), or control. The dish was then placed on an IMT-2 inverted microscope (Olympus America, Melville, NY, USA) and cells were viewed under 400x magnification. Individual cells were brought into close proximity with the probe (~5 – 10 µm away), as confirmed through visualization via the microscope (Fig. 4). Each cell was thus exposed to a different level of compressive strain depending on the initial distance between the probe and the cell. A
piezoelectric motor drove the probe a prescribed distance (12 – 16 µm) axially toward the cell at a rate of 4 µm/s. The probe was held at this position for 30 s and then returned to its original position. Following compression, chondrocytes were allowed to recover for 60 s. The entire compression and recovery event was recorded through an AVC-D7 CCD camera (Sony USA) connected to the microscope. Videos were saved as an AVI file at 640 x 480 resolution for subsequent analysis.

_Video analysis and biomechanical measurements_

Individual frames from the videos were extracted using Videomach 4.0.2 software (Gromada.com). Images of the initial cell-probe configuration, initial cell-probe contact, cell-probe contact at equilibrium compression, cell immediately after probe release, and cellular recovery every 4 s thereafter were examined. The dimensions of the cell and/or probe positions in each frame were marked using Microsoft Paint 5.1 (Microsoft Corporation, Redmond, WA, USA). A pixel-to-micron ratio of 7.0 was employed in all image subsequent analysis and the accuracy of the measurements was deemed to be 2 pixels.

The axial strain ($\varepsilon_a$) applied onto each cell was determined by differences in probe positions between initial cell contact and at equilibrium compression, divided by the initial cell height. Lateral strain ($\varepsilon_l$) was defined as the difference between initial cell width and cell width during equilibrium compression, divided by initial cell width. Using these values, apparent Poisson’s ratio was calculated as:

$$\nu_a = -\frac{\varepsilon_l}{\varepsilon_a}$$ (1)
Within each treatment group, apparent Poisson’s ratios were examined over the range of applied axial strains. An apparent Poisson’s ratio of 0.5 would suggest that the cell is incompressible, or does not incur volume change during compression.

Cantilever beam theory was employed to determine the reaction force of the cell onto the probe at peak compression:

\[ F = \frac{3EI}{L^3} \delta \]  

(2)

where \( E \) (Young’s modulus), \( I \) (moment of inertia), and \( L \) (cantilever beam length) are known parameters of the tungsten probe. The deflection of the cantilever (\( \delta \)) was determined by comparing the true displacement of the probe (via video analysis) with the prescribed piezoelectric displacement (between 12-16 \( \mu \)m). Applied stress was estimated as the cell’s reaction force divided by its initial cross-sectional area, as we have done previously.\(^{133, 173}\) Plots of stress vs. axial strain were generated for all experimental groups.

Cells were approximated as ellipsoids, with rotational symmetry about their z-axis (perpendicular to the plane of seeding), which is similar to previous methodologies.\(^{145, 173}\) Moreover, this basic shape was assumed to be maintained throughout the course of compression and cellular recovery. Thus, using the equation for an ellipsoid with two identical axes, cell volume was calculated as:

\[ V = \frac{1}{6} \pi h d^2 \]  

(3)

where \( h \) represents the cell’s height (or ellipsoid length in the z-direction) and \( d \) is the cell diameter (or ellipsoid length in the x- and y- directions). Cell volume was measured at initial setup \((V_0)\), immediately after compression \((V_f)\), and at equilibrium recovery \((V_r)\).
Using these volume measurements, a normalized volume change, \((V_0 - V_j)/V_0\), and recovered volume fraction, \(V_r/V_0\), were calculated and assessed as functions of \(\varepsilon_a\).

Upon release of the probe, the recovery strain history, \(\varepsilon(t)\), defined as change in cell height divided by its initial height, was monitored every 4 s and fit to a generalized time decaying exponential function using Matlab R2007b (Mathworks, Natick, MA, USA):

\[
\varepsilon(t) = Ae^{-t/\tau} + \varepsilon_r
\]

where \(A\) is the recovery coefficient, \(t\) is the time in seconds, \(\tau\) is the characteristic recovery time constant for the cell, and \(\varepsilon_r\) is the equilibrium residual strain. The latter was further compared to \(\varepsilon_a\) to investigate the capacity of the cell to regain its height from different magnitudes of compressive strain.

Data analysis

Power analysis prior to this study suggested a sample size of \(n = 30\) for experimental groups, based on expected deviations in cellular mechanical properties.\(^{173}\) All subsequent statistical analyses were performed using Microsoft Office Excel 2003 (Microsoft Corporation, Redmond, WA, USA). Linear regression was used to determine whether the equilibrium stress, apparent Poisson's ratio, normalized volume change, recovered volume fraction, and residual strain varied as functions of applied axial strain and to test the effects of the cytoskeletal disrupting agents on these functions. Change point analysis was further used to determine if a critical threshold of applied axial strain existed, whereby the biomechanical behavior of single cells irreversibly changed. As
previously described,\textsuperscript{145} this approach involved performing a series of linear regressions on overlapping segments of data (vs. applied strain) to look for potential changes in slope. If a discontinuity was apparent, the original data were separated into two subsets, each of which was analyzed with linear regression to identify if these resultant lines exhibited different correlations with applied strain. An effect of axial strain was considered significant if \( p \leq 0.05 \).

Results

In this study, a total of 128 cells were tested singly over a range of applied strains (30 for control, 34 for cytochalasin treatment, 30 for acrylamide treatment, and 34 for colchicine treatment). Cells were examined for their compressive properties and recovery behavior through video analysis of the mechanical event (Fig. 5). Representative recovery curves for control cells incurring either low or high applied strains are shown in Fig. 6, demonstrating the substantially different recovery behavior of cells below or above the critical strain threshold. Salient compressive properties and recovery behavior characteristics for each treatment group are summarized in Table 4.

Cytoskeletal disruption

Through immunocytochemistry, we confirmed that the chosen cytoskeletal disrupting agents, at their respective concentrations, were effective in disturbing their targeted cytoskeletal element (Fig. 7). Consistent with prior reports,\textsuperscript{175, 177, 178} cytochalasin treatment broke down actin microfilaments into spotty aggregates,
acrylamide collapsed the intermediate filament network, and colchicine substantially decreased overall intensity of microtubule staining.

**Cell size and morphology**

Generally, the acrylamide or colchicine treatment did not affect chondrocyte size or their rounded morphology, with cellular heights and widths typically varying between 8 and 12 μm. However, the initial width (w) and volume (vol), although not height (ht), of cytochalasin cells (ht = 10.8 ± 1.6 μm, w = 10.7 ± 1.0 μm, vol = 657 ± 195 μm³) was greater (p<0.05) than control cells (ht = 10.2 ± 1.3 μm, w = 9.7 ± 0.9 μm, vol = 512 ± 136 μm³), acrylamide cells (ht = 10.5 ± 1.0 μm, w = 9.8 ± 0.9 μm, vol = 547 ± 155 μm³), and colchicine cells (ht = 10.2 ± 2.0 μm, w = 9.8 ± 1.0 μm, vol = 522 ± 164 μm³).

**Cell stiffness**

A compressive modulus for each experimental group was estimated as the slope of equilibrium stress values plotted against the applied axial strain (Fig. 8). Control, cytochalasin, acrylamide, and colchicine cells all exhibited a significant linear correlation between stress and strain (p<0.001), with slopes of 1.63 kPa, 1.01 kPa, 1.69 kPa, and 1.39 kPa, respectively. A significant difference was observed between the compressive modulus of control and cytochalasin cells (p<0.05), as indicated by the 95% confidence intervals for those slopes.
**Cellular compressibility**

Control, cytochalasin, and colchicine cells all displayed compressive material characteristics, whereby volumetric changes were observed in response to the applied compressive load. Moreover, apparent Poisson's ratio (as an indicator of apparent compressibility) significantly decreased as a function of applied strain in the control, cytochalasin, and colchicine groups (Fig. 9). No differences in apparent Poisson's ratio were noted between control and cytochalasin cells. Colchicine treatment resulted in an overall increase in cellular compressibility compared to control cells. The intercept of the apparent Poisson's ratio for colchicine cells was significantly lower from that of control cells (p=0.05), as indicated by their 95% confidence intervals. In direct contrast to colchicine cells, treatment with acrylamide generally caused cells to be incompressible over the entire range of applied axial strains, as no significant linear correlation (p = 0.73) was observed and all apparent Poisson's ratio values were approximately 0.5. Lastly, the slope of the apparent Poisson's ratio values for acrylamide cells was significantly different from that of control cells (p<0.05), as indicated by the 95% confidence intervals for these slopes.

The results of normalized volume change demonstrated discontinuities in the compressive behavior of chondrocytes in response to a range of applied axial strains (Fig. 10). Control, cytochalasin, and colchicine cells exhibited discontinuities at 30%, 25%, and 20% applied strains, respectively. Prior to this discontinuity, no significant correlation was observed between normalized volume change and applied strain. Conversely, above the discontinuity, plots of cellular normalized volume change demonstrated a significant linear correlation with applied strain. In contrast to
cytochalasin and colchicine treatment groups, no distinguishable discontinuity in normalized volume change behavior existed for acrylamide cells. Moreover, all normalized volume change values for acrylamide cells were near zero, supporting the previous observation of incompressibility (or no volume loss) over the entire range of applied strains as a result of acrylamide treatment.

Cellular recovery behavior

The recovery behavior of cells from all groups was well approximated by the exponential decay function for strain recovered over time, with $R^2$ values generally between 0.90 and 0.99 (Fig. 6). Residual strains and recovered volumes were further measured once the cell was allowed to recover to equilibrium. Discontinuities in cellular recovery were observed for control, cytochalasin, acrylamide, and colchicine cells at 30%, 25%, 30%, and 20% applied axial strains, respectively. This discontinuity constituted a yield strain, whereby the biomechanical behavior of single chondrocytes was irreversibly changed. Below the yield strain, chondrocytes were able to recover their original dimensions, both in terms of volume (Fig. 11) and axial strain (Fig. 12); recovered volume fraction and residual strain values did not display a significant correlation with applied axial strain. However, above the yield strain, recovered volume fraction and residual strain values exhibited a significant linear correlation with applied strain, indicating a permanent loss in cellular volumes and height.

Figure 13 illustrates the effects of cytoskeletal disrupting agents on cell recovery time constants. Colchicine treatment resulted in the largest increase (approximately two times) in recovery time over control cells ($p<0.05$), demonstrative of a slowed cellular
recovery. Cytochalasin and acrylamide treatments also resulted in increases in recovery time constant of approximately 1.5 times that of control (p=0.09 for cytochalasin, p < 0.05 for acrylamide).

Discussion

Elucidating the role of the cytoskeleton in the mechanical characteristics of single cells may have important ramifications toward understanding cellular mechanotransduction, as well as cell structure and function. This study was designed to examine the contribution of actin, intermediate filaments, and microtubules to the mechanical properties and recovery behavior of individual chondrocytes, over a range of applied compressive strains. Several notable results were observed in this study pertaining to the compressive biomechanical nature of single cells. First, we identified actin microfilaments as the greatest contributor to bulk cell stiffness, as measured by the compressive modulus, during unconfined cytocompression. Second, intermediate filaments and microtubules were each found to play a central function in cellular compressibility, as measured by the apparent Poisson’s ratio. Third, microtubule disruption had the largest effect on the transition from recoverable to permanent cell deformation, as measured by the critical strain threshold in cellular recovery behavior. Lastly, it was shown that all of the cytoskeletal elements are needed to maintain the time for recovery from a compressive force, with microtubules exerting the most influence.

Actin microfilaments were observed to play a significant role in the compressive stiffness of single cells. The removal of actin decreased cell compressive modulus, compared to control, whereas microtubule and intermediate filament inhibition had little
effect on this parameter. Prior research has shown a correlation between actin inhibition and cell stiffness under tension,\textsuperscript{114, 179} or point indentation,\textsuperscript{169} but not under a bulk cell testing modality which considers the typical compressive \textit{in vivo} loading conditions in musculoskeletal tissues. Differences in the role of each cytoskeletal element in overall cell compressive stiffness may be due to their respective locations and structure. Actin is positioned primarily along the periphery of the cell, where it provides mechanical reinforcement for the cytoplasm.\textsuperscript{106, 116} Conversely, microtubules are formed from $\alpha$ and $\beta$-tubulin arranged into protofilaments and can be generally thought of as structural rods, as reviewed elsewhere.\textsuperscript{180} These rods, however, may be more loosely distributed, when compared to the tighter actin network that is found throughout the cell.\textsuperscript{106} Thus, this loose microtubule meshwork and tight actin network may be the underlying difference between the contributions to cell stiffness by these two cytoskeletal elements. In contrast, intermediate filaments form coiled-coil structures, known to have flexible regions at their head and tail,\textsuperscript{181} and act primarily in resisting tensile forces.\textsuperscript{179} Identifying the cytoskeletal contributors to cell stiffness is important since this property may influence cell behavior\textsuperscript{12, 31} and can be an indicator of cell pathology.\textsuperscript{146} Cellular stiffness can play a critical role in the cell’s interpretation of mechanical stimuli, which are known to precipitate regenerative\textsuperscript{182, 183} or degenerative pathways\textsuperscript{168}. Furthermore, by elucidating the contributors to bulk cell stiffness, research can be performed to enhance a cell’s ability to function under mechanical environments related to either regenerative or pathologic conditions.

To our knowledge, this is the first study to demonstrate that cellular compressibility is maintained through a balance of intermediate filaments and
microtubules. We observed that the removal of intermediate filaments caused cells to become incompressible \( (v_Q = 0.5) \) over the entire range of applied strains, while inhibition of microtubules induced an overall downward shift in the apparent Poisson’s ratio values. Mechanistically, this means that intermediate filaments serve as tensile elements, akin to ropes, that aid in “pulling” the cell together,\(^\text{113}\) thereby limiting the translation of axial to lateral strain. Conversely, microtubules act as solid rods,\(^\text{184}\) which facilitate an outward “push” during axial compression and thus, a greater retention of the original cell volume. Therefore, considering the pattern of decreasing apparent Poisson’s ratio values with increased compressive strains in control cells, our results suggest that this may be due to a breakdown of the microtubule network at high strains.\(^\text{185}\) Understanding the functional role of the cytoskeleton in cellular compressibility is of particular relevance since cell volume changes may influence cellular homeostasis and tissue matrix production.\(^\text{186}\) However, it is important to note that other factors besides the cytoskeleton may affect cellular compressibility, such as intracellular osmotic levels and active volume regulation mechanisms,\(^\text{10, 187}\) and induce an exudation of the cytosol. Hence, future research should examine interactions between intermediate filaments, microtubules, and transmembrane water channels on cellular volumetric changes.

Identifying the ranges of mechanical forces which induce changes in cellular behavior is critical toward developing appropriate loading regimens in functional tissue engineering or understanding disease etiology. After the observed critical threshold in all experimental groups, cellular residual strain and recovered volume fraction exhibited a significant dependence on applied strain. In terms of classical engineering mechanisms, this threshold represents a yield point wherein the cells incur a permanent or “plastic”
Conversely, below this threshold, cells retain their original volume and shape after compression; thus, they can be mechanically stimulated for regenerative purposes. Based on our results, this critical strain threshold in cell mechanical behavior may be primarily due to a loss of structural stabilization by microtubules. In this study, the inhibition of microtubules resulted in the largest shift of the critical strain threshold, from 30% to 20% compressive strain. It has been previously shown that microtubules aid in maintaining cell shape and serve as a ‘scaffold’ for other cytoskeletal elements. Thus, at this yield strain, microtubules may buckle, resulting in the inability for cells to fully recover from the applied force. Identifying microtubules as the largest contributor to cell recovery behavior may have important implications in regenerative medicine. Future researchers may employ biochemical agents to directly target microtubules to help cells recover from unphysiological loads.

All cytoskeletal elements appear to play a role in the recovery time for single chondrocytes in response to a compressive load, with microtubules exerting the largest influence. Removal of microtubules doubled the cellular recovery time constant, and inhibition of actin and intermediate filaments let to a 50% increase in this time constant. Each cytoskeletal element has a structural and functional aspect that aids in cell recovery. Microtubules, which are spread throughout the cell, are used as guides for the localization of other cellular structures and act as a trafficking highway for organelles. This is relevant as many components of the cell have been shown to move when undergoing a mechanical stimulus. Thus, in the absence of these microtubule pathways, organelles or other cytoskeletal elements may have trouble re-localizing to their initial position. In addition, microtubules are highly dynamic; the addition of α- or β- tubulin subunits to
these struts may push on other cytoskeletal elements to enhance cell recovery.\textsuperscript{195, 196} Conversely, actin and intermediate filaments can facilitate a quick recovery of cellular shape through tension.\textsuperscript{197} These elements will be stressed as the cell laterally expands during compression. Upon release of the compressing probe, actin and intermediate filaments may contract, thereby aiding cell recovery to its original shape. Understanding the contributors to cellular recovery time may provide insight into the mechanisms at play during dynamic compressive stimulation of both cells and tissues, which has been shown beneficial in numerous regenerative applications.\textsuperscript{189, 198} With this information, appropriate loading frequencies may be developed to mirror alterations of the cytoskeleton during phenotypic changes,\textsuperscript{199, 200} and thus promote neotissue growth.

While studying the mechanical characteristics of single cells may shed light on intracellular mechanisms and cellular homeostasis, the removal of cells from their native environment poses a limitation. Isolating individual cells disrupts connections between the cytoskeleton and the extracellular matrix,\textsuperscript{78, 201} as well as eliminates the role of the cellular microenvironment to appropriately transmit mechanical forces to the cell.\textsuperscript{102, 202} Notwithstanding this caveat, the cytoskeletal organization of single chondrocytes appears to be retained during isolation in monolayer culture.\textsuperscript{114} In addition, mechanical testing of individual cells is capable of discerning differences based on pathologic state,\textsuperscript{146, 171} phenotype,\textsuperscript{200, 203} and spatial origin within a tissue.\textsuperscript{31} Thus, single cell unconfined cytocompression may be a useful testing modality to examine mechanical contributions of various cytoskeletal elements, so as long as its results are placed within the appropriate context.
This chapter provides new insight into the role of the cytoskeleton in the compressive mechanical characteristics of single cells and the relationship between cellular structure and mechanical function (Fig. 14). Actin microfilaments were observed to be the largest contributor to bulk cell compressive stiffness and cell volume. Meanwhile, intermediate filaments play an important role in cellular compressibility, fettering transverse cell expansion over the entire range of applied axial strains. On the other hand, microtubules contribute to the incompressive nature of cells, as well as maintain the critical strain threshold and time constant in cellular recovery behavior. Discerning the role of the cytoskeleton in the mechanical properties and behavior of single cells facilitates a greater understanding of cellular biomechanical changes during tissue pathogenesis and regeneration,\textsuperscript{127, 203} as well as the mechanisms for force transmission within the cell.\textsuperscript{110}
Table 4: Salient compressive properties and recovery behavior characteristics for cells experiencing actin, intermediate filament, or microtubule disruption

Compressive moduli and Poisson ratios are presented as value ± 95% confidence boundaries. All others presented as mean ± standard deviation. (AF = actin microfilaments; IF = intermediate filaments; MT = microtubules; * = significance from control (p<0.05)).

<table>
<thead>
<tr>
<th>Biomechanical Characteristic</th>
<th>Mechanical Indicator</th>
<th>Control</th>
<th>AF disruption (cytochalasin)</th>
<th>IF disruption (acrylamide)</th>
<th>MT disruption (colchicine)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Stiffness</strong></td>
<td>Compressive modulus (kPa)</td>
<td>1.63 ± 0.31</td>
<td>1.01 ± 0.10*</td>
<td>1.69 ± 0.14</td>
<td>1.39 ± 0.19</td>
</tr>
<tr>
<td><strong>Initial compressibility</strong></td>
<td>Apparent Poisson's ratio @ εa = 0</td>
<td>0.49 ± 0.08</td>
<td>0.49 ± 0.07</td>
<td>0.50 ± 0.05</td>
<td>0.36 ± 0.06*</td>
</tr>
<tr>
<td><strong>Strain-dependent compressibility</strong></td>
<td>Apparent Poisson's ratio slope</td>
<td>-0.47 ± 0.25</td>
<td>-0.47 ± 0.22</td>
<td>-0.03 ± 0.22*</td>
<td>-0.33 ± 0.16</td>
</tr>
<tr>
<td><strong>Yield in recovery behavior</strong></td>
<td>Critical strain threshold (εcrit, %)</td>
<td>20</td>
<td>25</td>
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</tbody>
</table>

Generalized recovery behavior characteristics below critical threshold:

\[ ε(t) = Ae^{−t/T} + ε, \]

- Initial strain (for εa < εcrit)
- Recovery duration (for εa < εcrit)
- Permanent deformation (for εa < εcrit)

Generalized recovery behavior characteristics above critical threshold:

\[ ε(t) = Ae^{−t/T} + ε, \]

- Initial strain (for εa > εcrit)
- Recovery duration (for εa > εcrit)
- Permanent deformation (for εa > εcrit)

Recovery coefficient (A)

<table>
<thead>
<tr>
<th>Control</th>
<th>AF disruption</th>
<th>IF disruption</th>
<th>MT disruption</th>
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</thead>
<tbody>
<tr>
<td>0.17 ± 0.09</td>
<td>0.15 ± 0.05</td>
<td>0.17 ± 0.05</td>
<td>0.11 ± 0.03</td>
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<tr>
<td>1.6 ± 1.3</td>
<td>1.9 ± 0.8</td>
<td>2.9 ± 1.6*</td>
<td>4.1 ± 1.8*</td>
</tr>
<tr>
<td>7.4 ± 5.2</td>
<td>8.8 ± 7.2</td>
<td>5.9 ± 3.8</td>
<td>9.9 ± 4.2</td>
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</tbody>
</table>
Figure 4: Illustration of the cytocompression setup

A piezoelectric actuator drove a compressing probe axially toward articular chondrocytes seeded onto glass slides. Cells were exposed to compressive strains, generally ranging between 10% and 60%, for 30 s and then allowed to recover to equilibrium after the probe was removed. The entire mechanical event was recorded through a CCD video camera connected to the microscope. The figure is not drawn to scale.
Figure 5: Single cell compression and recovery behavior

The entire mechanical event was video recorded and subsequently analyzed to yield mechanical properties and indicators for recovery behavior. Differences can be observed between cells experiencing low strains (A- E) and high strains (F – J). The frames of initial experimental set up (A, F), initial probe contact (B, G), and equilibrium compression (C, H) provide information on the compressive properties of the cells by monitoring changes in cell shape and the movement of the probe. Upon release of the probe (D, I), the cell’s recovery behavior was tracked every 4 s until equilibrium was reached (E, J).
Figure 6: Representative recovery curves of single chondrocytes

Recovery strains were acquired every 4 s after the compressive force was removed, until the cell reached equilibrium (at around 60 s). For demonstration, the recovery behavior of untreated control cells is shown in response to axial strains ($\varepsilon_a$) either below or above the critical strain threshold. Below their critical threshold ("low"), cells typically fully recovered their initial height ($\varepsilon_r \sim 0$). Above their critical threshold ("high"), cells generally sustained a permanent loss in their initial height ($\varepsilon_r > 0$). Moreover, recovery time constants did not change as a function of applied strain within the same experimental group. The recovery behavior of cells from all groups was well approximated by the exponential decay function, with $R^2$ values generally between 0.90 and 0.99.

<table>
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<tr>
<th>Control Strain</th>
<th>$\varepsilon_a$</th>
<th>$\varepsilon_r$</th>
<th>$t$</th>
<th>$R^2$</th>
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<tr>
<td>&quot;low&quot; control strain</td>
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<td>0%</td>
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<td>&quot;high&quot; control strain</td>
<td>40.7%</td>
<td>11.5%</td>
<td>2.1 sec</td>
<td>0.99</td>
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</table>
Figure 7: Alterations in cytoskeletal network in response to specific cytoskeletal disrupting agents

Cytochalasin treatment was observed to break down actin microfilaments into spotty aggregates throughout the cell. Incubation with acrylamide collapsed the intermediate filament network to become localized only around the nucleus. Lastly, culture with colchicine decreased the intensity of microtubules to a faint staining. All fluorescent images for the same immunocytochemistry stain were taken at the same exposure time.
Figure 8: Cellular stiffness in response to cytoskeletal disrupting agents

Compressive moduli were measured based on the slope between equilibrium stress and applied axial strain. The lower and upper limits for 95% confidence interval for this slope are listed within the brackets. Control (A), cytochalasin (B), acrylamide (C), and colchicine (D) cells all exhibited a significant linear correlation between stress and strain. Treatment with cytochalasin significantly decreased the compressive modulus of chondrocytes compared to control cells (*p<0.05). No differences were observed between the other treatment groups and control cells.
Figure 9: Apparent Poisson’s ratios in response to cytoskeletal disrupting agents and applied axial strain

Apparent Poisson’s ratios significantly decreased as a function of applied strain in control (A), cytochalasin (B), and colchicine (D) cells. No differences in apparent Poisson’s ratio values were observed between control and cytochalasin cells, suggesting that actin does not play a major role in cellular compressibility. Acrylamide cells (C) were consistently incompressible (\( \nu \approx 0.5 \)) over the entire range of applied strains and exhibited a significantly different slope from control cells (\,*p<0.05\,*). The intercept for the apparent Poisson’s ratio values of the colchicine cells (D) was significantly lower than that of control cells (\,\#p=0.05\,), indicating an overall increase in cellular compressibility with colchicine treatment.
Figure 10: Normalized volume change behavior in response to cytoskeletal disrupting agents and applied axial strain

Discontinuities in the normalized volume change values were observed for control (A), cytochalasin (B), and colchicine (D) cells at 30%, 25%, and 20% applied strains, respectively. After the discontinuity, normalized volume change values exhibited a significant linear correlation with applied axial strain. No discernable discontinuity in normalized volume change behavior was observed for acrylamide cells (C), with all values near zero.
Figure 11: Recovered volume fraction behavior in response to cytoskeletal disrupting agents and applied axial strain

Discontinuities in recovered volume fraction were observed for control (A), cytochalasin (B), acrylamide (C), and colchicine (D) cells at 30%, 25%, 30%, and 20% applied axial strains, respectively. After the discontinuity, recovered volume fraction values exhibited a significant linear correlation with applied strain, indicating a permanent loss in cell volume.
Figure 12: Residual strain behavior in response to cytoskeletal disrupting agents and applied axial strain

Discontinuities in residual strain were observed for control (A), cytochalasin (B), acrylamide (C), and colchicine (D) cells at 30%, 25%, 30%, and 20% applied axial strains, respectively. After the discontinuity, residual strain values exhibited a significant linear correlation with applied strain, indicating a permanent loss in cellular heights.
Figure 13: Effect of cytoskeletal disrupting agents on recovery time constants

An increase in recovery time constant, averaged over the entire range of applied strains, was observed with cytochalasin treatment ($^\# p=0.09$), acrylamide treatment ($^* p<0.05$), and colchicine treatment ($^* p<0.05$) compared to control cells. A larger recovery time constant is indicative of a slowed cellular recovery to the compressive force.
Figure 14: Proposed role of actin microfilaments, intermediate filaments, and microtubules in cell compression and recovery

(A) When the cell is unperturbed, actin microfilaments are positioned cortically, intermediate filaments connect the nucleus to the cell membrane, and microtubules function as rigid struts. (B) Under compression, actin microfilaments reorganize themselves along the interface of the cell and probe to directly resist the compressive force. Meanwhile, intermediate filaments become tensed and exert an inward force, limiting the transverse expansion of the cell. Conversely, microtubules push outwards during compression, thereby supporting a greater maintenance of original cell volume. (C) During recovery, actin microfilaments and intermediate filaments pull the cell to its original shape through their tensile actions. Moreover, microtubules extend outwards and upwards through tubulin polymerization to enhance cell recovery, in addition to facilitating the re-localization of organelles or other cytoskeletal elements. Arrows indicate direction of the normal force generated by each cytoskeletal element during compression or recovery.
CHAPTER 3: *In situ* mechanical properties of the chondrocyte cytoplasm and nucleus*

Abstract

The way in which the nucleus experiences mechanical forces has important implications for understanding mechanotransduction. Knowledge of nuclear material properties and, specifically, their relationship to the properties of the bulk cell can help determine if the nucleus directly experiences mechanical load, or if it is signal transduction secondary to cell membrane deformation that leads to altered gene expression. Prior work measuring nuclear material properties using micropipette aspiration suggests that the nucleus is substantially stiffer than the bulk cell,\textsuperscript{118} whereas recent work with unconfined compression of single chondrocytes showed a nearly one-to-one correlation between cellular and nuclear strains.\textsuperscript{168} In this chapter, a linearly elastic finite element model of the cell with a nuclear inclusion was used to simulate the unconfined compression data. Cytoplasmic and nuclear stiffnesses were varied from 1 to 7 kPa for several combinations of cytoplasmic and nuclear Poisson’s ratios. It was found that the experimental data were best fit when the ratio of cytoplasmic to nuclear stiffness was 1.4, and both cytoplasm and nucleus were modeled as incompressible. The cytoplasmic to nuclear stiffness ratio is significantly lower than prior reports for isolated nuclei. These results suggest the nucleus may behave mechanically different *in situ* than when isolated.

Introduction

How mechanical forces are experienced by the nucleus has important consequences for understanding mechanotransduction. Mechanotransduction is the process by which mechanical loads induce changes in the gene expression profile of a cell, which can ultimately alter cellular physiology and homeostasis. It has also been shown that alterations in the physical dimensions of the nucleus, resulting from an applied load on the tissue or single cell, correlate with changes in gene regulation. Previous investigation into mechanical characteristics of isolated nuclei suggest they behave like a viscoelastic material and are significantly stiffer than the cell as a whole. However, these results are possibly influenced by the fact that the nuclei were removed from their in situ environment. The nuclear lamina, the framework for nuclear structure, is intimately linked to intermediate filaments positioned throughout the cytoplasm. Thus, mechanical properties of the nucleus may change when these connections are disrupted and the nucleus undergoes structural reorganization.

In this chapter, a finite element modeling approach was employed to obtain cytoplasmic and nuclear stiffness values which best match previously reported cellular and nuclear axial and lateral strains obtained during unconfined compression of single attached chondrocytes. Based on the nearly one-to-one correlation of cellular and nuclear strains observed in that study, we hypothesized the in situ nuclear stiffness is similar to that of the cytoplasm. Further, effects of changing both cytoplasmic and nuclear Poisson's ratios were explored to investigate the validity of the commonly used assumption of cellular incompressibility.
Methods

An axisymmetric model of the chondrocyte (height = 10 μm, width = 12 μm) with a nuclear inclusion (radius = 2.5 μm) was created using ABAQUS 6.7.1 (Fig. 15). The aforementioned geometric parameters were chosen to closely resemble an attached chondrocyte seeded for 3 hours. Both nucleus and cytoplasm were modeled as isotropic linearly elastic solids. This elastic model was chosen since the experimental data for cytoplasmic and nuclear strains were reported at equilibrium, corresponding to long-time behavior of a viscoelastic solid. Along the cell bottom, 4 μm of membrane was placed in frictionless contact with a rigid substrate. Preliminary analysis showed substrate adhesiveness had no effect on the cytoplasmic and nuclear mechanical properties determined. Finally, the cell membrane was placed in frictionless contact with the compression platen. A reference point was created for the platen to which a 25 nN load was applied. The cytoplasm and nucleus consisted of 522 and 144 axially symmetric four node reduced integration continuum elements (CAX4R), respectively. This was determined sufficient for convergence, as a model containing 2123 and 561 elements (cytoplasm and nucleus, respectively) yielded identical results.

To determine the combination of cytoplasmic and nuclear stiffnesses and Poisson’s ratios that best matched observed cytoplasmic and nuclear strains, a root-mean-square difference cost function was used, defined as:

$$\delta_{RMS} = \sqrt{\left(\varepsilon_{Axial}^{Model}(Nuc) - \varepsilon_{Axial}^{Experimental}(Nuc)\right)^2 + \left(\varepsilon_{Axial}^{Model}(Cyto) - \varepsilon_{Axial}^{Experimental}(Cyto)\right)^2 + \left(\varepsilon_{Lateral}^{Model}(Nuc) - \varepsilon_{Lateral}^{Experimental}(Nuc)\right)^2 + \left(\varepsilon_{Lateral}^{Model}(Cyto) - \varepsilon_{Lateral}^{Experimental}(Cyto)\right)^2}$$
\( \delta_{\text{RMS}} \) was calculated for each combination of material properties. Lower values of \( \delta_{\text{RMS}} \) indicate model output more closely matched the previously reported data.

Briefly, as reported by Leipzig and Athanasiou,\(^{168}\) cytoplasmic and nuclear strains were measured experimentally through the analysis of immunocytochemistry of cells fixed under a 25 nN compressive load. Cytomechanical testing was performed using a previously validated creep cytoindentation apparatus,\(^{31, 115, 133, 189, 205}\) which applies controlled compressive loads onto single adherent cells. Cell to platen contact was determined by a 5 nN preload, followed by compression to 25 nN. At equilibrium deformation, chondrocytes were fixed with paraformaldehyde. After fixation, a phalloidin stain was applied for the cytoskeleton and a Hoescht's stain was applied for the nucleus. Both loaded and unloaded (control) cells were imaged with a confocal microscope, followed by three-dimensional image reconstructions. The use of fluorescent staining to examine cellular and nuclear deformations and strains has been previously described in the literature.\(^5, 206\)

In an initial coarse search, cytoplasmic and nuclear Young's moduli were varied parametrically at 0.5 kPa increments from 1 to 7 kPa for combinations of Poisson's ratios shown in Table 5. These search parameters were guided by literature values for Poisson's ratios and cellular stiffness,\(^ {10, 31, 133, 137, 168}\) as well as preliminary analyses confirming Young's moduli less than 1 kPa and Poisson's ratios less than 0.3 yielded higher \( \delta_{\text{RMS}} \) values.

Based on results from the coarse search, Young's moduli were refined to increment 0.25 kPa from 3 to 5.5 kPa for \( v_{\text{Cyto}} = v_{\text{Nuc}} = 0.5 \) and \( v_{\text{Cyto}} = 0.4, v_{\text{Nuc}} = 0.5 \). Simulations were also performed for the cell without a nuclear inclusion to examine the
contribution of the nucleus. For these cases, the cell was considered to be an isotropic linearly elastic solid with the same initial physical dimensions as before. For the cell model, cost function values were calculated using only cytoplasmic axial and lateral strains.

Due to the inherent variability in any cell mechanics technique and analysis, the finite element model was examined for its sensitivity to slight changes in the experimental data of Leipzig and Athanasiou. The following three cases were studied: 1) increased axial and lateral cytoplasmic strains by 5%; 2) decreased axial and lateral nuclear strains by 5%; and 3) increased cytoplasmic strains by 5% and decreased nuclear strains by 5%. Finite element analysis was performed on the aforementioned cases, as before, for $\nu_{\text{Cyto}} = \nu_{\text{Nuc}} = 0.5$ to yield the in situ stiffness values for the cytoplasm and the nucleus.

Results

Table 5 shows minimum values computed from the cost function for each combination of Poisson's ratios, and the values of cytoplasmic and nuclear Young's moduli for which the minimum was obtained. From the initial coarse search, the ratio of cytoplasmic to nuclear stiffness was either $\sim 1.4$ ($E_{\text{Cyto}} = 3.5$ kPa, $E_{\text{Nuc}} = 5$ kPa) or $\sim 1.1$ ($E_{\text{Cyto}} = 4$ kPa, $E_{\text{Nuc}} = 4.5$ kPa) depending upon Poisson's ratios used. The minimum $\delta_{\text{RMS}}$, or case most closely matching the experimental data, occurred for $\nu_{\text{Cyto}} = \nu_{\text{Nuc}} = 0.5$, i.e., when both the cytoplasm and nucleus were considered incompressible. Figure 15 shows the cell in its undeformed and deformed states for this minimum $\delta_{\text{RMS}}$ case. When comparing the cases where the cytoplasmic and nuclear Poisson's ratios were held equal,
there was generally little effect of Poisson’s ratio on $\delta_{RMS}$, although the $v_{\text{Cyto}} = v_{\text{Nuc}} = 0.3$ case had a different “best fit” Young’s moduli than either the $v_{\text{Cyto}} = v_{\text{Nuc}} = 0.4$ or $v_{\text{Cyto}} = v_{\text{Nuc}} = 0.5$ cases.

Figure 16 shows a 3-D plot of $\delta_{RMS}$ as a function of $E_y^{\text{Cyto}}$ and $E_y^{\text{Nuc}}$ for $v_{\text{Cyto}} = v_{\text{Nuc}} = 0.5$. In this refined search, the minimum Young’s moduli were determined to be $E_y^{\text{Cyto}} = 3.75$ kPa and $E_y^{\text{Nuc}} = 5.25$ kPa, with $\delta_{RMS} = 0.02276$. In the refined search for $v_{\text{Cyto}} = 0.4$ and $v_{\text{Nuc}} = 0.5$, minimum Young’s moduli were determined to be $E_y^{\text{Cyto}} = 4.0$ kPa and $E_y^{\text{Nuc}} = 4.75$ kPa, with $\delta_{RMS} = 0.02572$. Without a nuclear inclusion, $E_y^{\text{Cell}} = 4.25$ kPa, which did not change for $v_{\text{Cell}} = 0.4$ or 0.5.

Finally, experimental strains were varied to ascertain the effects of the experimental measurements on the ratio of cytoplasmic to nuclear stiffnesses. Slight changes in the experimental data of Leipzig and Athanasiou$^{168}$ did not yield substantial differences in the calculated stiffness for the cytoplasm and nucleus. In the case where the experimentally measured axial and lateral cytoplasmic strains were increased by 5%, the Young’s moduli were determined to be $E_y^{\text{Cyto}} = 3.0$ kPa and $E_y^{\text{Nuc}} = 5.5$ kPa. In the alternative case, where the experimentally measured axial and lateral nuclear strains were decreased by 5%, the Young’s moduli were determined to be $E_y^{\text{Cyto}} = 3.5$ kPa and $E_y^{\text{Nuc}} = 6.0$ kPa. Finally, when the cytoplasmic strains were increased by 5% and nuclear strains decreased by 5%, the Young’s moduli were determined to be $E_y^{\text{Cyto}} = 3.0$ kPa and $E_y^{\text{Nuc}} = 6.0$ kPa, yielding a nuclear to cytoplasmic stiffness ratio of 2.0.
Discussion

Using a finite element approach, this chapter investigated *in situ* mechanical properties of the nucleus. Young’s moduli and Poisson’s ratio were parametrically changed for the cytoplasm and nucleus, and predicted cellular and nuclear strains were compared to known experimental results during unconfined cytocompression. It was found that the experimental data were best matched when $E_{Y_{\text{Cyto}}} = 3.75$ kPa, $E_{Y_{\text{Nuc}}} = 5.25$ kPa, and both cytoplasm and nucleus were incompressible. These results suggest that the ratio of nuclear to cytoplasmic stiffness is less than previously reported for single cells. Moreover, changing Poisson’s ratio had little effect on the model.

Examining nuclear mechanical properties is an important step toward understanding cellular mechanotransduction. Nuclear physical characteristics change in response to an applied load on native tissue or tissue engineered constructs. Enclosed within the nucleus, chromatin is organized by the nuclear lamina. Due to cellular deformations, mechanical linkages between cytoskeleton and nuclear lamina may lead to changes in chromatin conformation/alignment and/or 3-D spatial orientation of transcription factors. Thus, understanding how the nucleus senses and responds to forces provides insight into possible gene regulatory mechanisms.

The results presented in this chapter have applicability to current finite element models of cell – matrix interactions. These models predict the local mechanical environment of chondrocytes under various loading conditions. When considering the cell without a nuclear inclusion, cellular Young’s modulus was minimally greater than the cytoplasm itself. Moreover, the *in situ* difference between cytoplasmic and nuclear stiffnesses during compression is small, and variations in Poisson’s ratio had
little effect on Young's moduli (i.e. $E_{\text{Cyto}} = 3.75 \text{kPa}$, $E_{\text{Nuc}} = 5.25 \text{kPa}$ for $\nu_{\text{Cyto}} = 0.5$, $\nu_{\text{Nuc}} = 0.5$ versus $E_{\text{Cyto}} = 4 \text{kPa}$, $E_{\text{Nuc}} = 4.75 \text{kPa}$ for $\nu_{\text{Cyto}} = 0.4$, $\nu_{\text{Nuc}} = 0.5$). These results suggest that assumptions of cellular homogeneity and incompressibility may be valid simplifications for theoretical models describing chondrocytes. Supporting these simplifications, no volume change has been measured experimentally in single chondrocytes subjected to unconfined compression at strain levels below $\sim$30-35%.\textsuperscript{173}

Several explanations exist for why our results differ from previously reported nuclear and cellular stiffnesses. We observed a nuclear to cytoplasmic stiffness ratio of $\sim$1.1 or $\sim$1.4 depending on the assumed combination of Poisson's ratios, whereas prior results from micropipette aspiration testing of chondrocytes and isolated nuclei suggest this ratio is 3 to 4.\textsuperscript{118} However, in comparison to the free-floating state in micropipette aspiration, cytoskeletal rearrangements during cell attachment for unconfined cytocompression may cause alterations in nuclear structure and decreased stiffness. Moreover, when tested \textit{in situ}, connections between the nuclear lamina and cytoskeleton are intact, resulting in a more integrated mechanical framework between the cytoplasm and nucleus. Further, the cellular stiffness under compression is greater than previous micropipette aspiration results with single chondrocytes,\textsuperscript{146} in which tensile forces dominate. Prior literature has confirmed that cell stiffness is greater during bulk cell compression than during local aspiration of the cell membrane.\textsuperscript{31, 139, 146}

It is further important to note that the stiffness values for single chondrocytes calculated in this chapter coincide with measurements obtained by Knight and Bader\textsuperscript{206} for cells compressed within alginate constructs. In both unconfined cytocompression and cell compression within constructs, single cells must withstand compressive forces
applied onto the whole cell, and thus a similar mechanical response is expected. Additionally, the predicted stiffness value of the overall cell using our model is 2 to 3 times greater than previous unconfined compression results for single chondrocytes.\textsuperscript{31,133} Since our finite element model represents the cell as an ellipsoid, which more accurately resembles the geometry of attached chondrocytes than a cylinder,\textsuperscript{145} the increased stiffness is likely an effect of modeling the changing contact surface area. The contact area between the cell (represented by our geometry) and the platen in the compressed state at equilibrium is 28\% of that obtained assuming the entire cell’s cross-section is in contact under compression (cylindrical geometry). Thus, for the same applied force, the stress experienced by our cell would be approximately 3.6 times greater than that of a cell with an assumed cylindrical geometry. Smaller contact area results in increased stress and, hence, greater cell stiffness.

Several assumptions were made for this model which could limit its representation of physical reality. First, the assumption of a spherical nucleus can potentially affect the resultant properties of the cytoplasm and nucleus. Changes in nucleus size, morphology, uniformity, and connections with the cytoplasm may alter the stress distributions applied on the nucleus, thereby changing the calculated stiffness of the nucleus. For instance, a larger contact area between the nucleus and cytoplasm could decrease the stress in the nucleus, resulting in a lower nuclear stiffness. In addition, preferential interactions between the nucleus and cytoskeleton could result in non-uniform nuclear deformations, changing the calculated stiffness values. In this chapter, we choose to assume a simple spherical geometry because previous finite element modeling\textsuperscript{208} and experimental data\textsuperscript{119} have used or suggested, respectively, a spherical
nucleus. An additional limitation is that the cell is assumed to be in frictionless contact with the platen, which is inherently difficult to verify experimentally. This may result in variations in cell stiffness and, thus, different cytoplasmic to nuclear stiffness ratios. However, as mentioned in the previous paragraph, the high cell stiffness reported in this chapter may also be due to a more accurate representation of cellular geometry. Finally, this model assumed both the cytoplasm and nucleus to be isotropic materials. Differences in the Poisson’s ratio between principal directions can alter the axial and lateral strains and, thus, the calculated mechanical material properties. However, the experimental results presented by Leipzig and Athanasiou suggest a deviation from isotropy only at loads 50 nN or greater. At the 25 nN load case, examined in this chapter, the experimental Poisson’s ratio values for the cell and nucleus were 0.45 and 0.42, respectively. These values have been confirmed with other experimental testing modalities, generally yielding Poisson’s ratios between 0.3 and 0.5. Thus, based on previous experimental work at the low loads and strains examined in our model anisotropy was not considered. In the future, this model can be adapted to include consideration of varying nuclear morphologies, cytoskeletal and nuclear interactions, frictional contact between the cell and the compressing platen, and anisotropies.

In conclusion, this chapter elucidated a combination of chondrocyte cytoplasmic and nuclear stiffnesses and Poisson’s ratios which simulated previous results for unconfined cytocompression. In situ, nuclear stiffness was determined to be 40% more than the cytoplasm, which is lower than previously reported. Moreover, little effect of Poisson’s ratio on the model’s behavior was observed, and the incompressible case
best matched the prior experimental data. These results have implications in understanding the basis of cellular mechanotransduction.
Table 5: Effect of Poisson’s ratio combinations on cytoplasmic and nuclear stiffnesses

In the initial coarse search, cytoplasmic and nuclear stiffness values were each varied parametrically from 1 to 7 kPa, resulting in 169 different stiffness combinations for each set of Poisson’s ratio values. In the coarse search, it was observed that the $\nu_{\text{Cyto}} = \nu_{\text{Nuc}} = 0.5$ combination best fit the experimental data with the lowest cost function value, resulting in $E_{\gamma}^{\text{Cyto}} = 3.5$ kPa, $E_{\gamma}^{\text{Nuc}} = 5$ kPa. The similarity of “best fit” stiffnesses and cost function demonstrate that Poisson’s ratio had little effect on the ratio of cytoplasmic to nuclear stiffness.

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<td>Cytoplasm (kPa)</td>
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<td>0.3</td>
<td>4</td>
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Axisymmetric finite element depiction of a chondrocyte, with distinct nuclear and cytoplasmic regions, undergoing unconfined compression. In its undeformed state (A), the cell is considered to be 10 μm tall and 12 μm wide, with a spherical nuclear inclusion of radius 2.5 μm. The lateral and axial deformations of both the cytoplasm and nucleus were measured in response to a 25 nN load applied onto the cell (B). The linear elastic mechanical properties of the cytoplasm and nucleus were varied parametrically, and a cost function ($\delta_{RMS}$) value was calculated for each case in comparison to known experimental data.\(^{168}\) (ax = axial; lat = lateral)
Figure 16: Cost function plots for the case of $v_{Cyto} = v_{Nuc} = 0.5$.

In the initial coarse search, the elastic moduli of the cytoplasm and nucleus were varied from 1 kPa to 7 kPa, at 0.5 kPa increments. In the exploded view, the search was refined to range from 3 kPa to 5.5 kPa, at 0.25 kPa increments. A global minimum for $\delta_{RMS}$ was observed at $E_{Cyto}^* = 3.75$ kPa, $E_{Nuc}^* = 5.25$ kPa (indicated by the asterisk).
CHAPTER 4: Biomechanics of single chondrocytes under direct shear

Abstract

Articular chondrocytes experience a variety of mechanical stimuli during everyday activity. One such stimulus, direct shear when applied at the tissue level, is known to affect chondrocyte homeostasis and induce catabolic or anabolic pathways. Understanding how single chondrocytes respond biomechanically and morphologically to various levels of applied shear is an important first step toward elucidating tissue level responses and disease etiology. To this end, a novel videocapture method was developed in this study to examine the effect of direct shear on single chondrocytes, applied via the controlled lateral displacement of a shearing probe. Through this approach, precise force and deformation measurements could be obtained during the shear event, as well as clear pictures of the initial cell-to-probe contact configuration. To further study the non-uniform shear characteristics of single chondrocytes, the probe was positioned in three different placement ranges along the cell height. It was observed that the apparent shear modulus of single chondrocytes decreased as the probe transitioned from being close to the cell base (4.1 ± 1.3 kPa), to the middle of the cell (2.6 ± 1.1 kPa), and then near its top (1.7 ± 0.8 kPa). In addition, cells experienced the greatest peak forward displacement (~30% of their initial diameter) when the probe was placed low, near the base. Forward

cell movement during shear, regardless of its magnitude, continued until it reached a plateau at ~35% shear strain for all probe positions, suggesting that focal adhesions become activated at this shear level to firmly adhere the cell to its substrate. Based on intracellular staining, the observed height-specific variation in cell shear stiffness and plateau in forward cell movement appeared to be due to a rearrangement of focal adhesions and actin at higher shear strains. Understanding the fundamental mechanisms at play during shear of single cells will help elucidate potential treatments for chondrocyte pathology and loading regimens related to cartilage health and disease.
Introduction

Articular cartilage is a highly mechanical tissue that lines diarthrodial joints and functions to ensure proper joint movement. Due to its biomechanical nature, cartilage physiology is largely maintained by proper mechanical stimulation during normal activity. A variety of forces are present within cartilage and have been shown to affect tissue composition and integrity, including compression, tension, shear, and hydrostatic pressure. The particular stress and strain fields that the chondrocyte experiences within its microenvironment are influenced by its physical properties relative to its immediate tissue matrix surroundings. Cellular interpretations of these mechanical signals through mechanotransductive pathways may induce either catabolic or anabolic gene expression changes, thereby altering the essential extracellular matrix synthesis for cartilage tissue. In addition, chondrocyte mechanical characteristics will affect cellular deformations and the transmission of direct strain to the nucleus, which also alters biosynthetic activity. Therefore, much cartilage research has specifically focused on studying the mechanical properties and behavior of individual chondrocytes toward understanding the role of mechanical forces in precipitating tissue regeneration or degeneration.

Various single cell testing modalities have been developed to simulate certain aspects of the chondrocyte biomechanical milieu. While the exact in vivo stress and strain environments of chondrocytes can never be fully replicated with any existing apparatus, these approaches present the distinct advantage of identifying the precise levels of force necessary to initiate changes in cellular activity or physical characteristics. Moreover, the information gained through these experiments may be coupled with multi-scale finite
element models, which describe the local mechanical environment of chondrocytes, to gain insight to the response of cartilage to various loading conditions. The majority of previous research efforts have utilized such experimental modalities as atomic force microscopy, unconfined compression, or micropipette aspiration to yield indicators for single chondrocyte biomechanics under compression or tension. To also examine the adhesive strength of the connections between a cell and its substratum, recent efforts in our group have examined the maximum force necessary to detach a cell from various biomaterials. However, to date, little work has been performed to singly investigate the mechanical characteristics of chondrocytes under direct shear.

While previous research has demonstrated that shear forces directly influence cellular homeostasis and matrix production, the fundamental mechanisms at play during these processes remain elusive. Using a single cell approach, a lateral mechanical force can be applied onto a single adherent cell to simulate shearing behavior. Unlike fluid flow-induced shear that populations of cells experience in a flow or perfusion chamber, direct shear allows the precise measurement of cell strain and shear forces, as well as the control of the contact region for the cell experiencing the shear force. A concrete understanding of chondrocyte behavior under shear can also aid computational models that are predictive of intracellular signaling and structural changes under mechanical perturbations. In this manner, a detailed insight into the cellular responses and mechanical characteristics during shear may shed light on loading regimens related to cartilage health and disease.

The overall objective of this study was to investigate the biomechanical properties and intracellular structural changes during direct shear of single chondrocytes. A novel
videocapture method was developed to visualize the morphological alterations of the single cell under shear and to acquire precise measurements of force and strain. Individual chondrocytes were sheared using a probe placed at various positions along their height to further examine spatial variations in cellular shear properties. The intracellular organization of the actin cytoskeleton and focal adhesions under mechanical shear were additionally investigated toward understanding the response of single chondrocytes under shear. Based on prior observations demonstrating a correlation between cell stiffness and actin abundance, it was hypothesized that chondrocytes would exhibit non-uniformity in their mechanical properties related to changes in their actin network during shear. It was further hypothesized that focal adhesions and actin would reorganize to the trailing side of the cell, since this region is experiencing the highest levels of stress.

Methods

Cell isolation and seeding

Articular cartilage was harvested from the fetlock joint of mature steers obtained from a local abattoir (Doreck and Sons Packing Company, Santa Fe, Texas). Single chondrocytes were isolated from the middle/deep region of the tissue through overnight digestion in 0.2% collagenase and 0.3% dispase, as previously described. After tissue digestion, chondrocytes were counted and resuspended in supplemented DMEM (0.1 mM NEAA, 100U/ml penicillin/streptomycin, 0.25 μg/ml fungizone) at a concentration of 2.0 x 10^5 cells/ml. Cut pieces of glass microscopy slides (~5 x 20 mm) were placed in a
single well of a 6-well plate and covered with 3 ml of the cell suspension, yielding an areal cell seeding density of approximately $10^5$ cells/cm$^2$. The plates were incubated at $37^\circ$C and 10% CO$_2$ for 3h to allow for proper chondrocyte attachment.$^{31,176}$

**Cytoshear and videocapture**

Direct shearing of single chondrocytes was performed using a modified cytodetachment apparatus.$^{142,143}$ This device was adapted from its original setup to allow for the videocapture of cells experiencing mechanical shear. Essentially, the glass slides were transferred to a Petri dish which was placed on an IMT-2 inverted microscope (Olympus America, Melville, NY, USA), such that the cells could be viewed perpendicular to the original seeding plane at 400x magnification. Supplemented DMEM with 30 mM HEPES was added to the dish throughout the duration of the experiment to prevent pH changes in the ambient conditions. A 50.8 µm diameter tungsten probe (Advanced Probing Systems, Inc., Boulder, CO, USA) was then aligned to the side of an attached cell (Fig. 17). Three general ranges ($n = 8 - 10$ cells / group) were examined for the vertical distance of the probe from the glass: 20 – 35% of original cell height ("low"), 35 – 50% of original cell height ("medium"), and 50 – 65% of original cell height ("high"). Control over the distance between the bottom of the probe and the glass slides was achieved via fine linear positioners, as confirmed by visualization on the microscope. A piezoelectric motor then drove the probe a prescribed distance (chosen between 10 – 15 µm) laterally toward the cells at a rate of 4 µm/s. The entire shear event was recorded through an AVC-D7 CCD camera (Sony USA) connected to the microscope. Videos were saved as an AVI file at 640 x 480 resolution for subsequent analysis.
Video analysis and biomechanical measurements

Individual frames from the videos were extracted using Videomach 4.0.2 software (Gromada.com). Images of the initial cell-probe configuration, initial cell-probe contact, and the cytoshear event every 0.25 s thereafter were examined. The dimensions of the cell and probe positions in each frame were marked using Microsoft Paint 5.1 (Microsoft Corporation, Redmond, WA, USA). A pixel-to-micron ratio of 7.0 was employed in all image subsequent analysis and the accuracy of the measurements was deemed to be 2 pixels or 0.29 μm.

Immediately prior to initial cell-probe contact, the cell width \( W_C(0) \), cell height \( H_C \), and probe height from the base \( H_P \) were measured. The relative positive of the probe onto the cell was calculated as:

\[
\text{Probe position (\%)} = \frac{H_P}{H_C} \quad (1)
\]

Upon shear application, the probe indentation onto the cell was defined as the forward movement of the probe less the movement of backmost part of the cell (Fig. 18A). The shear strain \( (\varepsilon) \) at each time interval was then calculated as probe indentation divided by \( W_C(0) \). Cantilever beam theory was further employed to determine the reaction force of the cell at the various strain levels:

\[
F = \frac{3EI}{L^3} \delta \quad (2)
\]

where \( E \) (Young’s modulus), \( I \) (moment of inertia), and \( L \) (cantilever beam length) are known parameters of the tungsten probe. The deflection of the cantilever \( (\delta) \) was calculated by comparing the true displacement of the probe (via video analysis) with the
prescribed piezoelectric displacement for each time step. The cell-probe contact area for this reaction force was estimated as the upper half of an ellipse (Fig. 18B):

\[
A = \frac{1}{2} \pi \left( \frac{W_c(0)}{2} \right) \left( H_c - H_p \right)
\]

where \( \frac{W_c(0)}{2} \) and \( (H_c - H_p) \) represent major and minor radii of the ellipse, respectively.

Then considering only the significant linear portion of the force versus shear strain plot, this slope was normalized to the cell-probe contact area to yield an apparent shear modulus.

The cell’s width \( W_c(t) \) and position of the cell’s trailing edge at each time interval were also recorded as indicators for physical changes in the cell’s morphology and location during the shear event. In particular, the cell’s elongation was determined as:

\[
\text{Elongation} = \frac{W_c(0)}{W_c(t)} - 1
\]

In addition, the forward distance its trailing edge traveled during shear application, divided by the initial cell diameter, represented a normalized forward body movement (nFBM) for each cell.

**Immunocytochemistry**

Fluorescent staining was performed to observe alterations in focal adhesion organization and the actin cytoskeleton in response to direct shear. Chondrocytes were seeded using the protocol described above, except onto microscope slides that were etched with an indelible marker along their underside. Immediately prior to experimentation, a digital image was taken of the cell seeding pattern in one of the etched
regions on the slide, in order to identify specific chondrocyte locations. After 3 hrs of culture, the slides were placed flat inside a Petri dish on the IMT-2 microscope such that the cells could be viewed bottom-up. The same region of the slide that was previously imaged digitally was located on the microscope and a single chondrocyte within this region was selected for shearing and fixation. A probe was placed immediately adjacent to this cell and translated laterally toward the cell, similar to the protocol described above. The cell was held at a high shear strain level for 10 min, at which point 4% paraformaldehyde was carefully added to the dish and fixation was allowed to occur for an additional 10 min.

After the cell was fixed, the microscope slide was removed from the Petri dish, washed with PBS, blocked with 10% FBS, and permeabilized with 0.1% Triton X-100 for cell staining. Slides were then incubated with AlexaFluor 647 Phalloidin (Invitrogen, Carlsbad, CA) for actin visualization, mouse anti-Vinculin primary antibody (Sigma-Aldrich, St. Louis, MO) followed by a goat anti-mouse secondary antibody (AlexaFluor 488; Invitrogen, Carlsbad, CA) for focal adhesion imaging, and Hoescht’s dye (Invitrogen, Carlsbad, CA) for nuclei staining. The slide was viewed on a LSM 510 confocal microscope (Carl Zeiss, Oberkochen, Germany). Images of the sheared cell were acquired using a 63x objective with Z-stacks (at 0.2 μm) through the cell. Unperturbed cells were similarly imaged on the same slide for use as controls.

*Data analysis*

All statistical analysis was performed using Matlab R2007b (Mathworks, Natick, MA, USA). A single factor ANOVA was used with a Fisher’s Least Significant
Difference post-hoc test (when warranted) to identify differences in mechanical characteristics obtained with the different probe heights. Significance was defined as $p < 0.05$ throughout the study.

Results

In this study, single chondrocytes were examined for their biomechanical characteristics and morphological changes under shear through video analysis of the mechanical event (Fig. 19). Substantially different trends in the forward movement of the cell’s trailing edge and deflection of the probe (and thus, applied force) could be observed for probes placed in the low, medium, and high positions during the shear application.

*Mechanical response during shear*

Shearing cells at each probe position resulted in different trends in force accumulation (Fig. 20). Force values were generally negligible until around 15-20% applied strain for all groups, after which the force levels increased significantly. However, the slope of force versus shear strain was substantially different among cells being sheared from the three probe heights. The low probe height typically induced the greatest slope in force accumulation, while the high probe height generally led to a lower slope. After normalizing this slope of the linear region in each force plot to the cell-probe contact area, variations in apparent shear moduli along the cell’s height became evident (Fig. 21). The apparent shear moduli values were $4.1 \pm 1.3$ kPa, $2.6 \pm 1.1$ kPa, $1.7 \pm 0.8$
kPa for cells experiencing shear from probes in the low, medium, and high positions, respectively. Significant differences were observed between the moduli of all three groups (p<0.05).

**Physical cell alterations during shear**

The average height and diameter of chondrocytes were found to be 10.9 ± 1.2 μm and 11.4 ± 1.0 μm, respectively. Moreover, the cells consistently exhibited an initial elliptical morphology during all trials, as demonstrated by a bulging along the cell periphery.

Salient changes in the forward movement and elongation of the cell for the different probes heights are summarized in Table 6. Cells sheared from all three probe positions experienced forward movement of their trailing edge, as indicated by their nFBM. An accumulation of nFBM did not occur until soon after the probe crossed the original vertical plane of the cell’s trailing edge, typically around 15-25% strain. The nFBM for each cell then increased until it reached a plateau at approximately 35% strain (Fig. 22). This plateau strain did not change between probe heights. However, the magnitudes of the peak nFBM did vary among the three probe heights, with a significant difference observed between the low and medium probe positions. In addition, the time for the cell to reach this plateau changed as a function of initial probe height, with significant differences observed between the low and high probe positions. Moreover, the elongation of the cell significantly increased at greater applied strain levels and was unaffected by the initial probe position.
**Intracellular changes during shear**

Cells, either sheared or control, were subsequently imaged to identify alterations in their intracellular structure, with consistent changes observed among the sheared cells. Distinct modifications in the actin network and focal adhesion organization occurred as chondrocytes individually experienced direct shear (Fig. 23). Control cells typically displayed rounded morphologies at all focal planes through the cell’s height. In addition, actin filaments and focal adhesions were equally distributed throughout the control cells’ periphery. In contrast, the sheared cells generally sloped downward and became narrower along their trailing side. The actin network in the sheared cells was predominately identified behind the nucleus on the trailing side of the cell and near the cells’ base. Lastly, focal adhesions were consistently concentrated along the trailing edge of the cells.

**Discussion**

The study was designed to examine the effect of direct shear, which is prevalent within cartilage, on chondrocyte biomechanical characteristics. Confirming our hypotheses, this study presents several notable findings relating to chondrocyte biomechanics and demonstrates a clear connection between physical cellular properties and intracellular organization. First, we identify that the shear stiffness of individual chondrocytes, as measured by an apparent shear modulus, is highly non-homogeneous, increasing toward the cell’s base. Second, cell forward movement during shear continues until a critical strain is reached, consistent among all probe heights, wherein the cell becomes strongly adhered onto the substrate. This result suggests that focal adhesion
activation may be a strain-dependent process within chondrocytes. Lastly, this study describes the development of a novel videocapture methodology to study the cytoshear characteristics of single chondrocytes.

To our knowledge, this is the first study to examine the mechanical properties of single chondrocytes experiencing direct shear. This is a necessary step in understanding the complete biomechanical milieu of chondrocytes within cartilage, which includes shear forces, among other mechanical pressure modalities. It is found that the apparent shear modulus increases (~1.5-fold) as the probe is repositioned from the cell top to its middle, and again increases (~1.5-fold) as the probe is further repositioned toward the cell base. This observed non-uniformity in cell mechanical properties, along its height can be attributed to spatial variations in the concentration of the actin cytoskeleton during shear. Actin filaments are highly sensitive to mechanical loading and dynamically adapt to perturbations in the cellular microenvironment through Rho-kinase mediated signaling mechanisms. In our study, sheared chondrocytes exhibit a reorganization of their actin filaments, which become localized on the trailing side of the cell, near its base. Moreover, it has been previously established that actin contributes significantly to cell stiffness parameters under compression or tension testing modalities. Thus, based on the results in this study, similar connections can be drawn between actin filaments and the spatial shear characteristics within a single cell. Elucidating the mechanisms at play in determining chondrocyte shear stiffness may have important ramifications in future tissue engineering efforts, which employ shear stress as a means toward improving cellular viability and matrix production. Furthermore,
through a precise knowledge of the apparent shear moduli of single chondrocytes, regimens can be developed to induce desired cell strains.\textsuperscript{226}

Studying the range of shear strains that induce desired changes in cellular behavior is critical toward understanding the mechanosensitivity of single chondrocytes and identifying potential loading modalities that promote tissue growth. Single chondrocytes begin to exhibit a forward movement along their substrate at \( \sim 15 - 25\% \) applied shear, which continues until a critical strain of \( \sim 35\% \) is reached. This forward movement may involve both a rolling and sliding component as the cell displaces in the direction of the probe, which may intrinsically entail the breakage of some focal contacts between the cell and its substrate. While the peak nFBM for chondrocytes increases as the probe is repositioned closer to the cell’s base, the critical strain wherein the nFBM reaches its plateau is not affected by the probe height. At this critical strain, the cell-substrate bonds may strengthen through focal adhesion activation mechanisms,\textsuperscript{227} thereby preventing additional movement of the cell’s trailing edge during shear. This result is supported by cytoimmunochemical staining of chondrocytes experiencing shear above this critical strain, where a clear rearrangement of focal adhesions to the cell’s trailing side is evident.

Our observation that the critical strain in nFBM is not altered by probe position suggests that focal adhesion recruitment may occur primarily through strain-dependent, rather than force-dependent, mechanisms. Previous research has found that focal adhesion complexes are indeed sensitive to mechanical stimulation and may develop at the location of the applied load.\textsuperscript{227-229} In the scenario where a critical shear strain results in a plateau of forward movement, stretch-activated ion channels may induce intracellular
signaling pathways once a critical strain is reached, which then leads to the development of strong focal adhesion complexes\textsuperscript{227, 230, 231}. These results lay the groundwork for future research to examine the real-time reorganization of focal adhesions or specific signaling activation pathways prevalent during shear of single cells, toward a better understanding of the levels of strain and intracellular mechanisms affecting cellular physiology.

A novel experimental approach is presented in this study to examine the shear characteristics of single cells. Through video analysis of the mechanical event, clear measurements of cellular deformations and movements can be obtained as the cell experiences direct shear. These physical cell alterations are further correlated with applied stresses or strains to yield fundamental properties for single chondrocytes. Our current experimental modality improves upon previous work in our laboratory which studied the lateral detachment force for individual adherent chondrocytes\textsuperscript{142-144} by enabling control of the probe height and capturing cell morphological changes via video recordings. Despite technological advances, an inherent limitation remains that chondrocytes must be removed from their native mechanical microenvironment for single cell testing. In essence, this sidesteps the natural transmission of forces through the chondrocyte pericellular matrix\textsuperscript{102, 232}. However, chondrocytes have been shown to retain their cytoskeletal organization\textsuperscript{114} and the biophysical differences between pathologic or regenerative states\textsuperscript{146, 171, 200} when isolated \textit{in vitro}. Furthermore, our experimental approach presents the unique advantage of singly investigating how cellular shear properties may be altered on various biomimetic materials and attributed to specific intracellular structures. For instance, single chondrocytes may be seeded on different coated substrates, such as with aggrecan or collagen, which are frequently utilized in
tissue engineering scaffolds.\textsuperscript{233} Future experimental work may also employ cytoskeletal inhibitors or growth factors to study the specific role of the actin network or focal adhesions in spatial shear properties.\textsuperscript{114, 176, 227}

In addition, studying the biomechanics of single chondrocytes undergoing shear has the potential to benefit future computational studies of cellular behavior. A number of studies in recent years have utilized theoretical approaches to replicate fundamental biological phenomena, including contractility and spreading characteristics on various surfaces, with remarkable similarity to experimental measurements.\textsuperscript{234-236} Furthermore, computational models have focused specifically on single chondrocytes experiencing various mechanical perturbations, such as the application of a lateral force,\textsuperscript{237} compressive load,\textsuperscript{216} or local membrane aspiration.\textsuperscript{208} These combinations of experimental and computational approaches have yielded important observations regarding the cell-substrate interface and intracellular contribution to cell stiffness, which could not be demonstrated in the experimental results alone. Our current experimental method for cell shear can provide inputs for three-dimensional computational models which simulate the rearrangement of the cytoskeleton via intracellular signaling mechanisms. In particular, an active bio-chemical-mechanical model has been developed to simulate the stress-dependent stress fiber activation and active focal adhesion assembly in single cells.\textsuperscript{220} These models may then be used to examine the thresholds of shear strains exerted onto chondrocytes that induce a rearrangement of the cytoskeleton and potentially alter cellular homeostasis or biosynthetic capacity.\textsuperscript{145} Thus, the future utilization of active cell modeling in parallel with \textit{in vitro} examination of subcellular
biomechanical phenomena provides a powerful novel tool to elucidate the mechanisms underlying cellular responses to the physical environment.

This study provides new insight into the biomechanical characteristics of single chondrocytes under shear. The apparent shear modulus of individual adherent chondrocytes is non-homogeneous since it is observed to increase toward the cell’s base, correlating with alterations in the actin network during shear. In addition, chondrocytes exhibit a forward movement in the direction of the shear force, until an applied shear strain is reached, regardless of initial probe position. Above this critical strain level, focal adhesions reorganize into strong contacts along the cell’s trailing edge to prevent additional forward movement. These results improve our knowledge of the chondrocyte mechanical environment and intracellular changes in response to applied loads. A firm understanding of the physical characteristics of single chondrocytes may shed light on the role of mechanical forces in promoting cartilage regeneration or degeneration.$^{189, 215}$
Table 6: Physical alterations of single chondrocytes experiencing direct shear

<table>
<thead>
<tr>
<th></th>
<th>“Low” probe height (20-35%)</th>
<th>“Medium” probe height (35-50%)</th>
<th>“High” probe height (50-65%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cell forward movement</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak normalized forward body movement (nFBM)</td>
<td>0.31 ± 0.14^A</td>
<td>0.14 ± 0.10^B</td>
<td>0.05 ± 0.04^B</td>
</tr>
<tr>
<td>Time to plateau (s)</td>
<td>2.42 ± 0.48^A</td>
<td>2.06 ± 0.72^A,B</td>
<td>1.63 ± 0.26^B</td>
</tr>
<tr>
<td>Shear strain for plateau (e, %)</td>
<td>32.4 ± 5.6</td>
<td>34.0 ± 12.9</td>
<td>35.7 ± 7.6</td>
</tr>
<tr>
<td><strong>Cell forward expansion</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Elongation @ e = 25% ( % increase)</td>
<td>7.0 ± 4.4</td>
<td>6.4 ± 6.2</td>
<td>7.9 ± 6.4</td>
</tr>
<tr>
<td>Elongation @ e = 50% ( % increase)</td>
<td>21.8 ± 5.8</td>
<td>24.3 ± 7.9</td>
<td>22.9 ± 10.4</td>
</tr>
<tr>
<td>Elongation @ e = 75% ( % increase)</td>
<td>38.4 ± 6.1</td>
<td>37.3 ± 17.9</td>
<td>34.4 ± 15.6</td>
</tr>
</tbody>
</table>
Figure 17: Illustration of the cytoshear setup

A piezoelectric actuator drives a shearing probe laterally toward articular chondrocytes seeded onto glass slides. Through visualization on an inverted microscope, the probe height can be controlled during the experiment. The entire mechanical event is further recorded through a CCD video camera connected to the microscope. The figure is not drawn to scale.
Figure 18: Biomechanical analysis of cytoshear

Through an examination of the video for each mechanical event, precise measurements of cell dimensions and probe positions can be obtained as the cell experiences direct shear (A). In its initial configuration, the cell width \( W_c(0) \), cell height \( H_c \), and probe height \( H_p \) are recorded. Then, every 0.25 s after the probe contacts the cell, the cell’s width \( W_c(t) \) is measured and subsequently used in the calculation for cell elongation. The cell’s trailing edge is also tracked as an indicator for forward body movement. The probe indentation, defined as the forward-most position of the probe minus the trailing edge of the cell, is further measured over time and used in the determination of shear strain. In addition, the applied force at each time interval can be computed by comparing this forward-most position of the probe to the prescribed probe displacement. The force values are then normalized to an estimated contact area between the cell and the probe to yield an applied stress. Looking head-on (B), this contact area can be defined as the upper half of an ellipse, with half of the cell’s initial width and the distance of the probe base to cell top as major and minor radii, respectively.
**Figure 19: Alterations in cellular morphology and position during cytoshear**

The entire mechanical event is video recorded and subsequently analyzed to yield mechanical properties and indicators for cell movement. Differences can be observed when the probe is placed in its low position (A - C), medium position (D - F), and high position (G - I) during the time course of shear application. Most notably, the cell’s trailing edge experiences a substantial forward movement when the probe is in the low position compared to the high position. In addition, through this videocapture methodology, information on applied shear strain and the cell’s reaction force is obtained by tracking the movement of the probe over time and measuring the initial dimensions of the cell.

<table>
<thead>
<tr>
<th></th>
<th>&quot;Low&quot; probe position</th>
<th>&quot;Medium&quot; probe position</th>
<th>&quot;High&quot; probe position</th>
</tr>
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<tbody>
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<td><img src="C" alt="Image" /></td>
<td><img src="F" alt="Image" /></td>
<td><img src="I" alt="Image" /></td>
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</table>
Figure 20: Representative force plots of single chondrocytes experiencing shear from different probe heights

Force values are typically minimal until around 15-20% applied strain, after which a significant increase in force is observed. In addition, the slope of force versus shear strain is noticeably different among the three probe heights, with the low probe height generally inducing the greatest slope in force accumulation.
Figure 21: Apparent shear moduli as a function of probe height

Single chondrocytes are perceived to be stiffer when the probe is placed lower to their base, with significant differences (p < 0.05) observed among all three groups. This suggests that the cell is non-uniform in its mechanical properties along its vertical axis. Data presented as mean ± standard deviations.
Figure 22: Representative normalized forward body movement (nFBM) plots of single chondrocytes experiencing shear from different probe heights

The nFBM of each cell indicates the forward distance its trailing edge travels during the shear event, divided by the initial cell diameter. These values are typically minimal until around 15-25% applied strain, since the probe has not yet crossed the vertical plane of the cell's trailing edge. After this point, a substantial increase in nFBM can be observed until it plateaus at approximately 35% strain. However, the magnitudes of this movement are noticeably different among the three probe heights, with the low probe height generally inducing the greatest forward displacement during shear.
Figure 23: Alterations in cell structure in response to direct shear

The bright field image, nuclei (displayed in blue), actin network (displayed in red), and focal adhesions (displayed in green) are shown for a representative control and sheared cell at different focal planes in the cell’s height. A clear change in cellular morphology and intracellular organization of actin and focal adhesions can be observed for the cell experiencing the shear force. The control cell exhibits a rounded morphology, with actin and focal adhesions equally distributed along its periphery at all focal planes. In contrast, the sheared cell undertakes a ‘sloped’ morphology along its trailing side, which converges akin to a ‘comet’s tail’. The actin network in the sheared cell can be identified concentrated close to the cell's base, on the trailing side of the cell directly behind the nucleus. In addition, strong pockets of focal adhesions can be observed beneath the trailing edge of the cell, near its base. For representation, this sheared cell experiences 75% applied shear strain.
CHAPTER 5: Mechanical characterization of differentiated human embryonic stem cells

Abstract

Human embryonic stem cells (hESCs) possess an immense potential in a variety of regenerative applications. A firm understanding of hESC mechanics, on the single cell level, may provide great insight into the role of biophysical forces in the maintenance of cellular phenotype and elucidate mechanical cues promoting differentiation along various mesenchymal lineages. Moreover, cellular biomechanics can provide an additional tool for characterizing stem cells as they follow certain differentiation lineages, and thus may aid in identifying differentiated hESCs which are most suitable for tissue engineering. This chapter examined the viscoelastic properties of single undifferentiated hESCs, chondrogenically differentiated hESC subpopulations, mesenchymal stem cells (MSCs), and articular chondrocytes (ACs). hESC chondrogenesis was induced using either Transforming Growth Factor-β1 (TGF-β1) or Knock Out Serum Replacer (KOSR) as differentiation agents and the resulting cell populations were separated based on density. All cell groups were mechanically tested using unconfined creep cytompression. Analyses of subpopulations from all differentiation regimens resulted in a spectrum of mechanical and morphological properties spanning the range of hESCs to MSCs to ACs.

Density separation was further successful in isolating cellular subpopulations with distinct mechanical properties. The instantaneous and relaxed moduli of subpopulations from TGF-β1 differentiation regimen were statistically greater than those of undifferentiated hESCs. In addition, two subpopulations from the TGF-β1 group were identified which were not statistically different from native articular chondrocytes in their instantaneous and relaxed moduli, as well as their apparent viscosity. Identification of a differentiated hESC subpopulation with similar mechanical properties as native chondrocytes may provide an excellent cell source for tissue engineering applications. These cells will need to withstand any mechanical stimulation regimen employed to augment the mechanical and biochemical characteristics of the neotissue. Density separation was effective at purifying distinct populations of cells. A differentiated hESC subpopulation was identified with both similar mechanical and morphological characteristics as ACs. Future research may utilize this cell source in cartilage regeneration efforts.
Introduction

The biomechanical properties of single cells may significantly influence tissue development and homeostasis. The physical characteristics of individual cells play a vital role in the generation of local stress-strain fields within the cellular microenvironment and the forces in turn experienced by the nucleus. It has recently been observed that cellular mechanical properties may be indicative of phenotypic alterations within mesenchymal lineages. Hence, cell biomechanical techniques have emerged as potential tools for the characterization and identification of cell populations during various developmental or differentiation processes. The most common of these biomechanics methodologies are atomic force microscopy, micropipette aspiration, cytoindentation, and unconfined cytocompression, which can yield the elastic or viscoelastic material properties of single cells given the assumptions of isotropy, incompressibility, and homogeneity.

Examining the mechanical properties of single human embryonic stem cells (hESCs) is of particular interest due to the pluripotent nature of these cells and their clear potential in an array of regenerative medicine applications. The prospect for using an abundant alternative cell source, such as hESCs, in tissue engineering is particularly appealing since this would obviate the common concerns of donor tissue scarcity or of dedifferentiation during autologous cell expansion. Despite their potential, an examination of the mechanical properties of naïve and differentiated hESCs has yet to be undertaken. An understanding of the mechanical characteristics of undifferentiated stem cells can greatly aid research investigating the forces necessary to promote differentiation into various cell lineages.
Embryonic stem cells have recently been utilized in cartilage tissue engineering efforts. Studying the mechanical properties of chondro-induced hESCs may identify certain differentiated cell subpopulations that are most similar to native chondrocytes. It is believed that these cell subpopulations will be most suited for use in a tissue engineering approach for articular cartilage since they would be able to sustain similar in vivo mechanical loads. In addition, through an understanding of the mechanical properties of these differentiated cell groups, loading regimens can be determined which elicit favorable biochemical or behavioral responses, and thus promote neotissue growth.

Several differentiation strategies have been previously investigated to chondrogenically induce hESCs within embryoid body (EB) cultures. Biochemical agents, such as transforming growth factor-β (TGF-β), or media supplements, such as Invitrogen’s Knock Out Serum Replacer (KOSR), have been employed to promote the chondrogenic phenotype. However, a prevailing concern among the various approaches is the production of non-uniform cell populations post differentiation. Thus, cell purification techniques are necessary to ensure that tissue engineered constructs are formed with homogeneous, chondrogenically differentiated hESCs. One such methodology, a Percoll gradient system, is capable of separating articular chondrocytes (ACs) based primarily on cell density, resulting in populations that differ in cell morphology, nucleus size, and protein synthesis. Moreover, cell fractions originating from embryonic cells have shown significant differences in chondrogenic potential, both in monolayer and micromass cultures. Therefore, it is of
interest to examine potential differences in various chondro-induced hESC subpopulations, separated based on cell density.

The objectives of this chapter were to characterize the viscoelastic material properties of single hESCs and to identify mechanical differences between hESCs and their chondrogenically differentiated counterparts. Chondrogenesis was induced using two differentiation agents (TGF-β1 and KOSR), and the resulting cell populations were fractionated based on density. Mechanical properties of the undifferentiated hESCs and differentiated hESC cell subpopulations were measured using unconfined creep cytocompression. We hypothesized that density separation of differentiated hESCs would yield subpopulations with different mechanical characteristics. We further hypothesized that a chondrogenically differentiated hESC subpopulation can be identified with stiffness properties and morphologies similar to those of native mesenchymal stem cells (MSCs) or ACs.

Methods

Chondrogenic differentiation of human embryonic stem cells

The NIH-approved H9 hESC line (Wicell, Madison, WI) was cultured at passage 39 according to Wicell’s instructions on irradiated CF-1 mouse embryonic fibroblasts (MEFs, Charles River Laboratory, Wilmington, MA). Colonies were passaged using 0.1% type IV collagenase (Invitrogen, Carlsbad, CA) every 4-6 days. For the final passage prior to EB formation, colonies were passaged onto Matrigel (BD Biosciences, San Jose, CA) coated plates to reduce contamination of hESC colonies with feeder cells.
While on Matrigel, the colonies were given MEF-conditioned medium. Once the hESC colonies on Matrigel reached 70-80% confluence, dispase (0.1% w/v in DMEM/F-12) was applied for 10–15 min to lift the hESC colonies from the culture dish. This left MEFs behind to form EBs from the hESC colonies. After two washes with DMEM/F-12, the EBs were suspended in a chondrogenic medium containing either 1 ng/ml TGF-β1 (Peprotech, Rocky Hill, NJ) or 5% KOSR (Invitrogen, Carlsbad, CA). The base chondrogenic medium consisted of high-glucose DMEM (Invitrogen, Carlsbad, CA), $10^{-7}$ M dexamethasone, ITS+ Premix (6.25 ng/ml insulin, 6.25 mg transferrin, 6.25 ng/ml selenious acid, 1.25 mg/ml bovine serum albumin, and 5.35 mg/ml linoleic acid; Collaborative Biomedical, San Jose, CA), 40 μg/ml L-proline, 50 μg/ml ascorbic acid, and 100 μg/ml sodium pyruvate. The EBs were then distributed into Petri dishes (Fisher, Hampton, NH) containing 15 ml of medium per dish. EBs were cultured for 3 wks with media changes every 48 h.

**EB digestion and density separation**

After 21 days of differentiation, EBs were digested in 0.05% trypsin-EDTA (Invitrogen, Carlsbad, CA) for 1 h followed by up to 1 h of 0.15% collagenase II (Worthington Biochemical Corp., Lakewood, NJ) digestion until a suspension of single cells was created. Cells were counted with a hemocytometer, washed with DMEM containing 1% FBS, centrifuged at 200 x g, and resuspended in 2 ml of DMEM.

Density separation of differentiated hESCs was performed as described previously for chondrocytes and heart mast cells. Isotonic Percoll (Sigma, St. Louis, MO) was mixed with sterile PBS (HyClone, Logan UT) to produce a 60% stock solution.
The stock was further diluted with PBS to produce Percoll solutions of 10, 20, 30, 40, 50, and 60%. A pre-formed density gradient was created by sequentially layering 2 ml of each Percoll solution, starting with 60%, into a 15 ml conical bottom tube (VWR, Bridgeport, NJ). Two ml of DMEM containing the differentiated hESCs was carefully layered on top of the gradient, and the tube was centrifuged at 400 x g for 20 min. After centrifugation, the cells collect at the interface between Percoll layers, which relates to their cellular density. The interface between each density layer was then isolated along with 1 ml of Percoll above and below each interface, using a sterile pipette. The Percoll was diluted with 8 ml of DMEM, centrifuged at 200 x g, and the cell pellet was resuspended in 2 ml DMEM. Cells from each interface were counted with a hemocytometer.

MSC culture and articular cartilage isolation

Human MSCs from the bone marrow of one donor, age 35, were obtained from the Tulane Center for Gene Therapy (New Orleans, LA). Cells were seeded at 60 cells/cm² in T75 flasks (BD Biosciences, San Jose, CA) and cultured in α-MEM (Invitrogen, Carlsbad, CA) containing 16.5% Fetal Bovine Serum (‘FBS’, Atlanta Biologicals, Lawrenceville, GA), 4 mM L-glutamine, and 100 units/ml penicillin/streptomycin (Invitrogen, Carlsbad, CA). Cells were passaged every 7-10 days using 0.05% trypsin-EDTA (Invitrogen, Carlsbad, CA) and replated into T75 flasks. Undifferentiated MSCs were used for the experiment at passage 4.

Human ACs were isolated from healthy cartilage of one donor, age 25, excised as part of a surgery at M.D. Anderson Cancer Center (Houston, TX) to remove an
osteosarcoma (Rice University IRB approval #08-115X). Articular cartilage tissue was digested overnight using 0.2% collagenase type II (w/v) (Worthington Biochemical, Lakewood, NJ) in supplemented DMEM (0.1 mM NEAA, 100U/ml penicillin/streptomycin, 0.25 µg/ml fungizone) at 37°C and 10% CO₂.

Cell seeding

Isolated cells from each density layer were resuspended in their appropriate differentiation medium (TGF-β1 or KOSR) and seeded within a silicone isolator (PGC Scientifics, Gaithersburg, MD) onto a tissue culture dish to yield an approximate density of 3.8 x 10⁴ cells/cm². Culture plates were incubated for 3 – 5 h at 37°C and 10% CO₂ to allow for proper cell attachment prior to cytocompression testing. Previous studies in our laboratory have shown that seeding time after a minimum of 3 h does not affect the viscoelastic properties of single cells. The same seeding method was employed for undifferentiated hESC, human AC, and human MSC controls.

Creep cytocompression

Unconfined creep cytocompression experiments were performed on each experimental group (n > 10 cells / group) using the same medium as the seeding phase, supplemented with HEPES buffer (Fisher Scientific, Pittsburgh, PA) to prevent pH changes while the culture dish was exposed to ambient conditions. A previously validated creep cytoindentation apparatus was used to apply controlled stresses onto single adherent cells via a 50.8 µm diameter tungsten probe. Cells were positioned directly
below the probe, as confirmed through visualization on an inverted microscope. The compressing tip was driven toward the cell by vertical control over the far end of the probe using a piezoelectric actuator. A laser displacement meter simultaneously tracked the true position of the probe tip (Fig. 24). Before each trial, the system was calibrated by comparing known piezoelectric displacements with recorded measurements from the laser micrometer. During cytocompression, the deflection of the probe (δ) was calculated based on the differences in piezoelectric movement and laser displacement measurements. Cantilever beam theory was then used to calculate the reaction force by the cell, based on known physical parameters of the tungsten probe and the measured deflection distances. Finally, a closed-loop algorithm was employed to maintain a constant force level of 100 nN onto each cell for 30 s by appropriately moving the piezoelectric actuator. Cell diameter was measured with a reticle in the microscope objective. Applied stress was defined as the force divided by the maximum cell diameter. Cell height was determined by measuring the contact distance between the probe and the culture dish after each test, and comparing it to the contact distance of the cell.

Viscoelastic properties

The unconfined compression creep behavior of single cells was fitted to a standard linear viscoelastic solid model. Previous work has shown that the viscoelastic model accurately depicts the initial creep behavior of single chondrocytes. Briefly, this model considers the cell to be an isotropic, homogeneous, and incompressible viscoelastic solid undergoing small deformations. It yields three unique material properties: instantaneous modulus (E₀), relaxed modulus (Eₓ), and apparent viscosity (μ).
The experimental deformation behavior of single cells over time was analyzed using the following equations:

\[ u(t) = \frac{2\sigma h_0}{3E_\infty} \left[ 1 + \left( \frac{\tau_c}{\tau_\sigma} - 1 \right) e^{\frac{-t}{\tau_\sigma}} \right] H(t) \]  

\[ \frac{E_0}{\tau_c} = \frac{\tau_\sigma}{\tau_\epsilon} E_\infty \]  

\[ \mu = \tau_\epsilon (E_0 - E_\infty) \]

where \( u(t) \) is the cell deformation over time, \( \sigma \) is the applied constant stress, \( h_0 \) is the initial cell height, \( H(t) \) is the step function, and \( \tau_c \) and \( \tau_\sigma \) are the stress and creep relaxation time constants, respectively. Creep curves were fitted to this viscoelastic model using Matlab 6.5 (The MathWorks, Natick, MA), via the non-linear Levenburg-Marquardt method.

**Histology and immunohistochemistry**

To confirm cartilaginous differentiation of the hESCs, representative EBs from the TGF-β1 and KOSR groups were frozen in cryoembedding medium and sectioned at 12 μm thicknesses. Safranin-O and fast green staining was used to examine the presence of sulfated glycosaminoglycans (s-GAG). Additional slides were processed with immunohistochemistry (IHC) analyses to visualize collagen types I and II. Briefly, these slides were fixed in chilled acetone, quenched of exogenous peroxidase activity with 3% \( \text{H}_2\text{O}_2 \) in methanol, blocked with serum (Vectastain ABC kit, Burlingame, CA), and incubated with either mouse anti-collagen type I antibody (Axell, Westbury, N.Y.) or
rabbit anti-collagen type II antibody (Cederlane, Burlington, NC). The appropriate mouse or rabbit secondary antibody (Vectastain ABC kit) was applied, followed by the avidin-biotinylated enzyme complex (Vectastain ABC kit), DAB reagent (Vector Labs), and hematoxylin counterstain to visualize nuclei. Native tendon and articular cartilage served as positive and negative controls. To assess if the hESCs had differentiated along other mesenchymal lineages, additional slides were processed with Von Kossa and oil red O stains for mineralization and adipose tissue, respectively.

Data analysis

Analysis of variance was used to discern differences in mechanical properties among all differentiated hESC and control groups, with a Tukey’s post-hoc test when warranted. Significance was defined as \( p < 0.05 \) throughout the study.

Results

hESC differentiation and density separation

Positive staining was observed for s-GAGs, and collagen types I and II in EBs from both TGF-β1 and KOSR differentiation regimens (Fig. 25), suggesting a cartilaginous differentiation. Staining with Von Kossa and oil red O was negative (data not shown), indicating the absence of undesired differentiation. Subpopulations of chondrogenically differentiated hESCs were isolated based on cell density (Fig. 26). In the TGF-β1 group, cells were identified within 10 – 20% (TGF 10-20), 20 – 30% (TGF
20-30), 30 – 40% (TGF 30-40), 40 – 50% (TGF 40-50), and 50 – 60% (TGF 50-60) density interfaces, respectively. In the KOSR group, cells also fell within 10 – 20% (KOSR 10-20), 20 – 30% (KOSR 20-30), 30 – 40% (KOSR 30-40), 40 – 50% (KOSR 40-50), and 50 – 60% (KOSR 50-60) density interfaces, respectively. The percentage of cells isolated at each Percoll density interface for both differentiation regimens is given in Table 7. Due to the low cell yield in the TGF 10-20, KOSR 10-20, KOSR 40-50, TGF 50-60, and KOSR 50-60 groups, mechanical testing of these subpopulations was not possible.

*Viscoelastic properties and cell morphologies*

The deformation behavior of single cells in response to a 100 nN step load was fitted to a viscoelastic model (eqs. 1 – 3) to yield an instantaneous modulus, relaxed modulus, and apparent viscosity. Representative creep curves for undifferentiated and differentiated hESCs and native ACs are shown in Fig. 27. Creep cytocompression testing of cell subpopulations from both differentiation regimens resulted in a spectrum of mechanical properties ranging from undifferentiated hESCs to MSCs to ACs (Fig. 28).

The instantaneous moduli values were 0.53 ± 0.33 kPa, 1.03 ± 0.33 kPa, 1.71 ± 0.63 kPa, 1.83 ± 0.75 kPa, 0.85 ± 0.25 kPa, 0.52 ± 0.11 kPa, 1.16 ± 0.53 kPa, 1.33 ± 0.37 kPa, for hESC, TGF 20-30, TGF 30-40, TGF 40-50, KOSR 20-30, KOSR 30-40, MSC, and AC groups respectively. Differences in $E_o$ were observed between differentiation regimens, among the TGF-β1 subpopulations, and between hESC and all TGF-β1 groups. Moreover, both the TGF 30-40 and 40-50 groups were not different in $E_o$ from the AC group.
The relaxed moduli values were 0.37 ± 0.20 kPa, 0.71 ± 0.26 kPa, 1.04 ± 0.40 kPa, 1.09 ± 0.44 kPa, 0.63 ± 0.20 kPa, 0.44 ± 0.07 kPa, 0.73 ± 0.43 kPa, 1.14 ± 0.31 kPa, for hESC, TGF 20-30, TGF 30-40, TGF 40-50, KOSR 20-30, KOSR 30-40, MSC, and AC groups respectively. Differences in $E_\infty$ were observed between differentiation regimens, among the TGF-β1 subpopulations, and between hESC and all TGF-β1 groups and the KOSR 20-30 group. In addition, the TGF 30-40 and 40-50 groups were not different in $E_\infty$ from MSC and AC groups. Notably, the $E_\infty$ of ACs was also greater than MSCs.

The apparent viscosity values were 0.43 ± 0.44 kPa-s, 0.53 ± 0.38 kPa-s, 1.66 ± 1.63 kPa-s, 1.58 ± 1.48 kPa-s, 0.54 ± 0.49 kPa-s, 0.58 ± 0.48 kPa-s, 1.20 ± 0.93 kPa-s, and 0.99 ± 1.05 kPa-s, for hESC, TGF 20-30, TGF 30-40, TGF 40-50, KOSR 20-30, KOSR 30-40, MSC, and AC groups respectively. Differences in apparent viscosity were observed between hESC and TGF 30-40 and TGF 40-50 groups. In addition, the TGF 30-40 and TGF 40-50 groups were not different from the MSC and AC groups.

The creep time constant values were 2.32 ± 1.92 s, 2.50 ± 1.47 s, 6.03 ± 2.44 s, 5.97 ± 2.17 s, 2.75 ± 1.82 s, 3.84 ± 1.96 s, 5.13 ± 3.79 s, and 3.69 ± 2.95 s, for hESC, TGF 20-30, TGF 30-40, TGF 40-50, KOSR 20-30, KOSR 30-40, MSC, and AC groups respectively. The time constant values of the TGF 30-40, TGF 40-50, and MSC groups were found to be greater than the hESC group, suggestive of a longer time to reach equilibrium deformation under compression. In addition, only the TGF 30-40 group was different from the AC group.

The morphological characteristics of the hESCs, as described by the ratio of cell height to width, changed as a result of the differentiation process and fell within the range
of MSCs to ACs (Fig. 29). The cell height : width values were 0.34 ± 0.14, 0.63 ± 0.25, 0.63 ± 0.18, 0.72 ± 0.31, 0.63 ± 0.17, 0.65 ± 0.18, 0.44 ± 0.11, 0.82 ± 0.18, for hESC, TGF 20-30, TGF 30-40, TGF 40-50, KOSR 20-30, KOSR 30-40, MSC, and AC groups respectively. Moreover, the TGF 40-50 and KOSR 30-40 groups were not different from ACs. For comparison, cell height values were 7.30 ± 2.59, 8.89 ± 3.31, 8.12 ± 1.30, 8.73 ± 3.56, 8.23 ± 3.51, 10.3 ± 2.56, 6.92 ± 1.34, 9.41 ± 2.07, for hESC, TGF 20-30, TGF 30-40, TGF 40-50, KOSR 20-30, KOSR 30-40, MSC, and AC groups respectively.

Discussion

The use of hESCs in regenerative medicine is an exciting approach with direct applications to tissue engineering. This study was designed to examine the mechanical properties of hESCs and chondrogenically differentiated hESC subpopulations, using MSCs and ACs as controls. Two differentiation regimens (TGF-β1 or KOSR) were utilized, and the resulting subpopulations were separated based on cell density. Confirming our hypotheses, this study presents several notable findings relating to cellular mechanics and chondrogenic differentiation. First, the mechanical characteristics of single hESCs were investigated and directly compared to native human MSCs and ACs. Second, the density gradient technique was successfully employed to separate cell subpopulations with distinct mechanical properties. Finally, a subpopulation of differentiated hESCs was identified with similar mechanical and morphological properties as native chondrocytes.

To our knowledge, this is the first study to examine the mechanical properties of single hESCs, a necessary step toward understanding the role of mechanical factors in
cellular homeostasis and differentiation. It is well established that hESCs are mechanosensitive cells and respond differentially to applied forces or their three-dimensional mechanical environment. For instance, previous research has employed dynamic compression and hydrostatic pressure as successful differentiation agents for hESC chondrogenesis. In addition, changes in substrate rigidity and scaffold porosity, which are intimately linked with the transduction of forces on to single cells, can promote a desired embryonic stem cell differentiation. Therefore, an understanding of the mechanical properties of hESCs may greatly aid research toward identifying an appropriate loading regimen and mechanical environment which induce a favorable cellular differentiation. In the future, this information can be coupled with traditional biochemical differentiation agents, such as growth factors, to optimize hESC differentiation approaches in the laboratory.

The reported hESC characteristics were directly compared to MSCs and native ACs to yield the ‘mechanical range’ of single cells along the chondrogenic lineage. Phenotypic changes during chondrogenesis are manifested by changes in cytoskeletal structure and cellular morphology, which in turn contribute substantially to altered cellular mechanics. Examining mechanical changes during cartilage development on the single cell level sheds light on the role of biomechanical factors in healthy tissue formation and maintenance of the cellular microenvironment. In this experiment, cells became more rounded with differentiation to suggest continued rearrangement of the cytoskeleton. Moreover, parallels between the progression of cellular phenotype and mechanics can be drawn. It was found that the instantaneous modulus of hESCs was approximately 45% and 40% that of MSCs and ACs,
respectively. In terms of the relaxed modulus, increases were observed from hESCs to MSCs (~2-fold) and from MSCs to ACs (~1.5-fold). Thus, cellular stiffening appears to coincide with chondrodifferentiation. Moreover, the observed differences in stiffness properties between hESCs, MSCs, and ACs may be indicative of the types of forces each of the cells typically is exposed to in vivo. For instance, mature native ACs must withstand high compressive loading in articular cartilage\textsuperscript{22} and therefore need to be stiffer, while hESCs or MSCs experience primarily non-deformational hydrostatic forces, although at different levels, in the developing embryo or limb bud.\textsuperscript{48}

Density separation was successfully utilized to isolate cell subpopulations with different mechanical properties. It is of great interest to identify an effective methodology to purify nonhomogeneous cell populations for use in tissue engineering. Both differentiation methods yielded subpopulations with properties distributed along the 'mechanical range' from hESCs to ACs. For example, the instantaneous modulus of TGF 20-30 cells was akin to that of MSCs, while the TGF 40-50 subpopulation was similar only to ACs. Interestingly, no differences in cell morphology (e.g., cell height and diameter) were observed among the separated hESC subpopulations, suggesting that the mechanical differences were related to cytoskeletal and organelle densities. In light of this counterintuitive finding, mechanics may be a finer tool to detect differences among cell populations. Thus, it is particularly exciting to observe, for the first time, that the density separation can be utilized to isolate cells with unique mechanical properties. Therefore, future studies should compare this separation technique to more traditional cell sorting methodologies, such as Fluorescence-Activated Cell Sorting (FACS) and Magnetic-Activated Cell Separation (MACS).\textsuperscript{262,263}
Unconfined cytocompression was then employed to identify a subpopulation of differentiated hESCs potentially suitable for articular cartilage tissue engineering. While all of the differentiated hESC subpopulations were more rounded than undifferentiated hESCs, only the TGF 40-50 and KOSR 30-40 groups were not morphologically different from ACs. Of these two groups, only the TGF 40-50 subpopulation was similar to ACs with regards to all viscoelastic material parameters. Moreover, TGF 40-50 cells were 4-fold higher in apparent viscosity than undifferentiated hESCs, which is suggestive of a transformation from elastic to viscoelastic mechanical behavior. This coincides with previous research demonstrating the important role of vimentin intermediate filaments, minimally present in hESCs, in maintaining the chondrocyte phenotype and a viscoelastic response to an applied load. Identification of a differentiated hESC subpopulation with similar mechanical properties as native chondrocytes may provide utility in tissue engineering. These cells will need to withstand any mechanical stimulation regimen employed to augment the functional characteristics of engineered tissue, as well as the highly mechanical environment in the native joint. If the cells are too soft, they may experience non-physiologically high strain levels during normal loading activity. It has been shown that mechanical behavior of chondrocytes is strain-dependent and that beyond a critical point cells can no longer recover from the applied strain, suggestive of a breakdown in the cytoskeleton or other pathogenic changes. Conversely, if the differentiated hESCs are too stiff, the necessary levels of mechanical stimulation may not be reached to maintain chondrocyte homeostasis. Thus, the TGF 40-50 subpopulation may prove to be a valuable cell source for cartilage regeneration.
Creep cytocompression is a powerful methodology to infer changes in cell physiology, as long as the results are taken within the appropriate context. The behavior of individual cells seeded on a Petri dish can potentially be quite different to that of cells distributed within extracellular matrix. For instance, the microenvironment of chondrocytes has been shown to significantly influence the transmission of forces around individual cells. Moreover, testing single cells does not consider the role of cell communication and the transmission of signaling molecules in the response of cells to an applied force. An additional limitation is in regards to the assumptions necessary to yield viscoelastic properties to describe the cell. In this study, cells were considered to be homogeneous, isotropic, and incompressible materials. While all cell types are unquestionably complex arrangements of organelles, cytoskeletal structures, and nuclear components, the previous assumptions in material behavior facilitate ease in data analysis and allow for consistent comparisons across all experimental groups. Despite the aforementioned caveats, approaches in single cell mechanics have been successfully utilized to distinguish cells based on zonal arrangement within a tissue, pathogenic state, and phenotype. Thus, mechanical differences between single cell populations may be retained after isolation in vitro and may not be entirely dependent on the cellular microenvironment and biochemical factors. Moreover, the creep curves generated for all groups in this study fit well to the theoretical viscoelastic model employed. Taking these considerations in tandem, single cell mechanical testing may be a suitable method to identify changes indicative of hESC chondrogenesis.

This chapter identifies cellular mechanics as an important marker for phenotypic changes. We have elucidated mechanical and morphological differences between hESCs,
MSCs, and ACs, which may be indicative of changes in intracellular structures or the cellular mechanical environment during chondrogenesis. Using a density separation technique, we were able to distinctly isolate subpopulations with unique mechanical characteristics. Furthermore, from these subpopulations, we identified one group of differentiated hESCs with similar mechanical properties as native ACs, which may be useful in future cartilage tissue engineering efforts. An understanding of the mechanical characteristics of undifferentiated and differentiated hESCs may have implications toward elucidating the role of physical forces in promoting specific cellular phenotypes.
Table 7: Distribution of cells isolated at each Percoll gradient interface

Cell density distribution using either TGF-β1 or KOSR differentiation regiments. Values are given as a percentage of the total cells collected from the entire gradient.

<table>
<thead>
<tr>
<th>Percoll Interface</th>
<th>TGF</th>
<th>KOSR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Top (0-10%)</td>
<td>0.57</td>
<td>0.38</td>
</tr>
<tr>
<td>10-20%</td>
<td>2.82</td>
<td>6.54</td>
</tr>
<tr>
<td>20-30%</td>
<td>22.6</td>
<td>52.69</td>
</tr>
<tr>
<td>30-40%</td>
<td>50.28</td>
<td>27.31</td>
</tr>
<tr>
<td>40-50%</td>
<td>16.95</td>
<td>9.23</td>
</tr>
<tr>
<td>50-60%</td>
<td>4.52</td>
<td>3.08</td>
</tr>
<tr>
<td>Bottom (60%)</td>
<td>2.26</td>
<td>0.77</td>
</tr>
</tbody>
</table>
Figure 24: Illustration of the creep cytocompression apparatus

A piezoelectric actuator drives a 50.8 μm tungsten probe axially toward cells seeded onto a culture dish and the free end of the probe is simultaneously tracked by a laser micrometer. The difference in recorded displacement by the laser micrometer and piezoelectric motor results in a probe deflection (δ), which is correlated to a reaction force using cantilever beam theory. Through a negative feedback algorithm, the position of the probe is continuously altered to hold a step load of 100 nN onto the single cells. An inverted objective located below the stage is used to position the probe and the cell, as well as measure cell diameters.
Figure 25: Histological sections of embryoid bodies

Embryoid bodies (EBs) were differentiated with either TGF-β1 (column 1) or KOSR (column 2). Original magnification, 40X. Collagen type I, collagen type II, and s-GAGs well all present, indicating a cartilaginous differentiation of the hESCs.
Figure 26: Density separation of differentiated hESC subpopulations

Density separation was performed using the Percoll gradient technique. For this figure, chondro-induction was achieved with KOSR. Differentiated hESCs were centrifuged through Percoll solutions ranging from 10 to 60% and the cell interface between each density layer was counted and seeded for cytocompression testing. The majority of the KOSR cells (52.7%) fell within the 20-30% density interfaces.
Figure 27: Representative creep curves of single cells

The experimental data points were fitted to a viscoelastic model to yield an instantaneous modulus, relaxed modulus, apparent viscosity, and a creep time constant. Undifferentiated hESCs typically exhibited a greater deformation, suggestive of a lower stiffness, and a faster time to equilibrium, suggestive of a lower apparent viscosity and time constant, than differentiated hESCs (example shown from TGF 40-50 group) in response to the same applied load. In addition, the equilibrium deformation of single cells from the TGF 40-50 subpopulation was akin to that of native chondrocytes, indicative of their similar stiffness values. For clarity, only one out of every 1000 experimental data points is shown for each curve. Representative cells were of 12 μm height.
Figure 28: Viscoelastic properties of undifferentiated and differentiated single hESCs, and mesenchymal stem cell and articular chondrocyte controls.

Differences in instantaneous and relaxed moduli were observed between density interfaces (TGF 20-30 vs TGF 30-40 or TGF 40-50), differentiation regimens (TGF-β1 vs. KOSR), and differentiation state (hESC vs. MSC vs. TGF 40-50) (A). Moreover, differentiated cell subpopulations (TGF 30-40 and TGF 40-50) were identified which were not different than native ACs. In addition, the apparent viscosities of the TGF 30-40 and TGF 40-50 groups were greater than that of undifferentiated hESCs (B). Data presented as mean ± standard deviations.
Figure 29: Morphological properties of undifferentiated and differentiated single hESCs, and mesenchymal stem cell and articular chondrocyte controls.

The cell height-to-width ratios of all differentiated cell subpopulations were greater than that of undifferentiated hESCs and approaching 1.0, which is indicative of a more rounded cell morphology. The values for differentiated hESCs all fell between the range of MSCs to ACs. Moreover, there was no difference between the cell height-to-width ratio of the TGF 40-50 and KOSR 30-40 cells and native ACs. Data presented as mean ± standard deviations.
CHAPTER 6: Effects of TGF-beta1 and IGF-I on the compressibility, biomechanics, and strain-dependent recovery behavior of single chondrocytes

Abstract

The responses of articular chondrocytes to physicochemical stimuli are intimately linked to processes that can lead to both degenerative and regenerative processes. Toward understanding this link, we examined the biomechanical behavior of single chondrocytes in response to growth factors (IGF-I and TGF-β1) and a range of compressive strains. The results indicate that the growth factors alter the biomechanics of the cells in terms of their stiffness coefficient (~two-fold increase over control) and compressibility, as measured by an apparent Poisson’s ratio (~two-fold increase over control also). Interestingly, the compressibility decreased significantly with respect to applied strain. Moreover, we have again detected a critical strain threshold in chondrocytes at ~30% strain in all treatments. Overall, these findings demonstrate that cellular biomechanics change in response to both biochemical and biomechanical perturbations. Understanding the underlying biomechanics of chondrocytes in response to such stimuli may be useful in understanding various aspects of cartilage, including the study of osteoarthritis and the development of tissue engineering strategies.

Introduction

The biological responses of articular cartilage to mechanical forces play important roles in its normal physiology and disease. This phenomenon, known as mechanotransduction, may have important ramifications for the development of treatments for and understanding of debilitating musculoskeletal ailments, including osteoarthritis. For example, researchers have studied the effects of forces on tissue explants and engineered tissues, using modalities such as direct mechanical compression, shear, and hydrostatic pressure. These studies demonstrate that mechanical stimulation can have both beneficial and detrimental effects on both cells and tissues. Currently, the understanding of what elicits these different responses remains incomplete.

Particularly vexing is the connection between cellular biomechanics and biological processes. As one step to understanding this vital interplay, it is important to characterize the biomechanics of the chondrocyte and how they change in response to physicochemical stimuli, such as growth factors and mechanical perturbations, as previously discussed. Establishing a basic understanding of the mechanical nature of the chondrocyte aids theoretical models of cartilage that consider the cell and helps the development of new methodologies to study chondrocyte mechanobiology, such as a 'single cell' approach. This approach entails the use of instruments that can apply discreet forces to single adherent chondrocytes, such as the cytoindenter, as well as the assessment of a cellular response, such as cartilage-relevant gene expression. Studies of single cell biomechanics help direct these efforts by providing information on
the cellular characteristics of time constants for recovery, compressibility, and mechanical thresholds.

For example, when a possible change in chondrocyte mechanical behavior was identified between 25-30% compressive strain (termed the critical strain region), it was postulated that this represented a threshold akin to a yield strain. The significance of this critical region remains unclear. However, it is possible that permanent damage may be incurred by the chondrocyte beyond this critical region. This damage may occur due to high strains or strain accumulation after repeated loading, suggesting that this critical region would have important implications in directing appropriate in vitro mechanical stimulation regimens as well as for studies of mechanical damage to cartilage. Understanding the cause of this critical region also remains uncertain, but it is possible that cytoskeletal components such as microtubules, intermediate filaments, and F-actin rearrange or break down at high strains.

Considering that TGF-β1 and IGF-I increase F-actin levels and thereby increase cell stiffness two-fold over control, in this chapter, we tested the hypothesis that these same growth factors would alter the critical strain region. We were also motivated to investigate how these growth factors and mechanical strains affected the compressibility of the cell, as measured by an apparent Poisson’s ratio, since current computational models of cartilage assume this characteristic to be constant. Although we have previously observed that the apparent Poisson’s ratio does not change with respect to increasing strains, we hypothesized that the growth factors, due to their ability to reorganize the cytoskeleton and alter cellular mechanics, would increase the average apparent Poisson’s ratio of the cells compared to control. We also expected that
the growth factors would increase cell volume due to these effects on the cytoskeleton. Cell volume and shape are of interest since they are intimately tied to cell function. To test these hypotheses, single chondrocytes were exposed to IGF-I, TGF-β1, or no growth factors, and cells in each group were subjected to a range of compressive strains from 5-60% and their morphology, compressibility, stiffness, and recovery behavior were analyzed.

Methods

The basic protocols for this experiment were based on our previous work. These methods and a few improvements are briefly explained below. Cell culture supplies were obtained from Invitrogen (Carlsbad, CA, USA) unless specified otherwise.

Cell isolation and seeding

Articular cartilage was harvested from the fetlock joint of mature steers obtained from local abattoirs (Doreck and Sons Packing Company, Santa Fe, TX, USA; Kasper’s Meat Market, Weimar, TX, USA). Chondrocytes from 13 joints derived from 12 different animals were isolated from the middle/deep region of the tissue as previously described. The primary chondrocytes were counted, centrifuged, and resuspended in supplemented DMEM (0.1 mM NEAA, 100U/ml penicillin/streptomycin, 0.25 μg/ml fungizone) at a concentration of 200,000 cells/ml with either TGF-β1 (10 ng/ml), IGF-I (100 ng/ml), or no growth factor. The chondrocytes were then seeded on 15 mm x 2 mm glass slides, which were placed inside of a 6-well plate, and incubated for 3 hrs at 37°C
and 10% CO₂. The 3 hr seeding was previously established as a minimal time point that could achieve stable cell adhesion for cellular compression testing.

Cytocompression

Individual chondrocytes were subjected to a range of strains (~0.05 to 0.60) using a 50.8 μm diameter tungsten probe (Advanced Probing Systems, Inc., Boulder, CO, USA) attached to a modified cytodetacher device, as described previously. Glass slides were transferred from a 6-well plate to a Petri dish such that the cells could be viewed perpendicular to the original seeding plane using an IMT-2 inverted microscope (Olympus America, Melville, NY, USA) at 400x (Fig. 30). Supplemented DMEM (as above) with 30 mM HEPES and either TGF-β1 (10 ng/ml), IGF-I (100 ng/ml), or blank control was added to the dish. Individual cells were brought in close contact (~5-10 μm away) with the probe, and a piezoelectric motor drove the probe a prescribed distance (12-16 μm) toward the cells at a rate of 4 μm/s. The probe was held at its position for 30 s and then removed from the cell. Chondrocytes were given up to 90 s to recover and reach equilibrium.

Videocapture and image analysis

The compression and recovery of each cell were captured on an AVC-D7 CCD camera (Sony Corp., New York, NY, USA) and saved as an AVI file at 640 x 480 resolution. A pixel-to-micron ratio of 7.0 was employed in all subsequent image analysis. The accuracy of the measurements was deemed to be 2 pixels, or 0.29 μm. Individual
frames from the videos were extracted using Videomach 4.0.2 software (Gromada.com). Images of the initial cell-probe configuration, initial cell-probe contact, cell-probe contact at peak strain, cell immediately after probe release, and cellular recovery every 4 seconds thereafter were examined. The dimensions of the cell (see below) and probe positions in each frame were marked using Microsoft Paint 5.1 (Microsoft Corporation, Redmond, WA, USA).

**Biomechanical properties and characteristics**

Cells were approximated as ellipsoids, with rotational symmetry about the x-axis (perpendicular to the plane of seeding). The basic shape of the cell was assumed to remain unchanged through the duration of the compression and recovery events, as we have done before.\(^{279}\) Cell volume was therefore calculated using the equation for an ellipsoid with two identical axes:

\[
V = \frac{1}{6} \pi h d^2
\]

where \( h \) represents the cell’s height (or ellipsoid length in the x-direction) and \( d \) is the cell diameter (or ellipsoid length in the y- and z- directions). The volume of the cell was measured before compression (\( V_0 \)), immediately after compression (\( V_i \)), and at equilibrium recovery (\( V_r \)). A normalized volume fraction (\( (V_0 - V_i)/V_0 \)) and recovered volume fraction (\( V_r/V_0 \)) were calculated with these values. In contrast to our previous experiment,\(^{279}\) we assessed applied axial strain (vs. apparent strain) by measuring the difference in probe positions between initial cell contact and at peak compression, divided by the initial cell height.
Cantilever beam theory was employed to determine the reaction force of the cell onto the probe at peak compression:

$$F = \frac{3EI}{L^3} \delta$$  \hspace{1cm} (2)

where $E$ (Young’s modulus), $I$ (moment of inertia), and $L$ (cantilever beam length) are known parameters of the tungsten probe. The deflection of the cantilever ($\delta$) was determined by comparing the true displacement of the probe (via subsequent video analysis) with the prescribed piezoelectric displacement (between 12-16 μm).

The compressibility, as measured by an apparent Poisson’s ratio, of the cell was determined as described previously.\textsuperscript{278} Briefly, the physical dimensions (height, width) of the cell were measured before and immediately post-compression. The apparent Poisson’s ratio was calculated as:

$$\nu = \frac{d_1/d_o - 1}{1 - h_1/h_o}$$  \hspace{1cm} (3)

where $d_1$ and $d_o$ are the post-compression and initial diameters for the cell, respectively, and $h_1$ and $h_o$ are the post-compression and initial cell heights, respectively.

Upon release of the probe, the residual strain ($\varepsilon_r$) on the cell was defined as change in cell height divided by its initial height. Plots of residual strain versus time were fit to a generalized decaying exponential function using Matlab R2007b (Mathworks, Natick, MA, USA):

$$\varepsilon_r = Ae^{-\frac{t}{\tau}} + C$$  \hspace{1cm} (4)

where $A$ is the recovery coefficient, $t$ is the time in seconds, $\tau$ is the characteristic recovery time constant for the cell, and $C$ is the equilibrium residual strain.
Statistics

Statistical analysis was performed using JMP IN 5.1 (SAS Institute Inc., Cary, NC, USA). Linear regression was used to determine whether the apparent Poisson's ratio, equilibrium stress, normalized volume fraction, recovered volume fraction, and residual strain varied as functions of applied strain or equilibrium stress, as well as to test the effects of growth factors on these functions. Change point analysis was used to determine if critical levels of applied strain existed where biomechanical behavior changed, as performed previously. Briefly, this entailed performing a series of linear regressions on overlapping segments of data, where the resulting slopes were plotted against the applied strain to look for possible discontinuities. If a discontinuity was apparent, the original data were separated into two subsets, each of which was analyzed with linear regression. An effect was considered significant if $p < 0.05$.

Results

In this section, treatments will be referred to as control, IGF-I, and TGF-β1. A total of 83 cells were tested and analyzed for this experiment (26 for control, 26 for IGF-I, and 31 for TGF-β1). Figure 31 demonstrates how cells were analyzed.

Chondrocyte size

The different growth factor treatments had significant effects on the morphological measurements of the cell heights ($h$, $p=0.04$), diameters ($\text{dia}$, $p=0.01$), and volumes ($\text{vol}$, $p=0.005$). Specifically, TGF-β1 cells ($h=11.4\pm1.7 \ \mu m$, $\text{dia}=11.2\pm1.8 \ \mu m$, \text{vol}=0.005). Specifically, TGF-β1 cells ($h=11.4\pm1.7 \ \mu m$, $\text{dia}=11.2\pm1.8 \ \mu m$, \text{vol}=0.005).
vol=795±325 µm³) had significantly greater dimensions (p<0.05) in all three measurements compared to IGF-I cells (h=10.5±1.2 µm, dia=10.2±0.8 µm, vol=600±162 µm³), while control cells (h=10.7±1.2 µm, dia=10.6±0.8 µm, vol=638±137 µm³) were not different from either group, with the exception of having less volume than TGF-β1 cells (p<0.05).

**Compressibility**

The cells in each group exhibited characteristics of compressible materials, as volume changes were noted as a function of increasing strain. Plotting the normalized volume fraction (\(V_{\text{norm}}\)) against the applied strain (\(\varepsilon\)) showed significant linear correlations for each treatment (\(V_{\text{norm}} = 0.54\varepsilon - 0.05\) with \(R^2=0.41\) and \(p=0.0004\) for control, \(V_{\text{norm}} = 0.30\varepsilon - 0.02\) with \(R^2=0.71\) and \(p<0.0001\) for IGF-I, and \(V_{\text{norm}} = 0.31\varepsilon - 0.04\) with \(R^2=0.45\) and \(p<0.0001\) for TGF-β1).

The apparent Poisson’s ratio was significantly higher (p=0.0001) in the growth factor groups compared to control (0.18±0.11 for control, 0.31±0.13 for IGF-I, and 0.30±0.11 for TGF-β1). Additionally, the apparent Poisson’s ratio decreased significantly with applied strain for all treatments (Fig. 32, p=0.001 for control, p=0.003 for IGF-I, and p=0.003 for TGF-β1). The rate of decrease in Poisson’s ratio with applied strain was statistically the same for growth factors and control, but the intercept of the control regression line was significantly less than the growth factors (p<0.05), in line with the finding that the mean Poisson’s ratio was less for control.
Stiffness coefficient

Plotting the equilibrium stress data against the applied strain yielded a stiffness coefficient for each group (Fig. 33). Both growth factor treatments resulted in statistically significant linear correlations between stress and strain (p=0.002 for IGF-I and p<0.0001 for TGF-β1). On the other hand, the control cells did not have a significant linear correlation between stress and strain (p=0.1). The linear fits for each group indicated that the growth factor treatments had stiffness coefficients about two-fold higher than the control (1.77-fold for IGF-I and 2.03-fold for TGF-β1).

Recovery behavior

The recovery behavior of the single chondrocytes depended on the applied axial strain for all treatments. Figure 31 illustrates how the cells recovered over time at different levels of strain. Generally, under all treatments, cells recovered fully and quickly to their original heights and volumes at low strains. At increasing levels of strain, however, the cells in all treatments exhibited a slower recovery response that was not complete, having less height (residual strain) and a lower recovered volume compared to initial values. The exponential decay function (Eq. 4) approximated the recovery behavior of the cells in all treatments well, with R² values between 0.63 and 0.99. Residual strains from Eq. 4 agreed well with the measured residual strains from the digital image analysis, with linear coefficients of 0.995±0.005 for control (R²=0.99), 1.07±0.007 for IGF-I (R²=0.97), and 1.04±0.002 for TGF-β1 (R²=0.99). Each line had a significance level of p<0.0001. The time constants (τ) for recovery increased with
applied strain ($\varepsilon$) ($\tau = 28.58\varepsilon + 3.32$ with $R^2=0.14$ and $p=0.03$ for control, $\tau = 41.04\varepsilon + 6.00$ with $R^2=0.25$ and $p=0.009$ for IGF-I, $\tau = 48.00\varepsilon - 2.61$ with $R^2=0.36$ and $p=0.0004$ for TGF-$\beta$1).

Figures 34 and 35 (A-C) illustrate how the recovery characteristics of residual strain and recovered volume fraction varied with the applied strain. The residual strain increased with applied strain in all groups ($p<0.0001$ for control, $p<0.0001$ for IGF-I, $p=0.0006$ for TGF-$\beta$1), while the recovered volume fraction changed inversely with the applied strain in all groups ($p<0.0001$ for all groups).

**Biomechanical behavior changes**

The change point analyses for residual strain (Fig. 34 D-F) and recovered volume fraction (Fig. 35 D-F) each as functions of applied strain revealed a discontinuity at an applied strain of 0.30 to 0.35 for all groups. The linear fits for the data indicated no significant effects of strains less than 0.30 on the recovery behavior of the single chondrocytes in all groups. However, above strains of 0.30, the residual strains and recovered volume fractions exhibited significant linear correlations with the applied strains for all groups ($p<0.05$, Figs. 34 and 35).

**Discussion**

The growth factors used here are widely studied in various cartilage processes, including tissue engineering efforts and osteoarthritis research. Thus, it is of interest to understand their effects on the biomechanics and recovery behavior of chondrocytes,
since these characteristics relate to the effectiveness of mechanical stimulation regimens and the health and disease of cartilage. Additionally, it is important to understand how cellular biomechanics change with direct compressive strains, since this modality of mechanical stimulation has physiological relevance and influences cellular processes. This chapter offers several new findings relevant to chondrocyte mechanobiology. First, we show that, with all treatments, the apparent Poisson’s ratio decreases with applied strain. Second, TGF-β1 and IGF-I alter the measured Poisson’s ratio of the cells compared to control. Finally, we offer evidence that these growth factors do not alter the critical strain of chondrocytes.

The finding that compressibility changes with applied strain (Fig. 32) is a new and important finding, though it was surprising considering previous work. Key differences in the methods may account for this discrepancy, which are discussed in detail later. Our new finding suggests that with increasing strains, cells have an impaired ability to translate axial strain to transverse strain. Mechanistically, this change in compressibility may be related to both greater fluid exudation and cytoskeletal breakdown as applied strains increase. However, the apparent Poisson’s ratio measured in this study represents a bulk measurement of cellular compressibility, whereby changes in individual components of the cell, such as the actin cytoskeleton, can have profound effects on its structural properties. It is well documented that the growth factors used in this study cause reorganization of the actin cytoskeleton through specific signaling molecules, such as focal adhesion kinase and several others, that likely relate to enhanced adhesion processes. These growth factors also increase chondrocyte stiffness. Thus, it is not surprising that the apparent Poisson’s ratio was higher for
TGF-β1 and IGF-I compared to control, as these changes in cellular structure likely improved the ability of the cell to translate axial to transverse strains, even as applied strains increased (Fig. 32). Theoretical models of cartilage may need to consider the biochemical and biomechanical milieu of the tissue and take into account the observed changes in cellular compressibility. Additionally, this effect on cellular compressibility may be related to the observed detrimental effects on chondrocyte viability and matrix synthesis due to high magnitude mechanical stimuli (up to 50% compressive strain) on cartilage explants, as these may reduce cellular volume beyond the point of recovery.

In this chapter, we also identified a possible change in the recovery behavior of chondrocytes at approximately 30-35% applied compressive strain (Figs. 34 and 35). This critical region is slightly higher than our previous work, where we identified a possible change in behavior between 25-30% apparent strain (discussed below). Physiological strains in femoral head cartilage have been measured to be 2-10%, with native chondrocytes likely experiencing a two-fold increase in strain compared to the macroscopic tissue. Though there are differences between species and joints, it is interesting that these estimates of physiological levels of cellular strain fall below the observed critical strain for chondrocytes. An enticing possibility is a direct link between this critical strain and the determination of chondrocyte behavior towards either degenerative or regenerative/homeostatic processes.

We have previously observed a two-fold stiffening effect of the growth factors with a concurrent increase in F-actin levels. Though we again detected a two-fold stiffening effect of the growth factors, the data show that IGF-I and TGF-β1 did not
substantially alter the critical region of strain. Taken together, these results question the role of the actin cytoskeleton in determining the critical region. Future work involving the direct disruption or stimulation of other specific cytoskeletal components should help elucidate the underlying basis of the critical region. The role of other physical phenomena such as active volume regulation should also be closely examined since these mechanisms are minimized by the ambient conditions of these experiments. It is possible that different physical mechanisms are predominant depending on the level of applied strain. For example, below the apparent critical strain for cells, passive diffusion and cytoskeletal components may be most responsible for re-establishing cellular dimensions. Above the critical strain, it is possible that other mechanisms, such as active volume regulation, play a more important role. Establishing a fundamental understanding of chondrocyte biomechanics may be relevant to various cartilage processes. For example, changes in chondrocyte volume may be linked to matrix synthesis.

It is important to note improvements to our technique compared to previous work that may account for the discrepancy in findings for the compressibility and slight increase in the critical strain, as well as other observations. Serum, which contains a variety of growth factors at unknown concentrations, was judiciously excluded from these experiments so that the effects of the individual growth factors would be isolated. It appears that the media components (i.e., presence or absence of serum and growth factors) affect the biomechanics of the cells, as the stiffness coefficients for the groups was about 50% less than the analogous relaxed moduli measured in our previous work that used serum. Additionally, the mean control Poisson’s ratio (0.18) was about 40%
less than published values, while the growth factor groups (~0.30) were similar.\textsuperscript{278, 279, 299, 300} This result highlights the influence of growth factors, added exogenously or through the addition of serum, on the cytoskeleton and its role in determining these cellular properties. These alterations in cellular biomechanics are likely related to the higher recovery time constants observed in this chapter compared to our previous work.\textsuperscript{279} It is important to note, however, that we have again observed increases in recovery time constants with applied strains,\textsuperscript{279} indicating that high strain, high frequency stimulation regimens of cartilage may cause irreparable damage to the cell. While relative changes due to growth factors or other treatments within a study can be observed with or without serum, the marked changes in the absolute values of the cellular characteristics again emphasizes the need to consider the environment of the cell when using these values.

Another alteration in this methodology compared to our previous work\textsuperscript{279} involved the image analysis. All analysis in this study was based on the applied strain to the cell at equilibrium deformation, as opposed to an apparent strain that was recorded once the platen was removed. The apparent strain would be less than the applied strain and hence account for the differences in critical strain ranges.\textsuperscript{279} The apparent strain in the previous study was also directly used in the calculation of the apparent Poisson’s ratio, whereas our calculations for apparent Poisson’s ratio and applied strain in this study were independent. Other important comparisons include the cell morphologies. Cells in each group largely retained a rounded shape, and the volume measurements (~700 $\mu$m$^3$) are similar to previously reported values.\textsuperscript{285} The finding that TGF-$\beta$1 increased cell size (~25% increase in volume over control) agrees with our prior work, though IGF-I did not
alter the size as expected.\textsuperscript{276} It is possible that the effects of growth factors on single cells may be different when serum is present.

Our methodology has progressed in several important respects, but it is important to understand its limitations. In particular, the removal of the cell from its physiological environment alters cellular processes.\textsuperscript{301} Additionally, two-dimensional recordings of the cell may not fully capture its three-dimensional complexity, since 3D analysis of chondrocytes in compressed cartilage tissue has shown non-symmetrical deformation along split-lines.\textsuperscript{302} However, the results of this chapter are consistent with much of the literature, as discussed above. Moreover, the use of \textit{in vitro} methods to study articular cartilage remains an important tool so long that results are kept in context. It is clear that the isolation of cells from their matrix retains at least some characteristics, including differences in zonal chondrocyte biomechanics\textsuperscript{278} and osteoarthritic chondrocyte characteristics.\textsuperscript{303}

In summary, this chapter examined the effects of TGF-β1 and IGF-I on single chondrocyte morphology, compressibility, biomechanics, and recovery behavior. The results build upon our previous single cell studies by allowing for the measurement of applied strain and the stiffness coefficient as well as the observation of chondrocyte recovery behavior. Using this methodology we have demonstrated that cell compressibility changes with strain and with growth factors. We have also shown that the critical region of strain does not change with the administration of these growth factors. Characterizing how chondrocytes respond to these physicochemical stimuli and understanding their underlying biomechanics may help elucidate etiologies for osteoarthritis and offer new directions for tissue engineering efforts.
Figure 30: Cytocompression experimental setup

Articular chondrocytes were seeded onto a glass slide and exposed to IGF-I, TGF-β1, or no growth factor for 3 hrs. Individual cells from each group were subsequently compressed by a probe at 4 μm/s to a prescribed distance (12-16 μm). Measurements of the cell were taken before, during, and after the probe was released to provide information regarding cell morphology, biomechanics, compressibility, and recovery behavior. The figure is not drawn to scale.
Figure 31: Cytocompression and cellular recovery behavior

The compression and recovery events were analyzed through video recording. For demonstration, TGF-β1 cells are shown experiencing small (19%) strains (A – F), medium (29%) strains (G – L), and high (52%) strains (M – R). The initial frame (A, G, M), first probe contact (B, H, N), and equilibrium contact (C, I, O) provide information on applied strain and stress by tracking the movement of the probe and measuring the initial dimensions of the cell. Immediately upon release of the probe (D, J, P), cell dimensions are measured to provide an apparent Poisson’s ratio. Additionally, the recovery behavior of the cell is tracked. Shown are cells at 4 s after probe release (E, K, Q) and at equilibrium (F, L, R).
Figure 32: Changes in apparent Poisson’s ratio due to physicochemical stimuli

(A) The apparent Poisson’s ratio was measured by measuring the changes in height and diameter of each compressed cell. The growth factors increased the apparent Poisson’s ratio compared to control (p=0.0001). For demonstration, superimposed outlines of one cell from each group are shown at a strain of 0.5 before (light gray) and after (black) compression. The cell outlines illustrate the significant effect that the growth factors had on cellular compressibility. (B) The apparent Poisson’s ratio (ν) decreased as a function of applied strain (ε) for all treatments, with the intercept of the linear fit for the control cells (ν = -0.66ε + 0.40, R^2=0.37 and p=0.001) being significantly different (p<0.05) from both growth factor treatments (ν = -0.43ε + 0.45, R^2=0.31 and p=0.003 for IGF-I; and ν = -0.41ε + 0.45, R^2=0.27 and p=0.003 for TGF-β1).
Figure 33: Cellular stiffness in response to growth factors

(A) Control cells did not have a significant linear correlation between stress and strain. In contrast, equilibrium stress increased significantly with strain when either growth factor (B, C) was applied, indicating about a two-fold increase in the stiffness coefficient (i.e., the slope) over control.

A

Control cells

\[ y = 249.65x + 53.636 \]

\[ R^2 = 0.1061 \]

\[ p = 0.105 \]

B

IGF-I cells

\[ y = 442.67x + 92.443 \]

\[ R^2 = 0.3269 \]

\[ p = 0.002 \]

C

TGF-β1 cells

\[ y = 519.22x - 30.774 \]

\[ R^2 = 0.4937 \]

\[ p < 0.0001 \]
Figure 34: Residual strain behavior with applied strain

Residual strain ($\epsilon_r$) significantly increased in control (A), IGF-I (B), and TGF-β1 (C) groups. Change point analysis for each group (D, E, F) revealed possible discontinuities in all groups at approximately 30% applied strain ($\epsilon$). Linear fits for each data set above and below this discontinuity are shown. The overall linear fit for control cells was $\epsilon_r = 0.41\epsilon - 0.06$ ($R^2 = 0.61$, p<0.0001). IGF-I cells had an overall fit of $\epsilon_r = 0.24\epsilon - 0.02$ ($R^2 = 0.61$, p<0.0001). The overall fit for TGF-β1 cells was $\epsilon_r = 0.27\epsilon - 0.05$ ($R^2 = 0.34$, p=0.0006).
**Figure 35: Recovered volume fraction behavior with applied strain**

Recovered volume fraction ($V_r$) significantly decreased in control (A), IGF-I (B), and TGF-β1 (C) groups. Change point analysis for each group (D, E, F) revealed possible discontinuities in all groups at approximately 30% applied strain ($\varepsilon$). Linear fits for each data set above and below this discontinuity are shown. The overall linear fit for control cells was $V_r = -0.64\varepsilon + 1.11$ ($R^2 = 0.65$, $p<0.0001$). IGF-I cells had an overall fit of $V_r = -0.38\varepsilon + 1.04$ ($R^2 = 0.79$, $p<0.0001$). The overall fit for TGF-β1 cells was $V_r = -0.24\varepsilon + 1.04$ ($R^2 = 0.42$, $p<0.0001$).
CHAPTER 7: Matrix development in self-assembly of articular cartilage*

Abstract

Articular cartilage is a highly functional tissue which covers the ends of long bones and serves to ensure proper joint movement. A tissue engineering approach that recapitulates the developmental characteristics of articular cartilage can be used to examine the maturation and degeneration of cartilage and produce fully functional neotissue replacements for diseased tissue. This study examined the development of articular cartilage neotissue within a self-assembling process in two phases. In the first phase, articular cartilage constructs were examined at 1, 4, 7, 10, 14, 28, 42, and 56 days immunohistochemically, histologically, and through biochemical analysis for total collagen and glycosaminoglycan (GAG) content. Based on statistical changes in GAG and collagen levels, four time points from the first phase (7, 14, 28, and 56 days) were chosen to carry into the second phase, where the constructs were studied in terms of their mechanical characteristics, relative amounts of collagen types II and VI, and specific GAG types (chondroitin 4-sulfate, chondroitin 6-sulfate, dermatan sulfate, and hyaluronan). Collagen type VI was present in initial abundance and then localized to a pericellular distribution at 4 wks. N-cadherin activity also spiked at early stages of neotissue development, suggesting that self-assembly is mediated through a minimization

of free energy. The percentage of collagen type II to total collagen significantly increased over time, while the proportion of collagen type VI to total collagen decreased between 1 and 2 wks. The chondroitin 6- to 4- sulfate ratio decreased steadily during construct maturation. In addition, the compressive properties reached a plateau and tensile characteristics peaked at 4 wks. The indices of cartilage formation examined in this study suggest that tissue maturation in self-assembled articular cartilage mirrors known developmental processes for native tissue. In terms of tissue engineering, it is suggested that exogenous stimulation may be necessary after 4 wks to further augment the functionality of developing constructs.
Introduction

Articular cartilage is a specialized type of hyaline cartilage, providing a nearly frictionless surface along diarthrodial joints and acting to resist and distribute compressive forces. Its tensile and compressive properties are attributed to the presence of fibrillar collagens and negatively charged glycosaminoglycans (GAGs), respectively, in the tissue’s extracellular matrix (ECM). Articular cartilage lacks the ability to repair itself under conditions of wear and tear or traumatic injury, leading to osteoarthritis (OA) which afflicts millions of Americans and significantly affects the economy. In recent years, tissue engineering has become a promising option toward the treatment of OA, allowing researchers to produce functional replacements for diseased cartilage.

Toward this end, a self-assembling process has been developed in our laboratory to yield cartilage tissue constructs of clinically relevant dimensions and compressive mechanical properties approaching those of native tissue. Most notably, this approach does not involve the use of a scaffold, thereby bypassing the typical scaffold-related concerns of biodegradability, stress-shielding, and hindrance of cell-to-cell communication. While previous studies have focused on the end functionality of tissue constructs, an understanding of the development of neotissue within the self-assembling process remains incomplete. Studying the maturation of these cartilage constructs will yield valuable information regarding the developing biophysical environment of chondrocytes and elucidate intervention windows for biochemical or biomechanical stimulation.

An understanding of native articular cartilage development is of particular importance, as it will provide essential benchmarks for tissue growth in vitro. The various
ECM components, and their associated arrangement, can be used as indicators for the effectiveness of a myriad of tissue engineering approaches, including the self-assembling process, to recapitulate the different stages of the developmental process of articular cartilage and produce functional tissue constructs. Native articular cartilage is known to arise during an intricate process of joint development, first involving the formation of an interzone region through mesenchymal condensation at the future joint site, which then separates in a perichondrium-like layer and an intermediate layer consisting of softer tissues. Nascent cartilage begins to form with the chondrogenesis of mesenchymal progenitor cells located at the perichondrium regions and continues to develop until the formation of growth plates, which become the primary source of self-renewing, proliferating chondrocytes. Like many musculoskeletal tissues, the biochemical composition of articular cartilage undergoes substantial changes during its development, reflected particularly in modulating levels and spatial organization of specific collagen and GAG types. In particular, it has been observed that collagen type VI plays an essential role in cartilage development and the maintenance of the cellular microenvironment. Variations in chondroitin sulfation patterns may be further indicative of matrix remodeling, and thus affect the binding and activity of growth factors or cytokines within the tissue matrix. Correlating with these biochemical alterations, the compressive and tensile mechanical properties of articular cartilage also change during fetal and adolescent tissue maturation.

The purpose of this study was to examine the development of tissue within a self-assembling process for articular cartilage. Specific emphasis was placed on the relative levels of collagen types II and VI, and chondroitin 4-sulfate (CS-4) and chondroitin 6-
sulfate (CS-6), and mechanisms of cellular aggregation during the early stages of neotissue development. We hypothesized that the relative levels of specific collagens (types II and VI) and GAGs (CS-4 and CS-6) would follow known developmental trends for native articular cartilage. We further examined the temporal-spatial relationship of collagen types II and VI to identify the progression of a pericellular matrix (PCM) within the tissue. Additionally, this study investigated the compressive and tensile mechanical characteristics of the constructs, and their relationships to changing biochemical properties.

Methods

This study was performed in two phases to examine tissue development within the self-assembling process for articular cartilage. In the first phase, tissue constructs were assessed histologically and quantitatively for their total collagen and GAG content after 1, 4, 7, 10, 14, 28, 42, and 56 days of development. In the second phase, four time points from the first phase (7, 14, 28, and 56 days) were chosen to examine maturing structure-function relationships in the tissue. To this end, tensile and compressive mechanical properties of the tissue constructs were related to collagen and GAG levels. The specific types of collagen (types II and VI) and GAGs (CS-4, CS-6, dermatan sulfate, and hyaluronan) were further investigated to identify key matrix developmental trends. The seeding and culture techniques were the same for both phases of this study.
Cell isolation and seeding

Articular chondrocytes were isolated from the distal femur of 1 wk old male calves (Research 87 Inc., Boston, MA) less than 36 h post slaughter, via an overnight digestion in 0.2% collagenase type II (Worthington, Lakewood, NJ), as described previously. To reduce variability among animals, a mixture of cells were pooled together from six and seven animals for the first and second phases of the study, respectively, to yield a representative mixture of chondrocytes. The pooled cells were counted on a hemocytometer, and their viability was assessed using a trypan blue exclusion test. Each femur yielded approximately 150 million chondrocytes and a viability of >99% was determined for all specimens. Chondrocytes were frozen in culture medium supplemented with 20% FBS and 10% DMSO at -80°C for 2 days before use. After thawing, viability remained >80%. Chondrocytes were centrifuged at 1.2 x 10^3 rpm for 7 min and resuspended at a density of 5.5 x 10^7 cells/ml in a chemically-defined medium, consisting of DMEM with 4.5 g/L-glucose and L-glutamine, 100 nM dexamethasone, 1% fungizone, 1% penicillin/streptomycin (Biowhittaker/Cambrex, Walkersville, MD), 1% ITS+ (BD Biosciences, Bedford, MA), 50 mg/mL ascorbate-2-phosphate, 40 mg/mL L-proline, and 100 mg/mL sodium pyruvate (Fisher Scientific, Pittsburgh, PA). Articular cartilage constructs were seeded by adding 100 μl of this cell suspension to custom-made 5 mm agarose coated wells (described below). Time t = 0 was defined as this point of initial construct seeding. Cells were given 3 h to coalesce and then a remaining 400 μl of medium was added to each well. Medium was subsequently changed every 24 h. Based upon prior results in our laboratory demonstrating enhanced tissue mechanical properties, self-assembled constructs were removed from
confinement in the agarose well at 2 wks and transferred into 10-mm diameter wells coated with 2% agarose, where they remained up to 8 wks.

**Preparation of agarose wells for chondrocyte seeding**

Agarose coated wells were constructed as described previously. Briefly, a negative polysulfone mold consisting of 5-mm diameter x 10-mm long cylindrical prongs was constructed to fit into 6 wells of a 48-well plate (Costar, Corning, NY). Individual wells were constructed by pressing the negative mold into 1 ml of sterilized, molten 2% molecular biology grade agarose in phosphate-buffered saline (PBS) (Sigma, St. Louis, MO). The agarose was allowed to gel at room temperature for 1 h with the mold in place. To each agarose well, 500 µl of chemically-defined medium was added and changed twice to saturate the well by the time of cell seeding.

**Histology and immunohistochemistry**

Samples from both phases were frozen in cryoembedding medium and sectioned at a thickness of 12 µm. Safranin-O and fast green staining were used to visualize the GAG distribution within the constructs. Additional slides were processed for qualitative immunohistochemistry (IHC) examination of the presence and spatial arrangement of collagen type I, II, and VI, using a Biogenex i600 autostainer (San Ramon, CA). After fixation in 4°C acetone, the slides were washed with a solution of PBS containing tween, quenched of exogenous peroxidase activity with 1% hydrogen peroxide in methanol and blocked with serum (Vectastain ABC kit, Burlingame, CA).
The slides were then incubated with either mouse anti-collagen type I antibody (Axell, Westbury, N.Y.) at a 1:750 dilution in PBS, rabbit anti-collagen type II antibody (Cederlane, Burlington, NC) at a 1:500 dilution in PBS, rabbit anti-collagen type VI antibody (US Biological, Swampscott, MA) at a 1:300 dilution in PBS, or rabbit anti-collagen type X antibody (Abcam Inc., Cambridge, MA) at a 1:300 dilution in PBS. The appropriate mouse or rabbit secondary antibody (Vectastain ABC kit) was applied, followed by the avidin-biotinylated enzyme complex (Vectastain ABC kit) and DAB reagent (Vector Labs). Slides were removed from the autostainer, counterstained with hematoxylin, dehydrated in graded ethanol, and mounted with a coverslip. Slides stained without the addition of a primary antibody served as negative controls. Native meniscal fibrocartilage and articular cartilage served as positive controls for collagen types I and II, respectively. The presence of N-cadherins was also examined on slides from Phase I and in a non-seeded cell suspension. Briefly, slides were fixed in 4% paraformaldehyde, blocked with 10% FBS, and incubated with a rabbit anti-N-cadherin primary antibody (US Biological, Swampscott, MA) at a 1:240 dilution in 1% BSA in PBS, followed by detection with a goat anti-rabbit secondary antibody (Alexaflour 546). Nuclei were observed using a Hoescht’s stain. Porcine cardiac tissue served as a positive control for N-cadherin immunohistochemistry.

**Biochemical analysis**

Approximately 3 - 4 mg pieces of each construct (n = 6 per time point) were frozen for biochemical analysis in both phases. Frozen tissue pieces were lyophilized and digested in pepsin (10 mg/ml) followed by pancreatic elastase (1 mg/ml) in acetic acid
and Tris buffer solutions, respectively. Total DNA content was measured by Picogreen® Cell Proliferation Assay Kit (Molecular Probes, Eugene, OR). Total sulfated GAG was then quantified using the Blyscan GAG assay kit (Biocolor, Newtownabbey, Northern Ireland), based on the 1,9-dimethylmethylene blue binding. Samples were further assayed for total collagen content via hydrolysis in 2N NaOH, followed by a chloramine-T hydroxyproline reaction, as described previously. Specific levels of collagen type II were determined for Phase II samples using an Enzyme-Linked ImmunoSorbent Assay (ELISA) assay, developed by Chondrex, Inc. (Chondrex, Redmond, WA). Collagen type I was quantified via a sandwich ELISA using a monoclonal mouse anti-human capture antibody (USBiological) and polyclonal rabbit anti-human detection antibody (USBiological).

The relative levels of collagen type VI were examined through western blotting in Phase II. Based upon the results of the hydroxyproline assay, the equivalent amount of each tissue digest containing 20 µg of total collagen was precipitated for blotting. The samples, collagen type VI protein standard, and a prestained SDS-PAGE protein ladder (Bio-Rad) were loaded into a 10-20% Ready Gel Tris-HCl Gel (Bio-Rad) and run at 50 mA for 30 min. The gels were then transferred onto blotting paper at 350 mA for 60 min. Blots were blocked in 2% BSA for 3 h at room temperature and then incubated with a rabbit anti-COL6 antibody (USBiological, Swampscott, MA) at a 1:1500 dilution. A rabbit secondary antibody (Vectastain ABC kit) was applied, followed by the avidin-biotinylated enzyme complex (Vectastain ABC kit) and DAB reagent (Vector Labs) to visualize protein bands. The integrated optical density of each band was determined using GelPro® software (Media Cybernetics, Bethesda, MD).
Fluorophore-assisted carbohydrate electrophoresis (FACE) analysis was additionally performed on Phase II samples (n=6) to quantitatively determine changes in CS-4, CS-6, glucose, dermatan sulfate, and hyaluronan during construct development, as previously described.\textsuperscript{308, 316, 317} Separate tissue construct portions were frozen, lyophilized, and rehydrated in 100 mM ammonium acetate. Tissue pieces were digested in 10\% (w/v) proteinase-K (EMD Pharmaceutical, Durham, NC) overnight at 60°C. Ammonium acetate (100 mM) was subsequently added to each sample, and followed by digestion with either chondroitinase AC II alone or together with chondroitinase ABC (Associates of Cape Cod, Falmouth, MA). The difference in band intensity from the two digests yielded dermatan sulfate content. Samples were fluorescently tagged with 2-aminoacridone HCl (Molecular Probes, Eugene, Oreg., USA) and run on a carbohydrate electrophoresis gel at 400 mA for 45 - 60 min. Individual FACE gel bands were analyzed using GelPro\textsuperscript{®} software (Media Cybernetics, Bethesda, MD). Specific GAGs and total glucose were quantified via comparison to a fluorescently labeled maltotriose standard curve.

\textit{Mechanical assessment}

The compressive mechanical properties of Phase II samples (n=6 per time point) were evaluated with an indentation apparatus.\textsuperscript{318} A 3-mm punch was extracted from each construct, attached to the sample holder with cyanoacrylate glue, and submerged in PBS. The sample was positioned under the load shaft of the apparatus, such that the sample surface test point was perpendicular to the indenter tip. A tare load of 0.2 g (0.002 N) was applied using a 1-mm diameter rigid, flat-ended, porous indenter tip and samples were
allowed to reach tare creep equilibrium. A step load of 0.7 g (0.007 N) was then applied and sample displacement was measured until equilibrium was reached. The intrinsic mechanical properties of the samples, aggregate modulus ($H_A$), permeability ($k$), and Poisson's ratio ($\nu$), were determined using the linear biphasic theory.$^{134,319}$

Tensile tests were performed on Phase II samples ($n=6$) with an electromechanical materials testing system (Instron Model 5565, Canton, MA) using a 50 N load cell. Samples were cut into a dog-bone shape with an approximate gauge length of 1.5-mm and glued onto paper tabs for gripping. The thickness and width of each sample were appropriately measured to calculate the cross sectional area for the applied forces. Samples were pulled at a constant strain rate of $0.01 \text{ s}^{-1}$. Stress-strain curves were developed from the load-displacement curve and analyzed for the tissue's tensile Young's modulus ($E_Y$) and ultimate tensile strength (UTS).

**Statistical analysis**

A single factor ANOVA was used with a Tukey's post-hoc when warranted. Significance was defined as $p < 0.05$ throughout the study.

**Results**

Based upon statistical changes in total collagen/wet weight (WW) and GAG/WW levels within the constructs in Phase I, the following four time points were selected for further investigation in Phase II: Day 7, Day 14, Day 28, and Day 56.
**Gross appearance**

The physical characteristics of self-assembled engineered articular cartilage constructs followed similar trends in development during both experimental phases (Table 8). At day 1, constructs appeared non-uniform and did not have smooth surfaces. Beginning at day 7, the surfaces of the construct began to glisten, suggesting an increased hydration and presence of GAGs (Fig. 36). This glistening appearance consistently became more evident over time. Finally, from 4 wks onward, the tissue constructs took on a slight "bowl shaped" morphology.

**Histology and immunohistochemistry**

The spatial arrangement of GAGs and collagen type II and VI was modulated throughout the course of neotissue development (Fig. 36). Safranin-O staining for GAGs was observed in a pericellular distribution initially, with the staining intensity increasing over time throughout the construct. Collagen type II appeared evenly distributed within the ECM, with its staining intensity increasing uniformly over time. Interestingly, collagen type VI staining was observed in abundance initially throughout the tissue matrix and then could be seen localized around individual cells by 4 wks. Based on IHC, there was no collagen types I or X production at any time point, indicating no cellular dedifferentiation or hypertrophy in the constructs. Additionally, N-cadherin staining demonstrated a minimal presence of the protein in cell suspension. During the first day, however, the intensity of N-cadherin staining increased drastically and peaked at 4 days.
Minimal N-cadherin staining could be observed onwards of 2 wks. Similar histology pictures could be seen for Phase II (not shown) as was observed for Phase I.

**Biochemical analysis**

In the first phase, an increasing trend for GAG/WW was observed, with statistically significant increases occurring between days 7 and 14, days 14 and 28, and days 28 and 56. The GAG/WW values were 1.0 ± 0.6%, 1.3 ± 0.4%, 1.9 ± 0.2%, 2.1 ± 0.7%, 3.5 ± 0.8%, 7.5 ± 1.1%, 8.2 ± 1.2%, and 9.1 ± 0.3%, at days 1, 4, 7, 10, 14, 28, 42, and 56, respectively. Total GAG per construct also increased during tissue development, attaining a maximum value of 3.2 ± 0.2 mg at 8 wks. In contrast to GAG/WW, a decreasing trend was found for total collagen/WW, with statistically significant changes between days 28 and 56. However, total collagen per construct displayed an increasing trend over time reaching a value of 1.9 ± 0.3 mg at 8 wks. The collagen/WW values were 19.0 ± 6.2%, 19.0 ± 4.4%, 18.1 ± 4.4%, 12.5 ± 2.4%, 12.0 ± 2.7%, 9.6 ± 2.1%, 6.5 ± 0.9%, and 5.3 ± 0.9%, at days 1, 4, 7, 10, 14, 28, 42, and 56, respectively (Fig. 37). There were no statistical differences in construct cellularity during neotissue maturation.

Further investigation in Phase II into the specific levels of collagens type II and VI exposed differing trends. Through ELISA quantification, the levels of collagen type II/WW were 0.7 ± 0.2%, 1.4 ± 0.1%, 3.9 ± 0.1%, and 4.9 ± 0.7% for 1, 2, 4, and 8 wks, respectively, with statistical differences between 2 and 4 wks, and 4 and 8 wks. When normalized to total collagen, percentages of collagen type II to total collagen were 8.1 ± 2.0%, 13.8 ± 3.0%, 42.3 ± 3.9%, and 69.1 ± 12.9% for 1, 2, 4, and 8 wks, respectively (Fig. 38). Statistical changes were observed between 2 and 4 wks, and 4 and 8 wks.
Through western blotting, the proportion of collagen type VI to total collagen also changed during development (Fig. 38). In the example blot, the integrated optical density of the band decreased by approximately 40% between 1 and 2 wks. No change was observed between 2 and 4 wks, and a 15% increase in band density occurred between 4 and 8 wks. Similar trends were observed in all samples tested. Collagen type I was not detectable at any time point with the ELISA.

Examination into the specific GAGs in Phase II samples revealed a decreasing ratio of CS-6 to CS-4 over time (Fig. 38). At 1, 2, 4, and 8 wks the relative levels of CS-6 to CS-4 was $2.5 \pm 0.4$, $2.1 \pm 0.2$, $1.8 \pm 0.1$, and $1.4 \pm 0.3$, respectively. Statistical differences were observed between 1 and 4 wks, 1 and 8 wks, and 2 and 8 wks. The total amount of chondroitin (0-, 4-, and 6-) sulfate was further quantified to display an increasing molar concentration over time. The total chondroitin sulfate values were $7.4 \pm 4.5$ nmol/mg, $36.4 \pm 7.3$ nmol/mg, $48.4 \pm 12.6$ nmol/mg, and $64.5 \pm 5.3$ nmol/mg for 1, 2, 4, and 8 wks, respectively, and statistical increases were recorded between 1 and 2 wks and 4 and 8 wks. Conversely, the total dermatan sulfate levels were $1.4 \pm 1.3$ nmol/mg, $4.2 \pm 3.6$ nmol/mg, $7.5 \pm 3.1$ nmol/mg, and $5.3 \pm 1.7$ nmol/mg for 1, 2, 4, and 8 wks, respectively, with a statistical difference observed only between 1 and 4 wks. Moreover, the amount of glucose in each sample was $0.2 \pm 0.2$ nmol/mg, $0.6 \pm 0.2$ nmol/mg, $3.1 \pm 1.1$ nmol/mg, and $3.1 \pm 0.9$ nmol/mg at 1, 2, 4, and 8 wks, respectively, with a statistical increase observed between 2 and 4 wks. Finally, hyaluronan was not observed in a measurable quantity at any time point.

The second phase of this study also displayed similar values and trends for total GAG and collagen as the first phase (Fig. 39). The GAG/WW levels were $2.4 \pm 0.3\%$, 5.9
± 0.2%, 9.4 ± 0.7%, and 10.0 ± 0.1% for 1, 2, 4, and 8 wks, respectively. Statistical changes were observed for GAG/WW between 1 and 2 wks, and 2 and 4 wks. The collagen/WW levels were 8.9 ± 1.0%, 10.0 ± 1.6%, 9.4 ± 0.9%, and 6.9 ± 1.0% at 1, 2, 4, and 8 wks, respectively. A statistically significant drop in collagen/WW was observed between 4 and 8 wks.

**Mechanical assessment**

The compressive and tensile properties of the constructs were assessed in Phase II (Fig. 39). The aggregate modulus of the constructs was 37.2 ± 15.2 kPa, 85.7 ± 19.4 kPa, 280.3 ± 52.7 kPa, and 304.0 ± 72.6 kPa, for 1, 2, 4, and 8 wks, respectively, with a statistically significant increase between 2 and 4 wks. A significant linear correlation (R² = 0.96, p < 0.05) was further observed between the mean aggregate modulus and GAG/WW of each group. The permeability of the constructs was 1.5 ± 1.9 (x 10⁻¹⁵) m⁴/N-s, 8.5 ± 7.5 (x 10⁻¹⁵) m⁴/N-s, 6.0 ± 5.8 (x 10⁻¹⁵) m⁴/N-s, and 11.0 ± 4.5 (x 10⁻¹⁵) m⁴/N-s at 1, 2, 4, and 8 wks, respectively. A significant difference in permeability was observed between 1 and 8 wks. The Poisson’s ratio of the constructs also increased over time, with values of 0.1 ± 0.1, 0.2 ± 0.1, 0.3 ± 0.1, 0.4 ± 0.1 at 1, 2, 4, and 8 wks, respectively. Significant changes in Poisson’s ratio were observed between 1 and 4 wks, 1 and 8 wks, and 2 and 8 wks.

The Young’s modulus of the 4 wk constructs at 1035.4 ± 96.4 kPa was statistically greater than any other time point. The ultimate tensile strength of the 2 and 4 wk constructs at 293.5 ± 55.3 kPa and 342.7 ± 52.3 kPa, respectively, were significantly different from the 1 and 8 wk groups at 143.4 ± 38.7 kPa and 200.3 ± 51.1 kPa,
respectively. Univariate regression analysis revealed that total collagen/WW did not have a statistically significant relationship with either the ultimate tensile strength ($R^2 = 0.29$, $p = 0.46$) or the Young’s modulus ($R^2 = 0.001$, $p = 0.97$) of each group. Similarly, collagen type II/WW was not statistically correlated to the Young’s modulus ($R^2 = 0.49$, $p = 0.30$) or the ultimate tensile strength ($R^2 = 0.10$, $p = 0.68$) at each time point.

Discussion

This study was designed to examine the maturation of neotissue within a self-assembling process to engineer articular cartilage over an 8 wk culture period. It is important to understand neotissue development, since this may elucidate potential intervention windows for biochemical or biomechanical stimulation, as well as provide an essential characterization of structure and function of tissue replacements. This study presents several notable findings pertaining to the self-assembling process. 1) We demonstrate that collagen type VI is present throughout the ECM at early stages of neotissue growth and then localizes to a PCM around 4 wks, thereby recapitulating a major characteristic of in vivo cartilage development.\textsuperscript{160} 2) Our results suggest that the initial cellular aggregation and construct formation within the self-assembling process is mediated by cadherin-cadherin interactions. 3) The protein content for collagens type II and VI, and relative levels of CS-4 and CS-6, follow similar trends to that of native cartilage development during all 8 wks of culture. Similarly, tissue compressive and tensile mechanical properties mirrored developmental trends during the first 4 wks. 4) This study identifies 4 wks as a potential time point for exogenous stimulation to further augment the functional properties of the tissue constructs.
To our knowledge, this is the first study to demonstrate the localization of collagen type VI in a scaffold-less tissue engineering approach. Previous research has identified the protein in tissue engineered cartilage scaffolds, and chondrocyte-seeded agarose constructs, but not in a self-assembly cell-based approach which seems to capture the developmental nature of articular cartilage. Collagen type VI is the primary marker for the PCM. The PCM, together with its enclosed cell, is defined as the chondron and is considered to be the smallest metabolic and functional unit of articular cartilage. In particular, it is known that the PCM surrounding the chondrocyte organizes and constructs collagen fibrils, regulates cellular osmolarity, and modulates growth factor interactions with the enclosed cell. The PCM also acts as a biomechanical buffer of applied stresses and may play an important role in the mechanotransductive response of chondrocytes to exogenous stimulation within a tissue engineering approach. Therefore, future work may focus on investigating the three-dimensional structure of chondrons in self-assembled cartilage constructs to further characterize the chondrocyte’s developing microenvironment. In addition, chondrons can be isolated from cartilage constructs at various stages of development and directly tested to determine the mechanical properties of the newly-formed PCM.

We propose that the early stages of tissue development within the self-assembling process are mediated by cadherin-cadherin interactions, as described by the differential adhesion hypothesis. These interactions act to minimize the free energy of the population of cells by sorting cells with varying intracellular adhesiveness. In articular cartilage, it has been shown that N-cadherin activity spikes to its highest level during mesenchymal condensation and subsequently decreases around the central condensation
region, as the cells spread apart and begin to differentiate into chondrocytes. At later stages of joint development, N-cadherin staining is only evident in the perichondrium and not in mature cartilage tissue. A similar trend is observed in the maturation of self-assembled engineered cartilage, suggesting that our process captures this integral component of native articular cartilage development. N-cadherins are minimally present in the initial cell suspension, and then are observed with an intense staining upon 1 day of seeding. Therefore, it can be inferred that N-cadherin activity plays a significant role in cellular aggregation during the self-assembling process. Indeed, this may have broad implications for future tissue engineering studies attempting to recapitulate the zonal architecture of various cartilaginous tissues through co-cultures of cells with varying cadherin levels.

Parallel trends in collagen content can be observed in the maturation of tissue within the self-assembling process and native articular cartilage development. In our study, collagen type II represented a small fraction (8%) of the total collagen at early stages of neotissue maturation. Over time, collagen type II levels significantly increased, reaching 69% of the total collagen at 8 wks. Similarly, collagen type II is not found in native tissue until after partuition, both in terms of mRNA expression or protein production. In mature cartilage, however, collagen type II is observed throughout the ECM and composes approximately 85 – 90% of the total collagen. It was further observed in this study that collagen type VI was present in high abundance initially, and then decreased in proportion to total collagen, as the protein localized around individual cells. An analogous trend has been observed in native articular cartilage for collagen type VI. The expression of collagen type VI spikes at chondrocyte differentiation and is
present throughout matrix development.\textsuperscript{57, 160} This steep rise in collagen type VI expression in native tissue soon levels off at later stages of chondrogenesis, coinciding with an upregulation in collagen type II.\textsuperscript{58}

In terms of chondroitin sulfate, there are several similarities between the self-assembling process and native tissue maturation. The decreasing ratio of CS-6 to CS-4 (from 2.5 at 1 wk to 1.4 at 8 wks) observed in this study is mirrored in native cartilage tissue development. During embryogenesis, CS-6 can be found throughout the developing matrix, then from parturition onwards the staining intensity and relative abundance for the GAG increase uniformly.\textsuperscript{60} Conversely, CS-4 is observed in trace amounts in the developing ECM,\textsuperscript{60} and then increases in content at a greater rate than CS-6 post parturition.\textsuperscript{161, 322} However, it should be noted that species dependant sulfation patterns have been previously reported in developing tissues.\textsuperscript{163}

Similar trends can also be drawn between the maturing biomechanical properties of self-assembled articular cartilage and those of native tissue. Excitingly, this study observed analogous trends in compressive mechanical properties to reported results in the aggregate moduli of developing bovine articular cartilage. Our 2 wk (85 ± 19 kPa), 4 (280 ± 52 kPa), and 8 wk (304 ± 72 kPa) values are right on par with the aggregate moduli of third trimester fetal (110 ± 30 kPa), 1 - 3 wk old calf (270 ± 20 kPa), and young adult bovine (310 ± 30 kPa) articular cartilage explants, respectively, from the knee joint.\textsuperscript{310} However, previous studies have shown that the stiffness of mature bovine cartilage, as measured by creep indentation, ranges from 472 kPa to 899 kPa,\textsuperscript{319, 323} suggesting that further increases in the compressive properties of self-assembled articular cartilage constructs may be necessary. Additional comparisons can be made between the
changes in the emergent tensile properties of self-assembled cartilage constructs and the developing cartilage from the femoral condyle. Significant increases in the Young’s moduli of our constructs between 2 and 4 wks and decreases between 4 and 8 wks mirrored the changes observed between third trimester fetal, 1 - 3 wk old calf, and young adult bovine articular cartilage.\textsuperscript{311} These similarities in compressive and tensile properties are additionally noteworthy since our current study utilized immature bovine articular chondrocytes in the self-assembling process. Taken together with the observed trends in biochemical components, our results suggest that the self-assembling process embodies many of the known developmental characteristics for articular cartilage. Future studies, therefore, should look to identify key developmental signaling patterns, such as Wnt\textsuperscript{324, 325} or bone morphogenetic protein expression,\textsuperscript{326} to confirm this tissue engineering approach as an appropriate model for \textit{in vitro} cartilage development. Moreover, further research may examine matrix maturation during self-assembly of differentiated stem cells,\textsuperscript{243} which more closely resemble the phenotypic state of cells during the mesenchymal condensation phase of native articular cartilage development.

Statistically significant changes in the developing compressive and tensile mechanical properties of the neotissue were generally accompanied by concurrent alterations in GAG and collagen levels, respectively. Significant increases in GAG/WW between 1 wk (2.4%), 2 wk (5.9 %), and 4 wk (9.4%) coincided with a stiffening of the constructs (37.2 kPa to 85.7 kPa to 280.3 kPa). Moreover, a significant correlation was observed between GAG levels and aggregate moduli. Notably however, no change was observed in the aggregate modulus or GAG content after 4 wks, although the Poisson’s ratio did continue to increase. This suggests that other ECM components, possibly cross-
linking proteins collagen type IX or fibronectin, may influence the apparent compressibility of the constructs, and thus warrant further investigation. Similar relationships were observed between tensile properties and collagen content. Statistically greater ultimate tensile strength values at 2 and 4 wks (293.5 kPa and 342.7 kPa, respectively) over 1 and 8 wks (143.4 kPa and 200.3 kPa, respectively) followed similar trends in total collagen/WW. The statistically significant decrease in total collagen/WW between 4 and 8 wks also coincided with a significant drop in Young’s moduli (1035.4 kPa to 537.9 kPa). The significant increase in Young’s modulus between 2 and 4 wks, however, was not accompanied by changes in total collagen/WW. Therefore, it is possible that the observed tensile stiffening may be due to collagen organizational changes or increased cross-linking within the ECM. Additionally, this difference in tensile properties may be accounted for by an increased presence of collagen type II. Similarly, significant increases in Young’s moduli and collagen type II/WW were observed between 2 and 4 wks, indicating that the specific type of collagen, not just total collagen content, present in the neotissue may dramatically influence tensile properties. Moreover, this result confirms the substantial role that collagen type II has on tensile characteristics, and therefore a preferred target for growth factor treatments to augment these properties.

Examining the structural and biochemical changes during tissue construct growth within the self-assembling process sheds light on potential intervention strategies employing exogenous stimulation. At 4 wks, the GAG content of our constructs (9.4% GAG/WW) far exceeds that of native tissue (~5% GAG/WW). While it is important not to hinder the developing compressive properties, the application of small doses of
chondroitinase-ABC$^{329}$ or other treatments may be helpful to reorganize the tissue matrix and bring GAG levels closer to those of native tissue. This is essential for later in vivo work where a mismatch between the osmolarity of the constructs and the surrounding host tissue is undesirable. Potential mechanical stimulation windows can also be identified. Our current finding of PCM formation after 4 wks of growth suggests that this is potentially an appropriate time point for compressive mechanical stimulation, since the PCM is known to appropriately facilitate the transmission of mechanical stresses onto the enclosed chondrocytes.$^{78, 102, 232}$ Moreover, our observation that the aggregate modulus reached a plateau at 4 wks, and the tensile Young’s modulus decreased after 4 wks further warrants consideration that exogenous stimulation mechanisms may be necessary around that time point to further augment the functional characteristics of the neotissue. The statistical drop in total collagen/WW between 4 and 8 wks, coinciding with the decrease in tensile properties, is possibly due to increased activity of matrix metalloproteinases, and thus, exogenous tissue inhibitor of matrix metalloproteinases may be incorporated during this time period to prevent further breakdown of the collagen matrix. The slight increase in collagen type VI to total collagen between 4 and 8 wks may also signify greater matrix catabolism.$^{330}$ Moreover, the lack of hyaluronan production at any time point suggests that GAGs in our constructs are not being integrated into aggregan aggregates, which is likely essential for a continued increase in compressive mechanical properties beyond 4 wks. Therefore, future studies should consider mechanisms to initiate hyaluronan production, such as hypoxia,$^{331}$ growth factor stimulation,$^{332}$ or inducible overexpression systems.$^{333}$ Finally, a significant increase in glucose retention was observed in the constructs between 2 and 4 wks, with no change
thereafter. This drop in glucose consumption could be indicative of a concomitant decrease in chondrocyte metabolic activity during this period, which may necessitate dynamic mechanical stimulation\textsuperscript{334} or greater concentrations of ascorbic acid added to the culture medium\textsuperscript{335} at 4 wks.

In conclusion, the development of neotissue within the self-assembling process can be described in four phases (Fig. 40). In the first phase, cells are seeded at a high density in a non-adherent agarose mold. Minimal N-cadherin activity is present at this stage, as the cells have not yet begun to recognize each other. In the second phase, N-cadherin activity increases, suggesting greater cell-cell interactions. According to the differential adhesion hypothesis, cells begin to coalesce at this stage to minimize the free energy within the biological system. In the third phase, cells begin to migrate apart and produce an ECM consisting of primarily collagen type VI, a known developmental marker, for collagens and CS-6 for GAGs. In the fourth phase, separations within the ECM can be identified, with notable differences in the spatial distribution of collagen types II and VI. The mature tissue matrix consists predominately of collagen type II, while collagen type VI decreases in proportion to total collagen and presumes a pericellular localization. In addition, the chondroitin sulfation patterns change during neotissue development, as the CS-6 to CS-4 ratio follows a downward trend over time. The composition of this ECM can be directly related to the emergent compressive and tensile mechanical properties of the neotissue.
Table 8: Physical characteristics of self-assembled articular cartilage constructs

<table>
<thead>
<tr>
<th>Phase</th>
<th>Wet Weight (mg)</th>
<th>Thickness (mm)</th>
<th>Diameter (mm)</th>
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<tr>
<td></td>
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</tr>
<tr>
<td>Day 1</td>
<td>4.2 ± 2.8</td>
<td>0.4 ± 0.1</td>
<td>5.0 ± 0.2</td>
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<tr>
<td>Day 4</td>
<td>6.7 ± 1.3</td>
<td>0.4 ± 0.1</td>
<td>5.2 ± 0.3</td>
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<td>Day 7</td>
<td>7.0 ± 1.3</td>
<td>0.4 ± 0.1</td>
<td>5.5 ± 0.2</td>
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<tr>
<td>Day 10</td>
<td>8.9 ± 1.0</td>
<td>0.5 ± 0.1</td>
<td>5.3 ± 0.2</td>
</tr>
<tr>
<td>Day 14</td>
<td>10.1 ± 1.1</td>
<td>0.5 ± 0.1</td>
<td>5.3 ± 0.2</td>
</tr>
<tr>
<td>Day 28</td>
<td>15.9 ± 2.7</td>
<td>0.6 ± 0.2</td>
<td>5.9 ± 0.2</td>
</tr>
<tr>
<td>Day 42</td>
<td>25.7 ± 3.7</td>
<td>0.6 ± 0.2</td>
<td>6.3 ± 0.1</td>
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<tr>
<td>Day 56</td>
<td>35.9 ± 1.8</td>
<td>0.8 ± 0.2</td>
<td>7.3 ± 0.1</td>
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<td>Week 1</td>
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<td>0.3 ± 0.1</td>
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<tr>
<td>Week 2</td>
<td>8.9 ± 0.5</td>
<td>0.4 ± 0.1</td>
<td>5.0 ± 0.1</td>
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<tr>
<td>Week 4</td>
<td>18.6 ± 0.8</td>
<td>0.6 ± 0.1</td>
<td>5.9 ± 0.1</td>
</tr>
<tr>
<td>Week 8</td>
<td>34.3 ± 5.8</td>
<td>0.8 ± 0.1</td>
<td>7.1 ± 0.2</td>
</tr>
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</table>
**Figure 36: Gross morphology and histological sections of self-assembled cartilage constructs**

GAGs were initially concentrated pericellularly, but were observed throughout the tissue by 4 wks. Also at 4 wks, collagen type VI localized around the cells, while collagen type II appeared evenly distributed in the constructs. Original magnification, 40X.

<table>
<thead>
<tr>
<th>Day</th>
<th>Gross Morphology</th>
<th>GAG</th>
<th>Col II</th>
<th>Col VI</th>
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**Figure 37: GAG and collagen content in constructs during development**

In Phase I, significant differences in GAG/WW can be observed between 7 and 14 days, 14 and 28 days, and 28 and 56 days (A). In addition, significant differences can be observed in collagen/WW between 7 and 14 days, and 28 and 56 days (B). Increasing trends were observed for both total GAG per construct (C) and total collagen per construct (D). Data presented as mean ± standard deviations.
Figure 38: Major biochemical changes in cartilage neotissue maturation with the self-assembling process

The percentage of collagen type II to total collagen increased consistently over time, from 8.1% at 1 wk to 69.1% at 8 wks, with statistically significant increases between 2, 4, and 8 wks (A). In a western blot for collagen type VI, normalized to total collagen content, it is observed that the proportion of collagen type VI to total collagen decreased by approximately 40% between 1 and 2 wks and then increased slightly at 8 wks (B). Relative collagen type VI levels were determined by the integrated optical density of each band. The ratio of CS-6 to CS-4 decreased steadily during construct maturation, from 2.4 at 1 wk to 1.4 at 8 wks (C). Data presented as mean ± standard deviations.
Figure 39: Construct biomechanical and biochemical properties in Phase II

Large and significant increases in the aggregate moduli (A) were observed between 2 and 4 wks, corresponding to increases in GAG/WW (B). The Young’s modulus and ultimate tensile strength (C) increased during the first 4 wks, coinciding with increases in the percentage of collagen type II to total collagen. Both tensile mechanical properties then decreased significantly at 8 wks, concurrent with a similarly significant decrease in total collagen/WW (D). Data presented as mean ± standard deviations.
**Figure 40: Tissue development within the self-assembling process**

In the first phase of neotissue development, a high density cell suspension is seeded in an agarose mold. In the second phase, chondrocytes begin to recognize each other and coalesce through a minimization of the free energy in the system, as described by the differential adhesion hypothesis. In the third phase, tissue constructs begin to form as the cells migrate apart and secrete predominately collagen type VI and CS-6 to compose its extracellular matrix. In the fourth phase, extracellular matrix separations become apparent, as indicated by a PCM surrounding individual chondrocytes. The initially abundant collagen type VI decreases in proportion to total collagen and localizes around individual cells, as the percentage of collagen type II to total collagen increases. The mature tissue matrix consists predominately of collagen type II and a slightly greater percentage of CS-6 than CS-4.
CONCLUSIONS

This thesis presented work toward 1) improving our knowledge of articular chondrocyte mechanobiology, and 2) enhancing the ability of current tissue engineering techniques to recapitulate native cartilage structure and function. The burden of treating patients with injured cartilage is enormous. Each year, countless Americans suffer trauma to musculoskeletal tissues, resulting in billions in health care and related job loss costs. In particular, millions of Americans are afflicted with osteoarthritis, the most common form of arthritis, which affects their joint movement and quality of life. Thus, developing new approaches to treat damaged or degenerated articular cartilage would be a tremendous boon to the numerous patients with osteoarthritis, as well as have important economic ramifications. To this end, this thesis examined two avenues of cartilage research. The first is related to the mechanisms of cartilage degradation and regeneration by studying the mechanical properties and behavior of the single chondrocyte. The second corresponds to the microenvironment of chondrocytes by examining the development of engineered cartilage tissue.

The overall hypotheses of this thesis were that: a) biomechanical or biochemical stimuli may alter the mechanical characteristics of single chondrocytes; b) chondrocyte biomechanical properties and behavior are modulated by intracellular structural components; and c) extracellular matrix maturation in our ‘self-assembling’ tissue engineering approach would mirror the known developmental process of articular cartilage. To address these hypotheses, three Specific Aims were developed, which examined the biomechanical characteristics of single chondrocytes and their developing pericellular and extracellular matrices. The first two Aims of this thesis built upon
previous research in our laboratory to elucidate the underlying intracellular structures influencing a cell’s mechanical response to physical forces and how bulk cellular properties may change during regenerative processes. In addition, prior to this thesis, much work in our group had focused solely on characterizing the end point biochemical and mechanical properties of tissue cartilage engineered constructs. Thus, toward identifying potential intervention strategies to improve functional neotissue parameters, the third Specific Aim of this thesis investigated the emergent biophysical environment of chondrocytes and mechanisms of cellular aggregation within a self-assembling process for cartilage engineering.

Prior research has shown that alterations in chondrocyte mechanical properties may be indicative of degenerative or regenerative states in cartilage. While these changes in mechanical characteristics are thought to be associated with modifications in the cytoskeleton, the unique relationships of each cytoskeletal element, actin microfilament, intermediate filaments, and microtubules, to the compressive biomechanics of single chondrocytes previously remained unknown. Hence, Chapter 2 of this thesis was motivated to study the role of these intracellular elements in chondrocyte mechanical properties and behavior toward elucidating the cytoskeleton’s role in reinforcing cell structure and identifying potential treatments for restoring homeostatic conditions. Inhibition of actin microfilaments resulted in the largest decrease (~50%) in bulk compressive stiffness and increase (~30%) in cell volume. Moreover, chondrocytes exhibited strain-dependent characteristics in their volumetric changes, as measured through an apparent Poisson’s ratio, which can be attributed to a balance of intermediate filaments and microtubules. In particular, intermediate filaments contributed to cellular
compressibility (or volume loss), while microtubules played a role in cell incompressibility (or volume retention). Thus, the decrease in Poisson’s ratio at high strains may be due to a breakdown of the microtubule network. In addition, a critical threshold of applied strain was reported at approximately 30%, wherein chondrocytes could no longer regain their initial volume after compression. This was considered to be akin to a yield point in the stress-strain behavior of the cell and may be associated with degenerative characteristics in cartilage. Furthermore, the strain threshold in chondrocyte recovery behavior underwent the largest decrease (~33%) following microtubule disruption. Together with Poisson’s ratio data, these results highlight microtubules as an important target for biochemical agents to help cells recover from unphysiological loads.

Chapter 3 further investigated the role of subcellular structures in cell biomechanics by specifically studying the contribution of the nucleus to cellular compressive characteristics. It has been previously demonstrated that the nucleus undergoes substantial deformation during tissue loading,\textsuperscript{35} which is associated with gene expressions changes.\textsuperscript{120, 168} Thus, a concrete understanding of how the nucleus senses and responds to forces may provide insight into the basis of cellular mechanotransduction. Toward this objective, a finite element approach was utilized in this chapter to determine the \textit{in situ} material properties for the nucleus and cytoplasm, based on known strains for the chondrocyte under compression.\textsuperscript{168} The Young’s moduli of the cytoplasm and nucleus were varied parametrically from 1 to 7 kPa for several combinations of cytoplasmic and nuclear Poisson’s ratios. It was observed that changing the Poisson’s ratio had little effect on the behavior of the model, suggesting that incompressibility is a reasonable assumption for the cell at low strains (see Chapter 2). In addition, it was found that the
experimental data were best matched when the moduli of the cytoplasm and nucleus were 3.75 kPa and 5.25 kPa, respectively. Moreover, this presents a substantially lower ratio of cytoplasmic to nuclear stiffness than prior reports for isolated nuclei. In addition, examining nuclear properties in situ is a significant advantage over previous approaches, which studied these characteristics after the nucleus was isolated from the cytoplasm. A more integrated mechanical framework between the cytoplasm and nucleus results when the connections between the nuclear lamina and cytoskeleton remain intact, facilitating the appropriate transmission of force to the nucleus. Future research may examine if the mechanical characteristics of the nucleus change as a function of applied strain, similar to the approach taken in Chapter 2, to improve our knowledge of mechanical forces in cartilage mechanobiology.

While the previous two chapters focused on chondrocyte mechanics during compression, these cells also experience substantial shear forces during everyday activity. Thus, to further our knowledge of single chondrocyte biomechanics, a novel videocapture technique was developed in Chapter 4, which enabled the direct measurement of cell deformations and forces under applied shear. In this approach, cell shear was achieved via the controlled lateral displacement of a miniature probe and the entire event was video-recorded through a CCD camera connected to the microscope. To further examine potential non-uniformities in chondrocyte shear characteristics, the shear probe was placed in three different positions along the cell height. It was observed that the apparent shear modulus of single chondrocytes decreased (~50%) as the probe transitioned from the cell base to its top. This mechanical heterogeneity correlated well with spatial variations in the actin cytoskeleton during shear. In addition, chondrocytes
were observed to move along their substrate until reaching a yield point, wherein focal adhesions reorganized into strong pockets along the cells' trailing edge. While the cells experienced the greatest peak forward displacement (~30% their initial diameter) when the probe was placed near the base, the critical strain for this plateau in forward movement was unaffected by probe height. This suggests that focal adhesion activation in chondrocytes may occur primarily through strain-dependent, rather than force-dependent, means. The results presented in this chapter complement prior work which has shown that shear forces directly influence cellular homeostasis and matrix production in cartilage.\(^6, 82, 218, 219\) Therefore, understanding the fundamental mechanisms at play during these processes will aid future models for cartilage engineering and the study of osteoarthritis.

Another important component in cartilage engineering is the selection of an abundant cell type that is capable of producing cartilage-specific matrix proteins. With this consideration in mind, the mechanical characteristics of differentiated human embryonic stem cells (hESCs) were examined in Chapter 5 to provide insight into the role of biophysical forces in promoting chondrogenesis. hESCs hold tremendous promise as an alternative cell source since they have the ability to differentiate into any type of cell in the body. Applied to cartilage regeneration, these cells may be induced into a chondrocyte phenotype and utilized in tissue engineering modalities. Furthermore, it is important to identify groups of differentiated hESC with similar mechanical properties as native chondrocytes since these cells will inherently need to withstand any mechanical stimulation regimen employed in tissue engineering, as well as the highly mechanical environment in the native joint. Using two common differentiation regimens,
Transforming Growth Factor-β1 (TGF-β1) or Knock Out Serum Replacer (KOSR), single hESCs subpopulations were separated based on density and tested under unconfined compression for their viscoelastic properties. Compiling the results of the subpopulations from both TGF-β1 and KOSR differentiation regimens yielded a spectrum of mechanical characteristics spanning the chondrogenic range of undifferentiated hESCs to undifferentiated mesenchymal stem cells to native articular chondrocytes. However, one subpopulation of cells from the TGF-β1 group had similar compressive and morphological properties as native chondrocytes and may therefore prove to be a valuable cell source for cartilage tissue engineering.

Upon selecting an appropriate cell source for cartilage engineering, growth factors are frequently employed to promote robust extracellular matrix synthesis. Using a single cell approach, Chapter 6 discussed the effect of two prominent cartilage growth factors, TGF-β1 and Insulin-like Growth Factor-I (IGF-I), on chondrocyte mechanics. The results in this chapter demonstrated that the growth factors similarly affect chondrocyte biomechanics, with an approximately two-fold increase in both cell stiffness and apparent Poisson’s ratio. Moreover, the critical threshold in applied strain (see Chapter 2) was unaffected by growth factor treatment. Considering that TGF-β1 and IGF-I principally alter cell stiffness and compressibility, but not this critical strain, it is possible that these growth factors primarily target actin microfilaments and intermediate filaments (see Chapter 2). In addition, examining the influence of TGF-β1 and IGF-I on cellular mechanical properties has great implications for the use of these growth factors in future tissue engineering techniques. Indeed, prior research in our group has shown that TGF-β1 and IGF-I may substantially alter the functional properties of engineered cartilage.
Thus, by studying the specific effect of these biochemical agents on single chondrocyte characteristics, proper dosing regimens may be developed to elicit particular cellular responses and enhance tissue construct properties.

While past studies in our lab have focused on the end functionality of tissue constructs, an understanding of the development of neotissue within the self-assembling process previously remained incomplete. Information regarding the emergent cellular milieu in tissue engineered constructs will enable researchers to formulate models which accurately depict the transmission of biomechanical signals down to the single cell level\textsuperscript{78},\textsuperscript{138} and identify regimens of exogenous stimuli tailored to specific stages of construct growth. Toward this goal, Chapter 7 described a two-phased experimental approach in studying the development of specific extracellular matrix components within a self-assembling process for cartilage engineering, and their relationship to functional tissue properties. It was observed that chondrocytes initially aggregated and assembled through N-cadherin mediated interactions, which recapitulated a key mechanism of native cartilage growth.\textsuperscript{50, 51} A redistribution of collagen was also noted with time, as collagen type VI was initially abundant throughout the construct and then became localized to a pericellular distribution by 4 wks. A corresponding increase in collagen type II, dispersed throughout the construct, was observed on the same time scale. This collagen reorganization is reminiscent of native articular cartilage development.\textsuperscript{160} Similar developmental trends were echoed in decreasing ratios of chondroitin 6- to 4-sulfate over time.\textsuperscript{60} The composition of this extracellular matrix was directly related to the emergent compressive and tensile mechanical properties of the neotissue, which either reached a plateau or peaked at 4 wks, respectively. Moreover, these results open
the door to an array of future methods for employing exogenous stimulation toward improving cartilage tissue construct properties. For instance, pericellular matrix formation at 4 wks suggests this may be an appropriate window for direct mechanical stimulation. In addition, inhibitors of matrix catabolism may be incorporated at this same time period to reduce the loss of total collagen and tensile properties.

This thesis contributed significantly to our understanding of how chondrocyte mechanical characteristics change in the presence of biochemical agents indicative of regenerative processes or resulting from phenotypic modulation; and elucidated the underlying intracellular structural components at play during these tangible processes. Moreover, these results provide vital knowledge on cellular responses to mechanical forces and the loading regimens related to cartilage health and disease. In addition, through a fundamental understanding of the emergent biophysical environment of chondrocytes within the self-assembling process, this thesis presented several intervention strategies toward improving our current tissue engineering as a treatment for cartilage afflictions.
REFERENCES


