RICE UNIVERSITY

Optimizing a Scaffoldless Approach for Cartilage Tissue Engineering

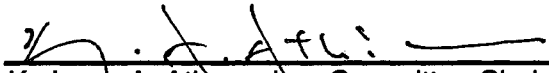
by

Benjamin Daniel Elder

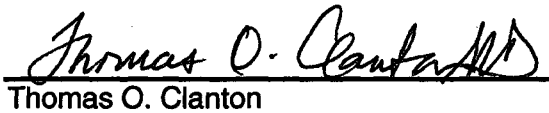
A THESIS SUBMITTED
IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE

Doctor of Philosophy

APPROVED, THESIS COMMITTEE:

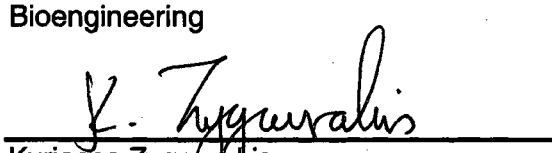


Kyriacos A. Athanasiou, Committee Chair
Karl F. Hasselmann Professor
Bioengineering



Thomas O. Clanton
Professor
Orthopaedic Surgery
UTHSC-Houston

Antonios G. Mikos
John W. Cox Professor
Bioengineering



Kyriacos Zygourakis
Chair, A.J. Hartsook Professor
Chemical and Biomolecular Engineering

HOUSTON, TEXAS
JANUARY, 2009

Abstract

Optimizing a Scaffoldless Approach for Cartilage Tissue Engineering

By Benjamin D. Elder

Articular cartilage has a poor intrinsic healing response, so tissue engineering provides a promising approach for cartilage regeneration. The major objective of this proposal was to enhance the self-assembling process, used in articular cartilage tissue engineering, by investigating the effects of construct confinement, hydrostatic pressure application, and growth factor addition. First, the effects of construct confinement in different directions and at different times were investigated. It was demonstrated that construct confinement resulted in enhanced biomechanical properties in the direction orthogonal to the confinement surface, either by enhancing collagen organization or by increasing collagen production. Next, the effects of hydrostatic pressure at different timepoints, magnitudes, and frequencies on the biomechanical and biochemical properties of self-assembled constructs were determined. It was demonstrated that the application of static hydrostatic pressure, at 10 MPa, for 1 h/day, from days 10-14 days led to significant increases in compressive and tensile properties, accompanied by significant increases in GAG and collagen content, respectively. To our knowledge, this was the first study to demonstrate increases in the biomechanical properties of tissue from pure HP application. Furthermore, the effects of exogenous application of growth factors, at varying concentrations, dosages, and combinations, with and without hydrostatic pressure, were

assessed on the biochemical and biomechanical properties of engineered constructs. A systematic approach was used to determine the effects of BMP-2, IGF-I, and TGF-β1, alone and in combination, on the functional properties of engineered constructs. This was the first study to demonstrate significant increases in both compressive and tensile biomechanical properties as a result of growth factor treatment. Also, for the first time, synergistic and additive effects on construct biomechanical and biochemical properties were found when combining growth factor treatment with hydrostatic pressure application. Finally, the effects of various decellularization treatments were examined, and it was determined that it was possible to remove cells while maintaining construct functional properties. The results presented in this thesis are exciting, as they have allowed for a better understanding of the self-assembling process, and have allowed the self-assembled constructs to mature into functional articular cartilage, as evidenced by biomechanical and biochemical properties spanning native tissue values.

Acknowledgments

I would first like to thank the support from the National Institute of Arthritis and Musculoskeletal and Skin Diseases, grant #R01 AR053286 for funding the work described in this thesis. Additionally, I would like to thank the National Institute of Health Biotechnology Training Program Fellowship for funding my final years of graduate school. Finally, I would like to acknowledge my funding from the Baylor College of Medicine Medical Scientist Training Program.

I am especially thankful for all of the support and help I have received from current and previous members of the Athanasiou lab. I especially would like to thank Dr. Jerry Hu and Dr. Chris Revell, without whom none of the work in this thesis would have been possible. They served as wonderful mentors, and were always available to help design experiments and analyze results. I would also like to thank Roman Natoli for his tireless aid with statistical design and analysis, as well as his incredible insight and imagination in designing new ways to study cartilage tissue engineering. Additional thanks go to Dr. Eugene Koay, Dr. Gwen Hoben, Dr. Deirdre Johns, and Naj Gunja for their help with tissue harvesting, running bioreactors, and designing future studies. I would like to thank Kerem Kalpakci for his help with tensile testing, as well as Gidon Ofek, Vincent Willard, and Dan Huey for their support throughout my time at Rice. Finally, I am thankful for the contributions of the rest of the members of the Athanasiou group, for their continuous insight.

I would like to thank the Baylor College of Medicine Medical Scientist Training Program Faculty and Staff for their help throughout my training. Specifically, Kathy Crawford has always gone out of her way to provide advice and help with any issues during my time at Baylor and Rice. Vanessa Hatfield has also been indispensable since joining the program. Additionally, I would like to thank Dr. Marty Matzuk and Dr. Jim Versalovic for their mentoring and advising during my time in the MSTP.

I am grateful to my committee members, Drs. Athanasiou, Clanton, Mikos, and Zygourakis, for their guidance and tutelage throughout my graduate training. I would like to thank Dr. Mikos for his mentorship and for teaching me the fundamentals of tissue engineering in his course. Much thanks to Dr. Clanton for serving on my committee and giving me more exposure to the field of Orthopaedic Sports Medicine. Additionally, I would like to thank Dr. Zygourakis for being a part of this committee. Finally, a very special thank you is to Dr. Athanasiou, who has been a more wonderful mentor and advisor than I could have hoped for, and who will always have a profound influence on my life. He continuously encouraged me in all of my academic pursuits, and was always available, whether discussing science or just life in general. I hope that one day I can be as great a scientist and mentor as the example he has set for me.

Finally, I would like to thank my family for their unending support and love throughout my life. I would like to thank my dad Dr. Jack Elder, who has

provided an excellent example of the surgeon I hope to become, and my mom, Dr. Judith Elder, who is always available for help, advice, or some loving words. They have always encouraged me to pursue my dreams. I would also like to thank my siblings, my brother Sam and sister-in-law Becca, my sister Katie and soon to be brother-in-law Matt, and finally my youngest sisters Allison and Abby. Most importantly, I would like to thank my wife, Bernadetta, who makes my life better at all times. While navigating graduate school, she has continuously provided love and support, and helped keep me sane during my training. To conclude, I would like to thank my soon to be daughter (name TBD), who's impending arrival has kept me focused on my work, and has provided a bright future to look forward to every day.

Table of Contents

List of Figures

List of Tables

Introduction

The overall objective of this thesis is to enhance the self-assembling process for *in vitro* tissue engineering of articular cartilage. The self-assembling process has been developed by our group and applied to engineer articular cartilage constructs. Motivated by this objective, it is hypothesized that 1) the self-assembling process can be enhanced by identifying suitable growth factors and mechanical forces; and 2) the effects of these exogenous factors individually or in combination will allow the formation of constructs *in vitro* resembling native tissue. To test these global hypotheses, three specific aims were employed:

1) **To determine the effects of radial and vertical confinement on the self-assembling process of articular cartilage.** This approach involves determining the effects of construct confinement in different directions and at different times on the self-assembling process. First, self-assembled constructs are radially confined in agarose wells for four different timepoints. Next, the constructs are confined for two weeks, and then transferred to incrementally larger wells for the third and fourth weeks of culture. Finally, the effects of vertical confinement, in the form of passive axial compression are examined. The engineered constructs are assessed histologically for collagen and glycosaminoglycan (GAG), and immunohistochemically for collagen types I and II. Additionally, the constructs are assessed biochemically to quantify DNA, total collagen, and GAG content. Finally, biomechanical evaluation of the constructs is performed using creep indentation and uniaxial tensile testing. *It is*

hypothesized that the application of confinement will enhance the mechanical properties of the constructs, and that confinement at different timepoints in construct development will have a significant effect on construct properties.

2) **To determine the effects of hydrostatic pressure on the self-assembling process of articular cartilage.** This approach involves the determination of hydrostatic pressure (HP) effects on the self-assembling process. Initially, an appropriate control for HP is selected at one application time. Next, the self-assembled constructs are placed under static physiologic-magnitude HP to determine the temporal effects of HP application. Additionally, the immediate and long-term effects of HP application are assessed. Finally, a full-factorial experimental design is used with two factors (magnitude, frequency) at three levels each. These factors are optimized, with "optimal" defined as the set of conditions producing properties closest to native tissue in terms of extracellular matrix (ECM) composition and biomechanical properties. The best two HP conditions are subsequently used in specific aim 3. *The hypotheses of this aim are that 1) there exist optimal conditions (magnitude, frequency, application time) for HP that improve the quality of self-assembled constructs and 2) these optimal conditions fall within the physiologic range of pressure magnitudes.*

3) **To determine:**

    a. **The effects of growth factors alone and in combination**

b. **The combined effects of growth factors and HP on the self-assembling process of articular cartilage.**

The first step of this approach involves the determination of growth factor effects on the self-assembling process. The self-assembled constructs are treated temporally with three individual growth factors at two concentrations, each delivered in the media continuously or intermittently. The best treatment for each growth factor, that produces construct properties closest to native tissue in terms of ECM composition and biomechanical properties, is carried forward and studied in combinations of two and three at a time to quantify growth factor interactions. The optimized growth factor combinations are then combined with the optimized HP conditions from specific aim 2 to examine their combined effects. *The specific hypotheses of this aim are that 1) there exist growth factor conditions that are most beneficial in the self-assembling process and 2) combining growth factor treatment with HP will result in additive and synergistic effects.*

Chapter 1 delivers an overview of the field of articular cartilage tissue engineering, while chapter 2 describes the prior use of HP in articular cartilage tissue engineering strategies. Chapters 3 through 6 focus on studies to enhance the functional properties of tissue engineered constructs such that they approach native tissue. Finally, Chapter 7 describes a method for the decellularization of scaffoldless tissue engineered constructs. Chapter 1 reviews the field of articular cartilage tissue engineering, with a specific focus on the design criteria for a

tissue engineered constructs, as well as highlighting the various parameters that must be addressed in cartilage tissue engineering studies, including scaffolds, growth factor and cytokine application, and the use of exogenous mechanical stimulation. This chapter motivates the use of the selected growth factors and mechanical stimulation strategies used in this thesis.

Chapter 2 reviews the prior work involving the application of HP to articular cartilage chondrocytes. This chapter focuses on prior HP studies in several areas, including HP bioreactor design and the use of HP in tissue engineering strategies, with a particular emphasis on examining the differences between HP application to chondrocytes in monolayer, in 3-D tissue engineered constructs, as well as explants. Furthermore, a comparison between the different effects of intermittent and static HP is made. Additionally, the chondroprotective effects of HP, the use of HP as a differentiation agent, the use of high magnitude HP, as well as the mechanotransduction pathways of HP application are examined. This chapter identifies four criteria, namely magnitude, frequency, duration of application, and application time in construct development, as parameters that may be altered in studies involving the effects of HP. These parameters are all examined in chapters 4 and 6.

Chapters 3-6 address work performed toward the completion of the Specific Aims of this thesis. Chapter 3 addresses Specific Aim 1, and describes the beneficial effects of radial and vertical confinement on construct properties. Chapter 4

addresses Specific Aim 2 and demonstrates the effects of temporal HP application by identifying a suitable control for HP application, determining the effects of HP application at different timepoints in construct development, and examining both the immediate and long-term effects of HP application. Specific Aim 3 is addressed in chapter 5, which determines the effects of multiple individual growth factors, including TGF-β1, IGF-I, and BMP-2, at different concentrations and different application times, on construct functional properties; additionally, this chapter demonstrates the effects of treatment with growth factor combinations. Chapter 6 addresses Specific Aims 2 and 3, and identifies the optimal magnitude and frequency for HP application. Additionally, this chapter examines the effects of combined treatment with HP and growth factors, and demonstrates synergistic and additive effects when combining TGF-β1 treatment with HP application.

Finally, chapter 7 describes a preliminary study to identify a method for construct decellularization, with the objective of eliminating the cells and DNA content of the tissue while preserving the biochemical and biomechanical properties of the tissue, particularly the GAG content and compressive stiffness.

Chapter 1: Paradigms of Tissue Engineering with Applications to Cartilage Regeneration

Benjamin D. Elder and Kyriacos A. Athanasiou

Chapter published as: Elder, BD and Athanasiou, KA. Paradigms of Tissue Engineering with Applications to Cartilage Regeneration. In: Musculoskeletal Tissue Regeneration: Biological Materials and Methods, Pietrzak, WS, Ed. Totowa, NJ: Humana Press 2008: 593-615.

ABSTRACT

Tissue engineering has been widely explored as an option for regeneration of various musculoskeletal tissues. This chapter examines the tissue engineering paradigm, or approach, with a focus on its application to cartilage tissue engineering. Since understanding the tissue engineering approach will require an understanding of cartilage physiology, a brief review of cartilage structure and function is provided. A discussion of current studies of the four parameters of the paradigm, namely scaffolds, cell sources, bioactive agents, and bioreactors is presented, along with the latest technologies that incorporate manipulation of several parameters in a single approach.

INTRODUCTION

Tissue engineering approaches currently are studied in order to repair many musculoskeletal tissues, including bone, vertebrae, knee meniscus, tendon, ligament, temporomandibular joint (TMJ) cartilage, and articular cartilage. This approach aims for functional tissue restoration and involves the use of cells, scaffolds, bioactive agents, and mechanical forces. The goal of tissue engineering is to create tissue with biomechanical and biochemical properties that match those of the native tissue.

Cartilage degeneration from injury or from osteoarthritis is one of the greatest problems currently faced in orthopedics, and is the second most common chronic condition reported in the United States.[1] According to the website www.arthritis.org,[2] approximately 21 million people in the US are affected with osteoarthritis, resulting in total annual costs of approximately $5700 per person living with osteoarthritis. Due to the prevalence of articular cartilage pathologies and the need for more effective methods for cartilage repair, tissue engineering has emerged as a promising approach for cartilage regeneration. This chapter will provide an overview of the paradigms of tissue engineering, predominantly exemplified by exploring the strategies currently used to engineer articular cartilage. To gain a better understanding of how tissue engineering approaches are applied to articular cartilage regeneration, a brief discussion of articular cartilage structure and function is provided.

BACKGROUND

Structure and Function of Articular Cartilage

Articular cartilage is a specialized form of hyaline cartilage that is essential for the proper function of diarthrodial joints. The main function of articular cartilage is to distribute forces between the subchondral bones. Also, along with synovial fluid, articular cartilage provides lubrication, friction reduction, and wear resistance for the joint.

Articular cartilage is avascular, aneural, and alymphatic, and is sparsely populated by cells called chondrocytes. Articular cartilage is considered to consist primarily of a solid phase and a fluid phase.[3] Water is the primary component of the fluid phase and accounts for 75-80% of the wet weight of the tissue. Additionally, electrolytes such as Na^+, Ca^{2+}, and Cl^- are found in the fluid phase. The solid phase is characterized by the extracellular matrix (ECM), consisting predominantly of collagen and proteoglycans, which surrounds the chondrocytes and provides structural support to the tissue. The ECM is composed of approximately 50%-75% collagen, and 30-35% proteoglycans.[4, 5]

Collagen is the primary constituent of the ECM of articular cartilage. As reviewed elsewhere,[5] collagen II accounts for 90-95% of the collagen in the matrix and is often used as a marker for chondrogenic differentiation in tissue engineering studies. The collagen II fibrils are largely responsible for the tensile strength of the tissue. Other types of collagen are present in the matrix in much smaller

amounts and serve varying roles. Collagen XI contributes to fiber formation with collagen II, while collagen VI, IX, and X contribute to the ECM structure.

Proteoglycans are glycoproteins that are characterized by long, unbranched, and highly charged glycosaminoglycan (GAG) chains.[6] Aggrecan, the most common proteoglycan in articular cartilage, is responsible for the compressive strength of the tissue. In addition to collagen II, the expressions of GAG and aggrecan are also used as specific markers for chondrogenic differentiation.

Mature articular cartilage has a distinct zonal arrangement in vertical sections (Fig. 1). Beginning with the articulating surface, it consists of the superficial, middle, deep, and calcified zones. These zones exhibit great differences in their properties.[7] The superficial zone comprises the first 10-20% of the thickness of the tissue, and is characterized by densely packed collagen II fibrils oriented in the direction of shear stress, along with flattened chondrocytes. The middle zone comprises the next 40-60% of the tissue thickness, and consists of randomly arranged collagen fibers and chondrocytes with a more rounded morphology. It also serves as a transition between the superficial and deep zones. The deep zone contains collagen fibers that extend into the calcified zone in order to reinforce the bond between cartilage and bone. The cells of the deep zone appear more ellipsoid in shape, and are aligned with the collagen fibers. A distinct tidemark separates the deep zone from the calcified zone. This tidemark is usually considered to be the boundary between cartilage and bone. The

calcified zone is composed of chondrocytes that are trapped in a calcified matrix.

Chondrocytes sparsely populate cartilage, comprising less than 10% of the volume of the tissue.[8] Chondrocytes differentiate from mesenchymal stem cells (MSCs) and are responsible for the maintenance and regulation of the ECM through the enzymatic degradation of existing ECM, the synthesis of new ECM, and the production of various bioactive agents such as growth factors. In healthy articular cartilage, chondrocytes do not proliferate. Since the tissue is relatively avascular, the chondrocytes exist in a low oxygen tension environment and must obtain oxygen and nutrients from the synovial fluid through diffusion.

Biomechanics of Articular Cartilage

As mentioned above, the aggrecan content of cartilage is largely responsible for its compressive properties. Aggrecan is negatively charged, leading to osmotic swelling and hydration of the tissue from the Donnan osmotic pressure.[9] When cartilage is compressed, the interstitial fluid pressure initially supports most of the applied load. The water is then pushed out of the matrix and into the synovial cavity; therefore, it moves from a loaded region to an unloaded region. The frictional force between the leaving water and the matrix leads to dissipation of the applied force, and the load eventually equilibrates. Upon removal of the load, fluid comes back into the aggrecan network. This process allows for the cushioning of an applied load without damage to the chondrocytes or ECM. The interaction between the matrix and the interstitial fluid of cartilage is modeled by

Mow et al.'s[3] biphasic theory. Applying the biphasic theory to articular cartilage in studies of creep indentation yields three material properties: the aggregate modulus, the Poisson's ratio, and the permeability of the porous solid phase, which measure the stiffness, the apparent compressibility, and the resistance to fluid flow respectively.[3] The mechanical properties of articular cartilage vary with the anatomic location of the joint. A review by Hu et al.[5] indicated that the aggregate modulus ranges from 0.53 MPa to 1.34 MPa, the Poisson's ratio from 0.00-0.14, and the permeability from 0.90×10^{-15} m^4/Ns to 4.56×10^{-15} m^4/Ns.

Articular cartilage is exposed to a wide variety of forces including hydrostatic pressure, compression, and shear forces. As reviewed elsewhere,[10] the force exerted on the knee has been found to be approximately 3.5 times body weight, while the ankle and shoulder experience loads of 2.5 times body weight and 1.5 times body weight respectively. In addition, contact pressures between 3-18 MPa have been observed in the human hip joint.[11] During loading of articular joints, synovial fluid inside the joint capsule generates hydrostatic pressure by transmitting force throughout the tissue. Compressive forces are generated in articular cartilage as a result of direct contact between the articulating surfaces. Likewise with compressive forces, shear forces are generated in the knee joint during loading as a result of direct contact between the articulating cartilage surfaces, as the two surfaces attempt to move past each other.

Repair of Articular Cartilage

As reviewed elsewhere,[12] injuries of articular cartilage can be classified as 1) chondral damage without visible tissue disruption, 2) cartilage damage alone such as chondral flaps and tears, and 3) cartilage damage accompanied by underlying bone damage (osteochondral fracture). As a result of the relatively nonexistent vascular supply, scarcity of chondrocytes in the tissue, and the lack of chondrocyte proliferation, the ability of articular cartilage to repair itself is intrinsically limited. As reviewed elsewhere,[13] in a chondral injury, the chondrocytes surrounding the defect show a limited ability to proliferate in order to repair the damaged site. In an osteochondral injury, MSCs from the bone marrow can migrate to the site for tissue repair. However, in both cases, the defect is repaired with fibrocartilage formation, which is predominantly collagen I and lacks the mechanical integrity of articular cartilage, thus leading to its relatively rapid degradation with normal loading of the joint.[14]

The current clinical options for treatment of patients with damaged articular cartilage are relatively limited. According to a recent review,[15] the most successful treatment options for restoring native hyaline cartilage have involved tissue grafting, where cartilage is removed from a less load-bearing region and is grafted to the defect site. However, this approach involves significant donor site morbidity and the result is often short-lived, as fibrocartilage fills the donor site and the area surrounding the graft. Autologous chondrocyte implantation is another treatment strategy that entails harvesting a limited supply of cartilage

cells from the individual, expanding the cells in culture, and injecting them in the defect site. The area is then covered with a periosteal flap. As reviewed elsewhere,[15] this procedure was intended to treat focal defects of the knee in the United States; however, it has also been used to treat focal defects in the ankle, shoulder, elbow, hip, and wrist. Although this procedure has yielded promising results,[16] 25% of the patients experienced graft failure, 22% experienced delamination, and 18% experienced tissue hypertrophy.[17] In addition to the reported clinical complications, a major drawback to this procedure and any other currently available is that it has only been used to treat focal defects and has not been used to treat entire osteoarthritic joints.

Tissue Engineering

Due to the poor ability of articular cartilage to heal itself, and the limited clinical treatment options, tissue engineering may provide the most promising approach to articular cartilage regeneration, potentially providing engineered tissue that is indistinguishable from native cartilage. As reviewed elsewhere,[18, 19] the biomechanical characteristics of engineered constructs are the most important quantitative indicators of the approximation of the regenerated tissue to native tissue, but biochemical analyses of the collagen and GAG content also yield important information. Tissue engineering aims to accomplish the regeneration of articular cartilage by manipulating four parameters: scaffold material, cell sources, bioactive agents (growth factors/cytokines), and mechanical forces (Fig. 2). Although significant steps have been made in the study of each parameter,

complete tissue regeneration likely will require the complex task of optimizing these parameters in combination.

TISSUE ENGINEERING PARADIGMS

Scaffolds

The main function of a scaffold in tissue engineering is to provide support and a temporary structure to cells as they begin to secrete and form an ECM. The engineered construct will eventually replace the scaffold as it slowly degrades over time. There are two approaches to employing a scaffold: immediate implantation of the cell-seeded scaffold or *in vitro* culture of the scaffold before implantation, and each of these approaches has different design concerns. A scaffold used *in vivo* for cartilage tissue engineering should contain internal channels that allow for the diffusion of nutrients and room for tissue growth.[20] Also, it should have adequate biocompatibility to prevent the release of toxic byproducts and a large immune response, and should exhibit biodegradation kinetics that match the rate of new tissue formation. In addition, a scaffold should have sufficient mechanical properties to allow for its immediate use *in vivo*, as the cells will not have had enough time to synthesize an ECM that will eventually replace the scaffold. Finally, the scaffold should allow for the attachment, proliferation, and differentiation of cells seeded on its surface.[20] However, if a scaffold will be cultured *in vitro* rather than implanted immediately, the inherent mechanical properties of the scaffold are not nearly as important, as

the degrading scaffold will be replaced by an engineered construct with its own mechanical properties before implantation.

In general, scaffold materials can be divided into two groups: natural and synthetic materials. Also, composite scaffolds, which are composed of multiple materials, have been used in tissue engineering. The use of these scaffold materials will be discussed further with a particular emphasis on successful approaches in cartilage tissue engineering.

Natural Polymers

Natural polymers have several properties making them advantageous for use as scaffolds. They often have excellent adhesion properties, adequate biocompatibility, and decreased toxicity during scaffold degradation.[21] Collagen, fibrin, chitosan, hyaluronan, alginate, and agarose have all been investigated with varying degrees of success in cartilage tissue engineering.

Collagen gels have been used extensively as a scaffold material, as collagen is a fundamental component of the ECM of cartilaginous tissues as well as various other connective tissues; therefore, collagen gels are expected to have low immunogenicity, although like all natural polymers, they must be purified before their use. In addition, as a widely abundant ECM component, collagen allows for excellent incorporation of both endogenous and exogenous cells from joint tissue, thus making it an excellent candidate for success in both *in vitro* cell-

seeding and *in vivo* integration. Nehrer et al.[22] compared type I and type II collagen gels, and found that chondrocyte-seeded type II collagen gels maintained the chondrocyte phenotype and had increased expression of GAGs. Although some success has been observed with collagen gels, as with all natural scaffolds, concerns regarding pathogen transfer have been expressed. Specifically, the increased incidence of prion diseases such as bovine spongiform encephalopathy has hindered the use of collagen from bovine sources.[23]

Fibrin has been used as both a delivery device and a stand-alone scaffold.[23] This material has the advantage of being injectable, which would allow for its non-invasive delivery. Passaretti et al.[24] demonstrated that chondrocytes expanded through passage one and seeded in a fibrin polymer that was injected subcutaneously in nude mice made an ECM resembling that of native cartilage. However, fibrin has poor mechanical properties and may lead to a host immune response.[23]

Chitosan is a polymer derived from the N-deacetylation of chitin, which is abundant in the exoskeleton of arthropods. The properties of chitosan make it extremely useful as a scaffold material. It has excellent biocompatibility,[25] is easily synthesized,[26] and its mechanical properties and degradation rates can easily be manipulated.

Hyaluronan, alginate, agarose, as well as various synthetic polymers are all used to create hydrogels, which are highly water-soluble polymers that can be cross-linked covalently or physically. They are highly swollen with water, usually containing >90% water. Often, hydrogels have excellent biocompatibility but weak mechanical properties. The main advantage of hydrogels is that as low viscosity, fluid-like solutions, they are injectable and can fill irregularly shaped defect sites. Once the defect site has been filled with a cell/polymer suspension, the hydrogel can be cross-linked, for example, by transdermal photopolymerization,[27] thus causing a fluid-solid transition to occur. This procedure permits the researcher to avoid many of the problems involving cell-seeding, and provides the clinician with a minimally invasive treatment for chondral defects that avoids surgical intervention. Another advantage of hydrogels is that they are excellent at maintaining the chondrogenic phenotype; this is probably because embedding the chondrocytes in the hydrogel preserves their round morphology. Furthermore, embedding the cells in a hydrogel is extremely useful when using mechanical stimulation, as it allows for uniform force transfer to the cells without the stress shielding that may be caused by other scaffold materials.

Hyaluronan is a naturally occurring polysaccharide that is an important component of articular cartilage. Burdick et al.[28] recently demonstrated that the mechanical properties and degradation rates of hyaluronan scaffolds could easily

be manipulated to encompass a wide range of desirable values, which could potentially allow for clinical use.

Alginate is a polysaccharide derived from algae that has excellent biocompatibility. As a hydrogel, alginate can be delivered with an injection, which allows for minimally invasive treatment of chondral defects. Alginate has also proven effective for maintaining or even inducing the chondrogenic phenotype. This is probably because the chondrocytes are embedded in the alginate hydrogel, which enables them to maintain their round morphology. Another exciting attribute of alginate hydrogels is their ability to be formed into different shapes,[29] which would allow for the production of a geometrically customized construct prior to implantation. However, the major downside of alginate hydrogels is the inability to modulate their long degradation time *in vivo*, which can hinder the growth of new tissue.[20]

Agarose is a polysaccharide derived from seaweed that is very similar to alginate in its properties. Like alginate, agarose has excellent biocompatibility, and helps to maintain the chondrogenic phenotype by preserving chondrocytes' round morphology. Agarose has been used extensively in *in vitro* studies,[23] although it shares the slow degradation kinetics of alginate. Another problem with agarose is that it may elicit a foreign body giant cell immune response *in vivo*.[30]

Synthetic Polymers

Synthetic polymers are fabricated in a laboratory, and offer several advantages over natural polymers. Their physical and mechanical properties can easily be modulated, thus allowing for degradation kinetics and mechanical properties that are optimized for a specific application. In addition, since they are not derived from organisms, there is no concern regarding pathogen transmission, and they can easily be synthesized in large quantities. Also, synthetic polymer scaffolds can undergo surface modifications, with peptides or bioactive molecules, that can enhance their biocompatibility and integration in defects. However, unless they are sufficiently small or synthesized to form a hydrogel, they must be surgically implanted into the recipient.

The most widely used materials are the poly(α-hydroxy esters), including polyglycolic acid (PGA), polylactic acid (PLA) and their copolymer poly(lactic-co-glycolic acid) (PLGA).[31-33] The biocompatibility of each polymer has been extensively studied, and allows for their use in various implantation applications.

PLA and PGA are often extruded into long polymer strands, which are then used to form a highly porous nonwoven fibrous mesh. The porous nature of the scaffold allows for cell to cell communication and nutrient diffusion, but leads to poor mechanical properties until tissue formation occurs. Many studies have demonstrated the efficacy of these polymers in cartilage ECM synthesis and the

maintenance of the chondrocyte phenotype,[20] as well as efficacy in *in vivo* studies.[23]

Copolymers of PLA/PGA are advantageous, as they allow for more control over the degradation kinetics, by varying the ratios of monomers used. PLGA scaffolds have shown promising results in *in vivo* studies,[23] but an exciting new approach has been to construct hydrogels out of the copolymer. Mercier et al.[34] created hydrogels out of PLGA microspheres that, when seeded with chondrocytes and injected in athymic mice, allowed for the production of cartilaginous ECM.

A recent approach has been the creation of composite scaffolds, in which multiple scaffold materials are used together in an effort to harness the advantages of each component. For example, Caterson et al.[35] demonstrated the efficacy of a PLA/alginate amalgam for the chondrogenic differentiation of MSCs.

"Scaffold-less" Approaches

Despite the promising results obtained using the various aforementioned scaffold materials, there are problems associated with using a scaffold. For example, scaffolds can hinder cell to cell communication, contribute to stress shielding, and alter the chondrogenic phenotype. Furthermore, they may be toxic or produce toxic byproducts during degradation, and their degradation rate must be

modulated to coordinate with new tissue formation.[36] As a result of these inherent problems, novel approaches to tissue engineering have been developed that do not employ the use of a scaffold. These approaches include pellet culture,[37] aggregate culture,[38] and a more recent approach, the self-assembling process.[36]

In the self-assembling process, calf articular chondrocytes were seeded at high density in 5mm diameter and 10mm deep agarose wells. After 24 hours of culture, the cells formed constructs that were not attached to the walls of the agarose wells. After 4 weeks of culture, the constructs were transferred to large wells, and following 12 weeks of culture, this process resulted in tissue engineered constructs of clinically relevant dimensions, at ~15mm in diameter and 1mm in thickness. The constructs resembled native articular cartilage morphologically, and had levels of collagen II and GAG approaching that of native tissue, with no collagen I production. Perhaps the most exciting result was that the self-assembled constructs reached over 1/3 the stiffness of native tissue. The self-assembling process has also been coupled with mechanical stimulation.[39] Hydrostatic pressure application under a treatment of 1 Hz and 10 MPa for 4 hours/day was shown to stimulate collagen production and aid in the retention of GAGs within constructs compared to static culture. Although more work still needs to be done in the characterization and optimization of the method, the self-assembling process is a promising approach towards functional tissue engineering of articular cartilage.

Cell Sources

An ideal cell source must satisfy several criteria: be easily accessible or available, demonstrate self-renewal or the ability to be expanded extensively, have the capacity to differentiate into the cell lineage of interest upon induction or remain differentiated in the cell lineage of interest, and exhibit minimal immunogenicity or tumorigenicity.[40] Progenitor cells such as MSCs and embryonic stem (ES) cells as well as fully differentiated chondrocytes have all been used as cell sources for engineered cartilage constructs. Certain advantages and disadvantages are inherent to approaches involving each cell type.

Primary chondrocytes from native cartilage are the most obvious cell source for tissue engineering of cartilaginous tissues. Immature chondrocytes are often used for studies due to their higher metabolic activity.[41] Chondrocytes can easily be isolated from freshly excised articular cartilage following an enzymatic digestion with collagenase. However, a large number of cells must be obtained to be seeded onto a three-dimensional scaffold. Since overharvesting chondrocytes can lead to further problems at the harvest site, serial passage of chondrocytes on monolayers is required to acquire the large cell density needed for seeding on a three-dimensional scaffold. Chondrocytes passaged in monolayer "dedifferentiate" and become more fibroblast-like in appearance and ECM production: they lose their round morphology and become more spindle-shaped, switch their collagen production from primarily type II collagen to type I

collagen, and they regain their ability to divide.[42-44] This loss of chondrogenic potential is associated with the suppressed activation of key signaling proteins in the Ras-mitogen-activated protein kinase pathway, which leads to apoptosis.[45] In a recent study, it was found that passaged articular chondrocytes in monolayer showed phenotype changes as early as one passage, and their chondrogenic phenotype could not be rescued even with 3-D culture in alginate beads.[46] Despite these limitations, primary chondrocytes continue to be used in clinical applications as several culture conditions, such as culture in agarose gels,[47] allow for the reexpression of the chondrocyte phenotype.

The study of stem cells has gained prominence in cartilage tissue engineering, as new chondrocytes, originating from host MSCs,[48] repair osteochondral defects. Adult MSCs are multipotent cells that can be induced to differentiate down multiple cell lineages such as chondrogenic, osteogenic, and adipogenic lineages. MSCs are advantageous as they are able to self-renew, and they can be obtained relatively non-invasively from tissues such as bone marrow aspirates,[49-54] adipose tissue,[55-60] synovial tissue,[61] as well as several other tissues. The chondrogenic phenotype is often characterized by the expression and synthesis of collagen II and proteoglycans, as well as by the upregulation of genes such as sox-9 which are markers of cartilage ECM production. MSCs used in cartilage engineering have been differentiated through the application of members of the transforming growth factor-β (TGF-β) family as well as dexamethasone. MSCs used in research studies so far have primarily come

from bone marrow. Several studies have indicated that a 3-D culture environment is important for chondrogenic differentiation, as it may help to maintain a rounded cell shape. An exciting finding from a study by Yoo et al.[51] was that the addition of TGF-β1 and dexamethasone maintained the chondrogenic potential of bone-marrow derived MSCs through 20 passages. This is an important finding as *in vitro* expansion through several passages often is required to generate sufficient cells for implantation. Human adipose-derived adult stem (hADAS) cells show great promise for cartilage tissue engineering as they can be isolated from various easily accessible sources such as from the inguinal fat pad[62], infrapatellar fat pad[63], and subcutaneous adipose tissue.[52] hADAS cells express markers characteristic of articular cartilage when cultured with TGF-β1, dexamethasone, and ascorbate.[57, 58, 60, 62] Although adult stem cells represent a promising cell source for articular cartilage engineering, more work needs to be performed to understand the developmental processes involved in differentiation so that these processes may be further manipulated to optimize *in vitro* cell expansion while maintaining chondrogenic differentiation; then, it may be possible to develop *in vivo* approaches for construct delivery and host integration.

As with the study of several other tissues, the use of embryonic stem cells is increasing in cartilage tissue engineering.[64-67] ES cells are derived from the inner cell mass of the embryonic blastocyst and are pluripotent. Following aggregation into embryoid bodies *in vitro*, they can differentiate into tissue of all three germ

layers. ES cells are capable of virtually infinite proliferation while remaining in an undifferentiated state. Because of their pluripotency and their provision of an unlimited cell source, their use is promising for many tissue engineering applications. Although ES cells appear to be an extremely promising cell source, their differentiation pathways must be better elucidated to manipulate them further for cartilage tissue engineering. In addition, the ethical and legal concerns regarding the source and means of collection of ES cells significantly complicate their use.

Finally, an exciting new cell source may derive from dermal fibroblasts which can be triggered to differentiate by culture on cartilage matrix proteoglycans.[68-70] This cell source could be extremely useful as the cells are both easily accessible and widely available.

Growth Factors

Growth factors are used in tissue engineering to modulate cellular differentiation and proliferation, as well as to modulate ECM synthesis. Articular cartilage displays dramatic changes when exposed to growth factors that are naturally present in the native environment. The effects of many of these growth factors alone and in combination have been studied for cartilage tissue engineering, including the transforming growth factor beta (TGF-β) family, insulin-like growth factor (IGF), fibroblast growth factors (FGFs), hepatocyte growth factor (HGF), and platelet-derived growth factor (PDGF). As reviewed elsewhere,[71] growth

factor studies generally are conducted *in vitro* in which the growth factor is delivered as a soluble factor in the media; therefore, the concentration and frequency of delivery can easily be manipulated. Growth factor effects have also been studied *in vivo*, albeit with variable results as it is far more difficult to control the interactions within the body as well as the concentrations.[71] However, as scaffold delivery vehicles and gene therapy approaches continue to improve, delivery of growth factors at more controlled doses and temporal increments may become a more achievable task.

Members of the TGF-β family are probably the most widely used growth factors to date. For cartilage tissue engineering, the notable members of the TGF-β family include TGF-β1, TGF-β3, and bone morphogenetic proteins (BMPs). TGF-β1[51-53, 56] and TGF-β3[50, 72] are both widely used as chondrogenic differentiation factors for MSCs and embryonic stem cells. In addition to uses in chondrogenic differentiation, TGF-β1 has been shown to upregulate ECM synthesis although there are conflicting reports on its effects. Several studies have shown that TGF-β1 increases collagen II expression in monolayer[73] and in 3-D scaffolds[74], while other studies have shown no effects on the gene expression of ECM proteins.[75] Possible explanations for the different observed effects of TGF-β1 include variable effects of TGF-β1 on the zonal populations of chondrocytes, as well as variations in the temporal application of the growth factor. The main effect of BMPs in cartilage tissue engineering is chondrogenic differentiation or the maintenance of differentiation. They have also seen

extensive use in bone tissue engineering for osteogenic differentiation and increased matrix synthesis. As reviewed elsewhere,[71] although other BMPs have been studied, BMP-2 has been the most commonly used BMP for studies involving cartilage. BMP-7 is another BMP that is beginning to be used in cartilage tissue engineering, and has also been used recently as a chondrogenic differentiation factor.[76]

Several other growth factors show potential for use in cartilage tissue engineering. IGF-I has a profound anabolic effect on chondrocytes *in vitro*,[77-79] and has been shown to increase GAG production, as well as aggrecan and collagen II gene expression in articular chondrocytes grown on monolayer.[80] Perhaps the most exciting *in vivo* effect of IGF-I use is the autoinductive autocrine/paracrine transcriptional response, which could potentially be harnessed to extend and amplify the effects of IGF-I on cartilage repair.[81] Basic fibroblast growth factor (bFGF) has been shown to stimulate chondrocyte proliferation and synthesis,[82-84] although it has also been used for fibrochondrocyte studies involving the knee meniscus and the temporomandibular joint.[33] FGF-18 has recently been shown to promote chondrogenic differentiation of limb bud mesenchymal cells.[85] HGF has been minimally studied in cartilage tissue engineering, but preliminary studies indicate that it may enhance or modulate chondrocyte proliferation.[86, 87] The effects of PDGF on proliferation and ECM synthesis have been minimally reported, but it has been shown to have an effect on chondrocyte proliferation.[88]

Although many growth factors show promising results when used alone, their use in combination has yielded exciting results, as synergism between many growth factors has been observed in several studies. BMP-2 and TGF-β1 work in concert for the chondrogenesis of periosteal cells; it was suggested that BMP-2 induces neochondrogenesis, while TGF-β1 modulates the terminal differentiation in BMP-2 induced chondrogenesis.[89] Combined treatments with TGF-β3 and BMP-6 or TGF-β3 and IGF-I were shown to be the most effective combinations for chondrogenic induction of bone marrow MSCs.[90] However, growth factor combinations do not always interact synergistically. For example, the addition of IGF-I and TGF-β in combination did not improve the histologic features or mechanical performance of tissue engineered cartilage constructs.[91]

Perhaps the most exciting new results have come from studying the synergism between growth factor application and mechanical stimulation. Bonassar et al.[92] found that the combination of IGF-I and dynamic compression led to a 290% increase in proteoglycan synthesis, a degree greater than that achieved by either stimulus alone. Also, Mauck et al.[93] showed that the combination of dynamic deformational loading with either TGF-β1 or IGF-I increased the stiffness of engineered constructs by 277% or 245%, respectively, with respect to untreated free-swelling controls.

Growth factor treatment has been extremely useful for cartilage tissue engineering, as it has allowed and maintained chondrogenic differentiation,[75, 94, 95] and has also increased production of ECM proteins in studies of articular cartilage,[80, 92] knee meniscus,[96, 97] and the TMJ.[33, 98] However, there are still properties of the growth factors that need to be investigated. In addition to the need to better characterize the roles of each growth factor, their effects on different cell types, the correct dosage frequency, and concentrations must be elucidated to optimize their use in tissue engineering.

Mechanical Loading

To serve its function as a biomechanical structure, articular cartilage is exposed to a wide variety of forces including hydrostatic pressure, compression, and shear forces. Chondrocytes are directly connected to their microenvironment by focal adhesions which are discrete regions of the cell's plasma membrane that bind to extracellular material.[21] In addition to their involvement in the structural integrity of the chondrocyte, focal adhesions are involved in the process of mechanotransduction, in which cells regulate transcriptional activities based on mechanical signals received at their surface. Although the exact mechanisms of mechanotransduction in the chondrocyte have not been completely elucidated, evidence suggests that elements of the cytoskeleton and integrins allow the coordination of mechanical forces and transcriptional changes.

Several studies have suggested that mechanical stimulation is necessary for maintaining and possibly improving the biomechanical function of articular cartilage. For example, during immobilization, articular cartilage undergoes changes characterized by a loss of function.[99, 100] Also, in a canine study, articular cartilage in the knee became significantly stiffer following loading in physiologic ranges as a result of running on a treadmill.[101] To investigate these issues further, many studies subjected cartilage explants to mechanical stimulation, and determined that mechanical stimulation served to maintain and even upregulate the production of ECM, and it was determined that *in vitro* loading conditions within the physiologic range of native hyaline cartilage were most beneficial.[102] Several methods have been used to deliver mechanical stimulation to articular cartilage; these include hydrostatic pressure, direct compression, and shear, and are the predominant forces present in the knee.

Hydrostatic Pressure

During loading of diarthrodial joints, synovial fluid inside the joint capsule generates hydrostatic pressure that is transmitted to cartilage. Direct compression of cartilage also generates hydrostatic pressure, as the majority of the force is absorbed by the water in the cartilage matrix. As the fluid tries to leave the cartilage matrix, it experiences resistance to its flow and therefore cannot easily leave as a result of the relative impermeability of cartilage. Since the water is somewhat "trapped" in the tissue, a uniform normal load or hydrostatic pressure is applied to the individual chondrocytes in the tissue as a

result of the interstitial fluid pressure. However, since the cartilage matrix is not completely impermeable, the water is eventually forced out of the tissue and into the synovial cavity. The energy of the applied load is then dissipated as water leaves the tissue and encounters resistance as it moves through the cartilage matrix. In diarthrodial joints during normal activity, the magnitude of this interstitial pressure is usually between 7 and 10 MPa.[103] Also, a normal adult cadence corresponds to frequencies of 0.6 to 1.1 Hz loading per leg during walking,[104] and >1.5 Hz during running.[105]

Two approaches have been used to combine the application of hydrostatic pressure with culturing techniques for tissue engineering.[106] In the first approach, the application of hydrostatic pressure is separated from culturing. The cells are grown in static culture, and are moved to a specialized chamber (Fig. 3A) at certain times to apply hydrostatic pressure. Following application of hydrostatic pressure, the cells are returned to their static culture conditions, and this process is repeated per the desire of the researcher. This approach is beneficial because it allows for the application of hydrostatic pressure only at certain times and for certain durations, rather than applying a continuous load. However, the major drawback of this approach is that there is an increased risk of contamination while transferring the cells between the static culture and hydrostatic pressure chamber. The second approach uses a semicontinuous perfusion system; a single device allows for medium to be delivered to the cells while hydrostatic pressure is applied. This approach is advantageous because it

minimizes the possibility of contamination, and it can be automated; however, the downside of this approach is that fluid shear is also introduced into the system.

When using hydrostatic pressure stimulation, the parameters that may be varied include the frequency of loading, the duration and magnitude of loading, as well as the time points at which the cells are subjected to loading. As reviewed elsewhere,[106] loads near the physiological range, between 0.1 and 15 MPa, and frequencies between 0.05 and 1 Hz, have yielded the most favorable results, although the majority of the hydrostatic pressure studies conducted so far have tested explants or monolayers. Research involving the use of hydrostatic pressure in 3-D culture of chondrocytes, especially at longer time points, is lacking. However, a recent study using hydrostatic pressure at previously tested ranges was shown to have beneficial effects on 3-D constructs.[39] In the study, 3-D self-assembled articular chondrocyte constructs (as described previously) were subjected to 10 MPa hydrostatic pressure at 1 Hz for 4 hours per day and 5 days a week for up to 8 weeks, which led to a significant increase in collagen content while preventing a decrease in GAG content relative to the unstimulated control group. However, no significant difference in mechanical properties was observed between the treatment groups.

Hydrostatic pressure does not always produce beneficial results. For example, several studies that investigated constant hydrostatic pressure found little or no improvement in ECM composition.[107-109] Also, when hydrostatic pressure is

above the physiological range, it may actually harm the cells, as decreased ECM production and expression of inflammatory mediators have been observed with these higher pressures.[108]

Although the results on using hydrostatic pressure stimulation seem promising at this point, far more work must be undertaken to elucidate the precise application conditions for optimizing biomechanical and biochemical properties, particularly in 3-D engineered articular cartilage constructs.

Direct Compression

During normal joint loading in a healthy person, compressive forces are generated in articular cartilage as a result of direct contact between the articulating surfaces and once hydrostatic pressure in the interstitial fluid subsides, as water is forced out of the loaded cartilage matrix. If no pathologic processes are present, articular cartilage is able to withstand compression many times per day without injury. In general, cartilage experiences deformation or strain in the range of 2-10%, which was determined under a load of five times body weight in the human hip.[110]

As with hydrostatic pressure, the application of direct compression is usually a two-step approach, in which the application of force is separated from culturing. The cells are grown in a static culture, and are moved to a specialized device (Fig. 3B) at certain times in order to apply direct compression. These devices

are generally designed so that a flat surface compresses the top of the construct at a specific load or displacement. Following application of the force, the cells are returned to their static culture conditions, and this process is repeated as desired.

When using direct compression, the parameters that may be varied include strain or magnitude, frequency, and the time points at which the constructs are subjected to loading. As reviewed elsewhere,[106] most studies have examined frequencies in the range of 0.0001 to 3 Hz, strains from 0.1 to 25%, loads from 0.1 to 24 MPa, and durations lasting hours to weeks, although these parameters are often limited by the equipment used.

As with constant hydrostatic pressure loading, cartilage responds negatively to static loading, most likely as a result of limited mass transport.[106] Therefore, studies using dynamic compression have produced the most positive results. Mauck et al.[111] found a 33% increase in GAG production and an aggregate modulus on the same order of magnitude as in native cartilage when subjecting cartilage constructs to 3% strain at 1 Hz and three times of 1 hour on, 1 hour off per day, 5 days per week, for 4 weeks. Also, in dynamic compression, the loading frequency is an extremely important parameter to be studied. Lee et al.[112] subjected constructs to compression at frequencies from 0.3 to 3 Hz and 15% strain, and found that GAG synthesis was significantly higher in the constructs subjected to 1 Hz compression. Interestingly, in addition to improved

biochemical properties, dynamic compression has recently been shown to enhance the chondrogenic differentiation of MSCs, again indicating that mechanical loading plays an important role in cartilage repair.[113]

Bioreactors and Shear Forces

The purpose of a bioreactor is to create an environment that will aid in the development of the desired tissue properties. In tissue engineering, the major uses of bioreactors have been in the application of shear forces or in examining the effects of media perfusion and gas exchange on constructs.

As with compressive forces, shear forces are generated in the knee joint during loading as a result of direct contact between the articulating cartilage surfaces, as the two surfaces attempt to move past each other. Although a thin layer of synovial fluid provides lubrication between the cartilage surfaces, shear forces continue to occur as the surface-to-surface contact is not completely frictionless. Several studies, discussed in [106], have shown a benefit to applying shear to cartilage constructs. The most widely used bioreactors in cartilage tissue engineering have been spinner flasks, perfusion bioreactors, and rotating-wall bioreactors.

Spinner flasks are perhaps the simplest bioreactors used, as a magnetic stir bar mixes oxygen and nutrients throughout the medium. Their primary use has been for cell-seeding of scaffolds, as mixing in spinner flasks has proven extremely

useful for uniformly seeding cells on scaffolds at high yields.[114] This approach involves attaching scaffolds to needles suspended from a stopper at the top of the flask. Cells in the medium are mixed in the flask due to the stir bar, and eventually are seeded onto the scaffold. Bueno et al.[115] recently used a modified spinner flask, called a wavy-walled bioreactor, which is designed to enhance the mixing of the medium while minimizing the shear. They found that the kinetics of chondrocyte aggregation were significantly improved over a spinner flask when using a wavy-walled bioreactor.

In a direct perfusion bioreactor, a scaffold is surrounded tightly by a medium chamber consisting of a hollow tube, and medium is forced through the fixed scaffold, from one end of the tube to the other. This design allows for a more uniform shear force and a more uniform concentration of nutrients to be delivered to the construct, as the cells located in the entire scaffold thickness are exposed both to convective solute transport and to a flow-induced mechanical stimulus.[116] Also, as used in some of the hydrostatic pressure systems, these bioreactors prevent the need to change the medium and therefore reduce the risk of contamination. Janssen et al.[117] recently used a direct perfusion bioreactor in the production of engineered bone constructs of clinically relevant dimensions. A further modification to these systems can allow for the recycling of used medium along with the addition of fresh medium, which allows beneficial proteins such as ECM constituents to be maintained in the medium.

The use of rotating-wall bioreactors has been a promising approach. The main improvement over a direct-perfusion bioreactor is that a low shear environment is created without sacrificing the high diffusion in the perfusion systems. Essentially, this device consists of two concentric cylinders separated by a space containing medium and scaffolds. The rotation rates of the cylinders can be modulated so as to create different flow and shear environments within the fluid. For example, to produce a low shear force, both cylinders are rotated slowly at the same rate or nearly the same rate. This technique has been coupled with other parameters, such as in the investigation of the effects of oxygen tension on cartilage constructs.[118] Since cartilage tissue is avascular and chondrocytes are exposed to a low oxygen tension environment *in vivo*, Saini and Wick[118] investigated the effects of oxygen tension on developing chondrocytes in a concentric cylinder bioreactor. They found that 5% oxygen tension led to constructs with double the GAG content of constructs cultured in 20% oxygen, with no effect on chondrocyte proliferation or collagen production. Interestingly, Wang et al.[58] found that 5% oxygen tension was also an extremely effective inducer of chondrogenesis in hADAS cells, as it led to increased protein, collagen, and GAG synthesis, with an inhibition of cell proliferation. This is a significant finding, as it may provide additional means of controlling the growth and metabolism of undifferentiated progenitor cells. However, culture in a rotating-wall bioreactor has not always proven beneficial; a recent TMJ disc tissue engineering study found little or no benefit when using a rotating-wall bioreactor as compared to static culture.[119]

The use of bioreactors in tissue engineering has yielded exciting results and possibilities. Future directions of bioreactor use will likely involve the combination of the hydrodynamic flow chambers with other sources of mechanical stimulation,[120] as well as with growth factor addition in the medium. As these technologies improve and the processes of growth factor addition and mechanical stimulation are optimized, it may become possible to create a large-scale cartilage bioreactor for mass production of engineered constructs.

FUTURE TRENDS AND NEEDS

Successful tissue engineering approaches that will be used in the clinic likely will require optimization of the four parameters of the tissue engineering paradigm. Scaffolds will need to exhibit adequate biocompatibility and mechanical properties and allow for diffusion of nutrients to the seeded cells, or a "scaffold-less" approach such as the self-assembling process will need to be employed. Stem cells, both adult and embryonic, represent a promising cell source for articular cartilage engineering; however, more work needs to be performed to understand the developmental processes involved in differentiation so that these processes may be further manipulated to optimize *in vitro* cell expansion while maintaining chondrogenic differentiation. Growth factor application also must be optimized for tissue engineering, through further characterization of the roles of each growth factor and their effects on different cell types, as well as elucidation

of the correct dosage frequency and concentrations. Finally, as scaffolds, cell sources, growth factor application, and mechanical stimulation are optimized, mass production of tissue-engineered constructs may become possible through the creation of large-scale bioreactors.

Current tissue engineering approaches strive to obtain a construct with mechanical, biochemical, and histological properties as close as possible to native tissue. However, since relatively few constructs have seen clinical use to date, it is unclear how closely the properties of the construct must mimic those of native tissue in order to prove clinically functional. It is likely that as the parameters of the tissue engineering paradigm are optimized to produce constructs that approach native tissue properties, constructs with a wide spectrum of properties will be produced along the way. Then, implantation studies may be performed to determine the optimal properties a construct must possess for *in vivo* use.

CONCLUSIONS

When cartilage is damaged, it has a limited ability to heal itself and clinical treatment is unable to fully restore tissue function. Therefore, tissue engineering is an ideal approach for successful cartilage regeneration, through the interaction of the selected scaffold, cell source, growth factors, and mechanical stimulation. Although many promising results have been attained thus far, tissue engineering

still has to overcome hurdles, as successful regeneration of cartilage cannot be realized until the four parameters of the tissue engineering paradigm have been optimized. Nonetheless, this is an exciting time as we are rapidly approaching widespread clinical use of tissue engineered cartilage constructs for treatment of articular cartilage, knee meniscus, and TMJ pathologies.

Chapter 2: The Use of Hydrostatic Pressure in Articular Cartilage Tissue Engineering

Benjamin D. Elder and Kyriacos A. Athanasiou

Chapter submitted as: Elder, BD and Athanasiou, KA. The Use of Hydrostatic Pressure in Articular Cartilage Tissue Engineering. *Tissue Engineering Part B.*

ABSTRACT

Cartilage has a poor intrinsic healing response, and the innate healing response as well as current clinical treatments cannot restore its function. Therefore, articular cartilage tissue engineering is a promising approach for the regeneration of damaged tissue. As cartilage is exposed to mechanical forces during joint loading, many tissue engineering strategies utilize exogenous stimuli in order to enhance the biochemical or biomechanical properties of the engineered tissue. Hydrostatic pressure is emerging as arguably one of the most important mechanical stimuli for cartilage. However, no optimal treatment has been established across all culture systems. Therefore, this review evaluates prior studies on articular cartilage involving the use of hydrostatic pressure to reach a consensus on the magnitudes, frequencies, and application times that should be pursued further. Additionally, this review addresses hydrostatic pressure bioreactor design, chondroprotective effects of hydrostatic pressure, the use of hydrostatic pressure for chondrogenic differentiation, the effects of high pressures, as well as hydrostatic pressure mechanotransduction.

INTRODUCTION

Injuries to articular cartilage generally result in the formation of mechanically inferior fibrocartilage, which will eventually degrade with use.[14] Additionally, current clinical treatments for articular cartilage injuries generally aim to enhance this intrinsic repair response, but may often result in the formation of fibrocartilage. Therefore, tissue engineering approaches provide tremendous promise for cartilage regeneration. A principal tenet of the cartilage tissue engineering approach is the use of exogenous mechanical stimulation to simulate joint loading and lead to enhanced chondrocyte metabolic activity and extracellular matrix (ECM) production. Hydrostatic pressure (HP) provides a robust method for chondrocyte stimulation, as it can be applied to chondrocytes in monolayer, 3-D engineered constructs, as well as explants.

Cartilage is a highly hydrated tissue, comprised of 75-80% water per wet weight. The high water content results as water is attracted to the negatively charged proteoglycan molecules within the tissue. During joint loading, a uniform perpendicular stress (Fig. 4) is imparted to the chondrocytes as the synovial fluid imparts a hydrostatic pressure on the fluid phase of the tissue. Additionally, as the tissue undergoes a compressive load, the pressurization of the fluid phase initially supports the applied load, as water is trapped within the solid matrix of the tissue due to its low permeability. Eventually, fluid is expelled from the tissue and the frictional force between the fluid and solid phases of the tissue dissipates energy from the applied load. In the joint, cartilage is typically exposed to

stresses between 3-10 MPa,[121] while stress as high as 18 MPa has been reported in the hip joint.[11] These stresses should be translated to hydrostatic pressure due to fluid phase pressurization, as described above. Additionally, the human walking cadence generally is up to 1 Hz.[104] As such, tissue engineering efforts have generally focused on magnitudes and frequencies within these physiologic ranges.

Although prior reviews have addressed the effects of intermittent HP on chondrocytes in monolayer,[122] and the effects of HP on chondrocyte mechanotransduction,[123] a comprehensive review discussing the effects of HP in articular cartilage tissue engineering studies does not exist. As such, this review addresses bioreactor design for the application of HP, different tissue engineering strategies involving the application of HP, the chondroprotective effects of HP, the use of HP towards chondrogenic differentiation, the effects of high pressures on cartilage, as well as the mechanotransduction mechanisms that explain the beneficial results from HP application in cartilage tissue engineering studies.

HYDROSTATIC PRESSURE BIOREACTORS

In general, there are two predominant methods of applying HP to cells, explants, or constructs, and they both offer advantages and disadvantages. In the first method, HP is applied by compressing a gas phase that transmits load through the medium to the cells. However, this method is limited as pressurizing the gas

phase may alter the gas concentration within the culture medium. For instance, Hansen et al.[124] observed a 0.36 decrease in the pH of the medium following 10 h of HP application. However, the advantage of this approach is that it allows for the controlled alteration of partial pressures within the medium, such as when examining the effects of HP at different oxygen levels.[125] Alternatively, a less complicated approach involves applying HP by compressing only the fluid phase, which limits any changes in gas solubility within the chamber. This method generally involves connecting a fluid-filled chamber by hose to a piston attached directly to a hydraulic press, controlled by a computer (Fig. 5). This is the selected setup in a large number of prior studies, as well as in our own work. Both types of bioreactors also include temperature control, generally by placing the chamber in a water bath, in order to maintain the culture temperature at 37°C. Finally, either type of bioreactor may be altered to allow for semicontinuous medium perfusion, as reviewed in detail previously.[106]

TISSUE ENGINEERING STRATEGIES WITH HYDROSTATIC PRESSURE

HP has seen extensive use as an agent for increasing the metabolic activity of chondrocytes in tissue engineering studies. In general, these studies have assessed the effects of HP on chondrocytes cultured in monolayer, cartilage explants, as well as chondrocytes in 3-D culture, both with and without a scaffold. In tissue engineering studies involving HP application, it is possible to vary the magnitude, frequency, and duration of application of HP. Additionally, in studies

involving 3-D engineered constructs, it is also possible to vary when HP can be applied in construct development. However, little consensus has been reached regarding the ideal levels of each of these parameters, particularly when different culture conditions are used.

In general, studies assessing the effects of HP on chondrocytes in monolayer have demonstrated beneficial effects of dynamic HP, while static HP has been found to have either no effect or a detrimental effect. For instance, Suh et al.[126] cultured young bovine chondrocytes in monolayer, and exposed them to 0.8 MPa, for 5 min on, 30 min off, 10 times. This treatment resulted in a 40% increase in proteoglycan synthesis as well as enhanced aggrecan mRNA. However, there was no change in collagen synthesis during pressurization. Also, when using juvenile bovine chondrocytes in monolayer, Jortikka et al.[127] demonstrated that HP at 5 MPa, 0.5 Hz, for 20 h, significantly increased sGAG incorporation, while 5 MPa static HP for the same application time had no effect on sGAG incorporation.

In addition to these studies using chondrocytes from 1-2 year old animals, several studies using adult chondrocytes have been performed, predominantly by Smith's group, and have demonstrated similar results. For example, Smith et al.[128] exposed adult articular chondrocytes in monolayer to 10 MPa HP, static or 1 Hz, for 4 h. They demonstrated that HP application at 1 Hz increased aggrecan and collagen II mRNA immediately after application, while static HP

decreased collagen mRNA levels. In a later study, Smith et al.[129] cultured normal adult bovine chondrocytes in monolayer, and applied HP at 10 MPa, 1 Hz, for up to 24 h for just 1 day, or for 4 h/day for 4 days. They found that aggrecan mRNA continued to increase up to 24 h of loading, while collagen II mRNA expression was increased maximally with 4 and 8 h of HP application. However, they demonstrated the importance of examining multiple loading profiles, as changing to an application of 4 h/day for 4 days led to even greater increases in both aggrecan and collagen II mRNA. Additionally, Ikenoue et al.[130] again demonstrated the importance of examining multiple application times, as they cultured normal adult human chondrocytes in monolayer, and exposed them to 1, 5, or 10 MPa HP, at 1 Hz, for 4 h/day, for 1 or 4 days. They demonstrated enhanced collagen II gene expression only for treatment with 5 and 10 MPa, for 4 days; also, while enhanced aggrecan expression was observed with all treatments, these groups resulted in the greatest enhancement of aggrecan gene expression. This study also indicates that magnitude and frequency have significant effects on chondrocyte metabolism, and it appears that collagen production may be more sensitive to the selected HP regimen. On the other hand, a study by Takahashi et al.[131] demonstrated beneficial effects when applying static HP to chondrocytes in monolayer, although it must be highlighted that a chondrosarcoma cell line was used rather than primary chondrocytes. They found that 1 and 5 MPa static HP for 2 h resulted in a significant increase in sGAG incorporation immediately after HP stimulation, and that 5 MPa static HP led to increased expression of TGF-β1 mRNA.

Studies assessing the effects of HP on chondrocytes in 3-D culture or in explants have demonstrated different results, as it has been suggested that chondrocytes in monolayer respond differently to HP than tissue, since the cell interaction with its ECM is likely involved in the HP response. For instance, Parkkinen et al.[132] observed enhanced sGAG incorporation in explants exposed to HP at 5 MPa, 0.5 Hz, for 1.5 h, while a significant inhibition of sGAG incorporation was found in monolayer cultures exposed to the same regimen. Furthermore, Carver and Heath[133] observed that adult and juvenile P3 equine chondrocytes in PGA meshes respond differently to HP at 0.25 Hz, for 20 min every 4 h for 5 wks. For adult cells, 6.87 MPa HP was required to increase GAG and collagen production, while for juvenile cells, either magnitude increased GAG production, but only 6.87 MPa increased collagen production, thus suggesting that collagen production may be more sensitive to the applied regimen. In a later study, applying HP at 3.44 MPa, 0.25 Hz, 20 min every 4 h for 5 wks to P3 juvenile bovine chondrocytes in PGA meshes resulted in significantly increased GAG production with no effect on collagen production; however, the results of both studies may stem from the use of passaged chondrocytes.[134] Finally, in our own work,[135] exposing immature bovine chondrocytes in scaffoldless constructs to HP at 10 MPa, 1 Hz, for 4 h/day, 5 days/wk for up to 8 wks with 10% FBS led to increased collagen content relative to control at 4 and 8 wks, and also prevented the decreased in GAG/construct observed in the control groups over time.

Additionally, contrary to the majority of studies involving chondrocytes in monolayer, static HP regimens in the physiologic range have generally demonstrated beneficial effects on chondrocytes in 3-D culture or cartilage explants. For example, in explants from 2 year old bovines, a 2 h application of 5-10 MPa static HP enhanced sulfated GAG incorporation while 5-15 MPa static HP increased proline incorporation.[102] Also, Mizuno et al.[136] exposed immature bovine chondrocytes in 3-D collagen sponges to static HP, at 2.8 MPa for up to 15 days, and observed increased GAG production at 5 and 15 days of culture. Similarly, Toyoda et al.[137] found that exposing immature bovine chondrocytes in 2% agarose gels to 5 MPa static HP for 4 h resulted in a 4-fold increase in aggrecan mRNA as well as a 50% increase in collagen II mRNA. In another study using the same constructs and HP regimen, Toyoda et al.[138] observed an 11% increase in GAG production as well as a 4-fold increase in aggrecan mRNA. Finally, in our own recent work,[139] using scaffoldless articular cartilage constructs as described previously,[36, 140] a full-factorial comparison was made between 3 magnitudes (1, 5, and 10 MPa) and 3 frequencies (static, 0.1, and 1 Hz) of HP for 1 h/day, from days 10-14 of construct development. It was determined that static HP at 5 or 10 MPa as well as cyclic HP at 10 MPa, 1 Hz, resulted in a significant increase in compressive stiffness and GAG production; however, only static HP at 5 or 10 MPa resulted in a significant increase in tensile stiffness and collagen production. An additional exciting finding of the study was additive and synergistic effects when applying HP and growth factors, as the combination of 10 MPa static HP and TGF-β1 resulted in 164% and 231% increases in

compressive and tensile stiffness, respectively, as well as 85% and 173% increases in GAG and collagen production, respectively.

Although physiological magnitudes clearly have beneficial effects on chondrocyte gene expression, protein production, and biomechanical properties, based on the varying results observed in these studies regarding effects of HP regimens, it is clear that magnitude, frequency, and application time must all be optimized for each system. It is possible that a regimen may yield beneficial effects in a 3-D culture system, while simultaneously resulting in little effect or a detrimental effect in a monolayer system. Additionally, the cell type used appears to play a significant role in the response to HP application, as immature bovine chondrocytes appear to have a greater metabolic response to HP stimulation than adult human chondrocytes. Finally, performing HP studies on 3-D constructs leads to additional issues, as the optimal time to begin applying HP during construct development must be determined. Perhaps application of HP very early in culture might yield similar results to monolayer studies due to the absence of abundant ECM, while studies later in construct development when a significant ECM is present may yield substantially different results.

CHONDROPROTECTIVE EFFECTS OF HYDROSTATIC PRESSURE

HP also appears to be useful in providing chondroprotective effects to chondrocytes subjected to an inflammatory stimulus. For instance, the

application of HP at 10 MPa, 1 Hz, for 12 or 24 h to human osteoarthritic chondrocytes in monolayer resulted in decreased expression of matrix metalloproteinase (MMP)-2, interleukin (IL)-6, and monocyte chemoattractant protein (MCP)-1.[141] Additionally, Lee et al.[142] demonstrated chondroprotective effects of HP on human osteoarthritic chondrocytes in monolayer. They found that applying HP at 10 MPa, 1 Hz, for 4 h, following an 18 h treatment with the known inflammatory mediator lipopolysaccharide (LPS), mitigated the damaging effects of LPS, as there were decreased nitric oxide and nitric oxide synthase levels, which are known to have deleterious effects on ECM production. There was also enhanced collagen II and aggrecan mRNA levels relative to unpressurized cells treated with LPS. A later study by Lee et al.[143] identified chondroprotective effects of HP following shear stimulation, as the application of HP at 10 MPa, 1 Hz to human osteoarthritic chondrocytes after shear stress inhibited nitric oxide release. Additional chondroprotective effects of HP on osteoarthritic chondrocytes were observed in work by Fioravanti et al.,[144] as coupling HP with exogenous application of hyaluronic acid resulted in significant chondroprotective effects from IL-1β treatment induced inflammation, as well as a significant increase in GAG production. Furthermore, Gavenis et al.[145] found that applying 40 kPa of HP at 0.0125 Hz to human osteoarthritic chondrocytes resulted in a 53.3% increase in GAG content by 14 days; however, the GAG/dry weight remained only 0.06%.

Finally, chondroprotective effects have also been demonstrated in primary chondrocytes, as HP at 5 MPa, 0.5 Hz, 3 h/day, for 3 days led to upregulation of TIMP-1 and downregulation of MMP-13 and collagen I gene expression in bovine chondrocytes cultured in alginate beads.[146] These results appear promising as they indicate that HP could possibly be used to delay the onset of osteoarthritis. Furthermore, primary osteoarthritic chondrocytes could potentially be used in tissue engineering strategies, which is exciting, as they would be somewhat of a readily available autologous cell source. However, it must be mentioned that Islam et al.[147] observed an increase in the number of apoptotic cells when applying HP at 5 MPa, 1 Hz, for 4 h to osteoarthritic human chondrocytes in monolayer, thus indicating that osteoarthritic chondrocytes may be quite sensitive to the selected HP regimen.

HYDROSTATIC PRESSURE AND DIFFERENTIATION

In addition to its wide use as an agent for mechanical stimulation in tissue engineering, HP has seen tremendous use as a method for differentiating cells towards a chondrogenic phenotype. For instance, Angele et al.[148] cultured adult human bone marrow mesenchymal stem cells (bmMSCs) in aggregate culture, and found that HP at 5 MPa, 1 Hz, 4 h/day, for 1 day had no effect, while 7 days of treatment resulted in a significant increase in both collagen and GAG content as early as 7 days after removal of the HP stimulus, but a maximal increase was observed 21 days after removal of the HP stimulus. This study suggests that multiple days of HP application are required for an effect, and as seen in our own

work with hydrostatic pressure,[139] the maximum effects of HP may be delayed until several weeks after removal of the stimulus. In a later study, Luo and Seedhom[149] seeded ovine bmMSCs on polyester scaffolds and demonstrated that following 4 wks of culture, HP at 0.1 MPa, 0.25 Hz, for 30 min/day, for 10 days resulted in both increased GAG and collagen content, while shorter timepoints had no effect on collagen content. However, as the constructs were assessed immediately after HP application, it is possible that the longer application time required to see effects on collagen content may actually just be due to the delayed effects of the earlier days of HP application, as discussed above. Additionally, Wagner et al.[150] seeded human bmMSCs in type I collagen sponges, and observed that HP at 1 MPa, 1 Hz, for 4 h/day, for 10 days resulted in increased aggrecan, collagen II, and sox9 mRNA and increased histological staining for GAGs. However, they also observed an increase in collagen I mRNA, which was possibly due to the use of an osteochondrogenic medium, which differs from prior work. This hypothesis is further supported as Scherer et al.[125] found that a chondrogenic medium was required for HP to promote chondrogenesis of bovine bmMSCs in high density monolayer.

As described above in tissue engineering studies, combined treatment with both growth factors and HP as agents for chondrogenesis appears promising. For example, Miyanishi et al.[151] cultured adult human bmMSCs in pellet culture, and exposed them to HP at 10 MPa, 1 Hz, 4 h/day, for up to 14 days, with and without 10 ng/ml TGF-β3. The combined treatment with HP and TGF-β3 resulted

in a significant increase in collagen II, aggrecan, and sox9 mRNA levels, that was greater than the increased levels from either treatment alone. In a follow-up study, Miyanishi et al.[152] created pellet cultures of adult human bmMSCs, and applied HP at 0.1, 1, and 10 MPa, 1 Hz, for 4 h/day, for 3, 7, or 14 days, along with 10 ng/ml TGF-β3. In this study, all magnitudes significantly increased aggrecan and sox9 mRNA, but only 10 MPa significantly increased collagen II mRNA. Furthermore, 10 MPa was the only treatment to significantly increase both GAG and collagen production, with the maximum effect observed after 14 days of HP application. Again, this observation may be due to the delayed effects of HP as discussed above, and it would be interesting to determine if similar results would be obtained if assessment was delayed until 5-10 days after removal of the HP stimulus.

HP has also been used as a method for chondroinduction of other cell types, such as fibroblasts and dedifferentiated chondrocytes. For instance, Elder et al.[153] found that 7200 cycles/day of HP at 5 MPa, 1 Hz, for 3 days, applied to murine embryonic fibroblasts in monolayer, resulted in an almost 200% increase in GAG production, along with an almost 225% increase in collagen synthesis. Additionally, Heyland et al.[154] cultured dedifferentiated porcine chondrocytes in alginate beads, and observed a 25% increase in GAG production as well as a 65% increase in collagen II production following HP application at 0.3 MPa, 1 Hz, for 6 h/day. Finally, Kawanishi et al.[155] grew pellet cultures of dedifferentiated bovine chondrocytes (P3), and demonstrated that HP application at 5 MPa, 0.5

Hz, for 4 h/day for 4 days led to a 5-fold increase in aggrecan mRNA and a 4-fold increase in collagen II mRNA. However, HP had a negligible effect on collagen I mRNA levels, as both control and HP treated pellets had similar decreases in collagen I mRNA. Based on these results, HP appears to be a promising method for differentiating cells to a chondrocytic phenotype, although in the case of dedifferentiated chondrocytes, HP may have a greater effect on enhancing collagen II production than diminishing collagen I production.

EFFECTS OF HIGH HYDROSTATIC PRESSURES

As described above, there has been extensive work demonstrating the beneficial effects of physiological magnitudes of hydrostatic pressures on the gene expression, biochemical, and biomechanical properties of chondrocytes in monolayer, engineered constructs, as well as explants. However, raising pressures above these physiological levels has been shown to have either limited or even detrimental effects. Also, although static HP has generally been shown to have beneficial effects when using physiological magnitudes for 3-D tissue engineering studies, it becomes far more detrimental than dynamic loading at higher pressures. For example, Hall et al.[102] examined the effects of 20-50 MPa static HP, for 20 s, 5 min, or 2 h on bovine explants, and found that short term application times had no effect on GAG and collagen synthesis rates, while 2 h application resulted in a significant decrease in both GAG and collagen synthesis. In another 3-D study, Nakamura et al.[156] seeded normal adult rabbit chondrocytes in alginate beads and found that 50 MPa static HP for 12 or 24 h

resulted in a significant increase in the number of apoptotic cells. Additionally, they found that 50 MPa static HP led to a dramatic increase in heat-shock protein 70 (hsp70) mRNA. In another study, Fioravanti et al.[157] studied the effects of high HP on normal human chondrocytes in alginate beads, and found that 24 MPa static HP applied for 3 h decreased mitochondria and golgi body number, and altered the actin and tubulin of normal chondrocytes such that they more closely resembled osteoarthritic cells in these characteristics.

Several studies on chondrocytes in monolayer have demonstrated similar detrimental results. For instance, Parkkinen et al.[109] assessed the effects of high pressures on bovine chondrocytes in monolayer, and found that a 2 h application of 30 MPa static HP, and to a lesser extent 15 MPa static HP, resulted in a microtubule dependent compaction of the golgi apparatus with a concomitant decrease in GAG synthesis. However, 15 and 30 MPa HP at either 0.05 or 0.125 Hz had no effect on the golgi apparatus or GAG synthesis. Similarly, in a later study, Parkinnen et al.[158] assessed the effects of 2 h of HP stimulation on bovine chondrocytes in monolayer, and found that 30 MPa static HP led to a reversible complete loss of stress fibers, while 30 MPa HP, at 0.05 or 0.125 Hz, just changed the appearance of the stress fibers. It was suggested that the altered stress fibers may be the result of very small strains on the cells or microfilaments, or possibly due to alterations in the intracellular ion concentrations, as described further below. In a similar study using bovine chondrocytes in monolayer, Lammi et al.[107] found that 30 MPa static HP resulted in a 37% decrease in GAG

synthesis accompanied by decreased aggrecan mRNA levels; this treatment also resulted in the production of atypically large aggrecan molecules. These results are interesting as the altered aggrecan size demonstrates that HP can affect production of ECM at the levels of both transcription and translation.

Detrimental effects of high HP have also been observed in other chondrocyte-like cell lines, cultured in monolayer. For example, Sironen et al.[159] assessed the effects of 30 MPa HP, static or 1 Hz, for up to 24 h on immortalized human chondrocyte cell lines and chondrosarcoma cells, cultured in monolayer. They found that static HP resulted in significantly increased hsp70, hsp40, Gadd45 and Gadd153 gene expression, all of which are genes associated with stress responses. Additionally, they demonstrated that static HP had a greater effect on the increased gene expression than HP at 1 Hz. In a separate study, Sironen et al.[160] used a cDNA array to assess the effects of a 6 h treatment with 30 MPa static HP on human chondrosarcoma cells. This treatment had negative effects on the ECM content, as it led to decreased osteonectin, fibronectin, and procollagen levels. Furthermore, Takahashi et al.[108] found that in a human chondrosarcoma cell line, a 2 h application of 50 MPa static HP significantly increased IL-6 and TNF-α mRNA, and also led to decreased expression of proteoglycan core protein; these results are indicative of osteoarthritic changes. Finally, Kaarniranta et al.[161] observed a doubling of hsp70 mRNA after 12 h of treatment with 30 MPa static HP, while treatment with 30 MPa, 0.5 Hz did not

change the level of hsp70. However, treatment with 4 MPa HP, either static or dynamic, did not alter the expression of hsp70.

Taken together, these results suggest that HP magnitudes outside of the physiologic range may result in a stress response, especially when using static HP, so tissue engineering strategies should focus on more physiological magnitudes. Although these higher pressures likely are not useful for cartilage tissue engineering strategies, they indicate that high pressures may play a role in the progression of osteoarthritis, as many osteoarthritic changes can be observed in chondrocytes exposed to these high pressures.

HYDROSTATIC PRESSURE MECHANOTRANSDUCTION

Unlike direct compression and shear mechanical stimulation, hydrostatic pressure does not result in macroscopic deformation of cartilage. According to the biphasic theory, the solid matrix of cartilage is intrinsically incompressible, and no tissue deformation will be observed under an external hydrostatic load, even though the tissue may be anisotropic. Bachrach et al.[162] tested this theoretical prediction on normal bovine cartilage explants, and found that static pressures in the physiological range, up to 12 MPa, did not result in measurable cartilage deformation. Similarly, Tanck et al.[163] found that physiological hydrostatic pressure magnitudes on fetal cartilage result only in extremely small deformations of approximately 2 μ-strain, as a result of the relative incompressibility of the solid matrix of articular cartilage. However, it must be

noted that even though there is only a minute tissue strain, this, along with the strain imparted by the compressibility of water itself, may be great enough to impart strain on the chondrocytes themselves, although it has previously been demonstrated that the cells are relatively incompressible at these physiological pressures.[164] Therefore, as HP generally produces a state of stress with no or very little strain, alternative mechanisms have been proposed to explain the mechanotransduction pathways of HP application.

Several studies have indicated that HP likely has direct effects on cell membrane ion channels (Fig. 6). Hall[165] examined the effects of static HP on isolated bovine chondrocytes for 20 s or 10 min, and found that the Na/K pump was substantially inhibited when going from 2.5-5 MPa, and this inhibition increased slightly when pressure was increased up to 50 MPa. For example, 10 MPa static HP for 10 min resulted in a 53% decrease in the activity of the Na/K pump, relative to control. Additionally, the Na/K/2Cl transporter was inhibited by increasing pressure up to 50 MPa, and it was found that increasing the magnitude of 10 s of static HP application from 7.5-15 MPa resulted in an almost 40% reduction in Na/K/2Cl transporter activity. An additional study by Browning et al.[166] examined the effects of static HP, ranging from 2-30 MPa for up to 180s on juvenile bovine chondrocytes, in monolayer. They found that both 20 and 30 MPa static HP application resulted in a significant increase in the activity of the Na/H pump. Furthermore, adding the kinase inhibitor staurosporine prevented the HP-induced stimulation of Na/H exchange, thus suggesting that direct activation of the

transporter is a phosphorylation-dependent process. In a similar study, Mizuno[167] assessed the effects of 5 min of static HP at 0.5 MPa on immature bovine articular chondrocytes in monolayer, and found that the application of HP to middle zone cells resulted in a 2-fold increase in intracellular calcium. It was determined that this increase was dependent upon direct effects of HP on stretch-activated calcium channels, as well as the release of intracellular calcium. Likewise, Browning et al.[168] assessed the effects of short term application of static HP on isolated juvenile bovine articular chondrocytes. They found that 30 s of static HP application at 30 MPa resulted in an approximately 3-fold increase in intracellular calcium, largely caused by calcium release from intracellular stores. Additionally, they found that this intracellular calcium release was dependent on IP_3 mediation, and that similar induction of IP_3-mediated calcium release occurred at more physiologic pressure magnitudes, such as 10 MPa.

As reviewed previously,[169] the direct effects of HP on transmembrane ion transporter function are likely due to the pressure's effects on the conformations of the transmembrane proteins. Although HP does not measurably deform cartilage due to the intrinsically incompressible nature of its phases, the transporter proteins themselves have void spaces created by their folding orientation that can be compressed. As these spaces undergo increased strain as pressure rises, the protein will eventually alter its orientation to achieve a lower energy folding state. Thus, as described above, a pressure-dependent change in intracellular ion concentrations will be observed. It is widely known

that alterations in intracellular ion concentrations result in changes in cellular gene expression and protein synthesis.[170] Thus, it is likely that specific pressures result in certain ion concentration changes that lead to the specific effects on gene expression, protein production, and eventually biomechanical properties, in the tissue engineering studies described previously.

CONCLUSIONS

Cartilage regeneration has been an extremely difficult problem due to the poor intrinsic healing capacity of the tissue. However, mechanical stimulation with HP has provided significant beneficial effects on gene expression and protein production of chondrocytes in monolayer, and has led to enhanced biochemical and biomechanical properties in engineered constructs. It is apparent that physiologic magnitudes, particularly between 5 and 10 MPa have beneficial effects on cartilage properties; however, there are substantial differences in the effects observed between monolayer and 3-D culture, as static HP regimens have little effect or are detrimental to chondrocytes in monolayer, while static HP in the physiologic range enhances the functional properties of 3-D engineered constructs. Additionally, work involving HP application to osteoarthritic chondrocytes demonstrates that osteoarthritic chondrocytes may be used in tissue engineering strategies, and that HP could potentially be used as a treatment modality to delay osteoarthritic changes. Furthermore, physiologic magnitudes of HP, particularly with intermittent loading frequencies can be used as a differentiation factor for MSCs, embryonic stem cells, as well as

dedifferentiated chondrocytes. Finally, it is clear that high hydrostatic pressures, particularly between 30-50 MPa but as low as 15 MPa, have detrimental effects on chondrocytes and generally result in a stress response and decreased metabolic activity. These detrimental effects are especially apparent with loading times exceeding 2 h.

Although the work performed up to this point appears promising, additional work must be performed in each system to optimize the magnitude, frequency, duration of application, and application time in construct development. Additionally, based on the additive and synergistic effects of HP and growth factor application, it is likely that following optimization, HP will need to be used in combination with other exogenous stimuli such as growth factors as well as with other mechanical stimuli such as direct compression in order to yield a construct with biochemical and biomechanical properties approaching those of native tissue.

Chapter 3: Effects of Confinement on the Mechanical Properties of Self-Assembled Articular Cartilage Constructs in the Direction Orthogonal to the Confinement Surface

Benjamin D. Elder and Kyriacos A. Athanasiou

Chapter published as: Elder, BD and Athanasiou, KA. Effects of Confinement on the Mechanical Properties of Self-Assembled Articular Cartilage Constructs in the Direction Orthogonal to the Confinement Surface. *Journal of Orthopaedic Research.* 2008, 26: 238-246.

ABSTRACT

During *in vivo* development, articular cartilage is exposed to several different forms of stress. This study examined the effects of radial confinement and passive axial compression-induced vertical confinement, on the biomechanical, biochemical, and histological properties of self-assembled chondrocyte constructs. The self-assembled constructs, engineered without the use of an exogenous scaffold, exhibited significant increases in stiffness in the direction orthogonal to that of the confinement surface. With radial confinement, the significantly increased aggregate modulus was accompanied by increased collagen organization in the direction perpendicular to the articular surface, with no change in collagen or glycosaminoglycan (GAG) content. Additionally, radial confinement was most beneficial when applied before t=2 wks. With passive axial compression, the significantly increased Young's modulus and ultimate tensile strength were accompanied by a significant increase in collagen production. This study is the first to demonstrate the beneficial effects of confinement on tissue engineered constructs in the direction orthogonal to that of the confinement surface.

INTRODUCTION

Cartilage degeneration, from injury or osteoarthritis, is a tremendous problem in current orthopaedic practice. Following injury or osteoarthritis, articular cartilage is unable to repair itself, resulting in a permanent defect or the formation of mechanically inferior fibrocartilage.[14] Therefore, tissue engineering is a promising approach for the treatment of articular cartilage injuries, as this approach may eventually allow for the production of engineered tissue indistinguishable from native cartilage.

A chondrocyte self-assembling process for tissue engineering articular cartilage was recently developed[36] that allowed constructs to reach 1/3 the stiffness of native cartilage. Additionally, the benefits of hydrostatic pressure stimulation on self-assembled constructs have been demonstrated,[135] as intermittent hydrostatic pressure applied at 10 MPa and 1 Hz, for 4 hrs per day and 5 days per wk was shown to increase collagen production. The self-assembling process avoids many of the problems associated with scaffold use, namely concerns over stress shielding, biocompatibility and biodegradation.

Articular cartilage is exposed to a variety of forces *in vivo* including compression, shear, and hydrostatic pressure. Additionally, mechanical stimulation is vital for maintaining the integrity of the tissue, as articular cartilage demonstrates changes representative of a loss of function when immobilized.[99, 100] Therefore, it is likely that some form of mechanical intervention will be required for further

refinement of tissue engineering techniques. Although the precise signaling pathways involved in mechanotransduction have not been fully elucidated, several studies have shown promising results involving the use of mechanical stimulation including dynamic compression,[111, 171-176] shear,[174, 177] and hydrostatic pressure.[39, 102, 178, 179]

Although coupling mechanical stimulation with the self-assembling process for tissue engineering articular cartilage represents a promising solution for treatment of injuries, several questions remain concerning this approach. Aside from the studies showing beneficial effects of dynamic compression and hydrostatic pressure, studies comparing the effects of passive confinement on the anisotropy of articular cartilage are lacking. Additionally, studies involving the effects of mechanical intervention at different times are limited.[180]

The purpose of this study was to examine the effects of construct confinement in different directions and at different times on construct mechanical properties. Radial confinement and passive axial compression-induced vertical confinement of self-assembled constructs were used. It was hypothesized that the application of confinement would enhance the mechanical properties of the constructs in the orthogonal direction. It was further hypothesized that confinement at different timepoints in construct development would have a significant effect on construct properties. To test these hypotheses, three experiments were performed (Fig. 7). First, self-assembled constructs were radially confined in agarose wells for 1,

2, 3, or 4 wks, after which the constructs were cultured unconfined for the remainder of the 4-wk study; the effects of confinement on compressive stiffness were investigated. Second, the constructs were cultured in the same wells used in the first experiment for 2 wks, after which they were transferred to incrementally larger wells for the 3rd and 4th wk of culture. Finally, the effects of vertical confinement, in the form of passive axial compression, on the tensile stiffness were examined.

METHODS

Chondrocyte Isolation and Seeding

Chondrocytes were isolated from the distal femur of wk-old male calves[93, 118, 181] (Research 87 Inc.) less than 36 hrs after slaughter, with collagenase type 2 (Worthington) in the culture medium. The medium was DMEM with 4.5 g/L-glucose and L-glutamine, 100 nM dexamethasone, 1% fungizone, 1% Penicillin/Streptomycin, 1% ITS+, 50 μg/mL ascorbate-2-phosphate, 40 μg/mL L-proline, and 100 μg/mL sodium pyruvate (termed chemically-defined medium). Each leg came from a different animal and yielded roughly 150 million chondrocytes. To reduce variability among animals, cells from all legs were pooled together to yield a mixture of chondrocytes; a mixture of cells from 8 legs was used in the 1st study, while a mixture of cells from 6 legs was used in the 2nd and 3rd studies (see descriptions below). The pooled cells were counted on a hemocytometer, and viability was assessed using a trypan blue exclusion test.

Viability was >99% for the pooled cells. Chondrocytes were frozen in culture medium supplemented with 20% FBS and 10% DMSO at –80°C for 2 wks to a month before use. After thawing, viability remained greater than 75%. A polysulfone die consisting of 5 mm dia. x 10 mm long cylindrical prongs was constructed to fit into 6 wells of a 48-well plate. Additional polysulfone die consisting of 6 mm dia. x 10 mm long cylindrical prongs and 7 mm dia. x 10 mm long cylindrical prongs were fabricated. To construct each agarose mold, sterile, molten 2% agarose was introduced into a well fitted with the polysulfone die. The agarose was allowed to gel at room temperature for 60 min. The agarose mold was then separated from the polysulfone die and submerged into two exchanges of culture medium to completely saturate the agarose well with culture medium by the time of cell seeding. To each agarose well, 5.5×10^6 cells were added in 150 µl of culture medium. The cells self-assembled within 24 hrs in the agarose wells and were maintained in the same well for a specified amount of time; t=0 was defined as 24 hrs after seeding.

1st Study: Radial Confinement of Self-Assembled Constructs

At t=1, 2, or 3 wks, self-assembled constructs (n=6) were removed from confinement in the 5 mm dia. agarose well, and placed in one well of a 6-well culture plate coated with 2% agarose (Fig. 7a). Each agarose-coated well contained 3-4 constructs, and 500 µl of medium per construct was changed daily (1.5-2 ml per well). At t=4 wks, all samples were tested for morphological, histological, biochemical, and biomechanical properties.

2nd Study: Maintenance of Radial Confinement of Self-Assembled Constructs

At t=2 wks, self-assembled constructs (n=5) were removed from confinement in the 5 mm dia. agarose wells, and transferred to 6 mm dia. agarose wells (Fig. 7b). At t=3 wks, these constructs were removed from confinement in the 6 mm dia. agarose wells, and transferred to 7 mm dia. agarose wells. A control consisted of constructs confined in 5 mm dia. agarose wells for 2 wks, and then maintained in agarose-coated wells, as described above. Each day, 500 µl of medium was changed. At t=4 wks, all samples were tested for morphological, histological, biochemical, and biomechanical properties.

3rd Study: Passive Axial Compression of Self-Assembled Constructs

At t=10 days, self-assembled constructs (n=5) were removed from confinement in 5 mm dia. agarose wells, and transferred to 6 mm dia. agarose wells. Vertical confinement, in the form of passive axial compression with a dead weight, was applied by placing a 5 mm dia. x 1 cm long, 1 g, porous, sintered steel cylinder on top of each construct in the 6 mm dia. wells (Fig. 7c). The dead weight corresponded to a stress of 0.5 kPa. At t=14 days, the porous cylinders were removed, and the constructs were transferred to agarose-coated wells for the remainder of the study. A control consisted of constructs cultured in 5 mm dia. agarose wells, then transferred to 6 mm dia. agarose wells at t=10 days, and finally maintained in agarose-coated wells for the remainder of the study.

Histology and Immunohistochemistry

Samples were frozen and sectioned at 14 μm. Safranin-O and fast green staining were used to examine GAG distribution.[182, 183] Picrosirius red was used for qualitative examination of collagen content. Polarized light microscopy of picrosirius red-stained sections was used to examine the collagen organization of the constructs. Slides were also processed with immunohistochemistry (IHC) to test for the presence of collagen types I (COL1) and II (COL2) on a Biogenex (San Ramon, CA) i6000 autostainer. After fixing in chilled acetone, the slides were rinsed with IHC buffer (Biogenex), quenched of peroxidase activity with hydrogen peroxide/methanol, and blocked with horse serum (Vectastain ABC kit, Vector Laboratories, Burlingame, CA). The slides were then incubated with either mouse anti-COL1 (Accurate Chemicals (Westbury, NY)) or mouse anti-COL2 (Chondrex (Redmond, WA) antibodies). The secondary antibody (mouse IgG, Vectastain ABC kit) was then applied, and color was developed using the Vectastain ABC reagent and DAB (Vector Laboratories).

Quantitative Biochemistry

Samples were digested with 10 mg/ml pepsin solution (Sigma) in 0.05 M acetic acid with 0.5 M NaCl at 4°C for 72 hrs, followed by 1 mg/ml pancreatic elastic in 1x TBS at 4°C overnight. Total DNA content was measured by Picogreen® Cell Proliferation Assay Kit (Molecular Probes). Total sulfated GAG was then quantified using the Blyscan Glycosaminoglycan Assay kit (Biocolor), based on

1,9-dimethylmethylene blue binding.[184, 185] After being hydrolyzed by 2 N NaOH for 20 min at 110°C, samples were assayed for total collagen content by a chloramine-T hydroxyproline assay.[186]

Indentation Testing

Samples were evaluated with an automated indentation apparatus.[187] A step mass of 0.7 g (0.007 N) was applied with a 1 mm flat-ended, porous indenter tip, and the specimens were allowed to creep until equilibrium, as described elsewhere.[36] Preliminary estimations of the Young's modulus of the samples were obtained using the analytical solution for the axisymmetric Boussinesq problem with Papkovich potential functions.[188, 189] The intrinsic mechanical properties of the samples were then determined using the linear biphasic theory.[3]

Tensile Testing

Samples were cut into 500 μm thickness and tested using a uniaxial materials testing machine (Instron 5565), as described elsewhere.[190] Samples were cut into dog-bone shapes that had approximately 1-mm-long gauge lengths. Cyanoacrylate glue was used to attach the samples to paper tabs for gripping, outside of the gauge length. A constant strain rate of 0.01 s^{-1} was used. The Young's modulus was equal to the slope of the linear region of the curve, the tensile strength equal to the maximum stress, the maximum strain was the strain corresponding to the maximum stress, and the energy was equal to the area under the curve (trapezoid rule) from zero strain to maximum strain.

Statistical Analysis

All samples were assessed biochemically and biomechanically (n=5 or 6). A single factor ANOVA was used to analyze the samples, and Tukey's *post hoc* test was used when warranted. Significance was defined as $p < 0.05$.

RESULTS

Gross Appearance and Histology

There were no differences in gross morphology between any of the groups. After 2 wks of culture, all constructs reached a diameter slightly below 6 mm. By 3 wks of culture, constructs reached a diameter slightly below 7 mm, and by 4 wks of culture, constructs reached a diameter approaching 7.5 mm. In the confinement study, there were no significant differences in thickness between the 2-wk confinement group and the 1, 3, or 4-wk confinement groups, with thicknesses of 1.05±0.05 mm, 1.02±0.06 mm, 1.07±0.17 mm, and 1.10±0.14 mm respectively. Likewise, in the passive axial compression study, there were no significant differences in thickness between treatment groups. The compressed group had a thickness of 0.73±0.09 mm and the control group had a thickness of 0.81±0.07 mm. Additionally, in the follow-up confinement study, there was no significant difference in thickness between the 2-wk confinement group and the group confined for 2 wks in 5 mm dia. wells, 1 wk in 6 mm dia. wells, and 1 wk in 7 mm dia. wells, with values of 0.58±0.09 and 0.51±0.04 respectively. At t=4

wks, all constructs stained positive for collagen throughout the thickness of the construct (Fig. 8b). Additionally, safranin-O staining for GAG was observed throughout the constructs (Fig. 8c). COL2 immunostaining was observed throughout the constructs, with no differences in production among the treatment groups (Fig. 8d). Based on IHC, there was no COL1 production for any constructs (Fig. 8e).

Polarized Light Microscopy

Polarized light microscopy was used to assess the collagen organization of the constructs in the confinement study. The constructs that were confined in 5 mm dia. wells for 2 wks and then unconfined and cultured in agarose-coated wells for the remaining 2 wks exhibited small-fiber collagen organization in the direction perpendicular to the construct surface as well as larger-fiber collagen organization in the direction parallel to the construct surface (Fig. 8a). The alignment of the small collagen fibers resembled struts. Small-fiber collagen organization was minimally observed in the other treatment groups (Fig. 8a), namely confinement in 5 mm dia. wells for 1, 3, or 4 wks.

The increased collagen organization was not observed in the passive axial compression study, as the constructs to which a dead weight was applied did not show any small-fiber collagen organization.

Quantitative Biochemistry

There was no significant difference in WW/construct, DNA/construct, GAG/WW, and collagen/WW between the 2-wk confinement group and the 1, 3, or 4-wk confinement groups. The 2-wk confinement group had a WW of 39±4 mg, while the 1-wk, 3-wk, and 4-wk confinement groups had a WW of 44±3 mg, 37±4 mg, and 41±2 mg respectively. The 2-wk confinement group had a DNA/construct of 49±2 μg, while the 1-wk, 3-wk, and 4-wk confinement groups had a DNA/construct of 44±11 μg, 46±13 μg, and 47±5 μg respectively. The 2-wk confinement group had a GAG/WW of 0.061±0.009 mg/mg, while the 1-wk, 3-wk, and 4-wk confinement groups had a GAG/WW of 0.067±0.014 mg/mg, 0.055±0.003 mg/mg, and 0.050±0.004 mg/mg respectively. The 2-wk confinement group had a collagen/WW of 0.039±0.006 mg/mg, while the 1-wk, 3-wk, and 4-wk confinement groups had a collagen/WW of 0.032±0.005 mg/mg, 0.041±0.009 mg/mg, and 0.036±0.008 mg/mg respectively. Additionally, in the follow-up confinement study, there was no significant difference in WW/construct, DNA/construct, GAG/WW or collagen/WW between the 2-wk confinement group and the group confined for 2 wks in 5 mm dia. wells, 1 wk in 6 mm dia. wells, and 1 wk in 7 mm dia. wells. These groups had WW values of 16±1 mg and 14±2 mg, DNA/construct values of 31±2 μg and 31±4 μg, GAG/WW values of 0.074±0.008 mg/mg and 0.065±0.006 mg/mg, and collagen/WW values of 0.071±0.019 mg/mg and 0.088±0.011 mg/mg respectively. The collagen/WW for the passive axial compression group at 0.067±0.009 mg/mg was significantly higher than the unloaded control group, which had a collagen/WW of

0.044±0.008 mg/mg. There was no significant difference in GAG/WW between the passive axial compression group and the control, with values of 0.070±0.006 mg/mg and 0.064±0.007 mg/mg respectively. Finally, the passive axial compression group had WW values of 20±1 mg and 29±3 mg respectively; there was no difference in DNA/construct between the passive axial compression group and the control group, with values of 41±7 μg and 40±2 μg respectively.

Mechanical Evaluation

The aggregate modulus of the 2-wk confinement group reached 225±32 kPa, and was significantly higher than the aggregate moduli of the 1, 3, or 4-wk confinement groups, with values of 120±43 kPa, 126±56 kPa, and 94±52 kPa respectively (Fig. 9). In the follow-up confinement study, the aggregate modulus of the 2-wk confinement group at 214±110 kPa was insignificantly higher than that of the group confined for 2 wks in 5 mm dia. wells, 1 wk in 6 mm dia. wells, and 1 wk in 7 mm dia. wells, at 177±96 kPa.

The tensile modulus of the passive axial compression group at 1.4±0.3 MPa was significantly higher than the tensile modulus of the control group at 1.0±0.1 MPa (Fig. 10a). Additionally, the ultimate tensile strength of the passive axial compression group at 339±86 kPa was significantly higher than the ultimate tensile strength of the control group at 200±71 kPa (Fig. 10b). However, there was no significant difference between the aggregate modulus of the passive axial

compression group and control group, with values of 101±48 kPa and 111±52 kPa respectively.

DISCUSSION

This study was designed to assess the effects of radial confinement and, separately, to determine the effects of passive axial compression-induced vertical confinement, on the mechanical properties of 3-D self-assembled articular cartilage constructs over a 4-wk culture period. Confining constructs for 2 wks in 5 mm dia. agarose wells led to a significantly increased aggregate modulus. This increased compressive stiffness was accompanied by increased collagen organization without a change in GAG or collagen content. However, confinement in 5 mm dia. wells for 2 wks, followed by confinement in 6 mm dia. wells for 1 wk and confinement in 7 mm dia. wells for 1 wk did not enhance the compressive properties of the constructs, and trended towards a decrease in aggregate modulus. The application of a 0.01 N dead weight to the constructs, corresponding to 0.5 kPa of stress, resulted in significant increases in both tensile modulus and ultimate tensile strength, as well as total collagen per wet weight. These results, discussed further below, support our hypothesis, as changes in mechanical properties were identified in a direction orthogonal to the *confinement surface in tissue-engineered articular chondrocyte constructs.* Additionally, this study demonstrates further refinement and characterization of the self-assembling process.

Radial confinement in 5 mm dia. agarose wells for 2 wks led to approximately a 2-fold increase in aggregate modulus at t=4 wks, relative to confinement for 1, 3, or 4 wks. There was no difference in ECM content among the different confinement groups; however, increased collagen organization in the direction parallel to that of the compression testing and orthogonal to the confinement surface was observed only in the group confined for 2 wks. The organized collagen fibers appeared to form struts that may help to increase the compressive stiffness of the constructs. These results were unexpected as the collagen of articular cartilage is typically responsible for the tensile stiffness, while the GAG content is responsible for the compressive stiffness. However, these results agree with those found in previous studies,[191, 192] that have demonstrated that total GAG and collagen content may not be an ideal indicator of mechanical properties; rather, ECM organization may play a significant role in predicting mechanical properties. A possible explanation is that the organization of the collagen fibers aids the proteoglycans in resisting compressive forces.

Confinement during the self-assembly process may lead to radial construct compression. We observed that at t=10 days of confinement, the constructs reached the wall of the 5 mm dia. wells. At t=2 wks of confinement, constructs slightly less than 6 mm dia. were unconfined from the 5 mm dia. wells. Therefore, we hypothesized that the constructs confined for 2 wks exhibited a higher aggregate modulus and increased collagen organization due to the effects

of a low-magnitude radial compression, as well as contact with the agarose well. This radial compression would neither be constant strain nor constant stress, since the constructs continued to grow radially while confined, thus resulting in potentially increasing strain and increasing stress with construct growth. This could account for the results observed in the other three confinement groups, as the constructs confined for 1 wk did not contact the walls of the well and therefore may not have been radially compressed. Additionally, at t=3 wks of confinement, constructs slightly less than 7 mm dia. were unconfined from the 5 mm dia. wells, and at t=4 wks of confinement, constructs approaching 7.5 mm dia. were unconfined from the 5 mm dia. wells; therefore, they may have experienced higher magnitudes of radial compression, which negated the positive effects of the lower-magnitude radial compression.

Since the aforementioned radial confinement-induced stress could not be quantified, it is possible that the constructs merely were radially confined rather than radially compressed. Perhaps, confinement may have diminished the nutrient supply through the lateral surface, potentially becoming more detrimental over periods longer than 2 wks. However, the wells were constructed of agarose, with a 98% fluid phase to allow for adequate nutrient diffusion to the edges of the constructs. Additionally, confinement did not affect the cellularity of the constructs, as there was no difference in histological images and DNA/construct between the 2-wk confinement group and the other confinement groups.

In a follow-up to this study, we examined the temporal effects of the radial confinement of the 2-wk confinement group, and found that t=10-14 days was the most beneficial time for constructs to be confined by the agarose well. To maintain radial confinement similar to that experienced by the 2-wk confinement group during t=10-14 days for a longer period, constructs were confined in incrementally larger agarose wells, to mimic the radial growth of the constructs with time and approximately allow the constructs to contact the edge of the wells from t=1.5 wks to 4 wks. Constructs only confined for 2 wks in 5 mm dia. wells and unconfined for the duration of the study were used as controls. Interestingly, maintaining confinement for 4 wks caused the aggregate modulus to trend lower than the 2-wk confined control, from 214±110 kPa to 177±96 kPa, although there was no significant difference between these values. These results demonstrate that the application of radial confinement between 1 and 2 wks was more beneficial than the maintenance of a similar level of radial confinement through later time-points, which may actually be detrimental to the constructs. However, due to the constraints of the experimental setup used for the confinement studies, we were unable to apply radial confinement before approximately t=1.5 wks, so it is possible that applying radial confinement at even earlier timepoints may be more beneficial.

As described above, radial confinement resulted in changes in the compressive stiffness of constructs. Therefore, the effects of vertical confinement, in the form

of a passive axial stress, on the tensile characteristics of the constructs were examined. The application of a dead weight from t=10-14 days increased the tensile properties of the constructs. To eliminate the effects of radial confinement, the control constructs were placed in incrementally larger agarose wells. At t=4 wks, the passive axial compression group demonstrated a 1.4-fold increase in Young's modulus, as well as a 1.7-fold increase in ultimate tensile strength, relative to the control group, again confirming our hypothesis that the application of confinement to self-assembled articular cartilage constructs affects the mechanical properties in the direction orthogonal to the confinement surface. In this case, the increased tensile strength of the passive axial compression group was accounted for by a significantly higher value of collagen/WW for the passive axial compression group vs. the control group, with minimal small-fiber collagen organization for either group. Interestingly, vertical confinement led to different changes in the construct ECM than found in radial confinement. This suggests that there may be different mechanotransduction pathways for radial confinement and passive axial compression, and future studies should be performed to elucidate these potential differences. Finally, the application of a dead weight had no effect on the cellularity of the constructs, as there was no difference in histological images or DNA/construct between the passive axial compression group and the control group.

To our knowledge, this study is the first to provide evidence of the benefits of confinement on mechanical properties in the direction orthogonal to the

confinement surface. It shows that an increased construct aggregate modulus can be accounted for by increased collagen organization in the direction orthogonal to the construct surface. Previous studies have also demonstrated a relationship between cartilage mechanical properties and collagen organization, as determined by polarized light microscopy. For example, Kiviranta et al.[193] found that in bovine knee osteochondral plugs, there was a significant correlation between Poisson's ratio and collagen organization, as assessed by quantitative polarized light microscopy. Additionally, Kelly et al.[192] found that dynamic deformational loading of chondrocyte-seeded agarose hydrogels led to an increased bulk Young's modulus with increased collagen organization in the radial direction relative to the free-swelling control. Although several other studies have examined collagen organization in cartilage explants, to our knowledge, this study is the only one to demonstrate a relationship between collagen organization and aggregate modulus in tissue engineered constructs.

Several prior studies have investigated the use of dynamic compression [111, 176, 177] and/or shear [174, 177] on the ECM of tissue-engineered cartilage constructs. These studies demonstrated 1.5-2.8-fold increases in GAG, and 1.4-fold increases in collagen with mechanical stimulation, which differ from the results of the radial confinement study, which demonstrated no change in ECM content, and the passive axial compression study, which demonstrated a 1.5-fold increase in collagen without an increase in GAG. Since the other studies all involved dynamic, rather than passive stimulation, it is possible that simultaneous GAG

and collagen increases may only be observed under dynamic mechanical stimulation as a result of the increased nutrient diffusion to the construct in dynamic stimulation, as noted elsewhere.[192] However, Waldman et al.[176] found that dynamic compression of 5% amplitude at 1 Hz, for 400 cycles every other day for 1 wk, resulted in increased collagen with no change in GAG content, which matches the results of the passive axial compression study.

Although many studies have examined the relationship between mechanical stimulation and construct mechanical properties, to our knowledge, this is the first tissue engineering study to indicate the beneficial effects of passive confinement for a short term. This result indicates the possibility of an adaptive response to confinement that either results in increased collagen organization as seen in the radial confinement experiment, or increased matrix synthesis as seen in the passive axial compression experiment. Consistent with this finding, although using immature bovine cartilage explants, Boustany et al.[194] found that static compression of <25% strain for 60 hours increased the biosynthetic rate of GAG and collagen production, although the mechanical properties of the explants were not examined.

Additional studies should be performed in the future to track construct development using electron microscopy in order to elucidate the mechanism leading to strut-like collagen organization, observed only in the 2-wk confined constructs. In addition, future studies should investigate the combination of

confinement with other modalities of mechanical stimulation such as hydrostatic pressure and direct compression, as the combination may result in synergistic effects on construct mechanical properties. Finally, future work should examine the effects of the addition of growth factors to the culture medium both before and after the application of confinement, in order to look for a synergistic effect between the growth factors and confinement.

In summary, this study permitted the examination of the hypothesis that the mechanical properties of self-assembled articular cartilage constructs are influenced by the application of stress in a direction orthogonal to the confinement surface. This study furthers our prior work involving the self-assembling process, by indicating that the ECM of self-assembled constructs may be modulated by both radial and vertical confinement. Also, this study provides evidence to support early (<2 wks) application of confinement and passive axial compression, and demonstrates the benefit of low-magnitude passive stress application.

Chapter 4: Effects of Temporal Hydrostatic Pressure on Tissue Engineered Bovine Articular Cartilage Constructs

Benjamin D. Elder and Kyriacos A. Athanasiou

Chapter accepted as: Elder, BD and Athanasiou, KA. Effects of Temporal Hydrostatic Pressure on Tissue Engineered Bovine Articular Cartilage Constructs. *Tissue Engineering Part A.* 2008

ABSTRACT

The objective of this study was to determine the effects of temporal hydrostatic pressure on the properties of scaffoldless bovine articular cartilage constructs. The study was organized in three phases: First, a suitable control for HP application was identified. Second, 10 MPa static HP was applied at three different timepoints (6-10 days, 10-14 days, and 14-18 days) to identify a window in construct development when HP application would be most beneficial. Third, the temporal effects of 10-14 days static HP application, as determined in phase II, were assessed at 2, 4, and 8 wks. Compressive and tensile mechanical properties, GAG and collagen content, histology for GAG and collagen, and immunohistochemistry for collagen types I and II were assessed. Using a culture control identified in phase I, in phase II HP application from 10-14 days resulted in a significant 1.4-fold increase in aggregate modulus, accompanied by an increase in GAG content, while HP application at all timepoints enhanced tensile properties and collagen content. In phase III, HP had an immediate effect on GAG content, collagen content, and compressive stiffness, while there was a delayed increase in tensile stiffness. The enhanced tensile stiffness was still present at 8 wks. For the first time, this study examined the immediate and long term effects of hydrostatic pressure on biomechanical properties, and demonstrated that HP has an optimal application time in construct development. These findings are exciting as HP stimulation allowed for the formation of robust tissue engineered cartilage; for example, 10 MPa static HP resulted in an

aggregate modulus of 273±123 kPa, a Young's modulus of 1.6±0.4 MPa, a GAG/wet weight of 6.1±1.4% and a collagen/wet weight of 10.6±2.4% at 4 wks.

INTRODUCTION

Cartilage is an avascular tissue and therefore has a limited intrinsic ability for repair following injury or osteoarthritis. Current treatments result in the formation of fibrocartilage, which is mechanically inferior to articular cartilage.[14] Due to the limitations of current therapies, tissue engineering has emerged as a promising approach for treating cartilage degeneration, as a result of injury or osteoarthritis.

Scaffoldless approaches for tissue engineering articular cartilage[36-38, 195] bypass several of the problems associated with scaffold use including stress shielding, biocompatibility, and biodegradation. In particular, the self-assembling process has allowed for significant increases in construct biochemical and biomechanical properties; however, these properties are still lacking in comparison to adult native tissue.[140] Therefore, the use of mechanical stimulation modalities such as hydrostatic pressure (HP) appears to be a promising approach for enhancing the biomechanical and biochemical properties of engineered constructs.

Articular cartilage is exposed to hydrostatic pressure *in vivo*, and efforts to stimulate chondrocytes with HP have focused on the physiological range of 3-18 MPa.[11, 121, 122, 129, 130, 132, 133, 135, 139, 196] It is believed that there are significant differences in the effects of static and intermittent HP. For example, several studies on human articular chondrocytes in monolayer have demonstrated that intermittent HP at 10 MPa, 1 Hz, results in increased aggrecan and collagen II mRNA,[128-130] while static HP was shown to have no effects on mRNA levels.[128]

In contrast, in other work using explants or immature chondrocytes in 3-D culture, the beneficial effects of static HP have been demonstrated. Hall et al.[102] demonstrated enhanced GAG production with static pressures in the physiological range, while both Hall et al.[102] and Lammi et al.[107] observed either no benefit or detrimental effects with pressures above the physiological range. In tissue engineered constructs with immature bovine chondrocytes, Toyoda et al.[137, 138] and Mizuno et al.[136] have also observed beneficial effects of static HP on GAG synthesis, aggrecan mRNA, and collagen type II mRNA. Finally, a recent study by our group compared the effects of 1, 5, and 10 MPa under static, 0.1 Hz, and 1 Hz conditions, and found that 10 MPa static HP significantly increased construct compressive and tensile properties, while 10 MPa, 1 Hz treatment only resulted in a significant increase in compressive properties.[139] Based on these results, 10 MPa static HP was selected for this study. Additionally, our own prior work has suggested 10-14 days to be a potentially suitable timeframe to apply mechanical stimulation,[140] while previous work by Ikenoue et al.[130] demonstrated that 4 days of HP application had a greater effect on aggrecan and collagen II mRNA than 1 day of HP application. Based on the results of these studies, a comparison among HP application times of 6-10, 10-14, and 14-18 days was made in this study.

Although several studies have been performed to assess the effects of HP on tissue engineered constructs, no studies have determined the optimal timepoint in construct development for the application of HP. Additionally, studies

assessing the immediate and delayed effects of HP on construct biomechanical properties are lacking. Therefore, the objective of this study was to determine when in construct development the biomechanical and biochemical properties were maximally sensitive to HP application. Furthermore, this study sought to examine the effects of HP on construct biochemical and biomechanical properties immediately after HP application, as well as up to 6 wks following HP application, in order to determine how long the beneficial effects of HP would last after removal of the stimulus. First, it was hypothesized that the bagging process used in applying HP would have no effect on construct properties. As prior studies involving mechanical stimulation demonstrated the benefit of application from 10-14 days, it was likewise hypothesized that HP would have an optimal application timepoint in construct development for the enhancement of construct biomechanical and biochemical properties. Finally, due to the slower turnover of collagen remodeling relative to GAG in the ECM, it was hypothesized that construct compressive properties would be increased immediately following HP application, while there would be a delayed increase in tensile properties. To test these hypotheses, three experiments were performed. First, 10 MPa static HP was applied to self-assembled constructs and compared to two different control groups. Second, 10 MPa static HP was applied to the constructs at three different times in construct development. Finally, the effects of HP application were assessed immediately at 2 wks, as well as at later timepoints of 4 wks and 8 wks.

MATERIALS AND METHODS

Chondrocyte Isolation and Seeding

Cartilage was obtained from the distal femur of wk-old male calves[93, 118, 181] (Research 87, Boston, MA) less than 36 hrs after slaughter, and was digested with collagenase type 2 (Worthington, Lakewood, NJ) to yield chondrocytes. To reduce variability among animals, each leg was obtained from a different animal, and cells from all legs were combined together to create a mixture of chondrocytes; a mixture of cells from at least 4 legs was used in each study. Cell number was assessed on a hemocytometer, and viability remained >90%, as determined by a trypan blue exclusion test. Chondrocytes were frozen in culture medium supplemented with 20% FBS (Biowhittaker, Walkersville, MD) and 10% DMSO at –80°C for 1-2 wks before use. Following thawing, viability remained greater than 85% in each study. A polysulfone mold consisting of 5 mm dia. x 10 mm long cylindrical prongs fit into 6 wells of a 48-well plate, and to construct each agarose well, sterile, molten 2% agarose was added to wells fitted with the polysulfone die. The agarose was allowed to gel at room temperature for 60 min, after which the mold was separated from the agarose. Culture medium was exchanged twice to completely saturate the agarose well with culture medium by the time of cell seeding. The medium was DMEM with 4.5 g/L-glucose and L-glutamine (Biowhittaker), 100 nM dexamethasone (Sigma, St. Louis, MO), 1% Fungizone/Penicillin/Streptomycin (Biowhittaker), 1% ITS+ (BD Scientific, Franklin Lakes, NJ), 50 μg/mL ascorbate-2-phosphate, 40 μg/mL L-proline, and

100 µg/mL sodium pyruvate (Fisher Scientific, Pittsburgh, PA). To each well, 5.5 x 10^6 cells were added in 125 µl of culture medium. The cells self-assembled within 24 hrs in the agarose wells and were maintained in the same well for a specified amount of time; t=0 was defined as 24 hrs after seeding.

Preparation for Specimen Pressurization

Both bagged control (BC) and HP constructs were loaded into heat sealable bags (Kapak/Ampak Flexibles, Cincinnati, OH) previously sterilized by ethylene oxide. To each bag, 40 ml medium was added, and any air bubbles adhering to the bottom of the bag were released. The bags were then heat-sealed without any bubbles inside.

Specimen Pressurization

BC specimens were placed into an opened pressure chamber maintained at 37°C, while pressure specimens were placed into a pressure chamber (Parr Instrument, Moline, IL), filled with water, and sealed underwater without any bubbles inside. The pressure chamber used has been described previously.[135] Briefly, for 5 consecutive days, the specimens were pressurized to 10 MPa static HP for 1 h. After the execution of the desired regimen, the pressure chamber was disassembled, and the pouches were sterilized with 70% ethanol. In a sterile culture hood, the pouches were opened with autoclaved scissors and the samples were returned to agarose coated wells of 6-well culture plates.

Phase I: Selection of HP Control

At 10 days, self-assembled constructs (n=6/group) were removed from confinement in 5 mm dia. agarose wells and exposed to 10 MPa static HP for 1 h/day, for 5 days. The constructs were then placed in one well of a 6-well culture plate coated with 2% agarose for the remainder of the study. A bagged control consisted of constructs removed from confinement in 5 mm dia. agarose wells at 10 days, and placed in the HP chamber for 1 h/day, for 5 days, but unpressurized. The constructs were then placed in one well of a 6-well culture plate coated with 2% agarose for the remainder of the study. A culture control (CC) consisted of constructs removed from confinement in 5 mm dia. agarose wells at 10 days, and cultured in one well of a 6-well culture plate coated with 2% agarose for the remainder of the study. 500 μl of medium per construct was changed daily, and all constructs were assessed at 4 wks.

Phase II: Temporal Effects of HP Application

At 6 days, 10 days, or 14 days, self-assembled constructs (n=6/group) were removed from confinement in 5 mm dia. agarose wells, and placed in one well of a 6-well culture plate coated with 2% agarose. The constructs unconfined at 6 days were exposed to 10 MPa static HP, 1 h/day, from 6-10 days, and were cultured unconfined in the 6-well plate for the remainder of the study. The 6-day culture control group (CC 6) remained unconfined in culture from 6 days until the conclusion of the study. The constructs unconfined at 10 days were exposed to

10 MPa static HP, 1 h/day, from 10-14 days, and were cultured unconfined in the 6-well plate for the remainder of the study. The 10-day culture control group (CC 10) remained unconfined in culture from 10 days until the conclusion of the study. The constructs unconfined at 14 days were exposed to 10 MPa static HP, 1 h/day, from 14-18 days, and were cultured unconfined for the remainder of the study. The 14-day culture control group (CC 14) remained unconfined in culture from 14 days until the conclusion of the study. 500 μl of medium per construct was changed daily, and all constructs were assessed at 4 wks.

Phase III: Short-term and Long-term Effects of HP Application.

At 10 days, constructs were removed from confinement, and 10 MPa static HP was applied for 1 h/day, from 10-14 days. A CC was treated as in phase I. Both HP and CC constructs (n=6/group) were assessed at 2 wks, 4 wks, and 8 wks.

Histology and Immunohistochemistry

Samples were frozen and sectioned at 14 μm. GAG distribution was examined with a safranin-O/fast green stain.[182, 183] To examine collagen content, picrosirius red was used. Slides were also processed with IHC to test for the presence of collagen types I and II on a Biogenex (San Ramon, CA) i6000 autostainer. Following fixation in chilled acetone, the slides were washed with IHC buffer (Biogenex), quenched of peroxidase activity with hydrogen peroxide/methanol, and blocked with horse serum (Vectastain ABC kit, Vector Laboratories, Burlingame, CA). The slides were then incubated with either mouse anti-

collagen type I (Accurate Chemicals, Westbury, NY) or rabbit anti-collagen type II (Cedarlane Labs, Burlington, NC) antibodies. Secondary antibody (anti-mouse or anti-rabbit IgG, Vectastain ABC kit) was applied, and color was developed using the Vectastain ABC reagent and DAB (Vectastain kit).

Quantitative Biochemistry

Samples were frozen overnight and lyophilized for 72 hrs, followed by re-suspension in 0.8 mL of 0.05 M acetic acid with 0.5 M NaCl and 0.1 mL of a 10 mg/mL pepsin solution (Sigma) at 4°C for 72 hrs. Next, 0.1 mL of 10x TBS was added along with 0.1 mL pancreatic elastase and mixed at 4°C overnight. From this digest, total DNA content was measured by Picogreen® Cell Proliferation Assay Kit (Molecular Probes, Eugene, OR). Total sulfated GAG was then quantified using the Blyscan Glycosaminoglycan Assay kit (Biocolor), based on 1,9-dimethylmethylene blue binding.[184, 185] After being hydrolyzed by 2 N NaOH for 20 min at 110°C, samples were assayed for total collagen content by a chloramine-T hydroxyproline assay.[186]

Indentation Testing

Samples were evaluated with an automated indentation apparatus.[187] A step mass of 0.7 g (0.007 N) was applied with a 1 mm flat-ended, porous indenter tip, and specimens were allowed to creep until equilibrium, as described elsewhere.[36] Preliminary estimations of the aggregate modulus of the samples were obtained using the analytical solution for the axisymmetric Boussinesq

problem with Papkovich potential functions.[188, 189] The intrinsic mechanical properties of the samples, including aggregate modulus, Poisson's ratio, and permeability were then determined using the linear biphasic theory.[3]

Tensile Testing

Tensile tests were performed using a uniaxial materials testing system (Instron Model 5565, Canton, MA) with a 50 N load cell as described previously.[197] Briefly, samples were cut into a dog-bone shape with a 1-mm-long gauge length. Samples were attached to paper tabs for gripping with cyanoacrylate glue outside of the gauge length. The 1-mm-long sections were pulled at a constant strain rate of 0.01 s^{-1}. All samples broke within the gauge length. Stress-strain curves were created from the load-displacement curve and the cross-sectional area of each sample, and Young's modulus was calculated from each stress-strain curve.

Statistical Analysis

All samples were assessed biochemically and biomechanically (n=6). For phase I, a single factor ANOVA was used to analyze the samples, and a Fisher LSD *post hoc* test was used when warranted. For phase II, a two-factor ANOVA was used to analyze the samples, and a Fisher LSD *post hoc* test was used when warranted. For phase III, a student's t test was used to compare the two groups at each timepoint. Significance was defined as $p < 0.05$.

RESULTS

Gross Appearance and Histology

In all studies, the construct diameter slightly exceeded 6 mm at 4 wks (Fig. 11a). In phase I, there were no differences in thickness among the HP, BC, and CC groups, with values of 0.46±0.06, 0.41±0.03, and 0.43±0.06 mm respectively (Fig. 11b). In phase II, there were no differences in thickness among the different groups, with values of 0.51±0.03 and 0.50±0.07 mm for the HP 6-10 and CC 6 groups, 0.52±0.06 and 0.48±0.07 mm for the HP 10-14 and CC 10 groups, and 0.54±0.06 and 0.51±0.09 mm for the HP 14-18 and CC 14 groups, respectively. In phase III, there were no differences in thickness among the groups, with values of 0.42±0.03 and 0.41±0.02 at 2 wks, 0.70±0.07 and 0.67±0.05 at 4 wks, and 0.93±0.14 and 0.85±0.16 mm at 8 wks for the CC and HP groups, respectively.

In each phase, all constructs stained positive for collagen throughout their thickness (Fig. 11c). Based on Safranin-O staining, GAG production was observed throughout the constructs (Fig. 11d). Based on IHC, collagen II was expressed throughout each construct (Fig. 11e), while there was no collagen I production (Fig. 11f).

Quantitative Biochemistry

In phase I, there were no differences in WW/construct or DNA/construct among the different treatment groups. The HP, BC, and CC groups had WW/construct values of 11.6±1.9, 12.2±0.6, and 12.0±1.9 mg, and DNA/construct values of 39.9±8.9, 41.0±11.0, and 36.9±11.4 μg, respectively. The HP group had a significantly higher GAG/WW than either the BC or CC groups, with values of 6.1±1.4, 4.4±0.5, and 4.1±0.1%, respectively (Fig. 12c). The HP treated group had a significantly higher collagen/WW than either the BC or CC groups, with values of 10.6±2.4, 6.2±1.9 and 6.7±0.6%, respectively (Fig. 12d).

In phase II, there were no differences in WW/construct or DNA/construct among the different treatment groups. The WW/construct values were 10.7±1.3 and 12.2±1.5 mg for the HP 6-10 and CC 6 groups, 11.1±1.2 and 11.0±1.5 mg for the HP 10-14 and CC 10 groups, and 11.9±1.7 and 11.0±1.6 mg for the HP 14-18 and CC 14 groups, respectively. The DNA/construct values were 34.3±7.5 and 32.3±1.4 μg for the HP 6-10 and CC 6 groups, 43.6±8.6 and 32.7±7.6 μg for the HP 10-14 and CC 10 groups, and 44.6±10.5 and 43.6±14.2 μg for the HP 14-18 and CC 14 groups, respectively. HP was a significant factor for GAG/WW and collagen/WW. HP application from 6-10, 10-14, and 14-18 days increased GAG/WW from 3.9±1.4 to 4.5±0.4%, 3.5±0.9 to 4.8±0.6%, and 4.3±0.7 to 5.1±0.8%, respectively (Fig. 13c). HP application from 6-10, 10-14, and 14-18 days increased collagen/WW from 7.4±1.7 to 10.4±1.7%, 8.0±0.9 to 10.8±1.2%, and 8.5±1.1 to 9.4±1.5%, respectively (Fig. 13d).

In phase III, the WW/construct values were 10.0±1.2 and 7.7±0.3 mg at 2 wks, 17.1±1.7 and 14.4±2.2 mg at 4 wks, and 28.5±5.6 and 27.7±3.9 mg at 8 wks for the CC and HP groups, respectively. There were no differences in cellularity at each time point with DNA/construct values of 35.7±4.7 and 34.0±3.8 μg at 2 wks, 37.1±7.3 and 34.3±2.0 μg at 4 wks, and 33.5±3.3 and 30.2±3.0 μg at 8 wks for the CC and HP groups, respectively. HP significantly increased GAG/WW from 4.4±0.9 to 5.7±1.3% at 2 wks, and from 6.2±0.3 to 8.1±0.4% at 4 wks. The GAG/WW was 7.8±0.6 and 8.5±0.7% for the CC and HP groups at 8 wks (Fig. 14c). HP significantly increased collagen/WW from 7.4±2.5 to 12.2±0.3% at 2 wks, and from 7.1±1.8 to 10.8±1.9% at 4 wks. The collagen/WW was 8.6±1.6 and 7.4±1.5% for the CC and HP groups at 8 wks (Fig. 14d).

Mechanical Evaluation

In phase I, the HP treated group had a significantly higher aggregate modulus than the BC or CC groups, with values of 273±123, 134±45, and 116±19 kPa, respectively (Fig. 12a). There were no differences among the groups in Poisson's ratio or permeability, with ranges of 0.14-0.19 and 3.94×10^{-14}-9.78×10^{-14}, respectively. Additionally, the HP treated group had a significantly higher Young's modulus than the BC or CC groups, with values of 1.6±0.4, 1.0±0.3, and 0.9±0.1 MPa, respectively (Fig. 12b).

In phase II, HP was a significant factor for aggregate modulus and Young's modulus. HP application from 10-14 days led to a significant increase in aggregate modulus from 101±32 to 238±131 kPa. HP application from 6-10 days increased aggregate modulus from 97±24 to 159±52 kPa, and HP application from 14-18 days decreased aggregate modulus slightly from 195±64 to 177±68 kPa (Fig. 13a). HP application did not significantly change Poisson's ratio from control for any group, with a range of 0.04-0.22. Additionally, there were no differences in permeability among the groups, with a range of $2.83x10^{-15}$-$2.04x10^{-13}$. HP application from 6-10, 10-14, and 14-18 days increased Young's modulus from 0.9±0.1 to 1.3±0.1 MPa, 0.9±0.2 to 1.4±0.3 MPa, and 0.8±0.2 to 1.3±0.2 MPa, respectively (Fig. 13b).

In phase III, HP significantly increased aggregate modulus from 113±16 to 158±28 kPa at 2 wks, and from 138±30 to 270±46 kPa at 4 wks. There was no difference at 8 wks, with values of 257±51 and 296±68 kPa for the CC and HP groups, respectively (Fig. 14a). There were no differences among the groups in Poisson's ratio or permeability, with ranges of 0.18-0.26 and $2.26x10^{-14}$-$6.78x10^{-14}$. There was no difference in Young's modulus at 2 wks, with values of 373±182 and 476±228 kPa for the CC and HP groups, respectively. HP significantly increased Young's modulus from 596±185 to 1144±281 kPa at 4 wks, and from 912±131 to 1404±442 kPa at 8 wks (Fig. 14d).

DISCUSSION

This study utilized a 3-phase approach to choose an appropriate control group for HP application, to determine the effects of temporal HP application, and to assess the temporal effects following HP application. To the best of our knowledge, this study is the first to assess the effects of HP application at different timepoints in construct development, and the first to examine short-term and long-term changes in construct properties following HP application.

In phase I, HP application significantly increased construct biomechanical and biochemical properties relative to both control groups, and the bagging process had no effect on construct properties. The application of 10 MPa static HP for 1 hr/day, from days 10-14 led to a 120% increase in aggregate modulus and a 60% increase in Young's modulus, accompanied by significant increases in GAG and collagen content, respectively. Additionally, there were no differences in biomechanical, biochemical, histological, or gross morphological properties between the BC and CC groups. These results support our hypotheses, as HP application led to a significant increase in both compressive and tensile properties, and the bagging process inherent to HP application was shown to have no effect on construct biomechanical and biochemical properties. A comparison between the BC and CC groups was necessary to determine the effects of the bagging and handling process requisite for HP stimulation. In our setup, it is impossible to apply HP under sterile conditions without handling and bagging the constructs. As the handling and bagging is inherent to HP

application, we consider HP application to include these inherent steps. Therefore, the CC group was selected for use in subsequent phases as it allows us to compare HP application in its entirety (including the handling and bagging process) to a control.

In phase II, it was determined that 10-14 days was the optimal time in construct development for HP application. HP application at all timepoints led to similar increases in tensile properties; however, HP application from 10-14 days had the greatest effect on aggregate modulus, a 140% increase. These results support our hypothesis, as HP application at a certain timepoint in construct development had the most beneficial effect on construct biomechanical properties. A 40% increase in GAG/WW accompanied the increased aggregate modulus of the 10-14 day HP group. On the other hand, HP application at all timepoints led to an approximately 0.5-fold increase in Young's modulus, accompanied by increases in collagen/WW. These results are interesting as they suggest that there may be different mechanisms for the effects of HP on compressive and tensile properties. Additionally, these results correlate with a prior study on self-assembled constructs that suggested that 10-14 days of construct development may be an important window for mechanical intervention.[140]

In phase III, HP application had immediate and delayed effects on construct properties. Application of static HP at 10 MPa for 1 hr/day significantly increased compressive properties, GAG/WW, and collagen/WW immediately after the 5

days of HP application at 2 wks, but the significant increase in tensile properties observed in the prior phases was delayed until 4 wks. These results support our hypotheses, as the aggregate modulus was enhanced immediately after 10-14 days of HP application, while there was a delayed increase in tensile properties. This result was expected due to the slower turnover of collagen remodeling relative to GAG in the ECM. Since collagen content was quantified with a hydroxyproline assay, it is also possible that the measured collagen at 2 wks was pro-collagen or immature collagen, which was not fully cross-linked or organized in the ECM until the next measurement at 4 wks. Additionally, by 8 wks, construct biomechanical and biochemical properties appear to level off, although a significant difference in tensile stiffness remains, likely as a result of the initial matrix formation present at the 4 wk timepoint.

The results of these studies correlate with those of previous studies involving the use of static HP in physiologic magnitude ranges. For instance, Mizuno et al.[136] found that the application of 2.8 MPa static HP to 3-D collagen sponges seeded with bovine articular chondrocytes led to a 3.1-fold increase in [(35)S]-sulfate incorporation in GAG. Additionally, Smith et al.[128] observed a 32% increase in GAG synthesis with 10 MPa static HP application to high density cultures of adult bovine articular chondrocytes. Likewise, Toyoda et al.[138] found that 5 MPa static HP, applied to bovine articular chondrocytes cultured in agarose gels, significantly increased GAG synthesis and increased levels of aggrecan mRNA 4-fold, while in a separate study, a 50% increase in the level of type II collagen

mRNA was recorded with this same regimen.[137] These results mirror the biochemical findings of the currently presented studies, as significant increases in both collagen content and GAG content were observed, that presumably led to significant increases in both compressive and tensile biomechanical properties.

Since HP application does not lead to cartilage deformation,[162] it is difficult to envisage a mechanism to explain the beneficial effects of HP on construct biomechanical properties. However, as reviewed elsewhere,[169] HP can deform the void spaces of cell transmembrane proteins, and at a certain pressure, the void space deformation leads to a change in protein conformation. This conformation change likely occurs in cell surface ion channels that act as "pressure sensors," theoretically occurring over the pressures at which we see effects. For example, in chondrocytes, the Na/K pump and Na/K/2Cl transporter were shown to be sensitive to 10 MPa static HP application.[165] Additionally, the Na/H pump[166] and stretch-activated calcium channels[167] in articular chondrocytes are affected by HP application. As ion concentration changes have been shown to alter protein synthesis,[170] different ion channel responses to HP likely stimulate signal transduction cascades that eventually lead to upregulation of ECM-specific genes. The increased gene expression likely leads to increased ECM protein production, eventually resulting in enhanced biomechanical properties as observed in this study. Alternatively, although water has a high bulk modulus, it is not incompressible. Therefore, it is possible that 10 MPa HP results in stimulation of additional mechanotransduction pathways as a result of the

compressibility of water, as this may result in small strains on the cells without a measurable construct deformation.

Although several studies have examined the effects of various HP regimens on construct gene expression and protein production, to our knowledge, this is the first study to assess the effects of temporal HP application, as well as the first to examine the immediate and long-term effects of HP on construct biomechanical and biochemical properties. Future studies should determine if combining HP with other mechanical stimulation, such as direct compression or shear, leads to additive or synergistic effects.

Chapter 5: Systematic Assessment of Growth Factor Treatment on Biochemical and Biomechanical Properties of Engineered Articular Cartilage Constructs

Benjamin D. Elder and Kyriacos A. Athanasiou

Chapter accepted as: Elder, BD and Athanasiou, KA. Systematic Assessment of Growth Factor Treatment on Biochemical and Biomechanical Properties of Engineered Articular Cartilage Constructs. *Osteoarthritis and Cartilage.* 2008

ABSTRACT

The objective of this study was to determine the effects of bone morphogenetic protein-2 (BMP-2), insulin-like growth factor (IGF-I), and transforming growth factor-β1 (TGF-β1) on the biochemical and biomechanical properties of engineered articular cartilage constructs under serum free conditions. A scaffoldless approach for tissue engineering, the self-assembly process, was employed. The study consisted of two phases. In the first phase, the effects of BMP-2, IGF-I, and TGF-β1, at two concentrations and two dosage frequencies each were assessed on construct biochemical and biomechanical properties. In phase II, the effects of growth factor combination treatments were determined. Compressive and tensile mechanical properties, glycosaminoglycan (GAG) and collagen content, histology for GAG and collagen, and immunohistochemistry (IHC) for collagen types I and II were assessed. In phase I, BMP-2 and IGF-I treatment resulted in significant, >1-fold increases in aggregate modulus, accompanied by increases in GAG production. Additionally, TGF-β1 treatment resulted in significant, ~1-fold increases in both aggregate modulus and tensile modulus, with corresponding increases in GAG and collagen content. In phase II, combined treatment with BMP-2 and IGF-I increased aggregate modulus and GAG content further than either growth factor alone, while TGF-β1 treatment alone remained the only treatment to also enhance tensile properties and collagen content. This study determined systematically the effects of multiple growth factor treatments under serum-free conditions, and is the first to demonstrate significant increases in both compressive and tensile biomechanical

properties as a result of growth factor treatment. These findings are exciting as coupling growth factor application with the self-assembly process resulted in tissue engineered constructs with functional properties approaching native cartilage values.

INTRODUCTION

Articular cartilage has a limited ability for self-repair, and injuries to articular cartilage result in the formation of mechanically inferior fibrocartilage.[14] Since current clinical treatments are limited, tissue engineering is a promising strategy for articular cartilage regeneration.

To alleviate some of the potential issues associated with scaffold use, our lab has developed and employed a scaffoldless process for tissue engineering, called the self-assembly process.[36, 135, 140] Using this process, the goal is to create engineered constructs with biochemical and biomechanical properties approaching those of native tissue. Growth factor application appears to be a promising approach for enhancing these properties.

Previous studies[74, 198] systematically assessed the effects of several growth factors at different concentrations on chondrocyte-seeded PGA scaffolds, and indicated that treatment with BMP-2 and IGF-I enhanced GAG production, while TGF-β1 enhanced collagen production. However, these studies employed fetal bovine serum (FBS) in the medium, potentially confounding the effects of exogenous growth factor application. Also, a prior study by Ng et al.[199] indicated beneficial effects from temporal application of IGF-I and TGF-β1.

Although many studies have demonstrated beneficial effects of growth factor application, no studies have systematically assessed the effects of growth factors

alone and in combination under serum-free conditions. Furthermore, no studies have examined growth factor effects on both compressive and tensile properties. The objective of this study was to determine the effects of growth factor application on the biomechanical and biochemical properties of self-assembled articular cartilage constructs. This study utilized a 2-phase approach to determine the effects of single growth factor treatments followed by the determination of the effects of combined growth factor treatments. Based on the results of prior studies,[74, 80, 150, 198, 200] in phase I, it was hypothesized that BMP-2 and IGF-I treatment would enhance compressive properties by increasing GAG production, and TGF-β1 treatment would enhance both compressive and tensile properties by increasing GAG production and collagen production respectively. It was further hypothesized that growth factor concentration and dosage frequency would have significant effects on construct biochemical and biomechanical properties, based on prior work.[201, 202] In phase II, it was hypothesized that combined growth factor treatment would have beneficial effects on construct properties, by increasing biochemical and biomechanical properties further than any growth factor alone. To test these hypotheses, three experiments were performed in phase I and one experiment was performed in phase II. In phase I, BMP-2, IGF-I, and TGF-β1 were all assessed at two concentrations and two dosage frequencies each, with the best treatment for each growth factor selected for use in phase II. In phase II, the growth factor treatments selected from phase I were assessed in combinations of two and three.

METHODS

Chondrocyte Isolation and Seeding

Chondrocytes were obtained from the distal femur of wk-old male calves[93, 118, 181] (Research 87, Boston, MA), and digested with collagenase type 2 (Worthington, Lakewood, NJ). Each leg yielded roughly 150 million chondrocytes, and animal variability was reduced by pooling cells from five legs of different animals to yield a mixture of chondrocytes for each study (see descriptions below). The pooled cells were counted on a hemocytometer, and viability >90% was found using a trypan blue exclusion test. Chondrocytes were frozen in culture medium supplemented with 20% FBS (Biowhittaker) and 10% DMSO at –80°C for 3 days before use in phase I, and for 3 wks before use in phase II. After thawing, viability remained greater than 85%. A polysulfone die consisting of 5 mm dia. x 10 mm long cylindrical prongs was used to construct each agarose mold. Sterile, molten 2% agarose was introduced into a well fitted with the polysulfone die. The agarose was allowed to gel at room temperature for 60 min, and two exchanges of culture medium were used to completely saturate the agarose well with culture medium by the time of cell seeding. To each well, 5.5 x 10^6 cells in 100 µl of culture medium were added. The cells self-assembled within 24 hrs in the agarose wells and were maintained in the same well for t=10 days; t=0 was defined as 24 hrs after seeding. The culture medium was DMEM with 4.5 g/L-glucose and L-glutamine (Biowhittaker/Cambrex, Walkersville, MD), 100 nM dexamethasone (Sigma, St. Louis, MO), 1% Fungizone/Penicillin/Streptomycin (Biowhittaker), 1% ITS+ (BD Scientific, Franklin Lakes, NJ), 50 µg/mL ascorbate-

2-phosphate, 40 μg/mL L-proline, and 100 μg/mL sodium pyruvate (Fisher Scientific, Pittsburgh, PA).

Phase I: Individual Growth Factor Effects

This phase included three separate studies to assess the individual effects of BMP-2, IGF-I and TGF-β1 at different concentrations and dosage frequencies. All growth factors were obtained from Peprotech Inc. (Rocky Hill, NJ), and were applied in the culture medium. For each growth factor, the effects of two concentrations (low and high) and two dosage frequencies were assessed, with separate no growth factor controls for each study, yielding a total of five treatment groups for each growth factor study (Fig. 15). The concentrations used were 10 and 100 ng/ml for BMP-2 and IGF-I, and 10 and 30 ng/ml for TGF-β1, selected from prior studies.[74, 198] The dosage regimens were 2 wks continuous application followed by 2 wks of no growth factor (continuous), or growth factor application only during the 1st and 3rd wk of culture (wk rotation), which were chosen based on pilot studies and current ongoing work in our group as well as adapted from prior studies using intermittent growth factor application by Lieb et al.[201, 202]

For all studies, at t=10 days, self-assembled constructs (n=6/group) were removed from confinement in 5 mm dia. agarose wells and transferred to individual 2% agarose coated wells of a 48-well culture plate for the remainder of the study. Per construct, 500 μl of medium was changed daily, and all constructs

were assessed at t=4 wks. The "best" treatment for each growth factor was selected, using a functionality index as described below, for use in phase II.

Phase II: Growth Factor Combination Effects

One treatment for each growth factor was selected from phase I to be compared individually, as well as in combinations of two and three in phase II (Fig. 15). The specific application treatments selected were 10 ng/ml continuous BMP-2, 10 ng/ml wk rotation IGF-I, and 30 ng/ml continuous TGF-β1. As in phase I, constructs were unconfined from agarose wells at t=10 days, and transferred to individual 2% agarose coated wells of a 48-well culture plate for the remainder of the study. Again, 500 μl of medium per construct was changed daily, and all constructs were assessed at t=4 wks.

Histology and Immunohistochemistry

Samples were frozen and sectioned at 14 μm. Safranin-O and fast green staining were used to examine GAG distribution.[182, 183] Picrosirius red was used for qualitative examination of collagen content. A von Kossa stain was used to assess for mineralization. Slides were also processed with IHC to test for the presence of collagen types I, II, and X. After fixing in chilled acetone, the slides were rinsed with IHC buffer (Biogenex), quenched of peroxidase activity with hydrogen peroxide/methanol, and blocked with horse serum (Vectastain ABC kit, Vector Laboratories, Burlingame, CA). The slides were then incubated with either mouse anti-collagen type I (Accurate Chemicals, Westbury, NY), rabbit

anti-collagen type II (Cedarlane Labs, Burlington, NC), or rabbit anti-collagen X (Abcam Inc., Cambridge, MA) antibodies. The secondary antibody (anti-mouse or anti-rabbit IgG, Vectastain ABC kit) was applied, and color was developed using the Vectastain ABC reagent and DAB (Vectastain kit).

Quantitative Biochemistry

Samples were frozen overnight and lyophilized for 72 hrs, followed by re-suspension in 0.8 mL of 0.05 M acetic acid with 0.5 M NaCl and 0.1 mL of a 10 mg/mL pepsin solution (Sigma) at 4°C for 72 hrs. Next, 0.1 mL of 10x TBS was added along with 0.1 mL pancreatic elastase and mixed at 4°C overnight. From this digest, total DNA content was measured by Picogreen® Cell Proliferation Assay Kit (Molecular Probes, Eugene, OR). Total sulfated GAG was then quantified using the Blyscan Glycosaminoglycan Assay kit (Biocolor), based on 1,9-dimethylmethylene blue binding.[184, 185] After being hydrolyzed by 2 N NaOH for 20 min at 110°C, samples were assayed for total collagen content by a chloramine-T hydroxyproline assay.[186]

Indentation Testing

Samples were evaluated with an indentation apparatus.[187] A step mass of 0.7 g (0.007 N) was applied with a 1 mm flat-ended, porous indenter tip, and specimens were allowed to creep until equilibrium, as described elsewhere.[36] Preliminary estimations of the aggregate modulus of the samples were obtained using the analytical solution for the axisymmetric Boussinesq problem with

Papkovich potential functions.[188, 189] The aggregate modulus (H_A), permeability, and Poisson's ratio of the samples were then determined using the linear biphasic theory.[3]

Tensile Testing

Tensile tests were performed using a uniaxial materials testing system (Instron Model 5565, Canton, MA) with a 50 N load cell as described previously.[197] Briefly, samples were cut into a dog-bone shape with a 1-mm-long gauge length. Samples were attached to paper tabs for gripping with cyanoacrylate glue outside of the gauge length. The 1-mm-long sections were pulled at a constant strain rate of 0.01 s^{-1}. Stress-strain curves were created from the load-displacement curve and the cross-sectional area of each sample, and Young's modulus (E_Y) was calculated from the linear region of each stress-strain curve.

Functionality Index (FI)

A functionality index (Eq. 1) was used to determine the "best" treatment condition for each growth factor in phase I, for use in phase II. The index was only used as a selection tool within each experiment, without making comparisons among experiments. It was weighted using normalized collagen and GAG content, tensile stiffness, and creep indentation compressive stiffness. The index served as a quantified comparison between the properties of the engineered constructs and native tissue. In the functionality index, G represents GAG/WW, C represents collagen/WW, E^T represents tensile modulus, and E^C represents

compressive stiffness (aggregate modulus). The subscripts nat and sac are used to denote native and self-assembled construct values, respectively. Using immature bovine cartilage explants, native tissue values were 5% and 15% for GAG/WW and collagen/WW respectively, and 213 kPa and 12.1 MPa for E^C and E^T respectively. Although different weights may be afforded to each component of the FI, they are equally weighted in this study. Since the eventual goal of our tissue engineering approach is *in vivo* construct implantation, as cartilage experiences both compressive and tensile loading in the joint, these properties are equally weighted. Furthermore, the biochemical characteristics are equally important as constructs with biochemical characteristics divergent from native tissue may present problems in construct integration with native tissue. However, due to the flexibility of the FI, the exact weights can easily be modified based on the results of future studies.

$$FI = \frac{1}{4}\left(\left(1-\frac{(G_{nat}-G_{sac})}{G_{nat}}\right)+\left(1-\frac{(C_{nat}-C_{sac})}{C_{nat}}\right)+\left(1-\frac{(E^T_{nat}-E^T_{sac})}{E^T_{nat}}\right)+\left(1-\frac{(E^C_{nat}-E^C_{sac})}{E^C_{nat}}\right)\right) \quad (1)$$

Statistical Analysis

All samples were assessed biochemically and biomechanically (n=6 or 7). In each phase, a single factor ANOVA was used to analyze the samples, and a Fisher LSD *post hoc* test was used when warranted. Significance was defined as $p < 0.05$.

RESULTS

Gross Appearance and Histology

Construct diameter was approximately 6 mm in all studies. In phase I, BMP-2 at all concentrations and dosages increased construct wet weight (WW) and thickness slightly, as demonstrated in Table I. IGF-I treatment led to a slightly decreased construct WW, with no differences in construct thickness, as shown in Table II. Finally, treatment with TGF-β1 resulted in a concentration dependent decrease in construct WW and thickness, as indicated in Table III. In phase II, there were no differences in construct WW or thickness among any of the treatment groups (Table IV). In all studies, constructs stained positive for collagen and GAG throughout their thickness (Fig. 16), and based on IHC, collagen II was expressed throughout each construct, with no collagen I production. Similar images can be observed in our previous work.[140] Additionally, no constructs demonstrated mineralization and no chondrocyte hypertrophy was noted with BMP-2 treatment.

Quantitative Biochemistry

In phase I, there were no differences in cells/construct among the different treatment groups in the BMP-2 study (Table I). Treatment with 10 ng/ml continuous BMP-2 led to the greatest increase in GAG/WW, although all BMP-2 treatments significantly increased GAG/WW (Fig. 17c). There were no differences in collagen/WW among any of the treatment groups (Fig. 17d). In the IGF-I study, there were no differences in cells/construct among any of the

treatment groups (Table II). All IGF-I treatments significantly increased GAG/WW, with the exception of 10 ng/ml continuous treatment (Fig. 18c). There were no differences in collagen/WW among any of the treatment groups (Fig. 18d). In the TGF-β1 study, 30 ng/ml treatment led to an approximately 14% increase in cells/construct (Table III). Additionally, 30 ng/ml TGF-β1, at either continuous or 2 wk rotation dosages, significantly increased both collagen/WW and GAG/WW (Figs. 19c and 19d).

In phase II, there were no differences in cells/construct among any of the treatment groups (Table IV). All growth factor treatments significantly increased GAG/WW, although combined BMP-2 and IGF-I treatment led to the greatest increase in GAG/WW (Fig. 20c). However, both treatment with TGF-β1 alone and combined application of all three growth factors significantly increased collagen/WW (Fig. 20d).

Mechanical Evaluation

In phase I, all BMP-2 treatments significantly increased aggregate modulus, although BMP-2 at 10 ng/ml continuous application led to the greatest increase (Fig. 16a). There were no differences in Poisson's ratio or permeability noted among the different groups, with ranges of 0.15-0.28 and 4.1×10^{-14}-1.2×10^{-13}, respectively. Furthermore, there were no differences in Young's modulus among any of the treatment groups in the BMP-2 study (Fig. 16b). In the IGF-I study, all IGF-I treatments except for 10 ng/ml continuous significantly increased

aggregate modulus, while application at 10 ng/ml wk rotation led to the greatest increase in aggregate modulus (Fig. 17a). There were no differences in Poisson's ratio or permeability noted among the different groups, with ranges of 0.19-0.26 and $8.0x10^{-14}$-$1.2x10^{-13}$, respectively. Additionally, there were no differences in Young's modulus among any of the treatment groups in the IGF-I study (Fig. 18b). In the TGF-β1 study, only 30 ng/ml continuous treatment significantly increased aggregate modulus (Fig. 19a). However, both TGF-β1 treatments at 30 ng/ml exhibited a significant increase in Young's modulus (Fig. 19b). There were no differences among the treatment groups for Poisson's ratio and permeability, with ranges of 0.09-0.22 and $2.3x10^{-14}$-$7.2x10^{-14}$, respectively.

In phase II, all three individual growth factor treatments significantly increased aggregate modulus (Fig. 20a), replicating the results of phase I. However, combined BMP-2 and IGF-I treatment led to the greatest enhancement of aggregate modulus. Only individual application of TGF-β1 significantly increased Young's modulus (Fig. 20b). There were no differences in Poisson's ratio or permeability among the treatment groups, with ranges of 0.09-0.26 and $5.1x10^{-14}$-$1.3x10^{-13}$, respectively.

DISCUSSION

The objective of this study was to assess systematically the effects of growth factors on the biochemical and biomechanical properties of self-assembled articular cartilage constructs. The study utilized a 2-phase approach to determine the effects of different growth factors, concentrations, and dosage frequencies, as well as to examine the effects of growth factor combination treatment. This approach allowed for a methodical growth factor examination under serum-free conditions. To the best of our knowledge, this study is the first to demonstrate significant increases in both compressive and tensile biomechanical properties as a result of growth factor treatment.

In phase I, all BMP-2 treatments led to significant increases in construct compressive stiffness and GAG/WW. The greatest enhancement was observed with 2 wk continuous treatment at 10 ng/ml, resulting in a 104% increase in compressive stiffness. Despite the increased compressive properties, no increases in tensile properties or collagen/WW were noted for any of the treatment groups. These results supported our hypothesis that BMP-2 would only increase the compressive properties of the constructs by increasing the GAG/WW, as increased GAG production without changes in collagen synthesis has previously been observed with BMP-2 treatment.[198, 203] BMP-2 treatment of 2 wk continuous dosage at 10 ng/ml was selected for use in phase II as it demonstrated the greatest increase in the functionality index.

Similarly, in phase I, all IGF-I treatments except for 10 ng/ml continuous application significantly increased construct compressive stiffness and GAG/WW. However, the greatest increase was observed with the wk rotation dosage at 10 ng/ml, with a 122% increase in compressive stiffness. As with BMP-2 treatment, no increases in tensile properties or collagen/WW were observed for any of the treatment groups. These results supported our hypothesis that IGF-I would increase only the compressive properties of the constructs by increasing the GAG/WW, as previous studies demonstrated enhanced GAG production without changes in collagen synthesis from IGF-I treatment in both tissue engineered constructs and explants.[74, 204, 205] IGF-I treatment of wk rotation dosage at 10 ng/ml was selected for use in phase II as it demonstrated the greatest increase in the functionality index.

Finally, in phase I, 30 ng/ml TGF-β1 treatment, at either dosage frequency, significantly increased tensile stiffness and collagen/WW, as well as GAG/WW. However, only 30 ng/ml TGF-β1 treatment at the 2 wk continuous dosage significantly increased compressive stiffness. These results demonstrate that the enhancement of compressive properties likely requires a lag period, as suggested previously,[199] following TGF-β1 treatment; both 30 ng/ml treatments increased GAG/WW, but only the 2 wk continuous application, with 2 wks between cessation of growth factor treatment and construct evaluation, demonstrated an increase in compressive stiffness. It is likely that the increased lag time is required to incorporate and organize the GAG and collagen into the

ECM.[199] Based on these results, 2 wk continuous TGF-β1 treatment at 30 ng/ml was selected for use in phase II as it demonstrated the greatest increase in the functionality index, and was the only treatment in phase I that increased both compressive and tensile properties. This result supported our hypothesis that TGF-β1 treatment would increase both compressive and tensile properties by increasing both GAG and collagen content, respectively. Additionally this finding corresponds with previous work that has demonstrated that TGF-β1 treatment increases collagen synthesis or gene expression,[73, 74, 206, 207] while TGF-β1 treatment only under serum free conditions increases proteoglycan synthesis.[206]

In phase I, the different dosage frequencies had profound effects on the biochemical and biomechanical properties of the constructs. For example, 10 ng/ml IGF-I applied at the 2 wk continuous dosage significantly increased compressive stiffness and GAG/WW, while 10 ng/ml IGF-I applied at the wk rotation dosage had no effect on compressive stiffness and GAG/WW. Additionally, as described above, only 30 ng/ml TGF-β1 treatment at the 2 wk continuous dosage increased the compressive stiffness. A possible explanation is that different dosages may mimic temporal patterns of growth factor expression during wound healing[208] as well as during chondrogenesis, as reviewed by Goldring et al.[209]

TGF-β1 and the combination of BMP-2 and IGF-I were identified as the winners in terms of construct functionality in this study. These results were primarily

obtained in phase II, where BMP-2, IGF-I, and TGF-β1 were applied at the selected conditions from phase I in combinations of one, two, or three. Combined BMP-2 and IGF-I treatment had beneficial effects, demonstrating the greatest increase in aggregate modulus (119%), accompanied by the greatest increase in GAG/WW (54%). However, as with the use of these growth factors individually, there was no difference in tensile properties or collagen/WW. As in phase I, only treatment with TGF-β1 alone led to a significant increase in tensile properties and collagen/WW. There was a disparity in values obtained for the individual growth factor treatments between phases I and II, likely as a result of different donor tissue from which the cells were isolated. However, although the values for the properties of the control constructs vary between the phases, similar percent increases in properties are observed for the individual growth factors in each phase.

It is also interesting to note that combining TGF-β1 with either of the other growth factors did not have additive or synergistic effects, negating the increased compressive and tensile stiffness observed with TGF-β1 treatment alone. This result agrees with prior work by Blunk et al.[74] which noted that combined TGF-β1 and IGF-I treatment decreased GAG and collagen fractions. Additionally, TGF-β1 has been shown to regulate the autocrine/paracrine axis of IGF-I,[210] and it is likely that combined growth factor treatment may alter these intracellular pathways, potentially leading to the reduced effects observed in this study. Prior work by Suzuki et al.[211] also supports our results, as it was demonstrated that

BMP-2 signal transduction was inhibited by application of TGF-β1. However, it is possible that there is a concentration-dependence of our results; for example, if TGF-β1 was applied at much higher or lower concentrations than used in the manuscript, IGF-I and BMP-2 may have different responses than what was reported in this study.

It is important to note that our results differ from several prior growth factors studies[74, 198] which have utilized culture medium containing FBS. This medium already contains growth factors, potentially confounding the effects of additional growth factor application. In this study, we utilized serum-free medium to control for any confounding from the presence of FBS in the medium and to enable us to look solely at the effects of the growth factor supplementation. The use of serum-free medium may explain some of the differences between our results and those of prior studies. Additionally, the self-assembly process may modulate some of the effects of growth factors differently. For example, Gooch et al.[198] found that treatment with BMP-2 at 100 ng/ml led to the presence of hypertrophic chondrocytes; however, we found no differences in chondrocyte morphology nor any other histological properties. Furthermore, it has previously been shown that growth factor application at higher concentrations significantly increases construct WW.[74, 198] We did not observe this WW increase, and in fact found that TGF-β1 treatment actually decreased the construct WW. It is possible that these responses are due to the combined effects of FBS and supplemental growth

factors, and that the use of growth factors in serum-free conditions mitigates the hypertrophic response at the concentrations used in the present study.

Although multiple studies have examined the effects of various growth factors on monolayer, explant, and engineered construct gene expression and biochemical properties, this study systematically assessed the effects of different growth factors, concentrations, dosages, and combinations, leading to construct biochemical and biomechanical properties in the range of native tissue values. Since most other investigations of engineered cartilage have not achieved the biochemical and biomechanical properties found in this study in only 4 wks, the results presented here likely are due to the combination of the self-assembling process, serum-free media, and the selected growth factor regimens. Only treatment with TGF-β1 was found to enhance both the compressive and tensile properties of engineered constructs, while combined treatment with BMP-2 and IGF-I led to adjunctive enhancement of construct compressive stiffness and GAG content. As previous studies have demonstrated beneficial effects of combined growth factor treatment and direct compression,[92, 93] future studies should assess the effects of these growth factor treatments when combined with mechanical stimulation, such as hydrostatic pressure and direct compression.

Chapter 6: Synergistic and Additive Effects of Hydrostatic Pressure and Growth Factors on Tissue Formation

Benjamin D. Elder and Kyriacos A. Athanasiou

Chapter published as: Elder, BD and Athanasiou, KA. Synergistic and Additive Effects of Hydrostatic Pressure and Growth Factors on Tissue Formation. *PLoS ONE*. 2008

ABSTRACT

Hydrostatic pressure (HP) is a significant factor in the function of many tissues, including cartilage, knee meniscus, temporomandibular joint disc, intervertebral disc, bone, bladder, and vasculature. Though studies have been performed in assessing the role of HP in tissue biochemistry, to the best of our knowledge, no studies have demonstrated enhanced mechanical properties from HP application in any tissue. The objective of this study was to determine the effects of hydrostatic pressure (HP), with and without growth factors, on the biomechanical and biochemical properties of engineered articular cartilage constructs, using a two-phased approach. In phase I, a 3 x 3 full-factorial design of HP magnitude (1, 5, 10 MPa) and frequency (0, 0.1, 1 Hz) was used, and the best two treatments were selected for use in phase II. Static HP at 5 MPa and 10 MPa resulted in significant 95% and 96% increases, respectively, in aggregate modulus (H_A), with corresponding increases in GAG content. These regimens also resulted in significant 101% and 92% increases in Young's modulus (E_Y), with corresponding increases in collagen content. Phase II employed a 3 x 3 full-factorial design of HP (no HP, 5 MPa static, 10 MPa static) and growth factor application (no GF, BMP-2 + IGF-I, TGF-β1). The combination of 10 MPa static HP and TGF-β1 treatment had an additive effect on both H_A and E_Y, as well as a synergistic effect on collagen content. This group demonstrated a 164% increase in H_A, a 231% increase in E_Y, an 85% increase in GAG/wet weight (WW), and a 173% increase in collagen/WW, relative to control. To our knowledge, this is the first study to demonstrate increases in the biomechanical

properties of tissue from pure HP application, using a cartilage model. Furthermore, it is the only study to demonstrate additive or synergistic effects between HP and growth factors on tissue functional properties. These findings are exciting as coupling HP stimulation with growth factor application has allowed for the formation of tissue engineered constructs with biomechanical and biochemical properties spanning native tissue values.

INTRODUCTION

Hydrostatic pressure plays an important role in the mechanoregulation of several tissues; including cartilage,[102, 122, 130, 132, 135, 136, 138, 165] knee meniscus,[212] temporomandibular joint disc,[178, 213] intervertebral disc,[213-215] bone,[216] bladder,[217] and vasculature.[218] In these studies, HP generally led to increased extracellular matrix (ECM) production. HP application appears particularly promising as a strategy in cartilage tissue engineering, as cartilage degeneration remains a tremendous problem.[1] Following injury, cartilage has a poor ability to self-repair due to its avascularity, and current clinical treatments for articular cartilage injuries result in the formation of mechanically inferior fibrocartilage.[14] Therefore, cartilage regeneration with tissue engineering strategies appears to be a promising approach. A scaffoldless approach to tissue engineering, the self-assembly process, has been developed and utilized by our group to produce engineered constructs with biochemical and biomechanical properties approaching native tissue values.[36, 135, 140]

Cartilage is typically exposed to pressures in the physiologic range of 3-18 MPa,[11, 121, 196] and tissue engineering efforts have generally focused on these physiologic pressures. Prior studies have shown complex effects from HP application, demonstrating both inhibition and enhancement of ECM protein production and gene expression depending on the selected HP regimen and culture system. For example, several pioneering studies by Smith et al.[128-130, 142, 143] on monolayers have demonstrated enhanced protein production and gene

expression when applying intermittent hydrostatic pressure at 10 MPa, 1 Hz to both normal human adult articular chondrocytes as well as to osteoarthritic chondrocytes. However, they found detrimental effects on collagen II mRNA production when applying 10 MPa static (0 Hz) HP to adult articular chondrocytes in monolayer.[128] On the other hand, Mizuno et al.[136] applied 2.8 MPa static HP to 3-D bovine chondrocyte seeded collagen sponges and found an increase in GAG production. Similarly, Toyoda et al.[137, 138] observed significantly increased GAG production, aggrecan mRNA, and type II collagen mRNA expression when applying 5 MPa static HP to bovine articular chondrocyte seeded agarose gels.

Several prior studies have also demonstrated the benefits of growth factors, including BMP-2, IGF-I, and TGF-β1, on construct functional properties.[74, 80, 198] In recent work (under review, Osteoarthritis and Cartilage), we have demonstrated the benefits of combined BMP-2 and IGF-I treatment on construct compressive properties and GAG production, as well as the benefit of TGF-β1 treatment on construct compressive and tensile properties, with corresponding enhancement of GAG and collagen production. Furthermore, previous work has demonstrated the benefits of combining growth factor application with direct compression mechanical stimulation on construct[93] and explant[92] functional properties.

Though several studies have been performed in assessing the role of HP in tissue biochemistry, to the best of our knowledge, no studies have demonstrated

enhanced biomechanical properties from HP application in any tissue. Furthermore, studies that systematically assess the effects of multiple HP magnitudes and frequencies on construct functional properties are lacking. Additionally, there is a dearth of studies demonstrating synergistic effects on tissue functionality from combining hydrostatic pressure and growth factors.

Using a scaffoldless cartilage tissue engineering model,[36, 140] this study sought to test the hypotheses that 1) a short-term application of static HP during construct development will have the greatest enhancement of construct biochemical and biomechanical properties, and that 2) there will be additive or synergistic effects when combining growth factors and HP stimulation. These hypotheses were assessed and supported using a two-phased approach. In phase I, a 3 x 3 full-factorial design of HP magnitude (1, 5, and 10 MPa) and frequency (0, 0.1, and 1 Hz) was used, and the best two treatments were selected for use in phase II. Phase II employed a 3 x 3 full-factorial design of HP (no HP, 5 MPa static, 10 MPa static) and growth factor application (no GF, BMP-2 + IGF-I, TGF-β1) for a total of nine treatment groups.

MATERIALS AND METHODS

Chondrocyte Isolation and Seeding

Cartilage from the distal femur of wk-old male calves was obtained[93, 118, 181] (Research 87, Boston, MA) and digested with collagenase type 2 (Worthington,

Lakewood, NJ) for 24 hrs, as described in detail previously.[140] A polysulfone die consisting of 5 mm dia. x 10 mm long cylindrical prongs that fit into 6 wells of a 48-well plate was used to construct each agarose mold, as described in detail previously.[140] The culture medium is a chemically defined medium that has been described previously.[140] To each agarose well, 5.5 x 10^6 cells were added in 100 µl of culture medium; t=0 was defined as 24 hrs after seeding.

Phase I: HP Magnitude and Frequency Selection

At t=10 days, self-assembled constructs (n=6/group) were removed from confinement in 5 mm dia. agarose wells and exposed to HP for 1 h/day, for 5 days. The study employed a 3 x 3 full-factorial design of magnitude (1, 5, 10 MPa) and frequency (0, 0.1, 1 Hz), for a total of 9 treatment groups. The constructs were then placed in individual agarose-coated wells of 48-well culture plates for the remainder of the study. A control (CC) consisted of constructs removed from confinement in 5 mm dia. agarose wells at 10 days, and cultured in individual wells of 48-well culture plates coated with 2% agarose for the remainder of the study. Per construct, 500 µl of medium was changed daily, and all constructs were assessed at t=4 wks.

Phase II: Combination of HP and Growth Factors

This study employed a 3 x 3 full-factorial design of HP (no HP, 5 MPa static, 10 MPa static) and growth factor application (no GF, BMP-2 + IGF-I, TGF-β1) for a total of nine treatment groups. The hydrostatic pressure regimens were selected

in phase I (please see results), while the growth factor treatments were selected from a prior study by our group (under review, Osteoarthritis and Cartilage). The HP regimens were applied as in phase I, for 1 hr/day, from t=10-14 days. The specific growth factor treatments were TGF-β1 (30 ng/ml) continuously from t=0-14 days, or a combined treatment of BMP-2 (10 ng/ml) continuously from t=10-14 days and IGF-I (10 ng/ml) from t=0-7 days and t=14-21 days. All growth factors were obtained from Peprotech Inc. (Rocky Hill, NJ), and applied in the culture medium. As in phase I, constructs were removed from confinement at t=10 days, and cultured in individual wells for the remainder of the study. Per construct, 500 μl of medium was changed daily, and all constructs were assessed at t=4 wks.

Specimen Pressurization

The procedure used has been described previously.[135] Briefly, constructs were placed into heat sealable bags (Kapak/Ampak Flexibles, Cincinnati, OH) with 35 ml medium, and the bags were heat-sealed without any bubbles inside. The chamber was maintained at 37° C during pressurization. From t=10-14 days, the constructs were pressurized at a specific regimen for 1 hr. Following HP application, the pouches were opened with autoclaved instruments and the samples were returned to individual agarose coated wells.

Histology and Immunohistochemistry

Samples were frozen and sectioned at 14 μm. Safranin-O and fast green staining were used to examine GAG distribution.[182, 183] Picrosirius red was used

for qualitative examination of collagen content. A von Kossa stain was used to examine mineralization. IHC was used to determine the presence of collagen types I and II, as described previously.[140]

Quantitative Biochemistry

Samples were frozen overnight and lyophilized for 72 hrs, followed by re-suspension in 0.8 mL of 0.05 M acetic acid with 0.5 M NaCl and 0.1 mL of a 10 mg/mL pepsin solution (Sigma) at 4°C for 72 hrs. Next, 0.1 mL of 10x TBS was added along with 0.1 mL pancreatic elastase and mixed at 4°C overnight. From this digest, total DNA content was measured by Picogreen® Cell Proliferation Assay Kit (Molecular Probes, Eugene, OR). Total sulfated GAG was quantified using the Blyscan Glycosaminoglycan Assay kit (Biocolor).[184, 185] Total collagen content was assessed by a chloramine-T hydroxyproline assay.[186]

Mechanical Testing

To obtain salient compressive properties, samples were evaluated under conditions of creep indentation,[187] which has been described in detail previously.[140] The aggregate modulus (H_A), permeability, and Poisson's ratio of the samples were then determined using the linear biphasic theory.[3] To obtain construct tensile properties, uniaxial tests were run on a materials testing system (Instron Model 5565, Canton, MA) with a 50 N load cell, as described previously.[197] Stress-strain curves were created from the load-displacement curve and the cross-sectional area of each sample, and Young's modulus (E_Y)

was calculated from the linear region of each stress-strain curve. Construct thickness was measured using digital calipers.

Statistical Analysis

Biochemical and biomechanical assessments were performed on all constructs (n=6 or 7). In each phase, a single factor ANOVA was used to analyze the samples, and a Fisher LSD post hoc test was used when warranted. Significance was defined as $p<0.05$. Additionally, in phase II, the interaction term of a two factor ANOVA was used to test for synergism, as described previously [219], with significance defined as $p<0.05$.

RESULTS

Gross Appearance and Histology

All constructs reached a diameter of approximately 6 mm at t=4 wks (Fig. 21a). In phase I, there were no differences in wet weight (WW) or thickness among the treatment groups, as demonstrated in Table V. However, as shown in Table VI, in phase II, there was a decrease in construct WW and thickness in all groups treated with TGF-β1.

In both studies, positive staining for collagen (Fig. 21b) and GAG (Fig. 21e) was observed throughout the construct thickness. Additionally, based on IHC, collagen II was expressed throughout each construct (Fig. 21c), with no collagen

I production (Fig. 21f). Finally, in phase II, there was no mineralization or chondrocyte hypertrophy observed with BMP-2 + IGF-I treatment.

Quantitative Biochemistry

In phase I, all values of cells/construct, GAG/WW, and collagen/WW are found in Table V. There were no differences in cells/construct among the different treatment groups. Several treatments resulted in significant increases in GAG/WW, but the greatest increases in GAG/WW were observed with the 5 MPa static, 10 MPa static, and 10 MPa, 1 Hz regimens (Fig. 22c), with GAG/WW values of 8.1±0.6, 8.1±0.4, and 9.1±0.8%, respectively. However, only 5 MPa static and 10 MPa static HP application significantly increased collagen/WW (Fig. 22d), with values of 9.4±2.5 and 10.8±1.9%, respectively.

In phase II, all values of cells/construct, GAG/WW, and collagen/WW are found in Table VI. There were no differences in cells/construct among the different treatment groups. All treatments exhibited a significant increase in GAG/WW (Fig. 23c); additionally, there was an adjunctive effect between 10 MPa static HP and TGF-β1, as their combination resulted in a greater GAG/WW, of 9.6±0.4%, than either treatment alone. Treatment with either HP regimen or with TGF-β1 significantly increased the collagen/WW (Fig. 22d). Furthermore, combined treatment with 10 MPa static HP and TGF-β1 led to a synergistic increase in collagen/WW to 15.3±2.9%; the increase in collagen/WW was statistically greater than the sum of either treatment alone.

Mechanical Evaluation

In phase I, all values of H_A and E_Y are found in Table V. The 1, 5, and 10 MPa static HP groups, as well as the 10 MPa, 1 Hz group all demonstrated a significant increase in H_A relative to the control group (Fig. 22a), with values of 268±45, 269±44, 270±46, and 287±82 kPa, respectively. However, only the 5 MPa static HP group exhibited significant increases in E_Y to 1196±271 kPa (Fig. 22b); a similar increase in E_Y to 1144±281 kPa was observed for the 10 MPa static HP group.

In phase II, all values of H_A and E_Y are found in Table VI. All treatments exhibited a significant increase in H_A (Fig. 23a), with the 10 MPa + TGF-β1 treatment group displaying the greatest increase, to 248±37 kPa. This increase indicated an additive effect between 10 MPa static HP and TGF-β1, as the effect of their combined use on H_A was equal to the sum of the effects of either treatment alone. Treatment with either HP regimen alone or with TGF-β1 significantly increased the E_Y; furthermore, combined treatment of 10 MPa static HP and TGF-β1 led to an additive increase in E_Y to 2048±266 kPa (Fig. 23b).

DISCUSSION

This study employed a 2-phased approach to choose an optimal HP loading regimen, as well as to determine the effects of combined growth factor and HP

application. To the best of our knowledge, this study is the first to 1) demonstrate increases in the biomechanical properties of tissue from pure HP application, using a cartilage model, 2) demonstrate additive or synergistic effects between HP and growth factors on tissue functional properties, and 3) systematically assess the effects of varying physiologic magnitudes and frequencies of HP on tissue functional properties.

In phase I, 5 MPa and 10 MPa static HP were the only regimens that increased both H_A and E_Y with parallel increases in GAG and collagen content. These results support our hypothesis, as static hydrostatic pressure was found to have the greatest effect on construct biochemical and biomechanical properties. Since 5 MPa and 10 MPa static HP were the only regimens to significantly increase the compressive and tensile stiffness as well as GAG/WW and collagen/WW, these two regimens were selected for use in phase II.

In phase II, the combination of 10 MPa static HP and TGF-β1 treatment had significant effects on construct biomechanical and biochemical properties, thus supporting the hypothesis that combined HP and growth factor treatment would have additive and synergistic effects on construct functional properties. The combined treatment of 10 MPa static HP and TGF-β1 had an additive effect on both H_A and E_Y, as the increases in compressive and tensile stiffness for the combined treatment were equal to the sum of the effects of the two individual treatments. Additionally, the combined treatment exhibited a synergistic increase

in collagen/WW, as the effect of the combined treatment was statistically greater than the sum of the effects of each individual treatment. Excitingly, the collagen/WW of this group, at 15.3%, spanned reported values for native articular cartilage.[4]

However, although 5 MPa and 10 MPa static HP have similar effects on construct properties when applied alone, 5 MPa static HP did not exhibit the same additive and synergistic effects when combined with TGF-β1 treatment. This result suggests that there are different cellular responses to varying HP magnitudes; for example, it can be speculated that increasing HP from 5 MPa to 10 MPa in the presence of TGF-β1 may activate additional intracellular pathways that lead to enhanced production of ECM proteins and increased biomechanical properties. Interestingly, a similar effect has been observed previously in work on chondrogenic differentiation of human mesenchymal stem cells (MSCs).[151] It was found that collagen II mRNA expression of MSCs cultured with TGF-β3 responded maximally to 10 MPa HP application.

It is also interesting to note that combining BMP-2 + IGF-I treatment with either of the HP treatments did not lead to further enhancement of construct properties, and actually negated the beneficial effects of HP alone on construct properties. It has previously been shown that HP modulates the level of TGF-β mRNA.[131] Additionally, combined treatment with TGF-β1 and IGF-I has detrimental effects on GAG and collagen content shown by Blunk et al.[74] and our own work (under

review, Osteoarthritis and Cartilage). Based on these prior studies, one can speculate that HP application may lead to the production of TGF-β1, which, when combined with the effects of exogenously applied IGF-I may have detrimental effects, as seen previously, although it is possible that enhanced TGF-β1 mRNA expression may not correspond to increased TGF-β1 production due to the extensive post-transcriptional and post-translational regulation of TGF-β1, as reviewed previously.[220] In future studies, it would be exciting to elucidate the pathways involved in HP signal transduction, and how they coincide with the growth factor signal transduction cascades. Since the exact pathways for HP signal transduction have not been elucidated, we can only speculate that the pathways leading to increased matrix synthesis are either further enhanced, when combining HP and TGF-β1, or perhaps inhibited, when combining HP and the BMP-2 + IGF-I combination.

By demonstrating the beneficial effects of static HP over cyclic HP application on construct biomechanical and biochemical properties, this study contradicts several prior studies that have shown positive effects from cyclic HP.[122, 128-130, 142, 143] Though when comparing these studies, it is important to note that HP was applied to chondrocytes in monolayer rather than 3-D constructs. Furthermore, these studies utilized adult or osteoarthritic chondrocytes which behave substantially differently than the immature bovine chondrocytes used in this study.[41] On the other hand, the results of this study agree with the conclusions of

several other studies that applied static HP to 3-D constructs and found beneficial effects on construct biochemical properties.[136-138]

When assessing the effects of combined HP and growth factor treatment on cartilage properties, the results presented here agree with prior studies that have combined these treatments as differentiation agents for mesenchymal stem cells.[151, 152] For example, Miyanishi et al.[151] found that combined HP application with TGF-β3 increased SOX9, collagen II, and aggrecan mRNA levels 1.9, 3.3, and 1.6-fold, respectively, more than treatment with TGF-β3 alone. It is also known that another form of mechanical stimulation, namely direct compression, exhibits synergistic effects when combined with growth factor treatment on articular cartilage constructs[93] and explants[92]. Specifically, Mauck et al.[93] found that combined treatment with dynamic compression and TGF-β1 resulted in a 277% increase in equilibrium aggregate modulus, while Bonassar et al.[92] observed a 290% increase in proteoglycan synthesis with combined dynamic compression and IGF-I treatment.

Physiologic HP does not deform cartilage;[162] therefore, the enhanced construct biomechanical properties observed in this study must be accounted for by other mechanisms. As reviewed elsewhere,[169] on the microscopic level, HP can compress void spaces within and around proteins on the cell surface. At a certain pressure, the compression of void spaces becomes great enough that the protein can achieve a lower energy state by changing its conformation. Cell

surface ion channels may serve as "pressure sensors," altering their conformations and thus changing the intracellular ion concentrations depending on the applied pressure. For example, Hall[165] found that in chondrocytes, the activity of the Na/K pump was suppressed substantially with 10 MPa static HP application for 10 min, while the Na/K/2Cl transporter was more sensitive to HP application. Also, Browning et al.[166] observed activation of the Na/H pump in bovine articular chondrocytes with HP application at approximately 10 MPa. Additionally, Mizuno[167] found that HP increases intracellular calcium through the activation of stretch-activated channels. Since protein synthesis is affected by intracellular ion concentrations,[170] it is envisioned that different ion channel responses to varying HP magnitudes alters the intracellular ion flux and stimulates signal transduction cascades for upregulation of ECM-specific genes, enhanced ECM protein production, and increased biomechanical properties as observed in this study. Growth factors may serve as an adjunctive method for stimulating similar downstream pathways, thus leading to additive and synergistic effects, as observed in this study.

The beneficial effects of HP on tissue biochemical properties are not confined merely to cartilage, and it is possible that the approach of this study, namely combining optimized HP and growth factor treatments, may be applicable to several other tissues. For example, Stover et al.[217] found that applying cyclic HP to bladder smooth muscle cells resulted in a proliferative response suggestive of tissue remodeling. Also, Reza and Nicoll[214] observed increased production of

collagen II in intervertebral disc cells from the outer annulus exposed to 5 MPa HP. Additionally, Almarza and Athanasiou[178] demonstrated increased collagen I gene expression and protein production when applying 10 MPa static HP to temporomandibular joint disc cells. Finally, Suzuki et al.[212] applied 4 MPa static HP to knee meniscal cells, and found a significant increase in collagen I mRNA and a significant decrease in matrix metalloproteinase -1, and -13. Although none of these studies assessed the effects of HP on biomechanical properties, it can be speculated that coupling these HP regimens with the application of exogenous bioactive agents specific to these tissues, may also result in additive and synergistic effects on the functional properties.

Multiple studies have assessed the effects of both static and intermittent HP regimens on gene expression and protein production. This study, which investigated the effects of multiple HP magnitudes and frequencies on construct functional properties, demonstrated enhanced biomechanical and biochemical tissue properties. Additionally, it systematically assessed the effects of combining HP and growth factors on construct functional properties, and indicated synergistic and additive effects. Future studies should determine the effects of temporal HP application during construct development, as well as examine the immediate and long-term effects of HP application on construct properties.

Chapter 7: Evaluating Five Extraction Techniques for the Decellularization of Scaffoldless Tissue Engineered Articular Cartilage Constructs

Benjamin D. Elder and Kyriacos A. Athanasiou

Chapter to be submitted as: Elder, BD and Athanasiou, KA. Evaluating Five Extraction Techniques for the Decellularization of Scaffoldless Tissue Engineered Articular Cartilage Constructs.

ABSTRACT

Several prior studies have been performed to determine the feasibility of tissue decellularization to create a non-immunogenic xenogenic tissue replacement for bladder, vasculature, heart valves, knee meniscus, temporomandibular joint disc, ligament, and tendon. However, to the best of our knowledge, no studies have examined the decellularization of either scaffoldless engineered constructs or articular cartilage tissue. The objective of this study was to assess the effects of 5 different decellularization treatments, for 1 h or 8 h, on scaffoldless tissue engineered articular cartilage constructs after 4 wks of culture. The specific treatments used were 1) 1% SDS, 2) 2% SDS, 3) 2% Tributyl phosphate, 4) 2% Triton X-100, and 5) Hypotonic followed by hypertonic solution, followed by a 3 h wash in PBS. Following this wash, the constructs were assessed histologically, biochemically for cellularity, GAG, and collagen content, and biomechanically for compressive and tensile properties. Treatment with 2% SDS for 1 h eliminated 33% of DNA, while maintaining or increasing biochemical and biomechanical properties. On the other hand, treatment for 8 h resulted in the elimination of 46% of DNA, although GAG content and compressive properties were significantly decreased. As all other treatments either did not result in significant decellularization, or else significantly compromised construct functional properties, 2% SDS appeared to be the most effective agent for cartilage decellularization. The results of this study are exciting as they indicate the feasibility of creating engineered cartilage that will be non-immunogenic as a replacement tissue.

INTRODUCTION

Injuries to articular cartilage, whether traumatic or from degeneration, generally result in the formation of mechanically inferior fibrocartilage, due to the tissue's poor intrinsic healing response.[14] As such, tissue engineering strategies have focused on developing replacement tissue *in vitro* for eventual *in vivo* implantation.

Although engineered articular cartilage tissue has recently been created with biochemical and biomechanical properties in the range of native tissue values,[139] there are currently two significant limitations to cartilage tissue engineering. First, human cells are scarce in number and difficult to procure, and passage of these cells leads to dedifferentiation. These issues make the use of autologous cells for cartilage repair extremely difficult. Additionally, the majority of cartilage tissue engineering approaches have employed bovine or other animal cells, and tissues grown from these cells are xenogenic and may result in a severe immune response following implantation that would preclude their use, though this has not been fully elucidated.

It is believed that a decellularized xenogenic tissue may be a viable option as a replacement tissue, as the antigenic cellular material will be removed while preserving the relatively non-immunogenic extracellular matrix (ECM), as described in an earlier review.[221] Ideally, this will also preserve the biomechanical properties of the tissue. For instance, an acellular dermal

matrix[222] has seen successful use clinically as the FDA approved Alloderm product. Additionally, acellular xenogenic tissues have been created for many musculoskeletal applications, including replacements for the knee meniscus,[223] temporomandibular joint disc,[224] tendon,[225] and ACL,[226] as well as in other tissues including heart valves,[227-233] bladder,[234] artery,[235] and small intestinal submucosa.[236, 237] However, studies demonstrating the effects of tissue decellularization on cartilage as well as on musculoskeletal tissue engineered constructs are lacking.

Therefore, the objective of this study was to determine the effects of multiple decellularization treatments on construct cellularity, biochemical, and biomechanical properties. It was hypothesized that cells could be removed from self-assembled constructs while preserving the biochemical and biomechanical properties. To test this hypothesis, self-assembled articular cartilage constructs were cultured for 4 wks, and then treated with 1% SDS, 2% SDS, 2% Tributyl Phosphate (TnBP), 2% Triton X-100, or a hypotonic/hypertonic solution, for either 1 or 8 h. The effects of the decellularization treatments were assessed on construct cellularity and functional properties.

MATERIALS AND METHODS

Chondrocyte Isolation and Seeding

Cartilage was harvested from the distal femur of wk-old male calves[93, 118, 181] (Research 87, Boston, MA) shortly after slaughter, and chondrocytes were

isolated following digestion with collagenase type 2 (Worthington, Lakewood, NJ). To normalize variability among animals, each leg came from a different animal, and cells from all legs were combined together to create a mixture of chondrocytes; a mixture of cells from five legs was used in the study. Cell number was determined on a hemocytometer, and a trypan blue exclusion test indicated that viability remained >90%. Chondrocytes were frozen in culture medium supplemented with 20% FBS (Biowhittaker, Walkersville, MD) and 10% DMSO at –80°C for 1 day prior to use. After thawing, viability was greater than 90%. A stainless steel mold consisting of 5 mm dia. x 10 mm long cylindrical prongs was placed into a row of a 48-well plate. To construct each agarose well, sterile, molten 2% agarose was added to wells fitted with the die. The agarose solidified at room temperature for 60 min, after which the mold was removed from the agarose. Two changes of culture medium were used to completely saturate the agarose well by the time of cell seeding. The medium was DMEM with 4.5 g/L-glucose and L-glutamine (Biowhittaker), 100 nM dexamethasone (Sigma, St. Louis, MO), 1% Fungizone/Penicillin/Streptomycin (Biowhittaker), 1% ITS+ (BD Scientific, Franklin Lakes, NJ), 50 μg/mL ascorbate-2-phosphate, 40 μg/mL L-proline, and 100 μg/mL sodium pyruvate (Fisher Scientific, Pittsburgh, PA). To seed each construct, 5.5 x 10^6 cells were added in 100 μl of culture medium. Constructs formed within 24 h in the agarose wells and were cultured in the same well until t=10 days, after which they were unconfined for the remainder of the study, as described previously;[238] t=0 was defined as 24 h after seeding.

Decellularization Treatments

At t=4 wks, self-assembled constructs (n=6/group) were removed from culture and exposed to one of five decellularization treatments, for either 1 h or 8 h. The decellularization treatments included:

1) 1% SDS
2) 2% SDS
3) 2% Tributyl Phosphate (TnBP)
4) Triton X-100
5) Hypotonic/Hypertonic Solution (half-time of each)
    a. Hypotonic: 10 mM Tris HCl, 5 mM EDTA, 1 μM PMSF
    b. Hypertonic: 50 mM Tris HCl, 1 M NaCl, 10 mM EDTA, 1 μM PMSF

All treatments included 0.5 mg/ml DNase Type I, 50 μg/ml RNase, 0.02% EDTA, and 1% P/S/F, in PBS. Both 1 h control and 8 h control groups were exposed to this same solution without detergent treatments. These treatments were applied at 37℃ with agitation. Following the 1 h or 8 h treatment, the constructs were washed for 3 h in PBS at 37℃ with agitation. Additionally, an untreated control was assessed immediately following removal from culture, without the treatment or wash steps.

Histology and Immunohistochemistry

After freezing, samples were sectioned at 14 μm. To determine construct cellularity, a hematoxylin & eosin (H&E) stain was used. A Safranin-O/fast green

stain was used to examine GAG distribution.[182, 183] To assess collagen content, picrosirius-red was employed. Immunohistochemistry was utilized to test for the presence of collagen types I and II on a Biogenex (San Ramon, CA) i6000 autostainer. Following fixation in chilled acetone, the slides were washed with IHC buffer (Biogenex), quenched of peroxidase activity with hydrogen peroxide/methanol, and blocked with horse serum (Vectastain ABC kit, Vector Laboratories, Burlingame, CA). The slides were then incubated with either mouse anti-collagen type I (Accurate Chemicals, Westbury, NY) or rabbit anti-collagen type II (Cedarlane Labs, Burlington, NC) antibodies. Secondary antibody (anti-mouse or anti-rabbit IgG, Vectastain ABC kit) was applied, and color was developed using the Vectastain ABC reagent and DAB (Vectastain kit).

Quantitative Biochemistry

Samples were frozen overnight and lyophilized for 48 h, followed by re-suspension in 0.8 mL of 0.05 M acetic acid with 0.5 M NaCl and 0.1 mL of a 10 mg/mL pepsin solution (Sigma) at 4°C for 72 h. Next, 0.1 mL of 10x TBS was added along with 0.1 mL pancreatic elastase and mixed at 4°C overnight. A Picogreen® Cell Proliferation Assay Kit (Molecular Probes, Eugene, OR) was used to assess total DNA content. GAG content was quantified using the Blyscan Glycosaminoglycan Assay kit (Biocolor), based on 1,9-dimethylmethylene blue binding.[184, 185] After hydrolysis with 2 N NaOH for 20 min at 110°C, total collagen content was determined using a chloramine-T hydroxyproline assay.[186]

Indentation Testing

Samples were assessed with an automated indentation apparatus, as described previously.[187] A 0.7 g (0.007 N) mass was applied with a 1 mm flat-ended, porous indenter tip, and specimens crept until equilibrium, as described elsewhere.[36] Preliminary estimations of the aggregate modulus of the samples were obtained using the analytical solution for the axisymmetric Boussinesq problem with Papkovich potential functions.[188, 189] The sample biomechanical properties, including aggregate modulus, Poisson's ratio, and permeability were then calculated using the linear biphasic theory.[3]

Tensile Testing

A uniaxial materials testing system (Instron Model 5565, Canton, MA) was employed to determine tensile properties with a 50 N load cell, as described previously.[197] Briefly, samples were cut into a dog-bone shape with a 1-mm-long gauge length. Samples were glued to paper tabs with cyanoacrylate glue outside of the gauge length. The 1-mm-long sections were pulled at a 1% constant strain rate. All samples broke within the gauge length. Stress-strain curves were created from the load-displacement curve and the cross-sectional area of each sample, and Young's modulus was calculated from each stress-strain curve.

Statistical Analysis

All samples were assessed biochemically and biomechanically (n=6). First, the three control groups were compared using a single factor ANOVA. As no

difference was noted, only the culture control was used in the final analysis. To compare treatment groups, a single factor ANOVA was used, and a Tukey HSD *post hoc* test was used when warranted. Significance was defined as $p< 0.05$.

RESULTS

Gross Appearance and Histology

In all groups, the construct diameter was approximately 6 mm at 4 wks. Treatment for 8 h with either 1% SDS or the hypotonic/hypertonic solution resulted in a significant decrease in construct thickness (Table VII). Additionally, treatment for 8 h with 1% SDS, 2% SDS, 2% Triton X-100, or the hypotonic/hypertonic solution resulted in a significant decrease in construct wet weight (Table VII).

Figure 24 displays the histological results of the study. Extensive staining for cell nuclei was observed in the H&E staining of the control group. 1% SDS treatment for 1 h reduced the number of cell nuclei, while treatment for 8 h eliminated all nuclei from the construct. The 2% SDS treatment had similar results. However, treatment with 2% TnBP or 2% Triton X-100, for either timepoint, had no effect on the number of nuclei. Both hypotonic/hypertonic treatments resulted in a slight reduction in number of cell nuclei. All decellularization treatments for 8 h resulted in a significant reduction or complete elimination of staining for GAGs. Additionally, 1 h treatment with the hypotonic/hypertonic solution reduced the GAG content. However, there were no apparent differences in GAG staining

among the 1 h treatments with 1% SDS, 2% SDS, 2% TnBP, 2% Triton X-100, and the control. Finally, all constructs demonstrated extensive staining for collagen, although the 8h decellularization treatments resulted in slight alterations in construct morphology.

Quantitative Biochemistry

Several decellularization treatments resulted in a significant reduction in construct DNA (Fig. 25). Treatment for 1 h with 2% SDS or the hypotonic/hypertonic solution, as well as 8 h treatment with 1 or 2% SDS or the hypotonic/hypertonic solution all resulted in a significant reduction of the DNA in the constructs. However, treatment with 2% TnBP or 2% Triton X-100 for either amount of time had no effect on construct DNA.

The effects of the decellularization agents on construct GAG content are found in Fig. 26. Treatment with 1% or 2% SDS for 1 h had no effect on GAG content, while all other treatments significantly reduced the GAG content of the constructs. Additionally, all 8 h treatments resulted in complete or nearly complete removal of GAG from the constructs. Finally, there were no significant changes in total collagen content following treatment with the decellularization agents (Fig. 27).

Biomechanical Evaluation

The effects of the various decellularization treatments on construct aggregate modulus are displayed in Fig. 28. Treatment for 1 h with 1% or 2% SDS as well as with 2% TnBP maintained the compressive stiffness. However, treatment for 8 h with 1% SDS, 2% TnBP, and 2% Triton X-100 significantly reduced the aggregate modulus. The groups treated for 8 h with either 2% SDS or the hypotonic/hypertonic solutions were too weak to be mechanically tested with creep indentation. Additionally, the effects of the various decellularization treatments on Poisson's ratio and permeability are displayed in Table VIII. A significant decrease in Poisson's ratio was noted for the groups treated for 8 h with 1% SDS, 2% TnBP, and 2% Triton X-100. Finally, only treatment for 8 h with 1% SDS resulted in a significantly decreased permeability.

Figure 29 indicates the tensile properties of the constructs treated with the various agents. Treatment for 1 h with 1% SDS, 2% TnBP, or 2% Triton X-100 maintained the tensile stiffness. A 1 h treatment with 2% SDS actually increased the Young's modulus. However, 8 h treatments with 2% SDS, 2% TnBP, and 2% Triton X-100 significantly decreased the Young's modulus. Similar trends are noted for the Ultimate Tensile Strength data, although a significant decrease was only noted for the hypotonic/hypertonic treatment.

DISCUSSION

To the best of our knowledge, this is the first study to decellularize articular cartilage tissue, in this case engineered cartilage. The objective of this study was to assess the effectiveness of multiple different decellularization protocols on self-assembled articular cartilage constructs. The study utilized a two-factor approach, in which five different treatments were examined at two application times each.

The results of this study indicated that SDS, at concentrations of either 1% or 2%, is an effective treatment for tissue decellularization, thus confirming our hypothesis that cells could be eliminated from engineered constructs while maintaining the biomechanical properties. An ionic detergent, SDS typically is able to solubilize the nuclear and cytoplasmic cell membranes. Although all SDS treatments led to cell removal, treatment with 2% SDS appeared the most promising, although application time also had significant effects. For instance, treatment with 2% SDS for 1 h resulted in a 33% decrease in cellularity, while maintaining both GAG and collagen content, as well as maintaining compressive stiffness. This treatment even resulted in an increase in tensile stiffness; a similar increase in tensile properties was observed in a study of ACL decellularization.[226] On the other hand, treatment with 2% SDS for 8 h led to complete histological decellularization, as well as a 46% decrease in DNA content. However, this treatment also resulted in loss of all GAG and compressive stiffness, as well as a decrease in tensile stiffness. Treatment with

2% SDS for 8 h also resulted in a significant decrease in construct WW, presumably as a result of the GAG loss, which would also decrease the tissue hydration.

As described above, it must be noted that although treatment with 2% SDS resulted in complete histological decellularization, it did not result in complete elimination of DNA. It appeared that SDS treatment was effective at achieving complete lysis of cell membranes and nuclear membranes, as an H&E stain did not reveal any indication of the presence of cell nuclei, while the DNAse treatment was not completely effective in degrading the DNA following membrane lysis. It is possible that a higher DNAse concentration is required to achieve more complete elimination of DNA. Additionally, as nucleases were only added during detergent treatment, it is possible that adding a nuclease during the 3 h wash step would enable the nucleases to more effectively destroy the remaining DNA.

However, the exact level of tissue decellularization requisite to eliminate an immune response, as well as the proper assessment of decellularization, is currently unclear. As it is believed that the joint space is relatively immune privileged, as reviewed previously,[239] it is possible that complete decellularization of the tissue is not required. Additionally, it is unclear if decellularization should be assessed histologically merely as elimination of cell nuclei, or if a more complete assessment involves quantifying the tissue's DNA content, as prior

studies have utilized differing approaches. For example, Lumpkins et al.[224] found that 1% SDS treatment for 24 h resulted in complete removal of cell nuclei, although they did not assess the DNA content of the tissue. On the other hand, Dahl et al.[235] examined the effects of a hypotonic/hypertonic treatment and found that there was complete removal of cell nuclei, but no decrease in DNA content. To study this issue further, *in vivo* studies are warranted to determine if there is threshold of decellularization at which an immune response is eliminated.

Although it was less effective than the 2% concentration, 1% SDS displayed similar effects. For example, treatment for 1 h resulted in a 15% decrease in DNA content, while maintaining GAG and collagen content as well as maintaining biomechanical properties. Additionally, treatment for 8 h resulted in a 37% decrease in DNA content, loss of all GAG and compressive stiffness, as well as a decrease in tensile stiffness.

On the other hand, treatment with Triton X-100 and TnBP did not appear promising, as they had a minimal effect on tissue decellularization, and resulted in a slight decrease in GAG content. Several prior studies have indicated the ineffectiveness of Triton X-100, although it was used in this study as it is believed to have minimal effects on protein-protein interactions.[221] For example, Dahl et al.[235] examined the effects of 1% Triton X-100 on porcine carotid arteries, and found that this treatment resulted in similar cellularity to control and no decrease in DNA content. In another study on tendon decellularization, Cartmell and

Dunn[225] examined the effect of 1% Triton X-100 for 24 h, and found that cell density remained similar to control. Contrary to our results, this study demonstrated complete decellularization with 1% TnBP, although a 48 h treatment was required. Therefore, it is possible that TnBP treatment may result in decellularization of self-assembled constructs at longer application times, although the GAG loss after as little as 8 h prevents the use of longer application times.

Finally, although a hypotonic/hypertonic treatment has been an effective decellularization agent in this study as well as prior studies,[226, 235] it did not appear to be a viable treatment for self-assembled cartilage constructs, as it had severely detrimental effects on construct functional properties. For instance, treatment for as little as 1 h resulted in nearly complete loss of compressive and tensile stiffness, while constructs treated for 8 h were untestable mechanically. Additionally, treatment at both application times resulted in nearly complete elimination of GAG content.

Based on these results, treatment with 2% SDS appears to be the most promising, and should be examined further in future studies. For example, it is possible that treatment with 2% SDS for an application time between 1 h and 8 h may result in the best compromise between decellularization and maintaining the GAG content and compressive properties. It would therefore be interesting to examine the effects of 2% SDS at varying timepoints between 1 and 8 h.

Additionally, it is possible that a more effective protocol would involve multiple rounds of 1 h SDS treatment followed by a 1 h wash step, as this would re-establish concentration gradients for the detergent and wash solution at the beginning of each step. Finally, although the majority of prior decellularization studies have been performed in PBS, it is possible that the constructs could maintain their GAG content during a longer detergent treatment if in media, as this would maintain an isomolar environment, and potentially eliminate the GAG loss down a concentration gradient. Although the results of this study did not result in a completely decellularized construct with maintenance of biochemical and biomechanical properties, the results are promising and indicate the potential of a decellularized articular cartilage construct that could be used to treat damaged cartilage tissue without eliciting an immune response.

Conclusions

This thesis demonstrates work towards enhancing the functional properties of tissue engineered articular cartilage constructs. Exogenous stimuli, including radial and vertical confinement, hydrostatic pressure, and growth factor application, as well as their combined use, resulted in constructs with biochemical and biomechanical properties in the range of native tissue values, thus enhancing the *in vivo* translatability of the self-assembling process. Additional translatability of our laboratory's scaffoldless approach to tissue engineering articular cartilage was illustrated, as it was possible to remove cells from the constructs without compromising the integrity of the constructs, thus paving the way for implantation of a xenogenic tissue engineered replacement tissue.

The global hypotheses of this thesis were that 1) the self-assembling process could be enhanced by identifying suitable growth factors and mechanical forces, and 2) the effects of these exogenous factors individually or in combination would allow for the formation of constructs *in vitro* resembling native tissue. Prior work in our laboratory has generally focused on characterizing the biochemical and biomechanical properties of native articular cartilage tissue in order to develop design criteria for tissue engineered constructs. Additionally, several prior studies have focused on developing and modulating the scaffoldless approach utilized in this thesis. However, in these prior tissue engineering studies, the functional tissue properties were significantly lacking when compared to native

tissue. Therefore, this thesis concentrates on enhancing the functional properties of self-assembled articular cartilage constructs such that they approach native tissue values.

Chapter 1 presented an overview of the paradigms of tissue engineering, focusing on its application to articular cartilage regeneration. Many prior studies have focused on modulating the four parameters of the cartilage tissue engineering paradigm, specifically scaffolds, cell sources, bioactive agents, and bioreactors. Both natural and synthetic materials have been utilized as scaffolds with promising results; however, several issues must be overcome when dealing with scaffolds. For instance, the scaffold degradation kinetics must be coordinated with the deposition of new tissue, and there is concern over scaffold toxicity or the toxicity of byproducts during degradation. Therefore, scaffoldless approaches such as the self-assembling process developed by our laboratory have demonstrated increased promise in recent studies. Additionally, this chapter addressed the inherent advantages and disadvantages to the use of different cell sources, and indicated that primary chondrocytes generally result in constructs with the most robust functional properties. Furthermore, the effects of growth factors on chondrocyte metabolism were reviewed, and it was determined that TGF-β1, BMP-2, and IGF-I all have beneficial effects on the biochemical and biomechanical properties of tissue engineered constructs. Finally, the effects of different mechanical stimulation modalities were assessed, including direct compression, hydrostatic pressure, and shear, and it was concluded that these

modalities showed promise as agents for enhancing construct functionality. However, despite the promise of prior results in the literature as well as in work performed in our own laboratory, successful tissue engineering strategies would need to optimize all parameters to generate constructs with properties approaching those of native tissue.

Chapter 2 delivered a more comprehensive overview of the use of hydrostatic pressure in tissue engineering strategies. This chapter indicated that hydrostatic pressure provides a promising method for enhancing the ECM production of chondrocytes in monolayer, in 3-D engineered constructs, as well as in tissue explants. It was apparent that pressure magnitudes within the physiologic range, generally between 3 and 10 MPa, had the most beneficial effects. Additionally, chondrocytes in monolayer seemed to respond maximally to regimens using dynamic frequencies, generally <1 Hz, while chondrocytes in explants and 3-D constructs typically responded favorably to an application of static pressure. Furthermore, this chapter demonstrated the potential use of hydrostatic pressure as a chondroprotective agent, thus suggesting its potential use as a treatment to delay the onset of osteoarthritis, as well as implying that hydrostatic pressure could enable the use of osteoarthritic chondrocytes in future tissue engineering studies. Also, hydrostatic pressure was found to be effective as a differentiation agent, for stem cells as well as dedifferentiated articular chondrocytes. Additionally, many studies have examined the use of hydrostatic pressures above the physiologic range, and have generally demonstrated detrimental

effects at these high magnitudes, particularly between 30 and 50 MPa, but also as low as 15 MPa. This indicated that tissue engineering studies should remain focused on utilizing pressures in the physiologic range for the enhancement of functional properties. Finally, as hydrostatic pressure does not result in macroscopic deformation of cartilage, it was postulated that hydrostatic pressure has a direct effect on cell transmembrane ion transporters, thus altering their activity and changing intracellular ion gradients. An altered intracellular ion concentration could activate signal transduction cascades and lead to upregulation of ECM-specific genes, and the eventually increased biochemical properties, as observed previously.

The temporal and directional effects of confinement on construct biomechanical properties were examined in Chapter 3. This was necessary as it would aid in identifying more optimal construct culture conditions. It was found that constructs confined radially for 2 wks demonstrated a significantly higher aggregate modulus than the other treatment groups, accompanied by extensive organization of collagen fibrils, forming struts in the direction perpendicular to the articular surface. Additionally, when maintaining radial confinement for a longer period, it was determined that the increased confinement time resulted in a slight decrease in aggregate modulus, thus indicating the importance of early (<2 wks) application of confinement, and possibly other forms of mechanical stimulation. Finally, passive axial compression during early construct growth resulted in increased tensile properties, accompanied by a significant increase in collagen

content. These studies indicated that mechanical stimulation, as evidenced by radial and vertical confinement, had significant effects on construct properties, as the biomechanical properties were influenced by an application of stress in the orthogonal direction.

Although construct confinement for 2 wks demonstrated beneficial results on compressive properties in Chapter 3, as this effect was highly dependent on construct growth rates, which were variable among different studies, all remaining studies involved construct confinement for only 10 days, thus controlling for the effects of confinement. However, the effects of mechanical stimulation, in the form of hydrostatic pressure, were assessed at similar timepoints to the windows observed in the confinement studies. As no prior studies had demonstrated the effects of hydrostatic pressure on construct biomechanical properties, the temporal effects of hydrostatic pressure on construct biomechanical and biochemical properties were examined in Chapter 4. This study consisted of two phases. In the first phase, a bagged control, consisting of constructs placed in an unpressurized HP chamber, was compared to a culture control, which remained in culture for the remainder of the study. It was determined that there was no difference between the controls, so the culture control was used in all subsequent HP studies in this thesis. In the next phase, the effects of 10 MPa static HP application were compared at three different times in construct development, from 6-10, 10-14, and 14-18 days. It was found that 10 MPa static HP enhanced both aggregate modulus and Young's modulus,

with parallel increases in GAG and collagen content, respectively. Additionally, it was determined that HP application from 10-14 days had the greatest effect on construct biomechanical and biochemical properties, so this application time was chosen for future HP studies in Chapter 6. Finally, in the third phase, the immediate and delayed effects of HP were assessed on construct properties, and an immediate increase in GAG production and aggregate modulus were demonstrated. However, there was an immediate increase in collagen content, but a delayed increase in tensile properties, likely as a result of the slower turnover of collagen relative to GAG production.

Although HP was shown to have significant effects on construct functional properties, they still lagged behind those of native tissue. Therefore, additional exogenous stimulation modalities, namely growth factor application, were examined in Chapter 5, as the effects of multiple growth factor treatments under serum-free conditions were assessed. Three growth factors, BMP-2, IGF-I, and TGF-β1 were examined alone and in combination, on the properties of engineered cartilage constructs. All growth factors were assessed at two concentrations each, low and high, and two dosage frequencies, in the media for the first 2 wks of culture or in the media during the first and third wks of culture. From these groups, the best treatment, in terms of construct functional properties was determined for each individual growth factor. It was determined that BMP-2, at a concentration of 10 ng/ml and the 2-wk continuous dosage, had the greatest effects on construct properties, leading to a significant increase in compressive

stiffness and GAG production. Treatment with IGF-I, at 10 ng/ml and the 2-wk rotation dosage, had a similar effect on construct properties, also leading to significant enhancement of construct compressive stiffness and GAG production. On the other hand, 30 ng/ml TGF-β1, at the 2-wk continuous dosage, led to a significant increase in compressive stiffness and GAG production, as well as a significant increase in tensile properties, accompanied by a significant increase in collagen production. These three individual treatments were then studied individually and in combinations of two and three. It was determined that the combination of BMP-2 and IGF-I resulted in the greatest increases in compressive stiffness and GAG content, while TGF-β1 alone was the only treatment that resulted in a significant increase in both compressive and tensile properties. Therefore, these two treatments were used for subsequent study, in combination with HP, in Chapter 6. These findings were exciting, as this was the only study to show increases in both compressive and tensile biomechanical properties as a result of growth factor treatment. In addition to combination with HP, future studies should assess the effects of combining these growth factor regimens with other mechanical stimulation modalities such as direct compression.

As no prior studies systematically assessed the effects of multiple physiologic regimens of HP on engineered cartilage constructs, a full-factorial study of three magnitudes, 1, 5, and 10 MPa, and three frequencies, static, 0.1, and 1 Hz, was conducted and described in Chapter 6. HP duration and application time were

selected in Chapter 4. HP application at 10 MPa, 1 Hz resulted in a significant increase in compressive stiffness and GAG content, while only static HP at 5 and 10 MPa resulted in significant increases in compressive and tensile stiffness, as well as GAG and collagen content. Therefore, these treatments were combined with the optimized growth factor treatments described in Chapter 5. It was determined that the combination of 10 MPa HP and TGF-β1 had additive and synergistic effects on tissue functional properties, with a 164% increase in compressive stiffness, a 231% in tensile stiffness, an 85% increase in GAG content, and a 173% increase in collagen content. Additionally, the collagen/WW of this group, at 15.3% was on par with native tissue. The findings of this study were extremely exciting, as engineered cartilage was produced for the first time with biochemical and biomechanical properties spanning native tissue values.

Finally, although the aforementioned results of this thesis appear very promising, all of the studies involved the use of bovine chondrocytes, which would lead to a xenogenic implant for treating articular cartilage injuries. Therefore, Chapter 7 assessed the feasibility of decellularizing self-assembled articular cartilage constructs, to create a non-immunogenic xenogenic tissue for *in vivo* implantation. The control constructs utilized in Chapters 4-6 were selected for use in this study. It was found that treatment with 2% SDS for 1 h resulted in a 33% decrease in DNA content, while maintaining GAG content, collagen content, and compressive stiffness, and even increasing tensile stiffness. On the other hand, 2% SDS treatment for 8 h led to complete histological decellularization,

with a 46% decrease in DNA content. However, there was loss of all GAG and compressive stiffness. Although complete tissue decellularization with maintenance of functional properties was not achieved in this study, the results indicate that this may be feasible with additional study.

Current tissue engineering approaches strive to obtain a construct with biomechanical, biochemical, and histological properties as close as possible to native tissue. The results described in this thesis have tremendous clinical implications. For example, the results of the growth factor and hydrostatic pressure studies outline an exogenous treatment protocol that could potentially be applied to any chondrogenically differentiated cell sources such as embryonic or mesenchymal stem cells for the creation of implantable cartilage constructs, potentially from a patient's own cells. This approach would alleviate the current issues involving the scarcity of primary chondrocytes for tissue engineering cartilage. Additionally, it is believed that this approach could be applied to enhance passaged autologous chondrocytes such as in the FDA approved autologous chondrocyte implantation procedures. Moreover, it is likely that self-assembled constructs created with bovine cells can be decellularized while maintaining their biomechanical properties, enabling the use of a xenogenic implant. To fully assess this possibility, *in vivo* studies will be performed shortly to determine the effectiveness of self-assembled constructs following implantation in a joint defect.

References

1. Benson V, Marano MA. Current estimates from the National Health Interview Survey, 1995. Vital Health Stat 10 1998(199):1-428.

2. Osteoarthritis Fact Sheet. In. http://www.arthritis.org/conditions/Fact_Sheets/OA_Fact_Sheet.asp.

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

4. Buckwalter JA, Hunziker EB, Rosenberg LC, Coutts R, Adams M, Eyre D. Articular Cartilage: Composition and Structure. 2nd ed. Park Ridge: American Academy of Orthopedic Surgeons; 1979.

5. Hu JC, Athanasiou KA. Structure and Function of Articular Cartilage. In: An YH, Martin KL, eds. Handbook of Histology Methods for Bone and Cartilage. 1st ed. Totowa, NJ: Humana Press Inc.; 2003:73-95.

6. Ruoslahti E. Structure and biology of proteoglycans. Annu Rev Cell Biol 1988;4:229-55.

7. Darling EM, Hu JC, Athanasiou KA. Zonal and topographical differences in articular cartilage gene expression. J Orthop Res 2004;22(6):1182-7.

8. Jeffery AK, Blunn GW, Archer CW, Bentley G. Three-dimensional collagen architecture in bovine articular cartilage. J Bone Joint Surg Br 1991;73(5):795-801.

9. Lai WM, Mow VC, Zhu W. Constitutive modeling of articular cartilage and biomacromolecular solutions. J Biomech Eng 1993;115(4B):474-80.

10. Mow VC, Flatow EL, Ateshian GA. Biomechanics. In: Buckwalter JA, Einhorn TA, Simon SR, eds. Orthopaedic Basic Science: Biology and Biomechanics of the Musculoskeletal System: American Academy of Orthopaedic Surgeons; 2000:140-2.

11. Hodge WA, Carlson KL, Fijan RS, Burgess RG, Riley PO, Harris WH, et al. Contact pressures from an instrumented hip endoprosthesis. J Bone Joint Surg Am 1989;71(9):1378-86.

12. Buckwalter JA. Articular cartilage injuries. Clin Orthop Relat Res 2002(402):21-37.

13. Hunziker EB. Articular cartilage repair: are the intrinsic biological constraints undermining this process insuperable? Osteoarthritis Cartilage 1999;7(1):15-28.

14. Buckwalter JA. Articular cartilage: injuries and potential for healing. J Orthop Sports Phys Ther 1998;28(4):192-202.

15. Alford JW, Cole BJ. Cartilage restoration, part 2: techniques, outcomes, and future directions. Am J Sports Med 2005;33(3):443-60.

16. Erggelet C, Browne JE, Fu F, Mandelbaum BR, Micheli LJ, Mosely JB. [Autologous chondrocyte transplantation for treatment of cartilage defects of the knee joint. Clinical results]. Zentralbl Chir 2000;125(6):516-22.

17. Wood JJ, Malek MA, Frassica FJ, Polder JA, Mohan AK, Bloom ET, et al. Autologous cultured chondrocytes: adverse events reported to the United States Food and Drug Administration. J Bone Joint Surg Am 2006;88(3):503-7.

18. Darling EM, Athanasiou KA. Biomechanical strategies for articular cartilage regeneration. Ann Biomed Eng 2003;31(9):1114-24.

19. Leipzig ND, Athanasiou KA. Cartilage Regeneration. In: Bowlin GL, Wnek G, eds. Encyclopedia of Biomaterials and Biomedical Engineering: Marcel Dekker, Inc. ; 2004:283-91.

20. Darling EM, Athanasiou KA. Bioactive Scaffold Design for Articular Cartilage Engineering. In *Biomedical Technology and Devices Handbook.* In: Moore J, Zouridakis G, eds. Biomedical Technology and Devices Handbook New York: RC Press; 2003:Ch 21 (1-16).

21. Athanasiou KA, Shah AR, Hernandez RJ, LeBaron RG. Basic science of articular cartilage repair. Clin Sports Med 2001;20(2):223-47.

22. Nehrer S, Breinan HA, Ramappa A, Shortkroff S, Young G, Minas T, et al. Canine chondrocytes seeded in type I and type II collagen implants investigated in vitro. J Biomed Mater Res 1997;38(2):95-104.

23. Frenkel SR, Di Cesare PE. Scaffolds for articular cartilage repair. Ann Biomed Eng 2004;32(1):26-34.

24. Passaretti D, Silverman RP, Huang W, Kirchhoff CH, Ashiku S, Randolph MA, et al. Cultured chondrocytes produce injectable tissue-engineered cartilage in hydrogel polymer. Tissue Eng 2001;7(6):805-15.

25. Lahiji A, Sohrabi A, Hungerford DS, Frondoza CG. Chitosan supports the expression of extracellular matrix proteins in human osteoblasts and chondrocytes. J Biomed Mater Res 2000;51(4):586-95.

26. Nettles DL, Elder SH, Gilbert JA. Potential use of chitosan as a cell scaffold material for cartilage tissue engineering. Tissue Eng 2002;8(6):1009-16.

27. Elisseeff J, Anseth K, Sims D, McIntosh W, Randolph M, Yaremchuk M, et al. Transdermal photopolymerization of poly(ethylene oxide)-based injectable hydrogels for tissue-engineered cartilage. Plast Reconstr Surg 1999;104(4):1014-22.

28. Burdick JA, Chung C, Jia X, Randolph MA, Langer R. Controlled degradation and mechanical behavior of photopolymerized hyaluronic acid networks. Biomacromolecules 2005;6(1):386-91.

29. Thornton AJ, Alsberg E, Albertelli M, Mooney DJ. Shape-defining scaffolds for minimally invasive tissue engineering. Transplantation 2004;77(12):1798-803.

30. Rahfoth B, Weisser J, Sternkopf F, Aigner T, von der Mark K, Brauer R. Transplantation of allograft chondrocytes embedded in agarose gel into cartilage defects of rabbits. Osteoarthritis Cartilage 1998;6(1):50-65.

31. Athanasiou KA, Agrawal CM, Barber FA, Burkhart SS. Orthopaedic applications for PLA-PGA biodegradable polymers. Arthroscopy 1998;14(7):726-37.

32. Almarza AJ, Athanasiou KA. Seeding techniques and scaffolding choice for tissue engineering of the temporomandibular joint disk. Tissue Eng 2004;10(11-12):1787-95.

33. Detamore MS, Athanasiou KA. Evaluation of three growth factors for TMJ disc tissue engineering. Ann Biomed Eng 2005;33(3):383-90.

34. Mercier NR, Costantino HR, Tracy MA, Bonassar LJ. A novel injectable approach for cartilage formation in vivo using PLG microspheres. Ann Biomed Eng 2004;32(3):418-29.

35. Caterson EJ, Li WJ, Nesti LJ, Albert T, Danielson K, Tuan RS. Polymer/alginate amalgam for cartilage-tissue engineering. Ann N Y Acad Sci 2002;961:134-8.

36. Hu JC, Athanasiou KA. A self-assembling process in articular cartilage tissue engineering. Tissue Eng 2006;12(4):969-79.

37. Furukawa KS, Suenaga H, Toita K, Numata A, Tanaka J, Ushida T, et al. Rapid and large-scale formation of chondrocyte aggregates by rotational culture. Cell Transplant 2003;12(5):475-9.

38. Stewart MC, Saunders KM, Burton-Wurster N, Macleod JN. Phenotypic stability of articular chondrocytes in vitro: the effects of culture models, bone morphogenetic protein 2, and serum supplementation. J Bone Miner Res 2000;15(1):166-74.

39. Hu JC, Athanasiou KA. The effects of intermittent hydrostatic pressure on self-assembled articular cartilage constructs. Tissue Eng 2006.

40. Song L, Baksh D, Tuan RS. Mesenchymal stem cell-based cartilage tissue engineering: cells, scaffold and biology. Cytotherapy 2004;6(6):596-601.

41. Takemitsu Y. The effect of age upon sulfate-S35 fixation of chondroitin sulfate in cartilage and bone of the normal white rats and S35-autoradiographic study of these tissues. Kyushu J Med Sci 1961(12):251-81.

42. Benya PD, Nimni ME. The stability of the collagen phenotype during stimulated collagen, glycosaminoglycan, and DNA synthesis by articular cartilage organ cultures. Arch Biochem Biophys 1979;192(2):327-35.

43. Benya PD, Padilla SR, Nimni ME. The progeny of rabbit articular chondrocytes synthesize collagen types I and III and type I trimer, but not type II. Verifications by cyanogen bromide peptide analysis. Biochemistry 1977;16(5):865-72.

44. Benya PD, Padilla SR, Nimni ME. Independent regulation of collagen types by chondrocytes during the loss of differentiated function in culture. Cell 1978;15(4):1313-21.

45. Schulze-Tanzil G, Mobasheri A, de Souza P, John T, Shakibaei M. Loss of chondrogenic potential in dedifferentiated chondrocytes correlates with deficient Shc-Erk interaction and apoptosis. Osteoarthritis Cartilage 2004;12(6):448-58.

46. Darling EM, Athanasiou KA. Rapid phenotypic changes in passaged articular chondrocyte subpopulations. J Orthop Res 2005;23(2):425-32.

47. Benya PD, Shaffer JD. Dedifferentiated chondrocytes reexpress the differentiated collagen phenotype when cultured in agarose gels. Cell 1982;30(1):215-24.

48. Shapiro F, Koide S, Glimcher MJ. Cell origin and differentiation in the repair of full-thickness defects of articular cartilage. J Bone Joint Surg Am 1993;75(4):532-53.

49. Butnariu-Ephrat M, Robinson D, Mendes DG, Halperin N, Nevo Z. Resurfacing of goat articular cartilage by chondrocytes derived from bone marrow. Clin Orthop Relat Res 1996(330):234-43.

50. Mauck RL, Yuan X, Tuan RS. Chondrogenic differentiation and functional maturation of bovine mesenchymal stem cells in long-term agarose culture. Osteoarthritis Cartilage 2006;14(2):179-89.

51. Yoo JU, Barthel TS, Nishimura K, Solchaga L, Caplan AI, Goldberg VM, et al. The chondrogenic potential of human bone-marrow-derived mesenchymal progenitor cells. J Bone Joint Surg Am 1998;80(12):1745-57.

52. Huang JI, Kazmi N, Durbhakula MM, Hering TM, Yoo JU, Johnstone B. Chondrogenic potential of progenitor cells derived from human bone marrow and adipose tissue: a patient-matched comparison. J Orthop Res 2005;23(6):1383-9.

53. Hegewald AA, Ringe J, Bartel J, Kruger I, Notter M, Barnewitz D, et al. Hyaluronic acid and autologous synovial fluid induce chondrogenic differentiation of equine mesenchymal stem cells: a preliminary study. Tissue Cell 2004;36(6):431-8.

54. Gronthos S, Zannettino AC, Hay SJ, Shi S, Graves SE, Kortesidis A, et al. Molecular and cellular characterisation of highly purified stromal stem cells derived from human bone marrow. J Cell Sci 2003;116(Pt 9):1827-35.

55. Nathan S, Das De S, Thambyah A, Fen C, Goh J, Lee EH. Cell-based therapy in the repair of osteochondral defects: a novel use for adipose tissue. Tissue Eng 2003;9(4):733-44.

56. Betre H, Ong SR, Guilak F, Chilkoti A, Fermor B, Setton LA. Chondrocytic differentiation of human adipose-derived adult stem cells in elastin-like polypeptide. Biomaterials 2006;27(1):91-9.

57. Awad HA, Wickham MQ, Leddy HA, Gimble JM, Guilak F. Chondrogenic differentiation of adipose-derived adult stem cells in agarose, alginate, and gelatin scaffolds. Biomaterials 2004;25(16):3211-22.

58. Wang DW, Fermor B, Gimble JM, Awad HA, Guilak F. Influence of oxygen on the proliferation and metabolism of adipose derived adult stem cells. J Cell Physiol 2005;204(1):184-91.

59. Li X, Lee JP, Balian G, Greg Anderson D. Modulation of chondrocytic properties of fat-derived mesenchymal cells in co-cultures with nucleus pulposus. Connect Tissue Res 2005;46(2):75-82.

60. Lin Y, Luo E, Chen X, Liu L, Qiao J, Yan Z, et al. Molecular and cellular characterization during chondrogenic differentiation of adipose tissue-derived stromal cells in vitro and cartilage formation in vivo. J Cell Mol Med 2005;9(4):929-39.

61. Nishimura K, Solchaga LA, Caplan AI, Yoo JU, Goldberg VM, Johnstone B. Chondroprogenitor cells of synovial tissue. Arthritis Rheum 1999;42(12):2631-7.

62. Huang JI, Beanes SR, Zhu M, Lorenz HP, Hedrick MH, Benhaim P. Rat extramedullary adipose tissue as a source of osteochondrogenic progenitor cells. Plast Reconstr Surg 2002;109(3):1033-41; discussion 42-3.

63. Wickham MQ, Erickson GR, Gimble JM, Vail TP, Guilak F. Multipotent stromal cells derived from the infrapatellar fat pad of the knee. Clin Orthop Relat Res 2003(412):196-212.

64. Keller G. Embryonic stem cell differentiation: emergence of a new era in biology and medicine. Genes Dev 2005;19(10):1129-55.

65. Kramer J, Hegert C, Guan K, Wobus AM, Muller PK, Rohwedel J. Embryonic stem cell-derived chondrogenic differentiation in vitro: activation by BMP-2 and BMP-4. Mech Dev 2000;92(2):193-205.

66. Levenberg S, Huang NF, Lavik E, Rogers AB, Itskovitz-Eldor J, Langer R. Differentiation of human embryonic stem cells on three-dimensional polymer scaffolds. Proc Natl Acad Sci U S A 2003;100(22):12741-6.

67. Levenberg S, Burdick JA, Kraehenbuehl T, Langer R. Neurotrophin-induced differentiation of human embryonic stem cells on three-dimensional polymeric scaffolds. Tissue Eng 2005;11(3-4):506-12.

68. French MM, Rose S, Canseco J, Athanasiou KA. Chondrogenic differentiation of adult dermal fibroblasts. Ann Biomed Eng 2004;32(1):50-6.

69. Yates KE, Mizuno S, Glowacki J. Early shifts in gene expression during chondroinduction of human dermal fibroblasts. Exp Cell Res 2001;265(2):203-11.

70. Mizuno S, Glowacki J. Chondroinduction of human dermal fibroblasts by demineralized bone in three-dimensional culture. Exp Cell Res 1996;227(1):89-97.

71. French MM, Athanasiou KA. Differentiation Factors and Articular Cartilage Regeneration. In: Ashammakhi N, Ferretti P, eds. Topics in Tissue Engineering; 2003:1-19.

72. Ahn JI, Terry Canale S, Butler SD, Hasty KA. Stem cell repair of physeal cartilage. J Orthop Res 2004;22(6):1215-21.

73. Galera P, Vivien D, Pronost S, Bonaventure J, Redini F, Loyau G, et al. Transforming growth factor-beta 1 (TGF-beta 1) up-regulation of collagen type II in primary cultures of rabbit articular chondrocytes (RAC) involves increased mRNA levels without affecting mRNA stability and procollagen processing. J Cell Physiol 1992;153(3):596-606.

74. Blunk T, Sieminski AL, Gooch KJ, Courter DL, Hollander AP, Nahir AM, et al. Differential effects of growth factors on tissue-engineered cartilage. Tissue Eng 2002;8(1):73-84.

75. Park Y, Sugimoto M, Watrin A, Chiquet M, Hunziker EB. BMP-2 induces the expression of chondrocyte-specific genes in bovine synovium-derived progenitor cells cultured in three-dimensional alginate hydrogel. Osteoarthritis Cartilage 2005;13(6):527-36.

76. Knippenberg M, Helder MN, Zandieh Doulabi B, Wuisman PI, Klein-Nulend J. Osteogenesis versus chondrogenesis by BMP-2 and BMP-7 in adipose stem cells. Biochem Biophys Res Commun 2006;342(3):902-8.

77. Tyler JA. Insulin-like growth factor 1 can decrease degradation and promote synthesis of proteoglycan in cartilage exposed to cytokines. Biochem J 1989;260(2):543-8.

78. Guenther HL, Guenther HE, Froesch ER, Fleisch H. Effect of insulin-like growth factor on collagen and glycosaminoglycan synthesis by rabbit articular chondrocytes in culture. Experientia 1982;38(8):979-81.

79. Luyten FP, Hascall VC, Nissley SP, Morales TI, Reddi AH. Insulin-like growth factors maintain steady-state metabolism of proteoglycans in bovine articular cartilage explants. Arch Biochem Biophys 1988;267(2):416-25.

80. Darling EM, Athanasiou KA. Growth factor impact on articular cartilage subpopulations. Cell Tissue Res 2005;322(3):463-73.

81. Nixon AJ, Saxer RA, Brower-Toland BD. Exogenous insulin-like growth factor-I stimulates an autoinductive IGF-I autocrine/paracrine response in chondrocytes. J Orthop Res 2001;19(1):26-32.

82. Arevalo-Silva CA, Cao Y, Weng Y, Vacanti M, Rodriguez A, Vacanti CA, et al. The effect of fibroblast growth factor and transforming growth factor-beta on porcine chondrocytes and tissue-engineered autologous elastic cartilage. Tissue Eng 2001;7(1):81-8.

83. Toolan BC, Frenkel SR, Pachence JM, Yalowitz L, Alexander H. Effects of growth-factor-enhanced culture on a chondrocyte-collagen implant for cartilage repair. J Biomed Mater Res 1996;31(2):273-80.

84. Fujimoto E, Ochi M, Kato Y, Mochizuki Y, Sumen Y, Ikuta Y. Beneficial effect of basic fibroblast growth factor on the repair of full-thickness defects in rabbit articular cartilage. Arch Orthop Trauma Surg 1999;119(3-4):139-45.

85. Davidson D, Blanc A, Filion D, Wang H, Plut P, Pfeffer G, et al. Fibroblast growth factor (FGF) 18 signals through FGF receptor 3 to promote chondrogenesis. J Biol Chem 2005;280(21):20509-15.

86. Amano O, Koshimizu U, Nakamura T, Iseki S. Enhancement by hepatocyte growth factor of bone and cartilage formation during embryonic mouse mandibular development in vitro. Arch Oral Biol 1999;44(11):935-46.

87. Grumbles RM, Howell DS, Wenger L, Altman RD, Howard GA, Roos BA. Hepatocyte growth factor and its actions in growth plate chondrocytes. Bone 1996;19(3):255-61.

88. Kieswetter K, Schwartz Z, Alderete M, Dean DD, Boyan BD. Platelet derived growth factor stimulates chondrocyte proliferation but prevents endochondral maturation. Endocrine 1997;6(3):257-64.

89. Hanada K, Solchaga LA, Caplan AI, Hering TM, Goldberg VM, Yoo JU, et al. BMP-2 induction and TGF-beta 1 modulation of rat periosteal cell chondrogenesis. J Cell Biochem 2001;81(2):284-94.

90. Indrawattana N, Chen G, Tadokoro M, Shann LH, Ohgushi H, Tateishi T, et al. Growth factor combination for chondrogenic induction from human mesenchymal stem cell. Biochem Biophys Res Commun 2004;320(3):914-9.

91. Kaplan BA, Gorman CR, Gupta AK, Taylor SR, Iezzoni JC, Park SS. Effects of transforming growth factor Beta and insulinlike growth factor 1 on the biomechanical and histologic properties of tissue-engineered cartilage. Arch Facial Plast Surg 2003;5(1):96-101.

92. Bonassar LJ, Grodzinsky AJ, Frank EH, Davila SG, Bhaktav NR, Trippel SB. The effect of dynamic compression on the response of articular cartilage to insulin-like growth factor-I. J Orthop Res 2001;19(1):11-7.

93. Mauck RL, Nicoll SB, Seyhan SL, Ateshian GA, Hung CT. Synergistic action of growth factors and dynamic loading for articular cartilage tissue engineering. Tissue Eng 2003;9(4):597-611.

94. Darling EM, Athanasiou KA. Retaining zonal chondrocyte phenotype by means of novel growth environments. Tissue Eng 2005;11(3-4):395-403.

95. Sailor LZ, Hewick RM, Morris EA. Recombinant human bone morphogenetic protein-2 maintains the articular chondrocyte phenotype in long-term culture. J Orthop Res 1996;14(6):937-45.

96. Pangborn CA, Athanasiou KA. Effects of growth factors on meniscal fibrochondrocytes. Tissue Eng 2005;11(7-8):1141-8.

97. Pangborn CA, Athanasiou KA. Growth factors and fibrochondrocytes in scaffolds. J Orthop Res 2005;23(5):1184-90.

98. Almarza AJ, Athanasiou KA. Evaluation of three growth factors in combinations of two for temporomandibular joint disc tissue engineering. Arch Oral Biol 2006;51(3):215-21.

99. Helminen HJ, Saamanen AM, Jurvelin J, Kiviranta I, Parkkinen JJ, Lammi MJ, et al. [The effect of loading on articular cartilage]. Duodecim 1992;108(12):1097-107.

100. Palmoski MJ, Colyer RA, Brandt KD. Joint motion in the absence of normal loading does not maintain normal articular cartilage. Arthritis Rheum 1980;23(3):325-34.

101. Jurvelin J, Kiviranta I, Tammi M, Helminen HJ. Effect of physical exercise on indentation stiffness of articular cartilage in the canine knee. Int J Sports Med 1986;7(2):106-10.

102. Hall AC, Urban JP, Gehl KA. The effects of hydrostatic pressure on matrix synthesis in articular cartilage. J Orthop Res 1991;9(1):1-10.

103. Hall AC, Horwitz ER, Wilkins RJ. The cellular physiology of articular cartilage. Exp Physiol 1996;81(3):535-45.

104. Waters RL, Lunsford BR, Perry J, Byrd R. Energy-speed relationship of walking: standard tables. J Orthop Res 1988;6(2):215-22.

105. Schwab GH, Moynes DR, Jobe FW, Perry J. Lower extremity electromyographic analysis of running gait. Clin Orthop Relat Res 1983(176):166-70.

106. Darling EM, Athanasiou KA. Articular cartilage bioreactors and bioprocesses. Tissue Eng 2003;9(1):9-26.

107. Lammi MJ, Inkinen R, Parkkinen JJ, Hakkinen T, Jortikka M, Nelimarkka LO, et al. Expression of reduced amounts of structurally altered aggrecan in articular cartilage chondrocytes exposed to high hydrostatic pressure. Biochem J 1994;304 (Pt 3):723-30.

108. Takahashi K, Kubo T, Arai Y, Kitajima I, Takigawa M, Imanishi J, et al. Hydrostatic pressure induces expression of interleukin 6 and tumour necrosis factor alpha mRNAs in a chondrocyte-like cell line. Ann Rheum Dis 1998;57(4):231-6.

109. Parkkinen JJ, Lammi MJ, Pelttari A, Helminen HJ, Tammi M, Virtanen I. Altered Golgi apparatus in hydrostatically loaded articular cartilage chondrocytes. Ann Rheum Dis 1993;52(3):192-8.

110. Armstrong CG, Bahrani AS, Gardner DL. In vitro measurement of articular cartilage deformations in the intact human hip joint under load. J Bone Joint Surg Am 1979;61(5):744-55.

111. Mauck RL, Soltz MA, Wang CC, Wong DD, Chao PH, Valhmu WB, et al. Functional tissue engineering of articular cartilage through dynamic loading of chondrocyte-seeded agarose gels. J Biomech Eng 2000;122(3):252-60.

112. Lee DA, Noguchi T, Frean SP, Lees P, Bader DL. The influence of mechanical loading on isolated chondrocytes seeded in agarose constructs. Biorheology 2000;37(1-2):149-61.

113. Angele P, Schumann D, Angele M, Kinner B, Englert C, Hente R, et al. Cyclic, mechanical compression enhances chondrogenesis of mesenchymal progenitor cells in tissue engineering scaffolds. Biorheology 2004;41(3-4):335-46.

114. Vunjak-Novakovic G, Obradovic B, Martin I, Bursac PM, Langer R, Freed LE. Dynamic cell seeding of polymer scaffolds for cartilage tissue engineering. Biotechnol Prog 1998;14(2):193-202.

115. Bueno EM, Bilgen B, Carrier RL, Barabino GA. Increased rate of chondrocyte aggregation in a wavy-walled bioreactor. Biotechnol Bioeng 2004;88(6):767-77.

116. Raimondi MT, Boschetti F, Falcone L, Migliavacca F, Remuzzi A, Dubini G. The effect of media perfusion on three-dimensional cultures of human chondrocytes: integration of experimental and computational approaches. Biorheology 2004;41(3-4):401-10.

117. Janssen FW, Oostra J, Oorschot A, van Blitterswijk CA. A perfusion bioreactor system capable of producing clinically relevant volumes of tissue-engineered bone: in vivo bone formation showing proof of concept. Biomaterials 2006;27(3):315-23.

118. Saini S, Wick TM. Effect of low oxygen tension on tissue-engineered cartilage construct development in the concentric cylinder bioreactor. Tissue Eng 2004;10(5-6):825-32.

119. Detamore MS, Athanasiou KA. Use of a rotating bioreactor toward tissue engineering the temporomandibular joint disc. Tissue Eng 2005;11(7-8):1188-97.

120. Seidel JO, Pei M, Gray ML, Langer R, Freed LE, Vunjak-Novakovic G. Long-term culture of tissue engineered cartilage in a perfused chamber with mechanical stimulation. Biorheology 2004;41(3-4):445-58.

121. Afoke NY, Byers PD, Hutton WC. Contact pressures in the human hip joint. J Bone Joint Surg Br 1987;69(4):536-41.

122. Smith RL, Carter DR, Schurman DJ. Pressure and shear differentially alter human articular chondrocyte metabolism: a review. Clin Orthop Relat Res 2004(427 Suppl):S89-95.

123. Wilkins RJ, Browning JA, Urban JP. Chondrocyte regulation by mechanical load. Biorheology 2000;37(1-2):67-74.

124. Hansen U, Schunke M, Domm C, Ioannidis N, Hassenpflug J, Gehrke T, et al. Combination of reduced oxygen tension and intermittent hydrostatic pressure: a useful tool in articular cartilage tissue engineering. J Biomech 2001;34(7):941-9.

125. Scherer K, Schunke M, Sellckau R, Hassenpflug J, Kurz B. The influence of oxygen and hydrostatic pressure on articular chondrocytes and adherent bone marrow cells in vitro. Biorheology 2004;41(3-4):323-33.

126. Suh JK, Baek GH, Aroen A, Malin CM, Niyibizi C, Evans CH, et al. Intermittent sub-ambient interstitial hydrostatic pressure as a potential mechanical stimulator for chondrocyte metabolism. Osteoarthritis Cartilage 1999;7(1):71-80.

127. Jortikka MO, Parkkinen JJ, Inkinen RI, Karner J, Jarvelainen HT, Nelimarkka LO, et al. The role of microtubules in the regulation of proteoglycan synthesis in chondrocytes under hydrostatic pressure. Arch Biochem Biophys 2000;374(2):172-80.

128. Smith RL, Rusk SF, Ellison BE, Wessells P, Tsuchiya K, Carter DR, et al. In vitro stimulation of articular chondrocyte mRNA and extracellular matrix synthesis by hydrostatic pressure. J Orthop Res 1996;14(1):53-60.

129. Smith RL, Lin J, Trindade MC, Shida J, Kajiyama G, Vu T, et al. Time-dependent effects of intermittent hydrostatic pressure on articular chondrocyte type II collagen and aggrecan mRNA expression. J Rehabil Res Dev 2000;37(2):153-61.

130. Ikenoue T, Trindade MC, Lee MS, Lin EY, Schurman DJ, Goodman SB, et al. Mechanoregulation of human articular chondrocyte aggrecan and type II collagen expression by intermittent hydrostatic pressure in vitro. J Orthop Res 2003;21(1):110-6.

131. Takahashi K, Kubo T, Kobayashi K, Imanishi J, Takigawa M, Arai Y, et al. Hydrostatic pressure influences mRNA expression of transforming growth factor-beta 1 and heat shock protein 70 in chondrocyte-like cell line. J Orthop Res 1997;15(1):150-8.

132. Parkkinen JJ, Ikonen J, Lammi MJ, Laakkonen J, Tammi M, Helminen HJ. Effects of cyclic hydrostatic pressure on proteoglycan synthesis in cultured chondrocytes and articular cartilage explants. Arch Biochem Biophys 1993;300(1):458-65.

133. Carver SE, Heath CA. Increasing extracellular matrix production in regenerating cartilage with intermittent physiological pressure. Biotechnol Bioeng 1999;62(2):166-74.

134. Carver SE, Heath CA. Semi-continuous perfusion system for delivering intermittent physiological pressure to regenerating cartilage. Tissue Eng 1999;5(1):1-11.

135. Hu JC, Athanasiou KA. The effects of intermittent hydrostatic pressure on self-assembled articular cartilage constructs. Tissue Eng 2006;12(5):1337-44.

136. Mizuno S, Tateishi T, Ushida T, Glowacki J. Hydrostatic fluid pressure enhances matrix synthesis and accumulation by bovine chondrocytes in three-dimensional culture. J Cell Physiol 2002;193(3):319-27.

137. Toyoda T, Seedhom BB, Kirkham J, Bonass WA. Upregulation of aggrecan and type II collagen mRNA expression in bovine chondrocytes by the application of hydrostatic pressure. Biorheology 2003;40(1-3):79-85.

138. Toyoda T, Seedhom BB, Yao JQ, Kirkham J, Brookes S, Bonass WA. Hydrostatic pressure modulates proteoglycan metabolism in chondrocytes seeded in agarose. Arthritis Rheum 2003;48(10):2865-72.

139. Elder BD, Athanasiou KA. Synergistic and additive effects of hydrostatic pressure and growth factors on tissue formation. PLoS ONE 2008;3(6):e2341.

140. Elder BD, Athanasiou KA. Effects of confinement on the mechanical properties of self-assembled articular cartilage constructs in the direction orthogonal to the confinement surface. J Orthop Res 2008;26(2):238-46.

141. Trindade MC, Shida J, Ikenoue T, Lee MS, Lin EY, Yaszay B, et al. Intermittent hydrostatic pressure inhibits matrix metalloproteinase and pro-inflammatory mediator release from human osteoarthritic chondrocytes in vitro. Osteoarthritis Cartilage 2004;12(9):729-35.

142. Lee MS, Ikenoue T, Trindade MC, Wong N, Goodman SB, Schurman DJ, et al. Protective effects of intermittent hydrostatic pressure on osteoarthritic chondrocytes activated by bacterial endotoxin in vitro. J Orthop Res 2003;21(1):117-22.

143. Lee MS, Trindade MC, Ikenoue T, Schurman DJ, Goodman SB, Smith RL. Intermittent hydrostatic pressure inhibits shear stress-induced nitric oxide release in human osteoarthritic chondrocytes in vitro. J Rheumatol 2003;30(2):326-8.

144. Fioravanti A, Cantarini L, Chellini F, Manca D, Paccagnini E, Marcolongo R, et al. Effect of hyaluronic acid (MW 500-730 kDa) on proteoglycan and nitric oxide production in human osteoarthritic chondrocyte cultures exposed to hydrostatic pressure. Osteoarthritis Cartilage 2005;13(8):688-96.

145. Gavenis K, Kremer A, Von Walter M, Hollander DA, Schneider U, Schmidt-Rohlfing B. Effects of cyclic hydrostatic pressure on the metabolism of human osteoarthritic chondrocytes cultivated in a collagen gel. Artif Organs 2007;31(2):91-8.

146. Wong M, Siegrist M, Goodwin K. Cyclic tensile strain and cyclic hydrostatic pressure differentially regulate expression of hypertrophic markers in primary chondrocytes. Bone 2003;33(4):685-93.

147. Islam N, Haqqi TM, Jepsen KJ, Kraay M, Welter JF, Goldberg VM, et al. Hydrostatic pressure induces apoptosis in human chondrocytes from osteoarthritic cartilage through up-regulation of tumor necrosis factor-alpha, inducible nitric oxide synthase, p53, c-myc, and bax-alpha, and suppression of bcl-2. J Cell Biochem 2002;87(3):266-78.

148. Angele P, Yoo JU, Smith C, Mansour J, Jepsen KJ, Nerlich M, et al. Cyclic hydrostatic pressure enhances the chondrogenic phenotype of human mesenchymal progenitor cells differentiated in vitro. J Orthop Res 2003;21(3):451-7.

149. Luo ZJ, Seedhom BB. Light and low-frequency pulsatile hydrostatic pressure enhances extracellular matrix formation by bone marrow mesenchymal cells: an in-vitro study with special reference to cartilage repair. Proc Inst Mech Eng [H] 2007;221(5):499-507.

150. Wagner DR, Lindsey DP, Li KW, Tummala P, Chandran SE, Smith RL, et al. Hydrostatic pressure enhances chondrogenic differentiation of human bone marrow stromal cells in osteochondrogenic medium. Ann Biomed Eng 2008;36(5):813-20.

151. Miyanishi K, Trindade MC, Lindsey DP, Beaupre GS, Carter DR, Goodman SB, et al. Effects of hydrostatic pressure and transforming growth factor-beta 3 on adult human mesenchymal stem cell chondrogenesis in vitro. Tissue Eng 2006;12(6):1419-28.

152. Miyanishi K, Trindade MC, Lindsey DP, Beaupre GS, Carter DR, Goodman SB, et al. Dose- and time-dependent effects of cyclic hydrostatic pressure on transforming growth factor-beta3-induced chondrogenesis by adult human mesenchymal stem cells in vitro. Tissue Eng 2006;12(8):2253-62.

153. Elder SH, Fulzele KS, McCulley WR. Cyclic hydrostatic compression stimulates chondroinduction of C3H/10T1/2 cells. Biomech Model Mechanobiol 2005;3(3):141-6.

154. Heyland J, Wiegandt K, Goepfert C, Nagel-Heyer S, Ilinich E, Schumacher U, et al. Redifferentiation of chondrocytes and cartilage formation under intermittent hydrostatic pressure. Biotechnol Lett 2006;28(20):1641-8.

155. Kawanishi M, Oura A, Furukawa K, Fukubayashi T, Nakamura K, Tateishi T, et al. Redifferentiation of dedifferentiated bovine articular chondrocytes enhanced by cyclic hydrostatic pressure under a gas-controlled system. Tissue Eng 2007;13(5):957-64.

156. Nakamura S, Arai Y, Takahashi KA, Terauchi R, Ohashi S, Mazda O, et al. Hydrostatic pressure induces apoptosis of chondrocytes cultured in alginate beads. J Orthop Res 2006;24(4):733-9.

157. Fioravanti A, Benetti D, Coppola G, Collodel G. Effect of continuous high hydrostatic pressure on the morphology and cytoskeleton of normal and osteoarthritic human chondrocytes cultivated in alginate gels. Clin Exp Rheumatol 2005;23(6):847-53.

158. Parkkinen JJ, Lammi MJ, Inkinen R, Jortikka M, Tammi M, Virtanen I, et al. Influence of short-term hydrostatic pressure on organization of stress fibers in cultured chondrocytes. J Orthop Res 1995;13(4):495-502.

159. Sironen R, Elo M, Kaarniranta K, Helminen HJ, Lammi MJ. Transcriptional activation in chondrocytes submitted to hydrostatic pressure. Biorheology 2000;37(1-2):85-93.

160. Sironen RK, Karjalainen HM, Torronen K, Elo MA, Kaarniranta K, Takigawa M, et al. High pressure effects on cellular expression profile and mRNA stability. A cDNA array analysis. Biorheology 2002;39(1-2):111-7.

161. Kaarniranta K, Elo M, Sironen R, Lammi MJ, Goldring MB, Eriksson JE, et al. Hsp70 accumulation in chondrocytic cells exposed to high continuous hydrostatic pressure coincides with mRNA stabilization rather than transcriptional activation. Proc Natl Acad Sci U S A 1998;95(5):2319-24.

162. Bachrach NM, Mow VC, Guilak F. Incompressibility of the solid matrix of articular cartilage under high hydrostatic pressures. J Biomech 1998;31(5):445-51.

163. Tanck E, van Driel WD, Hagen JW, Burger EH, Blankevoort L, Huiskes R. Why does intermittent hydrostatic pressure enhance the mineralization process in fetal cartilage? J Biomech 1999;32(2):153-61.

164. Wilkes RP, Athanasiou KA. The Intrinsic Incompressibility of Osteoblast-like Cells. Tissue Eng 1996;2(3):167-81.

165. Hall AC. Differential effects of hydrostatic pressure on cation transport pathways of isolated articular chondrocytes. J Cell Physiol 1999;178(2):197-204.

166. Browning JA, Walker RE, Hall AC, Wilkins RJ. Modulation of Na+ x H+ exchange by hydrostatic pressure in isolated bovine articular chondrocytes. Acta Physiol Scand 1999;166(1):39-45.

167. Mizuno S. A novel method for assessing effects of hydrostatic fluid pressure on intracellular calcium: a study with bovine articular chondrocytes. Am J Physiol Cell Physiol 2005;288(2):C329-37.

168. Browning JA, Saunders K, Urban JP, Wilkins RJ. The influence and interactions of hydrostatic and osmotic pressures on the intracellular milieu of chondrocytes. Biorheology 2004;41(3-4):299-308.

169. Kornblatt JA, Kornblatt MJ. The effects of osmotic and hydrostatic pressures on macromolecular systems. Biochim Biophys Acta 2002;1595(1-2):30-47.

170. Horowitz SB, Lau YT. A function that relates protein synthetic rates to potassium activity in vivo. J Cell Physiol 1988;135(3):425-34.

171. Hunter CJ, Imler SM, Malaviya P, Nerem RM, Levenston ME. Mechanical compression alters gene expression and extracellular matrix synthesis by chondrocytes cultured in collagen I gels. Biomaterials 2002;23(4):1249-59.

172. Sah RL, Kim YJ, Doong JY, Grodzinsky AJ, Plaas AH, Sandy JD. Biosynthetic response of cartilage explants to dynamic compression. J Orthop Res 1989;7(5):619-36.

173. AufderHeide AC, Athanasiou KA. Mechanical stimulation toward tissue engineering of the knee meniscus. Ann Biomed Eng 2004;32(8):1161-74.

174. Stoddart MJ, Ettinger L, Hauselmann HJ. Enhanced matrix synthesis in de novo, scaffold free cartilage-like tissue subjected to compression and shear. Biotechnol Bioeng 2006;95(6):1043-51.

175. Waldman SD, Spiteri CG, Grynpas MD, Pilliar RM, Hong J, Kandel RA. Effect of biomechanical conditioning on cartilaginous tissue formation in vitro. J Bone Joint Surg Am 2003;85-A Suppl 2:101-5.

176. Waldman SD, Spiteri CG, Grynpas MD, Pilliar RM, Kandel RA. Long-term intermittent compressive stimulation improves the composition and mechanical properties of tissue-engineered cartilage. Tissue Eng 2004;10(9-10):1323-31.

177. Waldman SD, Spiteri CG, Grynpas MD, Pilliar RM, Kandel RA. Long-term intermittent shear deformation improves the quality of cartilaginous tissue formed in vitro. J Orthop Res 2003;21(4):590-6.

178. Almarza AJ, Athanasiou KA. Effects of hydrostatic pressure on TMJ disc cells. Tissue Eng 2006;12(5):1285-94.

179. Lammi MJ, Elo MA, Sironen RK, Karjalainen HM, Kaarniranta K, Helminen HJ. Hydrostatic pressure-induced changes in cellular protein synthesis. Biorheology 2004;41(3-4):309-13.

180. Kelly TA, Wang CC, Mauck RL, Ateshian GA, Hung CT. Role of cell-associated matrix in the development of free-swelling and dynamically loaded chondrocyte-seeded agarose gels. Biorheology 2004;41(3-4):223-37.

181. Khalafi A, Schmid TM, Neu C, Reddi AH. Increased accumulation of superficial zone protein (SZP) in articular cartilage in response to bone morphogenetic protein-7 and growth factors. J Orthop Res 2007;25(3):293-303.

182. Shimizu M, Minakuchi K, Kaji S, Koga J. Chondrocyte migration to fibronectin, type I collagen, and type II collagen. Cell Struct Funct 1997;22(3):309-15.

183. Rosenberg L. Chemical basis for the histological use of safranin O in the study of articular cartilage. J Bone Joint Surg Am 1971(53):69-82.

184. Brown AN, Kim BS, Alsberg E, Mooney DJ. Combining chondrocytes and smooth muscle cells to engineer hybrid soft tissue constructs. Tissue Eng 2000;6(4):297-305.

185. Pietila K, Kantomaa T, Pirttiniemi P, Poikela A. Comparison of amounts and properties of collagen and proteoglycans in condylar, costal and nasal cartilages. Cells Tissues Organs 1999;164(1):30-6.

186. Woessner JF, Jr. The determination of hydroxyproline in tissue and protein samples containing small proportions of this imino acid. Arch Biochem Biophys 1961;93:440-7.

187. Athanasiou KA, Agarwal A, Dzida FJ. Comparative study of the intrinsic mechanical properties of the human acetabular and femoral head cartilage. J Orthop Res 1994;12(3):340-9.

188. Sneddon I. The relaxation between load and penetration in the axisymmetric Boussinesq problem for a punch of arbitrary profile. Int J Eng Sci 1965(3):47-57.

189. Hayes WC, Keer LM, Herrmann G, Mockros LF. A mathematical analysis for indentation tests of articular cartilage. J Biomech 1972;5(5):541-51.

190. Detamore MS, Athanasiou KA. Tensile properties of the porcine temporomandibular joint disc. J Biomech Eng 2003;125(4):558-65.

191. Mauck RL, Wang CC, Oswald ES, Ateshian GA, Hung CT. The role of cell seeding density and nutrient supply for articular cartilage tissue engineering with deformational loading. Osteoarthritis Cartilage 2003;11(12):879-90.

192. Kelly TA, Ng KW, Wang CC, Ateshian GA, Hung CT. Spatial and temporal development of chondrocyte-seeded agarose constructs in free-swelling and dynamically loaded cultures. J Biomech 2006;39(8):1489-97.

193. Kiviranta P, Rieppo J, Korhonen RK, Julkunen P, Toyras J, Jurvelin JS. Collagen network primarily controls Poisson's ratio of bovine articular cartilage in compression. J Orthop Res 2006;24(4):690-9.

194. Boustany NN, Gray ML, Black AC, Hunziker EB. Time-dependent changes in the response of cartilage to static compression suggest interstitial pH is not the only signaling mechanism. J Orthop Res 1995;13(5):740-50.

195. Waldman SD, Couto DC, Grynpas MD, Pilliar RM, Kandel RA. A single application of cyclic loading can accelerate matrix deposition and enhance the properties of tissue-engineered cartilage. Osteoarthritis Cartilage 2006;14(4):323-30.

196. Huberti HH, Hayes WC. Patellofemoral contact pressures. The influence of q-angle and tendofemoral contact. J Bone Joint Surg Am 1984;66(5):715-24.

197. Aufderheide AC, Athanasiou KA. Assessment of a bovine co-culture, scaffold-free method for growing meniscus-shaped constructs. Tissue Eng 2007;13(9):2195-205.

198. Gooch KJ, Blunk T, Courter DL, Sieminski AL, Vunjak-Novakovic G, Freed LE. Bone morphogenetic proteins-2, -12, and -13 modulate in vitro development of engineered cartilage. Tissue Eng 2002;8(4):591-601.

199. Ng KW, O'Conor CJ, Kugler LE, Ateshian GA, Hung CT. The response of engineered cartilage to a timed application of transforming and insulin-like growth factors. In: Transactions of the ORS 2008; 33:588.

200. Chubinskaya S, Hakimiyan A, Pacione C, Yanke A, Rappoport L, Aigner T, et al. Synergistic effect of IGF-1 and OP-1 on matrix formation by normal and OA chondrocytes cultured in alginate beads. Osteoarthritis Cartilage 2007;15(4):421-30.

201. Lieb E, Milz S, Vogel T, Hacker M, Dauner M, Schulz MB. Effects of transforming growth factor beta1 on bonelike tissue formation in three-dimensional cell culture. I. Culture conditions and tissue formation. Tissue Eng 2004;10(9-10):1399-413.

202. Lieb E, Vogel T, Milz S, Dauner M, Schulz MB. Effects of transforming growth factor beta1 on bonelike tissue formation in three-dimensional cell culture. II: Osteoblastic differentiation. Tissue Eng 2004;10(9-10):1414-25.

203. Luyten FP, Yu YM, Yanagishita M, Vukicevic S, Hammonds RG, Reddi AH. Natural bovine osteogenin and recombinant human bone morphogenetic protein-2B are equipotent in the maintenance of proteoglycans in bovine articular cartilage explant cultures. J Biol Chem 1992;267(6):3691-5.

204. Sah RL, Chen AC, Grodzinsky AJ, Trippel SB. Differential effects of bFGF and IGF-I on matrix metabolism in calf and adult bovine cartilage explants. Arch Biochem Biophys 1994;308(1):137-47.

205. Fortier LA, Lust G, Mohammed HO, Nixon AJ. Coordinate upregulation of cartilage matrix synthesis in fibrin cultures supplemented with exogenous insulin-like growth factor-I. J Orthop Res 1999;17(4):467-74.

206. Fortier LA, Nixon AJ, Mohammed HO, Lust G. Altered biological activity of equine chondrocytes cultured in a three-dimensional fibrin matrix and supplemented with transforming growth factor beta-1. Am J Vet Res 1997;58(1):66-70.

207. Galera P, Redini F, Vivien D, Bonaventure J, Penfornis H, Loyau G, et al. Effect of transforming growth factor-beta 1 (TGF-beta 1) on matrix synthesis by monolayer cultures of rabbit articular chondrocytes during the dedifferentiation process. Exp Cell Res 1992;200(2):379-92.

208. Bos PK, van Osch GJ, Frenz DA, Verhaar JA, Verwoerd-Verhoef HL. Growth factor expression in cartilage wound healing: temporal and spatial immunolocalization in a rabbit auricular cartilage wound model. Osteoarthritis Cartilage 2001;9(4):382-9.

209. Goldring MB, Tsuchimochi K, Ijiri K. The control of chondrogenesis. J Cell Biochem 2006;97(1):33-44.

210. Tsukazaki T, Usa T, Matsumoto T, Enomoto H, Ohtsuru A, Namba H, et al. Effect of transforming growth factor-beta on the insulin-like growth factor-I autocrine/paracrine axis in cultured rat articular chondrocytes. Exp Cell Res 1994;215(1):9-16.

211. Suzuki S, Nagano T, Yamakoshi Y, Gomi K, Arai T, Fukae M, et al. Enamel matrix derivative gel stimulates signal transduction of BMP and TGF-{beta}. J Dent Res 2005;84(6):510-4.

212. Suzuki T, Toyoda T, Suzuki H, Hisamori N, Matsumoto H, Toyama Y. Hydrostatic pressure modulates mRNA expressions for matrix proteins in human meniscal cells. Biorheology 2006;43(5):611-22.

213. Kasra M, Merryman WD, Loveless KN, Goel VK, Martin JD, Buckwalter JA. Frequency response of pig intervertebral disc cells subjected to dynamic hydrostatic pressure. J Orthop Res 2006;24(10):1967-73.

214. Reza AT, Nicoll SB. Hydrostatic pressure differentially regulates outer and inner annulus fibrosus cell matrix production in 3D scaffolds. Ann Biomed Eng 2008;36(2):204-13.

215. Wuertz K, Urban JP, Klasen J, Ignatius A, Wilke HJ, Claes L, et al. Influence of extracellular osmolarity and mechanical stimulation on gene expression of intervertebral disc cells. J Orthop Res 2007;25(11):1513-22.

216. Nowlan NC, Murphy P, Prendergast PJ. A dynamic pattern of mechanical stimulation promotes ossification in avian embryonic long bones. J Biomech 2008;41(2):249-58.

217. Stover J, Nagatomi J. Cyclic pressure stimulates DNA synthesis through the PI3K/Akt signaling pathway in rat bladder smooth muscle cells. Ann Biomed Eng 2007;35(9):1585-94.

218. Muller-Marschhausen K, Waschke J, Drenckhahn D. Physiological hydrostatic pressure protects endothelial monolayer integrity. Am J Physiol Cell Physiol 2008;294(1):C324-32.

219. Slinker BK. The statistics of synergism. J Mol Cell Cardiol 1998;30(4):723-31.

220. Govinden R, Bhoola KD. Genealogy, expression, and cellular function of transforming growth factor-beta. Pharmacol Ther 2003;98(2):257-65.

221. Gilbert TW, Sellaro TL, Badylak SF. Decellularization of tissues and organs. Biomaterials 2006;27(19):3675-83.

222. Chen RN, Ho HO, Tsai YT, Sheu MT. Process development of an acellular dermal matrix (ADM) for biomedical applications. Biomaterials 2004;25(13):2679-86.

223. Stapleton TW, Ingram J, Katta J, Knight R, Korossis S, Fisher J, et al. Development and characterization of an acellular porcine medial meniscus for use in tissue engineering. Tissue Eng Part A 2008;14(4):505-18.

224. Lumpkins SB, Pierre N, McFetridge PS. A mechanical evaluation of three decellularization methods in the design of a xenogeneic scaffold for tissue engineering the temporomandibular joint disc. Acta Biomater 2008;4(4):808-16.

225. Cartmell JS, Dunn MG. Effect of chemical treatments on tendon cellularity and mechanical properties. J Biomed Mater Res 2000;49(1):134-40.

226. Woods T, Gratzer PF. Effectiveness of three extraction techniques in the development of a decellularized bone-anterior cruciate ligament-bone graft. Biomaterials 2005;26(35):7339-49.

227. Liao J, Joyce EM, Sacks MS. Effects of decellularization on the mechanical and structural properties of the porcine aortic valve leaflet. Biomaterials 2008;29(8):1065-74.

228. Kasimir MT, Rieder E, Seebacher G, Silberhumer G, Wolner E, Weigel G, et al. Comparison of different decellularization procedures of porcine heart valves. Int J Artif Organs 2003;26(5):421-7.

229. Seebacher G, Grasl C, Stoiber M, Rieder E, Kasimir MT, Dunkler D, et al. Biomechanical properties of decellularized porcine pulmonary valve conduits. Artif Organs 2008;32(1):28-35.

230. Tudorache I, Cebotari S, Sturz G, Kirsch L, Hurschler C, Hilfiker A, et al. Tissue engineering of heart valves: biomechanical and morphological properties of decellularized heart valves. J Heart Valve Dis 2007;16(5):567-73; discussion 74.

231. Grauss RW, Hazekamp MG, Oppenhuizen F, van Munsteren CJ, Gittenberger-de Groot AC, DeRuiter MC. Histological evaluation of decellularised porcine aortic valves: matrix changes due to different decellularisation methods. Eur J Cardiothorac Surg 2005;27(4):566-71.

232. Meyer SR, Chiu B, Churchill TA, Zhu L, Lakey JR, Ross DB. Comparison of aortic valve allograft decellularization techniques in the rat. J Biomed Mater Res A 2006;79(2):254-62.

233. Meyer SR, Nagendran J, Desai LS, Rayat GR, Churchill TA, Anderson CC, et al. Decellularization reduces the immune response to aortic valve allografts in the rat. J Thorac Cardiovasc Surg 2005;130(2):469-76.

234. Rosario DJ, Reilly GC, Ali Salah E, Glover M, Bullock AJ, Macneil S. Decellularization and sterilization of porcine urinary bladder matrix for tissue engineering in the lower urinary tract. Regen Med 2008;3(2):145-56.

235. Dahl SL, Koh J, Prabhakar V, Niklason LE. Decellularized native and engineered arterial scaffolds for transplantation. Cell Transplant 2003;12(6):659-66.

236. Hodde J, Hiles M. Virus safety of a porcine-derived medical device: evaluation of a viral inactivation method. Biotechnol Bioeng 2002;79(2):211-6.

237. Hodde J, Janis A, Ernst D, Zopf D, Sherman D, Johnson C. Effects of sterilization on an extracellular matrix scaffold: part I. Composition and matrix architecture. J Mater Sci Mater Med 2007;18(4):537-43.

238. Elder BD, Athanasiou KA. Systematic assessment of growth factor treatment on biochemical and biomechanical properties of engineered articular cartilage constructs. Osteoarthritis Cartilage 2008.

239. Revell CM, Athanasiou KA. Success rates and immunologic responses of autogenic, allogenic, and xenogenic treatments to repair articular cartilage defects. Tissue Eng Part B (Submitted) 2008.

Appendix A: Figures

Chapter 1: Figures 1-3

Chapter 2: Figures 4-6

Chapter 3: Figures 7-10

Chapter 4: Figures 11-14

Chapter 5: Figures 15-20

Chapter 6: Figures 21-23

Chapter 7: Figures 24-29

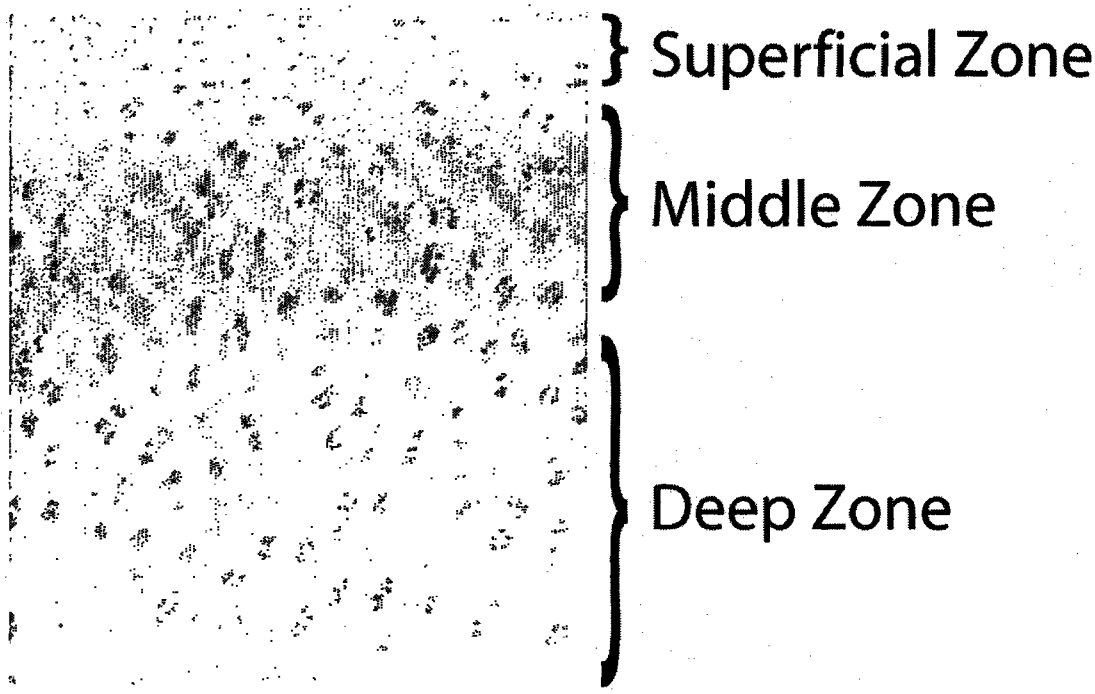


Figure 1: Zonal arrangement of cartilage.

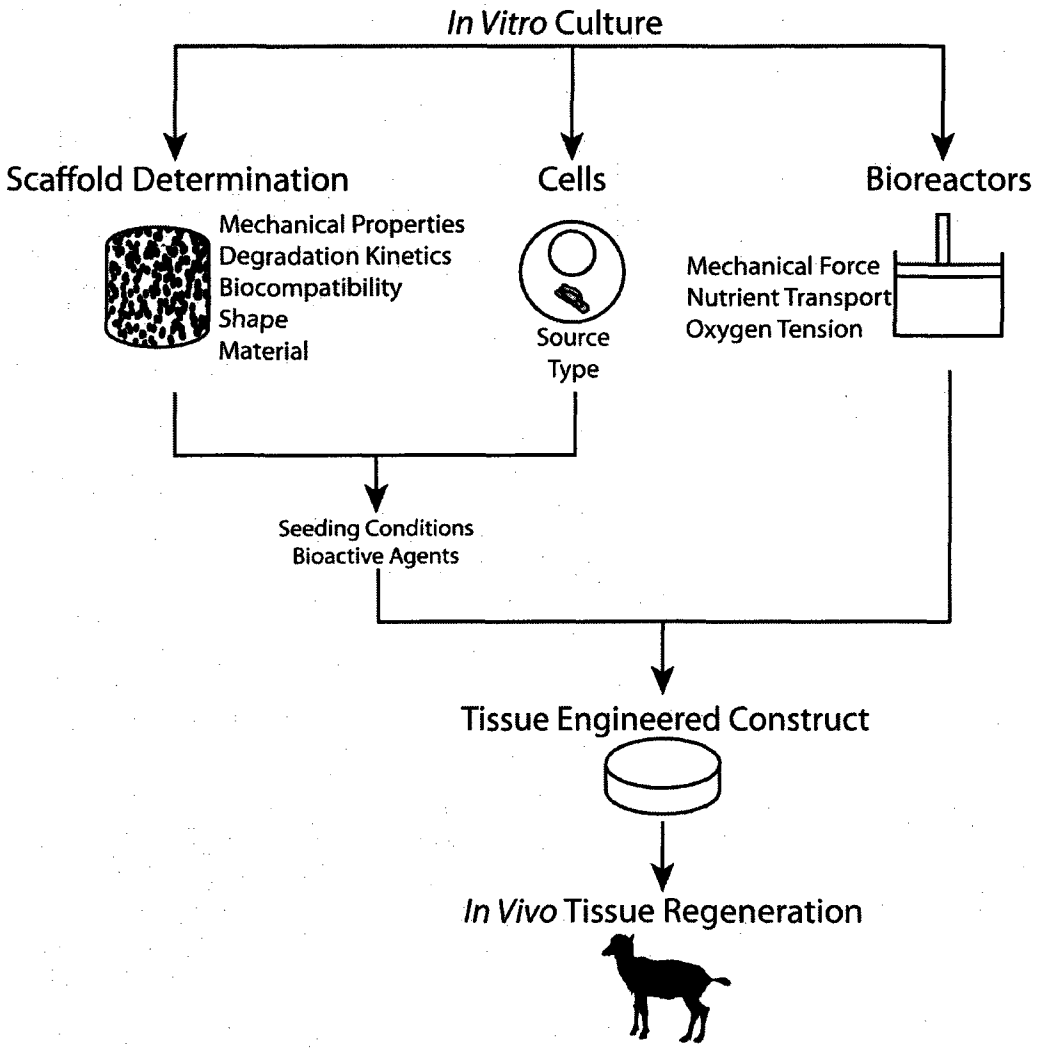


Figure 2: Tissue engineering paradigm.

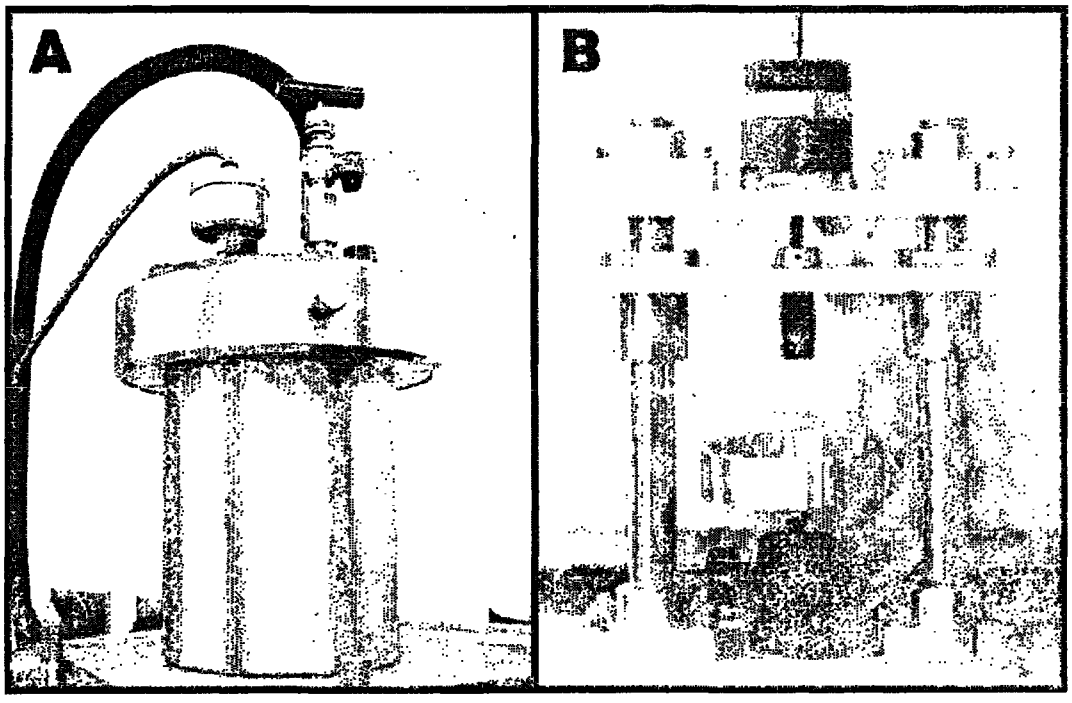


Figure 3: (A) Hydrostatic pressure chamber. (B) Direct compression device.

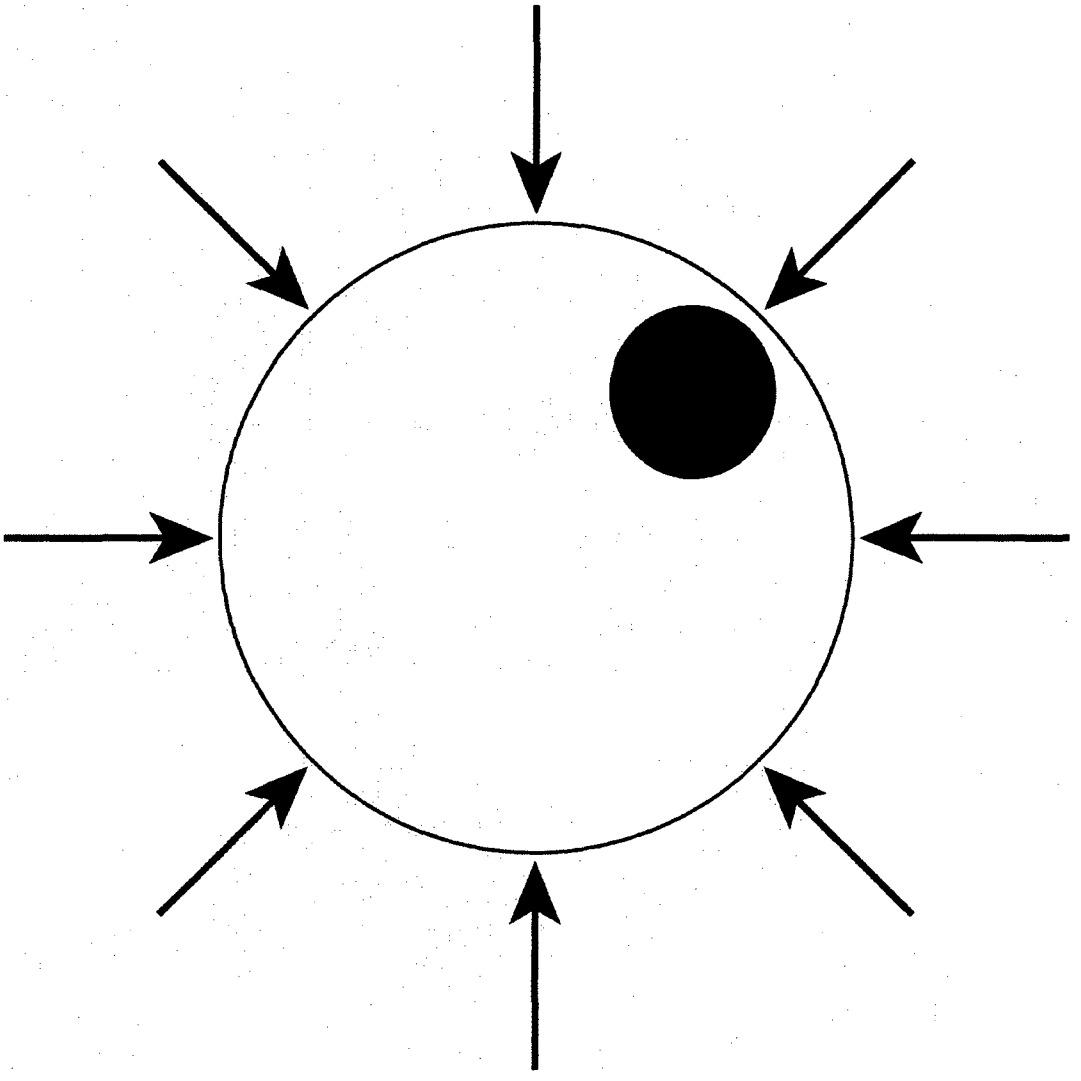


Figure 4: Illustration of a chondrocyte exposed to HP.
The cell experiences a uniform stress, without any measurable tissue strain.

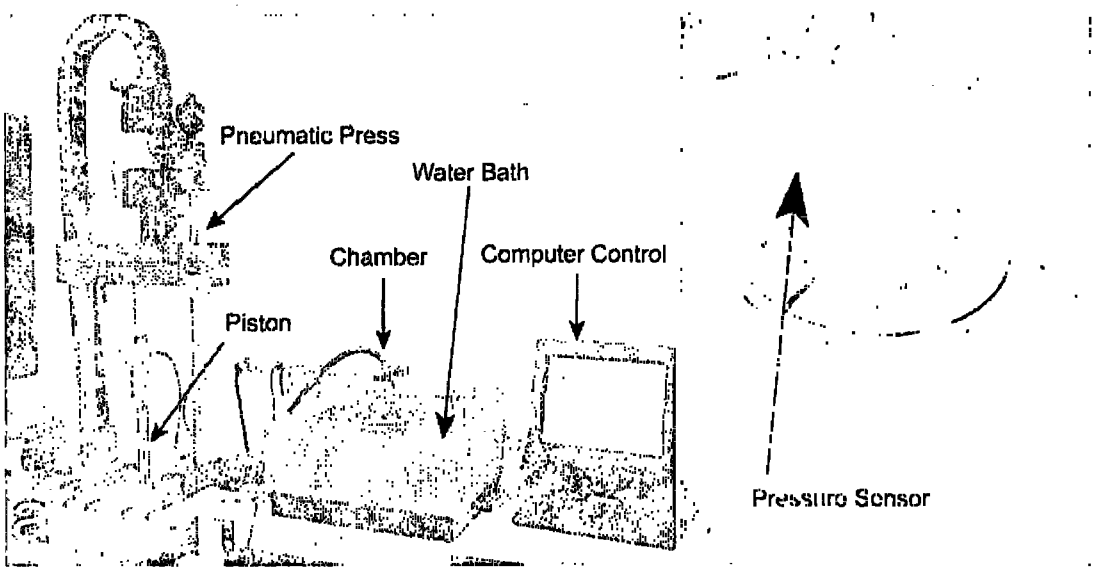


Figure 5: Representative HP bioreactor design.
(a) Computer controls Instron, which compresses piston and generates pressure within chamber. Chamber is placed in water bath to maintain temperature at 37°C. (b) HP chamber, with pressure sensor to verify pressures applied within the chamber.

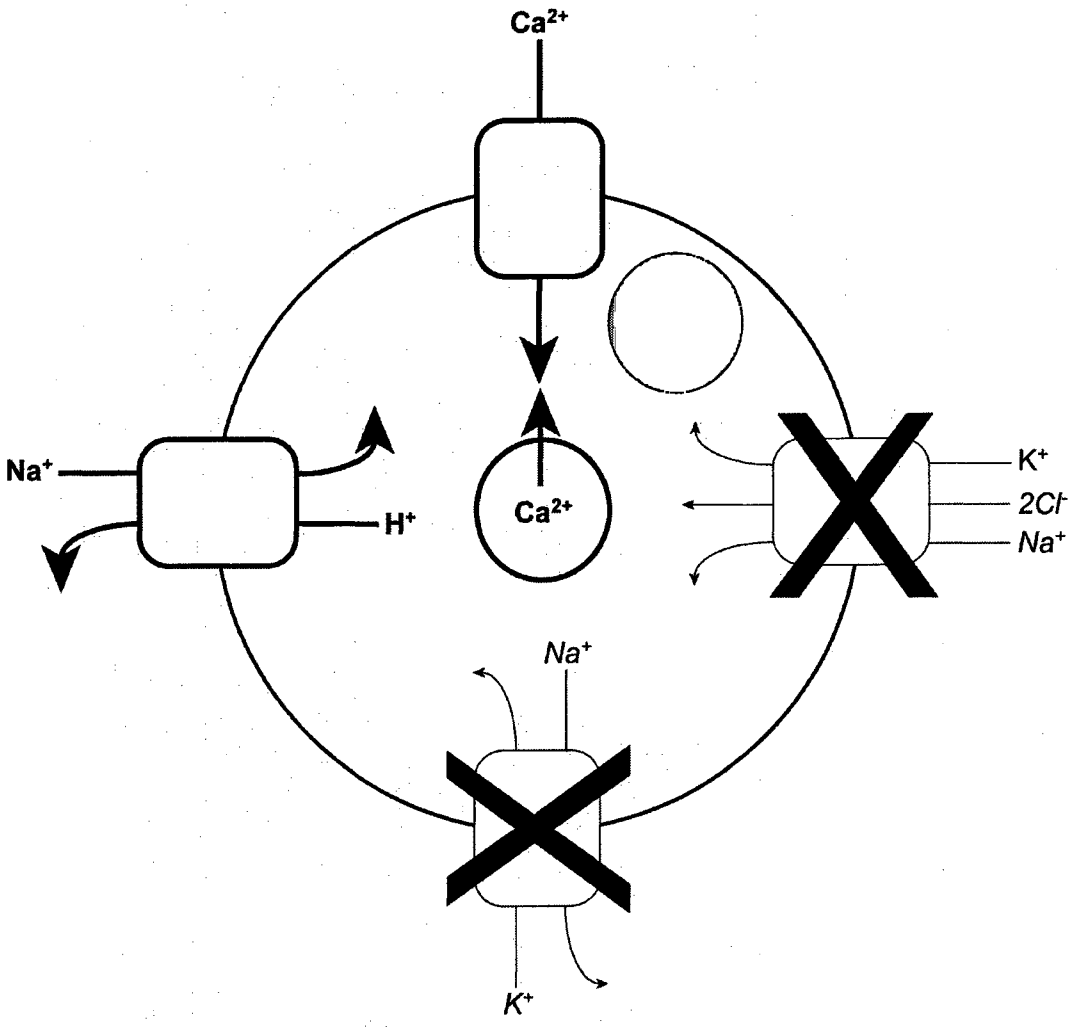


Figure 6: HP mechanotransduction.
Pressurization inhibits Na/K and Na/K/2Cl channels, while it activates Na/H and stretch-activated Ca channels, and triggers release of intracellular Ca stores.

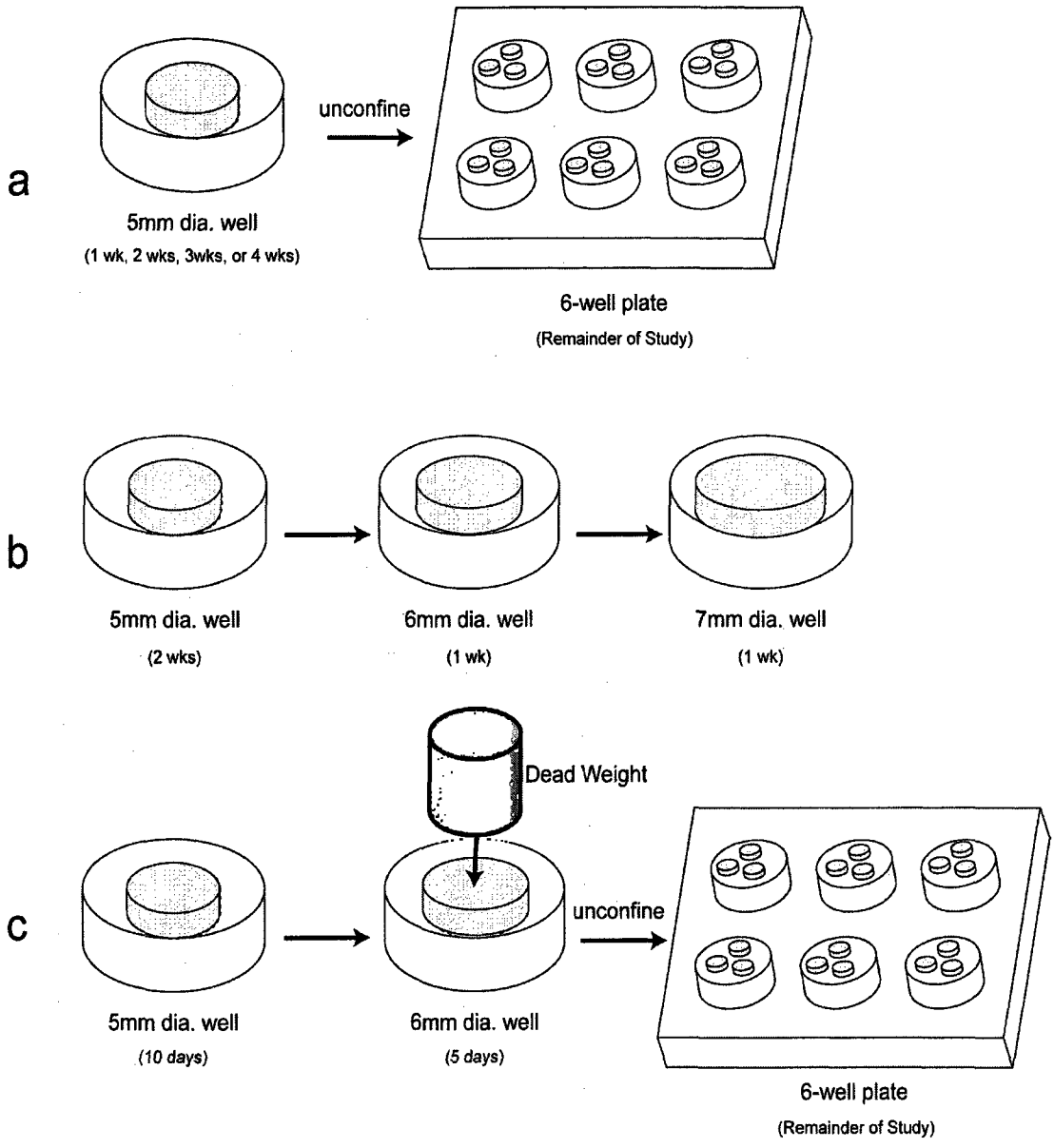


Figure 7: The experimental design.
(a) 1st study: Radial confinement of self-assembled constructs; (b) 2nd study: Maintenance of radial confinement of self-assembled constructs; (c) 3rd study: Passive axial compression of self-assembled constructs.

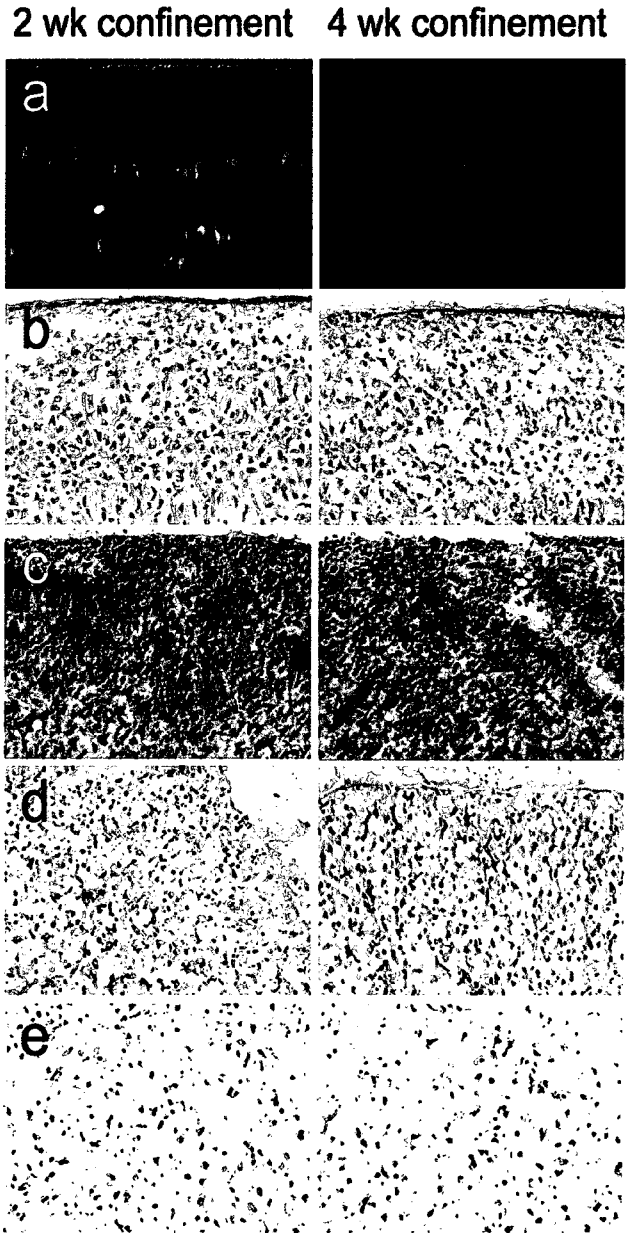


Figure 8: Histology of 2-wk and 4-wk confined constructs at 4 wks. Original magnification, 10x. (a) Polarized light microscopy images with the construct surface at the top. Two-wk confined group demonstrated organization of collagen fibrils perpendicular to the surface. (b) Picrosirius-red. (c) Safranin-O. (d) Collagen II IHC. (e) Collagen I IHC.

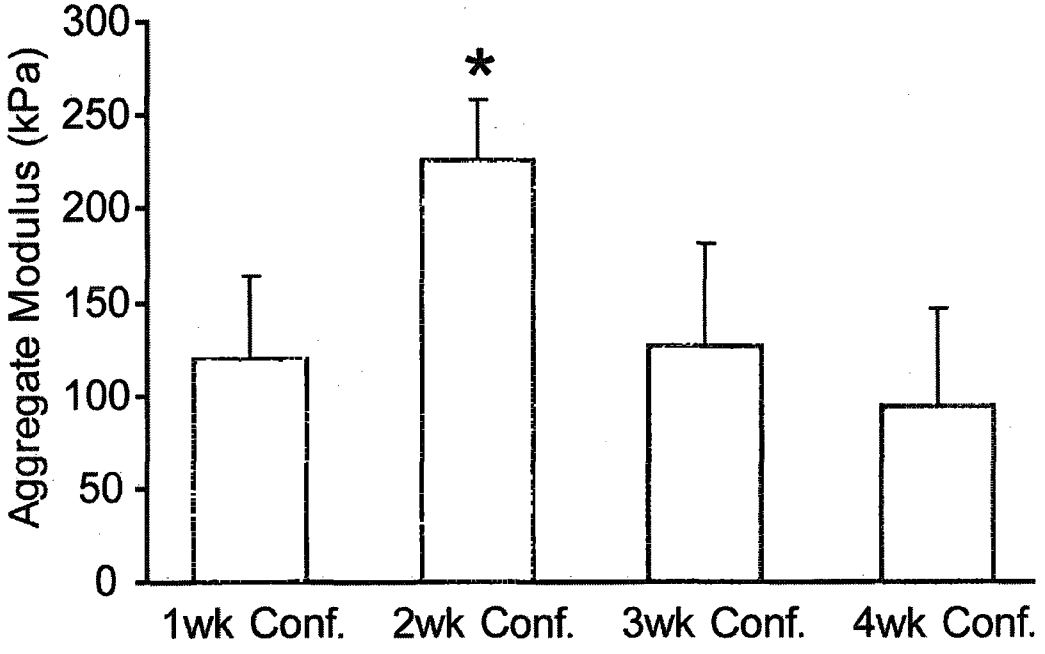


Figure 9: Mechanical properties of constructs in radial confinement study. Constructs confined for 2 wks demonstrated significantly higher aggregate modulus than the other groups. Means and standard deviations.

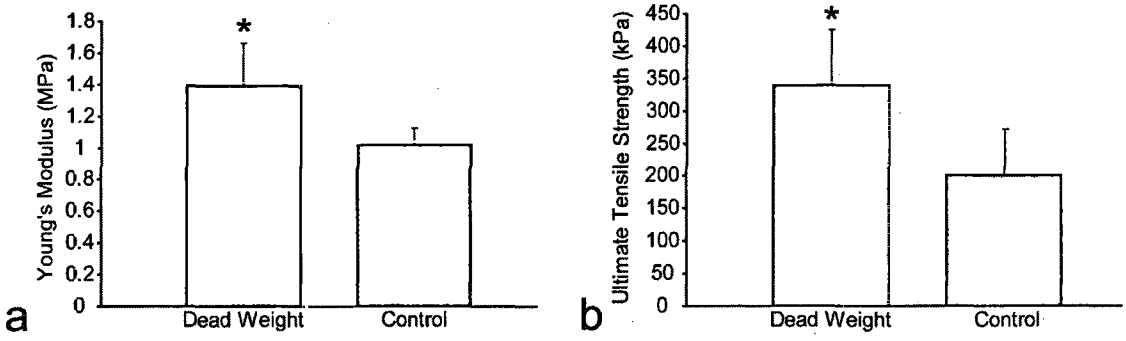


Figure 10: Mechanical properties of constructs in passive axial compression study.
(a) Passive axial compression group exhibited significantly higher Young's modulus than control group. (b) Passive axial compression group exhibited significantly higher ultimate tensile strength than control group. Means and standard deviations.

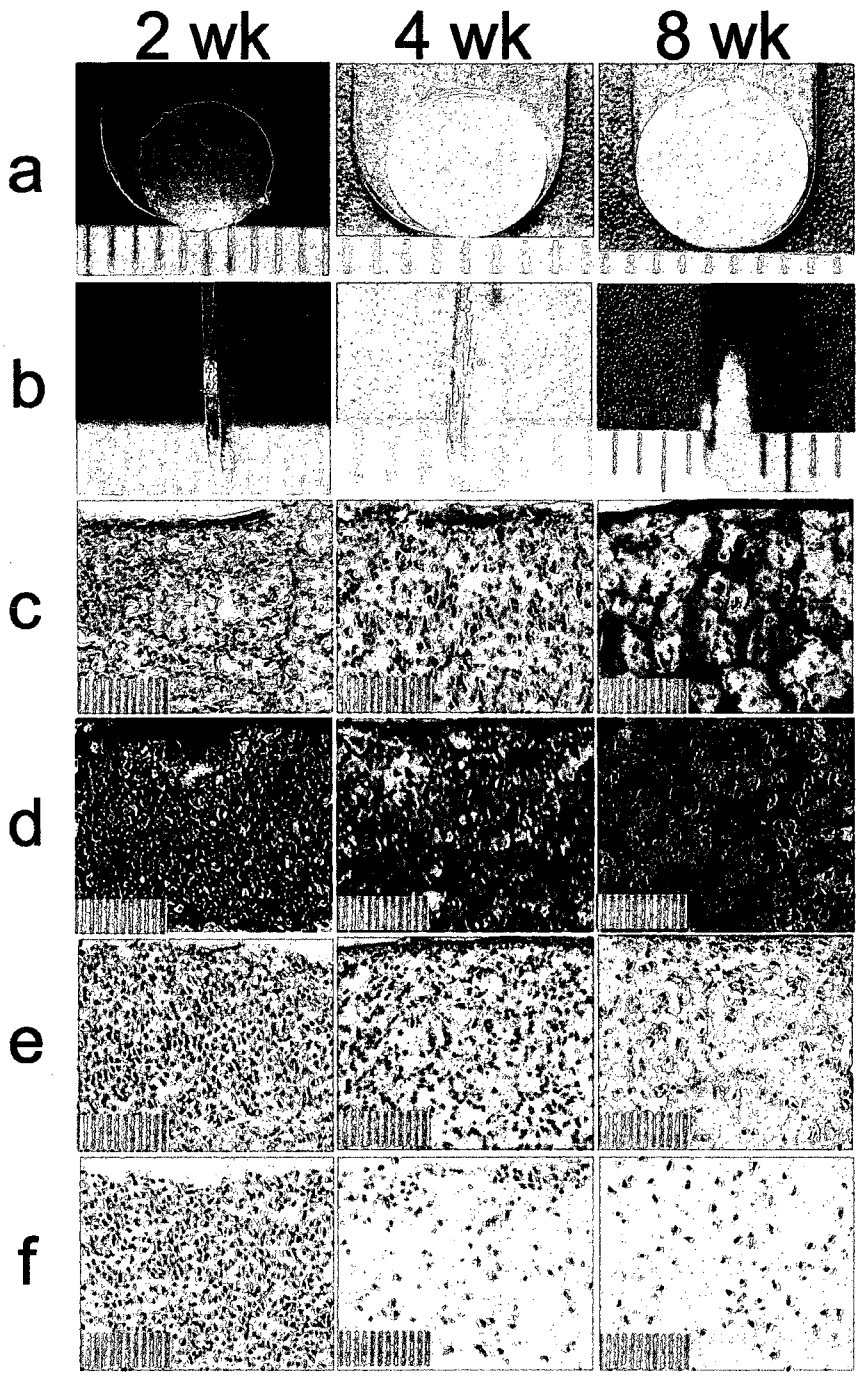


Figure 11: Histological and immunohistochemical images representative of all self-assembled constrcuts at 2, 4, and 8 wks.
10x original magnification, scale bar marks are 10 μm. (a) Gross morphology. (b) Gross morphology profile. (c) Picrosirius-red stained sections. (d) Safranin-O/Fast green stained sections. (e) Collagen II IHC sections. (f) Collagen I IHC sections.

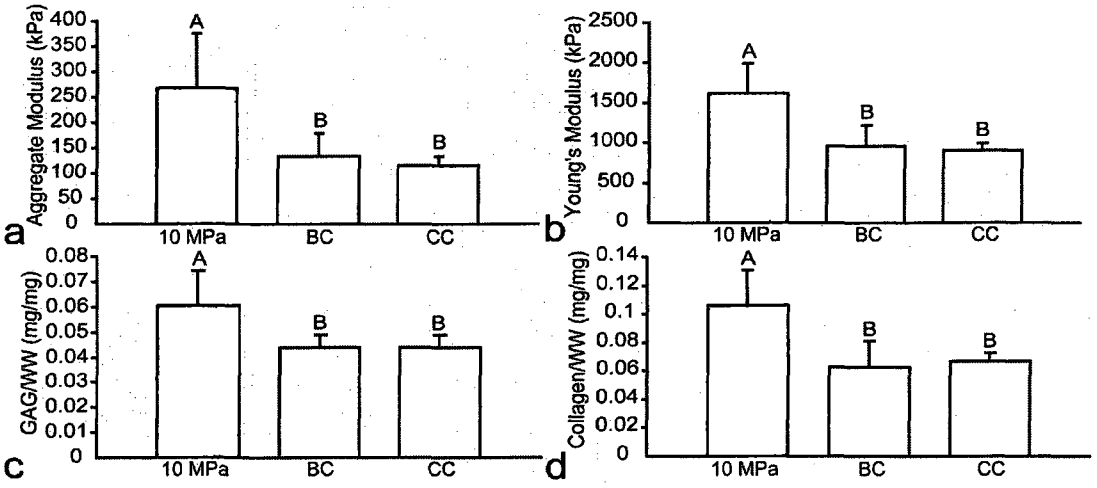


Figure 12: Biomechanical and biochemical properties of self-assembled constructs in phase I.
The HP treated group exhibited a significantly higher (a) aggregate modulus, (b) Young's modulus, (c) GAG/WW and (d) collagen/WW than BC or CC groups. Columns and error bars represent means and standard deviations. Groups denoted by different letters are significantly different ($p<0.05$).

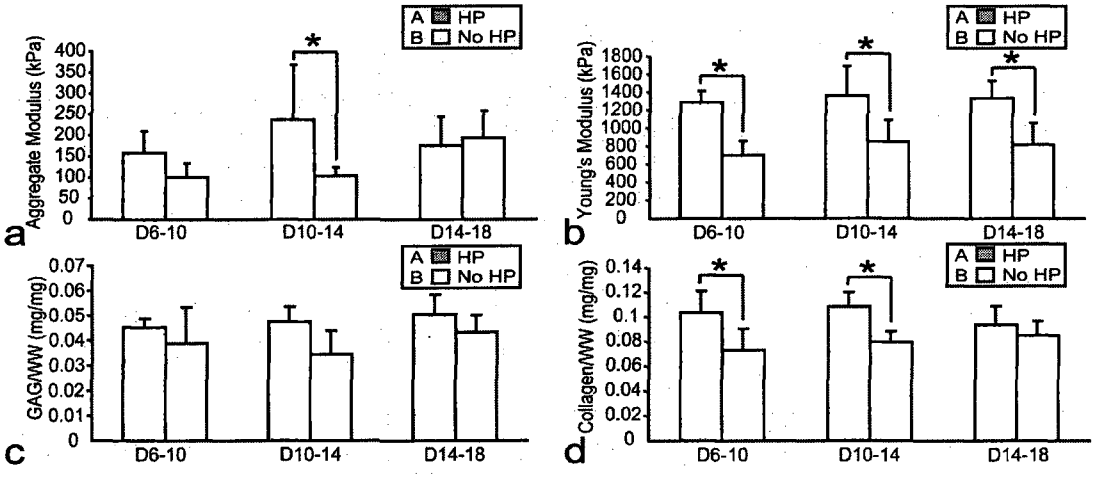


Figure 13: Biomechanical and biochemical properties of self-assembled constructs in phase II.

HP treatment was a significant factor for (a) aggregate modulus, (b) Young's modulus, (c) GAG/WW and (d) collagen/WW. Columns and error bars represent means and standard deviations. Groups denoted by different letters are significantly different ($p<0.05$) in the two-factor ANOVA (HP and application times). (*) indicates significant difference from control ($p<0.05$), based on the *post-hoc* analysis comparing each individual group.

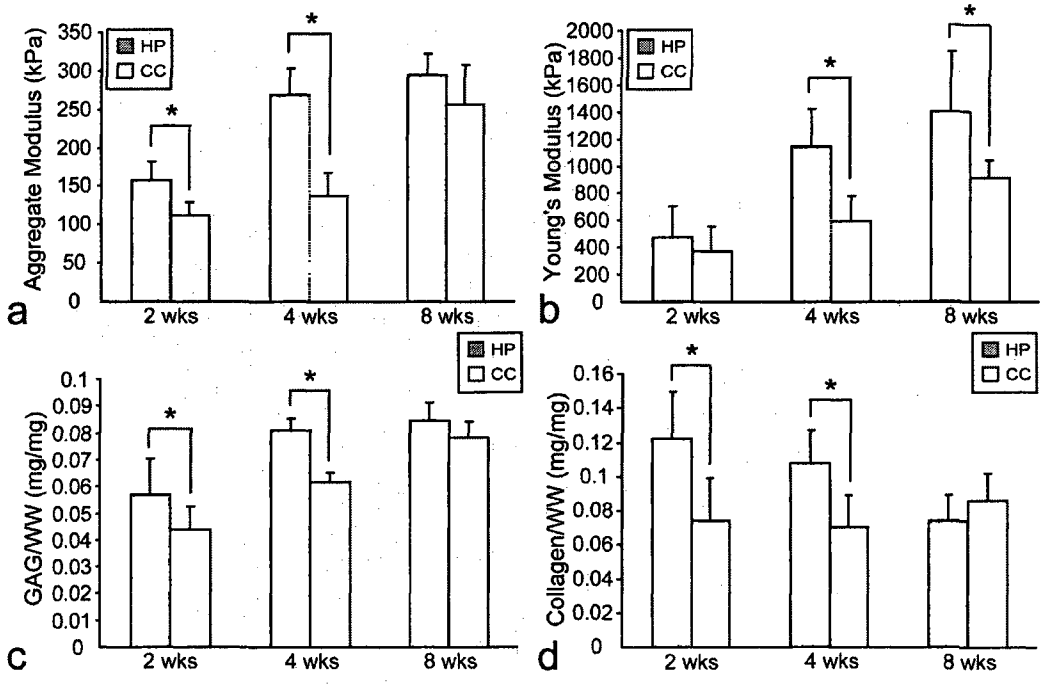


Figure 14: Biomechanical and biochemical properties of self-assembled constructs in phase III.

(a) Aggregate modulus was significantly increased by HP at 2 wks and 4 wks. (b) Young's modulus was significantly increased by HP at 4 wks and 8 wks. (c) GAG/WW and (d) collagen/WW were significantly increased at 2 wks and 4 wks by HP application. Columns and error bars represent means and standard deviations. (*) indicates significant difference from control ($p<0.05$).

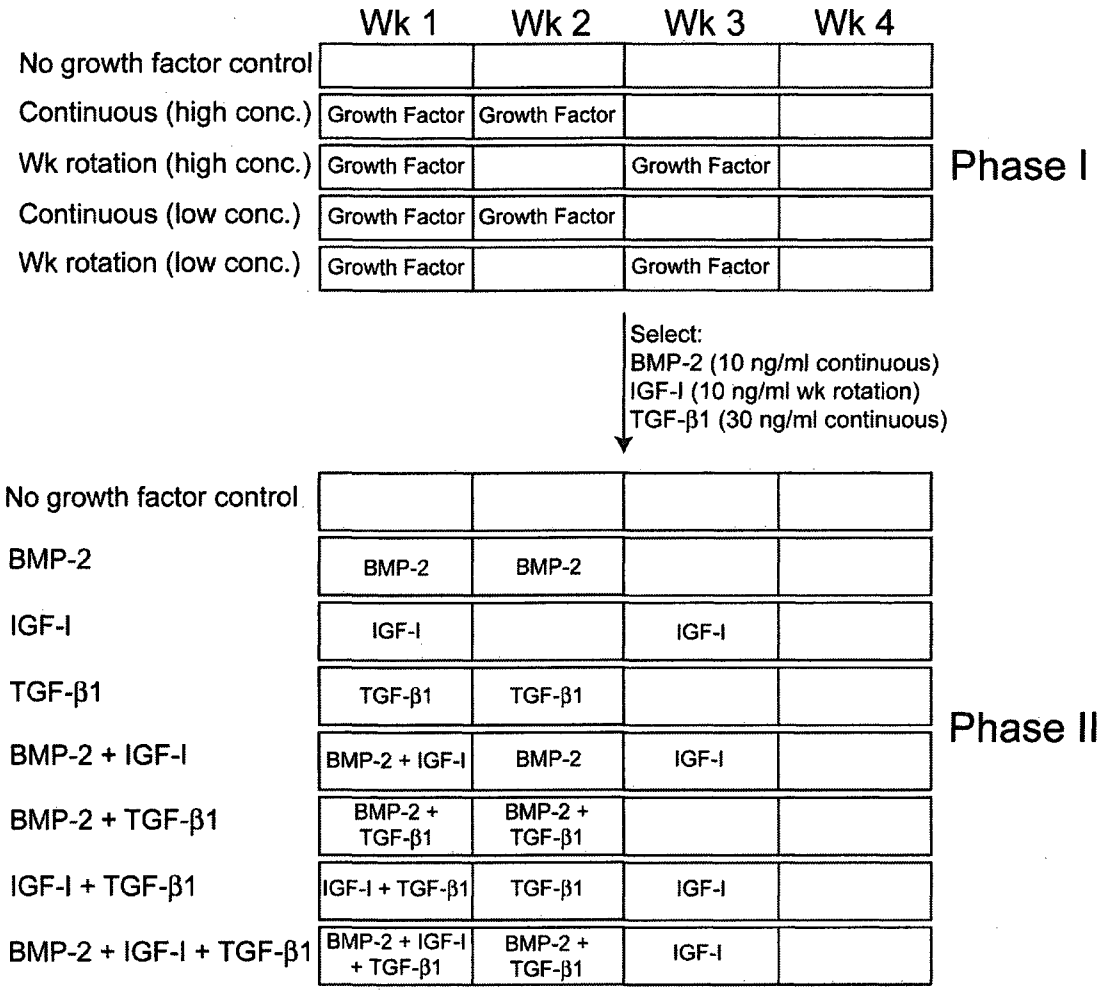


Figure 15: Schematic diagram indicating experimental designs of phases I and II.
The experimental design depicted in phase I was carried out for each individual growth factor separately (blocked by growth factor). The best treatment for each growth factor was selected for phase II. Phase II assessed the effects of each growth factor individually and in all combinations of two and three.

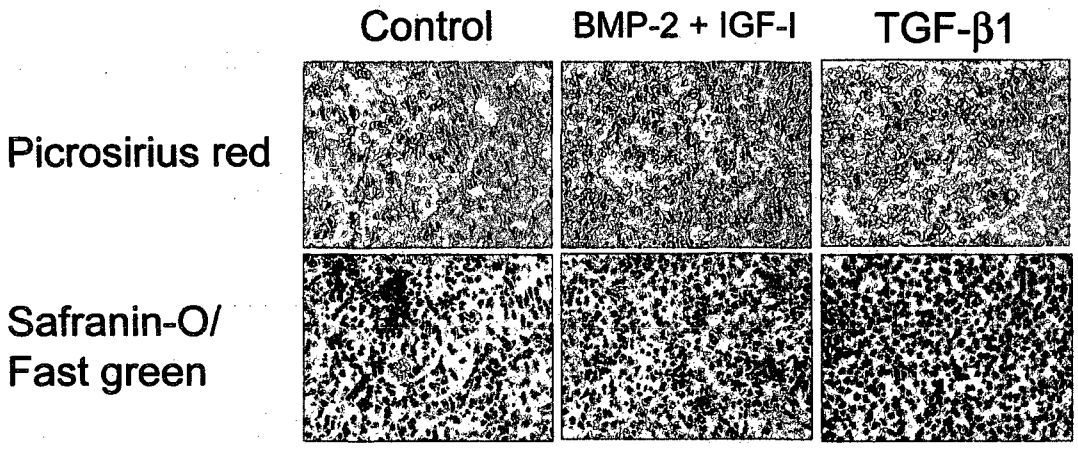


Figure 16: Photomicrographs of collagen and GAG staining.
No growth factor control constructs, BMP-2 + IGF-I constructs, and TGF-β1 *treated constructs. 10x original magnification.*

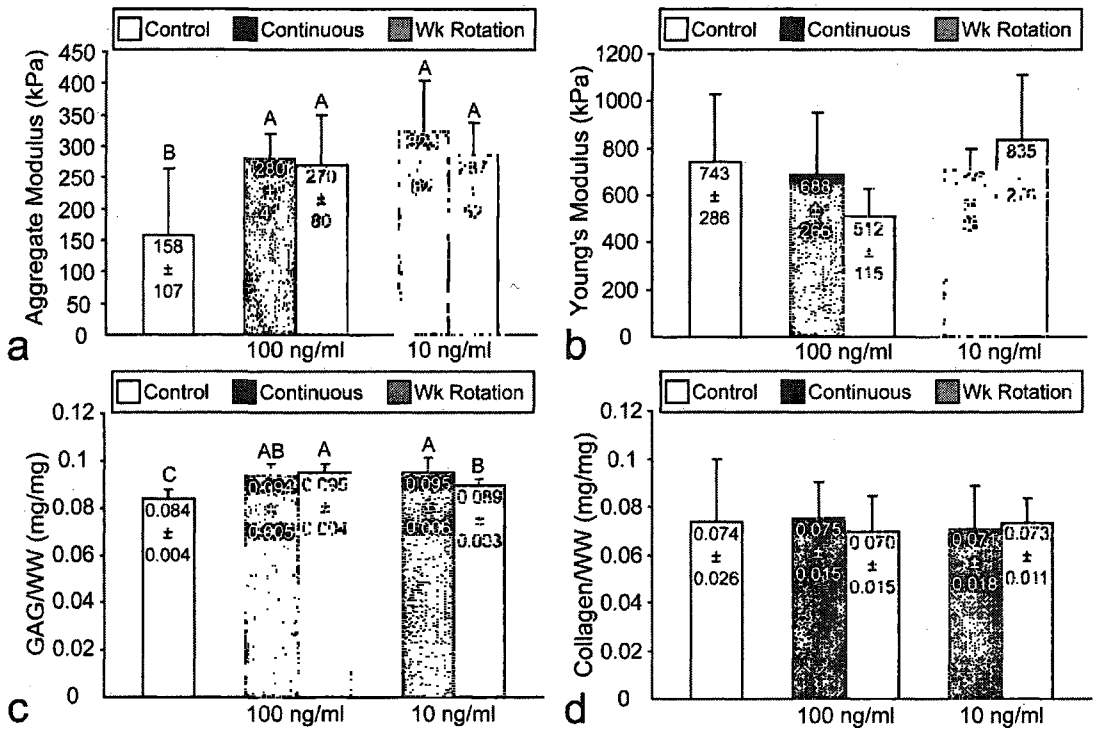


Figure 17: Biomechanical and biochemical properties of BMP-2 treated constructs in phase I.

All BMP-2 treatments significantly increased (a) aggregate modulus with no effects on (b) Young's modulus. Likewise, all BMP-2 treatments significantly increased (c) GAG/WW with no effect on (d) collagen/WW. Columns and error bars represent means and standard deviations. Groups denoted by different letters are significantly different ($p<0.05$).

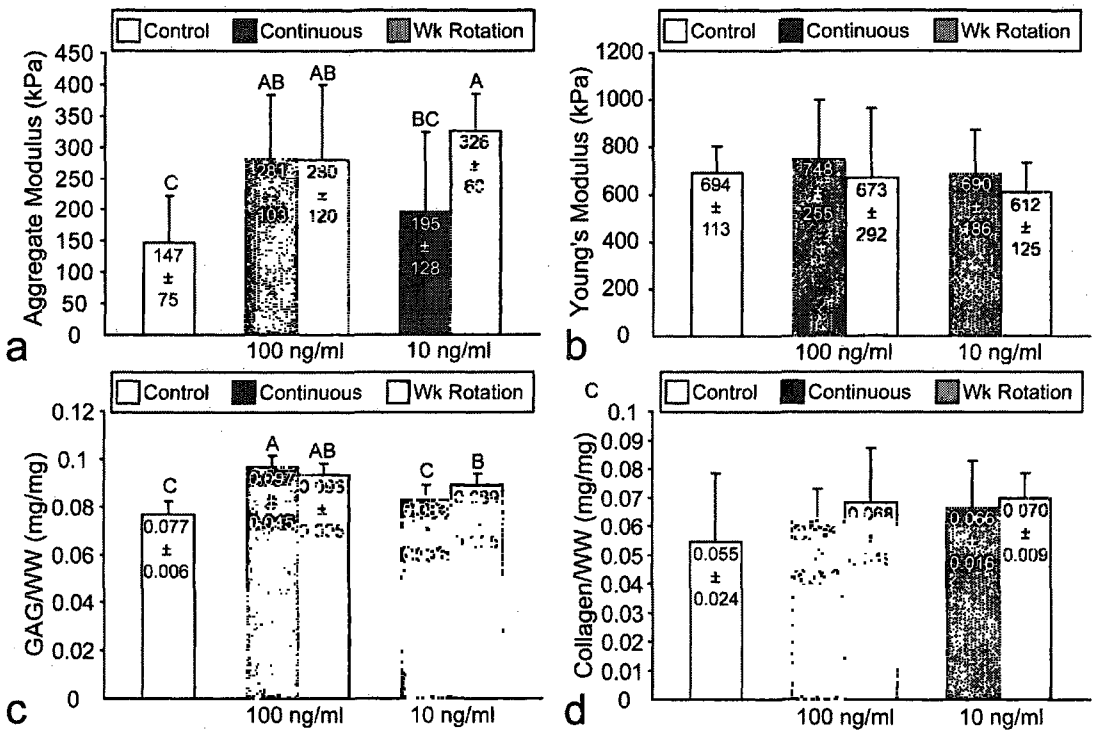


Figure 18: Biomechanical and biochemical properties of IGF-I treated constructs in phase I.

All IGF-I treatments, except 10 ng/ml continuous, significantly increased (a) aggregate modulus with no effect on (b) Young's modulus. Likewise, all IGF-I treatments, except 10 ng/ml continuous, significantly increased (c) GAG/WW with no effect on (d) collagen/WW. Columns and error bars represent means and standard deviations. Groups denoted by different letters are significantly different ($p<0.05$).

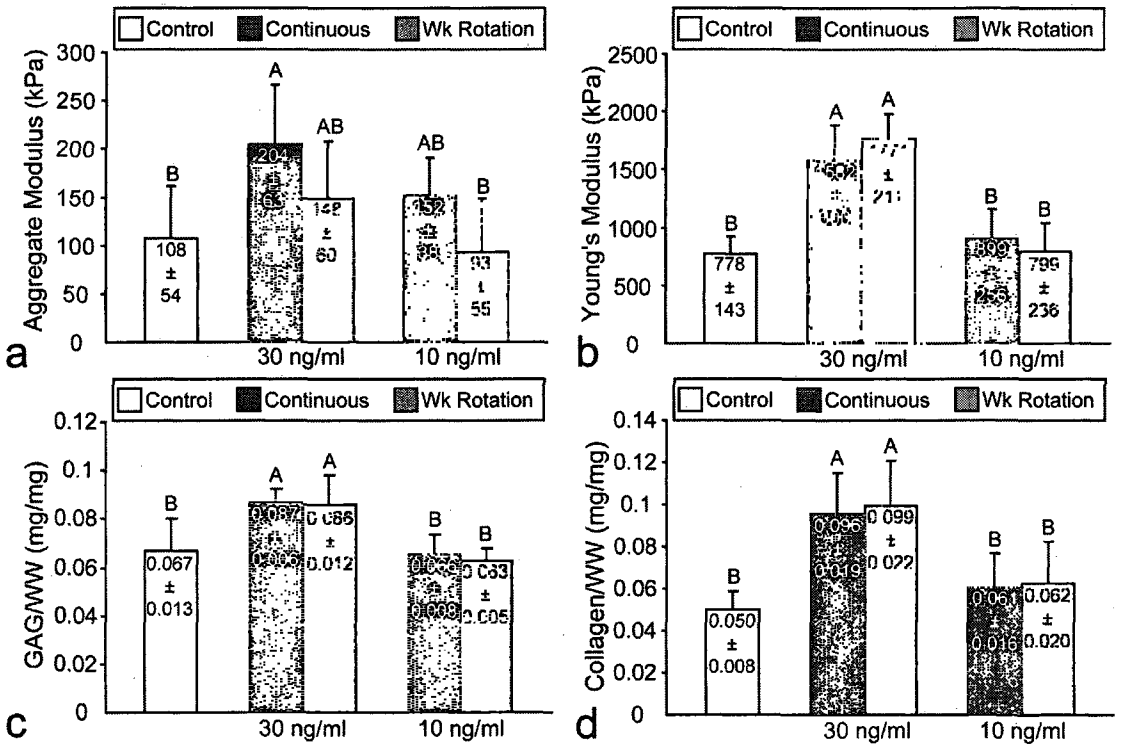


Figure 19: Biomechanical and biochemical properties of TGF-β1 treated constructs in phase I.

TGF-β1 treatment at 30 ng/ml and 2-wk continuous dosage significantly increased (a) aggregate modulus and (b) Young's modulus, with corresponding increases in (c) GAG/WW and (d) collagen/WW. Columns and error bars represent means and standard deviations. Groups denoted by different letters are significantly different ($p<0.05$).

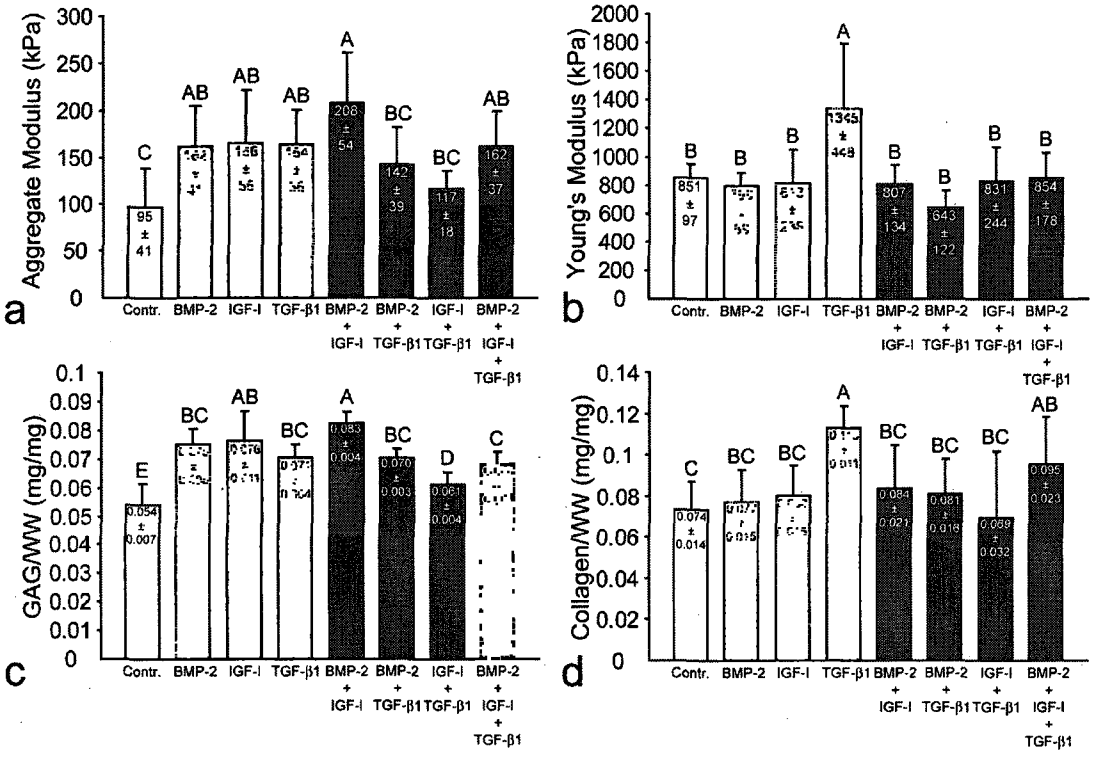


Figure 20: Biomechanical and biochemical properties of constructs in phase II.

Combined treatment with BMP-2 and IGF-I led to the greatest enhancement of aggregate modulus and GAG/WW, while TGF-β1 alone was the only treatment to enhance both compressive and tensile stiffness. (a) aggregate modulus, (b) Young's modulus, (c) GAG/WW and (d) collagen/WW. Columns and error bars represent means and standard deviations. Groups denoted by different letters are significantly different ($p<0.05$).

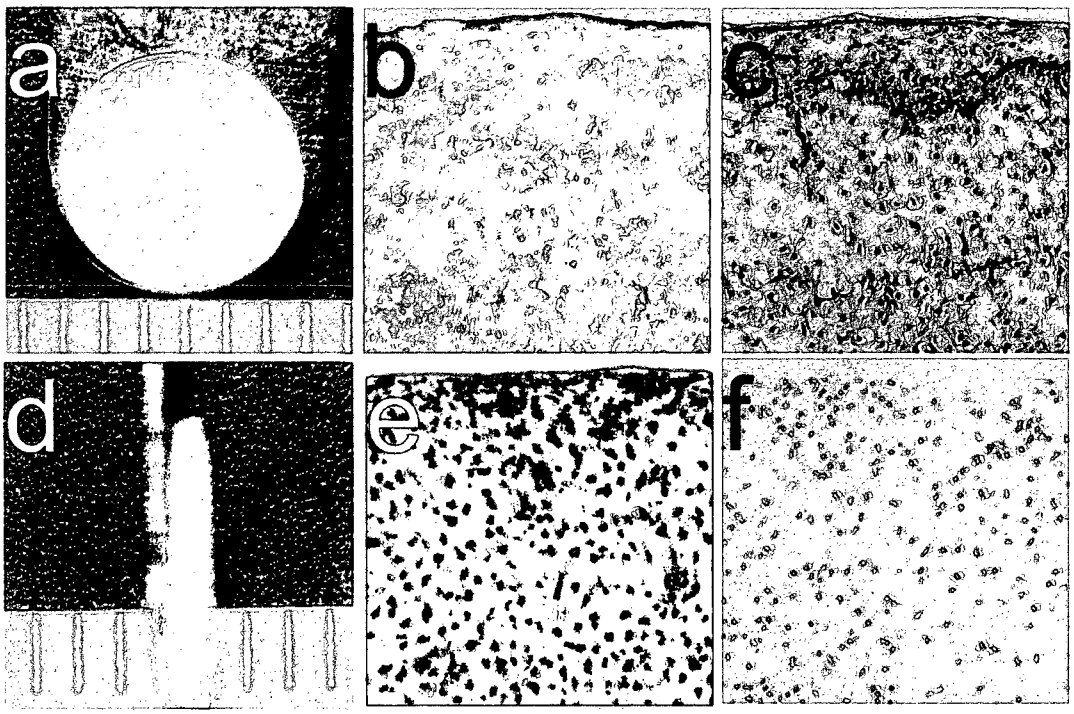


Figure 21: Histological and immunohistochemical images representative of all self-assembled constructs.
10x original magnification. (a) Gross morphology. (b) Picrosirius-red stained sections. (c) Collagen II IHC sections. (d) Gross morphology profile. (e) Safranin-O/fast green stained sections. (f) Collagen I IHC sections.

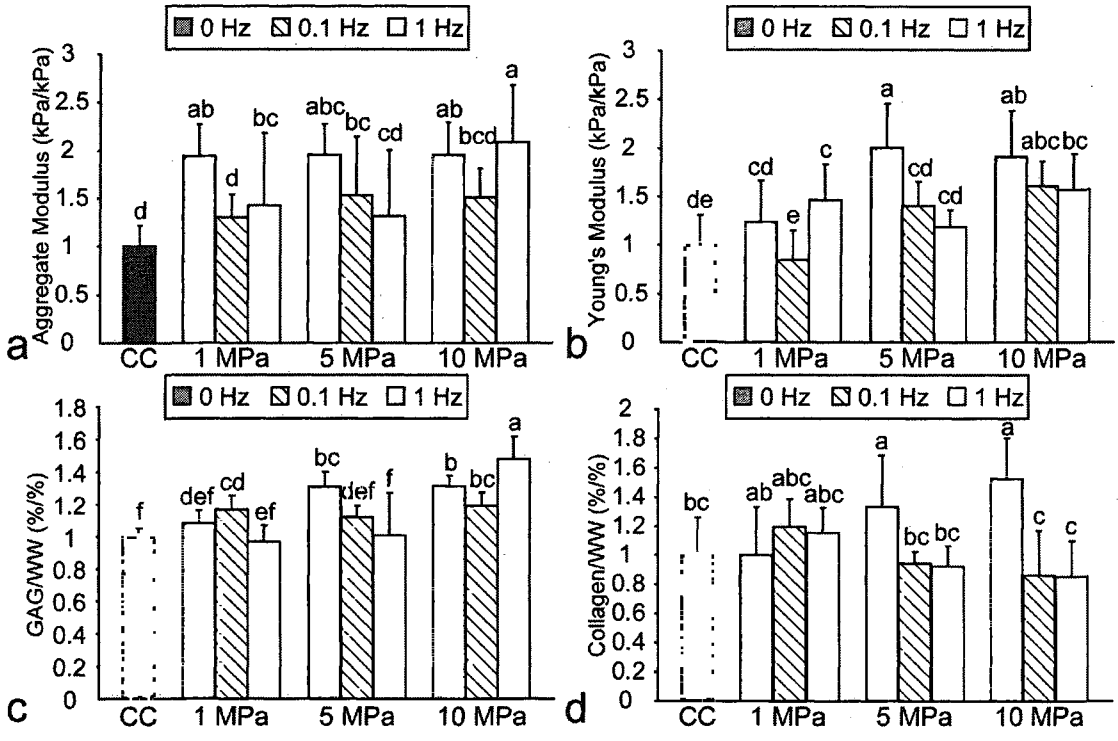


Figure 22: Biomechanical and biochemical properties of self-assembled constructs in phase I, normalized to control values.

HP application at 5 or 10 MPa, 0 Hz, resulted in a significantly higher (a) aggregate modulus, (b) Young's modulus, (c) GAG/WW and (d) collagen/WW than control. Columns and error bars represent means and standard deviations. Groups denoted by different letters are significantly different ($p<0.05$).

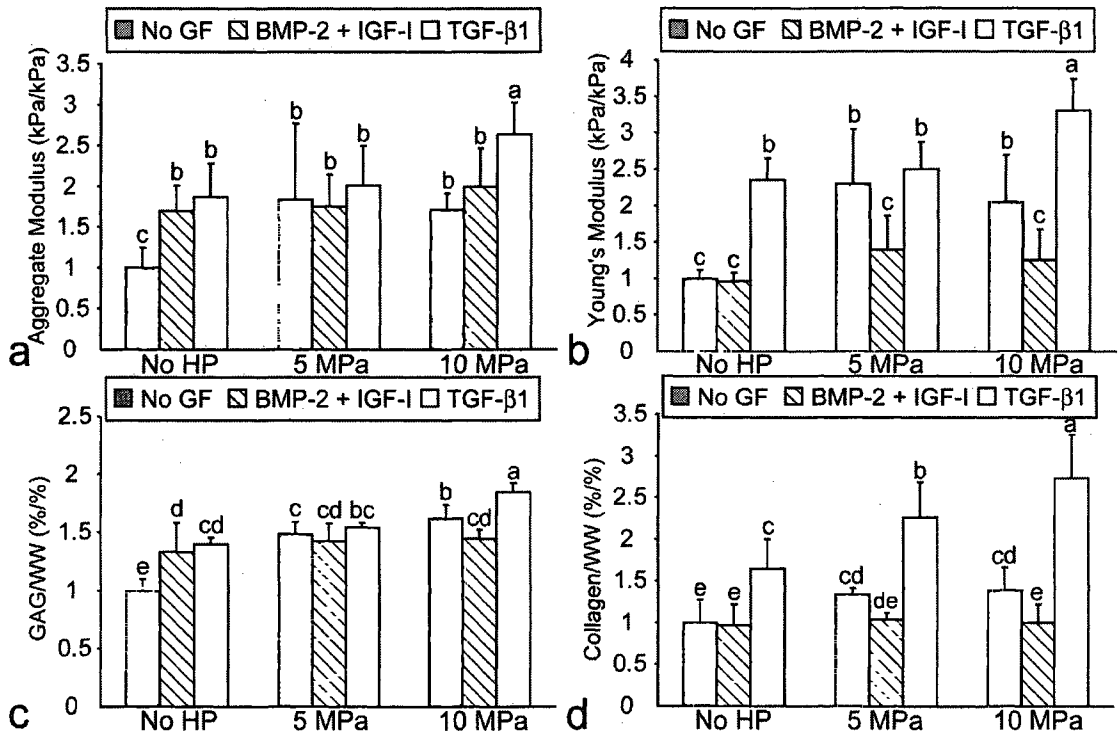


Figure 23: Biomechanical and biochemical properties of self-assembled constructs in phase II, normalized to control values.
(a) aggregate modulus, (b) Young's modulus, (c) GAG/WW and (d) collagen/WW. Combined treatment with 10 MPa static HP and TGF-β1 led to additive increases in aggregate modulus and Young's modulus, and a synergistic increase in collagen/WW. Columns and error bars represent means and standard deviations. Groups denoted by different letters are significantly different ($p<0.05$).

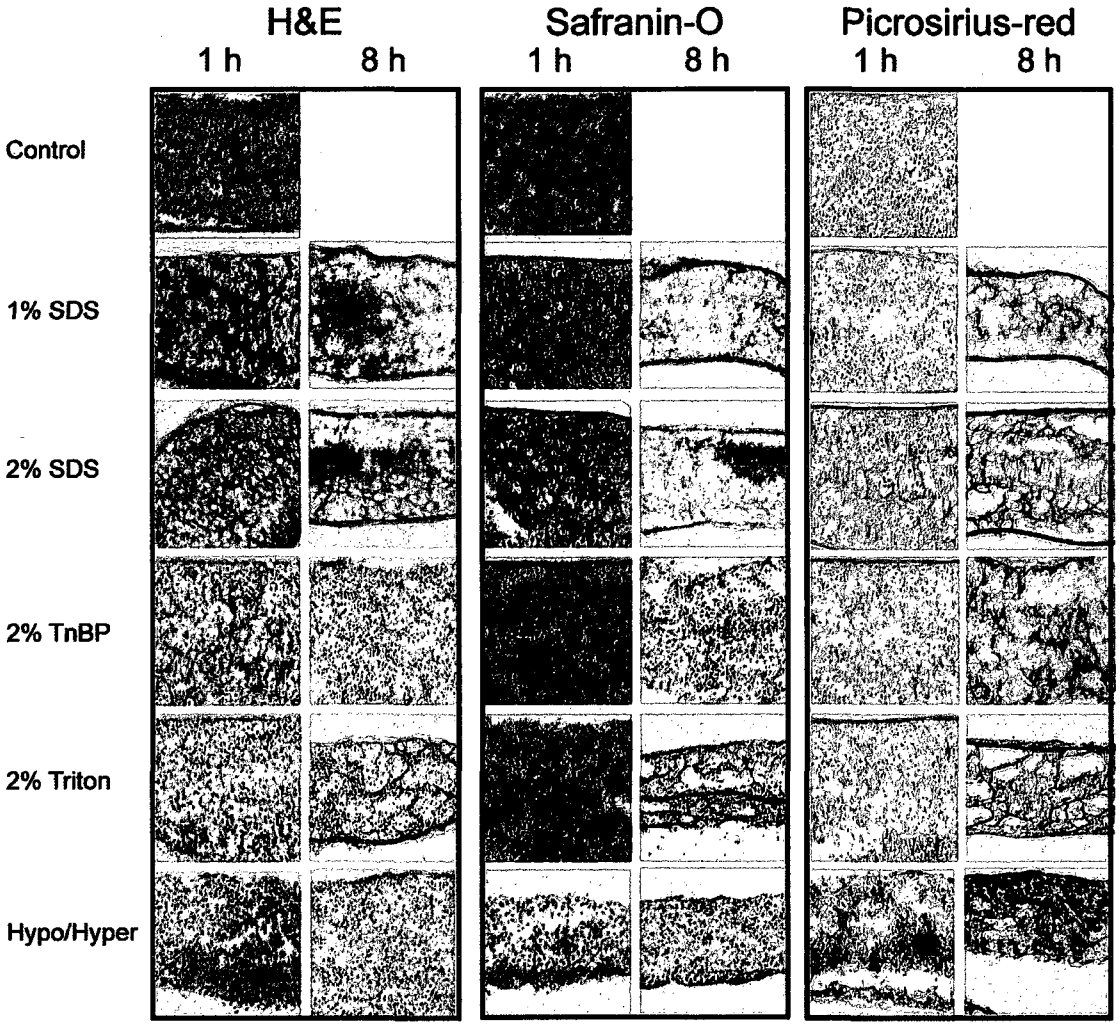


Figure 24. Photomicrographs demonstrating construct cellularity, GAG content, and collagen content for various treatment groups.
10x original magnification. Treatment with 2% SDS for 1 h decreased cellularity while preserving GAG content, while treatment for 8 h eliminated all nuclei, but also eliminated all GAG.

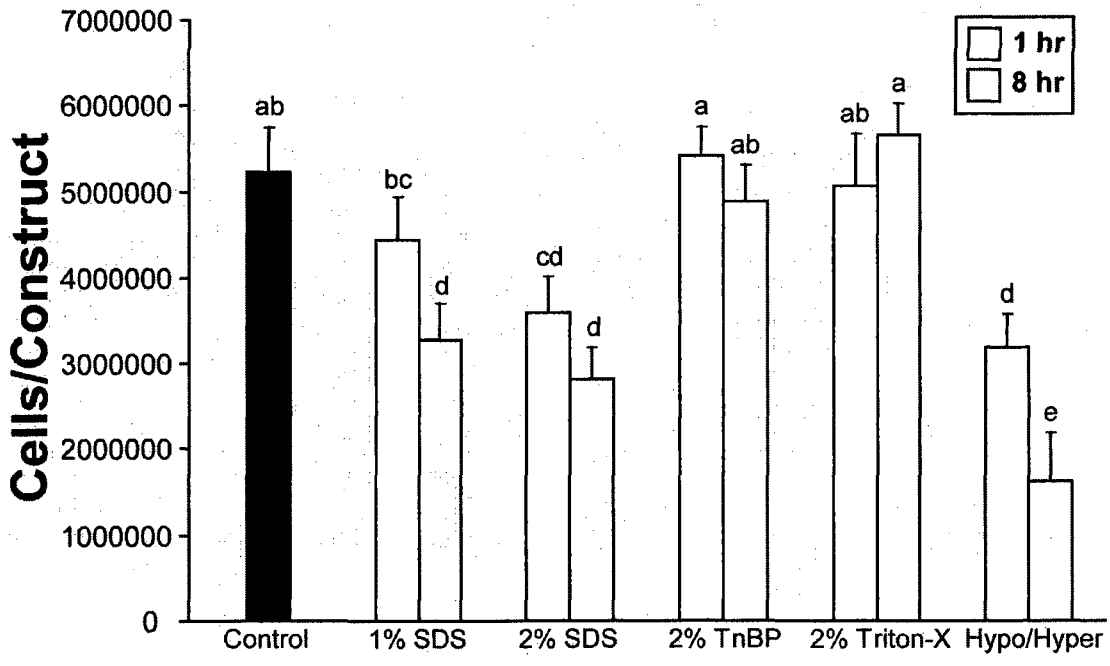


Figure 25. Cellularity (DNA content) of constructs following decellularization treatment.
Treatment with 2% SDS or the hypotonic/hypertonic solutions at either application time significantly decreased construct cellularity. Columns and error bars represent means and standard deviations. Groups denoted by different letters are significantly different ($p<0.05$).

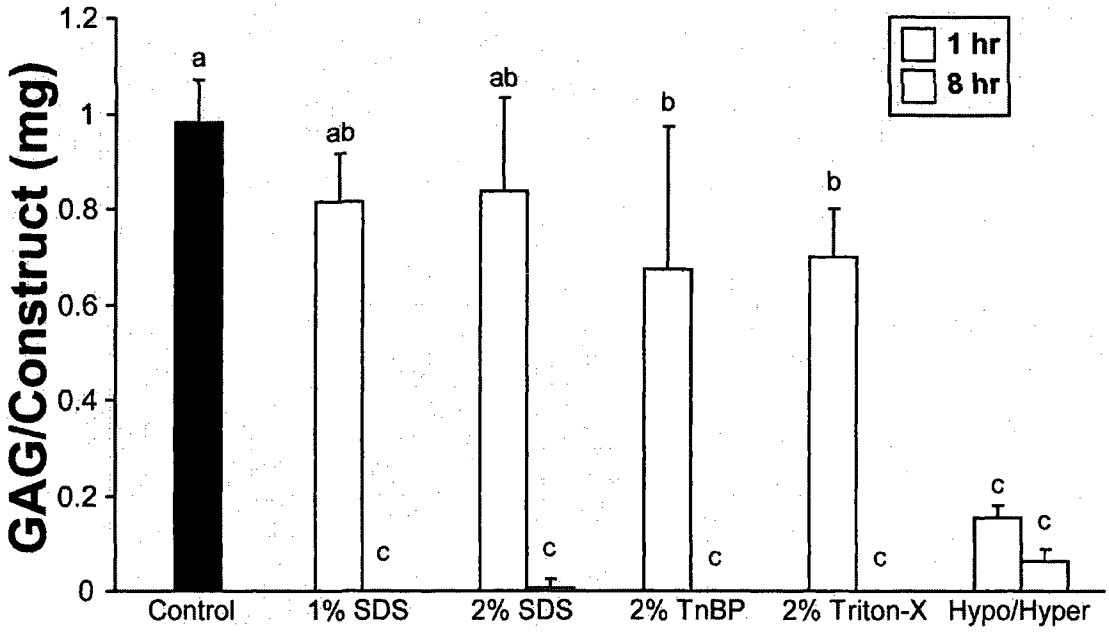


Figure 26. Construct GAG content following decellularization.
All 8 h treatments resulted in nearly complete GAG removal, while both 1% and 2% SDS for 1 h maintained GAG content. Columns and error bars represent means and standard deviations. Groups denoted by different letters are significantly different ($p<0.05$).

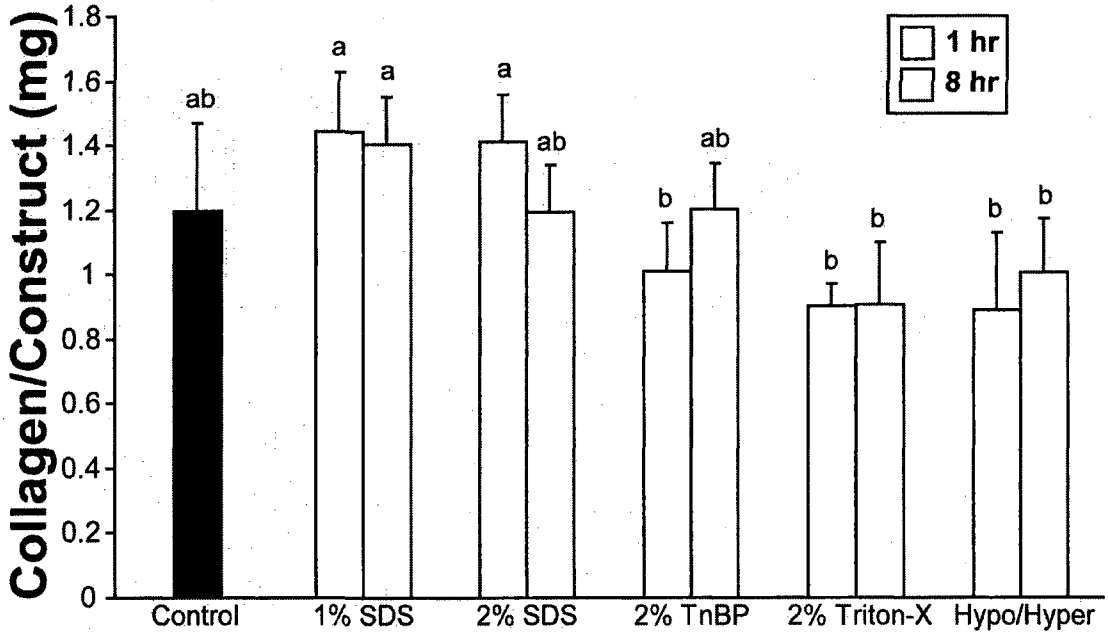


Figure 27. Construct collagen content following decellularization. Treatment with SDS or TnBP maintained collagen content, while treatment with Triton X-100 or the hypotonic/hypertonic combination significantly reduced total collagen content. Columns and error bars represent means and standard deviations. Groups denoted by different letters are significantly different ($p<0.05$).

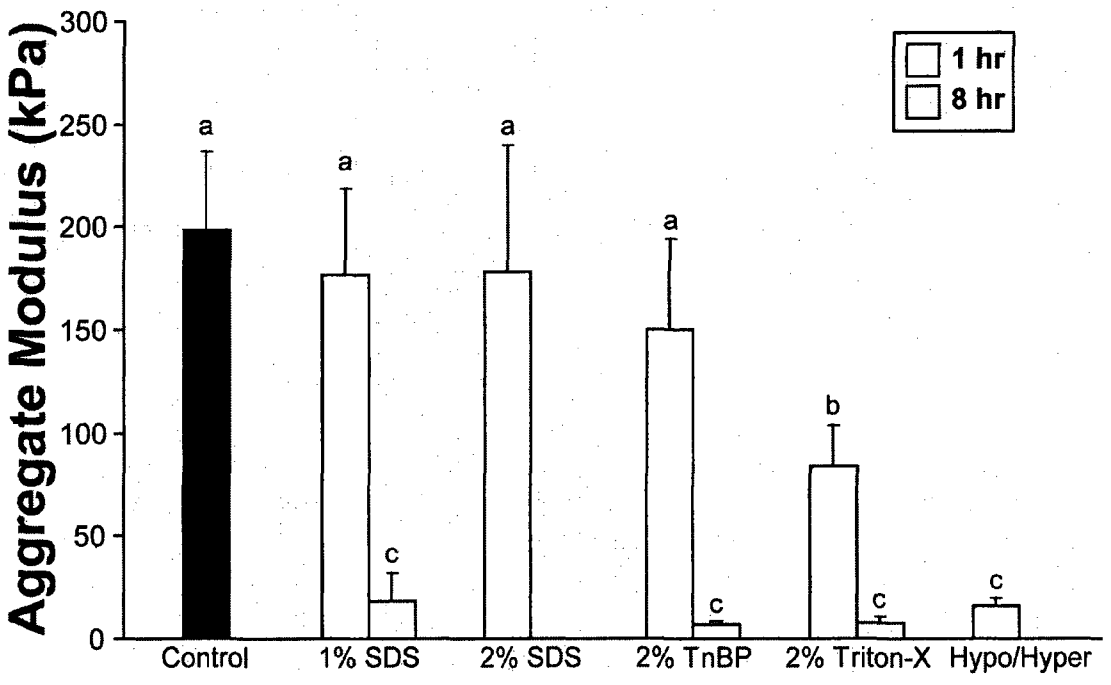


Figure 28. Construct compressive properties following decellularization. All 8 h treatments either significantly reduced compressive stiffness, or were untestable. However, treatment for 1 h with 1% or 2% SDS, or 2% TnBP maintained compressive stiffness. Columns and error bars represent means and standard deviations. Groups denoted by different letters are significantly different ($p<0.05$).

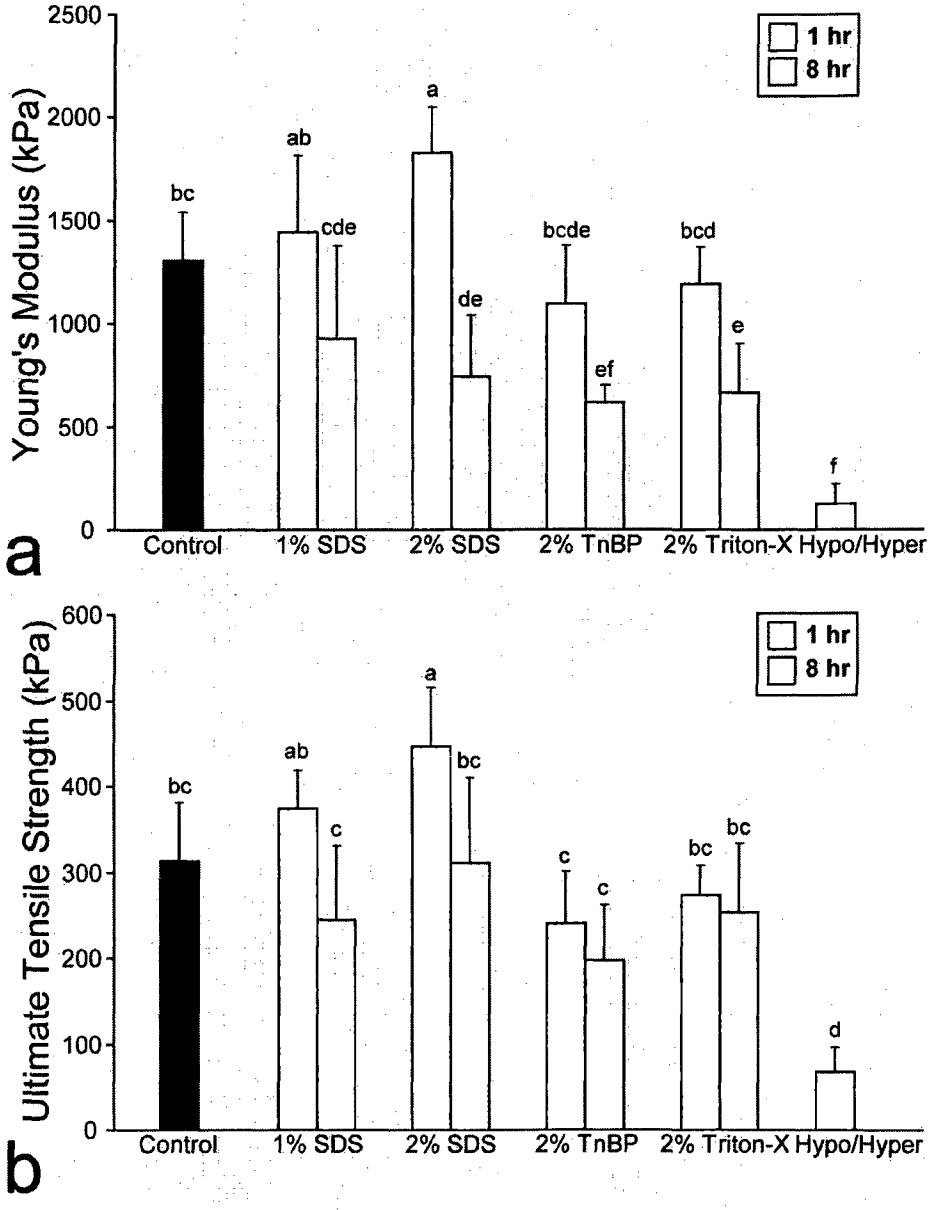


Figure 29. Construct tensile properties following decellularization.
(a) Treatment with 1% SDS for 1 h maintained tensile stiffness, while treatment with 2% SDS for 1 h increased tensile stiffness. The 8 h detergent treatments resulted in a slight decrease in tensile stiffness. (b) Similar trends were observed for ultimate tensile strength. Columns and error bars represent means and standard deviations. Groups denoted by different letters are significantly different ($p<0.05$).

Appendix B: Tables

Chapter 5: Tables I-IV

Chapter 6: Tables V-VI

Chapter 7: Tables VII-VIII

Table I: Properties of constructs treated with BMP-2 in phase I.

Group	WW (mg)	Thickness (mm)	Total Cells ($x10^6$)	FI
Control	21.8±2.4	0.67±0.04	4.6±0.5	0.75±0.18
100 ng/ml Continuous	23.7±1.8	0.71±0.07	5.3±0.3	0.94±0.06[a]
100 ng/ml Wk Rotat.	22.6±1.9	0.71±0.04	4.8±0.9	0.96±0.08[a]
10 ng/ml Continuous	23.3±2.0	0.73±0.07	4.4±1.0	0.97±0.10[a]
10 ng/ml Wk Rotat.	23.8±1.3	0.72±0.04	4.8±0.3	0.92±0.06[a]

[a]Significantly different from control
Wk Rotat., 2-wk rotation dosage

Table II: Properties of constructs treated with IGF-I in phase I.

Group	WW (mg)	Thickness (mm)	Total Cells ($x10^6$)	FI
Control	25.7±1.0	0.74±0.06	5.2±0.6	0.59±0.19
100 ng/ml Continuous	24.5±1.4	0.65±0.08	5.3±0.7	0.93±0.12[a]
100 ng/ml Wk Rotat.	22.7±1.6[a]	0.71±0.09	5.0±0.4	0.92±0.14[a]
10 ng/ml Continuous	23.2±1.7[a]	0.75±0.10	4.6±0.6	0.78±0.19[a]
10 ng/ml Wk Rotat.	24.1±1.9	0.75±0.12	5.0±0.3	0.96±0.08[a]

[a]Significantly different from control
Wk Rotat., 2-wk rotation dosage

Table III: Properties of constructs treated with TGF-β1 in phase I.

Group	WW (mg)	Thickness (mm)	Total Cells ($x10^6$)	FI
Control	24.9±2.8	0.88±0.14	5.7±0.3	0.60±0.08
30 ng/ml Continuous	12.6±0.7[a]	0.57±0.06[a]	6.6±0.4[a]	0.82±0.07[a]
30 ng/ml Wk Rotat.	13.9±0.6[a]	0.57±0.02[a]	6.5±0.8[a]	0.81±0.14[a]
10 ng/ml Continuous	17.8±1.3[a]	0.64±0.09[a]	5.7±0.4	0.62±0.07
10 ng/ml Wk Rotat.	17.9±4.6[a]	0.76±0.17	5.8±0.8	0.55±0.06

[a]Significantly different from control
Wk Rotat., 2-wk rotation dosage

Table IV: Phase II construct properties.

Group	WW (mg)	Thickness (mm)	Total Cells ($x10^6$)	FI
Control	13.3±1.3	0.45±0.09	5.6±0.5	0.53±0.06
BMP-2	15.0±1.6	0.55±0.06	5.2±0.4	0.76±0.07[a]
IGF-I	13.3±3.1	0.55±0.05	5.7±0.9	0.73±0.12[a]
TGF-β1	14.5±1.6	0.58±0.05	5.5±0.3	0.72±0.04[a]
BMP-2 + IGF-I	16.7±1.3[a]	0.59±0.05	5.9±0.4	0.80±0.08[a]
BMP-2 + TGF-β1	14.9±1.4	0.57±0.04	5.8±0.6	0.66±0.02[a]
IGF-I + TGF-β1	14.2±1.4	0.57±0.06	5.5±0.3	0.59±0.04
BMP-2 + IGF-I + TGF-β1	13.0±1.3	0.53±0.08	6.1±0.4	0.70±0.05

[a]Significantly different from control

Table V: Phase I construct properties.

Group	WW (mg)	Thickness (mm)	H_A (kPa)	E_Y (kPa)	GAG/WW (%)	Col./WW (%)	Total Cells ($\times 10^6$)
No HP, No GF	32.1±0.7	0.98±0.09	94±24	619±73	5.2±0.5	5.6±1.5	5.0±0.5
No HP, BMP-2+IGF-I	33.0±1.8	1.09±0.11	160±29	596±70	6.9±1.3	5.4±1.4	5.1±1.5
No HP, TGF-β1	16.2±1.1	0.69±0.06	176±38	1460±182	7.3±0.3	9.2±2.0	5.0±4.5
5 MPa, No GF	29.0±1.7	1.01±0.15	173±87	1424±465	7.8±0.6	7.5±0.5	5.7±0.3
5 MPa, BMP-2+IGF-I	32.0±2.5	1.03±0.14	165±37	862±293	7.4±0.8	5.8±0.4	5.2±0.5
5 MPa, TGF-β1	15.4±1.0	0.65±0.06	189±46	1545±235	8.1±0.2	12.6±2.4	5.1±0.4
10 MPa, No GF	27.8±0.8	0.94±0.13	161±19	1268±404	8.5±0.6	7.8±1.5	5.6±0.4
10 MPa, BMP-2+IGF-I	31.4±1.3	1.06±0.09	187±45	776±260	7.5±0.4	5.6±1.2	5.6±0.1
10 MPa, TGF-β1	14.8±0.4	0.69±0.08	248±37	2048±266	9.6±0.4	15.3±2.9	5.5±0.4

Col., total collagen

Table VI: Phase II construct properties.

Group	WW (mg)	Thickness (mm)	H_A (kPa)	E_Y (kPa)	GAG/WW (%)	Col./WW (%)	Total Cells ($\times 10^6$)
No HP, No GF	32.1±0.7	0.98±0.09	94±24	619±73	5.2±0.5	5.6±1.5	5.0±0.5
No HP, BMP-2+IGF-I	33.0±1.8	1.09±0.11	160±29	596±70	6.9±1.3	5.4±1.4	5.1±1.5
No HP, TGF-β1	16.2±1.1	0.69±0.06	176±38	1460±182	7.3±0.3	9.2±2.0	5.0±4.5
5 MPa, No GF	29.0±1.7	1.01±0.15	173±87	1424±465	7.8±0.6	7.5±0.5	5.7±0.3
5 MPa, BMP-2+IGF-I	32.0±2.5	1.03±0.14	165±37	862±293	7.4±0.8	5.8±0.4	5.2±0.5
5 MPa, TGF-β1	15.4±1.0	0.65±0.06	189±46	1545±235	8.1±0.2	12.6±2.4	5.1±0.4
10 MPa, No GF	27.8±0.8	0.94±0.13	161±19	1268±404	8.5±0.6	7.8±1.5	5.6±0.4
10 MPa, BMP-2+IGF-I	31.4±1.3	1.06±0.09	187±45	776±260	7.5±0.4	5.6±1.2	5.6±0.1
10 MPa, TGF-β1	14.8±0.4	0.69±0.08	248±37	2048±266	9.6±0.4	15.3±2.9	5.5±0.4

Col., total collagen

Table VII: Phase II construct properties.

Treatment Group	Construct Wet Weight	Thickness
Control	14.8±1.1	0.49±0.03
1% SDS, 1 h	14.3±1.0	0.50±0.02
1% SDS, 8 h	8.8±1.2[a]	0.38±0.04[a]
2% SDS, 1 h	12.3±1.1	0.43±0.05
2% SDS, 8 h	9.3±2.6[a]	0.47±0.08
2% TnBP, 1 h	15.2±1.1	0.53±0.06
2% TnBP, 8 h	12.2±1.2	0.49±0.04
2% Triton X-100, 1 h	13.7±1.2	0.47±0.05
2% Triton X-100, 8 h	11.2±1.7[a]	0.47±0.08
Hypo/Hyper 1 h	15.0±3.0	0.40±0.09
Hypo/Hyper 8 h	7.0±1.3[a]	0.35±0.04[a]

[a]Significantly lower than control ($p<0.05$)

Table VIII: Phase II construct properties.

Treatment Group	Poisson Ratio	Permeability
Control	0.30±0.07	14.3±3.9
1% SDS, 1 h	0.26±0.04	15.6±8.0
1% SDS, 8 h	0.07±0.09[a]	2.0±1.6[a]
2% SDS, 1 h	0.26±0.10	12.6±6.3
2% SDS, 8 h	Not testable	Not testable
2% TnBP, 1 h	0.24±0.13	5.5±3.1
2% TnBP, 8 h	0.04±0.03[a]	7.3±7.5
2% Triton X-100, 1 h	0.16±0.11	4.3±2.6
2% Triton X-100, 8 h	0.04±0.04[a]	5.1±4.7
Hypo/Hyper 1 h	0.14±0.14	14.9±6.6
Hypo/Hyper 8 h	Not testable	Not testable

[a]Significantly lower than control ($p<0.05$)