A Tissue Engineering Strategy for Integrative Cartilage Repair

by

Keri A. Mroszczyk

B.S. Biomedical Engineering
Boston University, 2012

Submitted to the
Department of Mechanical Engineering
in Partial Fulfillment of the Requirements for the Degree of

Master of Science in Mechanical Engineering

at the

Massachusetts Institute of Technology

June 2014

Signature redacted

Signature of Author: _____
Department of Mechanical Engineering
May 23, 2014

Signature redacted

Certified by: _____
Alan J. Grodzinsky
Professor of Biological, Electrical, and Mechanical Engineering
Thesis Supervisor

Signature redacted

Accepted by: _____
David E. Hardt
Professor of Mechanical Engineering
Graduate Officer

A Tissue Engineering Strategy for Integrative Cartilage Repair

by

Keri A. Mroszczyk

Submitted to the Department of Mechanical Engineering
on May 23, 2014 in Partial Fulfillment of the
Requirements for the Degree of
Master of Science in Mechanical Engineering

ABSTRACT

Tissue engineering for cartilage repair is a promising approach for improving the healing of articular defects, as biomaterials and growth factors can be supplied directly to a focal lesion. However, integrating neo-tissue with native cartilage to provide mechanical and biological continuity at the interface remains a challenge due to the limited regenerative capacity of the tissue.

In an effort to improve integration, enzyme treatments were investigated as a means of functionally grafting engineered tissue to native tissue. Using an *in vitro* model of defect repair, this study develops a repair strategy that employs both a hydrogel (KLD) functionalized with HB-IGF-1 and an enzyme pre-treatment of the cartilage surrounding a defect to provide local delivery of the pro-anabolic factor and allow for functional integration of cartilage neo-tissue.

Results indicate that the rate of proteoglycan synthesis was elevated in cartilage explants into which KLD pre-mixed with HB-IGF-1 had been cast; both the explants that had received the enzyme pre-treatment and those that were left untreated had a two-fold increase compared to the explants exposed to non-functionalized KLD. Similarly, GAG content was favorably elevated in the chondrocyte-seeded gels exposed to the growth factor. While GAG was depleted selectively within the inner annulus of the explants receiving the pre-treatment, no negative effect was observed on the rate of proteoglycan synthesis or GAG content compared to that in explants left untreated. Further, mechanical tests suggest that the combination of KLD functionalized with HB-IGF-1 together with an enzyme pre-treatment is able to increase interfacial strength between engineered tissue and native matrix.

Taken together, a repair strategy combining an enzyme pre-treatment of a defect with a peptide hydrogel functionalized with pro-anabolic HB-IGF-1, as developed in this study, is a promising approach for enhancing integration. Stimulating the surrounding tissue with the growth factor and allowing for functional integration of newly synthesized matrix promotes continuity at the interface between new and native tissue, ultimately improving the overall quality of repair.

Thesis Supervisor: Alan J. Grodzinsky
Title: Professor of Biological, Electrical, and Mechanical Engineering

Acknowledgements

First and foremost I would like to thank my advisor, Alan Grodzinsky, for his mentoring throughout this project, which enabled me to develop a greater understanding of cartilage biomechanics, biological transport, and tissue engineering. I am deeply appreciative of all the meetings, guidance, and encouragement he invested in my research over the past two years. I would also like to express my gratitude to all the members of the Grodzinsky Lab for their support – to Han-Hwa Hung for embedding, sectioning, and staining the histology and to Eliot Frank for his help with the mechanical testing. I would especially like to thank Paul Liebesny for his patience in teaching me the general tissue engineering protocols and for the many discussions of methods and results. Additionally, I am extremely grateful for the technical assistance, perspective, and most importantly the friendship of Yang Wang and Ambika Bajpayee.

I would especially like to thank Hannah Zlotnick, who worked as a UROP on this project, for her help with the histological analysis of the enzyme pre-treatment and for her extra hands during the *in vitro* cultures and mechanical testing. Further, I appreciate the consultations with Dr. David Frisbie and Dr. John Kisiday regarding clinically relevant enzyme pre-treatments and repair strategies.

For the coordination and preparation of the peptide hydrogel, I would like to acknowledge Dr. Lisa Spirio. For the supply of HB-IGF-1, I would like to thank Dr. Parth Patwari and Dr. Rich Lee. I would also like to thank Dr. Emily Florine and Dr. Paul Kopesky for their insights on protocol development.

This project was supported in part by NIH-NIAMS Grant AR060331.

Table of Contents

Glossary

ACI – autologous chondrocyte implantation

ACL – anterior cruciate ligament

ANOVA – analysis of variance

BMSCs – bone marrow stem cells, bone marrow stromal cells, bone marrow-derived mesenchymal stem cells

CS – chondroitin sulfate

ECM – extracellular matrix

FBS – fetal bovine serum

GAG – glycosaminoglycan

sGAG – sulfated glycosaminoglycan

HB-IGF-1 – a fusion protein of IGF-1 and the positive Heparin Binding (HB) domain of HB-EGF (epidermal growth factor)

IGF-1 – insulin-like growth factor-1

KLD – self-assembling peptide hydrogel with repeating lysine (K), leucine (L), and aspartic acid (D) amino acid sequences.

MCL – medial cruciate ligament

OA – osteoarthritis

OAT – osteochondral autograft transplantation

PBS – phosphate-buffered saline

PGA – polyglycolic acid

PTOA – post-traumatic osteoarthritis

RAD – self-assembling peptide hydrogel with repeating arginine (R), alanine (A), and aspartic acid (D) amino acid sequences.

List of Figures

II. BACKGROUND

III. METHODS

IV. RESULTS

I. Introduction

1.1 Problem Statement

Articular cartilage is a specialized tissue that simultaneously allows the transfer of loads from one bone to another and the articulation of the load-bearing surfaces with low friction. These biomechanical properties are attributed to the highly organized, extracellular matrix (ECM), which is produced by chondrocytes and is predominantly composed of collagen fibers and aggrecan proteoglycans. However, this avascular tissue has limited capacity for regeneration should a defect occur [1]. Focal defects to the articular surface typically result from a pivot on a bent knee, or an injury to the meniscus, MCL, or ACL and, if left untreated, more than 40% of people who experience such a traumatic joint injury will develop osteoarthritis (OA) [2]. The current gold standards for treatment include surgical implantation of autologous chondrocytes and microfracture at the lesion [3, 4], which largely aim to relieve joint pain but do not regenerate a continuous surface of hyaline tissue, leading to the formation of fibrocartilage that fails to restore the native biomechanical environment.

Methods of tissue engineering have sought to improve cartilage repair by creating a microenvironment in the lesion that allows for endogenous cell migration, chondrocyte proliferation, and ECM synthesis [5-9]. To further improve repair, HB-IGF-1, a fusion protein of IGF-1 and the positive Heparin Binding (HB) domain of HB-EGF, has recently been shown to provide local delivery of pro-anabolic IGF-1 to cartilage upon release from a self-assembling peptide hydrogel regulated by charge-charge interactions within the gel [6]. However, the challenge of integrating the neo-tissue with native cartilage has not been adequately addressed. In an effort to improve integration, enzyme treatments have been investigated as a means of enhancing the cellularity at the edge of a defect [10-12] and functionally grafting engineered tissue to native tissue [13]. A repair strategy that employs both a hydrogel functionalized with HB-IGF-1 to stimulate encapsulated cells as well as native tissue and an enzymatic pre-treatment of the defect surface is proposed to better promote biological and mechanical continuity at the interface.

1.2 Specific Aims

The overall goal of this study was to develop an improved tissue engineering strategy for integrative cartilage repair using a self-assembling peptide hydrogel

functionalized with HB-IGF-1 coupled with an enzyme pre-treatment in an *in vitro* model of defect repair. In support of this goal, this study aimed to 1) determine an optimal enzyme cocktail and pre-treatment duration to selectively deplete GAG at the edge of the defect, 2) assess the ability of HB-IGF-1 to stimulate encapsulated cells as well as surrounding, pre-treated cartilage tissue, and 3) quantify the release of HB-IGF-1 from the gel to determine the extent of sustained, local delivery.

1.3 Scope of Report

The first phase of this study investigated the ability of two different enzymes to deplete GAG content in explants of cartilage tissue. Annuli of cartilage were harvested from the femoropatellar grooves of 1-2-week-old calves, and the inner surfaces were exposed to chondroitinase ABC at 1 or 2 U/ml or trypsin at concentrations ranging from 50 μg/ml to 25 mg/mL, for 0.5 to 30 minutes. The explants were imaged following histological preparation, and a protocol was developed to process the images in MATLAB to quantify the areas of depleted GAG content. The trends in the average depth of enzymatic degradation with respect to concentration and incubation time allowed an evaluation of the utility of the enzyme pre-treatments for the integrative cultures using an *in vitro* model of defect repair.

Secondly, this study tested a novel repair strategy that coupled the release of HB-IGF-1 from a self-assembling peptide hydrogel with an enzyme pre-treatment of the cartilage tissue surrounding the defect. Both acellular and chondrocyte-seeded peptide suspensions were cast into cartilage annuli, and the resulting cartilage-gel constructs were then cultured for 7, 8, and 15 days. Four conditions were prepared: (i) Control: KLD cast into untreated explants, (ii) Enzyme treated: KLD cast into enzyme pre-treated explants, (iii) HB-IGF-1: KLD pre-mixed with HB-IGF-1 and cast into untreated explants, and (iv) Enzyme treated plus HB-IGF-1: KLD pre-mixed with HB-IGF-1 and cast into pre-treated explants. In conditions (iii) and (iv), prior to gel casting, HB-IGF-1 was mixed into the unassembled peptide such that HB-IGF-1 was delivered from the functionalized gel to the cartilage interface as a single dose. The rate of biosynthesis, the sGAG content, and the strength of the gel-explant interface were measured and compared among conditions.

Finally, the release of HB-IGF-1 from the hydrogel was quantified using an IGF-1 ELISA kit. Samples of the medium from the *in vitro* cultures were taken every 24 hours

for nine days after which the gels and explants were separated and incubated in 10x PBS for five days during which samples of the PBS were also taken. The mass of HB-IGF-1 in each of the medium and high-salt samples was quantified to determine the extent of diffusion of the growth factor out of the gel to the surroundings.

1.4 Summary of Results

Histological analysis of the enzyme-treated specimens indicated that exposing the explants to 2 U/mL of chondroitinase and 50 μg/ml of trypsin for two minutes resulted in a depletion of GAG within 200 μm of the inner surface of the tissue, which was used for optimization of the subsequent experiments. Even though GAG content was selectively depleted at the interface between the hydrogel and cartilage annulus, the enzyme treatment did not have a negative effect on the rate of sulfate incorporation in the surrounding tissue. Following 7, 8, and 15 days of culture, the rate of proteoglycan synthesis was elevated in cartilage explants into which KLD pre-mixed with HB-IGF-1 had been cast; both the explants that had received the enzyme pre-treatment and those that were left untreated had a two-fold increase compared to the explants exposed to non-functionalized KLD ($p<0.05$). Further, the GAG content in the chondrocyte-seeded gels cast into the explants was significantly greater ($p<0.05$) in the conditions exposed to the growth factor compared to the hydrogels that were not functionalized with HB-IGF-1. These two outcomes led to an increased interfacial strength between the hydrogel and native tissue.

There was a limited release of HB-IGF-1 to the medium over the course of culture suggesting that the majority of the HB-IGF-1 had diffused laterally into the cartilage as evident by the elevated rate of biosynthesis. The cumulative release of HB-IGF-1 to the medium from the acellular hydrogels accounted for an average of 4% of the total mass initially added at the time of casting, whereas 61% was recovered from the cartilage tissue following desorption in 10x PBS. Taken together, these findings demonstrate that combining an enzyme pre-treatment of a defect surface and a peptide hydrogel functionalized with pro-anabolic HB-IGF-1, as presented here, can enhance integration by stimulating surrounding tissue and allowing functional integration of newly synthesized matrix.

II. Background

2.1 Traumatic Joint Injury

Articular cartilage is the shiny, white surface of tissue that covers the ends of long bones, allowing for smooth articulation of joints, such as the knee. Torsional loading on a bent knee, a direct blow, or an injury to the meniscus, MCL or ACL, can result in an injury to this surface in the form of a focal defect [2]. If left untreated or poorly repaired, this kind of injury can lead to severe inflammation and progressive degeneration of the joint, ultimately resulting in post-traumatic osteoarthritis (PTOA), which is characterized by severe joint pain and affected mobility [2, 14]. Even with the current treatments for acute joint injuries, approximately 5.6 million individuals in the United States are affected by PTOA and seek care by an orthopedic reconstructive surgeon. The corresponding aggregate financial burden of this disease was estimated in 2006 to be $3.06 billion annually [15]. A repair strategy that promoted regeneration of cartilage tissue within focal defects soon after injury would restore the native biomechanical environment of the articular surface, prevent degeneration of the joint, and ultimately aim to reduce the prevalence of osteoarthritis.

2.2 Cartilage Biology and Repair

The ECM of articular or hyaline cartilage is predominantly composed of aggrecan proteoglycans and type II collagen fibers, which afford the smooth mechanical function of synovial joints. Aggrecan contains a core protein with sulfated glycosaminoglycan (sGAG) chains, which have a fixed, negative charge. These negatively charged GAGs, including chondroitin sulfate (CS) and keratan sulfate, are the main molecules contributing to the tissue's compressive stiffness, while the collagen fibers bear the tensile loads in the ECM [1]. The negatively charged GAG chains attract positive ions, and the resulting electrostatic repulsion forces between adjacent GAG molecules allow the tissue to resist static or low frequency compression without large changes in the swelling pressure [16-18]. At higher loading rates (frequencies), the densely packed GAGs are the main barrier to intra-tissue flow fluid, and the resulting low hydraulic permeability enables increased poroelastic self-stiffening beyond that of cartilage's equilibrium compressive stiffness [19]. The organization of the collagen fibers into a tight network promotes a cohesive matrix by mechanically sequestering the

proteoglycans and resisting the osmotic pressure maintained by the GAGs [20]. Integrity of this inert matrix is critical for supporting a peak dynamic load of 15 to 20 MPa that knees experience upon climbing stairs, at compressive strains as high as 10-20% [21, 22] as well as static loads of 3.5 MPa with strains up to 45% [23]. Upon injury to the articular surface, the mechanical stiffness of the tissue is compromised due to an initial loss of GAG content, which is the precipitating event of PTOA, and further damage to the collagen matrix promotes greater degeneration of the tissue – a point of no return for therapeutic intervention [2, 24-27].

Should such a loss of GAG content occur, due to an injury for example, cartilage has a limited capacity for regeneration. The maintenance of the ECM is controlled by chondrocytes, which synthesize the macromolecules and assemble the proteoglycans and collagen fibers into an organized matrix, altering the framework as necessary in response to mechanical and biochemical cues. In turn, the matrix serves to bind the chondrocytes to the macromolecular framework [28]. However, these specialized cells are sparsely distributed throughout the matrix and have relatively low metabolic and mitotic activity, reducing the tissue's regenerative capacity [29-31]. Further, the transport of molecules and biochemical signals is controlled strictly by diffusion through the extracellular matrix, as the tissue lacks vasculature, nerves, and lymphatic vessels, hindering an adequate reparative response that is possible in other orthopaedic tissues [32].

Instead of a hyaline repair, the innate physiological response to a focal defect is the formation of fibrocartilage. The structure of this new tissue differs greatly compared to that of the native cartilage, as it is denser and more fibrous due to its limited GAG content and the prevalence of type I instead of type II collagen (Fig. 1). It is unable to withstand the compressive and hydrostatic stresses of everyday activities, making it susceptible to degeneration over time [33]. Currently, efforts to surgically repair defects include: microfracture, osteochondral autograft transplantation (OAT), and autologous chondrocyte implantation (ACI). Microfracture is the least invasive and most cost effective of the surgical procedures and is the current gold standard for treatment. This procedure aims to induce an improved endogenous repair by puncturing the subchondral bone to provide the defect with a blood supply and cell source from the underlying marrow. These surgical strategies, however, fail to provide a hyaline repair [3, 4, 9, 34-

36], do not address the challenge of integrating the neo-tissue with native cartilage, and ultimately lead to degradation of the surface. A treatment that enhanced the microfracture procedure by controlling the microenvironment in the lesion, such that cells could undergo chondrogenesis [37] and synthesize hyaline ECM, would better repair the articular surface.

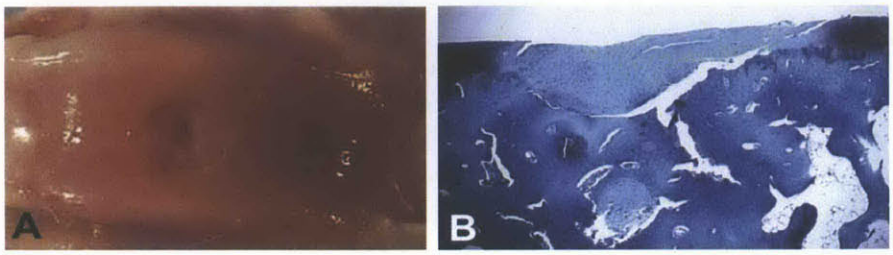

Figure 1. A) Innate repair of a defect that was made in the trochlear groove of a rabbit, which differs from the smooth, shiny hyaline surface of the surrounding tissue. B) A histological section in the sagittal plane of the same defect shows fibrous repair, as it lacks the GAG specific Safranin-O red stain of the adjacent tissue [9].

2.3 Motivation for Tissue Engineering

Methods of tissue engineering have sought to improve cartilage repair by creating a microenvironment that allows for cell migration, chondrocyte proliferation, proteoglycan synthesis, and ECM assembly *in vivo* such that new hyaline cartilage forms to fill the defect. Tissue engineering strategies take advantage of endogenous cell sources, employ biomaterials that allow delivery of pro-chondrogenic, pro-migratory, and pro-anabolic factors, and commonly provide a non-invasive method for tissue regeneration.

Many biomaterials have been studied with the intent of engineering new tissue within a scaffold to fill a defect and restore the mechanical integrity of the surface [38]. While agarose, decellularized ECM, and polymer-based scaffolds have been studied extensively, self-assembling peptide hydrogels including those with repeating sequences of $(RADA)_4$ or $(KLDL)_3$ (known as RAD and KLD, respectively) are of particular interest [39, 40]. These scaffolds are especially promising, as they are biocompatible, bioabsorbable, and gel *in situ*. Further, they are able to capture bone marrow stromal cells (BMSCs), stimulate chondrogenesis, and produce new hyaline-like cartilage tissue [5-9, 41-44].

Even though both self-assembling peptide hydrogels used in previous studies were matched for mechanical characteristics [8, 39, 40], KLD was chosen over RAD for additional investigation due to its greater ease of preparation and handling. KLD has a backbone composed of positively charged lysines (K), hydrophobic leucines (L), and negatively charged aspartic acids (D), and these alternating hydrophobic and hydrophilic residues (Fig. 2A) promote beta sheet formation into interweaving fibers (Fig. 2B) [39]. This nanofiber scaffold gels *in situ* upon exposure to physiologic pH and ionic strength (Fig. 2C) and has been shown to improved repair of articular defects in rabbits [9]. The nanofiber-based structure is attractive in that it allows for functionalization with growth factors, due to favorable electrostatic interactions at local charge groups, potentially supporting an enhanced repair [5, 6, 45].

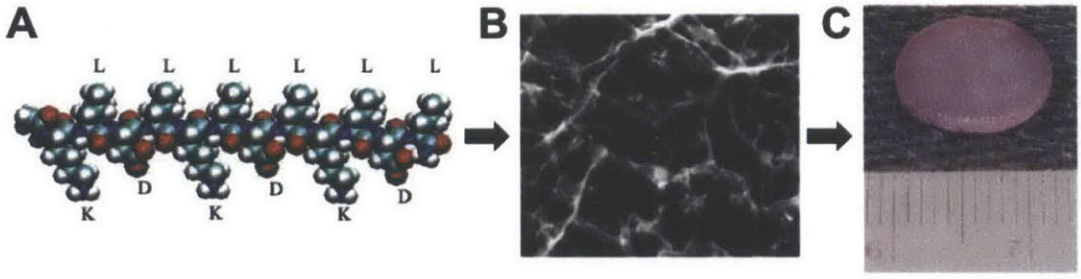

Figure 2. A) Molecular structure of KLD showing the repeating sequence of amino acids [7]. B) The alternating hydrophilic and hydrophobic residues promote beta sheet formation into interweaving fibers [39]. C) A chondrocyte-seeded hydrogel 12 mm in diameter that gelled *in situ* upon exposure to physiological pH and ionic strength [7].

Tissue engineering is advantageous in that it allows endogenous tissue regeneration by cells present in the defect. A previous study has shown that applying a biomaterial with pre-encapsulated cells did not improve repair *in vivo* while a hydrogel alone provided superior regeneration, under the assumption that cells were recruited from the subchondral bone [9]. Mesenchymal stem cells, otherwise known as bone marrow stromal cells (BMSCs), are easily acquirable at the site of injury and have the ability to differentiate into multiple phenotypes, including chondrocytic, osteocytic, and adipocytic lineages [46]. While stem cells can be isolated from other sources, such as adipose tissue, BMSCs have proven to provide superior matrix production compared to adipose-derived progenitor cells [36]. Further, even though synovial MSCs have a greater chondrogenic potential that BMSCs [42, 47], fostering migration of these cells into the scaffold to fill

the defect would be challenging. By inducing blood flow from the marrow into the hydrogel and fostering chondrogenesis of the BMSCs that migrate into the scaffold, new cartilage tissue can be regenerated *in situ*. Inducing chondrogenesis and maintaining a hyaline cartilage phenotype without progression to hypertrophy and fibrosis or calcification is critical [37]. The self-assembling peptide hydrogel alone, without functionalization with chondrogenic factors, has been shown to induce chondrogenesis in BMSCs [7, 9, 42]. Not only does it promote differentiation of BMSCs into chondrocytes, the large water content (>99%), provides adequate space for BMSCs to migrate and for new matrix to form and support of the mechanical load on the articular surface.

The aim of this project is to develop an approach to repair defects by implanting a molecular-engineered scaffold for an augmented microfracture procedure [48]. This involves the formation of holes in the subchondral bone to create a blood supply, implanting the gel containing certain pro-migratory factors to stimulate the migration of mesenchymal cells from the bone marrow to populate the scaffold. The challenge of promoting migration of mesenchymal stem cells into the scaffold and inducing chondrogenesis will not be addressed in this work. The *in vitro* model developed here concerns a post-migratory phase of repair in which cells have filled the scaffold and undergone chondrogenesis. At this point in the repair process, it is advantageous to enhance the production of matrix by adding a pro-anabolic factor to the gel to deliver directly to the defect.

2.4 Insulin-like Growth Factor-1

The metabolic activity of chondrocytes is modulated by a number of growth factors including insulin-like growth factor-1 (IGF-1), transforming growth factor-β1 (TGF-β1), bone morphogenic protein-7 (BMP-7), and platelet-derived growth factor (PDGF) during pre-natal and post-natal development [49]. IGF-1 is of particular interest as it is the predominate regulator of chondrocyte metabolism and activity [50]. It is synthesized locally within cartilage tissue by chondrocytes to increase the production of collagen, proteoglycans, and other matrix macromolecules as well as by the liver [50, 51]. In addition to its pro-anabolic effects, IGF-1 has been shown to have anti-catabolic effects, aiding to prevent degeneration by proteinases [52, 53], and to improve the biomechanics of the tissue [54]. This growth factor is also of particular interest as it

promotes chondrogenesis from progenitor cells [55] and promotes the deposition of type II collagen from recruited stem cells when released from a fibrin composite in an articular defect *in vivo* [56]. Due to its small size as a 7.6-kDa polypeptide, IGF-1 is quickly cleared from the joint, causing concern of systemic effects if administered exogenously and limiting its capacity to affect cartilage tissue locally.

In an effort to prolong the delivery of IGF-1 to cartilage tissue, HB-IGF-1, a fusion protein of IGF-1 and the positive Heparin Binding (HB) domain of HB-Epidermal Growth Factor (HB-EGF) has been developed (Fig. 3) [57]. The positively charged HB domain allows for exploitation of favorable interactions with the negatively charged GAGs in the ECM of intact tissue to prolong retention and activity in chondrocytes. Upon administration to explants for 48 hours followed by washout of excess growth factor, HB-IGF-1 increased the rate of proteoglycan synthesis in cartilage disks for up to 6 days [57]. Further, HB-IGF-1 outperforms IGF-1 as it is retained in human and rat cartilage through binding to CS [58]. It is also retained in the self-assembling peptide hydrogels due to non-specific electrostatic interactions with local charge groups [6].

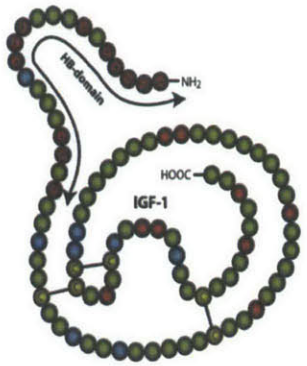

Figure 3. Representation of HB-IGF-1 showing the amino acid sequence of IGF-1 and the positive Heparin-Binding domain where red circles are positive amino acids, blue circles are negative amino acids, and yellow circles are cysteine [57].

Efforts have been made recently to use hydrogels as a method to provide a sustained release of antibodies [59] and growth factors [45] . In particular, an *in vitro* study undertaken to assess the ability of the self-assembling peptide hydrogel RAD, to provide sustained, local delivery of HB-IGF-1 to neighboring cartilage has demonstrated that HB-IGF-1 released from the hydrogel can enhance matrix synthesis in an explant cultured adjacent to the growth factor-functionalized gel (Fig. 4) [6]. Explants were cultured on top of RAD into which HB-IGF-1 had been pre-mixed prior to gel assembly, such that the growth factor was delivered in one dose. After 10 days, explants cultured in this condition had elevated rates of proteoglycan synthesis compared to explants cultured on gels pre-mixed with one dose of IGF-1 and on control gels without exposure to a

growth factor. In a separate experiment, chondrocyte-seeded hydrogels pre-mixed with HB-IGF-1 had greater GAG content compared to control gels after 10 days. Results of these studies demonstrate the potential use of KLD functionalized HB-IGF-1 to enhance neo-tissue production within the hydrogel scaffold and also stimulate the surrounding tissue, for improved integration of engineered matrix with native cartilage. Because diffusion of HB-IGF-1 would have to occur laterally to affect native cartilage after acting on encapsulated cells in a focal defect *in vivo,* to prevent loss of the growth factor to the synovial fluid, additional investigation of the functionalized hydrogel was warranted.

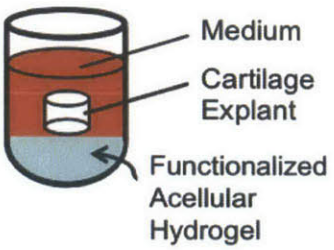

Figure 4. Culture system used to assess ability of RAD to provide sustained released of HB-IGF-1 to an adjacent cartilage explant [6].

2.5 Integration and Enzymatic Treatments

Despite developments in repair strategies employing tissue engineering, the challenge remains to integrate engineered cartilage tissue with native matrix surrounding the defect. For a hydrogel to remain secure in the defect, it must provide both vertical integration with the subchondral bone and lateral integration with the surrounding tissue. Vertical integration with the underlying bone is often achieved; however, lateral integration with native cartilage is difficult [60-62]. Without addressing the need to join engineered tissue with native tissue, micromotion can occur between an implant and native tissue [63], and a stress concentration at the interface threatens hyaline repair, fostering degradation to the mechanically inferior fibrocartilage.

Prior work has employed an *in vitro* model of defect repair for the purpose of studying integration and has shown that simply adding chondrocytes to a cartilage annulus without any treatment of the interface does not promote the joining of neo-tissue and native tissue [64, 65]. The clinical application of sutures or staples to close the gap between a graft of cartilage and native tissue fails to repair the wound, generates additional damage, and remains in the tissue long-term [66]. Fibrin glue has often been used for vertical and lateral fixation of implants within defects for orthopaedic repairs, but its success is mostly confined to the integration of osteochondral implants with osseous healing [67]. In an effort to improve the lateral adhesion of biomaterials and

cartilage tissue, a glue-like adhesive was developed with a chondroitin sulfate-based solution. This CS solution was designed such that two functional arms were generated: one arm to covalently bond to a biomaterial scaffold and the second arm to bond to the tissue surface [68]. This adhesive has been tested *in vivo* with a photoreactive polymer implant and was shown to both increase tissue fill compared to controls and alleviate pain [69]. Integration was measured based on the gap distance between implant and native tissue as determined from MRI, though the extent of mechanically functional integration was not directly determined. An additional adhesive glue, tissue transglutaminase, has been shown to improve adhesive strength between cartilage-cartilage constructs [70], but does not address adhesion between a tissue engineering scaffold and native tissue. Such adhesives are "painted" on a defect and do not penetrate the tissue. In general, these approaches aim to simply bond two materials, and while the integrity of the bond may surpass that of an "unpainted" surface, ideally a well-integrated tissue should restore a biologically continuous surface as well as a mechanically continuous surface.

To promote functional integration of engineered cartilage tissue with native tissue, such that new matrix knits together with native matrix, pre-treating the edge of the defect with an enzyme has been pursued as an alternative. Depleting a portion of the proteoglycan content at the edge of a defect exposes the collagen network as well as other proteins, which has been shown to improve cell-adhesion properties of the native tissue [10, 11, 71-74]. Enzyme pre-treatments also increase cell density at the wound edge [75-78], increase coverage by repair cells [10, 11], and do not negatively affect cell-associated proteoglycan deposition [12]. Pre-treating the edge of a defect with collagenase and hyaluronidase increases the interfacial strength between cartilage-cartilage constructs compared to untreated defects [76]. Further, by histological examination, it is evident that extracellular matrix fibers are able to cross over from enzyme-treated parts to untreated parts in cartilage-cartilage constructs [75, 77]. While pre-treatments have been shown to improve the integration between cartilage grafts and native tissue, of greater interest is the integration of engineered cartilage with the surrounding matrix. In one study, chondrocyte-seeded, biodegradable polyglycolic acid (PGA) scaffolds showed greater integration with explant annuli that had been pre-treated with trypsin compared to untreated explants [13]. Therefore, it is advantageous to expose

the defect to a pre-treatment and allow controlled degradation for a limited amount of time to create these desirable pro-integrative effects but avoid global degradation of ECM that would prevent a favorable healing response. However, there has been little investigation of using both a tissue-engineered scaffold containing pro-anabolic factors and an enzyme pre-treatment to promote integration and to enhance repair.

This thesis project evaluated the effects of both an enzyme treatment of the tissue at the edge of a defect and KLD functionalized with pro-anabolic HB-IGF-1 to provide continuity at the hydrogel-cartilage interface. By comparing the distance of depleted GAG content in cartilage annuli treated by different enzyme cocktails at several different incubation times, this study developed a quantitative guideline for determining the extent of degradation and the incubation period required to obtain an optimal depletion within a defect *in vitro*. Additionally, this project assessed the ability of a self-assembling peptide hydrogel to deliver HB-IGF-1 to native tissue, stimulate production of ECM of encapsulated cells, and promote integration between neo-tissue and native matrix. The overall objective of this study was to develop an improved tissue engineering strategy for integrative cartilage repair using a self-assembling peptide hydrogel functionalized with HB-IGF-1 coupled with an enzyme pre-treatment using an *in vitro* model of defect repair.

III. Methods

3.1 Materials

The self-assembling peptide hydrogel, KLD12, with the sequence AcN-$(KLDL)_3$-CNH_2, hereafter known as KLD, was donated by 3-D Matrix (Waltham, MA). Hydrogels were prepared at a concentration of 0.35% (w/v) for all experiments, consistent with previous literature [8, 43]. The supply of HB-IGF-1 was provided by Dr. Parth Patwari (Brigham & Women's Hospital, Boston, MA). All other materials were purchased from the suppliers noted below.

3.2 Enzyme Pre-treatment

3.2.1 Cartilage Explant Harvest

Cartilage explant disks 8 mm in diameter and 1.4 mm thick consisting of middle zone tissue were obtained from the femoropatellar grooves of immature bovine calves (Research 87, Boylston, MA) using an 8 mm biopsy punch. Middle zone tissue was considered to be within a depth of 1.4 mm after the first 0.7 mm

of tissue, including the superficial zone, was removed from the surface of full thickness plugs using a custom jig. Concentric circular holes were then cut in the 8 mm disks using a 6 mm biopsy punch to create annuli of cartilage tissue similar to previous studies [13, 65, 76, 79] (Fig. 5A). These explant rings were allowed to rest for 48 hours in serum-free, basal medium consisting of high glucose Dulbecco's modified Eagle's medium (DMEM) (Mediatech, Inc, Manassas, VA) supplemented with penicillin-streptomycin-amphotericin (PSA), 4-(2-hydroxyethyl)-1-piperzaineethanesulfonic acid (HEPES) (Invitrogen, Carlsbad, CA), proline, ascorbate, sodium pyruvate, and non-essential amino acids (NEAA) (Sigma-Aldrich).

3.2.2 Controlled Depletion of GAG

Following equilibration in basal medium, the annuli were sealed to the bottom of a 24-well plate using 3% agarose such that the annuli were level and centered within the wells, and the centers of the explants were able to retain a volume of liquid (Fig. 5B). Chondroitinase ABC (Sigma-Aldrich) and trypsin derived from bovine pancreas (Sigma-Aldrich) were prepared at the given concentrations at room temperature (20°C) in phosphate-buffered saline (PBS; pH 7.4). Chondroitinase pre-treatments were prepared at 1 U/mL and 2 U/mL, consistent with prior work [10-12], while the trypsin pre-treatments were prepared at concentrations of 10 mg/mL (1%) [13], 25 mg/mL (2.5%) [11, 12], 50 μg/mL [80], 200 μg/mL, and 500 μg/mL.

The pre-treatment was applied by adding 50 μl of the enzyme to the center of an annulus, allowing the enzyme to incubate in the explant for a given time at room temperature, suctioning out the enzyme, and thoroughly flushing the annulus with 1ml of PBS 5 times (Fig. 5B). The treatments lasted 0.5-30 minutes for each enzyme concentration, and each treatment was applied to 1-2 explants. For the trypsin treatments, prior to washing with PBS, 50 μl of fetal bovine serum (FBS) (ThermoScientific, Logan, UT) was added to the defect following the removal of the enzyme and was allowed to incubate for the same time for which the enzyme was applied, in order to neutralize any remaining enzyme. The defect was then thoroughly flushed with PBS as was done for the chondroitinase pre-

treatments. For trypsin pre-treatments at concentrations of 10 mg/mL and 25 mg/mL, the neutralization step was not performed. For this pre-treatment study, the hydrogel was not cast into the center of the explants, only the enzyme was applied to the defect (step 1 in Fig. 5B). The hydrogel was cast following pre-treatments in subsequent in vitro cultures. Additional explants were prepared as controls, to which no enzyme pre-treatment was applied, to determine the extent of GAG depletion attributable to the initial creation of the defect.

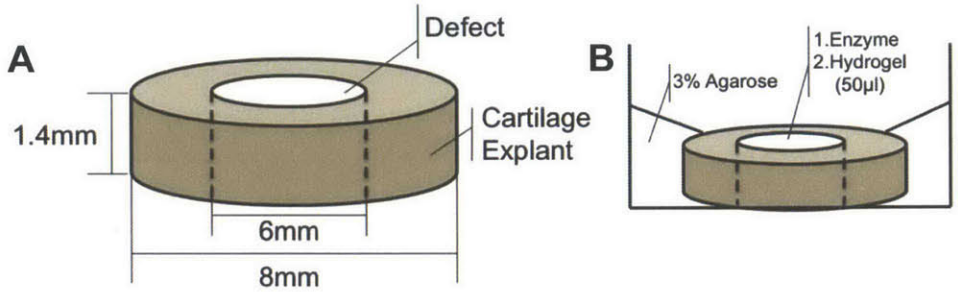

Figure 5. A) Cartilage annuli were made by coring cylinders of tissue 8 mm in diameter from the medial and lateral ridges of the trochlear grooves of 1-2 week old calves, removing the first 0.7 mm of tissue from the surface of the of the plug, and cutting a 1.4 mm-thick disc from the remaining cylinder using a custom jig. A concentric, circular hole 6 mm in diameter was then cut in the disk using a biopsy punch, to model a focal defect. B) An annulus was sealed to the bottom of the well of a 24-well plate with 3% agarose to allow the ring to hold 50 µL of the enzyme for the duration of the pre-treatment (B; Step 1).

3.2.3 Histology

The explants were immediately fixed in 10% neutral-buffered formalin for 24 hours at 4°C following the pre-treatment. The formalin was removed, and the explants were dehydrated in 70%, 80%, 95%, and 100% ethanol followed by xylene and paraffin. The specimens were then sliced into sections, deparaffinized, and stained with toluidine blue in order to visualize the GAG content [8]. The explant rings were sectioned axially to allow visualization of the entire circumference of the inner surface of the annulus (Fig. 6).

Figure 6. A representation of the plane used to section the annuli of cartilage for visualization of the GAG depletion at the inner surface.

3.2.4 Image Processing

A cross-section of each annulus, which had been sectioned halfway through the height of the cylinder, was

imaged using a digital camera kit attached to a light microscope. Each image was made at ten times magnification, using maximum light with the light polarizer. For each treatment, four images were taken of the explant – one in each quadrant of the ring – in order to average the distance of depletion over the inner circumference (Fig. 7A).

A. Pre-Processing

Each RGB image was then imported into ImageJ (NIH). Given the gradation of toluidine blue staining corresponding to the gradation of GAG content at the edge of the defect (Fig. 7B), the contrast was enhanced to define a clear boundary of GAG depletion. The contrast was enhanced based on a 50% cutoff threshold on each of the 8-bit RGB color channels, and the RGB image was then converted to an 8-bit gray scale image. This adjustment resulted in 1 to 2 zones of depletion: the inner-most zone was defined as an area of totally depleted GAG, and the next zone was considered to be partially depleted – where the enzyme had time to only partially degrade the proteoglycan content prior to removal and/or neutralization (Fig. 7C). The inner edge of the defect was marked manually, and the images were rotated such that the inner edge of the annulus was as near vertical as possible to facilitate quantification.

B. Quantification

The rotated images were loaded into MATLAB (MathWorks, Inc., Natick, MA), and using a custom script, the distance of GAG-depletion was semi-automatically calculated (Fig. 7D) (see Appendix A for detailed code). Briefly, the user was prompted to manually define two regions of interest (ROI): 1) total depletion and 2) total plus partial depletion. The horizontal distances between the edges of the ROI were then computed by counting the number of pixels and converting to the image length scale. The average distance of the depletion was reported for each image, and these distances were averaged over the 4 images for each specimen. The standard deviation was then calculated as a measure of the variability across the averages from the images.

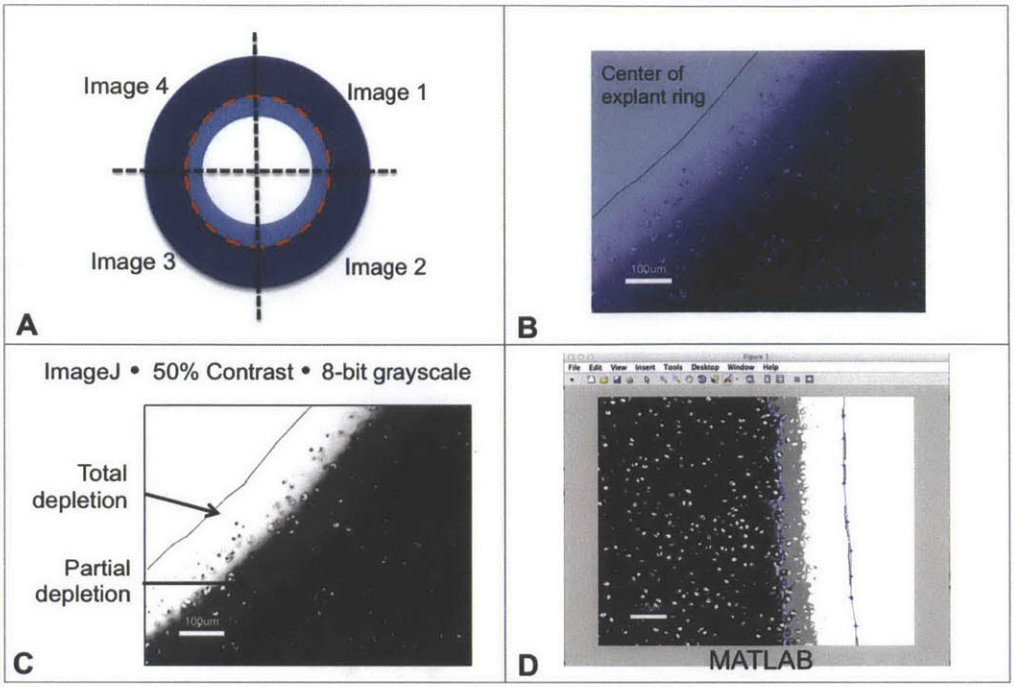

Figure 7. A summary of the histological analysis of the enzyme pre-treatment. A) Sections were digitally imaged in each of the 4 quadrants of the annulus in order to create 4 images representative of the depletion over the circumference of the defect. B) These RGB images showed a gradation of toluidine blue staining in proportion to the gradation of GAG content. C) To resolve a boundary to which the enzyme penetrated, the images were loaded into ImageJ and the contrast was enhanced to reveal two zones of depletion. D) The images were then loaded into MATLAB to measure the distance of depletion.

3.3 *In Vitro* Integration Model

3.3.1 Cartilage Explant Harvest

In the same manner that cartilage annuli were prepared for the pre-treatment study, cartilage explant disks 8 mm in diameter and 1.4 mm thick consisting of middle zone tissue were obtained aseptically from the femoropatellar grooves of immature bovine calves (Research 87, Boylston, MA). Concentric circular holes were cut in the disks using a 6 mm biopsy punch to create annuli of cartilage tissue. These explants rings were allowed to rest for 48 hours in 2 ml of basal medium.

3.3.2 Chondrocyte Harvest

Chondrocytes were harvested from the same immature bovine calves from which the cartilage explants were harvested. Cartilage tissue from the femoral condyles was collected aseptically and digested in basal medium with 2 mg/mL

proteinase (Sigma-Aldrich) for 1 hour, rinsed with PBS, and further digested with 0.25 mg/mL collagenase (Sigma-Aldrich) in basal medium for 24 hours at 37°C. After 24 hours, the solution was diluted to twice the volume with basal medium and maintained at 37°C for another 24 hours in order to match the timing of the explant equilibration. After the 48 hours, chondrocytes were filtered from the remaining tissue using cell strainers with pore sizes of 70 and 40 μm [7, 81]. The resulting cells were then encapsulated within the hydrogel for culture on the same day, as described below.

3.3.3 Construct Assembly and Culture

Following equilibration in basal medium, the explant rings were sealed to the bottom of the wells of a 24-well plate using 3% agarose so that the annuli were level and centered within the wells. 50 μl of a given enzyme was applied for a specified time at room temperature to the center of explants receiving the pre-treatment. The enzyme solution was then removed from the defect, any remaining enzyme was neutralized with FBS and/or the explant was thoroughly flushed with PBS as was performed in the pre-treatment study.

Hydrogel disks with 50 μL initial volume were cast into the neutral-buffered explants to initiate self-assembly and cultured in 2 mL of basal medium for 8 to 15 days. Four conditions were prepared for culture: (i) Control: KLD cast into untreated explants, (ii) Enzyme treated: KLD cast into enzyme pre-treated explants, (iii) HB-IGF-1: KLD pre-mixed with HB-IGF-1 and cast into untreated explants, and (iv) Enzyme treated plus HB-IGF-1: KLD pre-mixed with HB-IGF-1 and cast into pre-treated explants (Fig. 8). In conditions (iii) and (iv), prior to gel casting, HB-IGF-1 was mixed into the unassembled peptide at a concentration of 615 nM (see Appendix A for determination of concentration). In this manner, HB-IGF-1 was delivered from the functionalized gel to the cartilage interface as a single dose. KLD was cast both as A) acellular and B) chondrocyte-seeded gels, in which chondrocytes were encapsulated prior to assembly at a concentration of 30 million cells/mL. The medium was changed every 2 days for the duration of culture in all conditions.

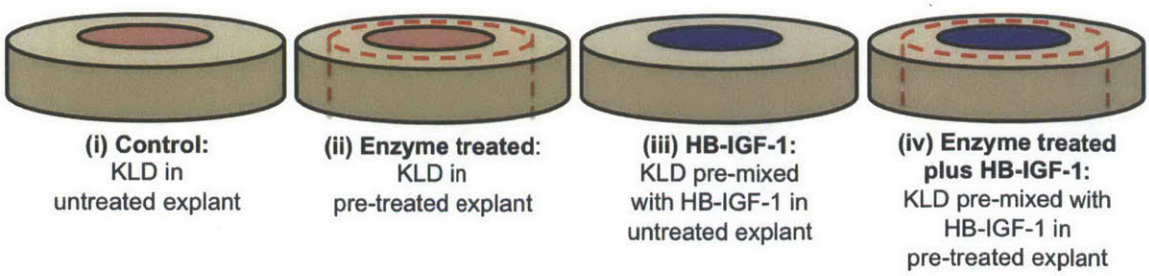

Figure 8. Four conditions were prepared to study integration *in vitro*. The conditions receiving the pre-treatment had the enzyme applied strictly to the inner core of the explant prior to gel casting. In the conditions exposed to HB-IGF-1, KLD was pre-mixed with 615 nM of HB-IGF-1 and cast into the explant. In two separate phases of the experiment, KLD was cast as A) acellular gels and as B) chondrocyte-seeded gels.

3.3.4 Viability Staining

To assess the viability of the annuli and KLD-encapsulated chondrocytes for all culture conditions, sections of the explant and gels were stained with 4 μg/mL fluorescein diacetate (FDA) (live cells) and 35 μg/mL propidium iodide (dead cells). The sections were viewed using a Nikon Eclipse TE-300 fluorescence microscope at the commencement and completion of culture.

3.3.5 Preliminary Mechanical Testing

Prior to digestion for biochemical analysis, the integration strength of the chondrocyte-seeded hydrogels to the cartilage explants was determined following the 15-day cultures using a push-out test similar to those described elsewhere [13, 65, 76, 79]. The hydrogel was pushed out from the explant with a 4.7 mm plunger while the cartilage annulus was supported on a rigid, annular platform with a 6.2 mm inner diameter (Fig. 9). The tests were performed with a Dynastat mechanical spectrometer (IMASS, Hingham, MA) by leading the push-out rod on top of the 6 mm hydrogel disc through the cartilage explant at 10 μm/s. The specimens were kept moist with PBS prior to initiating the test. During the test, both displacement and load were monitored at a sample frequency of 20 Hz. The maximum force achieved before separation of the tissues was normalized by the lateral area of the core, with the resulting value considered representative of the interfacial strength [79].

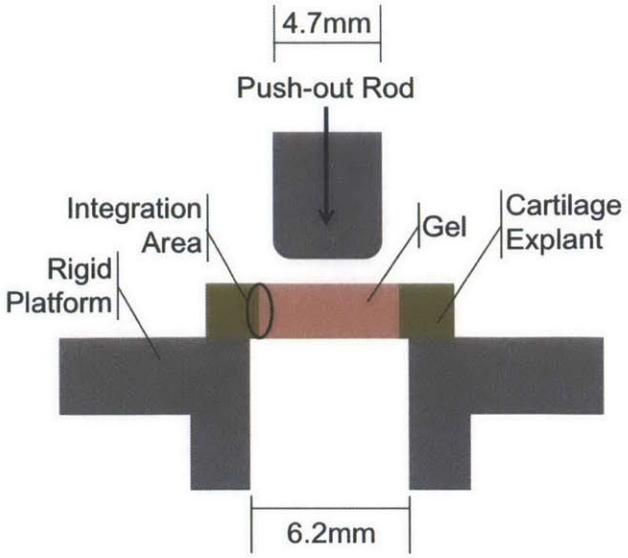

Figure 9. Side-view representation of the push-out setup. The displacement transducer and load cell were connected to the rigid push-out rod.

3.3.6 Biochemical Assays

During the final 24 hours of culture for both the 8-day and 15-day timepoints, the medium was supplemented with 5 μCi/mL of ^{35}S-sulfate (Perkin Elmer, Inc., Waltham, MA) to measure cellular biosynthesis of proteoglycans. Upon completion of culture, four 30-minute rinses of PBS with excess of non-labeled sulfate were performed for all samples to wash out free radiolabel. The gels were then separated from the explants, and each sample was digested in 0.25 mg/mL proteinase-K (Roche Applied Science, Indianapolis, IN) for 48 hours at 60°C. Digested samples were assayed for total retained sGAG content by 1,9-dimethyl-methylene blue (DMMB) dye binding assay [82], DNA content by Hoechst dye binding [83], and radiolabel incorporation with a liquid scintillation counter.

3.3.7 Statistical Analysis

Due to the small sample size of these studies, normality of the data was first assessed using the Levene test for unequal variances. All experiments consisting of explants from a single animal were considered non-normal. Therefore, non-parametric analyses of variance (ANOVAs) were performed on

the ranks of the data for all measurements. The average rate of sulfate incorporation and average sGAG content were compared among the four conditions using a one-factor ANOVA (JMP 11, SAS Institute Inc., Cary, NC). The average adhesive strength at the disk-ring interface of hydrogel-explant constructs was also compared among the four conditions in the same manner. Tukey *post hoc* tests were performed following the ANOVAs to determine differences among the four treatment groups with a threshold for significance of $p<0.05$. Data that included two animal repeats were analyzed using a mixed effects model of variance with animal source as a random variable followed by a Tukey *post hoc* test (JMP 11).

3.4 HB-IGF-1 Release

3.4.1 Construct Assembly and Culture

Cartilage annuli were created from explants harvested from the trochlear grooves of bovine calves as described previously. Acellular hydrogel disks with 50 μL initial volume were cast into the neutral-buffered explants to initiate self-assembly and cultured in 2 mL of basal medium. These constructs were cultured at 37°C for 9 days. Every 24 hours, 500 μL of medium was removed from each well and replaced with fresh medium. The medium samples were stored in low-binding microcentrifuge tubes (Sigma-Aldrich). As a control release experiment, IGF-1 was also incorporated into acellular hydrogel disks and cast into neutral-buffered explants. Medium was sampled in the same manner as was for the constructs containing HB-IGF-1.

On day 9, the entire volume of medium was removed, and the explants and gels were separated into different wells. 1 ml of 10x PBS was added to each specimen in order to disrupt any HB-IGF-1 or IGF-1 remaining in both the gel and explant due to nonspecific electrostatic interactions within the scaffold and cartilage tissue, allowing for desorption of the growth factor. 500 μL of the high-salt solution was sampled on days 11, 14 and 27, and this volume was replaced with 500 μL of fresh 10x PBS. Additional cartilage annuli, which were not exposed to HB-IGF-1, were incubated in 10x PBS for 48 and 96 hours, after which samples of the 10x PBS were taken. These explants were considered

control samples, as they determined if the high-salt solution caused desorption of confounding factors, such as IGF-1 binding proteins, which would elicit a signal in the assay.

3.4.2 HB-IGF-1 Detection

The amount of HB-IGF-1 released to the media and desorbed into the 10x PBS was measured using an IGF-1 enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's protocol (R&D Systems). Standard curves were made in both the basal medium and high-salt solution using HB-IGF-1 and IGF-1. The standard curves were created using concentrations of 30, 20, 10, 5, 2, 1, 0.5, and 0 ng/mL HB-IGF-1 and applying a 2-part exponential fit using the curve-fitting toolbox in MATLAB. For the control study with IGF-1, the standard curves were created using concentrations of 10, 8, 6, 4, 2, 1, 0.5, and 0 ng/mL IGF-1 and applying a 1-part exponential fit using the curve-fitting toolbox in MATLAB.

IV. Results

4.1 Enzyme Pre-treatment

Histomorphological analysis of the cartilage annuli subjected to enzyme pre-treatments indicated that GAG content could be selectively degraded at the edge of the defect. Qualitatively, for each of the cartilage explants, the distance of GAG depletion increased with longer incubation times and higher concentrations. By varying the type of enzyme, concentration, and duration of treatment, GAG was depleted from 100 μm to 1000 μm – the full thickness of the explant ring used in this *in vitro* defect model.

In control annuli that were not exposed to an enzyme pre-treatment, the creation of the 6 mm defect within the explants caused little to no loss of GAG around the inner circumference. These explants showed rich toluidine blue-GAG staining and, following contrast enhancement, no zones of depletion. Therefore, the depletion evident in the pre-treated specimens must be solely the result of the enzymatic activity (Fig. 10).

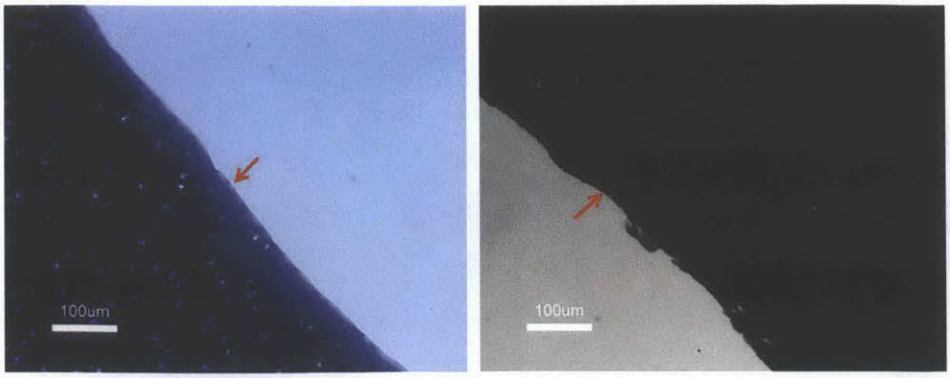

Figure 10. In control explants not exposed to an enzyme pre-treatment, there was no considerable GAG loss at the edge of the defect (red arrows) resulting from the creation of the annulus with a biopsy punch. The injury to the explant did not result in the depletion of proteoglycan content based on qualitative histological examination.

4.1.1 Chondroitinase ABC

As an enzyme pre-treatment, chondroitinase was slow-acting and was inconsistent in the distances of GAG depletion it created among explants. Qualitatively, following treatments at a concentration of 2 U/mL lasting 1, 5, and 10 minutes, 100-200 μm of the GAG content was degraded, evident by a lack of toluidine blue stain at the inner edge of the explant (Fig. 11). However, with 30 minutes of treatment, GAG was depleted as far as 1 mm into the annulus (Fig. 11).

As with 2 U/mL, a lower concentration of 1 U/ml showed around 200 μm of GAG depletion for treatments lasting 1-10 minutes (Fig. 12). However, a 30-minute treatment at this lower concentration resulted in a similar extent of degradation compared to the shorter treatments; it did not cause the 1000 μm of GAG depletion that was evident with the 30-minute treatment at the higher concentration (Fig. 12). Given the large molecular weight of chondroitinase, and its specificity for GAG chains, it slowly depleted the GAG content in the extracellular matrix at the edge of the defect to a distance of around 200 μm in 10 minutes or less. Further, this enzyme caused little to no partial depletion of GAG content (Fig. 12). For optimization of the *in vitro* culture system, a pre-treatment of 2 U/mL for 2 minutes was chosen to achieve around 200 μm of degradation.

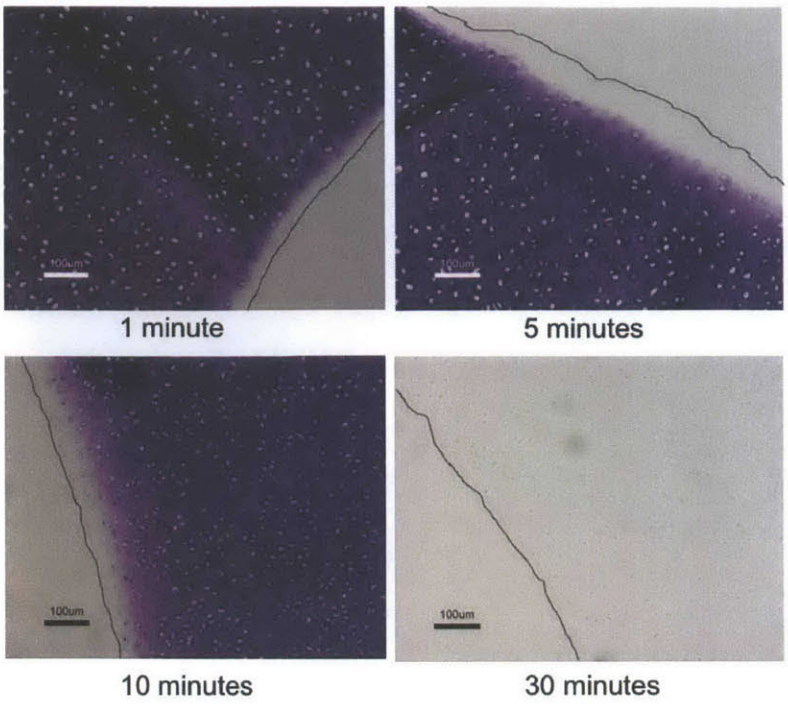

Figure 11. Representative histological sections of explants treated with 2 U/mL chondroitinase for 1 to 30 minutes. The blue/purple staining is indicative of GAG content remaining in the explant while the gray line marks the inner edge of the defect, to which the enzyme was exposed. Within 30 minutes, most of the GAG had been depleted.

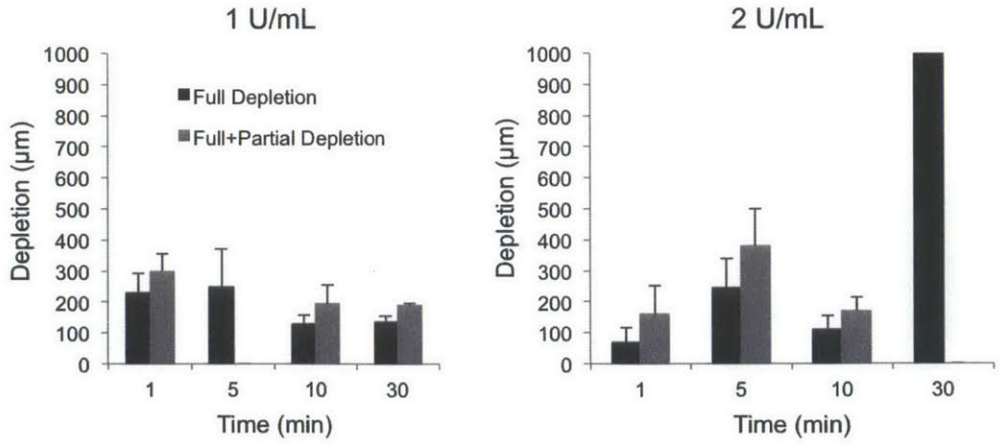

Figure 12. Quantification of the distance of GAG depletion showing the extent of "total" and "partial plus total" zones of degradation for 1 U/mL chondroitinase and 2 U/mL chondroitinase. Time points with a single bar had explants that showed only a totally depleted zone, following contrast enhancement. Values indicate an average (+ standard deviation) over 4 images per explant for 1-2 explants.

4.1.2 Trypsin

Trypsin pre-treatments were more aggressive in that the enzyme, which is five times smaller than chondroitinase, degraded both GAG chains and core proteins and caused greater distances of GAG depletion with shorter incubation times. Qualitatively, following contrast enhancement, two zones of GAG depletion – a "totally depleted" zone shown in white and a "partially depleted" zone shown in gray – consistently increased as the length of exposure increased (Fig. 13).

Initially, concentrations of 10 and 25 mg/mL were applied to the explants. However, without neutralization of the enzyme, these high concentrations caused total depletion of the GAG content through the entire thickness of the annulus (Appendix B, Fig. B.1). As a result, subsequent enzyme pre-treatments with trypsin were followed by an application of FBS to neutralize the enzymatic activity. Trypsin pre-treatments were then prepared at 50, 200, and 500 μg/mL and a consistent distance of 200-400 μm of GAG degradation was achieved for treatments lasting 30 seconds to 5 minutes (Fig. 14). When explants were exposed to 500 μg/mL of trypsin for 2 minutes, 400 μm of depletion was achieved (200 μm of total plus an additional 200 μm of partial depletion) (Fig. 13 and Fig. 14). For optimization of *in vitro* integration cultures, a pre-treatment of 50 μg/mL for 2 minutes was chosen to deplete GAG content up to 200 μm into the tissue (Fig. 14).

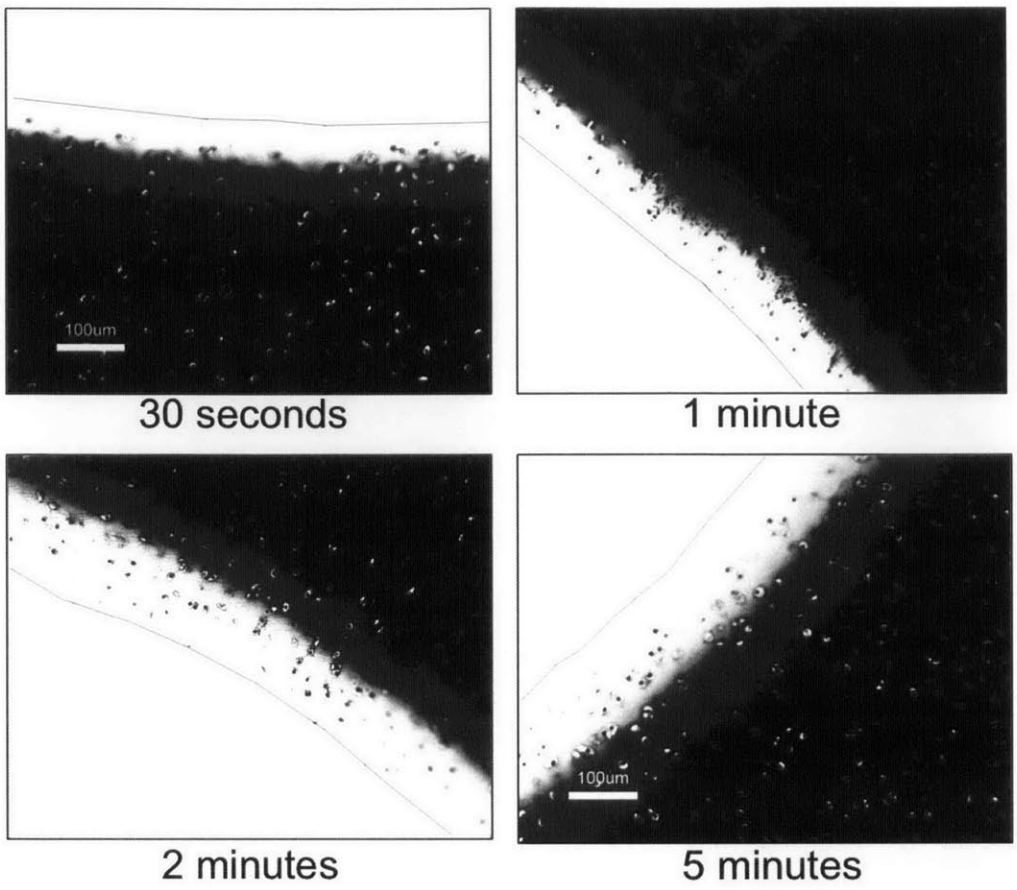

Figure 13. Representative histological sections following contrast enhancement showing the two zones of GAG depletion following treatment with 500 μg/mL trypsin for 0.5 to 5 minutes. As time increased, the extent of GAG depletion increased.

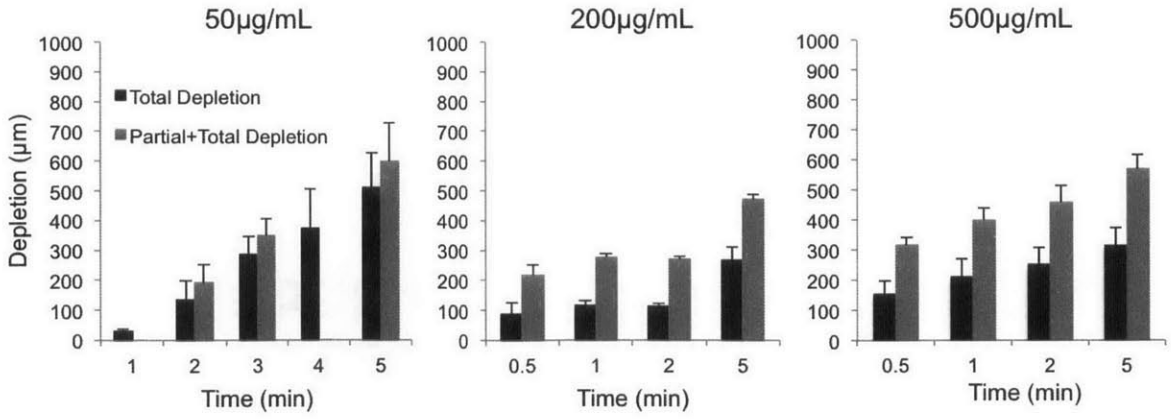

Figure 14. Quantification of the distance of GAG depletion showing the extent of "total" and "partial plus total" zones of degradation for three concentrations of trypsin pre-treatments. Time points with a single bar indicate explants that only showed a totally depleted zone following contrast enhancement. Values indicate an average (+ standard deviation) over 4 images per explant for 1-2 explants.

4.2 *In Vitro* Integration Model

4.2.1 Acellular Hydrogels

After 7 days of culture, explants into which KLD pre-mixed with HB-IGF-1 had been cast showed a two-fold increase in the rate of sulfate incorporation compared to the two conditions with explants that were not exposed to HB-IGF-1 (Fig. 15A). Explants that received the enzyme pre-treatment were exposed to 2 U/mL chondroitinase for 2 minutes, and there was no negative effect on biosynthesis of proteoglycans in explants receiving this pre-treatment.

The sGAG content in the explants was elevated in each of the three treatment conditions compared to control, though no difference was observed in the sGAG content between the explants receiving the pre-treatment and the explants exposed to HB-IGF-1 (Fig. 15B).

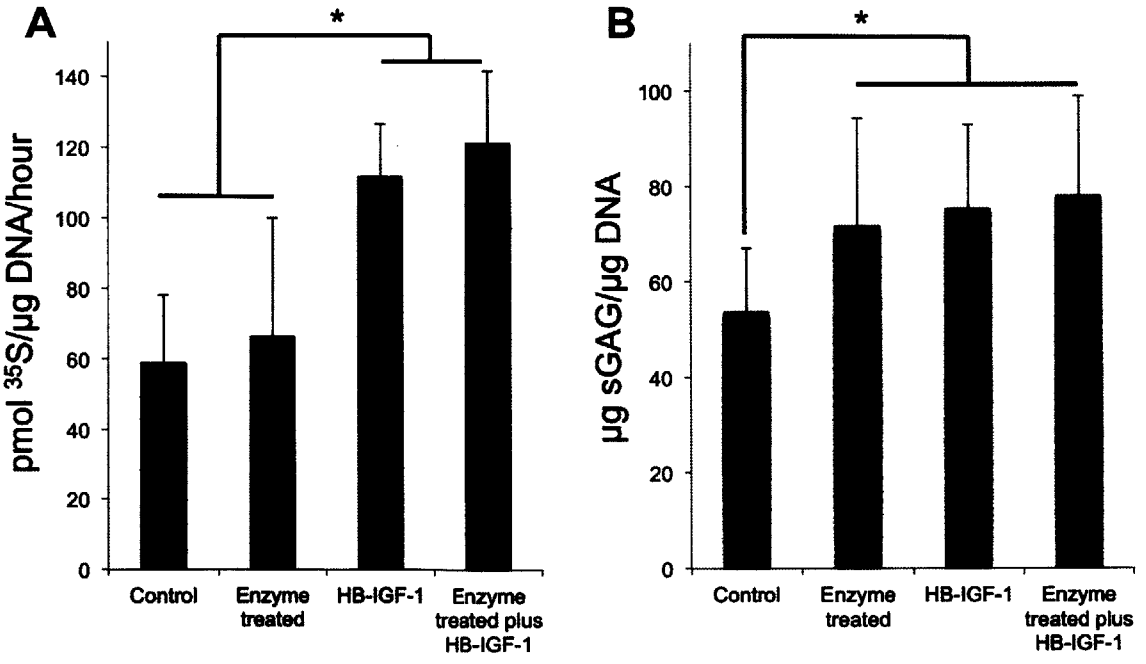

Figure 15. A) Rate of biosynthesis of sulfated GAG and B) sGAG content in explants into which acellular KLD was cast, normalized by DNA content. The gel-explant constructs were cultured in basal medium for 7 days, after which solely the explants were analyzed for extracellular matrix and cellular content. Explants that received the enzyme pre-treatment were exposed to 2 U/mL chondroitinase for 2 minutes. Values are mean + standard deviation for n=11 per condition, (5-6 explants x 2 animals). * indicates $p<0.05$.

4.2.2 Chondrocyte-seeded Hydrogels

A. Enzyme Pre-treatment: 2 U/mL Chondroitinase

Following 8 days of culture, the rate of proteoglycan synthesis was elevated in cartilage explants into which KLD pre-mixed with HB-IGF-1 had been cast; both the explants that had received the enzyme pre-treatment and those that were left untreated had a greater rate of sulfate incorporation compared to the explants exposed to non-functionalized KLD (Fig. 16A). The sGAG content in the three treatment conditions was not different from control, though explants that received the enzyme pre-treatment and were exposed to KLD pre-mixed with HB-IGF-1 showed significantly higher sGAG content compared to the explants receiving the pre-treatment alone (Fig. 16B).

The chondrocytes seeded in the hydrogels, however, showed no difference in biosynthesis rate of proteoglycans among all four conditions after 8 days of culture (Fig. 16C). Exposure to HB-IGF-1 did have a significant effect on the sGAG content in the gels. The two conditions in which the growth factor was pre-mixed into the gel showed twice the sGAG content compared to the conditions that were not exposed to HB-IGF-1 – both the gels cast into untreated explants and those cast into pre-treated explants (Fig. 16D).

Following 15 days of culture, in explants harvested from a different animal, the rate of sulfate incorporation was elevated in explants into which KLD pre-mixed with HB-IGF-1 had been cast, as was evident after 8 days (Fig. 17A). Again, the enzyme pre-treatment did not have an effect on the rate of sulfate incorporation. Explants that received the pre-treatment showed no difference in sGAG content compared to the untreated control condition (Fig. 17B). However, the sGAG content was elevated in the two conditions exposed to HB-IGF-1 compared to the pre-treated explants not exposed to the growth factor, but was not elevated compared to the untreated control condition.

The chondrocyte-seeded gels cast into pre-treated explants showed no difference in the rate of sulfate incorporation compared to control (Fig. 17C). However, the biosynthesis rate was elevated in the gels containing HB-IGF-1 that were cast into untreated explants compared only to the control condition. Further,

the chondrocyte-seeded gels containing the growth factor that were cast into untreated explants had a greater sGAG content than those cast without HB-IGF-1 (Fig. 17D).

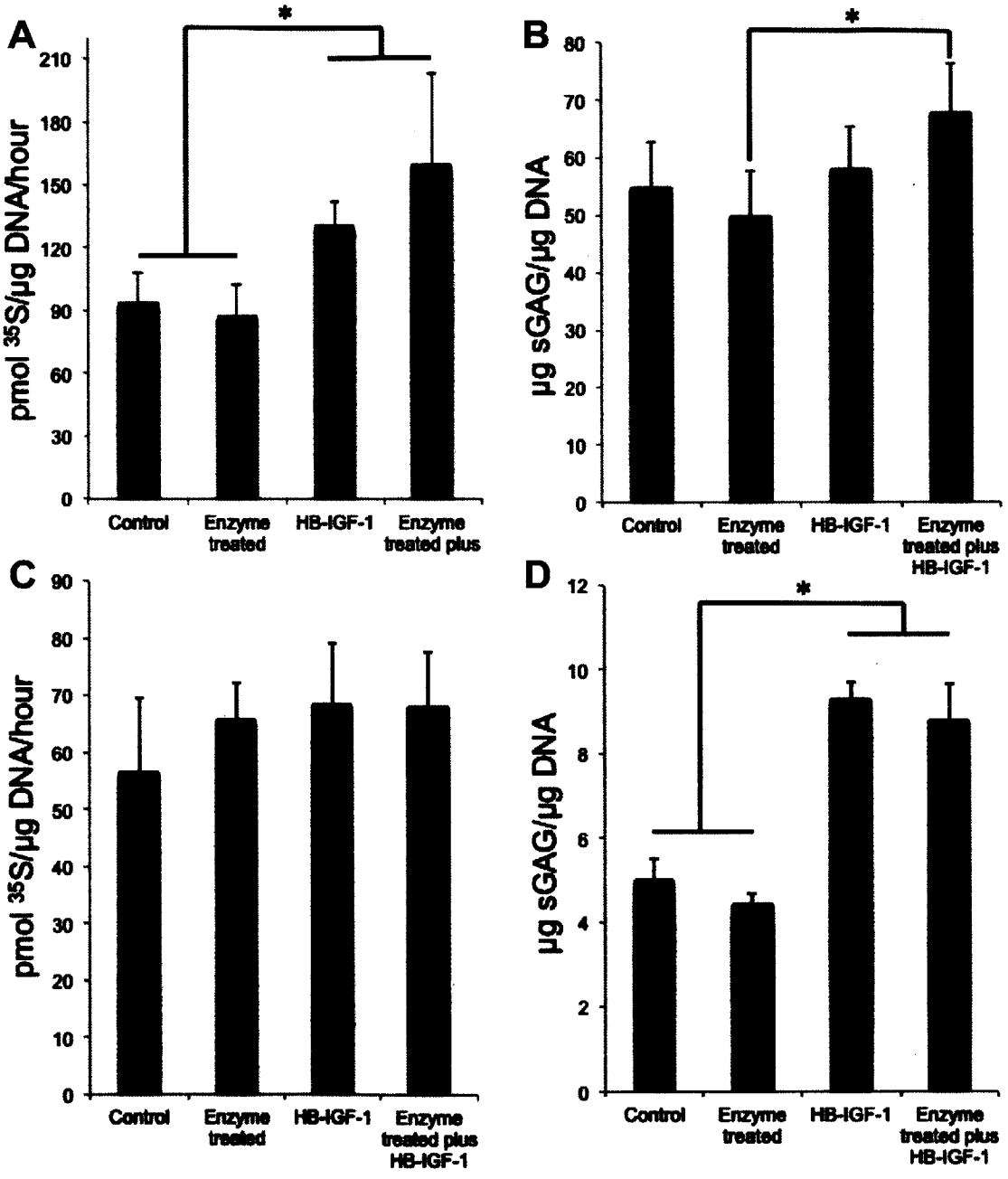

Figure 16. A) Rate of biosynthesis of sulfated GAG and B) sGAG content in explants into which chondrocyte-seeded KLD was cast, for which the C) the rate of biosynthesis of sulfated GAG and D) sGAG content in the gels were also analyzed. The gel-explant constructs were cultured in basal medium for 8 days, after which the explants and gels were analyzed separately for extracellular matrix and cellular content. Explants that received the enzyme pre-treatment were exposed to 2 U/mL chondroitinase for 2 minutes. Values are mean + standard deviation for n=4-5 per condition, with explants and chondrocytes harvested from a single animal. * indicates $p<0.05$.

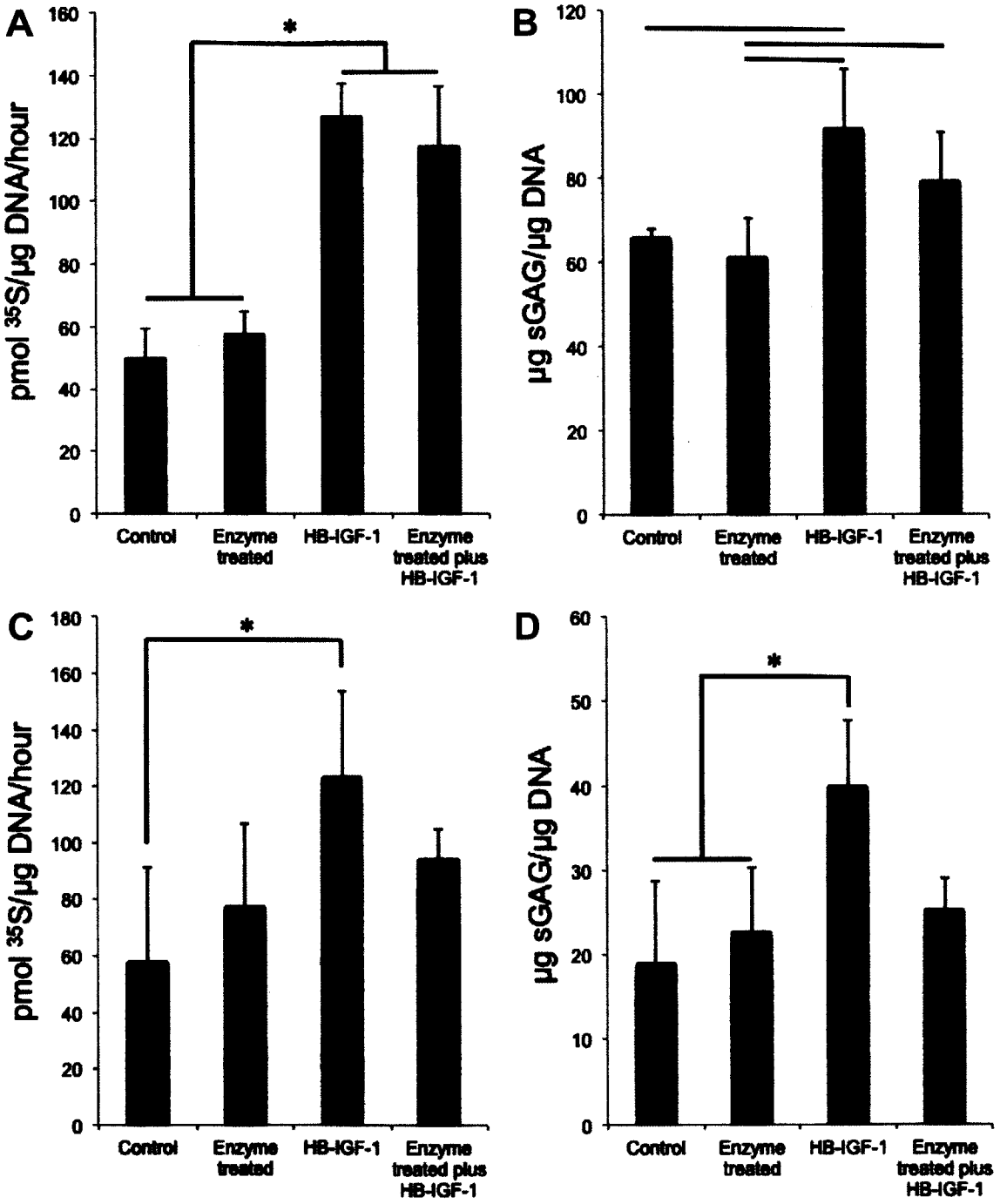

Figure 17. A) Rate of biosynthesis of sulfated GAG and B) sGAG content in explants into which chondrocyte-seeded KLD was cast, for which the C) the rate of biosynthesis of sulfated GAG and D) sGAG content in the gels were also analyzed. The gel-explant constructs were cultured in basal medium for 15 days, after which the explants and gels were analyzed separately for extracellular matrix and cellular content. Explants that received the enzyme pre-treatment were exposed to 2 U/mL chondroitinase for 2 minutes. Values are mean + standard deviation for n=6 per condition, with explants and chondrocytes harvested from a single animal. For (A,C,D) * indicates $p<0.05$, and for (B), lines indicates a significant difference between two conditions with $p<0.05$.

B. Enzyme Pre-treatment: 50 μg/mL Trypsin

Similar to cultures which used chondroitinase as the enzyme pre-treatment, following 15 days of culture, the rate of sulfate incorporation was elevated in cartilage explants exposed to KLD containing HB-IGF-1; both the explants that had received the trypsin pre-treatment and those that were left untreated were greater than explants exposed to non-functionalized KLD (Fig. 18A). This trend was maintained when explants from a second animal were cultured under the same conditions (Appendix B, Fig. B.2A). There was no difference in the sGAG content of the explants from the three treatment conditions compared to control (Fig. 18B). However, the sGAG content in the explants exposed to the HB-IGF-1-containing KLD was greater compared to the pre-treated explants exposed to non-functioned gel.

In the chondrocyte-seeded hydrogels, the rate of sulfate incorporation was significantly lower in the gels that were cast without the growth factor into pre-treated explants, compared to the other three conditions (Fig. 18C). As was seen in cultures using the chondroitinase pre-treatment, the gels that were pre-mixed with HB-IGF-1 had a greater sGAG content compared to those that were not exposed to the growth factor (Fig. 18D). Additionally, the gels that received HB-IGF-1 and were cast into untreated explants had a greater sGAG content compared to the gels receiving the growth factor and were cast into pre-treated explants. Further, the sGAG content in non-functionalized gels cast into pre-treated explants were significantly lower than the other three conditions (Fig. 18D).

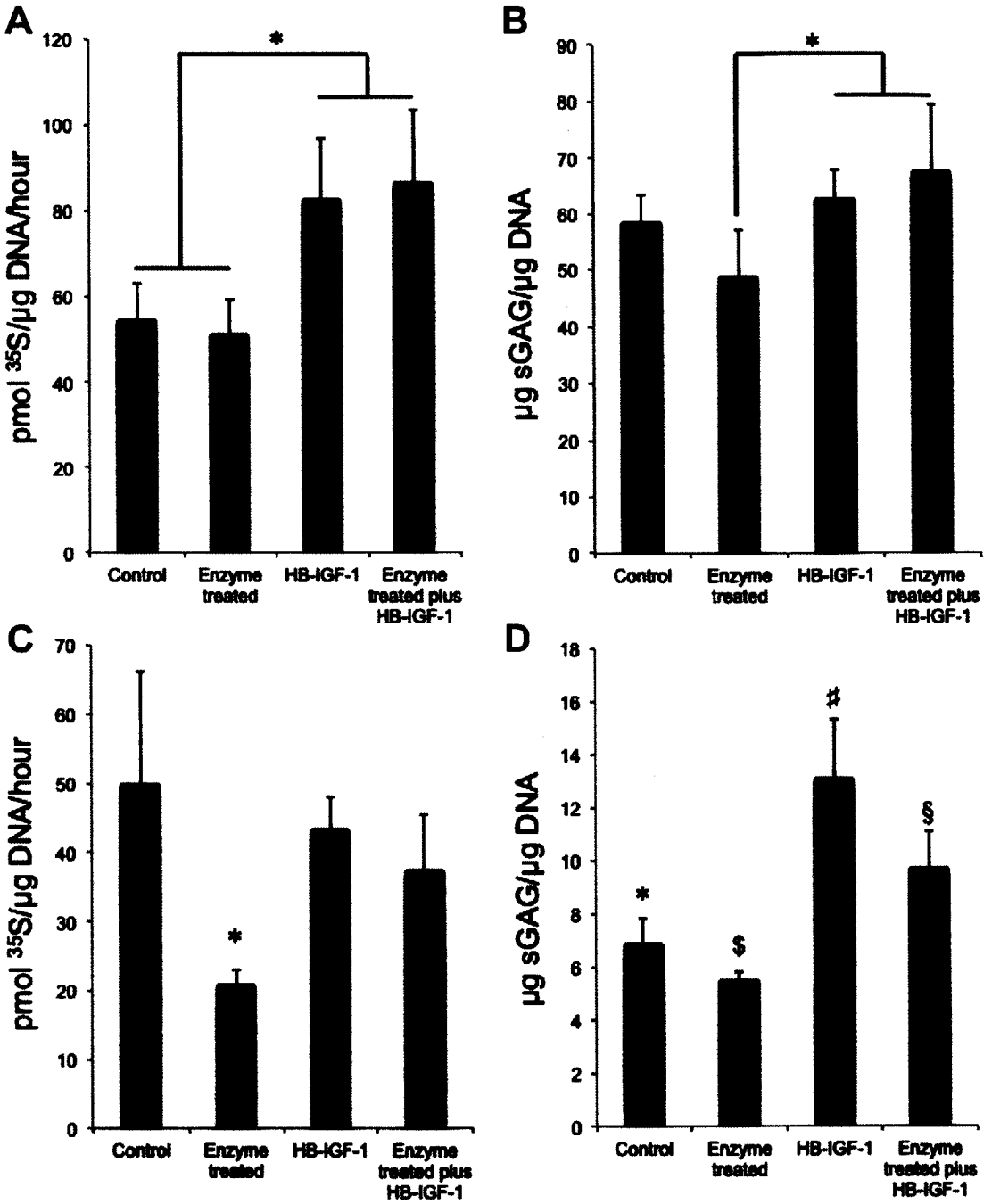

Figure 18. A) Rate of biosynthesis of sulfated GAG and B) sGAG content in explants into which chondrocyte-seeded KLD was cast, for which the C) the rate of biosynthesis of sulfated GAG and D) sGAG content in the gels were also analyzed. The gel-explant constructs were cultured in basal medium for 15 days, after which the explants and gels were analyzed separately for extracellular matrix and cellular content. Explants that received the enzyme pre-treatment were exposed to 50 µg/mL trypsin for 2 minutes followed by FBS for an additional 2 minutes to neutralize the enzyme. Values are mean + standard deviation for n=6 per condition, with explants and chondrocytes harvested from a single animal. For (A,B,C) * indicates $p<0.05$, and for (D), *, $, #, and § indicate that each condition is significantly different from the other three with $p<0.05$.

C. Mechanical Strength of Integration

The adhesive strength between the gels and the explants for the 15-day, chondrocyte-seeded cultures showed high variability within conditions for both the chondroitinase and trypsin pre-treated explants. However, there was a trend of increasing strength with the addition of the pre-treatment and growth factor for both pre-treatment types. Most notably, the integration strength was over three times higher in the constructs receiving the trypsin pre-treatment that contained gels pre-mixed with HB-IGF-1 compared to the control condition (Fig. 19).

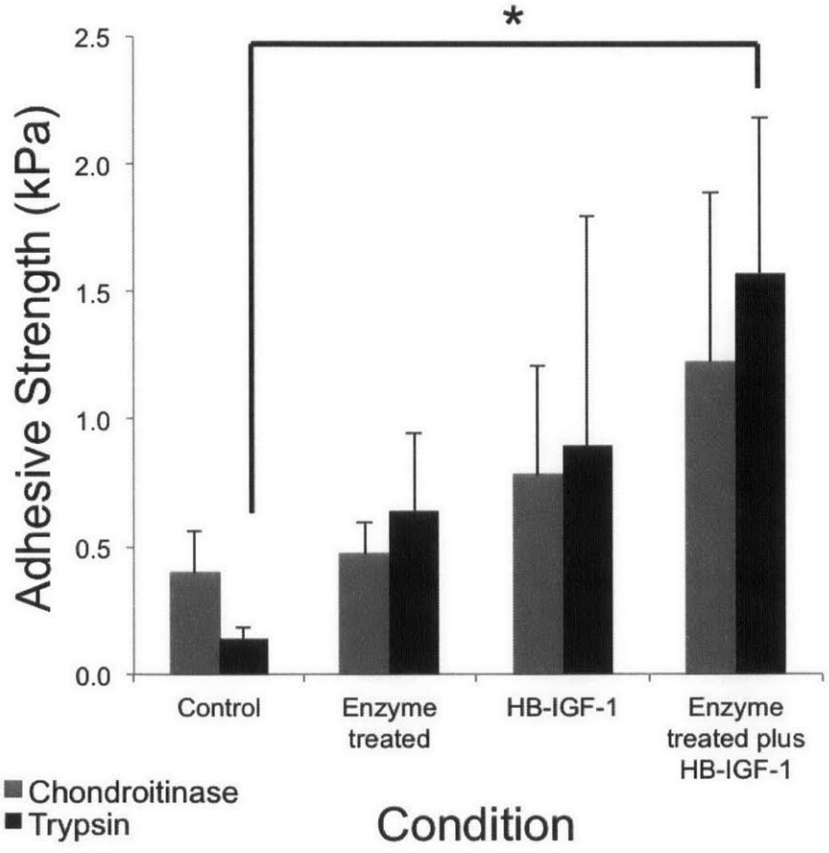

Figure 19. Adhesive strength of the gel to the explant at the integration interface, calculated as the peak force measured from the push out test normalized by the surface area of the inner core of the annulus. Data is shown from two separate experiments, in which 2 U/mL of chondroitinase or 50μg/mL of trypsin was applied for 2 minutes as the enzyme pre-treatment. Values show mean + standard deviation, n=4-6 for each condition per treatment type, and * indicates $p<0.05$.

4.3 HB-IGF-1 Release

There was a limited release of HB-IGF-1 to the medium over the course of culture, suggesting that the majority of the HB-IGF-1 had diffused laterally into the cartilage as evident by the elevated biosynthesis rates. Over nine days of culture, less than 1% of the total HB-IGF-1 that was initially pre-mixed into acellular gels on day 0 was released daily to the medium (Fig. 20A). The cumulative release of HB-IGF-1 to the medium from the acellular hydrogels accounted for an average of 4.2% release of the total amount initially added at the time of casting (Fig. 21). On day 9, when the explant and gels were separated and the remaining HB-IGF-1 was desorbed by incubation in 10x PBS, less than 2% was recovered from the gels while over 40% and 15% of the mass was recovered from the cartilage explants on days 11 and 14 respectively. Additional mass was recovered from the explants on day 27 with further desorption. In total, 67% of the initial mass of the HB-IGF-1 was recovered, and 61% was recovered from the cartilage specifically. The 10x PBS sampled from explants that were not exposed to HB-IGF-1 did not elicit a signal on the IGF-1 ELISA following 72 and 144 hours of incubation in the high salt solution.

As a separate control experiment, the release of IGF-1 from acellular hydrogels over nine days of culture was monitored in the same manner. 44% of the total IGF-1 that was initially pre-mixed into acellular gels on day 0 was released to the medium (Fig. 21) whereas 1% was recovered from the explant (Fig. 20B). In total, 50% of the initial mass was recovered from all samples.

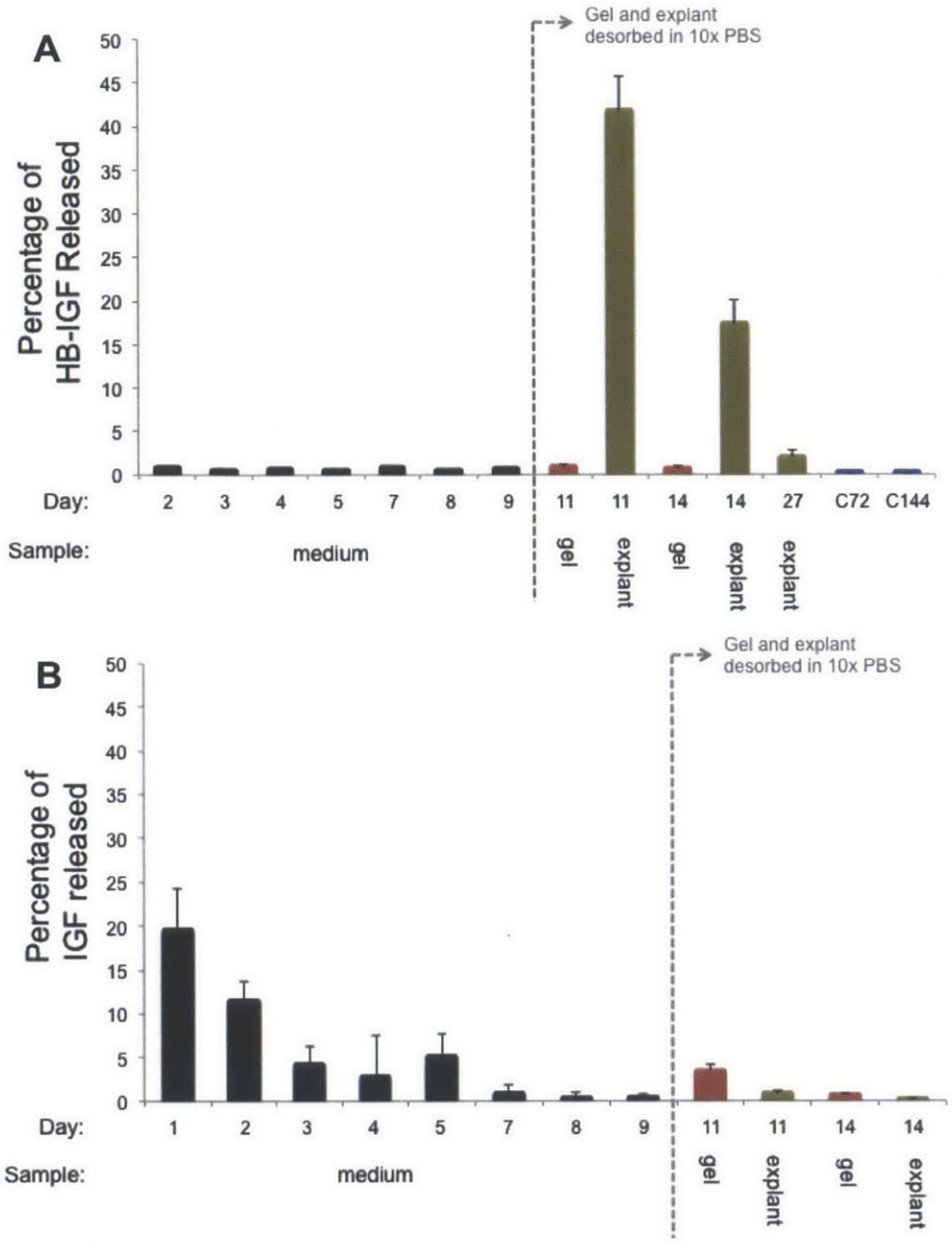

Figure 20. A) The release of HB-IGF-1 from acellular hydrogels into which 312.5 ng of HB-IGF-1 (615 nM) was initially loaded and cast into explant annuli, shown as a percentage of the total that was pre-mixed into each gel. For the first 9 days, the medium was sampled for HB-IGF-1 content that was released from the gels. After 9 days, the medium was removed, and the gels and explants were desorbed separately in 10x PBS, which was sampled on days 11, 14, and 27. C72 and C144 indicate control explants, which were not exposed to HB-IGF-1, that were incubated in 10x PBS for 72 and 144 hours, respectively, after which samples of the high salt solution were taken. Bars indicate average of 5 gel/explant constructs + standard deviation. B) The release of IGF-1 from acellular hydrogels into which 233.5 ng of IGF-1 (615 nM) was initially loaded and cast into explant annuli, shown as a percentage of the total that was pre-mixed into each gel. For the first 9 days, the medium was sampled for IGF-1 content that was released from the gels. After 9 days, the medium was removed, and the gels and explants were desorbed separately in 10x PBS, which was sampled on days 11 and 14. Bars indicate average of 5 gel/explant constructs + standard deviation.

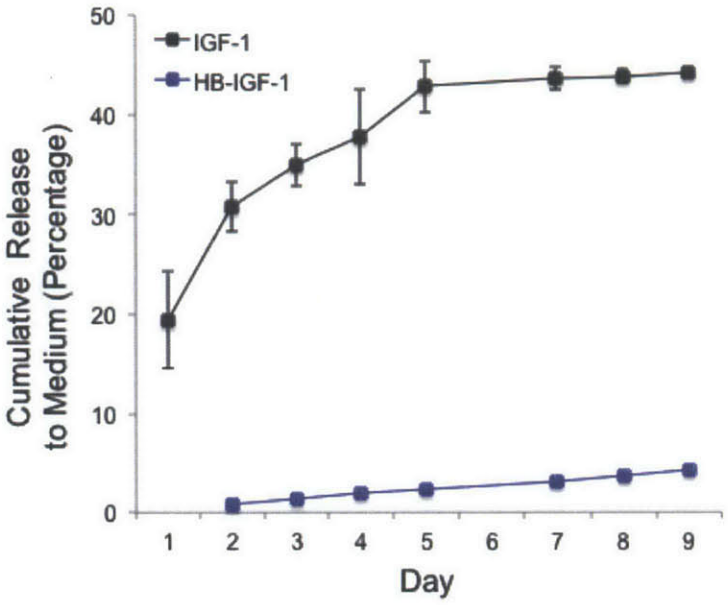

Figure 21. The release of HB-IGF-1 and IGF-1 to the medium from acellular hydrogels into which 312.5 ng of HB-IGF-1 and 233.5 ng of IGF-1 (both 615 nM) was initially loaded and cast into explant annuli, shown as a cumulative percentage of the total that was pre-mixed into each gel. Points indicate average of 5 gel/explant constructs ± standard deviation.

V. Discussion

5.1 Enzyme Pre-treatment

Depleting a portion of the proteoglycan content at the edge of a defect exposes the collagen network as well as other proteins, which has been shown to improve the adhesion of cells to native tissue [10, 11, 71-74]. Previous studies have also reported that enzyme pre-treatments increase cell density at the wound edge [75-77], enhance coverage by repair cells [10, 11], and do not negatively affect cell-associated proteoglycan deposition [12]. Exposing the defect to a pre-treatment to allow controlled degradation of proteoglycan content is thus advantageous, but for a limited amount of time to incur these desirable effects but avoid global degradation of ECM that would hinder overall repair. This study, therefore, provided a systematic evaluation of two different pre-treatments at 7 total concentrations using an *in vitro* defect model for cartilage repair.

Depleting GAG content 200 μm into the native cartilage was hypothesized to allow sufficient space for cell migration, adhesion, and deposition of new matrix without damaging the native tissue beyond repair for *in vitro* optimization of our integrative

repair strategy. Initially, a chondroitinase pre-treatment was of interest given its extensive use in previous studies [10, 11, 74] and its specificity for chondroitin sulfate (CS) GAG chains. In restricting the degradation to CS, this non-proteolytic pre-treatment was considered a milder approach than trypsin, which degrades GAG content in addition to core proteins, or than collagenase, which would disrupt the underlying structural network and could cause irreparable damage [14, 24, 84] (Fig. 22). The enzymatic activity of chondroitinase, however, was inconsistent in comparing the 100-200 μm of depletion achieved with 1-10 minutes and the 1000 μm achieved with 30 minutes at a concentration of 2 U/mL (Figs. 11 and 12). With the exception of that 30-minute time point, there was little variation in the distance of depletion with time, even though one might have expected degradation to increase with duration of exposure. Since chondroitinase specifically targets GAG chains and is 120 kDa, its diffusion through the tissue is size-limited [85] – it must degrade part of the matrix before further penetrating the ECM. For the *in vitro* cultures, the 2-minute 2 U/mL pre-treatment was chosen to allow about 200 μm of depletion to assess effects of the pre-treatment on integrative repair.

Trypsin was pursued as an alternative enzymatic pre-treatment, which, at 23 kDa, would more easily diffuse into the tissue to provide more uniform degradation of the ECM with shorter treatment times. As a pre-treatment, trypsin has been shown to similarly improve coverage of the defect by repair cells [10, 11], and since it degrades both core proteins and GAG chains, it provides greater clearing of ECM in a shorter incubation time. Trypsin can also be neutralized with serum, which is an advantage over chondroitinase for clinical application. A pre-treatment of 50 μg/mL for 2 minutes was chosen for further study in the *in vitro* cultures as it repeatedly provided 200 μm of partial plus total depletion (Fig. 14) to match the depletion achieved with the chondroitinase pre-treatment. This concentration was initially investigated based on prior work which applied this concentration of trypsin to explants without noticing a changes in swelling of the tissue [80]. Additional concentrations of trypsin were analyzed in order to investigate shorter durations of exposure, which are relevant for clinical translation.

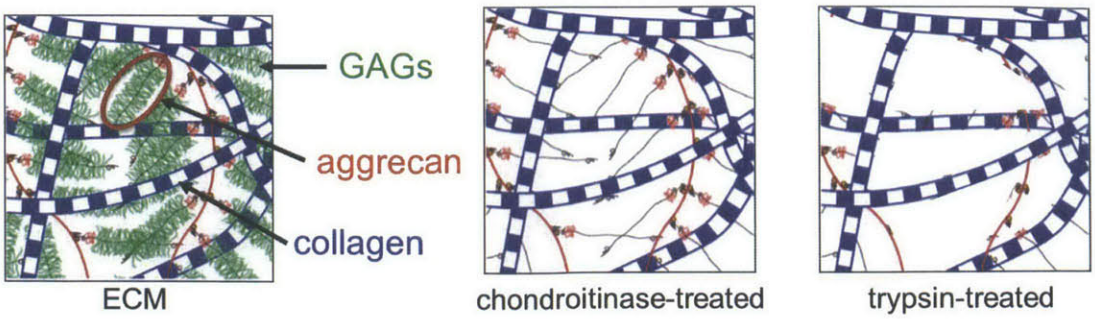

Figure 22. Depiction of the main constituents on cartilage ECM and the effects of chondroitinase, which clears GAG chains, and trypsin, which degrades both GAG chains and the core proteins of aggrecan. Both enzymes leave the collagen network intact [19].

After consulting with an operating physician, Dr. David Frisbie from the Equine Orthopaedic Research Center at Colorado State University, we determined that a clinically relevant pre-treatment would have to last 5 minutes or less. In an effort to decrease duration of treatment, higher concentrations of 200 μg/mL and 500 μg/mL were investigated. All additional explants followed similar temporal patterns of GAG degradation – greater degradation with higher concentration and longer incubation times (Figs. 13 and 14). These concentrations, while stronger than 50 μg/mL, showed similar magnitudes of degradation for 2-minute treatments. However, importantly, these concentrations provided equal depths of partial and total depletion for a given incubation time, which was considered advantageous over chondroitinase and over the lower concentration of trypsin; the totally depleted regions left the collagen network intact and provided room for synthesis of new matrix while the partially depleted region provided anchoring GAG and adhesive proteoglycans (Fig. 22). A 2-minute treatment of 500 μg/mL of trypsin followed by 2 minutes of neutralization with serum was chosen for future *in vivo* studies, as it provided 200 μm of total depletion plus an additional 200 μm of partial depletion with a total treatment time of 4 minutes.

Results indicate that enzyme pre-treatments involving 1% and 2.5% trypsin caused greater than 1 mm of depletion, the entire thickness of the annulus in the present study, when applied for as little as 1 minute. This extent of depletion was a more severe degradation than was desired and more than had previously been observed, where a depth of 0.6-1.2 mm was achieved with 1% trypsin in similar femoropatellar cartilage from 2-3 week old calves [13]. Further, chondroitinase pre-treatments at 1 U/mL and 2 U/mL for 1

to 10 minutes caused a depletion of GAG content 100-200 μm into the surrounding tissue, contrary to previous reports in which a treatment of 1 U/mL for 4 to 5 minutes caused proteoglycan depletion to a depth of 1 μm, even though chondrocyte density appeared to decrease within a 100 μm region next to defects in the femoropatellar grooves of skeletally mature rabbits as viewed with electron micrographs [10, 11]. The differences between this present study and prior work are due in part to the source of cartilage tissue. Immature bovine cartilage is more porous and is laden with blood vessels, allowing greater diffusion of the enzyme into the tissue and increasing degradation compared to skeletally mature specimens. Additionally, the method by which the depletion was detected – electrograph versus histology stained with GAG-specific toluidine blue – accounts for differences in the reported depletion of proteoglycan content, as toluidine blue loses its ability to bind glycosaminoglycans when 42% or less of the oligosaccharides are lost [86]. Therefore, the extent of depletion reported in the present study describes areas of tissue with less than 42% of GAG content.

5.2 *In Vitro* Integration Model

Previous work has employed similar culture systems modeling a focal defect [13, 65, 76, 79]. However, no model has yet combined KLD, HB-IGF-1, and an enzyme pre-treatment to improve matrix synthesis for the purpose of integrative repair. The results indicate that KLD functionalized with HB-IGF-1 can provide an extended release of the growth factor locally to adjacent cartilage as well as to encapsulated cells to enhance matrix biosynthesis for 7 to 15 days and improve interfacial strength between engineered tissue and native cartilage. While GAG was depleted within the inner annulus, in general, no detrimental effect was observed on the rate of proteoglycan synthesis or GAG content compared to explants left untreated.

The culture system was first established with an acellular gel to isolate the effects of the growth factor on surrounding tissue in this specific *in vitro* model of defect repair, as it has been shown previously to be released from the gel and affect synthesis in a neighboring explant after 6 and 10 days [6]. After matching the ratio of HB-IGF-1 to cartilage volume of this prior study, it was evident that HB-IGF-1 can also elevate sulfate incorporation in native cartilage tissue in the *in vitro* defect model (Fig. 15A). The elevated rate of sGAG synthesis in explants exposed to KLD pre-mixed with the growth

factor suggests that the HB-IGF-1 was released laterally into the cartilage where it affected native chondrocytes, as HB-IGF-1 is specifically retained in cartilage through its binding to chondroitin sulfate (CS) [58]. It is unlikely that the HB-IGF-1 was released to the medium where it then acted on the explant, as medium must have a concentration of 100 ng/mL of IGF-1 in order to stimulate the tissue [87]. If the entire mass of HB-IGF-1 was released to the medium, a concentration of 156 ng/mL could be achieved, but the entire volume of medium was replaced every other day, which makes achieving and sustaining 100 ng/mL in the medium unlikely. Importantly, the chondroitinase pre-treatment did not negatively affect biosynthesis of proteoglycans or the GAG content in the explant, even though approximately 10-20% of the tissue had been depleted [88, 89]. Even though the growth factor increased matrix biosynthesis and increased the GAG content compared to control, the GAG content was also elevated in the explants exposed to the pre-treatment alone compared to control (Fig. 15B). This unexpected result may have been due to a reparative response by the chondrocytes in response to the local depletion of extracellular matrix [12].

After observing the positive effects of HB-IGF-1 from acellular KLD on sGAG synthesis in the explants, chondrocytes were then encapsulated in the hydrogel to model a post-migratory repair model, in which mesenchymal stem cells from the marrow have migrated into the gel and undergone chondrogenesis. While the growth factor did not have an effect on the rate of sulfate incorporation in the chondrocyte-seeded gels after 8 days (Fig. 16C) and little effect after 15 days (Figs. 17C and 18C), there was a two-fold increase in sGAG content in the gels exposed to HB-IGF-1 compared to the two conditions not exposed to the growth factor after 8 days (Fig. 16D) and 15 days (Figs. 17D and 18D). These results agree with previous work in which biosynthesis was unchanged in chondrocyte-seeded gels upon exposure to HB-IGF-1 after 6 and 10 days in culture, but, after 10 days, the growth factor increased sGAG content compared to control [6]. Further, the results demonstrate that HB-IGF-1 can have a prolonged effect on the GAG content on the gels up to two weeks, which has not been previously shown [6, 58]. The GAG content is the most applicable indication that HB-IGF-1 acted on the chondrocytes, as the gels were initially cast with no GAG content, and the radiolabel

incorporation assay strictly measured the activity of the cells during the last 24 hours of culture, even if the synthesis was elevated prior to digestion.

Globally, the results from the chondrocyte-seeded gels agree among the three animal donors for 8 and 15 days. However, subtle differences illustrate important considerations. After 8 days, there were no changes in the rate of sulfate incorporation in the gels, but, after 15 days, the biosynthesis was elevated in the chondrocytes exposed to HB-IGF-1 and cast into untreated explants compared to control, which corresponds to the trend in GAG content in the chondrocyte-seeded gels (Figs. 17C and 17D). While GAG content was elevated in the HB-IGF-1-only condition compared to those that were not exposed to the growth factor, the constructs receiving both the pre-treatment and HB-IGF-1 had gels with GAG content similar to those cast without HB-IGF-1 (Fig. 17D). The differences in proteoglycan biosynthesis is not immediately disconcerting, as it represents one point in time from the duration of culture, but the gels receiving HB-IGF-1 and cast into pre-treated explants were expected to have similarly elevated GAG content to the gels cast with HB-IGF-1 into untreated explants, compared to control. The difference may be an artifact of sample size or the signaling in the pre-treated explant may have counteracted the effect of HB-IGF-1, as other work has shown that the presence of neighboring tissue affects GAG content in chondrocyte-seeded gels [65]. Similarly, chondrocyte-seeded gels cast into explants pre-treated with trypsin had less GAG content after 15 days compared to the functionalized gels cast into untreated explants (Fig. 18D), though both conditions that received HB-IGF-1 had greater GAG content compared to the control as expected. The depressed rate of proteoglycan biosynthesis in gels cast into pre-treated explants may have also been a result of altered signaling due to the local degradation of the surrounding tissue.

Not only did the growth factor affect the encapsulated chondrocytes, it also diffused laterally into neighboring tissue and increased proteoglycan biosynthesis two-fold after 8 (Fig. 16A) and 15 days (Figs. 17A and 18A), as was observed in the 7-day acellular culture. The increased rate of sulfate incorporation in the explants exposed to KLD pre-mixed with HB-IGF-1 was sustained for 15 days, which is longer than has been shown previously [6] and which further supports the hypothesis that HB-IGF-1 is retained in the cartilage by CS [58]. This trend was maintained between both 15-day

cultures: those using chondroitinase as the pre-treatment and those using trypsin (Figs. 18A and 18A). Therefore, switching to the trypsin pre-treatment did not negatively affect the proteoglycan synthesis of the explant and will be pursued for future experiments.

The enzyme pre-treatments did not negatively affect the rate of sulfate incorporation in the explants, despite the initial depletion of proteoglycan content. Given the relatively large initial volume of cartilage tissue in the explants, the overall GAG content was also not expected to change with 200 μm of degradation. After 8 days, the GAG content in the explants from the three treatment conditions were not different from the control (Fig. 16B), as was observed in the acellular culture, but the HB-IGF-1 did increase the GAG content in explants receiving the pre-treatment compared to those exposed to the pre-treatment alone. More notably, after 15 days, the exposure to the growth factor compensated for the depletion of the GAG in the enzyme pre-treated explants (Figs. 17B and 18B). With greater a sample size and a longer duration of culture, the GAG content in both conditions exposed to HB-IGF-1 is expected to be greater than the two conditions without the growth factor.

Taken together, the enzyme pre-treatment is a promising addition to hydrogel repair strategies as it does not negatively affect the rate of proteoglycan biosynthesis in native tissue and it provides space for newly synthesized tissue to integrate as well as for HB-IGF-1 to anchor and transiently bind within the surrounding cartilage [58], potentially leading to superior interfacial strength. The incorporation of HB-IGF-1 into KLD is also a critical component to the repair, as it increases matrix content within the gel and stimulates biosynthesis in the surrounding native cartilage. Biomechanical assessment can ultimately assess the extent to which the combination of an enzyme pre-treatment of a defect surface and a peptide hydrogel functionalized with pro-anabolic HB-IGF-1 can enhance integration by stimulating surrounding tissue and allowing functional integration of newly synthesized matrix. Preliminary tests on 15-day cultures employing the trypsin pre-treatment demonstrated that only when the pre-treatment is combined with KLD containing HB-IGF-1 can the strength between the engineered tissue developing within the gel and the surrounding native tissue be improved compared to the non-functionalized KLD cast unto untreated explants (Fig. 19). The trend in increasing interfacial strength with application of the enzyme pre-treatments is consistent with

previous work employing pre-treatments [13, 76], though this study demonstrates a significant difference between conditions after 15 days of culture whereas previous work has shown improved strength between cartilage-cartilage constructs after 4-5 weeks [13, 76]. This present study is the first of its kind to demonstrate improved integration between engineered tissue and native tissue after only two weeks. This repair strategy, employing the pre-treatment and HB-IGF-1, is therefore a promising approach for improving integration. While constructs employing the chondroitinase pre-treatment did not show differences in adhesive strength among conditions, a greater sample size may elucidate a difference among conditions. However, it is also likely that trypsin, which provided degradation of both GAG content and core proteins, has superior integrative potential.

The mechanical tests performed in this thesis were initial measurements aimed to validate the integrative potential of the improved repair strategy. The preliminary measurements allowed setup and evaluation of the test configuration that has been reported in previous studies [13, 65, 76, 79, 90]. The initial results are encouraging but suggest that additional considerations need to be addressed. The measurements of adhesive strength showed a large amount of variability. The magnitude of strengths were significantly lower than the MPa magnitude of those previously reported for cartilage-cartilage constructs [13, 76], though closer to the tens of kPa that have been reported for interfacial repair tissue that forms after 3 weeks [90], 6 weeks [65], and after 20 days in agarose gels [79]. The disparity between previous work and the results in this present study are largely attributed to the use of the peptide hydrogel, whose mechanical properties are strictly determined by the tissue that develops within it [7], and to the relatively short duration of culture. The interfacial strength is expected to increase with longer culture periods.

There were several limitations to this study that are important to note. Firstly, the push-out rod used in this experiment was not beveled as is depicted in the ideal setup (Fig. 9) and will be adjusted for future experiments [91]. The sharp edge of the rod could have caused fracture of the gel rather than failure at the gel-cartilage interface, which would have resulted in a measurement of the stiffness of the gel alone. This limitation is also a concern based on the limited time of culture and the fact that the gel itself lacks the

mechanical integrity of cartilage. An additional experiment might test the force required to push through a construct composed of gel alone.

While the explant annuli were secured to the bottom of a 24-well plate with 3% agarose, there was considerable warping of the explant tissue that was especially noticeable in the 15-day cultures. This warping, which was more visible in the conditions exposed to HB-IGF-1, pulled the explant away from the gel, disrupting any integrative tissue that may have been forming over a portion of the interface, and, in some cases, the gel was freed from the explant. Matrix growth within the gel also causes contraction of the gel, which may have contributed to the disruption of the gel-cartilage interface. While constructs that were completely disengaged were removed from the experiments, samples that experienced mild warping were largely responsible for the variability in the mechanical measurements and some of the spread in the data evident in the measurements of matrix synthesis (Appendix B, Fig. B2). The growth factor enhanced the rate of sulfate incorporation and increased tissue volume, which perhaps was due to swelling from increased production of proteoglycans [16-18, 49, 92, 93]. Future cultures would be improved by better confining the explant with steel mesh or a custom culture dish. The mechanical tests were performed after only 15 days of culture to assess the functional effects of the enhanced biosynthesis, however sufficient tissue growth at the interface is expected to take at least 4 weeks based on previous studies [13, 65, 79]. In calculating the interfacial strength, the change in volume of the tissue was not measured as the surface area of the annulus was assumed to remain constant throughout culture, despite some visible changes in swelling of the tissue. In general, the results described here are limited to young bovine tissue and it is unclear whether these results will be evident in tissue donors of different age or species [94]. These results also do not account for the presence of serum that would be present *in vivo*, and may shadow the effects of HB-IGF-1 that were evident in this culture system.

Additional studies are needed to address remaining questions. Future work should include animal repeats to improve the statistical power of the promising results shown here, in which HB-IGF-1 enhanced matrix production in encapsulated chondrocytes and surrounding tissue. While pre-treatments of 2 U/mL chondroitinase and 50 μg/mL of trypsin did not negatively affect matrix synthesis, the effects of the clinically relevant

pre-treatment of 500 μg/mL of trypsin will have to be verified *in vitro* prior to *in vivo* studies. The control experiments in these studies did not include the incorporation of IGF-1 since previous studies have shown that HB-IGF-1 outperforms its counterpart [6, 58]. However, including IGF-1 pre-mixed into KLD would provide an additional control for these cultures and demonstrate the superiority of HB-IGF-1 in this model system. In addition to measuring the mechanical strength of the constructs, histological sectioning of the constructs would allow histomorphological examination of the tissue growth at the interface. To further improve tissue growth at the interface, and more closely model *in vivo* conditions, oscillatory compression of 1 Hz could be applied to the constructs after a period of maturation, which has shown to stimulate synthesis above static control levels [95]. Future studies might also investigate the development of collagen across the scaffold-cartilage interface as collagen deposition is correlated with mechanical strength at the interface [96]. Ultimately, while the culture system developed here is a post-migratory model, KLD encapsulated with BMSCs and pro-migratory factors would more closely elucidate the effects of the repair strategy *in vivo*. Despite the limitations and potential for future work, results confirm that the combination of an enzyme pre-treatment of a defect surface and a peptide hydrogel functionalized with pro-anabolic HB-IGF-1, as presented here, can enhance integration by stimulating surrounding tissue and allowing mechanical and biological continuity at the interface.

5.3 HB-IGF-1 Release

Given the large quantity of CS present in articular cartilage, we suspect that the majority of HB-IGF-1 initially added to the gel diffused laterally into and remained within the surrounding tissue as evident in the elevated rates of sulfate incorporation. By testing the media for HB-IGF-1 throughout a 9-day culture with acellular gels, the extent and location of delivery of the growth factor in this system could be quantified. Over 9 days, the cumulative loss of HB-IGF-1 to the medium accounted for about 4% of the mass initially loaded into the gel (Fig. 21). After desorption, it was evident that little HB-IGF-1 remained in the gel. Rather, the growth factor had diffused into the native tissue where it remained for at least 9 days as evident by the 61% recovery after disrupting non-specific electrostatic binding in the cartilage tissue with a high salt solution (Fig. 20).

These findings support the hypothesis that the HB-IGF-1 diffused laterally into the cartilage instead of vertically into the medium. These results indicate that HB-IGF-1 is superior to IGF-1 as a gel additive as 44% of IGF-1 was released to the medium in 9 days, compared to the 4% of HB-IGF-1. Further, little IGF-1 was recovered from the cartilage explant, unlike its HB-fused cousin, indicating that IGF-1 does not provide a sustained release from the hydrogel and will be ineffective in stimulating the cartilage tissue for integrative repair, as suggested elsewhere [6] (Figs. 20 and 21). While only 67% of the total HB-IGF-1 and 50% of the IGF-1 was recovered, it is suspected that the remaining mass was occupied by IGFBPs, adsorbed to the 24-well plate, and adsorbed to test tubes, despite their low-retention properties.

Additional studies will be needed to determine exactly how long HB-IGF-1 remains in the cartilage, as the release profile presented here is specific for the 9-day time point. Further, while studies have shown that IGF-1 is cleared after at least 6 days of culture [6] and after 9 days as presented here, a control experiment incorporating IGF-1 into the gel would provide a definitive comparison of the two growth factors on the effects of proteoglycan synthesis to corroborate the release studies. Future work may aim to account for all the mass of the growth factors added to the hydrogel and determine a definitive loading capacity of the growth factors in the gel. A comprehensive transport and uptake study with HB-IGF-1 should be further pursued by fluorescently or radioactively labeling the growth factor to assess real-time kinetics [97].

VI. Conclusion and Future Directions

Taken together, the results of this study constitute significant progress towards the development of a repair strategy using a nanofiber-based scaffold that better integrates engineered cartilage tissue with native matrix. Prior to this project, a method of integrating neo-tissue with native tissue was limited to surface adhesives and enzyme pre-treatments combined with non-functionalized PGA scaffolds, with much investigation of the effects of isolated pre-treatments on innate repair. However, the effects of combining an enzyme pre-treatment and a self-assembling peptide hydrogel containing pro-anabolic HB-IGF-1 in an *in vitro* model of defect repair had not yet been established. This present study assessed each of these factors and demonstrated the integrative potential of the repair strategy.

The trends in GAG depletion, enzyme concentration, and incubation time that were identified by the investigation of enzymatic pre-treatments led to an improved understanding of the extent of proteoglycan degradation that is possible within a clinically relevant time. As a result of this assessment, two separate treatments – 2 U/mL chondroitinase for 2 minutes and 50 μg/mL trypsin for 2 minutes – were identified to achieve 200 μm of depletion for *in vitro* studies. Further, for clinical application, a treatment of 500 μg/ml of trypsin for 2 minutes, followed by 2 minutes of neutralization with serum, was determined to consistently create 200 μm of total depletion plus an additional 200 μm of partial depletion for clinical implementation. The identification of a shorter time of treatment from previous studies can potentially improve cell viability and allow greater ease of application [13].

Additionally, the application of chondrocyte-seeded KLD hydrogels functionalized with HB-IGF-1 to both pre-treated and untreated annuli favorably elevated the rate of proteoglycan synthesis in the surrounding tissue as well as the GAG content in the gels, compared to constructs containing non-functionalized gels. Importantly, preliminary mechanical tests indicate that a strategy employing both a pre-treatment and a hydrogel containing HB-IGF-1 provides mechanical integration of tissue, evident by an elevated adhesive strength between the hydrogel and the native tissue. In general, the pre-treatment did not cause negative effects on the rate of sulfate incorporation or GAG content in the explants. Importantly, it was determined that the HB-IGF-1 diffused laterally out of the hydrogel and remained in the cartilage explants by nonspecific electrostatic interaction with GAG chains; the growth factor elevated the rate of proteoglycan synthesis in the explant while also acting on encapsulated cells to increase GAG content within the scaffold. HB-IGF-1 remained within the constructs for at least 14 days whereas IGF-1 was released to the medium within the first week of culture (Figs. 20 and 21).

Overall, HB-IGF-1 released from a self-assembling peptide hydrogel can enhance matrix synthesis in the native tissue of a defect and, if the hydrogel were to encapsulate bone marrow stromal cells, enhance matrix production of engineered tissue. This combined effect could improve regeneration and integration through the local, prolonged delivery of IGF-1. Further, an enzyme pre-treatment would allow cartilage neo-tissue to

better integrate with native tissue to achieve a continuous interface, potentially improving integrative repair should the strategy be applied in an enhanced microfracture procedure. Future work should monitor the integrative potential over longer periods of culture and encapsulate BMSCs, to better assess mechanical strength at the interface and determine the extent of delivery and effects of HB-IGF-1. The improved repair strategy prepared in this thesis will need to be assessed *in vivo* using a rabbit model of defect repair in collaboration with Colorado State University. Additional studies may also assess the effects of pro-chondrogenic or pro-migratory factors on encapsulated cells and surrounding tissue. In order to definitively assess the transport and release of HB-IGF-1 in the hydrogel and cartilage tissue, a thorough transport study will be required.

The results of this study provide a significant expansion of the application of self-assembling peptide hydrogels, HB-IGF-1, and enzyme pre-treatments for integrative cartilage repair. This project has shown that it is possible to deplete proteoglycan content in tissue surrounding a defect without negatively affecting proteoglycan synthesis and to use this pre-treatment to improve interfacial strength between the native tissue and engineered tissue when pro-anabolic HB-IGF-1 is delivered to the defect within KLD. Because the regeneration of a continuous articular surface is critical for the long-term, efficacious repair of focal cartilage defects, such a repair strategy will prove to be a powerful tool in improving integration of engineered cartilage tissue

VII. References

[1] R. B. Martin, D. B. Burr, and N. A. Sharkey, *Skeletal Tissue Mechanics*. New York, NY: Springer-Verlag, 1998.

[2] D. D. Anderson, S. Chubinskaya, F. Guilak, J. A. Martin, T. R. Oegema, S. A. Olson, and J. A. Buckwalter, "Post-traumatic osteoarthritis: improved understanding and opportunities for early intervention," *J Orthop Res,* vol. 29, pp. 802-9, Jun 2011.

[3] G. Knutsen, L. Engebretsen, T. C. Ludvigsen, J. O. Drogset, T. Grontvedt, E. Solheim, T. Strand, S. Roberts, V. Isaksen, and O. Johansen, "Autologous chondrocyte implantation compared with microfracture in the knee. A randomized trial," *J Bone Joint Surg Am,* vol. 86-a, pp. 455-64, Mar 2004.

[4] J. R. Steadman, K. K. Briggs, J. J. Rodrigo, M. S. Kocher, T. J. Gill, and W. G. Rodkey, "Outcomes of microfracture for traumatic chondral defects of the knee: average 11-year follow-up," *Arthroscopy,* vol. 19, pp. 477-84, May-Jun 2003.

[5] P. W. Kopesky, E. J. Vanderploeg, J. D. Kisiday, D. D. Frisbie, J. D. Sandy, and A. J. Grodzinsky, "Controlled delivery of transforming growth factor beta1 by self-assembling peptide hydrogels induces chondrogenesis of bone marrow stromal cells and modulates Smad2/3 signaling," *Tissue Eng Part A,* vol. 17, pp. 83-92, Jan 2011.

[6] E. M. Florine, R. Miller, R. T. Lee, P. Patwari, and A. J. Grodzinsky, "Delivering HB-IGF-1 with Self-Assembling Peptide Hydrogels," *Journal of Tissue Engineering A,* 2014.

[7] J. Kisiday, M. Jin, B. Kurz, H. Hung, C. Semino, S. Zhang, and A. J. Grodzinsky, "Self-assembling peptide hydrogel fosters chondrocyte extracellular matrix production and cell division: implications for cartilage tissue repair," *Proc Natl Acad Sci U S A,* vol. 99, pp. 9996-10001, Jul 23 2002.

[8] P. W. Kopesky, E. J. Vanderploeg, J. S. Sandy, B. Kurz, and A. J. Grodzinsky, "Self-assembling peptide hydrogels modulate in vitro chondrogenesis of bovine bone marrow stromal cells," *Tissue Eng Part A,* vol. 16, pp. 465-77, Feb 2010.

[9] R. E. Miller, A. J. Grodzinsky, E. J. Vanderploeg, C. Lee, D. J. Ferris, M. F. Barrett, J. D. Kisiday, and D. D. Frisbie, "Effect of self-assembling peptide, chondrogenic factors, and bone marrow-derived stromal cells on osteochondral repair," *Osteoarthritis Cartilage,* vol. 18, pp. 1608-19, Dec 2010.

[10] E. B. Hunziker and E. Kapfinger, "Removal of proteoglycans from the surface of defects in articular cartilage transiently enhances coverage by repair cells," *J Bone Joint Surg Br,* vol. 80, pp. 144-50, Jan 1998.

[11] E. B. Hunziker and L. C. Rosenberg, "Repair of partial-thickness defects in articular cartilage: cell recruitment from the synovial membrane," *J Bone Joint Surg Am,* vol. 78, pp. 721-33, May 1996.

[12] T. M. Quinn and E. B. Hunziker, "Controlled enzymatic matrix degradation for integrative cartilage repair: effects on viable cell density and proteoglycan deposition," *Tissue Eng,* vol. 8, pp. 799-806, Oct 2002.

[13] B. Obradovic, I. Martin, R. F. Padera, S. Treppo, L. E. Freed, and G. Vunjak-Novakovic, "Integration of Engineered Cartilage," *Journal of Orthopaedic Research,* vol. 19, pp. 1089-1097, 31 January 2001.

[14] J. A. Buckwalter and H. J. Mankin, "Articular cartilage: degeneration and osteoarthritis, repair, regeneration, and transplantation," *Instr Course Lect,* vol. 47, pp. 487-504, 1998.

[15] T. D. Brown, R. C. Johnston, C. L. Saltzman, J. L. Marsh, and J. A. Buckwalter, "Posttraumatic osteoarthritis: a first estimate of incidence, prevalence, and burden of disease," *J Orthop Trauma,* vol. 20, pp. 739-44, Nov-Dec 2006.

[16] M. D. Buschmann and A. J. Grodzinsky, "A molecular model of proteoglycan-associated electrostatic forces in cartilage mechanics," *J Biomech Eng,* vol. 117, pp. 179-92, May 1995.

[17] W. M. Lai, J. S. Hou, and V. C. Mow, "A triphasic theory for the swelling and deformation behaviors of articular cartilage," *J Biomech Eng,* vol. 113, pp. 245-58, Aug 1991.

[18] P. J. Basser, R. Schneiderman, R. A. Bank, E. Wachtel, and A. Maroudas, "Mechanical properties of the collagen network in human articular cartilage as measured by osmotic stress technique," *Arch Biochem Biophys,* vol. 351, pp. 207-19, Mar 15 1998.

[19] H. T. Nia, I. S. Bozchalooi, Y. Li, L. Han, H. H. Hung, E. Frank, K. Youcef-Toumi, C. Ortiz, and A. Grodzinsky, "High-bandwidth AFM-based rheology reveals that cartilage is most sensitive to high loading rates at early stages of impairment," *Biophys J,* vol. 104, pp. 1529-37, Apr 2 2013.

[20] J. A. Buckwalter, V. C. Mow, and A. Ratcliffe, "Restoration of Injured or Degenerated Articular Cartilage," *J Am Acad Orthop Surg,* vol. 2, pp. 192-201, Jul 1994.

[21] S. K. Van de Velde, J. T. Bingham, A. Hosseini, M. Kozanek, L. E. DeFrate, T. J. Gill, and G. Li, "Increased tibiofemoral cartilage contact deformation in patients with anterior cruciate ligament deficiency," *Arthritis Rheum,* vol. 60, pp. 3693-702, Dec 2009.

[22] S. K. Van de Velde, T. J. Gill, L. E. DeFrate, R. Papannagari, and G. Li, "The effect of anterior cruciate ligament deficiency and reconstruction on the patellofemoral joint," *Am J Sports Med,* vol. 36, pp. 1150-9, Jun 2008.

[23] C. Herberhold, S. Faber, T. Stammberger, M. Steinlechner, R. Putz, K. H. Englmeier, M. Reiser, and F. Eckstein, "In situ measurement of articular cartilage deformation in intact femoropatellar joints under static loading," *J Biomech,* vol. 32, pp. 1287-95, Dec 1999.

[24] S. Grenier, M. M. Bhargava, and P. A. Torzilli, "An in vitro model for the pathological degradation of articular cartilage in osteoarthritis," *J Biomech,* vol. 47, pp. 645-52, Feb 7 2014.

[25] M. A. DiMicco, P. Patwari, P. N. Siparsky, S. Kumar, M. A. Pratta, M. W. Lark, Y. J. Kim, and A. J. Grodzinsky, "Mechanisms and kinetics of glycosaminoglycan release following in vitro cartilage injury," *Arthritis Rheum,* vol. 50, pp. 840-8, Mar 2004.

[26] Y. Wang and A. J. Grodzinsky, "The Response of Cartilage to Injury, in Post-Traumatic Arthritis: Pathogenesis, Diagnosis, and Management," in *Post-Traumatic Arthritis*, F. Guilak and S. Olson, Eds., ed New York, NY: Springer, 2014 (in press).

[27] R. Brocklehurst, M. T. Bayliss, A. Maroudas, H. L. Coysh, M. A. Freeman, P. A. Revell, and S. Y. Ali, "The composition of normal and osteoarthritic articular cartilage from human knee joints. With special reference to unicompartmental replacement and osteotomy of the knee," *J Bone Joint Surg Am,* vol. 66, pp. 95-106, Jan 1984.

[28] J. A. Martin and J. A. Buckwalter, "The role of chondrocyte-matrix interactions in maintaining and repairing articular cartilage," *Biorheology,* vol. 37, pp. 129-40, 2000.

[29] R. A. Stockwell, "The cell density of human articular and costal cartilage," *J Anat,* vol. 101, pp. 753-63, Sep 1967.

[30] R. A. Stockwell, "Chondrocytes," *J Clin Pathol Suppl (R Coll Pathol),* vol. 12, pp. 7-13, 1978.

[31] M. B. Aydelotte, B. L. Schumacher, and K. E. Kuettner, *Articular Cartilage and Osteoarthritis*. New York, NY: Raven Press, 1992.

[32] J. A. Buckwalter, E. B. Hunziker, L. C. Rosenberg, and e. al., "Articular Cartilage: Composition and Structure," in *Injury and Repair of the Musculoskeletal Soft Tissues*, S. L. Woo and J. A. Buckwalter, Eds., ed Park Ridge, IL: American Academy of Orthopaedic Surgeons, 1988, pp. 405-425.

[33] A. J. Freemont and J. Hoyland, "Lineage plasticity and cell biology of fibrocartilage and hyaline cartilage: its significance in cartilage repair and replacement," *Eur J Radiol,* vol. 57, pp. 32-6, Jan 2006.

[34] A. Gobbi, R. A. Francisco, J. H. Lubowitz, F. Allegra, and G. Canata, "Osteochondral lesions of the talus: randomized controlled trial comparing chondroplasty, microfracture, and osteochondral autograft transplantation," *Arthroscopy,* vol. 22, pp. 1085-92, Oct 2006.

[35] B. D. Harman, S. H. Weeden, D. K. Lichota, and G. W. Brindley, "Osteochondral autograft transplantation in the porcine knee," *Am J Sports Med,* vol. 34, pp. 913-8, Jun 2006.

[36] K. Mithoefer, T. McAdams, R. J. Williams, P. C. Kreuz, and B. R. Mandelbaum, "Clinical efficacy of the microfracture technique for articular cartilage repair in the knee: an evidence-based systematic analysis," *Am J Sports Med,* vol. 37, pp. 2053-63, Oct 2009.

[37] M. B. Goldring, K. Tsuchimochi, and K. Ijiri, "The control of chondrogenesis," *J Cell Biochem,* vol. 97, pp. 33-44, Jan 1 2006.

[38] L. A. Solchaga, J. E. Dennis, V. M. Goldberg, and A. I. Caplan, "Hyaluronic acid-based polymers as cell carriers for tissue-engineered repair of bone and cartilage," *J Orthop Res,* vol. 17, pp. 205-13, Mar 1999.

[39] A. L. Sieminski, C. E. Semino, H. Gong, and R. D. Kamm, "Primary sequence of ionic self-assembling peptide gels affects endothelial cell adhesion and capillary morphogenesis," *J Biomed Mater Res A,* vol. 87, pp. 494-504, Nov 2008.

[40] A. L. Sieminski, A. S. Was, G. Kim, H. Gong, and R. D. Kamm, "The stiffness of three-dimensional ionic self-assembling peptide gels affects the extent of capillary-like network formation," *Cell Biochem Biophys,* vol. 49, pp. 73-83, 2007.

[41] J. D. Kisiday, M. Jin, M. A. DiMicco, B. Kurz, and A. J. Grodzinsky, "Effects of dynamic compressive loading on chondrocyte biosynthesis in self-assembling peptide scaffolds," *J Biomech,* vol. 37, pp. 595-604, May 2004.

[42] J. D. Kisiday, P. W. Kopesky, C. H. Evans, A. J. Grodzinsky, C. W. McIlwraith, and D. D. Frisbie, "Evaluation of adult equine bone marrow- and adipose-derived progenitor cell chondrogenesis in hydrogel cultures," *J Orthop Res,* vol. 26, pp. 322-31, Mar 2008.

[43] P. W. Kopesky, H. Y. Lee, E. J. Vanderploeg, J. D. Kisiday, D. D. Frisbie, A. H. Plaas, C. Ortiz, and A. J. Grodzinsky, "Adult equine bone marrow stromal cells produce a cartilage-like ECM mechanically superior to animal-matched adult chondrocytes," *Matrix Biol,* vol. 29, pp. 427-38, Jun 2010.

[44] R. E. Miller, P. W. Kopesky, and A. J. Grodzinsky, "Growth factor delivery through self-assembling peptide scaffolds," *Clin Orthop Relat Res,* vol. 469, pp. 2716-24, Oct 2011.

[45] S. Koutsopoulos, L. D. Unsworth, Y. Nagai, and S. Zhang, "Controlled release of functional proteins through designer self-assembling peptide nanofiber hydrogel scaffold," *Proc Natl Acad Sci U S A,* vol. 106, pp. 4623-8, Mar 24 2009.

[46] M. F. Pittenger, A. M. Mackay, S. C. Beck, R. K. Jaiswal, R. Douglas, J. D. Mosca, M. A. Moorman, D. W. Simonetti, S. Craig, and D. R. Marshak, "Multilineage potential of adult human mesenchymal stem cells," *Science,* vol. 284, pp. 143-7, Apr 2 1999.

[47] J. Fan, R. R. Varshney, L. Ren, D. Cai, and D. A. Wang, "Synovium-derived mesenchymal stem cells: a new cell source for musculoskeletal regeneration," *Tissue Eng Part B Rev,* vol. 15, pp. 75-86, Mar 2009.

[48] C. Erggelet, M. Endres, K. Neumann, L. Morawietz, J. Ringe, K. Haberstroh, M. Sittinger, and C. Kaps, "Formation of cartilage repair tissue in articular cartilage defects pretreated with microfracture and covered with cell-free polymer-based implants," *J Orthop Res,* vol. 27, pp. 1353-60, Oct 2009.

[49] A. Asanbaeva, K. Masuda, E. J. Thonar, S. M. Klisch, and R. L. Sah, "Regulation of immature cartilage growth by IGF-I, TGF-beta1, BMP-7, and PDGF-AB: role of metabolic balance between fixed charge and collagen network," *Biomech Model Mechanobiol,* vol. 7, pp. 263-76, Aug 2008.

[50] K. D. Osborn, S. B. Trippel, and H. J. Mankin, "Growth factor stimulation of adult articular cartilage," *J Orthop Res,* vol. 7, pp. 35-42, 1989.

[51] P. Pavasant, T. Shizari, and C. B. Underhill, "Hyaluronan synthesis by epiphysial chondrocytes is regulated by growth hormone, insulin-like growth factor-1, parathyroid hormone and transforming growth factor-beta 1," *Matrix Biol,* vol. 15, pp. 423-32, Dec 1996.

[52] T. I. Morales, "Transforming growth factor-beta and insulin-like growth factor-1 restore proteoglycan metabolism of bovine articular cartilage after depletion by retinoic acid," *Arch Biochem Biophys,* vol. 315, pp. 190-8, Nov 15 1994.

[53] J. A. Tyler, "Insulin-like growth factor 1 can decrease degradation and promote synthesis of proteoglycan in cartilage exposed to cytokines," *Biochem J,* vol. 260, pp. 543-8, Jun 1 1989.

[54] R. L. Sah, A. C. Chen, A. J. Grodzinsky, and S. B. Trippel, "Differential effects of bFGF and IGF-I on matrix metabolism in calf and adult bovine cartilage explants," *Arch Biochem Biophys,* vol. 308, pp. 137-47, Jan 1994.

[55] G. Maor, M. Silbermann, K. von der Mark, D. Heingard, and Z. Laron, "Insulin enhances the growth of cartilage in organ and tissue cultures of mouse neonatal mandibular condyle," *Calcif Tissue Int,* vol. 52, pp. 291-9, Apr 1993.

[56] A. J. Nixon, L. A. Fortier, J. Williams, and H. Mohammed, "Enhanced repair of extensive articular defects by insulin-like growth factor-I-laden fibrin composites," *J Orthop Res,* vol. 17, pp. 475-87, Jul 1999.

[57] T. Tokunou, R. Miller, P. Patwari, M. E. Davis, V. F. Segers, A. J. Grodzinsky, and R. T. Lee, "Engineering insulin-like growth factor-1 for local delivery," *FASEB J,* vol. 22, pp. 1886-93, Jun 2008.

[58] R. E. Miller, A. J. Grodzinsky, K. Cummings, A. H. Plaas, A. A. Cole, R. T. Lee, and P. Patwari, "Intraarticular injection of heparin-binding insulin-like growth factor 1 sustains delivery of insulin-like growth factor 1 to cartilage through binding to chondroitin sulfate," *Arthritis Rheum,* vol. 62, pp. 3686-94, Dec 2010.

[59] S. Koutsopoulos and S. Zhang, "Two-layered injectable self-assembling peptide scaffold hydrogels for long-term sustained release of human antibodies," *J Control Release,* vol. 160, pp. 451-8, Jun 28 2012.

[60] I. M. Khan, S. J. Gilbert, S. K. Singhrao, V. C. Duance, and C. W. Archer, "Cartilage integration: evaluation of the reasons for failure of integration during cartilage repair. A review," *Eur Cell Mater,* vol. 16, pp. 26-39, 2008.

[61] M. B. Pabbruwe, E. Esfandiari, W. Kafienah, J. F. Tarlton, and A. P. Hollander, "Induction of cartilage integration by a chondrocyte/collagen-scaffold implant," *Biomaterials,* vol. 30, pp. 4277-86, Sep 2009.

[62] C. D. Hoemann, J. Sun, M. D. McKee, A. Chevrier, E. Rossomacha, G. E. Rivard, M. Hurtig, and M. D. Buschmann, "Chitosan-glycerol phosphate/blood implants elicit hyaline cartilage repair integrated with porous subchondral bone in microdrilled rabbit defects," *Osteoarthritis Cartilage,* vol. 15, pp. 78-89, Jan 2007.

[63] H. Guo, T. Chen, R. F. Warren, and S. A. Maher, "Inhomogeneous Aggregate Modulus Of The Native Cartilage Causes Micromotion Between Native And Tissue-engineered Cartilage Under Loading," presented at the 60th Annual Meeting of the Orthopaedic Research Society, New Orleans, LA, 2014.

[64] J. T. Enders, T. J. Otto, H. C. Peters, J. Wu, S. Hardouin, B. R. Moed, and Z. Zhang, "A model for studying human articular cartilage integration in vitro," *J Biomed Mater Res A,* vol. 94, pp. 509-14, Aug 2010.

[65] T. Vinardell, S. D. Thorpe, C. T. Buckley, and D. J. Kelly, "Chondrogenesis and integration of mesenchymal stem cells within an in vitro cartilage defect repair model," *Ann Biomed Eng,* vol. 37, pp. 2556-65, Dec 2009.

[66] E. B. Hunziker, "Articular cartilage repair: basic science and clinical progress. A review of the current status and prospects," *Osteoarthritis Cartilage,* vol. 10, pp. 432-63, Jun 2002.

[67] G. Kaplonyi, I. Zimmerman, A. D. Frenyo, T. Farkas, and G. Nemes, "The use of fibrin adhesive in the repair of chondral and osteochondral injuries," *Injury,* vol. 19, pp. 267-72, Jul 1988.

[68] D. A. Wang, S. Varghese, B. Sharma, I. Strehin, S. Fermanian, J. Gorham, D. H. Fairbrother, B. Cascio, and J. H. Elisseeff, "Multifunctional chondroitin sulphate for cartilage tissue-biomaterial integration," *Nat Mater,* vol. 6, pp. 385-92, May 2007.

[69] B. Sharma, S. Fermanian, M. Gibson, S. Unterman, D. A. Herzka, B. Cascio, J. Coburn, A. Y. Hui, N. Marcus, G. E. Gold, and J. H. Elisseeff, "Human cartilage repair with a photoreactive adhesive-hydrogel composite," *Sci Transl Med,* vol. 5, p. 167ra6, Jan 9 2013.

[70] K. Jurgensen, D. Aeschlimann, V. Cavin, M. Genge, and E. B. Hunziker, "A new biological glue for cartilage-cartilage interfaces: tissue transglutaminase," *J Bone Joint Surg Am,* vol. 79, pp. 185-93, Feb 1997.

[71] A. M. Rich, E. Pearlstein, G. Weissmann, and S. T. Hoffstein, "Cartilage proteoglycans inhibit fibronectin-mediated adhesion," *Nature,* vol. 293, pp. 224-6, Sep 17-23 1981.

[72] C. H. Jo, E. M. Kim, H. J. Ahn, H. J. Kim, S. C. Seong, and M. C. Lee, "Degree of degeneration and chondroitinase ABC treatment of human articular cartilage affect adhesion of chondrocytes," *Tissue Eng,* vol. 12, pp. 167-76, Jan 2006.

[73] M. C. Lee, K. L. Sung, M. S. Kurtis, W. H. Akeson, and R. L. Sah, "Adhesive force of chondrocytes to cartilage. Effects of chondroitinase ABC," *Clin Orthop Relat Res,* pp. 286-94, Jan 2000.

[74] J. C. Lee, H. J. Min, S. Lee, S. C. Seong, and M. C. Lee, "Effect of chondroitinase ABC on adhesion and behavior of synovial membrane-derived mesenchymal stem cells in rabbit partial-thickness chondral defects," *J Orthop Res,* vol. 31, pp. 1293-301, Aug 2013.

[75] L. M. Janssen, C. D. In der Maur, P. K. Bos, J. A. Hardillo, and G. J. van Osch, "Short-duration enzymatic treatment promotes integration of a cartilage graft in a defect," *Ann Otol Rhinol Laryngol,* vol. 115, pp. 461-8, Jun 2006.

[76] J. van de Breevaart Bravenboer, C. D. In der Maur, P. K. Bos, L. Feenstra, J. A. Verhaar, H. Weinans, and G. J. van Osch, "Improved cartilage integration and interfacial strength after enzymatic treatment in a cartilage transplantation model," *Arthritis Res Ther,* vol. 6, pp. R469-76, 2004.

[77] P. K. Bos, J. DeGroot, M. Budde, J. A. Verhaar, and G. J. van Osch, "Specific enzymatic treatment of bovine and human articular cartilage: implications for integrative cartilage repair," *Arthritis Rheum,* vol. 46, pp. 976-85, Apr 2002.

[78] D. Seol, Y. Yu, H. Choe, K. Jang, M. J. Brouillette, H. Zheng, T. H. Lim, J. Buckwalter, and J. Martin, "Effect of Short-Term Enzymatic Treatment on Cell Migration and Cartilage Regeneration; In Vitro Organ Culture of Bovine Articular Cartilage," *Tissue Eng Part A,* Jan 15 2014.

[79] C. J. Hunter and M. E. Levenston, "Maturation and integration of tissue-engineered cartilages within an in vitro defect repair model," *Tissue Eng,* vol. 10, pp. 736-46, May-Jun 2004.

[80] L. J. Bonassar, E. H. Frank, J. C. Murray, C. G. Paguio, V. L. Moore, M. W. Lark, J. D. Sandy, J. J. Wu, D. R. Eyre, and A. J. Grodzinsky, "Changes in cartilage composition and physical properties due to stromelysin degradation," *Arthritis Rheum,* vol. 38, pp. 173-83, Feb 1995.

[81] P. M. Ragan, V. I. Chin, H. H. Hung, K. Masuda, E. J. Thonar, E. C. Arner, A. J. Grodzinsky, and J. D. Sandy, "Chondrocyte extracellular matrix synthesis and turnover are influenced by static compression in a new alginate disk culture system," *Arch Biochem Biophys,* vol. 383, pp. 256-64, Nov 15 2000.

[82] R. W. Farndale, C. A. Sayers, and A. J. Barrett, "A direct spectrophotometric microassay for sulfated glycosaminoglycans in cartilage cultures," *Connect Tissue Res,* vol. 9, pp. 247-8, 1982.

[83] Y. J. Kim, R. L. Sah, J. Y. Doong, and A. J. Grodzinsky, "Fluorometric assay of DNA in cartilage explants using Hoechst 33258," *Anal Biochem,* vol. 174, pp. 168-76, Oct 1988.

[84] J. Iqbal, J. L. Bird, A. P. Hollander, and M. T. Bayliss, "Effect of matrix depleting agents on the expression of chondrocyte metabolism by equine chondrocytes," *Res Vet Sci,* vol. 77, pp. 249-56, Dec 2004.

[85] A. Maroudas, "Distribution and Diffusion of Solutes in Articular Cartilage," *Biophys J.* , vol. 10, pp. 365–379, 1970.

[86] A. R. Poole, "The relationship between toluidine blue staining and hexuronic acid content of cartilage matrix," *Histochem J,* vol. 2, pp. 425-30, Sep 1970.

[87] L. J. Bonassar, A. J. Grodzinsky, A. Srinivasan, S. G. Davila, and S. B. Trippel, "Mechanical and physicochemical regulation of the action of insulin-like growth factor-I on articular cartilage," *Arch Biochem Biophys,* vol. 379, pp. 57-63, Jul 1 2000.

[88] D. Huang, "Effect of extracellular chondroitin sulfate on cultured chondrocytes," *J Cell Biol,* vol. 62, pp. 881-6, Sep 1974.

[89] A. M. Nahir, D. Shomrat, and M. Awad, "Chondroitinase ABC affects the activity of intracellular enzymes in rabbit articular cartilage chondrocytes," *J Rheumatol,* vol. 22, pp. 702-7, Apr 1995.

[90] E. S. Reindel, A. M. Ayroso, A. C. Chen, D. M. Chun, R. M. Schinagl, and R. L. Sah, "Integrative repair of articular cartilage in vitro: adhesive strength of the interface region," *J Orthop Res,* vol. 13, pp. 751-60, Sep 1995.

[91] W. J. Dhert, C. C. Verheyen, L. H. Braak, J. R. de Wijn, C. P. Klein, K. de Groot, and P. M. Rozing, "A finite element analysis of the push-out test: influence of test conditions," *J Biomed Mater Res,* vol. 26, pp. 119-30, Jan 1992.

[92] A. I. Maroudas, "Balance between swelling pressure and collagen tension in normal and degenerate cartilage," *Nature,* vol. 260, pp. 808-9, Apr 29 1976.

[93] S. R. Eisenberg and A. J. Grodzinsky, "Electrokinetic micromodel of extracellular matrix and other polyelectrolyte networks," *Physicochemical Hydrodynamics,* vol. 10, pp. 517–539, 1988.

[94] J. A. Martin, S. M. Ellerbroek, and J. A. Buckwalter, "Age-related decline in chondrocyte response to insulin-like growth factor-I: the role of growth factor binding proteins," *J Orthop Res,* vol. 15, pp. 491-8, Jul 1997.

[95] C. J. Hunter and M. E. Levenston, "The influence of repair tissue maturation on the response to oscillatory compression in a cartilage defect repair model," *Biorheology,* vol. 39, pp. 79-88, 2002.

[96] M. A. DiMicco and R. L. Sah, "Integrative cartilage repair: adhesive strength is correlated with collagen deposition," *J Orthop Res,* vol. 19, pp. 1105-12, Nov 2001.

[97] A. M. Garcia, N. Szasz, S. B. Trippel, T. I. Morales, A. J. Grodzinsky, and E. H. Frank, "Transport and binding of insulin-like growth factor I through articular cartilage," *Arch Biochem Biophys,* vol. 415, pp. 69-79, Jul 1 2003.

Appendix A: Protocols Related to Methods

i. Quantification of GAG Depletion in MATLAB

5/20/14 4:55 PM /Users/kmrosz.../GAGdepletion Histology.m 1 of 2

```
% Average distance of enzyme penetration/GAG depletion from histology
% Keri Mroszczyk
% January 15, 2014

clear all
close all

% Select .tiff image from sample folder:
[fn, pn] = uigetfile('*.*','Choose Image file');

% Add the directory of the selected file to the search path
addpath(pn);

% Pixel to micron conversion 1.5 pixels= 1 um
scale=1.5;

% Read image
A=imread(fn);

% Positive is counterclockwise, Negative values of rotation are clockwise
% A_rot = imrotate(A,□200,'bilinear','crop'); % Rotate image

% Define vectors for the average distanc ein pixels and in microns
AveDistPx=[0,0];
AveDistUm=[0,0];


for k = 1:2 % Repeated for the 2 different degrees of degradation
    % The first polygon you draw will be the larger of the two (the "black"
    % to "white") and the second will be the the smaller/complete distance
    % of degradation (the "blue/pink" to "white")

    % If there is no "second" region you can just draw a random square in
    % the image and ignore the second value in the AveDistPx vectors

    a=A; % Make two different variables with the image "A" stored
    a1=A;

    figure(1), b=imshow(a); % Displays the image

    ydata = get(b,'XData'); % Deliberately switched since 'XData' retieves the column
coordinates

    xdata = get(b,'YData');

    % Trace the boundary of each area of GAG depletion, starting with the
    % larger degree of degradation
    h = impoly;

    vertices=wait(h); % Double click to proceed, allows user to modify polygon

    % Mask the region of interest

    masked=1□poly2mask(vertices(:,1),vertices(:,2),xdata(2),ydata(2));% The masked
region will have all zeros whereas outside the masked region, there will be all 1's
    % The masked region is our region of interest (ROI), the area of GAG depletion

    % We need to find the left edge of the polygon, where 1's first turn to
    % 0's and the right edge, where 0's turn to 1's

    [r,c]=size(masked); % FInd dimensions of the masked image
    var=zeros(r,2); % Allocates a vector with two columns and the same number of rows as
the masked image
    for i=1:(r); % Loops through to find the coordinates of the pixel where the value
switches from 0 to 1
        for j=1:(c□1);
            if masked(i,j)==1 && masked(i,j+1)==0
                var(i,1)=j;
```

```
            end
            if masked(i,j)==0 && masked(i,j+1)==1
                var(i,2)=j;
            end
        end
    end

    % Now find the distance in pixels
    distance=var(:,1)-var(:,2);
    AveDistPx(1,k)=abs(round(mean(distance)));

    % Converts to microns
    AveDistUm(1,k)=abs(round(mean(distance)/scale));


    % The rest of the script just displays an image quickly to show you the
    % masked region that was generated, but is not critical for the
    % distance calculations

    [row,col]=find(masked==1); %find all values outside polygon


    %*****************************************************************

    for i=1:length(row) %Set everything outside polygon as -20000

        a1(row(i),col(i),:)=-20000;

    end

    figure(k+1), b=imshow(a1);
end

AveDistUm
AveDistPx
```

ii. Determining Dose of HB-IGF-1

Previous studies with HB-IGF-1 and a self-assembling peptide hydrogel used 50 nM of the growth factor in a 100 µl acellular gel, which significantly elevated the synthesis of proteoglycans in a neighboring disc of cartilage tissue 3 mm in diameter and 1 mm in height (Florine et al., 2014). In using the *in vitro* model of defect repair developed in this thesis, with the gel cast into an explant annulus, the volume of tissue was over four times greater than used previously. Thus, a gel with 50 nM of HB-IGF-1 had no detectable effect on the proteoglycan synthesis in the explant annulus. For all experiments thereafter, the concentration was increased to exceed that used in previous work, such that an effect on proteoglycan synthesis could be detected and that the gel could serve as a reservoir of growth factor for *in vivo* studies.

Previously: 50.8 ng of HB-IGF-1 loaded into 100 µl of gel adjacent to 7.07 mm^3 of tissue

My study: 312.5 ng of HB-IGF-1 loaded into 50 µl of gel adjacent to 30.8 mm^3 of tissue

iii. Harvesting, Isolation, and Casting Protocols

Harvesting Cartilage Explants

Procedure must be done under sterile conditions
Materials:
-To cut the joint: tissue forceps, regular forceps, 2 blade holders
-To cut cores: 8mm biopsy punch, rod to push plug out
-To slice cores: brain cutter, blades
-To punch the slices: 6mm punches
-Other: bottle for PBS, bottle for medium

Medium: hg DMEM, sodium pyruvate, HEPES, NEAA, PSA, asc, proline

1. Wear lab coat. Set up cutting room with gauzy paper shiney side up, tape, garbage bag taped to counter.
2. Cut meat away around the ball of the femur. The nonsterile knife can be used for this.
3. Saw off the ball joint and mount the joint in the holders. Tighten the set screws with the allen wrench. Rinse off the saw.
4. Cut the joint inside the hood. Cut off the outer tissue, then switch scalpel blades. Once the tibia is removed, switch blades again.
5. Expose the femoropatellar groove.
6. Punch 8mm cores using a biopsy punch and loosen with a fresh scalpel.
7. If the cartilage core gets stuck to the bit, use the glass pipette to push it out.
8. Place the cores into a 24 well plate with PBS+PSA.
9. Keep track of where the cores are from (medial vs lateral, top to bottom), get 5-6 cores from the lateral side and 4-5 on the medial side. Can also get some from the center of the groove.
10. Throw the joint away and rinse out the mount.
11. To get a 1.4 mm slice: use the brain cutter and two razor blade. Cut off the first 0.7mm (one notch in the brain cutter) and then cut another 1.4mm (two notches) and keep that disc in PBS.
12. Need to get a flat surface first, then can keep slices.
13. When all the discs are cut, suction out the PBS an punch a 6mm concentric hole in the discs with a biopsy punch and add 2ml of basal medium to equilibrate for 48hrs.

Chondrocyte Isolation Procedure

Materials:
-tissue forceps, flat forceps, scalpel handles
-long spatulas
-foil (~18" long) to cover joint
-spinner flask with stir bar

Medium for isolation: hg DMEM, HEPES, NEAA, PSA, proline, ascorbate, sodium pyruvate

14. Make 50mL of 2mg/mL pronase (=proteinase) in isolation medium. Sonicate for 20-30 mins and vortex. Sterile filter.
15. Make 50mL of 0.25mg/mL collagenase in isolation medium. Vortex to mix. Sterile filter.
16. Add 15-20mL PBS+PSA to a 10cm Petri dish and weigh. Record weight on lid and put in hood.
17. Expose femoral head about 1.5" from ball joint, remove head with saw, and mount joint in holder. Spray joint with ethanol and move into the hood.
18. Switch gloves.
19. Remove bulk tissue and expose chondyles by removing outer fascia and cutting medial and lateral ligaments to loosen joint. Pull down the tibia and cut the ACL and PCL and the rest of the tissue connecting the tibia and femur. Replace blades often to avoid contamination.
20. Switch gloves.
21. Place sterile foil under joint and use a fresh scalpel handle and blade to remove cartilage from femoral chondyles. Cut at a shallow angle to avoid cutting too deep so you avoid blood vessels. Start on the right chondyle and move left to avoid touching the surface. Place cartilage shavings into Petri dish with sterile PBS+PSA. Foil is sterile, so shavings that fall can be used.
22. Weigh the Petri dish with cartilage in it to determine grams of tissue harvested. Dice the tissue to achieve better extraction (but makes it harder to get out of the dish and can lose during pipetting).
23. Use sterile spatula to transfer cartilage from dish to spinner flask.
24. Add pronase and place on stir plate in incubator. Set on low and make sure the bar is rotating evenly. Digest for 1 hour at 37C.
25. Aspirate the pronase using a pipet.
26. Rinse the cartilage with sterile PBS, mix it thoroughly, and aspirate the PBS.
27. Rinse again.
28. Add collagenase solution to spinner flask.
29. Return flask to stir plate and incubate at 37C overnight.
30. The next morning check to see if the tissue is completely digested. If not, wait 30 mins. If still not fully digested can wait more or add fresh collagenase, or can proceed.
31. Filter solution into a tube with a 70um strainer. Filter again into a tube with a 40um strainer.

32. Centrifuge for 8min at 1900xg.
33. Aspirate media and resuspend in 10mL PBS.
34. Centrifuge for 8min at 1900xg.
35. Aspirate PBS and resuspend in PBS.
36. Mix PBS well to disrupt pellet.
37. Centrifuge suspension for 8min at 1900xg
38. Aspirate PBS
39. Resuspend in medium at 37C.
40. Count cells
    a. 100uL cell suspension and add 400uL PBS.
    b. Take 50uL cell solution from a and mix with 50uL Trypan blue
    c. Mix well
    d. 10uL to hemocytometer
    e. The average count per subsquare divided by 10 gives cell density in millions of cells/mL
41. Calculate total yield and cells/g tissue (Should get 50-80 million cells

Radiolabeling

1. Have media stock made up and split into two groups: media for radiolabeling, and media without radiolabel. Do not split up the radiolabeling media into subgroups because then you need standards for each subgroup.
2. Have tubes labeled for saving media from gels that will be radiolabeled and two labeled red top freeze tubes for radiolabel standards.
3. Put radiolabel tape on tubes that will have radiolabeled media in them to avoid confusion. Place radiolabel tape on the plates that will contain labeled samples as well. Get bin for radiolabeled tips to be discarded into. DO NOT put in regular sharps container.
4. Use the calculation sheet for solutions to determine the amount of isotopes needed (35S).
    a. Check the logbook to see what label number/letter we are on and how much is left.
    b. Make sure that the calibration date in the spreadsheet is correct.
    c. Enter 2 mL per plug and number of plugs.
    d. Enter 0.5 mL for the standard.
    e. Keep concentrations of 35S at 5 auCi/mL respectively.
    f. Print the sheet of what you are using to keep for your records.
5. Remove watch and other metal items when working with isotopes.
6. Remove appropriate vials from locked fridge. Check the labels to make sure they match the logbook records. Note which label you are using on the printed sheet.
7. Only use pipet tips with the radiolabel or labeled media. Do not use 5 or 10 mL pipets because they are difficult to dispose of (must be broken in half to fit in jugs). Do not aspirate radiolabeled media into the aspirator.
8. ***Add the 35S isotope. Mix well. ***Take 0.5 mL of media for the 35S standard. If you forget to do this, you are toast.
9. BE EXTRAORDINARILY CAREFUL not to spill anything that is radiolabeled because cleanup is a pain. Close bottles ASAP and try not to touch the sides of tubes with isotope in them with the pipetter.
10. When working with labeled solutions or the isotopes themselves, try not to set the lids down on the hood surface because it can get contaminated. Hold onto the lids and place them back on top of the vials.
11. As soon as you are done with the stock isotopes, put them back into the locked fridge and lock it. Record in the logbook what you used and how much is left.
12. Note the time when you begin labeling the gels on the Rx sheet you printed.
13. Once the radiolabeled media is added to the plate, put the radiolabel tape on it and mark which wells are labeled.
14. Once you are done with the tubes that had radiolabeled media in them, to clean up pour any leftover media into the bin in the wet lab. Rinse the tubes with a small amount of DI water and pour into bin. The tube then goes into the radiolabeled trashcan for 35S. Fill out the tags on both the bin and the trashcan.
15. Radiolabel incorporation and gel takedown will end exactly 24 hours after radiolabel was added. Before this, have the following ready: labeled tubes for removing the radioactive media; labeled red top freeze vials that have been

weighed for storing the actual gel and measuring wet weight; Rx wash; and sterilized spatulas. To make Rx wash, you take a 1 mL alloquat from the fridge and add 249 (250-1) mL of PBS (can take 225 mL ultrafiltered water and add 25 mL of 10x PBS). (To make up more alloquats follow the formula on the solution spreadsheet where add proline and sodium sulfate to PBS I think.)

16. Once at 24 hour point, remove media on radiolabeled tubes to save. Fill in wells with Rx wash (1 mL/plug). The Rx wash should be in a 50 mL tube so that you aren't pipetting in and out of the stock 1 L bottle. Move non-radiolabeled samples on the plate to a different plate along with the media that they were in (so they don't go in the fridge). Use sterilized tools to do this. **The tips you are using need to go into a radiolabel bin.
17. Every 30 mins take off old wash solution and add fresh for a total of 4 washes. Place waste wash in 50 mL tube labeled with radiolabel tape. As needed, empty waste wash into radiolabel container in wet lab.
18. Place plate in the fridge during the washes to slow cell metabolism and keep from dying.
19. After the last wash, separate the gels from the rings.
    a. For KLD: use curved spatula to get gel and ring out of well, use scalpel to cut ring and straight spatula to move gel out of ring
20. Move gels into appropriate pre-labeled 1.5ml vials.
21. Add 1ml proteinaseK to the gels and 1.5mL to the explants, put in the 60 degree water bath for 48 hours.
22. Run standard ECM assays.

Appendix B: Data Related to Results

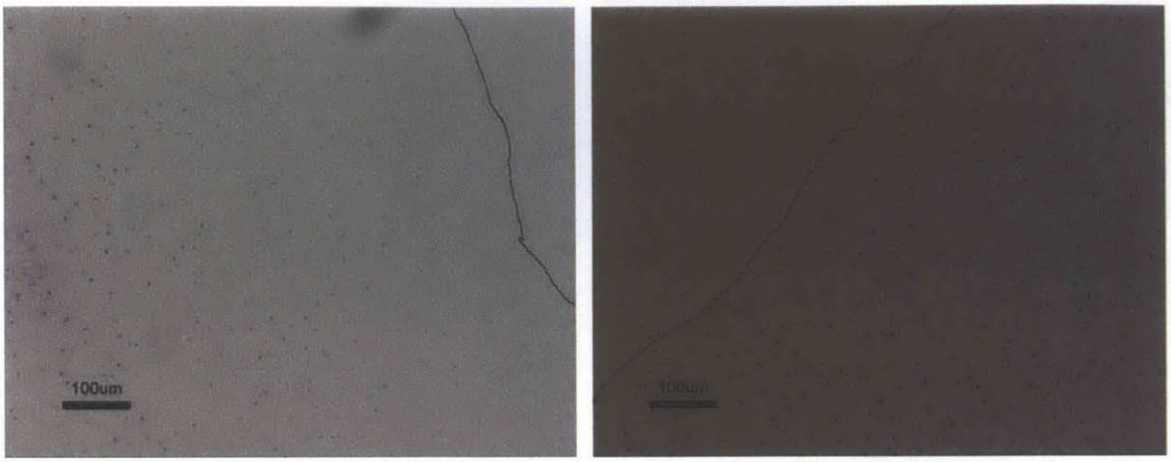

Figure B1. Representative histological sections showing complete GAG depletion following treatment with 1% (10 mg/mL) and 2.5% (25 mg/mL) trypsin for 1 minute. The gray line marks the inner surface of the annulus to which the enzyme was exposed. The lack of toluidine blue staining indicates total degradation of the GAG content.

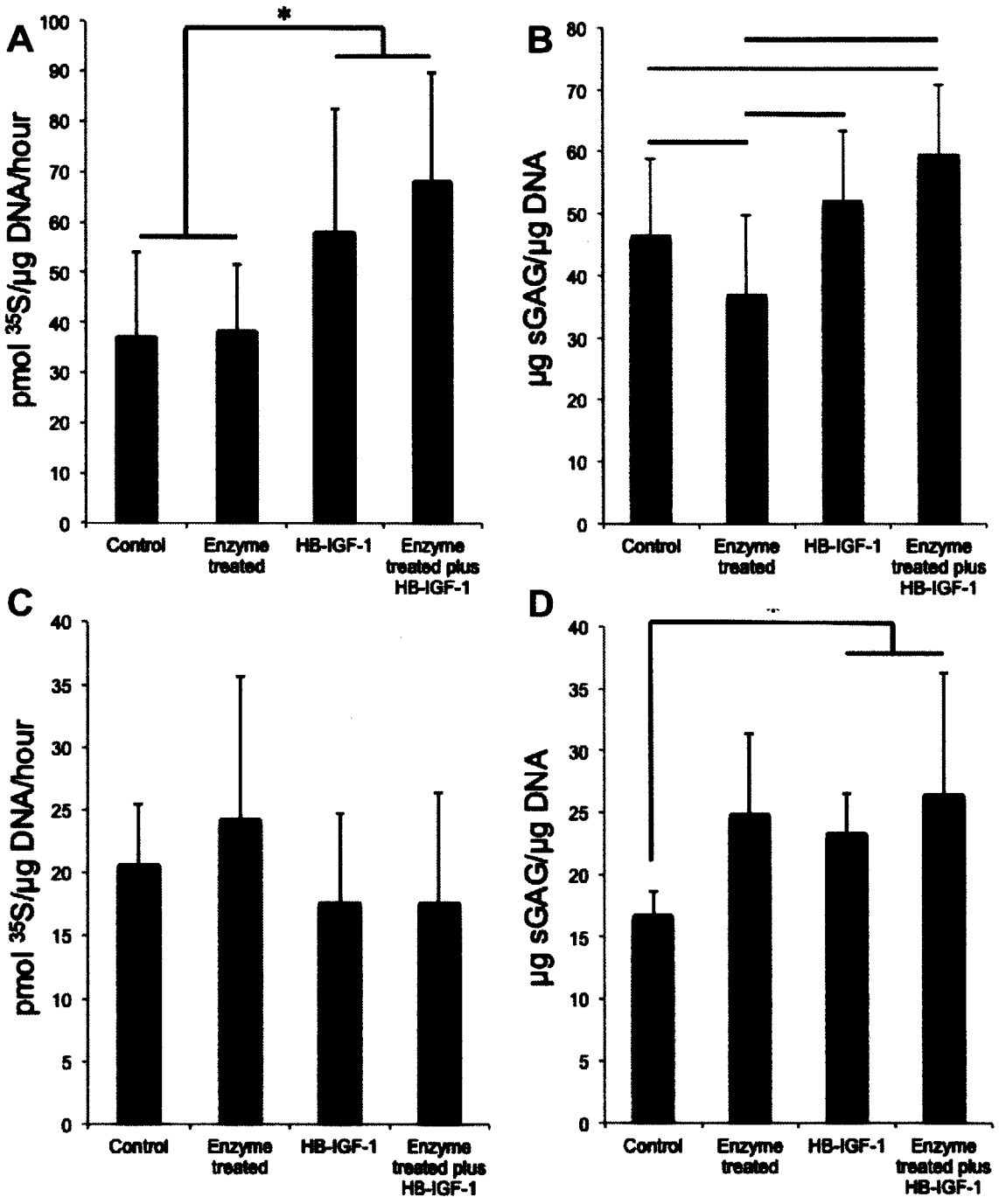

Figure B2. A) Rate of biosynthesis of sulfated GAG and B) sGAG content in explants into which chondrocyte-seeded KLD was cast, for which the C) the rate of biosynthesis of sulfated GAG and D) sGAG content in the gels were also analyzed. The gel-explants constructs were cultured in basal medium for 15 days, after which the explants and gels were analyzed separately for extracellular matrix and cellular content. Explants that received the enzyme pre-treatment were exposed to 50 µg/mL trypsin for 2 minutes followed by FBS for an additional 2 minutes to neutralize the enzyme. Values are mean + standard deviation for n=14 per condition, (6-8 explants x 2 animals). * indicates $p<0.05$, and for (C), bars indicate difference between two conditions with $p<0.05$.