In vitro and in vivo Growth Factor Delivery to Chondrocytes and Bone-Marrow-Derived Stromal Cells in Cartilage and in Self-Assembling Peptide Scaffolds

by

Rachel E. Miller

B.S. Bioengineering
The Pennsylvania State University, 2005

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Signature of Author _______________________________________________________

Department of Biological Engineering
May 20, 2010

Certified by ____________________________________________________________

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

Accepted by ____________________________________________________________

Peter C. Dedon
Professor of Toxicology and Biological Engineering
Associate Head, Department of Biological Engineering
Chair, Course XX Graduate Program Committee
Thesis Committee

Dr. Alan J. Grodzinsky
Thesis Advisor
Professor of Biological, Electrical, and Mechanical Engineering
Massachusetts Institute of Technology

Dr. Linda G. Griffith
Thesis Committee Chair
Professor of Biological and Mechanical Engineering
Massachusetts Institute of Technology

Dr. Richard T. Lee
Professor of Medicine
Harvard Medical School

Dr. Parth Patwari
Associate Biophysicist, Brigham and Women’s Hospital
Instructor in Medicine
Harvard Medical School

Dr. David B. Schauer
Professor of Biological Engineering and Comparative Medicine
Massachusetts Institute of Technology
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Rachel E. Miller

Submitted to the Department of Biological Engineering on May 20, 2010 in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Biological Engineering at the Massachusetts Institute of Technology

ABSTRACT

The inability of articular cartilage to repair itself after acute injury has been implicated in the development of osteoarthritis. The objective of this work was to develop methods for delivering growth factors to cartilage and to test the ability of a self-assembling peptide scaffold, (KLDL)_3, with or without growth factors to augment repair. Delivery methods included growth factor adsorption, scaffold-tethering, and modification of growth factor structure.

(KLDL)_3 was modified to deliver IGF-1 and TGF-β1 to chondrocytes and bone-marrow-derived stromal cells (BMSCs), respectively, by adsorption and by biotin-streptavidin tethering. This study showed that while TGF-β1 can be effectively delivered by adsorption, IGF-1 can not. Additionally, while tethering these factors provided long-term sequestration, tethering did not stimulate proteoglycan production in vitro.

A full-thickness, critically sized, rabbit cartilage defect model was used to test the ability of (KLDL)_3 with or without chondrogenic factors (TGF-β1, dexamethasone, and IGF-1) and BMSCs to stimulate cartilage regeneration in vivo. (KLDL)_3 alone showed the greatest repair after 12 weeks with significantly higher Safranin-O, collagen II immunostaining, and cumulative histology scores compared to untreated contralateral controls. Ongoing studies include the evaluation of (KLDL)_3 in a clinically relevant sized equine defect co-treated with microfracture and subjected to strenuous exercise.

A fusion protein was created by adding a heparin-binding domain to IGF-1 (HB-IGF-1), converting IGF-1 from a short-acting growth factor to one that can be retained and locally delivered in articular cartilage in vivo. It was shown that HB-IGF-1 is retained in cartilage through binding to negatively charged glycosaminoglycan chains, with chondroitin sulfate the most prevalent type in cartilage. HB-IGF-1 was shown to bind adult human cartilage and to be preferentially delivered and retained in rat articular cartilage after intra-articular injection. In contrast, unmodified IGF-1 was not detectable after intra-articular injection. These results suggest that modification of growth factors with heparin-binding domains may be a clinically relevant strategy for local delivery to cartilage.

Taken together, these results show that (KLDL)_3 self-assembling peptide hydrogels are customizable for growth factor delivery and can promote cartilage repair in vivo. In addition, the fusion protein HB-IGF-1 is preferentially retained in cartilage tissue compared to un-modified IGF-1.

Thesis Supervisor: Alan J. Grodzinsky
Title: Professor of Biological, Electrical, and Mechanical Engineering
EDUCATION
Ph.D. in Biological Engineering, concentration in Biomaterials, May 2010
Massachusetts Institute of Technology, Cambridge, MA 02139
Thesis Title: *In vitro* and *in vivo* Growth Factor Delivery to Chondrocytes and Bone-
Marrow-Derived Stromal Cells in Cartilage and in Self-Assembling Peptide Scaffolds
Thesis Advisor: Prof. Alan Grodzinsky
GPA: 4.9/5.0

B.S. and Honors in Bioengineering (Materials Science concentration) with highest
distinction, May 2005
The Pennsylvania State University, University Park, PA 16802
Thesis Title: The Development of Nano-engineered Hemocompatible Biomaterials
Thesis Supervisor: Prof. Erwin Vogler
GPA: 3.99/4.0

Annville-Cleona High School, Salutatorian, June 2001
Annville, PA 17003

AWARDS
Siebel Scholar, Class of 2010
National Science Foundation (NSF) Graduate Research Fellowship (2008 – 2010)
Student Marshal – PSU College of Engineering – 2005 graduating class
Astronaut Scholarship Foundation Scholarship (2004 – 2005)
National Dean’s List (2004)
Evan Pugh Scholar Award (top 0.5% of my class 2003-2005)
Schreyer Honors College Scholarship (2001 – 2005)
President’s Freshman Award (Spring 2002)
Undergraduate Dean’s List (All Semesters)

REFEREED JOURNAL ARTICLES


REFEREED CONFERENCE PRESENTATIONS


TEACHING EXPERIENCE

Teaching Assistant: Fields, Forces, and Flows in Biological Systems (20.430), MIT Fall 2006

UNIVERSITY SERVICE

Biological Engineering Graduate Student Board, 2006-2009
Biological Engineering Retreat Committee, 2006-2009
Mentor: Undergraduate Research Opportunities Program, 2009-2010
Biomedical Engineering Society, 2003-2005
Mentor: Schreyer Honors College, PSU, Fall 2002, 2003, 2004
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As a kid in elementary school, I imagined being an archaeologist, meteorologist, or marine biologist, but I only discovered what being a bioengineer meant in high school: the ability to blend my loves of math, science, and medicine. To be writing my doctoral thesis at MIT is still unbelievable. I owe many people thanks for helping me to attain this dream, but my family tops the list. My parents always encouraged my curiosity and taught me to make the most of every opportunity; my brother helped me to develop my rebellious adventurous side. I would also like to thank my high school calculus and computer programming teacher, Mr. Kreiser, for pushing me to challenge myself, and my undergraduate research advisor, Dr. Vogler, for introducing me to research and for making me apply to MIT.

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# Table of Contents

List of Tables 10  
List of Figures 11  

## Chapter 1  
Introduction  
1.1 Cartilage Biology 13  
1.2 Cartilage Pathogenesis and the Current Strategies for Repair 14  
1.3 Cartilage Tissue Engineering 15  
1.4 Insulin-like Growth Factor – I 18  
1.5 In vivo Animal Models 20  
1.6 Thesis Outline 21  
1.7 References 22  

## Chapter 2  
Growth Factor Delivery via Self-assembling Peptide Scaffolds 28  
2.1 Introduction 29  
2.2 Materials and Methods 31  
2.3 Results 34  
2.4 Discussion 37  
2.5 Acknowledgements 40  
2.6 References 41  
2.7 Figures 45  

## Chapter 3  
Effect of Self-assembling Peptide, Chondrogenic Factors, and Bone-Marrow-Derived Stromal Cells on Osteochondral Repair 49  
3.1 Introduction 50  
3.2 Materials and Methods 53  
3.3 Results 58  
3.4 Discussion 62  
3.5 Acknowledgements 66  
3.6 References 67  
3.7 Tables 71  
3.8 Figures 80  

## Chapter 4  
Intra-articular Injection of HB-IGF-1 Sustains Delivery of IGF-1 to Cartilage through Binding to Chondroitin Sulfate 85  
4.1 Introduction 86  
4.2 Materials and Methods 88  
4.3 Results 93  
4.4 Discussion 97  
4.5 Acknowledgements 99  
4.6 References 100
List of Tables

Chapter 3: Effect of Self-assembling Peptide, Chondrogenic Factors, and Bone Marrow Derived Stromal Cells on Osteochondral Repair

Table 3.1 Treatment groups with amounts delivered per animal 71
Table 3.2 Gross Observations Scoring System 72
Table 3.3 Modified O’Driscoll Histological Scoring System 75
Table 3.4 Gross Scores 77
Table 3.5 Histological Scores 79

Appendix B

Table B1 Treatment groups not included in Chapter 3 131
# List of Figures

## Chapter 2: Growth factor delivery via self-assembling peptide scaffolds

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>High-affinity tethering prolongs retention of IGF-1</td>
<td>45</td>
</tr>
<tr>
<td>2.2</td>
<td>bIGF-1 is bioactive</td>
<td>46</td>
</tr>
<tr>
<td>2.3</td>
<td>Soluble IGF-1, but not adsorbed or tethered IGF-1, stimulates sGAG production</td>
<td>47</td>
</tr>
<tr>
<td>2.4</td>
<td>Soluble and adsorbed TGF-β1, but not tethered, promotes sGAG production</td>
<td>48</td>
</tr>
</tbody>
</table>

## Chapter 3: Effect of Self-assembling Peptide, Chondrogenic Factors, and Bone Marrow Derived Stromal Cells on Osteochondral Repair

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1</td>
<td>Gross necropsy photographs of treated and untreated joints in KLD, KLD+CF, and KLD+CF+BMSCs</td>
<td>80</td>
</tr>
<tr>
<td>3.2</td>
<td>Gross pathologic observation of joints comparing KLD, KLD+CF, and KLD+CF+BMSCs treated and contralateral untreated defects</td>
<td>81</td>
</tr>
<tr>
<td>3.3</td>
<td>Histologic scores comparing KLD, KLD+CF, and KLD+CF+BMSCs treated and untreated defects</td>
<td>82</td>
</tr>
<tr>
<td>3.4</td>
<td>Safranin-O staining</td>
<td>83</td>
</tr>
<tr>
<td>3.5</td>
<td>Immunohistochemistry scores</td>
<td>84</td>
</tr>
</tbody>
</table>

## Chapter 4: Intra-articular Injection of HB-IGF-1 Sustains Delivery of IGF-1 to Cartilage through Binding to Chondroitin Sulfate

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.1</td>
<td>Retention of HB-IGF-1 in bovine cartilage explants following enzymatic digestion of glycosaminoglycans</td>
<td>104</td>
</tr>
<tr>
<td>4.2</td>
<td>Retention of HB-IGF-1 on cells lacking heparan sulfate</td>
<td>105</td>
</tr>
<tr>
<td>4.3</td>
<td>Binding analysis of HB-IGF-1 and IGF-1 to isolated glycosaminoglycans</td>
<td>106</td>
</tr>
<tr>
<td>4.4</td>
<td>Retention of HB-IGF-1 in vivo</td>
<td>107</td>
</tr>
<tr>
<td>4.5</td>
<td>Retention of HB-IGF-1 in human cartilage explants</td>
<td>108</td>
</tr>
</tbody>
</table>

## Appendix A

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>Effect of soluble IGF-1 and bIGF-1 on GAG and DNA synthesis of chondrocytes encapsulated in KLD</td>
<td>115</td>
</tr>
<tr>
<td>A2</td>
<td>Effect of soluble IGF-1 and bIGF-1 on sulfate incorporation of chondrocytes encapsulated in KLD</td>
<td>116</td>
</tr>
<tr>
<td>A3</td>
<td>Effect of tethered IGF-1 on BMSCs encapsulated in KLD</td>
<td>117</td>
</tr>
<tr>
<td>A4</td>
<td>Example flow cytometry images</td>
<td>119</td>
</tr>
<tr>
<td>A5</td>
<td>Effect of casting order on cell death</td>
<td>120</td>
</tr>
<tr>
<td>A6</td>
<td>Chondrocytes encapsulated in KLD with or without tethered IGF-1 were evaluated for cell death and apoptosis</td>
<td>121</td>
</tr>
<tr>
<td>A7</td>
<td>TUNEL staining on chondrocytes encapsulated in KLD</td>
<td>124</td>
</tr>
</tbody>
</table>
Appendix B

Figure B1 Circular dichroism spectroscopy to monitor assembly 128
Figure B2 In situ peptide assembly and cell viability 129
Figure B3 In situ filling and joint articulation using 4 mg/mL KLD 130
Figure B4 In situ filling and joint articulation using 3.2 mg/mL KLD 130
Figure B5 Gross necropsy photographs of joints in Groups 4 and 5 131
Figure B6 Gross and histologic effects comparing defects treated with KLD+BMSCs+1.4 ng TGF-β1 in group 3 to defects treated with the same combination in group 4 132
Figure B7 Immunohistochemistry scores (0-4) 133

Appendix C

Figure C1 Western blots on conditioned medium using anti-HS stub 136

Appendix D

Figure D1 Luminex data on mouse joints 144
Chapter 1. Introduction

1.1 Cartilage Biology

Adult articular cartilage is composed of one cell type, the chondrocyte, which accounts for approximately 1-5% of the tissue volume with the remaining filled by extracellular matrix (ECM) (15-40%) and water (60-85%)\(^{20,47,51,60}\). Cells maintain a spherical morphology, though somewhat flattened in the superficial zone, and do not directly contact other cells\(^{58}\). Cartilage lacks a nervous and vascular supply, resulting in chondrocytes in adult articular cartilage remaining relatively inactive metabolically and having a poor repair capacity\(^{20,48}\).

Chondrocytes synthesize all ECM components, which include collagens (10-30% wet wt), proteoglycans (PGs) (3-10% wet wt), and non-collagenous proteins and glycoproteins\(^{51,60}\). The collagen network provides tensile strength and helps to retain proteoglycans, which offer compressive resistance. Type 2 collagen is the predominant type found in articular cartilage with types 9 and 11 incorporated within the basic structure of this heteropolymer\(^{13}\). The major PG in the extracellular matrix is aggrecan, which consists of a protein backbone to which glycosaminoglycan (GAG) side chains of chondroitin sulfate (CS) and keratan sulfate (KS) are covalently attached\(^{60}\). The negatively charged GAGs of the PGs give cartilage its high fixed-charge density, controlling ionic equilibrium in the tissue\(^{50}\). Aggrecan molecules bind non-covalently to hyaluronic acid (HA) chains, stabilized by link protein\(^{26}\). Chondrocytes are directly surrounded by a pericellular matrix about 2 \(\mu\)m wide\(^{58}\) composed of molecules that can
interact with the cell including type 6 collagen, HA, a heparan sulfate and chondroitin sulfate containing proteoglycan (perlecan), and decorin^57.

1.2 Cartilage Pathogenesis and the Current Strategies for Repair

Cartilage injuries generally occur when the knee is impacted or subjected to sudden twisting, often concurrent with meniscal or ligament tears^6,^27. These types of injuries occur in both children and adults^55, with roughly 3 million knee injuries reported each year in the United States^1. These injuries result in an acute inflammatory environment (presence of IL-1β, TNF-α, IL-6, among others) in the joint, which dissipates after 1-2 weeks^30, but remains elevated above normal levels for months to years^4,^44,^45. These changes in cytokine levels, in combination with changes in loading of the joint^69, are implicated in the progression of a majority of people who sustain these injuries to develop osteoarthritis (OA) within 10-20 years^24,^45.

OA is a musculoskeletal disorder and the most common form of arthritis, affecting 21.5% of the U.S. population^20,^73. The disease is characterized by progressive destruction of articular cartilage; symptoms include pain and impaired joint movement leading to disability^20. Current treatments include drug therapy for pain relief, surgical approaches (e.g., microfracture, drilling, and mosaic-plasty), biological approaches (e.g., autologous chondrocyte implantation (ACI)), and as a final recourse, joint replacement. None of these treatments has yielded lasting results or regeneration of tissue equivalent to normal cartilage^20,^60. Microfracture, which creates hemorrhage to induce the migration of potential repair cells, results in fibrocartilage tissue that is bio-mechanically inferior to
native tissue. ACI is not indicated for the treatment of cartilage damage associated with OA and results have not been shown to be significantly different than microfracture treatment when used to repair focal defects within joint surfaces. Joint replacement is currently the only option for severe articular cartilage degeneration; however, it is not a permanent solution and often, additional surgery is required. Recently it has been shown in vitro that it is possible for cartilage explants to recover from initial aggrecanase-mediated GAG loss but not from collagen loss. This suggests that early intervention following cartilage injuries may be key to halting progression to OA, likely with a combination of anabolic, anti-catabolic, and defect-filling treatments.

1.3 Cartilage Tissue Engineering

In 1997, a panel held by the National Science Foundation defined tissue engineering as “… the application of the principles and methods of engineering and life sciences toward the fundamental understanding of structure-function relationships in normal and pathological mammalian tissue and the development of biological substitutes to restore, maintain, or improve function”. From the previous section, this suggests that cartilage repair may be improved (1) by augmenting native repair attempts through filling defects in situ or (2) by implanting more mature constructs grown in vitro and developing methods for integrating this engineered tissue with native tissue. In the case of (1), this could be particularly beneficial in combination with marrow stimulation techniques in that larger defect sizes may be able to be treated when an external scaffold is supplied to encourage cellular infiltration and differentiation. This strategy will be the focus of this thesis work. Many groups have begun efforts towards the development of tissue-
engineered cartilage; however the growth of cartilage that is comparable to native tissue and is able to successfully integrate with native tissue has not yet been achieved (see \textsuperscript{8, 18, 28, 41, 67} for review). The challenge for successful tissue engineering remains the discovery of an optimal combination of scaffold, biological signaling, and cells for tissue regeneration that is also practical for clinical use.

The scaffold should ideally serve as a microenvironment that promotes ECM production by either endogenous or exogenous cells. A variety of natural and synthetic materials have been proposed for use in cartilage tissue engineering. As some of the few materials approved by the Food and Drug Administration (FDA) for use in the body, polyglycolic acid (PGA), polylactic acid (PLA), and polylactic-co-glycolic acid (PLGA) have been widely investigated, but these polymers degrade quickly in the body and have been shown to produce toxic monomers\textsuperscript{70}. Natural polymers that undergo enzymatic hydrolysis, such as hyaluronic acid, collagen, fibrin, and chitin have also been used in a variety of combinations as scaffolds\textsuperscript{28, 70}. While these natural materials are all able to support maintenance of the chondrocyte phenotype, none has been shown to be an ideal substrate for cartilage formation with biochemical and mechanical properties comparable to native tissue. Hydrogels are an appealing scaffold choice due to their high water content, similar to the articular cartilage environment. This attribute also allows for the encapsulation of chondrocytes and proteins within the network, and these gels have been shown to maintain the spherical morphology and gene expression of chondrocytes\textsuperscript{74}. Hydrogels have been developed from alginate, polyethyleneglycol (PEG), chondroitin sulfate, self-assembling peptides, and other materials\textsuperscript{41}.
Self-assembling peptides offer the opportunity to create customizable scaffolds that can be induced to assemble upon placement in a physiological environment. In addition, scaffolds composed of nanofibers have been shown to produce better outcomes than microfiber-based scaffolds. The family of self-assembling peptides all satisfies the principle that there is no net charge, but an equal number of ionizable basic and acidic amino acids that promote β-sheet formation upon self-assembly, which can be initiated by a solution at physiological pH and ionic strength. The peptide KLD is composed of the sequence AcN-KLDLKDLKDL-CNH₂ and is based on the work of Zhang et al. KLD has been demonstrated to support the synthesis and accumulation of cartilage-like ECM by embedded chondrocytes and bone marrow derived stromal cells (BMSCs).

This peptide also offers flexibility in design that allows for the opportunity of growth factor delivery for local, specific, prolonged biological stimulation – key to achieving optimal tissue induction, especially since growth factors typically have a short half-life once they are introduced into the body. A variety of growth factors have been shown to be important in chondrogenesis and in stimulation of matrix production by chondrocytes, including: TGF-β1, IGF-1, and members of the BMP and GDF families (see review). Recently, Davis et al. developed a system for tethering IGF-1 to another self-assembling peptide, RAD16-II, by a “biotin-sandwich” technique. By adding a biotin linkage to the end of the peptide sequence and incorporating a minority of this biotinylated peptide in a solution of un-modified peptide, self-assembly was not
disrupted. This allowed for the attachment of biotinylated IGF-1 molecules through the inclusion of streptavidin. This system provided controlled delivery of IGF-1 to myocardial tissue in vivo. The non-specific “biotin-sandwich” strategy should be able to be applied to other self-assembling peptides such as KLD and offers the potential for multiple growth factors to be incorporated into a hydrogel for local tissue delivery.

The final parameter to be considered for successful tissue engineering is cell source. There are three main types of cells that have been considered for use in cartilage tissue engineering: primary chondrocytes, adult bone-marrow-derived stromal cells (BMSCs), and embryonic stem cells. There is a limited availability of primary chondrocytes in adult cartilage, and expansion in culture often leads to de-differentiation. Embryonic stem cells, while offering great potential for the differentiation into multiple cell types, are associated with many ethical and safety concerns. Therefore, adult BMSCs may present the best choice for the future of cartilage tissue engineering. Autologous BMSCs are obtainable from a patient’s own bone marrow, and they have been shown to be able to undergo expansion and chondrogenesis without risk of tumorigenesis. Much current research is being directed towards the development of optimal expansion and differentiation conditions.

1.4 Insulin-like Growth Factor – I

IGF-1 plays a primary role in cartilage homeostasis by stimulating PG synthesis, promoting cell survival, and inhibiting cartilage degradation. IGF-1 is produced primarily by the liver, but other cell types, including chondrocytes, are also able to synthesize IGF-
1. In cartilage, IGF-1 can act both in a paracrine and autocrine manner. It is a basic, 7.6 kDa protein that binds the IGF-1 receptor (IGF-1R) with a $K_D$ of 0.2-1 nM. IGF-1Rs exist on the cell surface as disulfide-linked dimers and studies have shown that a single molecule of IGF-1 is able to activate the receptor with approximately 10,000 receptors on the surface of each chondrocyte. Human OA chondrocytes express and produce increased levels of IGF-1 but are hypo-responsive to it. An explanation for this may be the increased production of IGF binding proteins (IGFBPs), which can associate with the cell surface or ECM and may be responsible for decreased amounts of IGF-1 available to bind IGF-1Rs. Therefore, IGF-1 growth-factor or gene therapy offers a potential treatment for diminished IGF-1 activity, but to be effective, amounts of IGF-1 delivered within the joint must exceed the binding capacity of local IGFBPs.

IGF-1 has also been used as treatment in a variety of cartilage injury models. Inclusion of IGF-I in fibrin-chondrocyte constructs enhanced chondrogenesis in equine cartilage defects, including incorporation into surrounding cartilage. Cartilage explants treated with IL-1 were partially rescued from proteoglycan degradation by co-culture with synovial cells transduced with AdIGF-1. Madry et al. demonstrated that chondrocytes transfected with AdIGF-1 included in alginate scaffolds can enhance repair of rabbit cartilage defects. In addition, Goodrich et al. transplanted equine chondrocytes genetically modified with AdIGF-1 into equine femoral trochlea defects and found that repair of the defect with hyaline-like cartilage was accelerated. Direct injections of AdIGF-1 into the metacarpophalangeal (MCP) joints of horses were also shown to result in significant elevations of IGF-I in synovial fluid for approximately 21 days with
minimal detrimental effects\textsuperscript{21}. A critical factor that must be considered in any IGF-1 delivery strategy is that systemic delivery of IGF-1 can cause significant side effects\textsuperscript{31} as well as the potential to promote diabetic retinopathy\textsuperscript{72} and cancer\textsuperscript{7}. These studies suggest that IGF-1 is a critical component of cartilage repair, yet an optimal delivery technique has yet to be developed.

1.5 \textit{In vivo} Animal Models

While \textit{in vitro} cell work is important for the initial screening and development of tissue-engineered products, the effectiveness of constructs \textit{in vivo} must also be considered. To this end, several animal models have been used for the testing of constructs designed to regenerate cartilage. Typically, the initial screening for the safety and \textit{in vivo} efficacy of such constructs are performed in a small animal model. Nude mice have been used for the testing of cartilage growth potential by subcutaneous implantation\textsuperscript{2, 33, 56, 62, 63}, however, this model does not allow for immunological screening of potential constructs nor does it evaluate the ability for healing of a cartilage defect or for the ability to integrate with native tissue. An alternative immuno-competent small animal model is the rabbit. Rabbits offer many of the same advantages of mice in that they are available at low cost with minimal housing requirements. Appropriate defect models in weight-bearing areas of the medial femoral condyles of rabbit knee joints have been developed for the testing of tissue-engineered constructs\textsuperscript{11, 17, 23, 61}.

Once basic testing has been completed, true construct functionality must be evaluated in a large animal model such as a dog, sheep, goat, pig, or horse. While no defect model is
directly applicable to humans due to physiological and anatomical differences between the human joint and model animal joints\textsuperscript{28}, the horse seems to most closely approximate human cartilage based on the thickness of its articular cartilage\textsuperscript{16}, its susceptibility to osteoarthritis\textsuperscript{15}, and similar cartilage biochemical composition\textsuperscript{68}. A model for inducing OA in equines has been developed\textsuperscript{15}, and several studies have used manufactured defects as models for testing cartilage repair strategies\textsuperscript{3, 43, 53, 71}. It must be kept in mind, however, that positive results obtained from \textit{in vivo} animal testing must be viewed with caution as they are not necessarily predictive of success in humans\textsuperscript{19, 25}.

1.6 Thesis Outline

The objective of this work was to develop methods for delivering growth factors to cartilage and to test the ability of a self-assembling peptide scaffold, (KLDL)\textsubscript{3}, with or without growth factors to augment repair. Delivery methods included growth factor adsorption, scaffold-tethering, and modification of growth factor structure.

In Chapter 2, IGF-1 and TGF-\(\beta\)\textsubscript{1} were adsorbed and tethered to the self-assembling peptide scaffold KLD and the effects on matrix production by chondrocytes and BMSCs, respectively, were observed.

In Chapter 3, the effects of KLD, chondrogenic factors, and allogeneic BMSCs on the \textit{in vivo} repair of a critically sized full-thickness rabbit cartilage defect were examined.
In Chapter 4, binding of a novel fusion protein, heparin-binding IGF-1 or HB-IGF-1, in cartilage tissue was investigated. The mechanism by which HB-IGF-1 is retained in cartilage was examined and the ability of HB-IGF-1 to provide sustained growth factor delivery to cartilage in vivo and to human cartilage explants was determined.

Finally, in Chapter 5, the main findings and conclusions are discussed and new questions motivated by this thesis are explored.

1.7 References


Chapter 2. Growth Factor Delivery via Self-assembling Peptide Scaffolds

The optimal strategy for delivering growth factors to cells for the purpose of cartilage tissue engineering remains an unmet challenge. The purpose of this study was to engineer self-assembling peptide scaffolds (i.e., (KLDL)_3 or KLD) to deliver growth factors to encapsulated cells. Chondrocytes or bone marrow stromal cells were encapsulated in KLD with or without IGF-1 or TGF-β1, respectively. The growth factors were either (1) pre-mixed with peptide solution prior to assembly to adsorb them to KLD, or (2) tethered to KLD through biotin-streptavidin bonds. Fluorescently tagged streptavidin was used to determine IGF-1 kinetics; sGAG and DNA content was measured. Tethering IGF-1 to KLD significantly increased retention of IGF-1 in KLD compared to adsorption, but neither method increased sGAG or DNA accumulation above control. Adsorbing TGF-β1 significantly increased proteoglycan accumulation above control, but again, tethering did not affect sGAG levels. Increasing the amount of tethered TGF-β1 negatively impacted DNA levels. This study provided an initial evaluation of methods for delivering growth factors to cells within a KLD hydrogel scaffold and showed that while TGF-β1 can be effectively delivered by adsorption, IGF-1 can not. Additionally, while tethering these factors provided long-term sequestration, tethering was not effective in stimulating proteoglycan production. Self-assembling peptides are a clinically relevant material that can be injected in vivo. While tethering growth factors to KLD results in long-term delivery, tethering does not result in the same bioactivity as soluble delivery, indicating that presentation of proteins is vital when considering a delivery strategy.
2.1 Introduction

Acute cartilage defects remain a challenge to repair. Currently, interventions such as microfracture or autologous chondrocyte implantation remain the standard of care, but these methods still result in mechanically inferior repair tissue. Cartilage tissue engineering has emerged as a possible avenue for improving repair. As such, many combinations of scaffolds, cells, and growth factors have been proposed.

Growth factors including TGF-β1, IGF-1, and members of the GDF and BMP families are known to be important in inducing repair, attracting migration of repair cells, and stimulating chondrogenesis, proliferation, and production of matrix. Due to the reality that growth factors act on multiple tissues and can have detrimental side effects if applied systemically, local delivery is necessary. Although intra-articular injections may present a simple approach for delivery directly to the joint, often much higher than physiologic concentrations must be used along with multiple injections in order to overcome the fact that growth factors have short half-lives and may be cleared rapidly from the synovial fluid. Therefore, delivery methods using scaffolds have been proposed to protect growth factors from degradation and to deliver them over longer periods of time. Despite the advantages hydrogels offer as scaffolds, growth factors quickly diffuse out of them. Therefore, most successful delivery strategies to date have incorporated growth factor-loaded microspheres within the hydrogel, so that release of the factors can be controlled by the degradation rate of the microspheres. While this has shown positive results, high loading concentrations of growth factors within these microspheres are still required. This creates the possibility of high localized doses...
through initial bolus release \emph{in vivo}, which is particularly dangerous for TGF-\(\beta\)1 since it has been shown to cause side effects such as inflammation and osteophyte formation\textsuperscript{3}.

Hydrogel scaffolds made from the self-assembling peptide sequences (RADA)\textsubscript{4} and (KLDL)\textsubscript{3}, which we refer to as RAD and KLD, respectively, have been shown to support maintenance of the chondrocyte phenotype\textsuperscript{24,34} and chondrogenesis of bone marrow stromal cells\textsuperscript{26}. These peptides assemble into hydrogels upon contact with solutions of physiologic pH and ionic strength\textsuperscript{49}, enabling them to be injected into tissues \emph{in vivo}, where they have been shown to encourage cell infiltration\textsuperscript{7}. In addition, bioactive sequences have been appended to the RAD sequence without disrupting assembly\textsuperscript{14,17,47}. RAD has also been shown to support growth factor delivery: PDGF-BB and other proteins have been adsorbed to RAD\textsuperscript{18,27}, SDF-1 was inserted directly onto the RAD peptide sequence\textsuperscript{39}, and IGF-1 was tethered to RAD through biotin-streptavidin-biotin bonds\textsuperscript{8}.

The purpose of this study was to engineer the self-assembling peptide, KLD, to deliver the growth factors IGF-1 and TGF-\(\beta\)1 to encapsulated chondrocytes or bone marrow stromal cells (BMSCs), respectively, in the context of cartilage tissue engineering. The growth factors were either (1) pre-mixed with the peptide solution prior to assembly to adsorb them to the hydrogel, or (2) tethered to the hydrogel with high-affinity prior to assembly\textsuperscript{8}. 
2.2 Materials and Methods

Materials: KLD peptide with the sequence AcN-(KLDL)$_3$-CNH$_2$ and biotinylated-KLD (bKLD) peptide with the sequence biotin-(aminocaproic acid)$_3$-(KLDL)$_3$ was synthesized by the MIT Biopolymers Laboratory (Cambridge, MA) using an ABI Model 433A peptide synthesizer with FMOC protection or received as a gift from 3DM (3DM, Inc., Cambridge, MA). Biotin-conjugated IGF-1 (bIGF-1) (immunological and biochemical testsystems GmbH, Reutlingen Germany) and biotin-conjugated TGF-β1 (bTGF-β1) (R&D Systems, Minneapolis, MN) were purchased and used as described below.

Chondrocyte Isolation and Encapsulation: Chondrocytes were isolated from 1-2 week old bovine calves (Research 87, Marlborough, MA) as described previously$^{38}$. Chondrocytes were encapsulated in KLD using acellular agarose molds to initiate self-assembly as outlined below$^{26}$, resulting in 6mm diameter, 50 µL peptide gel disks. To verify bioactivity of bIGF-1, chondrocytes were encapsulated at 12x10$^6$ cells/mL in KLD peptide (0.35% w/v) alone and cultured in IGF-1-free basal medium supplemented with either soluble IGF-1 (PeproTech Inc., Rocky Hill, NJ), soluble bIGF-1, or soluble bIGF-1/streptavidin at indicated concentrations (ng/mL); n=3-4. Basal medium consisted of serum-free high-glucose Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 0.003% ITS+1 (Sigma-Aldrich, St. Louis, MO), 1 mM sodium pyruvate, 10 mM HEPES buffer, 0.1 mM nonessential amino acids, 0.4 mM proline, 20 µg/mL ascorbic acid, 100 units/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin B. This choice of concentration of ITS+1 is equivalent to 5 nM.
insulin, termed mini-ITS$^{2,30}$, and chosen in order to avoid cross-talk of insulin with the IGF-1 receptor.

For high-affinity tethering of IGF-1, control, soluble, and tethered gels were created by pre-mixing KLD peptide (0.35% w/v) with 0.0035% w/v bKLD and encapsulating chondrocytes at 3x10$^6$ cells/mL; n=4. For tethered gels, bIGF-1 and fluorescent streptavidin-AlexaFluor 488 (Invitrogen) were pre-mixed at equimolar concentrations and added to the KLD/bKLD mixture prior to adding cells. For adsorbed gels, bIGF-1 and fluorescent-streptavidin were pre-mixed as for the tethered gels, but no bKLD was used in this case. Chondrocyte gels were cultured in basal medium and supplemented with soluble IGF-1 for the soluble condition (soluble IGF-1 replenished at each medium change).

BMSC Isolation: Bone marrow was harvested from 1-2 week old bovine calves (Research 87) and stromal cells (BMSCs) were isolated as described previously$^6,26$. BMSCs were selected via differential adhesion and expanded two passages in low glucose-DMEM with 10% ES-FBS (Invitrogen), 10mM HEPES, 100 units/mL penicillin G, 100 µg/mL streptomycin, 0.25 µg/mL amphotericin B, and 5 ng/mL FGF-2 (R&D Systems).

BMSC Encapsulation: BMSCs were encapsulated in KLD mixtures at 3x10$^6$ cells/mL using acellular agarose molds to initiate self-assembly$^{26}$. For no TGF (n=8) and soluble TGF (n=12) conditions, BMSCs were encapsulated in KLD peptide (0.35% w/v) alone.
For adsorbed gels (n=16), 0.35% KLD was pre-mixed with 100 ng/mL TGF-β1 (R&D Systems) and 100 nM dexamethasone (Sigma-Aldrich, St. Louis, MO) prior to adding cells. For tethered gels (n=4), 0.35% w/v KLD was pre-mixed with 0.0035% w/v bKLD, 2.1 µg/mL streptavidin (Pierce Biotechnology, Rockford, IL) and 100 or 500 ng/mL bTGF-β1 prior to adding cells. The resulting 6mm diameter, 50 µL peptide gel disks were cultured in high glucose-DMEM (Invitrogen) supplemented with 1% ITS+1 (Sigma-Aldrich), 1 mM sodium pyruvate, 37.5 µg/mL ascorbate-2-phosphate (Wako Chemicals, Richmond, VA), PSA (100 U/mL penicillin, 0.1 mg/mL streptomycin, 0.25 µg/mL amphotericin), 10 mM HEPES, 0.4 mM proline, and 0.1 mM non-essential amino acids. Soluble gels had additional supplementation of 10 ng/mL recombinant human TGF-β1 (R&D Systems) (replenished at each medium change). No TGF, soluble, and tethered gels had additional supplementation of 100 nM dexamethasone (replenished at each medium change).

**Cell Viability and Cell-Seeded Hydrogel Biochemistry:** Viability was determined by staining with FDA (live) and ethidium bromide (dead). There were no differences in viability among conditions for chondrocyte or BMSC experiments, and all experiments had >75% viability. At each timepoint, gels were digested with 250 µg/mL proteinase-K (Roche Applied Science, Indianapolis, IN) overnight at 60 °C. sGAG in each hydrogel sample was assessed via DMMB dye binding\(^{12}\); DNA was quantified by Hoechst dye assay\(^{23}\). For chondrocyte gels, digests were analyzed for presence of fluorescent streptavidin via fluorometer reading at 485/535 nm.
**Statistical Analyses:** All data are presented as mean ± standard error of the mean (SEM). Chondrocyte data were analyzed by one-way ANOVA at each timepoint. BMSC data were analyzed using a mixed model of variance at each timepoint with animal cell-source as a random factor. Residual plots were constructed for dependent variable data to test for normality and data were log transformed if necessary to satisfy this assumption. *Post hoc* Tukey tests for significance of pairwise comparisons were performed with a threshold for significance of *p*<0.05.

### 2.3 Results

**IGF-1 can be tethered to peptide hydrogels:** Using a biotin-sandwich approach as previously described for a related peptide sequence, RAD₈, we tethered IGF-1 to KLD scaffolds by including 1:100 ratio by weight of biotinylated KLD (bKLD):KLD and pre-mixing streptavidin with a biotinylated version of the growth factor (bIGF-1). Multivalent streptavidin and bIGF-1 were mixed in equimolar amounts so that the predominant complex formed would consist of a single biotinylated growth factor bound to a single streptavidin molecule, allowing streptavidin to bind bKLD with one of its three remaining binding sites. Excess bKLD (>130:1 molar ratio bKLD:streptavidin) was used to ensure that all of the delivered growth factor was tethered. To validate this tethering approach relative to simple adsorption, we used fluorescently tagged streptavidin to track the streptavidin/bIGF-1 complex in peptide hydrogels with encapsulated chondrocytes. Although adsorbed and tethered gels were loaded with equivalent amounts of streptavidin/bIGF-1, tethered gels retained significantly more bIGF-1 immediately after encapsulation (Fig 2.1A). After one day, adsorbed streptavidin/bIGF-1 had decreased to
10-17% of the day 0 levels, and by day 8 it was only 2-6% of day 0 (Fig 2.1B,C).

Tethering allowed amounts to remain at 20-27% of the day 0 levels even by day 8 (Fig 2.1B,C). In contrast, when TGF-β1 is adsorbed to KLD peptide hydrogels ~40% is retained at day 21 (data not shown).

**bIGF is biologically active:** To ensure that bIGF-1 had the same biological activity as normal IGF-1, chondrocytes encapsulated in KLD were stimulated with 100 ng/mL of soluble bIGF-1 or soluble IGF-1 for 4 days. sGAG content of the gels treated with IGF-1 and bIGF-1 was shown to be equivalent, and both conditions produced significantly more sGAG than gels incubated without IGF-1 (Fig 2.2A). DNA content was not significantly different among the treatments (Fig 2.2B). In addition, 100 ng/mL of soluble bIGF-1 was compared to soluble complexes of 100 ng/mL bIGF-1 pre-mixed with an equimolar amount of streptavidin to rule out any effect of binding to streptavidin on sGAG production. Again, after 4 days, bIGF-1 with and without streptavidin produced equivalent amounts of sGAG, significantly more than gels incubated without IGF-1 (Fig 2.2C), and DNA was equivalent among the treatments (Fig 2.2D). The bioactivity of bTGF-β1 was also confirmed in similar experiments (data not shown).

**Adsorbed and Tethered IGF-1 with chondrocytes:** Next, bIGF-1/streptavidin complexes were either adsorbed to KLD prior to chondrocyte encapsulation or tethered to KLD prior to encapsulation. Concentrations of bIGF-1 in the KLD hydrogel were varied for the adsorbed and tethered conditions such that cells would be exposed to amounts known to stimulate proteoglycan synthesis (>50 ng/mL)\(^{13}\). Although concentrations up to
1000 ng/mL were tested for adsorbed and tethered bIGF-1, neither method of delivering IGF-1 was able to stimulate sGAG production over no IGF-1 levels. This outcome for adsorption of IGF-1 is consistent with Fig 1, which showed adsorbed bIGF-1/streptavidin rapidly diffused out of KLD (Fig 2.3A). But the lack of bioactivity for tethering was unexpected since sufficient levels were present throughout the culture period. Gels treated with 50 ng/mL and 300 ng/mL of soluble IGF-1 produced significantly greater amounts of sGAG than control IGF-1-free gels at all timepoints, as expected (Fig 2.3A). DNA levels increased over time but were not different than control IGF-1-free gels for any treatment at any timepoint (Fig 2.3B).

**Adsorbed and Tethered TGF-β1 with BMSCs:** We next tested the hypothesis that since TGF-β1 adsorbed more strongly than IGF-1, it would result in greater proteoglycan stimulus than no TGF-β1; we also investigated whether tethered TGF-β1 would promote proteoglycan synthesis. BMSCs were stimulated with either soluble TGF-β1 and soluble dexamethasone, adsorbed TGF-β1 and adsorbed dexamethasone, or tethered TGF-β1 with soluble dexamethasone. In contrast to IGF-1, adsorbed TGF-β1 was able to stimulate BMSC sGAG production significantly higher than no TGF-β1 at all timepoints. By day 21, adsorbed TGF-β1 stimulated 31% as much sGAG as soluble TGF-β1 (Fig 2.4A). Tethering TGF-β1 at up to 500 ng/mL did not stimulate sGAG production by BMSCs, similar to results seen with chondrocytes exposed to tethered IGF-1. In addition, tethering at 500 ng/mL significantly inhibited DNA accumulation compared to TGF-β1-free gels at days 14 and 21 (Fig 2.4B). Soluble TGF-β1 stimulated an increase in DNA
over TGF-β1-free gels by day 21; adsorbed gels were not different from TGF-β1-free gels at any timepoint (Fig 2.4B).

2.4 Discussion

The optimal strategy for delivering growth factors to cells for the purposes of cartilage tissue engineering and cartilage repair remains an unmet challenge. Self-assembling peptides are a clinically relevant material that can be injected in vivo7, 8 and assemble on contact with solutions of physiologic pH and ionic strength49. We explored the ability of the self-assembling peptide hydrogel KLD to deliver IGF-1 and TGF-β1 to cells through two methods, tethering and adsorption of the growth factors to the peptide. While these growth factors can be tethered to KLD, they do not promote the same stimulation of matrix accumulation by chondrocytes or BMSCs compared to soluble delivery of these factors. In addition, both IGF-1 and TGF-β1 are able to be adsorbed to KLD, but only TGF-β1 is retained at sufficient quantity and duration to promote proteoglycan production.

Although biotin-streptavidin tethering was shown by Davis et al. to be effective in delivering IGF-1 in order to reduce apoptosis of implanted cardiomyocytes8, it was not effective in delivering IGF-1 or TGF-β1 to stimulate proteoglycan production by chondrocytes or BMSCs, respectively, in the context of cartilage tissue engineering. Due to the strength of the non-covalent biotin-streptavidin bond (K_D = 4x10^{-14} M)^16 it is unlikely that the growth factors are released by this tether. Instead, the loss of bIGF-1 by
day 8 shown in Fig 1 is probably due to the degradation of the gel resulting in loss of the bKLD fibers to which the growth factors are attached. A difference in peptide gel degradation rates caused by differences in sequence, (RADA)$_4$ vs. (KLDL)$_3$, and by differences in cell type could therefore partly explain the differences between this study and that of Davis et al.$^8$. Cartilage tissue also differs from myocardial tissue in that large amounts of extracellular matrix are produced, as early as one day after encapsulation. This matrix synthesis and secretion may prevent the stimulation of chondrocytes and BMSCs by immobilized factors by sterically blocking receptor-ligand binding. In addition, high-affinity tethering could block internalization of the ligand-receptor complex, which could limit bioactivity. Internalization of receptors is not required for TGF-β1 Smad2 signaling$^{31}$, while IGF-1 signaling is thought to involve both cell surface and endosomal signaling$^4$. However, inhibition of internalization may lead to altered signaling, resulting in lack of proteoglycan stimulation. Finally, while the density of tethered growth factors may also affect bioactivity$^{42}$, both IGF-1 and TGF-β receptors exist as functional complexes on cell surfaces, and binding of one ligand can initiate signaling$^{10, 41}$. Therefore, the distance between these immobilized growth factors should not have inhibited their effectiveness. Increasing the seeding density of chondrocytes (data not shown) or increasing the amount of growth factors immobilized within the scaffold had no effect.

Growth factors, including TGF-β1, have been covalently immobilized or tethered onto other polymer scaffolds or hydrogels$^{1, 9, 21, 22, 29, 35}$, although sometimes with altered signaling$^{11, 36, 40}$. There have been a limited number of other attempts at delivering
tethered growth factors for the purposes of cartilage tissue engineering\textsuperscript{37}. A possible solution to increase bioactivity of our tethering may be to include cleavable links in our peptide sequence. Lutolf et al. have proposed using MMP-cleavable links to deliver growth factors in poly(ethylene glycol)-based hydrogels upon catabolic events\textsuperscript{32, 33}. In addition, Segers et al. directly inserted the protein SDF-1 onto the RAD peptide sequence with an MMP-cleavable linkage\textsuperscript{39}. This idea may be adapted to our system by including such sequences appended to bKLD so that upon MMP cleavage, the growth factor complexes would become soluble. Other sequences may be more applicable for chondrogenic purposes, including sequences cleavable by the aggrecanases, ADMATS-4/5. Finally, using tethers with a lower binding affinity may allow this method to result in better biological activity.

Other methods for slow release of IGF-1 and TGF-\(\beta\textsubscript{1}\) have been developed (see\textsuperscript{28, 45} for review), but IGF-1 remains difficult to effectively deliver \textit{in vivo} due to its small size and rapid diffusion out of tissue. A new fusion protein made by adding the heparin-binding domain of heparin-binding EGF to IGF-1 (i.e. HB-IGF-1) has been found to be amenable to delivery in mature articular cartilage\textsuperscript{46}. A single dose of HB-IGF-1 resulted in sustained delivery and stimulation of proteoglycan synthesis for at least 8 days. The use of HB-IGF-1 with the KLD peptide system could be enabled by mixing in heparin, heparan sulfate, or the heparan sulfate proteoglycan, perlecan, with KLD. This approach has been shown to be effective in delivering other heparin-binding growth factors such as FGF-2 and BMP-2 to cells within fibrin\textsuperscript{19}, collagen type 1\textsuperscript{48}, and PLGA\textsuperscript{20} scaffolds. Although adsorption of TGF-\(\beta\textsubscript{1}\) to KLD effectively increased proteoglycan production in
our study, changing the degradation rates of KLD by changing the concentration or adding cross-links may offer a way of improving delivery.

This study provided an initial evaluation of methods for delivering growth factors to cells within the self-assembling peptide hydrogel KLD, and showed that while TGF-β1 is able to be delivered by adsorption, IGF-1 is not. Additionally, while tethering these factors through a biotin-streptavidin bond provided long-term sequestration, tethered growth factors were not effective in stimulating proteoglycan production. Therefore, while peptide sequences are readily functionalized, the manner in which growth factors are delivered affects bioactivity and varies for specific growth factors and biological systems of interest.

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2.6 References


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2.7 Figures

**Figure 2.1.** High-affinity tethering prolongs retention of IGF-1: Fluorescently labeled streptavidin and biotinylated-IGF-1 were either adsorbed or tethered to KLD prior to encapsulation of chondrocytes at 3×10⁶ cells/mL. Background from the peptide was measured on gels without fluorescent streptavidin and subtracted from fluorescence counts. (A) Streptavidin retained immediately after gel assembly. (B) Streptavidin retained after 1, 4, or 8 days of culture. * vs. corresponding concentration adsorbed gel, p<0.05. mean ± SEM. (C) Fluorescence microscopy on gels at day 8 on gels without bIGF-1/fluorescent streptavidin, gels with 1000 ng/mL adsorbed bIGF-1/fluorescent streptavidin, or gels with 1000 ng/mL tethered bIGF-1/fluorescent streptavidin. Scale bar = 250 µm.
Figure 2.2. bIGF-1 is bioactive: Chondrocytes were encapsulated in KLD at 12x10^6 cells/mL and cultured in medium with different soluble factors for four days. (A) sGAG and (B) DNA retained after culture in medium with soluble IGF-1 or soluble bIGF-1. (C) sGAG and (D) DNA retained after culture in medium with soluble bIGF-1 or soluble bIGF-1 + soluble streptavidin. mean ± SEM, * vs. No IGF, p<0.05.
Figure 2.3. Soluble IGF-1, but not adsorbed or tethered IGF-1, stimulates sGAG production: Biotinylated IGF-1 and streptavidin were either adsorbed or tethered to KLD at the indicated concentrations prior to encapsulation of chondrocytes at 3x10^6 cells/mL. No IGF-1 control gels were cultured in IGF-1-free medium. (A) sGAG and (B) DNA retained in gel. mean ± SEM, * vs. No IGF, p<0.05.
Figure 2.4. Soluble and adsorbed TGF-β1, but not tethered, promotes sGAG production: TGF-β1 and dexamethasone were adsorbed to KLD prior to assembly or biotinylated TGF-β1 and streptavidin were tethered to KLD prior to encapsulation of BMSCs at 3x10^6 cells/mL. No TGF control gels were cultured in TGF-β1-free medium. Soluble dexamethasone (100 nM) was included in the medium for all conditions but adsorbed. (A) sGAG and (B) DNA retained in gel. mean ± SEM, * vs. No TGF, p<0.05.
Chapter 3. Effect of Self-assembling Peptide, Chondrogenic Factors, and Bone-Marrow-Derived Stromal Cells on Osteochondral Repair

The goal of this study was to test the ability of an injectable self-assembling peptide (KLD) hydrogel with or without chondrogenic factors (CF) and allogeneic bone marrow stromal cells (BMSCs) to stimulate cartilage regeneration in a full-thickness, critically sized, rabbit cartilage defect model *in vivo*. We used CF treatments to test the hypotheses that CF would stimulate chondrogenesis and matrix production by cells migrating into acellular KLD (KLD+CF) or by BMSCs delivered in KLD (KLD+CF+BMSCs). Three groups were tested against contralateral untreated controls: KLD, KLD+CF, and KLD+CF+BMSCs, n=6-7. TGF-β1, dexamethasone, and IGF-1 were used as chondrogenic factors (CF) pre-mixed with KLD and BMSCs before injection. Evaluations included gross, histological, immunohistochemical and radiographic analyses. KLD without CF or BMSCs showed the greatest repair after 12 weeks with significantly higher Safranin-O, collagen II immunostaining, and cumulative histology scores than untreated contralateral controls. KLD+CF resulted in significantly higher aggrecan immunostaining than untreated contralateral controls. Including allogeneic BMSCs+CF markedly reduced the quality of repair and increased osteophyte formation compared to KLD alone. These data show that KLD can fill full-thickness osteochondral defects in situ and improve cartilage repair as shown by Safranin-O, collagen II immunostaining, and cumulative histology. In this small animal model, the full-thickness critically sized defect provided access to the marrow, similar in concept to abrasion arthroplasty or
spongialization in large animal models, and suggests that combining KLD with these
techniques may improve current practice.

3.1 Introduction

Repair of articular cartilage injuries remains a challenge, despite the development of
surgical treatments such as microfracture, abrasion arthroplasty, and spongialization,
which are used with the goal of recruiting marrow-derived cells by penetration of the
subchondral bone. While these techniques promote increased short-term healing, long-
term repair still consists of mechanically inferior fibrocartilage. Recent research has
focused on tissue engineering strategies using scaffolds to improve cartilage repair and
regeneration. In particular, hydrogels made from materials such as chitosan-glycerol
phosphate, polyethyleneglycol (PEG), fibrin, polyglycolic-co-lactic acid (PLGA), and
collagen have been explored with the goal of improving the accumulation of
extracellular matrix produced by cells (e.g., bone marrow stromal cells (BMSCs))
migrating into the scaffold from marrow.

Stimulation of BMSC chondrogenesis in vitro and in vivo has received much attention. In
vitro studies have shown that transforming growth factor-β1 (TGF-β1), dexamethasone,
and insulin-like growth factor-1 (IGF-1) promote chondrogenesis of BMSCs, and methods for delivering these chondrogenic factors have been developed, often in
conjunction with scaffolds. There have been a number of in vivo studies
performed delivering IGF-1, TGF-β1, or the combination of IGF-1 and TGF-β1.
β1, 17, 19, 40 to cartilage defects in order to stimulate chondrogenesis of endogenous BMSCs, but to our knowledge, no in vivo studies have incorporated dexamethasone.

The hypothesis that delivery of exogenous BMSCs to the joint can enhance cartilage regeneration has prompted the exploration of a wide variety of growth factor and scaffold combinations to stimulate BMSC chondrogenesis13, 20, 37. In vivo studies have attempted to deliver BMSCs alone12, 35, BMSCs encapsulated in scaffolds30, 43, 48, and BMSCs encapsulated in scaffolds with the inclusion of TGF-β14, 7, 14, 15. Despite the in vitro promise shown by 3D-cultured BMSCs, most long-term in vivo treatments with BMSCs have resulted in sub-optimal cartilage repair tissue12, 20, 43, 48. Improving BMSC chondrogenesis in vivo is likely dependent on several factors that are not well understood, including cell delivery, microenvironment, and a combination of pro-chondrogenic longitudinal signaling. In addition, an ideal clinical approach would minimize or obviate the in vitro culture duration and be performed with a single arthroscopic procedure.

Recent studies have shown that hydrogels made from the self-assembling peptide sequences (RADA)₄ and (KLDL)₃ (hereafter referred to as KLD) can maintain the chondrocyte phenotype26 and stimulate chondrogenesis of BMSCs in vitro6, 27, 29. These hydrogels have the ability to rapidly assemble when exposed to physiological pH and ionic strength50 and have pore sizes in the range of 100-500 nm42. These synthetic peptides have been used in vivo without immunogenic reaction3. Furthermore, TGF-β1 has been shown to adsorb to KLD when pre-mixed with the peptide solution prior to assembly, resulting in extended delivery of TGF-β1 to BMSCs and stimulating
chondrogenesis in vitro, promoting sGAG production and accumulation comparable to continuous medium supplementation of TGF-β1 over 21 days\textsuperscript{28}. In addition, including dexamethasone with TGF-β1 in medium supplementation during the in vitro culture of BMSCs in RADA results in less catabolic cleavage of aggrecan compared to culture with TGF-β1 alone\textsuperscript{8}. Finally, IGF-1 can be tethered to peptide scaffolds via biotin-streptavidin bonds; this tethered IGF-1 has been shown to remain biologically active and to promote cell survival in rat cardiomyocytes over 28 days\textsuperscript{3}.

The goal of this study was to test the ability of an injectable KLD hydrogel with or without BMSCs and chondrogenic factors (CF) to stimulate cartilage regeneration in vivo in a critically sized rabbit full-thickness cartilage defect model. This model provides access to the marrow, analogous to abrasion arthroplasty or spongialization in large animal models. We used CF treatments (IGF-1, TGF-β1, and dexamethasone) to test the hypotheses that CF would stimulate chondrogenesis and matrix production (1) by cells migrating into acellular KLD and (2) by P2 passaged allogeneic BMSCs delivered in KLD. IGF-1 was tethered to the peptide with a biotin-streptavidin bond\textsuperscript{3} to stimulate long-term production of cartilage ECM, while TGF-β1 and dexamethasone were pre-mixed with KLD prior to BMSC encapsulation to stimulate chondrogenesis and initial matrix production. A 12-week timepoint enabled evaluation of mid-term benefits of the treatment compared to contralateral untreated defects.
3.2 Materials and Methods

Materials: KLD peptide with the sequence AcN-(KLDL)_3-CNH₂ was synthesized by the MIT Biopolymers Laboratory (Cambridge, MA) using an ABI Model 433A peptide synthesizer with FMOC protection. Human recombinant TGF-β1 (R&D Systems, Inc., Minneapolis, MN), dexamethasone (Sigma-Aldrich, St. Louis, MO), sucrose (Sigma-Aldrich), biotinylated-IGF-1 (bIGF-1) (immunological and biochemical test systems GmbH, Reutlingen Germany), streptavidin (Pierce Biotechnology, Inc, Rockford, IL), biotinylated-KLD (biotin-(aminocaproic acid)_3-(KLDL)_3 or b-KLD) (MIT Biopolymers Laboratory), FBS (Invitrogen, Carlsbad, CA), and FGF-2 (R&D Systems, Inc.) were purchased and used as described.

Cell Isolation: Bone marrow was harvested and pooled from four rabbits used for an initial pilot study, and BMSCs were isolated as previously described²⁹. BMSCs were selected via differential adhesion to plastic and expanded two passages in alphaMEM with 10% FBS and 2 ng/mL FGF-2, 10 mM HEPES, and PSA (100 U/mL penicillin, 100 µg/mL streptomycin, and 250 ng/mL amphotericin), resulting in a total of approximately four population doublings. Each passage was conducted by seeding at a concentration of 12x10³ BMSCs/cm² and incubating for two days to allow BMSCs to grow to ~75% confluence.

In Vivo Study Design: All procedures were approved by the Animal Care and Use Committees at Colorado State University and Massachusetts Institute of Technology. Twenty skeletally mature, retired, female breeder New Zealand White rabbits (average
age 11 months and body weight 4.7 kg) were used for this study (Myrtle’s Rabbitry, Thompson Station, TN). One rabbit died during the study due to neurologic problems post-surgery and was not included in the analysis. The n values shown in Table 1 do not reflect this animal. Three different groups were tested against contralateral, untreated, empty controls: (1) KLD, (2) KLD+chondrogenic factors (CF), and (3) KLD+CF+BMSCs (Table 3.1). For all groups, KLD was resuspended in 10% sterile sucrose, and the final KLD concentration was kept constant at 3.2 mg/mL. For groups 2 and 3, KLD peptide (48 µg) was pre-mixed with a CF mixture consisting of 1.4 ng TGF-β1, 0.6 ng dexamethasone, 4.1 ng biotinylated-IGF-1 (bIGF-1), 30.7 ng streptavidin, and 0.48 µg b-KLD. For group 3, 150x10^3 BMSCs were encapsulated in the KLD/CF mixture. Encapsulation of BMSCs in vitro in this manner resulted in 80-90% viability. In vitro studies indicated that this amount of TGF-β1 would result in a concentration of TGF-β1 inside the scaffold sufficient to stimulate chondrogenesis. At the same time, if all of the TGF-β1 were to be released from the scaffold at once into the joint space in vivo, it would amount to approximately 1 ng/mL concentration in the joint space (1.4 ng / 1.4 mL joint space volume), compared to the native concentration of 52.3 pg/mL found in adult rabbits. Streptavidin and bIGF-1 were mixed at a 1:1 molar ratio in order to achieve, on average, binding of one bIGF-1 to each streptavidin allowing streptavidin to still bind b-KLD (in 100x molar excess of streptavidin and bIGF-1 to ensure homogeneous distribution of IGF-1 throughout the gel). The ability of this tethering to occur using these molar ratios has been shown in vitro and in vivo. The amount of bIGF-1 tethered was chosen to provide a local concentration of 300 ng/mL inside the scaffold,
which is above the threshold shown to be sufficient for chondrocyte stimulation of proteoglycan synthesis *in vivo* \(^9\).

**Defect Creation and Gel Injection:** All surgical procedures were performed under inhalation general anesthesia. A medial-parapatellar arthrotomy approach to the femoropatellar joint was performed, and the patella was luxated laterally. A 3mm-diam x 2mm-deep full-thickness, critically sized defect was created in the central region of the femoral trochlear groove (Fig 3.1). Direct pressure was applied with a surgical sponge to ensure all bleeding was stopped prior to application of the peptide, which was delivered as a liquid. Defects were filled with designated treatments (15 µL volume) or left untreated, as dictated by group assignment. The liquid peptide suspension could easily be seen filling the defect, and defects were filled until visually full. At this time, Lactated Ringer’s Solution was added to the joint periphery to gently fill the joint and cause polymerization of the peptide, which was visually inspected to ensure retention of the implant. Dorsal-caudal and lateral-medial 90° radiographs of each stifle joint were obtained immediately after surgery. At 12 weeks, rabbits were euthanized with pentobarbital after sedation. Post-surgical radiographic views were repeated following euthanasia.

**Gross Pathologic Observations of Joints:** The limbs and joints were examined and graded by a blinded observer (DDF) unaware of treatment group (Table 3.2). For the muscle wastage measurement, the limbs were shaved and a flexible tape measure was
used in a similar anatomic location of the stifle to make this assessment. This scoring system was chosen in order to compare to other studies performed by Frisbie et al.11

**Synovial Membrane Histology:** Synovial membrane was harvested and placed in neutral-buffered 10% formalin, embedded in paraffin, 5-µm sections created and stained with hematoxylin and eosin (H&E). Sections were evaluated blindly (DDF) for cellular infiltration, vascularity, intimal hyperplasia, subintimal edema and subintimal fibrosis on a scale of 0-4 (0=none, 1=slight, 2=mild, 3=moderate, 4=severe)10.

**Articular Cartilage Histology:** Femoral sections for histology were fixed in neutral-buffered 10% formalin, decalcified, embedded in paraffin, sectioned at 5 µm, and stained with either H&E or Safranin-O, fast green (SOFG) (ThermoScientific/Shandon VeriStain Gemini ES stainer) for proteoglycan visualization. Sections stained with H&E were evaluated blindly (DDF) using a modified O’Driscoll scoring system11, 39 (Table 3.3) with a maximum cumulative histology score of 28 and a higher score indicating a repair more like native cartilage. Sections stained with SOFG were evaluated blindly (DDF) for intensity of staining on a scale of 0-3 (Table 3.3) and were included in the cumulative histology score.

**Articular Cartilage Immunohistochemistry:** For immunohistochemical analyses, femoral sections were snap frozen in OCT using liquid nitrogen and sectioned at 8 µm. Each section was incubated in 0.25 U/mL chondroitinase ABC (Sigma-Aldrich) for 15 min before incubation in primary antibody solution (Collagen I (1:10) #M-38-s and II
(undiluted) # II-II6B3-s, Hybridoma Bank; Aggrecan (1:100, Alexis Biochemicals) #ALX-803-313; or rabbit IgG as control). Endogenous peroxidase was blocked using 0.3% H2O2 in methanol. Sections were incubated in goat anti-mouse HRP secondary antibody solution (1:500, Jackson Immunoresearch, Westgrove, PA)), stained with Vector Nova RED (Vector Laboratories, Burlingame, CA) and counterstained with Fast Green. Controls gave no signal. Non-calcified tissues were evaluated blindly (DDF) for the percentage of repair tissue stained positive (0=no stain, 1=1-25%, 2=26-50%, 3=51-75%, 4=76-100%).

**Radiographic Analysis:** Radiographs and photographs were taken of bone segments from femoral sections. Radiographs were graded for presence of lysis, bony proliferation, osteophyte presence, and patellar luxation, and a total radiographic score was calculated by summing these scores (maximum score of 16). All grading was done on a 0-4 scale (0=none, 1=slight, 2=mild, 3=moderate, 4=severe). Additionally, radiographs post-euthanasia were scored for healing of the defect (0=no healing, 1=slight healing, 2=mild bone filling, 3=lesion is visible but difficult to measure, 4=lesion not visible to measure), sclerosis around the defect, and other sclerosis (0-4, none-severe). Sclerosis is defined as the increase in density of bone seen radiographically and was subjectively scored by MFB in a blinded fashion.

**Statistical Analyses:** Scores were evaluated for inter- and intra-group differences using an ANOVA framework with PROC GLIMMIX (fits generalized linear mixed models) of SAS (Cary, NC) with rabbit as a random variable. For all joint pathologic, histological,
immunohistochemical, and radiographic analyses, intra-group differences were analyzed with treatment set as the main effect (one-way); differences among groups were analyzed using treatment and group as main effects (two-way). Untreated controls were therefore analyzed separately depending on what treatment the contralateral joint received in order to account for possible systemic effects\textsuperscript{18}. When main or interaction effects had p-values that were considered significant (p-value < 0.05) or a trend (0.05 < p-value < 0.10), individual comparisons were made using the least square means procedure\textsuperscript{12}. P-values presented in the text include which main effect or interaction term they are referring to if quoted for comparisons among groups. All data are presented as mean ± standard error of the mean (SEM). Non-parametric analyses were also performed when appropriate. The authors were able to reach similar conclusions based on either analysis. Because the authors feel many of the biologic outcome parameters represent more of a continuum rather than defined categories, the parametric analysis of the data is presented.

3.3 Results

**Radiographic Analyses:** Radiographic analyses pre-treatment did not show any sclerosis in any of the rabbits. Treated defects post-treatment showed some slight osteophyte formation (0.333±0.139, treated; 0.056±0.143, untreated) (treatment p=0.087). There was no difference among groups (group p=0.490).

**Gross Observation of Joints:** Upon necropsy, joints and incision areas in all experimental groups appeared normal by gross examination (Fig 3.1), and no inflammation or infection was noted, indicating no adverse immune reaction to the
treatments. Repair cartilage appeared to be moderately to normally attached to surrounding cartilage in all defects with no differences between treatments (treatment p=0.299) or groups (group p=0.541) (Table 3.4:XIII). Similarly, repair tissue ranged from normal to slightly soft compared to surrounding tissue when assessed for attachment to the subchondral bone, with no differences between treatments (treatment p=0.192) or groups (group p=0.970) (Table 3.4:XIV). Rabbits receiving KLD+CF+BMSCs (group 3) demonstrated mild osteophyte formation, with an increased score compared to KLD-alone (group 1) (group p=0.029) (Fig 3.2A). Treated defects in group 3 also had better attachment to subchondral bone compared to untreated defects in group 3 (p=0.030) (Table 3.4:XIV). Overall, treated defects had an increased defect volume filled (treatment p= 0.032) and the level of treated defects was higher than contralateral untreated defects (treatment p=0.050) (Fig 3.1; Fig 3.2B,C). Untreated defects also had more muscle wastage at the site of incision (assessed by circumferential measurement at the proximal aspect of the patella), with group 1 and 3 treated defects different from their respective controls (treatment p=0.001, interaction p=0.032) (Fig 3.2D). Treated defects were scored higher in grade of repair compared to contralateral untreated defects, equivalent to a score of good (3.040±0.219, treated vs. 2.476±0.219, untreated, on a scale of 0-4) (treatment p=0.061).

**Histologic Examinations:**

*Synovial Membrane H&E*
Evaluation of the synovial membrane by H&E staining showed some mild intimal hyperplasia and some mild to moderate vascularity and subintimal fibrosis, but there were no differences among any groups or treatments.

**Articular Cartilage H&E**

Assessment of the articular cartilage H&E staining showed that KLD (group 1) had a higher cumulative histology score compared to contralateral untreated controls when looking at that group alone (p=0.034) (Fig 3.3A). When comparing among groups and treatments, KLD and KLD+CF (groups 1 and 2) had higher cumulative scores than KLD+CF+BMSCs (group 3) (group p=0.030) (Fig 3.3A). Defects treated with KLD received the highest score for nature of predominant tissue in the defect (see Table 3.3:1 and Table 3.5:1), 1.00±0.276, or similar to fibrocartilage. The other groups and treatments ranged between 0 and 1, indicating presence of some fibrocartilage and some non-chondrocytic cells; none of the groups or treatments were significantly different, however (group p=0.749, treatment p=0.264). Group 3 had significantly lower surface regularity (Table 3.3: II) with scores indicative of some fissuring of the surface (group p=0.005, Fig 3.3B) and more degenerative change (Table 3.3: VIII) in cartilage surrounding the defect showing mild to moderate hypocellularity (group p=0.015) compared to KLD and KLD+CF (Fig 3.3C). Group 2 had more reconstitution of subchondral bone than group 3 (group p=0.030), but all groups were still below normal subchondral bone levels (Fig 3.3D; Table 3.3: IX). Group 2 also showed the highest score for bonding to adjacent cartilage (Table 3.5:V), but no groups or treatments were significantly different (treatment p=0.161, group p=0.226). Treated defects had increased cellularity (Table
3.3:VI) compared to contralateral untreated defects (treatment p=0.031) showing normal to slight hypocellularity in the treated defects repair tissue (1.778±0.110 vs. 1.556±0.110); group 3 trended higher than the other groups (2.00±0.164, group 3; 1.50±0.177, groups 1 and 2) (group p=0.081).

**Articular Cartilage SOFG:**
Defects treated with KLD-alone (group 1) had increased Safranin-O staining (scores of slight to moderate staining) than the contralateral untreated defects or treated defects in groups 2 and 3, which had only none to slight staining (interaction p=0.011) (Fig 3.4A). There was no overall effect of treatment or group on this measure (treatment p=0.286, group p=0.604). As shown in Fig 3.4B, treatment with KLD-alone resulted in greater staining throughout the repair tissue.

**Immunohistochemistry Evaluations:** Looking at only group 1, defects treated with KLD showed increased collagen II immunostaining compared to contralateral untreated defects (p=0.028); although aggrecan immunostaining for defects treated with KLD received a score of 2.7, it was not different from the contralateral untreated control (p=0.526) (Fig 3.5A,B). This is in contrast to the difference observed in Safranin-O staining between defects treated with KLD and the contralateral untreated defects. In group 2, KLD+CF treatment elicited a tissue with higher aggrecan detected by immunostaining vs. the contralateral untreated control (p=0.041). In addition, although defects treated with KLD+CF received the highest collagen II immunostaining score of all the groups, 3.4, this was not different from its contralateral untreated control
Comparing all defects, treated defects had more collagen II (treatment $p=0.001$) and increased aggrecan (treatment $p=0.056$) compared to untreated defects; there were no differences among groups (group $p=0.293$). There were similar levels of collagen I immunostaining found in all the defects (treatment $p=0.471$, group $p=0.919$), consistent with the observation of mostly fibrocartilage seen in the gross scoring (Fig 3.5C).

3.4 Discussion

Treatment of full-thickness articular cartilage defects with the self-assembling peptide KLD (group 1) markedly improved cartilage regeneration, as seen by significant increases in cumulative histology score, Safranin-O staining, and collagen II immunostaining, compared to critically sized contralateral untreated defects. KLD has several advantages as a material due to its ability to be injected arthroscopically into a defect, assemble on contact with tissue, and promote cartilage regeneration without inducing an immune response. Adding TGF-$\beta$1, dexamethasone, and IGF-1 to KLD (group 2) resulted in increased aggrecan immunostaining, but in general did not result in any additional beneficial or deleterious effects compared to KLD alone. Motivated by in vitro results supporting BMSC chondrogenesis$^{27-29}$, we delivered these factors with allogeneic BMSCs in KLD in vivo (group 3). However, this treatment resulted in a poorer repair than with KLD or KLD+CF. This is similar to other reports of fibrous tissue formation after BMSC treatment$^{35, 43, 48}$, interpreted as a negative result.
In our rabbit model, the presence of a full-thickness defect allowed bone marrow to infiltrate the defect\textsuperscript{41} and act as a cell source, similar to abrasion arthroplasty and subchondral bone microfracture surgical techniques. The positive healing response seen with KLD treatment (group 1) demonstrates that the scaffold supports cell migration and further chondrogenesis of these cells \textit{in vivo}. The high porosity of this scaffold (\textasciitilde 99.6\% water content\textsuperscript{26}) is in agreement with a recent \textit{in vivo} study in rabbits by Ikeda et al.\textsuperscript{21} demonstrating that scaffolds with >85\% porosity promoted migration of bone marrow cells into polymer scaffolds and with an \textit{in vitro} study by Wang et al.\textsuperscript{45} demonstrating endothelial cell migration into several types of self-assembling hydrogels.

Regarding group 2, KLD+CF treatment, our results raise the issues of whether the CF dose was appropriate to improve endogenous cell response and whether cells migrating into the scaffold \textit{in vivo} respond differently to these CFs than \textit{in vitro} and thereby require different stimulation. The inclusion of CFs did not show increased chondrogenesis over KLD alone (group 1) as determined by cumulative histology score, Safranin-O, aggrecan, and type II collagen immunostaining. Similar to these results, Holland et al.\textsuperscript{17} reported that TGF-\(\beta\)1 and IGF-1 delivered in gelatin microparticles within an acellular oligo(poly(ethylene glycol) fumarate) (OPF) scaffold resulted in repair no different than in empty defects, despite positive results \textit{in vitro}. That study also delivered IGF-1 alone and found that this growth factor alone resulted in a significantly improved repair. In addition, other studies have looked at delivering IGF-1 alone \textit{in vivo} and have reported similar positive results\textsuperscript{36,40}. While we did not test the effects of IGF-1 alone in this study,
our results emphasize the need for further understanding of the combination of IGF-1 with other CF in vivo in order to take advantage of its characteristic anabolic properties.

The intra-articular use of TGF-β1 remains controversial due to the pro-inflammatory response by the synovium seen at certain concentrations. Van der Kraan and van den Berg et al. have studied the interaction of TGF-β1 with various joint tissues and have shown that while TGF-β1 can stimulate proteoglycan production in cartilage, when it is exposed to synovial tissue, synovial fibrosis can occur; TGF-β1-induced osteophyte formation is also common. Mi et al. showed that injection of recombinant adenoviral vector for hTGF-β1 into the knee joint space through the patellar tendon (resulting in ~8.75 ng TGF-β1) dramatically increased joint inflammation, though this dose is more than 6-fold higher than our total dose of 1.4 ng. Accordingly, we did not observe these effects when TGF-β1 was added to the peptide alone (group 2). In contrast, when TGF-β1 was added in combination with BMSCs (group 3), osteophyte formation increased, suggesting an interaction of TGF-β1 with these cells was responsible for the increased osteogenesis rather than the presence of TGF-β1 alone in the joint (group 2). This finding is similar to results recently published by Guo et al., in which 600 ng of TGF-β1 per mL of hydrogel was used in combination with BMSCs in an OPF scaffold. Furthermore, we observed a detrimental increase in osteophyte formation and distortion of normal joint anatomy when, in the same rabbit, 1.4 ng TGF-β1+BMSCs were placed in one joint and 0.7 ng TGF-β1+BMSCs in the contralateral joint. The authors were surprised at this result given the total body dose of TGF-β1 was only 1.5x what was used in group 2 or group 3 and the cell numbers 2-fold greater. While the amount of TGF-β1 delivered in
group 3 of our study was lower than that in the Mi\textsuperscript{32} study, and we attempted to deliver TGF-β1 in a controlled fashion, it is still possible that this TGF-β1 level was too high to beneficially induce chondrogenesis when combined with BMSCs, despite the inclusion of dexamethasone, which has been shown to enhance chondrogenesis compared to TGF-β1 alone\textsuperscript{25, 34}. A study by Fan et al. using only 0.8 ng of TGF-β1 with BMSCs in a gelatin-chondroitin-hyaluronate tri-copolymer scaffold reported improved rabbit cartilage defect healing compared to treatment with BMSCs implanted in a scaffold without TGF-β1. Taken together, these studies suggest that while the amount of TGF-β1 we chose combined with BMSCs effectively induces chondrogenesis in vitro\textsuperscript{28}, additional research is needed to determine a successful strategy for optimizing chondrogenesis of BMSCs in vivo, and interactions with synovial tissues must be considered.

Due to the often noted ability of cartilage defects in young/skeletally immature rabbits to heal well naturally\textsuperscript{41}, we used skeletally mature rabbits with average age of 11 months and critically sized defects in order to test the ability of treatment to improve adult animal healing. Although full-thickness defects were used, defects only entered the subchondral/trabecular bone, resulting in contralateral untreated defects that do not heal spontaneously by 12 weeks. These results are similar to other studies using similar-aged rabbits and defect sizes. A limitation of our model is the lack of enough tissue to perform direct biochemical assessment in addition to histological and immunohistochemical measurements in this study. However, the purpose of the present study was to perform an initial trial prior to a larger animal study where such additional measures will be utilized, since ample tissue will be available.
In summary, the self-assembling peptide hydrogel KLD offers a new material suitable for further testing in a clinically relevant defect in a large animal. We demonstrated improved filling of osteochondral defects and improved cartilage repair, as seen by cumulative histology score, Safranin-O staining, and type II collagen immunostaining. In this small animal model, the full-thickness defect provided access to the marrow, similar in concept to abrasion arthroplasty or spongialization in large animal models (goat, sheep, horse, human), and suggests that combining KLD with these techniques may offer an improvement over current practice. Ongoing studies include the evaluation of KLD in a clinically relevant sized equine defect co-treated with microfracture and subjected to strenuous exercise, compared to defects treated with microfracture alone.

3.5 Acknowledgements

I would like to thank Alan J. Grodzinsky, Sc.D. and Eric J. Vanderploeg, Ph.D. for their helpful discussions in planning and analyzing this study. I would like to thank my collaborators at Colorado State University for their animal model expertise: Christina Lee, Ph.D., Dora J. Ferris, DVM, Myra F. Barrett, DVM, John D. Kisiday, Ph.D., and David D. Frisbie, DVM, Ph.D., Diplomate ACVS. Finally, this work would not be possible without the following funding sources: NIH-NIBIB Grant EB003805; NDSEG and NSF graduate fellowships.
3.6 References


3.7 Tables

**Table 3.1.** Treatment groups with amounts delivered per animal.

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>KLD (µg)</th>
<th>bKLD (µg)</th>
<th>TGF-β1 (ng)</th>
<th>bIGF (ng)</th>
<th>Streptavidin (ng)</th>
<th>Dex (ng)</th>
<th>BMSCs (x10³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (KLD)</td>
<td>6</td>
<td>48</td>
<td>0.48</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2 (KLD+CF)</td>
<td>6</td>
<td>48</td>
<td>0.48</td>
<td>1.4</td>
<td>4.1</td>
<td>30.7</td>
<td>0.6</td>
<td>0</td>
</tr>
<tr>
<td>3 (KLD+CF+BMSCs)</td>
<td>7</td>
<td>48</td>
<td>0.48</td>
<td>1.4</td>
<td>4.1</td>
<td>30.7</td>
<td>0.6</td>
<td>150</td>
</tr>
</tbody>
</table>
### Table 3.2. Gross Observations Scoring System.

<table>
<thead>
<tr>
<th>Feature</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>I. Joint Observation</strong></td>
<td></td>
</tr>
<tr>
<td>Normal appearance</td>
<td>0</td>
</tr>
<tr>
<td>Slight inflammation</td>
<td>1</td>
</tr>
<tr>
<td>Moderate inflammation</td>
<td>2</td>
</tr>
<tr>
<td>Severe inflammation</td>
<td>3</td>
</tr>
<tr>
<td><strong>II. Incision Appearance</strong></td>
<td></td>
</tr>
<tr>
<td>Normal appearance</td>
<td>0</td>
</tr>
<tr>
<td>Slight inflammation</td>
<td>1</td>
</tr>
<tr>
<td>Moderate inflammation</td>
<td>2</td>
</tr>
<tr>
<td>Severe inflammation</td>
<td>3</td>
</tr>
<tr>
<td>Slight dehiscence (incision basically intact)</td>
<td>4</td>
</tr>
<tr>
<td>Marked dehiscence (requires intervention)</td>
<td>5</td>
</tr>
<tr>
<td>Active infection</td>
<td>6</td>
</tr>
<tr>
<td>Healing infections</td>
<td>7</td>
</tr>
<tr>
<td><strong>III. Muscle wastage</strong></td>
<td></td>
</tr>
<tr>
<td>Circumferential measurement at the proximal aspect of the patella (cm)</td>
<td></td>
</tr>
<tr>
<td><strong>IV. Angle of Stifle: Change in normal angle (if any)</strong></td>
<td></td>
</tr>
<tr>
<td>None, within normal limits</td>
<td>0</td>
</tr>
<tr>
<td>Abnormal</td>
<td>1</td>
</tr>
<tr>
<td><strong>V. Inflammation/Swelling</strong></td>
<td></td>
</tr>
<tr>
<td>None, within normal limits</td>
<td>0</td>
</tr>
<tr>
<td>Abnormal</td>
<td>1</td>
</tr>
<tr>
<td><strong>VI. Trauma/Damage</strong></td>
<td></td>
</tr>
<tr>
<td>None, within normal limits</td>
<td>0</td>
</tr>
<tr>
<td>Abnormal</td>
<td>1</td>
</tr>
<tr>
<td><strong>VII. Infection/Discharge</strong></td>
<td></td>
</tr>
<tr>
<td>None, within normal limits</td>
<td>0</td>
</tr>
<tr>
<td>Abnormal</td>
<td>1</td>
</tr>
<tr>
<td><strong>VIII. Presence of Osteophytes and other Osteoarthritic Changes</strong></td>
<td></td>
</tr>
<tr>
<td>None, within normal limits</td>
<td>0</td>
</tr>
<tr>
<td>Abnormal</td>
<td>1</td>
</tr>
<tr>
<td><strong>IX. Articular Surface Integrity, Contour, and Congruity</strong></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>0</td>
</tr>
<tr>
<td>Abnormal</td>
<td>1</td>
</tr>
<tr>
<td><strong>X. Presence of Loose Bodies in Synovial Fluid</strong></td>
<td></td>
</tr>
<tr>
<td>None, within normal limits</td>
<td>0</td>
</tr>
<tr>
<td>Abnormal</td>
<td>1</td>
</tr>
<tr>
<td><strong>XI. Injury to Apposing Articular Surface</strong></td>
<td></td>
</tr>
<tr>
<td>None, within normal limits</td>
<td>0</td>
</tr>
<tr>
<td>Abnormal</td>
<td>1</td>
</tr>
<tr>
<td><strong>XII. Synovial Membrane Appearance</strong></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>0</td>
</tr>
<tr>
<td>Slight</td>
<td>1</td>
</tr>
<tr>
<td>Mild</td>
<td>2</td>
</tr>
<tr>
<td>Moderate</td>
<td>3</td>
</tr>
</tbody>
</table>
**XIII. Cartilage Attachment**
This category describes on average the defect repair tissue attachment with the surrounding normal cartilage. Possible responses were:
- Normal Attachment: 0
- Moderate Attachment: 1
- Mild Attachment: 2
- Slight Attachment: 3
- No Attachment: 4

**XIV. Bone Attachment**
This category describes the firmness of the repair tissue attachment to the bone at the base of the defect. Possible responses were:
- Similar to Surrounding Cartilage: 0
- Slightly Soft vs. Surrounding Cartilage: 1
- Mildly Soft vs. Surrounding Cartilage: 2
- Moderately Soft vs. Surrounding Cartilage: 3
- Marked Softening vs. Surrounding Cartilage: 4

**XV. Firmness**
This category describes on average the firmness of the repair tissue to normal surrounding articular cartilage. Possible responses were:
- Similar Compared to Surrounding Cartilage: 0
- Slightly Soft Compared to Surrounding Cartilage: 1
- Mildly Soft Compared to Surrounding Cartilage: 2
- Moderately Soft Compared to Surrounding Cartilage: 3
- Marked Softening Compared to Surrounding Cartilage: 4

**XVI. Blood**
This category describes the presence or absence of hemorrhage associated with the defect area or its periphery. Possible responses were:
- Fresh Blood, Active Hemorrhage at Time of Surgery: 1
- Old Blood, No Active Hemorrhage at Time of Surgery: 2
- No Blood Visualized at Time of Surgery: 3

**XVII. Shape**
This category describes the margin of the defect as it relates to the original geometry at time 0 (creation). Possible responses were:
- No Apparent Change in Damage Tissue Beyond Defect: 0
- Degeneration in Tissue Beyond Defect: 1

**XVIII. Grade (Overall quality of repair)**
This category describes the overall subjective evaluation of the repair tissue by the evaluator. Criteria used to determine the grade were:
1. Attachment of repair tissue to the surrounding normal articular cartilage
2. Level (height) and undulation of the repair tissue surface as compared to the surrounding normal articular cartilage
3. Color of the repair tissue, where white homogenous tissue without a fibrous like appearance is used as the “gold standard.”
Possible responses were:
- Tissue Not Present to Grade: 0
- Poor: 1
- Fair: 2
XIX. Level
This category describes the level of repair tissue filling in association with the surrounding normal articular cartilage.
Possible responses in relation to the surrounding normal articular cartilage were:
- Mildly Recessed
- Slightly Recessed
- Leveled
- Slightly Elevated
- Mildly Elevated
- Moderately Elevated

XX. Color
This category describes the color of the repair tissue. When repair tissue is characterized by two colors, the predominate color is indicated first. Possible responses were:
- Red
- White/Red
- Yellow
- Yellow/White
- White/Yellow
- White

XXI. Surface
This category describes the relative undulation of the repair tissue surface. Possible responses were:
- Non-Undulating
- Slightly Undulating
- Mildly Undulating
- Moderately Undulating

XXII. Area
Percent of Surface Area Filled in Defect (0-100%)

XXIII. Volume
Percent of Volume Filled in Defect (0-100%)
## Table 3.3. Modified O’Driscoll Histological Scoring System

<table>
<thead>
<tr>
<th>Feature</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>I. Nature of the Repair Tissue</strong></td>
<td></td>
</tr>
<tr>
<td>Some Fibrocartilage, mostly nonchondrocytic cells</td>
<td>0</td>
</tr>
<tr>
<td>Mostly Fibrocartilage</td>
<td>1</td>
</tr>
<tr>
<td>Mixed Hyaline and Fibrocartilage</td>
<td>2</td>
</tr>
<tr>
<td>Mostly Hyaline Cartilage</td>
<td>3</td>
</tr>
<tr>
<td>Hyaline Cartilage</td>
<td>4</td>
</tr>
<tr>
<td><strong>II. Surface Regularity</strong></td>
<td></td>
</tr>
<tr>
<td>Severe Disruption, Including Fibrillation</td>
<td>0</td>
</tr>
<tr>
<td>Fissures</td>
<td>1</td>
</tr>
<tr>
<td>Superficial Horizontal Lamination</td>
<td>2</td>
</tr>
<tr>
<td>Smooth and Intact</td>
<td>3</td>
</tr>
<tr>
<td><strong>III. Structural Integrity</strong> (morphologic zone reconstitution)</td>
<td></td>
</tr>
<tr>
<td>Severe Disintegration</td>
<td>0</td>
</tr>
<tr>
<td>Slight Disruption, Including Cysts</td>
<td>1</td>
</tr>
<tr>
<td>Normal</td>
<td>2</td>
</tr>
<tr>
<td><strong>IV. Thickness</strong></td>
<td></td>
</tr>
<tr>
<td>0% - 50% of Normal Cartilage</td>
<td>0</td>
</tr>
<tr>
<td>50% - 100% of Normal Cartilage</td>
<td>1</td>
</tr>
<tr>
<td>100% of Normal Adjacent Cartilage</td>
<td>2</td>
</tr>
<tr>
<td><strong>V. Bonding to Adjacent Cartilage</strong></td>
<td></td>
</tr>
<tr>
<td>Not Bonded</td>
<td>0</td>
</tr>
<tr>
<td>Bonded at One End or Partially at Both Ends</td>
<td>1</td>
</tr>
<tr>
<td>Bonded at Both Ends of Graft</td>
<td>2</td>
</tr>
<tr>
<td><strong>VI. Hypocellularity</strong></td>
<td></td>
</tr>
<tr>
<td>Moderate Hypocellularity</td>
<td>0</td>
</tr>
<tr>
<td>Slight Hypocellularity</td>
<td>1</td>
</tr>
<tr>
<td>Normal Cellularity</td>
<td>2</td>
</tr>
<tr>
<td><strong>VII. Chondrocyte Clustering</strong></td>
<td></td>
</tr>
<tr>
<td>25% - 100% of the Cells</td>
<td>0</td>
</tr>
<tr>
<td>&lt; 25% of the Cells</td>
<td>1</td>
</tr>
<tr>
<td>No Clusters</td>
<td>2</td>
</tr>
<tr>
<td><strong>VIII. Freedom from Degenerative Changes in Adjacent Cartilage</strong></td>
<td></td>
</tr>
<tr>
<td>Severe Hypocellularity, Poor or No Staining</td>
<td>0</td>
</tr>
<tr>
<td>Mild or Moderate Hypocellularity, Slight Staining</td>
<td>1</td>
</tr>
<tr>
<td>Normal Cellularity, Mild Clusters, Moderate Staining</td>
<td>2</td>
</tr>
<tr>
<td>Normal Cellularity, No Clusters, Normal Staining</td>
<td>3</td>
</tr>
<tr>
<td><strong>IX. Reconstitution of Subchondral Bone</strong></td>
<td></td>
</tr>
<tr>
<td>No Subchondral Bone Reconstitution</td>
<td>0</td>
</tr>
<tr>
<td>Minimal Subchondral Bone Reconstitution</td>
<td>1</td>
</tr>
<tr>
<td>Reduced Subchondral Bone Reconstitution</td>
<td>2</td>
</tr>
<tr>
<td>Normal</td>
<td>3</td>
</tr>
<tr>
<td><strong>X. Inflammatory Response in Subchondral Bone Region</strong></td>
<td></td>
</tr>
<tr>
<td>Severe</td>
<td>0</td>
</tr>
<tr>
<td>Moderate</td>
<td>1</td>
</tr>
<tr>
<td>None / Mild</td>
<td>2</td>
</tr>
<tr>
<td><strong>XI. Safranin-O Staining</strong></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>Slight</td>
<td>1</td>
</tr>
<tr>
<td>--------</td>
<td>---</td>
</tr>
<tr>
<td>Moderate</td>
<td>2</td>
</tr>
<tr>
<td>Normal</td>
<td>3</td>
</tr>
<tr>
<td><strong>XII. Cumulative Histology Score (sum of above scores)</strong></td>
<td><strong>0-28</strong></td>
</tr>
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</table>
Table 3.4. Gross Scores. One-way p-values for within group comparisons are listed in the untreated columns. Two-way p-values for differences among groups are in a separate column (TXT = treatment, Group = group, TXT*Group = interaction term). P-values are not shown when all the scores being compared are zero.

<table>
<thead>
<tr>
<th>Gross Scores</th>
<th>KLD</th>
<th>KLD+CF</th>
<th>KLD+CF+BMSCs</th>
<th>Two-way P-values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Treated</td>
<td>untreated</td>
<td>treated</td>
<td>untreated</td>
</tr>
<tr>
<td>I. Joint Observation</td>
<td>0±0</td>
<td>0±0</td>
<td>0±0</td>
<td>0±0</td>
</tr>
<tr>
<td>II. Incision Appearance</td>
<td>0±0</td>
<td>0±0</td>
<td>0±0</td>
<td>0±0</td>
</tr>
<tr>
<td>III. Muscle wastage</td>
<td>12.8±0.3</td>
<td>12.4±0.3, p=0.010</td>
<td>12.8±0.3, p=0.530</td>
<td>13.0±0.3, p=0.049</td>
</tr>
<tr>
<td>IV. Angle of Stifle: Change in normal angle (if any)</td>
<td>0±0</td>
<td>0±0</td>
<td>0±0</td>
<td>0±0</td>
</tr>
<tr>
<td>V. Inflammation/ Swelling</td>
<td>0±0</td>
<td>0±0</td>
<td>0±0</td>
<td>0±0</td>
</tr>
<tr>
<td>VI. Trauma/Damage</td>
<td>0±0</td>
<td>0±0</td>
<td>0±0</td>
<td>0±0</td>
</tr>
<tr>
<td>VII. Infection/Discharge</td>
<td>0±0</td>
<td>0±0</td>
<td>0±0</td>
<td>0±0</td>
</tr>
<tr>
<td>VIII. Osteophytes and other Osteoarthritic Changes</td>
<td>0±0</td>
<td>0±0</td>
<td>0±0</td>
<td>0±0</td>
</tr>
<tr>
<td>IX. Articular Surface Integrity, Contour, and Congruity</td>
<td>0±0</td>
<td>0±0</td>
<td>0±0</td>
<td>0±0</td>
</tr>
<tr>
<td>X. Presence of Loose Bodies in Synovial Fluid</td>
<td>0±0</td>
<td>0±0</td>
<td>0±0</td>
<td>0±0</td>
</tr>
<tr>
<td>XI. Injury to Opposing Articular Surface</td>
<td>0±0</td>
<td>0±0</td>
<td>0±0</td>
<td>0±0</td>
</tr>
<tr>
<td>XII. Synovial Membrane</td>
<td>0±0</td>
<td>0±0</td>
<td>0±0</td>
<td>0±0</td>
</tr>
<tr>
<td>XIII. Cartilage Attachment to surrounding cartilage</td>
<td>0±0</td>
<td>0±0</td>
<td>0±0</td>
<td>0±0</td>
</tr>
<tr>
<td>XIV. Bone Attachment</td>
<td>0±0</td>
<td>0±0</td>
<td>0±0</td>
<td>0±0</td>
</tr>
<tr>
<td>XV. Firmness</td>
<td>0±0</td>
<td>0±0</td>
<td>0±0</td>
<td>0±0</td>
</tr>
<tr>
<td>XVI. Blood</td>
<td>3.0±0.1</td>
<td>3.0±0.1, p=1.000</td>
<td>3.0±0.1, p=1.000</td>
<td>3.0±0.1, p=1.000</td>
</tr>
<tr>
<td>XVII. Shape</td>
<td>0±0</td>
<td>0±0</td>
<td>0±0</td>
<td>0±0</td>
</tr>
<tr>
<td>XVIII. Grade (Overall quality of repair)</td>
<td>3.2±0.4</td>
<td>2.2±0.4, p=0.076</td>
<td>2.7±0.4</td>
<td>2.8±0.4, p=0.818</td>
</tr>
<tr>
<td>XIX. Level</td>
<td>2.7±0.3</td>
<td>2.0±0.3, p=0.102</td>
<td>2.5±0.3</td>
<td>2.3±0.3, p=0.695</td>
</tr>
<tr>
<td>XX. Color</td>
<td>5.8±0.5</td>
<td>4.7±0.5, p=0.126</td>
<td>4.8±0.5</td>
<td>4.8±0.5, p=1.000</td>
</tr>
<tr>
<td>XXI. Surface</td>
<td>1.8±0.3</td>
<td>2.2±0.3, p=0.465</td>
<td>1.5±0.3</td>
<td>2.0±0.3, p=0.296</td>
</tr>
<tr>
<td>-------------</td>
<td>---------</td>
<td>------------------</td>
<td>---------</td>
<td>------------------</td>
</tr>
<tr>
<td>XXII. Area</td>
<td>94.2±6.6</td>
<td>90.0±6.6, p=0.383</td>
<td>95.7±6.6</td>
<td>90.0±6.6, p=0.441</td>
</tr>
<tr>
<td>XXIII. Volume</td>
<td>91.7±7.6</td>
<td>71.7±7.6, p=0.058</td>
<td>88.2±7.6</td>
<td>80.8±7.6, p=0.477</td>
</tr>
</tbody>
</table>
Table 3.5. Histological Scores. One-way p-values for within group comparisons are listed in the untreated columns. Two-way p-values for differences among groups are in a separate column.

<table>
<thead>
<tr>
<th>Feature Scores</th>
<th>KLD</th>
<th>KLD+CF</th>
<th>KLD+CF+BMSCs</th>
<th>Two-way p-values</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Nature of the Repair Tissue</td>
<td>1.0±0.3</td>
<td>0.7±0.3,</td>
<td>0.7±0.3,</td>
<td>TXT: 0.264</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p=0.363</td>
<td>p=1.000</td>
<td>Group: 0.749</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>TXT*Group: 0.710</td>
</tr>
<tr>
<td>II. Surface Regularity</td>
<td>2.5±0.3</td>
<td>2.2±0.3,</td>
<td>2.0±0.3</td>
<td>TXT: 0.130</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p=0.175</td>
<td>p=0.465</td>
<td>Group: 0.005</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>TXT*Group: 0.906</td>
</tr>
<tr>
<td>III. Structural Integrity</td>
<td>1.3±0.2</td>
<td>1.0±0.2</td>
<td>1.2±0.2</td>
<td>TXT: 0.284</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Group: 0.118</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>TXT*Group: 0.655</td>
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<tr>
<td>IV. Thickness</td>
<td>1.7±0.2</td>
<td>1.2±0.2,</td>
<td>1.2±0.2</td>
<td>TXT: 0.583</td>
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<tr>
<td></td>
<td></td>
<td>p=0.237</td>
<td>p=1.000</td>
<td>Group: 0.478</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>TXT*Group: 0.396</td>
</tr>
<tr>
<td>V. Bonding to Adjacent Cartilage</td>
<td>1.2±0.3</td>
<td>0.9±0.3,</td>
<td>0.6±0.3</td>
<td>TXT: 0.0161</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p=0.363</td>
<td>p=1.000</td>
<td>Group: 0.226</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>TXT*Group: 0.580</td>
</tr>
<tr>
<td>VI. Hypocellularity</td>
<td>1.7±0.2</td>
<td>1.3±0.2,</td>
<td>1.3±0.2</td>
<td>TXT: 0.031</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p=0.175</td>
<td>p=1.000</td>
<td>Group: 0.081</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>TXT*Group: 0.259</td>
</tr>
<tr>
<td>VII. Chondrocyte Clustering</td>
<td>1.7±0.2</td>
<td>1.3±0.2,</td>
<td>1.3±0.2</td>
<td>TXT: 0.364</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p=0.175</td>
<td>p=1.000</td>
<td>Group: 0.397</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>TXT*Group: 0.809</td>
</tr>
<tr>
<td>VIII. Freedom from Degenerative Changes in Adjacent Cartilage</td>
<td>1.7±0.2</td>
<td>1.8±0.2,</td>
<td>1.2±0.2</td>
<td>TXT: 0.658</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p=0.363</td>
<td>p=0.175</td>
<td>Group: 0.015</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>TXT*Group: 0.720</td>
</tr>
<tr>
<td>IX. Reconstitution of Subchondral Bone</td>
<td>1.8±0.3</td>
<td>2.2±0.3,</td>
<td>2.2±0.3</td>
<td>TXT: 0.074</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p=0.363</td>
<td>p=0.363</td>
<td>Group: 0.030</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>TXT*Group: 0.556</td>
</tr>
<tr>
<td>X. Inflammatory Response in Subchondral Bone Region</td>
<td>2.0±0.1</td>
<td>2.0±0.1,</td>
<td>2.0±0.1</td>
<td>TXT: 0.036</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p=0.363</td>
<td>p=1.000</td>
<td>Group: 0.358</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>TXT*Group: 0.358</td>
</tr>
<tr>
<td>XI. Safranin-O Staining</td>
<td>1.2±0.2</td>
<td>0.3±0.2,</td>
<td>0.3±0.2</td>
<td>TXT: 0.286</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p=0.012</td>
<td>p=0.337</td>
<td>Group: 0.011</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>TXT*Group: 0.541</td>
</tr>
<tr>
<td>XII. Cumulative Histology Score (sum of above scores)</td>
<td>17.2±1.1</td>
<td>14.5±1.1,</td>
<td>16.2±1.1,</td>
<td>TXT: 0.140</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p=0.034</td>
<td>p=0.915</td>
<td>Group: 0.030</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>TXT*Group: 0.541</td>
</tr>
</tbody>
</table>
Figure 3.1. Gross necropsy photographs of treated and untreated joints in KLD, KLD+CF, and KLD+CF+BMSCs.
Figure 3.2. Gross pathologic observation of joints comparing KLD, KLD+CF, and KLD+CF+BMSCs treated and contralateral untreated defects. * p<0.05, † p=0.0292.

(A) Gross osteophytes (scored as 0-1, where 0 = normal, 1 = abnormal); (B) Volume % Filled (scored as 0-100%); (C) Level of treated defects (scored as 1-6, where <3 is recessed, 3 is level with surrounding cartilage, and >3 is elevated); (D) Muscle wastage (circumference proximal to patella).
Figure 3.3. Histological scores comparing KLD, KLD+CF, and KLD+CF+BMSCs treated and untreated defects. Higher scores indicate more similar to native cartilage. (A) Cumulative histology (scored as 0-28); (B) Surface regularity (scored as 0-3); (C) Freedom from degenerative change of cartilage surrounding defect (scored as 0-3); (D) Reconstitution of subchondral bone (scored as 0-3); * p<0.05.
Figure 3.4. Safranin-O staining. (A) Scored as 0-3; * p<0.05. (B) Images showing histological evaluation of representative treatment groups and representative contralateral control knees. Treatment and contralateral pictures were representative of the mean scores and were taken from different animals for KLD and KLD+CF+BMSCs, and from the same animal for KLD+CF. Scale bar = 1 mm.
Figure 3.5. Immunohistochemistry scores (0-4). A) Aggrecan. B) Collagen II. C) Collagen I. * p<0.05.
Chapter 4. Intra-articular Injection of HB-IGF-1 Sustains Delivery of IGF-1 to Cartilage through Binding to Chondroitin Sulfate

IGF-1 stimulates cartilage repair but is not a practical therapy due to its short half-life. We have previously modified IGF-1 by adding a heparin-binding domain and have shown that this fusion protein (HB-IGF-1) stimulates sustained proteoglycan synthesis in cartilage. Here, we first examined the mechanism by which HB-IGF-1 is retained in cartilage. We then tested whether HB-IGF-1 provides sustained growth factor delivery to cartilage in vivo and to human cartilage explants. Retention of HB-IGF-1 and IGF-1 was analyzed by Western blotting. The requirement of heparan sulfate (HS) or chondroitin sulfate (CS) glycosaminoglycans for binding was tested using enzymatic removal and cells with genetic deficiency of HS. Binding affinities of HB-IGF-1 and IGF-1 proteins for isolated glycosaminoglycans were examined by surface plasmon resonance and ELISA. In cartilage explants, chondroitinase treatment decreased binding of HB-IGF-1, whereas heparitinase had no effect. Furthermore, HS was not necessary for HB-IGF-1 retention on cell monolayers. Binding assays showed that HB-IGF-1 bound both CS and HS, whereas IGF-1 did not bind either. After intra-articular injection in rat knees, HB-IGF-1 was retained in articular and meniscal cartilages, but not in tendon, consistent with enhanced delivery to CS-rich cartilage. Finally, HB-IGF-1 but not IGF-1 was retained in human cartilage explants. After intra-articular injection in rats, HB-IGF-1 is specifically retained in cartilage through its high abundance of CS. Modification of growth factors with heparin-binding domains may be a new strategy for sustained and specific local delivery to cartilage.
4.1 Introduction

Insulin-like growth factor-I (IGF-1) is known to be an important anabolic factor in cartilage homeostasis\textsuperscript{14}. IGF-1 not only promotes synthesis of aggrecan, link protein, and hyaluronan\textsuperscript{3,6,34}, it also inhibits proteoglycan degradation\textsuperscript{19,29,47}. IGF-1 is primarily produced by the liver and reaches cartilage through the synovial fluid\textsuperscript{33,42,43}, acting on chondrocytes through both autocrine and paracrine mechanisms\textsuperscript{27,36}. In multiple animal models of cartilage injury, viral delivery of IGF-1 has been successfully used to enhance cartilage repair\textsuperscript{15,30}.

While IGF-1 may therefore be a potential therapeutic for cartilage repair, a clinically useful technique for non-viral IGF-1 delivery to cartilage has yet to be developed. A successful IGF-1 delivery strategy must overcome two major obstacles. First, IGF-1 has a short half-life of 8-16 hours \textit{in vivo} when delivered systemically\textsuperscript{23}. Second, systemic delivery of IGF-1 must be minimized since long-term excess circulating IGF-1 has been linked to increased risk for cancer\textsuperscript{5} and high-dose systemic IGF-1 administration causes significant adverse events\textsuperscript{21}. Studies delivering IGF-1 directly to the joint through fibrin constructs\textsuperscript{11,20,37} have been promising, but rapid clearance of IGF-1 from the joint has prevented intra-articular injections of IGF-1 without a carrier from being effective\textsuperscript{42}, and has been a limiting factor in delivery methods proposed to date.

We have focused on the family of heparin-binding growth factors as a model for sequestration and sustained local delivery of growth factors to cartilage. Basic fibroblast growth factor (bFGF or FGF-2), vascular endothelial growth factor (VEGF), heparin-
Motivated by these considerations, we have designed a new strategy for local delivery of IGF-1 in various tissues: we added the heparin-binding domain of HB-EGF to the amino-terminus of IGF-1 to create a new heparin-binding IGF-1 fusion protein, HB-IGF-1. We have previously shown that HB-IGF-1 produces long-term delivery of bioavailable IGF-1 to bovine cartilage explants and a single dose stimulates a sustained increase in proteoglycan synthesis compared to IGF-1. However, the mechanism by which HB-IGF-1 is retained in tissues is not yet clear. Heparin-binding domains are all highly positively charged but the rigidity of their secondary structure varies, leading to different specificities for binding to heparan sulfate as opposed to other negatively charged sulfated glycosaminoglycans. Cartilage extracellular matrix (ECM) contains...
primarily chondroitin sulfate (CS), while the pericellular matrix is rich in heparan sulfate (HS)\textsuperscript{9,50}.

We hypothesized that HB-IGF-1 is retained in cartilage by binding heparan sulfate proteoglycans in the matrix and at the cell surface. In the present study, we tested this hypothesis by measuring release of bound HB-IGF-1 following chondroitinase or heparitinase treatment of cartilage explants, binding of HB-IGF-1 to cells unable to produce heparan sulfate, and the binding affinities of HB-IGF-1 for isolated heparan sulfate and chondroitin sulfate. Surprisingly, we found that HB-IGF-1 was retained primarily by binding to chondroitin sulfate, whereas heparan sulfate was not required. This result led us to test whether intra-articular injection of HB-IGF-1 allows sustained \textit{in vivo} delivery preferentially to CS-rich rat knee cartilage and whether HB-IGF-1 can bind adult human cartilage.

\textbf{4.2 Materials and Methods}

\textit{Protein Production:} HB-IGF-1 and IGF-1 were expressed in \textit{E. coli} as Xpress and hexahistidine tagged proteins and were purified by Ni-NTA affinity followed by reverse-phase chromatography, as previously described in detail\textsuperscript{46}.

\textit{Binding in Bovine Cartilage with GAG-ase Treatments:} Cartilage disks (3 mm diameter, 0.5 mm thick) from calf femoropatellar grooves were cultured in serum-free low glucose-DMEM with 500 nM HB-IGF-1 or IGF-1 for 2 days. At Day 2, disks were washed with PBS and treated with either no enzyme, chondroitinase ABC (E.C. 4.2.2.4, Associates of
Cape Cod, Inc., East Falmouth, MA) (0.4 U/mL), or heparitinase (4:1 mixture of heparitinase I and II, E.C. 4.2.2.8, Associates of Cape Cod, Inc.) (0.036 U/mL). These enzymes have been shown to be specific for chondroitin sulfate\textsuperscript{38} and heparan sulfate\textsuperscript{44}, respectively. At Day 4, half of the chondroitinase-treated disks were treated with heparitinase (0.036 U/mL); all other disks were incubated in enzyme-free medium (n=4). On Day 6, disks were flash frozen and protein was extracted by pulverization and by incubation with 100 mM NaCl, 50 mM Tris, 0.5% TritonX-100, pH 7.0 with protease inhibitor cocktail (Roche, Basel, Switzerland) rotating at 4 °C overnight. Protein was quantified by BCA assay (Thermo Fisher Scientific Inc., Rockford, IL) and equal amounts of protein were loaded on a 4-12% SDS-PAGE gel and analyzed by Western blot with anti-IGF-1 (1:500, Abcam Inc., Cambridge, MA, recognizes both HB-IGF-1 and IGF-1\textsuperscript{46}). 5 ng of recombinant HB-IGF-1 was loaded as a protein standard and equal protein of an explant incubated with 500 nM IGF-1 for 2 days without enzyme treatment or washing was loaded as a positive control. HB-IGF-1 released to the medium was analyzed by ELISA as previously described\textsuperscript{46}. Heparitinase activity was confirmed by assaying conditioned medium using an anti-HS-stub antibody 3G10 (1:500, Associates of Cape Cod, Inc.) by Western blot. This antibody has been shown previously to only detect neoepitopes generated by heparitinase cleavage and not by chondroitinase\textsuperscript{7}. Chondroitinase activity was confirmed by assaying for GAG loss in treated explants using the DMMB dye binding assay, which showed that >75% sGAG was removed after 48 h.
Binding to Chinese hamster ovary (CHO) cell surfaces: Mutant CHO cells unable to produce heparan sulfate (strain pgsD-677)\(^{26}\) and wildtype CHO (K1) cells were cultured in F12 medium supplemented with 10% FBS. At confluence, cells were washed with PBS and incubated in serum-free medium with 100 nM HB-IGF-1 or IGF-1. After 3 hours, cells were washed 3x10 min with PBS and lysed with 50 mM Tris/HCl, 150 mM NaCl, 10 mM EDTA, 1% Triton X-100, 1% Igepal CA-630 (Sigma-Aldrich, St. Louis, MO), 2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor cocktail. Protein was quantified by BCA assay, and equal amounts of protein were analyzed by Western blot using anti-IGF-1 as described above. 5 ng of recombinant IGF-1 was loaded as a control.

Biotinylation of Glycosaminoglycans (GAGs): Heparan sulfate (HS) (bovine kidney, Sigma, #H7640, 0.88 sulfates/disaccharide\(^{25}\)) and chondroitin sulfate C (CS) (CS-C from shark cartilage, Sigma, #C4384, 0.99 sulfates/disaccharide\(^{25}\)) (0.5 mg) were biotinylated mid-chain with Ez-Link-biotin hydrazide (Thermo Fisher Scientific Inc.) as previously described\(^{12}\) and purified following manufacturer instructions. Biotinylation was confirmed by dot blot using anti-biotin (1:500, Cell Signaling Technology, Danvers, MA).

Binding Analysis via Surface Plasmon Resonance: All binding experiments were performed at room temperature at a flow rate of 20 µL/min on a Biacore2000 system (GE Healthcare, Buckinghamshire, UK). Biotinylated HS and CS were immobilized on separate flow cells of a streptavidin-coated Biacore chip (GE Healthcare, Piscataway, NJ) and coated with ~600 RU. Another flow cell was left untreated as a control. HB-IGF-1 or
IGF-1 was injected in running buffer consisting of 0.01M HEPES, 0.15M NaCl, 3mM EDTA, 0.005% Tween20, pH 7.4. KinInject was used to inject each IGF-1 over the chip with association and dissociation times of 5 min. The surface was regenerated by flowing 1 M NaCl over the chip between experiments. Three to four concentrations of each IGF-1, with three repeats at each concentration, were performed for kinetic analyses. Control flow cell curves were subtracted from all binding curves in order to account for non-specific binding and refractive index change. Association and dissociation rate constants (k$_a$ and k$_d$ respectively) were determined by fitting the measured binding curves globally with a 1:1 binding model using BIAevaluation software v4.1 and floating Rmax as a local parameter$^{40}$. The equilibrium dissociation constant (K$_D$) was calculated as k$_d$/k$_a$.

**ELISA Analysis of Binding to Biotinylated GAGs:** Coating, blocking and washing buffers, secondary antibody, substrate, and stop solutions were purchased from KPL, Inc. (Gaithersburg, MD). Streptavidin-coated microplates (R&D Systems, Inc., Minneapolis, MN) were coated with biotinylated HS and CS at 20 µg/mL overnight at 4 °C. Plates were blocked for 15 min at room temperature before incubation with 0-500 nM HB-IGF-1 or IGF-1 for one hour. Plates were washed three times and incubated with rabbit anti-IGF-1 (10 µg/mL) (Abcam Inc.) for one hour at room temperature to detect protein bound to the biotinylated GAGs. After additional washes, anti-rabbit HRP (1:500) was applied for one hour at room temperature. Following final washes, color was developed by addition of ABTS peroxidase substrate solution. Absorbance was measured at 405 nm after quenching the wells with stop solution.
**Rat Intra-articular Injection:** 10 µg HB-IGF-1 in 50 µl saline, 10 µg IGF-1 in 50 µl saline, or 50 µl saline alone was injected into the right knee joint of 2-month-old male Sprague-Dawley rats. After one day, joint tissues were harvested, extracted, and analyzed by Western blot. 5 ng of recombinant HB-IGF-1 or IGF-1 was loaded as standards. All animal procedures were approved by the Harvard Medical Area Standing Committee on Animals.

**Human Cartilage Binding Assay:** Joints from 4 human subjects were obtained postmortem from the Gift of Hope Organ and Tissue Donor Network (Elmhurst, IL). Cartilage disks (3 mm diameter, 0.8 mm thick) were harvested from femoropatellar grooves of 26-year old (Collins grade 0), 49-year old (Collins grade 0), and 42-year old female (Collins grade 2) knee joints and from a 28-year old (Collins grade 0) male knee joint and cultured in 1% ITS serum-free high glucose DMEM supplemented with 500 nM HB-IGF-1 or IGF-1. After 48 h (Day 0), disks were washed with PBS and incubated in IGF-1 free medium. Disks were collected on Days 0, 1, 2 and 4, and protein was extracted and analyzed for IGF-1 bound by Western blot using anti-IGF-1. 5 ng of recombinant HB-IGF-1 or IGF-1 was loaded as standards. Procedures were approved by the Office of Research Affairs at Rush–Presbyterian–St. Luke’s Medical Center and the Committee on Use of Humans as Experimental Subjects at Massachusetts Institute of Technology.

**Statistical Analyses:** All data are presented as mean ± standard error of the mean. Surface plasmon resonance binding constants were log-transformed and evaluated by Student’s *t-*
test without assuming equal variances. Densitometry data for HB-IGF-1 binding to human cartilage were rank-transformed and evaluated by paired $t$-test. Densitometry data for HB-IGF-1 binding to CHO cells were rank-transformed and evaluated by Student’s $t$-test without assuming equal variances. HB-IGF-1 loss to medium ELISA data were analyzed by one-way ANOVA. Immobilized GAG ELISA data were analyzed by two-way ANOVA followed by Posthoc Tukey tests. All tests were performed with acceptance level $\alpha=0.05$.

4.3 Results

*HB-IGF-1 Is Retained in Cartilage Explants through Binding to Chondroitin Sulfate:* To determine which GAG was more important for HB-IGF-1 retention in cartilage, we first tested whether removal of HS or CS affects HB-IGF-1 retention in cartilage explants. Explants were first incubated with 500 nM HB-IGF-1 or IGF-1 for 48 h (days 0-2, Fig 4.1A). After washing the explants, they were then treated with either chondroitinase ABC, heparitinase, or no enzyme for another 48 h (days 2-4). To rule out the possibility that the high abundance of CS in the tissue could sterically block penetration of heparitinase into cartilage and therefore block heparitinase action, at day 4 half of the explants treated with chondroitinase were incubated with heparitinase for another two days. All other explants were incubated in medium with no added enzymes during this time (days 4-6). All explants were collected after this final incubation period (on day 6) and were analyzed by Western blot for HB-IGF-1 or IGF-1 remaining in the explant.
As shown previously⁴⁶, cartilage explants incubated with IGF-1 in the medium for two
days retained no IGF-1 in the tissue after 4 days of incubation in the absence of
exogenous IGF-1 (Fig 4.1B). In contrast, in the absence of enzymatic treatment, HB-IGF-
1 was strongly retained in cartilage explants. Unexpectedly, treatment with heparitinase
had no effect on the retention of HB-IGF-1 in the cartilage, whereas treatment with
chondroitinase ABC caused a substantial decrease in HB-IGF-1 remaining in the tissue.
While some HB-IGF-1 remained in the explants after treatment with chondroitinase,
treatment of these explants with heparitinase did not reduce retention of HB-IGF-1 any
further (Fig 4.1B, “C’ase+H’ase”). ELISA of conditioned medium confirmed that
significantly more HB-IGF-1 was released from chondroitinase-treated cartilage than
from heparitinase-treated cartilage, indicating comparatively low amounts of HB-IGF-1
bound to heparan sulfate ex vivo (Fig 4.1C).

_Heparan Sulfate is Not Required for Retention of HB-IGF-1 on Cell Monolayers:_ To
confirm that heparan sulfate is not required, we tested retention of HB-IGF-1 and IGF-1
to mutant Chinese hamster ovary (CHO) cells (strain pgsD-677) that are unable to make
HS due to a genetic defect in HS chain polymerization²⁶. The mutant cells upregulate
synthesis of CS and thus produce similar amounts of total sulfated GAG as the wild-type
cells²⁶. After incubation of CHO cells with 100 nM HB-IGF-1 or IGF-1 in serum-free
medium, cells were washed and lysed for analysis by Western blot. HB-IGF-1 was
retained in the wildtype CHO cells after washing, whereas no IGF-1 was retained (Fig
4.2A,B). However, HB-IGF-1 was also retained in the HS-deficient cells, confirming that
HB-IGF-1 can be retained by binding to CS in the absence of HS (Fig 4.2A,B).
HB-IGF-1 but not IGF-1 Binds Immobilized Glycosaminoglycans (GAGs): In order to quantify the binding affinities of HB-IGF-1 and IGF-1 for immobilized CS and HS, we used surface plasmon resonance. Comparable levels of biotinylated HS and CS were attached to a streptavidin-coated Biacore chip. A representative sensorgram is shown in Fig 4.3A, demonstrating that while IGF-1 does not bind to either GAG (response units (RU) <10), HB-IGF-1 binds to both HS and CS. Kinetic analysis of the surface plasmon resonance curves confirmed that although HB-IGF-1 bound HS significantly more strongly ($K_D = 21 \pm 6$ nM) than CS ($K_D = 160 \pm 12$ nM) ($p = 0.012$), the $K_D$ for the binding of HB-IGF-1 to CS is well within the range of reported receptor-ligand affinities$^{24}$. This difference in $K_D$ values resulted from significantly different association rate constants, ($k_a = 16 \times 10^4 \pm 6.6 \times 10^4$ (1/M·s) for HS vs. $k_a = 1.5 \times 10^4 \pm 0.06 \times 10^4$ (1/M·s) for CS, $p=0.035$); the dissociation rates $k_d$ were similar ($2.7 \times 10^{-3} \pm 0.93 \times 10^{-3}$ (1/s) for HS vs. $2.5 \times 10^{-3} \pm 0.16 \times 10^{-3}$ (1/s) for CS), $p=0.85$).

The relative differences in binding affinities of HB-IGF-1 and IGF-1 to HS and CS were confirmed by a sandwich ELISA. Binding of HB-IGF-1 to immobilized HS and CS increased with concentration, with more binding to HS at the same given concentration of HS or CS contained in each well (Fig 4.3B). Therefore, although HB-IGF-1 binds HS with higher affinity, the data suggest that binding to CS would dominate in cartilage tissue, where CS is ~500-1000 more abundant than HS$^{16,50}$. 
*HB-IGF-1 Is Preferentially Retained in Cartilage after Intra-articular Injection:* The ability of HB-IGF-1 to bind CS led us to hypothesize that it would be retained preferentially in the GAG-rich cartilage tissues if delivered by intra-articular injection. Consistent with this hypothesis, we found that one day after intra-articular injection in a rat knee, HB-IGF-1 remained strongly detectable in articular cartilage extracts (Fig 4.4, Articular Cartilage), despite stronger immunoreactivity of the IGF-1. HB-IGF-1 was also slightly detectable in extracts of the fibrocartilaginous meniscus (Fig 4.4, Meniscus). In contrast, HB-IGF-1 was not detectable in patella, patellar tendon, or muscle extracts (Fig 4.4). IGF-1 was not detectable in any of the tissues one day after injection (Fig 4.4).

*HB-IGF-1 Is Retained in Human Cartilage:* To examine whether HB-IGF-1 may be relevant as a strategy for clinical delivery of growth factors to cartilage, we tested whether HB-IGF-1 could also be retained in post-mortem adult human cartilage explants. Human knee cartilage obtained from three Collins grade 0 donor joints (both male and female) and one Collins grade 2 joint (female) was incubated with 500 nM HB-IGF-1 or IGF-1 for two days, washed with PBS, and incubated in no-IGF-1 medium for an additional 0, 1, 2, or 4 days. Binding of IGF-1 was only detectable immediately after washing out the IGF (Fig 4.5A, Day 0). In contrast, HB-IGF-1 was retained after further incubation for up to 4 days (Fig 4.5A). Analysis of Western blots from the four donor cartilages by densitometry demonstrated that retention of HB-IGF-1 was significantly higher than IGF-1 after 1, 2, and 4 days of incubation (Fig 4.5B).
4.4 Discussion

We demonstrate here that adding a heparin-binding motif to IGF-1 converts it from a short-acting growth factor to one that can be locally delivered and retained in articular cartilage in vivo. Contrary to our initial expectations, HB-IGF-1 does not require heparan sulfate for retention in either cell culture or in cartilage explants. While we show that HB-IGF-1 does have a higher affinity for heparan sulfate, its affinity for chondroitin sulfate is within an order of magnitude. Therefore, in cartilage, where CS concentrations are 500-1000 times higher than HS, binding to CS dominates and retention of HB-IGF-1 is independent of HS.

These results led us to hypothesize that HB-IGF-1 could be preferentially delivered and retained in articular cartilage due to its high concentration of sulfated glycosaminoglycans. We tested this hypothesis in rats and showed that after intra-articular injection, HB-IGF-1 remains bound to the CS-rich articular cartilage, but not to the adjacent patella, patellar tendon, or muscle tissue. In contrast, unmodified IGF-1 is not able to bind either CS or HS, and was not detectable in either tissue after intra-articular injection. Finally, we demonstrated that HB-IGF-1 is retained in adult human cartilage whereas unmodified IGF-1 is not. Taken together, the results suggest that modification of growth factors with heparin-binding domains may be a clinically relevant strategy for local delivery to cartilage.

Our finding that HB-IGF-1 is retained primarily by chondroitin sulfate contrasts with the extensive work demonstrating that the heparin-binding domain of FGF-2 binds primarily
to heparan sulfate proteoglycans in cartilage\textsuperscript{17,50}. This is likely explained by differences in the binding specificities of the heparin-binding domains. In general, specificity for HS depends on not only the heparin binding domains, but also on the secondary and/or tertiary structure of the native proteins. The heparin-binding domain we used here to make HB-IGF-1 came from HB-EGF. Structure-function analysis of HB-EGF has shown that in addition to the heparin-binding domain, a portion of the EGF-like domain\textsuperscript{45} is required for binding specifically to heparan sulfate. Similarly, FGF-2 affinity for HS has been shown to depend on the spatial distribution of basic amino acids within the heparin-binding loops of this molecule and on the specific conformation and topological arrangement of these loops\textsuperscript{39}. Since we added only the heparin-binding domain of HB-EGF to IGF-1, it is likely that charge plays a primary role in the interactions of HB-IGF-1, allowing it to bind other negatively charged glycosaminoglycans such as CS.

FGF-2 also appears to be retained in pericellular matrix as a reservoir of the growth factor that does not activate the chondrocytes until released by matrix mechanical stimulation or trauma. While we have not shown specific binding locations within cartilage, we have previously demonstrated that retention of HB-IGF-1 is accompanied by a sustained increase in \textsuperscript{35}S-sulfate incorporation\textsuperscript{46}, strongly suggesting that factors retained by chondroitin sulfate represent a pool that remains available to stimulate chondrocyte receptors at the cell surface.

There may be additional mechanisms that are contributing to the differences seen here between HB-IGF-1 and un-modified IGF-1. In particular, previous work has shown that
IGF-1 binds primarily to IGF binding proteins (IGFBPs) in cartilage, and not to ECM constituents\textsuperscript{2,13}. Cartilage from osteoarthritic patients is known to be less responsive to IGF-1\textsuperscript{14}, in part due to increased levels of IGF binding proteins (IGFBPs)\textsuperscript{8,14,28,31,32}. IGFBPs are present at a binding site density of 30-150 nM in bovine cartilage\textsuperscript{13} and can bind IGF-1 with an equilibrium affinity (K\textsubscript{D}) of ~5 nM\textsuperscript{13}. The K\textsubscript{D} found for HB-IGF-1 binding to heparan sulfate is on the order of that found for IGF-1 binding to IGFBPs, indicating that it may be able to compete for IGFBP binding, possibly resulting in more bioactivity than normal IGF-1 in addition to better delivery. Moreover, the modification of the amino-terminus of IGF-1 by inclusion of the heparin-binding domain may decrease the affinity of HB-IGF-1 to IGFBPs, as the deletion of the first three amino acids in the case of the mutated analog des(1-3)IGF-1 decreased binding to IGFBPs by 100-fold\textsuperscript{1}.

In conclusion, HB-IGF-1 may be a new therapeutic for sustained and relatively specific local delivery of IGF-1 to cartilage through its preferential retention in CS-rich tissues. Modification of growth factors by addition of heparin binding domains may therefore be a novel strategy for targeted delivery to cartilage after intra-articular injection.

\textbf{4.5 Acknowledgements}

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4.6 References


Figure 4.1. Retention of HB-IGF-1 in bovine cartilage explants following enzymatic digestion of glycosaminoglycans. (A) HB-IGF-1 (HB) or IGF-1 (I) was incubated with cartilage disks for two days (Day 0 to Day 2) followed by treatment with no enzyme (No Enz), chondroitinase (C’ase), or heparitinase (H’ase) for an additional two days (Day 2 to Day 4). At Day 4, a subset of chondroitinase-treated disks was incubated with heparitinase (C’ase+H’ase) for two days while all remaining disks were kept in enzyme-free medium. (B) Western analysis of HB-IGF-1 or IGF-1 remaining in the cartilage tissue at Day 6. The blot shown is representative of four repeats. (C) ELISA of HB-IGF-1 released to the medium following 48-hour enzyme treatment of cartilage explants (Days 2-4 for No enzyme, C’ase, and H’ase conditions; Days 4-6 for C’ase + H’ase). mean ± SEM, * vs. No enzyme, n=4, p<0.001.
Figure 4.2. Retention of HB-IGF-1 on cells lacking heparan sulfate. (A) HB-IGF-1 (“HB”) and IGF-1 (“I”) were incubated with mutant CHO cells unable to produce heparan sulfate (“no HS”) and wildtype CHO cells (“WT”), then washed in PBS. Western analysis of the cell lysates for IGF showed that HB-IGF-1 remained bound to cells with or without the presence of heparan sulfate, whereas IGF-1 binding was not detectable. (B) Densitometry of Western blots from four repeated experiments, each normalized to wild-type HB-IGF-1 binding. mean ± SEM, * vs. WT HB, p<0.005.
Figure 4.3. Binding analysis of HB-IGF-1 and IGF-1 to isolated glycosaminoglycans. (A) Representative sensorgram for Biacore kinetic analysis over HS or CS surfaces using 250 nM HB-IGF-1 or IGF-1. HB-IGF-1 is shown in the top two curves (black) with corresponding equilibrium binding constants, $K_D$, determined from a minimum of three concentrations used during three experimental repeats. IGF-1 (bottom two curves, grey) was unable to bind either surface (RU < 10). (B) Sandwich ELISA detecting absorbance at a given HB-IGF-1 (solid line, black) or IGF-1 (dashed line, grey) concentration resulting from binding HS or CS. Representative of two repeats, each with duplicate wells. $n=2$, mean ± SEM, * vs. CS, $p<0.05$. 
Figure 4.4. Retention of HB-IGF-1 in vivo. Western blot showing retained IGF-1 (I) or HB-IGF-1 (HB) in rat cartilage, meniscus, patella, patellar tendon, or muscle extracts one day after intra-articular injection of IGF-1, HB-IGF-1, or saline.
Figure 4.5. Retention of HB-IGF-1 in human cartilage explants. Human cartilage (grade 0 to 2) was incubated with HB-IGF-1 or IGF-1 for two days (Day -2 to 0), washed, and incubated in IGF-free medium for 4 days (Days 0-4). (A) Amount of HB-IGF-1 or IGF-1 remaining in cartilage after 0-4 days. The experiment was performed on cartilage from four donors and a representative Western blot is shown. (B) Analysis of four Western blots by densitometry, normalized to the density of 5 ng of the respective protein standard. mean ± SEM, * vs. IGF-1, p<0.05.
Chapter 5. Conclusions and Future Directions

The inability of articular cartilage to repair itself after acute injury has been implicated in the development of osteoarthritis. Providing anabolic stimulation through delivery of growth factors may improve the ability of endogenous and exogenous cell sources to initiate repair. Additionally, providing a chondrogenic microenvironment may further stimulate repair by native or delivered bone-marrow-derived stromal cells. The objective of this work was to develop methods for delivering growth factors to cartilage and to test the ability of a self-assembling peptide scaffold, (KLDL)₃, with or without growth factors to augment repair. Delivery methods included growth factor adsorption, scaffold-tethering, and modification of growth factor structure.

In Chapter 2, two different modes of growth factor delivery were investigated: adsorption and tethering. IGF-1 and TGF-β₁ were pre-mixed with the self-assembling peptide hydrogel, (KLDL)₃ or KLD, solution to adsorb the growth factors to the scaffold during the assembly process. Biotinylated versions of the growth factors were tethered to the peptide through streptavidin and biotinylated KLD. Adsorption was able to deliver TGF-β₁ in sufficient quantities to induce chondrogenesis of bone marrow derived stromal cells (BMSCs); IGF-1 diffused too quickly out of the scaffold to stimulate extracellular matrix production by chondrocytes. Additionally, while tethering these factors through a biotin-streptavidin bond provided long-term sequestration, tethered growth factors were not effective in stimulating proteoglycan production. Therefore, while self-assembling peptide sequences are readily functionalized, the manner in which growth factors are
delivered affects bioactivity and varies for specific growth factors and biological systems of interest.

To improve tethering, one could use a lower affinity tethering system to allow reversible tethering, or one could incorporate cleavable links in the peptide sequence. For instance, one could use an MMP- or ADAMTS- cleavable link before the growth factor linkage, so that upon upregulation of these enzymes the growth factor would be released.

In Chapter 3, the self-assembling peptide hydrogel KLD with or without chondrogenic factors and allogeneic BMSCs was evaluated in a full-thickness, critically sized rabbit cartilage defect model for ability to stimulate cartilage repair. Delivering KLD alone resulted in improved filling of osteochondral defects and improved cartilage repair, as seen by cumulative histology score, Safranin-O staining, and type II collagen immunostaining. In this small animal model, the full-thickness defect provided access to the marrow, similar in concept to abrasion arthroplasty or spongialization in large animal models (goat, sheep, horse, human), and suggests that combining KLD with these techniques may offer an improvement over current practice. Ongoing studies include the evaluation of KLD in a clinically relevant sized equine defect co-treated with microfracture and subjected to strenuous exercise, compared to defects treated with microfracture alone.

Even though the chondrogenic factors and BMSCs selected for this study proved sufficient to induce chondrogenesis in vitro, they did not have the intended effect when
delivered in vivo, highlighting the necessity of further in vivo testing. The length of time the peptide hydrogel is present in the defect and the degradation kinetics should be also addressed in future in vivo and in vitro studies.

In Chapter 4, it was shown that adding a heparin-binding motif to IGF-1 converts this protein from a short-acting growth factor to one that can be locally delivered and retained in articular cartilage in vivo. HB-IGF-1 does not require heparan sulfate for retention in either cell culture or in cartilage explants. While HB-IGF-1 does have a higher affinity for heparan sulfate, its affinity for chondroitin sulfate is within an order of magnitude. Therefore, in cartilage, where CS concentrations are found to be 500-1000 times higher than HS, binding to CS dominates and retention of HB-IGF-1 is independent of HS.

These results led to the hypothesis that HB-IGF-1 could be preferentially delivered and retained in articular cartilage due to its high concentration of sulfated glycosaminoglycans. This hypothesis was tested in rats in vivo, and it was shown that after intra-articular injection, HB-IGF-1 remained bound to the CS-rich articular cartilage, but not to the adjacent patella, patellar tendon, or muscle tissue. In contrast, unmodified IGF-1 was not able to bind either CS or HS, and was not detectable in either tissue after intra-articular injection. Finally, it was demonstrated that HB-IGF-1 was retained in adult human cartilage, whereas unmodified IGF-1 is not. Taken together, the results suggest that modification of growth factors with heparin-binding domains may be a clinically relevant strategy for local delivery to cartilage. Ongoing studies focus on
evaluating the ability of HB-IGF-1 to promote proteoglycan synthesis in rat cartilage *ex vivo* following intra-articular injection.

While injection of the protein alone has been shown to be adequate to deliver HB-IGF-1 to cartilage for up to four days, and sustained proteoglycan synthesis for up to eight days, to attain longer-term delivery, a carrier may be necessary. One option would be to use a hydrogel such as KLD with incorporation of chondroitin sulfate or heparan sulfate to increase binding and modulate the release kinetics. Another option would be to encapsulate HB-IGF-1 in polymer microspheres and deliver the microspheres in a hydrogel to the joint. In addition, radiolabel studies with $^{125}$I-HB-IGF-1 will enable better characterization of the uptake into KLD and the further delivery into cartilage tissue.

In conclusion, the work in this thesis provides a basis for future growth factor delivery studies in the context of cartilage and suggests that the self-assembling peptide KLD and the fusion protein HB-IGF-1 may be further developed to aid in cartilage repair.
Appendix A – Protocols and data related to Chapter 2

Encapsulating cells in KLD with or without biotin-streptavidin tethering of growth factors

Use tissue-culture treated 24-well plates if using chondrocytes, non-tissue cultured if using BMSCs (BMSCs tend to crawl out of the gels and stick to the bottom of tissue-culture treated plates).

Agarose ring molds:
Fill wells of a 24-well plate with 0.6 mL of 2-3% sterile low-melting point agarose. Let agarose solidify at 4 °C for a few hours. In the hood, use a sterile 6 mm punch to make holes in the center of each well. Use a sterile spatula to remove these holes. Use a sterile 12 mm punch to make holes centered around the 6 mm holes you just created. Again, use a sterile spatula to remove the agarose around the hole you just punched so you are left with a ring with an outer diam of 12 mm and an inner diam of 6 mm.

Casting with KLD:

You will need the following solutions:
10% sterile sucrose
10% sterile sucrose + 2.5 mM HEPES
HGDMEM + 25 mM HEPES
Culture medium (usually 1% ITS+1 with HGDMEM unless doing IGF-1 experiments, then mini ITS+1 (0.003% ITS+1))

And the following materials:
KLD (6 mg per round of casting)
For tethering: biotinylated KLD (bKLD), streptavidin, biotinylated IGF-1 (bIGF-1)
2 mL tubes with 0.9 mL Tris buffer in them
Repeat pipettor with a 1 mL sterile syringe for each round of casting

Fill the wells of the 24-well plate with HGDMEM+HEPES so that the rings get saturated with medium in order to aid in self-assembly of the peptide.

Resuspend KLD at 4.5 mg/mL with sterile 10% sucrose. If tethering, you may want to increase this to 4.6 mg/mL to account for the extra volume you will be adding. For this protocol, I am assuming a final concentration of ~3.5 mg/mL. You can safely use a final concentration in the range of 3.2 – 4 mg/mL. Sonicate for about 60 min, vortexing and spinning down intermittently.

If tethering, resuspend bKLD at 2 mg/mL with sterile 10% sucrose. Sonicate for at least 30 min – bKLD at this concentration goes into solution much faster than KLD.

When KLD is in solution, transfer 1.2 mL to a polypropylene 5 mL tube for each round of casting you plan to do. (1.165 mL if tethering). Put tube in sonicator.
If tethering, add 27 µL bKLD to each tube (1:100 concentration of bKLD:KLD).

Pre-mix streptavidin and bIGF-1 in equimolar amounts. If you want 300 ng/mL bIGF-1 inside the peptide, combine 4.5 µL of bIGF at 100 ug/mL and 3.34 µL of streptavidin at 1 mg/mL. Add this mixture to your peptide tube with bKLD in it about 5-15 minutes before casting. (If you want to adsorb growth factors, you would just add your growth factor directly to the peptide mixture at this point without including the streptavidin or bKLD.)

When all peptide tubes look like they have a low viscosity and few bubbles, spin down your tube of cells (200g for 8 min for chondrocytes, 100g for 5 min for BMSCs).

While the cells are spinning, aspirate the medium out of the centers of your agarose rings in your 24-well plates. Make sure that the rings are not leaking. If they are, just remove all of the medium from the well.

When the cells are finished, aspirate the supernatant and resuspend your cells in 0.3 mL of sucrose+HEPES. Make sure the pellet is broken up completely as cell clumps generally result in death.

Pull up all of the cells and put them into your peptide tube. Use your thumb to cover the top of the tube and vortex gently and briefly (~ speed 4) to homogenize the cells and peptide mixture.

Pull up the cell/peptide solution in your repeat pipettor set to 50 µL. Click the dispenser button once so that the volume numbers stop blinking.

Now quickly put 50 µL of cells/peptide in the middle of each ring (generally get 19-20 gels per round of casting).

When you are finished, use a P200 to put 50 µL of leftover cell/peptide solution in tubes containing 900 µL Tris buffer for use as day 0 DNA standards.

About 2-5 minutes after casting, you should put either HGDMEM+HEPES or culture medium in all of your wells so that the gels finish assembling and the pH is neutralized.

Repeat this process for the remaining rounds of casting.
Dose-response with soluble IGF-1 and bIGF-1 on chondrocytes encapsulated in KLD

**Figure A1.** Effect of soluble IGF-1 and bIGF-1 on sGAG and DNA content of chondrocytes encapsulated in KLD. Chondrocytes were encapsulated in KLD and cultured for 4 days in soluble IGF-1, soluble bIGF-1, or 1% ITS+1. Treatments were started on day 1 post encapsulation. sGAG and DNA content per gel are shown. mean±SEM. n=4.
Figure A2. Effect of soluble IGF-1 and bIGF-1 on $^{35}$S-sulfate incorporation of chondrocytes encapsulated in KLD. Chondrocytes were encapsulated in KLD and cultured for 4 days in soluble IGF-1, soluble bIGF-1, or 1% ITS+1. Treatments were started on day 1 post encapsulation; Radiolabeled on day 4; Ended experiment on day 5. mean±SEM. n=4.
Effect of tethered IGF-1 on BMSCs encapsulated in KLD

**Figure A3.** Effect of tethered bIGF-1 on BMSCs encapsulated in KLD. TGFβ-1 (100 ng/mL) and dexamethasone (100 nM) were adsorbed to KLD gels with or without 300 ng/mL biotinylated-IGF-1/streptavidin/biotinylated-KLD. (A) sGAG retained in the hydrogels. (B) DNA content of the hydrogels. (C) 35S-sulfate incorporation over the last 24 hours. (D) 3H-proline incorporation over the last 24 hours. n=16 (3 animals, 4 experiments, n=4 per experiment). mean ± sem. * vs. Day 14, † vs. Day 21, p<0.05 (linear mixed model with animal as a random factor on log transformed data).
Flow Cytometry
Invitrogen - #V13241 – Vybrant Apoptosis Assay Kit #2
Accuri C6 Flow Cytometer – Schauer and Lauffenburger labs each have this machine
Can reuse and autoclave syringe filters and plastic filter holders
PI (propidium iodide, red fluorescence): >670 nm (FL3)
AlexaFluor 488-Annexin V: 488 nm (FL1)

Some annexin binding buffer comes with this kit, but you can make more of your own:

ABB Recipe (500mL) - 10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl₂, pH 7.4
1.192g HEPES
4.0908g NaCl
0.184g CaCl₂*2H₂O
~2mL of 2M NaOH

1. Assemble syringes/filters by putting a filter between a top and bottom plastic part and screwing them together. Put this on the bottom of a 1 mL syringe with the plunger removed.
2. Transfer 1-2 gels from the same condition to 1 mL syringe.
3. Add 300-500 µL PBS to wash the gel(s) down and push them through the syringe with the plunger. Collect the output in a large Eppendorf tube. You can use more PBS if necessary, the important part is to get the gel(s) washed down and in liquid so that when you push the plunger through, the gel is actually pushed through the filter and not getting stuck to the sides of the syringe.
4. Centrifuge the large Eppendorf tube at 200g for 8 min.
5. Keep pellet (cells). Some of the peptide will collect near the pellet but still in the supernatant, so you can discard that.
6. Reconstitute with ~400 µL annexin binding buffer to get ~1x10⁶ cells/mL. You should count the cells the first time you do this and optimize it for your experiment.
7. Put 100 µL of each sample in new Eppendorf tube.
8. Add 1 µL of working stock PI to each tube.
9. Add 3 µL AlexaFluor488-Annexin V to each tube.
10. Use leftover cells to make up 3 types of controls:
    a) Combine equal amounts of each type of sample into 3 tubes for total of 100 µL/tube.
    b) Keep one control unstained. Stain one control with red only. Stain one control with green only.
11. Vortex lightly to mix.
12. Stand at room temp for 15 min in the dark.
13. Add 0.4 mL annexin-binding buffer to each tube. Keep samples on ice.
14. Run through flow cytometer. Run controls first to set gates and adjust the compensation. You should not have to adjust the compensation more than a few % to get the green control samples all in the lower right quadrant instead of some in the upper right quadrant. Keep some cell only samples (cells that were never encapsulated in peptide) if possible to identify what is peptide and what is cells in the side scatter vs forward scatter plot.
15. Population should separate into 3 quadrants: lower left = alive; lower right = green, apoptotic; upper right = red and green, dead.

Example pictures of side vs forward scatter plots:

- **chondrocytes alone**
- **BMSCs alone**
- **RAD peptide without cells**
- **KLD peptide with chon**
- **RAD peptide with BMSCs**

Three controls, FL3 vs FL1 plots:

- **Unstained**
- **unstained**
- **red**
- **green**

And looking at the chondrocyte gate for the staurosorine positive control:

**Figure A4.** Example flow cytometry images.
Figure A5. Effect of casting order on cell death. There was a slight increase with time, but overall it seems the largest variability is between different rounds of casting and with cell density.
**Figure A6.** Chondrocytes encapsulated in KLD with or without tethered IGF-1 were evaluated for cell death and apoptosis by staining with propidium iodide and annexin v and performing flow cytometry at days 1, 2, 3, and 6 post encapsulation. Medium was supplemented with No IGF-1, soluble IGF-1 (300 ng/mL), or staurosporine (1 µM). mean ± SEM. n=5 experiments (2 gels averaged for each experiment) for day 1. n=2 for day 2. n=4 for day 3. n=1 for day 6. Day 1 values ranged from 20-41% death for no IGF-1. Intra-experiment variation was <3% per condition. Stats: ANOVA on days 1 and 3 data with Tukey post-hoc, * vs. staurosporine, p<0.05.
Casting Alginate Beads with Cells
Adapted from Delphine Dean and Bobae Lee.

Autoclave:
- 50 mL beaker
- 3-500 mL bottles (for solutions below)
- 1 spatula (to move beads)

Solutions:
- 2% alginate in 0.9% NaCl
- 0.9% NaCl: 9 g in 1000 mL DI water
- 150 mM NaCl (FW 58.44): 4.38 g in 500 mL DI water
- 102 mM CaCl$_2$ (FW 111) in 0.9% NaCl: 5.66 g CaCl$_2$ in 500 mL of 150 mM NaCl
- 55 mM Na Citrate (FW 294.10) in 150 mM NaCl: 8.09 g Na Citrate in 500 mL of 150 mM NaCl

Filter all solutions through a 200 µm filter and into the sterile bottles.

To make the beads
1. Fill the sterile 50 mL beaker with 30 mL of 102 mM CaCl$_2$ solution.
2. Get a cell count of cells using the hemocytometer. Spin cells down (if needed). 1800 rpm, 10 min. Suction off supernatant (being careful not to suck up any cells.) Gently tap bottom of vial to break up cells. May need to add 1 mL sterile PBS (no Mg$^{++}$ and Ca$^{++}$, pH 7) to help break up the cells.
3. Add appropriate amount of alginate. [ideal conc: 10-15x10$^6$ cells/mL; can be as high as 20x10$^6$ cells/mL] (I did 20 million cells/mL and it worked fine.)
4. Use 16G needle and 3 mL syringe to suction up the alginate-chondrocyte solution. Remove as many air bubbles as possible from the syringe.
5. Remove 16G needle and replace with 22G needle. Hold the syringe at a 45° angle with the hole facing downward.
6. With steady pressure, drop even-sized droplets into the CaCl$_2$ solution. Halfway-through you may want to exchange the CaCl$_2$ solution with fresh solution so the Ca$^{++}$ does not get depleted.
7. After 10 minutes, suction off the CaCl$_2$ and rinse twice with 20 mL sterile PBS (no Mg$^{++}$ and Ca$^{++}$). Let each rinse go for 1 min. (Note: CaCl$_2$ + PBS causes a precipitate to form, so only do 1 min rinses or use DMEM instead of PBS.)
8. Replace PBS with culture medium.
9. Fill a 12-well plate with 2 mL culture medium in each well. Using a sterile spatula, transfer 6-8 beads/well.
10. Do a cell viability assay on one of the beads.
11. Keep alginate-cell beads in an incubator.

To dissolve the beads – I used the first method
1. Put 4 beads into a 1.5 mL eppendorf tube. Add 1.2 mL NaCitrate. Place in a 37 °C water bath or incubator for 10 minutes. Shake gently to mix. Do not vortex.
2. Spin 3000-4000 rpm (~1000 rcf) in Eppendorf centrifuge 5415C for 5 minutes.
3. Remove supernatant. Add 1 mL 0.9% NaCl. Centrifuge 3000-4000 rpm, 5 min. Repeat.
4. Resuspend final pellet in PBS (if doing a cell viability assay) or culture medium at desired concentration (~0.5 ml -> gave me about 1 million cells/mL).
5. Do a cell viability assay – should get <5% dead.

OR

1. Put 15 beads into a 15 mL conical tube. Add 5 mL NaCitrate. Place in 37 °C water bath for 10 minutes. Shake gently to mix. 5 more minutes with frequent shaking if the beads are not dissolved. Do not vortex.
2. Spin <1000 rpm (with CBE centrifuge) for 6 min.
3. Remove supernatant. Add 3 mL of 0.9% NaCl to rinse. Spin <1000 rpm. Resuspend in medium.
TUNEL staining (courtesy of Dr. Bodo Kurz, University of Kiel)

![TUNEL staining](image)

**Figure A7.** TUNEL staining on chondrocytes encapsulated in KLD. Percent chondrocytes stained positive for TUNEL when encapsulated in KLD with or without tethered bIGF-1. Cells were incubated in No IGF-1, IGF-1 (300 ng/mL), or staurosporine (1 µM). Day 3 post encapsulation. Gels were fixed in 10% formalin overnight, paraffin embedded, and shipped to Dr. Bodo Kurz for TUNEL staining. Stats: Mixed model with animal as a random factor, n=18 (3 animals), Tukey post-hoc test, mean ± SEM, * vs No IGF, † vs Staurosporine, p<0.05.

References below state that TUNEL is not specific for apoptosis and that results must be confirmed with morphology. Dr. Kurz has done this by electron microscopy and we have determined that the results are more consistent with the flow cytometry data showing no difference with soluble IGF or tethered IGF compared to no IGF gels, indicating the TUNEL staining was not representative of apoptosis in this case.


**Western blots on cells encapsulated in peptide hydrogels**
Adapted from Diana Chai and Nora Szasz protocols

**Gel Lysis Protocol**

Lysis Buffer:
50mM Tris/HCl, pH 7.2 (0.151375g in 25mL)
150mM NaCl (0.21915g in 25mL)
10mM EDTA (0.09306g in 25mL)
1% Triton X-100 (0.25mL)
1% equiv of NP-40 (0.25mL)
-Sonicate to dissolve.

-Day of add the following to 0.85 mL of lysis buffer:
- 10 µL Na₃VO₄ (0.7356 g/20mL dH₂O pH 10 for 20 vials of 100x)
- 100 µL NaF (0.8398 g/20mL dH₂O for 20 vials of 10x)
- 40 µL protease inhibitor cocktail (stock at 25x)

1. Rinse each peptide gel with ice-cold PBS.
2. Put each gel in a large eppendorf tube with 30 µL lysis buffer in it and mechanically break up by pipetting up and down.
3. Sonicate briefly, sit on ice for 15 min.
4. Centrifuge at 10000g for 10 min.
5. Freeze supernatant.

Run a BCA assay to determine the total protein content in the gel. Try up to a 1:10 dilution.

**Western blot**

Use 15 well (1.5mm thick) Invitrogen bis-tris gel.
Choose the % gel and MOPS/MES buffer depending on the MW of your protein of interest.
Can run up to 25 µL per well.
For each sample:
5 µL – 4x running buffer
2 µL – 10x reducing agent
up to 13 µL sample (load equal amounts of total protein, typically ~10-15 µg)
- I like to make a stock of 4x running buffer and reducing agent and then add 7 µL to each of my samples.

After prepping samples, boil for 5 minutes, spin down, and load into your gel.
Run at 200 V for 30-45 min. If apparatus heats up, can surround with ice or run at 4 °C.

Transfer for 60 minutes @ 70V (time and voltage may change depending on protein of interest) with stir bar in cold room in transfer buffer without SDS (3.03 g tris base + 14.4 g Glycine, 200 mL methanol). (Can add 0.1% SDS and reduce methanol to 10% if transferring large proteins >80 kDa). Use PVDF membrane in most cases.
Block for 1 hr in 5% milk+PBST (or TBST) at room temp on shaker plate.

Incubate gel in 1:500 to 1:1000 primary antibody overnight at 4 °C on shaker plate.

The following day, wash in PBST 3x10 min at room temp on shaker plate.

Incubate in secondary anti-species of your primary antibody (1:2000) for 1 hr at room temp on shaker plate.

Wash in PBST 3x10 min or longer last rinse if high background is an issue at room temp on shaker plate.

Image with chemiluminescent kit.
**Figure A8.** Phospho AKT and phospho ERK Western blots. Chondrocytes were encapsulated in KLD with or without 300 ng/mL tethered IGF-1 (bIGF). Gels were cultured in No IGF-1 (No and bIGF) or 300 ng/mL IGF-1 (IGF) supplemented medium for 1, 2, 3, or 6 days post encapsulation. BMSCs were encapsulated in KLD with 100 ng/mL TGF-β1 and 100 nM dexamethasone adsorbed to the scaffold (SDT+dex) with or without 300 ng/mL tethered IGF-1 (bIGF). Gels were cultured in basal medium for 7 days post encapsulation. At each timepoint, gels were lysed and protein measured by BCA assay. Equal amounts of protein were loaded (5-10 µg) and a Western blot for either (A) pAKT (Cell Signaling #9271) or (B) pERK (Cell Signaling #9101) was performed. Membranes were stripped and reprobed for either AKT (Cell Signaling #9272) or ERK (Cell Signaling #9102).
Appendix B. Protocols and data related to Chapter 3

**Figure B1.** Circular dichroism spectroscopy to confirm secondary structure of the peptide and to monitor assembly. Top: Dissolved KLD in 10% sucrose to 0.35 mg/mL. Added 2 or 5 µL of 0.2 N NaOH to 200 µL KLD solution to get pH 7.5 and 9, respectively. As pH increases, the peptide self-assembles. Bottom: 25 µL of PBS was added to 275 µL of 0.35% RAD.
**In situ peptide assembly**

Made 4 defects, front two were subchondral (5 mm diam), back two were 5 mm diam x 2 mm deep cartilage-only defects with vertical edges.

![Diagram of peptide assembly](image)

**Figure B2. In situ peptide assembly and cell viability.** Used 2 peptide concentrations for subchondral defects, front-most one was 6.3 mg/mL, back one was 3.5 mg/mL. At 5 min, starting to gel but still liquid. At 8 min, the 6.3 mg/mL gel was assembled. At 12 min, the 3.5 mg/mL gel was assembled. (Used 2 µL of Coomassie blue to make the peptide purple in order to see it in the defect.) These gels stayed in the defect even after tilting the joint to fill the other defects. PBS did not wash the gels out. Used 2 peptide concentrations for non-chondral defects, front one was 6.3 mg/mL but we added 100 µL more sucrose to it since starting to clump in falcon tube, ended up looking like original 6.3 mg/mL but actually probably more like 4 mg/mL. Back defect was 3.5 mg/mL. At 8 min, both defects had assembled nicely and did not get disrupted by stream of PBS or by articulation of joint. Chondrocytes in KLD survived this procedure (Lower right).

**Notes:**
Blood from subchondral defect did not affect self-assembly process but these were not actively bleeding defects, so this may not reflect the surgical situation.
Able to self-assemble KLD in 8 min and flow PBS over gels without displacement.
Joint Articulation/KLD retention study

**Figure B3.** In situ filling and joint articulation using 4 mg/mL KLD with Trypan blue. Made 15 mm square defect, 1.5 mm deep on trochlear groove of bovine. Filled to edge of defect. Dripped in PBS to initiate self-assembly. Lubricated joint surface (not defect area) with PBS. Articulated joint 3 times and KLD remained in defect. Additional 3 articulations had no effect on KLD. Upper left, before fill; Upper right: fill; Lower left: after articulations.

**Figure B4.** In situ filling and joint articulation using 3.2 mg/mL KLD with Trypan blue. Did same as above and peptide stayed in defect, but didn’t seem as sticky as 4 mg/mL. Left: fill; Right: after articulation.
Other conditions of Rabbit Study

Table B1. Treatment groups not included in Chapter 3.

<table>
<thead>
<tr>
<th>Condition</th>
<th>n</th>
<th>KLD</th>
<th>BMSCs</th>
<th>TGF-β1</th>
<th>bIGF/dex</th>
<th>HA</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 (left)</td>
<td>7</td>
<td>+</td>
<td>+</td>
<td>1.4 ng</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>4 (right)</td>
<td></td>
<td>+</td>
<td>+</td>
<td>0.7 ng</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>5 (left)</td>
<td>6</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>5 (right)</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

Figure B5. Gross necropsy photographs of joints in Groups 4 and 5.
**Figure B6.** Gross and histologic effects comparing defects treated with KLD+BMSCs+1.4 ng TGF-β1 in group 3 to defects treated with the same combination in group 4. Defects in group 3 had contralateral empty defects while defects in group 4 had contralateral defects treated with KLD+BMSCs+0.7 ng TGF-β1.
Figure B7. Immunohistochemistry scores (0-4). A) Aggrecan. B) Collagen II. C) Collagen I. * p<0.05.
**Results:** Radiographic analyses pre- and post-treatment did not reveal any differences among groups 1-3, but comparing treated defects in group 3 to the 100 ng/mL TGF-β1 defects in group 4 showed more radiographic pathology for the group 4 knees (p<0.057) demonstrating higher amounts of lysis, bony proliferation, osteophyte formation and patellar luxation in that group.

Upon necropsy, joints in groups 1-3 and 5 appeared normal, while three rabbits that received treatments in both knees (group 4) had mild to severe inflammation. Group 4 also demonstrated bony proliferation along the trochlear ridges and patellar luxation. Those receiving an intra-articular injection of BMSCs in one knee (group 5) also showed mild osteophyte formation, while rabbits with both knees treated (group 4) had mild to moderate osteophyte formation. Those receiving an intra-articular injection of BMSCs in one knee (group 5) also showed mild osteophyte formation, while rabbits with both knees treated (group 4) had mild to moderate osteophyte formation. Comparing group 3 and group 4 defects treated with the same amount of TGF-β1 (100 ng/mL), group 4 revealed significantly worse repair based on total grade (the overall quality of the repair tissue taking into account all observed factors) (p<0.019), color (p<0.019), and synovial membrane (p<0.0496), while incision appearance, inflammation and swelling, and articular surface integrity approached significance (p<0.055).

Within group 4, comparing the defect with 50 ng/mL TGF-β1 to the contralateral defect receiving 100 ng/mL TGF-β1, there were no significant differences for any of the scores. Defects receiving an intra-articular injection of BMSCs (group 5) appeared more yellow in color than those receiving HA alone (p<0.028).

Similar to gross evaluation, there were no significant differences within group 4. Knees with 100 ng/mL TGF-β1 in group 4 had worse repair tissue than treated defects in group 3 with more hypocellularity (p<0.055) and less repair tissue thickness (p<0.064). There were no significant differences within group 5, although the nature of predominant tissue and reconstitution of subchondral bone scores trended towards a worse repair for the BMSC-treated defects (p<0.07).

Comparing group 3 treated defects to group 4 defects with 100 ng/mL TGF-β1, collagen II scoring was not significant, but trended (p<0.1) towards higher scores for group 3. Within groups 4 and 5 there were no significant differences.

**Discussion:** In addition, treating two knees in one rabbit with BMSCs and different doses of TGF-β1 (group 4) resulted in an increased inflammatory response and bony reaction, as seen by the comparison between this group and the treated defects in group 3 in which only one knee received TGF-β1. This finding suggests possible systemic effects of treatment and that negative effects were caused by increasing the total body dose of TGF-β1 (2.25 ng total for rabbits receiving two treatments and 1.5 ng for those with one treatment). Finally, treating defects with an intra-articular injection of BMSCs in HA (group 5) offered no advantage over injection of HA alone.
Appendix C. Protocols and data related to Chapter 4

Western blot on medium following enzyme treatment of cartilage explants
HS stub antibody detects cleavage following heparatinase treatment.

Use 4-12% bis-tris 15 well (1.5mm thick) Invitrogen mini-gel. Used Novex sharp protein standard from Invitrogen.

Can run up to 25 µL per well.
5 µL – 4x running buffer
2 µL – 10x reducing agent
13 µL conditioned medium
Boil all samples for 5 min.

Ran Gel in MOPS running buffer at 200V for 45-60 min

Transferred for 80 minutes @ 75V with stir bar in cold room in transfer buffer with 0.1% SDS (1 g SDS) and 10% methanol (3.03 g tris base + 14.4 g Glycine, 100 mL methanol). Used PVDF membrane.

Antibody Incubation:
Blocked for 1 hour in 5% milk+PBST.
Incubated gel 1:500 with 3G10ab (Seikagaku) O/N @ 4 °C .

Following day, washed in PBST 3x10 min.
Incubated in secondary: 1:1000 Anti-mouse 60 minutes @ RT.
Wash in PBST 3x10 minutes
Imaged using chemiluminescence kit.
Figure C1. Western blots of conditioned medium following no enzyme (N), heparitinase (H), chondroitinase (C), or chondroitinase followed by heparitinase (C+H) treatment of cartilage explants. Used anti-HS stub (3G10ab) antibody.
**Biotinylation of GAGs**

Biotin hydrazide biotinylates GAGs on carboxyl groups. I first attempted to use Sulfo-NHS-LC-Biotin, which normally biotinylates at the primary amine of proteins, but this didn’t seem to work for the GAGs even though others have reported doing it this way.

Pierce EZ-Link Biotin-LC-Hydrazide kit: #21340
Pierce Zeba Desalt Spin Columns: #89891
Heparin: Sigma, #H3149
Heparan sulfate: Sigma, #H7640
Chondroitin sulfate: Sigma, #C4384

1. Dissolved GAG in 0.1 M MES at 2.5 mg/mL. Used 200 µL (0.5 mg).
2. Added 12.5 µL of 50 mM biotin hydrazide. (at least 1 mg biotin in 54 µL DMSO).
3. Added 0.5 µL of 0.5 M EDC. (at least 5 mg in 50 µL MES).
4. Mix with constant shaking (taped to rotary shaker plate and put on high) O/N at room temperature.
5. Desalt using spin columns and equilibrate in water. I would use 2 different columns to make sure you get rid of all the unincorporated biotin. I only used one column and one of the times I did this it seemed like I had a lot of free biotin left in my sample when I was finished. You can store at 4 °C for at least several weeks. An alternative would be to lyophilize and store in -80 °C for long-term storage.
6. Confirm biotinylation using HABA/Avidin assay or dot blot using anti-biotin.

**Sandwich ELISA (biotinylated GAGs attached to streptavidin-coated ELISA plate; HBIGF/XIGF in solution; amount bound detected by anti-IGF-1)**
Used KPL ELISA kit (54-62-15)

1. Use pre-coated R&D streptavidin plate (CP003). Coat ELISA plates with 100 µL of 20 µg/mL biotinylated GAGs in coating buffer. Incubate overnight at 4 °C. Need 6.5 mL coating buffer.
2. Block with kit for 15min
   1. **Make BSA diluent for rest of steps.**
   2. Add 300 µL BSA Diluent/Blocking Solution to each well.
   3. Incubate 15 minutes at room temp, empty plate and tap out residual liquid.
3. Incubate with varying amounts of HBIGF.
   1. Add 100 µL Standard/Sample to each well. (do 2 wells per sample)
   2. React at room temperature for 1 hour.
   3. Empty plate, tap out residual liquid.
4. Wash plates.
1. Fill each well with 1X Wash Solution.
2. Invert plate to empty, tap out residual liquid.
3. Repeat 3 - 5 times.

5. Incubate with anti-IGF for 1 h at 10 µg/mL at room temp.
   1. Add 100 µL Detection Antibody Solution to each well. (anti-IGF at 10 µg/ml)
   2. React 1 hour, room temperature.
   3. Empty plate, tap out residual liquid and wash as above.

6. Wash plates.
7. Incubate with anti-rabbit HRP for 1 h at 1:500 at room temp.
   1. Add 100 µL Secondary Antibody Solution to each well.
   2. React 1 hour, room temperature, in dark.
   3. Empty plate, tap out residual liquid and wash as above.
   4. Give final 5 minute soak in Wash Solution; tap residual liquid from plate.

8. Wash plates.
9. **Read Substrate**
   1. Dispense 100 µL Substrate Solution into each well.
   2. After sufficient color development (read every 1 min), add 100 µL Stop Solution to each well.
   3. Read plate with plate reader at 405 nm.
Surface Plasmon Resonance (Biacore) Protocols

Conditioning Buffer:
1M NaCl in 50 mM NaOH.

Running Buffer:
0.01M HEPES pH 7.4 -> 1 mL in 100 mL
0.15M NaCl -> 0.8766 g
3mM EDTA -> 0.111672 g
0.005% Tween20 -> 5 µL
Filter and Degas.

Regeneration Buffer:
1M NaCl in PBS, pH 7.4

Start-up Protocol:
Load chip.
Put in Running Buffer.
Dock.
Prime, ignore errors.

Shutdown Protocol:
Undock. Take out chip, store in 50 mL conical with 2 mL water in bottom at 4 °C.
Put in maintenance chip. Dock.
Put tubes in water instead of running buffer.
Prime into water (7 min).
Fill 2 vials with 3 mL SDS (1) or 3 mL glycine (2). Put in rack.
Run desorb (22 min).
Prime water 2x.
Take out water. Put in kim wipe. Prime with air.
Undock.
Take out maintenance chip.

First run, attach GAGs to chip:
Run sensorgram. Fc-1-2-3-4 measure all 4.
Switch to Fc-4. Flow 20 µL/min. NaOH 3x 1min.
Wash needle 2x.
Manual inject. Load bHeparin, 26 µL in F4, saturated at 10 µL.
Switch to Fc-3. Quick inject 20 µL NaOH 3x.
Wash needle 2x.
Switch to 5 µL/min. Load bHS, saturated in few µL.
Switch to Fc-2.
Flow 20 µL/min NaOH 3x1min.
Wash needle 2x.
Flow 10 µL/min. Manual inject. Load bCS, saturated at 5 µL.
Switch to Fc-1.
Flow 20 µL/min. Quick inject 20 µL NaOH 3x. Wash needle 2x.
Put 1 µM (90 µL) HBIGF. Switch to Fc-1-2-3-4. 5 µL/min.
KinInject 25 µL = 300 s. Dissoc 300 s.
Put in 0.1M NaCl in PBS. Change to 20 µL/min.
Quick inject 20 µL x2.
Wash needle 2x.
Switch to 5 µL/min. Put in 100 nM (70 µL) HBIGF.
Kininject 25 µL = 300 s. Dissoc 300 s.
20 µL/min. Quick inject 0.1M NaCl in PBS. 20 µL 1x.

To acquire kinetic data:
Docked chip. Primed into RB.
Run 1: Flow rate 20 µL/min. Run with RB. Quick Inj 1M NaCl in PBS. Run with RB.
Run 2: 100 nM fresh HBIGF at 20 µL/min for 300 s. Dissociation 300 s.
  Quick Inj 2x with 1M NaCl 20 µL.
  Wash needle 2x.
Run 3: 250 nM HBIGF
Etc.
Densitometry (semi-quantitative analysis of Western blots)

with FluorChem software program:
Hit cancel when it asks for a serial number.

Make sure your file name is short or the file will not open.

On the contrast enhancement panel, hit reverse and auto contrast to view your gel. You can further adjust the black, white, and gamma channels to improve your view. Doing this does not affect the pixels the software program uses to calculate density.

On the toolbox panel, choose the Analysis Tools tab. Then choose the Spot Denso tab.

Make sure that if you select a band and select background that the background has a lower value. If this is not the case, click the invert button. In most cases, when you are starting with a black gel with white bands, you should not have to select invert.

Use the object buttons to outline each band you wish to analyze separately.

If you have different amounts of background on your gel, also draw small boxes under each band of interest using the background buttons. Then select the background box and corresponding band box while holding the shift key. Hit link bkgd. This should change both boxes to the same color.

To copy your data, click output and choose copy to clipboard.

Paste data into excel.

The average column = IDV/area. If your boxes are different sizes, you should use the IDV column to analyze your data.

In order to average data from multiple gels, you must have a common condition you can normalize to on each gel.
**Culture of CHO (Chinese Hamster Ovary) Cells**
Adapted from Robin Prince of Rich Lee’s lab

CHO cells grow in F-12 media supplemented with 10% FBS. 677 cells do not produce heparan sulfate and appear more boxy compared to the CHOK1 (WT) cells which look more spindle shaped.

**Starting culture**
Thaw cells in 37 °C water bath. – Do this fast (2 min or less)
Put one vial in 10 cm dish with 10 mL warmed F-12 + 10% FBS media.
Change medium next morning.
It may take several days for the cells to become confluent. (Because these cell lines are very stable it is not necessary to maintain them in drug-containing media).

**Splitting confluent cells**
Warm pbs, medium, and trypsin to 37 °C.
Aspirate off the media from a 10 cm dish of cells.
Add 2 mL trypsin and let incubate for 5 min at 37 °C.
Add 8 mL medium to stop trypsin, pipette up and down to de-clump.
Spin down cells at 125g for 5 min.
Resuspend in 10 mL medium, pipet up and down.
Typically split 1:4 or 1:5, can go up to 1:8 safely.

**Freezing cells**
You should do this during the first passage to keep your stock. You can take half the confluent cells in a 10 cm dish to freeze and replate the rest depending on your needs.

Rinse 10 cm dish with PBS. Put in 2 mL trypsin (37 °C for 5 min). Add 8 mL F12+10% FBS to stop. Spin down, resuspend in 10 mL F12+10% FBS. Count undiluted and should get ~6 million cells. Take 5 mL (3 million cells) to freeze down and put in a separate tube. Spin that down and resuspend in 0.6 mL (5 million cells/mL) of F12+20%FBS+10%DMSO. Put in cryovial and put in Mr. Frosty in -80 °C overnight. Transfer to liquid nitrogen storage.
Appendix D. Luminex Pilot Study

Adapted from Megan McBee and Arek Racynski of David Schauer’s lab

**Joint Extraction on Mice**

**Materials:**

We used the Biorad Lysis buffer kit.

1. 500 mM AEBSF (12 mg/100 µL water). Make 100 µL fresh.
2. **Lysing Solution (from Biorad kit)**
   = 40 µL Factor 1 + 20 µL Factor 2 + 9.9 mL Cell Lysis Buffer. Vortex gently. Add 40 µL 500 mM AEBSF per 10 mL Lysing Solution. Make 10 mL (for 20 samples)
3. Diluent = 0.05% Tween-20, 0.1% BSA in PBS. Make 50 mL fresh

**Methods:**

1. The joints were harvested by careful dissection to remove all attached tissues (particularly muscle). The "whole joint" preparation includes bone ends, patellar without proximal muscle, and all intra-articular structures (cartilage, cruciates, synovium, meniscus etc) and fluid. This preparation was flash frozen and then pulverized as a single sample. If possible, weigh the amount of tissue you collect prior to flash freezing.
2. Pulverize by 12 hammer blows.
3. Recover powder to tube and immediately add 350 µL (record known volume) of "Lysing Solution" (Reagent 2 above) to sample. Parafilm top of closed tubes to prevent any possible leakage. Rotate samples for 1-2 h at 4 °C for extraction.
4. Centrifuge samples at 4500g for 20 min at 4 °C.
5. Collect supernatant without disturbing pellet and remove clear supernatant. Weigh how much you collect.
6. Store at -80 °C.

**Before doing Luminex Assay:**

1. Thaw samples and perform BCA assay. Refreeze. (We used this to normalize and to get an idea of how much sample to load.)

**Luminex Assay:**

1. We used the 23-plex mouse cytokine pro kit which includes magnetic beads to be used in combination with a magnetic plate washer. It comes with very detailed instructions which we followed. We used the same lysis buffer to dilute samples and make up standard curves as explained a bit more below.

2. From the BCA assay our samples were in the range of 2.3-4.7 mg/mL which is a little on the high side of what to load, but since this was our first try with this we used either 50 µL straight sample in duplicates or 25 µL sample + 25 µL lysis buffer in duplicates. We also used both the high and low PMT standard curves for the same reason that we didn’t know what to expect. To use both of the standard curves you have to run the plate twice, but we didn’t notice any issues with doing this.
In the end, the data we used came from using the undiluted samples and the high PMT curves.

3. To make sure that all of the standards and samples were incubated with the beads for about the same amount of time, we pipetted everything into a separate 96 well plate and then used a multichannel pipet to load the samples/stds onto the actual plate. This will make more sense when you read the biorad protocol.

4. Also, you should change tips for everything as the assay is very sensitive.

5. Finally, we used n=3 since this was a pilot study, but this was probably too low to get significance on some of the cytokines.

Figure D1. Methods: IACUC approval was given by Rush University. Ten week old C57Bl/6 male mice were subjected to a surgical Destabilization-Induced Joint Injury (DMM) model developed by Glasson et al. alone, to treadmill (TM) running alone (14 days 15° incline, 32 cm/sec, 20 min per day, for 2 weeks), or to DMM surgery followed by TM running beginning on day 4 post-surgery. For DMM surgery, the anterior medial meniscotibial ligament was completely severed. Control mice were subjected to only cage activity. At 3 weeks post surgery, mice were euthanized and joints were stained with India Ink for gross observations or protein was extracted for Luminex (23-plex mouse cytokine kit), n=3. Statistics: Data were log-transformed and analyzed by ANOVA with Tukey post-hoc test, p<0.05. Luminex data were normalized to the total protein extracted per joint and to the control mice.