# Optimizing Chondrogenic Factors and Protein Delivery Methods for Cartilage Repair

by

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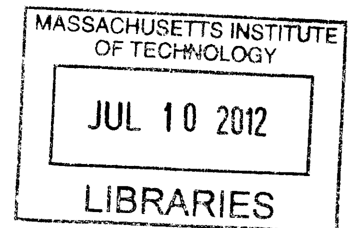
B.S.E. Biomedical Engineering
Tulane University, 2007

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

**Doctor of Philosophy in Biological Engineering**
at the
Massachusetts Institute of Technology

June 2012

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ABSTRACT

Joint injuries are common and often result in damage to cartilage, which has a limited ability to repair itself. Tissue engineering is a promising approach for improving cartilage healing in which biomaterials and chemical factors are supplied to direct cells to create a new tissue. The objective of this thesis was to optimize cartilage-like extracellular matrix production by investigating the effects of Dexamethasone (Dex) and HB-IGF-1 (heparin-binding insulin-like growth factor-1) on cells encapsulated in the self-assembling peptide RAD and agarose hydrogels.

Dex is a synthetic corticosteroid that has been shown to improve cartilage-like tissue production by bone marrow stromal cells (BMSCs), but the mechanisms underlying BMSC response to Dex are not understood. The hypothesis that the addition of Dex to chondrogenic medium would affect matrix production and aggrecanase activity of human and bovine BMSCs in RAD and agarose hydrogels was tested. The effects of Dex were dependent on the hydrogel material and the species/age of the BMSCs. Importantly, Dex reduced aggrecanase-mediated degradation of matrix in both agarose and RAD hydrogels and for both young bovine and adult human BMSCs.

HB-IGF-1, a fusion protein of the heparin binding domain of HB-EGF and IGF-1, can be retained in cartilage matrix and stimulate proteoglycan synthesis with a single dose, whereas unmodified IGF-1 easily diffuses out of cartilage tissue. The RAD peptide was used as a scaffold for retaining growth factor to stimulate encapsulated chondrocytes and adjacent cartilage tissue. RAD was modified by adsorption of HB-IGF-1 before and after RAD assembly, as well as adsorption of heparan sulfate (HS) and IGF-1. The RAD material retained HS adsorbed pre-assembly and HB-IGF-1 delivered in both adsorption methods. Adsorbed HB-IGF-1 and IGF-1 led to increased aggrecan content regardless of the method of adsorption. A trend was found for increased proteoglycan synthesis in adjacent explants as well.

RAD self-assembling hydrogels are a promising material for culturing BMSCs undergoing chondrogenesis, retaining, and delivering HB-IGF-1. Dex decreases aggrecanase activity of differentiating BMSCs and adsorbed HB-IGF-1 appears to enhance aggrecan production by encapsulating chondrocytes and adjacent tissue. These findings show potential for improving cartilage repair in vivo.

Thesis Supervisor: Alan J. Grodzinsky
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## Acknowledgements

I decided in 8th grade that I wanted to be a biomedical engineer. I loved math and science, especially biology (leading to a previous leaning towards veterinary medicine), and I wanted my job to help people. An engineer from Intel came to my school and talked about how engineers solved problems for companies, which led me to investigate all the varieties of engineering and to find the field that would become the focus of my college studies and beyond. Many people have helped me along this path and I would like to specifically thank some of them below.

During my summers at the Oregon Medical Laser Center, Kathy McKenna and Becki Rowe introduced me to cell culture and the importance of thoughtful experimental design (controls!). They were among my first role models of female scientists and I am lucky to have benefited from their advice and experience, as well as the support of everyone at OMLC. Both my undergraduate advisers, Darryl Overby and Michael Caplan, encouraged me to pursue a PhD, and to apply to MIT in particular.

The BE faculty and students make the department here a stimulating and supportive place to work. Doug Lauffenburger explained the "cue, signal, response" paradigm to me using linear algebra concepts during my interview weekend and opened a whole new way of thinking for me. All of the BE faculty have so much passion and enthusiasm for research and when I left that weekend, I was excited about returning to learn more.

Al Grodzinsky has been a fantastic adviser throughout my time at MIT. He provides space for designing a research path and learning on your own, but his door is always open for guidance. I have benefited greatly from interactions with my fellow lab members, especially the tissue engineering group. Rachel Miller, Paul Kopesky, and especially Eric Vanderploeg taught me all the skills I needed for cartilage tissue engineering, patiently answered my questions, and taught me how to think about scientific problems from multiple points of view.

Darrell Irvine, Chris Evans, John Kisiday, and Parth Patwari have provided important perspective to my work as members of my thesis committee and collaborators. I thank them for their time and ideas, which have encouraged me to think about new hypotheses.

The entire class of 2007 has helped to make the time at MIT fun and rewarding. I am grateful to have such great colleagues and friends. During my first semester at MIT, Michelle Sukup Jackson and Edgar Sanchez were my constant companions and their friendship and support helped me survive the long nights in the dungeon.

My parents, sister, and boyfriend have been unwavering in their support throughout my PhD, listening to the details of failed experiments and sharing in my enthusiasm for the ones that worked. Thank you for helping me achieve the most challenging goal I've ever attempted.

My work was funded in part by a Graduate Research Fellowship from the National Science Foundation, the National Institutes of Health, and Presidential and Whitaker Health Sciences Fund Fellowships from MIT.

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# Chapter 1: Introduction*

Bone marrow stromal cells (BMSCs) are an established cell choice for cartilage repair because they are easily harvested, expanded, and differentiated into a cartilage phenotype characterized by aggrecan and type II collagen production. Transforming growth factor β (TGF-β), insulin-like growth factor 1 (IGF-1), and Dexamethasone (Dex) all influence the process of chondrogenesis. Although difficult challenges remain for optimizing the use of BMSCs for cartilage tissue engineering, in vitro culture systems present an excellent opportunity for studying chondrogenesis and understanding how progenitor cells respond to their biological, chemical, and mechanical microenvironment.

## 1.1 Motivation for Cartilage Tissue Engineering

Cartilage is an avascular, aneural tissue composed of a sparse population of chondrocytes and dense extracellular matrix. Joint injuries are common and often involve damage to cartilage, which is a risk factor for osteoarthritis. This joint disease affects ~27 million people and is a growing concern in the United States due to the aging population.[1] Cartilage has a limited ability to repair itself after damage caused by injury or disease. Current therapies for treating cartilage defects include microfracture and autologous chondrocyte implantation, which result in short-term pain relief, but not long-term efficacy.[2] The last resort for joint pain caused by osteoarthritis is complete joint replacement. Cartilage tissue engineering has emerged as a promising method for aiding the body's natural healing process by supplying a combination of cells, scaffold, and/or other stimuli (chemical, mechanical) to encourage tissue regeneration. Research to determine the optimal cell source, scaffold material, and growth factors is ongoing.[3] The focus of this chapter is on the primary chondrogenic factors

* This chapter has been published as Florine, E.M. and A..J. Grodzinsky, *The role of chondrogenic factors in differentiation of bone marrow stromal cells to the cartilage lineage*. Stem Cells and Cancer Stem Cells, ed. M.A. Hayat. Vol. 7. 2012: Springer.

that are used to initiate and enhance chondrogenesis of BMSCs for cartilage repair: growth factors TGF-β and IGF-1, and the pro-chondrogenic, anti-inflammatory corticosteroid Dex.

### 1.2 Bone Marrow Stromal Cells

Bone marrow stromal cells (BMSCs) are most commonly isolated by separating the cellular content of the bone marrow from the fat and plasma components of the tissue via centrifugation, and then separating the BMSCs from the cell population by plastic adhesion.[3] The adherent cells grow in colonies and have the ability to differentiate into multiple phenotypes, including chondrocytic, osteocytic, and adipocytic lineages.[4] BMSCs can be easily harvested from a variety of marrow sites. It should be noted that these cells have been and continue to be referred to by other names such as bone marrow stem cells (also BMSCs) or mesenchymal stem cells or stromal cells (MSCs). The International Society for Cytotherapy established a set of minimal criteria for MSCs, including positive and negative surface molecule expression profiles, but a cell population meeting these criteria is still heterogeneous.[5] The BMSC cell population consists of cells with different proliferation and differentiation capacities, whose composition can be affected by culture conditions.[6] Despite the heterogeneity and lack of definition of the BMSC population, these cells have proven to be capable of expansion and differentiation and are a valuable tool for tissue regeneration.

Bone marrow stromal cells offer several advantages over chondrocytes for cartilage regeneration therapies in that they can be expanded for several passages in vitro without loss of differentiation potential and their harvest does not involve damage to the joint, whereas cartilage biopsies performed to harvest chondrocytes can potentially initiate cartilage degradation at the harvest site. Adult progenitor cells have been found in a wide variety of tissues and have been compared to BMSCs. The different progenitor cell populations have

been found to have different chondrogenic potentials under the same culture conditions. Several studies have found synovial MSCs to have higher chondrogenic capacity than BMSCs,[7] but harvesting synovial cells does involve surgery on the joint. In a comparison of BMSCs and adipose-derived progenitor cells (ADPCs), it was found that BMSCs were superior to ADPCs in terms of protein synthesis of aggrecan and type II collagen when cultured with TGF-β1.[8]

### 1.3 Chondrogenesis

Chondrogenesis, or the differentiation of progenitor cells along the chondrocytic lineage, is dependent on a three-dimensional culture environment and presence of chondrogenic factors.[5] In embryonic development, mesenchymal cells condense, differentiate, produce extracellular matrix, and proliferate. Cells that will become the growth plate and bone begin to undergo hypertrophy, ultimately leading to mineralization, chondrocyte apoptosis, and vascularization of the tissue.[9] A challenge in BMSC chondrogenesis is maintaining a stable hyaline cartilage phenotype and not progressing into hypertrophic cartilage. Expression of type X collagen, matrix metalloproteinase-13 (MMP-13), and alkaline phosphatase activity are characteristic signs of hypertrophy.[5] Type X collagen gene expression and protein synthesis occur during BMSC chondrogenesis, and implanting these constructs ectopically in severe combined immunodeficiency (SCID) mice can result in mineralized tissue.[10] A better understanding of hypertrophy regulation will lead to strategies for implementing a stable hyaline cartilage phenotype.

Chondrogenesis is defined functionally in terms of gene expression and protein synthesis. Extracellular matrix molecules that make up the majority of cartilage include the abundant compression-resistant proteoglycan aggrecan and tensile load-bearing type II collagen fibrils,

so these are natural markers of chondrogenesis. In addition to these molecules, a rise in SRY (sex determining region Y)-box 9 (Sox9) expression early in the differentiation process and suppression of genes associated with other phenotypes (such as peroxisome proliferator-activated receptor (PPAR) -γ for adipogenesis or osteocalcin for osteogenesis) is expected.[11] Type I collagen expression is expected to be much lower than type II collagen expression since type I collagen is produced during the development process, but is not found in mature hyaline cartilage. Thus, collagen typing helps to differentiate between fibrocartilage, which contains collagen I, and hyaline cartilage, which contains no collagen I. Avoiding fibrocartilage repair in vivo remains a key challenge in cartilage tissue engineering.[12]

### 1.4 Transforming Growth Factor-β

Transforming growth factor-β (TGF-β) is a commonly used growth factor for inducing chondrogenic differentiation of BMSCs in vitro. There are several isoforms of TGF-β, with TGF-β1, 2, and 3 capable of inducing upregulated aggrecan synthesis in BMSCs. A study comparing different isoforms of TGF-β on pellet cultures of human BMSCs showed that TGF-β3 was superior to TGF-β1 for chondrogenesis in that system,[13] though both TGF-β1 and 3 are commonly used. In humans, TGF-β is produced in latent form due to its binding to a latency-associate peptide; TGF-β can be released by protease activity or changes in pH.[14]

TGF-β initiates a signaling cascade by binding to TGF-β type II receptors, which then recruit and phosphorylate the TGF-β type I receptors. These receptors are serine/threonine kinases. TGF-β binding to the TGF-β receptors induces the phosphorylation of Smad2 and Smad3, which then form homo-oligomers, recruit Smad4, and enter the nucleus where they can bind to DNA or interact with other proteins to influence transcription.[14] While the intracellular signaling that leads to chondrogenic differentiation following TGF-β stimulation is still being

investigated, the mitogen-activated protein kinases (MAPK) p38, extracellular signal-regulated kinase-1 (ERK-1), c-Jun N-terminal kinase (JNK), and Wnt pathways appear to be involved.[15] TGF-β induces the gene and protein expression profiles described above that are associated with chondrogenesis.[11] Importantly, TGF-β also stimulates chondrocyte proliferation and extracellular matrix production and has been shown to inhibit cartilage matrix degradation.[16]

TGF-β has been delivered to BMSCs in a variety of ways, with the most common being in soluble form in the culture medium for in vitro studies. While soluble delivery of TGF-β enhances chondrogenesis of BMSCs and therefore has the potential to aid in cartilage healing, delivery of TGF-β into joints in vivo can have negative effects. For example, adenovirus-mediated intra-articular gene delivery of TGF-β1 resulted in fibrogenesis, muscle edema, nitric oxide production, and chondrogenesis of the synovial lining.[17] In a recent rabbit study, TGF-β1 and other chondrogenic factors were adsorbed to a self-assembling peptide scaffold and delivered with or without allogenic BMSCs in a full-thickness model of cartilage repair. Joints receiving only TGF-β1 and chondrogenic factors adsorbed to the peptide were not statistically different from joints receiving peptide alone, but both demonstrated better healing than untreated contralateral controls. Joints that received BMSCs, TGF-β1, and chondrogenic factors demonstrated poorer healing quality than joints that received only TGF-β1 and chondrogenic factors. Interestingly, when two joints in the same animal were treated with BMSCs, TGF-β1, and chondrogenic factors, evidence of inflammation and osteophytes were present.[12] Taken together, these examples show that understanding the dose-dependent effects that result from TGF-β interacting with various cell types, cytokines, and growth factors in the joint is critical for using TGF-β for therapeutic purposes.

Due to the short half-life of TGF-β in vivo and the potential for negative effects, alternative delivery methods have been studied. Researchers have investigated the strategies of loading TGF-β into microspheres that are embedded within a scaffold, TGF-β-binding proteins, and TGF-β gene delivery, among other methods, to provide sustained, local delivery of the growth factor (see **Fig. 1.1A**).[18-20] These methods have shown success both in vitro and in vivo. Aside from the goal of local delivery, the length of time that TGF-β is supplied to BMSCs also appears to be important. There is evidence that supplying TGF-β3 for a limited time during the culture duration results in more matrix production than continuous delivery.[21] Work by Kopesky *et al.*[22] has shown that only four days of TGF-β1 supplementation to the culture medium was sufficient for young bovine and adult equine BMSCs to accumulate ~60% of the aggrecan content of self-assembling hydrogels receiving TGF-β1 continuously at day 21. When TGF-β1 was adsorbed to agarose, the accumulation of G1-NITEGE aggrecan fragments generated by aggrecanase (ADAMTS-4/5) activity was reduced compared to continuous TGF-β1 supplementation.[22] These studies motivate further research into the mechanisms of how transient TGF-β delivery affects chondrogenesis and catabolic pathways.

### 1.5 Insulin-like Growth Factor-1

Insulin-like growth factor (IGF) has two isoforms, with IGF-1 capable of stimulating increased proteoglycan synthesis and growth and IGF-2 acting as a glucose regulator and growth stimulator.[23] IGF-1 is known to be an essential growth factor for maintaining cartilage homeostasis. It is synthesized by the liver and enters the circulation, and it can also be produced in other tissues like cartilage. IGF binding proteins (IGFBPs) can sequester IGF-1 within cartilage matrix and thereby regulate transport of the growth factor within the tissue and to the target cell receptors. These IGFBPs can also be found in the circulation and

in the synovial fluid. Proteases that act on the IGF binding proteins and IGF-1 itself add another layer of complexity.[23] Levels of IGF-1 in the synovial fluid are increased in patients with osteoarthritis, but the chondrocytes are hyporesponsive to it, which may be a result of increased IGF binding protein production and the resulting immobilization of IGF-1.[16, 23]

IGF-1 can bind to the IGF-1 receptor (IGF-1R) or the insulin receptor. IGF-1Rs on the cell membrane form dimers and signal via a tyrosine kinase domain. IGF-1 binding to IGF-1R results in activation of the phosphoinositide 3-kinase (PI3K)/Akt pathway, which results in upregulation of proteoglycan synthesis, and the ERK pathway.[24] IGF-1 has been found to play a role in cartilage development[25] and is also important in more mature cartilage tissue, where it upregulates type II collagen and proteoglycan synthesis, reduces proteoglycan degradation, and promotes cell survival.

IGF-1 has been delivered in an equine cartilage repair model by loading it into a fibrin clot. This strategy was found to enhance healing compared to fibrin clots without IGF-1, possibly through stimulation of native progenitor cells in the subchondral bone.[25] Intra-articular injections of IGF-1 have shown little efficacy, but have not caused any harm either.[26] Adenoviral gene delivery of IGF-1 to BMSCs has not proven efficacious.[20] Studies of BMSCs undergoing chondrogenesis in the presence of IGF-1 have been mixed, which may be explained in part by the variable culture conditions, including cell species, IGF-1 delivery method, scaffold, and presence of other growth factors. Several studies have investigated the potential of TGF-β and IGF-1 to synergistically enhance chondrogenesis with some success. Worster *et al.*[27] found that equine BMSCs grown in monolayer in the presence of TGF-β1 and then seeded into fibrin disks with adsorbed IGF-1 had increased proteoglycan production and procollagen II mRNA levels compared to either growth factor alone. Despite some

success with IGF-1 delivery, further investigation of the interactions between IGF-1 and other growth factors, as well as the timing and specific cell source for optimal tissue repair is needed.

Similar to TGF-β, IGF-1 easily diffuses out of the joint due to its small size, allowing for the possibility of negative systemic side-effects and limiting its ability to affect cartilage repair. A new strategy for sustained and local delivery of growth factors, such as IGF-1, to cartilage is to add a heparin-binding domain to the growth factor. As an example, HB-IGF-1 is a fusion protein consisting of the heparin-binding domain of heparin binding epidermal growth factor-like growth factor (HB-EGF) and IGF-1 (see **Fig. 1.1B**).[28] The positively charged HB domain binds reversibly to the negatively charged chondroitin sulfate glycosaminoglycan chains of aggrecan proteoglycans in the cartilage matrix, allowing HB-IGF-1 to be retained in cartilage.[29] HB-IGF-1 has been shown to cause sustained upregulation of proteoglycan synthesis in cartilage explants six days after washing unbound growth factor out of the culture.[28] In addition, intraarticular injection of HB-IGF-1 into rats has also resulted in retention and sustained bioactivity. Studies to investigate the binding and release of this molecule from self-assembling peptide hydrogels and to optimize its delivery to chondrocytes are ongoing.

## 1.6 Dexamethasone

Dexamethasone (Dex) is a synthetic glucocorticoid, analogous to cortisol, which is frequently added to cartilage tissue engineering cultures in vitro because of its demonstrated ability to enhance TGF-β-induced chondrogenesis.[30] Glucocorticoids (GCs) pass through the lipid bilayer of cell membranes and can bind to the glucocorticoid receptor (GR) in the cytoplasm. The binding causes release of chaperone proteins and translocation of the GC/GR complex to

the nucleus where it can bind to DNA and/or interact with transcription factors to regulate transcription. The mechanisms and interactions between GCs and other cellular components are not completely understood,[31] though the work of Derfoul *et al.*[32] did show that the actions of Dex on chondrogenesis were mediated by the GR.

Dex, in combination with TGF-β1, has been shown to stimulate pellet cultures of rabbit BMSCs to grow larger than those cultured in TGF-β1 alone.[30] Dex on its own is not sufficient to induce chondrogenesis of BMSCs, however. Gene expression and protein synthesis of aggrecan by bovine BMSCs in agarose hydrogel culture are elevated further by TGF-β1 and Dex than by TGF-β1 alone.[33] Polymeric microspheres delivering a combination of TGF-β3 and Dex have been studied in vitro and in vivo; this approach has successfully promoted gene expression and protein synthesis consistent with the chondrogenic phenotype (see **Fig. 1.1C**).[34] Dex has been shown to have anti-catabolic and pro-anabolic properties. In self-assembling peptide hydrogels, the combination of TGF-β1 and Dex dramatically reduced the aggrecanase-generated aggrecan fragment G1-NITEGE compared to TGF-β1 alone.[35] This work also showed that the effects of Dex on chondrogenesis are scaffold-dependent. In agarose, Dex increased proteoglycan content, as expected, but caused a modest decrease in proteoglycan content in self-assembling peptide hydrogels. Further studies have demonstrated that the influence of Dex is also dependent on cell species/age.

## 1.7 Discussion

Cartilage tissue engineering is an evolving field with many open questions. Researchers continue to optimize interactions between cells, growth factors, and scaffold materials in attempts to obtain a neotissue that can effectively replace native cartilage. This chapter has focused on BMSCs as an important progenitor cell type and the well-studied chondrogenic

factors TGF-β, IGF-1, and Dex. It is important to remember that there are many other growth factors of interest as well, including bone morphogenic proteins (BMPs), fibroblast growth factors (FGFs), and platelet-derived growth factor (PDGF).[2] In addition, cartilage is a mechanically-functional tissue and researchers have had a long-standing interest in understanding how chondrocytes and their progenitors respond to mechanical stimuli. Much remains to be learned about mechanotransduction in BMSCs, but initial studies have shown that dynamic compression can further enhance ECM gene expression and protein synthesis by BMSCs during later stages of chondrogenic stimulation by TGF-β and Dex.[33]

While BMSCs stimulated with growth factors such as TGF-β, IGF-1, and Dex show promising in vitro results, many challenges remain for translation to successful in vivo cartilage repair. Obtaining a regenerated tissue that integrates well with the surrounding tissue, maintains a stable hyaline cartilage phenotype over time, and has the same mechanical properties as the native tissue is for now an elusive goal. Nevertheless, these in vitro culture systems provide great potential for studying the underlying biology of progenitor cells and how interactions with their environment change their behaviour. Harnessing this knowledge will allow us to move forward in the growing field of regenerative medicine.

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## 1.9 Figures

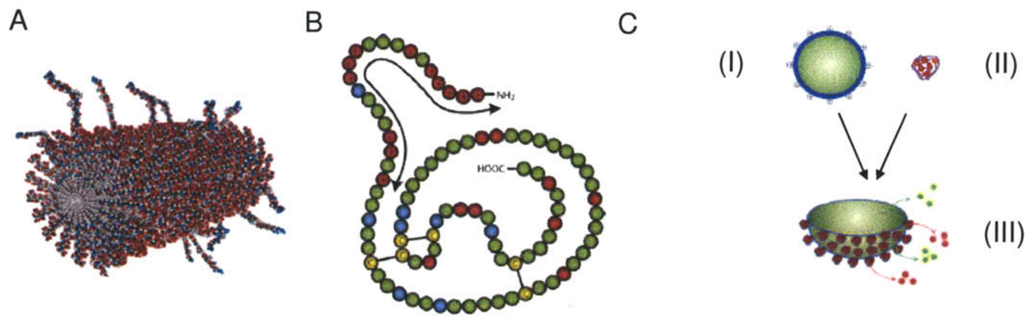


**Figure 1.1**: Growth Factor Delivery Methods. (A) Illustration of TGF-β1-binding and non-bioactive peptide amphiphile co-assembly with binding epitopes exposed on the surface (Shah, *et al.*, 2010). (B) Schematic of HB-IGF-1 amino acid sequence with arrow selecting the HB-domain, red circles as positive amino acids, blue circles as negative amino acids, and yellow circles as cysteine (Tokunou, *et al.*, 2008). (C) Outline of fabrication of microparticles delivering Dex and TGF-β. (I) PEI coated FITC-bound DEXA-PLGA microsphere, (II) Cy5.5-bound heparanized TGF-β, (III) nanoparticle coated microsphere 3D scaffold for dual release of dexamethasone and growth factor (Park, *et al.*, 2009).

## Chapter 2: Effects of Dexamethasone on Mesenchymal Stromal Cell Chondrogenesis and Aggrecanase Activity: Comparison of Agarose and Self-Assembling Peptide Scaffolds*

Dexamethasone (Dex) is a synthetic glucocorticoid that has pro-anabolic and anti-catabolic effects in cartilage tissue engineering systems, though the mechanisms by which these effects are mediated are not well understood. We tested the hypothesis that the addition of Dex to chondrogenic medium would affect matrix production and aggrecanase activity of human and bovine bone marrow stromal cells (BMSCs) cultured in self-assembling peptide and agarose hydrogels. We cultured young bovine and adult human BMSCs in $(RADA)_4$ self-assembling peptide and agarose hydrogels in medium containing TGF-β1±Dex and analyzed extracellular matrix composition, aggrecan cleavage products, and the effects of the glucocorticoid receptor antagonist RU-486 on proteoglycan content, synthesis, and catabolic processing. Dex improved proteoglycan synthesis and retention in agarose hydrogels seeded with young bovine cells, but decreased proteoglycan accumulation in peptide scaffolds. These effects were mediated by the glucocorticoid receptor. Adult human BMSCs showed minimal matrix accumulation in agarose, but accumulated ~50% as much proteoglycan and collagen as young bovine BMSCs in peptide hydrogels. Dex reduced aggrecanase activity in $(RADA)_4$ and agarose hydrogels, as measured by anti-NITEGE Western blotting, for both bovine and human BMSC-seeded gels. The effects of Dex on matrix production are dependent on cell source and hydrogel identity. This is the first report of Dex reducing aggrecanase activity in a tissue engineering culture system.

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* This chapter will be published as Florine, E.M., Miller R.E., Porter, R.M., Evans, C.H., Kurz, B., and Grodzinsky, A.J., *Effects of Dexamethasone on Mesenchymal Stromal Cell Chondrogensis and Aggrecanase Activity: Comparison of Agarose and Self-Assembling Peptide Scaffolds*. Cartilage. (In Press).

## 2.1 Introduction

Tissue engineering using bone marrow-derived stromal cells (BMSCs) is an attractive strategy for healing cartilage defects. BMSCs have been shown to differentiate to a cartilage lineage in a variety of scaffolds and create cartilage-like extracellular matrix (ECM).[1-4] These cells can be extracted without the need for further damage to the joint and expanded for several passages without losing differentiation potential.[5,6] Additionally, BMSCs have been found to synthesize ECM with higher dynamic stiffness, longer sulfated glycosaminoglycan (sGAG) chains, and longer aggrecan core proteins than matrix synthesized by chondrocytes, regardless of donor age.[7] Despite progress in cartilage tissue engineering, further optimization is needed to improve integration with native tissue, mechanical function, and maintenance of the chondrocyte phenotype.[8,9]

One strategy for improving our understanding of these complex systems is to investigate the specific effects of chemical factors added to the cellular microenvironment. Dexamethasone (Dex) is a synthetic glucocorticoid frequently added to culture medium for chondrogenesis studies, motivated by research demonstrating pellet cultures of rabbit BMSCs stimulated with TGF-β1 and Dex (TGF+Dex) grew larger than those cultured with TGF-β1 alone.[2] Additional studies found that TGF+Dex increased aggrecan biosynthesis and gene expression in bovine and human BMSCs over TGF-β1 alone.[10,11]

Dex also has anti-catabolic properties. The aggrecanases ADAMTS-4/5 (a disintegrin and metalloproteinase with thrombospondin motifs-4/5) are key destructive enzymes in human osteoarthritis progression and are involved in cytokine-induced aggrecanolysis in cartilage explants.[12-14] ADAMTS-4/5–generated aggrecan fragments are also found in BMSC-seeded hydrogels.[3] Recently, Dex was found to reduce sGAG loss and rescue proteoglycan synthesis

in cartilage explants exposed to inflammatory cytokines.[15] The mechanisms through which Dex mediates pro-anabolic and anti-catabolic effects is not well understood, though evidence that the glucocorticoid receptor mediates the increase in aggrecan mRNA levels caused by Dex has been reported.[16]

We have used self-assembling peptide hydrogels for cartilage tissue engineering because they support TGF-β1-induced chondrogenesis of BMSCs in vitro[3] and have been successfully used for studies of cartilage repair in animal studies.[17, 18] Additionally, self-assembling peptides have been used to deliver growth factors,[18-21] which is important for designing an optimal chondrogenic microenvironment as well as controlling sustained, local delivery of growth factors. While scaffold-free, or pellet, cultures offer important culture systems for studying chondrogenesis, our objective was to investigate BMSC matrix production and catabolism in hydrogel scaffolds motivated by the long-term translational challenges of growth factor delivery and integration between neocartilage and adjacent native cartilage. We tested the hypothesis that chondrogenesis of human and bovine BMSCs in self-assembling peptide hydrogels, as well as subsequent matrix production and aggrecanase activity, would be responsive to chondrogenic medium supplemented with Dex. We compared the influence of Dex on chondrogenesis of young bovine BMSCs and adult human BMSCs in $(RADA)_4$ self-assembling peptide hydrogels and agarose hydrogels. By analyzing accumulation of sGAG, DNA, and collagen, the effects of the glucocorticoid receptor antagonist RU-486, and aggrecan cleavage products, we found that cell source and scaffold environment changed the BMSC response to Dex, emphasizing the importance of optimizing these variables for neocartilage generation. Dex reduced ADAMTS-4/5 activity in $(RADA)_4$ self-assembling peptide and agarose hydrogels for both cell types.

## 2.2 Methods

*Bovine BMSC Isolation and Expansion*

Bovine BMSCs were extracted from the femora and tibiae of four 1-2 week old calves (Research 87, Marlborough, MA) as described previously.[3] Briefly, marrow was isolated aseptically, homogenized in phosphate-buffered saline (PBS), centrifuged, and the cell fraction was plated on tissue culture plastic for 30 minutes to remove rapidly adhering cells. The remaining cell population was plated into flasks at $1 \times 10^6$ mononuclear cells/cm$^2$. Remaining red blood cells were removed by a medium change two days after plating. Colonies were expanded in low glucose Dulbecco's modified Eagle's medium (DMEM) (Mediatech, Inc, Manassas, VA) with 10% fetal bovine serum (Invitrogen, Carlsbad, CA or ThermoScientific, Logan, UT), penicillin streptomycin amphotericin (PSA) (Sigma-Aldrich, St. Louis, MO), 4-(2-hydroxyethyl)-l-piperzaineethanesulfonic acid (HEPES) (Invitrogen), and 1ng/mL basic fibroblast growth factor (bFGF) (R&D Systems, Minneapolis, MN) until reaching 80% confluence as described previously.[3] Cells were then removed by 0.05% trypsin/0.53mM ethylenediaminetetraacetic acid (EDTA) (Invitrogen) and frozen for future use (passage 0, P0). After thawing frozen P0 aliquots, cells were seeded at 6000 cells/cm$^2$ and expanded two passages consisting of 3 days each in expansion medium with 5ng/mL bFGF. BMSCs were then seeded into hydrogels for chondrogenesis studies.

*Human BMSC Isolation and Expansion*

Human BMSCs (hBMSCs) were isolated from intramedullary aspirate generated using a Reamer Irrigator Aspirator device during surgical procedures performed at Brigham and Women's Hospital and Massachusetts General Hospital (Boston, MA).[22] The three patients whose cells were used in this study (two males, one female, ages 81, 51, and 37) granted informed consent and surgical procedures were preapproved by the local Institutional Review

Board. The bulk aspirate was centrifuged, the red blood cell fraction lysed briefly in a buffer of 155mM $NH_4Cl$, 10mM $KHCO_3$, and 0.1mM EDTA (pH 7.2), and BMSCs isolated from the remaining nucleated cell fraction by differential adhesion to tissue culture plastic as above. These donor cell populations have been confirmed to undergo chondrogenic differentiation in pellet cultures in response to TGF-β1 and Dex.[23] Frozen aliquots of P1 or P2 cells were thawed and seeded at 1000 cells/$cm^2$ in expansion medium with 5ng/mL bFGF. Cultures received supplements of bFGF on day 4, passaged on day 6, given more bFGF on day 9 or 10, and were seeded into hydrogels on day 12. One patient's cells required further expansion and were passaged on day 12, given more bFGF on day 14, and cast on day 17.

*Hydrogel Encapsulation and Culture*

Following BMSC expansion in monolayer, cells were encapsulated in one of two hydrogel materials: $(RADA)_4$ self-assembling peptide hydrogel (RAD, also known as PuraMatrix, a gift from 3DM, Cambridge, MA) and low melting point agarose (Invitrogen, catalog number 15517-022). BMSCs were encapsulated in 0.5% (w/v) RAD or 2% (w/v) agarose at $10^7$ cells/mL. These hydrogel concentrations were chosen to match previous studies.[3, 4, 11, 24, 25] The cell/hydrogel mixture was cast as disks (~6mm diameter, 1.5mm thickness) into the center of rings of acellular agarose pre-equilibrated in chondrogenic medium consisting of high glucose DMEM, 1% ITS+1 (Sigma-Aldrich; insulin, transferrin, sodium selenite, bovine serum albumin, and linoleic acid), proline (Sigma-Aldrich), ascorbate-2-phosphate (Wako Chemicals, Richmond, VA), HEPES, PSA, non-essential amino acids (NEAA) (Sigma-Aldrich), and sodium pyruvate (Invitrogen), as described previously.[3] The cell-seeded hydrogel disks were cultured in chondrogenic medium with 10ng/mL recombinant human TGF-β1 (R&D Systems) with or without 100nM Dexamethasone (Sigma-Aldrich), labeled TGF+Dex or TGF, respectively. Hydrogels were cultured for up to 21 days with medium

changes every 2-3 days. Some hydrogels were immediately cast into buffer containing 50mM tris(hydroxymethyl)aminomethane (Tris) and 1mM $CaCl_2$ to measure day 0 DNA levels.

*Viability Staining*

To assess viability of BMSCs for all culture conditions, hydrogels were viewed using a Nikon Eclipse TE-300 fluorescence microscope at ~day 4 with 4μg/mL fluorescein diacetate (live cells) and either 0.875μg/mL ethidium bromide or 35μg/mL propidium iodide (dead cells).

*Glucocorticoid Receptor Antagonist Culture*

RU-486 (Mifepristone; Sigma-Aldrich), a glucocorticoid receptor antagonist with partial agonist activity,[26, 27] was added to the culture medium to determine whether observed responses to Dex were mediated by the glucocorticoid receptor. A dose-response study confirmed that 1μM RU-486 would not interfere with BMSC viability or sGAG synthesis. This dose has been used previously.[16] Bovine BMSCs were encapsulated in $(RADA)_4$ or agarose hydrogels as described above and cultured in medium with 10ng/mL TGF-β1 or 10ng/mL TGF-β1 with 100nM Dex with and without RU-486 for 21 days.

*Hydrogel Biochemistry*

For the final 24 hours of culture, hydrogels used for biochemical analysis were radiolabeled with 5μCi/mL $^{35}S$-sulfate and 10 or 20μCi/mL $^{3}H$-thymidine (Perkin Elmer Inc, Waltham, MA) to measure proteoglycan and DNA synthesis, respectively. Unincorporated radiolabel was removed, hydrogels were weighed wet, lyophilized, weighed dry, and digested with Proteinase-K (Roche Applied Science, Indianapolis, IN) as described previously.[3] Digested

samples were assayed for sGAG content by 1.9-dimethylmethylene blue (DMMB) dye binding,[28] DNA content by Hoescht dye binding,[29] and radiolabel incorporation by liquid scintillation counting. Hydroxyproline content, as a measure of total collagen, was measured by reaction with p-dimethylaminobenzaldehyde.[30] sGAG released to the culture medium was measured by DMMB.

*Histology and Immunohistochemistry*

Day 21 hydrogels from one experiment were fixed in 4% paraformaldehyde for 2 hours at room temperature and overnight at 4℃. Osteochondral explants were collected from the distal femurs of 1-2 week old bovine calves for collagen staining controls. Explants were fixed in 4% paraformaldehyde, transferred to 70% ethanol, and demineralized. Gels and explants were then embedded in paraffin, sliced into sections, deparaffinized, and stained as described previously in detail.[3] Briefly, for immunohistochemistry, samples were stained for type I or type II collagen; negative controls where incubated without primary antibodies.[31] Additional sections were stained for sulfated proteoglycans using toluidine blue dye solution.[32] For detection of apoptotic cells, sections were stained with haematoxylin. Cells with and without nuclear blebbing were counted using a Zeiss Axiophot microscope with 40x objective (Zeiss, Wetzlar, Germany). For quantification of nuclear blebbing,[33] sections from three different gels per experimental group were evaluated by counting cells in three adjacent, distinct fields of vision in the center of each section. For each individual gel/sample the percentage of apoptotic cells was calculated and the mean value for each experimental group was given (n=3).

*Aggrecan Extraction and Western blot Analysis*

Additional hydrogels were cultured for 21 days, soaked in PBS with Complete Protease Inhibitors (Roche) for ~2 hours, and frozen at -20°C until extraction. Hydrogels were rotated in 4M guanidine hydrochloride with 100mM sodium acetate plus Protease Inhibitors for two days at 4°C to extract proteoglycans. After centrifugation at 13,000 *g* for 30 mins, the supernatant was removed and its sGAG content was measured by DMMB. Aggrecan extract was then run through microcentrifuge tubes with a 10,000 MW cut-off (Millipore, Billerica, MA). Retained protein was washed twice with buffer containing 0.05M Tris, 0.05M sodium acetate, and 0.01M EDTA and resuspended in this buffer at a concentration of 1μg sGAG/μL. The aggrecan was then deglycosylated using protease-free chondroitinase ABC (30mU/100μg sGAG), keratanase II (0.5mU/100μg sGAG), and endo-β-galactosidase (0.5mU/100μg sGAG) (Seikagaku Biobusiness Corporation, Tokyo, Japan). sGAG was loaded into a 4-12% Bis-Tris gel (Invitrogen) and run at 200V for 45 mins. Proteins were transferred to a polyvinylidene fluoride membrane and probed with the anti-NITEGE monoclonal antibody AGG-C1[34] (a gift from Dr. Carl Flannery, Pfizer) and the anti-G1 antibody G1-2 (a gift from Dr. John Sandy, Rush University).[35] Some membranes were stripped following anti-NITEGE imaging and re-probed with anti-G1 antibody. To ensure the removal of the NITEGE antibody following stripping, membranes were exposed to the secondary antibody again and imaged to ensure no signal.

*Statistical Analysis*

Results are reported as mean±standard error of the mean. A linear mixed model of variance with animal/patient as a random factor and medium condition and timepoint as fixed effects was used to analyze data for experiments testing the effects of Dex on sGAG content, DNA content, proteoglycan synthesis, DNA synthesis, sGAG retention, and hydroxyproline content

for bovine and human BMSCs. Data from the agarose and RAD scaffolds were analyzed separately and not compared statistically. Data from experiments with RU-486 were analyzed using the same model with only the medium condition as a fixed effect. Residual plots for all of the above comparisons were investigated and data were transformed as necessary to ensure normality. Apoptotic cell data were analyzed using a general linear model with medium condition as an independent variable. A Kolmogorov-Smirnov test was used to ensure normality and data were transformed as necessary. Tukey post hoc tests with $p<0.05$ were used to evaluate statistical significance for all pairwise comparisons. Statistical tests were performed using Systat 12 software.

## 2.3 Results

*Matrix and Cellular Content of Bovine BMSC-Seeded Hydrogels*

sGAG content increased with time in both scaffolds seeded with bovine BMSCs, though there was no statistical difference between days 14 and 21 in agarose hydrogels. By day 21, sGAG content of RAD hydrogels was nearly 150% of agarose hydrogels. Consistent with previous literature,[11] TGF+Dex significantly increased sGAG accumulation over TGF alone in agarose hydrogels (**Fig. 2.1A**). In RAD, a decrease in sGAG accumulation was observed with Dex compared to TGF alone on days 14 and 21. DNA content did not change significantly between days 7 and 21 for either scaffold (**Fig. 2.1B**). In agarose, the addition of Dex increased DNA content at all timepoints. Whereas little or no proliferation was observed in agarose hydrogels, RAD hydrogels showed a 2.5 fold increase in DNA content over day 0 levels by day 7. DNA content and sGAG normalized to DNA are also reported in Supplementary figure S2.1.

Proteoglycan synthesis normalized to DNA content increased from day 7 to days 14 and 21 in RAD and remained elevated with TGF+Dex, while synthesis dropped at day 21 with TGF alone (**Fig. 2.1C**). Proteoglycan synthesis showed a trend of decreasing over time in agarose. TGF+Dex showed a significant increase compared to TGF alone for agarose at days 14 and 21, consistent with sGAG content. DNA synthesis rates normalized to DNA content were not statistically different over time for the TGF+Dex condition in RAD, while the TGF alone condition showed a significant decrease at day 21 (**Fig. 2.1D**). In agarose, DNA synthesis decreased with time in both conditions. Dex supplementation significantly increased DNA synthesis in agarose at day 7 and day 21.

Dex had a greater effect on sGAG retention in agarose than in RAD hydrogels (**Fig. 2.1E**). In agarose, sGAG retention decreased from day 14 to day 21 and the addition of Dex significantly increased retention at all timepoints. In contrast, sGAG retention was generally higher in RAD than in agarose at all times with or without Dex. Dex increased sGAG retention over TGF alone in RAD at day 7. Total sGAG produced and total sGAG normalized to DNA are also reported in Supplementary figure S2.1.

Hydroxyproline content increased over time in both scaffolds (**Fig. 2.1F**), similar to the trends in sGAG content. In RAD, addition of Dex decreased hydroxyproline content at all timepoints compared to TGF alone. In agarose, there was no statistical difference between TGF and TGF+Dex at any timepoint.

*Histology and Immunohistochemistry*

Toluidine blue staining of day 21 hydrogels (**Fig. 2.2A**) was consistent with the quantitative sGAG content (**Fig. 2.1A**), with TGF+Dex and TGF gels showing similar levels of staining

in RAD and TGF+Dex agarose gels showing darker staining than TGF alone. Interestingly, addition of Dex led to more diffuse staining throughout the agarose scaffold compared to the largely pericellular staining for TGF alone.

To investigate the types of collagen present in day 21 hydrogels, immunohistochemistry was performed. RAD hydrogels were positive for collagen type II staining, but agarose gels were not (**Fig. 2.2B**). After analyzing multiple sections of RAD gels, a trend of more collagen type II staining in TGF alone gels compared to TGF+Dex gels was seen in RAD, based on the distribution of matrix areas that were stained positively. This was consistent with higher levels of hydroxyproline in RAD gels with TGF alone compared to TGF+Dex (**Fig. 2.1F**). Collagen type I staining was not seen in either agarose or RAD hydrogels (data not shown).

Day 21 hydrogels were also analyzed for nuclear blebbing as an indicator of apoptosis by haematoxylin staining.[33] Representative images showed little apoptosis in RAD gels with either medium condition, while agarose gels showed much higher levels of blebbing (**Fig. 2.2C**). After reviewing multiple sections, the percentage of apoptotic cells was calculated for each condition (**Fig. 2.2D**). Agarose hydrogels showed 20-30% of total cells being apoptotic, while RAD gels were near 2%. TGF+Dex showed a trend of less apoptosis than TGF alone in agarose (19.0% vs 31.8%, p=0.186).

*Glucocorticoid Receptor Antagonist Studies*

RU-486 was added to the culture medium in ten-fold excess of Dex to determine whether the previous responses were mediated by the glucocorticoid receptor. At day 21, TGF plus RU-486 (TGF+RU) was not significantly different from the TGF alone condition in either scaffold, as expected (**Fig. 2.3A**). Importantly, the addition of RU-486 to the TGF+Dex

condition significantly reduced sGAG levels such that there was no difference between TGF+Dex+RU and TGF alone in agarose. In RAD, a decrease in sGAG content was observed for TGF+Dex+RU compared to TGF+RU and TGF alone. DNA content was not significantly different among media conditions in either scaffold (data not shown).

Addition of RU-486 to the TGF+Dex condition in agarose reduced proteoglycan synthesis levels to that below TGF alone levels (**Fig. 2.3B**), consistent with the results of total sGAG content analysis in Fig. 3A. In RAD, addition of RU-486 lowered the TGF+Dex production of sGAG to that below the TGF alone level.

*Bovine Aggrecan Western Blot*

Western blots of aggrecan extracted from day 21 hydrogels were performed to analyze G1-NITEGE neoepitope fragments generated by ADAMTS-4/5 cleavage as well as all fragments containing a G1 domain. In agarose, both TGF and TGF+Dex showed NITEGE fragments (**Fig. 2.4A**), though the staining was reduced in the TGF+Dex condition. In RAD, NITEGE fragments were found in the TGF alone condition, but were dramatically reduced for TGF+Dex. Addition of the RU-486 GR antagonist resulted in the reappearance of full-intensity NITEGE bands when added to the TGF+Dex condition in both scaffolds. All conditions in both scaffolds showed full-length aggrecan as a dominant species, with NITEGE fragments also staining strongly in the agarose hydrogels (**Fig. 2.4B**). The ~140kDa band is consistent with m-calpain activity,[36, 37] although the activity of this enzyme was not investigated specifically in this study. The bands between 40-50kDa are consistent with link protein, which shares close homology to the aggrecan G1 domain.[38, 39]

*Matrix Content and Aggrecan Western Blot for Human BMSC-Seeded Hydrogels*

To investigate the applicability of these results to a more clinically relevant scenario, the response of human BMSCs (hBMSCs) to Dex when cultured in RAD and agarose scaffolds was also tested. hBMSCs produced minimal sGAG in agarose hydrogels (**Fig. 2.5A**), whereas sGAG accumulation in RAD hydrogels seeded with hBMSCs was ~50% of that of young bovine BMSCs in RAD gels. In agarose there was an increase in sGAG with Dex supplementation at both timepoints, while in RAD Dex had no significant effect. In agarose there was no significant increase in sGAG with time, whereas in RAD sGAG content increased significantly from day 14 to 21. DNA content and sGAG normalized to DNA are also reported in Supplementary figure S2.2.

Neither scaffold showed high levels of proliferation as measured by a fold change in DNA compared to day 0 (**Fig. 2.5B**). In RAD, TGF+Dex showed more proliferation than TGF alone at both timepoints. In agarose there was no significant effect of Dex. Neither scaffold showed a significant change in DNA content from day 14 to 21.

Proteoglycan synthesis normalized to DNA content in RAD increased with time and was higher in the TGF alone condition than TGF+Dex at both timepoints (**Fig. 2.5C**). Proteoglycan synthesis in agarose increased with time and showed no significant effect of Dex.

Hydroxyproline content in RAD hydrogels increased with TGF+Dex compared to TGF alone at both timepoints (**Fig. 2.5D**). Hydroxyproline content increased with time in RAD for both media conditions. Agarose showed identical statistical differences between conditions, though the levels of hydroxyproline content were less than ~33% of those found in RAD.

sGAG retention levels were ~50% in RAD with a significant increase for TGF+Dex compared to TGF alone (**Fig. 2.5E**). sGAG retention levels did not change significantly over time in RAD. In agarose, sGAG retention decreased with time and was increased by the addition of Dex. Total sGAG produced and total sGAG normalized to DNA are also reported in Supplementary figure S2.2.

Consistent with Western blots performed on hydrogels seeded with bovine BMSCs, aggrecan extracted from day 21 hBMSC-seeded RAD hydrogels showed NITEGE fragments with TGF alone, but a dramatic decrease in NITEGE fragments with TGF+Dex (**Fig. 2.5F**). Both conditions showed a strong full-length aggrecan band in the G1 blot. Aggrecan from hBMSCs cultured in agarose gels was not analyzed by Western blotting due to the limited quantity of sGAG produced.

### 2.4 Discussion

We hypothesized that Dex would improve matrix production and reduce ADAMTS-4/5 activity in agarose and RAD hydrogels seeded with adult human and young bovine BMSCs. We tested this hypothesis by comparing sGAG, DNA, and hydroxyproline accumulation, apoptosis, and ADAMTS-4/5-generated NITEGE fragments for self-assembling peptide and agarose hydrogels cultured in TGF-β1±Dex. To our knowledge, this is the first study to report that Dex affects ADAMTS-4/5 activity in a tissue engineering system. We found that Dex reduced ADAMTS-4/5 activity across both hydrogel types and cell sources, but the overall effects of Dex on chondrogenesis depended on the donor species/age and the type of hydrogel. We have demonstrated chondrogenic differentiation through cartilage-like matrix production, including aggrecan and type II collagen. Others have investigated the effects of

Dex on TGF-β-induced chondrogenesis at the gene expression level for both bovine and human BMSCs and have found evidence of the pro-anabolic effects of Dex which are consistent with our results.[3, 11, 16, 40]

For young bovine BMSCs in agarose, Dex caused a two-fold increase in sGAG content, consistent with previous literature.[11] This increase was due to the increase in proteoglycan synthesis per cell caused by Dex. Although statistical comparisons were not made between agarose and RAD hydrogel results, as this was the focus of our previous work,[3] it is interesting to note one additional finding. Bovine BMSCs proliferated to a greater extent in RAD than agarose, which ultimately resulted in the accumulation of more total sGAG even though sGAG per cell (as measured by sGAG normalized to DNA) was lower in RAD than agarose for the TGF+Dex condition (Fig S1A). sGAG was better retained in bovine BMSC-seeded agarose hydrogels with TGF+Dex compared to TGF alone. A trend of decreasing apoptosis was found in the presence of Dex for young bovine BMSCs in agarose at day 21, while RAD hydrogels with bovine BMSCs showed very little apoptosis in either condition. RAD gels seeded with young bovine BMSCs had reduced sGAG and hydroxyproline content in the presence of Dex compared to TGF-β1 alone, but the overall levels of these matrix components were still higher than in agarose hydrogels in the presence of Dex at day 21. An increase in proteoglycan synthesis with Dex was observed on day 21; therefore it is possible that longer culture duration with TGF+Dex could have resulted in increased sGAG accumulation. The effects of Dex on sGAG accumulation and synthesis in both scaffolds were mediated by the signaling of Dex through the glucocorticoid receptor, which correlates well with the ability of RU-486 to reverse Dex-induced aggrecan gene expression and type II collagen production by human mesenchymal progenitor cells derived from bone.[16]

Hydrogels seeded with adult human BMSCs showed less matrix production overall compared to young bovine-BMSC-seeded gels. In RAD hydrogels with hBMSCs, the addition of Dex increased DNA and hydroxyproline content, but not sGAG content. We were surprised to find very little evidence of chondrogenic differentiation by hBMSCs in agarose. A previous study of hBMSCs in agarose showed evidence of proteoglycan and type II collagen production at the protein level, but only one cell cluster was shown.[25] hBMSCs have been successfully cultured in other scaffold materials, though pellet culture remains one of the most common culture methods for these cells.[10, 16, 41-44]

ADAMTS-4/5 activity, as measured by anti-NITEGE Western blotting, was decreased by Dex in RAD and agarose hydrogels seeded with young bovine BMSCs and for RAD gels seeded with adult human BMSCs. It is exciting that this finding was consistent across scaffold type and cell species/age. A recent study has shown that Dex does not regulate ADAMTS-4/5 activity at the gene expression level,[15] but suggests the possibility that Dex could be involved in regulating the activation of latent ADAMTS-4/5 enzymes. This is an exciting research area that is the focus of ongoing studies. Use of the glucocorticoid receptor antagonist RU-486 confirmed that the decrease in ADAMTS-4/5-generated NITEGE fragments was mediated by the glucocorticoid receptor. This is an interesting finding that should be investigated further.

There were several limitations to this study. First, apoptosis was only investigated at early timepoints to ensure high viability, and at day 21 to compare to other histology samples. Further study of cell death over the entire culture duration comparing across conditions could aid in optimizing tissue engineering systems. Secondly, proteoglycan and DNA synthesis data were normalized to DNA content (**Fig 2.1, 2.3, 2.5**), which underestimates the synthesis

levels in agarose, since the DNA levels include live and dead cells. Third, hydroxyproline content reflects multiple collagen types and the type present in agarose gels seeded with bovine BMSCs (**Fig 2.1F**) was not determined in this study. Since the matrix in agarose was largely pericellular (**Fig 2.2A**) and the pericellular matrix (PCM) of primary chondrocytes cultured for 21 days in agarose was found to be rich in type VI collagen,[45] we believe this is an abundant constituent of the PCM. Finally, our results encompassing young bovine and adult human BMSCs do not allow us to conclude whether differences in outcomes were associated with donor age or species. Both of these factors are important, and given the conflicting evidence about the importance of donor age for BMSC therapies, further studies are warranted.[46] Recent work by Erickson et al. has shown that BMSCs from young bovine tissue produce cell aggregates with higher sGAG and collagen content than BMSCs from skeletally mature adult donors, bringing forth the hypothesis that the difference in cell donor ages could be a contributing factor in this study.[47] Despite the limited number of human donors used in this study, we did find reproducible trends, which allowed us to find the statistically significant differences between conditions presented here. The differences we found between the cell types used here highlight the importance of considering the effects of species and age differences when translating in vitro studies into a clinical setting.

Several questions for future work remain, including how cell interactions with the scaffold microenvironment affect differentiation, proliferation, matrix production and remodeling, and cell death. This question is especially interesting given the lack of cartilage-like matrix produced by human BMSCs in agarose. Variables that may affect the response of BMSCs to Dex in different scaffolds include scaffold mechanical properties, scaffold interactions with newly-synthesized matrix, and cell-mediated scaffold compaction. We previously reported that BMSCs maintain a rounded morphology in agarose throughout the culture duration,

whereas BMSCs in RAD spread and elongate to achieve a networked morphology with extensive cell-cell contact early in culture.[3] This is likely an important factor since cell-cell contact is an essential aspect of chondrogenesis in limb bud formation.[48]

Dex does affect matrix production by BMSCs in agarose and RAD peptide scaffolds, but the specific results depend on the cell source and the scaffold type. These findings highlight the importance of choosing a scaffold, cell type, and growth factor combination carefully since the interactions between these variables can change the outcome. Dex reduced ADAMTS-4/5 activity in both types of hydrogels and both cell sources, suggesting an exciting new avenue for investigating interactions between Dex and ADAMTS-4/5.

## 2.5 Acknowledgments

The authors would like to thank Rita Kirsch for her excellent technical support.

## 2.6 Informed Consent and Human/Animal Rights

The three patients whose cells were used in this study granted informed consent and surgical procedures were preapproved by the local Institutional Review Board at either Brigham and Women's Hospital or Massachusetts General Hospital (Boston, MA).

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## 2.8 Figures

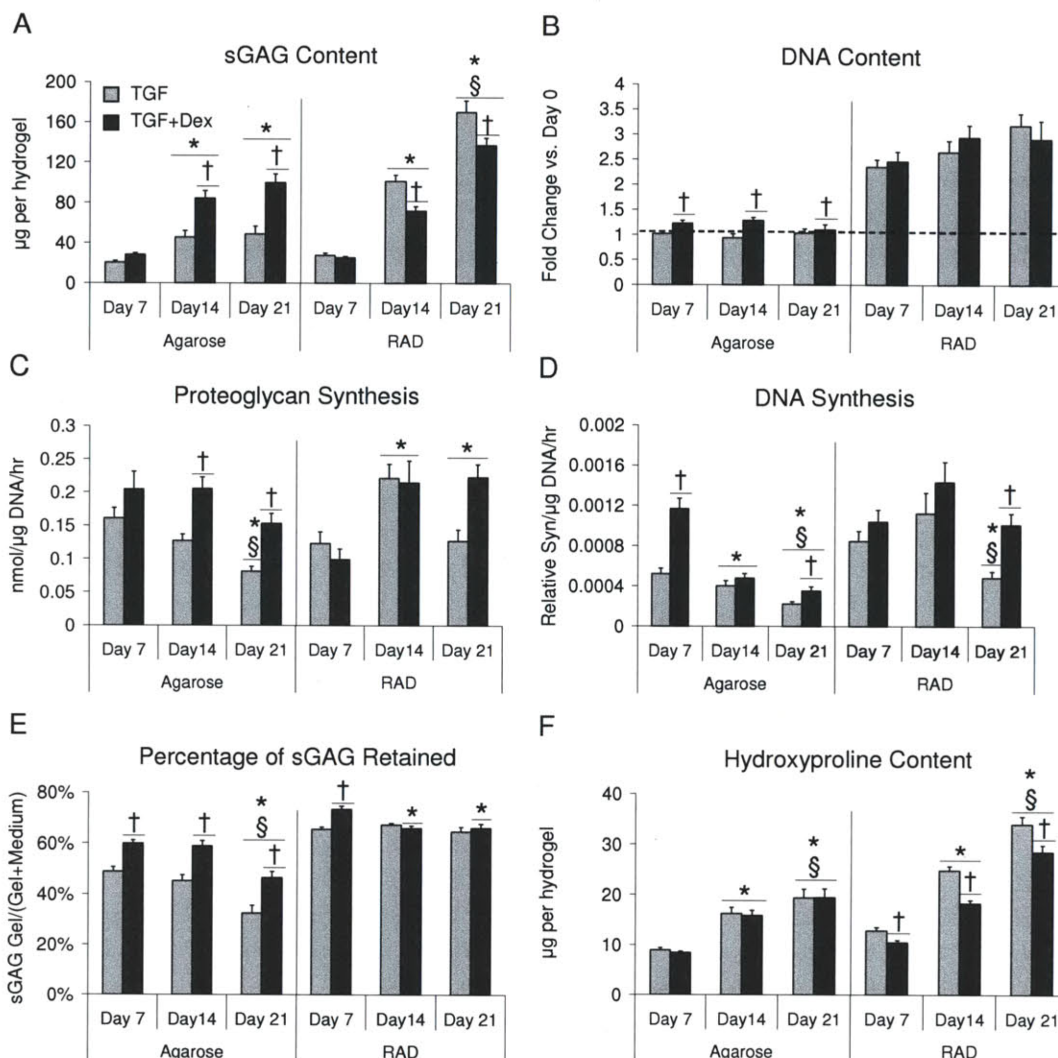



**Figure 2.1**: Extracellular matrix and cellular content in agarose and RAD hydrogels seeded with bovine BMSCs and cultured in TGF or TGF+Dex medium. (A) sulfated glycosaminoglycan (sGAG) content, (B) DNA content, (C) proteoglycan synthesis, (D) DNA synthesis, (E) percent sGAG retained, (F) hydroxyproline content. Values are mean ± standard error of the mean. n=13-16 (3-4 hydrogels x 4 animals). † versus TGF, * versus day 7, § versus day 14, $p<0.05$

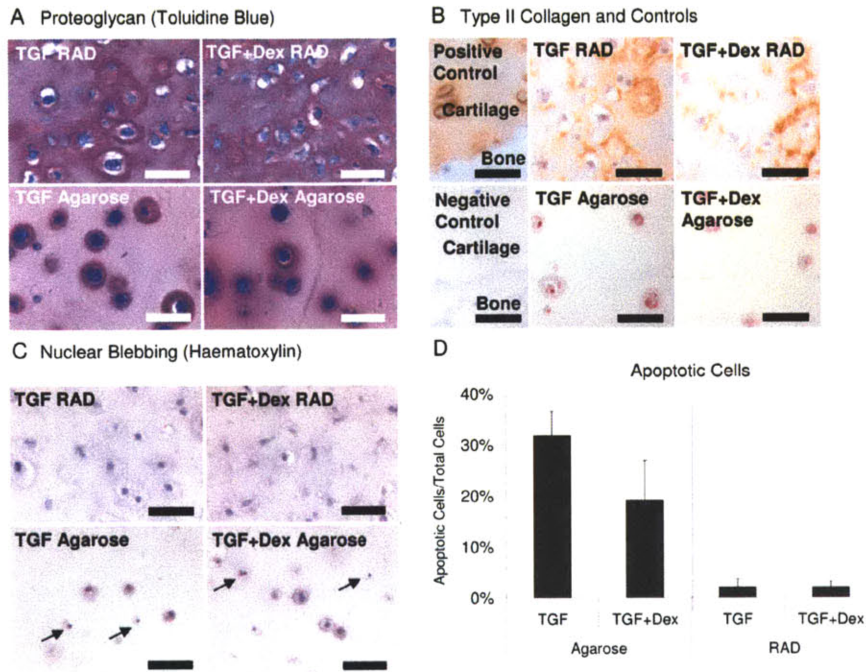



**Figure 2.2**: Representative staining of day 21 agarose and RAD hydrogels seeded with bovine BMSCs and cultured with TGF or TGF+Dex medium. (A) Toluidine blue staining for proteoglycans, (B) type II collagen immunohistochemistry, (C) haematoxylin staining for nuclear blebbing (arrows indicate apoptotic cells), and (D) percentage of apoptotic cells, n=3, values are mean ± standard error of the mean. Controls performed on bovine osteochondral explants. Scale bar is 30μm.

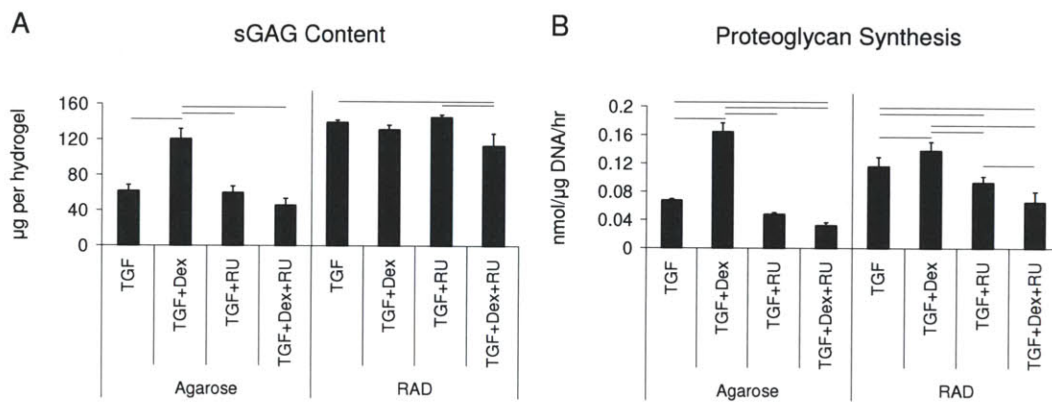



**Figure 2.3**: Effects of RU-486 on matrix production in agarose and RAD hydrogels seeded with bovine BMSCs and cultured in TGF medium ± RU-486 and TGF+Dex ± RU-486 medium for 21 days. (A) sulfated glycosaminoglycan (sGAG) content and (B) proteoglycan synthesis. Values are mean ± standard error of the mean. n=8 (4 hydrogels x 2 animals). Line indicates significant difference between two conditions, $p<0.05$

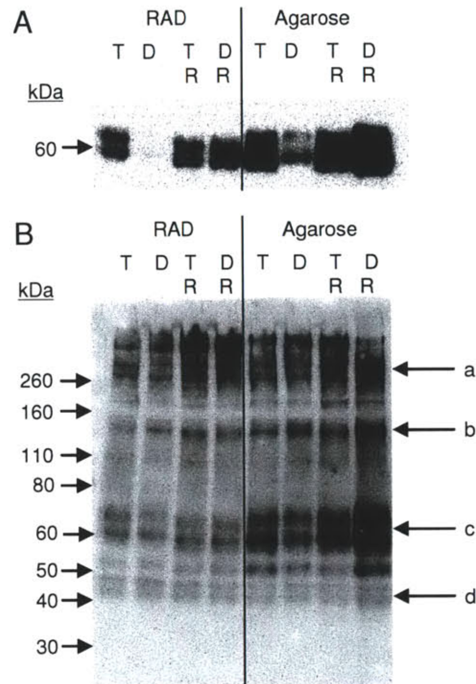



**Figure 2.4**: Analysis of aggrecan cleavage products extracted from day 21 agarose and RAD hydrogels seeded with bovine BMSCs and cultured in TGF medium (T) ± RU-486 (R) and TGF+Dex (D) ± RU-486 medium. 10μg sGAG loaded per lane. (A) anti-NITEGE Western blot and (B) anti-G1 Western blot. Arrows in (B) correspond to (a) full-length aggrecan, (b) potential m-calpain cleavage fragment, (c) G1-NITEGE fragment, and (d) potential link protein. Anti-NITEGE blot was stripped and re-probed with anti-G1 antibody to obtain image 4B. Representative of two repeats for each experiment type.

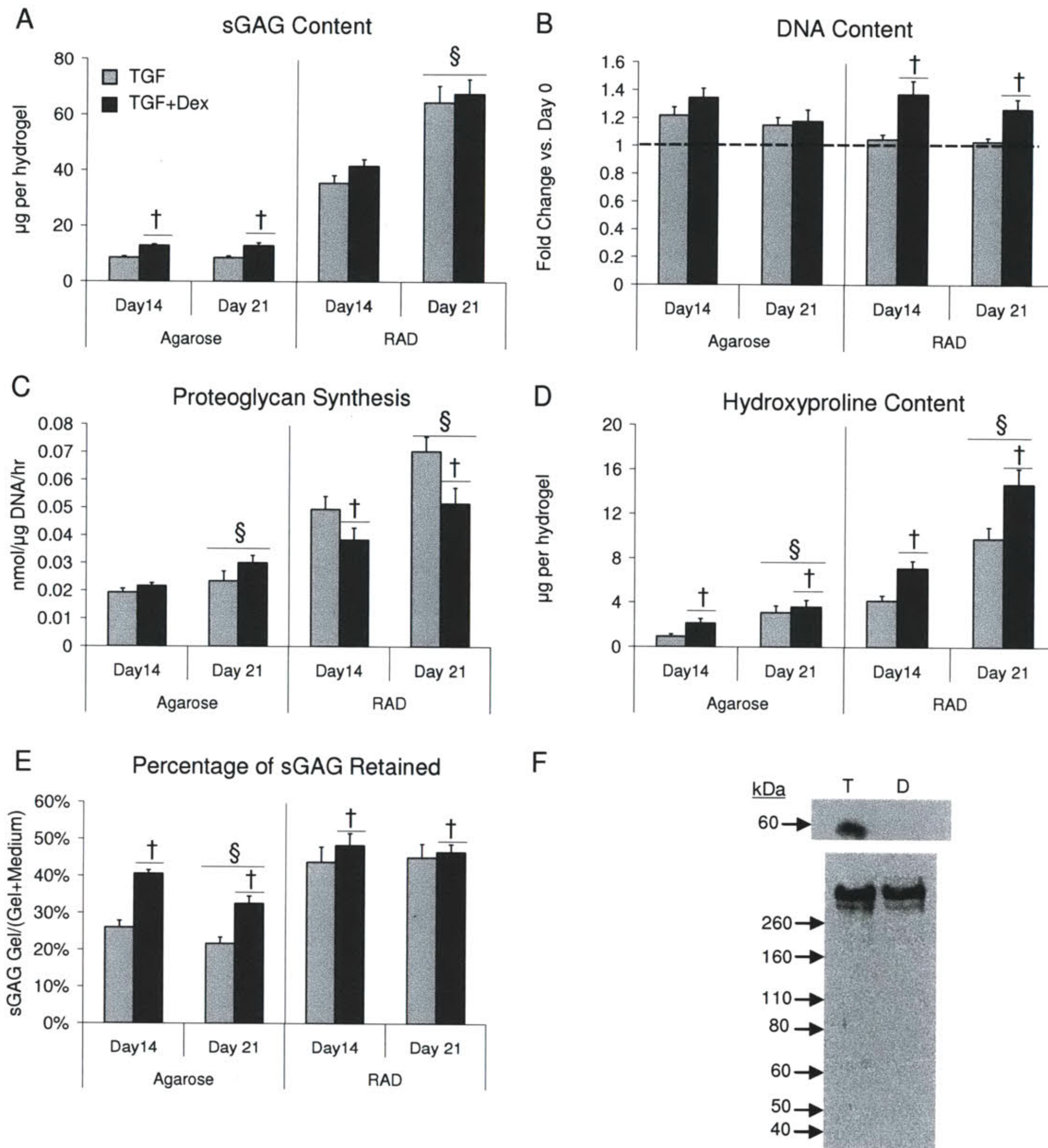
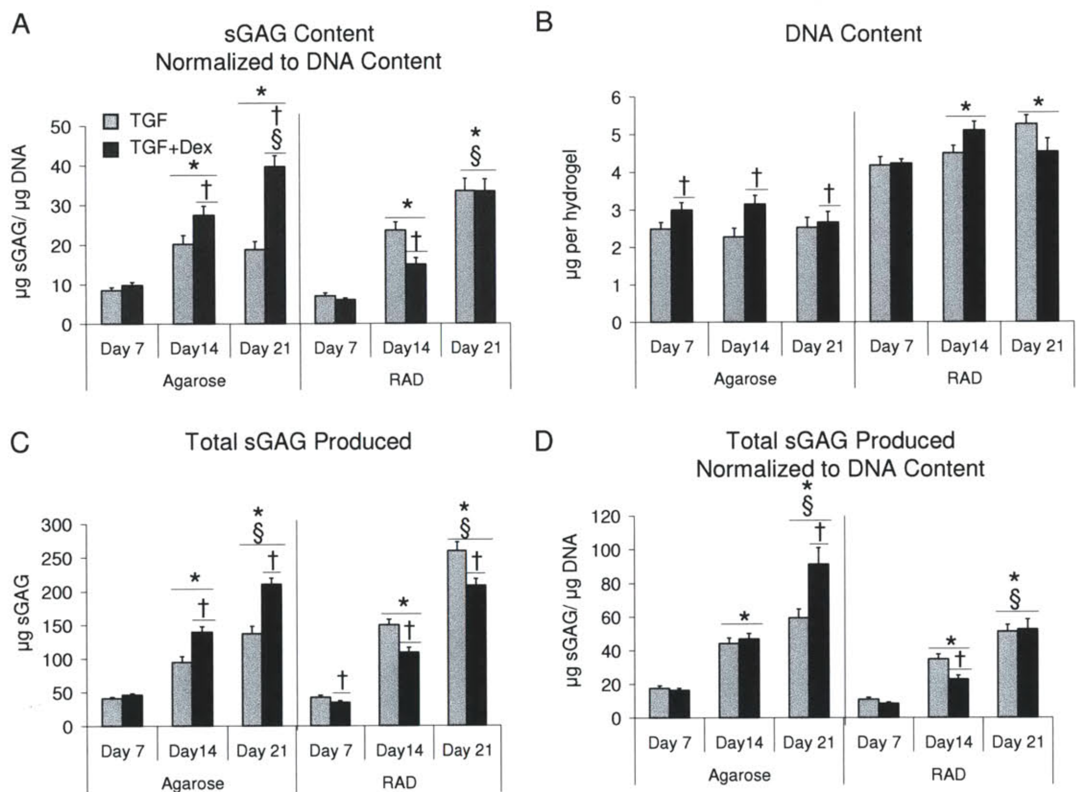
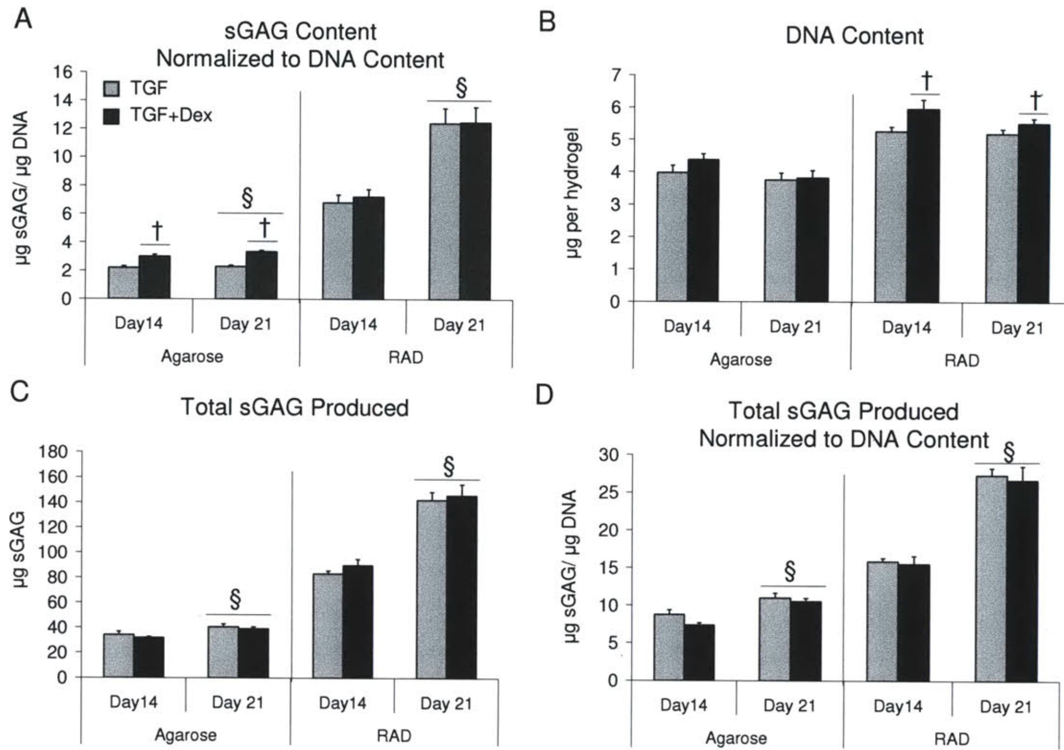



**Figure 2.5**: Extracellular matrix and cellular content in agarose and RAD hydrogels seeded with human BMSCs and cultured in TGF or TGF+Dex medium. (A) sulfated glycosaminoglycan (sGAG) content, (B) DNA content, (C) proteoglycan synthesis, (D) hydroxyproline content, (E) percent sGAG retained, (F) Western blot of aggrecan extracted from day 21 RAD gels seeded with human BMSCS and cultured in TGF (T) and TGF+Dex (D) medium. Top blot is anti-NITEGE, lower blot is anti-G1. 20μg sGAG loaded per lane. Values are mean ± standard error of the mean. n=11-17 (3-6 hydrogels x 2-3 patients). † versus TGF, § versus day 14, $p<0.05$




**Supplemental Figure 2.1**: Extracellular matrix and cellular content in agarose and RAD hydrogels seeded with bovine BMSCs and cultured in TGF medium versus TGF+Dex medium. (A) sulfated glycosaminoglycan (sGAG) content retained in the hydrogel normalized to DNA content, (B) DNA content, (C) total sGAG content produced (retained in gel plus released to medium), (D) total sGAG content produced normalized to DNA content. Values are mean ± standard error of the mean. n=13-16 (3-4 hydrogels x 4 animals). † versus TGF, * versus day 7, § versus day 14, $p<0.05$




**Supplemental Figure 2.2**: Extracellular matrix and cellular content in agarose and RAD hydrogels seeded with human BMSCs and cultured in TGF medium versus TGF+Dex medium. (A) sulfated glycosaminoglycan (sGAG) content retained in the hydrogel normalized to DNA content, (B) DNA content, (C) total sGAG content produced (retained in gel plus released to medium), (D) total sGAG content produced normalized to DNA content. Values are mean ± standard error of the mean. n=11-17 (3-6 hydrogels x 2-3 patients). † versus TGF, § versus day 14, $p<0.05$

# Chapter 3: Delivering HB-IGF-1 with Self-Assembling Peptide Hydrogels

Heparin binding insulin-like growth factor 1 (HB-IGF-1) is a fusion protein of the HB domain of HB-EGF and IGF-1 that has been shown to bind specifically to cartilage and promote sustained upregulation of proteoglycan synthesis in cartilage explants with a single dose. We investigated methods for adsorbing HB-IGF-1 to self-assembling peptides in order to deliver the growth factor to encapsulated chondrocytes and adjacent cartilage explants. We tested whether heparan sulfate (HS) and HB-IGF-1 could be retained in self-assembling peptide hydrogels when added to unassembled peptides in solution. Additionally, we tested HB-IGF-1 retention when adsorbed to assembled hydrogels. These conditions were then tested for their ability to stimulate encapsulated chondrocytes and adjacent cartilage tissue to synthesize proteoglycans. HS and HB-IGF-1 were found to be retained in peptide hydrogels, with both adsorption pre- and post-assembly retaining HB-IGF-1. All HB-IGF-1/HS combinations tested were found to stimulate increased sGAG content of chondrocyte-seeded hydrogels compared to basal controls. A trend of increased sGAG synthesis in cartilage explants cultured adjacent to functionalized hydrogels was observed. Additional repeats of these experiments are ongoing and will aid in interpretation of the differences between conditions. Delivery of HB-IGF-1 using self-assembling peptide hydrogels is a promising technique that could aid in cartilage repair via enhanced matrix production and integration with native tissue.

### 3.1 Introduction

Injuries to cartilage are common and have a limited ability to self-repair. One approach to enhancing the native healing response is to deliver pro-anabolic growth factors to the damaged area using a biomaterial scaffold such that the growth factor is delivered locally at

the site of injury and in a sustained manner over time. Insulin-like growth factor 1 (IGF-1) is known to enhance aggrecan production and minimize aggrecan catabolism, making it an exciting candidate for delivery to cartilage tissue.[1-3] Unfortunately, unmodified IGF-1 has a short half-life and easily diffuses out of the joint, creating the potential for unwanted side effects.[4-6]

Recently, we have shown that a fusion protein combining the heparin-binding domain of HB-EGF and IGF-1, called HB-IGF-1, is a promising candidate for sustained, local delivery to cartilage. HB-IGF-1 binds to heparan sulfate (HS) with a $K_D$ of 21nM and chondroitin sulfate with a $K_D$ of 160nM, whereas IGF-1 does not bind to either.[7] The mechanism of binding is thought to be via interactions between the positively charged HB domain of HB-IGF-1 and the negatively charged sulfated glycosaminoglycan (sGAG) chains. An intraarticular injection of HB-IGF-1 into the joints of rats showed that the growth factor bound to articular cartilage and the meniscus, but was not detected in tendon, patella, or muscle.[7] In addition, HB-IGF-1 bound to cartilage explants was able to stimulate increased proteoglycan synthesis six days after the washout of unbound growth factor.[8]

Given the promising interactions between HB-IGF-1 and cartilage tissue, we investigated several methods of delivering HB-IGF-1 using the self-assembling peptide $(RADA)_4$, or RAD. We have found RAD to be advantageous in a rabbit model of cartilage repair,[9] supportive of chondrogenesis for bone marrow stromal cells,[10] and capable of delivering growth factors in vitro.[11] The RAD peptide sequence consists of alternating hydrophobic and hydrophilic amino acids, where the hydrophilic amino acids alternate between positive and negative charges. Since we hypothesized that the interaction between HB-IGF-1 and chondroitin sulfate chains was based on charge, we further hypothesized that we could take

advantage of the charged amino acids in RAD to bind negatively charged HS chains or positively charged HB-IGF-1 alone to deliver HB-IGF-1. We previously found that tethering of IGF-1 to RAD via a biotin-streptavidin link was not effective in stimulating cells,[12] so we tested different methods of adsorption, which was a successful method for delivering TGF-β1 from self-assembling peptide hydrogels.[11]

We developed methods to adsorb HB-IGF-1 to RAD peptide in solution prior to self-assembly and, separately, to RAD hydrogels after assembly. When adding HB-IGF-1 to unassembled RAD, we were concerned about the possibility of growth factor denaturation given the low pH of the unassembled peptide (~2) and the possibility that after assembly of the hydrogel, the encapsulated growth factor might not be bioavailable. We note, however, that our previous experience with adsorption of TGF-β into similar self-assembling peptide hydrogels resulted in sustained release of bioactive growth factor.[11] Nevertheless, in addition to this method, we also adsorbed HB-IGF-1 to RAD after its assembly into hydrogels. We tested the hypothesis that HB-IGF-1 adsorbed to RAD with or without HS could stimulate increased sGAG accumulation in chondrocyte-seeded hydrogels and increased proteoglycan synthesis in explants co-cultured with functionalized hydrogels. Before we pursued our final hypothesis, we investigated whether HS alone could be retained in RAD, and also whether IGF-1 and HB-IGF-1 with or without HS could be retained in RAD.

## 3.2 Methods

*Materials*

$(RADA)_4$ self assembling peptide, hereafter called RAD and also known as PuraMatrix, was a gift from 3DM (Cambridge, MA). Hydrogels were formed at a concentration of 0.5% (w/v) for all experiments, consistent with previous literature.[10, 13] IGF-1 (Increlex) and HB-IGF-1

proteins were from Ipsen/Biomeasure, Inc (Basking Ridge, NJ). Bovine joints were from Research 87 (Marlborough, MA) and heparan sulfate from bovine kidney (0.88 sulfates/disaccharide[14]) (catalog number H7640) was from Sigma-Aldrich (St. Louis, MO).

*Cartilage Explant Harvest*

1mm thick, 3mm diameter middle zone cartilage explants were harvested from the femoropatellar grooves of immature bovine calves and allowed to rest for 24 hours in basal medium with low glucose Dulbecco's modified Eagle's medium (DMEM) (Mediatech, Inc, Manassas, VA) supplemented with penicillin streptomycin amphotericin (PSA), 4-(2-hydroxyethyl)-1-piperzaineethanesulfonic acid (HEPES) (Invitrogen, Carlsbad, CA), proline (Sigma-Aldrich), ascorbate-2-phosphate (Wake Chemicals, Richmond, VA), and non-essential amino acids (NEAA) (Sigma-Aldrich), as described previously.[15]

*Chondrocyte Harvest*

Cartilage from the femoral chondyles of immature bovine calves was collected and digested with pronase for 1 hour and collagenase overnight.[16] Chondrocytes were filtered from remaining tissue with cell strainers with pore sizes of 70 and 40μm and seeded into hydrogel culture on the same day, as described below.

*HB-IGF-1 Dose Response*

After a 24 hour rest period in basal medium, cartilage explants had the medium changed and replaced with new basal medium, 50nM IGF-1, or 0.5, 5, 50, or 100nM HB-IGF-1 for a culture period of 48 hours. At the end of this period, soluble growth factor was removed by washing with PBS and replaced with basal medium. Media changes with basal medium were performed every 2 days thereafter. See **Figure 3.1A** for graphic showing experimental

procedure. For the final 24 hours of culture on day 8, explants were radiolabeled with 5μCi/mL $^{35}$S-sulfate (Perkin Elmer Inc, Waltham, MA) to measure proteoglycan synthesis, as described previously.[17] Unincorporated radiolabel was removed and explants were weighed wet and digested with Proteinase-K (Roche Applied Science, Indianapolis, IN). Digested samples were analyzed for radiolabel incorporation by liquid scintillation counting.

*Heparan Sulfate Release*

Heparan sulfate (HS) was mixed into RAD at a concentration of 5μM. 100μL of phosphate-buffered saline (PBS) with PSA was layered on top of 50μL of HS-functionalized RAD. These volumes were chosen to allow predicted levels of HS release to be detectable. After 15 minutes, the bath was removed and replaced with a bath of either PBS+PSA ("PBS alone"), PBS+PSA with 1M NaCl added ("1M NaCl"), or PBS+PSA with the pH adjusted to 9 with NaOH ("pH 9"). Hydrogels were cultured at 37℃. 80μL of the bath from each sample was collected every 24 hours for days 1-7. The HS content of the bath was measured by 1.9-dimethylmethylene blue (DMMB) dye binding.[18]

*IGF-1 and HB-IGF-1 Release when Adsorbed to Unassembled Peptide*

50nM IGF-1 or 50nM HB-IGF-1 was allowed to form a complex with 50nM HS for 15 minutes before mixing with RAD peptides in solution. As separate conditions, 50nM IGF-1 or 50nM HB-IGF-1 alone were mixed with RAD peptides in solution. 50μL of hydrogel were assembled by and cultured with a 750μL overlying bath of PBS+PSA at 37℃ in low-binding microcentrifuge tubes. These volumes were chosen to match parallel experiments using chondrocyte-seeded hydrogels. The bath was replaced every 24 hours for 6 days. Hydrogel samples were frozen at each timepoint after removal of the bath. Determination of growth factor retention was made by Western blotting. Hydrogel samples were mechanically

disrupted and loaded directly into a 4-12% Bis-Tris gel (Invitrogen) and run at 200V for 45 minutes. Proteins were transferred to a polyvinylidine fluoride membrane and probed with an anti-IGF-1 antibody (catalog number 9572, Abcam, Cambridge, MA), which recognizes both IGF-1 and HB-IGF-1.[8]

*IGF-1 and HB-IGF-1 Release when Adsorbed to Assembled Peptide*

50μL RAD hydrogels with or without 50nM HS mixed in were allowed to assemble for 24 hours in 750μL PBS+PSA at 37°C in low-binding microcentrifuge tubes. After 24 hours, the bath was replaced with PBS+PSA with 50nM HB-IGF-1. 24 hours after addition of the HB-IGF-1 bath, soluble growth factor was washed out of the cultures using PBS and the bath was replaced with PBS+PSA. See **Figure 3.1B** for a graphic showing the experimental procedure. Hydrogel samples were frozen after bath removal every 24 hours. Growth factor retention was assessed by Western blotting, as described above.

*IGF-1 and HB-IGF-1 Delivery to Peptide Seeded with Bovine Chondrocytes*

Bovine chondrocytes were encapsulated in RAD at a density of 30 million cells/mL. The RAD was either unaltered RAD (for "Basal," "IGF soluble," "HB soluble," and "HB adsorb" conditions), or had one of the following adsorbed prior to cell encapsulation: 50nM HS (for "HB adsorb HS" condition), 50nM IGF-1 (for "IGF mix" condition), or 50nM HB-IGF-1 (for "HB mix" condition). The cell/hydrogel mixture was cast as 6mm diameter, 1.5mm thick disks into agarose rings pre-equilibrated in the basal medium described above, as described previously.[10] Some hydrogels were cast into 50mM tris(hydroxymethyl)aminomethane (Tris) and 1mM $CaCl_2$ at the time of casting to measure day-0 DNA levels. The hydrogel disks were cultured in basal medium for 24 hours to allow peptide assembly. After 24 hours, a medium change was performed on all hydrogels. Hydrogels for the "IGF soluble" and "HB

soluble" conditions received 50nM IGF-1 and HB-IGF-1, respectively, in the medium through the entire culture period. Hydrogels for the "HB adsorb" and "HB adsorb HS" conditions received medium with 50nM HB-IGF-1 for 24 hours from day 1 to day 2 and the soluble growth factor was washed out with PBS on day 2. For days 3-10, these hydrogels were cultured in basal medium. Hydrogels for the "Basal," "IGF mix," and "HB mix" conditions received basal medium for the entire culture duration. Hydrogels were cultured for up to 10 days with medium changes as shown in **Figure 3.1C**.

For the final 24 hours of culture, hydrogels were radiolabeled with 5μCi/mL $^{35}$S-sulfate and 10μCi/mL $^{3}$H-proline (Perkin Elmer Inc) to measure proteoglycan and protein synthesis, respectively. Unincorporated radiolabel was removed and hydrogels were weighed wet, lyophilized, weighed dry, and digested with Proteinase-K (Roche Applied Science), as described previously.[10] Digested samples were analyzed for radiolabel incorporation by liquid scintillation counting, for sGAG content by DMMB dye binding,[18] for DNA content by Hoechst dye binding,[19] and hydroxyproline content as a measure of total collagen by reaction with p-dimehtylaminobenzaldehyde.[20]

*IGF-1 and HB-IGF-1 Delivery to Explants Cultured with Peptide*

Bovine cartilage explants were harvested and placed in basal medium to rest. One day after the harvest, 100μL RAD hydrogels were cast into 2mL cryo-tubes and assembled with 300μL of basal medium. The hydrogel and medium conditions as well as the schedule of medium changes were identical to those described for the bovine chondrocyte experiment described above. The cartilage explants received new basal medium on day 1 and were added to the cryo-tubes with the acellular peptide on day 2 after PBS washing.

Explants were radiolabeled with 5μCi/mL $^{35}S$-sulfate (Perkin Elmer Inc) for the final 24 hours of culture to measure proteoglycan synthesis. Explants were washed to remove unincorporated radiolabel, weighed wet, and digested with Proteinase-K (Roche Applied Science). Digest samples were analyzed for radiolabel incorporation by liquid scintillation counting and DNA content was measured by Hoechst dye binding.

*Statistics*

Results are presented at mean ± standard error of the mean. A general linear model with medium condition as an independent variable was used to analyze HB-IGF-1 dose response data. HS release data were rank transformed and analyzed using a general linear model with medium condition and day as independent variables. A general linear model with culture condition and time as independent variables was used to analyze sGAG, DNA, proteoglycan synthesis, protein synthesis, and hydroxyproline content data for chondrocyte-seeded hydrogel experiments and growth factor stimulation of explant experiments. A Kolmogorov-Smirnov test was used to test for normality and data were transformed as necessary. Tukey post hoc tests with $p<0.05$ were used to evaluate statistical significance for all pairwise comparisons. Systat 12 software (Systat, Chicago, IL) was used for all statistical analyses.

## 3.3 Results

*Determining a Dose of HB-IGF-1*

To determine a dose of HB-IGF-1 to use for future experiments, a dose response study was performed on cartilage explants. Explants received medium with either soluble IGF-1 or HB-IGF-1 at various doses for 48 hours and then unbound growth factor was removed by washing with PBS. Six days later, explants were radiolabeled to measure proteoglycan synthesis. See **Figure 3.1A** for a diagram of the experimental procedure. The data presented

in **Figure 3.2** is proteoglycan synthesis normalized to explant wet weight and to the no growth factor "0" condition.

Six days after removing soluble growth factor, the 50nM IGF-1 condition ("IGF 50") was not statistically different from the no IGF control, consistent with previous reports.[8] Both the 50nM and 100nM doses of HB-IGF-1 showed significantly greater proteoglycan synthesis than the no IGF control and the 0.5nM dose of HB-IGF-1. There was a trend on increasing proteoglycan synthesis as the HB-IGF-1 dose increased, although 5, 50, and 100nM HB-IGF-1 were not statistically distinct. A dose of 50nM HB-IGF-1 was chosen for future experiments as it was the lowest dose found to be statistically different from the no IGF control.

*Heparan Sulfate Release from Peptide*

Heparan sulfate (HS) was adsorbed to unassembled RAD and cultured with a bath of either PBS alone, PBS plus 1M NaCl ("1M NaCl"), or PBS with the pH adjusted to 9 by NaOH ("pH 9"). Release of HS from the peptide was measured over a 7 day period. In PBS alone, less than 15% of the total HS was released from the RAD by day 7. The addition of 1M NaCl or alteration of the pH to 9 resulted in increased release of HS compared to PBS alone, with the 1M NaCl bath releasing more than the pH 9 bath (**Figure 3.3**).

*IGF-1 and HB-IGF-1 Release when Adsorbed to Unassembled Peptide*

50nM IGF-1 or HB-IGF-1 with or without 50nM HS was mixed into unassembled RAD, PBS was added to initiate assembly, and the PBS bath was then changed daily. Each day, hydrogels were frozen after the bath was removed and their growth factor content assessed by Western blotting. HB-IGF-1 was strongly retained in the hydrogel with or without HS for the

entire culture duration (**Figure 3.4A**). IGF-1 was also retained for the entire culture duration with or without HS (**Figure 3.4B**).

*IGF-1 and HB-IGF-1 Release when Adsorbed to Assembled Peptide*

50nM HB-IGF-1 was allowed to adsorb for 24 hours to assembled hydrogels of RAD alone or RAD with 50nM HS mixed in prior to assembly. Soluble growth factor was then washed out of the culture using PBS (**Figure 3.1B**). Timepoints were taken daily to assess retention of HB-IGF-1. HB-IGF-1 was retained in RAD (**Figure 3.5A**) and RAD functionalized with HS throughout the culture period (**Figure 3.5B**). Lower levels of protein were seen at day 8 in both conditions, perhaps suggesting release of the adsorbed HB-IGF-1.

*IGF-1 and HB-IGF-1 Delivery to Peptide Seeded with Bovine Chondrocytes*

We next tested the ability of HB-IGF-1 adsorbed before or after RAD assembly to stimulate bovine chondrocytes encapsulated in the peptide. For the "HB mix" and "IGF mix" conditions, 50nM HB-IGF-1 or IGF-1, respectively, were adsorbed to unaltered RAD prior to peptide assembly and cultured in basal medium. For the "HB adsorb" and "HB adsorb HS" conditions 50nM HB-IGF-1 was adsorbed to assembled unaltered RAD or RAD with 50nM HS adsorbed pre-assembly. For these conditions, the RAD was allowed to assemble in basal medium for 24 hours, medium with 50nM HB-IGF-1 was then added for 24 hours, and unbound growth factor was washed out and the gels were cultured in basal medium for the rest of the culture period (**Figure 3.1C**). Unaltered RAD was cultured in basal medium as a negative control ("Basal") and unaltered RAD was cultured in 50nM IGF-1 or 50nM HB-IGF-1 delivered as soluble proteins in the medium as positive controls ("IGF soluble" and "HB soluble," respectively).

sGAG content increased from day 6 to day 10 (**Figure 3.6A**). All conditions except HB adsorb HS had significantly greater sGAG content compared to the Basal condition at both timepoints. HB adsorb and HB mix were not statistically different from the HB soluble condition. The HB adsorb HS condition did not proliferate to the same extent as all other conditions at either timepoint (**Figure 3.6B**). The DNA content of the IGF mix and HB mix conditions decreased with time.

HB mix showed lower proteoglycan synthesis at day 6 than all other conditions (**Figure 3.6C**). Proteoglycan synthesis decreased from day 6 to day 10 for all conditions except HB mix. Protein synthesis was elevated over Basal for IGF soluble, HB soluble, HB adsorb, and HB adsorb HS at both timepoints (**Figure 3.6D**). IGF mix and HB mix had protein synthesis levels not statistically different than the Basal condition at both timepoints.

All conditions showed increased hydroxyproline content compared to the Basal condition at day 6 (**Figure 3.6E**). At day 10 only the soluble conditions and HB mix were significantly elevated compared to the basal condition. The hydroxyproline content of the hydrogels increased from day 6 to day 10 for all conditions.

*IGF-1 and HB-IGF-1 Delivery to Explants Cultured with Peptide*

To ensure that the growth factor adsorbed to the RAD was bioactive and bioavailable, and also to test the hypothesis that HB-IGF-1 could be released from the peptide to stimulate nearby cartilage, we repeated the above experiment with acellular functionalized peptides co-cultured with bovine cartilage explants. All conditions showed a trend of having higher proteoglycan content compared to the Basal condition at both timepoints, although the only statistically significant difference was between Basal and HB adsorb HS (**Figure 3.7**). The

lack of statistical significance is likely due to a smaller "n" and increased variability in this experiment, which will be resolved with additional repeats.

### 3.4 Discussion

We tested the hypothesis that HB-IGF-1 adsorbed to RAD with or without HS could stimulate increased sGAG accumulation in chondrocyte-seeded hydrogels and increased proteoglycan synthesis in explants co-cultured with functionalized hydrogels. We established a dose response of HB-IGF-1 and confirmed that IGF-1 does not stimulate increased proteoglycan synthesis in cartilage explants after washing unbound growth factor out of the culture.[8] We found that HS could be retained in RAD when added to unassembled RAD. Since the HS was minimally released into a bath of PBS at pH 7.4, but could be released by increasing the pH or adding salt to the bath, we conclude that HS is retained in RAD via ionic interactions, e.g., the positively charged amino acids. Given the strong retention of HS in RAD and the nanomolar binding constant between HS and HB-IGF-1, we hypothesized that HS could be used to retain HB-IGF-1. Because the interaction between HS and RAD was based on charge, we also thought the positively charged HB-IGF-1 might be retained by the negatively charged amino acids in RAD by charge interactions without the presence of HS. We therefore pursued both of these delivery methods. HB-IGF-1 was retained in RAD with or without HS and regardless of whether it was adsorbed to the peptide before or after it was assembled.

Having established that HB-IGF-1 could be retained in the peptide, we tested whether the growth factor could stimulate encapsulated cells and adjacent cartilage explants. We found that both adsorption methods resulted in increased sGAG content in chondrocyte-seeded hydrogels compared to the basal condition, though IGF-1 adsorbed pre-assembly also

stimulated increased sGAG content. The lower sGAG content seen in the HB adsorb HS condition was likely due to a dramatically lower level of cell proliferation. Another repeat of this experiment is ongoing and will aid in interpretation of the differences between experimental conditions. In the explant experiment, there was a trend for increased proteoglycan synthesis when co-cultured with functionalized RAD over the basal peptide. Due to increased variability and a lower number of specimens per condition, the trend was not statistically significant except for HB adsorb HS. Additional repeats for this experiment are also ongoing and should further clarify these results.

We have shown that HB-IGF-1 adsorption to RAD self-assembling peptide hydrogels is a promising method for delivering the pro-anabolic, anti-catabolic growth factor IGF-1 to cells encapsulated in the hydrogels and adjacent tissue. Even though current results suggest that IGF-1 could be delivered by the same method, we have shown here and previously that IGF-1 does not stimulate cartilage tissue after soluble growth factor is removed, where HB-IGF-1 results in sustained upregulated proteoglycan synthesis. We therefore suggest that HB-IGF-1 delivered via the peptide hydrogel to adjacent cartilage at the site of a focal defect in vivo would be capable of binding to sGAG chains within the cartilage and thereby enable sustained bioactivity. In contrast, hydrogel-delivered IGF-1 may stimulate the adjacent cartilage at short times, but likely not in a sustained manner. The hypothesis that HB-IGF-1 would enhance the integration of peptide hydrogel-mediated neocartilage with adjacent native cartilage at the defect interface will be tested in future animal studies.

While this study demonstrates that HB-IGF-1 is retained in RAD hydrogels, further studies are needed to understand the release of HB-IGF-1 from the peptide in both acellular and cellular culture systems in vitro. We attempted to study this using an IGF-1 enzyme-linked

immunosorbent assay (ELISA). We found that the levels of growth factor released were below our limit of detection, even for IGF-1 adsorbed to unassembled peptide, which previous results suggest should be released over a 24 hour period.[12] It is possible that the growth factors were lost to adsorption to the microcentrifuge tubes, despite their being low-binding tubes, or that the initial amount of protein loaded into the hydrogels and the rate at which it was released caused the concentration in the bath to be below the limits of detection for the assay. To address these problems, the volume of the hydrogel and bath could be adjusted to raise the concentration of the growth factor when it is released. Another approach is to create radiolabeled HB-IGF-1 to determine how it binds and releases from the peptide spatially and over time. This could also be used in vivo to demonstrate HB-IGF-1 being released from the hydrogel and binding to surrounding cartilage tissue.

Future work also remains for understanding how the RAD self-assembling hydrogels assemble. It is unclear how HS and HB-IGF-1 interact with unassembled and assembled RAD fibers. Though we hypothesize that the interactions are based on charge, the stoichiometry and how the interactions might change as self-assembly proceeds are unknown.

To further improve on the methods described here, the peptide could be modified to create more binding sites for HB-IGF-1 and additional matrix components could be encapsulated to encourage cell performance. RAD hydrogels have been modified previously to include a sequence from collagen type IV that binds heparin,[21] which could provide additional binding sites for HB-IGF-1. Additionally, we have found that mixing collagen types I and VI into self-assembling peptides resulted in increased sGAG synthesis and accumulation by encapsulated bovine bone marrow stromal cells (BMSCs).[22] Therefore, we propose that self-assembling peptides can be further optimized by including collagens in addition to HB-IGF-1

to enhance matrix production by BMSCs and chondrocytes encapsulated in the hydrogel and integration between native tissue and the new repair tissue at the defect interface. Self-assembling peptide hydrogels provide an exciting tissue engineering scaffold for enhancing cartilage repair.

### 3.5 Acknowledgements

The authors would like to thank Dr. Rachel Miller, Dr. Parth Patwari, and Dr. Richard Lee for helpful conversations and supplies.

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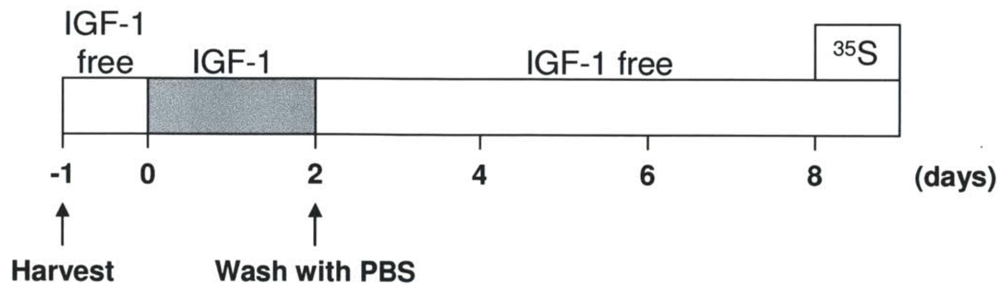
### 3.7 Figures

A

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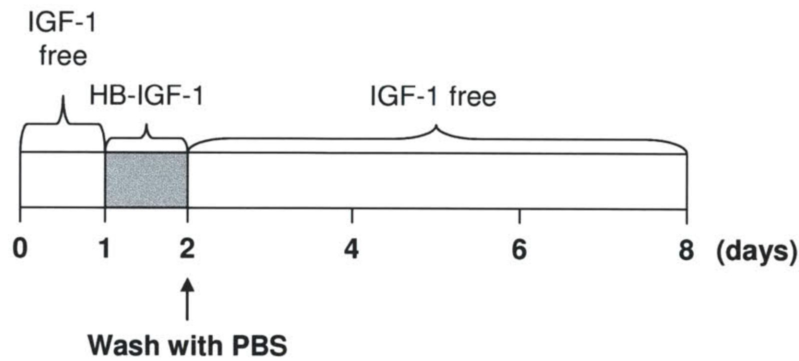


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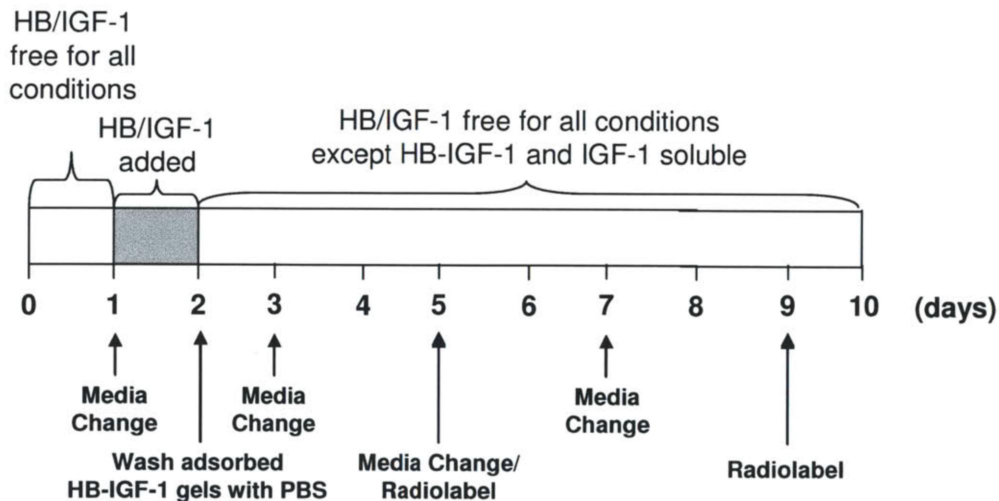


**Figure 3.1**: Timelines of experimental procedures. (A) timeline of experimental procedure for HB-IGF-1 dose response on cartilage explants (B) timecourse of culture conditions for HB-IGF-1 retention to assembled peptide, (C) timecourse of experimental procedure for bovine chondrocytes in regular or functionalized peptide cultured in various media conditions.

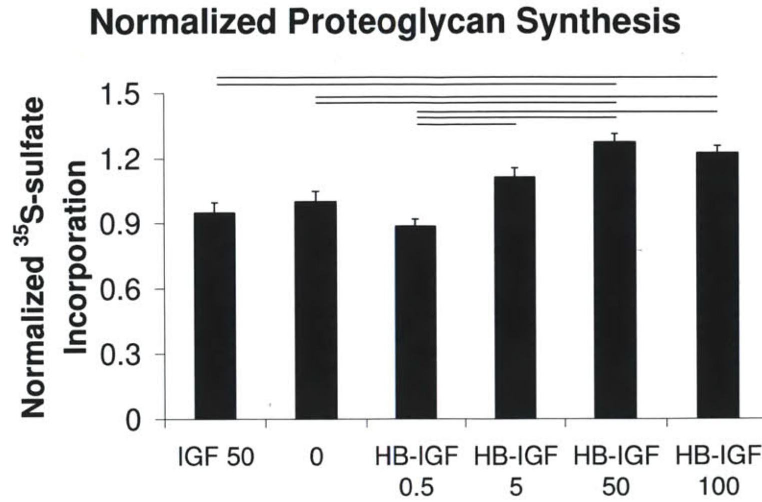


**Figure 3.2**: HB-IGF-1 dose response on cartilage explants at day 8 following wash-out procedure. Proteoglycan synthesis normalized to wet weight and the no IGF "0" condition. See Figure 3.1A for timeline of experimental procedure. Values are mean ± standard error of the mean. N=6. Line indicates significant difference between two conditions, $p<0.05$.

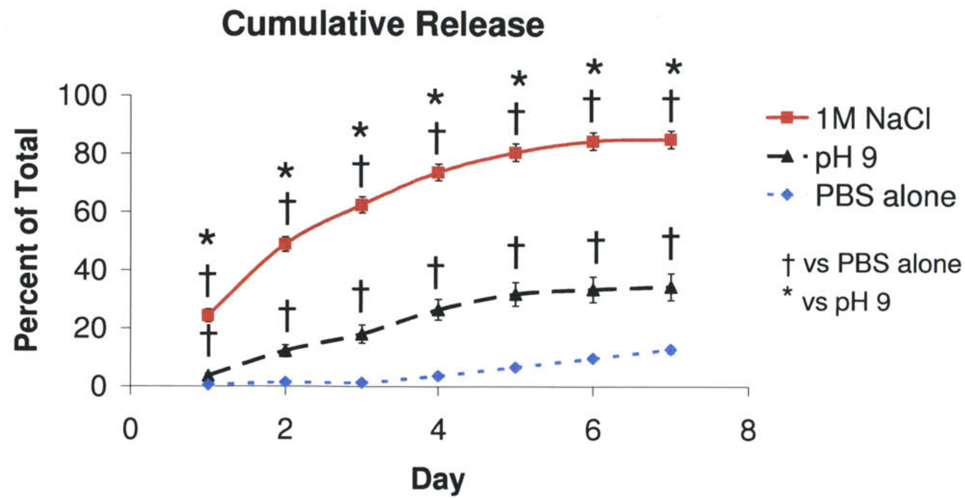


**Figure 3.3**: Heparan sulfate release from acellular peptide scaffold. Peptide with 5μM heparan sulfate cultured in 100 μL bath of PBS, PBS with 1M NaCl ("1M NaCl), or PBS with a pH of 9 (adjusted using NaOH, "pH 9"). Values are mean ± standard error of the mean. N=2-8. † versus PBS alone, * versus pH 9, $p<0.05$.

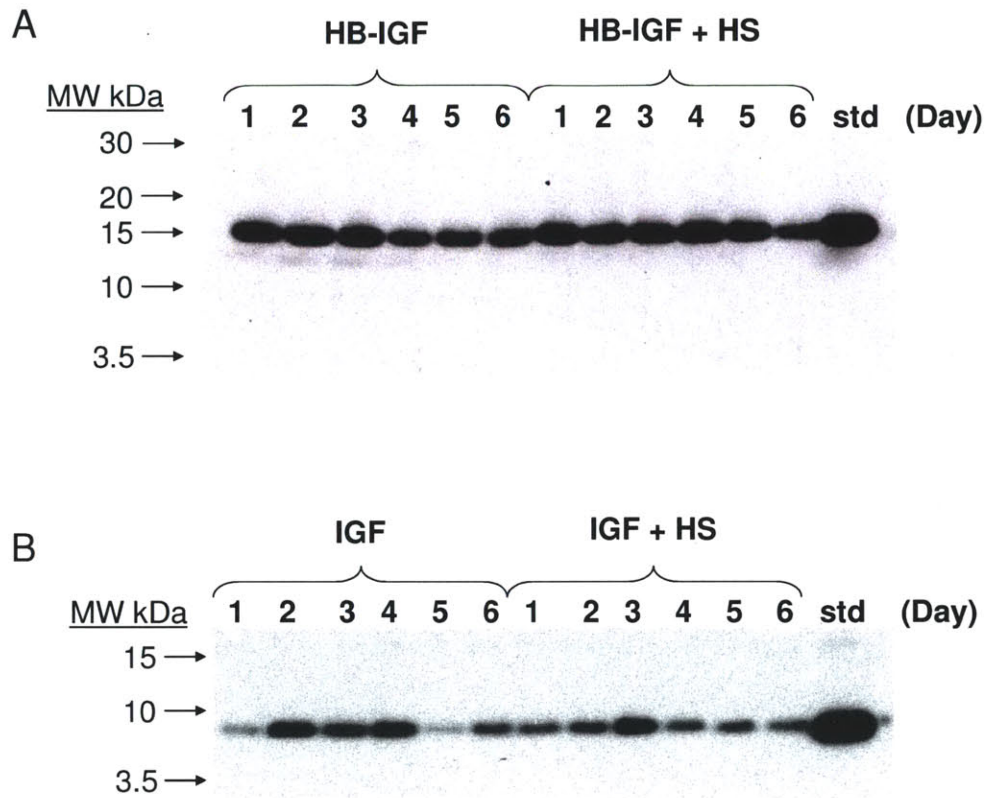


**Figure 3.4**: Growth factor retention when mixed into unassembled acellular peptide. IGF-1 Western blots of (A) HB-IGF-1 ± HS and (B) IGF-1 ± HS retained in peptide on days 1 through 6. "Std" is a standard of 5ng HB-IGF-1 or IGF-1.

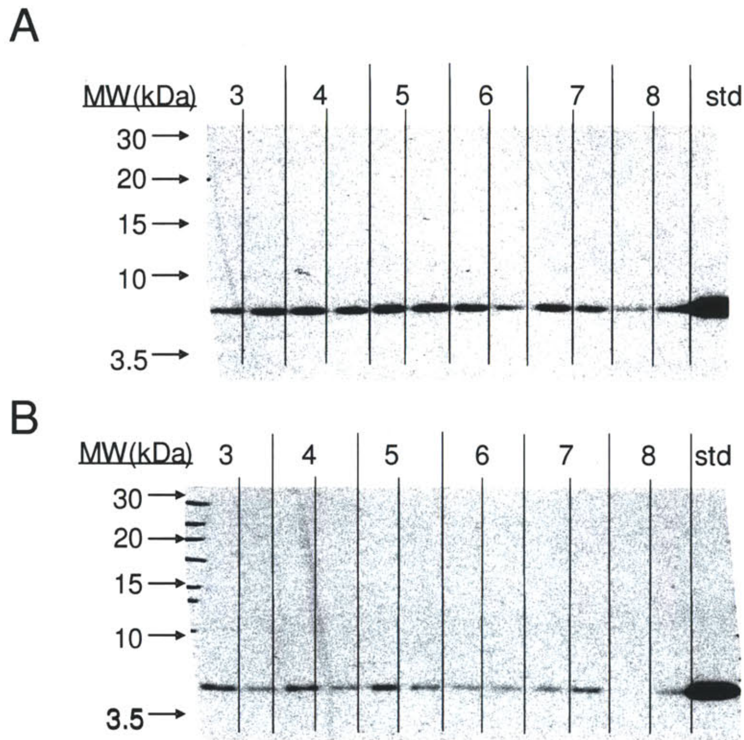


**Figure 3.5:** HB-IGF-1 retention when adsorbed to acellular assembled peptide. IGF-1 Western blots of (A) HB-IGF-1 retained after adsorption to peptide alone and (B) HB-IGF-1 retained after adsorption to peptide mixed with heparan sulfate in peptide at day 3-8. "Std" is a standard of 5ng HB-IGF-1. See Figure 3.1B for timecourse of culture conditions.

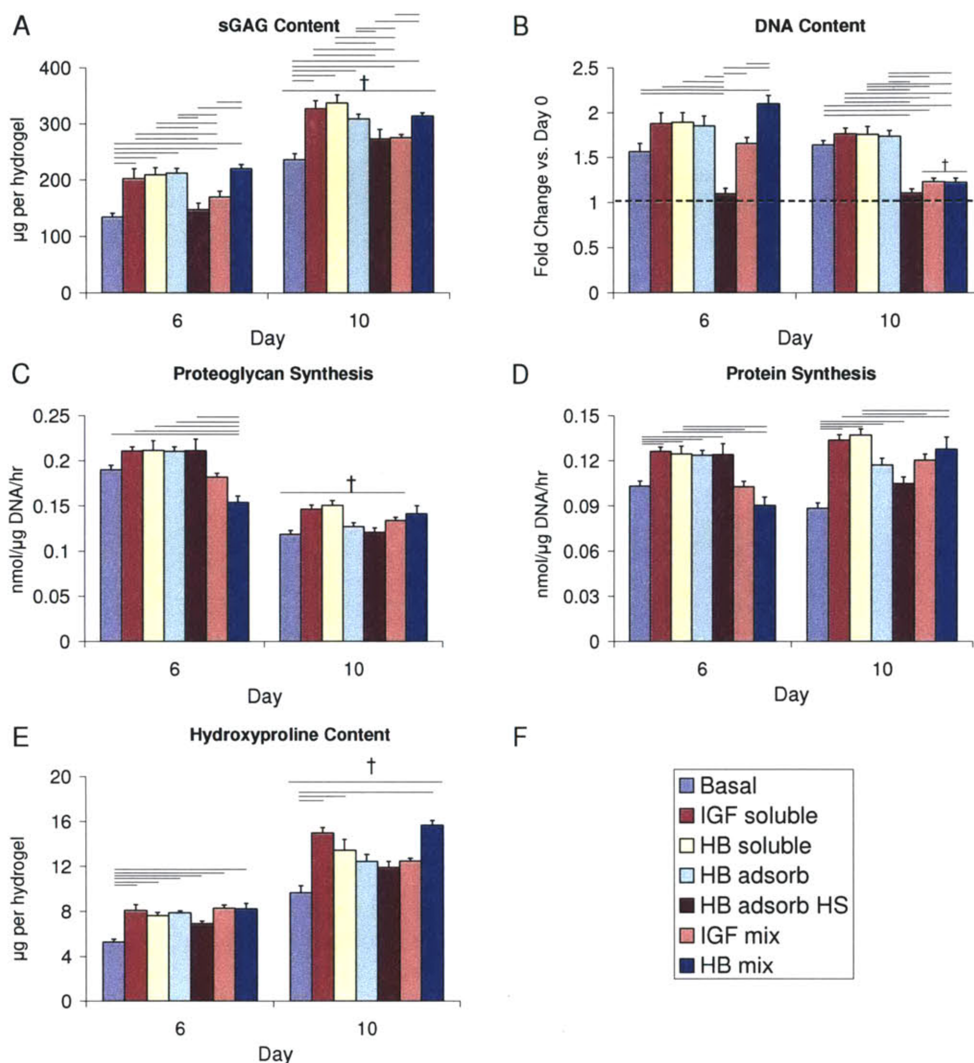


**Figure 3.6:** Extracellular matrix and cellular content in peptide hydrogels seeded with bovine chondrocytes. (A) sulfated glycosaminoglycan (sGAG) content, (B) DNA content, (C) proteoglycan synthesis, (D) protein synthesis, (E) hydroxyproline content, and (F) key of culture conditions. Values are mean ± standard error of the mean. N=5-6. Line indicates significant difference between two conditions, † versus day 6, $p<0.05$. See Figure 3.1C for timecourse of experimental procedure.

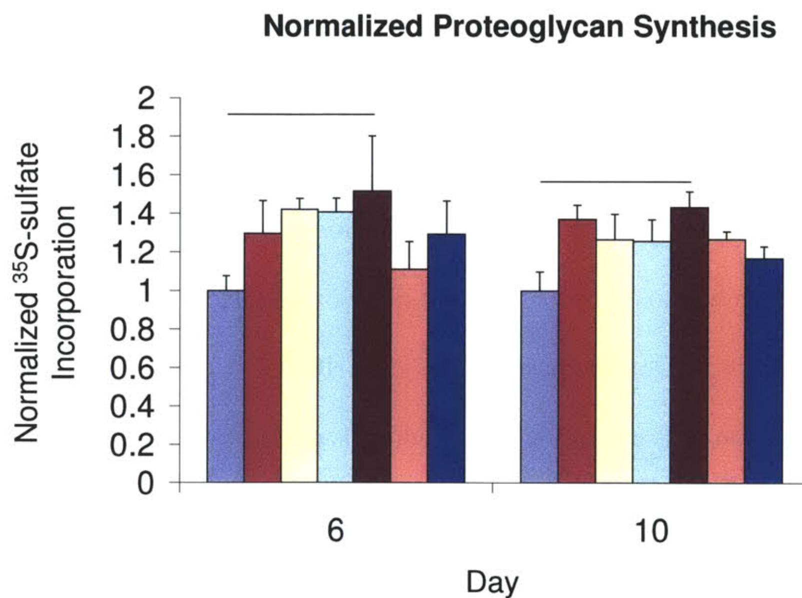


**Figure 3.7:** Proteoglycan synthesis by explants cultured with functionalized peptide. Proteoglycan synthesis data were normalized to DNA content and the "Basal" condition. Values are mean ± standard error of the mean. N=4. Line indicates significant difference between two conditions, $p<0.05$.

# Chapter 4: Conclusions and Future Directions

Injuries to cartilage are an important and unsolved problem in medicine. Cartilage is an avascular tissue with limited ability to self-repair, necessitating outside intervention in order to achieve complete, functional healing. Tissue engineering combines cells, materials, and external stimuli such as growth factors to create a microenvironment for tissue repair. The objective of this thesis was to optimize cartilage-like extracellular matrix production by investigating the effects of Dexamethasone (Dex) and HB-IGF-1 (heparin-binding insulin-like growth factor-1) on cells encapsulated in hydrogels made from the self-assembling peptide RAD and from agarose.

In Chapter 2, the effects of Dex on matrix production and catabolic processing by young bovine and adult human bone marrow stromal cells (BMSCs) undergoing chondrogenesis in these two complementary hydrogels was investigated. Dex enhanced proteoglycan synthesis and accumulation for bovine BMSCs in agarose hydrogels, but decreased accumulation in RAD hydrogels. These effects were found to be mediated by the glucocorticoid receptor, since the presence of the glucocorticoid receptor antagonist RU-486 reversed the effects of Dex. Addition of Dex to the culture medium decreased apoptosis for bovine BMSCs in agarose. Adult human BMSCs showed minimal protein production in agarose hydrogels, but produced ~50% of aggrecan and collagen levels found in RAD hydrogels seeded with bovine BMSCs. Importantly, Dex reduced the activity of the catabolic enzyme aggrecanase in both agarose and RAD scaffolds seeded with young bovine BMSCs and in RAD scaffolds seeded with adult human BMSCs. Since this result was consistent across cell species/age and across different scaffold materials, it may suggest a conserved interaction between Dex and proteins

regulating aggrecanase activity. In order to translate this work into a clinical model, a method for delivering Dex locally to avoid unwanted systemic side effects is needed.

The cause of the differences in BMSC behavior observed in RAD and agarose is unknown, and provides many avenues for future investigations. BMSCs seeded in RAD hydrogels experienced higher levels of proliferation than in agarose and contracted the scaffold during culture, perhaps enhancing proteoglycan retention and increasing the local cell density. Agarose hydrogels are also stiffer than RAD hydrogels, and this variable is known to affect MSC differentiation. Agarose is also an inert material, while it is possible that the $(RADA)_4$ sequence of the peptides interacts with integrins or other cellular components and alter intracellular signaling pathways leading to different cell phenotypes.

Along this line of thinking, one might also consider how differences in the amino acid sequence of self-assembling peptides might affect differentiation and matrix production. BMSCs seeded in RAD hydrogels have a networked morphology in the early stage of culture, whereas BMSCs in KLD, with a sequence of $(KLDL)_3$, exhibit a clustered morphology. While these differences did not correlate with differences in chondrogenic gene expression or matrix production, they provide an interesting clue towards understanding how cells interact with these materials.

In Chapter 3, HB-IGF-1 was adsorbed to RAD self-assembling peptides in solution and to assembled hydrogels to test whether these functionalized hydrogels could stimulate increased proteoglycan synthesis by encapsulated bovine chondrocytes and adjacent cartilage explants. Heparan sulfate (HS) was retained in RAD hydrogels via ionic interactions and could be released by altering the bath to high salt concentrations or high pH. HB-IGF-1 was retained

for the full culture duration in RAD when adsorbed prior to or following peptide self-assembly and with or without the presence of HS as a linking molecule. IGF-1 was also retained in RAD when adsorbed prior to peptide assembly. HB-IGF-1 adsorbed to RAD by both adsorption methods stimulated increased proteoglycan accumulation in chondrocyte-seeded hydrogels compared to the basal condition. Furthermore, cartilage explants showed a trend of enhanced proteoglycan synthesis when cultured with functionalized peptide. Additional repeats of these experiments are ongoing to aid in interpretation of the differences between conditions.

Optimizing delivery of chondrogenic factors is challenging. Alteration of the cell type, species, age and material type can change a result that is thought to be broadly applicable into one that is context-dependent. These are important variables to consider for in vitro studies when the objective is translating the results into a clinical setting. An additional challenge is attempting to control a local chemical environment in a scaffold without releasing growth factors into the systemic circulation, where they can cause negative side-effects. Delivering these factors to prevent post-traumatic osteoarthritis (OA) is complicated further by the long time period over which OA develops. It is unknown whether a short-term therapy could alter the biologic response of the cells in the joint sufficiently to avoid OA progression or if the treatment would need to be chronic.

While we have established the beneficial effects of Dex and HB-IGF-1, future work remains before animal studies are attempted. Dex is a ~400Da, hydrophobic, potent molecule whose delivery needs to be carefully controlled to avoid the numerous side effects associated with chronic corticosteroid use. HB-IGF-1 is a ~10kDa protein retained well by RAD hydrogels, but the characteristics of its release profile are currently unknown. Development of

radiolabeled HB-IGF-1 would allow for transport studies both in vitro and in vivo. In addition, it would also be very useful to do a comprehensive study of growth factor interactions with RAD peptide hydrogels to determine which growth factors adsorb to the material and with what affinity. This could allow further optimization of the material to only bind factors that enhance chondrogenesis and matrix production.

In conclusion, delivery of Dex and HB-IGF-1 to cartilage is a beneficial pro-chondrogenic, pro-anabolic, anti-catabolic strategy for reducing aggrecanase activity and encouraging proteoglycan synthesis by encapsulated cells and adjacent native tissue.

## Appendix A: Data and Protocols Related to Chapter 2

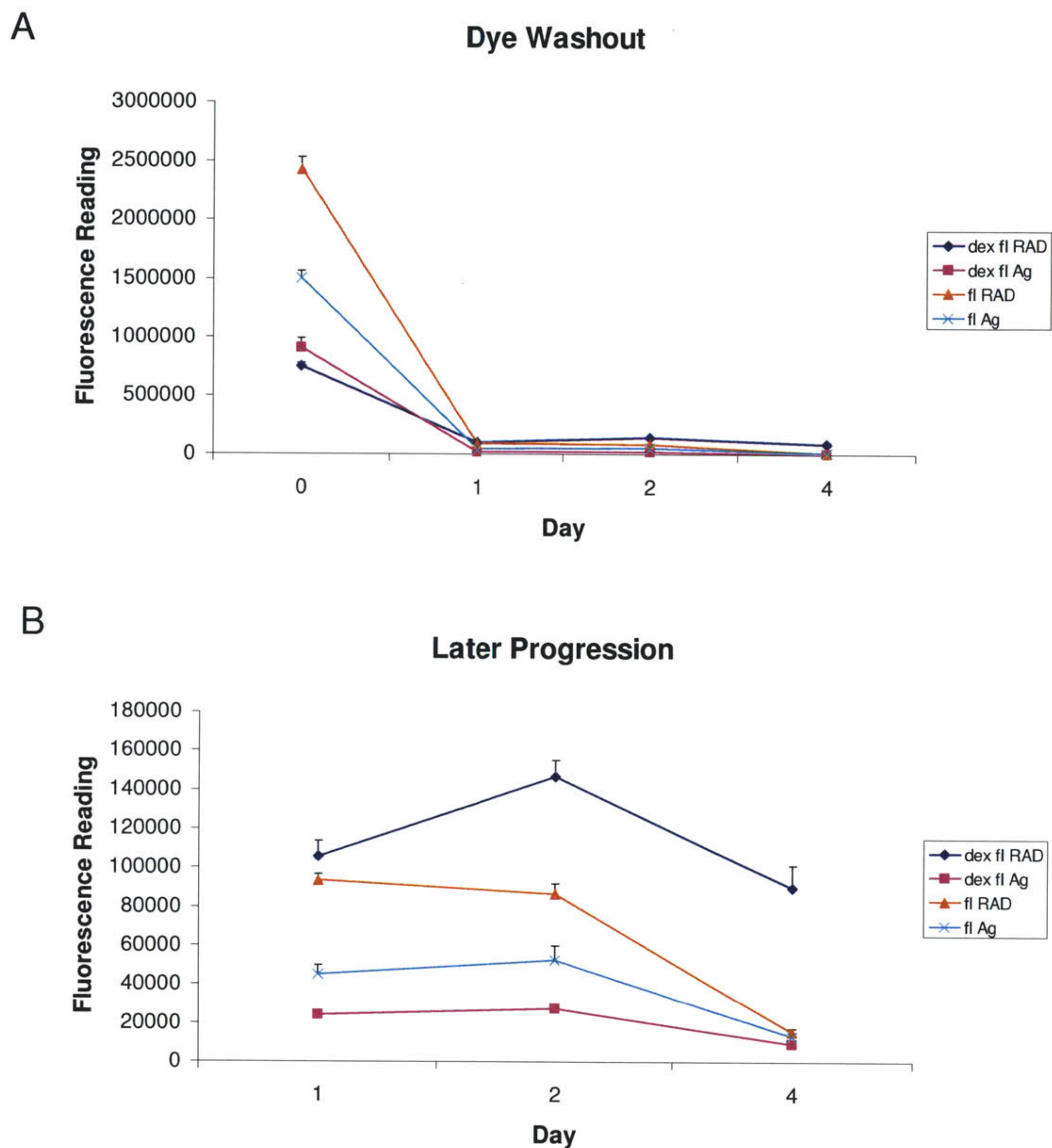


**Figure A1**: Fluorescently-labeled Dex is not retained in RAD or agarose hydrogels. Acellular hydrogels were loaded with 10 μM fluorescein (fl) or Dex-fluorescein (dex fl) and cultured in PBS that was changed on day 2. Note: fluorescein alone has a higher baseline level of fluorescence than Dex-fluorescein. (A) fluorescence readings from hydrogels mechanically disrupted in PBS over entire experiment duration, (B) readings from days 1-4 only. Values are mean ± standard error of the mean. N=6.

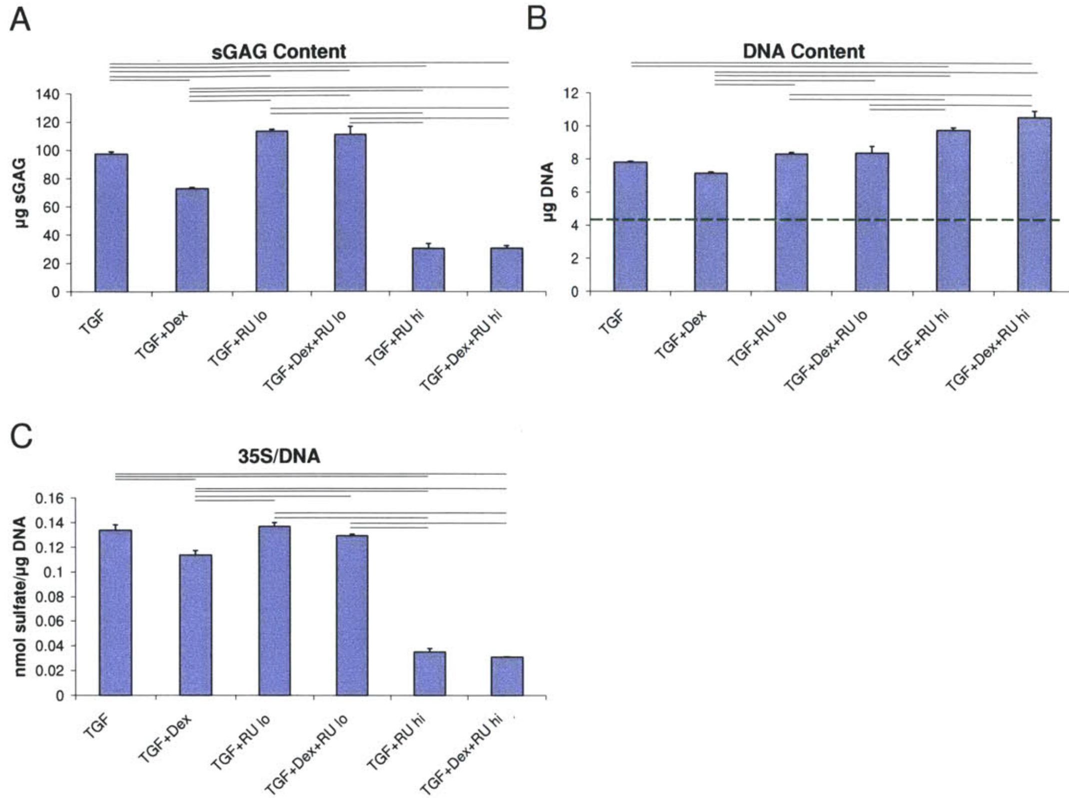


**Figure A2**: High doses of RU-486 inhibit sGAG synthesis. Extracellular matrix and cellular content in RAD hydrogels seeded with bovine BMSCs and cultured in medium with TGF-β1 ± Dex with or without RU-486 at 1µM (lo) or 10 µM (hi) at day 14. (A) sGAG content, (B) DNA content (line indicated day 0 DNA level), (C) proteoglycan synthesis. Values are mean ± standard error of the mean. N=3-4. Line indicates significant difference between conditions, $p<0.05$

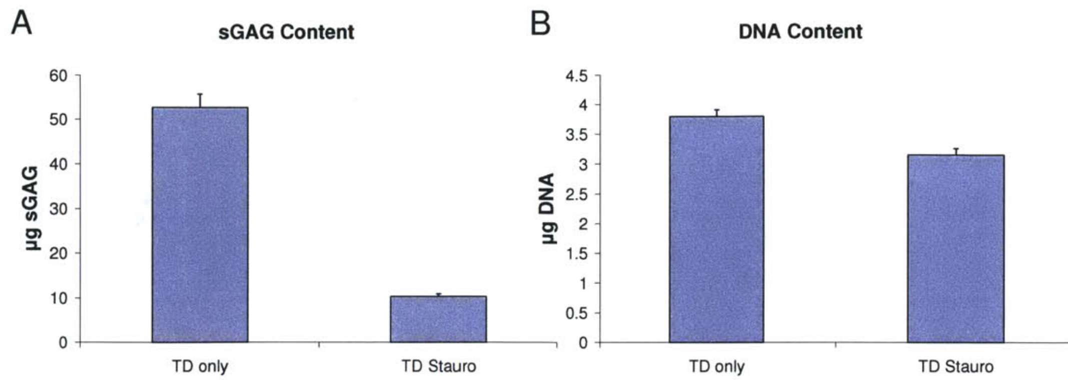


**Figure A3**: DNA from dead cells is retained in agarose hydrogels. Extracellular matrix and cellular content at day 14 in agarose hydrogels seeded with bovine BMSCs and cultured in medium with TGF-β1 and Dex (TD) with or without 1μM staurosporine added at day 7 (TD Stauro). (A) sulfated glycosaminoglycan (sGAG) content, (B) DNA content. Values are mean ± standard error of the mean. N=4.

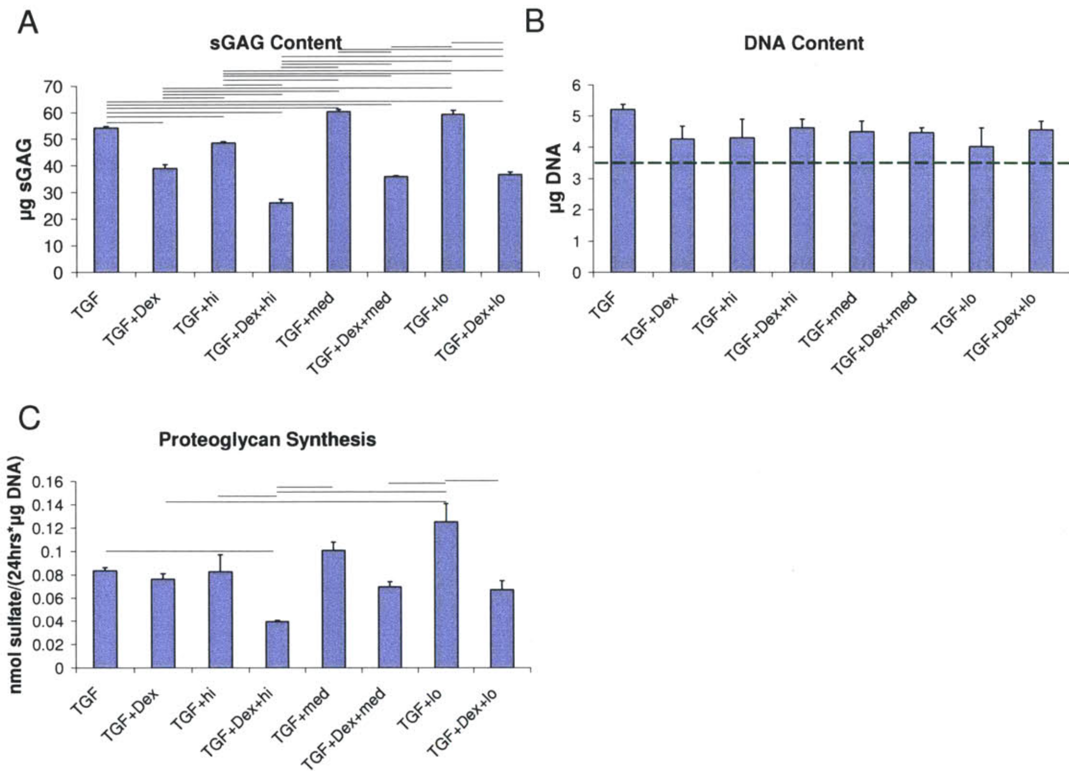


**Figure A4**: High doses of aggrecanase inhibitor slightly inhibit sGAG synthesis. Extracellular matrix and cellular content in RAD hydrogels seeded with bovine BMSCs and cultured in medium with TGF-β1 ± Dex with or without aggrecanase inhibitor AGG-523 at 0.1μM (lo), 1μM (med), or 10μM (hi) at day 14. (A) sulfated glycosaminoglycan (sGAG) content, (B) DNA content (line indicated day 0 DNA level), (C) proteoglycan synthesis. Values are mean ± standard error of the mean. N=3. Line indicates significant difference between conditions, $p<0.05$.

Protocol to Make Agarose Rings

1. Autoclave tools needed: 2 forceps (1 pair curved and 1 pair flat), 2 spatulas (1 straight, one curved), and the 3 parts needed for making the ring mold (100 of each of: thin metal rings, fat metal washers, white plastic posts; makes 4 24 well plates with 4 extras)
2. Make the agarose:
   a. Set water bath at 70℃ (or 66℃ for one in injury room).
   b. Make agarose at 3%. For 100 mL put 3 grams of low melting point agarose into 100 mL of PBS. (Agarose rings use 2% agarose, for cell-seeded hydrogels need 3% so at 2% once add cells in media.)
   c. To get agarose to dissolve, microwave for 30 second intervals until gets to near boiling. DO NOT boil over or will lose water and change concentration. Or instead of microwaving, can put in 70℃ water bath for a few hours. (Don't forget it there.)
   d. Once the agarose is dissolved, swirl the bottle around to mix and autoclave it on the liquid cycle.
3. Make the agarose rings (**Need agarose at 2%**):
   a. Place agarose in 70℃ water bath to melt.
   b. Use curved forceps to put thin rings into wells of a 24 well plate (**Non-tissue culture treated plates**) with shiny side up. After thin pieces are in, place fat washers in with shiny side down (so shiny sides face each other).
   c. Place white pegs in center of each mold with flat forceps.
   d. Line up holes of fat washers so are all horizontal (makes casting rings easier).
   e. Take 5 mL of agarose from big bottle and put in round-bottom Falcon tube so repeat pipetter can reach bottom.
   f. Pull full volume into repeat pipetter tip (1.25 mL repeater pipet tips, sterile), do one click out on setting 1. Switch to setting 5 and inject into ring molds in little hole in washer. Inject agarose slowly and evenly. Each ring will be 125 μL and can do 8 rings with each pipet-ful. Do not reuse tip after pausing to put plates in fridge. Tip can be immediately reused to fill a plate with multiple castings in a row.
   g. Repeat step f until all molds are filled.
   h. Let rings sit in fridge for 5-10 mins (NOT longer) so agarose sets. Return agarose to water bath while waiting.
   i. Disassemble mold in opposite order as were assembled.
   j. Fill wells with PBS to cover rings.

Incomplete rings can be removed and have a new mold put in to form a new ring. On the day of casting, aspirate out any stray agarose in center of rings.

To clean up: Put metal pieces in beakers with about 300 mL of DI water and put in 70℃ water bath for 30 mins. Then set out to dry. Throw away posts and use new ones next time. Re-autoclave metal parts and 100 new posts in beakers covered with foil.

Protocol for Casting BMSCs in Peptide and Agarose Hydrogels

Requirements:
- Solutions for casting
- Agarose rings
- Cells
- Media calculations for feeding after casting

Important Notes:
- Agarose for gels needs to be made up in PBS, not water, so that the cells will be at the right osmolarity.
- Things that get added to the peptide (like SD Dex) need to be in water, not PBS, so that they don't assemble the peptide.

Procedure:
1. Look at your cells to get an idea of the confluence and how many cells you expect.
2. Make up any solutions for casting that weren't prepared previously.
3. Warm up PBS, FBS media, and other solutions. Thaw trypsin.
4. Remove PBS from agarose rings, replace with high glucose/HEPES media. Put plates in incubator.
5. Make up gel solutions. For agarose gels, put 3% agarose in water bath at 66°C in injury room. For RAD, follow RAD prep Excel sheet to determine amount of RAD and sucrose solutions need and how to split up. For KLD, follow Excel sheet in MSC expansion file (end at 4.38 mg/mL aliquots). These protocols assume you are not adding anything other than cells to the peptide. For the RAD, sonicate and vortex it for 10 minutes in the original vial. Then add sucrose and aliquot it out. Then put it back in the sonicater. For KLD, add 10% sucrose to the lyophilized powder, sonicate and vortex, and put in mini-centrifuge to move bubbles to the top. Pool peptide into one tube and put that in the sonicator. Spin down small tubes again to get remaining peptide out of the bottom. Aliquot so have 1.2 mL peptide each for full casts.
6. Put 900 μL Tris in each day 0 vial. Buffer assembles peptide.
7. Bring mini-centrifuge to TE room.
8. Trypsinize cells from flasks, spin down as usual (200 x g for 8 minutes).
9. Aspirate media from the tubes, leaving enough media to resuspend the pellet in. Resuspend cells in the FBS media they were originally in using the P1000. Combine all cells together into one 50 mL tube. Count. Do two counts, one from each of two different pulls from the original cell resuspension.
10. Split cells up so have 15 million in each tube (15 million cells in 1.5 mL total volume gives 10 million cells/mL). Keep in fridge until ready to use. If doing PCR, save aliquots of cells for monolayer comparison.
11. When ready to use, spin cells down in small centrifuge at 0.1 x 1000 @ rcf setting (so is 100 g) for 5 mins. Put plastic top on or it whistles.
12. Aspirate media from center of agarose rings. Leave in outer edge. If rings are leaking media into the center quickly, remove the media from the outer edge as well.
13. Put tip on repeat pipetter. (For old one, twist the tip on in order to get a good seal and avoid bubbles.) Set to correct setting.
14. Once cells are pelleted, remove media and resuspend in 300 μL of 10% sucrose with 2.5 mM Hepes for peptide, for agarose do 500 μL (so 1 mL 3% agarose plus 500 uL cell solution gives a 2% agarose gel).

15. If doing an agarose casting, place 1 mL aliquots of agarose in water bath at 42℃ after the cells are trypsinized. Keep the agarose in the water bath until right before you are ready to add the cells. If adding something to the hydrogel, add that.
16. Add cells to 1.2 mL of peptide (so 1.5 mL total volume). Vortex about 3 times on setting 4. Note: AVOID VORTEXING ON MAX SPEED FOR RAD TO AVOID CLUMPS OF CELLS. DO ON SETTING 4.
    a. Pull up peptide slowly and don't get stuck to the bottom of the tube to avoid bubbles.
    b. For old pipetter: Pull into repeat pipetter on setting 2 (so 50 uL per gel). Cast into agarose rings and into day 0 tubes. Can get 23 gels total from 1 pull including day 0's, usually $24^{th}$ short.
    c. For new pipetter: Will say 50 uL at the top once tip is attached. Pull up the full volume and click out once back into the vial. Cast 20 gels (for agarose last one will be bubbles). Take 50μL day 0 gels from volume remaining in tube. Vortex day 0 vials.
17. Top off gels with about 300 uL high glucose/HEPES media after waiting one casting. The gels need to be covered. If this is done too soon after casting, you can damage the gels.
18. Repeat steps 11 to 17 for remaining casts.
19. Once all gels are cast, change media on gels to culture media (750 μL per gel). Make sure medias were made correctly, with growth factors added.
20. Turn the water bath back to 37.1℃ if needed.

**Adjustments for casting chondrocytes**:

-Follow end of chondrocyte isolation protocol to remove cells from digested tissue and move into basal medium instead of trypsinizing flasks of BMSCs.

-Cast at a density of 30e6 cells/mL, so make aliquots of 45e6 cells to be added to 1.5mL total volume of RAD for full casts.

-Spin cells down at 1000 rcf for 7 mins because they are smaller than BMSCs.

Protocol to Do Live/Dead Assay on Fluorescence Microscope

Materials:

- Sterile spatulas (one curved, one flat)
- PBS
- Chamber slide
- Ethidium bromide and FDA solutions
- 20 μL pipetter and tips
- Waste container
- Scalpel handle and blade
- Large Petri dish

1. Check no one is using the microscope and that the Mercury lamp hasn't been turned off recently. You can also sign up in the Google calendar if you know a date in advance.
2. Prep the samples
   a. For agarose:
      i. Fill each well of the chamber slide with 1 mL PBS.
      ii. Scoop ring and gel out of plate with sterile curved spatula into large Petri dish.
      iii. Use scalpel to cut agarose ring outside of hood. Use straight spatula to move agarose ring away from gel.
      iv. Inside the hood, use spatulas to transfer the gel into the well of the chamber slide. Keep track of which sample is where.
      v. Repeat for all gels.
   b. For peptide:
      i. Try to nudge peptide out of ring so that peptide floats out and can scoop up into chamber slide. **Must go directly into PBS in chamber slide and NOT onto a Petri dish or you will never get it off.**
      ii. If the peptide won't come out of the ring, try moving the ring and peptide into the chamber slide to get the peptide to fall out.
3. Turn on microscope equipment
   a. Mercury lamp on first, if clicks is not fully on yet. Once the lamp is on it needs to be on for at least 30 mins. Write when the lamp got turned on, your name, etc in the log book.
   b. Turn on other three boxes (camera and light to the microscope). Camera must be on before software is opened or it won't connect.
   c. Open the OpenLab software and open the video controls and untitled layers windows under the "Window" and then "Palettes" menus.
   d. Place the eye icon in the layers window so that it is on video preview to see what the microscope is showing. (Placing the eye on other pictures allows you to look at other pictures you've taken.)
   e. Block bright field light with lens cap placed in light path.
   f. Set light allocation knob to setting "D" to see through eyepiece and camera.
   g. If the fluorescent light doesn't come through, make sure that the shutter button in the upper LHS of the little box on the right has the red light on.
   h. Make sure the phase ring is on 1, all filters at the top are to the left, and the lamp is turned to a reasonable level.

4. Adding the live/dead dye
    a. To make stock dye: 1mL PBS, 1 uL FDA (green dye, stains cytoplasm) and 3.5 uL of ethidium bromide (red dye, stains nucleus) per sample.
    b. Remove 1mL PBS and add 1 mL dye to the one gel you're imaging.
    c. Tips go into waste container.
    d. Wait a few minutes. (After first set can add pictures together for red/green before start next sample.)
5. Taking Pictures
    a. Using the 10x objective (objectives are turned by hand) and the green filter, find the sample. Note you can use the 4x objective to get a more widescale view of the entire sample. Best to do this before taking other pictures.
    b. Once a good spot is located, focus on the cells there. Switch to the red filter to make sure is no debris there.
    c. Switch back to green filter. Change light allocation knob to "C" so all light is to camera.
    d. Click the "AE" button in the video controls panel to autoexpose the image. Focus the image a bit so it looks more clear on the screen. Do autoexpose again.
    e. Click the camera icon on the LHS of the screen to take the picture. It will show up as a new layer. (Camera is black and white only.)
    f. WITHOUT CHANGING THE FOCUS, change to the red filter. Click autoexpose again. Take the picture. Watch the exposure time (around 1 second), because as gets longer picks up more background.
    g. DO NOT CHANGE THE FOCUS. Move the filter slider to the middle slot (neither red nor green, for dapi). Remove the lens cap to allow the brightfield light through. Click autoexpose again. Take the picture.
    h. Find another spot to take pictures and repeat b through g until 3 sets of pictures are gathered for the sample.
    i. Save the pictures by going to "save" in the file menu. Under the desktop, find the folder with your name. Name the pictures appropriately (day x of expt, date pictures taken, sample condition). All the pictures will be saved under that one name. You can name each individual picture by double-clicking on it.
    j. Repeat steps 4 and 5 for all samples.
    k. To turn off the microscope: Turn off the top two boxes first and the Mercury lamp last. Once the lamp is off, it needs to stay off for 30 mins. Log the time and that you turned the lamp off in the log book.
6. Getting your pictures off the computer
    a. Open the file for a particular condition (with multiple pictures in it).
    b. Go to Image, Contrast Enhancement, and select Best Guess.
    c. In the LHS menu, click on the color box and select FITC for green live photos, Rhod LP for red dead photos. Bright field pictures do not need to be colored.
    d. Save the file. (The above actions can be undone. Can go to reset under the contrast menu and greyscale on the color box.)
    e. Select all of the pictures in that file with the shift key.
    f. Go to Image, Depth, and change the depth to Millions of Colors and change the "Change" setting to "Selected Layers."
    g. Click ok. It will say it can't undo the actions, click ok anyway.
    h. Go to File, Save as Multiple to save each picture as an individual file.

i. Make sure saves to correct file. Under Naming click the box for start with document name, unclick the box for append layer name (unless you have named the layers), keep the numerical subscript (and count from 1), append with .tif, and save selected layers only. Hit Save.
j. Close the file and do not save it (because it's easier to work with the file when it's not set on millions of colors).
k. Repeat a-j for all files
l. Copy the OpenLab files as well as the newly created TIF files to a USB drive.
m. Save the files on the USB drive to the computer in the scintillation room and on your desk computer. Make sure the PC can read the files.
n. Don't store too many pictures on the computer in the microscope room. When you create a new set, delete the old set once you're sure they are saved.

7. Combining red and green pictures
    a. Can be done in the OpenLab software by highlighting both pictures and dragging them to the "New" icon at the top of the layers window.
    b. Can be added together in Photoshop:
        i. Open the two pictures you want to combine.
        ii. Go to Image, Apply Image, select "Difference" under the Blending menu and make the Source the file you want to add on.
        iii. Save the combined image as a new name.
8. To go to a greyscale picture, go to "Image", "Mode", and select "Greyscale."
9. To save the pictures as smaller files to put in Powerpoint:
    a. Open the TIFF image
    b. Go to "Image," "Image Size," and change the size from 1344 to 800 to shrink the picture size down.
    c. Save the image using Save As (DO NOT save over the original TIFF file), and save it as a jpeg. Select "high" quality in the box that comes up after you save.

<u>Notes:</u>
-You can set the exposure time by double-clicking on the time box. The entries are in ms, s, min, hr.
-You can delete pictures by dragging them to the trash icon at the top of the layers window.
-You can combine two pictures by highlighting them both and dragging them to the "New" icon at the top of the layers window.
-If something seems wrong with the camera, go to the menu furthest to the right, go to video manager, and select reset camera.

-Take pictures in the same order each time to avoid confusion (green, red, bright field).

Protocol to Extract Aggrecan from Peptide Gels

1. Make up 4M guanidine HCl in 100mM sodium acetate.
    a. Determine the amount of guanidine HCl powder and sodium acetate powder you need. Want to make about 1 mL of solution for every condition. Doesn't hurt to have extra, can use for making up GAG stds, etc.
    b. Measure out the sodium acetate powder and dissolve it in water to get 100 mM sodium acetate.
    c. Measure out the guanidine HCl powder IN THE FUME HOOD (it's a chaotropic agent).
    d. Add some volume of the 100 mM sodium acetate that is significantly less than the final volume you want to the guanidine powder. This is because the guanidine has a significant volume effect. The guanidine will dissolve easily. (Can pour extra sodium acetate solution down the sink.)
    e. pH the solution to 7.2 (make 10x dilution of acid or base so less drastic changes)
2. Add appropriate amount of protease complete tablet to inhibit proteases, add right before you're about to use the solution. DO NOT SONICATE!
3. Dice up your samples with a scalpel and put them back in their 2 mL tubes.
4. Add 1 mL solution with protease inhibitor per condition.
5. Place tubes on rotating machine at 4℃ for 48 hours to extract aggrecan.
6. Spin the tubes down in the microcentrifuge in the cold room at 13,000 x g for 30 mins.
7. Remove the supernatant and put it in a new 2 mL tube. Discard the pellet.
8. Measure the sGAG content of each sample. Note: the standards you use for the GAG assay must be made up in guanidine! If the amount of GAG is around 100, you must dilute the sample to be sure the measurement is not above the standard curve (do 1:2 and 1:5 dilutions).
9. Make up Chase buffer. Will need enough to resuspend 100μg GAG in 100μL Chase buffer. To make 10 mL of solution do:
    i. 60 mg Tris base (MW 121.1), which is 0.05 M Tris
    ii. 41 mg sodium acetate (MW 82.03), which is 0.05 M
    iii. 37.2 mg EDTA (MW 372.2), which is 0.01 M
    iv. dissolve in water
    v. pH to 7.6
10. Run 0.5mL of sample (max volume for tube) through microcentrifuge filter by centrifugation for 30mins at 14000 x g (max speed) in the cold room. Microcentrifuge tube product info: Amicon Ultra Centrifugal Filters, 0.5mL 10K (kDa) cutoff, cat no: UFC501024, 24 pack, made by Millipore
11. Measure volume in bottom of tube and discard (measuring is to get an idea of how much volume is left in the filter portion).
12. Repeat steps 10 and 11 for last 0.5mL of sample.
13. Run 0.5mL of Chase buffer through the filter by centrifugation for 30mins at 14000 x g and measure volume at bottom and discard.
14. Repeat step 13 (can do more rinses to dilute salt more if desired, downside is potential for adsorption to the filter and loss of protein from your sample).
15. Get a new tube to put the filter in. This will be your final storage for the protein, so label it well.
16. Insert the filter into the new labeled tube so that the sample currently in the filter will spin into the tube (so invert the filter from how you had it before).

17. Run the centrifuge for 2 mins at 1000 x g.
18. Measure the volume and add Chase buffer to achieve 100μg in 100μL.
    a. Remeasure GAG concentration here to get more accurate loading or to test protein retention.
    b. Digest the resuspended samples with protease-free chondroitinase ABC (30 mU/100 μg GAG) (Warner) for 3 hours in a water bath of 37℃. Only digest the amount of GAG you need to do ~5 blots. Chondroitinase is at 5mU/uL.
    c. Add keratanase II (0.5 mU/100 ug GAG) and endobetagalactosidase (0.5 mU/100 μg GAG) (Seikagaku # 100455, #100812 respectively . . . 1 mU/uL when rehydrated). Note: Dilute 2.5 μL into 20 μL water, gives 0.5 mU/5 μL. Incubate 2-3 hours at 37℃.
    d. Can measure protein with BCA assay if you're unsure the isolation was successful.

Now you have extracted the aggrecan from the plugs, measured how much you have, removed the guanidine salt, and digested away the sGAG chains. Then you can aliquot it into 10 μg aliquots (or smaller) and freeze those in the -20 until you're ready to run the Western blot.

Protocol for Running Western Blots

1. Have a plan
    a. Volume of reagents to mix with sample volumes
        i. 4-12% 15 well 1.5 mm thick Invitrogen mini-gel tolds 25μL per well max, usually load 20μL of volume
        ii. Purple sample buffer above western bench is 4x, so total volume per lane divided by 4.
        iii. 10x reducing agent, so total volume/10.
        iv. Volume of sample (5-10 μg seems standard, but can vary)
        v. Water to make up rest of volume
    b. Type of gel want to use (4-12% vs 10%) (smaller proteins get resolved better on bigger % gels, thicker gels allow to load more volume, fewer wells also leads to more volume)
    c. Order of lanes to run (put ladders on edges so know where gel ends)
    d. Antibody using, it's dilution, which animal to use for secondary
    e. If doing a strip and re-probe need to plan which antibody to do first (ie do NITEGE before G1 because G1 covers NITEGE).
2. Get a bucket of ice to keep samples and reducing agent cold.
3. Get NuPAGE Sample Reducing Agent (10x) from the fridge in the injury room and a Novex Sharp pre-stained protein standards vial from the western shelf in the freezer.
4. Set water to boil behind the shaker plate on the Western bench.
5. Make the running buffer: make 500 mL total, is 25 mL of 20x MOPS above western bench, then add rest of volume in DI water. Mix by covering top of graduated cylinder with parafilm and inverting. (If doing smaller proteins can use MES instead of MOPS)
6. Aliquot the appropriate amount of sample into new labeled tubes.
7. Make the sample buffer stock of 4x sample buffer, 10x reducing agent, and water. Add the appropriate volume to each sample. Vortex and spin down each sample tube. Set to boil for 5 mins (denatures proteins).
8. Get a gel from the cold room.
9. Put the electrophoresis machine together
    a. The clear box with the gold post is the main unit
    b. Put the squarish piece with the 2 gold posts in, the side with the post facing down goes in the slot on the right.
    c. The piece with the lever goes in the back
    d. The blank plastic piece goes between the gold post piece and the lever piece, or could run a second gel here.
    e. The gel goes in the front of the gold post piece. **Take the white tape off of the gel before you put it in.
    f. Remove the comb from the gel, CAREFULLY.
    g. Tighten the lever in the back so the gel is held tight.
10. Pour running buffer behind the gel in the middle compartment between the gel and the back plate until the liquid level is over the white bar and covers all the wires. If the buffer leaks out of the middle compartment into the outer parts, you did something wrong. Pour the rest on the buffer into the outer part of the box.
11. Use the special super-fine pipette tips to squirt some buffer over the top of the lanes to make sure there's no gunk blocking them.
12. Once samples are done boiling, vortex them and spin them down again.

13. Load the protein standards ladders (10μL) and your samples with the super-fine tips. Set the pipetter above the full volume you expect to pull up so you get everything. If the tip gets clogged, you can throw it out or cut it to try and salvage the sample. (Note: If running 2 gels at a time, you may want to run the gel in the cold room or on ice and add more running buffer outside the gel. You would want to load the samples in the cold room if you were going to run the gel there. Second gel should face OUT away from the center of the box.)
14. Once all samples are loaded, put the top on the electrophoresis box and plug the cords into the power supply (red to red, black to black). Run the gel at 200V for about 45 mins. If you don't see a bunch of bubbles when you turn the power on, something is wrong. Make sure protein of interest is going to be a bit away from the edge of the gel and separated well when you stop running the gel.
15. While the gel is running, make up the transfer buffer (1000 mL total)
    a. Measure out 3.03 g Tris base and 14.4 g glycine. (If have had problems with transfer in past can add 0.5 g SDS.)
    b. Add 800 mL water
    c. pH the solution to 8.3
    d. Add 200 mL methanol (stored under the waste fume hood).
16. After you're done running the gel (turn off the power supply and unplug the electrophoresis box), you need to transfer the separated proteins to a membrane. To do this, get the aluminum tray and place a transfer cassette black side down, slide side up. Get 2 sponges and place 1 on the black part, one in the tray next to the cassette. Get 2 pieces of blotting paper and put one on each sponge. Pour transfer buffer over each blotting paper.
17. Remove the gel from the electrophoresis machine and crack open the plastic casing with the spackle applicator-looking tool. You want the gel to stick to the side with the black writing when you pull the plastic pieces apart. Cut the comb and the two side pieces off the top of the gel with a razor blade.
18. Put the gel on top of the blotting paper and get the gel to come off the plastic and stick to the blotting paper. You want to make sure that the part of the gel you care about most (the top for full-length aggrecan) is fully on the blotting paper and not right on the edge. Cut off the bottom ridge of the gel. Add additional transfer buffer. Avoid any bubbles.
19. Assemble the transfer cell by placing the red and black plastic piece in the middle of the clear plastic box with the black part towards the middle. Also make sure you have a green lid for the box and a small stir bar in the middle of the red and black piece.
20. Cut a piece of PVDF membrane (not nitrocellulose) to fit inside one of the little lids the blot will go into. The paper also should not be bigger than the gel or it messes up the voltage. Do not touch the white membrane, only the blue papers.
21. Saturate the white membrane only (not the blue papers) in methanol in a tip lid. Rinse with DI water in another tip lid.
22. Place the membrane on top of the gel, avoiding bubbles and avoiding moving the membrane once it's been laid down.
23. Place the other piece of blotting paper on top of the membrane, then add the other sponge. Roll over the sandwich with a 50 mL tube to squeeze out bubbles.
24. Maintain pressure on the stack while closing the cassette and sliding the top closed. Place the cassette into the transfer cell (black to black). You may need to jam it in a bit. Put an ice pack in the back of the transfer cell (from the top of the freezer/fridge in the tissue engineering room). Pour in a bunch of the transfer buffer (including the stuff in the aluminum tray), but not too full so you don't spill.

25. Take the transfer cell to the cold room. Add the rest of the transfer buffer, move the cell on top of the stir plate, and set the level to 2.5 (or maybe a little above to get the stir bar spinning). Put the top on the transfer cell (red to red, black to black) and plug the cords into the power supply (red to red, black to black). If one power supply doesn't work, try the other one. Other options are to switch lids and add more buffer. Set the voltage to 75V and transfer for 60 minutes (or a bit more if had problem before, can overtransfer small proteins).
26. To clean up the electrophoresis equipment, pour the MOPS buffer down the sink and rinse off all of the machine parts.
27. While the proteins are transferring, make up the blocking solution. Can either do milk or BSA in TBST or PBST. Usually do 5% milk in TBST. To make 50 mLs weigh out 2.5 g Carnation instant non-fat dry milk and add TBST. There is a volume effect, so don't add the full 50 mLs. Add some volume, mix it to break up the clumps and then sonicate it. Add the rest of the volume of TBST afterwards. (Note: TBST= Tris buffered saline + Tween, Tween is a detergent. Rachel makes up 10x TBS because it takes a long time to dissolve and pH. 10x TBS is 24.23g Tris base, 80.06g NaCl, 800 mL water. Then pH to 7.6 by adding HCl, and fill to 1L with water. Make up 500 mL of 1x TBST at a time. Tween is viscous so use wide mouth pipette tips. To make up 500 mL of TBST take 50 mL of 10x TBS and add 450 mL water. Then add 250 μL of Tween (is 0.5%).)
28. Once the transfer is complete, turn off the power supply and bring the transfer cell back to the western bench. Remove the cassette from the machine and open it, clear side up. Remove the PVDF membrane and place into one of the small lids so that the protein side (the side facing the gel) is up. Rinse the membrane off with TBST (about 8-10 mLs). Dump out the TBST and cover with 10 mL milk.
29. Place on stir table for 1 hour to block against non-specific binding.
30. To clean up the transfer materials, throw the gel into the big plastic jar (unless you want to do a Coomassie stain), throw away the blotting paper, and just rinse out everything else.
31. After the blocking is complete, make up the primary antibody solution. The total volume will be 8 mL because that's the minimum volume to cover the membrane in the small boxes. So, for a 1:1000 dilution add 8 μL of the antibody, or for a 1:2000 dilution add 4 μL of the antibody. Usually make up antibody in whatever solution you use to block (sometimes this is specified for a particular antibody).
32. Put all 8 mL on the membrane and put it on a stir table in the cold room to incubate overnight.
33. The next morning, do one quick rinse with TBST and then wash the membrane 3 times in TBST on a stir table in the lab for 10 minutes each. If you anticipate doing a similar Western soon, you can save the primary antibody solution and reuse it. The staining will be less intense.
34. Make up the secondary antibody (1:2000 anti-rabbit for example) and incubate for 60 minutes at room temperature.
35. Do one quick rinse of the membrane with TBST to get the milk off. Wash the membrane 3 times with TBST for 10 minutes each again.
36. Set up the gel dock for visualizing the gel: Open the FluorChem software and set it to filter 1. Adjust so that you get all of the light coming in (top to 2.8) and adjust the focus so you see the inner square (middle is rough focus, bottom is fine focus).
37. Get the ECL kit from the fridge (chemiluminescence) and make up a 1:1 mix of the two solutions in the box. Make up 5 mL total to cover the whole membrane. **Only do this right before you're ready to use this solution!

38. Put the liquid directly on the membrane and let the ECL mix sit for 1 minute on the membrane. After the one minute is up, pick up the membrane with tweezers and dab one corner onto a paper towel.
39. Place the membrane on a plastic sheet, cover it without creating bubbles.
40. To image the membrane:
    a. Do a 1 minute supersensitive picture first, then a 5-10 minute at normal (longer if is grainy).
    b. Take a white image to see the ladders. Set to normal/high, autoexpose, expose preview, see the green light flash, and hit acquire image. Save. Remove from computer with USB key.
41. Save membrane in plastic wrap and keep in fridge. Or, to strip and re-probe:
    a. Rinse twice with TBST for 5 minutes each.
    b. Strip for 15 minutes with the stripping buffer (brown bottle on bottom shelf to left above western bench)
    c. Rinse twice with TBST for 5 minutes each.
    d. Block for 1 hour with milk.
    e. Add secondary antibody again to ensure no signal after stripping
    f. Do a quick rinse and 3 10min washes.
    g. Repeat imaging with ECL solution
    h. Do 3 10 min washes and block again.
    i. Incubate overnight with primary antibody in cold room, so go from step 31 back to 40.
42. To analyze the pictures in Photoshop:
    a. Image, adjust, invert
    b. Image, adjust, brightness/contrast and slide the contrast around
    c. You want the blot to be dark enough so you can tell where the gel is, not underexposed.
    d. Match up the MW standards with lane on end and put arrows where they are.
    e. To get the pictures into powerpoint:
        i. Image, mode, 8 bit
        ii. Save for web
        iii. Save as jpeg
        iv. Can import into powerpoint

Coomassie Staining (for the gel or the membrane):

- Coomassie dye is 1 g dye in 500 mL methanol and 500mL water. For staining the gel, take dye and add 5-10% acetic acid to fix the proteins in the gel. Put the staining gel on a stir plate for 30 minutes.
- Coomassie waste goes in the chemical hood.
- Rinse gel a few times with DI water.
- Destaining solution is 7.5% acetic acid and 10% methanol in water. Cover the gel with destaining solution with a Kimwipe on one side. Rinse 2x with destaining solution and new Kimwipes.
- If taking pictures, let destain overnight. Flip down the white board in the imaging machine and select transluminator in the software.

Coomassie is pretty irreversible. Panceau S is another dye to show proteins but is reversible. For Panceau S, stain for 30-60 mins and destain with water. Can add to gel before you transfer.

# Appendix B: Data and Protocols Related to Chapter 3

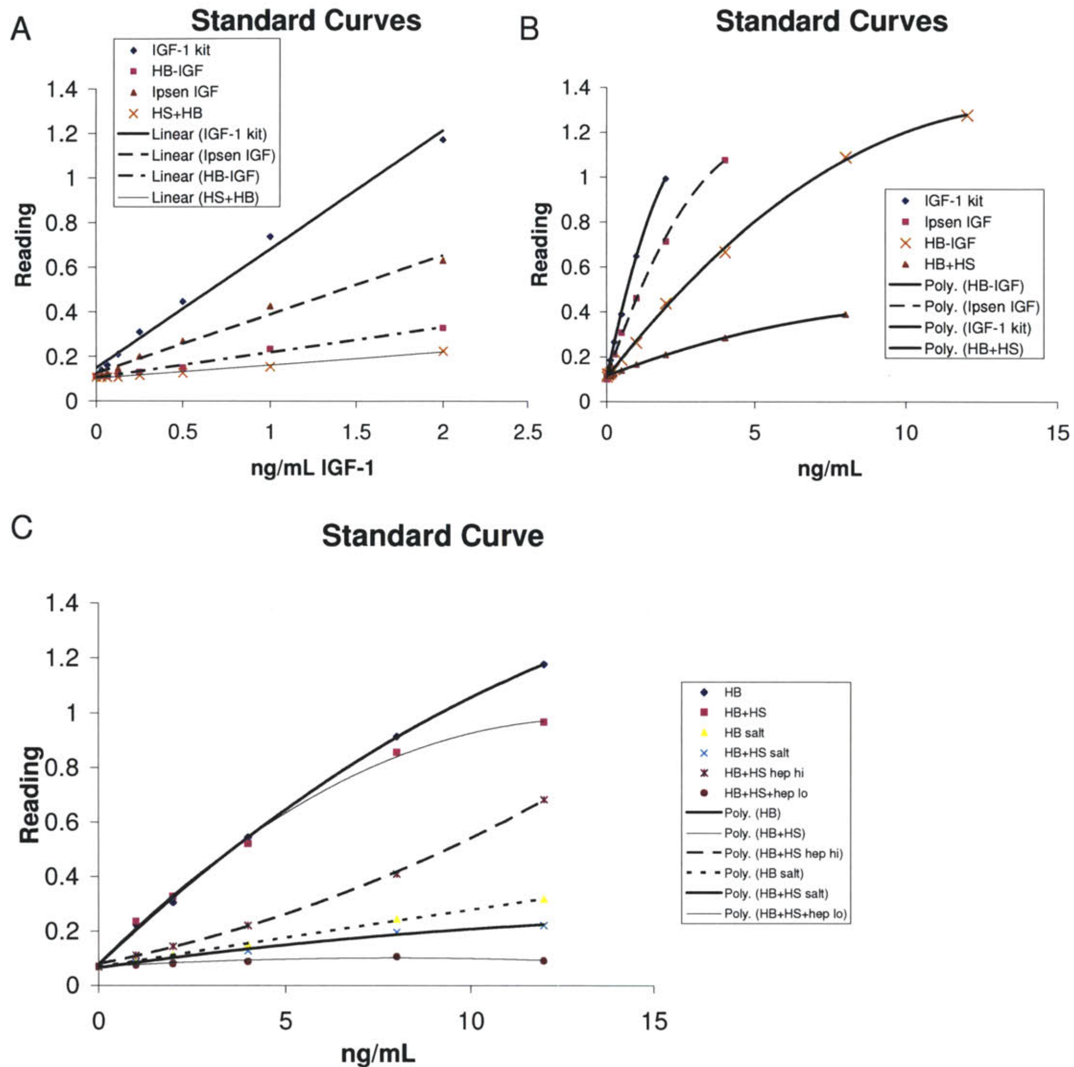


**Figure B1**: IGF-1 ELISA standard curves. (A) comparison of standard curves for IGF-1 from ELISA kit, IGF-1 from Ipsen, HB-IGF-1, and HB-IGF-1 mixed with heparan sulfate (HS), (B) standard curves from (A) but extended to higher concentrations, (C) comparison of standard curves of HB-IGF-1 with or without HS, HB-IGF-1 with or without HS in a high salt buffer, and HB-IGF with HS and two concentrations of heparitinase, 0.01mU/μL (lo) and 0.1mU/ μL (hi). Heparitinase digestion took place for 3 hours at 37°C.

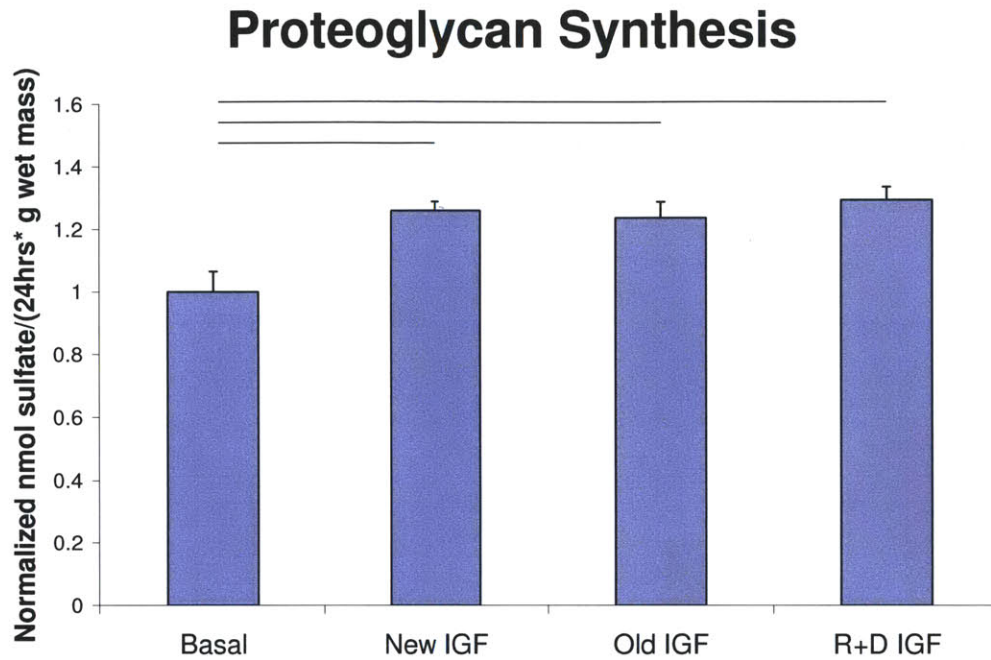


**Figure B2**: Test IGF-1 stability on cartilage explants. Proteoglycan synthesis at day 5 in middle zone cartilage explants cultured in basal medium or with 50nM new Ipsen IGF-1 (New IGF), Ipsen IGF-1 that had been frozen and stored (Old IGF), or IGF-1 from R+D (R+D IGF). Values are mean ± standard error of the mean. N=6. Line indicates significant difference between conditions, $p<0.05$.

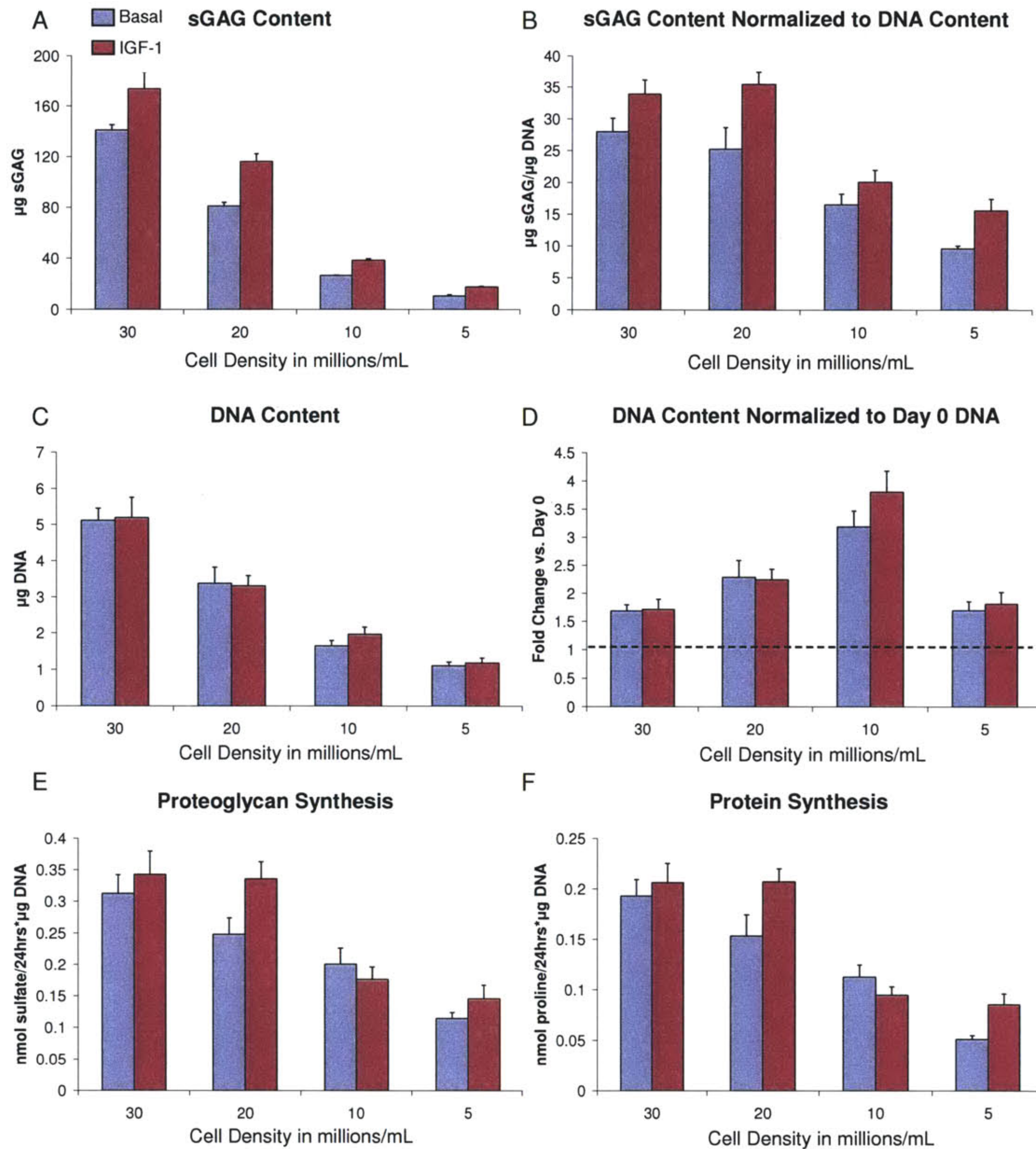


**Figure B3**: Effects of cell density on response to IGF-1 in RAD hydrogels. Extracellular matrix and cellular content in RAD hydrogels seeded with bovine chondrocytes at day 5 cultured in basal medium or with 50nM IGF-1. (A) sulfated glycosaminoglycan (sGAG) content, (B) sGAG content normalized to DNA content, (C) DNA content, (D) DNA content normalized to day 0 DNA values, (E) proteoglycan synthesis, and (F) protein synthesis. Values are mean ± standard error of the mean. N=4.

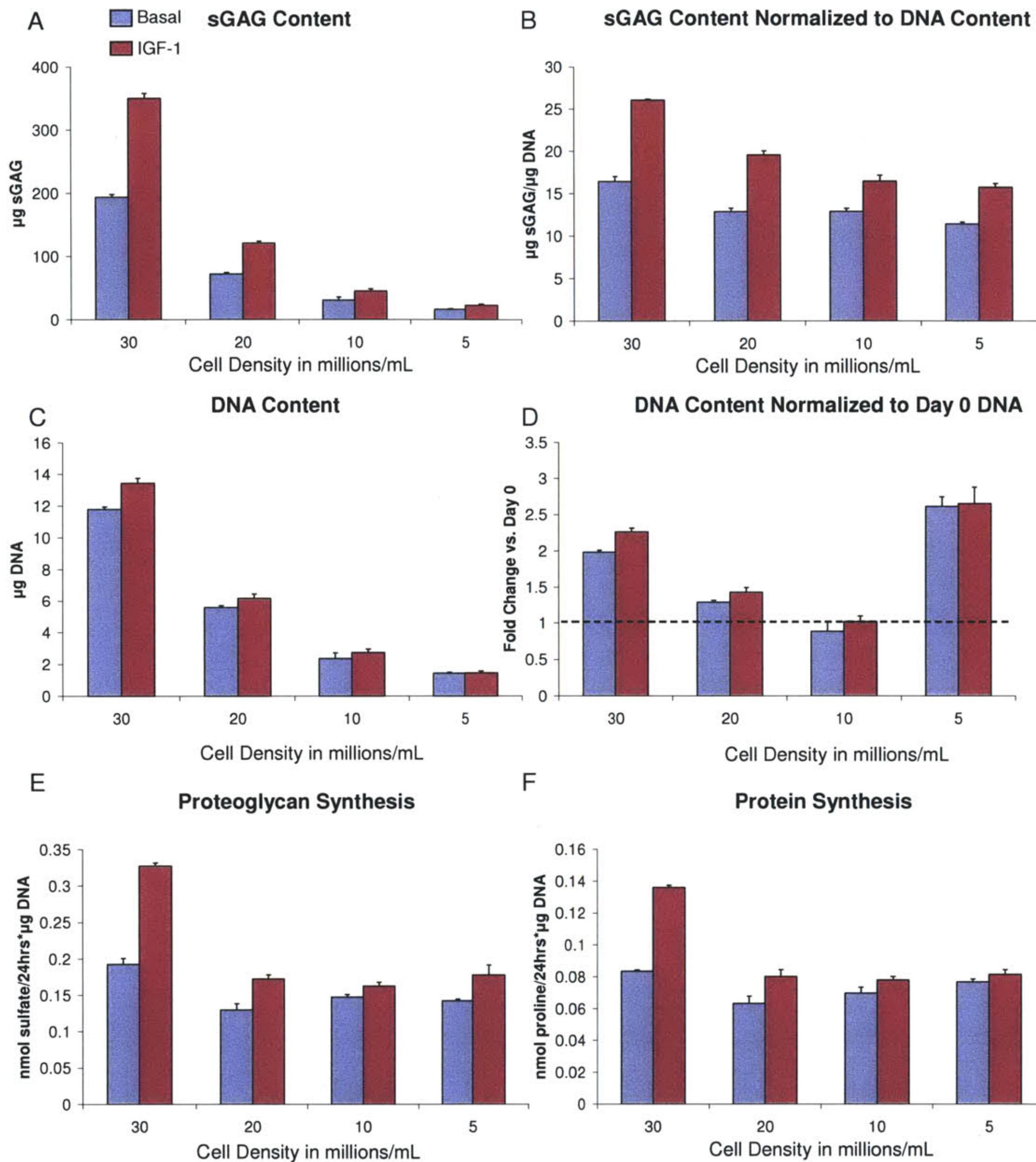


**Figure B4**: Effects of cell density on response to IGF-1 in agarose hydrogels. Extracellular matrix and cellular content in agarose hydrogels seeded with bovine chondrocytes at day 5 cultured in basal medium or with 50nM IGF-1. (A) sulfated glycosaminoglycan (sGAG) content, (B) sGAG content normalized to DNA content, (C) DNA content, (D) DNA content normalized to day 0 DNA values, (E) proteoglycan synthesis, and (F) protein synthesis. Values are mean ± standard error of the mean. N=3-4.

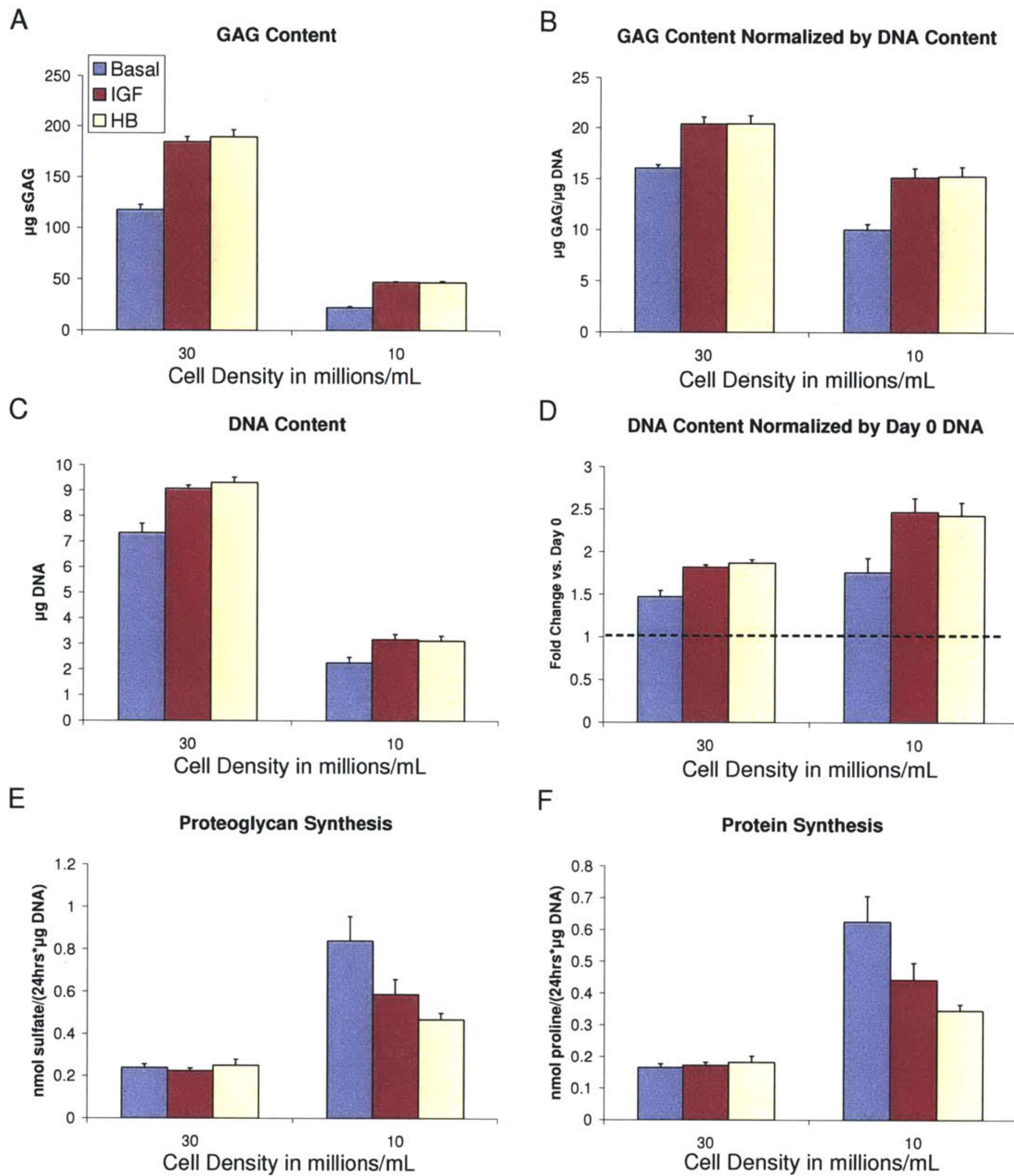


**Figure B5**: Effects of cell density on response to IGF-1 and HB-IGF-1 in RAD hydrogels. Extracellular matrix and cellular content in RAD hydrogels seeded with bovine chondrocytes at day 5 cultured in basal medium or with 50nM IGF-1 or HB-IGF-1. (A) sulfated glycosaminoglycan (sGAG) content, (B) sGAG content normalized to DNA content, (C) DNA content, (D) DNA content normalized to day 0 DNA values, (E) proteoglycan synthesis, and (F) protein synthesis. Values are mean ± standard error of the mean. N=6.

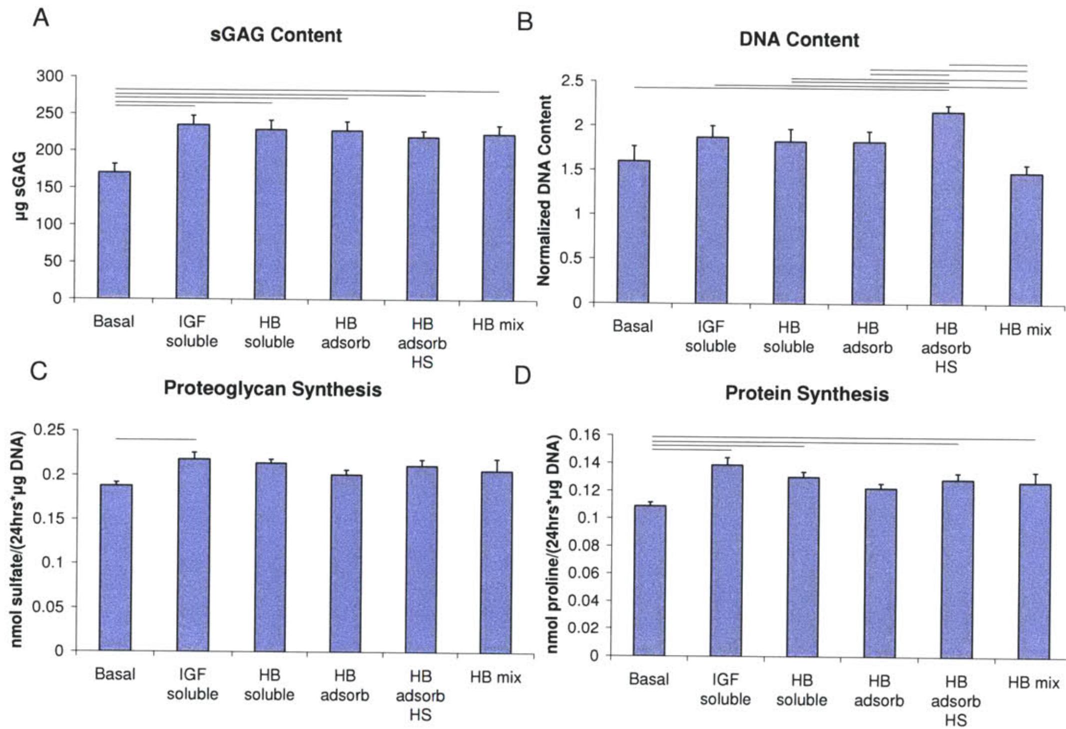


**Figure B6**: HB-IGF-1 delivery to encapsulated chondrocytes by functionalized RAD. Extracellular matrix and cellular content at day 6 in RAD hydrogels seeded with bovine chondrocytes. (A) sulfated glycosaminoglycan (sGAG) content, (B) DNA content, (C) proteoglycan synthesis, (D) protein synthesis. Values are mean ± standard error of the mean. N=12 (6x2 animals). Line indicates significant difference between two conditions, $p<0.05$.