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Abstract

Cartilage injury, which can lead to arthritis, currently lacks any effective treatment, and so the design of chondroinductive biomaterials holds a compelling appeal for the field of regenerative medicine. Although chondroinductive signaling pathways are conventionally activated by cellular communication with growth factors and extracellular matrix (ECM) components, these natural molecules have translational limitations such as immunogenicity, lack of reproducibility, and high cost. As a result, designing acellular chondroinductive biomaterials with no animal-derived elements presents a clearly superior approach for commercial applications.

Growth factor and ECM molecules often contain cell-binding sequences that could potentially be reproduced in synthetic peptides, and this idea provides the rationale for the current dissertation: evaluation of the chondroinductivity of synthetic peptides to mimic the effects of growth factors and ECM components in cartilage repair. I identified the peptide candidate, SPPEPS, as a matching sequence of two chondroinductive molecules, aggrecan proteoglycan and transforming growth factor-β_3 (TGF-β_3). The N-terminal subunit of TGF-β_3 is known as the latency-associated protein (LAP) and the SPPEPS peptide sequence belongs to the LAP region of TGF-β_3 which is known to be a ligand for a number of integrins and integrins play a critical role in cartilage regeneration. First, the properties of SPPEPS and Link N peptides were evaluated in rat bone marrow

mesenchymal stem cells (rBMSCs), demonstrating their potential as chondroinductive sequence. Next, I compared the properties of the SPPEPS peptide in isolation and when incorporated with RGD into pentenoate-functionalized hyaluronic acid (PHA) hydrogels, thereby demonstrating the chondroinductive potential of PHA hydrogels. Finally, chondroinductivity was evaluated for SPPEPS or RGD peptides *conjugated into* PHA hydrogel networks and demonstrated the potential for hydrogels with crosslinked SPPEPS peptides to regenerate tissue with prevalent collagen type II production, which is a faithful reproduction of native hyaline cartilage. Future studies will focus on evaluating the chondroinductivity of PHA hydrogel with different concentrations of SPPEPS peptide, in addition to designing of bifunctional materials with desirable mechanical integrity.

Chapter 1: Introduction

Cartilage injury lacks effective treatment and it may eventually lead to further degeneration of the tissue, including conditions such as arthritis. This unfortunate situation motivates the work described in this dissertation: the design of a chondroinductive (i.e., cartilage regenerating) biomaterial to assist in the repair of cartilage defects. Although natural molecules such as growth factors and extracellular matrix components could be incorporated into biomaterials to emulate natural cellular environments, thereby stimulating cartilage regeneration, such molecules present significant barriers to commercial adoption: they are expensive, lack reproducibility, and are prone to risks associated with immunogenicity. The main focus of this dissertation was therefore to investigate whether small *synthetic* peptides can mimic the effects of cell adhesion motifs from natural molecules, and if such peptides can affect cellular differentiation, specifically chondrogenic differentiation. Chondroinductive peptide sequences, if indeed they exist, might therefore be incorporated into chondroinductive biomaterials for the treatment of cartilage injury.

At the beginning of this thesis, we did not have any peptide candidates. In an exploratory and high-risk/high-reward journey, my thesis tells the story of the thought process behind parallel approaches to identify potential candidates, their evaluation, and the ultimate selection of a leading candidate that is currently the basis of a provisional U.S. patent application.

The story begins by looking outside of the field of regenerative medicine. Receptor-binding peptides, and methods to identify them, are a major focus in cancer research. By taking our cue from cancer therapy research, we may potentially create 100% synthetic biomaterials with the following steps: 1) Identify the receptors involved in cartilage regeneration; 2) Design peptides that bind the receptors and activate desired signaling pathways; 3) Incorporate these peptides into suitable biomaterials. If successful, this approach could revolutionize the treatment of injured cartilage. With these design steps in mind, the Specific Aims for this thesis were: (1) to evaluate the effects of synthetic peptides on chondrogenic differentiation of rat bone marrow-derived mesenchymal stem cells (rBMSCs), (2) to evaluate the chondroinductivity of synthetic peptides conjugated to pentenoate-functionalized hyaluronic acid (PHA) hydrogels *in vitro.* (3) to evaluate the potential of the synthetic peptides conjugated to PHA in regenerating hyaline-like cartilage tissue *in vivo.*

In my Aim 1, various candidate peptides were either dissolved in the cell culture medium or used as coatings for cell culture plates. The superior peptide was chosen for further studies in Aim 2, where chondroinductivity was evaluated when the peptide was crosslinked to pentenoate-functionalized hyaluronic acid (PHA) hydrogel networks *in vitro*. Cognizant of the differences between chondrogenic effects in a laboratory experiment and in a physiological context, my Aim 3 was to evaluate the efficacy of the peptide in regenerating a cartilage-like tissue when conjugated to PHA hydrogels *in vivo*.

Chapter 2 is a review of the literature that introduces the idea of designing biomaterials tailored to desired cell-matrix interactions by incorporation of peptides. Moreover, this review introduces the methods of identifying the peptides and the important role of cell adhesion peptides and cell adhesion integrins in cartilage. This review chapter provides the foundation for my vision for the study design in the chapters that follow. The review addresses the limitations of the approaches that use natural molecules as components for biomaterials in regenerative medicine and provides suggestions for new strategies for identifying chondroinductive peptide sequences focused on integrin-peptide interactions which may lead to chondrogenic differentiation of stem cells.

Chapter 3 addresses Aims 1 and 2, i.e., the *in vitro* evaluation of two peptides (i.e., Link N and SPPEPS) in terms of chondroinductivity. I identified (Ser-Pro-Pro-Glu-Pro-Ser, SPPEPS) as a matching sequence of two chondroinductive molecules, the core protein of the proteoglycan aggrecan, and transforming growth factor-β_3 (TGF-β_3). The chondroinductivity of the peptide was evaluated for the first time and the encouraging results led to proteomics analysis on the rBMSCs exposed SPPEPS to further evaluate the protein profile and signaling pathways upregulated by the peptide. In addition, SPPEPS was conjugated to PHA hydrogels and the chondroinductivity was evaluated by qPCR for rBMSCs cultured on top of the hydrogels. PHA hydrogel was chosen as a carrier system due to its fast crosslinking time (1-2 minutes) which is attractive for surgical applications. In

Chapter 3, the SPPEPS peptide supported chondrogenic differentiation *in vitro* and therefore was chosen for further *in vivo* evaluation in Chapter 4.

Chapter 4 addresses Aim 3, i.e., the *in vivo* potential of a PHA hydrogel when it is conjugated to either SPPEPS or RGD in regenerating a hyaline-like tissue, using a rabbit femoral condyle model. The regenerated tissue was assessed based on the gross morphology, Hematoxylin & Eosin staining, Alcian Blue staining and collagen II immunohistochemistry.

Chapter 5 concludes this thesis with a summary of key results and interpretations. I provide not only recommendations for further analysis, but also possible future research directions in the field to design the next generation of materials for articular cartilage regeneration.

In this thesis, the study designs and all the experiments were done by myself except the proteomics experiment in the first aim, which was conducted by the Laboratory for Molecular Biology and Cytometry Research at the University of Oklahoma Health Sciences Center. However, the identification of analysis methods and the raw data evaluation was done by myself. For the third aim, the study protocols for Institutional Animal Care and Use Committee (IACUC) approval, the study design, surgical room preparation, monitoring the animal's wellbeing, harvesting and preparing the tissue for histology analysis was done by myself. The sectioning, Alcian Blue and H&E staining of the tissues were

performed by the Stephenson Cancer Tissue Pathology Core at the University of Oklahoma Health Sciences Center and the Immunohistochemistry staining was done by myself.

Overall the work presented in the current dissertation introduced a novel approach in designing chondroinductive biomaterials and provided the first step in the design of acellular synthetic chondroinductive biomaterials with the goal of developing cost-effective and highly translatable treatments.

Chapter 2: Chondroinductive Peptides: Drawing Inspirations from Cell-Matrix Interactions[1]

Abstract

In the field of regenerative medicine, creating a biomaterial device with the potential to alone affect cellular fate is a desirable translational strategy. Native tissues and growth factors are attractive candidates to provide desired signals in a biomaterial environment; however, these molecules can have translational challenges such as high cost, complicated regulatory pathways, and/or limitations with reproducibility. In regenerative medicine, there is a burgeoning community of investigators who seek to overcome these challenges by introducing synthetic peptides to mimic the desirable signals provided by growth factors and tissue matrices. Since in cartilage tissue, cell-adhesion signaling mediates cell migration, growth, and differentiation, synthetic peptides that mimic a desired cell-adhesion sequence may help to control cellular fate. This chapter emphasizes the value of the signaling ability of peptides, specifically in the cartilage regeneration field. The primary challenge in cartilage regeneration is to regenerate true hyaline cartilage instead of a fibrous tissue. The vision is to create materials that take advantage of the signaling abilities of peptides and that themselves induce chondrogenesis without the need for tissue-derived matrix or growth factors, which can potentially revolutionize arthritis prevention and treatment.

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Introduction

It is estimated that 78 million adults aged 18 years or older in the U.S. (26% of the population) will be diagnosed with arthritis by the year 2040 (Centers for Disease Control and Prevention; Nov 20, 2016). Despite arthritis being one of the leading causes of disability, it does not yet have an effective treatment and may develop following a cartilage injury. There are surgical treatments that aim to repair cartilage tissue such as autologous chondrocyte implantation (ACI), microfracture, osteochondral transplantation (mosaicplasty), and allograft implants. Although these treatments may provide clinical improvement, they are generally not successful in producing a tissue with the same long-term mechanical and chemical properties of native articular cartilage. The deficiency of the treatments to produce an effective cartilage tissue has created an impetus for the regenerative medicine community to establish strategies that lead to restoration of a fully functional hyaline cartilage.[1]

To design a bioactive material, one approach includes the incorporation of natural components such as extracellular matrix (ECM) molecules or growth factors in a scaffold structure. Although natural components might be able to mimic the native environment for the cells and enhance cartilage formation, disadvantages such as high cost, limitations of reproducibility, and the risks involved with immunogenicity can be significant limitations for commercialization. Therefore, a synthetic product with the potential of inducing cartilage regeneration without the need for human- or animal-derived elements (if indeed such a material

exists) would present a more attractive translational option. Synthetic biomaterials may be utilized for acellular applications where the cartilage regeneration does not rely on cell-based therapies,[2-4] although there are applications where a cell-based strategy may be required, for example in regeneration of the entire joint surface,[5, 6] and a chondroinductive biomaterial would be advantageous in either case. In cartilage regeneration, the goal of designing an all-synthetic biomaterial is to create scaffolds with desired mechanical integrity and the ability to lead endogenous mesenchymal stem cells (MSCs) toward chondrogenic differentiation and prevent chondrocyte dedifferentiation. The properties of synthetic biomaterials may be tailored by changing the polymer composition to achieve desirable failure properties (e.g., maximum stress, strain, and toughness);[7] however, the remaining challenge is incorporating all-synthetic cellular signals to replace ECM molecules and growth factors to lead endogenous stem cells to chondrogenic differentiation and prevent chondrocyte dedifferentiation.

Peptides have the potential to mimic ligands and act through desired cellular signaling pathways. Identifying the bioactive peptides that are chondroinductive and then incorporating them in a biomaterial may lead to 100% synthetic chondroinductive biomaterials, which may potentially revolutionize the field of cartilage injury treatment.

Perhaps the regenerative medicine community may take a cue from the cancer field, as identifying receptor-binding peptides is a crucial research focus for

oncological therapy. Delivery of a therapeutic with receptor-binding peptides decreases the side effects of the therapeutic compared to chemotherapeutic methods, in which the drugs are given to the patients at concentrations approaching maximum body tolerance with low efficiency.[1] The regenerative medicine community may likewise design synthetic bioactive materials by incorporating receptor-binding peptides in the material structure to take advantage of their signaling abilities.

In regenerative medicine, there are a select group of pioneering studies that have employed peptides as direct signals.[8-13] In addition, there are review papers that have eloquently covered receptor-binding peptides;[1, 14] however, they have not focused on peptide applications for regenerative medicine nor the approaches of identifying peptides that may help with cellular differentiation.

This chapter provides an overview of cartilage regeneration studies that have taken advantage of bioactive peptides for their potential of affecting cellular fate. Furthermore, we emphasize cell-matrix adhesion, adhesion receptors, and methods of identifying adhesion receptor-binding peptides as a potential strategy to affect cellular differentiation and induce chondrogenic differentiation.

Potential Chondroinductive Peptides in Cartilage Regeneration

The most common methods of incorporating receptor-binding peptides in biomaterials are using peptides as a coating for scaffold surfaces and conjugating the peptides to polymers in scaffolds. Incorporating small peptides in biomaterials for regenerative medicine is attractive because short peptide sequences can be synthesized reproducibly in large quantities, their small size reduces the chance of non-specific binding, and they have the potential to affect cellular fate. Arginine-glycine-aspartate (RGD) peptides are attractive because of their cell-adhesion properties and as a result, RGD is the most commonly used receptor-binding peptide across a variety of applications. However, there are only a few peptides that have been employed in the cartilage regeneration field for their capacity to potentially induce chondrogenic differentiation. Given below is a concise overview of each of these studies.

Link N Peptide

Link protein is a glycoprotein that stabilizes the non-covalent interaction of hyaluronate and aggrecan molecule G1 domain[15] and the Link N sequence (DHLSDNYTLDHDRAIH) is the amino-terminal peptide of the link protein. In 2013, Wang *et al.*[16] indicated that the Link N sequence acts through the BMP type II receptor. There are several studies that investigated the potential of Link N sequence in improving cartilage tissue regeneration both *in vitro* and *in vivo*.[17]

In 2003, Mwale and co-workers showed that adding 100 ng/mL Link N to the culture medium of both nucleus pulposus (NP) and annulus fibrous (AF) cells

isolated from bovine intervertebral discs (IVDs) every two days increased the glycosaminoglycan (GAG) and collagen types II and IX accumulation after 20 days. Type II collagen and type IX collagen contents were measured by ELISAs and GAG content was measured by the DMMB assay.[15] In another study, Gawri *et al*. reported that injecting 50 µL Link N with concentration 20 mg/mL into healthy intact IVDs isolated from 13 adult human donors promoted aggrecan synthesis compared to the control group (50 µL $^{35}SO_4$). Aggrecan synthesis was assayed by estimating $^{35}SO_4$ incorporation in intact IVDs after 48 hours.[18] Injecting Link N with concentration 10 mg/mL into 5 mm deep annular punctures in the center of NP of 19 New Zealand White rabbit IVDs significantly increased the aggrecan expression for both NP and AF after two weeks.[19]

GFOGER Peptide

The GFOGERGVEG-POGPA peptide sequence was identified for its ability to bind the $\alpha_2\beta_1$ integrin. The peptide was recognized by comparing the binding abilities of overlapping peptides from collagen I, α_1(I)CB3 domain to the $\alpha_2\beta_1$ receptor.[20, 21] In 2000, Knight and co-workers[22] reported that removal of the GER sequence from GFOGERGVEG-POGPA sequence stopped the peptide from binding to $\alpha_2\beta_1$; however, the removal of its C-terminal had no evident effect on the level of the peptide adhesion to $\alpha_2\beta_1$ integrin. This study further indicated the ability of the GFOGER peptide to bind to the $\alpha_2\beta_1$ receptor by demonstrating that the adhesion of collagen I to the receptor was inhibited by GFOGER.

In 2010, Liu *et al.*[23] incorporated GFOGER into poly(ethylene glycol) (PEG) hydrogels through Michael addition chemistry. Human MSCs were encapsulated in PEG hydrogels with and without GFOGER. After 9 days, the relative gene expressions of collagen types II and X and aggrecan were higher in the groups with the GFOGER peptide.

B2A2 Peptide

The B2A2 peptide consists of a receptor targeting domain, a hydrophobic spacer domain, and a heparin-binding domain. The peptide was designed in 1999 to bind to heparin and endothelial cell proteoglycans.[24] In 2005, the peptide was recognized for its ability to bind to the BMP-2 receptor.[25, 26] Since BMP receptors are involved in chondrocyte formation, in 2012 Lin *et al.*[27] hypothesized that the B2A2-K-NS peptide could play a role in cartilage repair. To test their hypothesis, they added 10 µg/mL of the peptide to murine embryonic stem cell medium every three days. After seven days, gene expression of SOX9 and collagen type II increased compared to the non-treated group (i.e., medium without the peptide). To identify the effects of the peptide on cartilage regeneration *in vivo*, osteoarthritis was chemically induced in the knees of six adult rats by injecting monoiodoacetate (MIA) into the synovial space. At days 7 and 14, 500 ng of the peptide was injected to the knees. After 21 days, the histological analyses indicated that the B2A2 treatment enhanced cartilage repair compared to untreated knees, which received saline instead of the peptide.

KIPKASSVPTELSAISTLYL Peptide

The KIPKASSVPTELSAISTLYL sequence, which is known as the BMP_2 peptide, corresponds to residues 73–92 of the knuckle epitope of bone morphogenetic protein-2 (BMP-2). This peptide was identified in 2003 as a potential candidate to improve bone formation through raising alkaline phosphatase activity compared to the other overlapping peptides of knuckle epitope of BMP-2.[28-30] In 2012, the ability of the peptide to direct chondrogenesis was investigated when it was added to the human MSC medium (100 ng/mL) three times per week. The gene expressions for aggrecan (after three days) and SOX9 and collagen type II (both after one week) were significantly higher than the negative control (no growth factor in the medium).[31]

N-Cadherin Mimetic Peptide

Cell-cell adhesion plays an important role in initiating chondrogenesis. In several studies, peptides that mimic cell-cell interactions have been used as a strategy for designing chondroinductive biomaterials.[32, 33] In 2012 it was shown that functionalization of hyaluronic acid (HA) hydrogels with N-cadherin mimetic peptides increased GAG and total collagen content of encapsulated MSCs after 28 days, when compared to MSCs encapsulated in HA hydrogels functionalized with scrambled N-cadherin mimetic peptides.[34, 35]

Self-Assembled Peptides

Several studies investigated the potential of self-assembled peptide hydrogels in modulating chondrogenesis. Self-assembled peptide hydrogels have been the focus of regenerative medicine applications due to their favorable physical properties; these hydrogels are made from natural building blocks and they may provide safe degradation products. Their physical properties such as pore size, fiber thickness, and mechanical performance can be tuned, and may improve a material's biological properties if biologically relevant peptides are used.[36-44] In the cartilage regeneration field, it was shown that encapsulating MSCs for 21 days in self-assembled peptides resulted in higher glycosaminoglycan content and more spatially uniform proteoglycan and collagen type II deposition when compared to MSCs encapsulated in agarose hydrogels.[45, 46]

Cell-Matrix Adhesion and Chondrogenic Differentiation

It is widely known that adhesion molecules are crucial in mediating multiple cellular signaling pathways,[47, 48] and have a high impact on critical cellular processes such as gene expression, cell cycle, and programmed cell death.[50]

Key components of cell-adhesion can be divided into three main groups: adhesion receptors, ECM proteins, and cytoplasmic plaque membrane proteins. The adhesion receptors have a critical role in mediating cell-cell and cell-ECM signaling, and they are typically classified using four groups: integrins, cadherins, immunoglobulins, and selectins. These receptors have been the subject of

significant interest, as reflected by the thousands of publications that describe them in detail.[49, 50] In this review, we provide only a brief overview given that more extensive descriptions are available elsewhere.

Adhesion Receptors

Integrins are known as cell receptors for ECM proteins and mediate cell-ECM adhesion. The structure of each integrin consists of an α and a β subunit, with both subunits composed of three main domains: an extracellular domain, a cytoplasmic region, and a single membrane-spanning domain. There are at least 16 α and eight β subunits known for integrins, and the specific subunit pairing determines explicit ligand-integrin binding properties.[49, 51-53] Cadherins mediate cell-cell adherence junctions and communicate with a group of linking proteins called catenins, which are crucial for cadherin function. Cadherins have different types of N-, P-, R-, B-, and E-cadherins, all with essential roles in both tissue formation and signaling cascade regulation.[54-58] Immunoglobulin cell-adhesion molecules (Ig-CAMs) are a member of the immunoglobulin superfamily and studies show that some are critically engaged in T and B cell activation.[59, 60] Selectins are smaller families of adhesion receptors that mediate rolling interactions of leukocytes on vascular endothelial cells, a therefore play a critical role in controlling inflammatory diseases.[61-64]

Adhesion Receptors and Cartilage Regeneration

Cartilage has an ECM-rich environment with a limited number of chondrocytes for which the value of ECM as a survival factor has been demonstrated.[65, 66] Integrins therefore play a critical role in cartilage regeneration because integrins mediate cell-ECM signaling pathways. To design chondroinductive biomaterials, it is valuable to identify the integrins expressed by chondrocytes and the changes of integrin expression during chondrogenic differentiation. Incorporating integrin-specific sequences in biomaterials may help to better control the cellular differentiation through activating desired adhesion signaling pathways. Here we cover the studies focused on identifying chondrocyte-specific integrins and the studies that aimed to monitor the integrin expression modifications during chondrogenic differentiation of stem cells.

Integrin Expression of Chondrocytes

In 1994, Woods *et al.*[67] investigated the integrins expressed by chondrocytes of human healthy knee cartilage. Cartilage tissue was harvested with biopsies from adult donors and the chondrocytes were isolated from the tissue. The integrin expression of the cells was analyzed by immunohistochemistry using monoclonal antibodies against different integrins, which indicated that normal human articular chondrocytes display a substantial quantity of $\alpha_1\beta_1$, $\alpha_5\beta_1$ and $\alpha_v\beta_5$ integrins as well as limited quantities of $\alpha_3\beta_1$ and $\alpha_v\beta_3$ integrins. The expressions of $\alpha_1\beta_1$, $\alpha_3\beta_1$, and $\alpha_5\beta_1$ were highlighted in 1995 by Loeser and co-workers[68] by immunostaining chondrocytes harvested from bovine articular cartilage.

In 1998, Camper *et al*.[69] described $\alpha_{10}\beta_1$ expression by human chondrocytes via immunostaining of chondrocytes in tissue sections of human cartilage tissue. In 1995, it was shown that the integrin expression of fetal chondrocytes is different from that of adult chondrocytes;[70] the immunostaining of fetal healthy knee sections demonstrated that fetal chondrocytes expressed α_6 and α_2, which are not expressed by adult chondrocytes. In addition, the fetal chondrocytes did not express β_3 and α_3, which were expressed by adult chondrocytes in previous studies.

Integrin Expression during Chondrogenic Differentiation

The variation of integrin expression during chondrogenic differentiation was investigated for the first time in 2005 by Goessler *et al*.[71] In this study, the integrin expression of human bone marrow MSCs was analyzed with microarray hybridization. During the 21 days of chondrogenic differentiation, the expression of integrin $\alpha_5\beta_1$ was downregulated while the expression of other integrins remained constant. In 2006, the important role of the α_5 integrin subunit was further emphasized by Connelly and co-workers[72] when anti-α_5 antibody was added to the medium of bone marrow MSCs harvested from the tibiae and femora of an immature calf, producing a reduction in GAG accumulation after 6 days relative to the untreated control group. In 2007, Goessler *et al.*[73] studied the integrin expression of both bone marrow- and adipose-derived human MSCs before and after 20 days of chondrogenic differentiation. Microarrays and immunohistochemistry indicated that the integrin $\alpha_5\beta_1$ was expressed by

undifferentiated MSCs, and its expression rose during chondrogenic differentiation of both types of MSCs. A 2013 study indicated the importance of the integrin β_8 subunit during chondrogenic differentiation: β_8 expression was upregulated after 21 days of chondrogenic differentiation of human MCSs when measured by quantitative PCR. The knockdown of the β_8 receptor via an shRNA lentiviral construct resulted in no collagen type II expression, which further supported the importance of the β_8 receptor in chondrogenic differentiation.[74]

In summary, the important role of adhesion molecules in regulating cellular signaling is widely recognized. The roles of integrins in cartilage tissue and chondrocyte signaling are of special importance given the ECM-rich nature of the tissue, and the role of integrins in mediating cell-ECM signaling, as cartilage has an ECM-rich environment with low cell density. The integrins that affect cartilage regeneration are typically categorized into two main groups; the integrins expressed by differentiated chondrocytes, and the integrins expressed during chondrogenic differentiation.

For the biomaterials that target chondrocytes, utilizing ligands specific to integrins of chondrocytes may possibly prevent chondrocyte dedifferentiation and contribute in mediating the integrity of cartilage tissue. For the biomaterials that target MSCs for chondroinductivity, identifying and incorporating ligands specific to the integrins that change expression during chondrogenic differentiation may

further improve cartilage tissue development. This approach can therefore produce biomaterials with the capability to activate cartilage regeneration.

The current studies in the field of cartilage biology may reveal valuable information about the chondrogenic role of integrins, but more extensive studies are required to elucidate the contributions of a wider range of integrins and to identify the specific role of each integrin during chondrogenic differentiation.

Adhesion Receptor-Binding Peptides

In 1984, Pierschbacher and Ruoslahti[75] indicated that the cell-binding potential of fibronectin can be duplicated with the cell attachment domain of fibronectin, a peptide sequence with three amino acids (Arg-Gly-Asp, i.e., RGD). RGD was subsequently recognized for the ability to bind to 12 integrins.[76-80] The fact that RGD is the cell attachment site to many other adhesion proteins has given this peptide a distinguished position in cell adhesion biology, and numerous regenerative medicine and pharmaceutical applications are taking advantage of this sequence to design cell adhesion materials.[81] The RGD example underscores the notion that the binding site of a receptor can indeed be recapitulated by a relatively short peptide sequence, which can be used instead of growth factors, antibodies, and ECM proteins.[82-85] Replacing entire adhesion molecules with a short adhesion peptides is attractive because such peptides are easy to synthesize in large quantities. Moreover, their small size reduces the chance of any nonspecific binding, and they can be designed to obtain a desired cell response.

It is important to keep in mind that several distinct proteins can bind to an individual receptor.[86] For instance, the $\alpha_v\beta_3$ integrin has been reported to bind to collagen VI, laminin, fibronectin, vitronectin, thrombospondin, von Willebrand factor, and fibrinogen.[87]

In addition, a peptide that binds to a desired integrin may be unable to target a specific pathway and even inhibit the binding of the other suitable sequences. In 2006, Connelly *et al.*[72] demonstrated the inhibition of chondrogenesis in RGD-modified alginate gels. In that study, bovine bone marrow-derived MSCs were encapsulated in either RGD- or RGE-modified alginate gels. After 7 days, the chondrogenic medium significantly stimulated chondrocytic gene expression (i.e., collagen II, aggrecan, and SOX9) in the RGE-modified gels but not in the RGD-modified gels. The results indicated that RGD interactions significantly inhibit the chondrogenic response in terms of gene expression for this particular system.

In cancer therapy, the limitations of current chemotherapeutics make identification of adhesion receptor-binding peptides an attractive research topic. In chemotherapeutic procedures, the drugs are typically not of maximal efficiency and are given to the patients at the highest level of body tolerance. Identifying peptides that bind to adhesion receptors of cells, and incorporating them in cancer therapy drugs, may reduce side effects as the therapeutic would only target the tumor cells. Table 2.1 shows a selected group of adhesion receptor-binding peptides that have been discovered for cancer therapy applications.[1]

In summary, the success of the cancer therapy field in identifying peptides that bind to specific adhesion receptors indicates opportunities for the regenerative medicine community to discover peptides that induce or facilitate chondroinductivity by targeting integrins. In addition, the extensive research surrounding the RGD sequence indicates the potential of peptides to affect cellular fate. Although adhesion receptor-binding peptides have the potential to change cellular fate, it is important to keep in mind that the ability of a peptide to bind to the target integrin may not always lead to activating the desired signaling pathways and may even inhibit the preferred cellular activities. However, an adhesion peptide sequence may hold tremendous value for retaining endogenous cells in the construct *in vivo*, and may play a crucial role in a combined strategy with a differentiation peptide.

Methods of Identifying Adhesion Receptor-Binding Peptides

If the regenerative medicine community is to pursue the recognition of novel peptide sequences to create desirable cell responses, then eventually the methods of identifying them must be understood. Investigators have applied many different methods for discovering adhesion receptor-binding peptides, from synthesizing overlapping or random sequences of an adhesion protein to using complex peptide libraries.

Peptide libraries provide the opportunity to screen the binding ability of a given protein in a high throughput manner. Peptide libraries are categorized based

on their method of display and can be categorized into two main groups: biological, and nonbiological. Biological libraries use DNA, or genotype, to encode peptide sequences,[1] whereas nonbiological libraries use peptides synthesized *in vitro*. To screen the binding ability of a desired protein or cell line, it is incubated along with the peptide library, and unbound peptides are subsequently washed away. The remaining peptides are then screened according to their design method. Biological libraries include bacterial, bacteriophage, ribosome, mRNA, yeast, cDNA, retrovirus, baculovirus, and mammalian cell display. Bacterial and phage libraries are applied for adhesion receptor-binding peptide isolation. In the bacterial libraries, peptides are genetically connected to the membrane flagella and fimbriae proteins and are displayed on the surface of bacteria. The DNA of the isolated bacterial clone is sequenced to reveal the identity of the adhesion receptor-binding peptide.[88, 89] In the phage display libraries, the DNA sequence of a desired peptide is embedded into the DNA of a phage coat protein to encode the peptide. After the incubation process, the DNA of the resulting phage is sequenced to discover its peptide content.[90, 91] Positional scanning synthetic peptide combinatorial libraries (PS-SPCLs) and one-bead one-compound (OBOC) libraries are the popular non-biological libraries for recognition of adhesion receptor-binding peptides. In OBOC libraries, the peptides are synthesized on beads that are approximately 80–100 μm in diameter; the split mix synthesis method generates the library and after incubation of the protein of interest and washing away of the unbound proteins, mass spectrometry or Edman's sequencing determines the peptide content of adhesion receptor-binding beads.[92, 93] In PS-SPCLs, the peptides are synthesized

individually. To synthesize individual peptides, one amino acid is held fixed while the remaining amino acids of the peptide vary. Holding a different amino acid constant and alternating others creates combinational libraries. There are various screening methods for PS-SPCLs such as using specific cellular effects as the readout or fluorescent tagging.[94] The detailed explanation of each peptide library could be the focus of an entire review, and indeed there are many excellent reviews about them that we recommend.[88, 89, 92-100] These methods have been utilized to identify adhesion receptor-binding peptides for other applications than regenerative medicine. For example, Table 2.1 shows a selected group of peptides from the field of cancer therapy, identified by panning *in vivo*, OBOC and phage display libraries and indicates the feasibility of the proposed methods for identifying receptor-binding peptides.

In summary, the methods of identifying adhesion receptor-binding peptides may be an excellent start to recognize bioactive sequences, although further investigation would be required to confirm the ability of the sequence to mimic the desired binding and to activate the target signaling pathways.

Discussion

Chondroinductive biomaterials are categorized into two broad groups: natural, and synthetic. Natural materials are attractive candidates to be incorporated in scaffold structure because they have the potential to communicate with cells not only through chemical signaling but additionally through mechanical signaling, which is beneficial to tissue-dependent cells. The regenerative

properties of natural materials may be attractive for biomaterial design, but their disadvantages, such as reproducibility and the risks involved with immunogenicity, may complicate regulatory pathways. As a result, natural materials may be less attractive for commercialization if 100% synthetic materials are capable of eliciting a comparable biological response, which indeed makes designing all-synthetic biomaterials a more appealing translational strategy.

The main challenge in designing an all-synthetic biomaterial is to identify chemical signals that mimic the adhesion receptor-binding site of ECM or growth factors and lead the stem cells to the desired lineage. In the case of cartilage regeneration, the cell-ECM signaling is crucial for both chondrocyte survival and chondrogenic differentiation. Cell-ECM signaling is mediated by ECM interactions with adhesion receptors, namely integrins. Therefore, the integrins expressed by chondrocytes and the integrins that change expression during chondrogenic differentiation may play a role in mediating cartilage tissue development through interaction with ECM. As a result, the ligands specific to these integrins may have the potential to provide chemical signals for the cells and to produce chondroinductivity. Bioactive peptides are attractive candidates to mimic integrin-ligand binding because of favorable properties such as reproducibility and low cost.

The integrin-binding peptides, such as RGD, are mainly employed in biomaterials for their cell-adhesion properties. In the field of cartilage regeneration, a few studies have employed bioactive peptides for their signaling abilities,

although none have employed strategies to identify these peptides based on their ability to bind to chondrocyte integrins or integrins expressed during chondrogenic differentiation.

Chapter 3: Effects of a Bioactive SPPEPS Peptide on Chondrogenic Differentiation of Mesenchymal Stem Cells [2]

Abstract

A synthetic 'chondroinductive' biomaterial that itself could induce chondrogenesis without the need for growth factors, extracellular matrix, or other pre-seeded cells, could revolutionize orthopedic regenerative medicine. The objective of the current chapter was thus to introduce a synthetic SPPEPS peptide and evaluate its ability to induce chondrogenic differentiation. In the current chapter, dissolving a synthetic chondroinductive peptide candidate (100 ng/mL SPPEPS) in the culture medium of rat bone marrow-derived mesenchymal stem cells (rBMSCs) elevated collagen type II and aggrecan gene expressions compared to the negative control (no growth factor or peptide in the cell culture medium) after 3 days. In addition, proteomic analyses indicated similarities in pathways and protein profiles between the positive control (10 ng/mL TGF-β_3) and peptide group (100 ng/mL SPPEPS), affirming the potential of the peptide for chondroinductivity. Incorporating the SPPEPS peptide in combination with the RGD peptide in pentenoate-functionalized hyaluronic acid (PHA) hydrogels elevated the collagen type II gene expression of the rBMSCs cultured on top of the hydrogels compared to using either peptide alone. The evidence suggests that

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SPPEPS may be a chondroinductive peptide, which may be enhanced in combination with an adhesion peptide.

Introduction

Cartilage tissue does not have the ability to regenerate on its own. Minor injury to cartilage tissue may therefore lead to further degeneration and eventually to arthritis, which is the leading cause of disability worldwide. The current surgical treatments for cartilage injury such as autologous chondrocyte implantation (ACI), microfracture, osteochondral transplantation (mosaicplasty), and allograft implants, do not reproducibly lead to tissue with mechanical and structural properties comparable to native articular cartilage.[3] The failure of current treatments to reproducibly regenerate a fully integrated and healthy cartilage tissue has motivated the regenerative medicine community to investigate strategies that lead to the creation of fully functional hyaline cartilage.[1, 2] Cell-based approaches may be promising in terms of hyaline cartilage formation, but inherent translational challenges motivate the identification of acellular alternatives.[3, 4] Acellular materials may support chondroinductivity if natural components such as extracellular matrix (ECM) molecules or growth factors are added to their structure. While natural components may mimic the native cartilage enviroment and be chondroinductive, their limitations (e.g., cost, reproducibility, and potential for immunogenicity or disease transmission) present challenges for commercial adoption. Therefore, an all-synthetic biomaterial, providing chondroinductive capabilities without the need for animal-derived components or cells, may offer a superior alternative.

To regenerate cartilage tissue with an all-synthetic material, the ideal scaffolding biomaterial must have mechanical integrity suitable for weight-bearing application and the ability to induce chondrogenic differentiation of endogenous mesenchymal stem cells (MSCs). The desirable failure properties (e.g., maximum stress and strain, and toughness)[7] of the material may be achieved via polymer selection and composition; identifying all-synthetic cellular signals for chondroinduction would then be the remaining challenge. In the field of regenerative medicine in general, there are several studies that have aimed to identify peptides as signals for cells.[8-13] In addition, there are review papers on receptor-binding peptides; however, they have not focused on regenerative medicine applications.[14]

The chondroinductivity of synthetic peptides has been explored by a few pioneering groups. For example, the Link N peptide was identified as potentially chondroinductive in 2003, with 100 ng/mL of the peptide added to the culture medium of both nucleus pulposus (NP) and annulus fibrous (AF) cells, elevated accumulation of collagen type II and IX (measured by ELISA).[15, 19] In 2010, it was shown that the GFOGER peptide increased relative gene expressions of aggrecan and collagen types II and X of human mesenchymal stem cells encapsulated in poly(ethylene glycol) (PEG) hydrogels when the GFOGER peptide was chemically incorporated in the hydrogels by Michael additional chemistry.[23] The B2A peptide with a sequence of (H-AISMLYLDENEKVVLKK(H-AISMLYLDEN-EKVVLK)-Ahx-Ahx-AhxRKRLDRIAR-NH_2)[101] was designed in 1999 and consists of a receptor

targeting domain, a hydrophobic spacer domain and a heparin-binding domain.[24] In 2012, it was shown that adding 10 µg/mL of the B2A peptide to murine embryonic stem cells medium increased Sox9 and collagen type II gene expressions.[27] In 2012, the BMP_2-mimic peptide sequence (KIPKASSVPTE-LSAISTLYL) was added to human MSCs medium (100 ng/mL), which resulted in increased Sox9 and collagen type II gene expressions.[31]

Identifying peptides capable of chondroinduction, and designing biomaterials that incorporate them, may lead to 100% synthetic, chondroinductive biomaterials – which could be revolutionary in cartilage injury treatment. In the current study, we explored the chondroinductivity of a peptide candidate with rat bone marrow-derived mesenchymal stem cells (rBMSCs) in 2D culture. The peptide was then conjugated to a pentenoate-functionalized hyaluronic acid (PHA) hydrogel, and the chondroinductivity of the resultant hydrogels (via 2D culture on their surface) was evaluated by real-time quantitative polymerase chain reaction (rt-PCR).

We identified the peptide candidate (Ser-Pro-Pro-Glu-Pro-Ser, SPPEPS) as a matching sequence of two chondroinductive molecules, aggrecan (a proteoglycan found in abundance in cartilage matrix that is known to induce chondrogenic differentiation[102]) and transforming growth factor-β3 (TGF-β_3, known to play a major role in cartilage development).[103, 104] TGF-β_3 has 390 to 414 amino acids and consists of two polypeptide chains, i.e., C-terminal and N-terminal

subunits. The N-terminal subunit is known as the latency-associated protein (LAP),[105] and the SPPEPS peptide sequence belongs to this LAP region of TGF-β_3. It is known that LAP is a ligand for a number of integrins.[106-108] It is widely known that integrins mediate cell-ECM signaling pathways and play a critical role in cartilage regeneration. Moreover, the binding sequences of integrins can be duplicated by bioactive peptides and the peptides have the potential to activate the desired signaling pathways. Replacing growth factors and extracellular proteins with their respective peptide binding sequences may potentially revolutionize the field of regenerative medicine, as we recently reviewed in more detail.[109]

In the current study, the chondroinductivity of the SPPEPS peptide sequence was evaluated with rBMSCs when the SPPEPS peptide was coated onto the culture surface or was dissolved in cell culture medium at different concentrations. For further analysis, the chondroinductivity of SPPEPS was evaluated when the peptide was conjugated to PHA hydrogels and rBMSCs were cultured on top of the hydrogels. The peptide was conjugated to PHA with or without the RGD adhesion peptide. Our hypothesis was that the SPPEPS peptide, as a matching sequence found in two chondroinductive molecules (i.e., TGF-β_3 and aggrecan), would induce initial chondrogenic differentiation of rBMSCs superior to that induced by TGF-β_3.

Methods and Materials

Cell Culture and SPPEPS Peptide Incorporation

rBMSCs from the tibias and femurs of 8 week-old male Sprague–Dawley Rats (ScienCell, Carlsbad, CA) were thawed and cultured to passage 4 in cell culture medium (minimum essential medium-α (Cat# 12561072, Thermo Fisher Scientific, Waltham, MA) supplemented with 10% fetal bovine serum (FBS, Cat# 16000044, Thermo Fisher Scientific) and 1% penicillin/streptomycin (Cat# 15140-122, Thermo Fisher Scientific)). The cells were used immediately after passage 4. The six peptides (Table 3.1) were custom-ordered from Biosynthetic (Lewisville, TX), who synthesized the peptides with a PTI Symphony peptide synthesizer (Tucson, AZ) using solid-phase synthesis with Fluorenylmethyloxycarbonyl protecting group (Fmoc) chemistry, which means Fmoc was used for the temporary protection of the N-terminus, and then cleaved from the resin using standard methods. Biosynthetic confirmed peptide identities by mass spectroscopy, and purity was shown to be >95% using analytical high-performance liquid chromatography (HPLC, Shimadzu, Columbia, MD).

In one set of groups, the SPPEPS peptide was adsorbed to the well plate surface. The SPPEPS peptide was dissolved in sterile DI water (20 µg/mL), sterile-filtered (0.22 µm), and added to sterile 96-well tissue culture treated (TCT) plates to reach the desired coating concentrations (0.5, 1 and 3 µg/mm^2) before overnight water evaporation at room temperature in sterile conditions. The rBMSCs were thawed and after passage 4 were cultured on the plates at 150,000 cells/well (i.e.,

23.5 x 10^6 cells/cm^2) with the aforementioned cell culture medium, and the cell culture medium was changed every second day. Uncoated TCT plates served as the control group.

In a separate set of groups, the SPPEPS peptide was included in *soluble form* in the medium instead of being coated on the surface. rBMSCs were cultured on 96-well plates (150,000 cells/well) with cell culture medium containing SPPEPS (50, 100 and 500 ng/mL). Fresh peptide was added with every medium change every second day.

Description of Experimental Design

In a preliminary study, the chondroinductivities of Link N and SPPEPS with rBMSCs were evaluated at two plate coating densities (0.5 and 1 µg/mm^2) and two soluble concentrations (10 and 100 ng/mL) after 3 days by rt-PCR (n=3). The chondroinductivity was compared with the negative control (no growth factor or peptide in the cell culture medium) and positive control groups. The positive control was a known chondrogenic growth factor in lieu of the SPPEPS peptide, i.e., 10 ng/mL (dissolved in rBMSCs cell culture medium) human transforming growth factor-β_3 (TGF-β_3, R&D systems, Minneapolis, MN). The collagen type II gene expression of SPPEPS 100 ng/mL group was 2.3 times higher than in the control group in our preliminary study ($p < 0.05$) (Fig. 3.8), which led us to further analyses on the chondroinductivity of the SPPEPS peptide for 50 to 500 ng/mL peptide concentrations in cell culture medium and 0.5 to 3 µg/mm^2 coating concentrations.

In the current study, rBMSCs were cultured either on TCT plastic or on a hydrogel surface. For the cells cultured on TCT plates, three different concentrations of SPPEPS peptide (50, 100 and 500 ng/mL) in cell culture medium, three different coating concentrations (0.5, 1 and 3 µg/mm^2) and a control group (no growth factor or peptide in the cell culture medium) were evaluated (n=6) and the peptide chondroinductivity was evaluated by rt-PCR after 3 days (see gene expression sub-section below).

The 100 ng/mL concentration of SPPEPS was selected for proteomic analysis due to its superiority in inducing chondrogenic differentiation (see Proteomics sub-section below). Another group of rBMSCs from the same source described above (i.e., tibias and femurs of 8 week-old male Sprague–Dawley Rats (ScienCell, Carlsbad, CA)) were purchased and the samples were prepared using the cell culture methods described above. The positive control was 10 ng/mL TGF-β_3 in cell culture medium and the negative control group was a group with no peptide or growth factor in the cell culture medium (n=4). All proteomics data were normalized to the control.

For the analysis of the rBMSCs cultured on the hydrogel surface, a new group of rBMSCs from the same source described above (i.e., tibias and femurs of 8 week-old male Sprague–Dawley Rats (ScienCell, Carlsbad, CA)) was purchased and prepared until passage 4 using the cell culture methods described above. The

rBMSCs on tissue culture plates served as the baseline control group and the chondroinductivities of PHA hydrogels were analyzed by rt-PCR after 3 days for various peptides, which were conjugated to the PHA hydrogel (n=4). A PHA (5% (w/v) pentanoate-functionalized hyaluronic acid) group with no peptide conjugation (see next sub-section for supplier and synthesis information), along with PHA+RGD (PHA conjugated to 1.5 mM GCGYGRGDSPG), PHA+SP (PHA conjugated to 1.5 mM GCGYGSPPEPS), PHA+PS (PHA conjugated to 1.5 mM GCGYGPSEPSP (scrambled SPPEPS)), PHA+RGD+SP (PHA conjugated to 1.5 mM GCGYGRGDSPG and 1.5 mM GCGYGSPPEPS) were chosen for analyses. The PHA+PS group served as a scrambled sequence control for the PHA+SP group.

Synthesis of Pentenoate-Functionalized Hyaluronic Acid (PHA)

PHA was synthesized as previously described.[110] Briefly, hyaluronic acid (HA, Mw = 60 kDa, Lifecore Biomedical, Chaska, MN) was fully dissolved in DI water to a 5% (w/v) concentration before dropwise addition of dimethylformamide (DMF) to reach a 3:2 ratio of water:DMF. Simultaneously, 5M pentenoic anhydride (Cat# 471801, Sigma-Aldrich, St. Louis, MO) was slowly added to the solution in excess relative to HA. When adding the DMF and pentenoic anhydride was complete, the solution pH was maintained at 8-9 by slow dropwise addition of 1M NaOH during constant monitoring of the PH for 5 hours. The conjugation reaction

was completed overnight to form PHA. The next day, solid NaCl was added to the solution to reach the final concentration of 0.5M NaCl. After the salt was fully dissolved, the polymer was precipitated by adding acetone (water/acetone (v/v) ratio of 1:4) and centrifuging the solution at 7,000 x g for 5 minutes. After centrifuging, the PHA was completely precipitated in the form of pellets. The pellets were dissolved in DI water and dialyzed against DI water for 48 hours, exchanging the DI water every 12 hours. After dialysis, dry PHA was frozen, lyophilized, and stored at –20°C.

PHA Hydrogel Preparation, Peptide Conjugation, and Surface Analysis

To prepare PHA hydrogels (without peptide incorporation), a solution of phosphate-buffered saline (PBS) containing 2.3 mM 2-Hydroxy-4′-(2-hydroxyethoxy)-2-methylpropiophenone (Irgacure 2959, Cat# 410896, Sigma-Aldrich) and 1% dithiothreitol (DTT, Cat# D0632, Sigma-Aldrich) was prepared and sterile-filtered (0.22 µm). The PHA was removed from the freezer and brought to room temperature and then sterilized using ethylene oxide gas (AN74i, Anderson Anprolene, Haw River, NC) and mixed with the PBS solution to reach 5% (w/v) PHA concentration. The solution was left at room temperature for 2 hours to fully dissolve the PHA particles. 100 µL of this precursor solution was added to each well of a 48 well plate and briefly centrifuged before hydrogel crosslinking with a handheld 312 nm UV-light at 9 mW/cm^2 for 2 minutes (EB-160C, Spectroline, Westbury, NY).

To prepare PHA hydrogels with conjugated peptides, hydrogels were made in two steps. First, PHA was mixed with PBS solution containing 1 mM Irgacure and 1.5 mM thiolated peptide to achieve a 2% (w/v) PHA solution. The solution was conjugated with UV light for 2 minutes, and the conjugation of the peptide to the PHA network was confirmed with H^1NMR (Varian Mercury VNMRS 400 MHz spectrometer, Fig. 3.9). This solution was then mixed with more PHA (to achieve final concentration of 5% (w/v)) and was mixed with PBS solution containing Irgacure and DTT to achieve 2 mM Irgacure concentration and 1% w/v DTT. The solution was again crosslinked for 2 minutes to create the final hydrogels. rBMSCs were cultured with the same methods explained above until passage 4 and then were cultured on top of the hydrogels (30,000 cells/well, i.e., 47 x 10^6 cells/cm^2) and the medium (minimum essential medium-α supplemented with 10% FBS and 1% penicillin/streptomycin) was changed every second day for 3 days (n=4).

The DNA contents of all the samples (Control, PHA, PHA+RGD, PHA+SP, PHA+PS and PHA+RGD+SP) were measured after 3 days (n=4). The samples were digested overnight at 65°C in 200 µL papain mixture (125 mg/mL papain from papaya latex, 5 mM N-acetyl cysteine, 5 mM EDTA, and 100 mM PBS) and the DNA content of the samples was measured using a PicoGreen assay kit (Thermo-Fisher Scientific, Waltham, MA, P7589) according to the manufacturer's instructions. All the samples were evaluated in triplicate.

Gene Expression

The gene expressions were analyzed for all the rBMSCs samples cultured on the TCT plates after 3 days (n=6) and all the rBMSCs cultured on the hydrogel surface after 3 days (n=4). The gene expressions of each sample were evaluated in triplicate. To prepare the samples for gene expression, the mRNAs were extracted after 3 days using Qiagen RNeasy mini kit (Valencia, CA) following the kit instructions, before reverse transcription using a cDNA kit (Invitrogen, Carlsbad, CA). rt-PCR was performed with a qTOWER^3 Thermal Cycler (Upland, CA) using TaqMan Col2A1, Sox9, ACAN and GAPDH primers (Invitrogen, Carlsbad, CA). GAPDH was used as an endogenous control, with relative gene expression measured using the $2^{-\Delta\Delta Ct}$ method. For cells on both surfaces, the calibrators were the rBMSCs at passage 4 before transferring them to the 96-well plates.

Proteomics

For the proteomics analyses, the samples were prepared at 7 and 14 days (n=4). The samples were washed with PBS three times and lysed in 50 µL modified RIPA buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1% Na-deoxycholate and 1% IGEPAL) containing phosphatase inhibitors and protease inhibitors (Roche, Indianapolis, IN). The lysates were centrifuged for 10 minutes at 12,000 g and the supernatant collected for mass spectrometry-based proteomics.

The protein lysate was subjected to the FASP protocol. Briefly, the cysteines were reduced and alkylated and the protein digested overnight with

trypsin in 50 mM ammonium bicarbonate. The resulting peptides were dried and resuspended in 10 mM ammonium formate pH 10.0. Liquid chromatography tandem mass spectrometry was performed by coupling a nanoAcquity UPLC (Waters Corp., Manchester, UK) to a Q-TOF SYNAPT G2S instrument (Waters Corp., Manchester, UK). Each protein digest (about 100 ng of peptide) was delivered to a trap column (300 µm × 50 mm nanoAcquity UPLC NanoEase Column 5 µm BEH C18, Waters Corp, Manchester, UK) at a flow rate of 2 µl/min in 99.9% solvent A (10 mM ammonium formate, pH 10, in HPLC grade water). After 3 min of loading and washing, peptides were transferred to another trap column (180 µm × 20 nanoAcquity UPLC 2G-V/MTrap 5 µm Symmetry C18, Waters Corp., Manchester, UK) using a gradient from 1% to 60% solvent B (100% acetonitrile). The peptides were then eluted and separated at a flow rate of 200 nL/min using a gradient from 1% to 40% solvent B (0.1% formic acid in acetonitrile) for 60 min on an analytical column (7.5 µm × 150 mm nanoAcquity UPLC 1.8 µm HSST3, Waters Corp, Manchester, UK). The eluent was sprayed via PicoTip Emitters (Waters Corp., Manchester, UK) at a spray voltage of 3.0 kV and a sampling cone voltage of 30 V and a source offset of 60 V. The source temperature was set to 70 °C. The cone gas flow was turned off, the nano flow gas pressure was set at 0.3 bar and the purge gas flow was set at 750 ml/h. The SYNAPT G2S instrument was operated in data-independent mode with ion mobility (HDMSe). Full scan MS and MS2 spectra (m/z 50 - 2000) were acquired in resolution mode (20,000 resolution FWHM at m/z 400). Tandem mass spectra were generated in the trapping region of the ion mobility cell by using a collisional energy ramp from

20 V (low mass, start/end) to 35 V (high mass, start/end). A variable IMS wave velocity was used. Wave velocity was ramped from 300 m/s to 600 m/s (start to end) and the ramp was applied over the full IMS cycle. A manual release time of 500 µs was set for the mobility trapping and a trap height of 15 V with an extract height of 0 V. The pusher/ion mobility synchronization for the HDMSe method was performed using MassLynx V4.1 and DriftScope V2.4. LockSpray of Glufibrinopeptide-B (m/z 785.8427) was acquired every 60 s and lock mass correction was applied post acquisition. The data was analyzed with PLGS and QIP (Waters Corp., Manchester, UK). Proteomic data were further analyzed using the Database for Annotation, Visualization and Integrated Discovery (DAVID, https://david.ncifcrf.gov/) through Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis.

Statistical Analyses

For the statistical analyses, Tukey's HSD method was used for inter-group comparisons, and Dunnett's test was employed to compare the groups with the negative control. Calculations were performed with GraphPad Prism (Graphpad Software Inc., La Jolla, CA), with $p < 0.05$ considered significant. The results for rt-PCR and PicoGreen were reported as the mean ± standard deviation, with proteomics results reported as median ± standard deviation.

Results

DNA Content

In the hydrogel surface analysis (Fig. 3.1), the DNA contents were in the range of 0.8 to 1.1 µg, with 34% and 27% higher DNA contents in the PHA and PHA+SP+RGD groups compared to the control (rBMSCs on TCT plates), respectively ($p < 0.05$). In addition, comparing the DNA contents of hydrogel groups indicated that the DNA content of the PHA group was 36% and 32% higher compared to the PHA+RGD and PHA+SP groups respectively ($p < 0.05$).

Gene Expression

In the preliminary study, the collagen type II gene expression of SPPEPS 100 ng/mL group was 2.3 times higher than in the control group ($p < 0.05$) (Fig. 3.8), which led us to further analyses on the chondroinductivity of the SPPEPS peptide. In the current study, for the rBMSCs on TCT plates, the Sox9 gene expression from control group was 4.0, 4.2, and 4.5 times higher than all of the coating groups (SPPEPS 0.5, 1 and 3 µg/mm^2), respectively ($p < 0.05$). Comparing the Sox9 gene expressions of the peptide groups indicated that the Sox9 gene expressions of SPPEPS 50 and 500 ng/mL groups were 4.5 - 5.2 times and 3.2 - 3.6 times higher than SPPEPS 0.5, 1 and 3 µg /mm^2 groups, respectively ($p < 0.05$) (Fig 3.2). The collagen type II gene expressions from SPPEPS 0.5, 1 and 3 µg/mm^2 and SPPEPS 100 ng/mL groups were 61, 74 and 75% lower and 1.5 times higher than in the control group, respectively ($p < 0.05$). Collagen type II gene

expressions from peptide-in-medium groups (i.e., SPPEPS 50, 100 and 500 ng/mL) were 2.5 to 6.5 times higher than peptide-coating groups (i.e., SPPEPS 0.5, 1, 3 μg /mm^2) ($p < 0.05$). In addition, collagen type II gene expression from SPPEPS 100 ng/mL group was 1.5 and 1.6 times higher compared to SPPEPS 50 and 500 ng/mL, respectively ($p < 0.05$) (Fig. 3.2). No other differences in collagen II gene expressions were statistically significant. The aggrecan gene expressions from SPPEPS 3 μg/mm^2, and 50, 100 and 500 ng/mL groups were 31, 34, 35, 31% higher compared to the control group, respectively ($p < 0.05$) (Fig. 3.2). There were not any other significant differences among the groups.

In the hydrogel surfaces study, the Sox9 gene expressions from the hydrogel groups were 20 to 100 times higher than from the TCT plate group, although none of the differences were significant (Fig. 3.3). The combined effect of the SPPEPS and RGD peptides was especially evident in collagen II gene expression, as the collagen type II gene expression from the PHA+SP+RGD group was 300 times higher compared to the TCT plate group and 3 to 7 times higher compare to the PHA, PHA+RGD, PHA+SP and PHA+PS groups, respectively ($p < 0.05$). There were no other significant differences among the collagen type II gene expressions, although the collagen type II gene expressions of the PHA, PHA+RGD, PHA+SP and PHA+PS groups were 40 to 90 times higher than TCT plate group (Fig. 3.3). Interestingly, the aggrecan gene expression of TCT plate group was 2.6 times higher than PHA+PS group ($p < 0.05$) (Fig. 3.3). There were no other significant differences among the gene expressions.

Proteomics analysis

The proteomics data monitored the expression of 1446 proteins in each group (positive TGF-β_3 control, negative control and SPPEPS: days 7 and 14). In the positive control day 7, SPPEPS day 7, positive control day 14, and SPPEPS day 14, there were 248, 132, 206 and 148 proteins with higher expression levels compared to the negative control, respectively ($p < 0.05$), and there were 192, 297, 166 and 127 proteins with a lower expression level compared to the negative control, respectively ($p < 0.05$) (Fig. 3.4). Among the hundreds of upregulated proteins, there were 60 common proteins to both the SPPEPS and positive control groups at day 7 ($p < 0.05$). KEGG pathway analysis of these 60 proteins indicated that the "Insulin Signaling Pathway" was activated through the GSK-3β gene (Fig. 3.7). GSK-3β has been identified to be an essential gene for early stage chondrocyte differentiation.[111] In addition, this gene participates in regulation of Wnt/β-Catenin signaling,[112] which is a key player signaling pathway for cartilage development.[113] Among the 60 common upregulated proteins, collagen type XIα1 was identified as well, which plays a critical role in cartilage formation (Fig. 3.6).[114] At day 14, 26 common proteins were upregulated in both the positive control and SPPEPS groups ($p < 0.05$) (Fig. 3.5). Gene ontology analysis of these 26 proteins with DAVID indicated the upregulation of the ENPP1 gene, which is shown to be expressed by chondrocytes.[115] In addition, the CLIC4 gene was upregulated, which plays an important role in the regulation of TGFβ signaling.[116]

Discussion

This is the first study to introduce the amino acid sequence SPPEPS as a potentially chondroinductive peptide. Supportive evidence included the higher expression of collagen II and aggrecan genes with 100 ng/mL of soluble SPPEPS on TCT plates compared to the negative control. Coating the TCT plate surface with SPPEPS was a less effective method than including in soluble form for promoting chondrogenesis, as the only gene upregulation compared to the negative control was aggrecan for the 3 $\mu g/mm^2$ group. This difference in response due to method of presentation may be dose-dependent, and/or might have been due to temporal variability for gene expression for peptide in medium vs. peptide as plate coating.

The SPPEPS (100 ng/mL) group was chosen for proteomics analysis due to superior chondroinductivity and consistent patterns of inducing higher collagen type II gene expression for rBMSCs compared to the negative control and inducing higher expression of aggrecan gene for rBMSCs compared to the negative control. Our use of proteomics represents a powerful advance in the analysis of such phenomena, providing a better understanding of changes that occur in cellular pathways and the whole profile of the proteins when the peptide is introduced to cell culture medium.

With the databases available, there are many different methods to analyze the proteomics data and to infer more information regarding cellular profiles. In the

current study, we performed data analysis with DAVID on the common proteins among the study groups that expressed at significantly higher levels compared to the negative control. At 7 days, the activation of “Insulin Signaling” pathways through an essential gene for cartilage development (i.e., GSK-3β) in both the positive control and SPPEPS groups in addition to the upregulation of collagen type XIα supported the potential of the SPPEPS peptide in regulating cartilage regeneration. In addition, 60 of the 132 proteins that were expressed higher in the SPPEPS group (compared to the negative control) were expressed in the positive control, indicating similarities of protein expression in both groups. However, 72 proteins were expressed in the SPPEPS group, but not in the positive control, indicating the necessity of further analysis to clarify the effects of these proteins on other cellular functions.

Among the 26 common upregulated proteins in both of the groups at 14 days, the upregulations of ENPP1 and CLIC4 genes and their role in chondrocyte differentiation and regulation of TGFβ signaling, respectively, indicated promising potential for the SPPEPS peptide in terms of inducing chondroinductivity. At 14 days, the increased expression of 26 common proteins, in both the positive TGF-β_3 and the SPPEPS groups, may be consistent with the SPPEPS peptide inducing chondroinductivity; however, 122 of the 148 higher-expressed proteins in the SPPEPS group were *not* changed in the TGF-β_3 positive control, and thus once again more investigation is required regarding the perhaps unintended effects of the SPPEPS peptide on other cellular functions.

For the positive control, 188 of the 248 proteins at day 7 and 180 of the 206 proteins at day 14 have not been detected in the SPPEPS peptide groups. This indicates that the peptide alone cannot completely replace TGF-β_3, which may be a positive finding if deleterious effects are avoided. Therefore, future research is required to identify additional chondroinductive sequences and to study their synergistic effects on chondrogenic differentiation.

In the hydrogel surface analysis, both Sox9 and collagen type II gene expressions of all the hydrogel groups were 20 to 300 times higher compared to the TCT groups, which indicated the high impact of PHA for chondroinductivity whether the peptides were conjugated to the hydrogel or not. Although the differences were not identified as significant (except collagen type II gene expression for PHA+RGD+SP compared to the TCT plate group) in statistical analysis, because of the low sample number in the study, the data still emphasize the high impact of PHA on chondrogenic differentiation. One of the most striking findings of the study was that the collagen type II gene expression for the PHA group containing both the RGD adhesion peptide and the experimental SPPEPS peptide was 3 to 7 times higher than all the other PHA groups, including those with the RGD or SPPEPS alone, which suggests that synergistic adhesion and differentiation peptides may be an attractive general area of investigation for chondrogenic differentiation.

Conclusion

This chapter demonstrated the potential chondroinductivity of the SPPEPS peptide, supported by gene expression in different settings and by proteomic analysis in response to soluble peptide. Adding the SPPEPS peptide (identified as a matching sequence of two chondroinductive molecules) to the cell culture medium of rBMSCs led to a significant increase of chondrogenic markers. Although our results support the potential of the SPPEPS peptide in cartilage regeneration, we acknowledge that proteins were identified that were expressed in the positive control but not in the peptide group, and likewise proteins expressed in the peptide group but not in the positive control, which indicates that the peptide alone cannot exactly mimic TGF-β_3. Although the proteomics analysis was not performed for the hydrogel-surface culture, the gene expression data indicate the high potential of the hydrogels when both the RGD adhesion peptide and the SPPEPS peptide were conjugated in inducing enhanced chondroinductivity. Overall, the opportunity to identify chondroinductive peptides is an exciting burgeoning area of investigation in orthopedic regenerative medicine, with SPPEPS being an attractive candidate for future investigation.

Chapter 4: Chondroinductive Peptide in Hyaluronic Acid Hydrogels for Hyaline Cartilage Regeneration[3]

Abstract

The primary challenge in cartilage regeneration is regenerating true hyaline-like cartilage. Acellular biomaterials have advantages compared to cell-based therapies including a single surgical procedure and cost savings but are generally limited in their ability to achieve true hyaline cartilage. We therefore endeavor to identify biomaterials that are themselves inherently chondroinductive, without the need for extracellular matrix, growth factors or exogenous cells. In the current study, our hypothesis was that incorporation of a bioactive SPPEPS peptide into a hyaluronic acid hydrogel would provide superior cartilage regeneration compared to unadulterated hydrogels. For this purpose, thiolated RGD or thiolated SPPEPS (matching peptide sequence of aggrecan core protein and transforming growth factor-beta 3 (TGF-β_3)) peptides were conjugated to pentenoate-functionalized hyaluronic acid (PHA). The resultant hydrogels were implanted into defects in the femoral condylar cartilage of rabbits before photocrosslinking with UV light. After 12 weeks, although differences in gross morphological scoring were not statistically significant, the SPPEPS peptide indicated the potential of inducing chondrogenic differentiation based on the strong collagen II immunostaining and cell morphology relative to the PHA-only control. The collagen II production and

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evidence of rudimentary columnar organization of chondrocyte-like cells in lacunae in the SPPEPS group supported our hypothesis and encourage future exploration of synthetic chondroinductive peptides for orthopedic regenerative medicine.

Introduction

There are several treatments that aim to repair or regenerate injured cartilage, such as autologous chondrocyte implantation (ACI), microfracture, osteochondral transplantation (mosaicplasty), and allograft implants, but none have successfully, unequivocally, and reproducibly produced tissue with the mechanical and chemical properties of hyaline cartilage.[3] In recent years, different regenerative medicine approaches have aimed to produce hyaline-like cartilage tissue; however, their translational limitations have not created a comprehensive business model to develop across the board technologies.[3, 4, 117] The key limitations such as high cost associated with the use of stem cells and regulatory challenges associated with the use of animal-derived growth factors or extracellular matrix (ECM) components, has inspired us to aim to develop acellular strategies without the use of animal-derived components by incorporating bioactive signals in the structure of biomaterial scaffolds.[1, 2] Our unpublished *in vitro* work has suggested that a new SPPEPS peptide may have the ability to induce chondrogenesis, i.e., to possess chondroinductive character, which we endeavored to evaluate *in vivo* in the current study.

In recent years, a precious few studies have examined the *in vivo* bioactivity of peptides in terms of chondroinductivity. For example, Link N peptide at a concentration of 10 mg/mL was injected into 5 mm deep annular punctures in the center of the nucleus pulposus (NP) of New Zealand White rabbit intervertebral discs (IVDs), and aggrecan expression for both NP and annulus fibrous (AF) cells increased after 2 weeks.[19] In another study, 500 ng B2A peptide with a sequence of (H-AISMLYLDENEKVVLKK(H-AISMLYLDEN-EKVVLK)-Ahx-Ahx-AhxRKRLD-RIAR-NH_2) was injected into the synovial space of the knee joint of rats with osteoarthritis (chemically induced). The authors concluded that B2A enhanced cartilage repair after 21 days according to Alcian Blue and H&E histological analyses.[27]

In the current study, we identified SPPEPS as a potentially chondroinductive peptide given that it is a matching amino acid sequence of the aggrecan core protein and transforming growth factor-beta 3 (TGF-β_3), both of which are known to play roles in chondrogenesis. Here, we conjugated the SPPEPS peptide to a pentenoate-functionalized hyaluronic acid (PHA) hydrogel network. PHA hydrogels are attractive for surgical application due to their faster crosslinking time (1-2 minutes) compared to other hyaluronic acid-based hydrogels.[110, 118, 119] In addition, hyaluronic acid nanoparticles (HAnp) were incorporated into the hydrogels to increase the hydrogel precursor yield stress for surgical placement.[120] The performance of the PHA hydrogels with conjugated SPPEPS were compared in regeneration of rabbit femoral condylar cartilage

defects to PHA hydrogels alone (negative control) and to PHA hydrogels conjugated with the well-known RGD adhesion peptide.

The goal of the current study was to design a biomaterial that could induce hyaline-like cartilage production by guiding chondrogenic differentiation of endogenous bone marrow-derived mesenchymal stem cells (BMSCs) without the need to harvest/seed cells of any kind or to incorporate any growth factors. Our hypothesis was that the SPPEPS peptide in PHA hydrogels would lead to a more hyaline-like cartilage tissue compared to PHA hydrogels alone.

Materials and Methods

Synthesis of Hyaluronic Acid Nanoparticles (HAnp)

HAnp were made by utilizing carbodiimide crosslinking chemistry, which is further explained elsewhere.[121] Briefly, 300 mg HA (MW = 16 KDa, Lifecore Biomedical, Chaska, MN) was dissolved in 120 mL DI water, and then after the HA was fully dissolved, 200 mL acetone was added. 60 mg adipic acid dihydrazide (AAD) and 140 mg 1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) (Thermo Scientific, Rockford, IL) were each dissolved in 1 mL DI water and were then added to the solution after 15 and 10 minutes, respectively. After 20 minutes, 200 mL acetone was added to the solution. The reaction was completed

in 3 hours. The solution was dialyzed against DI water for 2 days, frozen, lyophilized and stored at –20°C.

Synthesis of Pentenoate Functionalized Hyaluronic Acid (PHA)

PHA was prepared as our group has described previously.[110] Briefly, 5% (w/v) solution of HA (MW = 60 KDa, Lifecore Biomedical) in DI water was prepared, DMF was added to the solution to reach a water to DMF ratio of 3:2 while pentenoic anhydride (Cat# 471801, Sigma-Aldrich, St. Louis, MO) was slowly added to the solution in 5 M excess relative to HA. The pH of the solution was monitored for 5 hours and was maintained at pH 8-9 by adding 1M NaOH to the solution. The reaction was completed overnight. NaCl was added to the solution to reach 0.5M concentration of NaCl, and the polymer precipitated by adding 4 volumes of acetone and centrifuging for 5 minutes (7,000 x g). The precipitated PHA was dissolved in DI water and dialyzed for 48 hours. The final product was 17% functionalized with the pentenoate group in relation to the repeating disaccharide unit. Functionalization was confirmed with ^{1}H-NMR (Varian Mercury VNMRS 500 MHz spectrometer, Fig. 4.9) by comparing the integration of the alkene peaks on the functional group to the acetyl methyl group on the HA. PHA was frozen, lyophilized, and stored at –20°C.

Hydrogel Preparation

The hydrogel precursor material was prepared the day before the surgery. PHA was sterilized using ethylene oxide gas (AN74i, Anderson Anprolene, Haw River, NC). A solution of 2.3 mM 2-Hydroxy-4′-(2-hydroxyethoxy)-2-methylpropio-

phenone (Irgacure 2959, Cat# 410896, Sigma-Aldrich) and 1% dithiothreitol (DTT, Cat# D0632, Sigma-Aldrich) was prepared in phosphate-buffered saline (PBS) and was sterile-filtered. To prepare the hydrogels in the PHA group, PHA and HAnp were added to the PBS solution to make a 5% (w/v) concentration of each. To incorporate peptides (i.e., SPPEPS or RGD) in the hydrogel network, a solution with concentration 2% (w/v) PHA and 5% (w/v) HAnp was made by adding PHA and HAnp to PBS solution containing 1 mM Irgacure and 1.5 mM thiolated SPPEPS or thiolated RGD. The solutions were fully dissolved (2 hours) and the peptides were conjugated to the PHA after exposure to 312 nm UV light (EB-160C, Spectroline) for 2 minutes. Afterward, the solutions were mixed with additional PHA and PBS solution containing Irgacure and DTT to achieve the final concentrations of 5% (w/v) PHA, 2 mM Irgacure and 1% DTT. When the PHA and HAnp were completely dissolved (2 hours), the solutions were pipetted into sterile syringes, protected from light with tin foil, and kept sterile until the surgery. Thiolated peptides were custom-ordered (Biosynthetic, Lewisville, TX) and synthesized on a PTI Symphony peptide synthesizer (Tucson, AZ). Each peptide's purity was shown to be >95% using analytical high-performance liquid chromatography (HPLC, Shimadzu, Columbia, MD).

Description of Experimental Groups

In the current study, four different study groups were investigated (n=6). In all the groups, defects were created in the medial femoral condyles, and three holes were microdrilled into the subchondral bone. In the sham control group, no material was placed in the defect. The other groups were the PHA, PHA+RGD and PHA+SP groups (i.e., 5% PHA+5% HAnp, 5% PHA+5% HAnp containing 1.5 mM RGD, and 5% PHA+5% HAnp containing 1.5 mM SPPEPS, respectively).

Surgical procedure

All animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Oklahoma Health Sciences Center (Protocol # 17-007-SS A). 12 female New Zealand white rabbits, each 6 months old and between 4 to 4.5 kg (Robinson Service, Inc (RSI), Mocksville, NC), were monitored for normal and healthy behavior a week prior to the surgery. Following analgesic delivery, stable general anesthesia, and antibiotic administration, the area around each knee was shaved and prepared (including 3 rounds of alternating chlorhexidine scrub and alcohol solution with a final betadine spray). Before the procedure, lactated ringers injection (LRS) was provided at multiple injection sites around the neck and shoulders to ensure proper hydration and to maintain blood pressure. After surgical anesthesia was established, the rabbits were laid in the supine position and a medial parapatellar incision was made sufficient to allow exposure of the medial femoral condyle. The tibia was lightly pushed to displace it

laterally to allow the exposure of the medial femoral condyle, and an osteochondral defect of 3.5 mm diameter was drilled in the medial femoral condyle cartilage with the depth of 1-2 mm, essentially a full-thickness cartilage defect that penetrated into subchondral bone. An initial pilot defect was created with a 1 mm drill bit, and gradually enlarged to a 3.5 mm defect using 2 and 3.5 mm drill bits. The joint was washed of debris with sterile saline before three smaller holes (0.4 mm diameter) were microdrilled into the subchondral bone of the defect area to mobilize local bone marrow to the site of cartilage lesion. Before placing the hydrogels, the blood in the defect was removed with sterile gauze. The hydrogel precursor material was placed in the defect area with a syringe and a needle and exposed to 312 nm UV-light at 9 mW/cm^2 (EB-160C, Spectroline) for 5 minutes to form a crosslinked network (Fig. 4.1). The joints were washed with sterile saline, and the articular capsule and bursae were closed with absorbable suture before binding of the skin with intradermal absorbable suture. The rabbits were placed on a recirculating hot water blanket during anesthesia and recovery and use of analgesic agents continued for 3 days after the surgery. Rabbit sternal recumbency was monitored until they could rest unassisted in their home cage.

Post-surgical Care

After both knee procedures were finished, rabbits were returned to their cages and their knee joints were allowed unconstrained post-operative movement. Each rabbit's condition was monitored to detect post-operative complications.

Animals were examined three times a day for the first 3 days, once a day for 7 days, and every other day for the remaining 10.5 weeks to ensure that they exhibited normal patterns of behavior, were active and inquisitive, had normal posture and movements, and were eating and drinking. Physical examinations were performed to look for signs of infection or distress. All the rabbits displayed normal behavior one day after the surgery and no signs of inflammation were observed, except for rabbit #04, who had inflammation of the left knee (PHA group) after surgery, but fully recovered within 5 days.

Gross Morphological Assessment

The animals were euthanized after 12 weeks by intravascular administration of concentrated barbiturate (200 mg/kg) in an ear vein after isoflurane anesthesia via masking (as approved by the IACUC protocol). The depth of anesthesia was analyzed by measuring heart rate, respiration rate, and pedal withdrawal. After the joint retrieval, the knees were photographed. Gross morphology was assessed from the images by three independent scorers. The scoring criteria were developed from the ICRS scoring chart [122] (Table 4.2), based on edge integration of the boundaries of regenerated tissue and native cartilage, smoothness of the repair surface, degree of filling at the cartilage surface, color of the regenerated cartilage, and the percent of repair tissue relative to the total area.

Histological Preparation and Staining

After joint retrieval, knees were placed in formalin for 36 hours and then decalcifying solution for 48 hours (Cat # P7589, Richard-Allan Scientific™ Decalcifying Solution, Thermo Fisher Scientific, Waltham, MA), before samples were washed in running water for 1 hour. The embedding, sectioning, and staining of tissue was performed by the Tissue Pathology Core at the University of Oklahoma Health Sciences Center. Briefly, the tissues were embedded in paraffin and sectioned to a thickness of 4 to 8 µm before mounting on positively charged slides. The slides were dried overnight at room temperature and incubated at 60°C for 45 minutes. Staining with Hematoxylin & Eosin or Alcian Blue (Cat # 3801571, 3801616, 38016SS3DG, Leica Biosystems, Wetzlar, Germany) was performed utilizing a Leica ST5020 Automated Multistainer following manufacturer protocols. The stains were scored blindly by three different scorers base on the grading system described in Table 4.3.

Immunohistochemistry

The slides were processed similar to the basic histology as described above up through incubation in 60°C for 45 minutes**.** The slides were cleared with xylene for 10 minutes and slowly rehydrated in 100%, 95%, and 70% ethanol for 4, 3, and 3 minutes respectively. After incubating the slides in distilled water (dH_2O) for 3 minutes, the sections were exposed to 0.3% hydrogen peroxide solution (Cat # ab94666, Abcam, Cambridge, MA) for 30 minutes to suppress endogenous peroxidase activity. The slides were soaked in PBS+Tween (Cat # P3563, Sigma

Aldrich, St Louis, MO) for 5 minutes and were incubated in proteinase K (Cat # ab64220, Abcam) for 15 minutes in 37°C and 10 minutes in room temperature and then were soaked in PBS tween for 5 minutes. Sections were blocked first with a sequence of Avidin and then Biotin blocking solutions (Cat # SP-2001, Vector Laboratories, Burlingame, CA) each 15 minutes, and then with 3% blocking horse serum (Cat #S-2012, Vector Laboratories) for 20 minutes. The collagen type II primary antibody (Cat # 08631711, MP Biomedicals, CA, USA, dilution 1:150) incubation followed for 1 hour at room temperature and then overnight at 4°C. The next day, the slides were soaked in PBS+Tween for 5 minutes and were exposed to horse anti-mouse IgG biotinylated secondary antibody and ABC reagent (Cat #PK-6102, Vector Laboratories) for 60 and 30 minutes, respectively. Visualization was accomplished with ImmPact DAB peroxidase substrate (Cat #SK-4100, Vector laboratories) followed by exposure to DAB enhancing solution (Cat #H2200, Vector laboratories) and then Hematoxylin QS solution (Cat #H-3404, Vector Laboratories) for 10 seconds and 1 minute, respectively. The slides were dehydrated in graded ethanol, cleared in xylene and mounted (Permount, SP15-500 Fair Lawn, NJ).

Statistical Analyses

The data are shown as mean ± standard deviation where applicable. The statistical analyses were performed using ANOVA followed by Tukey's HSD method for comparisons (Graphpad Software Inc., La Jolla, CA), the statistical significance threshold was 0.05 in all the analyses (i.e., $p < 0.05$).

Results

Gross Morphological Observations

At the time of tissue retrieval, visual inspection revealed no signs of inflammation, swelling, or redness at the defect sites and joint surfaces (Fig. 4.2). The percentage of the repaired tissue for the control group was between 56 and 100% (average 86 ± 16%), between 30 and 98% for the PHA group (average 74 ± 22%), between 26 and 100% (average 84 ± 26%) for the PHA+RGD group, and between 15 and 100% (average 74 ± 35) for the PHA+SP group. No statistically significant differences were detected among the groups (Fig. 4.3). The gross morphological scores (Fig. 4.3), where 10 represents the highest quality of regeneration (Table 4.2), were between 4 and 9 (average 7.8 ± 1.9) for the control group, between 2.6 and 8.6 (average 6.2 ± 2.1) for the PHA group, between 1.6 and 10 (average 7.6 ± 3.2) for the PHA+RGD group, and between 1.3 and 8.6 (average 5.6 ± 3.2) for the PHA+SP group. PHA+RGD was the only group that had condyles (6R and 8L) with a total score of 10 out of 10 for gross morphology.

Histomorphometric Observations

The morphometric analyses of the H&E staining indicated that in 50% of the condyles from the control group, the cells were mostly chondrocytes, 33% of the defects had mixed chondrocytes and fibrocartilage differentiated in the cartilage zone and 17% had mostly fibrocartilage appearance. For the PHA group, we observed that 17% of the defects had mostly chondrocytes, 50% had mixed

chondrocytes and fibrocartilage and 33% had mostly fibrocartilage. In the PHA+RGD group, for 17% of the condyles, most of the cells in the defects were chondrocytes; for 67% of the defects, the cells were a mix of fibrocartilage and chondrocytes; and 17% of the defects mostly had a fibrocartilage appearance. In the PHA+SP group, the cells were mostly chondrocytes in 33% of the defects. We observed a mix of fibrocartilage and chondrocytes in 50% of the defects, and mostly fibrocartilage in 17% of the defects.

The scoring of the cartilage thicknesses indicated that the cartilage thicknesses of 17% of the condyles from the control group were similar to the surrounding native cartilage, for 50% and 33% of the condyles the cartilage thicknesses were greater and less than the healthy cartilage, respectively. For the PHA group 33% of the condyles showed no cartilage thickness, 17% had greater cartilage thicknesses compared to the surrounding tissue, and 50% had smaller cartilage thicknesses compared to the surrounding. The cartilage thicknesses in the defect areas of PHA+RGD condyles were greater than the surrounding for 50% of the defects and less than the surrounding cartilage for the rest. 17% of the condyles from the PHA+SP group had cartilage thicknesses similar to the surrounding cartilage. The cartilage thicknesses were greater and less than the surrounding cartilage for 17% and 66% of the condyles, respectively.

In the condyles of the control group, 66%, 17%, and 17% had edge integrations on both ends, on one end, and on neither end, respectively. In the

PHA group, 33%, 50%, and 17% of the condyles had cartilage integrations on both ends, on one end, and on neither end, respectively. The integrations of the regenerated tissues were at the both ends for 33% and at one end for 67% of the knees for PHA+RGD condyles. 50% of the defect integrations were at both sides and the rest were integrated on one side in the PHA+SP condyles.

The scoring of the regenerated subchondral bone in the defect areas indicated that 50% of the condyles from the control group showed normal subchondral bone regeneration. 33% and 17% of the condyles had reduced and minimal reconstruction of subchondral bone compared to the native surrounding tissue, respectively. In the PHA group, 33% of the samples showed no subchondral bone reconstruction, 17% of the condyles had normal reconstruction of subchondral bones compared to the surrounding tissue, and 50% of them showed no reconstruction of the subchondral bone. In the PHA+RGD group, 17%, 50%, and 33% of the condyles had normal, reduced, and minimal reconstruction of subchondral bones, respectively. In the PHA+SP group, for 50% of the condyles, the reconstruction of the subchondral bones appeared normal, and for the rest of the knees the reconstructed bones were reduced compared to the surrounding tissue.

In the control group, the Alcian Blue staining of the knees was intense for the 2R, 3L and 11R condyles, whereas the regenerated cartilage in the 1L, 10L and 12R condyles did not stain intensely (Fig. 4.6). From the morphometric score

distribution (Fig.4. 4), we observed that 50% of the knees were normally stained and 50% were moderately stained. In the PHA group, we did not observe any intense Alcian Blue staining, 50% of the condyles had moderate staining and 50% of them had slight staining. In the Alcian Blue staining of the PHA+RGD group, we observed that the 8L condyle had normal staining (i.e., similar to healthy tissue) in the defect area and the rest of the condyles had slight or moderate staining. The scoring of the PHA+SP group indicated that 50% of the condyles had normal staining, 17% of them had moderated and 33% had slight staining.

Collagen II Immunostaining

In the control group, collagen II staining was prevalent with intensity similar to the surrounding cartilage tissue for the 2R and 3L condyles. In the rest of the control group condyles, the collagen II immunostaining did not cover the whole cartilage region of the regenerated tissue. The 1L and 10L condyles had some staining at the edges of the defects. In the 11R condyle, the staining was not observed at the cartilage surface, and the 12L condyle had mild staining at parts of the cartilage-bone border (Fig. 4.7). Furthermore, closer observation indicated that in the 2R and 3L condyles, columnar lacunae formations were observed, although in the cartilage zone of the 3L condyle, several fibrocartilage cells clusters were observed where no collagen II staining was present (Fig. 4.8).

For the PHA group, the collagen II staining was slight in the defect areas of the 1R, 5R, and 8R condyles, and the staining in the 10R, 7L, and 4L condyles

was observed at the edges of the defects (Fig. 4.7). In addition, the 4L condyle had a small patch of collagen II staining in the middle of the defect. Closer observation of the defects indicated that the regenerated tissues of the 5R and 8R condyles had some lacunae in the defect site, but with a poor cartilage and bone structure (Fig. 4.8). All the defects of the PHA+RGD group had non-prevalent collagen II staining, although the stained regions of the 8L and 9R condyles had the same stain intensity as their surrounding healthy cartilage. The collagen II immunostaining in the 5L and 11L condyles was present at the edges of the defects but not in the center, and staining in the 6R condyle was only observed in the cartilage-bone boarder. For the 2L, 8L, and 9R condyles, no staining was observed at the cartilage surface.

The most prevalent collagen II immunostaining was in the PHA+SP group. From this PHA+SP group, the 3R, 7R, and 9L condyles especially had prevalent collagen II staining, and the intensities were the same as the surrounding cartilage. Staining in the 6L and 12R condyles was only observed in the cartilage-bone border and at the center of the regenerated tissue, respectively. The 4R condyle staining was observed as a small patch in the middle of the defect. Closer observation of the defects (Fig. 4.8) indicated that the lacunae of the 3R and 7R condyles had some columnar formations but not in all the defect region. The staining for the 7R condyle was intense around several clustered columnar lacunae (Fig. 4.8).

Discussion

The current study was the first time that potential chondroinductivity has been demonstrated *in vivo* by conjugating/immobilizing a peptide sequence to a biomaterial implant for cartilage regeneration. Specifically, the potential of PHA+SP hydrogels in developing a hyaline-like tissue as an acellular technology was demonstrated without the need for exogenous cells, growth factors, or human/animal-derived extracellular matrix, supporting the hypothesis of the study.

The fact that 66% of the knees had >80% regeneration in the defect indicated good regeneration rate in the majority of the condyles, which may be due to microdrilling of the defects and BMSCs infiltration to the defects. The lower regeneration rates in some of the condyles could potentially be due to mechanical failure of the hydrogel, for example an anomaly due to a particular individual rabbit's physical stress placed on the material. Another possibility for variations in healing may be the variations in surgical technique, as a full-thickness chondral-only defect with a 3.5 mm diameter was difficult with the natural curvature of the femoral condyle surface, and so there may have been some variation in the extent of subchondral bone disruption. In addition, microdrilling of course resulted in bleeding of the defect, which in turn caused expected difficulties in placing the hydrogel precursor into the defect. Although the blood was removed with sterile gauze, mixing of blood and the hydrogel precursor material occurred in some of the samples, which changed the fluid mechanics of the hydrogel precursor prior to

crosslinking. This limitation can be avoided by increasing the percentage of HAnp to produce more paste-like precursor solutions.[120]

Incorporation of the peptides (PHA+SP and PHA+RGD) elevated the structural integrity of the regenerated cartilage compared to PHA group. However, a significant finding was the poorer collagen II production in the PHA+RGD group relative to the PHA+SP group, which was likely due to the fact that although RGD encourages cellular adhesion, it does not provide any chondrogenic-specific signal in the absence of chondrogenic growth factors.[72, 123]

Creating true, functional hyaline cartilage has been the elusive goal of cartilage regeneration community, and there are indeed a small handful of approaches in the literature that have evaluated a hyaline-like tissue regeneration by showing superior collagen II IHC staining of the treated groups compared to the non-treated groups in rabbit models. Superior collagen II staining was shown in a few cell-based approaches. For example, in 2008, Funayama *et al.*[124] showed collagen II production when a type II collagen solution mixed with allogeneic chondrocytes was injected into patellar groove osteochondral defects (5 mm diameter, 4 mm depth) of rabbits (n=2) after 24 weeks. In 2016, Kuo *et al*.[125] implanted chitosan/chondroitin-6-sulfate/hyaluronan (GCH) cryogel scaffolds seeded with allogeneic chondrocytes in patellar groove defects (4 mm in diameter and 2 mm in thickness) of rabbits (n=4), and after 12 weeks the collagen II IHC of

the defects supported collagen II production. In 2017, Park *et al*.[126] implanted a composite of HA and human umbilical cord blood-derived mesenchymal stem cells into the patellar groove defects (3 mm in diameter and 3 mm in depth) of rabbits (n=10), and after 16 weeks collagen II production was shown in the defects. In addition to the cell-based approaches the incorporation of growth factors in the scaffolds have shown collagen II production in the regenerated tissue of rabbit models; in 2017, Betz *et al*.[127] press-fitted autologous muscle tissue fragments (treated with BMP-2) in the medial femoral condyle defects (diameter 4 mm, depth 5 mm) of rabbits (n=7). After 8 weeks collagen II production confirmed with IHC was observed in the in the defects. In 2016, implanting alginate gels containing stromal cell-derived factor (SDF)-1 in patellar groove defects (4.5 mm in diameter and 3 mm in depth) of rabbits (n=5) led to superior collagen II production (confirmed by IHC) after 16 weeks compared to the untreated defects.

Incorporation of cells in cartilage regeneration approaches has therefore shown promising results and might be necessary for treatments of large cartilage defects; however, the translational limitations of cell-based therapies such as high cost, the need for two separate surgeries, and regulatory challenges have inspired the biomaterials community to develop alternative strategies. Employing chondroinductive growth factors in cartilage regeneration approaches might be advantageous to overcome the cell-based therapy limitations and the clinical approval of the number of growth factor based products demonstrate a significant step for overcoming their regulatory barriers.[117] However, the drawbacks

associated with growth factors—in addition to their high cost and the associated regulatory classification as a combination product—is their potential for eliciting ectopic tissue formation *in vivo.* In addition, their short half-life suggests that they need a carrier system to enhance their tissue regeneration potential. The bottom line is that growth factor inclusion, with all translational risks aside, still requires the design of a delivery system that ensures targeted and on demand delivery of the therapeutic is necessary.

The current study was the first *in vivo* analysis of the SPPEPS peptide, conducted with a fast-crosslinking PHA hydrogel as the biomaterial platform. With this proof of concept in place, the results of the current study provide the motivation and rationale for follow up studies with larger animal numbers to allow for additional tastings. In the current study, only one concentration of the SPPEPS peptide was analyzed. It would be valuable to explore not only different concentrations of the SPPEPS sequence, but further to evaluate the chondroinductivity of the peptide in combination with adhesion peptides such as RGD or other potential chondroinductive sequences such as Link N or B2A. Furthermore, different biomaterial carrier systems may be explored to support osteochondral regeneration. In addition, larger animal numbers will allow for functional mechanical testing that would have been premature to include in the current study.

Conclusion

The current study investigated the chondroinductivity of a novel bioactive peptide (SPPEPS) conjugated to a PHA hydrogel. Although the gross morphology did not identify significant differences among the groups, the collagen II immunostaining demonstrated the potential of the SPPEPS peptide in enhancing chondrogenic differentiation of the infiltrated BMSCs and production of a hyaline-like cartilage tissue. Identifying bioactive peptides and incorporating them in cartilage regeneration strategies may lead to the design of chondroinductive materials without the need for adding cells of any kind or growth factors, which would be tremendously beneficial from a translational perspective.

Chapter 5: Conclusion

The current dissertation has successfully introduced the capabilities of a novel synthetic peptide, SPPEPS, in inducing chondrogenesis *in vitro* and *in vivo*. In regenerative medicine, developing strategies that do not rely on cell sources or natural stimuli to produce the targeted tissue, are advantageous for translational purposes. The cell-based therapies have limitations for the patients such as high cost, long recovery time and need for two separate surgeries (harvesting and implanting the cells). In addition, the need for GMP facilities and the surgeons' training add more obstacles for developing across-the-board strategies. Incorporation of animal-derived components such as growth factors have regulatory complications (i.e., combination product) in addition to their high cost. The lack of specificity in natural components may lead to ectopic tissue formation or tumorigenesis. One classic example of ectopic tissue formation happened with bone morphogenetic protein-2 (BMP-2). The application of BMP-2 for posterior lumbar interbody fusion (PLIF) was halted by the Federal Food and Drug Administration (FDA) when their trial study indicated that the application of BMP-2 elevated the incidence of ectopic bone formation in the neural canal.[128] Small peptides reduce the chance of nonspecific binding, which means that they may be incorporated in biomaterials to elicit a desired and targeted cell response. Furthermore, peptides are easy to synthesize in large quantities with lower price compared to natural molecules and can easily be immobilized to the carrier biomaterial to avoid challenges with controlled release.

The cell-binding ability of synthetic peptides was first shown in 1984,[75] when fibronectin cell-binding was duplicated with a three amino acid peptide, Arg-Gly-Asp (RGD), which is found in the cell attachment domain of fibronectin. Later on, it was shown that RGD is the cell attachment site of many other adhesion proteins.[76-79, 129] RGD is a good example that demonstrates a specific binding sequence can be reduced to a short peptide, and the peptide can be used instead of growth factors, antibodies, and ECM proteins.[82-85, 129] To successfully draw out a desired response from synthetic peptides, understanding the cellular signaling networks is crucial. The mechanisms of many cellular interactions are not fully understood; however, the advance of high-throughput measurements and genome sequencing enable us to collect comprehensive data sets, which can eventually lead us to theory formation and computational prediction of the cellular signaling networks.[130]

In this thesis, my goal was to find a peptide that specifically affects chondrogenic differentiation, which led us to the matching sequence of chondroinductive molecules and for further analysis the peptide was crosslinked to pentenoate-functionalized hyaluronic acid (PHA) hydrogels.

The physical properties of hydrogels have made them attractive for many different applications in regenerative medicine. Properties such as capabilities of

filling any shape of defect, the crosslinking after placing the material, which keeps the material in place and the fact that other molecules can be encapsulated in a hydrogel network. Hyaluronic acid (HA) hydrogels specifically have been a focus area of interest in regenerative medicine because HA has a crucial role in different cellular and tissue functions[66, 131-134] and HA hydrogels mimic salient elements of native extracellular matrices (ECMs) and can support cell adhesion.[133]

The current dissertation successfully demonstrated the potential of the SPPEPS peptide in inducing chondroinductivity when the peptide significantly enhanced the expression of chondrogenic markers; however, the proteomics analysis indicated that the peptide alone was not sufficient for mimicking transforming growth factor-beta 3 (TGF-β_3). Identifying more chondroinductive sequences and studying their synergistic effects might be a promising route for designing biomaterials with the potential of expressing a protein profile similar to the one stimulated by TGF-β_3.

The *in vitro* evaluations do not always translate successfully to *in vivo* models, the changes in cellular environment and mechanical forces are oversimplified in *in vitro* models. The costs related to use of animal models does not make the *in vivo* analysis always possible. For the future analysis, improvement of the *in vitro* models such as 3D cell culture or simulating mechanical forces *in vitro* might help to develop better *in vitro* models.

Despite the TCT plates study, the *in vitro* analysis of the hydrogels indicated no significant difference of chondrogenic marker expression between the groups with or without the SPPEPS conjugation. However, the hydrogels with both RGD and SPPEPS peptides conjugated to PHA showed superiority in terms of supporting chondrogenic differentiation, which further indicated the role of cellular adhesion in supporting chondrogenic differentiation. In the hydrogel study only one concentration of the peptide was tested; therefore, analyzing PHA hydrogels with various concentrations of the peptide may be advised in the future.

For the *in vivo* study, the scoring of the gross morphology did not indicate any significant differences among the groups, although the collagen II immunohistochemistry indicated the potential of the SPPEPS peptide in inducing chondrogenic differentiation in the defects; however, the results were not consistent in all the samples, which may suggest that larger sample numbers might be needed. In addition, the mechanical properties of the regenerated tissue were not evaluated, which can be considered for future analysis. The mechanical properties of hydrogels do not necessarily mimic the cartilage ECM. This limitation might be addressed by designing multi-material constructs where one material supports the mechanical integrity and the other mimics the biological properties of cartilage ECM. The material can further be custom-made, and 3D printed for irregular shapes.

The current dissertation has successfully created a new route for designing chondroinductive biomaterials. Replacing natural molecules with synthetic peptides may lead to designing of biomaterials tailored to receptor–peptide interactions, which will be the next generation of biomaterials for cartilage regeneration; however, this would not be possible without fully understanding of the mechanism of chondrogenic differentiation, which might be achieved by high through-put analysis of cellular networks.

This dissertation has successfully identified a chondroinductive peptide sequence (SPPEPS) and demonstrated its potential in inducing chondroinductivity in 2D cell culture for *in vitro* study when the peptide was conjugated to hydrogels for *in vitro* and *in vivo* study,

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Appendix A: Figures

Chapter 1: No Figures

Chapter 2: Figure 1.1

Chapter 3: Figures 3.1-3-9

Chapter 4: Figures 4.1-4.9

Chapter 5: No Figures

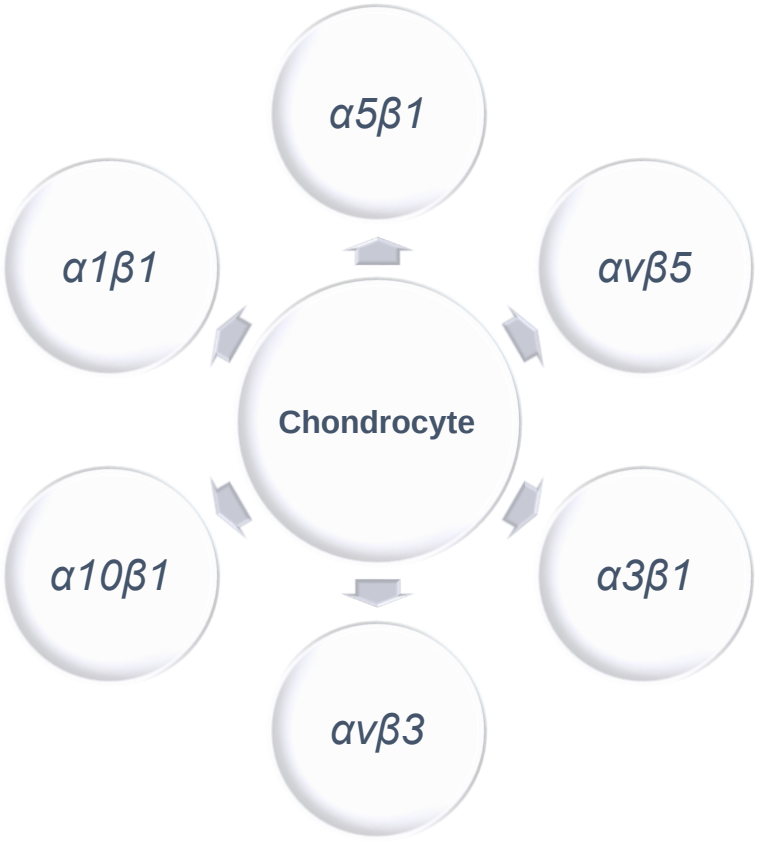

Figure 2.1: Chondrocyte Integrin Expression

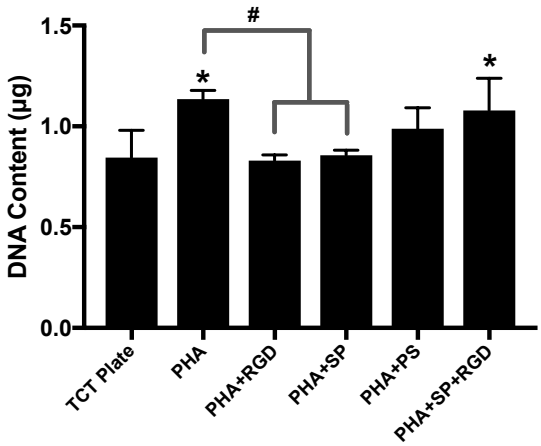

Figure 3.1. DNA Content Analysis of the rBMSCs on Hydrogel Surface after 3 Days.

Individual conjugation of SPPEPS and RGD to PHA decreased the cell content; however, the cell contents are less than the PHA group when RGD and SPPEPS peptides are incorporated in the hydrogels individually, however; the cell content is comparable with PHA group, when both SPPEPS and RGD peptides are incorporated in the hydrogels. PHA = 5% (w/v) pentanoate-functionalized hyaluronic acid with no peptide conjugation, PHA+RGD = PHA conjugated to 1.5 mM GCGYGRGDSPG, PHA+SP = PHA conjugated to 1.5 mM GCGYGSPPEPS, PHA+PS = PHA conjugated to 1.5 mM GCGYGPSEPSP (scrambled SPPEPS), PHA+RGD+SP = PHA conjugated to 1.5 mM GCGYGRGDSPG and 1.5 mM GCGYGSPPEPS. (* and $^{\#}$) = $p < 0.05$ compared to the TCT plate group and the PHA group, respectively. Data are reported as mean + standard deviation (n=3).

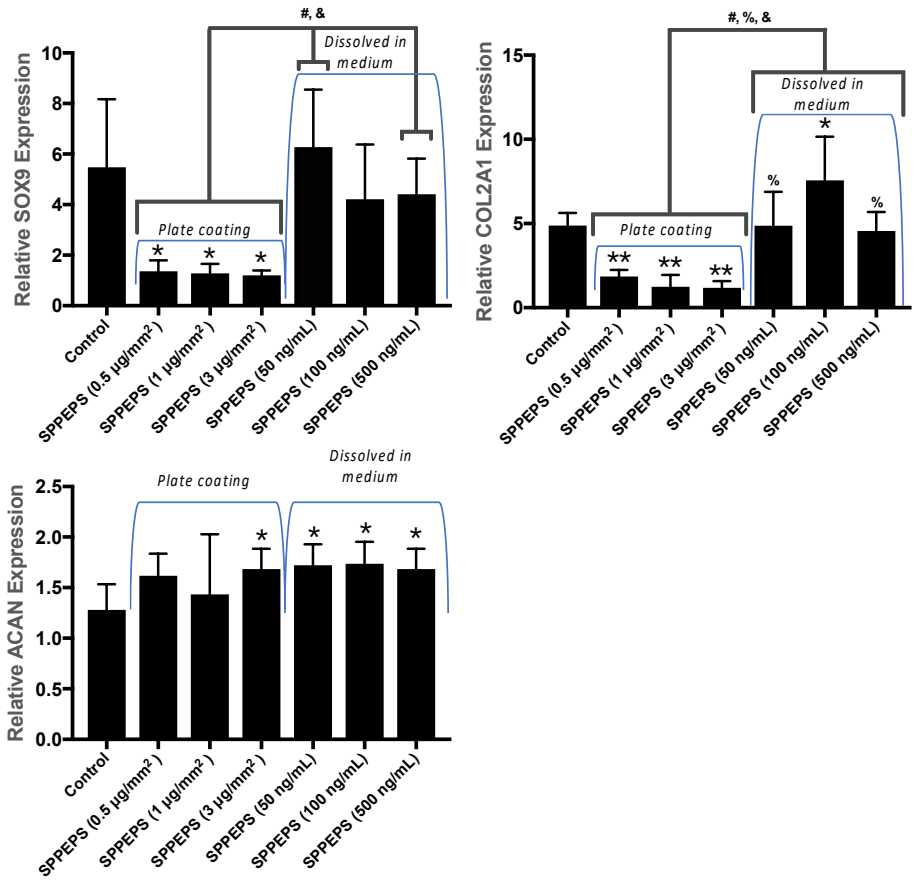

Figure 3.2. Gene Expression Analyses of rBMSCs on TCT Plates Measured by rt-PCR after 3 Days.

For the SPPEPS 100 ng/mL group, the collagen type II and aggrecan gene expressions were 1.5 times and 35% higher than the control group, respectively. (*, #, % and &) = $p < 0.05$ compared to the Control, SPPEPS 50, 100 and 500 ng/mL respectively. (**) = $p < 0.001$ compared to the Control. Data are reported as mean + standard deviation (n=6).

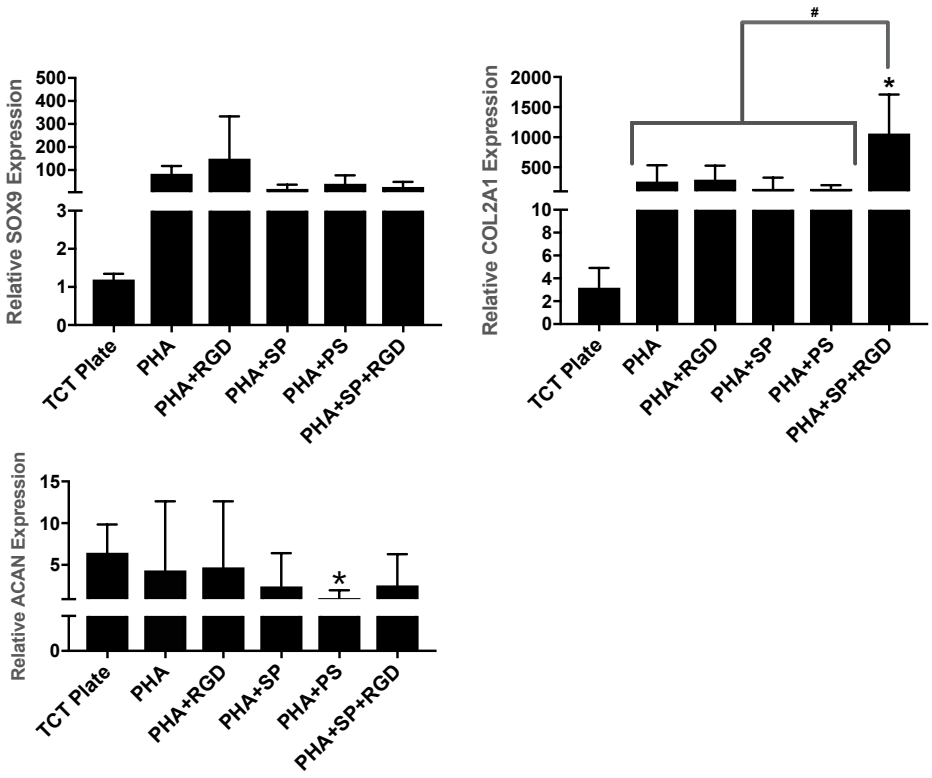

Figure 3.3. Gene Expression Analysis of rBMSCs on Hydrogel Surfaces Measured by rt-PCR after 3 Days.

Combined effects of SPPEPS and RGD conjugation induced collagen type II gene expression far superior to either peptide individually. PHA = 5% (w/v) pentanoate-functionalized hyaluronic acid with no peptide conjugation, PHA+RGD = PHA conjugated to 1.5 mM GCGYGRGDSPG, PHA+SP = PHA conjugated to 1.5 mM GCGYGSPPEPS, PHA+PS = PHA conjugated to 1.5 mM GCGYGPSEPSP (scrambled SPPEPS), PHA+RGD+SP = PHA conjugated to 1.5 mM GCGYGRGDSPG and 1.5 mM GCGYGSPPEPS. (* and $^{\#}$) = $p < 0.05$ compared to the TCT plate group and PHA+SP+RGD respectively. Data are reported as mean + standard deviation (n=4).

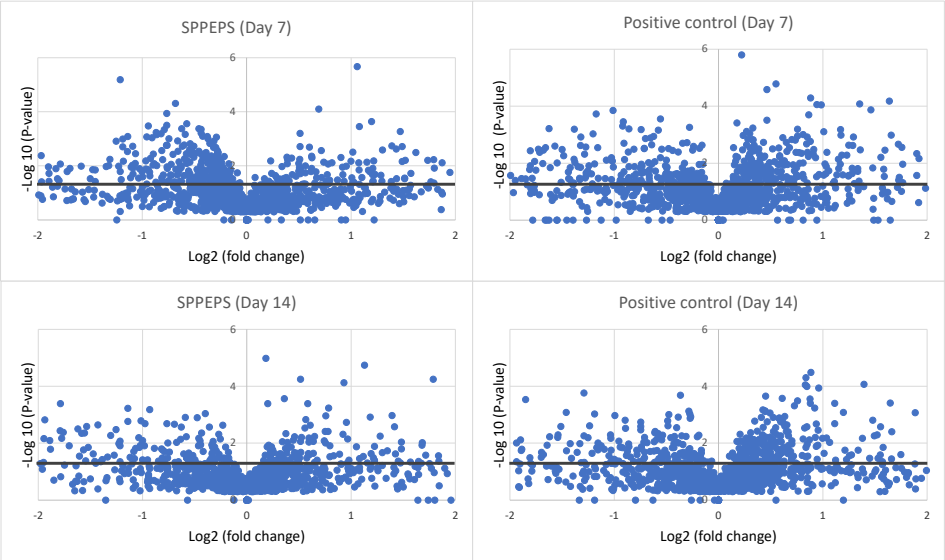

Figure 3.4. Proteomics Analysis, Volcano Plots for 7 and 14 Days.

440, 429, 372 and 275 proteins were expressed in positive control day 7, SPPEPS day 7, positive control day 14, and SPPEPS day 14, respectively (each with a significant change compared to the negative control). Positive control=TGF-β_3 (10 ng/mL). SPPEPS=SPPEPS 100 ng/mL. –Log 10 (P-value) > 1.3 is considered to be significant (i.e., Log 10 (0.05) =1.301), (n=4).

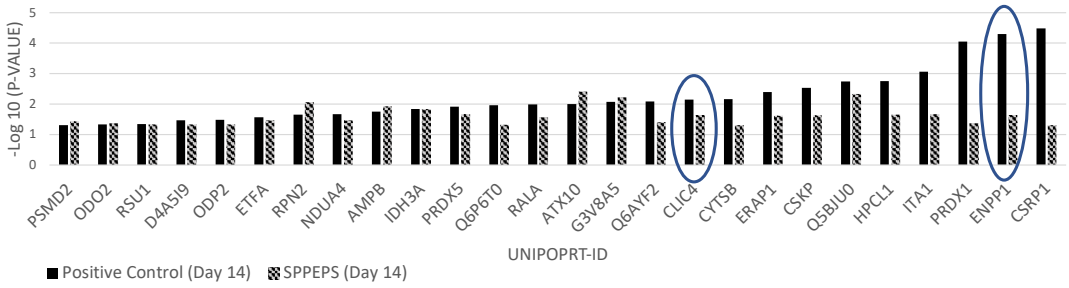

Figure 3.5. Proteomics Analysis. Common Proteins Expressed in SPPEPS and Positive Control Groups at 14 Days.

26 common proteins were expressed in SPPEPS and Positive Control Groups at 14 days, with a significant increase compared to the Negative Control. The blue circles represent CLIC4 and ENPP1 genes that play important roles in chondrogenic differentiation. SPPEPS = SPPEPS 100 ng/mL, positive control = TGF-β_3 (10 ng/mL). –Log 10 (P-value) > 1.3 is considered significant (n=4).

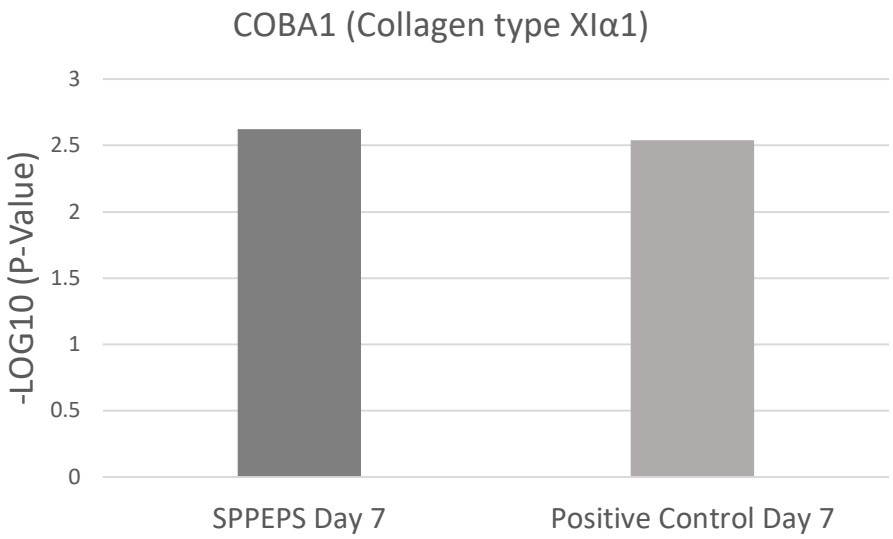

Figure 3.6. Proteomics Analysis, collagen type XIα1 Expression.

Collagen XIα1 content significantly increased in both the positive and SPPEPS groups when the data were normalized to the negative control. SPPEPS = SPPEPS 100 ng/mL, positive control = TGF-β_3 (10 ng/mL). –Log 10 (P-value) > 1.3 is considered significant (n=4).

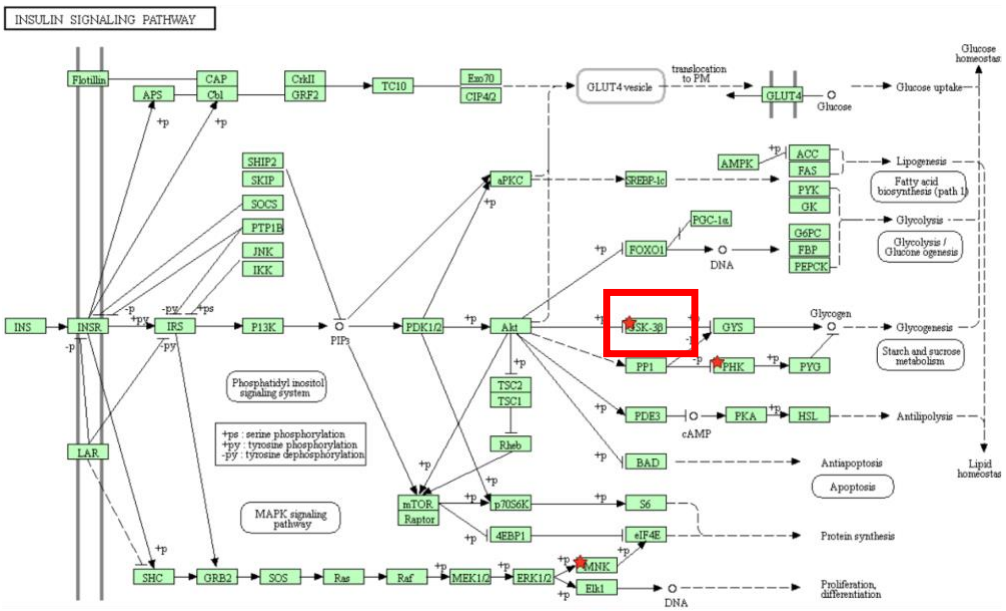

Figure 3.7. Proteomics Analysis, Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathways.

At 14 days, in both SPPEPS 100 ng/mL and TGF-β_3 (10 ng/mL) groups, KEGG pathway analysis of the proteins that were significantly higher from negative control indicate that the “Insulin Signaling Pathway” was activated through the GSK3-β gene, which is known to be an essential gene for early stage chondrocyte differentiation.

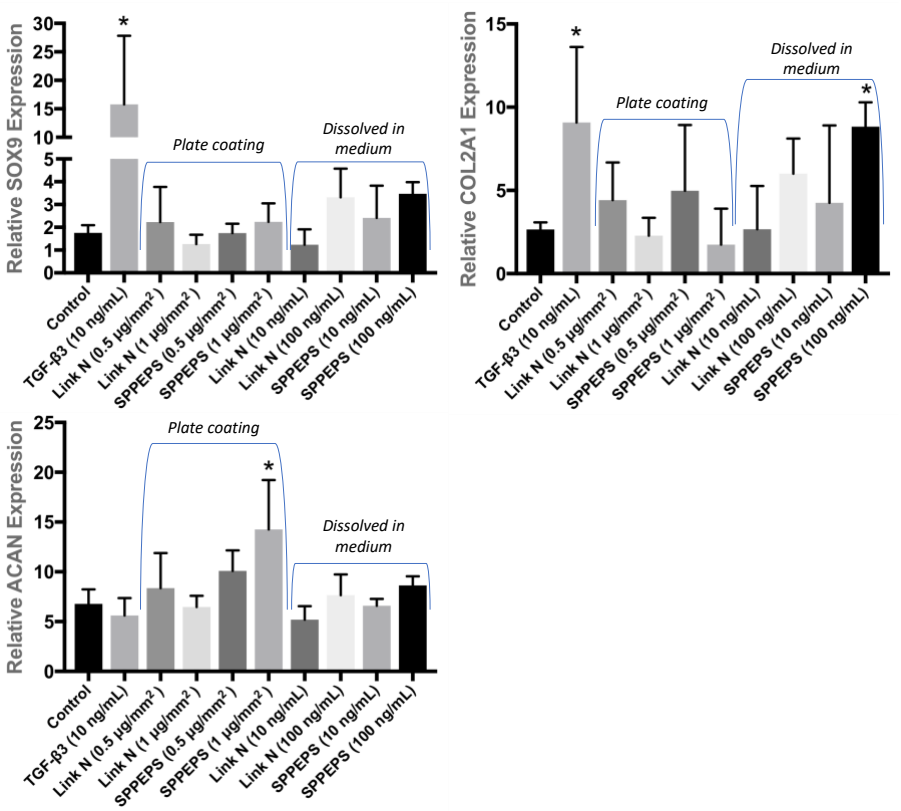

Figure 3.8. Preliminary Study, Gene Expression Analyses of rBMSCs on TCT Plates Measured by rt-PCR after 3 Days.

Collagen type II gene expression for SPPEPS (100 ng/mL) was 3.3 times higher compare to the control group. Control = rBMSCs on TCT plates with no peptide or growth factors in the cell culture medium. TGF-β_3 = transforming growth factor-β_3 dissolved in cell culture medium. Link N = Link N (ASP-HIS-LEU-SER-ASP-ASN-TYR-THR-LEU-ASP-HIS-ASP-ARG-ALA-ILE-HIS, DHLSDNYTLDHDRAIH) peptide sequence, SPPEPS = Ser-Pro-Pro-Glu-Pro-Ser peptide sequence, matching sequence of aggrecan proteoglycan and TGF-β_3. (*) = $p < 0.05$ compared to the control. Data are reported as mean + standard deviation (n=3).

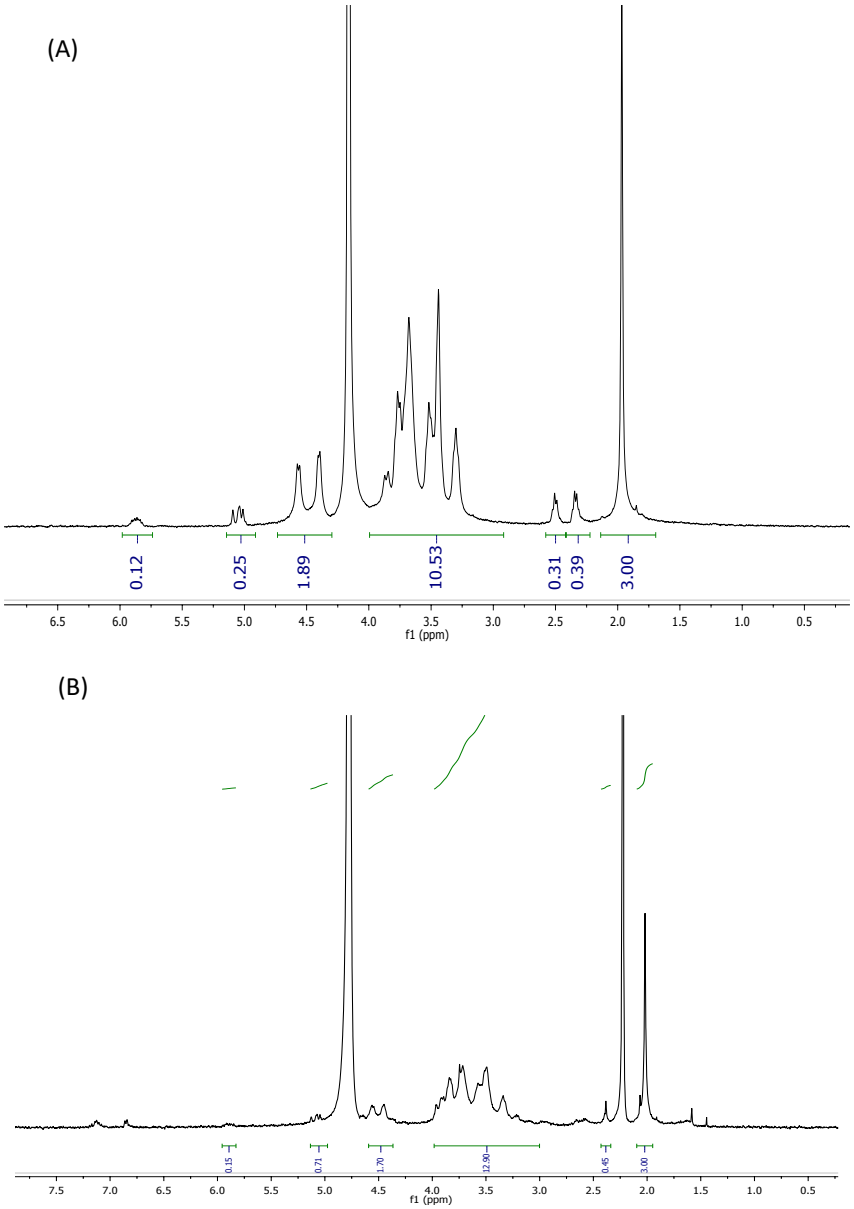

Figure 3.9. H ^{1}NMR analysis

(A) 60 kDa PHA, and (B) 60 kDa PHA with conjugated GCGYGRGDSPG. (Varian Mercury VNMRS 400 MHz spectrometer).

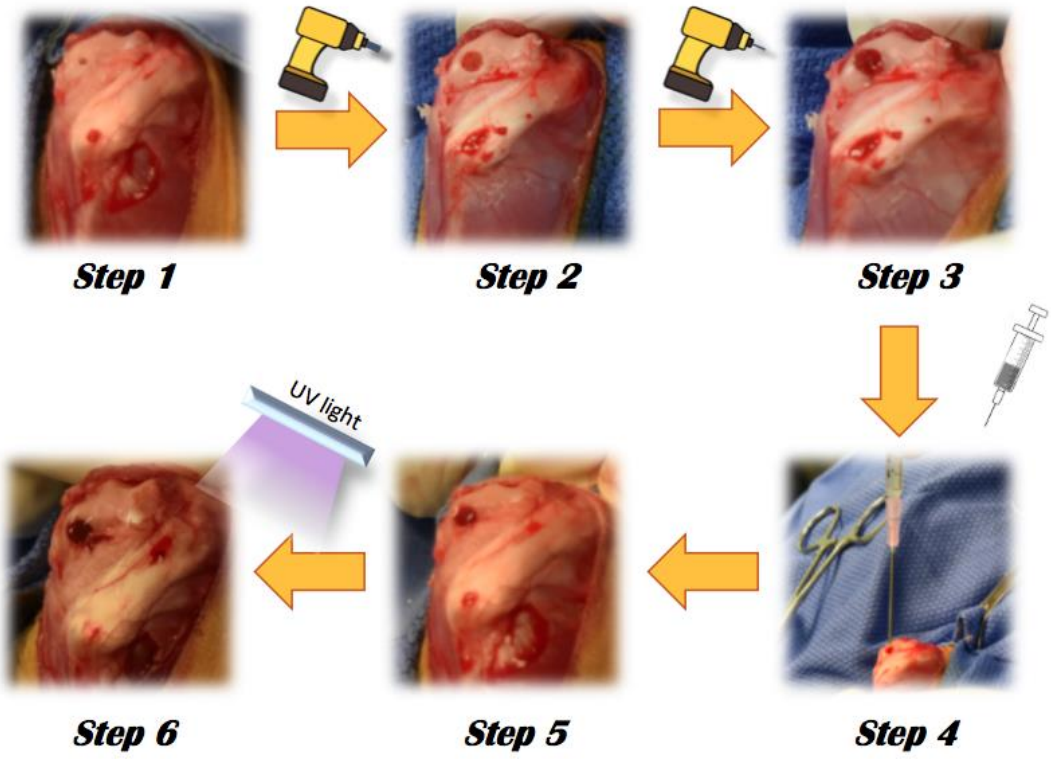

Figure 4.1: Surgical Procedure (Sequence of Steps)

(1) Pilot hole (1 mm diameter) was created in the femoral condylar cartilage. (2) The hole was enlarged to 3.5 mm diameter. (3) Microdrilling of 4 holes (0.4 mm in diameter) in the subchondral bone stimulated bone marrow to the defect area. (4 and 5) The hydrogel precursor was implanted in the defect with needle and syringe. (6) The hydrogel was photocrosslinked for 5 minutes.

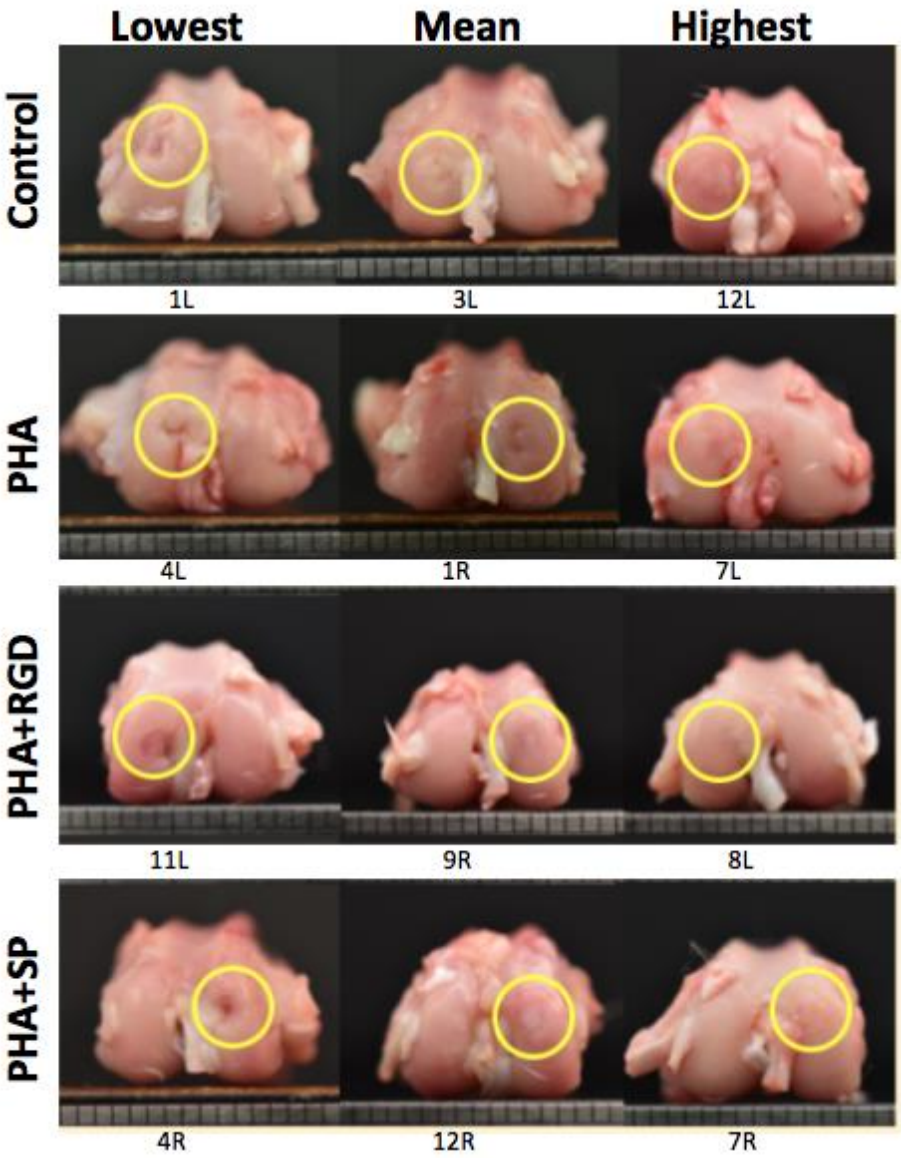

Figure 4.2: Representative Gross Morphological Images

The images represent the worst, intermediate and best gross morphology of rabbit condyle defects 12 weeks post-surgery. For all the groups, we observed variability in the regenerated tissues; however, the best PHA+RGD sample regenerated the tissue most similar in appearance to the healthy cartilage. Control = The defect with no implanted material, PHA = 5% pentanoate-functionalized hyaluronic acid + 5% hyaluronic acid nanoparticles, PHA+RGD = PHA conjugated to 2 mM RGD, PHA+SP = PHA conjugated to 2 mM SPPEPS. The text under each image represent rabbit number and knee side. (e.g., 1R = Rabbit #1, right knee). Each interval on the rulers indicates 1 mm.

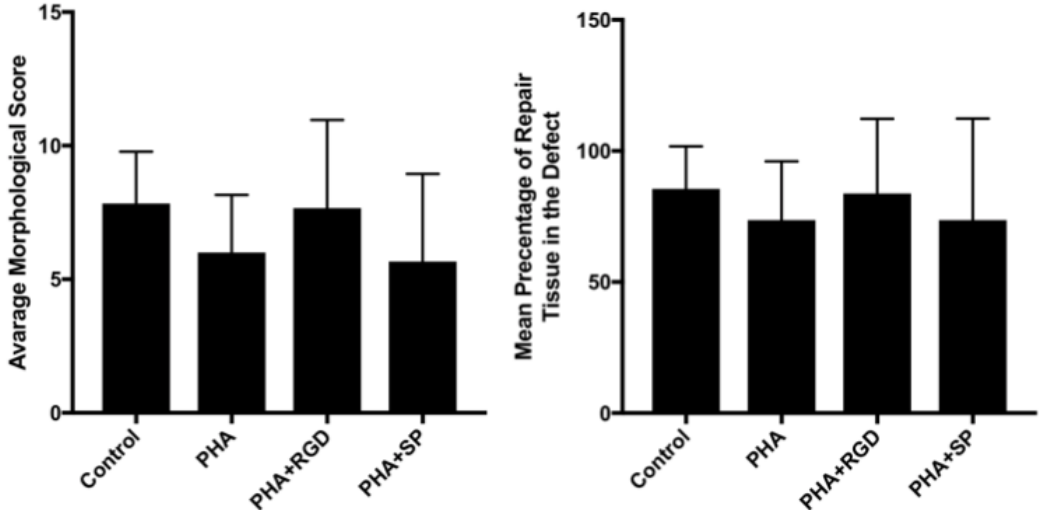

Figure 4.3: Gross Morphology Scoring

Average morphological score (max score = 10), and mean percentage of repaired tissue in rabbit condyle defects 12 weeks post-surgery (n=6). Only two condyles scored 10 for average morphological and had 100% regenerated tissue and both the were from the PHA+RGD group. No significant differences were observed among the groups. Control = The defect with no implanted material, PHA = 5% pentanoate-functionalized hyaluronic acid + 5% hyaluronic acid nanoparticles, PHA+RGD = PHA conjugated to 2 mM RGD, PHA+SP = PHA conjugated to 2 mM SPPEPS.

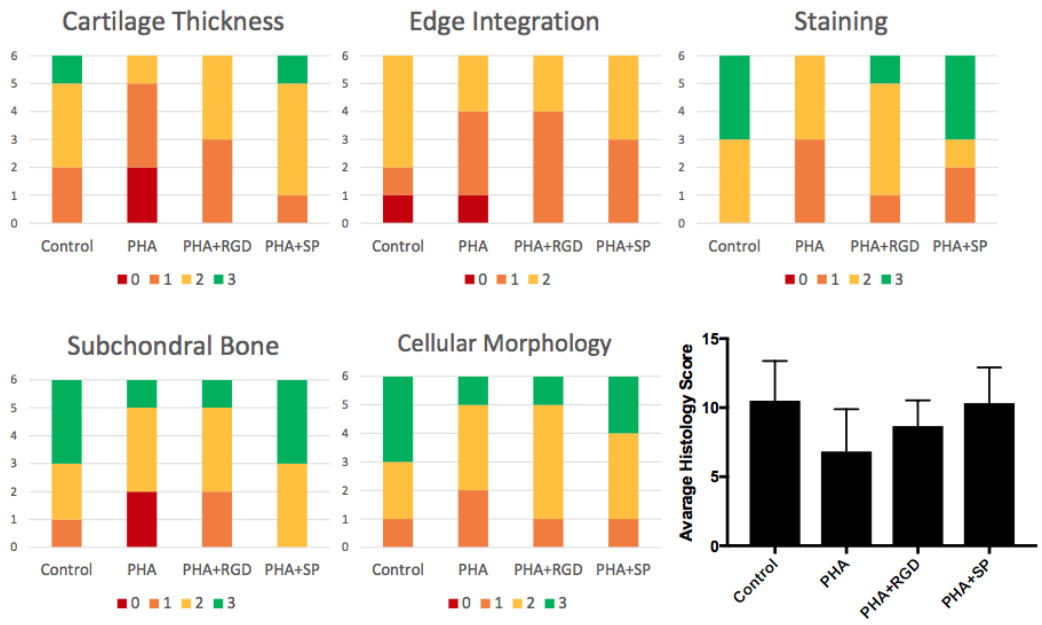

Figure 4.4: Histology Score Distribution

Stacked column plot compared each of the histological scores and the average histology score for each group, values represent mean + standard deviation (n=6). The histology scoring system is explained in Table 3. There was a more frequent presence of the high score (e.g., 3) for the PHA+SP and Control groups, and there were no examples of "0" score for either of the peptide groups. Control = The defect with no implanted material, PHA = 5% pentanoate-functionalized hyaluronic acid + 5% hyaluronic acid nanoparticles, PHA+RGD = PHA conjugated to 1.5 mM RGD, PHA+SP = PHA conjugated to 1.5 mM SPPEPS.

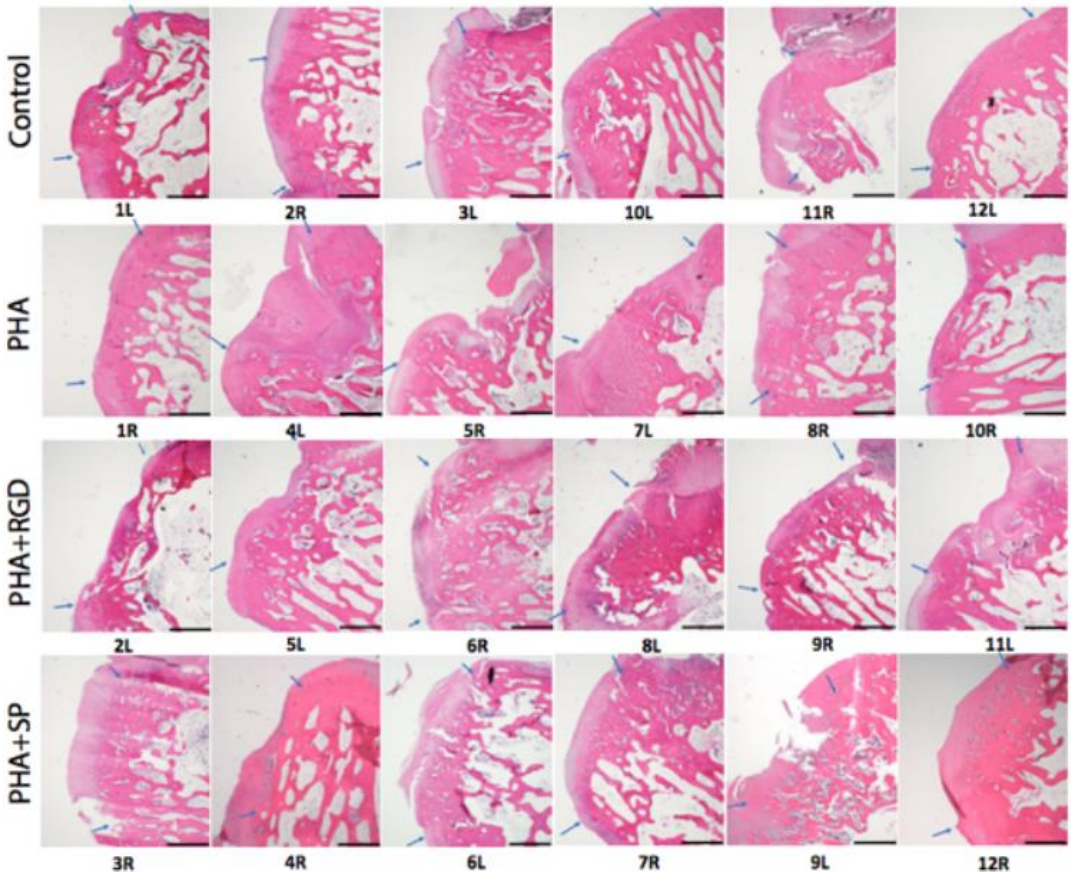

Figure 4.5. H&E Staining

Histological analysis of rabbit condyle defects at 12 weeks post-surgery (n=6). Sections were taken in the frontal plane and were stained with hematoxylin and eosin (H&E). The microscopic observations indicated the structural variability of the regenerated tissue within all the groups; however, we observed that incorporation of the peptides (PHA+SP and PHA+RGD groups) elevated the structural integrity compared to the PHA group. Control = The defect with no implanted material, PHA = 5% pentanoate-functionalized hyaluronic acid + 5% hyaluronic acid nanoparticles, PHA+RGD = PHA conjugated to 1.5 mM RGD, PHA+SP = PHA conjugated to 1.5 mM SPPEPS. The text under each staining represent rabbit number and knee side. (e.g., 1R = Rabbit #1, right knee) The arrows are pointed at the edges of the defects. Scale bars = 1 mm.

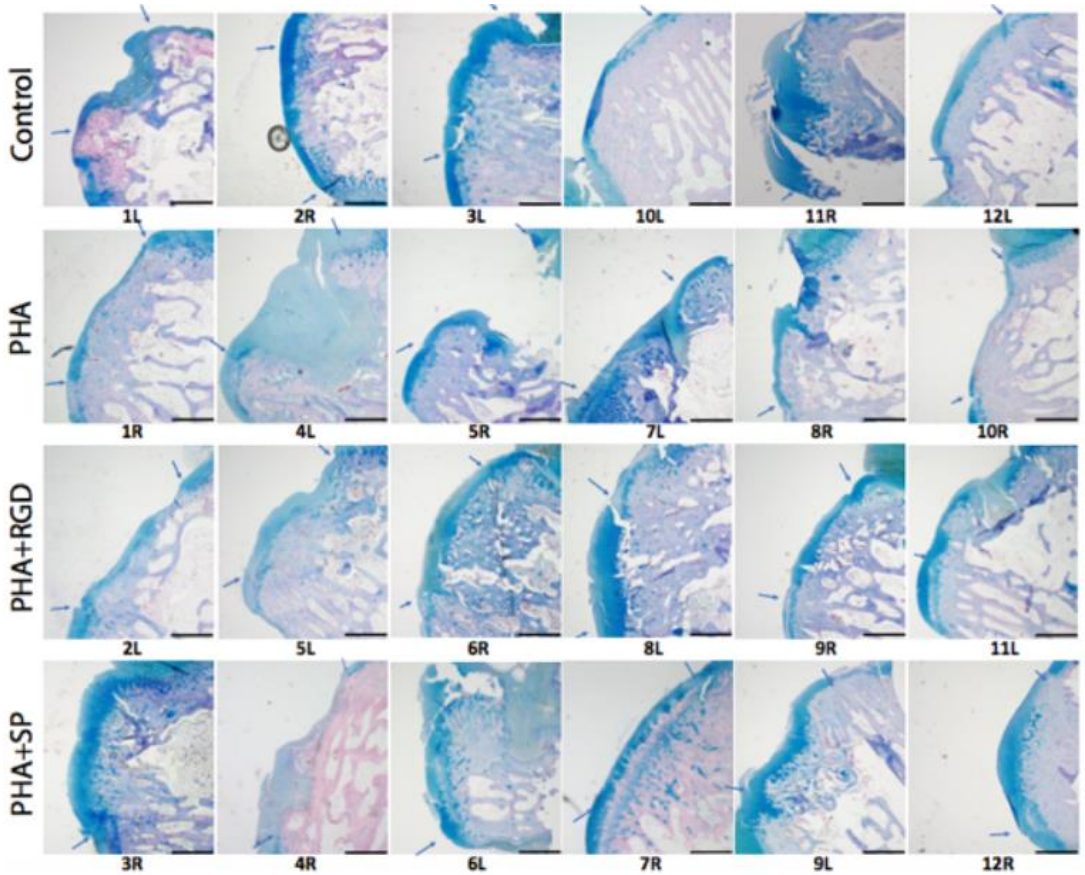

Figure 4.6. Alcian Blue Staining

Histological analysis of rabbit condyle defects at 12 weeks post-surgery (n=6). Sections were taken in the frontal plane and were stained with Alcian Blue. The stain intensities were superior in the control and PHA+SP group compared to the other groups. Control = The defect with no implanted material, PHA = 5% pentanoate-functionalized hyaluronic acid + 5% hyaluronic acid nanoparticles, PHA+RGD = PHA conjugated to 1.5 mM RGD, PHA+SP = PHA conjugated to 1.5 mM SPPEPS. The text under each staining represent rabbit number and knee side. (e.g., 1R = Rabbit #1, right knee). Arrows indicate the edges of the defects. Scale bars = 1 mm.

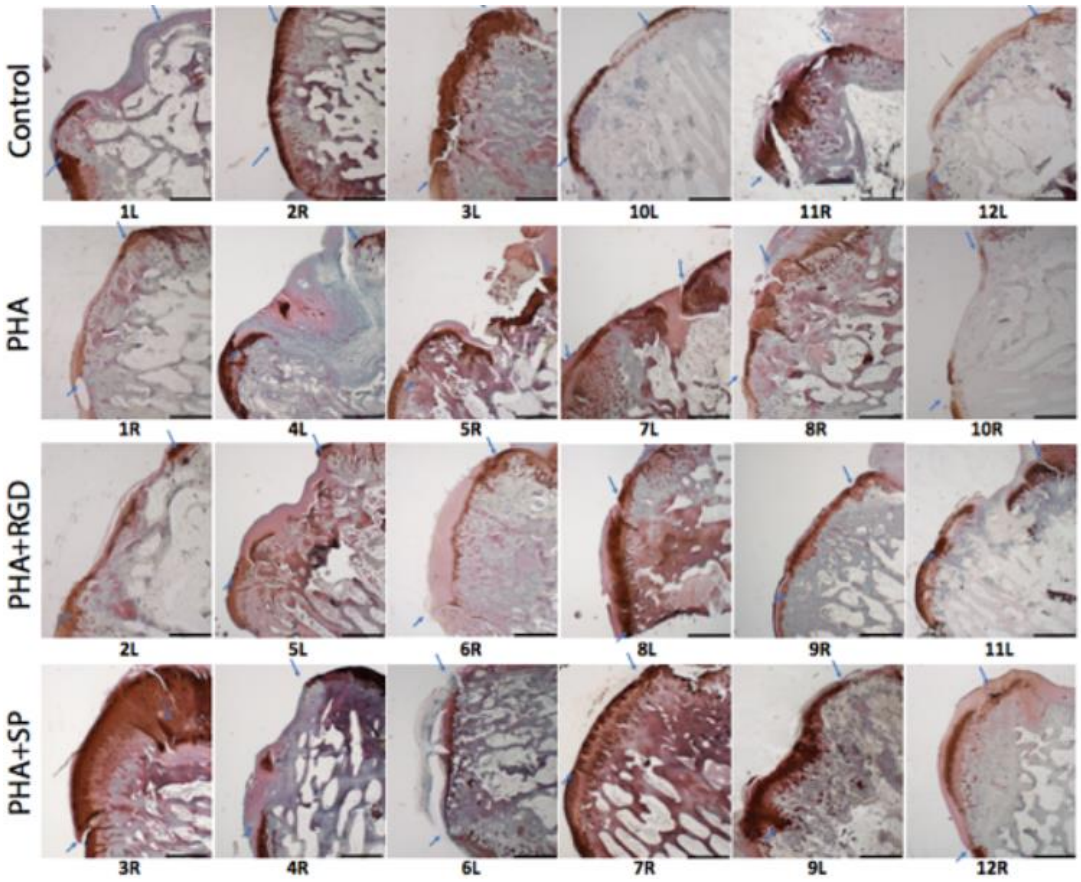

Figure 4.7. Collagen II Immunohistochemistry

Histological analysis of rabbit condyle defects at 12 weeks post-surgery (n=6). Sections were taken in the frontal plane and were stained with collagen type II antibody. 3R, 7R and 9L condyles from PHA+SP group and 2R condyles from Control showed the most intense and prevalent collagen II staining, which indicated the superior potential of the PHA+SP group in collagen type II production. Control = The defect with no implanted material, PHA = 5% pentanoate-functionalized hyaluronic acid + 5% hyaluronic acid nanoparticles, PHA+RGD = PHA conjugated to 1.5 mM RGD, PHA+SP = PHA conjugated to 1.5 mM SPPEPS. The text under each staining represent rabbit number and knee side. (e.g., 1R = Rabbit #1, right knee). The arrows are pointed at the edges of the defects. Scale bars = 1 mm.

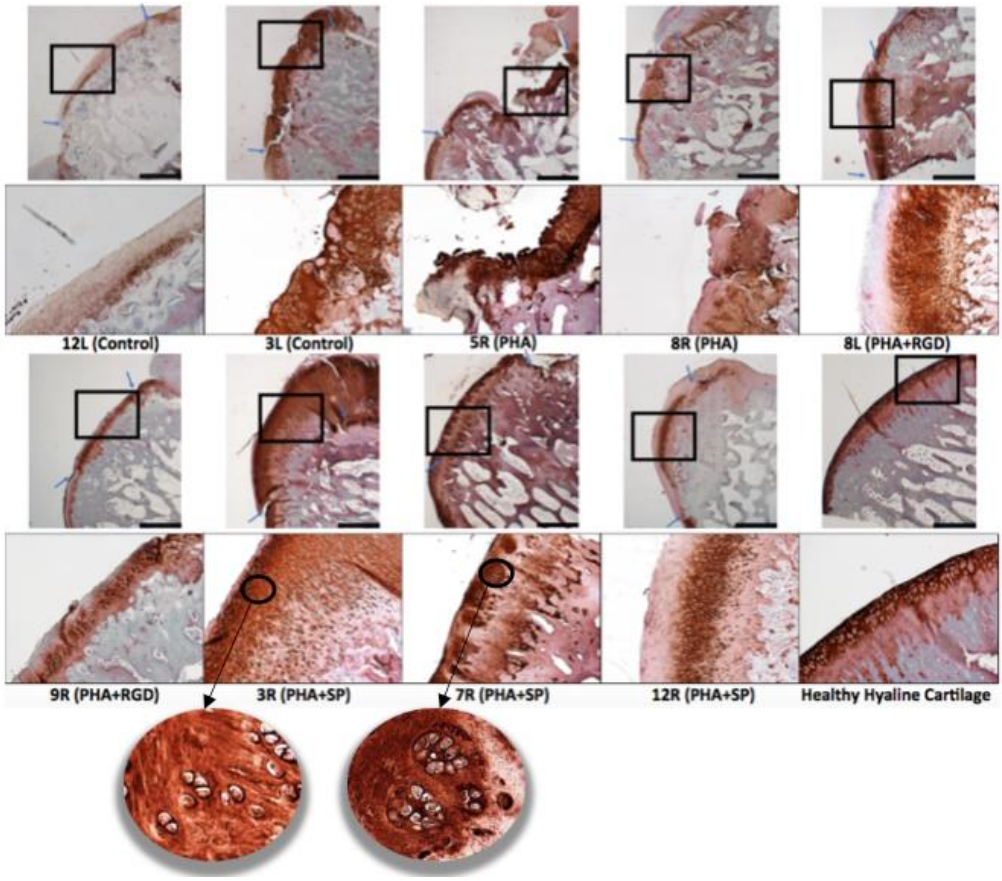

Figure 4.8: Representative Magnified Collagen II IHC

Histological analysis of rabbit condyle defects after 12 weeks post-surgery (n=6). Sections were taken in the frontal plane and were stained with collagen type II antibody. Magnified images shown below each overall section correlate to the box above. The magnified image of 3L condyle revealed the unstained cellular clusters. Magnified microscopic images of 3R and 7R condyles showed the chondrocyte formation in the cartilage zones, with staining being more intense around regions of chondrocyte-like cells in some areas. Control = The defect with no implanted material, PHA = 5% pentanoate-functionalized hyaluronic acid + 5% hyaluronic acid nanoparticles, PHA+RGD = PHA conjugated to 1.5 mM RGD, PHA+SP = PHA conjugated to 1.5 mM SPPEPS. The text under each staining represent rabbit number and knee side. (e.g., 1R = Rabbit one, right knee) The arrows are pointed at the edges of the defects. Scale bars = 1 mm.

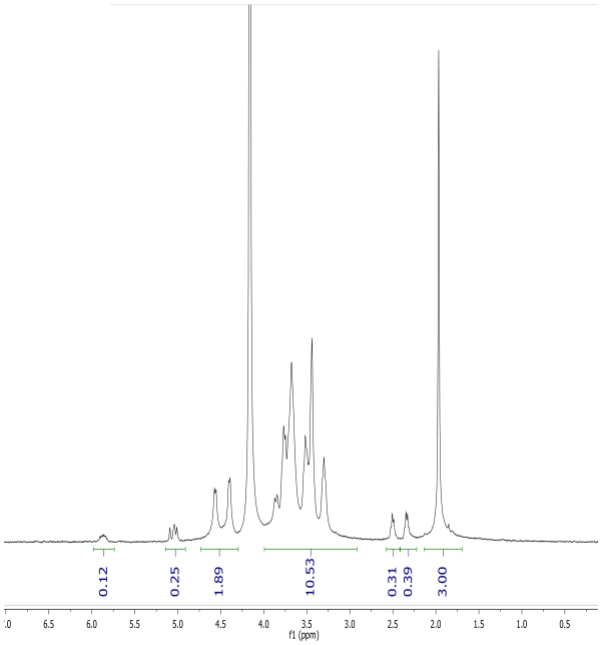

Figure 4.9. H ^{1}NMR Analysis of 60 kDa PHA

Appendix B: Tables

Chapter 1: No Tables

Chapter 2: Table 2.1

Chapter 3: Table 3.1

Chapter 4: Table 4.1-4.3

Chapter 5: No Tables

Table 2.1 Integrin-Binding Peptides identified for cancer therapy applications

Discovery Method	Receptor	Sequence	Application
OBOC	$\alpha_4\beta_1$	LTGpLDI[135]	Leukemia
	$\alpha_3\beta_1$	cdGLGBNc[136] (LXYI)	Breast cancer
Panning *in vitro*	$\alpha_v\beta_3$	CQQSNRGDRKRC[137](RMS-I)	Rhabdo-myosarcoma cancer
	$\alpha_v\beta_6$	RGDLATLRQLAQEDGVVGVR[138]	Lung cancer
	$\alpha_4\beta_1$	CPLDIDFYC[139] (pIII)	Leukemia
Panning *in vivo*	$\alpha_3\beta_1$	SWKLPPS[140]	Gastric Cancer
	$\alpha_v\beta_3$	CRGDKGPDC[141]	Prostate Cancer
Phage display library	$\alpha_v\beta_6$	RTDLDSLRTYTL[142]	Ovarian cancer
	$\alpha_v\beta_3$	CDCRGDCFC [143, 144]	Breast Cancer

Table 3.1: List of Synthesized Peptides

Peptide	Sequence	MW	Description
1	GRGDSP	587.59	Adhesion peptide
2	GCGYGRGDSPG	1025.06	RGD with spacer
3	SPPEPS	612.63	Chondroinductive peptide
4	GCGYGSPPEPS	1050.10	SPPEPS with spacer
5	GCGYGPSEPSP	1050.10	Scrambled SPPEPS with spacer
6	DHLSDNYTLDHDRAIH	1921.99	Link N sequence

Table 4.1 List of Group Placement and Outcome Analysis for Each Rabbit Knee

Animal no	Left Knee	Right Knee	Time (Weeks)	Analysis
Rabbit 1	Control	PHA	12	Morphology and histology
Rabbit 2	PHA+RGD	Control	12	Morphology and histology
Rabbit 3	Control	PHA+SP	12	Morphology and histology
Rabbit 4	PHA	PHA+SP	12	Morphology and histology
Rabbit 5	PHA+RGD	PHA	12	Morphology and histology
Rabbit 6	PHA+SP	PHA+RGD	12	Morphology and histology
Rabbit 7	PHA	PHA+SP	12	Morphology and histology
Rabbit 8	PHA+RGD	PHA	12	Morphology and histology
Rabbit 9	PHA+SP	PHA+RGD	12	Morphology and histology
Rabbit 10	Control	PHA	12	Morphology and histology
Rabbit 11	PHA+RGD	Control	12	Morphology and histology
Rabbit 12	Control	PHA+SP	12	Morphology and histology

Table 4.2: Scoring Table for Morphological Analysis

Feature	Score
Repair tissue or test article present in implant site	
Full presence	2
Partial	1
None	0
Edge integration (New tissue relative to native artilage)	
Full	2
Partial	1
None	0
Smoothness of repair surface	
Smooth	2
Intermediate	1
Rough/Missing	0
Cartilage surface degree of filling	
Flush	2
Slight depression	1
Depressed/Overgrown	0
Color of cartilage (opacity/translucency of repair issue)	
Translucent	2
Opaque	1
Missing	1
Amount of repair tissue relative to total area of defect (estimated)	% present in defect.

Table 4.3: Histology Grading System

Feature	Feature
Cellular morphology	
Hyaline cartilage	4
Mostly hyaline cartilage	3
Mixed hyaline and fibrocartilage	2
Mostly fibrocartilage	1
Some fibrocartilage and mostly nonchondrocytic cells	0
Staining	
Normal to nearly normal	3
Moderate	2
Slight	1
None	0
Cartilage thickness	
Similar to the surrounding cartilage	3
Greater than the surrounding cartilage	2
Less than the surrounding cartilage	1
No cartilage	0
Reconstruction of subchondral bone	
Normal	3
Reduced subchondral bone reconstruction	2
Minimal subchondral bone reconstruction	1
No subchondral bone reconstruction	0
Edge integration	
Bonded at both ends of graft	2
Bonded at 1 end or partially at both ends	1
Not bonded	0