Regulation of Human Nucleus Pulposus Cell Phenotype and Behavior by Laminin-Mimetic Peptide Substrates

by

Devin Thomas Bridgen

Department of Biomedical Engineering
Duke University

Date: ______________________

Approved:

___________________________
Lori A. Setton, Supervisor

___________________________
Jun Chen

___________________________
Farshid Guilak

___________________________
Brenton D. Hoffman

___________________________
George A. Truskey

Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Biomedical Engineering in the Graduate School of Duke University

2015
ABSTRACT

Regulation of Human Nucleus Pulposus Cell Phenotype and Behavior by Laminin-Mimetic Peptide Substrates

by

Devin Thomas Bridgen

Department of Biomedical Engineering
Duke University

Date:____________________

Approved:

___________________________
Lori A. Setton, Supervisor

___________________________
Jun Chen

___________________________
Farshid Guilak

___________________________
Brenton D. Hoffman

___________________________
George A. Truskey

An abstract of a dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Biomedical Engineering in the Graduate School of Duke University

2015
Abstract

Intervertebral disc (IVD) disorders can cause pain and disability for affected individuals. A subset of IVD disorders are thought to originate in the nucleus pulposus (NP) region of the IVD, due to alterations in tissue structure and composition with age and injury. Cells of the NP undergo a phenotypic and behavioral shift with age, changes that are thought to disrupt tissue homeostasis and lead to disc degeneration. There is significant interest in developing biomaterials and strategies to revert adult degenerate NP cells to healthy, young NP cell phenotypes with increased biosynthesis and cell clustering. Studies demonstrate that healthy porcine NP cells interact with laminin proteins through specific integrin subunits on soft substrates in a process that regulates cell morphology and biosynthesis. The central hypothesis of this dissertation is that the engagement of cell-surface adhesion receptors, using laminin-mimetic peptides on a controlled stiffness material, can revert adult degenerate NP cellular phenotype and behaviors to their healthy, biosynthetically active form.

In the first part of this dissertation, integrin subunits used by adult degenerate human NP cells to attach to laminin were revealed using flow cytometric analyses, function blocking antibodies, and immunohistochemistry. Secondly, NP cell recognition peptides were identified by screening laminin-mimetic peptides for cell attachment. Finally, human NP cells were cultured on peptide functionalized polyacrylamide gels to
examine the effect of ligand and substrate stiffness in regulating adult human NP cell phenotype and biosynthesis.

Findings reveal that adult human NP cells express and utilize integrin subunits α3, α5, and β1 to attach to laminins, in contrast to integrin α6β1 found previously for healthy porcine NP cells. This difference between current and previous findings likely arises from aging-associated difference in NP cells between human and porcine tissues. Select laminin-mimetic peptides, chosen from the literature and informed by NP cell integrin expression, were found to promote significant NP cell attachment in a concentration dependent manner. Finally, a subset of laminin mimetic peptides were found to promote a young NP cell phenotype by increasing cell clustering on soft (0.3 kPa) and stiff (14 kPa) substrates as well as increasing proteoglycan synthesis on soft substrates.

The studies presented in this dissertation demonstrate that adult degenerate human NP cells attach to laminin proteins in an integrin dependent manner. Furthermore, laminin-mimetic peptides are able to mediate human NP cell attachment at levels comparable to full-length laminin, increase cell clustering when presented on soft and stiff substrates, and can increase NP cell biosynthesis when presented on soft substrates. Utilizing laminin-mimetic peptides may allow for the design of biomaterials that promote a healthy young NP phenotype for a variety of disc therapies.
To my father, Mark, and my mother, Margot
# Contents

Abstract .......................................................................................................................... iv

List of Tables ...................................................................................................................... xi

List of Figures .................................................................................................................... xii

Acknowledgements ......................................................................................................... xvi

1. Introduction .................................................................................................................. 1
   1.1 Motivation .................................................................................................................. 4
   1.2 IVD Structure, Degeneration, and the Role of NP Cells ............................................. 5
   1.3 Role of Laminins in Regulating Cell Behaviors .......................................................... 9
   1.4 Role of Matrix Elasticity in Regulating Cell Behaviors .............................................. 12

2. Human Nucleus Pulposus Cell Adhesion and Integrin-Mediated Interactions with Extracellular Matrix Proteins ................................................................. 16
   2.1 Introduction ................................................................................................................. 16
   2.2 Methods and Materials ............................................................................................... 19
      2.2.1 Cell Isolation and Culture .................................................................................... 19
      2.2.2 Flow Cytometry ................................................................................................... 20
      2.2.3 Integrin Blocking Assays ..................................................................................... 22
      2.2.4 Immunohistochemical Detection of Integrins in IVD Tissue ................................. 24
      2.2.5 Statistical Analyses .............................................................................................. 25
   2.3 Results ....................................................................................................................... 25
      2.3.1 Flow Cytometry ................................................................................................... 25
      2.3.2 Integrin Blocking Assays..................................................................................... 27
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.3.3</td>
<td>Immunohistochemistry</td>
<td>32</td>
</tr>
<tr>
<td>2.4</td>
<td>Discussion</td>
<td>33</td>
</tr>
<tr>
<td>2.5</td>
<td>Conclusions</td>
<td>39</td>
</tr>
<tr>
<td>3.</td>
<td>Human Nucleus Pulposus Cell Adhesion to Laminin-Mimetic Peptides</td>
<td>42</td>
</tr>
<tr>
<td>3.1</td>
<td>Introduction</td>
<td>42</td>
</tr>
<tr>
<td>3.1.1</td>
<td>Peptide Selection</td>
<td>44</td>
</tr>
<tr>
<td>3.2</td>
<td>Methods</td>
<td>48</td>
</tr>
<tr>
<td>3.2.1</td>
<td>Cell Isolation and Culture</td>
<td>48</td>
</tr>
<tr>
<td>3.2.2</td>
<td>Cell Attachment Quantification Assay</td>
<td>49</td>
</tr>
<tr>
<td>3.2.3</td>
<td>Statistical Analyses</td>
<td>50</td>
</tr>
<tr>
<td>3.3</td>
<td>Results</td>
<td>50</td>
</tr>
<tr>
<td>3.3.1</td>
<td>Cell Attachment Quantification</td>
<td>50</td>
</tr>
<tr>
<td>3.4</td>
<td>Discussion</td>
<td>53</td>
</tr>
<tr>
<td>3.5</td>
<td>Conclusion</td>
<td>58</td>
</tr>
<tr>
<td>4.</td>
<td>Regulation of Human Nucleus Pulposus Cell Phenotype and Behavior by Laminin-Mimetic Peptide Functionalized Substrates</td>
<td>60</td>
</tr>
<tr>
<td>4.1</td>
<td>Introduction</td>
<td>60</td>
</tr>
<tr>
<td>4.1.1</td>
<td>Studies of Matrix Elasticity with Polyacrylamide Gel Substrates</td>
<td>60</td>
</tr>
<tr>
<td>4.1.2</td>
<td>Laminin Mimetic Peptide Selection</td>
<td>61</td>
</tr>
<tr>
<td>4.1.3</td>
<td>Young, Healthy Nucleus Pulposus Markers</td>
<td>63</td>
</tr>
<tr>
<td>4.1.4</td>
<td>Summary</td>
<td>65</td>
</tr>
<tr>
<td>4.2</td>
<td>Methods</td>
<td>66</td>
</tr>
</tbody>
</table>
4.2.1 Cell Isolation and Culture ................................................................. 66
4.2.2 Polyacrylamide Gel Substrates .......................................................... 66
4.2.3 Mechanical Characterization of Gel Substrates .................................. 69
4.2.4 Characterization of Peptide-Polyacrylamide Conjugation ..................... 70
4.2.5 Analysis of NP Cell Clustering ............................................................. 72
4.2.6 NP Cell Clustering Optimization ......................................................... 74
4.2.7 Sulfated Glycosaminoglycan Production ........................................... 74
4.2.8 mRNA Extraction and Quantification .................................................. 75
4.3 Results ................................................................................................. 77
4.3.1 Mechanical Characterization of Gel Substrates .................................. 77
4.3.2 Characterization of Peptide-Polyacrylamide Conjugation ..................... 81
4.3.3 NP Cell Clustering Optimization ......................................................... 84
4.3.4 Analysis of NP Cell Attachment and Clustering ................................. 87
4.3.5 Sulfated Glycosaminoglycan Production ........................................... 92
4.3.6 mRNA Quantification ......................................................................... 92
4.4 Discussion .......................................................................................... 95
4.4.1 Polyacrylamide Gel Methods .............................................................. 95
4.4.2 Characterization of Polyacrylamide Gel Substrates .............................. 98
4.4.3 NP Cell Behavior on LM-Mimetic Substrates ..................................... 101
4.4.4 Porcine Versus Human Cell Attachment ........................................... 103
4.4.5 Summary ......................................................................................... 104
4.5 Conclusion ......................................................................................... 105
List of Tables

Table 1: Monoclonal antibodies for human integrin subunits and IgG controls used in immunohistochemical staining, flow cytometry analysis, and function blocking experiments..................................................................................................................22

Table 2: Peptides screened for cell attachment ..................................................................................45
List of Figures

Figure 1: Organization of the intervertebral disc, with staining for AF (actin) and NP (vimentin) cells

Figure 2: Photograph of a 12 year old intervertebral disc (IVD) (a) and magnetic resonance images of human IVDs at increasing stages of disc degeneration (b-d). Modified from Chen et al. and Smith et al. [5, 22]

Figure 3: Immunostaining for laminin α5 (top) and γ1 (bottom) chains in young rat NP (left) and AF (right) tissues. Green = laminin, red = cell nuclei. Modified from Chen et al., [5]

Figure 4: Vacuolated morphology of immature porcine NP cells attached to LM-111 (A), or attached to LM-111 after blocking adhesion receptor integrin α6. Note that blocking integrin α6 selectively inhibits attachment of the larger vacuolated cells

Figure 5: Schematic of LM-111 structure adapted from Miner et al., 2004

Figure 6: Immature porcine NP cells grown for seven days on soft 210 Pa polyacrylamide gels functionalized with either laminin-rich basement membrane extract (BME)(top) or collagen type II (bottom). Adapted from Gilchrist et al., 2011

Figure 7: Immature porcine NP cells grown for seven days on either soft laminin-rich basement membrane extract (BME) gels (top) or stiff BME-coated tissue culture plastic (bottom). Adapted from Gilchrist et al., 2011

Figure 8: Immature porcine NP cells grown on a soft gel produced 1.7 times more sGAG over a 12 day period. Adapted from Gilchrist et al., 2011

Figure 9: Flow cytometric analysis of integrin expression for human NP cells: (a) Mean ± SE of mean fluorescence intensity (MFI) averaged across all samples for each anti-integrin antibody. Data are normalized by values for β1 integrin expression on a per-sample basis; and (b) mean ± SE of the percentage of cells positive for each integrin subunit averaged across all samples for each anti-integrin antibody. Conditions labeled with different letters are significantly different from each other and from the IgG controls (ANOVA, p < 0.05)
Figure 10: Cell attachment to different protein coatings compared to the BSA control. Conditions labeled with different letters are significantly different from each other (ANOVA, p < 0.05).

Figure 11: The effect of integrin function-blocking antibodies on NP cell attachment to (a) LM-111 or (b) LM-511 normalized to the IgG control (mean ± SE). Conditions labeled with different letters are significantly different from each other (ANOVA, p < 0.05).

Figure 12: The effect of integrin function-blocking antibodies on NP cell attachment to type II collagen normalized to the IgG control (mean ± SE). Conditions labeled with different letters are significantly different from each other (ANOVA, p < 0.05).

Figure 13: The effect of 10 µM EDTA on cell attachment to LM-111, LM-511, or type II collagen (mean ± SE). Conditions labeled with different letters are significantly different from each other (two-way ANOVA, p < 0.05).

Figure 14: Representative images of immunostaining adult human tissues showing the pattern for positive labeling with antibodies against integrin subunits β1, α3, and α5. Green = antibody reactivity and red = propidium iodide counterstain.

Figure 15: Concentration dependent attachment curves for integrin α3 binding peptides (a), integrin α6 binding peptides (b), and other LM-mimetic peptides (c). BSA is used as a negative control in all graphs, while LM-111 at 20 µg/mL and LM-511 at 10 µg/mL are used as positive controls. Error bars removed for clarity.

Figure 16: Percent cell attachment (± SE) for all ligands, with peptides at 200 µg/mL. Bars with letters are significantly different from BSA and each other (p < 0.05).

Figure 17: Blocking cell attachment to peptide AG73, plated at 100 µg/mL, with either 10 µg/mL EDTA or 50 µg/mL heparin (Sigma). Cells from a 59 year-old patient were allowed to attach, in triplicate, for 1 hour.

Figure 18: Modified from Nomizu, et al. (1995) [95]. Effect of integrin blocking on rhabdomyosarcoma cell spreading on AG10, AG32, AG73, and LM-111 substrates. Blocking conditions include unblocked (a), anti-integrin β1 (b), anti-integrin α2 (c), anti-integrin α3 (d), anti-integrin α6 (e), and IgG (f).

Figure 19: Diagram showing the assembly of the media chamber and polyacrylamide gel into a culture chamber.
Figure 20: Representative AFM data from an 8% acrylamide, 0.15% bis acrylamide gel probed 36 times in a 50 µm x 50 µm grid. Force-indentation curves (blue dotted lines) closely correlate to the Hertz spherical indentation model (red line) with an R² of 0.9981 (A). At the same gel location, a surface height and stiffness map is shown (B)..............78

Figure 21: The mean elastic moduli (± SD) of polyacrylamide gel substrates for gels with 8% or 5% acrylamide and 0.15%, 0.1%, 0.04%, or 0.03% bis-acrylamide. Conditions not connected by the same letters are significantly different from each other (p<0.05, ANOVA, Tukey’s HSD post-hoc)........................................................................................................79

Figure 22: The mean elastic moduli (± SD) of gels with 8% or 5% acrylamide and 0.15%, 0.1%, or 0.03% bis-acrylamide from two different pre-gel solution batches (A and B). No detectable differences were found between batches (p>0.05, 2-way ANOVA)...............80

Figure 23: Variation in surface height of different polyacrylamide gel formulations (± SD). Conditions not connected by the same letters are significantly different from each other (p<0.01, ANOVA, Tukey’s HSD post-hoc).................................................................................................81

Figure 24: Amount of peptide (µg) conjugated to 0.3 kPa and 14 kPa polyacrylamide gels for varying amounts of applied peptide (0, 0.01, 0.05, and 0.1 mM). There is no detectable difference in conjugated peptide between 0.3 kPa and 14 kPa gels (p>0.5, 2-way ANOVA, Tukey’s HSD post-hoc)...........................................................................................................82

Figure 25: Representative confocal z-stack images of fluorescently labeled peptides conjugated to a 14 kPa gel. Peptides (green, FITC) and cells (red, CMV-mCherry reporter) are shown in relation to each other, superimposed over a scaled diagram of the polyacrylamide gel (A). A three-dimensional isometric confocal image illustrates the even distribution of peptides throughout the gel (B). .................................................................83

Figure 26: Representative images visualizing the effect of cell seeding density on human NP cell attachment and clustering. Green = F-actin (phalloidin) and blue = cell nuclei (propidium iodide counterstain). ........................................................................................................85

Figure 27: The effect of cell seeding density on (A) percent cell attachment to soft and stiff LM-111 functionalized substrates and (B) cell cluster size on a 0.3 kPa LM-111 substrate. ..............................................................................................................86

Figure 28: Day 4 cell attachment as a percent of initially seeded cells. Peptides P4 and AG10 at 14 kPa have a significant decrease in cell attachment relative to LM-111 at 14 kPa (p<0.01, 2-way ANOVA, Dunnett’s post hoc).................................................................................88
Figure 29: Day 4 cell clustering. Percent of cells in clusters on each substrate (A) and percent of cells in a cluster with >9 cells (B). Conditions with * have a significant increase compared to 14 kPa LM-111 (p<0.05, 2-way ANOVA (ligand, stiffness), Dunnett’s post hoc). ................................................................. 89

Figure 30: Representative day 4 images showing the effect of substrate stiffness and ligand for NP cell morphologies. Over 50% of attached cells interact to form multi-cell clusters on all substrates except 14 kPa LM-111 and 0.3 kPa AG73. Spread cells with stress fibers were only present on stiff LM-111 substrates. Green = F-actin (phalloidin) and blue = cell nuclei (propidum iodide counterstain). ................................................................. 91

Figure 31: Day 4 NP cell sGAG production normalized to DNA content. Substrates 0.3 kPa p678 and 0.3 kPa AG10 produce a significant increase in sGAG/DNA compared to 14 kPa LM-111 (p<0.05, 2-way ANOVA, Dunnett’s post hoc). ................................................................. 92

Figure 32: Gene level expression (±SE) of aggrecan (A), n-cadherin (B), type I collagen (C), type II collagen (D), and GLUT-1 (E) for each substrate as determined by qRT-PCR. Significance compared to 14 kPa LM-111 is denoted by * (p<0.05) and # (p<0.1) (2-way ANOVA, Dunnett’s post hoc) ................................................................. 94

Figure 33: Schematic of polyacrylamide gel chemistry using the crosslinker N2 to introduce amine-reactive NHS groups. ........................................................................................................ 97

Figure 34: Percent attachment of human and porcine NP cells to 14 kPa polyacrylamide gels functionalized with LM-111, AG73, P4, p678, or AG10. Conditions not connected by the same letters are significantly different from each other (p<0.05, ANOVA, Tukey’s HSD post-hoc) ........................................................................ 104

Figure 35: A heat map comparing the findings of this chapter for the peptide substrates, with color scaling performed across all substrates within each tested variable. Percent of seeded cells attached to each substrate, percent of attached cells in a cluster, and percent of attached cells in a large cluster (>9 cells) are shown in (A). sGAG biosynthesis and gene expression of aggrecan, n-cadherin, type I collagen, type II collagen, and GLUT-1 as a fold change relative to 14 kPa LM-111 are shown in (B). ........................................................................ 105
Acknowledgements

I want to acknowledge and thank the many people who have contributed to the work presented in this dissertation, as well as to my development as a scientist and engineer. First and foremost, I would like to thank my advisor, Dr. Lori Setton, for the opportunity to work at Duke and for her guidance these past six years. Dr. Setton has trained me to be a better scientist, to intelligently test hypotheses, and to critically analyze problems. She trained me to communicate scientific knowledge in a clear and impactful manner for a variety of audiences. I would also like to thank my committee members Dr. Jun Chen, Dr. Farsh Guilak, Dr. Brent Hoffman, and Dr. George Truskey for all of their positive feedback and valuable recommendations.

The work presented here would not be possible without the support of several collaborators. I’d like to acknowledge several orthopaedic surgeons, including Dr. Bill Richardson, Dr. Rob Isaacs, Dr. Chris Brown, and Dr. Melissa Erickson for their support providing tissue samples. Dr. Johannah Sanchez-Adams was essential for performing atomic force microscopy with me. Dr. David Alcorta was patient and informative as he taught me the basics of molecular biology and viral reporter constructs.

I want to acknowledge all of the Setton Lab members who have been colleagues and friends. Dr. Chris Gilchrist laid the groundwork for the hypotheses in this dissertation, and his support when I first arrived at Duke allowed me to hit the ground running. Liufang Jing was always willing to lend a hand, an ear, and share her amazing
dumplings. I had the pleasure of mentoring Pratt Fellow Kristie Yang, who contributed to multiple areas of study and was a source of eternal energy. Senior lab members Dr. Aubrey Francisco and Dr. Michael Sinclair introduced me to Duke BME, provided invaluable scientific and life advice, and always kept lab exciting. Timothy Mwangi has been a close friend and colleague, and I cherish our many memories from these past 6 years together. Thanks to all the lab members who have provided valuable input during our lab meetings, including Dr. Chris Gilchrist, Dr. Aubrey Francisco, Dr. Michael Sinclair, Dr. Timothy Mwangi, Dr. Priscilla Hwang, Elizabeth Leimer, Dr. Kyle Allen, Dr. Robby Bowles, Dr. Bailey Fearing, Richard Bell, Liufang Jing, and Dr. Jun Chen.

Finally, thanks to all the personal relationships that have made the past six years amazing. Thanks to my family, their love and support has been a rock throughout my PhD. Thanks to my amazing girlfriend Brittany, you have filled the past three years with smiles, joy, and adventure, and I can’t wait to see where we end up next! And of course, thanks to my fellow graduate students at Duke for their friendship, support, and constant entertainment. You have made my time here at Duke unforgettable, and I deeply treasure our friendships.
1. Introduction

The intervertebral disc (IVD) is a heterogeneous structure that provides load support and flexibility in the spine. The IVD is composed of the outer anulus fibrosus (AF) and the inner nucleus pulposus (NP) tissues. The NP is a soft, gelatinous, and highly hydrated material that is composed largely of glycosaminoglycans (GAGs), collagen proteins, and water [1]. The NP tissue is sparsely populated by large, vacuolated cells that are biosynthetically active in generating the NP extracellular matrix (ECM) during development and growth. The IVD tissue degenerates with age and maturation, presenting as a decrease in disc height, increase in stiffness, decreased and altered GAG composition, decreased water content, and reduced cell viability [2-4]. The protein laminin (LM) is of particular interest for its unique expressing in the NP region of the disc and decreasing presence with age and degeneration [5, 6]. The NP cell phenotype becomes more fibroblast-like as early as the first decade of life, with changes that are thought to disrupt the maintenance of tissue homeostasis and contribute to changes in the ECM and eventually disc degeneration [7-9].

The composition and organization of the ECM environment of the NP provides critical cues to the resident NP cells through presentation of ECM binding partners, regulation of physical properties, and regulation of nutrient supply and waste transport. ECM characteristics such as substrate stiffness and ligand presentation have been shown to guide cell behavior and survival in multiple cell types, and including NP cells.
prior studies of ECM factors that regulate maintenance of young healthy NP cells, results showed that young, porcine NP cells interact with laminin ECM proteins through specific integrin subunits [5, 6, 10-12]. In particular, these results revealed that young healthy NP cells make use of the α6 integrin subunit and its binding partners to attach to laminin in a process that regulates cell morphology and sulfated GAG biosynthesis. In separate studies, the interaction with laminin is further regulated by the stiffness of the substrate, as observed for laminin-coated polyacrylamide substrates [12]. In that work, it was shown that laminin promoted cell attachment and cell clustering for young NP cells as well as elevated GAG synthesis, but only when laminin was adhered to substrates of stiffness less than 720 Pa. Thus, it is these interactions with both laminin ligands and substrate that appear to be capable of promoting a healthy and biosynthetically active NP phenotype.

These prior studies of cell-ECM interactions for the NP motivate the current work to develop a peptide functionalized biomaterial that is capable of engaging NP cell-receptors and regulating cell morphology and biosynthesis. Cell recognition peptides are able to provide portions of the functionality of full-length proteins, and are chosen for study here because of their increased specificity and stability. While previous work focused on NP-ECM interaction in the healthy young NP cell, little is known how adult degenerate human NP cells attach and interact with key ECM proteins such as laminins. Identification of the cell-surface adhesion receptors used by adult human NP
cells is a prerequisite to developing peptide functionalized biomaterials targeting NP-specific cell receptors.

The overarching hypothesis of this dissertation is that a young NP cell phenotype can be promoted in adult degenerate human NP cells by mimicking the ligand and elastic properties of the young NP tissue in a biomaterial scaffold. Our central hypothesis is that the engagement of cell-surface adhesion receptors, using ECM-mimetic peptides in a soft biomaterial, regulates NP cellular phenotype and biosynthesis. We hypothesize that human NP cells use integrins to bind to the ECM: engaging these cell-surface adhesion receptors with specific peptides on soft surfaces will promote healthy, young, and biosynthetically active NP phenotype. Additionally, we hypothesize that peptide specificity and substrate stiffness will influence biosynthesis and the expression of NP-specific molecular markers.

This dissertation first identifies the integrin subunits used by adult, human NP cells to attach to key ECM proteins. Using primary adult degenerate human NP cells, we identified integrin subunits α3, α5, and β1 as key cell-surface adhesion receptors that NP cells express and use to attach to the ECM ligand, laminin. Second, this work demonstrated that cells from adult degenerate human NP cells are able to attach to select laminin-mimetic peptides, including peptides reported to bind to either integrin α3 or integrin α6. Third, peptides promoting maximal cell attachment were incorporated into soft (0.4 kPa) and stiff (14 kPa) polyacrylamide gels to identify substrate conditions
that promote a younger biosynthetically active NP phenotype for NP cells from adult degenerate tissues. The findings of these studies demonstrate that both ligand and substrate stiffness impact adult human NP behavior, including sGAG biosynthesis, cell clustering, and NP marker expression. Furthermore, the ligands identified in these studies may be useful for developing tissue-mimetic cell-based therapies for regenerating the NP.

1.1 Motivation

In the United States, between 50% and 75% of the population will experience back pain or disability at some point in their lives, costing the US over $100 billion per year in 2005 [13, 14]. The intervertebral disc (IVD) degenerates with age, leading to conditions such as disc herniations, spinal stenosis, and degenerative spondylolisthesis, accounting for 47% of the hospital visits for patients with back problems [13, 14]. Disc degeneration is thought to stem from changes in the nucleus pulposus (NP) region of the disc, characterized by a loss of water content, the formation of fissures, and decreased disc height [14]. Current surgical intervention to treat degenerative disc disease includes discectomy and spinal fusion, but these procedures reduce range of motion and can accelerate disc degeneration on adjacent discs [15]. Tissue engineering strategies are a promising approach to regenerating the IVD, but further information is needed to formulate a functional tissue [9]. The goal of this proposal is to determine the impact of the extracellular matrix (ECM) environment, specifically ligand presentation and
substrate elasticity, on NP cell behavior. These data will be used to develop a peptide-functionalized biomaterial designed to promote a healthy young NP cell phenotype, with increased ECM production and cell clustering, as an approach to generating functional NP tissue.

1.2 IVD Structure, Degeneration, and the Role of NP Cells

The IVD, composed of the outer anulus fibrosus (AF) and the inner NP tissues (Figure 1), consists of a dense ECM rich in collagens, proteoglycans, and non-collagenous molecules [2, 14]. The AF is a stiff tissue consisting of concentric fibrocartilage lamellae, which serves to contain the NP and increase force dissipation [16]. In contrast, the inner NP is a gelatinous region containing a high concentration of water (60-90% by weight), a variety of proteins, and a diverse population of glycosaminoglycans (GAGs) that contribute to a high interstitial swelling pressure [1, 17]. GAG content is one of the defining properties of NP tissue, with a GAG to collagen (hydroxyproline) ratio of 27:1, compared to only 2:1 in hyaline cartilage [18, 19]. This ECM provides a host of mechanical and biochemical signals to the resident NP cells that may promote cell survival, ECM production, and regulate cell morphology and differentiation [2, 14].
Figure 1: Organization of the intervertebral disc, with staining for AF (actin) and NP (vimentin) cells

With aging and disc degeneration, the human NP undergoes changes characterized by a decrease in cell viability, water content, viscoelasticity, proteoglycan content, and a shift in cell phenotype, all of which contribute to a loss of disc height and nucleus pressurization [2-4, 20-22]. The decrease in disc height, decrease in water content, and development of tissue fissures is visualized in Figure 2 [22]. Cell death, and the inability for cells to repair damaged ECM, disrupts tissue homeostasis, leading to IVD degeneration. Degeneration of the ECM can then, in turn, accelerate cell death and decrease ECM production, creating further degradation of the NP. By maintaining healthy cells, using ligand and substrate elasticity cues from the ECM, we hope to halt further degradation and increase ECM biosynthesis.
Figure 2: Photograph of a 12 year old intervertebral disc (IVD) (a) and magnetic resonance images of human IVDs at increasing stages of disc degeneration (b-d). Modified from Chen et al. and Smith et al. [5, 22]

Healthy young NP cells produce a distinct ECM compared to older and degenerate NP cells, and this specific ECM may be necessary for functional NP tissue [5, 23]. Prior studies have identified multiple laminin isoforms present in the NP but not the AF of animal and young human IVD tissues (Figure 3) [5, 6, 10]. Unique interactions between NP cells and LM proteins, further discussed in Chapters 1.3 and 2, highlight laminins as a potential basis for therapeutic biomaterials.
Figure 3: Immunostaining for laminin α5 (top) and γ1 (bottom) chains in young rat NP (left) and AF (right) tissues. Green = laminin, red = cell nuclei. Modified from Chen et al., [5]

Healthy young NP cells are highly vacuolated, with a large diameter between 20 and 50μm (Figure 4a), and organized in cell clusters throughout the NP tissue [24, 25]. NP cell clusters have been shown in increase cell viability, biosynthesis, and allow for cell-cell communication that may influence cell function [26, 27]. When screening NP cell therapies, cell clusters may be a useful marker for a healthy NP phenotype. NP cells lose their young phenotype with age, becoming smaller (loss of vacuoles), elongated, and more fibroblast-like, coinciding with changes in the ECM of the NP [1, 3]. Loss of the immature NP cell phenotype may be one of the first steps in IVD degeneration [8]. While humans lose their young healthy cells by the age of 10, animal models such as pigs and
rats retain the immature NP cell phenotype significantly past skeletal maturity [28]. These animals are useful as *ex vivo* models of healthy young NP cells, though this morphological presentation is lost after expansion on tissue culture plastic [29]. Using healthy animal NP cells as a model, there is interest in pushing adult human NP cells towards a healthy young phenotype to reduce cell death and promote ECM production, thereby retarding IVD degeneration. We show that modifying the niche of NP cells in vitro, using specific peptides and appropriate substrate elasticity, promotes a healthy young cell phenotype.

![Image](image.png)

**Figure 4:** Vacuolated morphology of immature porcine NP cells attached to LM-111 (A), or attached to LM-111 after blocking adhesion receptor integrin α6. Note that blocking integrin α6 selectively inhibits attachment of the larger vacuolated cells.

### 1.3 Role of Laminins in Regulating Cell Behaviors

The phenotype and survival of NP cells may be regulated by cell-matrix interactions, motivating the need for appropriate ligands that promote a healthy NP cell...
phenotype. Healthy young NP cells have a unique ECM environment, with high expression of, and interactions with, laminin proteins (LM-111, LM-332, and LM-511) [5, 6, 10]. Laminins are heterotrimeric proteins composed of α, β, and γ polypeptide chains that combine to form at least sixteen different isoforms (Figure 5) [30]. Laminins have been shown to control a wide variety of cell behaviors, including differentiation, migration, proliferation, cell-cell interactions, and ECM production [31, 32].

![Figure 5: Schematic of LM-111 structure adapted from Miner et al., 2004](image)

Due to the influence of laminins on cell behaviors, laminins have been incorporated into biomaterials for a variety of tissue engineering purposes [33-40]. Basement membrane extract (BME), a laminin-rich soluble form of basement membrane typically purified from Engelbreth-Holm-Swarm tumor, is commonly used to functionalize surfaces with laminins, most frequently for epithelial, endothelial, and smooth muscle cell culture. The interactions between NP cells and multiple laminin
isoforms suggest laminins are important for region-specific IVD biology and cell phenotype. For example, immature porcine NP cells were shown to maintain a rounded, clustered phenotype when grown on soft (210 Pa) polyacrylamide gels functionalized with laminin-rich BME as compared to collagen type II that promoted a fibroblastic phenotype (Figure 6) [12]. Both the unique NP-LM interactions, as well as the impact of laminins on cell behavior, indicate laminin derived biomaterials may be useful for NP tissue engineering approaches.

Figure 6: Immature porcine NP cells grown for seven days on soft 210 Pa polyacrylamide gels functionalized with either laminin-rich basement membrane extract (BME)(top) or collagen type II (bottom). Adapted from Gilchrist et al., 2011
Most ECM proteins are large molecules that can mediate cell attachment through multiple mechanisms [41, 42]. For example, there are many different cell receptors that can engage laminins, including integrins (α1β1, α2β1, α3β1, αVβ3, α6β1, α6β4, α7β1) and non-integrin receptors such as syndecans, α-dystroglycan, CD151, and lutheran (CD239) [42-45]. Due to the variety of cell receptors a single laminin protein can engage, it is difficult to control the activation of specific cell receptors when using the full length proteins for tissue engineering applications. Additionally, laminins are large proteins with quaternary structure [30] that are subject to wide variations in bioactivity due to isolation, processing, and storage conditions that can alter its conformation and presentation of cell-receptor ligands. For example, the protein LM-332 is able to present different integrin-binding ligands depending on its conformation [43, 46]. When fully intact, the protein primarily exposes the integrin α3β1 binding region, promoting cell migration in the epidermis for wound healing [47]. However, after processing by serine proteolysis, the cryptic α6β4 ligand becomes exposed and promotes a stable monolayer formation [48-52]. The complexity and non-specificity of full-length laminins motivates our desire to use biomaterials functionalized with laminin-mimetic peptides for tissue engineering of the NP.

1.4 Role of Matrix Elasticity in Regulating Cell Behaviors

In addition to ECM ligands, matrix elasticity has been shown to be a key ECM variable. Without some degree of stiffness, integrin engagement alone will not generate
a complete cellular response such as focal adhesion formation [53-55]. Matrix elasticity has been shown to modulate many cell behaviors, including cell adhesion, spreading, clustering, and motility [56-63]. Additionally, substrate elasticity has been shown to have a large impact on stem cell fate and differentiation [58, 64, 65]. More compliant substrates have been shown to decrease cell adhesion strength, increase cell motility, alter cell-matrix adhesions, reduce cell spreading, and promote cell clusters [56, 57]. Despite a wide range of substrate stiffness for culturing different cell types, the optimal stiffness for maintaining cell phenotype is often similar to the elasticity of the native tissue the cells are sourced from [66]. Young NP cells are embedded in a soft tissue, with elasticities ranging from 0.3 to 5 kPa \textit{in vivo} [67, 68], informing our hypothesis that soft substrates will promote an immature NP phenotype.

Polyacrylamide gels are a common tool for studying matrix elasticity due to the physiologically tunable range of elasticity (0.1-35 kPa), ability to covalently attach proteins, and non-fouling surface [56, 62, 69]. Previous work in the Laboratory has used both laminin-rich BME gels and protein functionalized polyacrylamide gels to show that soft laminin-rich substrates promote porcine NP cell-cell interactions (Figure 7) as well as increased biosynthesis (Figure 8) [12]. On stiffer substrates, the young porcine NP cells lose their morphology, spread out, and become more fibroblast-like. In contrast, soft substrates increased GAG ECM production, cell-cell interactions, and a NP cell
phenotype [12]. These findings suggest that the mechanical properties of substrates, in addition to ligand presentation, are critical to the behavior of NP cells.

Figure 7: Immature porcine NP cells grown for seven days on either soft laminin-rich basement membrane extract (BME) gels (top) or stiff BME-coated tissue culture plastic (bottom). Adapted from Gilchrist et al., 2011
Figure 8: Immature porcine NP cells grown on a soft gel produced 1.7 times more sGAG over a 12 day period. Adapted from Gilchrist et al., 2011
2. Human Nucleus Pulposus Cell Adhesion and Integrin-Mediated Interactions with Extracellular Matrix Proteins


2.1 Introduction

The extracellular matrix (ECM) of the intervertebral disc (IVD) provides a host of mechanical and biochemical signals to the resident nucleus pulposus (NP) cells that may promote cell survival, ECM production, and regulate cell morphology and differentiation [2, 14]. Cells use surface molecules to engage ECM ligands in their surroundings, and these cell-matrix interactions can impart ligand-specific signaling cascades. Understanding the mechanisms that regulate cell interactions with the ECM is essential to understanding how ECM changes affect cell survival, matrix synthesis, and metabolism, with consequences for understanding IVD function and regeneration with aging.

Integrins are a class of cell surface molecules that mediate cell interactions with the ECM, including adhesion and migration [70-73]. Additionally, integrin-ECM interactions can regulate cell signaling, modulating cell functions such as cell survival, cell proliferation, and protein production [74]. Structurally, integrins are heterodimers composed of α and β units that cooperate to interact with different ligands. Initial
research has documented the presence of specific α and β subunits in rat IVD during development [75], including the integrin subunits α5 and β1 known to mediate interactions with collagens and fibronectin. Immunostaining of human IVD tissues has also documented the presence of these integrin subunits in NP or anulus fibrosus regions, and further identified the presence of α1, α3, α5, α6, αV, β1, β3, and β5 subunits [5, 11]. Immunostaining has also confirmed detection of these same subunits in porcine NP tissues along with higher expression levels for the α6 and β4 integrin subunits in the porcine NP [5, 6, 11]. While a functional role for these integrin subunits has been studied for cells of articular cartilage, fibrocartilage, and porcine NP [6, 76, 77], limited information is available about how these integrin subunits may regulate cell attachment, survival, function, and more in the adult human intervertebral disc.

For cells of the intervertebral disc, studies have shown the α5β1 integrin heterodimer regulates interactions with fibronectin [6, 78], and that these same integrin subunits are involved in the onset of cell pathobiology following exposure to degraded fragments of fibronectin [79]. Studies of rat NP cells have shown that attachment to type II collagen is mediated by the α2 integrin subunit in a process that involves activation of ERK [80], while porcine NP cells were instead shown to use the α1 integrin subunit to attach to type II collagen [6]. Studies by our group have focused on intervertebral disc cell interactions with laminin (LM) proteins, which may play a key role in healthy NP tissue [5, 6, 10, 11, 81]. Laminins are heterotrimeric proteins composed of α, β, and γ
polypeptide chains that combine to form at least sixteen different isoforms [30]. Porcine NP cells have been shown to interact with laminins LM-111 and LM-511 through integrin mediated mechanisms [5, 6]. Specifically, porcine NP cells have been found to use integrins α6 and β1 to attach to LM-111 [6] with the finding that blocking integrin α6 selectively prevents attachment of larger, notochordal-like porcine NP cells to LM-111 [6]. Together, these findings begin to reveal a role for specific integrin subunits in mediating NP cell-ECM interactions.

Mature human NP cells lose many phenotypic traits of young and healthy NP cells, and NP cell-matrix interactions may be altered with maturity or aging [20]. One study has shown that mechanotransduction of healthy adult NP cells was dependent on RGD-mediated interactions with ECM for cells embedded in alginate, although this interaction was lost for cells from degenerate NP tissue [82]. Another study has reported elevated levels of α5 and β1 integrin subunits in tissues from herniated disc, with no evidence of differences in α1, α2 and αV and β3 subunits [83]. Understanding which integrin subunits an adult human cell uses to attach to the ECM is critical to first understanding mechanisms used for survival and regulation of cell function, as well as their changes with degeneration and aging.

For this work, it was hypothesized that adult human NP cells would attach to laminin proteins 111 and 511 in an integrin-dependent manner (Hypothesis 2.A). Based on the shift in human NP cell phenotype with degeneration and age, it was additionally
hypothesized that the laminin-binding integrin receptors used by NP cells from adult degenerate tissues would differ from those used by healthy young porcine NP cells (Hypothesis 2.B). To investigate these hypotheses, expression levels of integrin subunits for adult human NP cells were determined, both in vivo and in vitro. Function blocking antibodies against integrins were used to inhibit cell attachment to ECM proteins, revealing the set of integrin subunits used by adult human NP cells to attach to each protein. These data identify the cell-surface adhesion receptors that are most significant for regulating adult human NP cell attachment to LM proteins for comparison to prior studies of the young and healthy NP cell phenotype.

2.2 Methods and Materials

2.2.1 Cell Isolation and Culture

Cells from the NP region of to-be-discarded degenerative disc surgical waste (ages 21-69) were isolated using a pronase-collagenase digestion [84]. NP tissue was separated from the surgical sample, which also contains anulus fibrosus and cartilaginous tissue, and rinsed in wash medium (Dulbecco’s Modified Eagle’s Medium (Gibco, Thermo Fisher Scientific, Waltham, MA) with 5 mL of 10 mg/mL Kanamycin (Sigma-Aldrich, St. Louis, MO), 2.5 mL of 250 µg/mL Amphotericyn B (Gibco, Thermo Fisher Scientific), and 1.65 mL of 50 mg/mL Gentamycin (Gibco, Thermo Fisher Scientific)). The NP tissue was digested for 2-4 hours at 37°C, until the majority of the tissue was dissolved, in 25 mL of digestion solution per gram of NP tissue (Wash
medium with 5% heat inactivated FBS (Hyclone, GE Healthcare Life Sciences, Logan, UT), 0.3% collagenase type II (Worthington Biochemicals, Lakewood, NJ), and 0.2% pronase (Roche Applied Science, Mannheim, Germany)). The resulting cell suspension was passed through a 70 µm cell strainer (BD Falcon, Bedford, MA) to remove any tissue debris and spun at 400g for 10 minutes to collect the cell pellet. The cells were plated onto tissue culture plastic and expanded up to passage 2 at 5% CO₂ and 37 °C in F-12 media (Invitrogen, Thermo Fisher Scientific) supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin (Sigma-Aldrich). For experimental use, cells were detached from flasks using 0.025% trypsin/EDTA (Lonza, Basel, Switzerland) and immediately washed in 1 mg/mL soybean trypsin inhibitor (sigma) in serum-free F-12 media. The soybean trypsin inhibitor prevents excess enzymatic digestion without exposing the cells to the extracellular matrix proteins found in serum-containing media. Cells were used for both flow cytometry and integrin blocking experiments as detailed in the following sections.

2.2.2 Flow Cytometry

Cells from monolayer culture (tissues aged 21-63, n=6) were allowed to recover following detachment in suspension with 10% FBS for 2 hours. NP cells suspended at 1-2 million cells/mL were then incubated for 30 minutes at 4 °C in solution with primary integrin monoclonal antibodies recognizing integrin subunits α1, α2, α3, α5, α6, β1, and β4, or negative isotype controls IgG1 and IgG2a (Table 1). Integrin subunits were
selected for analysis based on reported integrin binding motifs on LM-111 and LM-511 [42-45]. After washing the cells twice in 1X PBS, the cells were incubated for an additional 30 minutes at 4 °C with the appropriate secondary antibody (10 μg/mL Alexa 488 anti-mouse or anti-rat, Invitrogen). Cells were then rinsed twice with PBS and the mean fluorescence intensity (MFI) and percent of labeled cells was measured via flow cytometry (Accuri C6, BD Accuri Cytometers Inc., Ann Arbor, MI). Negative isotype controls were used to establish a gate containing 98% of the negative cells; the percent of cells above this gate were considered positively labeled. Results indicated that the β1 integrin MFI values were consistently greater than any other condition. For this reason, MFI values were normalized by MFI for β1 integrin on a per sample basis to remove inter-subject variability. A minimum of 10,000 events were counted for each flow cytometry reading.
Table 1: Monoclonal antibodies for human integrin subunits and IgG controls used in immunohistochemical staining, flow cytometry analysis, and function blocking experiments

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Working Concentration</th>
<th>Isotype Control</th>
<th>Clone/Part Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>α1</td>
<td>10μg/ml</td>
<td>Mouse IgG1</td>
<td>FB12, MAB1973Z</td>
</tr>
<tr>
<td>α2</td>
<td>10μg/ml</td>
<td>Mouse IgG1</td>
<td>P1E6, MAB1950Z</td>
</tr>
<tr>
<td>α3</td>
<td>10μg/ml</td>
<td>Mouse IgG1</td>
<td>ASC-1, MAB2056</td>
</tr>
<tr>
<td>α5</td>
<td>10μg/ml</td>
<td>Mouse IgG1</td>
<td>P1D6, MAB1956</td>
</tr>
<tr>
<td>α6</td>
<td>40μg/ml</td>
<td>Rat IgG2a</td>
<td>GoH3, MAB1378</td>
</tr>
<tr>
<td>β1</td>
<td>10μg/ml</td>
<td>Mouse IgG1</td>
<td>4B4, 6603113</td>
</tr>
<tr>
<td>β4</td>
<td>10μg/ml</td>
<td>Mouse IgG1</td>
<td>ASC-3, MAB2058Z</td>
</tr>
<tr>
<td>IgG1</td>
<td>10μg/ml</td>
<td>-</td>
<td>DD7, CBL600</td>
</tr>
<tr>
<td>IgG2a</td>
<td>10μg/ml</td>
<td>-</td>
<td>DD13, CBL605</td>
</tr>
</tbody>
</table>

2.2.3 Integrin Blocking Assays

Ninety-six-well assay plates (half-well Costar plates, 3688, Corning, Corning, NY) were overlaid with 40 μl of either chicken type II collagen (40 μg/mL, Sigma-Aldrich), mouse LM-111 (20 μg/mL, Millipore, Billerica, MA), or mouse LM-511 (10 μg/mL, Sigma-Aldrich) and incubated overnight at 4 °C. These protein concentrations were chosen from a cell attachment screen that varied protein concentrations to identify the maximum cell attachment saturation point. The minimum amount of protein above the saturation point was used for the following experiments to minimize ligand cost. The wells were blocked with 3.75% bovine serum albumin (BSA) for 3 hours at 37 °C, then rinsed with 1X PBS. Wells without protein but blocked with BSA were negative controls to identify non-specific cell binding. Calibration experiments using a BSA
incubation below 2 hours would significantly increase non-specific cell attachment to tissue culture plastic. NP cells (tissues aged 30-69, n = 4 or 5) were detached from tissue culture plastic as described above and incubated for 10-15 minutes in suspension with soybean trypsin inhibitor (T9777, Sigma-Aldrich). Cells were then incubated in serum free F-12 media (SFM) with function blocking integrin antibodies (Table 1) for 30 minutes at 37 °C.

Cell attachment to type II collagen was blocked with the following combinations of antibodies at the given concentrations recognizing integrin subunits: α1 (10 μg/mL), α2 (10 μg/mL), α1 + α2, β1 (10 μg/mL), or IgG1 (10 μg/mL). Cell attachment to LM-111 and LM-511 was blocked with the following combinations of antibodies recognizing integrin subunits: α1 + α2, α3 (10 μg/mL), α6 (40 μg/mL), α3 + α6, α5 (10 μg/mL), α3 + α5, α1 + α2 + α3 + α5, β1, β4 (10 μg/mL), IgG2a (10 μg/mL), or IgG1. Antibody concentrations were selected using manufacturer recommendations and published methodologies. Cells in solution with the antibodies were then placed in protein and BSA coated wells (4,000 cells/well), allowed to attach for 1 hour, and washed twice with serum-free media to remove unattached cells. Attached cells were then counted using the CellTiter-Glo luminescent cell viability assay (Promega, Madison, WI). The CellTiter-Glo assay measures the number of viable cells by quantifying the ATP present.

In separate experiments, the role of cations in cell attachment was determined by incubating NP cells (tissues aged 30-69, n=5) with 10 μM of the chelator
ethylenediaminetetraacetic acid (EDTA) for 30 minutes at 37 °C. Cells were then similarly plated on substrates coated with LM-111, LM-511 or type II collagen and allowed to attach for 1 hour, washed twice with serum-free F-12 media, and finally counted using the CellTiter-Glo luminescent cell viability assay. Control NP cells that were not incubated with EDTA represented the maximum attachment values for each substrate.

The unblocked controls (IgG for antibody blocking) were used to determine the maximum cell attachment to each substrate for each sample. Cell attachment for each blocking condition was performed in triplicate for cells from each sample, averaged, and then normalized to the unblocked cell attachment value. Any decrease in cell attachment from the unblocked control was considered evidence of blocking.

2.2.4 Immunohistochemical Detection of Integrins in IVD Tissue

Tissue samples (n=4-8, ages 30-74) from each patient were embedded (OCT compound, Tissue-Tek), flash frozen in liquid nitrogen, and stored frozen at -80 °C until sectioning. Cryosections (7 μm) were fixed in 4% formaldehyde, incubated with blocking solution (5% goat serum + 3.75% BSA), and then incubated with antibodies against integrin subunits α1, α2, α3, α5, α6, or β1 (1:100 dilution, 1.5 hours) with appropriate IgG controls (Table 1). After primary antibody incubation, the slides were washed, incubated with a pre-diluted secondary antibody, and co-stained with propidium iodide (1 mg/mL, Sigma). Anti-mouse AlexFluor 488 secondary antibody
was used for α1, α2, α3, α5, β1, and IgG1 antibodies, and an anti-rat AlexFluor 488 secondary antibody was used for α6 and IgG2a antibodies. Stained sections were imaged using confocal laser scanning microscopy (Zeiss LSM 510, 63X water immersion objective, Carl Zeiss, Thornwood, NY, USA). For each tissue sample, visual fields across the entire tissue section were manually inspected for positive staining, and representative positive and negative images were acquired.

### 2.2.5 Statistical Analyses

One-way analysis of variance (ANOVA) with post-hoc Tukey’s Test was used to test for differences in both normalized MFI and the percent of positive cells amongst groups labeled with antibodies or IgG. Normalized cell attachment was tested for a difference amongst integrin subunits using a two-factor ANOVA (integrin subunit, substrate). The normalized cell attachment for EDTA was analyzed with a two-factor ANOVA (blocking condition, substrate). Post-hoc Tukey’s test was used to find differences amongst all blocking conditions.

### 2.3 Results

#### 2.3.1 Flow Cytometry

Human NP cells were found to express integrin subunits α1, α3, α5, and β1 with MFI values that were significantly higher than IgG1 controls (Figure 9a; ANOVA, p<0.05). On average, MFI values for integrin subunits α3 and α5 were 34% and 33% of β1 values, respectively. A majority of NP cells (72-94%) were positive for integrin subunits
α3, α5, and β1 (Figure 9b), while only 38% and 26% of all NP cells were positively labeled for subunits α1 and α2, respectively.

Figure 9: Flow cytometric analysis of integrin expression for human NP cells: (a) Mean ± SE of mean fluorescence intensity (MFI) averaged across all samples for each anti-integrin antibody. Data are normalized by values for β1 integrin expression on a per-sample basis; and (b) mean ± SE of the percentage of cells positive for each integrin subunit averaged across all samples for each anti-integrin antibody. Conditions labeled with different letters are significantly different from each other and from the IgG controls (ANOVA, p < 0.05).
2.3.2 Integrin Blocking Assays

Human NP cells adhered to LM-111, LM-511, and type II collagen II in similar numbers (47%-55% of cells plated); in comparison, cell attachment to BSA control substrates was only 7% (Figure 10). Cells readily attached to each substrate individually and began spreading, as determined by visual inspection with light microscopy. On surfaces coated with LM-111 or LM-511, blocking integrin subunit β1 reduced the number of adherent NP cells to 31% and 42% of IgG control values, respectively (Figure 11; ANOVA, p<0.05). There was no detectable difference in normalized cell attachment between the BSA control and the integrin β1 blocking condition (data not shown; ANOVA, p>0.05), suggesting that the integrin subunit β1 is necessary for all NP cell attachment to LM-111 and LM-511. Blocking integrin subunits α1 + α2, α3, or α5 did not significantly reduce cell attachment to LM-111 or LM-511 (Figure 11; ANOVA, p>0.05). In contrast, blocking α3 and α5 integrin subunits together significantly reduced cell attachment to 58% and 52% of IgG control values for LM-111 and LM-511 respectively (Figure 11; ANOVA, p<0.05). Blocking integrin subunits α1, α2, α3, and α5 simultaneously further reduced cell attachment to 37% and 28% of IgG control values for LM-111 and LM-511 respectively.
Figure 10: Cell attachment to different protein coatings compared to the BSA control. Conditions labeled with different letters are significantly different from each other (ANOVA, p < 0.05).
Figure 11: The effect of integrin function-blocking antibodies on NP cell attachment to (a) LM-111 or (b) LM-511 normalized to the IgG control (mean ± SE). Conditions labeled with different letters are significantly different from each other (ANOVA, p < 0.05).
There was no detectable decrease in NP cell attachment to type II collagen when integrin subunits α1 and/or α2 were blocked as compared to IgG control values (ANOVA, p>0.05). In contrast, blocking integrin subunit β1 reduced NP cell attachment to type II collagen to 39% of control values (Figure 12; ANOVA, p<0.05).

Figure 12: The effect of integrin function-blocking antibodies on NP cell attachment to type II collagen normalized to the IgG control (mean ± SE). Conditions labeled with different letters are significantly different from each other (ANOVA, p < 0.05).
Pre-incubation of NP cells with EDTA reduced cell attachment to LM-111, LM-511, and type II collagen to 6%-11% of the unblocked control values, showing NP cell attachment is cation-dependent (Figure 13; ANOVA, p<0.05).

![Bar graph showing the effect of 10 µM EDTA on cell attachment to LM-111, LM-511, or type II collagen (mean ± SE). Conditions labeled with different letters are significantly different from each other (two-way ANOVA, p < 0.05).](image)

Figure 13: The effect of 10 µM EDTA on cell attachment to LM-111, LM-511, or type II collagen (mean ± SE). Conditions labeled with different letters are significantly different from each other (two-way ANOVA, p < 0.05).
2.3.3 Immunohistochemistry

NP tissue from degenerate human IVDs exhibited positive and intense staining for integrin subunit β1 in every visual field examined (Figure 14). In contrast, immunopositivity of NP tissue for integrin subunits α3 and α5 was less frequently identified across tissue sections, and was often less intense compared to that observed for the integrin subunit β1. Over half of the tissue sections stained negative for integrin subunits α1, α2, and α6 in every visual field, while the remaining tissue sections stained slightly positive in only select visual fields. It should be noted that the adult human NP tissue generally had very low cellularity such that there were usually only a few cells in each visual field. These low cell numbers may decrease the power needed to detect infrequent positive staining via immunohistochemistry.
Figure 14: Representative images of immunostaining adult human tissues showing the pattern for positive labeling with antibodies against integrin subunits β1, α3, and α5. Green = antibody reactivity and red = propidium iodide counterstain.

2.4 Discussion

The results of this chapter identify functional integrin subunits that mediate interactions between extracellular matrix (ECM) proteins of the intervertebral disc (IVD) and adult human nucleus pulposus (NP) cells. This study analyzed adult degenerate human NP cell attachment to the ECM proteins LM-111, LM-511, and type II collagen, with a focus on laminins due to their increased expression in the NP compared to the AF
of young and healthy IVD tissue [5, 6]. Results of this study show that adult degenerate human NP cells express laminin-binding integrins α1, α2, α3, α5, and β1 and use integrin β1 to attach to LM-111, LM-511, and type II collagen. Attachment to LM-111 and LM-511 is additionally mediated by a combination of integrin subunits α3 and α5, in contrast to integrin subunit α6 for healthy porcine NP cells [6]. Adult NP cells attach to LM-111 and LM-511 at levels comparable to Collagen II, despite a significant loss of laminin protein from NP tissue with age and degeneration [5].

Incubating NP cells with EDTA completely blocks cell adhesion to LM-111, LM-511, and type II collagen, suggesting cell attachment is cation dependent. EDTA can be used to block cell-cell adhesion through cadherins, but visual inspection revealed the NP cells were attaching to each substrate individually, leaving integrins as the likely target of EDTA inhibition. That the integrin β1 function-blocking antibody and EDTA each block a majority of cell attachment to the tested substrates suggests that integrins are critical to NP cell attachment to these ECM proteins. The integrin subunit β1 mediates cell attachment to ligands using multiple α-subunit binding partners, including subunits α1, α2, α3, α4, α5, α6, α7, α8, α9, α10, α11, and αV [74]. The results of the integrin blocking assays performed here show that integrin subunits α3 and α5, as well as α1 and α2, may contribute to NP cell attachment to the dominant laminin isoforms within the young and healthy IVD, LM-111 and LM-511. Since these alpha subunits are only known to dimerize with subunit β1 [74], it can be concluded that adult human NP cells
may use integrins $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$ and $\alpha_5\beta_1$ combined to interact with LM-111 and LM-511.

The results of flow cytometric analyses confirm NP cell expression of integrin subunits $\alpha_1$, $\alpha_2$, $\alpha_3$, $\alpha_5$, and $\beta_1$. These results corroborate prior findings for protein expression via immunohistochemistry [11] and mRNA expression via PCR for $\alpha_1$, $\alpha_2$, $\alpha_5$, and $\beta_1$, only [82]. The findings in the current study of immunopositivity for integrin subunit $\alpha_3$ and no evidence of subunit $\beta_4$, is consistent with our prior report for very young human NP tissue expression via immunohistochemistry and flow cytometry [5], but not that for younger adult NP tissue expression levels via immunohistochemistry [11]. This difference could be due to differences in antibody reactivity, the sensitivity of different methods (histology vs. flow cytometry), the difference in tissue ages, or an alteration in integrin expression induced by cell isolation and a brief period of culture. In contrast to young human and porcine NP cells [5, 6], the current study demonstrates that adult human NP cells do not express the $\alpha_6$ integrin subunit or use it to attach to LM-111 or LM-511. This indicates integrin $\alpha_6$ expression and utilization may have a relationship with aging or degeneration of the NP.

The immunopositivity for integrin subunits $\alpha_3$, $\alpha_5$, and $\beta_1$ via histochemical staining corroborates findings via flow cytometry and indicates that the subunits most highly expressed in vivo are also most highly expressed after cell culture through passage 2. Not every visual field examined was found to be positive for integrin
subunits α3 and α5 using immunohistochemistry, most likely due to the low cellularity of human NP tissue combined with the lower incidence of α3 and α5 expression. Many tissue sections had very few cells as identified by propidium iodide, making it difficult to obtain enough random images to quantify integrin expression since most fields of view would be empty. Instead, cells were actively identified using the nuclear staining and representative images were taken of the integrin expression. The tissue findings highlight a limitation of histological analyses when studying the largely hypocellular adult human NP tissue, and may provide an explanation for variances between this work and that reported previously for younger human NP tissues [11].

Despite demonstrating that integrins are critical for NP cell attachment to ECM proteins using EDTA and integrin β1 blocking, no single integrin alpha subunit was found to block the majority of cell attachment to any of the tested substrates. Instead, we found evidence for multi-integrin alpha subunit cooperation by blocking multiple integrin subunits simultaneously. Blocking subunits α3 and α5 or α1, α2, α3, and α5 together significantly reduced cell attachment to laminins at levels similar to blocking subunit β1 alone. Despite a lack of statistical significance between blocking α3 + α5 and α1 + α2 + α3 + α5, it appears subunits α1 + α2 contribute to cell attachment, decreasing cell attachment an additional 36-47% compared to α3 + α5 alone. Thus it can be concluded that multiple alpha subunits demonstrate cooperativity to mediate NP cell attachment to LM-111 and LM-511 in studies as performed here. When one alpha
subunit is blocked, another can compensate to prevent loss of cell attachment, in a process that deviates from a simple additive or subtractive effect.

Cell attachment to type II collagen has been previously reported to be mediated by integrin α1β1 for human chondrocytes and porcine NP cells [6, 70], and integrin α2β1 for rat NP cells [80]. Loeser and co-workers found that human chondrocytes most highly expressed integrins α1β1 and α5β1, as compared to the findings reported here that human NP cells most highly express integrin subunits α3, α5, and β1. Human chondrocytes were reported to attach to type II collagen through the use of integrin subunits α1 and β1, blocking 38.3% and 98% of cell attachment, respectively [70]. For human NP cells, only anti-integrin β1 antibody reduced attachment to type II collagen in a significant manner, inhibiting 61% of all NP cells attaching as compared to IgG controls. The absence of a significant effect of alpha integrin subunits regulating attachment to type II collagen for human NP cells, but not chondrocytes, was found despite using a similar cell attachment time (60 minutes versus 45 minutes), the same chick type II collagen from Sigma, a similar protein concentration (40 μg/mL versus 10 μg/mL), and obtaining a similar unblocked cell attachment efficiency (49% versus ~50%) across these studies [76]. The data presented does not confirm that adult degenerate human NP cells attach to type II collagen through α1 or α2 integrin subunits. It must be concluded that human NP cells use a different set of integrin alpha subunits than
chondrocytes and young and healthy NP cells to attach to type II collagen, possibly the collagen-binding subunits α10β1 or α11β1 [85, 86].

In a study by Le Maitre and co-workers, NP cell attachment to the ECM binding domain, RGD, was found to be important in mechanosensing for healthy, but not degenerate, NP cells [82]. Specifically, they found that blocking with an RGD peptide altered the ability of NP cells from non-degenerate discs to sense mechanical stimulation when embedded in an artificial matrix. RGD is a critical peptide for mediating cell binding to fibronectin, largely through an α5β1 integrin-mediated mechanism. Fibronectin was not studied here, as porcine NP cells were not previously found to favor binding to fibronectin over laminins and collagens [10], which were the focus of the current study. Nevertheless, the reported change in mechanosensitivity suggests an altered cell-ECM interaction for degenerate IVD cells, supporting our findings identifying different functional integrin subunits in adult degenerate human NP cells compared to prior studies of young and healthy NP cell binding [6].

Limitations to these data include cell source variability, antibody functionality, and cell receptor damage. The use of primary human cells is inherently variable due to inter-patient differences. The cells used here, isolated from degenerate tissue, have a range of variables including age, race, degree of degeneration, and disc level. Additionally, the tissue is isolated manually, separating the NP tissue from anulus fibrosis tissue, bone, and cartilage. While care is taken to prevent contamination from
these other tissues, other cell types may make up a portion of the cells tested here, even after sorting cells based on cell size. Antibody functionality is another study limitation due to the variation in antibody-ligand affinity for different antibodies. Function-blocking antibodies have a range of affinities for their specific ligand as well as a range of function-blocking efficacy. Variation in both antibody-ligand affinity and antibody function-blocking efficacy lead to an overall decrease in blocking efficiency for certain antibodies. For example, if an antibody was only 50% efficient, a 50% decrease in cell attachment in an integrin blocking assay may actually indicate that specific integrin is entirely responsible for cell attachment. In these studies, we used the most widely used function-blocking anti-integrin antibodies to minimize the inefficiencies due to a lack of ligand-antibody affinity. Lastly, the extracellular portion of the integrins may have been damaged during cell detachment with trypsin. Care was taken to minimize cell exposure to trypsin, but in the process of cell detachment some integrin receptors may have been damaged, reducing the ability for antibodies to bind. The possible reduction in antibody binding would decrease flow cytometry signal and function blocking ability.

2.5 Conclusions

The results in this chapter identify functional integrin subunits used by adult human NP cells to interact with the ECM, providing a foundation for developing bioconducive biomaterial interfaces that engage these cell receptors. Adult human NP cells express and utilize different integrin receptors as compared to porcine and rat NP
cells, highlighting a significant limitation of animal models when designing therapies for the IVD. Human NP cell attachment information will enable an understanding of the mechanisms that regulate cell survival, cell phenotype, and mechanosensing, allowing for improved therapeutic interventions for the treatment of IVD disorders. The main conclusions of this chapter include:

1. **Adult human NP cells highly express integrin subunits β1, α3 and α5 both in vitro and in vivo.** Over 70% of isolated cells expressed these integrin subunits, flow cytometric analysis showed highest expression of subunit β1, and all three subunits were identified in vivo. Low levels of integrin subunits α1 and α2 were additionally identified in isolated cells using flow cytometry.

2. **Adult human NP cells engage laminin isoforms LM-111 and LM-511 as well as type II collagen in an integrin-dependent manner, confirming Hypothesis 2.A.** The majority of cell attachment to all three proteins is inhibited when broadly blocking integrin function using EDTA and specifically blocking integrin β1 using function blocking antibodies.

3. **Attachment to LM-111 and LM-511 uses a combination of integrin alpha subunits α3 and α5, as well as possibly α1 and α2, in contrast to the single α6 subunit for healthy porcine cells, confirming Hypothesis 2.B.** Results showed no single alpha subunit could decrease cell
attachment from IgG controls, but combinations could inhibit cell attachment to levels comparable to blocking integrin β1. This finding suggests a cooperation between different integrin subunits when attaching to a large protein such as laminin.

4. Adult human NP cells did not show a preference for attaching to LM-111 or LM-511 over type II collagen, as had been shown previously with healthy porcine NP cells. This finding shows the shift in cell-protein engagement mechanism corresponds with altered cell attachment behavior.
3. Human Nucleus Pulposus Cell Adhesion to Laminin-Mimetic Peptides

3.1 Introduction

Work in the Setton laboratory has previously shown that soft substrates presenting laminin-111 (LM-111) promote maintenance of an immature NP cell phenotype for porcine NP cells, including increased matrix synthesis and expression of NP-specific markers, with implications for cell-mediated regeneration of NP tissue [12, 87]. There are many different cell receptors that can engage laminins, including integrins (α1β1, α2β1, α3β1, αVβ3, α6β1, α6β4, α7β1) and non-integrin receptors such as syndecans, α-dystroglycan, CD151, and lutheran (CD239) [42, 43, 45]. Our lab has identified integrin subunits that porcine and human (Chapter 2) NP cells use to engage LM-111, including integrins α3β1 and α6β4. For this work, we initially screened a variety of LM-derived and integrin-binding peptides for their ability to promote adhesion of NP cells to surfaces (Table 1).

Peptides have long been used as cell recognition motifs to increase cell attachment and elicit specific cell responses [88]. Peptides have many beneficial properties compared to full length proteins, such as receptor specificity, increased stability, ease of coupling, and cost effectiveness [89]. Additionally, a higher density of peptides can be attached to a surface compared to the full length protein [90]. The most common peptide sequence in tissue engineering, RGD, was derived from fibronectin in 1984 [91]. RGD primarily recognizes integrins α5β1 and αVβ3, and has been shown to
additionally interact with up to 12 different integrins [89-92]. RGD has repeatedly been conjugated to different surfaces to increase cell attachment and alter cell behavior of specific cell types [88-90, 93, 94]. Since the isolation of the RGD sequence, many other receptor-specific peptides have been the subject of study. Cell recognition peptides are most commonly isolated from protein digestions, where the peptide fragments are screened for an ability to promote attachment and impact on cell behavior and phenotype. For example, Nomizu et al. have been screening laminin chains since 1995 for active peptide sequences, resulting in a library of laminin-derived bioactive peptides [45, 95-97]. Due to these and other similar studies, there is a large selection of peptides available that have been shown to engage specific integrin and non-integrin cell surface receptors, providing a powerful resource for generating tightly controlled ECM environments.

The literature has shown that presenting cells with the appropriate set of integrin-binding peptides can control cell behavior. For example, two different groups were able to use varied combinations of peptides binding integrins α2β1, α5β1, αVβ5, αVβ3, α6β1, and/or α9β1 to maintain the phenotype and proliferation of embryonic stem cells [98, 99]. Biomaterials modified with cell recognition peptides have been used to culture a large number of cell types, including epithelial cells, endothelial cells, smooth muscle cells, islet cells, fibroblasts, among others [33, 34, 93, 100, 101]. Despite widespread use of cell recognition peptides in the field of tissue engineering, a role for
cell recognition peptides in modulating NP cell function has not been studied. With application to other cell types, groups have functionalized materials with multiple peptides to impart aspects of LM-111 functionality [102, 103]. Due to the importance of LM to the NP, this LM-mimetic approach may be applicable to NP tissue engineering strategies. We plan on using peptides to engage NP cell receptors with the goal of imparting the functionality of full-length laminin proteins in a controlled manner.

3.1.1 Peptide Selection

The initial set of peptides screened (Table 1) was chosen from the literature based on an ability to mediate cell attachment. It should be noted, however, that most of the literature demonstrated cell attachment for cell types significantly different from NP cells (keratinocytes, melanoma cells, osteosarcoma cells, etc.). Peptide selection was additionally constrained by using peptides derived from laminins and/or peptides shown to engage integrin α3, α5, or α6. Peptides were manufactured with a cysteine-glycine-glycine sequence at the N-terminus, with the glycine amino acids acting as a spacer and the cysteine residue providing a terminal sulfhydryl group for future material chemistries allowing surface conjugation.
Peptides GD6, P4, p678, and cNGQGEO were all chosen for screening based on their ability to engage integrin α3 [47, 104-106]. GD6 is derived from the LG domain of murine laminin and has been shown to promote attachment of keratinocytes and melanoma cells [104, 107]. P4, derived from the α3 laminin chain, has been shown to promote keratinocyte cell adhesion, spreading, and cell signaling through focal adhesion kinase phosphorylation [47]. P4-coated chitin matrices were shown to accelerate wound healing in rat and rabbit cutaneous wound models by accelerating fibroblast proliferation and reducing inflammatory cell infiltration [108]. p678 is derived from thrombospondin-1 and has been shown to mediate migration, attachment, and spreading of breast carcinoma cells as well as adhesion and neurite-like outgrowth of small cell lung carcinoma cells [105, 109, 110]. Lastly, the cyclic peptide cNGQGEQ was identified using screening of a large peptide combinatorial library, and was shown to mediate attachment of 3 different carcinoma cell lines [106].

<table>
<thead>
<tr>
<th>Peptide Name</th>
<th>Amino Acid Sequence</th>
<th>Reported Cell Receptors</th>
</tr>
</thead>
<tbody>
<tr>
<td>GD6</td>
<td>CGG(KQNCLSSRAS)FRGCVRNRLRSR</td>
<td>Integrin α3</td>
</tr>
<tr>
<td>P4</td>
<td>CGGPPFMLMLKGSTR</td>
<td>Integrin α3</td>
</tr>
<tr>
<td>p678</td>
<td>CGGFQGVLQNVRFVF</td>
<td>Integrin α3</td>
</tr>
<tr>
<td>cNGQ</td>
<td>CGGcMGQGEO (cyclic)</td>
<td>Integrin α3</td>
</tr>
<tr>
<td>AG32</td>
<td>CGGTWYKIAFQRNRK</td>
<td>Integrins α2, α6, β1</td>
</tr>
<tr>
<td>AG10</td>
<td>CGGNRWHSIYITRG</td>
<td>Integrins α2, α5, α6, β1</td>
</tr>
<tr>
<td>T1</td>
<td>CGGTTSWSQCSKS</td>
<td>Integrin α6</td>
</tr>
<tr>
<td>AG73</td>
<td>CGGRKRLQVQLSIRT</td>
<td>Syndecans</td>
</tr>
<tr>
<td>RGD</td>
<td>CGGRGDS</td>
<td>Integrins α5, αV, α8</td>
</tr>
<tr>
<td>IKVAV</td>
<td>CSRARKQAASIKVAVSADR</td>
<td>Integrins</td>
</tr>
</tbody>
</table>

Table 2: Peptides screened for cell attachment
Peptides AG32, AG10, and T1 have been shown to engage integrin α6. AG10 and AG32 are from the LG1 and LG2 domains of the laminin α1 chain, respectively. The peptides have been shown to promote cell attachment, spreading, tumor invasion, and have been used to maintain the stem-ness of [95, 99, 111]. Human embryonic stem cell attachment to peptide AG10 was shown to be inhibited by function blocking antibodies against integrin α5, α6, β1, and α2β1, indicating AG10 is not specific to integrin subunit α6 [99]. The peptide T1 is derived from the full-length protein angiogenesis inducer T1, and has been shown to promote attachment of embryonic stem cells (human and mouse), human foreskin fibroblasts, and human umbilical vein endothelial cells [98, 112, 113]. Peptide T1 has additionally been shown to support early mesodermal differentiation of human embryonic stem cells when presented in conjunction with RGD [113], as well as maintain stem-ness of murine embryonic stem cells in conjunction with ligands for integrins α5β1, αVβ5, and α9β1 [98]. Prior work has shown that integrin α6β1 is a critical cell surface receptor for porcine NP cells, mediating attachment of large immature NP cells to LM-111 [6]. While adult human NP cells were not found to express integrin α6β1 (Chapter 2), a material presenting this ligand may be necessary for support of immature NP cells.

Peptide IKVAV is one of the most commonly studied laminin-derived peptides. It has been shown to mediate attachment through integrins, promote cell adhesion (melanoma cells, adipose derived stem cells, astrocytes, fibroblasts, endothelial cells),
and neurite outgrowth [114-117]. The peptide RGD was chosen both for its ability to bind integrin α5β1 as well as its widespread use in biomedical engineering. RGD primarily recognizes integrins α5β1 and αVβ5, although it has also been shown to interact with up to 12 different integrins [89-92]. RGD has repeatedly been conjugated to different surfaces to increase cell attachment of specific cell types [88-90, 93, 94].

Peptide AG73 (RKRLQVQLSIRT, derived from the α1 chain of mouse laminin) is the only non-integrin binding peptide in this screen, and has been shown to elicit a variety of cell behaviors, including cell attachment, by binding to syndecan cell-surface receptors [95, 96, 118-121]. Syndecans are transmembrane proteoglycans composed of a protein core with heparin sulfate or chondroitin sulphate glycosaminoglycan sugar chains attached to the extracellular domain [122]. The syndecan family of receptors, which is composed of four isoforms (syndecan 1, 2, 3, and 4), bind a variety of molecules including ECM proteins and growth factors. They have been shown to cooperatively bind with different integrin subunits to differentially modulate cell behaviors, including focal adhesion formation and organization as well as Rho signaling [122].

Fibroblast cell behavior has previously been shown to be sensitive to substrate elasticity when interacting with AG73-conjugated materials, with soft substrates promoting cell clusters and stiffer substrates promoting cell attachment and cell spreading [123]. AG73 has been used in combination with integrin-binding peptides to tailor protein-mimetic substrates. In combination with an integrin α2β1 peptide, the
focal contact organization of fibroblasts was altered as the ratio of peptides was varied; a molar ratio of 1:9 (AG73:α2β1 peptide) promoted a cell morphology similar to that of cells grown on laminin substrates [116]. The engagement of syndecans, individually and in combination with integrins, has been shown to promote a wide variety of cell behaviors. For a more complete review of syndecan function, we recommend the review by Mark Morgan et al. [122]. The potential for syndecans to contribute to NP cell-matrix signaling motivated our use of the peptide AG73 when screening cell recognition peptides.

For this work, it was hypothesized that adult human NP cells would attach to laminin-mimetic peptides in a concentration-dependent manner (Hypothesis 3.A). To investigate these hypotheses, LM-mimetic peptides were obtained and evaluated for their ability to mediate adult degenerate NP cell attachment at different ligand concentration. These findings identify peptides that support maximal human NP cell attachment that may have value for biomaterial incorporation in future studies. A select subset of these peptides will be the subject of further characterization in Chapter 4.

3.2 Methods

3.2.1 Cell Isolation and Culture

Cells from the NP region of to-be-discarded degenerative disc surgical waste (ages 30-77) were isolated using a pronase-collagenase digestion, as previously described in Chapter 2.2.1. The cells were plated onto tissue culture plastic and
expanded up to passage 2 at 5% CO₂ and 37 °C in F-12 media supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. For experimental use, cells were detached from flasks using 0.025% trypsin/EDTA (Lonza) and immediately washed in 1 mg/mL soybean trypsin inhibitor (sigma) in serum-free F-12 media.

3.2.2 Cell Attachment Quantification Assay

The cell attachment substrates were prepared by adding 40 μl of each peptide solution (table 2) to one well each of a 96 half-well assay plate (Corning). The peptides were manufactured at over 95% purity (American Peptide Co., Inc., Sunnyvale, CA) and solubilized in ultrapure water at 1 mg/mL; peptides p678 and AG10 were the exception, requiring 20% acetonitrile to fully solubilize at 1 mg/mL. Stock peptides were stored at -80°C until use. Experimental peptide solutions were prepared from the stock by dilution with ultrapure water. Peptides were plated as described by addition of 40 µL of experimental peptide solutions at 0.1, 1, 10, 100, and 200 µg/mL on the plates. This range was chosen to encompass all concentrations typically seen in the cell-peptide literature. Controls included wells with no protein (PBS only), mouse LM-111 at 20 µg/mL, or mouse LM-511 at 10 µg/mL. Laminin protein concentrations, the same as used in Chapter 2.2.3, were chosen based on the saturation point promoting maximal NP cell attachment. The plates were incubated overnight at 4 °C to adsorb the peptides to the surface of the plastic. The wells were then blocked with 3.75% bovine serum albumin
(BSA) for 3 hours at 37 °C followed by one rinse with 1X PBS. Wells without protein but blocked with BSA were negative controls to identify non-specific cell binding.

NP cells (tissue ages 30-77, n=9 patients) were detached from tissue culture plastic as described above, placed in wells (4,000 cells/well), allowed to attach for 1 hour, and washed twice with serum-free media to remove unattached cells. Attached cells were lysed and counted using the CellTiter-Glo luminescent cell viability assay (Promega).

Cell attachment for each blocking condition was performed in triplicate for cells from each sample and averaged. Percent attachment was determined by dividing the number of attached cells by 4000, the number of cells initially added to each well.

3.2.3 Statistical Analyses

Differences in percent cell attachment amongst culture substrates were detected via one-factor ANOVA (substrate) and Tukey’s HSD post hoc analysis for each plated peptide concentration.

3.3 Results

3.3.1 Cell Attachment Quantification

All peptides mediating cell attachment were found to be concentration dependent (Figure 15) with a uniformly consistent monotonic increase in percent cells attached with increasing peptide concentration. Peptides AG73, GD6, P4, IKVAV, and AG10 were able to mediate levels of attachment comparable to full-length LM-111
protein when plated at 200 µg/mL (Figure 16). Peptide AG73 supported the highest level of cell attachment at all concentrations, peaking at 78% attachment at 200µg/ml. Of the integrin α3 binding peptides, P4 mediated the most attachment at a concentration of 200µg/ml (59%), followed closely by GD6 (56%) and p678 (41%), while the cyclic peptide cNGQGEQ had no significant increase (p > 0.05) in cell attachment compared to the BSA control. Integrin α6 binding peptides AG10 and AG32 both mediated high levels of cell attachment (59% and 42%, respectively, at 200µg/ml). RGD, unexpectedly, did not promote any increase in cell attachment over the BSA control at any concentration (p > 0.05).

At lower peptide concentrations, there were fewer differences between substrates. For peptide ligands plated at 10 µg/mL, only GD6, p678, IKVAV and AG73 had a significant increase in cell attachment compared to the BSA control (ANOVA, p<0.05). At this concentration, AG73 had the highest cell attachment that was significantly higher than GD6, p678, and IKVAV and was comparable to LM-111 and LM-511. For ligands plated at 1 µg/mL, only peptides AG73 and p678 had a significant increase in cell attachment as compared to BSA (ANOVA, p<0.05). However, they were also significantly lower than positive controls LM-111 and LM-511 (ANOVA, p<0.05).
Figure 15: Concentration dependent attachment curves for integrin α3 binding peptides (a), integrin α6 binding peptides (b), and other LM-mimetic peptides (c). BSA is used as a negative control in all graphs, while LM-111 at 20 µg/mL and LM-511 at 10 µg/mL are used as positive controls. Error bars removed for clarity.
3.4 Discussion

This chapter identifies laminin-mimetic peptides, including some reported to bind the integrin receptors identified in Chapter 2, which are able to promote adult degenerate human NP cell attachment. The cell attachment substrates were prepared by adsorbing the peptides at varying concentrations to stiff tissue culture plastic. Of the ten tested peptides, seven are able to increase the percent cell attachment over the BSA negative control for at least one plated concentration. These seven include peptides reported to bind to integrin α3 (p678, GD6, and P4), integrin α6 (AG10 and AG32), syndecans (AG73), and multiple integrins (IKVAV). Of these, p678, P4, AG10, and AG73
were selected for further screening and characterization for biomaterial development (chapter 4).

Of all the tested peptides, AG73 promotes the greatest percent cell attachment at every plated concentration. There is a significant increase in cell attachment when AG73 is plated as low as 1 µg/mL (29% vs 7% for BSA). At 10 µg/mL, AG73 promotes cell attachment comparable to LM-111 and LM-511 (62% vs. 67% for LM-111 and 60% for LM-511). Cell attachment peaks at 78% of the maximum peptide concentration of 200 µg/mL. These findings are somewhat surprising due to our earlier findings that adult degenerate human NP cells attach to full length LM-111 and LM-511 in an integrin-specific manner, whereas peptide AG73 is reported to bind in a syndecan-dependent manner [118]. To confirm that NP cells are attaching to AG73 through a non-integrin cell receptor, we found that cell attachment to AG73 is inhibited with heparin, but not EDTA (Figure 17). The results from Chapter 2 indicate that syndecans do not play a role in cell adhesion to LM-111 or LM-511. Taken together, these results indicate that non-integrin binding ligands may be sufficient, though not necessary, for human NP cell adhesion. One possible explanation for these findings is that the AG73 motif, located in the LG domain of laminin, is not typically exposed on full-length LM-111 or LM-511. However, under certain conditions the motif may be exposed, and these results indicate that it can interact powerfully with adult human degenerate NP cells.
Figure 17: Blocking cell attachment to peptide AG73, plated at 100 µg/mL, with either 10 µg/mL EDTA or 50 µg/mL heparin (Sigma). Cells from a 59 year-old patient were allowed to attach, in triplicate, for 1 hour.

The peptides reported to bind through integrin α3 all promote cell attachment, as compared to the BSA control, except for peptide cNGQ. It is unclear why cNGQ is not able to mediate cell attachment while the other peptides did. The peptide cNGQ is a cyclic peptide, so it may adsorb differently than the others. Additionally, cNGQ was identified through the screening of a fragment library, and was only tested against carcinoma cell lines. It’s possible that the peptide is too specific to the carcinoma cells. The other three integrin α3 binding ligands promote high levels of cell attachment. Peptides GD6 and P4 achieve the highest cell attachment efficiency, 56% and 59% respectively (at 200 µg/mL). In contrast, peak cell attachment for peptide p678 is only 41%. However, p678 is the only integrin-binding peptide that is able to mediate a
significant increase in cell attachment at the low concentration of 1 µg/mL (15% cell attachment vs 7% for BSA, ANOVA, p<0.05).

Of the integrin α6 binding peptides, only AG10 and AG32 increase cell attachment significantly (59% and 42%, respectively, at 200 µg/mL). At peptide concentrations of 10 µg/mL or below, neither of these peptides are able to mediate cell attachment. Peptide T1 did not have a significant increase in cell attachment as compared to the BSA control (p>0.05). However, the percent cell attachment appears to trend higher with higher T1 peptide concentration, and it is possible a critical concentration of T1 peptide was not adsorbed for effective cell attachment. It is initially somewhat surprising that adult degenerate human NP cells are able to attach to integrin α6 ligands since the cells do not express integrin α6, as shown in Chapter 2. However, the tested peptides are likely not specific to only integrin subunit α6. It was demonstrated that blocking antibodies against integrin α6 and β1 could individually block rhabdomyosarcoma cell spreading for peptides AG10 and AG32, as well as antibodies against integrin α2 for peptide AG32 (Figure 18) [95]. Separately, it was shown that blocking antibodies against integrins α5, α6, β1, and α2β1 would partially inhibit human embryonic stem cell attachment to peptide AG10 [99]. It is perhaps the lack of integrin subunit specificity that enables peptides AG10 and AG32 to mediate cell attachment in a 1-hour period when T1 cannot.
Interestingly, RGD was not able to support attachment of human NP cells at any of the tested concentrations. Based on the results of Chapter 2, we expected RGD to mediate some cell attachment through the use of integrin α5. The lack of cell attachment to RGD suggests that either the conformation of the RGD peptide is inactive, the peptide is not adsorbing onto the surface of the tissue culture plastic, or that human NP cells won’t attach to the sequence. We conjugated the RGD sequence to a hydrogel, from Chapter 4, to test if covalent attachment to a surface alters RGD’s ability to mediate human NP cell attachment. However, we still saw no NP cell attachment to RGD. Lastly,
we ordered a second, cyclic, RGD peptide (c(Arg-Gly-Asp-DPhe-Val). This peptide was also unable to mediate NP cell attachment. Previous studies have shown human NP cell interaction with RGD peptides in specific instances. NP cell interactions with RGD was previously was found to be important in mechanosensing for healthy, but not degenerate, NP cells [82]. The group found that blocking with an RGD peptide altered the ability of NP cells from non-degenerate discs to sense mechanical stimulation when embedded in an artificial matrix.

Limitations to these data, as in Chapter 2, include cell source variability. Additionally, adsorption of peptides to a surface is an imprecise method of material functionalization. Though commonly used in the literature, it does not control the total amount of peptide that attaches to the surface. One peptide sequence may be adsorbing at a greater rate than another due to variables such as hydrophobicity and net charge. This may partially limit comparisons between peptides with different sequences, though a wide enough range of concentrations was tested to ensure cell attachment for adhesion-promoting peptide sequences.

3.5 Conclusion

The results in this chapter identify specific laminin-mimetic peptides that are able to promote adult degenerate human NP cell attachment at levels comparable to full-length laminin proteins. Cell attachment is a critical first step when developing bioconducive biomaterial interfaces, and the identification of these peptides provides a
set of ligands for further material integration and behavioral testing (Chapter 4). The main conclusions of this chapter include:

1. **Adult human NP cells attach to select laminin-mimetic peptides at rates comparable to full-length laminins 111 and 511, confirming Hypothesis 3.A.** Peptides AG73, IKVAV, GD6, P4, and AG10, plated at 200 µg/mL, promote cell attachment at levels similar to full-length LM-111 plated at 20 µg/mL.

2. Adult human NP cell attachment to laminin-mimetic peptides occurs in a concentration-dependent manner, with an undetectable increase in cell attachment for all peptides plated at 0.1 µg/mL.

3. Despite a lower cell attachment efficiency compared to laminins, peptide p678 is of interest due to its ability to mediate cell attachment at plated concentrations as low as 1 µg/mL.

4. Peptides RGD, cNGQ, and T1 do not promote a significant increase in cell attachment over the BSA control for any of the tested peptide concentrations. This is particularly surprising for RGD, which has been previously shown to interact with degenerate human IVD cells.
4. Regulation of Human Nucleus Pulposus Cell Phenotype and Behavior by Laminin-Mimetic Peptide Functionalized Substrates

4.1 Introduction

Healthy, young nucleus pulposus (NP) cells inhabit a soft, gelatinous, and highly hydrated tissue that contains glycosaminoglycans (GAGs), collagen proteins, and laminin proteins [1]. Young NP cells are biosynthetically active cells that become more fibroblast-like with age and degeneration, a change that appears to correlate with structural changes in the NP tissue, including a decrease in disc height, increase in stiffness, decreased and altered GAG composition, and decreased water content [2-4]. Environmental cues from the surrounding tissue, including extracellular matrix stiffness and ligand presentation, are known to be critical regulators of cell behavior and function. Studies demonstrate that healthy porcine NP cells interact with laminin on soft (<0.7 kPa) substrates, but not stiff, in a process that increases cell clustering and biosynthesis [12]. Motivated by this work, we investigate the effect of substrate stiffness and ligand presentation on the behavior and biosynthesis of adult degenerate human NP cells.

4.1.1 Studies of Matrix Elasticity with Polyacrylamide Gel Substrates

A polyacrylamide (PAAm) gel culture system was used in this chapter to create two dimensional substrates with a range of mechanical stiffnesses. PAAm hydrogels are useful for studying cell-matrix mechanical interactions due to their ease of
manipulation, range of linear elasticities, optical transparency, and resistance to nonspecific binding [85, 122]. Ligands can be covalently attached to the gel, which is otherwise non-adhesive, isolating the effects of the attached ligand [56, 124]. The gel is formed by reacting acrylamide with bis-acrylamide, with the ratio of polyacrylamide to bis-acrylamide regulating gel elasticity. The gels can be tuned to a wide, physiologically relevant, range of linearly elastic moduli [56, 62]. Previous studies have used PAAm gels to identify the role of substrate stiffness for a variety of cell behaviors such as mobility, spreading, integrin clustering, and differentiation [56, 58, 62, 63, 125].

Based on the prior work identifying 0.7 kPa as a stiffness threshold for a shift in healthy porcine NP cell behavior, we formulated and tested gel formulations with elastic moduli on either side of the 0.7 kPa threshold. In this chapter we evaluate the effects of this shift in substrate stiffness on the behavior and biosynthesis of adult degenerate human NP cells.

4.1.2 Laminin Mimetic Peptide Selection

Of the peptides screened in Chapter 3, p678, P4, AG10, and AG73 were selected for further characterization in this chapter. Peptides P4 and p678 were selected for their reported ability to bind integrin subunit α3, a critical integrin used by adult human NP cells to attach to laminins (Chapter 2). Peptide P4 promoted the highest cell attachment of all the integrin α3-binding peptides while p678 was the only integrin-binding peptide able to promote a significant increase in cell attachment at a low concentration of 1
µg/mL. Peptide AG10 was selected for its reported ability to bind integrin α6, the functional integrin subunit used by healthy porcine cells to attach to LM-111 [6]. Additionally, peptide AG10 promoted greater cell attachment then AG32 at concentrations of 100 and 200 µg/mL. Finally, AG73 was select for its superior ability to promote cell attachment. AG73 promoted greater cell attachment than all of the other tested substrates, including LM-111 and LM-511. Though a non-integrin binding peptide, its ability to promote high levels of cell attachment and its derivation from laminin led us to select AG73 as our final peptide ligand in this chapter.

Peptides P4 (CGGPPFLMLLGSTR) and p678 (CGGFQGVLQNVRFVF) are reported to engage integrin α3, and in Chapter 3 were shown to mediate up to 59% and 41% of cell attachment [47, 104, 105]. While neither peptide has been used to promote progenitor cell differentiation, both have been shown to modulate cell behaviors. P4, derived from laminin, has been shown to promote keratinocyte cell adhesion, spreading, and focal adhesion kinase phosphorylation as well as increasing fibroblast proliferation and reducing inflammatory cell infiltration [47, 108]. P678 is derived from thrombospondin-1 and has been shown to mediate migration, attachment, and spreading of breast carcinoma cells as well as adhesion and neurite-like outgrowth of small cell lung carcinoma cells [105, 109, 110].

Peptide AG10 (CGGNRWHSIYITRFG), derived from the LG1 domain of the laminin α1 chain, promoted 59% of cell attachment in Chapter 3 and is reported to
promote cell attachment and spreading through integrin α6β1 [95]. AG10 has additionally been shown to partially promote human embryonic stem cell attachment through integrins α5 and α2β1 [99]. Peptide AG73 (CGGRKRLQVQLSIRT), derived from the α1 chain of mouse laminin) is the only non-integrin binding peptide in this screen. AG73 has been shown to elicit a variety of cell behaviors, including cell attachment, by binding to syndecan cell-surface receptors [95, 96, 118-121]. In combination with peptide C-16 (CGGKAFDITYVRLKF), AG10 and AG73 were able to recapitulate the functionality of the laminin-rich substrate Matrigel and promote human embryonic stem cell adhesion, maintenance, and proliferation [99].

4.1.3 Young, Healthy Nucleus Pulposus Markers

In this chapter, we examine the effect of ligand and substrate stiffness in regulating adult human NP cell phenotype and biosynthesis, with the goal of promoting a young and healthy set of behaviors. To this end, it is essential that young and healthy NP cell markers are defined. For this work, we defined a young and healthy NP cell phenotype as promoting cell clustering, increasing proteoglycan (sGAG) biosynthesis, and altering gene expression (upregulation of aggrecan, type II collagen, n-cadherin, and glucose transporter GLUT-1 gene, downregulation of type I collagen).

Proteoglycan content, as defined by sulfated glycosaminoglycans (sGAG), is one of the defining properties of NP tissue, with a GAG to collagen (hydroxyproline) ratio of 27:1, compared to only 2:1 in hyaline cartilage [18, 19, 126]. sGAG production is often
used as a marker of successful NP regenerative medicine therapies, and consensus has recently been reached on its usefulness in this regard [126]. Studies with immature porcine NP cells have shown that sGAG production can be increased when culturing NP cells on a soft laminin substrate [12, 27, 127]. It is unknown if this is true for adult degenerate human NP cells.

NP cell clusters are another marker of a healthy NP cell, and have been shown to increase cell viability, biosynthesis, and allow for cell-cell communication that may influence cell function [26, 27]. Studies have shown that soft laminin-functionalized substrates induce cell clustering for porcine NP cells, and this coincides with an increase in sGAG production. When porcine NP cell clustering on soft laminin substrates is inhibited by blocking the cell-cell adhesion glycoprotein n-cadherin, sGAG production decreases back to levels seen on stiff laminin [27]. This work additionally identifies n-cadherin as a marker of a healthy NP cell, with a four-fold increase in gene expression on soft laminin substrates relative to stiff. While n-cadherin expression is seen in young human cells, its expression is found to decrease with degeneration, and it is now an accepted marker of the healthy NP phenotype [126, 128].

Using qRT-PCR, we looked at gene expression of five markers of a healthy NP, including the previously discussed aggrecan and n-cadherin, as well as types I and II collagens and glucose transporter GLUT-1. Collagens are a primary component of the IVD and a shift in collagen isotype content can signify disc degeneration [2, 14, 128, 129].
With degeneration, there is an initial upregulation of type II collagen, thought to be an attempt at tissue repair. However, with more severe degeneration, there is a loss of type II collagen and an increase in type I [130, 131]. Finally, consensus has been reached that GLUT-1 is a marker of the NP [126]. It has been found to be expressed in the NP, but not the AF, of rat and human NP tissues, and has additionally been shown to decrease with progressive disc degeneration [132, 133].

4.1.4 Summary

In this study, select laminin-mimetic peptide ligands and substrate stiffnesses were examined for their ability to regulate adult human NP cell phenotype and biosynthesis. It was hypothesized that culturing adult human NP cells on soft substrates presenting laminin-mimetic peptides would increase cell clustering and proteoglycan production over four days in culture (Hypothesis 4.A). Additionally, it was hypothesized that varying peptide ligand and substrate stiffness combinations would modulate the size distribution of cell clusters (Hypothesis 4.B). Finally, it was hypothesized that soft, laminin-mimetic substrates would upregulate gene expression of aggrecan, n-cadherin, type II collagen, glucose transporter GLUT-1, and down-regulate gene expression of type I collagen (Hypothesis 4.C).
4.2 Methods

4.2.1 Cell Isolation and Culture

Cells from the NP region of to-be-discarded degenerative disc surgical waste tissues (ages 36-67, n=5) were isolated using a pronase-collagenase digestion, as described in Chapter 2.2.1. The cells were plated onto tissue culture plastic and expanded up to passage 2 at 5% CO₂ and 37 °C in F-12 media supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. For experimental use, cells were detached from flasks using 0.025% trypsin/EDTA (Lonza) and immediately washed in culture media (F-12 with 10% FBS and penicillin/streptomycin).

A subset of cells were infected with a CMV-mCherry lentiviral construct to visualize the cells as they were cultured on different substrates. The constitutive reporter was generated using a 2nd generation lentiviral system and the CMV-mCherry sequence was custom made by GenScript (Piscataway, NJ). For infection, primary human NP cells were plated at 80% confluence in a t-25 flask and overlaid with the transduction medium (150 µL of viral solution, 6 mL media, and 4 µg/mL polybrene) overnight. Robust mCherry expression was seen via fluorescent microscopy after a few days in culture.

4.2.2 Polyacrylamide Gel Substrates

Thin PAAm gel substrates with “tunable” mechanical properties were created by polymerizing acrylamide with bis-acrylamide crosslinker at various concentrations. A pre-gel solution was prepared using acrylamide (5% or 8% weight/volume final
concentration, 40% stock solution, Bio-Rad, Hercules, CA), bis-acrylamide (0.03 – 0.15% weight/volume final concentration, 2% stock solution, Bio-Rad), HEPES buffer (30 mM final concentration, 1 M stock, Gibco, Life Technologies), and N,N,N',N'-tetramethylethylene-diamine (TEMED, 0.4% final concentration, Bio-Rad). The pH of the solution was brought to 6.0 with 5N HCl. This pre-gel solution was stored at 4°C up to 4 weeks before use.

Immediately before forming the gels, acrylic acid N-hydroxysuccinimide ester dissolved in ethanol (N2, 20 mM final concentration, CovaChem, Loves Park, IL) was added to the pre-gel solution. The solution was degassed for 15 minutes under vacuum in a sonicator bath (Branson B1510, Danbury, CT). Polymerization was initiated by adding ammonium persulfate (1:200, 10% stock solution, Bio-Rad). A 120 µL quantity of the solution was quickly aliquoted onto a pre-cleaned microscope slide (Superfrost Plus, Fisher Scientific) and flattened using a 24 x 60 mm coverglass (Corning). Gels were allowed to polymerize for 15 minutes, submersed in ice-cold water, and the top coverslip was gently removed with a razor blade. The 24 x 60 mm PAAm gel was partitioned into 8 distinct 0.7 cm² gels by clamping an 8-well media chamber (Millicell EZ Slide, Millipore) onto the microscope slide (Figure 19). A rubber gasket is affixed to the bottom of the Millicell EZ Slide media chamber, which prevents fluid flow between wells of the culture chamber.
Figure 19: Diagram showing the assembly of the media chamber and polyacrylamide gel into a culture chamber.

Gels were functionalized by immediately adding 200 µL of the desired ligand to each well (0.1 mM peptide or 120 µg/mL LM-111 in cold 50 mM HEPES with 5 mM EDTA at pH 8.5 unless otherwise noted) and incubated at 4°C overnight. As compared to Chapters 2 and 3, this chapter uses a higher concentration of LM-111 for functionalizing surfaces (120 µg/mL vs. 20 µg/mL). The laminin concentration was increased to accelerate the conjugation reaction with the NHS before the NHS hydrolyzed and became inert. Care was taken to insure a short time (~30 min for degassing and polymerization) between adding the N2 crosslinker to the monomer pre-gel solution and adding the ligand to the polymerized gel due to the hydrolysis of the amine-reactive NHS on the N2 molecule ($t_{1/2} = 4-5$ hours at pH 7.0 and 0°C) [134]. After ligand incubation, gels were rinsed with 1X PBS twice, sterilized in UV light for 20 minutes, and immediately used for experiments.
4.2.3 Mechanical Characterization of Gel Substrates

The mechanical properties of PAAm gels functionalized with peptide AG73 were measured using atomic force microscopy (AFM) indentation (MFP-3D, Asylum Research, Santa Barbara, CA), as described previously [12]. A cantilever with a nominal stiffness of 60 pN/nm and a 5 µm borosilicate spherical tipped probe (Novascan Technologies Inc.) was used to indent the PAAm gels of interest at a constant indentation rate of 15 µm/sec. The generated force-indentation curves were fit to the Hertz contact model for spherical indentation of a flat surface \( F = \left[ \frac{4ER_1^2}{3(1-v^2)} \right] \delta^{3/2} \) \[135\], where \( F \) is the normal indentation force, \( E \) is the Young’s modulus, \( R \) is the probe radius, \( v \) is Poisson’s ratio, and \( \delta \) is the indentation distance. A Poisson’s ratio of 0.45 was used based on previous tests by Engler et al. [62] and the curve fitting was done using MATLAB (Mathworks, Natick, MA).

For each gel formulation, at least three different gels were tested, at least two separate locations were tested on each gel, and 36 indentations were made in a 50 µm by 50 µm grid at each location, resulting in a minimum of 216 indentations per gel formulation. Elastic moduli were compared amongst gel formulations using a one-factor ANOVA (substrate) and Tukey’s HSD post-hoc test. Gel moduli were additionally measured for gels made from two different batches of pre-gel acrylamide solution for three acrylamide formulations (8%/0.15%, 5%/0.1%, and 8%/0.03% acrylamide/bis-acrylamide) to determine whether gel stiffness varied significantly with solution
preparation. Differences were tested using a 2-factor ANOVA (substrate, batch) and Tukey’s post-hoc test (n=3 gels per batch).

The AFM displacement measurements were additionally used to determine the surface roughness of each gel formulation. The standard deviation of the gel surface height for each 50 µm by 50 µm grid tested was calculated and averaged for each gel formulation. A larger average standard deviation in surface height indicates a more variable surface topography. Differences were tested using a 1-factor ANOVA and Tukey’s post-hoc test (n ≥ 6 per gel formulation).

For all following experiments, only 14 kPa and 0.3 kPa PAAm gel formulations were used to test the effect of NP cell biosynthesis and phenotype upon substrate stiffness.

4.2.4 Characterization of Peptide-Polyacrylamide Conjugation

Polyacrylamide gels (8%/0.15% or 5%/0.03% acrylamide/bis-acrylamide) were functionalized with 0.1, 0.05, 0.01, or 0 mM peptide GD6 conjugated to the fluorophore FITC (CGG(KQNCLSSRAS)FRGCVRNLRLSR – FITC, American Peptides). After conjugation, the gels were washed four times for 5 minutes each to ensure complete removal of unattached peptide.

Total peptide attached to each gel was determined using a plate reader to quantify fluorescence (n=4). Each functionalized gel was detached from the glass microscopy slide with a razor blade, finely diced, and suspended in 80 µL PBS and
placed in a clear 96-well plate. On the same plate, a standard curve of known fluorescent peptide concentrations (ranging from 100 µM to 1.5 µM) was plated in duplicate. The fluorescence intensity of each well was read using a Perkin Elmer EnSpire plate reader (490 nm excitation, 520 nm emission). All sample readings, except for the gels with no peptide attached, were above the lower limit of detection (LLOD = 3 µM) as determined by the linear portion of the standard curve. Peptide concentration for each sample was determined from the standard curve and total peptide per gel was calculated by dividing by the dilution factor.

To determine total gel height and depth of peptide conjugation, Z-stack images were obtained using confocal laser scanning microscopy (Zeiss LSM 510, Carl Zeiss, Thornwood, NY, USA) with 1µm thick optical slices (n≥3 gels). The gain for the green channel (488 nm excitation, 517 nm emission), used to image the FITC tagged peptide, was set to ensure maximal peptide concentration would not saturate any pixels. Peptide functionalization depth was calculated as the z-distance that the average pixel intensity for each planar image (each 1 µm thick) was over 25% of the image slice with maximum average pixel intensity. To determine the planar variation in peptide conjugation density, the standard deviation of pixel intensity for three 1 µm image slices per gel was calculated and averaged. An increase in standard deviation indicates more irregular planar peptide densities. Total gel height was determined by subtracting the z-
coordinate of initial FITC fluorescence from the z-coordinate of initial autofluorescence from the bottom glass microscope slide.

To visualize cell interaction with peptide conjugated gels, human NP cells expressing a CMV-mCherry constitutive reporter construct were seeded onto the gels at 20,000 cells/cm² and allowed to attach for 2 days. Representative z-stack images were taken on the confocal microscope to qualitatively identify if the cells penetrated the upper surface of the gel.

The final set of ligands used for all following experiments are LM-111 plated at 120 µg/mL and peptides AG73, P4, p678, and AG10 plated at 0.1 mM.

4.2.5 Analysis of NP Cell Clustering

Cell attachment and clustering was determined on soft (0.3 kPa) and stiff (14 kPa) substrates functionalized with five different ligands (LM-111, AG73, P4, p678, or AG10). Human NP cells (n = 4 patients, ages 54 - 63) were seeded onto the gels at 60,000 cells per cm² in culture media (F-12 media with 10% FBS and penicillin/streptomycin). For a discussion on the rationale for this cell seeding density, see Chapter 4.4.3. Cells were cultured on the gels at 5% CO₂ and 37 °C for four days in 400 µL of culture media before cell analysis.

To visualize the attached cells, the nuclei and filamentous actin (F-actin) were stained with propidium iodide and phalloidin, respectively. The gels were rinsed with PBS to remove unattached cells followed by a 10 minute incubation with 4%
formaldehyde at room temperature to fix the cells. The cells were then permeabilized with 0.2% triton for 5 minutes. Cell F-actin structure was labeled with Alexa 488 phalloidin (Invitrogen) at a 1:200 dilution for 20 minutes at room temperature. Cell nuclei were counterstained with propidium iodide (Sigma) at 1 mg/mL. Stained slides were imaged using confocal laser scanning microscopy (Zeiss LSM 510, Carl Zeiss). For each gel, images from three fields of view were captured.

The number of cells attached to each gel was determined by counting the nuclei in each field of view, averaging the three values for each gel, and extrapolating for the entire gel area (0.85mm² field of view, 0.7cm² gel area). Percent cell attachment was determined by dividing the attached cells by the total cells seeded per well (42,000 cells), averaged for all the gels, and differences between substrates was determined with a 2-way ANOVA (stiffness, ligand) and Tukey’s HSD post-hoc test.

Cell clusters were manually outlined and counted using Image J software (National Institute of Health, USA). The .lsm images from the confocal microscope were imported to Image J and converted to two-channel RGB image stacks. Cell clusters were defined as having at least 3 cells, no gap between cells in a cluster, and at least 25% of each cell’s perimeter was touching another cell in the cluster. The percent of attached cells in clusters, as well as the percent of attached cells in large clusters (>9 cells per cluster), was quantified for each field of view. These values were averaged for all gels of a given substrate, and differences between conditions was determined with a 2-way
ANOVA (stiffness, ligand). Tukey’s HSD post hoc test was used to test for differences between all conditions, and Dunnett’s post hoc test was used to test for differences as compared to 14 kPa LM-111.

4.2.6 NP Cell Clustering Optimization

During cell clustering protocol development, it became clear that cell seeding density plays a critical role in determining the number of cells that attach to laminin functionalized PAAm gels as well as the degree of cell clustering that occurs on soft laminin substrates. Past work investigating porcine NP cell attachment and clustering used a cell seeding density of 18,000 cells/cm$^2$ [12] for LM-111 functionalized PAAm gels and 31,500 cells/cm$^2$ for basement membrane extract (Matrigel) gels [27]. To determine the optimal cell seeding density for the experiments in this dissertation work, human NP cells (from 63-year-old NP tissue) were seeded onto 14 kPa and 0.3 kPa gels functionalized with LM-111 at 20,000, 40,000, 80,000, or 120,000 cells/cm$^2$ for four days. At day 4, the total cells attached and the size of each cell cluster was visualized and counted, as described previously (Chapter 4.2.5). Two gels were tested per condition and eight fields of view were taken per gel.

4.2.7 Sulfated Glycosaminoglycan Production

The production of sulfated glycosaminoglycans (sGAG) was determined on soft (0.3 kPa) and stiff (14 kPa) substrates functionalized with five different ligands (LM-111, AG73, P4, p678, or AG10). Human NP cells (n = 4 patients, ages 32 - 63) were seeded
onto the gels at 60,000 cells per cm² in culture media (F-12 media with 10% FBS and penicillin/streptomycin). Cells were cultured on the gels at 5% CO₂ and 37 °C for four days before cell analysis. The production of sGAG by NP cells was quantified using the dimethymethylene blue (DMMB) spectrophotometric assay [12]. At day four, media from each well (400 µL) was collected while the cells and substrates remaining in each well were digested in papain solution (125 µg/mL in PBS with 5 mM EDTA and 5 mM cysteine) for 4 hours at 55°C. As a control, media and a papain digest were collected from three cell-free wells (PAAm only). sGAG was quantified for each sample by mixing 40 µL of the sample with 125 µL DMMB dye and reading the absorbance at 535 nm on a plate reader (Perkin-Elmer Enspire Multimode Reader). The concentration of sGAG was determined using standard curves of chondroitin-4-sulfate (Sigma-Aldrich) diluted in either media or papain and corrected using reading from the cell-free control samples. The sGAG production was then normalized to DNA content (Quant-iT PicoGreen dsDNA kit, Invitrogen) for each sample. Differences in sGAG/DNA (µg/µg) amongst substrates were detected with a two-factor ANOVA (stiffness, ligand) and Dunnett’s post hoc analysis.

4.2.8 mRNA Extraction and Quantification

Cell from patients (ages 36-67, n=5) were plated at 60,000 cells/cm² on gels prepared from each of the ten experimental substrates. Eight wells were used per experimental substrate to obtain enough mRNA to test multiple PCR targets. The cells
were cultured for four days in culture media (as for Section 4.2.5). After four days, gels were gently rinsed with 1X PBS to remove unattached cells. Cells were detached with 0.025% trypsin/EDTA, spun down, and lysed with 350 µL of RLT buffer (Qiagen, Valencia, CA) with 1% β-Mercaptoethanol (Fisher Scientific). RLT buffer was not added directly to the gel substrates since the substrate would dissolve and inhibit subsequent steps. The cell lysate was stored at -80°C for up to 2 weeks before mRNA extraction and conversion to cDNA.

Total RNA was extracted with the RNAeasy Micro kit plus DNase I digestion (Qiagen). mRNA quality and concentration was determined on the ND-1000 Spectrophotometer (NanoDrop, Thermo Fisher Scientific) and reverse transcribed into cDNA using the iScript cDNA synthesis kit (Biorad). cDNA samples were diluted to a final concentration of 10 ng/µL using RNAse and DNase-free water.

qRT-PCR was performed on cDNA obtained from NP cells after four days of culture on each of the 10 substrates of interest. The cDNA was used to identify aggrecan (ACAN, Hs00153936_m1), type I collagen (Col1A1, Hs00164004_m1), type II collagen (Col2A1, Hs00156568_m1), N-cadherin (CDH2, Hs00983056_m1), and glucose transporter (GLUT) 1 (SLC2A1, Hs00892681_m1) (Life Technologies, Thermo Fisher Scientific). The housekeeping gene GAPDH (NM_002046.3) was used as an internal control for each sample. Duplicate PCR reactions were performed for each target gene for each RNA sample using the StepOnePlus real-time PCR system (Applied
Biosystems, Waltham, MA). Each well contained 12.5 µL 2x universal master mix (Applied Biosystems), 1.25 µL Taqman primer probes, and 9.25 µL ddH2O, and 2 µL cDNA. Relative gene expression changes were quantified amongst the ten substrate groups by comparing ΔCt values (Target Ct – GAPDH Ct). Fold change in expression was calculated using the delta-delta Ct method (Fold change = 2^ΔΔCt). The second Δ accounted for the fold change over cells cultured on LM-111 at 14 kPa. Differences in expression level for each gene was tested with a 2-way ANOVA (stiffness, ligand) with Dunnett’s post hoc analysis (Both p<0.1 and p<0.05 are noted). Additionally, differences between peptide and laminin substrates were tested for each gene (ANOVA)

4.3 Results

4.3.1 Mechanical Characterization of Gel Substrates

The atomic force microscopy (AFM) indentation curves correlated strongly with the Hertz model with a minimum R² value of 0.98 for all tests. Sample force-displacement curves for one 50 µm by 50 µm test grid are shown in Figure 20a.
Figure 20: Representative AFM data from an 8% acrylamide, 0.15% bis acrylamide gel probed 36 times in a 50 µm x 50 µm grid. Force-indentation curves (blue dotted lines) closely correlate to the Hertz spherical indentation model (red line) with an $R^2$ of 0.9981 (A). At the same gel location, a surface height and stiffness map is shown (B).

The elastic modulus of the tested hydrogels was found, as expected, to increase with increasing acrylamide and bis-acrylamide concentration. The tested gel formulations of 8% acrylamide with 0.15% bis-acrylamide, or 5% acrylamide with 0.1%, 0.04%, or 0.03% bis-acrylamide yielded elastic moduli of 14 kPa, 2.4 kPa, 0.5 kPa, and 0.3 kPa, respectively (Figure 21).
Figure 21: The mean elastic moduli (± SD) of polyacrylamide gel substrates for gels with 8% or 5% acrylamide and 0.15%, 0.1%, 0.04%, or 0.03% bis-acrylamide. Conditions not connected by the same letters are significantly different from each other (p<0.05, ANOVA, Tukey’s HSD post-hoc).

To test for batch-to-batch variability of pre-gel solutions, the elastic modulus was determined for two separate batches of 8% acrylamide/0.15 bis-acrylamide, 5% acrylamide/0.1% bis-acrylamide, and 5% acrylamide/0.03 bis-acrylamide gels. No differences between batches were detectable (p>0.05, ANOVA) (Figure 22). Elastic moduli magnitudes for the batch variation testing are different than the final elastic moduli (Figure 21) due to the use of an earlier gel protocol for this particular experiment (decreased peptide incubation time and a different glass slide).
Figure 22: The mean elastic moduli (± SD) of gels with 8% or 5% acrylamide and 0.15%, 0.1%, or 0.03% bis-acrylamide from two different pre-gel solution batches (A and B). No detectable differences were found between batches (p>0.05, 2-way ANOVA).

Previous work has detailed an increase in polyacrylamide gel surface creasing with decreasing elastic moduli [136]. To determine the surface roughness for different gel formulations, the standard deviation of the surface height for each 50 µm by 50 µm test location was calculated and averaged (n ≥ 6 per gel formulation) (Figure 23). There was no detectable difference in surface height variation between gel formulations with 8%/0.15%, 5%/0.1%, or 5%/0.04% Acrylamide/Bis (± 0.67, 0.69, and 0.77 µm, respectively). However, the 5% acrylamide 0.03% bis acrylamide formulation had a significant increase in surface height variation (± 1.47 µm, p<0.01, 1-way ANOVA (substrate), Tukey’s HSD post hoc).
4.3.2 Characterization of Peptide-Polyacrylamide Conjugation

The amount of the peptide covalently attached to PAAm gels increases with the concentration of the peptide applied to the gel for conjugation (Figure 24). There was no detectable difference in total conjugated peptide per gel between 14 kPa and 0.3 kPa gels (p>0.5, 2-way ANOVA (stiffness, applied peptide), Tukey’s HSD post-hoc). Gels functionalized with 0.1mM peptide, as used in the remainder of this dissertation, were found to have 4.98 ± 0.82 µg (1,522 ± 250 picomoles) of peptide conjugated to each gel. This amount equates to 7.6% of the total peptide applied to the gel during functionalization.
Figure 24: Amount of peptide (µg) conjugated to 0.3 kPa and 14 kPa polyacrylamide gels for varying amounts of applied peptide (0, 0.01, 0.05, and 0.1 mM). There is no detectable difference in conjugated peptide between 0.3 kPa and 14 kPa gels (p>0.5, 2-way ANOVA, Tukey’s HSD post-hoc).

The average depth of peptide functionalization was found to be 11.3 ± 2.1 µm from the surface of the gel (Figure 25). There was no detectable difference in peptide conjugation depth between 14 kPa and 0.3 kPa gels (p>0.4, ANOVA). The variation in peptide density, as measured by the planar variation in fluorescence intensity, was found to be 8 ± 2%. There was no significant difference in peptide density variation between 14 kPa and 0.3 kPa gels (p>0.3, ANOVA). The total gel thickness was found to be 84.6 ± 6.9 µm, with no difference in thickness between 14 kPa gels and 0.3 kPa gels (p>0.5, ANOVA).
Figure 25: Representative confocal z-stack images of fluorescently labeled peptides conjugated to a 14 kPa gel. Peptides (green, FITC) and cells (red, CMV-mCherry reporter) are shown in relation to each other, superimposed over a scaled diagram of the polyacrylamide gel (A). A three-dimensional isometric confocal image illustrates the even distribution of peptides throughout the gel (B).

The surface density of peptide can be approximated based on the known volume of peptide, total peptide conjugated, and the assumption that cells can only penetrate the gel by 1 nm [124]. While the imaging is not precise enough to measure 1 nm, Figure 25 demonstrates there is no qualitatively significant penetration of the attached cells into the gel. Plugging in the appropriate values (Total peptide = 1,522 picomole, peptide depth = 11.3 µm, gel area = 0.7 cm², cell penetration depth = 1 nm), we find a surface peptide concentration of 0.19 pM/cm² or 1,160 peptides/µm².
4.3.3 NP Cell Clustering Optimization

We found evidence of substantial differences in cell attachment and clustering when varying the initial cell seeding density (Figure 26). The percent cell attachment remains constant (49% ±5%) for the 0.4 kPa substrate for each cell seeding density; however, the percent cell attachment decreased with increasing cell seeding density for the 14 kPa substrate from 106% ±8% at 20,000 cells/cm$^2$ to only 20% ±1% at 160,000 cells/cm$^2$ (Figure 27a). In addition to percent cell attachment, the percent of cells in a cluster and the size of the cell clusters on the soft 0.3 kPa substrate was dependent on the cell seeding density (Figure 27b). Seeded at 20,000 cells/cm$^2$, 43% of the cells were single cells and only 15% of the cells were in a cluster composed of 6 or more cells. With increasing cell seeding density, there was a decrease in the number of single cells on each gel. The number of medium sized cell clusters (6-20 cells per cluster) peaked at a cell seeding density of 40,000 cells/cm$^2$ (57% of attached cells). At higher cell seeding densities, the number of cells in large clusters (>20 cells per cluster) increased, with a maximum at a cell seeding density of 160,000 cells/cm$^2$ (22% of attached cells). In summary, the cell seeding density affects the percentage of cells that attach to a substrate as well as the size of cell clusters on substrates that promote cell clustering. Note that single cells on both soft and stiff substrates are counted the same when looking at cluster size, despite obvious differences in morphology (cell spreading). It may be beneficial to additionally characterize the shape factor of single cells in future work.
Figure 26: Representative images visualizing the effect of cell seeding density on human NP cell attachment and clustering. Green = F-actin (phalloidin) and blue = cell nuclei (propidum iodide counterstain).
Figure 27: The effect of cell seeding density on (A) percent cell attachment to soft and stiff LM-111 functionalized substrates and (B) cell cluster size on a 0.3 kPa LM-111 substrate.

Based on these findings, we selected a cell seeding density of 60,000 cells/cm² for subsequent experiments. This seeding density ensures a similar percent cell attachment
for soft and stiff substrates while enabling significant cell clustering on soft substrates. The ability for a soft substrate to engage over twice as many cells per gel may be an important design consideration when developing future biomaterial therapies aiming to maximize cell delivery.

**4.3.4 Analysis of NP Cell Attachment and Clustering**

Greater than 50% of the initially seeded human NP cells attached to all substrates, except for peptides P4 and AG10 at 14 kPa, after four days of cell culture (Figure 28). Peptides P4 and AG10 at 14 kPa, with 13% and 39% cell attachment respectively, had significantly lower cell attachment as compared to LM-111 at 14 kPa (p<0.01, 2-way ANOVA (ligand, stiffness), Dunnett’s post hoc). LM-111 at 14 kPa had the highest cell attachment (77%), followed by 14kPa AG73, 0.3 kPa p678, 0.3 kPa AG73, and 0.3 kPa LM-111 (67%, 65%, 62% and 59% attachment, respectively).
Figure 28: Day 4 cell attachment as a percent of initially seeded cells. Peptides P4 and AG10 at 14 kPa have a significant decrease in cell attachment relative to LM-111 at 14 kPa (p<0.01, 2-way ANOVA, Dunnett’s post hoc)
Figure 29: Day 4 cell clustering. Percent of cells in clusters on each substrate (A) and percent of cells in a cluster with >9 cells (B). Conditions with * have a significant increase compared to 14 kPa LM-111 (p<0.05, 2-way ANOVA (ligand, stiffness), Dunnett’s post hoc)
Only 10% of cells were clustered on 14 kPa LM-111 substrates, consistent with expectations based on prior work of porcine NP cells on "stiff" laminin-rich PAAm gels [12]. All other substrate conditions, except 0.3 kPa AG73 (27% clustering), had a significant increase in cell clustering relative to 14 kPa LM-111 (p<0.05, 2-way ANOVA (ligand, stiffness), Dunnett’s post hoc) (Figure 29a). 0.3 kPa P4 promoted the greatest amount of cell clustering (81% of cells), a significant increase compared to 0.3 kPa p678, 0.3 kPa AG10, 0.3 kPa AG73, 14 kPa P4, and 14 kPa LM-111 (40%, 37%, 27%, 35%, and 10%, respectively) (p<0.05, 2-way ANOVA (ligand, stiffness), Tukey’s HSD post hoc). There was a significant increase in the percentage of cells in large clusters (>9 cells per cluster) for substrates 14 kPa AG73, 14 kPa p678, and 0.3 kPa P4 (37%, 25%, 23% of cells, respectively) relative to 14 kPa LM-111 (Figure 29b, p<0.05, 2-way ANOVA (ligand, stiffness), Dunnett’s post hoc). Qualitatively, only 14kPa LM-111 promoted cells to spread and form stress fibers (Figure 30), with a majority of cells upon other substrates attaching in rounded morphologies.
Figure 30: Representative day 4 images showing the effect of substrate stiffness and ligand for NP cell morphologies. Over 50% of attached cells interact to form multi-cell clusters on all substrates except 14 kPa LM-111 and 0.3 kPa AG73. Spread cells with stress fibers were only present on stiff LM-111 substrates. Green = F-actin (phalloidin) and blue = cell nuclei (propidum iodide counterstain).
4.3.5 Sulfated Glycosaminoglycan Production

Soft substrate stiffness significantly increases sGAG production relative to 14 kPa substrates (p<0.03, ANOVA, Dunnett’s post-hoc) (Figure 31). In comparison to 14 kPa LM-111 (5.7 µg/µg), peptides p678 and AG10 at 0.3 kPa produce significantly more sGAG/DNA (17.1 and 20.8 µg/µg, respectively) (p<0.05, 2-way ANOVA (ligand, stiffness), Dunnett’s post hoc).

![Graph showing sGAG production normalized to DNA content for different substrates](image)

**Figure 31**: Day 4 NP cell sGAG production normalized to DNA content. Substrates 0.3 kPa p678 and 0.3 kPa AG10 produce a significant increase in sGAG/DNA compared to 14 kPa LM-111 (p<0.05, 2-way ANOVA, Dunnett’s post hoc)

4.3.6 mRNA Quantification

qRT-PCR data revealed that the tested substrates are able to modulate expression of aggrecan, n-cadherin, and type I collagen, but not type II collagen and GLUT-1 (Figure 32). All peptide substrates except 14 kPa AG73 and 14 kPa P4 significantly reduced type I collagen expression relative to 14 kPa LM-111(p < 0.05, 2-way ANOVA
(ligand, stiffness), Dunnett’s post hoc). Cells on 0.3 kPa AG73 gels had a significant increase in aggrecan expression (p=0.053, 2-way ANOVA, Dunnett’s post hoc). There was a significant increase in aggrecan (p<0.05), n-cadherin (p=0.051) and a decrease in type I collagen (p<0.01) when looking at all peptide substrates compared to LM substrates (ANOVA). There was no detectable difference in type II collagen and GLUT-1 expression between all substrates.
Figure 32: Gene level expression (±SE) of aggrecan (A), n-cadherin (B), type I collagen (C), type II collagen (D), and GLUT-1 (E) for each substrate as determined by qRT-PCR. Significance compared to 14 kPa LM-111 is denoted by * (p<0.05) and # (p<0.1) (2-way ANOVA, Dunnett’s post hoc)
4.4 Discussion

4.4.1 Polyacrylamide Gel Methods

Polyacrylamide gels are frequently used when studying cell-substrate interactions [12, 56, 58, 61]. However, in our experiences in the laboratory, the typical PAAm gel fabrication method introduces significant variation in gel stiffness and functionalization. In this work we set out to reduce the variability of each gel.

The first design parameter we focused on optimizing was the method for covalently attaching ligands to the surface of the gel. Most of the PAAm work in the literature (such as [12, 56, 58, 61]) uses the ultraviolet (UV) light-activated heterobifunctional crosslinker Sulfo-SANPAH. On one end, a photoactivatable nitrophenyl azide is used to attach the crosslinker to the PAAm gel. On the other end, an amine-reactive N-hydroxysuccinimide (NHS) ester is used to attach a primary amine-presenting ligand to the gel.

In our experience, we found this crosslinker to be unreliable. After applying Sulfo-SANPAH to the surface of a PAAm gel and exposing it to UV light, there was a clearly visible irregular distribution of the Sulfo-SANPAH on the surface of the gel (Sulfo-SANPAH is colored a bright red). Additionally, the process of exposing the Sulfo-SANPAH solution to UV light wastes critical time during which the NHS ester can hydrolyze. At a pH of 8.0 and 25°C the half-life of the NHS group is only 1 hour, and that decreases to only 10 minutes at pH 8.6 at 0°C [134]. The half-life can be increased by
decreasing the pH, with a half-life of 4-5 hours at pH 7.0 and 0°C [134]. However, for the NHS group to react with a primary amine on your ligand of choice, the solution should be between pH 7 and 9.

In preliminary experiments we tested the conjugation of LM-111 and select peptides to PAAm gels using sulfo-SANPAH by plating NP cells on the different substrates. There was an unacceptably high variation in cell attachment rate and cell morphology from gel to gel in these experiments. We hypothesized that the variation may have been caused by an irregular distribution of the sulfo-SANPAH crosslinker on the gel and/or varying amounts of active sulfo-SANPAH on the gel when the ligands were added.

To improve ligand conjugation to our PAAm substrates, we adopted a functionalization protocol used by Dr. Cynthia Reinhart-King and Dr. Daniel Hammer [124]. They use the bifunctional crosslinker N-succinimidyl ester of acrylamideohexanoic acid (N6). This crosslinker is put into the pre-gel acrylamide solution at pH 6.0 and the acrylamide portion of the crosslinker covalently attaches to the gel as it polymerizes. The amine-reactive NHS groups are now evenly distributed throughout the volume of the PAAm gel, while the low pH preserves the NHS group until ligand is added to the gel at pH 8.5. For our work, we used the crosslinker acrylic acid N-hydroxysuccinimide ester (N2), which has the same functional groups as the N6 crosslinker, but with no spacer between the acrylamide and the NHS group. We custom ordered the N2 molecule from
CovaChem in pre-aliquoted 15 mg vials packaged under nitrogen gas. A fresh 15 mg vial of N2 was opened for each batch of gels to prevent the NHS group from hydrolyzing over time due to moisture in the air. The pre-aliquoted N2 linker additionally decreased variability due to weighing errors. Figure 33 illustrates the chemistry used for our experiments in Chapter 4.

Figure 33: Schematic of polyacrylamide gel chemistry using the crosslinker N2 to introduce amine-reactive NHS groups.

The second design parameter we optimized was the method for shaping the PAAm gel and encompassing it in a media chamber for cell culture. There is the opportunity for significant error introduction when placing and removing the coverslip used to flatten the polymerizing acrylamide solution onto a glass slide. Too much application pressure can result in lost acrylamide volume. Variations in the force vector
used to remove the coverslip after polymerization can visibly alter the surface
topography of the gels, particularly for gels with an elastic modulus under 1 kPa. Most
PAAm protocols use small circular coverslips, between 12 and 25mm in diameter, to
form individual gels that are then placed in wells for tissue culture. To decrease gel
formation variability, we formed larger gels (24 x 60 mm) and then clamped on 8-well
media chambers (Millicell EZ slide, Millipore). This simple change allows gels to be
made eight times faster and with fewer chances for human error.

4.4.2 Characterization of Polyacrylamide Gel Substrates

Mechanical characterization of the PAAm gels showed highly repeatable elastic
moduli, as determined by atomic force microscopy (AFM), for each gel formulation. The
elastic moduli for each of our gel formulations were of comparable values to those seen
in the literature for similar PAAm gel formulations [12, 137, 138]. The slight differences
in values could be from multiple factors including gel formation methods (gelation time,
functionalization chemistry, pre-gel degassing protocol, etc.) and measurement details
(indentation rate, AFM probe, Poisson ratio used, e.g.).

The standard deviation of the moduli for our gels is higher than reported by
some in the literature [12]. However, the previous work only tested one gel in many
locations per gel formulation. Here, we determined the elastic modulus for multiple
locations per gel, multiple gels per batch of pre-gel acrylamide solution, and multiple
batches of pre-gel acrylamide solution per gel formulation. This thorough testing
enables our standard deviations to give a better idea of true modulus variation for each
gel formulation. For a given test location, the elastic modulus was found to be uniform,
with a standard deviation under 10% for all gel formulations except the softest 0.3 kPa
gel (±18%). The soft 0.3 kPa gels also showed a slight but significant increase in surface
roughness relative to the other gel formulations (±1.5 μm vs ±0.8 μm). The increase in
variability of the elastic modulus and surface roughness for 0.3 kPa gels could be due to
the actual gel mechanics or the measurement methods. Difficulties were encountered
when measuring the elastic modulus of the 0.3 kPa PAAm gels due to the AFM probe
tip occasionally sticking to the gel. The force-displacement curve would be clearly
shifted when sticking occurred, with the force going negative when the probe tip was
lifted. When this occurred, we would simply re-test that gel location and often the probe
would not stick on the second try. The sticking events may have been due to gel voids or
surface features that were able to entrap the 5 μm diameter AFM probe tip.

Of the tested gel formulations, 8% acrylamide/0.15 % bis acrylamide and 5%
acrylamide/0.03% bis acrylamide were selected for use with peptide functionalized
experiments. These two stiffnesses were selected to maximize the range of elastic moduli
tested. Previous work in Dr. Setton’s laboratory has shown a shift in porcine NP cell
phenotype on laminin functionalized materials at around 0.7 kPa [12]. While 5%
acrylamide/0.04% bis acrylamide was below this threshold (0.5 kPa), the standard
deviation indicated about 10 percent of the gel would have an elastic modulus above the
0.7 kPa threshold. In contrast, less than 0.1 percent of the 5% acrylamide/0.03% acrylamide gel is above the 0.7 kPa threshold. This benefit was determined to outweigh the potential drawbacks of using a gel with a slight increase in surface roughness.

The gels, incubated with 0.1 mM peptide, were found to have a surface peptide concentration of 0.19 pM/cm² or 1,160 peptides/µm². This peptide density is comparable to the values found by Dr. Reinhart King for similar PAAm gels [124]. Ligand density is a critical parameter for modulating cell attachment [139]. Previous work had determined a minimum peptide density of 6 peptides/µm² for cell spreading and 60 peptides/µm² for the formation of focal adhesions when fibroblasts were seeded on RGD functionalized glass [140]. Our peptide density is well above these values, so cells should be capable of attaching and spreading if the substrate stiffness and ligand permit. On non-fouling interpenetrating polymers, others have looked at the impact of RGD density on endothelial cell function [141]. The work found an increase in cell adhesion and spreading up to 9,000 peptides/µm², a plateau, and then a further increase beyond peptide densities of 17,000 peptides/µm². A minimum peptide density of 1,200 peptides was found to activate extracellular signal-related kinase (ERK). This work by Patel and co-workers suggests our final peptide density might not maximize cell spreading, but is high enough to activate certain cell signals such as ERK.
4.4.3 NP Cell Behavior on LM-Mimetic Substrates

Both ligand and substrate stiffness were found to influence human NP cell clustering. All substrates, except peptide AG73 at 0.3 kPa and LM-111 at 14 kPa, increased the percentage of clustered cells relative to 14 kPa LM-111. The results for LM-111 correspond with the earlier finding for young porcine NP cells, which showed an increase in cell clustering for substrates under 0.7 kPa [12]. A decrease in substrate stiffness has previously been shown to promote cell-cell interactions and cell clustering [12, 57, 142]. This shift in cell clustering was not seen for peptides P4, p678, and AG10, and was the reverse for peptide AG73. While the mechanism for this effect was not directly studied, cells generally cluster when cell-cell adhesion is preferred over cell-ECM interactions. The shift in cell clustering for peptides may be due to a shift in receptor expression levels between soft and stiff substrates. Regardless, these peptides could be a useful tool for promoting cell clustering, if desired, on stiffer substrates. Future studies should test stiffer substrates to see if there is a stiffness threshold for these peptides that would induce cell spreading instead of clustering.

Of the cells in clusters, certain substrates were found to promote larger cluster sizes (Peptides AG73 and p678 on 14 kPa gels and peptide P4 on 0.3 kPa gels). This may be due to altered ligand-receptor affinity on these substrates, promoting cell migration and eventually cell-cell adhesions instead of more numerous cell-matrix adhesions.
Soft substrates functionalized with peptides p678 or AG10 significantly increase sGAG/DNA production as compared to 14 kPa LM-111 (17.7 and 20.8 µg/µg vs. 5.7 µg/µg for 14 kPa LM-111), despite limited differences in cell clustering morphology. Though not significant, there appears to be an increase in sGAG production for LM-111 on soft gels (9.1 µg/µg) as compared to stiff (14 kPa) LM-111 (5.7 µg/µg). The values are comparable to those shown previously for young porcine NP cells on soft laminin substrates (between 10 and 40 µg/µg) [12, 27, 127].

qRT-PCR additionally suggests a shift in NP behaviors for select substrates. There was a significant increase in aggrecan and n-cadherin mRNA and a significant decrease in type I collagen mRNA on peptide substrates relative to laminin substrates. No significant differences in type II collagen or GLUT-1 expression levels were observed with variation in substrate stiffness or peptide; however, there appears to be a trend towards higher type II collagen expression for AG73 and 0.3 kPa AG10, p678, and P4. Previous work showed a ~4-fold increase in n-cadherin and aggrecan as well as a ~2-fold increase in type II collagen expression for porcine NP cells cultured on soft versus stiff laminin substrates [27]. The values found in this dissertation are of similar magnitudes, though significance was not detected. Greater sample variation is expected in primary human cell samples, and a larger sample size is likely necessary to detect further differences in gene expression between substrates.
4.4.4 Porcine Versus Human Cell Attachment

The work in this dissertation discusses the behavior of adult human NP cells on a variety of substrates, and proceeds to compare the findings with previous studies with healthy porcine NP cells. As a comparison, we looked at porcine and human NP cell attachment to stiff (14 kPa) PAAm gels functionalized with LM-111, AG73, P4, p678, or AG10 (Figure 34). NP cells from two patients and two pig spines were seeded at 80,000 cells/cm² on each condition (n=3 gels per patient/pig) and cells were counted after four days of culture using microscopy (n=5 fields of view per gel). The cell seeding density is higher than the other work in this dissertation due to this experiment taking place before cell clustering optimization was completed.

A significantly lower number of porcine cells attached to peptides P4 and p678 as compared to human cells to those same substrates (p<0.05, 2-way ANOVA (ligand, cell type), Tukey’s HSD post-hoc). We hypothesize this is due to the differential mechanism for porcine and human NP cell attachment to laminin proteins. Porcine NP cells may use integrin α6 to attach to peptide AG10, but a deficiency in integrin α3 maybe inhibit their ability to attach to peptides P4 and p678. Porcine NP cells readily attach to peptide AG73, indicating syndecans play a role in the ECM environment of porcine NP tissues.
Figure 34: Percent attachment of human and porcine NP cells to 14 kPa polyacrylamide gels functionalized with LM-111, AG73, P4, p678, or AG10. Conditions not connected by the same letters are significantly different from each other (p<0.05, ANOVA, Tukey’s HSD post-hoc).

4.4.5 Summary

The findings of this chapter indicate both substrate ligand and substrate stiffness play a role in regulating human NP cell behavior. With the goal of identifying substrate conditions that promote healthy, young NP cell behaviors, it is important to consider the findings of this chapter as a whole. To that end, Figure 35 displays a heat map of the major findings for cells attaching to varying peptide-conjugated surfaces to help visualize trends between substrates. Cell attachment (% cells) and clustering (% cells in clusters or in large clusters) are visualized in Figure 35a, with higher values for each quantity colored green. Phenotypic markers of a healthy/young NP cell are shown in Figure 35b, with green indicating values characteristic of a ‘healthier’ cell phenotype.
The numbers shown in the Figure 35b cells indicate the fold change for each marker relative to 14 kPa LM-111. Most apparent is the fact that all conditions promote a healthier NP cell phenotype as compared to 14 kPa LM-111, though 14 kPa LM-111 promotes the highest degree of cell attachment. There does not appear to be large differences for peptide ligands on soft versus stiff substrates. Of the peptides tested, AG73 appears to be one of the most supportive of the “healthy” NP cell phenotype at both 0.3 kPa and 14 kPa while P4 is one of the worst performing peptides.

<table>
<thead>
<tr>
<th></th>
<th>14 kPa</th>
<th></th>
<th>0.3 kPa</th>
</tr>
</thead>
<tbody>
<tr>
<td>AG73</td>
<td>67%</td>
<td>52%</td>
<td>65%</td>
</tr>
<tr>
<td>P4</td>
<td>13%</td>
<td>39%</td>
<td>69%</td>
</tr>
<tr>
<td>p678</td>
<td>62%</td>
<td>51%</td>
<td>55%</td>
</tr>
<tr>
<td>AG10</td>
<td>14%</td>
<td>25%</td>
<td>23%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>14 kPa</th>
<th></th>
<th>0.3 kPa</th>
</tr>
</thead>
<tbody>
<tr>
<td>AG73</td>
<td>2.6</td>
<td>1.8</td>
<td>3.1</td>
</tr>
<tr>
<td>P4</td>
<td>1.3</td>
<td>2.4</td>
<td>1.5</td>
</tr>
<tr>
<td>p678</td>
<td>1.7</td>
<td>1.5</td>
<td>1.4</td>
</tr>
<tr>
<td>AG10</td>
<td>3.1</td>
<td>4.8</td>
<td>3.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 35: A heat map comparing the findings of this chapter for the peptide substrates, with color scaling performed across all substrates within each tested variable. Percent of seeded cells attached to each substrate, percent of attached cells in a cluster, and percent of attached cells in a large cluster (>9 cells) are shown in (A). sGAG biosynthesis and gene expression of aggrecan, n-cadherin, type I collagen, type II collagen, and GLUT-1 as a fold change relative to 14 kPa LM-111 are shown in (B).

4.5 Conclusion

The results in this chapter identify specific laminin-mimetic peptides and substrate stiffnesses that are able to promote adult degenerate human NP cell clustering, increased sGAG production, and changes in the expression of key genes.
Polyacrylamide gels were able to tightly control ligand presentation and substrate stiffness, isolating the cellular effects of both variables. Identification of these ligands and substrate stiffnesses that promote a younger, healthier NP phenotype provide important design considerations for a variety of intervertebral disc therapies. The main conclusions of this chapter include:

1. ** Culturing adult human NP cells on soft substrates presenting laminin-mimetic peptides increases cell clustering and proteoglycan production, confirming Hypothesis 4.A. ** NP cells attach to soft (0.3 kPa) and stiff (14 kPa) polyacrylamide gels functionalized with peptides AG73, P4, p678, and AG10 at levels comparable to LM-111. All peptide substrates, except peptide AG73 at 0.3 kPa, increased the percentage of clustered cells relative to 14 kPa LM-111. Additionally, soft substrates significantly increase sGAG production relative to stiff substrates, with peptides p678 and AG10 at 0.3 kPa increasing sGAG/DNA production over three-fold relative to 14 kPa LM-111.

2. ** Ligand presentation and substrate stiffness modulate the size of cell clusters after four days of culture, confirming Hypothesis 4.B. ** Peptides AG73 and p678 on 14 kPa gels and peptide P4 on 0.3 kPa gels induced a significant increase in large cluster (>9 cells/cluster) formation compared to 14 kPa LM-111.
3. Peptides are able to increase gene expression of aggrecan, n-cadherin, and decrease expression of type I collagen, partially confirming Hypothesis 4.C. None of the tested substrate ligands or stiffnesses were able to impact the gene expression of type II collagen or glucose transporter GLUT-1.
5. Conclusions and Future Directions

The extracellular matrix environment is a key regulator of cell behaviors. Previous work has demonstrated that ligand and substrate stiffness can direct organization, biosynthesis, adhesion, migration, and phenotype for a variety of cell types. Healthy, young NP cells have been shown to interact with laminin proteins in an integrin-dependent manner, with an increase in cell clustering and proteoglycan production when cultured on soft laminin-rich substrates. It was unknown if soft laminin substrates could similarly induce a young and healthy phenotype for adult degenerate human NP cells. The central hypothesis of this dissertation is that the engagement of cell-surface adhesion receptors, using laminin-mimetic peptides on a controlled stiffness material, can revert adult degenerate NP cellular phenotype and behaviors to their healthy, biosynthetically active form. This hypothesis was tested in the following studies:

1. In Chapter 2 of this dissertation, integrin subunits expressed by adult human NP cells were identified via flow cytometry and immunohistochemistry. Functional integrin subunits used by adult human NP cells to interact with the IVD protein laminin 111 and 511 were investigated using function-blocking antibodies.

2. In Chapter 3, the role of laminin-mimetic peptide ligands in modulating adult human NP cell attachment was examined. Laminin-mimetic
peptides were chosen from the literature, informed by NP cell integrin expression (Chapter 2), and screened for increased NP cell attachment. Peptides were adsorbed at a range of concentrations on stiff tissue culture plastic and the number of cells attached to these substrates was determined after one hour of cell attachment.

3. Finally, in Chapter 4, the role of substrate ligand and stiffness in modulating adult human NP cell behavior was investigated by culturing NP cells on soft (0.3 kPa) and stiff (14 kPa) polyacrylamide (PAAm) hydrogels functionalized with select laminin-mimetic peptides. Cell behavior was quantified after four days of culture on these substrates by analyzing cell clustering, proteoglycan production, and gene expression.

Chapter 2 demonstrated that adult human NP cells highly express integrin subunits β1, α3 and α5 both in vitro and in vivo. Human NP cells engage laminin isoforms LM-111 and LM-511 as well as type II collagen in an integrin-dependent manner, with EDTA and anti-integrin β1 antibodies inhibiting the majority of cell attachment to these proteins. Attachment to LM-111 and LM-511 was found to not use one integrin alpha subunit, but a combination of subunits α3 and α5, as well as possibly α1 and α2. This finding is in contrast to the single α6 subunit for healthy porcine cells, indicating a shift in cell phenotype with age and/or degeneration. These studies
confirmed that human NP cells engage ECM proteins in an integrin-dependent manner. Furthermore, the identification of integrin subunits that are both expressed and used by NP cells was used to help inform the selection of peptides to be screened in Chapter 3.

Chapter 3 found that adult human NP cells attach to select laminin-mimetic peptides (AG73, IKVAV, GD6, P4, and AG10) in a concentration dependent manner and at rates comparable to full-length laminins 111 and 511. This result identifies peptides of interest for further study in Chapter 4 for their effect on NP cell behaviors when conjugated to soft and stiff gel substrates.

Finally, Chapter 4 found that both peptide ligand and substrate stiffness impact human NP cell behavior. PAAm gels were able to tightly control ligand presentation and substrate stiffness, enabling the detection of differences in cell behavior for each substrate condition. Culture of NP cells on different PAAm substrates revealed an increase in cell clustering on most peptide functionalized substrates, an increase in large cell clusters on specific peptides at 14 kPa, and an ability to increase proteoglycan production on soft (0.3 kPa) substrates. Lastly, peptide substrates were able to promote a healthier NP cell gene expression for aggrecan, n-cadherin, and type I collagen.

The findings presented in this dissertation prompt several future areas of study:

1. Studies in Chapter 2 demonstrate that adult human NP cells use multiple integrin subunits to attach to LM-111 and LM-511. It is unknown how
each of the subunits contributes to cell morphology and behavior beyond initial cell attachment. Integrin inhibition experiments, possibly through the use of CRISPER constructs, could shed light on the down-stream effects of individual integrin subunits. This information could further assist with biomaterial design for degenerate IVD therapies.

2. Studies in Chapter 3 identify peptides, mostly reported to bind specific integrin subunits, which are able to promote adult human NP cell attachment. However, it is unknown what integrin, or non-integrin, cell adhesion receptors are used by human NP cells to bind to each peptide. As reported by others, some of the peptides may be able to bind multiple cell adhesion receptors. Further function blocking experiments, as performed in Chapter 2, could further illuminate the mechanisms used.

3. Chapter 4 identifies substrate conditions that are able to promote aspects of a healthy NP cell over a four day period. While an important first step, it will be important to look at cell behavior at longer time-points. The majority of potential therapies that would take advantage of the design criteria discussed in this dissertation would require long-term cell culture, and it is important that these therapies would provide a beneficial effect throughout their use.
4. The ligands in Chapter 4 were all studied individually, and there is the potential for improved therapeutic activity with the presentation of multiple peptide ligands simultaneously. For example, peptides AG73 and p678 seem to complement each other on soft substrates, with one increasing cell clustering and gene expression while the other induces higher levels of sGAG biosynthesis. A study looking at the effect of peptide combinations on NP cell behavior, based on the results shown in Chapter 4, may lead to substrates with an improved ability to promote a young/healthy NP cell phenotype.

5. The markers of a healthy NP cell used in Chapter 4 have all been shown to be important in the field of IVD regenerative medicine. However, their clinical importance is poorly understood. For further material development, it will be important to look at criteria known to be relevant to the clinic. One possible approach would be to look at cell survival and behavior during and after a variety of challenges that would be present in an IVD undergoing surgery, such as high levels of cytokines. The growth condition in this dissertation have all been optimized for NP cell culture, and do not accurately portray the diseased IVD environment.

6. The work in this dissertation is all performed in two dimensions. However, most cell therapies for the IVD would require the use of three-
dimensional tissue constructs. It is unknown if the findings of the work presented here will translate to three dimensions. One possible approach would be to functionalize polyethylene glycol (PEG) hydrogels with the peptides in Chapter 4. PEG gels are frequently used for three dimensional tissue constructs, and are similar to PAAm in that it is a non-fouling material with tunable stiffnesses in a physiologically appropriate range. Understanding the effects of LM-mimetic peptides in a three-dimensional material with further the field’s ability to design NP-specific biomaterials.

In summary, this dissertation demonstrated that the engagement of cell-surface adhesion receptors, using laminin-mimetic peptides on PAAm gels with controlled stiffnesses, may promote a young and healthy phenotype in adult degenerate human NP cells as determined by cell clustering, proteoglycan production, and gene expression. This finding highlights the importance a cell’s surrounding extracellular matrix environment when designing culture systems, particularly for clinical therapies. The work here identified laminin mimetic peptides and substrate stiffnesses that may be useful for therapies aiming to revert the degeneration of IVD tissues with age and use.
References


Biography

Devin Thomas Bridgen was born on November 29th, 1984 in Manchester, Connecticut to Dr. Mark Bridgen and Margot Bridgen. He attended Cornell University and earned a Bachelor of Science degree in Biological Engineering Cum Laude in 2007. Later that year, he earned a Masters of Engineering degree from Cornell University, where he studied the mechanical effects of crosslinking collagen gels with riboflavin.

After Cornell, he worked briefly as an R&D engineer at a startup company Sadra Medical, developing a bovine pericardium trans-aortic valve implant. He was awarded an International Whitaker Fellowship in 2008 to work with Dr. Ivan Martin at University Hospital Basel, Switzerland, where he developed a mini perfusion bioreactor to study the relationship between fluid flow and cell growth in three dimensions.

While at Duke, Devin was named a James McElhaney Fellow in Biomedical Engineering. His publications include:


In addition to his research efforts, Devin held several offices in graduate student government. He was a Board of Trustees Academic Affairs Committee member, the BME department’s representative to the Graduate and Professional Student Council, and the Executive Treasurer for the Engineering Graduate Student Council. In his free time, Devin enjoys playing competitive volleyball and adventuring in the North Carolina wilderness.