BIOMOLECULARLY AND MECHANICALLY INSTRUCTIVE MATERIALS FOR GUIDING CELL-SUBSTRATE INTERACTIONS

BY

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DISSERTATION

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Abstract

Techniques that enable the creation of instructive biomaterials have the potential to advance our understanding and ability to guide cell fate and function. Benzophenone (BP) photolithography is uniquely suited to generating chemically and mechanically tailored materials because of its molecularly general and spatially modulated characteristics. Here, we describe the application of this methodology to investigate two important biological processes in the context of mechanical and biochemical cues. First, we generated gradients of cellular adhesion molecules that allowed us to understand the mechanism by which bromelain affects cell rolling during the early steps of inflammation. The latter part of this work utilized BP-mediated immobilization on two substrates, collagen-glycosaminoglycan membranes and polyacrylamide hydrogels, to evaluate stem cell differentiation. In summary, we have developed a photoreactive platform that provides the capability to control biomolecule density separately from mechanical properties. This strategy can be used on a variety of biorelevant substrates to replicate the properties of the native extracellular matrix. Our results shed light on the combinatorial influence of multiple cues and have the potential to identify key parameters to specifically alter cell response with a view towards tissue regeneration.
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Chapter 1
Introduction and Background

1.1 The extracellular matrix: a complex and dynamic environment

The extracellular matrix (ECM) is a hydrated network of proteins surrounding cells that provides biomolecular, mechanical, and spatial cues. It consists mainly of fibrillar structural proteins (e.g. collagen) mixed with glycosaminoglycan sugars and additional adhesive proteins (e.g. fibronectin). The ECM is synthesized and remodeled to some extent in healthy tissue through the action of matrix metalloproteinases (MMPs), but dysregulation is associated with tumor metastasis, cardiovascular disease, and other pathological states. Matrices play an important role in tissue development and function by controlling cell growth, migration, and differentiation. In addition to providing physical support, the ECM mediates cytokine signaling via sequestration of soluble growth factors. Furthermore, the cues described are not simply “on” or “off,” but have precise spatiotemporal patterns critical to proper tissue development and function analogous to embryonic development.

Cells in the human body constantly encounter an array of stimuli that influence their behavior and tissue-specific processes. The interaction of cells with adhesive surfaces is especially complex due to its reciprocal nature. Underlying substrate stiffness affects cell adhesion, spreading, and differentiation. Cells adhere to their surroundings through specific integrin receptors, which recruit focal adhesion complexes that bind the actin cytoskeleton to the ECM. Moreover, growth factors have been shown to modulate the activity of integrins in the cell membrane; for example, platelet-derived growth factor (PDGF)-BB stimulates integrin synthesis, and modulates integrin mobilization and focal adhesion dissociation. In turn, integrins can repress growth factor receptor availability through internalization (e.g. integrin-mediated bone morphogenetic protein receptor endocytosis). Recent studies indicate that control of cell spreading, through manipulation of substrate stiffness or adhesion geometry or area, strongly influences cell growth and differentiation. Unraveling the influence of physical factors and growth factors, which are known to
activate the same signaling pathways inside the cell, is a key challenge in understanding and regulating cell fate.

Regenerative medicine, through controlling and directing the healing process, has tremendous potential in the clinical setting. The body has the capacity to respond to tissue injury through inflammation and subsequent repair. Both the inflammatory and regenerative processes are tightly regulated through release and recruitment of growth factors coordinated by the ECM. A better understanding of cellular response in tissue inflammation and repair, leading to more effective strategies to harness this knowledge for therapeutic applications, is the motivation behind this dissertation. Improved methods for generating instructive materials will be critical for studying the molecular basis of cellular behavior. The ability to create biochemically defined material surfaces is an important tool for advancing this research.

1.2 Surface modification techniques to create custom cell microenvironments

A key challenge in current biomaterials research is to quantitatively determine cell response to individual and multi-parameter inputs. Early in vitro studies relied on supplementation with freely soluble factors to explore cell response; however, this approach is limited by diffusion away from the desired area and does not recapitulate the native immobilization of bioactive molecules to the ECM. Furthermore, immobilized biomolecules have been shown to have enhanced bioactivity compared to controlled release of soluble factors.\(^\text{11}\) Importantly, immobilized biomolecules have the potential to be spatially patterned in a manner characteristic of the microenvironment in which cells live. A myriad of techniques have been developed to achieve localized biomolecule presentation. Physical adsorption is the simplest approach, but may not be stable over time. Self-assembled monolayers (SAMs) of alkanethiols present an attractive strategy for controlling surface modification.\(^\text{12}\) Spatial patterning can be achieved by directly depositing biomolecules using an AFM tip (dip-pen nanolithography, DPN). DPN allows for extremely high-resolution patterns, but it is slow and not multiplexable, so its usefulness is limited to applications requiring nanoscale features.\(^\text{13}\) Additionally, SAMs are limited to gold or silver surfaces. Patterns may also be created using microcontact-based printing, the use of which has since been expanded beyond SAMs to substrates such as polystyrene.\(^\text{14}\) Another direct-write patterning method, inkjet printing, allows immobilization without surface contact and can be applied to more biologically relevant surfaces.\(^\text{15}\) Yet none of these techniques offer the capability to generate discrete, immobilized, bioactive patterns and gradients of different classes of biomolecules without elaborate experimental configurations.
Photolithography is a powerful approach that combines excellent throughput, the flexibility to create features of arbitrary shape and size, and high resolution (below the scale of single cells). To create patterns, a photoreactive compound is exposed to light in a spatially defined manner resulting in covalent attachment of the ligand of interest. Other lithography techniques, such as electron beam or ion beam lithography, require exposure to extreme environments (e.g., high vacuum)\textsuperscript{16} and so cannot be used for bioactive molecules without affinity-based schemes that require exogenous functionalization of the molecules to be patterned.\textsuperscript{17} Like other affinity-based conjugation techniques, the number of species that can be bound is limited. In contrast, photolithography can be carried out without the need for functionalizing the molecule of interest. All of the work described in this dissertation relies on photolithography to generate instructive materials.

1.3 Surface patterning and benzophenone: using light as a reagent to enable instructive biomaterials

A number of ligands useful for generating covalent bonds in response to light have been explored in photoaffinity labeling literature. Among the available classes of molecules that could be incorporated into biomaterials, benzophenone (BP) compounds have a number of distinct advantages. Benzophenone undergoes an $n \rightarrow \pi^*$ transition upon excitation with UV light, forming a triplet diradical state. The excited species then abstracts a hydrogen from a nearby C-H bond and recombines, which is the most favorable conjugation. Alternatively, an electron transfer followed by proton abstraction and radical recombination may occur (e.g., from amines).\textsuperscript{18} Ideally, the photoreactive moiety should not require any specific functional group but instead have rather broad reactivity; hence, the preferential reaction of benzophenone with C-H bonds is valuable for attaching a variety of biomolecules. The absorption of UV light around 360 nm is advantageous, since shorter, more energetic wavelengths could damage sensitive biomolecules, while higher wavelengths would cause instability in ambient light. An important advantage of photolithography is the ability to create overlapping patterns by serial exposure of the substrate using different photomasks. The ability of benzophenone to relax to the ground state and be re-excited is essential to this application. In contrast, many other photoprobes undergo irreversible excitation. Furthermore, benzophenone is more stable than diazo esters, aryl azides, and diazirines.\textsuperscript{18} Overall, benzophenone-mediated photocova lent attachment is optimal as a molecularly general technique for immobilization on bioactive surfaces.

Photolithography using benzophenone has been shown to be a useful surface patterning methodology for immobilizing lipids, proteins, and antibodies,\textsuperscript{19} among other components;\textsuperscript{20–22} however, authors have not utilized the generated patterns for biological applications. BP-mediated biomolecular patterns generated
in our lab have validated control over surface density while maintaining the bioactivity of patterned molecules: direct immobilization of overlapping patterns or opposing gradients of protein and carbohydrate moieties, and recognition of immobilized P-selectin by its ligand P-selectin glycoprotein ligand-1 (PSGL-1) in solution and via engagement with HL-60 cells through cell-surface-expressed PSGL-1. The basic scheme for producing patterns is to functionalize the substrate surface with benzophenone, incubate the substrate in an aqueous solution of the molecule of interest, and expose the specimen to 365 nm laser light through a photomask. Unlike methods that involve copolymerization of biomolecules within the matrices themselves, photolithography allows spatial modulation of immobilization. This process has the advantage of separating material fabrication from both biomolecule patterning and subsequent cell incorporation.

Most studies that focus on cell-material interactions have been centered around manipulation of a single factor, which presents only a small fraction of the total interactions that take place in the microenvironment. Given that biochemical cues act in concert with physical stimuli to directly affect cells, the respective contributions and interactions of these cues is difficult to distinguish. Due to the complexity of forces cells experience, multi-parameter control of material characteristics will be necessary to elucidate the interplay of these factors. This added level of control over substrate stiffness, combined with the benzophenone methodology for creating overlapping biomolecular patterns, allows systematic investigation of cell behavior. Development of such modular biocompatible materials is crucial to recapitulate the complex ECM and enable new insights in tissue regeneration.

1.4 Untangling the cellular microenvironment: overview of work presented herein

This thesis presents an overview of the important role of the extracellular matrix, strategies for mimicking its properties, and finally the development and application of benzophenone photolithography to illuminate the biology of inflammation and stem cell fate through instructive materials.

Chapter 2 describes the application of BP photolithography to investigate the anti-inflammatory mechanism of the proteinase bromelain using in vitro cell rolling assays on two-dimensional glass substrates. This study builds on previous work reported by our group, showing that multi-component surface-immobilized gradients can be used to understand the dynamic transition from leukocyte rolling to leukocyte adhesion during the early stages of the inflammation process. The results reported in this chapter establish how bromelain influences leukocyte rolling by specific cleavage of PSGL-1 on the cell surface and subsequent reduction in P-selectin binding activity.
It has increasingly become clear that substrates are not simply inert surfaces, but regulators of cell behavior through microstructure and mechanical properties. Therefore, the majority of the work discussed in this dissertation focuses on moving away from planar, non-compliant materials and towards mechanically and chemically tailored materials. Chapter 3 focuses on the interplay of growth factors and substrate stiffness on collagen-glycosaminoglycan scaffolds. Collagen is the most plentiful protein in the human body, and as the major structural ECM component it supports cell adhesion and growth. Despite the wide use of collagen scaffolds in tissue engineering, there existed no suitable method for generating spatially controlled multi-component biomolecular patterns in collagen scaffolds. Additionally, owing to their porosity, collagen-glycosaminoglycan materials are not amenable to many of the direct surface patterning methods described above. Preliminary findings from our lab established this technique as a viable method for attaching proteins while maintaining cell viability. Here we report the application of BP photolithography on collagen membranes to reveal combined effects of proliferative factors, osteogenic factors and substrate stiffness on mesenchymal stem cell fate decisions. Results suggest that a complex interplay of these factors is responsible for controlling cell activity.

Despite their success, natural materials such as collagen have some disadvantages as biomaterials. Collagen presents native cues that cannot be overlooked, and its bulk mechanical stiffness is orders of magnitude higher than native tissue. In chapter 4, we chose to apply the BP methodology to synthetic bioinert hydrogels. Polyacrylamide hydrogels provide a blank slate with which to investigate cell behavior and are mechanically tunable to achieve a closer approximation of the elastic modulus of tissues. Taken together, the studies in chapters 2, 3, and 4 show the utility of benzophenone photolithography to elucidate cell-material interactions and accelerate fundamental biological studies needed to lead to medical applications. The concluding chapter will discuss the implications of this work to enable the fabrication of even more sophisticated biomaterials.
1.5 References


Chapter 2

Bromelain Decreases Neutrophil Interactions with P-Selectin In Vitro by Proteolytic Cleavage of P-Selectin Glycoprotein Ligand-1

This chapter has been adapted from the article “Bromelain Decreases Neutrophil Interactions with P-Selectin, but Not E-Selectin, In Vitro by Proteolytic Cleavage of P-Selectin Glycoprotein Ligand-1” and used here with permission from PLOS in accordance with the Creative Commons Attribution License © 2013. The original article (Banks, J. M., Herman, C. T., and Bailey, R. C. PLoS ONE, 8(11):e78988, 2013.) can be accessed online at http://dx.doi.org/10.1371/journal.pone.0078988.

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2.1 Abstract

Stem bromelain, a cysteine protease isolated from pineapples, is a natural anti-inflammatory treatment, yet its mechanism of action remains unclear. Curious as to whether bromelain might affect selectin-mediated leukocyte rolling, we studied the ability of bromelain-treated human neutrophils to tether to substrates presenting immobilized P-selectin or E-selectin under shear stress. Bromelain treatment attenuated P-
selectin-mediated tethering but had no effect on neutrophil recruitment on E-selectin substrates. Flow cytometric analysis of human neutrophils, using two antibodies against distinct epitopes within the P-selectin glycoprotein ligand-1 (PSGL-1) active site, revealed that bromelain cleaves PSGL-1 to remove one of two sites required for P-selectin binding, while leaving the region required for E-selectin binding intact. These findings suggest one molecular mechanism by which bromelain may exert its anti-inflammatory effects is via selective cleavage of PSGL-1 to reduce P-selectin-mediated neutrophil recruitment.

2.2 Introduction

Inflammation is a complex physiological process involving numerous receptor-ligand interactions between leukocytes and the endothelial lining of the blood vessel that ultimately lead to the trafficking of leukocyte subsets throughout the body.\(^1\) Numerous diseases are associated with dysregulated inflammation, including rheumatoid arthritis, asthma, psoriasis, thrombotic disorders, cancer, and autoimmune disease.\(^2\)

Bromelain is a mixture of several cysteine proteases isolated from pineapple extracts, and is taken as a complementary anti-inflammatory treatment.\(^3\) Bromelain is known to alter multiple cell surface molecules involved in the adhesion and activation of leukocytes\(^4\) leading to anti-inflammatory, fibrinolytic, and anti-thrombotic effects in vivo and in vitro.\(^5\) Previous reports have investigated the effects of bromelain treatment on neutrophil migration in response to chemokines.\(^4\) However, bromelain’s ability to alter cell surface molecules involved in the initial tethering and rolling of leukocytes on the inflamed endothelium has not been investigated to date. Among the enzymes present in bromelain extract is stem bromelain, which was used in this study and will be simply referred to as bromelain herein.

Endothelial-expressed P-selectin and E-selectin play central roles during the initiation of an inflammatory response. When the endothelium receives distress signals from underlying tissue, P-selectin is the first biomolecule deployed from intracellular storage pools to the luminal surface.\(^9\) The interaction between P-selectin and its primary leukocyte-expressed ligand, P-selectin glycoprotein ligand-1 (PSGL-1), supports neutrophil rolling along the surface of the blood vessel.\(^11\) E-selectin, which binds to PSGL-1 among other ligands\(^12\) is also presented on the endothelium during the inflammatory response, but its expression is largely controlled by translation\(^13\) and its presentation temporally lags behind that of P-selectin in vivo.\(^9\) Given these temporal selectin expression dynamics, we felt it would be beneficial to independently probe bromelain’s effects on neutrophil interactions with substrates presenting each of these glycoproteins. To achieve this, we utilized a photochemical surface modification strategy\(^14\) developed in our lab to generate substrates presenting controlled densities of P-selectin or E-selectin\(^15\), and then used these substrates to
investigate the effect of bromelain treatment on the ability of human neutrophils to tether and roll in flow assays (Figure 2.1).

Neutrophils are among the first responders that rapidly accumulate at sites of inflammation and thus a potential key player in the initial steps of the immune response. Flow cytometric expression analysis of two ligands involved in leukocyte recruitment mediated by P-selectin and E-selectin, PSGL-1 and cutaneous lymphocyte antigen (CLA), respectively, was performed to determine the effects of bromelain treatment on ligand expression. The results reveal a site-specific proteolysis through which bromelain treatment abolishes interactions between neutrophils and immobilized P-selectin, but not E-selectin, under conditions of physiological shear stress in vitro, suggesting another molecular mechanism through which bromelain may act as an anti-inflammatory agent.

### 2.3 Results and discussion

Interestingly, we found that bromelain treatment of neutrophils nearly eliminated their ability to interact with P-selectin presented on substrates in vitro, while E-selectin-mediated interactions are unaffected. The observations from neutrophil flow assays were complemented by dose-dependent effects of bromelain treatment on the expression of P-selectin and E-selectin ligands using flow cytometry. Our findings suggest that the anti-inflammatory effects of bromelain may be attributed in part to its ability to proteolytically process PSGL-1 and thereby reduce the number of cells interacting with P-selectin presented on the inflamed endothelium during the initial phases of an inflammatory response.

Using a molecularly general method previously developed in our lab for the covalent and controllable photochemical immobilization of biomolecules on planar glass substrates, we generated and characterized substrates presenting defined high and low levels of P- or E-selectin (Figure 2.1, Figure 2.2, and Figure 2.3). Substrates were characterized with fluorescence imaging and average fluorescence intensity values were converted into biomolecule site densities using an established fluorescence-radioactivity correlation for P-selectin and E-selectin (Figure 2.4).

Figure 2.5 shows neutrophil flow assay results using primary human neutrophils. For the first set of flow assays, human neutrophils were isolated (>90% pure, >95% viable) and flowed at a constant shear stress over substrates presenting immobilized P-selectin at high and low site densities. The number of interacting cells was significantly reduced when neutrophils were treated with 50 µg/mL bromelain, compared to a control sample (p<0.05). Incubation of control cells with saturating levels of a monoclonal antibody (mAb) against PSGL-1, clone KPL-1, which recognizes an epitope encompassing three sulfated tyrosines in the
active site of PSGL-1, similarly resulted in a decrease in the number of interacting cells, suggesting that the decreased interactions are due to reduced PSGL-1 association with underlying P-selectin. In the case of bromelain treatment, this is caused by proteolytic cleavage of PSGL-1 so as to remove the region of the molecule required to engage P-selectin.

It is well established that PSGL-1 is the predominant and most well-characterized binding partner of P-selectin. However, we wanted to validate the specific targeting of PSGL-1 by bromelain as an effector of rolling and tethering on P-selectin substrates and therefore performed an additional control experiment. This experiment utilized neutrophil-like HL-60 promyelocytes, which also tether and roll on P-selectin surfaces via PSGL-1, and a monoclonal anti-PSGL-1 blocking antibody (clone KPL-1) with and without bromelain treatment. As shown in Figure 2.6, treatment with the blocking antibody alone led to a marked decrease in the number of tethering events on both high and low P-selectin substrates. Importantly, HL-60 cells that were treated with bromelain after being blocked with the antibody did not show a further reduction in substrate interaction, confirming the specific action of bromelain on PSGL-1 as it leads to reduced interactions with P-selectin surfaces.

P-selectin is the primary molecular player that controls initial neutrophil interactions with the inflamed endothelium; however E-selectin is also known to play a key role in mediating leukocyte rolling. PSGL-1 is a ligand for both P- and E-selectin and therefore we wanted to see whether bromelain treatment similarly abolished neutrophil interactions on E-selectin. Isolated human neutrophils were flowed over substrates presenting two different site densities of immobilized E-selectin. In contrast to experiments on P-selectin-presenting substrates, bromelain treatment had no significant effect on neutrophil tethering to immobilized E-selectin. The cutaneous lymphocyte antigen (CLA) glycan moiety pendant on PSGL-1 and other ligands, plays a role in leukocyte tethering and rolling mediated by E-selectin. To see if neutrophil interactions with immobilized E-selectin could be altered by blocking CLA on all E-selectin ligands, we incubated neutrophils with saturating levels of HECA-452, a widely used mAb that targets CLA. Not surprisingly, this blocking antibody reduced tethering to immobilized E-selectin (Figure 2.5c-d), though the effect was not as significant as that for the anti-PSGL-1 blocking on P-selectin substrates. The incomplete inhibition of tethering on E-selectin after anti-CLA blocking reflects the fact that E-selectin, unlike P-selectin, has a diverse range of potential binding partners and thus simply blocking one binding epitope does not abolish all interactions, a finding consistent with the literature. Overall, neutrophil flow assays with E-selectin-presenting substrates reveal that bromelain does not proteolytically abolish all interactions between neutrophils and E-selectin. Importantly, the sulfated tyrosine residue epitope targeted by KPL-1 is not required for recognition of PSGL-1 by E-selectin and so the unaffected interactions with this substrate
after bromelain treatment is consistent with a molecularly specific cleavage of PSGL-1, but also supports E-selectin recognition by ligands besides PSGL-1.

Neutrophils flowed across simple human serum albumin-blocked substrates, without P- or E-selectin, showed no interactions which established that observed cell-substrate interactions were the result of specific receptor-ligand interactions.

To further investigate the molecular mechanism by which bromelain treatment regulates the initial phases of neutrophil tethering, we performed flow cytometric analyses to determine how expression of PSGL-1, which binds to both P-selectin and E-selectin, is modulated by bromelain treatment. There are two distinct structural domains of PSGL-1 that are required for interactions with each of the selectins and our flow cytometric analysis utilized two mAbs specific for these regions. Antibody KPL-1 recognizes a region that contains the sulfated tyrosine motif required for interactions with P-selectin, but not E-selectin. This specificity was previously demonstrated as incubation of leukocytes with the KPL-1 antibody completely blocked interactions with P-selectin without affecting leukocyte recognition of E-selectin. Antibody CHO131 recognizes a sialyl-Lewis\textsuperscript{x} bearing a core 2 O-glycan that is required for interaction of neutrophils with both P-selectin and E-selectin.

Flow cytometric analysis using these antibodies suggests that bromelain is able to specifically cleave PSGL-1 at a site in between the epitopes for clones KPL-1 and CHO131 (Figure 2.7). Analysis with clone KPL-1 revealed that the level of the sulfated tyrosine motif is reduced by \(~80\%\) as the dose of bromelain exposure was increased from 0 to 100 \(\mu\)g/mL. However, analysis with clone CHO131 showed that the levels of this epitope were not significantly decreased at all concentrations of bromelain tested. In fact, CHO131 epitope expression moderately increased at low bromelain concentrations before showing a slight decrease at higher concentrations. While we do not completely understand the significance of this increase, it is clear that bromelain is able to directly and dramatically attenuate the sulfated tyrosine motif needed for P-selectin tethering while leaving significant expression of the sLe\textsuperscript{x} glycan needed for E-selectins interaction with PSGL-1. Treatment of human neutrophils with deactivated bromelain had no significant effect on neutrophil expression of PSGL-1 (Figure 2.8), suggesting that enzyme activity is required to induce the changes in surface expression of PSGL-1 that we observed following treatment with active bromelain. The molecularly-specific processing of PSGL-1 at a position that down-regulates interactions with P-selectin, yet upstream of the sialyl-Lewis\textsuperscript{x} bearing core 2 O-glycan structure involved in E-selectin interactions, suggests that bromelain may selectively affect the very initial phases of neutrophil recruitment.

As additional verification of PSGL-1 processing by bromelain, we performed Western blot analysis of the protein after exposure to the enzyme. Figure 2.9 compares the 100-150 kDa band of an immunoblot
of untreated PSGL-1 and PSGL-1 treated with bromelain. When probed with a polyclonal antibody, no statistically significant difference in intensity was observed but an overall reduction in molecular weight was apparent. When visualized by staining with anti-PSGL-1 clone KPL-1, which recognized the N-terminal portion of the protein that present the sulfated tyrosine residues, there was a significant reduction in the band intensity for bromelain treatment compared to the untreated sample \(p < 0.05\). These results are consistent with flow cytometry analysis that showed a marked reduction in the N-terminal component of PSGL-1 using the same antibody clone. As a control, human IgG1 Fc was left untreated or treated identically with bromelain and immunoblotted alongside the PSGL-1 Fc chimera. In both blots, no bands were observed in either IgG1 Fc lanes, confirming that changes in PSGL-1 band intensity are not due to interference from the Fc chimera portion of the recombinant protein.

We also determined the effects of bromelain treatment on expression levels of CLA, a carbohydrate epitope shared by sialyl-Lewis\(^x\) and sialyl-Lewis\(^a\) structures that has been found to be present on PSGL-1 and other E-selectin ligands.\(^{20}\) To characterize CLA expression on neutrophils, we used clone HECA-452, and this analysis revealed that CLA expression declines in a nearly linear fashion by up to 20% as the dose of bromelain increases from 0 to 100 \(\mu\)g/mL (Figure 2.10). Similar to flow cytometry analysis of PSGL-1 expression, treatment of human neutrophils with deactivated bromelain caused no significant changes in the surface levels of CLA (data not shown).

In this study we used a combination of molecular biology and controlled chemical surface modification to reveal new insight into one potential molecular mechanism by which bromelain can regulate immune response. We found that bromelain cleaves PSGL-1, P-selectin's primary ligand that is involved in the onset of selectin-mediated leukocyte rolling during inflammation, resulting in the attenuation of neutrophil recruitment on immobilized P-selectin, but not E-selectin, in vitro. While the levels of bromelain utilized in this study are higher than that known to be achievable in serum, previous studies on the effects of bromelain treatment on leukocyte migration and inflammation established the value of in vitro assays using similarly high relative bromelain concentrations.\(^{4,5}\) Therefore the basic mechanistic insight gleaned from this study, as well as future efforts to understand neutrophil-selectin interactions both in vitro and in vivo, may reveal additional key insights into the molecular mechanism underlying bromelains anti-inflammatory properties.
2.4 Methods

2.4.1 Ethics statement

Blood was obtained from healthy adults who gave informed consent. Written consent was obtained and recorded from all volunteers. This protocol and consent procedure was approved as “Isolation of human blood cells for rolling and adhesion studies and molecular analysis” by the Institutional Review Board at the University of Illinois at Urbana-Champaign entitled (IRB #11117).

2.4.2 Materials

All chemicals were purchased from Sigma-Aldrich, unless otherwise noted.

2.4.3 Photochemical immobilization of P-selectin or E-selectin on glass substrates

Substrates presenting two regions of distinct site densities of immobilized P-selectin or E-selectin were prepared using a previously reported photopatterning method. Briefly, cleaned glass microscope slides were functionalized with 4-(triethoxysilyl)butyl aldehyde (Gelest), incubated with 20 mM 4-benzoyl benzylamine hydrochloride (Matrix Scientific) and 200 mM NaCNBH₃, followed by immersion in aldehyde-blocking buffer (200 mM ethanolamine, 0.1 M Tris, pH 7), rinsing water, methanol, and ethanol. P-selectin and E-selectin (R&D Systems) were freshly diluted to 2.5 µg/mL from concentrated stocks into PBS with Ca²⁺ and Mg²⁺ (Sigma). BP-modified substrates were assembled into a rectangular parallel-plate flow chamber (GlycoTech) with a silicone gasket (127 µm thickness). Protein photoimmobilization was enabled through the use of an Ar ion laser (Coherent Innova 90-4, Laser Innovations, 351.1-363.8 nm), whose Gaussian beam profile was converted to a flat-top profile using a -Shaper (MT-Berlin), and expanded to a 1 cm² area using beam-expanding optics (ThorLabs, 14 mW/cm²). At least six replicate substrates were generated presenting areas of high (650-750 molecules/µm²) and low (250-400 molecules/µm²) protein site density by exposing two distinct regions of each substrate for 3 or 30 seconds (for P-selectin) and for 6 or 60 seconds (for E-selectin). Substrates for flow assays were stored in 0.5% human serum albumin (HSA) in Hanks Buffered Saline Solution with Ca²⁺ and Mg²⁺ and 10 mM HEPES (pH 7.4, HBSS/HEPES) until use, while the remaining replicate substrates were incubated with fluorescently labeled antibodies and visualized with a fluorescence slide scanner (Figure 2.3, GenePix 4000B, MDS Analytical Technologies), as previously reported for P-selectin. Data for E-selectin quantitation was obtained for this study (Figure 2.4).
2.4.4 Neutrophil flow assays on substrates presenting P-selectin and E-selectin

Substrates presenting P-selectin or E-selectin (Figure 2.2 and 2.3) were utilized in flow assays with human neutrophils using a previously described methodology,\textsuperscript{15} shown schematically in 2.1. Human neutrophils were isolated from whole blood by density centrifugation using Ficoll-Paque, followed by selection with Easy-Sep Human Neutrophil Enrichment Kit (STEMCELL Technologies). Neutrophil viability was determined with trypan blue staining. Neutrophil purity was determined by flow cytometry using two fluorescently labeled antibodies against neutrophil surface markers CD16 and CD66b (STEMCELL Technologies, Figure 2.11). One sample was resuspended in a solution of 50 µg/mL bromelain in RPMI, and the other two samples were resuspended in RPMI. All samples were incubated for 30 min at 37°C, washed with HSA/HBSS/HEPES, and pelleted for 5 min at 500 rcf. One of the RPMI-treated neutrophil samples was resuspended in 30 µg/mL anti-PSGL-1 (clone KPL-1) or anti-CLA (clone HECA-452) and incubated at room temperature for 30-60 min before being diluted to $0.5 \times 10^6$ cells/mL for flow assay experiments. The remaining cell pellets were immediately resuspended in HBSS/HEPES with 0.5% HSA to $0.5 \times 10^6$ cells/mL. All samples were used in flow assay experiments within 2-3 hours of isolation.

Neutrophils were introduced into the chamber at 3 mL/min (9.3 dyn/cm$^2$) for 1 min, then the flow rate was dropped to 400 L/min (1.28 dyn/cm$^2$). After 1 min, 20-30 sec videos were acquired at 4-5 positions in each area of the substrate. Neutrophils from three donors were used to perform independent experiments on three replicate substrates for each protein. For each substrate, the number of new tethers formed per unit area and time was determined by counting the number of cells that interacted with the surface. Cells that were already rolling or adhered upon the surface were not included in the analysis. HSA-blocked BP-modified substrates were used as control substrates to verify that the observed interactions were due to neutrophil interactions with immobilized P-selectin or E-selectin.

2.4.5 Differentiated HL-60 flow assays on substrates presenting P-selectin

HL-60 promyelocytes (ATCC) were differentiated for 6 days following addition of 1.3% dimethyl sulfoxide. Cell viability and treatment with RPMI, bromelain, or anti-PSGL-1 (clone KPL-1) was performed as described above for neutrophil flow assays. For double treatment with bromelain and anti-PSGL-1 (clone KPL-1), the sample was resuspended in 30 µg/mL antibody and incubated at room temperature for 10 min before incubation with bromelain for 30 min at 37°C. Flow chamber assays were performed as described for neutrophils on three replicate P-selectin substrates. Flow cytometric analysis of bromelain's effect on cell surface molecules Human neutrophils were isolated from whole blood as described above. Neutrophils were prepared for analysis of expression of PSGL-1 and CLA following treatment with 0, 10, 25, 50, or 100 µg/mL
bromelain in RPMI cell culture medium for 30 min at 37 °C. In some experiments, an additional aliquot of cells were treated with chemically deactivated bromelain, which was prepared by reacting bromelain with dithiothreitol and iodoacetamide, followed by treatment with a proteinase cocktail inhibitor solution containing E-64, according to manufacturers protocols. Following incubation with bromelain or RPMI, cells were washed with HSA/HBSS/HEPES and were pelleted for 5 min at 500 rcf. Neutrophils were then blocked for 15 min with 3% HSA in HBSS/HEPES and incubated for 30 min with primary monoclonal antibodies and fluorescently labeled secondary antibodies at final concentrations of 20 and 10 µg/mL, respectively. For detection of cell-surface PSGL-1, we used mouse anti-human PSGL-1 clone KPL-1 (Millipore), and mouse anti-human PSGL-1 clone CHO131 (R&D Systems). For detection of CLA, clone HECA-452 (BioLegend) was used. For all samples, we used secondary antibody PE-conjugated goat anti-mouse IgG (Invitrogen). Control samples were incubated with the PE-labeled secondary antibody alone. Solutions composed of primary and secondary antibodies were preincubated for at least 1 hr prior to incubation with cells. Cells were analyzed with a BD FACSCanto II cytometer (BD Biosciences). Fluorescence data from neutrophil populations were plotted in histogram form (FCS Express, BD Biosciences), and the average fluorescence intensity from each sample was plotted as the percentage of the antigen expression relative to the RPMI-treated sample as a function of bromelain concentration.

2.4.6 Western blot analysis of proteolytic processing of recombinant PSGL-1 by bromelain

Equimolar amounts of recombinant human PSGL-1 Fc chimera (R&D Systems) or recombinant human IgG1 Fc (R&D Systems) were treated with bromelain at a final concentration of 10 µg/mL for 30 minutes at 37 °C. PSGL-1 was left untreated under the same conditions. Samples were electrophoresed on a 4-20% SDS-polyacrylamide gel (Bio-Rad) and immunoblotted on PVDF membranes (Millipore). The 100-150 kDa band of PSGL-1 (consistent with manufacturer specifications) was visualized with anti-PSGL-1 antibody clone KPL-1, which recognizes the sulfated tyrosine residues near the N-terminus, or a polyclonal PSGL-1 antibody (R&D Systems). After incubation with relevant secondary HRP-linked antibody, the blot was visualized using enhanced chemiluminescent substrate (Pierce). Digital images were taken using an ImageQuant LAS 4010 biomolecular imager (GE) and analyzed using ImageQuant TL software (GE).

2.4.7 Statistical Analysis

Groups were compared using one-way ANOVA with Tukeys HSD test for post-hoc comparison of more than two groups. For western blot data in 2.9c, students unpaired t test was used to compare results. Data were
analyzed with GraphPad Prism software and p-values are provided.
2.5 Figures

Figure 2.1: Substrates for cell interactions studies were fabricated via direct, photochemical functionalization. Glass slides chemically-modified to present benzophenone moieties were immersed in a solution containing the protein of interest—here P- or E-selectin. Photoimmobilization is initiated by exposure to 365 nm light, which results to covalently bound protein through a radical generation mechanism. Importantly, the density of immobilized protein can be systematically defined by carefully controlling the exposure time. Substrates, prepared in batches, were then subsequently used to quantify deposition density (via fluorescence or radioimmunoassay) or utilized to study the effects of bromelain treatment on primary human neutrophil-selectin interactions.
Figure 2.2: Fluorescence images and plots of average site density from substrates presenting photoimmobilized selectins. (a) Sites densities for P-selectin substrates were determined using a previously reported radioimmunoassay\textsuperscript{15}. (b) A similar radioimmunoassay for E-selectin (2.3) was used to determine site densities of E-selectin substrates. Scale bar = 1 mm.
**Figure 2.3**: Average fluorescence intensity and site density determination for P-selectin and E-selectin substrates. Substrates were incubated with fluorescently labeled antibodies, and fluorescence intensity units were converted to site density (molecules/µm²) using previous reported calibration curves.¹⁵

<table>
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<th>Substrate</th>
<th>F.I., low-3 sec</th>
<th>Molecules/µm²</th>
<th>F.I., High-30 sec</th>
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**Approximations:**
- Low P-selectin: 250 molecules/µm²
- High P-selectin: 600 molecules/µm²

<table>
<thead>
<tr>
<th>Substrate</th>
<th>F.I., Low-6 sec</th>
<th>Molecules/µm²</th>
<th>F.I., High-60 sec</th>
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**Approximations:**
- Low E-selectin: 400 molecules/µm²
- High E-selectin: 750 molecules/µm²

For **P-selectin**:
- Site density = 2.96(F.I.) - 194.07, \( R^2 = 0.92 \)

For **E-selectin**:
- Site density = 5.97(F.I.) - 1597.20, \( R^2 = 0.91 \)
Figure 2.4: The relationship between fluorescence intensity and site density was determined for photoimmobilized E-selectin on BP-modified substrates. Substrates were generated in triplicate via flood UV exposures in the presence of protein solution, resulting in homogeneous substrates presenting a wide range protein site density levels. Fluorescence measurements were acquired after incubating substrates with fluorescently labeled antibodies. Briefly, we generated six replicate substrates presenting varying levels of immobilized E-selectin in a defined area on BP-modified substrates and split the substrates into two groups: one group for fluorescence analysis and the other for radioactivity analysis. For site density determination, substrates were incubated with saturating concentrations of primary monoclonal antibody (mAb) and $[^{125}\text{I}]$-labeled secondary polyclonal antibody (pAb). Control substrates were used to account for non-specific mAb and pAb binding. The data from both studies were correlated to determine the relationship between fluorescence intensity and site density for each protein. Using the equation from the linear regression for the resulting calibration curve, immobilized E-selectin substrates were quantified by converting data from fluorescence analyses to site density. Data is plotted as the average ($\pm$ 95% C.I.) for n=3 substrates.
Figure 2.5: Data from flow assays with neutrophils treated with RPMI or bromelain. Bromelain attenuates neutrophil tethering on immobilized P-selectin but has no effect on E-selectin-mediated tethering. (a) Snapshots from representative flow assay videos show neutrophils interacting with substrates presenting immobilized P-selectin. Arrows point to cells interacting with the substrate under conditions of shear stress. (b) Data from primary human neutrophil flow assays with P-selectin substrates reveal that the number of interacting cells is decreased when neutrophils are treated with 50 µg/mL bromelain in RPMI on regions of “high” and “low” immobilized P-selectin. (** p < 0.05) Treatment with saturating levels of anti-PSGL-1 clone KPL-1 significantly decreased P-selectin mediated tethering on high P-selectin substrates (** p < 0.05). (c) Snapshots from representative flow assay videos show neutrophils interacting with substrates presenting immobilized E-selectin with arrows indicating interacting cells. (d) Data from primary human neutrophil flow assays with E-selectin substrates reveal that the number of interacting cells is not significantly affected when neutrophils are treated with 50 µg/mL bromelain in RPMI on regions of “high” or “low” immobilized E-selectin. Treatment with saturating levels of anti-CLA clone HECA-452 had no significant effect on neutrophil tethering to immobilized E-selectin. Neutrophils failed to interact with HSA-blocked control substrates. Data represent the average of n=3 donors (±SEM). Scale bar: 80 µm.
Figure 2.6: Blocking of PSGL-1 with a monoclonal anti-PSGL-1 antibody prevents subsequent proteolytic cleavage by bromelain thereby preventing further reduction in tethering on P-selectin. Data from flow assays with differentiated HL-60 promyelocytes treated with bromelain, blocking antibody, both, or untreated. For both “high” (**p < 0.001) and “low” (* p < 0.10) P-selectin substrates, bromelain, blocking antibody, and combination treatments reduced substrate interactions. In both cases, combination treatment did not significantly further reduce interactions beyond that of bromelain treatment alone. Data represent the average of n=3 independent P-selectin substrates (±SEM).
Figure 2.7: Flow cytometric analysis reveals bromelain cleaves PSGL-1 within its active site in a dose-dependent manner. To analyze neutrophil expression of PSGL-1, the primary ligand for P-selectin, we used two anti-PSGL-1 antibodies that recognize distinct structural motifs within the PSGL-1 active site. (a) Representative data from an analysis with clone KPL-1 reveals an 80% decrease in PSGL-1 expression following treatment with 100 µg/mL bromelain. (b) Representative data from an analysis with clone CHO131 reveals a slight increase followed by a decrease in PSGL-1 expression back to initial levels as bromelain concentrations increase from 0 to 100 µg/mL. (c) The average PSGL-1 expression levels of neutrophils from n=3-4 donors (±SEM) plotted as a function of bromelain concentration, suggesting that bromelain cleaves PSGL-1 at a position between the two epitopes recognized by the site-specific antibodies.

Figure 2.8: Flow cytometry analysis of PSGL-1 and CLA expression on neutrophils treated with RPMI or deactivated bromelain reveals that deactivated bromelain (1000 µg mL⁻¹) has no significant effect on PSGL-1 or CLA expression levels. Data presented is from 2 or 3 donors, and has been normalized to account for differences in the flow cytometry instrument parameters used in each analysis.
Figure 2.9: Bromelain proteolytically cleaves PSGL-1. Western blot analysis of untreated PSGL-1 and PSGL-1 treated with bromelain. (a) Western blot probed with a polyclonal antibody recognizing PSGL-1 reveals a decrease in molecular weight after treatment with bromelain. (b) Western blot probed with antibody KPL-1 after treatment with bromelain reveals a 39% decrease in the amount of PSGL-1 corresponding to cleavage of the KPL-1 epitope. (c) Quantification of band intensity shown in figure 2.9b reveals that bromelain treatment significantly reduces PSGL-1 band intensity ($** p<0.001$). Data are expressed as the mean ±SD of n=4 replicates. Data are representative of results obtained in three independent experiments.
Figure 2.10: Flow cytometric analysis of CLA levels on human neutrophils following bromelain treatment. (a) Representative data from an analysis with anti-CLA clone HECA-452 reveals a slight decrease in PSGL-1 expression as bromelain concentration increases from 0 to 100 µg mL\(^{-1}\). (b) A plot of the average CLA expression levels of n=6 donors (±SEM) following bromelain treatment ranging from 0 to 100 µg mL\(^{-1}\) reveals that bromelain causes average CLA expression to decrease by ∼20%.

Figure 2.11: Flow cytometry analysis of human neutrophils for neutrophil markers (a) CD66b and (b) CD16 revealed >90% neutrophil purity in all cell samples isolated for neutrophil flow assay experiments (representative data shown). Neutrophils were isolated using traditional Ficoll-Paque density centrifugation followed by magnetic bead separation. Control sample (black) were incubated with fluorescently labeled isotype control antibodies.
Figure 2.12: Bromelain proteolytically cleaves PSGL-1. SDS-PAGE analysis and silver staining of untreated PSGL-1 and PSGL-1 treated with bromelain reveals that bromelain treatment decreases the observed molecular weight.
2.6 References


Chapter 3

The Combined Effects of Matrix Stiffness and Growth Factor Immobilization on the Bioactivity and Differentiation Capabilities of Adipose-Derived Stem Cells

This chapter has been adapted from the article “The combined effects of matrix stiffness and growth factor immobilization on the bioactivity and differentiation capabilities of adipose-derived stem cells” and used here with permission from Elsevier © 2014. The original article (Banks, J. M., Mozdzen, L. C., Harley, B. A. C., and Bailey, R. C. Biomaterials, 35(32):8951-8959, 2014.) can be accessed online at http://dx.doi.org/10.1016/j.biomaterials.2014.07.012.

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3.1 Abstract

Biomaterial designs are increasingly incorporating multiple instructive signals to induce a desired cell response. However, many approaches do not allow orthogonal manipulation of immobilized growth factor signals and matrix stiffness. Further, few methods support patterning of biomolecular signals across a biomaterial in a spatially-selective manner. Here, we report a sequential approach employing carbodiimide crosslinking and benzophenone photoimmobilization chemistries to orthogonally modify the stiffness and immobilized growth factor content of a model collagen-GAG (CG) biomaterial. We subsequently examined the singular and combined effects of bone morphogenetic protein (BMP-2), platelet derived growth factor (PDGF-BB), and CG membrane stiffness on the bioactivity and osteogenic/adipogenic lineage-specific gene expression of adipose derived stem cells, an increasingly popular cell source for regenerative medicine studies. We found that the stiffest substrates direct osteogenic lineage commitment of ASCs regardless of the presence or absence of growth factors, while softer substrates require biochemical cues to direct cell fate. We subsequently describe the use of this approach to create overlapping patterns of growth factors across a single substrate. These results highlight the need for versatile approaches to selectively manipulate the biomaterial microenvironment to identify synergies between biochemical and mechanical cues for a range of regenerative medicine applications.

3.2 Introduction

The development of biomaterial tools for a range of tissue engineering applications increasingly relies on the coordinated presentation of multiple instructive signals. These efforts are often inspired by the native extracellular matrix (ECM), where cells receive cues from an assortment of solution phase\textsuperscript{1–9} and substrate-supported stimuli\textsuperscript{10–15}. The ECM is a complex biomolecular network secreted by cells that serves as a mechanical and structural support within which biomolecular cues can be presented in spatially\textsuperscript{16–19} and temporally\textsuperscript{20,21} defined manners. Given the regulatory role played by the ECM in a multitude of important processes such as tissue development\textsuperscript{22} and repair\textsuperscript{19,23}, it is not surprising biomaterials platforms are increasingly trying to regulate the coordinated presentation of multiple classes of ECM-inspired signals. However, untangling the interactions between mechanical and biochemical cues is often complicated. New approaches are needed to both elucidate the effects of and then recapitulate the functional properties of such
complicated in vivo microenvironments.

In the context of musculoskeletal regeneration, a wide range of studies have concentrated on coordinated presentation of biomolecular signals. Efforts initially focused on presenting a bioactive dose of a given single factors (tenogenesis: IGF-1, PDGF, GDF, bFGF; osteogenesis: BMP-2; angiogenesis: VEGF). Our previous work using collagen-GAG biomaterials demonstrated enhanced tenocyte chemotaxis (via IGF-1) and proliferation (via PDGF-BB) in a dose dependent manner. Moving towards more complex tissue injuries, Thomopoulos et al. demonstrated single factor (BMP-2) supplementation can promote healing at the tendon-bone interface. More recently, efforts have begun to explore the potential of coordinated presentation of multiple cues. Borselli et al. showed a synergistic response in muscle cells with combined delivery of angiogenic and myogenic growth factors. We previously reported advantages of coordinated growth factor presentation for promoting phenotypic stability and proliferation, but also found unintended consequences of multi-factor supplementation in the context of MSC-guided regenerative medicine applications. There are often trade-offs in stem cell proliferation and differentiation, which suggests that multiple cues may need to work in tandem to elicit the desired response. Notably, Tan et al. demonstrated that GDF-5 induced tenogenic differentiation in MSCs without negatively impacting proliferation, while other studies have looked at multiple cues to promote proliferation (i.e. PDGF) and tenogenesis simultaneously.

While solution phase supplementation in the media is the most straightforward method to provide instructive biomolecular cues in vitro, it is limited by diffusion and a lack of spatial localization. Inspired by the biomolecular tethering to the native ECM, recent efforts have suggested that immobilized growth factors can enhance bioactivity. A range of methods (e.g., carbodiimide cross-linking, biotin-avidin linkages, and ‘click’ chemistries) have recently been reported for covalently attaching growth factors to collagen-based scaffolds. However, these approaches did not include the ability to control the spatial distribution of these biomolecules. In addition, the same cross-linking techniques which are used to immobilize growth factors can also increase biomaterial stiffness. However, biomaterial mechanical properties can have both direct and indirect effects on cell, and particularly MSC, response. The mechanical properties of the matrix are also known to play profound roles impacting cell fate, with a range of stem cells showing particular sensitivity to mechanical stiffness, making it potentially difficult to assess the individual impact of matrix-immobilized growth factors.

Increasing evidence suggests that mechanical and biomolecular signals may act in a more coordinated manner. Recently Allen et al. reported that matrix stiffness differentially primed the TGF-β signaling pathway in the context of MSC-chondrogenesis, suggesting the mechanical stiffness of the environment impacts...
how sensitive a cell may be to an exogenous factor. Further, Zouani et al. and Tan et al. both demonstrated
different types of coordinated responses for MSCs to matrix stiffness and BMP-2 presentation. Notably,
Zouani et al. demonstrated a minimum stiffness (3.5 kPa) for MSCs to be responsive to BMP-2 with no
synergy between matrix stiffness and BMP-2 dose, while Tan et al. reported a synergetic effect of ma-
trix stiffness and BMP-2; however in this case, matrix stiffness was modified via hydrogel density, which
likely significantly altered the microstructural cues presented to the cells. Both of these studies support the
contention that MSCs integrate multiple extrinsic signals in the context of fate decisions. However,
these reports also motivate the development of new classes of biomaterials able to orthogonally modify ma-
trix structure, stiffness, and biomolecule incorporation, as well as approaches that are amenable to spatial
control over biomolecule incorporation, in order to better control stem cell differentiation and proliferation.
Such platforms offer unique potential for the field of tissue engineering and regenerative medicine to more
efficiently and effectively utilize structural, mechanical, and biomolecular signals that have yet to be fully
realized.

Herein we report a strategy that allows for independent manipulation of the mechanical properties and
 spatially controlled presentation of biomolecular cues using a collagen-GAG (CG) biomaterial platform. CG
biomaterials have been used for a range of soft (e.g., skin, peripheral nerve) and hard/musculoskeletal
(e.g., bone, cartilage, tendon) tissue engineering applications, making them an attractive target for
technologies to selectively incorporate exogenous biomolecular signals. We have recently demonstrated
approaches to orthogonally modify the microstructural and mechanical properties of, as well as app-
roaches to transiently or covalently modify growth factor presentation within these CG biomaterials.
Here we build on a previously reported benzophenone (BP) photolithography approach to spatially con-
trol immobilization of biomolecules to a CG biomaterial. In this work, we explore the integration of a
separate crosslinking approach to allow orthogonal manipulation of matrix stiffness of the density of immo-
ibilized biomolecular signals (BMP-2, PDGF-BB). We subsequently describe the individual and combined
impacts of matrix mechanical (elastic modulus) cues and immobilized BMP-2/PDGF-BB growth factors on
adipose-derived mesenchymal stem cell (ASC) bioactivity. This combined technological approach provides
an enabling platform to map synergies between multiple biochemical and mechanical cues across a single
biomaterial platform for a range of tissue engineering applications.
3.3 Materials and methods

All reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted. Phosphate buffered saline buffer (PBS) was reconstituted and pH adjusted to 7.4.

3.3.1 Preparation of CG membranes

CG membranes were prepared via a previously described evaporative process. Briefly, a CG suspension was prepared from type I collagen (1.0% w/v) isolated from bovine Achilles tendon and chondroitin sulfate (0.1% w/v) derived from shark cartilage in 0.05 M acetic acid. The suspension was homogenized at 4 °C to prevent collagen gelatinization during mixing. The CG suspension was degassed, pipetted into a petri dish, and allowed to evaporate under ambient conditions to produce a film. Circular membrane specimens (8 mm dia.) were cut using a biopsy punch (Integra-Miltex, York, PA) and stored in a desiccator until use.

3.3.2 Chemical crosslinking of CG membranes to modulate stiffness

Prior to use, CG membranes were hydrated in ethanol followed by PBS. They were subsequently crosslinked using carbodiimide chemistry for 1 hour in a solution of 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) and N-hydroxysulfosuccinimide (NHS) at molar ratios of 1:1:5, 5:2:5, 5:20.7:1, 5:2:1 EDC:NHS:COOH where COOH represents the amount of collagen in the scaffold. A control group of membranes were not cross-linked (NX). After crosslinking, membranes were rinsed and stored in PBS until further use.

3.3.3 Covalent immobilization of growth factors via benzophenone photolithography

Benzophenone (BP) was immobilized to the CG membrane using a previously described approach. Briefly, benzophenone-4-isothiocyanate was synthesized as previously reported and dissolved in dimethyl formamide (DMF) to a final concentration of 20 mM. To that solution, N,N-diisopropylethylamine was added to a final concentration of 0.5 M. CG membranes were submerged in this solution and allowed to react for 48 hours protected from light. Membranes were then rinsed in DMF, ethanol, and PBS to remove unreacted benzophenone reagent, and subsequently stored in PBS at 4 °C in the dark until use.
3.3.4 Biomolecular photoimmobilization

Stock solutions of bone morphogenetic protein 2 (BMP-2, R&D Systems, Minneapolis, MN) and platelet derived growth factor BB (PDGF-BB, R&D Systems) were prepared according to the manufacturers instructions and stored at −20 °C until use. In preparation for patterning, CG membranes were soaked in a solution containing 5 µg/mL protein (PDGF-BB or BMP-2) in PBS. Membranes were subsequently transferred to a glass slide, covered with a glass coverslip, and exposed to 20 mW/cm² of 365 nm light provided by a beam of an argon ion laser (Coherent Innova 90-4 with UV optics, Laser Innovations, Santa Paula, CA) that had been expanded and homogenized using refractive beam shaping optics (π-Shaper, Molecular Technologies, Berlin, Germany). Membranes were exposed for a defined time (1 or 5 minutes). After UV exposure, membranes were rinsed in a solution of 0.2% pluronic F-127 in PBS. For patterning multiple proteins, membranes were further rinsed in PBS then soaked in the second protein solution and subsequently irradiated as described above. After patterning, membranes were stored in PBS at 4 °C until use.

3.3.5 Fluorescent visualization of biomolecular patterns

In order to visualize biomolecule attachment, membranes containing immobilized proteins were transferred into a solution of 1% bovine serum albumin (BSA) in PBS to block non-specific antibody binding. A rabbit anti-PDGF-BB antibody (AbCam, Cambridge, MA) was pre-incubated with Alexa Fluor 647-conjugated goat anti-rabbit IgG (Invitrogen, Carlsbad, CA) at a concentration of 1 µg/mL in 1% BSA-PBS. Similarly, a rabbit anti-BMP-2 antibody (AbCam) was pre-incubated with Alexa Fluor 647-conjugated goat anti-rabbit IgG (Invitrogen) at a concentration of 1 µg/mL in 1% BSA-PBS. Membranes were placed in the relevant antibody staining solutions overnight at 4 °C, after which they were rinsed for at least 1 hour in PBS and visualized using a fluorescence slide scanner (Axon Instruments-Molecular Devices, Sunnyvale, CA). Total immobilized protein was estimated using a calibration curve generated from known concentrations of factor\textsuperscript{57,62}.

3.3.6 Mechanical characterization

Tensile mechanical tests were performed on hydrated CG membranes (10 mm gauge length x 6 mm wide; 0.6 mm thickness,) at a rate of 1.0 mm/min using a Bose Electroforce BioDynamic 5110 with a 1000 g load cell. Data was collected with Bose WinTest software. The elastic modulus was calculated from the linear region of the stress-strain curve for each sample.
3.3.7 Culture of adipose-derived mesenchymal stem cells and CG membrane seeding

ASCs were isolated according to published procedures and stored in liquid nitrogen until use. Two million ASCs were thawed and plated in 75 cm² tissue culture flasks at a density of 6,000 to 7,000 cells/cm² containing high glucose Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% fetal bovine serum, 1% penicillin g-streptomycin, and 5.0 mg/L amphotericin B. Cells were expanded at 37 °C and 5% CO₂, with the culture medium changed every 3 days. Cells were rinsed with PBS and harvested using 0.25% trypsin-EDTA followed by addition of an equal volume of complete medium. ASCs were centrifuged at 200 g for 5 minutes and resuspended to a concentration of 1×10⁵ cells per 20 μL in DMEM. Cells were seeded onto the CG membranes using a previously described static seeding method. Briefly 10 μL of cell-laden media was pipetted onto one side of each CG membrane in ultra-low attachment 6-well plates (Corning Life Sciences, Lowell, MA) and incubated at 37 °C with 5% CO₂ for 30 minutes. Membranes were flipped and the remaining 10 μL of cells was added to the membrane. After 30 minutes of incubation at 37 °C to allow cell attachment, 5 mL of complete DMEM was added to each well. The culture medium was changed every 3 days.

3.3.8 Quantifying cell attachment and proliferation

Total number of cells attached to the CG membranes were monitored using Hoechst 33258 dye (Invitrogen, Carlsbad, CA) which fluorescently labels double-stranded DNA. Total cell number was determined at day 0 (initial cell attachment) as well as days 1, 4 and 7 (subsequent proliferation) using a fluorescence spectrophotometer (Tecan Group Ltd., Männedorf, Switzerland)

3.3.9 Characterizing cell bioactivity

The mitochondrial metabolic activity of ASCs seeded on the CG membranes were quantified via alamarBlue (Invitrogen). Membranes were incubated in the alamarBlue solution with gentle shaking for 1 hour. Viable cells reduce the resazurin in the alamarBlue solution to resorufin, which produces fluorescence. Fluorescence was measured (excitation 540 nm, emission 580 nm) on a fluorescent spectrophotometer (Tecan). Results were compared to a prepared standard to compute equivalent cell number. Results (n = 3/timepoint) were reported as the relative metabolic activity compared to the number of originally seeded cells.
3.3.10 RNA isolation, cDNA synthesis, and quantitative real time polymerase chain reaction

The expression of osteogenic, adipogenic, and matrix synthesis markers was determined via qPCR using previously described methods\textsuperscript{52,66}. Membranes were rinsed in PBS to remove unattached cells. Total RNA was isolated using the RNeasy plant kit (Qiagen Inc., Valencia, CA) and converted to cDNA using a QuantiTect reverse transcription kit (Qiagen Inc., Valencia, CA), both according to the manufacturers instructions. Gene expression profiles were determined for Alkaline phosphatase (ALP), type 1 collagen alpha-1 (COL1A1), osteocalcin (OCN), and peroxisome proliferator-activated receptor gamma (PPARG), with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) used as a housekeeping gene. Previously validated primer sequences were chosen from the literature (Table 3.1) and purchased from Integrated DNA Technologies (Coraville, IA). Gene expression profiles were determined via three independent replicates of each experimental condition by quantitative real-time polymerase chain reaction (qRT-PCR). qRT-PCR was performed using a 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). The cDNA was amplified according to the following conditions: 50 °C for 2 minutes and 95 °C for 10 minutes, then 95 °C for 15s and 60 °C for 60s for 40 amplification cycles. Amplification was monitored by SYBR Green and a dissociation melting curve was performed to confirm a single PCR product. Results were analyzed using SDS Software and the transcripts of interest were normalized to the housekeeping gene GAPDH. Relative fold change (fold ∆) was calculated using the delta-delta Ct method.

3.3.11 Statistical analysis

One-way analysis of variance (ANOVA) was performed on biomolecular immobilization data (Figure 3.1B-C), tensile mechanical properties (Figure 3.2), and orthogonal control of biomolecular presentation and CG membrane stiffness (Figure 3.3), followed by Tukey’s post-hoc test. Two-way ANOVA was performed on all other data sets followed by a Tukey post-hoc test. Independent factors included crosslinking and immobilized growth factor (PDGF-BB, BMP-2). Pairwise comparisons were performed as necessary. Mechanical characterization used at least n = 7 membranes per group while cell number, metabolic activity and gene expression experiments used n = 3 membranes per group, estimation of biomolecule immobilization as a function of exposure time used n = 4 membranes per group, while biomolecule immobilization as a function of EDC crosslinking used n = 10 membranes per group. Significance was set at p<0.05. Error bars are reported as standard error of the mean unless otherwise noted.
3.4 Results

3.4.1 Characterization of functional patterns of growth factors on CG membranes

Both BMP-2 and PDGF-BB were successfully immobilized on CG membranes, either in discrete patterns of stripes or squares (Figure 3.1A). Increasing the UV exposure time (+: 1 min.; ++: 5 min.) resulted in significant \( p < 0.05 \) increase in immobilization of both PDGF-BB (Figure 3.1B) and BMP-2 (Figure 3.1C) versus no UV exposure. Total immobilized protein within exposed areas was determined for PDGF (+: 0.03 ng/mm\(^2\); ++: 0.05 ng/mm\(^2\)) and BMP-2 (++: 0.10 ng/mm\(^2\)).

Further, while only one side of the CG membrane was directly exposed to UV light during photoinmobilization, identical biomolecular patterns were observed on both sides (Figure 3.4). Therefore for all subsequent bioactivity assays, cells were seeded on both sides of the membranes after BP-photopatterning.

3.4.2 Mechanical properties of crosslinked CG membranes

CG membranes were crosslinked using a range of carbodiimide crosslinking intensities. Increasing the EDC:NHS:COOH crosslinking ratio (NX: non-crosslinked; 1:1:5; 5:2:5; 5:20.7:1; 5:2:1) led to a significant \( p < 0.05 \) increase in membrane stiffness. Here, three crosslinking chemistries (NX; 5:20.7:1; 5:2:1) were identified that led to significant \( p < 0.001 \) differences in membrane stiffness (2.85 \( \pm \) 0.22, 3.86 \( \pm \) 0.14, 5.05 \( \pm \) 0.24 MPa) between all groups (Figure 3.2).

3.4.3 Orthogonal control of biomolecular patterning and CG membrane stiffness

To confirm the capacity to orthogonally manipulate mechanical stiffness and immobilized biomolecules, we assessed the coordinated changes of EDC crosslinking and BP photoimmobilization. Notably, while UV exposure has been used to crosslink collagen biomaterials, increasing UV exposure (even to the level of 20 minutes, far beyond the maximal 5 minute exposure used for BP photoimmobilization) did not impact EDC crosslinked membrane stiffness (Figure 3.3A). Further, increasing EDC crosslinking did not impact BP photoimmobilization (Figure 3.3B). Together, these suggest orthogonal control of biomolecular patterning and CG membrane stiffness for the range of stiffness and biomolecular pattern densities reported here.
3.4.4 Cellular bioactivity and gene expression on CG membranes patterned with PDGF

We then explored the coordinated impact of substrate stiffness (2.85, 3.86, 5.05 MPa) and immobilized PDGF (−, +, ++) on the proliferation and metabolic activity (Figure 3.5; Figure 3.6) as well as changes in osteogenic gene expression profile (Figure 3.7; Figure 3.12) of ASCs. Notably, ASC metabolic activity was significantly (p<0.001) impacted by immobilized PDGF regardless of membrane stiffness (Figure 3.5A). Here ASC showed an approximately 2-fold increase in metabolic activity by day 7 in the presence of photoimmobilized PDGF, but showed no response to changes in matrix stiffness. ASC proliferation showed similar response (Figure 3.5B). Two-way ANOVA suggested significant (p<0.05) interaction between matrix stiffness and PDGF immobilization, though comparison between all groups identified a significant (p<0.001) influence of immobilized PDGF on ASC proliferation. The greatest increase in cell number was on moderately stiff membranes, suggesting an optimal stiffness for cell proliferation (Figure 3.5B). Examining gene expression profiles of the ASCs, more complex relationships between matrix stiffness and immobilized PDGF appeared (Figure 3.7). Expression of type I collagen alpha-1 (COL1A1; Figure 3.7A) was significantly (p<0.05) downregulated with increasing stiffness and increasing PDGF immobilization, while alkaline phosphatase (ALP; Figure 3.7B) was significantly (p<0.05) downregulated only with increasing PDGF immobilization. Comparatively, osteocalcin (OCN; Figure 3.7C) expression was significantly (p<0.001) upregulated with matrix stiffness, but significantly (p<0.001) downregulated with increasing PDGF immobilization. However, no interactions were observed between matrix stiffness and immobilized PDGF.

3.4.5 Cellular bioactivity and gene expression on CG membranes patterned with BMP-2

We then examined the coordinated impact of substrate stiffness (2.85, 3.86, 5.05 MPa) and immobilized BMP-2 (−, +, ++) on the proliferation and metabolic activity (Figure 3.8; Figure 3.9) as well as changes in osteogenic gene expression profile (Figure 3.10; Figure 3.13) of ASCs. The metabolic activity and total number of ASCs was not impacted by either substrate stiffness or immobilized BMP-2, with the exception of a significant (p<0.05) increase in ASC number with BMP-2 on the softest substrate (Figure 3.8B). In addition, ASCs showed a significant (p<0.05) upregulation in the osteogenic marker alkaline phosphatase (ALP) and a decrease in the adipogenic marker peroxisome proliferator-activated receptor gamma (PPARG) with substrate stiffness, regardless of BMP-2 immobilization (Figure 3.10A).
3.4.6 Gene expression on membranes patterned in quadrants of PDGF and BMP-2

We subsequently examined the interaction between BMP-2 and PDGF, creating variants of CG membranes by sequentially immobilizing half the membrane with BMP-2, rotating the membrane 90 degrees, then photoimmobilizing PDGF to create a single substrate with all possible protein combinations in 4 quadrants (BMP-2/PDGF, BMP-2 only, PDGF only, no factor; Figure 3.11A). Region-specific differences in collagen 1 were observed (Figure 3.11B). Confirming observations using monolithic patterns (Figure 3.7), collagen 1 expression was downregulated with photoimmobilized PDGF (Figure 3.11B). However, photoimmobilized BMP-2 was sufficient to both increase collagen 1 expression when presented alone as well as to rescue collagen 1 expression in the presence of PDGF and BMP-2 (Figure 3.11B).

3.5 Discussion

This work describes the development of a collagen-GAG membrane platform that enables independent manipulation of mechanical properties and biomolecule immobilization as well as spatial control over the location of biomolecule immobilization. It has been increasingly recognized that mechanical, structural, and biochemical cues all impact cell bioactivity. This constellation of substrate properties has the potential to act synergistically or destructively, or may differentially prime cell response to other signals, making strategies to orthogonally control these cues an important design target. However, orchestrated control of these properties is challenging. Many studies have focused on controlling only one property at a time, such as PDGF to induce cell proliferation, BMP-2 to promote a pro-osteogenic phenotype, or the use of IGF-1 or TGF-β to promote a pro-tenogenic phenotype. And while some recent approaches have begun to explore the combinatorial effects of matrix stiffness and biomolecular signals, strategies to incorporate spatial control over biomolecular signals remains poorly developed.

In this work we describe integration of carbodiimide crosslinking and benzophenone photolithography methods to investigate independent and combined effects of biophysical signals (stiffness) and multiple biomolecular cues (BMP-2, PDGF-BB) on porcine adipose derived stem cells using a model collagen-GAG biomaterial. We have previously reported the utility of benzophenone-based biomolecule immobilization within the collagen-GAG biomaterial platform as well as on glass, and have also demonstrated that photoimmobilized biomolecules retain their activity; however, we had not explored the potential of BP-photolithography to facilitate orthogonal manipulation of matrix stiffness and growth factor immobilization. In this study, PDGF and BMP-2 were chosen as model growth factors due to their known potential
to impact cell proliferation\textsuperscript{6,9} and osteogenesis\textsuperscript{29,45,53}, respectively. While tracking proliferation and gene expression profiles of porcine adipose derived stem cells is well defined\textsuperscript{63}, the relative impact of PDGF and BMP-2 on these processes remained unknown.

We first demonstrated that both factors could be covalently immobilized to the CG membrane in defined patterns, and that these patterns extended through the depth of the membranes (Figure 3.1, Figure 3.4). We subsequently demonstrated the utility of a combined photolithography-carbodiimide crosslinking strategy to orthogonally manipulate matrix stiffness and biomolecule immobilization. Similar to previous observations\textsuperscript{54,55,78}, increasing EDC:NHS:COOH ratios increased collagen-GAG stiffness. Here we identified 3 crosslinking treatments (Figure 3.2) and created substrates with significantly different elastic moduli. Crosslinking treatments were chosen so that all substrate were kept in a closely related range of stiffness (2.85–5 MPa). Importantly, UV exposure during BP photolithography did not significantly impact mechanical properties of the membranes and EDC crosslinking did not impact later surface bioconjugation (Figure 3.3). Previously, we demonstrated that BP photoimmobilization does not impact the crystallinity or structural properties of the collagen fibers within the CG biomaterial\textsuperscript{57}. Together, these results suggest that sequential use of EDC-crosslinking and BP-photolithography provides a platform to orthogonally manipulate the elastic modulus and presence of immobilized biomolecules within a model CG-biomaterial.

We subsequently investigated the individual and coordinated impact of matrix stiffness and biomolecule (PDGF, BMP-2) attachment on adipose-derived mesenchymal stem cell bioactivity (Figures 4–7). While many of these results are consistent with previous findings in the literature that investigated the monolithic impact of PDGF\textsuperscript{3,6,9,79}, BMP-2\textsuperscript{29,30}, or matrix stiffness\textsuperscript{38,40}, results reported here serve as an important proof-of-principle of a coordinated approach to examine the intersection between mechanical and biomolecular signals. We observed significantly increased metabolic activity and cell proliferation on substrates with low and high doses of immobilized PDGF as compared to controls (Figure 3.5). We also found that matrix stiffness contributed to cell proliferation, which peaked at intermediate substrate stiffness at the highest dose of PDGF. Matrix stiffness has previously been shown to increase growth-factor-dependent Erk activation through focal adhesion assembly\textsuperscript{80}, providing an explanation for our observed differential response in PDGF-induced proliferation. The facile identification of a combination of PDGF density and substrate stiffness to increase proliferation highlights the potential of our approach. Similar to our previous observations of examining tenocyte bioactivity in response to PDGF supplementation\textsuperscript{6}, there appeared to be a tradeoff between cell proliferation and phenotype with PDGF supplementation (Figure 3.7). While COL1A1, ALP, and OCN expression were all upregulated with increasing stiffness, this effect was lost with addition of the pro-growth signal, PDGF (Figure 3.7). We subsequently examined the effects of BMP-2 immobilization and matrix stiff-
ness on ASC bioactivity and gene expression. To simplify analysis, we chose to examine the presence/absence of photoimmobilized BMP-2 rather than dose-dependent results. Consistent with previous findings in the literature\(^{79,81}\), we found cell bioactivity was not correlated with BMP-2, and additionally noted a lack of coordinated interaction between BMP-2 and substrate stiffness (Figure 3.8). Although BMP-2 is a known pro-osteogenic factor, we observed that photoimmobilized BMP-2 had little effect on lineage-specific gene expression. Instead, stiffness had a major effect on ASCs which exhibited increasing pro-osteogenic ALP expression and decreasing pro-adipogenic PPARG expression with increasing substrate stiffness (Figure 3.10). The impact of matrix stiffness on changes in pro-osteogenic vs. pro-adipogenic gene expression profiles is consistent with previous observations in the literature\(^{82}\). While initially surprising, the lack of an effect of BMP-2 on pro-osteogenic gene expression profiles suggest that over the range of stiffness and BMP-2 density tested here, ASC response is dominated by matrix mechanical properties. Ongoing efforts are exploring a wider range of matrix stiffness and immobilized BMP-2 densities. Further, as BP-photoimmobilization has been shown to be effective within fully-3D CG scaffolds, future efforts will likely explore the coordinated impact of matrix stiffness, pore size, and BMP-2 immobilization in the context of regenerative medicine approaches for critical size bone defects.

To demonstrate the greater potential for integrating BP photolithography with the CG biomaterial platform, we lastly examined spatially-defined interactions between PDGF and BMP-2 (Figure 3.11). Such an effort is a demonstration of principle for ongoing efforts exploring a wider range of pattern densities and their resultant impact on ASC response. Here, we examine the impact of the highest immobilization density of both PDGF and BMP-2 on the substrate with the greatest stiffness. No significant difference in metabolic activity or cell number was observed after 7 days (data not shown); however, expression of COL1A1 was strongly increased with the presence of BMP-2 alone or in combination with PDGF (Figure 3.11). Substrates containing PDGF alone, or no immobilized growth factors, showed marked downregulation of COL1A1, consistent with observations for monolithic PDGF immobilization (Figure 3.7). Given the observed interactions between matrix stiffness and biomolecule immobilization, future efforts will leverage this approach to explore a wider range of combinatorial environments. Further, given recent observations that matrix stiffness can impact MSC receptivity to biomolecular signals\(^{44}\), the system described here may offer unique potential to rapidly assess signal transduction and cell response for a range of matrix environments across a single substrate via immunofluorescence-based assays. Here, efficient identification of combinations of matrix mechanical and biomolecular signals able to support enhanced ASC bioactivity in the absence of media supplementation provides the foundation for developing instructive biomaterials containing selective alterations to mechanical and compositional properties to enhance multi-lineage ASC specification in a spatially
3.6 Conclusions

This work establishes the ability to separate fabrication and biomolecule patterning via a molecularly-
general biomolecule photoimmobilization method using a CG biomaterial. We demonstrated sequential use
of carbodiimide crosslinking and benzophenone photoimmobilization chemistries to independently control
immobilization of multiple biomolecular species (PDGF, BMP-2) as well as the mechanical properties of a
model CG substrate. Notably, this approach allows us to identify the coordinated impact of mechanical and
biochemical cues on ASC metabolic activity, proliferation, and gene expression profile. Overall, our results
highlight how the bioactivity and lineage commitment of adipose stem cells depend on a variety of cues whose
optimal combinations are difficult to predict. The approach described here may prove particularly valuable
for high throughput screening of biomaterials to identify environments supportive of a range of stem cell
phenotypes. Given the certain complexity of the chemical and mechanical environments that determine stem
cell fate and lineage commitment, this capability to independently control both classes of cue presentation
should enable more facile engineering of customized scaffold materials applicable to a wide range of tissue
engineering goals. These efforts are informing ongoing work in our lab to enhance the regenerative potential
of spatially-gradated CG scaffolds for regenerative repair of orthopedic insertions such as the osteotendinous
junction.
3.7 Figures

Figure 3.1: (A) Representative image of photoimmobilized PDGF (stripe) and BMP-2 (square) on CG membranes. Scale bar: 500 µm. (B) Immobilization of PDGF-BB as a function of UV exposure time (1, 5 min) normalized versus non-irradiated control. (C) Immobilization of BMP-2 as a function of UV exposure time (1, 5 min) normalized versus non-irradiated control. *: significant increase versus non-irradiated control.
Figure 3.2: Elastic modulus of crosslinked CG membranes as a function of EDC:NHS crosslinking intensity. *: significant difference between groups.

Figure 3.3: Orthogonal control of biomolecular patterning and CG membrane stiffness. (A) Elastic modulus of CG membranes as a function of UV exposure. (B) Mean fluorescence intensity of photoimmobilized PDGF (UV: 5 min.) as a function of EDC crosslinking intensity. Results reported as mean ± standard deviation.
Figure 3.4: Representative images of both sides of a single CG membrane. PDGF was immobilized via UV exposure on only one side of the membrane. However, stripes of immobilized PDGF are visible on both the (A) front and (B) back of the membrane. Scale bar: 500 µm.
Figure 3.5: (A) ASC metabolic bioactivity and (B) overall cell number at day 7 on CG membranes as a function of substrate stiffness and photoimmobilized PDGF. †: significant increase compared to non-PDGF functionalized substrate of identical stiffness. Individual comparisons (substrate stiffness, PDGF immobilization) between all groups are shown in Figure 3.6.
Figure 3.6: (A) ASC metabolic bioactivity at a defined PDGF immobilization (−, +, ++) with increasing stiffness and (B) at a defined substrate stiffness (2.85 MPa, 3.86 MPa, 5.05 MPa) with increasing PDGF immobilization. (C) ASC cell number at a defined PDGF immobilization (−, +, ++) with increasing stiffness and (D) at a defined substrate stiffness (2.85 MPa, 3.86 MPa, 5.05 MPa) with increasing PDGF immobilization. *: significant difference between groups.
Figure 3.7: Gene expression profiles of (A) collagen 1 (COL1A1), (B) alkaline phosphatase (ALP), and (C) osteocalcin (OCN) for ASCs cultured on CG substrates as a function of stiffness and photoimmobilized PDGF. *: significant increase compared to softest substrate with identical immobilized protein concentration. †: significant down-regulation compared to non-PDGF functionalized substrate of identical stiffness. Individual comparisons (substrate stiffness, PDGF immobilization) between all groups are shown in Figure 3.12.
Figure 3.8: (A) ASC metabolic bioactivity and (B) overall cell number at day 7 on CG membranes as a function of substrate stiffness and photoimmobilized BMP-2. †: significant difference compared to substrate of equal stiffness but without BMP-2. Individual comparisons (substrate stiffness, BMP-2 immobilization) between all groups are shown in Figure 3.9.
Figure 3.9: (A) ASC metabolic bioactivity at a defined BMP-2 immobilization (−, ++) with increasing stiffness and (B) at a defined substrate stiffness (2.85 MPa, 3.86 MPa, 5.05 MPa) with increasing BMP-2 immobilization. (C) ASC cell number at a defined BMP-2 immobilization (−, ++) with increasing stiffness and (D) at a defined substrate stiffness (2.85 MPa, 3.86 MPa, 5.05 MPa) with increasing BMP-2 immobilization. *: significant difference between groups.
Figure 3.10: Gene expression profiles of ASCs cultured on CG substrates as a function of stiffness and photoimmobilized BMP-2. (A) Alkaline phosphatase (ALP) expression is upregulated with substrate stiffness. *: significant decrease relative to stiffest membrane with no protein. (B) Adipogenic peroxisome proliferator-activated receptor gamma (PPARG) expression decreases with increasing substrate stiffness. Individual comparisons (substrate stiffness, BMP-2 immobilization) between all groups are shown in Figure 3.13.
Figure 3.11: Schematic of 4-quadrant overlapping pattern of PDGF and BMP-2 generated on CG membranes. (B) Gene expression levels of collagen 1 (COL1A1) normalized versus ASCs on control substrates (−/−).
Figure 3.12: Gene expression profiles of (A, B) collagen 1 (COL1A1), (C, D) alkaline phosphatase (ALP), and (E, F) osteocalcin (OCN) for ASCs cultured on CG substrates at (A, C, E) a defined PDGF immobilization (−, +, ++) with increasing stiffness and (B, D, F) at a defined substrate stiffness (2.85 MPa, 3.86 MPa, 5.05 MPa) with increasing PDGF immobilization. *: significant difference between groups.
Figure 3.13: Gene expression profiles of (A, B) alkaline phosphatase (ALP) and (C, D) peroxisome proliferator-activated receptor gamma (PPARG) for ASCs cultured on CG substrates at (A, C) a defined BMP-2 immobilization (−, ++) with increasing stiffness and (B, D) at a defined substrate stiffness (2.85 MPa, 3.86 MPa, 5.05 MPa) with increasing BMP-2 immobilization. *: significant difference between groups.
### 3.8 Tables

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3.9 References


Chapter 4

A Tunable, Photoreactive Hydrogel System to Probe Synergies Between Mechanical and Biomolecular Cues on Adipose-Derived Mesenchymal Stem Cell Differentiation

We acknowledge funding for this work from the National Science Foundation Division of Material Research under award 1105300. Porcine adipose-derived stem cells were a gift from Prof. M. Wheeler (UIUC) with assistance from A. Maki. We thank C. Herman for synthesis of the benzophenone methacrylamide monomer. We thank Prof. H. Kong (UIUC) for use of the mechanical testing system. We also acknowledge A. Alsop for helpful discussions.

4.1 Abstract

Here we describe the incorporation of the photoreactive molecule benzophenone into polyacrylamide hydrogels, allowing for orthogonal control over spatial incorporation of biomolecules and selective modulation of matrix stiffness. We examine differentiation of adipose-derived stem cells (ASCs) cultured on matrices whose elastic moduli are tuned to \(~5\)kPa, \(~14\)kPa, and \(~37\)kPa combined with an immobilized growth factor, bone morphogenic protein 2 (BMP-2). We observe that BMP-2 has the most pronounced effect at the intermediate stiffness, while the lowest and highest stiffness hydrogels are directed by elasticity alone. Together, this approach describes a facile platform for fundamental studies of cell fate decisions in the context of both mechanical and biochemical cues, and may lead to improved insight and effectiveness of stem cell therapies.

4.2 Introduction

Stem cell therapy holds great promise for the treatment of injuries and diseases, including many applications in tissue regeneration.\(^1\) In order to fully utilize stem cells in such applications, biomaterials that facilitate study over the individual and combined effects of multiple external parameters on cell fate and function is necessary.\(^2,3\) In the human body, cells receive and integrate a complex and diverse series of stimuli presented
by the microenvironment. The extracellular matrix mediates the presentation of both adhesive proteins and
growth factors. Recent studies have also shown that mechanical stimuli exert a profound effect on stem cell
renewal and differentiation. Understanding the combined effects of the matrix environment on cell fate
is of particular importance for the field of regenerative medicine, as injury may lead to changes in matrix
biomolecular and mechanical properties; for example, fibrotic scarring and subsequent local stiffening is often
observed in response to tissue damage.

Physical and chemical factors not only play key individual roles, but also act cooperatively to orchestrate
cell behavior. Crosstalk between integrin and growth factor signaling pathways is known to direct cell
migration, proliferation, and differentiation. McBeath and coworkers showed that mechanotransduction
via RhoA/ROCK-mediated contractility controls stem cell differentiation into adipocytes or osteoblasts.
Furthermore, the role of BMP/Smad signaling in studies by Du et. al suggested that stiffness-mediated
activation of β1 integrin and subsequent integrin-mediated internalization of BMP-2 receptor accounts for
inhibited osteogenic differentiation on soft substrates. Activation of both RhoA/ROCK and FAK signaling
mediated by differential integrin expression on stiffer substrates was also shown to regulate osteogenesis via
the ERK1/2 pathway. Such intersecting network signaling has been implicated in priming growth factor
signaling on stiffer substrates. However, despite intensive research efforts, much remains to be elucidated
about the complex interplay of physical and chemical signals as combinations of these factors can have
unpredictable or unintended consequences. For example, we previously reported a tradeoff between stem
cell growth and differentiation that was remedied by combined presentation of proliferative and osteogenic
factors, highlighting the fact that new approaches are needed to create defined biomaterials to orthogonally
modulate both the mechanical and biomolecular microenvironment.

Polymer hydrogels have been used extensively as substrates for fundamental studies of cell-material
interactions owing to their inertness, high water content, permeability, synthetic tenability, and tissue-
like elasticity. Although hydrogels consist mostly of water, incorporation of biochemical cues has been a
common approach to elucidate fundamental aspects of cellular function. A range of techniques have been
applied to modify hydrogels with bioactive moieties, including covalent conjugation (e.g. thiol, azide)
or non-covalent affinity interactions (e.g. heparin, biotin), but these methods often lack the capacity to
control the spatial organization of these cues. Further, they often require exogenous labeling of bioactive
moieties or the presence of binding partners. Moreover, soft nonplanar substrates are not typically
amenable to flow or contact-based patterning approaches and often do not allow for modular control over
the presentation of multiple factors.

Our group, and others, have recently investigated the use of direct immobilization of biomolecules using
benzophenone photolithography, a simple technique that provides control over the amount and location of biomolecule immobilization regardless of surface topology.\textsuperscript{26–31} Here, BP molecules are covalently incorporated into a biomaterial. Subsequent UV exposure creates a transient benzophenone diradical that either abstracts a hydrogen from a nearby C-H bond available on a solution-phase biomolecule and recombines—resulting in a covalent C-C bond—or relaxes to the ground state from which it can be re-excited.\textsuperscript{32} Increasingly complex biomolecule patterning would otherwise require multiple orthogonal chemical modifications, but the broad C-H bond reactivity of benzophenone allows immobilization of multiple molecules without the need for (bio)chemical modification. Importantly, the benzophenone-based spatial patterning strategy allows for the post-synthetic modification of various biomaterial constructs in a way that does not perturb the intrinsic mechanical properties.\textsuperscript{16} This is in contrast to alternative strategies that either require the incorporation of biomolecular cues during the creation of the scaffold itself (e.g. during hydrogel polymerization) or covalent crosslinking chemistries that often lead to substrate stiffening.\textsuperscript{33}

In order to derive meaningful information from cellular interactions with the surrounding environment, it is highly desirable to control both the physical and biomolecular properties of a biomaterial. Unlike ECM-derived materials, synthetic hydrogels offer increased control over chemical composition and modifiable mechanical properties more similar to native tissue. Here, we apply a simple photolithographic method to create matrices from polyacrylamide, a non-fouling polymer that will allow specific control of only the chosen molecules on the surface. With the additional spatial control provided by using light as a reagent, the integration of the benzophenone-based derivatization approach offers significant opportunities for improving the efficiency and biomimetic complexity of screening cell-substrate interactions. As a first demonstration, we explore combining these cues on a porous two-dimensional substrate; however, this strategy could be easily translated to three-dimensional architectures that more closely resemble the ECM structure. To determine and decouple the collective roles that biochemical and physical cues play in modulating the differentiation of mesenchymal stem cells, we report the application of BP photolithography to study the effect of mechanical properties in the presence and absence of BMP-2, a potent osteogenic factor.\textsuperscript{34}

### 4.3 Materials and methods

All general laboratory supplies were purchased from Fisher Scientific and reagents purchased from Sigma-Aldrich unless otherwise noted.
4.3.1 Synthesis of N-[3-[(4-benzoylphenyl) formamido]propyl] methacrylamide (BPMAC)

The synthesis of benzophenone methacrylamide monomer BPMAC was adapted from previously described conditions,\(^{35}\) and shown in Figure 4.1. Briefly, 4-benzoylbenzoic acid (BBA) was reacted with N-(3-aminopropyl)methacrylamide (APMA) in the presence of 4-(dimethylamino)pyridine (DMAP) and (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) in dimethylformamide (DMF). BBA (1.3 mmol) was mixed with the other reactants in DMF at a molar ratio of 1.2:1.2:1:1 EDC:DMAP:BP:APMA and stirred overnight at room temperature. The solution was poured into 90 mL \(\text{CH}_2\text{Cl}_2\) and washed five times with \(\text{H}_2\text{O}\) (30 mL), followed by 5 washes with 50 mM \(\text{NH}_4\text{C}_2\text{H}_3\text{O}_2\), (30 mL, pH 4.5). The organic phase was dried over \(\text{MgSO}_4\) and solvent was removed under vacuum. The synthesis yield was 75\% and the white solid was confirmed to be consistent with the literature by 1H NMR and positive ion ESI MS. The pure material was stored in 100 mM aliquots in DMSO at \(-20\) °C.

4.3.2 Fabrication of hydrogels

Polyacrylamide gels were fabricated from monomer solutions containing acrylamide and bisacrylamide (GE Life Sciences) in ratios of 10:0.3, 10:0.1, or 10:0.03 acrylamide:bis-acrylamide (%w/v) in pH 7.4 phosphate buffered saline (PBS, Corning). When indicated, benzophenone methacrylamide was added from 100mM stock solution in DMSO at a 1:100 volume ratio. These monomer solutions were filtered using 0.2 \(\mu\)m filters and degassed under vacuum immediately prior to use. Free radical polymerization was initiated by adding 1:10 total volume ammonium persulfate solution (8mg/mL, Bio-Rad) and 1:100 total volume tetramethylethylenediamine (Bio-Rad) to gel solutions. Gels were cast between two glass plates treated with Repel-Silane (GE Life Sciences) separated by a 1 mm spacer. After complete polymerization (~1 h), gels were removed from the plates, swollen in PBS overnight, and punched into 8 mm discs using a dermal biopsy punch (Integra-Miltex). Gels were then stored protected from light in PBS at 5 °C until use.

4.3.3 Biomolecule photoimmobilization and visualization

Prior to photopatterning, hydrogels were first incubated with the protein of interest; bone morphogenetic protein 2 (BMP-2, R&D Systems) and/or fibronectin (R&D Systems) at 5 \(\mu\)g/mL in PBS for 1 hour. Gel disks were then exposed to UV light (351-364 nm) through a photomask using a beam expanded and intensity homogenized (\(\pi\)-shaper, Molecular Technologies, Berlin, Germany) Argon ion laser (Coherent Innova 90-4, Laser Innovations). For gradient immobilization, a translation stage (ThorLabs) was programmed to move a
shutter so as to gradually reveal the substrate over a defined time period during illumination. For substrates containing both fibronectin and BMP-2, the proteins were uniformly and sequentially deposited one at a time with PBS rinsing in between. Hydrogels for cell culture experiments were rinsed for 1 hour in PBS and stored at 5 °C until use. Hydrogels used to visualize the immobilized biomolecule were rinsed in 0.2% pluronic F-127 in PBS and blocked in 1% BSA-PBS for 1 hour each before staining with primary rabbit anti-BMP-2 antibody (AbCam) and secondary goat anti-rabbit IgG AlexaFluor-647 antibody conjugate (Invitrogen) in 1% BSA-PBS overnight. Samples were rinsed in PBS before being imaged using a microarray scanner (Molecular Devices).

4.3.4 Mechanical characterization

Substrate stiffness was evaluated by measuring the elastic modulus of 8 mm hydrogel disks following incubation in PBS overnight at room temperature. Samples were tested by uniaxial compression at a rate of 1 mm/min using a mechanical testing system (Insight, MTS Systems) and data collected and analyzed using TestWorks software. The elastic modulus was calculated by fitting the linear regime (i.e. the first 10%) of the stress-strain curve.

4.3.5 Adipose-derived mesenchymal stem cell culture

Adipose-derived mesenchymal stem cells (ASCs) were harvested from porcine donors as previously described and preserved in liquid phase nitrogen. ASCs were cultured in standard flasks at 5,000 -10,000 cells/cm² with Dulbecco’s High Glucose Modified Eagles Medium (DMEM) supplemented with Penicillin-Streptomycin, amphotericin B, and 10% fetal bovine serum (Cell Media Facility, UIUC School of Chemical Sciences). Prior to culture experiments, hydrogel specimens (post-functionalization) were sterilized with UV, placed in low-attachment 24-well plates (Corning) and incubated in serum-free DMEM at 37 °C for at least 30 minutes prior to cell seeding. Cells were collected using trypsin-EDTA and resuspended in complete DMEM with serum at a concentration of ~4,500 cells per 15 µL. Substrates were seeded at low density to reduce cell-cell interactions by placing 15 µL of cells onto each specimen. Cells were allowed to attach for 1 hour, followed by addition of complete DMEM. Media, which was changed every 3-4 days.

4.3.6 Metabolic activity

Following incubation with alamarBlue reagent (Invitrogen) for 2 hours, resorufin fluorescence was measured using a fluorescence microplate reader (Tecan). Relative cell metabolic activity was determined and reported as a percentage of the control (without BP, protein, or UV exposure).
4.3.7 Cell staining and fluorescence microscopy

Cell-laden hydrogels were rinsed in PBS and then fixed in 10% neutral buffered formalin for 30 minutes followed by rinsing three times in PBS. For fluorescence staining, samples were permeabilized in a solution of 1% Triton X-100 in PBS for 30 minutes, blocked in a solution of 1% bovine serum albumin in PBS for 30 minutes, and then incubated with rhodamine-phalloidin (diluted 1:500) and/or DAPI (diluted 1:1000) for 30 minutes. Samples were rinsed and stored in PBS at 5 °C. For Oil Red O staining, a working solution was freshly prepared from a 0.3% (w/v) solution of Oil Red O in isopropanol by diluting 3 parts of the stock solution to 2 parts deionized water and filtering through filter paper. Samples were incubated twice with 60% isopropanol for 5 minutes, and then incubated in the Oil Red O solution for 5 minutes before rinsing thoroughly with PBS. All fluorescence and brightfield cell images were taken using an EVOS microscope (Advanced Microscopy Group).

4.3.8 Alkaline phosphatase (ALP) activity

Alkaline phosphatase activity was measured on day 7 using p-nitrophenyl phosphate (pNPP, AbCam), a colorimetric substrate that turns yellow when dephosphorylated by the enzyme. Cell culture medium was collected, incubated with pNPP solution, and the resulting absorbance read at 405 nm. Activity in glycine units was determined by comparison with a standard curve. To ensure activity differences were not due to larger numbers of cells, the cells on hydrogels were lysed in RIPA buffer (Invitrogen), diluted in Tris-EDTA buffer, and measured for consistent DNA content using Hoechst 33342 via fluorescence measurement using a microplate reader (Tecan).

4.3.9 Real-time quantitative reverse transcriptase PCR (qPCR)

Quantification of lineage-specific RNA targets was performed via real-time quantitative reverse transcriptase PCR (qPCR). Samples were rinsed in PBS and the RNA isolated using an RNeasy mini kit (Qiagen). RNA was converted to cDNA in a 20 µL volume using QuantiTect reverse transcription kit according to the manufacturer’s instructions. PCR primers (see Table 4.1) for alkaline phosphatase (ALP), type 1 collagen alpha-1 (COL1A1), osteocalcin (OCN), peroxisome proliferator-activated receptor gamma (PPARG), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were selected from those previously validated in the literature.36 qPCR was performed using a QuantiTect SYBR Green RT-PCR kit (Qiagen) according to the manufacturer’s instructions on a 7900HT Fast Real-Time PCR system (Applied Biosystems) and the cycle threshold data collected using SDS software. Data was converted to relative expression using the ∆∆CT method with GAPDH as a housekeeping gene and log transformed for graphical representation.
4.3.10 Statistical analysis

All data are presented as mean (n=3) ± standard deviation unless otherwise specified. Statistical significance was determined using t-test for pairwise comparisons or ANOVA followed by Tukey’s Multiple Comparison Test or uncorrected Fisher’s LSD for groups of three or more.

4.4 Results and discussion

The extracellular matrix is a network presenting immobilized growth factors and physical cues that direct cell behavior. Exciting progress has been made in deciphering the complexity of the ECM; however, much of this work has focused individually on a single class of cell stimuli: microstructure, mechanics, or presented biomolecules. However, the multifactorial studies probing synergistic effects across these different classes have been relatively underexplored. In this work, we sought to systematically examine mesenchymal stem cell fate decisions in response to the osteogenic factor bone morphogenic protein 2 (BMP-2) while simultaneously varying the hydrogel stiffness.

4.4.1 Hydrogel fabrication and characterization: Independent control over biomolecular presentation and mechanical properties

We have previously reported the use of benzophenone photolithography in immobilizing bioactive molecules on planar and non-planar glass and collagen substrates. Here we incorporated the benzophenone moiety into a crosslinked polyacrylamide hydrogel system to enable presentation of biomolecules in a spatially defined manner as shown in Figure 4.2a. This attachment strategy allowed us to selectively incorporate fibronectin for cell adhesion as well as BMP-2, an osteoinductive factor, on the surface of polyacrylamide hydrogels. Here we were able to control both the location of protein immobilization via a photomask to define an area of illumination, while creating gradients in local BMP-2 dose via the use of an opaque shutter mounted to a translation stage (Figure 4.2b). We quantified the increase in biomolecule immobilization with increasing UV exposure time (Figure 4.2c, p ≤ 0.001), and observed negligible protein attachment to a UV and BMP-2 exposed control hydrogel that lacked benzophenone (−BP).

A major benefit of the hydrogel system is that in addition to tailoring local biomolecule presentation, the elastic modulus of the substrates can also be separately controlled by varying the amount of bisacrylamide crosslinker incorporated into the hydrogel. We created hydrogels of low, medium, and high bulk stiffness (∼5kPa, ∼14kPa, and ∼37kPa) corresponding to reported moduli of adipose, muscle, and bone tissue. The ratios of 10:0.3, 10:0.1, and 10:0.03 acrylamide:bis-acrylamide (%w/v) were found to produce these desired
substrate stiffnesses as shown in Figure 4.3a and Figure 4.4. The modulus increased significantly (p ≤ 0.001) with increasing amounts of crosslinker, consistent with previous reports. Importantly, we found that neither the incorporation of benzophenone nor UV exposure significantly affected the mechanical properties of the hydrogels. This is significant because it demonstrates the ability of this approach to modulate stiffness independent of the presentation of biomolecular cues.

4.4.2 Cell viability on benzophenone hydrogels

We confirmed that cell viability on BP-containing substrates was not adversely affected compared to substrates with no growth factor (BMP-2) or no BP. We observed no significant difference in the metabolic activity of ASCs on each substrate at day 1 or day 7 in culture (Figure 4.3b). Microscopic imaging of cell morphology of ASCs cultured on BP-hydrogels confirmed healthy spreading and proliferation, as visualized by nuclear and actin cytoskeleton staining in Figure 4.3c.

4.4.3 Influence of stiffness on ASC differentiation

In the absence of adhesion ligands, non-fouling polyacrylamide hydrogels do not support cell adhesion. Therefore, in order to determine the effect of stiffness alone on cell differentiation, adipose-derived mesenchymal stem cells were seeded onto substrates of low, medium, and high elastic moduli onto which fibronectin was photoimmobilized to promote cell adhesion. Gene expression analysis after 7 days focused on a combination of bone-specific (ALP, COL1A1, and OCN) and a fat-specific marker (PPARG) as shown in Figure 4.5a. We observed that the highest stiffness substrates led to significantly elevated expression of ALP and OCN (p ≤ 0.05), and COL1A1 showed a significant linear trend (p ≤ 0.05) in expression with increasing stiffness. Conversely, the bone-specific PPARG showed a significant decrease in expression with increasing substrate stiffness (p ≤ 0.05). These results suggest that cells cultured on ~37kPa substrates are inclined towards an osteogenic phenotype and a reduction in adipose character, consistent with reports in the literature.

This trend towards increasing osteogenic character with increasing substrate stiffness was further confirmed through assays for functional alkaline phosphatase activity, which signifies bone-like tissue formation. After 7 days of culture, the stiffest substrates showed the highest ALP activity, followed by medium and then low stiffness substrates (Figure 4.5b, p ≤ 0.05).
4.4.4 Combined influence of stiffness and immobilized BMP-2 on ASC differentiation

To probe the combined effects of mechanical and biomolecular cues, we immobilized BMP-2 on substrates of varying stiffness and utilized qPCR and ALP activity assays to determine relative osteogenic versus adipogenic lineage character. After 7 days in culture, medium stiffness substrates showed the greatest fold expression changes compared control substrates in expression of lineage-specific marker genes (Figure 4.6a). Expression levels of ALP and OCN were $\sim 11$ and $\sim 7$ fold higher, respectively, in the medium stiffness condition versus control. The magnitude of fold change was significantly higher on medium substrates as compared to low and high stiffness conditions ($p \leq 0.05$) and adipogenic marker PPARG was significantly decreased ($p \leq 0.05$). In the stiffest substrates, we observed less pronounced upregulation of ALP, OCN, and COL1A1 compared to the control lacking BMP-2, likely because the expression was already substantial in the baseline condition. Furthermore, the lowest stiffness substrates showed little change compared to the non-functionalized control. Interestingly, these results suggest that synergistic combinations of stiffness and BMP-2 have the power to optimize specific cell differentiation outcomes. Given the interdependence of mechanical and biochemical properties to mediate cell behavior, this platform could be a valuable tool for control of key stem cell niche attributes.

Alkaline phosphatase activity was measured as a function of stiffness and BMP-2 immobilization, supporting the trend observed in the gene expression studies. Similar to the ALP results discussed above, ALP activity tends on increase with stiffness (Figure 4.6b). Compared to substrates without immobilized growth factor, medium stiffness substrates showed the greatest relative increase in ALP activity with the addition of BMP-2, as shown in Figure 4.6c. An increase in ALP activity was also observed for the high stiffness group; however, the relative increase due to the addition of BMP-2 was slightly less in magnitude. Conversely, low stiffness substrates showed a considerably smaller increase in ALP activity with BMP-2 supplementation, which was significantly lower than medium or high stiffness groups ($p \leq 0.05$).

As a final method of probing the combined mechanical and biomolecular effects within this hydrogel system, we examined accumulation of the lipid dye Oil Red O as a marker of adipogenesis (Figure 4.7; black arrowheads indicate positive Oil Red O staining; white arrowheads indicate cells negative for Oil Red O staining). Consistent with gene expression and ALP activity assays, Oil Red O staining revealed significant lipid droplet staining in cells cultured on soft substrates regardless of BMP-2 supplementation. Conversely, the stiffest substrates appeared to have negligible Oil Red O staining with or without BMP-2, consistent with the observation that cells on stiff membranes undergo osteogenic differentiation without the need for growth factor supplementation. However, the medium stiffness substrates showed pronounced decreases in
Oil Red O staining in the presence of the BMP-2, compared to non-functionalized substrates.

Taken together, these results show that cells cultured on substrates having particular stiffness are poised to be extra responsive to the presence of immobilized biomolecule cues, suggesting that synergistic environmental factors work together to optimize specific cell differentiation outcomes. In this case it is particularly interesting that an intermediate stiffness between that of native fat and bone tissue is the most influenced by biomolecular cues.\(^{42}\)

In considering how tissues develop from the earliest stages where mechanical environments are not fully established, a more heightened responsiveness to biomolecular cues might serve as a mechanism for eliciting greater local control over cell differentiation, proliferation, and lineage commitment. Additionally, most reports in the literature focus on efforts to drive bone-derived mesenchymal stem cell differentiation. Notably, our findings show that adipose-derived stem cells maintain flexibility to differentiate towards osteogenic or adipogenic lineage especially at intermediate stiffnesses, in the same fashion as bone-derived cells. These results suggest strongly the need for approaches to tease apart the effect of mechanical, biomolecular, and microstructural guidance cues on cell fate in order to continue to shed light on the complex synergies that underlie tissue development and regeneration. Versatile biomaterials platforms, such as the phototunable hydrogel system described here that offer independent control over multiple classes of cues, will play a significant role in future efforts to elucidate multifactorial control elements that regulate the stem cell niche.

### 4.5 Conclusions

In this work, we have demonstrated the utility of benzophenone-containing hydrogels as a tunable platform for screening the combined effects of local physical and chemical cues on adipose stem cell fate. Integrating benzophenone methacrylamide into hydrogels of varying crosslinking density allowed us to tune mechanical and biochemical properties independent of one another. This photoreactive hydrogel provides a facile strategy for fabricating patterned and gradient designs into a hydrogel network without the need for biomolecule labeling. We found that increasing hydrogel stiffness alone induced an osteogenic phenotype, but lineage commitment was amplified particularly at moderate stiffness by the addition of BMP-2. This study shows the potential of orthogonally tunable biomaterials to elucidate cell fate decisions in a combinatorial manner and represents an important step towards building in the complexity of the native extracellular matrix. Improving our understanding of these stimuli may lead to the development of more sophisticated materials for regenerative medicine.
4.6 Figures

Figure 4.1: Synthesis of benzophenone methacrylamide monomer.
Figure 4.2: Biomolecule immobilization and characterization. (A) Schematic demonstrating the process to introduce and utilize photoreactive monomer in polyacrylamide hydrogels to create biomolecular patterns and gradients. (B) Fluorescence micrograph and quantitation of hydrogel substrate presenting immobilized gradient of BMP2 in a vertical stripe pattern visualized using AlexaFluor647-labeled anti-BMP-2 (red, scale bar=500 µm). (C) Photochemical immobilization of BMP-2 corresponds to UV exposure time. Data are mean ± SD, n=3, (*) significantly different than all other exposure time groups, p ≤ 0.001; (#) control is significantly different than all other exposure time groups, p ≤ 0.05.
Figure 4.3: Mechanical characterization and biocompatibility of polyacrylamide hydrogel substrates. (A) Elastic modulus of hydrogel discs with and without benzophenone monomer; before and after UV exposure. Stiffness is varied through monomer:crosslinker ratio — Low, 10:0.03; Medium; 10:0.1; High, 10:0.3 (%w/v). Data are mean ± SD, n=3, ns=no statistical significance (p >0.05); † significantly greater than Low group, p ≤ 0.01; ‡ significantly greater than Low and Medium groups, p ≤ 0.001; § significantly greater than Low group, p ≤ 0.001. (B) Metabolic activity is not affected by the presence of benzophenone methacrylamide (BP), BMP-2 or UV light. Data are mean ± SD, n=4, ns=no statistical significance (p >0.05). (C) Fluorescence micrograph of ASCs cultured on benzophenone-containing hydrogels after 7 days. Blue: nuclei (DAPI); Red: actin (rhodamine phalloidin); Scale bar: 200 µm
Figure 4.4: Representative stress-strain curves of low, medium, and high stiffness hydrogels with or without benzophenone.
Figure 4.5: Increased substrate stiffness induces osteogenic differentiation. (A) Osteogenic (ALP, COL1A1, OCN) and adipogenic (PPARG) gene expression of cells cultured on varying stiffness substrates without BMP-2 normalized relative to the softest substrate. Data are mean ± SEM, n=3, (*) significantly different than other two experimental groups, p ≤ 0.05; (∨) significant linear trend with decreasing stiffness, p ≤ 0.05. (B) ALP activity, expressed as glycine units (U), of cells cultured on substrates of varying stiffness without BMP-2 after 7 days. Data are mean ± SD, n=3, (*) significantly different than other two experimental groups at the same time point, p ≤ 0.05.
Figure 4.6: Combined effects of immobilized BMP-2 and stiffness on osteogenic differentiation. (A) Relative osteogenic (ALP, COL1A1, OCN) and adipogenic (PPARG) gene expression after 7 days as a function of stiffness compared to soft substrate without BMP-2. Data are mean ± SEM, n=3, (*) significantly different than other two experimental groups, p ≤ 0.05. (B) ALP activity, expressed as glycine units (U), of cells cultured on varying stiffness substrates containing immobilized BMP-2 after 7 days. (C) Relative ALP activity on day 7 of cells cultured on varying stiffness substrates containing immobilized BMP-2 normalized relative to the substrate without BMP-2. Data are mean ± SD, n=3, (**) significantly different than other two experimental groups, p ≤ 0.01.
Figure 4.7: Representative optical micrographs of cell-seeded hydrogels stained with Oil Red O after 7 days. Lipid droplets, characteristic of adipogenic lineage, decrease with higher stiffness and BMP-2. Overlay of fluorescent and transmitted light images. Red: Oil Red O; Blue: DAPI (nuclei); Black arrows indicate cells negative for Oil Red O staining; Scale bars: 100 µm

Figure 4.8: Schematic of photoreactive hydrogel concept used to orthogonally tune mechanical and biomolecular properties.
### 4.7 Tables

Table 4.1: Gene-specific primer sequences.

<table>
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<th>Gene</th>
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<th>Reverse primer sequence (5’→3’)</th>
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<td>ALP</td>
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</tr>
</tbody>
</table>
4.8 References


Chapter 5

Conclusions

The work described in this dissertation set out to develop enabling tools for guiding cell-substrate interactions in a systematic and controllable manner. Despite significant research investment and activity, clinical advances in cell-instructive materials and the field of tissue engineering in general have not kept pace with expectations. Although it is widely recognized that cellular function is determined by a constellation of cues, much of the scientific literature still focuses on the role of a single factor or class of signals. In order to gain and apply insight from the native cell microenvironment, biomaterials of increasing complexity and spatial heterogeneity are required. A simple, universal strategy that allows presentation of a wide range of biomolecules along with tunable mechanical properties has significant potential to recapitulate key characteristics of the ECM.

The benzophenone photolithography method overcomes important limitations in the design of biomaterials. The promise of this approach was demonstrated in Chapter 2, in which neutrophil flow assays on P- and E-selectin substrates shed light on the anti-inflammatory effect of bromelain. Chapter 3 illustrated the importance of mimicking functional characteristics of tissue to investigate stem cell differentiation in the context of multiple chemical cues and variable mechanical properties on collagen-glycosaminoglycan membranes. In Chapter 4, I extended the utilization of benzophenone to softer substrates using hydrogels to examine the interrelationship of mechanical and biochemical signaling on osteogenesis.

Taken together, the work presented here highlights the utility of this method; however, much remains to be understood about the complex microenvironment. There is a need to better understand the answers to fundamental biological questions, such as:

- How do cells integrate competing or synergistic signals?
- Do spatial variations (i.e. gradients) and organized heterogeneity in signaling matter?
- Is there a critical threshold for mechanical or biochemical cues?
- How do cell characteristics in three-dimensional environments differ from those cultured in two dimensions?
• Are cell fate and function decisions reversible?

Overall, these studies have contributed to a more quantitative understanding of how cells process the co-operative effects of multiple factors. Researchers must continue to systematically investigate multiparametric cell behavior in order to develop and fully realize therapies that imitate nature.
Appendix A

A Photocleavable Linker for User-Mediated Release of Growth Factors

A.1 Introduction

The work described thus far has demonstrated control of cell-material interactions through immobilization of multiple biomolecules as well as tissue-matched mechanical properties. Future directions in creating cell instructive materials must extend to controlling biomolecule activity in time and space to fully recapitulate the complex ECM.

Towards the creation of dynamically responsive materials, we sought to make a tri-functional linker that can be coupled to the biomaterial surface (e.g. CG scaffold), conjugate the desired growth factor (e.g. protein or peptide), and release the growth factor at the desired time. Using a photolithographic strategy for selectively cleaving chemical cues provides the same advantages as demonstrated in the body of this dissertation: facile control of the location and amount of photocleavage via modulation of light exposure. Thus this strategy would allow the researcher to turn “off” the signal at a later time point or spatial location by removing the bioactive molecule. Alternatively, release of the molecule could allow its subsequent delivery. The complete linker and UV-mediated nitrobenzyl cleavage is shown in Figure A.1A. The heterofunctional linker consists of hydrazinonicotinamide (HyNic) and sulfhydryl moieties for conjugation on either end.

A.2 Materials and methods

A.2.1 Solid phase synthesis of tri-functional linker

The photocleavable linker was constructed using solid phase peptide synthesis on 0.1mmol scale starting with Fmoc-Cys(Trt) Wang resin. Resin (Chem-Impex Inc, 0.55 mmol/g) was swollen in dimethylformamide (DMF), deprotected using 20% piperidine in DMF, and coupled to Fmoc-Peg2-OH (Chem-Impex) using 1:1:2 ratio of amino acid/HCTU/N-Methylmorpholine. Three more coupling steps were carried out: Fmoc-2-nitro-β-phenylalanine (Chem-Impex), then Fmoc-Peg2-OH, and finally Boc-HyNic (Solulink) were added sequentially. Finally, the resin was washed with DMF, washed with ethanol, and cleavage from the solid
support carried out in a separate flask. The resin was removed by filtration, TFA evaporated from the filtrate, and ice cold diethyl ether added to extract the peptide. The resulting viscous yellow oil was analyzed by ESI-MS. Initially, the deprotection was performed using TFA/DTT/water/triisopropylsilane (88/5/5/2) for 1.5 hours; however, this resulted in species 40 mass units less than expected due to Boc deprotection producing the free hydrazone on the N terminus (Figure A.1B). The cocktail TFA/acetone/water/TIS (92.5/2.5/2.5/2.5) produced the acetone hydrazone with expected molecular weight (Figure A.1C). This cocktail was tested with added DTT, which appeared to improve the observed yield of desired product (Figure A.1D). Note: the presence of acetone is critical.

### A.2.2 Linker conjugation to substrate and payload

Growth factors of interest can be readily modified through primary amines using NHS-maleimide, which can be attached to one side of the linker through its cysteine moiety. Sulphydryl groups occur in very low abundance in type I collagen, so maleimides will not be conjugated nonspecifically to collagen substrates. The other end of the linker, HyNic, can be covalently attached to an aryl aldehyde modified surface (e.g. collagen scaffold treated with succinimidyl-4-formylbenzamide).

To prepare collagen membranes, the dry membranes were weighed, then soaked in ethanol for several hours, then soaked in 100 mM phosphate buffered saline pH 7.4 overnight. The correct amount of sulfo-S-4FB reagent (Solulink) was determined according to the manufacturer’s protein/peptide conjugation calculator. Briefly, a 3-fold molar excess of Sulfo-S-4FB was reacted with the membranes by adding 21.2 µL of sulfo-s-4FB stock solution (5 mg/mL in DMF) to the membranes in 7 mL PBS (“concentration”=dry membrane mass/final volume=2.5 mg/mL; collagen mw=175 kDa). The membranes were incubated at room temperature for 2 hours, then rinsed three times with PBS and stored in PBS in the dark at 5 °C until use.

Next, the photocleavable linker was attached to collagen-4FB membranes (n=6) by incubating 174 µg peptide linker for 2 hours in a solution of 100 mM PBS + 20mM aniline at pH 6.

As a first step to illustrate attachment and release using the linker peptide, we attached the fluorophore fluorescein-maleimide (Fisher) to the end of the linker. Membranes were incubated with fluorescein-5-maleimide (25-fold molar excess dye with respect to the total amount of linker added) overnight at 5 °C.
A.3 Preliminary progress towards light-controlled growth factor delivery

The substrate was exposed to UV light ($\lambda$=350-365 nm, power 50mW/cm$^2$) for 4 minutes. Substrates were rinsed in PBS, and the fluorescence of the supernatant and the collagen surface were measured to determine released and immobilized fluorescein (Figure A.2). Preliminary results suggest that UV exposure does cause dye to release from the membrane. Considerable fluorescein release was measured in control substrates incubated with fluorescein-maleimide only (no peptide linker), suggesting that further rinsing may be necessary to remove nonspecific attachment to the collagen membranes.

Overall, the photocleavable linker was successfully synthesized and shown to mediate the release of a fluorescent dye from collagen membranes. This user-defined removal strategy could be a useful approach to temporally control the release of growth factors in order to orchestrate changes in cell behavior via dynamic biochemical signaling.
Figure A.1: Synthesis of nitrobenzyl-containing photocleavable linker.
Figure A.2: Characterization of fluorescein dye released from collagen membrane.