TUNEABLE COLLAGEN SCAFFOLDS FOR THE DIRECTED DIFFERENTIATION OF EMBRYONIC

STEM CELLS TOWARDS NEURAL LINEAGES

by

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ABSTRACT OF THE DISSERTATION

Tunable Collagen Scaffolds for the Directed Differentiation of Embryonic Stem Cells Towards

Neural Lineages.

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This thesis aims to develop and understand the role of peptide modified collagen scaffolds in

tissue engineering and stem cell differentiation. The extracellular matrix and microenvironment

surrounding cells plays a critical role in controlling phenotypic and genotypic behavior of cells. It

is important to elucidate this role in stem cell differentiation to develop regenerative therapies.

Here in, we developed a method to alter, positively and negatively, the adhesion migration and

reorganization of scaffolds by fibroblast and smooth muscle cells on and in collagen. By grafting

an adhesive peptide 'RGD' and a scramble non-adhesive peptide 'RDG' to collagen we were able

to modulate in a dose dependent manner the adhesion of cells to collagen and establish the

bimodal dependence of migration on adhesion in and on collagen scaffolds. We then extended

this approach to understand the role of cell adhesion on differentiation of stem cells; we

assayed the differentiation of sox-1 reporting murine embryonic stem cells on these scaffolds.

Sox-1 expression was found to be inversely related to cell-matrix adhesion. Sox-1 reporting stem

cells cultured on RDG grafted collagen scaffolds had a significantly greater population of cells

that were sox-1 positive relative to controls. However, the difference between conditions was at

most 10%. To further evaluate the ability of matrix cues to guide the differentiation of stem cells we grafted peptide mimics of neural stem cell niche carbohydrates, human natural killer-1 and polysialic acid to collagen and assayed the differentiation of stem cells towards cell types from all three germ layers. Cells cultured on human natural killer-1 and polysialic acid grafted collagen preferentially differentiated towards neuroectoderm lineage cells as indicated by qPCR and Immunohistochemistry. Identifying matrix cues and environments that support and direct the differentiation of stem cell is essential for regenerative medicine. Scaffolds that support and direct the differentiation of stem cells may eventually be used as therapies for degenerative diseases.

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Dedication

To my parents, Mom your love and support always comforts me Dad your strength and vision wills me forward

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Chapter 1

1. Introduction

1.1. Embryonic Stem Cells and Stem Cell Differentiation

Embryonic stem cells continue to excite the medical community. Embryonic stem cells are capable of virtually unlimited proliferation in vitro while maintaining the ability to differentiate into any cell type, under appropriate conditions [1, 2], thus, making them potentially invaluable for cell replacement therapies. The promise of stem cells has been heard for over a decade, but several social and scientific factors have prevented the successful application of embryonic stem cell based therapies to humans. Sources for embryonic and stem cell shave raised ethical, legal, religious, and policy questions. However, with the recent advances made in induced pluripotent stem cells[3-7], several of the arguments against stem cells use have been subdued. From a scientific standpoint the enigma that is a stem cell is not completely understood. Researchers are only beginning to recognize how environmental cues determine phenotypic behavior of cells and cell fates. The majority of work done to decipher stem cell differentiation in vitro largely comprises the use of various growth factors and chemical cues exposed to stem cells grown in a monolayer to obtain a particular cell phenotype [8-16]. While these advances are important, it is very likely that several chemical and physical cues that exist and work in concert to direct stem cell fate will be missed.

In vivo, stem cells exist in an extracellular matrix and receive physical and chemical cues from surrounding cells and tissues. The majority of the work done to decipher stem cell fate regulation however, has been on 2D tissue culture plastic. In particular, the role of stem cell

extracellular matrix interactions in differentiation is unclear and has been relatively neglected in studies of stem cell differentiation, especially in terms of a natural or biomimetic environments. This thesis focuses on understanding the relation between stem cell-matrix adhesion and differentiation into neural lineages. We have developed a method to alter positively and negatively the adhesiveness of collagen gels. We quantified the adhesion, migration and remodeling of cells on and in these scaffolds. We then assayed the differentiation of murine embryonic stem cells towards neural lineages on these gels. Additionally, using similar techniques, we characterized the differentiation of mouse embryonic stem cells toward neural lineages on collagen gels grafted with specific peptides that mimicked carbohydrates present in neural stem cell niches.

1.2. Extracellular Matrix and Stem cells

Stem cells in vivo exist in an extracellular matrix. The extracellular matrix was once thought to function merely as a scaffold to support the growth of cells and maintenance of tissue and organ structure, but has now been identified to regulate many aspects of cell behavior, including cell shape, migration, adhesion, proliferation and growth, cell survival and differentiation. In terms of a physical scaffold the extracellular matrix consists of several structural proteins such as collagen, laminin, fibronectin, and elastin that are susceptible to degradation and reassembly. The most abundant of the ECM proteins is collagen. Bovine type I collagen is biocompatible – fibroblast-contracted collagen gels are the structural basis for the first living tissue engineered product on the market in the United States (Apligraf, Organogenesis, Inc., Canton, MA). Collagen is extremely versatile and strong. It self-assembles into a fibrous hydrogel mesh upon incubation at 37°C and physiological pH that can provide orientation for regeneration and can entrap cells, can be electrospun into fibers, freeze-dried into a porous sponge, and cross-linked with a variety

of techniques. Collagen and other structural proteins of the ECM are often closely associated with and even linked to proteoglycans and glycoproteins. These specialized molecules provide specific signaling and adhesive cues that regulate downstream signaling and cell behavior. Cells interact and attach to the ECM via integrins. Integrins link the actin cytoskeleton of the cell to the ECM via cytoplasmic binding proteins allowing for cells to respond, probe and remodel the ECM surrounding it. Matrix remodeling is particularly noticeable during development.

Embryonic Germ Layer	Cell Type
Ectoderm	Neurons Oligodendrocytes Astrocytes Epidermis
Mesoderm	Epithelial cells Adipocytes Cardiomyocytes Chondrocytes Hematopoietic (stem) cells Endothelial cells Osteoblasts Striated and Smooth-muscle cells
Endoderm	Hepatocytes Pancreatic-like islets Insulin-producing cells Lung cells

Table 1: Differentiation of mouse embryonic stem cells in vitro.

Pluripotent embryonic stem cells can give rise to all three embryonic germ layers— endoderm mesoderm**,** and ectoderm**.** These three germ layers are the embryonic source of all cells of the body.

During the normal process of development, multipotent stem cells make many cell fate decisions and migrate to form the familiar layers and patterns in a blastula and subsequently the embryo **(Table 1)**. These choices are determined by a combination of intrinsic and extrinsic signals. The genetic make up of the cells determine the intrinsic cues. The intrinsic cues can be regarded as the set of instructions activated and modulated by the spatial and temporal organization of extrinsic factors. Extrinsic cues are signals received by the cell that elicit a response that mediates a translation of genetic material to proteins Extrinsic cues may be either soluble or contact mediated; their sources can be local or distant. Contact cues include physical cues such as matrix stiffness and adhesion both of which are perceived by cells via integrins. Several groups have extensively studied the importance of matrix stiffness in the regulation of stem cells[17-

26]. Engler et al. have shown that the elastic modulus of the substrate stem cells are grown on is a key regulator for differentiation of mesenchymal stem cells to a particular lineage[18, 25, 26]. Stem cells will preferentially differentiate towards cells from tissues that the substrate modulus they are cultured on mimics. To a lesser degree, the influence of cell matrix adhesion has only indirectly been suggested to direct the differentiation of stem cells. Hakuno et al have shown that the stabilization of focal adhesions preferentially differentiates stem cells towards cardiacmyocytes [27], cells that are derived from the endoderm germ layer. The discrepancy between the number of studies investigating stem cell matrix stiffness and adhesion is particularly surprising given the direct correlation of focal adhesion formation and matrix stiffness[28]. The coupling of the matrix stiffness and adhesion makes it important to elucidate the response of stem cells to each factor.

1.3. Cell-Matrix Interactions

Cells interact with their matrix via integrins. Integrins bind to adhesive site in the matrix to form cell adhesions. Cell adhesions are coupled to the cytoskeleton of the cell via coupling proteins, allowing for the continual probing and remodeling of their matrix. Increasing or decreasing adhesion of cells to their matrix has profound effects to its normal function [29, 30]. For example, cell migration is a ubiquitous process that is of fundamental importance in tissue morphogenesis,

Figure 1: Cell Migration vs. Increasing Adhesion.

Cell Migration is a ubiquitous process that is of fundamental importance in tissue morphogenesis, wound healing, and tissue engineering. Different cells employ distinct mechanisms of migration, but each, requires the transfer of internal cytoskeletal forces to external tractional forces. These forces, exerted on the substratum via specific cell surface receptors and complimentary cell adhesion molecules, are balanced by cell migration and/or matrix reorganization.

wound healing, and tissue engineering. For effective migration, cells need to be able to successfully convert internal cytoskeletal forces to external tractional forces against an extracellular substratum. Tractional forces are exerted on the substratum via specific cell surface receptors and complimentary cell adhesion molecules present in the substratum. Tractional forces exerted by the cells are balanced by cell migration and/or matrix reorganization. Increasing adhesion increases tractional forces and the ability for a cell to pull itself forward. However, if tractional forces are increased too high, the matrix becomes 'too sticky' for migration, often resulting in the activation of apoptosis pathways.

Both specific and non-specific binding sites regulate adhesion of cells to matrix structural proteins. The sequence Arg-Gly-Asp (RGD) is a ubiquitous extracellular matrix adhesion binding site to several different cell types. RGD is predominantly present on fibronectin and to a lesser degree present on collagen and other matrix proteins as well. Studies have indicated that the sequence RGD is involved in many cellular processes, including tissue repair, embryogenesis, blood clotting, and cell migration/adhesion. Mesodermal cell migration during gastrulation can be blocked by injection of RGD tripeptides that block cellular integrins (Winklbauer). Specific adhesion on the other hand is regulated by sequence specific receptors, as in the cases of the laminin derived peptides IKVAV and YIGSR which preferentially bind to neurons. Molecular markers that are not part of primary protein backbone can also modulate specific adhesion of cells to matrix. These molecules include sugars, glycoproteins and carbohydrates.

1.4. Polysialic Acid and Human Natural Killer-1

Of the several carbohydrates available to modify integrin binding and signaling are two particularly interesting ones: polysialic acid (PSA) and L2/human natural killer-1 (HNK). PSA and HNK carbohydrate epitopes are expressed mainly in developing neural niches but also in restricted areas even in adulthood. They are present as glycosilations on neural cell adhesion molecules and attenuate their binding, promoting cell migration and axon fasciculation. These phenotypic behavioral changes have significant impact in nervous system development and function. The long, negatively charged polysialic acid chains that attach to NCAM confer anti-adhesive properties on the molecule. Polysialic acid attenuates the binding properties of NCAM by creating a negatively-charged hydrated sphere around the molecule, preventing NCAM-binding sites from efficiently contacting by keeping NCAM molecules and cell membranes physically separated from each other [31]. Human natural killer-1 is a carbohydrate that was originally identified on human natural killer cells (abo and balch, 1981), hence the name. However, it is highly expressed in the developing and adult nervous systems and is generally accepted as a marker for neural crest cells[32-36]. Human natural killer-1 epitopes are present on several neural adhesion molecules, including L1, neural cell adhesion molecule, J1, and the myelin-associated glycoprotein. It is a sulfated glucuronic acid attached to n-acetyllactosamine on the non-reducing termini of glycans [37] and is involved in cell-cell and as cell-matrix interactions [35, 38] as well. However, their role in the differentiation of stem cells has not been studied.

1.5. Peptide Mimics

Several factors have contributed to the lack of studies investigating the role of carbohydrates in stem cell differentiation. Carbohydrates are particularly difficult to isolate without denaturing and synthesis of carbohydrates using recombinant

technologies is not trivial and very cost prohibitive. Additionally, carbohydrates in themselves are difficult to use and require complex chemistries to manipulate. Fortunately, Schachner's research group has developed peptide mimics to HNK and PSA carbohydrates using phage display[39-44]. These mimics have been functionally validated [40, 42, 43] and are easier to handle, using standard chemistry techniques these peptides can be grafted onto tissue equivalents commonly used in tissue engineering applications and the differentiation of stem cells on these scaffolds may be assayed.

1.6. Thesis Overview

This thesis focuses on understanding the relation between the biophysical adhesion of stem cells and their matrix environment. The current chapter provides a brief introduction to stem cells and matrix biology and outlays the need to understand the relationship between stem cell biology and matrix interactions. In Chapter 2 we developed a method to alter positively and negatively the adhesiveness of collagen gels. During normal functioning collagen matrices provides optimal level of adhesion for resident cells. However, during injury or remodeling these properties may not be most favorable. Here in, we quantified the adhesion and migration of cells and remodeling of matrix by fibroblast and smooth muscle cells on and in collagen hydro gels grafted with a fibronectin derived peptide and a scrambled version of the same. We chose to use fibroblasts and SMCs to develop the peptide grafted collagen gel system because of the robustness of the cell types and the abundance of literature available on the biohoysical interaction of these cells with extracellular matrices. In chapter 3, we assayed the differentiation of murine embryonic stem cells towards neural lineages on these gels. We used a

sox-1 reporting stem cell line to quantify the populations of cells that differentiate towards neural lineages on scaffolds of varying degrees of adhesion. In Chapter 4 we extended these techniques to characterize the differentiation of mouse embryonic stem cells toward neural lineages on collagen gels grafted with specific peptides that mimic carbohydrates present in neural stem cell niches during development. Immunohistochemistry and Quantitative PCR methods were used to assay the differentiation of ESD3 murine stem cells towards the formation of cell types from all three germ layers. Chapter 5 summarizes the challenges faced in guiding stem cell differentiation based on scaffold properties and presents potential future applications and implications for regenerative medicine.

1.7.References

1.   Evans, M.J. and M.H. Kaufman, *Establishment in culture of pluripotential cells from mouse embryos.* Nature, 1981. **292**(5819): p. 154-6.

2.   Martin, G.R., *Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells.* Proc Natl Acad Sci U S A, 1981. **78**(12): p. 7634-8.

3.   Okita, K., T. Ichisaka, and S. Yamanaka, *Generation of germline-competent induced pluripotent stem cells.* Nature, 2007. **448**(7151): p. 313-7.

4.   Nakagawa, M., et al., *Generation of induced pluripotent stem cells without Myc from mouse and human fibroblasts.* Nat Biotechnol, 2008. **26**(1): p. 101-6.

5.   Yokoo, N., et al., *The effects of cardioactive drugs on cardiomyocytes derived from human induced pluripotent stem cells.* Biochem Biophys Res Commun, 2009. **387**(3): p. 482-8.

6.   Ohnuki, M., K. Takahashi, and S. Yamanaka, *Generation and characterization of human induced pluripotent stem cells.* Curr Protoc Stem Cell Biol, 2009. **Chapter 4**: p. Unit 4A 2.

7.   Belmonte, J.C., et al., *Induced pluripotent stem cells and reprogramming: seeing the science through the hype.* Nat Rev Genet, 2009. **10**(12): p. 878-83.

8.   Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., Walter, P., *Molecular Biology of the Cell*. 4 ed, ed. G. S. 2002, New York, NY: Garland Publishing Co.

9.   Galvin-Parton, P.A., D.C. Watkins, and C.C. Malbon, *Retinoic acid modulation of transmembrane signaling. Analysis in F9 teratocarcinoma cells.* J Biol Chem, 1990. **265**(29): p. 17771-9.

10.   Strickland, S., K.K. Smith, and K.R. Marotti, *Hormonal induction of differentiation in teratocarcinoma stem cells: generation of parietal endoderm by retinoic acid and dibutyryl cAMP.* Cell, 1980. **21**(2): p. 347-55.

11.   Mummery, C.L., et al., *Characteristics of embryonic stem cell differentiation: a comparison with two embryonal carcinoma cell lines.* Cell Differ Dev, 1990. **30**(3): p. 195-206.

12.   Rudnicki, M.A., et al., *Actin and myosin expression during development of cardiac muscle from cultured embryonal carcinoma cells.* Dev Biol, 1990. **138**(2): p. 348-58.

13. Ventura, C. and M. Maioli, *Opioid peptide gene expression primes cardiogenesis in embryonal pluripotent stem cells.* Circ Res, 2000. **87**(3): p. 189-94.

14. Sharma, N.S., et al., *Sodium butyrate treated embryonic stem cells yield hepatocyte-like cells expressing a glycolytic phenotype.* Biotechnol Bioeng, 2006.

15. Rambhatla, L., et al., *Generation of hepatocyte-like cells from human embryonic stem cells.* Cell Transplant, 2003. **12**(1): p. 1-11.

16. Germain, L., M.J. Blouin, and N. Marceau, *Biliary epithelial and hepatocytic cell lineage relationships in embryonic rat liver as determined by the differential expression of cytokeratins, alpha-fetoprotein, albumin, and cell surface-exposed components.* Cancer Res, 1988. **48**(17): p. 4909-18.

17. Leipzig, N.D. and M.S. Shoichet, *The effect of substrate stiffness on adult neural stem cell behavior.* Biomaterials, 2009. **30**(36): p. 6867-78.

18. Engler, A.J., et al., *Myotubes differentiate optimally on substrates with tissue-like stiffness: pathological implications for soft or stiff microenvironments.* J Cell Biol, 2004. **166**(6): p. 877-87.

19. Breuls, R.G., T.U. Jiya, and T.H. Smit, *Scaffold stiffness influences cell behavior: opportunities for skeletal tissue engineering.* Open Orthop J, 2008. **2**: p. 103-9.

20. Nirmalanandhan, V.S., et al., *Effect of scaffold material, construct length and mechanical stimulation on the in vitro stiffness of the engineered tendon construct.* J Biomech, 2008. **41**(4): p. 822-8.

21. Chokalingam, K., et al., *Tensile stimulation of murine stem cell-collagen sponge constructs increases collagen type I gene expression and linear stiffness.* Tissue Eng Part A, 2009. **15**(9): p. 2561-70.

22. Hoffer, J.A. and S. Andreassen, *Regulation of soleus muscle stiffness in premammillary cats: intrinsic and reflex components.* J Neurophysiol, 1981. **45**(2): p. 267-85.

23. Rowlands, A.S., P.A. George, and J.J. Cooper-White, *Directing osteogenic and myogenic differentiation of MSCs: interplay of stiffness and adhesive ligand presentation.* Am J Physiol Cell Physiol, 2008. **295**(4): p. C1037-44.

24. Pek, Y.S., A.C. Wan, and J.Y. Ying, *The effect of matrix stiffness on mesenchymal stem cell differentiation in a 3D thixotropic gel.* Biomaterials. **31**(3): p. 385-91.

25. Engler, A.J., et al., *Matrix elasticity directs stem cell lineage specification.* Cell, 2006. **126**(4): p. 677-89.

26.   Engler, A.J., et al., *Extracellular matrix elasticity directs stem cell differentiation.* J Musculoskelet Neuronal Interact, 2007. **7**(4): p. 335.

27.   Hakuno, D., et al., *Focal adhesion kinase signaling regulates cardiogenesis of embryonic stem cells.* J Biol Chem, 2005. **280**(47): p. 39534-44.

28.   Pelham, R.J., Jr. and Y. Wang, *Cell locomotion and focal adhesions are regulated by substrate flexibility.* Proc Natl Acad Sci U S A, 1997. **94**(25): p. 13661-5.

29.   Hannigan, G.E., Leung-Hagesteijn, C., Fitz-Gibbon, L., Coppolino, M.G., Radeva, G., Filmus, J., Bell, J.C., Dedhar, S., *Regulation of cell adhesion and anchorage-dependent growth by a new beta 1-integrin-linked protein kinase.* Nature., 1996. **379**(6560): p. 91-6.

30.   Koblinski, J.E., Wu, M., Demeler, B., Jacob, K., Kleinman, H.K., *Matrix cell adhesion activation by non-adhesion proteins.* J Cell Sci., 2005. **118**(13): p. 2965-74.

31.   Yang, P., X. Yin, and U. Rutishauser, *Intercellular space is affected by the polysialic acid content of NCAM.* J Cell Biol, 1992. **116**(6): p. 1487-96.

32.   Vincent, M., J.L. Duband, and J.P. Thiery, *A cell surface determinant expressed early on migrating avian neural crest cells.* Brain Res, 1983. **285**(2): p. 235-8.

33.   Vincent, M. and J.P. Thiery, *A cell surface marker for neural crest and placodal cells: further evolution in peripheral and central nervous system.* Dev Biol, 1984. **103**(2): p. 468-81.

34.   Kruse, J., et al., *Neural cell adhesion molecules and myelin-associated glycoprotein share a common carbohydrate moiety recognized by monoclonal antibodies L2 and HNK-1.* Nature, 1984. **311**(5982): p. 153-5.

35.   Nieke, J. and M. Schachner, *Expression of the neural cell adhesion molecules L1 and N-CAM and their common carbohydrate epitope L2/HNK-1 during development and after transection of the mouse sciatic nerve.* Differentiation, 1985. **30**(2): p. 141-51.

36.   Schachner, M., *Families of neural adhesion molecules.* Ciba Found Symp, 1989. **145**: p. 156-69, discussion 169-72.

37.   Yu, R.K. and M. Yanagisawa, *Glycobiology of neural stem cells.* CNS Neurol Disord Drug Targets, 2006. **5**(4): p. 415-23.

38.   Kunemund, V., et al., *The L2/HNK-1 carbohydrate of neural cell adhesion molecules is involved in cell interactions.* J Cell Biol, 1988. **106**(1): p. 213-23.

39.   Simova, O., et al., *Carbohydrate mimics promote functional recovery after peripheral nerve repair.* Ann Neurol, 2006. **60**(4): p. 430-7.

40.   Simon-Haldi, M., et al., *Identification of a peptide mimic of the L2/HNK-1 carbohydrate epitope.* J Neurochem, 2002. **83**(6): p. 1380-8.

41.   Lahrtz, F., et al., *VASE-encoded peptide modifies NCAM- and L1-mediated neurite outgrowth.* J Neurosci Res, 1997. **50**(1): p. 62-8.

42.   Torregrossa, P., et al., *Selection of poly-alpha 2,8-sialic acid mimotopes from a random phage peptide library and analysis of their bioactivity.* J Biol Chem, 2004. **279**(29): p. 30707-14.

43.   Marino, P., et al., *A polysialic acid mimetic peptide promotes functional recovery in a mouse model of spinal cord injury.* Exp Neurol, 2009. **219**(1): p. 163-74.

44.   Hall, H., T. Vorherr, and M. Schachner, *Characterization of a 21 amino acid peptide sequence of the laminin G2 domain that is involved in HNK-1 carbohydrate binding and cell adhesion.* Glycobiology, 1995. **5**(4): p. 435-41.

Chapter 2

2. Positively and Negatively Modulating the Adhesivity of Type I Collagen Via Peptide Grafting

2.1. Abstract

The biophysical interactions between cells and type I collagen are controlled by the level of cell adhesion, which is dictated primarily by the density of ligands on collagen and the density of integrin receptors on cells. The native adhesivity of collagen was modulated by covalently grafting glycine-arginine-glycine-aspartic acid-serine (GRGDS), which includes the bioactive RGD sequence, or glycine-arginine-aspartic acid-glycine-serine (GRDGS), which includes the scrambled RDG sequence, to collagen with the heterobifunctional coupling agent 1-ethyl-3-(3-Dimethylaminopropyl) carbodiimide (EDC). The peptide-grafted collagen self-assembled into a fibrillar gel with negligible changes in gel structure and rheology. Rat dermal fibroblasts (RDFs) and human smooth muscle cells (SMCs) demonstrated increased levels of adhesion on gels prepared from RGD-grafted collagen, and decreased levels of adhesion on RDG-grafted collagen. Both cell types demonstrated an increased ability to compact free-floating RGD-grafted collagen gels, and an impaired ability to compact RDG-grafted gels. RDF migration on and within collagen was increased with RDG-grafted collagen and decreased with RGD-grafted collagen, and dose-response experiments indicated a biphasic response of RDF migration to adhesion. SMCs demonstrated similar, though not statistically significant trends. The ability to both positively and negatively modulate cell adhesion to collagen increases the versatility of this natural biomaterial for regenerative therapies.

2.2. Introduction

Cell migration is a ubiquitous process that is of fundamental importance in tissue morphogenesis, wound healing, and tissue engineering. Different tissue cells can demonstrate distinct morphologies and mechanisms during migration. Fibroblasts, which exhibit relatively slow migration, explore the direction of migration by extending a leading edge, attaching to the matrix, strengthening attachments by the formation of focal adhesion sites, contracting actin bundles to advance the cell body, and finally releasing rear attachments to propel forward [1-3]. In contrast, keratinocytes, which can migrate more quickly, deploy a treadmilling-mechanism of myosin contraction of the actin cytoskeleton–integrin–matrix links to give the appearance of a gliding motion [4]. For each mechanism, however, effective migration requires the successful transfer of internal cytoskeletal forces to external tractional forces against an extracellular substratum. Traction is exerted on the substratum via specific cell surface receptors and complimentary adhesion ligands present in the substratum, and is balanced by translocation of the cell and/or reorganization of the matrix.

The strength of cell adhesion to the substratum contributes critically to the balance of external and internal forces that governs cell migration [5, 6]. In 2D in vitro systems, cells exhibit a biphasic response of migration with respect to adhesion strength, with the highest migration coefficients occurring at intermediate levels of available ligands and/or receptors [5-7]. If adhesion strength is low or too few adhesions are made, the cell is unable to develop sufficient traction to propel. If adhesion strength is too great or adhesions are too plentiful, ligand-receptor dissociation is impeded and cells cannot remove themselves from the substrate to effectively locomote. A similar biphasic relation between adhesion and cell migration has been demonstrated and modeled in three-dimensional (3D) matrices [1, 8-11].

Biopolymeric gel-based in vitro assays – particularly type I collagen systems – provide an especially valuable platform to examine cell migration. Collagen is a ubiquitous ECM protein that forms the structural framework for many soft tissues. Solutions of collagen can be used to coat two-dimensional (2D) substrates, or can self-assemble into fibrillar three-dimensional hydrogels that offer a better representation of the microenvironment of living tissues when compared to traditional 2D systems. When tissue cells are entrapped in an entanglement of collagen fibers to form so-called 'tissue equivalents', cells can attach to and exert traction on the fibers, which can both compact the matrix and/or propel the cells [12, 13]. As a natural ECM substrate, collagen provides a significant basal level of adhesivity via several peptide sequences, including RGD and GFOGER [14, 15], that can vary among cell types depending on the nature of integrin expression, and therefore places different cells at different locations on the biphasic curve relating cell migration to cell adhesion.

Physiologically, the strength and specificity of adhesion can be modulated by environmental factors to enhance cell migration, such as during wound healing, when resident fibroblasts are stimulated by cytokines and growth factors released at the wound site to migrate into the provisional matrix [16]. In vitro, fibroblasts in collagen gels exhibit minimal migration under standard culture conditions, but increase in motility significantly when exposed to growth factors such as the BB isoform of platelet-derived growth factor (PDGF-BB) [17], basic fibroblast growth factor (bFGF) [18], or epidermal growth factor (EGF) [19]. Clinically, the ability to increase cell migration has broad applications in regenerative therapies, where repopulating damaged or engineered tissues with host cells can accelerate healing and recovery, including wound healing [20] and peripheral nerve and spinal cord regeneration [21]. Although cell migration can generally be enhanced via exogenous soluble factors [22, 23], genetic reprogramming [24, 25], or by blocking adhesion with soluble, competitive ligands [26], from a

tissue engineering perspective, the ability to enhance cell migration through collagen scaffolds via intrinsic properties of the biomaterial without the inclusion of technology for the controlled release of soluble factors or the genetic manipulation of cells would have great clinical potential. The specificity and strength of collagen adhesivity have been increased by covalently grafting bioactive peptide sequences associated with integrin-mediated binding to the collagen backbone [8, 27, 28]. However, to increase the speed or motility of many cell types on or within collagen, a decrease in the adhesivity is required [7, 29].. Herein we show that grafting GRDGS, nominally a scrambled, non-adhesive peptide control for GRGDS [26], decreases fibroblast and smooth muscle cell adhesion to collagen gels. The decrease in cell adhesion decreased the ability for these cells to compact free-floating collagen gels, and, at low-to-moderate concentrations of grafted peptide, increased cell migration.

2.3. Methods and Materials

2.3.1. Cell Culture

Experiments were performed with rat dermal fibroblasts (RDFs) and human aortic smooth muscle cells (SMCs). RDFs were obtained using a primary explant technique from rat pups that constitutively express green fluorescent protein via an actin promoter (tissue generously provided by the W.M. Keck Center for Collaborative Neuroscience). Cell lines were initiated for culture by thawing an aliquot of cells and centrifuging at 2,000 rpm for 2 min at 4°C. The pellet of cells was resuspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with penicillin-streptomycin (pen-strep) (1% v/v) and L-glutamine (1% v/v) (Sigma, St. Louis, MO). Cells were plated in T25 flasks using 4 ml of DMEM with 10% fetal bovine serum (FBS), 1% pen-strep, and 1% L-glutamine and kept in a humidified CO_2 incubator at 37°C. Trypsin/EDTA (Sigma) was used to passage the cells once a week at a 1:8 dilution. Flasks (75-90% confluent) were harvested with 0.5% Trypsin/EDTA and washed twice with DMEM supplemented with 10% FBS (Atlanta Biologicals, Atlanta, GA), 1% pen-strep, and 1% L-glutamine. All experiments were conducted before the twelfth passage, at which point a new culture was initiated from frozen cells. SMCs (a gift from Dr. Gary Nackman at the University of Medicine and Dentistry of New Jersey – UMDNJ) were cultured in MCDB-131 medium with SmGM-2 SingleQuot (Cambrex/Clonetics, Walkersville, MD). SMCs were stored and cultured in a manner similar to RDFs. All experiments with SMCs were conducted before passage eight.

2.3.2. Conjugation of Peptides to Collagen Backbone

Two peptide sequences, GRGDS (RGD) and GRDGS (RDG) were custom synthesized (Genscript, Piscataway, NJ) and were conjugated to the backbone of collagen in suspension. A hetero-

bifunctional coupling agent, 1-ethyl-3-(3-Dimethylaminopropyl) carbodiimide (EDC, Sigma), was

used to activate the carboxylic group of the peptide by mixing 1ml of a 1M solution of EDC in

MES buffer (pH 2-4, Sigma) with 2 mg of peptide for ten minutes at 37°C. The peptide-EDC

mixture was added to 5ml of a 3mg/ml suspension of type I collagen (Elastin Products,

Owensville, MO) in 0.02N acetic acid. The activated peptide covalently bound to free amines on

residues on the collagen backbone via nucleophilic attack. A low pH buffer was used while

coupling peptides to the collagen backbone to avoid self-assembly of collagen fibers. Peptide-

EDC-collagen mixtures were incubated on a shaker overnight at 4°C and then dialyzed against

0.02N acetic acid for 12 hours using snakeskin dialysis tubing with a 10kD molecular weight

cutoff (Pierce Biotechnology, Rockford, IL) to remove unconjugated peptide. Dialyzed peptide-

grafted collagen was lyophilized at -150°C and 50mTor for 12 hours to remove all water.

Lyophilized product was re-suspended in 0.02N acetic acid to make a 3mg/ml solution of grafted

collagen. The efficiency of peptide grafting was measured indirectly by grafting peptides that

included a FITC tag during synthesis (Genscript) and comparing the fluorescence intensity of

grafted collagen after reconstitution to a standard curve created by admixing fluorescent

peptide into collagen solution. Efficiency ranged between 50-60%, resulting in 67-80μg

peptide/mg collagen.

2.3.3. Collagen Gel Preparation

Type I collagen gels were prepared by mixing 20 μl 1M Hepes buffer (Sigma), 140 μl 0.1N NaOH

(Sigma), 100μl 10X Minimum Essential Medium Medium (MEM) (Sigma), 52 μl M199 (Invitrogen,

Carlsbad, CA), 1 μl pen-strep ((5000 units of penicillin and 5000 mg streptomycin/ml in 0.85%

saline) (Sigma), 10 μl L-Glutamine (29.2 mg/ml in 0.85% saline) (Sigma), and 677 μl of 3.0 mg/ml

collagen (grafted or native) to make a 2.0 mg/ml collagen solution. To prepare gels with

different concentrations of grafted collagen, peptide-grafted collagen solutions were diluted with native collagen solutions at varying ratios. The native and grafted collagen solutions self-assemble into a gel upon incubation at 37°C. For assays with RDFs, three concentrations of grafted RGD or RDG peptides were investigated: a 'high' (H) concentration of 133μg/ml (~0.25mM), a 'medium' (M) concentration of 67μg/ml (~0.125mM), and a 'low' (L) concentration of 33.5μg/ml. For experiments with SMCs, only the 'medium' concentrations of grafted RDG and RGD were assayed.

2.3.4. Fibril Size and Density

High-magnification confocal microscopy was used to estimate the effects of the peptide grafting on collagen fiber size and density. Straight 1cm long, 500μm wide, and 100μm deep channels were generate in poly(dimethyl siloxane) (PDMS) (Fisher Scientific, Pittsburgh, PA) using standard soft lithography and were bonded to glass coverslips. The channels were filled with grafted or native collagen solution spiked with FITC-labeled collagen (10% v/v; Elastin Products), to allow for visualization of fibers. Devices were transferred to a Leica TCS SP2/MP confocal microscope (Leica Microsystems, Exton, PA). Images were taken at 63X with a 2X digital zoom at 488nm excitation with a 500- 535nm emission bandpass filter. All image frames underwent two line and frame averaging. Three images were taken at random in each device. Each image was divided into nine equal squares. The average number and diameter of fibers was determined in three of the nine squares with the image analysis software. The analysis was repeated for 3 gels in each condition, and results were compared with ANOVA (significance set at $P < 0.05$).

2.3.5. Mechanical Testing of Scaffolds

Potential effects of the grafting procedure on the mechanical properties of the resulting self-assembled gels were assessed via parallel plate rheometry using a Rheometrics SR- 2000 rheometer (TA Instruments, New Castel, DE), as previously described [30]. Briefly, A 25-mm diameter hole was punched in a 4-mm thick layer of PDMS. Collagen solution (800 µL) was pipetted into the punched well and transferred to a 37°C incubator to induce self-assembly. The gels were carefully removed with a spatula and transferred to the bottom plate of the rheometer. The top plate was lowered to a height of 0.8 mm. The dynamic storage and loss moduli of the gel were evaluated at 1% shear strain amplitude at frequencies ranging from 0.1 to 5 Hz for three samples prepared from separate batches of native collagen and RGD-grafted collagen. The storage moduli were compared with ANOVA at discrete frequencies. Significance levels were set at $P < 0.05$.

2.3.6. Adhesion Assay

RGD- and RDG-grafted collagen solutions at low, medium, and high concentration, as well as native collagen solutions (200µl), were pipetted into separate wells of a 24-well plate in triplicate and allowed to self-assemble at 37 °C and 100% humidity. RDFs (100 µl of a 50,000 cells/ml suspension) were seeded on the gels and allowed to settle and attach for 1 hour. Wells were rinsed three times with 1ml PBS in five-minute intervals. The remaining cells were stained with Calcein AM (Invitrogen) using an Olympus IX81 inverted epifluorescent microscope (Olympus, Melville, NY) with a 4X objective and a FITC filter. Images were captured digitally (Hamamatsu ORCA, Hamamatsu City, Japan) using Olympus Microsuite

software). The number of cells in each field of view were counted using Image Pro Plus

(Version 5.1 for windows, Media Cybernetics, MD). Three randomly selected fields were

imaged and quantified for each well. The data were analyzed statistically with ANOVA. Post

hoc pairwise comparisons were performed with Tukey's test. Significance levels were set at

$P < 0.05$. The adhesion assay was repeated with SMCs for the RGD-M, RDG-M, and the

control collagen conditions. Images and data were collected and analyzed in a similar

manner.

2.3.7. Gel Compaction Assay

The effects of peptide grafting on the ability of cells to exert traction on the fibrillar collagen

network were indirectly evaluated by measuring cell-mediated compaction of free floating

collagen disks. RDFs (50,000 cells/ml) were suspended in type I collagen solutions with a

specified volume fraction of RDG- or RGD-grafted collagen. A PDMS annulus (4.75mm ID, 4mm

height) was placed in a well of a 24-well plate and was filled with 35 µl of the cell/collagen

suspension. The plate was transferred to a humidified incubator operating at 37ºC and 5% CO_2

to induce self-assembly of the collagen and entrap the cells within the forming fibrillar network

[31]. The collagen did not adhere to the PDMS, and following self-assembly, addition of 500 µl of

DMEM to the well caused the gelled disc to float out of the ring. The plate was then returned to

the incubator. Without a mechanical constraint to compaction, traction is exerted isotropically

[12, 32], and as such, the cell-mediated compaction resulted in a uniform reduction in the size of

the disk. An image of the compacting collagen disc was taken at day 0 and then every 24hrs for 6

days using a dissection microscope (Carl Zeiss, Inc., Thornwood, NY) equipped with a digital

camera (MatrixVision, Oppenweiler, Germany). The area of the collagen disc was measured

from the images using ImageJ (NIH, Bethesda, MD). Each condition was done in (at least)

triplicate on a particular day, and the design was repeated 4 times. The amount of compaction was determined by comparing the area of the disk to its initial area at day 0. Statistical significance was evaluated with a one-way ANOVA. Significance levels were set at $P < 0.05$. The compaction assay was repeated with SMCs for the RGD-M, RDG-M, and control collagen conditions. Images and data were collected and analyzed in a similar manner.

2.3.8. Migration Assay

The effects of peptide-grafting on cell migration both on collagen gels (2D migration) and within collagen gels (3D migration) were assayed using methods similar to Shreiber et al. [13] A 200µl aliquot of collagen solution was pipetted into wells of a 24-well plate and allowed to self-assemble at 37°C and 100% humidity. RDFs or SMCs (5,000/well) were seeded on the gels. SMCs were pre-stained with Lysotracker Red (Invitrogen) to visualize the cells using TRITC optics during time-lapse epifluorescent microscopy. A custom-built on-stage incubator was placed on the computer-controlled stage of the Olympus IX81 to maintain temperature and humidity during the time lapse experiment. RDFs were visualized using FITC optics via the constitutively expressed GFP. For experiments in collagen gels (which were not free-floating, but rather were adherent to the tissue culture plastic at the bottom and around the perimeter of the gel) 1,000 cells/well were introduced into the collagen prior to casting the gel, similar to the compaction studies described above. Fewer cells were used in gels than on gels to limit cell-mediated compaction of the gel, which introduces a significant convective component to cell position that complicates quantifying cell migration. FITC-fluorescent beads were included in two experiments to assess convective movement of collagen gels, which was judged to be minimal (data not shown). The plates were returned to the incubator, and the cells were allowed to attach and spread for 3 hours prior to initiating time-lapse microscopy.

Time-lapse microscopy of cell migration was performed via a computer-controlled stage of an Olympus IX81 inverted microscope with a temperature-controlled incubation chamber set to maintain 37°C (TA Instruments, New Castle, DE). Air buffered media (medium 199 - M199, (Gibco) with Hanks' salts supplemented with 10% FBS, 1% pen-strep, and 1% L-glutamine) was used for the duration of the time-lapse experiment. Images for the migration assay were captured digitally using Olympus Microsuite Imaging Software. Three fields of view were imaged in each well with a 4x objective at 10-15 minutes for 12–14 hours, which captured the X-Y position of cells within the field. The Z-position of cells, even within gels, was not recorded.

Images were processed using Matlab's image processing toolbox (Mathworks, Natick, MA); original images were filtered and binarized to subtract background fluorescence, when required. Processed images were imported into Image Pro Plus (Media Cybernetics, MD) to determine two dimensional spatial cell tracks for each cell in view, which were then verified manually. At least 50 cells in each well were tracked. The average mean squared displacement (MSD - $<d^2(t)>$) for each well was calculated over all time intervals using overlapping intervals [33]. The resulting $<d^2(t)>$) vs. time interval (t) data were fit to a persistent random walk model assuming two dimensional migration,

$$\left\langle d^2(t) \right\rangle = 4\mu \left[t - P\left(1 - e^{-t/P} \right) \right]$$

to determine the cell motility coefficient μ and the persistence time, P [34].

For RDFs within collagen and SMCs on collagen, three conditions (RDG-M, RGD-M, and collagen) were assayed in each experiment in triplicate, and the design repeated three times.

For the dose-response RDF experiments on collagen, separate experiments were performed

with RDG (RDG-L, RDG-M, RDG-H, and collagen) and with RGD (RGD-L, RGD-M, RGD-H, and

collagen), with each condition performed in triplicate and each design repeated three times. The

cell motility coefficients and persistence times were analyzed for statistical significance using a

one-way ANOVA. Significance levels were set at $P < 0.05$.

2.4. Results

2.4.1. Physical Characteristics of Collagen Gels

Figure 2 Confocal Micrographs of Collagen

Confocal micrographs of (A) native and (B) peptide-grafted collagen (RGD-M shown). No overt differences were apparent in collagen gels formed following peptide-grafting, and no significant differences were identified in estimates of fiber number (ANOVA, P = 0.702) or size (ANOVA, P = 0.538). (Bar = 10 m).

The effects of peptide grafting on the collagen fiber microstructure were estimated from high magnification confocal micrographs (**Figure 2**). No differences in the average number of collagen fibers (ANOVA, P=0.702) or the average thickness of fibers (P=0.538) were detected between peptide-grafted and native collagen. The effects of peptide grafting on the mechanical properties of the collagen post-self assembly were evaluated with parallel plate rheometry (Figure 2). Profiles of storage and loss moduli vs. shear strain frequency for peptide-grafted and untreated collagen gels were consistent with our previous reports [30]. No significant differences were detected in storage or loss moduli at 0.1, 1, 2.5, or 5Hz (ANOVA, min P = 0.70). Together, these results indicate that grafting peptides to collagen oligomers does not interfere

Figure 3 Cone Plate Rheology Results- Native and Grafted Collagen

Average storage and loss moduli (+/- standard error) for native and peptide-grafted collagen. The procedure for grafting peptides on to collagen had little effect of the rheological properties of the resulting fibrillar gel, as measured with parallel plate rheology. No significant differences in storage or loss moduli were detected across the frequency sweep (max P = 0.703)

significantly with self-assembly into a fibrillar hydrogel, which is consistent with previous studies employing similar techniques [27].
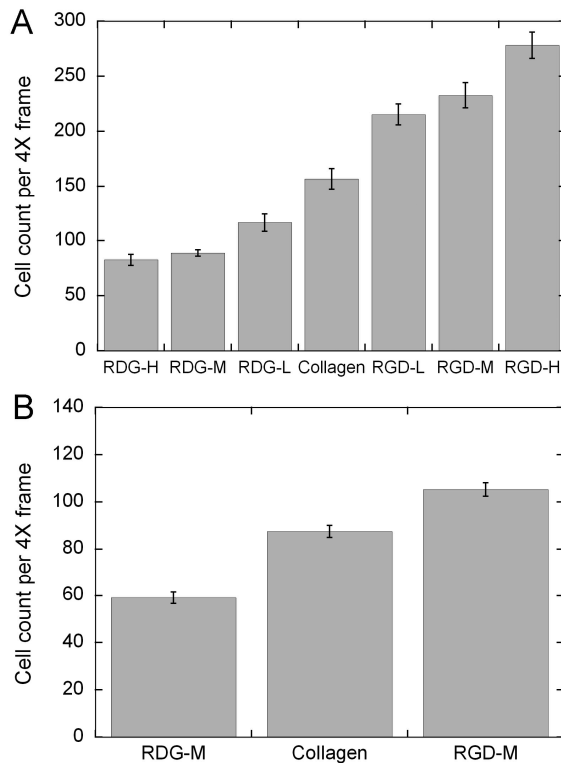
Figure 4 Effects of peptide-grafting on (A) RDF adhesion and (B) SMC adhesion.

Cell attachment (average +/- standard error) was increased on RGD-grafted gels and decreased on RDG-grafted gels. RDF adhesion was assayed at 3 concentrations of RDG and RGD, and demonstrated a dose-response relationship (ANOVA, $P<0.001$). SMC adhesion was assayed at the medium concentration of grafted peptides, and also demonstrated a significant dependence on peptide grafting (ANOVA, $P < 0.001$).

2.4.2. Adhesion

Grafting bioactive and scrambled peptides significantly affected cell adhesion to collagen gels. RDF adhesion (**Figure 4 A**) and SMC adhesion (**Figure 4 B**) increased above controls on collagen grafted with RGD, and decreased below controls on collagen grafted with RDG (ANOVA, $P<0.001$). For RDFs, adhesion was tested at varying concentrations of grafted RGD and RDG, which together modulated cell adhesion in a roughly sigmoidal, dose-dependent manner (ANOVA, $P<0.001$). Post hoc analysis with Tukey's test demonstrated that all pairwise comparisons were significantly different (max $P = 0.009$) except the following pairs: RDG-H and RDG-M ($P = 0.995$), RDG-M and RDG-L ($P = 0.061$), and RGD-L and RGD-M ($P = 0.530$).
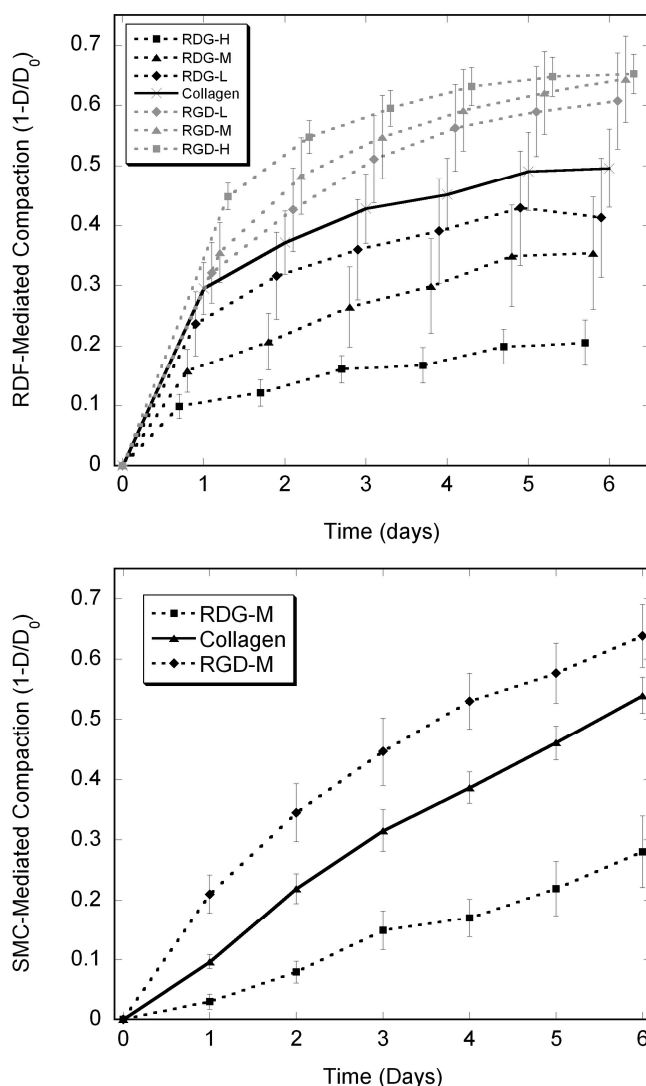
2.4.3. Compaction

Figure 5 Effects of peptide-grafting on cell mediated compaction of free-floating collagen gels

Effects of peptide-grafting on (A) RDF- and (B) SMC-mediated compaction of free-floating collagen gels (average degree of compaction +/- standard error). RDF-mediated compaction demonstrated a dose-response dependence on the concentration of grafted RGD or RDG peptide (ANOVA, $P < 0.001$.) Compaction was increased in gels prepared with RGD-grafted collagen and decreased in gels prepared with RDG-grafted collagen. Similar significant trends were identified with SMCs at the medium concentration of peptide grafting (ANOVA, $P = 0.007$).

The effects of modulating cell adhesion to collagen via peptide-grafting on cell-mediated compaction of collagen gels were evaluated in cell-populated, free-floating collagen gels over a six-day period (**Figure 5**). Increasing cell adhesion via grafting of RGD peptides increased the degree of cell-mediated compaction by both RDFs and SMCs. Conversely, decreasing cell adhesion via grafting of RDG peptides decreased the ability of these cells to compact collagen gels. Significant differences were found in compaction among conditions at each of the 6 days following cell seeding for SMCs (ANOVA, max $P=0.009$) and RDFs (ANOVA, max $P<0.001$). In general, decreasing adhesion (via RDG-grafting) had a

greater negative effect on compaction than the positive effect induced by increasing adhesion (via RGD-grafting).

2.4.4. Migration

The influence of modulating adhesion to collagen gels via peptide grafting on cell migration was assayed in two and three dimensional systems with RDFs, and in a two dimensional system with SMCs. Separate sets of RDF dose-response experiments were performed with RGD-grafted collagen and with RDG-grafted collagen, each with native collagen controls. Cell motility was significantly decreased below controls for RGD-grafted collagen (P =0.025). No significant differences were detected among the RGD conditions (Pairwise comparisons with Tukey's test, min P = 0.99) (**Figure 6** A). In contrast, cell motility demonstrated a statistically significant (ANOVA, P=0.003), bimodal dependence on the concentration of RDG that was grafted to collagen, first increasing at low and medium concentrations of grafted RDG, and then decreasing to near control levels when adhesion was further decreased via a high concentration of grafted RDG (**Figure 6 B**). Tukey's test revealed significant differences between RDG-L and collagen (P = 0.005) and RDG-L and RGD-H (P = 0.024). No other comparisons were significantly different (min P = 0.077). Cell migration assays were also performed with the RDG-M and RGD-M conditions for RDFs migrating within a collagen gel (**Figure 7 A**) and for SMCs migrating on a collagen gel (**Figure 7 B**). RDF migration within collagen gels significantly depended on peptide-grafting (P=0.005). Migration was increased in RDG-grafted gels and decreased in RGD-grafted gels compared to collagen controls. Similar trends were observed with SMCs migrating on collagen gels, but these differences were not statistically significant (P = 0.157). No significant differences were observed in the persistence time of each cell population on or in the various scaffolds (min P = 0.256).
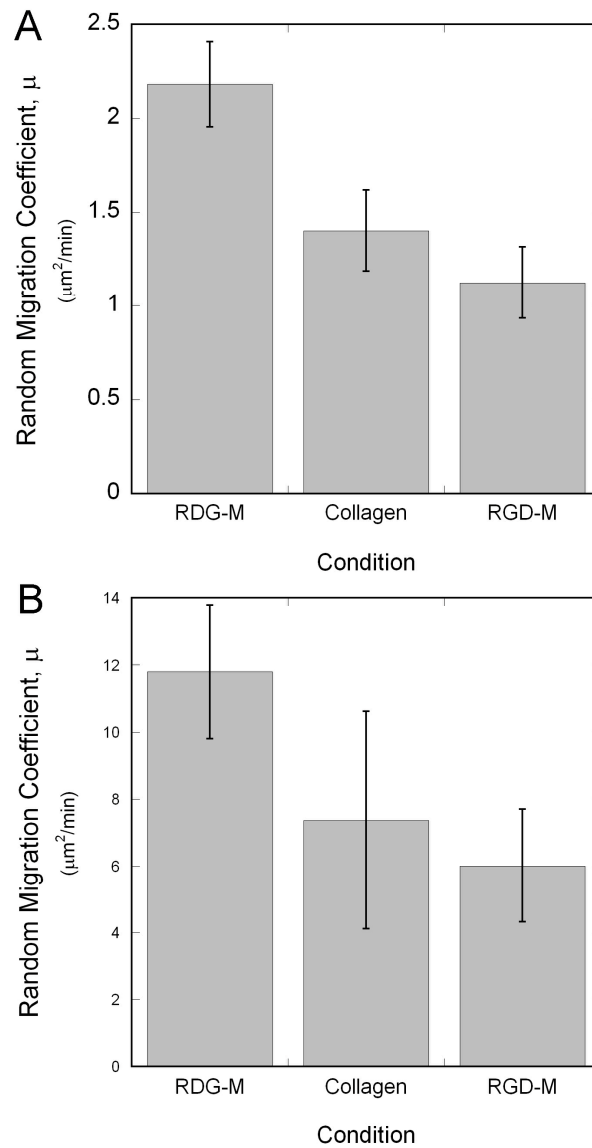
Figure 6 Effects of peptide-grafting on cell migration on collagen gels

Effects of peptide grafting on (A) RDF migration in collagen gels and (B) SMC migration on collagen gels. Both cells demonstrated increased migration with RDG-M collagen and decreased migration with RGD-M collagen. The differences were statistically significant (ANOVA, $P = 0.005$) for RDFs migrating in collagen gels, but not significant ($P = 0.157$) for SMCs migrating on collagen gels.
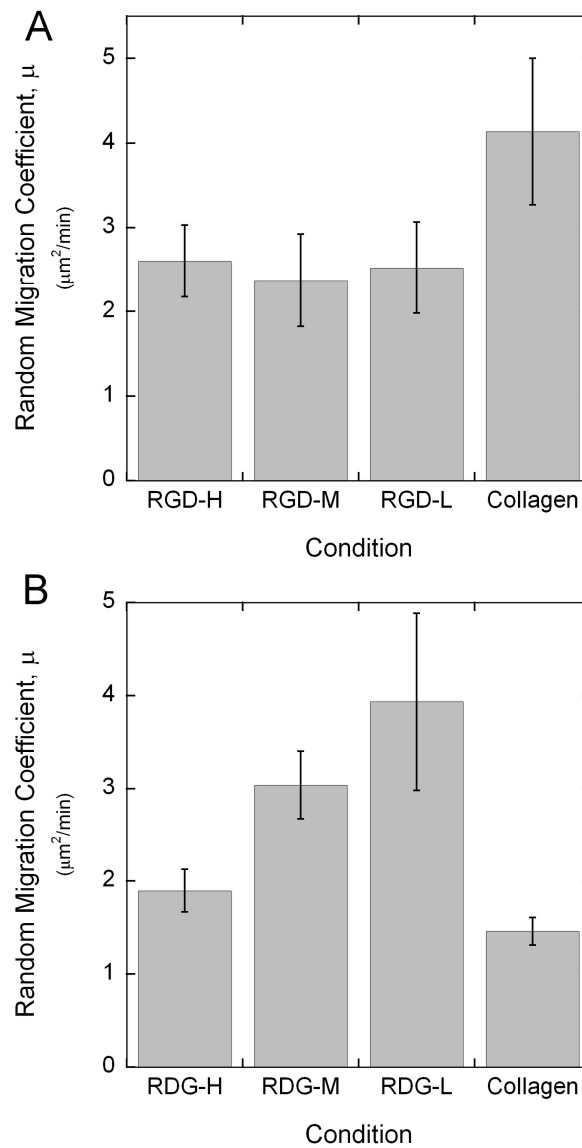
Figure 7 Dose-response effects of peptide grafting on the random migration coefficient

Dose--response effects of peptide grafting on the random migration coefficient (average +/- standard error) for RDFs migrating on collagen gels. (A) RGD-grafted collagen; (B) RDG-grafted collagen. Cell migration was decreased on RGD-grafted gels compared to collagen (ANOVA, $P = 0.025$), but no dose-dependence was identified in post-hoc tests (Scheffe's test, min $P = 0.99$). Cell migration was significantly affected by RDG-grafting (ANOVA, $P = 0.003$). Post hoc tests revealed that migration on the RDG-L condition was significantly greater than RDG-H ($P = 0.042$) and collagen ($P = 0.010$), but was not distinguished from RDG-M ($P = 0.547$). The RDG-M condition was not significantly different than collagen ($P = 0.119$) or RGD-H ($P = 0.360$).

2.4.5. Adhesion-Migration-Compaction Relationships

To examine the relationships among cell adhesion, cell migration, and cell traction via cell-mediated compaction, data from RDF dose-response adhesion, compaction (after 24hrs), and migration experiments were plotted on the same sets of axes. First, data sets from individual experiments were normalized to the average control condition for that day, and the normalized values were averaged across all experiments. These data were then grouped according to the grafting condition (RGD-L, M, H or RDG-L, M, H) and plotted as [adhesion, migration] and [adhesion, compaction] X-Y pairs. Figure 8 demonstrates the observed biphasic response of migration to cell adhesion and the monotonic response of gel compaction.

2.5. Discussion
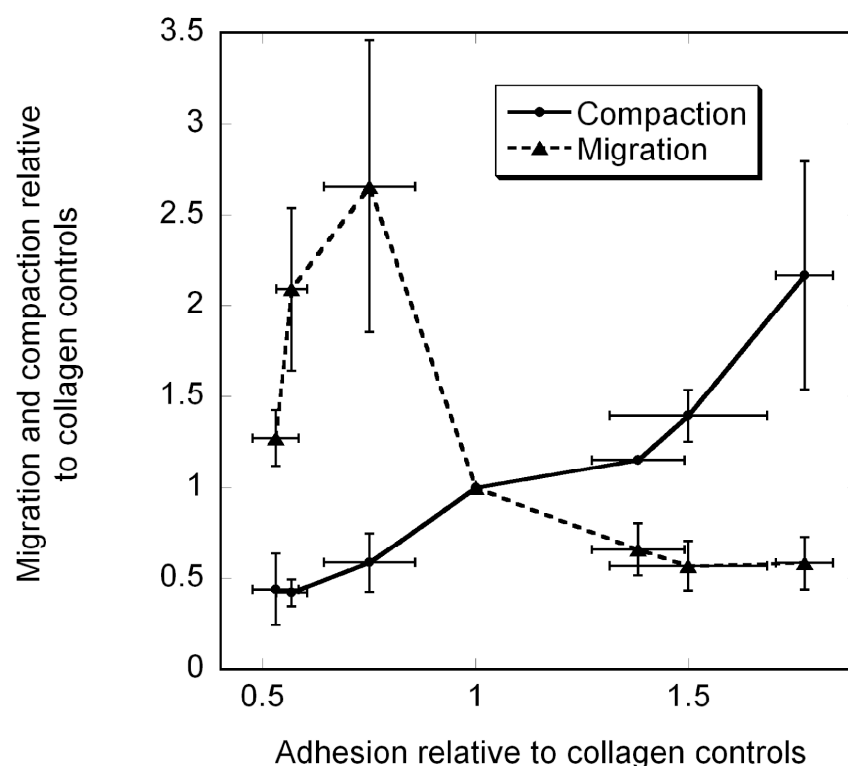
Figure 8 Inferred dependence of cell migration and cell-mediated compaction on the level of cell adhesion.

For the dose-response RDF experiments, the average adhesion, migration, and compaction (after 24hrs) from each experimental set was normalized by the average response in the control conditions for that set. These normalized values were then averaged across all experiments and plotted as (migration, adhesion) and (compaction, adhesion) X-Y pairs (+/- standard error). At low levels of adhesion, both cell migration and cell-mediated gel compaction (via cell traction) are inhibited. As adhesion increases, an optimal level is reached for cell migration, after which the elevated adhesive forces limit the ability for cells to detach from the substrate, which is observed as a decrease in migration. Conversely, the elevated adhesive forces increase the ability for the cells to exert traction and compact the fibrillar collagen network.

It is generally accepted that a biphasic relationship exists between cell adhesion and cell migration, where an optimum density of integrin-ligand binding creates the most favorable force balance for cell migration [7]. We demonstrated that this can be accomplished for naturally adhesive biomaterials by covalently grafting non-adhesive peptide sequences to mask native adhesion. Addition of non-adhesive RDG decreased adhesion and cell-mediated compaction, and increased cell migration, whereas addition of bioactive RGD to collagen increased adhesion and cell-mediated compaction, but had little effect on cell migration.

In vitro, bioactive peptides are commonly applied to functionalize a synthetic, non-adhesive surface [35]or are added in a soluble form to inhibit adhesions [36-38]. These peptides can elicit cell behavior though synergistic adhesion-mediated phenomena [36] and binding-mediated intracellular pathways [39]. Scrambled sequences or sequences with a substituted peptide are often used as controls for these experiments, and cells are typically blind to these scrambled versions [40-43]. Using heterobifunctional coupling systems, covalent grafting of the bioactive peptides to naturally bioactive biomaterials, including collagen, has been employed to increase the level and specificity of adhesion without significantly altering the physical structure of the assembled network of collagen fibers or the mechanical properties [27]. In general, increasing adhesion will result in increased cell traction [11, 44], which can be inferred from measurements of gel compaction . Our compaction results with RGD-grafted collagen are consistent with this notion. However, rarely are the results of grafting control peptides reported. Notable exceptions are reports from Burgess et al. [8] and Myles et al [27], who demonstrated a monotonic increase in adhesion of K1735 melanoma cells to increasing concentrations of GRGDSPC grafted to type I collagen, but a biphasic response in cell migration within a 3D collagen gel. No changes were noted in adhesion or migration with the scrambled peptide, GRGESPC.

The contradictory results from Myles et al. and Burgess et al. compared to those presented herein are likely explained by important differences in the respective cell types and/or coupling agents. Although melanoma cells express $\alpha 1\beta 1$ and $\alpha 2\beta 1$ integrins, which have a high affinity for collagen and are generally accepted to recognize the peptide sequence GFOGER [14], the motility of the highly metastatic K1735 cells is mediated by expression of $\alpha v\beta 3$ integrins [45, 46], which primarily recognizes RGD sequences in vitronectin and fibronectin. As such, increasing adhesion via addition of RGD to collagen shifts the position of the cells on the curve towards the right, first towards the peak of the curve and an optimal adhesion level for

migration, and then to a level of adhesion too great for effective migration. Surprisingly,

Burgess et al. found that, while the K1735 cells demonstrated a biphasic relationship between

cell migration and cell adhesion, the relationship was not due to a similar response in cell speed,

but rather a biphasic response in persistence time. Instead, cell speed was found to decrease

monotonically with increasing adhesion. This result may be indicative of the colonizing nature of

the metastatic K1735 cells. Burgess et al. also note potential distinctions between assaying

migration on a 2D surface and within a 3D network. In a 3D system, increased cellular

interactions with and reorganization of the matrix is involved, and the adhesivity, stiffness, and

porosity can work in concert to affect migration [44, 47]. However, we observed an inverse

correlation between adhesion and migration for cells both in and on gels.

In our experiments, we used normal RDFs and SMCs. Both of these cells express $\alpha1\beta1$ and

$\alpha2\beta1$ integrins and employ these integrins in regulating the collagen network and cellular and

tissue functions [48-51]. Moreover, neither cell is naturally motile unless stimulated by a change

in environmental cues, as occurs, for instance during wound healing [20], and both the RDFs and

SMCs would reside naturally on the right end of the biphasic relationship between adhesion and

migration. Accordingly, further increasing adhesion via addition of RGD has a negative to

minimal effect on migration, and a disruption in adhesion is required to increase migration. A

decrease in adhesion would also be reflected in gel compaction, where the ability to exert

traction sufficient to compact the collagen gel is diminished.

The other chief difference between the studies was the reagent(s) used to couple the

peptides to collagen. In Myles et al and Burgess et al., sulfosuccinimidyl 6-[30-2-(pyridyldithio)-

propionamido] hexanoate (Sulfo-LC-SPDP) was used to conjugate RGD peptides to collagen,

whereas we used EDC [8, 27]. Neither technique appears to overly influence the mechanical or

structural properties of the fibrillar gels. We note that the confocal technique, with a resolution limit of ~200nm, offers a less precise estimate of structure than the scanning electron micrography (SEM) used by Myles, but when combined with the rheological data and dose-response adhesion data, provides reasonable assurance that the observed differential response of the cells to the gels was due to adhesive binding (or lack thereof).

The Sulfo-LC-SPDP reacts with the short collagen oligomers to form an amine bond, and then the 2-pyridil disulfide group is reacted with the sulfhydryl group of the cysteine residue on GRGDS or GRGES to form a disulfide bond. As a result, a 15Å spacer is introduced between the conjugated peptides and the collagen [52] , and the non-adhesive, scrambled peptide may not interfere with binding to nearby ligands or with integrin clustering. The spacer may also influence the traction exerted on the collagen fibers via the grafted RGD by providing a potentially compliant structure between the peptide and fiber. Conversely, covalent coupling via EDC leaves no spacer [53], and the grafted scrambled peptide may mask natural adhesion sites more efficiently, and allow more direct transfer of traction for RGD grafted collagen. Hern and Hubbell have reported that fibroblast spreading on RGD-modified poly(ethylene glycol) diacrylate networks, which were naturally non-adhesive, was significantly affected by including a PEG spacer between the network and grafted peptide [35], though that spacer was ~10x the molecular weight of the Sulfo-LC-SPDP spacer. Similar results were demonstrated by Park et al with a non-adhesive, thermoreversible N-isopropylacrylamise hydrogel [54].

Type I collagen has been extensively researched as a biomaterial for a variety of tissue engineered regenerative therapies, including skin, blood vessels, ligaments and tendons, and peripheral nerves and spinal cords. The ability to manipulate the intrinsic adhesivity of collagen, and thereby modulate both cell migration and cell traction, can provide valuable flexibility to

these approaches, particularly if spatial control of differentially-grafted collagen can be achieved to provide distinct regions that promote adhesion or promote contraction. It should be recognized that the cells in these different applications may require different degrees of peptide grating (scrambled and/or bioactive) to achieve the desired properties. Additionally, the relationships among migration, adhesion, and traction become more complex when considering cells within a three-dimensional system capable of cell-mediated reorganization, such as a collagen or fibrin gel, where the stiffness of the gel and the resultant change in porosity and cell density can also contribute to the observed behavior [11]. Our technique allows the adhesivity of these systems to be altered without affecting significantly the initial structure or stiffness, and may therefore provide a valuable platform to further elucidate these complex relationships.

2.6. Acknowledgements

2.7. References

1. Even-Ram, S. and K.M. Yamada, Cell migration in 3D matrix. Curr Opin Cell Biol, 2005. 17(5): p. 524-32.

2. Vicente-Manzanares, M., D.J. Webb, and A.R. Horwitz, Cell migration at a glance. J Cell Sci, 2005. 118(Pt 21): p. 4917-9.

3. Beningo, K.A., M. Dembo, and Y.L. Wang, Responses of fibroblasts to anchorage of dorsal extracellular matrix receptors. Proc Natl Acad Sci U S A, 2004. 101(52): p. 18024-9.

4. Galbraith, C.G. and M.P. Sheetz, Keratocytes pull with similar forces on their dorsal and ventral surfaces. J Cell Biol, 1999. 147(6): p. 1313-24.

5. Palecek, S.P., J.C. Loftus, M.H. Ginsberg, D.A. Lauffenburger, and A.F. Horwitz, Integrin-ligand binding properties govern cell migration speed through cell-substratum adhesiveness. Nature, 1997. 385(6616): p. 537-40.

6. Gupton, S.L. and C.M. Waterman-Storer, Spatiotemporal feedback between actomyosin and focal-adhesion systems optimizes rapid cell migration. Cell, 2006. 125(7): p. 1361-74.

7. DiMilla, P.A., K. Barbee, and D.A. Lauffenburger, Mathematical model for the effects of adhesion and mechanics on cell migration speed. Biophys J, 1991. 60(1): p. 15-37.

8. Burgess, B.T., J.L. Myles, and R.B. Dickinson, Quantitative analysis of adhesion-mediated cell migration in three-dimensional gels of RGD-grafted collagen. Ann Biomed Eng, 2000. 28(1): p. 110-8.

9. Zaman, M.H., Understanding the molecular basis for differential binding of integrins to collagen and gelatin. Biophys J, 2007. 92(2): p. L17-9.

10. Hazgui, S., N. Bonnet, J. Cutrona, B. Nawrocki-Raby, M. Polette, L. Chouchane, P. Birembaut, and J.M. Zahm, 3D culture model and computer-assisted videomicroscopy to analyze migratory behavior of noninvasive and invasive bronchial epithelial cells. Am J Physiol Cell Physiol, 2005. 289(6): p. C1547-52.

11. Zaman, M.H., L.M. Trapani, A.L. Sieminski, D. Mackellar, H. Gong, R.D. Kamm, A. Wells, D.A. Lauffenburger, and P. Matsudaira, Migration of tumor cells in 3D matrices is governed by matrix stiffness along with cell-matrix adhesion and proteolysis. Proc Natl Acad Sci U S A, 2006. 103(29): p. 10889-94.

12. Knapp, D.M., T.T. Tower, R.T. Tranquillo, and V.H. Barocas, Estimation of cell traction and migration in an isometric cell traction assay. Aiche Journal, 1999. 45(12): p. 2628-2640.

13. Shreiber, D.I., V.H. Barocas, and R.T. Tranquillo, Temporal variations in cell migration and traction during fibroblast-mediated gel compaction. Biophys J, 2003. 84(6): p. 4102-14.

14. Knight, C.G., L.F. Morton, A.R. Peachey, D.S. Tuckwell, R.W. Farndale, and M.J. Barnes, The collagen-binding A-domains of integrins alpha(1)beta(1) and alpha(2)beta(1) recognize the same specific amino acid sequence, GFOGER, in native (triple-helical) collagens. J Biol Chem, 2000. 275(1): p. 35-40.

15. Mineur, P., A. Guignandon, A. Lambert Ch, M. Amblard, M. Lapiere Ch, and B.V. Nusgens, RGDS and DGEA-induced [Ca2+]i signalling in human dermal fibroblasts. Biochim Biophys Acta, 2005. 1746(1): p. 28-37.

16.   Mutsaers, S.E., J.E. Bishop, G. McGrouther, and G.J. Laurent, Mechanisms of tissue repair: From wound healing to fibrosis. International Journal of Biochemistry & Cell Biology, 1997. 29(1): p. 5-17.

17.   Shreiber, D.I., P.A. Enever, and R.T. Tranquillo, Effects of pdgf-bb on rat dermal fibroblast behavior in mechanically stressed and unstressed collagen and fibrin gels. Exp Cell Res, 2001. 266(1): p. 155-66.

18.   Kilarski, W.W., N. Jura, and P. Gerwins, An ex vivo model for functional studies of myofibroblasts. Lab Invest, 2005. 85(5): p. 643-54.

19.   Wells, A., M.F. Ware, F.D. Allen, and D.A. Lauffenburger, Shaping up for shipping out: PLCgamma signaling of morphology changes in EGF-stimulated fibroblast migration. Cell Motil Cytoskeleton, 1999. 44(4): p. 227-33.

20.   Clark, R.A., K. Ghosh, and M.G. Tonnesen, Tissue engineering for cutaneous wounds. J Invest Dermatol, 2007. 127(5): p. 1018-29.

21.   Schmidt, C.E. and J.B. Leach, Neural tissue engineering: strategies for repair and regeneration. Annu Rev Biomed Eng, 2003. 5: p. 293-347.

22.   Liu, G., S.G. Eskin, and A.G. Mikos, Integrin alpha(v)beta(3) is involved in stimulated migration of vascular adventitial fibroblasts by basic fibroblast growth factor but not platelet-derived growth factor. J Cell Biochem, 2001. 83(1): p. 129-35.

23.   Heldin, C.H., A. Ostman, and L. Ronnstrand, Signal transduction via platelet-derived growth factor receptors. Biochim Biophys Acta, 1998. 1378(1): p. F79-113.

24.   Cunningham, C.C., T.P. Stossel, and D.J. Kwiatkowski, Enhanced motility in NIH 3T3 fibroblasts that overexpress gelsolin. Science, 1991. 251(4998): p. 1233-6.

25.   Taylor, W.R., A.H. Greenberg, E.A. Turley, and J.A. Wright, Cell motility, invasion, and malignancy induced by overexpression of K-FGF or bFGF. Exp Cell Res, 1993. 204(2): p. 295-301.

26.   Knapp, D.M., E.F. Helou, and R.T. Tranquillo, A fibrin or collagen gel assay for tissue cell chemotaxis: assessment of fibroblast chemotaxis to GRGDSP. Exp Cell Res, 1999. 247(2): p. 543-53.

27.   Myles, J.L., B.T. Burgess, and R.B. Dickinson, Modification of the adhesive properties of collagen by covalent grafting with RGD peptides. J Biomater Sci Polym Ed, 2000. 11(1): p. 69-86.

28.   Chen, R. and J.A. Hunt, Biomimetic materials processing for tissue-engineering processes. Journal of Materials Chemistry, 2007. 17(38): p. 3974-3979.

29.   DiMilla, P.A., J.A. Stone, J.A. Quinn, S.M. Albelda, and D.A. Lauffenburger, Maximal migration of human smooth muscle cells on fibronectin and type IV collagen occurs at an intermediate attachment strength. J Cell Biol, 1993. 122(3): p. 729-37.

30.   Sundararaghavan, H.G., G.A. Monteiro, N.A. Lapin, Y.J. Chabal, J.R. Miksan, and D.I. Shreiber, Genipin-induced changes in collagen gels: Correlation of mechanical properties to fluorescence. J Biomed Mater Res A, 2008.

31.   Bell, E., S. Sher, and B. Hull, The living skin-equivalent as a structural and immunological model in skin grafting. Scan Electron Microsc, 1984(Pt 4): p. 1957-62.

32.   Barocas, V.H. and R.T. Tranquillo, An anisotropic biphasic theory of tissue-equivalent mechanics: the interplay among cell traction, fibrillar network deformation, fibril alignment, and cell contact guidance. J Biomech Eng, 1997. 119(2): p. 137-45.

33.   Dickinson, R.B. and R.T. Tranquillo, Optimal estimation of cell movement indices from the statistical analysis of cell tracking data. AIChE Journal, 1993. 39: p. 1995-2010.

34.   Dunn, G.A. and A.F. Brown, A unified approach to analysing cell motility. J Cell Sci Suppl, 1987. 8: p. 81-102.

35.   Hern, D.L. and J.A. Hubbell, Incorporation of adhesion peptides into nonadhesive hydrogels useful for tissue resurfacing. J Biomed Mater Res, 1998. 39(2): p. 266-76.

36.   Wu, P., J.B. Hoying, S.K. Williams, B.A. Kozikowski, and D.A. Lauffenburger, Integrin-binding peptide in solution inhibits or enhances endothelial cell migration, predictably from cell adhesion. Ann Biomed Eng, 1994. 22(2): p. 144-52.

37.   Yamada, K.M., S.K. Akiyama, T. Hasegawa, E. Hasegawa, M.J. Humphries, D.W. Kennedy, K. Nagata, H. Urushihara, K. Olden, and W.T. Chen, Recent advances in research on fibronectin and other cell attachment proteins. J Cell Biochem, 1985. 28(2): p. 79-97.

38.   Yu, X., G.P. Dillon, and R.B. Bellamkonda, A laminin and nerve growth factor-laden three-dimensional scaffold for enhanced neurite extension. Tissue Eng, 1999. 5(4): p. 291-304.

39.   Lafrenie, R.M. and K.M. Yamada, Integrin-dependent signal transduction. Journal of Cellular Biochemistry, 1996. 61(4): p. 543-553.

40.   Schor, S.L., I. Ellis, J. Banyard, and A.M. Schor, Motogenic activity of IGD-containing synthetic peptides. J Cell Sci, 1999. 112 (Pt 22): p. 3879-88.

41.   Tosatti, S., Z. Schwartz, C. Campbell, D.L. Cochran, S. VandeVondele, J.A. Hubbell, A. Denzer, J. Simpson, M. Wieland, C.H. Lohmann, M. Textor, and B.D. Boyan, RGD-containing peptide GCRGYGRGDSPG reduces enhancement of osteoblast differentiation by poly(L-lysine)-graft-poly(ethylene glycol)-coated titanium surfaces. J Biomed Mater Res A, 2004. 68(3): p. 458-72.

42.   Hsu, S.H., S.H. Chang, H.J. Yen, S.W. Whu, C.L. Tsai, and D.C. Chen, Evaluation of biodegradable polyesters modified by type II collagen and Arg-Gly-Asp as tissue engineering scaffolding materials for cartilage regeneration. Artif Organs, 2006. 30(1): p. 42-55.

43.   Feng, Y. and M. Mrksich, The synergy peptide PHSRN and the adhesion peptide RGD mediate cell adhesion through a common mechanism. Biochemistry, 2004. 43(50): p. 15811-21.

44.   Zaman, M.H., R.D. Kamm, P. Matsudaira, and D.A. Lauffenburger, Computational model for cell migration in three-dimensional matrices. Biophys J, 2005. 89(2): p. 1389-97.

45.   Li, X., B. Chen, S.D. Blystone, K.P. McHugh, F.P. Ross, and D.M. Ramos, Differential expression of alphav integrins in K1735 melanoma cells. Invasion Metastasis, 1998. 18(1): p. 1-14.

46.   Li, X., J. Regezi, F.P. Ross, S. Blystone, D. Ilic, S.P. Leong, and D.M. Ramos, Integrin alphavbeta3 mediates K1735 murine melanoma cell motility in vivo and in vitro. J Cell Sci, 2001. 114(Pt 14): p. 2665-72.

47.   Kuntz, R.M. and W.M. Saltzman, Neutrophil motility in extracellular matrix gels: Mesh size and adhesion affect speed of migration. Biophysical Journal, 1997. 72(3): p. 1472-1480.

48.   Fujimura, T., S. Moriwaki, G. Imokawa, and Y. Takema, Crucial role of fibroblast integrins alpha2 and beta1 in maintaining the structural and mechanical properties of the skin. J Dermatol Sci, 2007. 45(1): p. 45-53.

49.   Ogle, B.M. and D.L. Mooradian, The role of vascular smooth muscle cell integrins in the compaction and mechanical strengthening of a tissue-engineered blood vessel. Tissue Eng, 1999. 5(4): p. 387-402.

50.   Wang, R., M.H. Stromer, and T.W. Huiatt, Integrin expression in developing smooth muscle cells. J Histochem Cytochem, 1998. 46(1): p. 119-26.

51.   Jarvelainen, H., R.B. Vernon, M.D. Gooden, A. Francki, S. Lara, P.Y. Johnson, M.G. Kinsella, E.H. Sage, and T.N. Wight, Overexpression of decorin by rat arterial smooth muscle cells enhances contraction of type I collagen in vitro. Arterioscler Thromb Vasc Biol, 2004. 24(1): p. 67-72.

52.   Carlsson, J., H. Drevin, and R. Axen, Protein thiolation and reversible protein-protein conjugation. N-Succinimidyl 3-(2-pyridyldithio)propionate, a new heterobifunctional reagent. Biochem J, 1978. 173(3): p. 723-37.

53.   Grabarek, Z. and J. Gergely, Zero-length crosslinking procedure with the use of active esters. Anal Biochem, 1990. 185(1): p. 131-5.

54.   Park, K.H., K. Na, and H.M. Chung, Enhancement of the adhesion of fibroblasts by peptide containing an Arg-Gly-Asp sequence with poly(ethylene glycol) into a thermo-reversible hydrogel as a synthetic extracellular matrix. Biotechnol Lett, 2005. 27(4): p. 227-31.

Chapter 3

3. Sox-1 Expression of Embryonic Stem Cells in Positively and Negatively Modulated Adhesive Collagen Gels

3.1. Introduction

Stem cells in vivo exist in an extracellular matrix. The extracellular matrix once thought to function merely as a scaffold to support the growth of cells and maintenance of tissue and organ structure has been now identified to regulate many aspect of cell behavior, including cell shape [1-5], migration [6, 7], adhesion [8], proliferation and growth [1], cell survival and differentiation[9-17]. It consists of several structural proteins such as collagen, laminin, fibronectin and elastin that are susceptible to degradation and reassembly. Present on these structural proteins are unique sequences of peptides that dictate preferential cell binding. These specialized sequences provide specific signaling and adhesive cues that regulate down stream signaling and cell behavior. Cells attach to the ECM via integrins and these cell specific adhesion sites. Integrins link the actin cytoskeleton of the cell to the ECM via cytoplasmic binding proteins allowing for cells to rprobe, remodel and respond to the ECM surrounding it [18-21]. Matrix remodeling is particularly noticeable during development.

During the normal process of development, multipotent stem cells make many cell fate decisions and migrate to form the familiar layers and patterns in a blastula and subsequently the embryo. These choices are determined by a combination of intrinsic and extrinsic signals. The genetic make up of the cells determine the intrinsic cues. The intrinsic cues can be regarded as the set of instructions activated and modulated by the spatial and temporal organization of extrinsic factors. Extrinsic cues are signals received by the cell that elicit a response that

mediates a translation of genetic material to proteins. Extrinsic cues may be either soluble or contact mediated; their sources can be local or distant. Contact cues include physical cues such as matrix stiffness and adhesion, both of which are perceived by cells primarily via integrins. Several groups have extensively studied the importance of matrix stiffness in the regulation of stem cells [22-27]. For example, Engler et al. have shown that the elastic modulus of the substrate stem cells are grown on is a key regulator for differentiation of mesenchymal stem cells to a particular lineage[22]. Stem cells will preferentially differentiate towards cells from tissues that the substrate modulus they are cultured on mimics. To a lesser degree, the influence of cell matrix adhesion has only indirectly been suggested to direct the differentiation of stem cells. Hakuno et al have shown that the stabilization of focal adhesions preferentially differentiates stem cells towards cardiacmyocytes, cells that are derived from the endoderm germ layer [28]. The discrepancy between the number of studies investigating stem cell matrix stiffness and adhesion is particularly surprising given the direct correlation of focal adhesion formation and matrix stiffness[29]. The coupling of the matrix stiffness and adhesion makes it important to elucidate the response of stem cells to each factor. Herein, we assayed the differentiation of sox1 reporting murine embryonic stem cells towards Neuroectoderm formation by increasing and decreasing adhesion of cells to collagen while maintaining the stiffness constant. Sox-1 is part of the SRY-box containing gene family. Within the developing CNS sox-1 maintains neural cells in an undifferentiated state and is often used as a marker for neural stem cells [30].The adhesion of cells to collagen was modulated as in chapter 2. The adhesion of cells to collagen gels was non-specifically increased by grafting a ubiquitous matrix peptide binding site 'RGD' onto the collagen backbone. Grafting a scrambled version of the same, 'RDG' retarded cell adhesion. The results from this study indicate stem cells can respond to ubiquitous ECM adhesive cues and integrate the biophysical signal to regulate its

differentiation. Increasing adhesion differentiates murine embryonic stem cells away from neural lineages while decreasing adhesion preferentially increases sox-1 expression over a period of ten days.

3.2. **Methods and Materials**

3.2.1. Grafted and Collagen Gel Preparation

Peptide grafted and native collagen gels were prepared as in Monteiro et al. [31]. Briefly, Type I collagen gels were prepared by mixing 20 µl 1M Hepes buffer (Sigma), 140 µl 0.1N NaOH (Sigma), 100µl 10X Minimum Essential Medium Medium (MEM) (Sigma), 52 µl M199 (Invitrogen, Carlsbad, CA), 1 µl pen-strep ((5000 units of penicillin and 5000 mg streptomycin/ml in 0.85% saline) (Sigma), 10 µl L-Glutamine (29.2 mg/ml in 0.85% saline) (Sigma), and 677 µl of 3.0 mg/ml collagen (grafted or native) to make a 2.0 mg/ml collagen solution. Native and grafted collagen solutions self-assemble into a gel upon incubation at 37°C.

3.2.2. Cell Culture

Experiments were performed using a Sox1 reporting knock in murine embryonic stem cell line [32], these cells developed by Dr. Austin Smith of the Institute for Stem Cell Research at the University of Edinburgh. They have has been previously differentiated into cells from all three germ layers as well as have been terminally differentiated into astrocytes, neurons and oligodendrocytes [32]. When cultured in the neural differentiating media the GFP reported for sox-1 is increasingly expressed for 7 days and then is reduced on days 8 thru 10. Cells were a gift from Dr. Biju Parekkadan at Harvard University. All cell cultures were maintained in a humidified 37ºC, 5% CO_2 environment. The mES cells were maintained in an undifferentiated state in T-75 gelatin-coated flasks (Biocoat, BD-Biosciences, Bedford, MA) in Knockout Dulbecco's modified

Eagles medium (Gibco, Grand Island, NY) containing 15% knockout serum (Gibco, Grand Island, NY), 4 mM L-glutamine (Sigma–Aldrich, St. Louis, MO), 100 U/ml penicillin (Sigma–Aldrich, St. Louis, MO), 100 U/ml streptomycin (Sigma–Aldrich, St. Louis, MO), 10 mg/ml gentamicin (Gibco, Grand Island, NY), 1,000 U/ml ESGRO™ (Chemicon, Temecula, CA), 0.1 mM 2-mercaptoethanol (Sigma–Aldrich, St. Louis, MO). ESGRO™ contains leukemia inhibitory factor (LIF), which prevents ES cell differentiation. Every 2 days, media was aspirated and replaced with fresh media. Flasks (75-90% confluent) were harvested with 0.5% Trypsin EDTA (Sigma–Aldrich, St. Louis, MO), following media aspiration and washing with 6 ml of phosphate buffered solution (PBS) (Gibco, Grand Island, NY). Cells were detached following incubation with 3 ml of trypsin (Gibco, Grand Island, NY) for 3 min, resulting in a single cell suspension. Trypsin was neutralized by 4 mls of proliferation media. Harvested cells were then either re-plated in gelatin-coated T-75 flasks at a density of 1 million cells/ml or seeded on native and grafted collagen scaffolds one hour after gel self-assembly. ES cells at 75% confluence, cells were washed with PBS and placed in N2B27 medium, which is a 1:1 mixture of DMEM/F12 medium supplemented with N2 and Neurobasal medium supplemented with B27 (all from Invitrogen, US). The differentiation (N2B27) medium was changed every two days for the duration of the experiment. Cells were seeded at a density of 50K/ ml on scaffolds. 200 ul of cells in were added to each well of a 24 well plate.

3.2.3. Adhesion Assay

RGD- and RDG-grafted collagen solutions at low, medium, and high concentration, as well as native collagen solutions (100μl), were pipetted into separate wells of a 24-well plate in triplicate and allowed to self-assemble at 37 °C and 100% humidity. ES cells (200 μl of a 50,000 cells/ml suspension) were seeded on the gels and allowed to settle and attach for 3 hours. Wells were

rinsed three times with 1ml PBS in five-minute intervals. The remaining cells were stained with DAPI (Invitrogen) and imaged using an Olympus IX81 inverted epifluorescent microscope (Olympus, Melville, NY) with a 4X objective and a DAPI filter. Images were captured digitally (Hamamatsu ORCA, Hamamatsu City, Japan) using Olympus Microsuite software. The number of cells in each field of view were counted using Image Pro Plus (Version 5.1 for windows, Media Cybernetics, MD). Three randomly selected fields were imaged and quantified for each well. The data were analyzed statistically with ANOVA. Post hoc pair wise comparisons were performed with Tukey's test. Significance levels were set at $P < 0.05$.

3.2.4. Fluorescence Activated Cell Counting

The BD FACS CaliburTM (San Jose, CA) system was used to quantify GFP expression of Sox-1 positive cells. Cell medium was aspirated from 24 well plates, cells were washed with PBS and trypsinized for 1 min and re-suspended in PBS. Cells were then analyzed using histogram plots measuring count values of FL-1 (green fluorescence). A fluorescence threshold was set at the base of a bell shaped distribution of FL-1 fluorescence of undifferentiated sox-1 transfected stem cells cultured in the presence of LIF. Cells above the threshold were considered positive. GFP expression was measured for 3 independent experiment sets in duplicate every other day for 10 days. Data was analyzed for statistical significance using an ANOVA. Significance levels were set at P<0.05. Pair wise comparisons were done using a Tukey's post hoc test.

3.3.   Results

3.3.1. Adhesion

Grafting bioactive and scrambled peptides significantly affected cell adhesion to collagen gels. Sox 1 expressing ES cell adhesion **(Figure 9)** increased above controls on collagen grafted with RGD, and decreased below controls on collagen grafted with RDG (ANOVA, P<0.001). Post hoc analysis with Tukey's test demonstrated that all pair wise comparisons were significantly different (max P = 0.007) except the following pairs:

Figure 9 Embryonic Stem cell adhesion to peptide grafted collagen

Sox 1 expressing embryonic stem cell adhesion increased above controls on collagen grafted with RGD, and decreased below controls on collagen grafted with RDG (ANOVA, P<0.001). Cell adhesion to tissue culture plastic varied considerably between experimental sets.

Collagen: RGD Collagen and RGD Collagen: TCP. The number of cells seen attaching to TCP varied considerably between experimental sets. On average however, the TCP condition had the greatest number of cells that remained attached to scaffolds after rinses.

3.3.2. Fluorescence Activated Cell Counting

For all conditions the number of sox1 positive cells that expressed GFP increased on days 2,4 and 6 and decreased on days 8 and 10. Grafting bioactive and scrambled peptides significantly affected the sox-1 expression of cells as measured on day 4 (ANOVA .009), day 6 (ANOVA .001), day 8 (ANOVA 0.001) and day 10 (ANOVA 0.001). On

Figure 10 Percentage of Sox-1 related GFP expressing embryonic stem cells on various substrates.

For all conditions the number of sox1 positive cells that expressed GFP increased on days 2,4 and 6 and decreased on days 8 and 10. Grafting bioactive and scrambled peptides significantly affected the sox-1 expression of cells as measured on day 4 (ANOVA .009), day 6 (ANOVA .001), day 8 (ANOVA 0.001) and day 10 (ANOVA 0.001). On average the number of sox 1 expressing cells were highest on the RDG grafted gels each day except for days 0 and 2.

average the number of sox 1 expressing cells were highest on the RDG grafted gels each day except for days 0 and 2. Post hoc analysis with Tukey's test demonstrated that following pairwise comparisons were significantly different; day 4 RDG grafted collagen: collagen, day 6 RDG grafted collagen and RGD grafted collagen and on days 8 and 10 all pair wise comparisons were different with RDG grafted collagen having the highest number of sox-1 positive cells.
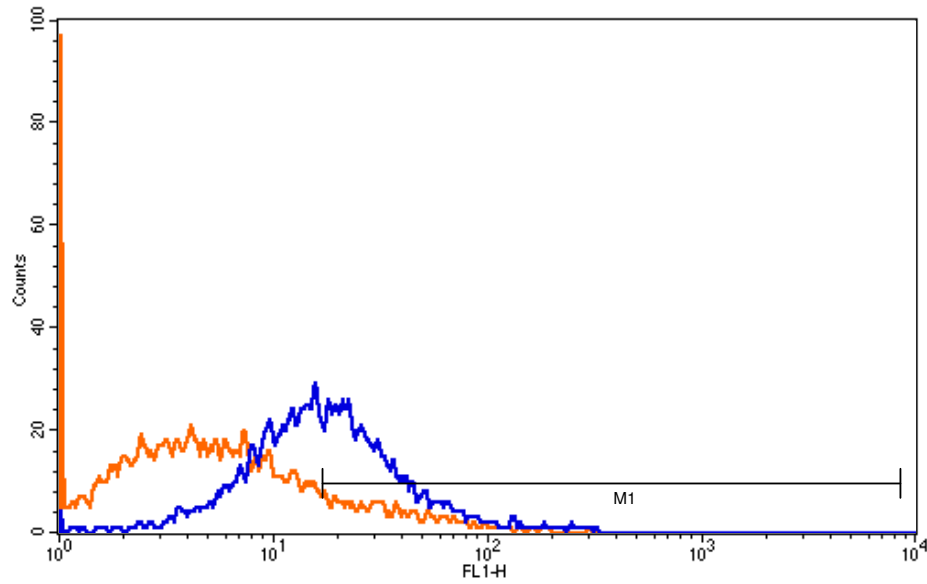
Figure 11 FACS analysis of Sox-1 expression on Day 4.

FACS analysis of sox-1 expression in N2B27 treated stem cells cultured on tissue culture plastic (blue) compared to day 4 cells cultured on tissue culture plastic in the presence of leukemia inhibiting factor. At its highest levels there was a 60% increase in FITC positive cells. Threshold for positive cells were set at base of control, indicated by M1.
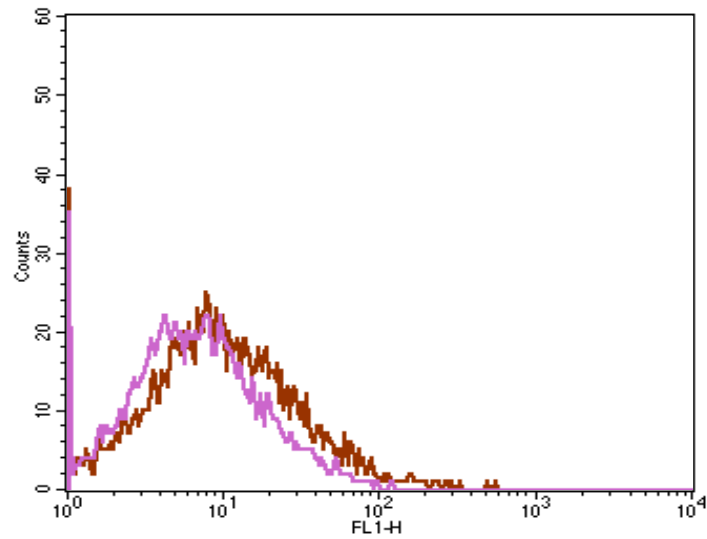
Figure 12 FACS analysis of Sox-1 expression of stem cells cultured on RGD Vs RDG, day 6.

FACS analysis of sox-1 expression in N2B27 treated stem cells cultured on RGD grafted collagen (pink) compared to stem cells cultured on RDG grafted collagen (brown).
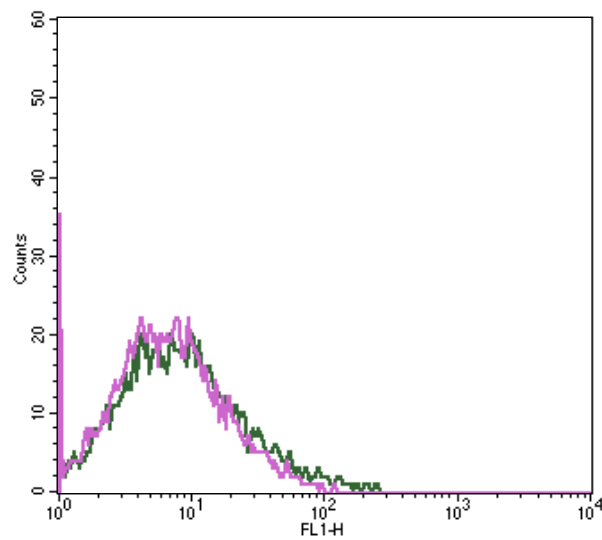
Figure 13 FACS analysis of Sox-1 expression of stem cells cultured on RGD Vs native collagen, day 6.

FACS analysis of sox-1 expression in N2B27 treated stem cells cultured on RGD grafted collagen (pink) compared to stem cells cultured on native collagen (green).
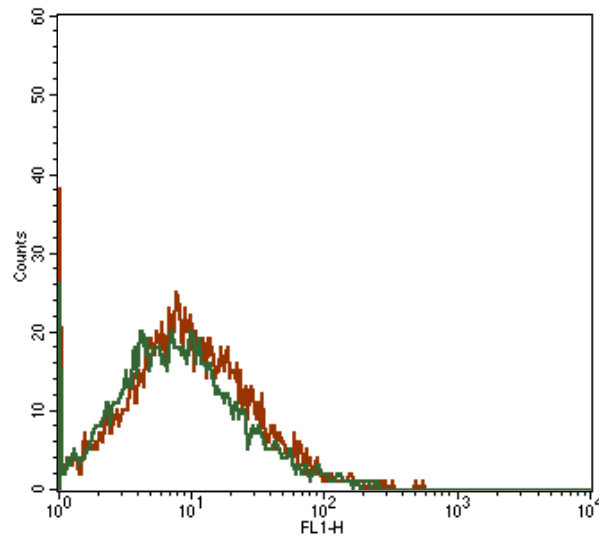
Figure 14 FACS analysis of Sox-1 expression of stem cells cultured on Collagen Vs RDG, day 6.

FACS analysis of sox-1 expression in N2B27 treated stem cells cultured on RDG grafted collagen (brown) compared to stem cells cultured on native collagen (green).
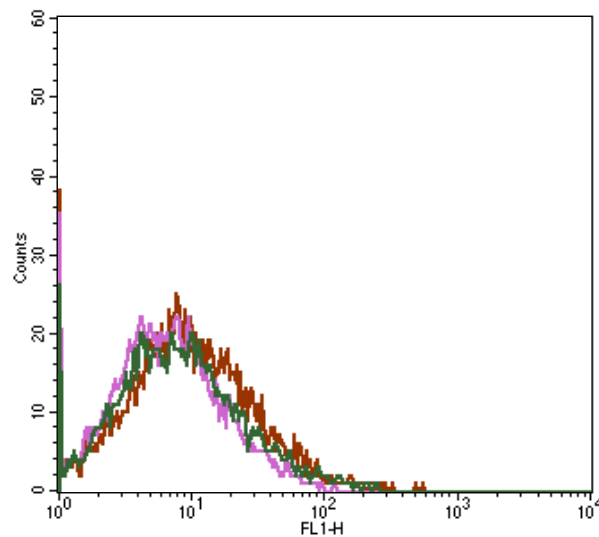
Figure 15 FACS analysis of Sox-1 expression of stem cells cultured on various collagen substrates

FACS analysis of sox-1 expression in N2B27 treated stem cells cultured on RDG grafted collagen (brown), on native collagen (green), and RGD grafted collagen pink. Sox-1 expression as reported by GFP is inversely proportional to cell adhesion to matrix. Increasing adhesion by grafting the ubiquitous matrix peptide RGD increases cell adhesion and decreases sox1 expression. Alternatively, decreasing adhesion by grafting a scrambled version of the peptide, 'RDG' decreases cell adhesion and increases sox-1 expression as noted by FACS.

3.4.   Discussion

Stem cells, capable of becoming any cell within the body, are of particular interest as a potentially endless source of versatile cells for regenerative purposes. However, the in vivo maturation of stem cells into tissues and then into organ systems that comprise living organisms is a complex process involving coordinated physical and chemical cues to determine their lineage. The poor understanding of the in vivo microenvironments that direct the differentiation of stem cells towards specific lineages presents a major challenge to incorporating stem cells in regenerative medicine practices. To better understand the relationship between stem cells and their microenvironment, here in, we studied the role of cell adhesion to a ubiquitous ECM protein collagen, to guide the differentiation of stem cells towards neuronal lineages. In chapter 2 we developed an approach to alter, positively as well as negatively, the specific and non-specific adhesion of cells to collagen and in the current chapter we assayed the differentiation of stem cells towards neuronal lineages on these scaffolds.

To assay the differentiation of stem cells we used a stem cell line that was developed by Dr. Austin Smith at the University of Edinburgh. These cells were genetically modified to with a GFP reporter for sox-1 expression. Previous research has shown that these cells are cable of differentiation to cells from all germ layers as well as terminal differentiation towards neurons, astrocytes and oligodendrocytes{Ying, 2003 #5}. When cultured in the presence of a neural differentiation media that comprised of a 1:1 mixture of DMEM F12 and Neural basal media on tissue culture plastic, Dr. Smiths group have reported GFP expression within 95% of the population. However, in our hand at best a 60% GFP expressing population was noted. The significant difference in these GFP expressing populations is attributed to the more restrictive

expression criteria used in these experiments. GFP expression used in FACS analysis was considered positive only if the relative fluorescence was above 7.5% of the basal level. The rationale for using this criterion was to avoid the inclusion of false positive data. Using this criterion a modest increase in differentiation was seen between conditions. At its highest levels there was 20% difference in the number of sox-1 positive cells that expressed GFP between conditions. This correlated to a 5% difference in the total population of cells. However, it should be noted that this increased differentiation was seen in the presence of a neural inducing media. Basal media was not used to avoid cell-matrix interactions with extracellular components present in the serum component of basal medias.

Grafting RDG peptides to collagen reduced the cell-matrix adhesions in the absence of serum containing media, however, promoted cell- cell aggregation. Like most adherent cell types, stem cells as well when cultured on non-adherent substrates in the absence of serum will cluster to form larger aggregates. The autocrine and paracrine signaling within these environments are difficult to control and may play a role in cell differentiation as well. Previously Parekkadan, et al have shown that Sox-1 expressing stem cells when cultured juxtaposed to neural differentiating stem cells will follow cell-cell mediated cues and differentiae towards neural lineages as well [33]. It is therefore possible that stem cells cultured on RDG grafted collagen scaffolds that aggregated to form larger clusters were directed to differentiate towards neural lineages via cell-mediated contact and paracrine signaling pathways. Nonetheless, scaffolds like the RDG grafted collagen environment that reduce cell adhesion to cell matrices may be used as delivery mechanisms for in vitro transplantation of stem cells within damaged or diseased neural tissue.

Ultimately, any therapy that utilizes stem cells for regenerative purposes will require their delivery and maintenance at the injury site for prolonged periods of time. The cues present on these delivery systems must be specific to guide the differentiation of resident or implanted stem cells towards the lineage and cell types of choice. As we understand more and more of the role that the matrix plays in stem cell differentiation we can incorporate these cues into therapeutic scaffolds to create a stem cell niche optimal for specific regenerative purposes.

3.5.   References

1.   Huet, C., et al., *Extracellular matrix regulates ovine granulosa cell survival, proliferation and steroidogenesis: relationships between cell shape and function.* J Endocrinol, 2001. **169**(2): p. 347-60.

2.   Abbott, L.A., S.M. Lester, and C.A. Erickson, *Changes in mesenchymal cell-shape, matrix collagen and tenascin accompany bud formation in the early chick lung.* Anat Embryol (Berl), 1991. **183**(3): p. 299-311.

3.   McDonald, J.A., *Matrix regulation of cell shape and gene expression.* Curr Opin Cell Biol, 1989. **1**(5): p. 995-9.

4.   Young, W.C. and I.M. Herman, *Extracellular matrix modulation of endothelial cell shape and motility following injury in vitro.* J Cell Sci, 1985. **73**: p. 19-32.

5.   Bethea, C.L. and S.L. Kozak, *Effect of extracellular matrix on PC 12 cell shape and dopamine processing.* Mol Cell Endocrinol, 1984. **37**(3): p. 319-29.

6.   Ebendal, T., *Extracellular matrix fibrils and cell contacts in the chick embryo. Possible roles in orientation of cell migration and axon extension.* Cell Tissue Res, 1977. **175**(4): p. 439-58.

7.   Lofberg, J., K. Ahlfors, and C. Fallstrom, *Neural crest cell migration in relation to extracellular matrix organization in the embryonic axolotl trunk.* Dev Biol, 1980. **75**(1): p. 148-67.

8.   Hannigan, G.E., et al., *Regulation of cell adhesion and anchorage-dependent growth by a new beta 1-integrin-linked protein kinase.* Nature, 1996. **379**(6560): p. 91-6.

9.   Rozario, T. and D.W. Desimone, *The extracellular matrix in development and morphogenesis: A dynamic view.* Dev Biol, 2009.

10.   Guilak, F., et al., *Control of stem cell fate by physical interactions with the extracellular matrix.* Cell Stem Cell, 2009. **5**(1): p. 17-26.

11.   Zhang, Y., et al., *Tissue-specific extracellular matrix coatings for the promotion of cell proliferation and maintenance of cell phenotype.* Biomaterials, 2009. **30**(23-24): p. 4021-8.

12.   Ricard-Blum, S., *"Regenerative medicine: stem cells and extracellular matrix".* Pathol Biol (Paris), 2009. **57**(4): p. 281.

13.   Badylak, S.F., D.O. Freytes, and T.W. Gilbert, *Extracellular matrix as a biological scaffold material: Structure and function.* Acta Biomater, 2009. **5**(1): p. 1-13.

14.   Nieponice, A., et al., *An extracellular matrix scaffold for esophageal stricture prevention after circumferential EMR.* Gastrointest Endosc, 2009. **69**(2): p. 289-96.

15.   Daley, W.P., S.B. Peters, and M. Larsen, *Extracellular matrix dynamics in development and regenerative medicine.* J Cell Sci, 2008. **121**(Pt 3): p. 255-64.

16.   Valentin, J.E., et al., *Extracellular matrix bioscaffolds for orthopaedic applications. A comparative histologic study.* J Bone Joint Surg Am, 2006. **88**(12): p. 2673-86.

17.   Li, F., et al., *Cellular and nerve regeneration within a biosynthetic extracellular matrix for corneal transplantation.* Proc Natl Acad Sci U S A, 2003. **100**(26): p. 15346-51.

18.   Hynes, R.O., *Integrins: bidirectional, allosteric signaling machines.* Cell, 2002. **110**(6): p. 673-87.

19.   Hemler, M.E., et al., *Structure of the integrin VLA-4 and its cell-cell and cell-matrix adhesion functions.* Immunol Rev, 1990. **114**: p. 45-65.

20.   Ager, A. and M.J. Humphries, *Integrin alpha 4 beta 1: its structure, ligand-binding specificity and role in lymphocyte-endothelial cell interactions.* Chem Immunol, 1991. **50**: p. 55-74.

21.   Bennett, J.S., *Integrin structure and function in hemostasis and thrombosis.* Ann N Y Acad Sci, 1991. **614**: p. 214-28.

22.   Engler, A.J., et al., *Myotubes differentiate optimally on substrates with tissue-like stiffness: pathological implications for soft or stiff microenvironments.* J Cell Biol, 2004. **166**(6): p. 877-87.

23.   Shearn, J.T., et al., *Mechanical stimulation of tendon tissue engineered constructs: effects on construct stiffness, repair biomechanics, and their correlation.* J Biomech Eng, 2007. **129**(6): p. 848-54.

24.   Nirmalanandhan, V.S., et al., *Effect of scaffold material, construct length and mechanical stimulation on the in vitro stiffness of the engineered tendon construct.* J Biomech, 2008. **41**(4): p. 822-8.

25.   Leipzig, N.D. and M.S. Shoichet, *The effect of substrate stiffness on adult neural stem cell behavior.* Biomaterials, 2009. **30**(36): p. 6867-78.

26.   Chokalingam, K., et al., *Tensile stimulation of murine stem cell-collagen sponge constructs increases collagen type I gene expression and linear stiffness.* Tissue Eng Part A, 2009. **15**(9): p. 2561-70.

27.   Pek, Y.S., A.C. Wan, and J.Y. Ying, *The effect of matrix stiffness on mesenchymal stem cell differentiation in a 3D thixotropic gel.* Biomaterials. **31**(3): p. 385-91.

28.   Hakuno, D., et al., *Focal adhesion kinase signaling regulates cardiogenesis of embryonic stem cells.* J Biol Chem, 2005. **280**(47): p. 39534-44.

29.   Pelham, R.J., Jr. and Y. Wang, *Cell locomotion and focal adhesions are regulated by substrate flexibility.* Proc Natl Acad Sci U S A, 1997. **94**(25): p. 13661-5.

30.   Nitta, K.R., et al., *Expression of Sox1 during Xenopus early embryogenesis.* Biochem Biophys Res Commun, 2006. **351**(1): p. 287-93.

31.   Monteiro, G.A., et al., *Positively and Negatively Modulating Cell Adhesion to Type I Collagen Via Peptide Grafting.* Tissue Eng Part A, 2009.

32.   Ying, Q.L., et al., *Conversion of embryonic stem cells into neuroectodermal precursors in adherent monoculture.* Nat Biotechnol, 2003. **21**(2): p. 183-6.

33.   Parekkadan, B., et al., *Cell-cell interaction modulates neuroectodermal specification of embryonic stem cells.* Neurosci Lett, 2008. **438**(2): p. 190-5.

Chapter 4

4. Defining Stem Cell Niches to Direct Mouse Embryonic Stem Cell Differentiation towards Neuroectoderm Lineages

4.1.   Introduction

The ability for embryonic stem cells to differentiate into functionally competent cells forms the basis for their use in tissue replacement strategies. In vivo, this is dictated in large part by the niche surrounding stem cells. Stem cell niches provide a myriad of physical, chemical, and genetic cues that act in concert to retain pluripotency or guide differentiation, and understanding and recreating these niches has increasingly become a centerpiece of research for tissue engineering and regenerative medicine applications. Biomaterials are proving to be a valuable component of these endeavors.

Biomaterials comprise natural and/or synthetic materials, and form the framework for tissue engineering scaffolds, which provide a three dimensional architecture on which cells can adhere, proliferate, differentiate, and secrete extracellular matrix (ECM) to form functional tissues. A number of biomaterial and scaffold properties influence cell behavior and tissue growth through biophysical interactions, such as surface charge, stiffness, and topography, or through direct ligand binding. These properties may also be tuned to design specific environments for controlling stem cell niches and understanding stem cell differentiation. At the same time, stem cells provide a powerful cell source that can be used in combination with biomaterials towards tissue engineering strategies for regenerative medicine applications,

particularly in tissues where generating significant populations of host cells by traditional culture means has proven to be difficult to impossible, such as neural, pancreatic, and hepatic tissues.

In this study, we aim to augment the ubiquitous ECM cues provided by a naturally occurring protein – type I collagen – with specific ligands to direct embryonic stem cell differentiation towards a neural lineage. Neural tissues present a particularly complex and dynamic environment to its cellular constituents during and post-development. Carbohydrates are among the components that directly interact with cell adhesion molecules contribute to the regulation of cell and tissue differentiation. Herein, we functionalized collagen scaffolds with peptide mimics of two carbohydrates present in neural stem cell niches in vivo – polysialic acid and L2/human natural killer-1.

Polysialic acid is present in developing vertebrae embryos as a glycosylation on neural cell adhesion molecules [1, 2]. The degree of polysialication generally decreases as development progresses, but is preserved in regions that retain their ability to generate neurons such as the subventricular zone, the rostral migratory stream, the olfactory bulb, and the dentate gyrus [3, 4]. The human natural killer-1 carbohydrate epitope is also capable of regulating NCAM adhesion and several other adhesion molecules [5, 6]. This carbohydrate was first indentified on human natural killer cells [7]. More recently, however, its presence on neural crest stem cells has established its role in neural development. Several neural recognition molecules as well as integrins recognize the human natural killer-1 carbohydrate epitope, which functionally links it to the extracellular matrix and likely regulates behavior and differentiation [8]. In the developing peripheral nervous system (PNS), the human natural killer-1 (HNK-1) antigen interacts and regulates the migration of neural crest cells, cell populations containing stem cells [9].

Studies identifying the role of these molecules in stem cell differentiation have been limited

due to the difficulties associated with isolating and mass-producing carbohydrates. Recently,

however, functional peptide mimics to these carbohydrates have been identified using phage

display followed by functional assays [10-12]. These peptide glycomimics are more cost effective

and easier to produce than their carbohydrate counterparts, and are manipulated using simpler

chemistries. As such, the peptide mimics can be incorporated into biomaterial strategies to

improve the bioactivity of stem cell niches in vitro, and, potentially, in vivo. We have previously

demonstrated that we can alter the bioactive adhesivity of collagen without altering the overall

mechanical properties of the scaffold by grafting functional peptide sequences from matrix

proteins such as fibronectin and laminin [3,4]. Herein, we hypothesized that we can tailor

collagen to guide the differentiation of embryonic stem cells towards a neural lineage by

immobilizing PSA and HNK onto the collagen backbone to recreate the inclusion of these

carbohydrates in the natural neural stem niche. (In this chapter the abbreviations PSA and HNK

will be used to refer specifically to the peptide forms of polysialic acid and human natural killer-

1, respectively.)

4.2.   Materials and Methods

4.2.1. Cell Culture

Experiments were performed with the murine embryonic stem cell line D3 (ATCC, Manassas,

VA). All cell cultures were incubated in a humidified 37ºC, 5% CO2 environment. The mES cells

were maintained in an undifferentiated state in T-75 gelatin-coated flasks (Biocoat, BD-

Biosciences, Bedford, MA) in Knockout Dulbecco's modified Eagles medium (Gibco, Grand

Island, NY) containing 15% knockout serum (Gibco, Grand Island, NY), 4 mM L-glutamine

(Sigma–Aldrich, St. Louis, MO), 100 U/ml penicillin (Sigma–Aldrich, St. Louis, MO), 100 U/ml

streptomycin (Sigma–Aldrich, St. Louis, MO), 10 mg/ml gentamicin (Gibco, Grand Island, NY),

1,000 U/ml ESGRO^{TM} (Chemicon, Temecula, CA), 0.1 mM 2-mercaptoethanol (Sigma–Aldrich, St.

Louis, MO). ESGRO^{TM} contains leukemia inhibitory factor (LIF), which prevents ES cell

differentiation. Every 2 days, media was aspirated and replaced with fresh media. Flasks (75-

90% confluent) were harvested with 0.5% Trypsin EDTA (Sigma–Aldrich, St. Louis, MO), following

media aspiration and washing with 6 ml of phosphate buffered solution (PBS) (Gibco, Grand

Island, NY). Cells were detached following incubation with 3 ml of trypsin (Gibco, Grand Island,

NY) for 3 min, resulting in a single cell suspension. Trypsin was neutralized by 4 mls of complete

media defined below. Harvested cells were then either re-plated in gelatin-coated T-75 flasks at

a density of 1 million cells/ml or seeded on native and grafted collagen scaffolds one hour after

gel self-assembly. Cells were seeded at a density of 50K/ ml on scaffolds. 100ul of cells and 100

ul of differentiating media were added to each well of a 48 well plate. Cell differentiation was

induced using complete media and the withdrawal of LIF. Complete media consists of Iscove's

modiﬁed Dulbecco's medium Gibco, Grand Island, NY) containing 20% fetal bovine serum

premium select (Atlanta Biologicals, Lawrenceville, GA), 4 mM L-glutamine, 100 U/ml penicillin,

100 U/ml streptomycin, 10 mg/ml gentomicin. No additional growth factors were used to induce

differentiation of stem cells into any lineage. Media was replaced every 2 days for the duration

of the experiment. Cells from passages 10 through 22 were used in the experiments.

4.2.2. Peptide Grafting and Collagen Gel Preparation

Peptide grafted and native collagen gels were prepared as in Monteiro et al. [13]. Briefly, HNK,

PSA, and control peptide sequences, were custom synthesized (Genscript, Piscataway, NJ) and

were conjugated to the backbone of collagen using 1-ethyl-3-(3-Dimethylaminopropyl)

carbodiimide (EDC, Sigma) Chemistry. EDC was used to activate the carboxylic group of the

peptide by mixing a 1mM solution of EDC in MES buffer (pH 2-4, Sigma) with the peptide of

interest for ten minutes at 37°C. The peptide-EDC mixture was added to a 3mg/ml suspension of type I collagen (Elastin Products, Owensville, MO) in 0.02N acetic acid. The activated peptide covalently binds to free amines on residues on the collagen backbone via nucleophilic

Peptide	Sequence
HNK	**FLHTRLFV**
PSA	**SSVTAWTTG**
HNK Control	**TVFHRFLL**
PSA Control	**GTTWATVSS**

Table 2: Peptide Sequences and scrambled controls grafted onto collagen conditions.

attack. Peptide-EDC-collagen mixtures were incubated on a shaker overnight at 4°C and then

dialyzed against 0.02N acetic acid for 12 hours using snakeskin dialysis tubing with a 10kD

molecular weight cutoff (Pierce Biotechnology, Rockford, IL) to remove unconjugated peptide.

Dialyzed peptide-grafted collagen was lyophilized at -150°C and 50mTor for 12 hours to remove

all water. Lyophilized product was re-suspended in 0.02N acetic acid to make a 3mg/ml solution of grafted collagen. The efficiency of peptide grafting was measured indirectly by grafting peptides that included a FITC tag during synthesis (Genscript) and comparing the fluorescence intensity of grafted collagen after reconstitution to a standard curve created by admixing fluorescent peptide into collagen solution. Efficiency ranged between 50-60%, resulting in 67-80 µg of peptide/mg collagen.

Experimental Conditions
HNK Grafted Collagen
PSA Grafted Collagen
HNK Control Grafted Collagen
PSA Control Grafted Collagen
HNK-PSA Grafted Collagen
Native Collagen (No peptide)
Tissue Culture Plastic

Table 3 Collagen grafted conditions on which embryonic stem cell differentiation towards neural lineages were assayed.

To prepare collagen gels, acid solubilized collagen (grafted or native) was neutralized to

physiologically relevant pH and ionic strength with 1M Hepes buffer (Sigma), 0.1N NaOH (Sigma)

and 10X Minimum Essential Medium (Sigma) and supplemented with M199 (Invitrogen,

Carlsbad, CA), pen-strep ((5000 units of penicillin and 5000 mg streptomycin/ml in 0.85% saline)

(Sigma) and L-Glutamine (29.2 mg/ml in 0.85% saline) (Sigma) to make a 2.0 mg/ml collagen

solution. Native and grafted collagen solutions (100 μl) were pipetted into individual wells of a

48-well plate and allowed to self-assemble into hydrogels upon incubation at 37°C. Mouse

embryonic stem cells were seeded (5,000 cells/well) on the native and grafted collagen scaffolds

one hour after gel self-assembly, and were maintained in differentiation media. In all seven

conditions were assayed.

4.2.3. Immunocytochemistry

Cells were stained immunohistochemically in 48-well plates for proteins and transcription

factors listed in table 3. Collagen gels were fixed with either 4% paraformaldehyde for 15

minutes at room temperature when staining for cytoskeletal proteins, or with 100% methanol

for 2 minutes at –20°C when staining for transcription factors. A rinse buffer comprising 1%

bovine serum albumin and 0.5% Triton in phosphate buffered saline (PBS) was used to wash

wells when staining for cytoskeletal proteins. Triton was not included in wash buffer when

staining for transcription factors. Wells were rinsed three times for 15 minutes. Normal goat

serum (10%) and bovine serum albumin (5%) in PBS were used for blocking non-specific

antibody binding. Cells were blocked with blocking solution for one hour, and incubated in

primary antibody solutions table 3 overnight at 4°C. Wells were then rinsed 3 times with wash

buffer and incubated in a 1:400 dilution of goat anti-mouse or rabbit Alexa 488 or 568 secondary

antibody (Molecular Probes/Invitrogen, Eugene, OR) for two hours. A DAPI dye was used to label

cell nuclei. Wells were washed a final time and then transferred to an inverted epifluorescence

microscope for imaging (Olympus IX 81, City, State).

Antibody	Source	Purpose	Concentration used	Species
Mouse anti-Oct4	Abcam	Pluripotent Stem Cell Marker	0.25ug/ml	Rabbit
Brachyury / Bry	Abcam	Mesoderm Marker	0.5ug/ml	Rabbit
Gata4	Millipore	Endoderm Marker	0.5ug/ml	Rabbit
Foxa2	Abcam	Endoderm Marker	0.5ug/ml	Rabbit
Nestin	Millipore	Ectoderm/Neural Stem Cell Marker	1.0ug/ml	Mouse
IgG Control		Staining Control	1.0ug/ml	Mouse/Rabbit

Table 4 Summary of Antibodies Used to Label Germ Layers

4.2.4. Quantitative Reverse transcription polymerase chain reaction (qPCR)

The fold change in mRNA expression of four genes – Oct4, Nestin, Brachyury, and Gata-4 were assayed at day 7 and day 14. Total RNA was extracted from cells cultured on various conditions using an Omega Bio-tek EZNA kit (VWR). Each washing step was repeated twice to minimize the amount of protein present in the extracted RNA. Immediately following the extraction, RNA was reverse transcribed to cDNA using a Perfecta cDNA synthesis kit (Quanta Bioscience) with included random hexamers. At least three distinct cDNA templates from seven different culture conditions for each of the two time pointswere used for qPCR with previously optimized conditions. qPCR was performed on a Roche Lightcyler system using a Perfecta SYBR Green fastMix kit (Quanta Bioscience). Each template was run in duplicate and only runs with single product peaks as observed in the melting curve cycle were tabulated. 18S was used as a

housekeeping gene. Results were normalized to gene expression of cells cultured on collagen for

the same time point. The parameters used for PCR were as follows: 10 min activation at 95 °C,

50 cycles of amplification (95°C for 5 seconds, 57 °C for 25 seconds and 72 °C for 30 seconds)

and a melting curve that went from 50 °C to 99 °C at a rate of 20 °C minute. Primers used are

listed in Table 4. A crossing threshold of 45 was used for gene expression for samples condition

in which no increase in SYBR green fluorescence intercalation was noted during PCR cycling.

Gene	Primer	Source
18S	CCCGAGCCACCTGGATAC	Jayaraman [14]
	CCAGTCGGCATCGTTTAT	
Oct4	GGAAAGCCGACAACAATGA	Parashurama [15]
	CAAGCTGATTGGCGAATGT	
Nestin	CGCTGGAACAGAGATTGGAAGG	Parashurama [15]
	GTCTCAAGGGTATTAGGCAAG	
Gata-4	CCCTACCCAGCCTACATGG	Wang [16]
	ACATATCGAGATTGGGGTGTCT	
Brachyury	GCTGGATTACATGGTCCCAAG	Wang [16]
	GGCACTTCAGAAATCGGAGGG	

Table 5 Summary of Primers Used for qPCR

4.3.   Results

4.3.1. Immunocytochemistry and qPCR Results
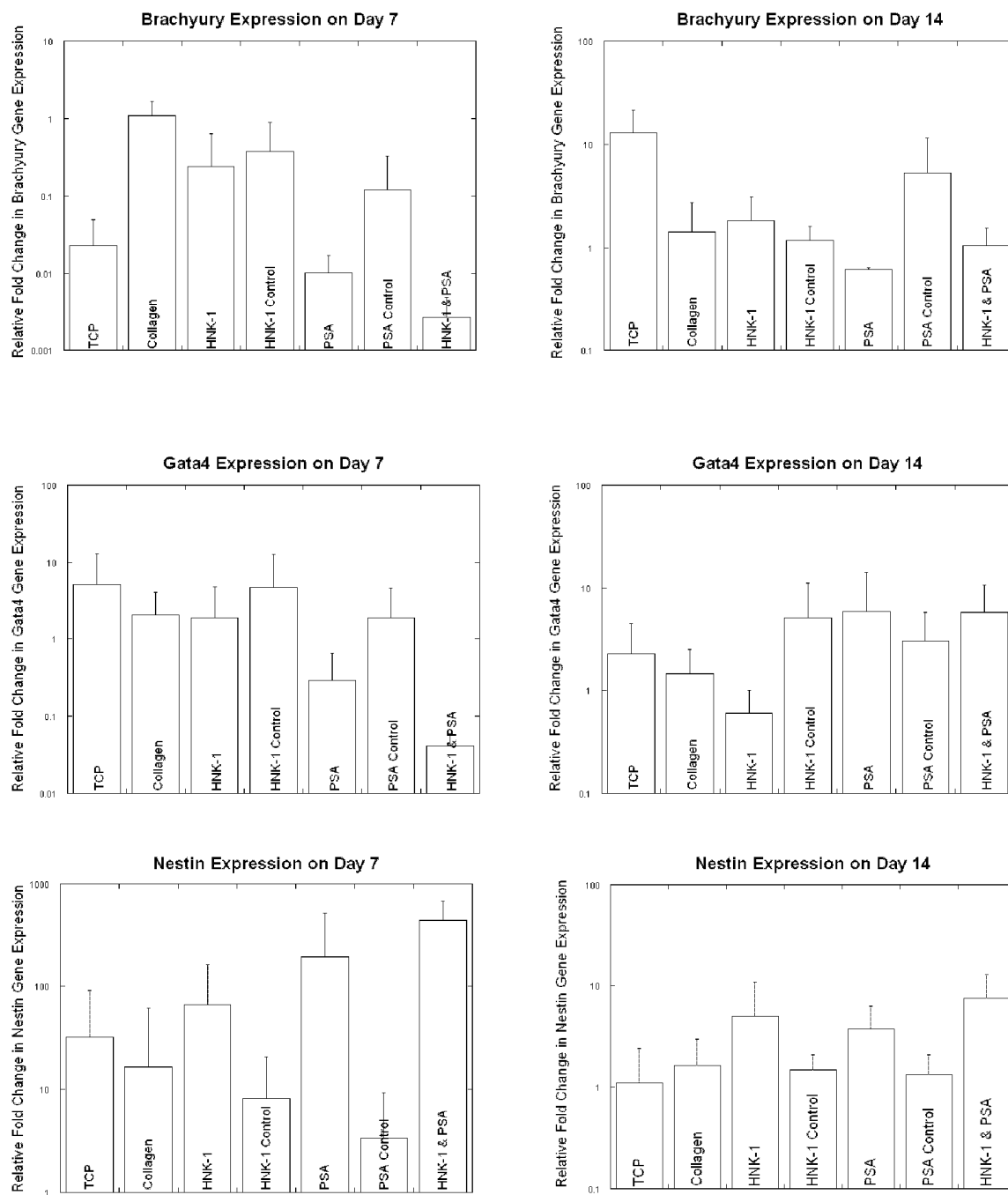
Figure 16 qPCR results for germ layer expression on days 7 and 14 post seeding

Germ layer-specific molecular marker expression in ES cells under the indicated conditions on day 7 or day 14 (Brachyury: Mesoderm, Gata4: Endoderm, Nestin: Ectoderm). Expression was normalized to ungrafted collagen conditions for each time point. On day 7 Mesoderm and Endoderm marker expression was significantly down regulated in HNK-PSA grafted collagen and PSA grafted collagen conditions while Nestin, a Neuroectoderm marker was up regulated during the same time period. There were no significant differences in gata4 expression between conditions at the day 14-time point; however, brachyury expression was unregulated in the TCP condition. On average Nestin expression was upregulated in the HNK, PSA and HNK-PSA grafted collagen conditions when compared to scrambled peptide and ungrafted control collagen and TCP conditions on day 14.

Oct4

The expression of Oct4 was significantly different between conditions 7 days post seeding cells in differentiating media (ANOVA, P<0.001). Oct 4 expression was highest in cells cultured on tissue culture plastic.. No expression of Oct 4 was noted before 45 cycles of amplification during the PCR cycling for any of the collagen or grafted collagen conditions. Tukey's post hoc analysis demonstrated Oct4 expression was significantly different in TCP cultures when compared to all other conditions (max p < 0.01). There was no difference in the Oct 4 expression between any of the grafted or native collagen conditions. Immunocytochemistry confirmed Oct4 PCR results. Oct 4 staining was present in large populations of cells cultured on Tissue culture plastic seven days post seeding. No Oct 4 staining was seen in cells cultured on collagen or grafted collagen conditions. On day 14 Oct4 gene expression was down regulated in all conditions when compared to day 7. No oct4 expression was noted in either the PCR or Immunocytochemistry assays for all conditions.

ⴰ
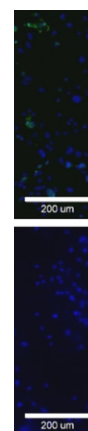
stic 7 days

ʼs following the
antly reduced

Nestin

Differentiation into neuroectoderm was evaluated via nestin expression. Nestin gene expression was measured using 18S as an internal control. Fold change in nestin gene expression is relative to that of cells on native collagen. An ANOVA comparison of the mean fold change in nestin mRNA levels from cells cultured on various conditions revealed a significant difference (P<0.003). Post hoc analysis using Tukey's test demonstrated that the HNK- PSA grafted conditions was significantly greater than all other conditions (max. p=0.018) except for PSA grafted collagen (p<0.281). Immunocytochemistry revealed nestin protein labeling in cells cultured on PSA- and HNK/PSA-grafted collagen 7 days post seeding in differentiation media. Fewer nestin positive cells were seen in tissue culture plastic and control grafted collagen conditions.

On Day 14 mouse embryonic stem cells differentiated towards a Neuroectoderm lineage within all conditions as denoted by nestin mRNA expression and Immunocytochemistry. The extent of differentiation was significantly different between grafted collagen conditions, as suggested by qPCR (p<0.047). A pair wise comparison using a Tukeys post hoc analysis did not reveal any pair wise differences. On average however, there was a 7.5 fold increase in nestin mRNA expression in the HNK-PSA, a 5 fold increase in the HNK and a 3.75 fold increase in the PSA conditions over mRNA expression from cells cultured on native collagen. In the HNK and PSA conditions positive nestin expression was noted both within spontaneously formed embryoid bodies as well as region away from embryoid bodies.   On the other hand, mES cells cultured on TCP, native collagen or control peptide grafted collagen had nestin expression primarily limited to embryoid bodies when present.
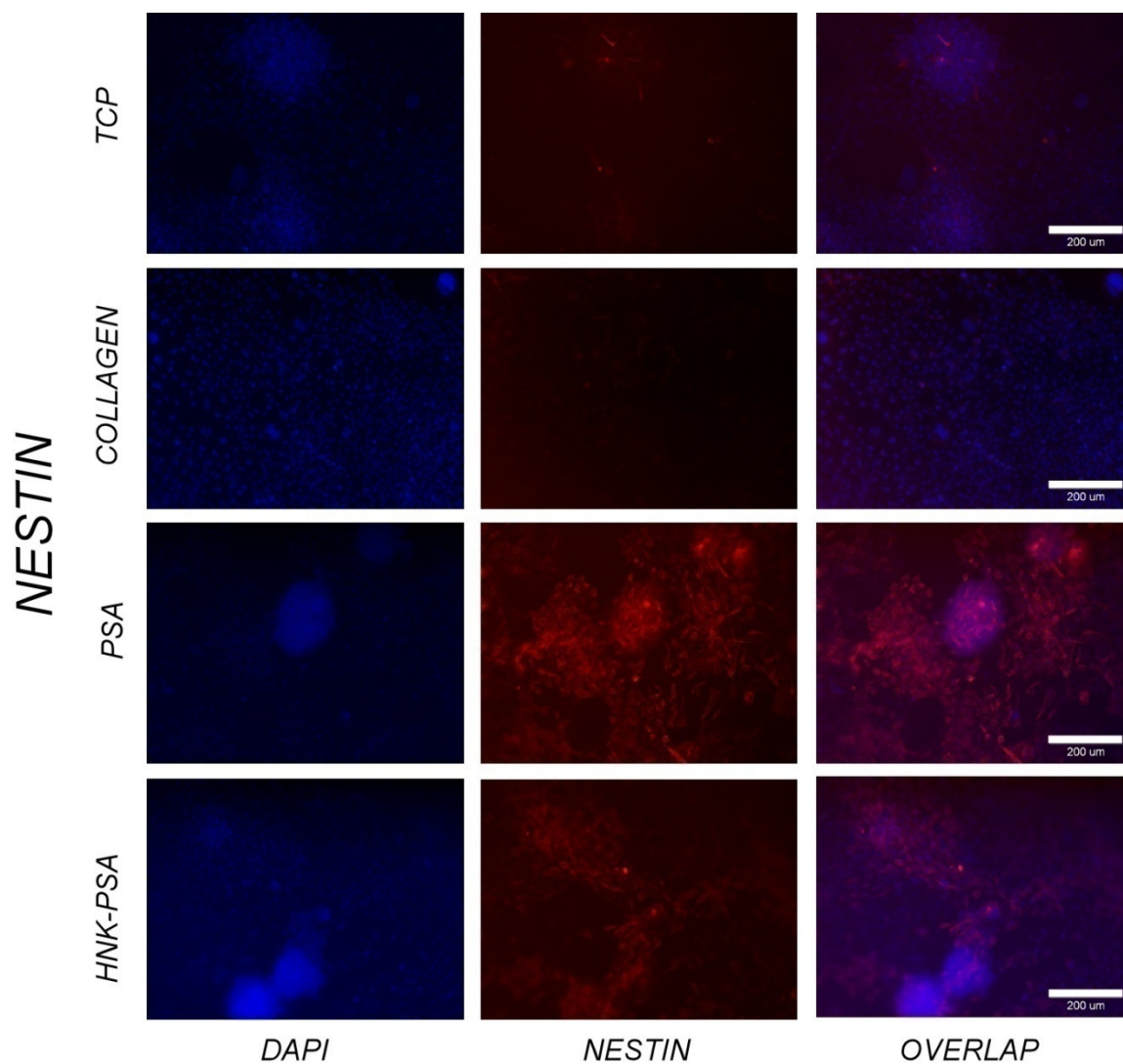
Figure 18 Nestin expression of stem cells cultured on collagen and grafted collagen conditions 7 days post seeding on gels.

Nestin, an intermediate filamentous protein was used as a marker for Neuroectoderm differentiation of stem cells. Nestin expression was up regulated in the PSA and HNK-PSA conditions 7 days following a change to differentiation medium Nestin expression in the PSA and HNK-PSA conditions were noted in both cells that aggregated into clusters as well as those away from clusters. Add different kinds of arrows and maybe a high mag image? Bar = 200 µm.
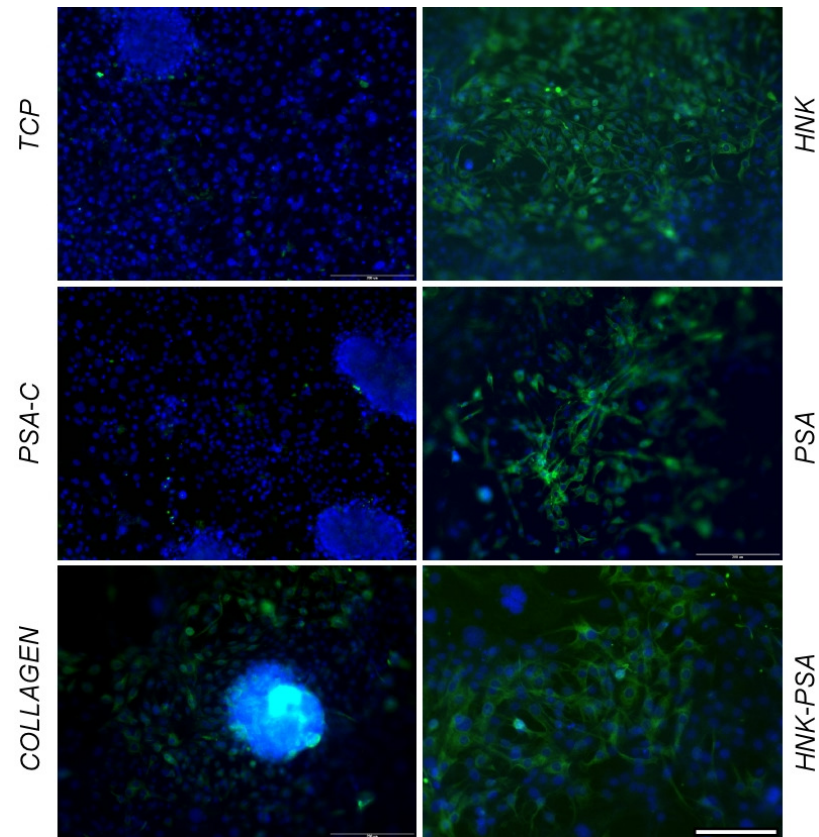
Figure 19 Nestin expression of stem cells cultured on collagen and grafted collagen conditions 14 days post seeding on gels.

Day 14 Nestin Expression in ES cells cultured on listed experimental conditions. Nestin filament expression was present in all conditions; however, nestin expression was up-regulated on collagen grafted with HNK, PSA and HNK-PSA carbohydrate mimics 14 days following the withdrawal of LIF. Extensive nestin positive staining was noted in regions away from cell clusters in the HNK, PSA and HNK-PSA carbohydrate mimic conditions. (Green: Nestin, Blue: Dapi, Bar = 200 μm.)

Brachyury

Brachyury gene expression was measured similar to Nestin expression on day 7. An ANOVA was used to compare mean fold change in brachyury gene expression between conditions, a significant difference was observed (p< 0.020). A pair wise comparison using Tukey's post hoc analysis revealed brachyury mRNA expression in cells cultured on native collagen was significantly different from all conditions except HNK and HNK control grafted collagen. The HNK-PSA grafted condition had the lowest mean level of brachyury mRNA expression. Immunocytochemistry resultson day 7 revealed brachyury preotein expression in native collagen conditions only (Figure 20). Bracuhyury mRNA expression level varied significantly between culture conditions (p=0.025). A pair wise comparison between conditions demonstrated that Brachyury mRNA expression in cells cultured on TCP for 14 days was significantly different when compared to all other conditions (max p<0.05) except HNK grafted collagen. TCP cultured stem cells had the highest average incidence of brachyury expression. This up-regulation in expression may be a result of the differentiation of cells that had previously remained undifferentiated as seen in Oct4 expression of ES cells on TCP on day 7.
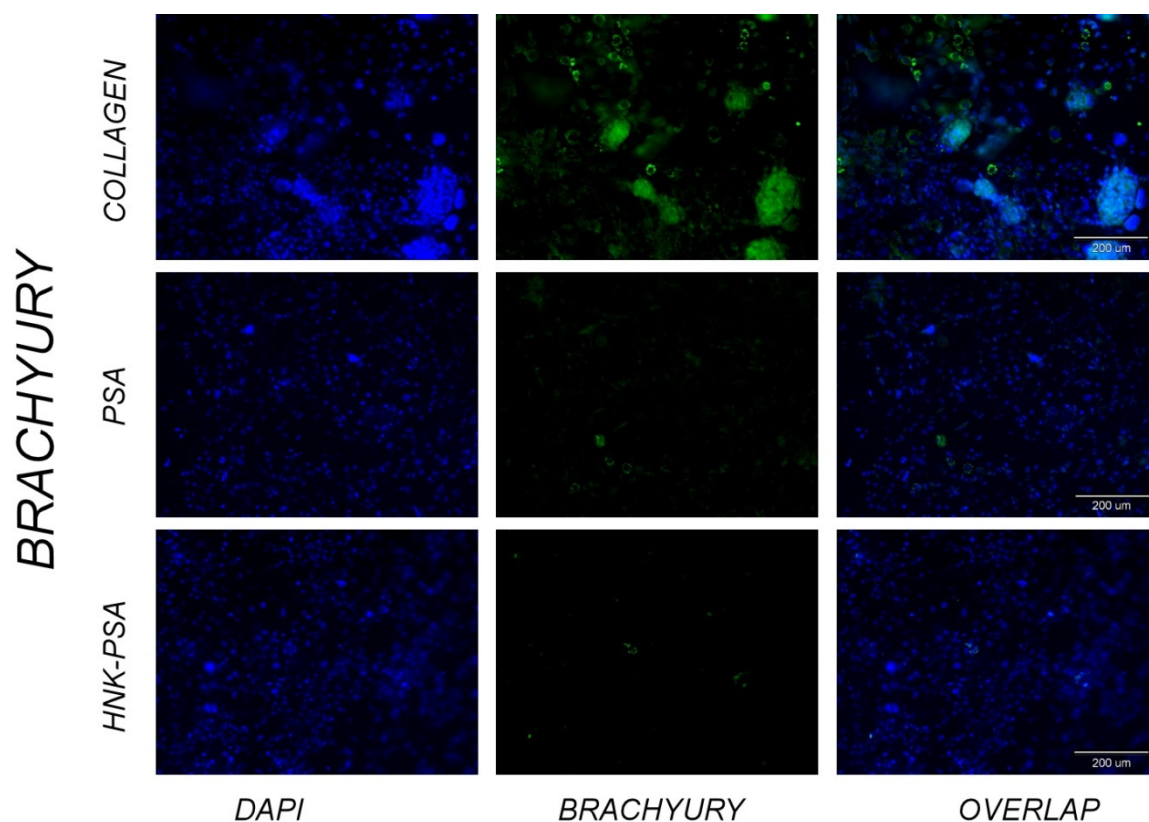
Figure 20 Brachyury Expression of Stem cells cultured on peptide grafted adn native collagen gels on day 7.

Brachyury expression of stem cells cultured on Collagen, PSA-grafted collagen and HNK-PSA grafted collagen. Samples were fixed and stained 7 days following the withdrawal of LIF. Brachyury expression was significantly reduced on carbohydrate-grafted collagen conditions. Dapi was used to label the nuclei, Bar = 200 μm.
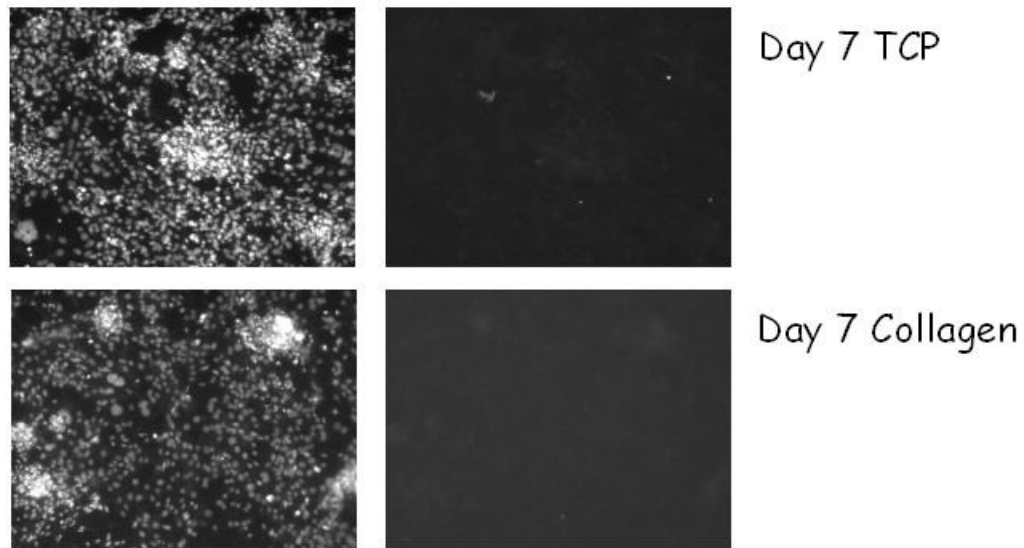
Gata4

Figure 21 Gata 4 Expression on day 7 post seeding of ES cells on substrates

Gata-4 expression (C and D) with corresponding Dapi staining (A and B). No staining was noted in any of the experimental conditions.

Gata4 gene expression was measured to assay the differentiation of mES cells towards an endodermal lineage. No significant differences in Gata4 gene expression between conditions were noted on day 7 (ANOVA, p<0.49). Immunocytochemistry did not reveal gata4 protein expression in any condition. Foxa2 expression however was noted in TCP and collagen conditions. Fox a2 was co-localized with dapi staining in the TCP condition where as in the collagen condition Foxa2 was present punctuated and perinuclear. No other condition stained positive for Foxa2 protein on day 7.
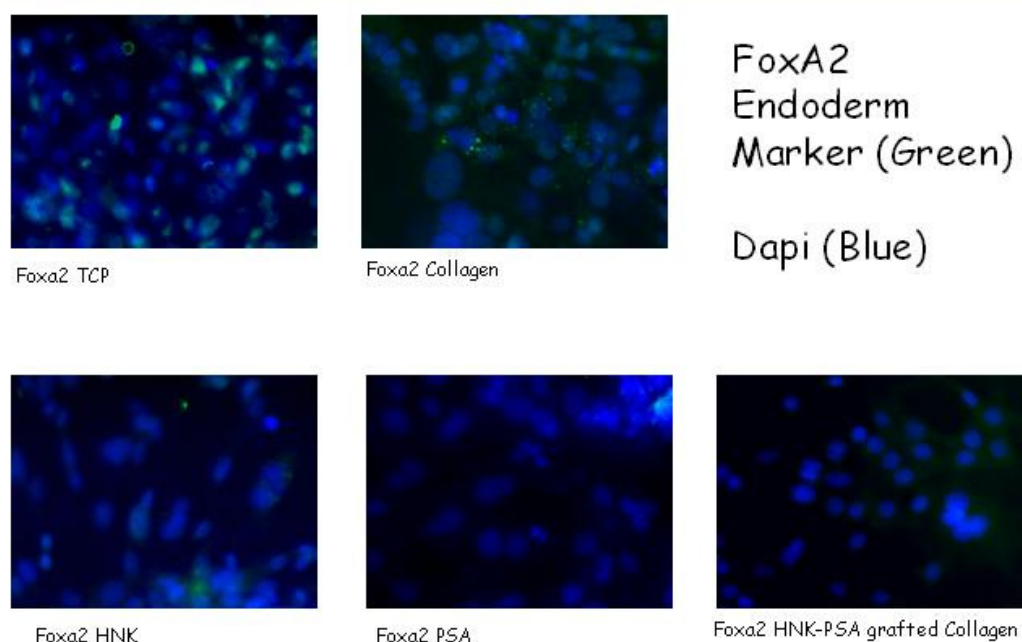
Figure 22 FoxA2 Staining for endoderm lineage cells.

FoX A2 staining was used to verify non-staining of gata4 marker. Fox a2 was co-localized with dapi staining in the TCP condition where as in the collagen condition Foxa2 was present punctuated and perinuclear. No other condition stained positive for Foxa2 protein on day 7

No significant differences were noted in the endoderm germ layer marker Gata4 on day 14.

4.4.   Discussion

Recent advances in developmental biology have demonstrated the role of carbohydrates in neural stem cell niches. Both Human natural killer-1 and polysialic acid are present in the developing CNS as well as in regions of the adult CNS that retain the ability to regenerate. Knock in and Knock out experiments targeting Human natural killer-1 and polysialic acid formation have identified their roles in spatial learning, memory formation and overall Nervous system development (REF). Encouraged by these finding and the concurrent development of peptide mimics to these otherwise complex carbohydrates we assayed the differentiation of the embryonic stem cells towards a Neuroectoderm lineage in collagen scaffolds with PSA and HNK mimitopes grafted to them.The results of this study showed preliminary evidence that both HNK and PSA carbohydrate mimics grafted to collagen can influence the differentiation of stem cells towards Neuroectoderm lineage and when used together have some synergistic effects, as indicated by nestin protein and gene expression 7 and 14 days after initial seeding of cells on scaffolds. We did not add NGF or other known chemokines or chemical reagents to the culture medium in order to exclude the action of the growth factor that would induce neural differentiation. However, the serum containing media used in these experiments may be responsible for cells differentiating towards other lineages.

4.4.1. Polysialic Acid and Human Natural Killer-1

Polysialic Acid and Human Natural Killer-1 are carbohydrates present in neural stem niches that appear to have important roles in cell-cell or cell-matrix adhesions during neurogenesis. The long, negatively charged polysialic acid chains that attach to ncam confer anti-adhesive properties on the molecule. Polysialic acid attenuates the binding properties of ncam by creating a negatively-charged hydrated sphere around the molecule, preventing ncam-binding sites from efficiently contacting by keeping ncam molecules and cell membranes physically

separated from each other (yang et al., 1992). Human natural killer-1 is a carbohydrate that was originally identified on human natural killer cells (abo and balch, 1981), hence the name. However, it is highly expressed in the developing and adult nervous systems and is generally accepted as a marker for neural crest cells. (tucker et al., 1984; vincent et al., 1983). Human natural killer-1 epitopes are present on several neural adhesion molecules, including l1, neural cell adhesion molecule, j1, and the myelin-associated glycoprotein. It is a sulfated glucuronic acid attached to n-acetyllactosamine on the non-reducing termini of glycans [9] and is involved in cell-cell and as cell-matrix interactions [5, 6] as well.

The peptide versions of these carbohydrates used in this study were identified using a phage display technology and functional studies [10, 17]. We attribute the observed effects to the structural similarity of the mimic peptides with the HNK-1 and PSA epitopes, and thus to carbohydrate–related functional effects. Lack of effects in control peptide grafted collagens is one argument in favor of peptide–related specificity. The signal transduction mechanisms underlying the functions of the HNK-1 and PSA epitope are unknown, so we can only speculate on the action of the mimics. However, given the lack of NCAMs on the surface of stem cells it suggests alternate pathways of signaling must be in play. It may be that these molecules activate non-specific integrin pathways that may play a role in regulating differentiation. Alternatively, differentiation may not be directed by these peptides but rather upon differentiation towards neuroectoderm lineage cells do these peptides become important in propagating and maintaining neuroectoderm lineage cells.

4.4.2. Collagen Vs. TCP

It should be noted that cells cultured on TCP retained pluripotent ES cell markers 7 days into differentiation protocols. Oct 4 expression was not seen in any of the collagen grafted or native

collagen conditions at that time point. Several stem cell differentiating preferences have been identified in various collagen sub types that may initiate/promote specialization of stem cells. Collagen IV induces trophoectoderm differentiation of mouse embryonic stem cells [18]. Chondrogenic differentiation of human mesenchymal stem cells is promoted in collagen type I hydrogels[19]. In addition, the physical information such as the stiffness or the mechanical compliance of the collagen matrix sensed by mES cells and those on TCP are significantly different. Collagen based substrates provide a more realistic mimic to in vivo differentiation. Traditional tissue culture plastic materials offer a substrate and surface that is physiologically removed.

4.4.3. Advantages of grafting peptides to scaffolds to direct differentiation

There are several advantages to grafting niche peptides onto scaffolds to direct differentiation of stem cells. It provides for the continuous presentation of a signaling molecule without the need for significant amounts of replenishment over time. Additionally, as more peptides and signaling cues are identified they can be easily incorporated within materials using similar techniques. Small peptide sequences derived from ECM proteins allow for a greater concentration of active groups on a surface compared to protein adsorption. All of which can be incorporated towards developing implantable biomaterials for regeneration purposes.

4.4.4. Future Implications

As stem cell replacement therapies slowly but surely become commonplace in treatments for degenerative diseases, there is an increasing need for the development of delivery materials that support the attachment, differentiation and proliferation of stem cells in vitro and in situ. Natural biomaterials inherently support proliferation and attachment of stem cells but are limited in their ability to direct differentiation. Masking these materials with directive bioactive cues may allow

for the development of biomaterial niches that offer a unique platform to study differentiation

niches in vitro and transplant the differentiation niche in vivo.

4.5.   References

1.   Yang, P., D. Major, and U. Rutishauser, *Role of charge and hydration in effects of polysialic acid on molecular interactions on and between cell membranes.* J Biol Chem, 1994. **269**(37): p. 23039-44.

2.   Yang, P., X. Yin, and U. Rutishauser, *Intercellular space is affected by the polysialic acid content of NCAM.* J Cell Biol, 1992. **116**(6): p. 1487-96.

3.   Fryer, H.J. and S. Hockfield, *The role of polysialic acid and other carbohydrate polymers in neural structural plasticity.* Curr Opin Neurobiol, 1996. **6**(1): p. 113-8.

4.   Kiss, J.Z. and G. Rougon, *Cell biology of polysialic acid.* Curr Opin Neurobiol, 1997. **7**(5): p. 640-6.

5.   Kunemund, V., et al., *The L2/HNK-1 carbohydrate of neural cell adhesion molecules is involved in cell interactions.* J Cell Biol, 1988. **106**(1): p. 213-23.

6.   Nieke, J. and M. Schachner, *Expression of the neural cell adhesion molecules L1 and N-CAM and their common carbohydrate epitope L2/HNK-1 during development and after transection of the mouse sciatic nerve.* Differentiation, 1985. **30**(2): p. 141-51.

7.   Abo, T. and C.M. Balch, *A differentiation antigen of human NK and K cells identified by a monoclonal antibody (HNK-1).* J Immunol, 1981. **127**(3): p. 1024-9.

8.   Hall, H., S. Carbonetto, and M. Schachner, *L1/HNK-1 carbohydrate- and beta 1 integrin-dependent neural cell adhesion to laminin-1.* J Neurochem, 1997. **68**(2): p. 544-53.

9.   Yu, R.K. and M. Yanagisawa, *Glycobiology of neural stem cells.* CNS Neurol Disord Drug Targets, 2006. **5**(4): p. 415-23.

10.   Simon-Haldi, M., et al., *Identification of a peptide mimic of the L2/HNK-1 carbohydrate epitope.* J Neurochem, 2002. **83**(6): p. 1380-8.

11.   Simova, O., et al., *Carbohydrate mimics promote functional recovery after peripheral nerve repair.* Ann Neurol, 2006. **60**(4): p. 430-7.

12.   Mehanna, A., et al., *Polysialic acid glycomimetics promote myelination and functional recovery after peripheral nerve injury in mice.* Brain, 2009.

13.   Monteiro, G.A., et al., *Positively and Negatively Modulating Cell Adhesion to Type I Collagen Via Peptide Grafting.* Tissue Eng Part A, 2009.

14.   Jayaraman, A., M.L. Yarmush, and C.M. Roth, *Dynamics of gene expression in rat hepatocytes under stress.* Metab Eng, 2000. **2**(3): p. 239-51.

15. Parashurama, N., et al., *Activin alters the kinetics of endoderm induction in embryonic stem cells cultured on collagen gels.* Stem Cells, 2008. **26**(2): p. 474-84.

16. Spandidos, A., et al., *A comprehensive collection of experimentally validated primers for Polymerase Chain Reaction quantitation of murine transcript abundance.* BMC Genomics, 2008. **9**: p. 633.

17. Torregrossa, P., et al., *Selection of poly-alpha 2,8-sialic acid mimotopes from a random phage peptide library and analysis of their bioactivity.* J Biol Chem, 2004. **279**(29): p. 30707-14.

18. Schenke-Layland, K., et al., *Collagen IV induces trophoectoderm differentiation of mouse embryonic stem cells.* Stem Cells, 2007. **25**(6): p. 1529-38.

19. Noth, U., et al., *Chondrogenic differentiation of human mesenchymal stem cells in collagen type I hydrogels.* J Biomed Mater Res A, 2007. **83**(3): p. 626-35.

Chapter 5

5. Conclusion and Future Applications

5.1. Regenerative Medicine and Stem Cells

The fundamental goal of regenerative medicine and tissue engineering is to replace damaged, lost or scar tissue, often seen in diseased or injured organs, with a replacement engineered biological substitute that can restore and maintain the normal functioning of the organ[1-4]. Stem cells, capable of becoming any cell within the body, are of particular interest as a potentially endless source of versatile cells for regenerative purposes[5-11]. However, the in vivo maturation of stem cells into tissues and then into organ systems that comprise living organisms, is a complex process involving coordinated physical and chemical cues to determine their lineage. The poor understanding of the in vivo microenvironments that direct the differentiation of stem cells towards specific lineages presents a major challenge to incorporating stem cells in regenerative medicine practices. To better understand the relationship between stem cells and their microenvironment, here in, we studied the role of cell adhesion to a ubiquitous ECM protein collagen, to guide the differentiation of stem cells towards neuronal lineages. We have developed an approach to alter, positively as well as negatively, the specific and non-specific adhesion of cells to collagen and assayed the differentiation of stem cells towards neuronal lineages on these scaffolds. We then extended this approach to assay the regulation of stem cell differentiation by specific neural niche carbohydrates, HNK and PSA by grafting peptide mimics of these carbohydrates onto collagen. Using these approaches we are able to manipulate the differentiation of stem cells towards ectoderm lineage cells. Combining stem cell therapies with tissue engineering approaches offers

unique opportunities to deliver in vivo as well as direct the differentiation of potentially

therapeutic stem cells.

5.2. Thesis limitations.

This thesis aims to develop and understand the role of peptide modified collagen scaffolds in

tissue engineering and stem cell differentiation. Stem cells are capable of differentiating into

cells from all three germ layers. What cell types they eventually become is directed by the

surrounding environment and signaling cues they receive.   Here in our goal was to understand

the role of cell adhesion on differentiation. To do so we developed a method that positively and

negatively attenuates the adhesion of cells to collagen, in chapter two. Fibroblast and smooth

muscle cells were used to develop this method. Fibroblast and smooth muscle cells are relatively

hardy when compared to embryonic stem cells and are often associated with a dense basement

membrane rich in collagen. The adhesion of cells to collagen was extended by adding a

ubiquitous integrin binding peptide 'RGD' to the collagen fibers, while grafting a scrambled

version of the same peptide 'RDG' was used to retard the adhesion of cells to collagen. These

approaches were then used in chapter 3 to assay the differentiation of stem cells on collagen

scaffolds of varying adhesion. Although stem cells in vivo exist in a three-dimensional matrix, in

this thesis we assayed the differentiation of stem cells on the surface of collagen scaffolds of

varying adhesion. Cells may be easily entrapped within these collagen scaffolds and a more

realistic three dimensional matrix may be realized, however, cells entrapped within collagen

scaffolds are difficult to assay using traditional experimental techniques such as

immunohistochemistry and polymerase chain reaction. For convenience purposes, stem cell

differentiation in chapter 3 was assayed using a knock in reporter for sox-1 gene expression.

Additional germ layer markers were not assayed. The rationale for omitting the assessment of

other germ layer markers was twofold; one, the stem cells line used in this chapter had been previously characterized and established by several other research groups and two, basal neural differentiating media was used in all experimental protocols in chapter 3. A more thorough and complete characterization of stem cell differentiation was carried out in chapter 4. In chapter 4 we assayed the regulation of stem cell differentiation by specific neural niche carbohydrates, human natural killer-1 and polysialic acid by grafting peptide mimics of these carbohydrates onto collagen. Again, stem cell differentiation was assayed only on the surface of these scaffolds. However, as opposed to chapter 3, in chapter 4 a complete media was used in experimental protocols. We did not add NGF or other known chemokines or chemical reagents to the culture medium in order to exclude the action of the growth factor that would induce neural differentiation.

5.3. Advantages of using peptide grafted collagen scaffolds for regenerative purposes

The advantage of using peptides grafted to collagen as scaffolds to study the differentiation of stem cells is several fold:

- Collagen is a naturally occurring ECM protein that is abundantly available and very biocompatible. It has been extensively used in the past for various clinical applications and wide-ranging clinical data is available with regard to its use in vivo.

- Grafting peptides to collagen allows for the continuous presentation of signaling cues that would otherwise need to be replenished every so often.

- Grafting peptides onto any substrate allows for a controlled presentation of the active sites to cells, by manipulating the degrees of freedom with which a peptide is attached to the surface of a structural protein.

- Several peptides can be presented simultaneously. As more and more peptides/proteins that regulate the differentiation of stem cells are identified they can be easily grafted/incorporated into this system and their effects assayed. Additionally, the synergistic and antagonistic effects of various peptide combinations can be readily deduced.

5.4. Disadvantages of using peptide grafted collagen scaffolds for regenerative purposes

Grafting peptides to collagen for regenerative therapies has its disadvantages as well:

- Collagen is a ubiquitous extracellular matrix component. Present on collagen are several adhesion and differentiating cues that may regulate the normal differentiation of stem cells. These natural cues may interfere with the peptides being used to guide the differentiation of stem cells for regenerative purposes.

- Grafting peptides on collagen presents scale up challenges. Peptides sequences can be difficult to synthesize and purify. Longer sequences present greater challenges when compared to shorter sequences. Every method presents a short coming. P*eptide coupling* makes joining large peptide segments efficiently and with sufficient yields challenging. In s*olid-phase synthesis* the challenge is application of polar supports and alternative N α-protection. In the p*rotecting groups* approach the challenge is to achieve quantitative cleavage at the end of synthesis [12]. Additionally challenges exist in

separating and purification of peptides after they are synthesized. New and alternative

approaches need to be developed before large-scale synthesis of peptides is possible.

- Chemicals used to graft peptides to collagen can alter both the peptides as well as the

  natural architecture of collagen fibers; these changes may present additional variables

  that may regulate differentiation.

- The majority of grafting techniques create a covalent bond between the peptide and the

  collagen backbone. These bonds are relatively stable and difficult for cells to digest. The

  formation of covalent bonds between peptides and the collagen backbone mitigates the

  possibility of temporal changes in peptides presented on the surface of the collagen.

- Form a clinical perspective the addition of peptides to a matrix scaffold classifies the

  scaffold as a drug. The regulatory pathways set forth by the FDA to bring a new drug to

  market are significantly more expensive when compared to that of a medical device.

5.5. Addressing Temporal and Spatial Changes in Matrix Compositions

During the normal process of development, multipotent stem cells make many cell fate decisions based on combinations of intrinsic and extrinsic signals that are regulated spatially and temporally. For example, during embryo development in vivo opposing gradients of noggin and BMP signaling persists from the dorsal side to the ventral front [13-18]. It is believed that these gradient cues are pertinent for the formation of the germ layers that give rise to specific endoderm and mesoderm cell types as well as the formation of the central cavity at the focal point. The effects of such spatial patterns of signaling cues have not been considered in these experiments. Future work with peptide-grafted matrices should incorporate these variables. One approach to study the effects of spatial patterning of signaling cues within matrices may be achieved by using microfluidic technologies[19, 20]. Using micro channels and a low Reynolds number flow controlled gradients of peptide-grafted collagen have been created. Sundararaghavan et al. used this approach to assay the migration of neurities across spatial gradients of laminin derived peptides. These techniques can be readily extended to assay the

differentiation of stem cells in spatially controlled environments. Initial experiments to recreate the environment have been outlined in Figure 23.

In addition to spatial changes in matrix signaling cues, another variable that the current work does not address are the temporal changes in matrix composition that occur during development. Temporal changes in matrix composition during development may provide a bigger challenge to replicate in vitro. These methods may include the introduction and removal of engineered cells into in vitro stem cell niches. Methods used to manipulate the matrix temporally should not interfere with the regulation pathways of differentiation. Creating an artificial extracellular matrix by the inclusion of engineered cells in the scaffold matrix that secrete signaling cues or proteins of interest when triggered [21] may provide a means to temporally alter the extracellular matrix. Triggers may be chemically, physically or even light induced. The ability to potentially present multiple signals in a combination is another advantage of using peptides grafted to proteins. This would perhaps be a more accurate simulation of stem cell niches. Furthermore, the ability to entrap cells in 3D matrices that may be cross linked to alter stiffness could be used to generate three dimensional structures to further replicate the natural spatial organization and signaling patterns.

5.6. L1 adhesion molecule and L1 over expressing embryonic stem cells.

One potential candidate that may be explored in the future is the L1 adhesion molecule. The L1 adhesion molecule is a member of the immunoglobulin superfamily shared by neural and immune system cells. In the nervous system L1 can mediate cell binding by a *homophilic* and *heterophilical* mechanism. L1 is expressed by neurons but not by astrocytes and oligodendrocytes in the CNS and by neurons and Schwann cells in the peripheral nervous system

[22-24] . L1 promotes neuronal survival, neurite outgrowth and synaptic plasticity both in vivo and in vitro [25-31]. Its role in neural stem cell proliferation and differentiation was identified by Dihné et al [32-34]. L1 coated substrates led to an approximately twofold higher number of β-tubulin^{+} neurons after differentiation when compared with PLL substrates. In addition, the number of GFAP[+] astrocytes was reduced by $\approx$33%. Furthermore, L1 has been shown to promote functional recovery in adult rats after contusion-induced spinal cord injury, although the mechanism by which L1 induced locomotor recovery is still unclear [35].

Additionally, Schachner et al. have engineered a murine embryonic stem cell line constitutively expressing L1 at all stages of neural differentiation. They have demonstrated that L1-transfected cells show decreased cell proliferation in vitro, enhanced neuronal differentiation in vitro and in vivo, and decreased astrocytic differentiation in vivo [32, 33]. L1 over expression also resulted in an increased yield of GABAergic neurons and enhanced migration of embryonic stem cell-derived neural precursor cells into lesioned striatum [32, 33] . Work to assess the therapeutic benefit of L1 over expressing stem cells in SCI mice is currently ongoing.

5.7.Conclusion

As stem cell replacement therapies slowly but surely become commonplace in treatments for degenerative diseases, there is an increasing need for the development of delivery materials that support the attachment, differentiation and proliferation of stem cells in vitro and in situ. Natural biomaterials inherently support proliferation and attachment of stem cells but are limited in their ability to direct differentiation. Masking these materials with directive bioactive cues may allow for the development of biomaterial niches that offer a unique platform to study differentiation niches in vitro and transplant the differentiation niche in vivo.

5.8.References

1.   Blelloch, R., *Regenerative medicine: short cut to cell replacement.* Nature, 2008. **455**(7213): p. 604-5.

2.   Schneider, M.D., *Regenerative medicine: Prometheus unbound.* Nature, 2004. **432**(7016): p. 451-3.

3.   Koike, N., et al., *Tissue engineering: creation of long-lasting blood vessels.* Nature, 2004. **428**(6979): p. 138-9.

4.   Chien, K.R., *Regenerative medicine and human models of human disease.* Nature, 2008. **453**(7193): p. 302-5.

5.   Song, H., et al., *Regenerative Medicine Special Feature: Interrogating functional integration between injected pluripotent stem cell-derived cells and surrogate cardiac tissue.* Proc Natl Acad Sci U S A, 2009.

6.   O'Brien, T. and F.P. Barry, *Stem cell therapy and regenerative medicine.* Mayo Clin Proc, 2009. **84**(10): p. 859-61.

7.   Nelson, T.J., et al., *Stem Cell Platforms for Regenerative Medicine.* Clin Transl Sci, 2009. **2**(3): p. 222-227.

8.   Alvarez, A., et al., *Stem cell and regenerative medicine.* Curr Stem Cell Res Ther, 2009. **4**(4): p. 287-97.

9.   Jain, K.K., *Stem cell technologies in regenerative medicine.* Expert Opin Biol Ther, 2002. **2**(7): p. 771-3.

10.   Audet, J., *Stem cell bioengineering for regenerative medicine.* Expert Opin Biol Ther, 2004. **4**(5): p. 631-44.

11.   Moody, S.A., *Stem cells: cell and developmental biology in regenerative medicine.* Biol Cell, 2005. **97**(2): p. 111.

12.   Meienhofer, J., *Why peptide synthesis continues to remain a formidable challenge.* Biopolymers, 1 Feb 2004. **20**(9): p. 1761 - 1784.

13.   Dale, L. and F.C. Wardle, *A gradient of BMP activity specifies dorsal-ventral fates in early Xenopus embryos.* Semin Cell Dev Biol, 1999. **10**(3): p. 319-26.

14.   Jones, C.M. and J.C. Smith, *Establishment of a BMP-4 morphogen gradient by long-range inhibition.* Dev Biol, 1998. **194**(1): p. 12-7.

15.   Wilson, P.A., et al., *Concentration-dependent patterning of the Xenopus ectoderm by BMP4 and its signal transducer Smad1.* Development, 1997. **124**(16): p. 3177-84.

16.   Knecht, A.K. and R.M. Harland, *Mechanisms of dorsal-ventral patterning in noggin-induced neural tissue.* Development, 1997. **124**(12): p. 2477-88.

17.   Dale, L. and C.M. Jones, *BMP signalling in early Xenopus development.* Bioessays, 1999. **21**(9): p. 751-60.

18.   Liem, K.F., Jr., T.M. Jessell, and J. Briscoe, *Regulation of the neural patterning activity of sonic hedgehog by secreted BMP inhibitors expressed by notochord and somites.* Development, 2000. **127**(22): p. 4855-66.

19.   Sundararaghavan, H.G., et al., *Neurite growth in 3D collagen gels with gradients of mechanical properties.* Biotechnol Bioeng, 2009. **102**(2): p. 632-43.

20.   Saadi, W., et al., *Generation of stable concentration gradients in 2D and 3D environments using a microfluidic ladder chamber.* Biomed Microdevices, 2007. **9**(5): p. 627-35.

21.   Marquez, Y.D., M.Y. Wang, and C.Y. Liu, *Cellular signaling in neural stem cells: implications for restorative neurosurgery.* Neurosurg Focus, 2005. **19**(3): p. E2.

22.   Lindner, J., F.G. Rathjen, and M. Schachner, *L1 mono- and polyclonal antibodies modify cell migration in early postnatal mouse cerebellum.* Nature, 1983. **305**(5933): p. 427-30.

23.   Rathjen, F.G. and M. Schachner, *Immunocytological and biochemical characterization of a new neuronal cell surface component (L1 antigen) which is involved in cell adhesion.* Embo J, 1984. **3**(1): p. 1-10.

24.   Kamiguchi, H. and F. Yoshihara, *The role of endocytic l1 trafficking in polarized adhesion and migration of nerve growth cones.* J Neurosci, 2001. **21**(23): p. 9194-203.

25.   Hoschouer, E.L., F.Q. Yin, and L.B. Jakeman, *L1 cell adhesion molecule is essential for the maintenance of hyperalgesia after spinal cord injury.* Exp Neurol, 2009. **216**(1): p. 22-34.

26.   Li, Y.L., et al., *Cell surface sialylation and fucosylation are regulated by L1 via phospholipase Cgamma and cooperate to modulate neurite outgrowth, cell survival and migration.* PLoS ONE, 2008. **3**(12): p. e3841.

27.   Shimada, T., et al., *Shootin1 interacts with actin retrograde flow and L1-CAM to promote axon outgrowth.* J Cell Biol, 2008. **181**(5): p. 817-29.

28.   Schmid, R.S., W.M. Pruitt, and P.F. Maness, *A MAP kinase-signaling pathway mediates neurite outgrowth on L1 and requires Src-dependent endocytosis.* J Neurosci, 2000. **20**(11): p. 4177-88.

29. Kamiguchi, H. and V. Lemmon, *Neural cell adhesion molecule L1: signaling pathways and growth cone motility.* J Neurosci Res, 1997. **49**(1): p. 1-8.

30. Hulley, P., M. Schachner, and H. Lubbert, *L1 neural cell adhesion molecule is a survival factor for fetal dopaminergic neurons.* J Neurosci Res, 1998. **53**(2): p. 129-34.

31. Kristiansen, L.V., et al., *Homophilic NCAM interactions interfere with L1 stimulated neurite outgrowth.* FEBS Lett, 1999. **464**(1-2): p. 30-4.

32. Chen, J., et al., *Cell adhesion molecule l1-transfected embryonic stem cells with enhanced survival support regrowth of corticospinal tract axons in mice after spinal cord injury.* J Neurotrauma, 2005. **22**(8): p. 896-906.

33. Bernreuther, C., et al., *Neural cell adhesion molecule L1-transfected embryonic stem cells promote functional recovery after excitotoxic lesion of the mouse striatum.* J Neurosci, 2006. **26**(45): p. 11532-9.

34. Dihne, M., et al., *A new role for the cell adhesion molecule L1 in neural precursor cell proliferation, differentiation, and transmitter-specific subtype generation.* J Neurosci, 2003. **23**(16): p. 6638-50.

35. Roonprapunt, C., et al., *Soluble cell adhesion molecule L1-Fc promotes locomotor recovery in rats after spinal cord injury.* J Neurotrauma, 2003. **20**(9): p. 871-82.

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Sundararaghavan, H.G., et al., Neurite growth in 3D collagen gels with gradients of mechanical properties. Biotechnol Bioeng, 2009. 102(2): p. 632-43.

Sundararaghavan, H.G., et al., Genipin-induced changes in collagen gels: correlation of mechanical properties to fluorescence. J Biomed Mater Res A, 2008. 87(2): p. 308-20.