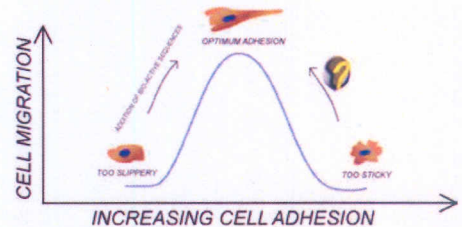# Optimizing Adhesion to Enhance Cell Migration in Collagen Gels

Monteiro, G., **Sundararaghavan, H. Fernandes, A. and Shreiber, D.**
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Introduction: Cell migration is a ubiquitous process that is of fundamental importance in tissue morphogenesis, 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. Several studies have determined that the specificity and adhesivity of collagen can be enhanced by the covalent grafting of bioactive peptides onto collagen. However, in some cases cell adhesion is so permissive the substrate is 'too sticky' for a cell to be able to migrate. Herein we show that grafting GRDGS, a non-adhesive peptide control for GRGDS, decreases fibroblast 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.

Methods: Two peptide sequences – GRGDS, which includes the cell-binding domain RGD, and its scrambled, non-adhesive version GRDGS (RDG) were grafted onto collagen using EDC. Peptides were grafted to soluble collagen, which was then dialyzed to remove unbound peptides. Grafting efficiency was between 50-60%. Rat dermal fibroblast (RDF) adhesion, matrix reorganization, and migration were assayed with three grafted concentrations of RGD or RDG – High: 0.14mg/ml, Medium: 0.07mg/ml and Low: 0.035mg/ml and native 2.0mg/ml collagen gels using methods detailed below.

Adhesion Assay: RDFs (50K/ml) that constitutively express GFP were seeded on collagen gels and allowed to attach for 3hrs. Following rinsing, the number of cells were counted under epifluorescent microscopy.

Matrix Compaction Assay: Cell traction was indirectly assayed by measuring compaction of free-floating, disc-shaped collagen gels seeded with 50Kcells/ml over a period of 6 days. Compacting gels were imaged under bright field and disc cross-sectional area was measured every 24 hours.

Cell Migration Assay: RDF migration was assayed on and in grafted and ungrafted collagen gels with computer-controlled microscopy. Selected fields were imaged every 10-15 minutes for 12-14 hours. Migration coefficients were determined using a persistence random walk model from two dimensional spatial cell tracks determined by Image Pro Plus.

Results and Discussion: 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. We demonstrated that this could 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. From a biomaterials perspective, the inclusion of soluble factors to affect cell adhesion – which often entails complex, drug-releasing materials or transfection of cells – can be avoided. Incorporating non-adhesive peptide sequences on to collagen is a simple, yet elegant means of manipulating traction mediated behavior.
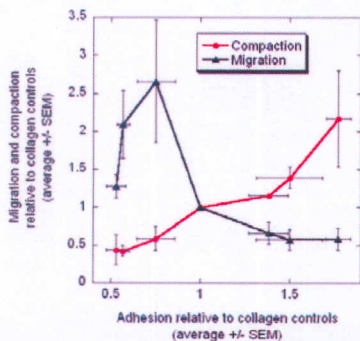
Three Dimensional Scaffolds for Embryonic Stem Cell Differentiation and Delivery.

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Introduction: Traditional 2D tissue culture (TC) methods have demonstrated that adhesive cues from extracellular matrix (ECM) molecules significantly influence the phenotypic behavior of cells. Although they provide models for investigating aspects of ECM-cell interaction, they do not replicate the 3D environment experienced by embryonic stem cells (ESCs), which is critical when studying embryoid body (EB) formation and differentiation. Previously, we demonstrated that the growth and size of EBs was significantly different in a 3D collagen scaffold vs. on that same scaffold [1]. In this study we have further investigated the role of 3D matrix stiffness in differentiation of embryonic stem cells into nestin positive cells, which is a marker of precursor cells capable of differentiating into ectodermal lineage specifically neurons. We believe that the 3D cues presented by a biomaterial scaffold to resident ESCs - specifically the mechanical stiffness and nature and density of cell adhesion binding sites - combine direct differentiation into specific lineages.

Methods: Day 2 murine EBs, cultured using standard hanging drop techniques, were seeded in free floating collagen scaffolds. The mechanical stiffness of the collagen scaffolds were altered using genipin, a cell tolerant crosslinking agent that has been previously characterized in our lab [2]. Collagen scaffolds were incubated in 0mM, 0.1mM, 0.5mM and 1.0mM of genipin for 12 hours. Following incubation in genipin, collagen scaffolds were washed and EBs were allowed to differentiate in standard differentiation media for 11days without the addition of any growth factors. Media was changed every 2 days. On day 11 EBs were fixed, cryosectioned and immunolabeled for the presence of nestin, an intermediate filament protein, that marks progenitor cells of the CNS [3].
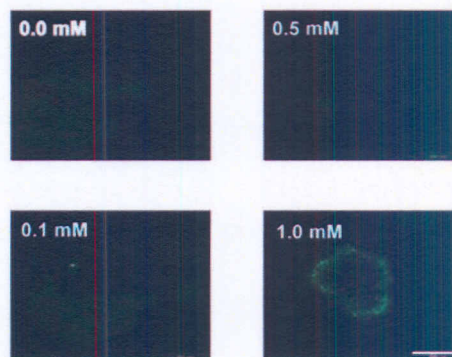

Figure 1: 20 micron sections of embryoid bodies seeded in collagen gels of varying stiffness. Collagen gels were cross-linked with 0mM, 0.1mM, 0.5mM and 1mM genipin. Embryoid bodies seeded in collagen scaffolds of 1mM Genipin showed the highest incidence of nestin positive cells. Scale Bar: 200 microns

Results: EBs cultured in collagen scaffolds with the 1mM genipin showed the highest incidence of nestin positive cells. EBs cultured in collagen scaffolds that were cross linked with 0.5mM genipin had a smaller less organized population of nestin positive cells, while, EBs cultured in 0mM and 0.1mM genipin had no cells that were nestin positive.

Discussion: The results indicate that the simple manipulation of the mechanical properties of collagen gels with genipin goes a long way in influencing the differentiation of ESCs within EBs into nestin positive cells. These nestin positive cells can be selectively harvested and differentiate into a number of cells types, including neural and pancreatic progenitors.

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GUIDING STEM CELL DIFFERENTIATION INTO NEURAL LINEAGES WITH TUNABLE COLLAGEN BIOMATERIALS

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INTRODUCTION

The long-term objective of this research is to develop tunable collagen-based biomaterial scaffolds for directed stem cell differentiation into neural lineages to aid in CNS diseases and trauma. Type I collagen is a ubiquitous protein that provides mechanostructural and ligand-induced biochemical cues to cells that attach to the protein via integrin receptors. Previous studies have demonstrated that the mechanical properties of a substrate or tissue can be an important regulator of stem cell differentiation. For example, the mechanical properties polyacrylamide gels can be tuned to induce neural differentiation from stem cells [1, 2]. Mesenchymal stem cells (MSCs) cultured on ployacrylamide gels with low elastic modulus (0.1--1 kPa) resulted in a neural like population. MSCs on 10-fold stiffer matrices that mimic striated muscle elasticity ($E_{muscle} \sim 8$--17 kPa) lead to spindle-shaped cells similar in shape to myoblasts. Still stiffer gels (25--40 kPa) resulted in osetoblast differentiation. Based on these observations, collagen gels may provide an ideal material for differentiation into neural lineages because of their low compliance.

However, collagen also presents adhesive ligands that direct stem cells to other lineages. In this study we aim to determine if the mechanical cues provided by collagen gels can be augmented by covalently grafting cues to the collagen backbone that are specific for neural differentiation. We have previously demonstrated that we can alter the ahesivity of collagen without altering the overall mechanical properties of the scaffold by grafting bioactive peptide sequences from matrix proteins such as fibronectin and laminin [3,4]. Herein, we aim to determine if the simple functionalization of collagen scaffolds with peptide mimics of carbohydrates known to influence neural differentiation – HNK-1 (Human Natural Killer-1) and PSA (Polysialic Acid) – to improve the efficacy of the collagen gels as substrates for differentiation. HNK-1 and PSA are carbohydrates present on the surface of cells as well as in the ECM of developing nervous systems. Both carbohydrates are known to participate in cell-cell and cell-substrate interactions in the development of the nervous system[5, 6]. Previous research has identified and characterized specific peptide sequences that mimic the function of these carbohydrates in vivo [7]. We used these peptide sequences to alter the bioactivity of collagen while preserving its mechanical properties.

METHODS

Peptide mimics of HNK-1, PSA, or control, scrambled versions, were grafted on to soluble collagen using a heterobifunctional coupling agent, 1-ethyl-3-(3-Dimethylaminopropyl) carbodiimide (EDC). EDC activates the carboxylic group of the peptide, which in turn is covalently bound to the collagen backbone via nucleophilic attack. We have previously shown that the grafting of peptides does not alter the mechanical properties of the collagen post self-assembly [3]. Peptides were grafted to soluble collagen, which was then dialyzed to remove ungrafted peptides and reconstituted with 0.02N acetic acid. Grafting efficiency was between 50-60% as determined by including fluorescently tagged peptides and calibrating fluorescence to a standard curve. Mouse embryonic stem (mES) cell differentiation into the primary germ layers was assayed with the peptide-grafted collagen, untreated collagen , and tissue culture plastic (TCP). mES cells were cultured on the surface of collagen gels grafted with HNK-1, PSA and HNK-1 and PSA control peptides for 14 days. Immunocytochemistry was performed to identify differentiation into endodermal (anti-Foxa2) or neuroectodermal (anti-nestin) lineages. Nestin staining was confirmed with quantitative real-time PCR (QPCR).

RESULTS

Differentiation was evaluated immunocytochemically after 14 days in culture. Foxa2, a member of the forkhead transcription family that is an early endoderm marker, was expressed most prominently in mES cells grown on TCP (Fig 1). Nestin, a filamentous protein present in ectodermal lineage cells was most prevalent on cells cultured on collagen grafted with PSA (Fig 2). Cells cultured on HNK-1 grafted collagen had fewer Foxa2 positive cells when compared to TCP and PSA grafted collagen. However, the extent of nestin positive cells was also reduced.
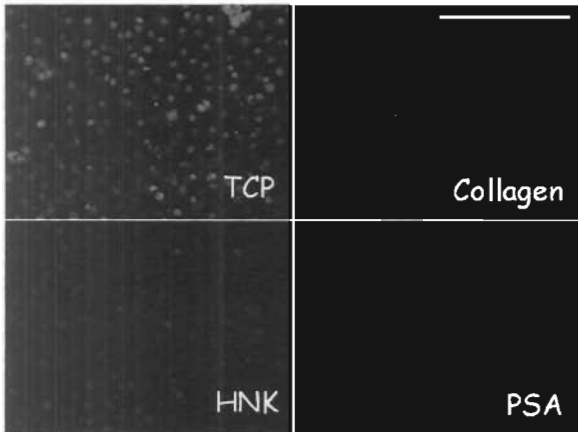

Figure 1: Representative images of mouse embryonic stem cells (mES), differentiated on collagen gels grafted with varying peptides. mES stained with Foxa2 (Green) , an early marker for endodermal differentiation and Dapi (blue). Scale bar = 200μm
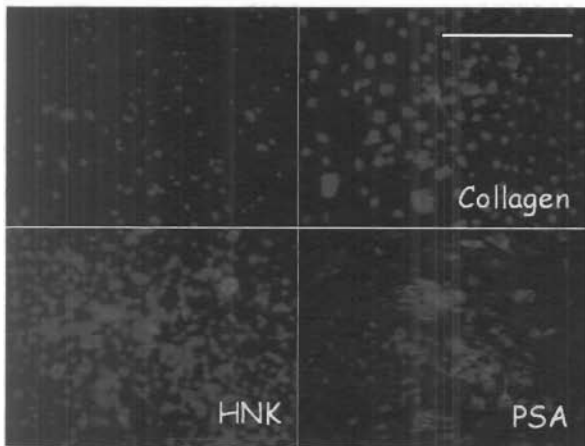

Figure 2: mES cells stained with nestin (Red), a filamentous protein present in neuroectoderm lineage cells. Nuclei are counterstained with Dapi (blue).

PCR results for nestin gene expression on day 14 correlate with the ICC results (Fig 3). There is an increase in nestin gene expression in both the PSA and HNK-1 grafted collagen conditions when compared to the control peptide, untreated collagen, and TCP conditions. The increase in expression and staining for untreated collagen vs. TCP is likely indicative of the response to the mechanical properties of the compliant gel vs. the stiff plastic. All PCR data is
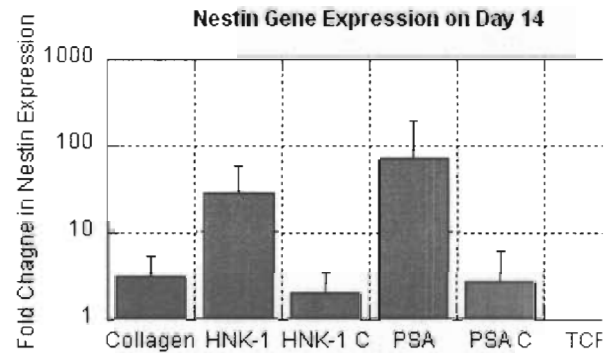

Figure 3: Fold change in nestin gene expression was greatest for the PSA grafted collagen conditions on

normalized to 18S expression, as a housekeeping gene, and expressed as folds change over the TCP condition on day 14 (N=4).

CONCLUSIONS

Although the mechanical properties of a substrate have been suggested to be potent regulators of differentiation, the adhesive ligand through which a cell senses those mechanical properties and receives biochemical signaling is also a primary controlling factor. Immunocytochemistry and PCR results indicate that the simple functionalization of collagen scaffolds with HNK-1 and PSA peptide mimics can dramatically improve the ability of the biomaterials to dictate cell fate. For neural differentiation, the ability to tune higher order interactions with biomaterials is especially critical, because of the expansive number terminally differentiated states, such as astrocytes, oligodendrocytes, or one of the vast specific neuronal types. As the specificity of differentiating factors are discovered, these molecules can be functionalized to collagen to provide a highly versatile differentiating material.

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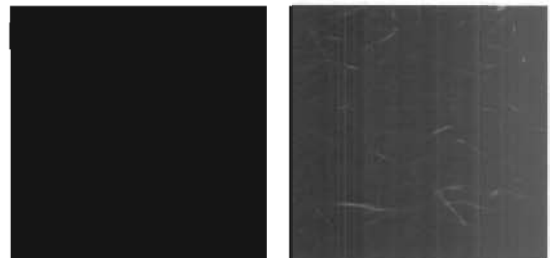# Directed Stem Cell Differentiation in Tunable Collagen Gels

Monteiro, G., Sundararaghavan, H. Fernandes, A. and Shreiber, D.
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Introduction: The long-term objective of this research is to develop a tunable biomaterial scaffold for directed stem cell differentiation into neural lineages to aid in spinal cord regeneration, post trauma. This study focuses on understanding the relationship between the biophysical properties of stem cells and their three-dimensional matrix environment. We aim to determine the matrix adhesion and stiffness that preferentially differentiates mouse embryonic stem cells (ESCs) into neural-ectoderm lineages. The control of stem cell differentiation via biomaterial mechanostructural and adhesive properties presents intriguing and exciting possibilities for regenerative therapies following spinal cord injury, CNS diseases and disorders, and other tissue engineering applications. A range of soluble factors, extracellular matrix proteins, mechanical loading or cell-cell mediated interactions may encode signals in the microenvironment surrounding stem cells by integral membrane proteins. Extracellular matrix proteins and their principal cell surface receptors, integrins, are known to transduce adhesion and mechanical signals primarily through extracellular regulated kinases. Identifying an optimal three-dimensional tissue culture environment for directed differentiation will lead to development of bioreactors for large-scale cultures and generation of large, uniform populations of cells.

Methods: In order to study the effect of the matrix microenvironment on stem cell differentiation, we cultured ESCs in a range of mechanical properties and adhesivities. Stiffness was modulated using genipin. Genipin is a naturally occurring crosslinking agent with a relatively short incubation period and low cell cytotoxicity. Collagen gels were crosslinked with 0, 1 and 5 mM genipin for 12 hours after which 100 µl of a 50K cell/ml solution was added to each well. Adhesivity of the collagen gels was modulated using a peptide mimic of a carbohydrate (HNK1) known to regulate ESC differentiation into neural lineages. A scrambled version of the same peptide was used to retard the adhesion of cells. 1,2, ethyl dimethyl carbodiimide (EDC), a hetro-bi-functional coupling agent activates the carboxylic group of peptides which are then covalently bound via nucleophilic attack to primary amines on collagen. The concentration of peptides used to modulate collagen gels ranged from 0 mg/ml to 0.07mg/ml of collagen. mESCs were cultured in modulated collagen scaffolds for 7,11 and 14 days after which the extent of differentiation was assayed using immunohistochemistry and qtPCR techniques.

Grafting HNK peptides to collagen does not alter the micro-structure within the gel. A) Fl-peptide grafted collagen gel. B) Fl-collagen gels

Results and Discussion: ESCs cultured on collagen gels with the no crosslinking showed the highest incidence of nestin positive cells for the stiffness conditions. ESCs cultured on HNK grafted collagen showed area maps with the most nestin staining. The results indicate that the simple manipulation of the mechanical properties of collagen gels with genipin or adhesive peptides goes a long way in influencing the differentiation of ESCs into nestin positive cells. These nestin positive cells can be selectively harvested and differentiate into a number of cells types, including neural and pancreatic progenitors.