L1-GUIDED DIFFERENTIATION OF EMBRYONIC STEM CELLS TOWARDS NEURAL LINEAGES

by

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ABSTRACT OF THE THESIS

L1-guided differentiation of embryonic stem cells towards neural lineages

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Embryonic stem cells are pluripotent cells that have the ability to differentiate into cell lineages from all three germ layers. However, the use of stem cells in therapeutics relies on the ability to control their differentiation. Studies have shown that implantation of undifferentiated ES cells into an injury site leads to their spontaneous differentiation and potential tumor formation. One method to control stem cell differentiation is through the design of biomaterials that mimic the natural microenvironment during development. Biomaterials can provide a microenvironment in which host as well as replacement therapeutic cells can reside. Controlling this microenvironment provides opportunities to present specific physical and soluble cues that control cell and tissue fate. Herein, we conjugate the cell adhesion molecule L1 to type I collagen to allow for its sustained, physiologically relevant presentation. L1 is a member of the immunoglobulin superfamily shared by neural and immune cells and has been shown to promote neurite extension as well as functional recovery in adult rats after contusion-induced spinal cord injury. In this study, we will investigate the role of L1 on mouse embryonic stem cells. We will assay the effects of L1 presentation on cell adhesion, proliferation, and most

importantly differentiation of embryonic stem cells (mESCs). As L1 has a homophilic binding domain, we will study the effects of using a genetically modified mESCs that overexpress L1 in combination with our L1-grafted biomaterial. Collectively, these studies will provide greater insight into the role of designing materials to guide the differentiation of stem cells. These materials may be used as delivery mechanisms for stem cell therapeutics or scaffolds on which ones own stem cells can differentiate towards a particular required cell type or lineage.

Acknowledgement

This thesis is not complete without me thanking everyone that helped me in the past 2 years. It's the great teamwork that made this work possible. "*Teamwork is the ability to direct individual accomplishments towards organizational objectives. It is the fuel that allows common people to attain uncommon results".* In a way we are very similar to stem cells. A single stem cell might not have the ability to cure any disease, but when a group of cells work in concert, the potential is unlimited. Teamwork is a learned technique and having the fortune to join this lab, I think I learned it well. Dr. Shreiber constantly encouraged everyone in the lab to work with each other and share our research progress, and it became a habit to provide every possible resource we could to help in each other's research. Sometimes it is easy to lose track and forget why you are doing what you are doing. But during times like that I'd remember the first thing Dr. Shreiber said to me during our first meeting: "it's not about what you do but the mindset to finish what you are doing". We might not be so fortunate to be working on something fun and interesting all the time, but times like that provide us with the opportunity to practice this mindset. Thank you, Dr. Shreiber, for making me a better scientist and a better person.

And here I would like to thank my parents, for their support in every possible way. They always believe that I can accomplish anything that I set my mind to do, even when some times I didn't believe in myself. My father, who taught me to bravely pursue true knowledge and never stop learning. His accomplishment will always be the highest standard to me and I'll spend my life trying to meet it. My mom, who taught me to love what I do and to never give up, carried me forward during some of the hardest times during research. And lastly I'd like to thank my wife Maggie. Being a researcher means many long hours in the lab and not being able to spend time with you. But you stayed with me and gave me strength and comforts whenever I needed them the most. Words fail to express my gratitude for everything you've done for me.

Dedication

Brave & wisdom
Passion & patience

To my parents, without your knowledge, wisdom, and guidance, I would not have the goals I have to strive for and be the best to reach my dreams

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Chapter 1

1. Introduction:

1.1. Biomaterials and Embryonic Stem Cell Differentiation

Biomaterials are used in tissue engineering as scaffolds to facilitate tissue regeneration and wound repair. Frequently, ubiquitous extracellular matrix (ECM) proteins molecules such as collagen or hyaluronic acid have been used to create porous structures that act as delivery mechanisms or scaffolds for therapeutic cells such as stem cells [1]. However, these materials may not have specialized or specific cues that can work in concert with stem cells to provide accelerated healing or regeneration of lost or damaged tissues. Combining stem cells with biomaterials that include directive cues may potentially provide a platform to deliver therapeutic stem cells and have them differentiate towards site-specific lineages required for repair. These materials may be effective in treating injuries/diseases such as stroke or spinal cord injury (SCI), where huge numbers of neurons and glial cells are lost and cannot be regenerated as neurons are post-mitotic and cannot be endogenously replaced.

Being able to control the differentiation of implanted stem cells is important not only to provide a road map for regeneration but also to limit and potentially minimize spontaneous differentiation and tumorgenesis [2]. One way in which biomaterials can be used to control the differentiation of stem cells is by including within these materials physical or chemical cues that are known to guide the differentiation of stem cells towards specific cell and tissue fates. Our broad long-term objective is to design biomaterials that can be used to control the differentiation of stem cells towards specific

lineages *in vivo*. In particular, we would like to develop implantable biomaterials that will guide the differentiation of embryonic stem cells toward neural lineages.

1.2. L1 Cell Adhesion Molecules

Among the many differentiating cues that have been identified, L1 has shown some promising results. L1 is a single pass type I transmembrane protein of the immunoglobulin (Ig) superfamily. The structure of L1 contains six Ig repeats followed by five fibronectin-like (FN) repeats (Figure 1). L1 can interact with itself (homophilic) but also with a variety of heterophilic ligands such as integrin, CD9 and other members of the neural cell adhesion family. It is abundantly expressed by neurons in the central nervous system (CNS) and Schwann cells in the peripheral nervous system (PNS), but is absent from astrocytes and oligodendrocytes [3-6]. L1 is a potent promoter of neurite outgrowth and elicits specific growth cone behavior. L1 interacts with the actin cytoskeleton via an ankyrin linkage and promotes specific distribution of F-actin within the growth cone. Study have shown that mutations in the L1 gene in human results in abnormalities in the development of the corticospinal tract and corpus callosum [7]. Some roles of L1 in neural stem cell proliferation and differentiation were identified by Dihne et al [8-10]. L1-coated

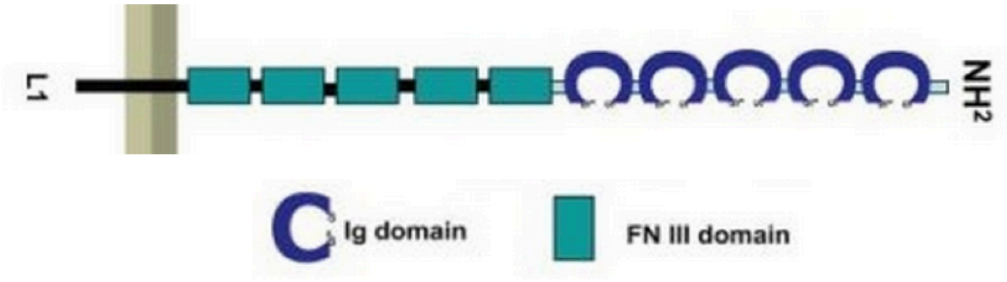

Figure 1: Structure of L1

L1 is a single pass transmembrane protein of the Ig superfamily, the structure of L1 consist of six Ig repeats followed by five fibronectin-like repeats

substrates led to an approximately two fold higher number of β-tubulin+ neurons after differentiation when compared to poly-lysine (PLL) substrates. In addition, the number of GFAP astrocytes was reduced by 33%. In this study, we will investigate the role of L1 in the differentiation of mouse embryonic stem cells (mESCs) cultured on our biomaterials.

1.3. L1 Peptide Mimics and L1-Overexpressing ES Cells

L1 peptide mimics and L1 overexpressing stem cells have been developed in the laboratory of Dr. Melitta Schachner. Using phage display technology they identified peptide sequences that have high binding activity for L1, and are functionally active in that they can either block or enhance the functions of L1. Peptides have the added advantage of being cost effective and easily mass produced. Additionally, functional groups may be added to the end of peptides to facilitate their attachment to various substrates and materials.

Using plasmid transfection technologies, Dr. Schachner's lab also developed an L1-overexpressing embryonic stem cell line that constitutively expresses L1. Recent findings show that the L1-overexpressing stem cells increased the number of surviving cells, enhanced neuronal differentiation *in vivo* and *in vitro*, and reduced glial differentiation compared to the parental stem cells [8, 11]. L1 overexpression also resulted in an increased yield of GABAergic neurons and enhanced migration of embryonic stem cell-derived neural precursor cells into leisioned striatum [8, 11].

1.4. Peptide Grafted Type I Bovine Collagen

Collagen is a biomaterial that is readily available and has been extensively used in our laboratory. Soluble type 1 bovine collagen is extremely versatile and can self-assemble into a fibrous hydrogel. It can be electrospun into fibers, freeze-dried into a porous

sponge, and cross-linked with a variety of techniques. In previous studies, we have demonstrated that grafting peptide sequences to collagen backbone does not significantly alter its structural capacity but allows control over adhesion-mediated behavior of cells [12].

Using similar methods, we believe we can conjugate L1 peptides onto the collagen structure and use these materials to guide the differentiation of stem cell. We hypothesize that L1 grafted collagen can be used to direct the differentiation of stem cells. Further, L1 peptide mimics grafted onto collagen will have a synergistic effect in directing the differentiation of L1 overexpressing stem cells towards neural lineages. Specifically, we will assay the differentiation of parental stem cells on collagen substrates with and without L1 peptides and compare them to the differentiation of L1 overexpressing cells towards neural lineages.

Chapter 2

2. Methods and Materials

Nomenclature	**Cell Type**
P-ES	Parental embryonic stem cells
L1-ES	L1-overexpressing embryonic stem cells
	Collagen Condition
L	L1-grafted collagen
S	L1-scramble-grafted collagen
C	Native collagen
x	Cultured on (e.g. P-ESxL = Parental embryonic stem cells cultured on L1 collagen)

Table1: Nomenclature

2.1. Cell Culture

Two embryonic stem cell lines, Parental Stem cells (P-ES) and L1-overexpressing stem cells (L1-ES), were cultured and assayed for the differentiation towards neural lineage cell types. Both cell lines were a gift from Dr. Schachner. Briefly, cell lines were initiated for culture by thawing an aliquot of cells and centrifuging at 2,000 rpm for 2 minutes at 4°C. The pellet of cells was suspended in ES media (Knock-Out Dulbecco's modified Eagle's medium (KO-DMEM) supplemented with Knockout Serum (16% v/v), L-glutamine (1% v/v), penicillin and streptomycin (0.1% v/v), Sigma, St. Louis, MO), and ESGRO (0.025% v/v)). Cells were plated on gelatin-coated T25 flasks with 4 ml of the same media with 4 μL of 2% B-mercaptoethanol (Sigma) and placed in a humidified CO_2 incubator at 37°C. Media was aspirated and replaced every two days. When flasks

reached 70-85% confluence, flasks were re-passaged or harvested. Following media aspiration and washing with 1 ml Phosphate buffer solution (PBS; Gibco, Grand Island, NY), 1 ml of 0.5% Trypsin EDTA (Sigma-Aldrich, St. Louis, MO) was added. Cells were detached following incubation for 3 minutes, resulting in a single cell suspension. Trypsin was then neutralized with 1 ml of complete media. The harvested cells were then re-plated on gelatin-coated flasks at ratios of 1:10. The remainders of the cells from a flask were used for experimental work. All experiments were performed with cells from passages that ranged between 4 and 15.

2.2. Conjugation of Peptides to Collagen

The L1 peptide sequence (ELEDITIFNSSTVLVRWRPVDKKC) and a scrambled version of the peptide (LTWPDIVTCLNRVSFREKVKIEDS) with equal molecular weight and peptide length 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) carbodimide (EDC, Sigma), was used to activate the carboxylic group of the peptides by mixing 1 ml of 1M solution in MES buffer (ph 2-4, Sigma) with 2 mg of peptide for ten minutes at 37°C. The solution was mixed carefully with 5 ml of Collagen suspension (3mg/ml) (Cat #C857, acid extracted from calf skin with > 95% purity; Elastin Products, Owensville, MO) in 0.02 N acetic acid at 4°C overnight. The solution was dialyzed against 0.02 N acetic acid using snakeskin dialysis tubing with a 10kDA molecular weight cutoff (Pierce Biotechnology, Rockford, IL) to remove unconjugated peptides. After 12 hours of dialysis, the solution was transferred to a 15 ml tube and placed in a lyophilizer at -150°C and 50 mTorr to

remove all water. The peptide-grafted collagen was re-suspended to 3mg/ml using 5 ml 0.02N acetic acid and mixed overnight at 4°C.

2.3. Collagen Gel Preparation

Type 1 collagen gels were prepared by mixing 140 µL 0.1 N NaOH (Sigma), 100 µL 10X Minimum Essential Medium (Sigma), 62 µL M199 (Invitrogen, Carsbad, CA), 20 µL 1M HEPES buffer (Sigma), 10 µL L-glutamine, and 1 µL penicillin and streptomycin from a stock solution of 5,000 units of penicillin and 5,000 mg streptomycin/ml in 0.85% saline (Sigma) with 667µL collagen (L1, L1 scramble, or native) to make a 2.0mg/ml collagen solution. 50 µL of collagen solution were placed in each well of a 24-well plate and incubated at 37°C for a minimum of 1 hour to allow self-assembly. If gels were not immediately used for seeding cells, 500 µL of PBS were added to each well after gels formed and kept at 4°C to prevent them from dehydrating.

2.4. Cell Differentiation

For cell differentiation, cells were plated at a density of 50,000 cells/ml with 150 µL of differentiation media (Iscove's Modified Dulbecco's Medium (Sigma), 20% fetal bovine serum premium select (Atlanta Biological, Lawrenceville, GA), 4 mM L-glutamine, 100 U/m Penicillin/Streptomycin, 10 mg/ml gentamicin). Cells were plated in 48-well plates coated with 40 µL of different types of collagens (L1, L1-scramble and native collagen). Media was replaced every 2 days for the duration of the experiment. On day 3, 5, 10, or 14, samples were fixed for immunofluorescence analysis.

2.5. Adhesion Assay

L1, L1-scramble, and native collagen were pipetted into separate wells of a 24-well plate in duplicate and allowed to self-assemble at 37°C and 100% humidity. L1-ES and P-ES

were seeded (250 μL of a 50,000 cell/ml suspension) with ES media (LIF included) on the self-assembled gel and allowed to settle and attach for 2 or 3 hours. Wells were rinsed three times with 1 ml PBS in 5-minute intervals. The remaining cells were stained with Dapi and imaged using an Olympus IX81 inverted epifluorescent microscope (Olympus, Melville, NY) with a 4X objective and Dapi filter. Images were captured digitally using Metamorph software. The number of cells in each field of view were counted using ImageJ and confirmed by a hand count.

2.6. Proliferation Assay

Regular type 1 collagen and L1-grafted collagen were tested for their ability to influence ESC proliferation. L1-ES and P-ES were seeded to each condition with ES media. After incubation in 37°C and 100% humidity for 3 hours, 1, 2, and 3 days, wells were rinsed with PBS and fixed with 4% paraformaldehyde. The cells were then stained with Dapi and captured with 4X objective and Dapi filter. The number of cells in each 4X field of view were counted using ImageJ.

2.7. Immunocytochemistry

2.7.1. Immunostaining

Cells were stained immunocytochemically in 48-well plates for proteins and transcription factors listed in Table2. Cells seeded on collagen gels for 3 days, 5 days, 10 days and 14 days were fixed at room temperature with 4% paraformaldehyde for 15 minutes, washed with immunobuffer (1% Triton, 1% bovine serum albumin (BSA) in PBS) for 5 minutes. Normal goat serum (10%) and BSA (5%) in PBS were used for blocking non-specific antibody binding. Cells were blocked with blocking solution for 1.5 hours and incubated with primary antibodies (Table 2) at 4°C overnight. Cells were then washed with

immunobuffer for 1 hour, and then secondary (goat anti mouse, 1:1000) antibodies were added and incubated at 4°C for 4 hours. At each staining, rat dermal fibroblasts (RDFs) were also labeled as a negative control. 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).

Antibody	Source	Purpose	Concentration	Dilution Used	Reactive Species
Oct4 (rabbit anti Oct4)	Abcam	Pluripotent Embryonic stem cell marker	0.25ug/ml	1:250	Rabbit
Nestin (ms anti Nestin)	Abcam	Ectoderm/Neural Stem cell marker	1.0ug/ml	1:250	Mouse
Tuj1 (ms anti Tuj1)	Covance	Neuron specific Class III β-tubulin	1.0ug/ml	1:400	Mouse

Table 2: Summary of antibodies used

2.7.2. Quantification of Neural Precursor Cells

Five 10X images from each of the two replicates (Dapi + Nestin) were taken from each well. The total number of cells in each image were counted using Dapi with ImageJ. All the images were normalized with the same brightness and contrast settings. All the Nestin-positive cells were hand counted and divided by the total number of cells in each image to calculate the percent of Nestin-expressing cells at each stage during differentiation.

2.8. Conditioned Media

To evaluate if differentiation was primarily affected by soluble factors secreted by the ESCs, cells were cultured in conditioned media. P-ES were seeded on L1-grafted collagen (L) and native collagen (C) with differentiation media (IMDM, FBS) in 24-well plates. The incubation media was then collected into separate 15 ml tubes 3 days after

seeding the cells. Equal volumes of fresh differentiation media were added and then stored at 4°C. P-ES and L1-ES were seeded in 48-well plates at 100,000 cells/ml in duplicate with 150 μL of differentiation media, conditioned media from P-ES seeded on native collagen (C), and conditioned media from P-ES seeded on L1-grafted collagen (L). Media was changed every 2 days and the cells were fixed on day 5 for immunostaining.

2.9. Discrete Microenvironment Gel Assay

Collagen scaffolds with concentric microenvironments of L1 grafted collagen and native collagen were created in a 24-well plate by allowing the two solutions of collagen to self-assemble juxtapose to each other. A circular plastic divider was placed in the center of a well. 30 μL of native collagen solution (spiked with FITC conjugated collagen) was pipetted on the outside of the divider, and 30 μL of L1 collagen solution (no fluorescent label) was placed on the inside of the divider. The set up was then allowed to self assemble at 100% humidity and 37˚C for 1 hour. Following self-assembly, the divider was removed. Parental cells were then seeded on the collagen scaffold for 3 days, at which time the cells were then fixed and stained for Nestin expression.

Chapter 3

3. Results

3.1. Morphological Differences

On day 0, P-ES and L1-ES demonstrated similar morphologies. Both cell types appeared spindle shaped, although the L1-overexpressing stem cells appeared slightly smaller (Figure 2a, 2b). Overt differences among the conditions were apparent as early as 2 days after incubation in differentiation media. On Day 2, L1-ESxL (L1-expressing ESCs seeded (x) on L1-grafted collagen) and P-ESxL (parental ESCs seeded on L1-grafted collagen) showed the most distinctive differences in morphology. Cells changed from the spindle shaped to a more rounded cell body with extending processes on the two ends of the cell. On the same day, none of the P-ESxC cells showed such morphology. A few cells showed similar neuronal morphology on L1-ESxC and P-ESxL conditions but not as abundantly as L1-ESxL. The morphology change observed in the L1-ESxL matches closely with the description for neural precursor cells (as shown in Figure 2d).

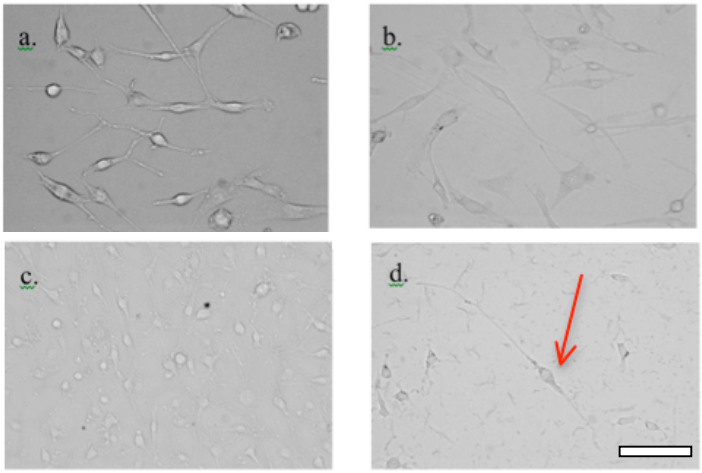

Figure 2: Morphology change of cells seeded on native and L1-grafted collagen (bar = 200µm)

a) Day 0 P-ES on tissue culture plastic (TCP). b) Day 0 L1-ES on TCP. **c)** Day 3 P-ES on native collagen (P-ESxC) **d)** Day 3 P-ES on L1-grafted collagen (P-ESxL). No overt differences were observed between the two cell types before in TCP. As early as 3 days growing on L1-grafted collagen, P-ES started extending and became neuro-precursor cell like (red arrow).

3.2. Adhesion

Cell adhesion was significantly affected on both L1 and scrambled peptide-grafted collagen. Results are summarized in Figure 3. A one-way ANOVA showed significant differences among the collagen conditions at both 2 hours and 3 hours (ANOVA $p < 0.0001$). Post hoc analysis with Tukey's test showed that L1 grafted collagen significantly increased the adhesion to both L1-ES and P-ES compared to native collagen. The L1-scrambled peptide grafted collagen showed a significant decrease in cell adhesion for both cell types, which is consistent with our previous findings by Monteiro et al. [13]. By covalently grafting L1-scrambled peptide on collagen, we may have blocked regular binding sites normally available on the collagen scaffold [12]. Furthermore, Dapi images of L1 cells cultured on scrambled collagen (Figure 4) showed several cluster of cells. It is generally accepted that cells will adhere to other cells when the substrate they are cultured on is less-adhesive (Differential Adhesion Theory) [14] .

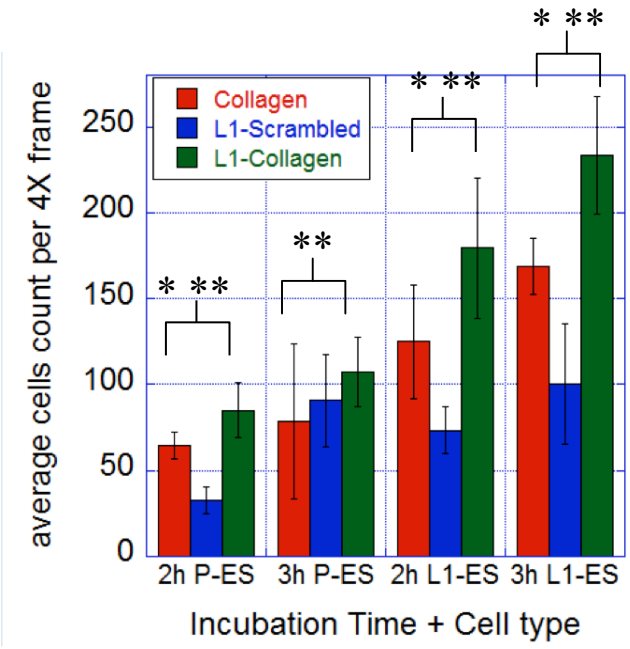

Figure 3: Adhesion changes in native, L1 and L1-scramble grafted collagens (average +/- stdev)

Cell attachment was significantly increased on L1-grafted gels in both time points (2 hours, ANOVA, $P<0.0001$), (3 hours, ANOVA, $P<0.0001$) and significantly decreased on L1-scrambled grafted gels compared to adhesion on native collagen. L1-ES seeded on L1-collagen showed the highest adhesion rate, which is likely due by L1's homophilic binding characteristic. P-ES, which does not express L1 also adheres better. This is likely caused by L1's heterophilic binding with molecules such as integrin or CD9 (embryonic marker). (*sig diff between C vs. S, **sig diff between C vs. L).

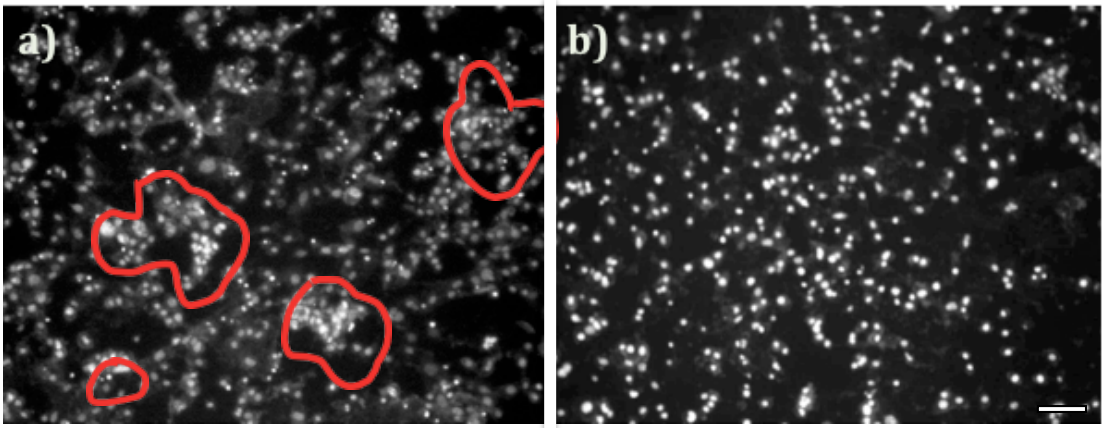

Figure 4: Cell aggregation on less adhesive surfaces

a) 4X Dapi image of 5th day L1-ESxS (L1 expressing cell seeded on L1-scrambled peptide) (cell count = 525) **b)** 4X Dapi image of 5th day L1-ESxL (cell count = 475). On non-adhesive surfaces, cells tend to from clusters and aggregations (circled in red), whereas on adhesive surfaces (L1-collagen), cells are more evenly distributed.

3.3.Proliferation

Proliferation assays were conducted to compare the rate of proliferation between the two cell types. Cell proliferation was assayed on all three collagen conditions. Briefly, cells were seeded on collagen hydrogels of the various grafting conditions. Knockout media with LIF was used to maintain cells in a pluripotent state. Proliferation of cells was measured at four time points (3 hours, 1 day, 2 days, and 3 days). Results from the proliferation assay are graphically represented in Figure 5. A one-way ANOVA with Tukey's post hoc test was conducted to measure differences in proliferation between cell and substrate combinations. To ensure equal seeding of cells in the various wells, a 3-hour time point was included in the study. No significant differences (ANOVA $p = 0.129$) were noted across any of the conditions at 3 hours, which suggests statistically equal seeding densities. At the day 1 time point significant differences (ANOVA $p<0.0001$) were noted in all combinations of cells and substrates except for L1-ESxL vs P-ESxC and P-ESxC vs P-ESxL. On Day 2 significant differences (ANOVA $p < 0.0001$) were noted between all combinations of cell and substrate except, L1-ESxL vs L1-ESxC

and P-ESxL vs P-ESxC. On day 3 significant differences (ANOVA $p < 0.0001$) were noted between all combinations of cell and substrate except, L1-ESxL vs L1-ESxC.

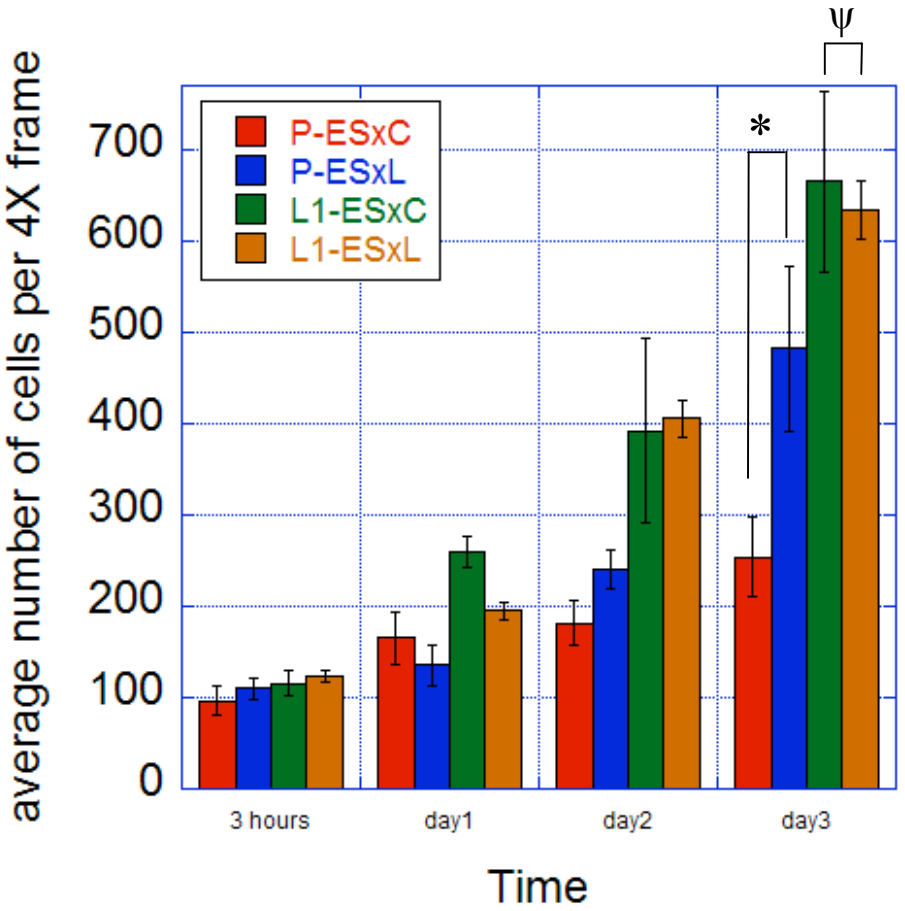

Figure 5: Proliferation Assay
No significant difference in proliferation for L1 cells on different collagens was observed ($^{\psi}p = 0.97$). However, P-ES showed an increase in proliferation when seeded on L1-grafted collagen compared to native collagen ($^{*}p < 0.0001$).

3.4.Immunocytochemistry

3.4.1. Pluripotent marker: Oct4

Prior to assaying differentiation of cells, cells from the same passage were plated on tissue culture plastic, fixed, and then stained for Oct4 expression after 3 hours of incubation to confirm that the cells were Oct4 positive. Both P-ES and L1-ES showed Oct4 expression on day 0 (Figure 6). Following confirmation of pluripotency, cells from the same batch was cultured for 3, 5, 10, or 14 days. As early as day 3, the expression of

Oct4 was significantly different among the various conditions. On day 3 (Figure 7), while partial Oct4 expression was still observed in conditions P-ESxC and P-ESxS, no Oct4 expression was observed in any condition that included L1 (P-ESxL, L1-ESxL, L1-ESxC, L1-ESxS). No Oct4 expression was noted in any conditions after day 5.

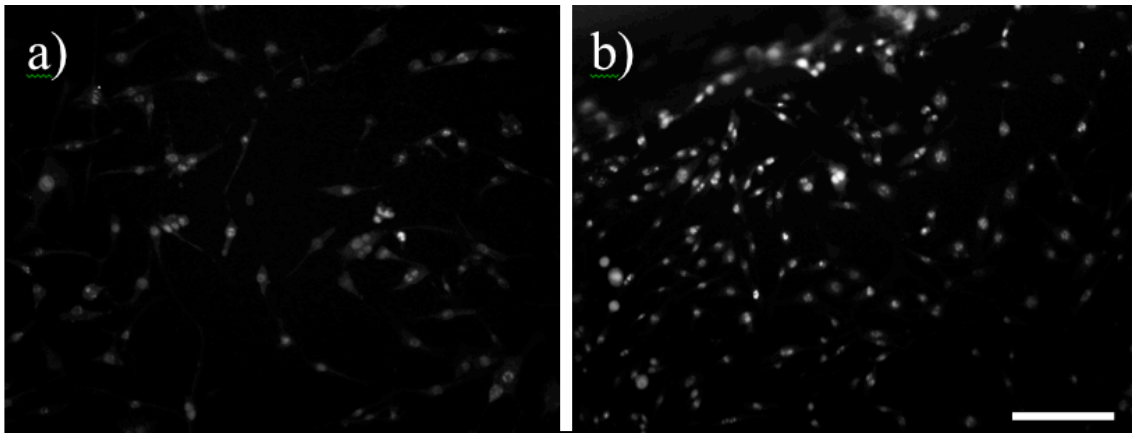

Figure 6: Day 0 Oct expression on tissue culture plastic

a) P-ES b) L1-ES on TCP. Virtually every cell is Oct4 positive, confirmed the cell's pluripotency.

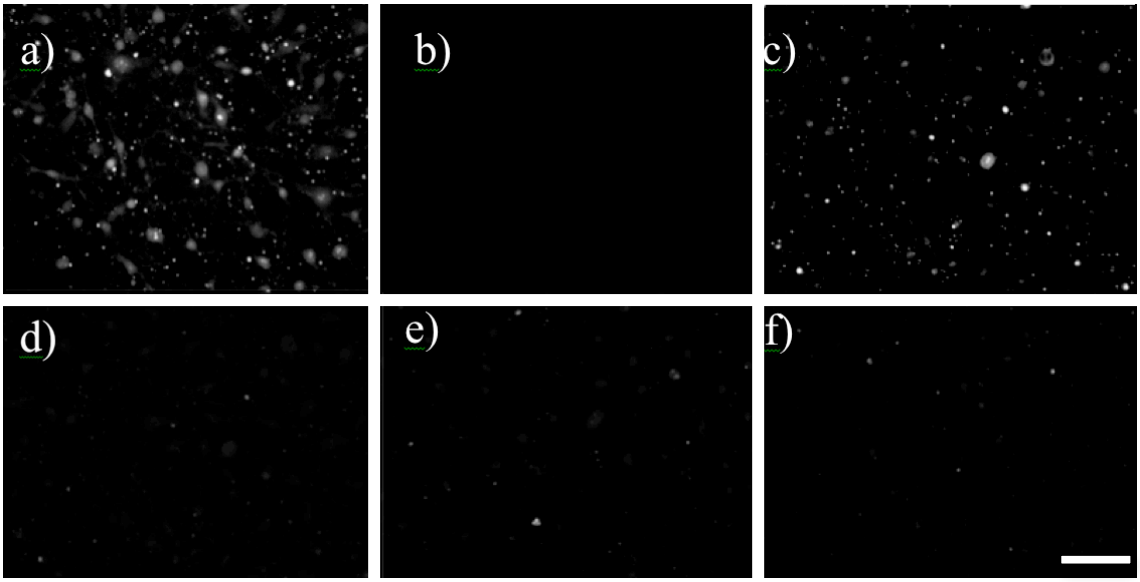

Figure 7: Day 3 Oct4 expression

a) P-ESxC **b)** P-ESxL **c)** P-ESxS **d)** L1-ESxC **e)** L1-ESxL **f)** L1-ESxL. 72 hours after incubating in differentiation media, P-ESxC and P-ESxL showed partial Oct4 expression, the rest of the conditions (conditions with either L1-ES or cells seeded on L1-collagen) showed no Oct-4 expression. (Bar = 200µm)

3.4.2. Neuroectoderm Marker: Nestin

3.4.2.1. Immunostaining

Nestin was used as a marker for differentiation into neuroectoderm germ layers. Before the experiment, Day 0 cells (P-ES, L1-ES) were stained for Nestin to test the antibody's specificity. No Nestin staining was noted on the day 0 time point. Pronounced Nestin expression was observed in many conditions as early as day 3 (Figure 8), and an overt difference was observed between the L1-ESxL and P-ESxC. By day 5 (Figure 9), Nestin expression is up-regulated for both P-ES and L1-ES seeded on L1-collagen. Cells seeded on scrambled collagen also showed Nestin expression, but the expression was more obvious around the cell clusters. On day 10 (Figure 9), the highest expression conditions from day 5 (L1-ESxL and P-ESxL) appeared to reduce Nestin expression, while the other conditions seemed to remain the same.

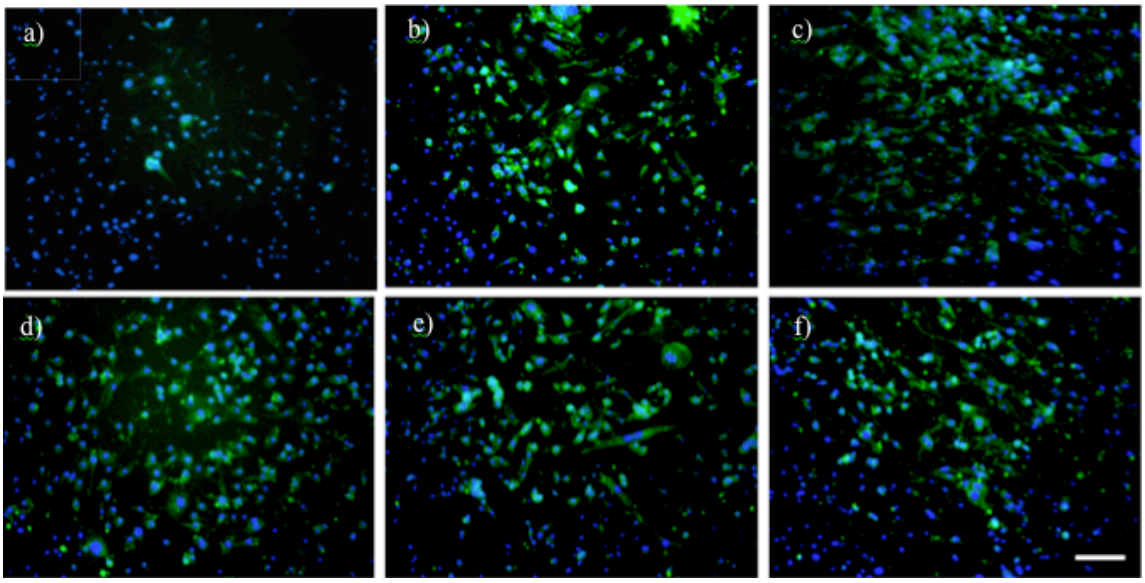

Figure 8: Day 3 Nestin expression (Green: Nestin, blue: Dapi, Bar = 200 µm)

a) P-ESxC **b)** P-ESxL **c)** P-ESxS **d)** L1-ESxC **e)** L1-ESxL **f)** L1-ESxS. Nestin, an intermediate filamentous protein was used as a marker for neuroectoderm differentiation of stem cells. Nestin expression was observed in all conditions as early as day 3 except for a) P-ESxC and c) P-ESxS.

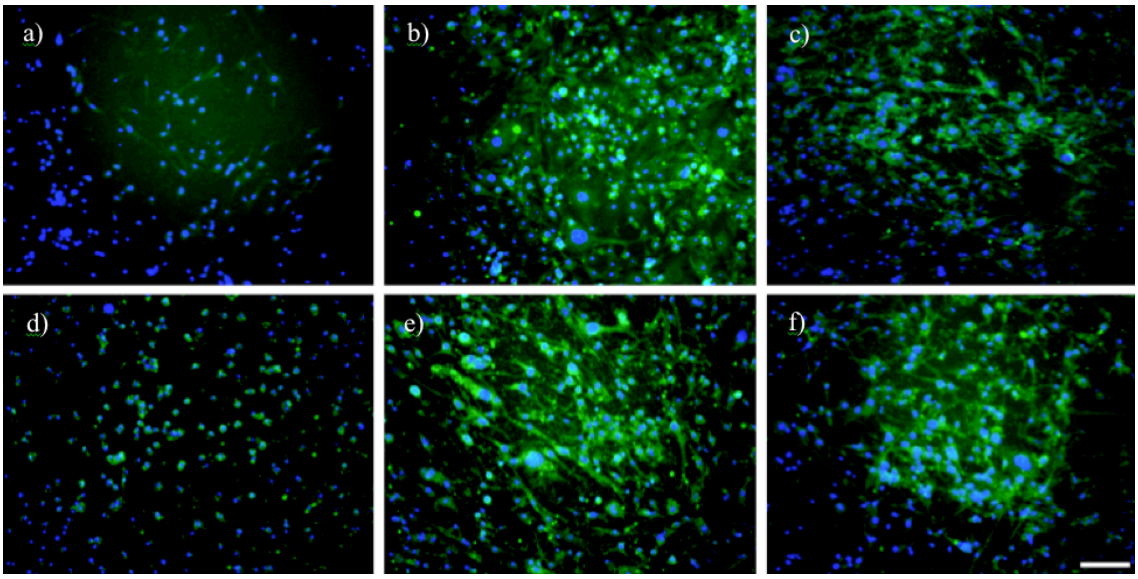

Figure 9: Day 5 Nestin expression (Green: Nestin, Blue: Dapi, Bar = 200 µm)

a) P-ESxC **b)** P-ESxL **c)** P-ESxS **d)** L1-ESxC **e)** L1-ESxL **f)** L1-ESxS. Nestin filament expression was up-regulated on collagen grafted with L1 peptide mimics 5 days post seeding on gels. Cells seeded on scrambled peptide showed more Nestin expression than cells seeded on native collagen. The result is likely due to the higher cell-cell interaction within the cell clusters.

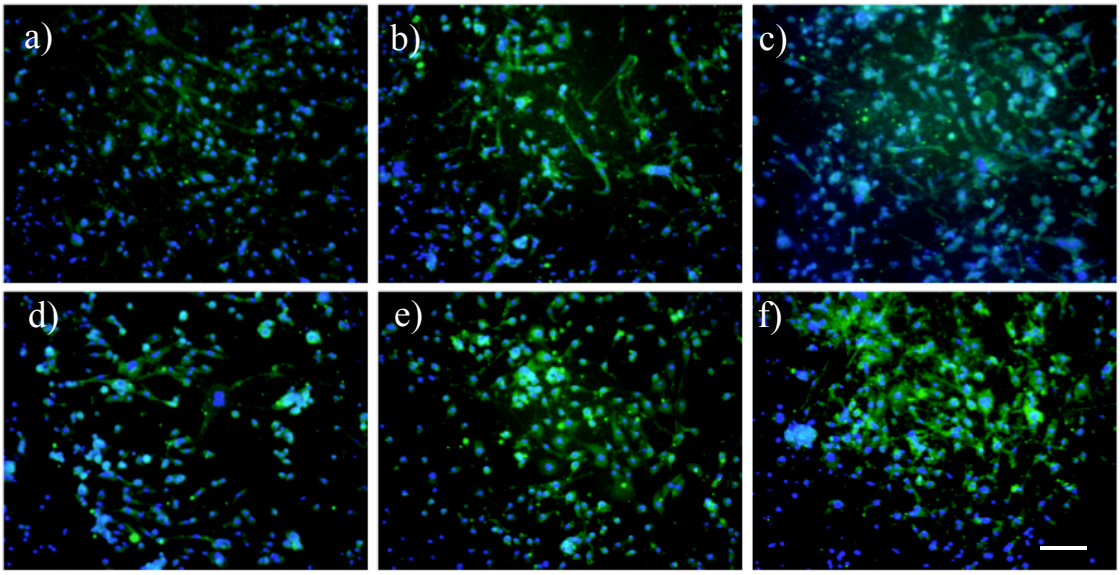

Figure 10: Day 10 Nestin expression (Green: Nestin, Blue: Dapi, Bar = 200 µm)

a) P-ESxC **b)** P-ESxL **c)** P-ESxS **d)** L1-ESxC **e)** L1-ESxL **f)** L1-ESxS. Cells on L1-collagen which showed the high expression on day 5 show reduced expression by day 10, suggesting that cells continue to differentiate and lose nestin as they express mature neural markers (e.g. Tuj1).

3.4.2.2. Quantification of Nestin Immunocytochemistry

3.4.2.2.1. P-ES

One-way ANOVA showed significant differences in Nestin expression for P-ES seeded on different collagens in all the days recorded (p=0.0009). On both day 3 and day 5, highest Nestin expression was observed on L1-collagen and lowest Nestin expression was observed on native collagen. On the same days, significant differences in Nestin expression was noted between collagen and scrambled collagen. As we have seen in the immunostained images, the Nestin expression reduced on day 10 for cells seeded on L1 collagen.

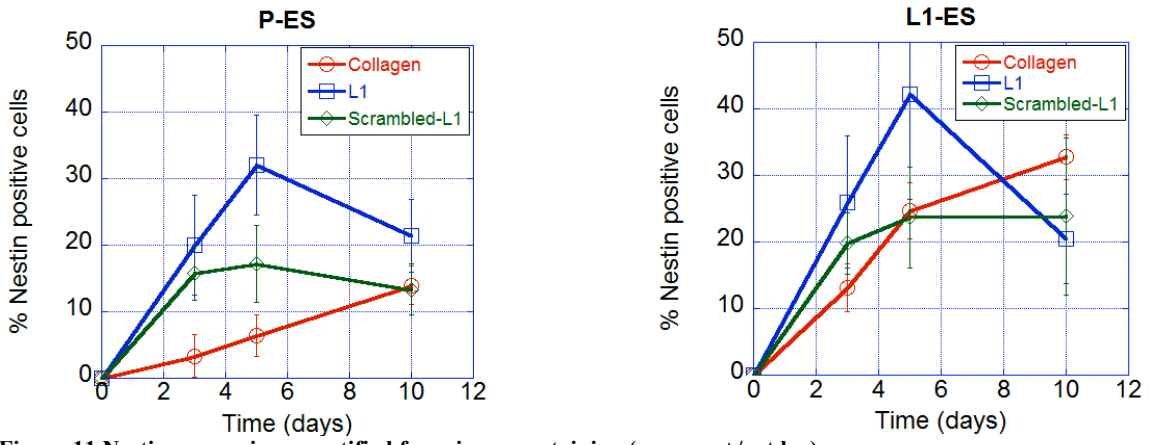

Figure 11 Nestin expression quantified from immunostaining (average +/- stdev)

Grafting L1 and scrambled-L1 peptides significantly affected the Nestin expression as measured on day 3, day 5 and day 10.The rise in Nestin expression was observed early in the L1-ESxL compared to P-ESxC. The drop in Nestin level on day 10 seen in the two conditions seeded on L1-collagen are likely due to continued differentiation, Nestin is down regulated and being replaced by matured neural markers (E.g. Tuj1)

3.4.2.2.2. L1-ES

For L1-ES cells, the overall Nestin expression trend is similar to that of P-ES Cells. Significant differences were noted on day 3 and day 5 between all conditions but not for day 10. On day 3 and day 5 the highest number of Nestin positive cells was again observed on L1-collagen, but Tukey's post hoc test did not show significant differences

between L1-collagen vs. native Collagen or L1-collagen vs. scrambled collagen conditions. Notably, when compared to P-ESxL, L-ESxL on average showed 10% more Nestin positive cells. No significant difference in the percentage of Nestin positive cells on the various conditions was observed on day 10.

3.4.3. Neuron Marker Tuj1

To determine whether the reduction in Nestin expression observed on day 10 was caused by mature neuron marker replacement, cells were labeled with anti-Tuj1. The antibody was raised against microtubules derived from rat brain. It is well characterized and highly reactive to neuron specific Class III β-tubulin (βIII) and it does not identify β-tubulin found in glial cells. Due to neuronal extensions and processes, it was difficult to quantify the percentage of Tuj1+ cells. Qualitatively, we see a greater number of cells in P-ESxL and L1-ESxL. These conditions showed a decrease in Nestin expression by day 10.

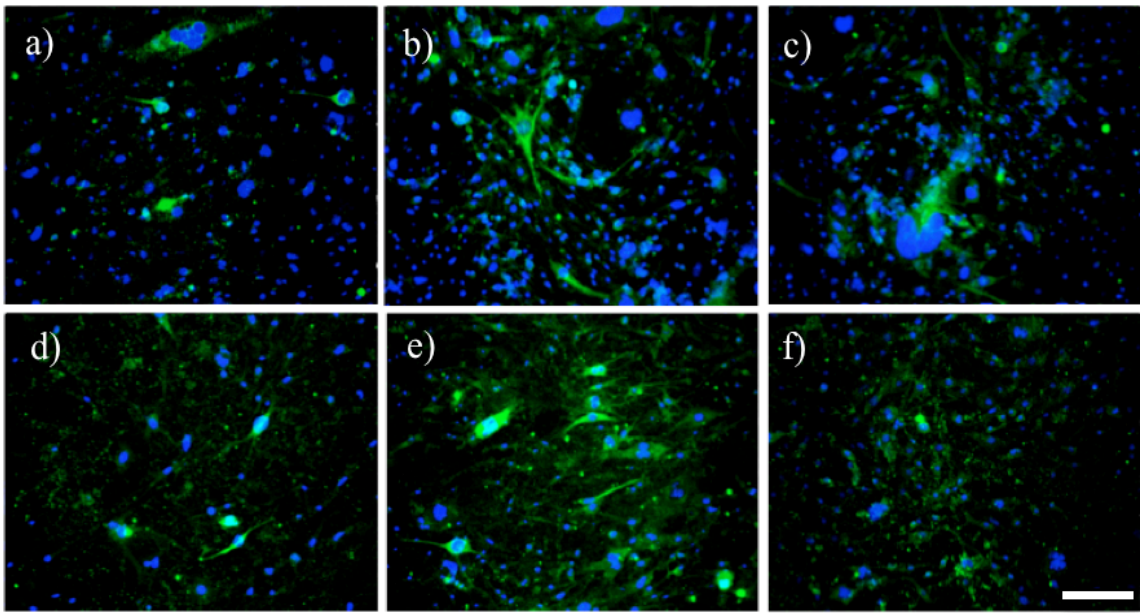

Figure 12 Day 10 Tuj1 expression (Bar = 200µm)

a) P-ESxC **b)** P-ESxL **c)** P-ESxS **d)** L1-ESxC **e)** L1-ESxL **f)** L1-ESxS
Strong Tuj1 expression proved the cells have differentiated terminally into neurons. The antibody was raised against microtubules derived from rat brain. It is well characterized and highly reactive to neuron specific class III B-tubulin and it does not identify B-tubulin found in glial cells.

These cells have a neuron-like morphology with extending processes as shown in Figure 12. The shift from Oct4 to Nestin expression and then towards Tuj1, strongly suggests that the cells are differentiating towards the neuronal lineages.

3.5.Conditioned Media

An experiment with conditioned media was conducted to determine whether the difference we observed in Nestin expression was primarily caused by soluble factors secreted by the cells during. A two-way ANOVA showed significant differences between the different cell/collagen combination (ex. P-ESxL vs P-ESxC) ($p < 0.0001$), but no significant differences were observed among the 3 conditions media used (ex. P-ESxC incubated with fresh IMDM vs P-ESxC incubated with P-ESxL conditioned media) in each condition (P=0.1006).

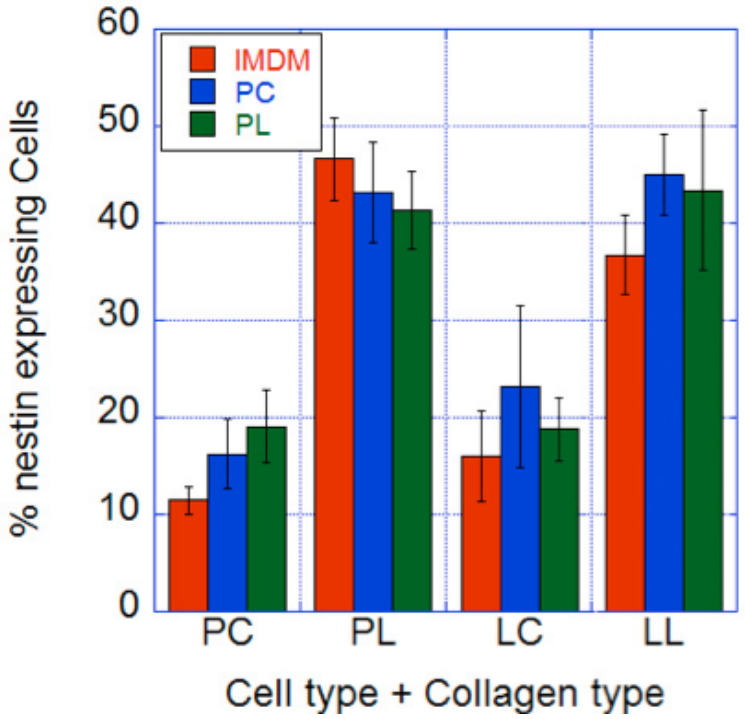

Figure 12: Conditioned media experiment (average +/- stdev)

No differences were observed between the different conditioned media conditions (ANOVA max p = 0.1006). The result suggests the differences in Nestin expression were caused by direct interaction with L1 rather than soluble factors secreted by cells during differentiation.

3.6.Discrete Microenvironment Gels

To assay whether the mechanism of action for the L1-guided differentiation of stem cells is mediated via insoluble cues, we cultured parental stem cells on a collagen gel with two discrete microenvironments. The inner ring consisted of L1-grafted collagen and the outer ring consisted of native collagen spiked with FITC-grafted molecules for visualization. Figure 13 represents an image that was taken at the interface of the two gels. Cells that were cultured in the L1 collagen region differentiated towards Nestin positive cells (green arrows) within 3 days. Cells cultured in the native collagen condition were Nestin negative (red arrows). These results suggest that bioactive adhesive cues are responsible for the observed differentiation.

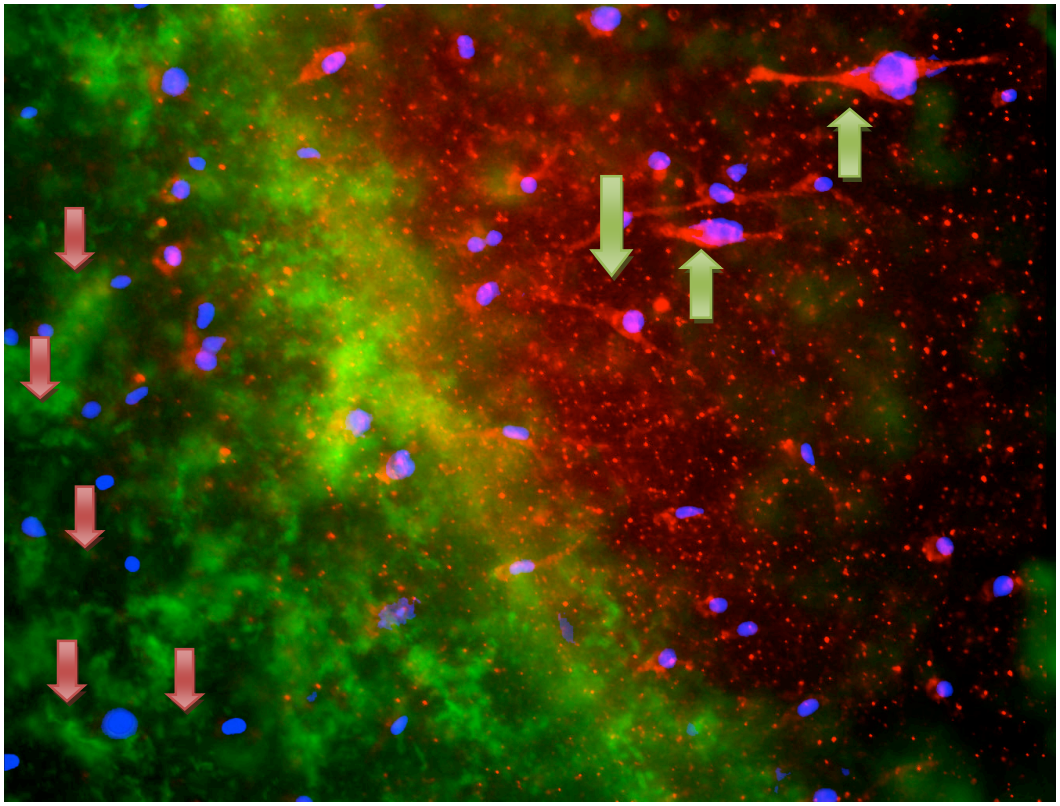

Figure 13: Discrete microenvironment collagen gels

Discrete environment collagen gel set up allows cells to be seeded on different type of collagen in the same well. Cells that were cultured in the L1 collagen region differentiated towards Nestin positive cells within 3 days (red = Nestin staining). Cells that were cultured in the native collagen (spiked with FITC) condition were Nestin negative.

Chapter 4

4. Discussion

Embryonic stem cells are a potentially limitless cell source, and could provide significant therapeutic benefits to various types of diseases that result from degeneration of tissue or loss of specific cells types. Biomaterials are used as scaffolds to house as well as deliver various therapeutic cells for tissue engineering and tissue regeneration purposes. Combing these therapies may provide potential synergistic effects that may accelerate and promote healing of tissues that were otherwise not possible.

In this study we attempted to direct the differentiation of embryonic stem cell towards a neural lineage using a tailored biomaterial. To tailor collagen, a ubiquitous ECM protein to guide the differentiation of stem cells towards neural lineages we grafted L1 peptide mimics onto it. L1 overexpressing stem cells were used as a bench mark to assay against. Parental ES cells with no germ layer preference were used to assay the differentiation of stem cells towards neural lineages. This study showed preliminary evidence that L1-peptide grafted collagen improved differentiating efficiency, and directed the differentiation of mouse embryonic stem cells toward neuroectoderm.

4.1. Cell Adhesion and Proliferation

As shown in section 3.2, grafting L1 peptide to collagen significantly increased the adhesion of L1-ES as well as P-ES. In the case of L1, it is likely that adhesion is mediated via homophilic binding. In the parental cells on L1 collagen, binding may be regulated via heterophilic binding between L1 peptide and cell surface adhesion molecules such as CD9 (embryonic stem cell marker). In the L1-scramble peptide grafted collagen, a decrease in cell adhesion was noted. This is expected, as the scrambled

peptide cannot induce L1-L1 homophilic binding nor heterophilic ligands. Additionally, the attachment of scrambled peptides onto the backbone of collagen may have blocked some of the regular binding sites on the collagen, as suggested by Monteiro et al [13]. It was also noted that L1-overexpresssing cells cultured on collagen grafted with scrambled peptide formed cell aggregates. The formation of cell-cell aggregates is consistent with the differential adhesion theory presented by Foty et al [14]. Aggregate formation was not evident in parental cells. Within the aggregated cell clusters, an increase in cell-to-cell interaction may occur. The increase in cell-cell interaction likely played a role in cell differentiation but it is difficult to control without changing other variables in the culture such as the seeding density or the addition of β-mercaptoethanol during incubation.

The proliferation of stem cells was different between the two cell lines. L1 cells proliferated significantly more than parental cells. These results are contrary to previous work published by Dr. Schachners lab where decreased proliferation was noted with L1 cells. However, several key differences exist between the two studies. In our studies LIF was used to prevent differentiation of cells. It is widely accepted differentiation slows down proliferation. Second, peptides were presented attached to the matrix in our studies. This markedly changes the signaling response. Biomechanical signaling is turned on when adhesion molecules are presented on the surface as opposed to when presented in solutions, as was the case in Schachner et al [11].

4.2. Directing to Neuroectoderm

Differentiation of stem cells was regulated by the presence of L1. L1 peptide grafted to collagen, as well as L1-overexpressing cells, influenced the kinetics of differentiation of stem cells towards neural lineages. The Oct4 staining suggested that the presence of L1

increased the speed of differentiation of stem cells away from pluripotency. By quantifying the immunostained images of Nestin expression, we were able to determine the percent of cells differentiating into Nestin positive cells, and were further able to compare the differences in Nestin expression between each condition. Both L1-ES and L1-collagen directed the differentiation towards Nestin positive cells. The highest number of Nestin positive cells at each time point was noted when cells were cultured on L1 collagen. This was consistent across both cell types and was observed up to, but not including day 10. Significantly higher Nestin expression was seen when P-ES were seeded on L1 scrambled collagen compared to native collagen. This is likely due to the increase in cell-cell interaction by aggregation as shown in the adhesion study. However, the difference between cells seeded on L1-scrambled collagen and native collagen was masked by L1-ES. The results suggested the L1-L1 homophilic binding between cells directs the differentiation towards Nestin positive cells. The drop in Nestin expression on day 10 on P-ESxL and L1-ESxL was likely due to the continued differentiation of the cells. Upon differentiation, Nestin becomes down regulated and is replaced by tissue-specific intermediate filament proteins. The staining for Tuj1 proved that this is indeed the case. Day 10 Tuj1 staining confirmed that a great number of cells in the 2 conditions have differentiated terminally into neurons.

4.3. Direct Interaction With the Adhesive Cues

In the conditioned media experiment, no significant differences were observed between the different conditioned media used. In the discrete environment collagen gel assay, cells that were cultured in the L1 collagen region differentiated towards Nestin positive cells within 3 days. Cells cultured in the native collagen condition were Nestin negative. These

results suggest that L1 differentiation of stem cells was generated by adhesive ligand binding in our system. If the differentiation was mediated via soluble factors, cells across the gel with distinct microenvironments should have differentiated towards neural lineages. Results from the conditioned media experiment corroborate these findings. If soluble factors were responsible for differentiation, conditioned media should have influenced differentiation of cells on P-ESxC to differentiate towards neural lineages.

4.4.Limitations

The aim of the thesis was to develop and understand the differentiation of embryonic stem cells. This study showed some preliminary positive results of L1 on the differentiation of stem cells into neuronal lineages. However, the quantification method used in the experiment relies greatly on manual counting and therefore might create a bigger margin of error, as the results might vary from person to person. A more precise and objective method, such as quantitative polymerase chain reaction (qPCR), can possibly be used. Our efforts to utilize qPCR to quantify the expression of cells did not materialize into meaningful results. Additionally, markers for cells in the other two germ layers (mesoderm and endoderm) could have been used to confirm that while higher percentage of cells are differentiating towards ectoderm, the number of cells differentiating into the other two layers are decreasing simultaneously. Although results from these studies show proof of concept of L1 direction of stem cells, cells *in vivo* exist in a three-dimensional (3D) space. The presentation of cues in a 3D environment is significantly different from those presented in this study. The level and complexity of interaction in the 3D environment might cause a different outcome for the cell's differentiation. However, as collagen absorbs fluorescence during the staining process, it

would be difficult to assay the difference in 3D using traditional methods such as immunohistochemistry. A recent study showed the L1 expression levels were correlated with breast cancer stage of progression in established data sets of clinical samples, so it is possible that improper use of L1 might lead to an even higher chance of tumor formation [15]. But the result of this study suggests that similar results might be achievable with the use of a different Ncam with homophilic binding characteristic.

4.5. Future Applications

The results from this study can be applied into a variety of techniques. If we are able to control the cell's differentiation with adhesive cues on the collagen, it might be possible to create a multi-purpose biomaterial patterned with different types of cues. The application is not limited to neural cells. As more cues are identified in the future, the same techniques can be applied. By grafting L1 peptide in combination with other signaling cues, it might be possible to specifically direct regions of areas on the same surface into all three germlayers. Potential future uses and developments of this research also include, but are not limited to, creating a bioreactor to efficiently produce precursor cells for transplantation. There are many advantages to using the peptide grafted collagen scaffold to create the bioreactor. For example, it allows for the continuous presentation of signaling cues that would otherwise need to be replenished every so often. Also, several peptides can be presented simultaneously to generate cells from the different layers. However, there are also disadvantages by associated with peptide grafted biomaterial. For example, 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 sequence. Every method presents a short coming. Eventually,

in vivo studies can be performed by transplanting the stem cells with the functionalized biomaterial (or just the functionalized biomaterial) into the injury/disease of neurodegenerative diseases. Though L1-overexpressing stem cells in combination with L1 grafted collagen showed synergistic effect and had the most number of cells differentiating into neurons, the use of engineered stem cell in human body is risky as they tend to be more unstable, especially with a molecule that is associated with tumor growth [16]. Obviously much more understanding of the mechanisms of cells behavior on the biomaterial *in vivo* will be required for possible future clinical applications.

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