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Engineering Biomaterials for Stem Cell Culture through the Identification of Novel Peptides

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Engineering Biomaterials for Stem Cell Culture through the Identification of Novel Peptides

by

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Abstract

Engineering Biomaterials for Stem Cell Culture through
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Professor David Schaffer, Chair

Stem cells are defined by two processes: self-renewal and differentiation. The balance of these two processes is determined by the signals—including extracellular matrix (ECM) proteins, soluble molecules, and other cells—in the microenvironment of the stem cell. To culture stem cells for cell replacement therapies, the microenvironment needs to support long-term self-renewal, including both the maintenance of multipotency or pluripotency and the proliferation, of the stem cells. Since defined medium conditions have been developed for many stem cells, including neural stem cells (NSCs) and human embryonic stem cells (hESCs), the focus of this work has been on the adherent substrates used during culture.

Many biomaterials—using natural, synthetic, or a combination of both types of materials—have been developed for neural stem cells. For example, natural materials such as collagen, other ECM proteins, and calcium alginate have been studied. Depending on the media conditions used, some of these natural materials promoted the self-renewal of neural stem cells. However, since most of these natural systems are not homogeneous because of the many isoforms of often impure ECM proteins present, synthetic materials have also been developed and investigated for NSCs. While synthetic materials have morphology and composition that is easier to control, in general synthetic materials lack the bioactive motifs necessary to actively engage and communicate with cells, resulting in low cell viability or premature differentiation. Accordingly, the field has been increasingly biofunctionalizing materials with motifs, such as peptides, that are capable of binding to cell adhesion recpetors, and studies with a biomaterial system utilizing an arginine-glycine-aspartic acid (RGD) peptide have shown that this material can support neural stem cells similar to their standard culture conditions.

Development of biomaterials for human embryonic stem cells (hESCs) has focused on finding natural and synthetic alternatives to Matrigel. Matrigel, a highly heterogeneous mixture of proteins including collagen IV and laminin, is still the typical adherent substrate for culture. Even though natural and synthetic materials have been explored as replacements for Matrigel, none of these materials has been shown to have the capacity for maintaining long-term self-renewal of hESCs similar to Matrigel. For example, materials containing RGD peptides do not support the growth of hESCs, as these cells utilize non-RGD-binding integrins for attachment to adherent substrates such as Matrigel-coated surfaces. Thus, there is a need to develop a method

to find novel peptides that attach to a cell population such as human embryonic stem cells. Once developed, this method could find candidate peptides that could be used in biomaterials to replace Matrigel.

Since neural stem cell culture is supported by materials using a RGD peptide, this cell type was used as a model to develop and validate a method for finding novel peptides. The method developed for finding novel peptides included using selections with an unbiased bacterial peptide display library to find candidate peptides followed by further characterization of synthetic versions of some of the peptides for their ability to support the culture of neural stem cells. Using this general method with adult neural stem cells (NSCs), 44 high-binding bacterial clones were found. Of these clones, four contained RGD motifs commonly found in integrin binding domains, and three had homology to extracellular matrix proteins. Three synthetic analogs of peptides were chosen from the biomimetic ligand selections, grafted onto interpenetrating polymer network (IPN) surfaces, and adsorbed on tissue culture polystyrene (TCPS). These three peptides were found to support cell proliferation to different extents, but all three supported self-renewal of the NSCs on IPN surfaces, while all three peptides supported both proliferation and self-renewal when adsorbed on TCPS. This library-based approach, unbiased towards any particular motif, was shown to yield peptides that supported the culture of neural stem cells and that contained motifs that are known to bind to cell adhesion receptors, such as integrins. Now that our method using bacterial peptide display selections was developed, we applied this method to human embryonic stem cells.

Using the method developed for neural stem cells, many peptides were found that bound with high-affinity to hESCs. When four of these peptides were adsorbed on TCPS, one peptide supported the short-term self-renewal of hESCs as indicated by proliferation and maintenance of pluripotent markers. In addition to finding general cell-binding ligands, selection was then targeted to a particular adhesion receptor, in this case the $\alpha_6\beta_1$ integrin, to recapitulate engagement with laminin. From the targeted selections, many peptides with high affinity for hESCs were found. Of the five tested when adsorbed on TCPS, two of them supported short-term self-renewal of hESCs. Overall, the development of a method utilizing bacterial peptide display selections to find novel peptides successfully found peptides that supported the culture of neural stem cells and human embryonic stem cells, with the best performing peptides being obtained from selections targeted for the $\alpha_6\beta_1$ integrin on hESCs.

I dedicate this dissertation to my family and friends for helping me through the tough times and for inspiring me in the good times. I especially want to thank my mom, dad, and brother for being there whenever I've needed anything.

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List of Symbols and Abbreviations

α_x	integrin subunit, x = 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, v, IIb, L, M, X, D, or E
β_x	integrin subunit, x = 1, 2, 3, 4, 5, 6, 7, or 8
3D	three-dimensional
ANOVA	analysis of variance
BLAST	basic local alignment search tool
BMP	bone morphogenetic protein
BDNF	brain-derived neurotrophic factor
CNS	central nervous system
CPX	circularly permutated OmpX protein
DMEM	Dulbecco's modified eagle medium
DMSO	dimethyl sulfoxide
EBs	embryoid bodies
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EVAL	poly(ethylene-co-vinyl alcohol)
FACS	fluorescence-activated cell sorter
FGF	fibroblast growth factor
GFAP	glial fibrillary acidic protein
GFP	green fluorescent protein
HA	hyaluronic acid
hESCs	human embryonic stem cells
IKVAV	isoleucine-lysine-valine-alanine-valine motif
IPN	interpenetrating polymer network
LAS	lysine-alanine sequential polymer
MEFs	mouse embryonic fibroblast (feeder) cells
NPCs	neural precursor cells
NSCs	neural stem cells
PEG	poly(ethylene glycol)
PDMS	poly(dimethyl siloxane)
PGA	poly(glycolic acid)
PLGA	poly(lactic-co-glycolic acid)
PLLA	poly(L-lactic acid)
PS	polystyrene
PVA	polyvinyl alcohol
PVDF	poly(vinylidene fluoride)
QCM	quartz crystal microbalance
RGD	arginine-glycine-aspartic acid motif
RWV	rotating wall vessel
SAM	self-assembled monolayer
SEM	scanning electron microscopy
sIPN	semi-interpenetrating polymer network
SSEA	stage-specific embryonic antigen
SVZ	subventricular zone

TCPS	tissue culture polystyrene
TEG	triethylene glycol
TGF	transforming growth factor
YIGSR	tyrosine-isoleucine-glycine-serine-arginine motif
X-gal	5-bromo-4-chloro-3-indoyl β-D-galactopyranoside

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Chapter 1:
Introduction

1.1 Stem Cell Niche

Stem cells are uniquely suited for multiple cell-based therapies because they can either proliferate and/or remain in the immature "stem-like" state, undergoing a process termed self-renewal, or differentiate into a specialized cell type.[1-3] On the other hand, differentiation is the process by which stem cells respond to various environmental and intrinsic control signals and develop into a specialized, mature cell type. For example, adult neural stem cells (NSCs) can differentiate into neurons, neural support cells called glia, or endothelial cells.[4, 5] On the other hand, human embryonic stem cells (hESCs) can likely differentiate into any cell type of the adult human body.[6, 7] In the long term, the proper understanding of stem cell culture for both self-renewal and differentiation can enable the cells lost from diseases like Parkinson's, Alzheimer's, and various spinal cord injuries to be replaced via stem cell therapies and potentially cure millions of patients each year.[2, 3, 8]

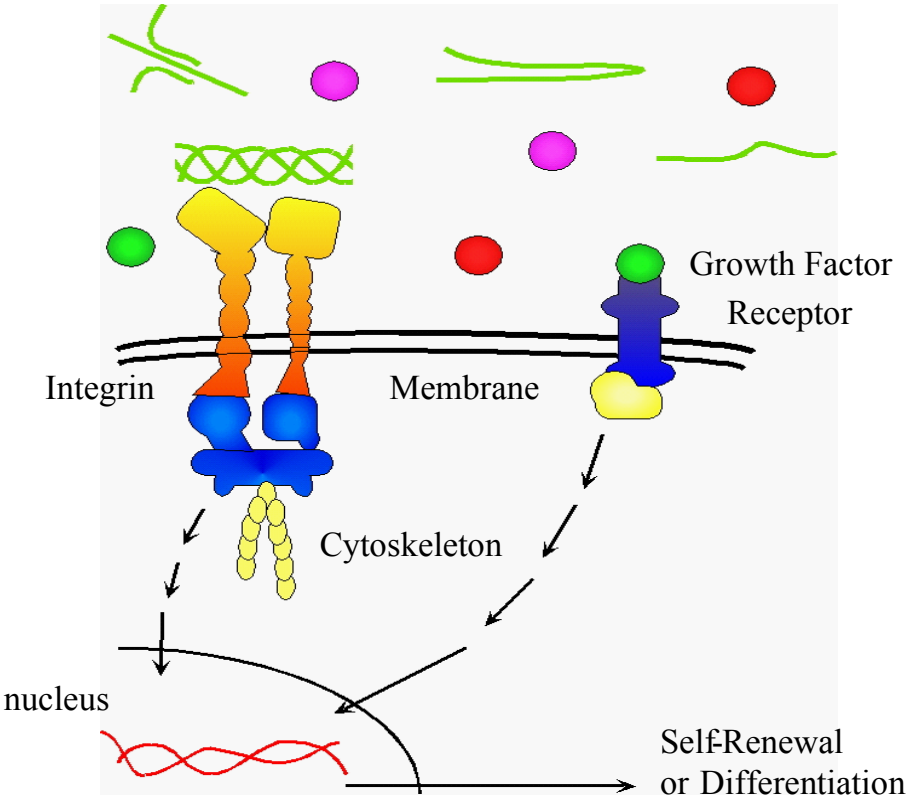

Figure 1-1: Schematic of cellular interaction with ECM proteins and growth factors. Cells can interact with ECM proteins (green lines) such as laminin and collagen through adhesion receptors like integrins. Integrins can intracellularly recruit signaling proteins and parts of the cytoskeleton leading to changes in gene expression. Growth factors (colored circles) interact with cells via growth factor receptors (blue). As with integrins, signaling proteins are recruited leading to changes in gene expression. The combined effects of cellular interaction with ECM proteins and other extracellular factors like growth factors regulate cell behaviors such as self-renewal or differentiation.

Inside organisms, development and replacement of cells in tissues are regulated by the stem cell niche, a natural microenvironment that regulates their self-renewal and differentiation to create new specialized cells during organismal development, or replace cells in adult organisms.[1] The niche is created through the interaction of cells with other components of the

environment, including extracellular matrix (ECM) proteins, other cells, and soluble factors including growth factors.[9]

Growth factors are proteins that interact with cells via binding to specific receptors, which recruit specific complements of signaling proteins leading to changes in gene expression ultimately driving changes in cell behavior such as self-renewal or differentiation (Figure 1-1). Similarly, ECM proteins are a group of molecules, including collagen and laminin, which interact with cells through various adhesion receptors on the cell surface. These adhesion receptors, such as members of the integrin family, then activate internal cell signaling via the recruitment of signaling proteins and elements of the cytoskeleton, leading to changes in gene expression and cell behavior (Figure 1-1).[10-13]

Integrins are one of the most studied classes of cell adhesion receptors. Each integrin is composed of two units, an α and a β subunit. There are 8 β and 18 α subunits, though integrins only make 24 different combinations as most integrin pairs involve the β_1 integrin.[12] ECM proteins typically bind to multiple integrins through various motifs in the protein. For example, laminin binds to 7 different integrins while fibrinogen binds to just 4.[12, 13] An example of some of these motifs include the arginine-glycine-aspartic acid or RGD motif—found on collagen, fibronectin, bone sialoprotein, and vitronectin—along with the YIGSR and IKVAV motifs found on laminin.[14, 15] Small peptides can be designed around these sequences, but often these peptides are unable to recapitulate the effects of the protein as a whole. Since these receptors are important for the attachment of many cells types—including stem cells—to a surface during cell culture,[16] it would be desirable to find methods to find synthetic peptides that could target them.

1.2 Current Methods for Growing Stem Cells

Efforts to use stem cells for tissue engineering, as well as for applications in high-throughput pharmacology and toxicology screening, hinge upon the ability to extract them from their natural niche in the body and control their behavior in cell culture. Control over their self-renewal and differentiation are best achieved by assembling the complement of signals that naturally regulate cells, i.e. imitating the stem cell niche. To achieve this goal, stem cells are typically cultured with animal cells, animal proteins, or even human proteins that either supply ECM or factors that encourage growth and control stem cell fate.

In part because adult neural stem cells were isolated earlier than human embryonic stem cells, methods for growing NSCs are better developed and involve better defined components. NSCs have been grown on animal laminin-coated plates with a defined media containing recombinant human FGF-2, or Fibroblast Growth Factor 2, a protein that encourages self-renewal of these cells.[4, 17, 18] On the other hand, human embryonic stem cells were initially grown in serum-based medium on gelatin-coated plates with mouse embryonic fibroblast (MEF) cells,[7] and very shortly thereafter in medium supplemented with FGF-2.[19]

FGF-2 encourages self-renewal of these cells and also causes the MEFs to secrete growth factors from the TGF-β family that encourage the growth and self-renewal of the stem cells.[20] Furthermore, gelatin as well as the ECM proteins secreted by the MEFs allow for hESC attachment. Conditioned medium from mouse embryonic feeder cells has also been shown to support MEF-free growth of hESCs on Matrigel.[21] Matrigel is a complex mixture of ECM proteins, primarily composed of ~60% laminin and ~30% collagen IV, extracted from a mouse tumor.[21-24] Conditioned medium is cell culture medium that has been previously used to grow the MEF cells, and it has thus been "conditioned" to contain the soluble factors secreted by those cells.

However, the use of animal or human-derived cells or proteins is highly problematic for numerous reasons. First, animal components/molecules can be transferred to the human cells, resulting in the potential immune reactions when stem cells are administered to patients.[25] Second, animal and in particular human proteins are expensive due to supply limitations and could potentially contain pathogens.[26, 27] Furthermore, even relatively pure ECM proteins are not highly defined since there are numerous forms of these enormous proteins (e.g. laminin has a molecular weight of 850,000 Da and comes in 10 isoforms, and numerous glycoforms). In addition, there can be considerable lot to lot variability in quality and purity, posing considerable problems for process development and scale-up.[28] Because of all of these problems, more defined systems for growing hESCs and NSCs are required.

Numerous defined liquid culture media formulations have been developed for hESCs, and almost all utilize FGF-2 to maintain self-renewal of the cells, including one with 100 ng/mL FGF-2.[29, 30] Some formulations also utilize members of the TGF-β family, such as Activin A and TGF-β1.[20, 31-34] For example, one somewhat defined system involved DMEM with FGF-2, TGF-β1, and several additional factors.[26] Another formulation utilized a defined, commercially available medium (X-Vivo) supplemented with 80 ng/mL FGF-2.[24] However, despite progress in the development of defined liquid culture medium, each of these systems used animal or human protein ECM substrates, often including the highly variable animal ECM mixture Matrigel.

To overcome the problems with ECM proteins, there have been recent efforts to develop synthetic substrates for NSCs and hESCs, which could then be coupled with the defined liquid media to create a fully chemically defined culture system that is reproducible and scaleable. The major goal of these synthetic adherent systems is to mimic the complex signals found in the natural stem cell niche.[9, 35] Imitating the stem cell niche is challenging for several reasons, including the fact that highly complex ECM proteins are difficult to emulate, and that all of the necessary mechanisms for maintaining self-renewal and differentiation are not even known or completely understood.

The overall objective of this thesis is to develop a method for finding novel peptides that bind to stem cells and to evaluate the peptides found through this method for their ability to recapitulate the signals from ECM proteins used in stem cell culture. In this thesis, Chapters 2 and 3 will explore specific biomaterials that have been developed for NSCs and hESCs, respectively. Although some synthetic materials have been developed for both of these cell types, none of these materials have replaced the ECM proteins used in culture. The remaining chapters with explore the development and execution of a method to find new peptides that can be used to replace the adherents proteins and protein mixtures that are used to culture stem cells. With this method, many promising peptides that can potentially replace the ECM proteins used in stem cell culture were found.

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Chapter 2:
Engineering Biomaterials for Synthetic Neural Stem Cell Microenvironments

2.1 Introduction

Neural stem cells (NSCs) have been isolated from various species—such as mice, rats, and human—and from numerous regions in the developing and adult nervous system—including the subventricular zone (SVZ), the subgranular zone of the hippocampus, the cortical neuroepithelium, and the spinal cord.[1-8] *In vivo*, the NSC is encompassed by a microenvironment or niche that presents it with a repertoire of diffusible factors,[6, 9, 10] cell-cell interactions,[11, 12] and extracellular matrix (ECM) ligands that bind to cellular receptors and thereby modulate signaling and gene expression (Figure 2-1).[13-15] Ultimately, these soluble and solid-phase components of the niche collectively regulate cell behavior and function—including mitosis, apoptosis, migration, and differentiation.[6, 16-24]

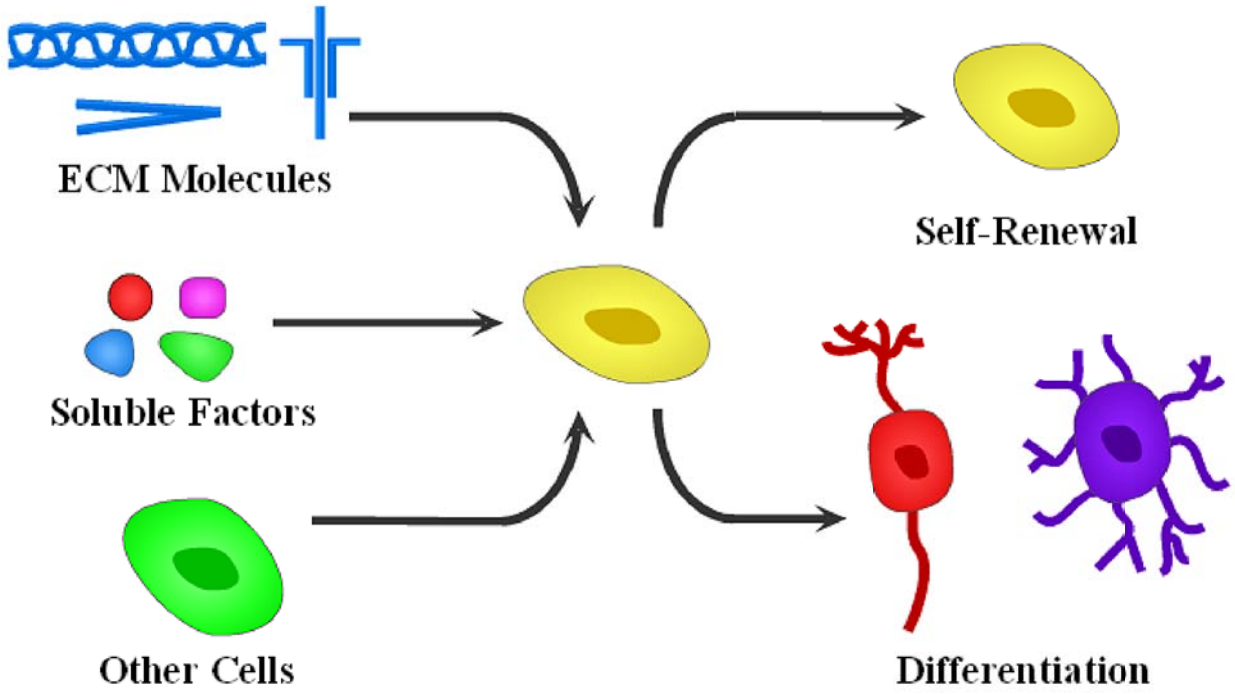

Figure 2-1. Influencing components in the stem cell microenvironment. Stem cells are influenced by many components of their microenvironment including ECM molecules, soluble factors, and other cells. The combination of all of these signals determines whether the cell undergoes self-renewal or differentiation.

NSCs have therapeutic potential to treat disorders and injuries such as Huntington's disease, multiple sclerosis, Parkinson's disease, stroke, and diseases and injuries of the spinal cord.[25-32] In cell transplantation therapies, NSCs have survived in various regions of the central nervous system (CNS), including the striatum, hippocampus, ventricles, SVZ, olfactory bulb, and cerebellum,[26, 33-37] and have shown promising results when implanted at the injured/diseased sites in animal models for numerous diseases and injury, such as Sly disease, myelin degeneration, Parkinson's disease, and spinal cord injury.[36-41]

In general, successful novel cell transplantation-based therapies will hinge upon the ability to isolate stem cells, expand them in an undifferentiated state, induce their differentiation into a specific neuronal cell type or types, and engraft them *in vivo* in a manner that ensures their functional integration into the affected tissue. Each of these stages requires precise control over cellular behavior, which will therefore entail the successful development of systems that emulate the natural stem cell niche, that is, synthetic stem cell microenvironments. For example, *ex vivo*

systems that support stem cell expansion and differentiation in a safe, scaleable, and economical fashion will be needed. In addition, in general, only a small fraction of stem cells or their progeny survive when implanted,[42-47] so there is a need to develop new systems or synthetic microenvironments that encourage successful incorporation, survival, and integration of NSCs into diseased or injured regions of the CNS.

Synthetic microenvironments have two major components: soluble and solid phases. For clinical applications, both components should be biochemically defined, reproducibly generated, non-immunogenic[48] (and therefore human in origin), and not pose risks of pathogen transfer. The soluble phase, therefore, should avoid the use of serum, a poorly defined collection of hundreds of proteins and other components that can suffer from lot-to-lot variability. Fortunately, there has been considerable progress in identifying and utilizing defined soluble factors to modulate stem cell behavior, leading to the development of defined serum-free media for culturing human embryonic stem cells. As these important advances have been discussed elsewhere,[49-54] this chapter will focus on the solid phase, specifically on the development of various materials for NSC culture including natural, semi-synthetic, and fully synthetic materials.

Although ECM molecules are a major component of the cellular niche, exploiting these molecules to construct controlled stem cell microenvironments has been comparatively difficult because they are extremely large (e.g., laminin is 850,000 Da), have multiple isoforms and glycoforms, are difficult to purify to homogeneity, and may be difficult to obtain from large-scale and high-quality sources. However, for clinical applications, matrices or substrates used for stem cell culture or implantation must satisfy many of the same criteria as soluble components. That is, they should be biochemically well-defined, purified to near or complete homogeneity, be bioactive via the presentation of key regulatory signals, nontoxic, non-immunogenic, and not pose risks of pathogen transfer. In addition, just as serum adds a complex mixture of poorly defined components to the soluble medium, serum proteins can also adsorb onto cell culture surfaces and thus complicate the development of fully defined soluble and solid-phase systems for stem cell culture.

An increasingly employed approach for emulating the ECM involves identifying bioactive motifs present in these molecules and grafting synthetic analogues of these signals onto a material. For example, cells engage with ECM ligands via receptors such as integrins, a major family of heterodimeric adhesion cell receptors, composed of α and β subunits, whose downstream signaling can regulate growth, differentiation, and survival.[17] Integrins are known to bind to several common polypeptide motifs such as arginine-glycine-aspartic acid (RGD),[55, 56] and chemically synthesized peptides containing this signal have been broadly used in biomaterials engineering, as discussed below.[57, 58]

In addition, it is becoming increasingly clear that not only the biochemical but also the mechanical properties of the microenvironment can modulate the cytoskeleton, the adhesion and growth of cells, and even the differentiation of stem cells;[59-61] therefore, it would be desirable to be able to finely tune the mechanical properties of the culture system. Collectively, the biochemical and mechanical signals of proteins or materials mimicking the solid phase of the native stem cell microenvironment will play a major role in controlling first the expansion and then the differentiation of stem cells for clinical applications.

Table 1: Chapter references organized by class of material utilized in each study. NSCs and NPCs isolated from various locations in the CNS of rats, mice, and humans – and from various stages of development - were used in these studies. Finally, given the strong influence that serum can exert on both the soluble and substrate components of the cellular microenvironment, the type of medium utilized is listed.

Type of Surface	Species	Stage of Develop-ment	Location of NSCs	Medium	Reference
Natural	Rat	E	Cortex	Serum-Free	72
	Rat	E	Cortex	Serum-Free	73
	Rat	E	Cortex	Serum-Free	71
	Rat	E	Cortex	Serum-Free	70
	Rat	A	SVZ	Serum-Free	76
	Mouse	E	Cortex	Serum-Free	80
	Human	N	Cortex	Serum-Free	
	Human	F	Cortex	Serum-Free	81
	Rat	F	Striatum	Serum-Free	82
	Rat	A	Hippocampus	Serum-Free	84
	Mouse	E	Cortex	Serum-Free	
	Rat	A	Hippocampus	Serum-Free	85
	Mouse	E	Hippocampus	Serum-Free	88
	Rat	A	Spinal Cord	Serum	89
	Rat	A	Hippocampus	Serum-Free	90
Semi-Synthetic	Rat	E	Cortex	Serum	91
	Rat	E	Forebrain	Serum-Free	99
	Rat	F	NR	Serum-Free	101
Synthetic	Rat	A	Hippocampus	Serum-Free	106
	Mouse	E	Cortex	Serum-Free	104
	Mouse	A	NR	NR	115
	Rat	A	Hippocampus	Serum	119
	Rat	E	Cortex	Serum-Free	122
	Mouse	N	Cerebellum	Serum	121
	Rat	E	Cortex	Serum-Free	132
	Rat	E	Cortex	Serum-Free	135
	Rat	A	Hippocampus	Serum-Free	144
	Rat	F	Hippocampus	Serum-Free	151
	Rat	A	Spinal Cord	Serum	153
	Mouse	N	Cerebellum	Serum	154
	Mouse	N	Cerebellum	Serum	150

NR = not reported, E = Embryonic, F = Fetal, N = Neonatal, A = Adult

2.2 *In Vitro* Studies

Although cells that can be expanded *in vitro* and undergo multipotent differentiation into neurons and/or glial cells have been isolated from numerous regions of an organism,[62] this review will focus on stem cells isolated directly from the CNS (Table 1). The terms "neural stem cell", "neural progenitor", and "neural precursor" have often been used interchangeably in the literature. We will use the term "neural stem cell" to refer to a population of cells with the capacity for extended self-renewal or proliferation in an immature state, as well as multipotent

differentiation into neurons and glial cells. In addition, the term neural progenitors or precursors (NPCs) refers to cells that exhibit multipotent differentiation but only have a more limited capacity for self-renewal. All of these cell populations can be grown either as neurospheres—cell aggregates in suspension—or as an adherent monolayer.

Extensive *in vitro* studies have developed two-dimensional surfaces or three-dimensional (3D) gels for culturing either relatively uniform NSC populations or to a lesser extent CNS tissue explants. In particular, these efforts have focused on engineering substrates, sometimes in conjunction with growth or other soluble factors that support or regulate specific cellular behaviors such as proliferation, differentiation into either neurons or glia, or neurite growth from neurospheres. The development of materials for *in vitro* cell culture is important for stem cell expansion and differentiation and can also serve as a first step toward the design of materials that can support the survival and engraftment of stem cells *in vivo* upon implantation.

2.2.1 Natural Surfaces and Gels

Numerous surfaces and gels have been generated from natural components such as collagen, other ECM proteins, and calcium alginate. These materials contain native biochemical signals and have enabled the attachment and expansion of many other cell types,[63-66] perhaps related to the fact that several ECM molecules including fibronectin, laminin, thrombospondin, and collagen IV are known to be present in close proximity to NSCs *in vivo*.[67-69] However, natural components can face several challenges. For example, it can be difficult to tune the mechanical properties of natural materials, and it is generally not possible to independently tune the mechanical and biochemical signals of these systems. Natural components, such as ECM proteins, also have problems with purity and the availability of large-scale sources of the materials, particularly if human proteins are involved. Regardless, studies with these natural materials provide highly valuable information and aid in the elucidation of design criteria for synthetic cellular microenvironments.

2.2.1.1 Collagen

Collagen, a triple helix protein that accounts for approximately 30% of all protein found in vertebrate animals, is present in skin, connective tissue, and many other regions throughout the body.[63] There are at least 21 types of collagen with varying biochemical and physical properties that comprise many distinct structures ranging from cornea to cartilage. Type I collagen is predominantly found in skin, bone, and tendon, where larger forces are exerted, suggesting a role in the mechanical integrity of tissue.[63]

Numerous efforts have used 3D type I collagen, which can form gels, to culture rat embryonic cortical NSCs.[70-73] In one study, O'Conner *et al*.[73] cultured neurospheres on the top of collagen I gels and found that cells were able to migrate and disperse from the spheres and subsequently extend neurite processes. Cells that migrated in the first 10 days were primarily neurons, while later migrating cells were primarily glial cells.[73] In a subsequent study, neurons in these gels were able to form networks exhibiting synaptic transmission with the neurotransmitter GABA.[72]

Ma *et al*.[71] further explored the use of the collagen I gels with embryonic cortical NSCs. Most cells remained attached to and proliferated on the gel surface during the first week of culture, and the cells that did differentiate during this initial time gave rise primarily to neurons that showed the capacity to form synapses. During the second week of culture, however, the remaining NSCs differentiated into glial cells.[71] In addition, two-dimensional gels show that

collagen supported cell attachment and culture, but 3D gels may better mimic the geometry experienced *in vivo*. Therefore, cells have been added to a collagen I solution, which was then allowed to gel to create a 3D system. Many of the neurospheres in the resulting 3D gels contained high levels of dead cells due to limited nutrient and oxygen transport, but cell viability was improved by using a rotating wall vessel (RWV) reactor.[70] Cells were first seeded into collagen I gels, and the gels were then placed into the reactor. The rotating wall reactor allowed cells to create tissue-like structures with differentiated neurons and astrocytes intermingling in the middle of the gel and NSCs closer to the surface.[74, 75] These studies utilizing collagen gels show the promise of using a 3D environment to create complex structures of differentiated cells.

2.2.1.2 Other ECM Molecules

ECM molecules other than collagen have also been used to prepare surfaces for the culture and differentiation of NSCs. For example, Matrigel is a complex mixture of laminin, collagen IV, and heparan sulfate,[52, 76] whereas E-C-L attachment matrix is a combination of entactin, collagen IV, and laminin. Both Matrigel and E-C-L, as well as single ECM molecules other than collagen, have been extracted from animal sources and used to create culture microenvironments *in vitro*. In addition, soluble factors have been tested in conjunction with these various mixtures of ECM proteins.

Whittemore *et al*.[76] explored the effects of combinations of ECM and growth factors on adult rat SVZ NPC propagation.[76] While epidermal growth factor (EGF)-treated NPCs attached to uncoated polystyrene (PS) plates and plates coated with E-C-L, laminin, and fibronectin, the cells did not expand on any of these surfaces. By contrast, NPCs grown with fibroblast growth factor-2 (FGF-2) attached to and proliferated on all surfaces except PS. Finally, NPCs exposed to FGF-2 plus heparin—which aids in FGF-2 signaling by binding to both FGF-2 and its receptor[77-79]—formed non-adherent neurospheres on plastic and attached as a monolayer to the remaining surfaces.[76] Collectively, these results demonstrated that precursor cells propagated with the same mitogen can exhibit a different behavior as a function of the substrate.

Neurospheres of postnatal human cortical NSCs and mouse embryonic cortical NSCs have been analyzed on various ECM proteins adsorbed to glass surfaces.[80] NSCs migrated from neurospheres seeded on the various surfaces, with more migration observed on laminin and Matrigel than on fibronectin or poly-L-ornithine. To stimulate cell differentiation, cultures were exposed to brain-derived neurotrophic factor (BDNF), fetal bovine serum, FGF, and all-*trans*retinoic acid. A larger number of astrocytes and neurons differentiated from NSCs on laminin and Matrigel; however, longer neurite growth was observed on fibronectin. Additionally, the α_6 integrin was also shown to be functionally important for cell attachment to laminin.[80] Once again, this study showed the importance of tuning the mixture of soluble factors and substrates to elicit specific cellular behaviors.

These studies are examples of the fact that ECM and other factors combine to regulate cell behavior, which raises the experimental difficulty of exploring many possible combinations of factors. To address this challenge, cellular microarrays, in which cells are plated on an array of "printed" features or islands of ECM and/or soluble factors, are powerful tools to test many combinations of signaling factors in parallel. A combinatorial microarray of ECM, growth factors, and morphogens was developed to analyze synergistic effects in regulating human fetal cortical NSCs function.[81] The cortical NSCs were able to form 3D web-like structures on fibronectin but only grew in a monolayer on laminin, vitronectin, and Matrigel. Laminin and soluble Wnt3A encouraged neuronal fate, while transforming growth factor-β (TGF-β) and bone

morphogenic protein-4 (BMP-4) drove glial differentiation.[81] This important study demonstrated that in constructing a microenvironment to regulate cell function, synergistic effects of signaling factors on cell behavior may be difficult to predict based on the effects of each individual component. However, complex combinations of factors, including ECM, may be necessary to achieve tight control over cell function.

Nakajima *et al*.[82] developed cellular microarrays that analyzed various ECM components, ProNectin F or ProNectin L, and different growth factors. ProNectin F and ProNectin L are recombinant proteins that form β-sheets displaying an RGD sequence from fibronectin or an IKVAV sequence from laminin at the ends of the intervening loops, respectively.[82, 83] Rat fetal striatal NPCs adhered well to fibronectin, laminin, ProNectin L, and Pronectin F but not to features/spots with just growth factors. Conditions that included EGF elicited higher proliferation rates and cellular expression levels of the intermediate filament protein nestin, a marker for NPCs. In addition, more cells differentiated into neurons on fibronectin and ProNectin L, while more NPCs differentiated into astrocytes on ProNectin F and laminin.[82] This study once again demonstrated that microarrays can yield substantial information on the combinatorial effects of substrate and soluble factors on cell function, results that will aid the development of bioactive, synthetic microenvironments.

A cellular microarray has also recently been developed for functional genomics screening in NSCs.[84] The microarray was synthesized via patterning a gold surface with a poly(dimethyl siloxane) (PDMS) stamp coated with 11-mercaptoundecanoic acid and then immersing the "stamped" surface in tri(ethylene glycol)undecane-thiol. This synthesis resulted in a surface with small regions where cells could attach, surrounded by regions containing ethylene glycol that resist cell attachment. When cells were seeded at a low density along with laminin on the microarrays, the resulting clonal populations of NSCs could be analyzed for any number of cell behaviors, including survival, proliferation, intracellular signaling, and differentiation.[84] NSCs were infected with viral vector carrying a cDNA library, and the cellular microarray was used to screen the resulting NSC population for cDNA clones that enabled cell proliferation in low growth factor concentrations.[84] This study demonstrates that a variety of functional genomic screens can be implemented on high-throughput microarrays for gene discovery. The implementation of such high throughput gene function screens on cellular microarrays coated with a variety of ECM molecules could further elucidate connections between ECM-related signaling and cell behavior.

In addition to high-throughput screens, surface patterning can be used to analyze the effects of spatially organized signaling factors on cellular behavior. For example, adult rat hippocampal NSCs have been cultured on laminin-coated surfaces that were first patterned via photolithography on silicon wafers and then transferred to PS via solvent casting. The surfaces exhibited parallel strips of alternating heights that were 13 μm wide, 4 μm high, and 16 μm apart.[85] While NSCs that were differentiated on unpatterned surfaces had randomly aligned processes, the surfaces with parallel wells yielded differentiated neurons with processes aligned along the direction of the grooves. When co-cultured with astrocytes on these surfaces, NSCs extended processes along the cytoskeletal filaments of the astrocytes, while the astrocytes spanned and thereby made contact with neurons on different grooves. Furthermore, prior studies have shown that astrocytes can promote neurogenesis.[86, 87] In the co-cultures of astrocytes and NSCs on these patterned substrata, more of the NSCs differentiated into neurons, which in turn exhibited longer processes.[85] This study demonstrated the effects of both substrate patterning

and cell co-cultures on cell differentiation and alignment, findings that could potentially be applied toward the assembly of implantable neural prostheses and cell-based devices.

2.2.1.3 Calcium Alginate

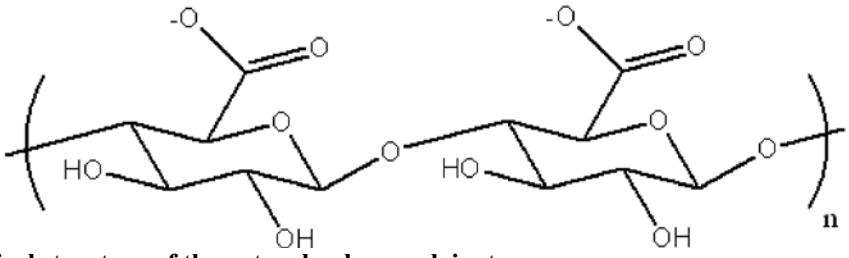

Figure 2-2. Chemical structure of the natural polymer alginate.

Alginates, polyanionic polysaccharides that are isolated from brown sea algae and contain mannuronic and guluronic acids (Figure 2-2), gel in the presence of bivalent cations such as calcium and barium.[64-66] Because alginates are both biocompatible and inexpensive, they have been broadly explored in cell encapsulation and tissue-engineering applications.[64] Recently, Li *et al*. encapsulated mouse embryonic hippocampal NPCs in calcium alginate microcapsules. The cells proliferated and maintained nestin expression along with the ability to differentiate into neurons and glial cells.[88]

In another study, rat adult spinal cord NSCs expressing green fluorescent protein (GFP) were grown in 3D calcium alginate gels with capillary channel features, which were formed by the oriented diffusion of copper ions during gel formation prior to the addition of cells. This geometry was designed to promote directional axonal growth through a site of injury and thereby aid axon regeneration.[89] ECM proteins adsorbed on the gels did not significantly change the density of axons or the length of axon ingrowth into the channels as compared to channels without ECM proteins. To assess the performance of the biomaterial in an organotypic culture, NSCs were seeded in calcium alginate gels for 7 days in serum-containing medium and then transplanted into the region between the entorhinal cortex and the hippocampus in rat brain slice cultures. The resulting brain slices exhibited GFP-expressing glial cells and neurons with axons aligning along the capillary features of the gel.[89]

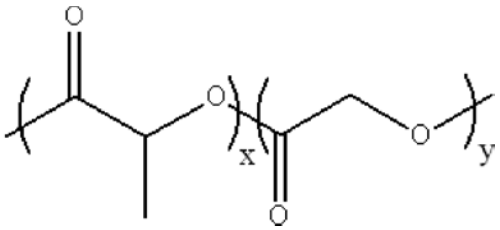

Figure 2-3. Chemical structure of the repeating unit for the copolymer poly(lactic-co-glycolic acid) (PLGA).

Another system developed by Ashton *et al*.[90] explored alginate hydrogels, embedded with poly(lactide-co-glycolide) PLGA (Figure 2-3) microspheres containing the enzyme alginate lyase, for NSCs transplantation. Because alginate hydrogels are not naturally degraded enzymatically *in vivo* in mammals, the addition of encapsulated alginate lyase allows for the controlled degradation of the alginate hydrogel.[90] Without the lyase, the NSCs exhibited elongated processes, while gels with the encapsulated lyase supported proliferating NSC

neurospheres. These studies show the potential of calcium alginate for engineering microenvironments for NSCs. Furthermore, these results indicate that when encapsulated in some materials, cells can presumably provide their own signals and therefore do not require the addition of ECM molecules, although adding exogenous signals may afford more control over cell behavior.

2.2.2 Semi-synthetic Surfaces and Gels

Surfaces and gels have also been developed using a blend of synthetic and natural components. The natural component in these blends is typically an ECM protein that is adsorbed to the synthetic component and presents signals to modulate cell attachment, growth, and differentiation. Moreover, the addition of a synthetic component enables control over the architecture and mechanics of the materials. These bioactive, modular materials can therefore be viewed as an intermediate step toward developing completely synthetic materials, although the ECM protein still poses challenges for purity, immunogenicity, scaleability, and other considerations.

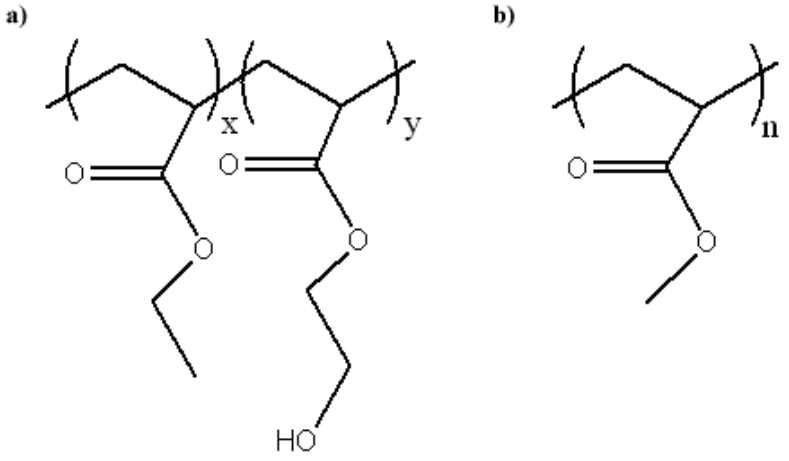

Figure 2-4. Chemical structure of repeating units of (a) poly(ethyl acrylate)-co-poly(hydroxyethyl acrylate) and (b) poly(methyl acrylate).

Soria *et al.*[91] tested the behavior of rat embryonic neural explants from the medial ganglionic eminence and ventricular zone of the cerebral cortex on various hydrophobic and hydrophilic polymers coated with laminin. The polymers that best supported cell adhesion and differentiation were poly-(ethyl acrylate)-co-poly(hydroxyethyl acrylate) (Figure 2-4a), poly(methyl acrylate) (Figure 2-4b), and chitosan, which are polymers with an intermediate degree of hydrophobicity. Chitosan is a *N*-deacetylated derivative of chitin, a component in the exoskeletons of many insects (Figure 2-5).[92, 93] All three materials exhibited differentiated neurons and glial cells, although it was not clear whether these mature cells arose from precursor cells or were already present in the explant. Importantly, the adsorbed layer of laminin was necessary for successful explant culture, and the conformation of the laminin adsorbed on each polymer was likely a key factor in the relative success of each material, as the orientation of ECM proteins can greatly affect cellular behavior, including attachment and proliferation.[91, 94-98]

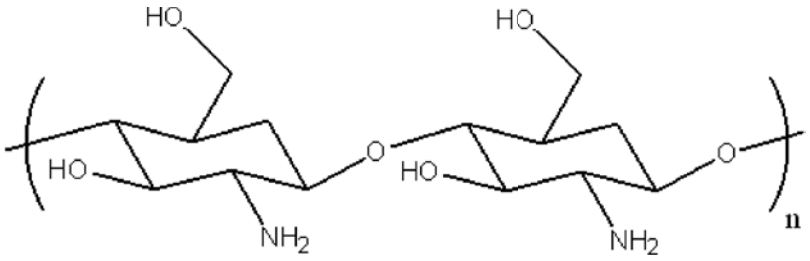

Figure 2-5. Chemical structure of chitosan, a natural polymer derived from chitin.

Other ECM proteins such as collagen have also been used in conjunction with a synthetic polymer. Rat embryonic forebrain NPCs were cultured on a triblock copolymer, consisting of poly(ethylene glycol) (PEG) (Figure 2-6) flanked by poly(lactic acid) blocks.[99] NPCs grown on this material in the presence of FGF-2 exhibited increased cell growth, although the addition of collagen only to the cell suspension prior to cell plating had no real effect, indicating that collagen does not exhibit an effect without FGF. When FGF-2 and collagen were used in combination, apoptosis decreased and metabolism increased, although the total number of cells and the relative level of β-tubulin content did not significantly change as compared to when no FGF-2 or collagen was used.[99] Finally, FGF-2 and collagen conditions produced neurons with longer processes as compared to the other conditions with either FGF-2 or collagen.

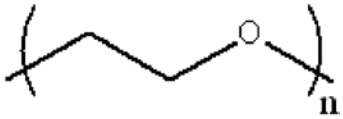

Figure 2-6. Chemical structure of the repeating unit of PEG.

As a final example of a semi-synthetic material employing a non-ECM protein, a mixed self-assembled monolayer (SAM) composed of 16-mercapto-1-hexadecanoic acid and (1-mercaptoundec-11-yl) triethylene glycol (TEG) thiol was generated on gold. After the carboxylic acid of the SAM was esterified, Ni-NTA was chelated to the surface followed by the addition of a recombinant hexahistidine-tagged EGF.[100, 101] Although cell attachment was initially weak, cells attached specifically to the surface via the EGF receptor (EGFR), as the addition of soluble EGF blocked attachment.[101] Fetal NPCs could be maintained on the surface for five days and retain their multipotency, as they were still able to differentiate into both neurons and glia. This maintenance may be related to previous work showing that EGFR and nestin expression can be correlated.[102] These studies collectively demonstrate that natural components can provide biochemical signals necessary to support cell attachment, proliferation, and differentiation when presented from a synthetic substrate. Promising semi-synthetic materials also provide a promising basis for the development of fully synthetic materials that avoid some challenges of using isolated proteins, as these can potentially be replaced with recombinant or synthetic signals.

2.2.3 Fully Synthetic Surfaces and Gels

Natural ECM proteins offer the important advantage of presenting both identified and likely unidentified motifs that bind to cellular receptors and thereby regulate cell behavior. However, natural components have the potential to elicit an immune response if implanted, can transfer immunogenic molecules to stem cells,[48] can pose a risk of pathogen transfer, and often do not offer the capacity to readily control the mechanical properties of the material. By

comparison, materials composed of primarily synthetic components offer advantages including low immunogenicity, reproducible and scaleable synthesis, and the ability to tune mechanical and biochemical properties, an important consideration for stem cells.[59-61] However, bio-functionalizing synthetic materials to present signals to support cell survival, proliferation, and differentiation can be challenging and may involve elaborate synthesis or conjugation schemes. Furthermore, it can be difficult to generate synthetic analogues of complex bioactive motifs, particularly when the intricate signals present within large ECM proteins may not be fully characterized.

2.2.3.1 Self-Assembling Peptides and Peptide Amphiphiles

Specific polypeptide sequences have the capacity to self-assemble into various structures, ranging from assembly of β-sheets via hydrogen bonding to cylindrical micelles via hydrophobic interactions.[83, 103, 104] To build upon these capabilities for creating bioactive matrices, the self-assembling peptide sequences can be synthesized as fusions to motifs found in ECM proteins, including RGD and IKVAV from fibronectin and laminin,[56, 105] respectively, to create self-assembled structures that can engage cellular adhesion receptors. These synthetic peptides also offer the advantage of being able to display a broad diversity of natural and even unnatural side chains from the peptide backbone, enabling the creation of multifunctional assemblies.

One example of such a self-assembling material is a triblock protein containing an RGDS motif.[106] The protein is designed with a random coil region flanked by two identical amphiphilic leucine zipper sequences that allow for the formation of helices that can multimerize with the termini of other copies of the polypeptide, allowing for self-assembly into a gel.[107-109] Incorporation of the RGDs into the random coil region allowed for better adhesion of individual adult rat hippocampal NSCs, while cells formed non-adherent neurospheres on surfaces of gels lacking the RGD motif.[106] Silva *et al*.[104] developed an IKVAV-containing peptide amphiphile unit that self-assembles into micelle nanofibers, via hydrophobic forces, for use with E13 mouse embryonic cortical neuronal precursor cells.[104, 110, 111] In nanofiber scaffolds, the neuronal precursors differentiated into neurons with extensive processes, while very few cells differentiated into astrocytes.[104, 112-114]

Another self-assembling peptide system has been developed using motifs from ECM proteins, such as YIGSR, RGDS, and IKVAV, and bone-homing peptides.[103, 115-117] The latter had previously been isolated via *in vivo* phage display, in which a phage library is injected into animals for the identification and isolation of displayed peptides that mediate viral localization to a specific tissue, in this case bone marrow. The bone-homing peptides were employed based on reports that bone marrow-derived cells could differentiate into neuron-like cells.[118] Peptides composed of self-assembly domains fused to bioactive motifs and formed a 3D fibrous structure driven by β-sheet formation in the presence of salt, similar to structures seen via scanning electron microscopy with Matrigel.[103, 116, 117] NPCs were seeded on top of and subsequently infiltrated into the scaffold. The cells differentiated into neurons and astrocytes on all functionalized peptide networks, and the highest cell viability was observed on the self-assembling peptides with the bone-homing peptides.[115]

A study using peptides that assemble into fibrous structures via β-sheet formation showed that this scaffold encouraged putative neural stem or precursor cells from adult rat hippocampal slices to migrate away from tissue explants laid on top of the scaffold.[119] Cells expressing nestin, as well as larger number of neurons and glia, were found in the scaffold following this migration. In addition, the cells that infiltrated into the scaffold could subsequently be recovered and

cultured on laminin-coated plates.[119] This system could therefore represent a useful method for extracting stem cells from tissue slices, although additional characterization will be required to validate that the isolated cells are true stem cells, that is, capable of self-renewal and multipotent differentiation.

Collectively, these self-assembling materials represent a unique set of building blocks that form complex structures in conjunction with presenting biochemical polypeptide signals similar to the ECM molecules mentioned earlier. These highly modular systems can be designed to incorporate multiple biochemical signals, with peptides that assemble into other complex geometries or into structures whose mechanical properties can be tuned.

2.2.3.2 Synthetic Polymers

NSCs have also been cultured on numerous synthetic polymers, many of which have previously been used with other cell types for many applications including tissue engineering and controlled drug delivery.[12, 120, 121] Optimizing these materials may lead to the development of reproducible, scalable, nontoxic, and non-immunogenic materials for *in vitro* expansion or differentiation, as well as *in vivo* implantation, of NSCs.

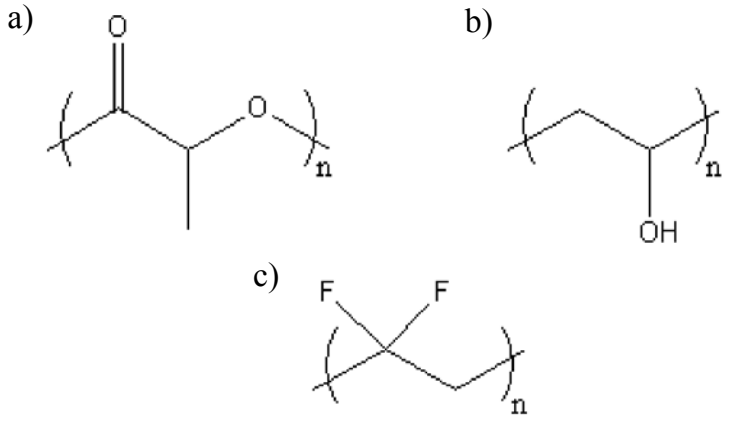

Figure 2-7. Chemical structure of the repeating unit for (a) poly(L-lactic acid) (PLLA), (b) poly(vinyl alcohol) (PVA), and (c) poly(vinylidene fluoride) (PVDF).

Wang *et al.*[122] used PS surfaces coated with synthetic poly-D-lysine or lysine-alanine sequential (LAS) polymers for culturing rat embryonic cortical NSCs.[122-124] The LAS copolymers were designed with repeating units of lysine-alanine to form an ordered copolymer chain.[123, 124] When a low density of neurospheres was plated on these surfaces, cells attached and extended processes that were sufficiently long to interconnect the spheres. However, when higher numbers of neurospheres were seeded on the surfaces, cells migrated from the spheres and differentiated into astrocytes but did not form processes to connect the spheres.[122] Analogous distinct behavior as a function of cell density has been observed in other studies with neurons and NSCs.[125-127]

Electrospinning is a technique that applies a strong electric field across an extruding polymer solution to greatly elongate the solution stream, thereby depositing thin filaments of polymer onto an underlying surface. To create a fiber network, a spinning disk is used as the collection surface, and a dense nanofiber mat is created from the single fiber. The fiber dimension and size, as well as the polymer composition, can be precisely controlled.[128] Poly(L-

lactic acid) (PLLA) (Figure 2-7a) has been used previously for nerve tissue regeneration because of its biocompatibility and biodegradability.[129] To extend upon these capabilities, mouse neonatal cerebellar NPCs were cultured on electrospun PLLA nano- and microfiber scaffolds and subsequently differentiated into cells with neurites aligned with the fibers. Importantly, cells exhibited longer neurites on nanofibers than on microfibers,[121] demonstrating that the size and topology of scaffold features can modulate cell differentiation and morphology.

Young *et al*.[12] studied rat embryonic cortical NSC behavior on the hydrophobic polymer polyvinyl alcohol (PVA) (Figure 2-7b) as well as amphiphilic poly(ethylene-co-vinyl alcohol) (EVAL) surfaces,[12, 130, 131] based in part upon prior work with differentiated cortical neuronal cultures on EVAL.[132-134] Cells, either as single cells or as neurospheres grown in serum-free medium, were not able to attach or survive on the PVA surface, while single cells attached to but did not proliferate on the EVAL. In addition, neurospheres at lower density attached to EVAL surfaces and extended neuritis between the spheres, while a higher density of neurospheres did not attach to the surface or differentiate.[12] This study again shows the important effect of biomaterial chemistry—specifically the relative hydrophobicity of the polymer—along with cell density, on cellular behavior.

Rat embryonic cortical NSCs have also been grown in serum-free medium on PVDF (Figure 2-7c) and chitosan surfaces.[135] The PVDF material has previously been used as a biomaterial to aid in nerve regeneration, in part due to its mechanical strength.[136-138] On both surfaces, cells within neurospheres extended processes between the spheres, with shorter processes formed on PVDF.[135] PVDF surfaces also biased cell differentiation toward astrocytes, while chitosan surfaces favored a neuronal fate. Finally, a population of proliferating cells was maintained on PVDF but not on chitosan.[135] Future work may elucidate the chemical differences between these surfaces that elicit this distinct behavior.

Bio-functionalized interpenetrating polymer networks (IPN) have also been used to culture stem cells.[139] This IPN is composed of polyacrylamide interpenetrated with a PEG and poly(acrylic acid) network.[140, 141] The IPN modulus can be controlled by tuning the amount of cross-linker incorporated into the polyacrylamide network, and biochemical signals can be grafted to polymer termini in the PEG network, which also prevents nonspecific protein adsorption. Importantly, the mechanics and chemical signals can therefore be tuned independently to create a non-fouling surface.[142] For example, the material has been functionalized with a number of synthetic peptides and even recombinant proteins.[143] When presenting a peptide motif derived from bone sialoprotein, bsp-RGD(15), the surface could support either the proliferation or the differentiation of NSCs in serum-free medium, depending on the soluble media conditions.[139] This system is therefore the first fully chemically and biochemically defined NSC culture system.

In summary, fully synthetic, bio-functionalized materials can support cell proliferation, and the addition of differentiating media leads to multipotent differentiation. Future work may explore the extent to which the substrate can guide cell lineage commitment. Furthermore, the use of thick gels can enable studies of the effects of matrix mechanics on NSC proliferation and differentiation.[144]

2.3 *In Vivo* Studies

There have been extensive efforts to implant neural stem or progenitor cells for neural repair in the absence of a scaffold.[145-147] Previous studies without materials have shown that the implanted cells can aid in tissue repair via several mechanisms, including paracrine delivery of

therapeutic molecules, thereby reducing the toxic nature of the microenvironment, and in some cases potential functional cellular integration into host tissue.[148-150] However, a major problem is that generally only a small fraction of stem cells or their differentiated progeny survives when implanted, often due to inflammation and hypoxia present at the site of injury or disease.[44, 45, 47, 148] Current methods of scaffold synthesis can allow for considerable control over the cellular microenvironment, which can serve as the basis of implantable materials to enhance the survival of engrafted cells.

Wu *et al*.[151] implanted rat fetal hippocampal NPCs encapsulated in an alginate gel into the spinal cords of rats, as this material has supported the growth and survival of NPCs *in vitro* and *in vivo*.[151, 152] When monolayer-grown cells were implanted, there was not successful incorporation into host tissue. However, when neurosphere-grown NPCs were dissociated, incorporated into an alginate sponge, and implanted, the cells integrated well into the host tissue and extended processes into the surrounding tissue. Most cells differentiated into astrocytes, with some neurons.[151]

Another study implanted a mesh of poly(glycolic acid) (PGA)—which was saturated with pluronic F127 and covered with a hydroxymethylcellulose membrane—into a transected spine.[153] When the material was co-implanted with rat adult spinal cord NSCs, the resulting graft developed neurons along with some astrocytes, and axons from the NSC-derived neurons were able to extend beyond the area of injury and potentially help the injured animals regain partial coordinated use of their hind limbs 4 weeks post-surgery. Animals implanted with just the polymer scaffold regained some use of their hind limbs, but the movements were not coordinated, as seen in the animal group implanted with scaffold and cells. By contrast, animals implanted without scaffold had the formation of scar tissue and astrocytes surrounding the scar, with very little behavioral improvement.[153]

A unique scaffold using two synthetic material layers was analyzed for the ability to aid the regeneration of injured spinal cord. Both layers were made of PLGA and a block copolymer of PLGA-polylysine; however, one was seeded with murine neonatal cerebellar NPCs, whereas the other contained long axially aligned pores to allow for axonal guidance.[154] The bilayer material was implanted such that the first layer lay against the exposed gray matter in a midline lateral hemisection of the spinal cord. The scaffold, with and without cells, mediated recovery of hindlimb function, although using both cells and scaffold mediated the best overall improvement. The scaffold also allowed for axon extension beyond the site of implantation.[154] This work thus successfully used a combination of polymer chemistry and macroscale structure to yield a therapeutic result.

Park *et al*.[150] explored the implantation of a scaffold made of woven PGA fibers, which had been used previously as a transplant scaffold for cartilage repair, with neonatal murine cerebellar NPCs into mice with hypoxic-ischemic injury.[150, 155] On this scaffold *in vitro*, the NPCs spontaneously differentiated into both neurons and glia that extended processes to wrap around several PGA fibers. After 4 days of culture and subsequent implantation, this scaffold exhibited differentiated neurons and glial cells and even host neuron and oligodendrocyte cell infiltration into the scaffold. After degradation of the biodegradable scaffold, vascularization was seen in the graft. Furthermore, donor neurons were able to establish long-distance connections to the corpus callosum, and the scaffold showed little evidence of an immunological response.[150]

Each of these *in vivo* studies shows the promise of PGA, PLGA, and alginate materials in tissue engineering for the spinal cord and brain. Donor NSCs were able in some cases to aid in

recovery from the injury and differentiate *in vivo* into different proportions of glial and neuronal cells. In addition, these observations were dependent on the chemical microenvironment created by the material, as well as its topological structure, since different results were seen with and without the use of a scaffold. Furthermore, host neurons and glial cells were even able to incorporate into one of the scaffolds. Finally, results were generally better when both the cells and the scaffold were used, showing the combined promise of biomaterials and NSCs in tissue regeneration. Future work may explore the potential of bioactive materials to actively engage cellular signaling, as well as materials with controlled biodegradation properties.

2.4 Conclusions

Neural stem cells are very promising for the treatment of neurodegenerative disorders and injuries of the CNS. Engineered materials containing natural and/or synthetic components can support the expansion and potentially in the future induce the lineage-specific differentiation of NSCs *in vitro*, with a variety of applications ranging from cell replacement therapy to *in vitro* diagnostics and screens. Furthermore, highly modular systems that enable the independent variation of mechanical and multiple biochemical signals have strong potential for the application of reductionist biology approaches to understand fundamental mechanisms of stem cell behavioral regulation. However, a number of challenges remain in the design of materials that are non-immunogenic, scalable, mechanically tunable, and bioactive in their presentation of key regulatory signals to cells. Synthetic materials have considerable promise for offering these capabilities, although challenges remain in the development of synthetic analogues of complex biochemical signals such as ECM proteins. If these challenges can be overcome, however, bioactive materials can be designed to present a microenvironment that can not only support cells *in vitro* but also protect them in the harsh environment of a diseased or injured region of the CNS and thereby greatly aid stem cell-based regenerative medicine.

2.5 References

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Chapter 3:
Biomaterials for Culture of
Human Embryonic Stem Cells

3.1 Matrigel

Human embryonic stem cells (hESCs) were first propagated on mitotically-inactivated mouse embryonic fibroblasts (MEFs),[1] but quickly other options for non-cellular substrates were developed. The first major development for hESC growth without MEFs was the use of Matrigel[2, 3]—a commercially available mixture of collagen IV, laminin, and many other proteins extracted from Engelbreth-Holm-Swarm sarcoma cells—with MEF-conditioned medium, or medium exposed to cultured MEFs for 24 hours.[4] While this method still relied on MEFs to generate factors for hESC colony maintenance in the cell medium, the system moved beyond the need for direct cell-cell contact.

Other groups have explored using Matrigel on other substrates besides on tissue-culture polystyrene (TCPS). For example, Matrigel has been reported as a coating for 3D culture of aggregates of hESCs in microwells.[5] These microwells were made using PDMS stamps on glass slides that were then filled with polyurethane. Self-assembled monolayers (SAMs) were formed on all surfaces except for the bottom of each microwell, which was then coated with Matrigel. hESCs were able to stay in these microwells for weeks and maintain expression of pluripotent markers such as Oct-4, but it was not reported if the cells proliferated over this period of time. Since uniform cell aggregates can be formed with this system, this system has promise for the generation of uniform embryoid bodies (EBs) for differentiation, perhaps more so than for culturing hESCs.[5]

Kohen, *et al*. explored the use of Matrigel coated onto TCPS, polystyrene (PS), and on glass.[6] The difference between TCPS and PS is that TCPS has been functionalized with oxygen groups from an oxygen plasma to render the surface more hydrophilic and thus more conducive for cell attachment and growth. While glass and TCPS have similar hydrophilic properties, Matrigel-coated glass surfaces were not able to support cell proliferation and self-renewal of hESCs as Matrigel-coated TCPS surfaces were. PS surfaces coated with Matrigel were shown to have much less cell attachment, and also did not support the culture of hESCs. Using scanning electron microscopy (SEM) and a quartz crystal microbalance (QCM), Matrigel was shown to deposit fibrillar networks on TCPS and glass, but on PS a globular network was seen. The higher density network on glass compared to on TCPS, along with the different network morphologies, were suggested as possible explanations for the observations seen with hESCs on these Matrigel-coated surfaces.[6] The findings of this study support the current use of Matrigel on TCPS, but not on other polymers, even ones with similar hydrophilicity to polystyrene.

Although Matrigel is the still the standard substrate in hESC culture, there are several problems with its use including the potential for immune reactions, the risk of transmitting animal pathogens, and the variability in the material.[7, 8] Specfically, exposure of hESCs to mouse proteins, such as those found in Matrigel, cause the cells to express non-human sialic acids on the cell surface. As antibodies for these sugars are present in human blood, the risk of immune reactions to hESCs propagated on Matrigel is quite high.[7] In addition, the variability in lots of Matrigel is not surprising given the complex nature of the process (the material is composed of protein extract from the tumor generated after murine engraftment of a cell line), and because this mixture contains several hundred different proteins.[8] Although Matrigel coated onto TCPS plates has been the standard substrate for hESC culture, other natural and synthetic alternatives have been developed, but these still haven't replaced Matrigel as the default substrate for culture.[9]

3.2 Natural Alternatives to Matrigel

One alternative to using Matrigel was using plates coated with human serum.[10] Plates coated with human serum were shown to have similar crystal-like structures as Matrigel using SEM, and were able to support the growth and self-renewal of hESCs when used with conditioned medium from fibroblast-like cells differentiated from hESCs for up to 21 passages.[10] Although this system avoids the use of non-human proteins, human serum is even more heterogeneous than Matrigel. In addition, the use of a conditioned media is not ideal, since there is variability in the factors in these media depending on the viability of the cells used for conditioning.

Another approach for using natural materials to replace Matrigel has been the use of a single or combination of several different extracellular matrix (ECM) proteins. Li, *et al*. explored the use of human laminin as a substrate for hESC culture for use with their defined medium of X-Vivo 10 medium supplemented with fibroblast growth factor 2 (FGF-2) and transforming growth factor β 1 (TGF-β1).[11] Although hESCs were shown to proliferate and maintain pluripotency with human laminin, there is considerable variability in this material as laminin is not one molecule, but several molecules with different isoforms and glycoforms. In addition, the use of human materials has a high risk of transmitting human pathogens.[12]

A recent study has examined the various laminin chains excreted by hESCs and found that hESCs synthesize laminin α_1, α_5, β_1, and γ_1 chains, indicating that they produce laminin-111 and laminin-511.[13] Interestingly, laminin-511 was able to support the short-term growth of hESCs similar to Matrigel.[13] One of the more interesting studies in developing defined culture condtions for hESCs was one using a combination of collagen IV, fibronectin, laminin, and vitronectin in combination with a defined medium called mTeSR1 utilizing FGF-2 and TGF-β1.[12] While the mTeSR1 medium has been commercialized, the defined substrate currently recommended for use with it is Matrigel.

Calcium alginate, a biomaterial derived from algae that gels in the presence of divalent cations,[14-16] has been used with human embryonic stem cells. Two studies have used calcium alginate to encapsulate hESC aggregates.[17, 18] Similar to the 3D Matrigel microwell system, calcium alginate allows for creation of more uniform EBs for differentiation as these systems have not been reported to have similar cell proliferation as seen with Matrigel.[17, 18] Finally, hyaluronic acid (HA) has been used as a hydrogel to maintain hESCs as aggregates.[19] HA is a glycosaminoglycan that is present during early embryogenesis and also regulates proliferation and gene expression of hESCs *in vivo*.[20, 21] Unlike the calcium alginate systems, the hyaluronic acid hydrogels were reported to support proliferation similar to Matrigel.[19]

Although several natural alternatives to Matrigel have been explored, a recent study comparing various biomaterials for hESC culture including human serum, various mixtures of ECM proteins, and Matrigel found that Matrigel was a far superior substrate for hESC culture in defined medium.[9] This study highlights that even though different natural materials have some advantages compared to Matrigel, they cannot support the long-term culture of hESCs as Matrigel does. Hence the development of synthetic substrates, which have many advantages over natural materials, is needed to replace Matrigel for long-term hESC culture.

3.3 Synthetic Alternatives to Matrigel

Synthetic materials have some advantages over natural materials, including lower risk of immune reactions and pathogen transmission as well as the decoupling of mechanical and chemical signals.[22] It is increasingly clear that cells respond to both biochemical and mechanical

cues. In systems based on natural molecules such as ECM proteins, these two cues are presented by the same molecule, while many synthetic materials allow for these variables to be controlled independently.[23] A final advantage of synthetic materials is that they are more homogeneous than Matrigel. As previously found with other cells, however, most polymers on their own do not support the culture of hESCs, so many of these systems use synthetic peptides as biochemical signals.[24, 25] However, unlike NSCs, simple RGD-containing peptides do not appear sufficient to support more than short term hESC self-renewal,[25] so that alternative peptide ligands must be identified.

One way to test many different synthetic polymers or combinations of polymer to see which ones promote the culture of a cell type is to use microarrays. Anderson, *et al*. developed a microarray with many combinations of 24 different polymers.[26] This proof-of-concept study showed the promise of microarrays to study many polymers at once. A second synthetic surface investigated with hESCs was a 3D matrix made of peptide-grafted nanofibers to replace the ECM proteins used previously. The peptides were found through binding-based selections of hESCs against self-assembled monolayer (SAM) surfaces with laminin-based peptide brush layers.[24] Although the surface was completely synthetic, the medium used was serum-based. The use of SAMs for testing the peptides also had the disadvantage that the peptides were presented at a high density, allowing cells to only have access to the end of the peptides such that the degree of specific interaction between the peptide and the cell is somewhat unclear. In addition, only a very narrow selection of peptides was investigated.

A later study with these SAM arrays utilized phage display to find new peptides to display instead of using ECM-based peptides.[27] To assess the effectiveness of library selection, a lacZ/X-gal assay was developed for use in the selections. Wild-type phage not containing the lacZ gene were mixed at a constant ratio in each round of selection, and after the panning step the resulting phage were infected on bacteria on plates containing X-gal. Library phage turned blue, while the wild-type stayed white, allowing for assessment of the phage recovered.[27] Several of the selected peptides were tested on the SAM arrays, and were shown to support short-term growth of hESCs. The main drawback of this study was the selected peptides were not shown to bind significantly to integrins, as occurs on Matrigel with hESCs,[25] based on little inhibition due to ethylenediaminetetraacetic acid (EDTA).[27]

Another peptide-based synthetic matrix investigated with hESCs was a semi-interpenetrating network (sIPN) of several polymers. The sIPN was made of N-isopropylacrylamide crosslinked with an acrylated peptide along with RGD peptide-grafted linear polyacrylic acid chains penetrating into the network.[28, 29] hESCs were able to attach well to and maintain short-term self-renewal on the sIPN, though there was a large variability in the colony morphology.[28] Additional work with hESCs focused on identifying the specific integrins expressed and important for attachment of hESCs to a Matrigel surface.[25] Combinations of adsorbed peptides on TCPS were then tested for the ability to mimic the integrin attachment profile seen on Matrigel. The best combination, with three peptides, activated the $\alpha_2\beta_1$ and $\alpha_v\beta_3$ integrins similar to Matrigel, but failed to significantly activate the $\alpha_6\beta_1$ integrin as seen with hESCs on Matrigel.[25]

The difficulty with developing synthetic culture systems for stem cells including hESCs is finding novel peptides that bind to cellular receptors and encourage cellular signaling. Although the RGD peptide was able to support self-renewal and allow for differentiation of NSCs,[30] this same peptide only supported short-term self-renewal of the hESCs. Finding novel peptides that bind to and encourage self-renewal of both NSCs and hESCs would be beneficial

for creating defined, scaleable systems for cell expansion, as well as for enhancing our knowledge of self-renewal signaling mechanisms. Since an RGD peptide does support the culture of NSCs, it would be beneficial to use this cell type to verify a method for finding novel peptides. Once developed, this method could then be used for finding novel peptides for hESCs that can be used in materials to replace Matrigel.

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Chapter 4:
Selection of Novel Peptides to Functionalize Biomaterials for Neural Stem Cells

4.1 Introduction

Many cell types, including stem cells, require interactions with extracellular matrix (ECM) molecules for survival and proliferation.[1, 2] ECM molecules play many roles including acting as an element in structures including the basement membrane, providing a scaffold for cell migration, and acting as ligands for cell receptors, including integrins.[3-5] Integrins are heterodimeric transmembrane receptors that transduce ECM molecule signals to the intracellular cytoskeleton.[6-8] As well as anchoring cells to matrix molecules or to other cells, integrins act as mechanosensors for their environment.[8] Exploring these receptors by learning more about their ligands, including ECM proteins or integrin-binding peptides, could aid in understanding the signaling mechanisms of integrins. Although animal-derived ECM molecules are used in cell culture, the use of animal-derived proteins for ECM is highly problematic for numerous reasons. Natural ECM molecules are enormous (e.g. ~500,000 MW fibronectin and ~850,000 MW laminin), are extremely complex (with numerous isoforms, splice variants, and glycoforms), and have numerous signaling motifs that are not yet fully understood.[9] In addition, there is considerable lot to lot variability in animal-derived ECM because of the many forms present.[10, 11] Therefore, there is a need to develop a system that allows reproducibility for scientific studies and clinical processes.

A potentially powerful alternative approach is to design and develop synthetic platforms that are grafted with synthetic peptide ligands to replace ECM proteins typically used in cell culture.[11, 12] Development of these new materials could aid basic investigation of ECM signal transduction and could help promote the development and production of cell-based therapies for the clinic.[13-17] One problem with developing these biomaterials is finding an appropriate peptide or combination of peptides that can recapitulate the complex signals from these ECM molecules. Although various cell-binding domains—such as RGD and IKVAV motifs—[18, 19]have been identified in many of these proteins, many of these domains when presented as small peptides are not as bioactive as the ECM proteins as evidenced by the fact that few of them support the culture of cells.[11, 18-24] In addition, many of the important signaling domains in ECM proteins may not be known, and for example only recently has the α_v binding domain in fibronectin been identified.[25] Finally, in general there is no guarantee that the optimal peptide ligand for a given receptor exactly matches a portion of the sequence of its natural ECM ligand. Therefore, there is a need to develop a general method to identify new candidate peptides that can be grafted onto biomaterials to replace these ECM proteins**.**

Because of the large number of potential peptide sequences possible, even for a very short peptide, rationally designing a peptide ligand is very challenging, but library-based screening methods can overcome this problem. With libraries, many peptides with random sequences can be tested simultaneously and thus can facilitate finding novel peptides even with little knowledge of the motifs required for adhesion receptor engangement.[26, 27] While phage display libraries have been used in many studies, as they were developed earlier, newer display libraries using bacteria as a display platform do not require infection steps for amplification in the selection process and are easier to manipulate for library creation.[26-30] Also, previous phage display studies focused on finding cell adhesion receptors antagonists, including for integrins, rather than identifying peptides agonists for these receptors.[31-35] In addition, it is easy to incorporate fluorescent proteins in bacteria that can be helpful in analyzing and selecting the libraries.[36-38] Using this method to target adult hippocampal neural stem cells (NSCs), we have shown that this method yields peptides containing homology to ECM proteins. As peptides adsorbed on tissue culture polystyrene (TCPS) or grafted to an interpenetrating polymer network

surface, several of these short ligands were able to support self-renewal of NSCs. Overall, this method produces a group of peptides, including some that seem to act as cell receptor agonists, for a desired cell type.

4.2 Results

To find novel peptide candidates to replace ECM proteins, we used bacterial peptide display selections, which are a versatile method for novel peptide ligand selection for several reasons. Unlike bacteriophage libraries that require viral infections for amplification, a selected population of bacteria can be amplified by simple culturing, and the peptide valency on bacteria can be adjusted to change the selection stringency.[27, 37, 38] Bacterial plasmids are also easy to manipulate, allowing for easy library creation with each library containing bacteria with random peptide sequences on the bacterial surfaces. In addition, with the expression of fluorescent proteins in the library bacteria, mammalian cells with bound bacteria can be analyzed or sorted with flow cytometry for library analysis and rapid selection.[36-38]

4.2.1 Description of the Method

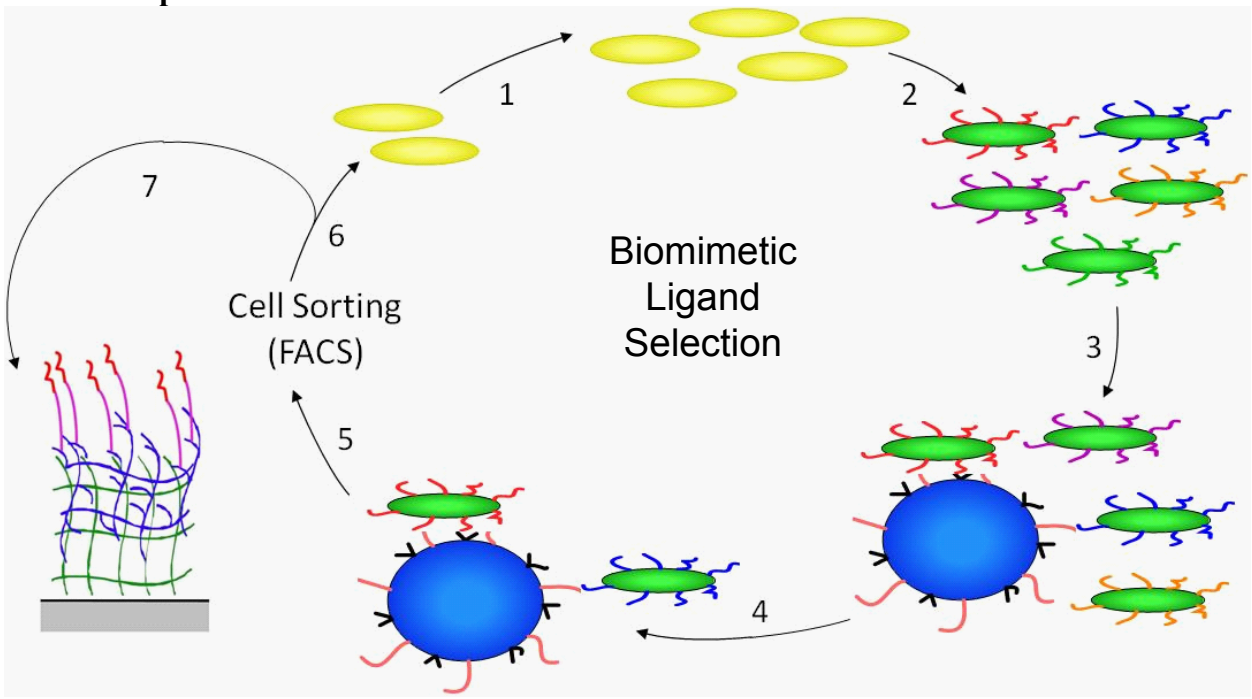


Figure 4-1. Schematic of biomimetic ligand selection and incorporation into biomaterials. (1). Bacterial libraries were expanded. (2). Co-expression of green fluorescent protein and bacterial outer membrane protein CPX with the displayed peptide are induced with arabinose. (3). Stem cells are added to bacterial libraries in a co-incubation step in which the bacteria can bind to the stem cell surface. (4). Non-adherent bacteria are washed away with low speed co-centrifugation. (5). For third round selections or analysis of bacterial populations, samples of the stem cells are sorted or analyzed on a fluorescence activated cell sorter or flow cytometer. (6). Bacteria populations are frozen or plated for further selection or analysis. (7). Peptides from clones are sequenced. Synthetic versions of these peptides are then conjugated on biomaterials.

Our method (Figure 4-1) integrates bacterial peptide display library selection with biomaterials engineering. Two types of peptides were used: linear X_{15} (i.e. random 15mer peptides) and looped $X_2CX_7CX_2$ (i.e. 7C peptides), consisting of a random 7mer constrained by

two cysteine residues that form a disulfide bond to yield a looped peptide. Both types of peptides were displayed on an engineered outer membrane protein, CPX.[39] In total, three libraries were pursued: one composed of only linear X_{15} clones, one composed only of 7C clones, and one containing half of each peptide type before any selection called the combined library.

Each round of biomimetic ligand selection involves a series of six steps (Figure 4-1). First, the libraries bacterial cultures were grown and then induced with arabinose to induce green fluorescent protein (GFP) and CPX expression in the bacteria. The CPX-expressing libraries were then co-incubated with the desired mammalian cells at 37ºC in their native media for one hour, and non-adherent clones were washed away with low speed co-centrifugation. For third round selections, library populations were subsequently sorted on a fluorescence-activated cell sorter (FACS) to isolate non-fluorescent mammalian cells with bound, GFP-expressing library bacteria. Selected bacteria were then expanded by growing in bacterial medium. The resulting populations from these selections were then either plated or frozen for further selection or later analysis. After all selections, some of the resulting bacterial clones with high affinity to the mammalian cell surface of interest were sequenced. Synthetic versions of a few of the selected peptides were commercially obtained, and peptides were either adsorbed on tissue culture polystyrene (TCPS) or conjugated onto our model biomaterial, the interpenetrating polymer network, described below.

4.2.2 Novel Peptides that Bind to Neural Stem Cells

For selections using the method described above with bacterial peptide display selections, we used adult rat hippocampal neural stem cells (NSCs).[1, 40] Representative histograms of GFP fluorescence are given for CPX alone, the unselected 7C library, the selected 7C library populations after each of the three selection rounds (Figure 4-2a). These histograms showed the NSC-binding activity of the library populations increased as progressive rounds of biomimetic ligand selection were performed. After three rounds of selection, the 7C library showed two clear populations, NSCs without bacteria bound at low GFP levels and NSCs with bound bacteria at high GFP levels, indicating that multiple bacteria are binding many of the NSCs. In addition, there were more cells with bound bacteria than without bound bacteria.

When the percentages of NSCs with bound bacteria are quantified for the various library populations with flow cytometry (Figure 4-2b), we observe low binding affinity for empty CPX with no peptide, unselected libraries, post round 1 libraries, and the post round 2 15mer library. However, with all other library populations, a statistical increase in binding is observed relative to bacteria expressing CPX alone. For instance, for the post round 3 libraries, there is a significant difference between the three libraries in terms of bacterial bound with the 7C library having the most NSCs with bacteria bound followed by the combined library and the 15mer library, respectively. These results indicate that biomimetic ligand selection increases the proportion of bacteria binding to NSCs.

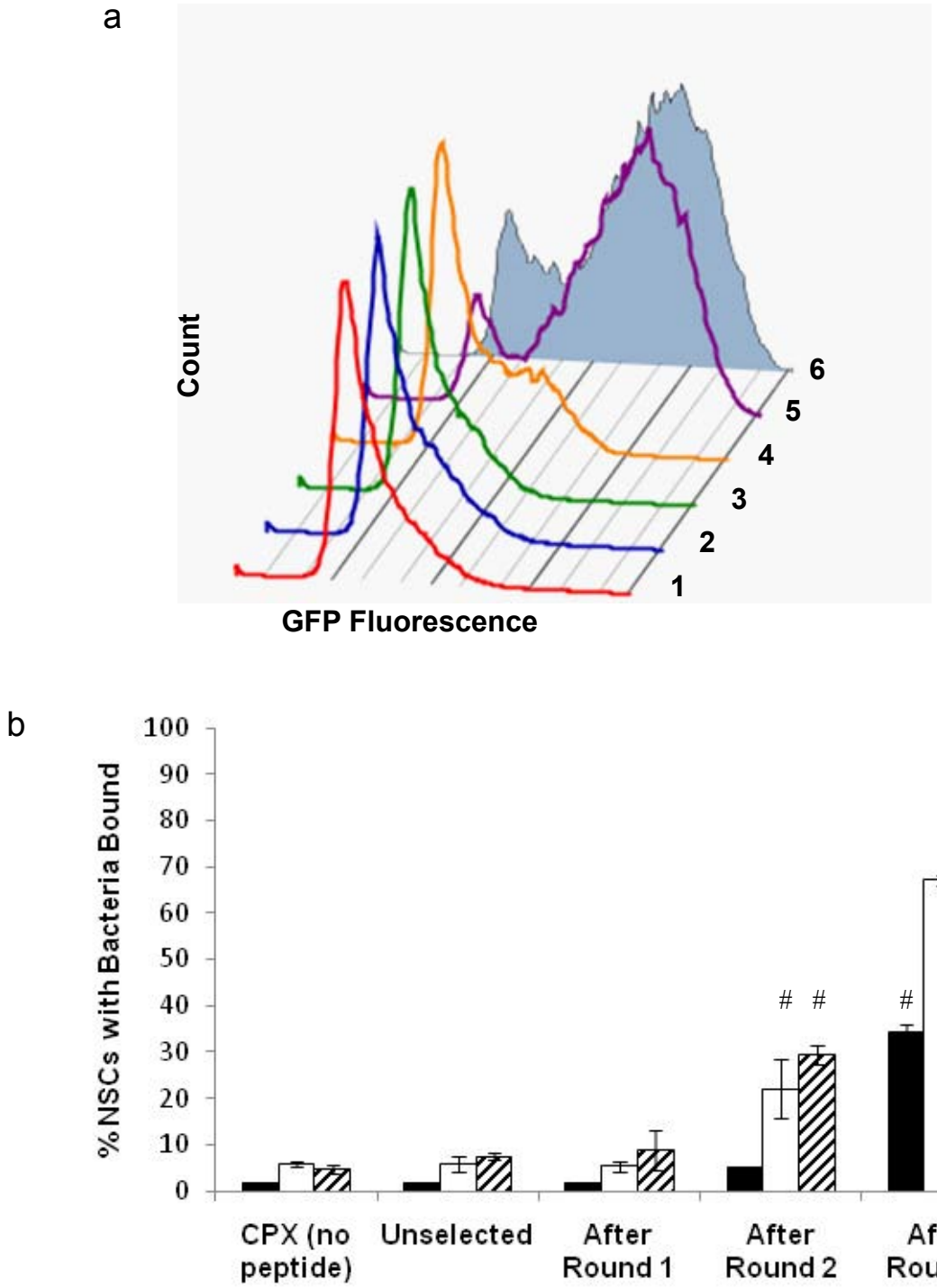


Figure 4-2: Binding capacity of peptide display library populations with neural stem cells. (a). Example histograms of library and clonal populations of the bacterial peptide display libraries binding to neural stem cells. (1) CPX (no peptide), (2) unselected 7C library, (3) 7C library after Round 1, (4) 7C library after Round 2, (5) 7C library after Round 3, and (6) high affinity clone 15-2. (b). Quantification of library populations. Three libraries were tested: (■)15mer library composed of peptides with the sequence X_{15}, (◪) 7C library composed of peptides with the sequence $X_2CX_7CX_2$, and (□) combined library containing both types of peptide clones. All unselected and post round 1 library populations showed similar binding as bacteria expressing CPX, the outer membrane display protein, but no peptide. After rounds 2 and 3, there

was significantly more bacteria binding to the neural stem cells with the 7C library having the highest amount of binding. The 15mer and combined libraries exhibited the higher binding with the combined library having the higher binding of the two. Data represent mean ± standard deviation. Library populations not in the same group (#, $, or ‡) were statistically different from one another ($p < 0.05$ using ANOVA between groups with Tukey-Kramer significant difference post hoc test).

Table 4-1: List of clones found with relatively high affinity for neural stem cells. Several of the peptides had homology to ECM proteins including 7C-15, 15-52, and 7C-24 with homologies to collagen, fibrinogen, and fibronectin, respectively. The integrin-binding motif arginine-glycine-aspartic acid (RGD) was found in several peptides are is in bold. The peptides used in further studies are highlighted in gray. The clone name indicates the library containing the clone and the data represent mean ± standard deviation.

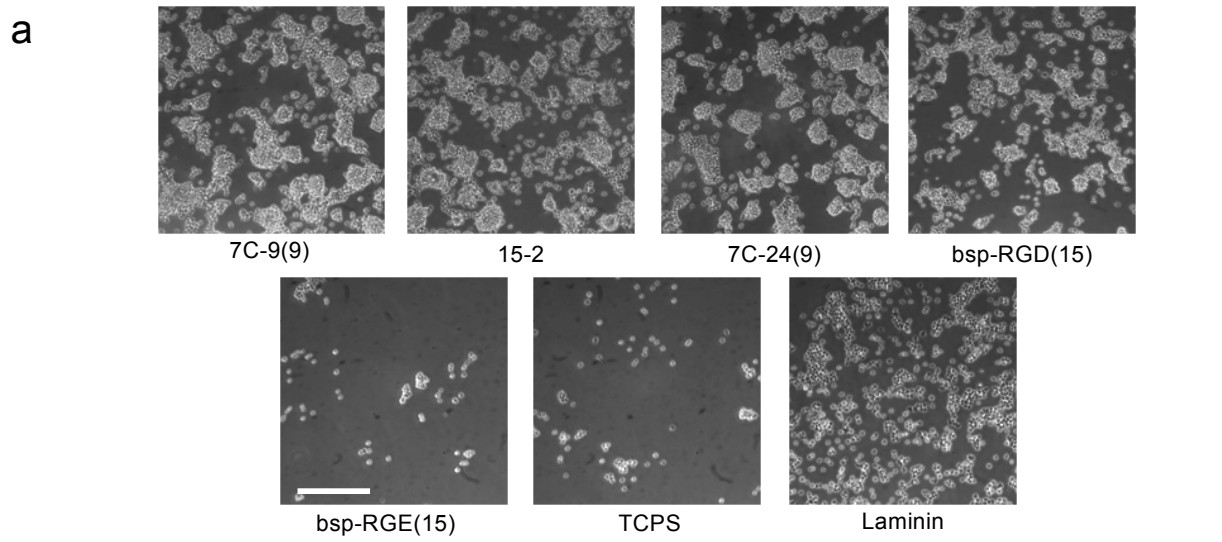
Clone	% NSCs with Bacteria (average ± S. D.)	Peptide Sequence
15-50	86.3 ± 0.8	GFVLVWSYTCRCWGK
7C-15	83.4 ± 0.8	QCCQLRGDAVCNC
7C-3	83.1 ± 0.7	WFCLLGRSAYCVR
7C-8	82.3 ± 2.1	WLCLDKNCMACVW
15-52	82.3 ± 5.0	ESGLKVMCMKYYCMA
7C-5	80.8 ± 1.5	IWCGSRFGCWCKP
15-32	80.5 ± 2.0	RRELVRMTDWVWVSG
Co-11	80.2 ± 3.6	LECPGESKYYCIY
7C-21	80.0 ± 2.6	WNCIKGSSWACVW
7C-1	79.4 ± 1.0	WYCFREN KYVCVM
7C-24	78.9 ± 1.7	WWCDMRGDSRCSG
7C-4	78.8 ± 2.4	YMCMSRGDATCDV
Co-10	78.4 ± 1.4	WRCLGDGYHACVR
Co-1	77.4 ± 4.2	SLCAAYNRWACIW
Co-21	77.3 ± 5.6	MYCERDSKYWCIH
Co-17	76.3 ± 3.0	WECAEESKFWCVF
Co-22	75.7 ± 0.8	VWCGMFGKRRCVT
Co-23	75.6 ± 0.9	LVCNRQNPWVCYI
7C-12	75.4 ± 3.2	FWCIRGEYWVCDR
Co-20	74.7 ± 4.1	RLCCWKTQYFCEI
7C-17	73.8 ± 3.4	WLCKGSNKYMCEW
15-2	73.7 ± 5.8	DHKFGLVMLNKYAYAG
7C-22	73.4 ± 0.7	WMCSGVQPNACVW
7C-9	73.4 ± 1.2	KLCCFDKGYYCMR
Co-16	72.9 ± 1.2	QGCAFVTYWACIF
Co-9	72.7 ± 3.9	SKCWGWTPYYCVA
Co-2	71.7 ± 3.4	WSCPKVNQYACFW
15-59	69.5 ± 0.7	DLCTYGHLWLGNGRP
15-16	69.4 ± 1.2	SDWSVLLSCERWYCI
Co-19	68.7 ± 6.1	WQCGRFWCIHCLW
Co-3	68.1 ± 7.4	GGCRWYAKWVCVW
Co-18	65.6 ± 5.9	WWCKKPEYWYCIW
7C-7	65.5 ± 2.3	LECTERGDFNCFV
Co-13	65.5 ± 8.0	STCSWVSSYVCIM
Co-8	64.6 ± 5.2	WTWESAFAGRWEVGD
7C-6	63.1 ± 3.2	GECFYYVMNTCVW
Co-5	62.5 ± 7.0	WDCGKKNAWMCIW
7C-2	58.9 ± 8.8	ESCWYQIMYKCAN
Co-12	54.7 ± 2.1	WVCLWRHRGDCSI
7C-14	53.3 ± 6.6	LNCAMYNACIW
7C-20	52.4 ± 3.3	WVCIWERFKSCNE
7C-11	50.7 ± 11.0	LCCESYICALCHY
Co-15	45.2 ± 7.2	WVCNDLIHHFCVW
7C-19	36.6 ± 3.7	WVCNKLGVYACEY

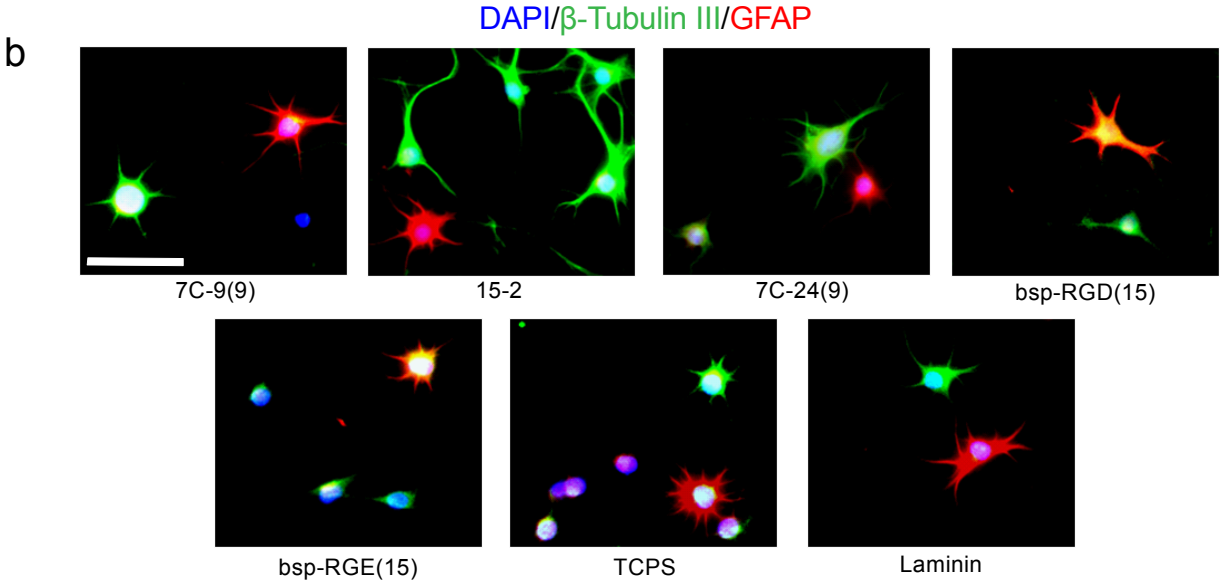
The binding capacities of individual bacterial clonal populations were next analyzed to determine whether clonal populations recapitulated the high binding of the selected libraries. After examining 60 clones, 44 were determined to be high binding, i.e. ones that have at least 30% of the NSCs with bacteria bound after co-incubation. Afterward, the plasmids encoding the CPX protein displaying the novel peptides were isolated and subjected to DNA sequencing. Several of these clones expressed peptides with homology to known ECM proteins based on small sequence protein BLAST searches. The full list of peptides found in the selections is given in Table 4-1, and the peptides used in surface studies are highlighted in grey. These clones have various binding capacities ranging from 36.6% to 86.3%. Interestingly, clones 7C-15, 15-52, and 7C-24 have homology to collagen, fibrinogen, and fibronectin, respectively. In addition, four clones contained an RGD motif, which is known to bind to a subset of integrins.[6, 8] These clonal data suggest that the library-based biomimetic ligand selections, which are unbiased towards any ECM or integrin binding domains, yield peptides that bind to cell receptors, possibly including integrins.

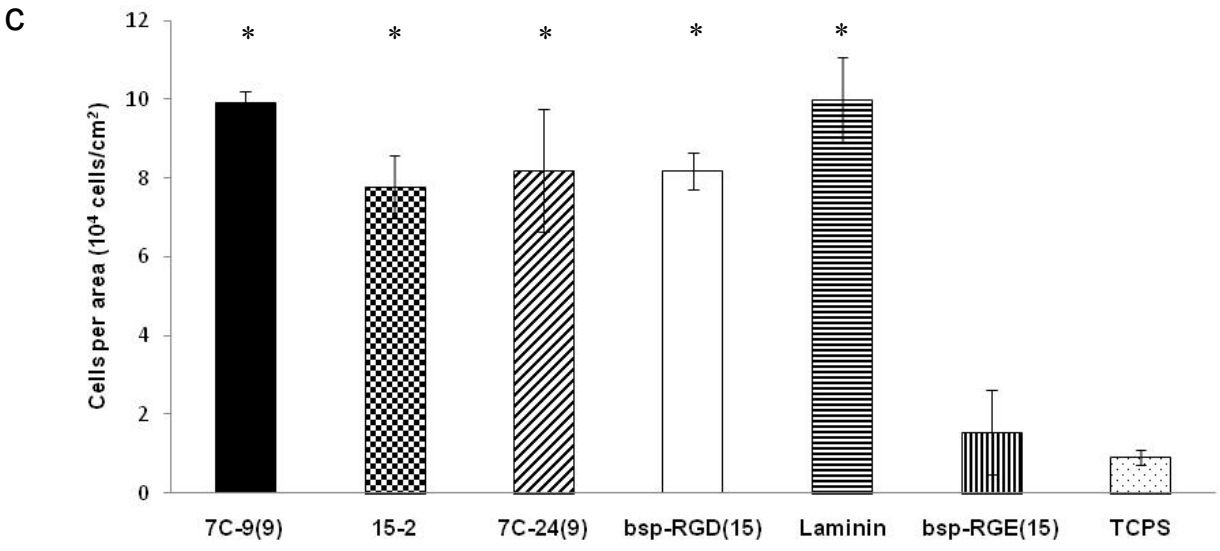
4.2.3 Synthetic Peptides Adsorbed on Tissue Culture Polystyrene

Synthetic versions of three of the peptides resulting from biomimetic ligand selections (indicated in grey in Table 4-1), were chosen for their high affinity for NSCs and predicted water-solubility based on the ratio of charged to hydrophobic residues in the sequence, and were commercially synthesized to assess their ability to support attachment and growth of NSCs. All three were tested both as adsorbed peptides on TCPS and chemically conjugated to the IPN. Two high-binding looped peptides, 7C-9 and 7C-24, were synthesized as a cyclic peptide—formed through a bond attaching the N- and C-termini—of the form CX_7G where X_7 are the amino acids from the middle of the sequenced clone. The cysteine residue was left to allow for the conjugation to the IPN and the glycine residue was added to mimic the size of the loop in the 7C peptide clones. The terminal residues outside of the loop from the 7C peptide clones were not incorporated synthetically since as these residues were theorized to not be important for NSC binding since they were so close to the bacterial cell surface. These synthetic peptides will be referred to as 7C-9(9) and 7C-24(9).

A high-binding linear peptide, 15-2, was synthesized with a cysteine residue on the N-terminus to allow for biomaterial conjugation. bsp-RGD(15)—a peptide previously shown to mimic NSC behavior on laminin—[11]and bsp-RGE(15)—a peptide that does not bind NSCs due to the insertion of an additional methylene in the D to E substitution that renders the peptide unable to bind integrins—were used as positive and negative controls, respectively. All of these synthetic peptides were adsorbed onto tissue culture polystyrene (TCPS), and NSCs were cultured on the resulting surfaces for 5 days (Figure 4-3a). All surfaces with the three selected peptides and bsp-RGD(15) encouraged NSC attachment as clumps, while bsp-RGE(15) and TCPS with no adsorbed peptide had little cell attachment. When proliferation after 5 days is quantified on these surfaces (Figure 4-3c), there is little cell expansion on bsp-RGE(15) and TCPS. In contrast, the other peptide-dried surfaces had similar levels of proliferation.


a
7C-9(9)
15-2
7C-24(9)
bsp-RGD(15)
bsp-RGE(15)
TCPS
Laminin


DAPI/β-Tubulin III/GFAP
b
7C-9(9)
15-2
7C-24(9)
bsp-RGD(15)
bsp-RGE(15)
TCPS
Laminin


c
Cells per area (10⁴ cells/cm²)
0
2
4
6
8
10
12
*
7C-9(9)
15-2
7C-24(9)
bsp-RGD(15)
Laminin
bsp-RGE(15)
TCPS


d
80
70
60
50
*

Figure 4-3: Neural stem cells on peptide-adsorbed TCPS surfaces. Peptides, including (■) 7C-9(9), (◩) 7C-24(9), (▩) 15-2, (□) bsp-RGD(15), (▤) Laminin, (▥) bsp-RGE(15), and (⊡) TCPS alone, were dissolved at 100 µM in synthesis-grade water or DMSO for 15-2, and then peptides were dried on TCPS. (a). Brightfield micrographs of the neural stem cells after 4 days of culture on the adsorbed surfaces exhibited similar attachment and clumping of cells on the surface on the 7C-9(9), 7C-24(9), 15-2, and bsp-RGD(15) surfaces while the bsp-RGE(15) and TCPS surfaces had significantly less cells. The scale bar represents 250 µm. (b). NSCs grown under differentiating conditions were assessed for expression of GFAP (red), a cytoskeletal marker for astrocytes, and β-Tubulin III (green), a cytoskeletal marker for neurons. All cells were stained with DAPI (blue) for the nucleus. All surfaces had astrocytes and neurons under differentiating conditions. All scale bars represent 100 µm. (c). Quantification of the number of cells on the surface with the Cyquant cell counting assay showed similar numbers of cells on all surfaces except the bsp-RGE(15) and TCPS surfaces, which had significantly less cells. (d). Quantification of differentiation markers, β-Tubulin III and GFAP, on peptide-adsorbed surfaces. All library-selected and bsp-RGD(15) peptide surfaces had similar percentages of neurons and astrocytes compared to laminin, while bsp-RGE(15) and TCPS surfaces had less neurons and more astrocytes. (Data represent mean ± standard deviation. Library populations not in the same group (*) were statistically different from one another ($p < 0.05$ using ANOVA between groups with Tukey-Kramer significant difference post hoc test).

Next, neural stem cell differentiation was tested on the adsorbed peptides in mixed differentiation conditions that encouraged commitment to both neuronal and astrocytic fates. After 5 days, both neurons and asytrocytes were found on all surfaces as determined by β-tubulin III and glial fibrillary acid protein (GFAP) staining, respectively (Figure 4-3b). When the percentages of neurons and astrocytes were quantified, all library-selected peptides were found to yield similar percentages of neurons and astrocytes to that seen on bsp-RGD(15) and laminin surfaces. In contrast, the TCPS and bsp-RGE(15) surfaces had fewer neurons and more astrocytes than laminin and the library-selected peptides (Figure 4-3d). In addition, under the differentiating conditions, bsp-RGE(15) had the lowest number of cells, even in comparison to plastic surfaces with no peptide (Figure 4-4) indicating that the results seen on the library peptides was distinguishable from that of a peptide that does not support NSC culture. This initial study indicated that all three peptides obtained using biomimetic selections were able to encourage NSC proliferation while maintaining the NSCs in a multipotent state similar to laminin, making them good candidate peptides for biomaterial conjugation.

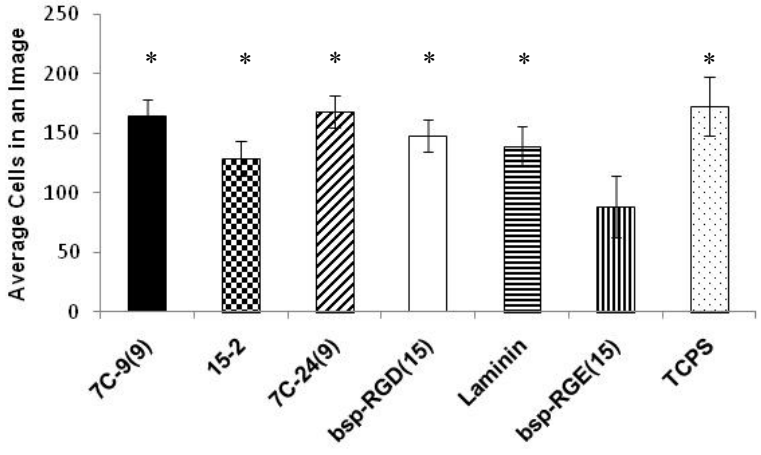


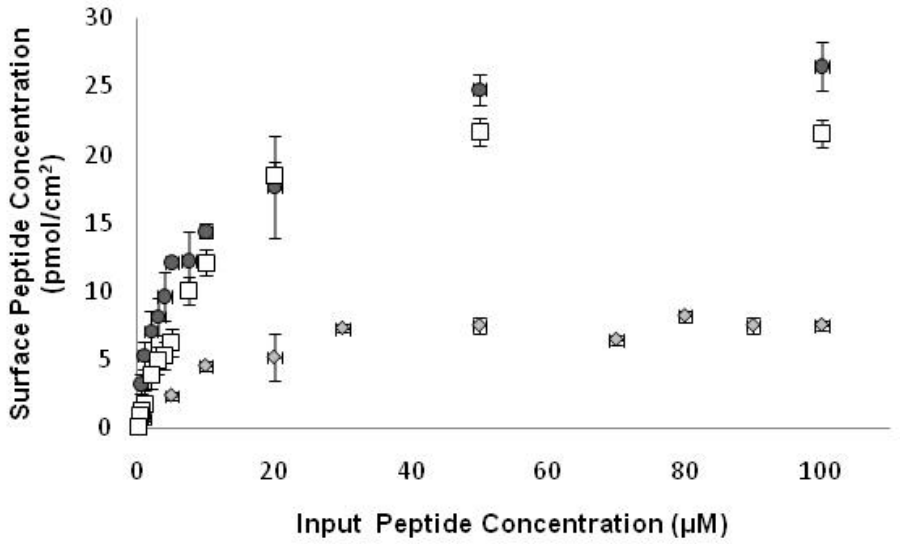
Figure 4-4: Neural stem cells attached under differentiating conditions. Peptides, including (■) 7C-9(9), (◩) 7C-24(9), (▩) 15-2, (□) bsp-RGD(15), (▤) Laminin, (▥) bsp-RGE(15), and (⊡) TCPS alone, were dissolved at 100 µM in synthesis-grade water or DMSO for 15-2, and then peptides were dried on TCPS. The number of cells was determined by counting cells from micrographs of NSCs after immunocytochemistry.

Cells were determined with DAPI, a stain for the cell nucleus. Data represent mean ± standard deviation. Samples not in the same group (*) were statistically different from one another ($p < 0.05$ using ANOVA between groups with Tukey-Kramer significant difference post hoc test).

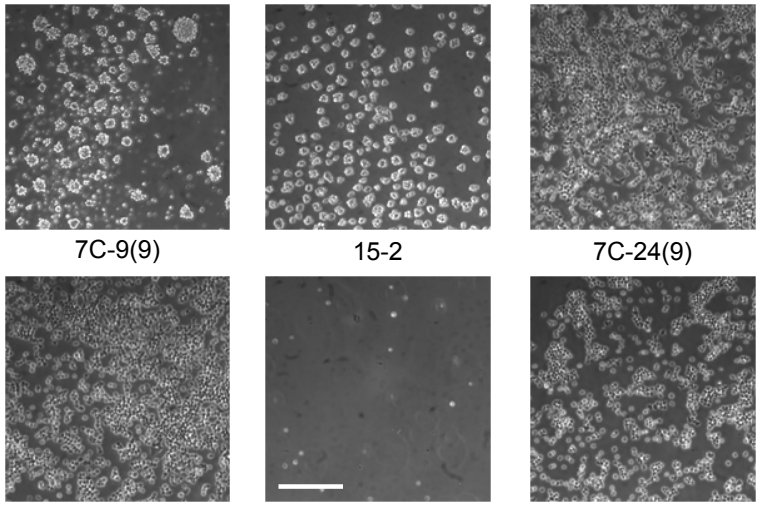
4.2.4 Synthetic Peptides on a Model Biomaterial

Finally, we analyzed the activity of peptides grafted an interpenetrating polymer network (IPN) composed of acrylamide and poly ethylene glycol (PEG) networks.[41, 42] This material was utilized because of its modularity in controlling material variables—such as ligand density, ligand orientation, and mechanical modulus—and because previous studies have shown that IPNs conjugated with bsp-RGD(15) were able to mimic the effects of laminin.[11] In addition, the IPN has been used to grow many cell types including osteoblasts and hippocampal neural stem cells, and it has also been used as a coating on titanium stents.[11, 14, 43, 44] Because of the modularity of the IPN in addition to previous success with growing NSCs on peptide-conjugated IPNs, the IPN was chosen as the model biomaterial for this study. To ensure that peptide surface concentration on the IPN was consistent for all peptides, fluorescently tagged peptides were used to determine the peptide concentration at which the peptide surface concentration becomes saturated (Figure 4-5a).[45] The linear peptides, 15-2 and bsp-RGD(15), had a saturation level around 20-25 pmol/cm^2. In contrast, the looped peptide 7C-9(9) had a maximum surface peptide density of 8 pmol/cm^2, as anticipated since looped peptides cannot pack as closely together on surface as linear ones. For all subsequent surface experiments, the peptides were used at 8 pmol/cm^2 or below, thereby delivering the same level of signal to the cells for all samples.

a


Surface Peptide Concentration (pmol/cm²)
30
25
20
15
10
5
0
0
20
40
60
80
100
Input Peptide Concentration (μM)

b


7C-9(9)
15-2
7C-24(9)
bsp-RGD(15)
bsp-RGE(15)
Laminin

c

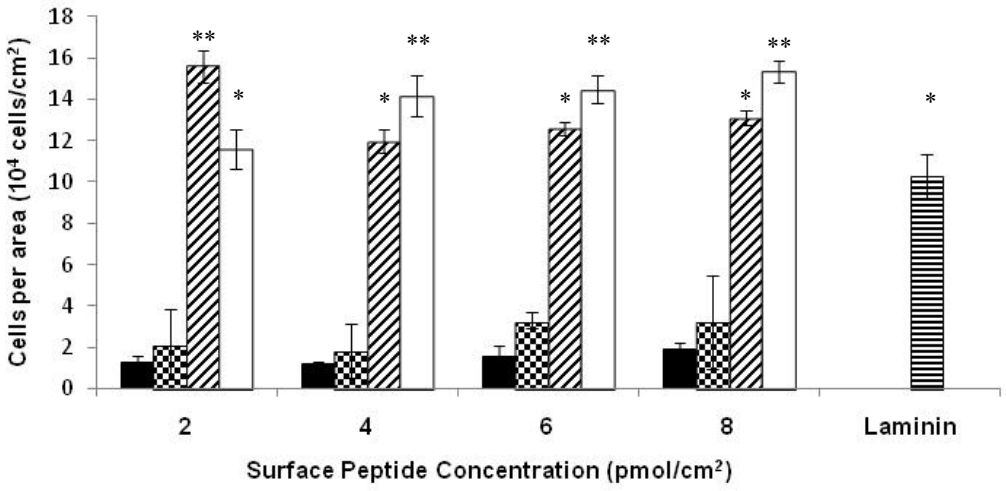

Cells per area (10⁴ cells/cm²)
18
16
14
12
10
8
6
4
2
0
**
*
2
4
6
8
Laminin
Surface Peptide Concentration (pmol/cm²)

Figure 4-5: Peptide grafting and cell proliferation on IPN surfaces. (a). Peptide density on interpenetrating polymer network (IPN) surfaces. Peptide densities on IPN surfaces were determined by grafting on a FITC-tagged peptide and digesting the FITC from the peptide with chymotrypsin. Fluorescent measurements then allowed for the calculation of the surface peptide concentration. Three peptides, (●) bsp-RGD(15), (□) 15-2 and (◇) 7C-9(9), were examined. bsp-RGD(15) and 15-2 exhibited saturation behavior with saturation around 25 and 20 pmol/cm^2, respectively, while the looped 7C-9(9) peptide showed saturation around 8 pmol/cm^2. (b). Neural stem cells were cultured on interpenetrating polymer networks (IPNs) conjugated with peptides including (■) 7C-9(9), (◩) 7C-24(9), (▣) 15-2, (□) bsp-RGD(15). For comparison, neural stem cells were also cultured on (▤) laminin. Brightfield images of the cells after 4 days illustrated that cells on the 7C-9(9) and 15-2 surfaces either attached in clumps or remained as non-adherent neurospheres. 7C-24(9) and bsp-RGD(15) surfaces showed similar cell morphology and growth to the Laminin control surface. The bsp-RGE(15)-conjugated surface showed little cell attachment. All surfaces had peptides at 8 pmol/cm^2. The scale bar represents 250 µm. (c). The amount of cells on each surface after 5 days was quantified with Cyquant. 7C-24(9) and bsp-RGD(15) surfaces had cell proliferation at or above the amount of laminin at all peptide surface concentrations. The 7C-9(9) and 15-2 surfaces had a lot less cells than all other surfaces, which was expected since the cells mainly formed neurospheres instead of attaching to the surface. Results from the bsp-RGE(15) and unconjugated IPN surfaces were below the detection limit of the assay. Data represent mean ± standard deviation. Library populations not in the same group (* or **) were statistically different from one another ($p < 0.05$ using ANOVA between groups with Tukey-Kramer significant difference post hoc test).

All three test peptides selected from the libraries—7C-9(9), 15-2, and 7C-24(9)—along with bsp-RGD(15) and bsp-RGE(15) were grafted onto IPN surfaces at concentrations ranging from 2 to 8 pmol/cm^2 (Figure 4-5b and Figure 4-6). NSCs grown on IPNs with peptides 7C-9(9) or 15-2 formed neurospheres, aggregates of cells in suspension, with a few clumps of cells attaching to the surface. However, the RGD-containing peptide 7C-24(9) had NSCs attached as a monolayer similar to laminin and bsp-RGD(15). IPNs conjugated with bsp-RGE(15) had very few cells attached. When proliferation after 5 days was quantified, two novel peptides showed very little proliferation, while the bsp-RGE(15) surfaces were below the detection limit of the assay (Figure 4-5c). 7C-24(9) had proliferation at or above the level of proliferation on laminin, and bsp-RGD(15) surfaces exhibited similar amounts of cell proliferation to 7C-24(9). While two of the library-selected peptides did not support proliferation to the same extent as laminin, 7C-24(9) was able to support proliferation at or above the level of laminin, indicating the selection process successfully identified peptide sequences allowing cell adhesion.

For the looped peptides, we investigated how the size of the loop would affect cell attachment and proliferation. Looped peptides of the form CX_7GG denoted as (10) at the end of the peptide name were tested. Both 7C-9(10) and 7C-24(10) had similar cell attachment behavior as their smaller looped counterparts. NSCs attached on 7C-9(10) surfaces formed neurospheres with some cell clumps attached, while cells on 7C-24(10) surfaces attached as a monolayer (Figure 4-7a). The cell proliferation after 5 days on these peptide-conjugated IPNs were compared with the smaller looped peptides (Figure 4-7b). The smaller loops supported proliferation at or above the proliferation of the larger loops, indicating that the smaller loops provided a better biomimetic surface.

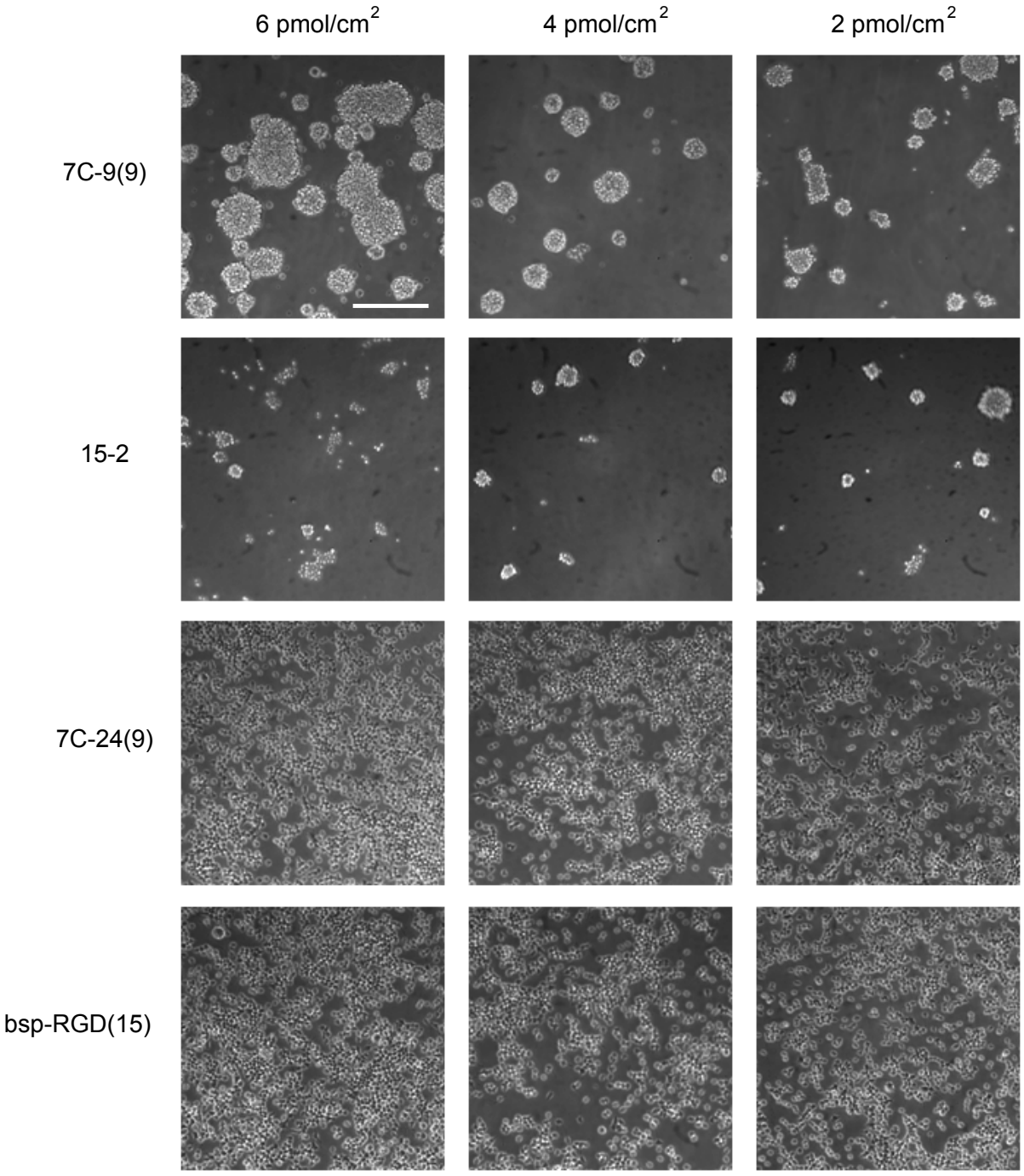


Figure 4-6: Brightfield images of cells grown on peptide-conjugated IPN surfaces at peptide concentrations between 2 pmol/cm^2 and 6 pmol/cm^2. Cell attachment on the smaller peptide density surfaces showed similar results to those seen on IPNs with 8 pmol/cm^2 peptide. The scale bar represents 250 μm.

The final study of these biomimetic ligand-selected peptides on IPNs was to assess the expression of cell markers under proliferating and differentiating conditions (Figure 4-8a). Under proliferating conditions with 20 ng/mL FGF-2, most NSCs on all peptide-conjugated IPNs expressed Nestin, a cytoskeletal maker for an NSC.[46] In addition, all peptide-conjugated IPNs supported both neuronal and glial differentiation of NSCs as indicated by expression of β-Tubulin III and GFAP (glial fibrillary acidic protein), respectively, when NSCs were exposed to

1 μM retinoic acid and 1% fetal bovine serum. When the numbers of neurons and glia were quantified under differentiating conditions (Figure 4-8b), there was no appreciable difference in the number of glial cells on different ligands. However, laminin surfaces had more neurons than all other IPNs, which had similar amounts of neurons. These data indicate that NSCs seeded on IPN surfaces conjugated with peptides obtained from biomimetic ligand selections are able to self-renew under proliferative conditions. Additionally, NSCs grown on these surfaces are able to differentiate into multiple lineages under differentiating conditions. In comparison to the peptides adsorbed on TCPS, only the 7C-24(9) peptide supported both the self-renewal and differentiation of NSCs when conjugated to the IPN although all three supported differentiation similar to laminin surfaces.

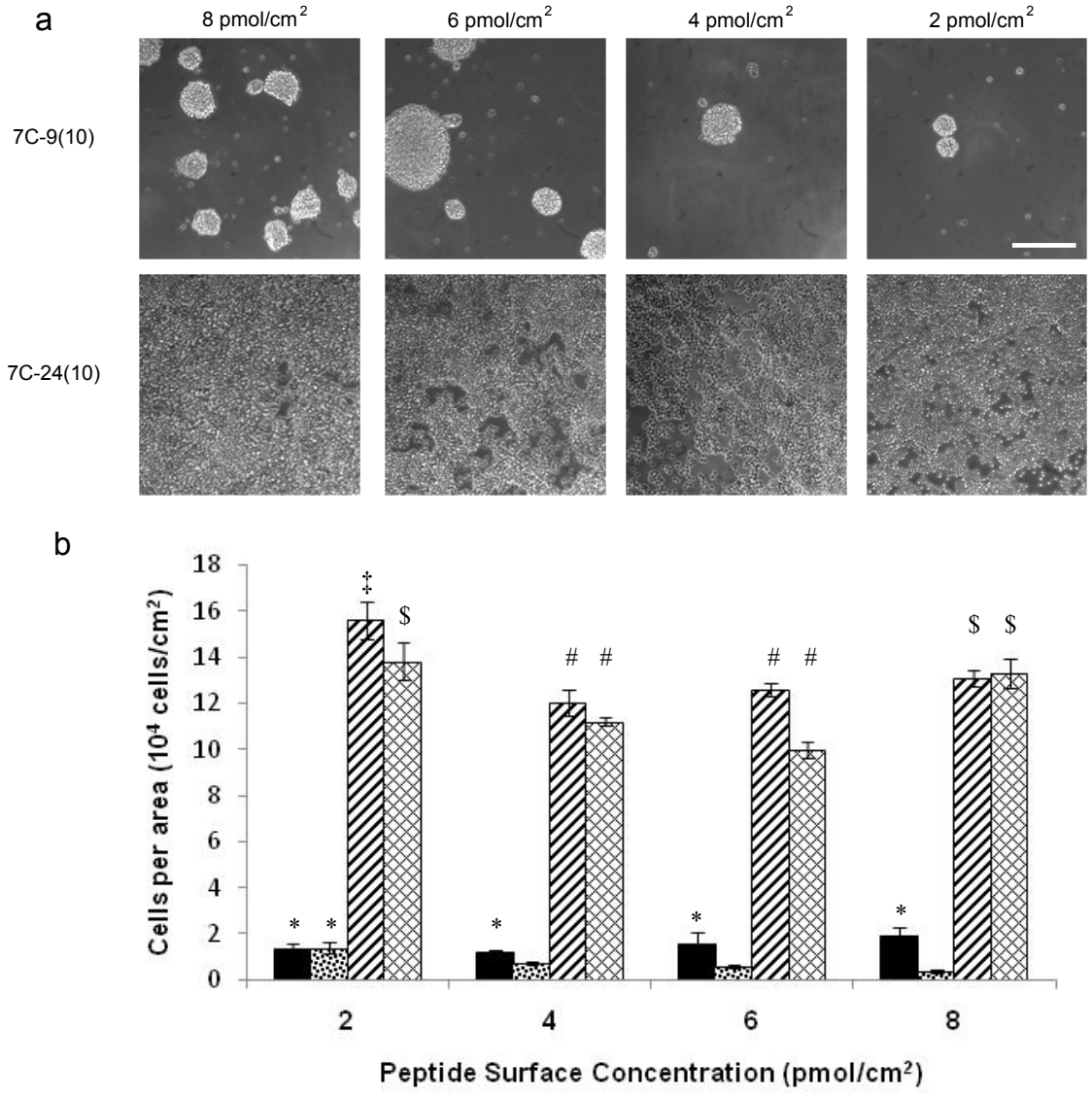


Figure 4-7: Neural stem cell growth on interpenetrating networks conjugated with larger cyclic peptides. A) Neural stem cells were cultured on peptide-grafted surfaces for 4 days. (a). Brightfield images of neural stem cells on surfaces with concentrations ranging between 2 and 8 pmol/cm^2 were taken. Similar to the smaller cyclic version of the peptides, 7C-9(10) showed cells mainly in neurospheres and 7C-24(10) showed cell attachment as a monolayer. The scale bar represents 250 μm. (b) Cells on these surfaces were quantified

after 5 days growth with Cyquant. (◩)7C-24(9) and (⊠)7C-24(10) exhibited more cell growth than (■)7C-9(9) and (⊞)7C-9(10) though all larger looped peptide surfaces had less or close to the same amount of cells as the smaller looped peptides. Data represent mean ± standard deviation. Samples not in the same group (*, #, $, or ‡) were statistically different from one another ($p < 0.05$ using ANOVA between groups with Tukey-Kramer significant difference post hoc test).

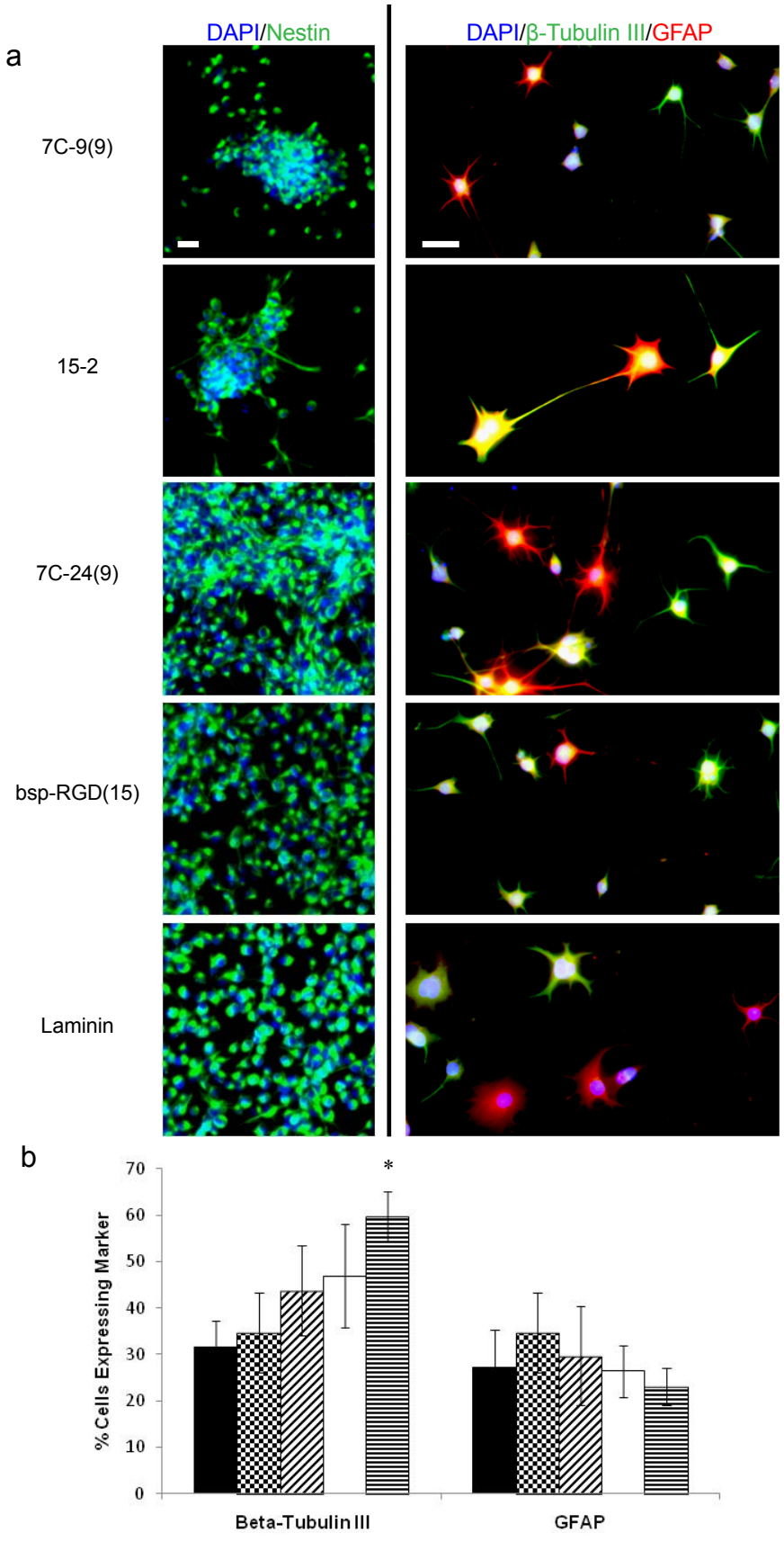

a
DAPI/Nestin
DAPI/β-Tubulin III/GFAP
7C-9(9)
15-2
7C-24(9)
bsp-RGD(15)
Laminin
b
% Cells Expressing Marker
70
60
50
40
30
20
10
0
*
Beta-Tubulin III
GFAP

Figure 4-8: Expression of cell markers under proliferative and differentiating conditions on peptide-conjugated interpenetrating polymer networks (IPNs). Neural stem cells were cultured on the surfaces for 5 days either under proliferative conditions with 20 ng/mL basic Fibroblast Growth Factor (bFGF) or with 1% fetal bovine serum and 1 μM retinoic acid. (a). NSCs grown under proliferative conditions were assessed for the expression of Nestin (green), a cytoskeletal marker for a neural stem cell, while NSCs grown under differentiating conditions were assessed for expression of GFAP (red), a cytoskeletal marker for astrocytes, and β-Tubulin III (green), a cytoskeletal marker for neurons. All cells were stained with DAPI (blue) for the nucleus. All surfaces had most of the cells staining for Nestin under proliferative conditions, and all surfaces had astrocytes and neurons under differentiating conditions. All scale bars represent 100 μm. (b). Quantification of differentiation markers, β-Tubulin III and GFAP, on (■) 7C-9(9), (▩) 15-2, (◪) 7C-24(9), (□) bsp-RGD(15), or (▤) laminin. Laminin had significantly more cells expressing β-Tubulin III than any other surface, but all other surfaces had similar β-Tubulin III expression. With GFAP expression there was no significant difference in expression on any surface. Data represent mean ± standard deviation. Library populations not in the same group (*) were statistically different from one another ($p < 0.05$ using ANOVA between groups with Tukey-Kramer significant difference post hoc test).

4.3 Discussion

Stem cells in general, and neural stem cells in particular, often require ECM proteins for their maintenance of self-renewal in cell culture.[3-5, 47] However, animal and human ECM proteins are expensive to produce, potentially contain human pathogens, and have considerable variability in composition.[11, 48, 49] All of these concerns cause considerable problems for process development and scale-up of stem cell therapies. In addition, as ECM molecule signals are not well understood, developing a biomaterial with a peptide that mimics ECM proteins could elucidate more about their roles in cellular signaling processes and might help identify signaling domains on ECM proteins. However, in part because such ECM signaling is not completely understood, designing peptides to mimic ECM proteins is difficult.

As one examples of the inherent difficulty in identifying ECM-like peptide ligands, while fibronectin has been known for many years to have binding domains for integrins, only recently was the domain for α_v integrin binding found in this large ECM protein.[3, 25] Additionally, even when a receptor-binding region within an ECM protein is known, very few peptides synthesized to match the sequence of this ECM motif are capable of serving as agonists for that receptor.[18, 19, 22] By using libraries in our method for finding peptides with signaling properties similar to ECM proteins, we have sampled a large peptide sequence space in an unbiased manner that does not require prior knowledge of the signals required for cell growth and maintenance. In this study, we have shown that the utilizing biomimetic ligand selection is a very powerful tool for identifying promising candidate peptides that can be conjugated to biomaterials surfaces for the growth of a desired cell type, including stem cells, indicating that these peptides may act as receptor agonists.

Although phage peptide display libraries have been used to find many peptides that bind to various purified integrins, most of these peptides were shown to bind to, but not to activate, the integrins.[31-35, 50] Cell experiments with phage display-derived novel peptides that bind to integrins have either focused on cell spreading or inhibition of cell binding to ECM proteins, but longer-term characterization of these peptides with cells were not performed.[32, 35] Yeast peptide display libraries have been used to target the $\alpha_v\beta_3$ integrin with RGD peptides, but a method that is unbiased towards any particular motif is also desirable since it can allow the investigation of novel peptides for non-RGD binding cellular receptors.[50] Recent studies with phage display have found peptides that support the short-term growth of hESCs.[51] However, these peptides were not shown to activate integrins or other cellular adhesion receptors. Besides finding a list of novel peptides that bind to NSCs, we have also explored several of these peptides on two

different biomaterial platforms and showed these peptides were able to mimic the effect of laminin, the ECM protein typically used in NSC culture.

With adult neural stem cells, this method yielded peptides that had homology to small sequences in ECM proteins such as collagen, fibronectin, and fibrinogen (Table 4-1). Even though the libraries were unbiased towards any particular ECM motifs, four of the selected peptides had an RGD motif, which is known to bind to a large number of integrins.[18] Since our biomimetic ligand selections yielded peptides with homology to ECM proteins, we hypothesize that some of the novel peptides that were selected may also target integrins or other important cell adhesion receptors. Synthetic peptides based on the sequences obtained using the selection process encouraged attachment and proliferation of NSCs to different extents in two different contexts: adsorbed on TCPS and conjugated to IPN surfaces. These synthetic peptides were also able to support self-renewal, or proliferation with maintenance of multipotency, of the NSCs as indicated by studies under proliferating and differentiating conditions, respectively.

All three library-selected peptides supported both self-renewal and differentiation of NSCs when adsorbed on TCPS, but only one of these supported both processes when conjugated on the IPN. This different behavior observed between the same peptides when adsorbed on TCPS and conjugated on IPNs seems to indicate that the orientation of the peptide is important, as all peptides have the same orientation on the IPN, as constrained by the linkage chemistry, while the adsorbed peptide surfaces theoretically allow all possible orientations. In comparison to surfaces with an RGE peptide that did not support proliferation of NSCs, all of the library-selected peptides supported proliferation of NSCs and thus most likely activate cell adhesion receptors. The peptide from the selections that performed the best on the IPN contained an RGD residue. When the peptides were adsorbed on TCPS, all peptides supported proliferation and differentiation of NSCs similar to laminin indicating that the peptide orientation was an important factor in cell behavior. Overall, this method is quite versatile and it can be applied to any desired cell type for any biomaterial grafted with peptides. This general approach should be helpful in finding candidate peptides that mimic ECM signals for more complicated systems such as human embryonic stem cells.

4.4 Methods

4.4.1 Cell Culture

Neural stem cells from the hippocampal region of the adult rat brain were isolated and cultured on polyornithine laminin plates tissue culture polystyrene plates as described elsewhere.[1, 40] These cells were grown in DMEM/F12 media (Invitrogen) supplemented with N2 (Invitrogen) and 20 ng/mL recombinant human fibroblast growth factor 2 (FGF-2) (Peprotech).

4.4.2 Bacterial Peptide Display Libraries

The bacterial display libraries were generated in MC1061 *E. coli* with a pBAD33 plasmid containing alajGFP and the CPX membrane protein with random peptides of the forms X_{15} (15mer) and $X_2CX_7CX_2$ (7C) at the N-terminus.[38] CPX was circularly permutated from OmpX, a common bacterial display protein, to locate the N- and C-terminus on the extracellular side.[39] alajGFP is a bright fluorescent protein engineered for high expression in *E. coli*.[36] Both genes were under the control of an arabinose-inducible promoter. Three libraries were used: one containing only 15mer clones (15), one containing only 7C clones (7C), and a third with initially equal parts of 15mer and 7C clones (combined).

4.4.3 Bacterial Peptide Display Selections

The selections were performed in three rounds based on the method of Dane *et al*.[38] In each round, a frozen stock of the library was grown overnight in LB supplemented with 34 μg/mL chloramphenicol (Sigma) and 0.2% D-glucose (Sigma). The library was then sub-cultured 1:50 with LB and 34 μg/mL chloramphenicol. After two hours, it was induced at 30°C with 0.02% L-arabinose (Sigma) to initiate expression of alaj GFP[36] and CPX. Neural stem cells removed from their plates with 2 mM Na_2EDTA (Fisher) in phosphate-buffered saline (PBS) were then co-incubated with the library bacteria in a shaker for 1 hour in DMEM/F12 media.

For the first round, 100-fold more bacteria than NSCs were used, and 50-fold more bacteria were used for the latter two rounds. Washing steps were then performed by centrifuging the samples at 3500 rpm for 4 min for the first round and 1600 rpm for 30 s for the subsequent rounds. The resulting pellet was then grown overnight in LB supplemented with 34 μg/mL chloramphenicol and 0.2% D-glucose. For the third round selections, FACS was performed on the samples after the washing. Clonal and library analysis was performed with flow cytometry. All libraries were analyzed by expanding 10^8 clones of each library. Representative flow cytometry data were analyzed with FlowJo software.

4.4.4 Synthetic Peptides

Peptides for all subsequent studies were purchased from American Peptide Company, Inc. Linear peptides had an additional cysteine residue on the N-terminus to allow for attachment on IPN surfaces. Cyclic peptides from the 7C library were ordered as either CX_7G - denoted as (9) - or CX_7GG - denoted as (10) - where X_7 are the residues in the middle of the cysteine-cysteine loop from the sequenced library clones; these peptides were cyclized through an amide linkage between the N- and C-termini of the peptides. bsp-RGD(15) and bsp-RGE(15), which are used as positive and negative controls in this study, have the sequences CGGNGEPRGDTYRAY and CGGNGEPRGETYRAY, respectively.[11]

4.4.5 Adsorbed Peptide Surfaces

Peptides were dissolved at 100 μM in synthesis grade water, or DMSO for peptide 15-2. For adsorption to TCPS plates, solutions were sterile filtered and dried onto the plates for 3 hours at room temperature in a sterile biohazard hood. NSCs were detached from laminin plates with Accutase (Innovative Cell Technologies, Inc.) and added at 30,000 cells/cm^2 in DMEM/F12 supplemented with 20 ng/mL FGF-2. Cells were incubated at 37ºC for 5 days with media replacement every other day. After 5 days, all media were removed, and plates were frozen at -80ºC for Cyquant (Invitrogen) cell counting, as per the manufacturer's instructions.

4.4.6 Interpenetrating Polymer Network Synthesis

Interpenetrating network surfaces was made as described previously.[41, 42] TCPS plates were first cleaned with 1.5 M sodium hydroxide (Sigma) dissolved in 70% ethanol (Sigma) for 1 hour, followed by washing with synthesis grade water and sonication for 30 minutes. Before synthesis, plates were functionalized in an oxygen plasma for 5 min. The first network of the IPN was synthesized by addition of 0.1485 g/mL acrylamide (Polysciences) and 0.0015 g/mL N,N-ethylenebisacrylamide (Polysciences) as a cross-linker initiated with 0.01 g/mL [3-(3,4-Dimethyl-9-oxo-9H-thioxanthen-2-yloxy)-2-hydroxypropyl]trimethylammonium chloride (QTX, Sigma Aldrich) as a photoinitiator dissolved in 97% isopropyl alcohol (Sigma) and 3% synthesis grade water, and plates were incubated on a UV light table for 4.5 min. The second network was

formed with 0.02 g/mL poly(ethylene glycol) monomethyl ether (MW 1000) (Polysciences) and 0.01 g/mL N,N-ethylenebisacrylamide as a crosslinker with 0.005 g/mL QTX as a photoinitiator. Acrylic acid (Polysciences) at 0.0162 mL per mL solution was also added to provide a functional site for subsequent peptide conjugation. The second network was polymerized on a UV light table for 6 min. Chains of amine terminated PEG (MW 3400) (Laysan) at 0.150 g/mL were then grafted to the acrylic acid in the second network with 0.0025 g/mL N-hydroxysulfosuccinimide (Sulfo-NHS, Pierce) and 0.005 g/mL 1-Ethyl-3-[3-dimethylaminopropyl]carbiodiimide hydrochloride (EDC, Pierce) dissolved in 0.5 M 2-(N-Morpholino)ethanesulfonic acid buffer at pH 7.0. Sulfosuccinimidyl 4-[N-maleimidomethyl]cyclohexane-1-carboxylate (Sulfo-SMCC, Pierce) was attached at 0.0005 g/mL in Sodium Borate Buffer at pH 7.5. Peptides were then attached in 0.1 M sodium phosphate buffer at pH 6.6 overnight at 4ºC via the free thiol on the terminal cysteine residue to the Sulfo-SMCC (Bearinger, et. al., 1997). Surfaces were washed with 0.1 M sodium phosphate buffer to remove unattached peptide.

For peptide 15-2, succinimidyl 4-[N-maleimidomethyl]cyclohexane-1-carboxylate (SMCC) was first dissolved in DMSO and then diluted 1:5 in sodium borate buffer to a final concentration of 0.0005 g/mL. The peptide 15-2 was similarly dissolved first in DMSO then diluted 1:5 in sodium phosphate buffer. Surfaces were then washed with 1% SDS to remove unattached peptide.

4.4.7 Peptide Density Determination

For peptide surface density determination, peptides with a fluorescein isothiocyanate (FITC) tag, synthesized by American Peptide Company, were attached to IPN surfaces as described above. The fluorescent peptide sequences used were CCFDK(FITC)GYYG, CDHKFGLVMLNK(FITC)YAYAG, and CGGNGEPRGDTYRAYK(FITC)GG for 7C-9(9), 15-2, and bsp-RGD(15), respectively, where the FITC residue is only attached to the lysine side chain and where the 7C-9 peptide is cyclized through the N- and C-termini. After washing away unattached peptide, surfaces were incubated for 2 hours with 1546 U/mL Chymotrypsin (Calbiochem) in 10 mM Tris-HCl buffer (Invitrogen) supplemented with 1.47 mg/mL $CaCl_2 \cdot 2H_2O$ (Fisher) adjusted to pH 8.0. Fluorescent measurements were then taken on a Spectra MAX Gemini XS to determine the final peptide concentration for each surface.

4.4.8 Cell Proliferation

For cell studies, surfaces were sterilized with penicillin/streptomycin (Invitrogen). Immediately before use, IPN surfaces were further sterilized in 70% ethanol and washed with PBS four times to remove any traces of ethanol. For proliferation studies, NSCs were detached from laminin plates and added at 30,000 cells/cm^2 in DMEM/F12 supplemented with 20 ng/mL FGF-2. Cells were incubated at 37ºC for 5 days with media changes every other day. After 5 days, plates were frozen at -80ºC and then assayed with CyQuant (Invitrogen).

4.4.9 Stem Cell Differentiation and Immunocytochemistry

NSCs were seeded at 10,000 cells/cm^2 in media supplemented with 20 ng/mL FGF-2 for proliferative conditions, or at 18,000 cells/cm^2 in media supplemented with 1 μM retinoic acid (Calbiochem) and 1% fetal bovine serum (Invitrogen) for differentiating conditions. Cells were incubated at 37ºC for 5 days with media changes every other day. Cells were then stained as previously described.[52, 53] Primary antibodies were incubated with cells for 48 hours at 4ºC at with the following dilutions: Nestin antibody (BD Pharmingen) at 1:500 for proliferating

conditions, while GFAP (Advanced Immunochemical, Inc.) and β-Tubulin III (Sigma) were used at 1:1000 and 1:250 for differentiating conditions, respectively. Secondary antibodies were used at a 1:250 dilution. All images were taken on a Nikon Eclipse TE2000-E microscope.

4.4.10 Quantification of Differentiation Pictures

All cells in a picture were identified and counted via DAPI staining. Cells were then manually scored as either β-tubulin III positive, GFAP positive, or indeterminate in comparison to control cells.

4.4.11 Statistics

All statistical analysis was carried out using ANOVA with a Tukey HSD post-hoc test. The results were considered significant at $p < 0.05$.

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Chapter 5:
High-throughput selection of novel peptides for human embryonic stem cell culture

5.1 Introduction

Human embryonic stem cells, as well as many other cell types, require interactions with extracellular matrix (ECM) molecules to support their survival, proliferation, and functions.[1, 2] For example, Matrigel is a complex mixture of components – including ~60% laminin, ~30% collagen IV, and hundreds of other proteins[3] – that is extracted from a mouse tumor and commonly used as an adherent substrate for hESCs cell culture.[4-7] In general, ECM molecules engage with cells via binding to adhesion receptors, including integrins[8-10], which are a well-studied class of heterodimeric transmembrane adhesion receptors that functionally link ECM to the intracellular cytoskeleton.[11-13] In addition to their functions in anchoring cells to matrix molecules or to other cells, integrins act as cell mechanical sensors for their environment.[13] Exploring these receptors by learning more about their ligands, including ECM proteins or peptides that mimic these proteins, could aid in understanding integrin signaling mechanisms. Although animal-derived ECM molecules, such as those found in Matrigel, are used to culture cells, the use of animal cells for animal-derived proteins for stem cell culture is particularly problematic for numerous reasons. Natural ECM molecules are large (e.g. ~500,000 MW fibronectin and ~850,000 MW laminin), extremely complex (with numerous isoforms, splice variants, and glycoforms), and have numerous signaling motifs that are not yet fully understood.[14] For example, we recently found that Matrigel interacts with numerous integrins, including ones beyond the canonical arginine-glycine-aspartic acid (RGD) integrin-binding motif. In addition, animal and human ECM can suffer from considerable lot to lot variability, can contaminate cultured cells with immunogens that potentially result in a downstream immune response,[15] and can potentially transfer pathogens to the stem cells or their progeny prior to their engraftment in a patient.[16] Therefore, there is a strong need to develop a defined, reproducible, and safe system to aid both basic biological investigation of cell-matrix interactions as well as the development of stem cell bioprocesses for biomedical application.

An emerging alternative to natural ECM is to develop synthetic platforms that are functionalized with bioactive, synthetic peptide ligands that emulate the activities of ECM proteins such as those found in Matrigel. These peptides could aid the functional investigation or dissection of ECM protein signaling and the development and production of cell-based therapies for the clinic.[17-21] The primary difficulty with developing these biomaterials, however, is identifying the appropriate peptide or group of peptides that mimic the complex signals from the extracellular matrix. Although cell-binding domains have been identified in a number of ECM proteins, when presented as small peptides many of these domains are not as bioactive as the ECM proteins or even function as antagonists, and it is thus challenging to develop defined substrates to support the complex functions of cells such as stem cells.[16, 22-29] Therefore, the development a general method to identify novel candidate peptides that mimic the biological activities and thereby replace the use of complex ECM blends would have broad implications for biology and medicine**.** Moreover, in situations where the identity of a functionally important receptor is known, but a peptide agonist for it is not, methods to create such a ligand are important.

The large number of potential peptide sequences for even a short ligand renders rational peptide ligand design extremely difficult; however, peptide display library based methods can overcome this problem as a large variety of unbiased peptides can be screened simultaneously. This problem is extremely important for cells such as hESCs, which are known to attach through non-RGD binding integrins and thus cannot be sustained solely on a peptide containing RGD in the long-term.[29] Although bacteriophage display libraries were developed earlier and used in

several studies, these focused on finding antagonists rather than agonists to cell adhesion receptors such as integrins.[30-34] A recent phage display study found many peptides for hESCs, but these peptides had little or no interaction with integrins.[35] Furthermore, new display technologies such as ones that utilize bacteria as a platform are easier to manipulate for library creation and require fewer steps in the selection process.[36-40] In addition, it is easy to incorporate other markers such as fluorescent proteins in bacteria that can be helpful in selecting or analyzing the libraries.[41-43] We have developed two methods utilizing bacterial peptide display libraries: the first finds novel peptides that bind to a given cell type, while the second method allows for the targeting of a specific molecule on the cell surface. Applying these methods to hESCs yielded many novel peptides — some of which bind a specific targeted adhesion receptor — that can support the short-term self-renewal of these important pluripotent cells.

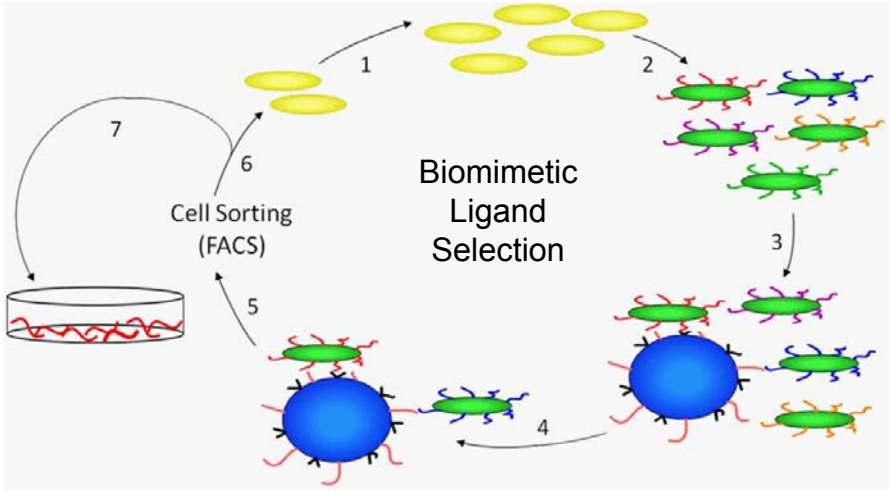
5.2 Results

To find novel peptides that could be used to replace ECM proteins, we used bacterial peptide display technology, which is a versatile platform for peptide selection for numerous reasons. The selected population of bacteria can be amplified by simple culturing, whereas bacteriophage require viral infections, and the peptide valency can be varied to modulate selection stringency.[37, 42, 43] In addition, bacterial plasmids are easy to manipulate, allowing for facile creation of libraries of bacteria with random peptide sequences displayed on their cell surfaces, as well as recovery of the peptide sequences following selection. Furthermore, because bacteria can be engineered to express fluorescent proteins, mammalian cells with bound bacteria can be isolated or characterized by high-throughput flow cytometry for rapid selection or clonal analysis.[41-43] Our method is the first the use bacterial peptide display to identify novel peptides for use in bioactive materials. We used two classes of library: X_{15} (15mer linear peptides) and $X_2CX_7CX_2$ (7C peptides, constrained by two cysteine residues that form a disulfide bond to yield a cyclic peptide), both of which were displayed on an engineered outer membrane protein CPX.[43, 44] In total, three libraries were pursued: one of exclusively 15mer clones, one of exclusively 7C clones, and one containing half of each peptide type (combined library). Each library had a diversity of 2×10^9, and have been used to find peptides binding to breast cancer, but not regular breast cells.[43]

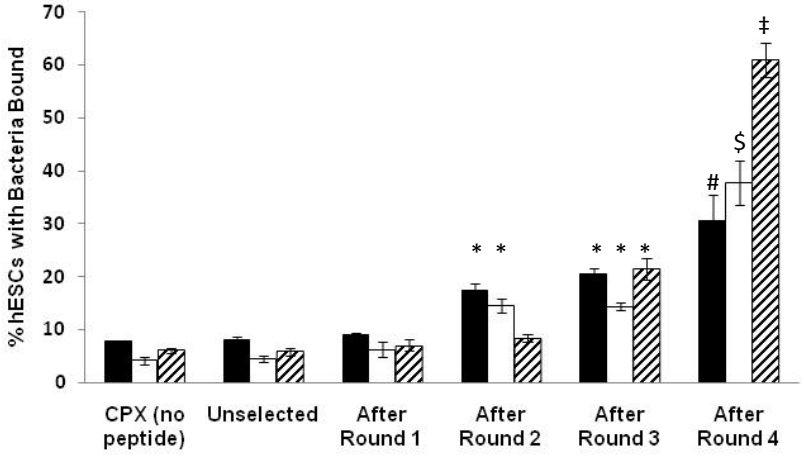
5.2.1 Biomimetic Ligand Selection

In the first of 6 steps involved in the biomimetic ligand selection (Figure 5-1a), the libraries were prepared by growing bacterial cultures followed by arabinose induction of green fluorescent protein (GFP) and CPX-peptide expression in the bacteria. The resulting libraries were then co-incubated with human embryonic stem cells at 37ºC in their native media, and in the first two rounds non-adherent clones were washed away with low speed co-centrifugation after one hour. Selected bacteria were then expanded by addition of bacterial medium. For the third and fourth round selections, fluorescence-activated cell sorter (FACS) was used to specifically isolate the (non-fluorescent) mammalian cells with bound, GFP-expressing bacteria.

a



b



c

Clone	% hESCs with Bacteria (avg. ± S. D.)	Frequency	Peptide Sequence
15-48+	87.3 ± 1.1	1	LEQRVGREMHSKWKR
Co-9+	86.9 ± 4.9	1	GMRFMQMYFKQGNRR
Co-16+	83.7 ± 5.5	1	GKDSGWGRRKYWESN
7C-7+	80.4 ± 1.8	1	DWCFCKGHYWCCW
7C-22+	79.6 ± 4.0	2	IGCYFPPLWVCTA
7C-23+	72.7 ± 1.2	4	YLCIRTWKGVFAM
7C-10+	67.6 ± 1.4	4	RCSFQTWQWVCGN
7C-1+	67.3 ± 4.6	1	FSCDFGKLWSCNK
7C-11+	63.1 ± 5.2	1	RCSFKTWKRWCGD
Co-10+	60.8 ± 3.9	1	KQRGLRDQRKSMWGT
7C-20+	58.4 ± 0.4	1	ECCYVQWEWRCRS
15-13+	53.7 ± 2.8	1	EKGLTTIPCSNRFAV
15-21+	53.0 ± 5.7	1	LCQGRNVYYGRRKYE
15-38+	52.6 ± 3.8	1	RATRWILIKLREWQE
15-17+	51.7 ± 3.6	1	NGHKWLDKRAKRKKP

Figure 5-1: Schematic of method and binding capacity of peptide display library and clonal bacterial populations with hESCs. (a) Schematic of biomimetic ligand selection and use as biomaterials. (1). Bacterial libraries were expanded. (2). Co-expression of green fluorescent protein and bacterial outer membrane protein CPX with the displayed peptide are induced with arabinose. (3). Stem cells are added to bacterial libraries in a co-incubation step in which the bacteria can bind to the stem cell surface. (4). Non-adherent bacteria are washed away with low speed co-centrifugeation. (5). For later round selections or analysis of bacterial populations, samples of the stem cells are sorted or analyzed on a fluorescence activated cell sorter or flow cytometer. (6). Bacteria populations are frozen or plated for further selection or analysis. (7). Peptides from clones are sequenced. Synthetic versions of these peptides can then be conjugated to biomaterials, cell culture apparatus, or implantable materials. (b) Quantification of library binding capacity of untargeted libraries with hESCs. Three libraries were tested: (■) 15mer library composed of peptides with the sequence X_{15}, (◩) 7C library composed of peptides with the sequence $X_2CX_7CX_2$, and (□) combined library containing both types of peptide clones. The affinity of the libraries to our hESCs was determined by flow cytometry with the use of the GFP expressed by the library bacteria. After three and four rounds of selections, all libraries had significantly greater binding capacity than unselected libraries or bacteria expressing CPX, but no peptide. Of the fourth round library populations, the 7C library had the greatest binding capacity with hESCs followed by the combined and 15mer library, respectively. (c) Complete list of clones found with binding at least 50% hESCs for untargeted libraries. Clones were analyzed with flow cytometry to quantify the percentage of human embryonic stem cells that had bacteria bound after co-incubation with the clonal bacteria population. Peptide sequences were determined via sequencing of the plasmid DNA from the bacteria. The peptides used in further studies are highlighted in gray. The clone name indicates the library containing the clone, and the frequency indicates the number of separate clonal populations analyzed that had the same peptide sequence. Data represent mean ± standard deviation. Library populations not in the same group (*, #, $, or ‡) were statistically different from one another ($p < 0.05$ using ANOVA between groups with Tukey-Kramer significant difference post hoc test).

5.2.1.1 Finding Novel Peptides for hESCs with Biomimetic Ligand Selection

Panning the bacterial peptide display libraries against hESCs for four successive rounds with all three libraries yielded populations that bound to hESCs with high efficiency compared to unselected libraries or to bacteria expressing CPX, the display protein, containing no peptide (Figure 5-1b). Between the three libraries, the 7C constrained library had the highest binding capacity, followed by the combined library, and then the 15mer linear library. To find the peptides with high-affinity to hESCs, we picked colonies after the last round of selection and panned them against hESCs. These clones were analyzed for their affinity to hESCs with flow cytometry, and high-affinity clones were then sequenced. After analyzing clonal bacterial populations from all three selected libraries with flow cytometry, we found 15 unique bacterial clones with peptide sequences having high affinity to hESCs, which we defined as binding to greater than 50% of hESCs after co-incubation but ranged as high as 87.3% (Figure 5-1c). Three of these sequences appeared more than once in our clonal screening indicating that the diversity of the library had decreased significantly over the course of the selections. Of the fifteen sequences found, four were chosen for further analysis because they bound to hESCs with relatively high affinity, were predicted to be water-soluble, and came from different selected libraries.

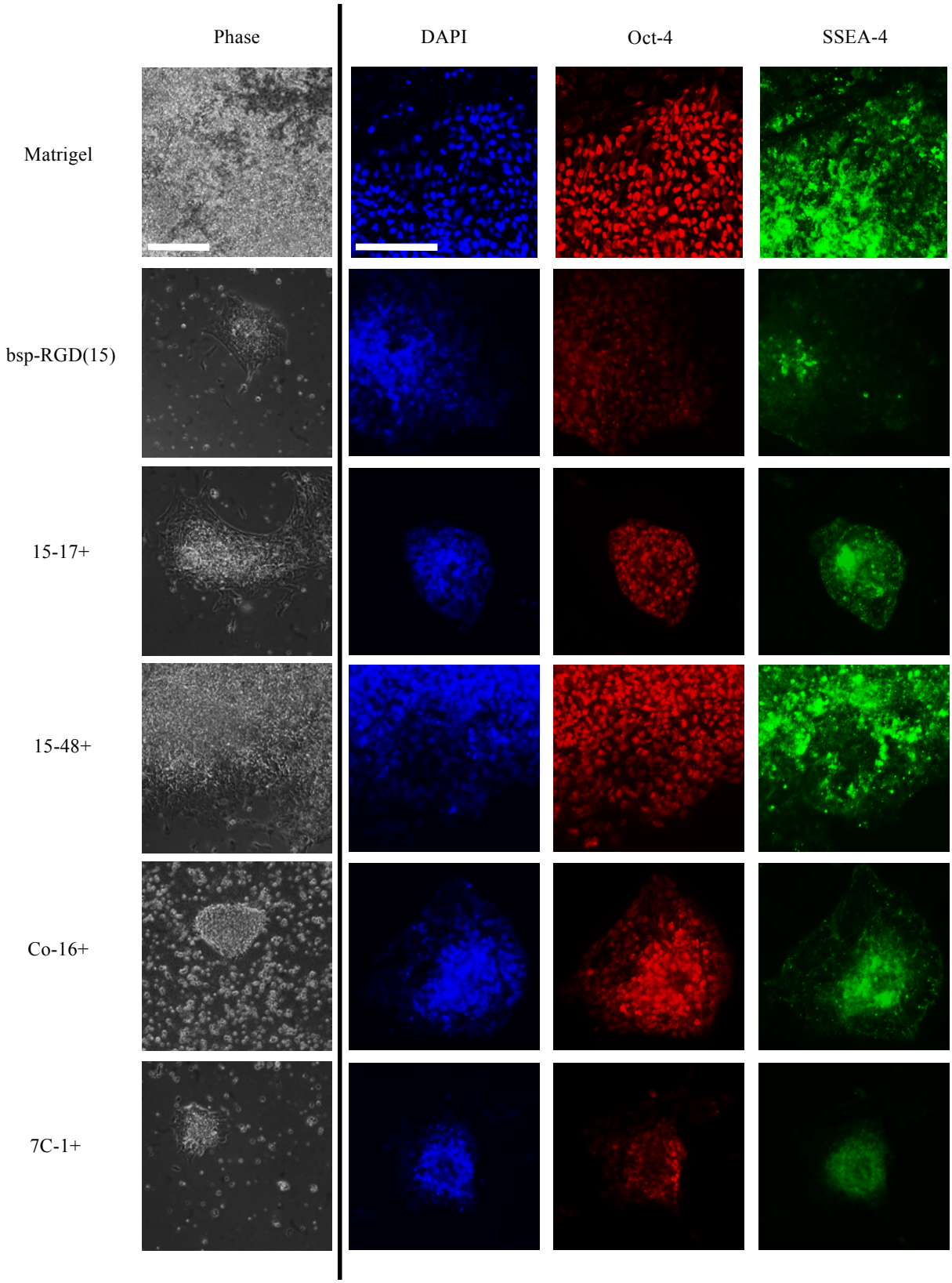


Figure 5-2: Brightfield and fluorescent immunocytochemistry micrographs of human embryonic stem cells cultured on peptide-adsorbed surfaces for 5 days. All library-selected peptide-adsorbed surfaces exhibited hESC colonies, though only 15-48+ adsorbed surfaces had similar colony size and morphology to Matrigel,

while the other peptides had similar colony size to bsp-RGD(15). Immunocytochemistry for pluripotent markers—DAPI (blue), Oct4 (red) , and SSEA-4 (green)—on hESCs grown on peptide-adsorbed surfaces after 5 days exhibited expression of both markers Oct4 and SSEA-4, with the highest expression of these two seen on Matrigel and 15-48+ surfaces and the least on 7C-1+ and bsp-RGD(15). Phase pictures were taken from different frames than the immunocytochemistry pictures for the same peptide or control surface. The scale bar indicates 250 µm.

5.2.1.2 Testing Peptides from Biomimetic Ligand Selections as Adherent Biomaterials

The four chosen peptides—15-17+, 15-48+, Co-16+, and 7C-1+—were adsorbed to TCPS surfaces for 3 hours. Because all of our studies were conducted with serum-free media, the cell behavior on these surfaces was due to the nature of the peptides adsorbed on the surface rather than for example deposition of serum components. In comparison, we also used Matrigel and TCPS surfaces adsorbed with bsp-RGD(15), a peptide previously used with hESCs containing an RGD motif known to bind several integrins including $\alpha_v\beta_3$ and $\alpha_5\beta_1$.[17, 26, 27, 29] When cells were placed on the surfaces as single cells and allowed to attach for 2 hours, all peptides—including bsp-RGD(15)—had slightly less cells attached in comparison to Matrigel, though they all had more cells attached than plain TCPS (Figure 5-3a). Five days after seeding, all peptide-adsorbed surfaces exhibited colonies. However, only 15-48+ exhibited colony morphology and size similar to those on Matrigel (Figure 5-2), whereas all other surfaces and in particular the bsp-RGD(15) surface had smaller colonies than those seen on Matrigel, while bare TCPS surfaces did not have any appreciable number of cells attached. Quantification of cells attached on all these surfaces confirmed that 15-48+ and Matrigel surfaces had similar numbers of cells after 5 days, while 15-17+, Co-16+, and bsp-RGD(15) adsorbed surfaces had significantly fewer (Figure 5-3a). 7C-1+ supported even fewer, and bare TCPS surfaces had no detectable cells.

Although several surfaces supported cell proliferation, it is critical that they also maintain hESC marker expression. Oct4 and SSEA-4 expression after five days on all surfaces were examined via immunocytochemistry, with the exception of bare TCPS, which no longer had cells attached after 5 days. All peptide surfaces exhibited colonies that expressed both Oct4 and SSEA-4, with Matrigel, Co-16+, and 15-48+ surfaces having the highest levels of both markers (Figure 5-2). Surfaces adsorbed with bsp-RGD(15) and 15-17+ exhibited modest levels of both markers, while 7C-1+ had the lowest expression of both markers. Importantly, quantification of these markers with flow cytometry confirmed the imaging results (Figure 5-3b). For both Oct4 and SSEA-4, Matrigel had the greatest expression of both markers, though 15-48+ and Co-16+ had slightly lower levels of Oct4, and 15-17+, 15-48+, and Co-16+ had slightly lower levels of SSEA-4 in comparison to Matrigel surfaces. For both markers, 7C-1+ surfaces exhibited the lowest levels of both markers (Figure 5-3b).

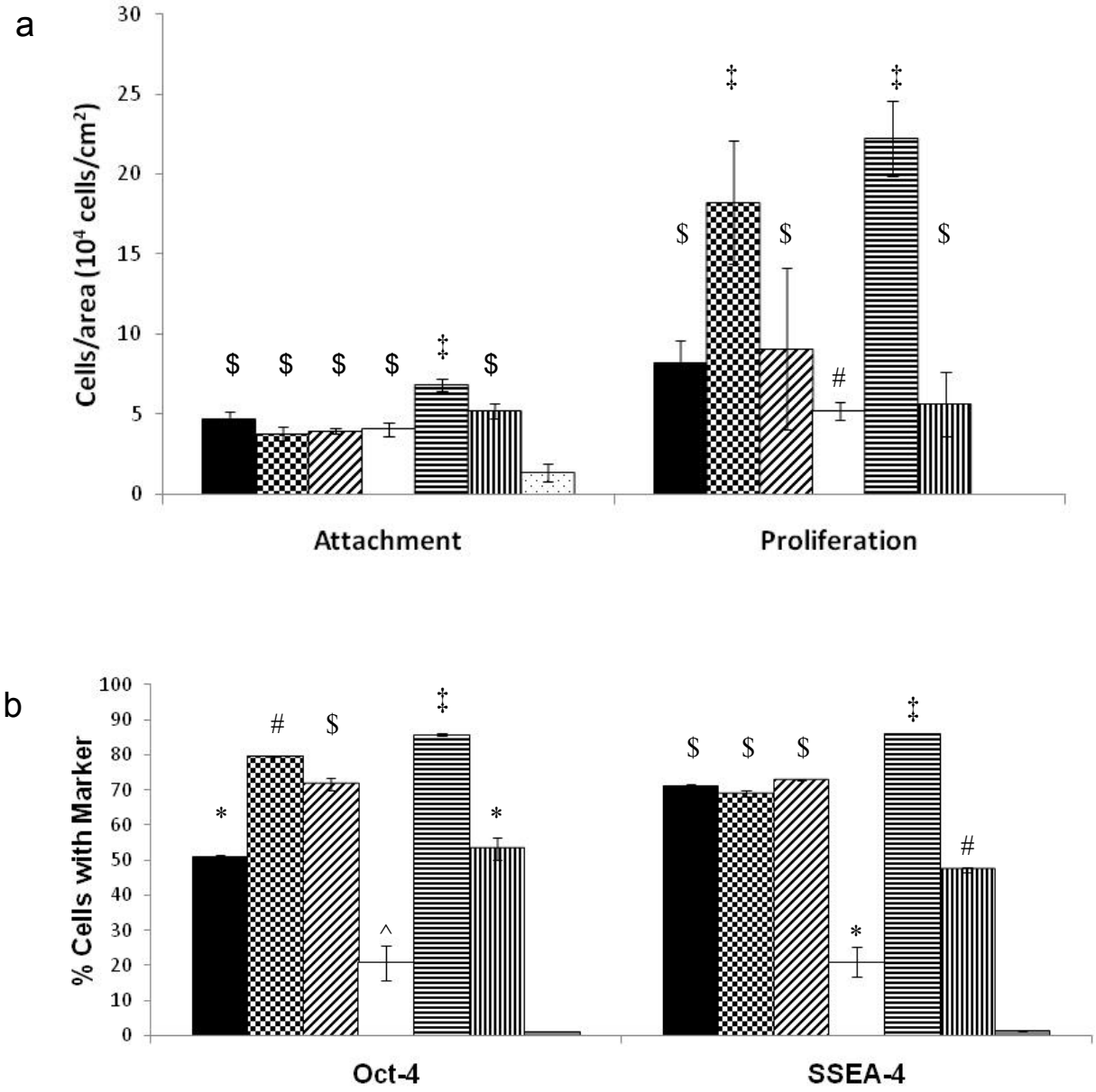


Figure 5-3: Quantification of attachment, growth, and expression of pluripotent markers of human embryonic stem cells on peptide-adsorbed and control surfaces. Samples, including (■) 15-17+, (▩) 15-48+, (▨) Co-16+, (□) 7C-1+, (▤) Matrigel, (▥) bsp-RGD(15), and (▩) TCPS alone were tested. (a) Attachment and proliferation of hESCs on peptide-adsorbed and control surfaces. hESCs as single cells were allowed to attach on peptide-adsorbed surfaces for 2 hours. Matrigel had higher cell attachment than any peptide-adsorbed surface, though all peptide-adsorbed surfaces had greater cell attachment than TCPS surfaces with no peptide. After 5 days proliferation on peptide-adsorbed and control surfaces, Matrigel and 15-48+ had the greatest proliferation. All other peptides had greater proliferation than TCPS with no adsorbed peptide, though 7C-1+ surfaces had the lowest cell proliferation of all the peptide surfaces. TCPS was below the detection limit of the assay. (b) Quantification of Oct4 and SSEA-4 expression of hESCs grown on control and peptide-adsorbed surfaces for 5 days. All surfaces had greater expression of Oct4 and SSEA-4 in comparison to isotype (▩). For both cell markers, Matrigel surfaces had the highest expression. SSEA-4 expression was slightly lower than Matrigel on 15-17+, 15-48+, and Co-16+ surfaces, while only 15-17+ and 15-48+ had slightly lower Oct4 expression in comparison to Matrigel. 7C-1+ surfaces had the lowest expression of both markers, while bsp-RGD(15) surfaces had modest levels of both markers. Data represent mean ± standard deviation. Samples not in the same group (‡, *, #, $, or ^) were

statistically different from one another ($p < 0.05$ using ANOVA between groups with Tukey-Kramer significant difference post hoc test). Statistical comparisons were only performed for the same cell marker.

5.2.2 Targeted Biomimetic Ligand Selections

Through the use of anti-integrin blocking antibodies, we recently found that several integrins are important for hESC attachment to Matrigel-coated substrates, namely $\alpha_v\beta_3$, $\alpha_2\beta_1$, and $\alpha_6\beta_1$.[29] However, while the $\alpha_6\beta_1$ integrin was shown to be the most important of these three,[29] and although this integrin is expressed highly on hESCs (Figure 5-4a),[29] we have previously found no effective peptide ligands for this important integrin. We therefore hypothesized that the biomimetic ligand selection could be adapted to target a specific cell surface receptor or antigen.

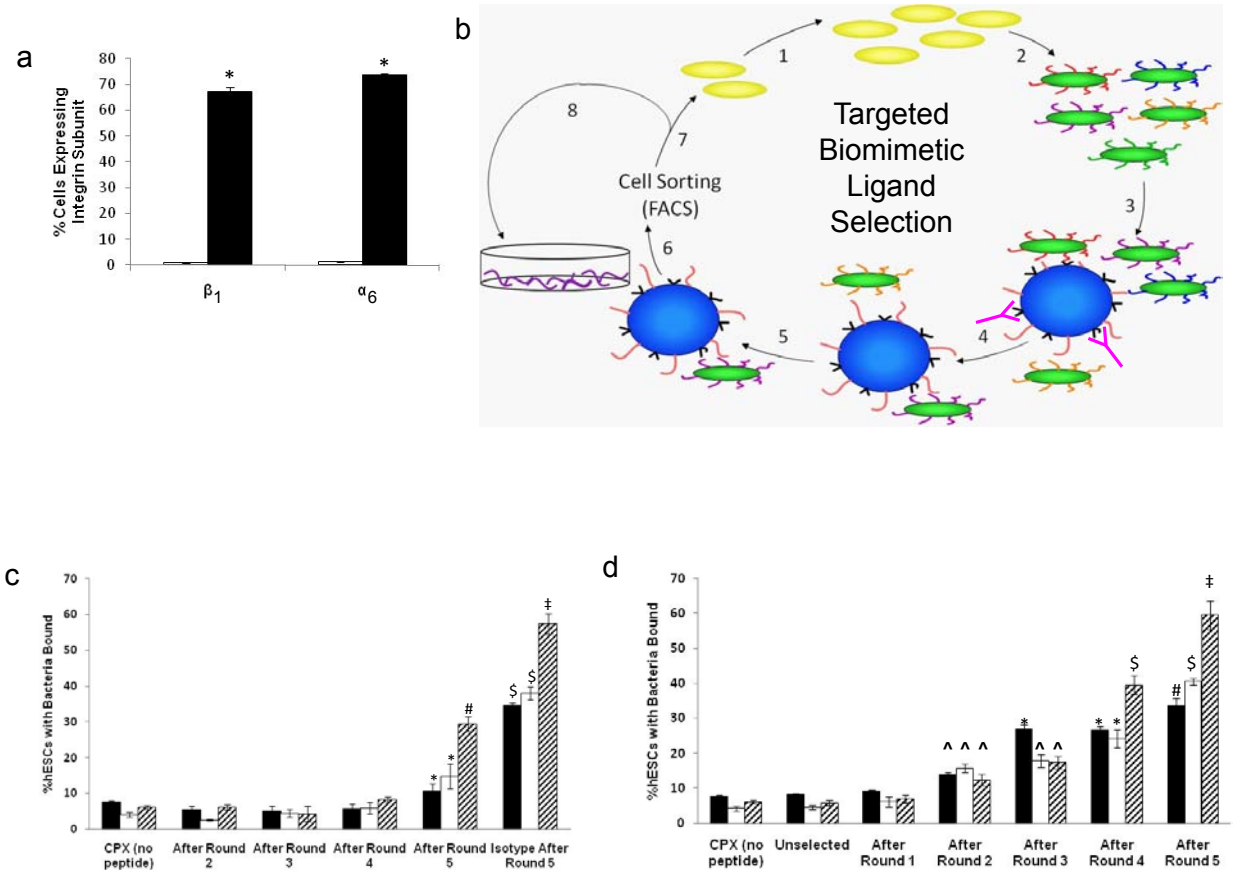
For these Targeted Biomimetic Ligand Selections (Figure 5-4b), we first incubated hESCs with blocking antibodies specific to the α_6 and β_1 integrins, then added the bacterial library. During this first co-incubation, the bacteria would have the opportunity to bind to all molecules on the cell surface except α_6 and β_1 integrins. After this negative selection, the bacteria that <u>did not</u> attach to these cells during this first co-incubation were then added to unblocked hESCs, to positively select them for the capacity to bind to α_6 and β_1 integrins, and the cells were washed or sorted as before (Figure 5-1a).

5.2.2.1 Finding Novel Peptides that Target the $\alpha_6\beta_1$ Integrin on hESCs

Using this Targeted Biomimetic Ligand Selection method, we panned the three libraries against hESCs in five rounds of selection. The dual positive/negative selections were performed in rounds two through four with antibodies for the α_6 and β_1 integrin subunits, while in the fifth round the double selection was conducted only with the α_6 integrin subunit to make the selection more specific for the $\alpha_6\beta_1$ integrin, as the α_6 integrin pairs with only two different β subunits, while β_1 pairs with 12 different α subunits.[13]

After the five rounds of selection, all three libraries had greater binding to hESCs than unselected libraries or bacteria expressing CPX with no peptide (Figure 5-4c). Similarly to the untargeted selections, the 7C library had higher affinity to the hESCs than the combined or 15mer library as detected by flow cytometry. In addition, when library populations were co-incubated with hESCs blocked with antibodies for α_6 and β_1 integrins, but not with isotype control antibodies, there was much less binding of the libraries to the hESCs, indicating that these libraries were binding to these integrins (Figure 5-4d).

Clonal bacterial populations were examined to individual peptides that mediated binding to greater than 50% of hESCs after co-incubation. High-binding peptides were then sequenced and nineteen unique clones were found from all three libraries, with many duplicates of several clones found during the screening process (Figure 5-4e). As with the selected libraries, these individual clonal populations were co-incubated with hESCs pre-blocked with $\alpha_6\beta_1$ integrin antibodies to determine how specific each peptide sequence was to these integrins. All but 6 of the clones—7C-26+/-, 7C-27+/-, 7C-20+/-, 7C-1+/-, Co-6+/-, and 7C-13+/-—had at least a 20% decrease in the percentage of hESCs bound with bacteria upon antibody blocking. Five of these peptides—Co-34+/-, 15-40+/-, 15-36+/-, Co-11+/-, and Co-24+/-—were then chosen for analysis of their ability to maintain self-renewal of hESCs, because they bound to unblocked hESCs with high efficiency, had higher reduction in binding upon antibody blocking compared to other peptides, and were predicted to be water soluble.



e

Clone	% hESCs with Bacteria (avg. ± S. D.)	Decrease in % hESCs with Bacteria when cells were preblocked (avg. ± S. D.)	Frequency	Peptide Sequence
15-21+/-	86.6 ± 3.5	23.8 ± 2.7	2	MKKQSAEMVKFKSKQ
Co-26+/-	85.4 ± 2.0	35.4 ± 0.6	1	HYCNLSLGGGCNY
Co-34+/-	83.5 ± 2.4	29.9 ± 2.6	1	NQKEGEKVWYVVRRF
7C-26+/-	83.1 ± 0.8	11.9 ± 1.4	2	FYCRRYSGNKCVT
Co-35+/-	82.5 ± 5.4	27.9 ± 2.4	1	HGCSVRDKISCWW
Co 32+/-	82.0 ± 3.9	28.4 ± 0.2	1	SRFKEVTSRQCGG
15-40+/-	82.0 ± 4.6	38.2 ± 2.9	1	EWRTGQLVTNKNTMK
15-25+/-	79.9 ± 3.8	28.8 ± 2.3	1	RRGAEEQLADVDVELG
7C-27+/-	78.5 ± 4.0	2.4 ± 0.3	1	KSCQHGWLCNCTA
15-36+/-	78.0 ± 4.3	49.7 ± 1.0	1	FKKRDRDKPPHRKYM
7C-20+/-	75.9 ± 1.5	11.1 ± 3.6	1	RLCARSEIELCRQ
15-35+/-	74.6 ± 3.6	46.8 ± 3.3	2	KAVIENDRAVAEGPPREVDGR
7C-31+/-	74.2 ± 6.4	26.5 ± 4.7	1	WGCYFMSGRQCMS
7C-1+/-	74.1 ± 1.7	6.4 ± 5.2	6	GSCLSDGRPRCEW
Co-6+/-	69.1 ± 6.7	7.3 ± 3.0	1	GGCGLQQQFQCKI
7C-13+/-	66.7 ± 1.8	-0.9 ± 0.3	1	GRCATRQNDRCKN
Co-11+/-	65.5 ± 5.8	34.5 ± 0.6	1	EGAWRNGSRPSPAR
Co-24+/-	62.6 ± 5.3	41.3 ± 1.9	1	RAERKGIFHVCVMEK
15-13+/-	57.1 ± 4.9	32.9 ± 1.8	1	DWKAMTFTGMHFNPR

Figure 5-4: Schematic of targeted biomimetic ligand selection and binding capacity of targeted peptide display library and clonal bacterial populations with hESCs. (a) Expression of α_6 and β_1 integrin subunits. Compared to isotype, there was significant expression of the α_6 and β_1 integrin subunits. (b) Schematic of targeted biomimetic ligand selection and use as biomaterials. (1). Bacterial libraries were expanded. (2). Co-expression of green fluorescent protein and bacterial outer membrane protein CPX with the displayed peptide are induced with arabinose. (3). Stem cells pre-blocked with antibodies are added to bacterial libraries in a co-incubation step in which the bacteria can bind to the all molecules on

the stem cell surface except the blocked molecule. (4). Non-adherent bacteria from the first co-incubation are incubated with unblocked cells to allow bacteria to bind to the cells. (5). Non-adherent bacteria are washed away with low speed co-centrifugeation. (6). For later round selections or analysis of bacterial populations, samples of the stem cells are sorted or analyzed on a fluorescence activated cell sorter or flow cytometer. (7). Bacteria populations are frozen or plated for further selection or analysis. (8). Peptides from clones are sequenced. Synthetic versions of these peptides are then conjugated on biomaterials. Three libraries were tested: (■) 15mer library composed of peptides with the sequence X_{15}, (◩) 7C library composed of peptides with the sequence $X_2CX_7CX_2$, and (□) combined library containing both types of peptide clones. (c) Quantification of library binding capacity of $\alpha_6\beta_1$ integrin targeted libraries with hESCs preblocked with α_6 and β_1 integrin subunit antibodies. Binding capacity of the libraries with preblocked cells was similar to binding with CPX. Round 5 libraries had higher binding with preblocked cells than other library populations, but this was still significantly lower than library binding with hESCs incubated with isotype antibodies. (d) Complete list of clones found with binding at least 50% hESCs for $\alpha_6\beta_1$ integrin targeted libraries. Clones were analyzed with flow cytometry to quantify the percentage of human embryonic stem cells that had bacteria bound after co-incubation with the clonal bacteria population. In addition, clonal populations were tested for binding with hESCs preblocked with antibodies for the $\alpha_6\beta_1$ integrin. The decrease in binding was determined by subtracting the % hESCs bound with bacteria for preblocked cells from the % hESCs bound with bacteria for unblocked cells. Peptide sequences were determined via sequencing of the plasmid DNA from the bacteria. The peptides used in further studies are highlighted in gray. The clone name indicates the library containing the clone, and the frequency indicates the number of separate clonal populations analyzed that had the same peptide sequence. Data represent mean ± standard deviation. Library populations not in the same group (^, *, #, $, or ‡) were statistically different from one another ($p < 0.05$ using ANOVA between groups with Tukey-Kramer significant difference post hoc test).

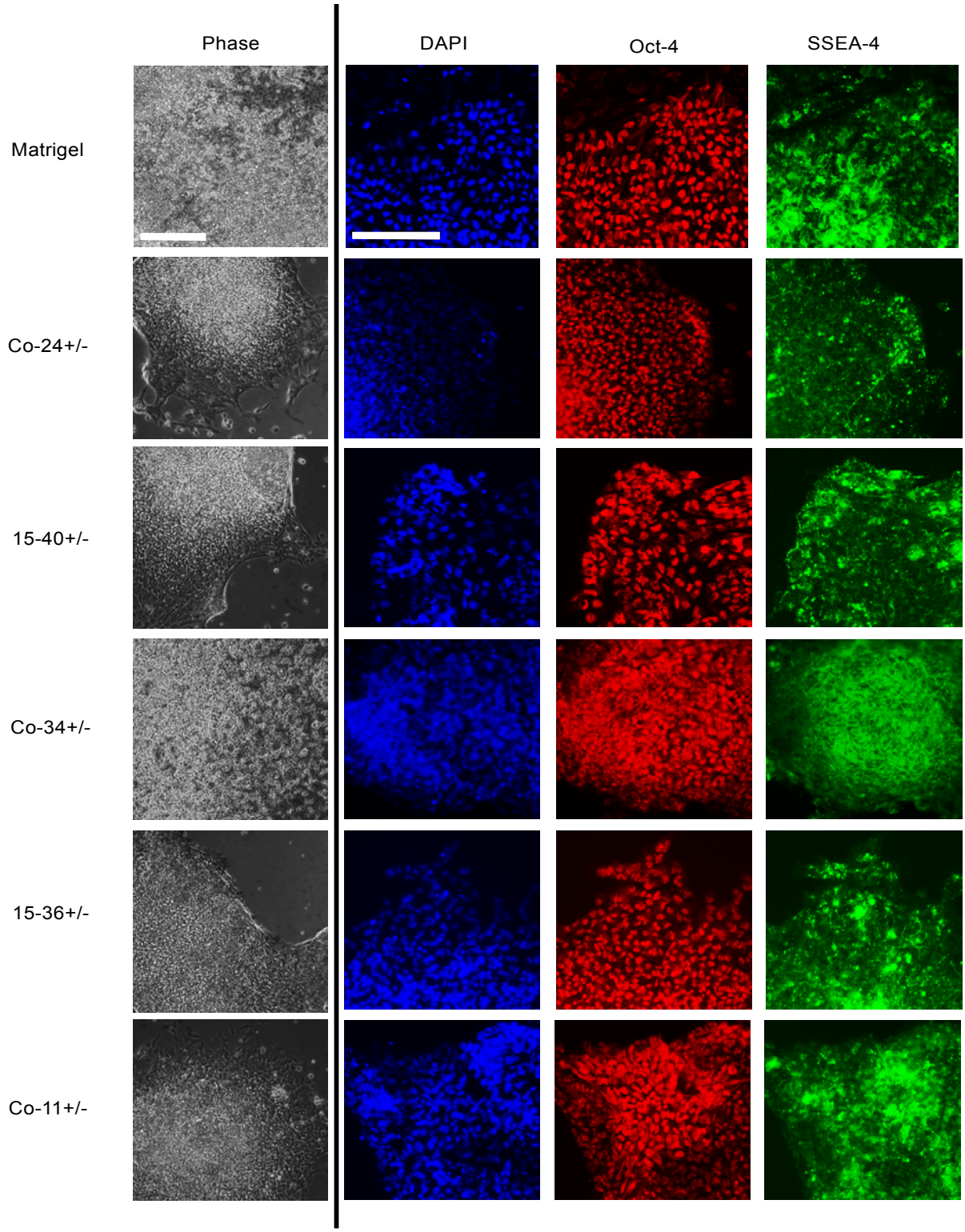
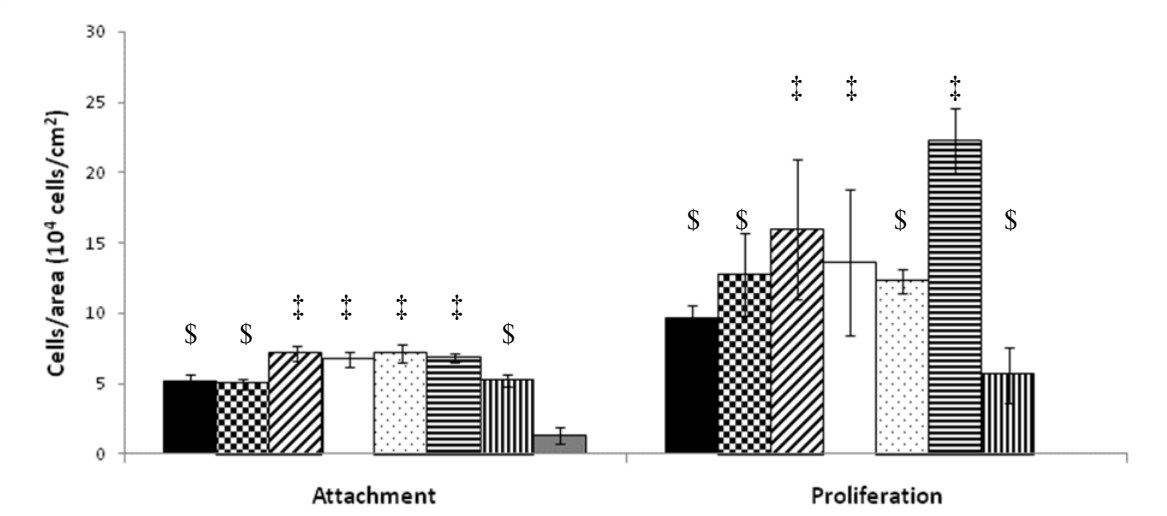


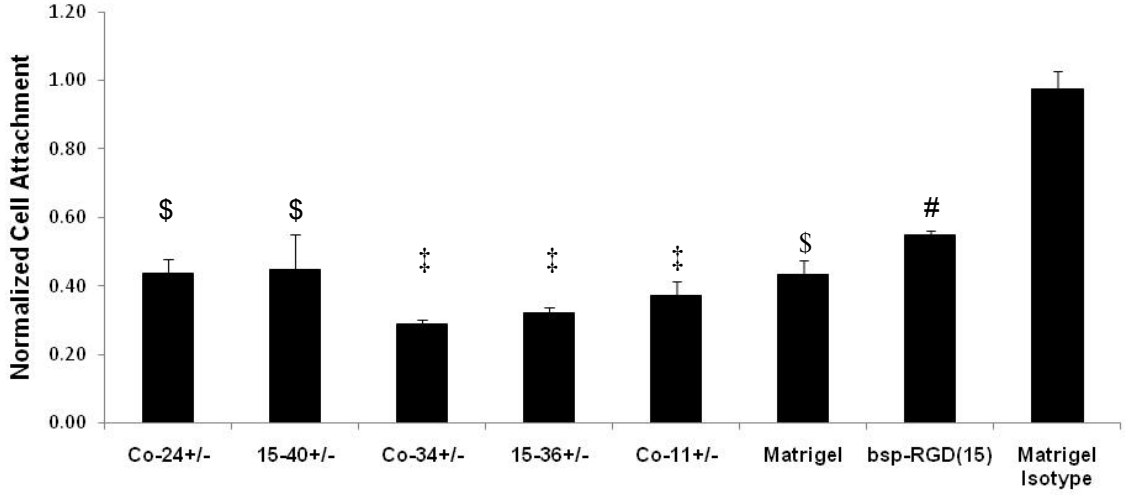
Figure 5-5: Brightfield and fluorescent immunocytochemistry micrographs of human embryonic stem cells cultured on targeted peptides adsorbed on polystyrene surfaces for 5 days. All targeted peptide-adsorbed surfaces exhibited hESC colonies with similar colony morphology to Matrigel. Immunocytochemistry for pluripotent markers—DAPI (blue), Oct4 (red) , and SSEA-4 (green)—on hESCs grown on peptide-adsorbed surfaces after 5 days exhibited expression of both markers Oct4 and SSEA-4, with the high expression of these two markers seen on Matrigel and all targeted peptide surfaces. Phase pictures were taken from

different frames than the immunocytochemistry pictures for the same peptide or control surface. The scale bar indicates 250 µm.

a


Cells/area (10^4 cells/cm^2)
30
25
20
15
10
5
0
Attachment
Proliferation

b


Normalized Cell Attachment
1.20
1.00
0.80
0.60
0.40
0.20
0.00
Co-24+/-
15-40+/-
Co-34+/-
15-36+/-
Co-11+/-
Matrigel
bsp-RGD(15)
Matrigel Isotype

c

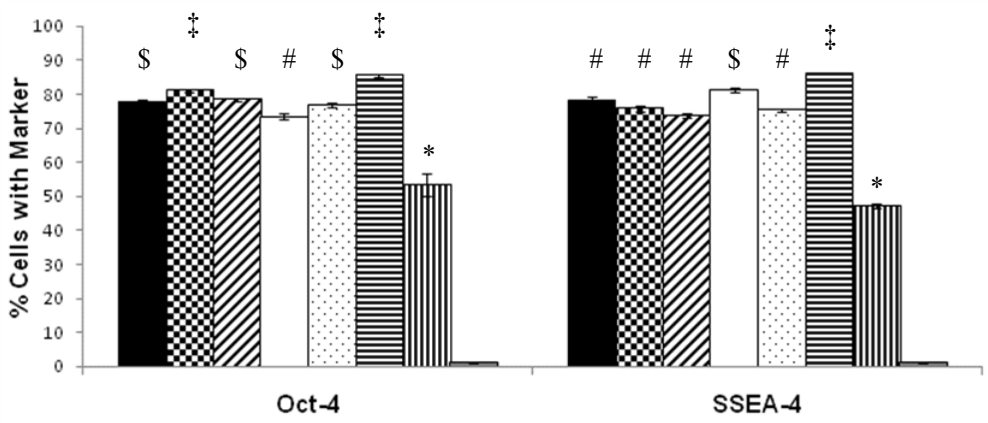

% Cells with Marker
100
90
80
70
60
50
40
30
20
10
0
Oct-4
SSEA-4

Figure 5-6: Quantification of attachment, growth, and expression of pluripotent markers of human embryonic stem cells on targeted peptides adsorbed on polystyrene surfaces and control surfaces. Samples, including (■) Co-24+/-, (▩) 15-40+/-, (◪) Co-34+/-, (□) 15-36+/-, (⊡) Co-11+/-, (▬) Matrigel, and (▥) bsp-RGD(15), (▩) TCPS alone were tested. (a) Attachment and proliferation of hESCs on peptide-adsorbed and control surfaces. hESCs as single cells were allowed to attach on peptide-adsorbed surfaces for 2 hours. Matrigel had similar cell attachment to Co-34+/-, 15-36+/-, and Co-11+/-, while Co-24+/- and 15-40+/-, and bsp-RGD(15) had slightly less cell attachment. After 5 days proliferation on peptide-adsorbed and control surfaces, Matrigel, Co-34+/-, and 15-36+/- had the greatest cell proliferation. All other peptides had greater proliferation than TCPS with no adsorbed peptide. TCPS was below the detection limit of the assay. (b) Attachment of hESCs preblocked with α_6 and β_1 integrin subunit antibodies on peptide-adsorbed and control surfaces. All surfaces were normalized to attachment on the same surface with unblocked cells. Compared to cells pre-incubated with isotype antibodies, all peptide-adsorbed surfaces and Matrigel had significantly less attachment when cells were preblocked with either α_6 or β_1 integrin subunit antibodies, indicating that the $\alpha_6\beta_1$ integrin is important for attachment on all these surfaces. The most significant decrease in attachment was on the targeted peptides Co-34+/-, 15-36+/-, and Co-11+/-. Matrigel, Co-24+/-, and 15-40+/- surfaces had higher attachment, but this was significantly less than the bsp-RGD(15). (c) Quantification of Oct4 and SSEA-4 on hESCs grown on control and peptide-adsorbed surfaces for 5 days. All surfaces had greater expression of Oct4 and SSEA-4 in comparison to isotype. For Oct4, Matrigel and 15-40+/- had similar Oct4 expression, closely followed by Co-24+/-, Co-34+/-, 15-36+/-, and Co-11+/-. For the marker SSEA-4, Matrigel had the highest expression overall, with all targeted peptides having slightly lower SSEA-4 expression than Matrigel. bsp-RGD(15) surfaces had cells with modest expression of both pluripotent markers. Data represent mean ± standard deviation. Samples not in the same group (‡, *, #, $, or ^) were statistically different from one another ($p < 0.05$ using ANOVA between groups with Tukey-Kramer significant difference post hoc test). Statistical comparisons were only done for the same cell marker.

5.2.2.1 Testing Peptides from Targeted Biomimetic Ligand Selections as Adherent Biomaterials

Similar to the untargeted peptides, the $\alpha_6\beta_1$ integrin targeted peptides were adsorbed on TCPS and compared with Matrigel and bsp-RGD(15) adsorbed surfaces. When hESCs were allowed to attach on the surfaces as single cells for 2 hours, Co-34+/-, 15-36+/-, and Co-11+/- had similar cell attachment in comparison to Matrigel. Co-24+/- and 15-40+/- had similar cell attachment as bsp-RGD(15), but much greater cell attachment than TCPS with no peptide (Figure 5-6a). When cells were pre-blocked with α_6 and β_1 integrin blocking antibodies, the normalized cell attachment after 2 hours decreased on all surfaces, with the greatest decreases seen on Co-34+/-, 15-36+/-, and Co-11+/- (Figure 5-6b). Matrigel, Co-24+/-, and 15-40+/- surfaces had smaller decreases in normalized cell attachment, though all surfaces including bsp-RGD(15) had decreased cell attachment compared to cells pre-blocked with isotype antibodies on Matrigel. All cell attachment was normalized to the attachment on that same surface for cells that were not pre-blocked. In contrast to the untargeted peptides, all targeted peptides exhibited similar colony morphology to Matrigel surfaces after 5 days of culture (Figure 5-5). Proliferation results after 5 days showed that two of the targeted peptides, Co-34+/- and 15-36+/-, supported growth rates similar to Matrigel (Figure 5-6a), where as the other three exhibited similar proliferation to bsp-RGD(15).

Finally, the expression of pluripotent cell markers was examined via immunocytochemistry on the targeted peptide-adsorbed surfaces and Matrigel after 5 days of cell culture. All surfaces exhibited hESCs expressing those markers at qualitatively similar levels to Matrigel (Figure 5-5). The expression of these markers was quantified with flow cytometry, and 15-40+/- surfaces were shown to have a similar amount of Oct4 expression as compared to Matrigel, though the other targeted peptide surfaces had just slightly lower levels of Oct4, and much higher in comparison to bsp-RGD(15). SSEA-4 levels for all the targeted peptides were only slightly lower than on Matrigel, but the levels for all were considerably higher than on bsp-RGD(15).

5.3 Discussion

Numerous classes of stem cells require engagement with extracellular matrix proteins to support their proliferation, self-renewal, and differentiation.[8-10, 45] However, animal and human proteins are problematic due to their variability, difficulty of purification, potential immunogenicity, and possible contamination with pathogens.[15, 16, 46, 47] Furthermore, from a basic stem cell and matrix biology viewpoint, the investigation of key ECM domains and their cognate cellular receptors that support and drive various cell functions is challenging due to the size, variability, and complexity of ECM protein families.

For our screening method, we first used biomimetic ligand selection to find novel peptides that bind to hESCs without targeting any specific molecule on the cell surface. We found 15 unique peptides that bound with high affinity to hESCs, and four of these were tested for the ability to support short-term self-renewal. Of the four tested, only 15-48+ supported the attachment, growth, and maintenance of pluripotent markers similar to Matrigel indicating that this peptide supported self-renewal in the short-term. Next we modified our biomimetic ligand selections with blocking antibodies for the $\alpha_6\beta_1$ integrin to target our selections to this adhesion receptor. From these selections, we found 19 unique peptides that bound to our cells. Five of the peptides that were shown to bind to the $\alpha_6\beta_1$ integrin were then tested for their ability to support self-renewal of hESCs. Two of these peptides, Co-34+/- and 15-36+/-, exhibited the ability to support short-term self-renewal of hESCs similar to Matrigel in addition to binding to the $\alpha_6\beta_1$ integrin on a surface.

Overall, these methods are quite versatile, and they can be applied to any desired cell type to identify peptides used in the design of biomaterials. We have used these methods with hESCs, but these methods can easily be applied to iPS or other stem cells to replace the ECM proteins used in their culture. In addition, the targeting biomimetic ligand selection could be used for any molecule on the cell surface that has an available blocking antibody. This is quite valuable especially for cell receptor targets that do not have many known bioactive peptides such as the $\alpha_6\beta_1$ integrin, a non-RGD-binding integrin.[29] Past phage display studies have found antagonistic peptides to integrins, but these peptides were not shown to act as agonists.[30-34] Cell experiments with novel peptides binding to integrins from phage display have included examining cell spreading or inhibition of cell binding to ECM proteins, but longer-term cell studies with these peptides were not performed.[31, 34] A recent study has used phage display screening to find peptides for use in biomaterials, but the peptides found did not bind significantly to integrins even though they are used in the binding to Matrigel surfaces.[35] A recent study with yeast peptide display libraries focused on targeting the $\alpha_v\beta_3$ integrin with RGD peptides, but there is also a need for a method that is unbiased towards any particular motif and that can allow the investigation of novel peptides or other cellular receptors.[48]

By targeting a specific integrin or other cell receptor, many candidate peptides specific to that target can be found that can then be tested for activation of the cell receptor. Since there are very few peptides that are known to mimic ECM-derived signals,[24, 25, 27] targeting a cell receptor such as an integrin can aid in finding more bioactive peptide ligands that mimic, and potentially replace, these ECM proteins. A key advantage to a library-based approach to searching for biomimetic ECM peptides is that it allows for sampling a larger peptide space without prior knowledge of the signals required for cell growth and maintenance. In this study, we have shown that the utilizing biomimetic ligand selection is a very powerful tool for identifying promising candidate peptides that can be used as biomaterials for the growth of a desired cell type, including stem cells, indicating that these peptides may be integrin agonists. These two general

methods we have described have been successful at finding many peptides that could potentially replace Matrigel, and thus aid in creating a defined culture system for hESCs and other cells that rely on ECM proteins during culture.

5.4 Methods

5.4.1 Cell Culture

HSF-6 human embryonic stem cells (University of California, San Francisco) were grown in X-Vivo 10 Media (Lonza) supplemented with 80 ng/mL FGF-2 (Peprotech) and 0.5 ng/mL TGF-β1 (R&D Systems) on hESC-qualified Matrigel (BD Biosciences), which was diluted and coated onto plates as per the manufacturer's instructions. This system has previously been found to support extended hES cell self-renewal.[6, 49]

5.4.2 Bacterial Peptide Display Libraries and Untargeted Bacterial Peptide Display Selections

The bacterial display libraries were generated in MC1061 *E. coli* with a pBAD33 plasmid containing alajGFP and the CPX membrane protein with random peptides of the forms X_{15} (15mer) and $X_2CX_7CX_2$ (7C) at the N-terminus.[43] CPX is a variant of the bacterial surface protein OmpX, which was previously circularly permutated to locate the N- and C-terminus on the extracellular side[44], and alajGFP is a bright fluorescent protein engineered for high expression in *E. coli*.[41] Both genes were expressed under the control of an arabinose-inducible promoter. Three libraries were used: one containing only 15mer clones (15), one containing only 7C clones (7C), and a third with initially equal parts of 15mer and 7C clones (combined).

The selections were performed in four rounds based on the method of Dane *et al*.[43] In each round, a frozen stock of the library was grown overnight in LB supplemented with 34 μg/mL chloramphenicol (Sigma) and 0.2% D-glucose (Sigma). The library was then subcultured 1:50 with LB and 34 μg/mL chloramphenicol. After two hours, it was induced at 30°C with 0.02% L-arabinose (Sigma) to initiate expression of alaj GFP[41] and CPX. Stromal or differentiated cells were first removed by incubating cells with 2 mM Na_2EDTA (Fisher) in phosphate-buffered saline (PBS) for 2-5 minutes at 37ºC followed by several washes with PBS. hESCs from the remaining colonies were separated into single cells with a longer incubation (at least 15 minutes) in 2 mM Na_2EDTA (Fisher) in PBS at 37ºC. The resulting single cells were then co-incubated with the library bacteria in a bacterial shaker at 37ºC for 1 hour in X-Vivo medium.

For the first round, 100-fold more bacteria than hESCs were used, and 50-fold more bacteria were used for the later rounds. Washing steps were then performed by centrifuging the samples at 3500 rpm for 4 min for the first round and 1600 rpm for 30 s for the subsequent rounds. The resulting pellet was then grown overnight in LB supplemented with 34 μg/mL chloramphenicol and 0.2% D-glucose. For the third through fifth round selections, FACS was performed on the samples after the washing. Clonal and library analysis were performed with flow cytometry after panning of the clonal or library populations with hESCs. All libraries were analyzed by growing up 10^8 clones of each library.

5.4.3 Quantification of α_6 and β_1 Integrin Expression

After being dissociated into single cells, hESCs were incubated with the antibody for the α_6 or β_1 integrin subunits (Millipore) for 1 hour. Cells were washed in 2% FBS in PBS twice and then incubated with Anti-Rat IgG Alexa 488 or Anti-Mouse IgG Alexa 488 secondary antibody

(Invitrogen) for 1 hour. Cells were then washed twice in 2% FBS in PBS and then resuspended in 2% FBS in PBS for analysis in a flow cytometer.

5.4.4 Targeted Bacterial Peptide Display Selections

For the targeted selections, single hESCs were incubated with 10 μg/mL of each blocking antibody for the α_6 and β_1 integrin subunits (Millipore)[29] in the second, third, and fourth rounds, but only with the α_6 integrin antibody for the 5th round. After incubation with the antibodies for 1 hour at 37ºC, the cells were incubated with the induced bacterial libraries at 37ºC with no shaking. The supernatant containing bacteria that did not bind to the integrin-blocked cells was then removed and incubated with hESCs not blocked with the integrin antibodies, to positively select for bacteria that bind these integrins. The samples were incubated at 37ºC for 1 hour in a bacterial shaker, and washing and sorting were conducted as for the untargeted selections.

5.4.5 Synthetic Peptide Adsorption to Surfaces and hESC Culture

Peptides for all subsequent studies were purchased from American Peptide Company, Inc. Linear peptides had an additional cysteine residue on the N-terminus to allow for attachment on IPN surfaces. Cyclic peptides from the 7C library were ordered as CX_7G, where X_7 are the residues in the middle of the cysteine-cysteine loop from the sequenced library clones; these peptides were cyclized through an amide linkage between the N- and C-termini of the peptides. bsp-RGD(15), a peptide previously studied with hESCs, has the sequence CGGNGEPRGDTYRAY.[26, 50]

Peptides were dissolved at 200 μM in synthesis grade water, these solutions were pipetted onto the plates, and the peptide was allowed to adsorb for 3 hours at room temperature in a sterile biohazard hood. Plates were washed twice with PBS before adding cells. hESCs on Matrigel plates were first incubated with 200 U/mL Collagenase IV (Invitrogen) for 5 minutes at 37ºC and then washed with PBS to remove stromal or differentiated cells from around the hESC colonies. The remaining colonies were then scraped off the plates and added at 100,000 cells/cm^2 in X-Vivo 10 supplemented with 80 ng/mL FGF-2 and 0.5 ng/mL TGF-β1. Cells were incubated at 37ºC for 5 days with media replacement every day except the day after plating. After 5 days, all media were removed, and plates were frozen at -80ºC for Cyquant (Invitrogen) cell counting, as per the manufacturer's instructions. For attachment studies, hESCs were incubated with 2 mM Na_2EDTA in PBS for at least 15 minutes after stromal cell removal. For antibody-blocked attachment, single hESCs were then incubated with 10 μg/mL of each blocking antibody for the α_6 and β_1 integrins at 37ºC for 1 hour before plating. Single hESCs were then added to peptide-adsorbed plates and incubated at 37ºC for 2 hours. After washing the surface once, plates were then assayed with Cyquant to count cells.

5.4.6 Immunocytochemistry for Oct4 and SSEA-4

On the fifth day after seeding on control or peptide-adsorbed surfaces, immunocytochemistry was performed on the samples for the POU family transcription factor Oct4 and for the cell surface marker Stage-specific Embryonic Antigen 4 (SSEA-4), both of which are highly specific and necessary markers for undifferentiated hES cells. Cells were fixed in 4% paraformaldehyde and then permeabilized with 0.1% Triton X-100. The cells were incubated with 0.5% SDS, blocked with 2% BSA, then with the Rabbit Oct4 antibody (Abcam) or SSEA-4 antibody (Millipore) overnight, and finally with anti-Rabbit Alexa 546 or anti-Mouse Alexa 488 secondary antibodies (Invitrogen). DAPI (4',6-diamidino-2-phenylindole)

(Invitrogen) was added to the cells, and images were captured on a Nikon Eclipse TE2000-E microscope.

5.4.7 Quantification of Oct4 and SSEA-4 Expression with Flow Cytometry

Oct4 and SSEA-4 Expression were quantified with flow cytometry (Cytomics FC 500, Beckmann Coulter). Prior to flow cytometry, cells were seeded and grown on peptide-adsorbed or control surfaces for 5 days, and cultures were incubated with 2 mM EDTA in PBS for 10-15 minutes for dissociation into single cells. Cells were then fixed in 2% formaldehyde in PBS for 20 minutes and washed with 2% Fetal Bovine Serum (FBS) (Invitrogen) in PBS twice. Cells were next permeabilized with 1 mg/mL Saponin (Fluka) in 10% Bovine Serum Albumin dissolved in PBS (SPB) for 15 minutes, then incubated with Oct4 Antibody (Abcam) for 1 hour. Cells were washed once with SPB then incubated with anti-Rabbit Alexa 488 antibody (Invitrogen) for 1 hour. Cells were then washed with SPB and then resuspended in 2% Fetal Bovine Serum in PBS for analysis in a flow cytometer.

The hES cells to be examined for SSEA-4 were incubated with SSEA-4 Antibody (Millipore) for 1 hour, immediately after the EDTA cell dissociation described above. Cells were then washed in 2% FBS in PBS twice and then incubated with Anti-Mouse Alexa 488 (Invitrogen) for 1 hour. Finally, cells were washed twice in 2% FBS in PBS and then resuspended in 2% FBS in PBS for analysis in a flow cytometer.

5.4.8 Statistics

All statistical analysis was carried out using ANOVA with a Tukey HSD post-hoc test. The results were considered significant at $p < 0.05$.

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Appendix A:
Relevant Protocols for Synthesizing and Evaluating Biomaterials for the Culture of hESCs and NSCs

A.1 Culturing hESCs on Matrigel

Modified from Geron Corp.[1]

Materials

X-Vivo 10 Medium, Lonza (catalog no. 04-743Q)
Human Basic Fibroblast Growth Factor (hbFGF or hFGF-2), Peprotech (catalog no. 100-18B)
Transforming Growth Factor Beta 1 (TGF-β1), R&D Systems (catalog no. 240-B)
Collagenase IV, Invitrogen (catalog no. 17104-019)
Knockout DMEM, Invitrogen (catalog no. 10829-018)
hESC-qualified Matrigel, BD Biosciences (catalog no. 354277)
Phosphate-Buffered Saline (PBS), Invitrogen (catalog no. 10010)
Human Serum Albumin (HSA), Sigma-Aldrich (catalog no. A1653)
InSolution Y-27632 (Rock Inhibitor), Calbiochem (EMD), (catalog no. 688001-500UG)

Matrigel Aliquoting Instructions

1) Thaw 5 mL undiluted bottle sent from the manufacturer at 4ºC overnight to completely thaw all of the Matrigel. Chill 1000 µL pipet tips at 4ºC overnight as well to keep Matrigel as cool as possible while aliquoting. (Alternatively: chill 1000 µL at -20ºC for at least 5 minutes before aliquoting Matrigel).

2) Prepare 1.5 mL centrifuge tubes in a tray for the Matrigel aliquots. Make sure you have more than enough tubes because the volume of Matrigel may be slightly greater than 5 mL.

3) Aliquot Matrigel into 1.5 mL centrifuge tubes changing tips for at least every 3 aliquots to keep Matrigel as cool as possible. Use the pre-chilled tips and aliquot Matrigel at the volume indicated in the product sheet (sent with Matrigel) for that particular lot. Try to work quickly since Matrigel can gel irreversibly above 4ºC.

4) Place Matrigel aliquots in -80ºC freezer until needed for making plates. Aliquots are good for up to 6 months at this temperature.

Making Matrigel Plates

1) Thaw an aliquot of Matrigel from -80ºC at 4ºC overnight to completely thaw the Matrigel.

2) Place 25 mL **cold** Knockout DMEM in a 50 mL conical tube.

3) Take a small portion of the Knockout DMEM aliquoting in Step 2, approximately equal in volume to the Matrigel aliquot, and add to the Matrigel aliquot tube. Pipet this liquid a few times to quickly dissolve the Matrigel. Add all of this liquid back into the 50 mL conical tube. Once the Matrigel aliquot is dissolved in the 25 mL Knockout DMEM, the Matrigel will no longer be able to make a 3D gel.

4) Mix the Matrigel and Knockout DMEM a few times creating as few bubbles as possible.

5) Add the Matrigel mixture to tissue-culture plates, approximately 8 mL for 10 cm dishes and 1 to 1.5 mL for a well of a 6-well plate. For other size plates, add enough Matrigel to cover the surface or scale with plate surface area according to the amounts given above.

6) Label all plates with your initials and date. Parafilm the edge of the plates to seal them, and store plates at 4ºC for up to 2 weeks (14 days). For best results, use Matrigel plates that have been incubated at 4ºC for at least one day, though cells stick attach relatively well to freshly made plates.

7) Before adding cells to plate, incubate Matrigel plate at room temperature for at least 20 minutes to allow Matrigel proteins to fully adsorb on the surface.

Making Collagenase IV Solution (200 U/mL)

1) Weigh out enough powdered collagenase IV to make 40 mL at a concentration of 200 U/mL.

2) Bring tube into sterile TC hood. Add 40 mL Knockout DMEM. Mix slightly either by shaking the tube or with a pipet.

3) Sterile filter collagenase IV solution in a sterile 50 mL tube with a syringe and .22 μm syringe filter.

4) Store collagenase IV solution at 4ºC for approximately a month, or at -20ºC for longer term storage. Frozen collagenase IV solution can be thawed at 4ºC overnight.

Making hbFGF Solution (100 ng/μL)

1) Remove frozen tube of hbFGF from -80ºC and thaw slightly at room temperature.

2) In a sterile TC hood, add enough plain X-Vivo 10 medium to make hbFGF at a concentration of 100 ng/μL. For example, a 100 μg bottle of hbFGF requires 1 mL X-Vivo 10. DO NOT STERILE FILTER. Since proteins stick to the filter membranes, you will lose a lot of your growth factor.

3) Aliquot the growth factor solution into appropriate size aliquots (~300 μL is recommended). Aliquots should be frozen at -20ºC until they are needed. If you are actively growing cells, an aliquot of hbFGF can be stored at 4ºC.

Making TGF-β1 Solution (1 ng/μL)

1) Remove frozen tube of TGF-β1 from -20ºC and thaw slightly at room temperature.

2) In a sterile hood, make up a solution for dissolving the TGF-β1 containing 4 mM HCl, 1 mg/mL HSA in plain X-Vivo 10 media in a conical tube. For 2 μg TGF-β1, use 1.33 μL of a

sterile 6 M HCl solution and 8 µL of a sterile 25% (by weight) HSA solution in 1.99 mL plain X-Vivo 10 media.

3) Mix this solution well. Add a small amount of this solution to the TGF-β1 in its bottle, and add of this back to the conical tube.

4) Aliquot the growth factor solution into appropriate size aliquots (~200 µL is recommended). Aliquots should be frozen at -20ºC until they are needed; aliquots are stable at -20ºC for about 3 months. If you are actively growing cells, an aliquot of TGF-β1 can be stored at 4ºC for about one month.

Making X-Vivo Media w/ 80 ng/mL hbFGF and 0.5 ng/mL TGF-β1

1) Aliquot 40 mL plain X-Vivo 10 media into a 50 mL conical tube.

2) Add 20 µL TGF-β1 and 32 µL hbFGF.

3) Mix aliquot a few times with a pipet. Aliquot can be stored at 4ºC for about a week.

Alternatively, you can add the appropriate amount of growth factors to a full or partial bottle of X-Vivo 10 if you are growing a lot of cells. For 500 mL X-Vivo 10, you will need to add 400 µL FGF-2 and 250 µL TGF-β1.

Passing Cells

1) Make fresh Matrigel plates or remove pre-made Matrigel plates from 4ºC refrigerator and incubate at room temperature at least 20 minutes to allow the Matrigel proteins to fully adsorb on the plate surface.

2) Aspirate medium off of cells completely.

3) Add 2-3 mL collagenase IV solution to a 10 cm plate. Incubate plate at 37ºC for about 2-5 minutes. (Note: cells do not need to be rinsed with PBS since there is no serum or serum-like component in the media). The goal of this step is to loosen the differentiated or stromal cells, but not the colonies.

4) Aspirate the collagenase IV solution. Rinse the plate by adding PBS dropwise all over the plate. Aspirate the PBS. If you are doing an experiment, this rinsing step can be done twice or three times to remove even more stromal cells. (Note: If your colonies appear to be coming off of the plate do not aspirate the PBS. Instead, scrape the cells into the PBS with a cell scraper, collect in a conical tube, and pellet the cells at 1000 rpm for 3-5 min in a table top centrifuge. Then resuspend the cells in X-Vivo 10 with factors. These steps will remove the collagenase, but prevent you from losing your hESCs.)

5) Add about 6 mL X-Vivo 10 media with factors to the plate.

6) Scrape the cells off the plate with a cell scraper. Make sure to keep the scraper at an angle to prevent contaminating your cells. It is also better to scrap the half of the plate away from you, turn the plate 180º, and then scrap the second half of the plate. You may use the same scraper for multiple plates if the plates have the same cells and if you haven't contaminated the scraper.

7) Remove the cells from the plate with a pipet and place the cells in a conical tube. Do not pipet over the plate; you want to break up the cells as little as possible.

8) Rinse the plate by tilting the plate and pipetting a few mL of X-Vivo 10 media with factors down the plate. Remove this liquid from the plate and add it to the conical tube. You can repeat this step if there are still a decent amount of cells on the plate.

9) Break up the colonies by pipetting the cell solution 4-5 times relatively vigorously with a 10 mL pipet.

10) Aspirate the Matrigel solution from the tissue culture plates. Do not aspirate the plate dry; you should see a pink film on the bottom of the plate from the Matrigel.

11) Add broken up hESCs to the plate. You should use about 1:3 to 1:8 of the cells from a confluent 10 cm to a new 10 cm, for example. (I usually prefer using 1:6). The total volume of media on a 10 cm should be around 10 mL (4 mL for a well of a 6-well); this higher volume of media helps to reduce differentiation by keeping the concentration of self-renewal factors high and diluting out differentiation factors given out by the cells on the plate. In addition, you should see small clumps of cells; if not, you broke up the cells too much.

12) Incubate the cells at 37ºC and 5% CO_2 to grow the cells.

13) Change the media on the cells every day except the day after passing. When changing the media, remove all of the media and replace with fresh X-Vivo 10 media with factors. I usually do not warm up media since water baths can contribute to contamination.

14) You should have to pass the cells every 5-7 days if you pass them at a ratio of 1:6. Cells are ready to passage when the plate is full of cells (both colonies and stromal cells), colonies are larger, and colonies are beginning to have layers (the colonies will look slightly "yellow" under the phase microscope because of the layers).

Freezing Cells

1) Follow the protocol to pass hESCs up through Step 8.

2) Add plain X-Vivo 10 media to almost fill up the 50 mL conical tubes.

3) Centrifuge the cells at 5 min at 1000 rpm.

4) Mix up the following cell freezing solutions (all amounts are per vial to freeze): X-Vivo Freezing Solution (1.3 mL plain X-Vivo 10 media + 48 μL sterile 25% HSA (by weight) + 12 μL

sterile 1 M HEPES), 1.5 M DMSO Freezing Solution (.27 mL X-Vivo Freezing Solution + 31 µL DMSO), and 2.0 M DMSO Freezing Solution (.52 mL X-Vivo Freezing Solution + 83 µL DMSO).

5) Aspirate the supernatant from the cells. Resuspend the cells in .3 mL X-Vivo Freezing Solution for each vial. Add 150 µL of 1.5 M DMSO Freezing Solution for each vial and incubate the cells for 5 minutes at room temperature.

6) Add an additional 150 µL of 1.5 M DMSO Freezing Solution for each vial and incubate the cells for 25 minutes at room temperature.

7) Label cryovials and pre-chill at -20ºC.

8) Add 600 µL of 2.0 M DMSO Freezing Solution for each vial and incubate the cells for 15 minutes at room temperature.

9) Pre-chill the cell solution in a slushy ice bath for 10 minutes.

10) Transfer 1.2 mL cell solution to each pre-chilled cryovial and freeze at -80ºC freezer (preferably in a alcohol-freezing container or in a foam container).

11) Transfer the cryovials to a box in a liquid nitrogen dewar for long term storage 24 hours after placing in the -80ºC freezer.

Note: For best results, have a lot of cells in each vial since a lot of cells die during the thawing process. I usually recommend making 2-3 vials per confluent 10 cm dish.

Thawing Cells

Note: The best viability is seen when cells were frozen at higher density and thawed less than a year later. If there aren't a lot of cells or the vial was frozen more than a year ago, the cells can still be thawed but may take up to a month to start growing at a normal rate.

1) Place Matrigel plates for cells at room temperature to allow the Matrigel proteins to adsorb to the plates.

2) For each vial you are thawing, prepare X-Vivo Thawing Media (14.25 mL plain X-Vivo 10 media + 0.6 mL sterile 25% (by weight) HSA + 0.15 mL sterile 1 M HEPES).

3) Remove the vial from liquid nitrogen and thaw quickly in a 37ºC waterbath until only a little bit of ice remains.

4) Carefully wipe off all of the water from the bath, spray with 70% ethanol.

5) Pipet the contents of the cryovial and add to a 50 mL conical tube. Add 1 mL X-Vivo Thawing Media. Incubate 5 minutes at room temperature.

6) Add 1 mL X-Vivo Thawing Media, and incubate 5 minutes at room temperature.

7) Add 2 mL X-Vivo Thawing Media, and incubate 5 minutes at room temperature.

8) Add 4 mL X-Vivo Thawing Media, and incubate 5 minutes at room temperature.

9) Add 6 mL X-Vivo Thawing Media, and incubate 5 minutes at room temperature.

10) Centrifuge cells at 1000 rpm for 5 minutes to pellet cells.

11) Aspirate off supernatant, and resuspend cells with X-Vivo 10 media with factors. For better results, add 10 μM Y-27632 (Rock Inhibitor) to aid in cell viability over the thawing process. (This can be added for the first passage or until the cells look "good.")

12) Remove the Matrigel from the plates and add thawed cells to the plate.

13) Incubate cells at 37ºC and 5% CO_2 to grow the cells.

14) Change the media every day except the day after thawing. You may need to pass the cells sooner than 5-7 days depending on how many cells survive the thaw. During the first passage, you'll probably want to pass the cells 1:1 to 1:3.

A.2 Transferring hESCs from Feeders to Matrigel

Note: Conditioned media can be used right away or frozen up to 1 month at -20ºC. For details on the media used with hESCs on feeders, please see the "Human Embryonic Stem Cell Culture Protocol Handbook."

Making Conditioned Media on Feeders[2]

1) Thaw mouse embryonic fibroblasts that have been irradiated or treated with mitomycin C onto gelatin coated plates in MEF media.

2) The next day, change the media to KSR media with FGF-2.

3) For the next seven days, collect the media on the plate in a tube. Add fresh KSR media with FGF-2 to the plate.

Transferring hESCs from Feeders to Matrigel

Note: Only start this protocol once the cells on feeders are growing well. If you are thawing cells, grow them for at least a couple passages. It is also recommended to karyotype cells before and after doing this procedure to ensure a normal karyotype of the resulting cell line.

1) Passage hESCs onto a Matrigel plate, aspirating the Matrigel before adding the cells. Plate the cells in a total of 10 mL feeder-conditioned media containing an additional 4 ng/mL FGF-2. Make sure to not break colonies up too much during the passage since Matrigel plates can support larger colonies than feeders. MEFs will gradually die out over the course of the next few passages since they can't divide.

2) Culture the cells, changing the media every day (including the day after passing).

3) When cells have filled the plate, passage the cells onto a new plate. Pass cells at a dense ratio (1:1 to 1:3). Cells will have a different morphology on Matrigel than on feeders with some cell lines not exhibiting borders (such as HSF-6 cells). In general, the colony of cells will be very dense where you can't tell each cell apart and stromal (or differentiated) cells will be elsewhere.

4) Grow cells on Matrigel in feeder-conditioned media for at least 6 passages so cells can adjust to growing on the new substrate.

5) Pass cells to a Matrigel plate in 90% feeder-conditioned media and 10% X-Vivo 10 media plus factors. Make sure to add the appropriate amount of growth factors for each type of media. Grow the cells for 1 or 2 passages in this media condition.

6) Pass cells to a Matrigel plate in 75% feeder-conditioned media and 25% X-Vivo 10 media plus factors. Grow the cells for 1 or 2 passages in this media condition.

7) Pass cells to a Matrigel plate in 50% feeder-conditioned media and 50% X-Vivo 10 media plus factors. Grow the cells for 1 or 2 passages in this media condition.

8) Pass cells to a Matrigel plate in 25% feeder-conditioned media and 75% X-Vivo 10 media plus factors. Grow the cells for 1 or 2 passages in this media condition.

9) Pass cells to a Matrigel plate in 10% feeder-conditioned media and 90% X-Vivo 10 media plus factors. Grow the cells for 1 or 2 passages in this media condition.

10) Pass cells to a Matrigel plate in 100% X-Vivo 10 media plus factors. Grow the cells for at least 3 passages in this media condition.

11) Get cells karyotyped to make sure the transformation process hasn't affected the karyotype. Freeze down stocks of cells for future use.

A.3 Bacterial Peptide Display Selections

Modified from Dane, et al.[3]

Note: "Desired selection cells" are the target cells for the library selections or the cells you want to find binding peptides for. "Negative selection cells" are the cells you are don't want to find binding peptides for. After all rounds of bacterial selection, you will have a library of bacteria expressing peptides that mostly bind to the desired selection cells, but not to the negative selection cells. Since Dane, et al. focused on finding peptides that bound to breast cancer cells, but not to regular breast cells, breast cancer cells were used as the desired selection cells and regular breast cells were used as the negative selection cells.

For the integrin targeting project, the negative selection cells were the desired selection cells incubated for 1 hour pre-selection with a blocking antibody for the desired integrin subunits. The purpose of this experiment was to find peptides that bound to that specific integrin.

Negative selection cells do not need to be employed in the selections if you are just looking for targets for a cell line or there is no obvious choice for the negative selection cells.

Materials

Luria Broth (LB) Media
Glucose, Sigma (catalog no. G7520), 20% in water
Chloramphenicol (CM), Sigma (catalog no. C-0378), 34 mg/mL in ethanol
Arabinose, Sigma (catalog no. A3256), 2% in water'
Ethylenediaminetetraacetic acid (EDTA), Fisher (catalog no. S312-500), 2 mM in PBS
Phosphate-Buffered Saline (PBS), Invitrogen (catalog no. 10010)
SOC Bacterial Media

Making Calibration Curve for a UV/Vis Machine

1) Grow up a bacterial culture in LB with the appropriate antibiotic to an optical density (OD) at 590 nm close to 1.

2) Make dilutions of the culture to check a range of OD values ranging from 0.1 to 1.0. The dilutions can be estimated using the equation $(OD_{desired}/OD_{total})$ * 200 μL = amount of culture to use in each 200 μL dilution. The remainder of each dilution should be plain LB.

3) Measure the OD of each dilution using 200 μL in a 96-well plate.

4) Make serial dilutions of the total culture, diluting the culture 1:10 in each dilution.

5) Plate 100 μL of the fourth (10^4) through the tenth (10^{10}) dilutions on separate LB-agar plates with the appropriate antibiotic.

6) Incubate the LB plates overnight at 37ºC.

7) Count the number of colonies on each plate. Some of the lower dilutions will have too many colonies to count.

8) From the amount of colonies on the counted plates, calculate the concentration of bacteria from the total culture yesterday. Then calculate the concentration of bacteria in each dilution used for an OD reading.

9) Plot the OD readings versus bacterial concentration. Find the equation for a trend line for the data. This equation is your calibration curve.

Round 1 Selections

1) Thaw one whole vial (containing $2 * 10^{10}$ cells) of the unselected library in 250 mL LB containing 2.5 mL Glucose, and 250 µL CM.

2) Grow the library at 37ºC in a bacterial shaker until the optical density (OD) at 590 nm is ~1.5. This should take 2.5 to 4 hours. Since OD values are most accurate between 0.1 and 1, dilute the culture 1:2 before measuring on UV/Vis spectrometer.

3) Using the calibration curve, calculate the volume of culture that contains 10^{10} cells. An example calibration curve is $(11.698*(OD_{590})-.1908)*10^8$ = bacterial cells/mL. This calibration curve was determined from the UV/Vis plate reader using 200 µL culture in a 96-well plate.

4) Spin down 10^{10} cells of the library in a centrifuge at 2500g for 10 minutes at 4ºC to remove the glucose, which inhibits the expression of the peptide display protein and green fluorescent protein (GFP).

5) Remove the supernatant and resuspend the library in 1 mL LB. Add the library to 100 mL LB containing 100 µL CM.

6) Grow the library in a bacterial shaker at 37ºC for 2 hours to subculture the library.

7) Induce the library for 2 hours shaking at room temperature or 30ºC by adding 1 mL 2% Arabinose.

8) During bacterial culture induction, detach desired selection cells with EDTA solution. Remove cells from plates in normal cell media, and break up cells into single cells.

9) Count selection cells and spin down $5*10^7 – 10^8$ cells per library at 1000 rpm for 3-5 minutes. Resuspend the cell pellet in 20 mL cell media per library. Place selection cells at 37ºC until library bacteria are added to them.

10) Measure the OD of the bacteria. Using the calibration curve, determine the volume containing 100x more bacteria than selection cells.

11) Spin down the volume of bacteria to add at 2500 g for 10 min at 4ºC.

12) Resuspend the bacterial pellet with media from the selection cells, and incubate the bacteria and selection cells together for 1 hour at 37ºC in a bacterial shaker.

13) After co-incubation, add PBS to make the total volume 50 mL. Spin down the cells at speed 3.5 for 4 minutes. Remove supernatant and place in a 50 mL tube for dilutions.

14) Do four more washes with 50 mL PBS each.

15) Resuspend the final pellet in 1 mL LB.

16) Make the dilutions for each wash and of the pellet, plating 100 µL of the following dilutions on LB-agar plates with CM:

Pellet (use 10 µL from 1 mL for 1:100): 10^3, 10^4, 10^5
Wash 1: 10^4, 10^5, 10^6, 10^7
Wash 2: 10^4, 10^5, 10^6
Wash 3: 10^3, 10^4, 10^5
Wash 4: 10^2, 10^3, 10^4
Wash 5: 10, 10^2, 10^3

Incubate plates at 37ºC overnight.

17) Add remaining resuspended pellet to 25 mL LB containing 250 µL glucose and 25 µL CM. Grow overnight.

18) Count bacterial plates to determine amount of bacteria in pellet and in each wash.

19) Freeze library with 10% glycerol at -80ºC.

Round 2

Note: If negative selection cells are not used, skip steps 7-10. Follow step 9 instead of steps 13 and 14.

1) Thaw a frozen aliquot of a Round 1 selected library in a heat block heated to 37ºC or at room temperature.

2) Make overnight culture with 25 mL LB, 25 µL CM, 250 µL glucose, and 500 µL Round 1 selected library stock. If the library was frozen down with dimethyl sulfoxide (DMSO) instead of glycerol, pellet the bacterial cells by spinning the tube in a microcentrifuge at 5000 rpm for 2 minutes. This pellet can then be resuspended in the overnight culture broth. Cultures frozen with glycerol do not need this additional step as the bacteria can metabolize the glycerol.

3) Grow culture overnight in a bacterial shaker at 37ºC.

4) Make subculture for library with 20 mL LB, 20 µL CM, and 400 µL overnight culture.

5) Grow the library in a bacterial shaker at 37ºC for 2 hours to subculture the library.

6) Induce the library for 2 hours shaking at room temperature or 30ºC by adding 200 µL 20% Arabinose.

7) During bacterial culture induction, detach negative selection cells with EDTA solution. Remove cells from plates in normal cell media, and break up cells into single cells.

8) Count negative selection cells and spin down $1*10^7 – 5*10^7$ cells per library at 1000 rpm for 3-5 minutes. Resuspend the cell pellet in 4 mL cell media per library. Place negative selection cells at 37ºC until library bacteria are added to them.

9) Measure the OD of the bacteria. Using the calibration curve, determine the volume containing 5x more bacteria than selection cells. Spin down the volume of bacteria to add at 5000 rpm for 2 min at if the volume of bacteria needed is greater than 20% of the desired selection cell media volume.

10) Incubate the bacteria and negative selection cells together for 1 hour at 37ºC with no shaking.

11) During negative selection cell co-incubation, detach desired selection cells with EDTA solution. Remove cells from plates in normal cell media, and break up cells into single cells.

12) Count desired selection cells and spin down $1*10^7 – 5*10^7$ cells per library at 1000 rpm for 3-5 minutes. Resuspend the cell pellet in 1 mL cell media per library. Place desired selection cells at 37ºC until library bacteria are added to them.

13) Carefully, remove almost all (~80-90%) of the supernatant from the negative selection tube. Do not remove all the liquid or disturb the cells at the bottom of the tube.

14) Centrifuge the supernatant collected at 4000 rpm for 5 minutes. Discard the supernatant and resuspend the pellet with the desired selection cells in their media.

15) Incubate the bacteria and desired selection cells together for 1 hour at 37ºC in a bacterial shaker.

16) After co-incubation, pellet the cells at 1600 rpm for 30 seconds. Remove supernatant and place in microcentrifuge tube for dilutions.

13) Do four more washes with 1 mL PBS each.

14) Resuspend the final pellet in 1 mL LB.

15) Make the dilutions for each wash and of the pellet, plating 100 µL of the following dilutions on LB-agar plates with CM:

Pellet (use 10 µL from 1 mL for 1:100): 10^3, 10^4, 10^5
Wash 1: 10^4, 10^5, 10^6
Wash 2: 10^3, 10^4, 10^5
Wash 3: 10^3, 10^4, 10^5
Wash 4: 10^2, 10^3, 10^4
Wash 5: 10, 10^2, 10^3

Incubate plates at 37ºC overnight.

16) Add remaining resuspended pellet to 5 mL LB containing 50 µL glucose and 5 µL CM. Grow overnight.

17) Count bacterial plates to determine amount of bacteria in pellet and in each wash.

18) Freeze library with 10% glycerol at -80ºC.

Round 3

Note: If negative selection cells are not used, skip steps 7-10. Follow step 9 instead of steps 13 and 14. Additional rounds of selection can be performed using the protocol for this round. For analysis of library or clonal populations, follow this protocol without negative selection cells through step 15, but analyze samples on a flow cytometer analyzer.

1) Thaw a frozen aliquot of a Round 2 selected library in a heat block heated to 37ºC or at room temperature.

2) Make overnight culture with 5 mL LB, 5 µL CM, 50 µL glucose, and 100 µL Round 2 selected library stock. If the library was frozen down with dimethyl sulfoxide (DMSO) instead of glycerol, pellet the bacterial cells by spinning the tube in a microcentrifuge at 5000 rpm for 2 minutes. This pellet can then be resuspended in the overnight culture broth. Cultures frozen with glycerol do not need this additional step as the bacteria can metabolize the glycerol.

3) Grow culture overnight in a bacterial shaker at 37ºC.

4) Make subculture for library with 5 mL LB, 5 µL CM, and 100 µL overnight culture.

5) Grow the library in a bacterial shaker at 37ºC for 2 hours to subculture the library.

6) Induce the library for 2 hours shaking at room temperature or 30ºC by adding 20 µL 20% Arabinose.

7) During bacterial culture induction, detach negative selection cells with EDTA solution. Remove cells from plates in normal cell media, and break up cells into single cells.

8) Count negative selection cells and spin down $2*10^6 – 1*10^7$ cells per library at 1000 rpm for 3-5 minutes. Resuspend the cell pellet in 2 mL cell media per library. Place negative selection cells at 37ºC until library bacteria are added to them.

9) Measure the OD of the bacteria. Using the calibration curve, determine the volume containing 5x more bacteria than selection cells. Spin down the volume of bacteria to add at 5000 rpm for 2 min at if the volume of bacteria needed is greater than 20% of the desired selection cell media volume.

10) Incubate the bacteria and negative selection cells together for 1 hour at 37ºC with no shaking.

11) During negative selection cell co-incubation, detach desired selection cells with EDTA solution. Remove cells from plates in normal cell media, and break up cells into single cells.

12) Count desired selection cells and spin down $2*10^6 – 1*10^7$ cells per library at 1000 rpm for 3-5 minutes. Resuspend the cell pellet in 200 µL cell media per library. Place desired selection cells at 37ºC until library bacteria are added to them.

13) Carefully, remove almost all (~80-90%) of the supernatant from the negative selection tube. Do not remove all the liquid or disturb the cells at the bottom of the tube.

14) Centrifuge the supernatant collected at 4000 rpm for 5 minutes. Discard the supernatant and resuspend the pellet with the desired selection cells in their media.

15) Incubate the bacteria and desired selection cells together for 1 hour at 37ºC in a bacterial shaker.

16) After co-incubation, add PBS to 1 mL then pellet the cells at 1600 rpm for 30 seconds.

13) Remove supernatant and do one more washes with 1 mL PBS.

14) Resuspend the final pellet in 0.4 mL LB.

15) Sort desired selection cells with bacteria bound (GFP+) on a flow cytometer cell sorter into 1 mL SOC media.

15) Plate 50 µL of SOC media after sorting on a LB/CM plate to determine bacterial recovery. Incubate plate at 37ºC overnight.

16) Add remaining SOC media with sorted cells to 4 mL LB containing 50 µL glucose and 5 µL CM. Grow overnight.

17) Count bacterial plate to determine yield of bacteria from sorting. Freeze library with 10% glycerol at -80ºC.

A.4 Predicting and Testing Peptide Solubility

Predicting Peptide Solubility in Water

1) Determine the percentage of nonpolar and charged amino acids in the peptide sequence. See below for a list of nonpolar and charged amino acids.

Nonpolar Amino Acids:	Alanine (A)
	Phenylalanine (F)
	Isoleucine (I)
	Leucine (L)
	Methionine (M)
	Proline (P)
	Valine (V)
	Tryptophan (W)
Charged Amino Acids:	Aspartic Acid (D)
	Glutamic Acid (E)
	Histidine (H)
	Lysine (K)
	Arginine (R)

2) Use the following sets of solubility rules to determine if the peptide is likely to be soluble in water. If the peptide is predicted to be soluble and soluble/might be soluble from the 1st and 2nd set of rules, then the peptide should be soluble in water.

Rule 1 (from Invitrogen): If the peptide contains $\leq$ 50% nonpolar amino acids and $\geq$ 20% charged amino acids, then the peptide is soluble in water.

Rule 2 (from Sigma-Aldrich): If the peptide contains $\leq$ 25% nonpolar amino acids and $\geq$ 25% charged amino acids, then the peptide is soluble in water. If the peptide contains 25-50% nonpolar amino acids and $\geq$ 25% charged amino acids, then the peptide might be soluble in water.

Testing Peptide Solubility

Note: Solvents to test peptide solubility include sodium phosphate buffer (see "Conjugating Peptides through a Carboxylic Acid" protocol), synthesis grade water, acetonitrile, and dimethyl sulfoxide (DMSO). Since IPN conjugation can be done with 10-20% acetonitrile or DMSO, test to see if the peptide will stay dissolved when the dissolved peptide in acetonitrile or DMSO is diluted with sodium phosphate buffer. For adsorbed peptide surfaces, there is no need to test dissolving in sodium phosphate buffer.

1) Weigh out ~1 mg peptide without wearing gloves to limit the amount of static created.

2) Place peptide in a 50 mL conical tube and dissolve with the appropriate amount of solvent. Usually peptide solubility should be tested between 100 μM and 0.01 μM, the concentrations of peptide used for conjugation. Sonication or vortexing may be necessary to dissolve some peptides.

3) Do serial dilutions of the peptide with the solvent to make solutions ranging from 100 μM to 0.01 μM.

4) Measure the absorbance at 280 nm on a UV/Vis spectrometer.

5) Plot the absorbance values versus the concentration. If the peptide is fully dissolved, the graph will be linear with a positive slope.

A.5 Peptide Surface Concentration Assay

Modified from Barber, et al.[4]

Note: Peptides should be designed so that the FITC tag will be released when digested with Chymotrypsin. Chymotrypsin cuts after Tyrosine (Y), Tryptophan (W), and Phenylalanine (F) amino acids if they are not followed by Proline (P).

Materials

FITC-conjugated Peptides, American Peptide
Chymotrypsin, Calbiochem/EMD (catalog no. 230834)
1M Tris-HCl Buffer, Invitrogen (catalog no. 15568-025)
DNase/RNase-Free Gibco Water, Invitrogen (catalog no. 10977-015)
$CaCl_2 \cdot 2H_2O$, Fisher (catalog no. C79-500)

Determination of Peptide Surface Concentration

1) Make digestion buffer by diluting Tris-HCl buffer (1:100) in DNase/RNase-Free Gibco water. Add 1.47 mg/mL $CaCl_2 \cdot 2H_2O$. If the $CaCl_2 \cdot 2H_2O$ is weighed very accurately, the pH will be close to 8.0.

2) Allow the salt to dissolve, and then pH the solution to 8.0.

3) Add chymotrypsin for a final concentration of 1546 U/mL. The dilution of chymotrypsin will depend on the lot of chymotrypsin bought.

4) Add 1 mL chymotrypsin in buffer to a well of a 24-well plate. Keep the plate covered in foil. Incubate the plate on a rocking table for 2 hours at room temperature.

5) Weigh out ~1 mg of the FITC peptide without gloves to reduce static.

6) Dissolve the peptide in sodium phosphate buffer at 100 μM.

7) Make serial dilutions of the peptides at concentrations of 10 μM, 1 μM, 0.1 μM, and 0.01 μM in 1.5 mL centrifuge tubes. Cover tubes with foil and incubate on rocking table for 2 hours at room temperature. It is also recommended to do two sets of dilutions.

8) Pipet 200 μL from each standard or sample well into separate wells of an opaque black 96-well plate. Do triplicates of each standard or sample if possible. Add 200 μL chymotrypsin in buffer to use as a blank.

9) Read fluorescence of all the samples on a fluorimeter plate reader with the following settings:

Excitation: 485 nm
Emission: 538 nm

Readings per well: 6

Data Analysis

1) Subtract blank from all standards and samples.

2) Plot Log (Concentration) versus Log (Fluorescence (RFU)) for the standards. This should yield a straight line with a positive slope. Fit an equation to the trend line. This equation should be of the form Log (Concentration) = A * Log (Fluorescence (RFU)) + B.

3) Use the following equation to calculate the surface concentration for each condition.

Surface Concentration (pmol/cm^2) = (10 ^ (A* Log (Fluorescence (RFU)) + B) * 200 μL * (total volume (μL) chymotrypsin per well/200 μL))/(surface area (cm^2))

If there were multiple samplings per well, use the average of those fluorescence in the equation. For multiple wells of the same condition, average the values of the surface concentration determined by the above equation.

The surface concentration values should created a Langmuir-type curve when plotted against the concentration used in the conjugation step.

A.6 Synthesizing Interpenetrating Polymer Networks (IPNs) on Tissue Culture Polystyrene (TCPS)

Modified from Greg Harbers[5, 6]

Materials

Acrylamide, Polysciences, Inc. (catalog no. 00019)
Polyethylene glycol (PEG) 1000 Monomethyl Ether ($H_2C=C(CH_3)CO_2(CH_2CH_2O)_nCH_3$), Polysciences, Inc. (catalog no. 16666)
N,N'-Methylenebisacrylamide (Bisacrylamide) ($(H_2C=CHCONH)_2CH_2$), Polysciences, Inc. (catalog no. 00719)
([3-(3.4)-Dimethyl-9-oxo-9H-thioxanthen-2-yloxy)-2-hydroxypropyl]trimethylammonium chloride) (QTX), Sigma Aldrich (catalog no. 406333) (note: no longer available for purchase)
Acrylic Acid, Polysciences, Inc. (catalog no. 00020)
2-propanol (Isopropanol or IPA), Fisher (catalog no. A416-500)
Sodium hydroxide (NaOH), Sigma Aldrich (catalog no. S8045)
Ethanol, Sigma Aldrich (catalog no. 362808)

Making Ethanol Cleaning Solution

Ethanol/NaOH Cleaning Solution: 120 g NaOH
1.4 L Ethanol
Synthesis Grade water to 2 L total
(will need to add more water as NaOH dissolves)

1) Fill a 2 L beaker with ethanol.

2) Place beaker on stir plate, and start stirring the liquid with a stir bar.

3) Slowly add the NaOH pellets to allow pellets to settle along the edge of the beaker. This will allow for easier/better mixing.

4) Slowly add water to 2 liters.

5) Stir the mixture until the ethanol is fully dissolved.

6) Add more water to make final volume 2 L.

Note: Do not store this solution as it can degrade over time. Since the ethanol concentration is high, be careful of any spills as the NaOH will leave a precipitate.

Cleaning Plates for Synthesis

1) Place TCPS plates in Ethanol Cleaning Solution for 1 hour to clean off any residue on the plate.

2) Remove plates from Ethanol Cleaning Solution, and dispose of cleaning solution in a waste container.

3) Rinse the plates 5 times in the beaker with synthesis grade water by filling the beaker almost all the way up each time.

4) Refill the beaker with synthesis grade water and sonicate for 30 minutes in a large waterbath sonicator.

5) Rinse the plates 3 times in the beaker with synthesis grade water.

Note: Plates may be stored in a parafilmed beaker full of synthesis grade water for a few days or synthesis can be done that day.

Prepping Samples for Synthesis

1) Rinse individual samples under synthesis grade water.

2) Tap out the excess water onto kimwipes with the sample face down.

3) Dry each sample with filtered dry nitrogen gas until it is completely dry.

4) Use oxygen plasma to clean and activate the polystyrene surface. Each machine will vary in operation, but run the plasma for 5 minutes once the light given off by the plasma is blue (purple or red light indicates there is Nitrogen in the gas plasma).

Note: Prepare the samples and complete the IPN synthesis in the same day for the best results.

Synthesizing the IPN Networks

Note: Use only Contrad cleaned glassware or new conical tubes to hold all reagents. Try to keep any solutions with QTX in the dark as much as possible before polymerization to minimize degradation.

Acrylamide Solution:	0.1485 g/mL acrylamide 0.0015 g/mL bisacrylamide 0.01 g/mL QTX in 3% IPA and 97% Synthesis Grade Water
PEG/Acrylic Acid Solution:	0.0200 g/mL PEG_{1000} 0.0100 g/mL bisacrylamide 0.005 g/mL QTX 16.2 μL/mL Acrylic Acid (add last) in 50% IPA and 50% Synthesis Grade Water

1) Turn on UV table and let it warm up for at least 30-45 minutes before the first polymerization. You can leave this on while doing the plasma cleaning of the surfaces.

2) Weigh out reagents to make acrylamide solution, keeping the QTX in the dark as much as possible to prevent degradation.

3) Dissolve the reagents with the IPA and synthesis grade water by sonicating the solution for at least 10 min or until all reagents are dissolved.

4) Add 0.75 mL to each well of a 12-well plate with either a pipet attached to a pipet aid or repeater pipette. Do this step as quickly as possible and right before adding the plates to the UV table. You may need to work in batches.

5) Allow the solution to adsorb onto the samples for 10 minutes. Turn off the table and place the samples on the UV table.

6) Polymerize the samples for 4.5 minutes after all UV bulbs have turned on.

7) After the polymerization is done, aspirate off the acrylamide solution and place samples in 2L beakers full of synthesis grade water.

8) After all the batches are done, pour off the soaking water and rinse the samples 4 or 5 times in the beaker with synthesis grade water.

9) Add fresh synthesis grade water to the samples and sonicate for 5 minutes.

10) Rinse the samples 2 or 3 more times with water in the 2L beaker.

11) Rinse each sample individually and tap out the excess water on kimwipes.

12) Dry the samples almost completely with filtered dry nitrogen.

13) Prepare the PEG/acrylic acid solution by weighing out the reagents, keeping the QTX in the dark as much as possible.

14) Dissolve the reagents with IPA and synthesis grade water and then add the acrylic acid. Sonicate the solution for at least 10 minutes to dissolve all the reagents and PEG.

15) Repeat steps 4 through 10 **except do the polymerization for 6 minutes.**

16) If samples are going to be conjugated, place the samples in a 2L beaker with fresh synthesis grade water. Ideally, conjugation should be done the day of IPN synthesis or the next day. For storage of unconjugated IPNs, dry the samples completely with filtered dry nitrogen and store in a nitrogen box.

A.7 Conjugating Peptides through a Carboxylic Acid

Modified from Greg Harbers[6]

Materials

Diamino Poly(ethylene glycol) (PEG) (MW 3400), Laysan Bio, Inc. (catalog no. NH2-PEG-NH2-3400)
1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC), Pierce/Thermo Scientific (catalog no. 22980)
N-hydroxysulfosuccinimide (Sulfo-NHS), Pierce/Thermo Scientific (catalog no. 24510)
Sulfosuccinimidyl 4-[N-maleimidomethyl]cyclohexane-1-carboxylate (Sulfo-SMCC), Pierce Thermo Scientific (catalog no. 22322)
2-[morpholino]ethanesulfonic acid (MES) (pre-weighed packets), Pierce/Thermo Scientific (catalog no. 28390)
Boric Acid (H_3BO_3), EMD Chemicals, (catalog no. BX0865-1)
Sodium Tetraborate Decahydrate ($Na_2B_4O_7 \cdot 10H_2O$), Fisher (catalog no. S246-500)
Sodium Phosphate Monobasic ($NaH_2PO_4 \cdot H_2O$), EMD Chemicals (catalog no. SX0710-1)
Sodium Phosphate Dibasic (Na_2HPO_4), EMD Chemicals (catalog no. SX0720-1)
Peptide for conjugation (with or without a FITC tag), Custom made by American Peptide Company

Making MES Solution (pH = 7.0), 0.5 or 0.1 M (make fresh the day of conjugation)

1) Dissolve each packet of MES into 100 mL synthesis grade water to make 0.5 M MES solution.

2) pH the MES solution to 7.0.

3) After doing the PEG step of the conjugation, dilute the solution 1:4 to make 0.1 M MES solution for the washes after the PEG step.

Making Sodium Borate Solution (pH = 7.5)

1) For every 500 mL solution desired, add 10 g Sodium Tetraborate Decahydrate and 14 g Boric Acid to 500 mL synthesis grade water.

2) Dissolve the powders, and pH the solution to 7.5.

3) Solution may be used the day it's made or stored for around 6 months at room temperature. Do not use the solution if there is any precipitate is in the bottom of the bottle.

Making Sodium Phosphate Solution (pH = 6.6)

1) For every 500 mL solution desired, add 4.31 g Sodium Phosphate Monobasic and 2.66 g Sodium Phosphate Dibasic to 500 mL synthesis grade water.

2) Dissolve the salts, and pH the solution to 6.6.

3) Solution may be used the day it's made or stored for around 6 months at room temperature. Do not use the solution if there is any precipitate is in the bottom of the bottle.

Conjugating Peptides to a Carboxylic Acid

Note: All incubation steps are performed on a rocker table at medium high speed to allow for good mixing during the incubation. The amounts given are for making 12-well plates. For FITC-tagged peptides, please do all steps after Step 11 in the dark to prevent photobleaching of the FITC tag and keep the samples wrapped in foil as much as possible.

Diamino PEG Solution:	0.150 g/mL $PEG(NH_2)_2$ 0.005 g/mL EDC 0.0025 g/mL Sulfo-NHS in 0.5 M MES Buffer (pH = 6.6)
Sulfo-SMCC Solution:	0.0005 g/mL Sulfo-SMCC in Sodium Borate Buffer (pH = 7.5)

1) Equilibrate samples with 0.5 M MES solution for 30 minutes, approximately 0.75 mL per well of a 12-well plate, on a rocker table at room temperature.

2) Weigh out reagents for the diamino PEG solution and dissolve in 0.5 M MES buffer. Do not make extra of this solution, since the diamino PEG will increase the volume of the solution.

3) pH the solution to 7.0 since the diamino PEG will change the pH of the solution after dissolving. Make sure the diamino PEG is fully dissolved before adjusting the pH.

4) Aspirate off the 0.5 M MES equilibrating solution, and add 0.5 mL of the diamino PEG solution to each well.

5) Place the samples on a rocker table for 1 hour at room temperature.

6) Aspirate the diamino PEG solution.

7) Rinse the samples 2-3 times with 0.1 M MES solution, incubating the samples with each wash around 5 minutes each.

8) Weigh out the reagents for the SMCC crosslinking solution and dissolve in Sodium Borate Buffer. You do not need to pH this solution as the Sulfo-SMCC does not affect the pH.

9) Rinse the samples 2 times with Sodium Borate buffer, incubating the samples with each wash around 5 minutes each.

10) Aspirate the liquid from the last wash.

11) Add 0.75 mL SMCC solution to each well, and incubate on the rocking table at room temperature for 30 minutes.

12) Make peptide solutions at the desired concentrations (usually between 0.1 μM and 100 μM) in Sodium Phosphate Buffer. Weigh the peptides on the scale without gloves to minimize the loss of peptide due to static from gloves. Once the peptide is dissolved in Sodium Phosphate Buffer, resume wearing gloves. When using FITC-tagged peptides, weigh the peptides in a dark room and place the peptides in foil-wrapped 50 mL conical tubes.

13) Rinse the samples 2-3 times with Sodium Borate buffer, incubating the samples with each wash around 5 minutes each.

14) Rinse the samples 2 times with Sodium Phosphate Buffer, incubating the samples with each wash around 5 minutes each.

15) Aspirate the liquid from the last wash.

16) Add approximately 0.75 – 1 mL peptide solution to the desired wells.

17) Parafilm the plate to reduce evaporation. For FITC-tagged peptides, also place foil around the plates to completely cover.

18) Incubate the plate in the refrigerator (4ºC) for at least 20 hours. No shaking is necessary for this step.

19) Aspirate off the peptide solution.

20) Wash the samples 4-5 times with Sodium Phosphate buffer, incubating the samples with each wash around 5 minutes each.

21) After the last wash, place the samples in a large beaker containing synthesis grade water. Sonicate 5 minutes.

22) Remove the samples from the water and rinse under a stream of synthesis grade water.

23) For storage in the nitrogen box, dry the sample completely with filtered Nitrogen gas.

Alternatively for cell studies, store the sample with penicillin-streptomycin, parafilm, and store at 4ºC until ready to place cells on the sample. Before adding cells, further sterilize plate with 70% ethanol; fill the wells and spaces between the wells completely. Spray the lid of the plate quite well, and let sit in a sterile TC hood for at least 30 minutes. Rinse 3 times with phosphate-buffered saline (PBS). Remove PBS and add cells in their media.

Sample can last for a few months in the fridge.

A.8 Conjugating IPN Surfaces with DMSO-Soluble Peptides

Note: Can also try 10% DMSO, but surfaces were more consistent with 20% DMSO.

Materials

Diamino Poly(ethylene glycol) (PEG) (MW 3400), Laysan Bio, Inc. (catalog no. NH2-PEG-NH2-3400)
1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC), Pierce/Thermo Scientific (catalog no. 22980)
N-hydroxysulfosuccinimide (Sulfo-NHS), Pierce/Thermo Scientific (catalog no. 24510)
Succinimidyl 4-[N-maleimidomethyl]cyclohexane-1-carboxylate (SMCC), Pierce Thermo Scientific (catalog no. 22360)
2-[morpholino]ethanesulfonic acid (MES) (pre-weighed packets), Pierce/Thermo Scientific (catalog no. 28390)
Boric Acid (H_3BO_3), EMD Chemicals, (catalog no. BX0865-1)
Sodium Tetraborate Decahydrate ($Na_2B_4O_7 \cdot 10H_2O$), Fisher (catalog no. S246-500)
Sodium Phosphate Monobasic ($NaH_2PO_4 \cdot H_2O$), EMD Chemicals (catalog no. SX0710-1)
Sodium Phosphate Dibasic (Na_2HPO_4), EMD Chemicals (catalog no. SX0720-1)
Peptide for conjugation (with or without a FITC tag), Custom made by American Peptide Company
Dimethyl sulfoxide (DMSO), Fisher (catalog no. D128-4)

Making Sodium Borate Solution (pH = 7.5) with 20% DMSO

1) For every 500 mL solution desired, weigh out 10 g Sodium Tetraborate Decahydrate and 14 g Boric Acid. Dissolve these powders in 400 mL synthesis grade water and 100 mL DMSO.

2) pH the solution to 7.5. Leave the pH probe in the solution as little as possible to avoid any degradation from DMSO.

3) Solution may be used the day it's made or stored for around 6 months at room temperature. Do not use the solution if there is any precipitate is in the bottom of the bottle.

Making Sodium Phosphate Solution (pH = 6.6) with 20% DMSO

1) For every 500 mL solution desired, weigh out 4.31 g Sodium Phosphate Monobasic and 2.66 g Sodium Phosphate Dibasic. Dissolve these powders in 400 mL synthesis grade water and 100 mL DMSO.

2) pH the solution to 6.6. Leave the pH probe in the solution as little as possible to avoid any degradation from DMSO.

3) Solution may be used the day it's made or stored for around 6 months at room temperature. Do not use the solution if there is any precipitate is in the bottom of the bottle.

Conjugating IPN Surfaces with DMSO-Soluble Peptides

Note: All incubation steps are performed on a rocker table at medium high speed to allow for good mixing during the incubation. The amounts given are for making 12-well plates.

SMCC Solution: 0.0005 g/mL SMCC
in Sodium Borate Buffer + 20% DMSO (pH = 7.5)

1) Follow the "Conjugating Peptides to a Carboxylic Acid" protocol through Step 7.

2) Weigh out the SMCC for the SMCC crosslinking solution. Dissolve the SMCC in DMSO using 1/5 the total desired volume for the solution.

3) Gradually add the remaining 4/5 desired volume with Sodium Borate Buffer (pH = 7.5) **without any DMSO** while vortexing the solution. The SMCC will come out of solution as you gradually add the buffer, but should go back into solution as it is mixed more.

4) Rinse the samples 2 times with Sodium Borate buffer containing 20% DMSO, incubating the samples with each wash around 5 minutes each.

5) Aspirate the liquid from the last wash.

6) Add 0.75 mL SMCC solution to each well, and incubate on the rocking table at room temperature for 30 minutes.

7) Make peptide solutions at the desired concentrations (usually between 0.1 μM and 100 μM). Weigh the peptides on the scale without gloves to minimize the loss of peptide due to static from gloves. Dissolve the peptide in DMSO using 1/5 the total desired volume for the solution. Gradually add the remaining 4/5 desired volume with Sodium Phosphate Buffer (pH = 6.6) **without any DMSO** while vortexing the solution. When using FITC-tagged peptides, weigh the peptides in a dark room and place the peptides in foil-wrapped 50 mL conical tubes.

8) Rinse the samples 2-3 times with Sodium Borate buffer with 20% DMSO, incubating the samples with each wash around 5 minutes each.

9) Rinse the samples 2 times with Sodium Phosphate Buffer with 20% DMSO, incubating the samples with each wash around 5 minutes each.

10) Aspirate the liquid from the last wash.

11) Add approximately 0.75 – 1 mL peptide solution to the desired wells.

12) Parafilm the plate to reduce evaporation. For FITC-tagged peptides, also place foil around the plates to completely cover.

13) Incubate the plate in the refrigerator (4ºC) for at least 20 hours. No shaking is necessary for this step.

14) Aspirate off the peptide solution.

15) Wash the samples with Sodium Phosphate Buffer with 20% DMSO for 10 minutes on a rocking table.

16) Wash the samples twice with 1% SDS, incubating the samples at least 10 minutes with each wash.

17) Wash the samples with Sodium Phosphate Buffer without DMSO for 10 minutes.

18) After the last wash, place the samples in a large beaker containing synthesis grade water. Sonicate 5 minutes.

19) Remove the samples from the water and rinse under a stream of synthesis grade water.

20) For storage in the nitrogen box, dry the sample completely with filtered Nitrogen gas. For cell experiments, surfaces can be stored as indicated in the "Conjugating Peptides to a Carboxylic Acid" protocol.

A.9 Adsorbing Peptides on Tissue Culture Polystyrene for Cell Studies

Modified from Meng, et al.[7]

Adsorbing Peptides

1) Weigh out peptides without using gloves.

2) Dissolve at 200 μM in sterile synthesis grade water or relevant solvent for the peptide. Do not sterilize peptide solutions as peptide will bind to the sterilizing membrane.

3) Add ~500 μL of the peptide solution to a well of a 12-well plate.

4) Allow peptides to adsorb to the plates in a sterile biohazard hood for at least 3 hours at room temperature.

5) Wash surface two or three times with sterile phosphate-buffered saline (PBS) to remove traces of any organic solvents and to prepare surface for cells.

6) Aspirate PBS from plate and add cells in desired media. Alternatively, surfaces can be stored parafilmed with surfaces covered in PBS with 1% penicillin-streptomycin at 4ºC for a few days.

A.10 Differentiaton and Immunocytochemistry of Neural Stem Cells (NSCs)

Modified from Palmer, et al., Hsieh, et al., and Hsieh, et al.[8-10]

Materials

DMEM/F-12 Media, Invitrogen, (catalog no. 11039-021), 5 mL N-2 supplement added to 500 mL media
N-2 Media Supplement, Invitrogen (catalog no. 17502-048)
Accutase, Innovative Cell Technologies (catalog no. AT-104)
Fetal Bovine Serum (FBS), Invitrogen (catalog no. 10437-028)
Trans-Retinoic Acid (RA), Calbiochem/EMD (catalog no. 554720-500 UG), 1000 μM in DMSO
Paraformaldehyde, Acros (catalog no. 41678-5000), 4%
Triton X-100, Fisher (catalog no. BP151-500)
Goat Serum, Sigma (catalog no. G9023)
Primary Antibodies
Isotype Antibodies
Secondary Antibodies
4',6-diamidino-2-phenylindole, dichloride (DAPI), Invitrogen (catalog no. D21490)
Phosphate-Buffered Saline (PBS), Invitrogen (catalog no. 10010)

Mixed Differentiation of NSCs

1) Aspirate the media off a plate of Neural Stem Cells. Wash the plate with PBS.

2) Aspirate the PBS, and add 2-3 mL accutase to a 10-cm dish of NSCs. Incubate at 37ºC for 5-10 minutes or until cells are coming off the plate.

3) Add 3-5 mL DMEM/F-12+N-2 to the plate. Pipet all over the plate to release the cells from the plate.

4) Place the cells in a conical tube and centrifuge at 1000 rpm for 2-3 minutes to separate the cells from the accutase.

5) Aspirate the supernatant and resuspend the cells in 1 mL DMEM/F-12+N-2 media. Further break up cells with a sterile flame-polished glass pipette.

6) Count the cells on a hemacytometer.

7) Plate NSCs (25,000 cells per well of an 8-well glass chamber slide or 70,000 cells per well of a plastic 12-well plate) in DMEM/F-12+N-2 with 1 μM RA and 1 % FBS. The RA will encourage differentiation to neurons, while the FBS encourages differentiation towards astrocytes. (Optional: For better results on a glass chamber slide, plate the cells in DMEM/F-12+N-2 and 20 ng/mL FGF-2 for one day. Change the media the day after seeding with DMEM/F-12+N-2 media with 1 μM RA and 1 % FBS.)

8) Incubate the cells at 37ºC for 4-5 days. Change the media (containing 1 μM RA and 1 % FBS) every other day.

Immunocytochemistry with NSCs

Note: All immunocytochemistry steps are done on a rocking/rotating table set at a low speed at room temperature outside of a biohazard hood unless otherwise specified. Plates as small as 12-well plates can be used for this protocol. For a 12-well plate, most steps can be done with 500 or 1000 μL though a smaller volume can be used with the primary and secondary antibody steps to reduce the amount of antibody used. For the best results, add/aspirate liquid slowly to/from the samples to reduce the amount of detached cells. In addition, I recommend removing most or all of the liquid in each step for the best pictures in the end. For glass chamber slides, only do full washes after fixing, incubation with primary antibody, and incubation with secondary antibody; otherwise do 80% washes.

1) Aspirate off NSC media, and fix cells with 4% paraformaldehyde for 15 minutes.

2) Wash the cells with PBS for 5 minutes.

3) Aspirate off the PBS, and permeabilize/block the cells with PBS-GST (PBS containing 5% goat serum and 0.3% Triton-X-100) for 30 minutes.

4) Aspirate off the PBS-GST, and incubate the cells with the desired primary antibodies diluted in PBS-GST at 4ºC, shaking is optional. This incubation should be done for at least 48 hours. The dilution of the primary antibody will vary, but most antibodies are effective around 1:250 to 1:1000. To check for background binding, use an isotype antibody (unconjugated antibody not specific to any protein, but of the same species and type as the primary) and incubate for the same time as the primary antibody on a separate sample.

8) Wash the cells two or three times with PBS-GST for 5 to 10 minutes each.

9) Aspirate off the PBS-GST, and incubate the cells with the desired secondary antibodies diluted in PBS-GST for 1 hour. For Alexa secondary antibodies, a ratio of 1:250 works well. Reduce the exposure of the secondary antibodies and samples to light by wrapping all tubes and plates in foil to reduce photobleaching. It is also a good idea to vortex the antibody and then centrifuge before use to remove antibody clumps that could affect pictures.

10) Wash the cells two or three times with PBS for 5 to 10 minutes each. In the first wash include DAPI to stain the nuclei of all the cells.

11) Cells may be imaged in PBS (approximately 200-300 μL per well) that day or the samples can be preserved by mounting with an anti-fade reagent and imaged later.

Example Antibodies

Primary Antibodies
α-β-Tubulin III (marker for a neuron), Mouse IgG2b, Sigma (catalog no. T8660), 1:500 dilution recommended
α-Nestin (maker for a neural stem cell), Mouse IgG1, BD Biosciences (catalog no. 556309), 1:250 dilution recommended
α-Glial Fibrillary Acidic Protein (GFAP, marker for an astrocyte), Rabbit IgG, Abcam (catalog no. ab7260), 1:1000 dilution recommended

Isotype Antibodies
Note: use the same concentration of antibody as the primary
Rat IgG2a, Invitrogen (catalog no. R2a00)
Mouse IgG1, Invitrogen (catalog no. MG100)
Mouse IgG2a, Invitrogen (catalog no. MG2a00)
Mouse IgG2b, Invitrogen (catalog no. MG2b00)
Mouse IgG3, Invitrogen (catalog no. MG300)
Mouse IgM, Invitrogen (catalog no. MGM00)

Secondary Antibodies
Goat α-Mouse IgG + Alexa 488, Invitrogen (catalog no. A11029)
Goat α-Rat IgG + Alexa 488, Invitrogen (catalog no. A11006)
Goat α-Rabbit IgG + Alexa 488, Invitrogen (catalog no. A11008)
Goat α-Rabbit IgG + Alexa 546, Invitrogen (catalog no. A11010)
Goat α-Mouse IgG3 +FITC, Invitrogen (catalog no. M32601)

A.11 Sorting hESCs on a Flow Cytometry Machine

Materials

X-Vivo 10 Medium, Lonza (catalog no. 04-743Q)
Human Basic Fibroblast Growth Factor (hbFGF or hFGF-2), Peprotech (catalog no. 100-18B)
Transforming Growth Factor Beta 1 (TGF-β1), R&D Systems (catalog no. 240-B)
Collagenase IV, Invitrogen (catalog no. 17104-019)
Knockout DMEM, Invitrogen (catalog no. 10829-018)
hESC-qualified Matrigel, BD Biosciences (catalog no. 354277)
Phosphate-Buffered Saline (PBS), Invitrogen (catalog no. 10010)
Human Serum Albumin (HSA), Sigma-Aldrich (catalog no. A1653)
InSolution Y-27632 (Rock Inhibitor), Calbiochem (EMD), (catalog no. 688001-500UG)
Ethylenediaminetetraacetic acid (EDTA), Fisher (catalog no. S312-500), 2 mM in PBS
Mouse Embryonic Fibroblasts (MEFs)
Penicillin-Streptomycin, Invitrogen (catalog no. 15140)

Preparation for Sorting hESCs

1) Plate MEFs in MEF media on a 6-well plate that has been coated with gelatin. (See the "Human Embryonic Stem Cell Culture Protocol Handbook" for more details). Grow the MEFs for a few days to allow them to deposit extracellular matrix proteins on the plate.

2) The day before sorting, change the media on the hESCs and add Rock Inhibitor (1:1000) to the media.

3) One hour before detaching the cells, change the media on the hESCs and add Rock Inhibitor (1:1000) to the media.

4) Aspirate media and add collagenase to remove stromal cells. Incubate at 37ºC for around 5 minutes. Wash with PBS two or three times to remove stromal cells, but not colonies. (Optional: Scrap a portion of the hESCs for plating on 10 cm dish; these cells will not be sorted.)

5) Add 2 mM EDTA in PBS to break up cells into single cells. Incubate at 37ºC for at least 15 minutes or until cells come off plates with some as single cells when the plate is shaken.

6) Add X-Vivo10 media (w/ growth factors and rock inhibitor) directly to EDTA-treated cells. Pipet the media all over the plate to release the cells from the plate and to break up the cell clumps into single cells.

7) Filter cells through a cell strainer placed on a new 50 mL tube.

8) Count the cells on a hemacytometer.

9) Add media (with growth factors and Rock Inhibitor) so cells are at a concentration of 10^6 cells/mL. Place cells in flow cytometry tubes for sorting.

10) Change media (use X-vivo with growth factors, penicillin-streptomycin, and rock inhibitor) on 6-well plate coated with MEFs.

Sorting hESCs and Post-Sort Treatment of hESCs

11) Use a negative control for setting up gates on the flow cytometer. Sort samples into the 6-well plate containing MEFs (one well per sample).

10) After sorting, you can add some naïve hESCs to the wells if your sorted cells have drug resistance. Change media every one or two days. Add penicillin-streptomycin for about 4 days and rock inhibitor for a week or two. The penicillin-streptomycin will help reduce contamination from the sorting process, and the rock inhibitor will increase cell viability.

11) Select cells with drug to remove naïve hESCs if they were added after sorting. Use a well of naïve hESCs as a negative control to gauge the progression of the selection.

A.12 Immunocytochemistry with hESCs

Materials

Paraformaldehyde, Acros (catalog no. 41678-5000), 4%
Triton X-100, Fisher (catalog no. BP151-500), 0.1% in PBS
Sodium Dodecyl Sulfate (SDS), Fisher (catalog no. BP166-500), 0.5% in PBS
Bovine Serum Albumin (BSA), Sigma (catalog no. A3803), 2% in PBS
Primary Antibodies
Isotype Antibodies
Secondary Antibodies
4',6-diamidino-2-phenylindole, dichloride (DAPI), Invitrogen (catalog no. D21490)
Phosphate-Buffered Saline (PBS), Invitrogen (catalog no. 10010)

Immunocytochemistry with hESCs

Note: All immunocytochemistry steps are done on a rocking/rotating table set at a low speed at room temperature outside of a biohazard hood unless otherwise specified. Cells can be cultured on glass or tissue culture polystyrene plates for at least 3 days. Plates as small as 12-well plates can be used for this protocol. For a 12-well plate, most steps can be done with 500 µL though a smaller volume can be used with the primary and secondary antibody steps to reduce the amount of antibody used. For the best results, add/aspirate liquid slowly to/from the samples to reduce the amount of detached cells. In addition, I recommend removing most or all of the liquid in each step for the best pictures in the end.

1) Aspirate off hESC media, and fix cells with 4% paraformaldehyde for 10 minutes.

2) Wash the cells with PBS for 5 minutes.

3) Aspirate off the PBS, and permeabilize the cells with 0.1% Triton-X-100 for 15 minutes.

4) Wash the cells with PBS for 5 minutes.

5) Aspirate off the PBS, and incubate with 0.5% SDS for 5 minutes. This step should be excluded if external cell markers are being targeted.

6) Wash the cells with PBS for 5 minutes.

7) Aspirate off the PBS, and incubate the cells with the desired primary antibodies diluted in PBS at 4ºC, shaking is optional. This incubation should be done at least overnight. The dilution of the primary antibody will vary, but most antibodies are effective around 1:250 to 1:1000. To check for background binding, use an isotype antibody (unconjugated antibody not specific to any protein, but of the same species and type as the primary) and incubate for the same time as the primary antibody on a separate sample.

8) Wash the cells two or three times with PBS for 5 minutes each.

9) Aspirate off the PBS, and incubate the cells with the desired secondary antibodies diluted in PBS for 1 hour. For Alexa secondary antibodies, a ratio of 1:250 works well. Reduce the exposure of the secondary antibodies and samples to light by wrapping all tubes and plates in foil to reduce photobleaching. It is also a good idea to vortex the antibody and then centrifuge before use to remove antibody clumps that could affect pictures.

10) Wash the cells two or three times with PBS for 5 minutes each. In the first wash include DAPI to stain the nuclei of all the cells.

11) Cells may be imaged in PBS (approximately 200-300 μL per well) that day or the samples can be preserved by mounting with an anti-fade reagent and imaged later.

Example Antibodies

Note: For more integrin antibodies, see supplemental information for Meng, et al.[7] For more information of tissue layer markers, see Genbacev, et al. and Lu, et al.[11, 12]

Primary Antibodies
α-Oct-4 (internal self-renewal marker for hESCs), Rabbit IgG, Abcam (catalog no. ab19857), 1:250 dilution recommended
α-SSEA-4 (external self-renewal marker for hESCs), Mouse IgG3, Millipore (catalog no. MAB4304), 1:250 dilution recommended
α-Tra-1-60 (external self-renewal marker for hESCs), Mouse IgM, Millipore (catalog no. MAB4360)
α-α_6 Integrin Subunit, Rat IgG2a, Millipore (catalog no. MAB1378)
α-β_1 Integrin Subunit, Mouse IgG1, Millipore (catalog no. MAB1987Z)
α-Smooth Muscle Actin (marker for mesoderm), Mouse IgG2a, Dako (catalog no. M0851)
α-Alpha-fetoprotein (AFP, marker for endoderm), Mouse IgG2a, Sigma (catalog no. A8452)
α-β-Tubulin III (marker for ectoderm or a neuron), Mouse IgG2b, Sigma (catalog no. T8660), 1:500 dilution recommended
α-Nestin (maker for a neural stem cell), Mouse IgG1, BD Biosciences (catalog no. 556309), 1:250 dilution recommended
α-Glial Fibrillary Acidic Protein (GFAP, marker for an astrocyte), Rabbit IgG, Abcam (catalog no. ab7260), 1:1000 dilution recommended

Isotype Antibodies
Note: use the same concentration of antibody as the primary
Rat IgG2a, Invitrogen (catalog no. R2a00)
Mouse IgG1, Invitrogen (catalog no. MG100)
Mouse IgG2a, Invitrogen (catalog no. MG2a00)
Mouse IgG2b, Invitrogen (catalog no. MG2b00)
Mouse IgG3, Invitrogen (catalog no. MG300)
Mouse IgM, Invitrogen (catalog no. MGM00)

Secondary Antibodies
Goat α-Mouse IgG + Alexa 488, Invitrogen (catalog no. A11029)
Goat α-Rat IgG + Alexa 488, Invitrogen (catalog no. A11006)
Goat α-Rabbit IgG + Alexa 488, Invitrogen (catalog no. A11008)
Goat α-Rabbit IgG + Alexa 546, Invitrogen (catalog no. A11010)
Goat α-Mouse IgG3 +FITC, Invitrogen (catalog no. M32601)

A.13 Immunocytochemistry with hESCs on Flow Cytometry for External Cell Markers

Materials

Collagenase IV, Invitrogen (catalog no. 17104-019), 200 U/mL in Knockout DMEM
Knockout DMEM, Invitrogen (catalog no. 10829-018)
Ethylenediaminetetraacetic acid (EDTA), Fisher (catalog no. S312-500), 2 mM in PBS
X-Vivo 10 Medium, Lonza (catalog no. 04-743Q)
Fetal Bovine Serum (FBS), Invitrogen (catalog no. 10437-028), 2% in PBS
Primary Antibodies
Isotype Antibodies
Secondary Antibodies
Phosphate-Buffered Saline (PBS), Invitrogen (catalog no. 10010)
InSolution Y-27632 (Rock Inhibitor), Calbiochem (EMD), (catalog no. 688001-500UG)
Ice bucket filled with ice

Immunocytochemistry with hESCs for flow cytometry

Note: For better cell viability, you can add Y-27632 to the cells (1:1000) 1 hour prior to cell detachment and with the resuspended cells in the last step.

1) Aspirate off hESC media, and incubate cells with collagenase for 2-5 minutes at 37ºC (approximately 2-3 mL per 10-cm dish).

2) Aspirate off the collagenase and wash a few times with PBS, adding it dropwise to remove stromal cells from plates.

3) Aspirate PBS and add 2-3 mL EDTA in PBS per plate and incubate for at least 15 minutes at 37ºC to detach the cells from the plate and help break up the cells. The cells have been incubated long enough when cells release from the plate when shaken.

4) Add X-Vivo 10 media to dilute the EDTA and lift the cells off the plate by pipetting over the surface many times.

5) Remove the media with cells to a 50 mL conical tube, and wash the plate with fresh X-Vivo 10 media to remove the remaining cells.

6) Add these washed cells to the 50 mL conical tube, and mix the cells well with a 10 mL pipet vigorously to break up cell clumps. If cells still look clumpy, you can put cells through a cell strainer.

7) Count the cells on a hemacytometer to determine the amount of cells.

8) Centrifuge enough cells for each sample at 1000 rpm for 5 minutes. Each sample should have $5 * 10^5$ cells.

9) Aspirate the supernatant from the cell pellet and resuspend with 500 μL 2% FBS in PBS (blocking buffer) containing the appropriate primary or isotype antibody for each sample. Incubate the samples in 1.5 mL centrifuge tubes on ice for 30 minutes. In general, a higher concentration of antibody is needed for flow cytometry staining in comparison to immunocytochemistry on plated cells. In general, the dilution should be 1:25 to 1:200 depending on the antibody.

10) Add 1 mL blocking buffer and centrifuge at low speed (around 2000 rpm) for 3-4 minutes.

11) Aspirate the liquid. Wash twice more with 1 mL blocking buffer each time and centrifuge at low speed (around 1000 rpm) for 5 minutes.

12) Aspirate the blocking buffer and resuspend the cell pellet with 500 μL blocking buffer containing the appropriate secondary antibody. In general, a dilution of 1:100 works well for Alexa secondary antibodies. Incubate the samples on ice for 30 minutes in the dark.

13) Add 1 mL blocking buffer and centrifuge at low speed (around 2000 rpm) for 3-4 minutes.

14) Aspirate the liquid. Wash twice more with 1 mL blocking buffer each time and centrifuge at low speed (around 1000 rpm) for 5 minutes.

15) Resuspend the cells in blocking buffer and keep on ice until the sample can be analyzed via flow cytometry.

Example Antibodies

Note: For more integrin antibodies, see supplemental information for Meng, et al.[7] For more information of tissue layer markers, see Genbacev, et al. and Lu, et al.[11, 12]

Primary Antibodies
α-SSEA-4 (external self-renewal marker for hESCs), Mouse IgG3, Millipore (catalog no. MAB4304), 1:100 dilution recommended
α-Tra-1-60 (external self-renewal marker for hESCs), Mouse IgM, Millipore (catalog no. MAB4360)
α-α_6 Integrin Subunit, Rat IgG2a, Millipore (catalog no. MAB1378)
α-β_1 Integrin Subunit, Mouse IgG1, Millipore (catalog no. MAB1987Z)

Isotype Antibodies
Note: use the same concentration of antibody as the primary
Rat IgG2a, Invitrogen (catalog no. R2a00)
Mouse IgG1, Invitrogen (catalog no. MG100)
Mouse IgG2a, Invitrogen (catalog no. MG2a00)
Mouse IgG2b, Invitrogen (catalog no. MG2b00)
Mouse IgG3, Invitrogen (catalog no. MG300)
Mouse IgM, Invitrogen (catalog no. MGM00)

Secondary Antibodies
Goat α-Mouse IgG + Alexa 488, Invitrogen (catalog no. A11029)
Goat α-Rat IgG + Alexa 488, Invitrogen (catalog no. A11006)
Goat α-Rabbit IgG + Alexa 488, Invitrogen (catalog no. A11008)
Goat α-Rabbit IgG + APC, Invitrogen (catalog no. A10931)
Goat α-Mouse IgG3 +FITC, Invitrogen (catalog no. M32601)

A.14 Immunocytochemistry with hESCs on Flow Cytometry for Internal Cell Markers

Materials

Collagenase IV, Invitrogen (catalog no. 17104-019), 200 U/mL in Knockout DMEM
Knockout DMEM, Invitrogen (catalog no. 10829-018)
Ethylenediaminetetraacetic acid (EDTA), Fisher (catalog no. S312-500), 2 mM in PBS
X-Vivo 10 Medium, Lonza (catalog no. 04-743Q)
Fetal Bovine Serum (FBS), Invitrogen (catalog no. 10437-028), 2% in PBS
Formaldehyde, VWR (catalog no. BDH0500-1LP), 2% in PBS
Saponin Buffer (1 mg/mL Saponin [Sigma (catalog no. 47036)], 1 % BSA in PBS)
Bovine Serum Albumin (BSA), Sigma (catalog no. A3803)
Primary Antibodies
Isotype Antibodies
Secondary Antibodies
Phosphate-Buffered Saline (PBS), Invitrogen (catalog no. 10010)
Ice bucket filled with ice

Immunocytochemistry with hESCs for flow cytometry

1) Aspirate off hESC media, and incubate cells with collagenase for 2-5 minutes at 37ºC (approximately 2-3 mL per 10-cm dish).

2) Aspirate off the collagenase and wash a few times with PBS, adding it dropwise to remove stromal cells from plates.

3) Aspirate PBS and add 2-3 mL EDTA in PBS per plate and incubate for at least 15 minutes at 37ºC to detach the cells from the plate and help break up the cells. The cells have been incubated long enough when cells release from the plate when shaken.

4) Add X-Vivo 10 media to dilute the EDTA and lift the cells off the plate by pipetting over the surface many times.

5) Remove the media with cells to a 50 mL conical tube, and wash the plate with fresh X-Vivo 10 media to remove the remaining cells.

6) Add these washed cells to the 50 mL conical tube, and mix the cells well with a 10 mL pipet vigorously to break up cell clumps. If cells still look clumpy, you can put cells through a cell strainer.

7) Count the cells on a hemacytometer to determine the amount of cells.

8) Centrifuge enough cells for each sample at 1000 rpm for 5 minutes. Each sample should have $5 * 10^5$ cells.

9) Aspirate the supernatant from the cell pellet and fix the cells with 250 μL 2% Formaldehyde for 15 to 30 minutes on ice.

10) Add 1 mL 2% FBS in PBS and centrifuge at low speed (around 2000 rpm) for 3-4 minutes.

11) Aspirate the supernatant and permeabilize the cells with 500 μL Saponin Buffer. Incubate the tubes at room temperature for 15 minutes.

12) Centrifuge the samples at low speed (around 2000 rpm) for 3-4 minutes.

13) Aspirate the supernatant from the cell pellet and resuspend with 100 μL Saponin Buffer containing the appropriate primary or isotype antibody for each sample. Incubate the samples in 1.5 mL centrifuge tubes on ice for 30 to 60 minutes. In general, a higher concentration of antibody is needed for flow cytometry staining in comparison to immunocytochemistry on plated cells. In general, the dilution should be 1:25 to 1:200 depending on the antibody.

14) Add 1 mL Saponin Buffer and centrifuge at low speed (around 2000 rpm) for 3-4 minutes.

15) Aspirate the liquid and resuspend the cell pellet with 100 μL Saponin Buffer containing the appropriate secondary antibody. In general, a dilution of 1:100 works well for Alexa secondary antibodies. Incubate the samples on ice for 30 to 60 minutes in the dark.

16) Add 1 mL Saponin Buffer and centrifuge at low speed (around 2000 rpm) for 3-4 minutes.

17) Resuspend the cells in 2% FBS in PBS and keep on ice until the sample can be analyzed via flow cytometry.

Example Antibodies

Note: For more integrin antibodies, see supplemental information for Meng, et al.[7] For more information of tissue layer markers, see Genbacev, et al. and Lu, et al.[11, 12]

Primary Antibodies
α-Oct-4 (internal self-renewal marker for hESCs), Rabbit IgG, Abcam (catalog no. ab19857), 1:100 dilution recommended

Isotype Antibodies
Note: use the same concentration of antibody as the primary
Rat IgG2a, Invitrogen (catalog no. R2a00)
Mouse IgG1, Invitrogen (catalog no. MG100)
Mouse IgG2a, Invitrogen (catalog no. MG2a00)
Mouse IgG2b, Invitrogen (catalog no. MG2b00)
Mouse IgG3, Invitrogen (catalog no. MG300)
Mouse IgM, Invitrogen (catalog no. MGM00)

Secondary Antibodies
Goat α-Mouse IgG + Alexa 488, Invitrogen (catalog no. A11029)
Goat α-Rat IgG + Alexa 488, Invitrogen (catalog no. A11006)
Goat α-Rabbit IgG + Alexa 488, Invitrogen (catalog no. A11008)
Goat α-Rabbit IgG + APC, Invitrogen (catalog no. A10931)
Goat α-Mouse IgG3 +FITC, Invitrogen (catalog no. M32601)

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