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Engineering Novel Peptide and Polymer Systems for Human Pluripotent Stem Cell Self-Renewal
and Differentiation

By

Anusuya M. Ramasubramanian

A dissertation submitted in partial satisfaction of the

requirements for the degree of

Joint Doctor of Philosophy

with University of California, San Francisco

in

Bioengineering

in the

Graduate Division

of the

University of California, Berkeley

Committee in Charge:

Professor Kevin E. Healy, Chair

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Spring 2017

Engineering Novel Peptide and Polymer Systems for Human Pluripotent Stem Cell Self-Renewal
and Differentiation

© 2017

by

Anusuya M. Ramasubramanian

University of California, Berkeley

Abstract

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University of California, Berkeley

Professor Kevin E. Healy, Chair

Developing synthetic materials that promote the expansion and differentiation of human pluripotent stem cells (hPSCs) has been a long-standing challenge. Unfortunately, most synthetic biomaterials either (1) lack bioactive motifs, resulting in low viability and premature stem cell differentiation, or (2) rely on a small subset of peptide motifs that promote few cell fates. To reliably guide hPSCs down their full repertoire of cell fates, including self-renewal, materials targeting a wider class of surface receptors are required. However, the development of such materials has been hindered by our limited understanding of stem cell surface receptors and their structural interactions with the extracellular matrix (ECM).

In Chapter 2, we used an unbiased bacterial display strategy to circumvent the dearth of structural information about receptor-ECM interactions and identify ligands for the highly expressed hPSC surface receptor, $\alpha_6\beta_1$ integrin. Using human embryonic stem cells (hESCs) as bait, theoretical library sizes of 10^{12} random yet unique 15mers were panned to identify 30 sequence-similar peptides that bind α_6 integrin. Characterization of adhesion via flow cytometry and centrifuge-based assays suggested that seven of our screened cyclic and linear peptides bound hPSCs with sub-micromolar K_d s similar to full-length ECM proteins like laminin. However, as identified in the literature, hPSCs rely on a plurality of motifs to mediate binding and maintain pluripotency over several passages. With this in mind, we were curious whether our novel peptides could interact synergistically with canonical binding motifs for $\alpha_v\beta_3$ integrin and syndecan-1 to enhance stem cell self-renewal. To that end, we adapted a high-throughput microculture platform to print self-assembled monolayers of peptides (SAMs) and screen combinations of both novel and canonical motifs for their capacity to maintain short-term hiPSC culture. By measuring each SAMs' capacity to promote cell proliferation, pluripotency and adhesive morphology, we were able to identify an optimal combination of three integrin- and syndecan-binding motifs that supported the self-renewal and expansion of hESCs and hiPSCs over a single passage.

In Chapter 3, we then asked if some of these peptides identified through screening and selection could be used to create scalable, thermoreversible polymer-peptide systems for multi-passage hPSC culture. Although several alginate, hyaluronic acid and agarose scaffolds have been generated in the past for the long-term culture of hPSCs, these gels have lacked degradable elements requisite for scalable passaging as well as adhesive motifs that promote hPSC adhesion. To develop 3D

materials more amenable to large-scale culture, we instead generated a hydrogel blend that was both thermoreversible and functionalizable. Using acrylated hyaluronic acid as the backbone, our screened integrin- and syndecan-binding peptides were conjugated via Michael addition chemistry. Conjugates were characterized via ^1H NMR for polymer acrylation and BCA assay for peptide conjugation. Peptide-decorated hyaluronic acid was then blended with a previously developed thermoreversible HyA-NIPAAm graft co-polymer to generate bioactive, scalable and defined 3D scaffolds for hPSC culture. Using these scaffolds, we were able to qualitatively show that blends incorporating our α_6 -integrin-binding cyclic peptides could support 12- to 15-fold expansion over a single passage.

While our results in Chapter 2 and 3 suggested the development of 2D and 3D peptide-based scaffolds for hPSC self-renewal, there remain many challenges and open questions regarding these targeted peptide-scaffolds and the utilization of high-throughput screening in other tissue engineering applications. In Chapter 4, we considered some of these challenges and alluded to future applications of display techniques for the generation of defined, synthetic extracellular matrices and purification agents.

To Ayya, for all that you have done and will continue to do for me

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List of Abbreviations

AcHyA	Acrylated Hyaluronic Acid
ADH	Adipic Acid Dihydrazide
APMAAm	Aminopropylmethacrylamide
ATs	Alkanethiols
BCA	Bicinchoninic acid
bFGF	Basic Fibroblast Growth Factor
BMP	Bone Morphogenetic Protein
BSA	Bovine Serum Albumin
bspRGD	Bone Sialoprotein RGD
CMC	Carboxymethyl chitosan
CPX	Circularly Permuted Outer Membrane Protein (Omp) X
DAPI	4',6-Diamidino-2-phenylindole
DARPin	Designed Ankyrin Repeat Proteins
DMEM/F12	Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12
DVS	Divinyl sulfone
EB	Embryoid Body
ECM	Extracellular Matrix
EDC	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
FACS	Fluorescence-Activated Cell Sorting
FBS	Fetal Bovine Serum
Fmoc	Fluorenylmethoxycarbonyl chloride
Fn	Fibronectin
GABA	γ -Aminobutyric acid
GFP	Green Fluorescent Protein
GPCRs	G-protein-coupled Receptors
HSA	Human Serum Albumin
hESCs	Human Embryonic Stem Cells
hFLG3	Human Fibroblast Growth Factor Receptor 3
hiPSCs	Human Induced Pluripotent Stem Cells
hMSCs	Human Mesenchymal Stem Cells

HOBt	Hydroxybenzotriazole
hPSCs	Human Pluripotent Stem Cells
HRG1β	Heregulin-1 β
HSCs	Hematopoietic Stem Cells
HyA	Hyaluronic Acid
HyA-vs	Hyaluronic Acid-vinyl sulfone
ICM	Inner Cell Mass
IGF	Insulin-like Growth Factor
IgG	Immunoglobulin
IPN	Interpenetrating Network
KLF4	Krueppel-like Factor 4
LCST	Lower Critical Solution Temperature
LDEV	Lactate Dehydrogenase Elevating Virus
Lm	Laminin
LUT	Lookout Table
MEFs	Mouse Embryonic Fibroblasts
MUA	Mercaptoundecanoic Acid
MWCO	Molecular Weight Cutoff
NAS	<i>N</i> -Acryloxysuccinimide
Neu5Gc	<i>N</i> -Glycolylneuraminic acid
NHS	<i>N</i> -Hydroxysuccinimide
Ni-NTA	Nickel-Nitrilotriacetic acid
NMR	Nuclear Magnetic Resonance (Spectroscopy)
NMWL	Nominal Molecular Weight Limit
NSCs	Neural Stem Cells
OCT4 (OCT3/4)	Octamer-binding Transcription Factor 4 (3/4)
PAO	Phenylarsine oxide
PAS	Peptide-Acrylate Surface
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PD	Polydopamine
PDMS	Polydimethylsiloxane
PEG	Polyethylene glycol

PLA	Poly(lactic acid)
PLGA	Poly(lactic- <i>co</i> -glycolic acid)
PMEDSAH	Poly[2-methacryloyloxy)ethyl dimethyl-(3-sulfopropyl)ammonium hydroxide]
pNIPAAm	Poly(<i>N</i> -isopropylacrylamide)
pNIPAAm-SH	Thiolated Poly(<i>N</i> -isopropylacrylamide)
PSCs	Pluripotent Stem Cells
PVE-alt-MA	Poly(methyl vinyl ether-alt-maleic anhydride)
QCM-D	Quartz Crystal Microbalance with Dissipation Monitoring
RGB	Red-Green-Blue
ROCKi	Rho-associated Protein Kinase Inhibitor
rpm	Rotations per minute
SAMs	Self-Assembled Monolayers
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SEC-MALS	Size Exclusion Chromatography and Multi-angle Light Scattering
SOX2	SRY(sex determining region Y)-box 2
SSEA3 (SSEA4)	Stage-specific Embryonic Antigen 3 (4)
TCPS	Tissue Culture Polystyrene
TEV	Tobacco Etch Virus
TGFβ	Transforming Growth Factor β
UPGMA	Unweighted Pair Group Method with Arithmetic Mean
Vn	Vitronectin
XPS	X-ray Photoelectron Spectroscopy

Acknowledgements

As I wrap up six years as a doctoral student, I am humbled by and grateful to the many people who have helped and inspired me during my time at Berkeley. A Ph.D. can be a very individualistic and, at times, isolating endeavor – a patchwork of a few “highs” and several “lows”. However, the tremendous support I received from the people mentioned here – an incredible tribe of mentors, colleagues, friends and family – have made this journey a truly memorable one.

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Chapter 1: Biomaterials for Human Pluripotent Stem Cell (hPSC) Culture

1.1 The ECM and the Pluripotent Stem Cell Niche

An Overview of the Stem Cell Niche and Its Components

Stem cells are a relatively rare class of cells within many organisms that have two important functional characteristics – (1) a capacity to self-renew extensively and (2) the capacity to differentiate into more specialized cell types^{1,2}. This dual ability to generate daughter cells that are either identical to themselves (self-renewal) or more restricted in their potential (differentiation) allows these stem cells to play a pivotal role in tissue growth, homeostasis and repair^{3,4}. Balancing the decision to self-renew or differentiate, quiesce or die, however, is complex^{5,6}. Stem cells must integrate a series of cell-extrinsic cues – usually instructive or permissive in nature – as well as cell-intrinsic signaling to make fate choices⁷. These cues or ‘inputs’ these cells experience do not occur in isolation. Rather, they have a physical analog in specific structures within the cellular microenvironment of the stem cell, known as the *stem cell niche*^{3,8,9} (**Fig. 1.1A**).

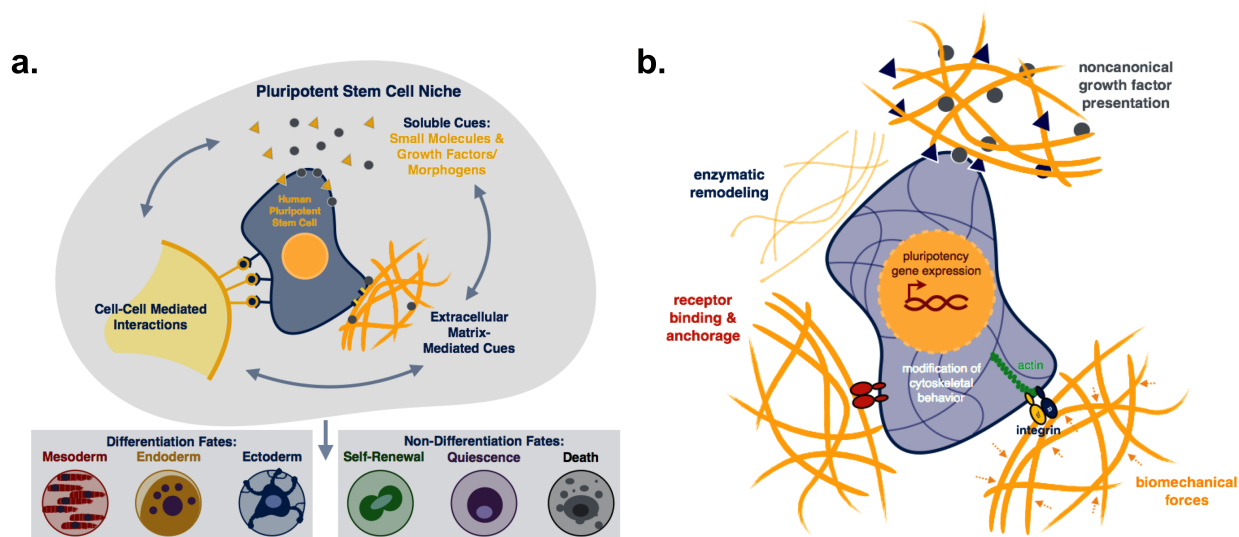


Figure 1.1: The Human PSC Niche. (a) A schematic of the human pluripotent stem cell niche. Several dynamic factors in the niche act in unison to drive stem cell fate choices. The dominant regulators of stem cell fate include soluble cues, extracellular matrix (ECM)-mediated cues (both biochemical and biophysical) and juxtacrine interactions with neighboring cells. (b) A schematic of the ECM-induced biophysical and biochemical cues. As the physical housing surrounding hPSCs, the ECM instructs these cells through the presentation of growth factors and adhesion motifs as well as changes in bulk biophysical properties. Changes in the ECM can drive modifications in cytoskeletal elements and thus the ECM itself can be a powerful driver of gene expression in hPSCs.

The Biological Context: The Stem Cell and Its Extracellular Matrix | It is easiest to grasp the concept of the stem cell niche by using the more familiar analogy of the “neighborhood”. In the archetypal community, land availability, demographics and real-estate development have a resounding impact on a given neighborhood’s personality as well as the resources and opportunities available to its residents and their descendants. Likewise, the physical, chemical, topographical and even electrical characteristics of the stem cell niche strongly influence the stem cells that reside within it^{10,11}. In fact, distinct stem cells populations with different capacities for differentiation arise over the course of development, influenced by the size and scale of their host tissue or organ, the resident support cells surrounding them and their niche’s proteinaceous infrastructure or extracellular matrix (ECM)⁸.

This last component of the cellular microenvironment, the ECM, is perhaps the most dynamic element within the niche. Under homeostatic conditions, the ECM experiences a constant turnover of proteins in direct response to matrix remodeling enzymes secreted by stem and supporting cells^{12,13}. As the matrix is degraded, constituent cells secrete their own compositionally unique matrix proteins at varying rates thus slowly changing both the bulk biophysical and chemical properties of their surroundings (**Fig. 1.1B**)^{12–15}. As an integrated whole, changes in the stiffness of the ECM as well as its ability to sequester and present noncanonical growth factors and adhesive motifs play a large role in the motility of the stem cell, its lineage commitment and, ultimately, future remodeling of the ECM itself. This is best illustrated in periods of homeostatic disruption, namely injury, disease and development, where rapid cell death, migration or metastasis can result in whole-sale changes to the chemistry and mechanics of ECM^{6,15}. Such disruptive events can make the microenvironment inhospitable to certain cell fate choices and, in other cases, conducive to differentiation¹⁵. From this vantage point, the ECM and its composition is an important window to understanding the mechanics of every stem cell – those adult stem cells that are maintained as stable populations over the lifespan of the organism as well as fetal and embryonic stem cells that play short but critical roles early in development⁵.

The Adult Stem Cell and the Embryonic Stem Cell | Adult or somatic stem cells – classes of unipotent, bipotent or multipotent stem cells in the adult organism with tissue-restricted potential¹⁶ – often have well-defined albeit diverse ECM that have been the subject of many detailed studies^{5,17,18}. For example, monopotent myosatellite cells remain quiescent in the basement membrane of the muscle fiber, primed to differentiate in response to injury through polarized cell surface receptors that interact with the stem cell’s two-dimensional sheet-like ECM, the basal lamina¹⁹. By contrast, the multipotent hematopoietic stem cell (HSCs) resides in a dramatically different environment. Encompassed by hydrated three-dimensional fibrillar polymer networks in the red bone marrow of the interior femur, HSCs respond to ECM stiffness and tethered signals – including a slew of growth factors – that influence their self-renewal and differentiation into myeloid and lymphoid phenotypes^{20–22}. While adult stem cells reside in diverse niches, their niches share one thing in common. They are often characterized by a stability that is required for maintaining these stem cell populations during long periods of self-renewal or even quiescence^{5,19}. Fetal or embryonic stem cell populations, on the other hand, lack similarly stable niches and instead encounter rapid developmental changes that actively restructure, reformulate and, in some cases, completely reconstitute their niche^{23,24}. This latter characteristic of embryonic stem cells makes it a difficult but also an exciting prospect to study these cells and their niches as we attempt to engineer synthetic alternatives to native ECM. In this chapter, I will review the canon of literature surrounding the human pluripotent stem cell (hPSC) and its *in vivo* niche while exploring the biomaterial platforms that have arisen over the last two decades to support the expansion of these cells *in vitro*.

In Vivo Matrix Microenvironment of Human Pluripotent Stem Cells (hPSCs)

There is no more vivid portrait of a transient stem cell population and its niche than that of the pluripotent stem cell – a class of stem cells with the extraordinary capacity to differentiate into all the cell types comprising the fetal and adult organism^{25–29}. Human embryonic stem cells (hESCs), the native human analog, arise four to five days after fertilization in the inner cell mass (ICM) of the blastocyst, an early-stage of the preimplantation embryo that contains 50 to 150 cells³⁰. Soon after fertilization, the embryo begins to undergo a rapid series of morphological changes known as gastrulation, making the blastocyst a highly dynamic niche for resident stem cells^{23,31}. This flux in the anatomy of the hESC niche presents important challenges not only in isolating hESCs but also in identifying the *in vivo* cues these stem cells receive.

Major protein players in the *In Vivo* hESC ECM | Despite these challenges in probing the hESC microenvironment *in vivo*, it is known that stem cells in the embryo are highly dependent not just on growth factor gradients, cell-cell interactions and epigenetic changes but also on the matrix proteins that house them¹⁰. This is well illustrated by disruptions in embryo development that result from perturbations of the ECM and ECM receptors. For example, loss-of-function studies in mice, where key ECM proteins like fibronectin (Fn)³², collagen³³ or laminin (Lm)³⁴ were deleted, result in embryonic lethality at various stages in development³⁵. Of these proteins, laminin, a high-molecular weight trimeric glycoprotein that appears as early as the two-cell stage of the embryo, is essential^{36,37}. Laminins are composed of an α , β , and γ -chain that have five, three, and three genetic variants respectively, resulting in sixteen different laminin isoforms³⁸ named according to the chain variants they contain (i.e. Lm-511 contains α 5, β 1, and γ 1 chains)³⁹ (**Fig. 1.2A**). Among these isoforms, Lm-111 and 511 appear to be particularly important. Deletory mutations in the α 1 or α 5 chains, in particular, are independently lethal early in embryogenesis^{34,40}.

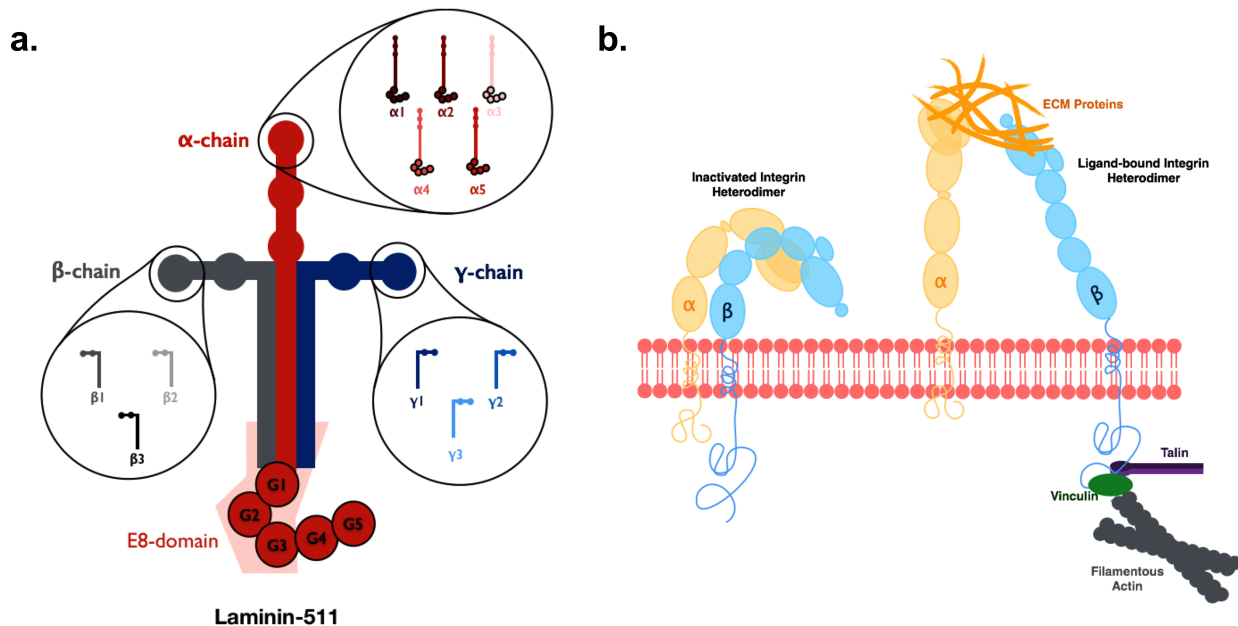


Figure 1.2: The Structure of Laminins and Integrins. (a) A schematic of the heterotrimeric ECM protein, laminin. Laminin is composed of three separate chains – an α -chain with five variants,

a β -chain with three variants and a γ -chain with three variants. Isoforms of laminin are named according to the chain variants they contain. For example, laminin-511, one of the key ligands for $\alpha_6\beta_1$ integrin, contains α_5 , β_1 , and γ_1 chains. In this laminin, putative interactions with integrin is thought to occur through the globular tail of the α_5 chain known as the E8 domain. This domain has been widely utilized, either in conjunction with the full-length protein or as an isolated protein fragment, to mediate hPSC adhesion *in vitro*. (b) A schematic of the heterodimeric membrane protein, integrin. Embedded in the cell membrane, integrins are one of the primary receptors on the surface of hPSCs. In their inactive state, the integrin headpieces remain folded. However, in an active or ligand bound state, the integrin head unfolds as the α and β integrins form a binding pocket for the ECM-derived ligand. This activation initiates downstream signaling including the formation of intracellular filamentous actin fibers.

Interactions with laminins and other matrix proteins occurs primarily through a class of transmembrane receptors known as integrins schematized in **Fig. 1.2B**^{41,42}. Integrins, along with receptors regulating Wnt, fibroblast growth factor (FGF) and transforming growth factor beta superfamily (TGF- β) signaling, comprise important initiators of intracellular signaling in the embryonic stem cell⁴³. An obligate heterodimer consisting of one of eighteen α and one of eight β subunits⁴², integrins regulate cell adhesion, shape, motility, the cell-cycle as well as anchorage via ECM proteins in human embryonic stem cells⁴⁴.

Table 1.1: Key Stem Cell Integrins, their Binding Partners and Phenotypes of Knockout Mice^{45,46}

Integrin or Integrin Heterodimer	Protein Ligand in the ECM	Phenotypes associated with mutation or knockout†
$\alpha 1$	Laminins, collagen	Defects in collagen synthesis, tumor angiogenesis; retinal degeneration and skin hardening
$\alpha 2\beta 1$	Laminins, collagen	Delayed platelet aggregation, mammary ductal gland branching and kidney development
$\alpha 3$	Laminins, collagen, fibronectin and entactin	Lung and kidney defects, skin blistering and aberrant neuronal pathfinding
$\alpha 4$	Fibronectin, VCAM-1	Defects in placental formation and cardiac development as hematopoietic cell maintenance
$\alpha 5$	Fibronectin	Defects neural crest and mesoderm layer formation
$\alpha 6$	Laminins	Skin blistering and aberrant brain cortex formation
αV	Fibronectin, vitronectin	Defects in blood-brain barrier, CNS formation and GI tract development
$\beta 1$	Laminins, collagen, fibronectin, vitronectin and entactin	Inner cell mass deterioration
$\alpha V\beta 3$	Fibronectin, fibrinogen, vitronectin, collagens, osteopontin, thrombospondin	Impaired cerebral blood vessel development; aberrant platelet formation and bone defects

†These summarize the key defects associated with each mutation or knockout. Those knockouts that lead to embryonic lethality are italicized. A larger summary of defects associated with each mutation can be found in Hynes *et al.*⁴⁷

As such, the deletion or mutation of integrins subunits can have important impacts on the viability of embryonic stem cells and their differentiated daughter cells. For example, deletion of the promiscuous β_1 integrin in early stages of development causes peri-implantation lethality and the deterioration of the embryo's ICM at E6.5 in mice⁴⁸. Meanwhile the deletion of less critical integrins can severely affect vascularization, epidermal or retinal development and induce symptoms of muscular dystrophy in the developing embryo⁴⁶. The implications of key *in vivo* integrin knockouts on embryonic development is summarized in **Table 1.1**. It is important to note that while many non-critical integrin mutations have limited effects on cell adhesion, differentiation and embryonic viability *in vivo*, *in vivo* models often mask the deleterious effects of integrin and ECM protein mutations through built-in compensatory mechanisms^{46,49}. For example, the expression of constitutively activated β_1 integrin subunits in embryonic stem cell-derived epidermal cells can inhibit keratinocyte differentiation and activate extracellular matrix adhesion – all hallmarks of tumorigenesis. However, similar effects are not observed *in vivo* without the exposure of these epidermal stem cells to chemical carcinogens^{13,50}. Thus, to better understand the role of matrix proteins in maintaining and instructing embryonic stem cells, it is important to observe the role of these proteins and their receptors *in vitro* as well.

In Vitro Culture of the Human Embryonic Stem Cell (hESCs) and Induced Pluripotent Stem Cell (hiPSCs)

The Human Embryonic Stem Cell: A Model for *In Vitro* Pluripotency | For more than a decade after the initial derivation of stem cells from mouse embryos⁵¹, mechanistic studies involving the *in vitro* culture of the human counterpart – human embryonic stem cells – were stymied by technical challenges of procuring, isolating and propagating cells of the ICM. In 1998, James Thompson and colleagues at the University of Wisconsin, Madison were the first group of researchers to develop a technique to successfully isolate and culture human embryonic stem cells (hESCs) *in vitro*⁵². Since the establishment of these initial five cell lines (two XX karyotype lines H7 and H9 and three XY karyotype lines H1, H13 and H14), thousands of other hESC lines have been established worldwide through a standard set of practices. In short, the pluripotent ICM of a fresh or frozen blastocyst is isolated via mechanical dissection⁵³, laser dissection⁵⁴ or a technique of selective lysis known as immunosurgery^{52,55,56}. Subsequent to ICM dissection, these isolated pluripotent stem cells are cultivated as two dimensional colonies in a media and surface that mimic and, in some cases, contain proteins like laminin that are found in the native stem cell niche.

Over the years, most of the methodologies for the downstream propagation of isolated human pluripotent stem cells have focused on culturing these cells in two dimensions on feeder layers⁵², protein and peptide surfaces^{57,58} as well as synthetic polymers⁵⁹. Regardless of the method used to propagate these cells, however, hESCs grown on *in vitro* surfaces adopt morphologies and marker expression profiles that are distinct from their *in vivo* counterparts. Unlike the three-dimensional cluster of cells in the ICM, hESCs cultured on two dimensional substrates *in vitro* form pancake-like colonies with high ratios of nucleus to cytoplasm and prominent nucleoli (shown in **Fig. 1.3A**)⁵². Moreover, these hESCs have a surprising reliance on preexisting material scaffolding for their proliferation. Whereas, throughout normal development, organ structures and tissues form through densely cellular structures surrounded by sparse networks of self-synthesized extracellular matrix^{60,61}, embryonic stem cells *in vitro* lack this “build-as-you-go” approach to ECM generation⁵². Rather, they require a complex of array of fully formed extracellular matrix networks to provide the structural and functional cues for their proliferation⁴¹ – a requirement that makes *in vitro* cultures less self-sustaining than their *in vivo* analog.

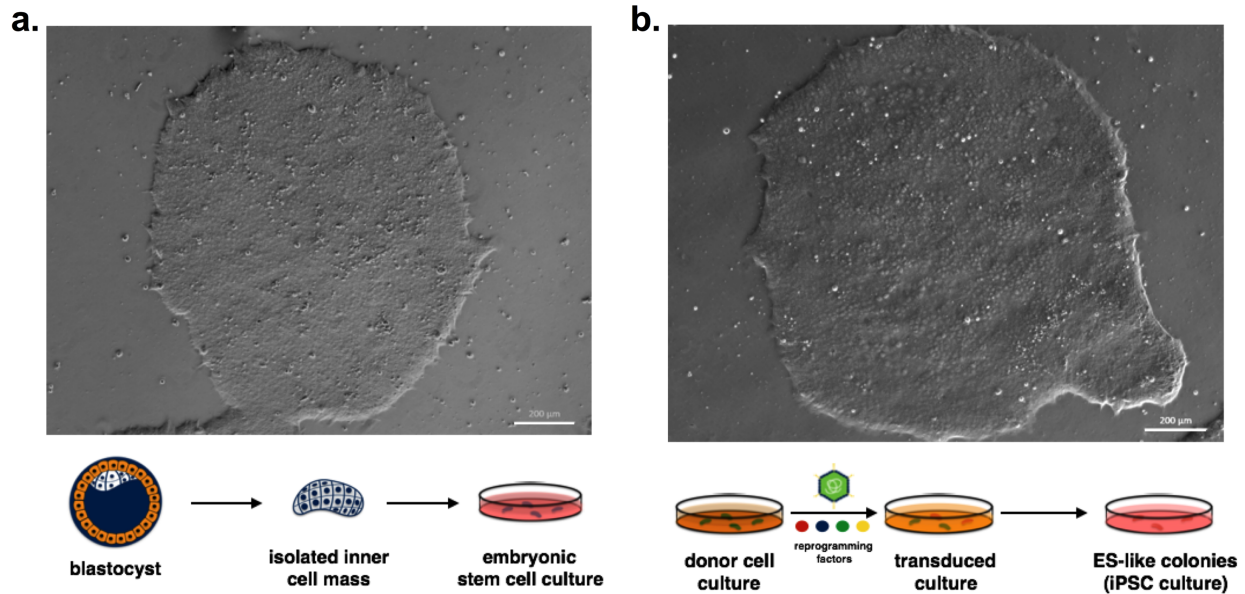


Figure 1.3: Derivation and Culture of hESCs and hiPSCs. (a) Human embryonic stem cell (hESC) colony grown on a 2D Matrigel surface (upper panel). In 2D culture, hESCs adopt a pancake-like morphology with a high nuclear to cytoplasmic ratio and prominent nucleoli. Colony outlines are smooth and slightly raised in appearance, producing plaque-like colonies that can range in size from tens of microns to several hundred microns. To generate these *in vitro* cultures, the pluripotent ICM of a fresh or frozen blastocyst is isolated through a variety of techniques and cultured as two dimensional colonies on a feeder layer or animal-derived protein matrix (lower panel). (b) Human adipose stem cell derived-induced pluripotent stem cell (hiPSC) colony grown on a 2D Matrigel surface (upper panel). These colonies adopt a near identical appearance to hESCs *in vitro*, generating large, smooth colonies of tightly packed cells. Induced pluripotent stem cells are generally derived from an isolated culture of donor cells. These cultures can be transduced with key stem-cell associated genes (i.e. reprogramming factors) using viral vectors. Cells that are effectively transduced within this culture (red) can be harvested and cultured on feeder layers or animal-derived protein matrices to generate an ES-like cell type known as iPSCs. Scale bars represent 200 μm .

However, this is not to say that *in vitro* hPSCs bear no functional similarity to cells in the ICM. While morphologically dissimilar, hESC colonies do show some of the same functional behavior observed in stem cells *in vivo*. More specifically, hESCs grown *in vitro* as 2D cultures on protein and polymer surfaces retain a capacity to differentiate into all three germ layers as well as to self-renew and maintain expression of specific embryonic stem cell markers: transcription factors Oct4, Nanog, Sox2; tumor rejection antigens Tra-1-81 and Tra-1-60; and stage-specific-antigens SSEA3 and SSEA4⁵². As hallmarks of pluripotency, these markers are routinely used to verify the presence of bona fide hPSC populations in *in vitro* cultures.

Advances in Tissue Scaffolds and Pluripotent Cell Models | For ten to twelve years after the initial establishment hESC cell lines *in vitro*, almost all stem cell and differentiation models relied on successive passaging and marker-based verification of static two-dimensional hESC cultures. This heavy reliance on 2D culture techniques and hESCs posed two essential problems. First, static two-dimensional cultures are often inadequate for supplying sufficient quantities of stem cells or stem-cell-

derived tissue to meet the therapeutic demands of most diseases^{62–64}. Second, the procurement of embryonic stem cells, through the isolation of the ICM and collateral destruction of the embryo, poses important ethical considerations^{65,66}. For the last decade, the major challenges and advancements in stem cell biology have revolved around (1) scaling the production of viable hESC cultures and (2) developing alternative methods to procure pluripotent stem cells. To tackle the first challenge, novel material platforms have been synthesized to culture hESCs as three-dimensional colonies that are susceptible to the kinds of chemical and physical forces seen in the native ICM^{62,64}.

On the cellular side, remarkable discoveries into the mechanism of pluripotency have allowed researchers to not only develop strategies for isolating hESCs without destroying the embryo but also to reprogram somatic cells to take on a pluripotent phenotype. This latter discovery by Yamanaka and colleagues in 2007 showed that the introduction of four transcription factors – Oct4, Sox2, cMyc and Klf4 – into a somatic cell, at low efficiencies, produce a unique pluripotent population of cells dubbed ‘induced pluripotent stem cells’ or iPSCs⁶⁷ (**Fig. 1.3B**). Despite their different origins, iPSCs and hESCs are functionally similar – displaying the capacity to self-renew as well as differentiate into tissue from all three germ layers *in vitro* and *in vivo*. However, key differences do exist between these two hPSCs, particularly in the persistence of epigenetic memory from the somatic cells of origin in iPSCs^{68,69}, differential DNA methylation patterns⁷⁰ as well as a different extent of genetic aberrations⁷¹. Thus, as we seek to implement therapies using PSCs, it is important to be cognizant of the differences that arise from the cell and the culture method that is leveraged⁷². The phenotypic differences between hESCs and hiPSCs (and possibly between cultures in 2D versus 3D) stress the importance of generating synthetic niches that promote self-renewal and directed differentiation in a broad range of PSC populations.

1.2 Considerations for Engineering hPSC Microenvironments

Before substantive efforts can be made to engineer fully functional, synthetic hPSC microenvironments, however, we need to have a strong understanding of the design principles – both physical and chemical – that undergird the *in vitro* stem cell niche. With the realization that somatic cells can be reprogrammed to generate hiPSC lines, the capacity to develop hPSC culture models and study niche cues, either in isolation or combination, has vastly expanded. In this section, I will summarize some of the key studies that have been performed in the last decade to understand the physical and chemical cues *in vitro* that inform the pluripotent stem cell. In doing so, I hope to shed light on how such cues might be holistically engineered into a synthetic *in vitro* hPSC niche.

The Biophysical ECM: Shape, Stiffness and Topography

Whether hPSCs are cultured on a 2D surface or in a 3D bulk material, their capacity to proliferate is tightly regulated by the physical properties, namely the shape, stiffness and microarchitecture, of the material in which they are housed⁷³ (**Fig. 1.4**). Recently, advances in material fabrication techniques have afforded new possibilities to independently tune each of these properties and explore its emergent effect on stem cell-ECM interactions at the resolution of single colonies or cells:

Micropatterning: Spatial Sensitivity in Self-Renewal & Differentiation | One such technique involves using nanolithography and microfluidics to selectively pattern islands of ECM proteins surrounded by regions of protein-resistant polymer brushes⁷⁴. By generating differently shaped or sized micropatterns that accommodate a single colony, the influence of substrate shape and spatial order on endpoint differentiation as well as dynamic behaviors like motility⁷⁵ can be systematically assessed.

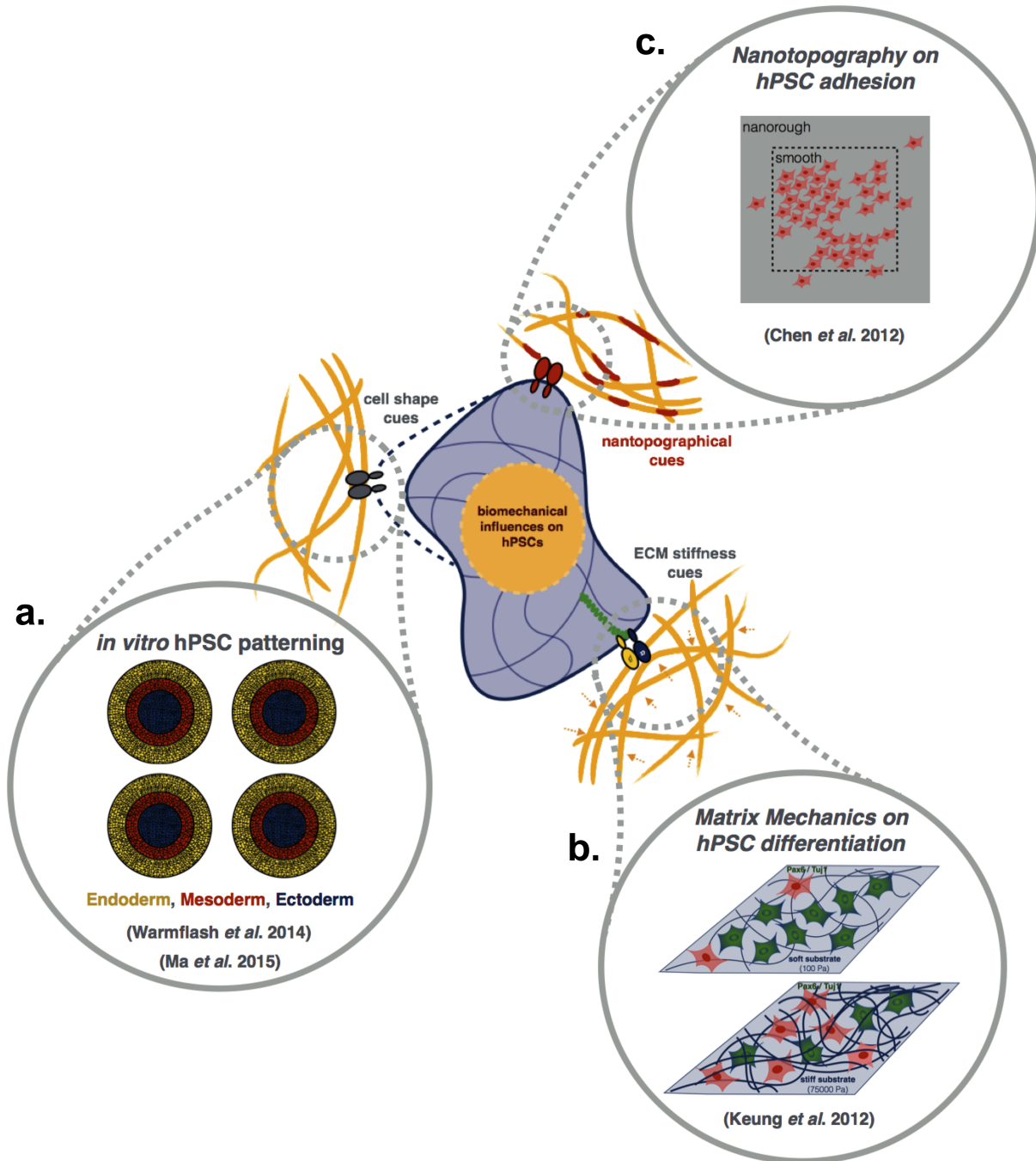


Figure 1.4: The Biophysical ECM & its Influence on hPSC Fate. The physical properties of the ECM – including shape, stiffness and microarchitecture – strongly affect the cell fate outcomes of the hPSCs it supports. Recent advances in material fabrication techniques have allowed these biophysical properties to be independently tuned in order to explore the emergent effects on hPSC fate. In this figure, we schematically highlight some of the key literature investigating the role of (a) micropatterning or spatial cues, (b) matrix mechanics or stiffness cues, and (c) nanotopographical or microarchitecture cues on hPSC self-renewal and differentiation. These individual studies are described in detail in the text.

Harnessing this technique, some of the earliest studies used micropatterning to explore the simple role of pattern size on the inhibition or promotion of self-renewal. As it turns out, niche size is one of the key regulators of hPSC fate. With changes in niche size, the balance therein between differentiation-inducing and –inhibiting factors changes and, as such, certain minimal pattern sizes are required to promote hPSC self-renewal⁷⁶.

While hPSCs are not alone in displaying a sensitivity for niche size^{77,78}, embryonic stem cells *in vivo* are unique in their capacity to migrate and form three germ layers in very specific yet reproducible spatial sequences. *In vitro* and in the absence of key spatial cues, these same cells differentiate at random in a heterogeneous and spatially disordered fashion. Recently however, Warmflash *et al.* showed that geometric confinement using patterned microislands can recapitulate native self-organizing capabilities *in vitro*⁷⁹. In their work, hESCs were grown on highly controlled Lm-521 micropatterns and treated with BMP4 – a key player in the embryonic signaling cascade that initiates gastrulation – to generate colonies that reproducibly differentiate into an outer trophectodermal band, an inner ectodermal core and a mesodermal annulus in between (**Fig. 1.4A**). Similarly, Ma and colleagues have shown that hESCs and hiPSCs grown on patterned Matrigel can be differentiated along the cardiac lineage to produce beating microchambers that are reminiscent of cardiac development *in vivo*⁸⁰.

Although much of the work with micropatterning and shape regulation has occurred in 2D culture, the work of Ma *et al.* hints at the possibility of using patterning techniques to generate more elegant 3D models that recapitulate tissue architecture. With the advent of tools like 3D bioprinting and microwell systems, the capacity to pattern hPSCs or hPSC-derived cells in differently shaped and sized 3D clusters is increasingly tractable⁸¹. In the last few years, significant progress has been made in using 3D microwells, in particular, to develop and directly differentiate embryoid bodies (EB) of regular size and shape. Several groups have used this strategy to understand how cluster size affects differentiation and demonstrated that EB size can bias clusters along a cardiogenic or endothelial pathway^{82–84}. Such studies, however, only mark the beginning of our explorations into size- and shape-mediated influences on hPSC fate in 3D. Ultimately, if we wish to construct better embryonic tissue mimics, it is important to consider how shape and size cues can be incorporated into a biomaterial to instruct hPSCs alongside other cues like stiffness and topography.

Matrix Mechanics Cues for Controlling hPSC Self-Renewal & Differentiation | Investigations into the role of niche-induced spatial cues on hPSC fate often naturally lend themselves to probing another important biophysical property of the niche – stiffness. Stiffness cues, particularly ECM stiffness, has long been known to influence the self-renewal and differentiation properties of numerous adult stem cell populations *in vitro* and *in vivo*⁸⁵. However, active investigations into the role of matrix stiffness on hPSCs are still in their nascency.

To understand how ECM stiffness may influence hPSCs, it is helpful to start by looking at the native embryo. During much of early development, the embryo resides in a very sparse and soft ECM (of ~400 Pa) that gradually grows and stiffens with increasing fate specification⁶¹. As with adult stem cells, both this matrix stiffening and the associated stem cell lineage specification inform and influence one another. With the caveat of 3D hPSC encapsulation^{64,86}, soft substrates that match the intrinsic softness of hESCs suppress focal adhesion formation and maintain self-renewal phenotypes⁸⁷. By contrast, as stem cells begin to differentiate in response to biochemical or biophysical cues, ECM stiffening drives directed differentiation and the production of tailored ECM – inducing further lineage specification.

This chatter between ECM stiffness cues and cellular mechanotransduction mechanisms is partly shown in the work of Keung *et al.* where both hESCs and hiPSCs were seeded on

polyacrylamide substrates of varying stiffnesses⁸⁸. While stiffness played little role in promoting pluripotency in this context, ECM mechanics was critical in regulating early differentiation. Softer substrates were better able to support neuroectodermal and neural differentiation as well as the eventual promotion of dopaminergic phenotypes than an analogous stiffer substrate (**Fig. 1.4B**). These findings were recapitulated by Sun and colleagues, who showed that soft PDMS micropost arrays could enhance the neural induction of hPSCs as well as the subsequent yield of functional motor neurons through the regulation of key mechanosensitive proteins in the Hippo pathway and actomyosin cytoskeletal integrity and contractility⁸⁹.

Ultimately, stiffness – the rigidity or softness of a substrate – represents only one facet of the mechanical cues exerted by the ECM on the stem cell. Along with stiffness cues, hPSCs and adult stem cell populations encounter shear^{62,90} as well as compressive and tensile loading^{63,91} that can be patterned in complex ways over space and time to regulate stem cell behavior. However, studying and regulating such dynamic biophysical cues in two-dimensions can be challenging and, for this reason, the advent of novel hydrogel systems that can spatially or temporally modulate matrix mechanics is particularly exciting. Recent studies, for example, show that hydrogel systems demonstrating dynamic substrate stiffening can either switch human MSCs from adipogenesis to osteogenesis⁹² or promote their cardiogenic differentiation⁹³ in two and three dimensions. Similarly, adult neural stem cells that are ‘pulsed’ with a stiffness cues through regulation of a dynamic oligonucleotide-crosslinked ECM show modulation of neurogenic commitment through Hippo pathway activation⁹⁴. Such studies, while still in their infancy, hint at the potential for understanding the role of matrix mechanics in pluripotent stem cells self-renewal and differentiation through novel material design and development.

ECM Architecture and its Influence on hPSC Fate | Aside from incorporating ECM stiffness cues into *ex vivo* materials, another important thrust within the field has been to understand and mimic the micro- and nanoscale architecture encountered by stem cells *in vivo* in *in vitro* culture systems. At the outset, much of this work has been done with adult stem cell populations, particularly hMSCs⁹⁵. However, mounting evidence suggests that PSCs – both murine and human – are equally sensitive to topography. For example, murine ESCs cultured on aligned electrospun nanofibers showed a proclivity towards neuronal differentiation⁹⁶. On the other hand, hPSCs cultured on nanorough coverslips, generated via reactive ion etching, showed a roughness-dependent propensity to adhere, spread and self-renew⁹⁷ (**Fig. 1.4C**). The capacity of roughness and topography to induce a wide-range of phenotypes – both self-renewing and differentiating – in pluripotent stem cells is surprising and suggests that these cues are at once complex and cell-dependent.

The Biochemical ECM: Growth Factors, Bioactive Motifs and Small Molecules

While material fabrication techniques have provided a toolkit for mimicking ECM mechanics, it is harder to imagine how we might develop tools to parse and recapitulate the ECM’s vast biochemical effects. As mentioned earlier in this chapter, hPSCs encounter chemical cues from the ECM – mostly in the form of tethered growth factors, adsorbed serum proteins and bioactive motifs – through key cell surface receptors: Wnt, FGF and TGF- β receptors as well as integrins, cadherins and G-protein coupled receptors (GPCRs)⁴³. *In vitro*, the effects of these tethered or adsorbed proteins can be amplified by small-molecules like inhibitors of Rho-associated protein kinase (ROCKi), which have been shown to influence hPSC behaviors like survival⁹⁸, differentiation⁹⁹ and reprogramming¹⁰⁰. In this section, we will briefly consider how key biochemical elements of the ECM can be decoupled from their native environments, explored and harnessed to promote specific fate outcomes:

The Role of Growth Factors in hPSC Pluripotency | ECM-tethered or adsorbed proteins like growth factors have long been considered as key players within the stem cell niche. For example, several early studies showed that basic FGF (bFGF) is essential for supporting long-term culture of hESCs on feeder-free ECM^{101–103}. More recently, proteomic screening tools have been leveraged to identify a larger group of players – including insulin growth factor (IGF)¹⁰⁴, Wnt^{105,106} and TGF- β ¹⁰⁷ – that cooperatively establish a regulatory biochemical niche for pluripotent hESCs *in vitro*.

Table 1.2: Culture Media Formulations used for *in vitro* hESC and hiPSC Culture²⁵

Medium	Source	Composition	Defined?	Xeno-free?
PluriStem	Millipore	DMEM/F12, HSA, Activin-A, TGF β 1, bFGF, lipids, insulin, transferrin, selenium, dorsomorphin (DM), IWP-2	N	Y
mTESR1†	STEMCELL Technologies	DMEM/F12/BSA, bFGF, TGF β , lipids, insulin, transferrin, cholesterol, lipids, pipercolic acid, GABA, β -mercaptoethanol	N	N
TESR2	STEMCELL Technologies	DMEM/F12/recombinant HSA, bFGF, TGF β , lipids, insulin, transferrin, cholesterol, lipids, pipercolic acid, GABA, β -mercaptoethanol	Y	Y
StemPro	Invitrogen	DMEM/F12/BSA, bFGF, TGF β , Activin, transferrin, LR3-IGF1, HRG1 β	N	N
NutriStem XF/FF	STEMGENT	Basal medium/HSA, bFGF, TGF β , insulin, transferrin	N	Y
E8/TESR-E8	GIBCO/STEMCELL Technologies	DMEM/F12, bFGF, TGF β , insulin, transferrin, selenium and ascorbic acid	Y	Y
X-Vivo 10	Lonza	Basal medium/HSA-pharmaceutical grade purification, bFGF, hFLG3, transferrin, β -mercaptoethanol	Y	Y
StemFit	Ajinomoto Co.	Basal medium/HSA, bFGF	N	Y
PSGro	Stem RD	DMEM/F12 with recombinant HSA, TGF β 1, bFGF, lipids, insulin, transferrin, selenium	Y	Y

†mTESR1 formulations were preceded by a TESR1 formulation that was identical mTESR1 but with an HSA fraction instead of BSA. TESR1 was eventually replaced by TESR2 which utilized recombinant HSA.

Understanding these key soluble regulators of the biochemical stem cell niche has been particularly important for the development and use of serum-free *in vitro* media formulations. In 2003, the first attempt at defining the soluble cues in culture medium resulted in several novel bFGF- and TGF- β 1- containing formulations that were capable of supporting hPSC cultures on human fibronectin¹⁰⁸. Over the years, these media formulations have been pared down to a defined and robust culture medium known as TESR Essential-8 (TESR-E8) that can support both hESCs and hiPSCs for ten or more passages^{109–112}. The composition of the TESR-E8 formulation and its variants are shown in **Table 1.2** and can be explored further in an excellent review on the topic²⁵. The development of defined growth-factor based media represents a significant advance in our efforts to standardize growth factors in *in vitro* culture systems. Nevertheless, we still lack a comprehensive understanding of how growth factors are presented to and the mechanism by which they act on their target.

In vivo growth factors and serum proteins are presented to cells in two forms: (1) as transiently present soluble ligands and (2) as more permanently bound ligands that are sequestered in the ECM through heparin-mediated binding, electrostatic interactions or physical entrapment¹¹³. Historically, in *in vitro* culture, the subtle differences in growth factor presentation have often been ignored, resulting in an undefined muddle of presentations. As a result, the bulk of defined 2D and 3D materials for hPSC culture incorporate few tethered ligands or proteins and instead rely on the bare polymer scaffold to promote pluripotency or differentiation⁶⁴. Although the success of naked polymers is difficult to assess, work by Habib and colleagues suggests that considering the presentation of tethered developmental signals – in this case the growth factor, Wnt – can be critical to directing cell fate in hPSCs¹¹⁴. Using Wnt-immobilized beads introduced to hESCs *in vitro*, they were able to show that Wnt-proximal daughter cells maintained high levels of pluripotency genes while daughter cells distal to the Wnt source acquired markers of differentiation. Studies like this highlight the importance of spatial presentation of growth factors within *in vitro* culture systems and underscore some of the methods by which we may be able to pattern oriented growth factors into novel materials.

Bioactive ECM Motifs as Mediators of hPSC Fate | Aside from sequestering growth factors and providing mechanical support, the ECM also has an intrinsic bioactivity that plays an important role in guiding hPSC fate. As mentioned earlier in this chapter, the native hPSC ECM is a diverse and dynamic proteinaceous milieu that incorporates isoforms of collagen, laminin, fibronectin and vitronectin^{12,13}. Although these matrix proteins serve largely as structural elements within tissue, they also have stretches of amino acids within their primary sequence that serve as ligands for adhesion receptors like integrins and cadherins¹¹⁵.

Over the last two decades, several bioactive motifs summarized in **Table 1.3**, like the adhesive RGD sequence in fibronectin¹¹⁶, the IKVAV sequence in laminin¹¹⁷ and differentiation-inducing mimetics from collagen (GFOGER)¹¹⁸, have been isolated from their native protein sequence and used to direct hPSC fate. With the realization that isolated motifs could be just as efficient as the full-length protein in mediating adhesive behaviors, proteins and peptides have been leveraged to activate key integrins $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_2\beta_1$, $\alpha_6\beta_1$ on undifferentiated hiPSCs or hESCs^{45,119}. Today, elements of full-length vitronectin – a mediator of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ ¹²⁰ – and laminin – a mediator of $\alpha_6\beta_1$ ¹²¹ – are commercially used to promote pluripotency and hESC clonal growth in two-dimensional culture.

In 3D scaffolds, however, the importance of engaging hPSC surface receptors through adhesive motifs remains largely unexplored⁶⁴. For the most part, while hPSCs can self-renew and maintain pluripotency in a number of 3D polymer materials like hyaluronic acid, they continue to have poor adhesive interactions with their scaffold⁸⁶. Although this has not stymied the adoption and translation of polymeric scaffolds, it is likely that as we gain a more nuanced understanding of the

ECM's biochemical influences, active efforts will be made to “dress” synthetic materials with peptide motifs that mimic native ECM proteins.

Table 1.3: Key Rationally Designed Adhesive Motifs used for hPSC Culture

Sequence	ECM Source	Reference †
KYGAASIKVAVSADR	Laminin	122,123
Ac-KGGNGEPRGDTYRAY	Bone sialoprotein	123,124
Ac-KGGPQVTRGDVFTMP	Vitronectin	123,125
PEO4-NGEPRGDTYRAY	Bone sialoprotein	122,123
GRGDSPK	Fibronectin	116,123
Ac-KGGAVTGRGDSPASS	Fibronectin	116,123
GSDPGYIGSR	Laminin	126
GKKQRFRHRNRKG	Vitronectin (heparin-binding)	126
FHRRIKA	Bone sialoprotein	126

†Includes both the initial identification of the motif as well as subsequent implementation for hPSC culture

1.3 Standard Biomaterials for the Self-Renewal & Expansion of hPSCs

Even a cursory investigation of the hPSC microenvironment – its biophysical and biochemical cues – highlights the dizzyingly complex network of signals required to maintain hPSCs in their self-renewing status quo. To realize therapeutic applications, it is nonetheless essential that these cues are not only reliably replicated but also standardized and scaled without compromising the biological performance of resident cells. Today, hPSCs are cultured *in vitro* in rigid, polystyrene culture vessels; tens of millions of which are manufactured via injection molding and coated with various natural and synthetic materials that promote cell adhesion¹²⁷. This section provides a brief overview of the polymeric and proteinaceous materials used as growth substrates for hPSCs and the steady progress that has been made over the last fifteen years to develop precisely defined culture systems:

Natural Scaffolds for hPSC Self-Renewal

Initial work in developing materials to expand hESCs *in vitro* relied heavily on feeder layers of irradiated mouse embryonic fibroblasts (MEFs) to support self-renewal phenotypes⁵². In an era when the composition and cues associated with the hESC ECM were poorly understood, MEFs were readily adopted because of their capacity to manufacture extracellular matrix proteins and growth factors – like Lm-511 and bFGF – that are essential to maintaining pluripotent cultures²⁵. Some of the earliest work by Thompson and colleagues leveraged MEF layers along with medium laden with fetal bovine serum (FBS) to support the long-term culture of hESCs *in vitro*⁵². Nevertheless, while xenogenic MEF layers and animal-derived cultured mediums are surprisingly efficient at supporting the clonal expansion of hESCs, these materials also represent an important source of pathogenic contamination. In fact, endotoxins, endogenous retroviruses and xenoepitopes like non-human sialic acid N-glycolneuraminic acid (Neu5Gc) can (1) be introduced to hPSCs via their xenogenic substrates and (2) elicit strong immune responses when these cells are transplanted *in vivo*¹²⁸. For this reason, the field as whole has moved towards feeder-free, natural scaffolds – many of which continue to be animal-derived.

Feeder-Free Systems: Animal-Derived Proteins | The need to develop feeder-free materials for hPSC self-renewal has generated numerous research initiatives over the last decade to explore how natural materials could be harnessed for this purpose. Among the early biological scaffolds to be explored was Matrigel, a decellularized and gelatinous protein admixture derived from Engelbreth-Holm-Swarm mouse sarcomas that consists of ECM proteins like laminin, entactin and collagen as well as proteoglycans and sequestered growth factors¹²⁹. Matrigel currently remains the gold-standard for hPSC culture and is widely used in *in vitro* studies of self-renewal and differentiation in spite of substantial drawbacks. Foremost among these drawbacks is batch-to-batch variability in composition, which makes it difficult to control and standardize substrate-induced signaling and receptor-ECM interactions in cells grown on Matrigel¹²⁷. While variations in cell-substrate interactions have limited consequences for hPSC self-renewal, they do have more serious implications on our ability to reliably and reproducibly differentiate hPSC cultures. Furthermore, Matrigel cultures have historically been susceptible to contamination by adventitious rodent pathogens like lactate dehydrogenase-elevating virus (LDEV)¹³⁰, harkening concerns about using Matrigel-derived hESCs in the *in vivo* context.

In part to address LDEV contamination and as an alternative to Matrigel, a similar cell-extract based coating known as Geltrex has also been widely utilized for hPSC culture. However, like Matrigel, Geltrex still contains unknown components and requires researchers to pretest batches to find those that are suitable for hPSC expansion.

Feeder-Free Systems: Single ECM Components | In light of these concerns with cell-extract systems, strong efforts have been made to identify single proteins or polymers – including hyaluronic acid⁸⁶, chitosan, alginate¹³¹ and matrix proteins^{57,58,121} – that can recapitulate the majority of Matrigel's functionality. Laminins, the main component of Matrigel, have been particularly promising stand-alone substrates for hPSC culture when used with a wide-range of defined and undefined media formulations. Early work culturing hESCs on Lm-332, Lm-511 and Lm-111 surfaces showed that these isoforms were competitive with Matrigel in promoting clonal expansion and pluripotency markers expression for ten or more passages¹³². Of these, only Lm-511, which binds through the highly expressed $\alpha_6\beta_1$ integrin, displayed adhesive properties for hESCs that exceeded those of Matrigel in xeno-free culture conditions⁵⁸. To explore how laminins might bind and promote hESC expansion, Miyazaki and colleagues isolated the globular E8 domain of Lm-511 and -338 isoforms (**Fig. 1.2A**) – thought to be the minimum fragment conferring integrin-binding capabilities – to see if individual E8 fragments could support hPSCs¹²¹. Unsurprisingly, both Lm-511E8 and Lm-338E8 were able to support the self-renewal of hESC and iPSC lines in mTESR1 medium.

While the bulk of commercialized single protein substrates for hPSCs utilize laminin or laminin fragments, proteins like vitronectin and fibronectin have also been shown to support hPSC self-renewal *in vitro*^{57,133}. However, like laminin isoforms, these proteins are produced recombinantly *in vitro* and, as such, their complex secondary and tertiary structures make it difficult to scale recombinant protein-based culture systems to meet therapeutic demands.

The Need for Defined Stem Cell Culture Systems

With the realization that somatic cells can be reprogrammed to generate iPSC lines, the therapeutic potential of hPSCs – previously limited by cell sourcing – has been vastly expanded⁶⁷. Human ESCs are now in clinical trials to treat eye diseases, diabetes and heart disease with other applications registered for clinical trial approval^{134–136}. Meanwhile, hiPSCs have been used in experimental procedures to treat macular degeneration¹³⁴ and are routinely used in the laboratory setting as *ex vivo* tools for disease modeling and drug testing⁷². In each of these applications, the

number of cells required for modeling and treatment is much larger than the throughput obtained from natural scaffold systems. For example, to treat the 1.1 million cases of myocardial infarction in the United States, close to 2×10^8 cells per infarct or forty football fields worth of 2D culture would be required¹³⁷. Likewise, the 100 million patients in the United States who suffer from central nervous system disorders¹³⁸ would each require a hundred thousand functional neurons, several million glial or support cells and significant organotypic reinnervation to ameliorate disease phenotypes. These quantitative estimates, while staggering in their own right, ignore the extensive necrosis and cell death accompanying stem cell transplantation – key roadblocks in the adoption of natural scaffold systems for *in vivo* needs.

Aside from the sheer number of cells required for disease models or cell-based therapies, natural scaffolds also present challenges due to their intrinsic heterogeneity. More specifically, each batch of cell-extract or single protein ECM have unique protein or protein isoform compositions that create variable signaling from one stem cell culture to the next. While this variability has little effect in low-throughput hPSC culture where maintenance of pluripotency markers is the key concern, scaling the production of complex protein mixtures to meet the demands of high-throughput stem cell expansion and differentiation remains costly and impractical¹²⁷.

Synthetic and Semi-Synthetic Biomaterials for Maintaining Undifferentiated hPSCs

Since the early 1960s and the advent of the plastics industry, synthetic materials have been generated in ever larger numbers. Initially, these materials were not intended for biomedical applications. However, with an increasing need for materials harboring the biological functionality of ECM proteins as well as the capacity for chemical modification and scalable synthesis, synthetic materials like polymers and peptides have become important scaffolds for tissue engineering. Unlike natural materials, synthetic and semi-synthetic biomaterials have two important properties that make them ideal scaffolds for hPSC culture: (1) their synthesis involves swappable monomer components that allow for the fine-tuning of biophysical properties like scaffold degradation and (2) their preparation often involves microfabrication techniques that afford exquisite control over biochemical properties at the molecular scale⁶⁴. In this section, I explore some of the work that has been done in the past five years to develop tunable and scalable, synthetic polymer and peptide scaffolds (**Fig. 1.5**):

Polymer Scaffolds | Synthetic and semi-synthetic materials that are used as hPSC scaffolds are typically derived from a handful of natural polymers like hyaluronic acid (HyA)⁸⁶, synthetic polymers like polyethylene glycol (PEG)¹³⁹ and polyesters like poly(lactic acid) (PLA) or poly(lactic-*co*-glycolic acid) (PLGA)¹⁴⁰. These materials can be decorated with active functional groups and polymerized or cross-linked to develop synthetic polymer-based coatings or bulk materials for hPSC expansion. In recent years, acrylate based polymers have been particularly promising scaffold systems because of acrylate monomer diversity and their capacity to undergo polymerization in the presence of UV/ozone. One such material, poly[2-(methacryloyloxy)ethyl dimethyl-(3-sulphopropyl) ammonium hydroxide or PMEDSAH was shown to support 2D hPSC expansion for more than ten passages in mTESR1⁵⁹. Using similar photopolymerization of acrylate and styrene groups, Irwin and colleagues showed that aminopropylmethacrylamide (APMAAm) could be formed to support and maintain 2D pluripotent stem cultures for over 20 passages in mTESR1¹⁴¹. Unfortunately, however, the biological effect associated with APMAAm surfaces could largely be attributed to the adsorption of bovine serum albumin (BSA) from the medium – making it difficult to utilize and scale these polymer coating in low-protein culture mediums.

Ultimately, to scale stem cell cultures to meet therapeutic demands, the polymer scaffolds that are generated must support 3D culture as well. In the past few years, many of the same acrylate- and

vinyl-mediated chemistries used in 2D culture have also been used to cross-link PEG- and natural polymer-modified 3D scaffolds to produce materials with a range of mechanical stiffnesses, degradation properties and biochemistry⁶⁴. While these bulk materials have shown enormous initial promise in maintaining and scaling hPSC culture, much work exists to make these polymer systems amenable to high-throughput culture and passaging. This includes the incorporation of enzyme-sensitive peptides to mediate degradation, adhesive peptides to enhance cell attachment in 3D and thermoreversible components to aid in passaging.

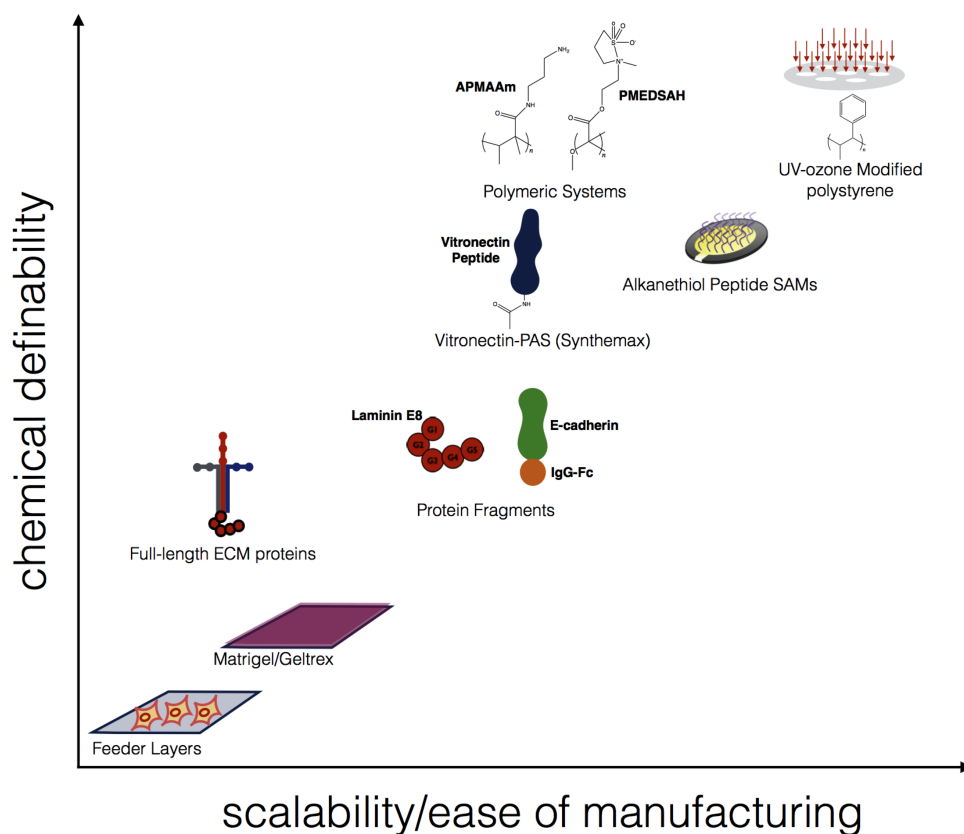


Figure 1.5: Definability and Scalability of hPSC Growth Substrates. A wide-range of growth substrates have emerged over the last two decades to support *in vitro* hPSC culture. On one end are poorly defined, poorly scaled substrates like mitotically-inactivated feeder layers (MEFs) and animal-derived ECM products. At the other end of the spectrum are well-defined, highly scalable polymer systems like aminopropylmethacrylamide (APMAAm) and chemical modification schemes such as UV/ozone treatment. While these later systems are highly scalable, they lack important bioactive motifs. A range of protein- and peptide-based scaffolds, that are both scalable, definable and bioactive, exist as well and employ a variety of –thiol and –amine mediated conjugation schemes to present adhesive motifs for stem cell culture.

Peptide-based Scaffolds and Substrates | Since many synthetic or semi-synthetic polymers lack the types of bioactive motifs found on natural polymers, there has been a simultaneous push to develop strategies for conjugating bioactive peptides to 2D polymer surfaces or 3D bulk materials. Acrylate-based chemistries, commonly leveraged for their tunable physical properties, have proven to be popular for peptide conjugation as well. Melkounian and colleagues, for example, were able to deposit carboxylic acid-containing acrylate groups onto tissue culture plastic (TCPS) and conjugate various RGD-amine peptides to this acrylate through 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide/*N*-hydroxysuccinimide (EDC/NHS) chemistry¹²³. Of the five peptide-acrylates or PAS surfaces generated, only two – full-length bone-sialoprotein (BP)-PAS and vitronectin (VN)-PAS – were able to support the long-term self-renewal of hPSCs, suggesting that short RGD sequences in isolation are likely insufficient for optimal PAS-stem cell interactions. The success of these two surfaces at both maintaining pluripotency and promoting colony expansion has led to their adaption as a commercial product – Synthemax^{123,142}. Along with StemAdhere¹⁴³, another substrate derived from full-length proteins, Synthemax has quickly captured the market as a synthetic alternative to Matrigel. Nevertheless, as suggested by Celiz *et al.* in their excellent review on materials for hPSC culture, both Synthemax and StemAdhere use recombinant protein components – making them more expensive than many natural scaffolds and prohibitively so for clinical scale-up¹²⁷.

1.4 Strategies for the Generation of Novel, Targeted Biomaterials

While marked improvements on natural materials, the synthetic and semi-synthetic hPSC culture systems surveyed above continue to present important challenges in terms of scalability, cost and effectiveness. Consequently, it is important that we broaden our search for materials that can support hPSC expansion and differentiation by mining the polymer and protein space for biologically functional “gems”. In many cases, limited mechanistic understanding of how these materials might mediate cell/surface interactions has made concerted, rational explorations of novel peptide and polymer candidates challenging. Increasingly, however, rational polymer or peptide design can be complemented with blind or “top-down” approaches to more efficiently probe and characterize new synthetic materials. This section explores some of the medium and high-throughput methodologies being used to individually and combinatorially screen material candidates that promote hPSC expansion.

Rational Design Strategies: “A Bottom-Up Approach”

Rational design – wherein a novel yet desired functionality is engineered into a known polymer or protein via the addition of orthogonal chemical groups – is a widely-utilized approach for developing new biologics, particularly protein vectors, small molecules and biomaterials. For years, such iterative strategies have been used in low-throughput to select bioactive motifs from full-length ECM proteins and tune the biophysical properties of bulk materials. However, with the exquisite control afforded by lithographic techniques and microarray platforms, we now have the power to evaluate and modify even larger numbers of putative material substrates for hPSC culture in parallel¹²⁷. Derda and colleagues were able to leverage one such microarray platform to spot-print self-assembled monolayers (SAMs) of eighteen thiolated laminin fragments onto a gold coverslip¹⁴⁴. Of these epitopes, only three – RNIAEIIKDI and TSIKIRGTYS from the γ -chain and LGTIPG from the β -chain – were able to support H1 and H9 hESC expansion over a single passage.

SAMs, like the ones from Derda *et al.*’s work, can be easily generated to activate specific cell surface receptors with a high spatial resolution. These two traits – ease of manufacturing and spatial control of biological function – make SAM surface modifications excellent tools for rapid ‘prototyping’

of rationally-designed peptide candidates. Recently, Klim and colleagues have used this same technique to generate eighteen bioactive peptide-alkanethiols (ATs) derived from both known protein-binding sequences and mined glycosaminoglycan sequences¹²⁶. Using a spot printing technique that was similar to that of Derda *et al.*, they were able to combine these ATs at varying surface densities to generate over 500 unique SAM surfaces for short-term microculture of hESCs and iPSCs. Their screen identified a single binding peptide from vitronectin (Vn), GKKQRFRHRNRKG, that could support the self-renewal of hPSCs when combined with ROCKi and a cyclic RGD peptide. Ultimately, however, SAMs are physically unstable under biological conditions¹⁴⁵, which limits their utility as long-term, scalable platforms for hPSC self-renewal and differentiation.

From this standpoint, the rational design and screening of polymer materials has produced more stable and scalable candidates for stem cell culture. More specifically, in work by Brafman and colleagues, 91 pre-designed and defined polymers were printed on microarrays and used as culture surfaces for two hESC and a single iPSC line¹⁴⁶. One polymer candidate from their screen, poly(methyl vinyl ether-alt-maleic anhydride) (PVE-alt-MA) proved capable of supporting the long-term propagation of these three undifferentiated hPSC lines for five passages.

High-throughput Materials Discovery: “A Top-Down Approach”

Polymer materials like PVE-alt-MA as well as peptide candidates like the Vn-derived GKKQRFRHRNRKG sequences represent the power of rational design approaches to comb extant biomaterials and ECM proteins for functional hits. However, given our limited mechanistic understanding of how hPSCs bind and interact with their substrates, these rationally designed or screened sequences likely represent only a fraction of the materials that could support hPSCs if synthesized. To sift through a wider swath of potential as-yet-synthesized materials, it can help to go “blind” and leverage high-throughput approaches.

On-slide synthesis of polymer microarrays, developed a little over a decade ago, has been one of these “blind” approaches to probe the functionality of polymers that may have been overlooked in rational design¹⁴⁷. Since its advent, this strategy has been used to contact-print and screen 496 unique materials derived by mixing sixteen acrylate “major” monomers and six “minor” monomers at random and polymerizing these mixtures through exposure to UV/ozone¹²⁰. As with most “top-down” approaches, the majority of these polymerized surfaces had physical properties – i.e. wettability, indentation elastic modulus, etc. – that were not conducive to 2D hPSC colony formation and expansion. However, the unbiased approach to synthesis does allow for patterns to be observed between major-minor monomer pairings that could be valuable for future rational design.

Aside from in situ high-throughput synthesis, another important family of techniques for novel biomaterial discovery is surface display¹⁴⁸. Historically used to pan antibody and antibody fragments, surface display strategies revolve around the unique presentation of 10⁸ or more peptide and protein fragments on the surface of bacteria¹⁴⁹ or phage¹⁵⁰. These peptide-displaying phage or bacteria can then be successively panned against a cellular or molecular target to identify high-affinity peptide binders. One of the first attempts to use peptide surface display strategies to identify ECM-mimics for a stem cell target was performed by Little *et al.*¹⁵¹. Using bacterial display, they screened two libraries, each composed of 10¹² cyclic or linear 15mer peptides, for their capacity to bind adult neural stem cells (NSCs). Although fewer than 1% of displayed peptides showed any affinity for NSCs, Little and colleagues were able to iteratively pan and identify a single cyclic sequence, WWCDMRGDSRCSG, that bound NSCs with high avidity. Synthetic analogs of this sequence could subsequently be grafted onto interpenetrating polymer network (IPN) to generate a scalable culture platform for the expansion of NSCs *in vitro*. We have since adapted this technique within our group

to identify novel adhesion peptides for hESCs and engineer their presentation within scalable polymer systems. The succeeding chapters describe some of our efforts in this space and highlight the unique advantage of using unbiased, high-throughput strategies for applications in biomaterials engineering.

1.5 Dissertation Scope and Outline

Developing synthetic materials for human pluripotent stem cell (hPSC) expansion has been a long-standing challenge in regenerative medicine. In this body work, I have used unbiased selection strategies to identify peptide mimetics for Lm-511 – an important component of hPSC extracellular matrices (ECM)^{58,121}. Using bacterial display and microculture techniques, we panned massive peptides libraries to identify high-affinity binders of the Lm-511 receptor, $\alpha_6\beta_1$ integrin. Through thiol-mediated chemistries, these peptides can be self-assembled into monolayers or functionalized onto thermoreversible biopolymers to generate 2D and 3D hPSC culture systems. By outlining an unbiased strategy for generating viable synthetic ECM for hPSC self-renewal, this work could represent an important advance in how we leverage unbiased strategies to generate novel biomaterials for other cellular engineering applications.

This dissertation is organized into four sections describing two distinct aims (Chapters 2 and 3). A concluding chapter (Chapter 4) outlines the main findings from the dissertation and highlights possible future directions for this project:

- **Chapter 2: Identification & Characterization of Novel α_6 -integrin Adhesion Peptides.**
In this chapter, we describe how bacterial display was used to identify ligands that bind α_6 integrin in hESCs. In conjunction, we outline the biophysical methods used to characterize candidate peptides, particularly their avidity for hESCs and their ability to maintain short-term hESC adhesion. Finally, this chapter describes the high-throughput microculture, surface modification and imaging platforms used to identify peptides candidates that could be presented alongside other canonical binding motifs to create a nascent hPSC culture surface.
- **Chapter 3: Development of 3D Peptide-presenting Polymer Systems for hPSC Culture.**
In this chapter, we describe how optimized peptide combinations identified in Chapter 2 have been leveraged to create a scalable, thermoreversible polymer-peptide system for long-term hPSC culture. In doing so, we outline the synthesis strategies currently used to generate the polymer backbone, the chemistry used to graft selected peptides to these backbones and the characterization strategies employed to assess the chemical and mechanical properties of these peptide-polymer conjugates. Finally, we show that the presence of peptides within these polymer systems significantly enhances their capacity to support undifferentiated hPSC expansion.
- **Chapter 4: Novel Strategies & Applications for Peptide & Small Protein Screening.**
This final chapter summarizes the dissertation’s main findings and explores possible next steps for using unbiased strategies to generate peptide materials for stem cell and other cellular engineering applications.

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Chapter 2: Identification & Characterization of Novel α_6 integrin Adhesion Peptides

2.1 Abstract

Human pluripotent stem cell (hPSCs) provide a theoretically unlimited source of cells for applications in regenerative medicine. However, before hPSCs can meet their therapeutic potential, synthetic yet scalable substrates that promote their faithful expansion *in vitro* are needed. Unfortunately, current synthetic substrates for hPSCs either lack bioactive motifs or rely on a subset of peptides that promote limited self-renewal. To reliably maintain hPSC pluripotency, synthetic materials targeting a wider class of surface receptors are required. Until now, the *de novo* development of such new materials has been hindered by our limited mechanistic understanding of stem cell receptor-ECM interactions.

Here, we overcame this challenge by using an unbiased bacterial display strategy to identify peptide mimics for the multimeric Lm 511 – a key component of current culture systems and a ligand for the highly expressed hPSC surface receptor, $\alpha_6\beta_1$ integrin. Using hESCs as a bait, vast libraries of short, random peptides were panned to identify sequence-similar variants that bind α_6 integrin. Biophysical characterization of adhesion via flow cytometry and centrifuge-based assays suggested that several peptides bind hPSCs with sub-micromolar K_d s similar to full-length laminin. We then harnessed a high-throughput microculture and imaging platform to identify peptides in this subset that could be presented alongside other canonical integrin- and syndecan-binding motifs to create a nascent hPSC culture surface. When presented as 2D SAMs, optimized peptide combinations supported the culture of both hiPSCs and hESCs over a single passage. Our results highlight how unbiased screening strategies can be used to identify novel, receptor-specific adhesion peptides that support the short-term and possibly long-term culture of hPSCs.

2.2 Introduction

Given their capacity to differentiate and form tissue from all three germ layers, human embryonic (hESCs) and induced pluripotent stem cells (hiPSCs) hold enormous potential as customizable cell sources for cell-replacement therapies¹, human disease modelling and drug discovery². However, such clinical applications often require the reliable, large-scale *in vitro* expansion and lineage-specific differentiation of hPSCs – a need that current, xeno-derived culture systems do not readily accommodate^{3,4}. While defined, xeno-free culture mediums have become a mainstay in standard-scale hPSC culture^{5,6}, feeder-free substrates continue to rely on ill-defined, animal-derived proteins. The wide-spread use of one such natural scaffold material, Matrigel, presents several unique challenges that hinder the clinical translation of pluripotent stem cell-based therapies⁷. Derived from the decellularized matrix of Engelbreth-Holm-Swarm sarcomas in mice, Matrigel is a complex mixture of growth factors, ECM proteins and proteoglycans that are loosely defined and, as such, susceptible to batch-to-batch variability in composition⁷. Furthermore, as with many cell-derived materials, Matrigel represents a potential source of endogenous pathogens and xenoepitopes that can induce an immune response if inadvertently introduced alongside transplanted hPSCs *in vivo*⁸.

To sidestep the translational challenges associated with cell-derived matrices, individual extracellular matrix proteins that mimic the functionality of Matrigel have been used as substrates for

hPSC self-renewal. Of these, three isoforms of the heterotrimeric laminin, the principal component of Matrigel – laminin-111, laminin-511, and laminin-332 – have been shown to activate the highly expressed hPSC surface receptor, $\alpha_6\beta_1$ integrin, and support the undifferentiated expansion of hESCs *in vitro*^{9,10}. Although Lm-511 and fragments derived from it display adhesive properties on par with that of Matrigel under xeno-free culture conditions^{9,11}, such recombinant proteins come with numerous disadvantages. For one, recombinant versions of full-length ECM proteins can be tedious and expensive to produce^{12,13}. Perhaps more importantly, however, even single isoforms of ECM proteins have multiple adhesive domains that can independently and simultaneously engage with numerous stem cell surface receptors¹⁴. Thus, it can often be challenging to both identify key receptor-mediated interactions and reengineer reproducible hPSC culture conditions using these substrates *in vitro*.

In this work, we instead sought to identify short, scalable peptide ligands of $\alpha_6\beta_1$ integrin that were capable of supporting hPSC self-renewal under fully defined culture conditions. Unlike full-length ECM proteins, small, synthetic peptides offer important advantages. By virtue of their size, peptide mimics often contain a single, minimally bioactive domain that can initiate receptor signaling in precise and reproducible ways to generate homogenous stem cell cultures^{14–17}. Although a number of adhesive peptide motifs – including the ‘RGD’ domain from fibronectin^{17,18}, ‘IKVAV’ domain from laminin¹⁹ and novel glycosaminoglycan binders¹⁴ – have been identified through “rational design” approaches, none of these motifs have been able to specifically activate the key hPSC surface receptor, $\alpha_6\beta_1$ integrin.

Library-based screening and selection strategies, however, represent a method by which new peptide ligands of integrins – particularly those that remain poorly understood – can nevertheless be discovered. Unlike rational design approaches, library-based strategies like bacterial peptide display allow multiple peptides with random sequences to be simultaneously panned for ones that bind a target cell type or receptor without a prior understanding of the motifs required for receptor engagement^{20,21}. Furthermore, unlike phage and other display techniques, bacterial display eliminates the need for viral infection or complex enzymatic steps and can be analyzed through simple plasmid isolation and sequencing or quantitatively through flow cytometry²². In this work, we couple an unbiased bacterial display strategy with previously used microculture and high-content imaging platforms to identify novel peptide binders of $\alpha_6\beta_1$ integrin and synergistic peptide co-regulators of hPSC adhesion. In doing so, we show that high-throughput screening strategies offer unprecedented advantages in materials discovery and substrate optimization for short-term adhesion and expansion of undifferentiated hPSCs.

2.3 Materials and Methods

2D Human Pluripotent Stem Cell Culture

Human ESCs (WA01, Wicell, Madison, WI) and a previously described adipose stem cell-derived hiPSC line²³ were cultured at 37°C and 5% CO₂ in mTESR1 cell-culture medium (StemCell Technologies, Vancouver, Canada) on tissue culture surfaces precoated with hESC-qualified Matrigel (BD Biosciences, San Diego, CA). For routine passaging, cells underwent either mechanical selection and scraping or enzyme-free dissociation using ReLeSR (Stem Cell Technologies) followed by seeding on fresh Matrigel-coated surfaces in mTESR1 medium supplemented with 10 μ M Y-27632 (StemCell Technologies). Both the H1 hESC and the iPSC cultures were periodically tested for mycoplasma contamination via PCR.

Quantification of Pluripotency Marker Expression in hPSCs

For immunofluorescence staining of various intracellular pluripotency markers, human ESC and iPSC colonies plated in 6-well tissue culture plates (Corning, Corning, NY) were gently dissociated using mechanical selection and scraping, centrifuged for 5 min. at $300 \times g$, and permeabilized using a transcription factor staining kit, Mouse/Human Pluripotent Stem Cell Multicolor Flow Cytometry Kit (R&D Systems, Minneapolis, MN), according to the manufacturer's instructions. In short, each cell pellet was resuspended at 5×10^5 cells in 500 μ L Fixation/Permeabilization Buffer and incubated at 4°C for 30 min before repelleting. Cell pellets were subsequently washed with 100 μ L of Permeabilization/Wash Buffer, labeled with 2 μ L of a fluorochrome-conjugated antibody for Oct3/4, Sox2, SSEA4 or the corresponding isotype control and incubated at 4°C for 30 min. Following incubation, 2 mL of Permeabilization/Wash Buffer was added to each sample and samples were centrifuged at $300 \times g$ for 5 min. at 4°C. The obtained cell pellets were resuspended in 300 μ L of the Phosphate Buffered Saline (PBS) (Corning) and subjected to flow cytometric analysis on a Beckman Coulter FC 500 Analyzer (Beckman Coulter, Indianapolis, IN). All raw data was analyzed using the FlowJo Software Package (FlowJo LLC, Ashland, OR).

Bacterial Peptide Display Libraries & Untargeted Peptide Selections

Bacterial Peptide Display Libraries were previously generated in MC1061 *E. Coli* using a single pBAD33 plasmid containing alajGFP – a jellyfish-derived GFP variant engineered for high expression in *E. Coli* – and a CPX protein fused at the exteriorized N-terminus to short, peptide sequences²⁰. CPX is a circularly permuted variant of the barrel-shaped surface protein, OmpX, in which the former L2 loop within the protein is opened to expose a new N- and C- terminus to the exterior of the cell. Peptides fused to the N-terminus of the CPX protein were of two varieties – either linear 15mer sequences of the form X₁₅ (15mer) or cyclized 7mer sequences of the form X₂CX₇CX₂ (7C). Both the alajGFP and CPX-peptide fusion genes were expressed downstream of an arabinose-inducible promoter allowing for the regulation of surface peptide expression²⁰. In this work, three libraries were utilized: (1) a library composed entirely of 7C peptide variants, (2) a library expressing solely linear, 15mer peptides and (3) a library initially composed equally of 15mer- and 7C-expressing clones.

Untargeted selections were performed in four rounds as previously described²⁰. In brief, during each round of selection, frozen stocks of the library were grown overnight in TB media supplemented with 34 μ g/mL of chloramphenicol (Sigma-Aldrich, St. Louis, MO) and 0.2% D-glucose (Sigma-Aldrich) at 37°C. The library was then subcultured 1:50 in TB media supplemented with 34 μ g/mL of chloramphenicol at 37°C. After two hours, library cultures were induced at 30°C through the addition of 0.02% L-arabinose (by volume) (Sigma-Aldrich) to initiate the expression of alajGFP and CPX-peptide fusions. During the induction of surface peptide expression, target cells or H1 hESCs were gently dissociated via exposure to 2mM Na₂EDTA (ThermoFisher Scientific, Waltham, MA) in PBS at 37°C for 15 min. and subsequent mechanical selection and scraping. These singularized hESCs were then co-incubated with library bacteria in a bacterial shaker for one hour in mTESR1 media supplemented with 10 μ M Y-27632.

To generate the selective pressure needed to enrich for tight binders, the size of the bacterial population used in selection was reduced with each round. In the initial round of screening, 100-fold more bacteria than hESCs were used while in subsequent rounds, selective pressure for binding was increased and only 50-fold more bacteria were present during selection. Following selection, bacteria-bound hESCs were pelleted via centrifugation at $1000 \times g$ for 5 min. and washed with PBS until the

supernatant had little phenol red-containing media and low turbidity (an OD less than 0.03). The resulting pellet was resuspended in LB media supplemented with 34 μ g/mL of chloramphenicol and 0.2% D-glucose and cultured overnight as both liquid culture, to generate sizable bacterial populations for further rounds of selection, and a solid culture for library characterization. For the third through fifth rounds of selection, washed hESC pellets were resuspended in PBS and subjected to FACS analysis (BD Influx, BD Biosciences, San Jose, CA) to identify bacteria-bound hESCs. Generational and clonal analyses were also performed via flow cytometry (BD LSR Fortessa X-20, BD Biosciences) after panning 10⁸-member library and clonal populations with hESCs. All raw data was analyzed using the FlowJo Software Package (FlowJo LLC, Ashland, OR).

Targeted Peptide Selections for α_6 integrin

Targeted selections were performed by incubating singularized hESCs with 10 μ g/mL of blocking antibodies for the α_6 integrin subunit during a fifth and final round of negative and positive selection. After this initial incubation with the blocking antibody at 4°C, antibody-blocked hESCs were pelleted and co-incubated with the bacterial library of interest for one hour at 4°C in mTESR1 containing 10 μ M Y-27632 and 1 μ M phenylarsine oxide (PAO) (Sigma-Aldrich) – a small molecule endocytosis inhibitor²⁴ that prevents the internalization of blocking antibodies during negative selection. The supernatant containing bacteria that did not bind to the antibody-blocked hESCs was subsequently removed and incubated with hESCs that were not exposed to antibody-mediated blocking of surface integrins - thereby identifying a subset of peptide-expressing bacteria that specifically bind via α_6 integrin. During this positive selection, the samples were incubated at 37°C for one hour on a bacterial shaker and subsequent washing and sorting were conducted as described for untargeted peptide selections.

Recombinant Peptide-GFP Fusion Protein Production

Individual peptide sequences identified as tight binders through untargeted and targeted selections were fused to *alajGFP* to generate peptide-GFP fusion constructs. These constructs were cloned into a pBAD33 vector and subsequently transformed into DH10B Competent *E. Coli* (ThermoFisher Scientific) and grown as individual clones on a chloramphenicol background. Peptide-GFP constructs, schematized in **Figure S1 in Appendix I**, consisted of the identified 15mer peptide sequence at the 5' end of the fusion construct followed by a short, flexible glycine-serine linker that joined the peptide to *alajGFP*. The 3' end of the fusion construct also contained a TEV cleavage site and 6X His tag to aid with the purification of the fusion protein after expression. As with peptide display on the bacterial surface, the expression of these peptide-GFP constructs was regulated by an arabinose-inducible promoter. To induce the expression of fusion proteins, individual DH10B clones were selected, grown for three hours in TB media supplemented with 34 μ g/mL of chloramphenicol at 37°C and induced at 30°C through the addition of 0.02% L-arabinose (by volume). After induction, these DH10B cultures were pelleted, frozen and lysed via sequential sonication in a sodium phosphate buffer. The lysed solution was passed through Ni-NTA agarose (Qiagen, Germantown, MD) gravity flow column to elute the recombinant peptide-GFP fusion proteins using an imidazole gradient. Protein solutions were concentrated more than two-fold and buffer exchanged with 10% glycerol/PBS using 10kDa NWML Amicon Ultra-15mL Filter (EMD Millipore) as per manufacturer's instructions. Purified and concentrated protein solutions was sterile filtered and qualitatively assessed

via SDS Page (**Figure S1**). Protein concentration were also determined using a BCA assay (ThermoFisher Scientific).

Centrifugation-Mediated Cell Adhesion Assay

Various peptide-GFP fusion proteins, diluted to a concentration of 10 $\mu\text{g}/\text{mL}$, were immobilized onto sterile Ni-NTA HisSorb Plates (Qiagen) according to manufacturer's instructions in a honeycomb pattern as described in **Figure 3**. Once the proteins were immobilized in the wells, wells were washed several times with a mild surfactant, PBS supplemented with 0.1% (v/v) Tween-20 (Bio-Rad, Hercules, CA), and allowed to dry. Wells with immobilized proteins were then seeded with H1 hESCs at 10,000 cells/well under standard conditions and cells were allowed to adhere to the protein surface for two hours. Plates were then subjected to a multispeed adhesion assay (200, 600, 1000, 2500, and 4000 rpm; ~ 6 to $2560 \times g$), modeled after methods by McClay and colleagues²⁵ and described in previous work from our group^{26,27}. Post-assay, the number of cells that remained adhered to the protein-Ni-NTA surface was quantified fluorescently via Cyquant (Molecular Probes, Eugene, OR) as described in the manufacturer's protocol. Plates were read using a Spectramax i3x (Molecular Devices, Menlo Park, CA; excitation/emission/cutoff 485/538/530nm).

Strength of Binding Quantification via Flow Cytometry

Cell surface heparan sulfate has a non-specific affinity for 6xHis tags within recombinant proteins²⁸. To remove heparan sulfate from the surface of hESCs, these cells were dissociated into single cells as previously described and incubated for 1.5 hours in a 20mM Tris-HCl (Sigma-Aldrich) and 7mM CaCl_2 (Sigma-Aldrich) buffer (pH 7.5) supplemented with 10 sigma units/mL heparinase III (Sigma-Aldrich) and 0.1 mg/mL bovine serum albumin (BSA) (Sigma-Aldrich). After incubation, the cells were pelleted, resuspended in a low-protein hPSC culture medium, TESR-E8 (Stem Cell Technologies), and aliquoted at 50,000 cells/well into a low-adhesion microwell plate (Corning). To each well containing cells, unique peptide-GFP fusion proteins was added at one of eight final concentrations (0 $\mu\text{g}/\text{mL}$, 0.5 $\mu\text{g}/\text{mL}$, 2.5 $\mu\text{g}/\text{mL}$, 5 $\mu\text{g}/\text{mL}$, 10 $\mu\text{g}/\text{mL}$, 25 $\mu\text{g}/\text{mL}$, 50 $\mu\text{g}/\text{mL}$ or 250 $\mu\text{g}/\text{mL}$ of protein). The hESCs were allowed to incubate with the protein solution for 1 hour at 4°C on a bacterial shaker to maximize binding events. Following incubation, samples were pelleted via centrifugation at 1000 $\times g$ for 5 min. and washed with PBS several times to minimize the presence of phenol-red containing medium and unbound fusion protein. Samples were ultimately resuspended in 300 μL of the Dulbecco's Phosphate Buffered Saline (DPBS) (Corning) and subjected to flow cytometric analysis for GFP fusion protein-bound hESCs on a BD LSR Fortessa X-20. All raw data was analyzed using the FlowJo Software Package and processed data was fit to a nonlinear one-site, specific-binding Hill Function using GraphPad Prism (GraphPad Software Inc., La Jolla, CA) to calculate dissociation constants, K_D .

Peptide Conjugation and Presentation Strategies

Several strategies were employed to present variations of a FITC-conjugated model peptide, CGGNGEPRGDTYRAYK-(FITC) (Thiolated bspRGD(15)-FITC, United Biosystems Inc., Herndon, VA), on tissue-culture polystyrene surfaces including polyphenol-thiol conjugations, biotin-avidin mediated coupling and thiol-gold monolayer generation. Methods described by Barber *et al.* were used to measure the efficiency of peptide conjugation²⁹. All peptide conjugation was performed

in sterile hood to allow surfaces to remain amenable for cell culture. To generate physically adsorbed peptide surfaces, thiolated bspRGD(15)-FITC was dissolved at one of four concentrations (200 nM, 600 nM, 1 μ M or 1 mM) in synthesis grade water, pipetted onto wells of a 96-well plate, and allowed to adsorb for three hours at room temperature.

Polyphenol-Mediated Peptide Conjugation | For polyphenol-mediated conjugations, the phenolic precursor, pyrogallol (Sigma-Aldrich), was freshly dissolved at 2mg/mL in pH 7.8, 100 mM bicine buffer with 600 mM NaCl. The phenolic solution was then sterile filtered, pipetted into individual wells of a 96-well plate and allowed to polymerize on the PS surface under mild agitation at room temperature. Following modification, wells were washed several times with sterile, ultrapure water (Corning) and dried under a sterile nitrogen stream. Incorporation of thiolated bspRGD(15)-FITC was achieved by incubating polyphenol-modified surfaces in either 200 nM, 600 nM, 1 μ M or 1 mM peptide solution in sterile pH 7.8 PBS for 2 hours at 4°C. Surfaces were subsequently washed with sterile PBS.

Neutravidin-Biotin Coupling | Conjugations of Biotin-CGGNGEPRGDTYRAYK-(FITC) (Biotin-bspRGD(15)-FITC, Bachem Americas Inc., Torrence, CA) to Pierce NeutrAvidin Coated 96-well plates was performed according to manufacturer's instructions using either 200 nM, 600 nM, 1 μ M or 1mM biotin-bspRGD(15)-FITC solutions in sterile pH 7.4 PBS. Plates were incubated in the biotinylated peptide solution for 2 hours at 4°C before being washed with sterile PBS.

Thiol-mediated Peptide Conjugation to PD/CMC Surfaces | To conjugate thiolated bspRGD(15)-FITC to polydopamine/carboxymethyl chitosan (PD/CMC) surfaces, PD/CMC surfaces were first prepared as described by Zhou *et al.*³⁰. Incorporation of thiolated bspRGD(15)-FITC was achieved through standard NHS/EDC chemistry. In brief, PD/CMC modified surfaces were exposed to sterile activation solution of 2mM EDC (Alfa Aesar, Tewksbury, MA) and 5mM NHS (Sigma-Aldrich) in 0.1M MES buffer (pH 5.6) (Sigma Aldrich) for 40 min. at room temperature. Activated PD/CMC wells were then washed with sterile DPBS several times and dried under vacuum. Six different concentrations of thiolated bspRGD(15)-FITC peptides (200 nM, 600 nM, 1 μ M or 1mM) were then dissolved in sterile DPBS and added to each freshly activated PD/CMC-modified well for incubation at 4°C overnight. Peptide-conjugated wells were subsequently washed with sterile DPBS to remove any unbound peptide.

Thiol-mediated Peptide SAM Formation | For the generation of thiol-mediated SAM surfaces, either 8-well glass chamber slides or a 532-pillar polystyrene microchips (PillarChip) (SEMCO, Vista, CA) were sputter-coated with a thin layer Au/Pd (150Å of gold-, 20Å of palladium) using Denton-382 SEM Coater (Nanoshift LLC, Berkeley, CA). To fabricate alkanethiol (AT) or thiol peptide SAMs, slides were washed with sterile, synthesis-grade water and exposed overnight at 4°C to 200 nM, 600 nM, 1 μ M or 1 mM solutions of either thiolated bspRGD(15)-FITC or an 11-mercaptopundecanoic acid-modified variant, (11-MUA)- CGGNGEPRGDTYRAYK-(FITC). All peptides were dissolved in sterile, synthesis-grade water. Following assembly of peptides SAMs via N-terminal thiols and 11-MUA, surfaces were washed several times with sterile water to remove any unbound peptide.

AT-peptide SAMs on PillarChips were generated in much the same way as thiol SAMs on glass slides. Three linear 11-MUA adhesion peptides (Genscript, Piscataway, NJ) were synthesized via standard Fmoc Chemistry on a peptide synthesizer by an outsider vendor: (11-MUA)-GGNGEPRGDTYRAYK-NH₂ or 11-MUA-bspRGD(15), (11-MUA)-GKPLRAKREITKLKFG-NH₂ or 11-MUA-Linear (15-23), (11-MUA)-GGRKLKRVQLSIRT-NH₂ or 11-MUA-Ag73. A cyclic

11-MUA adhesion peptide (CPC Scientific Inc., Sunnyvale, CA) was also synthesized via standard Fmoc chemistry and cyclized via a lactam bond between Asp(1) and Lys(9) to form (11-MUA)-GG-PEG₂-cyclo(DMGDGRPRK)-NH₂ or 11-MUA-Cyclic (7C-1). To generate AT-SAMs on individual pillars of the PillarChip, 850 nL of a 1 mM alkanethiol peptide or peptide combination (in sterile water) was spot-printed in a corresponding 532-well polystyrene microchip (WellChip) (SEMCO) in accordance with schematic shown in **Fig. 2.6D**. Wells were also printed with prediluted Matrigel, Synthamax (Corning) and mouse laminin protein (100 µg/mL) (ThermoFisher Scientific) as per manufacturer's instructions. Gold-coated PillarChips, sterilized via incubation in 75% ethanol, dried under a sterile nitrogen stream and treated for an hour with UV/ozone, were stamped into the printed WellChip. WellChip-PillarChip setups were stored in a humidified chamber at 4°C overnight to aid in SAM formation.

Materials Characterization of Peptide SAMs

SAM formation was confirmed by both X-ray photoelectron spectroscopy (XPS) and quartz crystal microbalance and dissipation monitoring (QCM-D). QCM-D experiments were carried out on a Q-sense Omega instrument (Biolin Scientific AB/Q-Sense, Västra Frölunda, Sweden). Gold-coated quartz sensors, with a fundamental frequency of 5 MHz, were obtained from Q-Sense (Biolin Scientific AB/Q-Sense). Since the Q-Sense Omega has four sets of independently controlled flow modules, four real time, simultaneous measurements of frequency and dissipation changes associated with the formation of different AT-SAMs or proteins surfaces were made. A peristaltic pump was used to control the flow rate of sample and measurements were made at the fundamental frequency of the sensor crystal (5 MHz) as well as the third, fifth, seventh, 11th and 13th overtones of this frequency. All measurements were started with ultrapure water, introduced at 50 µL/min, to establish a stable baseline on a clean sensor. Once established, solutions of peptide-ATs, Synthamax or Matrigel were introduced at a flow rate of 5 µL/min for 3 hours to allow for the formation of stable AT-peptide SAMs or protein coated surfaces. When frequency and dissipation changes had stabilized, the surfaces were rinsed once more with water to measure the amount of irreversibly adsorbed peptide or protein as well as its viscoelastic properties. Data was analyzed on QTools using the Voigt viscoelastic model.

SAM samples generated via QCM-D were dried under vacuum and subjected to XPS analysis using a PHI 5600 spectrometer (PerkinElmer, Waltham, MA) equipped with an Al monochromated 2 mm filament and a built-in charge neutralizer. The X-ray source operated at 350 W power, 14.8 V voltage, and 40 °C take-off angle. Survey scans were performed between 0 and 1100 eV electron binding energies. High resolution spectrum of the C1s, O1s and N1s regions were obtained between x-x, y-y, 395 and 410 eV respectively. Charge correction was performed by setting the C1s peak at 285.0 eV. Data analysis was conducted using MultiPak software version 9.6.015.

High-Throughput Microculture & High-content Imaging of hPSCs on Peptide SAMs

An enclosed MicroSys 5100-4SQ (Digilab Inc., Marlborough, MA) noncontact robotic microarray spotting system was used to print all surface coatings, cell suspensions and culture medium. When operated, the system maintained a relative humidity above 95% to reduce evaporation-induced effects. To prepare the microcultures, 850 nL of TESR-E8 medium (**Figure 2.6**) was printed into a fresh WellChip, covered with a gas-permeable sealing membrane (Diversified Biotech, Dedham, MA), and stored at 37°C in the incubator. Meanwhile, human ESC and iPSC colonies plated in 6-well tissue culture plates were gently dissociated using ReLeSR and mechanical scraping and selection. These cells were pelleted at 1000 × g for 5 min. and resuspended in TESR-E8 medium supplement with

10 μ M Y-27632 and 1% Penicillin-Streptomycin (ThermoFisher Scientific) at a working concentration of 10,000,000 cell/mL. The microarray system was then used to spot-print 150 nL of cell suspension onto each pillar of the previously coated PillarChip. Following printing, printed PillarChips were incubated “pillar side-up” for 30 min. at 37°C in a humidified chamber to allow cells to settle and attach to the coated surface of the pillar. Once the cells had adhered, PillarChips were stamped into the WellChip containing warmed growth medium and stored in a humidified chamber for culture. Chips were cultured for two days. Periphery wells were not analyzed to avoid the confounding effects of increased evaporation at the microchip periphery.

On-Chip Immunocytochemistry At the endpoint of the experiment, intracellular markers of interest were stained using on-chip immunocytochemistry. The PillarChip was carefully removed from the WellChip and placed it into a bath of 4% paraformaldehyde for 15 min. to fix cell cultures. The PillarChip was washed two times in PBS for 5 min. each and then placed in a bath of 0.25% Triton-X for 10 minutes to permeabilize cell membranes. Following Permeabilization, the PillarChip was washed five times in PBS for 2 min. each. To stain intracellular actin, the PillarChip was dried and then placed into a new WellChip that contained Alexa Fluor 568 Phalloidin (A22283, ThermoFisher, 1:40) for 1 hour. Then, the PillarChip was removed from the WellChip and washed in PBS twice for 5 min. each and placed into a new WellChip containing the primary antibody solution overnight. The PillarChip was then removed and washed twice for 5 min. each in PBS and placed into a new WellChip containing secondary antibodies and nuclear stain Hoechst 33342 (H3570, ThermoFisher, 1:2000) and incubated for 2 hours at 37°C. **Table S1 in Appendix I** lists the primary and secondary antibody used. After secondary staining, the PillarChip was washed twice in PBS for 5 min. each and then placed into a new WellChip containing PBS. The PillarChip was then sealed with a polypropylene film (GeneMate T-2452-1, VWR International, Radnor, PA) and stained microcultures were imaged with a 20X objective lens using a custom adapter and plate file in a Molecular Devices ImageXpressMicro (IXM) (Molecular Devices) high-throughput wide-field fluorescence microscope. Lamp exposure time was kept constant for all images.

Image Analysis Methods | Image analysis was performed using ImageJ software package (National Institutes of Health, Bethesda, MD). Both colony area and relative pluripotency were calculated for each microculture on the basis of the quantified masked area of colonies in the DAPI and OCT4 staining images. Multilayer morphology within microcultures was assessed by pseudocoloring gray-scale DAPI-stained images using a rainbow RGB (blue-red) lookup table (LUT). Thresholded pixels based on a pre-given color range could then be used to generate masks capturing regions of the culture with high intensity (i.e. multiple cell layers) versus low intensity (i.e. a single cell layer). The primary cytoskeletal parameter – the ratio of α 6 integrin to actin – was calculated by measuring the average fluorescent intensity of α 6 integrin in an imaged microculture and normalizing by the corresponding fluorescent intensity of actin. These measurements were then plotted and analyzed as a series of clustered box plots using the R software package (R Foundation for Statistical Computing, Vienna, Austria) or Python (Python Software Foundation, Delaware, USA). A hierarchical cluster dendrogram was generated in Python using the Seaborn visualization library; data were normalized using a z-score for each measurement variable (column). The Euclidean distance was used to measure pairwise distance between each observation (row) and the UPGMA algorithm was used to calculate the clusters. Principal Components were computed by singular value decomposition with the `prcomp` function the R *stats* package. The non-parametric Kruskal-Wallis test was used for statistical analysis followed by a *post-hoc* Dunn’s test to analyze specific sample pairs. $p < 0.05$ was regarded as significant.

Short-term hPSC Macrocultures on Peptide-SAMs

Tri-peptide AT-SAMs were generated by exposing gold-coated 8-well glass chamber slides to a 0.4 mM 11-MUA-Ag73, 0.4 mM 11-MUA-bspRGD(15) and 0.2 mM 11-MUA-Cyclic(7C-1) or Linear (15-23) solution overnight at 4°C as described. Following the assembly of the tri-peptide AT-SAMs, surfaces were washed several times with sterile DPBS to remove any unbound peptide. H1 hESCs and hiPSCs, previously dissociated using ReLeSR and mechanical scraping, were pelleted at $1000 \times g$ for 5 min. and resuspended in TESR-E8 medium supplemented with 10 μ M Y-27632 and 1% Penicillin-Streptomycin at a working concentration of 100,000 cell/mL. Cell suspensions were then pipetted into each well of the 8-well glass chamber slide and grown in TESR-E8 supplemented with Y-27632 and penicillin-streptomycin under standard culture conditions for five days. At the end of a single passage, cells were fixed with 4% (vol/vol) paraformaldehyde for 15 min., permeabilized with 0.2% Triton X-100 and blocked with 2% BSA, 4% donkey serum and 0.1% Triton X-100 for 30 min. The samples were then incubated with primary antibodies for 2h and secondary antibodies for 1.5 hours. A complete list of primary and secondary antibodies, vendors and dilutions used is presented in **Table S1 in Appendix I**. After incubation with secondary antibodies, samples were washed three times with DPBS prior to nuclear staining. To stain cell nuclei, 4,6-diamidino-2-phenylindole (DAPI) was used. Samples were imaged in PBS on an epifluorescent Zeiss inverted microscope (Zeiss AXIO Observer, Oberkochen, Germany) and ImageJ was used to process images.

Statistical Analysis

Unless otherwise noted, data were presented as mean $\pm$ s.d. For single comparisons, a two-sided Student's *t*-test was used. For multiple comparisons, one-way analysis of variance was used with *post hoc* Dunnett's correction. $P < 0.05$ was regarded as significant.

2.4 Results and Discussion

Identification of Novel Biomimetic Peptide Ligands for hESCs

Given the challenges of rationally designing materials that mimic the native hPSC niche, we used unbiased strategies to identify definable yet scalable adhesive protein sequences that could support hPSC self-renewal. Bacterial display, wherein small proteins can be presented on the bacterial surface for screening, has long been used to rapidly screen and evolve peptide-based vaccines and enzyme substrates with high throughput and fidelity^{21,22}. However, its application in biomaterials discovery has been limited until now²⁰. To develop *de novo* novel peptide scaffolds for stem cells, we adapted this bacterial display technique to identify both generic peptide ligands for hPSCs as well as specific ligands for prominent stem cell surface receptors as outlined in **Figure 2.1** and **Figure 2.2**. In the schema illustrated in **Figure 2.1A**, untargeted biomimetic ligand selection occurred in five discrete steps. Initially, bacterial libraries were cultured and induced to express GFP along with unique 15mer peptides presented at the N-terminus of their engineered outer membrane protein, CPX (as shown in the **inset of Fig. 2.1 A**). Expressed peptides were of two varieties: either random linear sequences (15mer), X₁₅, or looped peptides (7C), X₂CX₇CX₂, that consisted of random 7mer sequences constrained at either end by cysteine residues and cyclized by a disulfide bond (**Figure 2.1B**).

Both these cyclic and linear peptide-expressing bacteria were then panned against a target pluripotent stem cell – in this case, human embryonic stem cells – under native binding conditions.

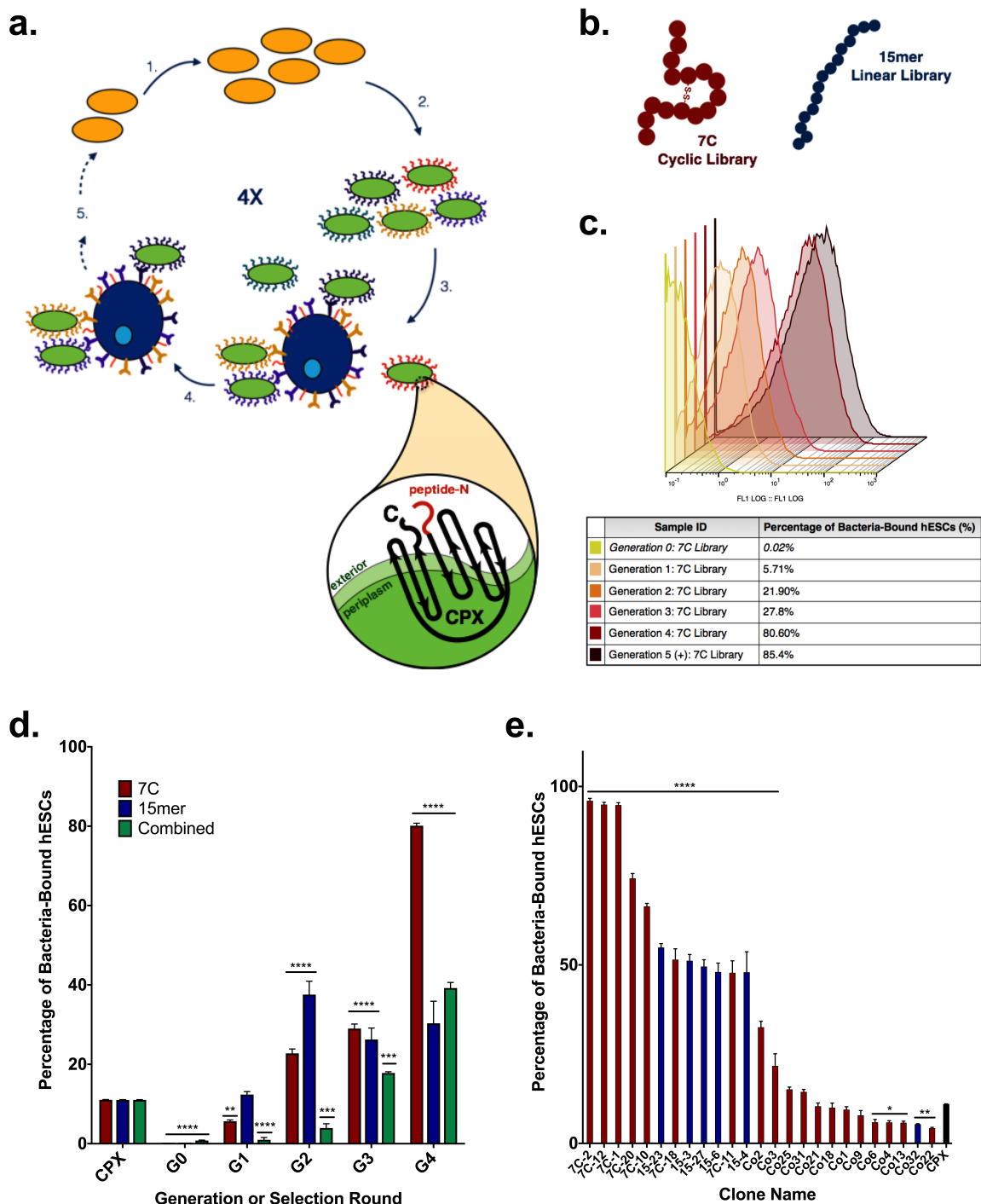


Figure 2.1: Untargeted Screening for hESC Binding Peptides. (a) The bacterial display scheme used to identify novel hESC binding peptides. Here, vast 10^{12} -member bacterial libraries were (1) subcultured and (2) induced to express alajGFP and a unique peptide variant on their surface. Each peptide, fused to the N-terminus of the circularly permuted outer membrane protein (CPX) (inset), was free to interact with receptors moieties on the surface of a target hES cell. Panning was performed by (3) incubating peptide-expressing bacteria with a target cell, (4) allowing for

binding events to occur and (5) eliminating unbound clones via centrifugation or fluorescence activated cell sorting (FACS). **(b)** Two peptide variants – cyclic peptides of the form $X_2CX_7CX_2$ and linear peptides of the form X_{15} – were affinity matured in three independent libraries – a library composed solely of cyclic peptides (7C), a library composed solely of linear peptides (15mer) and a library initially composed equally of cyclic and linear variants (Co). **(c)** A representative histogram overlay of generational 7C populations and their successively higher binding affinities for hESCs as captured by flow cytometry. **(d)** Quantitative binding analysis ($n = 3$) of each generational 7C, 15mer and Co library and its affinity for hESCs. All libraries were compared to ‘CPX’ or clones expressing the CPX scaffold in the absence of an N-terminal peptide. **(e)** Individual clones identified from fourth-generation (G_4) libraries via Sanger Sequencing were individually probed for the capacity to mediate binding to hESCs ($n = 3$). Nearly half of the clones probed displayed significantly higher affinities than the CPX scaffold alone (****, $p < 0.0001$) while a few displayed affinities significantly lower than that of the CPX scaffold (**, $p < 0.01$). One-way ANOVA plus a Dunnett’s multiple comparison analysis was conducted, with * indicating $p < 0.05$, ** indicating $p < 0.01$, *** indicating $p < 0.001$ and **** indicating $p < 0.0001$. All data is represented as means $\pm$ s.d.

Following binding, non-adherent clones were washed away via low-speed centrifugation and bound clones were isolated for further rounds of screening and selection. To increase the stringency of selection and identify high-affinity peptide binders through this scheme, library populations underwent successive rounds of screening under increasing selective pressure. High-affinity binders could be identified after each selection round via fluorescent-activated cell sorting (FACS) to isolate hESCs bound to GFP-expressing clones through surface-displayed peptides. Representative histograms of the relative affinity for hPSCs in the original and round-specific bacterial subpopulations of the 7C library are shown in **Figure 2.1C**. These histograms illustrate that with each successive round of biomimetic ligand selection, the affinity of the selected 7C library for hESCs increases and a larger proportion of the GFP-expressing library binds the non-fluorescent stem cell population.

To quantitatively assess the affinity maturation of peptide libraries after successive rounds of panning, the percentages of hESCs bound to bacteria for each selected library population and a control expressing just the CPX scaffold were measured (**Figure 2.1D**). While original, unselected libraries and those after a single selection round showed low affinities for hESCs, on par with or significantly lower than those of populations expressing the CPX scaffold alone, library binding affinities for hESCs rapidly increased after a second round of selection. In particular, the 7C library began to display affinities for hESCs that were significantly higher than that of the background CPX protein after two rounds of selection. Meanwhile, a library combining both the cyclic and linear variants showed significant affinity maturation after three rounds of selection against hESCs. By contrast, it is interesting to note that the libraries composed solely of linear peptides showed strongest affinity after two rounds of selection, binding approximately 40% of hESCs. Subsequent selection rounds, however, have little effect on affinity of 15mer libraries unlike their 7C counterparts, which showed a 60% increase in binding affinity between the second and fourth rounds of selection.

The prevalence of high affinity peptide binders within the 7C library is more readily apparent when the binding capacity of individual clonal populations is considered (**Figure 2.1E**). After analyzing thirty clones from each of the three fourth generation libraries, twenty-five unique sequences were identified via Sanger Sequencing. Of these, twelve were determined to be high-affinity binders (i.e. more than 50% of hESCs were bound to clonal populations of these high-affinity binders after co-incubation). **Table 2.1** summarizes both the generic binding capacity of individual clones for

hESCs and the peptide sequence that mediates this binding. It is of import that many of these clones expressed consensus sequences or conserved residues, underlined in black, that imply a shared adhesive motif that is critical in mediating specific binding to hPSCs. In some cases, these motifs were well-known sequences like ‘RGD’, found in the 7C-12 clone, that is a known binding partner of $\alpha_v\beta_3$ integrin¹⁸. In other cases, functionally unknown motifs along with larger segments of the screened peptides shared homology with known human ECM and structural proteins including chondroitin sulfate (7C-11), collagen type II (7C-18), collagen IV and fibrinogen (15-27)³¹. These generation and clonal data suggest that library-based screening techniques, although unbiased towards specific ECM or integrin binding domains, nevertheless yield peptides with a strong affinity for hESCs and possible specific affinities for stem cell surface receptors.

Table 2.1: Peptide-Expressing Clones with High Affinity for hESCs

Peptide Name	Sequence [†]	Binding Affinity for hESCs [*]
7C-1	FWCMG <u>DGR</u> P <u>R</u> CTG	94.8 ± 0.6%
7C-2	WFCLE <u>DGR</u> P <u>K</u> CDR	96 ± 0.7%
7C-10	SWC <u>R</u> YMLNGLCGL	66.4 ± 0.8%
7C-11	<u>KRCQ</u> <u>K</u> W <u>D</u> KSLCSS	47.8 ± 3.3%
7C-12	WNCVM <u>RGD</u> FWCPY	94.9 ± 0.6%
7C-18	<u>DGCS</u> <u>K</u> HGTH <u>K</u> CAL	51.5 ± 3.0%
7C-20	WLCLS <u>DGR</u> P <u>R</u> CYV	74.2 ± 1.4%
15-3	<u>RRCV</u> <u>S</u> <u>K</u> NNEDPYTNQ	51.1 ± 1.8%
15-4	ISSYGMNQL <u>R</u> W <u>G</u> RLN	48 ± 5.7%
15-6	<u>DK</u> WTCFLLT <u>F</u> W <u>R</u> DLM	48 ± 2.5%
15-23	<u>KPLRA</u> <u>K</u> <u>R</u> ET <u>T</u> KLKFG	55 ± 1.1%
15-27	QWEP <u>G</u> <u>R</u> NGLRMLV <u>W</u> E	50 ± 1.9%

[†]Sequences are color-coded to show negatively-charged (blue) and positively-charged (red) residues. Underlined residues represent motifs

^{*}Binding Affinity is represented by the percentage of hESCs that are bound to each clonal bacteria population

Targeted Identification of Binding Peptides for α_6 -integrin in hESCs

Given the homology between screened peptides and integrin-binding proteins like fibronectin and collagen, we anticipated that many of the untargeted peptides identified via screening may in fact have specificity for and mediate binding through integrins. However, identifying integrin binding motifs on screened peptides has been complicated by our limited understanding of the stem cell’s integrin landscape. While previous work from our lab had shown that hPSCs display a vast array of integrins, including $\alpha_6\beta_1$, $\alpha_2\beta_1$ and $\alpha_v\beta_3$ ³², few definitive interactions between ECM domains and integrin heterodimers have been studied outside of $\alpha_v\beta_3$ integrin’s affinity for the Arg-Gly-Asp

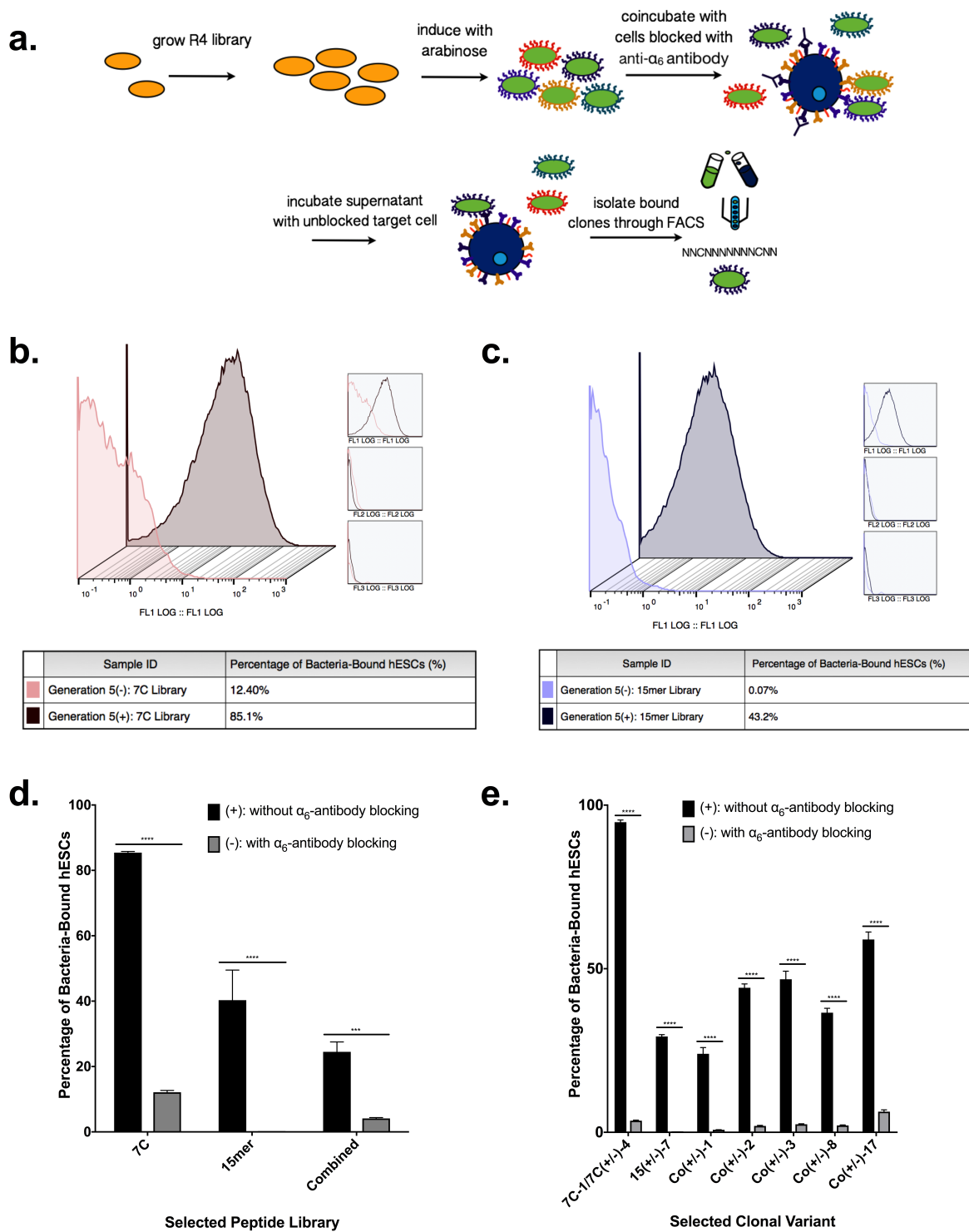


Figure 2.2: Targeted Screening for α_6 -integrin Binding Peptides. (a) The bacterial display scheme used to identify peptides that bind hESCs specifically via α_6 -integrin. This scheme is similar to the untargeted screening strategy illustrated in **Figure 2.1**. However, instead of a single

panning step against an hESC target, bacterial libraries are sequentially panned against two target hESCs – (1) hESCs with the target receptor, α_6 integrin, blocked by an antibody that ablates receptor binding (negative selection) and (2) unblocked hESCs (positive selection). Bacterial clones that do not bind the target during negative selection but do bind the target during positive selection are presumed to have α_6 integrin-specific binding. Representative histogram overlays of the targeted (b) 7C and (c) 15mer libraries show strong binding to unblocked hESCs with available α_6 integrin receptors (+) but negligible affinity for analogous blocked hESCs (-). (d) Quantitative binding analysis ($n = 3$) of each targeted 7C, 15mer and Co library and its affinity for unblocked (+) and blocked (-) hESCs. Comparisons were made within each library between blocked and unblocked groups. (e) Individual clones identified from these targeted fifth-generation (G_5 +/-) libraries via Sanger Sequencing were individually probed for their capacity to mediate binding to both unblocked and blocked hESCs ($n = 3$). All screened clones showed strong, significant ablation of the binding upon α_6 integrin-blocking (****, $p < 0.0001$) suggesting that these specifically and exclusively bind hESCs via α_6 integrin heterodimers including $\alpha_6\beta_1$ integrin. Two-way ANOVA plus a Sidak's multiple comparison analysis was conducted, with * indicating $p < 0.05$, ** indicating $p < 0.01$, *** indicating $p < 0.001$ and **** indicating $p < 0.0001$. All data is represented as means $\pm$ s.d.

sequence^{17,18}. For $\alpha_6\beta_1$ integrin, in particular, lack of structural information about the heterodimer's headpiece and ligand-binding domain has made it difficult to rationally design or identify putative binding partners^{33,34}. Thus, to identify effective peptide ligands of $\alpha_6\beta_1$ integrin, we conducted targeted biomimetic ligand selection as illustrated in **Figure 2.2A**. In these selections, hESCs were initially incubated with blocking antibodies specific to α_6 integrin. These blocked cells were subsequently co-incubated with fourth generation bacterial libraries, allowing for binding events to occur between bacteria and all cell surface moieties except for α_6 integrin. After this initial negative selection, bacteria that remained unbound during co-incubation were added to unblocked hESC cultures to identify clones capable of specifically binding α_6 integrin.

This targeted selection yielded a fifth generation of 15mer and 7C peptide-expressing clones that showed strong affinity for hESCs in the absence of the α_6 integrin blocking antibody. In the presence of a blocking antibody for α_6 integrin, however, peptide-mediated adhesion of both 15mer (**Figure 2.2B**) and 7C clones (**Figure 2.2C**) to hESCs was uniformly and significantly ablated (**Figure 2.2D**). As with our untargeted screens, we were interested in identifying individual clones within this targeted library that showed heightened and selective affinity for α_6 integrin on hESCs. After Sanger sequencing of thirty clones from each of these three fifth generation libraries, seven unique clones were identified that showed strong affinity for hESCs and markedly reduced affinities in the presence of an α_6 integrin-blocking antibody (**Figure 2.2E**) but not an isotype control. **Table 2.2** summarizes both the sequence and α_6 integrin-specific affinities of these targeted clones. Of the targeted clones examined, two unique clones – a cyclic sequence identified in both targeted and untargeted selections (7C-1/7C(+/-)-4) and a linear sequence (15(+/-)-7) – were chosen for further analysis and characterization. These sequences were chosen because they (1) mediated high-affinity binding to unblocked hESCs, (2) showed a strong reduction in binding upon α_6 integrin-specific blocking compared with other peptides, and (3) were predicted to be highly water soluble.

Table 2.2: hESC Affinities of Targeted, α_6 integrin-specific Clones

Peptide Name	Sequence [†]	Binding Affinity for Unblocked hESCs*	Binding Affinity for Blocked hESCs [§]	Frequency in Screened Population ^{††}
7C(+/-)-4/ 7C-1	FWCMG <u>DGR</u> P <u>R</u> CTG	94.8 $\pm$ 0.6%	3.55 $\pm$ 0.2%	11
15(+/-)-7	V <u>K</u> DWCVN <u>R</u> WICYW <u>E</u> <u>K</u>	29.3 $\pm$ 0.5%	0.12 $\pm$ 0.02%	5
Co(+/-)-1	WDCL <u>R</u> D <u>G</u> RQLCNF	24 $\pm$ 1.9%	0.78 $\pm$ 0.02%	4
Co(+/-)-2	GSCL <u>S</u> D <u>G</u> R <u>P</u> RCEW	44.2 $\pm$ 1.19%	1.95 $\pm$ 0.2%	10
Co(+/-)-3	YACMS <u>D</u> G <u>R</u> P <u>K</u> CRI	46.8 $\pm$ 2.4%	2.46 $\pm$ 0.2%	4
Co(+/-)-8	<u>D</u> E <u>C</u> L <u>Q</u> D <u>G</u> R <u>P</u> KCNN	36.5 $\pm$ 1.3%	2.09 $\pm$ 0.2%	1
Co(+/-)-17	IGC <u>R</u> G <u>D</u> AYY <u>R</u> CHQ	58.9 $\pm$ 2.3%	6.3 $\pm$ 0.6%	1

[†]Sequences are color-coded to show negatively-charged (blue) and positively-charged (red) residues. Underlined residues represent motifs

*Binding Affinity for Unblocked hESCs is represented by the percentage of hESCs that are bound to each clonal bacteria population in the absence of a blocking antibody

[§]Binding Affinity for Blocked hESCs is represented by the percentage of hESCs that are bound to each clonal bacteria population in the presence of an α_6 integrin blocking antibody

^{††} Frequency in the screened population represents the number of times the unique sequence appeared among the 30 clones screened in the targeted, fifth generation library.

Characterization of the Binding Strength of hESC-Binding Peptides

Although bacterial display represents an excellent scheme for rapidly identifying potential high-affinity ligands, peptides fused to a bacterial membrane protein are often presented at densities and orientations that poorly represent a long-term culture surface²⁰. To better characterize the capacity of screened peptides to bind and mediate short-term adhesion of hESCs under standard culture conditions, we generated and recombinantly-expressed peptide-GFP fusion proteins that were capped at the C-terminus by a polyhistidine-tag. Through non-covalent interactions between this tag and a Ni-NTA surface, peptides could be reliably and reproducibly presented at high densities on a culture surface for interactions with hPSC surface receptors.

In the scheme illustrated in **Figure 2.3A**, five peptide fusion proteins were generated based on clones identified through both untargeted and targeted screens – 7C-1 or 7C(+/-)-4, 7C-2, 7C-12, 15-23, and 15(+/-)-7. Along with Matrigel, Laminin and bspRGD-coated surfaces, these screened peptides were non-covalently attached via their polyhistidine tag to a Ni-NTA-coated surface, seeded with cells and exposed to a low-grade centrifugal force. **Figure 2.3B** shows the capacity of each peptide or protein-coated surfaces to maintain hESC adhesion in the presence of a detachment force. As expected, surfaces like Matrigel and Laminin showed characteristically strong adhesive profiles for hESCs while these same cells were quickly detached (>85%) from negative control (i.e. ‘Blank’ and ‘Control’) surfaces. Peptide surfaces, on the other hand, showed a wide range of unexpected affinities for hESCs. Both the targeted and untargeted 7C peptides as well as 15-23, displayed strong affinities for hESCs that were significantly above that of the ‘Blank’, GFP scaffold-only surface. However, on

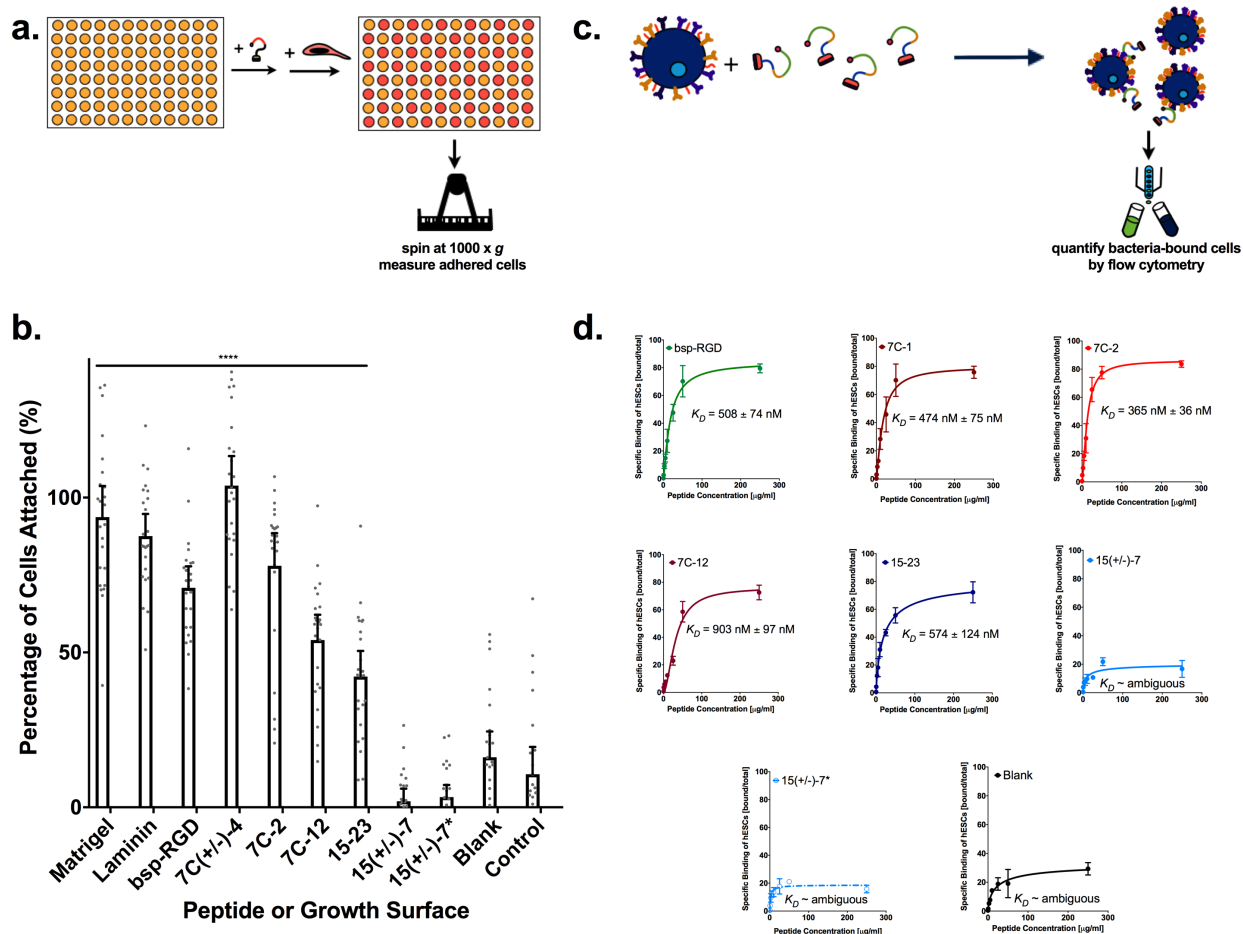


Figure 2.3: Biophysical Characterization of Peptide-hESC Binding. (a) Adhesion assay scheme used for characterizing the strength of binding between recombinantly-expressed peptide fusions and hESCs. Candidate peptides were expressed as fusion proteins with GFP and capped at the C-terminus with a polyhistidine tag. Polyhistidine tagged proteins could be coated on a 96-well Ni-NTA plate in a characteristic honey-comb pattern, seeded with hESCs and exposed to a constant detachment force of $10 \times g$. (b) The capacity of each surface to mediate the attachment of hESCs in the presence of the detachment force ($n = 45$). All surfaces were compared to a control surface, 'Blank', composed of just the GFP scaffold with no N-terminal peptide. Cyclic peptides, the linear 15-23 and control surfaces showed a significant capacity to maintain hESC adhesion (****, $p < 0.0001$). (c) Flow cytometry scheme for determining the dissociative binding strength of peptide fusions for the aggregate hESC receptor population. hESCs were incubated with sequentially larger quantities of fusion proteins and probed via flow cytometry. (d) Binding curves, fitted Hill Functions and dissociation constants for each peptide-fusion's interaction with hESCs. One-way ANOVA plus a Dunnet's multiple comparison analysis was conducted, with **** indicating $p < 0.0001$. Data is represented as means $\pm$ s.e.

surfaces containing the targeted linear peptide, 15(+/-)-7, more than 90% of hESCs detached. This linear sequence, VKDWCVNRWICYWEK, had intrigued us, in part, because of two cysteines – at the fifth and 11th positions – that sandwich five residues between them to possibly form a looped

structure. Given the prevalence of high-affinity cyclized peptides in our bacterial screens (**Figure 2.1** and **Figure 2.2**), we were curious about the capacity of these cysteines to form a highly constrained five-member loop that facilitates α_6 integrin-mediated adhesion. To better understand this sequence and its putative secondary structure, a second peptide sequence, 15(+/-)-7*, was generated in which the second cysteine was mutated into a glycine, thereby abolishing the capacity of the peptide to cyclize. However, this linearized version, 15(+/-)-7*, showed no marked difference in its strength of adhesion for hESCs when compared to 15(+/-)-7, suggesting that the presentation of this α_6 integrin-specific motif had little influence on hESC adhesion.

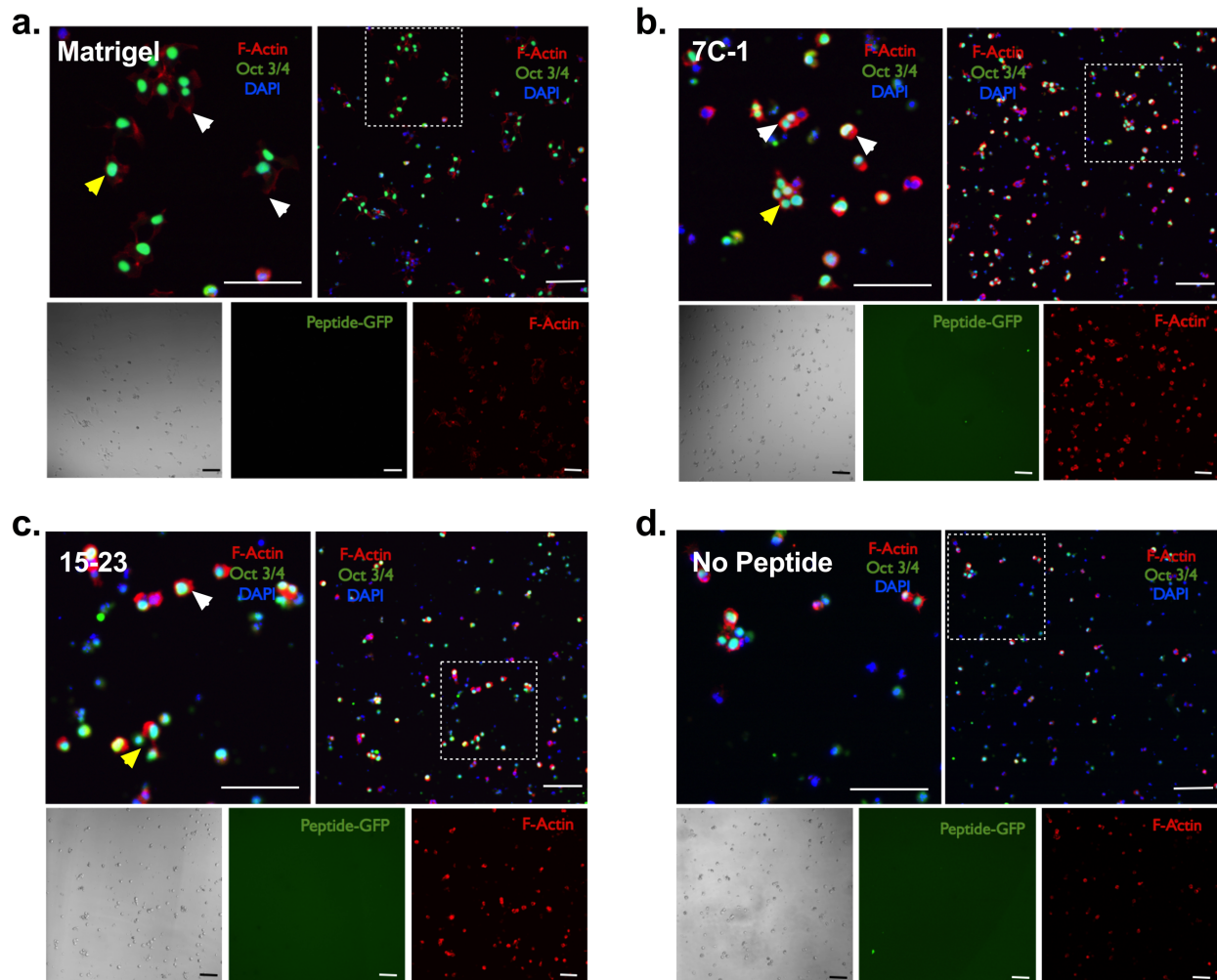


Figure 2.4: Qualitative Characterization of Peptide-hESC Binding. H1 hESCs cultured on (a) Matrigel, (b) cyclic (7C-1) peptide-GFP surfaces, (c) linear (15-23) peptide-GFP surfaces or (d) a GFP scaffold control for 6h and stained for markers of adhesion (F-actin, red) and pluripotency (Oct 3/4, green). Top left panel is the inset delineated by the white box in each top right panel. Bottom panels show the corresponding brightfield images, a confirmation of peptide-GFP presence and uniformity, and the F-actin stain alone. White arrows show dominant F-actin stains and yellow arrows illustrate regions of strong pluripotency marker expression. Scale bars represent 50 μm .

Strength of adhesion describes important biophysical properties of a cell's initial interfacing with its surface. However, this parameter provides a relatively crude image of the binding kinetics associated with the aggregate stem cell receptor population and its interaction with each candidate peptide. In **Figure 2.3C**, we describe a scheme for elucidating the dissociative binding strength of each peptide-GFP fusion protein for hESC surface receptors through flow cytometry. Here, hESCs were incubated with successively larger quantities of peptide-GFP proteins and probed for fluorescence via flow cytometry to generate a series of binding curves with fitted Hill Functions (**Figure 2.3D**). These fitted binding profiles could then be used to calculate an equilibrium dissociation constant, K_D , for the interaction between each peptide and the collective receptor population on the stem cell surface. As with the strength of adhesion data presented in **Figure 2.3B**, cyclic peptides, particularly 7C-1 (maroon curve in **Figure 2.3D**) and 7C-2 (red curve in **Figure 2.3D**), displayed binding strengths on par with canonical $\alpha_v\beta_3$ binding motifs like bspRGD (green curve in **Figure 2.3D**). These low K_D , high affinity peptides also match the binding profiles of other targeted, short peptide ligands for integrin heterodimers discovered via phage display.

Ultimately, identifying peptides that initiate and maintain high affinity interactions with hESCs is only a small part of the challenge associated with generating stem cell culture systems. Equally important considerations include whether the peptide is capable of maintaining viability, pluripotency and adhesion of hESCs. To answer this question, we considered how hESCs regulate pluripotency and cytoskeletal marker expression on our peptide-GFP fusion surfaces and corresponding controls (**Figure 2.4**) over short-term culture. On Matrigel (inset in **Figure 2.4A**), hESCs almost uniformly displayed strong expression of the pluripotency marker, POU5F1 or Oct 3/4, and a mature cytoskeletal architecture highlighted by a low intensity yet organized filamentous actin stain^{7,35}. By contrast, hESCs cultured on the GFP scaffold in the absence of a peptide mediator of adhesion (inset in **Figure 2.4D**) displayed little to no Oct 3/4 or F-actin expression after two hours of culture. The introduction of either a targeted cyclic (7C-1) (inset in **Figure 2.4B**) or untargeted linear (15-23) (inset in **Figure 2.4C**) peptide reversed some of the marker expression profiles seen on GFP scaffold surfaces with no peptide. For one, peptide surfaces were able to support Oct 3/4 expression on levels comparable to Matrigel. However, perhaps owing to the relatively sparse adhesive epitope density of peptide surfaces in comparison to Matrigel or laminin, the actin cytoskeleton formed by cells on both the cyclic and linear peptide surfaces were qualitatively less mature than those found on Matrigel. This suggests that while these peptide surfaces can support the adhesion of hESCs in the short-term, the development of a long-term culture surface with candidate peptides requires optimization of motif presentation, density and diversity.

Development and Characterization of Adherent Biomaterial Surfaces for hPSCs

To optimize peptide presentation for long-term hESC culture, a number of peptide conjugation strategies were explored as described in **Figure 2.5A**. Previous work had shown that hESCs, unlike many other cell types, require substrates with motifs that are presented at supraphysiological densities on the order of 600-900 pmol/cm² in order to achieve comparable growth rates to canonical Matrigel cultures¹⁶. In our case, five different conjugation strategies were employed to achieve surfaces with high peptide densities. Of these, only self-assembled monolayers or SAMs generated by incubating 1mM AT-bspRGD(15) solutions on a gold surface were able to achieve peptide densities on the order of several hundred pmol/cm² (**Figure 2.5A**). Alkanethiol (AT) peptides, consisting of a protein head group attached to a long thiol-terminated alkane chain, have the capacity to assemble on gold surfaces to form dense carpets³⁶. Due to van der Waals interactions between neighboring alkane chains during self-assembly, AT-peptides orient 45° relative to the

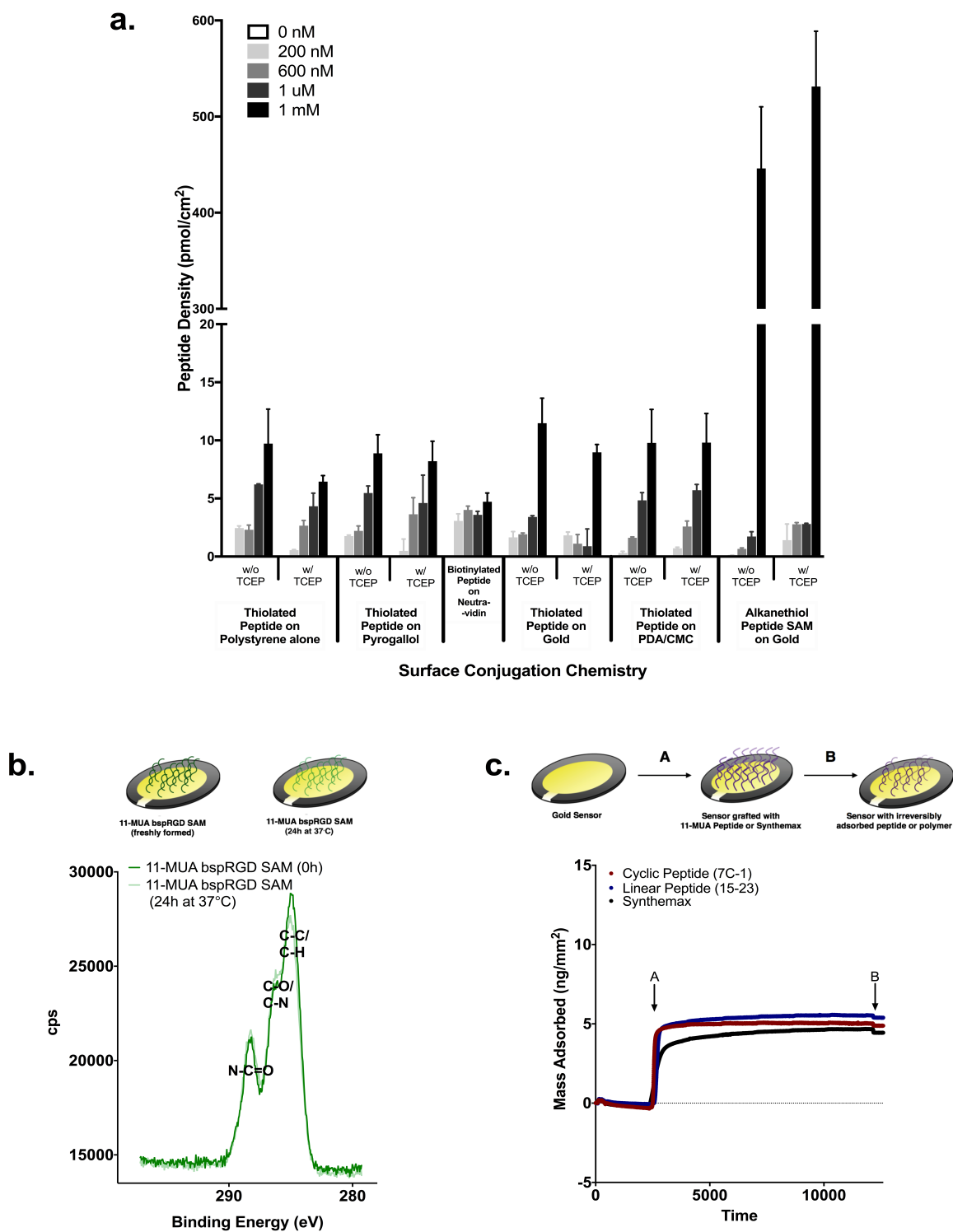


Figure 2.5: Development and Characterization of Peptide Conjugation Strategies. (a) Quantification of peptide presentation density (pmol/cm^2) associated with each conjugation strategies used to couple peptides to a polystyrene surface. Other than AT-SAMs, no other con-

-jugation strategy generated surfaces with peptide densities exceeding 500 pmol/cm². (b) XPS analysis of 11-mercaptoundecanoic acid (11-MUA) bspRGD SAMs that were either freshly formed and assessed (**dark green trace**) or incubated under isotonic PBS at 37°C and assessed after 24h (**light green trace**). Prominent carbon peaks are highlighted based on deconvolution data (not shown). (c) QCM-D traces of AT-SAMs generated using 11-MUA-functionalized cyclic (7C-1) (red trace) or linear (15-23) peptides (blue trace). A Synthemax-coated sample (black trace) is also shown. Clean gold sensor surfaces were exposed to peptide or protein at (A) to generate a surface coating. Once coating had stabilized after 2.5 hours at (B), surfaces were washed with water to remove reversibly bound peptide or protein and assess mass adsorbed.

substrate, exposing their peptide head groups for chemical interactions at the substrate surface³⁶. Previously, it was shown by Derda *et al.* and Klim *et al.* that native ECM-derived protein fragments could be presented on a gold surface via alkanethiol conjugation to create a viable ECM for short-term hPSC culture^{14,15,37}. However, as with most SAM surfaces, the stability and uniformity of these AT-SAMs is variable and can affect their capacity to support hPSC self-renewal³⁸.

To explore the short-term stability of the AT-peptide SAMs that we had generated, we probed two 11-mercaptoundecanoic acid (MUA)-RGD SAM samples via x-ray photoelectron spectroscopy or XPS. The first sample was immediately analyzed via XPS (**Figure 2.5B, dark green trace**) while the second was incubated under standard culture conditions – i.e. 37°C in an isotonic PBS solution – for 24 hours prior to analysis (**Figure 2.5B, light green trace**). The preservation of important carbon peaks between these two traces suggests that these SAMs not only formed but also experienced very little degradation under short-term culture conditions.

Table 2.3: Adsorbed Sauerbrey Mass of Rigid Adhesive SAMs and Protein Coatings

Alkanethiol Peptide SAM or Protein Coating	Adsorbed Sauerbrey Mass (ng/mm ²) [†]	Molecular Mass of AT-Peptide (Da)	Molecular Packing Density of SAM (molecules/mm ²)
Cyclic (7C-1) 11-MUA	5.08	1812.0	1.68×10^{12}
Linear (15-23) 11-MUA	5.62	1471.8	2.29×10^{12}
Synthemax	4.76	-	-

[†]Sauerbrey masses were determined by applying the Sauerbrey relationship³⁹, $m = -C \frac{1}{n} \Delta f$, to the change in sensor frequency at baseline prior to AT-peptide introduction (point A) and post-AT peptide introduction (point B).

To understand the formation kinetics and uniformity of these SAMs, 1mM cyclic (7C-1) (**red trace**) and linear (15-23) (**blue trace**) 11-MUA peptides were assembled on a gold quartz sensor and probed via quartz crystal microbalance and dissipation (QCM-D) (**Figure 2.5C**). In both cases, mature SAMs were rapidly formed following introduction of the AT-peptide (point A, **Figure 2.5C**) through irreversible adsorption (point B, **Figure 2.5C**). It is interesting to note that these peptide SAMs formed much more rapidly than the commercially available coating, Synthemax (**black trace**), although both Synthemax coatings and SAMs, once formed, generate a rigid, dense and irreversibly adsorbed protein layers that can be modeled as a Sauerbrey mass⁴⁰. **Table 2.3** outlines the adsorbed Sauerbrey masses associated with both cyclic and linear peptide SAMs as well as Synthemax. While

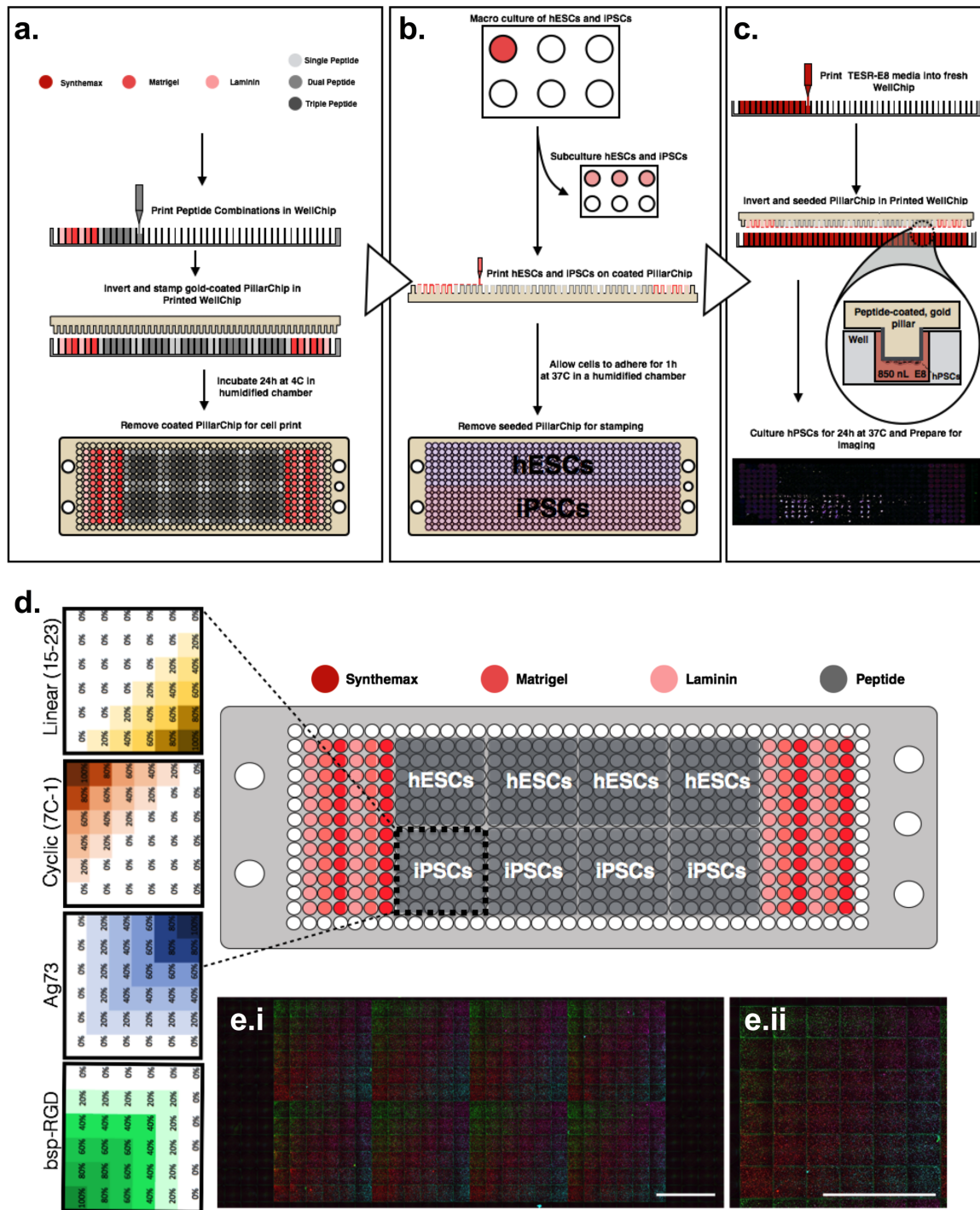


Figure 2.6: Scheme for High-Throughput hPSC Microculture on AT-Peptide SAMs. To generate hPSCs microcultures on AT-SAMs, combinations of 11-MUA variants of bspRGD, Ag73, cyclic and linear peptides were (a) printed onto WellChips and stamped onto gold-coated PillarChips to facilitate SAM formation on pillars. Both hESCs and iPSCs were (b) printed onto the PillarChips and (c) cultured for a single passage (2 days). (d) A schematic of the peptide print

strategy. Control coatings of Matrigel, Synthemax and Laminin were printed in two sets of columns at either end of the chip. Pillars in the center were coated with either one-, two- or three-alkanethiol peptides using the schematized gradient overlay scheme. (e) Print efficacy at (i) the chip and (ii) replicate level was verified by printing fluorescent bead-proxies for each peptide and high-throughput imaging. Scale bars represent 10 mm.

cyclic and linear 11-MUA peptides were similar in molecular weight, cyclic peptides SAMs had a smaller adsorbed Sauerbrey mass than equivalent linear SAMs. Consequently, linear SAMs displayed 1.4× higher molecular packing densities than cyclic SAMs. We attributed this to the looped secondary structure of cyclic peptides – a feature that makes it difficult to generate dense SAMs but likely easier for cell surface receptors to probe adhesive motifs as they initiate binding interactions with their matrix.

Optimization of Binding Peptide Combinations for Long-term hPSC Culture

Having identified a strategy for presenting our screened peptide motifs as self-assembled monolayers, we then set out to develop a formal optimization scheme to assess the capacity of our peptides to mediate hPSC self-renewal over a single passage. Previous work from our lab³² and others^{41–43} had illustrated that hPSCs rely on a plurality of motifs to mediate binding and maintain pluripotency over several passages. With this in mind, we were curious whether our novel cyclic and linear peptides could interact synergistically with canonical binding motifs – like bspRGD, a ligand for $\alpha_v\beta_3$ integrin, and Ag73, a syndecan-1 binding peptide^{44–46} – to enhance stem cell self-renewal.

Given the expansive combinatorial space associated with tri-peptide mixtures, we employed a previously-described high-throughput microculture platform^{47,48} for printing combinatorial peptide SAMs and examining each combinations' capacity to support undifferentiated hESCs and iPSCs over a single passage. **Figure 2.6A-C** illustrates the work flow for printing peptide combinations onto individual pillars of a gold-coated 536-pillar chip (PillarChip) to generate several unique AT-peptide SAM culture surfaces for hPSCs. To probe a large subset of peptide combinations over a small area of the chip, novel cyclic (7C-1) and linear (15-23) peptides, bspRGD and Ag73 were spot-printed in overlaying gradients that spread out from individual corners of a 36-pillar area as shown in **Figure 2.6D**. By overlaying the four peptide gradients on top of each other, thirty-six unique peptide combinations were printed in which the central 16 pillars were coated with a combination of three peptides while the 20 pillars that framed the periphery were coated with two or fewer peptides. The efficacy of this print strategy was verified with colored fluorescent beads as shown in **Figure 2.6E**.

Once pillars were coated with either AT-peptide SAMs or a control surface coating (i.e. Matrigel, Synthemax or Laminin), both H1 hESCs and iPSCs were printed onto the PillarChip, stamped into a corresponding WellChip and cultured for a single passage as shown in **Figure 2.6B** and **D**. While hESCs did not survive beyond the first few hours, iPSC microcultures proliferated extensively on these peptide SAMs. To determine the efficacy of each peptide SAM surface, seeded iPSCs were assessed on four individual parameters – colony area, relative colony pluripotency, the presence of multilayer morphologies within microcultures⁴⁹, and targeted integrin binding as quantified by the ratio of α_6 integrin stain intensity to F-actin stain intensity. **Figure 2.7A** shows how these four individual parameters interact with one another to inform two principal components (PC1 and PC2) that explain about 85% of the variation between peptide-SAM combinations tested. In particular, samples split into two rough clusters: (1) microcultures from central pillars (**teal dots**) that

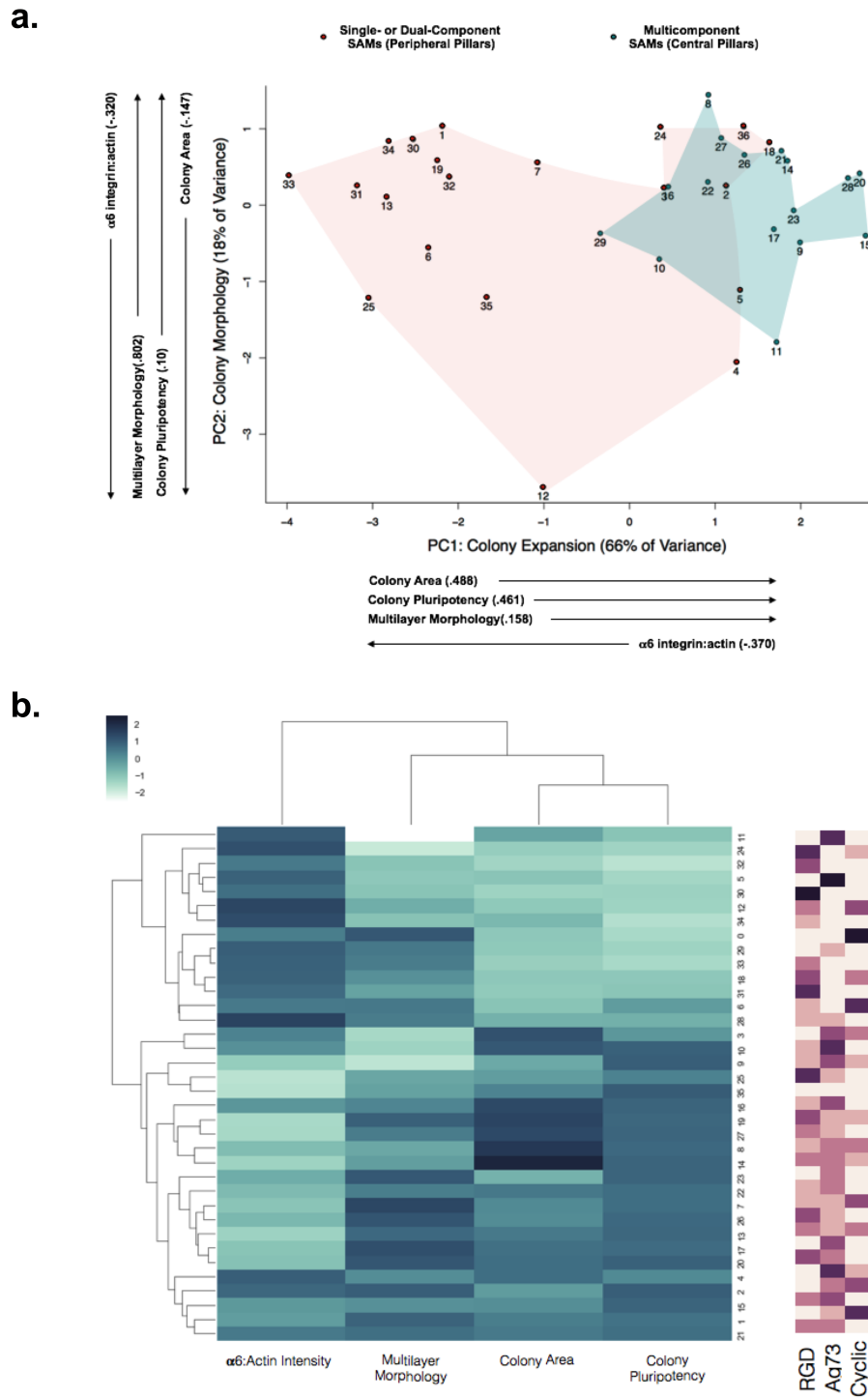


Figure 2.7: Partial Component & Hierarchical Clustering Analysis of hiPSC Microcultures.
 (a) Partial Component Analysis of hiPSC microcultures. Each of the 36 microcultures is plotted against two principal components – a colony expansion component and a colony morphology

component. The contribution of the four measured output parameters – colony area, colony pluripotency, multilayer morphology and α_6 integrin:actin intensity (a measure of targeted binding) – are listed for each component. Microculture samples ($n = 3$) are clustered based on the underlying SAM matrix contributions. Namely, cultures grown on SAM surfaces composed of three peptides are colored in teal while those grown on SAM surfaces with two or fewer peptide contributors are marked in red. Shaded boxes show regions of clustering. **(b)** Hierarchical-clustered dendrogram of hiPSC microculture data. The heatmap on the left shows the clustered performance of each microculture (labeled vertically) ($n = 3$) along the four measured output parameters (labeled horizontally). The scale bar on the right shows the corresponding peptide SAM supporting each microculture.

ranked highly in colony expansion metrics (PC1) and moderate to high in colony morphology metrics (PC2) and (2) samples from peripheral pillars (**red dots**) that showed poor colony expansion (PC1) and moderate to high multilayerism and targeted binding (PC2). This suggests that surface composition – particularly the number surface motifs available to stem cells – affects their capacity to attach, self-renew and maintain pluripotency in 2D culture.

To further explore the role of SAM surface composition on hiPSCs, the 36 unique microcultures were clustered in a dendrogram as shown in **Figure 2.7B**. Microcultures that had similar colony phenotypes, as determined by the measured colony expansion and morphology parameters, clustered close together in the dendrogram. The scale bar on the right shows the corresponding contribution of each peptide – 7C-1, 15-23, bspRGD or Ag73 – to the SAM surface matrix. As with PCA analysis, we discerned that SAMs composed of multiple peptides – marked by medium purple hues on the right-hand scale bar – generated microcultures with larger colonies and higher pluripotency marker expression (**darker shades of teal**). By contrast, targeted binding to the SAM surface via α_6 integrin in these colonies was lower. On surfaces where a single peptide dominated the SAM – delineated by primarily beige or deep purple notations – colony expansion and pluripotency marker expression were lower and targeted binding phenotypes were higher. Interestingly, the presence of multilayer colonies within the microculture showed no correlation to underlying SAM matrix composition.

We were, therefore, interested in investigating how specific trends in SAM composition affect microculture performance along each of the four output parameters measured. To study this, we parsed our microculture dataset along three SAM composition metrics – (1) the number of peptides composing each SAM surface (**Figure 2.8**), (2) the ratio of peptides used to generate the SAM (**Figure S2, Appendix I**) and (3) the percentage of Ag73 – the syndecan binding element – in each SAM (**Figure S3, Appendix I**). **Figure 2.8** shows how the number of peptides used to generate a SAM affects the resultant colony morphology and proliferation. In particular, we see that SAMs generated using three peptides performed significantly better than single or dual peptide SAMs in promoting colony expansion (**Figure 2.8A**) and pluripotency (**Figure 2.8B**). This is mirrored by the significant reduction in the ratio of α_6 integrin intensity to actin intensity – an indication that hiPSCs on triple peptide SAMs employed a more diverse subset of surface receptors to interact with their underlying matrix. **Figure 2.8E-G** shows a qualitative representation of this trend through representative stains on single, dual and triple SAM surfaces.

In global data analysis schemes (**Figure 2.7**), triple peptide surfaces showed a diversity of behavior with some multi-peptide SAMs showing a strong capacity to promote hPSC expansion (i.e. microculture **8**) while others showed a limited capacity to support proliferation (i.e. microculture **29**).

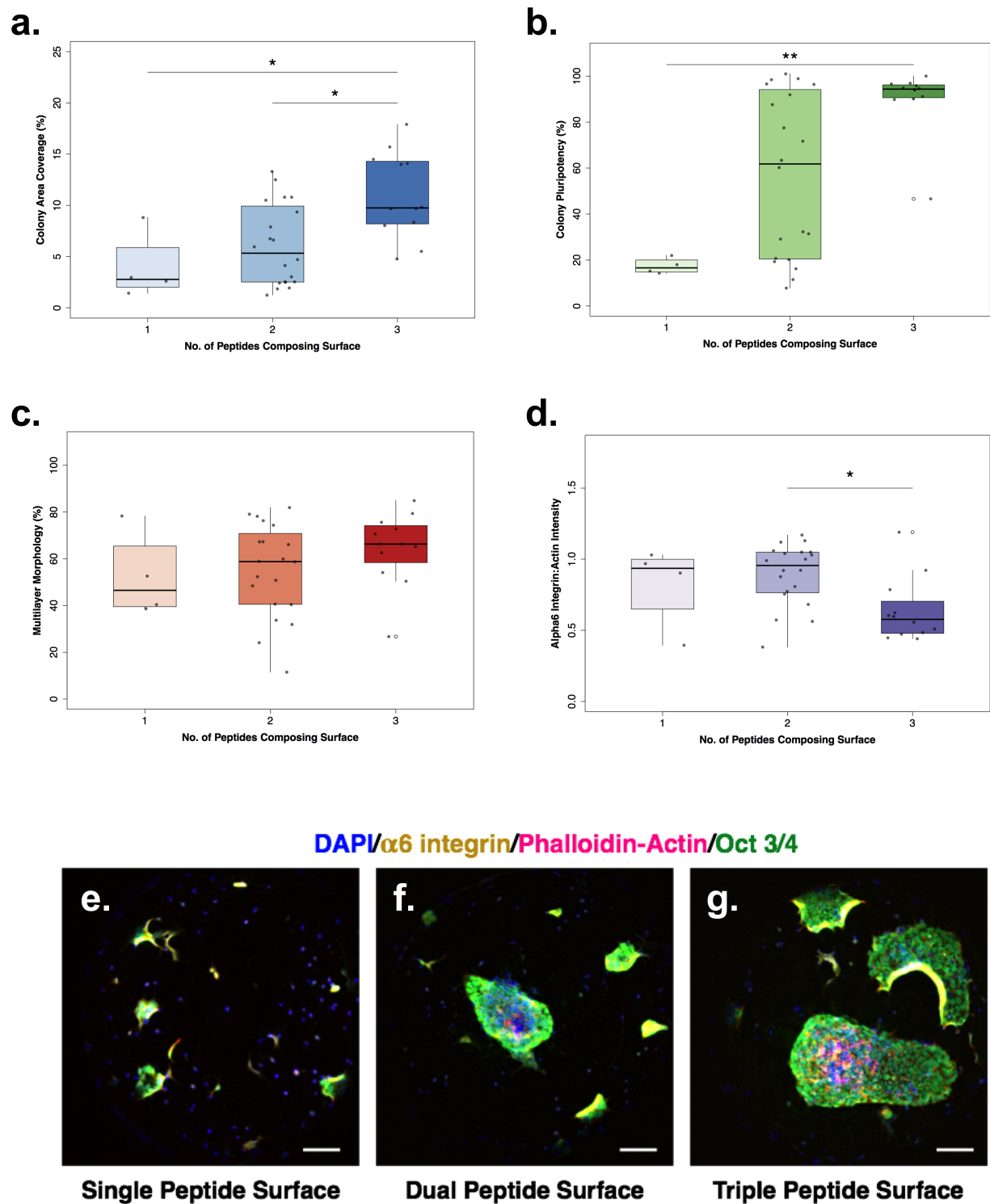


Figure 2.8: Role of Peptide Number in SAM functionality and hiPSC Microculture Phenotypes. Boxplots of (a) colony area, (b) relative colony pluripotency, (c) multilayer morphology and (d) the ratio of α_6 integrin intensity to F-actin intensity as a function of the number

of peptides composing the underlying SAM matrix. SAMs composed of three peptides ($n = 12$) showed a strong capacity to promote colony expansion in comparison dual peptide SAMs ($n = 19$) (*, $p < 0.05$) or single peptide SAMs ($n = 4$) (*, $p < 0.05$). These triple peptide SAMs were also more capable of maintaining Oct 3/4 marker expression in microcultures than single peptide surfaces (**, $p < 0.01$). Trends in colony area and pluripotency among these microcultures were mirrored by a decrease in targeted or α_6 integrin-exclusive binding in triple peptide surfaces (*, $p < 0.05$). Representative microcultures for a (e) single peptide surface, (f) dual peptide surface and (g) triple peptide surface. Scale bars represent $50 \mu\text{m}$. The non-parametric Kruskal-Wallis test was used for statistical analysis followed by a *post-hoc* Dunn's test to analyze specific sample pairs, with * indicating $p < 0.05$, ** indicating $p < 0.01$. All data is represented as means $\pm$ s.d.

The diversity of phenotypes generated on triple peptide SAMs led us to investigate how the peptide ratio employed to generate each SAM affected the resultant colony's phenotype (**Figure S2, Appendix I**). As with peptide number, peptide ratios that allowed for a more diverse and equitable presentation of adhesive motifs – i.e., 40-40-20% peptide distribution – produced more robust and expansive hiPSC cultures than SAMs that displayed a single predominant motif. We were, therefore, interested in seeing if the dominance of any particular novel or canonical peptide within this 40-40-20% SAM scheme influenced hiPSC proliferation and pluripotency. Although variations in cyclic, linear, bspRGD presence showed no significant influence on colony area, pluripotency or morphological markers, variations in Ag73 strongly and significantly influenced microculture outcomes (**Figure S3, Appendix I**). In particular, microcultures grown on SAMs where Ag73 was moderately expressed (i.e. constituting around 40% of the SAM) showed the highest levels of colony expansion and pluripotency marker expression.

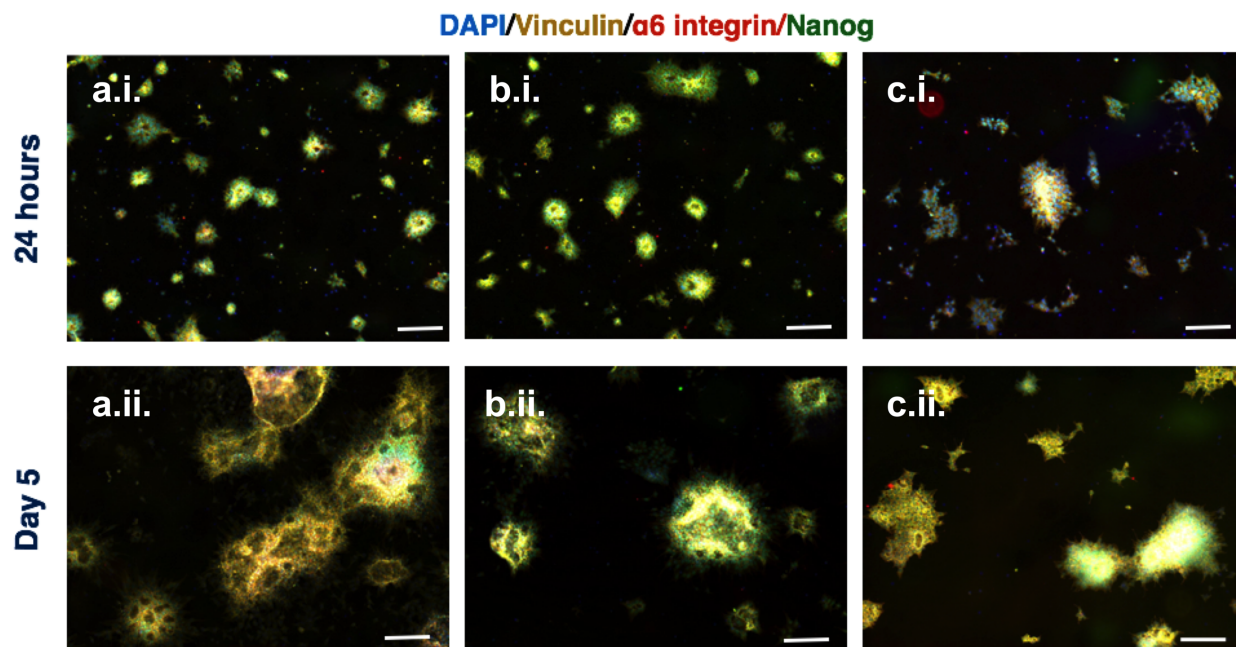


Figure 2.9: hPSC Macrocultures on 40-40-20% Peptide SAMs. (a) H1 hESCs and (b) hiPSCs

grown on 40% Ag73, 40% bspRGD and 20% Cyclic (7C-1) 11-MUA surfaces for (i) 24 hours or (ii) five days. For comparison, (c) H1 hESCs were also grown under similar conditions on Synthemax for (i) 24 hours and (ii) five days. All cultures were maintained under the presence of Rho-Kinase inhibitor, Y-27632. Both colony expansion and multilayer morphologies can be readily observed at five days after passaging on all surfaces. Scale bars represent 100 μm .

Having found a few optimal peptide compositions through high-throughput microculture screening, we sought to see if these surfaces were capable of supporting hESC and hiPSC macrocultures over a single passage. **Figure 2.9** illustrates the capacity of one optimized triple peptide SAM composed of 40% Ag73, 40% bspRGD and 20% Cyclic (7C-1) 11-MUA peptides to support both H1 hESCs (**A**) and hiPSCs (**B**) over a single 5-day passage. While hPSC macrocultures generated on this SAM relied on Rho-Kinase inhibitor, Y-27632, and formed significant multilayer or cystic regions that prevented clonal passaging, these colonies nevertheless expanded on par with hESCs grown under similar conditions on Synthemax (**Figure 2.9C**) and Matrigel. Together with data from our high-throughput studies, **Figure 2.9** highlights the capacity of selective peptide SAMs to support the short-term adhesion and self-renewal of hPSCs.

Given the large demand for stem cell-derived therapies in the clinic⁴, there has been an acute need for scalable, defined substrates to culture pluripotent stem cells *in vitro*^{16,50–52}. While surfaces like Geltrex and Matrigel have been the gold standard for hPSC culture at the bench^{7,35,53}, the batch-to-batch variability of these surfaces makes them unlikely candidates for clinically-relevant hPSC culture^{38,50}. Instead, attention in the field has shifted to synthetic materials – polymeric^{43,50,52,54} and peptidic^{14–16,37,51} – as *in vitro* analogs for the native ECM. Often derived from full-length ECM proteins like laminin^{9,11,55}, peptide surfaces, including peptide SAMs, have the capacity to present many of the same adhesive motifs as Matrigel. However, their minimalistic design makes these peptides scalable and definable mimics of ECM as well¹⁴. Past work creating scalable peptide-based substrates has been myopically focused on the generation of single-component substrates generated using a single, ‘rationally-designed’ adhesive motif^{17–19,44}. While these surfaces have met with some limited success, they have struggled to replicate the robust hPSC cultures seen on Matrigel and mitotically-inactivated mouse embryonic fibroblasts (MEFs)³⁸. In many cases, this can be attributed to the limited capacity of rationally-designed, single-peptide ECMs to activate a broad base of stem cell surface receptors.

In this work, we have introduced a new set of high-throughput screening technologies for identifying novel peptide binders that activate a broad range of stem cell surface receptors including the prominent laminin receptor, $\alpha_6\beta_1$ integrin¹⁰. Specifically, we have shown that both targeted and untargeted cyclic and linear motifs, identified via “blind” bacterial display strategies, can be combined with canonical integrin and syndecan binding motifs to generate viable culture surfaces for hPSCs. These surfaces, when equitably composed of a syndecan binding motif and at least two other integrin-binding domains, including novel peptides, can mediate robust short-term self-renewal of hPSCs on par with traditional culture substrates like Matrigel. Although our high-throughput microculture studies have shown these surfaces to only be viable in the short-term, it is likely that these peptides can also play an important role as long-term scaffolding elements as well. With the capacity to be coupled to polymeric backbones via simple thiol-mediated chemistry⁵⁶, these peptides combinations can be used to enhance the viability of extant 2D and 3D culture systems for both hPSCs⁵⁷ and a wide range of integrin-expressing cell types.

2.5 Conclusions

Developing synthetic materials for human pluripotent stem cell (hPSC) expansion has been a challenge in regenerative medicine³⁸. The work presented here highlights two key technologies – bacterial display and high-throughput microculture – that were harnessed to pare large peptides libraries, identify high-affinity binders of the laminin receptor, $\alpha_6\beta_1$ integrin, and optimize their presentation as a short-term hPSC culture surface. Given the efficacy of these optimized peptide surfaces at promoting the short-term adhesion and self-renewal of hPSCs, we anticipate that screened peptides can be adapted for a variety of other applications – as bioactive moieties on polymer culture systems and solitary purification agents for hPSCs and other α_6 integrin-presenting cells.

2.6 Acknowledgements

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Chapter 3: Development of Peptide-presenting 3D Polymer Blends for hPSC Culture

3.1 Abstract

Human pluripotent stem cells (hPSCs) hold vast promise as cell sources for tissue and organ engineering as well as human disease modelling and drug discovery. However, for each of these therapeutic applications, large quantities of pluripotent cells that faithfully preserve their hallmark capacities for self-renewal and differentiation are required – an insurmountable challenge for flat, two-dimensional (2D) culture systems. Three-dimensional (3D) biomaterials, which more closely mimic the natural 3D organization of tissues and organs, can, in theory, allow for the large-throughput expansion of hPSCs. However, in their current incarnations, 3D biomaterials often lack the functionalization required to engage adhesion molecules expressed by hPSCs and promote uniform self-renewal in the absence of exogenous growth factors.

Here, we present preliminary data on the role of engaging hPSC surface integrins via targeted binding peptides in 3D scaffolds. Using an acrylated hyaluronic acid polymer as the backbone, targeted binding peptides for integrins and syndecans were conjugated via Michael addition chemistry and biophysically and biochemically characterized. Peptide-decorated hyaluronic acid could then be blended with thermoreversible HyA-NIPAAm graft co-polymers to generate bioactive, scalable and defined 3D hPSC cultures. Unlike many previously described 3D culture systems, peptide-expressing HyA-NIPAAm blends showed an enhanced capacity to support hiPSCs over two passages under growth factor reduced conditions. Our results, thus, highlight how targeted-integrin adhesive motifs can be used to functionalize 3D polymeric culture systems and enhance their capacity to support the long-term culture of hPSCs.

3.2 Introduction

Human pluripotent stem cells, both embryonic stem cells (ESCs)¹ and induced pluripotent stem cells (iPSCs)^{2,3}, are characterized by two hallmark traits – (1) the capacity to self-renew extensively in an undifferentiated state and (2) the capacity to terminally differentiate into the ~200 cell types in the human body⁴. These two characteristics make hPSCs highly valuable and customizable cell sources for a wide range of research and translational applications including cell-replacement therapies⁵, human disease modelling⁶ as well as pharmacology and toxicology screens^{7,8}. However, many of these cited clinical challenges require the large-scale production of high-quality hPSCs *in vitro*⁶⁻⁹. For example, approximately 10⁵ to 10⁹ cells per patient of hPSC-derived, therapeutically-relevant cells are required to treat myocardial infarctions, Parkinson's Disease and type I diabetes¹⁰. These quantitative estimates, while staggering in their own right, ignore the poor engraftment of these cells *in vivo* upon transplantation as well as the growing demand for cell-replacement therapy⁵ – key challenges that highlight the need for large-scale stem cell expansion *in vitro*.

The growth and expansion of hPSCs both *in vivo* and *in vitro* is governed by key soluble cues, cell-cell interactions as well as substrate-mediated interactions from the extracellular matrix¹¹. During embryonic development – a natural example of large-scale stem cell expansion – cells not only interact with their neighboring cells but also with a surrounding three-dimensional ECM of laminins and select proteoglycans¹². These ECM interactions, governed by cell-surface receptors like integrins, syndecans

and cadherins, play an important role in regulating both cell-growth in the blastocyst inner cells mass as well as its eventual differentiation and tissue morphogenesis¹³.

Given the three dimensionality of the native stem cell niche and its capacity to regulate the expansive proliferation and differentiation of stem cells, there has been an increasing interest in developing biomaterials that mimic the structure and function of the native ECM^{4,14,15}. Over the last decade, a range of scalable and synthetic 3D suspension systems for hPSCs have been developed including those that rely on cell aggregates^{16–18}, polymeric and peptidic microcarriers¹⁹ and alginate-based microencapsulation¹⁰. While many of these systems are easy to synthesize via microfabrication techniques and have had some success for hPSCs culture, the majority have suffered from sizable disadvantages. These include (1) poor scalability due to the presence of undefined, animal-derived supplements or building blocks^{16,18–21}, (2) significant cell agglomeration, cell-death and unwanted differentiation due to poor adhesion of hPSCs to the scaffold¹⁸, and (3) limited capacity for long-term serial expansion of hPSCs^{16,18–21}.

Previous work from our group has shown that challenges associated with scalability and poor serial expansion can be tackled through the use of thermoreversible polymers as culture systems^{22,23}. In these systems, hPSCs are grown in a NIPAAm-modified derivative of the common natural polymer, hyaluronic acid (HyA)²⁴. At temperatures below its lower critical solution temperature (LCST), the HyA-NIPAAm graft copolymer is a liquid and can be mixed with hPSCs. Once cells are suspended in the HyA-NIPAAm solution, the temperature of the culture system can be raised above its LCST to solidify the HyA copolymer and promote clonal expansion of encapsulated hPSCs^{22,23}. Since these HyA systems are thermoreversible, they are readily amenable to serial expansion by liquefying the cell-laden gels at low temperature and passing expanded hPSCs into fresh polymer^{22,23}. However, while these thermoreversible platforms can support 3D hPSC expansion for over 60 passages²², hPSCs show poor adhesivity for these and other bare hyaluronic acid scaffolds²⁵. In the absence of key bioactive domains and tethered cues that can promote the adhesion and pluripotency of hPSCs, synthetic systems often rely on the modulation of exogenous growth factors including bFGF^{26,27} and TGF- β ^{17,28} that can hinder the scalability and reproducibility of synthetic culture systems. However, the importance of engaging hPSC surface receptors, particularly integrins, via tethered motifs in lieu of soluble factors remains largely unexplored⁴.

Here, we present preliminary data on the role of engaging hPSC surface integrins via targeted binding peptides in 3D scaffolds. Using an acrylated hyaluronic acid (AcHyA) polymer as the backbone, thiolated peptide ligands for $\alpha_v\beta_3$ and $\alpha_6\beta_1$ integrin and syndecan-1 were conjugated via Michael addition chemistry and biophysically and biochemically characterized by ¹H-NMR, colorimetric assays and size-exclusion chromatography. Peptide-decorated hyaluronic acid could then be blended with the HyA-NIPAAm graft co-polymers described²³ to generate a bioactive yet scalable hPSC culture system. Unlike many previously described 3D culture systems²⁵, peptide-expressing HyA blends showed an enhanced capacity to support the 10-fold expansion of both iPSCs and hESCs over a single passage. Our results, thus, highlight how targeted-integrin adhesive motifs can be used to functionalize 3D polymeric culture systems and enhance their capacity to support the long-term culture of hPSCs.

3.3 Materials and Methods

2D Human Pluripotent Stem Cell Culture

Human ESCs (WA01, Wicell, Madison, WI) and a previously described adipose stem cell-derived iPSC line²⁹ were cultured at 37°C and 5% CO₂ in mTESR1 cell-culture medium (StemCell Technologies, Vancouver, Canada) on tissue culture surfaces precoated with hESC-qualified Matrigel (BD

Biosciences, San Diego, CA). For routine passaging, cells underwent either mechanical selection and scraping or enzyme-free dissociation using ReLeSR (Stem Cell Technologies) followed by seeding on fresh Matrigel-coated surfaces in mTESR1 medium supplemented with 10 μ M Y-27632 (StemCell Technologies). Both the H1 hESC and the iPSC cultures were periodically tested for mycoplasma contamination via PCR.

Synthesis of Acrylated Hyaluronic Acid (AcHyA) & Peptide Functionalization

The functionalization of hyaluronic acid (HyA) with acrylate groups was performed in a two-step process adapted from a protocol described by Jha *et al*^{60,31}.

Generation of Acrylated HyA variants | Briefly, hydrazide groups were introduced to a 750 kDa hyaluronic acid (LifeCore Biomedical, Chaska, MN) dissolved in ultrapure, synthesis-grade water (100 mL, 3 mg/mL, Corning, Corning, NY) through the addition of 30 molar excess adipic acid dihydrazide (ADH, Sigma-Aldrich, St. Louis, MO). Following the addition of ADH, the HyA solution was adjusted to a pH of 6.8 using low molarity (0.1 mM) NaOH and HCl solutions to prevent the degradation of HyA chains. To promote the functionalization of HyA, 3 mmol EDC (Alfa Aesar, Tewksbury, MA) and 3 mmol HOBt (Sigma-Aldrich) dissolved in 1:1 DMSO/Water solution were sequentially added to the HyA-ADH solution and the pH was maintained at 6.8 for the first three hours. Once the pH had stabilized, the reaction was allowed to proceed overnight under constant low-grade stirring. Following the overnight reaction, the solution was dialyzed using a 100 kDa MWCO membrane (Fisher Scientific, Santa Clara, CA) for two days against synthesis grade water supplemented with 1% w/v NaCl (Sigma-Aldrich) and a single day against pure water. The dialyzed, hydrazide-functionalized HyA solution was then supplemented with NaCl to produce 5% w/v solution and precipitated as small batches in 200-proof ethanol (Decon Labs, King of Prussia, PA). Subsequently, the precipitated HyA was redissolved in 100 mL of ultrapure, synthesis grade water for the second step of synthesis.

At this stage, an excess of N-acryloxysuccinimide (NAS, 700 mg, Sigma-Aldrich) was added to the HyA-ADH solution to convert hydrazide groups to acrylate groups³². This reaction was allowed to proceed overnight in the dark under low-grade stirring. Following the overnight reaction, AcHyA solutions were exhaustively dialyzed against ultrapure water as previously described. Dialyzed solutions were then lyophilized, verified via ¹H-NMR for the presence of acrylate peaks at 5.0- 6.5 ppm and used for further modification. The relative ratio of hydrazide peaks to acrylate peaks in the HyA variant was used to determine the degree of acrylation³³.

Peptide Modification of AcHyA | For peptide modifications, thiolated variants of four targeted peptides identified via the screening methodology in Chapter 2 – Ag73 (Ac-CGGRKLRQVQLSIRT-NH₂), bspRGD(15) (Ac-CGGNGEPRGDTYRAYK-NH₂), the linear peptide 15-23 (Ac-CGKPLRAKREITKLKFG-NH₂) and the cyclic peptide 7C-1 (CMGDGRPRG, head to tail cyclization) – were synthesized using standard Fmoc chemistry (United Peptide, Herndon, VA). Peptides were grafted to the HyA backbone³¹ by incubating each thiolated peptide (10mg) with an AcHyA solution (25 mg in 10 mL of ultrapure water) overnight at 4°C. Following the grafting reaction, Peptide-AcHyA solutions were dialyzed extensively against ultrapure water and lyophilized for subsequent cell culture experiments. The presence of coupled peptides was confirmed according to ¹H-NMR and a bicinchoninic assay (BCA, ThermoFisher Scientific, Waltham, MA). The latter was performed according to the manufacturer's protocols.

Synthesis of Hyaluronic Acid-NIPAAm Co-polymer

The functionalization of hyaluronic acid (HyA) with poly N-isopropylacrylamide (pNIPAAm) occurs through a three-step process²³ (**Figure 3.2**): (1) the generation of a vinyl sulfone-presenting HyA (HyA-vs) precursor, (2) the generation of thiolated pNIPAAm precursor, and (3) the synthesis of HyA-NIPAAm graft co-polymers from these precursors via Michael addition chemistry. Both the HyA-vs and pNIPAAm-SH precursors were generated according to the protocol described by Ekerdt *et al.*²³. To ensure sufficient substitution of the HyA backbone in the HyA-vs precursor, molar excess ratios of 2.5x divinyl-sulfone (DVS) (Sigma-Aldrich) was used to deprotonate hydroxyl groups and generate HyA polymers with ~50% functionalized disaccharides for all downstream synthesis. ¹H NMR spectroscopy was utilized to determine the amount of vinyl sulfone groups attached to the HyA polymer as described by Ekerdt *et al.*²³. Similarly, the presence and quantification of bulk thiol content in pNIPAAm-SH precursors was analyzed via ¹H NMR spectroscopy and gel permeation chromatography (GPC) as previously described²³.

Generation and Characterization of HA-NIPAAm Graft Co-polymers | In order to graft pNIPAAm-SH to the HyA backbone via Michael addition chemistry, HyA-vs was dissolved overnight in 0.3 M triethanolamine (TEOA) buffer (Sigma-Aldrich) in PBS (Corning) at pH 8.0 and supplemented with 1mM EDTA (Sigma-Aldrich). Once dissolved, pNIPAAm-SH was added to the solution at a weight ratio of 20:1 of pNIPAAm-SH to HA-vs. The NIPAAm precursor was allowed to dissolve into solution at 4°C under constant mixing for 4-16 hours. Following the initial reaction of pNIPAAm-SH to the HyA backbone, a mild reducing agent – 20 mM TCEP-HCl (Sigma-Aldrich) – and 10mM EDTA were added to the solution and incubated for 1 hour to reduce any unwanted disulfide bonds between pNIPAAm units and promote pNIPAAm grafting efficiency. Capping of free vinyl sulfone groups on the HyA backbone was initiated by adding 10mM D-cysteine (Sigma-Aldrich) and allowing the cysteines to react with available vinyl sulfone groups for 3 hours at 4°C. The HyA-NIPAAm solution was then exhaustively dialyzed against PBS using a 100 kDa MWCO membrane for multiple days and lyophilized for storage. To confirm that the pNIPAAm had indeed grafted to the HyA backbone, HyA-NIPAAm copolymers were analyzed via ¹H NMR spectroscopy. Here, the relative ratio of HyA peaks to pNIPAAm peaks in the copolymer was determined to assess the presence and degree of NIPAAm substitution.

Additionally, copolymers were also preliminarily assessed via size exclusion chromatography and multiangle light scattering (SEC-MALS) to verify grafting and NIPAAm conjugation to the HyA backbone as previously described. The SEC-MALS setup used consisted of three parts: (1) an Agilent HPLC 1100, (2) a DAWN-Helios II multiangle light scattering detector and (3) an Optilab relative refractive interferometer (Wyatt Technologies, Santa Barbara, CA). Prior to analysis, HyA-NIPAAm graft copolymers or non-functionalized HyA controls were resuspended at 0.5-1 mg/mL in PBS. Samples were then sterile-filtered through 0.45 μ m filter and injected as 400 μ L samples at 0.2 – 0.5 mg/mL. Separation of HyA and pNIPAAm was performed using a Shodex OH pak SB-806 HQ column (Showa Denko America, New York, NY). For batch analysis, wavelength of the diode array detector was set to 280 nm to measure UV absorbance and refractive index changes were measured differentially with a 690 nm laser. To get a rough estimate of grafting efficiency, dn/dc values and UV extinction coefficients measured with a spectrophotometer for unmodified HyA were obtained from the literature. Data analysis to look at changes in molar mass between HyA-NIPAAm and unmodified HyA were carried out using the Astra VI software package (Wyatt Technologies).

Generating AcHyA-Peptide and HyA-NIPAAm Blends

To generate blends of AcHyA variants and HyA-NIPAAm for rheological analysis, lyophilized AcHyA-bspRGD or AcHyA alone was dissolved in PBS at 1, 2, or 3% (wt/vol) in the presence of 10% (wt/vol) HyA-NIPAAm. Polymers were allowed to dissolve at 4°C under constant mixing for 4-16 hours to ensure physical entanglement or blending. To ensure that samples were fully blended, HyA-NIPAAm solutions were molded into circular disks approximately 1-2 mm in thickness and visually inspected via microscopy for homogeneity. Blended hydrogels were also mechanically inspected using parallel plate rheometry.

For cell culture, both AcHyA-peptide or AcHyA alone and HA-NIPAAm were sterilized in ethanol for 2 hours. The ethanol was then allowed to evaporate in a sterile culture hood and the sterile AcHyA variants and HA-NIPAAm copolymers were blended as described above in TESR-E8 medium (Stem Cell Technologies) to produce 1% (wt/vol) AcHyA and 10% (wt/vol) HA-NIPAAm blended hydrogel for hPSC culture.

Rheology of AcHyA-Peptide and HyA-PNIPAAm Hydrogels

Hydrogel blends of 10 wt/vol% HyA-NIPAAm and either 1, 2 or 3 wt/vol% of AcHyA-bspRGD or AcHyA were synthesized as described above. Viscoelastic properties of the hydrogel blends were determined by an oscillatory rheometer (MCR 302 Modular Compact Rheometer, Anton Paar, Ashland, VA) with a parallel plate geometry (8 mm) under a constant strain (10%) and frequency (0.1 Hz). Temperature sweeps of 4°C to 40°C (at a rate of 1-2°C/min) were analyzed to determine blend compositions that closely match the viscoelastic properties of the native HyA-NIPAAm system²³.

Expansion and Maintenance of hPSCs in AcHyA-Peptide and HyA-NIPAAm Blends

Sterilized HyA-NIPAAm blends, generated as described above, were dissolved in cold TESR-E8 supplemented 10 μ M Y-27632. Both H1 hESCs and iPSCs derived from 2D culture on Matrigel were encapsulated within these hydrogel blends as described in the protocol by Lei *et al*²². Briefly, hPSCs grown on Matrigel were treated with Accutase (Invitrogen, Carlsbad, CA) for 5 min. at 37°C to release the cells from their culture surface. Following treatment with Accutase (StemCell Technologies), cell suspensions were collected and placed on ice. To singularize the cells, cell suspensions were vigorously pipetted with a low volume pipette and passed through a 40 μ m cell strainer (ThermoFisher Scientific). The singularized cell solution was then pelleted via centrifugation at 1000 $\times$ g for 5 min. and resuspended in the cold solutions of HyA-peptide blends or PEG-NIPAAm control, MeBiol (Cosmo Bio USA, Carlsbad, CA), at a density of 1 $\times$ 10⁶ cells per 100 μ L of gel. Cell-gel suspensions were then pipetted into cell culture inserts in 24-well culture plates and incubated at 37°C for approximately 15min. to allow the hydrogels to solidify. Once gels had solidified, TESR-E8 basal medium supplemented with 10 μ M Y-27632 and 1% penicillin-streptomycin (ThermoFisher Scientific) were added to insert and the insert well and replaced at 37°C for long-term culture. After a week of culture or when hPSC clusters reached a desired density, ice cold PBS was added to each well insert to reliquify the peptide-conjugated hydrogel blend. The liquefied cell-gel solution was then centrifuged for 1000 $\times$ g for 5 min. to pellet cell clusters. The gel was removed and the cell pellet was exposed to Accutase at 37°C for 12 min. to aid in breaking cell clusters. Cells were then singularized and recultured as described above. At the end of two passages, cell clusters in 3D gels were fixed with 4% (vol/vol) paraformaldehyde at 37°C for 30 min. Cultures stained with 4,6-diamidino-2-phenylindole (DAPI, ThermoFisher Scientific) according to manufacturer's protocol to visualize nuclei. Stained images were analyzed for via the ImageJ software package (National Institutes of Health, Bethesda, MD).

Statistical Analysis

Unless otherwise noted, data were presented as mean $\pm$ s.d. For single comparisons, a two-sided Student's *t*-test was used. For multiple comparisons, one-way analysis of variance was used with *post hoc* Dunnet's correction. $p < 0.05$ was regarded as significant.

3.4 Results and Discussion

Targeted Peptides can be conjugated to Acrylated Hyaluronic Acid (AcHyA)

The overall goal of this work was to generate a robust culture system with peptide-based adhesive motifs that could support the long-term adhesion and expansion of undifferentiated hPSCs. Previously, we had identified key, targeted peptides with a strong affinity for hESC integrin and syndecan receptors via bacterial display-mediated screening. Through microculture techniques, we had shown that these peptides could be presented as tightly-packed, self-assembled monolayers (SAMs) that mediate the 2D adhesion and proliferation of both hESCs and hiPSCs. However, while 2D culture systems represent a viable, initial platform for testing and optimizing binding peptide candidates, large-scale hPSC expansion requires volumetric growth. Over the last decade, several hydrogel materials – including alginate^{20,34}, agarose³⁵ and hyaluronic acid^{22,25} – have been investigated for both hPSC expansion and differentiation. As a naturally occurring component of the early embryo and a ligand for the cell surface glycoproteins²⁴, CD44³⁶ and CD168³⁷, hyaluronic acid (HyA) in particular has been widely utilized to generate hPSC culture systems²⁵. However, in most cases, these hyaluronic acid-based platforms are composed of unfunctionalized polymer variants and thus poorly mimic the highly adhesive and bioactive milieu of the inner cell mass.

To explore how the addition of four targeted peptides, shown in **Table 3.1**, could enhance the adhesivity of hyaluronic acid, we employed well-characterized functionalization strategies to acrylate hyaluronic acid and conjugate thiolated peptides to these acrylates via a Michael type addition reactions. The employed scheme is shown in **Figure 3.1**. To acrylate hyaluronic acid, a two-step synthesis strategy was leveraged. In the initial step, carboxylic acid groups on the 750 kDa hyaluronic acid were converted to hydrazide groups through the addition of ADH. These hydrazide groups were then further functionalized to form acrylate groups (shown in blue) through the addition of N-acryloxysuccinimide (**Figure 3.1A**). To quantify the efficiency of this reaction, acrylated hyaluronic acid was subjected to ¹H NMR analysis and the relative ratios of acrylate peaks – appearing at 5.5 to 6.5 ppm – and HyA peaks (at 2.0 ppm) were compared. Approximately 30% of carboxylic acid groups on the HyA backbone were converted to functional acrylate groups, allowing for sufficient downstream peptide conjugation.

Table 3.1: Integrin- and Syndecan-Binding Peptides conjugated to Acrylated HyA backbone

Peptide Name	Sequence	ECM Source	Binding Partner
bspRGD(15)	CGNGEPRGDTYRAYK	Bone sialoprotein	$\alpha_v\beta_3$ integrin ³⁸
Ag73	CGGRKLRQVQLSIRT	Laminin	Syndecan-1 ³⁹
7C-1	CMGDGRPRG	derived via screening	$\alpha_6\beta_1$ integrin
15-23	CGKPLRAKREITKLKFG	derived via screening	unknown

depicting the two-step chemical synthesis strategy utilized to generate acrylate hyaluronic acid (AcHyA). Initially, 750 kDa to 1MDa HyA was reacted with adipic acid dihydrazide (ADH) in the presence of EDC and HOBt to convert carboxylic acid groups on the HyA backbone into hydrazide moieties. Next, hydrazide-containing variants were reacted with N-acryloxysuccinimide (NAS) to generate an acrylated hyaluronic acid variant (acrylate group shown in blue). (b) Scheme for generating peptide-AcHyA conjugates. Via Michael addition chemistry, cysteine-modified target peptides could be reacted with AcHyA to generate conjugates with approximately 20% peptide substitution. (c) The efficiency of both acrylation and subsequent peptide functionalization could be qualitatively verified through ^1H NMR. Peaks associated with acrylation occur at 5.5 to 6.5 ppm (blue highlighted box) while those associate with bspRGD conjugation coincide with hyaluronic acid peaks (green highlighted box). Acrylation efficiencies can be surmised by comparing acrylate peaks to that of hyaluronic acid (gray highlighted box).

Acrylates represent a highly labile functional group and are particularly reactive to thiols^{30,31}. By adding N-terminal cysteines to our target peptide sequences, these peptides could then be coupled to the AcHyA backbone for biofunctional interactions within a 3D gel. By employing a Michael type addition reaction, we generated several AcHyA-peptide conjugates as shown in **Figure 3.1B**. AcHyA-bspRGD conjugates were characterized via ^1H NMR for the presence of peaks associated with functional, unreacted acrylate sites (blue box, **Figure 3.1C**) as well as bspRGD (green box, **Figure 3.1C**). Since bspRGD and other small peptides do not produce distinct and easily recognizable peaks for characterization via NMR, peptide coupling was also verified directly through a BCA assay and indirectly via acrylate peak reduction in the NMR spectra of peptide-conjugated AcHyA. Through BCA analysis, it was determined that 1 wt% AcHyA-bspRGD hydrogels contained approximately 8.5 mg/mL of peptide. Indirect analysis of acrylate peak reduction following peptide conjugation suggested that 20% of open acrylate sites were quenched by thiolated target peptides.

Having verified peptide conjugation, we now explored strategies for presenting peptide-HyA conjugates in a three-dimensional context. While available acrylate groups within these conjugates have the intrinsic capacity to cross-link in the presence of UV light or a bifunctional thiol crosslinker³¹, AcHyA gels generated through these strategies lack important mechanical and thermoreversible properties needed for long-term hPSC expansion. Namely, without thermoreversible components, these AcHyA gels must be degraded through use of hyaluronidase, making long-term, large-scale passaging of resident stem cells both cumbersome and untenable. To circumvent this challenge, we instead leveraged a hyaluronic acid-based thermoreversible graft copolymer designed by Ekerdt *et al*³. In this system, HyA is functionalized with NIPAAm through a Michael addition scheme and characterized via ^1H NMR, to confirm the presence of NIPAAm in the co-polymer, and SEC-MALS, to validate the physical engraftment of NIPAAm to the HyA backbone (**Figure S1 and S2, Appendix II**). Once generated, the HyA-NIPAAm conjugates undergo state transitions based on temperature⁴⁰. For example, below its lower critical solution temperature (LCST), the grafted NIPAAm hydrogen bonds with water generating an aqueous polymer solution. However, above its LCST, amide groups within the NIPAAm microphase separate, generating physical crosslinks between HyA chains⁴¹. This results in the bulk transition of the polymer from a liquid phase to a hydrogel state. Since this HyA-NIPAAm system does not naturally present growth factors or adhesive motifs, peptide-displaying AcHyA conjugates were blended in at low concentrations to generate bioactive gels of varying stiffness with preserved thermoreversible properties (**Figure 3.2B & C**). By tuning the relative concentration of each polymer component in the blend, the material properties of the hydrogel could then be optimized for the long-term expansion of both undifferentiated hESCs and hiPSCs.

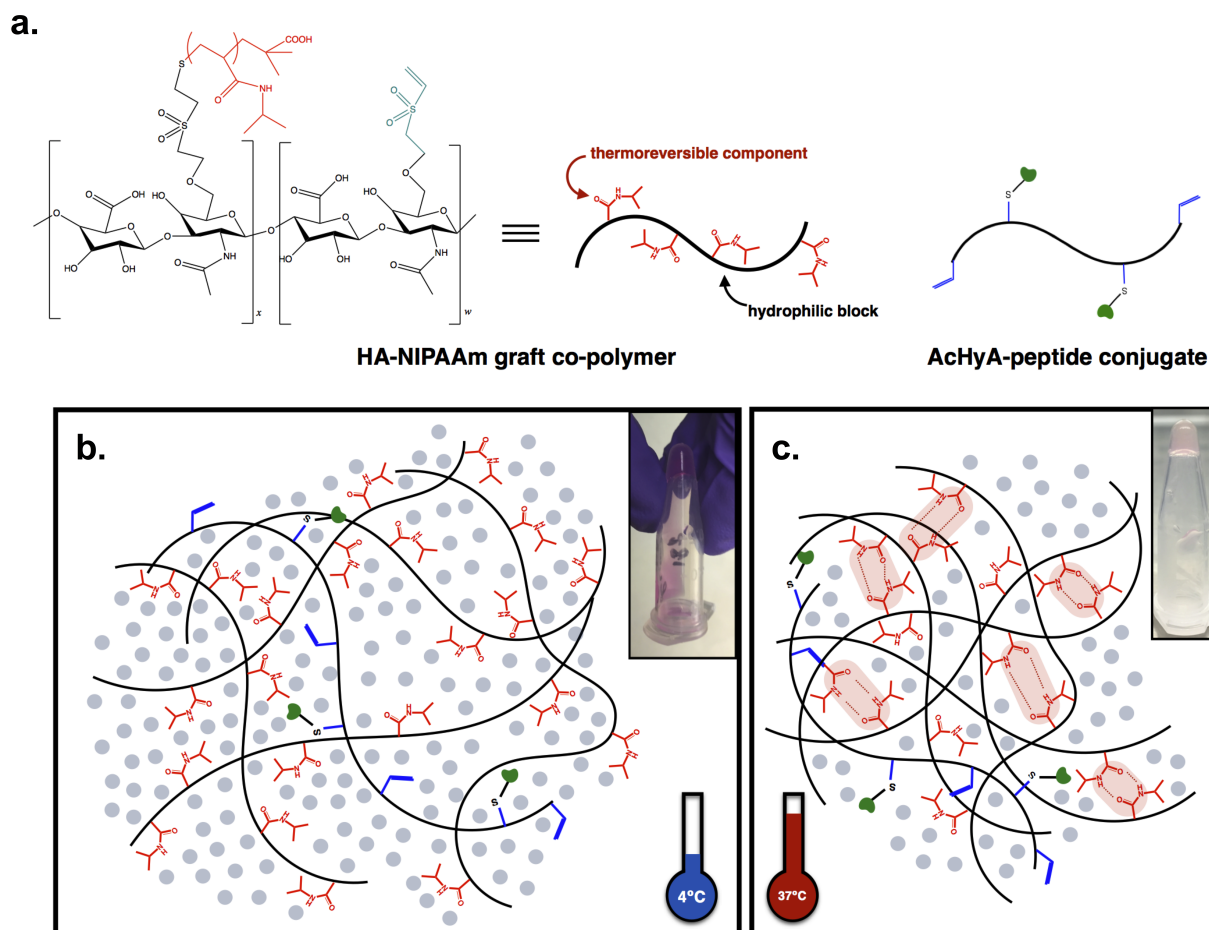


Figure 3.2: Generation of HyA-NIPAAm and AcHyA-peptide blends. (a) Schematic of the two components used to generate the blended hydrogel: (1) HyA-NIPAAm copolymer and (2) AcHyA-peptide conjugate. The HyA-NIPAAm is composed a thermoreversible NIPAAm graft and a hydrophilic backbone. Blended gels were generated by adding between 0.5-3wt% AcHyA-peptide conjugates to a 10 wt% HyA-NIPAAm gel. (b) Below its lower critical solution temperature (32°C), amide groups on the NIPAAm hydrogen bond with the surrounding water, creating an aqueous polymer solution (inset). (c) Above the lower critical solution temperature, the amide groups in the NIPAAm grafts hydrogen bond with one another, initiating a microphase separation into hydrophobic domains that exclude water. This causes the polymer to transition from a solution to a solid hydrogel (inset).

Material Properties of AcHyA-Peptide and HyA-PNIPAAm Blends

To generate thermoreversible HyA blends that were amenable to long-term hPSC culture, we utilized a 10wt% HyA-NIPAAm hydrogel²³ and titrated in varying concentrations (0.5-3 wt%) of our bioactive component – the AcHyA-peptide conjugates. These hydrogels were then subjected to rheological analysis to measure the storage and loss modulus of each blend as a function of temperature. Previous work with both hyaluronic acid and other 3D polymer systems had shown that the stiffness of the surrounding matrix is an essential emergent property of the culture system that

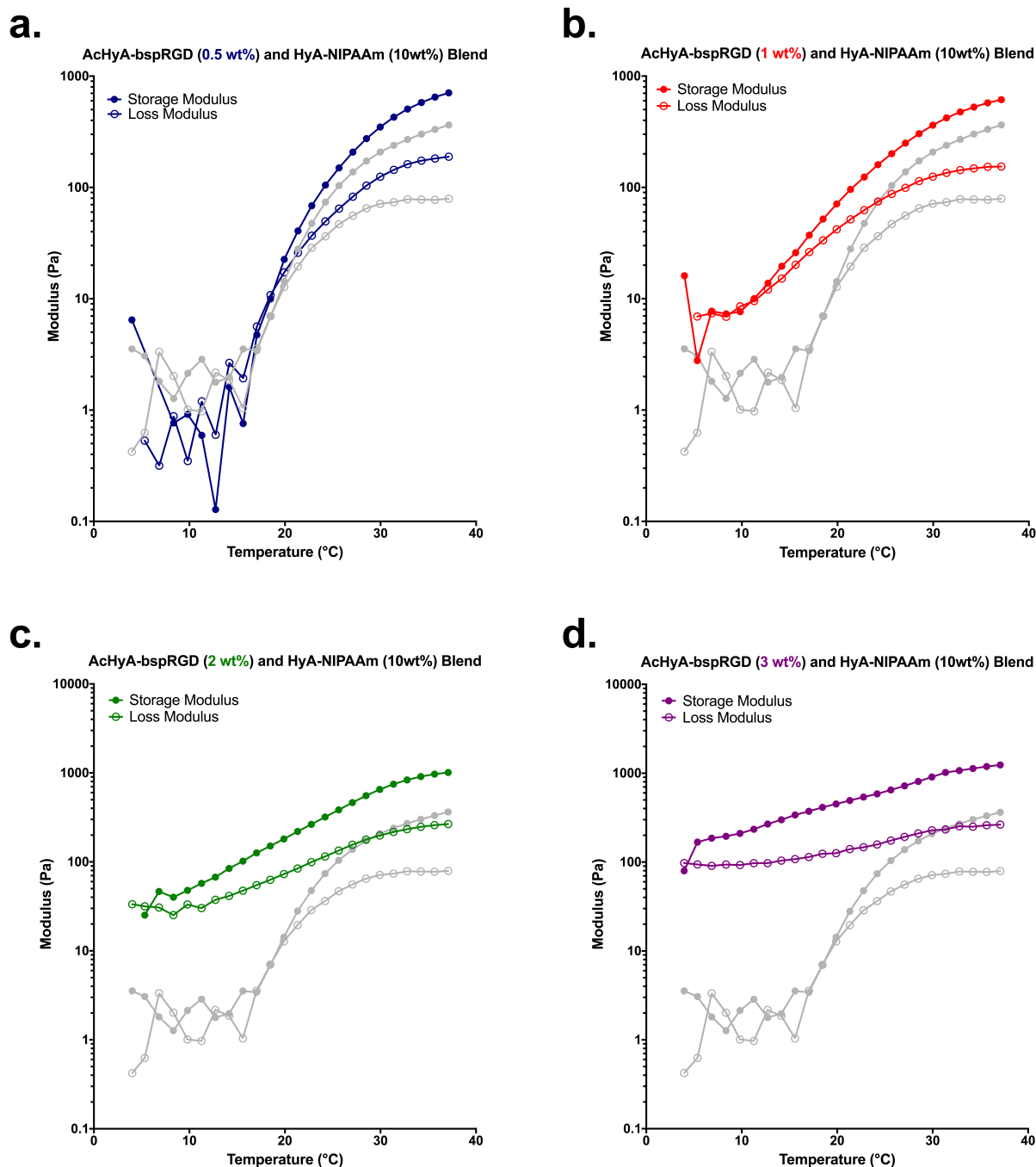


Figure 3.3: Rheological Characterization of HyA-NIPAAm and AcHyA-peptide blends. The storage (closed circle) and loss modulus (open circle) as a function of temperature for HyA-NIPAAm and AcHyA-bspRGD(15) blends containing (a) 0.5 wt% AcHyA-bspRGD(15), (b) 1 wt% AcHyA-bspRGD(15), (c) 2 wt% AcHyA-bspRGD(15) and (d) 3 wt% AcHyA-bspRGD(15). For comparison, the storage (closed circle) and loss (open circle) moduli of the base HyA-NIPAAm polymer (no added AcHyA-bspRGD) are shown in each panel in gray.

guides the viability, proliferation and differentiation of resident hPSCs^{42–45}. Interestingly, *in vivo*, hESCs reside in a soft, ECM-sparse microenvironment of about 400–600 Pa⁴⁶. However, viable polymeric culture systems *ex vivo* display storage and loss moduli that are either on par with that of the inner cell mass – 600 Pa polyacrylamide gels⁴³ – or considerably stiffer – 2 to 10 kPa methacrylated hyaluronic acid⁴⁷. The diversity in mechanical properties among *in vitro* polymeric culture systems suggests that while matrix stiffness is a key parameter affecting hPSCs, it likely synergizes or complements dominant chemical cues presented by the polymer or culture system to regulate hPSC fate¹¹.

Given the complexity of matrix mechanical cues within the *in vitro* hPSC niche, we sought to develop bioactive blends with either 0.5, 1, 2, or 3 wt% AcHyA-peptide that closely mimicked the mechanical properties of the previously optimized HyA-NIPAAm polymer system²³. **Figure 3.3** shows the storage modulus – a measure of a hydrogel’s elastic properties – and the loss modulus – a measure of the viscous properties of a hydrogel – for each polymer blend as a function of temperature. Unsurprisingly, all four blends showed storage and loss moduli that were slightly higher than the HyA-NIPAAm system at 37°C – a characteristic that can be attributed to the higher overall polymer concentration in these blends. More importantly, however, since these hydrogels were thermoreversible like their HyA-NIPAAm base, we expected to see each blend undergo a *sol-gel* transition and display a substantial increase in storage and loss moduli at its lower critical solution temperature (LCST). While this was the case for blended hydrogels containing lower concentrations of AcHyA (**Figure 3.3A & B**), hydrogel blends that had a more sizable fraction of the non-thermoreversible AcHyA-peptide conjugate displayed more subtle modulus changes as function of temperature (**Figure 3.3C & D**). The loss of stark thermoreversibility in 2 and 3 wt% AcHyA-peptide blends suggests that the dominant presence of peptide conjugates interferes with the capacity of NIPAAm grafts to hydrogen bond with another and microphase separate. Thus, to preserve the thermoreversible and mechanical functionality of our culture system, we used 0.5 wt% AcHyA-peptide and HyA-NIPAAm blends for further biological characterization.

Presence of Targeted Peptides in Blended HyA-peptide Hydrogels enhances hPSC Expansion

Having characterized the rheological properties associated with our peptide-expressing polymer blends, we now sought to identify if these hydrogels could serve as a long-term culture platform for hPSCs. In particular, given the poor adhesion of hPSCs to unfunctionalized HyA and HyA-NIPAAm backbones^{4,25}, we were curious if the presence of adhesive conjugates within our blended hydrogels could enhance hPSC expansion in TESR-E8, a low-growth factor medium. Previous work had shown that functionalization of two-dimensional culture surfaces with vitronectin-derived motifs that activate key integrins, namely $\alpha_v\beta_3$ and $\alpha_6\beta_1$ integrin, can support the five-fold expansion and clonal outgrowth of both H1 and H7 hESCs⁴⁸. Therefore, we were interested in investigating the role of four adhesive peptides, including two canonical motifs (bspRGD and Ag73) and a single novel peptide (7C-1), in supporting hPSC proliferation.

To qualitatively assess proliferation, singularized hiPSCs were encapsulated in HyA polymer blends containing a single target peptide and grown for two passages in TESR-E8. Since these cells grew clonally (**Figure 3.4A**) as three-dimensional clusters over the course of culture, hiPSC cluster size within each hydrogel blend could be used as a qualitative measure of proliferation. At the end of two passages, hiPSCs were fixed *in situ* and stained with 4,6-diamidino-2-phenylindole (DAPI) to visualize the encapsulated clusters. **Figure 3.4** illustrates the encapsulated and stained hiPSC clusters within each peptide-displaying HyA blend after a single passage. While HyA-NIPAAm itself promoted significant 10-fold expansion of hiPSCs (**Figure 3.4B**), the addition of non-peptide containing acrylated HyA to these polymer systems reduced the average size of encapsulated clusters

by three-fold (**Figure 3.4C**). Although TUNEL staining and other live-dead assays were not performed on encapsulated hiPSCs, it is likely that unquenched acrylates represented a cell-toxic element in these polymer blends. When these acrylates were quenched with any of the target peptides (**Figure 3.4D-F**) – bspRGD, Ag73 or 7C-1 – large cluster (i.e. $>1000 \mu\text{m}^2$ in projected area) phenotypes were rescued.

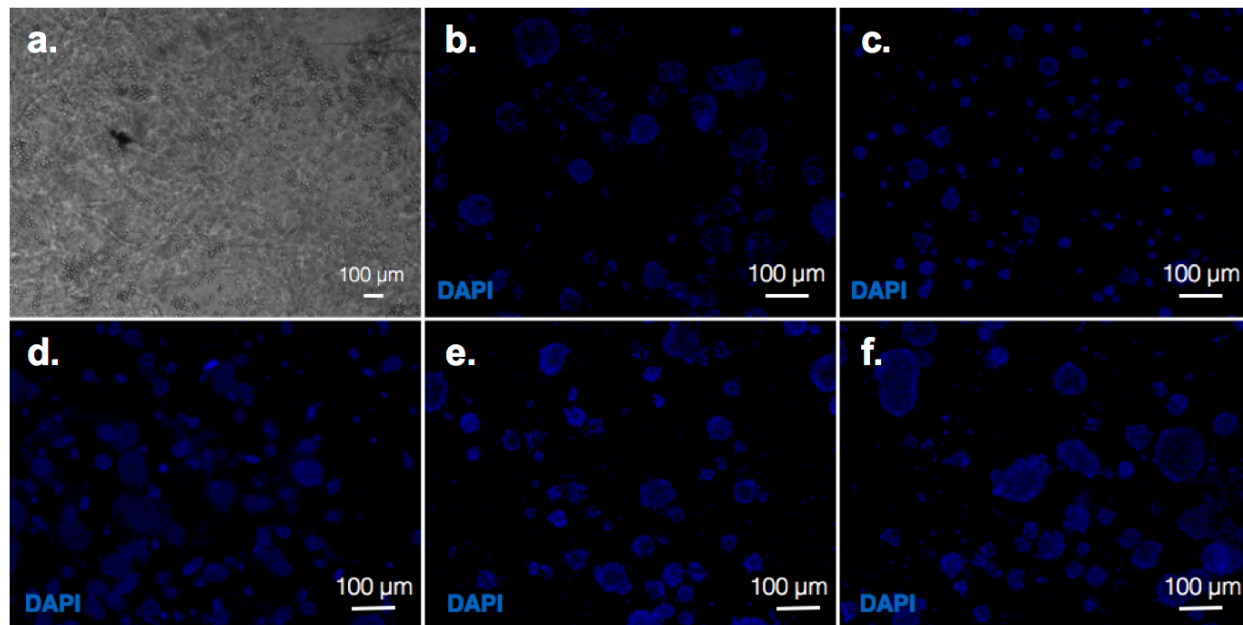


Figure 3.4: Human iPSC Culture in Peptide-Expressing HyA-NIPAAm Blends. Human iPSCs were encapsulated as (a) single cells at a density of 10^6 cells per $50 \mu\text{L}$ HyA-NIPAAm blended hydrogels and grown for two passages. (b-f) Human iPSC clusters after four days of culture in (b) HyA-NIPAAm alone, (c) HyA-NIPAAm with unconjugated AcHyA, (d) HyA-NIPAAm with AcHyA-bspRGD(15), (e) HyA-NIPAAm with AcHyA-Ag73 and (f) HyA-NIPAAm with AcHyA-cyclic(7C-1). Average cluster size was analyzed through ImageJ (data not schematized). Scale bars represent $100 \mu\text{m}$.

While blended hydrogels with target peptides supported hiPSC expansion for two passages, there were observable differences in both the size and morphology of clusters generated by each peptide-presenting hydrogel. For example, while bspRGD(15) represents a canonical $\alpha_v\beta_3$ -binding motif in fibronectin³⁸, its presence produced clusters with a cross-sectional or projected area of $2000 \mu\text{m}^2$ – 40% smaller than those generated in HyA-NIPAAm alone (**Figure 3.4D**). Surprisingly, Ag73, the syndecan-1 binding motif³⁹ that had proved to be essential in 2D self-assembled monolayer cultures, also generated clusters on par with those in HyA-NIPAAm controls (**Figure 3.4E**). By contrast, hydrogels presenting the $\alpha_6\beta_1$ integrin-binding cyclic peptide showed a strong capacity to support the generation of large hiPSC clusters with an average cross-sectional area of $3500 \mu\text{m}^2$ (**Figure 3.4F**). Although unexplored, it is likely that the effectiveness of the 7C-1 peptide in this 3D context can be attributed to its secondary structure and the increased accessibility of adhesive motifs within the constrained, cyclized sequence. In the 3D context, where motifs are more sparsely presented, secondary structure and the presentation of adhesive motifs may play a more critical role than in self-assembled monolayers where structural elements of a sequence can be masked by high

peptide density⁴⁹. Thus, these qualitative data suggest that while HyA-NIPAAm can support long-term hPSC culture, the presentation of key integrin-binding motifs – particularly cyclized ligands – can selectively enhance proliferation of hPSCs in these systems by 2- to 3-fold.

3.5 Conclusions

Developing three-dimensional (3D) biomaterials for *in vitro* human pluripotent stem cell (hPSC) expansion is key to a wide-range of therapeutic and research applications including cell-replacement therapies⁵, drug discovery and disease modeling⁶. While a number of polymeric culture systems exist^{22,23,25}, most of their constituent polymers are undecorated and lack critical bioactive motifs required for engaging stem cell surface receptors, particularly integrins. Without sufficient integrin engagement, hPSCs grown in these culture systems show poor long-term adhesion and proliferation^{4,25}. In this chapter, we highlighted some preliminary efforts to biofunctionalize an HyA-NIPAAm culture system previously shown to support the long-term culture of both hESCs and hiPSCs. By blending in acrylated hyaluronic acid functionalized with three target integrin-³⁸ and syndecan-binding³⁹ peptides – bspRGD(15), Ag73 and a novel $\alpha_6\beta_1$ integrin-binding motif (7C-1) identified through unbiased screens – we were able to show that the cyclized 7C-1 peptide could enhance hiPSC expansion in HyA-NIPAAm. Given the preliminary efficacy of these individual target peptides, we anticipate that optimization of the type and quantity of integrin-based motifs presented in HyA-NIPAAm can help generate a scalable yet bioactive blended culture system that promotes both the adhesion and the proliferation of undifferentiated hiPSCs.

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Chapter 4: Summary & Future Directions

4.1 Summary

This dissertation explored a fundamental question of how we might engineer complex yet scalable synthetic niches for the *ex vivo* culture of human pluripotent stem cells (hPSCs) – a cell type with enormous therapeutic potential in *in vitro* disease modeling¹ and *in vivo* cell replacement therapies². However, before we could embark on engineering such a synthetic microenvironment, it was important to glean the design principles associated with building a successful and viable ‘niche’. From this standpoint, understanding how human embryonic stem cells (hESCs) – the body’s only pluripotent cell type – build, adapt and respond to their native microenvironment within the early embryo was critical.

In Chapter 1, we reviewed the literature on these native niches and considered the soluble cues, cell-cell mediated interactions and extracellular matrix (ECM) signaling that guide hESC fate in early development³⁻⁵. Through these explorations, we highlighted some of the key elements constituting a successful stem cell niche including (1) biophysical cues intrinsic to the ECM as well as (2) biochemical cues in the form of ECM-tethered growth factors and adhesive motifs⁶. We then explored some of the attempts made over the last two decades to replicate these cues *ex vivo* and the animal derived ECM proteins⁷ as well as synthetic substrates⁸⁻¹² that have been generated as a result. While some of these materials have proved successful for research-scale expansion of hPSCs, we also considered the drawbacks associated with using these natural and synthetic matrices to meet clinical demands for stem cell-derived therapies. Chief among these drawbacks was the difficulty in reliably scaling the synthesis of these materials while preserving their bioactivity^{13,14}.

To tackle these challenges and produce scalable, bioactive culture materials, in Chapter 2, we explored a series of high-throughput strategies for identifying short peptides that could serve as nascent hPSC culture surfaces. While a number of adhesive motifs have been previously identified, such sequences have served as targeted ligands for a limited subset of integrins¹⁵⁻¹⁷. Meanwhile, studies from our lab have shown that hESCs display a diversity of cell surface receptors¹⁸ – principally $\alpha_6\beta_1$ integrin – with few known adhesive motifs. Without structural information to guide rational peptide design, we instead leveraged an unbiased technique^{19,20}, bacterial peptide display, to screen three 10^{10} -member libraries of novel peptides for those that could bind hESCs via α_6 integrin. Through this panning-based strategy, we were able to identify seven putative cyclic and linear peptide ligands that could engage the α_6 -integrin. We then harnessed biophysical methods^{21,22} to characterize these peptides, particularly their avidity for hESCs and their ability to maintain the short-term adhesion of these cells. However, as we had previously learned, hPSCs rely on a plurality of motifs to mediate binding and maintain pluripotency over several passages²³⁻²⁵. With this in mind, we were curious whether our novel cyclic and linear peptides could interact synergistically with canonical binding motifs for $\alpha_v\beta_3$ integrin and syndecan-1 to enhance stem cell self-renewal. To this end, we adapted a high-throughput microculture platform^{26,27} to print self-assembled monolayers of peptides (SAMs) and screen combinations of both novel and canonical motifs for their capacity to maintain short-term hiPSC culture. By measuring each SAMs’ capacity to promote cell proliferation, pluripotency and adhesive morphology, we were able to identify an optimal triple motif surface – composed of 40%

syndecan-1 binding motifs, 40% $\alpha_v\beta_3$ integrin binding peptides, and 20% $\alpha_6\beta_1$ integrin binding peptides – that supported the self-renewal and expansion of hESCs and hiPSCs over a single passage.

In Chapter 3, we then asked if some of these integrin- and syndecan-binding peptides identified through screening could be used to create scalable, thermoreversible polymer-peptide systems for multi-passage hPSC culture. Although several alginate^{28,29}, hyaluronic acid¹¹ and agarose³⁰ scaffolds have been generated in the past for the long-term culture of hPSCs, these gels have lacked degradable elements requisite for scalable passaging as well as adhesive motifs that promote hPSC adhesion. To develop 3D materials more amenable to large-scale culture, we instead generated a hydrogel blend that was both thermoreversible and functionalizable. Using acrylated hyaluronic acid as the backbone, our screened binding peptides were conjugated via Michael addition chemistry³¹. Conjugates were characterized via ¹H-NMR for polymer acrylation and BCA assay for subsequent peptide conjugation. Peptide-decorated hyaluronic acid was then blended with a previously developed thermoreversible HyA-NIPAAm graft co-polymer^{12,32} to generate bioactive, scalable and defined 3D scaffolds for hPSC culture. Using these scaffolds, we were able to qualitatively show that blends incorporating our α_6 -integrin-binding cyclic peptides could support 12- to 15-fold expansion over a single passage. Thus, our results suggested that these peptides could augment polymeric culture systems and allude to the eventual generation of 3D culture systems with multiple adhesive motifs for long-term hPSC expansion.

4.2 Future Directions and Ancillary Applications

The 2D peptide SAMs and the 3D peptide-functionalized hyaluronic acid blends described in this dissertation represent an important advance on traditional culture substrates like Matrigel and Geltrex. Nevertheless, both the high-throughput screening platforms used to identify these peptides as well as the peptides themselves are versatile and have applications outside of stem cell self-renewal. In this section, we identify some of the applications we are currently exploring for our α_6 -integrin-binding peptides and high-throughput materials screening platforms:

1. **Applications of α_6 -integrin-binding peptides as purification agents:** Since α_6 -integrin is a relatively rare integrin expressed on the surface of a handful stem and differentiated cells²⁴, it can be utilized as a marker for selecting specific cell types within a heterogeneous population. This is particularly important in differentiation cultures where α_6 -integrin-expressing hPSCs give rise to partially or terminally-differentiated cells with substantially different integrin expression profiles³³. By using our screened binding peptides or peptide-HyA conjugates as purification agents, undifferentiated stem cells expressing α_6 -integrin could either be enriched for or purified out of culture depending on the application. This has widespread implications for cell-replacement therapies², where the presence of undifferentiated or stem-like cells could lead to metastatic cancers, as well as *in vitro* cultures, where pure hPSC populations may be highly desired.
2. **Applications of α_6 -integrin-binding peptides in corneal wound healing:** Aside from human pluripotent stem cells, another key cell type expressing α_6 -integrin are epithelial cells in the outermost layer of the cornea³⁴. This corneal epithelium serves an important role in maintaining both the transparency and structural integrity of the cornea as well as protecting the ocular environment against infection³⁵. In normoglycemic individuals, cornea wound healing is naturally mediated and defects in this outer epithelial layer are quickly healed through a combination of soluble factors and substrate-mediated cues in the ECM beneath the epithelium. However, in

patients suffering from diabetes, ulcerations and defects in the epithelium can continue to persist in spite of therapies aimed at promoting wound healing³⁶. Although the biological mechanisms underlying persistent corneal defects is not entirely understood, it is thought that circulating glucose binds critical extracellular matrix proteins^{37,38}, particularly laminin³⁹. As a result, these ECM proteins form glycosylated complexes and are unable to interact with epithelial cell-surface receptors like α_6 -integrin³⁸. Currently, corneal defects are treated through semi synthetic methods that aim to protect the cornea from infection rather than accelerate its reepithelialization⁴⁰. However, keratoprosthesis or artificial cornea with laminin-mimetic elements that are resistant to glycation could restore the cell adhesive functionality of the basement membrane and accelerate reepithelialization. Since our screened target peptides are α_6 -integrin-specific and lack lysine-rich regions most susceptible to glycation^{37,39}, they could serve as ideal laminin-mimetics for promoting corneal wound healing in diabetic patients.

3. **Identification of high-affinity affibody binders using bacterial display:** Library-based screening and selection strategies, as outlined in Chapter 2, represent a method by which high-affinity protein ligands for key receptors can be discovered even in the absence of important structural and functional information about the receptor. In this dissertation, we focused on screening peptide libraries for high-affinity ligands of integrins. However, display techniques are capable of presenting a more versatile range of proteins including antibodies⁴¹, affibodies – a class of alpha helical peptides⁴² – and DARPins – a class of synthetic ankyrin repeat proteins^{43,44}. These complex proteins, because of their increased diversity of secondary structures and multiple adhesive motifs, can often serve as more potent binders of cellular and molecular targets than either cyclic or linear peptides. While both DARPins and antibodies have been screened via phage or yeast display in the past^{41,43,44}, little work has been done to adapt these screens to a bacterial platform. We have shown, as a proof of concept, that IgG-binding affibodies can maintain their capacity to mediate interactions with rabbit IgG when presented on the C-terminus of bacterial scaffold proteins (**Figure S3, Appendix II**). With the capacity to express affibodies on the bacterial surface, we can now envision generating libraries of more complex proteins and identifying specific binders with nanomolar-scale affinities for a wider range of receptors using bacterial display.

4.3 References

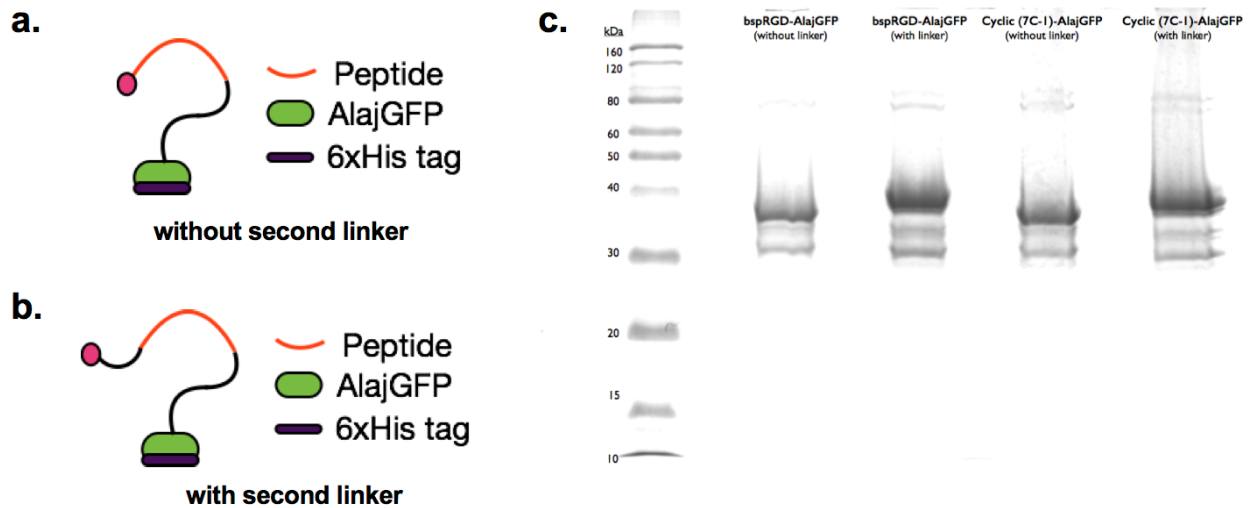
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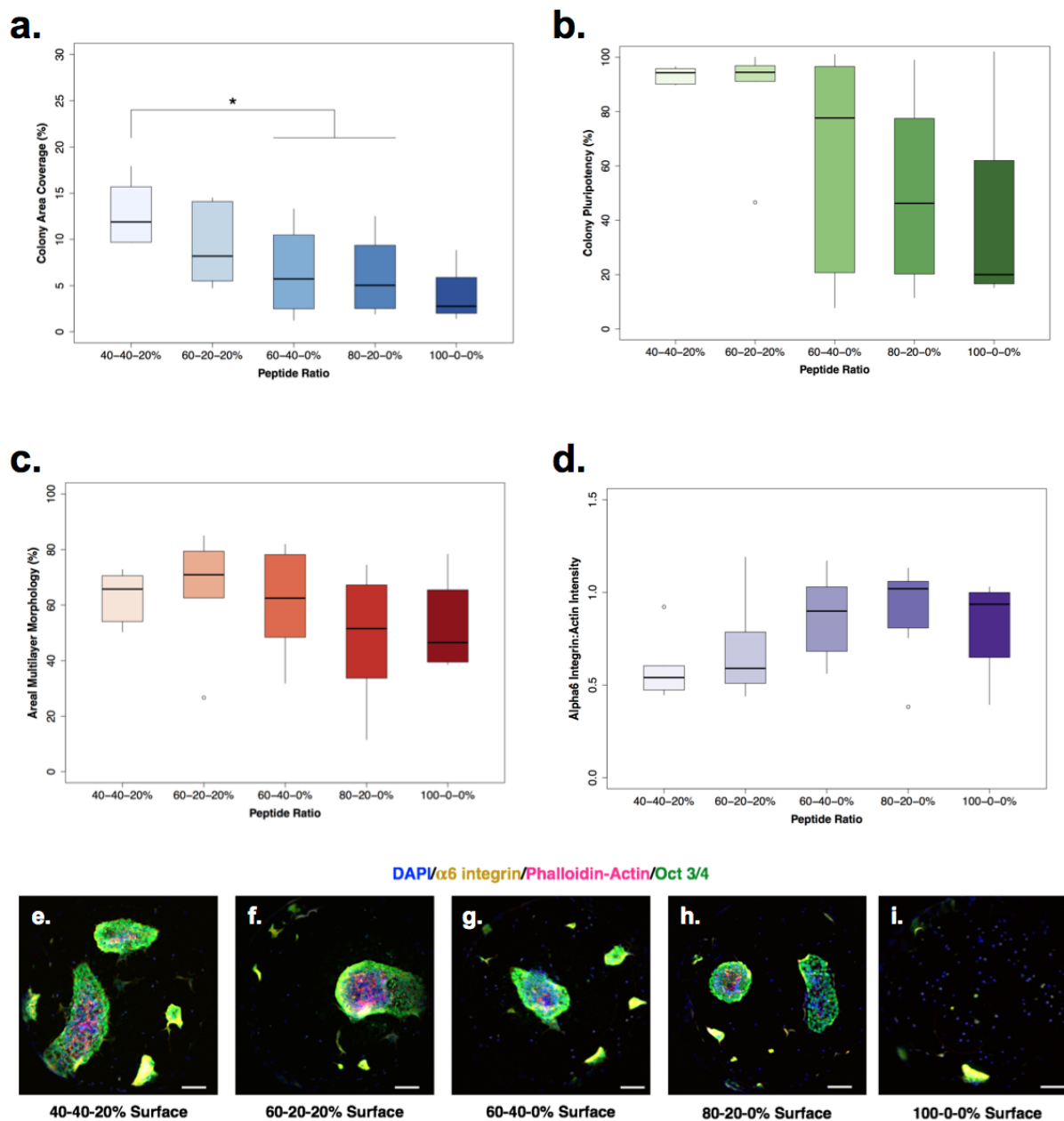
Appendix I: Supplementary Figures and Tables for Chapter 2



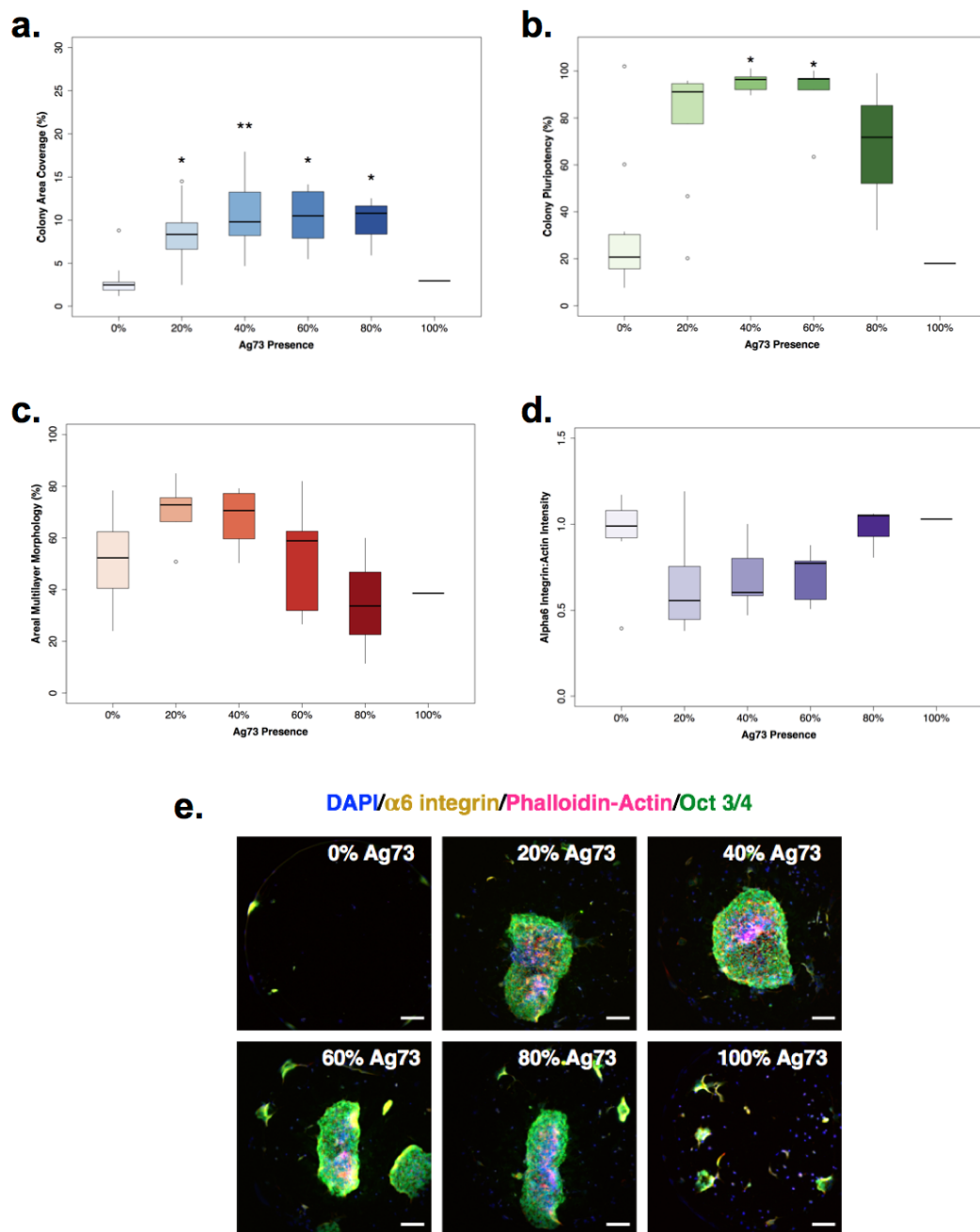
Supplementary Figure I.1: Peptide-AlaJGFP Fusion Proteins for Biophysical & Biochemical Characterization. To test the capacity of each screened candidate peptide to mediate hPSC adhesion, fusion constructs of candidate peptides and alaJGFP were generated. These fusion proteins were of one of two varieties: (a) One variant, denoted “without linker” had a biotinylated peptide (denoted by a fuchsia circle) at the N-terminus followed by a short, flexible Gly-Ser linker connecting the peptide to the AlaJGFP barrel protein. (b) A second variant, denoted “with linker”, presented the peptide between two Gly-Ser linkers. The linker at the N-terminus of the peptide was capped by a biotin while a Gly-Ser linker at the C-terminus joined the peptide to the AlaJGFP barrel protein. Both fusion proteins were capped at the C-terminus with a polyhistidine tag. This tag allowed for protein purification with a Ni-NTA column. (c) Following purification on a Ni-NTA column, the presence of the desired His-tagged fusion protein was confirmed via SDS-PAGE using an InVision™ His-Tag In-Gel Stain. Both cyclic (7C-1) and linear (bspRGD) fusion proteins with and without linker showed dominant banding around 36-38 kDa as expected. The “without linker” construct was used for all further work with recombinant fusion proteins.

Supplementary Table I.1: Antibodies and Dilutions used for Immunofluorescence Experiments

Antibody	Vendor	Catalog Number	Application	Dilution
Rabbit polyclonal anti-Oct3/4	Abcam	ab18967	IF (Fig. 2.4, 2.8)	1:1000
Alexa Fluor® 568 Phalloidin	Invitrogen	A12830	IF (Fig. 2.4, 2.8)	1:100
Mouse monoclonal anti- α_6 integrin	Abcam	ab194969	IF (Fig. 2.8, 2.9)	1:40
Rabbit polyclonal anti-vinculin	Sigma	SAB4503069	IF (Fig. 2.9)	1:200
Goat polyclonal anti-Nanog	Invitrogen	PA5-1406	IF (Fig. 2.9)	1:250
Donkey anti-mouse 488	Jackson	715-545-151	IF (Fig. 2.4, 2.8, 2.9)	1:250
Donkey anti-rabbit 647	Jackson	711-605-152	IF (Fig. 2.4, 2.8, 2.9)	1:250
Donkey anti-goat Rhodamine/TRITC	Jackson	705-025-033	IF (Fig. 2.4, 2.8, 2.9)	1:250

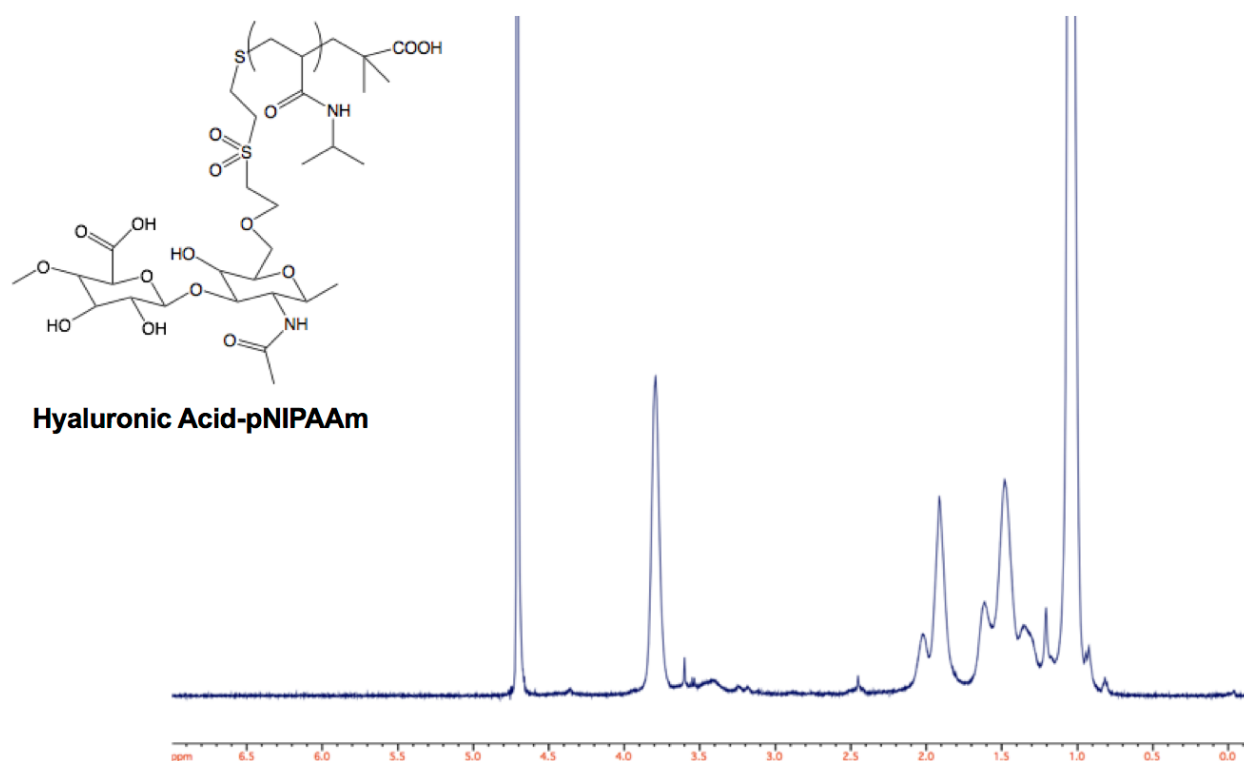


Supplementary Figure I.2: Role of Peptide Ratio on SAM Functionality and hiPSC Microculture Phenotypes. Boxplots of (a) colony area, (b) relative colony pluripotency, (c) multilayer morphology and (d) the ratio of α_6 integrin intensity to F-actin intensity as a function of the ratio of peptides composing the underlying SAM matrix. SAMs composed of a 40-40-20% peptide ratio showed a significantly enhanced capacity to support hiPSC expansion than 60-40-0% or 80-20-0% SAM surfaces (*, $p < 0.05$). Representative microcultures grown on (e) 40-40-20%, (f) 60-20-20%, (g) 60-40-0%, (h) 80-20-0% and (i) 100-0-0% SAM surfaces are shown. Scale bars represent 50 μm . The non-parametric Kruskal-Wallis test was used for statistical analysis followed by a *post-hoc* Dunn's test to analyze specific sample pairs, with * indicating $p < 0.05$. All data is represented as means $\pm$ s.d.

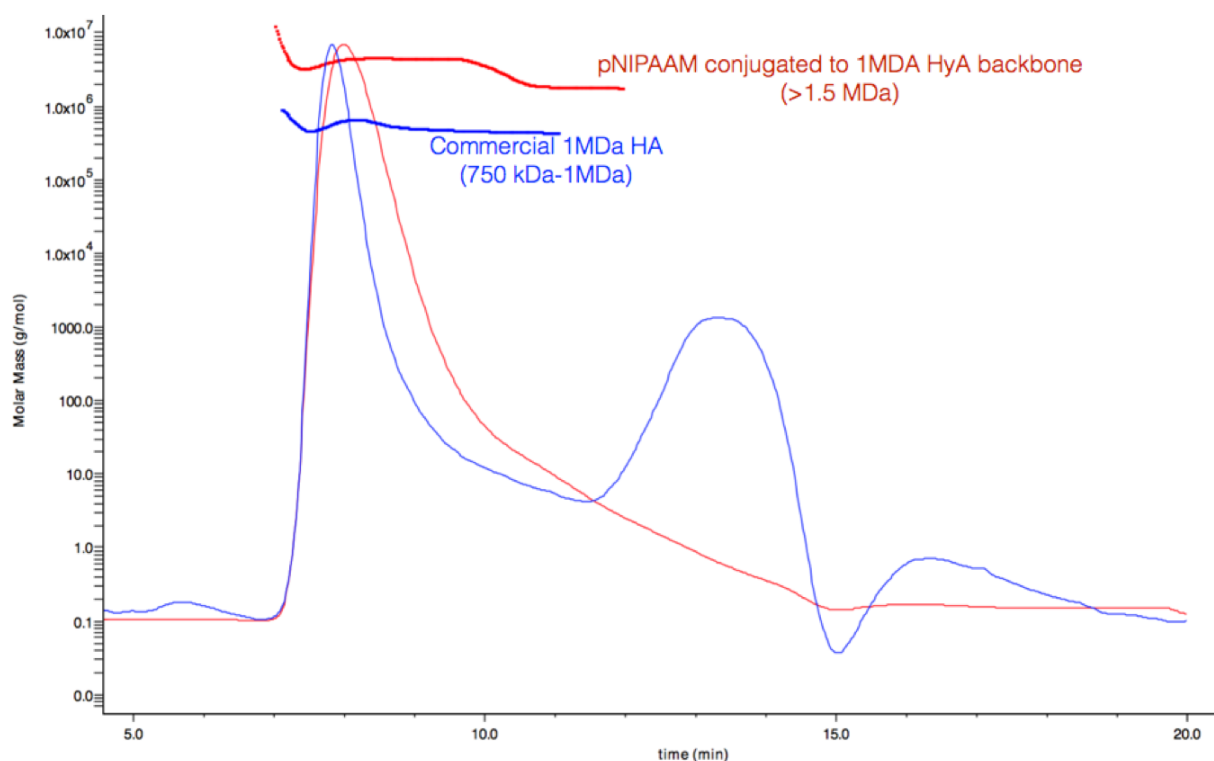


Supplementary Figure I.3: Role of Ag73 on SAM Functionality and hiPSC Microculture Phenotypes. Boxplots of (a) colony area, (b) relative colony pluripotency, (c) multilayer morphology and (d) the ratio of α_6 integrin intensity to F-actin intensity as a function of amount of syndecan binding peptide, Ag73, composing the underlying SAM matrix. SAMs composed of 40% Ag73 showed a significant capacity to support hiPSC expansion (**, $p < 0.01$) and pluripotency (*, $p < 0.05$) as did 60% Ag73 SAMs (*, $p < 0.05$). (e) Representative microcultures grown on SAMs with variable amounts of Ag73. The non-parametric Kruskal-Wallis test was used for statistical analysis followed by a *post-hoc* Dunn's test to analyze specific sample pairs, with * indicating $p < 0.05$, ** indicating $p < 0.01$. All data is represented as means $\pm$ s.d.

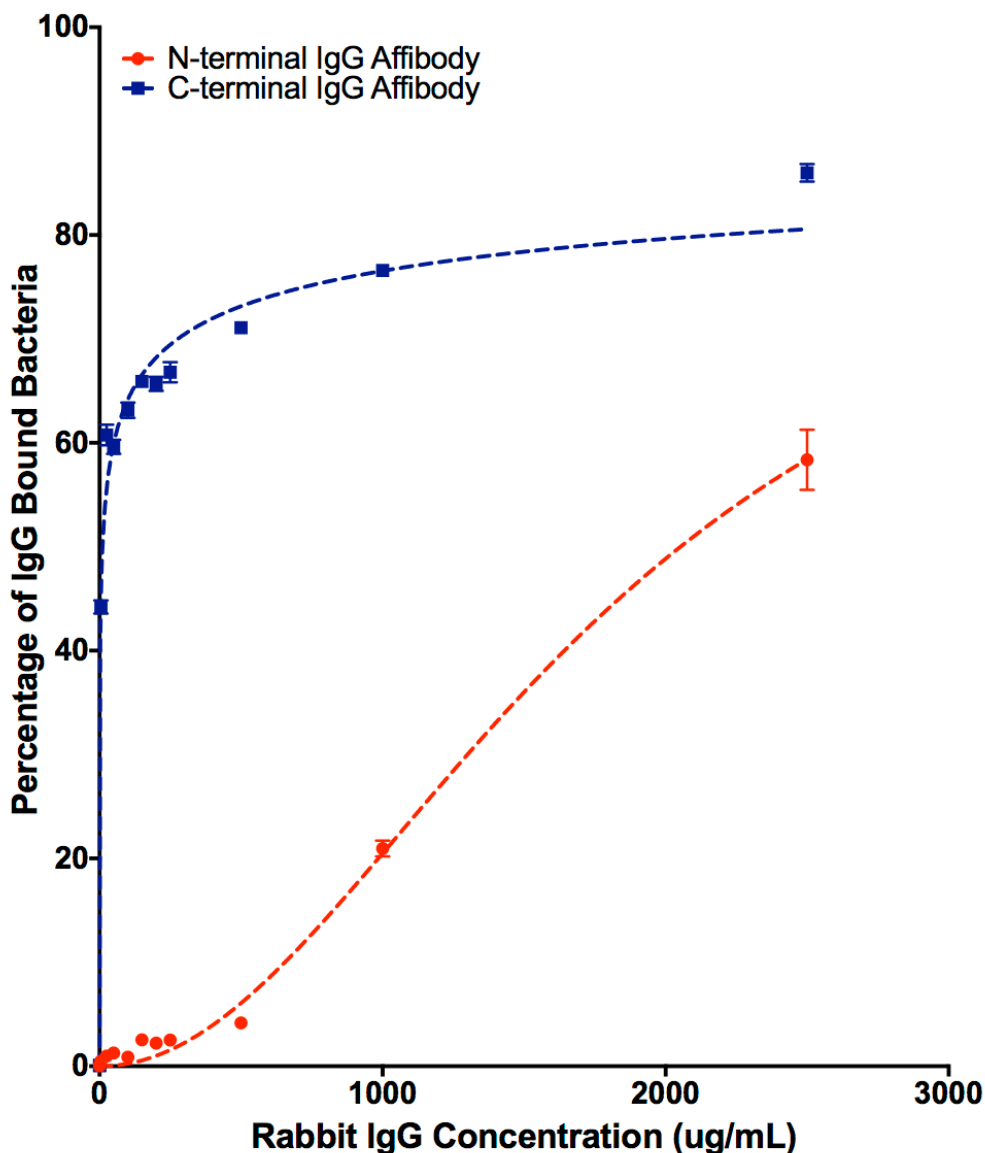
Appendix II: Supplementary Figures and Tables for Chapter 3 & 4



Supplementary Figure II.1: ¹H NMR Spectra for Hyaluronic Acid-pNIPAAm. Hyaluronic acid-pNIPAAm conjugates were generated by reacting vinyl sulfone-functionalized hyaluronic acid variants (50%) with thiolated pNIPAAm (pNIPAAm-SH). The presence of pNIPAAm in the conjugate could then be quantified qualitatively through ¹H NMR. pNIPAAm peaks occur at 3.8 to 4.0 ppm while those associated with hyaluronic acid occur at 2.0 ppm. Thus, by comparing NIPAAm peaks (at 4.0 ppm) with those of hyaluronic acid (at 2.0 ppm), a qualitative estimate of the degree and extent of NIPAAm grafting in the dialyzed HyA conjugate could be determined. The prominence of the pNIPAAm peak in the above spectra (at 3.8 ppm) indirectly suggests that pNIPAAm-SH grafted efficiently to the HyA-vs backbone to generate HyA-pNIPAAm copolymers.



Supplementary Figure II.2: Molecular Mass and NIPAAm Grafting by Multi-angle light scattering (MALS). Although there are a number of ways to determine molar mass or molecular weight of polymeric macromolecules, mutli-angle light scattering (MALS) is the only way to rapidly determine the absolute molecular weight of macromolecules in solution. Here, we utilized MALS to determine the molecular weight of both commercial 1MDa Hyaluronic acid (HyA) (blue trace), used as the conjugate backbone, as well as the presumptive pNIPAAm-conjugated HyA variant (red trace). Depending on the efficiency of the Michael addition reaction, NIPAAm-containing variants would be expected to elute earlier than commercial HyA and MALS would imply a higher molecular weight for the NIPAAm-HyA conjugates. In the above figure, we see that the pNIPAAm-conjugated HyA (red trace) did indeed elute earlier than commercial HyA (blue trace). Based on elution time, the pNIPAAm-conjugated HyA had a molecular mass of roughly 1.5 MDa, suggesting that every unit of HyA had approximately two functional pNIPAAm groups. Equally important, we see a trailing shoulder with decreasing molecular weight on the peak of pNIPAAm-conjugated HyA trace (red), suggesting polydispersity of pNIPAAm-conjugated HyA and the presence of unconjugated pNIPAAm contaminants.



Supplementary Figure II.3: IgG Binding Capacity of Bacterial Surface-displayed IgG Affibodies. To determine whether more complex proteins, such as affibodies and DARPins, could be displayed on the surface of bacteria and screened via flow cytometry, a previously-identified Rabbit IgG binding affibody was displayed as both an N- or C-terminal fusion of the bacterial scaffold protein, CPX. Once the affibody was expressed on the bacterial surface, its capacity to bind an Alexa 488 fluorophore-tagged rabbit IgG was determined via flow cytometry. Interestingly, affibodies expressed as C-terminal fusions of CPX maintained IgG binding functionality and 75% of these affibody-expressing bacteria bound rabbit IgG even at concentrations below 1 mg/mL. By contrast, N-terminal affibodies showed a reduced capacity to bind rabbit IgG at all concentrations, suggesting that these affibodies were incorrectly folded and displayed on the bacterial surface. This data suggests that complex proteins, when presented as C-terminal fusions of scaffold proteins, can maintain functionality for subsequent screening via bacterial display.