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Synthetic Surfaces for Human Embryonic Stem Cell Culture

Andrei G. Fadeev and Zara Melkoumian
Corning Incorporated
USA

1. Introduction

Human embryonic stem cells (hESCs) have two properties that distinguish them from other cell types: self-renewal, the ability to propagate indefinitely in culture, and pluripotency, the ability to differentiate into any type of specialized cells found in the human body. These properties provide the foundation for the development of hESC-derived cell-based therapeutics, where specific cell types derived by differentiation of hESCs become a therapeutic agent that cures the disease or restores the function of damaged organs or tissue. To make this a reality, several technologies must be developed to provide an unlimited and consistent supply of hESC-derived cells for clinical use. These include robust and scalable methods for production of undifferentiated hESCs, differentiation of the hESCs into desirable cell types, recovery, purification, storage and transportation of the derived cells to the location of use, and methods and techniques for delivery of the therapeutic cells to a human body to provide health benefits.

Since the derivation of the first hESC lines by Thomson, J. et al. (Thomson, 1998) and Reubinoff, B. et al. (Reubinoff et al., 2000), hundreds of new lines have been established and propagated under various cell culture conditions. Historically, hESCs were maintained in complex culture systems under poorly defined conditions comprising mouse or human feeder cell layers and medium containing fetal bovine serum (FBS) or serum replacement to provide an extracellular matrix (ECM)-rich environment for cell adhesion, as well as soluble growth factors for self-renewal. It is highly desirable that the cell culture systems utilized for therapeutic cells, including cell culture surfaces and the media, are well defined (all components are known and characterized and their abundance is controlled) and of non-animal origin or xeno-free (do not contain biological materials of a non-human nature).

Establishment of the first human embryonic stem cell line (Thomson, 1998) was accomplished by extending to hESCs a cell culture system developed for culturing mouse embryonic stem cells that is based on inactivated mouse embryonic fibroblasts (MEF) as a feeder layer. Soon after the first reports on isolation of human pluripotent cells came realization that feeder-free cell culture is essential for production of cells for transplantation (Donovan & Gearhart, 2001; Pera et al., 2000); (Pedersen, 2002). Back in 2000 this looked like a challenge that would require a very long time to overcome, as 19 prior years of using MEFs to support stem cell culture of non-human cells did not result in significant understanding of what exactly MEFs provide for stem cells. To make matters worse, there was experimental evidence showing that neither MEF conditioned medium nor ECM

secreted by MEFs alone was sufficient to produce the same outcomes (Thomson, 1998). Ten years later, a fully synthetic cell culture surface (Corning® Synthemax™ Surface), which allows xeno- and serum-free culture and differentiation of therapeutic quality stem cells under chemically defined conditions, became commercially available to address this problem.

In this chapter, we will review the evolution of hESC culture conditions from complex un-defined feeder layer surfaces and FBS-containing medium used initially to derive and maintain hESC in culture, to fully defined, xeno-free culture systems described by multiple independent studies in the past few years. The focus of our review will be a development of an element of the cell culture system, a fully defined, xeno-free cell culture surface, and the benefits of a fully synthetic cell culture surface for propagation and differentiation of human embryonic stem cells.

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2. Importance and significance of a fully defined cell culture system

The envisioned applications of hESCs, such as cell therapy and drug discovery, require growth and differentiation of hESCs on a large scale. Despite numerous ongoing clinical trials involving adult and human embryonic stem cells as a source of cells for a variety of therapies, there are fundamental challenges in large scale manufacture of hESCs that need to be addressed. These challenges include measurement metrics defining purity and the quality of the produced cells for a particular therapy application, complexity and variability of cell culture environment for different stem cells and their derivatives, and a need for automation of hESCs culture to bring the required consistency and repeatability into the cell therapy process (Thomas & Williams, 2009).

A recent review of cell culture procedures for hESCs (Fernandes et al., 2010) reports that in the vast majority (>80%) of cell culture studies published in 2009, hESCs were grown either on MEFs and cell lines derived from MEFs, or human feeder cells. In less than 18% of the studies the cells were cultured on other surfaces, ~16.5% of which used Matrigel™, an extracellular matrix isolated from Engelbreth-Holm-Swarm (EHS) mouse sarcoma (Becton Dickinson, Bedford, MA).

In the US, regulation of production and marketing of stem cell based therapies falls under FDA (Food and Drug Administration) jurisdiction. FDA repeatedly expressed concerns in regards to the potential use of stem-cell products derived from hESC that were isolated and cultured on MEFs (Halme & Kessler, 2006) and indicated the necessity of testing these cells for adventitious agents to meet FDA xenotransplantation guidelines. Another source of animal-derived biological material is non-human serum, which is frequently used in cell culture. FDA views fetal bovine serum (FBS) as a possible source of the prion that causes bovine spongiform encephalopathy and requires that FBS is produced in a country certified to be free of this disease (Kirschstein & Skirboll, 2001).

Cell culture surfaces, as a part of the cell culture system, should meet FDA guidelines and address hESCs process scalability issues. The desirable attributes for hESC cell culture surfaces for an animal product-free cell culture are an absence of animal derived materials and ideally even human derived materials (as those too may be subject to batch-to-batch variability and contamination with human pathogens), and compatibility with a defined, serum-free media. Other typical cell culture surface attributes include absence of

extractables, particulates, and other contaminants, compatibility with conventional sterilization techniques (typically gamma irradiation), stability at room temperature, similarity to common tissue culture treated (TCT) polystyrene and other cell culture surfaces, and, ideally, the surface needs to be ready for use, rather than require further preparation and manipulation.

3. Progress in hESC culture conditions

The first significant improvement in hESC culture conditions was reported by Geron Corporation in 2001 (Xu et al., 2001). When Matrigel and gelatine were tested as surfaces for hESC lines H1, H7, H9 and H14 using MEF conditioned medium, all cells attached to Matrigel, formed tight colonies separated by differentiated cells, and propagated. In contrast, the cells on gelatine showed poor survival and appeared differentiated after one passage. Since the major constituents of Matrigel are laminin, collagen IV, heparan sulfate proteoglycans, and entactin/nidogen (Kleinman et al., 1982, Kleinman, 1986 #244), the authors then explored individual ECM components of Matrigel and observed great similarity between Matrigel and laminin cultured cells.

Further investigation of hESC culture using media conditioned with cells other than MEFs (STO, an immortal mouse embryonic fibroblast; NHG190, a transfected mouse embryonic cell line; BJ5ta, an immortalized human foreskin fibroblast cell line; an immortalized human retinal epithelial cell line) revealed that only Matrigel (or laminin) and MEF conditioned medium maintain long-term propagation of undifferentiated hESCs, as cells under other conditions differentiated within 1 to 7 passages. There was no difference found in integrin expression profiles between cells cultured on MEFs, Matrigel, or laminin, and cells under all three of these cell culture conditions expressed $\alpha 6$ and $\beta 1$ integrins consistent with integrin mediated hESC attachment to laminin. The cultured cells were positive for Tra-1-60 and Tra-1-81, negative for SSEA-1, and formed teratomas with various differentiated cells in immunodefficient mice. These data demonstrated the capability of a MEF-free surface to support proliferation of hESCs in an undifferentiated state.

While Matrigel was a significant improvement over MEFs, its mouse origin and undefined and variable nature makes it highly undesirable as a substrate for therapeutic hESC scale up. It was also found that current hESC lines derived on MEF and cultured on Matrigel in the presence of animal serum have nonhuman sialic acid Neu5Gc, a potential immunogen, incorporated into their cell membranes (Martin et al., 2005). Exposure of the cells to human sera with antibodies specific for Neu5Gc resulted in binding of immunoglobulin and complement deposition, which would lead to cell killing in vivo, thus significantly reducing the efficacy of cell therapy. After reviewing several approaches, Martin et al. concluded that it is easier and safer to start over and derive hESC lines without exposing cells to animal products containing Neu5Gc.

The elimination of animal derived products from hESC culture requires a xeno-free cell culture surface and medium. The interdependence of the cell culture surface and medium in providing optimal conditions for cell culture performance made progress in this field iterative. Short term solutions involved human feeder cells (Genbacev et al., 2005), (Choo et al., 2004), and serum replacement or serum-free medium (Amit et al., 2000).

The next significant milestone in development of hECS culture was achieved in 2005 (Xu et al., 2005), when Geron demonstrated proliferation of H7 and H9 hESCs in serum-free

medium on Matrigel. Geron evaluated 15 growth factors and their combinations at various doses in a medium containing 80% Knockout–Dulbecco's modified Eagle's medium, 20% Knockout™ serum replacement (Invitrogen, Carlsbad, CA), 1 mM L-glutamine, 0.1 mM β-mercaptoethanol, 1% nonessential amino acids. Although the exact composition of Knockout™ formulation is a commercial secret, the product is a more-defined growth supplement (Price et al., 1998) that reduces the spontaneous differentiation of ESCs. Basic fibroblast growth factor (bFGF) was found to support growth of hESCs without conditioned medium.

This was followed up by a study conducted at Prof. J. A. Thomson's lab that disclosed a complete composition of serum- and xeno-free medium, TeSR1, for hESC culture (Ludwig et al., 2006). The medium is based on DMEM/F12 medium supplemented with bFGF, TGF-β1, LiCl, γ-aminiobutyric acid, and pipecolic acid. It contains only six polypeptides (Table 1) and none of them is of animal origin. Ludwig et al. also screened a variety of human ECM proteins individually and in combination to establish that a substrate coated with a mixture of collagen IV, fibronectin, laminin, and vitronectin can support growth of H1 and H9 hESC lines for at least 7 months. The stem cells cultured under these conditions did not test positive for the presence of nonhuman sialic acid. Despite these advances, the importance of further optimization of the cell culture surface was recognized, as the purified human matrix components are expensive and could potentially be contaminated with human pathogens.

When compared to different human ECM proteins, as well as human and animal sera matrices (Hakala et al., 2009), Matrigel was found to be a superior hESC culture surface. The comparison was performed using conventional and modified hESC culture media that included human foreskin fibroblast-conditioned culture medium, chemically defined mTeSR®1 medium and its xeno-free counterpart, TeSR1 medium. Judging by the maximum passage number attainable, hESC morphology, and expression of stem cell markers, Hakala concluded that a xeno-free, fully defined, and reproducible feeder cell–free hESC culture method still remained to be developed.

Polypeptide	Conc. mM
bFGF	$5.77\ 10^{-6}$
TGF β1	$2.35\ 10^{-8}$
Human Insulin	$3.92\ 10^{-3}$
Human Holo-Transferrin	$1.37\ 10^{-4}$
Human Serum Albumin	$1.95\ 10^{-1}$
Glutathione	$6.38\ 10^{-3}$

Table 1. Polypeptides in TeSR1, (Ludwig et al., 2006)

4. Synthetic surfaces for hESCs

Typically cells are cultured on a polystyrene surface that is rendered hydrophilic (e.g. TCT and Corning® CellBIND® culture ware) by various plasma treatments. It is recognized that cell attachment occurs through interactions between integrins, cell adhesion receptors, and extracellular matrix proteins. To enable cell attachment to a surface, ECM proteins need to be immobilized on that surface first.

The proteins adsorb onto a surface from the culture medium, in which case serum supplement is a source of ECM proteins. However, when hESCs are cultured on a TCT

surface in serum supplemented medium (80% Dulbecco modified Eagle's medium supplemented with 20% fetal bovine serum), they undergo differentiation within a passage or two (Thomson, 1998). Alternatively, the ECM could be secreted by the cultured cells. Unlike other cell types, hESCs do not secrete a sufficient amount of ECM to sustain themselves in an undifferentiated state (Braam et al., 2008). Therefore, development of a synthetic surface for hESC culture under serum free conditions is not a trivial task (Couture, 2010), (Elefanty & Stanley, 2010).

The approaches to develop a solution to the problem described above can be grouped into three categories. First is to design a surface that facilitates ECM protein adsorption. The second is to identify a specific extracellular matrix protein that could be manufactured at a significant scale and coated onto conventional cell culture surfaces. The third approach entails development of a biomimetic surface that mimics the function of extracellular matrix proteins.

4.1 Polymer surfaces facilitating ECM protein adsorption.

Attempts to develop a surface for hESC culture using conventional polymers involved investigation of improved processing methods for polystyrene (Mahlstedt et al.). When TCPS (tissue culture polystyrene) was etched using radio frequency oxygen plasma for 5 min at pressures below 20 mT, HUES7 and NOTT1 hESC lines attached and proliferated on the surface for at least 10-14 passages in MEF conditioned medium showing doubling times similar to those for cells cultured on Matrigel and expressing hESC pluripotency markers. BD Primaria™ and Corning® CellBIND® surfaces were tested in a similar experiment as controls and also demonstrated the ability to support hESC culture to some extent. Quartz crystal microbalance analysis showed an increase in the amount of adsorbed proteins from conditioned medium onto the plasma etched surface, which was related to the improved cell culture performance. The proposed underlying mechanism is an increased hydrophilicity of the surface that enables replacement of small albumin, which is the first protein to adsorb onto the surface due to its abundance in serum, over time with less abundant, higher molecular weight, cell adhesive proteins. Attempts to culture hESCs on this surface using mTeSR1 or StemPro® hESC defined media were unsuccessful, as these media do not contain cell adhesion ECM proteins.

Similar material properties were achieved through organic synthesis. Villa-Diaz (Villa-Diaz et al., 2010) identified a polymer, poly[2-(methacryloyloxy)ethyl dimethyl-(3-sulfopropyl)ammonium hydroxide, which, when coated on polystyrene, supported the undifferentiated proliferation of BG01 and H9 hESC lines for over 25 passages in MEF-conditioned medium. The surface did not support hESC culture in mTeSR1 medium, while mixed result were observed in StemPro medium, the composition of which is not disclosed.

A more recent study (Brafman et al., 2010) described screening of 90 polymers for their ability to support hESC culture. Brafman identified only one polymer, poly(methyl vinyl ether-alt-maleic anhydride) (PMVE-alt-MA), that HUES1 and HUES9 cells attached to and proliferated for five passages in StemPro medium. The expression levels of endogenous ECM proteins and integrins in hESCs grown on PMVE-alt-MA were found to be significantly higher compared to hESCs grown on Matrigel, suggesting that the cells were generating ECM proteins required for their attachment to the surface.

4.2 Extracellular matrix protein coatings

Multiple publications demonstrated that Matrigel could be replaced, under certain conditions, with a mixture of collagen, fibronectin, laminin, and vitronectin (Ludwig et al.,

2006), or even individual ECM proteins including laminin, fibronectin, or vitronectin (Table 2). As evidenced by the data, conditioned medium can support hESC proliferation in an undifferentiated state on most common ECM proteins. However, transition to defined media such as mTeSR1, TeSR1, X-VIVO™ 10 seems to restrict the choice to laminin or vitronectin.

Laminin is a major constituent of Matrigel, and it was the first pure ECM protein identified to support hESC attachment and proliferation of the hESCs (Xu et al., 2001). Fairly recently, a detailed study by Braam et al. demonstrated the ability of vitronectin to support hESC culture in mTeSR1 medium and investigated the underlying mechanism (Braam et al., 2008). E-Cadherin, a cell-cell adhesion glycoprotein, also was shown to enable attachment and propagation of H9 cells in mTeSR1 medium (Nagaoka et al., 2010).

Protein	Cell line	Medium	Passages	Reference
Laminin Collagen Fibronectin	H1,H9 H1 H1	MEF conditioned	6-7 6 6	(Xu et al., 2001),
Laminin	H1	X-VIVO 10 + GF	40	(Li et al., 2005)
Laminin	KhES-1, KhES-2, hES-3	MEF conditioned	10	(Miyazaki et al., 2008)
Laminin	HS420, HS207, HS401, H1, H9	Variants of TeSR1 and mTeSR1	5-20	(Rodin et al., 2010)
Fibronectin	H9, BG01	HESCO	8	(Lu et al., 2006)
Fibronectin	I-3, I-6, H9	Knockout SR	>50	(Amit et al., 2004)
Collagen I	H1, H9	SDEC conditioned	5	(Jones et al. 2010)
Collagen I	HUES-1 Shef 1	hESF9	21 15	(Furue et al., 2008)
Vitronectin	MEL1, MEL2, hES1	StemPro	>10	(Prowse et al., 2010)
Vitronectin	HUES1, HES2, HESC-NL3	mTeSR1	12	(Braam et al., 2008)
Vitronectin	H9	mTeSR1	10	(Rowland et al., 2010)
Vitronectin	HES-3, H1	mTeSR1	>30	(Yap et al., 2011)
Vitronectin	CHA6, H9	mTeSR1	>30	(Yoon et al., 2010)
E-Cadherin	H9	mTeSR1	37	(Nagaoka et al., 2010)

Table 2. ECM surfaces for hESC culture.

Miyazaki et al., (Miyazaki et al., 2008) investigated attachment of three hESC lines (KhES-1, KhES-2, and KhES-3) to various recombinant laminins (511, 411, 332, 211, and 111) in MEF conditioned medium and observed a significant discrepancy in cell attachment to these

laminins with laminin 332 showing the best performance across these cell lines. Others reported recombinant laminin-511 to be a good substrate for HS420, HS207, and HS401 cell lines (Rodin et al., 2010).

Although extracellular matrix protein coatings are frequently used for hESC culture in research labs, application of these coatings to hESC scale up is not straightforward. Recombinant proteins are fairly expensive to produce and purify. They are prone to batch-to-batch variabity, need to be coated onto a surface under aseptic conditions, and they can degrade or denature upon dehydration. Published protocols indicate that coatings of purified proteins require optimization (Yap et al., 2011); for example, a defined thickness threshold needs to be achieved to enable hESCs attachment and proliferation rates similar to those observed on Matrigel coatings. Formation of such a coating requires a significant period of time (hours) under aseptic conditions. Utilization of the protein for the coating is very inefficient, as >50% of it remains in the solution. All this makes purified protein cell culture surfaces expensive and limits their scalability.

4.3 Biomimetic surfaces for stem cells

The interest in synthetic biomimetic surfaces was initiated by a discovery of the RGD (arginine-glycine-aspartic acid) peptide sequence present in most ECM proteins. When coupled to a surface, the RGD sequence promotes cell attachment (Pierschbacher & Ruoslahti, 1984). A large variety of polymer materials incorporating RGD peptides have been designed and studied (Hersel et al., 2003).

Cells attach to the ECM through integrins, cell adhesion receptors (Humphries et al., 2006), (Hynes, 2002). Integrins support a broad spectrum of cellular functions including proliferation and differentiation, and can bind to such ECMs as collagen, fibronectin, laminin, vitronectin, and N-linked glycoproteins, (e.g. osteopontin and bone sialoprotein). They can also interact with other cells through vascular- or intracellular- cell adhesion molecules (VCAM and ICAM). The integrins are heterodimeric molecules consisting of α and β subunits. There are twenty four known αβ combinations, which can be divided into several subfamilies based on evolutionary relationships. The orthologs of human integrins recognizing the RGD amino acid sequence and laminins can be traced back to primitive organisms, while other integrins must have evolved with increasing organism complexity.

From such a perspective, it is not surprising that hESCs primarily express integrins recognising the RGD sequence and laminin (Braam et al., 2008), (Meng et al., 2010), (Rowland et al., 2010), (Prowse et al., 2011). Braam et al., evaluated integrin expression in three hESC lines (HES2, HUES1, and HESCNL3) using fluorescence-activated cell sorting analysis. While integrin chains α1, α4, α10, and β3 and integrins α9β1 and αVβ6 were either not detected or detected at very low levels, cells expressed integrin chains α2, α3, α5, α6, α11, β1 and integrin αVβ5. This leaves the following combinations of integrin chains to enable cell attachment: α2β1, α3β1, α5β1, α6β1, α11β1, αVβ5, and αVβ1. These combinations, designated in Figure 1 as small orange circles, comprise cell surface receptors for all major ECM components.

It was also found, as discussed in the previous section, that attachment through either laminin or RGD binding integrins (Li et al., 2005), (Rodin et al., 2010), (Wong et al., 2010), (Braam et al., 2008), (Rowland et al., 2010), (Yoon et al., 2010), (Yap et al., 2011) is sufficient to support undifferentiated proliferation of hECS in serum-free, chemically defined medium.

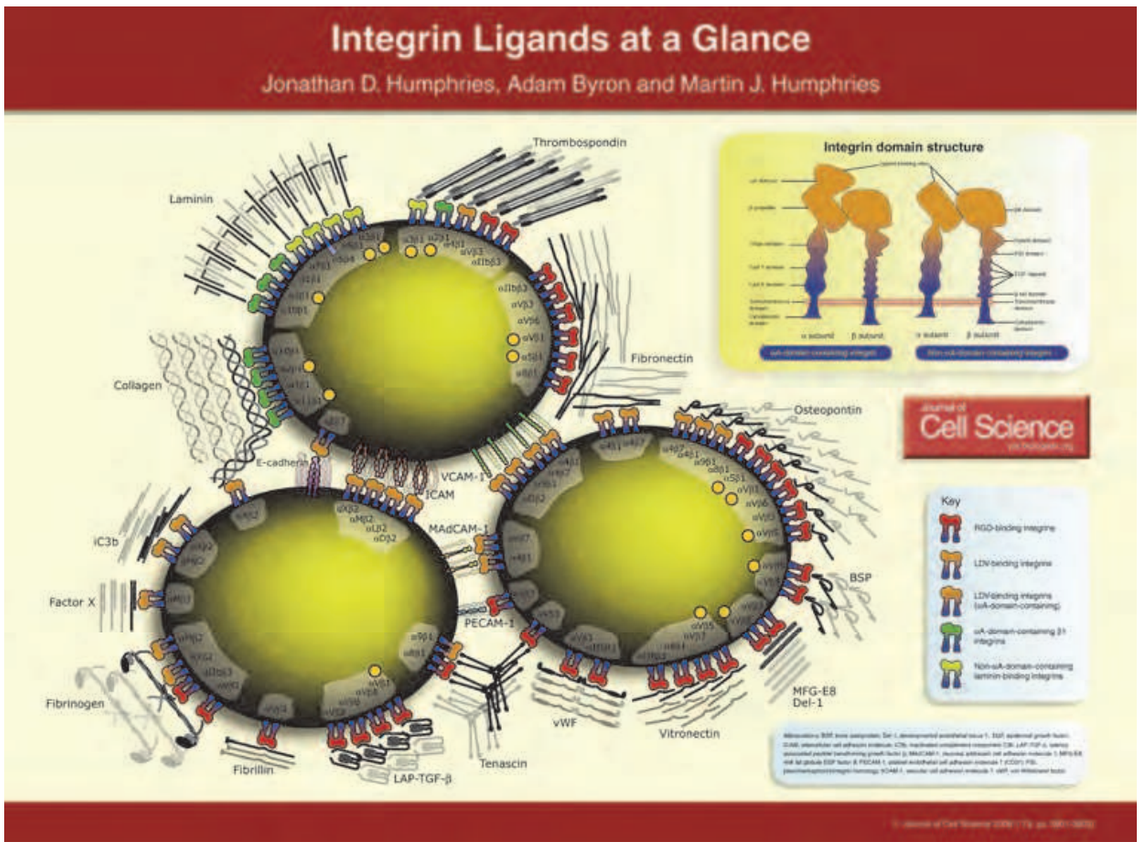

Fig. 1. Mammalian intergrins and their ligands (Humphries et al., 2006)

For these reasons, surfaces with immobilized laminin and RGD peptides were studied as synthetic surfaces for hESC proliferation (Li et al., 2006), (Derda et al., 2007), (Melkoumian et al., 2010) (Derda et al., 2010), (Klim et al., 2010). Li et al. was first to demonstrate that grafting the RGD containing peptide, Ac-KGGNGEPRGDTYRAY, from bone sialoprotein to acrylamide-acrylic acid copolymer can support short term attachment and proliferation of HSF-6 hESCs in MEF conditioned KSR-supplemented medium. The colonies of hESCs expressed Oct-4 and SSEA-4 markers of undifferentiated hESCs after five days in culture and maintained the same morphology as those cultured under the same conditions on Matrigel.
Derda et al. extensively studied laminin-derived peptides (Derda et al., 2007) in an attempt to identify specific sequences supporting hESC attachment. Using a self-assembled monolayers technique, the authors discovered several peptides enabling attachment and short term proliferation (6 days) of hESCs, again though, in MEF conditioned medium. The most important finding, which facilitated further advancement in the field, was an observation that hESCs, in comparison to other cells, require relatively high surface peptide densities due to low expression levels of corresponding integrins.
This suggests a hypothesis where a critical number of integrins on the cell surface are needed to be simultaneously engaged to enable optimal hESC adhesion. At low surface peptide density the probability of such engagement is reduced. We (Melkoumian et al., 2010) focused efforts on peptide-acrylate surfaces (PASs), as did others (Li et al., 2006), and pursued high peptide densities through optimization of acrylate polymer and peptide conjugation. The developed poly(hydroxyethyl methacrylate-co-carboxyethyl acrylate) coating provides a

significant amount of functional groups for peptide conjugation, while its swellable nature enhances presentation and accessibility of the conjugated peptide for integrin binding.
Co-conjugating a small amount of fluorescently labelled peptide together with a cell binding peptide and comparing the fluorescence intensities to a calibration curve generated by drying down the same mixture of peptides onto the acrylate surface, we estimated that a BSP peptide (Table 3) density of 6-9 pmol/mm^2 is sufficient to enable cell culture performance in X-VIVO 10 medium similar to Matrigel (Melkoumian et al., 2010), (Figure 2). An alternative approach in enabling hESC adhesion and proliferation was demonstrated by Kohlar, et al., (Kolhar et al., 2010), who employed cyclic RGD peptides to improve the strength of peptide – integrin binding to achieve a similar outcome. The reported peptide densities, 0.1–0.3 fmol/mm^2, are significantly lower. Nevertheless, stronger integrin binding to cyclic peptide allows development of a sufficient number of peptide-integrin interactions to enable cell attachment and proliferation.

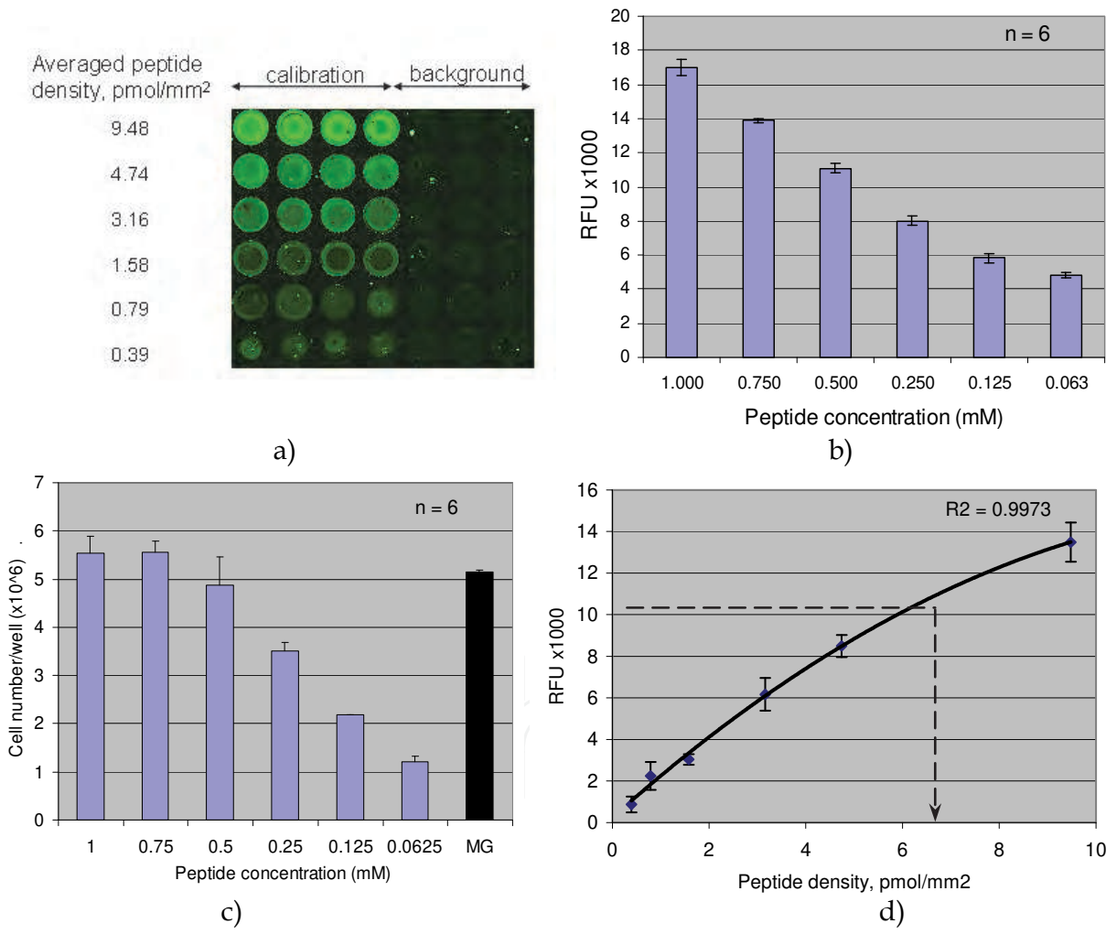

Fig. 2. Conjugated peptide surface density assessment; a) Fluorescence scan of dried down peptides representing various peptide densities; b) Fluorescence of conjugated peptide mixture; c) BSP peptide concentration (mM)-dependent H7 cell number after 5 days in culture in X-VIVO™ 10 + 80 ng/ml bFGF + 0.5 ng/ml TGFβ1 medium on PAS in 6-well plate, conjugated with serial dilution of BSP peptide. Cell seeding density was 1 x 10^6 cells per well; d) Peptide density calibration curve; (Melkoumian et al., 2010), supplementary Figure 1A and Figure 1B.

When a series of RGD peptides (Table 3) from various ECM proteins (bone sialoprotein, vitronectin, and fibronectin) was conjugated to the acrylate coating and tested for their ability to support hESC culture, only BSP and VN peptides supported attachment and proliferation of H1 and H7 hESCs similar to Matrigel (Melkoumian et al., 2010). None of the fibronectin peptides provided adequate cell attachment. According to Humphries et al., BSP protein engages the same integrins, αVβ5 and αVβ3, as vitronectin suggesting the same integrin activation by both BSP and VN peptides, (see Figure 1). Braam et al. (Braam et al., 2008) reported binding of hESCs to vitronectin through αVβ5 integrin and showed it being sufficient for cell self-renewal under chemically defined conditions. Since αVβ3 integrin was not detected in that study, it is highly likely that BSP and VN peptides enable hESC attachment through αVβ5 integrin, as it is the only remaining choice for BSP peptide, (Figure 1). This interaction is fairly specific and that specificity is governed by RGD flanking sequences.

Peptide based cell culture surfaces are not limited to integrin interactions, but can also be designed to take advantage of multiple attachment mechanisms. Recently Klim at el., (Klim et al., 2010) demonstrated self-assembled monolayer peptide biomimetic surfaces that included peptides binding to glycosaminoglycans. The cell culture surface presenting vitronectin peptide GKKQRFRHRNRKG was found to support attachment and proliferation of H9, H13, and H14 cells in mTeSR1 medium in the presence of ROCK inhibitor.

Sequence	Abbreviation	Reference
Ac-KGGNGEPRGDTYRAY	BSP	(Oldberg et al., 1988)
Ac-KGGPQVTRGDVFTMP	VN	(Suzuki et al., 1985)
GRGDSPK	FN-1	(Pierschbacher & Ruoslahti, 1984)
Ac-KGGAVTGRGDSPASS	FN-2	(Pierschbacher & Ruoslahti, 1984)
BSP, bone sialoprotein; VN, vitronectin; FN, fibronectin.		

Table 3. Peptide sequences conjugated to acrylate coatings; (Melkoumian et al., 2010)

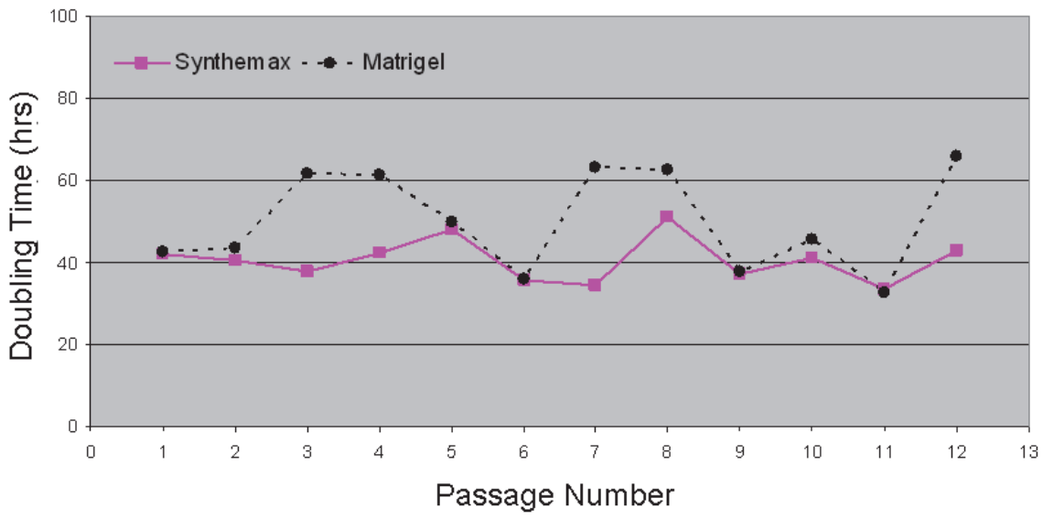

Fig. 3. Consistency of doubling time for H7 cells grown on Corning® Synthemax™ and Matrigel™.

The fact that hESCs can be cultured under defined conditions on a variety of substrates, from complex ECM protein mixtures to single peptide surfaces, demonstrates the native ability of undifferentiated hESCs to propagate without the need for a very precise and complex signalling through adhesion receptors (Klim et al., 2010), (Ying et al., 2008).
One of the desirable and anticipated advantages of synthetic surfaces is improved consistency and reproducibility of cell culture performance. Non-biological, small molecular weight compounds required for manufacture of synthetic surface can be consistently obtained in high quantity and purity and can be manipulated in a conventional industrial setting. When compared to proteins, short peptides can be easily manufactured via chemical synthesis. Purification and quality assurance are dramatically less complex for peptides. Short peptides do not denature and exhibit higher stability towards chemical and physical agents, thus, overall providing a scalable manufacturing platform for hESC cell culture surfaces, enabling cell therapy applications. This will translate into industrial processes resulting in consistent cell culture surface, as it did for tissue culture treated polystyrene surfaces. We observed a clear improvement in cell culture consistency at the early stages of surface development (Figure 3).

Cell Line	Culture Medium	Surface	Doubling Time (h)	%Oct-4 + Cells	%Tra1-60 + cells	%SSEA-4 + cells	Passages
H7	X-VIVO 10	Synthemax	41 ± 5	92 ± 4	85	100	21
		Matrigel	50 ± 12	92 ± 3	96	100	
	mTeSR1	Synthemax	55 ± 21	90 ± 4	66	-	11
		Matrigel	69 ± 26	83 ± 8	56	-	
	StemPro	Synthemax	31 ± 6	96 ± 0	-	-	5
		Geltrex	31 ± 5	97 ± 0	-	-	
	NutriStem™	Synthemax	32 ± 9	92 ± 3	-	-	5
		Matrigel	35 ± 4	92 ± 6	-	-	
H1	X-VIVO 10	Synthemax	46 ± 8	82 ± 5	78 ± 4	91	14
		Matrigel	53 ± 16	80 ± 8	83 ± 6	84	
H9	mTeSR1	Synthemax	43 ± 3	93 ± 5	73 ± 4	-	5
		Matrigel	44 ± 6	95 ± 4	76 ± 2	-	
BG01v	mTeSR1	Synthemax	40 ± 6	86 ± 6	-	-	11
		Matrigel	44 ± 6	89 ± 5	-	-	

Table 4. hESC long-term expansion on Synthemax™ in defined media: X-VIVO™ 10 (Lonza, + 80ng/ml bFGF + 0.5ng/ml TGFb1); mTeSR®1 (Stem Cell Technologies); NutriStem™ (StemGent); StemPro® hESC SFM (Invitrogen).

Since the publication in Nature Biotechnology (Melkoumian et al., 2010) gamma sterilization methods were developed for the peptide acrylate surface and the surface was commercialized under the Corning® Synthemax™ Surface trade name. It is being offered in two grades (therapeutic and research) in a number of formats including 6-well plates, T-75 and T-225 flasks. The utility of the surface has been demonstrated for additional cell lines including H9 and BGO1v. In addition to X-VIVO 10 and mTeSR1, the surface was found compatible with StemPro and NutriStem media. Multipassage hESC data on Synthemax surface is summarized in Table 4.

For hESC-based therapeutics it is critical to have a defined, scalable culture system for both the expansion and differentiation stages of cell production. The picture would not have been complete without testing Synthemax surface for hESC differentiation. After passaging cells 14 times on PAS surface, cells were treated with Activin A and BMP4 as previously described (Laflamme et al., 2007) to investigate differentiation into cardiomyocytes. The experiment resulted in spontaneously beating cell aggregates expressing the cardiomyocyte-specific markers, Nkx2.5 and α-actinin, and shows that the BSP peptide acrylate surface supports both expansion and differentiation of hESC to therapeutic progenitor cells, (see Figure 4).

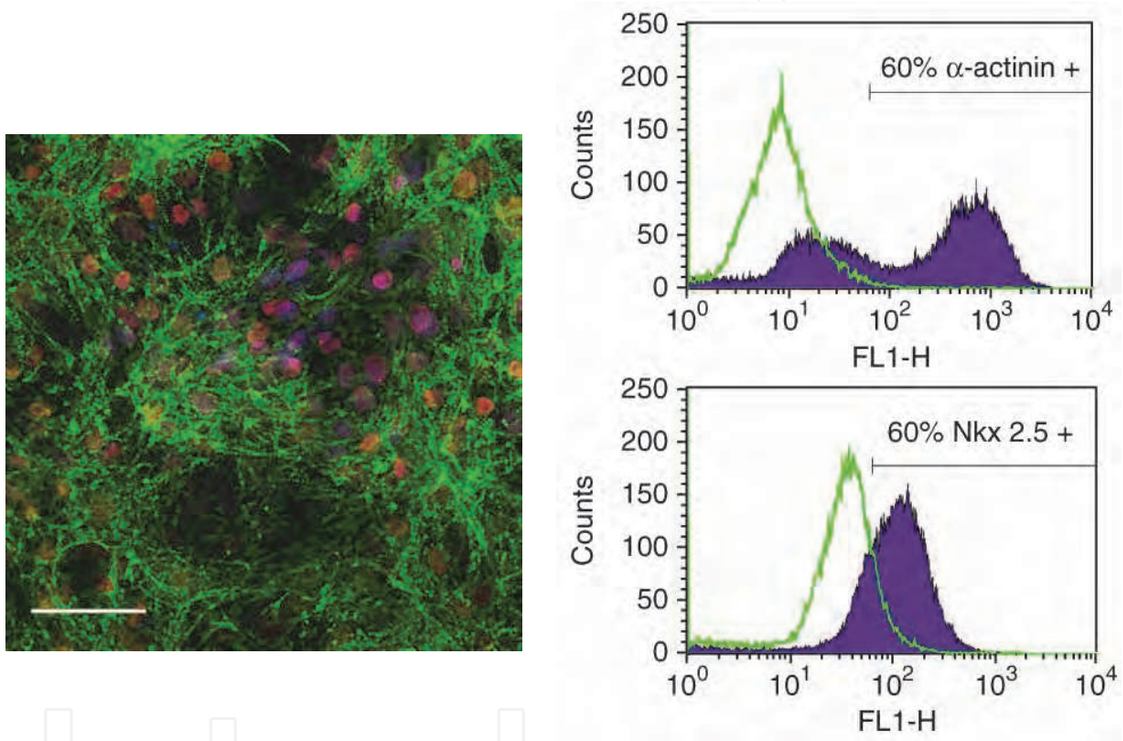

Fig. 4. Cardiomyocyte differentiation of H7 hESC on BSP-PAS surface: Immunofluorescent staining (a) and flow cytometry analysis (b) of differentiated cells for cardiomyocyte-specific markers, Nkx 2.5 shown in red and α-actinin shown in green.

5. Conclusions

Although a variety of recombinant protein coatings in combination with a defined serum-free cell culture medium have been shown to provide a good cell culture system for hESCs, the limited scalability of most of these systems limits their use for cell therapy applications. Peptide acrylate surfaces based on a pure synthetic chemistry approach offer consistency, reproducibility, scalability, safety, and eventually lower cost for hESC-derived cell therapy applications.

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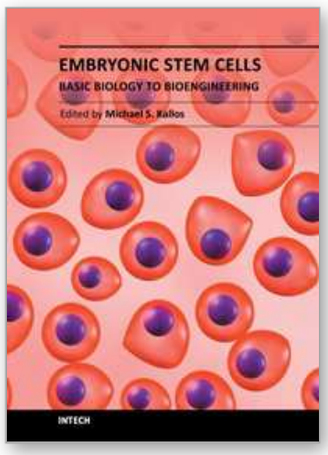

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Embryonic stem cells are one of the key building blocks of the emerging multidisciplinary field of regenerative medicine, and discoveries and new technology related to embryonic stem cells are being made at an ever increasing rate. This book provides a snapshot of some of the research occurring across a wide range of areas related to embryonic stem cells, including new methods, tools and technologies; new understandings about the molecular biology and pluripotency of these cells; as well as new uses for and sources of embryonic stem cells. The book will serve as a valuable resource for engineers, scientists, and clinicians as well as students in a wide range of disciplines.

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Phone: +385 (51) 770 447
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