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REVIEW

Substrates for clinical applicability of stem cells

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Abstract

The capability of human pluripotent stem cells (hPSCs) to differentiate into a variety of cells in the human body holds great promise for regenerative medicine. Many substrates exist on which hPSCs can be self-renewed, maintained and expanded to further the goal of clinical application of stem cells. In this review, we highlight numerous extracellular matrix proteins, peptide and polymer based substrates, scaffolds and hydrogels that have been pioneered. We discuss their benefits and shortcomings and offer future directions as well as emphasize commercially available synthetic peptides as a type of substrate that can bring the benefits of regenerative medicine to clinical settings.

Key words: Human pluripotent stem cells; Extracellular matrix protein; Synthetic substrate; Peptide; Polymer; Scaffold; Hydrogel

Core tip: This review article highlights numerous extracellular matrix proteins, peptide and polymer based substrates, scaffolds and hydrogels that have been pioneered for human pluripotent stem cell self-renewal for stem cell-based therapy. The benefits and shortcomings of these substrates as well as future direction that can bring the benefits of regenerative medicine to clinical settings are discussed.

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INTRODUCTION

Ever since the derivation of human embryonic stem cells (hESCs) from the inner mass of the blastocyst[1], great effort has been placed on extending the benefits of these pluripotent cells to regenerative medicine[2,3]. However, human pluripotent stem cells (hPSCs) which include human induced pluripotent stem cells (hiPSCs) and hESCs, have traditionally been cultured on mouse fibroblast feeder layers in the presence of animal cell-conditioned media or on Matrigel that pose inherent risk of pathogenic contamination[4] as well as the presence of non-human immunogenic epitopes such as *N*-glycolylneuraminic acid (Neu5Gc)[5]. Consequently, research has focused on developing xeno-free, chemically defined media and substrates that are

compliant with current good manufacturing practice (cGMP), scalable, maintain high degree of hPSC purity, colony homogeneity and pluripotency. Here, we review substrates that have been developed for maintenance of hPSCs for clinical applications.

EXTRACELLULAR MATRIX PROTEINS

Feeder free cultures for hPSC expansion rely on Matrigel which constitutes the basement membrane components derived from mouse Engelbreth-Holm Swarm (EHS) tumor. The EHS tumor can easily yield a hundred grams of basement membrane components that are abundantly found in Matrigel such as collagen Ⅳ, laminin, heparin sulfate proteoglycans and nidogen/entactin[6]. In addition, minor components can also be found in Matrigel which include proteases such as 72 kDa matrix metalloproteinase-2, 92 kDa matrix metalloproteinase-9, uruokinase, tissue-type plasminogen activator as well as growth factors, like transforming growth factor beta, fibroblast growth factor, epidermal growth factor, platelet-derived growth factor, insulin-like growth factors, and proteins (Amylase, Transferrin, Clusterin) in an undefined composition. Matrigel has been used as a substrate to study the long-term stability (40 passages) of hESCs[7] as well as to derive and maintain new hESC lines for over 70 passages[8]. Other studies have used Matrigel as an extracellular matrix (ECM) to induce hepatic differentiation of hESCs using hepatic growth hormone and activin A[9], to expand hESCs on Matrigel-coated cellulose microcarriers in 3D suspension cultures[10] and to study the effects of bone morphogenetic protein 4 on hESCs differentiation[11]. As Table 1 highlights, the batch-to-batch variability and xenogeneic-origin makes Matrigel unsuitable for quality control or cGMP production of hPSCs. Consequently, various ECM protein components have been sought as alternatives to Matrigel.

Vitronectin

The N-terminal of the 75 kDa vitronectin protein consists of the binding sites for integrins *via* the Arg-Gly-Asp RGD sequence, which allows cell-vitronectin interaction[12]. Braam *et al*[13] have shown that recombinant vitronectin is as effective as plasma purified vitronectin for hESC expansion and mediates cell attachment *via* the αVβ5 integrins. In mTeSR1 medium, recombinant vitronectin supported several hESC lines for five passages while maintaining normal morphology, karyotype, differentiation potential and cell surface markers for stemness. However, other ECM proteins such as laminin, collagen Ⅰ, collagen Ⅳ and fibronectin were only able to support cell growth in mouse embryonic fibroblast-conditioned media (MEF-CM)[13]. The use of mouse myeloma cell line NS0 to obtain the recombinant vitronectin holds potential risk of sialic acid contamination but others have been able to produce recombinant vitronectin in *Escherichia colivia* plasmid vectors[14]. Prowse *et al*[14] obtained recombinant somatomedin B (SMB) domain along with the RGD sequence of vitronectin (rVN SMB) and anchored the substrate to tissue culture plate through vitronectin's polyhistidine linker sequence.

Recently, a fully-defined, serum- and feeder-free medium called StemPro was developed for hPSC expansion and differentiation. The hESCs grown in an ascorbate (vitamin C) free StemPro media for over 10 passages showed similar binding affinity to another ECM protein purified from EHS tumor (called Geltrex) and pluripotency markers comparable to cultures grown on whole purified vitronectin and Geltrex[14]. The surface density of human purified plasma vitronectin necessary for long term (> 30 passages) hESCs attachment, proliferation and differentiation was determined by Yap *et al*[15] to be 250 ng/cm^2. However, the hESCs cultured on purified plasma vitronectin in mTeSR media showed slower (35 h) doubling time than Matrigel (25 h) but expressed normal pluripotency markers and karyotype[15]. Another study examined the scale up potential of vitronectin and laminin-coated three dimensional (3D) substrates from traditional two dimensional (2D) tissue culture polystyrenes (TCPs) to 3D polystyrene microcarriers (MCs)[16]. In this study, vitronectin was adsorbed onto TCPs and reached a surface density saturation of 510 ng/cm^2 compared with laminin at 850 ng/cm^2. Coating vitronectin and laminin to MCs allowed for a surface density saturation of 450 and 650 ng/cm^2, respectively. Even though, the two extracellular matrices showed similar adhesion and growth kinetics, the scale up to 3D culture resulted in a slower growth kinetics on the polystyrene MCs. This was explained by the hydrophobicity of MCs as well as the formation of large compact cell aggregates which deprive essential nourishments to the cells that are located in the center.

Laminins

Laminin provides adequate growth for hESCs *via* the integrin α6β1 laminin specific receptor since it is the major type of integrin present on hESCs[17,18]. The effect of various recombinant human laminin (rhLM) isoforms on hESC attachment and proliferation has been investigated. Miyazaki *et al*[18] showed that hESC line KhES-1 cells adhere markedly to rhLM-332 as well as to rhLM-511 and rhLM-111 (least adherence) when compared to non-adherence on rhLM-211 and rhLM-411. In the study, KhES-3 cells were able to adhere to rhLM-211 and rhLM-411 but their growth rate was markedly lower on rhLM-332, rhLM-511 and rhLM-111[18]. The primary reason for such a marked difference in adherence and growth is because rhLM-211 and rhLM-411 possess affinity for α3β1 and α7β1 which are laminin-binding integrin isoforms that are expressed on hESCs in a relatively low amount compared to α6β1 integrins[18]. This receptor specificity

Table 1 Benefits and shortcomings of Matrigel, extracellular matrix proteins, synthetic peptides, synthetic polymers and hydrogels

Substrate	Advantages	Disadvantages
Matrigel	Allows feeder-free cell culture	Xenogeneic origin[6]
	Inexpensive	Undefined components[6]
	Long-term hESCs culture[7,8]	Pathogenic contamination risk[4]
		Neu5Gc immunogenic epitope[5]
		Batch-to-batch variability[6]
ECM proteins	See subsections below	Batch-to-batch variability
Vitronectin		Degradation upon sterilization
rhLM-332		Pathogenic contamination risk
LM-E8		Not-Scalable[30]
rh E-cadherins-Fc protein		High production cost[30]
Fibronectin		Immunogenicity risk[17]
Vitronectin	Long-term hESC culture (> 30 passages)[15]	Degradation upon sterilization
	αVβ5 integrin receptor mediated cell attachment[13]	Not-Scalable[16,30]
		High production cost[30]
rhLM-332	High α6β1 integrin affinity[18]	
LM-E8	Smaller, easily purified, higher purity *vs* 780 ku laminins[20]	Not-Scalable[30]
	Better stem cell adhesion than Matrigel and intact laminins[20]	High production cost[30]
	ROCK inhibitor Y-27632 not needed[20]	
rh E-cadherins-Fc protein	hESC self-renewal, maintenance and pluripotency comparable to Matrigel™[26]	Low cell adherence *vs* Matrigel[26]
Synthetic peptides	No batch-to-batch variation[36]	High production costs[47,48]
	Immunogenicity risk avoided[37]	Sterilization difficulties[47]
	Since chemically synthesized	Easily degradable[47]
	Long-term hESCs culture[32,37,44]	Labor intensive cell passaging
		Limited scale-up potential of 2D platform[51]
Synthemax surface	Gamma irradiation sterilization[39]	
	2 yr shelf-life[39]	
	hESCs cryopreserved and thawed on substrate[38]	
	Scalable[38]	
	Long-term hESCs culture[38-40]	
Synthetic polymers	Inexpensive[45,47]	Limited scale-up potential of 2D platform
PMVE-alt-MA	Easy and rapid fabrication[45,49]	
PMEDSAH	Highly manipulable[47]	
APMAAm	Long-term substrate stability[46]	
Polyacrylates		
Chitosan-alginate polymers		
(pDTEc) polymer scaffolds		
Hydrogels	*In-vivo* 3D type environment[58]	Difficult to analyze cells embedded in hydrogels
	Thermoresponsive and pH sensitive properties[54,55,58]	
(AEtMA-Cl)- DEAEA based		Enzymatic release of cells from hydrogel[57]
PDEAAm-based		
HA-based		
Alginate-collagen based		
PEG-based		
PPP-based		

hESC: Human embryonic stem cell; ECM: Extracellular matrix; LM: laminins; PMVE-alt-MA : Poly(methyl vinyl ether-alt-maleic anhydride); PMEDSAH: poly[2-(methacryloyloxy)ethyl dimethyl-(3-sulfopropyl)ammonium hydroxide]; APMAAm: Aminopropyl methacrylamide; pDTEc: Poly(desaminotyrosyl tyrosine ethyl ester carbonate); AEtMA-Cl: 2-(acryloyloxyethyl) trimethylammonium chloride; HA: Hyaluronic acid; PDEAAm: Poly(N,N-diethylacrylamide); PEG: Polyethylene glycol; PPP: Platelet poor plasma; 2D: Two dimensional.

is further established by the fact that although laminin-332 is not expressed on hESCs, rhLM-332 was an adequate substrate for hESCs adherence and proliferation due to its affinity for α6β1 integrins[18].

Since laminin isoform-511/-521 are the major laminins expressed on hESCs, another study investigated hESC self-renewal of cell lines HS420, HS207 and HS401 on a rhLM-511 substrate in the mTeSR1 variant (O3) and xeno-free TeSR1 variant (H3) media[19]. The cells proliferated for 28 passages and normal karyotype was observed after 20 passages while exhibiting OCT4, SOX2 and Nanog pluripotency markers at a higher level than Matrigel and comparable to those grown on feeder cells. Importantly, rhLH-511 affinity of the cells was higher than cell-cell adhesion as demonstrated by cell spreading when the cells were passaged as individual clumps and this highlights the role of rhLM-511 in cell motility.

Further refinement to laminin-coated substrates has been achieved by using laminin E8 fragments (LM-E8) that are composed of the α, β and γ chains' C-terminal regions[20]. These truncated proteins are smaller, easily purified in a short time period and yield a higher purity compared with the 780 kDa LMs. Miyazaki *et al*[20] used LM-511-E8 and LM 332-E8 to achieve greater hESC and hiPSC adhesion than Matrigel™ and intact LM-511

and 332 in a TeSR2 medium. The three hESC lines and two hiPSC lines cultured on LM-E8-coated substrates underwent proliferation for 30 and 10 passages, respectively, where disassociated cells attached strongly to LM-E8 substrates as compared to weak adhesion on Matrigel, fibronectin and vitronectin substrates[20]. Importantly, LM-E8 substrates did not require the use of ROCK inhibitor γ-27632 which is needed for some recombinant protein-based substrates.

E-cadherin

E-cadherin, which is a transmembrane glycoprotein is involved in calcium-dependent cell-cell adhesion in epithelial as well as embryonic stem cells[21]. It is essential for the maintenance of pluripotent state of stem cells due to their critical role in cell-cell adhesions[22] as well as cell survival and renewal[23]. This E-cadherin mediated pluripotency is quickly lost, as measured by the levels of Oct4, Nanog and Sox2, when siRNA is used to suppress E-cadherin expression on cells[24]. This in turn causes a decrease in hESC proliferation[25] and emphasizes the critical role that E-cadherins play in cell-cell adhesion, survival, renewal, proliferation and pluripotency. Nagaoka *et al*[26] used recombinant E-cadherin-Fc-coated culture dishes (rhE-cad-Fc) where E-cadherins are fused with an IgG-Fc domain to coat tissue culture plates on which hESCs were examined for their pluripotency and propagation characteristics. The study demonstrated that both hESCs and hiPSCs that were cultured on rhE-cad-Fc protein-coated surface for > 13 passages in mTeSR1 medium were indistinguishable from those that were cultured on Matrigel as far as the rate of proliferation, cell morphology, maintenance of pluripotency and the ability to differentiate into multiple cell types were concerned. However, the ability of cells to adhere was decreased as a result of protease digestion cocktails using Accutase. This problem can be circumvented by using enzyme-free Cell Dissociation Buffer for passaging the cells. Therefore, rhE-cad-Fc can be considered superior to Matrigel as a substrate because of the use of more defined chemical conditions that yield similar results for hESC self-renewal, maintenance and pluripotency. E-cadherins have also been linked with matrix rigidity where hESCs on rigid substrates were shown to co-express E-cadherins and were $OCT4^+$. In contrast, the softer substrates had large hESC aggregates that had an increased tendency to differentiate. Furthermore, hESCs on rigid substrates exhibit increased cytoskeleton contractility related to E-cadherin expression among cells[27].

Fibronectin

The 220 kDa fibronectin dimer interacts with hESCs *via* the α5β1 integrin receptor such that only 25 percent of adsorbed fibronectin is needed for hESC renewal[28]. However, the 120 kDa central-binding domain of fibronectin is necessary for attachment and maintenance of hESCs pluripotency. Using a high throughput screening of various ECM proteins, Brafman *et al*[29] discovered that high concentrations of fibronectin and laminin (500 μg/mL) supported hESC proliferation commensurate with Matrigel in a concentration dependent manner and fibronectin, collagen I and laminin were each able to support a high degree of pluripotency.

SYNTHETIC PEPTIDES

Even though, the protein based substrates such as recombinant vitronectin, laminin and rhE-cad-Fc coated tissue plates may be feeder free, they are not xeno-free and as a result pose significant immunogenic and pathogenic hazards[4]. Additionally, such substrates are not scalable and have significant manufacturing costs associated with them[30]. Being protein-based, they can also undergo degradation following common sterilization techniques. Consequently, peptide-based substrates offer better alternatives for maintaining hPSCs pluripotency, self-renewal, growth, scalability and clinical applicability.

Heparin binding peptides promote cell adhesion and spreading through their interaction with cell-surface glycosaminoglycans (GAGs) which are involved in cell-cell recognition and adhesion, cell-matrix interactions and receptor-signal complexes[31]. In a study conducted to determine the optimal peptides for hESC self-renewal, Klim *et al*[32] discovered that surfaces which display heparin binding peptide GKKQRFRHRNRKG, derived from vitronectin, supported adhesion and self-renewal of hESCs at the lowest peptide surface density (0.5%) while other surface presenting various heparin binding peptides did not allow for cell attachment. These heparin binding peptides exhibit increased levels of Oct-4 and SSEA-4 expression, exhibit similar rates of growth, long term stem cell propagation (17 passages) and differentiation capabilities that are equal to Matrigel. The heparin binding peptide GKKQRFRHRNRKG has been used in polyacrylamide hydrogels of varying stiffness where H9 hESCs were cultured for 12 passages in mTeSR1 medium supplemented with 10 μmol/L ROCK inhibitor Y-27632[33]. Not only was the hESC self-renewal confirmed but it was found that GAG binding on stiffer materials conveyed mechanosensing information that allowed for F-actin polymerization and enabled cell adhesion. Additionally, the nuclear localization of transcriptional co-activators Yes-associated protein and transcriptional coactivator with PDZ-binding motif (TAZ) on stiffer matrices allowed for hESCs pluripotency[33].

Self-assembled monolayers (SAMs) with peptide sequence TVKHRPDALHPQ and LTTAPKLPKVTR have also been shown to promote hESC adhesion and proliferation for 3 passages[34]. However, non-clonal abnormalities were detected in cells cultured on TVKHRPDALHPQ SAMs while LTTAPKLPKVTR SAMs

did not exhibit karyotypic abnormalities. Similarly, 18 different laminin-derived peptides were also screened and five (including RNIAEIIKDI) were capable of supporting hESCs proliferation and pluripotency comparable to Matrigel™[35]. The presence of relatively low receptor concentration on cells requires the presentation of laminin-derived peptides at a higher concentration despite being presented in a uniform density and specific orientation.

More recently, Au-coated glasses have been modified with poly[oligo(ethylene glycol) methacrylate] polymer brushes through surface initiated polymerization and peptides that are derived from vitronectin[36]. A 0.75 mmol/L vitronectin peptide concentration is deemed necessary for hiPSC expansion for up to 10 passages in mTeSR1 medium. Using micro-contact printed polydimethylsiloxane stamp, cell adhesion was easily regulated only on the vitronectin peptide and pluripotency markers and self-renewal capabilities were observed to be similar to Matrigel. A more potent version of the RGD sequence in the form of cyclic RGD peptide has been used to modify surfaces on which hESCs can adhere more prominently than on linear RGD peptides at a 4 fold higher rate[37]. Normal pluripotency markers, karyotype and differentiation potential similar to Matrigel have been observed for cells cultured for 10 passages as well as in mTeSR1 medium.

Peptide acrylate surfaces

Peptide acrylate surfaces (PAS) are synthetic surfaces that are composed of acrylates which are conjugated to biologically active peptides[38]. One such platform is the so called Synthemax Surface where the synthetic surface is manufactured by covalently linking the vitronectin-derived RGD sequence to the acrylate surface[39]. Melkoumian *et al*[38] have used this technique to deposit various peptide sequences, as well as the vitronectin sequence, onto acrylate surfaces in a process that deposits carboxylic acid containing acrylate onto the surfaces of cultures and then conjugates amine-containing peptides to these surfaces using 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide/*N*-hydroxysuccinimide. Here, bone sialoprotein-PAS and vitronectin-PAS were used to culture hESC line H7 for more than 10 passages in a defined medium. The authors concluded that the hESCs grown on PAS system were not different to the cells that were grown on Matrigel when comparing cell viability, colony morphology, doubling time and hESC marker expression of OCT4, TRA-1-60 and SSEA-4. Furthermore, hESCs can be cryopreserved and thawed onto PAS in addition to their ability to undergo directed differentiation into functional cardiomyocytes. This research demonstrated the scalability of feeder free, xeno-free and chemically defined PAS substrate that are suitable for clinical applications. Jin *et al*[40] further examined the expansion and differentiation capabilities of hiPSCs on synthemax surface. The authors concluded that there is no significant difference between the doubling time and pluripotency marker expressions for hiPSCs cultured on Matrigel and Synthemax substrate. However, the cells cultured on the Synthemax substrate showed more compact morphology than Matrigel. The researchers were able to demonstrate definitive endoderm differentiation of hiPSCs and were able to decipher the specific binding of $\alpha V\beta 5$ receptors for Synthemax Surface while the Matrigel substrate bound to various integrin receptors. Lastly, cell-cell interfaces on Synthemax Surface showed broad and dense actin filaments as well as the up-regulation of zyxin which demonstrates the involvement of various cytoskeletal proteins in hiPSCs attachment and proliferation.

Synthemax Surface has also been used for retinal differentiation of hiPSCs[41] and to show efficient single cell passaging of hPSCs, using ROCK inhibitor Y-27632, similar to the results obtained on LM-E8 substrates[42]. It has been used to obtain oligodendrocyte progenitor cells (OPCs) from hESCs that express higher levels of OPC specific proteoglycan, NG2, as compared to OPCs grown on xenogeneic-derived ECM[43]. The researchers concluded that Synthemax Surface can replace the xenogeneic-derived ECM for hESCs differentiation into OPCs. Recently, Synthemax Surface was used to expand hESCs for 10 passages and to obtain insulin-producing β cells within 21 d in a defined serum-free media[44].

Nevertheless, the high production costs, degradation upon sterilization, labor intensive cell passaging and limited scale-up potential of 2D peptide surfaces have caused researchers to seek alternative substrates in the synthetic polymer arena.

SYNTHETIC POLYMERS

Poly(methyl vinyl ether-alt-maleic anhydride)

Brafman *et al*[45] used a high throughput screening approach to identify supports for hPSCs self-renewal as well as optimal conditions for pluripotency and proliferation for hPSCs. The authors examined 90 polymers with different functional groups, chemical compositions and molecular weights. They discovered that poly(methyl vinyl ether-alt-maleic anhydride) (PMVE-alt-MA) is most capable of supporting both short and long term maintenance of hESCs for HUES1 and HUES9, as well as one hiPSC line for over five passages. PMVE-alt-MA and poly(acrylic acid) cause greatest hPSC proliferation rates at discrete molecular weights of 1.25×10^6 Da and 4.5×10^5 Da, respectively. Of the 16 polymers, cells on PMVE-alt-MA did not exhibit detachment or differentiation. Furthermore, one hiPSC line and two HUES 1/9 lines were successfully cultured for over five passages with expression of normal pluripotency markers and differentiation capabilities and showed an increased expression of $\alpha 5$ and αv integrin receptors. Importantly, the authors showed that hPSCs can

further secrete endogenous ECM proteins in the absence of exogenous ECM proteins in defined medium thereby highlighting the role of ECM proteins in hPSC self-renewal.

Poly[2-(methacryloyloxy)ethyl dimethyl-(3-sulfopropyl)ammonium hydroxide]

Long term hESC growth has been demonstrated on poly[2-(methacryloyloxy)ethyl dimethyl-(3-sulfopropyl) ammonium hydroxide] (PMEDSAH)-coated TCPs *via* surface initiated graft polymerization technique that grafts PMEDSAH onto ozone-activated surfaces of TCPs[46]. For 25 passages, H9 and BG01 cells that were grown on PMEDSAH attached, proliferated, exhibited long term growth along with normal genetic and proteomic makeup as exhibited through Nanog, OCT3/4 and SOX2 expression and possessed differentiation potential. H9 cells exhibited higher cell-aggregate adhesions when grown in human cell conditioned medium as compared to BG01 cells. However, both these cell lines showed comparable cell doubling times, pluripotency and karyotype when compared with cells grown on Matrigel[46]. Consequently, variability exists in the response of these cell lines to various media conditions. H9 hESCs grown on PMEDSAH were able to be cultured on chemically defined StemPro media for 10 passages. Additionally, hiPSCs grown on PMEDSAH have also been shown to proliferate in defined xeno-free conditions for 15 passages while retaining pluripotency markers, normal morphology and karyotype[47]. However, the hiPSC-derived mesenchymal stem cell unequally preferred osteogenic and chondrogenic differentiation rather than adipogenic lineage.

Aminopropyl methacrylamide

Other synthetic polymer substrates such as the methacrylamide-based aminopropyl methacrylamide have been used to culture hESCs for over 20 passages in mTeSR1 media[48]. The H9-hOct4-pGZs cells showed higher expression of pluripotency markers than Matrigel as well as higher proliferation rate at passage 1 and 22 but the proliferation rate slowed down at latter passages. Importantly, bovine serum albumin (BSA) remains crucial for hESC attachment, growth and proliferation since it was shown to be adsorbed onto the APMAA surfaces in an unfolded state[48].

Polyacrylates

One study applied a high throughput screening approach to high-density polymer microarrays to obtain polymers with either 2-carboxyethyl acrylate or 2-(methylthio) ethyl methacrylate containing monomers that allowed cell adhesion at specific ratios[49]. Furthermore, an equal ratio of 4-tert-butylcyclohexyl acrylate and n-butyl methacrylate was found in polymers supporting greatest adhesion. The study demonstrated short term (5 passages) pluripotency potential of hESCs cultured on these surfaces.

Chitosan-alginate polymers

As natural polymers that mimic GAG structure, chitosan-alginate (CA) 3D scaffolds have been used for hESC self-renewal[50]. The cells grown on these scaffolds exhibit three times higher alkaline phosphatase activity and the expression of pluripotency markers were similar to hESCs grown on human fibroblast feeder layers. Additionally, hESCs which are recovered *via* EDTA and K_2HPO_4 solution express over 95% cell viability and the CA scaffolds allow for easy passaging of cells. Another study used alginate-chitin polymer based scaffold, which mimic the GAGs units of the ECM[51]. Combined conditions of high cell seeding density, avoidance of fluid shear stresses *via* encapsulation as well as increased surface area to volume ratio of encapsulated cells, and cryopreservation with high cell viability (> 75%) allows for long term culture (10 passages) and quick non-cytotoxic harvesting of cells through enzymatic disassociation of microfibers with further scalability.

Poly(desaminotyrosyl tyrosine ethyl ester carbonate) polymer scaffolds

Synthetic scaffolds composed of poly(desaminotyrosyl tyrosine ethyl ester carbonate)[52], a tyrosine derived polycarbonate polymer[53], have been used to study the effects of geometries on hESCs self-renewal and proliferation. Carlson *et al*[52] discovered that microfibrous architecture of poly-D-lysine pretreated scaffolds supports hESC survival and colony formation as this geometry allows cell-cell and cell-matrix contact and extensive ECM deposition of laminin and collagen Ⅳ with some collagen Ⅰ deposition. The endogenous production of laminin was an essential factor for hESC adhesion and survival.

HYDROGELS

Although synthetic polymers represent a more economical approach with more quality control over the manufacturing process, the 2D topography does not mimic the *in vivo* cell-cell and cell-ECM interactions. Hydrogels, serve as viable alternatives to 2D cultures that hold the potential for clinical scale hESC production. Using acrylate and acrylamide monomers, one study used 2-(acryloyloxyethyl) trimethylammonium chloride (AEtMA-Cl) and 2-(diethylamino) ethyl acrylate (DEAEA) in a 3:1 ratio to construct thermoresponsive hydrogels[54]. The undifferentiated colonies can be passaged without enzymatic disassociation by reducing the temperature from 37 ℃ to 15 ℃ for 30 min, followed by gentle pipetting since hydrogel swelling alone was not sufficient for hESC removal. The hydrogel adsorbed BSA and cells that were grown on the hydrogel demonstrated slower growth and lower total expansion rate over 5 d compared to Matrigel. Additionally, microdeletions and duplications on some chromosomes were present in both Matrigel and hydrogel culture conditions. Nonetheless, hESCs were able to be cultured

for over 20 passages in mTeSR1 medium on glass coverslips coated with the hydrogel and expressed pluripotency markers.

Another acrylamide-based Poly(N,N-diethylacrylamide) (PDEAAm) thermoresponsive hydrogel uses pentapeptide YIGSR-NH_2 to mimic B1 chain amino acid sequence of laminin[55]. Here, the hydrogel was further modified with $(NH4)_2SO_4$ salt to yield highly porous interconnected $(NH4)_2SO_4$-PDEAAm hydrogels that were able to support adhesion and growth of hESCs better than large NaCl generated random pores. The polyacrylamide hydrogels can also be used to direct the differentiation to specific lineages in microwells since microwells accumulate molecules above the hydrogel cut off range of 40 to 70 kDa[56]. As a result, microwells direct stem cells to be differentiated into neural, ectodermal and endodermal lineages while relatively small mesodermal inducing factors diffuse away and large mesodermal inducing factors accumulate in the microwells.

Hyaluronic acid (HA) based hydrogels were used by Gerecht *et al*[57] to culture hESCs in MEF-CM. The encapsulated hESCs formed colonies of varying sizes and maintained doubling time similar to 2D cultures. After 20 d, undifferentiated colony morphology was observed but a high cell seeding density between 5-10 x 10^6 is necessary to prevent apoptosis. The hydrogel allowed for enzymatic release (hyaluronidase) of cells that achieved cell viability of 76.5% ± 8%[57]. Additionally, Ikonen *et al*[58] used HA with hydrophilic pH-sensitive hydrogels to demonstrate adhesion and expansion of hESC cardiomyocytes. Here, the collagen-mimicking hydrogel nanofibers allowed for cell adhesion and growth due to their hydrophilicity but HA further augmented the cell survival and provided a more structurally sound hydrogel. Furthermore, the thinnest nanofibers (4.2 nm) were supportive of hESC cardiomyocytes culture.

Clinical scale applications require microenvironments that not only self-renew hESCs but are also able to direct their differentiation. To this end, Dixon *et al*[59] have used alginate-collagen hydrogels such that self-renewal of hESCs is sustained on alginate dominated state but differentiation can be induced by "switching" to a collagen predominant microenvironment *via* EDTA/sodium citrate based treatment. This process further changes the elasticity of the matrix from approximately 21.37 ± 5.37 kPa to ~4.87 ± 1.64 kPa. Moreover, the early switching (day 3) correlates with ectodermal differentiation while day 5 switching results in mesodermal and endodermal commitment. This hydrogel configuration offers advantages, namely the preservation of hydrogel structure and the relatively high hESC cell density (approximately 2 x 10^7) obtained before differentiation[59].

Polyethylene glycol (PEG)-based hydrogel functionalized with vinyl sulfone (VS) macromers with multiarms have been shown to maintain hESC self-renewal where the 8 multiarm PEG-VS hydrogel (10% PEG) proved to be ideal for hESC self-renewal and stemness expression[60]. However, the hydrogel needs to be optimized for specific cell lines since some of the cells lines demonstrated weak stemness markers. Another PEG-based thermoresponsive substrate utilized recombinant protein factors as a poly(N-isopropylacrylamide)-co-poly(ethylene glycol) (PNIPAAm-PEG) hydrogel[61]. Here, 10^{72} fold expansion over 60 passages was achieved in hPSCs using single cell passaging and stem cells were able to be further differentiated into dopaminergic neurons at even higher numbers.

Another novel hydrogel is derived from human platelet poor plasma (PPP) gelled in the presence of DMEM media, which contains calcium ions[62]. The resulting coagulation cascade forms a stable hydrogel. HESCs were able to be cultured for 25 passages and fibronectin was speculated to play a role in hESC adhesion *via* $\alpha5\beta1$ integrins[63]. Despite being relatively inexpensive, the PPP-based hydrogel was not xeno-free due to the N2 and B27 supplements in the media. Furthermore, scalability may be an issue being that the system is donor dependent.

CONCLUSION

Although many substrates exist for the self-renewal and expansion of hPSCs, most of the substrates possess limitations hinder their clinical applicability. As summarized in Table 1, Matrigel is xenogeneic in origin, contains undefined components and can be immunogenic. The relatively high production costs, immunogenicity risks and difficulties with sterilization[16,17,30] of ECM proteins limit their scalability potential while synthetic polymers, although being inexpensive and easily fabricated, have shown limited 3D scale-up capabilities. Hydrogels address the need for 3D *in vivo*-like environment but are not easily scalable.

Lastly, synthetic peptides are easily degradable[47] and difficulties with sterilization and high production costs exist[47,48]. Nevertheless, Synthemax Surface has shown to be capable for clinical applications since it can be sterilized *via* gamma irradiation, has a long shelf life of two years and can be stored at room temperature[39]. Furthermore, hESCs can be cryopreserved and thawed on substrate and studies have demonstrated its capability for long term hESCs self-renewal and maintenance[38-40]. Its scalability has also been demonstrated in T75 flasks[38]. Therefore, we conclude that Synthemax Surface is an ideal substrate for clinical applicability.

FUTURE DIRECTION

Although feeder free, xeno-free and chemically defined media has been developed, the clinical applicability of hESCs depends on synthetic substrates that can easily and economically be manufactured, can undergo common sterilization methods without degradation (*i.e.*, reusability) and yield large number of cells as required for transplantation dosage (2 x 10^8 cells/kg per dose)[64]. Microcarriers and suspension cultures have

been used to meet these demands but their limitations in controlling aggregate size, passaging challenges, shear forces in stirred cultures and difficult cell extraction from microcarrier have caused researchers to seek alternatives. As a result, significant need exists for developing scalable synthetic substrates like Synthemax that can work with multiple cell lines (without conferring epigenetic modifications to cells), easily cryopreserved, are non-labor intensive (*i.e.*, automation), adaptable to induce differentiation conditions and require fewer exogenous factors to maintain hPSCs self-renewal and expansion.

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