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Production of Human Pluripotent Stem Cell Therapeutics Under Defined Xeno-free Conditions: Progress and Challenges

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Abstract

Recent advances on human pluripotent stem cells (hPSCs), including human embryonic stem cells (hESCs) and induced pluripotent stem cells (hiPSCs) have brought us closer to the realization of their clinical potential. Nonetheless, tissue engineering and regenerative medicine applications will require the generation of hPSC products well beyond the laboratory scale. This also mandates the production of hPSC therapeutics in fully-defined, xeno-free systems and in a reproducible manner. Toward this goal, we summarize current developments in defined media free of animal-derived components for hPSC culture. Bioinspired and synthetic extracellular matrices for the attachment growth and differentiation of hPSCs are also reviewed. Given that most progress in xeno-free medium and substrate development has been demonstrated in two-dimensional rather than three dimensional culture systems, translation from the former to the latter poses unique **difficulties**. These challenges are discussed in the context of cultivation platforms of hPSCs as aggregates, on microcarriers or after encapsulation in biocompatible scaffolds.

Keywords

Human pluripotent stem cells; chemically defined media; xeno-free biomaterials; 3D culture; stem cell processing

1. Introduction

Since their isolation and derivation in 1998 [1] hESCs have been considered a promising inexhaustible cellular source for treating currently incurable diseases such as diabetes, Parkinson and heart failure. Stem cells exhibit two fundamental attributes: extensive self-

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renewal and the potential for differentiation into all types of somatic cells. Ethical concerns relating to the derivation of hESCs from fertilized eggs have largely abated with the reprogramming of terminally differentiated adult cells to stem cells termed iPSCs [2-4]. Human iPSCs and hESCs share many key properties including pluripotency and prolonged self-renewal under appropriate conditions making feasible their propagation in traditional static cultures and scalable stirred-suspension vessels [5-9]. These cells also provide a means toward patient-specific therapies and disease model development [10, 11].

Therapeutic use of hPSCs necessitates their expansion and efficient differentiation in large-scale under well-defined conditions. Scalable production is necessitated by most current cell therapy protocols **and those under development** requiring 10^8-10^{10} cells per patient [12]. For example, myocardial infarction results in the damage or ablation of at least 1-2×10^9 myocytes [13, 14] and approximately 1.3×10^9 β-cells are required for insulin independence in diabetes patients [15, 16]. Stirred suspension bioreactor systems affording densities of 10^6-10^7 cells/ml are appealing for generating stem cell therapeutics, especially given the limitations for scale-up of traditional dish cultures.

Large-scale production of hPSC derivatives goes hand in hand with the development of xeno-free environments excluding animal-derived products such as serum and cytokines commonly used in traditional mammalian cell culture [17-21]. The promise of stem cells for regenerative medicine and the rapid advances in recent years have intensified efforts toward the development of xeno-free scalable systems for stem cell products.

Yet such advances are contingent upon addressing hPSC survival, proliferation, and differentiation issues whose exact dependencies on intracellular signals and extrinsic factors require further elucidation. The microenvironment – commonly referred to as the niche – imposes its effects mainly through soluble factors, cell-cell and/or cell-matrix contacts and mechanotransduction (Figure 1). Here, we summarize the progress on the development of defined xeno-free media. **Advances** in extracellular matrices and synthetic substrates for the maintenance of uncommitted hPSCs on 2D surfaces are **also** reviewed. In the latter part of the article, the challenges are discussed **for** developing clinically relevant scalable systems for the culture of hPSCs as aggregates, after scaffold encapsulation and on microcarriers.

2. Xeno-free media for hPSC culture

Limiting the aberrant differentiation of cultured hPSCs is a key consideration as the cells self-renew or their fate is directed along particular lineages. Our knowledge **of** appropriate conditions supporting hPSC self-renewal is based on heterologous systems of development, mainly **that** of the mouse embryo. A significant body of studies on signaling of hPSCs in culture has extended our understanding of the dependence of self-renewal on extracellular parameters. It is well accepted that the pluripotency of hESCs is controlled through common genetic networks of transcriptional factors [22-24]. Examples of such factors include Nanog, Pou5f1 (also known as Oct4) and Sox2 with cooperative interactions among them underlying the maintenance or loss of pluripotent state early in embryonic development and *in vitro* [23, 25, 26].

Multiple signaling pathways such as the transforming growth factor-beta (TGF-β) super family-activated cascades, receptor tyrosine kinase (RTK) signaling (downstream of the basic fibroblast growth factor (bFGF)), canonical Wnt signaling [22, 27], and pathways related to insulin or insulin-like growth factors (IGFs) [28, 29] regulate pluripotency gene levels [30, 31]. Based on signal transduction findings, a key approach to develop media for hPSCs is to identify and supply extrinsic growth factors which work through cascades with direct access to hPSC pluripotency programs. Bone morphogenetic proteins (e.g. BMP4) and the leukemia inhibitory factor (LIF; a JAK/STAT signaling activator) are sufficient to preserve the undifferentiated state of cultured mouse ESCs (mESCs) [32] even in serum-free conditions [33] but not of hESCs [1, 34]. Human PSC **pluripotency** depends on TGFβ signaling [35] with TGFβ1, Activin A and Nodal directly activating Nanog expression via a promoter site for SMAD2/3 binding [36, 37]. Because these molecules are produced by hPSCs to varying degrees, they are not part of all medium formulations.

Basic FGF though is a universal supplement which is critical for sustaining hESC self-renewal *in vitro* [38, 39]. For hPSC culture on mouse embryonic fibroblast (mEF) feeder cell layers [40] or in mEF-conditioned medium [41], the bFGF concentration (4 ng/ml) is lower than in feeder-free cultures (40-100 ng/ml) [38, 42, 43]. Interestingly, the BMP antagonist noggin supports the growth of undifferentiated hESCs in unconditioned medium with 40 ng/ml bFGF but does not appear to have an effect when bFGF is increased to 100 ng/ml [44].

Canonical Wnt/β-catenin signaling has also been implicated in hPSC self-renewal [45, 46]. Even so, others reported that recombinant Wnt3a is not sufficient to maintain hESCs undifferentiated without feeder cells and β-catenin-mediated transcriptional activity is upregulated during differentiation [47]. The effects of Wnt signaling in hESC pluripotency have been difficult to unravel because different hPSC lines exhibit disparate levels of endogenous Wnt activity. Further, Wnt has been implicated in the specification of stem and progenitor cells along multiple and often developmentally distant lineages suggesting that exposure of hPSCs to Wnt ligands should be finely customized.

These and other -often unidentified- factors are traditionally provided through supplementation of the medium with fetal bovine serum (FBS). Nonetheless, the use of non-human components (e.g. Neu5Gc; [48]) is incompatible with clinical applications driving efforts to design xeno-free culture systems for hPSCs and their products. Serum replacers (e.g. knockout serum replacer (KSR)) [49] have proprietary composition and may also contain animal-derived components such as bovine serum albumin (BSA).

Media composed of chemically defined, non-xenogeneic compounds for the propagation and differentiation of hPSCs are highly desirable [18, 30, 50, 51]. Approaches to develop defined media for hPSCs consist of identifying both a suitable basal medium and additional signaling factors promoting cell growth and preservation of pluripotency or induction of (directed) differentiation. Basal media such as DMEM and DMEM/F12 provide mainly glucose, vitamins and salts (at appropriate osmolarity) to cells whereas factors (e.g. bFGF) eventually activate or repress genetic programs for hPSC self-renewal or specification. For example, a defined medium based on DMEM/F12 with 100 ng/ml bFGF and components

such as **TGF-β**, LiCl, insulin, GABA and BSA or human serum albumin (HSA) is extensively used in hPSC cultivation [52, 53]. Other formulations are show in Table 1. DMEM/F12 with 20 ng/ml bFGF and B27, N2 and BSA has been used to maintain hESCs for over 27 passages. And in the absence of BSA, DMEM/F12 combined with N2, B27 and high concentration of bFGF (40-100 ng/ml) is adequate for hESC maintenance. The X-Vivo 10 medium supplied with recombinant bFGF, stem cell factor (SCF), LIF and Flt3 ligands has also been successfully used **for** hESC maintenance. Nonetheless, the aforementioned media typically contain BSA (or the more expensive HSA). Recently, a fully defined medium (E8) containing 8 factors (including bFGF) without BSA was described for the long-term propagation of hPSCs [54].

Despite the significant advances in the development of defined and xeno-free media, there are still unresolved issues. For instance, side-by-side comparison by our laboratory and others of the performance of commercially available xeno-free media indicates differences in the fold-expansion of cells over the same period, particularly over multiple passages. Although the reason(s) for such discrepancies are unclear, the quality of supplements used in these medium formulations may be a suspect. The generation even of recombinant growth factors and other proteins (e.g. recombinant albumin) requires separation steps (e.g. isolation from bacterial cultures, purification etc.) which do not always result in impurity-free preparations. Traces of impurities may affect the propagation of cells and their long-term potential.

Moreover, almost all current protocols for hPSC culture require daily medium replacement increasing the cost and associated labor. Fluctuation of growth factor levels in the medium contributes to the variability of hPSC cultivation. Soluble human or zebrafish bFGF loses most of its activity in culture after 24 hours [60]. This may be circumvented with the controlled release of bFGF (or other factors) in culture. Basic FGF-loaded PLGA microspheres added to hPSC cultures reduce the frequency of medium from daily to every three days or biweekly [61].

Hence, creating supplements with extended shelf life while keeping the cost low are highly desirable. Small molecules promoting hPSC self-renewal have been suggested as candidates which may fit the bill. Using high-content screening methods, small molecules such as trimipramine and ethopropazine which can **diffuse easily through** multi-layer cellular configurations and have much longer degradation times, **have been** reported to maintain the self-renewal of hESCs replacing exogenous bFGF [62, 63].

3. Extracellular matrices for hPSC cultivation

Despite the availability of chemically defined media for hPSC cultivation, the quest for relevant xeno-free substrates, particularly for **use in** large-scale production of **stem cell products of wide utility**. Beyond the obvious requirement for promoting cell adhesion, the design of defined surfaces is subjected to a unique constraint **of** unimpeded hPSC self-renewal and differentiation. Efforts in this direction are hampered by the incomplete knowledge of **the** regulation of human stem and progenitor cell fate within complex niches *in vivo*.

Traditionally, hPSCs have been maintained on layers of inactivated mEFs, which secrete factors supporting hPSCs. Thus, early efforts focused on feeder cell surrogates of human origin including human fetal foreskin fibroblasts [64-67], adult epithelial cells [68], bone marrow cells [69, 70] and placenta-derived feeder cells [71, 72]. Apparent difficulties in the sourcing – including variability due to donor age and condition [73], derivation, preparation and preservation of human feeder cells limit their use in stem cell culture. Importantly, co-culturing hPSCs with feeder cells adds a requirement for separation and removal of the latter thereby imposing significant technical and economic burdens on **envisioned bioprocesses**.

The introduction of the extracellular matrix protein (ECM) mixture Matrigel produced by Engelbreth-Holm-Swarm mouse sarcoma cells led to successful expansion of stem cells without the need for feeder cells. Matrigel contains laminin, collagen type IV, heparan sulfate, proteoglycans, entactin, and nidogen [1, 74], and its use as an hPSC substrate is fairly straightforward and not time-consuming. However, its undefined composition precludes its use in applications calling for the xeno-free production of stem cell progeny. **These facts have elicited efforts to develop defined substrates for the generation of therapeutic products from stem cell cultures**.

3.1 Human ECM protein-based substrata

Natural ECM glycoproteins such as laminin, fibronectin, vitronectin, entactin, tenascin and collagen influence stem cell adhesion, survival, growth and differentiation [75, 76] through their interactions with cell surface moieties. Each ECM component exhibits distinct domains for binding to surface receptors (e.g. integrins) mediating adhesion and triggering signaling cascades linked to cell fate selection [77-79]. **Among different motifs, the integrin-interacting arginine-glycine-aspartic acid ('RGD') motif is shared by various ECM proteins including laminin, vitronectin and fibronectin** [80, 81]. **In fact, mutations in the RGD sequence result in greatly reduced cell adhesion** [82]. **A mixture of recombinant human collagen IV, vitronectin, fibronectin and laminin supports the derivation and growth of undifferentiated hESCs over multiple passages** [52]. Along this vein, the use of ECM proteins and peptides from tissue isolates or in recombinant form has been investigated **extensively** for the culture of hPSCs.

Among the 24 different known integrin heterodimers, α1β1, α2β1, α3β1, α6β1, α6β4, α7β1, α9β1, and αvβ3 in various cell types have been reported to bind laminin [83]. The α6β1 integrin is expressed by hESCs and plays a significant role in adhesion [74] suggesting that laminin is a critical ECM protein for supporting hESC proliferation. Indeed, natural or recombinant laminin in lieu of Matrigel **was reported to maintain** the growth and pluripotency of hESCs in mEF-conditioned medium [74, 84]. However, human placenta-derived laminin **was shown to** only support hESC self-renewal for 3 passages in chemically defined medium [85] **while over longer periods** (>10 passages), hESCs grew significantly more slowly with evident spontaneous differentiation and poor adhesion [86]. The presence of ECM molecules (besides laminin) secreted by feeder cells may be a potential explanation for the discrepancy in the **findings of** these studies. **Moreover, although** laminin-511 (but not laminin-332) supports the culture of multiple hESC lines under xeno-free conditions

[87], laminin peptides **failed to promote** hESC attachment and growth **in** a concurrent study [88]. **Thus, the utility of laminin alone as hPSC substrate remains unsettled**.

Like laminin, the use of vitronectin has been explored for the culture of hPSCs. Vitronectin mediates hPSC adhesion through αVβ5 integrins as shown in integrin-blocking antibody experiments [86]. The proliferation of three hESC lines (**HUES1, HES2, HESC-NL3**) in mTESR1 medium was supported by vitronectin in a manner comparable to that of Matrigel **and** in contrast to fibronectin- (acting through the α5β1 integrin) coated surfaces **requiring feeder cell-conditioned (but not defined) medium for hESCs to grow. Interestingly enough**, Liu *et al*. reported that cultured hESCs could not be maintained on vitronectin for more than 7 days [55] in defined medium containing bFGF, N2 and B27 supplements. These results illustrate the complexity of pinpointing individual matrix components supporting hPSC culture and emphasize the need for considering multiple aspects of the culture system including the medium used for hPSC maintenance.

Nonetheless, vitronectin from human plasma promotes self-renewal **for over** 20 passages without compromising the potential of hPSCs for differentiation [89]. Notably, a threshold surface density of 250 ng of vitronectin/cm^2 was estimated for successful hPSC culture. This value applies to whole-molecule vitronectin and should be adjusted when utilizing vitronectin derivatives or fragments. **This may explain the differential support of cultured hPSCs by variants of vitronectin** [54]. For hESCs grown in E8 medium, two truncated vitronectin molecules (amino acids 62-398 and 62-478) **promote** initial attachment and survival of hESCs as single cells (with ROCK inhibitor or blebbistatin) and as clumps. A chimeric glycoprotein of vitronectin and IGF1 **also maintains** hESCs in defined medium [78].

Fibronectin also features the RGD domain **interacting** with α3β1, α5β1, α8β1, αvβ1, αvβ3, αvβ5, and αvβ6 integrins [90, 91]. Based on available studies, the role of fibronectin in stem cell adhesion and culture is still **unclear**. Human-plasma fibronectin **promotes** hESC proliferation and pluripotency in defined medium for at least 10 [85] to 13 passages [55]. Yet, cultivation of hESCs on fibronectin-coated surfaces **failed in** mTeSR1 but not **in** mEF-conditioned medium as mentioned above [86]. As with vitronectin, a threshold density of 80 ng/cm^2 of plasma-fibronectin was determined for hESC culture in a serum-free medium [92]. This density also applied to the 120 kDa fragment of fibronectin with the central cell-binding domain containing the RGD motif (1–10 type III repeats), while other fibronectin fragments did not support the maintenance of hESCs.

Capitalizing on the central role of the RGD domain of ECM molecules on hPSC adhesion, various groups implemented an approach of building ECM substrata with synthetic RGD-containing peptides [88, 93]. A cyclic RGD peptide covalently bound to tissue culture surface at 10-30 fmol/cm^2 was used to culture hESCs in conditioned medium (10 passages) or mTeSR1 (5 days) [94]. It should be noted however that such substrata should promote not only hPSC adhesion but self-renewal and unhindered differentiation as well. For example, one peptide featuring the YIGSR domain promotes hESC adhesion but cultured cells display significantly reduced expression of OCT4 and SSEA4 [95] in contrast to other integrin-binding peptides (e.g. GKKQRFRHRNRKG, FHRRIKA and GWQPPARARI). Peptides

derived from the bone sialoprotein and vitronectin (but not from fibronectin) and covalently attached onto acrylate-coated surfaces facilitate the adhesion of hESCs [96]. Thus, although the presence of binding (e.g., RGD) domains of natural ECM molecules may point to candidate peptides for hPSC culture, additional optimization of the whole peptide sequences is necessary for the development of appropriate substrata.

A summary of natural ECM proteins or their derivatives used as substrata for hPSC culture is presented in Table 2. The type of medium utilized in each study is also shown.

3.2 Synthetic ECMs

The varied performance of natural or recombinant human ECM proteins and the associated high cost have **motivated the development of** synthetic ECMs. Recent studies have demonstrated success in culturing hPSCs on such synthetically prepared polymer surfaces [98]. Synthetic polymers have been widely investigated as extracellular matrices for the cultivation of PSCs because of their low cost and high availability [99]. Synthetic ECMs that are biocompatible and mimic natural ECMs have been researched extensively. Li et al.,[100] synthesized a 3D hydrogel scaffold made of poly(N-isopropylacrylamide-co-acrylic acid) [p(NIPAA-co-AAc)] with Gln-Pro-Gln-Gly-Leu-Ala-Lys, an acrylated peptide crosslinker that can **be digested** by collagenase. Polyacrylic acid-graft-Ac-CGGNGEPRGDTYRAY-NH_2, a linear polymeric chain containing synthetic peptides also assisted in enhancing cell adhesion. **A more extensive collection of** 91 different polyacrylamide polymers **was also investigated. Sixteen of those** supported pluripotent hESCs (HUES9) for five days [99] with poly(methyl vinyl ether-alt-maleic anhydride) [PMVE-alt-MA] **exhibiting the best performance for p**ropagation of pluripotent stem cells (HUES7 and HUES9 hESCs and an unnamed iPSC line) for five passages. **Stem cells on PMVE-alt-MA expressed integrins α_5 (ITGA5) and α_v (ITGAV) more than cells cultured on Matrigel, thereby strengthening attachment to the matrix and were capable of giving rise to endoderm, mesoderm and ectoderm cells**. Other polymers successfully used as ECMs for PSC culture are listed in Table 3. In addition, polymers with ester ions and cyclic polymer ions **have also been shown to promote hESC adhesion** [101].

Taken together, both the composition and chemical structure of synthetic matrices considered for hPSC culture are important determinants of the attachment and maintenance of pluripotency or capacity for specification.

3.3 Substrates for 3D hPSC culture

Findings reviewed thus far pertain mostly to flat surfaces coated with ECM or synthetic molecules for hPSC culture. Considerable efforts however have been geared toward the design of 3D scaffolds, some of which mimic natural stem/progenitor cell niches. Hydrogels are commonly used to create 3D environments for stem cells in culture. For example, scaffolds of 2.4% (w/v) alginate and 2.4% (w/v) chitosan prepared by lyophilization [106] support BG01V hESCs over 21 days of culture. Human H1 ESCs encapsulated in alginate beads and cultured in dishes maintain their undifferentiated state expressing OCT4, NANOG, SSEA-4, TRA-1-60 and TRA-1-81 after 260 days [107]. These cells were also coaxed toward type II pneumocytes after 160 days of encapsulation, and to neuronal and

chondrogenic lineages after 200 days of culture. Scaffolds of alginate and chitin support HUES7, BG01V/hOG and hFib2-iPS4 cells for 10 passages [108]. **Human ESCs in 3D alginate capsules show more rapid commitment into midbrain dopamine neurons than in 2D cultures** [109]. **In fact, when combined with poly(γ-glutamic acid) (γ-PGA), alginate promotes neural differentiation of iPSCs in matrices coated with nerve growth factor (NGF)** [110]. Lastly, defined hydrogels consisting of hyaluronic acid, which is present during early embryo development, have also been used to maintain and differentiate hESCs [111].

In addition to the composition, the scaffold ultrastructure affects stem cell growth. Fibrous scaffolds support the proliferation of stem cells (H1, H9 hESCs) cultured for 14 days in poly(desaminotyrosyl tyrosine ethyl ester carbonate) (pDTEc) matrices coated with poly-D-lysine. The cells can be differentiated into neuronal, smooth muscle, and hepatic-like lineages [112]. Similarly, hESCs (HES3, Endeavour-1, Envy) adhere to 3D PLGA cylindrical (2 mm thick) matrix slices coated with laminin [113]. Two days after seeding, the cells within the scaffold can be coaxed to mesoderm. Poly(methacrylic acid)-coated carbon nanotubes, which are similar in scale to collagen and laminin moieties, promote neuronal differentiation of hESCs [114, 115].

From this discussion becomes obvious that there are many potential avenues for manufacturing materials for hPSC cultivation. Hybrid biomaterials are promising as they combine the adhesion properties and biological functionality of natural or bioinspired ECMs with the low cost and prolonged shelf life of synthetic molecules.

4. Scalable hPSC culture systems

The development of xeno-free culture media and substrates is driven largely by the therapeutic applications envisioned for hPSCs and their progeny. The generation of large quantities of cells under strictly defined conditions and in a reproducible fashion is a prerequisite for the use of hPSC products in the clinic. Moreover, the production of larger batches of cells is more economical motivating scale-up of stem cell cultivation.

Different designs of bioreactors offer alternatives for the large-scale culture of hPSC products [116, 117]. Among those, **automated systems afford the handling of higher culture volumes per run while eliminating operator errors. Such systems include the CompacT SelectT** [118] **and Cell^{host} systems** [119], **which were utilized for large-throughput culture of hPSCs in tissue culture flasks. Other automated platforms designed for general scalable mammalian cell culture such as the CELLROLL roller bottle system (Integra Biosciences, Hudson, NH) and the CellCube (Corning Inc., Acton, MA) may also be suitable for hPSC cultivation. Other bioreactor modalities have also been demonstrated in conjunction with PSC culture including rotary cell culture systems** [120] **and slow-turning lateral vessels** [121].

To that end, stirred suspension bioreactor is an appealing choice for large-scale cultures due to the homogenous environment and ease of operation and monitoring of culture. These bioreactors afford multiple culture modes including the cultivation of cells encapsulated, on microcarriers or as aggregates (Figure 2). These modes have been demonstrated for the

culture of hPSCs and will be discussed below in the context of xeno-free generation of stem cells.

It should be noted that selecting biomaterials for scalable application e.g. by mere translation of materials used statically might not be straightforward. In stirred suspension, both cells and scaffolds face a different hydrodynamic environment than that in static culture. Agitation is necessary to keep cells and scaffold suspended and ensures a homogeneous environment. However, stirring exposes cells and scaffolds to shear stresses. Apparently, biomaterials should bear certain mechanical properties for preservation of structural integrity under flow in the bioreactor. Shear stress induces cell removal from surfaces and reduces cell viability [17]. In a recent study, we reported that a peptide-conjugated polystyrene matrix, which supported attachment and proliferation of hiPSCs under static condition, was not sufficient to achieve the same goal under agitation [9]. Human ESCs cultured on vitronectin-coated microcarriers also show reduced growth rate compared to cells cultured on dishes coated with the same protein [122]. **In addition to desired** mechanical properties, affordable, biocompatible and biodegradable materials are highly preferred for large-scale bioprocessing aiming at serving future clinical applications.

4.1 Cultivation of hPSCs after encapsulation

Cells cultivated in stirred suspension after their encapsulation in matrices (typically hydrogels) are protected from hydrodynamic shear and excessive agglomeration of clusters. The materials employed for encapsulation allow control of their permeability and therefore of the molecules exchanged between cells and the culture environment. For example, tight control of the permeability of encapsulation materials aims to allow the transport of O_2 and nutrients while blocking the penetration of immune cells and antibodies. These cell-laden scaffolds may be transplanted directly with minimal immunological rejection [123] serving as a basis for scalable systems intended for expanding and differentiating hPSCs to therapy-grade cells.

The general procedure of encapsulation entails the formation of cell-gel droplets and gel cross-linking. In this respect, biocompatible materials requiring mild cross-linking conditions are advantageous. Alginate is the most common material used for encapsulation [124] with appealing attributes such as biocompatibility, inertness toward cells [125] and a relatively straightforward protocol for generating micron-size capsules laden with cells under physiologically relevant conditions. A cell suspension (a few million cells per milliliter) of sodium alginate solution (normally 1-2% (w/v)) is dispersed in droplets, which solidify upon contact with a $CaCl_2$ solution [126]. Cells can be maintained in solid or liquefied-core capsules with external coating. We previously demonstrated that both mESCs and hESCs can be entrapped in alginate beads coated with poly-L-lysine (pLL) and cultured in spinner flasks [127]. The pLL coating allows the liquefaction of the bead core using Ca^{+2}-chelating agents thereby facilitating the controlled aggregation of the cells. Besides enhancing the mechanical strength of the beads, the pLL layer is also permeable to soluble differentiation factors (e.g. Wnt3a, Activin A, BMP4) as shown with the coaxing of encapsulated hESCs to cardiomyocytes-like cells. Combining alginate microencapsulation with microcarriers allowed the hESC expansion in spinner flasks for two weeks noting a 20-

fold increase in concentration and a 3-fold improvement of post-thawing viability after cryopreservation [128]. Alginate can also be mixed with other materials for stem cell entrapment. For example, mESCs encapsulated in a mixture of 1.1% (w/v) alginate and 0.1% (v/v) gelatin have been cultured in a rotary high-aspect-ratio vessel (HARV) [129]. The cells were successfully induced into alveolar epithelial cells with shorter times of differentiation compared to dish cultures. Mouse ESCs cultured in rotary bioreactors have also been induced to cardiomyocytes [130] and osteogenic lineages [131].

Besides alginate, agarose is another choice for hydrogel encapsulation of ESCs. Mouse ESCs encapsulated in size-controlled agarose capsules can be cultured in stirred suspension at high density and become hematopoietic progenitors [132]. Agarose-encapsulated mESCs propagated in 250-ml spinner flasks have also been differentiated into cardiomyocytes [133].

Despite all the advantages that cell encapsulation offers, it may pose considerable hindrance to the transfer of O_2, nutrients, waste and factors as shown in Figure 3. Such limitations may affect the control of cell proliferation and/or the differentiation along particular lineages. If cell purification is required, the separation and harvesting of cells from the scaffolding material(s) not only increases the cost of the process but potentially contributes to the reduction in cell number and viability. Moreover, the use of UV for cross-linking certain gels after cell loading is another concern.

4.2 Microcarrier culture of hPSCs

Microcarrier bioreactors have been utilized since the early 1970's for the large-scale culture of different (particularly anchorage-dependent) cell types intended to generate a wide gamut of products including, viruses, vaccines and proteins [134, 135]. Microcarriers afford distinct advantages such as high surface-to-volume ratio and flexibility in accommodating the adhesion needs of various cells via surface modification [136] in conjunction with the benefits of stirred suspension bioreactors such as real-time monitoring and controlling of the culture environment. Microcarrier culture usually holds a higher volume fraction (ratio between cell and medium). Assuming an average volume of approximately 2000 μm^3 per human cell ($\sim$15 μm^3 diameter), the cellular volume fraction in microcarrier suspension culture is about 0.4% (for 2×10^6 cells/ml) compared to 0.2%-0.3% for a confluent dish culture. Compared to bioreactor aggregate cultures, hPSCs attached on microcarriers are also exposed more readily and uniformly to the medium bulk concentrations of oxygen, nutrients and factors (Figure 1).

Current embodiments of the microcarrier culture systems however, require the separation of cells from the beads unless secreted metabolites or other non-cell products are desired. This requirement increases the downstream processing time and overall cost and may reduce the recovery of cellular products. Furthermore, high levels of agitation-induced shear are detrimental to cells while the effects of stress from lower stirring speeds especially on stem cells are still **unclear. Compared** to other cell types (e.g. CHO or Vero cells) traditionally cultured on microcarriers, hPSCs exhibit a more pronounced tendency for cell-cell aggregation. Thus, multi-bead cell clusters may be formed at low agitation speeds. The presence of shear in microcarrier bioreactors hampers the direct application of xeno-free

substrates from 2D to 3D environments [9]. Indeed, much of the discussion in the previous sections centered on xeno-free matrices developed for hPSC cultured on flat surfaces (e.g. dishes) but there are significant differences between 2D and 3D substrata (e.g., with respect to curvature and elasticity affecting stem cell shape, spreading, and ultimately specification). For example, the growth rate of mesenchymal stem cells cultured on peptide-modified alginate beads is inversely related to the diameter of the beads **due to** differences in shear stresses acting on cells [137]. **Proliferation of hESCs on vitronectin (full molecule)-coated microcarriers was reportedly hampered compared to tissue culture dishes layered with the same protein** [122]. Attachment and proliferation of hESCs on microcarriers coated with laminin-111, which supports hESC growth on dishes, were sensitive to shear [122]. Human PSCs attach and spread on vitronectin-derived peptide conjugated-microcarriers in static culture. However, cells readily peel off of microcarriers and form aggregates in agitated suspension [9]. Taken together these findings demonstrate that surface modifications for 2D hPSC cultures do not translate directly to dynamic 3D cultures.

The composition of microcarriers affects the overall surface charge and functional group availability for cell adhesion thereby dictating largely their suitability for cultivation of particular cell types. Various commercially available microcarrier types have been tested in multiple reports for hPSC culture [8, 122]. Microcarriers layered with Matrigel exhibit consistent performance in stirred suspension bioreactors (Table 4) but the matrix's undefined composition and animal origin prevent its use in clinical-grade hPSC products.

Microcarriers with positive surface charges **appear to perform** better than those with negative or neutral charge [122, 145]. Indeed, microcarriers with surface-conjugated peptides support hPSC attachment and growth under agitation, only after coating with pLL, which is a positively charged synthetic polymer. Despite the lower seeding efficiency than on Matrigel-coated microcarriers (38% vs. 77%), a similar fold-increase (23.3 vs. 20.7) is achieved for hPSCs on pLL-coated, peptide microcarriers over multiple 6-day passages. The cells maintain a normal karyotype and consistent expression of pluripotency genes and proteins (Nanog, OCT4 and SSEA4) during 5 consecutive passages while subsequently **they** form embryoid bodies and their specification can be directed to all three germ layers [9].

In the future, functional modifications of microcarriers will aim to not only support the expansion of uncommitted hPSCs but also their lineage-specific differentiation so that the two culture segments can be integrated in a single process. To better meet the needs of clinical applications, materials should be utilized for microcarrier construction which are biocompatible and biodegradable allowing direct transplantation to patients thereby eliminating expensive downstream processing steps. Certain clinical applications, for example, may call for particular degradation rates, which can be adjusted by controlling the biomaterial composition, for better integration of the implanted cells with the host tissue. Obviously, such considerations should be viewed in conjunction with the overall bioprocess cost.

4.3 Cultivation of hPSCs as aggregates

Undifferentiated hPSCs aggregate forming embryoid bodies (EBs) on non-adhesive surfaces or in suspension. Methods for EB formation include suspension in low-affinity culture dishes and hanging drops [146] although control of the aggregate size can be challenging. Conversely, cells can be cultured in microwells of specific size [147] or microchannel devices [148] resulting in aggregates with a narrow size distribution. The scalability of most of these methods for producing large quantities of EBs for bioreactor culture is debatable. This issue may be addressed with the use of rotary orbital suspension culture systems yielding EBs which are homogeneous in size and shape [149]. Yet, single dispersed hPSCs can be seeded directly into suspension bioreactors [17, 150] in the presence of ROCK inhibitor (Y-27632) [151]. Since then, several reports emerged of hPSCs successfully expanded as aggregates in stirred suspension systems [152-154].

A major advantage of culturing hPSCs as aggregates in stirred suspension is the absence of extraneous scaffolds. This reduces the downstream separation steps for obtaining pure cell populations and make the whole process easy to set up and economic. However, aggregates formed by hPSCs are usually uneven in size, which can be caused by initial heterogeneous aggregate formation and agglomeration during culture. As EBs increase in size, cells near the aggregates' core are subjected to limited transport of nutrients and O_2. Spatial gradients may further modulate the propensity of stem cells for proliferation, differentiation and apoptosis [133, 155]. Shear stress encountered by aggregates when cultured under agitation **affects** cell proliferation and differentiation. It was reported that moderate shear (1.5 to 15 dyne/cm^2) promotes hematopoietic and endothelial differentiation of hESCs [156].

There have been a few reports combining the bioreactor culture of hPSC aggregates with materials for structural support of the clusters and for promoting cell adhesion, self-renewal and differentiation. During normal embryogenesis, stem cells form complex 3D structures and differentiate along disparate lineages. To that end, the EB system may serve as an *in vitro* platform of stem cells differentiation mimicking aspects of in vivo development [157]. Microparticles (10-15 μm diameter) can be incorporated into cell aggregates to affect cell fate decisions via controlled release of soluble factors. Such localized delivery of cues promotes differentiation by altering their local concentration. Incorporation of gelatin microparticles loaded with bone morphogenetic protein-4 (BMP4) and thrombopoietin (TPO) promotes mesoderm differentiation of hESCs compared to the traditional medium supplementation with soluble stimuli [158]. Microparticles made of different materials including agarose, PLGA and gelatin have been embedded within mESC aggregates. When mESC clusters are cultured with retinoic acid (RA)-releasing PLGA beads, the fate and organization of the cells changes compared to aggregates without the particles [159]. Vascular differentiation is also enhanced by PLGA microparticles (diameter of 0.24-25 μm) releasing vascular endothelial growth factor (VEGF), placenta growth factor (PLGF) and bFGF [160].

Conclusions

Our review of the current state of the art in defined xeno-free media and substrates for hPSC culture underlines the great advances noted in recent years but also the issues remaining to

be resolved. Tackling these challenges will require expanding our knowledge of mechanisms governing stem cell self-renewal and commitment and most importantly, how these mechanisms can be exploited in synthetic culture environments. It is also becoming apparent that bridging stem cell research with the commercial scale production of hPSC therapeutics in a good manufacturing practice (GMP) fashion can be done effectively and efficiently through multidisciplinary approaches. Such efforts will be instrumental in the design and development of bioprocesses for the standardized and cost-effective production of stem cell products.

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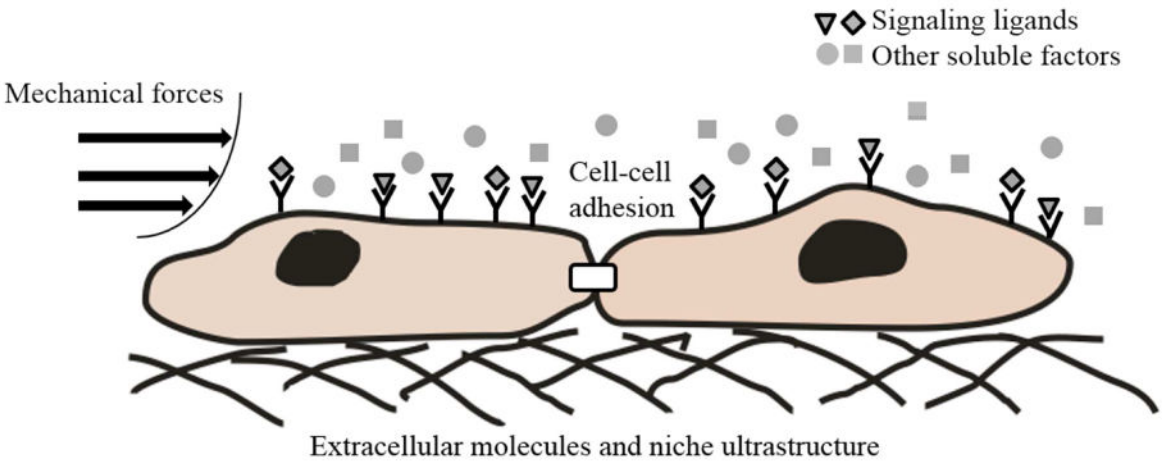

Figure 1.

Schematic representation of microenvironmental cues encountered by human PSCs. Such cues include signaling **ligands, other soluble factors, mechanical forces, interactions among cells and between cells and extracellular matrix molecules presented in a fashion dictated by the niche ultrastructure. Signaling ligands include bFGF (basic fibroblast growth factor), Wnt ligands, BMPs (bone morphogenetic proteins), activin A, and insulin. Other soluble factors include salts, vitamins and lipids**.

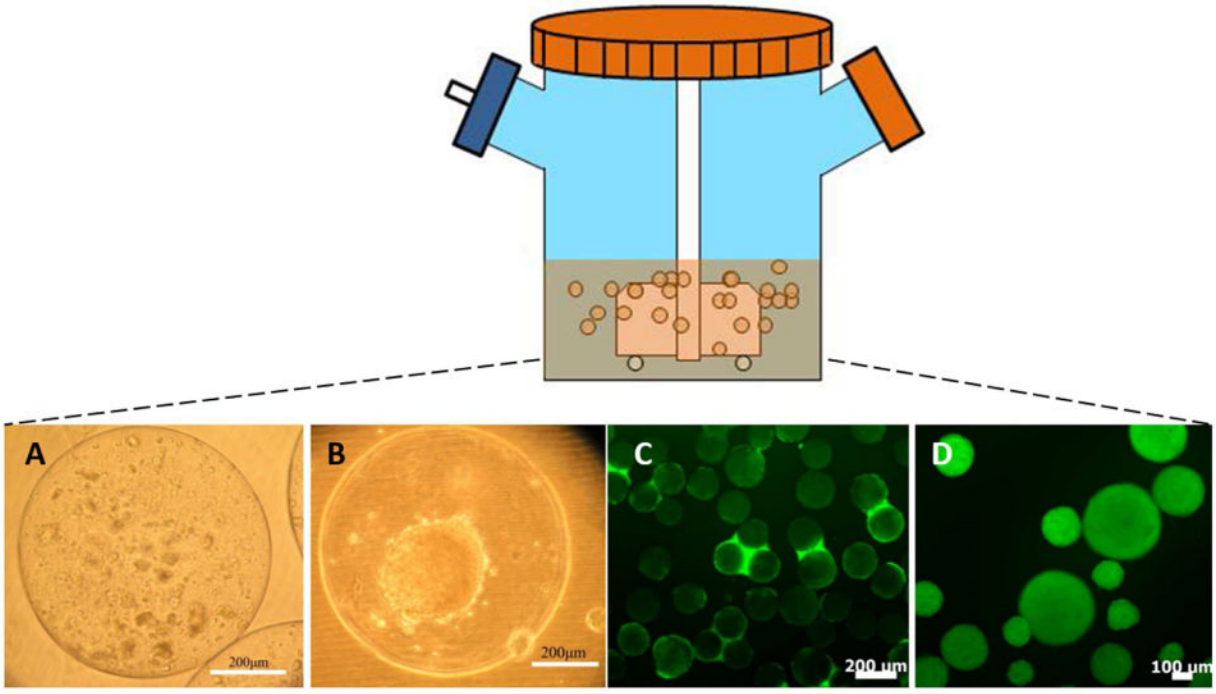

Figure 2. Different modes of hPSC cultivation in stirred-suspension vessels

(A-B) Human ESCs cultured in alginate capsules (A) without or (B) with a liquefied core. (C) Human ESCs cultured on vitronectin-coated microcarriers. (D) **Human ESCs cultured as aggregates**.

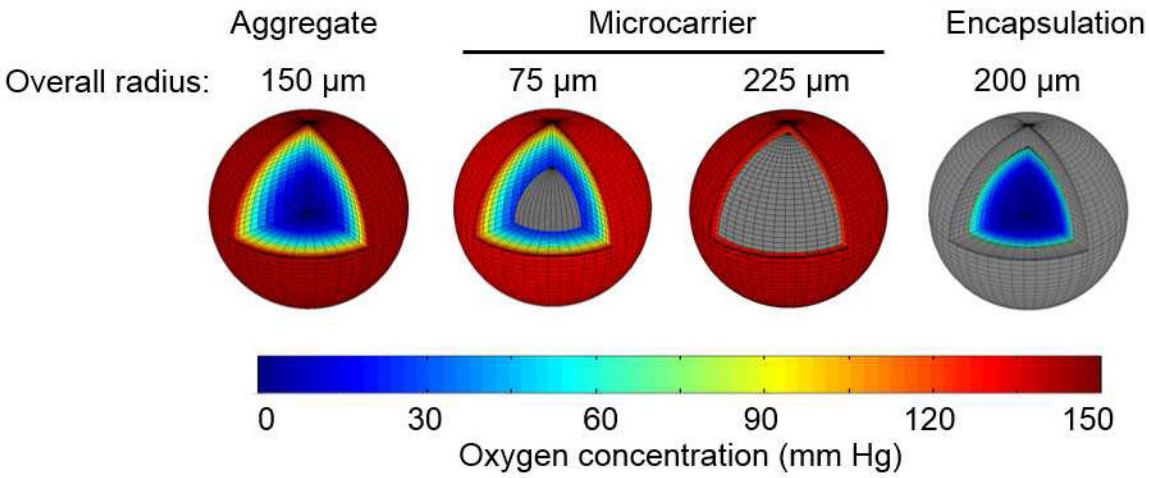

Figure 3.

Comparison of the profile of O_2 in different 3D culture modes, i.e. hPSCs cultured as aggregates, on microcarrier or after encapsulation in alginate beads. Profiles were generated using a reaction-diffusion model assuming Michaelis-Menten kinetics for O_2 consumption by cells and pertinent parameters for diffusion in 1% alginate matrices [161, 162]. Color regions represent the O_2 profile of hPSCs and gray regions indicate biomaterials (microcarrier or alginate). For aggregate culture, hPSC clusters with 150 μm radius were modeled. Two profiles are shown of hPSCs grown on the surface of microcarriers with radii of 75 and 200 μm. Alginate beads were taken having a radius of 200 μm with 1% composition encapsulating a 150 μm hPSC aggregate.

Table 1

Composition of defined and xeno-free media for hPSC culture.

Name	Basal medium	Select supplements	Xeno -free?	Ref.
		Commercially available media		
mTeSR	DMEM/F12	bFGF, TGFβ, Insulin, Transferrin, Cholesterol, Lipids, Pipecolic, BSA, GABA, LiCl, Amino acids, L-glutamine, β-mercaptoethanol	No	[53]
StemPro	DMEM/F12	bFGF, Activin A, Transferrin, Lipids, NEAA, L-glutamine, β-mercaptoethanol, HRG1β, LR3-IGF1	No	[28]
TeSR	DMEM/F12	bFGF, TGFβ, Insulin, Transferrin, Cholesterol, Lipids, Pipecolic, HSA, GABA, LiCl, Amino acids, L-glutamine, β-mercaptoethanol	Yes	[52]
E8	DMEM/F12	bFGF, TGFβ1, Insulin, Transferrin, Seleniun, L-ascorbic acid	Yes	[54]
		Other media		
	DMEM/F12	N2, B27, BSA, bFGF, L-glutamine	No	[55]
	IMDM/F12	bFGF, Activin A, Insulin, Transferrin, BSA, Activin A, L-glutamine, β-mercaptoethanol, Monothioglycerol	No	[39]
	DMEM/F12	bFGF, Insulin, Transferrin, Cholesterol, Lipid-rich BSA, L-glutamine, β-mercaptoethanol	No	[56]
	DMEM/F12	bFGF, N2, B27, BSA, L-glutamine, β-mercaptoethanol	No	[57]
	ESF	bFGF, Insulin, Transferrin, β-mercaptoethanol, 2-ethanolamine, selenite, Ascorbic acid, albumin conjugated with oleic acid	No	[58]
	XVIVO-10	bFGF, hFLT3*, L-glutamine, β-mercaptoethanol	Yes	[59]

*hFLT3: human FMS-like tyrosine kinase-3, Animal-origin components are underlined.

Table 2

Summary of extracellular protein based defined xeno-free substrates for hPSC culture on 2D surfaces.

Matrices	Medium	Cell line	Ref.
Human laminin (2 μg/cm^2, absorption)	NC-SFM	H1	[59]
Human fibronectin (25 μg/ml, absorption)	HESCO	H9, BG01	[56]
Human plasma fibronectin	DMEM/F12 plus N2, B27, bFGF, Activin A, and neurotrophin 4	MAN 1, HUES 1, HUES7	[85]
Recombinant human E-cadherin and mouse IgG_1 Fc domain fusion protein (absorption)	mTeSR	H1, H9, hiPSC2a*, hiPSC3a*, hiPSC6a*	[97]
Recombinant human laminin-511 (20 μg/ml, absorption)	O3 (a variant of mTeSR1) or H3 (a variant of TeSR1)	H1, H9, HS420, HS207, HS401, BJ#12* LDS1.4*	[87]
Recombinant human Vitronectin (5 ng/μl, absorption)	mTeSR1	HUES1, HUES2, HESCNL3	[86]
Combination of cyclic RGD and vitronectin derived heparin-binding peptides (Biotinylated peptides attached to streptavidin-coated surface)	mTeSR1	H1, H7, H9, H14, IMR-90-1*	[95]
a combination of human collagen IV, vitronectin fibronectin and laminin (10 μg/cm^2, 0.2 μg/cm^2, 5 μg/cm^2, 5 μg/cm^2, respectively, absorption)	TeSR1	H1, H7, H9, H14	[52]
Bone sialoprotein and vitronectin derived RGD containing peptides (peptides conjugated to acrylate-coated surface)	X-VIVO plus bFGF and TGF-β1	H1, H7	[96]
Recombinant Truncated forms of human Vitronectin	E8	H1, H9, IMR90*, iPSC-foreskin*, iPSC-DF19*	[54]

* hiPSC lines

Table 3

Synthetic polymers used for hPSC culture.

Surface coating	Cell line	Duration and success of pluripotency maintenance	Tested differentiation	Ref.
poly[2- (methacryloyloxy)ethy l dimethyl-(3-sulfopropyl) ammonium hydroxide] (PMEDSAH)	*H9, BG01 [102, 103], iPSCs [104]	25 passages with pluripotency maintained at levels similar to that of Matrigel [102]; 3 passages for BG01; 10 passages for H9[103]; 15 passages for iPSCs [104]	Yes; differentiated into mesenchy- mal stem cells that were later differentiated into adipogenic, chondrogenic and osteogenic lineages [104]	[102- 104]
aminopropylmethacryl amide (APMAAm)	H1s, H9-hOct4-pGZs,	10 passages for H1; 22 passages for H9-hOct4-pGZs; H1 cells attached to the substrate maintained pluripotency at levels similar with Matrigel cultures. The H9-hOct4-pGZs line had higher pluripotency levels on the substrate than on Matrigel. H1 cells: 63.3% higher attachment efficiency on the substrate than on Matrigel. H9-hOct4-pGZ cells, though attached less, proliferated faster than cells on Matrigel.	Yes; differentiated into embryoid bodies	[105]
poly(methyl vinyl ether-alt-maleicanhydride) [PM VE-alt-MA]	HUES7, HUES9 and unnamed hiPSC line	5 passages	Yes; differentiated to endoderm, mesoderm and ectoderm.	[99]

Table 4

Summary of microcarrier culture of hPSCs in stirred suspension bioreactors.

Microcarrier base material and/or brand	Coating	Cell line	Cell number fold increase/culture length (days)/passages	Ref.
cellulose	Matrigel	HES-2, HES-3	4 / 6 / 25	[138]
trimethyl ammonium coated polystyrene (Hillex II)	None	ESI017, HUES9	2.2/ 5 / 6	[139]
Collagen coated polystyrene (HyClone)	Matrigel	H1, H9	34 / 8 / 1	[140]
Cytodex 3	Matrigel	H1, H9	3.25 / 5	[8]
DE53(Watman)	Mouse laminin-111	HES2, HES3	10 passages	[122]
Cytodex 3	None	H9	6.8 / 14 / 1	[141]
Collagen(HyClone)	Matrigel	B12-3 (iPSC)	7 / 7 / 1	[17]
Cellulose (Whatman)	Matrigel	HES-2, HES-3	4 / 6 / 25	[142]
Cytodex 3	Matrigel	SCED461	15 / 14 / 1	[143]
Cultispher S	Gelatin	SHEF3	10 / 7 / 1	[144]
Peptide-conjugated polystyrene	Poly-L-lysine	IMR90	23.3 / 6 / 5	[9]