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Author manuscript
Ann Biomed Eng. Author manuscript; available in PMC 2015 July 01.

Published in final edited form as:
Ann Biomed Eng. 2014 July ; 42(7): 1357–1372. doi:10.1007/s10439-013-0921-4.

Efficient and scalable expansion of human pluripotent stem cells under clinically compliant settings: a view in 2013

Ying Wang[1,2], **Linzhao Cheng**[2,3,*], and **Sharon Gerecht**[1,4,*]

[1]Department of Chemical and Biomolecular Engineering and Institute for NanoBioTechnology, The Johns Hopkins University, 3400 N. Charles St., Baltimore, MD 21218

[2]Stem Cell Program, Institute of Cell Engineering, The Johns Hopkins University School of Medicine, Edward D. Miller Research Building, Room 747, 733 N. Broadway, Baltimore, MD 21205

[3]Division of Hematology, Department of Medicine, The Johns Hopkins University, Edward D. Miller Research Building, Room 747, 733 N. Broadway, Baltimore, MD 21205

[4]Department of Material Sciences and Engineering, The Johns Hopkins University. 3400 N. Charles St. Baltimore, MD 21218

Abstract

Human pluripotent stem cells (hPSCs) hold great promise for revolutionizing regenerative medicine for their potential applications in disease modeling, drug discovery, and cellular therapy. Many their applications require robust and scalable expansion of hPSCs, even under settings compliant to good clinical practices. Rapid evolution of media and substrates provided safer and more defined culture conditions for long-term expansion of undifferentiated hPSCs in either adhesion or suspension. With well-designed automatic systems or fully controlled bioreactors, production of a clinically relevant quantity of hPSCs could be achieved in the near future. The goal is to find a scalable, xeno-free, chemically defined, and economic culture system for clinical-grade expansion of hPSCs that complies the requirements of current Good Manufacturing Practices (cGMP). This review provides an updated overview of the current development and challenges on the way to accomplish this goal, including discussions on basic principles for bioprocess design, serum-free media, extracellular matric or synthesized substrate, microcarrier- or cell aggregate-based suspension culture, and scalability and practicality of equipment.

Keywords

Human pluripotent stem cells; large-scale expansion; cellular therapy; clinical trials

Correspondence: Prof. Sharon Gerecht, PhD, Department of Chemical and Biomolecular Engineering and Institute for NanoBioTechnology, The Johns Hopkins University. 3400 N. Charles St. Baltimore, MD 21218, Phone: (410) 516-2846; Fax: (410) 516-5510; gerecht@jhu.edu Or, Prof. Linzhao Cheng, PhD, Stem Cell Program, Institute of Cell Engineering, The Johns Hopkins University School of Medicine, Edward D. Miller Research Building, Room 747, 733 N. Broadway, Baltimore, MD 21205, Phone: (410) 614-6958; Fax: (443) 287-5611; lcheng2@jhmi.edu.

Competing financial interests
The authors declare no competing financial interests.

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1. Introduction

Human pluripotent stem cells (hPSCs), including human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs), are capable of not only indefinitely self-renewing but also differentiating into any mature cell type of the body. Thus, hPSCs hold great promise for revolutionizing regenerative medicine, disease modeling, and drug discovery. In the past several years, researchers have been inspired by the development of techniques for the derivation, expansion, and differentiation of hPSCs, as well as by the ever-increasing knowledge about their genetic, epigenetic, and functional properties. In spite of unsolved problems, these developments have moved us significantly closer to the ultimate goal of hPSC-based cell therapies. Manufacturing an hPSC-based product will comprise a number of complex steps, including cell isolation, initial purification and expansion, derivation of hPSC lines, creation of master and working cell banks, large-scale expansion, differentiation, purification, storage, distribution, and transportation of the final product, in addition to intensive quality control and testing for each step. This review focuses on one crucial hurdle impeding the realization of late-stage clinical trials and commercialization of hPSC regenerative medicine — the efficient and scalable expansion of hPSCs under clinically compliant conditions. We cover primarily the essential aspects of the commitment to clinical requirements, the development of culture media and substrates for expanding hPSCs, the demands and current situation of large-scale production.

2. Human pluripotent stem cells

First derived from the inner cell mass of the human blastocyst by James Thomson and colleagues in 1998,[109] hundreds of hESC lines have been generated and thoroughly studied. According to the NIH Human Embryonic Stem Cell Registry, the NIH has approved the registration of a total of 210 hESC lines, 49 of which contain disease-specific mutations. These hESC lines offer precious opportunities for the study of early human development, stem-cell biology, *in vitro* differentiation, and tissue formation. However, the derivation of hESCs requires the destruction of human embryos, which has raised an ethical controversy and led to stringent legal restrictions in the United States.[116] The limited sources of federal funding and the paucity of hESC lines representative of specific diseases, especially for somatic or aging-dependent diseases, have narrowed down the potential applications of hESCs in disease modeling, pathology, and cell therapy. Moreover, the allogeneic nature of hESC therapies requires that the donor and the patient have matching human leukocyte antigen (HLA) types to reduce immune rejections, further increasing the limitations.

Scientists have actively sought to use somatic-cell nuclear transfer (SCNT) technology to generate personalized hPSCs for patient-specific research, especially after the report of cloning of Dolly the sheep in 1997.[123] Noggle et al. generated a blastocyst by transferring the genome of an adult somatic cell into an oocyte with an intact nucleus, and then derived hESC lines from the blastocyst.[79] The resultant triploid cell line and, more generally, the limited availability of human oocytes have kept this technology from practical and widespread implementation. Very recently, Tachibana et al. reported rapid derivation of hESC lines from blastocysts they generated by optimized SCNT protocol that allowed to remove oocyte nucleus and to develop normal diploid blastocysts[103]. In addition to ethic

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controversy and practical difficulty to obtain sufficient eggs from female donors, the complexity and low efficiency of current SCNT technique will unlikely become a steady technology to generate autologous hPSCs in the near future.

After the momentous 2006 announcement that induced pluripotent stem cells (iPSCs) had been derived from mouse fibroblasts,[105] Yamanaka and colleagues reported altering human cell fates to generate hiPSCs from human fibroblasts by expression with only four transcription factor genes.[104] Thomson and colleagues achieved the same marvel by using slightly different 4 factors at the same time.[131] This revolutionary finding stimulated many follow-up studies and opened up a completely new field — the generation and use of hiPSCs in a wide variety of human biology and disease research.[89] In addition to skin fibroblasts, mononuclear cells in the peripheral blood of human adults were also successfully used to generate integration-free hiPSCs, offering an easier way to avoid skin biopsy operations to get donor samples from probably the most commonly accessible cell sources in clinic.[19,28,58,128]

Research showed that human iPSCs share equivalent phenotypical and functional properties with hESCs. They have identical morphologies; they grow indefinitely and exhibit telomerase activities; they can be positively stained for alkaline phosphatase activity; they express comparable levels of such pluripotency genes as *OCT4, SOX2*, and *NANOG*, as well as of such embryonic cell surface antigens as TRA-1–60, TRA-1–81, SSEA3, and SSEA4; and they can differentiate into cells from all three embryonic germ layers *in vitro* after induction. Their developmental pluripotency is also validated by their ability to form teratoma (in immune-deficient mice), a benign tumor consisting of cells of all the 3 embryonic germ layers that was uniquely formed by pluripotent cells. Recent studies of genome-wide gene expression and DNA methylation have revealed subtle but detectable differences between hiPSCs and hESCs (although variations between hESC or iPSC lines also exist).[125] Gene expression and DNA methylation revealed the epigenetic markers present in the parental somatic cells were not completed erased in derived iPSCs and remaining ones (i.e., the so-called epigenetic memory) do exist although diminish with serial passages. Evidence that hiPSC lines differentiated more efficiently into the cell types from which they were derived, or less to another cell type also existed;[7,29] this, from a positive point of view, could prove beneficial for providing more efficient differentiation on demand even than from hESCs.

Although many unknowns remain in the emerging field of hPSC research, pioneers have already cautiously started to explore potential clinical applications using hPSCs. One booming field is hPSC-based tissue engineering. Using hPSC-derived cells, researchers focus on generating fully or partially functional human tissues such as liver, bone, neuron, blood vessel, heart, eye, etc.[24, 25,52,85,94,106,132] These studies provide proof-of-concept for potential cellular therapies, and yet are still at the starting line of a long journey towards realistic clinical implementation.

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3. Clinically compliant settings

In order to examine the clinically compliant settings for hPSC expansion, we can learn more from approved clinical trials. In 2009, the U.S. Food and Drug Administration (FDA) approved the world's first human clinical trial of hESC-based therapy by Geron Corporation for treating spinal cord injuries. In this study, hESCs were differentiated into oligodendrocyte progenitor cells (OPCs). The patients, all of whom had severe spinal cord injuries, each received an injection of approximately 1.5 million hESC-derived OPCs in order to repair the myelin insulation around their nerve cells and restore spinal cord functions.[47] However, the company stopped this trial abruptly in 2012.[14] Although the exact reason(s) unclear, there were at least two possibilities: first, the cell dosage used in the study might not have been large enough to affect the patients' injured spinal cords; second, the process of expansion and differentiation of hESCs under clinical compliant settings was complex, causing dosage or unacceptable pricing problems. Currently, only three ongoing clinical trials of hPSC therapy have gained approval by FDA. All three of these phase I/II trials led by Advanced Cell Technology (ACT) Inc. are based on transplanting hESC-derived retinal pigment epithelial (RPE) cells for an eye disease.

3.1 Ongoing clinical trials of hESC therapies

RPE cells are critical for supporting photoreceptors in the human retina. Diseases such as advanced dry age-related macular degeneration (AMD) and Stargardt's macular dystrophy (SMD) can destroy RPE cells, causing vision impairment and even blindness. The researchers attempted to replace degenerate RPE cells and restore visual function by establishing an efficient method for differentiating hESCs into RPE cells followed by their retinal injection.[20] The phase I trials sought to study the efficacy, safety, and tolerability of this therapeutic approach to treating AMD and SMD. In their studies, the transplantation of 50,000 to 200,000 hESC-derived RPE cells constituted one dosage. Data gathered halfway through the clinical trials showed that patients having either of these two diseases experienced improved vision.[95] Because this treatment would work with small dosage sizes and because the final RPE cell product for implantation did not require the use of additional materials, such as scaffolds or nanoparticles, which may lead to complicated categories (combinations of Tissue, Device, and Biologic) that usually trigger slow regulatory process,[26,53] the FDA found it suitable to approve this hESC-based therapy ahead of other potential hPSC therapies. More recently, an institutional review board in Japan granted conditional approval of a planed clinical trial using RPE cells derived from hiPSCs, which is awaiting final approval from the Japanese government.[33]

3.2 Current applications of hiPSCs

With concern about safety issues, research exploiting the unique clinical properties of hiPSCs has focused mainly on establishing disease models for the study of pathology, toxicology, diagnosis, and drug screening,[30] giving an advantage to broad patient-specific cell sources.[68,86, 110] Another potential clinical application of hiPSCs, gene-correction based stem cell therapy,[61] would use the strategy of deriving an iPSC line from a patient with a disease caused by a genetic defect such as sickle cell disease.[127] Such diseases could then be treated by gene correction using zinc finger nuclease (ZFN),[134] transcription activator-like

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effector nuclease (TALENs),[39] or the newly reported CRISPR/Cas9 system[62] to repair the endogenic pathogenic mutations, after which they repaired genes would be expanded and differentiated into desired cell types for pathology study or transplantation purpose. For additional reading, please refer to thorough reviews about the use of hiPSCs in disease modeling and gene therapies by Robinton et al. and Merkle et al.[69, 89] To keep its focus on the expansion of hPSCs, this review will consider several prominent aspects for developing bioprocess of hPSC expansion.

3.3 Xeno-free conditions and chemically defined agents

According to the *Guidelines for the Clinical Translation of Stem Cells* (available at http://www.isscr.org/home/publications/ClinTransGuide) developed by the International Society for Stem Cell Research (ISSCR),[42] although the inclusion of animal materials in the hPSC manufacturing process does not prohibit using the cells in humans, the processes and products must undergo additional tests to exclude the transmission of animal pathogens, antigens, and mycoplasma into the products. This will increase the cost and time for clinical grade manufacture. One option is to find alternative components of human origin to avoid xenogeneic contamination. In any case, whether the products come from animal or human sources, they remain undefined because they contain a complex mixture of growth factors, hormones, extracellular matrix proteins, and many other functional and nonfunctional molecules that cannot be completely determined and excluded. They also have a high risk of exposure to viral contaminants, which will necessitate complex virus detection screening of the products.[12, 100] Moreover, due to the instability of the source tissue, these products tend to exhibit batch-to-batch variations, reducing the reproducibility and reliability of therapeutic cell products.[112] The preparation of feeder cells is also time-consuming and requires irradiation equipment and qualified personnel. All of these factors make it more practical and economical to use, where possible, chemically defined components that can reduce the variance and the uncertainty of generating cell-based products with consistent quality.

3.4 Current good manufacturing practices (cGMP)

The term cGMP is a “quality management system” for manufacturing and testing products intended for human use, assuring that they are “safe, pure, and effective” (FDA cGMP guidelines, http://www.fda.gov/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm064971.htm). All clinical trials in the U.S. must manufacture any cell products under FDA cGMP guidelines (Code 21 CFR 210–211, 312, 600, and 1271). Especially, human cells, tissues, and cellular- and tissue-based products (HCT/Ps) must comply with the current good tissue practices (cGTP) rules (Code 21 CFR 1271). Equivalent regulatory rules have also been established in Europe (EU Directive 2003/94/EC) and many other countries and regions internationally (ICHQ7). Thus, cGMP compatibility must receive specific consideration during the development of hPSC culture systems for clinical use. The manufacturing processes and analytical methods should be carefully developed, fully controlled, and exhaustively documented. This comprises many elements, such as valid protocols, control of work flow and cross-contamination, clean room operation, monitoring and control of critical environmental parameters (including temperature, O_2, and CO_2), and

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so on. The cGMP also requires the rigorous validation of facilities, critical utilities, and equipment, along with extensive training of staff and technicians.

Meticulous documentation management is extremely important for a cGMP process. All of these rules aim to avoid any possible contaminations and operational errors throughout the process and to retain the ability, for any problem that might occur, to trace its origin, correct the problem, and identify all possible impacts.

Many companies and research institutes have established cGMP-compliant hPSC culture facilities and relevant protocols, mainly for the purpose of cell banking, including the WiCell Research Institute (http://www.wicell.org), Cellular Dynamics International (http://www.cellulardynamics.com), the City of Hope National Medical Center[6], Upstate Stem Cell cGMP Facilities at the University of Rochester Medical Center (http://www.urmc.rochester.edu/upstate-stem-cell-facility), etc. These facilities provide cGMP-compliant services for all hPSC derivation, expansion, and differentiation for cell banking and clinical trials.

3.5 Demand of robust and scalable cultivation of hPSCs

Thus far, clinical trials with hPSCs have been limited to therapies that need only small dosages of cells. Apparently, the size of hPSC dosage required to treat a certain disease is a critical considerations for decision-makers choosing which disease to investigate using stem cell therapy. Although the desired scale of hPSC expansion in autologous therapies may be much smaller than in allogeneic therapies, autologous treatments for many other diseases and injuries still demand what are estimated to be large quantities of hPSCs and/or their progenies to replace cells that do not regenerate. For example, research has suggested that it would take at least 1×10^9 hPSCs to obtain enough islets for the transplantation treatment of type I diabetes;[96] an optimized dose for performing hematopoietic stem cell (HSC) transplantation for a 70-kg adult patient contains from 4.2×10^8 to 5.6×10^8 $CD34^+$ cells;[65] an engineered myocardial tissue should contain at least 1 to 2×10^9 to repair the damaged heart from a typical myocardial infarction;[45] a minimum number of 10^6 surviving tyrosine hydroxylase-immunopositive neurons per side of the brain were necessary to have a positive clinic response for Parkinson's disease treatment,[35] which may require 10^9 to 10^{10} hPSCs considering the low differentiation and survival rate.[73] Moreover, even for the small-dosage therapies, such as the clinical trials mentioned above for treating AMD, moving to phase II/III clinical trials can require the participation of hundreds to thousands of patients in multiple clinical sites, necessitating the development of batch cultures with the capacity to produce several hundred dosages, not to mention the requirements for the bulk manufacture of final commercial products. Clinical trials for these applications can only receive approval once the difficult problems in efficient (timely and economical) and scalable expansion and differentiation of hPSCs and their progenies for therapeutic purposes.

An optimal culture system would support the self-renewal of undifferentiated hPSCs with minimal selective pressure. Culturing cells under stressful conditions or for long periods may cause DNA deletions, rearrangements, and other genetic or epigenetic abnormalities that can lead to pathogenic disasters like cancer. Thus, hPSCs should be expanded vigorously for a short period and to reach the desired quantity in one batch of production.

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For a rough estimation as an example, a desired culture system for clinical applications should be able to expand 10^6 frozen hPSCs to a quantity of 10^{12} cells within 20 to 30 days in approximately 5–10 passages.

4. The evolution of feeder cells and substrates

One intrinsic feature of hPSCs is that the cells require tight cell-to-cell contact for survival and proliferation.[16,36] To provide the necessary signals and a substrate for cells to attach to, researchers have developed various feeder cells or substrate materials. Conventionally, mouse embryonic fibroblast (MEF) feeder cells were used to support the derivation and self-renewal of hPSCs in a two-dimensional (2-D) adhesion culture. For example, the hESCs used in the current RPE clinical trials were cultured on MEF feeder cells.[20] To eliminate xenogeneic contaminations, various types of human feeder cells from allogeneic muscle, skin, marrow, and endometrial cells, etc.,[18,40,88,133] as well as from autogenic hESC-derived fibroblasts,[102,118] were developed in the early 2000s. Some of these new feeder cells also proved capable of supporting the derivation of new hPSC lines.[40,118,133]

Since the first study, published more than a decade ago in 2001,[124] Matrigel (BD Biosciences) has been widely used as a substrate for the feeder-free adhesion culture of hPSCs. Matrigel — a mixture of extracellular matrix materials extracted from Engelbreth-Holm-Swarm (EHS) mouse sarcoma cells, contain (approximately) 60 percent laminin, 30 percent collagen IV, 8 percent entactin, multiple growth factors and indeterminate components (http://www.bdbiosciences.com/documents/BD_CellCulture_Matrigel_FAQ.pdf). It was established in the 1980s[49] and originally used for promoting differentiation and the outgrowth of differentiated cells from tissue explants.[48] It remains the most broadly used feeder-free substrate for 2-D and 3-D cell cultures. A similar product, Geltrex (Invitrogen) is also commercially available. However, beyond being xenogeneic, both of these materials suffer from lot-to-lot inconsistency, uncertainty of stable composition, risk of viral contamination, and inconvenient temperature sensitivity; all of these factors make them problematic for clinical grade manufacturing.

To address these concerns, many researchers have focused their efforts on developing defined, xeno-free substrates that support the derivation and expansion of hPSCs. The primary candidates were individual or simple mixtures of purified extracellular matrix (ECM) proteins from Matrigel. Initial studies showed that purified laminin could support the growth of undifferentiated hESCs, while culturing hESCs on fibronectin- or collagen IV-coated surfaces was not promising.[124] Thomson et al. made a breakthrough, finding that a combination of collagen IV, fibronectin, laminin, and vitronectin can replace Matrigel to derive and expand hESCs in defined conditions.[60] Purified laminin from human placentas was proved sufficient for long-term maintenance of hESCs in media containing activin A.[9] Recently, further studies have reported on the improvements to laminin-based substrates and the related mechanisms.[71, 72,90] Miyazaki and colleagues demonstrated that recombinant human laminin — specifically the isoforms laminin-511, laminin-332, and laminin-111 — support the attachment and long-term self-renewal of hPSCs in an undifferentiated state.[71,90] These studies found the binding and signaling activity of the ECM through the major cell surface integrin receptor, $\alpha 6\beta 1$, critical to interactions between the cell and the ECM. By

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keeping the essential binding sites of integrin α6β1, the truncated laminin fragments LM511-E8 and LM332-E8 were shown to be sufficient to support long-term expansion of hPSCs.[72] Recombinant human E-cadherin, which is recognized by integrin αEβ7[38] and α2β1,[122] has also exhibited the ability to act as an equivalent alternative to Matrigel.[74] Human fibronectin, which binds to integrin α5β1 and αVβ3,[21] was shown to support hESC expansion in a serum-free medium.[5] Another ECM component, vitronectin, was used to replace Matrigel, supporting the attachment and proliferation of hPSCs by mainly binding to integrin αVβ5.[10] Chen et al. enhanced the attachment and survival of hPSCs on vitronectin-coated surfaces by cutting off the N-terminal and/or C-terminal fragments from full-length vitronectin.[15] These recombinant human ECM-based substrates offered significant improvements in establishing defined and xeno-free culture conditions for adhesion cultures of hPSCs under clinically compliant settings. Most of them were successfully transferred into commercially available products, such as CELLstart (Invitrogen), Laminin-511, and Laminin-521 (BioLamina); and Vitronectin XF (STEMCELL Technologies). Furthermore, the development of truncated protein substrates can not only offers improvements of the supportive effects in culture, but also enhances the yield and simplifies the procedure of production and purification of large recombinant proteins, providing the sort of economical benefit that has become one of the major concerns in the production of therapeutic applications.[15,72].

An extreme condition for truncated ECM substrates is to cut off most of the protein, keeping only a small peptide sequence of the active domain. Polymers, such as acrylate,[67] or self-assembled molecules, such as alkanethiol,[50] were arranged on the tissue culture surface to form a monolayer. Such peptides can be easily conjugated on a synthesized acrylate or alkanethiol surface during manufacturing to avoid the coating process. By following this strategy, researchers found that several laminin peptides supported the growth of hESCs using an alkanethiol array.[23] Peptides originated from bone sialoprotein,[67] vitronectin,[50, 67] and laminin (such as Synthemax from Corning)[67] also proved capable of maintaining long-term undifferentiated cultures of hESCs. These peptides allow cells to attach and spread by binding to the glycosaminoglycans or integrins on the cell surface. However, the high price of peptide synthesis and the difficulty of bulk sterilization by γ- radiation due to degradation of the peptide remain the major hurdles to broad application of this technology. To make economical and ready-to-use culture surface, fully synthetic plastic surfaces without peptide fragments were recently studied.[44,66,75,113,114] Among all reported synthetic surfaces, poly[2-(methacryloyloxy)ethyl dimethyl-(3-sulfopropyl)ammonium hydroxide] (PMEDSAH)[113] and aminopropylmethacrylamide (APMAAm)[44] exhibited the best results, supporting robust and long-term expansion of multiple hPSC lines without compromising pluripotency or inducing abnormal karyotypes. Although how they do so remains not fully understood, both mechanical (including stiffness, wettability, and rigidity) and chemical properties of synthetic surfaces influenced cell attachment and proliferation by mimicking the biological microenvironment as other ECM substrates. For additional reading, please see an updated review by Villa-Diaz et al.[115] Appropriately treating the polystyrene surface with ultraviolet light and then coating with serum or vitronectin was also showed to improve hPSC compatibility.[93]

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Synthetic surfaces are suitable for large-scale robotic adhesion cultures because they eliminate the need for the coating procedure and allow storage and handling at room temperature. Furthermore, the compatibility with sterilization techniques used in bulk manufacture, such as electron beam- and γ-radiation,[115] is also a critical factor for making ready-to-use products. Generally, substrates or surfaces containing protein or peptide components are not suitable for large-scale sterilization by γ-radiation due to the possible denaturation or degradation of these components.[91] The radiation may also affect the physical and chemical properties of the synthetic or the plastic surface. Therefore, the surfaces that have been proven to be capable for bulk sterilization, such as PMEDSAH, facilitate using cGMP in the production of commercial disposable vessels for large-scale expansion of clinical grade hPSCs.

5. The evolution of culture media for hPSC expansion

Strictly speaking, a discussion of feeder cells and substrates should also consider the corresponding culture medium as a system. The culture media for hPSCs have also evolved rapidly in recent years. Initially, hESCs were derived and cultured in a basal medium substituted with a serum or serum replacement, such as KnockOut Serum Replacement (Invitrogen), N2 supplement, or B-27 supplement (Invitrogen), which mainly contain human or animal serum albumin combined with various cytokines and growth factors either on feeder cells or on Matrigel.[104,109,124] At the same time, researches using feeder-free cultures often used media conditioned with feeder cells (i.e., conditioned media, or CM), which typically had serum replacement added to it before its incubation with feeder cells and the many undefined factors that they secreted. For a long time, MEF-CM was considered the gold standard for feeder-free expansion of hPSCs and was usually used as a baseline for comparison during the development of hPSC culture media.

Over the past decade, to develop fully defined media without any unknown components or additives, researchers have systematically screened and optimized a variety of formulas.[5,15,32,55, 56,59,60,63,87,111,117,126] In these studies, both basic fibroblast growth factor (bFGF) and the TGF/Activin/Nodal pathways appeared to be indispensable for the stable self-renewal of undifferentiated hPSCs. Some other cytokines could also help to maintain the pluripotency of hPSCs by activating similar pathways, such as Smad2/3, AKT and ERK1/2.[80] Oh's review comprehensively summarizes the mainstream evolution of serum-free medium.[80] Table 1 provides an updated summary of the media and culture conditions with regard to clinically compliant settings. Among these formulations, mTeSR1 (STEMCELL Technologies) and StemPro (Invitrogen) — based on two publications in 2006[46] and 2007,[72] respectively — constituted significant milestones in the exploration of defined media. In 2010, the International Stem Cell Initiative Consortium performed an exhaustive study that documented the side-by-side validation of eight defined media.[43] Surprisingly, only mTeSR1 and StemPro media supported the attachment and undifferentiated expansion of most of the twelve different hESC lines tested during the ten-week test period in four different labs internationally. In other media tested, the cells either died due to low attachment or underwent progressive differentiation on Geltex or Matrigel.[43] Another side-by-side evaluation reported in 2010 introduced a comparison of the three most popular feeder-free culture systems: mTeSR1-Matrigel, StemPro-CELLStart and

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mTeSR1-vitronectin.[130] Many other individual studies also used mTeSR1 and StemPro as standard serum-free media on different xeno-free substrates (Table 1). However, both of these media contain bovine serum albumin (BSA) fragment V, an animal product that contains complex albumin and lipid components that are not fully defined. A xeno-free alternative for mTeSR1, which replaces BSA with human serum albumin, is also commercially available (TeSR2, STEMCELL Technologies), but its undefined nature and high price are still major drawbacks for clinical applications. Some studies have also used NutriStem XF/FF medium (Stemgent) as a xeno-free and feeder-free culture medium for hPSCs on Matrigel to remove MEF before other experiments, such as gene targeting.[70,134] These studies observed the preservation of marker expression and differentiation potential, but no systematic comparative study has reported an examination of the long-term culture of hPSCs with this media.

Impressively, Chen et al. recently reported a further refinement that finally eliminated BSA from their previous TeSR formula.[15] When reconsidering the necessity of the medium components in TeSR by double knockout, they found that BSA was not necessary in the absence of β-mercaptoethanol, a biological antioxidant. After further filtration, they simplified the formula to just eight essential components, including DMEM/F12 basal medium; the result, called E8 medium, is xeno-free and chemically defined. Combined with this group's separate finding that truncated vitronectin substrate supported the expansion of hPSCs, this system was shown to support the derivation and continuous culture of multiple hPSC lines with improved reprogramming efficiency, robust expansion (6 to 14 folds every 3 to 4 days), and well-kept pluripotency.[15] This low-protein medium (now commercially available as Essential 8 from Invitrogen and as TeSR-E8 from STEMCELL Technologies) has demonstrated reproducible and promising results when used with either Vitronectin XF or Matrigel in a number of studies.[78] The simplified formula reduced price of the medium by 25–30 percent compared to StemPro or mTeSR1, and even more dramatically by 50 percent compared to the xeno-free alternative TeSR2. Recently, our lab demonstrated that it could support a scalable suspension culture of hiPSCs.[119]

Compared to CM-MEF conditions, hPSC cultures in many feeder-free systems showed significantly higher expansion rates, lower differentiation rates, and homogenous populations — but with a simpler process. Typically when using these systems, about 5 to 15 folds of expansion can be achieved within 3 to 7 days with more than 80 – 90 percent of the hPSCs maintaining positive expression of undifferentiated state markers and normal karyotype for more than 20 passages. More importantly, combining many of these media with xeno-free substrates produced efficient systems for deriving hESC and hiPSC lines, showing promise as initial steps towards the clinical grade production of hPSCs in adhesion cultures.

6. Expanding hPSCs in suspension

The strategy of rapidly expanding hPSCs in adhesion was criticized for its intrinsic lack of scalability. Although the capacity of an adhesion culture can increase linearly to a certain level by adding more flasks in an automated system, a suspension culture system using conventional stirred-tank bioreactors is still preferred for its well-established geometric

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scalability and its compatibility with the online monitoring/control of temperature, O_2, CO_2, pH, glucose, lactate and biomass,[120] especially at the pilot scale or larger. Along with the evolution of culture media and substrates, the past decade has seen significant progress in the culturing of hPSCs in suspension. Generally, researchers have developed two distinct types of suspension cultures: one based on microcarriers (MCs), and the other based on cell aggregates.

6.1 Microcarrier-based suspension culture

In making the transition from adhesion to suspension cultures, initial studies focused on growing hPSCs on MCs in a "pseudo-suspension" culture condition. Coating the MCs with feeder cells or the other substrates mentioned above, the studies demonstrated that MCs provided large surface areas that hPSCs could attach to for expansion in agitating vessels such as spinner flasks. The MCs examined so far are spherical or cylindrical particles ranging in size from 60 to 800 μm and made of polystyrene, dextran, cellulose, or glass with similar density as the media.[8,13,31, 37,57,64, 76, 81, 84, 97] A conclusive review covering MC-based hPSC cultures was recently published by Chen et al.[14] In most studies, hPSCs were cultured in media conditioned with MEF or human feeder cells. Thus, in Table 2, we highlight only the two studies that were targeting clinical use, avoiding conditioned media and feeder-coated MCs. Importantly, Oh et al.[81] showed that MC-based suspension cultures could offer high-yield expansion of hESCs, reaching a maximum cell density of 3.5×10^6 cells ml^{-1} in a seven-day culture, as compared to 0.8 to 1.5×10^6 cells ml^{-1} in side-by-side adhesion cultures on Matrigel. Such high-yield cultures are preferable to the large-scale production of cells, because of the high conversion efficiency of media and labor. The authors hypothesized that, in a stirred bioreactor, MCs provide a larger surface area and better O_2 and nutrient transfer than adhesion cultures.[81] Heng et al.[37] demonstrated the use of human laminin to coat MCs, followed by culturing hESCs for 20 passages for average yields of approximately 1.5×10^6 cells ml^{-1} without losing pluripotency and normal karyotype. This remains the only reported study that has successfully cultured hPSCs on MCs coated with xeno-free substrate in a nonconditioned medium. Although the MC-based suspension culture has been scaled up to working volumes of 150 ml in spinner flasks, the experiments revealed several shortcomings. Because hPSCs tended to stick together rather than evenly distribute on the MC surface,[14] heterogeneous clustering might occur during the culture process, which could induce unexpected differentiation and selection of genetically abnormal populations due to the stress created by insufficient transfer of nutrients and O_2. Moreover, removing MCs at harvest required additional filtration that would be a cumbersome step for cGMP manufacturing.

6.2 Aggregate-based suspension culture

Inspired by the self-aggregation observed in the embryoid body differentiation of hESCs in suspension,[11,34,77,129] researchers sought to establish carrier-free suspension culture systems for expanding hPSCs (Table 3). Initially, they inoculated cells into suspension cultures as cell clumps, using mechanical or mild enzymatic passaging methods,[46,98] which resulted in relatively heterogeneous formations of cell aggregates and a reduced reproducibility. After the discovery of Rho-associated coiled-coil kinase (ROCK) inhibitors (Y27632, HA-100, etc.) and their function of diminishing apoptosis and permitting the survival of dissociated

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hPSCs,[36,121] several groups established suspension culture systems that, with the addition of ROCK inhibitors to the culture media, supported single-cell inoculation, uniform aggregation, and long-term proliferation in an undifferentiated state.[1,4,17,51,82,83,99,135] Zweigerdt et al. and Amit et al. reported detailed protocols for the adaptation and expansion of hPSCs in both static and dynamic suspension in a variety of vessel types.[4,135] Recently, Olmer et al. reported a high-yield culture in a fully controlled bioreactor system. Up to 2×10^8 of hiPSCs were obtained from a working volume of 100 ml in a single run of seven days.[83] Chen et al. moved one step forward to clinically compliant process showing long-term culture of hESCs in suspension with a calculated cumulative expansion of more than 1×10^{13}-fold within 21 passages (about three months). That paper also demonstrated a complete strategy for hESC banking under cGMP conditions using 500 ml disposable spinner flasks to expand cells.[17] Most recently, our group established a completely xeno-free system for the expansion and cryopreservation of hPSCs based on E8 medium, which showed a similar expansion rate, high viability, well-maintained pluripotency, normal karyotype, and resistance to multiple freeze-thaw cycles, but with only around 60 percent of the cost of protocols using mTeSR1 or StemPro as culture media.[119] Furthermore, Steiner et al. also claimed a complete strategy to derive, expand, and differentiate hESC lines all in suspension conditions,[101] making it possible to avoid any effects from feeder cells and substrates.

The key to efficient expansion in aggregate-based suspension cultures is to keep homogeneous aggregate formation to an appropriate size that allows the effective diffusion of nutrients. This can be accomplished by improving media formulation and passaging methods to enhance cell viability, as well as by designing bioreactors with optimized hydrodynamic properties that generate mild but sufficient shear stress in an evenly distributed velocity field. Different hPSC lines have varying adaptability to shear stress,[135] which may also constitute a limitation for universal applications. Furthermore, well-controlled one-direction shear flow showed to enhance hematopoietic and endothelial differentiation of PSCs in adhesion.[2,3] However, the shear force on the cell surface in the suspension culture system is uncontrolled and continuously changing due to the local unsteady flow and the rotation of the MC or the cell aggregate. The effect of this inconstant shear stress on *in vitro* cell differentiation has not been studies yet. Thus, optimization of the operation, including agitating speed, seeding density, media change, and split interval, is a necessary step for each particular hPSC line.

7. Culture scales and equipment

The scale of hPSC expansion for clinical use should be determined by the demands and specifications of individual therapies. The decision should consider the potential market, cells/dose, doses/year, lots/year, doses/lot and, eventually, determination of the total manufacturing requirement.[22]

Potential allogeneic therapies using hPSCs usually aim to provide cell products derived from a single donor to many other patients. Accordingly, the culture scale for this type of therapy could run from tens to hundreds of liters in order to be cost-effective. For hPSC cultures at this level, only a large-scale stirred-tank bioreactor system combined with aggregate-based

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suspension cultures might meet the requirement, although its development would remain very challenging.

In contrast, the strategy of autogenic therapies relies on providing cell products to treat only the patient who donated the parental cells, which adheres more closely to the principle of hiPSC therapies. Since the cultured products are for a single patient, a relatively small-scale hPSC culture should be adequate, as long as efficient differentiation and downstream processes are well established. For this purpose, the current level of development of highly efficient adhesion cultures might suffice, when combined with such multilayer vessels as CellCube and CellSTACK (Corning) and Cell Factory (Thermo Scientific Nunc), to obtain the needed cell dosages. A fully controlled, compact and closed planar culture system (Integrity Xpansion, ATMI) has also become commercially available. Achieving productions of hPSCs in large lot-size using adhesion cultures could also occur with the assistance of robotics technology to facilitate the handling of many culture flasks.[92] Successful studies have reported automated hESC adhesion cultures using the CompacT SelecT (TAP Biosystems) and the 3D Cellhost (Hamilton) systems.[107,108] The CompacT SelecT system can handle an estimated 90 T175 flasks per run, providing 15,750 cm^2 of surface area that can yield approximately 3×10^9 hPSCs in a single three-day culture in feeder-free conditions. For comparison, the largest volume used in current studies on suspension cultures of hPSCs was 250 ml. A three- to six-day culture can harvest approximately 2×10^6 cells ml^{-1}, making a total yield of 2×10^9 for four parallel vessels in one regular size CO_2 incubator. It should not be difficult to scale up to 500 ml in a 1-liter spinner flask, due to the compatible platform and bioreactor design. Comprehensive reviews by dos Santos et al.[27] and Want et al.[120] include useful information about bioreactor design and case studies of scale determination for clinical-grade expansion of hPSCs.

8. Clinical considerations for postexpansion downstream process

The differentiation efficiency of hPSCs is another critical hurdle for clinical applications, although not the focus of this review. After the differentiation process, the complete removal of undifferentiated hPSCs from the resultant product is strictly required to reduce the risk of tumor generation. For example, the clinical trial of RPE cells claimed that the product RPE dosage contained only 0.00008 percent parental hESCs. The cryopreservation method should also comply with the cGMP regulations, the expansion system, and the shipping and handling protocols. Hunt and Li et al. provided detailed reviews covering the development of cryopreservation for hPSCs under cGMP settings.[41,54]

9. Future directions

The methods for expanding hPSCs have improved significantly in recent years, facilitating robust and scalable clinical-grade hPSC production. Current knowledge of synthetic surfaces and suspension culture systems will likely continue to progress. More ready-to-use, chemically defined and xeno-free culture systems for culturing hPSCs using cGMP will follow the development of a deeper understanding of the chemical and biological mechanisms related to hPSC self-renewal. More studies using adhesion culture systems should focus on the optimization of the different combinations of synthetic surfaces and

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xeno-free serum-free media with only small molecule cocktails to achieve preferable culture systems that can be widely used to support the expansion of a broad range of hPSC lines. These culture systems should also be tested for their capability of supporting the derivation of new hPSC lines. Expansion systems specifically tailored for culturing hPSCs at full production scales — for example, tens or hundreds of liters — will benefit from better understanding questions at scales ranging from millimeters (MC engineering) to tens of meters (large bioreactor design). For MC-based culture, a synergetic development of synthetic surface technologies and microcarrier design (i.e. size, shape, porosity, etc.) may result in favorable MC system for hPSC expansion. Systematic optimization of seeding and feeding strategies could also help to overcome the hurdle of bad attachment and undesired agglomeration in large-scale bioreactors. For aggregate-based suspension culture, optimization of media composition, operation protocol, and bioreactor structure should be given the highest priority to ensure good formation and maintenance of the cell aggregates along the scale-up process. All developed systems should be tested for their capability to sustain pluripotency, as well as the phenotypic, genetic, and epigenetic stability of multiple hPSC lines. More importantly, successful development of high-efficient differentiation protocols to desired cell types is the rate-determining step. Differentiation with high efficiency in feeder-free and defined conditions should be an ultimate goal for the study on hPSC fate commitment. Using one or multiple hPSC-derived cell types to generate functional engineered tissue will take another leap to reach a whole new level of cell therapies. We can expect that, combined with other advances in derivation and differentiation technologies, more clinical trials using hPSCs and their progenies will be approved in the near future, working to reach the next generation of stem cell therapies.

Acknowledgement

This work is supported in part by the Edythe Harris Lucas and Clara Lucas Lynn Chair in Hematology (L.C.) and grants from the Maryland Stem Cell Research Fund (2011-MSCRFII-0088 to L.C.), the NIH (U01-HL107446 and 2R01-HL073781 to L.C.), and the NSF (grant 1054415 to S.G.).

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Table 1

Serum-free and feeder-free adhesion culture systems and their clinical compliant features

								Clinical compliance					
								Media			Substrate		
References	Media	Substrate	Cell line tested (#)[a]	Passage[b]	Folds (days)[c]	Charactrization[d]	New cell line derivation	XF	CD	Eco	XF	RTU[e]	Eco
Li et al.[55]	X-VIVO 10[f]	hLM	ES (1)	40	4.5–6.8 (5–7)	Full	No	●	●	○	○	○	○
Yao et al.[126]	N2B27[g]	Matrigel	ES (2)	22	6 (4–5)	Full	No	○	●	○	○	○	○
Ludwig et al.[60]	mTeSR1	Matrigel	ES (4)	25	7–15 (7)	Full	Yes	○	○	○	○	○	○
Wang et al.[117]	StemPro	Matrigel	ES (5)	25	3 (~5)	Full	No	○	○	○	○	○	○
Miyazaki et al.[71]	MEF-CM +bFGF	rh LM511	ES (3)	10	4–5 (4–5)	Partial	No	○	○	○	●	○	○
Braam et al.[10]	mTeSR1	rh VNT	ES (3)	5	3–4 (7)	Partial	No	○	○	○	●	○	○
Nagaoka et al.[74]	mTeSR1	rh E-cadherin	ES (2) iPS (3)	37	~10 (7)	Full	No	○	○	○	●	○	○
Yoon et al.[130]	StemPro	CELLStart	ES (2)	30	ND (5)	Partial	No	○	○	○	●	○	○
Saha et al.[93]	mTeSR1	VNT or serum coated UVPS	ES (3) iPS (2)	27	3 (6)	Full	No	○	○	○	●	○	●
Chen et al.[15]	E8	Truncated rh VTN	ES (1) iPS (5)	20	~10 (3–4)	Full	Yes	●	●	●	●	○	●
Miyazaki et al.[72]	mTeSR1 StemPro	LM511-E8 LM332-E8	ES (3) iPS (2)	30	~10 (4–6)	Full	No	○	○	○	●	○	○
	TeSR2	LM511-E8 LM332-E8	ES (1)	30	~10 (4–6)	Partial	No	●	○	○	●	○	○
Klim et al.[50]	mTeSR1	Heparin-binding peptide	ES (6) iPS (2)	20	10–12 (5–7)	Full	No	○	○	○	●	●	○
Mei et al.[66]	mTeSR1	Hit 9 polymer	ES (2)	10	3 (5–7)	Full	No	○	○	○	●	○	●
Melkoumian et al.[67]	X-VIVO10 mTeSR1	Synthemax	ES (2)	10	30–94 hours*	Full	No	● ○	● ○	○ ○	●	●	○
Irwin et al.[44]	mTeSR1	APMAAm	ES (2)	20	3–6 (3–5)	Partial	No	○	○	○	●	ND	●
Villa-Diaz et al.[113,114]	mTeSR1 StemPro	PMEDSAH	ES (5) iPS (1)	20	~38 hours*	Full	Yes	○	○	○	●	●	●

[a]Cell line tested (#): the number of cell line tested and the type of the cell line, ES or iPS.

[b]Passage: the passage number when the cells were karyotyped.

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[c]Fold (days): average fold expansion in the number of culture days per passage. When marked (*), means doubling time.

[d]Characterization: including expression of pluripotency markers, *in vitro* and *in vivo* differentiation assay and karyotyping.

[e]RTU: ready-to-use, indicating capability to be fabricated on culture vessel and sterilized as a single product, with no need for coating before use.

[f]X-VIVO10: X-VIVO10 medium supplemented with nonessential amino acid (NEAA), L-glutamine, b-mercaptoethanol, rh bFGF, rh stem cell factor (SCF), rh Flt3 ligand, and rh leukemia inhibitory factor (LIF).

[g]N2B27: DMEM/F12 medium supplemented with B2 supplement, B27 supplement, NEAA, L-glutamine, b-mercaptoethanol, BSA, and rh bFGF.

Abbreviations: XF, xeno-free; CD, chemically defined; Eco, economically efficient; RTU, ready-to-use; rh, recombinant human; hLM, human laminin; VNT, vitronectin; ND, not determined; UVPS, UV/ozone-treated polystyrene.

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Table 2

MC-based suspension culture systems for hPSCs and their clinical compliance

									Clinical compliance					
									Media			MC + coating		
References	Media	Micro-carriers	Coating	Stirring	Cell line tested (#)	Passage	Folds (days)	Charactrization	XF	CD	Eco	XF	RTU	Eco
Oh et al.[81]	mTeSR1 StemPro	DE-53	Matrigel	Spinner flask	ES (2)	25	5.8–10 (4–7)	Full	○	○	○	○	○	○
Heng et al.[37]	StemPro	Poly-styrene	hLM mVNT	Orbital shaker	ES (2)	20	8.5 (7)	Full	○	○	○	● ○	○	○

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Table 3

hPSC aggregate-based suspension culture systems in spinner flasks and their clinical compliance[a]

References	Media	Volume (ml)	Cell line tested (#)	Passage	Folds (days)	Max yield (cells ml^{-1})	Characterization	Undifferentiated (%)[b]	XF	CD	Eco
Krawetz et al.[51]	mTeSR1	100	ES (1)	4	5 (5–6)	1.6×10^6	Full	76 – 98	○	○	○
Singh et al.[99]	mTeSR1	50	ES (3)	5	2 (7)	$2–2.4 \times 10^6$	Full	85 – 98	○	○	○
Zweigerdt et al.[135]	mTeSR1	50	ES (3) iPS (2)	10	2–3 (4–7)	$2–3 \times 10^6$	Full	> 85	○	○	○
Amit et al.[4]	IL6RIL6[c]	50	ES (7) iPS (6)	20	17.7 (6)	1.89×10^6	Full	ND	○	○	○
Abbasalizadeh et al.[1]	HFF-CM[d]	100	ES (2) iPS (2)	20*	8 (7–10)	2.8×10^6	Partial	95 – 98	●	○	○
Olmer et al.[83]	mTeSR1	100	iPS (1)	2*	5.5 (7)	2×10^6	Partial	69 – 97	○	○	○
Chen et al.[17]	StemPro	60–250	ES (3)	21	3.2–4.3 (3–4)	1.1×10^6	Full	> 90	○	○	○
Wang et al.[119]	E8	50	iPS (2)	20	2.8–3.5 (3)	1.8×10^6	Full	95 – 99	●	●	●

[a] Meaning of items: see Table 1.

[b] Undifferentiated: undifferentiated population that was positively stained with markers indicating undifferentiated stage of hPSCs, including TRA-1-60, TRA-1-81, SSEA-3, SSEA-4, Oct-4, Nanog, Sox2, etc.

[c] IL6RIL6: DMEM/F12 medium supplemented with KO-serum replacement, nonessential amino acid (NEAA), L-glutamine, β-mercaptoethanol, rh bFGF, and rh full-length interleukin-6 and interleukin-6 receptor (IL6RIL6) chimera.

[d] HFF-CM: Human foreskin fibroblasts conditioned DMEM/F12, GlutaMAX, rh bFGF.

* Karyotype was not determined.

Abbreviations: XF: xeno-free; CD: chemically defined; Eco: economically efficient; ND: not determined.