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Developing Defined and Scalable 3D Culture Systems for Culturing Human Pluripotent Stem Cells at High Densities

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Abstract—Human pluripotent stem cells (hPSCs)—including embryonic stem cells (hESCs) and induced pluripotent stem cells (hiPSCs)—are very promising candidates for cell therapies, tissue engineering, high throughput pharmacology screens, and toxicity testing. These applications require large numbers of high quality cells; however, scalable production of human pluripotent stem cells and their derivatives at a high density and under well-defined conditions has been a challenge. We recently reported a simple, efficient, fully defined, scalable, and good manufacturing practice (GMP) compatible 3D culture system based on a thermoreversible hydrogel for hPSC expansion and differentiation. Here, we describe additional design rationale and characterization of this system. For instance, we have determined that culturing hPSCs as a suspension in a liquid medium can exhibit lower volumetric yields due to cell agglomeration and possible shear force-induced cell loss. By contrast, using hydrogels as 3D scaffolds for culturing hPSCs reduces aggregation and may insulate from shear forces. Additionally, hydrogel-based 3D culture systems can support efficient hPSC expansion and differentiation at a high density if compatible with hPSC biology. Finally, there are considerable opportunities for future development to further enhance hydrogel-based 3D culture systems for producing hPSCs and their progeny.

Keywords—Human embryonic stem cells, Induced pluripotent stem cells, 3D culture system, Thermoreversible hydrogel.

INTRODUCTION

Human pluripotent stem cells (hPSCs), including human embryonic stem cells (hESCs)[35] and induced human pluripotent stem cells (hiPSCs),[34] are being investigated for a broad range of biomedical applications because of their unique characteristics. Not only can they undergo effective long-term expansion *in vitro* to yield large quantities of cells, but they can also be differentiated into presumably all cell types in the adult body.[5] Thus, they are promising candidates in cell replacement therapies for various human degenerative diseases or injuries,[18,28] for generating engineered tissues or organs,[2] and for drug discovery and toxicity testing.[7,20]

All of these applications require a large number of cells.[2,7,20,28] In particular, the patient populations with degenerative diseases/injuries or organ failure are large, with for example ~ 8 million patients with myocardial infarction (MI), ~1–2.5 million with type I diabetes, and ~1 million with Parkinson's disease (PD) in the US alone.[27,29] In addition, to treat an individual with MI, type I diabetes, or PD, approximately 10^9 surviving cardiomyocytes, 10^9 β cells, or 10^5 dopaminergic (DA) neurons are required, respectively.[29] Furthermore, due to the low survival of transplanted cells *in vivo* (e.g. ~6% DA neurons or 1% cardiomyocytes have survived several months after transplantation in rodents[14,15]), even more cells will be necessary in reality. In addition, tissue engineering endeavors would require ~10^9 hepatocytes or cardiomyocytes to create an engineered human liver or heart, respectively.[2] Finally, for drug discovery, ~10^{10} cells are necessary to screen a library with a million compounds,[7] and there are many large chemical, peptide, and nucleotide libraries that can be screened against many types of cells derived from hPSCs.[41] In summary, a substantial number of hPSCs are necessary for current and future research and development.

Current strategies for producing hPSCs or their derivatives at a large scale generally involve three

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steps.[29] First, a working cell bank containing many hPSC aliquots is established and cryopreserved. Second, an aliquot is grown into the desired number of cells through a series of expansions. Finally, these cells are then differentiated into the targeted cell types. An efficient and scalable bioprocess is required for both the expansion and differentiation.[29] In addition, if the cells are being produced for clinical application, the bioprocess must comply with good manufacturing practices (GMP).[36] Currently, the most widely used systems involve the expansion and differentiation of hPSCs on 2D surfaces. Though significant advances have resulted in increasingly well-defined 2D culture systems (including a range of media and substrates), the production of cells on a large scale remains a challange.[29,38] For instance, at a typical density of ~5000 DA neurons/cm^2 or ~50,000 cardiomyocytes/cm^2, ~0.5 km^2 or 16 km^2 of cell culture surfaces are necessary to contain sufficient numbers of DA neurons or cardiomyocytes to treat PD or MI populations in the US, not to mention the surface area required to expand the parent hPSCs.

Thus, it may be desirable, and even unavoidable, to move from 2D to 3D for the large-scale hPSC production.[19,29] A number of 3D suspension culture systems have been investigated for hPSC culture during the past decade. Single or small clumps of hPSCs have been suspended and cultured as cell aggregates in liquid medium under continuous stirring or shaking.[1,6,32,42] Alternatively, hPSCs have been first seeded onto polymeric microspheres coated with matrix proteins and then cultured as a microcarrier suspension in a liquid medium.[4,22] While these 3D systems have achieved some degrees of success, many challenges have also been reported.[17] In particular, considerable cell agglomeration, which can lead to cell death or uncontrolled differentiation, is frequently observed in suspension cultures.[4,17,22] Apoptosis induced by shear forces resulting from the medium flow is also common.[1,4,6,22,32,42] As a result of such constraints, suspension systems often use low initial seeding densities and result in relatively low cell expansion and volumetric cell yields.[17,29] Encapsulating and culturing small clumps of hPSCs in a number of hydrogels have also been studied.[3,10,30,33] However, limited cell growth has been achieved to date, and uncontrolled differentiation can occur in such 3D culture systems.[17,29] In short, cost-effective production of hPSCs or their derivatives on a large scale and under well-defined conditions is very challenging.

An efficient 3D culture system for large-scale hPSC production should exhibit a number of features. First, it should support a high density hPSC culture at a high

FIGURE 1. 3D static suspension culture. Single iPS-Fib2s were cultured for 4 days in static liquid culture with mTeSR or E8 medium and RI (present for the full 4 days) at low, medium, or high seeding density (2.5 × 10^5, 1.0 × 10^6, or 2.5 × 10^6 cells/mL, respectively). (a and b) Cell morphologies on day 1, 2, 3, and 4 are shown with phase contrast images. (c, d and e) Mean diameter of the hPSC aggregates, fold expansion and cell densities at different days within the 4 days culture period. ***indicates statistical significance at a level of $p < 0.001$. Scale bar: 250 μm.

desirable for large-scale hPSC production. Likewise, the cell growth rate should be close or equal to the highest rate achieved on 2D surfaces. Second, the system should be well-defined such that production is reproducible and compatible with GMP. Third, the system should be simple, scalable, and easy to automate. Finally, it would be desirable to support single cell seeding to "synchronize" the environmental conditions that cells experience. While research has shown that cell dissociation promotes hPSC apoptosis,[23] single cell seeding offers the potential for more uniform and reproducible expansion and differentiation.[8,13,21]

We recently developed and reported a simple, well-defined, efficient, scalable 3D culture system, utilizing a thermoreversible hydrogel as a biomaterial scaffold, for both hPSC expansion and differentiation at high density.[17] In this paper, we describe additional characterization of the system as well as general design rationale for 3D systems that enable hPSC culture at a high density. Briefly, we found that under a typical set of conditions, we were unable to culture hPSCs as a suspension in a liquid medium with high volumetric yields. Substantial cell agglomeration was observed in these suspension cultures. The use of hydrogels as 3D scaffolds for hPSC culture was able to mitigate cell aggregation, and such scaffolds may also isolate cells from shear forces that accompany cell culture agitation and can lead to cytotoxicity.[1,4,6,22,32,42] Finally, the ability to adapt this system to also support cell differentiation—such as into neural lineages—is a useful feature, and future work may further enhance the ability of the system to promote economical cell expansion as well as differentiation into additional lineages.

MATERIALS AND METHODS

Reagents

hESC lines H1 and H9 were obtained from WiCell Research Institute. iPS-MSC[25] (derived from human mesenchymal stem cells) and iPS-Fib2[25] (derived from human dermal fibroblasts) were a kind gift from George Q. Daley at Children's Hospital Boston.

cell growth rate. Culturing hPSCs at a high density can significantly reduce the space, labor, and material necessary for cell expansion, and is thus highly

Essential 8 medium (E8), 0.5 mM EDTA, Accutase, ProLong® Antifade reagents, LIVE/DEAD® Cell Viability staining kit, Click-iT® EdU Alexa Fluor® 594

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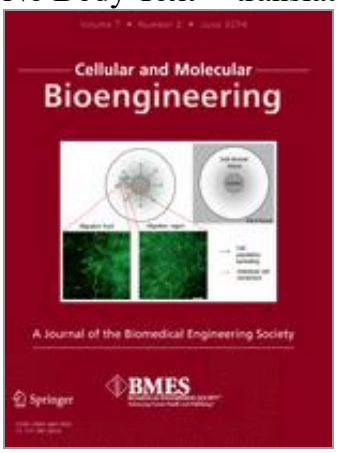

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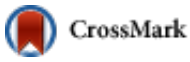

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