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A fully defined and scalable 3D culture system for human pluripotent stem cell expansion and differentiation

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Human pluripotent stem cells (hPSCs), including human embryonic stem cells and induced pluripotent stem cells, are promising for numerous biomedical applications, such as cell replacement therapies, tissue and whole-organ engineering, and high-throughput pharmacology and toxicology screening. Each of these applications requires large numbers of cells of high quality; however, the scalable expansion and differentiation of hPSCs, especially for clinical utilization, remains a challenge. We report a simple, defined, efficient, scalable, and good manufacturing practice-compatible 3D culture system for hPSC expansion and differentiation. It employs a thermoresponsive hydrogel that combines easy manipulation and completely defined conditions, free of any human- or animal-derived factors, and entailing only recombinant protein factors. Under an optimized protocol, the 3D system enables long-term, serial expansion of multiple hPSCs lines with a high expansion rate (∼20-fold per 5-d passage, for a 10^72-fold expansion over 280 d), yield (∼2.0 × 10^7 cells per mL of hydrogel), and purity (∼95% Oct4+), even with single-cell inoculation, all of which offer considerable advantages relative to current approaches. Moreover, the system enabled 3D directed differentiation of hPSCs into multiple lineages, including dopaminergic progenitor neurons with a yield of ∼8 × 10^3 dopaminergic progenitors per mL of hydrogel and ∼80-fold expansion by the end of a 15-d derivation. This versatile system may be useful at numerous scales, from basic biological investigation to clinical development.

Human pluripotent stem cells (hPSCs), including human embryonic stem cells (hESCs) (1) and induced pluripotent stem cells (iPSCs) (2), have the capacities for indefinite in vitro expansion and differentiation into all cell types within adults (3). They therefore represent highly promising cell sources for numerous biomedical applications, such as cell replacement therapies (4, 5), tissue and organ engineering (6), and pharmacology and toxicology screens (7, 8). However, these applications require large numbers of cells of high quality (4, 6–8). For instance, ∼10^5 surviving dopaminergic (DA) neurons, ∼10^5 cardiomyocytes, or ∼10^6 beta cells are likely required to treat a patient with Parkinson disease (PD), myocardial infarction (MI), or type I diabetes, respectively (9). Additionally, far more cells are needed initially because both in vitro cell culture yields and subsequent in vivo survival of transplanted cells are typically very low. As examples of the latter, only ∼6% of transplanted dopaminergic neurons or ∼1% of injected cardiomyocytes reportedly survive in rodent models several months after transplantation (10, 11). Furthermore, there are large patient populations with degenerative diseases or organ failure (9), including over 1 million people with PD, 1–2.5 million with type I diabetes, and ∼8 million with MI in the United States alone (12). Large numbers of cells are also necessary for applications such as tissue engineering, where for example ∼10^10 hepatocytes or cardiomyocytes would be required for an artificial human liver or heart, respectively (6). Additionally, ∼10^10 cells may be needed to screen a million-compound library once (8), and advances in combinatorial chemistry, noncoding RNAs, and investigations of complex signaling and transcriptional networks have given rise to large libraries that can be screened against many targets (13). Massive numbers of hPSCs may therefore be needed to deliver on the biomedical promise of these stem cells.

In general, hPSCs require key biological signals from their substrate, and from one another (14, 15), that promote cell survival and rapid proliferation and that culture systems must thus provide. Current 2D-based cell culture systems—which suffer from inherent heterogeneity and limited scalability and reproducibility—are emerging as a bottleneck for producing sufficient numbers of high-quality cells for downstream applications (9, 16). An attractive approach for scaling up production is to move cell culture from 2D to 3D (9, 17), and accordingly several 3D suspension systems have been probed for hPSCs production: cell aggregates (18–21), cells on microcarriers (22, 23), and cells in alginate microencapsulates (24) (SI Appendix, Table S1). Although these approaches have some attractive aspects, they also highlight significant challenges for 3D hPSC culture (9) (SI Appendix, Table S1) including the following: (i) the use of components from human or animal tissue (e.g., Matrigel, serum, and/or albumin), which limit reproducibility and/or scalability, pose risks for pathogen and immunogen transfer (18–24), and are thus problematic for good manufacturing practice (GMP) cell production (25); (ii) substantial cell agglomeration that can in some cases lead to differentiation and/or death (22, 23); (iii) shear forces in agitated cultures that can compromise cell viability (18–23); (iv) limited cell expansion rates and cell yields per volume (18–24); and (v) unclear potential for long-term serial expansion. As an example, in a recent culture of hPSCs within alginate hydrogel microspheres in mouse embryonic fibroblast-conditioned medium, 5% of the encapsulated single hPSCs remained viable after 7 d, and an ∼10^- to 20-fold expansion to a peak density of 3 × 10^6 cells per mL occurred after 20 d (24).

Significance

Human pluripotent stem cells can be cultured in vitro and differentiated into presumably all cell types of the human body, and they therefore represent highly promising cell sources for biomedical applications such as cell therapies, tissue engineering, and drug discovery. These applications require large numbers of high-quality cells, and we report an efficient, defined, scalable, and good manufacturing practice-compatible 3D system for the production of human pluripotent stem cells and their progeny. The ease of use and flexible scalability of this system makes it suitable for numerous applications from the laboratory toward the clinic.

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To address these challenges, we report an alternative system that expands and differentiates hPSCs within a thermoreversible hydrogel, composed of a polymeric solution that is liquid at low temperature but solidifies into an elastic hydrogel when warmed. Cells can thus be mixed with the liquid at low temperature, suspended and grown in a solid gel at 37 °C, and harvested and passaged by reliquefying the gel at low temperature. The resulting hydrogel offers many features that benefit hPSC biology and culture, including a 3D environment for rapid cell growth, prevention of large cell aggregate formation, isolation of cells from shear forces, and sufficient porosity for nutrient diffusion. Although thermoreversible materials have been used for culturing primary cells in the laboratory (26), large-scale hPSC culture presents numerous additional challenges and constraints, including apoptosis upon cell dissociation (14) and passing; low survival, limited proliferation, and loss of pluripotency in suboptimal culture conditions; and a strong preference for defined medium to support downstream biomedical applications. We have accordingly investigated these conditions and developed a chemically defined system with no matrix proteins that is capable of 10^3-fold expansion over 60 passages with strong maintenance of pluripotency.

**Results**

Several hydrogel materials—such as alginate (24, 27), agarose (28), and hyaluronic acid (29)—have been previously investigated for hPSC expansion or differentiation. However, to date thermoreversible materials have not been studied in hPSC culture, despite the fact that they offer a number of promising features for GMP-compatible, large-scale culture. In particular, they are synthetic and defined, biocompatible, and enable cell harvest or passaging by simply changing temperature at the range from 4°C to 37°C, a mild process for cells. Also, they can be readily processed (e.g., into spheres or fibers) for large bioreactors. After initial assessment of several synthesized or commercial available thermoreversible hydrogels, we found single hPSCs could survive and retain pluripotency marker expression in 8–10% (wt/vol) poly(N-isopropylacrylamide)-co-poly(ethylene glycol) (PNIPAAm-PEG) hydrogel (Mebiol Gel) (SI Appendix, Fig. S1).

Preliminary investigation showed that cell behavior in this material was significantly affected by multiple factors, including the addition of an inhibitor for the RhoA GTPase signaling effector Rho kinase (ROCK) (30), extent of cell dissociation, the culture medium (3), and initial seeding density. We therefore conducted a multifactoral analysis to assess the impact of this material relative to other materials or to cell-only suspensions, identify the optimal conditions under which it supports the biology and culture of hPSC, and study the potential interactions among numerous parameters. Thirty-six combinations of various factors, including seeding with single cells or clusters; use of ROCK inhibitor (RI); cell seeding density; culture medium; and with or without PNIPAAm-PEG hydrogel scaffold, were tested (Figs. 1 and 2). Specifically, cell dissociation promotes hPSC apoptosis via Rho GTPase signaling (14, 31, 32); however, single-cell seeding enables more reproducible expansion during large-scale hPSC production. hPSCs were thus dissociated into single cells and either directly encapsulated into the hydrogel (termed single-cell seeding) or—based on the poor viability observed for single-cell dissociation in 2D (30)—first cultured in suspension overnight to form small clusters that were subsequently encapsulated in the gel (termed precluster seeding) (Fig. 1A). Also, addition of RI for the first 24 h has been shown to support growth of single hPSCs in liquid suspension culture (19), and we included RI for the whole culture period (4-d RI) to assess its effects on cell expansion. Additionally, low seeding density (∼1–2.5 × 10^5 cells/mL) is often used (SI Appendix, Table S1), and we also included medium and high seeding densities (1.0 × 10^6 and 2.5 × 10^6 cells/mL, respectively). Furthermore, two media were used [mTeSR and completely defined Essential 8 (E8)]. Moreover, hPSC suspension in static liquid medium was also included for comparison with the hydrogel. Finally, iPSC–mesenchymal stem cells (MSCs), an iPSC derived from human MSCs (33), were used in this optimization.

The study revealed that (i) the hydrogel promoted high expansion rates and prevented cell agglomeration (Figs. 1B and 2A). Culturing single hPSCs in 10% (wt/vol) PNIPAAm-PEG with E8 in the presence of RI led to the formation of dense, uniform, and small spheroids with ∼8-, 10-, or 4.4-fold expansion over 4 d at low, medium, or high seeding density, respectively. However, adding RI for 1 d was insufficient to support single cells (Figs. 1B and 2A). When using mTeSR rather than E8, only moderate (∼1.6-fold) or no expansion was reached with 4-d or 1-d RI, respectively, for all of the seeding densities, indicating a negative interaction between mTeSR and the hydrogel scaffold (Figs. 1B and 2A). (ii) In the hydrogel and E8, preclustered hPSCs behaved similarly to single hPSCs. However, in the hydrogel and mTeSR with 4-d RI, preclustered hPSCs displayed significantly higher expansion (∼4.5-, 4.5-, 2.1-fold for low, medium, high density, respectively) than single hPSCs (Figs. 1B and 2B), indicating that mTeSR stresses single cells. (iii) For all static-suspension cultures in liquid medium without hydrogel, large cell aggregates were found, and only ∼2.8-, 1.6-, or 0.2-fold was achieved for low, medium, or high seeding density, respectively (Figs. 1B and 2C). Agglomeration and low expansion (<2.0-fold) were also found in a large dynamic suspension culture (a spinner flask) without hydrogel scaffold (SI Appendix, Fig. S2). No significant difference was found between using mTeSR and E8 or between with 1-d or 4-d RI for these static-suspension cultures (Figs. 1B and 2C). In summary, a combination of 10% (wt/vol) PNIPAAm-PEG, 4-d RI, defined E8, medium seeding density via single-cell or precluster seeding resulted in a high, 10.0-fold cell expansion in a single 4-d passage (Fig. 1B and C). Final, fine-tuning of conditions revealed that 48 h RI was sufficient to support this expansion for precluster seeding. 4-d RI was required for single-cell seeding, and 8% (wt/vol) hydrogel performed as well as 10% (wt/vol) hydrogel (SI Appendix, Fig. S3).

We also assessed whether other, nonthermoreversible materials systems—including UV cross-linked hyaluronic acid (29), agarose (28), and alginate hydrogel (24)—could support high levels of efficiency and hPSC expansion as described previously, in conjunction with E8 and RI. For single-seeded iPSC-MSCs, moderate expansion (∼2.8-fold) was seen only in alginate hydrogel with medium seeding density. A slightly higher, ∼5- or 6.5-fold expansion was achieved in agarose or alginate hydrogel for preclustered iPSC-MSCs, respectively (SI Appendix, Fig. S4), although these numbers do not approach the levels of expansion observed with the thermoreversible material.

The multifactorial optimization thus resulted in an effective, completely defined system for hPSC culture, which was then characterized in greater detail. For both single-cell and cluster seeding, live/dead staining revealed very high viability within the hydrogel or spheroids (Fig. 3A). Also, 5-ethyl-2-deoxyuridine (EdU) staining showed uniform cell proliferation across spheroids, suggesting effective transport of nutrients, oxygen, and/or protein factors within the hydrogel and spheroids (Fig. 3B). Microscopy also revealed that single-seeded cells expanded and grew into spheroids with a narrow size distribution (e.g., 40–120 µm in diameter at day 5, Fig. 3C), whereas precluster seeding resulted in spheroids with larger sizes and broader distribution (50–300 µm in diameter at day 5, Fig. 3C). For both seeding methods, 10-, 20-, or 21-fold expansion, with final yields of ∼1, 2.0, or 2.1 × 10^7 cells per mL hydrogel, was achieved for hPSCs on day 4, 5, or 6 of the culture, respectively (Fig. 3D). Furthermore, immunostaining showed 95% of cells expressed pluripotency markers octamer-binding transcription factor 4 (Oct4) and Nanog, and alkaline phosphatase expression was both high.
and homogeneous across spheroids (Fig. 3E–G). An additional 13-d culture was conducted to explore the sensitivity of cellular growth and pluripotency to spheroid size in this system. Single iPS-MSCs were cultured in PNIPAAm-PEG hydrogel with E8 and RI for 13 d without passaging. An extremely low density ($1.0 \times 10^5$ cells per mL) was used to provide sufficient space for cell
growth during the long 13-d culture. iPS-MSCs grew into spheroids with mean diameter of \( \sim 350 \mu m \), with the larger spheroids reached diameter up to \( \sim 750 \mu m \). Consistent cell growth and pluripotent marker expression were also seen in this size range (SI Appendix, Fig. S5). In summary, the culture system efficiently expanded hPSCs with high quality and density.

We next assessed the generality of this system for the long-term expansion of multiple hPSC lines (1, 33), which were continuously propagated for up to 60 passages or \( \sim 280 \) d (with passaging every 4 or 5 d). During each of these passages, iPS-MSCs, iPS-Fib2 iPSCs, and H9 hESCs expanded \( \sim 10 \) - or 20-fold over 4 or 5 d, respectively, and H1 hESCs expanded 7-fold over 4 d, indicating some differences in growth rates among the lines (Fig. 4A). However, during this long-term culture with an accumulated expansion up to \( \sim 10^{72} \)-fold, \( \sim 95\% \) of cells remained Oct4+ (Fig. 4B). Immunostaining again revealed uniform Nanog

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**Fig. 2.** Phase contrast images showing the cell morphologies for the factorial-designed experiment. iPS-MSCs were cultured in 10% PNIPAAm-PEG hydrogel via single cell (A) or precluster seeding (B) or in static liquid medium without hydrogel (static suspension) (C) for 4 days in mTeSR or E8 with 1d or 4d RI at low, medium or high seeding density (2.5x10^5, 1.0x10^6, or 2.5x10^6 cells per mL, respectively). (Scale bar: 250 \( \mu m \).)
and Oct4 expressions within spheroids (SI Appendix, Fig. S6). To our knowledge, this is the highest reported level of hPSC expansion in a culture system.

To assess whether cellular proliferation rates remained consistent over time, we reevaluated the growth kinetics of hPSCs after 10 passages in the gel. Notably, the expansion rate (∼10-, 20-, or 21-fold at days 4, 5, or 6, respectively) (SI Appendix, Fig. S7), pluripotency marker expression, and spheroid size distribution (SI Appendix, Fig. S7) were similar to those at passage 1 (Fig. 3).

To further establish maintenance of cell pluripotency, embryoid body (EB) differentiation in vitro and teratoma formation in vivo were conducted after long-term culture within the hydrogel. Each cell line (iPS-MSC, iPS-Fib2, H9, H1) could undergo differentiation into the three germ layers, with expression of endodermal (HNF3β), mesodermal (αSMA), and ectodermal (Nestin) markers (Fig. S4 and SI Appendix, Fig. S8). Additionally, each hPSC line formed teratomas after 6–12 wk in vivo, and neural rosettes and epidermis from ectoderm, cartilage and muscle from mesoderm, and gut-like structure from endoderm were observed (Fig. 5B and SI Appendix, Fig. S9). In addition, hPSCs remained karyotypically normal after long-term culture within the gel (Fig. 5C and SI Appendix, Fig. S10).

To further assess whether hydrogel culture maintained cell behavior in 2D, after every 40 d in 3D, a passage of the hPSCs was returned to standard 2D surfaces. Dissociated cells attached to surfaces coated with vitronectin and grew into compact colonies after 4 d (SI Appendix, Fig. S11A and B), and these cells subsequently underwent long-term expansion on 2D and maintain high Oct4+ levels (SI Appendix, Fig. S12). hPSCs could then be returned to the 3D culture system for additional expansion, indicating that cells can be interchanged between 2D and this 3D system as needed. Finally, cell banking is important for downstream applications, and hESCs expanded within the 3D system could be cryopreserved as single cells (SI Appendix, Fig. S11C).

Because suspension cultures are favored for large-scale production, we also demonstrated the hydrogel could readily be extruded into fibers (∼2 mm in diameter) with encapsulated cells. Suspension of these fibers in 3D liquid culture resulted in ∼19.5-fold expansion within 5 d for both single-cell and cell cluster seeding (SI Appendix, Fig. S13), yielding sufficiently high densities of encapsulated cell mass that the hydrogel fibers were opaque (SI Appendix, Fig. S13B). The pluripotency marker expression and spheroid size distribution (SI Appendix, Fig. S13 C and D) were indistinguishable from cells grown in hydrogels cast within a culture well (Fig. 3).

Biomedical applications for hPSCs entail differentiation into specific lineages, and we thus investigated whether cells after long-term culture in this system could then be directed into ectodermal, endodermal, or mesodermal lineages within the 3D material (Fig. 6). First, after a 4-d expansion in E8 medium and 9-d differentiation in neural induction medium with small molecule SMAD inhibitors in the 3D hydrogel (10), ∼80% of H9-derived cells became positive for the neural progenitor markers Nestin and Pax6 (Fig. 6A). In a parallel culture, a 4-d
expansion in E8 medium and 5-d differentiation in endoderm induction medium (4) in the 3D hydrogel resulted in ~95% of H9 or iPS-MSC–derived cells being positive for the endoderm progenitor marker forkhead box protein A2 (FOXA2) and SOX17 (Fig. 6B). Finally, a 4-d expansion in E8 medium and 9-d differentiation in cardiomyocyte induction medium (34) within the 3D hydrogel yielded beating spheroids 2 d after transfer to a fibronectin-coated plate (Movie S1).

Several clinical trials have demonstrated that fetal ventral midbrain tissue implants can alleviate motor symptoms in Parkinson patients under certain circumstances, although this approach is challenged by tissue supply and other considerations (35, 36). In recent important work, Kriks et al. (10) and Kirkby et al. (37) developed approaches for differentiating hPSCs into DA progenitors or neurons that could functionally integrate into the brains of mouse, rat, and nonhuman primate models of PD. We investigated whether the differentiation protocols—which included both defined (Noggin, Shh, FGF8, TGFβ3) and undefined protein components—could be adapted to 3D culture under defined conditions. DA induction of H9 spheroids (40–120 μm) within the hydrogel was initiated via dual SMAD inhibition, Shh, and a GSK3β inhibitor to activate Wnt signaling (Fig. 7A). On day 11, 81% of cells within the spheroids (~100–300 μm) were positive for the ventral midbrain DA progenitor markers FOXA2 and LMX1a (Fig. 7 B–D). These matched levels obtained in 2D; however, cells in 3D expanded ~80-fold over 15 d, resulting in 8 × 10^7 cells per mL of hydrogel, compared with only an approximately ninefold expansion on 2D surface (Fig. 7D). Finally, these DA progenitors could subsequently be cultured on 2D surfaces as described (37) for differentiation into mature TH+ DA neurons (Fig. 7E).

Discussion

Large numbers of hPSCs, or their differentiated progeny, are needed for many biomedical applications (9). For large-scale expansion, currently hPSCs from a cell bank are expanded into large numbers through progressive proliferation followed by directed differentiation into progenitors or mature cells. Efficient, scalable, and GMP-compliant culture systems are required for such processes (9). hPSC culture has made considerable progress, but current systems achieve only moderate expansion, yield, and quality (9) (SI Appendix, Table S1). The most effective system reported to date enabled approximately threefold to fourfold expansion of hESCs per passage in mTeSR medium containing 1% BSA, for a final yield of <1 × 10^6 cells per mL (18) (SI Appendix, Table S1). We have developed a 3D culture system—which combines a synthetic thermoreversible hydrogel and defined medium with an optimized protocol involving single-cell passaging—for simple, defined, scalable, and GMP-compliant hESC and hiPSC expansion (Figs. 1–5) and differentiation (Figs. 6 and 7) with high yield. Long-term culture (>280 d) with high expansion rate (20-fold over 5 d, or 6.4 × 10^7-fold over 1 mo, and 10^2–fold over 280 d), volumetric cell yield (2 × 10^7 cells per mL of hydrogel), and quality (~95% Oct4+) were achieved (Figs. 3 and 4). Additionally, the hPSCs were able to differentiate into all of the three germ layers after long-term culture in the 3D system (Fig. 5 and SI Appendix, Figs. S8 and S9). Finally, the 3D system also supported directed differentiation into neural progenitors, endoderm progenitors (Fig. 6), beating cardiomyocytes (Movie S1), or midbrain dopaminergic progenitors (Fig. 7) by simply replacing the expansion medium with the differentiation medium. This versatile system thus combines many advantageous and important features.

The survival and proliferation of hESCs and hiPSCs—which both exhibit characteristics typical of epiblast-like pluripotent stem cells (14)—depend on multiple extracellular cues. These include soluble protein growth factors [e.g., FGF2, TGF-β, and autocrine/paracrine cues (15)] that signal via a number of pathways to support cell survival and pluripotency factors [e.g., Nanog, Oct4, Sox2 (38–40)]. In addition, hPSCs engage in cell–matrix interactions (31), and cell–cell contacts via cadherins are particularly important for survival and pluripotency maintenance. Disruption of such cell–cell contacts leads to Abr-dependent activation of RhoA (and inactivation of Rac1), ROCK activation, and downstream actomyosin hyperactivation and apoptosis (14, 32). Other environ-

Fig. 4. Long-term, progressive expansion of hPSCs in the 3D hydrogel system. (A) The expansion rates of iPS-MSCs, iPS-Fib2s, H9s, and H1s at each passage (4 or 5 d) using single-cell or precluster seeding are shown. Passages with 4 d are indicated (dotted line). (B) Oct4 levels remained high during the long-term culture.
mental stress, such as mechanical forces (9, 41) or apparently redox conditions (3), can also adversely affect cell function. Through a combination of rational and empirical investigation, this culture system based on a thermoresponsive material apparently addresses a number of these needs. Importantly, multifactorial investigation indicated that the system’s properties may result from a combination of the synthetic PNIPAAm-PEG matrix, defined medium, and optimized protocol, as any of these elements alone yielded suboptimal outcomes (Figs. 1 and 2, and SI Appendix, Figs. S1–S5 and S13–S15). The culture medium provides key soluble cues (3), and the material may conceivably concentrate autocrine/paracrine factors near cells (15). RI ameliorates dissociation-induced apoptosis that otherwise occurs via cytoskeletal-associated pathways (14, 31, 32), and future investigation may elucidate whether this mechanically soft (SI Appendix, Fig. S14) thermoresponsive material may more favorably interface with these mechanosensitive pathways relative to other materials (14). The material also protects cells from shear-induced stress, yet this readily deformable hydrogel may more favorably allow single-cell division and subsequent cluster growth relative to chemically cross-linked hyaluronic acid, ionically associated alginate, or stiff agarose hydrogels. Finally, E8 unlike mTeSR lacks β-mercaptoethanol, an apparent stressor of hESCs, particularly in suboptimal culture environments (SI Appendix, Fig. S15). A combination of factors thus interacts to provide a permissive 3D system that supports hPSC biology, and future investigations may elucidate the mechanistic contributions of both biochemical and mechanical features of this microenvironment.

Fig. 5. hPSCs retained pluripotency and normal karyotype after long-term culture in the 3D hydrogel system. (A) EB differentiation in vitro. iPS-MSCs were expanded in the 3D hydrogel for 35 passages via single-cell seeding before EB differentiation. Immunostaining for the three germ layers (ectoderm: Nestin; mesoderm: aSMA; and endoderm: HNF3β) are shown. (B) Teratoma formation in vivo. iPS-MSCs expanded in 3D hydrogels for 35 passages via single-cell seeding were injected into mice s.c., and teratomas formed after 6–12 wk. Structures from all three germ layers (arrows) were identified, including ectoderm: neural rosette and epidermis; mesoderm: cartilage and muscle; and endoderm: gut-like-structure. (C) iPS-MSCs cultured in the 3D hydrogel for 35 passages via single-cell seeding retained normal karyotypes. (Scale bars: A, 100 μm; B, 50 μm.)
The system can also be adapted to multiple scales—from the laboratory toward the clinic—to support research in cell replacement therapies, artificial tissues and organs, and/or high-throughput drug screening/toxicity screening with hPSCs. For instance, ~50 mL of hydrogel would be sufficient to produce $10^9$ cells for preclinical animal studies, and a bioreactor with ~5L of hydrogel could yield $>10^{11}$ cells for clinical studies. The ability to move a single culture system through multiple scales may aid clinical development.

Advances in developmental and stem cell biology have enabled the development of approaches to effectively differentiate hPSCs into numerous additional cell types, such as neural crest...
cells (42), motor neurons (43), oligodendrocyte progenitors (44, 45), pancreatic progenitors (4, 46), hepatocyte-like cells (47), retinal cells (48), and others (49, 50). Several of these processes have even proceeded to clinical trials (51). Future work can explore integrating these advances into this defined 3D hydrogel culture system to aid efficient, economical, and reproducible cell production for multiple future applications.

Methods

Maintaining hPSCs on 2D Surface. Human ESC lines H1 and H9 were obtained from WiCell Research Institute. iPS-MSC (33) (derived from human MSCs) and iPS-Fib2 (33) (derived from human dermal fibroblasts) were gift from George Q. Daley (Children’s Hospital Boston, Boston). hPSCs were cultured on six-well plate coated with vitronectin (Invitrogen) in E8 medium (Invitrogen). Cells were passaged every 4 d with 0.5 mM EDTA (Invitrogen). To replate hPSCs on 2D surfaces following expansion within the 3D hydrogel, hPSC spheroids were dissociated with Accutase (Life Technologies) at 37 °C for 10 min, and cultured on six-well plate coated with vitronectin in E8 medium (supplied with 10 μM ROCK inhibitor, Y-27632, Selleckchem, for the first 24 h).

Expanding hPSCs in 3D Hydrogel. To transfer the culture from 2D to 3D, hPSCs on Matrigel or vitronectin-coated tissue culture plates were incubated with Accutase at 37 °C for 5 min and dissociated into single cells. For the single-cell seeding method, dissociated cells were mixed with PNIPAam-PEG (Cosmo Bio) solution dissolved in E8 medium at 4 °C and cast on tissue culture plate, then incubated at 37 °C for 15 min to form hydrogels before adding warm E8 medium containing 10 μM ROCK inhibitor. For the precluster seeding method, dissociated cells were cultured in suspension overnight in low adhesion plates to form small clusters that were subsequently encapsulated into the 3D hydrogel as mentioned above.

To passage hPSCs within 3D hydrogel, ice-cold PBS was added to the 3D culture at day 4 or 5 to dissolve the gel. Spheroids were collected by centrifuging at 200 × g for 3 min, incubated with Accutase at 37 °C for 10 min, and dissociated into single cells for reencapsulation as mentioned above. The NucleoCounter NC-200 (Chometec) was used to count cell number. To prepare hydrogel fibers, a 4 °C PNIPAam-PEG solution containing cells was extruded into room temperature E8 medium through a 2-mm-diameter tube. The resulting hydrogel fibers were cultured in suspension in E8 medium at 37 °C. Medium was changed daily for all cultures. To measure spheroid sizes, hPSCs were released from the hydrogel, and phase images were taken. The diameters of >2,000 spheroids were quantified with MetaXpress software (Molecular Devices). The frequency of spheroids within a diameter range was calculated with the Excel Histogram Plug-in. Expanded cells were cryopreserved as single cells in E8 medium with 10% (vol/vol) DMSO and 10 μM ROCK inhibitor in liquid N2.

Staining and Imaging. Cells cultured on 2D surfaces were fixed with 4% (wt/vol) paraformaldehyde (PFA) at room temperature for 15 min, permeabilized with 0.2% (vol/vol) Triton X-100 for 15 min, and blocked with 5% (vol/vol) goat serum for 1 h before incubating with primary antibodies at room temperature for 2 h. After extensive washing, secondary antibodies in 2% (wt/vol) BSA were added and incubated for another 1 h. Cells were washed with PBS for three times before imaging.

To assess the pluripotency marker expression of cells expanded in 3D hydrogels, hPSCs were dissociated into single cells with Accutase and stained in suspension. Cells were then placed in 96-well plates and analyzed with an ImageXpress (Molecular Devices). The percentage of Oct4+ or Nanog+ nuclei was quantified with MetaXpress software (Molecular Devices). This process was used to quantify the PAX6+ and Nestin+ cells after neural induction as well.

To stain spheroids, hPSCs were fixed with 4% (wt/vol) PFA at room temperature for 30 min, and then incubated with PBS plus 0.25% Triton X-100 plus 5% (vol/vol) goat serum plus primary antibodies at 4 °C for 4 h before washing, secondary antibodies in 2% (wt/vol) BSA were added and incubated at 4 °C for 4 h. Cells were washed with PBS for three times before imaging. Staining without primary antibodies was used as controls for all of the immunostainings.


Figure S1. Culturing iPSC-MSCs in 3D PNIPAAm-PEG hydrogel in mTeSR or E8. Small clumps of iPSC-MSCs were encapsulated in 10% hydrogel and cultured. (A) Spheroids in the hydrogel on day 1 or 5. (B) Live and dead cell staining of spheroids in the hydrogel on day 5. (C) The fold expansion and yield during a 6-day culture. (D) The % of Oct4+ or Nanog+ cells on day 4 or 6. *** indicates statistical significance at a level of p<0.001. Scale bar: 250 µm
Figure S2. Single iPS-MSCs were cultured in 60 ml E8 with RI at 1.0x10^6 cells/ml seeding density in a 125 ml spinner flask at 70-100 rpm for 4 days. (A) Phase images and (B) Fold expansion. Scale bar: 250 µm
Figure S3. Effect of RI and hydrogel concentration on hPSC expansion: iPS-MSCs were cultured in 8% or 10% hydrogels (1x10^6 cells/ml hydrogel) via single cell (A and B, 4-day culture) or precluster (C and D, 5-day culture) seeding in the presence of ROCK inhibitor (RI) for 1, 2, 4, or 5 days. The spheroid morphology (A, C), fold of expansion, and % of Oct4+ and Nanog+ cells (B, D) on day 4 (A, B) or 5 (C, D) are shown. Spheroids were released from 3D hydrogels before imaging. *** indicates statistical significance at a level of p<0.001. Scale bar: 250 µm
Figure S4. Culturing iPSC-MSCs in various hydrogels with E8 and RI at low or medium seeding density via single or precluster seeding. Acrylated hyaluronic acids (60 kDa) were UV crosslinked in the presence of 0.05% Irgacure 2959 in PBS with cells (PNAS, 2007, 11298). Low melting agarose (Sigma) was used for preparing the 1% agarose hydrogel (Cytotechnology, 2011, 227). Alginate (Sigma) solution in HEPES buffer was extruded into 100 mM CaCl$_2$ solution to prepare the alginate hydrogels (PloS One, 2011, e23212). Cells were cultured for 4 days in E8 + RI and live dead cell staining was done to evaluate the cell viability. For single cells, moderate expansion (~2.8-fold) was observed only in alginate hydrogel with medium seeding density. Using preclustered cells, ~5 or 6.5-fold expansion was achieved in agarose or alginate hydrogel, not as high as the 10-fold expansion achieved with the thermoreversible PNIPAAm-PEG hydrogel. *** indicates statistical significance at a level of p<0.001. Scale bar: 250 µm
Figure S5. A 13-day culture of iPS-MSCs in 3D PNIPAAm-PEG hydrogel. Single iPS-MSCs were seeded at low density (1.0x10^5 cells/ml) to provide sufficient space for cell growth during the 13-day culture. Cells grew into spheroids with mean diameter of ~350 µm, with larger spheroids reaching diameter of ~750 µm on day 13. Consistent cell growth and Oct4 expression were seen over the 13-day culture or diameter range. (A) Phase images. (B and C) Fold expansion and % of Oct4+ cells over spheroid diameter. Scale bar: 250 µm
Figure S6. Expression of pluripotent markers Nanog and Oct4 in spheroids. H9s, iPS-MSCs, H1s and iPS-Fib2s were cultured in PNIPAAm-PEG hydrogel with E8 medium via single cell seeding for 60, 60, 20 and 35 passages before immunostaining. Uniform expression of both markers were seen among spheroids. Scale bar: 100 µm
Figure S7. The expansion fold, yield (cells/ml hydrogel), % of Oct4+ cells and spheroid size distribution of iPS-MSCs (A) and H9s (B) on a 6-day culture at passage 10 in 3D hydrogel. *** indicates statistical significance at a level of p<0.001
Figure S8. EB differentiation. Immunostaining for the 3 germ layers (ectoderm: Nestin; mesoderm: αSMA and endoderm: HNF3β). hPSCs were long term cultured in 3D PNIPAAm-PEG hydrogel via single cell or precluster seeding before differentiation via EB. “Single, P10” indicates 10 passages via single seeding in 3D hydrogel before EB. Scale bar: 100 µm
Figure S9. Teratoma formation in vivo. hPSCs expanded in 3D PNIPAAm-PEG hydrogel for long term were injected into mice subcutaneously. Teratomas formed after 6-12 weeks. Structures (arrows) from all 3 germ layers were identified, including ectoderm: neural rosette and epidermis; mesoderm: cartilage and muscle; and endoderm: gut-like-structure. “Single, P10” indicates 10 passages via single seeding in 3D hydrogel before teratoma assay. Scale bar: 50 µm
Figure S10. (continued)
Figure S10. Karyotyping for long term cultured hPSCs. “H1, P10, single” indicates H1s were cultured in the 3D PNIPAAm-PEG gel for 10 passages via single cell seeding before karyotyping.
Figure S11. Phase contrast images or Oct4 staining for iPS-MSC (A) or H9 (B) cells expanding on 2D surfaces coated with vitronectin for 1 or 4 days. iPS-MSCs and H9s were cultured in the 3D PNIPAAm-PEG hydrogel for 10 passages before being replated on the 2D surface. (C) Oct4 staining of H9s at day 4 on 2D surface coated with vitronectin. H9s were expanded in 3D hydrogel for 10 passages via single cell or precluster seeding and cryopreserved in liquid N2 for 1 month before replating on 2D surfaces. Scale bar: 100 µm
Figure S12. Oct4 staining for iPS-MSC, H9, and H1 cells cultured on 2D surfaces coated with vitronectin for 4 days. hPSCs were cultured in the 3D hydrogel for 10 passages via single cell or precluster seeding, then replated on 2D surface for another 10 passages before staining. Scale bar: 100 µm
Figure S13. Suspension culture of hydrogel fibers with iPS-MSCs. PNIPAAm-PEG solutions with iPS-MSCs via single cell or precluster seeding were extruded into fibers and suspended in medium. (A) The hydrogel fibers (dashed lines highlight the fiber edges) with spheroids at day 3, 4, and 5 are shown, as well as spheroids harvested on day 5. (B) The fibers with cells on day 5 appeared white. The expansion rates (C) and Oct4 expression (D) for both seeding methods after 5 days culture were quantified. Scale bar: (A) 250 µm and (B) 2 mm
Figure S14. The storage modulus (G') of PNIPAAm-PEG, alginate, agrose and HA hydrogel in PBS at 37°C. *** indicates statistical significance at a level of p<0.001
Figure S15. Culturing iPS-MSCs in PNIPAAm-PEG hydrogel with E8 + 1% BSA + 100 µM β-mercaptoethanol. Single iPS-MSCs were encapsulated at low, medium or high density and cultured with 1d or 4d RI. (A) Phase images on day 4. No spheroids were formed. (B) A comparison on cell expansion under E8, mTeSR and E8 plus BSA and β-ME. Low expansions were seen in mTeSR and E8 plus BSA and β-ME. *** indicates statistical significance at a level of p<0.001. Scale bar: 250 µm
**Table S1: Comparison of 3D culture systems in the literature and the new 3D culture system (1)**

<table>
<thead>
<tr>
<th>Culture Method</th>
<th>Cell Lines</th>
<th>Medium</th>
<th>Seeding Method</th>
<th>Serial Expansion (no of passages)</th>
<th>Yield (cells/ml)</th>
<th>Fold of Expansion</th>
<th>Fold of Expansion in 30 days</th>
<th>Cell Quality</th>
<th>Differentiation Following Expansion?</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>(a) Suspension Culture of Cell Aggregates:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hPSC aggregates in spinner flask</td>
<td>hES2, hES3, ESI049</td>
<td>mTeSR</td>
<td>Single cells via TrypLE</td>
<td>3-5</td>
<td>~1x10^6</td>
<td>2x/7days</td>
<td>Cells started to differentiate after 5 passages</td>
<td>No</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hPSC aggregates in TCP</td>
<td>hES2, MF12, iP</td>
<td>mTeSR</td>
<td>Single cells via Collagenase</td>
<td>17</td>
<td>1-2x10^6</td>
<td>4-6x/4days</td>
<td>~1.7x10^6</td>
<td>No</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EBs in spinner flask</td>
<td>H1, H9</td>
<td>KSR</td>
<td>No</td>
<td>2-3x10^5</td>
<td>15x/21days</td>
<td>5-6% converted to hematopoietic progenitor after 14 days</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EBs in STLV bioreactor</td>
<td>H9.2</td>
<td>FBS</td>
<td>No</td>
<td>35x10^5</td>
<td>70x/28days</td>
<td>No</td>
<td>EB formation and Neural differentiation</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EBs in STLV bioreactor with perfusion and dialysis</td>
<td>VUB01, H9, HUES-9</td>
<td>KSR</td>
<td>No</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td><strong>(b) Stirred Tank Bioreactor Culture:</strong></td>
<td></td>
<td></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>hPSC aggregates in 125ml spinner flask</td>
<td>I3, I4, I6, H9.2</td>
<td>KSR</td>
<td>Small clumps via mechanical dissociation</td>
<td>20</td>
<td>9x10^5</td>
<td>25x/10days</td>
<td>~1.6x10^4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hPSC aggregates in 125ml spinner flask</td>
<td>H9</td>
<td>mTeSR, rapamycin</td>
<td>Single cells via accutase</td>
<td>3</td>
<td>4.5x10^3</td>
<td>1:5</td>
<td>&gt;90% Oct4+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EBs in</td>
<td>H9</td>
<td>KSR, FBS</td>
<td>No</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hPSC aggregates in TCP</td>
<td>HES1, HES2, H7</td>
<td>KSR</td>
<td>Small clumps via mechanical dissociation</td>
<td>10</td>
<td>&lt;5x/7days</td>
<td>~980</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hPSC aggregates in spinner flask</td>
<td>H9, H1, HES2</td>
<td>mTeSR</td>
<td>Single cells via accutase</td>
<td>21</td>
<td>&lt;1x10^5</td>
<td>4.1x/4days</td>
<td>~4.0x10^4</td>
<td>~27.5% converted to cardiomyocyte on Day 19</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### (b) Suspension Culture of Cells on Microcarriers

<table>
<thead>
<tr>
<th>Set</th>
<th>Microcarriers</th>
<th>Suspension Culture</th>
<th>Data</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Matrigel coated Cytodex™3 in Spinner flask, uncontrolled</td>
<td>SCED™4 61</td>
<td>MEF-CM</td>
<td>No</td>
<td>1.2x10⁶ 2.2x10⁶ 0.8x10⁶ 6x/11day 15x/11day 7x/12day</td>
</tr>
<tr>
<td>In spinner flask on DE53 coated with Matrigel</td>
<td>HES2, HES3</td>
<td>MEF-CM</td>
<td>Mechanical dissociation</td>
<td>1.43x10⁶ 1.37x10⁶ 10-15x/7day 10-15x/7day</td>
</tr>
<tr>
<td>Collagen coated Cytodex™3 in spinner flask</td>
<td>MEF-CM</td>
<td>No</td>
<td>1.5x10⁶ 6.8x/14days</td>
<td>a) 76% Oct4&lt;sup&gt;b&lt;/sup&gt; b) agglomeration seen</td>
</tr>
<tr>
<td>Polystyrene beads in spinner</td>
<td>StemPro</td>
<td>No</td>
<td>1.5x10⁶ 1.4x10⁶ 8.5x/7day 8.5x/7day</td>
<td>20% converted to definitive endoderm</td>
</tr>
<tr>
<td>Laminin coated beads in spinner flask</td>
<td>H1, H9</td>
<td>MEF-CM</td>
<td>Single cells via trypsin</td>
<td>11</td>
</tr>
<tr>
<td>Matrigel coated Cytodex™3 in TCP</td>
<td>H1, H9</td>
<td>MEF-CM</td>
<td>No</td>
<td>2.14x10⁹</td>
</tr>
<tr>
<td>Matrigel coated beads in spinner flask</td>
<td>HES2, HES3</td>
<td>mTeSR, StemPro</td>
<td>TrpLE + mechanical dissociation</td>
<td>6 month</td>
</tr>
<tr>
<td>Trisethylammonium coated polystyrene bead in TCP</td>
<td>ESI-017</td>
<td>CM</td>
<td>Collagenase + mechanical dissociation</td>
<td>No</td>
</tr>
<tr>
<td>CultispHERE S in spinner flask</td>
<td>SHEF3</td>
<td>KSR, CM</td>
<td>1</td>
<td>3.5x10⁶ 10x/7days</td>
</tr>
</tbody>
</table>

### (c) Suspension Culture of Cells in Alginate Microencapsulation

<table>
<thead>
<tr>
<th>Set</th>
<th>Alginate Microencapsulation</th>
<th>Data</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>hESCs on microcarriers in 1.1% alginate microbeads</td>
<td>SCED™4 61</td>
<td>MEF-CM</td>
<td>No</td>
</tr>
<tr>
<td>hESCs in PLL-coated liquid core microcapsules in static and spinner flask</td>
<td>H9</td>
<td>KSR, MEF-CM, FBS</td>
<td>No</td>
</tr>
<tr>
<td>hESC clusters in 1.1% calcium alginate microbeads in static culture</td>
<td>SCED™4 61</td>
<td>MEF-CM</td>
<td>No</td>
</tr>
</tbody>
</table>

### (d) The new culture system introduced in the paper<sup>4</sup>
<table>
<thead>
<tr>
<th>hPSCs in 3D thermoreversible hydrogel</th>
<th>iPS-MSC, iPS-Fib2, H9, H1</th>
<th>E8</th>
<th>Single cells via Accutase</th>
<th>&gt;60</th>
<th>&gt;2.0x10^7</th>
<th>10x4days</th>
<th>20x5days</th>
<th>6.4x10^7</th>
<th>~95% Oct4^+</th>
<th>&gt;80% converted to neural lineages</th>
</tr>
</thead>
</table>

### Advantages

(a) Suspension culture of cell aggregates
- Scalable
- Easy handling
- Reproducible
- Good 3D cell-cell, cell-matrix interaction

(b) Suspension culture of cells on microcarriers
- Scalable
- Reproducible
- Good mass and gas diffusion

(c) Suspension culture of cells in alginate microencapsulation
- Scalable
- Reproducible
- Protection from shear force
- Prevents agglomeration of cells
- Good 3D cell-cell, cell-matrix interaction

(d) The new 3D culture system in the paper
- Scalable
- GMP compliant
- Easy handling (hydrogel forms at room temperature and becomes solution at 4ºC)
- Protection from shear force
- Prevents agglomeration of cells
- High expansion rate
- High cell yield
- Effective mass and gas transport
- Good 3D cell-cell, cell-matrix interaction
- Compatible with many types of bioreactor

### Disadvantages

(a) Suspension culture of cell aggregates
- Difficult to control aggregate size
- Difficult to control cell purity
- Shear-force-induced cell death
- Relatively low expansion rate
- Relatively low cell yield (cells/volume)

(b) Suspension culture of cells on microcarriers
- Severe agglomeration of microcarriers and cells
- Shear-force-induced cell death
- Relatively low expansion rate
- Relatively low cell yield
- The processes of coating bead, seeding cells, harvesting cells and separating beads from cells are time-consuming and cost ineffective

(c) Suspension culture of cells in alginate microencapsulation
- Ineffective mass and gas transport within the alginate hydrogel
- Low expansion rate
- Low cell yield
- The processes of encapsulation and decapsulation (w/ EDTA) are time-consuming and cost ineffective

### Abbreviations:
- CM, conditioned medium; MEF-CM, mouse embryonic fibroblast conditioned medium; KSR, knockout serum replacement; FBS, fetal bovine serum; TCP, tissue culture plate; STLV, slowly turning lateral vessel; EB, embryonic body; hPSC, human pluripotent stem cell

(i) if serial expansion was demonstrated in the paper, the theoretical Fold of Expansion in 30 days was calculated as: (Fold of Expansion per passage)^(30)/(days per passage)

(ii) For these with a range instead of an accurate number, the median was taken for the calculation

(iii) mTeSR1 and StemPro medium contain 1% and 2% BSA respectively.
References:

Movie S1. H9s cultured for 20–30 passages in the 3D system were used for the cardiomyocytes differentiation. Single H9s were cultured in the hydrogel for 4 d before differentiation. On day 9, spheroids were released from the gel and plated on fibronectin-coated plate. The movie was taken at day 11. (Scale bar: 250 μm.)

Other Supporting Information Files

SI Appendix (PDF)