Dynamic suspension culture for scalable expansion of undifferentiated human pluripotent stem cells

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Human pluripotent (embryonic or induced) stem cells (hPSCs) have many potential applications, not only for research purposes but also for clinical and industrial uses. While culturing these cells as undifferentiated lines, an adherent cell culture based on supportive layers or matrices is most often used. However, the use of hPSCs for industrial or clinical applications requires a scalable, reproducible and controlled process. Here we present a suspension culture system for undifferentiated hPSCs, based on a serum-free medium supplemented with interleukins and basic fibroblast growth factor, suitable for the mass production of these cells. The described system supports a suspension culture of hPSC lines, in both static and dynamic cultures. Results showed that hPSCs cultured with the described dynamic method maintained all hPSC features after 20 passages, including stable karyotype and pluripotency, and increased in cell numbers by 25-fold in 10 d. Thus, the described suspension method is suitable for large-scale culture of undifferentiated hPSCs.

INTRODUCTION

Human pluripotent stem cells (hPSCs), embryonic or induced, are frequently cultured in medium supplemented with serum replacement and basic fibroblast growth factor (bFGF), as well as with supportive layers such as mouse embryonic fibroblasts or foreskin fibroblasts[1,2]. The culture systems used for hPSCs are traditionally adherent by nature, although any future therapeutic utilization of these cells will require their mass production as undifferentiated cells in a defined, reproducible and animal-free culture system. Most industrial scale-up systems are based on stirred suspension cultures with cells that were previously cultured solely in suspension, such as hybridoma cells; adherent cells that underwent adaptation to the suspension environment; or cells transferred onto microcarriers. However, when transferred from adherent culture to suspension, hPSCs spontaneously form aggregates known as embryoid bodies (EBs), in which differentiation into cells representative of the three embryonic germ layers initiates[3–6].

The protocol presented here for culturing hPSCs in suspension overcomes this natural tendency of the cells to aggregate into differentiating EBs and allows their continued culture as undifferentiated cell spheres in suspension without microcarriers[7]. As such, it represents a novel approach to propagating undifferentiated hPSCs using suspension cultures. The base medium used is supplemented with serum replacement, a low concentration of bFGF, and full-length interleukin-6 and interleukin-6 receptor chimera (IL6RIL6 chimera)[7]. Thus far, several cell lines from various sources have undergone successful adaptation to this system, including hESC lines I3, I4 and I6 (ref. 8); H9.2 (ref. 1); H9, H7 and H14 (ref. 9); as well as induced PSC (iPSC) lines iF4 and J1.2.3 (ref. 10); C3 and C2 (ref. 11); and KTN7 and KTR13 (ref. 12). Results for the adaptation of I3, I4, I6, H9.2, iF4 and J1.2.3 cell lines were reported in reference 7; results for the adaptation of H7, H9, H14, C3, C2, KTN7 and KTR13 are unpublished (M.A., I.L., Y.M., K.S., M.P. and J.I.-E., unpublished data).

The cells expanded in a prolonged suspension culture (over 1 year) and remained pluripotent, as was evident by EB and teratoma formation, expression of typical surface markers and karyotype stability. The suspension culture can be achieved in two main ways: either by suspending the cells in static Petri dishes or by stirring or rotating the cells dynamically in Erlenmeyer or spinner flasks. Examples of the morphology of cells cultured in suspension are presented in **Figure 1**. Although both methods allow a prolonged suspension culture that preserves all the hPSCs features, the dynamic method enables a scale-up of 25-fold in 10 d[7]. Although seeding concentrations and medium metabolites should still be optimized, these results demonstrate that the method used can serve as a basis for developing a controlled process for the mass production of hPSCs in bioreactors for therapeutic or industrial applications[7].

The use of full-length IL6RIL6 chimera as the main factor added to the culture medium is not trivial. Although leukemia inhibitory factor (LIF) was found to be involved in the maintenance of mouse ESC self-renewal[13–17], accumulating data on hESCs indicate that LIF fails to maintain hESCs as undifferentiated cells without the use of a supportive layer[9,18–20]. In these studies, it was shown that STAT3 phosphorylation, a key factor in the LIF signaling pathway, is either inconsistent or weak in hESCs[18,20]. Therefore, we chose to use a more potent LIF-related cytokine, i.e., the full-length IL6RIL6 chimera. Indeed, while adding this factor to the culture medium, we were able to expand undifferentiated hPSCs, both in the feeder layer–free culture using fibronectin as the matrix and in the suspension culture[7]. Additional factors found to support the culture of undifferentiated hPSCs in suspension and that yield the same results include the IL6 and its soluble receptor, and high concentrations of LIF[7].

The first attempts to culture hPSCs in suspension were based on the use of coated microbeads[21]. Overall, this system demonstrated a decreased ability to expand the cells in culture for an extended time (up to 6 weeks) and raised difficulties with respect to developing scale-up processes[21]. One main contributor to these disadvantages was a technical feature related to the constant need to upload and remove the cells from the beads in every passage. This resulted in massive cell loss and the need to coat the beads

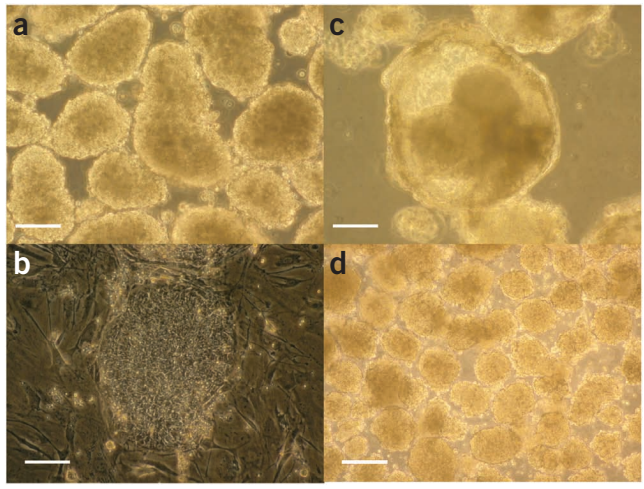

Figure 1 | Sphere morphology. Examples of spheres created by hPSCs cultured for prolonged periods in suspension. (**a**) Sphere of I3 hESC cell line cultured in a Petri dish for 43 passages. (**b**) Colony formed by iPSC line J-1.2.3 after being cultured for 30 passages in suspension and then recultured on MEFs. Note that the cell grain displays the typical morphology of hPSCs, i.e., small and round cells, with spaces between the cells and a high ratio of nucleus to cytoplasm. (**c**) An example of a differentiating clump formed by I3 hESC line after being cultured for two passages in suspension. Note the formation of a cyst, similar to EB formation. (**d**) Sphere of H14 hESC line cultured for 2 weeks in a spinner flask after being cultured for 27 passages in suspension in a Petri dish. The resultant spheres are smaller and more homogeneous. Scale bars, 100 μM.

with animal and nondefined materials, such as Matrigel, to improve the cells' adherence to the beads[21]. An additional study by Oh *et al.*[22] presented a stable system for expanding hPSCs with Matrigel-coated microbeads and a commercial medium free of animal components and serum. This system operated for 6 months and achieved an expansion two- to fourfold higher than that obtained by the standard 2D culture[22]. To date, several studies demonstrated a successful culture of hPSCs using microbeads in suspension[23–26].

Recently, a few methods have been proposed for culturing undifferentiated hPSCs as spheres without the use of beads[7,27–29]. Two of the proposed systems are based on a similar medium: a commercially available defined medium supplemented with Rho kinase (ROCK) inhibitor Y-27632 (refs. 27,28). The data presented in these studies demonstrate that the hPSCs maintained pluripotency and proliferation capacity after prolonged culture. However, both systems require continuous exposure to ROCK inhibitor, either for routine splitting[28] or as an integral constituent in the culture medium[27], which may pose a long-term negative effect on the cells. Additional disadvantages of these studies relate to the proliferation rates. Singh *et al.*[28] demonstrated that ~40% of the cells were lost in each passage and that adaptation of one out of three tested hESC lines (clinical grade one) failed, raising questions about the proposed system's reproducibility and the possibility of using this method for the mass production of hPSCs. In the method presented by Olmer *et al.*[27], single-cell dissociation resulted in 30% cell loss in each passage. In addition, background differentiation rates increased when passaging was carried out after the fourth day[27]. However, it should be mentioned that for the establishment of a reproducible culture system with less variable clump size, as required for clinical and industrial applications of hPSCs, a homogenous hPSC population is required[30]. This should ideally be achieved by enzymatic splitting and single-cell dissociation[7,27,28], and not by mechanical splitting. In contrast, the system presented by Steiner *et al.*[29], based on a complex serum-free medium, can be applied using several hESC lines. Thus, it was found to be suitable for the derivation of new hESC lines in suspension, enabling an increase of approximately ten fold during 2 weeks of culture[29]. The protocol described in this manuscript uses a simple serum-free medium with low concentrations of the factors used, thereby allowing the highest increase in vital cell numbers of 25-fold in 10 d[7]. It also proposes two ways for splitting, i.e., either based on mechanical splitting, which results in non-homogeneous spheroid size, or on enzymatic splitting, which allows reduced spheroid size variations[7]. However, the described protocol is based on culturing the cells as clumps rather than single cells, which hinders the genetic manipulation of the cells when cultured in suspension.

Experimental design

This is the first description of a highly efficient method for the continued long-term expansion of undifferentiated hPSCs in suspension and shaking cultures, which may constitute a significant step forward in facilitating the implementation of hPSC technologies. In this manuscript, we describe in detail how to establish a suspension culture of undifferentiated hPSCs in static and dynamic cultures. To establish a suspension culture, we recommend starting with a stage of spheroid formation in Petri dishes, as shown in **Figure 1a**. This step allows the smooth adaptation of the cells to the new culture conditions, without exposure to sheer stress. In our experience, an adaptation period of three to five passages is sufficient and will be reflected in a background differentiation of <5%, as measured either by cell morphology (shown in **Fig. 1c**), whereby the differentiated cells will form EBs rather than spheres, or by cell staining. During the adaptation period, it is often necessary to remove differentiating cells. Some cell lines are quicker to adapt (such as I4 and I3) and some need up to five passages before they finally acclimatize (such as iPSCs derived form keratinocytes KTN7 and KTR13). If desirable, the cells could be cultured for extended periods in Petri dishes and maintain hPSC features at concentrations of 1 million to 10 million cells per Petri dish. Testing the ability of suspension cultured cells to form teratomas after prolonged culture is demonstrated in **Figure 2**. This setup is applicable to many research strategies, as it allows feeder-layer-free culture of undifferentiated hPSCs at a reasonable cost and effort. Transferring the cells from Petri dishes to dynamic systems is simple and does not require an adaptation period. The cells can be transferred into shaking Erlenmeyer or spinner flasks. The volume of medium required for dynamic cultures can range from 25 ml to over a liter, depending on the size of the vessel. This applies to scalable procedures, in which cell numbers can reach hundreds of millions, depending on the volume of the medium. Spheroids in the dynamic system were more homogenous and had an average diameter of approximately 112 ± 14 μm[7]. An example of the morphology of spheroids in a dynamic culture is provided in **Figure 1d**. When all the required optimization processes are completed, this system could be prospectively used to prepare cells for controlled bioreactors as a last-stage step to generate cells in industrial-scale quantities for clinical applications. As controls, we recommend to use adherent cells cultured with MEFs in the same serum-free medium.

MATERIALS

REAGENTS

Cell lines

- hESC lines I3, I4, I6 (Technion-Israel Institute of Technology[8]); H9.2 (Technion-Israel Institute of Technology[1]); and H9, H7, H14 (WiCell international stem cells bank[9])
- iPSC lines iF4, J1.2.3 (Children's Hospital Boston and Dana-Farber Cancer Institute, Harvard Medical School[10]); C3, C2 (Technion-Israel Institute of Technology[11]); and KTN7 and KTR13 (Technion-Israel Institute of Technology[12])

Culture medium

- Dulbecco's modified Eagle's medium F12 (DMEM/F12, Biological Industries, cat. no. 01-170-1A)
- Knockout (KO)-serum replacement (Invitrogen, cat. no. 10828028)
- Defined FBS (HyClone, cat. no. SH30070.03)
- Nonessential amino acid (Invitrogen, cat. no. 11140035)
- L-glutamine (Biological Industries, cat. no. 03-020-1B)
- β-mercaptoethanol (Invitrogen, cat. no. 31350010) **! CAUTION** β-mercaptoethanol is a toxic material. Avoid inhalation, ingestion and skin contact.
- Human recombinant basic fibroblast growth factor (bFGF, R&D systems, cat. no. 233FB-CF) **▲ CRITICAL** The ED_{50} of the bFGF should be in the range of 0.1–0.6 ng ml^{-1}. If different, change the concentration accordingly.
- Human recombinant full-length IL6RIL6 chimera. Information regarding its structure and method of synthesis can be found in ref. 31
- Human recombinant IL6 (R&D systems, cat. no. 206-IL-050)
- Human recombinant IL6 soluble receptor (R&D systems, cat. no. 227-RS-025)
- Human recombinant leukemia inhibitory factor (LIF, Chemicon, cat. no. LIF1010)
- DMEM (Invitrogen Corporation, cat. no. 41965039)

Enzyme

- Collagenase type IV (Wordington, type IV, cat. no. 4189, activity of 220–320 U mg^{-1}) **▲ CRITICAL** In case the activity of the collagenase batch is out of range, the enzyme concentrations should be corrected accordingly, in order to avoid damage to the cells due to the incubation length.
- Trypsin-EDTA (0.25% vol/vol, Biological Industries, cat. no. 03-052-1A)

Chemicals and general reagents

- Rho kinase inhibitor Y-27632 (ROCK inhibitor, Mercury, cat. no. 688000)
- DMSO (Sigma, cat. no. D2650) **! CAUTION** DMSO is a toxic material. Avoid inhalation, ingestion and skin contact.

EQUIPMENT

- Petri dishes (58 mm; Greiner, cat. no. 60-628103)
- Erlenmeyer (125 ml; Corning, cat. no. 431405)
- Orbital shaker
- Spinner flasks (100ml and 250 ml; Integra, cat. nos QC182023 and QC182026, respectively)
- Magnetic palate (Integra, cat. no. QC183001)
- Incubator maintained at 37 °C, 90% humidity and 5% CO_2
- Biosafety cabinet with aspirator for tissue culture
- Water bath maintained at 37 °C
- Centrifuge, preferably refrigerated at 4 °C, for 15 ml and 50 ml tubes
- Disposable sterile filter systems (0.22 μM, 100–1,000 ml; Millipore, cat. no. 59-FB1000)
- Falcon conical tubes (15 and 50 ml)
- Glass Pasteur pipette, sterilized using 180 °C oven
- Plastic disposable pipettes for 1, 5, 10, 25 and 50 ml
- Cryovials (1 ml, Nalgene, cat. no. 5000-1012)
- Freezing box (Nalgene Labware, cat. no. 5100-0001)
- Pipette aid (Dramond, cat. no. 25PA)
- Micropipette (200 μl, Gilson, cat. no. P200)
- Inverted light microscope with phase contrast (×4, ×10, ×20 and ×40 objectives)
- Dissecting microscope (×1,000)

REAGENT SETUP

Suspension culture medium The suspension culture medium consists of 85% (vol/vol) DMEM/F12; 15% (vol/vol) KO-serum replacement; 1% (vol/vol) nonessential amino acid; 2 mM L-glutamine; 0.1 mM β-mercaptoethanol; 4 ng ml^{-1} human recombinant bFGF; and 100 pg ml^{-1} human recombinant full-length IL6RIL6 chimera. Filter the medium with a 0.22-μM filter and store at 4 °C until use. It can be used for up to 5 d after preparation. **▲ CRITICAL** The IL6RIL6 chimera can be replaced with one of the following two options: (i) 25 ng ml^{-1} human recombinant IL6 and 25 ng ml^{-1} human recombinant IL6 soluble receptor; or (ii) 3,000 U ml^{-1} human recombinant LIF. These two alternatives can be used to substitute the IL6RIL6 chimera and are equally able to support suspension culture provided that the same culture protocols are used.

Splitting medium The splitting medium consists of 1 mg ml^{-1} collagenase type IV in DMEM. Filter the splitting medium with a 0.22-μM filter and store at 4 °C until use. The splitting medium can be used for up to 3 d after preparation. **▲ CRITICAL** The concentration of collagenase is suitable for enzyme activity of 280–350 U ml^{-1}. Other activity measurements require modification of the concentration. **▲ CRITICAL** If small brown oil drops are floating on the medium, the collagenase cannot be used.

Trypsin-based splitting medium The splitting medium consists of trypsin-EDTA. **▲ CRITICAL** When trypsin is used for splitting, 10 μM of ROCK inhibitor should be added to the suspension culture medium from 1 h before splitting and until the following morning (i.e., overnight). The ROCK inhibitor stock solution should be maintained at −20 °C at a concentration of 10 mM and is diluted to 10 μM when added to the culture medium.

Freezing medium The freezing medium consists of 60% (vol/vol) DMEM; 20% (vol/vol) DMSO; and 40% FBS. The FBS can be replaced with 40% (vol/vol) serum replacement. Filter the freezing medium using a 0.22-μM filter and store at 4 °C until use. **▲ CRITICAL** The freezing medium can be used for up to 24 h after preparation.

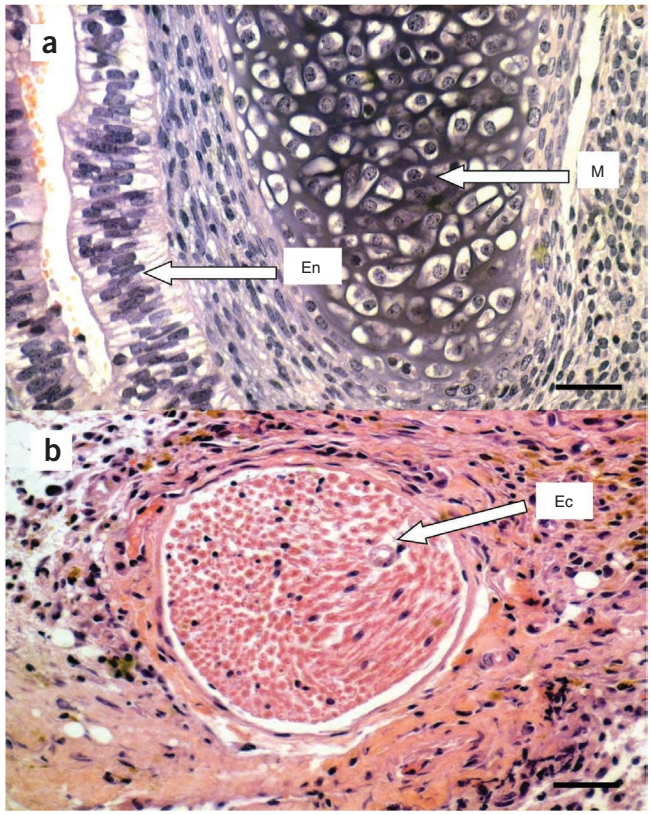

Figure 2 | Teratoma formation. (**a**,**b**) Histological sections of a teratoma, formed by I4 hESC line cultured for nine passages in suspension before injection into severe combined immunodeficiency mice, which include tissues representative of the three embryonic germ layers: (**a**) cartilage tissue (M) and columnar epithelium (En), and (**b**) myelinated nerve (Ec). Scale bar in **a**, 90 μm. Staining with H&E. Scale bar in **b**, 70 μm. Permission was obtained from the Technion institutional regulatory board for the teratoma assay.

BOX 1 | CHANGING THE MEDIUM OF hPSCs CULTURED IN SUSPENSION ● TIMING 15 MIN (UP TO FOUR PLATES)

1. Collect the cells and place them in a conical tube.
2. Centrifuge for 3 min at 80*g* at a recommended temperature of 4 °C.
3. Aspirate the medium from the tube without harming the cell pellet, add 5 ml of fresh suspension medium and then incubate at 37 °C throughout the cell culture.

▲ CRITICAL STEP The suspension medium should be preheated to room temperature (20 °C).

PROCEDURE

Creating the hPSC suspension culture ● TIMING 2 h

1| Aspirate the medium from the dishes containing the cells that are to be transferred from adherent to suspension culture. Add splitting medium to cover the wells (0.5 ml for 10 cm^2) and incubate at 37 °C for 20–40 min.

▲ CRITICAL STEP The cells should be split using the same method used routinely for splitting. If collagenase is not being used, the protocol should be changed accordingly. Most colonies will float after the incubation.

▲ CRITICAL STEP For initiating the suspension culture, we recommend using 5×10^6 to 5×10^7 undifferentiated hPSCs from adherent culture for one 58-mm Petri dish (see Step 5). The optimal starting concentration is 10^6 cells per ml. If it is higher, then 1 d after creating the cultures, the cells should be split among several plates until a concentration of 10^6 cells per ml is obtained.

? TROUBLESHOOTING

2| Add 1 ml of the suspension culture medium without removing the splitting medium and gently collect the cells into a conical tube.

3| Centrifuge for 3 min at 80*g* at the recommended temperature of 4 °C.

4| Aspirate the medium from the tube without harming the cell pellet. Resuspend the cells in 700 µl of fresh suspension medium and dissociate the cells by pipetting up and down five to ten times with a 200-µl tip.

▲ CRITICAL STEP Overdissociation of the cells can reduce the success rates of transferring cells to suspension culture.

? TROUBLESHOOTING

5| Transfer the cells to a 58-mm Petri dish, add 5 ml of fresh suspension medium and incubate at 37 °C throughout the cell culture. The medium should be changed on a daily basis (as described in **Box 1**).

▲ CRITICAL STEP Use a Petri dish or any other non-culture-treated dish, because if the cells attach to the plastic, then the experiment will fail.

Splitting hPSCs in suspension

6| Cells can be split using the mechanical (option A) or enzymatic (option B) method.

(A) Mechanical splitting of hPSCs in suspension ● TIMING 20 min

(i) Collect the cells cultured in suspension and place in a conical tube, and then repeat Steps 3–5.

▲ CRITICAL STEP The anticipated splitting rates should be between one plate into two and one plate into four, with the latter being the typical rate. The cells should be split every 5–7 d.

? TROUBLESHOOTING

(B) Splitting hPSCs in suspension with trypsin ● TIMING 1.5 h

(i) Add ROCK inhibitor (ROCK inhibitor stock solution of 10 mM, diluted in the culture medium to a final concentration of 10 µM) to the plates for splitting and incubate at 37 °C for 1 h.

(ii) Collect the cells and place them in a conical tube.

(iii) Centrifuge for 3 min at 80*g* at 4 °C.

(iv) Aspirate the medium from the tube without harming the cell pellet, add 1 ml of trypsin and incubate at 37 °C for 7–10 min. Monitoring the cells during the trypsin incubation will help determine the incubation time; when most cells are detached into single cells, the trypsin incubation can be terminated.

▲ CRITICAL STEP Do not exceed the incubation time, as excessive trypsinization can damage the cells.

? TROUBLESHOOTING

(v) Centrifuge for 3 min at 80*g* at 4 °C.

BOX 2 | FREEZING AND THAWING hPSCs IN SUSPENSION

Freezing hPSCs in suspension ● **TIMING 30 min**

1. To freeze the cells 1 d after splitting, collect the cells cultured in suspension and place them in a conical tube.

? TROUBLESHOOTING

2. Centrifuge for 3 min at 80*g* at a recommended temperature of 4 °C.
3. Aspirate the medium from the tube without harming the cell pellet.
4. Resuspend the cells in the suspension culture medium. The final volume of medium in each vial to be frozen in step 6 should be 0.5 ml, with a 1:1 ratio of suspension culture medium and freezing medium (added in step 5). The volume of suspension medium to be added to the tube in this step should be the number of expected vials multiplied by 0.25 ml.
5. Add an equivalent volume of freezing medium drop by drop and mix gently.

▲ **CRITICAL STEP** Adding the freezing medium drop by drop is crucial for cell recovery.

6. Pour 0.5 ml into a 1-ml cryogenic vial. A freezing ratio of 2×10^6 cells per vial is recommended.

▲ **CRITICAL STEP** It is important to not leave the cells at room temperature after adding the freezing solution.

7. Freeze overnight at −80 °C in freezing boxes.
8. Transfer to liquid nitrogen on the following day.

! CAUTION Liquid nitrogen is a hazardous material. Use suitable gloves to protect your hands and a suitable mask to protect your eyes.

Thawing hPSCs in suspension ● **TIMING 20 min**

1. Remove the vial from the liquid nitrogen.

! CAUTION Liquid nitrogen is a hazardous material. Use suitable gloves to protect your hands and a suitable mask to protect your eyes.

2. Gently swirl the vial in a 37 °C water bath.
3. When a small pellet of frozen cells remains, wash the vial in 70% (vol/vol) ethanol.

▲ **CRITICAL STEP** Do not leave thawed cells at room temperature while they are exposed to the freezing solution; move quickly to the following step.

4. Pipette the contents of the vial up and down once to mix.

▲ **CRITICAL STEP** Do not dissociate the cells; the aim of the mixing is to ensure collection of all the cells.

5. Place the contents of the vial in a conical tube and add 2 ml of suspension culture medium drop by drop.

▲ **CRITICAL STEP** Adding the suspension medium drop by drop is crucial for cell recovery.

6. Centrifuge for 3 min at 80*g* at a recommended temperature of 4 °C.
7. Remove the supernatant, resuspend the cells in 5 ml of suspension medium and transfer to a 58-mm Petri dish. Incubate at 37 °C throughout the cell culture.

(vi) Aspirate the trypsin without harming the cell pellet. Resuspend the cells in 700 µl of fresh suspension medium supplemented with 10 µM ROCK inhibitor and dissociate the cells further by pipetting up and down three to five times with a 200-µl tip.

(vii) Transfer the cells to a new 58-mm Petri dish, add 5 ml of fresh suspension medium supplemented with 10 µM ROCK inhibitor and incubate at 37 °C throughout the cell culture.

▲ **CRITICAL STEP** The ROCK inhibitor should be removed on the following day, by changing the medium to a fresh one (see **Box 1**).

7| Cells can be frozen 1 d after splitting (see **Box 2**), or you can proceed directly to Step 8 and transfer the cells to dynamic culture.

8| Cells can be transferred to dynamic suspension culture using an Erlenmeyer flask (option A) or spinner flasks (option B):

(A) Culturing hPSCs in a dynamic system using Erlenmeyer flasks ● **TIMING 10 min**

(i) Transfer cell spheres cultured in 1–3 Petri dishes (10^6 to 6×10^6 cells) for at least one passage in static suspension culture (from Step 6 or **Box 2**) to a 125-ml Erlenmeyer flask.

(ii) Add suspension culture medium to a final volume of 25 ml.

(iii) Shake continuously at 60–90 r.p.m. using an orbital shaker and place in the incubator at 37 °C. The medium should be changed three times a week. The recommended starting or post-splitting concentration should be 10^6 cells per ml for efficient yield without daily medium change, although a lower concentration of up to 3×10^4 cells per ml can also be used.

? TROUBLESHOOTING

(B) Culturing hPSCs in a dynamic system using spinners ● **TIMING 10 min**

(i) Transfer cell spheres cultured in 1–5 Petri dishes (10^6 to 6×10^7 cells) for at least one passage in suspension (from Step 6 or **Box 2**) to a 100-ml glass ball spinner flask in 50 ml of suspension culture medium.

BOX 3 | CHARACTERIZATION OF SUSPENSION CULTURE PLURIPOTENCY

Cells cultured in suspension should be examined for pluripotency after every 25 passages of continuous culture. The standard assays for testing hPSC pluripotency are EB and teratoma formation.

REAGENT SETUP

Differentiation medium: 80% (vol/vol) DMEM/F12; 10% (vol/vol) KO-serum replacement; 10% (vol/vol) FBS; 1% (vol/vol) nonessential amino acid; 2 mM L-glutamine; and 0.1 mM β-mercaptoethanol. If it is maintained at 4–8 °C, the medium can be used up to 5 d after preparation.

FBS medium (20%): 80% (vol/vol) DMEM; 20% (vol/vol) FBS; 1% (vol/vol) nonessential amino acid; 2 mM L-glutamine; and 0.1 mM β-mercaptoethanol. If it is maintained at 4–8 °C, the medium can be used up to 5 d after preparation.

PROCEDURE

EB formation ● TIMING 21 d

1. Collect the cells cultured in suspension and place them in a conical tube. Use cells cultured either in static culture (Step 5 of PROCEDURE) or dynamic culture (Step 8 of PROCEDURE) at a recommended concentration of 5 × 10^5 cells per ml.
2. Centrifuge for 3 min at 80*g* at a recommended temperature of 4 °C.
3. Aspirate the medium from the tube without harming the cell pellet. Resuspend the cells in 700 µl of fresh differentiation medium and dissociate the cells by pipetting up and down, using a 200-µl tip five to ten times.

▲ **CRITICAL STEP** Overdissociation of the cells may lower the success rates of EB formation.

4. Transfer the cells to a new 58-mm Petri dish, add 5 ml of fresh differentiation medium and incubate at 37 °C throughout the cell culture. Recommended seeding concentration is 2 × 10^6 cells per ml.
5. After 1 week, replace the medium with 20% (vol/vol) FBS medium. EBs should emerge, followed by the formation of notable cysts 10–14 d later. We recommend culturing EBs for at least 21 d to obtain markers of the three embryonic germ layers.

Teratoma formation ● TIMING 10 weeks

1. Collect a total of 5 × 10^6 to 10^7 cells from either static culture (Step 5 of PROCEDURE) or dynamic culture (Step 8 of PROCEDURE) dishes and place them in a conical tube.
2. Centrifuge for 3 min at 80*g* at a recommended temperature of 4 °C.
3. Aspirate the medium from the tube without harming the cell pellet. Resuspend the cells in 100 µl of fresh suspension medium.
4. Inject the cells into the hind limb muscles of 4-week-old severe combined immunodeficiency (SCID)/beige male mice using an insulin syringe and a needle of 18–21 gauge. Cells do not need to be dissociated before injection.
5. At 10 weeks after the injection, resultant teratomas should be harvested and prepared for histological analysis as follows: resultant teratomas should be fixed in 10% neutral-buffered formalin, dehydrated in graduated ethanol (70–100%) and embedded in paraffin. For histological examination, 5-µm sections should be deparaffinized and stained with H&E[32]. This protocol yields teratomas in 80% of the mice. We therefore recommend using two mice for each examined culture.

(ii) Shake continuously at 60–90 r.p.m. using a magnetic plate and place in the incubator at 37 °C. The medium should be changed twice a week. The recommended post-splitting cell concentration is 1 × 10^6 cells per ml. A lower concentration of up to 3 × 10^4 cells per ml can also be used. Higher concentrations are possible provided that the medium is changed daily.

? TROUBLESHOOTING

9| Cells cultured in suspension can be characterized using standard immunostaining procedures[7]**.** Pluripotency can be tested by EB or teratoma formation (as described in **Box 3**), and karyotype stability can be assessed as previously described[2].

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solution
1	Poor cell recovery of <60%	Low concentration of surviving cells after splitting	Use more than 10^7 cells in a 58-mm Petri dish, but 1 d after establishment of the experiment, divide the plate to achieve a concentration of 10^6 cells per ml. Alternatively, reduce incubation time with collagenase to 20 min
4	Poor cell recovery of <60%	Cell death due to clump breakage	Use a 1,000-µl Gilson tip and pipette up and down three to five times. At least 70% of cells should recover. Adapted cultures should have 16–20% of apoptotic cells 1 d after splitting

(continued)

TABLE 1 | Troubleshooting table (continued).

Step	Problem	Possible reason	Solution
6A(i)	Too high or too low splitting rates	If too high, indicative of karyotype problem If too low (1/1), could be attributable to cell adaptation or culture medium	Send a sample of the cells for karyotype examination; check the medium preparation to ensure no mistakes; try to dissociate the cells gently on next passage
6B(iv)	Poor cell recovery of <60%	Long incubation with trypsin	Reduce the incubation time to <5 min. Adapted cultures should have 20–30% of apoptotic cells at 1 d after splitting
8A(iii), 8B(ii)	Poor cell recovery	Shear stress	Reduce the shaking speed (no less than 50 r.p.m.). The cells in adapted cultures should proliferate to a concentration of at least 3×10^6 cells per ml
Box 2, step 1	Poor cell recovery of hiPSCs after freezing	Some iPSC lines have poor recovery rates after freezing (including iF4, KTN7 and KTR13)	Use ROCK inhibitor while freezing and thawing, using the same method as for trypsinization. Adapted cultures should have at least a 65% recovery rate

● TIMING

Steps 1–5, Creating the hPSCs suspension culture: 2 h
Step 6A, Mechanical splitting of hPSCs in suspension: 20 min
Step 6B, Splitting hPSCs in suspension with trypsin: 1.5 h
Step 7: Variable
Step 8A, Culturing hPSCs in a dynamic system using Erlenmeyer flasks: 10 min
Step 8B, Culturing hPSCs in a dynamic system using spinners: 10 min
Box 1, Changing the medium of hPSCs cultured in suspension: 15 min (up to four plates)
Box 2, Freezing hPSCs in suspension: 30 min
Box 2, Thawing hPSCs in suspension: 20 min
Box 3, EB formation: 21 d
Box 3, Teratoma formation: 10 weeks

ANTICIPATED RESULTS

Twenty-four hours after being placed in the suspension culture, the hPSCs will create spheroid clumps or disc-like structures. Examples of sphere morphology are illustrated in **Figure 1**. Histological examination of these spheres reveals a homogenous population of small cells with large nuclei. **Figure 1a** describes the morphology of undifferentiated spheres, whereas **Figure 1c** shows a differentiated sphere. These spheroids should be split mechanically every 5–7 d while maintaining their morphology, thereby allowing expansion of the suspension cultures. Although the choice of splitting day is flexible, one should take into consideration that a delay of more than 7 d would result in cell concentration higher than recommended and thus the need for more frequent medium change, whereas an early split would decrease cell yield. Splitting cells with trypsin is optional and will result in more homogeneous sphere size. However, it must be considered that after 48 h, some of the formed clump will attach to each other. Thus, the resulting spheres do not initiate from a single cell. While splitting cells with trypsin, the intervals between splits increase to 7–10 d. When the spheres are recultured with MEFs, all of the spheroids should be adhered to the MEFs and, after 24–48 h, should show typical morphology of an hPSC colony (see **Fig. 1b**). If cells are frozen, as described in **Box 2**, after thawing, at least 50% of thawed cells should recover, thereby resulting in detectable spheroids 24 h after thawing.

When cells are transferred to dynamic culture, using a typical 25-ml Erlenmeyer flask, the total number of spheroids increases from $1.33 \times 10^4 \pm 461$ on the seeding day to $3.5 \times 10^5 \pm 2.8 \times 10^4$ after 10–11 d, i.e., a 25-fold increase[7]. When cells are transferred to dynamic culture using spinner flasks, the total number of cells per ml increases from $1.06 \times 10^5 \pm 2.05 \times 10^3$ on the seeding day to $1.89 \times 10^6 \pm 3.94 \times 10^4$ after 6 d, i.e., a 17.7-fold increase (M.A., I.L., Y.M., K.S., M.P. and J.I.-E., unpublished data).

Similar to hPSCs cultured as adherent cells, cells cultured in suspension should be strongly positive for stage-specific antigen 4, tumor recognizing antigen (TRA)-1-60, TRA-1-81 and Oct 4. The staining procedure[7] does not require any special modification. In addition, the cells should express high levels of *POU5F1* (POU class 5 homeobox 1, also known as *OCT4*),

NANOGP3 (Nanog homeobox pseudogene 3; also known as *NANOG*), *SOX2* (SRY-box 2), *ZFP42* (zinc finger protein 42 homolog (mouse); also known as *REX1*), and *FGF4* (fibroblast growth factor 4), similar to those in hPSCs cultured with MEFs. The hPSC pluripotency after culture in suspension can be examined using EB and teratoma formation. The protocols for creating EBs and teratoma are similar to those used for hPSCs cultured as adherent cells and are detailed in **Box 3**. For teratoma formation, the cells should be injected as spheroids without passaging, and for EB formation, the cells should be split as described before transferring the serum-containing medium[7]. Examples of histological sections from teratomas formed by cells cultured in suspension are provided in **Figure 2**. Lastly, the karyotype of the hPSCs cultured in suspension should remain stable. We recommend testing karyotype stability with G-banding[2] every 20 passages in suspension; we suggest analyzing at least 20 metaphases. Our results show karyotype stability after a year of continuous culture in suspension[7].

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1. Amit, M. *et al.* Clonally derived human embryonic stem cell lines maintain pluripotency and proliferative potential for prolonged periods of culture. *Dev. Biol.* **227**, 271–278 (2000).
2. Amit, M. *et al.* Human feeder layers for human embryonic stem cells. *Biol. Reprod.* **68**, 2150–2156 (2003).
3. Doetschman, T.C., Eistetter, H., Katz, M., Schmidt, W. & Kemler, R. The *in vitro* development of blastocyst-derived embryonic stem cell lines: formation of visceral yolk sac, blood islands and myocardium. *J. Embryol. Exp. Morphol.* **87**, 27–45 (1985).
4. Itskovitz-Eldor, J. *et al.* Differentiation of human embryonic stem cells into embryoid bodies comprising the three embryonic germ layers. *Mol. Med.* **6**, 88–95 (2000).
5. Takahashi, K. *et al.* Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* **131**, 861–872 (2007).
6. Yu, J. *et al.* Induced pluripotent stem cell lines derived from human somatic cells. *Science* **318**, 1917–1920 (2007).
7. Amit, M. *et al.* Suspension culture of undifferentiated human embryonic and induced pluripotent stem cells. *Stem. Cell. Rev. Rep.* **6**, 248–259 (2010).
8. Amit, M. & Itskovitz-Eldor, J. Derivation and spontaneous differentiation of human embryonic stem cells. *J. Anat.* **200**, 225–232 (2002).
9. Thomson, J.A. *et al.* Embryonic stem cell lines derived from human blastocysts. *Science* **282**, 1145–1147 (1998).
10. Park, I.H. *et al.* Reprogramming of human somatic cells to pluripotency with defined factors. *Nature* **451**, 141–146 (2008).
11. Germanguz, Y. *et al.* Molecular characterization and functional properties of cardiomyocytes derived from human inducible pluripotent stem cells. *J. Cell Mol. Med.* **15**, 38–51 (2009).
12. Novak, A. *et al.* Enhanced reprogramming and cardiac differentiation of human keratinocytes derived from plucked hair follicle, using single excisable lentivirus. *Cell. Reprogram* **12**, 665–678 (2010).
13. Smith, A.G. *et al.* Inhibition of pluripotential embryonic stem cell differentiation by purified polypeptides. *Nature* **336**, 688–690 (1988).
14. Williams, R.L. *et al.* Myeloid leukemia inhibitory factor maintains the developmental potential of embryonic stem cells. *Nature* **336**, 684–687 (1988).
15. Conover, J.C. *et al.* Ciliary neurotrophic factor maintains the pluripotentiality of embryonic stem cells. *Development* **119**, 559–565 (1993).
16. Rose, T.M. *et al.* Oncostatin M (OSM) inhibits the differentiation of pluripotent embryonic stem cells *in vitro*. *Cytokine* **6**, 48–54 (1994).
17. Niwa, H. *et al.* Self-renewal of pluripotent embryonic stem cells is mediated via activation of STAT3. *Genes Dev.* **12**, 2048–2060 (1998).
18. Daheron, L. *et al.* LIF/STAT3 signaling fails to maintain self-renewal of human embryonic stem cells. *Stem Cells* **22**, 770–778 (2004).
19. Humphrey, R.K. *et al.* Maintenance of pluripotency in human embryonic stem cells is STAT3 independent. *Stem Cells* **22**, 522–530 (2004).
20. Sato, N. *et al.* Maintenance of pluripotency in human and mouse embryonic stem cells through activation of Wnt signaling by a pharmacological GSK-3-specific inhibitor. *Nat. Med.* **10**, 55–63 (2004).
21. Phillips, B.W. *et al.* Attachment and growth of human embryonic stem cells on microcarriers. *J. Biotechnol.* **138**, 24–32 (2008).
22. Oh, S.K. *et al.* Long-term microcarrier suspension cultures of human embryonic stem cells. *Stem Cell Res.* **2**, 219–230 (2009).
23. Storm, M.P., Orchard, C.B., Bone, H.K., Chaudhuri, J.B. & Welham, M.J. Three-dimensional culture systems for the expansion of pluripotent embryonic stem cells. *Biotechnol. Bioeng.* **107**, 683–695 (2010).
24. Kehoe, D.E., Jing, D., Lock, L.T. & Tzanakakis, E.S. Scalable stirred-suspension bioreactor culture of human pluripotent stem cells. *Tissue Eng. Part A* **16**, 405–421 (2010).
25. Chen, A.K., Chen, X., Choo, A.B., Reuveny, S. & Oh, S.K Expansion of human embryonic stem cells on cellulose microcarriers. *Curr. Protoc. Stem Cell Biol.* **14**, 1C.11.1–1C.11.14 (2010).
26. Lecina, M., Ting, S., Choo, A., Reuveny, S. & Oh, S.K. Scalable platform for hESC differentiation to cardiomyocytes in suspended microcarrier cultures. *Tissue Eng. Part C Methods* **16**, 1609–1619 (2010).
27. Olmer, R. *et al.* Long term expansion of undifferentiated human iPS and ES cells in suspension culture using a defined medium. *Stem Cell Res.* **5**, 51–64 (2010).
28. Singh, H., Mok, P., Balakrishnan, T., Rahmat, S.N. & Zweigerdt, R. Up-scaling single cell-inoculated suspension culture of human embryonic stem cells. *Stem Cell Res.* **4**, 165–179 (2010).
29. Steiner, D. *et al.* Derivation, propagation and controlled differentiation of human embryonic stem cells in suspension. *Nat. Biotech.* **28**, 361–364 (2010).
30. Couture, L.A. Scalable pluripotent stem cell culture. *Nat. Biotech.* **28**, 562–563 (2010).
31. Chebath, J. *et al.* Interleukin-6 receptor-interleukin-6 fusion proteins with enhanced interleukin-6 type pleiotropic activities. *Eur. Cytokine. Netw.* **8**, 359–365 (1997).
32. Silva, G.A., Moreira, A. & Alves, J.B. Histological processing of teeth and periodontal tissues for light microscopy analysis. *Methods Mol. Biol.* **689**, 19–36 (2011).