Maintenance of hPSCs under Xeno-Free and Chemically Defined Culture Conditions

Jung Jin Lim1,*, Hyung Joon Kim1,*, Byung-ho Rhie1, Man Ryul Lee2, Myeong Jun Choi3, Seok-Ho Hong4, Kye-Seong Kim1,5

1Department of Biomedical Science, Graduate School of Biomedical Science and Engineering, Hanyang University, Seoul, Korea
2Soochunhyang Institute of Medi-bioscience, Soochunhyang University, Cheonan, Korea
31st Research Center, Acceso Biopharma Co., Ltd., Yongin, Korea
4Department of Internal Medicine, School of Medicine, Kangwon National University, Chuncheon, Korea
5College of Medicine, Hanyang University, Seoul, Korea

Previously, the majority of human embryonic stem cells and human induced pluripotent stem cells have been derived on feeder layers and chemically undefined medium. Those media components related to feeder cells, or animal products, often greatly affect the consistency of the cell culture. There are clear advantages of a defined, xeno-free, and feeder-free culture system for human pluripotent stem cells (hPSCs) cultures, since consistency in the formulations prevents lot-to-lot variability. Eliminating all non-human components reduces health risks for downstream applications, and those environments reduce potential immunological reactions from stem cells. Therefore, development of feeder-free hPSCs culture systems has been an important focus of hPSCs research. Recently, researchers have established a variety of culture systems in a defined combination, xeno-free matrix and medium that supports the growth and differentiation of hPSCs. Here we described detailed hPSCs culture methods under feeder-free and chemically defined conditions using vitronectin and TeSR-E8 medium including supplement bioactive lysophospholipid for promoting hPSCs proliferation and maintaining stemness.

Keywords: Embryonic stem cells, Induced pluripotent stem cells, Feeder-free, Chemically defined conditions, Extracellular matrices

Introduction

The first derivation in 1998 of human embryonic stem cell (hESC) line was achieved by culturing inner cell mass on mouse embryonic fibroblasts (mEF) in the medium containing chemically undefined components such as fetal bovine serum (FBS) (1). Human induced pluripotent stem cells (hiPSCs) were also established in the same conditions as hESCs (2). These undefined culture conditions with animal-derived components hamper analysis of molecular mechanisms that control pluripotency, self-renewal and differentiation as well as the possible clinical application of human pluripotent stem cells (hPSCs) (3). Thus, the hPSC field gradually shifted to the development of chemically defined culture system...
by removal of feeder layers, serum and growth factors and replacement of Matrigel to human recombinant matrices (See Table 1) (1, 4-9). Despite these innovative advances, hPSC cultures still exhibit phenotypic and functional heterogeneity, and remain to be optimized for assurance of functionality and genetic stability over long-term maintenance (10, 11). Here, we describe the major improvements of hPSC culture system and chemically defined culture protocol for long-term maintenance. In addition, we briefly introduce the beneficial effect of bioactive lysophospholipid in maintaining hPSCs.

**From feeder to feeder-free system for hPSCs culture**

The first establishment of hESCs has described by using with inactivated mEFs as feeder cells and FBS-containing culture medium (1). Feeder-cells such as mEFs support the self-renewal of hESCs by the secretion of essential growth factors, cytokines and extracellular matrices (ECM) such as transforming growth factor β (TGFβ), activin A, laminin-511 and vitronectin (12). Those feeder-dependent systems support the stem cells by the mimetic microenvironment. Like other hESCs, hiPSCs depend on the support of feeder cells such as mEFs (Fig. 1A) or human fibroblast. There are certain drawbacks to the feeder-dependent culture systems, most notably the introduction of animal-derived cells to the human stem cell environment. Next, FBS (Fig. 1Aa) was replaced with knockout serum replacement (KSR) and basic fibroblast growth factor 2 (FGF2) (Fig. 1Ab). mEF was also changed with human feeder cells (13). Although the use of human feeder cells circumvented the use of animal-derived feeder cells, this culture system is fully dependent on the condition of human feeder cells and the function of the human feeder cells in the hPSCs co-culture system was still not fully understood.

The first major step in optimizing the hPSCs culture environment was to culture the stem cells independently from the feeder cells. To achieve reliable and safe production of hPSCs, it is desirable to use reagents that are defined, qualified, and preferably derived from a non-animal source. Furthermore, it should need an appropriate matrix to achieve the feeder free system. Therefore, development of feeder-free human PSCs culture systems has been an important focus of stem cell research. In 2006, researchers in WiCell reported that feeder-independent hESC culture that includes protein components solely derived from recombinant sources or purified from human material. They described the derivation of new hESC lines in these defined culture conditions. This first defined medium significantly improved hESC culture, and was commercially released as TeSR1, becoming the most widely-published feeder-free medium, used in over 1,100 peer-reviewed publications. This media is composed of a DMEM/F12 base supplemented with human serum albumin, vitamins, antioxidants, trace minerals, specific lipids and cloned growth factors (7).

### Table 1. Major improvements of hPSC culture protocols

| hPSC types | Culture condition | Point of improvement | Reference |
|------------|-------------------|----------------------|-----------|
| hESCs      | Feeder: Inactivated mEFs Medium: DMEM12 + 10%FBS | First establishment of human pluripotent stem cells | Thomson et al. 1998 (1) |
| hESCs      | Coating: Laminin (vs Matrigel) Medium: MEF-CM | Feeder-free culture system | Xu et al. 2001 (4) |
| hESCs      | Feeder: Inactivated human fetal and adult fibroblast Medium: DMEM12 + 20%FCS | Long-term propagation on human feeder cells | Richards et al. 2002 (5) |
| hESCs      | Feeder: Inactivated human fetal and adult fibroblast Medium: KO-DMEM12 + 20%HS | First establishment and propagation of hESC line in xeno-free conditions | Richards et al. 2003 (6) |
| hESCs      | Coating: Human matrix (Laminin, Vitronectin, Collagen, Fibronectin) Medium: TeSR1 | Derivation and propagation in defined culture condition | Ludwig et al. 2006 (7) |
| hiPSCs     | Coating: Vitronectin | Derivation and propagation in chemically-defined condition | Chen et al. 2011 (8) |
| hiPSCs     | Medium: E8 | | |
| hiPSCs     | Coating: Laminin, Vitronectin Medium: E8 + Kenpaullone (0.75 μM) + ID-8 (0.5 μM) + Tacrolimus (5–200 pM) | Derivation and propagation in chemically-defined and growth factor-free condition | Yasuda et al. 2018 (9) |

FCS: Fetal calf serum, HS: Human serum, KO: KNOCKOUT.
TeSR2 is a next version of mTeSR1 derived from TeSR1 media family which provides improved culture conditions for feeder-free maintenance of hPSCs while enabling a more defined and xeno-free culture environment for basic research, high-throughput gene profiling studies and preclinical applications. TeSR2 combines the advantages of a feeder-free culture system with the added value of being free of xenogenic components. Complete TeSR2 contains recombinant human basic FGF and recombinant human TGF-β. Addition of further growth factors is not required (14). More recently, Thomson and coworkers developed chemically defined TeSR-E8 medium (E8 medium), which is a derivative of mTeSR1 containing eight components, that lacks both serum albumin and β-mercaptoethanol. This E8 medium, combined with EDTA passaging, may be suitable for culturing a broad range of hPSC lines, particularly to improve episomal vector-based reprogramming efficiencies as well as experimental consistency (8). Currently available commercial hPSC culture media and their components are listed in Table 2.

Matrices for feeder-free system

The ECM is synthesized, secreted, and structured by embryonic cells from initial steps of developmental process. Composition, structure and function of ECM has been identified in detail recently and has revealed that the microenvironment including ECM is critically important for survival, cell growth, morphogenesis and differentiation (15). The extracellular matrix of many (16, 17), if not all, composed of certain unique macromolecules, including, laminin (18, 19), a heparan sulfate proteoglycan (20), type IV collagen (16) and entactin (21). Among those, Laminin is the first extracellular matrix protein expressed in two- to four-cell stage mouse embryos and is the main component of basement membranes of all basal laminae in mammals (22, 23). Interacting with integrin heterodimers such as α1β1, α2β1, α3β1, α6β1, and α6β4 on the cell surface, laminin induces signals for promoting cell adhesion, growth, and migration. Previous reports indicated that the laminin receptor was found to be highly expressed on ESCs and embryonal carcinoma cells (24, 25).

1. Matrigel, which is a commercially available protein mixture extracted from a whole mouse sarcoma tissue, has been one of the most widely used extracellular components for feeder-and serum-free culture of hPSCs (Fig. 1 Ba, 1Ca). It contains mostly types I and IV collagens, laminin, entactin, heparan sulfate proteoglycan, matrix metalloproteinases, undefined growth factors, and chemical compounds (26-29). Although it is widely used for research applications, it is important to note that Matrigel, which is a semi-chemically defined, xenogeneic substrate, cannot be used for generation of clinical-grade hPSCs. Previous studies have shown that Matrigel contains various growth factors including TGF-β, epidermal growth factor (EGF), insulin-like growth factor 1, bFGF, and platelet-derived growth factor (PDGF). Despite its availability and ease of use, Matrigel is not ideal for potential
Vitronectin is an adhesive glycoprotein (75 kDa) of the hemopexin family which is abundantly found in humans such as serum, ECM, and bones (30, 31). It is composed of 459 amino acid residues of human VTN gene and classed in three domains; which are B domain (N-terminal) and two hemopexin domains; which are B domain (N-terminal) and two hemopexin domains (central and C-terminal) (32).

Vitronectin recognizes the cells expressing its receptors: in-homology domains (central and C-terminal) (32). Vitronectin also contains an RGD (5, 6, 9) sequence, which is a binding site for membrane-bound integrins, e.g., vitronectin receptor, which serves to anchor cells to the ECM. In addition, vitronectin binds to the integrin αvβ5. Especially, the integrin αvβ5 of vitronectin receptor can facilitate cells not only in binding to the matrix, but also in maintaining human self-renewal and pluripotency, even when the cells are blocked from binding to integrin β1 (35). rhVitronectin was a defined functional alternative to Matrigel or laminin for supporting sustained self-renewal and pluripotency of hPSC. When used with Essential 8 medium, rhVitronectin has demonstrated the ability to maintain pluripotency and the normal growth characteristics in multiple human PSCs (Fig. 1Bb, 1Cb). rhVitronectin also has shown to support hiPSCs growth for a long-term cultivation without any karyotypic abnormalities and to maintain the pluripotency (36).

2. Vitronectin is an adhesive glycoprotein (75 kDa) of the hemopexin family which is abundantly found in humans such as serum, ECM, and bones (30, 31). It is composed of 459 amino acid residues of human VTN gene and classed in three domains; which are B domain (N-terminal) and two hemopexin homology domains (central and C-terminal) (32). Vitronectin recognizes the cells expressing its receptors: integrins αvβ1, αvβ3, αvβ5 or αIIbβ3 (33). rhVitronectin (recombinant human vitronectin) is a recombinant, full or partial length human single-chain and monomeric protein. Recently, several studies of functionality of integrins on hESCs have revealed that integrin families are important for the attachment of hESCs in in vitro condition (34).

Clinical application of hPSCs because it is animal-derived and xenogenic pathogens can be transmitted through culture even though no feeder cells are present. In addition, it also varies extensively from batch to batch. Clinical application of hPSCs because it is animal-derived and xenogenic pathogens can be transmitted through culture even though no feeder cells are present. In addition, it also varies extensively from batch to batch.

### Table 2. Commercial media for hPSC culture

| Medium                  | Formula                                                                 | Extracellular matrix                                                                 | XF/CD          | Brand        |
|-------------------------|------------------------------------------------------------------------|--------------------------------------------------------------------------------------|----------------|--------------|
| mTeSR<sup>TM</sup>1     | DMEM/F12, BSA, bFGF, TGF β, Insulin, Transferrin, Cholesterol, Lipids, Pipericolic acid, GABA, β-mercaptoethanol | Matrigel®, Vitronectin                                                              | NA             | STEMCELL     |
| TeSR<sup>TM</sup>2       | DMEM/F12, HSA, bFGF, TGF β, Insulin, Transferrin, Cholesterol, Lipids, Pipericolic acid, GABA, β-mercaptoethanol | Matrigel®, Vitronectin                                                              | XF             | STEMCELL     |
| Essential 8<sup>TM</sup> | DMEM/F12 bFGF, TGF β, Insulin, Transferrin, Selenium, Ascorbic acid     | Matrigel®, Vitronectin                                                              | CD             | Thermo Fisher Scientific |
| TeSR<sup>TM</sup>-EB     | DMEM/F12, bFGF, TGF β, Insulin, Transferrin, Selenium, Ascorbic acid    | Matrigel®, Vitronectin                                                              | XF             | STEMCELL     |
| StemPro<sup>®</sup>      | DMEM/F12, BSA, bFGF, TGF β, Activin, Transferrin, LR3-HGF1, HRG1β       | Geltrex®                                                                            | NA             | Thermo Fisher Scientific |
| PluriSTEM<sup>TM</sup>   | DMEM/F12, HAS, Activin A, TGF β1, bFGF, Lipids, Insulin, Transferrin, Selenium | Not defined                                                                          | XF             | Millipore    |
| StemMACS<sup>TM</sup>    | DMEM/F12, NaHCO3, L-Ascorbic Acid, Selenium, Growth factors              | Matrigel®, Geltrex®, Laminin-511, iMatrix-511, Vitronectin                           | XF             | Miltentiy    |
| iPS-Brew XF              | Transferrin, Insulin, bFGF                                              | Lamin-521, iMatrix-511, Vitronectin                                               | CD             | Biotec       |
| StemFit<sup>®</sup>      | DMEM/F12, NaHCO3, L-Ascorbic Acid, Selenium, Trace minerals               | iMatrix-511, Matrigel®, Geltrex®                                                 | XF             | Ajinomoto    |
| Basic02/03               | Transferrin, Insulin, bFGF, 21 Amino acids, five trace minerals and growth factors (including bFGF) | Laminin-521                                                                         | CD             | Company      |
| StemFlex<sup>TM</sup>    | Undisclosed                                                             | Geltrex<sup>TM</sup> (for clump cell), rhLaminin-521 (for single cell)            | NA             | Thermo Fisher Scientific |
| Cellartis<sup>®</sup>    | Undisclosed                                                             | TaKara Clontech iMatrix-511, Corning Synthemax                                     | XF             | Takara       |
| DEF-CS<sup>TM</sup>      | Undisclosed                                                             |                                                                                      | CD             |              |

NA: not available (neither XF nor CD), XF: xeno-free, CD: chemically defined medium.

Jung Jin Lim, et al: Human PSCs Culturing Methods 487
LN-521 has revealed that more cells survive on LN-521, suggesting that other parts of these large multi-domain laminin molecules interact with, and influence, the cells (40). Both LNs also have strong interaction with the integrin α6β1, which regulate focal adhesion kinase (FAK) signaling in hPSCs, and disruption of this pathway results in hPSC differentiation (41). We have confirmed that hiPSCs can be maintained in an undifferentiated state on recombinant LN-521-coated plates after dissociation and passaging (Fig. 1Bc, 1Cc). However, LN-521 can be used in fully animal-component-free and efficient culture systems for hiPSCs, LN-521 still remains with a high price and possible batch-to-batch variation.

Test of chemically defined system for hPSCs culture

Without feeder cells, the culture is entirely dependent on the quality of the medium for maintenance and proliferation of healthy stem cells. The first chemically defined medium formulation TeSR1 (7) substantially improved the culture of human PSCs, and was commercially released as mTeSR1 (Fig. 2 upper a, lower a). The combination of mTeSR1 medium and Matrigel substrate creates a rich environment to support hPSCs. However, mTeSR1 medium contains animal-derived components and relies on substantial amounts of bFGF to maintain undifferentiated hPSCs (Table 1). Recently, a more streamlined medium for feeder-free cell culture was developed, called NutriStem (Corning, USA), iPS-Brew XF (Miltenyi Biotec, USA), StemFit hPSC Medium (AJINOMOTO, Japan). Those hPSCs medium are more defined, xeno-free, and contains very low levels of growth factors and other proteins and/or HSA, including bFGF (Fig. 2 upper cd, lower cd, Table 1). The low protein composition avoids potential bias, inhibition, or other effects on subsequent differentiation of the cells. TeSR-E8 medium which developed by Chen et al. (8) is a complete defined, feeder-free medium compare to mTeSR1 or NutriStem formulation for the growth and expansion of human PSCs. TeSR-E8 medium contains only the eight components most needed to maintain pluripotent stem cells. Cultures are grown complete with their vitronectin (VTN-N) substrate, which together with TeSR-E8 medium make an effective feeder-free culture system for pluripotent stem cells (Fig. 2 upper b, lower b, Table 1). As mentioned above, rhVitronectin (VTN-N) has been shown to support human PSCs attachment and survival better than wild-type vitronectin. Combination of recombinant biomaterials and complete chemically defined medium may offer a fully defined culture system with a lower cost and higher consistency. In this study, we described detailed protocols to support the culture of hPSCs using TeSR-E8 medium on rhVitronectin-coated culture plate with small addition of bioactive lysosphospholipid.

Supplement of bioactive lysosphospholipid for improving proliferation of hPSCs

A lipid mediator sphingosine-1-phosphate (S1P) is known to exert multiple responses, such as proliferation, survival and cytoskeletal rearrangement, via its G protein-coupled receptor (GPCR) in many cell types (42, 43). Enhancement of PSCs growth rate under fully defined conditions is an important goal to facilitate the robust proliferation of cells to a clinical standard and to enable controlled differentiation of human PSCs (44). Previous studies have implicated S1P as a valuable component of defined culture medium to maintain PSCs. Supplementing S1P in the defined culture system led to a dose-dependent reduction in the level of apoptosis in PSCs while increasing their proliferation rate (45). Phytosphingosine-1-phosphate (P1P) is derived from plants, and is structurally similar to S1P, an endogenous signal lipid in mammalian cells (46, 47). Here we show that an enhanced synthetic sphingolipid, O-cyclic phytosphingosine-1-phosphate (cP1P), has a potential role in increment of proliferation and decrease of apoptosis and/or modulate self-renewal potential of the hPSCs. cP1P treatment appears to improve pro-
Fig. 3. Supplementation of cP1P for improving proliferation of hPSCs. cP1P treatment appears to regulate proliferation of hPSCs by the expansion of total cell populations associated with cell cycle progression (Fig. 3A∼D). cP1P also enhances protein expression of pluripotency markers (OCT4, REX-1, and KLF4) (Fig. 3E).

### Materials and Methods

#### Cell lines

- hESCs (H9, WiCell Research Institute, USA; CHA-hES 15, CHA Stem Cell Institute, Korea).
  - Note: These cell lines were initially derived and expanded on mEF cells and later adapted to culture on vitronectin.
- hiPSCs (CMC-hiPSC-003, Catholic Medical Center, Korea; Registered in National Stem Cell Bank at Korea National Institute of Health).
  - Note: This cell line was generated by non-integrating Sendai virus encoding OCT4, KLF4, SOX2 and c-MYC and propagated on vitronectin.

#### Reagents

- ROCK Inhibitor Y-27632 (Tocris, 10 mg, cat. no. 1254, UK)
- Vitronectin XF™ (STEMCELL Technologies, 2 ml, cat. no. 07180, Canada)
- CellAdhere™ Dilution Buffer (STEMCELL Technologies, cat. no. 07183, Canada)
- TeSR™-E8™ Kit for hESC/hiPSC Maintenance (STEMCELL Technologies, 05940, Canada) contains TeSR™-E8™ Basal Medium (cat. no. 05991) and TeSR™-E8™ 25X Supplement (cat. no. 05992)
- DPBS (PAN BIOTECH, cat. no. P04-36500, Germany)
- Cryostor CS10 (STEMCELL Technologies, cat. no. 07930, Canada)
- TrypLE™ Select Enzyme (Life Technologies, cat. no. 12605-010, US)
- Gentle Cell Dissociation Reagent (STEMCELL Technologies, cat. no. 07174, Canada)
- STEMdiff™ Trilineage Differentiation kit (STEMCELL Technologies, 05250, Canada)
- O-cyclic Phytosphingosine-1-phosphate (cP1P, AXCESO BIOPHAMA, Korea)
- 70% Ethanol (MERCK, cat. no. 1.00974.1011, Germany)
- Isopropanol (MERCK, cat. no. 1.09634.2511, Germany)

#### Equipment

- Safety bench, Class II (ILJIN HI-TECH, Korea)
- Refrigerator
- Freezer, −20/−80°C (Panasonic, MDF-u54-v-pxk, Japan)
- Micropipettes (Gilson, USA)
- Sterile pipette tips, 100, 200 and 1,000 μl (SARSTEDT, Germany)
- CO₂ incubator (Thermo Scientific, HERA CELL 240i, USA)
- Water bath, 37°C (SAMHEUNG ENERGY, WB-11GDN, Korea)
- Centrifuge (Eppendorf, 5810R, Germany)
- Hood equipped with stereomicroscope (OLYMPUS, SZ61, Japan)
- Light microscope (OLYMPUS, CKX41, Japan)
• Liquid nitrogen storage tank (CRYO Industries, USA)
• Conical tube, 15 ml (SPL, cat. no. 50015, Korea)
• Conical sterile polypropylene tube, 50 ml (SPL, cat. no. 50050, Korea)
• Sterile serological pipettes, 5, 10 and 50 ml (SPL, cat. no. 91005, 91010 and 91050, Korea)
• 35×10 mm dish (BD FALCON, cat. no. 353001, USA)
• 6-well plate (CORNING, cat. no. CT-3516, USA)
• Cryogenic handling gloves (Honeywell, USA)
• Forceps (WORLD PRECISION INSTRUMENTS, USA)
• Isopropanol freezing containers (Thermo Scientific, cat. no. 5100-0001, USA)
• Cryovials, 2.0 ml (CORNING, cat. no. CC-430488, USA)
• Cryo 1°C freezing container (Nalgene, cat. no. 52100-0001)

**Equipment setup**

**Complete TeSR™-E8™ medium:** Thaw TeSR™-E8™ 25× supplement at room temperature (15 ~ 25°C) or overnight (2 ~ 8°C). Add 20 ml of TeSR™-E8™ 20× supplement to 480 ml of TeSR™-E8™ Basal Medium and mix them thoroughly. Store the complete medium at 2 ~ 8°C for up to 2 weeks. If not used immediately, divide the medium into aliquots and store at −20°C for up to 6 months.

- **Vitronectin XFTM** is supplied as a 250 μg/ml concentrated stock solution. Thaw the vial of Vitronectin XFTM at room temperature (15 ~ 25°C) and dilute in CellAdhere™ dilution buffer or DPBS to reach a final concentration of 10 μg/ml (i.e. dilute 2 ml of Vitronectin XFTM stock solution in 48 ml of dilution buffer). If the entire vial will not be used immediately, divide the stock solution into aliquots and store at −20°C or −80°C for long-term storage.

- **Matrix and medium preparation (outlining how to coat six wells of non-tissue culture-treated 6-well plate):**
  1. Thaw an aliquot (240 μl) of Vitronectin XF™ stock solution at room temperature.
  2. Dilute 240 μl of Vitronectin XF™ stock solution in 5.76 ml of CellAdhere™ Dilution Buffer or DPBS to make 6 ml of a working solution (10 μg/ml) and mix gently (Do not vortex).
  3. Add 1 ml of diluted working solution to the center of each well of 6-well plate and gently rock the plate to evenly distribute the solution across the surface.
  4. Incubate the plate at room temperature for a minimum 2 h prior to use.
  5. While incubation of the coated plates, warm complete TeSR™-E8™ medium at room temperature (15 ~
25℃). Do not warm medium in a 37°C water bath.

► Note: The complete TeSR™-E8™ medium is light and temperature sensitive. Do not leave medium at room temperature for longer than 2 h and avoid exposure to light to prevent degradation of medium components.

6) To prepare the plates for passaging or thawing of hPSCs, remove the excess vitronectin from the plates and wash with CellAdhere™ dilution buffer or DPBS.

7) Aspirate wash solution and add 2 ml of pre-warmed TeSR™-E8™ medium per well.

8) Place the plates in a 37°C, 5% CO₂ incubator 1 h before thawing or passaging.

**Thawing hPSCs:** 1) Take the cryovial out of liquid nitrogen and move them to the tissue culture room on ice.
2) Quickly thaw the cells by gently swirling the vial in a 37°C water bath.
3) When only a small particle of ice is left in the vial, wipe the outside of the cryovial with 70% (v/v) ethanol to disinfect, dry and bring it under a biosafety cabinet.
4) Gently transfer the aggregates suspension into a 15 ml conical tube using a 1 or 2 ml serological pipette to minimize breakage of cell aggregates.
5) Add 5~7 ml of pre-warmed TeSR™-E8™ medium in a drop-wise manner to the tube.
► Note: While adding drops, gently rock the tube back and forth to distribute the aggregates evenly.
6) Centrifuge the mixture at 150 g for 3 min at room temperature.
7) Discard the supernatant and re-suspend the aggregates in 1 ml of pre-warmed TeSR™-E8™ medium.
► Note: While slowly adding warm TeSR™-E8™ medium, gently tap the tube to dislodge the cell pellet.
8) Plate the aggregates into one to two wells of vitronectin-coated plate and gently move the plate side to side, back and forth to distribute the aggregates evenly.
► Note: Supplement the medium with a stock solution of ROCK inhibitor to maintain 10 μM final concentration (add 1 μl of 10 mM ROCK inhibitor for 1 ml of medium) for enhancing cell survival after plating.
9) Incubate the plate at 37°C, 5% CO₂ incubator and do not disturb the plates for 24 h after plating.

**Passaging of hPSCs:** The following are the instructions for passaging hPSCs maintained in TeSR™-E8™ medium in cell aggregates (1. Passaging as cell aggregates) or single cell suspensions (2. Passaging as single cell suspensions). hPSC cultures can be passaged when they are 70~100% confluent. Before passaging, we recommend removal of spontaneously differentiating area of any colonies using a pipette tip (Fig. 4A).

1. **Passaging as cell aggregates**

Prepare vitronectin-coated plate 1 day before passaging (see Procedure section. Matrix and medium preparation).
1) Remove spent medium from the culture vessel and rinse with DPBS.
2) Add 1 ml of Gentle Cell Dissociation Reagent (GCDR) solution and incubate for 2~6 min at room temperature (15~25°C).
► Note: Observe cells under microscope during incubation to remove the GCDR solution before the colonies are completely detached. The incubation times may vary depending on cell lines.
3) Aspirate the GCDR solution and rinse with 0.5 ml of pre-warmed TeSR™-E8™ medium.
4) Add fresh 1 ml of warm TeSR™-E8™ medium.
5) Gently scrape the colonies with a 5 ml serological pipette or cell scraper and transfer the detached aggregates to a 15 ml conical tube using a 2 ml serological pipette.
6) Triturate the aggregates to create appropriate size (approximately 50~200 μm) for plating with a 200 μl or 1 ml pipette tips.
7) Plate the small aggregates at the desired density onto vitronectin-coated plates containing the complete TeSR™-E8™ medium.
► Note: The split ratio should be determined depending on the confluency at the day of passaging and the growth rates of cell lines.
8) Gently move the plate side to side, back and forth to distribute the aggregates evenly.
9) Incubate the plate at 37°C, 5% CO₂ incubator and do not disturb the plates for 24 h after plating.
10) Feed daily with fresh medium and observe regularly cell growth and morphology (Fig. 4B).

2. **Passaging as single cell suspensions**

1) Remove spent medium from the culture vessel and rinse with DPBS.
2) Add 1 ml of pre-warmed TrypLE to wells and incubate for 5 min at 37°C, 5% CO₂ incubator.
3) Aspirate the TrypLE solution and rinse with 0.5 ml of pre-warmed TeSR™-E8™ medium.
► Note: The rinsing should be done carefully as the colonies are loosely attached after the TrypLE treatment for 5 min.
4) Add fresh 1 ml of warm TeSR™-E8™ medium and break the colonies to single cell suspensions by gentle trituration with a 1 ml pipette tip.
5) Count the cells using a hemocytometer. Plate the cells at densities of 30,000~50,000 cells per cm² onto vitronectin-coated plates containing the complete TeSR™-
**E8™ medium.**

► Note: Addition of 10 mM ROCK inhibitor (1 μl of 10 mM ROCK inhibitor per 1 ml of medium) is recommended when plating single cell suspensions for enhancing cell survival.

► Note: Seeding density is critical for the outcome of the passaging. Thus, optimal seeding density should be adjusted empirically for each culture condition (matrix, medium, and culture ware) and each cell line.

6) Gently move the plate side to side, back and forth to distribute the single cell suspensions evenly.

7) Incubate the plate at 37°C, 5% CO₂ incubator and do not disturb the plates for 24 h after plating.

8) Feed daily with fresh medium and observe regularly cell growth and morphology (Fig. 4C).

**Freezing of hPSCs (outlining how to freeze hPSCs that maintained under above culture conditions in cell aggregates):** Cultures must be cryopreserved when in their log phase of growth (approximately 70~80% confluent) to enhance survival upon thawing. We recommend freezing the cell aggregates from one well of one 6-well plate per cryovial. Keep the freezing container and cryoprotectant in a refrigerator 1 day before freezing.

1) Remove spent medium from the culture vessel and rinse with DPBS.

2) Add 1 ml of GCDR and incubate for 6~12 min at 37°C, 5% CO₂ incubator.

► Note: Observe cells under microscope during incubation to remove the GCDR before the colonies are completely detached. The incubation times may vary depending on cell lines.

3) Aspirate the GCDR and add fresh warm TeSR™-E8™ medium.

4) Gently scrape the colonies with a 5 ml serological pipette or cell scraper.

► Note: Do not use a pipette to avoid breaking the cell aggregates into small pieces or single cells. Leave the aggregates as large as possible.

5) Gently transfer the aggregates suspension into a 15 ml conical tube using a 1 or 2 ml serological pipette.

► Note: Do not use a pipette to avoid breaking the cell aggregates into small pieces or single cells. Leave the aggregates as large as possible.

6) Centrifuge at 150 g for 3 min at room temperature.

7) Gently aspirate the supernatant and re-suspend the aggregates in 1 ml of cold CryoStor® CS10.

► Note: While slowly adding cryoprotectant, gently tap the tube to dislodge the cell pellet. Care must be taken to minimize the breakup of the aggregates.

8) Transfer the 1 ml suspension of aggregates in a cryovial using a 1 or 2 ml serological pipette.

9) Immediately place the cryovials into a pre-chilled freezing container and keep the aggregates at −20°C for 2 h, followed by −80°C for 2 h.

10) Transfer the aggregates into a liquid nitrogen tank for long-term storage.

► Note: A standard slow rate-controlled cooling protocol can be used for freezing (i.e. Reduce temperature at approximately −1°C/min, followed by long-term storage in liquid nitrogen).
Routine characterization for long-term maintenance of hPSCs: The longer hPSCs are maintained, the more likely they have some defects in terms of growth, developmental capacity and genomic stability. Defects in developmental capacity refer to changes in the pluripotency of the cells that may occur with time in long-term culture. Thus, it is useful to check periodically hPSC cultures to ensure maintenance of pluripotency during long-term culture using several methodologies. Here, we briefly describe commonly used in vitro approaches to assess the pluripotency of hPSCs.

1. Pluripotency marker expression by immunofluorescence staining (Fig. 5A, 5B)
   1) Aspirate the spent medium.
   2) Fix the cells with 4% PFA in PBS pH 7.4 for 10 min at room temperature.
   3) Incubate the samples for 10∼30 min with PBS containing 0.1∼0.4% Triton X-100. Optimal percentage of Triton X-100 should be determined for each protein of interest.
   4) Incubate cells with 1% BSA in PBS for 1 h to block unspecific binding of the antibodies. Alternative blocking solution may use 1∼10% serum from a goat or donkey; see antibody datasheet for recommendations.
   5) Incubate cells in the diluted primary antibody with blocking solution in a humidified chamber for 1 h at room temperature or overnight at 4℃.
   6) Discard the solution and wash the cells three times in PBS, 5 min each wash.
   7) Incubate cells with the secondary antibody in PBS for 1 h at room temperature in the dark.
   8) Repeat step 6.
   9) Counter staining with 0.1∼1 μg/ml Hoechst or DAPI for 5 min in the dark.
   10) Rinse with PBS.
   11) Analyze samples.

2. Pluripotency marker expression by flow cytometry (Fig. 5C)
   1) Harvest, wash the dissociate cells (1∼5×10⁶ cells/ml) that were fixed in 100 μl of 1% PFA with 0.1∼0.4% Triton X-100 or Tween 20 in cold PBS to each sample, then mix gently and incubate for 10∼15 min.
   2) Cell down (centrifuge 400∼600 ×g for 5 min at room temperature) and discard the supernatant.

---

Fig. 5. Characterization of hPSCs cultured in chemically defined medium. (A) Representative BF images of hPSCs grown on vitronectin-coated plate in TeSR-E8™ medium for 5 days and typical colony morphology of hPSCs with positive AP staining. (B) Immunofluorescence staining of hPSCs for pluripotency markers (OCT3/4, red; SOX2, green). Cell nuclei were counterstained using DAPI (blue). (C) Flow cytometry analysis of cultured hPSC for REX-1, E-Cadherin, and OCT3/4. (D) hPSCs were differentiated into three germ-layers using the STEMdiff™ Trilineage Differentiation Kit. During differentiation, the shape of each differentiated cell types was clearly different (a: Endoderm, b: Mesoderm and c: Ectoderm lineage). On day 7, the cultured and differentiated-hPSCs were analyzed by immunofluorescence staining (E) and flow cytometry (F). All scale bar, 100 μm. Flow cytometry analysis of differentiated-hPSCs for AFP (endodermal lineage), Brachyury (mesodermal lineage) and MAP2 (ectoderm lineage).
3) Incubate cells with 1% BSA in cold PBS for 1 h to block unspecific binding of antibodies on ice.
4) Repeat step 2.
5) Re-suspend the cell pellet in 100 µl of 1% BSA-PBS with directly conjugated primary antibody and incubate for 30 ∼ 60 min at room temperature. Protect form light.
6) Repeat step 2.
7) Re-suspend stained cells in an appropriate volume of PBS or FACS buffer.
8) Analyze samples.

3. In vitro differentiation potential

The hPSCs have the potential to form derivatives of all three embryonic germ layers. In vitro, the hPSCs were directly differentiated to all three germ layers using the STEMdiff™ Trilineage Differentiation Kit (see manufacturer’s instructions at https://www.stemcell.com/stemdiff-trilineage-differentiation-kit.html), which is commercially available and provides a simple culture assay (ectoderm, mesoderm and endoderm) (Fig. 5D). Immunofluorescence staining and flow cytometry analysis showed that ATF (endoderm), Brachyury (mesoderm) and MAP2 (ectoderm) were strongly expressed in differentiated hPSCs (Fig. 5E, 5F).

Anticipated Results

In this protocol report, we have tested various culture mediums, ECMs and agents for improving hPSCs culture and suggest that xeno-free and chemically defined TeSR™-E8™ in combination with rhVitronectin is suitable for stable maintenance of hPSCs. In addition, the supplementation of bioactive lysophospholipid (cP1P) to the culture conditions improves the proliferation of hPSCs. Xeno-free and chemically defined culture conditions in this protocol is ideal for reducing lot-to-lot variation and increasing reproducibility, which can ensure consistency for the use of hPSCs in both basic research and clinical applications. Long-term maintenance of hPSCs without compromising safety and functionality in this defined culture system would reduce the economic burden for large-scale expansions for various applications.

Acknowledgments

This research was supported by a grant from the Korea Centers for Disease Control and Prevention (2017ER-610300) and the Bio and Medical Technology Development Program (2017M3A9B3061830) of the National Research Foundation (NRF). We are also grateful to Axcesso Biopharma Co., Ltd. for providing cP1P and for excellent technical assistance.

Potential Conflict of Interest

The authors have no conflicting financial interest.

References

1. Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM. Embryonic stem cell lines derived from human blastocysts. Science 1998;282:1145-1147
2. Yu J, Vodyanik MA, Smuga-Otto K, Antosiewicz-Bourget J, Frane JL, Tian S, Nie J, Jonsdottir GA, Ruotti V, Stewart R, Slukvin II, Thomson JA. Induced pluripotent stem cell lines derived from human somatic cells. Science 2007;318:1917-1920
3. Dalhore S, Nayer B, Hasegawa K. Human pluripotent stem cell culture: current status, challenges, and advancement. Stem Cells Int 2018;2018:7396905
4. Xu C, Inokuma MS, Denham J, Golds K, Kundu P, Gold JD, Carpenter MK. Feeder-free growth of undifferentiated human embryonic stem cells. Nat Biotechnol 2001;19:971-974
5. Richards M, Fong CY, Chan WK, Wong PC, Bongso A. Human feeders support prolonged undifferentiated growth of human inner cell masses and embryonic stem cells. Nat Biotechnol 2002;20:933-936
6. Richards M, Tan S, Fong CY, Biswas A, Chan WK, Bongso A. Comparative evaluation of various human feeders for prolonged undifferentiated growth of human embryonic stem cells. Stem Cells 2003;21:546-556
7. Ludwig TE, Levenstein ME, Jones JM, Berggren WT, Mitchen ER, Crandall LJ, Daigh CA, Conard KR, Llanas RA, Thomson JA. Derivation of human embryonic stem cells in defined conditions. Nat Biotechnol 2006;24:185-187
8. Chen G, Gulbranson DR, Hou Z, Bolin JM, Ruotti V, Probasco MD, Smuga-Otto K, Howden SE, Diol NR, Propson NE, Wagner R, Lee GO, Antosiewicz-Bourget J, Teng JM, Thomson JA. Chemically defined conditions for human iPSC derivation and culture. Nat Methods 2011;8:424-429
9. Yasuda SY, Ikeda T, Shahsavarani H, Yoshida N, Nayer B, Hino M, Sharma NV, Suemori H, Hasegawa K. Chemically defined and growth-factor-free culture system for the expansion and derivation of human pluripotent stem cells. Stem Cell Reports 2016;6:330-341
10. Phadnis SM, Loewke NO, Dimov IK, Pai S, Amwake CE, Solgaard O, Baer TM, Chen B, Pera RAR. Dynamic and social behaviors of human pluripotent stem cells. Sci Rep 2015;5:14209
11. Jacobs K, Zambelli F, Mertzianidou A, Smolders I, Geens M, Nguyen HT, Barbe L, Sermon K, Spits C. Higher-density culture in human embryonic stem cells results in DNA damage and genome instability. Stem Cell Reports 2016;6:330-341
12. Hongisto H, Vuoristo S, Mikhaailova A, Suuronen R, Virtanen I, Otonkoski T, Skottman H. Laminin-511 ex-
pression is associated with the functionality of feeder cells in human embryonic stem cell culture. Stem Cell Res 2012;8:97-108

13. Inzunza J, Gertow K, Strömberg MA, Matilainen E, Blennow E, Skottman H, Holbank S, Ahrlund-Richter L, Hovatta O. Derivation of human embryonic stem cell lines in serum replacement medium using postnatal human fibroblasts as feeder cells. Stem Cells 2005;23:544-549

14. Viswanathan P, Gaskell T, Moens N, Culley OJ, Hansen D, Gervasio MK, Yeap YJ, Danovi D. Human pluripotent stem cells on artificial microenvironments: a high content perspective. Front Pharmacol 2014;5:150

15. Clause KC, Barker TH. Extracellular matrix signaling in morphogenesis and repair. Curr Opin Biotechnol 2013;24:830-833

16. Kefalides NA. Structure and biosynthesis of basement membranes. Int Rev Connect Tissue Res 1973;6:63-104

17. Timpl R, Rohde H, Risteli L, Ott U, Robey PG, Martin GR. Laminin. Methods Enzymol 1982/82 Pt A:831-838

18. Timpl R, Rohde H, Robey PG, Rennard SI, Foidart JM, Martin GR. Laminin—a glycoprotein from basement membranes. J Biol Chem 1979;254:9933-9937

19. Chung AE, Jaffe R, Freeman IL, Vergnes JP, Braginski JE, Carlin B. Properties of a basement membrane-related glycoprotein synthesized in culture by a mouse embryonal carcinoma-derived cell line. Cell 1979;16:277-287

20. Hassell JR, Robey PG, Barrach HJ, Wilczek J, Rennard SI, Martin GR. Isolation of a heparan sulfate-containing proteoglycan from basement membrane. Proc Natl Acad Sci U S A 1980;77:4494-4498

21. Carlin B, Jaffe R, Bender B, Chung AE. Entactin, a novel basal lamina-associated sulfated glycoprotein. J Biol Chem 1981;256:5209-5214

22. Cooper AR, MacQueen HA. Subunits of laminin are differentially synthesized in mouse eggs and early embryos. Dev Biol 1983;96:467-471

23. Ekbom P, Vestweber D, Kemler R. Cell-matrix interactions and cell adhesion development. Annu Rev Cell Biol 1986;2:27-47

24. Hierck BP, Thorsteinsdóttir S, Niessen CM, Freund E, Iperen LV, Feyen A, Hogervorst F, Poelmann RE, Mummery CL, Sonnenberg A. Variants of the alpha 6 beta 1 integrin. J Biol Chem 1993;268:33-53

25. Cooper AR, TAMURA RN, Quaranta V. The major laminin receptor of mouse embryonic stem cells is a novel isoform of the alpha 6 beta 1 integrin. J Cell Biol 1991;115:843-850

26. Kleinman HK, McGarvey ML, Liotta LA, Robey PG, Tryggvason K, Martin GR. Isolation and characterization of type IV procollagen, laminin, and heparan sulfate proteoglycan from the EHS sarcoma. Biochemistry 1982;21:6188-6193

27. Kleinman HK, McGarvey ML, Hassell JR, Martin GR. Formation of a supramolecular complex is involved in the reconstitution of basement membrane components. Biochemistry 1983;22:4969-4974

28. Mackay AR, Gomez DE, Cottam DW, Rees RC, Nason AM, Thorgerisson UP. Identification of the 72-kDa (MMP-2) and 92-kDa (MMP-9) gelatinase/type IV collagenase in preparations of laminin and Matrigel. Biotechniques 1993;15:1048-1051

29. Vukicevic S, Kleinman HK, Layten FP, Roberts AB, Roche NS, Reddi AH. Identification of multiple active growth factors in basement membrane Matrigel suggests caution in interpretation of cellular activity related to extracellular matrix components. Exp Cell Res 1992;202:1-8

30. Bortk TK. It’s the matrix! ECM, proteases, and cancer. Am J Pathol 2004;164:1141-1142

31. Li J, Bardy J, Yap LY, Chen A, Nurcombe V, Cool SM, Oh SK, Birch WR. Impact of vitronectin concentration and surface properties on the stable propagation of human embryonic stem cells. Biointerphases 2010;5:FA132-FA142

32. Doolittle JM, Gomez SM. Structural similarity-based predictions of protein interactions between IIIV-1 and Homo sapiens. Virol J 2010;7:82

33. Juliano RL, Varner JA. Adhesion molecules in cancer: the role of integrins. Curr Opin Cell Biol 1993;5:812-818

34. Braam SR, Zeinstra L, Litjens S, Van Oostwaard D, van den Brink S, van Laake L, Lebrin F, Kats P, Hochstenbach R, Passier R, Sonnenberg A, Mummery CL. Recombinant vitronectin is a functionally defined substrate that supports human embryonic stem cell self-renewal via alphavbeta5 integrin. Stem Cells 2008;26:2257-2265

35. Pulido D, Hussain SA, Hohenester E. Crystal structure of the heterotrimeric integrin-binding region of laminin-111. Structure 2017;25:530-535

36. Chen KG, Mallon BS, McKay RD, Robey PG. Human pluripotent stem cell culture: considerations for maintenance, expansion, and therapeutics. Cell Stem Cell 2014;14:13-26

37. Li Y, Powell S, Brunette E, Leblowski J, Mandalaram R. Expansion of human embryonic stem cells in defined serum-free medium devoid of animal-derived products. Biotechnol Bioeng 2005;91:698-698

38. Domogatskaya A, Rodin S, Boutaud A, Tryggvason K. Laminin-511 but not -332, -111, or -411 enables mouse embryonic stem cell self-renewal via alphavbeta5 integrin. Stem Cells 2008;26:2257-2265

39. Pulido D, Hussain SA, Hohenester E. Crystal structure of the heterotrimeric integrin-binding region of laminin-111. Structure 179:25:530-535

40. Chen KG, Mallon BS, McKay RD, Robey PG. Human pluripotent stem cell culture: considerations for maintenance, expansion, and therapeutics. Cell Stem Cell 2014;14:13-26

41. Li Y, Powell S, Brunette E, Leblowski J, Mandalaram R. Expansion of human embryonic stem cells in defined serum-free medium devoid of animal-derived products. Biotechnol Bioeng 2005;91:698-698

42. Domogatskaya A, Rodin S, Boutaud A, Tryggvason K. Laminin-511 but not -332, -111, or -411 enables mouse embryonic stem cell self-renewal in vitro. Stem Cells 2008;26:2800-2809

43. Rodin S, Domogatskaya A, Ström S, Hansson EM, Chien KR, Inzunza J, Hovatta O, Tryggvason K. Long-term self-renewal of human pluripotent stem cells on human recombinant laminin-511. Nat Biotechnol 2010;28:611-615

44. Rodin S, Antomsson L, Hovatta O, Tryggvason K. Monolayer culture and cloning of human pluripotent stem cells on laminin-521-based matrices under xeno-free and chemically defined conditions. Nat Protoc 2014;9:2354-2368

45. Villa-Diaz LG, Kim JK, Laperle A, Palecek SP, Krebsbach PH. Inhibition of focal adhesion kinase signaling by integrin alpha6beta1 supports human pluripotent stem cell self-renewal. Stem Cells 2016;34:1753-1764
42. Pyne S, Pyne NJ. Sphingosine 1-phosphate signalling and termination at lipid phosphate receptors. Biochim Biophys Acta 2002;1582:121-131
43. Spiegel S, Milstien S. Sphingosine 1-phosphate, a key cell signaling molecule. J Biol Chem 2002;277:25851-28584
44. Draper JS, Fox V. Human embryonic stem cells: multilineage differentiation and mechanisms of self-renewal. Arch Med Res 2003;34:558-564
45. Inniss K, Moore H. Mediation of apoptosis and proliferation of human embryonic stem cells by sphingosine-1-phosphate. Stem Cells Dev 2006;15:789-796
46. Kim MK, Park KS, Lee H, Kim YD, Yun J, Bae YS. Phytosphingosine-1-phosphate stimulates chemotactic migration of L2071 mouse fibroblasts via pertussis toxin-sensitive G-proteins. Exp Mol Med 2007;39:185-194
47. Pata MO, Hannun YA, Ng CK. Plant sphingolipids: decoding the enigma of the Sphinx. New Phytol 2010;185:611-630