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Fabrication of synthetic polymer coatings and their use in feeder-free culture of human embryonic stem cells

Himabindu Nandivada^{1,11,12}, Luis G Villa-Diaz^{2,11}, K Sue O'Shea³, Gary D Smith^{4,5,6,7}, Paul H Krebsbach^{2,8}, and Joerg Lahann^{1,9,10}

¹Department of Chemical Engineering, University of Michigan, Ann Arbor, Michigan, USA

²Department of Biologic and Materials Sciences, University of Michigan, Ann Arbor, Michigan, USA

³Department of Cell and Developmental Biology, University of Michigan, Ann Arbor, Michigan, USA

⁴Department of Obstetrics and Gynecology, University of Michigan, Ann Arbor, Michigan, USA

⁵Department of Urology, University of Michigan, Ann Arbor, Michigan, USA

⁶Department of Molecular and Integrated Physiology, University of Michigan, Ann Arbor, Michigan, USA

⁷Department of Reproductive Sciences Program, University of Michigan, Ann Arbor, Michigan, USA

⁸Department of Biomedical Engineering, University of Michigan, Ann Arbor, Michigan, USA

⁹Department of Materials Science and Engineering, University of Michigan, Ann Arbor, Michigan, USA

¹⁰Department of Macromolecular Science and Engineering, University of Michigan, Ann Arbor, Michigan, USA

Abstract

The culture of human embryonic stem (hES) cells in defined and xenogeneic-free conditions will contribute substantially to future biotechnological and medical applications. To achieve this goal, we developed the first fully defined synthetic polymer coating poly[2-(methacryloyloxy)ethyl dimethyl-(3-sulfopropyl)ammonium hydroxide] (PMEDSAH) that sustains long-term growth of hES cells in different culture media. Here we describe a detailed protocol for the reproducible fabrication of PMEDSAH coating on tissue culture polystyrene dishes, and for the feeder-free culture of hES cells on PMEDSAH coating in defined culture medium. this culture system represents a key step toward the fully defined and xenogeneic-free culture of hES cells.

Correspondence should be addressed to J.L. (lahann@umich.edu), G.D.S. (smithgd@umich.edu) or L.G.V.-D. (luisv@umich.edu)..

¹¹These authors contributed equally to this work.

¹²Present address: BD Biosciences–Discovery Labware, Bedford, Massachusetts, USA.

AUTHOR CONTRIBUTIONS This protocol was developed and written by H.N. and L.G.V.-D. after obtaining the material from all authors. H.N. designed the 'Fabrication of PMEDSAH-coated TCPS dishes' portion of the protocol; L.G.V.-D. designed the 'Establishment of hES cell cultures on PMEDSAH-coated TCPS dishes' portion of the protocol. K.S.O., G.D.S., P.H.K. and J.L. supervised the project.

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INTRODUCTION

Human embryonic stem (hES) cells have the potential to differentiate into all cell types of the body, and thus hold promise for cell replacement strategies and tissue engineering¹. However, to realize this potential, an improved understanding of culture conditions, directed differentiation, genomic stability and genetic modification of hES cells is required. The development of a fully defined microenvironment for the culture of hES cells will have a profound impact on this goal, because current practices to maintain hES cells in an undifferentiated state typically depend on the support of feeder cells derived from mouse embryos¹ or in a feeder-free manner by coating cell culture vessels with animal-derived protein substrates such as Matrigel². These culture conditions are undefined, vary between lots, require time-consuming testing, and, most notably, limit our ability to interpret mechanistic studies, and the xenogenic components also hinder the use of these stem cells for clinical applications³. Thus, identifying a chemically defined matrix that can support hES cell self-renewal in serum-and feeder-free conditions is an essential requirement for both basic stem cell biology and regenerative medicine. Although major progress has been made toward the development of defined and/or xenogeneic-free media for hES cells⁴⁻⁷, only recently has the development of cell culture matrices void of xenogeneic components been described by us⁸ and by other independent groups⁹⁻¹³ (Table 1).

Our report described the use of a chemically defined polymer coating for the long-term culture of hES cells⁸. This coating is synthesized by surface-initiated graft polymerization of PMEDSAH on tissue culture polystyrene (TCPS) dishes. Human ES cells grown on PMEDSAH-coated TCPS dishes remain in an undifferentiated state; maintain pluripotency with the ability to differentiate into endoderm, ectoderm and mesoderm derivatives; and preserve a normal karyotype. Conditioned medium (CM) from human cells and defined serum-free medium support hES cell culture on PMEDSAH, and their use in conjunction with PMEDSAH-coated TCPS dishes represents a major step toward achieving a clinically compliant hES cell culture system. Synthetic polymer coatings are reproducible to fabricate, inexpensive and highly manipulable compared with substrates based on biological components, such as recombinant proteins⁹⁻¹¹ and peptides^{12,13}.

Here we describe a protocol that details the procedure for the fabrication of PMEDSAH coatings and the preparation of culture medium. It is important to emphasize that successful establishment of hES cell cultures on PMEDSAH-coated TCPS dishes, as with other feeder-free conditions, depends on the rigorous and continuous selection of high-quality undifferentiated hES cells. In this protocol, we describe mechanical passaging of hES cell aggregates, as opposed to enzymatic methods that have been associated with an increased incidence of karyotypic abnormality¹⁴⁻¹⁶. However, an investigator may choose to modify this protocol by replacing the mechanical harvesting step with enzymatic methods. Human ES cells can be cultured on Matrigel², and these cells can be used as a control for the characterization of hES cells cultures on PMEDSAH-coated TCPS dishes. Finally, in this protocol, we focused entirely on hES cell culture and have not yet optimized the use of PMEDSAH-coated TCPS dishes for the culture of other stem cells.

MATERIALS

REAGENTS

Polymer synthesis reagents

- [2-(methacryloyloxy)ethyl]dimethyl-(3-sulfopropyl)ammonium hydroxide] (MEDSAH; Sigma-Aldrich, cat. no. 537284)
- Anhydrous ethanol (Fisher Scientific, cat. no. A405-P-4)

- Sodium chloride (Fisher Scientific, cat. no. S271-3)
- Deionized (DI) water (Crown Solutions)
- Argon gas (BOC Gases)

hES cells and culture reagents

- CHB-8 cells (Children's Hospital Corporation, cat. no. NIHhESC-09-0007)
- CHB-10 cells (Children's Hospital Corporation, cat. no. NIHhESC-09-0009)
- WA09 (H9) cells (WiCell Research Institute, cat. no. NIHhESC-10-0062)
- WA07 (H7) cells (WiCell Research Institute, cat. no. NIHhESC-10-0061)
- BG01 cells (BresaGen)
- Irradiated CF-1 mouse embryonic fibroblasts (MEF, passage 3; GlobalStem, cat. no. GSC-6001G)
- Gelatin type A, porcine (Sigma, cat. no. G1890; see REAGENT SETUP)
- Matrigel hESC-qualified Matrix (BD Biosciences, cat. no. 354277; see **Box 1**)
- Water (Sigma, cat. no. W3500)
- Dulbecco's phosphate buffered saline (D-PBS, without Ca^{2+} or Mg^{2+} ; GIBCO, cat. no. 14190)
- Dulbecco's modified Eagle medium (DMEM, high glucose; GIBCO, cat. no. 11965)
- DMEM/F12 (with L-glutamine and 15 mM HEPES; GIBCO, cat. no. 11330)
- KnockOut Serum Replacement (KOSR; GIBCO, cat. no. 10828) **▲ CRITICAL**
Store aliquots of KOSR at -20°C . Thaw at 4°C overnight. Use immediately after thawing.
- Heat-inactivated FBS (HI-FBS; GIBCO, cat. no. 10082)
- L-glutamine (GIBCO, cat. no. 25030)
- Non-essential amino acids (GIBCO, cat. no. 11140)
- Penicillin/streptomycin (GIBCO, cat. no. 15140)
- BSA (GIBCO, cat. no. 15561)
- Trypan blue solution (Sigma, cat. no. T-8154)
- 2-Mercaptoethanol (Sigma, cat. no. M7522; see REAGENT SETUP)

! CAUTION 2-Mercaptoethanol is toxic. Avoid inhalation, ingestion and skin contact during use. Personal protection includes gloves, safety glasses and good ventilation.

- Basic fibroblast growth factor (bFGF, human recombinant; Invitrogen, cat. no. 13256-029; see REAGENT SETUP)
- Trypsin-EDTA (0.25% wt/vol; GIBCO, cat. no. 25200)
- Human ES cell medium (see REAGENT SETUP)
- MEF-conditioned medium (MEF-CM; see **Box 2**)
- MEF medium (see REAGENT SETUP)

- Human Cell Conditioned Medium, Serum-Free (hCCM; GlobalStem, cat. no. GSM-9200; see REAGENT SETUP)
- StemPro hESC SFM kit (GIBCO, cat. no. A10007-01; see REAGENT SETUP) containing 500 ml of DMEM/F12 with GlutaMAX (GIBCO, cat. no. 10565-018), 10 ml of StemPro hESC supplement (Invitrogen, cat. no. A10006-01) and 40 ml of 25% (wt/vol) BSA (GIBCO, cat. no. A10008-01).

EQUIPMENT

Polymer synthesis equipment

- Easy-Grip cell culture dish (tissue culture-treated polystyrene; 35 mm; BD Falcon, cat. no. 353001; referred to as TCPS dishes in this protocol)
- UV Ozone cleaner (Jelight company, model no. 342) ! **CAUTION** This instrument produces ozone. Good ventilation is required.
- Branson 52 ultrasonic cleaner (50/60 Hz, Branson)
- Cylindrical reaction vessel (500 ml; Chemglass, cat. no. CG-1920-01)
- Reaction vessel lid (3 × 24/40 necks; Chemglass, cat. no. CG-1940-01)
- Viton O-ring (Chemglass, cat. no. CG-147-21)
- Reactor clamp (Chemglass, cat. no. CG-141-02)
- Support clamp for reaction vessel (Chemglass, cat. no. CG-1947-01)
- Vacuum/air manifold (Chemglass, cat. no. AF-0450-02)
- Condenser (24/40 Joint, 300-mm Jacket Length; Chemglass, cat. no. CG-1214-05)
- Heating mantle (Chemglass, cat. no. CG-10000-07)
- Heating mantle controller (Chemglass, cat. no. CG-15005-01)
- Stir plate (VWR, cat. no. 82002-572)
- Teflon-coated magnetic 1" stir bar (VWR, cat. no. 58949-034)
- Thermometer (Fisher Scientific, cat. no. 1504140)
- 24/40 Thermometer adapter (Chemglass, cat. no. CG-1042-01)
- 24/40 Glass stopper (Chemglass, cat. no. CG-3000-05)
- Beaker (1 liter, VWR, cat. no. 13912-604)
- Filtering flask (1 liter, Chemglass, cat. no. CG-8514-1L)
- General-purpose forceps (Fisher Scientific, cat. no. 10-316A)

hES cell culture equipment

- Cell culture incubators set at 37 °C, 95% humidity, 5% CO₂ (Hera Cell 150, Thermo Scientific) Class-II Biosafety cabinet with aspirator for tissue culture (SteriGARD III Advance, The Baker Company)
- Laminar flow hood (Edgegard EG-6230, The Baker Company)
- Stereomicroscope (Leica M295, Leica) with dark-field capability, LK 1500 LCD light source (Leica) and ThermoPlate (Leica Mats, Leica)

- Inverted microscope with phase contrast with $\times 5$, $\times 10$, $\times 20$ and $\times 40$ objectives (Leica DM IL, Leica) with a Leica DFC480 (Leica) digital camera
- Inverted microscope with phase contrast and epifluorescence capability (Leica DMIRB, Leica) with an Olympus DP-30 (Olympus) digital camera
- Benchtop centrifuge with aerocarrier rotor adapters for 15- and 50-ml tubes (IEC Centra CL2, Thermo Scientific) Microcentrifuge (MiniSpin plus, Eppendorf)
- Water bath (Isotemp 202S; Fisher Scientific)
- Bottle-top filter systems (150, 250 and 500 ml with 0.2- μ m pore size; Nalgene, cat. nos 09-740-46A, 09-740-59 and 09-740-46B, respectively)
- Easy-Grip cell culture dish (tissue culture-treated polystyrene; 35 mm; BD Falcon, cat. no. 353001; also used for PMEDSAH coating)
- Cell culture dish (150 mm, BD Falcon, cat. no. 351058)
- Polypropylene conical tubes (15 and 50 ml; BD Falcon, cat. nos 352096 and 352098, respectively)
- Desiccator jar (Sybron Corporation)
- Eppendorf tubes (1.5 ml; Fisher Scientific, cat. no. 05-402-25)
- Sterile square media bottle (PETG; Nalgene, cat. no. 2019-0125)
- Nonpyrogenic serological pipettes (2, 10, 25 and 50 ml; Fisher Scientific)
- Pipet-Aid (Drummond)
- Pipet-Lite for 2, 10, 20, 100, 200 and 1,000 μ l (Rainin)
- StemPro EZPassage disposable stem cell passaging tool (Invitrogen, cat. no. 23181-010)

REAGENT SETUP

Gelatin (0.1% (wt/vol))—Prepare a stock solution of 1% (wt/vol) gelatin with water (Sigma) and autoclave on liquid cycle for 45 min. When cooled, store at 4 °C. To prepare the working solution of 0.1% (wt/vol) gelatin, warm the 1% stock solution in a 37 °C water bath and, under sterile conditions, add 55 ml of 1% stock solution to 500 ml water (Sigma). Mix well by swirling and store at 4 °C. Warm to 37 °C before coating TCPS dishes to ensure that gelatin is melted. Gelatin-coated plates should be prepared fresh before use.

bFGF stock—Prepare 0.1% (wt/vol) BSA with D-PBS and filter through a 0.2- μ m pore size filter unit, aliquot and store at -20 °C. Spin the lyophilized bFGF vial briefly to bring the contents down. Under sterile conditions, dissolve 10 μ g of bFGF in 5 ml of 0.1% (wt/vol) BSA to obtain a final concentration of 2 μ g ml $^{-1}$. Dispense 250 μ l aliquots into sterile Eppendorf tubes and store them at -20 °C up to 6 months. ▲ **CRITICAL** Thaw and add the required concentration of bFGF to the culture medium just before use. Once thawed, discard the unused portion of the growth factor.

2-Mercaptoethanol—2-Mercaptoethanol is supplied at a concentration of 14.3 M. Prepare a stock of aliquots with 10 μ l each of 1 M and 14.3 M 2-mercaptoethanol. To prepare 1 M 2-mercaptoethanol stock, dilute 70 μ l of 14.3 M 2-mercaptoethanol in 1 ml of D-PBS and store at -20 °C up to 6 months.

Human ES cell medium—Prepare a solution of L-glutamine and 7 μ l of 14.3 M 2-mercaptoethanol. To prepare 250 ml of hES cell medium, mix the following ingredients in a sterile 250 ml bottle-top 0.2- μ m pore size filter: DMEM/F12 (200 ml), KOSR (50 ml), L-glutamine/2-mercaptoethanol solution (1.25 ml), non-essential amino acids (2.5 ml). After filtering, add 0.5 ml of bFGF stock to obtain a final concentration of 4 ng ml⁻¹. Store in the dark at 4 °C and use within 2 weeks. Penicillin/streptomycin can be added to this and the entire culture medium if necessary. **▲ CRITICAL** L-glutamine/2-mercaptoethanol solution should be prepared fresh immediately before use. Unused solution should be discarded.

MEF medium—To prepare 500 ml of MEF medium, mix the following ingredients in a sterile 500 ml bottle-top 0.2- μ m pore size filter: DMEM (440 ml), HI-FBS (50 ml), non-essential amino acids (5 ml) and L-glutamine (5 ml). Store in the dark at 4 °C and use within 2 weeks.

Human cell CM—To prepare hCCM, thaw the 100-ml bottle of hCCM overnight at 4 °C and add 250 μ l of bFGF stock. There is no need to filter the medium. Store in the dark at 4 °C and use within 2 weeks of preparation.

Stem Pro—StemPro hESC SFM is provided as a kit that contains 500 ml of DMEM/F12 with GlutaMAX, 10 ml of StemPro hESC supplement and 40 ml of 25% (wt/vol) BSA. In addition, bFGF and 2-mercaptoethanol must be added. **▲ CRITICAL** Replace DMEM/F12 with GlutaMAX with DMEM/F12 containing L-glutamine and 15 mM HEPES, to better maintain the physiological pH of the medium while the cells are outside the incubator. The StemPro hESC supplement is supplied as a frozen sample. Thaw it, dispense in 2 ml aliquots in conical tubes and store at – 20 °C up to expiration date provided by manufacture.

To prepare 100 ml of ready-to-use StemPro, mix 90.8 ml of DMEM/F12, 2 ml of StemPro hESC supplement, 7.2 ml of 25% (wt/vol) BSA, 200 μ l of bFGF stock and 10 μ l of 1 M 2-mercaptoethanol. Store in the dark at 4 °C and use within 2 weeks.

PROCEDURE

Fabrication of PMEDSAH-coated TCPS dishes • TIMING 1–2 d

- 1| Assemble the 500-ml cylindrical reaction vessel and three-necked reaction vessel lid with the O-ring and reactor clamp. Place a Teflon-coated magnetic stir bar in the reactor assembly. Attach the condenser to one of the necks of the reaction vessel and turn on the water through the condenser. Close the other two necks with glass stoppers. Place the assembled reactor on a heating mantle and on top of a stirring plate (setup shown in Fig. 1).

! CAUTION The PMEDSAH coating reaction should be performed in a chemical fume hood and proper protective equipment must be used when handling reactants and solvents. Furthermore, appropriate waste disposal procedures must be followed.

- 2| Attach the reactor to a vacuum/air manifold with vacuum and gas connections.
- 3| Degas the reactor setup three times by alternately applying vacuum and argon gas. Finally, leave the argon gas flowing through the system until end of the reaction.
- 4| Prepare 500 ml of solvent mixture with water and ethanol (4:1 (vol/vol)) in a 1-liter filtering flask and close the top with a rubber stopper. Degas this solvent mixture by applying vacuum for 30 min¹⁷.

- 5| Open the reactor clamp and lift the vessel lid slightly, while flowing argon gas through the system, to add 27 g of MEDSAH monomer and 500 ml of the degassed water-ethanol solvent mixture to the reactor setup for a final concentration of 0.25 M monomer. Close the reactor and seal it with the clamp.
- 6| Turn on the heating mantle controller and the stirring plate to begin heating and mixing the monomer solution.
- 7| Monitor the monomer solution temperature by inserting a thermometer through a thermometer adapter.
- 8| While the monomer solution is heating, place the TCPS dishes on the loading tray of a UV ozone cleaner and activate for 40 min.
▲ CRITICAL STEP For maximum activation, place the TCPS dishes as close to the UV source as possible. In practice, because of the raised edge of 35-mm TCPS dishes, the inner surface of the TCPS dish is at least 15–20 mm away from the UV lamp in the UV Ozone cleaner.
- 9| When the monomer solution reaches 65–70 °C, load surface-activated TCPS dishes into the reactor by opening the reactor clamp and lifting the reactor vessel lid slightly. Ensure that all surface-activated TCPS dishes are completely immersed in the heated monomer solution and re-close the reactor. Maintain monomer solution temperature at 80 ± 3 °C for 2.5 h.

? TROUBLESHOOTING

- 10| To stop the reaction, turn off the heating mantle controller and let the reaction solution temperature decrease to 50 °C.
- 11| In the meantime, heat salt solution (1% (wt/vol) NaCl in DI water) to 50 °C in a 1-liter beaker.
- 12| Use forceps to take the polymer-coated TCPS dishes out of the reactor; rinse them with the warm salt solution prepared in the previous step.
- 13| Leave the polymer-coated TCPS dishes in salt solution overnight (at least 12 h) at 50 °C.

CRITICAL STEP Washing with salt solution enables the removal of unreacted monomer and any unattached polymer gel from the TCPS dishes.

- 14| Finally, clean the PMEDSAH-coated TCPS dishes by ultrasonication in DI water and dry under a stream of nitrogen gas.

■ PAUSE POINT PMEDSAH-coated TCPS dishes can be stored in dry conditions in a desiccator jar until use. We have used PMEDSAH-coated TCPS dishes up to 3 months after fabrication for successful hES cell culture.

Establishment of hES cell culture on PMEDSAH-coated TCPS dishes •TIMING 2 weeks

- 15| *Day 1.* Irradiate PMEDSAH-coated TCPS dishes with UV light in a tissue culture hood overnight (or at least 30 min) to sterilize the surface.

▲ CRITICAL STEP This and all subsequent steps should be carried out in a tissue culture hood. Proper safety precautions must be taken when handling biological components.

▲ CRITICAL STEP Human ES cells can be cultured on Matrigel as previously described², and these cells can be used as control for the characterization of hES cell cultures on PMEDSAH-coated TCPS dishes. We

apply the following procedure to establish cultures on PMEDSAH-coated TCPS dishes in the presence of the feeder-free medium (medium conditioned by MEFs (MEF-CM) or by human cells (hCCM) or defined medium (StemPro)). The morphology and cell-population doubling time of hES cell colonies cultured on PMEDSAH-coated TCPS dishes is similar, regardless of the medium used⁸.

- 16| *Day 2.* Wash the PMEDSAH-coated TCPS dishes with D-PBS twice, then add 1 ml of the required medium (MEF-CM, hCCM or StemPro), and place PMEDSAH-coated TCPS dishes in the cell culture incubator.

▲ CRITICAL STEP This preparation is done at least 4 h before cell seeding; however, improved results have been observed when TCPS dishes are prepared 48 h before cell seeding. If it has been left in the incubator for more than a week, refresh the medium in the preconditioned dish and put it back in the incubator until use.

- 17| *Day 4.* Dispense the required amount of culture medium into a conical tube and pre-equilibrate it in the cell culture incubator.

- 18| Replace the medium in the TCPS dishes containing the 'ready-to-passage' hES cell colonies with pre-equilibrated fresh medium.

▲ CRITICAL STEP As in standard hES cell culture, undifferentiated and ready-to-passage hES cell colonies have clear and well-defined borders, may have centers that are denser and brighter compared with the edges and may be beginning to merge with other colonies (Fig. 2).

- 19| Use the stereomicroscope placed inside a laminar flow hood to identify ready-to-passage colonies and cut them mechanically into small cell aggregates with a sterile pulled glass pipette (Fig. 2). Alternatively, use the StemPro EZPassage tool to achieve high yield and uniform size cell aggregates.

▲ CRITICAL STEP We perform mechanical passaging of hES cells on PMEDSAH-coated TCPS dishes to avoid karyotypic abnormalities associated with enzymatic passaging^{14–16}.

? TROUBLESHOOTING

- 20| Working inside the tissue culture hood, collect and transfer 50–100 hES cell aggregates to each previously prepared PMEDSAH-coated TCPS dish, and then return the PMEDSAH-coated TCPS dishes to the cell culture incubator (passage 1). The following day, take no action.

▲ CRITICAL STEP After passaging hES cell aggregates to PMEDSAH-coated TCPS dishes, adherent colonies and embryoid bodies (EBs) can be observed. Some EBs will be floating, whereas others will be attached to PMEDSAH-coated TCPS dishes. If the latter are left attached, undifferentiated colonies will form.

? TROUBLESHOOTING

- 21| Forty-eight hours after passaging, while working under the stereomicroscope, remove any differentiated colonies and cells from PMEDSAH-coated TCPS dishes using a sterile pulled glass pipette. Replace the spent medium with 1 ml of pre-equilibrated fresh medium.

? TROUBLESHOOTING

- 22| Every other day, repeat the procedure described in Step 21.

- 23| After 7–10 d, several colonies will be ready to passage (Fig. 2). Split them mechanically and transfer 50–100 aggregates to each newly prepared PMEDSAH-coated TCPS dish (passage 2). Please note that it is important to feed the PMEDSAH-coated TCPS dish containing the original cells (passage 1), as colonies not ready for passage and cell aggregates that were not transferred to the new PMEDSAH-coated TCPS dish will grow and can be picked when they reach the status of ready-to-passage colonies.

Culture expansion on PMEDSAH-coated TCPS dishes for hES cell characterization • TIMING 4 weeks

- 24| When the colonies are ready for passage, split them mechanically into 50–100 aggregates per new PMEDSAH-coated TCPS dish. For instance, at passage 5, pick colonies and split them mechanically to propagate to passage 6, while using the remaining colonies for immunofluorescence analysis of pluripotency-associated transcription factors (OCT4 and SOX2) and surface markers (SSEA-3, SSEA-4, TRA1-60 and TRA1-81). Assess the differentiation potential *in vitro* by the formation of EBs.
- ▲ **CRITICAL STEP** Characterize hES cells every five passages to verify the continuous expression of pluripotency-associated transcription factors and surface markers, as well as to assess normal karyotype and differentiation potential *in vitro*.
- 25| Trypsinize the EBs and extract RNA to evaluate the presence of representative genes from ectoderm, mesoderm and endoderm by reverse transcription PCR.
- 26| Perform karyotype analysis.⁸

? TROUBLESHOOTING

Troubleshooting advice can be found in Table 2.

•TIMING

Steps 1–14, Fabrication of PMEDSAH-coated TCPS dishes: 1–2 d

Steps 15–23, Establishment of hES cell culture on PMEDSAH-coated TCPS dishes: 2 weeks

Steps 24–26, Culture expansion on PMEDSAH-coated TCPS dishes for hES cell characterization: 4 weeks

ANTICIPATED RESULTS

The culture of hES cells on chemically defined substrates, such as PMEDSAH, eliminates variables associated with feeder cells and naturally derived protein coatings; these can range from batch-to-batch inconsistencies to biosafety issues. By following the protocol described here, researchers can obtain consistent and easy-to-handle cultures of hES cells. As described previously by other researchers¹⁸ and in our research⁸, differences exist between hES cell lines, and this may be reflected in the outcome. We observed that BG01 cells could not be passaged beyond three passages on PMEDSAH-coated TCPS dishes in StemPro medium; however, we reported ten continual passages of H9 cells in the same conditions. Subsequently, other cells lines, such as, H1 (data not shown), H7, CHB-8 and CHB-10 have been successfully cultured on PMEDSAH-coated TCPS dishes for more than five passages (Fig. 2). Spontaneous differentiation of hES cells on PMEDSAH-coated TCPS dishes occurs as frequently as observed on feeder cells and other feeder-free culture systems, such as

Matrigel. Thus, as mentioned before, successful sustenance of hES cell cultures on PMEDSAH-coated dishes depends on careful selection of high-quality undifferentiated hES cells achieved by mechanical passaging methods.

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BOX 1 | COATING DISHES WITH MATRIGEL •TIMING 2–3 D

Prepare Matrigel solution at a concentration of 0.1 mg ml^{-1} in DMEM/F12 as given below:

1. *Day 1:* Thaw a 5-ml vial of Matrigel hESC-qualified matrix overnight at 4°C on ice.
2. Prepare 160- μl aliquots of Matrigel in Eppendorf tubes and store them at -70°C .
3. Thaw one aliquot of Matrigel on ice, overnight at 4°C .
4. *Day 2:* Working inside the tissue culture hood and on ice, dilute the contents of the Matrigel vial with 12.5 ml of ice cold DMEM/F12.
5. Immediately after mixing, dispense 1 ml of Matrigel-DMEM/F12 solution per each 35-mm TCPS dish.
6. Swirl the TCPS dishes to ensure that the surfaces are completely covered with the Matrigel-DMEM/F12 solution.
7. Incubate the TCPS dishes overnight at 4°C or for at least 2 h at room temperature.
8. Immediately before plating the cells, aspirate the Matrigel-DMEM/F12 solution and wash the dishes with D-PBS.

BOX 2 | PREPARATION OF MEF-CM • TIMING 4 D

1. *Day 1:* Dispense 15 ml of 0.1% (wt/vol) gelatin into a 150-mm cell culture dish and incubate it for at least 30 min at room temperature.
2. Warm MEF medium at 37 °C.
3. Thaw one vial of irradiated CF-1 MEFs at 37 °C in the water bath for 45–90 s.
4. Resuspend cells in the vial with 5 ml of warm MEF medium in a 15-ml conical tube. Count the number of viable cells and centrifuge them for 5 min at 1,000 r.p.m. Use Trypan blue solution to distinguish and count live MEFs.
5. Aspirate supernatant, tap the conical tube to dislodge the pellet and gently resuspend cells in the appropriate volume of MEF medium to obtain 6×10^6 irradiated MEFs in 60 ml of solution.
6. Remove excess gelatin solution from the 150-mm cell culture dish and add the 60 ml of medium containing the MEFs. Distribute cells evenly over the surface of the dish by gentle agitation (back-forth and right-left), and then place the dish in the cell culture incubator.
7. *Day 2:* Remove the MEF medium from the dish and wash the dish with warm D-PBS.
8. Immediately add 60 ml of warm hES cell medium and place the dish back in the cell culture incubator for 24 h.
9. *Day 3:* Collect the 60 ml of hES cell medium conditioned by MEFs and replace it with 60 ml of fresh hES cell medium. Store the collected MEF-CM at –20 °C.
10. *Days 4 and 5:* Repeat steps 8 and 9.
11. To prepare 125 ml of MEF-CM for feeder-free cell culture, thaw the stock medium and supplement with 1.25 ml of L-glutamine and 12.5 µl of 1 M 2-mercaptoethanol; mix all three components in a sterile 150 ml bottle-top 0.2-µm pore size filter and then add 250
12. 5 µl of bFGF. Store in the dark at 4 °C and use within 2 weeks of preparation.

▲ **CRITICAL STEP** MEF-CM should be collected only for 3 d because secretion of growth factors and cytokines by older irradiated MEFs change from supporting hES cell self-renewal to nonsupporting factors¹⁹.

Figure 1. Reactor setup used for surface-initiated graft polymerization of PMEDSAH on TCPS dishes. All reagents (solvent, monomer and TCPS dishes) are added by opening the reactor clamp and slightly lifting the reactor lid while purging the system with argon gas.

Figure 2. Culture of hES cells on PMEDSAH-coated TCPS dishes. (a–f) Representative micrographs of hES cell lines on PMEDSAH. Arrows indicate ready-to-passage colonies (e), which are cut into small pieces and transferred to new PMEDSAH-coated plates where (f) new colonies appear after 24–48 h. (g–l) Expression of pluripotency-associated transcription factors (OCT4 and SOX2) and surface markers (SSEA-3, SSEA-4, TRA1-60 and TRA1-81). In histograms, gray area is fluorochrome control.

TABLE 1

Defined substrates developed for long-term growth of hES cells in a feeder-free microenvironment.

	Method of preparation	Authors
<i>Biological components</i>		
Recombinant E-cadherin	Absorption by surface	Nagaoka <i>et al.</i> ⁹
Recombinant laminin-511	Absorption by surface	Rodin <i>et al.</i> ¹⁰
Recombinant vitronectin	Absorption by surface	Braam <i>et al.</i> ¹¹
RGD and vitronectin heparin-binding peptide	Biotinylated peptides bound to streptavidin-coated surface	Klim <i>et al.</i> ¹²
RGD domains of bone-sialoprotein and vitronectin	Peptides bound to acrylate-coated surface	Melkounian <i>et al.</i> ¹³
<i>Synthetic components</i>		
PMEDSAH	Polymer synthesis by surface-initiated graft polymerization	Villa-Diaz <i>et al.</i> ⁸

Two different groups of defined substrates have been developed based on biological and synthetic components. Compared with substrates based on biological components, synthetic polymer coatings can be fabricated reproducibly, are inexpensive and are highly manipulable.

TABLE 2

Troubleshooting table.

Step	Problem	Possible reason and solution
9	Changes in the optical transparency of PMEDSAH-coated TCPS dishes	The temperature of the monomer solution increased above 85 °C during the polymer reaction. Adjust the heating mantle controller and carefully monitor the solution temperature
19–21	Low attachment of hES cells	<p>The colonies passaged were not ready to passage. Take care to select colonies with denser and brighter centers compared with edges</p> <p>Cell-aggregate size is too small. In our experience, cell aggregates acquired from ready-to-passage colonies containing >50 cells attach well to PMEDSAH-coated TCPS dishes</p> <p>The medium was changed too soon. Take no action on the day after passage. If low attachment is observed after 48 h, add 0.5 ml of fresh pre-equilibrated medium and wait an additional 24 h before proceeding to Step 21</p>
19–21	Presence of differentiated cells	<p>Poor selection of undifferentiated colonies during passages and culture. Undifferentiated and ready-to-passage hES cell colonies have clear and well-defined borders and centers that are denser and brighter than the edges</p> <p>Cell culture medium has expired. Use medium within 2 weeks after preparation; use MEF-CM collected during the first 3 d of conditioning</p>