Feeder-free culture of human embryonic stem cells in conditioned medium for efficient genetic modification

Stefan R Braam[1,2], Chris Denning[3], Elena Matsa[3], Lorraine E Young[3], Robert Passier[1,2] & Christine L Mummery[1,2]

[1]Hubrecht Institute, Developmental Biology and Stem Cell Research, Uppsalalaan 8, 3584 CT, Utrecht, The Netherlands. [2]Leiden University Medical Centre, Department of Anatomy and Embryology, Leiden, Postal Zone S-1-P, P.O. Box 9600, 2300 RC Leiden, The Netherlands. [3]Wolfson Centre for Stem Cells, Tissue Engineering and Modelling (STEM), Centre for Biomolecular Sciences, University of Nottingham, Nottingham NG7 2RD, United Kingdom. Correspondence should be addressed to C.D. or C.L.M. (chris.denning@nottingham.ac.uk or c.l.mummery@lumc.nl).

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Realizing the potential of human embryonic stem cells (hESCs) in research and commercial applications requires generic protocols for culture, expansion and genetic modification that function between multiple lines. Here we describe a feeder-free hESC culture protocol that was tested in 13 independent hESC lines derived in five different laboratories. The procedure is based on Matrigel adaptation in mouse embryonic fiboblast conditioned medium (CM) followed by monolayer culture of hESC. When combined, these techniques provide a robust hESC culture platform, suitable for high-efficiency genetic modification via plasmid transfection (using lipofection or electroporation), siRNA knockdown and viral transduction. In contrast to other available protocols, it does not require optimization for individual lines. hESC transiently expressing ectopic genes are obtained within 9 d and stable transgenic lines within 3 weeks.

INTRODUCTION

Human embryonic stem cells (hESCs) hold great promise as models for human development and disease, as well as for drug discovery and cell-replacement therapies. Progress toward these goals has been impeded by technical issues, exemplified by the lack of generic strategies to culture multiple hESC lines in a format that is permissive to high-efficiency genetic manipulation. Most protocols are optimized on individual hESC lines and so do not readily translate effectively between independently derived lines or between laboratories. It is not surprising that most optimization is restricted to specific hESC lines given the labor intensiveness of maintaining multiple lines and the desire to select lines with greater propensity to differentiate toward particular lineages that are relevant to the research goals of the laboratory. For example, expression of the endodermal marker, α-fetoprotein, was up to 3,000-fold higher in differentiating HUES-8 cells than 16 other HUES lines derived, cultured and differentiated in parallel conditions by the same group[1].

Recently a variety of methods for genetic manipulation of hESC have been described including siRNA knockdown and transient and stable overexpression. DNA delivery was often inefficient and largely dependent on viral vectors (reviewed in ref. 2). To date only four labs have reported successful gene targeting in hESC[3–7]. In general all of these methods have relied on the use of feeder cells to maintain the hESC in an undifferentiated state. Drug selection has consequently necessitated the use of either drug resistant feeders or resupplementation of feeders during the procedure to compensate drug-induced feeder loss. Feeder-layers also limit the transfection efficiency[8] and are a major source of variability, as illustrated by a recent study in which a single plasmid transfection protocol applied to several independently derived lines resulted in transfection efficiencies ranging from 3 to 35% (ref. 9).

To develop highly efficient generic transfection in hESCs, we tested protocols in 13 different lines (BG01, HES-2, ENVY, HUES-1, -5, -7, -15, HESC-NL1, -2, -3, -4, NOTT-1 and -2). These lines were derived in five independent laboratories and grown under the most diverse conditions we had available: mechanical passage on mouse embryonic fibroblasts (MEFs) in serum-containing medium, mechanical passage on human feeders in KnockOut-serum replacement medium and enzymatic passage on MEFs in KnockOut-serum replacement medium. hESC lines were temporarily transferred to feeder-free conditions at high density, where they adapted quickly in the absence of gross karyotypic changes (tested by G-banding for HUES-7, HESC-NL-1,-2 and NOTT-1,-2). The expression of high levels of stem cell markers was reproducible in all lines[8], making the cells particularly suitable for studying stemness and signal transduction. Furthermore we found hESC grown under these conditions particularly suitable for proteomics studies[10]. Replating hESC at lower densities resulted in a substantial increase in genetic modification efficiency, enabling efficiencies of up to 90% for chemical transfection and viral transduction and 50% for electroporation in all hESC lines tested. The culture conditions also supported clonal growth. Stably transfected cells could then be returned to their original growth conditions, if required, where they retained their differentiation capacity[8].

The protocols described here result in a robust, reproducible, simple and efficient platform for hESC culture that allows highly efficient transfection/transduction without altering self-renewal and pluripotency. The major difference from procedures described by others previously is the use of Matrigel in combination with feeder cell-conditioned medium (CM) to culture the cells in a true monolayer rather than in tight colonies of multilayered cells. Although our protocol is not based on potentially clinically compliant, xenoreagent free growth media and substrates recently described by others[11,12], for all nontherapeutic uses of hESC, requiring transient or sustained gene expression, the method will be extremely useful. Applications include genetic lineage marking using tissue-specific promoter–reporter constructs to select subpopulations of (differentiated) cells, introduction of gene constructs for targeting and knockin strategies, ectopic overexpression

and siRNA-mediated knockdown, including high-throughput approaches using siRNA or gene expression libraries. The protocol is applicable to any research requiring high-efficiency introduction of genes or gene constructs into hESC.

Experimental design

DNA construct and transfection. Vectors for use in hESCs can be generated using either conventional restriction enzyme-based plasmid cloning or recombineering[13]. If a source of genomic DNA is required in the cloning process, bacterial artificial chromosome DNA is recommended because of its high quality.

Successful expression of the transgenic cassette is particularly dependent on the heterologous promoter and the use of the phosphoglycerate kinase (PGK) or CAG (chicken β-actin/CMV hybrid) promoter is recommended. However even with these promoters, locus-dependent silencing can occur. This is likely related high-level expression of *de novo* DNA methyltransferases in hESCs, causing methylation of CpG islands and rendering the promoter inactive[14]. This problem can be partially resolved by using bicistronic cassettes that enable continuous drug selection of the cells retaining transgene over expression. For example, we have successfully used the PGK-green fluorescent protein-internal ribosome entry site-neomycin phosphotransferase (pPGK-GFP-IRES-Neo) or pCAG-GFP-IRES-PAC (puromycin-N-acetyltransferase) cassettes[8,15], which allow selection by neomycin/G418 or puromycin, respectively. These expression cassettes also provide validated controls for performing transient and stable transfections/transductions. Alternatively, for vector-based microRNA gene knockdown, we have successfully used the pcDNA6.2-GW/EmGFP-mIR from Invitrogen. Although green fluorescent protein (GFP) and the microRNA are both driven by a CMV promoter, this vector is suitable for transient (but not stable) transfections.

MATERIALS

REAGENTS

- HUES1, -5, -7, -15 (supplied by Harvard University)[16]
- NOTT1 and NOTT2 (derived by the University of Nottingham[17] and available from the UK stem cell bank)
- BG01 (National Stem Cell Bank, NSCB)[18]
- HES2[19] (NSCB)
- Envy[20] (ES Cell International)
- HESC-NL LINES1, -2, -3, -4 (derived by the Hubrecht Institute; ES Cell International)
- HEK293T (ATCC, cat. no. CRL-11268)
- MEF (strain CD1; 13.5 d post coitum (for protocol, see ref. 21))
- PBS (Invitrogen, Gibco, cat. no. 14040)
- PBS, without $MgCl_2$ and $CaCl_2$ (Invitrogen, Gibco, cat. no. 14190)
- Opti-MEM I reduced-serum medium (Invitrogen, Gibco, cat. no. 31985)
- Genejammer (Stratagene, cat. no. 204130)
- Lipofectamine 2000 (Invitrogen, cat. no. 11668-019)
- AllStars negative control siRNA (20 nmol), Alexa488 conjugated (Qiagen, cat. no. 1027292)
- Matrigel growth factor, reduced (BD, cat. no. 354230)
- 0.05% Trypsin-EDTA (Invitrogen, Gibco, cat. no. 25300)
- Geneticin (Invitrogen, Gibco, cat. no. 11811)
- Puromycin (Invivogen, cat. no. ant-pr-1)
- Plasmocin (Invivogen, cat. no. ant-mpt)
- Mycoalert kit (Cambrex, cat. no. LT07-118)
- psPAX2 (Addgene, cat. no. 12260)
- pMD2G (Addgene, cat. no. 12259)
- pWPI (Addgene, cat. no. 12254)
- KaryoMAX Colcemid Solution, liquid (10 μg ml^{-1}), in PBS (Invitrogen, Gibco, cat. no. 15212) ! **CAUTION** Wear safety glasses and protective gloves.
- DMEM/F12 (1:1) (1×), liquid—with GlutaMAX (Invitrogen, Gibco, cat. no. 31331)
- KnockOut Serum Replacement (Invitrogen, Gibco, cat. no. 10828)
- Nonessential amino acids (Invitrogen, Gibco, cat. no. 11140)
- Penicillin/streptomycin (Invitrogen, Gibco, cat. no. 15070)
- β-mercaptoethanol (Invitrogen, Gibco, cat. no. 31350-010)
- Basic fibroblast growth factor (bFGF; Peprotech, cat. no. 100-18b) ▲ **CRITICAL** Specific activity of bFGF may vary among companies.
- DMEM (Invitrogen, Gibco, cat. no. 11960)
- FCS (Sigma, cat. no. F7524)
- GIn (Invitrogen, Gibco, cat. no. 25030)
- Hybrimax DMSO (Sigma, cat. no. D2650) ! **CAUTION** Keep away from sources of ignition. Take measures to prevent the build up of electrostatic charge. Wear safety glasses and protective gloves.
- Mitomycin C (Sigma, cat. no. M0503) ! **CAUTION** Do not breathe dust. Do not get in eyes, on skin, on clothing. Avoid prolonged or repeated exposure. Use respirators and components tested and approved under appropriate government standards such as NIOSH (US) or CEN (EU). Wear compatible chemical-resistant gloves and chemical safety goggles.
- Methanol (Sigma, cat. no. 322415) ! **CAUTION** Avoid contact with skin and eyes. Avoid inhalation of vapor or mist. Keep away from sources of ignition. Take measures to prevent the build up of electrostatic charge. Work in a chemical hood, wearing safety glasses and gloves.
- Glacial acetic acid (Sigma, cat. no. 695084) ! **CAUTION** Do not breathe vapor. Do not get in eyes, on skin, on clothing. Avoid prolonged or repeated exposure. Work in a fume hood wearing compatible chemical-resistant gloves and chemical safety goggles.
- Leishman's stain (Sigma, cat. no. L6254)
- Sodium citrate (Fisher, cat. no. S/3380/53)
- Di-sodium-hydrogen-ortho-phosphate (Na_2HPO_4; Fisher, cat. no. P285-500)
- Potassium-di-hydrogen-ortho-phosphate (KH_2HPO_4; Fisher, cat. no. BP332-500)

EQUIPMENT

- Tissue culture incubator, humidified 5% CO_2 atmosphere
- Tissue culture hood
- Stereomicroscope (Leica, cat. no. MZ7.5)
- *In vitro* fertilization (IVF) organ dishes (Falcon, cat. no. 353037)
- 6-well culture plates (Greiner, cat. no. 657160)
- 12-well culture plates (Greiner, cat. no. 665120)
- 24-well culture plates (Greiner, cat. no. 662160)
- 25 cm^2 Tissue culture flask (Greiner, cat. no. 690160)
- Cryo Ampoules (Greiner, cat. no. 123263)
- Electroporator (Gene pulser; Bio-Rad)
- Electroporation cuvettes (Eurogentec, cat. no. ce-004-06)
- Nalgene* 'Mr. Frosty' Freezing Container (Fisher Scientific, cat. no. 15-350-50)
- Amicon Ultra-15 Filter Unit (100 NMWL; Millipore, cat. no. UCF910008)

REAGENT SETUP

hESC medium DMEM/F12 (1:1) (1×), liquid—with GlutaMAX, 15% (vol/vol) KnockOut Serum Replacement, 10 mM nonessential amino acids, 0.1% (vol/vol) penicillin/streptomycin, 100 μM β-mercaptoethanol, 4 ng ml^{-1} bFGF. The medium can be stored at 4 °C for 1 month.

Medium for MEFs and HEK293T cells DMEM, 10% (vol/vol) FCS, 0.5% (vol/vol) penicillin/streptomycin, 1% (vol/vol) GIn, 10 mM nonessential amino acids. The medium can be stored at 4 °C for 1 month.

Freezing medium 20% Hybrimax dimethylsulphoxide, 80% (vol/vol) FCS. Prepare fresh.

MMC treatment MEFs Confluent MEFs are mitotically inactivated for 2.5 h with mitomycin C (10 μg ml^{-1} in MEF medium). Cells are washed with MEF medium and then twice with PBS, trypsinized (0.05% trypsin for 2–3 min. This should yield a single cell suspension) and seeded at 6.4 × 10^4 cells/cm^2 in MEF medium.

Karyotype fixative Five parts methanol: one part glacial acetic acid. Prepare fresh.

Leishman's Stain 1.5 g Leishman's stain added to 1 l of methanol. The stain is left to 'mature' for several days at room temperature (18–20 °C) before use. To produce a working solution, dilute Leishman's stain 1 in 5 with Sorenson's buffer immediately before use.

Sorenson's buffer 9.47 g di-sodium-hydrogen-ortho-phosphate and 9.08 g potassium-di-hydrogen-ortho phosphate made up to 1 l with deionized water. The solution can be stored at room temperature for 1 month.

Trypsin for G-banding 1.2 g of trypsin dissolved in 1 l of Sorenson's buffer for 20 min. Decant solution into 20 ml aliquots and store at −20 °C until use.

PROCEDURE

MEF conditioning ● TIMING Day 1–8

1| Allow mitomycin C-inactivated MEFs (see REAGENT SETUP) to attach for a minimum of 4 h, preferably 24 h. Wash with PBS and replace medium for hESC medium. Use 25 ml for a T75 flask.

2| After 24 h, harvest MEF CM and replace with fresh unconditioned hESC medium. Add 4 ng ml^{-1} bFGF to the fresh CM. Harvest the CM for up to seven consecutive days. Filtration is not essential but helps to remove any dead fibroblast cells. CM can be used fresh or stored frozen at −20 °C or −80 °C for up to 6 months.

Aliquotting Matrigel ● TIMING 15 min

3| Thaw one bottle of Matrigel overnight at 4 °C in at least 500 g ice.

4| Transfer the bottle on ice to a tissue culture hood.

5| Pippette 500 µl Matrigel to each sterilized prechilled Eppendorf tube using prechilled pipettes.
▲ CRITICAL STEP Keep Matrigel on ice because it naturally polymerizes as the temperature rises above 4 °C.

6| Freeze aliquots immediately at −20 °C or −80 °C for up to 6 months.

Matrigel coating ● TIMING 1 h

7| Thaw and dilute one aliquot of Matrigel (0.5 ml) by repetitive pipetting in 50 ml of cold DMEM/F12 (directly from the fridge).

8| Pipette the diluted Matrigel immediately into culture vessels and allow to polymerize for at least 45 min at room temperature. Note that the layer of polymerized Matrigel is only a few micrometers thick and should not be visible even under the microscope. Appearance of lumpy areas indicates premature polymerization. Use 0.5 ml for 24-well plates, 1 ml for IVF organ culture and 12-well plates, 2 ml for 6-well plates, 5 ml for T25 and 12 ml for T75.

9| Plates can be used immediately or stored at 4 °C. Before use, aspirate excess medium and unpolymerized Matrigel, and then rinse once with PBS.
▲ CRITICAL STEP Never let Matrigel dry out as this causes irreversible loss of extracellular matrix properties.
■ PAUSE POINT For storage, wrap plates and dishes with Parafilm to prevent contamination and drying of the Matrigel. Use the plates within 4 weeks of preparation.

Transfer of hESC to feeder-free culture ● TIMING Day 2–7

10| Start with a high-quality undifferentiated hESC culture (**Fig. 1a,b**). Using a glass needle, slice 10 colonies from one IVF organ dish (**Fig. 1c**), release the cells by vigorously pipetting with a P1000 Gilson pipette and transfer them to two Matrigel-coated IVF organ dishes containing 1 ml CM (from Step 2). At this stage dispase may be used to release the cells from the feeders. In our experience dispase helps to release the cells but is not necessary if the cells are cultured, for example, on human foreskin fibroblast feeders.
▲ CRITICAL STEP Steps 10–15 are specifically for cells maintained by mechanical 'cut and paste' passaging. For optimal maintenance of genetic stability, hESC cultures should, in our experience, be routinely maintained by mechanical passaging[7,22] and scaled-up for experimentation for up to 10–20 enzymatic passages under feeder-free conditions. For cells already adapted to enzymatic passage, go to Step 12.

11| Refresh CM daily for 4–5 d while cells are spreading and growing (**Fig. 1d,e**) until colonies start to touch each other. When confluent, remove 3D differentiated areas from the middle of a colony with a glass needle or P200 Gilson pipette (**Fig. 1f**).

12| Wash remaining attached undifferentiated cells with PBS, add 200 µl trypsin and incubate for 2–3 min at 37 °C.
▲ CRITICAL STEP Short incubation will not release cells while too long will damage the cells, decrease cell survival and may result in premature karyotypic change.

13| Add 1 ml hESC medium and resuspend the cell suspension vigorously with a P1000 to release the cells. Collect the cells into a 15-ml centrifuge tube, add another 4 ml hESC medium to dilute the trypsin and pellet the cell suspension (180*g*, room temperature, 4 min).
▲ CRITICAL STEP Trypsinization and resuspension should yield a cell suspension of smaller and larger hESC clumps.

14| After centrifugation, resuspend the cells in 1 ml CM and replate at very high density ($\sim$1–2.10^5/cm^2), i.e., from two organ dishes to one Matrigel-coated IVF organ dish. A small proportion of cells die during this first enzymatic passage.

15| The next day replace the medium with 1 ml fresh CM and carefully check morphology of individual cells at $\times$100–400 magnification. Keep refreshing the medium daily until the cells form a confluent monolayer. Generally, this takes 1–2 d. Confluent cells growing in a monolayer should be morphologically similar to individual cells in colonies (**Fig 1g**).
▲ CRITICAL STEP The medium may be very acidic (yellow). This is normal and does not influence the results.
? TROUBLESHOOTING

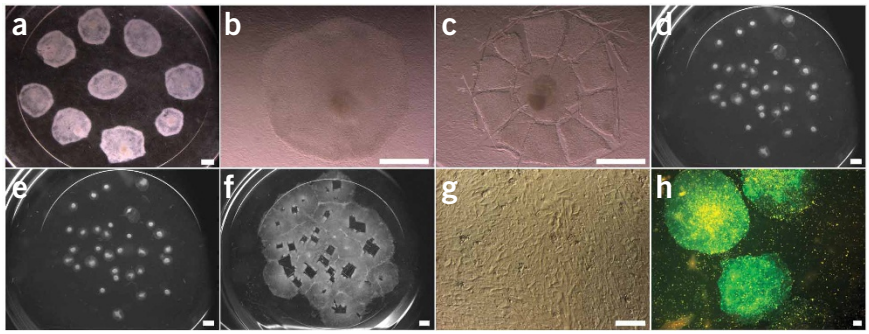

Figure 1 | Photographs of human embryonic stem cell (hESC) at different stages. (**a**,**b**) Day 7 hESC colonies grown on mouse embryonic fibroblasts (MEFs). (**c**) Day 7 hESC colony sliced before dislodgement and transfer to Matrigel. (**d**) hESC grown on Matrigel in conditioned medium at day 1. (**e**) hESC grown on Matrigel in conditioned medium at day 2. (**f**) hESC grown on Matrigel with the central area removed; these cultures are ready for trypsinization. (**g**) hESC monolayer culture on Matrigel. (**h**) Green fluorescent protein positive primary hESC colonies after Genejammer transfection with a GFP vector. Scale bars: 1 mm (**a**–**f**,**h**) and 100 μm (**g**).

16| When confluent, wash cells with PBS and add 200 μl trypsin for exactly 1.5 min. Dilute cells/trypsin in at least 5 ml of hESC medium and centrifuge immediately to remove the trypsin (180*g*, room temperature, 4 min). Resuspend in 2 ml CM and seed at appropriate split ratio; depending on cell line, split ratios are usually 1 in 3 and should reach confluency after 48–72 h. Some fast-growing lines can be split occasionally in a 1 in 5 ratio.
▲ CRITICAL STEP Cells split at low density that are cultured for at least 72 h might require slightly longer trypsin incubations. In general, using a 1 in 3 split max, 1.5 min Trypsin should be enough to yield a cell suspension with some single cells and clumps of 3–10 cells.
? TROUBLESHOOTING

17| Scale culture up to T25 flasks without exceeding a 1 in 3–5 split ratio and with daily replacement of CM. This culture method preserves genetic integrity for at least 10–20 passages for the lines tested in longer-term culture (HUES7, NOTT-1, -2, HESC-NL-1, -2). hESC cultured in this system have been shown to be highly positive ($>$90%) for all stem cell markers tested (OCT3/4a, SOX2, GCTM2, Tra-1-60, SSEA4)[8]. The culture is now ready for further manipulation or cryopreservation (as described in **Box 1**).
? TROUBLESHOOTING

18| The following options can be used to transfect/transduce the cultured hESCs with desired sequences to obtain stably transfected transgenic cells. Option A can be followed for siRNA transfection, option B for Genejammer plasmid transfection, option C for electroporation and option D for lentiviral transduction.
▲ CRITICAL STEP Some hESC clones will progressively silence stably transfected constructs even when an appropriate promoter is used. It is highly recommended to include a reporter construct (e.g., fluorescent protein or drug resistance marker) in the vector to allow visualization or continuous drug selection of transgene expression.

(A) siRNA transfection ● TIMING 3 d

(i) The day before transfection, trypsinize cells as described in Step 16 and plate in CM at a density of 1–2 $\times$ 10^5 cells per well onto a Matrigel-coated 12-well plate.

(ii) For each well to be transfected, prepare siRNA-Lipofectamine complexes as follows: dilute 3 μl siRNA in 75 μl of Opti-MEM I medium and mix by flicking the tube.
Note: The stock siRNA solution should be made at 20 μM, according to the manufacturers' instructions.

(iii) Mix Lipofectamine 2000 by inversion before use and then dilute 1.5 μl in 75 μl of Opti-MEM I medium. Mix gently and incubate for 5 min at room temperature.

BOX 1 | CRYOPRESERVING CELLS ● TIMING 30 MIN

1. Trypsinize a confluent T25 culture flask as described in Step 16, resuspend in hESC medium and pellet cells.
2. After centrifugation, resuspend the cell pellet in 750 μl of 100% FCS on ice.
3. Add 750 μl freezing medium, mix gently and divide between three ampoules (final concentration of DMSO is 10%).
4. Immediately place ampoules in a Nalgene cryopreservation container containing propan-2-ol. Transfer to −80 °C and, after 24 h, to liquid nitrogen for long-term storage.

(iv) After the 5-min incubation, combine the diluted siRNA with the diluted Lipofectamine 2000; total volume is 154.5 µl. Mix gently and incubate for 20 min at room temperature to allow the siRNA:lipid complexes to form.
▲ CRITICAL STEP It is highly recommend to use a fluophore-conjugated siRNA as control to monitor transfection efficiency.
(v) Aspirate medium from target cells and replace with 450 µl of CM.
(vi) Add the 154.5 µl of siRNA:Lipofectamine complexes dropwise to each well. This gives a final concentration of 100 nM siRNA. Mix gently by rocking the plate back and forth.
(vii) Add 1.5 ml CM after 4 h.
(viii) Incubate the cells at 37 °C for 48 h changing CM daily, and analyze 1–5 × 10^4 cells by fluorescence-activated cell sorting (FACS)[23] or fluorescence microscopy[24].
? TROUBLESHOOTING

(B) Genejammer plasmid transfection ● TIMING 3 d

(i) The day before transfection, trypsinize cells as described in Step 16 and plate cells in CM at a density of 1–2 × 10^5 cells per well onto a Matrigel-coated 12-well plate.
(ii) For each transfection sample, prepare DNA-Genejammer complexes by mixing 5.25 µl Genejammer to 75 µl Opti-MEM I and incubate at room temperature for 10 min. Add 1.75 µg target vector to the solution and mix gently. Incubate at room temperature for a further 10 min.
▲ CRITICAL STEP It is highly recommended that a vector encoding a fluorescent protein (e.g., pPGK-GFP-IRES-Neo or pCAG-GFP-IRES-PAC) be used as a control to monitor transfection efficiency.
(iii) Replace medium with 400 µl CM.
(iv) Add the 82 µl of DNA:Genejammer complexes dropwise to each well. This gives a final concentration of 3.6 ng μl^{-1} plasmid. Mix gently by rocking the plate back and forth.
(v) Add 1.5 ml CM after 4 h.
(vi) Incubate the cells at 37 °C for 48 h changing CM daily, and analyze 1–5 × 10^4 cells by FACS[23] or fluorescence microscopy[24].
? TROUBLESHOOTING

(C) Electroporation ● TIMING 2 d

(i) Trypsinize a confluent T25 culture flask as described in Step 16.
(ii) After centrifugation (180*g*, room temperature, 4 min), resuspend the cells in 800 µl CM containing 15–50 µg linearized DNA.
(iii) Transfer the 800 µl cell/DNA solution to a 4 mm gap electroporation cuvettes and incubate for 5 min at room temperature.
(iv) Flick the cuvette to ensure a homogeneous cell suspension and electroporate at 320 V/240 micro Faradays (µF).
▲ CRITICAL STEP It is highly recommended that a vector encoding a fluorescent protein (e.g., pPGK-GFP-IRES-Neo or pCAG-GFP-IRES-PAC) be used as a control to monitor transfection efficiency.
(v) Incubate for 5 min at room temperate and resuspend the cells in at least 10–15 ml of CM.
▲ CRITICAL STEP If smaller numbers of cells are electroporated, resupend cells in at least 5 ml CM to prevent cell death due to debris and DNA toxicity.
(vi) Plate cells on 2–3 Matrigel-coated 60 mm dishes. This is largely dependent on growth characteristics of a particular hESC line.
(vii) Incubate the cells at 37 °C for 48 h changing CM daily, and analyze 1–5 × 10^4 cells by FACS[23] or fluorescence microscopy[24].
? TROUBLESHOOTING

(D) hESC lentiviral transduction ● TIMING 6 d

(i) To prepare lentiviral particles for hESC transduction, first seed a T175 culture flask with 5 × 10^6 HEK293T cells in 20 ml of medium and allow the cells to attach overnight in the incubator. HEK293T cells are used as a packaging cell line for the production of a viral supernatant that contains infectious particles.
(ii) For each flask of HEK293T cells to be transfected, combine into 2 ml of Opti-MEM I medium, 50 µg of packaging plasmid (e.g., psPAX2), 20 µg of envelope plasmid (e.g., pMD2G) and 67 µg transfer plasmid (e.g., GFP-expressing plasmid such as pWPI).
▲ CRITICAL STEP These plasmids will generate second-generation, replication-defective lentiviral particles. Ensure the transfer plasmid to be used is second generation as first- and third-generation systems will require different combinations of plasmid.
(iii) To a separate tube, add 40 µl of Lipofectamine 2,000 to 2 ml of Opti-MEM I medium and mix gently. Incubate for 5 min at room temperature.

(iv) After the 5-min incubation, combine the diluted plasmid DNAs with the diluted Lipofectamine 2000. Mix gently and incubate for 20 min at room temperature to allow the DNA:lipid complexes to form.
(v) Aspirate medium from target HEK293T cells and replace with 16 ml of Opti-MEM I medium.
(vi) Add the DNA:lipid complexes (~4 ml) to each T175 flask. Mix gently by rocking the plate back and forth, and incubate for 4 h.
(vii) Aspirate the transfection medium and replace with 20 ml HEK293T medium.
(viii) Incubate for 48 h, during which time lentiviral particles will be produced and released into the medium.
(ix) Trypsinize hESC as described in Step 16 and plate cells in CM at a density of 5×10^4 cells per well onto a Matrigel-coated 6-well plate. Allow the cells to attach for 4–6 h in the incubator.
(x) To harvest lentiviral particles for the transduction of hESCs, harvest the medium (20 ml) from the HEK293T cells. This supernatant contains the replication-defective lentiviral particles.
(xi) Pass the lentiviral supernatant through a 0.2-μm filter to eliminate any detached HEK293T cells.
(xii) Concentrate the virus by transferring to an Amicon Ultra-15 Filter Unit by centrifuging at 4,000*g* for 20 min at room temperature. This will provide 200 μl of concentrated viral supernatant.
▲ CRITICAL STEP For best results, use the virus fresh. Storage at 4 °C or −80 °C can reduce the number of active transducing units in the supernatant.
(xiii) Aspirate medium from the hESC and replace with 4 ml CM. Add increasing amounts of the concentrated virus (i.e., add no virus to well 1 of the 6-well plate of hESCs, 1 μl to well 2, 3.3 μl to well 3, 10 μl to well 4, 33 μl to well 5 and 100 μl to well 6).
(xiv) Incubate the cells at 37 °C for 72 h changing CM daily and analyze 1–5 × 10^4 cells by FACS[23]. First, plot FACS-based transduction efficiency on the *y*-axis of a graph and volume of viral supernatant on the *x*-axis. Second, from a linear point on the curve, read off the transduction efficiency and volume of viral supernatant. Put these figures into the following equation:

$$\text{No. of transducing particles}/\mu\text{l} = \frac{\%\ \text{transduction efficiency} \times \text{starting hESC number}}{100\ (\text{converts percentage}) \times \text{volume of viral supernatant}}$$

For example, if 20 μl of viral supernatant resulted in 50% transduction efficiency of 5×10^4 cells, then:

$$\begin{aligned}\text{No. of transducing particles}/\mu\text{l} &= \frac{50 \times 5 \times 10^4}{100 \times 20} \\ &= 1,250/\mu\text{l} \\ &= 1.25 \times 10^6/\text{ml}\end{aligned}$$

▲ CRITICAL STEP Since a minimum of 1 active transducing particle is required to infect 1 cell, knowing the number of transducing particles that are present allows the correct numbers of active transducing particles to be incubated with the target cell population.
? TROUBLESHOOTING

Selection of stably transfected cells ● TIMING **14–21 d**

19| Apply selection 48 h after electroporation, Genejammer transfection or viral transduction while changing CM daily. By this stage the dish should be ~60–90% confluent. The recommended final concentration for the antibiotic G418 is 50 μg ml^{-1} whereas puromycin is 300 ng ml^{-1}.
▲ CRITICAL STEP If cells are too confluent, the action of the antibiotics will be delayed or ineffectual and death of untransfected cells will not be observed.
▲ CRITICAL STEP The antibiotic may need to be titrated and optimized for different hESC lines.

20| Suspend antibiotic selection when ~50–80% of the cells are killed. This slows the kill rate so that residual untransfected cells act as feeder cells to the stably transfected cells to reduce the stress of clonal growth.
▲ CRITICAL STEP When cells are growing extremely fast this step may not be necessary. However it greatly improves clonal growth, especially with slower growing cells.

21| Restart antibiotic selection after an additional 2 d, while changing CM daily. It is advisable to maintain selection thereafter to eliminate cells that may silence the transgene.
? TROUBLESHOOTING

22| After ~14 d individual colonies should be visible (**Fig. 1h**).
? TROUBLESHOOTING

Colony transfer ● TIMING 24 colonies can be picked in ~ 30 min

23| Let the colonies grow until they are ~3 mm in size or until they start to touch each other. Using a P200 pipette or glass needle, slice colonies into a grid motif and transfer to 12- or 24-well plates. RNA or DNA analyses can be performed directly on lysed colony fragments or later from the expanded cell populations. Cells can be cultured further on Matrigel or feeders, as required.

24| Check clones for their karyotype, as below, and differentiation potential[12].

Karyotype procedure for G-band analysis ● TIMING Harvesting cells takes 2 h. Total time 2 d

25| To hESC cultures in log phase growth, add KaryoMAX Colcemid to the medium at a 1:100 dilution to give a concentration of 100 ng ml^{-1} (50 µl into 5 ml). Incubate at 37 °C for 60 min.

26| Remove medium + KaryoMAX + floating cells to a tube. Passage as in Step 12, add cells to the same tube as before and collect by centrifugation.

27| Aspirate and resuspend the pellet in 0.3 ml hESC medium with a P1000 pipette.
▲ **CRITICAL STEP** Pipetting ensures that a single-cell suspension is produced to prevent clumping and to maximize swelling in the hypotonic solution.

28| Add 8 ml of fresh hypotonic solution (0.6% sodium citrate in H_2O) dropwise while vortexing.
▲ **CRITICAL STEP** Adding the hypotonic solution dropwise reduces the risk of osmotic shock.

29| Incubate cells for 20 min at room temperature to swell.

30| Centrifuge the cell suspension at 300*g* for 5 min. Aspirate and resuspend in 0.3 ml of hypotonic solution with a P1000 pipette.
▲ **CRITICAL STEP** Pipetting ensures that a single-cell suspension is produced to prevent clumping in the fixative.

31| Add 8 ml karyotype fixative dropwise while vortexing.

32| Spin the cell suspension (300*g*, room temperature, 4 min) and resuspend the pellet in fixative. Then repeat this step twice more.

33| Centrifuge and resuspend the pellet in a 0.5–1 ml of fix.
■ **PAUSE POINT** Cells may be stored at −20 °C.

34| Drop cells from a height of ~30 cm onto polished slides to burst the cells and spread the metaphase chromosomes. Incubate slides at 75 °C overnight before G-banding.

35| Treat the slides with trypsin for 3 min and then with diluted Leishman's stain (diluted 1 in 5 with Sorenson's buffer immediately before use, see REAGENT SETUP) for 2 min. This is carried out in a Coplin jar, which takes a volume of approximately 100 ml. Finally, rinse with water and air dry.

36| Analyze 30 metaphase spreads per sample line to determine karyotype to >95% confidence limits. This highly specialized procedure is best performed by a clinical cytogenetics service division.
? TROUBLESHOOTING

● TIMING
See flowchart (**Fig. 2**).

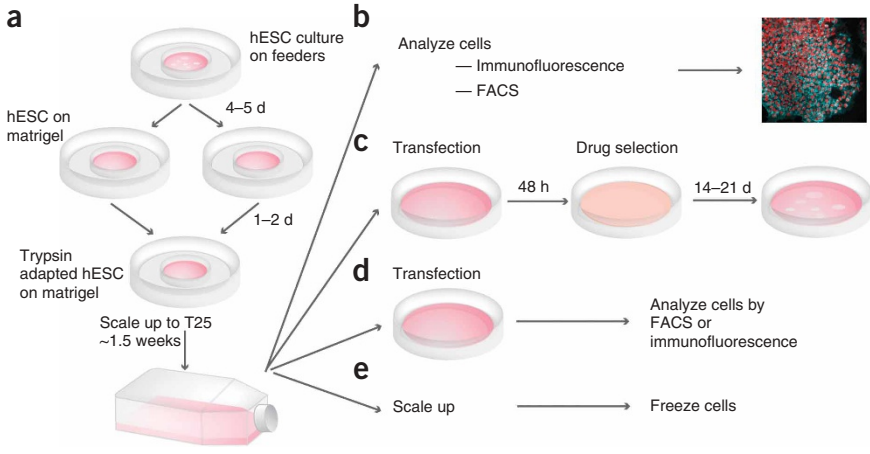

Figure 2 | Flowchart for experimental procedures. (**a**) hESCs grown on feeders are plated on Matrigel and grown for an additional 4–5 d. Differentiated 3D structures are removed and hESCs are trypsinized, replated in MEF-conditioned medium at high density by combining cells from two equivalent-size plates, grown for another 1–2 d and split several times to scale up to at least a T25 culture flask. These cells can now be used for (**b**) immunofluorescence/FACS, (**c**) stable transfection, (**d**) transient transfection or (**e**) upscaling and freezing.

? TROUBLESHOOTING

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

TABLE 1 | Troubleshooting table.

Step	Problem	Solution
15–17	Cells dying or differentiating	Cell density too low. Culture cells until they are confluent and fully compacted. hESC interact with Matrigel, which leads to cell spreading and loss of their typical morphology in colonies. High-density cultures should have a similar morphology as hESC in colonies. Split cells 1:1 or max 1:2 until they look healthy. At this point hESC may be split 1:3–1:5 max
		Bad quality conditioned medium. In general, when feeders are capable of supporting hESC they should be suitable for feeder conditioning
		Mycoplasm infection. Check cultures regularly using a mycoalert kit (Cambrex LT07-118). If positive it is best to dispose of cultures as the infection can rapidly spread to other culture flasks. However, if the culture is very precious, it may be possible to cure the infection with plasmocin
		Low specific activity of bFGF. It is recommended that new batches bFGF be compared side by side with the previous batch. If problem persists, increasing the bFGF concentrations to 10 ng ml^{-1} may help
		Differentiating cultures can sometimes be rescued by stepwise trypsinization. Compacted hESC colonies are usually more difficult to trypsinize compared with differentiated (mesenchyme-like) cells. Trypsinize cells under a stereomicroscope and wash away differentiated cells using PBS. Use fresh trypsin to release the undifferentiated cells and seed them on a new Matrigel-coated dish. A 1:1 split is recommended at this stage
18A–D	Low transfection efficiency	Check for Mycoplasm infection regularly using a mycoalert kit (Cambrex, cat. no. LT07-118). Treatment as indicated for Steps 10–17
		Cell density too high. Lower cell densities gives generally much higher transfection efficiencies[8]. Lower the cell density
18B	Transfection induced toxicity	Use pure plasmid DNA from either a midi- or maxiprep. Endotoxin-free plasmid purification kits (e.g., from Qiagen) may also be beneficial
19–22	No drug resistant colonies	Use an appropriate promoter, such as phosphoglycerate kinase or chicken β-actin/cytomegalovirus hybrid promoter (CAG), which has been successfully used to make stable lines in our labs. The cytomegalovirus hybrid promoter alone is progressively silenced and gives no drug resistant clones
		Confirm that the expression vector works in another cell type, for example HEK293 or COS
		Cell culturing may be suboptimal. Ensure conditioned medium and Matrigel batches support routine hESC cultures
19–21	Cells not dying during drug selection	Increase drug concentration. It is recommended to titrate the antibiotic dose for each cell line and every new drug batch
		Cell density at onset of drug treatment too high. Reduce to ~60% confluence in next experiment
36	Karyotypic abnormalities	If normal cells are present, single-cell cloning can be considered. Otherwise restart the process, ensuring that the culture is karyotypically normal and growing exponentially

ANTICIPATED RESULTS

Starting with any high-quality undifferentiated culture of hESC cells and following Steps 1–17, hESC should yield a robust growing monolayer culture within 2 weeks. These cells should express high levels of hESC markers (e.g., OCT4, SOX2, NANOG, SSEA4, TRA1-81, GCTM2) and be amenable for genetic manipulation.

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