Growth of Human Pluripotent Stem Cells using Functional Human ECM

Andres Sanz-Garcia[1,2], Miodrag Stojkovic[3,4], and Carmen Escobedo-Lucea[1,2]

1. Tissue Engineering Group. Division of Pharmaceutical Biosciences, Faculty of Pharmacy, University of Helsinki, Finland
2. Institute of Advanced Biomedical Engineering and Science, Tokyo Women's Medical University TWINS. Tokyo, Japan
3. SPEBO Medical, Leskovac, Serbia
4. Department of Human Genetics, Faculty of Medicine, University of Kragujevac, Serbia

Correspondence: *Carmen Escobedo-Lucea, PhD*
1 Tissue Engineering Group. Centre of Drug Research (CDR). Division of Pharmaceutical Biosciences. Faculty of Pharmacy. .University of Helsinki, Viikinkaari 5 E (P.O. Box 56), 00014 Helsinki, Finland
2 Institute of Advanced Biomedical Engineering and Science. Tokyo Women's Medical University TWINS. 8-1 Kawada-cho, Shinjuku-ku, Tokyo 162-8666, Japan
carmen.escobedo-lucea@helsinki.fi

The final publication is available at link.springer.com

Human Embryonic Stem Cell Protocols

http://link.springer.com/protocol/10.1007/7651_2014_154

Abstract

The use of animal products in the derivation and maintenance of human pluripotent stem cells (hPSCs) limits their possible applications in research and in clinics. Thus, one of the major goals in regenerative medicine is the establishment of animal-free conditions to support the culture and differentiation of human stem cells. Human fibroblasts produce an extracellular matrix (ECM) which can be extracted without the use of detergents, sterilized, and then used to coat tissue culture plates.

We have shown that human embryonic stem cells (hESCs) grown on this matrix maintain their pluripotency in the presence of medium conditioned by fibroblast cells, and that these cells maintain expression of surface proteins (SSEA4, Tra1-60, Tra1-81), alkaline phosphatase activity and specific intracellular markers (Nanog, Oct-4, Tert, FoxD3) in hESCs. This growth system reduces exposure of hPSCs to feeder layers and animal ingredients, thereby limiting the risk of pathogenic contamination and additionally, facilitating their manipulation. Herein we present an improved version of our previous protocol for extracting ECM from human foreskin fibroblast using a different buffer. Our new hypotonic shock method is detergent-free, reduces costs, and preserves the integrity of the extracted ECM. This improved protocol has been validated for undifferentiated-state hPSC maintenance (more than 40 passages), stem cell differentiation and for cell migration assays.

Keywords: human embryonic stem cells; human pluripotent stem cells, *in vitro* growth; extracellular matrix, hypo-osmotic lysis buffer

1. Introduction

The stem cell biology field is continually transforming, especially in recent years with the introduction of several validated protocols for obtaining human-induced pluripotent stem cells (hiPSCs) ***(1,2)***, which have created new avenues for studying different diseases and regenerative medicine applications. Several human embryonic stem cell (hESC) and hIPSC lines have been derived and grown using mouse feeder layers, Matrigel®, or other animal reagents ***(3,4)***. However, recent successes using pluripotent stem cell (PSC) derivatives in clinical trials ***(5,6)*** have emphasized the need for improved quality standards before these derivatives are applied in cell therapies. In particular, new culture methods must be developed that circumvent the need for animal products because these might provoke infections or immune rejection following transplantation into patients ***(7)***. Thus, chemically defined culture systems that are devoid of nonhuman substances will greatly facilitate the use of human stem cells (hSCs) in therapies.

Extracellular matrix (ECM) modulates cellular adhesion processes and signaling inside stem cell niches and is essential for maintaining their structure and supportive qualities ***(8,9)***. An attractive alternative to the use of feeder cells, and the complications associated with them, is the development of an animal-free ECM, and there would be many advantages to maintaining PSCs on such supports. Indeed, undefined mixtures of natural and synthetic matrix proteins are already used as a coating for in vitro human stem cell culture ***(10)***, but unfortunately, most of these reagents are cost-prohibitive for many laboratories. Decellularization procedures are traditionally used to isolate ECM from cells and organs ***(11)***, facilitating remodeling in animal models and humans. However, the procedures used to remove the cells, especially detergent-based methods, can alter the native structure of the ECM ***(12,13)***.

We previously published a protocol which allows long-term growth of undifferentiated hESCs on human functional foreskin ECM (hffECM), extracted by using RIPA buffer and cultured using human foreskin conditioned medium, in the *Methods in Molecular Biology* series ***(14)***. Here we present a modification of our previous protocol which replaces RIPA with a simple, low-cost hypotonic buffer without detergents. The hffECM obtained is capable of maintaining the pluripotency of hESCs for more than 40 passages ***(12)***. Additionally, our hffECM has also been validated as a coating for directed-differentiation experiments by using both hESCs and mesenchymal stem cells (MSCs) and as a basis for cell migration studies ***(12,15,16)***.

2. Materials

DOI 10.1007/7651_2014_154 *Springer Science & Business Media New York*

2.1. Culture of Human Foreskin Fibroblast for Derivation of Conditioned Media and Human Functional Foreskin-derived Extracellular Matrix (hffECM)

1. Human foreskin fibroblasts (ATCC, Catalogue No. CRL-2429, Passages 11--18; *see* **Note 1**).
2. Iscove's medium (Sigma, St. Louis, MO), supplemented with 10% human serum (HS), 1% Glutamax (GIBCO, Invitrogen, Carlsbad, CA).
3. Gelatin (1.5%; Sigma). In a sterile bottle, add embryo-tested water (Sigma) to gelatin. Warm the mixture to 37º C in a water bath using a shaker. Store at 4º C or make aliquots and keep them frozen at -20º C. These aliquots can be stored for up to 6 months. Before proceeding with tissue culture, thaw the aliquots and dilute them to 0.01% with sterile Dulbecco's PBS (DPBS) (Invitrogen). Pass the solution through a 0.22 μm filter (Nalgene, Hereford, UK). Coat the culture surfaces by pipetting 1 mL/well into 6-well plates (BD, San Jose, CA) or 12 mL into a 75 cm^2 flask (Iwaki, Ibaraki, Japan). Allow the gelatin to settle at 37º C for 30 min. Plates may be used immediately or stored at 4º C to prevent evaporation.
4. Mitomycin C (Fluka, Buchs, Switzerland) is dissolved in Iscove's medium (Sigma) at 1 mg/mL, stored at 4º C and then added to the cultures as required.
5. Triple Select (GIBCO, Invitrogen) is used instead of trypsin to detach cells from the tissue culture plates.
6. DPBS without Ca^{2+} or Mg^{2+}, pH 7.4 (GIBCO, Invitrogen).
7. Trypan Blue (Sigma) to count and evaluate cell viability (*see* **Note 2**).
8. Neubauer haemocytometer (Brand, Wertheim, Germany).

2.2. Derivation of Conditioned Media

As previously described ***(17)***, TESR1 medium is usually used as the base media for human foreskin fibroblast conditioning, using passage 11 to 18 cells.

1. TERS1 conditioned media: after collection media can be frozen at -80º C for up to 6 months (*see* **Notes 3** and **4)**.

2.3. Preparation of Human Functional Foreskin-derived Extracellular Matrix (hffECM)

1. Osmotic lysis buffer Tris-EDTA (TE) pH 7.4: 10mM TrisHCl pH 7.4, 1mM EDTA pH 8 (all from Sigma). For preparation details *see* **Note 5**.
2. Cell scrapers (Falcon, BD Biosciences, Madrid).
3. DPBS (GIBCO, Invitrogen).

2.4. Preparation of Samples for Transmission Electron Microscopy (TEM)

1. PB (EMS).

2. Glutaraldehyde (3%): dilute glutaraldehyde (Electron Microscopy Science [EMS], Hattfield, PA) from a 25% (v/v) stock in PB.

3. Petri dish (Falcon, BD).

4. Disposable Pasteur pipettes (Falcon).

5. Osmium 1% (Sigma): prepared by diluting osmium in PB.

6. Uranyl acetate (2%) diluted in 70% ethanol.

7. Preparation of alcohol gradient: use the appropriate volume of absolute ethanol (Merck), mixed with distilled water to prepare 30%, 50%, 70%, and 90% ethanol solutions.

8. Resin LR-White (EMS).

See **Note 6** for safety measures that must be adopted to perform this technique.

2.5. Preparation of Samples for Atomic Force Microscopy (AFM)

1. 0.1 M PBS (Gibco)

2. 2% paraformaldehyde/2.5% glutaraldehyde/PB (all from EMS).

3. Series of ethanol/water solutions (30%, 50%, 70%, 90%, 96%). All from Sigma.

4. Absolute ethanol (Sigma).

2.6. Culture and Maintenance of Undifferentiated hESC lines

1. H9 and H1 hESC lines (WiCell, Madison, WI).

2. TERS1 conditioned media.

3. TGF-β1 (Invitrogen): reconstituted with sterile 4 mM HCl (Sigma) containing 1 mg/mL of human serum albumin (Sigma) to a final stock solution concentration of 40 ng/mL. Store in 50 μL aliquots at -20º C.

4. Human recombinant basic fibroblast growth factor (bFGF; Invitrogen) is dissolved in 1 mL DMEM Knockout Medium (Invitrogen) and stored in 100 μL aliquots at -20º C.

2.7. Analysis of Undifferentiated hESC Markers

2.7.1. Staining of Pluripotency Cell Surface Markers by Immunocytochemistry

Antibodies that detect specific cell-surface hESC markers are commercially available from Chemicon and antibodies for detection of the ECM component fibronectin are available from Sigma. Secondary antibodies are all commercially available from Invitrogen. *See* **Tables 1** and **2** for recommended dilutions and providers.

1. DPBS without Ca^{2+}, Mg^{2+} (GIBCO, Invitrogen).

2. 4% paraformaldehyde (*see* recipe at 2.7.2.2).

3. 0.05% sodium azide (Sigma) in DPBS.

4. Triton-X-100 (Sigma): prepare a dilution of 1% Triton-X-100 in DPBS to permeabilize the hESCs.

5. Blocking solution: 4% serum in DPBS. Serum for the blocking solution should be of the same origin/animal as the secondary antibody.

6. Prolong gold anti-fade reagent with DAPI (Invitrogen). *See* **Note 7**.

2.7.2. Alkaline phosphatase (AP) detection kit (Chemicon Millipore, Billerica, MA)

1. This kit provides two components for AP detection: Fast Red Violet solution (0.8 g/L stock) and naphthol AS-BI phosphate solution (4 mg/mL) in AMPD buffer (2 mol/L), pH 9.5.

2. Paraformaldehyde 4% in PBS: Prepared fresh with distilled water. To prepare 100 mL, heat 50 mL distilled water to 60º C on a hot plate in a fume hood (do not exceed 65º C) and add 4 g of paraformaldehyde powder. Stir the solution until it becomes clear (a few drops of NaOH can be added). After that, filter the solution through a 0.22 μm filter and add 50 mL of sterile PBS at pH 7.4 (*see* **Note 8**).

3. TBST 1× Rinse Buffer: prepared fresh with 20 mM Tris-HCl, pH 7.4, 0.15M NaCl, 0.05% Tween-20 (all from Sigma).

2.7.3. Intracellular Markers by RT-PCR and QPCR Analysis

See **Table 3** for the complete list of primers and reaction conditions.

1. RNeasy mini kit (74104, Qiagen).

2. Deoxyribonuclease (DNase) I (RNasa-free DNase SET; 79254; Qiagen).

3. High Capacity cDNA RT kit (PN 4368814; Applied Biosystems).

4. FastStart PCR Master (04710436001; Roche).

5. Power SYBR® Green PCR master mix (4367659; Applied Biosystems).

6. PCR microplates.

7. 1.5 mL sterile microcentrifuge tubes (Eppendorf, Hamburg, Germany).

8. Absolute ethanol (Merck, Darmstadt, Germany).

9. Standard table-top microcentrifuge capable of 13,000 × g centrifugal force.

10. Standard thermal cycler.

11. NanoDrop spectrophotometer or similar to evaluate the synthetized cDNA quality.

2.7.4. Telomerase Activity

1. TRAPEZE Telomerase Detection Kit (Chemicon, Billerica, MA).

2. PBS without Ca2+, Mg2+ (GIBCO).

3. 10--20% nondenaturing polyacrylamide precasted gels.

4. 10× TBE (BioRad, Hercules, CA).

5. SYBR green (Molecular Probes).

6. Loading buffer 10× (BioRad).

7. Nondenaturing 10--20% polyacrylamide gel (15% precasted gels from BioRad).

3. Methods

Extracellular matrix compounds play important roles in cell adhesion, attachment, cell interactions, and proliferation ***(18,11)***. Previous studies have demonstrated that the components of ECM support undifferentiated growth of hESCs. Extracellular matrix is generally organized into a three-dimensional fibrous structure and therefore, it is crucial to extract the ECM samples from human fibroblasts rapidly and at 4º C which prevents conformational changes and protein degradation. Transmission electron microscopy (TEM) and AFM can be used to assess the integrity of hffECM after the obtention. To validate functionality, it is very important to evaluate the ability of the extracted ECM to maintain hESCs in the undifferentiated state. This can be assessed by long-term growth of hESCs on this ECM and by routine analysis of pluripotency using the following assays: RT-PCR, immunocytochemistry, determination of alkaline phosphatase, and telomerase expression and analysis of spontaneous differentiation ability ***(19)***.

3.1. Preparation of Fibroblast Cells and Conditioned Media

1. Human foreskin fibroblasts are grown in Iscove's Medium and the cells are split using Triple Select every 5--7 days.

2. When confluent, the cells were inactivated using mitomycin C at 37°C in an incubator with 5% CO_2 for 3 h.

3. They are then washed with DPBS three times (5 min), digested, and counted (*see* ***Note 2***).

4. Seed fibroblast at a density of 6×10^6 cells in a T75 flask coated with 0.1% gelatine, and culture at 37°C with 5% CO_2 for 24 h before adding TERS1 medium.

5. TERS1 conditioned medium is collected every day until day 7, and stored at -80°C for up to 6 months (*see* **Notes 3** and **4**).

3.2. Preparation of Extracellular Matrix (ECM)

1. Human foreskin fibroblasts are grown in an appropriate medium and the cells are split using Triple-select every 5--7 days. When the cells reach 100% confluence, they are inactivated by treatment with mitomycin C (10μg/mL) for 3 h, and then washed three times with DPBS.

2. Cells are detached, counted and seeded (2×10^5 cells/per well) in a six-well plates, coated with 0.1% gelatin, and cultured at 37°C and 5% CO_2 for 24 h.

3. Inactivated cell cultures are maintained for 7--8 days, with a media change every second day.

4. Foreskin cultures are treated on day 7, following the recommendations described in ***(15)***.

5.Cells are washed twice with DPBS without Ca^{2+} and Mg^{2+} and subsequently lysed by osmotic shock with Tris EDTA buffer (10 mM Tris, 1 mM EDTA, PH 7.4) adding, 1.5 mL per 10^6 cells.

6. After addition of osmotic shock buffer, cells are incubated o.n. at 4°C using an orbital shaker, after which time the Tris-EDTA buffer is removed by aspiration (*see* **Note 9**).

6. Lysed cells are eliminated from the plates by rinsing six times (5 min each) with DPBS. Plates containing the remaining hffECM are stored at 4º C or dried and sterilised using standard methods. Examples of foreskin fibroblast cells and ECM samples studied using TEM are shown in **Fig. 1A**.

3.3. Preparation of Samples for TEM (*see* **Notes 6 and 10 when Planning these Experiments**)

1. Wash samples twice with PBS for 2--3 min.

2. Fix for 30 min at 37º C with 3% glutaraldehyde. Add glutaraldehyde carefully until the plate surface is totally covered.

3. Incubate for 30 min and store at 37º C, keeping the samples inside a box or Petri dish to avoid release of carcinogenic vapor.

4. Aspirate glutaraldehyde with a disposable Pasteur pipette and discard adequately.

5. Wash 3--4 times for 3 min with PBS.

6. Cover the samples with PBS and maintain them at 4º C (*see* **Note 11**).

7. Fixation and contrasting: add 1 mL of 2% osmium to cover the plates and leave for 1 h.

8. Wash three times (5 min each) with distilled water at 4º C (do not exceed 15 min).

9. Dehydration procedure (all steps are performed at 4º C):

    9.1. Add 30% alcohol for 5 min.

    9.2. Wash with 50% alcohol (10 min) at 4º C (exceeding this time can cause protein damage).

    9.3. Wash twice with 70% alcohol (10 min each).

10. Add 1 mL of 2% uranyl acetate in 70% alcohol and incubate for 2 h at 4°C.

11. Continue with the dehydration process by using the following alcohol gradient:

    11.1 Add 70% ethanol 2 times × (10 min each).

    11.2. Wash with 90% ethanol 2 times × (10 min each).

12. To embed the samples in resin, use the following alcohol/LR white resin (EMS) gradient as follows:

    12.1. Incubate in 2 parts 90% ethanol: 1part resin for 45 min at RT.

    12.2. Replace the mixture using a Pasteur pipette and discard adequately.

12.3. Prepare a dilution 1part 90% ethanol: 2 parts resin, add to the samples and incubate for 45 min.

12.4. Remove the mixture and discard appropriately.

12.5. Mix 1 part of 100% ethanol and 2 parts resin and incubate for 45 min.

12.6. Replace the mixture by adding 100% LR white resin. Incubate o.n. in an orbital shaker.

13. To allow polymerization, transfer the samples to an oven and incubate them at 60º C for 24 h.

14. Detach and proceed with the sectioning.

15. Observe the samples under a transmission electron microscope.

3.4. Atomic Force Microscopy (AFM)

AFM provides detailed 3-dimensional images of ECM protein-surface interactions.

1. Samples should be washed in 0.1M PBS and fixed in 2% paraformaldehyde/2.5% glutaraldehyde/PB for 1 h at room temperature.

3. Samples are dehydrated using a series of ethanol/water solutions (30%, 50%, 70%, 90%, and 96%) for 10 min with final dehydration in absolute ethanol for 10 min twice.

3. The dehydrated samples were examined by AFM, operating in the tapped-air mode.

4. The ECM protein distribution (on glass) is estimated from AFM images. The amplitude set point should be 1.3 V with a drive amplitude of 300 mV.

5. Surface roughness is calculated by processing 10 representative AFM images.

Images of glass surfaces with and without hffECM captured using AFM are shown in **Fig. 1B**.

3.5. Growth of Undifferentiated Pluripotent Cells using Feeder-Free Conditions and hffECM

1. Once the ECM-coated plates are ready for use, conditioned medium which has been collected, should be added.

2. Add 2 mL of conditioned media containing 50 ng/mL and 100 ng/mL of fresh TGF-β1 and bFGF, respectively. Place the 6-well plates in an incubator at 37º C with 5% CO_2 for at least 3 h before seeding the new hESC colonies.

3. Wash the hESC colonies maintained on human feeders twice with prewarmed DMEM medium.

4. Dissect hESC colonies mechanically and collect them in a 15 mL polypropylene tube (*see* **Notes 12**and **13**).

5. Centrifuge the cells for 3 min at 200 × g and remove the supernatant.

6. Gently resuspend the pellet in conditioned medium supplemented with fresh TGF-ß1 and bFGF in the same 15 mL tube.

7. Add 1 mL of media containing 10--20 colonies to each well of the ECM-coated plates.

8. Incubate the plates at 37º C with 5% CO_2 and change the media every second day.

9. Undifferentiated hESCs should be transferred to new plates every 5--7 days and maintained at 37º C with 5% CO_2.

Examples of contrast phase microscope are shown in **Fig. 2A** and **Fig. 2B**.

3.6. Detection of Cell Surface Pluripotency Markers by Immunocytochemistry

1. Remove the culture medium.

2. Wash the cells once with DPBS without Ca^{2+} and $Mg2^{+}$. It is important to add the DPBS very gently and not directly to the cells.

3. Fix hESCs in 4% paraformaldehyde for 15 min at room temperature by adding 1.5 mL/well to the 6-well plates.

4. Wash 4 times with DPBS.

5. To permeabilize the hESCs, add 1.5 mL per well of 1%Triton-X-100, followed by an incubation of 10 min at room temperature.

6. Wash twice (5 min each) with DPBS.

7. Apply 4% of blocking solution at room temperature for 45 min. Serum for the blocking solution should be of the same species as the secondary antibody.

8. Remove the blocking solution but do not wash the cells.

9. Dilute the primary antibody blocking solution (*see* **Table 1**).

10. Add 1 mL of the corresponding primary antibody dilution to the cultures for at least 1 h at RT or overnight at 4º C.

11. Wash 3 times with DPBS. Cells can be left overnight before adding the secondary antibody.

12. Dilute the secondary antibody in DPBS and add 1 mL per well (*see* **Table 2**).

13. Incubate at room temperature for 60 min and in darkness as exposure to light may cause bleaching of fluorescent labels.

14. Wash the cells 4 times with DPBS.

15. If the cells are attached to a coverslip mount it on a slide using prolong gold antifade reagent with DAPI. Remove any bubbles that may have formed during mounting.

16. Let the slides dry for 15 min in conditions where they are protected from light.

17. After 90 min the samples can be observed using a fluorescence microscope. Examples of the morphology and undifferentiated hESC surface markers grown on plastic dishes coated by ECM derived from human fibroblasts and in the presence of conditioned medium are presented in **Fig. 2C**,**D** and **3**.

3.7. Preparation of Samples for RT-PCR and QPCR Analysis

Analyzes of mRNA expression by reverse transcription PCR (RT-PCR) and real-time quantitative PCR (QPCR) are carried out using standard protocols. An RT-PCR reaction is performed at 60º C and for 35 cycles, except for ß2-microglobulin (performed at 55º C for 35 cycles). The QPCR reaction is performed at 60º C (60 s) and 95º C (15 s) for 40 cycles. Primer sequences were designed using Primer3 software and synthesized by Sigma-Aldrich. All primer sequences and DNA fragments are listed in **Table 3**. Transcripts encoding the following proteins Oct-4, Tert, Nanog, and FoxD3 should be assessed for pluripotency, and AFP, DBH, and CAC for differentiation (specific lineage) markers. The ß2-microglobulin gene is used as an endogenous control (housekeeping) gene.

3.7.1. Extraction and Quantification of Total RNA

Total RNA extraction can be performed using 20 hESC colonies maintained on ECM. As a positive control, undifferentiated hESCs maintained on feeder cells should be used. We use the RNeasy mini kit (74104; Qiagen) following provider-recommended instructions with some modifications. To eliminate contamination by genomic DNA, the initial RNA pellet was incubated with deoxyribonuclease (DNase) I (RNase-free DNase SET; 79254; Qiagen).

1. Cut the cells from the culture dish with a needle and collect them in a centrifuge tube.
2. Add 350 μL of buffer RLT and vortex for 1 min.
3. Centrifuge the lysate for 3 min at maximum speed. Carefully remove the supernatant by pipetting out and discarding it.
4. Add 1 volume of 70% ethanol to the homogenized sample and mix well by pipetting only (do not centrifuge). The volume might be less than 350 μL due to loss during the homogenization process. The use of ethanol can trigger precipitates to form; however, their presence has no impact on the process.
5. Transfer up to 700 μL from the sample, including any precipitates, to an RNeasay mini spin column (pink) placed in a 2 mL collection tube, and close the lid.
6. Centrifuge at 8,000 × *g* (10,000 rpm) for 15 s, discard the flow-through and reuse the collection tube for step 7. Note: if the sample volume is greater than 700 μL, the aliquots must be centrifuged one after the other in the same column, discarding the flow-through after each centrifugation.
7. Add 350 μL RW1 buffer to the RNeasy mini spin column, close the lid, and centrifuge at 8,000 *x g* (10,000 rpm) for 15 s. Discard the flow-through and reuse the collection tube for step 10.

8. Add 10 µL DNase I stock solution to 70 µL RDD buffer. Mix by gently inverting the tube; centrifuge briefly to collect the liquid from the tube walls.

9. Add DNase I to the incubation mix (80 µL) and transfer it directly to the RNeasy column membrane, and it place on the benchtop at 20--30°C for 15 min.

10. Add 350 µL RW1 buffer to the RNeasy mini spin column, close the lid, and centrifuge at ≥ 8,000 *x g* for 15 s. Discard the flow-through.

11. Add 500 µL RPE buffer to the RNeasy mini spin column, close the lid, and centrifuge at ≥ 8,000 *x g* for 15 s. Discard the flow-through and reuse the collection tube.

12. Add 500 µL RPE buffer to the RNeasy mini spin column, close the lid, and centrifuge at ≥ 8,000 *x g* for 2 min to wash the column membrane.

13. Place the RNeasy mini spin column in a new 2 mL collection tube and centrifuge at full speed for 1 min to dry the membrane (optional step).

14. Place the RNeasy mini spin column in a new 1.5 mL collection tube and add 30--50 µL RNase-free water directly to the spin column membrane. Close the lid, and centrifuge at ≥ 8,000 *x g* for 1 min to elute the RNA.

15. If the expected RNA yield is >30 µg, repeat step 14 using another 30--50 µL RNase-free water, or the elute from **step 14** (if a high RNA concentration is required). Reuse the collection tube from **step 14**. Note: if the elute from **step 14** is used, the RNA yield might be 15--% lower than using a second volume of RNase-free water but the final RNA concentration will be higher (*see* ***Note 14***).

16. Quantify the extracted RNA and evaluate its quality using a NanoDrop spectrophotometer or a RNA-integrity gel. Either use the eluted RNA directly in following steps (RT-PCR and QPCR) or store the eluted RNA at -80º C for later analysis.

3.7.2. cDNA Synthesis

Use 50 µL from each sample obtained for total RNA to single-stranded cDNA reverse transcription (RT). We follow the recommendations from the High Capacity cDNA RT kit (PN 4368814, Applied Biosystems).

1. Place the 2× RT master mix on ice and mix gently.

2. Pipette 25 µL of 2× RT master mix into each well of an individual tube (*see* **Note 15**).

3. Pipette 25 µL of RNA sample into each well, pipetting up and down two times to mix (*see* **Note 16**).

4. Seal the tubes and briefly centrifuge them to spin down the contents and to eliminate any air bubbles.

5. Place the plate or tubes on ice until you are ready to load them into the thermal cycler.

6. Program the thermal cycler conditions with the following parameter steps: (i) step 1: 25 º C for 10 min; (ii) step 2: 37 º C for 120 min; (iii) step 3: 85 º C for 5 min; (iv) step 4: 4 º C for indefinite time.

7. Set the reaction volume to 20 μL.

8. Load the reactions into the thermal cycler and start the reverse transcription run.

9. Quantify the synthetized cDNA and evaluate its quality using a NanoDrop spectrophotometer or cDNA integrity gel.

3.7.3. PCR Analysis

Use a 50 μL reaction per sample to obtain the PCR products. We follow the recommendations from FastStart PCR Master (04710436001; Roche).

1. Prepare the microplates for PCR according to the instrument instructions.

2. Thaw the solutions and briefly spin the vials in a microcentrifuge before opening.

3. Mix the solutions by pipetting them up and down gently and store them on ice.

4. Prepare 10× concentration solutions of the PCR primers (see **Table 3**)

5. Prepare a PCR mix for the number of reactions to be run plus one additional reaction. The PCR mix contains the following products for each reaction in the following order: 25 μL 2× master mix, 5 μL forward primer (3 μM), 5 μL reverse primer (3 μM), and 10 μL PCR-grade water.

6. Mix the solution by pipetting it up and down (do not vortex).

7. Transfer 45 μL PCR mix into each reaction well of the PCR microplate.

8. Add 5 μL of template DNA (up to 100 ng cDNA) into each reaction well.

9. Prepare the microplate for the PCR reaction according to the instrument's instructions.

10. Program the thermal cycler conditions with following steps: (i) step 1: 1 cycle at 95º C for 4 min; (ii) step 2: 30 to 40 cycles at 95º C for 30 s, and 45--65º C for 30 s, and 72º C for 45--180 s; (iii) step 3: 1 cycle at 72 º C for 7 min; (iv) step 4: 4º C for an indefinite time.

7. Set the reaction volume to 50 μL.

8. Load the microplate into the thermal cycler and start the PCR reaction.

The results can be observed in the electrophoresis gels on **Fig. 4B** and **Fig. 4C**.

3.7.4. QPCR Analysis

1. Completely thaw the Power SYBR Green PCR Master Mix and gently mix the reagents.

2. In polypropylene tubes, prepare the PCR reagent mix for the desired number of PCR reactions. Each 20 μL PCR reaction contains 10 μL of the PCR Master Mix, 50--300 nM from each primer, 100 ng template, and a variable quantity of nuclease-free water (*see* **Note 17**).

3. Mix gently (do not vortex) and centrifuge briefly.

4. Prepare the microplate for the QPCR reactions according to instrument's instructions.

5. Program the thermal cycler conditions with the following steps: (i) step 1: 1 cycle at 95º C for 4 min; (ii) step 2: 30 to 40 cycles at 95º C for 30 s, 45--65º C for 30 s, and 72º C for 45--180 s; (iii) step 3: 1 cycle at 72 º C for 7 min; (iv) step 4 at 4 º C for an indefinite time.

6. Set the rest of the thermal cycler parameters (e.g., volume = 20 μL) following instrument user's manual to configure the plate documentation details.

7. Load the microplate into the thermal cycler and start the PCR reaction.

8. Analyze and export the results using a spreadsheet program with statistical features.

9. Calculate the average cycle time, the standard deviation, and the coefficient of variation for each group of replicates of any sample. Remove outlier points (Ct > 0.3 × standard deviation).

10. Apply the ΔΔCt method to obtain "fold changes" as desired output ***(20)***. The internal control selected is ß-2 microglobulin gene.

3.8. Telomerase activity assay

Telomerase activity is assayed using telomeric repeat amplification protocol using the Trapeze Kit (Chemicon) and according to the manufacturer's protocol but with some modifications.

3.8.1 Sample Preparation

1. For stem cell analysis, collect 30--100 colonies. Pellet cells at 400 × *g* for 5 min at 4º C. We highly recommend that the positive and negative controls provided with the kit and a negative control (a differentiated cell line and/or heat inactivated immortal/stem cells) be used.

2. Wash cell pellet with sterile Ca^{2+} and Mg^{2+} free PBS for 5 min at 400 × *g* at 4º C.

3. Resuspended the cell pellet with 5--20 μL CHAPS lysis buffer (Thermo Scientific) for stem cell colonies.

4. Place on ice for 30 min.

5. Pellet cells at high speed (12,000 rpm) at 4º C for 20 min.

6. Transfer the supernatant to a new tube. At this point the supernatants are kept at -80º C and can be stored for up to one year to be used for telomerase detection.

3.8.2 PCR reaction

1. Use 2--4 μL of sample per PCR reaction.

2. Heat inactivated negative controls must be subjected to 85º C for ten min. Then use the same 2--4 μL volume for the PCR reactions.

3. Prepare a master mix containing all these components except the templates, all the reagents are provided by the kit except Taq polymerase:

- 10× TRAP Reaction buffer 5 μL
- 50× dNTP mix 1 μL
- TS primer 1 μL
- TRAP primer mix 1 μL
- Taq polymerase (5 U/μL) 0.4 μL
- dH_2O 29.5 μL
- Template 2 μL

4. PCR program

- 1 cycle: 30º C 30 min
- 30--33 cycles: 94º C 30 s

59º C 30 s

3.8.1 Separation by Electrophoresis

1. For sample electrophoresis, use a 10--15 μl PCR reaction with a 2--5 μl loading buffer 10× (BioRad).

2. Load a nondenaturing 10--20% polyacrylamide gel (we use 15% precasted gels from BioRad).

3. Run the gel in TBE 0.5X until both color bands are out of the gel.

4. Prepare the SYBR green solution (1/10,000) in TBE 1X.

5. Stain the gel for 15--20 min in the dark.

6. Visualize the bands in a transilluminator (with the same wavelength as the ethidium bromide).

The results for telomerase activity are presented in **Fig. 4D**.

4. Notes

1. Conditioned media can be prepared using foreskin fibroblasts between passages 11--18, but in order to maintain the quality, we recommend that cells between passages 11 to 16 be used.

2. Cell counting: take 20 μL of cell suspension and dilute it with 70 μL of culture medium. Add 10 μL of Trypan Blue solution (Sigma), mix, and incubate for 1 min before counting viable (round, clear cells) and nonviable (blue) cells using a Neubauer haemocytometer and plate 6×10^6

cells per flask or 2×10^5 cells per well for 75 cm^2 flasks and 6-well plates respectively. More than 75% of cells should be viable.

3. Before using of conditioned TESR1 medium, add 100 ng/mL of bFGF and 50 ng/mL of TGF-β1. Conditioned medium can be kept at +4°C for 1 week or stored at -80º C for 6 months.

4. No differences between frozen and fresh conditioned media have been observed.

5. Lysis buffer preparation. The following is an example for 250 mL:

- 2.5 mL 1 M Tris-HCl pH 7.4
- 500 µL 0.5 M EDTA pH 8.0
- 247 mL H_2O

Stock Solutions:

- 1 M Tris-Cl, pH 7.4 (1 L):
  - 121 g Tris Base
  - 1 L H_2O

  Adjust pH
- 0.5 M EDTA (1 L):
  - 186.1 g EDTA Na_2H_2O
  - 1 L H_2O

  Adjust pH

6. TEM inclusion procedure: Glutaraldehyde is carcinogenic by inhalation, it is important to manipulate it in a fume hood. During the 37º C incubation it is very important to avoid inhaling these fumes, keeping the culture plates inside a petri dish in the special stove. Osmium is carcinogenic and toxic by inhalation and contact and so double gloves and a mask should be worn, in addition to working under fume hood. Uranyl acetate is carcinogenic and radioactive. Manipulation must be performed in an adequate installation under fume hood using double gloves and a mask. Given their toxicity, those residues need to be manipulated and discarded properly.

7. Try to avoid bubbles in the samples.

8. Do not use until the solution reaches room temperature and adjust the pH if necessary.

9. We have also performed this process by incubating with ice in the orbital shaker for 2.5 hours.

10. When planning to do TEM analysis, the seeding of hFFs, the cell lysis and subsequent pluripotent cell seeding needs to be done on Permanox® chambers.

11. At this point, the plates can be maintained for several days at 4°C by adding sodium azide.

12. hESCs were cultured and maintained as previously described ***(21)***. Cells were passaged mechanically and re-plated on ECM-coated plates.

13. The optimal size of hESCs before the splitting procedure is when the colonies completely cover the magnification field (x10).

14. If you use the elution obtained in step 10, the quantity of RNA obtained may be 15--30% less than using RNase-free water.

15. The kit reagents must always be thawed on ice.

16. Use up to 5 µg of total RNA per 50 µL RT reaction.

17. Include an extra volume to account for pipetting losses.

Acknowledgements:

The authors would like to thank Dr. Deborah Burks, Dr. Sonia Prado, Dr. X. Chen Xiong, Mrs. Vanessa Blanca, Dr. Angel Ayuso-Sacido, and Petra Stojkovic for their support. This work was supported by funds for research in the field of Regenerative Medicine from the Regional Government Health Department (Generalitat Valenciana); the Instituto Carlos III belongs to the Spanish Ministry of Health and Consumer Affairs. Drs. Carmen Escobedo-Lucea and Andres Sanz-Garcia are financed by Academy of Finland projects as follows: No. 273689 (Research Fellow), No. 266486 (FINSKIN), No. 276371 (VATURP), and No. 278153 (mobility grant).

Figure Captions

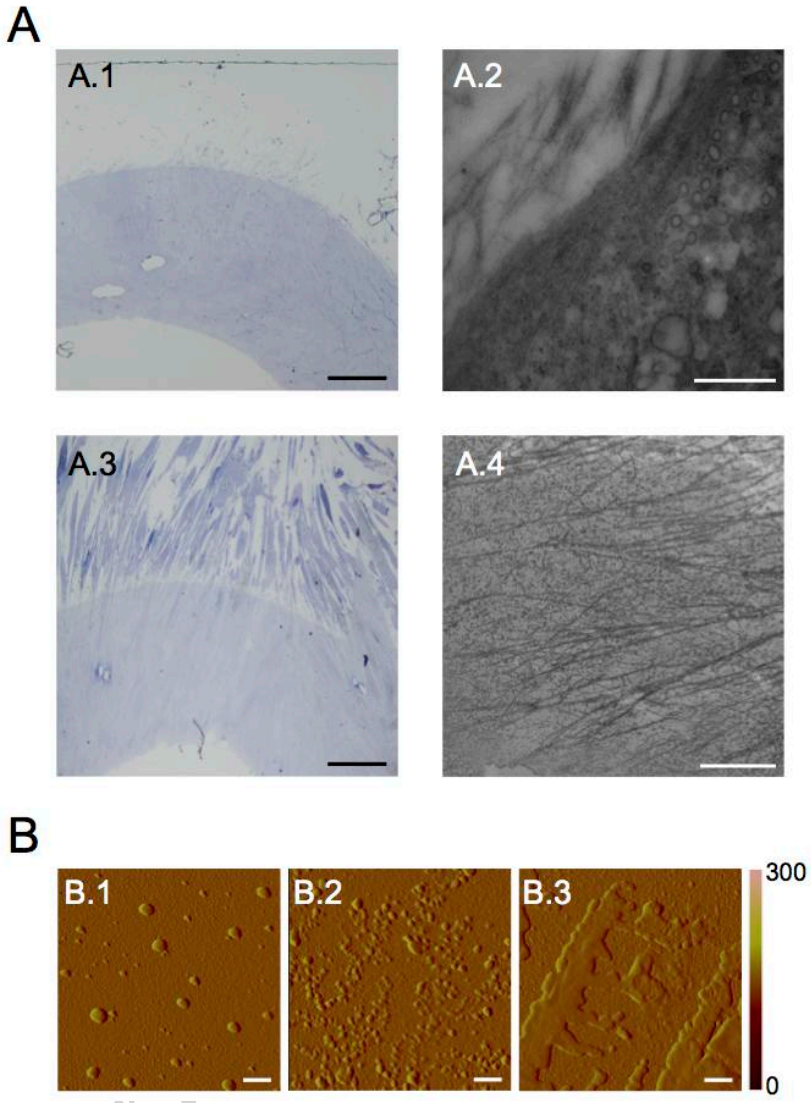

Fig. 1. Transmission electron microscopy (TEM) and atomic force microscopy (AFM) of the hffECM after the extraction to evaluate structural integrity and roughness. (**A**) Ultrastructural analysis of the efficiency of the extraction protocol using TEM. Plates of lysed cultures and their respective controls with intact foreskin fibroblasts were fixed and processed for TEM ultrastructural analysis. Human foreskin fibroblasts (hFFs) were maintained for the indicated times and then subjected to hypotonic lysis. (**A.1** and **A.3**) Semi-thin sections were prepared from control and lysed cultures and were stained with toluidine blue. (**A.2**) Representative TEM image of intact hFF control cells after 7 days in culture. (**A.4**) Representative TEM image of lysed culture plate after 7 days in culture. Scale bars, 200 μm (**A.1** and **A.3**), 1 μm (**A.2**), 500nm (**A.4**). (**B**) AFM analysis of ECM conformation on glass surfaces. The images show the amplitude mode with the same scale. (**B.1**) The smallest roughness corresponds to fibronectin that was used as

experimental control. The mean surface roughness observed demonstrates that the surface roughness is significantly lower on day 1 (**B.2**) than after hFFs culture on day 2 (**B.3**). Scale bars 250 nm (Figures 1.A.1 and 1.A.3 reproduced from ***(12)*** with permission from Springer).

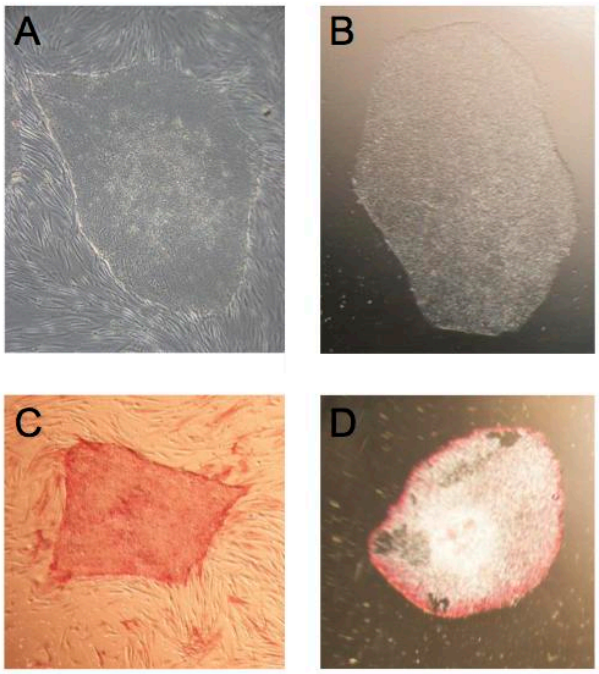

Fig. 2. (**A** and **B**) Morphology of undifferentiated hESCs grown for 11 passages over feeder cells and on plastic coated with hffECM using conditioned media respectively. (**C** and **D**) Images showing alkaline phosphatase activity in both feeder and hffECM conditions, indicating maintenance of pluripotency. Images were obtained using Zeiss Axiovert 200M microscope (magnifications ×100).

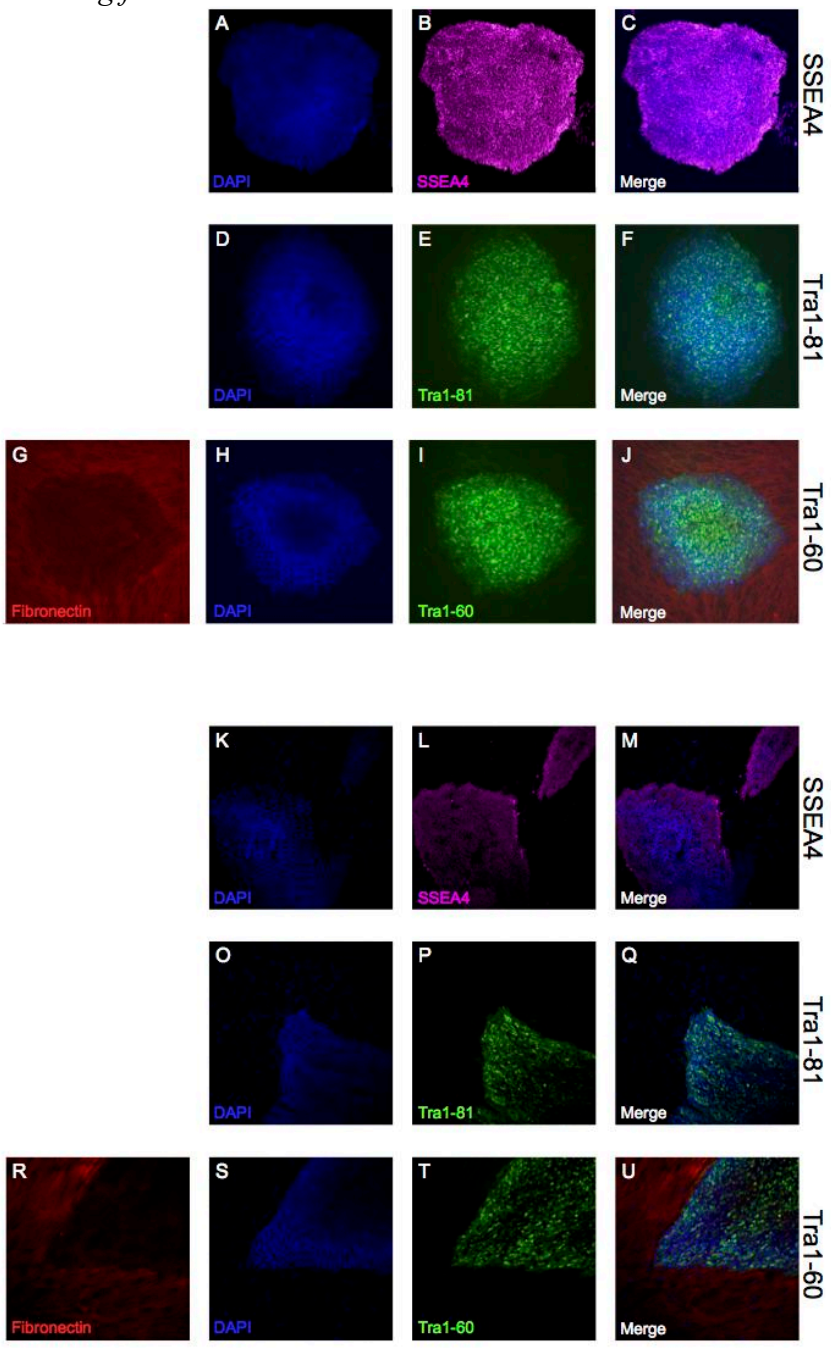

Fig. 3. Morphology and cell surface markers of undifferentiated hESC grown for 11 passages on plastic dishes coated with hffECM derived from human fibroblasts and in the presence of conditioned medium. Note round and compacted hESC colonies. The presence of SSEA4 (**A--C**, magenta), Tra1-81 (**D--F**, green), Tra1-60 (**H--J**, green), pluripotency markers were observed. Note the presence of specific fibronectin staining (**G--J** and **R-U**).

Morphology and cell surface markers of undifferentiated hESCs grown for 11 passages over hFFs feeder. The presence of SSEA4 (**K--M**, magenta), TRA-1-81 (**O--K**, green), TRA-1-60 (**S--U**, green), pluripotency markers were observed. Note the presence of specific fibronectin staining (**G** and **J**). Images were obtained using confocal microscope (10× magnifications).

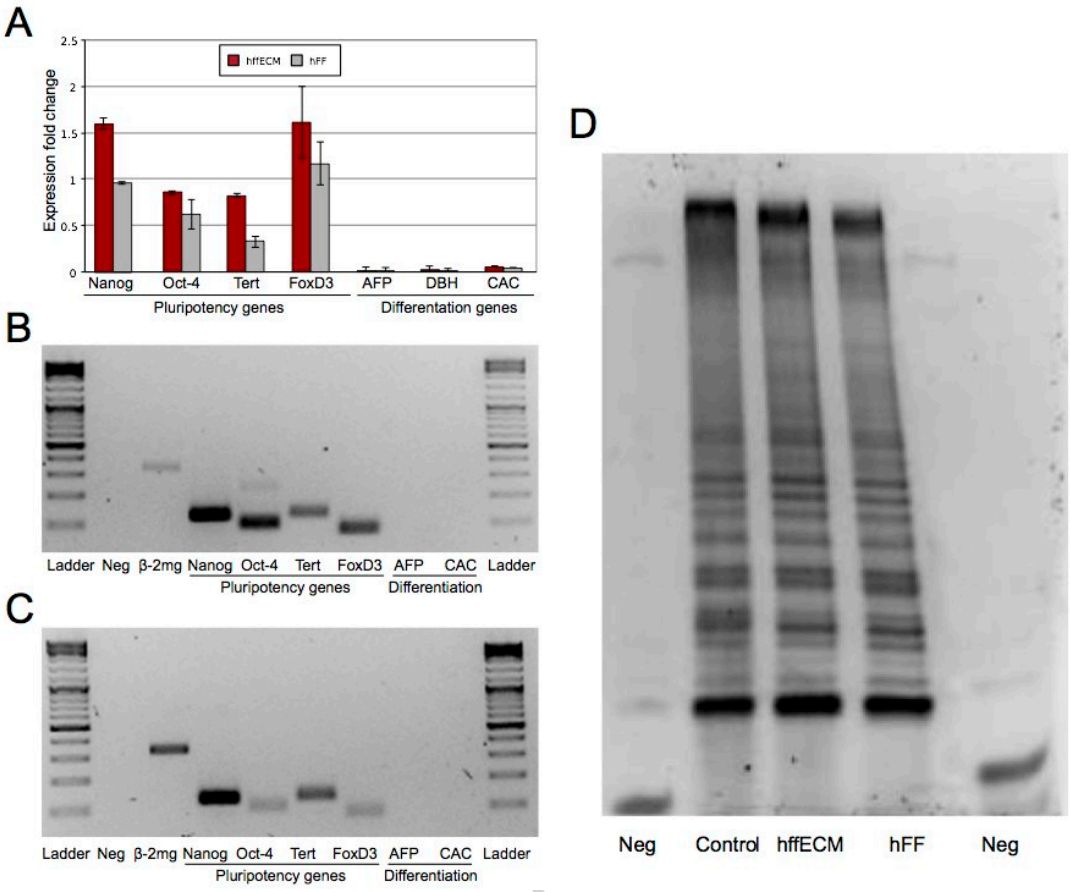

Fig. 4. Analysis of specific molecular hESC markers and telomerase activity. hESCs were grown on hffECM and in the presence of conditioned medium for 11 passages. Controls over foreskin feeder were also and grown at the same time. (**A**) QPCR comparing expression of pluripotency and differentiation genes in cells maintained on hffECM and hFF. The expression of characteristic pluripotent markers is higher in the cells maintained on hffECM than on hFF. This indicates that our system is highly selective and only cells with the best quality are able to adapt and survive. No expression of differentiation markers (AFP, DBH and CAC) was detected. (**B** and **C**) Cells maintained on hffECM and foreskin feeders respectively. Line 1 and 10: Ladders with 1Kb; 2. Corresponds to negative control; 3. Housekeeping gene ß-2 microglobulin. Cells express all the typical hESC markers 4. Nanog; 5. Oct-4; 6. Tert; 7. FoxD3. After 11 passages on ECM the cells were negative for differentiation markers AFP and CAC (lines 8 and 9, respectively. (**D**) Colonies retained telomerase activity after 11 passages on hffECM (line 3). Line 4 corresponds to their H9 counterparts maintained 11 passages over foreskin feeder cells. Line 1 and 6 are negative controls, and the line 2 the positive control.

Table

Table 1. List of primary antibodies

Primary antibody	Isotype	Working dilution	Catalogue number	Provider
SSEA4	IgG3	1:100	MAB 4304	Chemicon
Tra1-81	IgM	1:100	MAB 4381	Chemicon
Tra1-60	IgM	1:100	MAB 4360	Chemicon
Fibronectin	IgG1	1:100	F0916	Sigma

*SSEA4, Tra1-81, Tra1-60, and AP can also be acquired as a kit (SCR001; Chemicon).

Table 2. List of secondary antibodies

Secondary antibody	Working dilution	Catalogue number	Provider
Alexa Fluor goat against IgG	1:500	A11029	Invitrogen
Alexa Fluor against IgM	1:500	A21042	Invitrogen
Alexa Fluor against IgG1	1:500	A21124	Invitrogen

612
613 **Table 3.** List of primers and conditions used for both RT-PCR and qPCR
614
615

Gene Name	Primer Sequences	Annealing Temperature (° C)	Size (bp)	Detection
Housekeeping gene				
ß2-microglobulin	F: CTCGCGCTACTCTCTCTTTCTG R: GCTTACATGTCTCGATCCCACT	55	335	RT-PCR QPCR
Pluripotency assay genes				
Nanog	F: GGTGGCAGAAAAACAACTGG R: CATCCCTGGTGGTAGGAAGA	60	235	RT-PCR
Nanog	F: AATGGTGTGACGCAGAAGG R: ACTGGATGTTCTGGGTCTGG	60	157	QPCR
Oct-4	F: cctgtctccgtcaccactct R: CAAAAACCCTGGCACAAACT	60	128	RT-PCR QPCR
FoxD3	F: CAGAGCCCGCAGAAGAAG R: CGAAGCAGTCGTTGAGTGAG	60	133	RT-PCR
FoxD3	F: GCCCAAGAACAGCCTAGTGA R: GGGTCCAGGGTCCAGTAGTT	60	254	QPCR
Tert	F: GACCTCCATCAGAGCCAGTC R: CGCAAGACCCCAAAGAGTT	60	84	RT-PCR
Tert	F: GCGTTTGGTGGATGATTTCT R: AGCTGGAGTAGTCGCTCTGC	60	254	QPCR
Differentiation assay genes				
AFP	F: TGCGTTTCTCGTTGCTTACA R: GCTGCCATTTTTCTGGTGAT	60	81	RT-PCR
AFP	F: Acacaaaaagcccactccag R: ggtgcatacaggaagggatg	60	147	QPCR
DBH	F: tgactgggagaaaggtggtc R: tacgtgcaggaggtgatgag	60	160	QPCR
CAC	F: tgctgatcgtatgcagaagg R: gctggaaggtggacagagag	60	135	RT-PCR QPCR

616
617
618
619

References:

1. Takahashi K, Okita K, Nakagawa M et al. (2007) Induction of pluripotent stem cells from fibroblast cultures. *Nat Protoc* **2** (12), 3081--3089
2. Takahashi K, Tanabe K, Ohnuki M et al. (2007) Induction of Pluripotent Stem Cells from Adult Human Fibroblasts by Defined Factors. *Cell* **131** (5), 861--872
3. Klimanskaya I, Chung Y, Meisner L et al. (2005) Human embryonic stem cells derived without feeder cells. *Lancet* **7** (13), 1636--1641
4. Xu C, Inokuma M, Denham J et al. (2001) Feeder-free growth of undifferentiated human embryonic stem cells. *Nat Biotechnol* **19** (10), 971--974
5. Cyranoski D (2014) Next-generation stem cells cleared for human trial. *Nature*,
6. Schwartz SD, Regillo CD, Lam BL et al. (2014) Human embryonic stem cell-derived retinal pigment epithelium in patients with age-related macular degeneration and Stargardt's macular dystrophy: follow-up of two open-label phase 1/2 studies. *The Lancet*,
7. Martin MJ, Muotri A, Gage F et al. (2005) Human embryonic stem cells express an immunogenic nonhuman sialic acid. *Nature Medicine* **11**, 228--232
8. Kagami S, Kondo S, Löster K et al. (1998) Collagen type I modulates the platelet-derived growth factor (PDGF) regulation of the growth and expression of beta1 integrins by rat mesangial cells. *Biochem Biophys Res Commun* **252** (3), 728--732
9. Sjaastad MD, Nelson WJ (1997) Integrin-mediated calcium signaling and regulation of cell adhesion by intracellular calcium. *Bioessays* **19**, 47--55
10. Schuppan D, Cramer T, Bauer M et al. (1998) Hepatocytes as a source of collagen type XVIII endostatin. *Lancet* **352**, 879--880
11. Cukierman E, Pankov R, Stevens DR et al. (2011) Taking cell-matrix adhesion to the third dimension. *Science* **294** (5547), 1708--1712
12. Escobedo-Lucea C, Ayuso-Sacido A, Xiong C et al. (2012) Development of a human extracellular matrix for applications related with stem cells and tissue engineering. *Stem Cell Rev* **8** (1), 170--183
13. Grauss RW, Hazekamp MG, Oppenhuizen F et al. (2005) Histological evaluation of decellularised porcine aortic valves: matrix changes due to different decellularisation methods. *Eur J Cardiothorac Surg* **27** (4), 566--571
14. Escobedo-Lucea C, Stojkovic M (2010) Growth of human embryonic stem cells using derivates of human fibroblasts. *Methods Mol Biol* **584**, 55--69
15. Escobedo-Lucea C, Bellver C, Gandia C et al. (2013) A xenogeneic-free protocol for isolation and expansion of human adipose stem cells for clinical uses. *PLoS One* **8** (7), e67870
16. Prado-Lopez S, Conesa A, Arminan A et al. (2010) Hypoxia promotes efficient differentiation of human embryonic stem cells to functional endothelium. *Stem Cells* **28** (3), 407--418
17. Ludwig TE, Levenstein ME, Jones JM et al. (2006) Derivation of human embryonic stem cells in defined conditions. *Nat Biotech* **24** (2), 185--187
18. Werb Z, Chin JR (1998) Extracellular Matrix Remodeling during Morphogenesisa. *Ann N Y Acad Sci* **857** (1), 110--118
19. Thomson JA, Itskovitz-Eldor J, Shapiro SS et al. (1998) Embryonic stem cell lines derived from human blastocysts. *Science* **6** (282), 1145--1147
20. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2^[-delta delta C(T)] Method. *Methods* **25** (4), 402--408
21. Stojkovic P, Lako M, Stewart R et al. (2005) An autogeneic feeder cell system that efficiently supports growth of undifferentiated human embryonic stem cells. *Stem Cells* **23** (3), 306--314

669

670

DOI 10.1007/7651_2014_154 *Springer Science & Business Media New York*