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ORIGINAL ARTICLE *Reproductive biology*

Immortalized human skin fibroblast feeder cells support growth and maintenance of both human embryonic and induced pluripotent stem cells

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BACKGROUND: Feeder cells are frequently used for the early-stage of derivation and culture of human embryonic stem cell (hESC) lines.

METHODS: We established a conditionally immortalized human foreskin fibroblast line that secreted basic fibroblast growth factor (bFGF). These cells were used as feeder cells for hESC culture and induced pluripotent stem (iPS) cell derivation and expansion. This conditional immortalization was performed using lentiviral vector (LV) mediated transduction of Bmi-1 and human telomerase reverse transcriptase genes and the resulting cell line was further modified by LV-mediated transduction of a secreted form of bFGF gene product. Three different laboratories have tested whether this feeder cell line could support the maintenance of four different hESC lines.

RESULTS: Immortalized fibroblasts secreting stable amounts of bFGF supported the growth of all hESC lines, which remained pluripotent and had a normal karyotype for at least 10 passages. Even at high passage (p56), these modified cells, when used as feeders, could support iPS derivation and propagation. Derived iPS cells expressed pluripotency markers, had hESC morphology and produced tissue components of the three germ layers when differentiated *in vitro*.

CONCLUSION: These modified fibroblasts are useful as a genetically-defined feeder cell line for reproducible and cost-effective culture of both hESC and iPS cells.

Key words: feeder cells / human embryonic stem cells / induced pluripotent stem cells / human fibroblasts / immortalization

Introduction

Human embryonic stem cells (hESCs) are regarded as pluripotent since they have the ability to differentiate into almost all cell types. hESCs hold great promise for regenerative medicine and are a powerful tool for basic research (Thomson *et al.*, 1998; Reubinoff *et al.*, 2000). Pluripotent hESC lines are derived from the inner cell mass of surplus human blastocysts from day 5 to day 8 (Thomson *et al.*, 1998; Reubinoff *et al.*, 2000; Hovatta *et al.*, 2003; Inzunza *et al.*, 2005) or from 8-cell stage embryos (Strelchenko and Verlinsky, 2006). Conventionally, pluripotent hESCs are maintained directly on co-cultured feeder layers derived from either mouse or human sources (Reubinoff *et al.*, 2000; Richards *et al.*, 2002, 2003; Amit *et al.*, 2003; Hovatta *et al.*, 2003; Choo *et al.*, 2004). Human feeder

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cells also function in the derivation of hESC lines (Hovatta *et al*., 2003; Inzunza *et al*., 2005) and in supporting hESC and induced pluripotent stem (iPS) cell maintenance and propagation (Meissner *et al*., 2007; Nakagawa *et al*., 2007, 2008; Yu *et al*., 2007; Hanna *et al*., 2008; Lowry *et al*., 2008; Park *et al*., 2008). Of note, many groups have used feeder free systems and added high concentration of fibroblast growth factor (bFGF) showing maintenance of hESC pluripotency under these conditions (Wang *et al*., 2005a, b; Ludwig *et al*., 2006).

Self-renewal of both hESC and iPS cells are regulated by growth factors and feeder cells in culture (Nichols *et al*., 1998). Regardless of the source, feeders are derived from primary tissues and therefore have a limited life span in culture. Moreover, hESC cannot maintain pluripotency when co-cultured with mouse embryonic fibroblast (MEF) and human foreskin fibroblast (HFF) feeders that reached approximately 7–9 and 12–14 passages or more, respectively. Therefore, fresh batches of feeders have to be prepared on a routine basis, and then validated to account for batch-to-batch variation.

Immortalization of primary feeders would offer a possible solution to this problem, resulting in a sustainable, standardized and consistent source of feeders for the culture of hESC and iPS cells. Several methods have been used previously for immortalization, including the transduction of normal cells with simian virus 40 (SV40), Epstein–Barr virus or human papillomavirus (Bryan and Reddel, 1994; Jha *et al*., 1998; Sugimoto *et al*., 1999; Gudjonsson *et al*., 2002; Yamamoto *et al*., 2003). Recently, it was reported that the overexpression of the human telomerase reverse transcriptase (hTERT), can also extend the lifespan of somatic or differentiated cells (Bodnar *et al*., 1998; Xu *et al*., 2004).

Currently, culture conditions with or without feeders are laborious, time-consuming and/or expensive. There is also a need for large-scale culture for the genetic manipulation of hESC and iPS cells in basic research and for potential clinical applications. Until the optimal recipe for animal substance-free, feeder-free and cost-effective culture conditions is established, one possibility is to obtain Good Manufacturing Practice (GMP)-grade immortalized human feeder cells that can produce major growth factors such as bFGF, and to maintain large-scale cultures of hESC and iPS cells in a pluripotent and undifferentiated state, making stem cell research affordable.

Derivation of iPS cells from somatic cells such as mouse fibroblasts (Meissner *et al*., 2007; Nakagawa *et al*., 2007) and from fetal, neonatal and adult human skin fibroblasts (Takahashi *et al*., 2007; Yu *et al*., 2007; Nakagawa *et al*., 2008; Park *et al*., 2008) represents a clear breakthrough in regenerative medicine. Hence, it would be of great interest to establish optimal feeder and culture conditions that allow propagation and derivation of these pluripotent stem cells in clinically-compatible conditions.

Here, we show (i) the generation and establishment of immortal fibroblast cells, (ii) their supportive ability for growth and expansion of hESC lines and (iii) their potential for iPS cell derivation and propagation in the undifferentiated stage.

Materials and Methods

Human ESC and HFF cell cultures

HFFs (CCL-110 and CCL-2429; ATCC, Manassas, VA, USA) were used to prepare feeder dishes by inactivating the fibroblast cultures (between passages 4 and 10) mitotically using irradiation (35 Gy). Cells were plated on 0.1% gelatin-coated (Sigma, Switzerland) culture dishes (Nunclon, Roskilde, Denmark) at a density of 3.5×10^5 cells/cm^2 (Inzunza *et al*., 2005). The feeder cell culture medium was Dulbecco's modified Eagle's medium (DMEM) containing 2 mM glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin, 1 × MEM-amino acids (ICN Biomedicals, USA), 1 mM sodium pyruvate (Sigma, Switzerland) and 10% FCS. Feeder cell dishes were used within a week of plating. The original culture medium was changed to stem cell medium just before ES or iPS cells were added.

The hESC lines H1 and H9 from WiCell Research Institute Inc. (Madison, WI, USA, www.wicell.org), VAL3 from the Spanish National Stem Cell Bank (Prince Felipe Research Centre, Valencia, Spain) (Valbuena *et al*., 2006) and the HS181 line from the Karolinska Institutet were cultivated by passaging colonies of hESC once every 5–6 days on irradiated HFFs by means of mechanical dissociation. The propagation medium was composed of DMEM/F-12 or Knockout DMEM (Gibco/BRL, Paisley, Scotland, UK)+20% serum replacement, 1% penicillin-streptomycin, 1% non-essential amino acid (NEAA), β-mercaptoethanol (Sigma-Aldrich GmbH, Buchs, Switzerland, www.sigmaaldrich.com), L-glutamine and 4 or 20 ng/ml bFGF. All cell culture products were purchased from Gibco (Invitrogen AG, Basel, Switzerland, www.invitrogen.com) unless otherwise specified. Human ESC colonies were passaged mechanically every 5–6 days. The four hESC lines were grown concurrently in different laboratories and the same batches of irradiated transgenic HFFs and control irradiated non-transduced HFFs were used. The ratio of expansion was calculated by dividing the number of colonies in $P+1$ by the number of colonies in P, where P refers to passage. Fragment sizes obtained were similar in all four hESC lines, with 3–5 fragments from every colony cut.

Lentiviral vector production and HFF immortalization

The packaging constructs used in this study were the previously described pCMVΔR8.92 and the pCMVΔR8.93 plasmids (Dull *et al*., 1998). The vesicular stomatitis virus G envelope was the pM2DG plasmid (Zufferey *et al*., 1997). The pLOX-*TERT*-iresTK and pLOX-CWBmi1 expressing vectors for immortalization were kindly donated by Didier Trono (Salmon *et al*., 2000) and pWPI-bFGF-iresGFP by Jozef Kiss (Dayer *et al*., 2007). The pXL-EF1-spFGF2-i2TK::Sh LV-vector was generated by replacing Green Fluorescent Protein (GFP) with HSV1TK::ShBle fusion protein (originating from plasmid pMOD-HSV1TKSH from InvivoGen, USA). Maps and sequences of Lentiviral vectors (LVs) described in this study can be downloaded at http://medweb2.unige.ch/salmon/lentilab/.

Viral particles were produced using the transient transfection method (Zufferey *et al*., 1997). A total of 293T cells (2.8×10^6 cells in 10-cm tissue culture dishes) were co-transfected with either 10 μg of pLOX-*TERT*-iresTK, 3.75 μg of pCMVΔR8.92, 3.75 μg of pCMVΔR8.93, and 2.5 μg of pM2DG, or 10 μg of pLOX-CWBmi1, 3.75 μg of pCMVΔR8.92, 3.75 μg of pCMVΔR8.93, and 2.5 μg of pM2DG, or 10 μg of pWPI_SPbFGF-iresGFP, 3.75 μg of pCMVΔR8.92, 3.75 μg of pCMVΔR8.93, and 2.5 μg of pM2DG.

After transient transfection of the three plasmids in 293T cells by calcium phosphate precipitation, the supernatant was harvested, concentrated by ultracentrifugation and re-suspended in serum-free Cellgro medium (Boehringer Ingelheim Bioproducts, Heidelberg, Germany). Viral stocks were stored at −70°C. For immortalization, both LVs were added to the HFF target cells in six-well plates (10^4 cells/well) at a multiplicity of infection (MOI) of 2, and vector particles were left on the cells for 3 days. All strategies and the molecular constructions are shown in Fig. 1A.

Cloning of immortalized HFFs

After transduction, HFFs were clonally propagated and screened for Bmi and hTERT expression. Clones stably expressing both genes were then transduced at an MOI of 2 with LVs expressing bFGF either downstream of the TK::Sh selectable marker (pXL-EF1-spFGF2-i2TK::Sh) or downstream of the GFP (pWPI-SPbFGF), and selected with either Zeocin (InvivoGen, USA) or sorted for GFP fluorescence, respectively. Immortalized HFF expressing bFGF were then tested as feeders.

Throughout the text, HFFs transduced with pWPI_SPbFGF-iresGFP will be referred to as 'b-HFF', HFFs transduced with pLOX-CWBmi and pWPI_SPbFGF-iresGFP will be referred to as 'Bb-HFF', and HFFs transduced with pLOX-CWBmi, pLOX-*TERT*-iresTK, and pWPI_SPbFGF-iresGFP or pXL-EF1-spFGF2-i2TK::Sh will be referred to as 'I-HFF'.

HFF cell cycle

Normal and engineered HFFs were harvested and washed in 1× phosphate buffered saline (PBS), re-suspended in 500 μl 1× PBS containing 0.1% glucose and fixed with 70% cooled ethanol. Florescent-activated cell sorting (FACS) analysis was then performed after washing with PBS and incubating with propidium iodide solution and RNase A at 37°C for 45 min.

iPS generation and culture

Expression vectors of human Oct4, Sox2, Nanog and Lin28 genes were kindly provided by Addgene. iPS generation and lentiviral production are described elsewhere (Yu *et al*., 2007). Briefly, after LV production, HFF (CCL-110; ATCC, Manassas, VA, USA) were transduced with the four reprogramming genes and plated over irradiated I-HFF cells that were at passage 56. These cells were cultured in hESC culture medium. After 3 weeks, colonies with hESC morphology (iPS colonies) were isolated mechanically for expansion without antibiotic selection. The first two passages were mechanical, and then the enzymatic method using collagenase (200 U/ml) for expansion. Up to seven distinct iPS cell lines were named CHiPS 1–7 (CH stands for Confoederatio Helvetica).

Immunocytochemical analysis

Both undifferentiated hESC and iPS colonies were fixed in 4% paraformaldehyde (PFA) and subsequently permeabilized with 0.5% Triton X-100. After consecutive washing and blocking steps (using 5% bovine serum albumin, BSA), the cells were incubated with the primary antibodies SSEA-1 (1:25 dilution), SSEA-3 (1:50 dilution), SSEA-4 (1:50 dilution), NANOG (1:50 dilution), TRA-1-60 (1:50), TRA-1-81 (1:50) and OCT-4 (1:50 dilution) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). FITC- or TRITC-conjugated secondary antibodies were used for detection (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and nuclei were visualized by DAPI staining (Sigma, Switzerland).

Flow cytometry analysis

Cells were washed twice with PBS and dissociated with TrypLE Express (Invitrogen, USA) for flow cytometry analysis. To remove the feeder cells from hESC analysis, the mixed cell populations were seeded on gelatin-coated wells and incubated for 2 h at 37°C in standard hESC medium. Non-attached hES cells were subsequently incubated for 20 min at 4°C in DMEM containing 5% fetal bovine serum (FBS) with the following monoclonal antibodies: stage-specific embryonic antigen-3 (SSEA3), tumor-rejection antigen-1-60 (Tra1-60) and SSEA1 (kindly provided by Mark Jones from the Peter Andrews Laboratory, University of Sheffield, Sheffield, UK). The cells were incubated with the primary antibody at 1:10 dilution followed by a secondary Cy5-conjugated anti-rat IgM (for SSEA3) or anti-mouse IgG (for Tra1-60 and SSEA1) antibody (Chemicon, Temecula, CA, USA) at 1:200 dilution. Finally the cells were washed twice in PBS, centrifuged at 300 × *g* for 5 min, and re-suspended in DMEM containing 5% FBS. Non-viable cells were identified by their ability to incorporate propidium iodide (Sigma, Switzerland). For SSEA-4 phenotypic monitoring, VAL-3 and H9 colonies were mechanically dissected and dissociated to the single cell level with Accumax (Chemicon, Temecula, CA, USA) solution at 37°C. Cells were re-suspended to approximately 3×10^5 cells per sample and prepared in duplicate. The dissociated cells were then fixed with 4% PFA, stained with the mouse monoclonal anti-SSEA-4 (Chemicon, Temecula, CA, USA) at 1:300 and incubated with the secondary antibody Alexa Fluor 488 anti IgG (Molecular Probes, Invitrogen, Carlsbad, CA, USA).

Flow cytometry analysis was performed on a FACSCalibur (BD Biosciences, San Jose, CA, USA). Acquisition and analysis were performed with BD CellQuest™ Pro (BD Biosciences) and FlowJo (Tree Star, Ashland, OR, USA) software for SSEA3 and TRA-1-60 staining.

For SSEA-4 experiments, samples were analyzed with Cytomics CXP software on a Beckman Coulter Cytomics FC500 (Beckman-Coulter, Fullerton, CA, USA). Dead cells were excluded from acquisition using forward scatter characteristics. Negative controls were included in each set of experiments, foreskin fibroblasts being used for the primary antibody and isotype controls for secondary antibodies. All data were normalized by dividing the test mean by the control mean using an electronic gate.

In-vitro and *in-vivo* characterization of generated (iPS) cells

For *in vitro* characterization undifferentiated iPS cell colonies were transferred to suspension cultures and maintained in embryoid body (EB) medium (KnockOut-DMEM, 20% FCS, 1% PEST, 1% Glutamax, 1% NEAA, and 0.1 mM β-mercaptoethanol). EBs were cultured for 3 weeks in suspension cultures, after which half of the EBs were plated in culture dishes coated with gelatin and grown in the presence of EB medium and half were fixed in 4% PFA for sectioning. For plated EBs, the medium was changed every 2–3 days and after 14 days the cells were fixed in 4% PFA. For both experiments, cells were subjected to immunohistochemical evaluation using primary antibodies directed against α-smooth muscle actin, β-III-tubulin and Nestin (all from Santa Cruz Biotechnology, USA). FITC-conjugated secondary antibodies were used for detection (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

For the *in-vivo* characterization, 5 million of CH-iPS cells were collected from I-HFF feeder layers by collagenase IV and were re-suspended in a mixture of DMEM culture medium and Matrigel (ratio of 1:1). The cell mixtures were injected subcutaneously into flanks of SCID mice. Tumors were dissected from mice 5–8 weeks after injection and paraffin sections were processed and stained with haematoxylin and eosin.

Karyotype of immortalized HFFs and hESCs

Karyotyping of H1, H9, HS181, VAL-3, iPS cells and the immortalized HFF was performed by an independent laboratory in at least 20 metaphase spreads, using the GTG-banding method (Genetic Service, Geneva University Hospital, Switzerland).

Basic FGF secretion in the medium

For measurement of the secretion of bFGF by engineered HFFs, cells were mitotically inactivated by irradiation in the same way as feeders used for hESC culture and plated on 0.1% gelatin-coated (Sigma, Switzerland) culture dishes (Nunclon, Roskilde, Denmark) at a density of 3.5×10^5 cells/cm^2

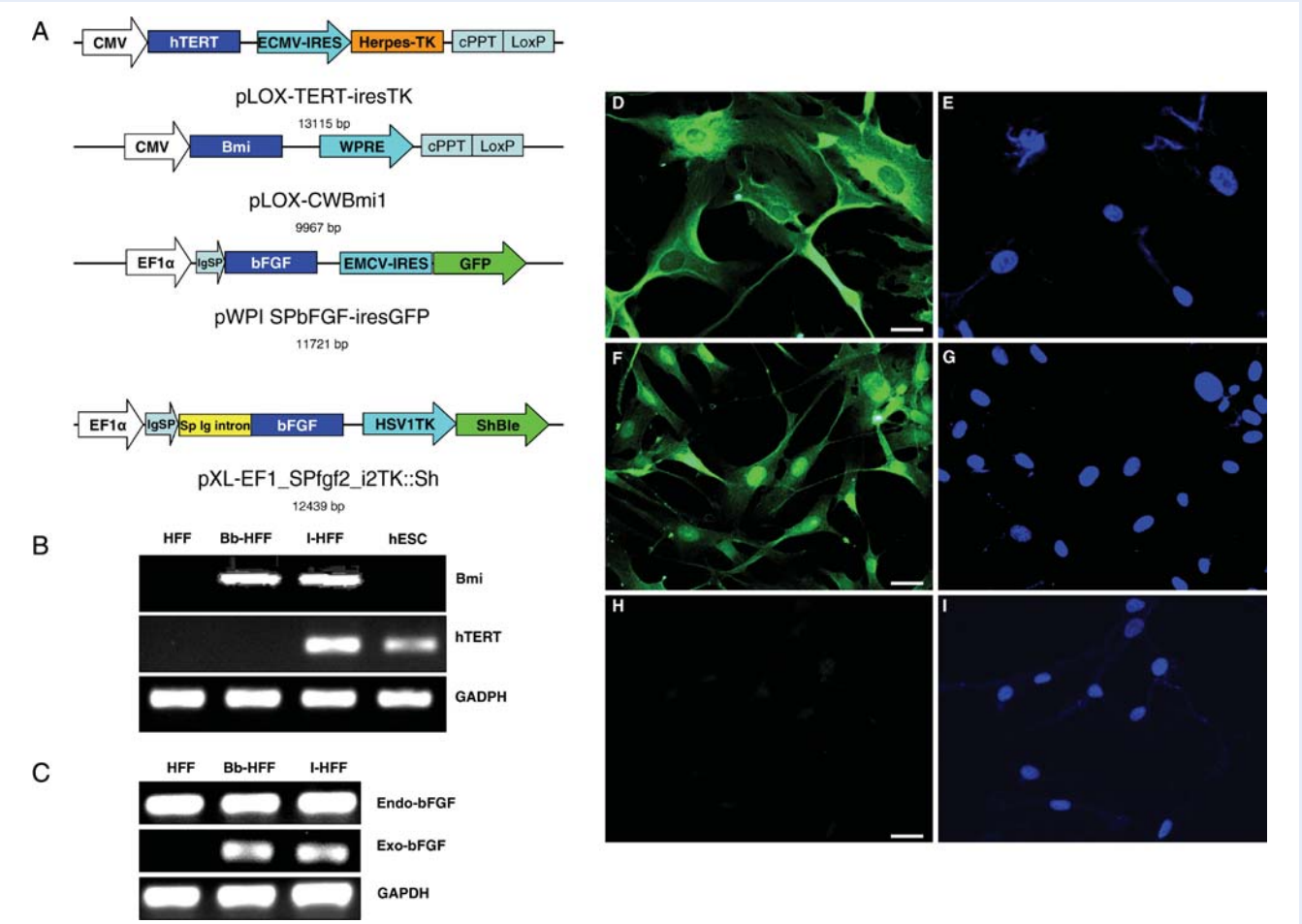

Figure 1 Schematic for the generation of transgenic feeders.
(**A**) HFFs were immortalized using LV constructs expressing hTERT and Bmi. (**B**) Expression of Bmi and hTERT. Upon transduction, clonally expanded cell lines were screened by RT–PCR to select those that constitutively expressed Bmi, hTERT or Bmi–hTERT. Foreskin fibroblasts expressing Bmi or hTERT/Bmi were then transduced with LV expressing bFGF fused to an Ig_Sp secretion signal sequence. Bmi and hTERT expression were analyzed by semi-quantitative RT–PCR in HFF, Bb-HFF, I-HFF and hESCs. (**C**) Endogenous versus exogenous bFGF expression in HFF, Bb-HFF, I-HFF. (**D**) Immunofluorescence staining of bFGF expression in HFF and I-HFF (**F**) (transduced with pXL-EF1-spFGF2-i2TK::Sh), (**E, G, I**) DAPI staining, (**H**) negative control. Bar = 20 μm.

[normal cell concentration used for hESC culture system (Hovatta *et al.*, 2003)]. Engineered feeders were cultured in the presence of hESC medium with or without bFGF. The medium was removed at several time-points, and the amount of bFGF secreted was quantified with a standard ELISA detection method (Quantikine, R&D, USA).

RNA isolation and reverse transcription

Total RNA was extracted using a Total RNA Extraction Kit (Qiagen, Hombrechtikon, Switzerland) according to the manufacturer's instructions for small-scale quantities of cells, and RNA concentration was assessed with a spectrophotometer (Biowave II, Biochrom, UK). Total RNA (1 μg) from each sample was used for first-strand cDNA synthesis with the Advantage RT–PCR Kit (Clontech; BD Biosciences, Switzerland) following the manufacturer's protocol. The cDNA from each sample was used as a PCR template to detect the expression of the genes listed in Table I. PCR reaction mixtures were prepared with 1 μg total cDNA as the template, and were then denatured at 94°C for 4 min, and cycled 32 times at 94°C for 30 s, 55–60°C for 30 s and 72°C for 1 min. A final extension at 72°C for 10 min was performed after cycling. PCR primers were designed with Vector NTI freeware (Invitrogen) to show possible genomic contamination. Negative control samples, including characteristic differentiation genes from ectoderm, mesoderm and endoderm, were included in each reaction. PCR products were resolved on 2% agarose gels, stained with ethidium bromide and visualized in a transilluminator.

Results

LV-mediated conditional immortalization of HFFs

The SV40 large T antigen (TAg) is extremely efficient at achieving immortalization, but induces a high degree of genetic instability that rapidly leads to chromosomal aberrations and profound phenotypic changes. In contrast, cells immortalized via the constitutive expression of hTERT and Bmi were devoid of these problems; we thus decided to

Table I Primers used for PCR and semi-quantitative RT–PCR analysis

Gene	Sense primer (5′–3′)	Anti-sense primer (3′–5′)
Oct3/4	GACAACAATGAAAATCTTCAGGAGA	TTCTGGCGCCGGTTACAGAACCA
Nanog	AAGACAAGGTCCCGGTCAAG	CCTAGTGGTCTGCTGTATTAC
REX1	GCGTACGCAAATTAAAGTCCAGA	CAGCATCCTAAACAGCTCGCAGAAT
hTERT	CGGAAGAGTGTCTGGAGCAAGT	GAACAGTGCCTTCACCCTCGA
PAX6	GAATCAGAGAAGACAGGCCA	GAGTAGGTATCATAACTCCG
NESTIN	GGCAGCGTTGGAACAGAGGTTGGA	CTCTAAACTGGAGTGGTCAGGGCT
GATA4	CTGGCCTGTCATCTCACTACG	GGTCCGTGCAGGAATTTGAGG
IHH	CTACGCCCCGCTCACAAAG	GGCAGAGGAGATGGCAGGAG
T(Brachyury)	GCAAAAGCTTTCCTTGATGC	ATGAGGATTTGCAGGTGGAC
AFP	GGGAGCGGCTGACATTATTA	CCCTCTTCAGCAAAGCAGAC
GAPDH	AGCCACATCGCTCAGACACC	GTACTCAGCGGCCAGCATCG
Endo-bFGF	GCAAAAACGGGGGATTCTTCCTG	TGCCCAGGTCCTGTTTTGGA
Exo-bFGF	ATGAAATGCAGCTGGGTTATC	TTGATGTGAGGGTCGCTCTT

use the latter strategy (Cudre-Mauroux *et al*., 2003). For reversible immortalization, commercially available HFFS and newly-derived foreskin fibroblasts were transduced simultaneously with loxP-containing human immunodeficiency virus (HIV)-derived LVs encoding hTERT and/or Bmi1 polycomb ring finger oncogene (Bmi) (Fig. 1A). HFFs transduced with a both LVs expressing hTERT and Bmi were subjected to cloning by limiting dilution. Clones arising from this procedure were screened for the presence of Bmi, *TERT* or Bmi/hTERT. Clones expressing either Bmi or both hTERT and Bmi were randomly selected, expanded, and further transduced with a LV expressing basic bFGF together with eGFP living color (pWPI_SPbFGF lentivector), or TK::Sh (pXL-EF1-SPfgf2-i2TK::Sh)-selectable markers. Notably, a secretion signal sequence was added to the coding sequence of bFGF in both constructs, and the cells obtained were polyclonal for bFGF expression and secretion.

To date, I-HFF cells have been maintained in culture for more than 1 year, namely more than 70 passages, which confirms their immortalized character. The LVs used in these experiments resulted in integrants flanked by loxP sites. As described previously for similarly immortalized liver endothelial cells (Salmon *et al*., 2000), the addition of Cre-recombinase led to the rapid growth arrest of these cells (data not shown). This demonstrated that their continuous proliferation was strictly dependent on the presence of immortalizing transgenes. Indeed, semi-quantitative RT–PCR analysis confirmed the presence of Bmi and exogenous hTERT in I-HFF compared with hESC that only express endogenous hTERT (Fig. 1B). Moreover, forced expression of Bmi does not activate endogenous hTERT.

All generated cells, HFF (original foreskin fibroblasts), Bb-HFF (transduced with the LVs expressing Bmi and bFGF) and I-HFF (transduced with the LVs expressing Bmi, hTERT and bFGF) were analyzed by RT–PCR for exogenous and endogenous bFGF expression. The HFF showed a basal expression of endogenous bFGF, whereas Bb-HFF and I-HFF expressed both (Fig. 1C).

Immunocytochemical analysis showed the presence of bFGF in the cytoplasm of HFF; in I-HFF, however, the expression of bFGF was both nuclear and cytoplasmic (Fig. 1D–F).

Characterization of conditionally immortalized HFFs

I-HFF showed an increased proportion of cells residing in the G2/M phase of the cell cycle (Fig. 2A). When b-HFF and Bb-HFF were compared with I-HFF at passage 20, the former two showed cell cycle arrest and accumulation in the G0/G1 phase of the cell cycle (Fig. 4E). Moreover, apart from I-HFF, all the other engineered cells showed an increased rate of apoptosis.

A karyotype analysis confirmed that I-HFF stably conserved the diploid state of control primary cells at passage 40 this was also stable in the hESC lines analyzed over the passages.

Using ELISA, we quantitatively assessed the time course secretion of bFGF in the medium by cultured HFF, B-HFF, Bb-HFF and I-HFF. Figure 2B showed that naïve HFF had steady basal low secretion of bFGF that did not exceeded 30 pg/ml. All transgenic feeders, however, secreted high concentrations of bFGF that reached 340 pg/ml at day 6 in Bb-HFF cells (Fig. 2B). Analysis of bFGF secretion by I-HFF and HFF in the presence of hESC did not reveal any significant difference between these two feeders (Fig. 2C). However, the decreased level of bFGF after addition of exogenous bFGF was significantly slower in the supernatant of hESC cultured on I-HFF than hESC cultured on HFF. H1 was also maintained and cultured in an undifferentiated state longer over I-HFF than over HFF in the absence of bFGF (Fig. 2D and E). Therefore, we decided to change the culture medium by adding exogenous bFGF at 4 ng/ml every 48–72 h as standard protocol for ESCs cultured on I-HFF.

Maintenance of pluripotency of various human ESC lines

The four hESC lines H1, H9, HS181 and VAL-3 from three laboratories were cultured on mitotically-inactivated immortalized bFGF-secreting feeder cells for 5–10 passages. Basic FGF was added to the culture medium of the modified feeders only twice a week. On these feeder cells, hESC lines H9 and HS181 maintained their

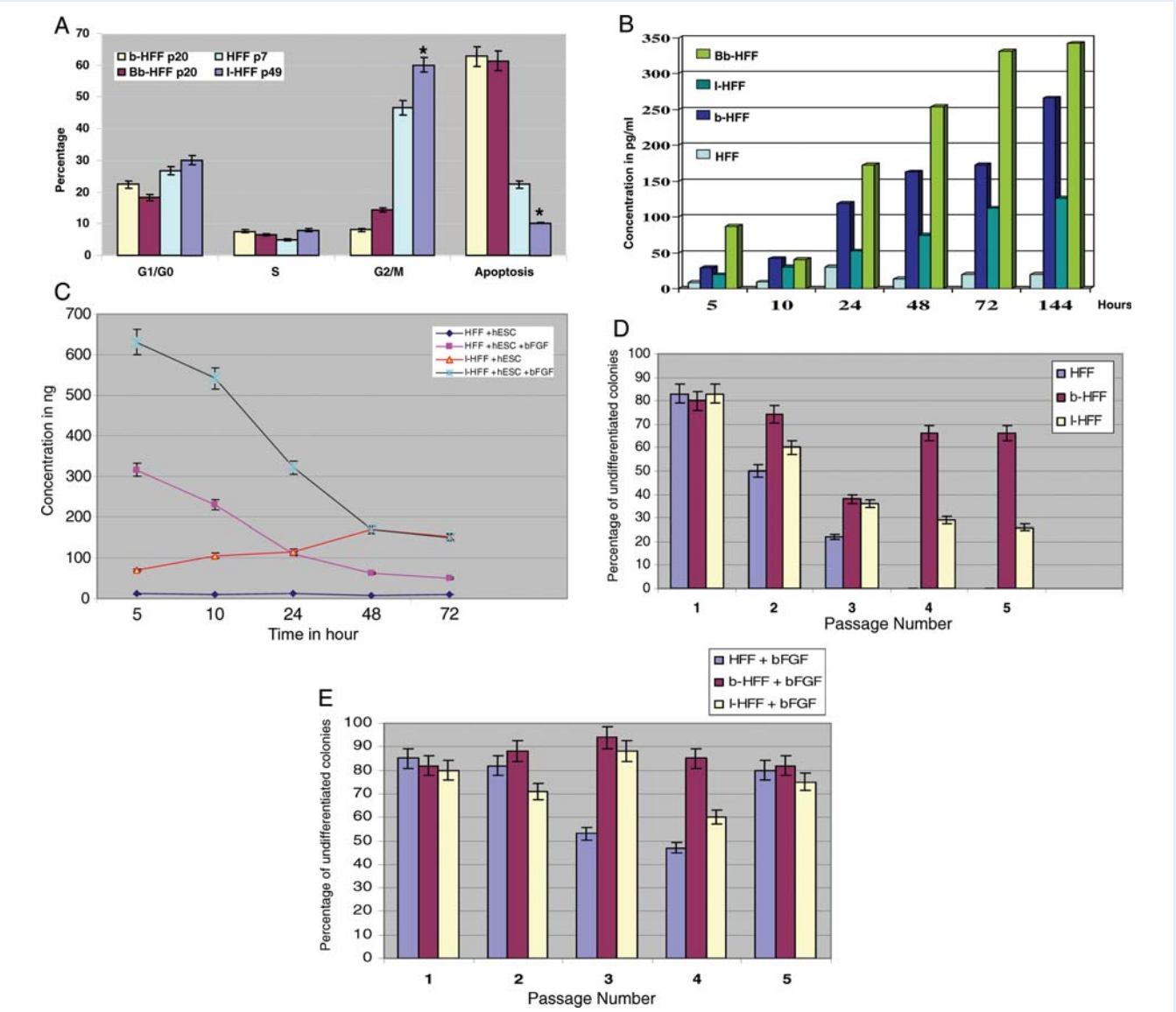

Figure 2 (**A**) Cell cycle analysis of HFFs at passages 7, Bb-HFF at passage 20, b-HFF at passage 20 and I-HFF at passage 49. About 60% of I-HFF cells were at G2/M phase, and they underwent less apoptosis at passage 49 when compared with HFF or Bb-HFF at passage 20. The asterisk (*) indicates that the difference is statistically significant ($P < 0.05$) when I-HFF was compared with the other cells. (**B**) ELISA assessment of bFGF secretion by the different engineered HFFs. (**C**) ELISA assessment of bFGF content in I-HFF and HFF medium in the presence of hESC supplemented or unsupplemented at $t = 0$ with exogenous bFGF at a concentration of 4 ng/ml. (**D**) Comparison of H1 expansion when cultured over HFF, b-HFF and I-HFF in the absence of bFGF. The same number of colonies was seeded over different feeders at the beginning of the experiment. As for I-HFF, b-HFF maintained H1 for at least five passages, but HFF could not maintain H1 for more than three passages. (**E**) The percentage of undifferentiated H1 colonies scored for five consecutive passages over HFF + bFGF was significantly different, whereas that for H1 over b-HFF and I-HFF + bFGF presented some random fluctuations.

undifferentiated state at passages 32 and 42 significantly longer than on non-modified feeders, as shown in Fig. 3. The expansion ratios of VAL-3 and H9 hESCs was higher on transgenic feeders than it was on non-transgenic feeders (Fig. 3A and B) when cultured for up to nine passages.

FACS analysis of H9 and HS181 lines after more than five passages over HFF and I-HFF demonstrated stable expression of pluripotency markers SSEA-3 and TRA 1-60 (Fig. 3E and G). In addition, both non-modified and modified foreskin fibroblasts also expressed SSEA-3, but not TRA-1-60 (Fig. 3D and F). HS181 and H9 also showed typical undifferentiating values for SSEA-4 staining by flow cytometry (not shown). All hESC colonies had typical undifferentiated ESC morphology with sharp edges (Fig. 4A) and expressed OCT4 (Fig. 4B) and NANOG, as revealed by immunofluorescence (Fig. 4C). A normal karyotype was detected in the hESC lines cultured on the immortalized feeders (Fig. 4E–G).

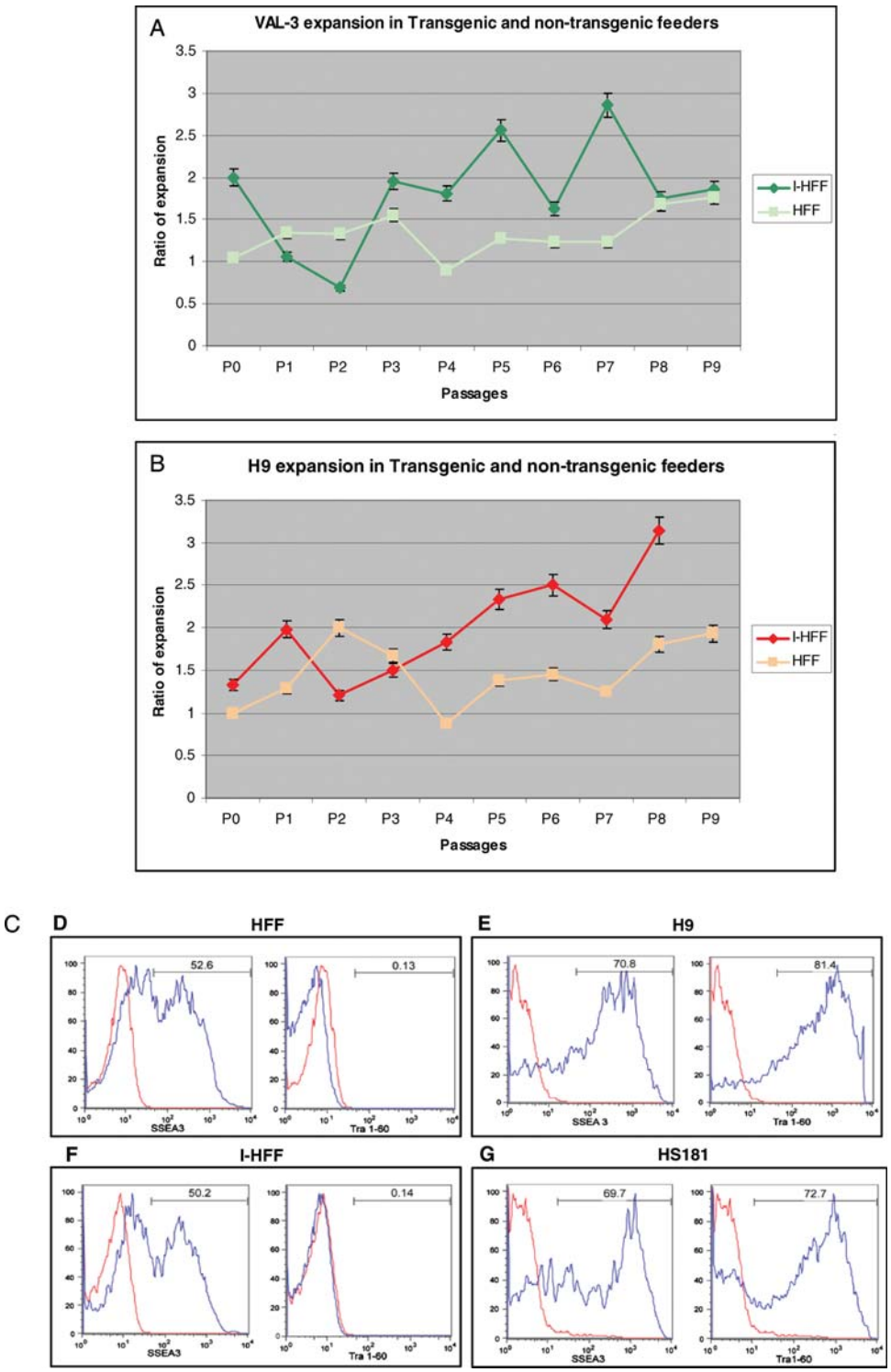

Figure 3 Pluripotency maintenance of different hESCs.
(**A**) Ratio of expansion of VAL3 (**B**) and H9 when cultured over HFFs and I-HFF. (**C**) Flow cytometry analysis of SSEA-3 and Tra-1-60 expression in HFF (**D**) and I-HFF (**F**) and in H9 (**E**) and HS181 (**G**) when cultured over HFF and I-HFF after more than five passages. The results showed a comparable level of expression of the pluripotency markers SSEA-3 and Tra-1-60 in both culture systems.

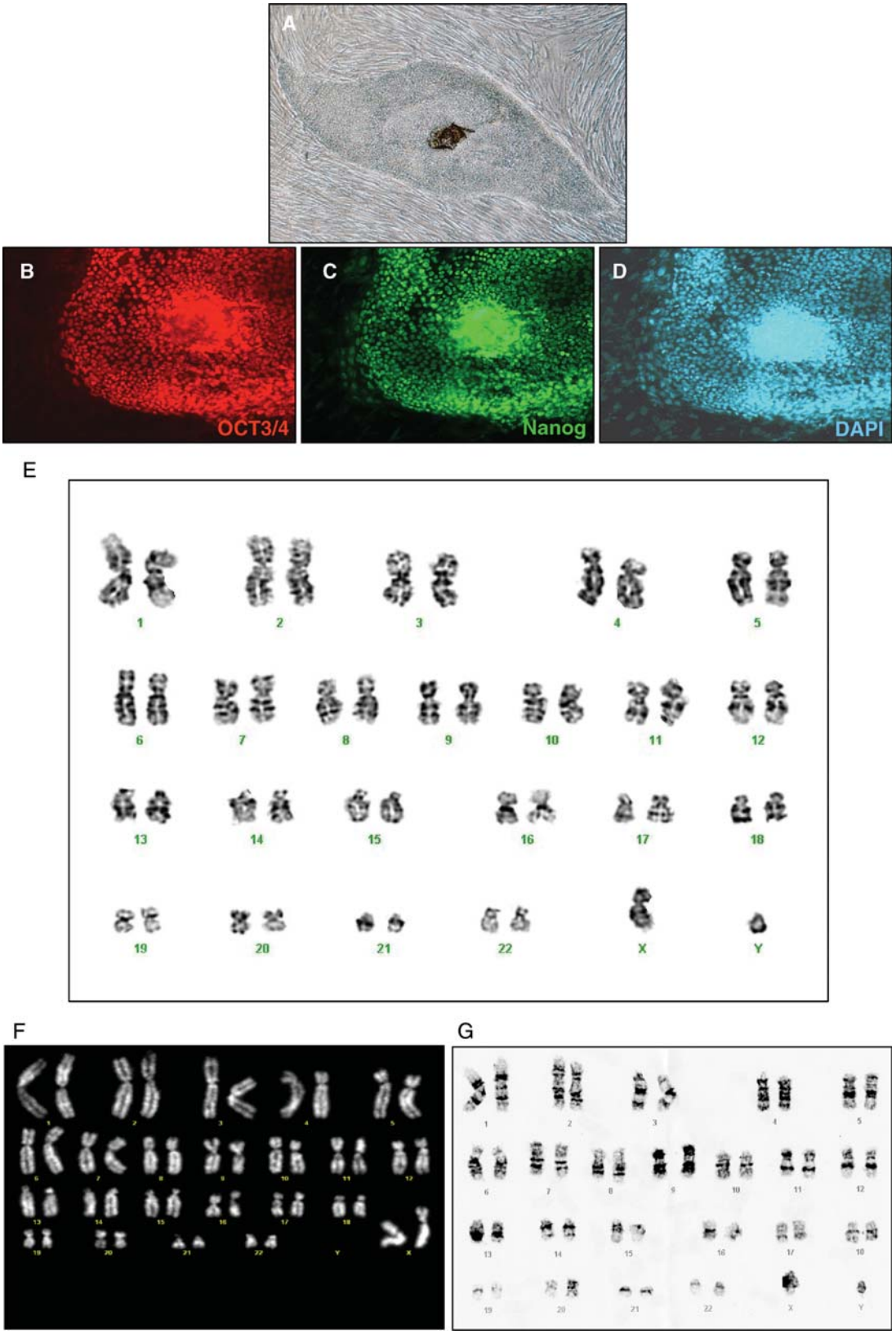

Figure 4 (**A**) H1 hESCs morphology when grown over I-HFF feeders.
(**B**) Oct4 and (**C**) Nanog expression in H1 hESC growing over I-HFF cells as feeders. (**D**) DAPI staining. Bar = 20 μm. (**E**) Karyotype at passage 40 of immortalized feeders constitutively expressing Bmi and hTERT as well the secreted form of bFGF. Karyotype analysis of (**F**) H1 and (**G**) H9 after five passages over the I-HFF.

Generation and characterization of iPS cells over I-HFF

The transduction of HFFs with LVs expressing Oct4, Nanog, Sox2 and Lin28, and the plating over irradiated immortalized feeders at a high passage (passage 56) proved successful. To facilitate observation of the new iPS cell colonies, we generated I-HFF expressing both bFGF and eGFP as a selection marker (Fig. 5C, F, I). Colonies formed by small stem cell-like cells appeared on the feeders about 20 days post-transduction (Fig. 5A, B, D, E). The small cells had typical hESC morphology with a large nucleus and scanty cytoplasm (Fig. 5L). The first two mechanical passages resulted in round iPS cell colonies on the feeders (Fig. 5G, K). Thereafter, enzymatic passage resulted in similar morphology.

At passage seven, the colonies displayed a normal karyotype (data not shown). When compared with the H1 hESC line, the majority of derived iPS lines expressed endogenous pluripotency markers REX1, SOX2, OCT4, NANOG, LIN28 (Fig. 6A). Moreover, despite the viral genes Oct4, Nanog, Sox2 and Lin28 were integrated in the genome of the different iPS lines (called CHiPS 1-7) as verified by PCR (Fig. 6B), no expression from exogenous transgene was detected by semi-quantitative PCR. Like CHiPS 4, 5, 7, the immunocytochemistry of CHiPS 6 showed the expression of OCT4, Nanog, SSEA4, SSEA3 and TRA-1-61 proteins (Fig. 6C). To eliminate the possibility that iPS cells were originally derived from I-HFF feeders which had integrated the reprogramming viruses, we performed a PCR on genomic DNA of CHiPS lines and showed that they did not contain and had not integrated any of the immortalizing vectors, namely Bmi and hTERT (Fig. 6D).

When differentiated into partially cystic EBs, tissue components expressed markers of the three germ layers (Fig. 7A). EBs resulting from different colonies of iPS cells expressed Nestin, GATA4, IHH, AFP and CK3 markers as revealed by semi-quantitative RT–PCR (Fig. 7B). CHiPS7, one of the cell sub-lines, also expressed Brachyury (Fig. 7B). The expression of the markers representative of the three germ layers, namely SMA, AFP and beta-3-tubulin, was demonstrated by immunohistochemistry of CHiPS 7 (Fig. 7C), and by immunofluorescence (Fig. 7D). Derived iPS cells also induced teratomas when injected into immuno-suppressed SCID mice (data not shown).

Discussion

We established immortalized bFGF-secreting human skin fibroblasts to be used as feeder cells for hESC and iPS cells. The cells supported the expansion of four different hESC lines in an undifferentiated state and also allowed the growth and derivation of iPS cells originating from the same parental fibroblasts. Several hESC and newly-established iPS cell lines were karyotypically normal after culture on these genetically modified feeders. Human ESCs remained undifferentiated and pluripotent on these feeders after 5–10 passages. These feeders thus offer a new standardized and effective culture system for hESCs.

Several types of feeder cells have been successfully used for hESC culture. At first, MEFs were used (Thomson *et al*., 1998; Reubinoff *et al*., 2000) followed by human fetal muscle and adult fallopian tube fibroblasts (Richards *et al*., 2002, 2003). HFFs were employed for both culture of existing hESC lines (Amit *et al*., 2003) and derivation of new ones (Hovatta *et al*., 2003). Fibroblasts differentiated from hESCs have been successfully used as feeder cells by several groups (Xu *et al*., 2004; Stojkovic *et al*., 2005; Wang *et al*., 2005a, b; Saxena *et al*., 2008). Human placental fibroblasts from early pregnancy were also functional (Genbacev *et al*., 2005; Simon *et al*., 2005) and adult bone marrow cells were employed by Cheng and colleagues (2003). The number of different cell types used as feeders reflects the fact that, although functional, none of these cell types as feeders have been optimal. MEFs bear a risk of infection and immune response (Martin *et al*., 2005), placental fibroblasts are not easy to isolate, and human skin cells vary in quality and do not support optimal growth after 20 passages (Unger *et al*., 2008a). There is also large batch-to-batch variation among these cell types (Eiselleova *et al*., 2008).

For the moment, bFGF remains a necessary growth factor for the undifferentiated growth of hESCs (Amit *et al*., 2003; Koivisto *et al*., 2004; Dvorak and Hampl, 2005). It regulates the expression of TGFβ1, Activin A and the BMP4 antagonist which are important for hESC growth and self-renewal (Dvorak and Hampl, 2005; Greber *et al*., 2006; Eiselleova *et al*., 2008). The fact that MEF and HFF lines secrete varying amounts of bFGF (Eiselleova *et al*., 2008) may partially explain why different batches of MEFs and HFFs support hESCs differently. Other growth factors such as Activin A and TGFβ1 also play a role (Eiselleova *et al*., 2008), and variability in the production of these factors affects their ability to support hESCs. Cai and colleagues (2007) generated immortalized feeder cells overexpressing Wnt3a that are supportive of hESC expansion and pluripotency maintenance. One fibroblast-like feeder cell line which differentiated spontaneously from hESCs supported hESC expansion particularly well (Saxena *et al*., 2008) up to passage 15. By secreting relatively high amounts of endogenous bFGF, these feeders required the addition of only 2 ng/ml bFGF to the culture medium instead of the usual concentrations of 4–20 ng/ml. In all cases, bFGF is expressed in its natural form, i.e. mostly un-secreted and cytoplasmic.

Therefore, in our study, we added a secretion signal to the bFGF, enabling its secretion, as demonstrated by Dayer and colleagues (2007). Moreover, as we detected bFGF in both the nucleus and the cytoplasm of I-HFF, we infer this by the existing sequence similarities between the secretion signal (Ig_sp) and known nuclear localization signals. As a result, our transduced immortalized bFGF-producing feeders (I-HFF) expressed higher levels of bFGF which were more suitable to hESC maintenance. Basic FGF is unstable in solution, which means that the medium has to be renewed daily to achieve an optimal propagation; besides being expensive, this is laborious and time-consuming. The use of bFGF-producing feeders standardizes and decreases the cost of cultures. By selecting and screening the best monoclonal I-HFF cell line for the secretion of bFGF, we may avoid the exogenous addition of this growth factor and will improve the standardization of embryonic stem cell culture conditions.

Immortalization enables the use of the same feeder cell line for prolonged periods of time in more standardized culture conditions, a critical issue in the maintenance and stability of hESCs (Skottman and Hovatta, 2006). Here we demonstrated that even at high passages (p49) our feeders secreted high concentrations of bFGF although in non-immortalized cells bFGF secretion decreased at much lower passage levels. Since I-HFF were clonal after immortalization with hTERT and Bmi and were not clonal for bFGF, a possible heterogeneity may explain the variation in its secretion. As a consequence, we added bFGF twice a week in order to further standardize our

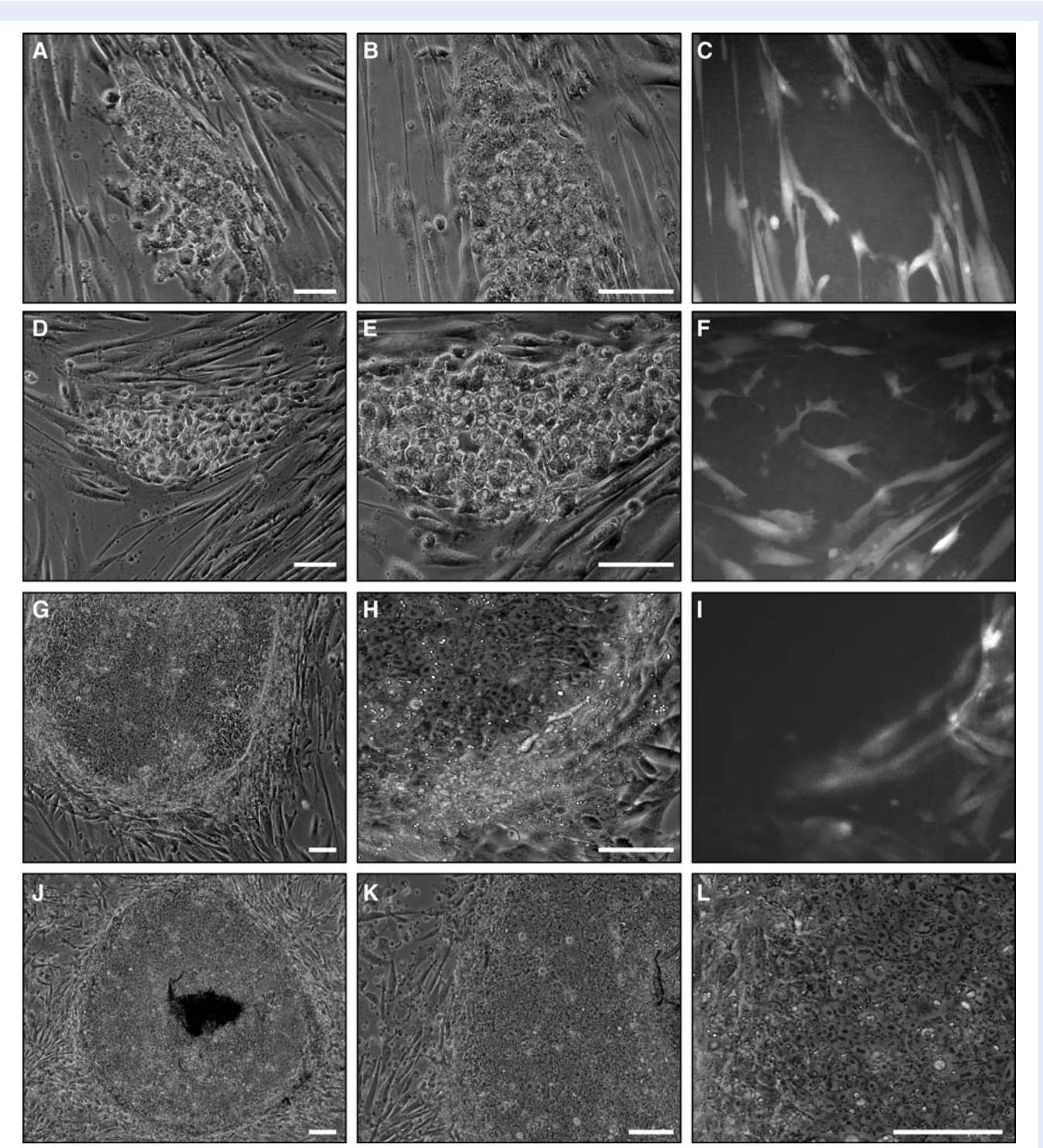

Figure 5 iPS cell generation over I-HFF (transduced with pWPI SPbFGF-iresGFP).
(**A, B, D,** and **E**) Morphology of reprogrammed colonies growing over I-HFF feeder cells. (**C, F**) Epifluorescent pictures showing that I-HFF cells were green fluorescent, allowing the easy identification of reprogrammed colonies. (**G, H**) Morphology of reprogrammed colony after the first passage when grown over I-HFF, (**I**) as shown with epifluorescent microscopy. (**J**) Typical morphology of an established iPS colony (4 × magnifications). (**K, L**) iPS cells displaying a hESC-like phenotype. Bar = 20 μm.

culture conditions. Notably, almost no senescence or apoptosis was seen in the immortalized bFGF-secreting feeders at passage 49, which was in strong contrast to HFF and Bb-HFF.

Xeno-free feeders would be necessary for clinical tests and treatment (Unger *et al.*, 2008b). We have already established a system for GMP-quality HFFs (Unger *et al.*, 2008a) and a transduction

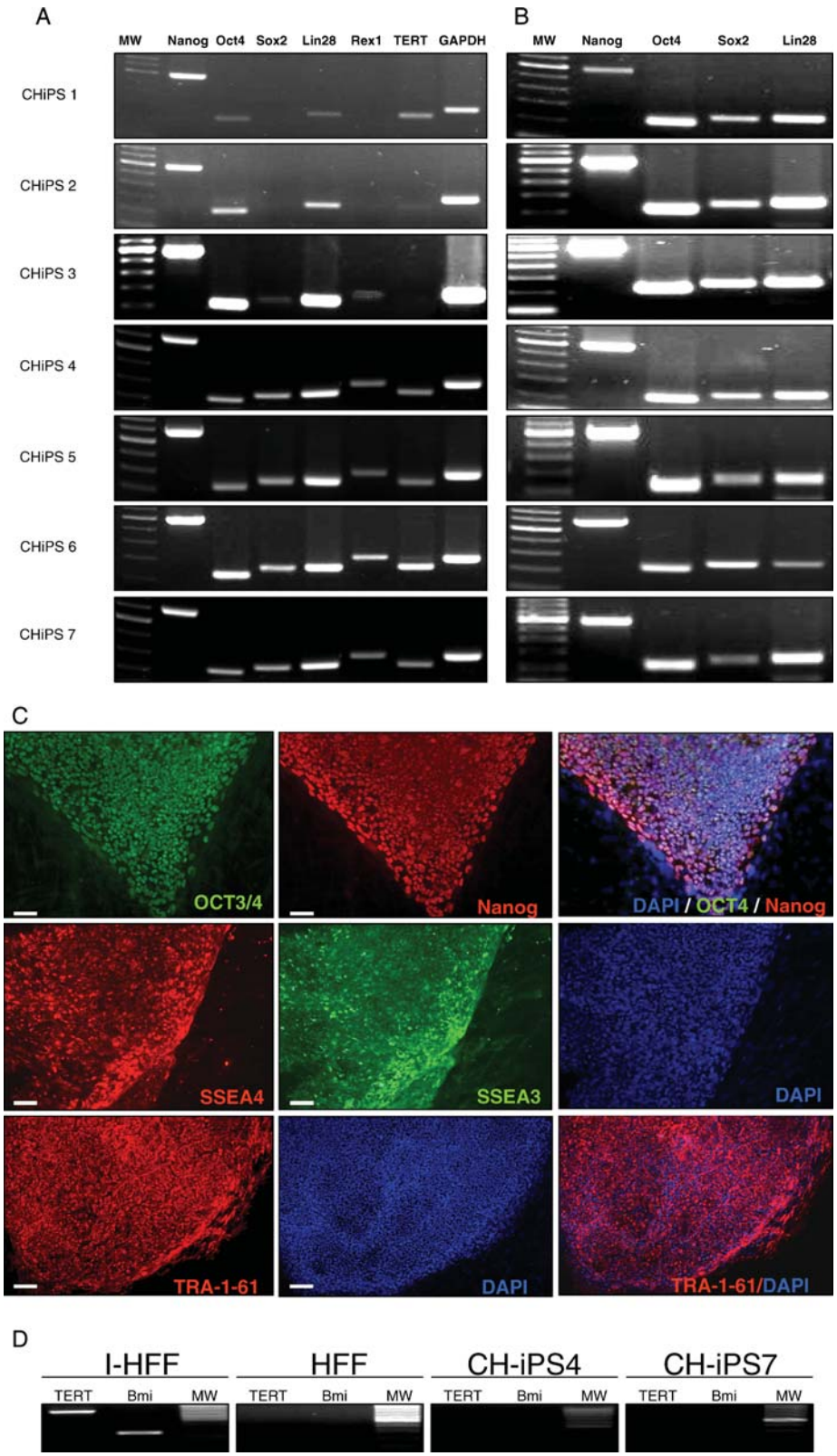

Figure 6 (**A**) Semi-quantitative RT–PCR expression profile of different endogenous pluripotency markers expressed in iPS cells when derived over the immortalized feeders.

Exogenous transgenes (Oct4, Nanog, Sox2) were not detected with semi-quantitative RT–PCR. (**B**) Integration of viral genes in genomic DNA of derived iPS determined by PCR. (**C**) Immuno-fluorescence staining of Oct4, Nanog, SSEA-3, SSEA-4 and Tra-1-60 in iPS cells derived using I-HFF as feeders. Bar = 20 μm. (**D**) Determination of integrated viral genes Bmi and hTERT in genomic DNA of I-HFF, HFF and CHiPS 4 and 7 by PCR.

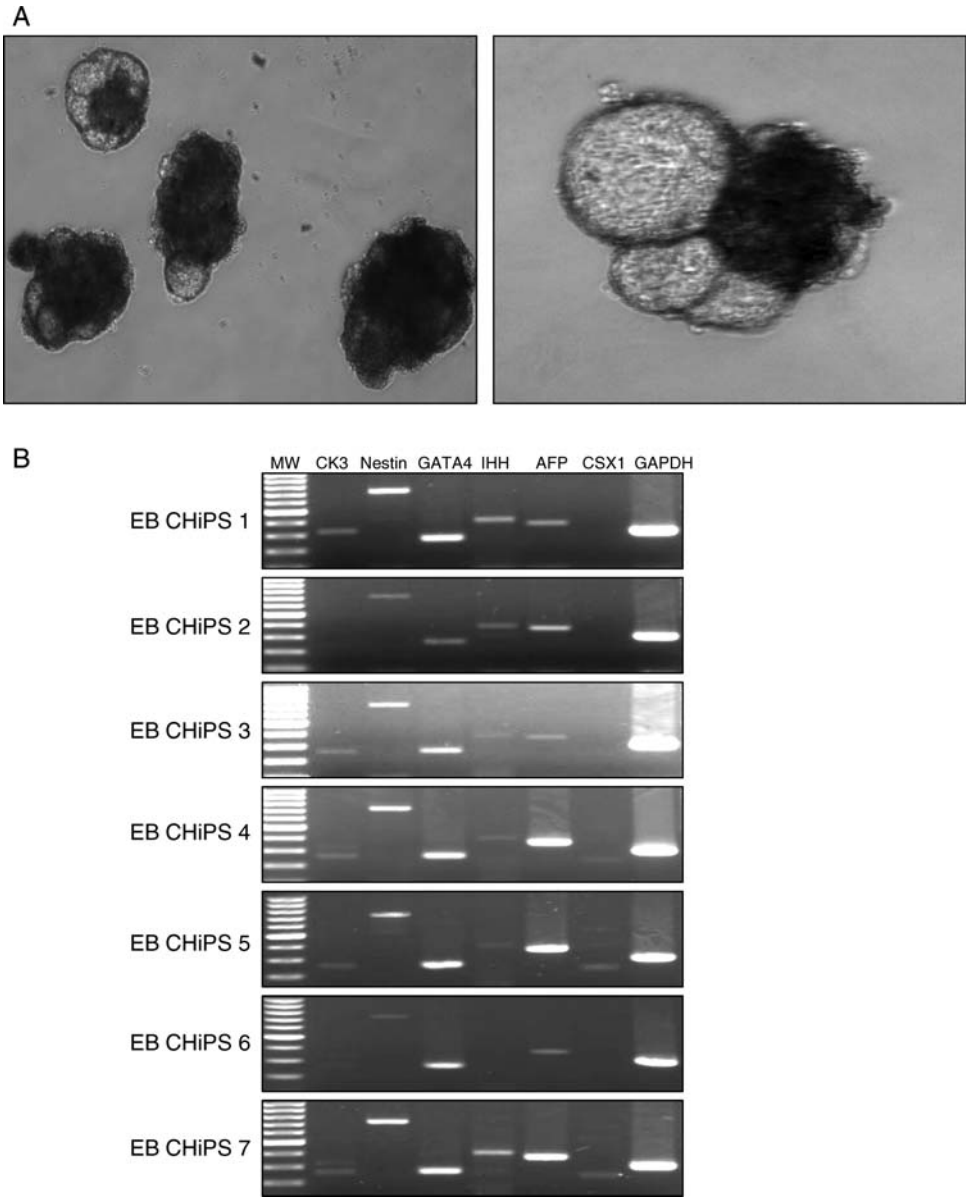

Figure 7 (**A**) Embryoid bodies formed from CHiPS 7 colonies after expansion over I-HFF feeders and growth in suspension. (**B**) Representative expression of three germ cell layers by semi-quantitative RT–PCR after 3 weeks of culture. (**C**) Immunohistological analysis of iPS cell-derived EBs. (a) Negative control, (b) SMA, (c) AFP and (d) β3-tubulin staining. (**D**) iPS cell differentiation potential. iPS cells were cultured in suspension as EB and plated over cover-slips coated with gelatin. As revealed by immunocytochemistry, differentiated cells expressed Nestin (top), β3-tubulin (middle, indicated by arrow) and SMA (bottom). Nuclei are stained by DAPI. Bar = 20 μm.

system similar to the one used in the present study can be applied to generate GMP-quality feeder cells. Such a procedure is currently being implemented.

Recently, the mechanisms governing the self-renewal of ESCs have become clearer (Saxena *et al.*, 2008). In the future, we may not require feeder cell support to establish stable hESC lines. By efficiently inhibiting differentiation during the propagation stage and releasing them to differentiate when desired, we may be able to obtain standardized controllable cells. Improved systems of feeder-free cultures are also under development.

Two teams have succeeded in creating feeder-free derivations of hESC lines. Klimanskaya and colleagues (2005) used a mouse-derived matrix which was neither xeno-free nor defined. Ludwig and colleagues (2006) derived two hESC lines on human tissue-derived fibronectin and laminin and in chemically-defined medium. One of these lines was karyotypically abnormal (47,XXY) from the beginning, and the other had gained an extra chromosome by passage 40. Although the first chromosomal abnormality may have resulted from an abnormal embryo, it was hypothesized that feeder-free conditions may promote culture adaptation of abnormal lines

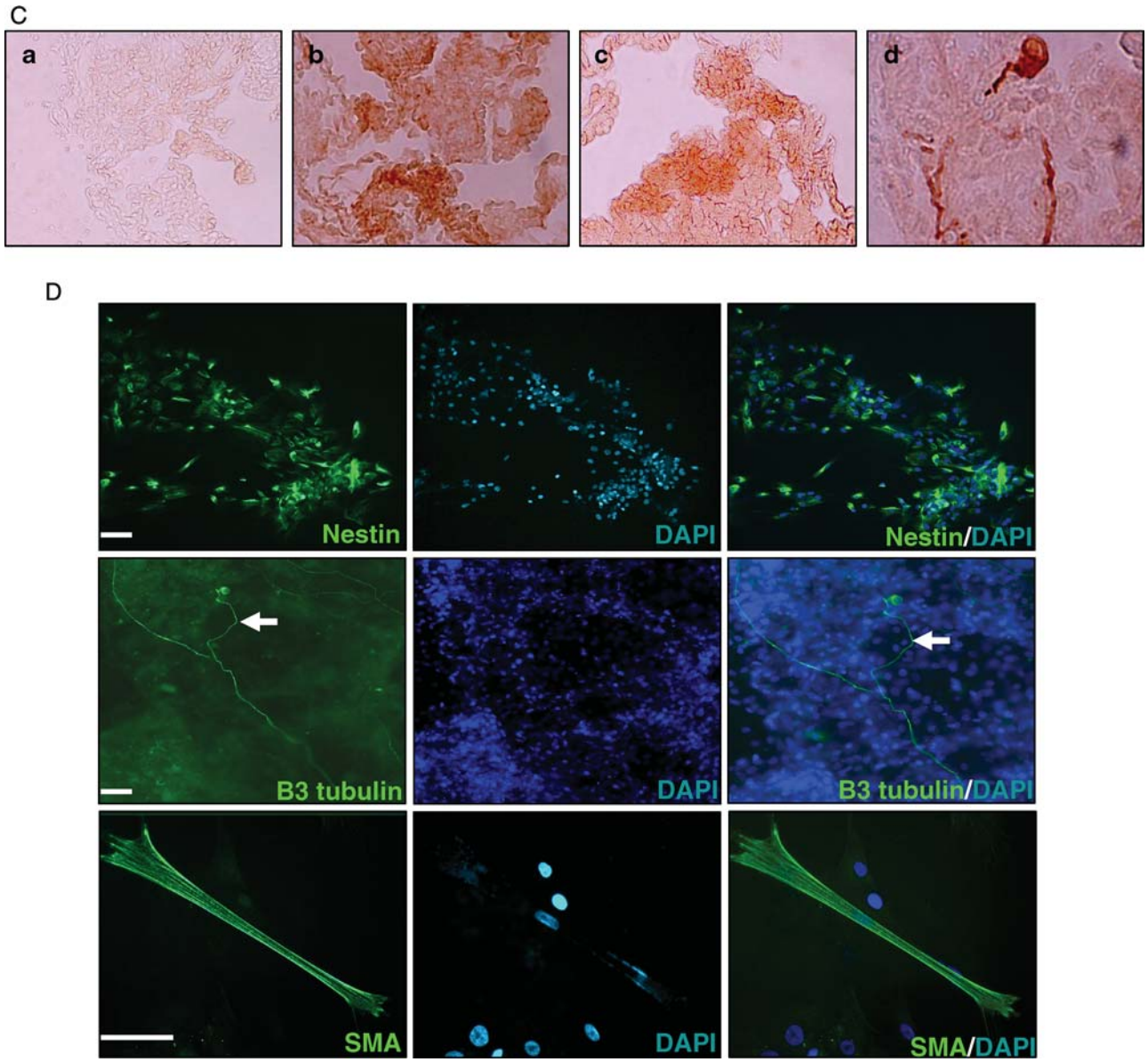

Figure 7 Continued

(Draper *et al*., 2004). For the time being, the use of feeder cells is still the safest and most cost-effective option to derive and propagate stable hESCs. For this purpose, our immortalized bFGF-producing foreskin fibroblasts offer a standardized functional solution, and the cells will be available to the scientific community. For certain experiments such as genetic studies and the generation of transgenic hESCs, feeder-free cultures are superior to co-cultures on feeders. Conversely, feeders offer a safe and cost-effective culture method for culturing and maintaining stable hESCs and for banking them.

Importantly, we also achieved the transduction of HFFs by over-expressing OCT4, SOX2, NANOG and LIN28 (Yu *et al*., 2007). The transduction procedure using non-replicating LVs proved successful in our laboratory. Even at passage 56 the immortalized bFGF-producing feeders appeared to support the growth of our iPS cell colonies very well.

Conclusion

We established the first well-functioning immortalized bFGF-producing foreskin fibroblast cell line. Although they have great potential as feeders for maintaining different pluripotent hESC lines, these conditionally immortalized foreskin fibroblasts proved to be useful for the derivation and the culture of iPS cell lines even at passage 56. Further studies are needed to determine whether the conditioned medium of these modified feeders can maintain hESC pluripotency in feeder-free culture conditions and improve reprogramming efficiency.

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