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SHORT REPORT

Human feeder cell line for derivation and culture of hESc/hiPSc

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Abstract We have generated a human feeder cell line from early second trimester Placental Stromal Fibroblasts (ihPSF) stably over-expressing the polycomb protein BMI-1. These feeder cells retain the ability to maintain human Embryonic Stem cells (hESc) over long-term culture whereas hTERT or BMI-1/hTERT immortalised feeder cell lines do not. ihPSFs were able to support the derivation of a new hESc line in near xenofree (free of non-human animal components) conditions and support continued culture of newly derived hESc and human induced Pluripotent Stem (hiPS) cell lines in complete xenofree conditions necessary for clinical use.

Introduction

The potentially inexhaustible supply of self-renewing pluripotent human embryonic stem (hES) cells holds promise as a target source of transplantation material for the treatment of a myriad of debilitating and degenerative diseases. To date, the vast majority of hES cell lines have been derived on Mouse Embryonic Feeder (MEF) cells but these lines are inappropriate for clinical use because of the necessity for xenofree processing. There have been numerous reports describing the maintenance of hES colonies on primary human feeder layers from adult, neonatal and foetal sources including term and pre-term placenta (Deleu et al., 2009; Ilic et al., 2008; Liu et al., 2010; Tecirlioglu et al., 2010). Alternatives to primary cells include autogeneic cultures of human feeders derived from hES cells (Choo et al., 2008; Li et al., 2011), feeder-free (Hernandez et al., 2010; Rodin et al., 2010; Thomas et al., 2009), feeder-conditioned

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(Escobedo-Lucea & Stojkovic, 2010) and most recently suspension culture (Larijani et al., 2011). Although clinical grade, xenofree hES cell lines have recently been successfully derived on primary human feeders (Unger et al., 2008) such feeders are subject to donor supply and variability.

A repeatable and scalable process of hES cell derivation and scale-up would require a xenofree, clinical grade feeder cell line. Choo et al. generated a hES feeder cell line using HPV E6/E7 immortalization but this line has not become widely used (Choo et al., 2006). More recently Unger et al. used a combination of BMI-1 and hTERT to generate a feeder cell line (Unger et al., 2009). The use of BMI-1 as an anti-senescing factor and hTERT as an immortalization factor has been reported for a number of cell types (Fulcher et al., 2009; Haga et al., 2007). However, hTERT immortalization has been shown to elicit a premalignant phenotype which could be deleterious in a feeder cell context (Milyavsky et al., 2003). We directly compared different modes of cell immortalization and found that BMI-1 over-expression alone resulted in a feeder cell line most able to maintain ES cell phenotype over multiple passages.

Results and discussion

Purified hPSF support hESc growth and maintenance

Mouse embryonic cells have been proven more capable of maintaining ESc colonies than adult mouse cells. To obtain a human equivalent to facilitate the xenofree culture and derivation of hESc we isolated and purified human Placental Stromal Fibroblasts (hPSF) from the conceptus derived chorionic villi of donated first and early second trimester placentas (a total of 6 placentae were obtained between 10 and 15 weeks post-conception). Purified primary hPSFs were cultured on human gelatin-coated plates for 3 passages (P3) then analysed using lineage specific markers. The proliferative adherent fraction persisting at P6 was confirmed to have a fibroblastic phenotype by immunohistochemistry (FSP^+, $CD45^-/CD31^-/K18^-$) (Fig. 1a). hPSF cultures were grown to P6 then mitotically inactivated alongside P3 MEF cultures and assessed for their ability to maintain pluripotent colonies of the hESc lines HUES7 (P42) and HES3 (P45). These were grown and maintained for at least 6 passages on both feeder cell types (Fig. 1b). There was no observable difference in HUES7 growth rates on either hPSFs or MEFs as defined by average colony area measured during real-time imaging (data not shown). Cells grown on hPSFs retained pluripotency to a comparable degree to those grown on MEFs as assessed by Nanog, OCT4, Tra-1-60 and Tra1-81 immunostaining (Fig. 1c) and were able to differentiate to cell-types representing all three germ lineages after Embryoid Body (EB) formation and re-plating (Supplementary Fig.1).

Generation of an ihPSF cell line

To introduce continuity into hESc derivation and continued culture we sought to create a human feeder cell line from hPSFs. We investigated a combination strategy of transgenically over-expressing the polycomb protein BMI-1 and the catalytic domain of human telomerase, hTERT, compared to individual expression (Supplementary Fig. 2). hPSF cultures were transduced at low multiplicity of infection (MOI ~ 10) and selected by antibiotic resistance (Retro-hTERTiresPuro) or GFP expression (Lenti-BMI-1iresGFP). However, it became apparent that over serial passages BMI-1/GFP expressing cells were selectively enriched, increasing from an initial transduction efficiency of 67 ± 7% GFP positive hPSFs to maintenance of a ~94 ± 2% GFP positive population for over 12 months (n = 6 ± s.e.m.) (Supplementary Fig. 3). This enrichment was not observed for GFP expressing cells suggesting that BMI-1 expression provides a growth or selection advantage.

Our new cell lines were analysed for transgene expression and functional activity at low passage (P6) and extended passage (P25). BMI-1 intrinsically inhibits cellular senescence by repressing p16(INK4a)-mediated cell senescence and is transcriptionally upregulated by the mitogen C-MYC (Guney et al., 2006). hTERT maintains chromosomal telomere length and extrinsically promotes cell cycle progression by inhibiting p21. However, recent reports show hTERT transcriptionally upregulates C-MYC which itself upregulates hTERT in a positive feedback loop that can ultimately result in apoptosis (Milyavsky et al., 2003). We observed increased BMI-1 expression at both the mRNA and protein level from P6 to P25 in BMI-1 and BMI-1/hTERT hPSFs (Fig. 2a and Supplementary Fig. 4a). This is not due to a proliferation advantage in BMI-1 expressing cells as the percentage of BMI-1/GFP positive cells peaked at P6 and remained constant to P25. To confirm functional activity of BMI-1 we assessed repression of the downstream target p16 by western blot. This revealed repression of p16 activity in BMI-1 expressing cells at P6 and P25 but a clear p16 immunoproduct was present in BMI-1/hTERT cells at P25. hTERT mRNA and protein remained relatively constant between P6 and P25 (Supplementary Fig. 4a and b) but telomerase activity was markedly reduced in BMI-1/hTERT cells compared to hTERT alone at P25 ($p < 0.001$; n = 6 ± s.e.m.) (Fig. 2b). Levels of p21 protein, which hTERT directly inhibits, were barely detectable throughout the experiment in all samples except those over-expressing BMI-1 alone (Supplementary Fig. 3b). These data indicate that after extended passage the hTERT transduced hPSFs lose telomerase activity in the presence of BMI-1 and conversely hTERT has a negative effect on BMI-1 inhibition of p16. We hypothesised that this effect could be caused by increased expression of the mitogen C-MYC leading to apoptosis in BMI-1/hTERT cells. Indeed, C-MYC mRNA increased in cells transgenically expressing hTERT over extended passage (Fig. 2c). We analysed the proliferation rate of P6 and P25 hPSF cells by cell counting over 14 days and BrdU incorporation during S-phase. There was no significant difference between the proliferation rate or percentage of cells in S-phase in any of our cell lines at P6 (Supplementary Fig. 5a and b). When cell proliferation was assessed at P25 there were significantly more cells after 14 days in BMI-1 cultures compared to BMI-1/hTERT (Fig. 2d, $p < 0.001$; n = 4 ± s.e.m.) but no significant difference in the number of cells entering S-phase (n = 4). TUNEL assays on our transgenic hPSF groups at P25 confirmed that there was a significant increase of apoptotic nuclei in BMI-1/hTERT cells compared to BMI-1 and hTERT alone (Fig. 2e $p < 0.05$; n = 6 ± s.e.m.). Collectively, these data indicate that separately, BMI-1 and hTERT contribute to perpetuating cellular proliferation. Combined expression of BMI-1/hTERT over extended

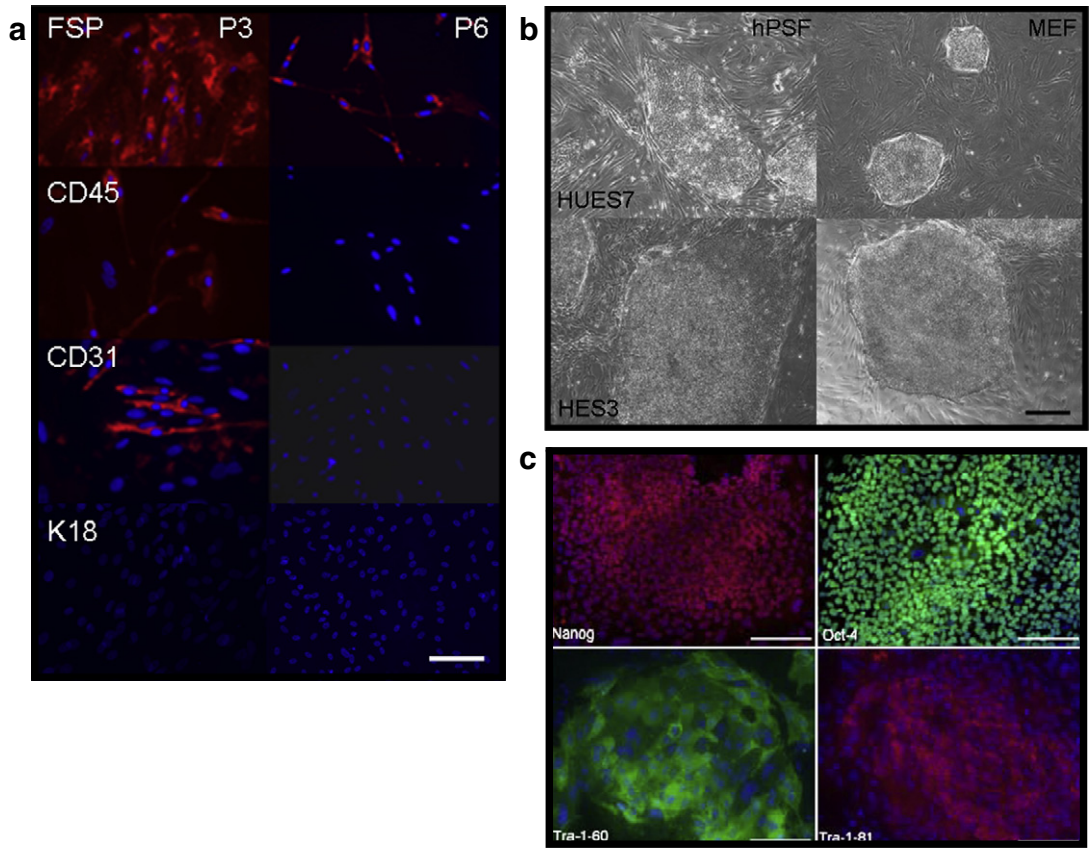

Figure 1 Primary hPSFs are able to support the growth and maintenance of hESc. Human Stromal Fibroblasts were isolated and purified from the chorionic villus fraction of first and second trimester placentae. (a) Adherent cells were immunostained for the presence of the lineage specific markers; fibroblast specific protein (FSP), haematopoietic marker CD45, endothelial marker CD31 and epithelial marker K18. Images are representative of 3 independent isolation experiments (size bar = 20 μm). (b) HUES7 and HES3 hESc colonies were serially mechanically passaged on mitotically inactivated hPSFs (P6) or MEFs (P3) for at least 4 passages in 3 independent experiments (size bar = 50 μm). (c) Colonies retained pluripotency as evidenced by Nanog, OCT4, Tra1-60 and Tra-1-81 immunostaining of HUES7 hESc ($n \geq 3$, size bar = 20 μm). Immunocytochemistry experiments were accompanied with the appropriate isotype antibody controls that were negative for all markers.

passage results in a loss of p16 inhibition and an increase in apoptosis that could be due to an increase in C-MYC expression.

Characterisation of ihPSF phenotype

We analysed growth factors (GF) and extra cellular matrix (ECM) components in an attempt to define the hESc maintenance factors perpetuated in ihPSFs. We compared P8 primary hPSFs which are unable to maintain hESc culture with P25 ihPSFs. A preliminary candidate approach showed no increase in transcripts for FGF2 or Activin A, GFs implicated in hESc maintenance (Supplementary Fig. 6a). However, a Coomassie stained PAGE analysis of ECM extract showed that in 3 of 4 cell cultures a substantial increase in protein bands around 250 kDa was evident in ihPSFs compared to hPSFs (Supplementary Fig. 6b). Subsequent western analysis showed that this corresponded to fibronectin which is known to be an important component of ECM capable of maintaining hESc (Fig. 2f i and ii). These data imply that BMI-1 expression protects the hESc maintenance integrity of ihPSFs critically in terms of ECM deposition and composition.

Prolonged maintenance of hESc/hiPSc colonies on ihPSFs

We first compared the ability of our transgenic hPSF cell lines to maintain the growth of the HES3 and HUES7 hESc lines and two hiPSc (iPSA and iPSB) lines. Mitotically inactivated feeder layers were prepared at P6, 12 and 25 (primary hPSFs terminally senesced beyond P12) then mechanically passaged hESc/hiPSc colonies for up to 6 passages on these feeder cells. Only BMI-1 and BMI-1/hTERT transduced hPSFs were able to maintain pluripotent hESc/hiPSc colonies at P12, while colonies began to differentiate (as ascertained by gross morphology) or die after 3 passages on primary, sham transduced and hTERT transduced hPSFs. Although both BMI-1 and BMI-1/hTERT transduced hPSFs maintained colonies at P12, by P25 only BMI-1 transduced hPSFs maintained hESc/hiPSc growth. hiPSc were maintained as colonies even on P12 hPSFs, unlike hESc, suggesting that hiPSc are more robustly

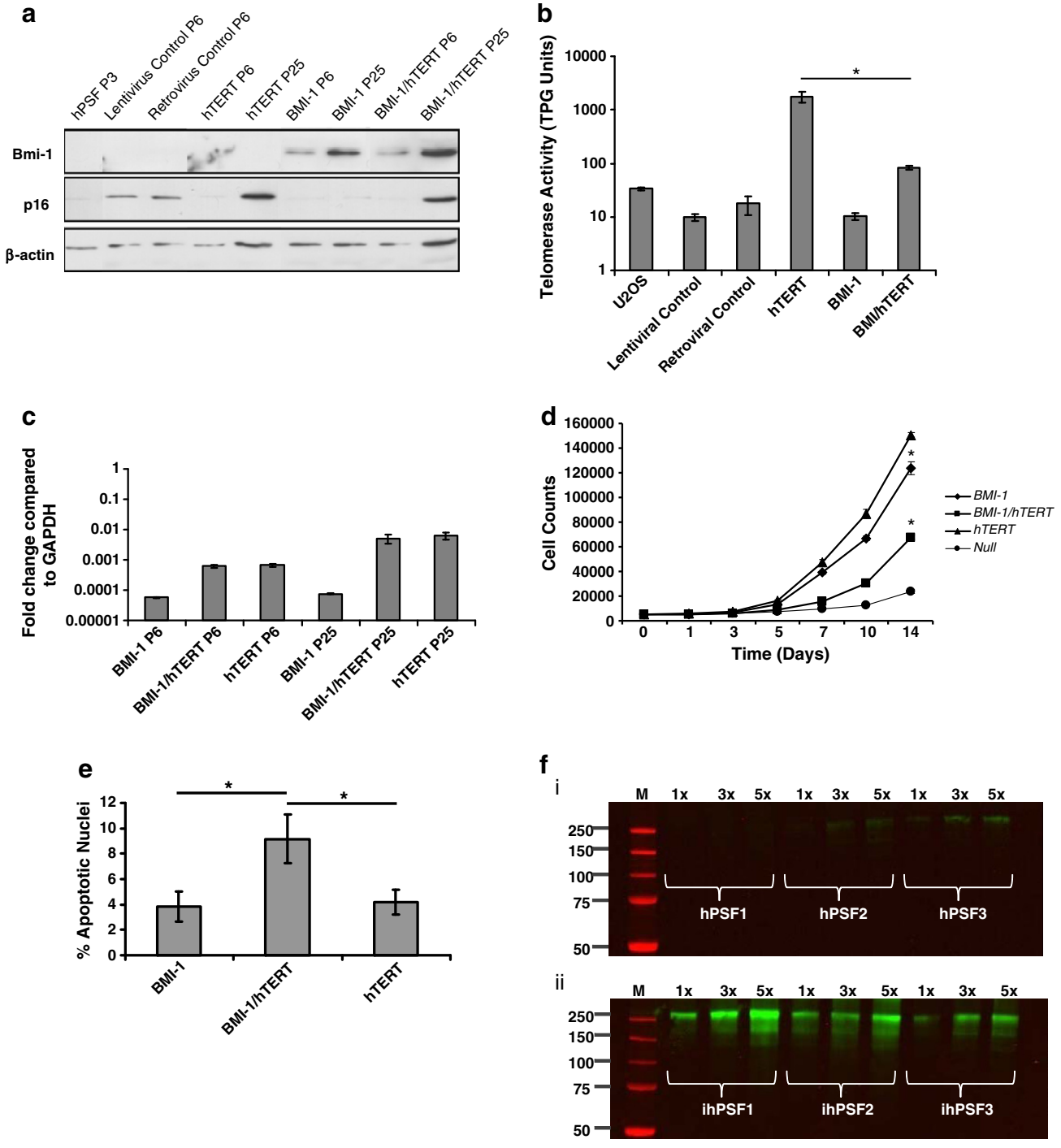

Figure 2 BMI-1 expression in hPSFs maintains feeder cell phenotypic integrity. (a) BMI-1 and p16 activity were assayed in transduced hPSFs at P6 and P25 by western blotting. Blots are representative of two independent experiments. (b) Telomerase activity was measured using a fluorescence Telomeric Repeat Amplification Protocol (TRAP). Data represent a single experiment where n=3. (c) C-MYC expression was quantified the cell lines at P6 and P25 using qRT-PCR (n=6±s.e.m.). (d) Cell proliferation was assessed at P25 by cell counting over 14 days. (e) Apoptotic index was calculated using the TUNEL assay. (f) A comparison of extracellular fibronectin secreted by hPSFs at P8 (i) and ihPSFs at P25 (ii) by western blot on extracellular matrix; 1×, 3×, 5× indicate relative protein loading. Unless otherwise stated all experiments were carried out at a minimum of n=4±s.e.m.

maintained than hESc (Table 1 and Supplementary Fig. 7). We isolated and amplified 10 BMI-1 transduced clones by serial dilution and colony picking in order to establish a homogeneous cell line (ihPSF). All 10 clones maintained the growth of HUES7 colonies so a single line was chosen at random to continue analyses. Three established hESc lines

Table 1 Maintenance of pluripotent HES3, HUES7, iPSA and iPSB colonies on early (P6) and mid- (P12) passage hPSFs. First column shows gene over expressed. None = pimary starting PSFs. Ticks indicate maintenance of pluripotency after at least 3 serial passages as evidenced by >90% OCT4 and Nanog positive immunostaining in all colonies analysed. A cross indicates <90% staining, NA indicates Not Analysed.

Transgene	HES3			HUES7			iPSA			iPSB		
	P6	P12	P25	P6	P12	P25	P6	P12	P25	P6	P12	P25
None	✓	x	NA	✓	x	NA	✓	✓	NA	✓	✓	NA
BMI-1	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
BMI-1/hTERT	✓	✓	x	✓	✓	x	✓	✓	✓	✓	✓	✓
hTERT	✓	x	x	✓	x	x	NA	NA	NA	NA	NA	NA
GFP	✓	x	x	✓	x	x	NA	NA	NA	NA	NA	NA
PuromycinR	✓	x	x	✓	x	x	NA	NA	NA	NA	NA	NA

(HUES1, HUES7 and HES3), two in-house lines (MAN-1 and MAN-2) and two hiPSc lines (iPSA and iPSB) were successfully maintained over 4–20 mechanical and/or trypsin-mediated passages on mitotically inactivated ihPSFs at P6 and P25 (Table 2 and Supplementary Movies 1 and 2). All hESc lines immunostained positive for the pluripotency markers OCT4 and SSEA3 and were karyotypically stable (data not shown).

GMP derivation and xenofree maintenance of hESc on ihPSFs

To move toward clinical viability, we attempted the derivation of a new hESc line from a donated blastocyst on ihPSFs under cGMP conditions. All components used were xenofree apart from Knockout Serum Replacement due to the lack of a viable alternative at the time. A line was successfully derived as outlined in Materials and methods (Fig. 3a i–iii). To our knowledge this is the first example of a hESc derivation on a clonal feeder cell line. We maintained ihPSFs and primary hPSFs in completely xenofree conditions over multiple passages to assess their long-term viability. The proliferation rate of primary hPSFs was significantly reduced after 14 days of xenofree culture compared to standard conditions ($p<0.05$; $n=6\pm$s.e.m.), whereas there was no significant difference for ihPSFs (Fig. 3b). Finally, multiple hESc colonies were cultured on inactivated ihPSF feeders for at least 4 passages. Colonies had distinct ES cell morphology and were positive for the pluripotency markers OCT4, Nanog and SSEA3 by immunohistochemistry (Fig. 3c) and positive for the FACS markers CD9 and EpCAM (Fig. 3d). Encouragingly, passage 25 ihPSFs maintained comparable levels of pluripotent colonies of HUES1 and HUES3 to early passage hPSFs and early passage MEFs (Table 3). Furthermore, HUES1 colonies grown on ihPSFs were able to form EBs and differentiate into cell types representing all three germ lineages as analysed by immunocytochemistry on replated cells (Fig. 3e).

We conclude that a clonal human Placental Stromal Fibroblasts cell line is able to support the derivation and maintenance of hESc/hiPSc. Transgenic overexpression of BMI-1 alone in hPSFs is sufficient to perpetuate a feeder cell phenotype capable of maintaining hESc/hiPSc cultures over at least 25 passages. The ectopic over expression of BMI-1 and hTERT is often used in cell biology to immortalise primary cells as well as retain their phenotypic integrity. However, our data imply that prolonged over-expression of these two transgenes creates a propensity toward apoptosis, possibly mediated through C-MYC, and ultimately degradation of the cell line. The clonal ihPSF cell line we have generated was used to successfully derive a new hESc line in near xenofree conditions in a clean room cGMP context. ihPSFs are amenable to continued xenofree culture and maintenance of both hESc and hiPSc making them an ideal feeder layer for clinical grade pluripotent stem cell derivation and scale-up.

Table 2 Passage of multiple cell lines on ihPSFs mitotically inactivated at low passage (P6) or extended passage (P25). M indicates mechanical passage, T indicates trypsin passage. Colonies were confirmed as being pluripotent by morphology and OCT4/Nanog staining. Passage given is maximum at which line tested.

hES cell line	hES passage no. (P6 ihPSF)	hES passage no. (P25 ihPSF)
MAN-1	P6 (M)	P6 (M)
MAN-2	P4 (M)	P6 (T/M)
HUES1	P6 (T/M)	P20 (M)
HUES7	P6 (T/M)	P6 (M)
HES3	P10 (M)	P10 (T), P20 (M)
hiPSA	N/A	P6 (T/M)
hiPSB	N/A	P6 (T/M)

Materials and methods

Isolation, purification, growth and maintenance of primary cells

Human Placental Stromal Fibroblasts

First and early second trimester placentas were donated after informed consent and under the guidelines set out according to the National Research Ethics Service (07/H1008/224). Chorionic villous tissue was dissected and washed in DMEM medium (Lonza, Basel, Switzerland) containing 2.5 μg/ml amphotericin B (Sigma-Aldrich, Poole, UK) and penicillin/streptomycin (Invitrogen, Paisley, UK) (wash medium),then cut into 5 mm^2 sections and subjected to 3 low-g (300×*g*) centrifugations in 50 ml of wash medium to

remove blood cells. It was treated with trypsin-EDTA (Invitrogen)/DNase I (Invitrogen) (0.125%/2 mg/ml final concentration) in 15 ml wash medium and incubated at 37 °C for 35 min with resuspension every 5 min. Released trophoblast was separated from tissue aggregates by filtration through a 100 μm MESH filter. Aggregates were washed repeatedly with wash medium on the MESH filter. The trypsin-EDTA-DNase I treatment was repeated to remove any remaining trophoblast and then human serum-containing medium added to neutralise the trypsin. Aggregates were then washed 3 times in wash medium prior to adding collagenase/hyaluronidase (Sigma-Aldrich) (1.5 mg/ml collagenase/2 mg/ml hyaluronidase) and incubation at 37 °C for 2 h with shaking. Tissue clumps were removed by a low-g (300×*g*) spin and the resultant single cell suspension decanted and then serially washed with wash medium followed by centrifugation at 1000×*g*. The cell pellet was resuspended in growth medium (DMEM) containing penicillin/streptomycin, L-glutamine and 10% human serum (Invitrogen) and plated onto 0.1% porcine gelatin (Sigma-Aldrich)-coated plates.

For xenofree growth hPSF and ihPSF cultures were trypsinised using Trypzean (Sigma-Aldrich), washed in PBS, pelleted at 600×*g*, resuspended in xenofree growth medium (DMEM (Lonza), 10% human serum (Lonza), penicillin/streptomycin) and plated on 0.1% human gelatin (Fibrogen, San Franscisco, CA, USA) coated plates. Cells were cryopreserved for long-term storage in xenofree Profreeze (Lonza) medium.

Mouse Embryonic Fibroblasts

Mouse Embryonic Fibroblasts (MEF) were isolated, purified and prepared as described by Michalska (2007). MEFs were routinely amplified on 0.1% porcine gelatin-coated plates in DMEM medium supplemented with 10% FCS, penicillin/streptomycin, L-glutamine.

Mitotic inactivation of hPSFs, ihPSFs and MEFs

Fibroblast feeder cells were plated and cultured as described in T-75 flasks until 80% confluent. Growth medium was replaced with growth medium supplemented with 10 μg/ml Mitomycin C (Sigma-Aldrich) and incubated for 3 h for MEFs and 6 h for hPSFs and ihPSFs at 37 °C, 5% CO_2. Cells were then serially washed 3 times in PBS, Trypsinised, counted using a hemocytometer, resuspended in Profreeze cryopreservation medium at 10^5 cells per vial and stored in liquid nitrogen.

Growth and maintenance of hES and hiPS cells

The hESc line HES3 was obtained from ES Cell International Pte Ltd (Singapore), HUES1 and 7 were a kind gift from Prof. Doug Melton (University of Harvard). The hESc lines MAN-1 and MAN-2 were derived at the North West Embryonic Stem Cell Centre (NWESCC), University of Manchester. Human iPSc lines iPSA and iPSB were generated at NWESCC using protocols detailed by Takahashi et al. Mitotically inactivated hPSFs and ihPSFs were plated onto 0.1% gelatin coated plastic (porcine or human) at a density of 2.5×10^4 cells/cm^2, MEFs were plated at 3.5×10^4 cells/cm^2 washed 3 times in PBS prior to applying hESc growth medium (KO-DMEM, 20% KO-serum replacement, 2 mM L-glutamine, 1× non-essential amino acids and 100 μM β-mercapoethanol (all Invitrogen) and 8 ng/ml FGF2 (Peprotec, London, UK)). All hESc/hiPSc lines were passaged by trypsin or mechanical cutting. Xenofree cultures of hESc were grown on inactivated xenofree PSFs grown on 0.1% human gelatin coated plastic in xenofree hESc growth medium (KO-DMEM (Invitrogen), 20% human serum replacement (Sigma-Aldrich), 2 mM L-glutamine, 1× non-essential amino acids and 100 μM β-mercapoethanol (all Invitrogen) and 8 ng/ml FGF2 (R&D Systems, Abingdon, UK)). All xenofree components were also cGMP compliant.

Embryoid body formation and replating

Embryoid bodies were generated using Aggrewell plates as per manufacturer's instructions (Stem Cell Technologies, Grenoble, France). EBs were plated onto standard tissue culture plates and grown in DMEM supplemented with 20% FCS for 14 days prior to analysis.

Derivation of a new hESc line

Early cleavage stage embryos were donated by couples undergoing IVF treatment at St Mary's Hospital after informed consent with approval of Central Manchester Research Ethics Committee and under licence R0171 from the Human Fertilisation and Embryology Authority. They were cultured in G1/2 sequential media until blastocyst formation. At the blastocyst stage, the zona pellucida of the embryos were removed by treatment with acid Tyrode's solution (pH 2.5, Sigma-Aldrich) before mechanical disruption of the trophectoderm (TE), with the aid of two pulled glass Pasteur pipettes, one with a cutting open end and the other with a closed round end. The part of the cell clump containing the inner cell mass (ICM) was gently triturated and washed in a fresh drop of ES medium, and transferred onto a layer of the mitotically inactivated (10 μg/ml of mitomycin C for 6 h) immortalised hPSFs in an organ culture dish. After 6–8 days culture or until outgrowths appeared (Passage 0), the outgrowth of ICM was split into several pieces by manual dissection and transferred to new feeder layers each 5–8 days thereafter.

Viral construction, production, transduction and selection of hPSFs

The MLV-based retroviral vector expressing hTERT (human Telomerase catalytic domain) in a bicistronic cassette with the puromycin resistance gene and control empty vector were kindly supplied by Cancer Research UK. The lentiviral vector expressing the human BMI-1 gene in a bicistronic cassette with eGFP and control eGFP vector were constructed using 2nd generation HIV-1 based lentiviral cassettes as previously described (Godfrey et al., 2005). Human BMI-1 full length cDNA was obtained from geneservice™ as a Mammalian Gene Collection (MGC) Clone, IMAGE Id number 40125787 within the pOTB7 vector. The ~3.2 kb BMI-1 cDNA was excised from this vector by restriction digest with B*am*HI and X*ho*I and ligated into the multiple cloning site (MCS) of the similarly cut lentiviral vector pHR-EF1α-MCS-IRES-GFP. All clones were validated by complete sequencing.

Viral preparation was carried out by transient transfection of 293T producer cells with the viral plasmid as detailed

below and the VSV-G encoding plasmid pMD.G2 and the viral helper plasmid; pCMVdelta8.74 for the lentivirus and pKat for the retrovirus. Briefly, 293T transfections were carried out using calcium phosphate precipitation. Virus containing supernatant was harvested after 48 h and filtered through a 0.45 μm nylon membrane (Millipore, Watford, UK). Transduction of hPSFs was carried out using application of unconcentrated viral supernatant after pre-incubation with 2 μg/ml polybrene applied over 4 h at 37 °C. Transduced hPSF cultures were then washed once with PBS and fed with growth medium. LNT-BMI-1iresGFP transduced cells selected naturally due to the increased proliferation/survival potential but MLV-hTERTiresPuro transduced cells were selected on the basis of puromycin resistance by supplementing growth medium with 0.5 μg/ml puromycin (Sigma-Aldrich) antibiotic for five days. When puromycin resistant cell lines

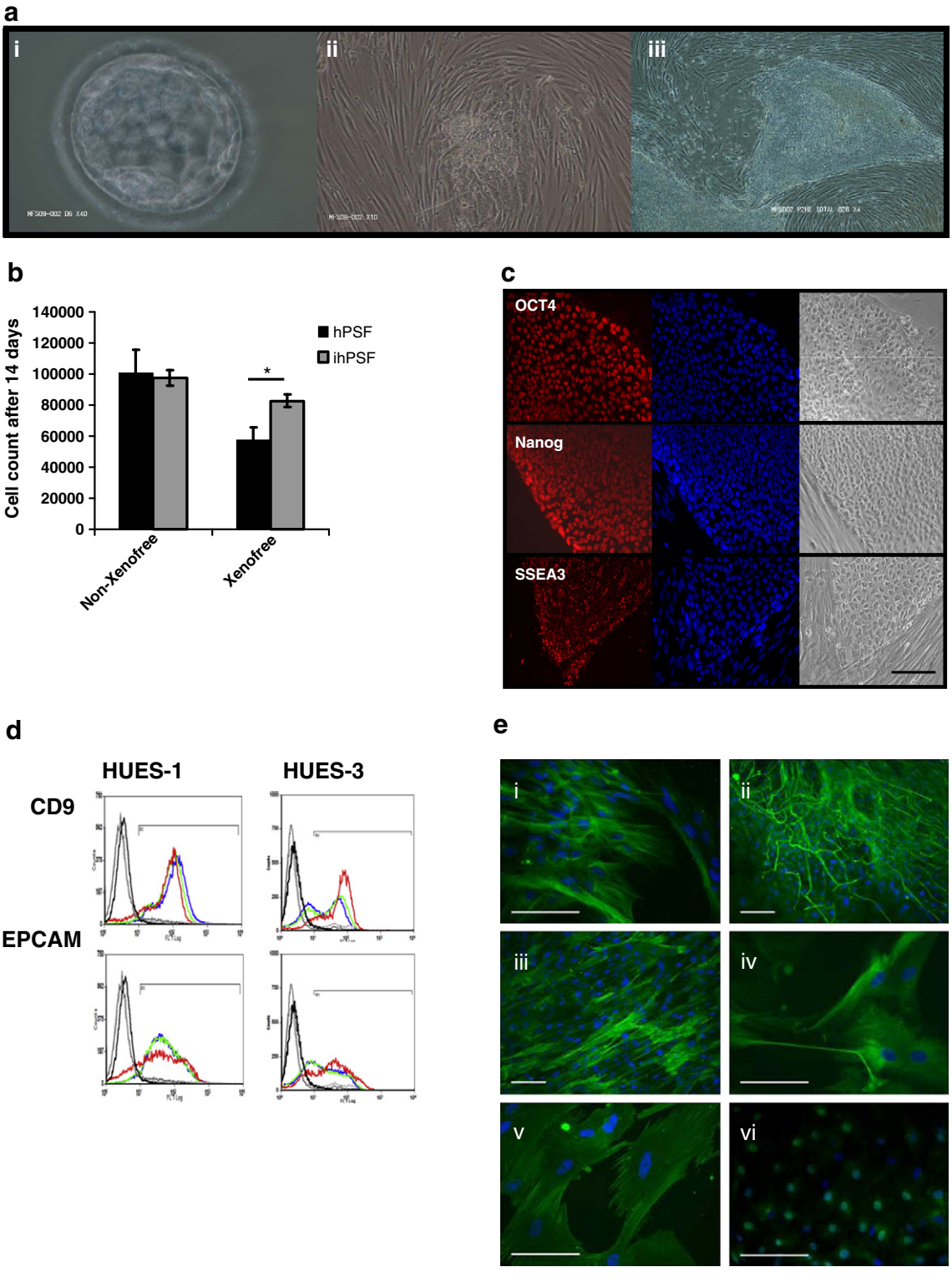

Table 3 A comparison of hESc colonies grown on ihPSFs, hPSFs and MEFs. hESc colonies were cultured on either immortalised PSFs (ihPSF; p25), primary early passage PSFs (hPSF; p4) or early passage MEFs (p4). Colonies were maintained for at least 4 passages then analysed with the pluripotency markers CD9 and EpCAM by FACS. Plots are shown in Fig. 3d.

		ihPSF	hPSF	MEF
HUES1	CD9	85.9	84.1	82.1
	EpCAM	82.6	81.7	74.4
HUES3	CD9	67.3	69.2	83.1
	EpCAM	69.2	66.7	74.1

were subjected to extended passage they were re-selected with 5 days of puromycin treatment after every 25 days.

Expression analyses

Immunocytochemistry

Monoclonal antibodies for human pluripotency markers (OCT4, Nanog and SSEA3), fibroblast specific protein, CD45, CD31 and K18 and appropriate FITC and TRITC labelled secondary antibodies were all purchased from R&D Systems. Immunocytochemistry was carried as follows. Briefly, cells were fixed with ice cold 4% paraformaldehyde for 20 min, washed 3 times in PBS, blocked with 10% goat serum in wash buffer (0.1% Triton in PBS) for 30 min. Primary antibody was applied at the manufacturer recommended concentration in wash buffer with 1% goat serum and incubated overnight at 4 °C. Cells were washed 3 times in wash buffer then secondary antibody applied in wash buffer containing 1% goat serum and incubated at room temperature for 2 h. Cells were again washed 3 times in wash buffer then counter-stained with DAPI containing mounting medium (Vector Laboratories, Peterborough, UK) a coverslip applied and visualised using an Olympus IX71 fluorescence microscope (Olympus, Watford, UK).

Flow cytometry for cell surface markers

Human ESc lines were grown for at least 4 passages on inactivated MEFs, hPSFs and ihPSFs. The cultures were treated with collagenase to eliminate the feeder cell population and the final single cell suspension was prepared by incubating the detached stem cell colonies with TrypLe (GIBCO) for 2 min at 37 °C. The cells were blocked with PBS + 0.1% FBS which were then incubated at 4 °C overnight with FITC-conjugated monoclonal mouse antibodies CD9 and EpCAM (both from R&D Systems) or mouse IgG (1:100) (Santa-Cruz). After the incubation the cells were washed 3 times with PBS + 0,1% FBS. The immunological control samples were incubated with AlexaFluor 488 (Invitrogen) for 1 h at room temperature which was followed by further washes with PBS + 0.1% FBS. All the samples were diluted in PBS for real-time analysis with Beckman Coulter Cyan ADP (The University of Manchester) and the data analysed using Summit V4.3.02 Build2451 Software.

Cell derived matrix preparation

Inactivated primary and immorto human placental fibroblasts were seeded at a density of 0.068×10^6 cells/cm^2 on 0.1% gelatin-coated plates and cultured for 14 days before extracting the matrices. Extracellular matrix was derived from the cells using 20 mM NH_4OH and 0.5% TritonX-100 extraction buffer. Matrix was then treated with 10 U/ml DNAase I (Promega) for 30 min at 37 °C and washed two times with PBS. Matrix was solubilised using 300 μl of pre-warmed 2× sample buffer prepared by diluting 5× sample buffer containing 10% SDS, 25% glycerol, 125 mM Tris–HCl pH 6.8, 0.01% Bromophenol Blue, 20% β-mercaptoethanol (Sigma) in distilled water. The matrix was removed from the plate using a cell scraper and the samples were stored at −80 °C for further use.

Western blotting

Protein expression was analysed by western blotting using standard methodology. Blots were blocked with 5% milk powder, 0.05% Tween in PBS. The BMI-1 human monoclonal antibody was purchased from Upstate Antibodies (Millipore), the monoclonal human Ink4a/p16, p21 and β-actin antibodies were purchased from AbCam (Cambridge, UK) and the fibronectin antibody was purchased from Sigma-Aldrich.

Telomerase assay

Telomerase activity was assayed as per manufacturer's instructions (TRAPeze® XL Telomerase Detection Kit, Chemicon International, (Millipore)). Briefly, lysate from 1×10^3 cells was added to a reaction mix containing a substrate oligonucleotide, onto which any active telomerase within

Figure 3 ihPSFs are able to support near xenofree derivation and maintain hESc/hiPSc in a xenofree environment. (a) Derivation of a new hESc line (MAN-3) was carried out on ihPSFs from a donated day 6 blastocyst (i). (ii) Attached outgrowth on inactivated ihPSFs at day 3 after plating; (iii) established colonies at P3. All standard operating procedures were carried out in a cGMP compliant environment with xenofree reagents except for Knockout Serum Replacement. (b) Their ability to maintain ihPSF proliferation in xenofree medium was assessed by cell counting over 14 days ($n = 4 \pm$ s.e.m.) prior to culturing HUES7 colonies for at least six passages in xenofree medium. (c) Pluripotency was confirmed by immunostaining for OCT4, Nanog and SSEA3 (right panel phase image). Images counterstained with DAPI and are representative of 3 separate staining experiments using HUES7. Similar results were achieved with MAN-1. (d) HUES1 and HUES3 colonies were grown for at least 4 passages on MEFS (red), hPSFs (green) and ihPSFs (p25) (blue) then assayed for the pluripotency markers CD9 and EpCAM. (e) The ability to differentiate to the 3 germ lineages was assayed by forming embryoid bodies, replating and immunostaining for the neurectoderm markers; (i) β-tubulin III and (ii) neurofilament, the mesoderm markers (iii) smooth muscle actin and (iv) vimentin, and the endoderm markers (v) alpha fetoprotein and (vi) SOX17. Immunostaining shown is for HUES7. Immunocytochemistry experiments were accompanied with the appropriate isotype antibody controls that were negative for all markers (size bars throughout represent 10 μm).

the lysates would add GGTTAG telomeric repeats. Subsequent PCR amplification of the extended products with Amplifluor® primers containing a 5′ fluorescein fluorophore and 3′ DABSYL quencher generated a fluorescence emission directly proportional to the amount of active telomerase in the cells. Heat treated lysate was used as a negative control and a standard curve was obtained using serial dilutions of the supplied control template. An internal control template utilising a sulforhodamine fluorophore removed artefactal differences due to PCR inhibition.

TUNEL assay

Apoptosis was assessed using the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick endlabeling (TUNEL). Briefly, HUES-1 cells were fixed at appropriate time points in 4% paraformaldehyde and permeabilised with 0.1% Triton X-100. As a positive control, cells were incubated in 50 μl of DNase 3 U/ml in 50 mM Tris–HCL (Roche, Burgess Hill, UK) for 20 min before TUNEL staining. TUNEL labelling was carried out using an In Situ Cell Death Detection Kit, FlTC conjugated (Roche) according to the manufacturer's instructions. After TUNEL labelling the cells were washed in PBS to remove any unbound material and mounted in Vectashield containing DAPI and photographed using an Olympus IX71 epifluorescence microscope (Olympus Optical Ltd., Tokyo, Japan).

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.scr.2011.04.005.

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