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REGULAR ARTICLE

Two new protocols to enhance the production and isolation of human induced pluripotent stem cell lines

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Abstract There are two critical stages in the retroviral reprogramming of somatic cells to produce human induced pluripotent stem cell (hiPSC) lines. One is the production of high titer virus required to reprogram somatic cells; the other is identification of true hiPSC colonies from heterogeneous cell populations, and their isolation and expansion to generate a sustainable, pluripotent stem cell line. Here we describe simple, time-saving methods to address the current difficulties at these two critical junctures. First, we have developed a method to increase the number of infectious viral units 600-fold. Second, we have developed a TRA-1-81-based positive selection column method for isolating "true" hiPSCs from the heterogeneous cell populations, which overcomes the labor-intensive and highly subjective method of manual selection of hiPSC colonies. We have used these techniques to produce 8 hiPSC lines from human fibroblasts and we believe that they are of considerable utility to researchers in the hiPSC field.

Introduction

Human induced pluripotent stem cell (hiPSC) lines can be maintained in their undifferentiated state and yet be differentiated into various cell types, providing a resource of considerable promise for the future of *in vitro* disease modeling and drug discovery. For example, disease-specific hiPSC lines from patients with spinal muscular atrophy (Ebert et al., 2009), familial dysautonomia (Lee et al., 2009), and amyotrophic lateral sclerosis (Dimos et al., 2008), all demonstrated disease phenotype in differentiated neurons. In addition, hiPSC-derived hepatocytes (Sullivan et al., 2010; Guguen-Guillouzo et al., 2010) and cardiomyocytes (Yokoo et al., 2009; Tanaka et al., 2009; Zwi et al., 2009) have applications for drug safety evaluation, bridging the gap

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between *in vitro* studies with heterologous cell lines and human clinical trials. Furthermore, hiPSC lines derived from patients carrying specific genetic diseases provide a novel and exciting platform to study drug/disease interactions, an approach that has not been previously possible.

Takahashi and Yamanaka (2006) first reported the establishment of murine iPSCs in 2006, by transducing mouse embryonic fibroblasts with retroviruses encoding c-Myc, Oct3/4, Sox2, and Klf4. Subsequently, the first hiPSC lines were derived in 2007 (Yu et al., 2007; Takahashi et al., 2007), again by employing retroviral transduction of fibroblasts. Since this pioneering research, the field of iPSC research continues to expand rapidly and several attempts have been made to develop methods that minimise the random integration of transgenes into the genome, which is considered necessary for clinical applications. These include excisable viral cassettes (Kaji et al., 2009; Woltjen et al., 2009), and nonintegrating adenoviral vectors (Zhou and Freed, 2009; Stadtfeld et al., 2008), together with nonviral methods such as repeated plasmid transfection (Okita et al., 2008) and protein transductions (Kim et al., 2009; Zhou et al., 2009). However, to date, these methods are associated with reprogramming efficiencies of as low as 0.0001–0.001%, which is between 1000-fold and 100-fold lower than retroviral transduction. Moreover, they are often restricted to embryonic or fetal tissue, or to mouse cells, whereas the desired target for clinical relevance would be cells isolated from adult humans. Therefore, for *in vitro* biomedical applications, such as disease modeling or drug screening where random viral integrations are of lesser significance, retroviral transduction is still the method of choice.

Although the use of retroviruses in hiPSC production is now well established, the method is constrained by the ability to cost-effectively produce lentivirus on a scale large enough to meet the requirements of the experiment. Furthermore, the common method for concentrating the virus by ultracentrifugation requires specialist equipment, and can be prohibitive due to this cost. Here we demonstrate that viral yields can be increased 100-fold by optimising the production conditions, and in addition, the transduction efficiency can be improved a further 6-fold by conjugating the virus produced to streptavidin superparamagnetic particles. This means that a single well within a 6-well plate can produce between 0.5×10^7 and 1×10^7 infectious units, enough for 10–20 hiPSC experiments in which 50 000 somatic cells are transduced with a multiplicity of infection of 10. As a result, the labor and cost burden of this element of hiPSC line production are greatly reduced.

An additional obstacle we have addressed in the production of sustainable hiPSC lines is the identification and subsequent isolation of truly reprogrammed hiPSCs from a heterogeneous derivation culture that also contains mouse embryonic fibroblast feeders, somatic cells, and partially reprogrammed cells of similar morphology to hiPSCs. This process remains highly subjective, but is critical for the production of high-quality hiPSC lines. Judgments are made on the basis of appearance with the "best looking," and often faster growing colonies being selected for propagation. In addition, this process of mechanical dissection and passage to culture the new hiPSC line is highly labor-intensive. Therefore, we have developed an isolation method involving the positive selection of "true" hiPSCs based on the cell surface expression of the pluripotency marker TRA-1-81, previously shown to be the most appropriate marker for identification of fully reprogrammed hiPSCs in culture (Chan et al., 2009). Not only does this eliminate the selection bias, but because manual identification and mechanical dissection are replaced with bulk trypsin passaging, the process is simplified and allows many lines to be derived at once.

In summary, the two methods we describe here are not only both time and cost-saving but they require no specialised equipment, they minimise technical skill, and, most importantly, they accelerate establishment of hiPSC lines.

Results and discussion

Optimisation of lentivirus production increases transduction efficiency by 600-fold

To optimise lentiviral production, we evaluated the influence of several parameters including density of packaging cells, transfection method, and DNA concentration. We also investigated harvesting times and frequencies, the influence of serum, and concentration methods to increase eventual viral titer. Finally, we investigated conjugation of virus to streptavidin superparamagnetic particles to determine the effect on transduction efficiency. Optimisation experiments were performed using a lentivirus encoding eGFP and titers were quantified by transducing both the fibroblasts and the hESC line, HUES7, with serial dilutions of GFP lentivirus, analysing by flow cytometry, and calculating the infection units within the linear range of the graph. The results are detailed in Fig. 1 and Supplementary Fig. 1.

Since previous reports utilise different cell numbers and DNA concentrations for viral production, we first evaluated the effect of these two factors on titer. This indicated that an increase in packaging cell number was proportional to the amount of virus produced. Moreover, the amount of total plasmid DNA used to make the virus was inversely proportional to the viral yield (Supplementary Fig. 1a). Under these conditions, transfection using Lipofectamine 2000 yielded more than twice the number of infectious units per milliliter (iu/ml) than calcium phosphate precipitation transfection methods (Supplementary Fig. 1b).

Next the timing and frequency of viral harvest were investigated. In most reports, transfection reagents are removed from the packaging cells 24 h post transfection and supernatant containing virus is collected once at 48–72 h post transfection. In our hands, this strategy led to a viral titer of $\sim2.5\times10^4$ iu/dish. However, by changing to a shorter incubation of 4–5 h for the transfection, and coupling this to multiple viral harvests at 20, 30, and 48 h, the pooled viral titer was increased over 10-fold to $\sim3.5\times10^5$ iu/dish, and when Lipofectamine 2000 was used this was further increased to $\sim1\times10^6$ iu/dish (Supplementary Fig. 1c). This could be due to several factors. The most likely explanation is that the half-life of lentivirus at 37 °C is around 17 h (Chilton and Le Doux, 2008), and therefore the number of infectious units of virus in the culture supernatant reaches an equilibrium, rather than continually increasing. Thus our procedure of harvesting at regular intervals and immediately storing the virus at -80 °C preserves the yield. In addition, the fresh nutrients provided by repeated medium changes

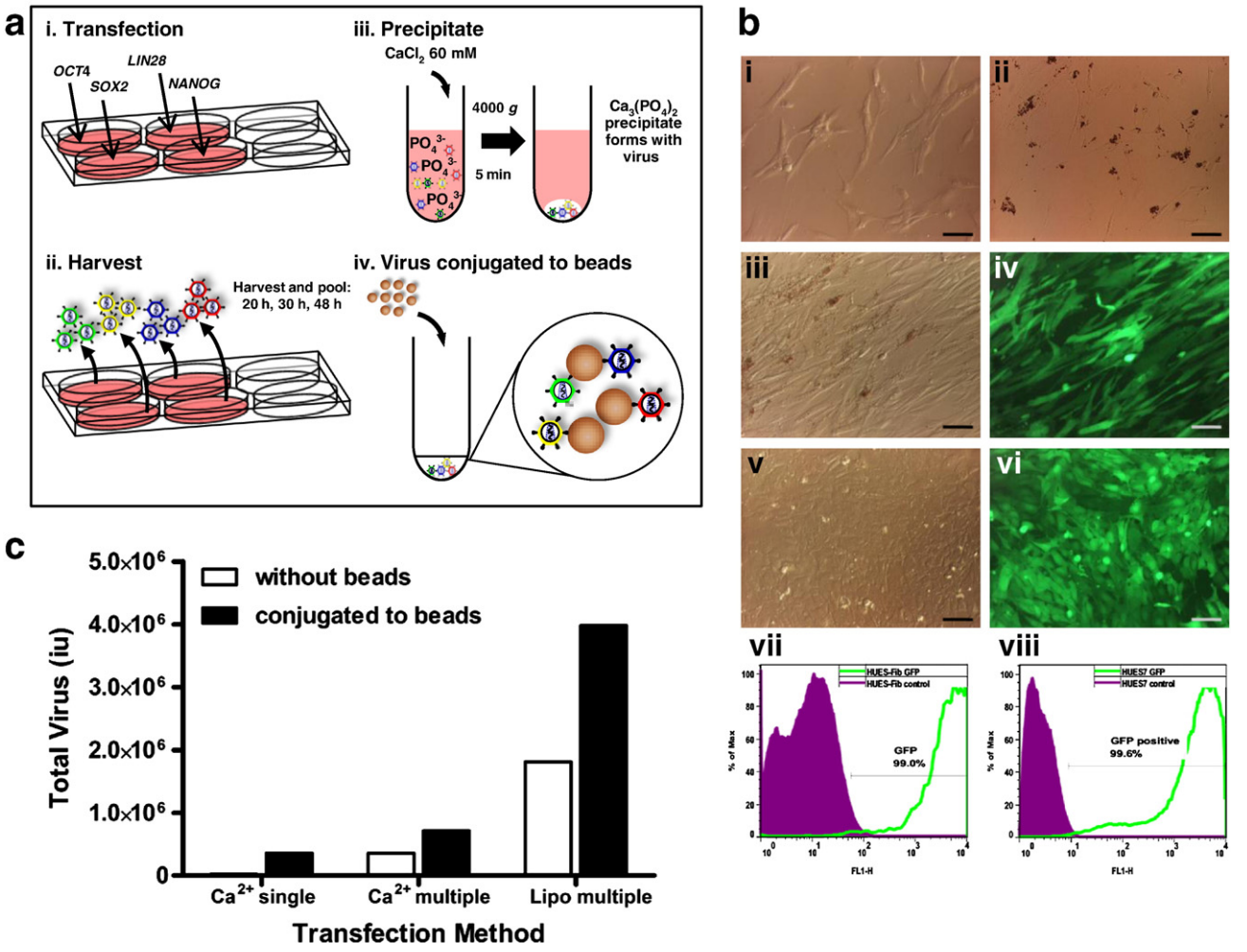


Figure 1 Optimisation of lentivirus production with a GFP-encoding lentivirus. (a) Schematic of lentivirus production in a single 6-well plate. BL15 cells were transfected with plasmids encoding *OCT4*, *SOX2*, *LIN28*, and *NANOG* together with the relevant packaging and envelope plasmids following preconditioning of the BL15 cells with biotin. Viral supernatants were harvested at 20, 30, and 48 h post transfection, pooled, and filtered, and the pH was adjusted to 7.7. Calcium chloride was then added to a final concentration of 60 mM to induce precipitation. The viral particles precipitate out of solution with the calcium phosphate, which is then collected by centrifugation. The viral particles, once resuspended, are then conjugated to streptavidin beads. (b) Infection of fibroblasts (i–iv, vii) and HUES7 (v, vi, viii) with a GFP-encoding lentivirus. Fibroblasts were transduced at an MOI of 10 and are shown pretransduction (i), 24 h posttransduction showing beads stuck to cells (ii), 96 h posttransduction the fibroblasts become confluent (iii) and express GFP with high efficiency (iv, vii). HUES7 cells were transduced at an MOI of 25 and also express GFP with high efficiency (v, vi, viii). Scale bars represent 100 μm. Using flow cytometry, transduction efficiency could be quantified to almost >99% in both cell types (vii, viii). (c) An experiment to optimise lentivirus production. Graph shows calcium phosphate precipitation transfection with single collection (single) or multiple harvest (multiple) and Lipofectamine 2000 transfection with multiple harvest (lipo multiple) both before and after bead conjugation.

likely enhance the viability of packaging cells. Furthermore, the inclusion of additional sodium pyruvate, as an energy source, in the culture medium during viral production maximises the packaging efficiency of the producer line, increasing the virus-making ability.

The method of viral concentration can have a major influence on titer. Here we used coprecipitation with calcium phosphate, coupled with virus conjugation to streptavidin superparamagnetic particles (Chan et al., 2005; Pham et al., 2001). In contrast to ultracentrifugation, which requires specialist equipment, and can damage viral vectors and reduce viral viability (Kamps et al., 1991), or spin concentration columns that are costly, this method is straightforward, consistent, and inexpensive. In order to induce precipitation from the phosphate-rich medium with calcium chloride, the viral supernatant must be serum free (Pham et al., 2001). Surprisingly, we found that removing serum from the virus production medium doubled the eventual viral yield achieved, even before concentration (Supplementary Fig. 1c). Conjugation of lentiviral particles to streptavidin superparamagnetic particles was made possible by employing a modified 293T packaging cell line, BL15 (Nesbeth et al., 2006), which express a biotin binding protein on their surface. When BL15 cells are preconditioned with biotin they metabolically couple biotin to a membrane-anchored biotin acceptor peptide (BAP). In the final assembly of a lentiviral particle, the virus is released from

the packaging cell and incorporates both viral and nonviral membrane-associated proteins. As a result of being produced in the BL15 cells, the surface of the virus particle is coated with a specific subset of proteins modified with biotin. The lentiviral particles can then be effectively bound to streptavidin superparamagnetic particles (Nesbeth et al., 2006) (schematic in Fig. 1a). In addition to the advantage of concentrating the viral supernatant into small volumes, the streptavidin superparamagnetic particles also enhance the transduction efficiency approximately 6-fold (6.37±0.17 [SEM]) (Fig. 1c), presumably by enhancing viral docking by bringing the cells in contact with the beads (Fig. 1b, ii). The beads did not appear to be toxic, although some persist in culture for several weeks; often reprogrammed colonies could be seen with beads attached round the edges. The lentivirus produced could be used to transduce both fibroblasts and HUES7 cells at >99% efficiency when used at a multiplicity of infection of 10 or 25, respectively (Fig. 1b). Furthermore, due to the optimisation steps, lentivirus production can be performed on a much smaller scale than previously documented (Park et al., 2008a). The flow cytometry method used to titer the virus allows calculation of the number of infectious virus particles in a given volume. As the virus was titered in the fibroblast target cell population, we were able to determine the amount of virus required for reprogramming experiments. Our preferred procedure is summarised in Fig. 1a and under Materials and methods. As a result, a single well of a 6-well plate, for each of the four iPSC factors, produced enough lentiviral particles for between 10 and 20 iPSC reprogramming experiments. This drastically reduces the burden of lentivirus generation, limiting the production phase of the experiment from as many as 6×10 cm plates per virus (i.e., a growth area of 360 cm^2) (Park et al., 2008a) down to a single well of a 6-well plate (i.e., 10 cm^2).

Isolation of hiPSCs from culture and derivation of stable lines

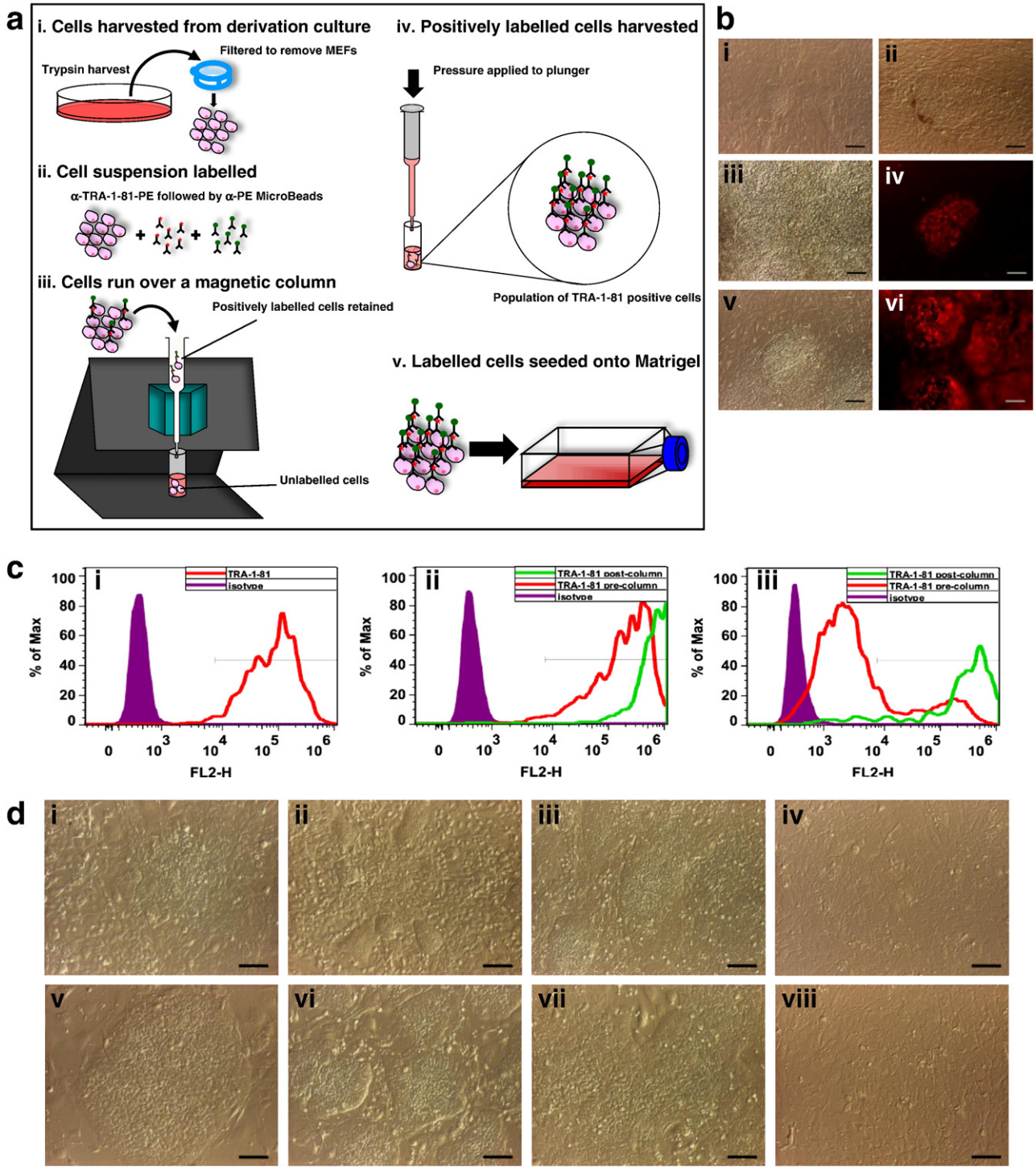
Following transduction with *OCT4*, *SOX2*, *NANOG*, and *LIN28*, cells were transferred to mitotically inactivated mouse embryonic fibroblast (MEF) feeder layers and cultured for 25–30 days with daily medium changes. Partially reprogrammed granular colonies were observed from Days 8–9 onward and small hiPSC colonies emerged from Day 11. After 20–30 days of somatic cell reprogramming, cultures typically comprised MEFs, supporting the proliferation of nonreprogrammed somatic cells, partially reprogrammed granular colonies, and a minority of fully reprogrammed hiPSC colonies. At this stage, identified hiPSC colonies could be mechanically dissected from derivation plates and transferred to fresh MEFs. However, this method is labor-intensive, time consuming, and highly subjective, with only the "best" colonies being selected for propagation. Moreover, this technique represents a major bottleneck in the derivation of new hiPSC lines. To overcome these issues, we developed a rapid, simple, and time-saving method for isolating true hiPSC from the heterogeneous cell populations that eliminates bias and which can be utilised immediately after somatic cell reprogramming (schematic Fig. 2a).

On Days 25–30, live cultures were stained with a phycoerthrin (PE)-TRA-1-81 antibody to identify true hiPSC colonies (Chan et al., 2009) (Fig. 2b). In some cultures, colonies were marked for mechanical dissection with a stem cell knife, and transferred to fresh mitotically inactive MEF feeders. Other cultures were harvested by trypsin/EDTA, stained with a PE-TRA-1-81 antibody, and then incubated with a secondary antibody conjugated to magnetic MicroBeads (Miltenyi Biotec). The cells were then run over a magnetic column, which retained the positively labeled cells, whereas unstained contaminating cells were washed through and discarded. The positively labeled cells could then be eluted from the column and be seeded directly onto Matrigel under feeder-free conditions (Fig. 2a). This has the advantage that cultures can be cryopreserved at high (~85%) viability using standard techniques rather than labor-intensive vitrification methods and can be expanded rapidly, compared to the mechanical dissection plates. The column enriched the cells to >95% TRA-1-81 positivity (Fig. 2c, iii). In fact, the method can also be used to enrich a cell population and isolate the cells expressing a specific marker at high levels only, by reduced incubation with the primary antibody (Fig. 2c, ii). In this regard, hiPSC line cultures were subjected to a second column after 2–3 passages to ensure almost complete removal of contaminating non-hiPSC (Fig. 2d). Overall, this method could be completed in 1 h and, due to its simplicity, many lines can be processed at once. Following harvest of cells from the magnetic column, and seeding to a T25 flask under feeder-free conditions on Matrigel, it was possible to yield a confluent flask (~3×10^6 hiPSCs) within a week. This meant that these cells could be cryopreserved on mass using DMSO as the protectant in just over a week after columnisation. In contrast, expanding hiPSC colonies by mechanical passage to produce this number of cells took several weeks and typically required 5–10 passages within multiple dishes. To bank these cells at early passage, the labor-intensive and highly technical vitrification method was used as it is better suited to freezing colonies (Reubinoff et al., 2001). Overall, the advantage of processing multiple lines simultaneously, and the ability to generate high numbers of cells relatively quickly, improves on the labor intensity of mechanical passage. A summary of our preferred methodology is described in Fig. 2a and under Materials and methods. It is important to note that with this method individual clones are not maintained independently, and are instead pooled to produce a polyclonal population. This method may have the drawback that individual clones cannot therefore be analysed for specific aspects, such as number of transgene integrations. However, it is well established in somatic and stem cells that as each clone expands, each of the daughter cells within the clone will acquire its own phenotype due to stochastic changes in, for example, epigenetic status (Baup et al., 2010) and proliferation rate (Smith and Whitney, 1980). Therefore, it is a slight misconception to assume that clonally derived cells are always best. Our polyclonal system has the advantages of accelerating the derivation process, averaging all clones, and making the workload of maintaining multiple patient samples more manageable. Nevertheless, if single clones are required, they can be derived by low density seeding at a later time.

Characterisation of hiPSC lines

The pluripotency of hiPSC lines produced by the positive selection method was assessed using accepted techniques (Yu

et al., 2007; Chan et al., 2009; Park et al., 2008b). Cell lines were grown to passage 15 under feeder-free conditions on Matrigel and analysed at p15–25 to normalise against a control hESC line, HUES7, which were typically used at p17–30. Cells were analysed for the expression of pluripotency markers by immunofluorescence and flow cytometry (Figs. 3a and b; Supplementary Fig. 2). All lines showed expression of proteins associated with pluripotency, including OCT4, SOX2, LIN28, NANOG, TRA-1-81, SSEA4, KLF4, DNMT3B, REX1, cMYC, and were negative for the differentiation marker SSEA1 and the fibroblast marker P4HB. Metaphase spreads at passages 15 and 25 confirmed that the cells showed normal karyotype (representative karyogram of 30 spreads/line/passage number, Fig. 3c). Cells were grown over several passages to determine their population doubling rate (Fig. 3d), and were found to have different growth rates, but all within the published range for hESCs (Cowan et al., 2004; Xu et al., 2001). To determine whether the hiPSC lines were pluripotent, cells were analysed to determine whether they had the ability to differentiate into three germ layers *in vitro* and form tematomas *in vivo*. In the *in vitro* assay, hiPSC were differentiated into embryoid bodies (EBs), as previously



described (Burridge et al., 2007), and fixed at 30 days in 4% paraformaldehyde. These EBs showed formation of ectoderm shown by the expression of β-III-tubulin, endoderm shown by α-fetoprotein, and mesoderm by the formation of beating clusters staining positive for α-actinin (Fig. 3e). In the *in vivo* assay, hiPSC were injected into the kidney capsule of NOD/SCID mice (Priddle et al., 2009). Teratomas were collected at Days 50–60, fixed, and embedded in paraffin wax. Sections were stained with hematoxylin and eosin and confirmed to express the three germ layers represented by early neuroectoderm, primitive gut endothelium (endoderm) and premature cartilage (mesoderm) (Fig. 3e). The methylation status of the *OCT4* and *NANOG* promoters was determined in primary fibroblasts, reprogrammed hiPSCs, and their differentiated progeny, using the HUES7 hESC line as a control, and were found to be unmethylated or hypomethylated in HUES7 and the hiPSC lines, while they were hypermethylated in primary fibroblasts and differentiated hiPSC progeny (Fig. 3g).

In conclusion, here we outline two key methods for the efficient and simplified production of hiPSC lines. The lines produced by this method are karyotypically stable and fulfill the accepted criteria to class them as true hiPSC10, 29. In addition, the methods described here are relevant to many other applications. For example lentivirus is widely used as a genetic modification technique, so simplified, high titer virus production is an invaluable technique. Furthermore, the positive selection method described here is generically applicable and can be adapted to purify any cell type based on surface marker expression. This includes enriching hESCs from cultures that contain differentiated cells and selection of specific cell lineages following differentiation.

Materials and methods

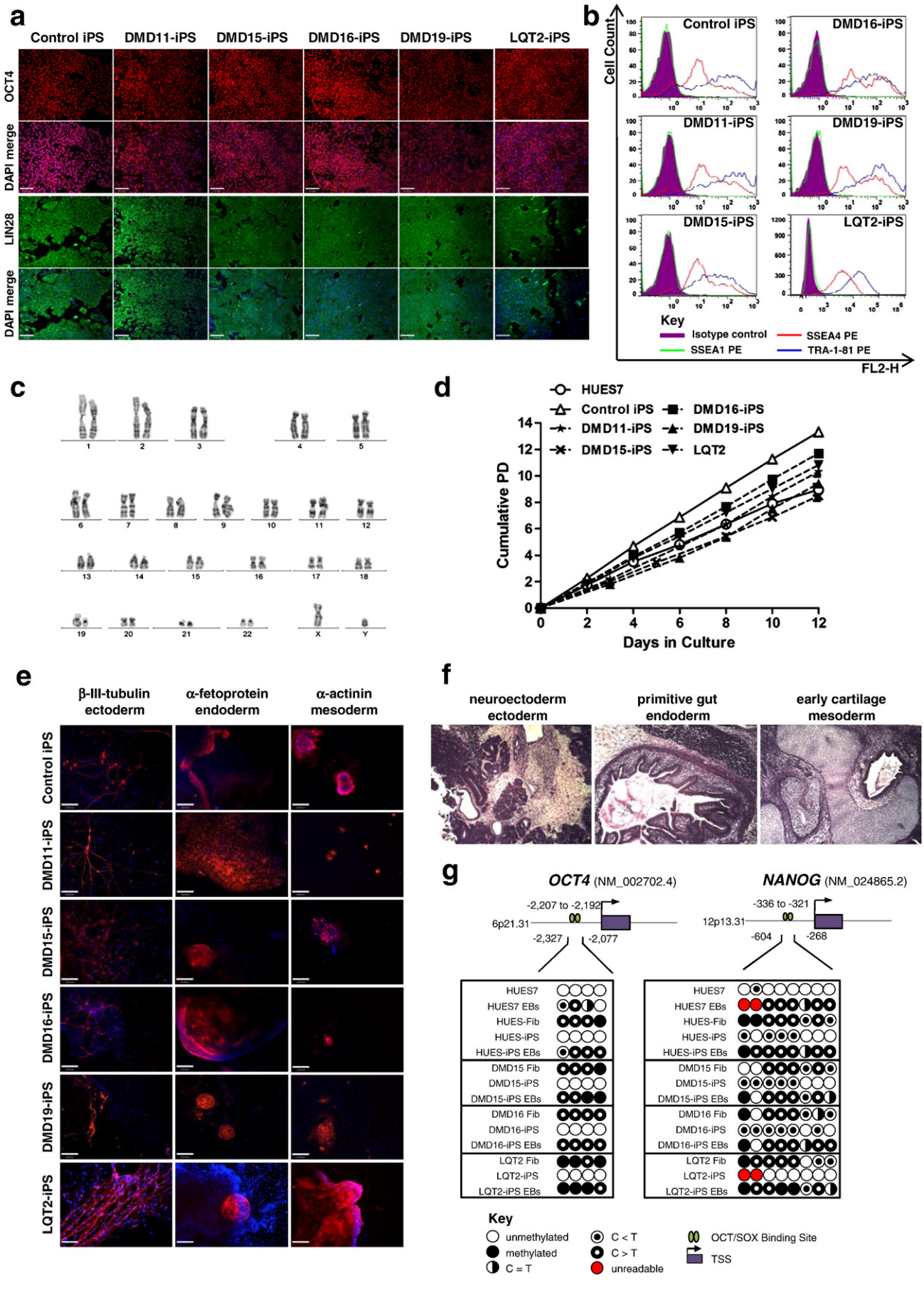
Optimised protocol for production of VSV-G enveloped lentivirus particles

Lentiviral expression plasmids encoding *OCT4*, *SOX2*, *LIN28*, and *NANOG* were sourced from Addgene (Yu et al., 2007). Lentiviral particles were produced using a 293T cell line, BL15, modified to express a biotin acceptor peptide domain on its cell surface (Chan et al., 2005; Nesbeth et al., 2006). BL15 cells were maintained in DMEM with 10% FCS. BL15 cells were pretreated with 100 μM A-biotin (Sigma) for 3 days, prior to viral production. On the day before transfection, BL15 cells were seeded at a density of 1×10^6 cells/well in gelatin-coated 6 well plates, in medium containing 100 μM biotin. One hour prior to transfection, medium was changed to Opti-MEM (Invitrogen) with 10% FCS supplemented with 100 μM biotin. Transfection mixes were set up as follows for each virus, for one well of a 6-well plate; mix A, 150 μl Opti-MEM plus 15 μl of Lipofectamine 2000 (Invitrogen); and mix B, 150 μl Opti-MEM and 0.5 μg pMD2.G (Addgene), 1.25 μg psPAX2 (Addgene), and 1.67 μg of the appropriate iPS factor expression plasmid. These were incubated separately for 5 min and then together for 20 min before adding to the relevant well of the 6-well plate. Transfection mixes were removed from the cells after 5 h and medium was replaced, after washing, with DMEM (serum-free) supplemented with 1 mM sodium pyruvate (Invitrogen). Viral supernatant was harvested at 20, 30, and 48 h post transfection, each time replacing the medium with DMEM supplemented with sodium pryuvate. Each collection was stored at -80 °C until use. After the final viral collection, harvests were thawed, pooled, and filtered, and the pH was adjusted to 7.7. To precipitate the virus particles, 1 M $CaCl_2$ was added to a final concentration of 60 mM. The viral supernatant was incubated at 37 °C for 30–40 min to form a calcium phosphate ($Ca_3(PO_4)_2$) precipitate, and then centrifuged at 4000 *g* for 5 min. The $Ca_3(PO_4)_2$/virus pellet was resuspended in 1–2 ml EDTA buffer (100 mM EDTA in 50 mM NaCl, pH 6.5). This virus could then be snap-frozen in aliquots at and stored at -80 °C with no significant loss of activity (Supplementary Fig. 1d). To facilitate the removal of the EDTA buffer and to enhance transduction efficiency virus was conjugated to streptavidin paramagnetic beads (Promega), using 600 μl of beads for the four pooled iPS viruses. Virus was conjugated to beads for 3–5 h at room temperature on a roller. Following this incubation, the supernatant was removed and the remaining beads were resuspended in DMEM with 10% FCS. Each well of a 6-well plate made approximately $0.5–1 \times 10^7$ infectious virus particles. Enough to transduce 10–20 plates of fibroblasts at an MOI of 10.

Culture and transduction of human dermal fibroblasts

Human dermal fibroblasts from patients with muscular dystrophy (DMD11, DMD15, DMD16, and DMD19) and Long

Figure 2 Isolation of hiPSCs by positive selection. (a) A schematic of positive selection of hiPSC over a magnetic column. Cells were trypsinised from the derivation plate, filtered, and incubated with TRA-1-81-PE antibody, followed by incubation with secondary antibody conjugated to magnetic MicroBeads. Labeled cells were run through a magnetic column where positively labeled cells were retained on the column. Finally the cells were expelled from the column and returned to culture under feeder-free conditions. (b) Representative images of hiPSC derivation; (i) granular type 1 colony on Day 11, (ii) small hiPSC colony with evidence of beads round the edge on Day 15, and (iii–vi) of live staining with a TRA-1-81-PE antibody at Day 25, under DIC microscope (iii, v) and a fluorescence microscope (iv, vi). (c) Representative flow cytometry histograms indicating column enrichment. Filled histograms show the isotype control with red lines showing precolumn and green postcolumn analysis. By way of control (i) shows HUES7 expressing TRA-1-81, and (ii) shows HUES-hiPSC line expressing TRA-1-81 and enrichment postcolumn of the cells expressing TRA-1-81 at a higher level. In (iii) a patient hiPSC line is shown pre- and postcolumn showing enrichment of the TRA-1-81-positive cells. (d) Representative images of cells, postcolumn enrichment and after transfer under feeder-free conditions (i–iv) and cells transferred under feeder-free conditions from MEFs (v–viii). First 24 h postcolumn (i) cells often did not show hESC morphology; however colonies began to emerge after a few days, seen in (ii). These cells grew to represent a large proportion of the culture (iii). Cells were often recolumnised after 2–3 passages after which a homogeneous population was observed under feeder-free conditions (iv). In manual passage culture, hiPSC colonies on MEFs were passaged to p7 (v) before transfer onto Matrigel (vi). Cells took several passages to settle under feeder-free conditions. Representative images show cells at p2 on Matrigel (p9: p7 on MEFs + p2 Matrigel) (vii), and p5 on Matrigel (p11: p7 on MEFs + p5 on Matrigel) (viii). Scale bars represent 100 μm.


a
Control iPS
DMD11-iPS
DMD15-iPS
DMD16-iPS
DMD19-iPS
LQT2-iPS
OCT4
DAPI merge
LIN28
DAPI merge
b
Cell Count
Control iPS
DMD16-iPS
DMD11-iPS
DMD19-iPS
DMD15-iPS
LQT2-iPS
Key
Isotype control
SSEA4 PE
SSEA1 PE
TRA-1-81 PE
FL2-H
c
d
HUES7
Control iPS
DMD16-iPS
DMD11-iPS
DMD19-iPS
DMD15-iPS
LQT2
Cumulative PD
Days in Culture
e
β-III-tubulin ectoderm
α-fetoprotein endoderm
α-actinin mesoderm
Control iPS
DMD11-iPS
DMD15-iPS
DMD16-iPS
DMD19-iPS
LQT2-iPS
f
neuroectoderm ectoderm
primitive gut endoderm
early cartilage mesoderm
g
OCT4 (NM_002702.4)
NANOG (NM_024865.2)
-2,207 to -2,192
6p21.31
-2,327
-2,077
-336 to -321
12p13.31
-604
-268
HUES7
HUES7 EBs
HUES-Fib
HUES-iPS
HUES-iPS EBs
DMD15 Fib
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LQT2 Fib
LQT2-iPS
LQT2-iPS EBs
Key
unmethylated
methylated
C = T
C < T
C > T
unreadable
OCT/SOX Binding Site
TSS

QT Syndrome type 2 (LQT2) were prepared from 4-mm skin punch biopsies, after informed consent was given by the patients. The studies are approved by the local ethics committee. DMD fibroblasts were obtained from the BioBank of the MRC Centre for Neuromuscular Diseases (Newcastle). Fibroblasts were grown in DMEM supplemented with 10% FCS, 0.1 mM nonessential amino acids (Invitrogen), and 2 mM L-glutamine (Invitrogen). An embryonic fibroblast line, derived from differentiation of the hESC line HUES7, was used as a control (HUES-Fib) and was cultured in the same medium. On the day of transduction fibroblasts were seeded into tissue culture-treated 6-well plates (NUNC) at a density of 50 000 cells/well and left to settle for 5–6 h. Once the cells had adhered to the plastic they were infected with the virus/bead mix at an MOI of 10, in the presence of 8 μg/ml Polybrene (Sigma-Aldrich). Medium was changed after 24 h with normal growth medium. After a further 24 h, cells were typsinised and resuspended in hiPSC culture medium (DMEM-F12 supplemented with 15% KSR, 100 μM β-mercaptoethanol, 10 ng/ml bFGF (Sigma-Aldrich), 4.5 mM GlutaMAX-1 (Invitrogen), and 0.1 mM nonessential amino acids). Cells were transferred to 90-mm dishes containing mitomycin C-inactivated MEFs at a density of 1.8×10^6 cells/plate. The medium was changed daily: for the first week with hiPSC culture medium, and then for the rest of the experiment, with MEF-conditioned medium (prepared as previously described (Braam et al., 2008)), supplemented with 10 ng/ml bFGF.

Live staining of hiPSC colonies

To mark true hiPSC colonies for manual dissection, culture plates were incubated with a phycoerythrin (PE)-conjugated TRA-1-81 antibody (BD) at a 1 in 200 dilution in culture medium for 15 min. Cells were then washed and then imaged under an inverted fluorescence microscope (Nikon Eclipse TE 2000).

Positive selection of true hiPSC

For derivation of hiPSC lines, colonies were either cut from the derivation plate and cultured on MEFs (as previously described Burridge et al., 2007) or trypsinised from the plate and isolated by positive selection over a magnetic column. For the positive selection method, the derivation plate was treated with 0.05% trypsin/EDTA to loosen the MEF layer, and the culture was transferred to a 50-ml tube as one sheet in approximately 20 ml culture medium. Using a 10-ml pipette, cells were forcibly pipetted up and down to recover the hiPSCs and other cells attached to the MEF layer. The resultant cell suspension was then filtered through a 40-μm cell strainer (BD) to remove the large MEF "clumps." The supernatant was spun at 300 *g* for 5 min and then washed in ice-cold column buffer (Hanks balanced salt solution, HBSS, with 2% FCS, pH 7.4). After the wash, the cell pellet was resuspended in 200 μl ice-cold column buffer containing 1 in 200 dilution TRA-1-81 PE-conjugated antibody, and incubated in the dark at 4 °C for 15 min. After the incubation, 5 ml of ice-cold column buffer was added to wash the cells and the solution was spun at 300 *g* for 5 min. The cell pellet was resuspended in 80 μl ice-cold column buffer plus 20 μl PE-MicroBeads (Miltenyi Biotec) and incubated for 15 min in the dark at 4 °C. After the incubation, 5 ml of ice-cold column buffer was added to wash the cells and the solution was spun at 300 *g* for 5 min. During the spin, a MS column (Miltenyi Biotec) was placed in its magnet attached to a magnet stand (Miltenyi Biotec) and primed with 500 μl ice-cold column buffer. The cell pellet was resuspended in 500 μl ice-cold column buffer and transferred to the column. The cell suspension was allowed to drip through the column, by gravity flow, with a tube catching the negative, flowthrough, population. The column was washed three times with column buffer and then the column was removed from the magnet and transferred to a fresh tube. Cells were ejected by applying 1 ml of column buffer to the column and then inserting the supplied plunger. Cells were spun at 300 *g* for 2 min, resuspended in MEF-conditioned medium, and then transferred under feeder-free conditions on Matrigel-coated dishes or flasks, as described previously (Denning et al., 2006).

Growth and maintenance of hESC and hiPSC

The HUES7 cell line and derived hiPSC lines were maintained on Matrigel as previously described (Denning et al., 2006).

Detection of pluripotency marker expression

For flow cytometry analysis, cells were harvested and fixed in 4% paraformaldehyde (PFA, Sigma-Aldrich), and then resuspended in PBS with 2% FCS. Cells were incubated with fluorescently conjugated antibodies, or relevant isotype controls, for 20 min in the dark at room temperature, washed, and run through the flow cytometer (FC500, BD Biosciences).

Figure 3 Characterisation of hiPSC lines. (a) Expression of pluripotency markers OCT4 and LIN28 in the 6 hiPSC lines by immunofluorescence; for a more extensive list see Supplementary Fig. 2. (b) Flow cytometry histograms of the hiPSC lines indicating the surface marker expression of TRA-1-81 and SSEA4, with no expression of SSEA1 which overlays the isotype control (filled histogram). (c) A representative karyogram of hiPSC lines; all of which showed a normal karyotype both at p15 and at p25. (d) The growth kinetics of the hiPSC lines all falling within the normal range of population doublings under feeder-free conditions. In (e) cells were differentiated *in vitro* to form EBs, and fixed at Day 30. The expression of β-III-tubulin, α-fetoprotein, and α-actinin indicates expression of ectoderm, endoderm, and mesoderm, respectively. *In vivo* differentiation assays also indicated the expression of the three germ layers with representative images shown in (f). Further information regarding teratomas, including larger annotated pictures, can be found in Supplementary Fig. 3. Finally, methylation status of the *OCT4* and *NANOG* promoters was investigated in several of the hiPSC lines, which showed the same pattern as the HUES7 control hESC line (g). Further information regarding the determination of methylation status can be found in Supplementary Fig. 4. Scale bars represent 130 μm.

For immunocytochemistry, cells were grown to confluence in chamber slides (NUNC) and then fixed in 4% PFA. Cells were permeabilised with 0.1% Triton X-100 (Sigma-Aldrich), and blocked with 8% goat or rabbit serum (Sigma-Aldrich) in PBS, as appropriate. Primary antibody incubations were performed overnight at 4 °C and secondary antibodies at room temperature for 1 h. Cells were imaged using a Nikon Eclipse 90i fluorescence microscope.

Determination of karyotype stability

Metaphase spreads for karyotype analysis were prepared as previously described (Braam et al., 2008). Cell karyotype was assessed at passage 15 after derivation and then again at passage 25 to determine stability.

Differentiation into three germ layers *in vitro*

The hiPSC lines were tested to determine whether they could form three germ layers *in vitro* following embryoid body formation as previously described (Burridge et al., 2007). EBs were fixed at Day 30 and analysed for the expression of proteins representing the three germ layers: β-III-tubulin for ectoderm, α-fetoprotein for endoderm, and α-actinin for mesoderm. Expression of these proteins was analysed by immunocytochemistry as described above.

Teratoma formation in NOD/SCID mice

The hiPSC lines were tested to determine whether they could form teratomas and consisting of the three germ layers *in vivo*. Teratomas were formed by injection of hiPSCs into the kidney capsule of NOD/SCID mice as previously described (Priddle et al., 2009). Tumors were collected between 50 and 60 days post injection and fixed in 4% PFA, embedded in paraffin wax, sectioned, and stained with hematoxylin and eosin as previously described (Priddle et al., 2009). The germ layers were identified under a light microscope.

Bisulfite sequencing of genomic DNA

Cell pellets from fibroblasts, reprogrammed hiPSC lines, and their differentiated progeny were analysed to determine the methylation status of the endogenous *OCT4* and *NANOG* promoters. Genomic DNA was extracted from frozen cell pellets of fibroblasts, reprogrammed iPSC lines, and their differentiated progeny (Qiagen DNeasy Blood and Tissue kit). Genomic DNA (1 μg) was digested overnight at 37 °C with *PacI* enzyme (1 μl of 10 U/μl; NEB), and subsequently bisulfite-treated as described previously (Kim et al., 2007). Bisulfite PCR primers (Invitrogen) were designed using MethPrimer software for *NANOG* (F, TGGTTAGGTTGGTTTTAAATTTTTG; and R, AACCCACCCTTATAAATTCTCAATTA) or were previously published for *OCT4* (Deb-Rinker et al., 2005). Bisulfite PCR was performed in a final volume of 25 μl, comprising Platinum Taq (0.125 μl Invitrogen), 5 μM forward and reverse primers (6 μL; Invitrogen), 10X Reaction buffer (2.5 μl; Invitrogen), 2.5 mM dNTPs (1.25 μl; Invitrogen), 1.5 mM $MgCl_2$ (0.75 μl; Invitrogen), and 1 μl of bisulfite converted DNA. The PCR cycling conditions were 95 °C for 10 min followed by 40 cycles (95 °C for 1 min, 63 °C for 1 min, 72 °C for 1 min) and a final extension step at 72 °C for 10 min. PCR products were purified (QIAquick gel extraction kit; Qiagen) and direct sequencing was performed to assess DNA methylation, using the forward primer for each gene. Results were represented using a five-category classification, as described in Supplementary Fig. 4.

Distinguishing between endogenous and lentiviral expression of *OCT4*, *SOX2*, *NANOG*, and *LIN28*

RNA extraction from frozen cell pellets was performed using the Qiagen RNeasy Mini kit (Qiagen), according to the manufacturer's instructions. Reverse transcription was performed using the First Strand cDNA Synthesis kit (GE Healthcare), with 100 ng RNA. RT-PCR was performed in a total volume of 25 μl, which was composed of Hotstar Taq polymerase (0.125 μl; Qiagen), 5 μM forward and reverse primers (2.5 μl; Invitrogen), 10x Reaction buffer (2.5 μl; Qiagen), 2.5 mM dNTPs (2.5 μl; Invitrogen) and 2 μl of cDNA. The PCR cycling conditions were 95 °C for 15 min followed by 35 cycles (95 °C for 1 min, 57 °C for 90 s, 72 °C for 1 min) and a final extension step at 72 °C for 5 min. Primers for RT-PCR are in Supplementary Table S1.

Statistics

Statistics were performed using Prism Software (Version 5.0 GraphPad, San Diego, CA).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.scr.2010.10.002.

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