ARTICLES

Reprogramming of human somatic cells to pluripotency with defined factors

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Pluripotency pertains to the cells of early embryos that can generate all of the tissues in the organism. Embryonic stem cells are embryo-derived cell lines that retain pluripotency and represent invaluable tools for research into the mechanisms of tissue formation. Recently, murine fibroblasts have been reprogrammed directly to pluripotency by ectopic expression of four transcription factors (*Oct4*, *Sox2*, *Klf4* and *Myc*) to yield induced pluripotent stem (iPS) cells. Using these same factors, we have derived iPS cells from fetal, neonatal and adult human primary cells, including dermal fibroblasts isolated from a skin biopsy of a healthy research subject. Human iPS cells resemble embryonic stem cells in morphology and gene expression and in the capacity to form teratomas in immune-deficient mice. These data demonstrate that defined factors can reprogramme human cells to pluripotency, and establish a method whereby patient-specific cells might be established in culture.

Pluripotency can be induced in somatic cells by nuclear transfer into oocytes[1] and fusion with embryonic stem cells[2], and for male germ cells by cell culture alone[3]. Ectopic expression of four transcription factors (*Oct4*, *Sox2*, *Klf4* and *Myc*) in murine fibroblasts is sufficient to yield iPS cells that resemble embryonic stem (ES) cells in their capacity to form chimaeric embryos and contribute to the germ lineage[4–7]. Direct, factor-based reprogramming might enable the generation of pluripotent cell lines from patients afflicted by disease or disability, which could then be exploited in fundamental studies of disease pathophysiology or drug screening, or in pre-clinical proof-of-principle experiments that couple gene repair and cell replacement strategies.

We attempted to use the original four reprogramming factors defined by ref. 4 (*OCT4*, *SOX2*, *KLF4* and *MYC*) to isolate iPS cells from human embryonic fibroblasts differentiated from H1-OGN cells, human ES cells that express the green fluorescence protein (GFP) reporter and neomycin (G418) resistance genes by virtue of their integration into the *OCT4* locus by homologous recombination (H1-OGN (ref. 8)). We differentiated H1-OGN cells *in vitro* for 4 weeks, and propagated a homogeneous population of fibroblast-like cells (dH1f, differentiated H1-OGN fibroblast; Fig. 1a). GFP expression was undetectable in dH1f cells, as assayed by flow cytometry (Supplementary Fig. 1). Expression of *OCT4*, *SOX2*, *NANOG* and *KLF4* was extinguished in dH1f cells, whereas *MYC* expression persisted at near-comparable levels to undifferentiated H1-OGN cells (Fig. 1b). The dH1f cells could be cultured readily for at least 14 passages, after which their proliferation slowed markedly. No dH1f cells survived selection in G418 (50 ng ml^{-1}), and no tumours formed after injection of dH1f cells into immune-deficient mice. Taken together, these data establish that dH1f cells represent differentiated human ES cell derivatives that have lost the essential features of pluripotency.

To ensure propagation of differentiated fibroblasts free of contamination by undifferentiated ES cells, we infected early passage dH1f cells with a lentiviral construct carrying the dTomato reporter gene, plated infected cells by serial dilution, and expanded individual

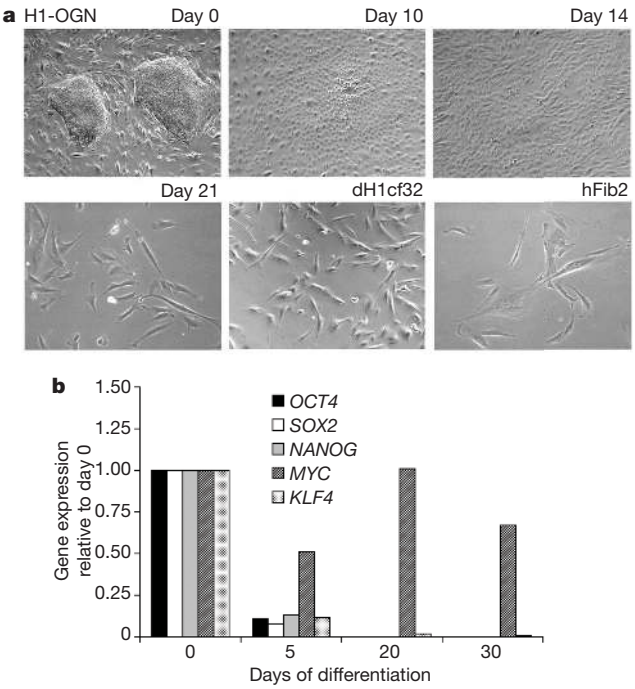

Figure 1 | Differentiation of human embryonic fibroblasts from human embryonic stem cells (H1-OGN). In the human ES cell line H1-OGN[8], the *OCT4* promoter drives expression of GFP-IRES-*neo*. **a**, Time course of differentiation of H1-OGN cells into a population of adherent fibroblasts, and subsequent expansion of a colony into a clonal fibroblast cell line (dH1cf32). The differentiated fibroblast derivatives of H1-OGN cells are morphologically indistinguishable from dermal fibroblasts cultured from an adult volunteer donor (hFib2). **b**, Quantitative real-time PCR demonstrates that the expression of a cohort of key pluripotency factors (*OCT4*, *SOX2*, *NANOG* and *KLF4*) is lost by the third week of differentiation, whereas expression of a fifth factor (*MYC*) persists.

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colonies. Southern hybridization confirmed distinct single or double lentiviral integration sites in three cell lines, thereby confirming their clonal derivation from single cells (cloned dH1cf16, dH1cf32 and dH1cf34; Supplementary Fig. 2). Proliferation of the cloned dH1cf cells began to slow markedly after an additional 4–5 passages. The dH1cf clones were G418 sensitive, negative for expression of *GFP*, *OCT4* and *NANOG*, and failed to induce tumours in immune-deficient mice (Supplementary Fig. 3 and data not shown).

Reprogramming of human ES-cell-derived fetal fibroblasts

We infected cultures of dH1f and cloned dH1cf cells with a cocktail of retroviral supernatants carrying human *OCT4*, *SOX2*, *MYC* and *KLF4*. Seven days after infection, cells were plated in human ES cell culture medium supplemented with the ROCK inhibitor Y27632, previously shown to enhance survival and clonogenicity of single dissociated human ES cells[9]. By 14 days after infection, cultures of infected dH1f cells showed distinct small colonies that were picked and expanded. The resulting cultures harboured colonies for which morphology was indistinguishable from the parental H1-OGN cells (Fig. 2a). Selection with G418 was not required to identify cells with ES-cell-like colony morphology; rather, morphology itself sufficed, as reported for identification of murine iPS cells[10,11]. We performed ten independent infections of 1×10^5 dH1f cells with the four factors, and consistently observed approximately 100 human ES-cell-like colonies, for a reprogramming efficiency of ~0.1% (Table 1). Interestingly, we obtained human ES-cell-like colonies when we eliminated either MYC or KLF4 from the cocktails, although with markedly lower efficiency (Table 1). Infection of different clones of dH1cfs revealed a lower efficiency and delayed appearance of ES-cell-like colonies (between 6–47 colonies per 10^5 cells after 21 days). Expanded cultures of human ES-cell-like colonies from dH1cf clones carried the identical lentiviral integration site as the parental cell line, thereby confirming their derivation from the original dH1cf clone, and eliminating the possibility that a contaminating undifferentiated H1-OGN cell had been re-isolated (Supplementary Fig. 2).

Reprogramming of fetal, neonatal and adult fibroblasts

We next tested a diverse panel of human primary cells available from commercial sources, as well as primary dermal fibroblasts isolated from a skin biopsy from a healthy volunteer, which were obtained following informed consent for reprogramming studies under a protocol approved by the Institutional Review Board and Embryonic Stem Cell Research Oversight Committee of Children's Hospital Boston.

We isolated cells with human ES-cell-like morphology from cultures of MRC5 fetal lung fibroblasts around 21 days after infection with the four transcription factors. We were also able to identify human ES-cell-like colonies by introduction of the four factors into Detroit 551 cells, another human primary cell culture derived from fetal skin (data not shown). In contrast to our results with human ES-cell-derived fibroblasts (dH1f, dH1cf) and primary fetal cells (MRC5, Detroit 551), transduction of the four transcription factors into more developmentally mature somatic cells, for example, neonatal foreskin fibroblasts (BJ1), adult mesenchymal stem cells (MSC) and adult dermal fibroblasts (hFib2), resulted in slowed proliferation and cellular senescence, and we failed to identify colonies with obvious ES-cell-like morphology from any of these infected cell cultures. We reasoned that adult human somatic cells might require additional factors to grow in continuous cell culture and to be reprogrammed to pluripotency, and thus we supplemented the four factors (*OCT4*, *SOX2*, *MYC* and *KLF4*) with genes known to have a role in establishing human cells in culture: the catalytic subunit of human telomerase, *hTERT*[12], and SV40 large T, which has potent anti-apoptotic activity[13]. When *hTERT* and SV40 large T were introduced together with the four transcription factors into BJ1, MSC and hFib2 cells, the cultures grew more rapidly but still showed significant cellular loss and sloughing into the media. However, against the background of adherent cells, we were able to recognize colonies with human ES-cell-like morphology (Fig. 2a and Table 1). Individual colonies of human ES-cell-like cells were picked and expanded. All ES-cell-like colonies shared DNA fingerprints with the line from which they derived, thereby ruling out the possibility of contamination with

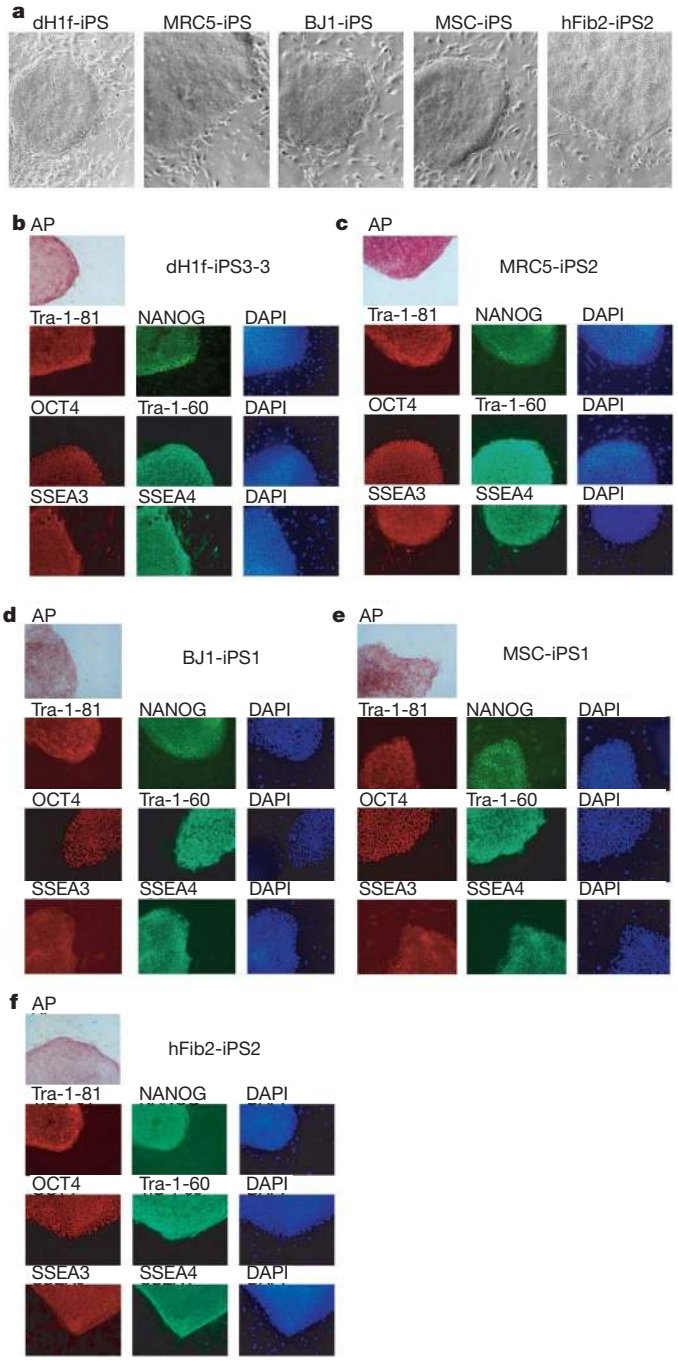

Figure 2 | Multiple cultured human primary somatic cells yield iPS cells. **a**, iPS cells produced from five independent human primary cell lines form colonies with a similarly compact, ES-cell-like morphology in co-culture with mouse embryonic feeder fibroblasts (MEFs). **b–f**, As shown via immunohistochemistry (IHC), human iPS cell colonies express markers common to pluripotent cells, including alkaline phosphatase (AP), Tra-1-81, NANOG, OCT4, Tra-1-60, SSEA3 and SSEA4. 4,6-Diamidino-2-phenylindole (DAPI) staining indicates the total cell content per field. Fibroblasts surrounding human iPS colonies serve as internal negative controls for IHC staining. dH1f-iPS3-3 (**b**, from H1-OGN differentiated fibroblasts), MRC5-iPS2 (**c**, from MRC5 human fetal lung fibroblasts), BJ1-iPS1 (**d**, from neonatal foreskin fibroblasts), MSC-iPS1 (**e**, from mesenchymal stem cells), hFib2-iPS2 (**f**, dermal fibroblast from healthy adult male).

existing human ES cells being carried in the laboratory (Supplementary Fig. 4).

Characterization of reprogrammed somatic cell lines

We analysed colonies selected for human ES-cell-like morphology from dH1f, MRC5, BJ1, MSC and hFib2 by immunohistochemistry, and detected expression of alkaline phosphatase, Tra-1-81, Tra-1-60, SSEA3, SSEA4, OCT4 and NANOG (Fig. 2b–f), all markers shared with human ES cells[14]. We also analysed gene expression by quantitative polymerase chain reaction (PCR) analysis, and noted that for derivatives of dH1f, dH1cf, MRC5, BJ1, MSC and hFib2, expression of *OCT4*, *SOX2*, *NANOG*, *KLF4*, *hTERT*, *REX1* and *GDF3* was markedly elevated over the respective fibroblast population, and comparable to the parental H1-OGN human ES cells (Fig. 3a–e). Expression of *MYC* did not vary markedly from the parental cell lines, suggesting that a consistent expression level was required to sustain cell proliferation in multiple cell types under our culture conditions (Fig. 3a–e). In murine iPS cells, retroviral expression of murine *Oct4*, *Sox2*, *Myc* and *Klf4* is silenced during iPS derivation and complemented by reactivation of expression from the endogenous gene loci[4–7]. We

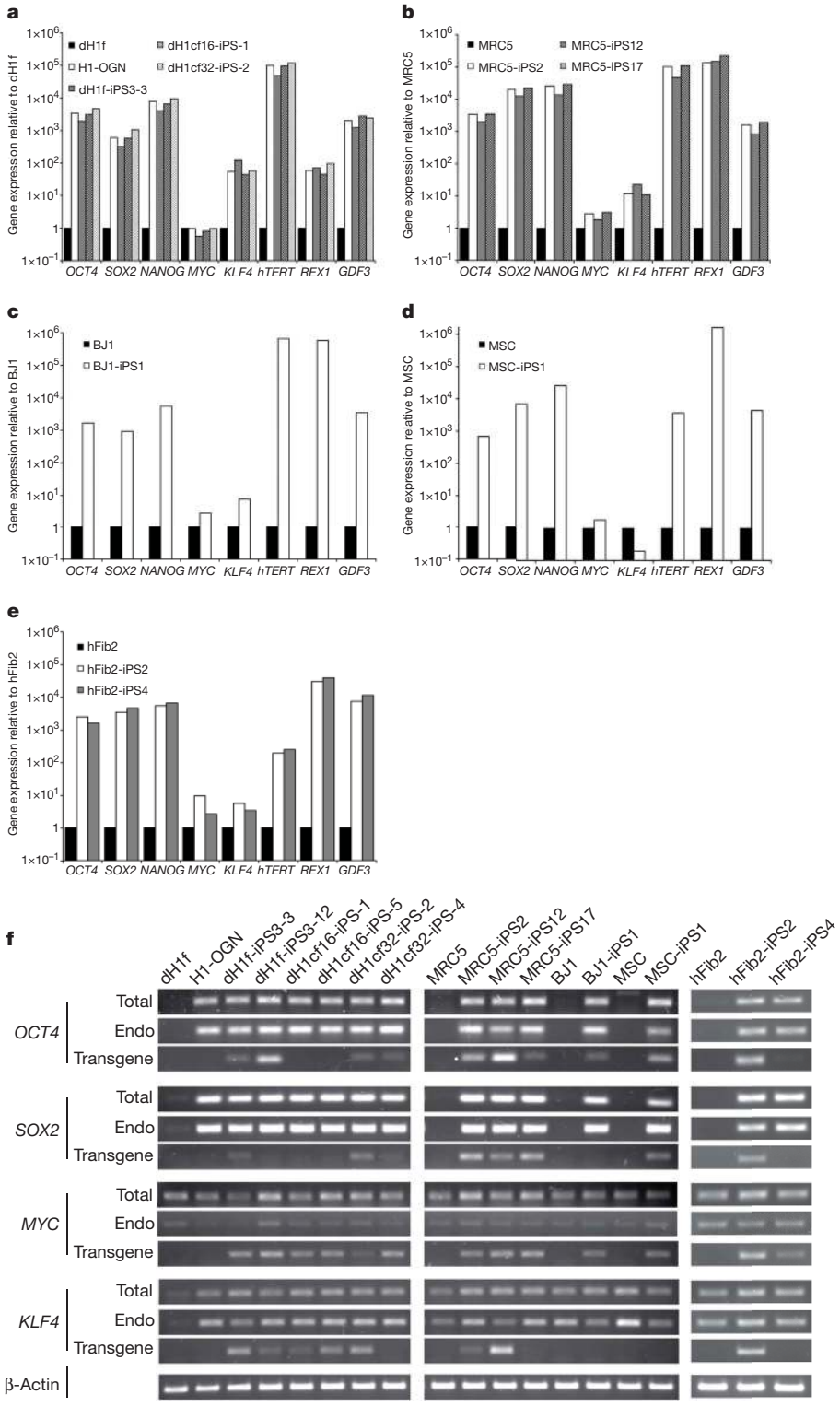

Figure 3 | Gene expression in human iPS cells is similar to human ES cells. **a**–**e**, Quantitative real-time PCR assay for expression of *OCT4*, *SOX2*, *NANOG*, *MYC*, *KLF4*, *hTERT*, *REX1* and *GDF3* in human iPS and parental cells. Individual PCR reactions were normalized against internal controls (β-actin) and plotted relative to the expression level in the parent fibroblast cell line. **a**, dH1f, dH1f-iPS3-3, dH1cf16-iPS-1 and dH1cf32-iPS-2 cells. **b**, MRC5-iPS2, MRC5-iPS12 and MRC5–iPS17. **c**, BJ1-iPS1. **d**, MSC-iPS1. **e**, hFib2-iPS2 and hFib2-iPS4. **f**, Transgene-specific PCR primers permit determination of the relative expression levels between total, endogenous (Endo) and retrovirally expressed (Transgene) genes (*OCT4*, *SOX2*, *MYC* and *KLF4*) via semi-quantitative PCR. β-Actin is shown as a positive amplification and loading control.

Table 1 | ES-cell-like colony formation with various donor cells and reprogramming factors

Cell line	OCT4 and SOX2	Three factors	Four factors	Six factors‡
ES-cell-derived fibroblasts dH1f	0	−OCT4*, 0; −SOX2†, 0; −KLF4, 63; −MYC, 11	118 ± 35	250
ES-cell-derived fibroblasts dH1cf (clones 16, 32, 34)	ND	ND	dH1cf16, 47; dH1cf32, 12; dH1cf34, 6	dH1cf16, 86; dH1cf32, 40; dH1cf34, 17
Fetal lung fibroblasts MRC5	ND	ND	39	ND
Neonatal foreskin fibroblasts BJ1	ND	ND	0	21
Mesenchymal stem cells	ND	ND	0	3
Adult dermal fibroblasts hFib2	ND	ND	0	7

The four factors were OCT4, SOX2, MYC and KLF4; the six factors were OCT4, SOX2, MYC, KLF4, hTERT and SV40 large T. Numbers are for colonies showing human ES-cell-like morphology per 10^5 infected cells. ND, not determined.
* No human ES-cell-like colonies but numerous (~10^2) colonies with flat morphology were observed.
† No colonies observed, not even the flat variety seen with the three-factor combination lacking OCT4.
‡ Only human ES-cell-like colonies scored, despite observation of frequent flat colonies.

analysed the expression of the endogenous loci and retroviral transgenes, and found that total expression of *OCT4*, *SOX2*, *MYC* and *KLF4* was comparable to human ES cells (Fig. 3f). Expression of the endogenous *OCT4* and *SOX2* loci was consistently upregulated relative to parental cells, and accompanied by variable levels of retroviral transgene expression, with silencing in some cells (Fig. 3f). These data suggest that expression of *OCT4* and *SOX2* is titrated to a specific range during selection in cell culture. There was variable but persistent expression of the retroviral *MYC* and *KLF4* transgenes (Fig. 3f). Single or multiple integrations (2–6 copies) of the *OCT4* and *SOX2* transgenes were detected by Southern blot analysis in different cell lines (Supplementary Fig. 5a, b).

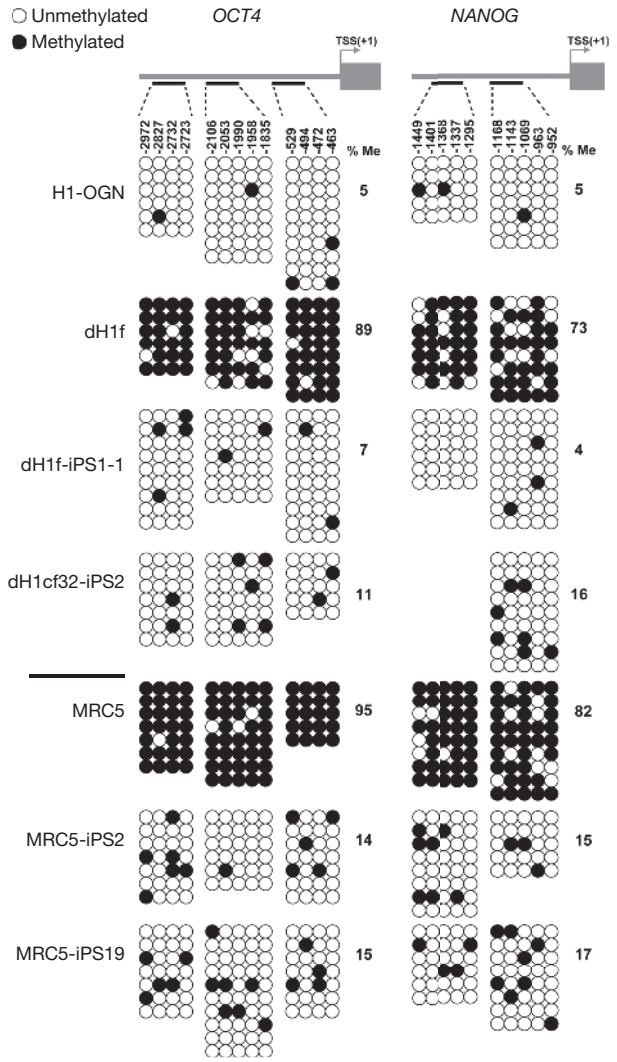

Figure 4 | iPS cells are demethylated at the *OCT4* and *NANOG* promoters relative to their fibroblast parent lines. Bisulphite sequencing analysis of the *OCT4* and *NANOG* promoters in H1-OGN human ES cells, dH1f differentiated fibroblasts, dH1f-iPS-1, dH1cf32-iPS2, as well as the MRC5 neonatal foreskin fibroblast line and its derivatives MRC5-iPS2 and MRC5-iPS19. Each horizontal row of circles represents an individual sequencing reaction for a given amplicon. White circles represent unmethylated CpG dinucleotides; black circles represent methylated CpG dinucleotides. The cell line is indicated to the left of each cluster. The values above each column indicate the CpG position analysed relative to the downstream transcriptional start site (TSS). The percentage of all CpGs methylated (% Me) for each promoter per cell line is noted to the right of each panel.

We were successful in recovering human ES-cell-like colonies from the postnatal BJ1, MSC and hFIB2 cells only when we used six factors in our retroviral cocktail (adding *hTERT* and SV40 large T to the original four factors). Although PCR analysis of genomic DNA from the bulk early post-infection cultures detected the respective retroviruses, the human ES-cell-like colonies that we ultimately isolated failed to show integration or expression of *hTERT* and SV40 large T (data not shown). We thus conclude that *hTERT* and SV40 large T are not essential to the intrinsic reprogramming of the recovered ES-cell-like cells. Because the six-factor cocktail showed a higher frequency of human ES-cell-like colony formation in all cell contexts tested (Table 1), we speculate that these factors may act indirectly on supportive cells in the culture to enhance the efficiency with which the reprogrammed colonies can be selected.

Reprogramming of somatic cells is accompanied by demethylation of promoters of critical pluripotency genes[2,15]. Therefore, we performed bisulphite sequencing to determine the extent of methylation at the *OCT4* and *NANOG* gene promoters for two parental cell lines and their reprogrammed ES-cell-like derivatives. As expected, H1-OGN human ES cells were predominantly demethylated at the *OCT4* and *NANOG* promoters. In contrast, the dH1f fibroblasts showed prominent methylation at these loci, consistent with transcriptional silencing in these differentiated cells. The ES-cell-like derivatives dH1f-iPS1-1 and dH1cf32-iPS2 revealed prominent demethylation, comparable to the state of these loci in H1-OGN human ES cells (Fig. 4, top). Similar data were obtained for MRC5 fetal lung fibroblasts, which showed prominent methylation of *OCT4* and *NANOG* loci, whereas analysis of the ES-cell-like derivatives MRC5-iPS2 and MRC5-iPS19 revealed prominent demethylation (Fig. 4, bottom). These data are consistent with epigenetic remodelling of the *OCT4* and *NANOG* promoters after retroviral infection, culture and selection for colonies with an ES-cell-like morphology.

Whereas expression analysis of a subset of genes by RT–PCR was consistent with reactivation of genes associated with pluripotency of human ES cells (Fig. 3), we performed global messenger RNA expression analysis on H1-OGN cells, parental fibroblast cells and their reprogrammed ES-cell-like derivatives. Clustering analysis revealed a high degree of similarity among the reprogrammed ES-cell-like derivatives (dH1f-iPS3-3, dH1cf16-iPS5, dH1cf32-iPS2, MRC5-iPS2 and BJ1-iPS1), which clustered together with the H1-OGN ES cells and were distant from the parental somatic cells, as determined by Pearson correlation (Fig. 5a). The differentiated dH1f and dH1cf derivatives of the H1-OGN human ES cells clustered tightly with the MRC5 fetal lung fibroblasts (Fig. 5a), suggesting their

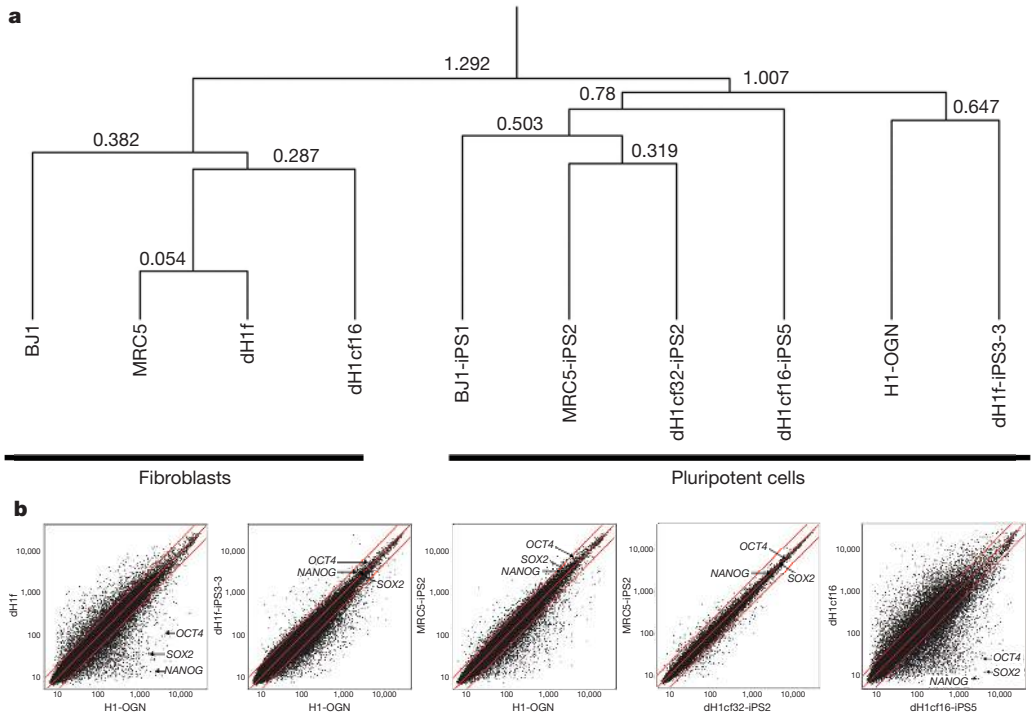

Figure 5 | Global gene expression analysis of iPS cells. a, A Pearson correlation was calculated and hierarchical clustering was performed with the average linkage method in H1-OGN, dH1f, dH1f-iPS3-3, dH1cf16, dH1cf-iPS cells (dH1cf16-iPS5 and dH1cf32-iPS2), MRC5, MRC5-iPS2, BJ1 and BJ1-iPS1 cells. The distance metric calculated by GeneSpring GX7.3.1 for comparisons between different cell lines is indicated above the tree lines. The fibroblast lines dH1f, dH1cf16, MRC5 and BJ1 cluster together, whereas iPS cells cluster together with the H1-OGN human ES cell line. **b**, Global gene expression patterns were compared between differentiated fibroblasts (dH1f, dH1cf16), reprogrammed somatic cells (dH1f-iPS3-3, MRC5-iPS2) and human ES cells (H1-OGN). Red lines indicate the linear equivalent and twofold changes in gene expression levels between the paired samples.

close resemblance to fetal fibroblasts. Analysis of scatter plots similarly shows a tighter correlation between reprogrammed somatic cells (dH1f-iPS3-3, MRC5-iPS2) and human ES cells (H1-OGN) than between differentiated fibroblasts (dH1f) and human ES cells (H1-OGN) or differentiated fibroblasts (dH1cf16) and their reprogrammed derivative (dH1cf16-iPS5) (Fig. 5b). Different lines of reprogrammed somatic cells are particularly well correlated (MRC5-iPS2 versus dH1cf32-iPS2) (Fig. 5b). Therefore, our data indicate that the cells reprogrammed from somatic sources are highly similar to embryo-derived human ES cells at the global transcriptional level.

Human ES cells will form teratoma-like masses after cell injection into immunodeficient mice, an assay that has become the accepted standard for demonstrating their developmental pluripotency[14,16,17]. We injected the human ES-cell-like cells derived from dH1f and dH1cf fibroblasts into $Rag2^{-/-}/\gamma c^{-/-}$ mice, and observed formation of well-encapsulated cystic tumours that harboured differentiated elements of all three primary embryonic germ layers (Fig. 6 and Supplementary Fig. 6). The human ES-cell-like cells derived from dH1f, dH1cf, MRC5 and MSCs differentiated *in vitro* into embryoid bodies, and RT–PCR of differentiated cells showed marker gene expression for all three embryonic germ layers: *GATA4* (endoderm), *NCAM* (ectoderm) and *Brachyury* and *RUNX1* (mesoderm; Supplementary Fig. 7). Some embryoid bodies manifest spontaneous beating, evidence of the formation of contractile cardiomyocytes with pacemaker activity[18] (data not shown). We dissociated embryoid bodies from human ES-cell-like cells derived from dH1f, dH1cf and

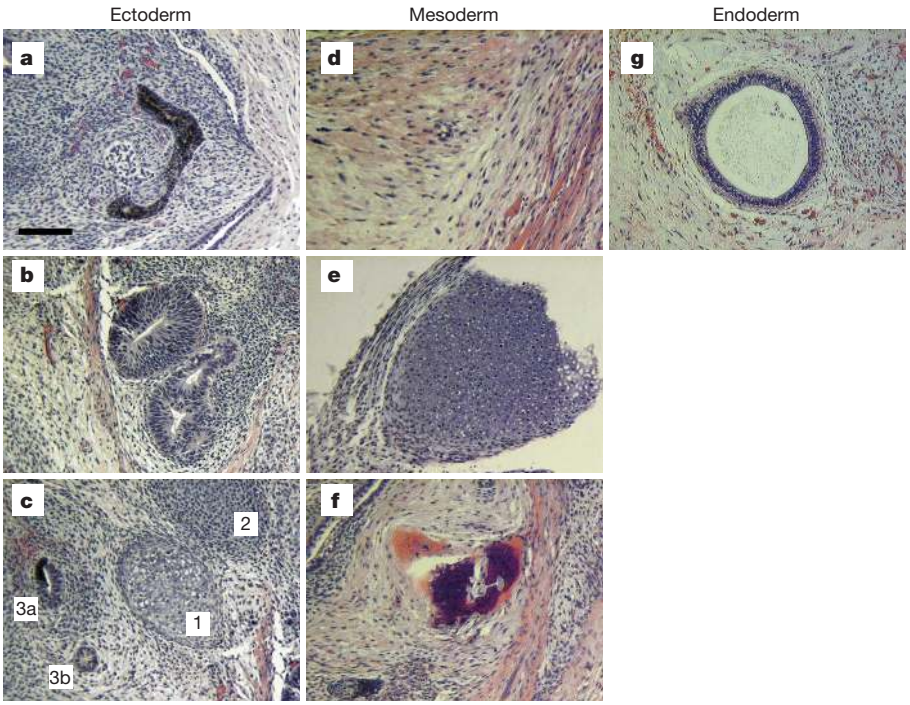

Figure 6 | Xenografts of human iPS cells generate well-differentiated teratoma-like masses containing all three embryonic germ layers. Immunodeficient mouse recipients were injected with human iPS cells (dH1f-iPS3-3) intramuscularly. Resulting teratomas demonstrate the following features in ectoderm, mesoderm and endoderm. Ectoderm: pigmented retinal epithelium (**a**), neural rosettes (**b**), glycogenated squamous epithelium (**c**); mesoderm: muscle (**d**), cartilage (**e**), bone (**f**); endoderm: respiratory epithelium (**g**). Of note, panel **c** contains all three germ layers: (1) glycogenated squamous epithelium, (2) immature cartilage, (3a) glandular tissue with surrounding stromal elements, and (3b) another small gland. All images were obtained from the same tumour. Tissue sections were stained with haematoxylin and eosin. Scale bar, 100 μm.

MSCs and plated cells in methylcellulose supplemented with haematopoietic cytokines, and detected robust formation of myeloid and erythroid colonies (Supplementary Fig. 8). Taken together, our analysis of the selected derivatives of the retrovirally infected cells suggests restoration of pluripotency. Hence, consistent with the precedent in the mouse, we labelled these cells human induced pluripotent stem (iPS) cells.

Conclusions

We observed that differentiated fibroblast derivatives of human ES cells, primary fetal tissues (lung, skin), neonatal fibroblasts and adult fibroblasts and MSCs can be reprogrammed to pluripotency using the same four genes (*OCT4*, *SOX2*, *KLF4* and *MYC*) that enable derivation of iPS cells from embryonic and adult fibroblasts in the mouse. When we eliminated single genes from the four-factor retroviral cocktail, we found that only *OCT4* and *SOX2* were essential, whereas *MYC* and *KLF4* enhanced the efficiency of colony formation (Table 1). As a significant percentage of mice carrying iPS cells develop tumours[6], eliminating these potentially oncogenic factors would be imperative before consideration of any clinical intervention with iPS cells. Taken together, our data demonstrate that OCT4, SOX2 and either MYC or KLF4 seem to be sufficient to induce reprogramming in human cells. Our data corroborate two recent reports published while this manuscript was under review[19,20]. Other combinations of factors, including novel factors, may also promote reprogramming, and indeed NANOG and LIN28 have been shown to complement OCT4 and SOX2 in reprogramming[20].

Our results establish the feasibility of reprogramming of human primary cells with defined factors, and furthermore we provide a method for obtaining, culturing and reprogramming dermal fibroblasts from adult research subjects, which should allow the establishment of human pluripotent cells in culture from patients with specific diseases for use in research. Clinical success with human iPS cells must await the development of methods that avoid potentially harmful genetic modification. Reprogramming with non-integrating virus or transient episomal gene expression, or more favourably, generation of iPS cells by biochemical means alone, is a worthy goal.

METHODS SUMMARY

Cell culture. The human ES cell line H1-OGN[8] was maintained in serum-free medium containing basic fibroblast growth factor (10 ng ml^{-1}). Differentiation medium was DMEM, 15% inactivated fetal calf serum (IFS), 1 mM Na-pyruvate, 4.5 mM monothioglycerol, 50 μg ml^{-1} ascorbic acid, 200 μg ml^{-1} iron-saturated transferrin and 50 units ml^{-1} penicillin/streptomycin. All fibroblasts were maintained in alpha-MEM, 10% IFS. Commercial fibroblast cell lines: MRC5 (from normal lung tissue of a 14-week-old male fetus; ATCC), BJ1 (neonatal foreskin; ATCC) and MSC (bone marrow mesenchymal stem cells, 33-yr-old male, Lonza).

Derivation of primary human fibroblast lines (hFib2). Primary skin fibroblasts were obtained via a 6-mm full-thickness skin punch biopsy from the volar surface of the forearm of a healthy volunteer male following informed consent (IRB and ESCRO, Children's Hospital Boston). Cultured outgrowths appeared after 7–14 days.

Retroviral production and human iPS cell induction. *OCT4*, *SOX2*, *KLF4* and *c-MYC* were introduced via the pMIG vector. SV40 large T was in pBABE-puro and *hTERT* was in pBABE-hygro (Addgene). dTomato was in lentivirus (provided by N. Geijsen). Viral infections were for 24 h and then seeded onto MEFs after 5 days. Human ES medium containing Y27632 (ref. 9) was substituted after 7 days. Chromosome counts (dH1f-iPS3-3, dH1cf32-iPS2, MRC5-iPS2, BJ-1-iPS1, BJ1-iPS3, MSC-iPS1 and hFib2-iPS1) were diploid. Karyotypes also indicated normal, diploid cells (Supplementary Fig. 9). The earliest cell line, dH1f-iPS3-3, has been in continuous culture for over 5 months (30 passages).

Bisulphite genomic sequencing. Bisulphite genomic DNA sample treatment and processing were performed simultaneously for all cell lines, with the exception of dH1f. Bisulphite conversion efficiency of non-CpG cytosines ranged from 80% to 99% for all individual clones for each sample.

Microarray analysis. RNA probes were prepared and hybridized to Affymetrix HG U133 plus 2 oligonucleotide microarrays according to the manufacturer's protocols (processed by the Biopolymer facility of Harvard Medical School). Microarrays were scanned and data was analysed using GeneSpring GX7.3.1.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Information The microarray data have been deposited in GEO and given the series accession number GSE9832. Reprints and permissions information is available at www.nature.com/reprints. Correspondence and requests for materials should be addressed to G.Q.D. (george.daley@childrens.harvard.edu).

METHODS

Cell culture. H1.1 human ES cells expressing *GFP* and *neo* integrated into the *OCT4* locus (H1-OGN[8]) were cultured in standard human ES cell culture medium (DMEM/F12 containing 20% KOSR, 10 ng ml^{-1} of human recombinant basic fibroblast growth factor, 1× NEAA, 5.5 mM 2-ME, 50 units ml^{-1} penicillin and 50 μg ml^{-1} streptomycin). H1-OGN cells were split into differentiation medium (DMEM containing 15% IFS, 1 mM sodium pyruvate, 4.5 mM monothioglycerol, 50 μg ml^{-1} ascorbic acid, 200 μg ml^{-1} iron-saturated transferrin, and 50 units ml^{-1} penicillin and 50 μg ml^{-1} streptomycin) for 4 weeks, with passaging every 3 to 4 days with 0.25% trypsin/EDTA. Differentiated fibroblasts (dH1f) and clones (dH1cf) were maintained in alpha-MEM containing 10% IFS. The following cell lines were obtained from commercial vendors and cultured in alpha-MEM containing 10% IFS: MRC5 (fibroblasts isolated from normal lung tissue of a 14-week-old male fetus; ATCC), BJ1 (neonatal foreskin fibroblast; ATCC) and MSC (mesenchymal stem cells cultured from bone marrow of a 33-yr-old male; Lonza). To form embryoid bodies, confluent undifferentiated iPS cells were mechanically scraped into strips and transferred to 6-well, low-attachment plates in differentiation medium consisting of knockout DMEM (Invitrogen) supplemented with 20% fetal bovine serum (Stem Cell Technologies), 0.1 mM non-essential amino acids (Invitrogen), 1 mM L-glutamine (Invitrogen) and 0.1 mM β-mercaptoethanol (Sigma).

Derivation of primary human fibroblast lines (hFib2). Procurement of skin tissue for use in reprogramming experiments was obtained via informed consent under a protocol approved by the Institutional Review Board and the Embryonic Stem Cell Research Oversight Committee of Children's Hospital Boston. Using sterile technique, a 6-mm full-thickness skin punch biopsy was obtained from the volar surface of the forearm of a healthy volunteer male. The biopsy was cut into 2 × 2 mm pieces. The pieces were plated in a 6-well plate and were trapped under a sterile cover slip to maintain them in place. Human fibroblast derivation media consisted of DMEM (Invitrogen), 10% FBS (Invitrogen) and penicillin/streptomycin (Invitrogen). A dense outgrowth of cells appeared after 7–14 days, which were passaged using 0.25% trypsin EDTA.

Retroviral production and human iPS cell induction. Human *OCT4*, *SOX2* and *KLF4* were cloned by inserting cDNA produced by PCR into the *Eco*RI and *Xho*I sites of the pMIG vector[21]. pMIG expressing *c-MYC* was provided by J. Cleveland[22]. SV40 large T in the pBABE-puro vector (plasmid 13970, T. Roberts) and *hTERT* in the pBABE-hygro vector (plasmid 1773, R. Weinberg) were obtained from Addgene. 293T cells in 10-cm plates were transfected with 2.5 μg of retroviral vector, 0.25 μg of VSV-G vector and 2.25 μg of Gag-Pol vector using FUGENE 6 reagents. Two days after transfection, supernatants were filtered through 0.45 μm cellulose acetate filter, centrifuged at 23,000 r.p.m. for 90 min and stored at −80 °C until use. Lentivirus expressing dTomato was provided by N. Geijsen. 1×10^5 of target somatic cells were plated in one well of a six-well plate and infected with retrovirus together with protamine sulphate. After 3 days of infection, cells were split into plates pre-seeded with mouse embryonic fibroblasts (MEFs). Medium was changed to human ES culture medium containing Y27632 7 days after infection. Chromosome counts of cell lines dH1f-iPS3-3, dH1cf32-iPS2, MRC5-iPS2, BJ-1-iPS1, BJ1-iPS3, MSC-iPS1 and hFib2-iPS1 all revealed a normal diploid number of 46. Normal karyotypes were documented for BJ1-iPS12, MRC5-iPS12 and hFib2-iPS4 (Supplementary Fig. 9). The earliest cell line derived, dH1f-iPS3-3, has been maintained in continuous cell culture for over 5 months (30 passages).

Surface antigen staining. Cells were fixed in 4% paraformaldehyde for 30 min, permeabilized with 0.2% Triton X-100 for 30 min, and blocked in 3% BSA in PBS for 2 h. Cells were incubated with primary antibody overnight at 4 °C, washed, and incubated with Alexa Fluor (Invitrogen) secondary antibody for 2 h. SSEA3, SSEA4, TRA-1-60 and TRA-1-81 antibodies were obtained from Millipore. OCT3/4 and NANOG antibodies were obtained from Abcam. Alkaline phosphatase staining was done per the manufacturer's recommendations (Millipore).

RT–PCR. RNA was isolated using an RNeasy kit (Qiagen) according to manufacturer's protocol. First-strand cDNA was primed via random hexamers and RT–PCR was performed with primer sets corresponding to Supplementary Table 1. For quantitative RT–PCR, Brilliant SYBR green was used (Stratagene).

Bisulphite genomic sequencing. Bisulphite treatment of genomic DNA (gDNA) was carried out using a CpGenome DNA Modification Kit (Chemicon) according to the manufacturer's protocol. Sample treatment and processing were performed simultaneously for all cell lines, with the exception of dH1f. Converted gDNA was amplified by PCR using *OCT4* primer sets 1, 4 and 7 (from refs 23, 24) and *NANOG* primer sets 1 and 2 (from ref. 24). PCR products were gel purified and cloned into bacteria using TOPO TA cloning (Invitrogen). Bisulphite conversion efficiency of non-CpG cytosines ranged from 80% to 99% for all individual clones for each sample.

Microarray analysis. Total RNA was isolated from cells using RNeasy kit with DNase treatment (Qiagen). RNA probes for microarray hybridization were prepared and hybridized to Affymetrix HG U133 plus 2 oligonucleotide microarrays according to the manufacturer's protocols (processed by the Biopolymer facility of Harvard Medical School). Microarrays were scanned and data were analysed using GeneSpring GX7.3.1.

Fingerprinting analysis. PCR was used to amplify across discrete genomic intervals containing highly variable numbers of tandem repeats (VNTR) in order to verify the genetic relatedness of iPS cell lines relative to their parent fibroblasts. A total of 50 ng of genomic DNA was used per reaction, cycled 35 times through 94 °C × 1 min, 55 °C × 1 min, and 72 °C × 1 min, and run on 2.5% agarose gels. Qualitative determinations were made based on differential amplicon mobility for each primer set: D10S1214, repeat $(GGAA)_n$, average heterozygosity 0.97; D17S1290, repeat $(GATA)_n$, average heterozygosity 0.84; D7S796, repeat $(GATA)_n$, average heterozygosity 0.95; and D21S2055, repeat $(GATA)_n$, average heterozygosity 0.88 (Invitrogen).

Southern hybridization. For Southern blots, gDNA was isolated using the DNeasy kit (Qiagen) according to the manufacturer's protocol, digested with *Xba*I (for dTomato), or *Spe*I and *Eco*RI (for *OCT4* and *SOX2*) and separated via agarose gel electrophoresis. Transfer to nylon membranes (Nytran Supercharge, Schleicher & Schuell Bioscience) was completed overnight in 10× SSC. Probes were labelled with ^{32}P-dCTP (Ready-to-Go DNA Labelling Beads, Amersham) and blots were hybridized (MiracleHyb, Stratagene) overnight to detect the presence of integrated viruses encoding dTomato, *OCT4*, or *SOX2*.

Assay for teratoma formation. For teratoma formation, 1×10^6 cells were resuspended in a mixture of DMEM, Matrigel and collagen (ratio of 2:1:1) and injected intramuscularly into immune-compromised *Rag2*$^{-/-}$/*γc*$^{-/-}$ mice. Xenografted masses formed within 4 to 6 weeks and paraffin sections were stained with haematoxylin and eosin for all histological determinations.

Haematopoietic colony-forming assays. Human iPS lines were differentiated for 14 days as embryoid bodies in culture media described above supplemented with SCF (300 ng ml^{-1}), Flt-3 ligand (300 ng ml^{-1}), IL-3 (10 ng ml^{-1}), IL-6 (10 ng ml^{-1}), G-CSF (50 ng ml^{-1}) and BMP4 (50 ng ml^{-1}). Embryoid bodies were disassociated and plated into methylcellulose colony-forming assay media containing SCF, GM-CSF, IL-3 and Epo (H4434, Stem Cell Technologies) at a density of 25,000 cells ml^{-1}.

Karyotype analysis. Chromosomal studies were performed at the Cytogenetics Core of the Dana-Farber/Harvard Cancer Center using standard protocols for high-resolution G-banding.

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