LETTERS

Direct reprogramming of human neural stem cells by *OCT4*

Jeong Beom Kim[1], Boris Greber[1], Marcos J. Araúzo-Bravo[1], Johann Meyer[2], Kook In Park[3], Holm Zaehres[1] & Hans R. Schöler[1]

Induced pluripotent stem (iPS) cells have been generated from mouse and human somatic cells by ectopic expression of four transcription factors (*OCT4* (also called *POU5F1*), *SOX2*, *c-Myc* and *KLF4*)[1–7]. We previously reported that *Oct4* alone is sufficient to reprogram directly adult mouse neural stem cells to iPS cells[8]. Here we report the generation of one-factor human iPS cells from human fetal neural stem cells (one-factor (1F) human NiPS cells) by ectopic expression of *OCT4* alone. One-factor human NiPS cells resemble human embryonic stem cells in global gene expression profiles, epigenetic status, as well as pluripotency *in vitro* and *in vivo*. These findings demonstrate that the transcription factor *OCT4* is sufficient to reprogram human neural stem cells to pluripotency. One-factor iPS cell generation will advance the field further towards understanding reprogramming and generating patient-specific pluripotent stem cells.

Induced pluripotent stem (iPS) cells have been generated from mouse and human somatic cells by overexpression of defined factors[1–7,9,10]. Mouse iPS cells are capable of differentiating into all three germ layers and have developmental potency of contributing to chimaeric mice and the germ line[2,3,9]. Human iPS cells are also very similar to human embryonic stem (ES) cells with regard to their differentiation potential *in vitro* and *in vivo*, which holds promise for research aimed at drug development and regenerative medicine to produce 'patient-specific iPS cells'. However, clinical applications of human iPS cells require avoiding viral transgenes, especially the oncogenes *c-Myc* and *KLF4*, from the host genome, as reactivation of the *c-myc* oncogene in mice frequently causes tumours in chimaeras and offspring derived from iPS cells[2]. It has become possible to generate iPS cells from various mouse and human somatic cells without the oncogenes *c-Myc* or *KLF4*[11–14]. Mouse neural stem cells (NSCs), which endogenously express *Sox2*, *Klf4* and *c-myc*, can be reprogrammed by two factors (*Oct4* and *Klf4*)[15] or one factor (*Oct4*)[8].

We continued to investigate human iPS cell generation from human NSCs to determine whether the single transcription factor *OCT4* alone, which is sufficient to reprogram mouse NSCs[8,15], can also reprogram human NSCs. We initially expanded human NSCs derived from human fetal brain tissue in serum-free NSC medium as previously described (Fig. 1a)[16,17]. Human NSCs were infected with retroviruses carrying human *OCT4* and *KLF4* (two factor, 2F) or *OCT4* (1F) and maintained in NSC medium for up to 7 days. Day 8 after infection, the cells were re-plated on to feeder layers in human ES cell medium containing 10 ng ml^{-1} bFGF and mouse embryonic fibroblast (MEF)-conditioned medium (CM) in a 1:1 ratio. Within 10–11 weeks after infection, we identified a colony resembling human ES cells in the *OCT4*-infected cultures. Notably, reprogrammed human NSCs did form a neural rosette in the centre of a colony (Fig. 1b). The colony grew larger, displaying typical human ES-cell-like morphology within another 5–6 days but the unreprogrammed neural rosettes still remained in the centre of the colony (Fig. 1c). Human NSCs may be reprogrammed to the pluripotent state through neural ectoderm. The neural rosettes were removed from the human ES-cell-like colony and the rest of the colony was mechanically divided and re-plated several times on to new feeder layers (passage 1 and 10, Fig. 1d, e).

We successfully established two clones out of three human ES-cell-like colonies by picking from *OCT4*-infected human NSCs (1F human NiPS clone A and C, reprogramming efficiency 0.004%), and three clones out of five human ES-cell-like colonies in *OCT4* and *KLF4*-infected human NSCs (2F human NiPS clones A, B and C, reprogramming efficiency 0.006%) within 7–8 weeks after infection. We next investigated the expression of pluripotency markers from non-transduced NSCs to work out the possibility that prolonged human ES cell culture conditions can induce pluripotency of human NSCs. These control human NSCs differentiated mostly into astrocytes and neurons (Supplementary Fig. 1a). In addition, polymerase chain reaction with reverse transcription (RT–PCR) analysis against pluripotency and neural markers demonstrated detection of neural markers, but absence of pluripotency marker expression (Supplementary Fig. 1b).

All iPS cell lines could be expanded in human ES cell culture conditions. The 1F human NiPS cells were morphologically similar to human ES cells and stained positive for alkaline phosphatase (Fig. 1e, f). Immunofluorescence staining confirmed that 2F and 1F human NiPS cells uniformly expressed human ES cell markers, including OCT4, SSEA4, TRA-1-60 and TRA-1-81 (Fig. 1g and Supplementary Fig. 2a). These results demonstrate that human iPS cells can be generated from human NSCs by *OCT4* and *KLF4* as well as by *OCT4* alone.

Next, we tested messenger RNA expression levels of pluripotency marker genes and known reprogramming factors in these iPS cells using quantitative RT–PCR (qRT–PCR). 2F and 1F human NiPS cells expressed human ES cell markers, including endogenous *OCT4* and *KLF4*, at similar levels as H9 and H1 human ES cells, whereas parental human NSCs showed endogenous expression of *SOX2* and *c-Myc* (Fig. 2a). Genotyping PCR demonstrated that 1F human NiPS cell clones only carried the *OCT4* transgene without contamination of transgenic *KLF4*, and 2F human NiPS cell clones contained the *OCT4* and *KLF4* transgenes in their genomes (Supplementary Fig. 3). We also confirmed that expression of transgenic *OCT4* or *KLF4* was significantly silenced in 2F and 1F human NiPS cell clones, except *OCT4* expression of 2F human NiPS cell clone B (Supplementary Fig. 4). Southern blot analysis confirmed three integrations of the *OCT4* transgene in 1F human NiPS cell clones and 2F human NiPS

[1]Max Planck Institute for Molecular Biomedicine, Department of Cell and Developmental Biology, Röntgenstrasse 20, 48149 Münster, NRW, Germany. [2]Hannover Medical School, Department of Experimental Hematology, Carl-Neuberg-Strasse 1, 30625 Hannover, Germany. [3]Department of Pediatrics, BK21 Project for Medical Sciences, Yonsei University College of Medicine, 250 Seongsanno, Seodaemoon-gu, Seoul 120-752, Korea.

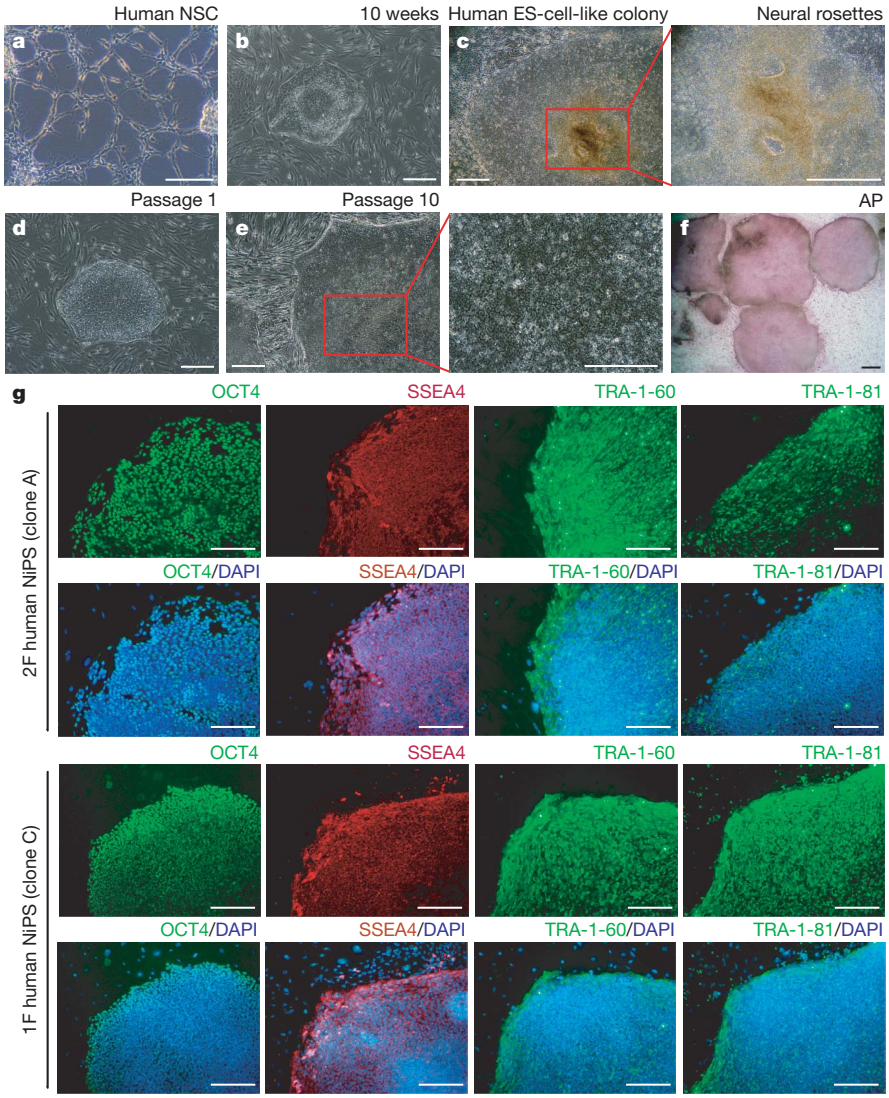

Figure 1 | 1F human NSC-derived iPS cell colony formation and cell line characterization. **a**, Morphology of human NSCs grown in NSC medium. **b**, Colony formation of human *OCT4*-infected cells 10 weeks after infection. **c**, The colony shows human ES-cell-like morphology but unreprogrammed neural rosettes remain in its centre. **d**, Typical human ES-cell-like iPS colony growing on a feeder layer after mechanical isolation at passage 1 (1F human NiPS clone C). **e**, High magnification of iPS colony at passage 10. **f**, 1F human NiPS colonies were stained for alkaline phosphatase (AP). Scale bars, 250 μm. **g**, Immunocytochemical analysis of pluripotency and surface markers (OCT4, SSEA4, TRA-1-60 and TRA-1-81) in 2F human NiPS (clone A) and 1F human NiPS (clone C) cells. Nuclei are stained with DAPI (blue). Scale bars, 250 μm.

cell clones (Supplementary Fig. 5). To exclude the possibility that iPS cell clones arose from human ES cell contamination in the laboratory, DNA fingerprinting analysis confirmed the genetic identity between human NiPS cells and donor human NSCs (Supplementary Table 1). To evaluate chromosomal abnormalities of human NiPS cells, we performed cytogenetic analysis by metaphase spread. 1F and 2F human NiPS cells maintained normal karyotype (46, XY) during the whole reprogramming period (Supplementary Fig. 6).

To confirm epigenetic remodelling in reprogrammed cells, we performed bisulphite sequencing analysis to determine the degree of DNA methylation of the *OCT4* and *NANOG* promoters (Fig. 2b). Similar to human ES cells, both promoter regions were demethylated in 2F and 1F human NiPS cells relative to the donor human NSCs. Taken together, a single transcription factor, *OCT4*, can reprogram human NSCs into iPS cells that are very similar to human ES cells at the molecular level.

Next, we tested *in vitro* pluripotency of 2F and 1F human NiPS cells by embryoid body (EB) differentiation and directed differentiation. During EB differentiation, human NiPS cells readily differentiated into vesicular structures with a variety of cell types through hanging drops including endoderm (AFP), mesoderm (α-SMA) and ectoderm (TUJ1) (Fig. 3a and Supplementary Fig. 2b). To enhance the differentiation towards all three germ layers, human NiPS cells were cultured by a directed differentiation protocol (see Methods). We then additionally confirmed the expression of markers for all three germ layers by qRT–PCR analysis (Fig. 3b).

To evaluate *in vivo* pluripotency of these human iPS cells, they were subcutaneously transplanted into severe combined immunodeficient (SCID) mice. Six-to-eight weeks after injection, 2F and 1F human NiPS cells gave rise to teratomas containing derivatives of all three germ layers, including respiratory epithelium, skeletal muscle, cartilage and neural epithelium (Fig. 4a and Supplementary Fig. 7). These results indicate that, like human ES cells, 2F and 1F human NiPS cells are pluripotent *in vitro* and *in vivo*.

Finally, we performed global gene expression analysis on human NSCs, 1F and 2F human NiPS cells derived from human NSCs, as well as H9 and H1 human ES cells using oligonucleotide microarrays. A heat map shows that the global 1F and 2F human NiPS cell expression profiles were overall similar to those in human ES cells, and different from parental human NSCs (Fig. 4b, left panel). Consistent with this, hierarchical clustering analysis showed that 1F and 2F human NiPS cells clustered closely with human ES cells and were distinct from

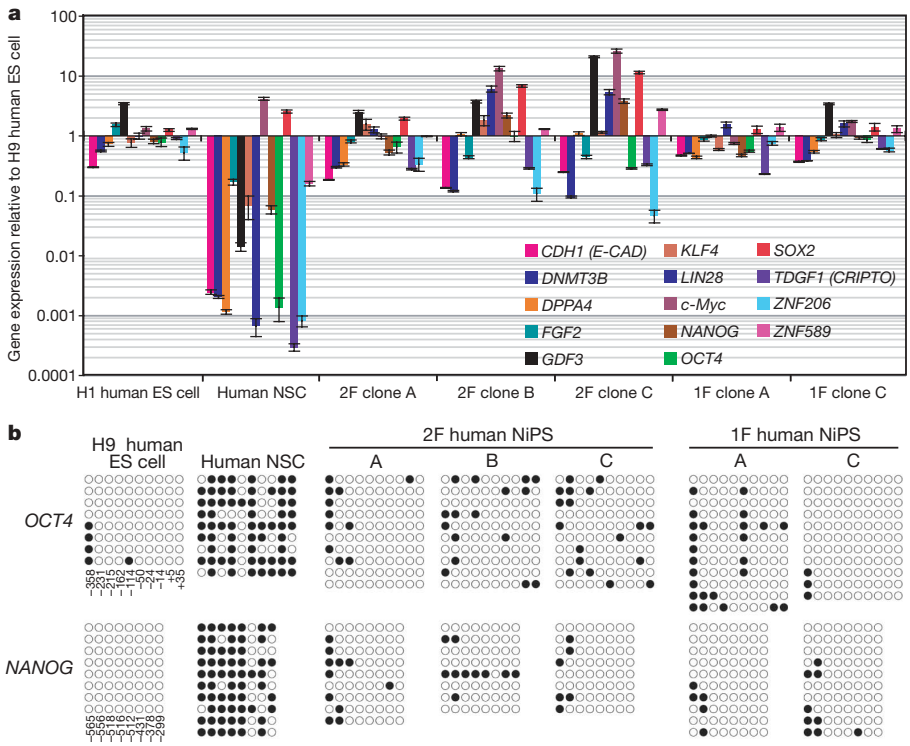

Figure 2 | Expression levels of pluripotent markers and DNA methylation analysis in human NSC-derived iPS cells. a, Quantitative PCR analysis for pluripotent markers in H1 human ES cells, human NSCs, 2F human NiPS clones (A, B and C) and 1F human NiPS clones (A and C). The expression levels are relative to H9 human ES cells using primers specific for endogenous transcripts, and on the logarithmic scale. Transcript levels were normalized to β-actin expression. Error bars indicate standard errors from duplicate samples ($n = 2$). **b**, Bisulphite sequencing analysis of *OCT4* and *NANOG* promoter regions in H9 human ES cells, human NSCs, 2F human NiPS clones (A, B and C) and 1F human NiPS clones (A and C). Each row of circles for a given amplicon represents the methylation status of CpG dinucleotides in one bacterial clone for that region. Open circles represent unmethylated CpGs; closed circles represent methylated CpGs. Bottom numbers of each column indicate CpG locations relative to the translational start site.

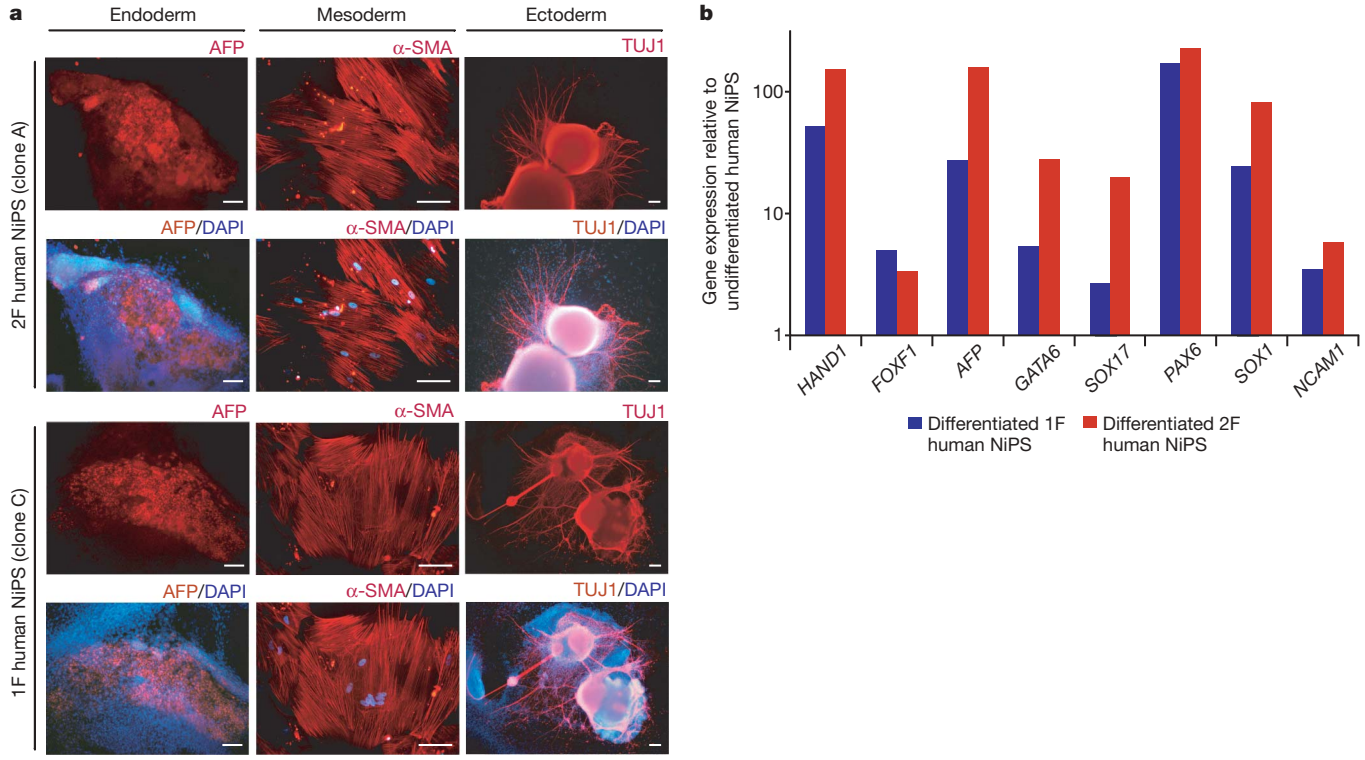

Figure 3 | *In vitro* differentiation of human NSC-derived iPS cells into all three germ layers. a, Immunofluorescence analysis of 2F human NiPS (clone A) and 1F human NiPS (clone C) derived cells of all three germ layers after EB differentiation: endoderm (α-fetoprotein; AFP), mesoderm (α-smooth muscle actin; α-SMA) and ectoderm (β-tublin lllb; TUJ1). Nuclei are stained with DAPI (blue). Scale bars, 100 μm. **b**, Quantitative PCR analyses of all three germ layer markers from differentiated 2F human NiPS (clone A) and 1F human NiPS (clone C) cells after directed differentiation: mesoderm (*HAND1*, *FOXF1*), endoderm (*AFP*, *GATA6*, *SOX17*) and ectoderm (*PAX6*, *SOX1*, *NCAM1*). Data denote β-actin-normalized fold changes relative to undifferentiated parental human NiPS cells.

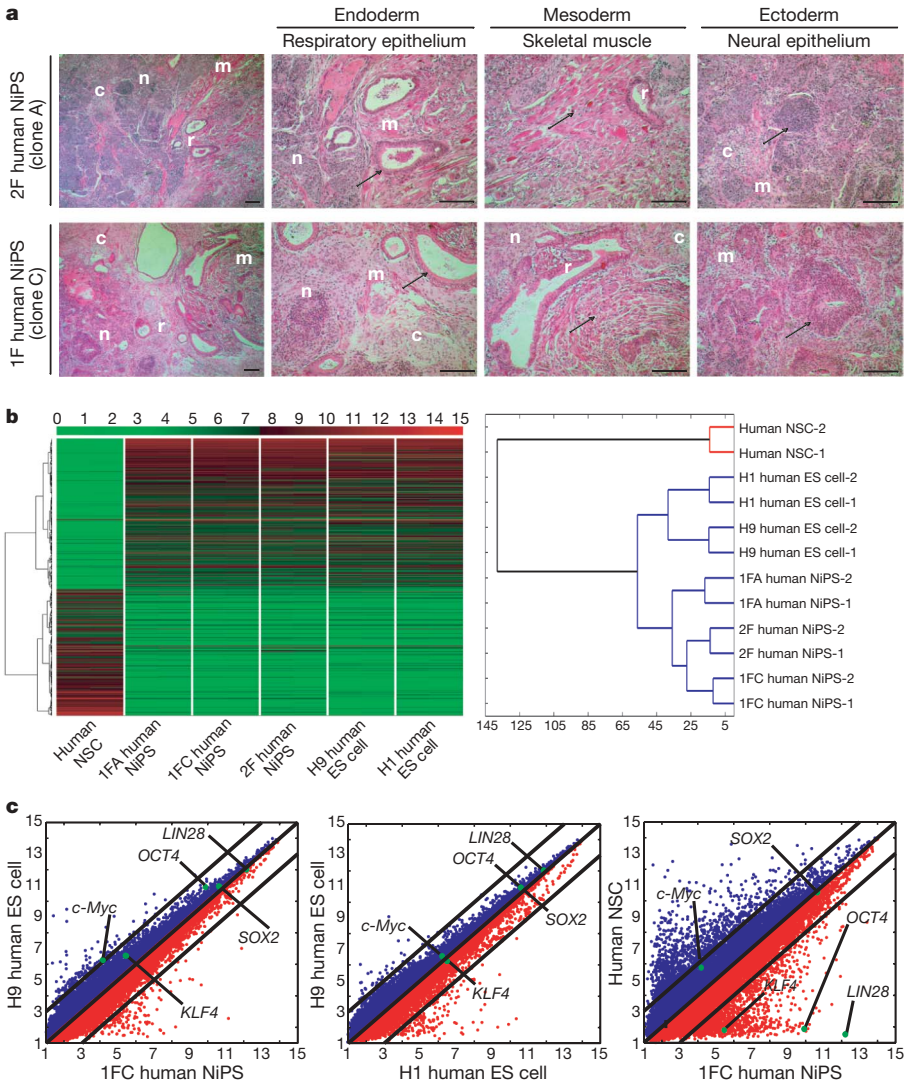

Figure 4 | *In vivo* pluripotency and global gene expression profile of human NSC-derived iPS cells. **a**, Teratoma formation after 6–8 weeks transplantation of 2F human NiPS (clone A) and 1F human NiPS (clone C) cells into SCID mice. Teratomas were sectioned and stained with haematoxylin and eosin at 6–8 weeks. Histological section of identified cells representing all three germ layers: endoderm (respiratory epithelium, r), mesoderm (skeletal muscle, m; cartilage, c) and ectoderm (neural epithelium, n). Enlargements of sections showing tissues are indicated by arrows. Scale bars, 100 μm. **b**, Heat map (left panel) and hierarchical cluster analysis (right panel) of global gene expression from human NSCs, 1F human NiPS (clone A and clone C), 2F human NiPS (clone A), H9 human ES cells and H1 human ES cells. A colour bar (top) indicates the colour code gene expression in $\log_2$ scale. The abscissa numbers in the hierarchical cluster correspond with the standardized Euclidean distance. **c**, Scatter plots comparing global gene expression profiles between H9 human ES cells and 1F human NiPS cells (clone C) (left panel), H9 human ES cells and H1 human ES cells (middle panel), and human NSCs and 1F human NiPS cells (clone C) (right panel). The black lines indicate twofold differences in gene expression levels between the paired cell populations. The transcript expression levels are on the $\log_2$ scale.

parental human NSCs (Fig. 4b, right panel). Scatter plot analysis demonstrated that the global expression differences between 1F human NiPS cells (clone C) and human ES cells were in the same range as those between two different human ES cell lines (H9 versus H1) (Fig. 4c). 1F (clone A) and 2F (clone A) human NiPS cells also showed high similarity with H9 and H1 human ES cells (Supplementary Fig. 8). Hence, global gene expression profiles revealed a high degree of similarity between 1F and 2F human NiPS cells and human ES cells.

Our study demonstrates the generation of one-factor (*OCT4*) and two-factor (*OCT4* and *KLF4*) human iPS cells that resemble human ES cells at the molecular level and with respect to their differentiation potential. The present study extends our findings on the feasibility of reprogramming from mouse to human NSCs with OCT4 alone. It demonstrates that endogenous expression of the reprogramming factors can complement exogenously added factors[8,15]. The feasibility to reprogram directly NSCs by *OCT4* alone might reflect their higher similarity in transcriptional profiles to ES cells than to other stem cells like haematopoietic stem cells or to their differentiated counterparts[18].

So far, small molecule approaches can be used to compensate for viral transduction of some of the four factors when the other factors are transduced[19]. Direct delivery of recombinant proteins also facilitated generation of human iPS cells[20]. Future studies will show if direct reprogramming is possible with small molecules or OCT4 recombinant protein alone. Knowing that specifically *Oct4* needs to be activated in NSCs might facilitate such an approach. It will be interesting to extend this study to human NSCs derived from other sources, such as dental pulp[21,22], as well as to other stem-cell types.

METHODS SUMMARY

Human NSCs were derived from fetal tissue and transduced with retroviral vectors expressing human OCT4 and OCT4 plus KLF4 as previously described[8,15,16,23]. The cells were re-plated on mouse feeder layers under human ES cell culture conditions until human ES-cell-like colonies could be mechanically isolated and further cultivated. Human iPS cells were characterized by

immunostaining, karyotyping, qRT–PCR, gene expression profiling by microarray, bisulphite sequencing, Southern blot analysis and short tandem repeat analysis. *In vitro* differentiation by EB formation and teratoma formation in SCID mice was used to measure the developmental potential of the human iPS cells.

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

Received 2 April; accepted 21 August 2009.
Published online 28 August 2009.

1. Takahashi, K. & Yamanaka, S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* **126,** 663–676 (2006).
2. Okita, K., Ichisaka, T. & Yamanaka, S. Generation of germline-competent induced pluripotent stem cells. *Nature* **448,** 313–317 (2007).
3. Wernig, M. *et al. In vitro* reprogramming of fibroblasts into a pluripotent ES-cell-like state. *Nature* **448,** 318–324 (2007).
4. Takahashi, K. *et al.* Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* **131,** 861–872 (2007).
5. Yu, J. *et al.* Induced pluripotent stem cell lines derived from human somatic cells. *Science* **318,** 1917–1920 (2007).
6. Park, I. H. *et al.* Reprogramming of human somatic cells to pluripotency with defined factors. *Nature* **451,** 141–146 (2008).
7. Lowry, W. E. *et al.* Generation of human induced pluripotent stem cells from dermal fibroblasts. *Proc. Natl Acad. Sci. USA* **105,** 2883–2888 (2008).
8. Kim, J. B. *et al.* Oct4-induced pluripotency in adult neural stem cells. *Cell* **136,** 411–419 (2009).
9. Maherali, N. *et al.* Directly reprogrammed fibroblasts show global epigenetic remodeling and widespread tissue contribution. *Cell Stem Cell* **1,** 55–70 (2007).
10. Dimos, J. T. *et al.* Induced pluripotent stem cells generated from patients with ALS can be differentiated into motor neurons. *Science* **321,** 1218–1221 (2008).
11. Nakagawa, M. *et al.* Generation of induced pluripotent stem cells without Myc from mouse and human fibroblasts. *Nature Biotechnol.* **26,** 101–106 (2008).
12. Aoi, T. *et al.* Generation of pluripotent stem cells from adult mouse liver and stomach cells. *Science* **321,** 699–702 (2008).
13. Wernig, M., Meissner, A., Cassady, J. P. & Jaenisch, R. c-Myc is dispensable for direct reprogramming of mouse fibroblasts. *Cell Stem Cell* **2,** 10–12 (2008).
14. Huangfu, D. *et al.* Induction of pluripotent stem cells from primary human fibroblasts with only *Oct4* and *Sox2*. *Nature Biotechnol.* **26,** 1269–1275 (2008).
15. Kim, J. B. *et al.* Pluripotent stem cells induced from adult neural stem cells by reprogramming with two factors. *Nature* **454,** 646–650 (2008).
16. Park, K. I., Teng, Y. D. & Snyder, E. Y. The injured brain interacts reciprocally with neural stem cells supported by scaffolds to reconstitute lost tissue. *Nature Biotechnol.* **20,** 1111–1117 (2002).
17. Kim, H. T. *et al.* Human neurospheres derived from the fetal central nervous system are regionally and temporally specified but are not committed. *Exp. Neurol.* **199,** 222–235 (2006).
18. Ramalho-Santos, M., Yoon, S., Matsuzaki, Y., Mulligan, R. C. & Melton, D. A. ''Stemness'': transcriptional profiling of embryonic and adult stem cells. *Science* **298,** 597–600 (2002).
19. Shi, Y. *et al.* A combined chemical and genetic approach for the generation of induced pluripotent stem cells. *Cell Stem Cell* **2,** 525–528 (2008).
20. Kim, D. *et al.* Generation of human induced pluripotent stem cells by direct delivery of reprogramming proteins. *Cell Stem Cell* **4,** 472–476 (2009).
21. Seo, B. M. *et al.* Investigation of multipotent postnatal stem cells from human periodontal ligament. *Lancet* **364,** 149–155 (2004).
22. Widera, D. *et al.* Adult palatum as a novel source of neural-crest related stem cells. *Stem Cells* **27,** 1899–1910 (2009).
23. Zaehres, H. & Daley, G. Q. Transgene expression and RNA interference in embryonic stem cells. *Methods Enzymol.* **420,** 49–64 (2006).

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

Acknowledgements We thank B. Schäfer for histology, T. Kitamura for the pMX retroviral vector and A. Malapetsas and S. Kölsch for editing. This work has been supported, in part, by a grant from the German Federal Ministry of Education and Research BMBF 01GN0811 and in part by a grant by the Deutsche Forschungsgemeinschaft DFG SCHO 340/4-1.

Author Contributions J.B.K., project design, generation and characterization of iPS cells, preparation of manuscript; B.G., M.J.A.-B., J.M., characterization of iPS cells; K.I.P., providing human NSCs; H.Z., project design, generation of iPS cells, preparation of manuscript; H.R.S., project design, preparation of manuscript.

Author Information The microarray data sets are available from GEO (Gene Expression Omnibus) under accession number GSE15355. Human NiPS cells are in the process of being deposited in the UK Stem Cell Bank. Reprints and permissions information is available at www.nature.com/reprints. Correspondence and requests for materials should be addressed to H.R.S. (office@mpi-muenster.mpg.de).

METHODS

Cell culture. Human fetal tissue from cadavers at 13 weeks of gestation was obtained with full patient consent and the approval of the research ethics committee of Yonsei University College of Medicine, Seoul, Korea. The methods of acquisition conformed to NIH and Korean Government guidelines. Human NSCs were derived from the telencephalon (HFT13) as previously described[17]. Briefly, telencephalon tissue was freshly dissected, dissociated in 0.1% trypsin for 30 min and seeded into 10 cm plates at a density of 200,000 cells ml^{-1} in NSC medium. These cells were cultured in NSC medium as previously described[16,17]. Human ES and iPS cells were maintained on mitomycin-C-treated CF1 mouse feeder layers (Millipore) in human ES cell medium, which contains knockout DMEM (Invitrogen) supplemented with 20% knockout serum replacement (Invitrogen), 1 mM L-glutamine, 1% non-essential amino acids, 0.1 mM β-mercaptoethanol, penicillin/streptomycin and 10 ng ml^{-1} human basic fibroblast growth factor (bFGF) (Invitrogen) as previously described[24].

Induction of 1F and 2F human NiPS cells. The pMX-based retroviral vectors encoding the human cDNAs of *OCT4* and *KLF4* were co-transfected with packaging-defective helper plasmids into 293 cells using Fugene transfection reagent (Roche) to produce vesicular stomatitis virus (VSV) G protein pseudotyped virus as previously described[4,23]. Viral supernatants were collected and concentrated by ultracentrifugation 48 h after transfection to infect human NSCs. For generation of iPS cells, human NSCs were seeded at a density of 5×10^4 cells per 6-well plate and incubated with virus-containing supernatants for *OCT4* or *OCT4* and *KLF4* supplemented with 6 μg ml^{-1} protamine sulphate (Sigma) for 24 h. The next day, the medium was replaced with fresh NSC medium at day 1 after infection and maintained up to day 7 after infection. Cells were further cultured in human ES cell medium from day 8 after infection. The iPS colonies were mechanically isolated at 2 months or 2.5 months after infection and were subsequently re-plated and maintained on CF1 mouse feeder layers (Millipore) in human ES cell medium.

qRT–PCR. Total RNA was isolated from bulk cell culture samples or hand-picked undifferentiated colonies using RNeasy columns (Qiagen) with on-column DNA digestion. cDNA was produced using oligo-dT_{15} priming and M-MLV reverse transcriptase (USB) according to the manufacturer's instructions at 42 °C for 1 h. About 50 ng of total RNA equivalent was typically used as template in 20 μl SYBR green PCR reactions (40 cycles of 15 s, 95 °C/60 s, 60 °C on Applied Biosystems 7300 instrumentation) that additionally contained 0.375 μM of each primer and 10 μl of SYBR green PCR mix (ABI). All primers used were confirmed to amplify the predicted product at close-to-optimal efficiency without side products. Primer sequences are given in Supplementary Table 2. Relative expression levels were calculated using the comparative C_t method, based on biological control samples and two housekeeping genes for normalization. Error bars reflect standard errors arising from biological replicates (marker gene expression data) or from using independent housekeeping genes for normalization (transgene silencing data).

Global gene expression analysis. For transcriptome profiling, 400 ng of total DNA-free RNA was used as input for labelled cRNA synthesis (Illumina TotalPrep RNA Amplification Kit, Ambion) following the manufacturer's instructions (IVT: 10 h). Quality-checked cRNA samples were hybridized as biological or technical duplicates for 18 h onto HumanRef-8 v3 expression BeadChips (Illumina), washed, stained and scanned following guidelines using materials/instrumentation supplied/suggested by the manufacturer. The microarray data are available from the GEO (Gene Expression Omnibus) website under accession number GSE15355.

Microarray data processing. The bead intensities were mapped to gene information using BeadStudio 3.2 (Illumina), background correction was performed using the Affymetrix Robust Multi-array Analysis (RMA) background correction model, variance stabilization was performed using the $\log_2$ scaling, and gene expression normalization was calculated with the quantile method implemented in the lumi package of R-Bioconductor. Data post-processing and graphics were performed with in-house developed functions in Matlab. Hierarchical clusters of genes and samples were performed with a standardized Euclidean metric and the Ward's linkage method.

Bisulphite sequencing. Genomic DNA was isolated from bulk cell culture samples or hand-picked undifferentiated colonies using DNeasy columns (Qiagen). A total of 300 ng was used as input for bisulphite conversion (EpiTect Bisulfite Kit, Qiagen). A total of 50 ng of converted DNA was used as a template for conventional nested PCRs amplifying 467- and 336-bp regions of the *OCT4* and *NANOG* promoters, respectively. Primers were specific for conversion of the sense DNA strand and are given in Supplementary Table 2. Purified PCRs were TA-cloned into pCR2.1-TOPO (Invitrogen). Insert sequences of randomly picked clones were analysed using the BiQ Analyser program, following its quality-check-based suggestions to drop individual clones if appropriate. Data from one CpG site at position +20 relative to the *OCT4* translation start codon is not shown as it was uninformative.

Teratoma formation. Human NiPS cells and human NSCs ($3–5 \times 10^6$ cells per mouse) were injected subcutaneously into the dorsal flank of SCID mice. Teratomas were fixed in 4% PFA overnight and embedded in paraffin after 6–8 weeks injection. Sections were stained with haematoxylin and eosin dyes.

Alkaline phosphatase and immunofluorescence staining. Alkaline phosphatase staining was performed with the ES Cell Characterization Kit (Chemicon) according to the manufacturer's protocol. Immunofluorescence staining was performed using the following primary antibodies: AFP (Sigma, 1:100), α-SMA (Sigma, 1:50), TUJ1 (Chemicon, 1:500), OCT4 (Santa Cruz, 1:200), SSEA4 (Chemicon, 1:200), TRA-1-60 (Chemicon, 1:200) and TRA-1-81 (Chemicon, 1:200).

***In vitro* differentiation of human iPS cells.** For immunocytochemistry, embryoid bodies (EBs) were generated from iPS cells with the hanging-drop method in MEF-conditioned medium. After 5 days, EBs were transferred to gelatin-coated plates and subsequently cultured for another 14 days in knockout DMEM (Invitrogen) supplemented with 20% FBS, 1 mM L-glutamine, 1% non-essential amino acids, 0.1 mM β-mercaptoethanol, and penicillin/streptomycin. For qRT–PCR, iPS colonies were mechanically isolated and re-plated on Matrigel-coated plates in MEF-conditioned medium. After 2 days, medium was replaced with medium for each of the three germ layers. Endodermal differentiation: RPMI1640 medium supplemented with 2% FBS, 100 ng ml^{-1} activin A (R&D Systems), L-glutamine and penicillin/streptomycin for 3 weeks[14]. For mesodermal differentiation: knockout DMEM supplemented with 100 μM ascorbic acid (Sigma), 20% FBS, 1 mM L-glutamine, 1% non-essential amino acids, 0.1 mM β-mercaptoethanol and penicillin/streptomycin for 3 weeks[25]. For ectodermal differentiation: the cells were maintained in N2B27 medium for 7 days and the medium replaced with N2 medium supplemented with 10 ng ml^{-1} bFGF2 (peprotech), 100 ng ml^{-1} Sonic Hedgehog (R&D Systems), 10 ng ml^{-1} PDFG (R&D Systems), L-glutamine and penicillin/streptomycin for 2 weeks. The medium was changed every other day. Primer sequences are given in Supplementary Table 2.

24. Thomson, J. A. *et al.* Embryonic stem cell lines derived from human blastocysts. *Science* **282**, 1145–1147 (1998).
25. Aasen, T. *et al.* Efficient and rapid generation of induced pluripotent stem cells from human keratinocytes. *Nature Biotechnol.* **26**, 1276–1284 (2008).