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Single Transcription Factor Reprogramming of Hair Follicle Dermal Papilla Cells to Induced Pluripotent Stem Cells

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ABSTRACT

Reprogramming patient-specific somatic cells into induced pluripotent stem (iPS) cells has great potential to develop feasible regenerative therapies. However, several issues need to be resolved such as ease, efficiency, and safety of generation of iPS cells. Many different cell types have been reprogrammed, most conveniently even peripheral blood mononuclear cells. However, they typically require the enforced expression of several transcription factors, posing mutagenesis risks as exogenous genetic material. To reduce this risk, iPS cells were previously generated with Oct4 alone from rather inaccessible neural stem cells that endogenously express the remaining reprogramming factors and very recently from fibroblasts with Oct4 alone in combination with additional small molecules. Here, we exploit that dermal papilla (DP) cells from hair follicles in the skin express all but one reprogramming factors to show that these accessible cells can be reprogrammed into iPS cells with the single transcription factor Oct4 and without further manipulation. Reprogramming was already achieved after 3 weeks and with efficiencies similar to other cell types reprogrammed with four factors. Dermal papilla-derived iPS cells are comparable to embryonic stem cells with respect to morphology, gene expression, and pluripotency. We conclude that DP cells may represent a preferred cell type for reprogramming accessible cells with less manipulation and for ultimately establishing safe conditions in the future by replacing Oct4 with small molecules. STEM CELLS *2011;29:964–971*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Embryonic stem (ES) cells are pluripotent cells derived from the inner cell mass of preimplantation stage embryos that can give rise to every cell type of the adult [1, 2]. The derivation and functional characterization of human ES cells have made them a powerful tool for regenerative medicine. However, immune rejection of transplanted cells and ethical concerns are critical issues in the application of human ES cells for clinical therapy. Remarkably, the generation of induced pluripotent stem (iPS) cells through direct reprogramming of somatic cells by defined transcription factors, typically Oct4, Sox2, Klf4, and c-Myc, opened a new avenue to avoid the controversy of using human ES cells [3, 4]. On the other hand, low reprogramming efficiency, random viral integration, and the use of oncogenic reprogramming factors (Klf4 and c-Myc) remain major challenges in the generation of iPS cells for regenerative medicine applications [5]. Kim et al. [6] recently demonstrated that mouse neural stem cells, which endogenously express Sox2, Klf4, and c-Myc, can be reprogrammed by either two factors (Oct4 and Klf4) or solely by one factor (Oct4) [7]. While this is a major advance toward identifying cells that can be reprogrammed more easily with less manipulation, the difficulty of isolating neural stem cells from patients makes these cells an impractical source for patient-specific derivation of iPS cells. In efforts to reprogram accessible cells, such as skin fibroblasts, with less factors, small molecule activators and inhibitors of epigenetic regulation and signaling pathways were used to replace individual reprogramming transcription factors [8, 9]. Most recently, skin-derived and mouse embryonic fibroblasts (MEFs) were successfully reprogrammed with Oct4 with simultaneous treatment of additional small molecules [9–11].

We have already identified several years ago that skin dermal papilla (DP) cells, a specialized mesenchymal cell type required for instructing epithelial stem cells during hair morphogenesis and regeneration [12], also express three of the four reprogramming factors, Sox2, c-Myc, and Klf4 [13]. Toward the goal of establishing a source of accessible somatic cells that can be more easily reprogrammed with less exogenous genetic material and without further manipulation, we

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recently demonstrated that DP cells can be reprogrammed into iPS cells by only two exogenous reprogramming factors, Oct4 and Klf4 [14]. Interestingly, we further found that DP cells have a much higher reprogramming efficiency compared to most other cell types [14].

In this study, we succeeded to reprogram accessible DP cells into iPS cells by transducing the single transcription factor Oct4 in the absence of additional modifiers of epigenetic regulation. Reprogramming DP cells with Oct4 alone occurred quite rapidly starting at 3 weeks and with efficiencies similar to most other cell types when they are reprogrammed with all four factors. Dermal papilla–derived one-factor iPS cells fulfil all pluripotency criteria, as determined by pluripotency gene expression, by their capacity to differentiate into cell types from all germ layers in vitro and by their robust contribution to the development of chimeric mice and to the germline in vivo. This suggests that hair follicle DP cells represent an accessible source of cells that can be reprogrammed into pluripotent stem cells with a single transcription factor. Therefore, DP cells may be a preferred cell type in the future, with which safe reprogramming may be accomplished without any exogenous genetic material.

Materials and Methods

Isolations of DP Cells

For preparations of DP cells, backskins of 4–6 days old Lef1-RFP/Oct4-GFP/Rosa26-LacZ pups were floated on dispase (Invitrogen, Carlsbad, CA, www.invitrogen.com) overnight at 4°C to separate epidermis from dermis. The dermis was then digested with 0.2% collagenase (Sigma-Aldrich, St. Louis, MO, www.sigmaaldrich.com) at 37°C for 40–60 minutes on an orbital shaker. Intact follicles and dermal cells were centrifuged at 300*g*, after which follicles were enriched at low speed centrifugation twice at 20*g*. Following trypsinization (0.25% trypsin/0.05 mM EDTA, Invitrogen) at 37°C for 5 min, single-cell suspensions were strained through 40 μm filters and pelleted at 300*g*. For DP cell isolations by fluorescence-activated cell sorting (FACS; BD Vantage and DAKO-Cytomation MoFlo sorters were used), hair cell suspensions were first stained for integrin α-9 (Itga9, 1:100, goat, R&D Systems, Minneapolis, MN, www.rndsystems.com) and donkey anti-goat APC-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA, www.jacksonimmuno.com 1:200) and then depleted for melanocytes (CD117) and endothelial cells (CD34) with biotinylated antibodies (BD Pharmingen, San Diego, CA, www.bdbiosciences.com) and magnetic anti-biotin microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany, www.miltenyibiotec.com) as previously described [14]. DP cells were selected as the RFP/APC-double positive cell population. For DP isolation under wild-type conditions, hair follicle cell suspensions were only stained with Itga9 as described above and FACS sorted them as the APC-positive population. All animal experiments were performed in accordance with the guidelines and approval of the Institutional Animal Care and Use Committee at Mount Sinai School of Medicine.

Production of Retrovirus, Infection, and Cell Culture

Retrovirus were produced and concentrated as previously described [14]. Briefly, Phoenix cells were plated at 18×10^6 cells per 150 mm dish. The next day, cells were transfected with pMX-based retroviral vectors encoding the human cDNA of Oct4, Sox2, Klf4, and c-Myc (kindly provided by the Christoph Schaniel/Lemischka Lab) by a standard $CaCl_2$ transfection method. Phoenix cells were cultured at 32°C 24 hours after transfection and virus supernatants were collected after additional 24, 48, and 72 hours, passed through 0.22-μm pore size filters (Millipore, Billerica, MA, www.millipore.com) and pelleted at 50,000*g* for 3 hours. Viruses were resuspended in 1× phosphate-buffered saline (PBS) buffer at ~200× concentration of the original volume and stored as aliquots at –80°C. DP cells were cultured in Amniomax C-100 medium (Invitrogen). For infections, freshly sorted DP cells were plated in 12-well plates and cultured until they reached 50%–60% confluence. Oct4 virus and 4 μg/ml of polybrene (Sigma) were added to the cells. Infection was enhanced by spinning the plates at 1,100*g* for 30 minutes. Two rounds of infections were performed within 24 hours, and 1 day after the second infection the medium was replaced by ES medium. The following day, DP cells were counted and seeded on irradiated MEFs in ES medium without any further selection. Oct4-green fluorescent protein (GFP)-positive colonies were picked and trypsin dissociated and replated onto irradiated MEFs in 96-well plates. All iPS cell lines and control Ainv15 ES cells (kind gift from Christoph Schaniel/Lemischka Lab) were cultured as previously described [14]. Before RNA and genomic DNA purification, iPS cells were depleted of feeder cells for two passages on 0.1% gelatin (Fisher Scientific International, Hampton, NH, www.fisherscientific.com).

Determination of iPS Cell Reprogramming Efficiency

The efficiency of formation of iPS cells is based on the number of Oct4-GFP-positive iPS cell colonies and the initial cell number of plated DP cells. The percentage of efficiency was determined by dividing the number of GFP-positive colonies by the number of cells before seeding on feeders.

RNA Isolation and Real-Time PCR

Total RNAs from FACS-sorted cells, cultured DP cells, ES cells, iPS cells, and all other cells in this study were purified using the Absolutely RNA Microprep kit (Stratagene, La Jolla, CA, www.stratagene.com), quantified with the NanoDrop spectrophotometer (Thermo Scientific, Pittsburgh, PA, www.thermo.com), and reverse transcribed (Superscript III First-Strand Synthesis System, Invitrogen) using oligo(dT) primers. Polymerase chain reactions (PCRs) were performed with a C1000 thermal cycler (Bio-Rad, Hercules, CA, www.bio-rad.com) at: 3 min at 94°C initial denaturing, 32–35 cycles of 15-second at 94°C denaturing, 30-second at 60°C annealing and 25-second at 72°C extension. Real-time PCR was run with a LightCycler 480 (Roche, Basel, Switzerland, www.roche.com) instrument with Lightcycler DNA master SYBR Green I reagents. Differences in gene expression between samples were calculated based on the $2^{-\Delta\Delta CT}$ method and normalized to GAPDH. Measurements were performed in duplicate. Primer sequences are listed in Supporting Information Table S2.

Immunofluorescence Staining

iPS cell and control ES cell cultures were fixed with 4% PFA in PBS (pH 7.4) after culture on LabTek eight-well chamber slides (Nalge Nunc International, Rochester, NY, www.nalgenunc.com) on MEF feeders, incubated in MOM blocking solution (Vector Laboratories, Burlingame, CA, www.vectorlabs.com) for 30 minutes, and stained with antibodies against Nanog (1:50, Abcam, Cambridge, United Kingdom, www.abcam.com), Oct4 (1:50, Santa Cruz Biotechnology, Santa Cruz, CA, www.scbt.com), Sox2 (1:100, Santa Cruz), and SSEA-1

(1:100, R&D Systems) overnight at 4°C. Human skin (7 μm sections) were deparaffinized in xylene, followed by treatment in 100% ethanol, and by serial hydration through 95% and 75% ethanol, and deionized H_2O. Sections were blocked with 3% normal donkey serum (Jackson ImmunoResearch), 0.1% Triton X-100 to reduce unspecific antibody binding. Sections were incubated with goat anti-Sox2 antibody overnight at 4°C. Slides were washed with PBS, incubated with species-specific Rhodamine Red-X conjugated secondary antibodies, washed and counterstained with 4′,6-diamidino-2-phenylindole (DAPI). The sections and cells were imaged using a Leica DM5500 upright fluorescence microscope and Leica LASAF software.

Microarray Analysis

RNAs were prepared from ES cell, DP cell, and iPS cell cultures with the Absolutely RNA microprep kit (Stratagene). Total RNA (200 ng) was labeled using the applause amplification and labeling system (NuGEN, San Carlos, CA, www.nugeninc.com), and Affymetrix GeneChip Gene ST 1.0 microarrays were hybridized according to the manufacturer's descriptions by the Genomics core facility. Raw data (cel files) were normalized with the Affymetrix Gene Expression Console to target values of 500 using the RMA algorithm. Expression values were log2 transformed and scatter plotted using Microsoft Excel 2007. For clustering analysis, gene lists were filtered based on a cutoff expression level of 300 and at least an expression level difference greater than twofold. The resulting gene expression lists were transformed into GCT files according to instructions of the GenePattern 2.0 webtool at http://genepattern.broadinstitute.org/. Hierarchical clustering was performed at the webserver using recommended settings: Pearson correlation coefficients were calculated for the population comparisons, and Euclidian distance calculations were used for gene level clustering. Data were visualized using GenePattern's hierarchical clustering online viewer.

In Vitro Differentiation

Embryoid bodies (EBs) were generated and differentiated into three germ layers as previously described [14]. Briefly, iPS cells were dissociated with trypsin and 5×10^5 cells were seeded in 10 cm^2 petri-grade dishes in serum-free differentiation (SFD) medium consisting of 75% Isocoves' modified Dulbecco's medium (GIBCO, Carlsbad, CA, www.invitrogen.com), 25% Ham's F12 medium (Cellgro, Mediatech, Herndon, VA, www.cellgro.com) with N2 and B27 supplements (GIBCO), 1% penicillin/streptomycin, 2 mM glutamine, 0.05% bovine serum albumin, 0.5 mM ascorbic acid (Sigma), and 4.5×10^{-4} M MTG (Sigma). EBs were harvested and dissociated at day 2. Cells were transferred to six-well low-cluster plates and treated with cytokines (R&D Systems) to induce differentiation into the three germ layers: 75 ng/ml Activin A for endoderm induction; 2 ng/ml Activin A, 5 ng/ml Wnt3a and 1 ng/ml BMP4 for mesoderm, and 10 ng/ml bFGF for ectoderm induction, respectively. After 2 days, EBs were replated on gelatin-coated 48-well plates and cultured in the following differentiation medium. For endoderm, SFD medium was supplemented with dexamethasone (10^{-7} M, Sigma), bFGF (10 ng/ml), hHGF (40 ng/ml), VEGF (10 ng/ml), mEGF (10 ng/ml), hTGFα (40 ng/ml), and hBMP4 (50 ng/ml). For mesoderm and ectoderm, SFD medium was supplemented with 15% serum. Differentiated cells were fixed with 4% PFA after 8 days. The cells were stained with anti-Foxa2 (1:50, Santa Cruz), anti-Vimentin (1:500, Biomeda, Foster City, CA), and Tubb3 (1:400, Covance, Princeton, NJ, www.covance.com). Secondary fluorescent antibodies (Alexa Fluor 488-conjugated donkey-anti-goat, Rhodamine Red-X-conjugated donkey-anti-rabbit and Rhodamine Red-X-conjugated donkey-anti-mouse at 1:200) were applied for 1 hour at room temperature. The cells were washed three times in PBS, and the nuclei were counterstained with DAPI for 5 minutes. The cells were imaged with a Leica DMI6000 inverted microscope and Leica LASAF software.

Viral Vector Integration Analysis

Genomic DNA was prepared with the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany, www.qiagen.com) according to the manufacturer's instructions. Integration PCR was performed with AmpliTaq polymerase (Applied BioSystems, Foster City, CA, www.appliedbiosystems.com) in PCR buffer and 0.2 μM dNTPs, as described previously [14]. Cycling conditions were as follows: 94°C for 5 min followed by 35 cycles of amplification (94°C denaturation for 1 minute, annealing for 30 second, 72°C elongation for 1 minute), with final incubation at 7°C for 7 minutes. Primers are listed in Supporting Information Table S2. For Southern blotting, genomic DNA (4 μg) was completely digested with BglII at 37°C overnight, separated on a 0.7% agarose gel, and transferred to a positive charged nylon membrane (Roche). The membranes were hybridized with individual DIG-labeled Oct4, Sox2, Klf4, and c-Myc cDNA probes at 42°C overnight. After hybridization, membranes were washed and incubated with anti-DIG-AP Fab fragments (1:10,000, Roche). Probe-target hybrids were then incubated with chemiluminescent CDP-Star substrates (Roche) and detected via exposure to X-ray film.

Bisulfite Sequencing

Sodium bisulfite treatment of purified genomic DNA was performed using the Zymo EZ-DNA Methylation Kit according to manufacturer's protocol with minor modifications. Briefly, 500 ng of DNA was converted at 65°C for 2.5 hours, desulphonated, purified and eluted in 10 μl elution buffer. For each amplicon, 2 ml of sodium bisulfite-treated DNA was PCR amplified using ZymoTaq DNA Polymerase under touchdown cycling conditions to reduce nonspecific amplifications. PCR products were then size fractionated in 2% TAE-agarose, extracted using the Qiaquick gel extraction kit (Qiagen), and cloned into the pGEM-T Easy Vector system (Promega, Madison, WI, www.promega.com). Blue-white selection was applied to reduce frequency of isolating false-positive clones. Twelve random clones were picked and sequenced with M13-rev primer and analyzed using VectorNTi software (Invitrogen). Bisulfite conversion efficiency of non-CpG cytosines was $>$95% for all individual clones for every sample. All clones with successful sequencing results are shown and no clones were selectively excluded. PCR primers are listed in Supporting Information Table S2.

Chimeric Mice

For the blastocyst injection, 4–5-week-old female mice (B6D2F1) were superovulated by administration of 5.0 IU of pregnant mare serum gonadotropin, followed by 5.0 IU of human chorionic gonadotrophin 2 days later via intraperitoneal injection and then mated with C57Bl/6J. All blastocysts were collected at day 3.5 after detection of vaginal plugs and flushed in FHM medium (Specialty Media, Promega). Then the blastocysts were washed in FHM medium and cultured in KSOM medium (Specialty Media) in the incubator (37°C, 5% CO_2 in air) until iPS cell injection. Fifty iPS cell colonies with good morphology were selected and picked in a stereomicroscope, transferred into a well with trypsin to obtain single cells. Cells were then transferred into the micromanipulation chamber in a drop of DMEM medium supplemented with 10% FCS and 2 mM HEPES. Cells (12–15) were injected

into each blastocyst-stage embryo. Injected embryos were then transplanted into the uterus of 2.5 dpc pseudopregnant Swiss Webster recipient females (12 injected blastocysts per female). Chimeric embryos were harvested at E14.5.

RESULTS

Generation of iPS Cell Colonies from DP cells by Oct4 Alone

We previously found that DP cells already endogenously express Sox2, Klf4, and c-Myc, three of the four classic Yamanaka reprogramming factors and that DP cells can be reprogrammed into iPS cells with Oct4 and Klf4 [13, 14]. To test whether Oct4 would be sufficient to reprogram DP cells into iPS cells, we isolated DP cells from transgenic reporter and wild-type mice and transduced them with Oct4 alone.

For this, we first stained hair follicle cell suspensions from skin of Lef1-RFP/Oct4-GFP/Rosa26-LacZ triple-transgenic mice with commercially available antibodies for the cell surface marker Itga9 and then isolated pure DP cells by FACS as the RFP-positive cells in conjunction with the cell surface marker Itga9, as previously described [13, 14]. DP cell isolations with this system typically had >97% purity, as determined with control enzymatic reactions for alkaline phosphatase (Supporting Information Fig. 1A), as described previously [14]. Alkaline phosphatase is an ES marker that is exclusively expressed in DP cells in mature hair follicles in postnatal day 4 backskin [15] (Supporting Information Fig. 1B). Oct4-GFP served as a robust reporter for endogenous activation of Oct4 expression [16], which is detected only in ES cells and germ stem cells in vivo [17, 18] and is not expressed in DP cells and in skin. Rosa26-LacZ was used as ubiquitously expressed marker to trace the cells in the in vivo pluripotency experiments.

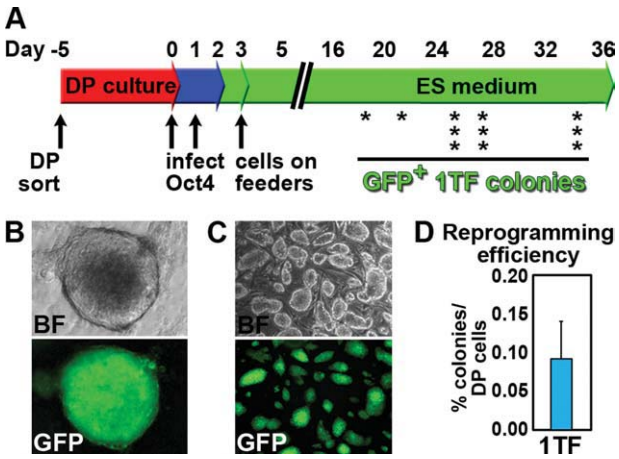

Figure 1. Generation of "one transcription factor" (1TF) induced pluripotent stem (iPS) cells from DP cells. **(A):** Reprogramming DP cells into iPS cells. Asterisks depict the time points of appearance of 1TF iPS cell colonies expressing Oct4-GFP. Note that the first 1TF colonies were detectable after only 18 days. **(B):** Morphology and Oct4-GFP expression in an original 1TF iPS cell colony before picking and transferring onto mouse embryonic fibroblasts. **(C):** Expansion of dermal papilla-derived Oct4-GFP positive 1TF iPS cell colonies (passage 3). **(D):** Reprogramming efficiency of 1TF iPS cells. Abbreviations: BF, bright-field; DP, dermal papilla; ES, embryonic stem cell; GFP, green fluorescent protein; 1TF, one transcription factor.

Primary DP cultures were then expanded for 5 days and infected on two consecutive days with retroviruses encoding Oct4 [3] (Fig. 1A). On the following day, these "one transcription factor" (1TF)-infected cultures were transferred onto feeder plates of MEFs and cultured in standard mouse ES cell medium containing leukemia inhibitory factor. The cultures were monitored daily for the appearance of GFP-positive colonies reporting activation of Oct4 expression under the endogenous promoter [19]. In control experiments with the classic 4TF—Oct4, Sox2, Klf4 and cMyc—we observed reprogrammed GFP-positive iPS cell colonies already 5 days after infection and approximately 400–700 colonies after 10 days, as described previously [14]. Remarkably, with Oct4 alone we also detected reprogrammed GFP-positive colonies, as early as 18 days after transduction (Fig. 1A). Most colonies typically appeared between 3 and 5 weeks. These DP-derived 1TF colonies showed well-defined ES cell–like morphology (Fig. 1B), and all picked colonies could be grown up and expanded as Oct4-GFP-positive subcultures on feeder cells (Fig. 1C), representing a subculturing efficiency of 100%. We expanded a total of eight independent 1TF lines for further analysis, as described below. In contrast to DP cells, regular skin fibroblasts and MEFs transduced with Oct4 in parallel control cultures never formed iPS cell colonies (not shown).

Finally, we calculated a reprogramming efficiency of 0.088% (Fig. 1D), which is approximately 10-fold higher than that of 1TF reprogramming of neural stem cells [7], and as high as reprogramming many other cell types with all 4TF, including MEFs and regular tail tip fibroblasts [3, 19, 20]. These results indicate that the molecular configuration of DP cells with the pre-existent endogenous expression of Sox2, Klf4, and cMyc (and also already AP) allows reprogramming into iPS cells with only Oct4. They also further suggest that reprogramming of DP cells to iPS cells generally is faster and more efficient than for most other cell types.

Having established that Oct4 alone can generate iPS cell colonies from pure DP cells from transgenic Lef1-RFP reporter mice, we next wondered whether we could effectively reprogram enriched but less pure DP cells obtained under wild-type conditions. For this, we FACS sorted a DP-enriched cell population from hair follicle preparations based on Itga9 labeling as described above but without the use of the RFP reporter (Supporting Information Fig. 2A). Itga9 is strongly expressed not only in DP cells within hair follicles but also present in other dermal cells. Alkaline phosphatase staining on isolated cells typically showed an enrichment of 50%–60% DP cells compared to 2%–3% presort (Supporting Information Fig. 2B). Overexpression of Oct4 in these cultures also resulted in multiple reprogrammed Oct4-GFP-positive iPS cell colonies after 4 weeks with typical ES cell ESC morphology (Supporting Information Fig. 1C). This suggests that DP cells can be effectively isolated and reprogrammed by Oct4 alone without the transgenic reporter from wild-type skin.

1TF iPS cells Generated from DP cells Express Pluripotency Genes and Turn-Off DP Genes

With immunofluorescence and real-time PCR, we further analyzed all eight individual DP-derived 1TF iPS cell lines for expression of typical pluripotency markers of ES cells. Consistent with their ES cell–like appearance, all 1TF iPS cell colonies stained positive for Nanog, Oct4, Sox2, and SSEA-1 (Fig. 2A). Real-time PCR analyses showed that selected pluripotency-associated genes, which are normally expressed in ES cells, were also robustly expressed in all DP-derived 1TF iPS cell lines (Fig. 2B). In contrast, these pluripotency genes were not detected in DP cells and MEFs, except for the expected

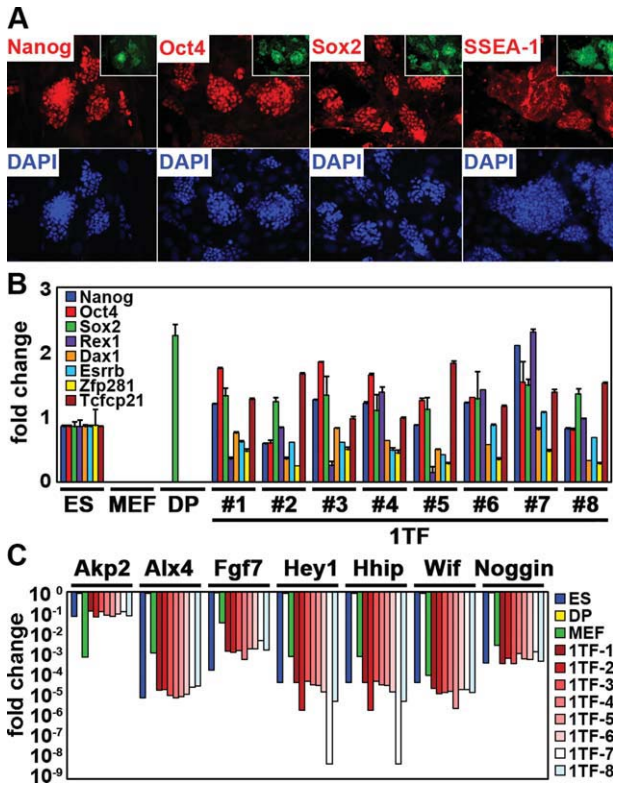

Figure 2. Molecular characterization of pluripotency features in dermal papilla (DP)-derived one transcription factor (1TF) induced pluripotent stem (iPS) cells. **(A):** Immunofluorescence staining of pluripotency markers. 1TF iPS cells were stained for pluripotency markers Nanog, Oct4, Sox2, and SSEA-1. The inset shows Oct4-green fluorescent protein (GFP) expression. DAPI staining highlighted all cells. **(B):** Real-time polymerase chain reaction (PCR) analysis of pluripotency genes. All DP-derived 1TF iPS cell lines express the tested pluripotency markers, at levels similar to embryonic stem (ES) cells. Expression levels were normalized to GAPDH and presented relative to ES cells. Note that mouse embryonic fibroblasts (MEFs) were negative for all genes and DP cells expressed only Sox2. **(C):** Real-time PCR analysis of DP signature genes in ES cells and DP cells, MEFs, and eight different 1TF iPS cell lines. DP-derived 1TF iPS cells strongly downregulated DP signature genes. Gene expression levels were normalized to GAPDH and presented relative to DP cells before reprogramming. Note that alkaline phosphatase (Akp2) is 10-fold lower in ES cells and iPS cells when compared with DP cells. Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; DP, dermal papilla cell; MEFs, mouse embryonic fibroblasts; 1TF, one transcription factor.

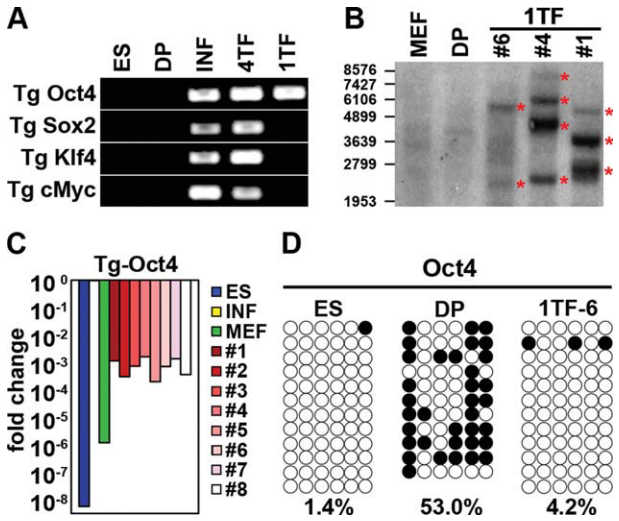

Figure 3. Viral vector integration analysis, transgene silencing an epigenetic reprogramming. **(A):** Genomic polymerase chain reaction (PCR) analysis verified that only Oct4 is inserted in the genome of one transcription factor (1TF) induced pluripotent stem (iPS) cells. Embryonic stem (ES) cell and dermal papilla cells (DP cells) were used as negative controls. Infected cells and 4TF iPS cells are positive controls containing all four transgenes. **(B):** Integration analysis of the Oct4 transgene by Southern blotting. Genomic DNA isolated from mouse embryonic fibroblasts (MEFs), DP cells, and three different 1TF iPS cell lines (#6, #4, and #1) was digested with Bgl II and hybridized with Oct4 cDNA probes. Red asterisks indicate the integration of the exogenous Oct4 transgene. Note that only 2–4 copies of the single trangene Oct4 are required to reprogram DP cells into iPS cells. **(C):** Real-time PCR analysis of Oct4 transgene expression. Virally expressed Oct4 was silenced in all eight tested iPS cell lines after three passages. Expression level of Oct4 was normalized to GAPDH and presented relative to infected cells. **(D):** Methylation analysis of the endogenous Oct4 promoter by bisulfate sequencing in ES cells, DP cells, and 1TF iPS cells. Each circle in horizontal rows represents an individual sequencing reaction and each vertical column represents a pair of CpG dinucleotides. Filled and open circles represent methylated and unmethylated sites, respectively. The percentage of methylation (%) is indicated below each cluster. Abbreviations: DP, dermal papilla cell; INF, infected cells; MEF, mouse embryonic fibroblasts; 1TF, one transcription factor; 4TF, four transcription factor; Tg, transgenes.

expression of *Sox2* in DP cells. Similarly, robust expression of pluripotency genes was also observed in 1TF iPS cells derived from DP-enriched cultures (Supporting Information Fig. 3A). Further characterization of DP-derived 1TF iPS cell lines confirmed that DP-specific signature genes were strongly downregulated after switching from a DP fate to a pluripotent stem cell fate (Fig. 2C).

Silencing of the Oct4 Transgene, Low Viral Integration, and Epigenetic Reprogramming of the Endogenous Oct4 Locus

After retroviral infection, viral transgenes become integrated into the host genome and effective transgene silencing is essential for the derivation of fully reprogrammed pluripotent iPS cells [5]. Genotyping PCR with virus-specific primers verified the integration of the Oct4 transgene (Fig. 3A and Supporting Information Fig. 3B). All 1TF iPS cell clones contained only the integration of the Oct4 transgene, thereby ruling out the possibility of cross-contamination with existing clones from wild-type ES cells or 2TF/4TF iPS cell lines in our laboratory. Furthermore, Southern blot analysis of three independent lines demonstrated that only two to four copies are integrated in a distinct pattern (Fig. 3B), suggesting that reprogramming of DP cells is effective without multiple copies even when Oct4 alone is the sole reprogramming factor. Importantly, real-time PCR for virus-specific Oct4 transcripts demonstrated that the Oct4 transgene was completely silenced after three passages (Fig. 3C and Supporting Information Fig. 3C). Furthermore, methylation analysis of the endogenous Oct4 promoter by bisulfite sequencing showed epigenetic reactivation of the endogenous locus confirming that reprogramming occurred at the epigenetic level (Fig. 3D).

Genome-Wide Gene Expression Analysis and Absence of Silencing of Imprinted Genes

We next performed global gene expression analysis of DP-derived 1TF iPS cells and compared them to DP cells, from

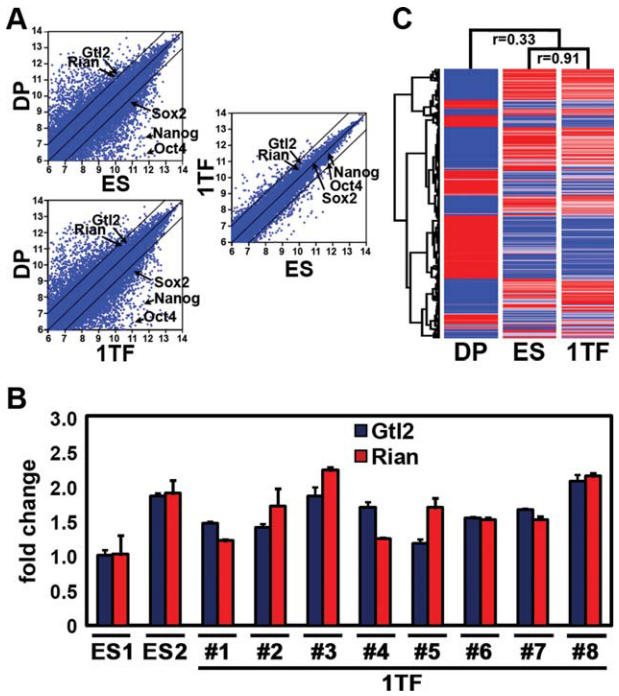

Figure 4. Genome-wide gene expression analysis of dermal papilla (DP)-derived one transcription factor (1TF) induced pluripotent stem (iPS) cells. **(A):** Scatter plots of global gene expression patterns comparing dermal papilla cells (DP cells), embryonic stem (ES) cells, and 1TF iPS cells by microarrays. Log2 expression values are shown. The central black line represents equal gene expression. The outer black lines indicate twofold different levels. Pluripotency genes *Nanog*, *Oct4*, and *Sox2* and imprinted genes *Gtl2* and *Rian* are highlighted. **(B):** Real-time polymerase chain reaction analysis shows similar expression levels of *Gtl2* and *Rian* in all tested 1TF iPS cell lines when compared with two different ES cell lines. ES-1 is the Ainv15 ES cell line. ES-2 is the CCE ES cell line. **(C):** Heat map analysis of DP cells, ES cells, and 1TF iPS cells. Increased gene expression is shown in red, decreased levels are in blue Abbreviations: DP, dermal papilla cell; 1TF, one transcription factor.

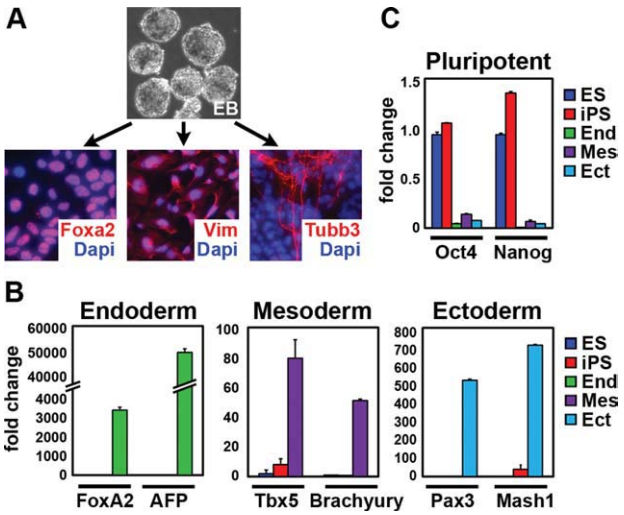

Figure 5. In vitro pluripotency potential of dermal papilla-derived one transcription factor (1TF) induced pluripotent stem (iPS) cells. **(A):** Differentiation of 1TF iPS cells into three germ layers in vitro. Embryoid bodies were generated from 1TF iPS cells and differentiated with germ layer specific differentiation media. Immunofluorescence analysis showed differentiated cells positive for endoderm (Foxa2), mesoderm (Vim), and ectoderm (Tubb3) markers. **(B):** Real-time polymerase chain reaction (PCR) analysis on undifferentiated and differentiated 1TF iPS cells showed upregulation of selected lineage markers for each germ layer. **(C):** Real-time PCR analysis on undifferentiated and differentiated 1TF iPS cells demonstrated simultaneous downregulation of pluripotency genes Oct4 and Nanog in differentiated cells. Gene expression levels were normalized to GAPDH and presented relative to embryonic stem (ES) cells. Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; EB, embryoid body; Ect, ectoderm; End, endoderm; Mes, mesoderm.

which they were generated, and to ES cells. Scatter plot analysis demonstrated a strong correlation of gene expression levels between iPS cells and ES cells, while ES cells and iPS cells were dissimilar to the parental DP cells (Fig. 4A). Interestingly, *Gtl2* and *Rian*, two imprinted genes from a locus that becomes silenced in partially reprogrammed iPS cells [21], showed similar expression between ES cells and 1TF iPS cells (Fig. 4A). Analysis of *Gtl2* and *Rian* expression in all eight iPS cell lines by real-time PCR highlighted that all of the lines seem to be fully reprogrammed, as neither gene was aberrantly silenced (Fig. 4B). Clustering analysis further showed high degrees of similarity between iPS cells and ES cells ($r = .91$), which were distant from the parental DP cells ($r = .33$) (Fig. 4C).

DP-Derived One-Factor iPS cells Give Rise to Cells from All Germ Layers in EB Differentiation Assays

We next functionally characterized the 1TF iPS cell lines by determining their developmental potential. We first investigated the differentiation potential of 1TF iPS cell lines in EB differentiation assays in vitro. DP-derived 1TF iPS cells effectively formed EBs that could be directed toward germ layer-specific differentiation (Fig. 5A), as determined by immunofluorescence staining for lineage markers, such as Foxa2 (endoderm), Vimentin (mesoderm), and Tubb3 (ectoderm). These results were also confirmed by real-time PCR for additional lineage-specific genes representing the three germ layers. Each lineage gene was strongly induced in the differentiation conditions for the expected germ layer (Fig. 5B). Concomitantly, the pluripotency-associated genes *Oct4* and *Nanog* were downregulated upon initiation of differentiation (Fig. 3C).

One-Factor Generated iPS Cells Widely Contribute to Somatic Tissues in Embryos and Give Rise to Healthy Adult Chimeric Mice and Viable Offspring

To test the pluripotency potential of iPS cells in vivo, we corroborated their contribution to chimeric embryos, in which 1TF iPS cells were injected into eight-cell stage embryos. After transferring the blastocysts into pseudopregnant females, we successfully obtained chimeric embryos and live pups using two independent 1TF iPS cell lines (Supporting Information Table S1). Fluorescence analysis of the Lef1-RFP transgene expression (Fig. 6A) and whole-mount X-Gal staining for the ubiquitous Rosa26-LacZ expression (Fig. 6B) revealed widespread contribution to the embryos. Moreover, we also observed Oct4-GFP expression in the fetal gonads of chimeric E14.5 embryos, suggesting potential contribution to the germline (Fig. 6C). In addition, several chimeric mice from two independent lines, as determined by PCR detection of the Oct4-GFP allele (Fig. 6D), were born and followed during postnatal development. All 17 chimeric mice (out of 43) developed into normal-appearing, healthy and fertile adult mice (up to 8 months old) without any gross abnormalities compared to wild-type mice. Besides Lef1-RFP expression in skin (not shown), they exhibited variable degrees of the expected agouti contribution to the coat color (Fig. 6E, Supporting Information Table S1).

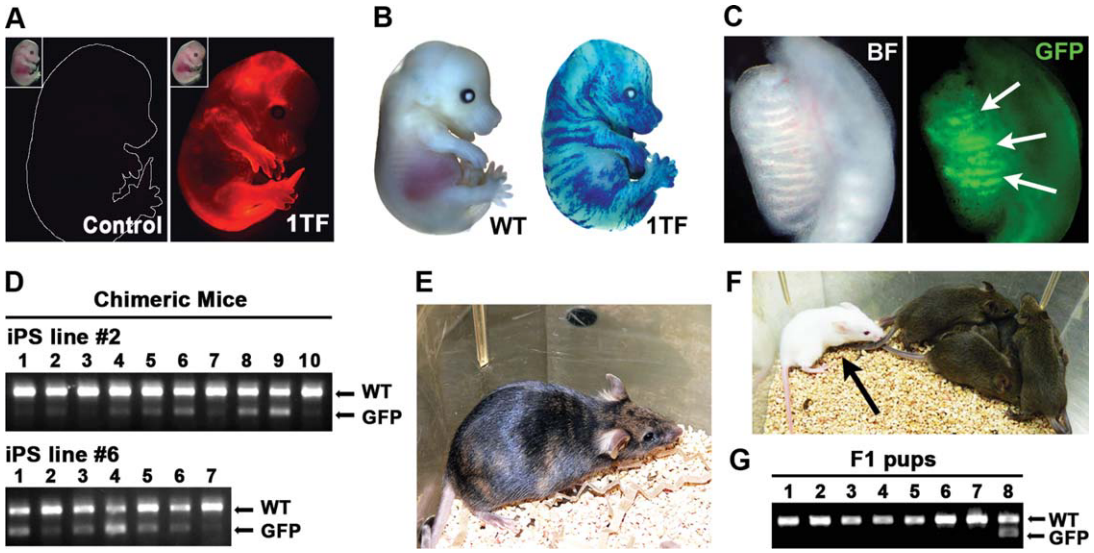

Figure 6. In vivo developmental potential of dermal papilla (DP)-derived one transcription factor (1TF) induced pluripotent stem (iPS) cells. **(A):** In vivo contribution of DP-derived 1TF iPS cells to developing embryos at E14.5. The chimeric embryo shows widespread expression of Lef1-RFP (right). Left: a control embryo at same exposure. The insets show the bright-field images of the embryos. **(B):** Whole-mount X-gal staining of same chimeric embryos. **(C):** Germline contribution of 1TF iPS cells as shown by Oct4-green fluorescent protein (GFP) expression in the gonads of E14.5 chimeric embryos (white arrows). **(D):** Genotyping polymerase chain reaction (PCR) of chimeric mice derived from 1TF lines #2 and #6. PCR analyses for the Oct4-GFP allele. The lower band is specific for the GFP knockin allele, the upper band is for the wild type (WT) allele. **(E):** A chimeric mouse with agouti coat color originating from 1TF #6 iPS cells at 8 weeks of age. **(F):** F1 generation litter of test matings with chimeric females. The white offspring (arrow) in this litter is derived from an iPS cell–generated oocyte from its chimeric mother. **(G):** Genotyping PCR of the F1 litter. PCR analysis detected the Oct4-GFP allele as the lower band; the upper band is for the WT allele. Abbreviations: BF, bright field; GFP, green fluorescent protein; 1TF, one transcription factor; WT, wild type.

To further verify the potential of 1TF iPS cells for germline transmission, we mated the chimeric mice with wild-type mice. We obtained several viable pups in the F1 generation from iPS cell–derived gametes, which are identified by the same uniform hair coat as the parental mice from which the DP cells were isolated for iPS cell generation (Fig. 6F, arrow). Lef1-RFP expression in skin (not shown) and PCR detection of the Oct4-GFP allele further confirmed the origin of the iPS cell–derived pups (Fig. 6F). None of the chimeric mice or the F1 pups developed tumors by the age of 8 months and 5 months, respectively. Taken together, these in vitro and in vivo data strongly suggest that the DP-derived 1TF iPS cell lines were bona fide pluripotent stem cells with a similar developmental potential as ES cells.

Discussion

In this study, we have successfully reprogrammed hair follicle-derived DP cells into fully functional iPS cells by ectopic expression of the single transcription factor Oct4. This is an important step toward the goal to develop safe and efficient protocols for generating patient-specific iPS cells from accessible cells for directed cell differentiation and transplantation. Recently, several groups have demonstrated reprogramming of very accessible cells, such as mononuclear cells from the peripheral blood [22–24]. However, to generate blood-derived iPS cells, all four classic TFs have to be infected, including the oncogenes c-Myc and Klf4. While generating iPS cells in this way is certainly useful for investigating disease mechanisms and testing novel drug treatments, the potential use for regenerative medicine is limited due to the inherent risk of cancer formation and of gene misregulation due to genomic integrations. These risks can be decreased by simply reducing the number of reprogramming factors. Using fewer than four transcription factors to reprogram skin fibroblasts was achieved by combining transcription factor expression with treatment of small molecule activators and inhibitors of epigenetic regulation and signaling pathways that could replace the activity of individual reprogramming transcription factors [8, 25]. This was very recently improved to the level that fibroblasts could be reprogrammed with Oct4 alone with simultaneous treatment of several additional small molecules [9–11]. To date, only neural stem cells can be reprogrammed by Oct4 alone without any further manipulation, but the difficulty of isolating these cells substantially diminishes their practical application.

In contrast, DP cells are easily accessible in the skin and can be easily isolated from preparations of hair follicles. In this study, we have performed proof-of-principle experiments in a robust genetic labeling system in the mouse to isolate pure DP cells (Lef-RFP/Itga9^{+}), report iPS cell reprogramming (Oct4-GFP), and trace the iPS cells in vivo (Rosa-LacZ). We demonstrate that murine DP cells are a cells source in skin that can be quite rapidly (3 weeks) and efficiently (~0.09%) reprogrammed into iPS cells by only Oct4 due to their endogenous expression of all other reprogramming factors. This suggests that DP cells could be the preferred starting population for iPS cell generation, and the ideal cell type for ultimately replacing the single final factor with small molecule modifiers of epigenetic regulation, such as the G9a histone methyltransferase inhibitor (BIX-01294), which was recently shown to replace Oct4 in the presence of the other three reprogramming factors [26].

In this study, we isolated and reprogrammed DP cells from skin of young mouse pups in which our isolation system was previously established [14]. At this stage, DP cells and hair follicles are fully mature and express all markers comparable to adult growing follicles. DP cells from mature human hair follicles can also be easily isolated and cultured from

individual follicles that are routinely prepared during hair transplantation surgeries [27] or from scalp skin discarded after facelifts [28]. In addition, one in 100 hairs plucked from the human head contains a DP [29], and only one to four microdissected DP cells are sufficient to initiate a successful culture. As human DP cells also endogenously express Sox2 (Supporting Information Fig. 4) [30], it is feasible that human DP cells have a similar potential for reprogramming by Oct4 alone.

Conclusion

In summary, in this study, we demonstrate that DP cells from hair follicles in the skin can be reprogrammed into iPS cells by the single transcription factor Oct4 and without the use of additional small molecules. As for future therapies it will be best to use cells that need the least manipulation for reprogramming and that are at the same time easily accessible, DP cells may be the optimal cell source for safe generation of pluripotent stem cells after replacing the final factor Oct4.

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Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflict of interest.

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