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Oct4 and Klf4 Reprogram Dermal Papilla Cells Into Induced Pluripotent Stem Cells

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ABSTRACT

Direct reprogramming of somatic cells into induced pluripotent stem (iPS) cells by only four transcription factors (Oct4, Sox2, Klf4, and c-Myc) has great potential for tissue-specific regenerative therapies, eliminating the ethical issues surrounding the use of embryonic stem cells and the rejection problems of using non-autologous cells. The reprogramming efficiency generally is very low, however, and the problems surrounding the introduction of viral genetic material are only partially investigated. Recent efforts to reduce the number of virally expressed transcription factors succeeded at reprogramming neural stem cells into iPS cells by overexpressing Oct4 alone. However, the relative inaccessibility and difficulty of obtaining neural cells in humans remains to be resolved. Here we report that dermal papilla (DP) cells, which are specialized skin fibroblasts thought to instruct hair follicle stem cells, endogenously express high levels of Sox2 and c-Myc, and that these cells can be reprogrammed into iPS cells with only Oct4 and Klf4. Moreover, we show that DP cells are reprogrammed more efficiently than skin and embryonic fibroblasts. iPS cells derived from DP cells expressed pluripotency genes and differentiated into cells from all germ layers in vitro and widely contributed to chimeric mice in vivo, including the germline. Our work establishes DP cells as an easily accessible source to generate iPS cells with efficiency and with less genetic material. This opens up the possibility of streamlined generation of skin-derived, patient-specific pluripotent stem cells and of ultimately replacing the remaining two factors with small molecules for safe generation of transplantable cells. STEM CELLS *2010;28:221–228*

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

INTRODUCTION

Embryonic stem (ES) cells are derived from the inner cell mass of blastocyst stage embryos and can be expanded in vitro, while retaining the pluripotency to give rise to all cell types of the embryo [1]. These features have made ES cells a powerful tool for potential applications in regenerative medicine by targeted differentiation into needed cells and tissues. However, even if such differentiated cells function perfectly well after transplantation into recipient patients, there is a significant risk of transplant rejection because of immunological donor/host incompatibilities of existing ES cell lines. A solution would be the generation of a large ES cell bank covering the entire immunological spectrum, which likely is an insurmountable task because of the sheer scope and, more importantly, the ethical implications of generating hundreds of new ES cell lines from human embryos.

A solution to these problems would be the de-differentiation and reprogramming of patients' own somatic cells into a stem cell-like state. Indeed, in a groundbreaking study, Yamanaka and Takahashi accomplished to reprogram mouse somatic cells into pluripotent ES cell-like cells by the simple retroviral overexpression of only four transcription factors (TFs) [2], Oct4, Sox2, Klf4, and c-Myc. These induced pluripotent stem (iPS) cells closely resemble ES cells since they restore the pluripotency-associated gene expression pattern and fulfill all major biological criteria for pluripotency, such as in vitro differentiation potential into cell types of all three germ layers, teratoma formation, integration into chimeric embryos, and germline transmission [3]. Subsequently, applying similar methods, several groups have successfully obtained human iPS cells from fibroblasts and other cell types [4–7], opening up the possibility of generating patient-specific pluripotent cells from adult somatic cells for autologous regenerative medicine. However, several limitations currently preclude the use of iPS cells in a clinical setting [3]. First, virus-mediated delivery of reprogramming factors permanently integrates the transgenes into the human genome, potentially altering genomic features and bearing a risk of viral transgene reactivation. Second, the reprogramming factors Klf4 and c-Myc are oncogenic [8]. Third, iPS cell reprogramming is very

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slow and inefficient [9]. To solve these issues, several groups have made considerable progress. Nonviral delivery and integration-free methods, such as transient expression of the reprogramming factors with adenoviral, plasmid, and episomal vectors [10–12], and with recombinant proteins [13], have been successfully employed, albeit at a very low efficiency. Also, genome-integrating but excisable systems have worked to remove the integrated transgenes [14–16]. iPS cells have also been generated without the oncogenic factors c-Myc or Klf4 [17–19]. Importantly, reducing the number of factors decreases the chance of retroviral insertional mutagenesis and might increase the likelihood of ultimately replacing the remaining factor(s) with small molecules, pointing to the need to identify cell types that can be more easily reprogrammed by fewer factors and with higher efficiency. Indeed, Schöler and colleagues recently demonstrated that mouse neural stem cells, which endogenously express Sox2, c-Myc, and Klf4, could be reprogrammed by only two factors (Oct4, Klf4) [20, 21] or by Oct4 alone [22]. However, the practical limitation is that neural stem cells are not an accessible source that can be easily isolated from patients.

To this end, we have identified that dermal papilla (DP) cells from the skin, which are specialized mesenchymal cells thought to instruct epithelial stem cells during hair morphogenesis and regeneration [23], already express three reprogramming factors, Sox2, c-Myc, and Klf4, and here we demonstrate that DP cells are an easily accessible source of somatic cells from skin that can be reprogrammed into iPS cells by only two exogenous programming factors, Oct4 and Klf4. We furthermore found that DP cells can be reprogrammed into iPS cells with four factors with much higher efficiency than with many other cell types. DP-derived two-factor iPS cells fulfill all pluripotency criteria, such as expression of pluripotency genes and differentiation into cell types from all germ layers in vitro and in chimeric mice in vivo, including potential contribution to the germ cells in the developing gonads. This suggests that hair follicle DP cells represent a cell type that can be easily accessible from skin to generate iPS cells efficiently and with less reprogramming factors.

Materials and Methods

Mice and Dermal Papilla Cell Isolations

Lef1-red fluorescent protein (RFP) transgenic mice [24] were crossed with Oct4-green fluorescent protein (GFP) knockin mice (obtained from The Jackson Laboratory, Bar Harbor, ME, http://www.jax.org, B6;129S4-Pou5f1tm2Jae/J, stock number: 008214 [25]) for several generations to obtain compound homozygous mice for using entire litters for DP cell isolations. 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. For cell preparations, back skins of 4- to 6-day-old Oct4-GFP/Lef1-RFP pups were floated on dispase (Invitrogen, Carlsbad, CA, http://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, http://www.sigmaaldrich.com) at 37°C for 40–60 minutes. Intact follicles and dermal cells were centrifuged at 300*g*, after which follicles were enriched at low-speed centrifugation two times at 20*g*. Following trypsinization (0.25% trypsin/0.05 mM EDTA; Invitrogen) at 37°C for 5 minutes, single-cell suspensions were strained through 40-μm filters and pelleted at 300*g*. Typically, we obtained a total of 40-50 $\times$ 10^6 hair cells per back skin and we used 4-6 pups per experiment. For DP cell isolations by fluorescence activated cell sorting (FACS, BD Vantage, and DAKO-Cytomation MoFlo) hair cell suspensions were first stained for integrin α-9 (Itga9, goat; R&D Systems Inc., Minneapolis, http://www.rndsystems.com) and donkey-anti-goat-APC conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA, http://www.jacksonimmuno.com) and then depleted for melanocytes (CD117) and endothelial cells (CD34) with biotinylated antibodies (BD Pharmingen, San Diego, http://www.bdbiosciences.com) and magnetic anti-biotin microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany, http://www.miltenyibiotec.com). DP cells were selected in FACS as the RFP/APC-double positive cell population. We routinely obtained 1–2 $\times$ 10^6 DP cells per sort. Mx, outer root sheath cells, a DF enriched in fibroblasts and melanocytes were FACS-isolated from Lef1-RFP/K14-H2BGFP mice as previously described [24].

Virus Production, Infection, and Cell Culture

Phoenix cells were plated at 18 $\times$ 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. Twenty-four hours after transfection, Phoenix cells were cultured at 32°C and virus supernatants were collected after an additional 24, 48, and 72 hours, passed through 0.22-μm pore size filters (Millipore, Billerica, MA, http://www.millipore.com) and pelleted at 50,000*g* for 3 hours. Viruses were resuspended in 1$\times$ phosphate-buffered saline at $\sim$200$\times$ 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, or passage 1 DP cells were seeded at a density of 5 $\times$ 10^4 cells in 6-well plates. Equal amounts of the four viruses and 4 μg/ml polybrene (Sigma-Aldrich) were added to the cells. Infection was enhanced by spinning 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 an ES medium (Dulbecco's modified Eagle's medium [DMEM] containing 15% fetal bovine serum [HyClone, Logan, UT, http://www.hyclone.com], leukemia inhibitory factor [Fisher Scientific International, Hampton, NH, http://www.fisherscientific.com], penicillin/streptomycin, L-glutamine [GIBCO, Carlsbad, CA, http://www.invitrogen.com], β-mercaptoethanol [Sigma-Aldrich], and nonessential amino acids [Sigma-Aldrich]). The following day, DP cells were counted and seeded on irradiated mouse embryonic fibroblasts (MEFs) in ES medium without any further selection. Oct4-GFP-positive colonies were picked and trypsin dissociated and replated onto irradiated MEFs in 96-well plates. All iPS lines and control Ainv15 ES cells (kind gift from Christoph Schaniel/Lemischka Lab) were maintained on irradiated MEF in the above-described standard ES medium. Before RNA and genomic DNA purification, iPS cells were depleted of feeder cells for two passages on 0.1% gelatin (Fisher). For blastocyst injections, iPS cells were permanently labeled after infection and FACS selection with a lentiviral vector expressing the red fluorescent protein td-tomato (kind gift from Matthias Stadtfeld/Hochedlinger Lab [10]).

Determination of iPS Cell Formation Efficiency

The efficiency of iPS cell formation is based on the number of Oct4-GFP-positive iPS colonies and the initial cell number (5 $\times$ 10^4 cells) of plated DP cells. The percentage of efficiency was determined by dividing the number of GFP-positive colonies by the number of cells seeded for infections.

Chimeric Mice

For the blastocyst injection, 4- to 5-week-old female mice (B6D2F1) were superovulated by administration of 5.0 IU pregnant mare serum gonadotropin, followed by 5.0 IU human chorionic gonadotropin (hCG) 2 days later via intraperitoneal injection and then mated with C57Bl/6J. Blastocysts were collected at day 3.5 after detection of vaginal plugs and flushed in FHM medium (Specialty Media, St. Louis, http://www.sigmaaldrich.com).

Blastocysts were then extensively 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 colonies with good morphology were selected and picked in a stereomicroscope and then transferred into a well with trypsin to obtain single cells. Cells were then transferred into the micromanipulation chamber in a drop of DMEM supplemented with 10% FCS and 2 mM HEPES. Twelve to fifteen cells 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 embryonic day 13.5–14.5.

RNA Isolation, Semiquantitative Reverse Transcription-Polymerase Chain Reaction (PCR) and Real-Time PCR, Immunofluorescence and Alkaline Phosphatase Staining, Bisulfite Sequencing, Viral Vector Integration Analysis, and In Vitro Differentiation

See supporting information materials and methods.

Results

We recently isolated pure DP cells and four other cell types from their immediate microenvironment (epithelial matrix and outer root sheath cells, melanocytes, and dermal fibroblasts) by FACS and determined their cell type-specific molecular signatures at a genomic level by microarray analysis [24]. In the DP signature we found Sox2, an essential ES cell transcription factor that is also instrumental in reprogramming somatic cells into iPS cells. Real-Time PCR with DP cells and the other four cell types confirmed specific expression of Sox2 in DP cells (Fig. 1A) and immunofluorescence staining at different hair follicle developmental stages showed exclusive expression of Sox2 in DP cells at the protein level (Fig. 1B). This is in agreement with a recent report of DP-specific Sox2 expression in skin [26]. We next tested whether the other three reprogramming factors (Oct4, Klf4, and c-Myc) are expressed in DP cells as well. Semiquantitative reverse transcription (RT)-PCR and real-time PCR with DP cells, ES cells, keratinocytes, MEFs, a DF enriched in regular fibroblasts, and the 3T3 fibroblast cell line confirmed, as expected, the exclusive expression of the pluripotency gene and reprogramming factor Oct4 and the pluripotency gene Nanog in ES cells (Fig. 1C, 1D). Sox2 was expressed in ES cells and DP cells, and absent in the other cell types. In addition to Sox2, however, Klf4 and c-Myc were also present in DP cells, both of which were found in keratinocytes and fibroblasts as well. Since DP cells express all but one transcription factor that are necessary to reprogram somatic cells into iPS cells, this strongly suggested that DP cells could represent an easily accessible cell type from skin that could potentially be reprogrammed into iPS cells by a reduced number of exogenous reprogramming factors.

To test the capacity and efficiency of DP cells to reprogram into iPS cells by a reduced number of reprogramming factors, we sought to isolate DP cells from Oct4-GFP reporter mice in which GFP is exclusively expressed in ES cells and germ cells under the control of the endogenous Oct4 promoter, and which report Oct4 activity in reprogrammed iPS cells [25]. To this end, we modified our previously established method of isolating pure RFP-positive DP cells from double-transgenic Lef1-RFP/K14-H2B-GFP mice [24] to purify DP cells from single transgenic Lef1-RFP mice crossed with Oct4-GFP mice by FACS. For this strategy to work, we had

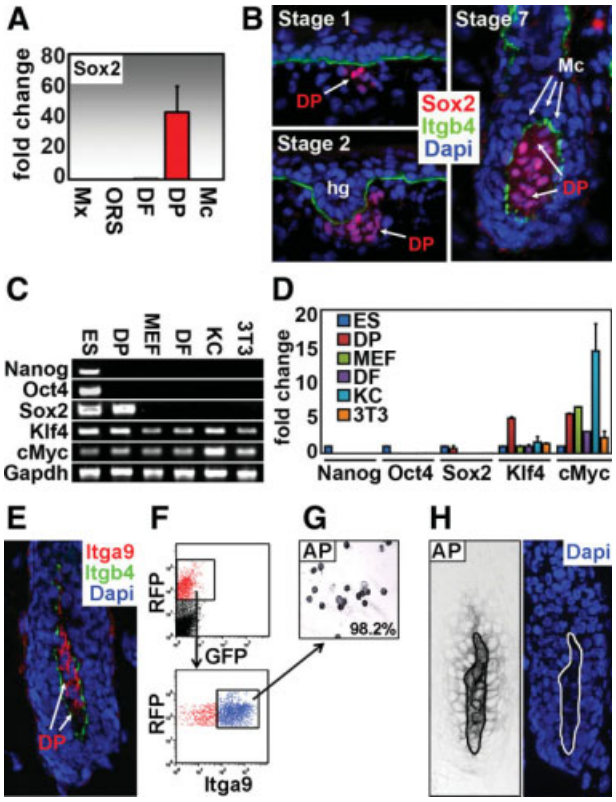

Figure 1. DP cells express the reprogramming factors Sox2, c-Myc, and Klf4. (**A**): Specific Sox2 expression in DP cells. RNAs were isolated, reverse-transcribed to cDNAs, and real-time polymerase chain reaction (PCR) was performed for Sox2 in freshly sorted DP cells and in four cell types from the hair follicle microenvironment. Expression levels were normalized to Gapdh and presented relative to the DF. (**B**): Immunofluorescence of Sox2 confirmed specific expression in the DP at different stages during hair development. Integrin beta 4 (Itgb4) staining demarcates the basement membrane that separates DP cells and dermis from the epithelium. Note that no Sox2 expression was detected in hair follicle Mc. (**C**): Semiquantitative reverse transcription (RT)-PCR of the four reprogramming factors, the ES cell gene Nanog, and Gapdh. (**D**): Real-Time PCR of reprogramming factors and the ES marker Nanog. Expression levels were normalized to Gapdh and presented relative to the ES cell fraction. Note that DP cells express three of the four reprogramming factors. (**E**): Single reporter isolation strategy of DP cells from Lef1-RFP/Oct4-GFP mice in combination with the DP cell surface marker integrin α-9 (Itga9). Itga9 is strongly expressed in DP cells. Itgb4 marks the border of the DP. (**F**): Single cells from hair follicle preparations of Lef1-RFP/Oct4-GFP mice were stained with Itga9 antibodies and then fluorescent activated cell sorted based on RFP and Itga9 expression. (**G**): The purity of DP isolations was confirmed by alkaline phosphatase (AP) staining. (**H**): AP is a DP-specific marker in the growing hair follicle in the skin. Abbreviations: AP, alkaline phosphatase; DP, dermal papilla; DF, dermal fraction; GFP, green fluorescent protein; hg, hair germ; KC, keratinocytes; Mc, melanocytes; MEF, mouse embryonic fibroblast; Mx, matrix cells; ORS, outer root sheath; RFP, red fluorescent protein.

to find a way to exclude non-DP cells that were previously RFP/GFP-double positive in the double-transgenic system and that would contaminate the DP isolation from Lef-RFP/Oct4-GFP mice. Turning to the DP signature, we identified the cell surface molecule integrin α-9 (Itga9), which is strongly expressed at the cell surface of all DP cells in immunofluorescence stainings (Fig. 1E), but also weakly expressed in dermal cells and endothelial cells (not shown). Using this marker, it was possible to positively label and isolate DP cells in Lef1-

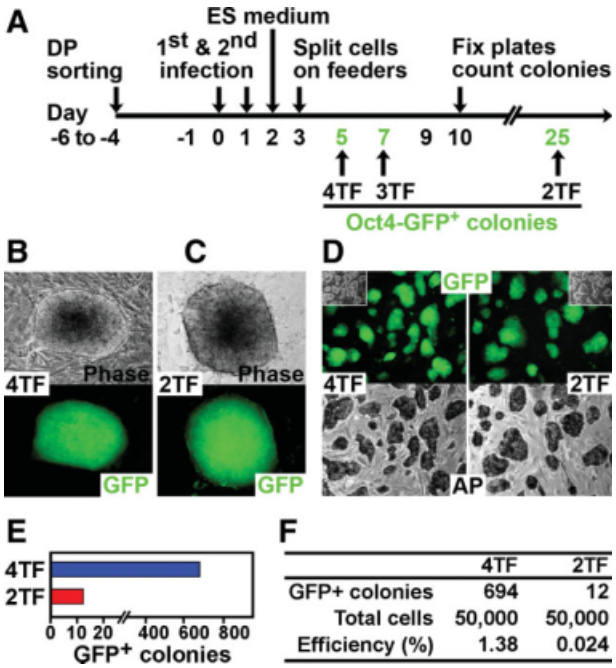

Figure 2. Generation of iPS cells from DP cells with four and two reprogramming factors. **(A)**: Timeline of reprogramming DP cells into iPS cells. DP cells that were sorted by FACS were infected two times with retroviruses expressing Oct4, Sox2, c-Myc, and Klf4 (4TF) or with Oct4 and Klf4 (2TF). DP culture medium was changed to ES cell medium at day 2. At day 3, DP cells were split onto MEF feeder cells. Note that already 2 days later at day 5, Oct4-GFP iPS colonies were formed with 4TF. Expressing 3TF, by omitting Sox2, showed colonies already after 7 days, and additional c-Myc omission yielded colonies after 25 days (2TF), indicating that endogenous Sox2 and c-Myc in DP cells was sufficient for reprogramming with only Oct4 and Klf4. **(B)**: Typical Oct4-GFP-positive iPS colony generated with 4TF reporting activation of endogenous Oct4 expression. **(C)**: Typical DP-derived 2TF iPS colony. **(D)**: Low magnification of 4TF and 2TF Oct4-GFP-positive iPS colonies (top). The same colonies are positive for the ES marker alkaline phosphatase (AP, bottom). The inset shows the morphology of phase-bright iPS colonies **(E)**: Generation of GFP-positive colonies at day 10 (4TF) and day 32 (2TF). **(F)**: Reprogramming efficiency of generating 4TF and 2TF DP-derived iPS cells. The efficiency is represented as the percentage of GFP-positive colonies per 50,000 plated cells. Abbreviations: AP, alkaline phosphatase; DP, dermal papilla; FACS, fluorescent activated cell sorting; GFP, green fluorescent protein; iPS, induced pluripotent stem cells; MEF, mouse embryonic fibroblasts; 4TF, four transcription factors; 2TF, two transcription factors.

RFP/Oct4-GFP mice (Fig. 1F). To confirm the purity of FACS isolations, DP cells were stained for the DP signature gene alkaline phosphatase (AP), which demonstrated that 98.2% of sorted cells were positive (Fig. 1G). AP is a well-established ES cell marker that is expressed also in skin, but exclusively in the DP cells of the hair follicles (Fig. 1H) [27].

We next freshly isolated DP cells from Lef1-RFP/Oct4-GFP mice and applied a reprogramming strategy similar to previously published methods [21]. As shown in the timeline in Figure 2A, 4-6 days after FACS isolation or 1 day after passaging the cells, approximately 50% confluent DP cells was infected with retroviruses expressing all four reprogramming transcription factors (4TF: Oct4, Sox2, Klf4, and c-Myc) on two consecutive days. We consistently infected 80%–90% DP cells as determined by parallel infections with a GFP control virus (not shown). One day after 4TF infections, the DP culture medium was changed to ES cell medium, and yet another day later DP cells were split onto irradiated MEF feeder cells. Only 2 days later, at day 5 of the reprogramming process, we already detected the first Oct4-GFP-positive colonies that exhibited the typical well-defined phase-bright borders with surrounding sheets of feeder cells and cells with high nuclear-cytoplasmic ratio (Fig. 2B). The early appearance of GFP-positive iPS colonies after only 5 days in DP cells is comparable to the timing observed with neural stem cells [22], and is several days ahead of reprogramming events with several other cell types [9]. These data suggest that DP cells represent a cell type that is amenable to effective pluripotency reprogramming.

Since DP cells express high levels of endogenous Sox2, we next infected DP cells with Oct4, c-Myc, and Klf4 (3TF), omitting exogenous Sox2. Again, Oct4-GFP-positive iPS colonies formed (supporting information Fig. 1), this time 2 days later at day 7 after infections (Fig. 2A). This demonstrated that endogenous Sox2 levels were sufficient for reprogramming with 3TF. This is similar to melanocytes, which were recently reprogrammed by 3TF without exogenous Sox2, although they did not appear before 21 days after infections [28]. DP cells also express high levels of the oncogene c-Myc (Fig. 1C, 1D), and thus we further omitted viral c-Myc overexpression to reprogram DP cells by Oct4 and Klf4 (2TF) alone. As shown in Figure 2C, we successfully generated Oct4-GFP-positive colonies with only 2TF that also exhibited the typical ES/iPS colony features. The appearance of 2TF was significantly delayed, however, with the first detectable colonies at day 25 after infections. Most of both 4TF and 2TF iPS colonies could be efficiently picked and propagated as Oct4-GFP-positive subcultures on feeders with typical ES/iPS morphology and robust expression of alkaline phosphatase (Fig. 2D). These results thus far suggested that the endogenous expression levels of Sox2 and c-Myc in DP cells are sufficient to induce pluripotency reprogramming, with a timing similar to that of neuronal stem cells [20].

In addition to the early appearance of 4TF iPS colonies, we noticed that iPS colonies formed very efficiently since we detected several hundred colonies within the first 2 weeks after infection. We fixed the cells and calculated the 4TF reprogramming efficiency at day 10. Of 5×10^4 plated DP cells we counted an average of $\sim$700 GFP-positive colonies with clear iPS morphology and calculated a nominal reprogramming efficiency of 1.38% (Fig. 2E), which is similar to neural stem cells and keratinocytes and $>$1000-fold higher than with many other cell types [2, 29, 30]. This suggested that DP cells are more prone to reprogramming than most other cell types that have been tested so far. The efficiency of iPS generation with 2TF, however, was greatly reduced to 0.024% (Fig. 2E), which is still comparable to reprogramming efficiencies with fibroblasts and other cells using 4TF. Because exogenous Sox2 and c-Myc were not required for reprogramming DP cells into iPS cells and DP cells express Klf4 as well (Fig. 1C, 1D), we also tried to derive iPS cells with Oct4 overexpression alone. Despite several attempts, we only observed a few morphological changes with weak Oct4-GFP expression; however, we did not detect the induction of colonies with convincing iPS morphology (not shown).

We next picked several 4TF, 3TF, and 2TF iPS colonies, expanded them, and characterized the acquisition of pluripotency features at the molecular level. Real-time PCR showed that all tested pluripotency genes, which are normally expressed in ES cells, were similarly expressed in all iPS cell lines (Fig. 3A). None of the pluripotency genes were expressed in DP cells and MEFs, except for the expected expression of Sox2 in DP cells, and of Klf4 and c-Myc in DP cells and MEFs. Interestingly, among all iPS lines, the gene expression levels in 2TF iPS cells were most similar to those in ES cells. In addition, we

confirmed the real-time PCR results at the protein level in immunofluorescence stainings for several markers such as Nanog, Oct4, Sox2, and SSEA-1 for the 2TF (Fig. 3B) and 4TF (supporting information Fig. 2) iPS lines.

After retroviral infection viral transgenes are integrated into the genome during iPS cell generation and successful reprogramming coincides with transgene silencing [8]. Therefore, we next tested the integration of the viral transgenes by genotyping PCR and silencing viral-specific transcripts by real-time PCR. The viral transgenes of all four factors were detected in 4TF iPS cells, whereas 3TF lacked the Sox2 transgene and 2TF iPS lines contained only the Oct4 and Klf4 transgenes, confirming that no cross-contamination occurred during the expansion of these clones (Fig. 3C). To verify that no cross-contamination occurred between individual 3TF and 2TF subclones, we corroborated the integration patterns and enumerated the integrated copy numbers by Southern blot analysis (supporting information Fig. 3). All tested iPS lines exhibited a distinct integration pattern and only one to four copies of each gene. Real-time PCR analysis with viral-specific primers further showed complete silencing of viral transcripts in 4TF, 3TF, and 2TF iPS lines with a 1,000-fold (4TF) to 100,000-fold (2TF) reduction of viral transgene expression levels already after only three passages (6 days) compared to freshly infected cells (Fig. 3D). Another hallmark of successful reprogramming of somatic cells into pluripotent stem cells is the epigenetic modification that needs to occur to reactivate ES genes that are silenced in differentiated cells by hypermethylation of gene promoters [8]. To this end, we analyzed the well-characterized Oct4 and Nanog promoter regions, which were methylated in DP cells and became demethylated in our iPS lines similar to control ES lines, demonstrating epigenetic reprogramming (Fig. 3E). Interestingly, the methylation status at the Nanog locus in DP cells was quite low (19.4%), which could account for the rapid and efficient reprogramming capacity of DP cells. Conversely, de novo hypermethylation was detected in iPS cells in the promoter region of the DP signature gene Fgf7 [24], which is unmethylated in DP cells (Fig. 3E). Furthermore, in addition to the de novo expression of pluripotency genes (Fig. 3A), all tested DP signature genes [24] were strongly downregulated in DP-derived 2TF iPS cells to levels similar to those of MEFs and ES cells, suggesting that the DP gene expression program was shut down in the conversion to a pluripotent cell fate (Fig. 3F). As expected, however, Akp2 continued to be expressed since AP activity is a marker of both DP and ES cells, which is typically stronger in DP cells (Fig. 3F and not shown).

To directly test the pluripotency of DP-derived iPS cells, we next assayed the capacity of 4TF, 3TF, and 2TF iPS cells to differentiate into cell types from all three germ layers in embryoid bodies in vitro. As shown in Figure 4A, DP-derived 2TF iPS cells effectively formed embryoid bodies, which differentiated into several cell types from endodermal, mesodermal, and ectodermal origins, as determined by real-time PCR (Fig. 4B). Immunofluorescence stainings for several lineage markers,

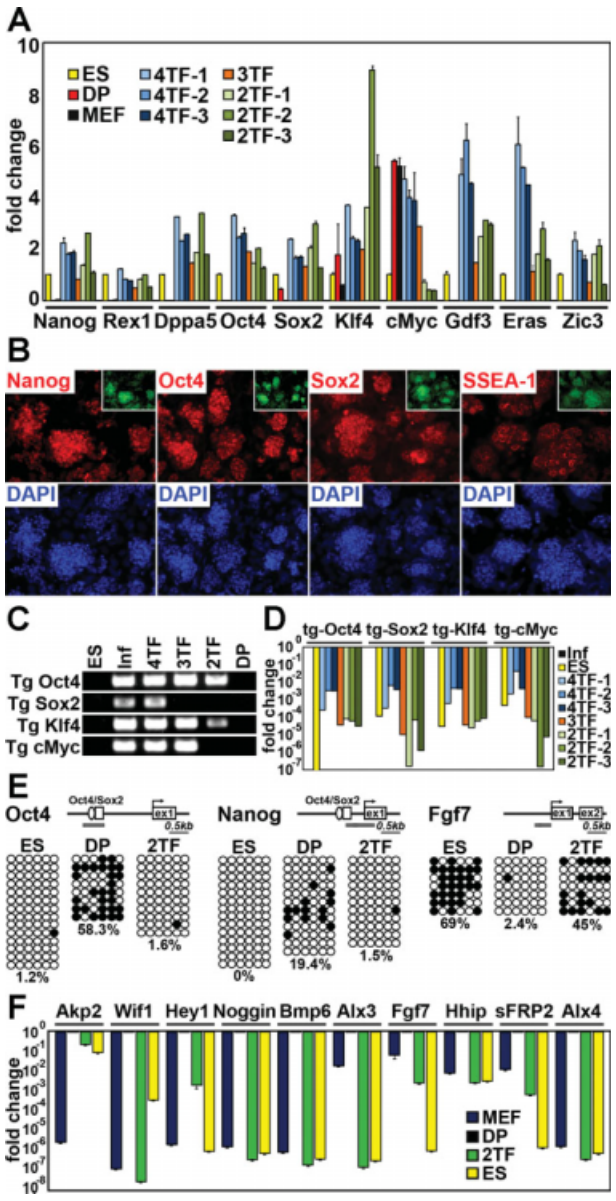

Figure 3. DP-derived 2TF iPS cells express pluripotency markers, silence the viral transgenes, and show epigenetic reprogramming. (**A**): Real-time polymerase chain reaction (PCR) of ES cell pluripotency genes and reprogramming factors. 4TF, 3TF, and 2TF iPS cells express all tested endogenous pluripotency genes. Note that 2TF iPS cells expression levels are most similar to ES cells. Levels were normalized to Gapdh and ES cells. (**B**): Immunofluorescence staining of pluripotency markers. 2TF iPS cells showed strong activation of endogenous pluripotency genes Nanog, Oct4, Sox2, and SSEA-1. The inset shows GFP expression from the Oct4 locus. DAPI staining highlighted all cells. (**C**): Genotyping PCR of viral transgene integration. Transgenes of all four factors were detected in 4TF iPS cells, whereas 3TF lacked the Sox2 transgene, and 2TF iPS cells contained only the Oct4 and Klf4 transgenes. ES and DP cells are negative controls. (**D**): Real-time PCR detecting viral-specific RNA expression confirmed efficient transgene silencing already after three passages (6 days). Levels were normalized to Gapdh and presented relative to freshly infected cells (Inf). (**E**): Methylation analysis of the Oct4, Nanog, and Fgf7 promoters by bisulfite sequencing in DP cells, DP-derived 2TF iPS cells, and ES cells. Schematic represents endogenous Oct4 and Nanog locus. The gray bar denotes the analyzed region. Each circle in horizontal rows represents an individual sequencing reaction and each vertical column represents a pair of CpG dinucleotides. Open circles and filled circles are unmethylated and methylated CpGs, respectively. The percentage of methylation (%) is indicated below each cluster. (**F**): Real-time PCR for a panel of DP signature genes [24] in MEFs, DP cells before reprogramming, reprogrammed 2TF iPS cells, and ES cells. All signature genes were strongly downregulated in iPS cells to levels similar to those in ES cells. Note that Akp2 remains to be expressed in iPS and ES cells as expected, albeit at lower levels. Levels were normalized to Gapdh and presented relative to DP cells. Abbreviations: DP, dermal papilla; iPS, induced pluripotent stem cells; MEF, mouse embryonic fibroblast; 4TF, four transcription factors; 3TF, three transcription factors; 2TF, two transcription factors.

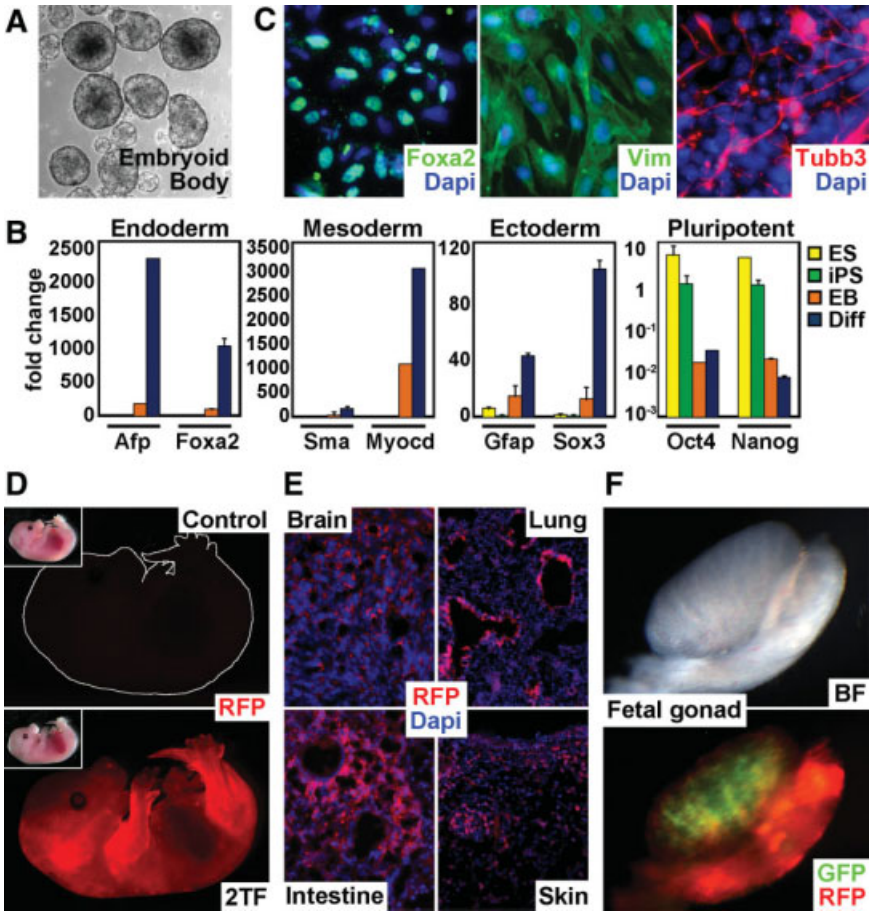

Figure 4. Two-factor DP-derived iPS cells give rise to lineages from all three germ layers in vitro and in vivo. **(A):** Embryoid bodies generated from 2TF iPS cells. **(B):** Real-time PCR of typical lineage markers after differentiation of embryoid bodies in lineage-specific culture conditions. Each marker is turned on in the appropriate differentiation environment. **(C):** Immunofluorescence analysis of differentiated embryoid bodies for markers of the three germ layers reveals expression of endodermal (Foxa2), mesodermal (Vim), and ectodermal (Tubb3) genes. **(D):** In vivo contribution of DP-derived 2TF iPS cells to developing embryos at E14.5. 2TF iPS cells were fluorescently labeled with dt-tomato (RFP) expressing lentiviruses and injected into blastocysts. Note the widespread contribution at the macroscopic level. **(E):** RFP detection in tissue sections confirmed contribution to organs from all germ layers, such as brain (ectodermal), lung and intestine (endodermal), and skin (mesodermal, ectodermal). **(F):** Germline contribution of 2TF iPS cells as shown by Oct4-GFP expression in the genital ridge of E14.5 embryos. Note also widespread detection of RFP from labeled iPS cells. Abbreviations: BF, brightfield; DP, dermal papilla; GFP, green fluorescent protein; iPS, induced pluripotent stem cells; RFP, red fluorescent protein; 2TF, two transcription factors; E14.5, embryonic day 14.5.

such as Foxa2 (endoderm), Vimentin (mesoderm), and Tubb3 (ectoderm), confirmed successful in vitro differentiation at the protein level (Fig. 4C).

The ultimate confirmation of pluripotency, however, involves successful in vivo contribution of iPS cells to developing blastocysts [8]. We injected DP-derived 2TF iPS cells into 8-cell-stage embryos, after permanently labeling the iPS cells by constitutive lentiviral expression of td-tomato, a RFP [10]. After transferring the blastocysts into pseudopregnant females, we obtained chimeric embryos and live pups with three independent iPS lines (supporting information Table 2). With the 2TF line #10 we obtained seven RFP-positive chimeric embryos out of a total of 17 embryos, suggesting that the integration of DP-derived 2TF iPS is very efficient. Fluorescence analysis of RFP expression showed that the iPS widely integrated throughout the embryo (Fig. 4D). Tissue sections confirmed iPS contribution to multiple organs, including brain, lung, intestine, and skin (Fig. 4E). Importantly, four out of seven chimeric embryos in line #10 showed germ cell integration as judged by robust detection of RFP and Oct4-GFP expression in the fetal gonads (Fig. 4F). Faithful GFP expression from the endogenous Oct4 locus in developing germ cells suggests that 2TF iPS cells potentially contributed to the germline.

Discussion

The goal of our study was to exploit our recent discovery that DP cells express all but one reprogramming factors [24] and to test whether easily accessible DP cells could be an improved source for effective iPS reprogramming. Our findings have three major implications: First, we established that DP cells are a cell type that is very rapidly and effectively reprogrammed into the pluripotent state (colonies form at day 5; 1.38% efficiency at day 10), suggesting that these cells could represent an improved starting population for generating iPS cells. DP cells are mesenchymal cells that specialize during embryonic development to induce de novo hair follicle fates from epidermal stem cells, to instruct stem cell progeny during hair growth, and to reactivate bulge stem cells to form a new follicle during the hair cycle [23, 31]. As such, DP cells are related in their mesodermal origin to mouse embryonic fibroblasts and skin fibroblasts, which undergo reprogramming at ~20- to 1400-fold lower efficiency (0.001%--0.08%) than DP cells [2, 6, 30]. This was surprising since it was previously suggested that cells of ectodermal origin, such as keratinocytes, neural stem cells, and melanocytes, might be reprogrammed faster and more efficiently because ES cells are embryonic ectodermal cells [28]. One possible explanation could be that DP cells already express high endogenous levels of c-Myc and Klf4 and also of Sox2 and AP, which might confer a more reprogrammable state. Interestingly, although DP cells were strongly methylated at the Oct4 locus, the Nanog promoter region was hypomethylated (19.4%), which is different from many other cell types that have been reprogrammed recently [21, 32], and might also explain why DP cells are very amenable to efficient reprogramming. In agreement with this notion, it was reported recently that Nanog is indispensable to transit to the pluripotent state [33] and the decreased methylation status at the Nanog promoter might facilitate rapid reactivation of the gene.

Second, DP cells can be reprogrammed into iPS cells by the two factors Oct4 and Klf4, and without exogenous Sox2

and the oncogenic factor c-Myc. DP-derived iPS cells generated with only two factors were similar to iPS cells produced with four factors both at the molecular and functional level, including the robust generation of chimeric mice. Omission of c-Myc from the reprogramming process is important since reactivation of the c-Myc virus can cause tumor formation [8]. Additional reduction of reprogramming factors to only two genes also decreases the likelihood of insertional mutagenesis, which is further reduced by our successful reprogramming with very low copy numbers of Oct4 and Klf4 (supporting information Fig. 3). Ultimately, reduction of reprogramming factors may also increase the likelihood of replacing the remaining virally delivered factors with temporal systems [10, 12] or with small molecules [17, 34].

The third main conclusion from our study is that to date DP cells are the only cells that are both easily accessible from skin and are reprogrammed into pluripotent cells without exogenous expression of Sox2 and c-Myc. In this regard, mouse neural stem cells, which also express high levels of Sox2, have been previously successfully reprogrammed by only two factors, Oct4 and Klf4, with similar speed and efficiency [20], yet isolating neural stem cells from humans would likely pose significant ethical and technical challenges. Remarkably, however, neural stem cells could be converted into iPS cells with Oct4 alone [22], which we could not accomplish so far, although DP cells express high levels of Klf4 as well. It is likely that we currently missed reprogramming events of DP cells with Oct4 alone that could occur much later than iPS generation with two factors, which is already significantly delayed by 3 weeks compared to four-factor reprogramming. We surmise that improvement of culture conditions should overcome this technical hurdle. In another recent attempt to define a cell type that is easily accessible from skin and that could also be reprogrammed with less factors, Utikal et al. showed that epidermal melanocytes could be converted into iPS cells without ectopic Sox2 expression because of endogenous Sox2 expression [28]. However, reprogramming was achieved only with three factors and was not possible without the oncogene c-Myc. In addition, reprogramming melanocytes into iPS cells appeared to be slower and less efficient than the iPS generation with DP cells (shown here). One possible explanation could be that the expression levels of Sox2 and c-Myc in DP cells are constitutively high (Fig. 1A--1D). Interestingly, we failed to detect endogenous Sox2 expression in hair follicle (Fig. 1A, 1B) and epidermal (not shown) melanocytes in vivo, suggesting that possibly low levels of Sox2 expression were induced in melanocytes in cell culture.

Several other studies recently reported that treatment with small molecules in combination with viral overexpression of Oct4 and Klf4 (OK) was sufficient to induce reprogramming in MEFs without exogenous or endogenous Sox2 expression [35, 36]. Although an inhibitor of histone methyl transferase (BIX) in combination with an L-channel calcium agonist (BayK) [35] or an inhibitor of transforming growth factor beta signaling [36] together with OK could reprogram MEFs without Sox2 at comparable efficiency to our experiments with OK in DP cells that already endogenously express Sox2, it is not clear whether chemical modulation with these small molecules could elicit unwanted off-target effects that could affect the long-term stability of reprogramming. In this context it appears to be a safer approach to use as starting population accessible cells, such as DP cells, that need as little manipulation as possible, because of their inherent gene expression that is preferential for pluripotency reprogramming. It is also worthwhile noting that all Sox2 replacement studies with small chemicals in combination with Oct4 and Klf4 alone were performed with MEFs, which are inherently different from primary adult fibroblasts [35–37]. In this regard it is interesting that replacement of Sox2 in primary fibroblasts seemed to work only by additional infection with the oncogene c-Myc [36, 37].

In this study, we have isolated pure DP cells from genetically labeled mice in combination with a new cell surface marker (Itga9) as a proof-of-principle that these accessible cells from skin can be reprogrammed efficiently and with only two factors. Although we used our established transgenic reporter system in combination with Itga9 to isolate near 100% enriched DP cells to unequivocally demonstrate that the reprogramming effects occur in DP cells, it is possible to isolate enriched DP cells (~60%) for further reprogramming studies by simply using our new cell surface marker in hair follicle preparations from wild-type mice (data not shown). It will also be interesting to test in the future whether DP cells isolated from human hair can be similarly reprogrammed by only two factors to translate our findings to a human setting. In this regard it is promising that human DP cells can also be easily isolated by simple microdissection from individual hair follicles [38] that are routinely prepared during hair transplantation surgeries. During this procedure thousands of follicles are isolated from a strip of skin removed from the back of the head [39]. Alternatively, 1 in 100 hairs plucked from the head contains a DP [40], and already 1-4 microdissected DPs are sufficient to initiate a successful culture [38, 40].

Conclusions

Our findings demonstrate that dermal papilla cells already express three of the four reprogramming factors and show that these cells are more reprogrammable than most other cell types, and simultaneously are easily accessible from hair follicles in the skin. This suggests that DP cells could represent an optimal source of cells for streamlined generation of skin-derived, patient-specific autologous pluripotent stem cells and for replacing the remaining two factors with small molecules for safe generation of transplantable cells.

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Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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