Polycistronic lentivirus induced pluripotent stem cells from skin biopsies after long term storage, blood outgrowth endothelial cells and cells from milk teeth

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A B S T R A C T
The generation of human induced pluripotent stem cells (hiPSCs) requires the collection of donor tissue, but clinical circumstances in which the interests of patients have highest priority may compromise the quality and availability of cells that are eventually used for reprogramming. Here we compared (i) skin biopsies stored in standard physiological salt solution for up to two weeks (ii) blood outgrowth endothelial cells (BOECs) isolated from fresh peripheral blood and (iii) children’s milk teeth lost during normal replacement for their ability to form somatic cell cultures suitable for reprogramming to hiPSCs.

We derived all hiPSC lines using the same reprogramming method (a conditional (FLPe) polycistronic lentivirus) and under similar conditions (same batch of virus, fetal calf serum and feeder cells). Skin fibroblasts could be reprogrammed robustly even after long-term biopsy storage. Generation of hiPSCs from juvenile dental pulp cells gave similar high efficiencies, but that of BOECs was lower. In terms of invasiveness of biopsy sampling, biopsy storage and reprogramming efficiencies skin fibroblasts appeared best for the generation of hiPSCs, but where non-invasive procedures are required (e.g. for children and minors) dental pulp cells from milk teeth represent a valuable alternative.

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1. Introduction

Human induced pluripotent stem cells (hiPSCs) generated from patients with genetic diseases hold great promise for disease modeling, safety pharmacology and drug discovery (Dambrot et al., 2011; Davis et al., 2011; Freund and Mummery, 2009).

This is particularly relevant for cells of the internal organs, for which biopsies are not routinely available and therefore analysis of the disease phenotype is hampered. hiPSCs are similar to human embryonic stem cells (hESCs) (Yamanaka, 2012) in that they self-renew and can differentiate into all somatic cell types of the human body.

Since the first derivation of hiPSCs in 2007 using fibroblasts cultured from skin biopsies and the retroviral expression of four pluripotency genes Oct3/4, Sox2, Klf4 and c-Myc (Takahashi et al., 2007), considerable research has been devoted to reprogramming other somatic cell types, also using other methods of gene delivery to the host cell. These include integrating methods (e.g. using lentiviruses or transposons) and a variety of non-integrating approaches (adenovirus, plasmid, protein, episomal vectors and RNA; reviewed in Tiscornia et al. (2011)). The obvious advantages of non-integrating methods are still limited by their relatively low efficiencies, high cost and labor intensity. In addition, general transfection methods require relatively large numbers of somatic cells. Integrating methods by contrast are reasonably efficient but the quality of the resultant iPSC lines may
be compromised by random integration of transgenes, altering endogenous gene expression, or resulting in incomplete silencing of transgenes after reprogramming (Mikkelsen et al., 2008; Sridharan et al., 2009; Takahashi and Yamanaka, 2006). Excissable systems for removing transgenes represent an important improvement in this respect.

In addition to skin fibroblasts, reprogramming of keratinocytes, hepatocytes, T-cells from peripheral blood, adipose tissue-derived stem cells, dental pulp from adult teeth (Tamaoki et al., 2010) and other cell types has been reported (reviewed in Dambrot et al. (2011)). Criteria for selecting which somatic cell type to reprogram include (i) tissue accessibility and invasiveness of the biopsy procedure, (ii) whether the tissue sample can be stored for prolonged periods during transport from clinic to laboratory, (iii) whether cell culture conditions are adequate for supporting proliferation of the somatic cells and (iv) the reprogramming efficiency of the particular somatic cell type.

Direct comparison of different methods is hampered by variability in reagents and experimental protocols. Here we studied the isolation and reprogramming efficiencies of three easily accessible somatic cell types with view to examining how normal constraints in the clinic impact the experimental outcome. For reprogramming we used an exciscable, polycistronic lentiviral vector, which can be easily produced in large quantities at low cost and only requires a small number of somatic cells. An additional dTomato reporter gene in the vector also enabled real-time monitoring of transduction efficiencies and silencing of transgenes. Skin fibroblasts and blood outgrowth endothelial cells (BOECs) were obtained by minimally invasive procedures (4 mm punch biopsy and collection of peripheral blood, respectively) and milk teeth were obtained after natural loss. Isolation of cells from all tissues was straightforward. More importantly, skin fibroblasts could be readily isolated at any time within two weeks after collection of the biopsy and reprogrammed with robust efficiencies even after storage of the biopsy for the entire period simply in cold physiological buffered saline. Similarly, cells from milk teeth could be easily isolated and reprogrammed at efficiencies comparable to skin fibroblasts. Isolation of BOECs showed patient-to-patient variability and required a longer period in culture than skin fibroblasts or dental pulp cells to obtain sufficient cells for reprogramming. Reprogramming of BOECs was also less efficient than that of skin fibroblasts or dental pulp cells. However, hiPSC lines from all three tissues displayed expression of typical markers of embryonic stem cells and differentiated readily into derivatives of ectoderm, endoderm and mesoderm in vitro. Although much of the methodology used is routine in many hiPSC laboratories, we have validated protocols here that (i) facilitate tissue collection and transport from distant sites and (ii) provide an additional non-invasive approach for use in minors.

2. Material and methods

2.1. Isolation of skin fibroblasts

Nine 4 mm punch biopsies were obtained from anonymously donated skin (from the Department of Dermatology, LUMC) and processed immediately (d0) as described below or stored in phosphate buffered saline (PBS) at 4 °C for 7 and 14 days (d7 and d14, respectively), before being processed (three biopsies per time point).

For fibroblast isolation, skin pieces were incubated overnight at 4 °C in 25 U/ml of dispase (Gibco) dissolved in Dulbecco’s Modified Eagle Media/F12 (DMEM/F12, Gibco). The next morning the biopsy was rinsed with PBS and the epidermis removed and discarded. Using a scalpel, the dermis was minced into small pieces of approximately 0.5 mm by 0.5 mm, then incubated in 0.75% collagenase A (Roche) and 2 U/ml of dispase (Gibco) in PBS for 1 h in a shaking water bath at 37 °C. The samples were mixed by gently vortexing every 10 min during incubation. During this period, the pieces disintegrated. At the end of incubation, fibroblast growth medium (Dulbecco’s Modified Eagle Media (DMEM) supplemented with 2 mM L-glutamine, 10 mM non-essential amino acids (NEAA), 75 U/ml penicillin, 75 μg/ml streptomycin, 50 μg/ml gentamicin, 1 mM sodium pyruvate (all Invitrogen), 10 μg/ml ascorbic acid and 10% fetal calf serum (FCS) (both Sigma)) was added. Cells were centrifuged for 5 min at 200 × g, resuspended in fibroblast growth media and plated in a 125 flask. Cells attached within one or two days. Upon reaching confluence, cells were passaged according to standard procedures using trypsin/EDTA (Gibco) at a split ratio of 1:3. Fibroblasts at passage 2 were used for reprogramming.

2.2. Isolation of dental pulp cells from milk teeth

Milk teeth from a 9 year old boy and a 10 year old girl were collected anonymously and stored dry at 4 °C overnight. Each tooth was washed with PBS, wrapped in Parafilm and plastic bags and mechanically crushed using a hammer. The pieces were incubated in a mixture of 4 mg/ml dispase (Gibco) and 3 mg/ml collagenase A (Roche) for 1 h in a shaking water bath at 37 °C, with gentle vortexing every 10 min. At the end of incubation, fibroblast growth media was added; the cells were centrifuged at 200 × g for 10 min and resuspended in fibroblast growth media containing 2.5 mg/L amphotericin B (Sigma). Cells were plated in a 6 cm dish until reaching confluence and subsequently split 1:3 using trypsin/EDTA according to standard procedures. Amphotericin B was removed after the first passaging. For reprogramming we used dental pulp cells at passage 3.

2.3. Isolation of BOECs

BOECs were isolated from anonymously donated peripheral blood, as described previously (Reinisch et al., 2009), with the exception that FCS (Sigma) was used instead of pooled human platelet lysate. In brief, 20 ml of blood was collected in a BD Vacutainer NH 170 IU and then mixed with complete EGM-2 media (Lonza) supplemented with 10% FCS and additional heparin (100 U/ml, Biochrom AG). After 24 h, erythrocytes were removed by gentle washes with PBS and the remaining cells cultured in EGM-2 with 10% FCS until colonies of cells with cobblestone morphology appeared 14–21 days later. Once colonies had reached a diameter of approximately 1 cm, cells were passaged using trypsin/EDTA. For reprogramming we used BOECs at passage 1–5. Ficoll-based isolation of BOECs was performed as described (Wang et al., submitted for publication).

2.4. Flow cytometry analysis of BOECs

BOECs were trypsinized and incubated with an antibody against CD31 (CD31-APC, Becton Dickinson) or CD34 (CD34 PerCP/Cy5.5, BD Pharmingen) for 30 min at 4 °C and subsequently analyzed with a LSRII FACS (BD Pharmingen).

2.5. Reprogramming vector and lentivirus production

Lentiviruses containing a self-inactivating polycistronic cassette encoding Oct3/4, Sox2, Klf4, and c-Myc (Warlich et al., 2011) were produced using polyethyleneimine to cotransfect HEK293T cells with the expression vector pRRL.PPT.SF.hOKSM.idTomato.-preFRT and the helper vectors pCMV–VSVG, pMDLg–RRE, and
The virus was harvested after 48 h. The amount of virus particles was measured by ELISA detecting HIV p24 using a kit (Zeptometrix) according to the manufacturer’s protocol and the virus titer was estimated by multiplying the p24 concentration (ng/ml) with a factor of 2500 (Carlotti et al., 2004).

2.6. Reprogramming skin fibroblasts

For lentiviral infection, $2 \times 10^4$ fibroblasts were seeded into one well of a 12-well plate and transduced 4 to 6 h later with the lentivirus at 0.4 multiplicity of infection (MOI) in the presence of 4 μg/ml polybrene (Sigma-Aldrich) in fibroblast media. The virus was removed after 24 h. Six days after transduction, the cells were harvested and subjected to flow cytometry analysis (LSRII FACS, BD) for quantification of dTomato expression. After determining efficiencies 10,000 fibroblasts were seeded on mouse embryonic fibroblasts (MEFs; $2 \times 10^6$ MEFs/10 cm dish) and cultured in hESC KOSR Medium (Dulbecco’s Modified Eagle Media (DMEM)/F12 supplemented with Glutamax, 10 mM NEAA, 25 U/ml penicillin, 25 μg/ml streptomycin, 100 μM β-mercaptoethanol, 20% knock-out serum replacement (KOSR; Invitrogen) and 10% FCS (Sigma)). Medium was changed every other day until the appearance of hESC-like colonies.

hiPSC lines were named according to the nomenclature suggested by Luong et al. (2011). The data shown in Figs. 1 and 2 were generated from hiPSC line C (LUMC0016iCTRL).

2.7. Reprogramming of dental pulp cells from milk teeth

Dental pulp cells from milk teeth were reprogrammed as described above for skin fibroblasts with MOIs of 0.5, 1 and 5. The hiPSC line G (LUMC0012iCTRL) was used to generate data, shown in Figs. 1 and 2.

2.8. Reprogramming of BOECs

BOECs were reprogrammed as described for skin fibroblasts, except that MEFs were added after attachment of transduced BOECs. Four days after transduction, BOECs were harvested and seeded on gelatinized 10 cm dishes in EGM-2/10% FCS medium. The following day, MEFs were added at a density of $1.7 \times 10^6$ cells/10 cm in MEF medium (DMEM supplemented with 2 mM L-glutamine, 10 mM non essential amino acids, 25 U/ml penicillin, 25 μg/ml streptomycin, and 10% FCS (Sigma)). After 24 h cells were cultured in hESC KOSR medium until the appearance of hiPSC colonies. The hiPSC line F (LUMC0018iCTRL) was used to generate data shown in Figs. 1 and 2.

2.9. Picking of hiPSC colonies and expansion

hESC-like colonies were picked manually, and cultured and expanded in mTESR1 according to the manufacturer’s protocol (Stem Cell Technologies).

Fig. 1. Characterization of hiPSCs derived from skin fibroblasts, dental pulp cells or BOECs. Bright field images of the somatic cells before transduction (column I). Undifferentiated hiPSCs express typical markers of hESCs (red: NANOG, TRA-1–81; green: OCT3/4, SSEA-4) as shown by immunofluorescent staining (columns II and III). Immunofluorescent stainings of spontaneous differentiation of hiPSCs into derivatives of the three germ layers (columns IV–VI: AFP (green): endoderm; βIII-Tubulin (red): ectoderm; CD31 (red): mesoderm; nuclei (DAPI, blue)). BOECs: Blood outgrowth endothelial cells and AFP: alpha feto-protein. Scale bar: 100 μm.
2.10. RT-PCR

Total RNA was isolated from undifferentiated hiPSCs using the NucleoSpin RNAII kit (Macherey–Nagel) according to the manufacturer’s instructions. cDNA was synthesized from 2 µg of RNA using the iScript cDNA synthesis kit (Invitrogen). Primers and PCR conditions were as previously described (Ohnuki et al., 2009). PCRs were performed with SilverStar Taq polymerase (Eurogentec). We used the Nkx 2-5eGFP/w hESC line (Elliott et al., 2011) as a standard for the expression of typical markers of undifferentiated pluripotent stem cells.

2.11. Microarray and PluriTest

For the whole genome microarray, total RNA was isolated from 10^6 cells with a RNA isolation kit (Qiagen) and hybridized on Illumina HT12v4 microarrays following the manufacturer’s protocol and as previously described (Müller et al., 2011). The raw microarray data was analyzed with the PluriTest algorithm as described (Müller et al., 2011).

2.12. Alkaline phosphatase (AP) staining and calculation of reprogramming efficiencies

Citrate–acetone–formaldehyde fixed colonies were exposed to an alkaline-dye mixture according to the manufacturer’s instructions. AP positive colonies were calculated as the number of AP positive colonies normalized to the number of cells seeded on MEFs after transduction.

2.13. Southern blot

DNA from hiPSC colonies was collected using phenol chloroform extraction. DNA was digested by SphI and run on a 0.7% TAE gel. The DNA was then transferred onto a nylon membrane and incubated with a 32P-labeled probe against RRE.

2.14. Spontaneous differentiation of hiPSCs into derivatives of the three germ layers

hiPSC colonies were passaged as described and pieces cultured in mTESR1 on Matrigel coated chamber slides (BD Falcon) for two days. Subsequently, cells were cultured in DMEM/F12 (Gibco) supplemented with 20% FCS to induce mesoderm or in DMEM/F12 supplemented with 1% FCS and 50 ng/ml Activin A (R&D systems) to induce both ectoderm and endoderm. The medium was changed every other day. After three weeks the cells were fixed with 2% PFA for 30 min at room temperature.

2.15. Immunofluorescent staining

Immunofluorescent staining was performed according to standard procedures. Briefly, PFA fixed cells were permeabilized with Triton X-100, blocked with 4% normal goat serum for 1 h before overnight incubation with the primary antibody (NANOG (1:500, Peprotech), SSEA-4 (1:30, Biolegend), OCT3/4 (1:100, Santa Cruz), TRA–1–81 (1:25, Biolegend), βIII–TUBULIN (1:2000, Covance), AFP (1:25, Quarrtet), CD31 (1:100, DAKO), von Willebrand factor (VWF, 1:1000 DAKO), and VE-cadherin (1:200 Santa Cruz)) at 4 °C. The next day, secondary antibodies labeled with Cy3 (1:200, Jackson Immuno Research), Alexa 568 (1:200, Invitrogen) or Alexa 488 (1:500, Invitrogen) were added for 1 h at room temperature. Nuclei were stained with DAPI before mounting slides with Mowiol (Calbiochem).

3. Results

3.1. Reprogramming cells from skin biopsies after long-term storage

Skin fibroblasts were isolated from three 4 mm punch biopsies under local anesthesia, which are regarded as minimally invasive and do not require stitching. Since immediate processing of biopsies is not always possible, for example, if patients are in clinics distant from the iPSC laboratory or even in different countries, we tested the
Table 1

<table>
<thead>
<tr>
<th>Day</th>
<th>Number of fibroblasts 7 days after isolation</th>
<th>Transduction efficiency (%)</th>
<th>Reprogramming efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0 (A)</td>
<td>1,090,000</td>
<td>3.2</td>
<td>0.02</td>
</tr>
<tr>
<td>Day 0 (B)</td>
<td>266,250</td>
<td>5.9</td>
<td>0.58</td>
</tr>
<tr>
<td>Day 0 (C)</td>
<td>236,250</td>
<td>11</td>
<td>0.84</td>
</tr>
<tr>
<td>Day 7 (A)</td>
<td>100,000</td>
<td>3.6</td>
<td>0.02</td>
</tr>
<tr>
<td>Day 7 (B)</td>
<td>109,375</td>
<td>4.7</td>
<td>0.33</td>
</tr>
<tr>
<td>Day 7 (C)</td>
<td>90,625</td>
<td>6.4</td>
<td>0.58</td>
</tr>
<tr>
<td>Day 14 (A)</td>
<td>100,000*</td>
<td>2.4</td>
<td>0.02</td>
</tr>
<tr>
<td>Day 14 (B)</td>
<td>37,500</td>
<td>2.6</td>
<td>0.18</td>
</tr>
<tr>
<td>Day 14 (C)</td>
<td>17,500</td>
<td>7.8</td>
<td>0.55</td>
</tr>
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</table>

Skin fibroblasts were isolated immediately after biopsy sampling (d0) or after 7 or 14 days of storage in PBS (d7 and d14) from three individuals (A, B, and C; left column). The total number of skin fibroblasts from each time point was counted one week after isolation (second column from left). Transduction efficiencies (% of cells expressing dTomato) were determined by flow cytometry analysis six days after transduction with 0.4 MOI (third column from left). Reprogramming efficiencies based on the number AP expressing colonies normalized to the number of cells seeded on MEFs (right column). AP: alkaline phosphatase; MOI: multiplicity of infection; MEFs: mouse embryonic fibroblasts.

* Number of cells after 14 days in culture.

effect of prolonged biopsy storage on fibroblast isolation and reprogramming efficiencies. In order to assess variability between skin samples of different origins, we took biopsies from three healthy individuals (samples A–C, Table 1). Fibroblasts were isolated from samples from each individual either immediately (d0) or after seven (d7) or 14 day (d14) storage in PBS at 4°C. At each time point, fibroblasts were isolated enzymatically, plated and counted after one week. As expected, fibroblasts grew readily from all d0 biopsies with samples yielding between ~2.5 × 10^5 cells and 1 × 10^6 cells (Table 1, samples A–C). Surprisingly, sufficient fibroblasts for reprogramming could also be isolated from biopsies after long-term storage, although the efficiency was clearly reduced. At d7 of processing, we obtained ~1 × 10^5 cells from each biopsy corresponding to 60% (Table 1, samples B and C) and 90% reduction compared to d0 processing (sample A). Fibroblast numbers were further reduced when the biopsy was processed after 14 days of storage (Table 1). Nevertheless, fibroblast numbers obtained after two weeks storage were sufficient for reprogramming since we only used 1–2 × 10^5 cells for lentiviral transduction. The lifespan of fibroblasts in culture is limited by the Hayflick’s factor (the maximum number of population doublings that a normal cell can undergo) and cells typically go into senescence after prolonged periods of culture (Esteban et al., 2010; Hayflick and Moorhead, 1961). Yet, a prerequisite for cellular reprogramming is that somatic cells are actively dividing (Banito and Gil, 2010; Chan et al., 2009). Since increased cell death during long-term storage of biopsies is likely to reduce the starting number of fibroblasts in the samples even before isolation, it can be inferred that a higher number of population doublings before reprogramming could take place. We therefore checked whether fibroblasts isolated from biopsies after long-term storage displayed any visible signs of senescence. As shown in Fig. 1, cells from d7 and d14 had typical spindle-like fibroblast morphologies similar to the d0 cells and did not show any obvious signs of senescence. We were thus able to obtain sufficient numbers of proliferating fibroblasts from small skin biopsies even after prolonged storage. Next, we tested whether cells isolated at different time points could be transduced with a polycistronic lentivirus carrying the four Yamanaka factors and a dTomato reporter (Warlich et al., 2011; Fig. 3A). Equal numbers of cells from d0, d7 or d14 were plated and transduced overnight at a MOI of 0.4. In an initial experiment we found that MOIs between 0.2 and 0.4 generate hiPSCs with one or two proviral integrations at favorable efficiencies (Supplementary Fig. 1). Fibroblasts were harvested six days post-transduction and subjected to flow cytometry for quantification of dTomato-expressing cells. In general the number of dTomato-positive cells did not exceed 1% (Table 1). For two out of three samples, transduction efficiencies in fibroblasts from d7 or d14 were lower compared to d0 fibroblasts with a maximal reduction of roughly 50% (Table 1, samples B and C). The transduction efficiency of sample A fibroblasts was low (d0 and d7) and was further reduced only at d14 (Table 1). Next, we tested whether fibroblasts isolated after long-term storage (d7 and d14) could still be reprogrammed to hiPSCs and whether the efficiencies differed from that of freshly isolated ones (d0). After flow cytometry analysis, transduced fibroblasts were seeded on MEFs and cultured in hESC media. When hESC-like colonies emerged, we used AP staining to quantify the number of hiPSC colonies. We found that AP staining closely overlaps with silencing of the transgenes (as determined by the absence of dTomato expression) indicating that AP staining can indeed serve as a preliminary marker to estimate the efficiency of reprogramming (Supplementary Fig. 2). Silencing of the transgenes is required for successful reprogramming (Chan et al., 2009). In a first experiment we reprogrammed fibroblasts from sample A. For freshly isolated cells (d0) we obtained a reprogramming efficiency of 0.02% (Table 1). Reprogramming efficiencies were similar for fibroblasts isolated from biopsies, which had been stored for 7 or 14 days (Table 1). Since the reprogramming efficiency for this particular sample was low compared to previous experiments (data not shown) we generated hiPSC lines from samples B and C. For both, reprogramming efficiencies were high (~0.6–0.8%, d0, Table 1). Reprogramming efficiencies were only slightly decreased when hiPSCs were generated from d7 or d14 fibroblasts (Table 1). Still, even at the lowest reprogramming efficiency (0.18%, 14d, sample B) we obtained 18 hiPSC colonies from 10,000 cells seeded on MEFs. This is far more than the average amount of three clones generally used to assess clonal variation. Colonies with hESC-like morphology were picked from d0, d7 and d14 samples between 28 and 42 days after transduction and could be readily expanded on Matrigel and mTESR1 (Fig. 3B). Undifferentiated hiPSCs derived from d0, d7 or d14 fibroblasts expressed markers typical of hESCs as assessed by immunofluorescent staining (Fig. 1) and RT-PCR (Fig. 2A). In addition, all lines were able to differentiate effectively into derivatives of the three germ layers in vitro (Fig. 1). In order to verify the pluripotent state, we used PluriTest to analyze undifferentiated hiPSCs derived from d14 fibroblasts. The most stringent assay of pluripotency in human pluripotent stem cells (PSCs) is generally regarded as teratoma formation after injection of undifferentiated cells into immunocompromised mice. Teratomas of hiPSCs should contain derivatives of all three germ layers. However, aside from requiring animal use and being time consuming, the results are difficult to quantify. By contrast, PluriTest is a bioinformatic assay which predicts pluripotency based on the comparison of the microarray data of a query sample with the expression profiles from more than 250 pluripotent stem cell lines as well as from non-pluripotent cells (Müller et al., 2011). The resulting “Pluripotency Score” indicates the extent to which the query sample contains a pluripotent signature whereas the “Novelty Score” reports whether the tested cells resemble normal PSCs (Müller et al., 2011). As shown in Fig. 2B, hiPSCs derived from d14 fibroblasts had a high Pluripotency Score, whereas their Novelty Score was low, indicating that they were indistinguishable from normal hESCs. In summary, we have developed an efficient protocol to generate hiPSCs from fibroblasts isolated from biopsies, even after prolonged storage in very simple and readily available conditions. To our knowledge, this is the first report showing that skin fibroblasts can be efficiently reprogrammed even when their isolation from biopsies is delayed for up to 14 days.
3.2. Reprogramming of blood outgrowth endothelial cells (BOECs)

Although punch biopsies are minimally invasive, the procedure can be unpleasant and not all patients agree to donate tissue in this way. Taking blood is even less invasive and often part of routine patient examinations. We therefore investigated whether peripheral BOECs could be reprogrammed into hiPSCs and how the efficiency compared with skin fibroblasts. To isolate BOECs, we used a method similar to that described by Reinisch et al. (2009). Briefly, 10–20 ml of blood was mixed at a ratio of 1:4 with EGM-2 endothelial growth media containing FCS and then cultured in endothelial growth media until colonies of cells with cobblestone-like morphologies emerged about three weeks later. Cells expressing the von Willebrand factor (VWF) and CD31 and were able to form typical tubular networks on Matrigel (not shown). Flow cytometry analysis revealed that the majority of the cells expressed CD31 (80%), whereas a smaller proportion was double positive for CD31 and CD34 (14%, data not shown). In the first experiment, we transduced BOECs at MOIs of 1, 10 or 25 and obtained a single hiPSC colony four weeks post transduction at 10 MOI, corresponding to a reprogramming efficiency of 0.001% (Table 2, sample D). The colony was picked and expanded on Matrigel in mTESR1 media. hiPSCs expressed typical markers of pluripotency, as above, and were able to differentiate into the three germ layers in vitro (not shown). The low reprogramming efficiency could have been caused by virus overload, thus we aimed at optimizing the amount of virus in the subsequent experiment: BOECs from a different individual were transduced with 1, 2 or 4 MOI. At 1 MOI 40% of the cells expressed dTomato and the percentage further increased up to 70% at 4 MOI (Table 2). The colony was picked and expanded on Matrigel in mTESR1 media. hiPSC colonies were transduced with increasing MOIs as indicated and transduction efficiencies determined by flow cytometry analysis of dTomato expressing cells (second column from left). Reprogramming efficiencies are based on the number of hESC-like colonies (BOECs) or AP expressing cells (dental pulp cells) normalized to the number of cells seeded on MEFs (right column). AP: alkaline phosphatase; MOI: multiplicity of infection; MEFs: mouse embryonic fibroblasts.

<table>
<thead>
<tr>
<th>MOI</th>
<th>Transduction efficiency (%)</th>
<th>Reprogramming efficiency (%)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>BOECs (D)</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>BOECs (E)</td>
<td>42</td>
<td>61</td>
</tr>
<tr>
<td>BOECs (F)</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>Tooth (G)</td>
<td>2.5</td>
<td>4.9</td>
</tr>
<tr>
<td>Tooth (H)</td>
<td>3.2</td>
<td>4.8</td>
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</table>

BOECs or dental cells were transduced with increasing MOIs as indicated and transduction efficiencies determined by flow cytometry analysis of dTomato expressing cells (second column from left). Reprogramming efficiencies are based on the number of hESC-like colonies (BOECs) or AP expressing cells (dental pulp cells) normalized to the number of cells seeded on MEFs (right column). AP: alkaline phosphatase; MOI: multiplicity of infection; MEFs: mouse embryonic fibroblasts.

Using the method for isolation of BOECs described above, we were unable to obtain BOECs from about half of the blood samples. In addition the number of BOEC-colonies clearly varied between patients and approximately $1 \times 10^5$ cells could be obtained in the best case. We therefore tried to isolate BOECs from 80 ml of blood with a standard Ficoll-based method. We were then able to obtain

![Fig. 3. Lentiviral vector and kinetics of somatic cell isolation and reprogramming. (A) Schematic of the polycistronic lentiviral vector used to generate iPSCs. (B) Isolation and reprogramming timelines for skin fibroblast, dental pulp cells, and BOECs, respectively. hiPSCs: human induced pluripotent stem cells; and BOECs: Blood outgrowth endothelial cells.](image-url)
BOECs in four out of five blood samples. Lower amounts of blood dramatically decreased the success rate for the isolation of BOECs (data not shown). For reprogramming, Ficoll-isolated BOECs were transduced with the lentivirus at 1, 2 or 4 MOI (Table 2, sample F). Although transduction efficiencies were low, we obtained two colonies at both 2 and 4 MOI, corresponding to a reprogramming efficiency of 0.018 and 0.022%, respectively. Again colonies at both 2 and 4 MOI, corresponding to a reprogramming efficiency of 16% at 5 MOI (Table 2). Transduced cells were plated on feeders and the number of hiPSC colonies was quantified with AP staining. Despite similar efficiencies, even if the skin samples had been stored for up to 14 months, the transduction was directly correlated with the amount of virus directly exposed to pathogens. Treatment with additional antibiotics and fungicides immediately after isolation can help overcome this problem.

Numerous reprogramming methods with different somatic cell types have been published (Eminli et al., 2009; Stadtfeld et al., 2008a; 2008b). Nevertheless, comparisons of efficiencies between laboratories remain difficult, even if the same reprogramming methods are used. The main reasons for this are differences in experimental procedures and the use of undefined cell culture components, which display high batch-to-batch variability. Although we did not reprogram skin fibroblasts, BOECs and dental pulp cells simultaneously, all of the hiPSC lines from the three tissue sources were generated with the same reprogramming method and under similar conditions that is with the same batches of FCS, lentivirus and MEFs. Our results show that skin fibroblasts and dental pulp cells can be isolated rapidly and are reprogrammable with similar kinetics and at similar efficiencies. As we had observed earlier for skin fibroblasts, low amounts of virus were sufficient to generate hiPSC with a maximum of two proviral integrations. For most of the clones, however, one transgene copy was sufficient for reprogramming. Since we used an excisable lentivirus, this offers the possibility of generating transgene-free hiPSCs by addition of the FLPe enzyme. This has been done routinely in independent experiments generating disease hiPSC lines (Dambrot et al., unpublished and Mikkers et al., unpublished).

Between the fibroblasts isolated from different individuals, we observed some variability in reprogramming efficiencies, although numbers of independent experiments were limited. For fibroblasts from individual A the efficiency of hiPSC generation was more than ten-fold lower compared to samples B and C. Nonetheless, transduction efficiencies were within the same range. This variability may therefore have been individual specific.

Compared to skin fibroblasts, dental pulp cells were derived in a completely non-invasive manner and thus represent an excellent source of somatic tissue for children. The reprogramming of dental pulp cells from adult teeth has been reported previously (Tamaoki et al., 2010), but the results here show that this is also efficient from juvenile teeth at the end of their lifespan even after dry storage at 4°C. This is important, since the time point of natural loss of teeth is often not precisely predictable and experimental planning may be more difficult for this type of tissue. We demonstrated that immediate processing is not required. In addition dental pulp cells can also be isolated from teeth, for which extraction for pathological reasons would require high amounts of EGM-2 media, which is expensive. As an alternative method we therefore tried to isolate BOECs from 80 ml of blood with a standard Ficoll-based method. Indeed this increased the efficiency for isolation of BOECs. Unfortunately, this would require high amounts of EGM-2 media, which is expensive. As an alternative method we therefore tried to isolate BOECs from 80 ml of blood with a standard Ficoll-based method. Indeed this increased the success rate for isolation of BOECs. Nevertheless, not every adult individual may agree to donate such a relatively large volume of blood. For pediatric samples a volume of 40 ml is regarded as the maximum. Thus, for children, blood as used here is not a likely tissue source for reprogramming. Another disadvantage of blood samples maybe their limited storage. For the isolation of BOECs, processing of the blood is initiated within 2 h after blood collection (Reinisch et al., 2009). In addition, culture of BOECs requires specific growth medium, which is relatively expensive. Importantly, compared to
fibroblasts and dental pulp cells, reprogramming of BOECs was slower and tended to be less efficient. Our experiments therefore indicated that BOECs may be less suitable for reprogramming than skin fibroblasts and dental pulp cells, at least with this lentiviral vector and experimental conditions used here. On the other hand, these cells could be ideal for reprogramming in other circumstances, for example, if differentiation of hiPSCs into the endothelial lineage is required. Effects of epigenetic memory, which predispose iPSCs to favor differentiation towards their cell of origin, have been reported for various cell types (Kim et al., 2011). In addition, BOEC-iPSCs lack irreversible rearrangements in genes involved in T cell and B lymphocyte function, contrary to iPSCs generated from these blood cell types (Loh et al., 2010). Finally BOECs are not exposed to UV irradiation of sunlight as in the case for skin fibroblasts. These cells may therefore have a lower risk of acquiring additional non-systemic mutations.

Since this work was completed, Geti et al. reported the generation of hiPSCs with retroviral vectors from late outgrowth endothelial progenitor cells (L-EPs, also called BOECs) from peripheral blood (Geti et al., 2012). Reprogramming efficiencies for L-EPs were higher compared to skin fibroblasts. In our hands the polycistronic lentivirus was able to reprogram skin fibroblasts and dental pulp cells at high efficiencies whereas hiPSC generation from BOECs tended to be low. The efficiency of reprogramming in individual cell types is likely to depend on the reprogramming method. Here we used a polycistronic lentivirus, which offers the possibility of transgene excision after reprogramming. In addition all reprogramming factors are on the same virus reducing the number of necessary integrations and eliminating potential variability between clones due to differences in factor stoichiometry.

Beside simple, inexpensive and fast isolation of cells and high reprogramming efficiencies for most samples, we show here that skin samples also offer the possibility of prolonged storage before processing. Although cell numbers were reduced, fibroblasts isolated after long-term storage in a simple salt solution, often the most readily available option in a standard hospital clinic, did not show any visible signs of senescence and were reprogrammed at similar or only slightly reduced efficiencies as fresh cells. Thus we have developed a robust protocol for obtaining actively dividing cells from small skin pieces which are equally suitable for reprogramming as freshly isolated samples. In some cases, immediate processing is impossible because of long distances between the patient center and the reprogramming facility. The ability to store skin samples overcomes this problem. Isolation of cell types from other somatic tissues, e.g. BOECs or T cells from blood, often requires almost immediate processing, excluding these tissues in such a case.

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Appendix A. Supporting information

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