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From teeth, skin, blood to heart : induced pluripotent stem cells as an in vitro model for cardiac disease

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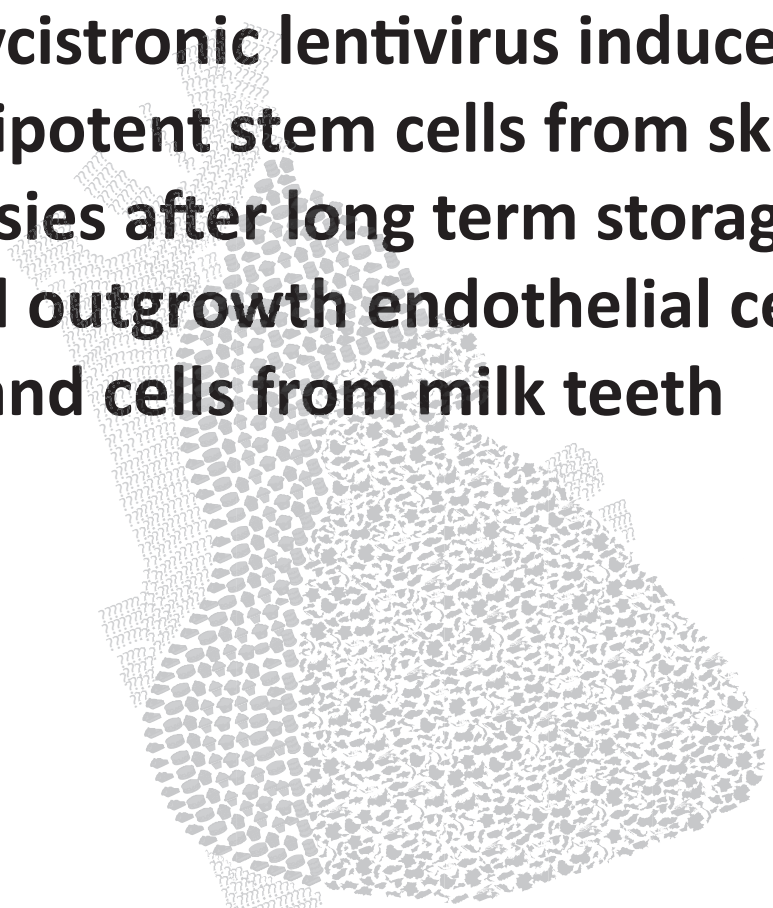
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Chapter 3:

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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Abstract

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 2 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.

Introduction

Human induced pluripotent stem cells (hiPSCs) generated from patients with genetic diseases hold great promise for disease modeling, safety pharmacology and drug discovery [1-3]. 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) [4] 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* [5], 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 [6]). 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 [7-9]. Excisable 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 [10] and other cell types has been reported (reviewed in [1]). 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 excisable, 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.

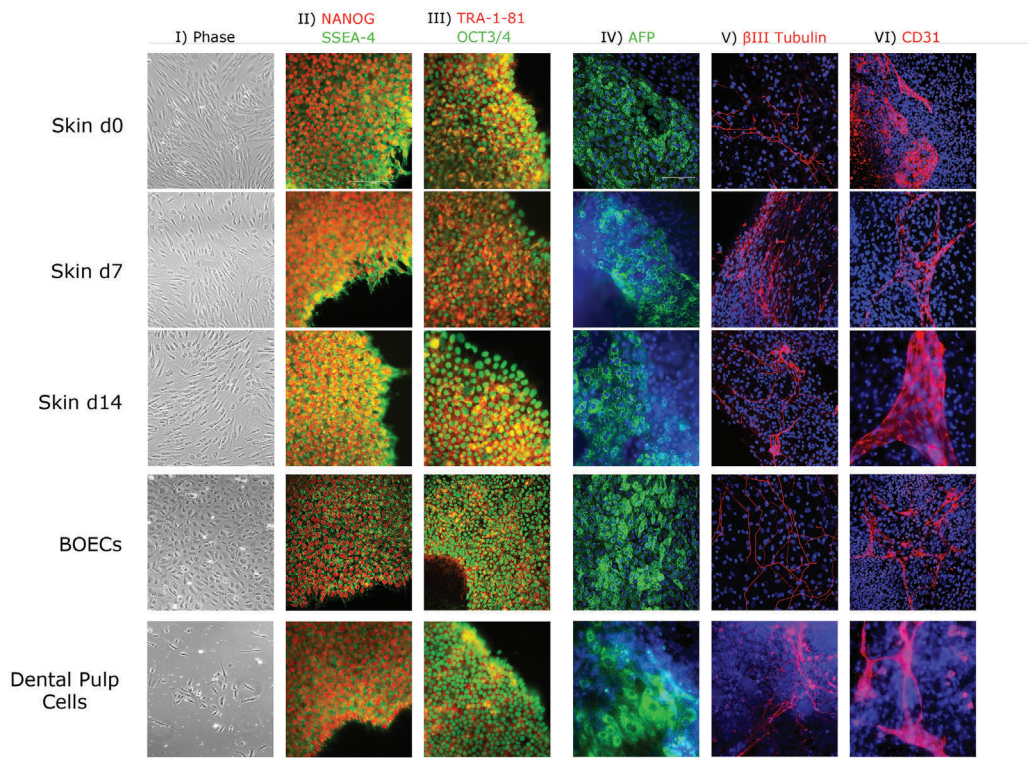


Figure 1: Characterization of hiPSCs derived from skin fibroblasts, dental pulp cells or BOECs. Bright field images of the somatic cells before transduction (column I). Undifferentiated hiPSCs express typical markers of hESCs (red: NANOG, TRA-1-81; green: OCT3/4, SSEA-4) as shown by immunofluorescent 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; β III-TUBULIN (red): ectoderm; CD31 (red): mesoderm; nuclei (DAPI, blue)). BOECs: Blood outgrowth endothelial cells; AFP: alpha feto-protein. Scale bar: 100 μ m.

Material and Methods:

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, 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 one hour in a shaking water bath at 37 °C. The samples were mixed by gently vortexing every ten minutes 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 μ g/ml streptomycin, 50 μ g/ml gentamicin, 1 mM sodium pyruvate (all Invitrogen), 10 μ g/ml ascorbic acid and 10 % fetal calf serum (FCS) (both Sigma)) was added. Cells were centrifuged for 5 min at 200 x g, resuspended in fibroblast growth media and plated in a T25 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.

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 hour in a shaking water bath at 37 °C, with gentle vortexing every 10 minutes. At the end of incubation, fibroblast growth media was added; the cells were centrifuged at 200 x g for 10 minutes 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.

Isolation of BOECs

BOECs were isolated from anonymously donated peripheral blood as described previously [11] 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 (100U/ml, Biochrom AG). After 24 hours 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 diameters of approximately 1 cm, cells were passaged using trypsin/EDTA. For reprogramming we used BOECs at passage 1 to 5. Ficoll-based isolation of BOECs was performed as described (Wang et al., submitted).

Flow cytometry analysis of BOECs

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

Reprogramming vector and lentivirus production

Lentiviruses containing a self-inactivating polycistronic cassette encoding *Oct3/4*, *Sox2*, *Klf4*, and *c-Myc* [12] were produced using polyethyleneimine to cotransfect HEK/293 T cells with the expression vector pRRL.PPT.SF.hOKSM.idTomato.preFRT and the helper vectors pCMV-VSVG, pMDLg-RRE, pRSV-REV. The virus was harvested after 48 hours. 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 [13].

Reprogramming skin fibroblasts

For lentiviral infection, 2×10^4 fibroblasts were seeded into one well of a 12-well plate and transduced 4 to 6 hours 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 hours. 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; 2×10^6 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 % knockout serum replacement (KOSR; Invitrogen) and 10 ng/ml basic FGF (PreproTech)). 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. [14]. The data shown in Figures 1 and 2 was generated from hiPSC line C (LUMC0016iCTRL).

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 Figures 1 and 2.

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 1.7×10^6 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, 10 % FCS (Sigma)) After 24 hours 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 Figures 1 and 2.

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).

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 [15]. PCRs were performed with SilverStar Taq polymerase (Eurogentec).

We used the Nkx 2-5^{eGFP/w} hESC line [16;16] as a standard for the expression of typical markers of undifferentiated pluripotent stem cells.

Microarray and PluriTest

For whole genome microarray total RNA was isolated from 1×10^6 cells with a RNA isolation kit (Qiagen) and hybridized on Illumina HT12v4 microarrays following the manufacturer's protocol and as previously described [17] The raw microarray data was analyzed with the PluriTest algorithm as described [17].

Alkaline phosphatase (AP) staining and calculation of reprogramming efficiencies

Citrate-acetone-formaldehyde fixed colonies were exposed to an alkaline-dye mixture according to the manufacture's instructions (Sigma-Aldrich, 86R-1KT). The reprogramming efficiencies were calculated as the number of AP positive colonies normalized to the number of cells seeded on MEFs after transduction.

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 probed against RRE.

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 3 weeks the cells were fixed with 2 % PFA for 30 min at room temperature.

Table 1: Effect of skin biopsy storage on reprogramming efficiencies.

	Number of fibroblasts 7 days after isolation	Transduction efficiency (%)	Reprogramming efficiency (%)
Day 0 (A)	1,090,000	3.2	0.02
Day 0 (B)	266,250	5.9	0.58
Day 0 (C)	236,250	11	0.84
Day 7 (A)	100,00	3.6	0.016
Day 7 (B)	109,375	4.7	0.33
Day 7 (C)	90,625	6.4	0.58
Day 14 (A)	100,000*	2.4	0.012
Day 14 (B)	37,500	2.6	0.18
Day 14 (C)	17,500	7.8	0.55
*number of cells after 14 days in culture			

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.

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 hour 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), β III-TUBULIN (1:2000, Covance), AFP (1:25, Quarttet), CD31 (1:100, DAKO), von Willebrand factor (VWF, 1:1000 DAKO), 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 hour at room temperature. Nuclei were stained with DAPI before mounting slides with Mowiol (Calbiochem).

Results:

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 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 $\sim 2.5 \times 10^5$ cells and 1×10^6 cells (Table 1, samples A-C).

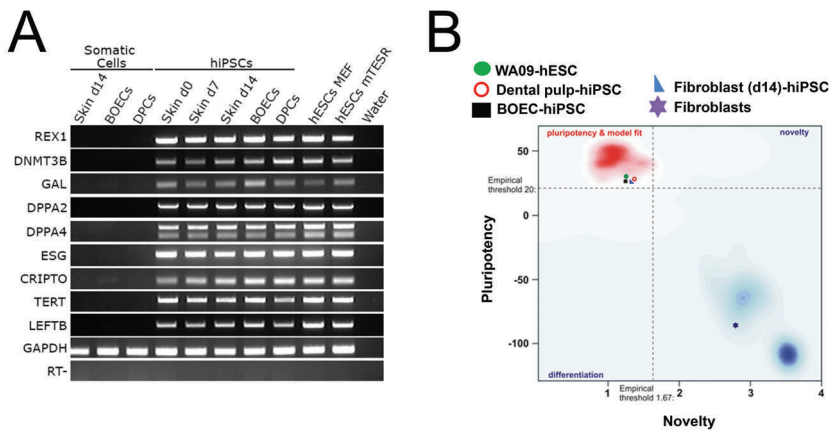


Figure 2: Undifferentiated hiPSCs express markers of pluripotent stem cells. A) RT-PCR analysis of the expression of pluripotent stem cell markers in somatic cells, hiPSCs and hESCs. GAPDH was used as a loading control. B) PluriTest analysis of hiPSCs derived from skin fibroblasts (d14), BOECs and dental pulp cells. All hiPSC lines have a high Pluripotency Score and a low Novelty Score indicating that they resemble normal pluripotent stem cells. The scores of a hESC line and fibroblasts, both from the reference pool are shown for comparison. BOECs: Blood outgrowth endothelial cells; DPCs: Dental Pulp Cells; hESCs: human embryonic stem cells; hiPSCs: human induced pluripotent stem cells; MEF: mouse embryonic fibroblasts.

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 $\sim 1 \times 10^5$ cells from each biopsy corresponding to 60 % (Table 1, samples B, 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 2 weeks storage were sufficient for reprogramming since we only used $1-2 \times 10^4$ 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 [18;19]. Yet a prerequisite for cellular reprogramming is that somatic cells are actively dividing [20;21]. 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 Figure 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 [12]. 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 11 % (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, 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 [21]. 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 eighteen 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 mTESR (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 hPSCs 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 [17]. 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 [17]. As shown in Figure 2B, hiPSCs derived from 14d 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.

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. [11]. 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 expressed 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 a 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). hiPSC colonies appeared late (4-7 weeks post transduction, Fig. 3B) and were again scarce. Due to the low numbers, we only quantified hiPSC colonies based on morphology and silencing of transgenes (absence of dTomato). Reprogramming efficiencies were generally low, with ~0.002 % at 1 MOI and a maximum of 0.008 % at 2 MOI (Table 2, sample E). hiPSC colonies were picked and expanded on Matrigel in mTESR1 media. Cells expressed the typical set of pluripotency markers and were able to differentiate into derivatives of all three germ layers in vitro (supplementary Fig. 3).

Table 2 :Transduction and reprogramming efficiencies for dental pulp cells and BOECs.						
	Transduction efficiency (%)			Reprogramming efficiency (%)		
	MOI			MOI		
	1	10	25	1	10	25
BOECs (D)	N/A	N/A	N/A	0	0.0011	0
	1	2	4	1	2	4
BOECs (E)	42	61	70	0.0019	0.0083	0.0058
BOECs (F)	2.5	4.9	16.2	0.012	0.064	0
	0.5	1	5	0.5	1	5
Tooth (G)	2.5	4.9	16.2	0.012	0.064	0
Tooth (H)	3.2	4.8	16.7	0.11	0.30	0.36

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 1×10^5 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 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 were pluripotent as assessed by immunofluorescent staining (Fig. 1) and RT-PCR (Fig. 2A) and readily differentiated into derivatives of the three germ layers (Fig. 1). In addition, BOEC-derived hiPSCs were pluripotent as assessed by PluriTest (Fig. 2B).

hiPSCs can thus be generated from BOECs isolated using different methods, although reprogramming efficiencies in general tend to be lower compared to skin fibroblasts under the same conditions.

Reprogramming cells from milk teeth

In the Netherlands and possibly elsewhere, medical ethics committees will generally not permit punch biopsies from children and minors unless part of a surgical procedure. In addition a volume of 40 ml is regarded as the maximum acceptable for pediatric blood collection. Previously reprogramming of cells obtained from dental pulp from adult individuals has been reported [10] Here we investigated whether milk teeth obtained from children after natural loss could also be used for this purpose. Fibroblast-like cells were isolated from teeth stored dry overnight at 4 °C and these expanded readily in culture reaching numbers between 4.5×10^5 and 7.5×10^5 cells after 16 or 19 days, respectively, with the capacity for further expansion as necessary for storage or tissue banking. Next we added increasing amounts of a lentivirus carrying the four Yamanaka factors and a dTomato reporter (0.5, 1 or 5 MOI). The transduction efficiency analyzed by flow cytometry at day 6 post transduction was directly correlated with the amount of virus although not linearly. Cells from both samples showed similar transduction efficiencies with a maximum of $\sim 16\%$ at 5 MOI (Table 2). Transduced cells were plated on feeders and the number of hiPSC colonies was quantified with AP staining. Despite similar transduction rates, we observed a patient-specific variation in reprogramming efficiencies (Table 2): Whereas one sample was reprogrammed at an efficiency similar to that of skin fibroblasts (sample (H), Table 2) the reprogramming efficiency of the second sample was about 5 times lower (sample G). hiPSC colonies from sample H (from all MOIs) were picked 32-42 days after transduction indicating that reprogramming kinetics of juvenile dental pulp cells and skin fibroblasts are approximately similar (Fig. 3B). Colonies expressed the typical set of pluripotency markers as assessed by immunofluorescent staining (Fig. 1) and RT-PCR (Fig. 2A) and differentiated readily into derivatives of the three germ layers in vitro (Fig. 1). Again PluriTest identified these hiPSCs as closely resembling normal hESCs (Fig. 2B). Thus hiPSCs can be generated from teeth obtained in a completely non-invasive manner from children at efficiencies similar to reprogramming of skin fibroblasts.

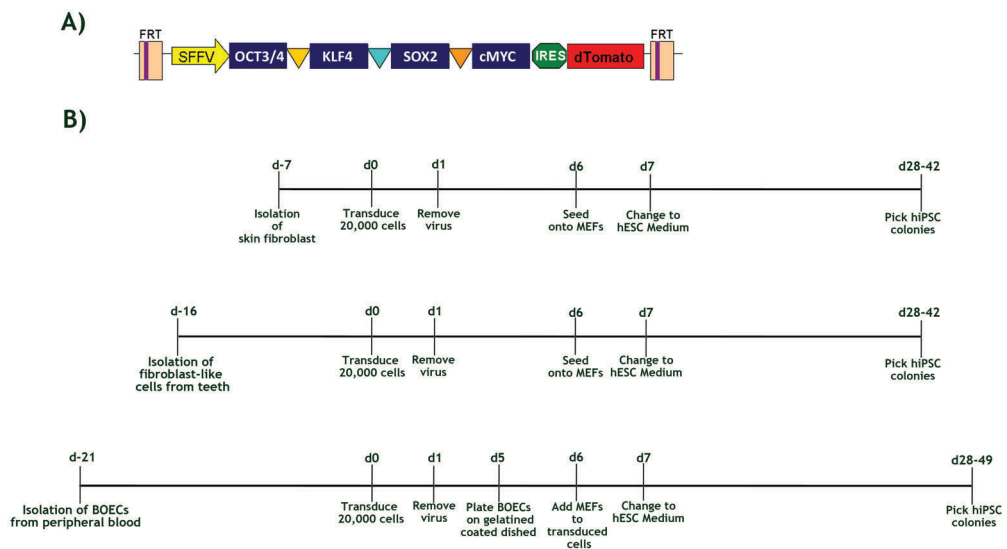


Figure 3: Lentiviral vector and kinetics of somatic cell isolation and reprogramming. A) Schematic of the polycistronic lentiviral vector used to generate iPSCs. B) Isolation and reprogramming timelines for skin fibroblast, dental pulp cells, and BOECs, respectively. hiPSCs: human induced pluripotent stem cells; BOECs: Blood outgrowth endothelial cells.

Discussion

In this study, we isolated cells from three easily accessible somatic tissue sources and compared reprogramming efficiencies using a polycistronic lentivirus. Skin fibroblasts and cells from milk teeth could be readily isolated and reprogrammed at similar efficiencies even if the skin samples had been stored for up to 14 days in a simple salt solution. By contrast the isolation efficiency of BOECs was variable from individual to individual in our hands and reprogramming efficiencies in general tended to be lower compared to the other two cell types.

Numerous reprogramming methods with different somatic cell types have been published [22-24]. 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. 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 tenfold 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 [10] 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 is scheduled. One disadvantage of this type of tissue however, is a higher risk of bacterial and fungal contamination since teeth are directly exposed to pathogens. Treatment with additional antibiotics and fungicides immediately after isolation can help overcome this problem.

Compared to the skin fibroblasts and dental pulp cells, isolation of BOECs from small amounts of blood was only successful in some of the samples and outgrowth of the cells was more time-consuming. The reason for this is possibly the low abundance of BOECs in peripheral blood. Indeed BOECs represent only a small fraction of mononuclear cells and their numbers vary considerably between donors [25]. This may be due to pathological conditions which influence the abundance of the cells in the blood [26]. It is likely that higher blood volumes would increase 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 two hours after blood collection [11]. 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 [27]. 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 [28]. 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-EPCs, also called BOECs) from peripheral blood [29]. Reprogramming efficiencies for L-EPCs 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.

Acknowledgements

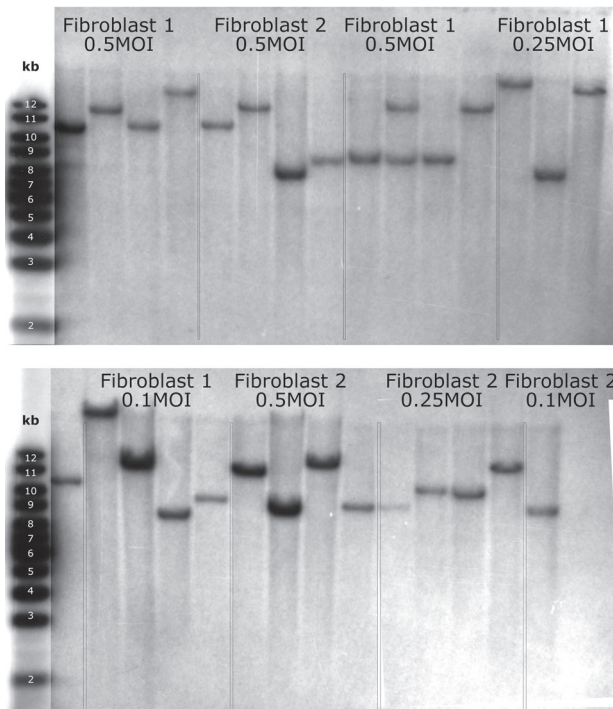
We thank A. Kraima (Anatomy and Embryology, Leiden University Medical Center (LUMC)) for collecting the blood samples, V. Orlova (Molecular Cell Biology, LUMC) for FACS analysis of the blood samples and M. Rabelink (Molecular Cell Biology, LUMC) for production of lentivirus. We also thank S. Commandeur, A. el Ghalbzouri (Dermatology, LUMC) for control skin samples; A. Riem (TandInZicht, Harmelen) for collection of milk teeth. We are grateful to J. Wang (Einthoven Laboratory for Experimental Vascular Medicine, LUMC) for assistance with the Ficoll-based isolation of BOECs. We thank C. Baum and A. Schambach (Department of Experimental Hematology, Hannover Medical School, Hannover, Germany) for the lentiviral vector used for reprogramming and the LUMC iPSC core facility for support. Financial support for hiPSC generation was provided by the Netherlands Institute of Regenerative Medicine (NIRM grant No. FES0908) and FP7 (Industem). The authors gratefully acknowledge the support of the Smart Mix Program of the Netherlands Ministry of Economic Affairs and the Netherlands Ministry of Education, Culture and Science. B. Brändl is supported by an Else-Kroener Fresenius grant to the ZIP stem cell lab.

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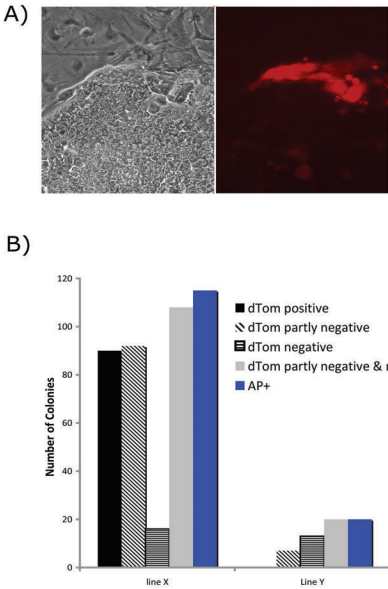
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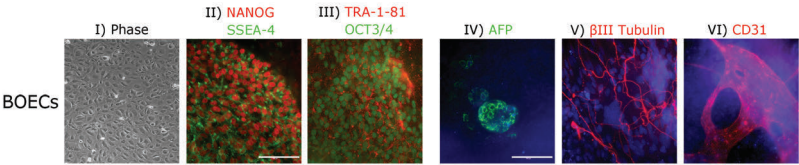
Supplementary Figures



Supplementary Figure 1: Transduction of skin fibroblasts with low MOI leads to the generation of hiPSC clones with a maximum of one or two proviral integrations. Southern blot of 29 hiPSC clones from 2 different fibroblast samples after transduction with MOI ranging from 0.1 to 0.5 confirming low integration number. MOI: multiplicity of infection.



Supplementary Figure 2: AP staining closely overlaps with silencing of the transgenes (absence of dTomato) A) An example of a partially dTomato negative colony is shown. These colonies are nevertheless likely to be fully reprogrammed because of their overall morphological similarity to hESCs. B) Quantification of colonies per 10 cm dish. Twenty four days after seeding, 10,000 transduced fibroblasts on a 10 cm dish with MEFs, the number of colonies which (i) expressed dTomato (“positive”), (ii) were partially negative or (iii) were completely negative for dTomato were counted. Subsequently AP-expressing colonies on the same plates were quantified. The sum of partially negative and negative colonies was similar (line X) or equal (line Y) to the number of AP expressing colonies. We therefore used AP staining as a preliminary marker to estimate reprogramming efficiencies in subsequent experiments. AP: alkaline phosphatase; dTom: dtomato.



Supplementary Figure 3: Characterization of hiPSC derived from BOECs (line E). 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 staining of hiPSCs following “spontaneous” differentiation into derivatives of the three germ layers (columns IV-VI: AFP (green): endoderm; β III-TUBULIN (red): ectoderm; CD31 (red): mesoderm; nuclei (DAPI, blue)). BOECs: Blood outgrowth endothelial cells; AFP: alpha feto-protein. Scale bar: 100 μ m.