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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 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.

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

Abbreviations: hESC, human embryonic stem cells; (h)iPSC, (human) induced pluripotent stem cell; BOECs, blood outgrowth endothelial cells; DMEM, Dulbecco's Modified Eagle Media; PBS, phosphate buffered saline; MEF, mouse embryonic fibroblast; AP, alkaline phosphatase; MOI, multiplicity of infection; NEAA, non-essential amino acids; FCS, fetal calf serum; KOSR, knock-out serum replacement; AFP, α-fetoprotein

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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). 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 (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 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.

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 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.

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, eBioscience) or CD34 (CD34 PerCPC/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 HEK/293T cells with the expression vector pRRL.PPT.SF.hOKSM.idTomato.-preFRT and the helper vectors pCMV–VSVG, pMDLg–RRE, and