Dental Pulp Stem Cells as Source for iPS Cell Banks

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ABSTRACT

Defined sets of transcriptional factors can reprogram human somatic cells to induced pluripotent stem (iPS) cells. In this study, we evaluated dental pulp stem cells (DPSCs) as a source of iPS cells. From all 6 DPSC lines tested with the conventional 3 or 4 reprogramming factors (*Oct3/4*, *Sox2*, *Klf4* with/without *c-Myc*), iPS cells were effectively established. Furthermore, determination of the human leukocyte antigen (HLA) types of 107 DPSC lines revealed 2 lines homozygous for all three HLA loci, *i.e.*, A, B, and DR, and showed that if an iPS bank will be established from these initial pools, the bank will cover approximately 20% of the Japanese population with perfect match. These data thus demonstrate a promising potential of DPSC collections as a source of iPS cell banks used for regenerative medicine.

INTRODUCTION

We previously showed that viral introduction of 4 transcription factors; *Oct3/4*, *Sox2*, *Klf4,* and *c-Myc*, reprogram mouse somatic cells into induced pluripotent stem (iPS) cells, which closely resemble embryonic stem (ES) cells (Takahashi and Yamanaka, 2006). The same or similar combination of factors can also generate human iPS cells (Takahashi *et al.*, 2007; Yu *et al.*, 2007; Aasen *et al.*, 2008; Lowry *et al.*, 2008; Park *et al.*, 2008; Loh *et al.*, 2009).

Direct reprogramming of patients' somatic cells would allow cell transplantation therapy free from immune-mediated rejections. An alternative approach is to establish an iPS cell bank consisting of various human leukocyte antigen (HLA) types. Safety issues have to be considered as to which types of somatic cells should be used for such iPS cell banks. Firstly, several methods of iPS cell generation without viral integration have been reported (Okita *et al.*, 2008; Stadtfeld *et al.*, 2008; Kaji *et al.*, 2009; Kim *et al.*, 2009; Soldner *et al.*, 2009; Woltjen *et al.*, 2009; Yu *et al.*, 2009; Yusa *et al.*, 2009; Zhou *et al.*, 2009). However, the efficiencies were extremely low in these non-integration induction methods. Thus, we have to carefully select cell sources to achieve reasonably high efficiency in generating non-integrated human iPS cells. A second important issue in constructing iPS cell banks is the availability of donor cells. Since we would have to collect donor cells from healthy volunteers, usage of medical waste, such as molar teeth, would be ideal. Furthermore, the

possibility of genetic abnormalities due to ultraviolet (UV) irradiation and/or reactive oxygen species in donor cells should be minimized. Lastly, the tumorigenisity of the neural cells derived from iPS cells generated from various tissues showed significant variation (Miura *et al.*, 2009). This novel issue also should be considered in selecting an appropriate source of the cells for generating iPS cell banks.

The current study was conducted to examine the potential use of collections of human dental pulp stem cells (DPSCs) as iPS cell banks. DPSCs are somatic stem cells obtained from human teeth (Gronthos *et al.*, 2000; Gronthos *et al.*, 2002; Takeda *et al.*, 2008). They have the capability for self-renewal and multi-lineage differentiation (Zhang *et al.*, 2006; Arthur *et al.*, 2008; Stevens *et al.*, 2008). Previously we isolated DPSCs from more than 180 patients, mostly from young patients with maturing wisdom teeth, and found that those cells are easy to handle, highly proliferative, and can be stored in liquid nitrogen for a long time by the conventional procedure (Takeda *et al.*, 2008). Since DPSCs can be obtained from removed wisdom teeth, no further procedures are required with respect to the donor. Wisdom teeth are routinely removed in many clinics, thus allowing a broad spectrum of HLA types to be easily obtained. Considering these characteristics of DPSCs, we evaluated the potential of DPSCs as a source of future iPS cell banks.

MATERIALS & METHODS

Cell Culture and iPS-DP Cells Generation

We collected normal human third molars from 6 patients under sufficient informed consent at Gifu University Medical Hospital, with the protocol having been approved by the Institutional Review Board. Isolation of DPSCs was performed according to our previous report (Takeda *et al.*, 2008). DPSCs were expanded in MSCGM (Lonza, Walkersville, MD, USA). We used these cells for the induction of iPS cells within 10 passages. Human dermal fibroblasts (HDFs) were cultured in DMEM with 10% FBS and penicillin/streptomycin. Human ES cell line (KhES01) was obtained from Kyoto University (Kyoto, Japan), and cultured on mitomycin C-treated SNL feeder layers (SNL cell line was obtained from Sanger Institute, Cambridge, UK) in Primate ES cell medium (ReproCell, Tokyo, Japan) supplemented with 4 ng/ml bFGF (Wako, Osaka, Japan). We generated iPS cells derived from DPSCs according to our previous protocol (Takahashi *et al.*, 2007).

Assessment of Reprogramming Efficiency

In order to determine the reprogramming efficiency of DPSCs, we counted ES-like colonies from 5×10^4 that had been induced with the reprogramming factors. After retroviral infection, we counted the number at 21 days post-infection obtained with the 4 factors and at 30 days in

the case of the 3 factors. Assays were performed in quadruplicate, and the average and standard deviation of the data were calculated. Student's *t*-test was used for statistical data interpretation.

Immunostaining

The cells were fixed with 4% paraformaldehyde for 10 min, and treated with PBS containing 5% normal goat or donkey serum, 1% BSA, and 0.2% TritonX-100 for 45 min. The primary antibodies and secondary antibodies have been described previously (Takahashi *et al.*, 2007). Nuclei were stained with 1 μg/ml Hoechst33342 (Invitrogen, Carlsbad, CA, USA).

RT-PCR and Real-Time PCR

Total RNA was purified with Trizol reagent (Invitrogen) and treated with Turbo DNA-free kit (Ambion, Austin, TX, USA) to remove genomic DNA contamination. RNA samples of human H9 ES cells were obtained from Dr. Kiichiro Tomoda (The Gladstone Institute, San Francisco, CA, USA). One microgram of total RNA was used for the reverse transcription reaction with Rever Tra Ace-α (Toyobo, Osaka, Japan) and dT_{20} primer (Toyobo), according to the manufacturer's instructions. PCR was performed with ExTaq (Takara, Shiga, Japan). For real-time PCR analysis, PCR amplification of cDNA was performed by using SYBR

Premix Ex Taq (Takara) and analyzed with Thermal Cycler Dice Real Time System (Takara). Primer sequences used for PCR are shown in Appendix Table 1.

Differentiation Assays *In Vitro* and *In Vivo*

Embryoid body-mediated differentiation was elicited as previously described (Takahashi *et al.*, 2007). Teratoma formation experiments were performed as in a previous study (Watanabe *et al.*, 2007), and the tumors were dissected and fixed with PBS containing 4% paraformaldehyde. Paraffin-embedded tissue was sliced and stained with hematoxylin and eosin.

RESULTS

Collection of DPSC Lines and Induction of iPS Cells with the Conventional Three or Four Reprogramming Factors

We have collected approximately 180 DPSC lines from young patients under informed consent at Gifu University Medical Hospital. Among them, we selected 107 lines as candidates for iPS cell generation (Table). Teeth were collected from patients mainly at age 13 to 20 and showed various developmental stages depending on the age of the patient (Appendix Fig. 1).

To evaluate the DPSC collection as a source for an iPS cell bank, we randomly selected 6 cell lines from the collection to induce iPS cells (Fig. 1A). DPSCs harvested from the dental pulps of the surgically extracted teeth (Appendix Fig. 1) showed a fibroblastic morphology (Fig. 2A). The generation of iPS cells from DPSCs was performed according to the methods previously described (Takahashi *et al.*, 2007); and we used one HDF cell line, described in our previous report (Takahashi *et al.*, 2007) as a control. By 14 days after infection of retroviruses expressing Oct3/4, Sox2, Klf4, and c-Myc, several ES-like colonies started to appear in 5 of the 6 DPSC lines (DP28, DP31, DP49, DP54, and DP87; Appendix Fig. 2). For convenience, hereafter we refer to iPS cells derived from DPSCs as iPS-DP, and those from HDFs as iPS-HDF. In contrast, DP75 and HDFs did not start to form ES-like colonies until 21 days. When the 3 factors without c-Myc were used for the induction, iPS-DP colonies could be observed at 20 to 25 days after retroviral infection for the 5 DPSC lines, as in the case with the 4 factors, DP75 required a longer time (30 days) for their induction. Next we compared iPS colony numbers among the DPSC lines. We observed significantly more iPS cell colonies with the five DPSC lines that showed faster colony formation than with DP75 or the control HDF line (Fig. 1B). For instance, with the 3 factors, the 5 DPSC lines showed higher efficiencies of iPS cell generation from 5×10^4 infected cells (0.01- 0.06%) than did DP75, which showed an extremely low efficiency (~0.002%). Despite

these differences in the reprogramming efficiency, there were no significant differences in infection efficiency between the DPSC lines and HDF (Appendix Fig. 3).

Previous reports suggested that the endogenous expression of the reprogramming factors affects the reprogramming efficiency and the requirement for these factors (Aasen *et al.*, 2008; Eminli *et al.*, 2008; Kim *et al.*, 2008; Maherali *et al.*, 2008). Among the reprogramming factors analyzed, endogenous Klf4 showed higher expression in DPSC lines than in HDFs (Fig. 1C, upper). Endogenous c-Myc expression in most DPSC lines was also slightly higher than that in HDFs (Fig. 1C, lower).

Characterization of iPS-DP Cells

To confirm the pluripotency of iPS-DP31 and iPS-DP75 generated with the 3 or 4 factors, we picked up several iPS-DP colonies (Fig. 2B) and clonally expanded them. Firstly, we confirmed that the iPS-DP clones had the same short tandem repeat (STR) types as the DPSCs from which these clones had originated (Appendix Table 2). By immunostaining, iPS-DP obtained with either the 3 factors (iPS-DP-3f) or the 4 factors (iPS-DP-4f) expressed undifferentiated human ES cell-specific cell markers such as SSEA-3, Tra-1-81, and Nanog, but did not express SSEA-1, a differentiation marker (Figs. 2C-2F). RT-PCR also showed that iPS-DP-3f and -4f cells expressed undifferentiated ES cell-marker genes such as *Oct3/4*, *Sox2*,

and *Nanog* at levels equivalent to those found in human ES cells and iPS-HDF (201B6), but that the DPSCs and HDFs did not express those genes (Fig. 2G). The pluripotency of iPS-DP cells was confirmed by EB-mediated differentiation (Figs. 3A-3F) and teratoma formation assay (Figs. 3G-3L). These data demonstrate that iPS-DP cells generated by the conventional reprogramming factors could differentiate into all three germ layers both *in vitro* and *in vivo*. Experiments performed to characterize the iPS-DP clones established in this study are summarized in Appendix Table 3.

HLA Typing

To estimate the coverage rate of our DPSC collection in the Japanese population, we determined the HLA types of 107 DPSC lines (Table) and identified 2 cell lines with homozygous HLA types at all 3 loci examined (A, B, and DR). The frequencies of haplotypes of these two homozygous cell lines were estimated to be 8.7% and 1.5% in Japanese population, based on the data provided by Japanese Red Cross Society (http://www.bmdc.jrc.or.jp/stat.html). This result suggests that our DPSC collection could cover approximately 20% of the Japanese population with a perfect match.

DISCUSSION

In the current study, we examined the potential of DPSC as a source for iPS cell banks. We found that iPS cells could be efficiently generated by the conventional reprogramming factors from DPSC lines derived from either the root-completed, root-forming or even early stage of root-completed stages. An advantage of DPSCs is that they can be isolated from removed wisdom teeth without any further surgical intervention with respect to the donor. Many cell lines can therefore easily be collected. Furthermore wisdom tooth DPSCs can be obtained from young patients with a low risk of bacterial contamination and genetic modification, because those teeth are aseptically obtained from the mandible and protected from UV and oxidative stress by surrounding hard tissues. These results demonstrate the promising potential of DPSCs as a source of iPS cell banks for use in regenerative medicine. With respect to safety, it is ideal not to use retrovirus vectors, as they integrate into the host DNA. Although we used retrovirus vector for delivery of the exogenous genes, the high efficiency of iPS cell generation from DPSCs would be advantageous even when non-integrating vectors or recombinant proteins will be used. In addition to the high efficiency of iPS generation and easy availability of donor cells, high proliferation and simple culture conditions would also encourage the generation of iPS cells from DPSCs with recent non-integrated methods.

Endogenous Klf4 expression seemed to correlate with high reprogramming

efficiency in DSPCs. However, it should be noted that the measured endogenous Klf4 expression level by real-time PCR did not completely correlate with the reprogramming efficiency of each DPSC line, suggesting that endogenous Klf4 expression may not be the sole factor responsible for reprogramming efficiency to DPSCs. Of the DPSC lines examined, only DP75 did not show iPS cell induction comparable to that of the other DPSC lines. DP75 was isolated from the oldest patient at the root-completed stage. Wisdom tooth maturation is generally completed around age 20. The other 5 DPSC lines were isolated from younger patients at the crown-completed, root-forming or just after root-completed stages. Possible explanations for the low reprogramming efficiency with DP75 are as follow: (1) DP75 may have contained fewer 'stem-like' cells than the remaining DP lines as the developmental stage proceeded. (2) There is a possibility that the low induction in DP75 was solely due to individual variation. Further studies are required to determine the precise mechanism for the relatively low efficiency of iPS cell generation of DP75.

It should be quite useful for regenerative medicine to establish iPS cell banks with a sufficient repertoire of HLA types, since the establishment of clinical-grade iPS cell lines from individual patients would require much time and high cost. Recently, Nakatsuji et al. estimated that a collection of 50 unique iPS cell lines having homozygous alleles of the 3 HLA loci (A, B, and DR) would cover ~90% of the Japanese population with a perfect match

of these loci (Nakajima *et al.*, 2007; Nakatsuji *et al.*, 2008). In this study, we determined the HLA types of 107 DPSC lines, and obtained 2 cell lines homozygous for these HLA loci, estimated to cover approximately 20% of the Japanese population with a perfect match. The easiness of isolation and handling of those cells will make it easy to expand the size of the bank in multiple institutes and even establish a number of iPS cell lines homozygous for 3 HLA loci.

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FIGURE LEGENDS

Figure 1. Generation of iPS cells from 6 DPSCs from our DPSCs collection. **(A)** Characteristics of DPSCs isolated from 6 patients whose cells were used for iPS cell generation. **(B)** Comparison of the number of ES-like colonies generated from 5 x 10^4 DPSCs and control HDFs, which had been transfected with retrovirus vectors expressing 4 factors (Oct3/4, Sox2, Klf4, and c-Myc) or 3 factors (Oct3/4, Sox2, and Klf4). We counted ES-like colonies induced by the 4 factors at 21 days and those by the 3 factors at 30 days post-infection. Mean numbers of colonies from quadruple experiments are shown, with error bars indicating SD. * $p < 0.05$, ** $p < 0.01$ vs. HDFs. **(C)** The expression levels of endogenous Klf4 and c-Myc were quantified by real-time RT-PCR. All DPSC lines expressed a significantly higher level of endogenous Klf4, and slightly higher level of endogenous c-Myc expression in most DPSC lines than did HDFs. Values were standardized to GAPDH, and then normalized to human ES (KhES01) cells (KhES). CC, crown-completed stage; RC, root-completed stage; RF, root-forming stage.

Figure 2. iPS-DP express ES cell-specific markers. **(A-B)** Typical morphology of DPSCs (A) and morphology of an iPS cell colony derived from DPSCs (B). Scale bar = 200 μm. **(C-F)** iPS-DP established from DP31 expressed pluripotency markers, SSEA-3 (D), Tra-1-81 (E), and Nanog (F), but not SSEA-1 (C), as judged by immunostaining. Nuclei were stained with Hoechst 33342. Scale bar = 100 μm. **(G)** RT-PCR analysis of ES cell-marker genes in iPS-DP cells obtained from DP31 (iPS-DP31) and DP75 (iPS-DP75) by using the conventional three (3f) or four (4f) factors. Numbers indicate different iPS-DP clones. Endogenous Oct3/4, Sox2, and Nanog were expressed in all the iPS-DP lines, as well as in human ES cells (H9 ES) and iPS-HDF (201B6), but not in DPSCs (DP31 and DP75) or HDFs. Nat 1 is an internal control. RT - indicate a negative control without reverse transcriptase.