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# Generation of human induced pluripotent stem cells from oral mucosa

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**Induced pluripotent stem (iPS) cells are one of the most promising sources for cell therapy in regenerative medicine. Using a patient's own genetically identical and histocompatible cells is the ideal way to practice personalized regenerative medicine. For personalized iPS cell therapy, the prerequisites for cell source preparation are a simple and safe procedure, no aesthetic or functional damage, and quick wound healing. Oral mucosa fibroblasts (OFs) may have high potential to fulfill these requirements. In this study, biopsy was performed in a dental chair; no significant incisional damage was recognized and rapid wound healing (within a week) was observed. We generated human iPS cells from the isolated OFs via the retroviral gene transfer of *OCT4*, *SOX2*, *c-MYC*, and *KLF4*. Reprogrammed cells showed ES-like morphology and expressed undifferentiated markers such as OCT4, NANOG, SSEA4, TRA-1-60, and TRA-1-81. Subsequent *in vitro* and *in vivo* analyses confirmed the pluripotency of resultant iPS cells, which matched the criteria for iPS cells. In addition, we found that the endogenous expression levels of *c-MYC* and *KLF4* in OFs were similar to those in dermal fibroblasts. Taken together, we propose that OFs could be a practical source for preparing iPS cells to achieve personalized regenerative medicine in the near future.**



[**Key words:** Oral mucosa fibroblasts; Induced pluripotent stem cells; Personalized medicine; Regenerative medicine]

Induced pluripotent stem (iPS) cells were originally developed from mouse dermal fibroblasts (DFs) via retroviral gene transfer with the 4 reprogramming factors Oct3/4, Sox2, Klf4, and c-Myc (1). However, it has been reported that both retroviral integration in the genome and use of the c-Myc oncogene may lead to tumor formation (2–4), which is a critical issue in regeneration therapy. New alternative methods for generating iPS cells using chemical additives or non-viral gene transfer have been recently reported to reduce the risk of tumor formation (5). Furthermore, characteristics of the cell source may also have some effects on the risk of tumor formation (6). Therefore, together with developing safe reprogramming methods, selecting a cell source is now considered an important issue for developing effective personalized iPS cell therapy.

Human cell sources for preparing iPS cells have been expanding rapidly; these cell sources include DFs (5), keratinocytes (7), bone marrow and dental tissue-derived mesenchymal stem cells (8,9), cord and peripheral blood (10,11), and neural and adipose stem cells (12,13). Higher efficiency for inducing iPS cells with fewer reprogramming factors has been demonstrated in somatic stem cells, although it is difficult to obtain and expand somatic stem cells (14). So far, DFs have been mainly used to produce iPS cells because these cells can be easily and safely obtained by surgery, and reproducibly reprogrammed (5).

Wounds in the oral mucosa are often experienced in daily life and are known to heal rapidly compared with other skin injuries. A possible explanation to rapid wound healing is that oral fibroblasts (OFs) produce high levels of active matrix metalloproteinase-2 (MMP-2) compared with DFs, and that MMP-2 may play an important role in rapid extracellular matrix reorganization and scarless wound healing (15,16). Therefore, we suggest that OFs might be an excellent cell source for iPS cells from patients themselves.

In this study, we successfully established iPS cells from human OFs using 4 reprogramming factors (OCT4, SOX2, c-MYC, and KLF4) via retroviral gene transfer. We propose that OFs could be a practical cell

*Abbreviations:* OFs, oral mucosa fibroblasts; DFs, dermal fibroblasts; iPS cells, induced pluripotent stem cells; MMP-2, matrix metalloproteinase-2; AP, alkaline phosphatase; EB, embryoid body; ES cell, embryonic stem cell; TiPS cells, tentative iPS cells.

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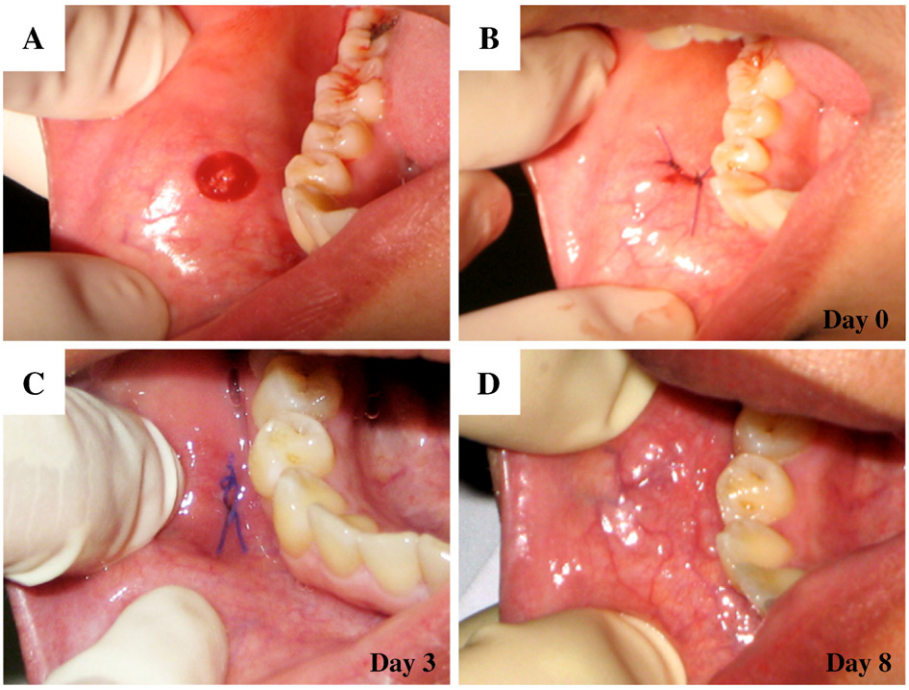


FIG. 1. Excision of oral mucosal tissue by punch biopsy. (A) Buccal surface immediately after dissection of oral mucosal tissue from the cheek of the volunteer by punch biopsy. (B) Wound closure. The wound was closed by two stitches of suture (day 0). (C) Buccal surface 3 days after surgery. The wound healed almost completely without a scar. (D) Buccal surface 8 days after surgery. The wound healed completely and became indistinguishable from the surrounding tissue.

source for preparing iPS cells to achieve personalized regenerative medicine in the near future.

## MATERIALS AND METHODS

**Cell cultures** Approval from the Institutional Research Ethics Committee of the University of Tokushima was obtained (Project No. 708). After receiving the agreement including an informed consent, human oral mucosa were obtained from healthy volunteers (aged: 26 to 35 years) in the Tokushima University Medical and Dental Hospital. The tissue was excised from the buccal mucosa using a 5-mm diameter disposable dermal punch (Nipro, Osaka, Japan), and the wound was sutured by 1–2 stitches. After the tissue was digested in 3 mg/ml dispase II (Roche, Mannheim, Germany) at 37 °C for 1 h with shaking, the epithelial cell sheet was removed from the mesenchymal tissue. OFs were migrated from mesenchymal tissue margin and began to proliferate in 3 to 4 days, cultured in Dulbecco's modified Eagle's medium (DMEM; Nissui) supplemented with 10% FBS (JRH Biosciences, Lenexa, KS, USA). At passage 3, OFs from a 27-year-old male were used for reprogramming experiments, and OFs from 27 to 31-year-old males were used for gene expression analysis.

Reprogrammed OFs were cultured on mitomycin C (Kyowa Hakko Kirin, Tokyo, Japan)-treated SNL feeder cells (17) (provided by Dr. Allan Bradley of the Sanger Institute), seeded on a type I collagen-coated dish (AGC Techno Glass, Tokyo, Japan) in hES medium (ReproCELL, Tokyo, Japan) supplemented with 4 ng/ml bFGF (Wako, Osaka, Japan). Reprogrammed OFs were cultured under 5% $CO_2$ in air at 37 °C and the medium was changed everyday. Passage of reprogrammed cells was used CTK solution instead of trypsin, and the cells were stored in DAP213 solution by a vitrification method (18).

Human normal skin fibroblasts (JCRB0534, TIG-114) were obtained from the Health Science Research Resources Bank (Osaka, Japan). The cells were maintained in Eagle's minimum essential medium (Nissui) supplemented with 10% FBS (JRH Biosciences), and harvested for RNA isolation at passage 3.

**Reprogramming of OFs** The reprogramming procedure was performed according to the protocol of Ohnuki et al. (19) with some modifications. In brief, the mouse ecotropic retrovirus receptor *Slc7a1* gene (Addgene, www.addgene.org) was introduced to 70% confluent human OFs by infection with lentivirus for 48 h. Transformants were selected by growth in 10 μg/ml blasticidin S (Funakoshi, Tokyo, Japan). Retrovirus production was carried out for 48 h in PLAT-E packaging cells (20) (provided by Dr. Toshio Kitamura, University of Tokyo) via transfection with pMIG-hOCT3/4, pMIG-hKLF4, pMIG-hSOX2, or pMXs-hc-MYC (Addgene). Blasticidin S-selected human OFs expressing the mouse *Slc7a1* gene were seeded at $1.3\times10^5$ cells/well in 6-well plates 1 day before transduction and introduced by retroviral infection that equally comprised 4 types of supernatants. After 2 days of transduction, the medium was replaced with DMEM supplemented with 10% FBS. After 6 days of transduction, infected OFs were reseeded at 4.3 to $4.45\times10^5$ cells per 100-mm culture dish on SNL feeder cells. The medium was replaced the following day with hES medium (ReproCELL) supplemented with 4 ng/ml bFGF and changed every 2 days. After 17 to 24 days of transduction, the colonies were transferred onto each SNL feeder in 24-well plates; we defined this stage as passage 1.

**Alkaline phosphatase staining and immunocytochemistry** Alkaline phosphatase (AP) staining and immunocytochemical procedure was performed as described previously (19) with some modifications. For immunocytochemistry, the primary antibodies were listed in Supplementary Table 1. The secondary antibodies, anti rabbit IgG, anti-mouse IgG, or anti-mouse IgM, were conjugated with Alexa Fluor-488 or -594 (1:500, Invitrogen).

**RNA purification and RT-PCR analysis** Total RNA was isolated using TRI reagent (Molecular Research Center, Cincinnati, OH, USA), treated with DNase I (Invitrogen), and used for cDNA synthesis with random primers using the RNA-PCR vol. 3.1 kit (Takara, Shiga, Japan) according to the manufacturer's protocols. RT-PCR analysis was performed using G*oTaq* polymerase (Promega, Madison, WI, USA). Gene-specific primers are given in Supplementary Table 2. Thirty cycles of PCR were performed for all genes except *GAPDH* (22 cycles). The annealing temperature was 58 °C.

**Embryoid body (EB) formation and in vitro differentiation** *In vitro* differentiation by EB formation was analyzed following the protocol of Ohnuki *et al.* (19) with some modifications. In brief, after 8 days of floating culture, tentative iPS (TiPS) clones formed EBs and were transferred to 0.1% gelatin-coated plates to induce further differentiation for 10 days. Differentiated markers such as α-fetoprotein for endoderm, α-smooth muscle actin for mesoderm, and neurofilament H for ectoderm were analyzed by immunocytochemistry.

**Karyotyping** Standard G-banding chromosome analysis was performed in the Nihon Gene Research Laboratories Inc. (Sendai, Japan). Selected iPS cells (TiPS-30) were analyzed at passage 19.

**Teratoma formation** Teratoma formation was examined following the protocol of Ohnuki *et al.* (19) with some modifications. In brief, nine 8-week-old SCID male mice (Nihon Crea, Tokyo, Japan) were anesthetized intraperitoneally with 2.5% tribromoethanol in butanol (0.014 ml/g body weight). Approximately 3.8 to 4.5Approximately $3.10^5$ TiPS cells were injected below the capsule of the testis. After 9 to 10 weeks of injection, tumors were dissected out and fixed with 4%

FIG. 2. Generation of tentative iPS cells (TiPS). (A) Time course for reprogramming oral fibroblasts (OFs) via retroviral gene transfer. (B) Phase contrast microscopy of original OFs in culture. (C) Established TiPS colonies on SNL feeder cells. (D) Blue color indicates AP staining in TiPS-30 clone. (E–J) Immunocytochemical analysis of pluripotent stem cell markers in TiPS-30 clone. (E) Oct 4; (F) Nanog; (H) SSEA4; TRA-1-60; TRA-1-81. (G) DAPI staining as a control for panels E and F. Panels H′, I′, and J′ indicate DAPI staining for panels H, I, and J, respectively. Scale bar indicates 1 mm in panels B–D and 200 μm in panels E–J.