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Induced pluripotent stem cell lines derived from human gingival fibroblasts and periodontal ligament fibroblasts

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Background and Objective: Human induced pluripotent stem (iPS) cells, which have similar properties to human embryonic stem (hES) cells, have been generated from neonatal and adult human dermal fibroblasts by reprogramming. iPS cells have high pluripotency and differentiation potential, and may be a potential autologous stem cell source for future regenerative therapy.

Material and Methods: iPS cell lines from human gingival fibroblasts and, for the first time, from periodontal ligament fibroblasts, were generated by reprogramming using a retroviral transduction cocktail of *OCT3/4, SOX2, KLF4* and c-*MYC*. iPS induction was investigated through expression of the embryonic stem cell markers SSEA4, OCT4, NANOG, GCTM-2, TG30 and TRA-1-60. Following *in vitro* differentiation, the expression of genes for differentiation markers for ectoderm (*SOX1*, *PAX6*), mesoderm [*RUNX1*, T(*Brachyury*)] and endoderm (*GATA4*, *AFP*) was assessed by real-time RT-PCR. The ability to form teratomas following implantation into mouse testes was assessed by histology.

Results: Human gingival fibroblast- and periodontal ligament fibroblast-derived iPS cells showed similar characteristics to hES cells. Both sets of iPS cells displayed colony morphology comparable to that of hES cells and expressed the hES cell-associated cell-surface antigens, SSEA3, SSEA4, GCTM-2, TG30 (CD9) and Tra-1-60, and the hES cell marker genes, *OCT4, NANOG* and *GDF3*. These iPS cells showed differentiation potential to form embryoid bodies *in vitro* and expressed genes for endoderm, ectoderm and mesoderm. Teratoma formation following implantation into mouse testes was observed.

Conclusion: These results demonstrate that iPS cells can be successfully generated from adult human gingival and periodontal ligament fibroblasts.

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Periodontitis is an infectious inflammatory disease causing the destruction of periodontal tissue and, in serious cases, leads to tooth loss (1). Once the periodontal tissue, including periodontal ligament and alveolar bone, is destroyed it is clinically difficult to reconstruct. As current procedures have shown unpredictable and limited potential for periodontal regeneration, the development of alternative strategies to regenerate

periodontal tissue is needed. Recently, periodontal ligament stem/stromal cells (PDLSCs) have been isolated from the periodontal ligament of extracted human teeth. These PDLSCs are capable of developing into adipocytes, osteoblast- and cementoblast-like cells *in vitro*, and demonstrate the capacity to produce cementum and periodontal ligament-like tissues *in vivo* (2). It is anticipated that utilizing the potential of PDLSCs to generate functional tissues may be an attractive new therapeutic option for periodontal regeneration (3,4). *In vivo* studies have recently demonstrated that autologous PDLSC implantation into periodontal defects can lead to the regeneration of periodontal tissue, without inflammation, in animal (5) and in human (6) trials. Furthermore, Sonoyama *et al*. (7) reported the generation of tooth-like constructs in a swine model using human PDLSCs in combination with different biomaterials and other stem cell populations isolated from the root apical papilla of human teeth. However, utilizing mesenchymal stem cells has limitations with regards to collecting sufficient cells from each patient as a result of variations in age, health condition and cell function.

In 2006, pluripotent stem cell populations, termed induced pluripotent stem (iPS) cells, were generated from mouse embryonic fibroblasts (MEFs) and adult mouse tail-tip fibroblasts by the overexpression of four transcription factors: *OCT3/4, SOX2,* c-*MYC* and *KLF4* (8). Mouse iPS cells display comparable properties to embryonic stem (ES) cells in terms of morphology, proliferation and gene expression *in vitro* and teratoma formation *in vivo*, and have the potential to generate viable chimeras after injection into blastocysts, demonstrating germline transmission (9–11). Recent studies have successfully generated iPS cells from neonatal and adult human dermal fibroblasts by the overexpression of *OCT4* and *SOX2* with the combination of either *KLF4* and *MYC* (12,13) or *NANOG* and *LIN28* (14). Human iPS cells, which have comparable capacity to human ES (hES) cells, are expected to be an alternative source of large quantities of pluripotent cells for regenerative research.

Apart from skin fibroblasts, iPS cells have been established from various cell types, such as mouse B lymphocytes, hepatocytes, gastric epithelial cells, neural stem cells, human blood cells and keratinocytes (15–21). Some current studies report that iPS cells generated from one tissue, such as blood cells, have a greater capacity to form cells from that tissue than fibroblast-derived iPS cells (22,23). This provides a good rationale for using periodontal ligament-derived iPS cells for periodontal regeneration. More recently, it has been reported that iPS cells can be established from mesenchymal-like stem/progenitor cells of dental tissue origin, such as stem cells from dental pulp cells (24,25), oral mucosa fibroblasts (26) and gingival fibroblasts (GFs) (27).

While iPS cells can theoretically be produced from any cell, it seems that finding an easily accessible source with high efficiency of iPS cell generation would be beneficial. Although the mechanisms of iPS cell generation are still unclear, it has been proposed that reprogramming may be more efficient from populations of undifferentiated stem cells within tissues (19). Because of the fetal-like qualities of adult gingiva (28–31), and the high turnover rate of periodontal ligament (32,33), we hypothesize that these two tissues may be a good alternative source of parent cells suitable for iPS cell induction.

Accordingly, in this study we investigated whether iPS cells could be established from fibroblasts derived from human periodontal ligament and gingivae by reprogramming these cells through retroviral transduction of *OCT3/4, SOX2, KLF4* and c-*MYC*.

Material and methods

Cell culture

Periodontal ligament fibroblasts (PDLFs) and GFs were obtained from healthy human premolars extracted and collected, with informed consent (Human Research Ethics Committee of the University of Adelaide; Approval Number H-112-2008), from healthy patients, both female, 15 (donor 1) and 32 (donor 4) years of age, undergoing orthodontic therapy in the University of Adelaide Dental Hospital. Periodontal ligament was scraped from the middle third of the root surface and gingival tissue was retrieved from the crown surroundings, as previously described (34). Both tissues were digested with collagenase I (3 mg/mL; Worthington Biochemical, Lakewood, NJ, USA) and dispase II (4 mg/mL; Roche Diagnostics, Indianapolis, IN, USA) for 2 h at 37°C to obtain single-cell suspensions. Explant cultures were maintained in α-minimal essential medium (α-MEM; Sigma-Aldrich, St Louis, MO, USA) containing 10% fetal calf serum (FCS; Thermo Electron, Melbourne, Vic., Australia), 2 mM L-glutamine (Sigma-Aldrich), 100 μM L-ascorbate-2-phosphate (Wako Pure Chemical Industries, Richmond, VA, USA), 1 mM sodium pyruvate (Sigma-Aldrich), 50 U/mL of penicillin G (JRH Biosciences, Lenexa, KS, USA) and 50 μg/mL of streptomycin (JRH Biosciences). *Ex vivo*-expanded PDLFs and GFs were maintained under the same culture conditions with 10% FCS, and used in this study between the third and sixth passages. During transduction steps, PDLFs, GFs and the Platinum-E Retroviral Packaging Cell Line (PLAT-E; Cell Biolabs, San Diego, CA, USA) were maintained in Dulbecco's modified Eagle's minimum essential medium (DMEM; Sigma-Aldrich) containing 10% FCS, 50 U/mL of penicillin G and 50 μg/mL of streptomycin. The cell line 293FT (Invitrogen, La Jolla, CA, USA) was maintained in DMEM containing 15% FCS, 6 mM L-glutamine, 0.1 mM non-essential amino acids (Sigma-Aldrich), 1 mM sodium pyruvate, 50 U/mL of penicillin G and 50 μg/mL of streptomycin. iPS cells were generated and maintained in hES cell medium, which is composed of 20% Knockout serum replacement (Invitrogen, Mulgrave, Vic., Australia)/DMEM/F12 (Invitrogen), 2 mM L-glutamine, 0.1 mM non-essential amino acids, 50 U/mL of penicillin G, 50 μg/mL of streptomycin, 90 μM β-mercaptoethanol (Invitrogen) and 10 ng/mL of basic

fibroblast growth factor-2 (FGF-2) (Millipore, Temecula, CA, USA).

Lentiviral production and transduction

The mouse Slc7a1 lentiviral vector was transduced into PDLFs and GFs following the Yamanaka laboratory method (12,35). 293FT cells (4×10^6 cells/100 mm dish) were plated and incubated at 37°C overnight. Cells were transfected with 3 μg of pLenti6/UbC/mSlc7a1 (Addgene, Cambridge, MA, USA) and 9 μg of Virapower packaging mix (Invitrogen) using Lipofectamine 2000 (Invitrogen). The next day, PDLFs and GFs were seeded at 8×10^5 cells/100 mm dish and incubated at 37°C overnight. Forty-eight hours after transfection, the culture media from the PDLFs and GFs was replaced with virus-containing supernatants of 293FT, which were collected and filtered through a 0.45-μm filter (Sartorius Stedim Biotech, Goettingen, Germany) supplemented with 4 μg/mL of polybrene (Sigma-Aldrich) and incubated overnight.

Retroviral production, reprogramming and iPS generation

Human *OCT3/4, SOX2, KLF4* and c-*MYC* retroviral vectors, previously used for dermal fibroblast cell reprogramming (12,35,36) were transduced into Slc7a1-transduced PDLFs and GFs. PLAT-E cells (8×10^6 cells/100 mm dish) were plated and incubated overnight. Cells were transfected with 9 μg of each pMXs plasmid DNA (encoding *OCT3/4, SOX2, KLF4* and c-*MYC*; Addgene) and Fugene 6 transfection reagent (Roche, Mannheim, Germany). The next day, PDLFs and GFs expressing the mouse *Slc7a1* gene were seeded at 8×10^5 cells/100 mm dish and incubated overnight. Forty-eight hours after transfection, the culture media from the PDLFs and GFs was replaced with equal amounts of PLAT-E supernatants containing each of the four retroviruses, which were collected and filtered through a 0.45-μm filter supplemented with 4 μg/mL of polybrene and incubated overnight. Retrovirus-containing supernatant was

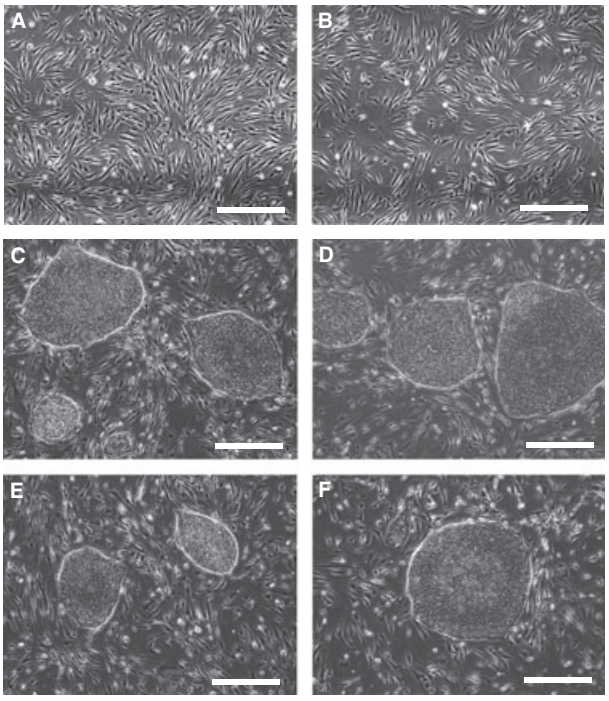

Fig. 1. Morphology of gingival fibroblast-induced pluripotent stem (GF-iPS) cells and periodontal ligament fibroblast-induced pluripotent stem (PDLF-iPS) cells. (A) Human GFs from donor 1 before retroviral transduction. (B) Human PDLFs from donor 1 before retroviral transduction. (C) Embryonic stem (ES) cell-like colonies originating from gingival fibroblasts (donor 1, passage 6). (D) ES cell-like colonies originating from PDLFs (donor 1, passage 6). (E) ES cell-like colonies originating from GFs (donor 4, passage 6). (F) ES cell-like colonies originating from PDLFs (donor 4, passage 6). Bars represent 500 μm.

Table 1. Primer sequences (12)

Primer	Sequence (5′–3′)	Applications
hOCT3/4-S944	CCCCAGGGCCCCATTTTGGTACC	Tg OCT4
hSOX2-S691	GGCACCCCTGGCATGGCTCTTGGCTC	Tg SOX2
hKLF4-S1128	ACGATCGTGGCCCCGGAAAAGGACC	Tg and endo KLF4
hMYC-S1011	CAACAACCGAAAATGCACCAGCCCCAG	Tg c-MYC
pMXs-AS3200	TTATCGTCGACCACTGTGCTGCTG	Tg
hOCT3/4-S1165	GACAGGGGGAGGGGAGGAGCTAGG	Endo OCT3/4
hOCT3/4-AS1283	CTTCCCTCCAACCAGTTGCCCCAAAC	
hSOX2-S1430	GGGAAATGGGAGGGGTGCAAAAGAGG	Endo SOX2
hSOX2-AS1555	TTGCGTGAGTGTGGATGGGATTGGTG	
hKLF4-AS1826	TGATTGTAGTGCTTTCTGGCTGGGCTCC	Endo KLF4
hMYC-S253	GCGTCCTGGGAAGGGAGATCCGGAGC	Endo c-MYC
hMYC-AS555	TTGAGGGGCATCGTCGCGGGAGGCTG	

Table 2. TaqMan gene-expression assays

Gene symbol	Assay ID	Application
POU5F1 (*OCT3/4*)	Hs01895061_u1	Endogenouse pluripotent
NANOG	Hs02387400_g1	gene expression
GDF3	Hs00220998_m1	
GATA4	Hs00171403_m1	Endoderm gene markers
AFP	Hs00173490_m1	
SOX1	Hs00534426_s1	Ectoderm gene markers
PAX6	Hs00240871_m1	
RUNX1	Hs00231079_m1	Mesoderm gene markers
T (*Brachyury*)	Hs00610080_m1	
GAPDH	Hs99999905_m1	Endogenous controls

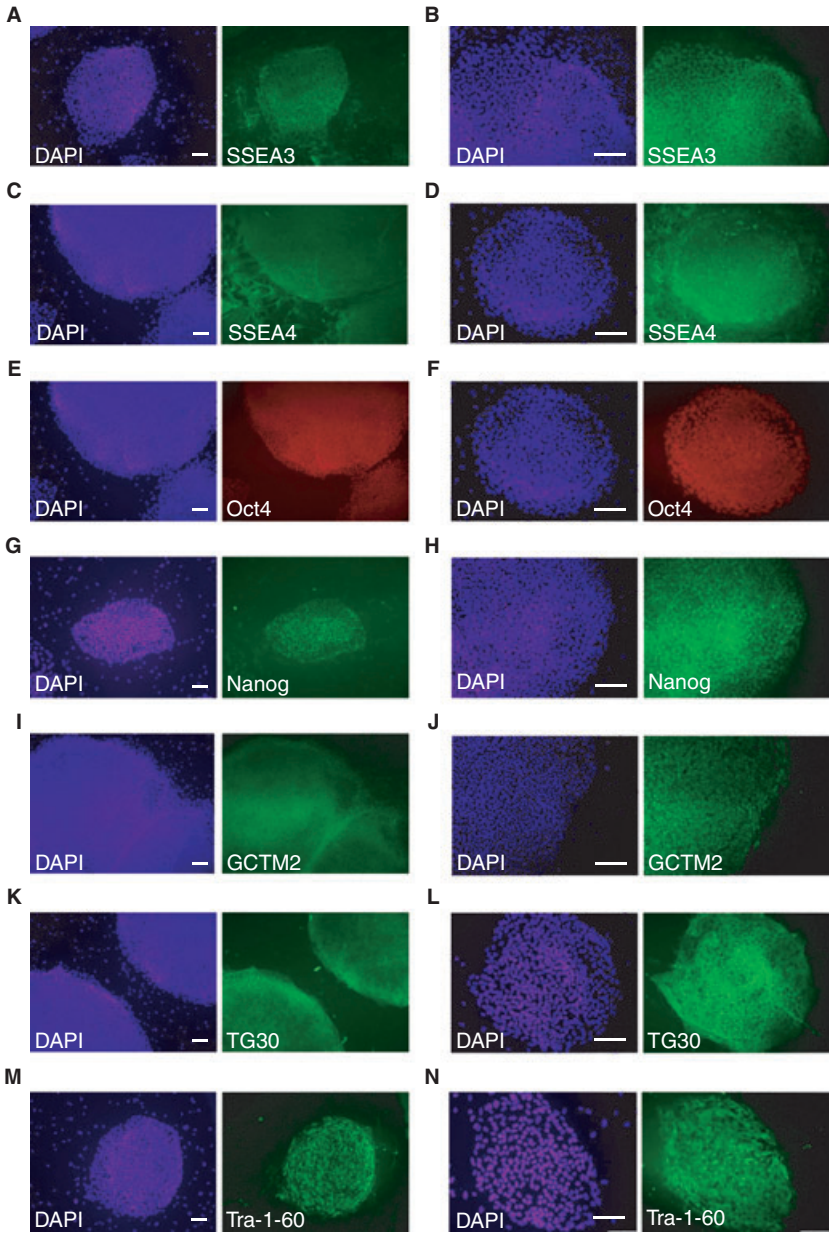

Fig. 2. Expression of human embryonic stem (ES) cell-associated proteins by gingival fibroblast-induced pluripotent stem (GF-iPS) cells and periodontal ligament fibroblast-induced pluripotent stem (PDLF-iPS) cells. GF-iPS cells from donor 1 and PDLF-iPS cells from donor 1 were stained with antibodies against proteins associated with the human ES cells SSEA3 (A,B), SSEA4 (C,D), OCT4 (E,F), NANOG (G,H), GCTM-2 (I,J), TG30 (K,L) and TRA-1-60 (M,N), and are shown as red and green fluorescence, respectively. 4′-6-Diamidino-2-phenylindole (DAPI) served as a blue nuclear staining control. Bars represent 200 μm.

replaced twice. Six days after transduction, cells were trypsinized and seeded at 5×10^4 or 5×10^5 cells/100 mm dish on a mitotically inactivated MEF feeder layer. The medium was changed to hES cell medium the following day and this culture medium was changed every other day. As the transduced cells became overconfluent before the iPS cells appeared, 2 wk after transduction, dishes of 5×10^5 transduced cells were passaged to fresh 100-mm MEF feeder dishes at a ratio of 1 : 3 using 1 mg/mL of collagenase I. To establish iPS cell lines, iPS colonies were selected 28–30 d after transduction, based on ES cell-like colony morphology. The iPS colonies were mechanically passaged into 10–12 pieces removing differentiated sections of colonies and separated from the MEF feeder layer with a glass capillary pipette under a stereoscopic microscope. Colony sections were transferred to an individual organ culture dish containing hES cell medium supplemented with 10 ng/mL of FGF-2. The initial passaging (to six passages) of new colonies was performed mechanically until the cells could be adapted to enzymatic dissociation (37). For subsequent passages, cells were dissociated with 4 mg/mL of collagenase I for 10–12 min at 37°C until the edges of the colonies curled up. The colonies were then detached, dissociated, broken into small clumps and plated on MEF flasks in the hES medium with FGF-2.

RT-PCR and quantitative real-time PCR analysis

Total RNA was purified using an RNeasy Mini kit with on-column DNase I digestion (Qiagen, Hilden, Germany). For RT-PCR, 1 μg of total RNA from each sample was used for cDNA synthesis using Superscript III Reverse Transcriptase and Oligo(dT) 12–18 primers (Invitrogen). PCR was performed using *Taq* DNA polymerase (Invitrogen) with the enhancer solution (Invitrogen) following the program: initial denaturation for 5 min at 94°C; 35 cycles of 94°C for 15 s, 55°C for 30 s and 68°C for 1 min; followed by a final incubation at 68°C for 7 min. For quantitative real-time PCR, cDNA was produced from 1 μg of total RNA from each sample using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). Quantitative PCR was performed using either TaqMan Universal PCR Master Mix or SYBR Green PCR Master Mix (Applied Biosystems) and analyzed with gene-specific primers using a 7500 Real-Time PCR System (Applied Biosystems). Relative gene expression was analyzed using software SDS1.2 (Applied Biosystems) and the $\Delta\Delta$Ct method. Primer sequences and

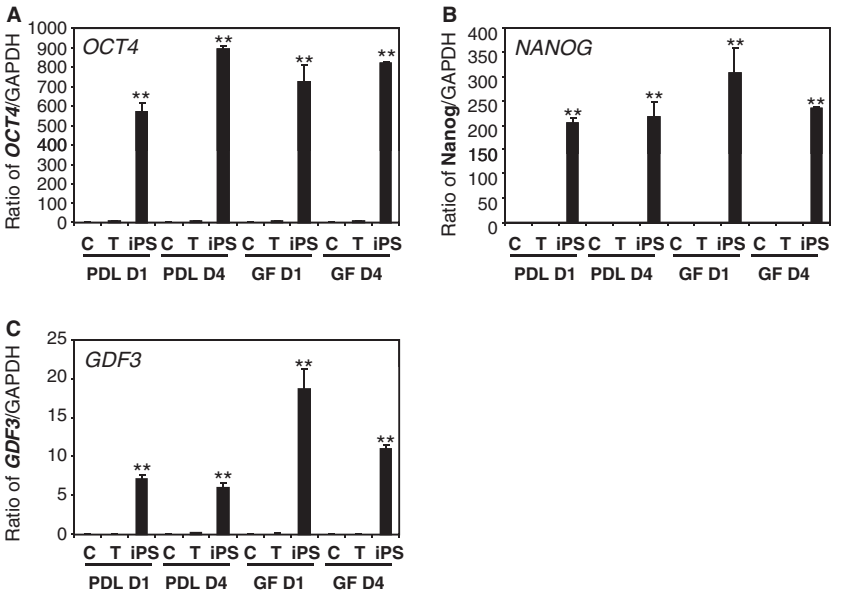

Fig. 3. Expression of *OCT4, NANOG* and *GDF3* genes in gingival fibroblast-induced pluripotent stem (GF-iPS) cells and periodontal ligament fibroblast-induced pluripotent stem (PDLF-iPS) cells. Real-time RT-PCR analysis of endogenous *OCT4* (A), *NANOG* (B) and *GDF3* (C) expression in GFs, PDLFs, GF-iPS cells and PDLF-iPS cells [from donor 1 (D1) and donor 4 (D4)]. C, GFs and PDLFs before retroviral transduction; T, GFs and PDLFs 1 d after retroviral transduction. The data are expressed as mean ± standard deviation of triplicate experiments (with samples taken from separate passages of each cell line. The level of gene expression in GF-iPS cells or PDLF-iPS cells were compared with each cell type, before or 1 d after transduction, using the Student's *t*-test, $^{**}p < 0.01$. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

information on TaqMan gene-expression assays are listed in Tables 1 and 2.

Immunofluorescent staining

Immunofluorescent staining was carried out as described previously (38). Briefly, human iPS cells were cultured on 12-well culture slides with an MEF feeder layer for 5 d in hES medium. The cells were fixed by immersion in precooled ethanol for 10 min. Primary antibodies and matched isotype controls were applied to each well and incubated at room temperature for 1 h. Immunofluorescent staining was performed using the following isotype-specific primary antibodies: SSEA3 (1 : 100 dilution; Millipore), SSEA4 (1 : 100 dilution; Millipore), OCT4 (1 : 50 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA), NANOG (1 : 50 dilution; eBioscience, San Diego, CA, USA), TRA-1-60 (1 : 100 dilution; Millipore), GCTM-2 (1 : 100 dilution; kindly provided by Dr Martin Pera, Center for Stem Cell and Regenerative Medicine, Keck School of Medicine, University of Southern California, Los Angeles, CA, USA), or TG30 (CD9; 1 : 500 dilution; Australian Stem Cell Centre, Clayton, Vic., Australia). Alexa Fluor 488- or 586-coupled secondary antibodies used were anti-mouse IgG2b for OCT3/4, anti-mouse IgG1 for NANOG, anti-mouse IgM for TRA-1-60 and GCTM2, anti-mouse IgG2a for TG30, anti-mouse IgG3 for SSEA4 (all at 1 : 1000 dilution; Invitrogen) and anti-rat Ig for SSEA3 (1 : 30 dilution; DAKO, Glostrup, Denmark). Cell nuclei were counterstained with 4′-6-diamidino-2-phenylindole (10 μg/mL; Sigma-Aldrich) and mounted in Vectashield (Vector Laboratories, Burlingame, CA, USA). Image capture was performed using a BX51 microscope (Olympus, Tokyo, Japan).

In vitro differentiation of human iPS cells

For spontaneous differentiation through embryoid body (EB) formation, human iPS cells were dissociated with 4 mg/mL of collagenase I and transferred to a low-attachment 6-cm Petri Dish (Simport, Beloeil, QC, Canada) in hES medium. After 7 d in suspension culture, EBs were collected. Total RNA was isolated for quantitative real-time PCR analysis to ascertain the presence of a panel of genes representative of differentiation to each of the three embryonic germ layers (Table 2).

Karyotyping

Standard G-banding chromosome analysis was performed at the Cytogenetics Laboratory, Monash Medical Centre (Clayton, Vic., Australia).

Teratoma formation

Teratomas were formed as described previously (39). Briefly, iPS cells grown on MEFs were harvested following collagenase (4 mg/mL) treatment and resuspended in phosphate-buffered saline at a concentration of 1×10^7 cells/mL. Severe combined immunodeficient (SCID) mice (5–6 wk of age) were anaesthetized, and 40 μL of the cell suspension was injected into the testis of each mouse. After 6–8 wk, teratomas were dissected and fixed in 4% paraformaldehyde. Samples were embedded in paraffin and processed for hematoxylin and eosin staining.

Results

GFs and PDLFs transduced with *OCT3/4, SOX2, KLF4* and c-*MYC* formed ES cell-like colonies

Human GFs and PDLFs from two adult donors transduced with the four factor genes, *OCT3/4, SOX2, KLF4* and c-*MYC*, were cultured on a layer of mitotically inactivated MEFs in hES media supplemented with FGF-2. Before transduction of the four genes, both GFs and PDLFs exhibited typical fibroblast spindle-like morphology (Fig. 1A,B). ES cell-like colonies appeared in the cultures of all GFs and PDLFs from both donors on days 14–21, which were further propagated. Interestingly, greater numbers of ES cell-like colonies emerged from

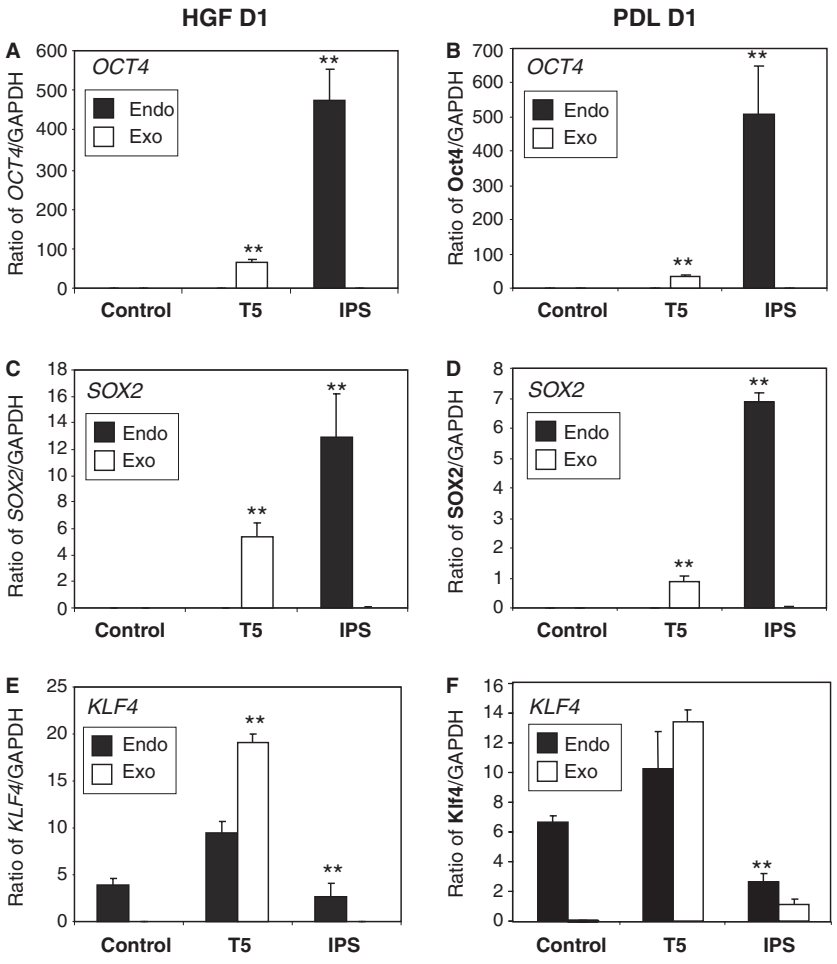

Fig. 4. Endogenous (Endo) and exogenous (Exo) expression of *OCT4, SOX2* and *KLF4* genes in gingival fibroblast-induced pluripotent stem (GF-iPS) cells and periodontal ligament fibroblast-induced pluripotent stem (PDLF-iPS) cells. Real-time RT-PCR analysis of endogenous and exogenous *OCT4* (A,B), *SOX2* (C,D) and *KLF4* (E,F) gene expression in GFs and GF-iPS cells from donor 1 (A,C,E), and in PDLFs and PDLF-iPS cells from donor 1 (B,D,F). Control: GFs and PDLFs before retroviral transduction. T5: GFs and PDLFs 5 d after retroviral transduction. The data are expressed as mean ± standard deviation of triplicate experiments. The level of endogenous gene expression (black column) in GF-iPS cells or in PDLF-iPS cells were compared with each cell type before or 5 d after transduction, and the level of exogenous gene expression (white column) in GFs or in PDLFs 5 d after retroviral transduction was compared with each cell type before retroviral transduction or iPS using the Student's *t*-test, $^{**}p < 0.01$.

transduced GFs, but not from PDLFs plated at low density (5×10^4 cells/dish) compared with those at high density (5×10^5 cells/dish) (data not shown). On day 30, 20 ES cell-like colonies from each cell type were collected and passaged manually. These colonies maintained their ES-like morphology following subculture (Fig. 1C–F). Non-ES cell-like colonies, which displayed granulated morphology, also emerged in all cell type culture (data not shown).

Expression of ES cell markers in iPS cells generated from GFs and PDLFs

The iPS cells generated from both GFs (GF-iPS) and PDLFs (PDLF-iPS) were found, by immunofluorescence staining, to express the human ES cell marker proteins, SSEA3 (Fig. 2A,B), SSEA4 (Fig. 2C,D), OCT4 (Fig. 2E,F), NANOG (Fig. 2G,H), GCTM-2 (Fig. 2I,J), TG30 (Fig. 2K,L) and TRA-1-60 (Fig. 2M,N). Moreover, real-time PCR analysis demonstrated that these iPS cells expressed mRNA for *OCT4* (Fig. 3A), *NANOG* (Fig. 3B) and *GDF3* (Fig. 3C), characteristic of human pluripotent cells. In contrast, GFs and PDLFs before transduction, and 1 d after transduction, expressed almost undetectable levels of mRNA for these ES cell markers (Fig. 3). To examine the level of retroviral silencing, the expression, by GF-iPS and PDLF-iPS cells, of endogenous and exogenous mRNA for *OCT4, SOX2* and *KLF4* was analyzed (Fig. 4). Both GFs and PDLFs, 5 d after transduction, expressed exogenous levels of all three pluripotency-associated genes but showed no endogenous gene expression of *OCT4, SOX2* and *KLF4*. Conversely, GF-iPS and PDLF-iPS expressed detectable levels of the three pluripotency-associated genes, but exogenous expression was decreased (Fig. 4). Control cultures of GFs and PDLFs before transduction showed undetectable expression of both endogenous and exogenous *OCT4* and *SOX2*, but not of endogenous *KLF4*.

EB formation by GF-iPS and PDLF-iPS cells *in vitro*

To examine the potential of GF-iPS and PDLF-iPS cells to differentiate into various cell types *in vitro*, EB formation assays were performed. The iPS cells derived from both GFs and PDLFs formed ball-shaped suspension cultures on day 7 (Fig. 5A), and expressed three different germ layer-associated genes, corresponding to the endoderm gene markers, *GATA4* and *AFP*, the ectoderm gene markers, *SOX1* (data not shown as fold change could not be calculated) and *PAX6*, and the mesoderm gene markers, *RUNX1* and T (*Brachyury*), determined by real-time PCR. Importantly, the expression of human ES cell-associated genes, *OCT4, NANOG* and *GDF3*, in EB derived from either GF-iPS or PDLF-iPS, was much lower than in the undifferentiated GF-iPS and PDLF-iPS cell cultures (Fig. 5B,C).

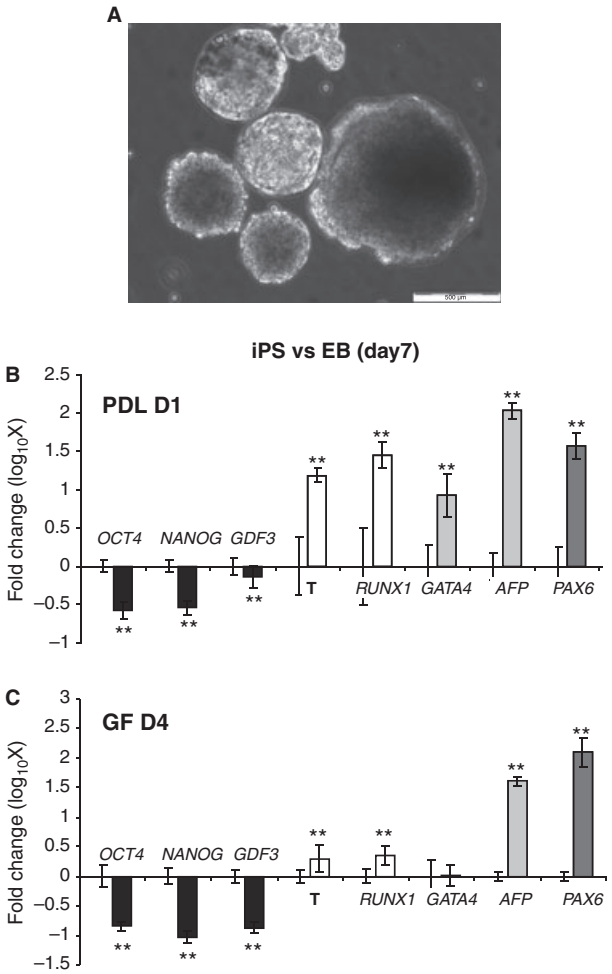

Fig. 5. Embryoid body (EB)-mediated differentiation of gingival fibroblast-induced pluripotent stem (GF-iPS) cells and periodontal ligament fibroblast-induced pluripotent stem (PDLF-iPS) cells. (A) Suspension culture of GF-iPS from donor 1 on day 7. Scale bar = 500 μm. (B,C) Real-time RT-PCR analysis of PDLF-iPS cells from donor 4 (B) and of GF-iPS cells from donor 1 (C) vs. EB cultures for markers associated with the undifferentiated human embryonic stem (hES) cells *OCT4, NANOG* and *GDF*, and markers associated with primitive ectoderm (*PAX6*), mesoderm (*RUNX1*, *Brachyury*) and markers associated with primitive endoderm (*GATA4* and *AFP*). Graphs show the fold change of expression for each gene marker in EB cultures relative to those in undifferentiated iPS cell cultures (X = EB/iPS). The data are expressed as mean ± standard deviation of triplicate experiments. The gene-expression level for each marker in EB cultures vs. GF-iPS or PDLF-iPS cell cultures was compared with the levels expressed in undifferentiated GF-iPS or PDLF-iPS cell cultures using the Student's *t*-test, $^{**}p < 0.01$.

GF-iPS and PDLF-iPS showed normal karyotypes

Karyotyping was performed to examine the chromosomes of GF-iPS and PDLF-iPS. A total of seven cell lines (one line of GF-iPS cells from donor 1, two lines of GF-iPS cells from donor 4, two lines of PDLF-iPS cells from donor 1 and two lines of PDLF-iPS cells from donor 4) out of eight lines showed normal karyotypes (Fig. 6).

Teratoma formation by GF-iPS and PDLF-iPS *in vivo*

Histological assessment of iPS cells implanted into the testes of SCID mice demonstrated teratoma formation after 8 wk. Tissues representative of tissues derived from embryonic ectoderm (neuronal tissue), mesoderm (cartilage) and endoderm (epithelial tissue) were demonstrated. Both GF-iPS and PDLF-iPS cell lines formed teratomas with regions representative of each of the three embryonic germ layers (Fig. 7).

Discussion

Fibroblasts derived from periodontal ligament and gingiva display differential expression of various mesenchymal stem cell markers and different differentiation capacity (40). In this study, we demonstrated the generation of iPS cell lines from human GFs and PDLFs using the Yamanaka cocktail of human pluripotency genes (41). The established GF-iPS and PDLF-iPS colonies expressed *SOX2, GDF3, OCT4, NANOG, SSEA3, SSEA4, TRA-1-60, GCTM-2* and *TG30* at mRNA or protein levels, and these have been reported as human ES cell-associated common marker genes and proteins (42). Among others, GCTM-2 and TG30 (CD9) have recently been reported to correlate with the presence of OCT4 protein in human ES cells (38), further supporting that our generated GF-iPS and PDLF-iPS cells possess ES cell-like characteristics.

Recent studies have reported that iPS cells can be established from mesenchymal cells originating from dental tissues, including dental pulp (24,25), oral mucosa (26) and gingiva (27), but not from periodontal ligament. Interestingly, Yan *et al.* (24) reported that human GFs were a poor candidate cell type for using to establish iPS cells. Our GF-iPS clones were generated by retroviral transduction using *OCT3/4, SOX2, KLF4* and c-*MYC* with mSlc7a1 lentiviral transduction following the protocol described by the Yamanaka laboratory (41). Conversely, Yan *et al.* tried to generate iPS cells from GFs using *LIN28, NANOG, SOX2* and *OCT3/4* without pretransduction of mSlc7a1, simian virus 40 (SV40) large T, or human Telomerase reverse transcriptase which Park *et al.* (13) pre-transduced before transduction of the four genes to establish iPS cells. mSLC7a1 is known as a cationic

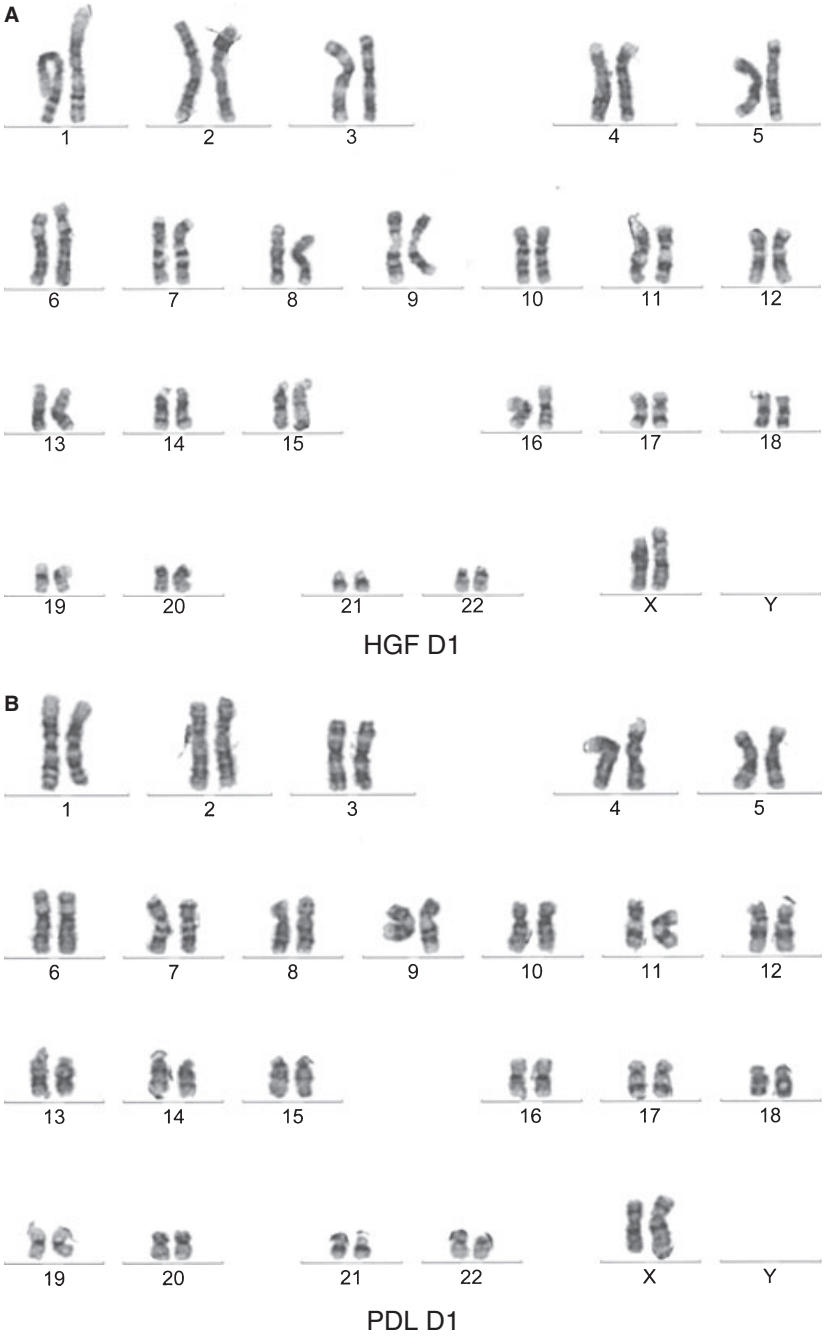

Fig. 6. Karyotyping of gingival fibroblast-induced pluripotent stem (GF-iPS) cells (HGF D1) and periodontal ligament fibroblast-induced pluripotent stem (PDLF-iPS) cells (PDL D1). Chromosomal analysis using G-banding revealed a normal karyotype for GF-iPS cells from donor 1 (A) and for PDLF-iPS cells from donor 1 (B). HGF D1, human gingival fibroblast-induced pluripotent stem cells from donor 1; PDL D1, human periodontal ligament fibroblast-induced pluripotent stem cells from donor 1.

amino acid transporter that mediates fusogenic interaction with the retroviral envelope protein Env (43,44), supporting efficient retroviral transduction to cells. The lack of mSlc7a1 transduction before retroviral transduction of GFs in the study of Yan *et al.* may have reduced the efficiency of the transduction, leading to failure in iPS cell generation. Production of iPS cells from PDLFs has not been reported previously. In our study, more than 20 colonies of both GF-iPS and PDLF-iPS cells emerged from each cell population, suggesting that both GFs and PDLFs could be relatively efficient cell sources for the generation of iPS cells.

In recent years, technologies for iPS generation, such as the use of nonintegrating genetic elements, small molecules or cytokines, have been advancing steadily. Some studies have demonstrated iPS generation using nonintegrating Epstein–Barr virus-derived episomal vectors (45) or integrating vectors that could be removed from the genome using piggy-BAC retrotransposition (46,47). More recently, other studies have demonstrated the successful reprogramming of MEF (48) and human newborn fibroblasts (49) using recombinant cell-penetrating reprogramming proteins to iPS cells. Although further development of these technologies is needed, the strategies to generate iPS cells without integrating exogenous sequences into the cell genome may support the development of iPS cell-generation methodology for clinical use.

We propose that because both GFs and PDLFs can be easily obtained during routine tooth extraction (for example, for orthodontic reasons or third molar removal) they represent a unique opportunity to generate iPS cells and could be a good alternative source for iPS cell banking (25).

Epigenetic memory usually stays with primary cells over several passages, but is eventually lost over time as pluripotency emerges. However, recent studies have shown that iPS cells maintain an epigenetic memory of their tissue of origin, which influences the subsequent differentiation potential of specific iPS cells (22). Interestingly, iPS cells derived from skin fibroblasts have been used to regenerate periodontal ligament in a mouse model (50). Therefore, we propose that iPS cells derived from both GFs and PDLFs represent an alternative source for developing pluripotent cell-based tissue-regenerative treatment for periodontitis. This may provide a significant advancement because previous studies, which have utilized a multiple adult stem cell populations, have had only limited success (7).

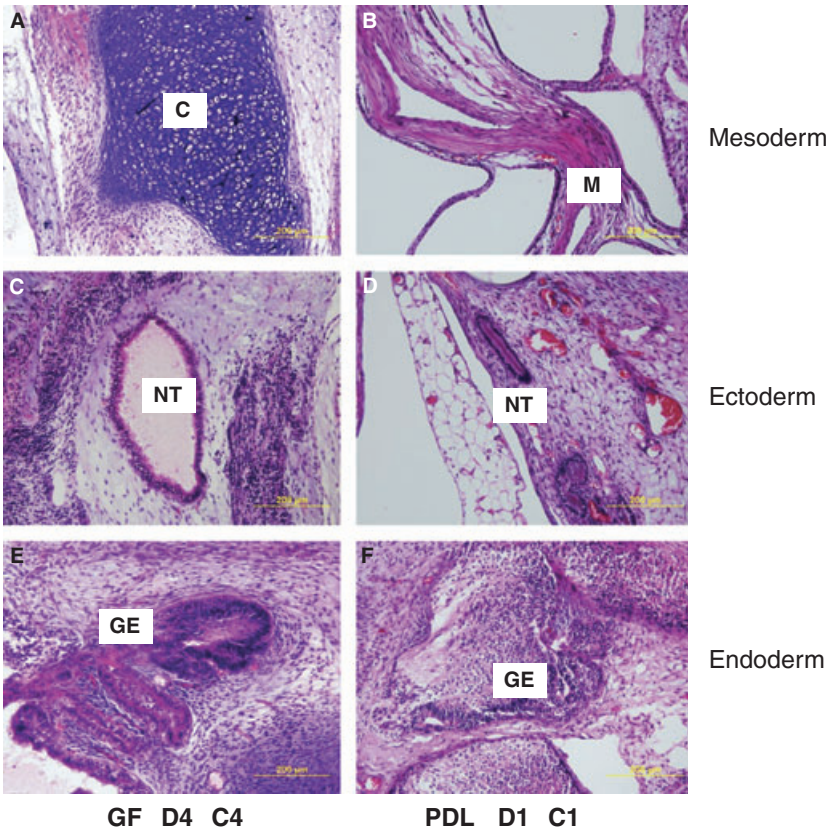

Fig. 7. Teratoma formation by gingival fibroblast-induced pluripotent stem (GF-iPS) cells and periodontal ligament fibroblast-induced pluripotent stem (PDL-iPS) cells. Histological assessment of teratoma formation following implantation of GF-iPS cells [donor 4, clone 4 (D4 C4)] and PDL-iPS cells [donor 1, clone 1 (D1 C1)] into the testes of severe combined immunodeficient (SCID) mice. (A) and (B) demonstrate tissues consistent with embryonic mesoderm origin [cartilage (C) and muscle (M)]; (C) and (D) demonstrate neuronal epithelial tissue (NT); (E) and (F) demonstrate gut-like epithelium (GE). Sections were stained with hematoxylin and eosin; the bar represents 200 μm. GF, gingival fibroblasts; PDL, periodontal ligament fibroblasts.

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