

Accelerated generation of human induced pluripotent stem cells from human oral mucosa using episomal plasmid vectors and maternal transcription factor *Glis1*

Abstract

Objective

Induced pluripotent stem cells (iPSCs) possess high pluripotency and differentiation potential and may constitute a possible source of autologous stem cells for clinical applications. However, the lengthy reprogramming process (up to one month) remains one of the most significant challenges facing standard virus-mediated methodology. The Gli-like transcription factor *Glis1* is highly expressed in unfertilized eggs and one-cell-stage embryos. In this study, iPSCs were generated using a combination of primary human oral mucosal fibroblasts (HOFs) and episomal plasmid vectors expressing transcription factors, including *Glis1*.

Materials and methods

HOFs were established from oral mucosal tissue 3 mm in diameter from a 23-year-old Asian male using a skin trephine. Human iPSCs were generated from the established HOFs using the following episomal plasmid vectors: pCXLE-hOCT3/4-shp53-F that expresses *OCT3/4* and short-hairpin RNA (shRNA) against *p53*, pCXLE-hSK that expresses *SOX2* and *KLF4*, pCXLE-hUL that expresses *L-MYC* and *LIN28*, and pCXLE-hGlis1 that expresses *Glis1*.

Results

Fifty colonies of human embryonic stem (ES)-like cells were observed as early as 20 days after initial episomal plasmid vector transduction. The resulting cell lines shared several characteristics with human ES cells, including morphology, pluripotency-associated gene and protein markers, karyotype analysis and the ability to differentiate *in vivo* into all three germ layers.

Conclusion

Our method, combining the use of HOFs and episomal plasmid vectors expressing *OCT3/4*, shRNA against *p53*, *SOX2*, *KLF4*, *L-MYC*, *LIN28* and *Glis1*, offers a powerful tool for safely and rapidly generating bona fide human iPSCs and facilitates the application of iPSC technology to biomedical research.

Keywords

iPSC, integration-free plasmid vector, *Glis1*, human oral mucosal tissue.

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Introduction

The successful reprogramming of human and mouse somatic cells into induced pluripotent stem cells (iPSCs) via ectopic overexpression of pluripotency-associated transcription factors is considered a major scientific breakthrough.¹⁻⁵ Similar to the characteristics of embryonic stem (ES) cells,⁶⁻⁸ human iPSCs can proliferate indefinitely, while retaining pluripotency, and can differentiate into all cell types found in the body. iPSCs have been generated from dermal fibroblasts,³ peripheral blood,⁹ dental pulp cells,¹⁰ gingival fibroblasts,¹¹ periodontal ligaments,¹² oral mucosa¹³ and mesenchymal stromal cells.¹⁴

Gingival tissue is routinely resected during general dental treatments, such as tooth extraction, periodontal surgery and dental implantation, and generally treated as biomedical waste.¹⁵ Egusa et al. successfully derived iPSCs from human gingival fibroblasts (HGFs) using retroviral transduction of transcription factors; they also reported that the reprogramming efficiency of mouse gingival fibroblasts was higher than that of dermal fibroblasts.¹¹ However, retroviral integration increases the risk of tumor formation, while integration-free methods decrease this potential risk.¹⁵ The development of novel approaches to generating integration-free iPSCs has eliminated the concern of integrating virus-associated genotoxicity in clinical applications.¹⁶ Integration-free human iPSCs have been generated using several methods.¹⁵ Okita et al. reported a simple method that uses *p53* suppression and nontransforming *L-MYC* to generate human iPSCs with episomal plasmid vectors.¹⁵ Our recent study demonstrated that iPSCs could be generated from a combination of primary HGFs and an episomal plasmid vector.¹⁷ However, the lengthy reprogramming process (up to one month) remains one of the most significant challenges facing standard virus-mediated methodology.

Maekawa et al. reported that the Gli-like transcription factor *Glis1* (Glis family zinc finger 1) markedly enhances the generation of iPSCs from both mouse and human somatic fibroblasts when it is expressed together with three transcription factors collectively known as OSK (*OCT3/4*, *SOX2* and *KLF4*) using retroviral transduction.¹⁸ However, little is known regarding whether *Glis1* can effectively promote direct reprogramming during iPSC generation using an episomal plasmid vector. In the current study, iPSCs were generated by combining primary

human oral mucosal fibroblasts (HOFs) with episomal plasmid vectors expressing *OCT3/4*, short-hairpin RNA (shRNA) against *p53*, *SOX2*, *KLF4*, *L-MYC*, *LIN28* and *Glis1*.

Materials and methods

Ethical statement

Approval for the sampling of human oral mucosa tissue, establishing iPSCs and genome/gene analysis was obtained from the Ethics Committee of Osaka Dental University, Hirakata, Japan (authorization No.: 110783; approval date: 30 September 2013) and the DNA Recombination Experiment Safety Committee of Osaka Dental University (authorization No.: 54; approval date: 18 July 2014). Written informed consent was obtained from the participant. The animal experiments followed a protocol approved by the Animal Committee of Osaka Dental University (authorization No.: 14-06002; approval date: 8 July 2014).

Cell culturing

HOFs were established from oral mucosal tissue 3 mm in diameter obtained using a skin trephine (derma punch, Maruho, Osaka, Japan) from a 23-year-old Asian male. Human oral mucosal tissue was placed in 35 mm tissue culture dishes and cultured in Dulbecco's Modification of Eagle's Medium (DMEM) containing 10% fetal bovine serum at 37°C and 5% CO₂.¹¹ The medium was replaced every three days. Once the HOFs had proliferated, the tissue was removed. When the cells reached subconfluence, they were dissociated using 0.25% trypsin (Invitrogen, Carlsbad, Calif., U.S.) and transferred to 60 mm tissue culture dishes (passage 1). HOFs were regularly passaged at a 1:3 ratio every three to four days.

Generation of iPSCs from HOFs with episomal vectors

One microgram of an expression episomal plasmid mixture containing pCXLE-hOCT3/4-shp53-F that expresses *OCT3/4* and shRNA against *p53*, pCXLE-hSK that expresses *SOX2* and *KLF4*, pCXLE-hUL that expresses *L-MYC* and *LIN28*, and pCXLE-hGlis1 that expresses *Glis1* (Addgene, Cambridge, Mass., U.S.) was electroporated into 6×10^5 primary HOFs (passage 5) with the Amaxa 4D-Nucleofector (Lonza, Basel,

Primer	Gene		Sequences (5' to 3')
Pluripotent marker	OCT3/4	Forward	GAAACCCACACTGCAGCAGA
		Reverse	TCGCTTGCCCTTCTGGCG
	NANOG	Forward	CTCAGCTACAAACAGGTGAAGAC
		Reverse	TCCCTGGTGGTAGGAAGAGTAAA
	SOX2	Forward	GGGAAATGGGAGGGGTGCAAAGAGG
		Reverse	TTGCGTGAGTGTGGATGGGATTGGTG
	KLF4	Forward	CGCTCCATTACCAAGAGCTCAT
		Reverse	CGATCGTCTTCCCCTCTTTG
	TERT	Forward	CGTACAGGTTTCACGCATGTG
		Reverse	ATGACGCGCAGGAAAAATGT
	C-MYC	Forward	GTTGGTCAGGCTGGTCTTGAA
		Reverse	CATGCGCCTGTAATCCTAGCA
Internal control	GAPDH	Forward	CCACTCTCCACCTTTGACG
		Reverse	ATGAGGTCCACCACCCTGTT

Table 1

List of primers used for qRT-PCR.

Switzerland) according to the manufacturer's instructions using program DT-130 (Lonza). These cells were then transferred on to mitomycin C-treated SNL 76/7 cells (cat. No. 07032801, lot No. 08F009; European Collection of Authenticated Cell Cultures, Porton Down, U.K.) at 5×10^4 cells per 100 mm dish. The following day, the culture medium was replaced with embryonic stem cell (ESC) culture medium consisting of DMEM/F12 medium (Sigma-Aldrich, St. Louis, Mo., U.S.) supplemented with 20% Knock-Out Serum Replacement (Gibco, Grand Island, N.Y., U.S.), 2 mM L-glutamine (Nacalai Tesque, Kyoto, Japan), 1% nonessential amino acids (Gibco), 0.1 mM 2-mercaptoethanol (Gibco) and 5 ng/mL fibroblast growth factor-2 (ReproCELL, Kanagawa, Japan). Thirty days subsequent to transduction, a number of colonies were mechanically picked and transferred to a 24-well plate. After several passages, ESC-like colonies were selected for further cultivation and characterization. iPSCs were generated and maintained in ESC culture medium. For routine passaging, iPSC colonies were detached with CTK solution (2.5 µg/mL trypsin, 1 mg/mL collagenase IV, 20% KSR, 1 mM CaCl₂/PBS, and 70% PBS) and split at a 1:3 ratio every four to five days.

Quantitative real-time reverse transcription-polymerase chain reaction

Total RNA was isolated using the RNeasy Micro Kit (Qiagen, Limburg, Netherlands) according to the manufacturer's protocol. Single-stranded complementary DNA was synthesized from a total of 500 ng RNA (DNase-treated) using the PrimeScript RT Master Mix (Takara, Shiga, Japan). Khes-1 RNA was provided by the Foundation for Biomedical Research and Innovation (Kobe, Japan). Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) was conducted in triplicate using SYBR Select Master Mix (Life Technologies, Grand Island, N.Y., U.S.) with a StepOnePlus system (Life Technologies) and the following PCR program: 95 °C for 10 min, then 40 cycles of 95 °C for 15 s, 60 °C for 1 min and 72 °C for 15 s. Specific primers are listed in **Table 1**. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was co-amplified as an internal standard. Gene expression was measured using the $\Delta\Delta C_T$ method.²⁰ Differences in gene expression between Khes-1, HOF-iPSCs and HOFs were evaluated by variance analysis with the Tukey test.

Table 2

Antibodies	Supplier	Cat. No	Dilution
OCT3/4	Santa Cruz Biotechnology, Dallas, Texas, U.S.	SC5279	1/100
NANOG	Cell Signaling Technology, Danvers, Mass., U.S.	3680S	1/100
SSEA-3	Abcam, Cambridge, Mass., U.S.	ab16286	1/100
SSEA-4	Millipore, Billerica, Mass., U.S.	MAB4360	1/100
TRA-1-60	Millipore, Billerica, Mass., U.S.	MAB4304	1/100
TRA-1-81	Millipore, Billerica, Mass., U.S.	MAB4381	1/100
DAPI	Invitrogen, Carlsbad, Calif., U.S.	D1306	5 µg/mL
Alexa Fluor 594 mouse	Invitrogen, Carlsbad, Calif., U.S.	A11062	1/500
Alexa Fluor 594 rat	Invitrogen, Carlsbad, Calif., U.S.	A21211	1/500

Surface antigen analysis

Cells (5×10^5) were obtained after treatment with 0.025% trypsin (Life Technologies). Cell surface antigen staining was performed in phosphate-buffered saline (PBS) with 2% human serum albumin (Mitsubishi-Tanabe Pharma, Osaka, Japan). The cell suspension was incubated with the antibodies listed in **Table 2** for 30 min at 4°C. Murine anti-human antibodies were used at the recommended concentrations. Primary antibodies and isotype controls are listed in **Table 2**. The stained cells were analyzed with FACSAria II (Becton Dickinson, Franklin Lakes, N.J., U.S.) and the data were analyzed using the FlowJo software (Tree Star, Ashland, Ore., U.S.).

Immunocytochemistry

For fixed staining of differentiation-specific markers, cells were fixed for 30 min in 4% paraformaldehyde at 4°C, followed by washing in PBS. The cells were then permeabilized for 15 min with 2% bovine serum albumin and 0.1% Triton X-100 (Sigma-Aldrich) and incubated overnight at 4°C with the primary antibodies diluted in PBS containing 2% bovine serum albumin. The cells were then

washed and incubated for 1 h with the appropriate fluorescence-conjugated secondary antibodies. Primary antibodies and secondary antibodies are listed in **Table 2**. The staining images were acquired with a ZOE Fluorescent Cell Imager (Bio-Rad Laboratories, Hercules, Calif., U.S.).

In vivo differentiation (teratoma formation)

NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ mice (Jackson Laboratory, Bar Harbor, Maine, U.S.) were anesthetized and iPSCs (1×10^6) were transplanted under the epidermal space of the neck. Two hundred microliters of saline was injected into a second epidermal space as a negative control. Mice were euthanized 12 weeks later and teratoma samples were collected and subjected to histological analysis. Teratomas were processed according to standard paraffin embedding and hematoxylin and eosin staining procedures by the Business Support Center for Biomedical Research Activities (Kobe, Japan).

Table 2

Antibodies used for flow cytometry and immunocytochemical staining of HOF-iPSCs.

Table 3

ESC-like colonies obtained from HOFs. The number of colonies per 5×10^4 cells after cell reprogramming with episomal vectors. These data were obtained from three independent induction experiments using HOFs from a donor.

	ES-like	Non-ES-like
HOFs	54	23
	52	24
	58	29

Table 3

Fig. 1

Excision of oral mucosal tissue by punch biopsy.

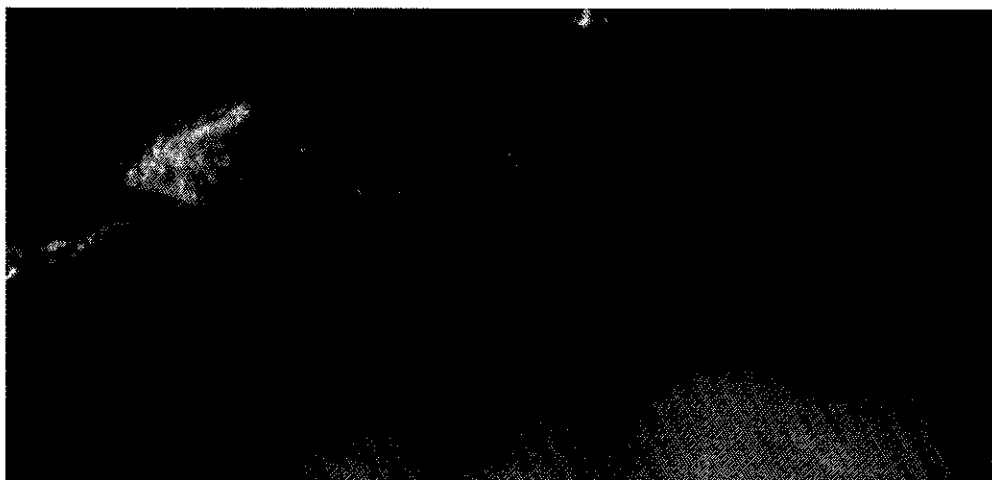


Fig. 1

Karyotype analysis

Chromosome G-band analysis was performed at Nihon Gene Research Laboratories (Sendai, Japan). At least 15 metaphases were analyzed.

picked at passage 1. Several days later, four ESC-like colonies were selected and expanded. All colonies were similar to ESCs in morphology and proliferative capacity and were named "HOF-iPSCs".

Results

Generation of iPSCs from HOFs using episomal plasmid vectors

Three lines of HOFs were established from the oral mucosa of the 23-year-old Asian male (Fig. 1). Homogeneous fibroblasts emerged from the oral mucosal tissue one week after the start of culturing. HOFs were exponentially expanded up to 30 passages; cells were counted at each passage and plated at 1.5×10^4 cells/cm². Colonies with a flat human ESC-like morphology and non-ESC-like colonies were counted at around day 20 after HOF transfection with episomal plasmid vectors expressing human *OCT3/4*, shRNA against *p53*, *SOX2*, *KLF4*, *L-MYC*, *LIN28* and *Glis1*. The average number of ESC-like colonies from three experiments was 54.7 ± 3.05 , with a reprogramming efficiency of approximately 1%; the average number of non-ESC-like colonies was 25.3 ± 3.21 (Table 3). A number of colonies obtained from the HOF cells were mechanically

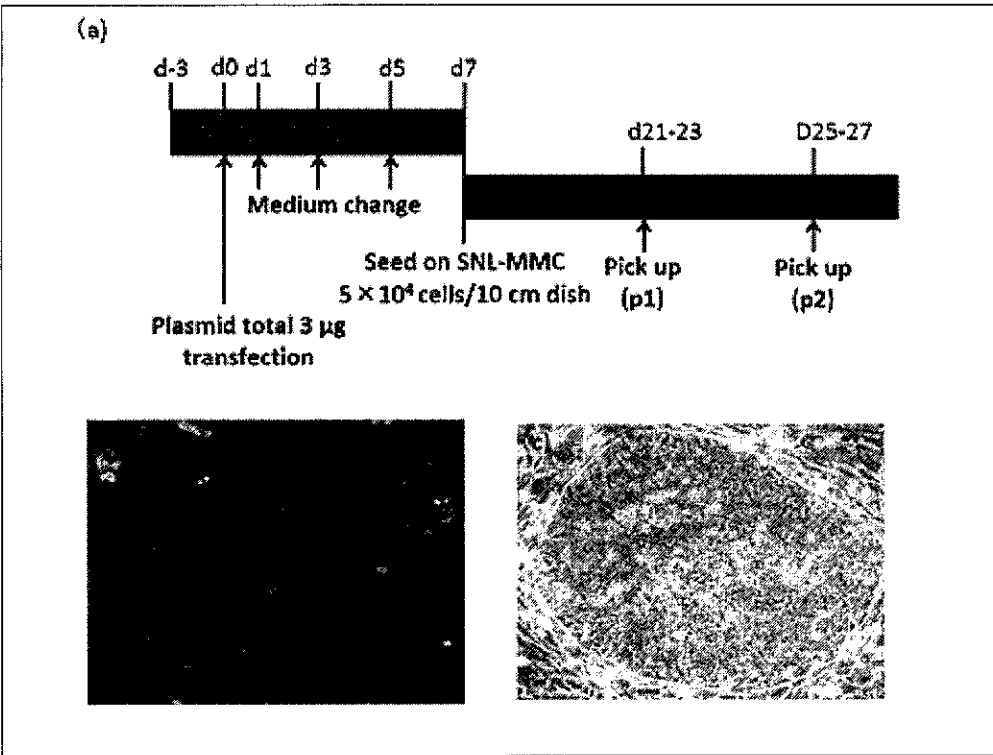
Expression of ESC-specific marker genes in HOF-iPSCs

HOF-iPSCs were selected for characterization from among the picked clones after 23 passages based on their higher level of proliferation and stability of the ESC-like morphology. The expression of the ESC-specific marker genes *OCT3/4*, *NANOG*, *SOX2*, *TERT*, *KLF4* and *C-MYC* in HOF-iPSCs was analyzed using qRT-PCR (Fig. 3). Expression of *NANOG* and *SOX2* was significantly higher and that of *C-MYC* and *TERT* was lower in KhES-1 cells compared with that in HOF-iPSCs (Figs. 3b–e). No significant difference was observed between KhES-1 and HOF-iPSCs for *OCT3/4* and *KLF4* expression (Figs. 3a & f). *KLF4* was the only gene to exhibit higher expression in HOF cells compared with both the KhES-1 cells and HOF-iPSCs (Fig. 3f).

Characterization of HOF-iPSCs

HOF-iPSCs were selected for characterization from among the picked clones after 20 passages

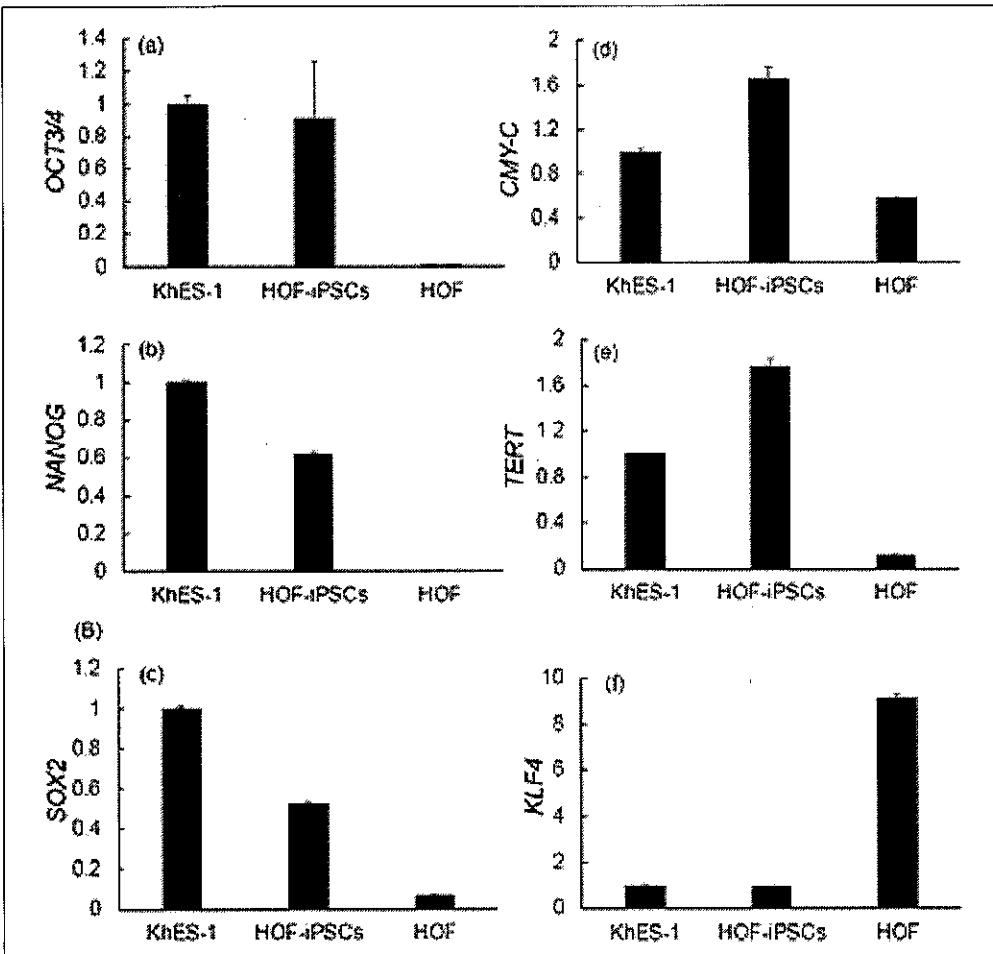
Fig. 2



Figs. 2 a-c

Generation of HOF-iPSCs. (a) Time course for HOF reprogramming. (b) Microscopy image of original HOFs in culture. (c) Generated HOF-iPSC colonies on SNL feeder cells.

Fig. 3



Figs. 3 a-f

QRT-PCR analysis of the expression of six pluripotency-related genes in HOF-iPSCs: (a) OCT3/4, (b) NANOG, (c) SOX2, (d) C-MYC, (e) TERT and (f) KLF4. KhES-1 cells (passage 23) were used as the positive control and HOFs (passage 6) as the negative control.

based on increased proliferation and stability of the ESC-like morphology. Expression of the ESC-specific surface markers SSEA-3, SSEA-4 and TRA-1-60 in HOF-iPSCs was analyzed using flow cytometry; all three markers were expressed (Fig. 4). HOF-iPSCs could be maintained beyond 20 passages and still demonstrated ESC-like morphology. In addition, HOF-iPSCs expressed ESC-specific surface markers, such as OCT3/4, SSEA-4, TRA-1-60 and TRA-1-81 (Fig. 5). Tumor formation was observed three months after the injection of HOF-iPSCs under the epidermal space in the neck of immunodeficient mice. Histological examination showed that the tumor contained various tissues, including cartilage (mesoderm), melanocytes (ectoderm), gut-like tube tissue (endoderm) and neural tissue (ectoderm; Fig. 6). Karyotype analysis of the tested clones showed a normal human karyotype (Fig. 7).

Discussion

Many strategies have been proposed for the management of large defects in oral tissue or organs such as due to congenital abnormalities, trauma or cancer treatment. Autogenous bone grafts are the gold standard for such reconstruction because of their osteoconductive, osteoinductive and nonimmunogenic properties.^{20, 21} Recently, cell therapy using stem cells combined with osteoconductive biomaterials or scaffolds has become a promising alternative to autogenous bone grafts.²² In order for cell therapy to efficiently treat large defects in oral tissue or organs, it is important to produce a sufficient number of cells that function similarly to primary islets. iPSCs, referred to as pluripotent stem cells, have been generated via retrovirus-mediated introduction of four transcription factors^{3, 4} and represent a potentially unlimited source of cells. iPSCs that can be efficiently generated from tissue easily accessible to dentists have great potential;²³ iPSCs have been generated from various oral mesenchymal cells²³ and these cells have been reported to possess higher reprogramming efficiency than skin fibroblasts do.¹⁰

Oral mucosal tissue is easily accessible and can be harvested by a simple and safe procedure. Oral mucosal wounds are characterized by rapid re-epithelialization and remodeling and are known to heal quickly compared with other skin injuries. This rapid re-epithelialization and remo-

deling is due to the increased production of active MMP-2 in oral mucosal fibroblasts compared with skin fibroblasts; MMP-2 may play an important role in rapid extracellular matrix reorganization and scarless wound healing.^{13, 24, 25} Therefore, we hypothesized that HOFs generated from patient tissue might provide a superior cell source for iPSCs. In the present study, we found that the endogenous expression level of *KLF4* was higher in HOFs than in ESCs or HOF-iPSCs. Endogenous *KLF4* has been shown to be expressed in gingival and periodontal fibroblasts derived from oral tissue.¹² Miyoshi et al. also found that HOFs express not only *KLF4* and *C-MYC* but also *NANOG* and *OCT4* at low levels, suggesting that HOFs possess a number of epigenetic advantages for reprogramming.¹³

Integrating virus-associated genotoxicity and tumor formation in iPSCs is of concern for clinical application.¹⁵ Integration-free human iPSCs have been generated using several methods.^{24, 26–30} Okita et al. used two of their findings to improve reprogramming efficiency using episomal plasmids;¹⁵ iPSC generation is markedly enhanced by *p53* suppression³¹ and *L-MYC* is more potent and specific than *C-MYC* during human iPSC generation.³² In our previous study,¹⁷ iPSCs were generated from HGFs using the above-mentioned method. The generated iPSCs expressed ESC-specific markers, as assessed by gene analysis and immunocytochemistry. Embryoid bodies and teratomas were formed from the iPSCs, demonstrating their ability to differentiate into three germ layers. However, 50 ESC-like colonies were obtained only 30 days post-HGF transfection. This lengthy reprogramming process (up to one month) is comparable to that of the standard virus-mediated methodology.³³

The maternal Gli-like transcription factor *Gli3* is highly expressed in unfertilized eggs and one-cell-stage embryos.¹⁸ Maekawa et al. showed that *Gli3*, but not *C-MYC*, increased iPSC tumorigenicity and markedly enhanced the generation of iPSCs from both mouse and human fibroblasts when expressed together with *OCT3/4*, *SOX2* and *KLF4*.¹⁸ In the present study, we observed 50 colonies of human ES-like cells as early as 20 days after initial episomal plasmid vector transduction. These results demonstrate that *Gli3* enhances the efficiency of iPSC generation using episomal plasmid vectors expressing *OCT3/4*, shRNA against *p53*, *SOX2*, *KLF4*, *L-MYC* and *LIN28*. However, iPSC generation from multiple donors will be required to establish the application of iPSC technology to biomedical research.

Fig. 4

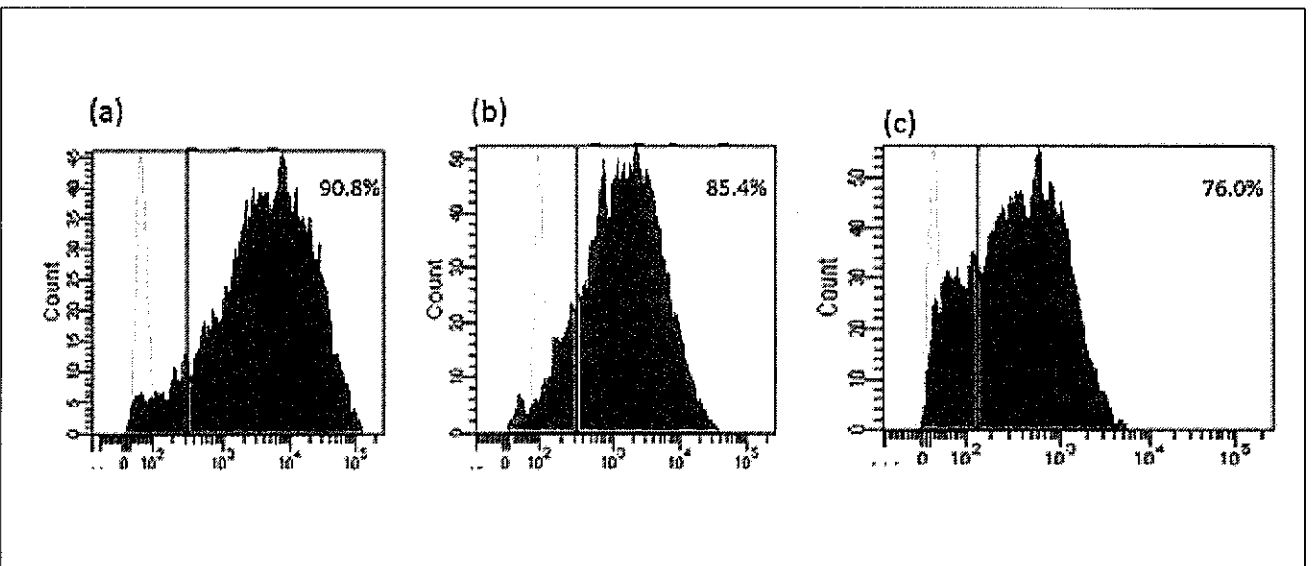
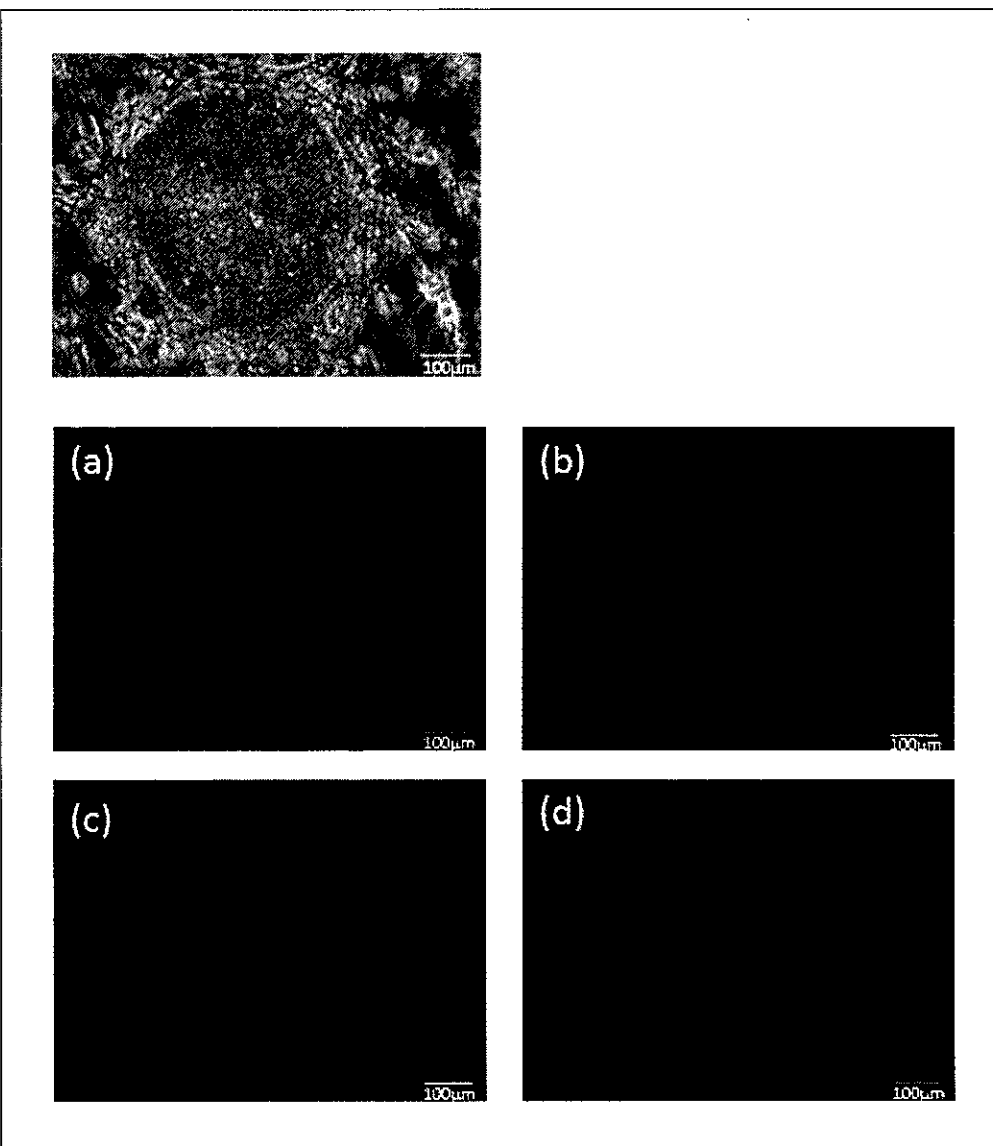


Fig. 5

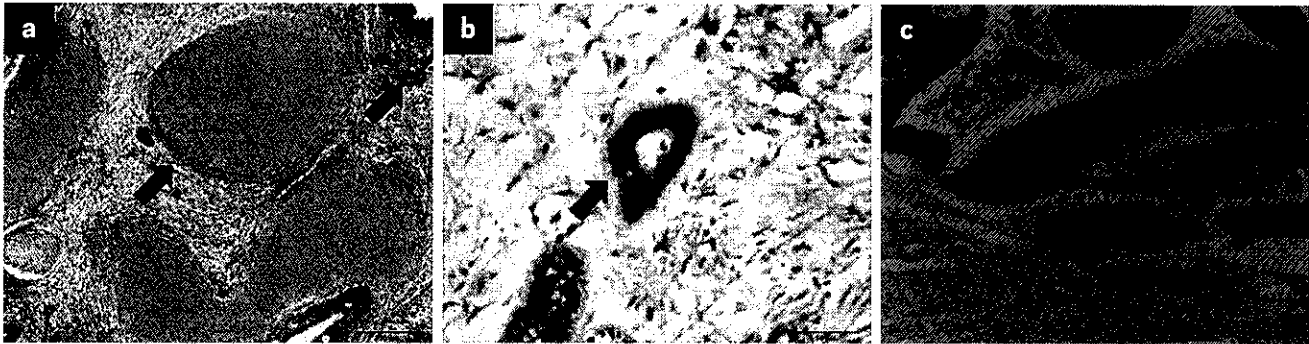


Figs. 4a–c

Flow cytometry analysis of pluripotent markers in HOF-iPSCs: (a) SSEA-3, (b) SSEA-4 and (c) TRA-1-60.

Figs. 5a–d

Generated HOF-iPSCs stained for (a) OCT3/4, (b) SSEA-4, (c) TRA-1-60 and (d) TRA-1-81; scale bar = 100 µm.



Figs. 6a-c

Figs. 6a-c

iPSCs have the potential to differentiate into three germ layers *in vivo*. Hematoxylin and eosin staining of teratomas derived from iPSCs at passage 20 revealed the presence of (a) cartilage (mesoderm; red arrow), melanocytes (ectoderm; black arrow), (b) gut-like tube tissue (endoderm; red arrow) and (c) neural tissue (ectoderm; red arrow).

Fig. 7

Karyotype analysis of iPSCs at passage 20 using G-band staining.

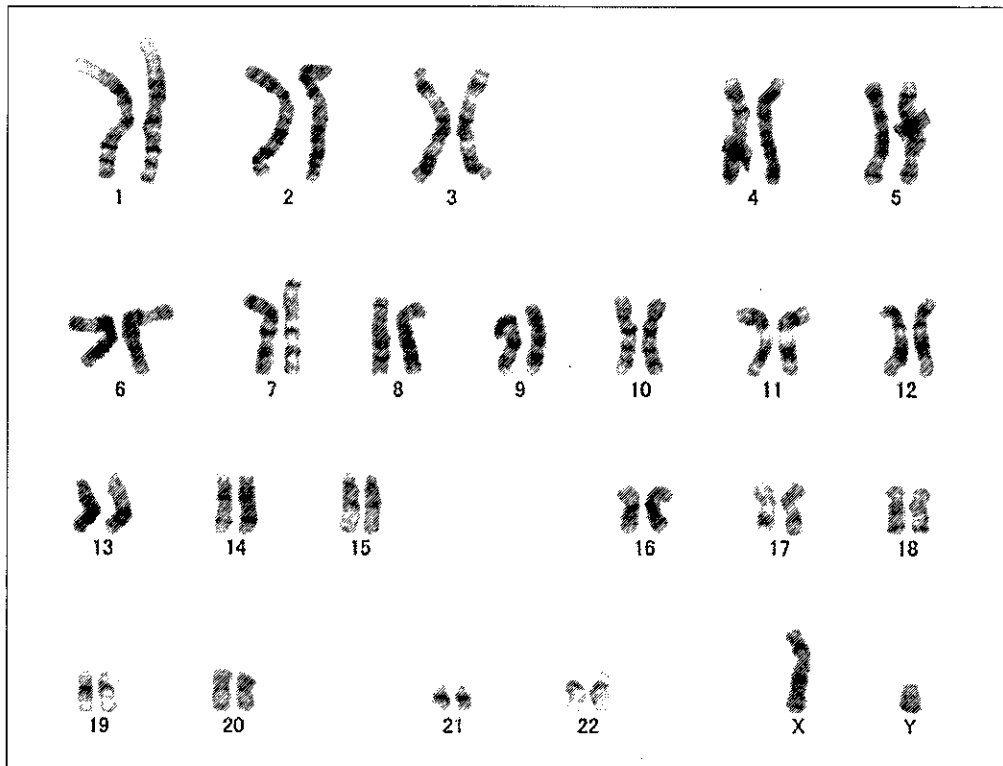


Fig. 7

Conclusion

Oral mucosal tissue can be conveniently obtained using a simple and safe procedure and possesses epigenetic advantages for reprogramming. We have successfully established a technique for rapidly and safely generating human iPSCs from oral mucosa using episomal plasmid vectors expressing *OCT3/4*, shRNA against *p53*, *SOX2*, *KLF4*, *L-MYC*, *LIN28* and *Glis1*. In order to repair large bone defects caused by trauma, tumors or congenital deficiency, it is necessary to combine sufficient cell numbers and biomaterials. The accelerated generation of integration-free human iPSCs would facilitate the application of clinical-grade iPSC technology for the treatment of large oral tissue or organ defects.

Competing interests

The authors declare that they have no competing interests.

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