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Expression of pluripotent stem cell factors in primary cells derived from human oral tissue

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Abstract

We previously reported that primary cells derived from rat dental pulp exhibit pluripotency, and that stem cell factors such as Stro-1, Ssea-1, Nanog, and Oct3/4 are expressed in these cells. In addition, downregulation of some factors is observed during lineage differentiation. We hypothesized that stem cell markers including *OCT3/4*, *SOX2*, and *NANOG* would be expressed in human oral tissue-derived primary cells. To further evaluate the expression of pluripotent stem cell factors in primary cells derived from human oral tissues, we quantitatively examined the expression of *OCT3/4*, *SOX2*, and *NANOG* in six primary cell lines derived from human gingival fibroblasts, human periodontal ligament fibroblasts, and human oral keratinocytes. Differential expression of *OCT3/4*, *SOX2*, and *NANOG* was detected in primary cells derived from various types of human oral tissue. Moreover, these cells were found to harbor multiple copies of all three genes. Levels of *OCT3/4*, *SOX2*, and *NANOG* correlated well with each other; in primary cells expressing low levels of *OCT3/4* and *SOX2*, the expression of *NANOG* also tended to be low. The expression of these factors in oral keratinocytes was higher than that in fibroblasts derived from human gingiva and periodontal ligament. This study indicates that primary cells derived from various types of human oral tissues express pluripotent stem cell factors. The results are expected to expand the future reservoir of potential cell sources that are suitable for cell therapy, replacing more controversial sources currently used.

Introduction

Primary cells derived from rodent dental pulp can differentiate into osteogenic, neurogenic, myogenic, and adipogenic lineages under *in vitro* induction conditions similar to those used for human bone marrow cells [1–5]. Several pluripotency markers have been identified, including *Oct3/4* [6–8], *Sox2* [9], and *Nanog* [10,11]. Previously, we reported that stem cell markers for rodents such as Stro-1, Ssea-1, Nanog, and Oct3/4, are expressed in rat dental pulp-derived cells [1]. Oct3/4, Sox2 and Nanog are transcription factors that are strongly expressed in embryonic stem cells [9–11], and their downregulation correlates with loss of pluripotency and self-renewal [12]. All are considered markers of primitive stem cells [13].

The epithelia consist of several layers of keratinocytes that have different characteristics. In the oral epithelium, oral keratinocyte stem cells reside close to the basal membrane and the vasculature of the lamina propria. These cells possess several attributes that distinguish them from other oral keratinocytes, including self-renewal potential, ability to differentiate into other cell types from the same tissue, quiescence, and long life span [14]. Oral keratinocyte progenitor cells can replace damaged epithelia with similar traits throughout the human body. Progenitor cells have been shown to regenerate the human cornea during transplantation procedures [15]. Although several studies have focused on the in vitro and in vivo behavior of oral fibroblasts following gingival augmentation, data on oral keratinocytes is scarce [16,17].

Therefore, we hypothesized that *OCT3/4*, *SOX2*, and *NANOG* would be expressed in primary cells derived from human oral tissues. To confirm this, we performed quantitative expression analysis using primary cells derived from various types of human oral tissues, such as human gingival fibroblasts, human periodontal ligament fibroblasts, and human oral keratinocytes.

Materials and methods

Cells and culture

Human gingival fibroblasts (HGnF) were isolated from human gingiva. They were cryopreserved at passage one and delivered frozen (ScienCell Research Laboratories, Inc., Carlsbad, CA, USA). Human periodontal ligament fibroblasts (HPLF) were isolated from human periodontal tissue. They were also cryopreserved at passage one and delivered frozen (ScienCell Research Laboratories, Inc.). Both HGnF and HPLF are characterized by their spindle morphology and were identified based on immunofluorescence with antibodies specific for fibronectin. HGnF and HPLF were cultured in Fibroblast Medium (ScienCell Research Laboratories, Inc.) supplemented with 10% (v/v) fetal calf serum (ScienCell Research Laboratories, Inc.) on Poly-D-Lysine-coated dishes at 37 °C in a humidified atmosphere containing 5% CO_2. Clonetics™ Human Periodontal Ligament Fibroblasts (HPdLF) were isolated from normal human periodontal tissue. Cryopreserved HPdLF were shipped at passage three (Lonza Walkersville, Inc., Walkersville, MD, USA). They were isolated from the ligament that fastens the molars to the jaw bone and stained negative for pan-cytokeratin. HPdLF were cultured in Stromal Cell Basal Medium (Lonza Walkersville, Inc.) supplemented with 0.1% (v/v) human fibroblast growth factor-B, 0.1% (v/v) human recombinant insulin,

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and 5% (v/v) fetal bovine serum (Lonza Walkersville, Inc.) on Poly-D-Lysine-coated dishes at 37 °C in a humidified atmosphere containing 5% CO_2. Human oral keratinocytes (HOK) were isolated from human oral mucosa. They were cryopreserved at passage one and delivered frozen (ScienCell Research Laboratories, Inc.). HOK were cultured in Oral Keratinocyte Medium (ScienCell Research Laboratories, Inc.) supplemented with 1% (v/v) Oral Keratinocyte Growth Supplement (ScienCell Research Laboratories, Inc.) on poly-D-Lysine-coated dishes at 37 °C in a humidified atmosphere containing 5% CO_2.

RNA extraction

Total RNA was extracted using a QIA shredder and RNeasy Mini Kit (QIAGEN Inc., Germantown, MD, USA) in accordance with the manufacturer's instructions. The extracted RNA was dissolved in UltraPure DNase/RNase-free distilled water (Life Technologies, Carlsbad, CA, USA), and the concentration of total RNA was measured using a NanoDrop spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). Experiment numbers C3 and C8 were assigned to total extracted RNA from different HGnF clones . C6 and C9 were assigned to total extracted RNA from HPLF and HPdLF, respectively. C1 and C11 were assigned to total extracted RNA from different HOK clones.

Quantitative real-time RT-PCR assay

cDNA was synthesized from total RNA using High Capacity RNA-to-cDNA Master Mix (Applied Biosystems, Foster City, CA, USA) in accordance with the manufacturer's instructions. λ polyA$^+$ RNA-A was added as an external reference during the reverse transcription process. As a reference to allow comparison between samples, 2.0×10^8 copies of λ polyA$^+$ RNA-A per 20 μL (Takara Bio Inc., Kusatsu, Shiga, Japan), diluted in EASY Dilution (Takara Bio Inc.), were added per 1 μg of total RNA during the cDNA synthesis process. Real-time RT-PCR was performed using the Fast SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) to examine the expression of each marker. Specific PCR primer sets were designed using the Perfect Real Time Support System (Takara Bio Inc.). The primer sets were as follows: 5′-GCAATTTGCCAAGCTCCTGAA-3′ and 5′- GCAGATGGTCGTTTGGCTGA-3′ for *OCT3/4*; 5′- GTGAGCGCCCTGCAGTACAA-3′ and 5′-GCTGCGAGTAGGACATGCTGTAG-3′ for *SOX2*; 5′- TCCAACATCCTGAACCTCAGCTA-3′ and 5′-AGGTTCCCAGTCGGGTTCAC-3′ for *NANOG*. PCR amplification was performed using the StepOne Plus system (Applied Biosystems) with an initial denaturation step of 20 sec at 95 °C, followed by 40 cycles each of 95 °C for 3 sec and 60 °C for 30 sec, finishing with an automatic melting curve stage. The values were adjusted using real-time primers for λ polyA (Takara Bio Inc.) as an internal control. The expression levels are presented as the fold change, comparing values after induction to those before induction, and were calculated using the $\Delta\Delta$Ct method.

Estimation of expressed copy number

λ polyA$^+$ RNA-A was diluted stepwise to a concentration of 1:10 using EASY Dilution (Takara Bio Inc). A quantitative standard curve was created using 1×10^1 to 1×10^6 copies of diluted λ polyA$^+$ RNA-A. Real-time primers for λ polyA (Takara Bio Inc.) were used for quantitative real-time RT-PCR, which was performed using the Fast SYBR Green PCR Master Mix (Applied Biosystems).

Results

Detection of pluripotent stem cell factors in human oral tissue-derived primary cells

The expression of *OCT3/4*, *SOX2* and *NANOG* was detected in two HGnF primary cell lines, two primary cell lines derived from human periodontal ligament fibroblasts, and two HOK primary cell lines. The differential expression levels are shown in Figure 1A, B, C.

Copy numbers of pluripotent stem cell factors in oral tissue-derived primary cells

Standards consisting of 10^1, 10^2, 10^3, 10^4, 10^5, and 10^6 copies had Ct values with mean ± standard deviation (SD) of 35.1 ± 0.52, 30.8 ± 0.13, 27.5 ± 0.03, 24.0 ± 0.01, 20.9 ± 0.02, and 17.5 ± 0.06, respectively. A standard curve was produced to directly compare copy number to Ct values for a copy number range of 1×10^1 to 1×10^6 (Figure 2). The mean Ct value was dependent on the serial dilution of λ polyA$^+$ RNA-A.

The mean Ct values for *OCT3/4*, *SOX2*, and *NANOG* for each primary cell line (derived from the various types of human oral tissues) are shown in Table 1. The estimated copy number for each marker was plotted on the standard curve. Using the standard curve, the copy numbers for *OCT3/4*, *SOX2*, and *NANOG* in each primary cell line were estimated and these values are presented in Table 2.

Discussion

Some reports suggest that cells positive for pluripotent stem cell factors such as *OCT3/4* and *SOX2* might represent reprogrammed cancer stem cells that induce oral carcinogenesis [18]. The expression of *OCT3/4*, *SOX2*, *NANOG*, and long non-coding RNAs, which regulate reprogramming, is associated with the development and prognosis of oral squamous cell carcinoma [19,20]. Side population (SP) cells have been identified as a side branch of Hoechst^{low} cells that comprise a darker fraction than cells in the G0/G1 phase in flow cytometry, and are present as cancer stem cells in many human cancers and cancer cell lines. We previously reported stem-like properties including pluripotency, self-renewal, and retention of an undifferentiated state in this subpopulation of human oral cancer SSC-4 cells. *OCT3/4* and *NANOG* levels were significantly higher in SP cells than in non-SP cells, suggesting that a subset of oral cancer cells has the potential to act as stem cells [21]. The levels of pluripotent stem cell factors have been well characterized in cancer stem cells; however, they require further characterization in normal cells derived from human oral tissues.

The present study found that *OCT3/4*, *SOX2*, and *NANOG*, pluripotent stem cell factors, are differentially expressed in primary

Table 1. Mean Ct values of *OCT3/4*, *SOX2*, and *NANOG* in each primary cell line derived from various types of human oral tissues.

	C1	C3	C6	C8	C9	C11
OCT3/4	31.3	33.6	32.9	32.0	32.2	29.8
SOX2	31.1	35.8	35.4	34.5	34.3	29.6
NANOG	34.3	34.3	34.6	34.1	33.1	33.3

Table 2. Copy numbers of *OCT3/4*, *SOX2*, and *NANOG* in each primary cell line derived from various types of human oral tissues.

	C1	C3	C6	C8	C9	C11
OCT3/4	90.2	19.5	31.1	56.6	49.6	245.0
SOX2	103.1	4.5	5.9	10.7	12.2	28.0
NANOG	12.2	10.0	14.0	27.2	23.8	11.8

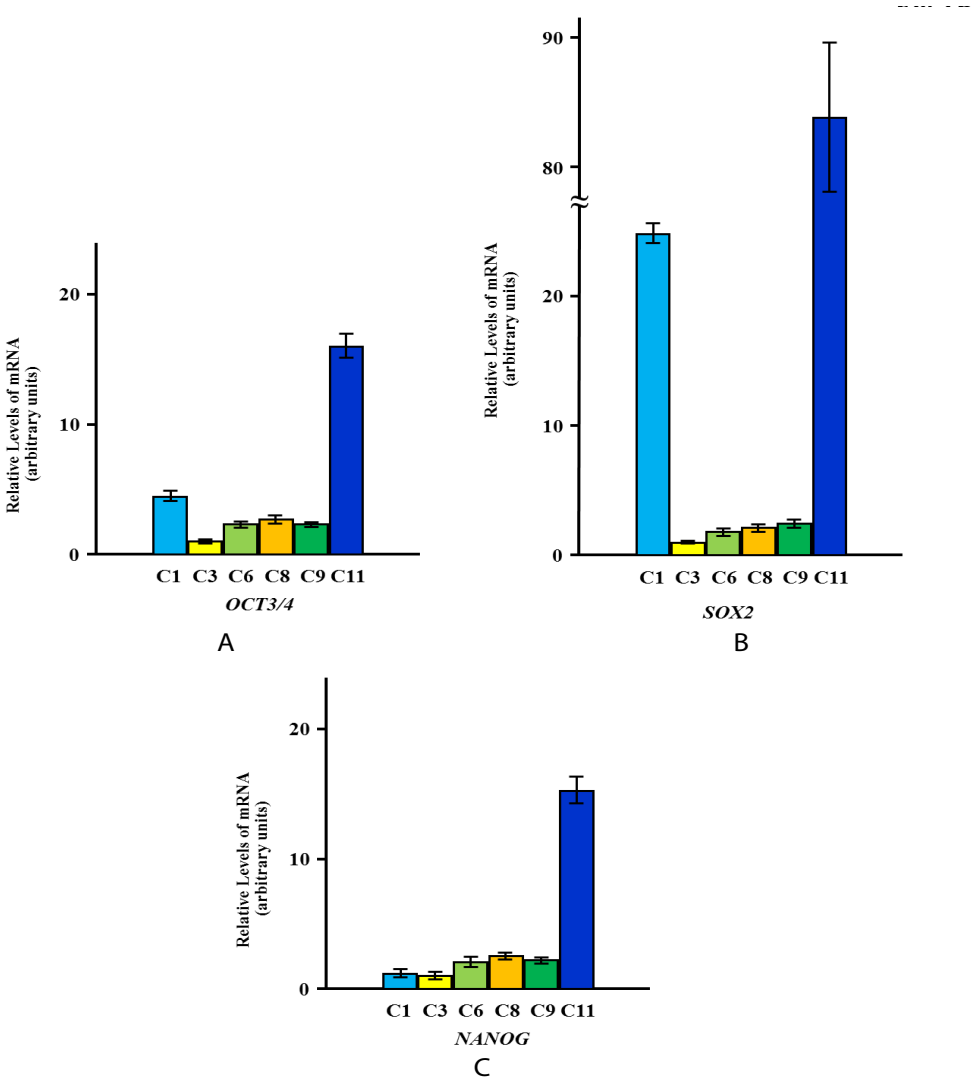

Figure 1. Quantitation of expression levels of *OCT3/4*, *SOX2*, and *NANOG* in primary cells derived from various types of oral tissues.

Expression levels of *OCT3/4* (A), *SOX2* (B), and *NANOG* (C) in primary cells derived from various types of oral tissues are shown. The yellow, green, and blue columns represent primary cells from human gingival fibroblasts (C3, C8), human periodontal ligament fibroblasts (C6, C9), and human oral keratinocytes (C1, C11), respectively. The relative mRNA expression levels are presented as the fold change in each cell vs. C3 as a reference, and represent the mean values of six independent assays for each cDNA sample. The values were calculated using Relative Quantity (RQ) Study Software and show statistical variability in terms of the calculation of each sample's RQ value. The RQmin/RQmax values are graphically represented as error bars.

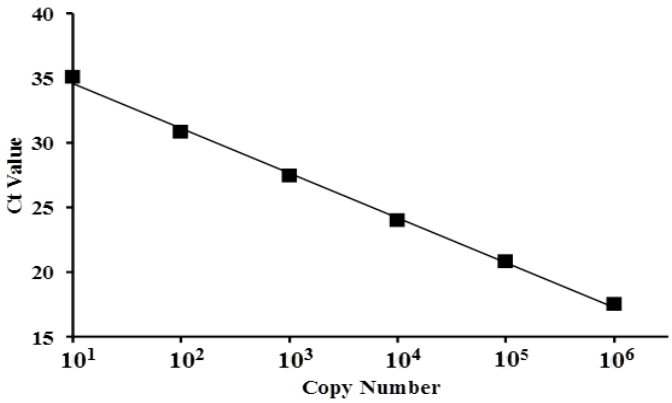

Figure 2. Scatter plot and standard curve of gene copy numbers based on λ polyA$^+$ RNA-A.

Ct values (represented by squares) were plotted for serially diluted samples of λ polyA$^+$ RNA-A. The x-axis represents copy number and the y-axis represents the Ct value. The standard curve for copy number ranged from 1×10^1 to 1×10^6 (depicted in the graph).

cells derived from various types of oral tissues. Copy numbers of *OCT3/4*, *SOX2*, and *NANOG* in these primary cells ranged from 19.5 to 245.0, 4.5 to 103.1, and 10.0 to 27.2, respectively. In primary cells expressing low levels of *OCT3/4* and *SOX2*, the level of *NANOG* also tended to be low. Although the expression of pluripotent stem cell factors in oral keratinocytes was relatively higher compared to that in fibroblasts derived from human gingiva and periodontal ligaments, the relationship between stem-like properties such as pluripotency or self-renewal and the expression of these factors remains unclear.

Recently, we reported that the direct conversion of dental pulp cells to cells of developmentally unrelated tissues involves miRNA-dependent regulation [22]. Pluripotent stem cell factors and intercellular signaling factors might be fundamentally regulated in a genetic and epigenetic manner, such that the expression of these factors might affect significant biological functions including stem-like properties. Highly efficient miRNA-mediated reprogramming of somatic cells has previously been reported [23,24]. The expression of the

miR-302/367 or miR-302/372 cluster rapidly and efficiently reprograms somatic cells into iPS cells without exogenous transcription factors such as *Oct3/4* or *Sox2*. It is possible that pluripotent stem factors are regulated by intracellular signaling via small RNAs from exosomes, for example. This study has expanded the potential cell sources suitable for regenerative medicine. Indeed, factors secreted by human dental pulp stem cells have been identified to have multifaceted therapeutic benefits for recovery after several types of injuries or diseases, such as those of the spinal cord, heart, and liver [25-27]. This study determined the exact copy numbers of pluripotent stem cell factors in primary oral cells. Therefore, primary cells derived from oral tissues expressing these factors or factors secreted by primary cells represent an attractive and promising tool in the field of tissue regeneration and engineering. We believe that the primary cells derived from various types of human oral tissues will become an acceptable source of cells for cell therapy in the future, replacing the more controversial sources currently used.

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Competing interest

The authors declare that there is no conflict of interest.

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