

Establishment of immortalized human periodontal ligament cells derived from deciduous teeth

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Abstract. Although periodontal ligament (PDL) cells have previously been isolated from permanent teeth, they have not been isolated from deciduous teeth. Here, we used human telomerase reverse transcriptase (*hTERT*) induction to establish the first immortalized PDL cell lines derived from deciduous teeth. Cells were transfected with plasmids containing *hTERT*. Single-cell cloning was then performed using the limited dilution method. Reverse transcriptase polymerase chain reaction and stretch PCR were used to detect *hTERT* expression in the clones. In order to determine whether the clones could differentiate into osteoblasts, we stimulated the cells with ascorbic acid and β -glycerophosphate. We successfully obtained 3 single-cell clones, and named them single cell derived from human deciduous PDL (SH) 9, 10 and 11. All the SH cells showed *hTERT* expression and stable proliferation after >80 population doublings and expressed the marker genes of PDL cells, including scleraxis, periostin, cementum-derived protein 23, and tenomodulin. Although all the clones expressed osteoblastic markers, only the clones from the SH 9 cell line differentiated into osteoblastic cells. This is the first report of the immortalization of PDL cells derived from deciduous teeth. These cells could be useful in studies investigating the cellular mechanisms and regenerative processes of human PDL cells.

Introduction

The periodontal ligament (PDL) is a highly specialized connective tissue that connects the cementum to the alveolar bone (1). PDL tissue consists of a heterogeneous cell population that includes fibroblasts, cementoblasts, osteoblasts, endothelial progenitor cells, epithelial cell rests of Malassez, macrophages, osteoclasts and progenitor/stem cells (2). As PDL is located between the tooth root and alveolar bone, it not only has an important role in supporting the teeth, but also contributes to tooth nutrition, homeostasis and to the repair of damaged tissue (3). However, it is unclear which cells are responsible for homeostasis and the regeneration of PDL tissue.

Fibroblasts are the predominant PDL cell type (4) and are thought to control PDL homeostasis and regeneration (5). Furthermore, we have previously reported that PDL fibroblasts contribute to the regulation of bone metabolism by expressing the receptor activator NF- κ B ligand and osteoprotegerin (6,7). Fibroblasts derived from the PDL have the ability to form bone-like tissues *in vitro*, similar to osteoblasts (8), and thus PDL cells function similarly to osteoblasts in hard tissue formation and express proteins with osteoblastic properties such as the runt-related transcription factor-2 (RUNX-2), osterix (9), osteocalcin (OSC) (10), osteonectin (OSN), type I collagen (Col I) (11), alkaline phosphatase (ALP) (12), parathyroid hormone receptor (PTHr) and periostin (13). Other studies have also shown that PDL fibroblasts express scleraxis (14), tenomodulin (15), and the cementum-derived protein-23 (CP-23) (16). However, the mechanism underlying the regulation of the differentiation of PDL cells is unclear.

PDL cells used in previous studies were a heterogeneous population, making it difficult to determine whether the cell types functioned alone or in cooperation with other cell types. These cells had a limited lifespan and were phenotypically unstable (17). Therefore, an immortal PDL cell line is required in order to clarify the functions of individual cells composing PDL.

PDL cell lines have previously been derived from permanent teeth (18,19), but not from deciduous teeth, as the cells are difficult to isolate. Exfoliated deciduous teeth lack their roots, which are needed to isolate the PDL cells, as these are resorbed during the eruption of permanent teeth. As PDL

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Abbreviations: hTERT, human telomerase reverse transcriptase; CP-23, human cementum-derived protein-23; TeM, tenomodulin; RUNX2, runt-related transcription factor-2; OSC, osteocalcin; OSN, osteonectin; Col I, type I collagen; ALP, alkaline phosphatase; PTHr, parathyroid hormone receptor

Key words: human periodontal ligament cells, deciduous teeth, human telomerase reverse transcriptase, immortalization, single cell clone, calcification

cells derived from deciduous teeth are thought to contain more progenitor/stem cells than those derived from permanent teeth, they could be more useful for research.

Most somatic diploid cells lose telomere length during cell replication, leading to replicative senescence (20). Telomere length is dependent upon telomerase, a cellular ribonucleoprotein complex consisting of 2 components, namely integral RNA that serves as the telomeric template, which has reverse transcriptase activity and an associated protein (21). The induction of the human telomerase reverse transcriptase (*hTERT*) results in telomere length elongation and in the extension of the *in vitro* replicative life span of human mortal somatic cells (22,23).

In this study, we describe how the induction of the *hTERT* gene allowed us to establish immortalized PDL cells derived from deciduous teeth. We also provide an evaluation of whether these cell lines have PDL properties, and the potential to differentiate into osteoblastic cells *in vitro*.

Materials and methods

Cell culture. PDL tissues were obtained from the middle third of the root surfaces of healthy human deciduous teeth, whose roots were not resorbed before the eruption of permanent teeth (obtained from 3 donors, aged 7 to 8 years), as previously described (6,7). Informed consent was obtained from the parents of the donors before tooth extraction, which was carried out in our hospital during the course of orthodontic treatment. The study protocol was approved by the Ethics Committee of Iwate Medical University, School of Dentistry (no. 01101).

A surgical blade was used to cut the PDL tissues into pieces so that they could be digested with collagenase (2 mg/ml) at 37°C for 30 min. The tissues were then washed with Dulbecco's phosphate-buffered saline (PBS), placed on culture dishes, and maintained in α -modified minimum essential medium (α -MEM; Gibco BRL, Gaithersburg, MD, USA) supplemented with 10% foetal bovine serum (FBS; Gibco BRL). Fibroblastic cells that grew from the PDL tissue culture were used as the PDL cells. When they reached confluence in 35-mm culture dishes, they were detached with 0.2% trypsin and 0.02% EDTA 4Na in PBS and subcultured at a 1:4 split ratio. All cultures were maintained at 37°C in a humidified atmosphere of 5% CO₂.

Transfection of the *hTERT* gene. All the PDL cells were transfected with a *pBABE-neo-hTERT* plasmid containing a neomycin-resistant gene (Addgene plasmid 1774, provided by Addgene Inc. Cambridge, MA, USA) and Lipofectamine™ LTX (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Cells were exposed to 10% FBS α -MEM containing 150 μ g/ml G418 (Gibco BRL) for 12-15 days. The surviving cells were trypsinized and allowed to grow in 100-mm culture dishes.

Single-cell cloning. Single-cell clones were obtained using the limited dilution method. After *hTERT* transfection and selection with G418, the surviving cells were seeded on a 96-well plate (Corning, NY, USA) at 0.5 cells per well, then incubated at 37°C in a humidified incubator in an atmosphere

Table I. Primers used in RT-PCR.

Gene name	Primer	Oligonucleotide sequence (5'-3')
<i>hTERT</i>	Forward	TCTGGGATGCGAACGGGC
	Reverse	TCCGGCTCAGGGGCAGC
Scleraxis	Forward	TGCGAATCGCTGTCTTTC
	Reverse	GAGAACACCCAGCCCAA
Periostin	Forward	TGTTGCCCTGGTTATATGAG
	Reverse	ACTCGGTGCAAAGTAAGTGA
CP-23	Forward	AACACATCGGCTGAGAACCCTCAC
	Reverse	GGATACCCACCTCTGCCTTGAC
TeM	Forward	TTGAAGACCCACGAAGTAGA
	Reverse	ATGACATGGAGCACACTTTC
RUNX2	Forward	CCCCACGACAACCGCACCAT
	Reverse	GTCCACTCCGGCCCCACAAATC
Osterix	Forward	CCTGGCTGCGGCAAGGTGT
	Reverse	GATCTCCAGCAAGTTGCTCTGC
OSC	Forward	CAGCAAAGGTGCAGCCTTTGT
	Reverse	TCCTGAAAGCCGATGTGGTC
OSN	Forward	CCGAAGAGGAGGTGGTGGCGG
	Reverse	ACGGGGTGGTCTCCTGCCTCC
Col I	Forward	ACTGGGGAAACCTGTATCCGG
	Reverse	AAGGGCAGGCGTGATGGCTTA
ALP	Forward	CTCGTTGACACCTGGAAGAGC
	Reverse	ACAGGATGGCAGTGAAGGGCT
PTHr	Forward	AGGAACAGATCTTCTGCTGCA
	Reverse	TGCATGTGGATGTAGTTGCGCGT
β -actin	Forward	CTGGCACCACCTTCTACAATG
	Reverse	AATGTACAGCACGATTTCCCGC

of 95% air and 5% CO₂. After the cells had grown for 10 days, they were treated with trypsin and subcultured into 24-well plates (Corning). This was repeated until confluency was reached at 20 days after single cell cloning. The population doubling (PD) level was defined as the number of doublings required for a single cell to reach confluence in a 100-mm culture dish. Both the PDs and the incubation day were considered to be zero when single-cell cloning was performed. PD was estimated at 20 for the PDL cell clones. Clones obtained after single-cell cloning in this study were named single cell derived from human deciduous PDL (SH).

Telomeric repeat amplification protocols. Telomerase activity was assayed by the stretch PCR method, using the TeloChaser (Toyobo, Osaka, Japan), according to the manufacturer's instructions. The TAG-U primer was mixed with crude cell extracts from SH or HeLa cells (2.5x10⁴ cells for each reaction) and was incubated at 37°C for 60 min to produce telomeric repeats (TTAGGG). After the telomerase reaction, DNA was purified and amplified with CTA-R primers. The PCR products were separated on a 10% acrylamide SDS-PAGE gel and stained with ethidium bromide. A HeLa cell extract was used as the positive control for telomerase activity. Heat-treated (60°C for 10 min) extracts of HeLa cells served as the negative control.

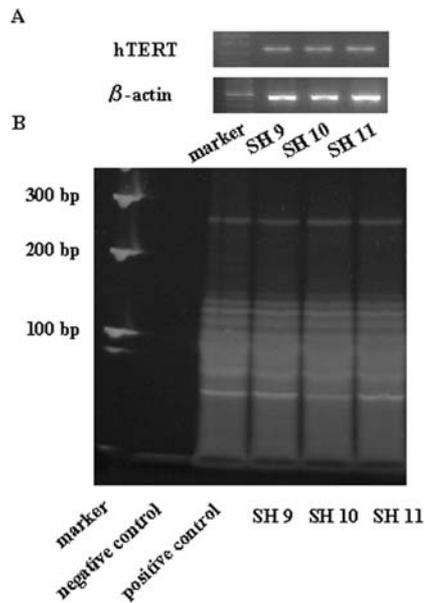


Figure 1. Expression of *hTERT* mRNA and *hTERT* activity in SH cells. (A) Telomerase activity was detected using stretch-PCR methods. (B) HeLa cells were used as the positive control. HeLa cells extract inactivated by heat treatment was used as the negative control.

Gene expression detection with reverse transcription polymerase chain reaction (RT-PCR). After 23 PDs, total RNA from the SH clones was isolated using TRIzol reagent (Invitrogen), according to the manufacturer's instructions. RNA sample (1 μ g) was reverse-transcribed to first-strand cDNA using a PrimeScript RT reagent kit (Takara Shuzo, Kyoto, Japan), according to the manufacturer's instructions. mRNA from cDNA samples was amplified with specific

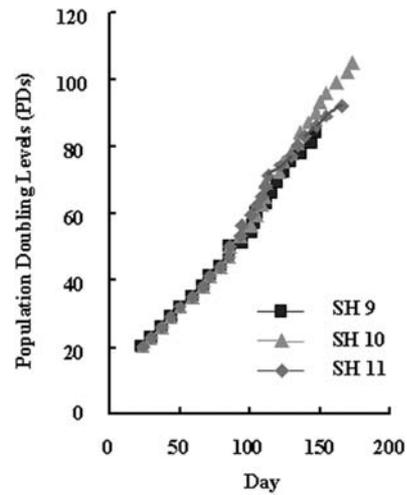


Figure 2. PD levels of SH cells.

primer pairs for β -actin, *hTERT* (19), scleraxis (15), periostin (24), CP-23 (16), and tenomodulin (TeM) (15) for the PDL markers, and RUNX2 (25), osterix (19), OSC, OSN, Col1a, ALP, and PTHR (26) for the osteoblastic cell markers. The primer sequences are shown in Table I.

Calcification assay. All the SH clones were cultured on 60-mm culture dishes with or without 10% FBS α -MEM containing 50 μ g/ml ascorbic acid (Nakarai Tesque, Kyoto, Japan) and 6 mM β -glycerophosphate (Sigma, St. Louis, MO, USA), a mixture referred to here as the differentiation medium (DM). Half the medium in each dish was exchanged every 2-3 days. After 4 weeks of culture, the cells were fixed with 10%

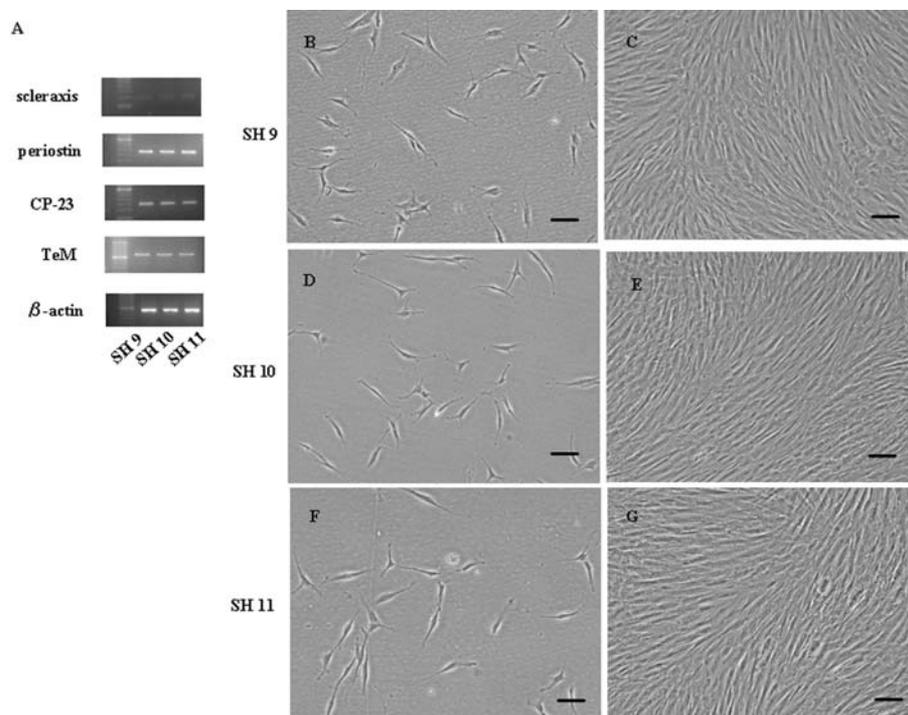


Figure 3. Results from RT-PCR analysis and comparison of SH cell morphology. (A) All the SH cell lines expressed mRNAs specific for PDL at 23 PDs. (B) There was no difference in the morphologies of the SH cell clones, either before or after confluence. The bars represent 100 μ m.

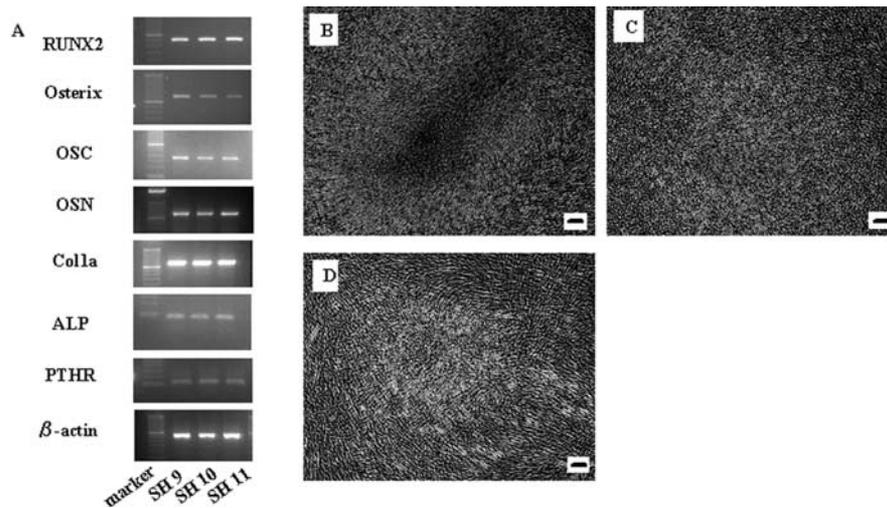


Figure 4. RT-PCR analysis and von Kossa staining in the SH cells cultured in DM for 4 weeks. (A) mRNA expression of RUNX2, osterix, OSC, OSN, Col I, ALP and PTHR in SH cells. Results of von Kossa staining using phase-contrast microscopy. The SH 9 cells formed mineralized nodules (B) though the SH 10 and 11 cells only formed multilayered nodules (C and D). The bars represent 100 μ m.

formaldehyde, then washed with distilled water and exposed to von Kossa stain, as previously described (27).

Results

Expression of *hTERT* in SH cell lines 9, 10, and 11. At PD 23, we observed the *hTERT* mRNA expression in the SH cell lines 9, 10 and 11 (Fig. 1A). The stretch PCR method indicated that there was telomerase activity in the SH extracts and the positive control, but not in the negative control (Fig. 1B). These results show that the SH cell line possesses telomerase activity.

Growth curve of SH cell lines 9, 10 and 11. All SH cell lines grew at a similar rate, \sim 1 PD every 2 days (Fig. 2). The SH cells divided at least 80 times and were passaged $>$ 40 times.

Characteristics of SH cell lines 9, 10, and 11. At PD 23, all the SH cells expressed scleraxis, periostin, CP-23, and TeM (Fig. 3A), at levels similar to those of the original primary PDL cells. However, the scleraxis expression was lower than that of the other genes.

The cells in all the 3 lines were spindle-shaped (Fig. 3B, D and F), a feature consistent with fibroblasts. After culture confluence, there were no morphological differences among the SH clones (Fig. 3C, E and G).

Osteogenic characterization. At PD 23, all the SH cell lines expressed bone-related genes, such as RUNX2, osterix, OSC, OSN, Col I, ALP and PTHR (Fig. 4A).

The SH 9 cells cultured in DM formed von Kossa-positive calcified deposits (Fig. 4B). The SH 10 and 11 cells cultured in DM formed multilayer nodules, but not calcified deposits (Fig. 4C and D).

Discussion

In this study, we established 3 PDL fibroblast cell lines immortalized by transfection with *hTERT*. SH 9 cells have

the characteristics of original primary cells with respect to morphological appearance, gene expression, and production of calcified deposits. Our results suggest that immortalized cells such as these could be useful tools for elucidating the biology and regenerative mechanisms of human PDL.

Normal cells have a limited capacity to divide, and cellular senescence occurs immediately *in vitro*. This is thought to result from telomere DNA loss with passage progression (28). Since cellular senescence is induced by telomere shortening in certain cell types, the overexpression of the *hTERT* gene, which prevents this process, facilitates the immortalization of cells (21,23). In the SH cell lines transfected with the *hTERT* gene (lines 9-11), we observed telomerase activity and growth that was not retarded over $>$ 80 PDs. The morphology of the SH cell lines was not altered. These results suggest that the *hTERT* gene was stably expressed. Additionally, the induction of the *hTERT* gene extended cell lifespan by preventing cellular senescence.

PDL cells have osteoblastic properties and the capacity to form bone-like tissue *in vitro* (8). In this study, only the SH 9 cells incubated in DM produced calcified deposits. However, the SH 10 and 11 cells also expressed RUNX2, osterix, OSC, OSN, Col I, ALP and PTHR, despite having no osteogenic ability (Fig. 4A). SH 10 and 11 formed multilayered nodules when cultured in DM, but did not produce calcified deposits (Fig. 4C and D). Based on these findings, we believe that we successfully isolated both osteogenic and non-osteogenic cell lines from the original PDL cells.

All the SH cell lines expressed scleraxis and TeM mRNA. Scleraxis, a basic helix-loop-helix transcription factor, is expressed in tendon progenitor populations and mature tendons (14,29). TeM is a late marker of tendon formation (30,31). PDLs have tendon-like features, and they usually do not differentiate into osteoblasts and form bone. This could be a mechanism used to maintain PDL space *in vivo*. Additionally, periostin is an extracellular matrix protein that is expressed in PDL and periosteum (32). CP-23 is expressed in PDL fibroblasts and cementoblasts but not in osteoblasts (16). Altogether, the presence of these products in our SH

cell lines indicates that the isolated cells have properties expected of PDL fibroblasts.

It has previously been reported that PDL cells from permanent teeth could not be immortalized with the *hTERT* gene alone (19). In this study, PDL cells from deciduous teeth were immortalized with single transfection of the *hTERT* gene. As we also failed to establish immortalized cells from dental pulp and PDL isolated from permanent teeth with the *hTERT* gene alone (data not shown), it has been suggested that certain systems such as tumor-suppression, could differ between deciduous and permanent teeth.

To our knowledge, this is the first study detailing how immortalized PDL cells can successfully be derived from deciduous teeth. We achieved this in 3 separate cell lines by transfecting them with the *hTERT* gene in order to elongate the telomeres. These immortalized cells could be useful tools for understanding the cellular functions and maintenance of PDL tissues. They could also be useful in regenerative therapies for periodontitis and oral injuries.

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