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**Original**

# Establishment of immortalized clonal cells derived from periodontal ligament cells by induction of the hTERT gene

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**Abstract: Since the periodontal ligament (PDL) contains a heterogeneous cell population, it is challenging to identify all cell types within the tissue and to determine whether they function alone to produce tissue components or interact with other cell types. Further, it is difficult to isolate and expand single cell clones from PDL cells, as normal cells have a limited life span and are phenotypically unstable. In the present study, we inserted the human telomerase reverse transcriptase (hTERT) gene, which encodes the catalytic subunit of the telomerase holoenzyme, into normal human periodontal ligament (HPL) cells and successfully obtained single cell clones. Expression of the inserted gene and telomerase activity in each of the clones was confirmed. Unlike the original HPL cells, at the end of the study (day 120), clone populations continued to actively double without phenotypic alteration. Osteogenic characteristics were present in some but not all clones. In conclusion, immortalization of HPL cells was successfully accomplished by transduction with the hTERT gene. This is the first report of immortalization of different cell types derived from PDL. (J. Oral Sci. 47, 177-184, 2005)**

Keywords: periodontal ligament cell; hTERT; immortalization; single cell clone.

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## Introduction

The periodontium is a highly specialized organ which supports the teeth. It comprises soft connective tissues, the periodontal ligament (PDL) and the gingiva, as well as cementum and alveolar bone. The PDL is a soft connective tissue embedded between the cementum and the inner wall of the alveolar bone socket, to sustain and help constrain the teeth within the jaw. PDL not only has an important role in supporting teeth, but also contributes to tooth nutrition, homeostasis, and repair of damaged tissue (1,2). The recognition that regeneration of these tissues can be achieved has resulted in increased attempts to understand the cellular and molecular mechanisms involved in regulating formation of these tissues during development and regeneration (3).

Spindle-shaped fibroblasts are the main cell type found in the PDL, and those derived from the PDL appear to display distinct functional activities to those found in the gingival tissues (4). The mammalian PDL cells comprise a heterogeneous cell population of connective tissue cells (5,6), including precursor cells that have the capacity to differentiate into osteoblasts and cementoblasts as well as fibroblasts (7-9).

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. Since these cells had a limited life span and were phenotypically unstable, the cells often showed changes in functional activity with prolonged culturing (10). Therefore, to clarify the functions of single cell types in PDL, an immortal cell line of PDL cells needs to be established.

d'Errico et al. (11) and Saito et al. (12) established a mouse PDL cell line, and Parker et al. (13) and Hoang et al. (14) succeeded in establishing a human PDL cell line, utilizing SV40 gene transduction in all cases . Infection with this DNA tumor virus inactivates the p53 and RB proteins (15) and frequently results in extension of cell life spans by about 20 population doublings (16). However, DNA tumor viruses are not able to immortalize human cells (17) because telomeres are shortened until the cell population reaches a state of crisis.

To avoid the crisis state, cells must have a telomere maintenance system (18). Cellular senescence of some cell types, such as human foreskin fibroblasts (BJ fibroblasts), appears to be induced only by telomere shortening, since overexpression of the human telomerase reverse transcriptase (hTERT) gene alone can allow cells to become immortalized (19). The hTERT gene encodes the catalytic subunit of the telomerase holoenzyme, and results in telomere length elongation and extension of the *in vitro* replicative life span of human mortal somatic cells (19). Kamata et al. (20) reported that immortalized cells could be derived from PDL tissue by transduction with the hTERT gene and HPV16 genes. However, immortalized clonal cells derived from normal human PDL by transduction with the hTERT gene alone have not been previously reported.

In the present study, we attempted to immortalize PDL cells by transduction with the hTERT gene to prevent cellular senescence during isolation and expansion.

## Materials and Methods

### Cell culture

Human periodontal ligament cells (HPL) were isolated from the non-inflamed human PDL tissues, collected from teeth extracted for orthodontic reasons. Informed consent from donors was obtained for use of the tissues.

Cells were cultured according to the method of Somerman et al. (21), with some modifications. Briefly, cells were grown in Dulbecco's modified Eagle's medium; DMEM (Sigma Chemicals, St. Louis, MO, USA), supplemented with 10% fetal bovine serum; FBS (Hyclone, Logan, USA) and antibiotic-antimycosis (Gibco, NY, USA). The cultures were kept at 37ºC in a humidified incubator (Forma $CO_2$ incubator MIP-3326, Sanyo Electric Medical System, Tokyo, Japan) in an atmosphere of 95% air and 5% $CO_2$. When cells had reached confluence in 35-mm culture dishes (Corning, NY, USA), they were trypsinized and passaged onto 100-mm cell culture dishes (Corning). These cells were considered as population doubling level 0 (0 PDs) and sub-cultured in the same medium as described above. The cells were also subcultured at a ratio of 1:4, permitting cells to be counted at 2 population doublings when the cells reached confluence.

### Transduction of hTERT gene into cells

The gene for hTERT was cloned into a pMSCVpuro retroviral vector (Clontech, CA, USA) using the EcoRI restriction enzyme. The cloned retrovirus vector was then transfected into the packaging cell line, RetroPack PT67 (Clontech) which was followed by selection with puromycin (1.8 μg/ml) (Sigma) 48 hours later. Two weeks after transfection, the surviving cells were trypsinized and allowed to continue to grow. The supernatants of the packaging cell lines were then collected and filtered through a 0.22 μm syringe filter, and polybrene (8 μg/ml) was added prior to their use as a retrovirus to transduce the hTERT gene into HPL cells.

HPL cells were plated on a T25 flask (Corning) at a concentration of $1.0 \times 10^5$ cells/ml 24 hours prior to infection. The packaged retrovirus (2 ml/dish) was then added to the medium of the T25 flask of target cells and incubated for 24 hours at 37ºC in a humidified incubator in an atmosphere of 95% air and 5% $CO_2$. The medium was changed, and the infected cells were then selected by treatment with 0.5 μg/ml puromycin (Sigma) for 7-10 days and subsequently used in the experiments.

### Cloning

Single cell clones were obtained using a limited dilution method. Following transduction with hTERT, HPL cells were seeded on a 96-well plate (Corning) at 0.5 cells per well, and then incubated at 37ºC in a humidified incubator in an atmosphere of 95% air and 5% $CO_2$. After the cells had grown for 14 - 21 days, they were treated with trypsin and sub-cultured on 100-mm cell culture dishes (Corning) until the cells reached confluence. The PD level, or the number of doublings required for a single cell to reach confluence in a 100-mm cell culture dish, was estimated at 30 for the clones.

### Telomeric repeat amplification protocol

Telomerase activity was determined using a PCR-based telomeric repeat amplification protocol (TRAP assay). TRAP assays were performed using a TRAPEZE telomerase detection kit (Chemicon International, Tenecula, CA, USA) according to the manufacturer's protocol. Telomerase-positive cells were used as a positive control, and lysis buffer was used as a negative control. The cells ($1.0 \times 10^5$) were lysed in 200 μl of cold TRAP lysis buffer. A quantity of extract representing $1.0 \times 10^3$ cells (2 μl of 1 % of an extract from $10^5$ cells) was incubated with 5.0 μl of 10 × TRAP reaction buffer, 1.0 μl of 50 × dNTP Mix,

1.0 µl of TS primer, 1.0 µl of TRAP primer Mix, 0.4 µl (2 Units) of Taq polymerase and 39.6 µl of $H_2O$. Fifty µl of reaction mix was incubated at 30ºC for 30 minutes and a 2-step PCR was performed for 30 cycles at 94ºC for 30 seconds and 59ºC for 30 seconds in a thermocycler (GeneAmp PCR System 9700, Applied Biosystems; Tokyo, Japan). The PCR products were separated by electrophoresis on a 12% polyacrylamide gel and stained with SYBR Green (Molecular Probes; Eugene, OR, USA).

## Alkaline phosphatase (ALP) activity

After nearly 30 PDs, a number of clonal cells were isolated and named A4, B6, C10, E10, F4 and H6. After nearly 70 PDs, the sample cells were frozen at -80ºC, then prior to use in experiments, cells were thawed and cultured for 4 more PDs. The cells were plated on 96-well plates at a density of $1 \times 10^5$ cells per well and cultured for 7 days. After the supernatant was removed, ALP activity was measured after incubation with 10 mM p-nitrophenylphosphate in 0.2 M Tris-HCL buffer (pH 10) at 37ºC for 30 minutes. Absorbance at 405 nm was read using a microplate reader (MIP-32, Corona, Ibaraki, Japan).

## Mineralization activity

The mineralization activity of the single cell clones A4 and C10 was examined using alizarin red S (ALZ) and von Kossa staining. Cells were cultured in DMEM containing 10% FBS supplemented with 50 µg/ml of ascorbic acid, 5 mM of β-glycerophosphate, and 10 nM of dexamethasone for 21 days in 6-well plates. For ALZ staining, cells were fixed for 30 min in 10% neutral buffer and rinsed with distilled water, after which they were stained with alizarin red S (Kanto Chemical, Tokyo, Japan) for 10 minutes. For von Kossa staining, cells were held in a silver nitrate (Kanto Chemical) solution for 10 minutes in the dark. After washing with water, the cells were exposed to bright light for 15 minutes and observe the formation of calcium phosphate deposition.

## RT-PCR analysis

Total cellular RNA was isolated from cells using an RNA extraction kit (QIAGEN, Tokyo, Japan) according to the manufacturer's instructions. cDNA was synthesized from 1 µg of total RNA using an omni script cDNA synthesis kit (QIAGEN). mRNA from the cDNA sample was amplified with specific primer pairs for β-actin, hTERT, ALP, core binding factor (Cbfa)-1, bone morphogenetic protein (BMP)-2, BMP-4, and osteocalcin (OCN). The primer sequences and product sizes were as follows:

hTERT (282 bp)
F:5'-CCTCTGTGCTGGGCCTGGACGATA-3', R:5'-ACGGCTGGAGGTCTGTCAAGGTAG-3'

ALP (196 bp)
F:5'-CCACGTCTTCACATTTGGTG-3', R:5'-AGACTGCGCCTGGTAGTTGT-3'

Cbfa-1 (270 bp)
F:5'-TGAGAGCCGCTTCTCCAACC-3', R:5'-GCGGAAGCATTCTGGAAGGA-3'

BMP-2 (440 bp)
F:5'-CCGCTGTCTTCTAGCGTTGC-3', R:5'-CCTGAAGCTCTGCTGAGGTG-3'

BMP-4 (339 bp)
F:5'-GTCCTGCTAGGAGGCGCGAG-3', R:5'-GTTCTCCAGATGTTCTTCG-3'

OCN (310 bp)
F:5'-CATGAGAGCCCTCACA-3',
R:5'-AGAGCGACACCCTAGAC-3'

β-actin (331 bp)
F:5'-ATGAGGATGCTCACGGAGCGCGGCTACAGC-3',
R:5'-ACACCACTGTGTTGGCGTACAGGTCTTTGC-3'.

The amplified PCR products were separated on 2% agarose/TAE gels and visualized by ethidium-bromide staining.

# Results

## Morphological and growth characterization of clonal cells

The original HPL cells grew at a rate similar to all the single cell clones (A4, B6, C10, E10, F4 and H6). However, by day 80, the growth of the original HPL cells began to plateau and they then ceased to proliferate, whereas the populations of each of the cell clones actively doubled up to the end-point of the study (day 120) (Fig. 1). The cell clones grew at a similar rate to the original HPL cells (approximately 1 PDs every 2 days) and their morphology was spindle-shaped, like the original HPL cells in the growth phase (Fig. 2-A). However, at confluence, the A4 clones became cuboidal in shape, while other clones maintained their fibroblastic-like spindle shape (Fig. 2-B).

## hTERT gene expression and telomerase activity

Following cloning of infected cells by limited dilution, we obtained 6 clones (A4, B6, C10, E10, F4 and H6), which were sub-cultured and continued to proliferate. The presence of hTERT mRNA and telomerase activity was confirmed. RT-PCR revealed expression of the hTERT gene in A4, B6, C10, E10, F4 and H6, whereas the original HPL cells did not express this gene (Fig. 3-A). Single-cell clones showed telomerase- specific expression in a TRAP assay, as did the positive control cells, whereas the original HPL cells did not (Fig. 3-B).

## Osteogenic characterization

ALP activity of A4, B6, C10, E10, F4 and H6 clones differed (Fig. 4). Among the clones, A4 showed the highest level of ALP activities and C10, the lowest. Additionally, the ALP activity pattern of each clone was nearly the same between 30 PDs and 70 PDs (Fig. 4-B).

To examine the osteogenic differentiation ability of the original HPL cells, as well as of the A4 and C10 clones, ALZ and von Kossa staining were performed. The HPL cells and A4 showed intense ALZ and von Kossa staining, whereas staining in C10 was negative (Fig. 5). Gene expression of ALP, cbfa-1, BMP-4, BMP-2 and OCN was also analyzed by RT-PCR (Fig. 6). The expression level of ALP mirrored the ALP activity. In addition, the RT-PCR findings showed that all of the genes were expressed in HPL cells and A4, whereas BMP-2 expression was weak and OCN expression was barely detectable in C10 (Fig. 6).

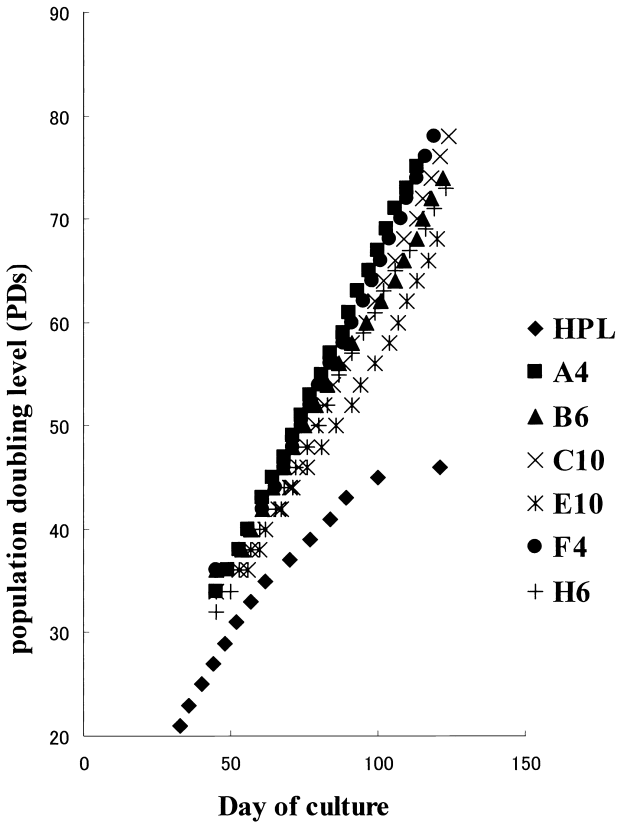


Fig. 1 Population doubling level (PDs) of HPL cells as well as A4, B6, C10, E10, F4 and H6 single cell clones are shown over 120 days of culture.

## Discussion

Normal cells have a limited capacity to divide, and cellular senescence soon occurs *in vitro*. This is thought to be the result of loss of telomeric DNA with passage progression in a culture setting (22). Telomeres, which are specialized structures at the ends of eukaryotic chromosomes that appear to function in chromosome protection, positioning, replication, and meiosis (23,24), are thought to control the entry into senescence. Human telomeres consist of repeats of the sequence

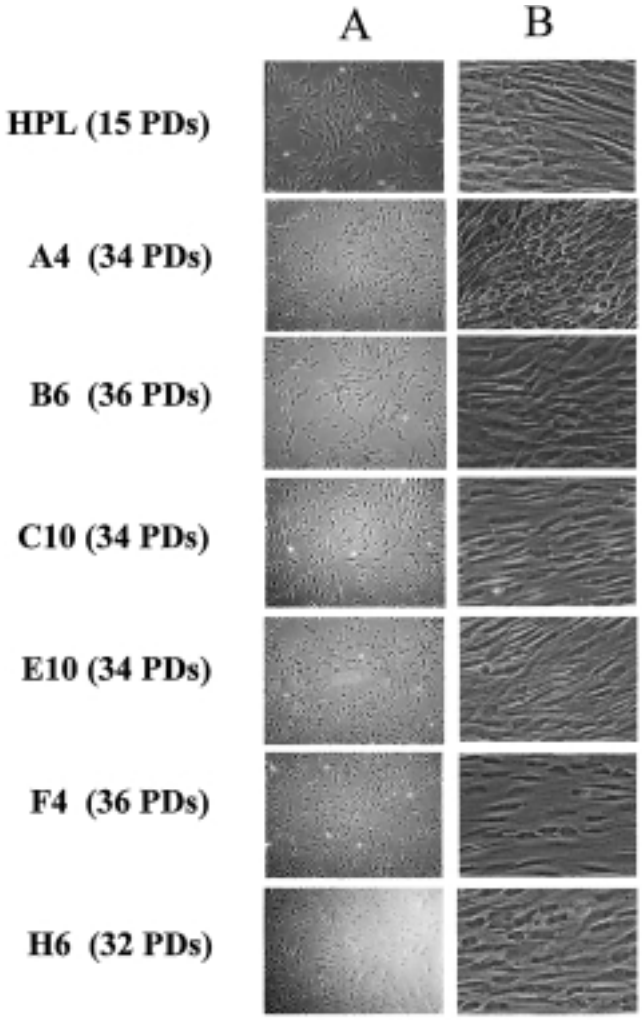


Fig. 2 Morphology of HPL cells and hTERT transduced single clones (A4, B6, C10, E10, F4 and H6). Cells are shown in the growth phase (A) and at confluence (B).

TTAGGG/CCCTAA at the chromosome ends and these repeats are synthesized by the ribonucleoprotein enzyme, telomerase (25). In the present study, retroviral infection with the catalytic subunit of telomerase, hTERT, and telomerase activity were induced in HPL single cell clones (A4, B6, C10, E10, F4 and H6). The growth of the telomerase-positive single cell clones was not retarded in comparison to telomerase-negative cells, and their morphology was not altered. These results suggest that stable expression of the hTERT gene was accomplished by retroviral infection. In addition, the transduction of HPL cells with the hTERT gene prevented cellular senescence and extended the cell life span.

PDL cells appear to have osteogenic characteristics; unlike gingival fibroblasts, PDL cells produce osteoblast-related extracellular matrix proteins and they also show higher ALP activity (26-28). In the present study, ALP activity differed between cell clones, suggesting that clones had been isolated during the differentiation stage. Further, the ALP activity of each clone was similar after freezing and prolonged culture up to nearly 70 PDs, showing that freezing and prolonged culturing had no effect on the cell line characteristics.

Murakami et al. reported that osteogenic PDL cells expressed ALP activity without differentiation factors and they could be distinguished from HPL cells that did not exhibit the osteoblast-like properties (26). In the present

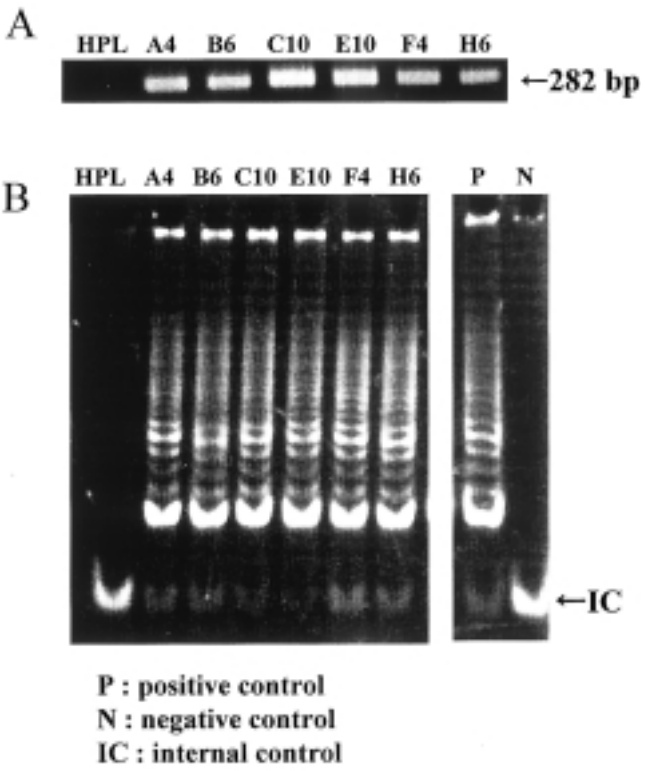


Fig. 3 hTERT mRNA and telomerase activity in HPL cells and hTERT transduced single clones. (A) Expression of hTERT mRNA in original HPL cells and hTERT-transduced single cell clones. (B) Telomerase activity in HPL cells and each of the single clones determined using the telomeric repeat amplification protocol (TRAP) assay. Telomerase-positive cells were used as a positive control. Lysis buffer was used as a negative control.

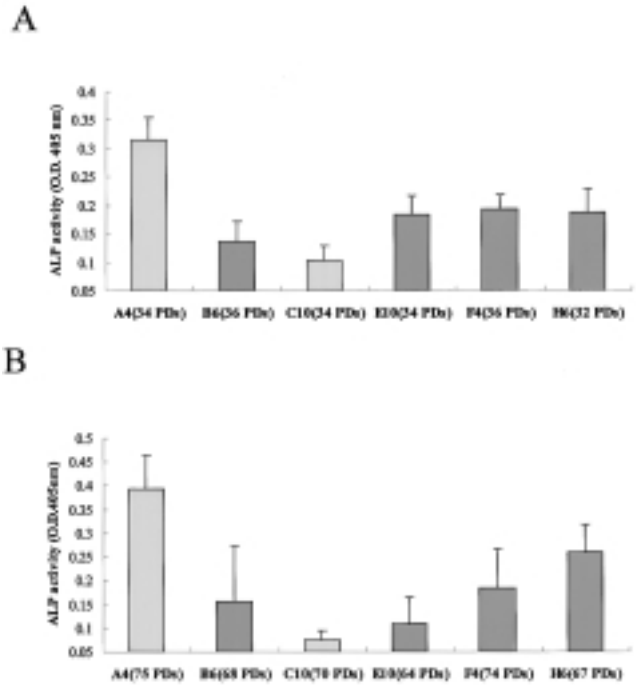


Fig. 4 Alkaline phosphatase (ALP) activity in HPL cells, as well as A4, B6, C10, E10, F4 and H6 clones, after nearly 30 PDs (A), and nearly 70 PDs (B). Values are the mean ± SD of results of 6 wells.

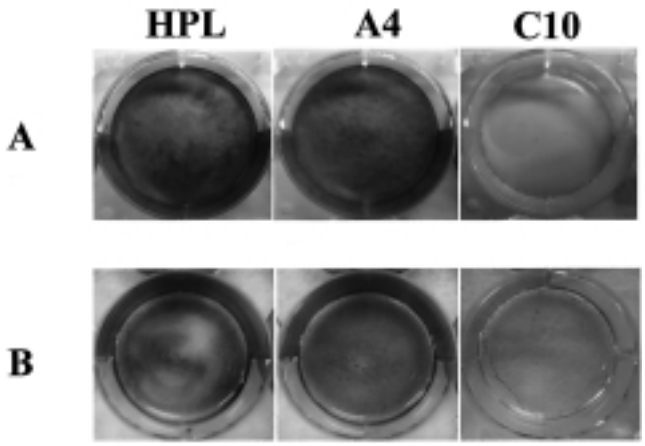


Fig. 5 Results of assessment of the mineralization activity of HPL cells, and the A4 and C10 clones using alizarin red (ALZ) (A), and von Kossa staining (B).

study, A4 (clone with highest level of ALP activity), had a cuboidal shape, whereas C10 (clone with lowest level of ALP activity) showed a spindle-like shape typical of PDL cells (Fig. 2-B). Based on these findings, osteogenic and non-osteogenic cell lines appeared to have been isolated from the original heterogeneous HPL population.

Since HPL cells form bone-like mineralized nodules in the presence of ascorbic acid, β-glycerophosphate and dexamethasone in monolayer cultures (29,30), we evaluated the mineralization activity of the original HPL cells and the 2 clones expressing the highest and lowest of ALP activity (A4 and C10, respectively). We also looked for mRNA expression of osteogenic markers ALP, Cbfa1, BMP-2, BMP-4, and OCN, each of which has a unique distribution within the periodontium, and plays important role in cementogenesis and osteogenesis (31,32). Like the original HPL cells, the A4 clone had an osteogenic phenotype (intense of ALZ and von Kossa staining). In addition, the mRNA expression of the osteogenic markers was evident in both A4 and original HPL cells. OCN is a protein found in the extracellular matrix of bone, though only in differentiated osteoblasts or odontoblasts, and appears to play a role in calcification (31). Our results suggest that the A4 clone cells have the potential to differentiate into mature osteoblast-like cells, like those found in the heterogeneous population of HPL cells.

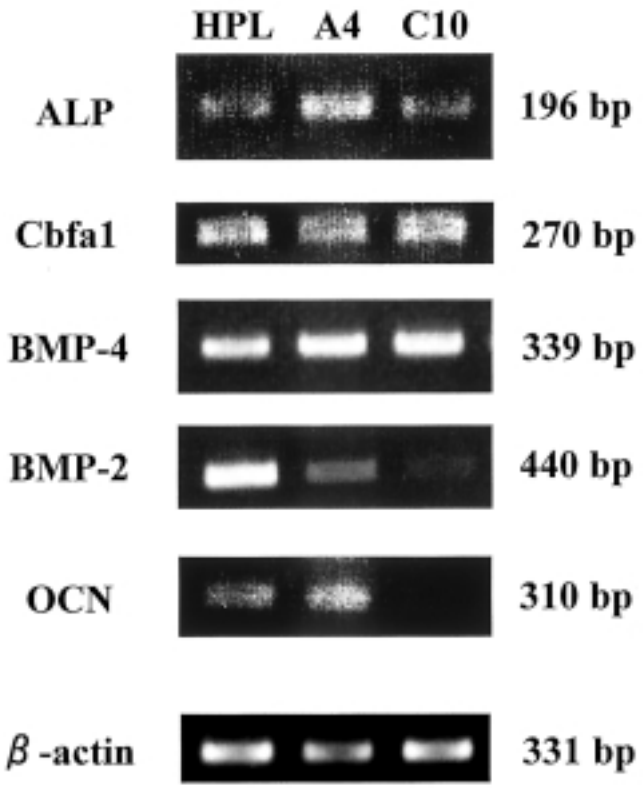


Fig. 6 mRNA expression of ALP, cbfa1, BMP-2, BMP-4, and OCN in HPL cells, as well as A4 and C10 clones. PCR products were separated on 2% agarose gel and stained with ethidium bromide.

The C10 clone was negative for ALZ and von Kossa staining. While mRNA expression of BMP-2 and OCN was weak, expression of ALP, BMP-4 and cbfa1 was detected. These results indicate that C10 does not have the ability to form a mineralization matrix, unlike A4 and the heterogeneous population of original HPL cells. Recently, Saito et al. (12) reported a clonal PDL cell line established from mice that did not produce mineralized matrix in mineralization medium, and the characteristics of the C10 clone appear similar. RT-PCR analysis in the present study demonstrated that, like HPL cells and the A4 clone cells, C10 expressed Cbfa1, a transcription factor necessary for the expression of bone-related proteins such as OCN. Cbfa1 is expressed early in mesenchymal and epithelial tissues destined to form the mineralized tissues of the teeth and periodontal tissues (33,34). However, expression of cbfa1 is not restricted to mineralized tissues.

Bone morphogenetic protein (BMP)-2 and 4 are members of a family of proteins have a unique ability to induce the formation of cartilage and bone, and play important roles in the development of a wide range of tissues (35). It has also been shown that BMP-2 increases the mRNA level of OCN in human bone marrow cells and human osteoblasts *in vitro* (36). In our study, BMP-2 expression was detected in the original HPL cells and A4 clone cells, but only weakly in C10 clone cells. These results suggest that the expression of BMP-2 in A4 may be linked to the regulation of mineralized matrix formation in the PDL tissues.

Since the PDL maintains its width during the tooth movement that results from mechanical stress or orthodontic forces, it is believed that the PDL cells possess a regulatory mechanism to induce and/or suppress mineralization. In the present study, A4 clone cells had the ability to induce mineralization, while C10 clone cells appeared to lack this ability or mineralization was inhibited. It is well known the fibroblast cells in the PDL are heterogeneous and comprise both osteogenic and non-osteogenic cells. However, to our knowledge, there is no previous report of both osteogenic and non-osteogenic cell lines established from normal human PDL tissues without an alteration of their phenotypes (up to nearly 70 PDs). This is the first report of distinct immortalized single cell clones established from PDL cells that retained their characteristics.

In conclusion, using HPL cells immortalized with the hTERT gene, we created at least 2 types of cell lines, one that was capable of forming a mineralized matrix, and another that was not. The HPL single cell clones A4 and C10 may be useful for additional studies of PDL tissues

to increase our understanding of the cellular functions of the PDL tissues, including those pertaining to maintenance of the biological functions of PDL tissues. They may also provide a powerful tool for the development of therapeutic strategies, such as cell therapy, and treatment for periodontitis.

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