Characterization of established cementoblast-like cell lines from human cementum-lining cells *in vitro* and *in vivo*

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Abstract

To Study cellular characteristics of human cementoblasts using a cellular model is important for understanding the mechanisms of homeostasis and regeneration of periodontal tissues. However, at present no immortalized human cementoblast cell line has been established due to limitation of the life span. In the present study, therefore, we attempted to establish human cementoblast-like cell lines by transfection with telomerase catalytic subunit *hTERT* gene. Two stable clones (HCEM-1 and -2) with high telomerase activity were obtained and they grew over 200 population doublings without significant growth retardation. The expression of mRNA for differentiation markers, type I collagen, alkaline phosphatase (ALP), runt related transcription factor 2, osteocalcin and bone sialoprotein was revealed in these clones by RT-PCR. Moreover, these cells showed high ALP activity and calcified nodule formation *in vitro*. Interestingly, HCEM-2 showed cementum like formation on the surface of hydroxyapatites granules by subcutaneous transplantation into immunodeficient mice with hydroxyapatite granules. Thus, we established human cementblast-like cell lines with high mineralization activity. We suggest that HCEM cell lines can be useful cell models for investigating the characteristics of human cementoblasts.

Key word: cementoblast; periodontal ligament; hTERT; immortalization, differentiation

Introduction

Cementum is a mineralized tissue produced by cementoblasts covering the roots of teeth that provides for the attachment of periodontal ligament to roots and surrounding alveolar bone [1]. A cellular model is very important for studying the detailed mechanisms of proliferation and differentiation of human cementoblasts to understand periodontal physiology and pathology, including periodontal tissue regeneration. Although several attempts have been made to obtain a population of cementoblasts and culture them for characterization [2-6], no immortalized cell lines of human cementoblasts have been reported and the detailed mechanisms regulating the proliferation and differentiation of human cementoblasts are not yet completely determined.

One of the reasons of the difficulty to obtain immortalized cell lines from normal tissues is that cells from non-neoplastic human tissue in culture display a limited capacity to divide and reach cellular senescence. Spontaneous escape from senescence and acquisition of indefinite life span are an exceptionally rare event in cultures of normal human cells [7]. Normal human fibroblasts have a limited proliferative capacity and undergo senescence after 50-80 doublings in culture [8, 9]. It is generally accepted that to establish human cell lines from non-neoplastic or benign tumor tissues is very difficult in comparison with rodent tissues, in which cells spontaneously immortalize with a relatively high frequency [10-13].

As normal human cells gradually lose telomeric DNA with passage progression in culture, telomeric erosion is thought to limit cellular life span [14].

Human telomeres consist of repeats of the sequence 5’-TTAGGG-3’ at chromosome ends, and these repeats are synthesized by a ribonucleoprotein enzyme, telomerase [15]. Ectopic expression of *hTERT* gene, which encodes the catalytic subunit of the telomerase holoenzyme, enables extension of life spans of normal human cells [16]. It has recently been shown that the ectopic expression of hTERT in combination with SV40 T and/or H-ras resulted in immortalization of human epithelial and fibroblastic cells [17]. We also immortalized human cemeto-ossifying fibroma cells by transfection with plasmids encoding temperature-sensitive *SV40 T-antigen* gene and *hTERT* gene [18]. Although ectopic expression of both hTERT and SV40 T are a useful method for immortalization of human cells, *SV40 T-antigen* gene may alter the nature cells such as growth with pile up and cell morphology [19-21]. In the present study, in order to establish human cementoblast-like cell lines, we first retrieved cementum-lining cells from root surface using our previously established enzymatic digestion method [22] and then infected with *hTERT* gene. We could obtain human cementobalst-like (HCEM) cell lines showing stable growth and high mineralization activities both *in vitro* and *in vivo*.

Materials and Methods

These studies were performed in compliance with regulations administered by the experimentation committee of the Graduate School of Biomedical Sciences, Hiroshima University.

Isolation of the Cementum-lining cells

We obtained the cells from a healthy premolar extracted for orthodontic treatment. To avoid contamination of experimental materials with gingival tissues, soft tissues attached to the cervical area of the tooth were carefully removed before and after extraction. The extracted tooth with periodontal ligament was rinsed once in Dulbecco's phosphate-buffered saline without calcium and magnesium (PBS, Nissui Pharmaceutical Co. Ldt., Tokyo, Japan). Then the tooth was immersed in a digestion solution to obtain the cells from the root surface, as previously reported [22]. Briefly, periodontal ligament attached to the extracted premolar was digested with 2 mg/ml collagenase and 0.25 % trypsin at 37 ˚C for 30 min. Our preliminary study showed that some cells observed on the root surface of human extracted premolar after 120-min-digestion were completely disappeared after 150- min-digestion. Based on this observation, five consecutive digestions of 30 to 150 min were performed each in a fresh digestive solution to release layers of cells from the periodontal ligament and cementum. Using phase-contrast microscopy, we have confirmed that cells were completely disappeared from the root surface after 150 min digestion. After that, the solutions were centrifuged to collect the released cells, and it was determined that the

last fraction after 150 min digestion exclusively included cementum-lining cells with high ALP activity and mineralization. These cells were then cultured in Minimum Essential Medium Alpha (α-MEM, Invitrogen, Grand Island, N.Y.) with 10% fetal bovine serum (FBS) plus penicillin G solution (10 UNITS/ml) and streptomycin (10 mg/ml) in a humidified atmosphere of 5 % CO_2 at 37 ˚C.

Transfection with hTERT gene

The gene for *hTERT* was cloned into the retroviral vector, pQCXIP (Clontech, Tokyo, Japan). The cloned retrovirus was transfected into the RetroPack PT67 Packaging Cell Line (Clonetech), where the vector was packaged into infectious, replication-incompetent retroviral particles. The packaged cells were maintained with puromycin (1.2 μg/ml) containing media for 1 week, retrovirus for hTERT was collected and add polybrane (8 μg/ml) and filterated with 0.20 μm filter. After puromycin selection for 1 week, packaging cells were maintained without puromycin. To infect the target cells, the packaged retrovirus (1 ml/dish) was then added to the media in a 25-mm^2 culture flask of target cells at 50 % to 60 % confluency. After 24 hours, infected cells were placed into media containing puromycin (0.5 μg/ml) for 2 to 3 weeks. Individual stable clones were selected by plating cells at low density and analyzed for telomerase activity by the telomeric repeat amplification protocol (TRAP), as described below.

Telomerase Assay

The telomerase assay was performed with a PCR-based modified TRAP assay [23, 24]. The cell pellet ($1x10^5$ cells) was suspended in 200 µl cold TRAP lysis buffer, incubated for 30 min on ice, and centrifuged at 15,000 rpm for 20 min at 4 ˚C. The extract equivalent of 1000 cells (2 µl) was incubated with 48 µl of 20 mM Tris-HCl (pH 8.3), 1.5 mM MgCl, 68 mM KCl, 0.05 % Tween 20, 1 mM EGTA, 50 mM dNTP, 5 µg of BSA, 2U of Taq DNA polymerase (Invitrogen, Carlsbad, CA), 10 attg internal telomerase assay standard (ITAS) DNA, and 0.1 µg of extra purified TS primer (5'-AATCCGTCGTCGAGCAGAGTT-3') at 20 ˚C for 30 min and then heated reaction. During this step, 0.1 µg (2 µl) of extra purified CXII primer (5'-CCCTTACCCTTACCCTTACCCT-3') was added, and the reaction mixture was subjected to 31 PCR cycles at 94 ˚C. Electrophoresis was performed on a 12 % nondenaturing acrylamide gel, stained with SYBR green (FMC Bioproducts, Rockland, ME), and bands visualized using a chemiluminence image analyzer.

RNA Preparation and Reverse Transcription-Polymerase Chain Reaction Analysis (RT-PCR)

Total RNA was isolated from cultures of confluent cells using the RNeasy Mini Kit (Qiagen, K.K., Kyoto, Japan) according to the manufacturer's instructions. Preparations were quantified and their purity was determined by standard spectrophotometric methods. cDNA was synthesized from 1 µg total RNA according to the Rever Tra Dash (Toyobo Biochemicals, Tokyo, Japan). The oligonucleotide RT-PCR primers for hTERT, type I collagen (COLI), alkaline phosphatase (ALP), runt

related transcription factor 2 (Runx2), osteocalcin (OCN), bone sialoprotein (BSP) and glyceraldehyde-3-phosphate (GAPDH) listed were purchased from Invitrogen (Table 1). Aliquots of total cDNA were amplified with 1.25 U of rTaq-DNA polymerase (Qiagen), and amplifications were performed in a PC701 thermal cycler (Astec, Fukuoka, Japan) for 30-32 cycles after an initial 30 sec denaturation at 94 ˚C, annealed for 30 sec at 56 ˚C, and extended for 1 min at 72 ˚C in all primers. The amplification reaction products were resolved on 1.5 % agarose/TAE gels (Nacalai tesque, Inc., Kyoto, Japan), electrophoresed at 100 mV, and visualized by ethidium-bromide staining.

Cell growth assay to assess the effect of hTERT tranfesction

Cells were plated into a 24 well multiwell plate (5000 cells/well). After incubation for 24 h (day 0), the culture media were then replaced with a fresh media and the number of trypsinized cells was counted using a cell counter (Coulter Z1, Coulter Corp, Hialeah, Florida) at 0, 1, 3, 5 and 7 days.

Measurement of ALP Activity

The quantitative analysis of ALP activity was performed biochemically by Bessey-Lowry enzymologic method using nitrophenyl phosphate as a substrate [25]. Cells were plated in 24 well culture plates (1×10^5 cells per well) and cultured in α-MEM containing 10 % FBS, penicillin G sodium (10 UNITS/ml) and streptomycin sulfate (10 mg/ml) for confluent that cells were plated for 1 week. The cells were washed with PBS and homogenized ultrasonically in 0.5 ml of 10 mM Tris-HCl buffer

(pH 7.4) containing 25 mM $MgCl_2$. Aliquots of the homogenates were used for quantification of ALP activity.

Mineralization Assay

Mineral nodule formation was detected by Dahl's method for calcium [26]. Cells were placed in a 24 well plate at a density of $1x10^5$ cells per well and cultured in α-MEM supplemented with 10% FBS, 50 μg/ml ascorbic acid, 10 mM sodium β-glycerophosphate and 10 nM dexamethasone at 37 ˚C for 4 weeks. The cells were fixed in 3.7 % formaldehyde neutral buffer solution, and then stained with alizarin red S (ALZ).

Transplantation of HCEM cells into mice

We transplanted HCEM cells into immunodeficient mice according to the previously described method [27, 28]. Briefly, the cells were inoculated subcutaneously into 4-week-old male BALB/cAnNcrj-nu mice (Charles River Japan, Inc., Kanagawa, Japan) after incubating the cells ($1.5x10^6$) in a mixture of 40 mg of hydroxyapatite granules (Apaseram, Pentax, Tokyo, Japan) and fibrin clot (mixture of mouse fibrinogen and thrombin, Sigma, St. Louis, MO). After 1 and 2 months, the transplants were retrieved and prepared for histological analysis.

Histological analysis

The transplants were fixed in a 10 % formaldehyde neutral buffer solution and

decalcified with 10 % EDTA in PBS for 1 week, and then embedded in paraffin. Sections were prepared and stained with hematoxylin-eosin for histological examination. To examine the origin of cells in the transplants, the sections were stained immunohistochemically with an anti-human mitochondria antibody (Chemicon International, CA). Endogenous peroxidase was quenched by incubating with 0.3 % H_2O_2 in methanol for 30 min. Nonspecific staining was blocked using Dako Protein Block Serum Free (Dako, Carpinteria, CA). The sections were incubated with the primary antibody (1:100) for overnight at 4 ˚C , and then incubated with a secondary antibody (Labelled Polymer, HRP, anti-mouse; Dako) for 30 min. For visualization, they were treated with Liquid DAB (3, 3'- diaminobenzidine) Chromogen Syatem (Dako) according to the manufacturer's protocol.

Statistical analysis

The results of cell growth analysis and quantitative ALP activity were shown as mean ± SE, and analyzed for significance using Wilcoxon's test for non-paired examination. *P* values of less than 0.05 were judged to be statistically significant.

Results

Immortalization of HCEM cell lines

Two clones (HCEM-1 and -2) were obtained by transfection with *hTERT* gene. These clones bypassed senescence and grew over 200 population doublings (PDs) without significant growth retardation. In contrast, non-transfected original cells entered crisis at about 60 PDs (Fig. 1A). High expression of hTERT was confirmed in HCEM-1 and -2, but not in original cells, by RT-PCR analysis (Fig. 1B). Both HCEM-1 and -2 showed remarkable telomerase activity, but the original cells did not (Fig. 1C). Even at 200 PDs, HCEM-1 and -2 were short and spindle-shaped, similar to original cells at 10 PDs (Fig. 1D). There were no histological findings indicating senescence or a neoplastic nature in HCEM-1 and -2. Cell growth speed of HCEM-1 and -2 was also similar to that of original cells (Fig. 1E).

Characterization of HCEM cell lines in vitro

To confirm that these clones with hTERT transfection maintain cellular characteristics of cementoblast-like original cells, we examined the expression of mRNA for COLI, ALP, Runx2, OCN and BSP by RT-PCR. In general, these genes are known to be expressed in cementoblasts. In addition, we also examined ALP activity by biochemical methods and nodule formation by ALZ staining. Original cells, HCEM-1 and -2 expressed COLI, ALP, Runx2 and OCN mRNA at the same levels (Fig. 2A). BSP mRNA was highly expressed in HCEM-1 and -2 in comparison with original cells (Fig. 2A). ALP activity in HCEM-1 and -2 tend to be higher than that in original cells.

However, we could not find statistical significance (Fig. 2B). Furthermore, HCEM-1 and -2 showed more intense ALZ staining than the original cells (Fig. 2C). In particular, HCEM-2 cells showed the most remarkable ALZ staining among them. We used HCEM-2 cells in the following study.

Cementum-like tissue formation by HCEM cell lines in vivo

To elucidate the capacity of differentiation of HCEM *in vivo*, HCEM-2 was transplanted subcutaneously into immunodeficient mice. On the other hand, the hTERT-transfected periodontal ligament cells (HPL) isolated from the middle position of the periodontal ligament of the same tooth by a shorter 30 min digestion were used as a control. After 1 month, a thin layer of hyalinous cementum-like material was observed at the surface of hydroxyapatite granules in the transplants (Figs. 3A a), and after 2 and 3 months, the cementum-like structures increased in thickness (Fig. 3A b and c). In contrast, deposition of cementum-like structures was not observed in control transplants of HPL (Figs. 3A d-f). We confirmed that the cells surrounding the hydroxyapatite granules in both HCEM-2 and HPL transplants reacted positively with the human anti-mitochondria antibody at 1 month (Figs. 3B a and b), indicating that transplanted HCEM-2 directly produce the cementum-like materials on the surface of hydroxyapatite granules.

Discussion

The periodontal ligament harbors progenitor cells that can differentiate into periodontal ligament fibroblasts, osteoblasts and cementoblasts. The progenitor cells in marrow spaces migrate into the perivascular area of the periodontal ligament and move to the bone and tooth surface, and then differentiate into osteoblasts or cementoblasts [29, 30]. It is reasonable to consider that periodontal ligament consists of different cell populations in various differentiation stages according to their position within the periodontal ligament. Seo et al. demonstrated that periodontal ligament cells contain stem cells that have the potential to generated cementum/ periodontal ligament like tissue [31]. In our previous study, we established a method to retrieve rat cementum-lining cells by using the sequential enzymatic digestion method [22]. These cementum-lining cells showed high activities of ALP and calcification and expressed BSP mRNA [22]. Moreover, we have succeeded in immortalizing rat cementoblast cell lines by transfection with *SV40 T-antigen* [32]. In the present study, we obtained cementum-lining cells from the human extracted tooth by using modified enzymatic digestion methods that we developed.

Recently, it has been reported that expression of hTERT and subsequent telomerase activity were successfully induced in normal cell lines and benign neoplasm cell lines [7, 33-37]. Here, telomerase activity caused by transfection with *hTERT* gene was successfully induced in cementoblast-like cell lines. Consequently, these transfectant cells bypassed senescence and grew over 200 PDs without significant growth retardation, while the original cells entered crisis at about 60 PDs (Fig. 1A).

These results indicate that transfection with *hTERT* alone can allow normal cementoblast-like cells to become immortalized. The aim of this study is to generate HCEM cell lines that can survive beyond normal programmed senescence and extend the usefulness of the cells for studying cellular mechanism involved in the proliferation and differentiation of human cementoblasts. The immortalized HCEM cells showed morphology similar to that of the original cells and no histological findings indicating senescence or neoplastic nature were observed even at 200 PDs (Fig. 1D). Furthermore, HCEM cell lines maintained cellular characteristics of cementoblasts including the mineralization related gene expression and high mineralization activity (Figs. 2A-C). This observation is supported by the finding that HCEM cell lines showed similar ALP activity to original cells. Although the expression levels of mRNA for COLI, ALP, Runx2 and OCN were same, higher BSP mRNA expression and more remarkable nodule formation were observed in HCEM cell lines, in comparison with original cells (Figs. 2A and 2C). Especially, HCEM-2 cells showed higher mineralization activity than HCEM-1. We suggest that these differences may result not from the influence of transfection with *hTERT* but from the heterogeneity of the original cells. It is generally accepted that progenitor cells located in prevascular area of periodontal ligament near bone as resident prevascular cells, they migrate to cementum site and differentiate into cementoblast at the surface of cementum [29, 30]. Therfore, we considered that the original cells contained cementoblast-lineage cells in various differentiation stages. The differences in mineralization activity between HCEM-1 and -2 may be responsible for their differentiation stages as cementoblasts.

In fact, we could get 4 clones after selection, HCEM-1 and -2 showed higher mineralized activities among them (data not shown). Overall, these results demonstrated that transfection with *hTERT* gene into cells did not alter the proliferation, morphology and differentiation of original cells.

As mentioned above, HCEM cell lines showed the expression of differentiation related genes, including COLI, ALP, Runx2, OCN and BSP, and high mineralization activities. These characteristics are essentially identical to those of cementoblasts. MacNeil et al. demonstrated that OCN and BSP, major components of cementum, are secreted by root lining cells using immunocytochemistry and in situ hybridization [3, 38, 39]. According to their study, BSP and OCN are selectively expressed by cells lining the root surface (cementoblasts), and not expressed by periodontal ligament cells. In fact, HCEM cell lines showed the expression of BSP mRNA, but HPL didn't (data not shown). Therefore, we believe that HCEMs established from root lining cells enriched with cemetoblasts are cementoblast lineage cells and useful cell models for investigating the characteristic of cementoblasts. Cementoblasts share many characteristics with osteoblasts, whereas there are clear differences in structure, function of cementum vs bone suggestive of difference between two cell types [40]. Further studies using HCEM cell lines are needed to determine whether cementobalsts are just positional osteoblasts or an unique cell type different from osteoblast.

In vitro study showed that cementum-like structures were observed on the surface of hydroxyapatite granules in HCEM-2 transplants, but not in HPL transplants

(Fig. 3A). Interestingly, cementum-like structures were not observed during the experimental period, when we transplanted only HCEM-2 without hydroxyapatite granules subcutaneously into immunodeficient mice (data not shown). This result suggests that the attachment of transplanted HCEM-2 cells to hydroxyapatite granules as the scaffold may be a critical for the production of cementum. The hydoxyapatite may provide a place on which the cells attach for differentiation into mature cementoblasts.

In summary, our findings suggest that HCEM cell lines (HCEM-1 and -2) can be useful cell models for studying the mechanism of proliferation and differentiation of human cementoblasts.

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Figure legends

Figure 1 (A) Population doublings (PDs) of non-transfected original cells (Original), and hTERT-transfected human cementoblast (HCEM-1 and -2). Original cells stopped at about 60 PDs, but HCEM-1 and -2 are immortalized and were observed to grow over 200 PDs. (B) Expression of hTERT gene by RT-PCR in Original, HCEM-1 and -2. High expression of hTERT gene was observed in HCEM-1 and -2. No expression of hTERT gene was seen in Original. (C) Telomerase activity of Original, HCEM-1 and -2 was determined by TRAP assay. Telomerase activity was present in HCEM-1 and -2, but not in Original. Internal control consisted of a 10 attg internal telomerase standard (ITAS) DNA. (D) The morphology of Original, HCEM-1 and -2 cells in culture. Original cells were short and spindle-shaped, and both HCEM-1 and -2 were also short spindles even at 200 PDs (scale bar = 100 μm). (E) Cell growth of Original, HCEM-1 and -2. Growth speeds of HCEM-1 and -2 at 60 PDs were same as that of Original at 10 PDs.

Figure 2 (A) Gene expression of COLI, ALP, Runx2, OCN and BSP by RT-PCR in Original, HCEM-1 and -2. Original, HCEM-1 and -2 expressed COLI, ALP, Runx2, OCN mRNA at comperable levels. BSP mRNA was strongly expressed in HCEM-1 and -2 in comparison with Original. (B) ALP activity by biochemical method in Original, HCEM-1 and -2. HCEM-1 and -2 showed a tendency to have higher ALP activity than Original at confluency. However, we could not find statistical significance. (C) ALZ staining in Original, HCEM-1 and -2. Cells were treated with culture medium supplemented with ascorbic acid, sodium ß-glycerophosphate

and dexamethasone for 4 weeks. HCM-1 and -2 showed more intense ALZ staining than Original.

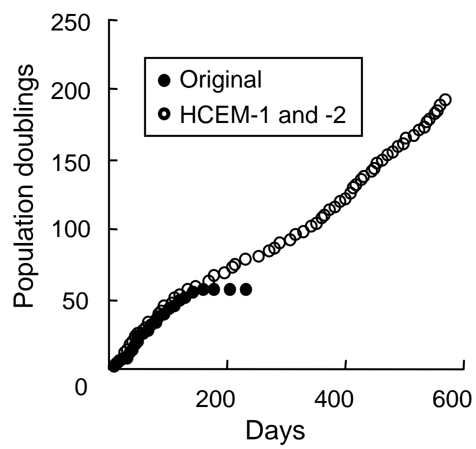
Figure 3 Histological analysis of transplants. (A) Staining by hematoxylin-eosin in HCEM-2 transplants (a-c), and HPL transplants (c-e). At 1 month (a, d), 2 months (b, e) and 3 months (e, f) after transplantation. Thin eosinophilic hyalinous layers (arrows) were observed at the surface of hydoxyapatite (*) after 1 month (a) and 2 months (b). After 3 months, much cementum like structures (triangles) were observed in HCM-2 transplants (c). In contrast, such a matrix formation was not seen in HPL transplants (d-f). (B) Immunohistochemical staining with human anti-mitochondria antibody in HCEM-2 transplants (a) and HPL transplants (b) at 1 month. The cells present in both HCEM-2 and HPL transplants were positively stained with human anti-mitochondria antibody (a, b). Original magnification x100.

Table1. Oligonucleotide primer sequences utilized in the RT-PCR

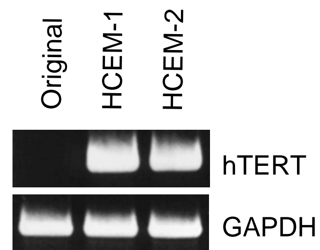
RT-PCR primer set		Sequence	Product length (bp)	Gnene bank Accession numbers
hTERT	F	5'-actttgtcaaggtggatgtgacgg-3'	493	NM_198253
	R	5'-aagaaatcatccaccaaacgcagg-3'		
COLI	F	5'-ctgaccttcctgcgcctgatgtcc-3'	300	XM_012651
	R	5'-gtctggggcaccaacgtccaaggg-3'		
ALP	F	5'-aagtactggcgagaccaagc-3'	214	XM_001826
	R	5'-agagggccacgaaggggaact-3'		
Runx2	F	5'-cgcattcctcatcccagtat-3'	462	NM_001015051
	R	5'-gactggcggggtgtaagtaa-3'		
OCN	F	5'-atgagagccctcagactcctc-3'	297	X53698
	R	5'-cgggccgtagaagcgccgata-3'		
BSP	F	5'-gaaccacttccccacctttt-3'	201	NM_004967
	R	5'-tctgaccatcatagccatcg-3'		
CP-23	F	5'-atgggcacatcaagcactga-3'	741	AY584596
	R	5'-ccccattagtgtcatcctgc-3'		
GAPDH	F	5'-tccaccaccctgttgctgta-3'	450	BC001601
	R	5'-accacagtccatgccatcac-3'		

FIGURE1

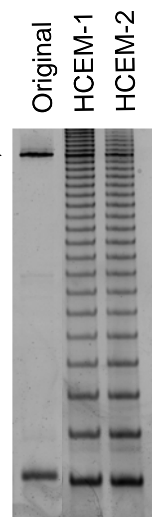
A



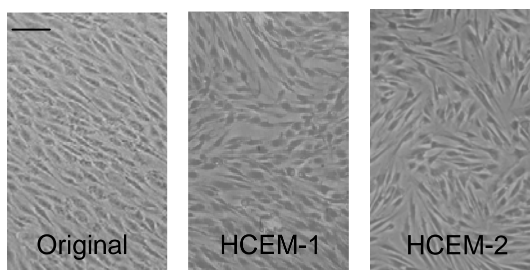
B



C



D



E

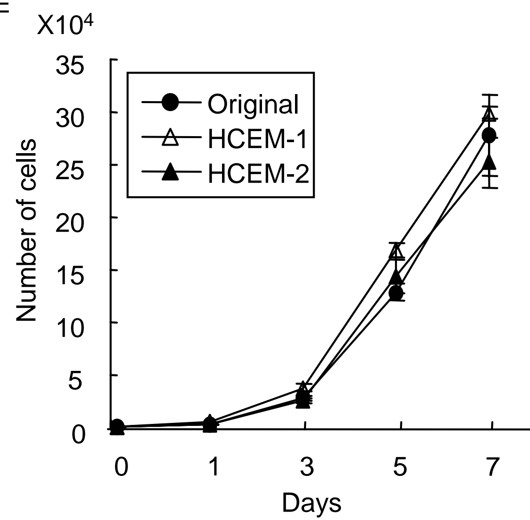


FIGURE2

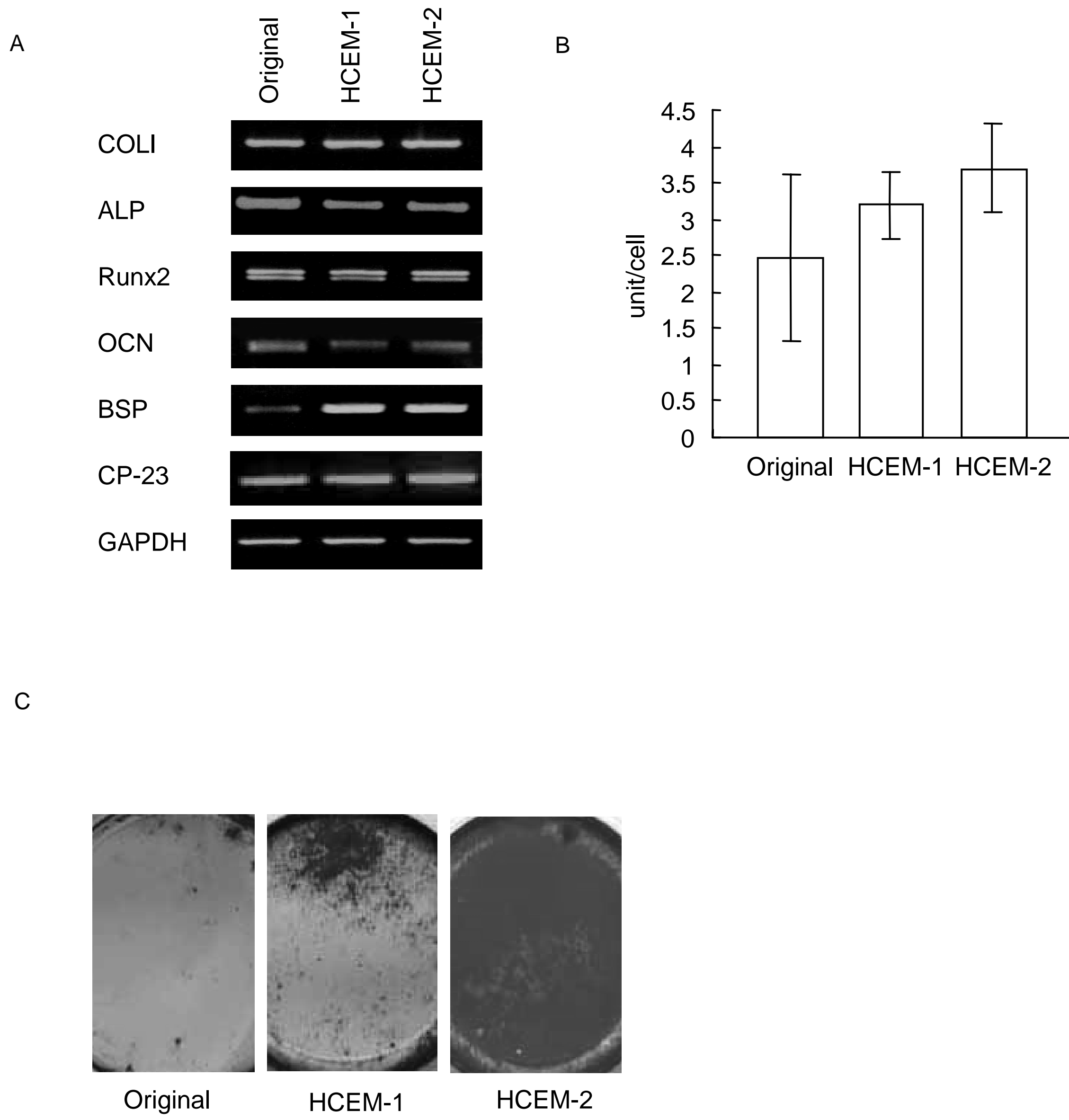


FIGURE 3