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Original Article

Immortalization and characterization of normal oral epithelial cells without using HPV and SV40 genes

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ABSTRACT

Background: As oral neoplasm often originates from epithelium, an immortalized epithelial cell line could be useful for the research of oral carcinogenesis. Although several oral epithelial cell lines were reported, they were either derived from cancer or immortalized by human papilloma virus or simian virus 40 genes, which have the potential to induce carcinogenesis.
Materials and methods: We established two immortalized cell lines from human oral epithelium by transducing mutant cyclin dependent kinase 4, cyclin D_1, and human telomerase reverse transcriptase with or without dominant-negative p53 into primary-cultured normal oral gingival epithelial cells using recombinant lentivirus vectors and named them MOE (mouth-ordinary-epithelium) 1a and MOE1b, respectively.
Results: MOE1 cells could be passaged for nine months or more, and the morphology of the cells did not change in comparison with that of fresh primary-cultured epithelial cells. MOE1 cells did not show epithelial–mesenchymal transition. MOE1b cells retain functional p53 and were considered to have less risk of genomic instabilities. Anchorage-independent growth was not observed in MOE1 cells. The expressions of cancer-associated genes including keratin-17 were not elevated in MOE1 cells, whereas oral cancer-derived HSC-2 cells showed overexpression of them. Furthermore, interleukin (IL)-1β, IL-6, IL-8, tumor necrosis factor-α, matrix metalloproteinase (MMP)-2, and MMP-9 were induced in response to lipopolysaccharide or heat-killed bacterium in MOE1 cells.
Discussion: MOE1 cells kept the characteristics of normal epithelial cells without acquiring typical features of cancer cells and they could be useful not only for the study of oral neoplasm but also for other oral diseases.

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1. Introduction

The surface of the oral cavity is covered with oral mucosa and the epithelium is the outer lining of the mucosa. These epithelial cells play a role in the physical defense against bacteria and viruses, but also participate in immune responses [1].

The mechanism of oral carcinogenesis in oral cancer is not fully understood. Given that most oral cancers derive from oral epithelium, normal oral epithelial cells are required as a control in studying oral carcinogenesis. Although primary oral epithelial cells can be cultured and used for cell biological study, these cells could not be maintained for longer than two months in general. Recently primary skin keratinocytes have been cultivated for a few months and applied to the treatment for severe heat burn in some institutes. However, primary oral epithelial cells are unsuitable for long-term experiments and do not guarantee reproducibility [2]. Therefore, it is useful to establish normal oral epithelium-derived cell lines for reproducible cell biological study in oral sciences.

Several studies of human oral diseases have been performed with normal oral epithelial cells immortalized by transduction with human papilloma virus (HPV) type 16 DNA or simian virus (SV) 40. HPVs are known to be associated with oral carcinogenesis and other oral diseases [3,4]. HPVs and SV40 have the potential to result in unnecessary and undesirable effects that disrupt DNA repair on virally immortalized cell lines [5,6]. Taken together, these facts indicate that establishing immortalized human normal oral

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epithelial cells without etiological factors should be useful in many fields.

In this study, we succeeded in the establishment of normal oral epithelium-derived cell lines (MOE1a and MOE1b) by the transduction of human telomerase reverse transcriptase (hTERT), cyclin D_1, and cyclin dependent kinase (CDK) 4R24C with or without p53C234 (which encodes carboxy-terminal 234 residues of p53 and functions as dominant-negative mutant) using recombinant lentiviral vectors. These two cell lines show neither epithelial–mesenchymal transition (EMT) nor transformation. Furthermore, they ensure bacterial stimulation as well as that of lipopolysaccharide (LPS) or heat-killed bacteria.

2. Materials and methods

2.1. Antibodies and reagents

Anti-p53 antibody which reacts with endogenous p53 and p53C234 was purchased from Calbiochem (Darmstadt, Germany). Anti-cyclin D_1, CDK4, and p16 antibodies were purchased from BD Biosciences (San Jose, CA, USA). Anti-p21 and caspase-3 antibodies were purchased from Cell Signaling Technologies (Danvers, MA, USA). Anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was purchased from Ambion Inc. (Austin, TX, USA). Anti-E-cadherin antibody was purchased from Covance (Princeton, NJ, USA). Anti-vimentin antibody was purchased from Dako (Carpinteria, CA, USA). Anti-cytokeratin-14 antibodies for immunohistochemistry and immunoblotting were purchased from Leica Biosystems (Newcastle, UK) and Dako, respectively. Agar noble was purchased from DIFCO (Detroit, MI, USA). Collagenase was purchased from Wako Pure Chemical Industries (Chuouku, Osaka, Japan). Defined keratinocyte serum-free medium (K-SFM) was purchased from Invitrogen Corp. (Carlsbad, CA, USA).

LPS and heat-killed bacteria were provided by Drs. H. Komatsuzawa and Y. Matsuda (Department of Oral Microbiology, Kagoshima University Graduate School of Medical and Dental Sciences).

2.2. Tissue samples and cell culture

Oral tissues were collected from patients at Kagoshima University and a volunteer. They provided written informed consent. The research protocol and consent form were approved by the institutional review board of Kagoshima University in advance. Primary culture of oral epithelium was performed as described previously [7]. In brief, diced oral epithelial tissues were washed with Dulbecco's modified Eagle's medium (DMEM) three times and treated with collagenase solution (20 mg/ml) at 37 °C for 2 h with shaking. Cells were washed with K-SFM (Invitrogen corp.) and seeded onto a tissue-culture flask for subsequent gene transduction using recombinant lentiviral vectors as described previously [8]. After viral transduction, cells were maintained with K-SFM in an atmosphere of 5% CO_2 at 37 °C. Mouse fibroblast-derived L cells were maintained with DMEM containing 10% fetal bovine serum (FBS). HFF2 human skin-derived cell line was established as described previously [9]. HFF2 cells were maintained with DMEM containing 8% FBS [9]. Human colon cancer-derived SW480 cells were maintained with Roswell Park Memorial Institute medium "1640" containing 10% FBS. Human oral cancer-derived HSC-2 cells were maintained with DMEM containing 10% FBS [10]. HeLa cells were maintained with DMEM containing 10% FBS. Microscopic images were obtained using ECLIPSE Ti-2000 (Nikon Corp., Tokyo, Japan), PowerShot A640 (Canon Inc., Tokyo, Japan), and TMU-100K imaging system (KOGAKU Co., Ltd., Osaka, Japan).

2.3. Vector construction and lentiviral transduction

Vector construction and lentiviral transduction of mutant CDK4 (CDK4R24C: a $p16^{INK4a}$-resistant form), cyclin D_1, p53C234, and hTERT were as described previously [8]. Briefly, hTERT (a catalytic subunit of the enzyme telomerase), human cyclin D_1, and human mutant CDK4 were recombined into a lentiviral vector, CSII-CMV-RfA [a gift from Dr. Miyoshi (RIKEN BRC, Tsukuba, Japan)], by LR reaction (Invitrogen Corp.) to generate CSII-CMV-hTERT, -cyclin D1, and -hCDK4R24C. The production of recombinant lentiviruses with the vesicular stomatitis virus G glycoprotein was as described previously [11]. Titers of recombinant viruses were estimated by quantitative reverse transcription-polymerase chain reaction (RT-PCR) and an appropriate volume of viral fluid was added onto primary-cultured human oral epithelial cells (passage 1) in the presence of polybrene (4–8 μg/μl). After incubation with viruses, the cells showing continuous proliferation were selected for further examination, as mock-infected cells stopped growing within 2 weeks.

2.4. Growth curve

3×10^5 cells were seeded onto 60-mm-diameter dishes and cultured for up to 25 days. At each assay point, cells were trypsinized, harvested using rubber policeman, washed with PBS, and counted under microscopic observation to rule out dead cells by trypan blue staining.

2.5. Immunohistochemistry

Immunostaining of cells was performed as described previously [10]. In brief, cells were fixed with 4% (w/v) paraformaldehyde in PBS and permeabilized with PBS containing 10% FBS and 0.1% (w/v) Triton X-100. After peroxidase activity was blocked with 3% (v/v) H_2O_2/methanol for 30 min, cells were incubated with normal goat blocking serum (Dako) for 30 min to block nonspecific antibody binding sites. Cells were incubated with anti-cytokeratin-14 or anti-vimentin antibodies overnight at 4 °C, and incubated with biotinylated goat anti-mouse IgG. Signals were amplified and visualized using the substrate-chromogen solution. The cells were also counterstained with 0.5% (w/v) hematoxylin.

2.6. Confocal microscopy

Confocal microscopy was performed as described previously [12]. In brief, cells were grown on coverslips and fixed in PBS containing 4% (w/v) paraformaldehyde for 10 min. The cells were washed with PBS three times and then permeabilized with PBS containing 0.1% (w/v) Triton X-100 and 2 mg/ml bovine serum albumin for 2 h. The cells were washed and incubated for 1 h with the anti-E-cadherin antibody. After being washed with PBS, they were further incubated for 1 h with Alexa 546-labeled antin-mouse IgG and 4′,6-diamidino-2-phenylindole. The coverslips were washed with PBS, mounted on glass slides, and viewed with a confocal laser-scanning microscope (LSM700, Carl-Zeiss, Jena, Germany).

2.7. Western blot analysis

Cells were scraped with a rubber policeman, washed, and analyzed using antibodies as described [12,13].

2.8. Telomeric repeat amplification protocol assay

Telomerase activity was detected using a non-radioisotopic method with a TRAPeze telomerase detection kit (Intergen, Purchase, NY, USA), according to the manufacturer's instructions as

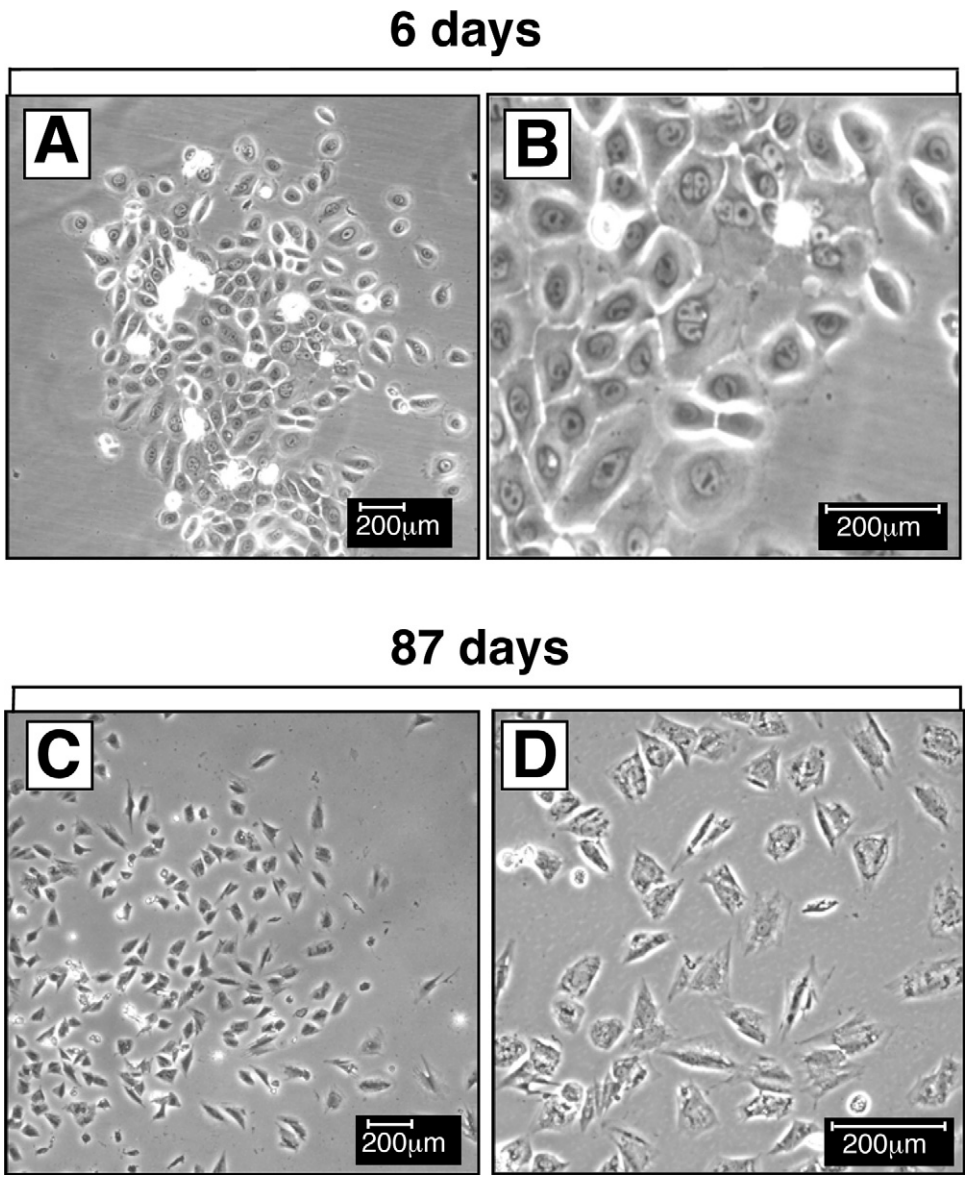

Fig. 1. Morphological changes of primary-cultured normal oral gingival epithelium cells. (A and B) Phase-contrast images of 6-day-culture cells. (C and D) Phase-contrast images of 87-day-culture cells. (B and D) Magnified images.

described [13]. In brief, 1 μm of cell protein lysed in the buffer containing 0.5% (w/v) 3-([cholamidopropyl]-dimethylammonio)-1-propanesulfonate was used for the assay. PCR products were separated on 12.5% (w/v) polyacrylamide gels, stained with SYBR Green I (Cambrex Co., East Rutherford, NJ, USA), and visualized with the LAS3000 CCD-Imaging System (Fujifilm Co. Ltd., Tokyo, Japan) on an ultraviolet trans-illuminator.

2.9. Anchorage-dependent growth

Anchorage dependence of cells was measured as described previously [14]. In brief, 1×10^4 cells were suspended in 1.5 ml of 0.3% (w/v) agarose in DH10 containing 10% FBS and overlaid on a layer of 5 ml of 0.5% (w/v) agarose in the same medium in 60-mm-diameter dishes. The cells were grown for 14 days and then they were photographed for magnified images.

2.10. Reactivity to LPS and heat-killed bacteria of MOE1 cells

MOE1 cells were plated onto 60-mm-diameter dishes and cultured overnight. Cells were treated with LPS (200 ng/ml) or heat-killed bacteria (*Aggregatibacter actinomycetemcomitans* IDH 781) [1×10^8 colony forming units (CFU)/ml] for 3–12 h. After treatment, the expression of interleukin (IL)-1β, IL-6, IL-8, tumor necrosis factor (TNF)-α, matrix metalloproteinase (MMP)-2, and MMP-9 mRNA were analyzed by real-time RT-PCR.

2.11. Real-time RT-PCR

Total RNA was extracted from cells using guanidine thiocyanate as described previously [15,16]. Total RNA was reverse-transcribed using First-strand cDNA Kit (Roche Diagnostics, Mannheim, Germany). Expression of each mRNA was estimated by real-time RT-PCR using Lightcycler Taqman master reaction mix® and Lightcycler 1.5®, Lightcycler software version 3.5 (Roche Diagnostics) according to the manufacturer's instructions. The series of primers used in this study were as follows: human IL-1β, forward, aaagcttggtgatgtctggtc, reverse, ggacatggagaacaccacttg; human IL-6, forward, gaaagtggctatgcagtttgaa, reverse, gaggtaagcctacactttccaaga; human IL-8, forward, agacagcagagcacacaagc, reverse, atggttccttccggtggt; TNF-α, forward, cagcctcttctccttcctgat, reverse,

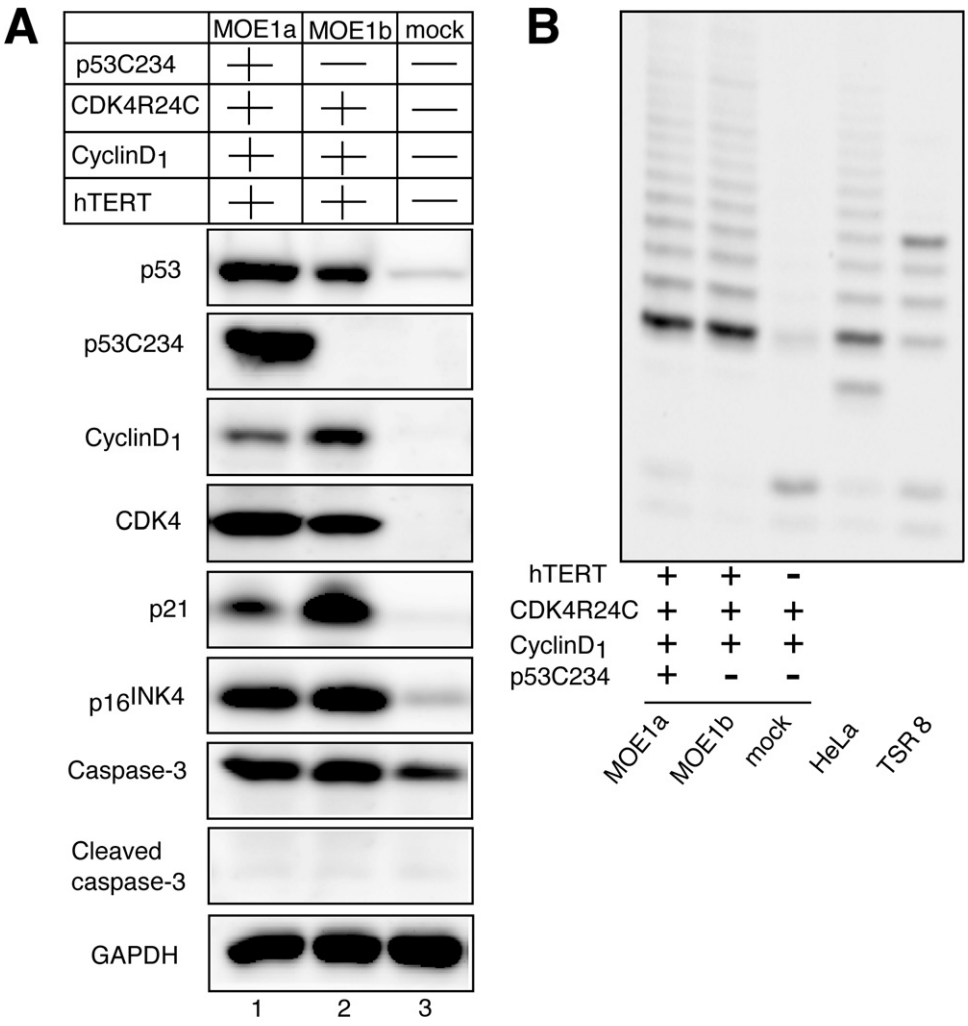

Fig. 2. Expression of virally induced and endogenous genes in MOE1a and MOE1b cells. Various combinations of genes of hTERT, CDK4R24C, cyclin D_1, and p53C234 were introduced into MOE1a and MOE1b cells using recombinant viral vectors as described. (A) Cell lysates were probed with anti-p53, cyclin D_1, CDK4, p21, p16, caspase-3, cleaved caspase-3, and GAPDH antibodies in MOE1 cells. Lane 1, MOE1a cells; lane 2, MOE1b cells; lane 3, mock. (B) Activation of telomerase in MOE1a and MOE1b cells was analyzed by telomeric repeat amplification protocol assay. TSR8, Eight tandem repeats of telomeric sequence (positive control).

gccagagggctgattagaga; MMP-2, forward, ataacctggatgccgtcgt, reverse, aggcacccttgaagaagtag; MMP-9, forward, gaaccaatctcaccgacagg, reverse, gccagagggctgattagaga; Keratin-17, forward, ttgaggagctgcagaacaag, reverse, agtcatcagcagccagacg; vascular endothelial growth factor (VEGF)-C, forward, tgccagcaacactaccacag, reverse, gtgattattccacatgtaattggtg; Wnt-5a, forward, attgtactgcaggtgtaccttaaaac, reverse, ccccttataaatgcaactgttc; Frizzled-2, forward, ggtgtcggtggcctacat, reverse, gagaagcgctcgttgcac.

3. Results

3.1. Mortality and morphological change of primary cultured oral epithelium

In primary culture after 6 days, normal oral epithelial cell colonies grew slowly and it took about several days for the number of cells to double. The primary culture was composed of large flat cells and small polygonal cells (Fig. 1A and B). When primary-cultured normal oral epithelial cells were cultured for 2 months (passaged 3 times), the number of small polygonal cells began to decrease, and spindle-shaped cells begin to increase in number. After 87 days, small polygonal cells were completely replaced by spindle-shaped cells (Fig. 1C and D), lost typical epithelial morphology, became senescent, and could no longer be passaged.

3.2. Expression of transduced genes and telomerase activity in immortalized normal oral epithelial cells

Early senescence of epithelial cells was considered to be due to activation of the p16/pRB pathway [13,17]. We transduced several genes into primary cultured cells for protection against cell-cycle arrest using recombinant lentivirus. We established two oral gingival cell lines named MOE (mouth-ordinary-epithelium) 1a and MOE1b by transducing CDK4R24C, cyclin D_1, and hTERT with or without p53C234, respectively. Transduction of hTERT and CDK4R24C genes did not generate clones with extended life span (data not shown). Although we tried to isolate MOE1 cells, isolated MOE1 cells easily entered into apoptotic state and were difficult to cultivate. This made us decide to treat MOE1 cells as population culture in this report (data not shown). Expressions of introduced genes were confirmed by immunoblotting (Fig. 2A). Expressions of p53, p21, and $p16^{INK4a}$ were augmented in MOE1a and MOE1b cells in comparison with those of primary-cultured oral epithelium. Furthermore, expression of caspase-3 was elevated in both cell lines, but activated cleaved caspase-3 was not detected in either cell line.

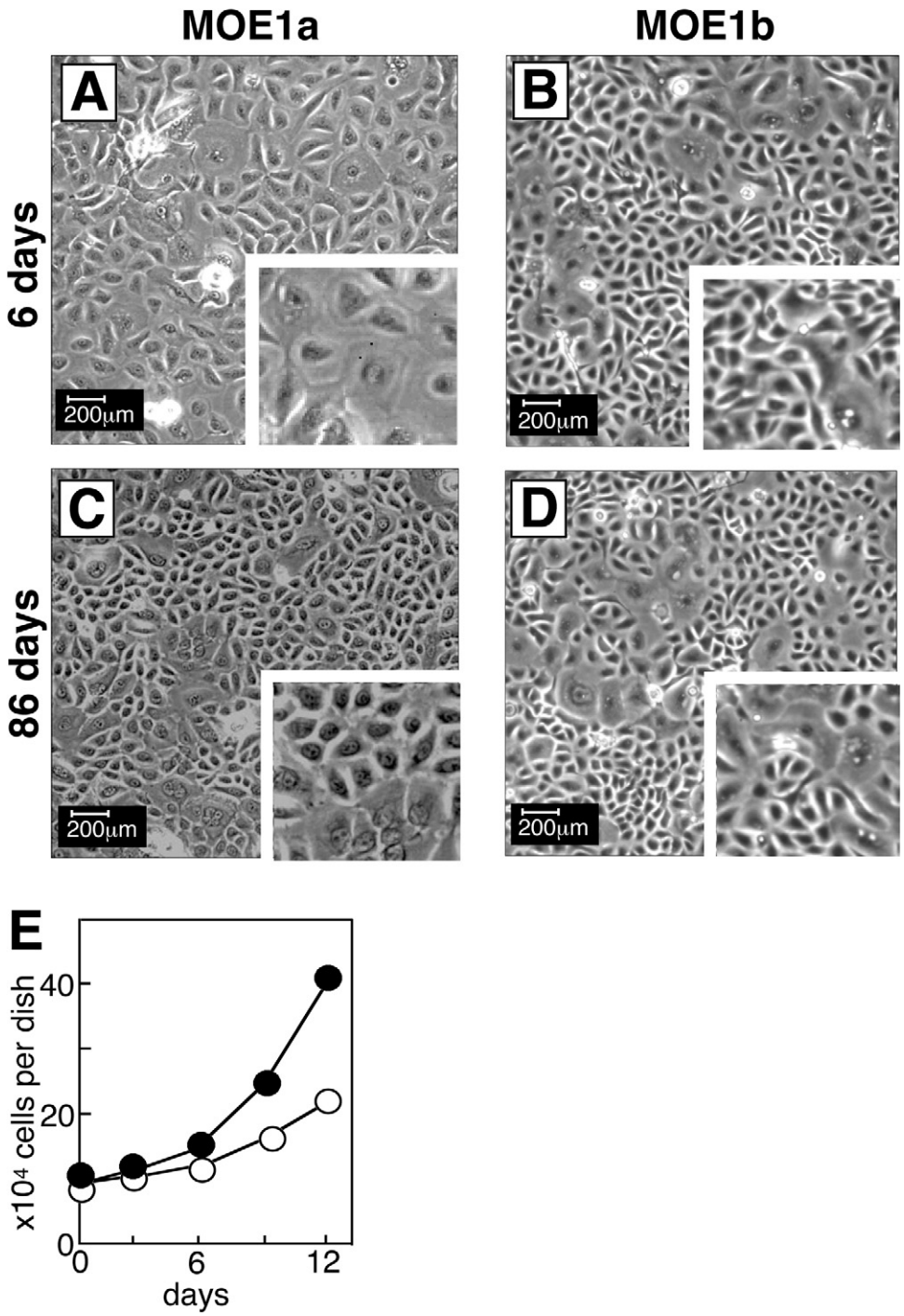

Fig. 3. Phase-contrast images of MOE1a and MOE1b cells. (A and B) Phase-contrast images of 6-day-culture MOE1a and MOE1b cells, respectively. (C and D) Phase-contrast images of 86-day-culture MOE1a and MOE1b cells, respectively. Inset, magnified images. (E) Growth curve of MOE1a and MOE1b. Closed circle, MOE1a; open circle, MOE1b.

These findings suggested that the expressions of cell-cycle progression factors (CDK4R24C, cyclin D_1) triggered accumulation of p53 and expression of cell-cycle inhibitors (p21 and $p16^{INK4a}$), but did not induce cell cycle arrest or massive apoptosis. Telomerase activation was detected by telomeric repeat amplification protocol assay in MOE1a and MOE1b cells in which hTERT were transduced (Fig. 2B).

3.3. Morphology of the immortalized normal oral epithelial cells

MOE1a and MOE1b cells exhibited extended life spans longer than nine months. They consisted of a large number of small polygonal cells and a small number of large flat cells. These findings implied that MOE1a and MOE1b maintained the morphology of normal primary-cultured oral epithelium even after 86 days of culture, which was different from the case of simply primary-cultured normal oral epithelial cells (Fig. 3A–D). MOE1a grew faster than MOE1b, suggesting a role of p53C234 in the prevention of cell-cycle arrest or apoptosis (Fig. 3E).

3.4. Expression of keratinocyte marker in MOE1 cells

MOE1a and MOE1b cells but not HFF2 expressed cytokeratin-14 and E-cadherin, which are epithelial markers. In contrast, HFF2 cells

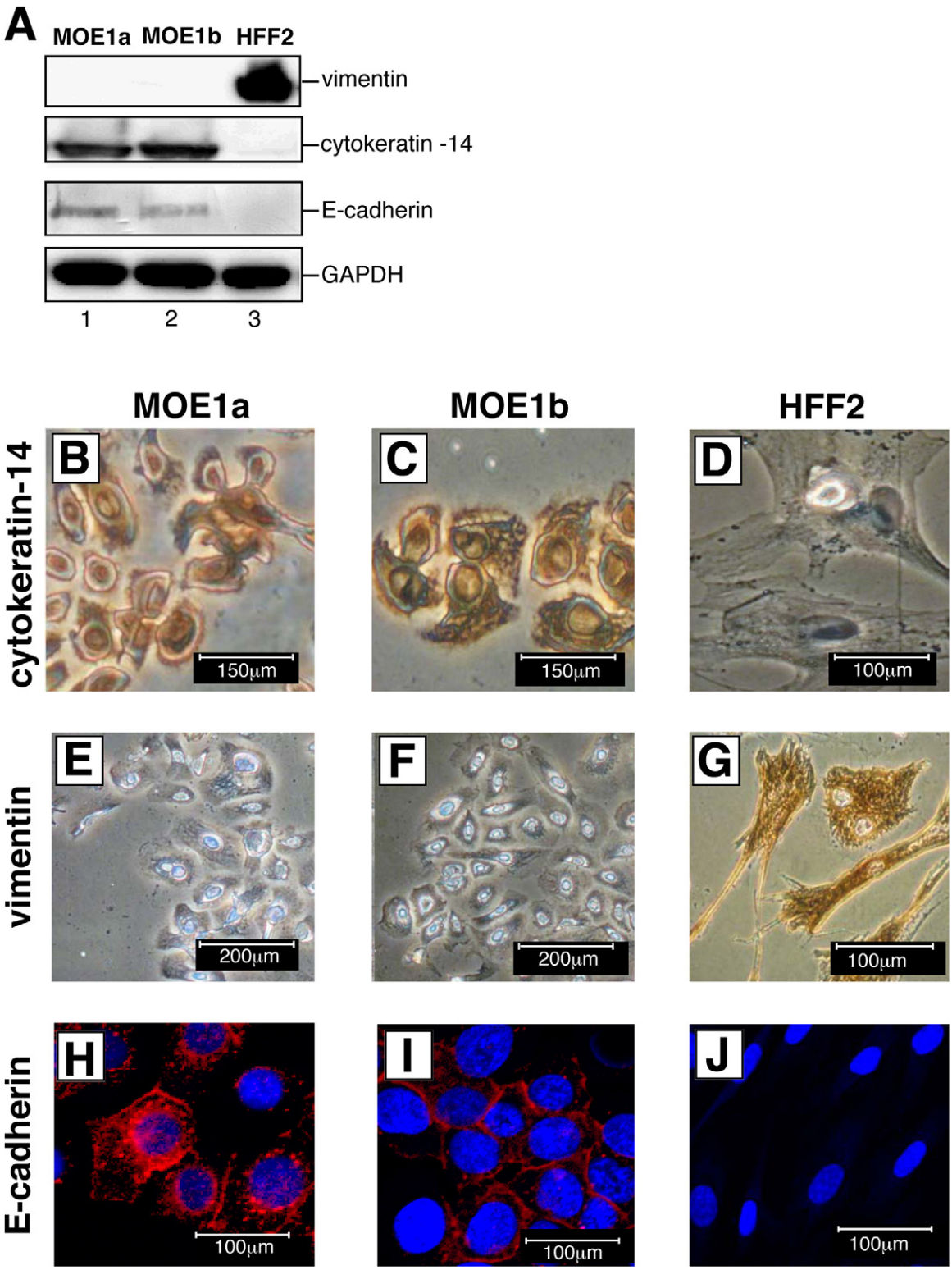

Fig. 4. Expression of epithelial and mesenchymal markers in MOE1 cells. (A) Cell lysates were probed with anti-vimentin, cytokeratin-14, E-cadherin, and GAPDH antibodies. Lane 1, MOE1a cells; lane 2, MOE1b cells; lane 3, immortalized human foreskin fibroblast HFF2 cells. (B–J) Immunocytochemical analysis of epithelial and mesenchymal markers. (B, E and H) MOE1a, (C, F and I) MOE1b, and (D, G and J) HFF2 cells were stained with (B–D) anti-cytokeratin-14, (E-G) anti-vimentin, or (H–J) anti-E-cadherin antibodies. Confocal laser microscopy was used for imaging for E-cadherin.

but not MOE1 cells expressed vimentin, a mesenchymal marker (Fig. 4). These findings suggested that MOE1a and MOE1b were derived from epithelial cells and that transgenes in MOE1 cells did not cause epithelial–mesenchymal transition, which is often related to tumorigenesis.

3.5. Anchorage dependence of MOE1 cells and expression of cancerous genes in MOE1 cells

It is well known that normal epithelial cells do not grow in an anchorage-independent manner, but transformed epithelial cells

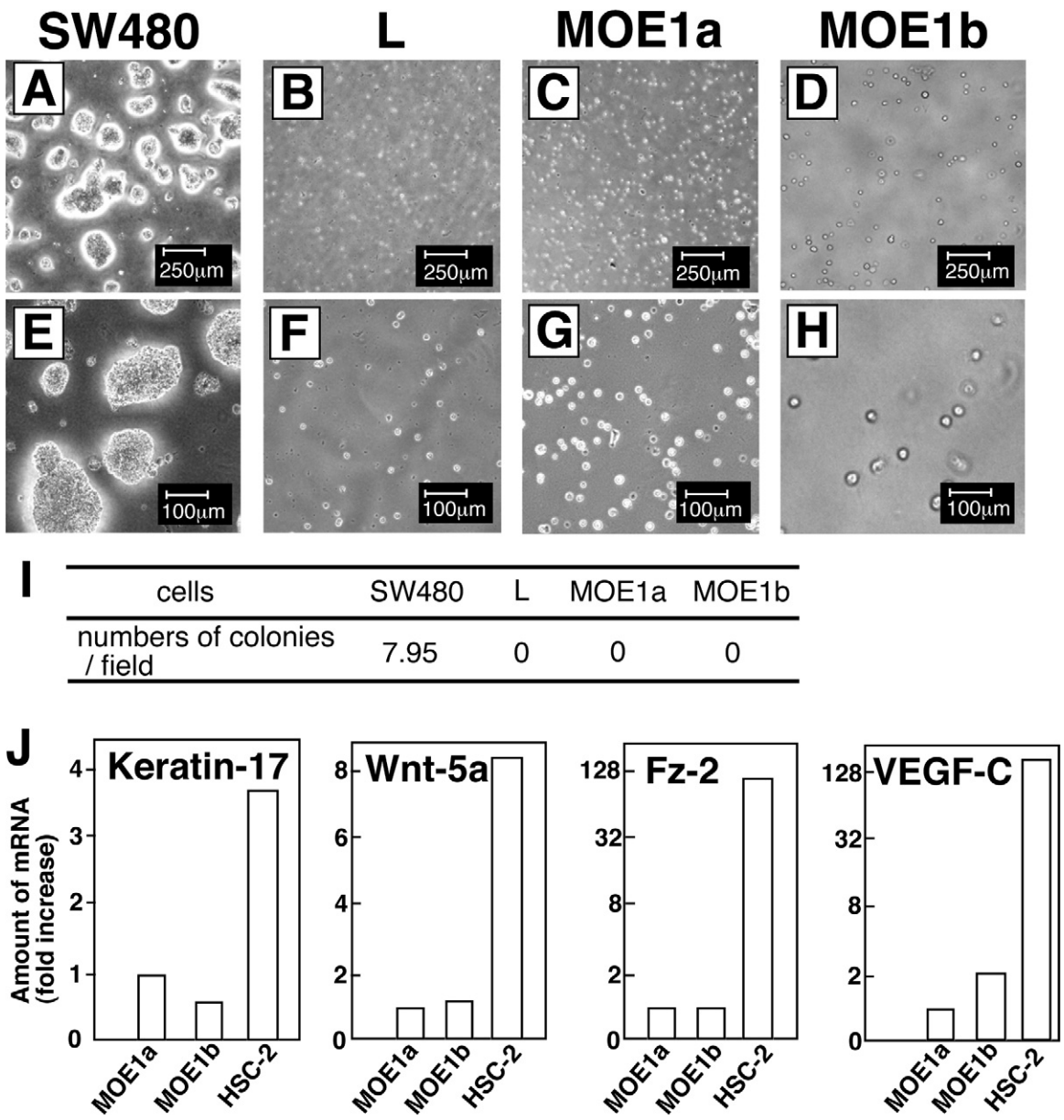

Fig. 5. Anchorage-dependent growth and expression of cancer-associated genes in MOE1 cells. Phase-contrast images of 14-day-culture (A and E) SW480, (B and F) L, (C and G) MOE1a, and (D and H) MOE1b cells. (E–H) Magnified images. (I) Colonies larger than 0.1 mm were counted. (J) Gene expression of Keratin-17, Wnt-5a, Frizzled-2 (Fz-2), and VEGF-C in MOE1 cells by RT-PCR.

show anchorage-independent growth and form colonies on an agar plate [14]. Colony formation assays were performed to confirm the absence of transformation (Fig. 5). SW480, a human colon cancer-derived cell, showed anchorage-independent growth on agar plate (Fig. 5A and E). MOE1a and MOE1b cells did not show anchorage-independent growth similar to mouse fibroblast-derived L cells (Fig. 5B–D and F–H). SW480 cells formed large colonies but other cell lines did not (Fig. 5I).

Reportedly, gene expression of Keratin-17, Wnt-5a, Frizzled-2, and VEGF-C was elevated in oral squamous cell carcinomas [10,18,19]. Expression of these genes was not elevated in MOE1 cells (Fig. 5J).

3.6. Reactivity to physiological stimulants of MOE1 cells

Epithelial cells produce cytokines and several secretory factors in response to LPSs [20]. MOE1 cells were examined in terms of whether they kept their responses to extracellular signals. They expressed IL-1β, IL-6, IL-8, TNF-α, MMP-2, and MMP-9 in response to LPS or heat-killed bacteria in a similar manner to primary cultured cells or other epithelial cell lines [21,22]. As there was no significant difference between the responses of MOE1a and MOE1b cells, transduction of p53C234 into cells did not affect these cellular responses (Fig. 6A and B). These results suggested that MOE1 cells maintained reactivity to physiological stimulants as well as primary-cultured cells or tissues in vivo and that they could be used as a model for oral cell biological approaches.

4. Discussion

Since the establishment of oral epithelial cell lines from HPV-induced cancer was reported in the 1990s, attempts were initiated to immortalize by artificial transduction of HPV genes. Several oral epithelial cell lines were established from normal oral epithelium, e.g. IHGK cells were established by transducing HPV16 DNA [23],

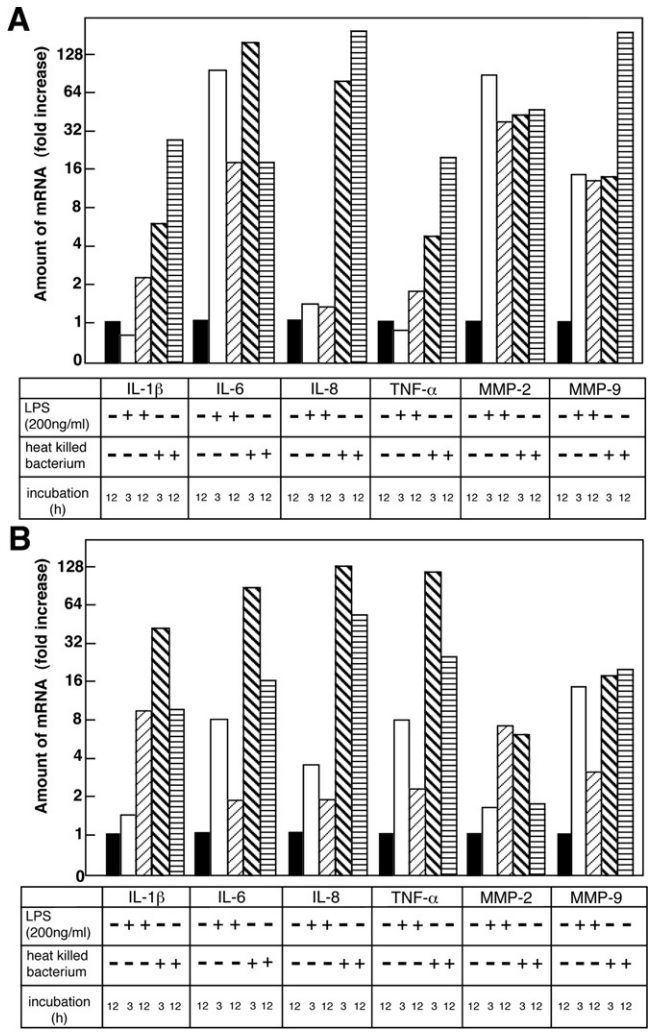

Fig. 6. Gene expression of secretory molecules induced by lipopolysaccharide (LPS) or heat-killed bacteria. (A) MOE1a cells were stimulated with LPS (200 ng/ml) or heat-killed bacteria (1×10^8 CFU/ml) for 3 or 12 h. Expression of IL-1β, IL-6, IL-8, TNF-α, MMP-2, and MMP-9 mRNA was evaluated by RT-PCR. (B) Result of the same assays using MOE1b cells.

while OBA-9 [20], and NDUSD1 cells [24] were established by transducing SV40 early genes.

The oral epithelium is exposed to external attack, which includes foods, chemical substances, alcohol, mechanical stresses, bacteria, and viruses, including HPV, which could be risk factors for oral cancer. EMT may be critical for neoplastic progression and the eventual tumorigenicity of epithelial cells. EMT and EMT-associated features were reported in human gingival keratinocytes immortalized with the E6/E7 oncogenes of HPV type 16, which induced degradation of several cellular proteins by ubiquitination [25].

The mechanisms behind virally induced cancer cells were analyzed and several HPV genes were found to interfere with the regulation of cell-cycle mechanisms [26,27]. The degradation of p53 by the E6 protein and the abrogation of the pRb functions by the E7 protein lead to the reactivation of the DNA synthesis machinery [25]. Although these studies support the possibility of normal immortalized cells without transformation, the fact that E6 and E7 induced transformation, inhibition of apoptosis, and accumulation of mutations in some cells implied that they had additional effects rather than immortalization [25].

We hypothesized that it is possible to immortalize oral epithelial cells by driving the cell cycle and relieving telomere reduction without using viral genes. We have shown that Rb/$p16^{INK4a}$ inactivation and telomerase activity are required to immortalize human epithelial cells and the method similar to this study was reported to be useful for generating normal or near normal diploid cells, whereas immortalization by viral oncogenes E6 and E7 showed a tendency for genomic abnormalities [13,17,28]. We attempted to immortalize oral epithelial cells by introducing CDK4R24C and hTERT. CDK4R24C is a mutant form of CDK4, which cannot bind to $p16^{INK4a}$ nor be inhibited by $p16^{INK4a}$. Although our first trial of oral epithelium was unsuccessful, we have successfully continued cell culture of human oral epithelial cells (MOE1b) by additional introduction of cyclin D_1 to promote cell-cycle progression. Expression of p53 was elevated in the MOE1 cells. Elevation of p53 levels was also reported for ovarian surface epithelial cells immortalized with the same set of the genes [8].

Besides our study, Rheinwald et al. reported immortalization of human keratinocytes without using HPV or SV40 [29]. They reported that the expression of p53 was increased in the oral keratinocytes sequentially transduced by hTERT, CDK4R24C, and that introduction of dominant negative p53 was necessary to make cells escape from senescence. We speculate that introduction of mutant CDK4 and CyclinD could continuously promote G1/S progression by active mutant CDK4/CyclinD complex formation and that this triggered accumulation of p53 and inhibitors of CDK/Cyclin complexes (p16 or p21) to retard G1/S progression. Since we were concerned that p53-elevation might lead to apoptosis, we generated another cell line (MOE1a) which was transduced with dominant-negative form of p53 (p53C234) in addition to CDK4R24C, hTERT, and cyclinD_1. Eventually, we were able to immortalize human oral epithelial cells regardless of the transduction of dominant negative p53 (p53C234) which is different from Ref. [29]. Our experiment in this study suggested that introduction of cyclin D_1 instead of dominant negative p53 could assist the progression of cell cycle and make cells escape from senescence in the p53-elevated condition. Although we found no apparent differences with regard to the characteristics between the two cell lines besides the faster growth rate of MOE1a cells, transduction of p53C234 might cause genomic instabilities in MOE1a cells.

Oral epithelium cells not only function as physical barriers to the outside environment but also play an active role in modulating interactions between the environment and the body. Oral epithelium cells act as the first step in immunological protection [1]. Oral epithelium cells synthesize and secrete a wide variety of cytokines, including IL-1β, IL-6, IL-8, and TNF-α [20].

MMPs are members of the family of zinc-dependent proteolytic enzymes. The expression and activity of MMPs in adult tissues is normally quite low, but increases significantly in various pathological conditions, such as tumor growth, metastasis, and periodontitis [30]. MMPs are upregulated in intestinal mucosa with inflammation by stimulation of LPS [31]. In our MOE1a and MOE1b cells, expression of IL-1β, IL-6, IL-8, and TNF-α mRNA was induced by these stimuli. Furthermore, expression of MMP-2 and MMP-9 mRNA was induced by stimulation of LPS or heat-killed bacterium. Although we did not use clonal cell lines, we repeatedly observed similar responses after several passages (data not shown). These reactions mimic the immune functions of normal oral epithelium.

MOE1 cells did not show anchorage-independent growth and overexpression of cancer-associated genes. Furthermore, expression of keratin-14 in MOE1 cells implies that immortalization of MOE1 cells was not based on an EMT-dependent mechanism [32]. These results suggest that MOE1 cells maintain several natural characters of normal epithelial cells without any evidence of malignant transformation, although anchorage-independent growth is not a common feature of oral cancer cell lines [33].

Finally, we believe that MOE1 cells could be useful for dental studies in various fields including oral cancer. In this study, we have shown a new strategy for establishing non-cancerous cell lines from

oral epithelium. Further studies will be necessary to immortalize oral epithelial cells "without inactivating the p53 pathway".

Acknowledgments

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