Immortalization and characterization of pleomorphic adenoma cells by transfection with the *hTERT* gene

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Abstract. Pleomorphic adenomas (PAs) of salivary glands are characterized by the mixed appearance of epithelial and mesenchymal-like components such as myxoid and chondroid tissues. Although various studies have examined PAs, thus far it is not clear how PAs make these multiple components. Thus, clarification of the histodifferentiation of this unique salivary gland tumor using not only tissues *in vivo* but also PA cells cultured *in vitro* is necessary. However, no *in vitro* model of PA has been reported, because normal and benign tumor cells tend to grow slowly and senesce quickly in culture. Therefore, we immortalized cells using transfection of the *hTERT* gene without otherwise altering the nature of those cells. The immortalized PA cells expressed mRNA of the pleomorphic adenoma gene 1 and showed epithelial and neoplastic myoepithelial characteristics by immunohistochemical immunofluorescence analyses and ultrastructural study. Our findings suggest that these cells will be a useful model to study the cellular differentiation of PA.

Introduction

Pleomorphic adenomas (PAs) of the salivary glands are characterized by the mixed appearance of epithelial and mesenchymal-like components such as myxoid and chondroid tissues. Although various studies have examined PAs (1,2), it is not clear how they make those multiple components. Thus, it is necessary to clarify the histodifferentiation of this unique type of salivary gland tumor using not only tissues *in vivo* but also PA cells cultured *in vitro*. However, no *in vitro* cell model of PA has been reported, because normal and benign PA tumor cells tend to grow slowly and senesce quickly in culture.

Generally, the loss of DNA from the telomeres of chromosomes that occurs with each replication shortens them to a critical minimal length after which the cells senesce (3). The parental PA cells used in this study entered the crisis state after 6 population doublings (PDLs). Telomerase is a ribonucleoprotein reverse transcriptase that stabilizes the telomere and chromosome structure by adding telomeric repeats that would otherwise be lost during replication (4-8). The telomerase enzyme consists of three major components, human telomerase RNA (hTR), human telomerase-associated protein (hTLP1) and human telomerase reverse transcriptase (hTERT), the latter being the catalytic subunit of telomerase (9). Normal and neoplastic cells usually express hTR and hTLP1 (10), although most normal cells and benign neoplastic cells, including PA, do not possess telomerase activity (11-13). However, malignant tumor cells exhibiting telomerase activity express high levels of hTERT, which seems to be sufficient for cells to regain telomerase activity (11).

In the present study, to generate a stable and standard culture of human PA cells, we established immortalized human PA cells by infecting them with a retroviral vector containing *hTERT*. We describe the cellular phenotypic characteristics of these immortalized PA cells, which will be a useful model to study the cellular differentiation of PA.

Materials and methods

All procedures were performed in compliance with regulations administered by Hiroshima University.

Cells and culture conditions. Surgical tissue fragments were obtained from a PA of the parotid gland of a 74-year-old Japanese female patient at Hiroshima University Hospital. The tissue specimen from the central part of the tumor was cut into small pieces, placed in 90-mm culture dishes with keratinocyte serum-free medium (Invitrogen, Grand Island, NY), and incubated in 5% CO_2 in air at 37˚C. Outgrowing

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cells were then subcultured in the same medium and used for analysis.

Transfection. The *hTERT* gene was cloned into the retroviral vector, pQCXIP (Clontech, Tokyo, Japan) with the *Hpa*I restriction enzyme. The cloned retrovirus was transfected into the RetroPack PT67 Packaging Cell Line (Clontech), where the vector was packaged into infectious, replication-incompetent retroviral particles. The packaged cells were maintained with puromycin (1.2 μg/ml) containing medium for 1 week. Retrovirus for hTERT was collected, polybrene was added (8 μg/ml) and the mixture was then filtered through a 0.20-μm filter. After puromycin treatment for 1 week, packaging cells were maintained without puromycin. To infect target cells, the packaged retrovirus (1 ml/dish) was added to the medium of a 25-mm^2 flask containing cultured target cells at 50-60% confluence. After 24 h, infected cells were placed in medium containing puromycin (0.5 μg/ml) for 2 to 3 weeks.

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 Inc., Valencia, CA) according to the manufacturer's instructions. Total RNA was quantified and its purity was determined by standard spectrophotometric methods. cDNA was synthesized from 1 μg total RNA using ReverTra Dash (Toyobo Biochemicals, Tokyo, Japan). The oligonucleotide RT-PCR primers listed in Table I were purchased from Invitrogen (Carlsbad, CA). Aliquots of total cDNA were amplified with 1.25 U of rTaq-DNA polymerase (Qiagen Inc.), and amplifications were performed using a PC701 thermal cycler (Astec, Fukuoka, Japan) for 30 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. 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. The sequences of the primers used were as follows: hTERT primers, F 5'-ACTTTGTCAA GGTGGATGTGACGG-3', R 5'-AAGAATCATCCACCA AACGCAGG-3'; PA gene (PLAG)1 primers, F 5'-CAAGT GCAGCCAGCAATTTA-3', R 5'-CATTTCCCAGAGATG CATGA-3; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers, F 5'-TCCACCACCCTGTTGCTGTA-3', R 5'-ACCACAGTCCATGCCATCAC-3'.

Telomerase assay. Telomerase activity was assayed with the PCR-based modified, telomeric repeat amplification protocol (TRAP) assay (13). Each cell pellet (1x10^5) was resuspended in 200 μl cold TRAP lysis buffer, incubated for 30 min on ice, and centrifuged at 15,000 x g for 20 min at 4˚C. The extract equivalent of 10 cells (2 μl) was incubated with 48 μl 20 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 68 mM KCl, 0.05% Tween-20, 1 mM EGTA, 50 mM dNTP, 5 μg BSA, 2 U Taq DNA polymerase (Invitrogen), 10-attg internal telomerase assay standard DNA, and 0.1 μg extra-purified TS primer (5'-AATCCGTCGTCGAGCAGAGTT-3') at 20˚C for 30 min and then heated at 90˚C for 3 min. During the above step, 0.1 μg (2 μl) of extra-purified CXII primer (5'-CCCTTACCC TTACCCTTACCCT-3') was added, and the reaction mixture was subjected to 31 PCR cycles at 94˚C. Electrophoresis was performed on 12% nondenaturing acrylamide gels, stained with SYBR-Green (FMC Bioproducts, Rockland, ME), and bands were visualized using a chemiluminescence image analyzer.

Immunohistochemistry. Cells were cultured in 35-mm^2 culture dishes until subconfluent, then fixed with 3.5% neutral buffered formalin for 1 h and washed 3 times with phosphate-buffered saline (PBS, 145 mM NaCl, 2.8 mM NaH_2PO_4, 7.2 mM Na_2HPO_4, pH 7.2). They were treated with 0.3% hydrogen peroxide in 100% methanol for 20 min, and washed 3 times with PBS. Immunostaining was performed using the Envision$^+$ system (DakoCytomation, Carpinteria, CA, USA). Primary antibodies against cytokeratin monoclonal antibodies (AE1/AE3, Biomeda and CAM5.2, Becton Deckinson immunocytometry system), vimentin monoclonal antibody (1:200, DakoCytomation), lactoferrin polyclonal antibody (1:200, DakoCytomation), S-100 protein polyclonal antibody (1:200, DakoCytomation), α-smooth muscle actin monoclonal antibody (1:200, DakoCytomation) and calponin monoclonal antibody (1:100, DakoCytomation) were applied at suitable dilutions and incubated overnight at 4˚C. Diaminobenzidine-tetrahydrochloride was used as a chromogen and Mayer's hematoxylin was used as a counterstain.

Immunofluorescence double staining. Cells were fixed with 10% neutral buffered formalin for 20 min at room temperature. To block non-specific binding, normal goat serum was applied for 30 min, and cells were then rinsed in 0.2% Triton X-100 in 0.01 M PBS for 5 min. Primary antibodies against cytokeratin polyclonal antibody (1:100, DakoCytomation), anti-vimentin monoclonal antibody (1:100, DakoCytomation), anti-ZO-1 monoclonal antibody (1:100, Zymed Laboratories, South San Francisco, CA) and anti-claudin-1 polyclonal antibody (1:100, Zymed) were applied at room temperature for 1 h. After washing, anti-rabbit IgG Alexa fluor® 488 (1: 200, Molecular Probes, Eugene, OR) and anti-mouse IgG Alexa fluor® 568 (1: 200, Molecular Probes) were applied at room temperature for 1 h. TO-PRO®-3 iodide (1:500, Molecular Probes) was used for nuclear counterstaining. The staining was observed using conventional fluorescence microscopy (Axiophot 2, Carl Zeiss, München-Hallbergmoos, Germany).

Ultrastructural study. Cells were fixed with modified Karnovsky fixative (2% paraformaldehyde buffer and 2.5% glutaraldehyde in 0.2 M cacodylate buffer) for 30 min at room temperature, and then were collected as cell pellets. Samples were post-fixed with 1% osmium in 0.2 M cacodylate buffer, dehydrated and embedded in EPON812 (TAAB Laboratories Equipment, Berkshire, UK). Ultra-thin sections were cut and double stained with uranyl acetate and lead citrate. Specimens were examined by transmission electron microscopy (H-7100, Hitachi, Tokyo, Japan).

Results

Histology of PA and cytology of PA cells. The tumor showed typical histological features of PA including an admixture of epithelial and mesenchymal-like components such as those found in myxoid and chondroid tissues (Fig. 1A). In culture,

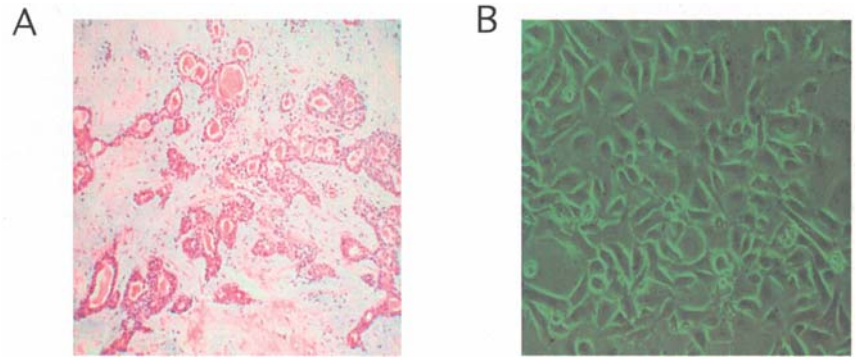

Figure 1. (A) Histological appearance of the original PA tumor *in situ*. The tumor consisted of epithelial and mesenchymal-like components such as those found in myxoid and chondroid tissues. (B) Morphology of non-transfected PA parental cells in culture. Cells were small and round or short and spindle-shaped.

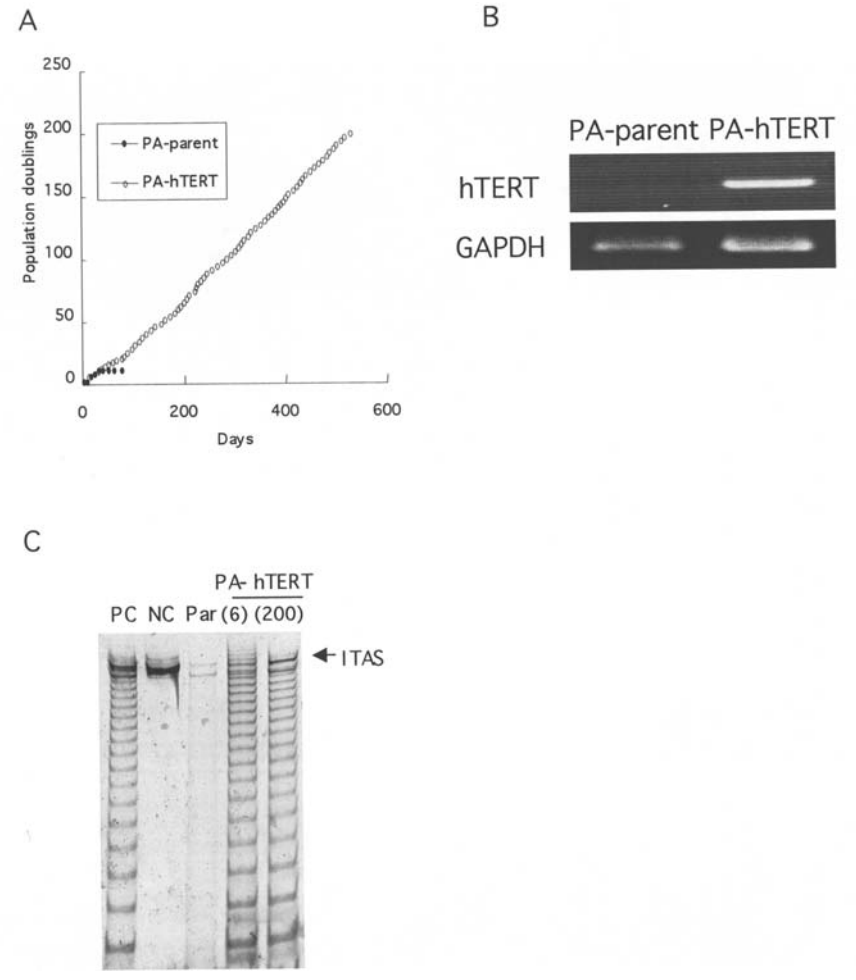

Figure 2. (A) PDLs of non-transfected PA-parent cells and hTERT-transfected PA (PA-hTERT) cells. PA-parent cells stopped growing after 6 PDLs, but PA-hTERT cells were immortalized and grew over 200 PDLs. (B) Expression of the *hTERT* gene by RT-PCR analysis in PA-parent cells and PA-hTERT cells. High expression of the *hTERT* gene was observed in PA-hTERT cells but not in PA-parent cells. (C) Telomerase activity of PA-parent cells and PA-hTERT cells was determined by the TRAP assay. Telomerase activity was present in PA-hTERT cells, but not in PA-parent cells. The internal control consists of a 10-attg internal telomerase standard (ITAS) DNA. PC, positive control (extract of an oral cancer cell line, HSC2); NC, negative control, without cell extract; Par, parental cells. Cells at 6 and 200 PDLs were used.

the appearance of PA cells included small round or short spindle-shaped cells (Fig. 1B).

Immortalization of PA cells. PA cells transfected with hTERT (PA-hTERT) bypassed senescence and grew over 200 PDLs without significant growth retardation (Fig. 2A). In contrast, non-transfected PA parental cells entered crisis at about 6 PDLs. We confirmed the high expression of hTERT in PA-hTERT cells, but not in parental cells by RT-PCR analysis (Fig. 2B). Although parental cells did not express telomerase activity, PA-hTERT maintained its telomerase activity even after 200 PDLs (Fig. 2C).

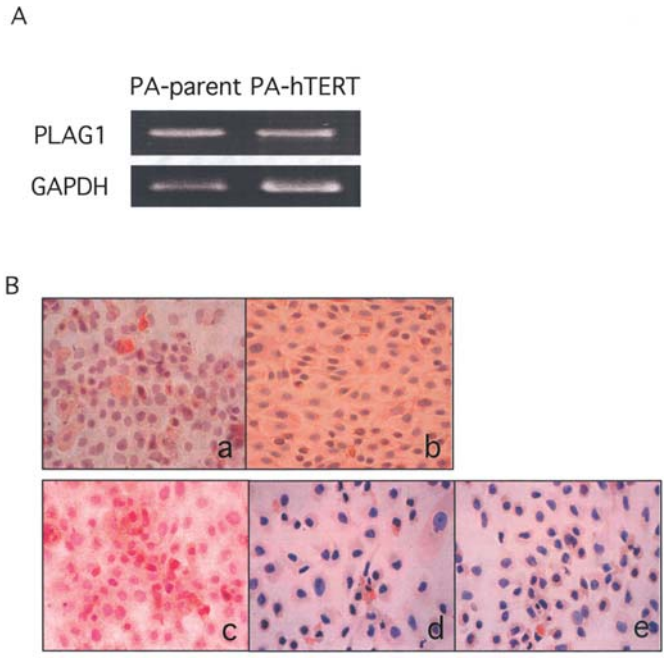

Figure 3. (A) Expression of the *PA1* (PLAG1) gene by RT-PCR in PA-parent cells and in PA-hTERT cells. Both cell lines expressed PLAG1 mRNA at the same level. (B) Immunohistochemical staining. PA-hTERT was strongly positive for cytokeratin antibody (a), vimentin antibody (b), LF antibody (c) and calponin (d), and S-100 protein (e) was weakly positive.

Expression of PLAG1 mRNA. To identify cells derived from PA, we examined the expression of PLAG1 mRNA using RT-PCR. PLAG1 is a zinc finger transcription factor gene, which is consistently rearranged and overexpressed in human PA of the salivary glands with 8q12 translocations (14,15). Parental PA cells and PA-hTERT cells expressed PLAG1 at the same level (Fig. 3A).

Immunohistochemical staining. The morphologic appearance of PA-hTERT cells was small and round or short and spindle-shaped, identical to the appearance of parental PA cells. Immunohistochemically, most PA-hTERT cells were strongly positive for cytokeratin (Fig. 3Ba) and vimentin (Fig. 3Bb). In addition, some cells were intensely positive for lactoferrin (Fig. 3Bc), α-smooth muscle actin or calponin (Figs. 3Bd). S-100 protein positive cells were also seen occasionally (Fig. 3Be).

Immunofluorescence double staining. Coexpression of cytokeratin and vimentin was distinct in the cytoplasm of PA-hTERT cells. Vimentin (red color) was distributed in the central areas of the cells surrounding the nuclei, while cytokeratin (green color) was distributed throughout the cytoplasm including peripheral areas. Nuclei were counterstained as dark red by TO-PRO (Fig. 4A). Also tight junction proteins were found in PA-hTERT cells. ZO-1 was observed at the cell-to-cell contact area clearly, and also with diffuse and weak staining in the cytoplasm (Fig. 4B). Coexpression of ZO-1 and claudin-1 was observed at the peripheral cell process. Expression of claudin-1 was observed as a continuous line and ZO-1 showed punctiform. Abundant expression of ZO-1 and claudin-1 were in the cytoplasm. Nuclei were indicated as dark red. (Fig. 4C)

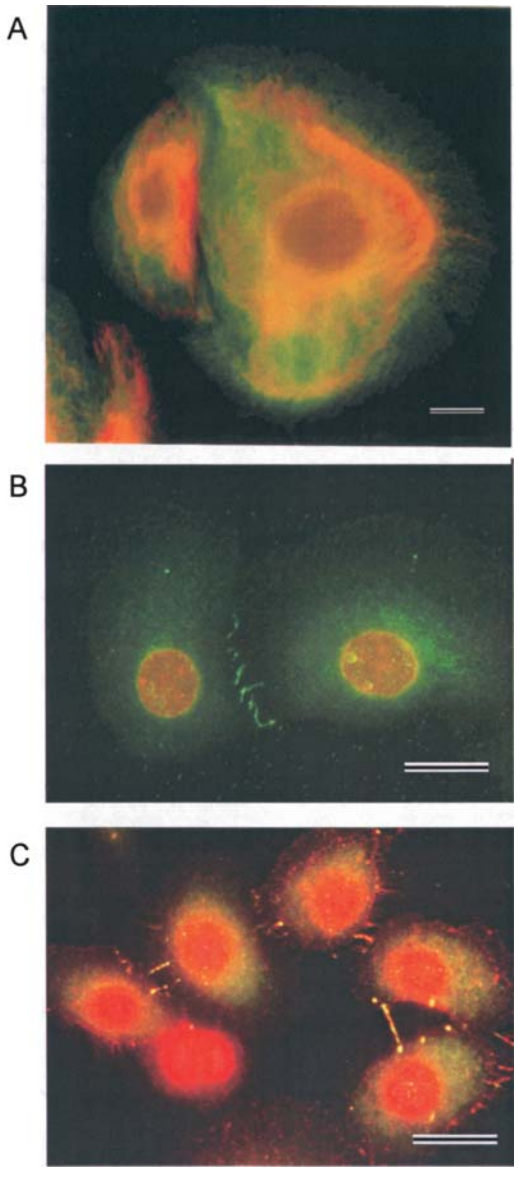

Figure 4. (A) Cytokeratin (green color) and vimentin (red color) were coexpressed distinctly in the cytoplasm. Vimentin was mainly distributed surrounding the nuclei, while cytokeratin was localized over the entire cytoplasm including the peripheral area (bar, 10 μm). (B) Expression of ZO-1 was observed distinctly at the cell-to-cell area discontinuously (original magnification x972; bar, 15.4 μm). (C) Coexpression of claudin-1 (red) and ZO-1 (green) was found at the peripheral cell process. While expression of claudin-1 was linear and continuous, ZO-1 showed punctiform. Both of them were also observed weakly and diffusely in cytoplasm. Nuclei were counterstained as dark red by TO-PRO (original magnification x680; bar, 22.0 μm).

Ultrastructural findings. Ultrastructurally, cytoplasmic organelles, including many bulging mitochondria, rough endoplasmic reticulum and Golgi apparatus, were recognized clearly. Many bundles of intermediate filaments and forming tonofilaments were observed surrounding the nuclei, and numerous microfilaments were localized in marginal areas of the cytoplasm and attached surfaces (Fig. 5A). A few incomplete amorphous, low-density secretory-like granules were also found, and some coated vesicles but not caveolae were aligned at the periphery of the plasma membrane (Fig. 5B). Golgi apparatus was also observed near the nuclei. At the cell junctional area, rich actin filaments were aligned

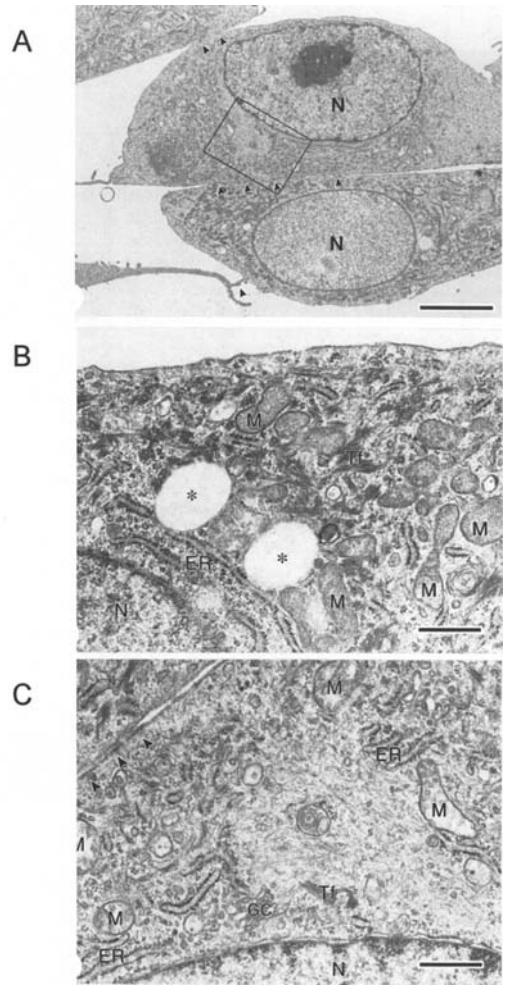

Figure 5. (A) Abundant cytoplasmic organelles and intermediate filaments were observed surrounding the nuclei (N) (bar, 3 μm). (B) Cytoplasmic organelles, rough endoplasmic reticulum (ER) and many bulging mitochondria (M) were recognized between the many bundles of tonofilaments (Tf). *A few incomplete amorphous low-density secretory-like granules were recognized. Coated vesicles but no caveolae were aligned at the periphery of the plasma membrane. (C) Golgi apparatus (GC) was also observed near the nuclei (N). At the cell junctional area, rich actin filaments were aligned beneath the plasma membrane, and adherence junctions or a desmosome-like structure (arrow head) were recognized (bar, 0.5 μm).

beneath the plasma membrane, and adherence junctions or a desmosome-like structure were recognized (Fig. 5C).

Discussion

PA, the most common type of salivary gland tumor, shows unique histopathologic features, i.e., a mixture of epithelial and mesenchymal-like components normally seen in myxoid or chondroid tissues. The presence of both epithelial and mesenchymal-like components has led to considerable discussion about the histogenesis of this tumor. At the present time, it has been widely accepted that PA is a purely epithelial tumor and that its mesenchymal-like components are composed of neoplastic myoepithelial cells and their products, such as mucopolysaccharide matrix, elastic fiber and basement membrane-like materials, based on ultrastructural and immunohistochemical analyses (1,2). However, the mechanism by which neoplastic myoepithelial cells form the mesenchymal-like components is not yet clarified, in part because there is no suitable *in vitro* model for PA. Therefore, the aim of this study was to create immortalized cells from PA that can grow stably and maintain the cellular properties of PA to allow analyses of their characteristic patterns of differentiation.

It is difficult to establish immortalized cells originating from benign tumors because of their slow growing nature and quick senescence in culture. Therefore, to prevent their senescence and to immortalize cell lines, some researchers use oncogenes or genes that are connected with immortalization, such as SV40, HPVE6/E7 and hTERT (16-19). However, SV40 and HPVE6/E7 may alter the nature of cells such as their contact inhibition and cell morphology (20). It has been reported that expression of hTERT and subsequent telomerase activity successfully immortalizes normal cells and benign neoplastic cells without otherwise altering the parent cells (18,19,21-23). In the present study, transfection with the *hTERT* gene immortalized cells from PA, a benign salivary tumor. Expression of the mRNA for the *hTERT* gene and telomerase activity as measured by the TRAP assay were recognized in the transfected PA-hTERT cells, but not in the parental PA cells. PA-hTERT cells survived beyond normal programmed senescence over 200 PDLs, which indicates that a stable transfection occurred. The extended life span of these cells makes them useful for studying mechanisms involved in the development and differentiation of PA. Both the PA-hTERT cells and parental PA cells showed small rounded or short spindle-shaped morphology and they expressed PLAG1 mRNA. PA-hTERT cells were positive for cytokeratins, a marker of epithelial cells, which confirmed their derivation from parenchymal cells, not from the stromal cells. In addition, immunohistochemical analyses revealed that PA-hTERT cells are composed of secretory epithelial cells positive for lactoferrin and myoepithelial cells positive for α-smooth muscle actin and calponin.

A few incomplete amorphous, low-density secretory-like granules were recognized, and many bundles of intermediate filaments forming tonofilaments and microfilaments were also observed in the cytoplasm of the cells, ultrastructurally. Furthermore, adherence junctions were also observed between the adjacent cells, and coexpression of ZO-1 and claudin-1 tight junction proteins was observed at the cell-to-cell contact area and peripheral cell process by double immunofluorescence staining. Tight junctions form well-developed networks of continuous intra-membranous strands, which consist of tight junction molecules, occluding (24), claudins (25) and peripheral membrane proteins including ZO-1 (26). Important roles of tight junctions were as barriers within intercellular spaces, and fences determining a boundary line between apical and basolateral plasma membranes, in addition to the maintenance of cellular polarity (27). From this study, it is not clear whether pleomorphic adenoma reveals only one single cell type with a myoepithelial character or whether there are different phenotypes. Studies are needed to explore the function of one cell in detail.

Hence, PA-hTERT cells are a mixed population of secretory epithelial cells and neoplastic myoepithelial cells similar to those of PA *in vivo*. These findings indicate that PA-hTERT cells, immortalized by transfection with hTERT, maintain their original phenotypic characteristics.

Overall, these findings suggest that these immortalized PA-hTERT cells will be useful for analyzing the mechanisms that regulate the differentiation of PA.

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