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DOI: <https://doi.org/10.1159/000363694>

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Journal Article

Originally published at:

Bao, Kai; Akguel, Baki; Bostanci, Nagihan (2014). Establishment and Characterization of Immortalized Gingival Epithelial and Fibroblastic Cell Lines for the Development of Organotypic Cultures. *Cells, Tissues, Organs*, 199(4):228-237.

DOI: <https://doi.org/10.1159/000363694>

Establishment and characterization of immortalized gingival epithelial and fibroblastic cell lines for development of organotypic cultures

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Running title: Gingival organotypic tissue

Key words: gingival epithelium; organotypic tissue; gingival fibroblast; human papillomavirus type 16.

Abstract:

In vitro studies using three-dimensional (3D) co-cultures of gingival cells can resemble their *in vivo* counterparts much better than 2D models that typically only utilize monolayer cultures with short living primary cells. However, the use of 3D gingival models is still limited by lack of appropriate cell lines. We aimed to establish immortalized cell line models of primary human gingival epithelium keratinocyte (HGEK) and gingival fibroblast (GFB) cells. Immortalized cell lines (HGEK-16 and GFB-16) were induced by E6 and E7 oncoproteins of human papillomavirus. In addition, 3D multilayered organotypic cultures were formed by embedding GFB-16 cells within a collagen matrix and seeded HGEK-16 cells on the upper surfaces. Cell growth analyses were subjected to both immortalized cell lines and their parental primary cells. The expressions of cell type specific markers (i.e.: cytokeratins (CK)10, CK13, CK16, CK 18, CK19 for HGEK-16 and collagen (Col) I, Col II for GFB-16) were evaluated by quantitative real-time PCR. Expansion of the primary cultures was impeded at early passages, while the transformed immortalized cell lines could be expanded for more than 30 passages. In 3D cultures, immortalized HGEK formed a multilayer of epithelial cells. qRT-PCR showed that the expressions of cell-specific markers in the 3D cultures were qualitatively and quantitatively closer to those in human gingival tissues than to monolayer cultures. These results indicate that immortalized gingival fibroblastic and epithelial cell lines can successfully form organotypic multi-layered cultures and therefore have potential to be useful tools for studying gingival tissue *in vitro*.

Introduction:

Gingival tissue comprises the superficial oral epithelium and underlying connective tissue. These tissues are the first locations to be affected by biofilms as well as the initiation sites for inflammatory processes. They are therefore recognized as the places for initiation of periodontal diseases, a widespread group of destructive oral infection-driven inflammatory diseases that affect 48.2% of the USA population aged ≥ 30 years [Albandar, 2011].

Based on structural and functional differences, gingival epithelial tissue has been classified as junctional epithelium, oral sulcular epithelium, and oral gingival epithelium. Oral junctional epithelium connects the tooth to the sub-epithelial connective tissues and sulcular epithelium lines the lateral wall of the gingival sulcus. Besides its supporting functions, the permeability of the epithelium allows emigration of granulocytes to the biofilm surface but also allows microorganisms to migrate from the biofilm [Sandros et al., 1994], which is believed to play an important role during the process of periodontal infection. In the clinic, the appearance of a periodontal pocket, an abnormal depth of the gingival sulcus, is considered to be an indicator for periodontal infection. Interestingly, disappearance of the junctional epithelium and ulceration of sulcular epithelium during the formation of the periodontal pocket has been observed [Nanci and Bosshardt, 2006], which means that gingiva is not able to form tight connections after the disease. Therefore, it is of importance to understand how healthy non-keratinized sulcular and junctional epithelium is breached by the oral biofilms; what changes occur to these structures during disease processing; and if the molecular mechanism of pristine status recover after the clinical improvements [Belibasakis et al., 2011]. In progressive periodontal diseases where the epithelium is ulcerated, the gingival connective tissues (mainly fibroblasts) are exposed to the bacterial biofilm directly. As connective tissues also influence the formation of gingival epithelia [Karring et al., 1975; Smola et al., 1998], concomitant studies using both epithelial and connecting tissues are crucial for understanding of the pathogenesis of periodontal diseases [MacNeil, 2007].

In vitro studies on gingival tissues have mainly been employed in monolayer cultures. Due to a lack of normal differentiation, gingival cells grown under these conditions

are deficient in differentiation-dependent cell polarization and cell–cell contacts [Radyuk et al., 2003]. Thus, the use of organotypic cell culture models that mimic the morphological and functional features of their *in vivo* counterparts is highly desirable despite their methodological complexity. One of the main methodological problems of establishing such 3D cultures is that primary cells have short life spans, as they only replicate for a few passages and therefore do not provide sufficient cell numbers for further use in organotypic cultures [Sacks, 1996; Pi et al., 2007]. To overcome this problem, previous work has made use of the E6/E7 oncoproteins from human papillomavirus type 16 (HPV16-E6/E7) to immortalize epithelial cells [Yeh et al., 2013].

In the present study, we used primary human gingival epithelial keratinocytes (HGEK) and human gingival fibroblasts (GFB) and aimed first to immortalize them through expression of oncogene HPV16-E6/E7. In addition, we aimed to establish a standardized organotypic gingival tissue model for mimicking oral junctional epithelial interface *in vitro*, using both immortalized gingival epithelial cells and gingival connective tissue. The characteristics of the established epithelia and fibroblasts in a 2D monolayer and organotypic culture were determined by histology and cell differentiation through quantification of cytokeratin expression and collagen expression using quantitative real time polymerase chain reaction (qRT-PCR).

Materials and Methods:

Primary cultures

GFBs were collected from clinically healthy gingiva as previously described [Belibasakis et al., 2005] and HGEKs were extracted following a published protocol [Guggenheim et al., 2009]. The ethical approval was granted from the Ethical Committee of the Dental Institute Board (StV Nr.08/14). The study was conducted according to the guidelines of the Declaration of Helsinki. Written and informed consent was obtained from each patient before enrolment in the study. HGEKs were cultivated and passaged in defined keratinocyte serum free medium (DKSFM, Gibco), supplemented with 100U/ml penicillin (Sigma), 100µg/ml streptomycin (Sigma), 2 mM L-glutamine (Sigma), and 0.25µg/ml fungizone (Sigma). GFBs were cultivated and passaged in Dulbecco's modified Eagle medium: nutrient mixture F-12

(DMEM/F-12) media (Sigma), supplemented with 1.2g/ml of Na-Bicarbonate (Sigma), 10 % foetal calf serum (FCS, Sigma), 100 U/ml penicillin (Sigma), 100µg/ml streptomycin (Sigma), 2mM L-glutamine (Sigma), and 0.05µg/ml fungizone (Sigma). Cell cultures were incubated at 37°C in a humidified atmosphere of 5% CO₂ in air. The medium was changed every two days. When the primary cell cultures reached confluence, cells were dissociated with trypsin/EDTA solution (Gibco, Life Technologies) just before transferring for experiments. Cell numbers were determined under light microscopy using hemocytometer. Population doubling was calculated as log₂ (cell number at subculture/cell number plated) for growth kinetics determination.

Immortalization and maintenance of cultures

For immortalization, gingival cells were retrovirally transduced with virus coding either for pLXSN empty vector control (pLXSN) or HPV16-E6E7 (pLXSN-HPV16-E6E7) as described previously [Akgul et al., 2010]. Briefly, primary cells were seeded at a cell density of 2×10^5 cells/cm² in 6cm dishes. Retroviral supernatants were mixed with an equal volume of DMEM in the presence of 5µg/ml of hexadimethrine bromide (Polybrene, Sigma) and added to the keratinocytes. Spin-infection was made by centrifugation at 300x g for 1h; the cells were then washed with PBS and cultured in corresponding fresh medium. After two days, cells were selected with G418 (Sigma-Aldrich) (100µg/ml for keratinocytes and 500µg/ml for fibroblasts) for seven days after which only infected cells survived. The use of pooled stable cell populations minimizes possible variations due to randomness of the viral integration site in the cellular chromosomes. Established HGEK-16 or GFB-16 cells were seeded at 3,333 cells/cm² cell and incubated at 37°C in a humidified atmosphere of 5% CO₂ in air for passaging. HGEK-16 cells were cultivated in DKSFM medium supplement with 100µg/ml G418. GFB-16 cells were cultivated in DF12 medium supplement with 250µg/ml G418.

Construction of 3D organotypic gingiva model

The method used to develop a 3D organotypic gingival model is a modification of previously reported models [Dongari-Bagtzoglou and Kashleva, 2006] with major changes described below. Briefly, the 3D organotypic gingiva models were constructed inside 13mm diameter Millicell cell culture inserts (Millipore) in 12-well

plates. Collagen mixtures were composed of DMEM (Sigma) medium, 10% FCS, 2mM L-glutamine, 0.22mg/ml sodium bicarbonate and 0.20mM HEPES 2.5mg/ml rat-tail collagen I. 0.2 ml of this collagen mixture was poured into each insert and allowed to gelify in the sterile hood to form acellular layers. 0.5 ml Collagen matrix containing 7.5×10^5 GFB-16 cells were later gelled above the acellular layers. The medium used to grow fibroblasts was then added to cover each gel for incubation at 37°C in a humidified atmosphere with 5% CO₂ until the collagen contracted (normally takes one or two days). The medium was then moved and the gels were plated with 1×10^6 HGEK-16 cells. The HGEK-16 cells were allowed to attach for two days in mixture of HGEK-16 and GFB-16 growth medium in a ratio of 1:1 with additional 10µg/ml epithelial growth factors. Then, inserts were placed onto sterile cottons to expose the gels to the air but still touched the medium. The medium used for this air-liquid interface was DMEM (4.5mg/ml glucose) and Ham's F-12 mixed with ratio of 3:1 containing 5µg/ml insulin, 0.4µg/ml hydrocortisone, 2×10^{-11} M 3,3',5-triiodo-L-thyronine (T3), 1.8×10^{-4} M adenine, 5µg/ml transferrin, 10^{-10} M cholera toxin, 2mM L-glutamine, 100U/ml penicillin, 100µg/ml streptomycin, 0.05µg/ml fungizone, 100µg/ml G418 antibiotic and 5% FBS. Cultures were maintained in air-liquid interface for 14 days with the medium changed every 2 days.

RNA extraction and cDNA synthesis

For RNA extractions from monolayer cultures, the cells were dissociated with 1x trypsin/EDTA and collected by centrifugation. Organotypic cultures were first washed with PBS, then resuspended in 600µl RLT buffer (Qiagen) and disintegrated at 25,000 rpm in Mini-Beadbeater-1 disrupter (BioSpec) with ice cooling between cycles and collected by centrifuge at 3,700rpm for 1min at 4°C. The supernatants were aliquoted into a clean tube and placed on ice for later usage. The pellets were resuspended with 590µl RNase-free water and incubated with 20µl proteinase K solution at 55°C for 5min. Mixtures were then centrifuged at 10,000rpm for 3min at 4°C and supernatants were combined with previous ones on ice for RNA extraction. Cell lysates from all strains were kept and extracted using the RNeasy Mini Kits (Qiagen) following the instructions of manufacturer. Clinically healthy gingiva for RNA extraction was obtained as previously described [Bostanci et al., 2009]. Briefly, gingival tissue biopsies, including both epithelium and connective tissue, were obtained from periodontally healthy subjects (n=3) during tooth extractions for

orthodontic reasons or crown-lengthening procedures. The gingival tissue biopsies were immersed in RNA stabilization reagent (RNA Later, Ambion, CA) and then frozen at -80°C. Total RNA was extracted using the AllPrep Kit (Qiagen, Basel, Switzerland). Moloney Murine Leukemia Virus Reverse Transcriptase (Promega) were used to synthesis cDNA following the instruction of the manufacturer.

Quantitative real-time polymerase chain reaction (qRT-PCR)

For characterization of gene expression in both 2D and 3D cultures, cytokeratin (CK) 10, CK13, CK14, CK16, CK 18, CK19, Collagen type I (Col I) and Collagen type II (Col II) were amplified using StepOnePlus™ Real-Time PCR Systems (Applied Biosystems) following standard instruction of the manufacture and data was analyzed by provided software. Oligonucleotide primer sequences are listed in Table 1. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as endogenous RNA control in the samples (house-keeping gene). The SYBR Green PCR Master Mix (Applied Biosystems) was used for the amplification reactions.

3D model treatments for immunohistology

Samples were rinsed twice with PBS for 5min, and then transferred into 3% paraformaldehyde+2% sucrose/PBS fixation medium for 2h. The samples were rinsed thrice with PBS, 20min each; dehydrated by immersion in 15% Sucrose/PBS for 2h at room temperature; then left in 30% Sucrose/PBS overnight at 4°C. The next day, samples were placed in 5ml of 5% (w/w) porcine-derived gelatine (Sigma, St. Louis, MO) solution with 5%(w/w) sucrose that was prewarmed to 45°C in models. The whole models were transferred in a cool 2-methylbutane (isopentane) and dry-ice bath at around -40°C until they were frozen. Specimens were stored at -80°C and sectioned at 8µm thickness using a HYRAX C 50 cryostat (Carl Zeiss).

Histological Staining and Image Composition

Cryosection slides from -80°C were dried at room temperature for 30min ~ 2h and then fixed in acetone (4°C) for 15min. After washing thrice with PBS, the specimens were blocked with 1% bovine serum albumin/ PBS for 10min. The specimens were then incubated with 4µg/ml mouse monoclonal keratin 14 Ab-1 (Clone LL002, Neomarker) or 4µg/ml Keratin 16 Ab-1 (CloneLL025, Neomarker) at 4°C for

overnight. After removal of the primary antibody, the Alexa Fluor® 488 Goat Anti-Mouse IgG (H+L) Antibody was applied to the samples and incubated for 45 min at room temperature. DAPI was later applied on the samples as a counterstain.

Statistical evaluation

The qRT-PCR analysis results for the expression levels of different transcripts in each sample were calculated by the comparative Ct method ($2^{-\Delta Ct}$ formula) after normalization to endogenous control GAPDH (i. e.: $\Delta Ct = Ct_{\text{target gene}} - Ct_{\text{GAPDH}}$). All data were expressed as the mean $\pm$ standard deviation (SD) for all of the determinations. Significance was calculated from log values of ΔCt between different conditions. Unpaired t-tests were used to test the differences between immortalized cell lines with their parent primary cells. Multiple comparison tests following one-way ANOVA were used to test the differences between 3D organotypic gingiva models, clinical samples and 2D monolayer cultures.

Results:

Characterization of immortalized human gingival epithelial and fibroblastic cell lines in 2D monolayer cultures

Immortalized cell lines show similar cell morphologies and growth kinetics with their parental cells in monolayer cultures.

Primary human gingival epithelial keratinocytes and gingival fibroblasts exhibited distinct morphologies under the light microscope (fig. 1). Keratinocytes exhibited characteristic epithelial cobblestone morphologies (fig. 1C-D) while fibroblasts exhibited typical spindle-shaped fibroblast morphologies (fig. 1 A-B). HPV16 immortalized clones of both keratinocytes and fibroblasts showed similar morphologies with their parental cells at early passages, although primary cells were more heterogeneous in their morphology.

The subculture time of primary gingival keratinocytes and fibroblasts remained similar in early passages but cells proliferated more slowly and became senescent at 4 and 18 population doublings respectively. In comparison, immortalized cells showed continuous proliferation and expansion over 30 population doublings whilst retaining

a similar cobblestone or spindle-shape phenotype. The growth behavior of the immortalized cell lines remained constant over time and required sub-cultivating every 4-5 days (fig. 1E).

Immortalized cell lines express stable levels of HPV16 E6/E7.

To test for the presence and expression levels of the HPV16 E6/E7, the mRNAs in HGEK-16 and GFB-16 were extracted from cells in different passages and examined by qRT-PCR (fig. 2). The analysis confirmed oncogene expression in all HPV16-E6/E7 transduced cells at all detected passages at a similar level. No oncogene expression was detected in the corresponding parental primary cells (data not shown).

Immortalized cell lines in monolayer cultures express similar levels of cell specific markers when compared with their parental cells.

Since CKs are the main intermediate filaments of gingival epithelia, the mRNA levels of selected CKs were examined as differentiation markers. Therefore, CK10, CK13, CK14, CK16, CK18 and CK19 were tested in primary HGEK and HGEK-16 cells by qRT-PCR (fig. 3 A). Essentially, HGEK and HGEK-16 cells all expressed these CKs although slight effects on CK expression by HPV16-E6/E7 were noted. Both cell types expressed low levels of CK10, CK13 and high levels of CK14, CK16, CK18 and CK19 (fig. 3A). Immunohistochemical analysis also confirmed strong expressions of CK14 in HGEK-16 in monolayer cultures (fig. 4C). As a marker of cornifying epithelial, CK10 was expressed at low level when compared to CK14, the marker of stratifying epithelium, which was expressed at highest levels. The marker of non-cornified epithelia, CK13, was presented but at lower levels. Magnitudes of gene expression of the selected CKs were compared between HGEK and HGEK-16. CK10 and CK13 were expressed at slightly higher levels in HGEK-16 (0.45-folds, $p=0.0237$; 3.60-folds, $p=0.0399$, respectively) compared with HGEK. In contrast, HGEK-16 expressed lower levels of CK14, CK16, CK18 and CK19 (7.44-folds, $p<0.00001$; 5.62-folds, $p=0.0002$; 1.58-folds, $p=0.0087$ and 40.13-folds, $p<0.00001$, respectively) compared with HGEK.

In addition, expression levels of extracellular matrix-related genes (i.e. collagen I (Col I) and collagen II (Col II)) were examined for GFB and GFB-16. Both fibroblast cell

types demonstrated strong expression of Col I and Col II (fig 3 B) with Col I expression in GFB-16 being slightly lower (1.62-folds, $p=0.024$) than in GFB.

Behavior of immortalized human gingival epithelial and fibroblastic cells in 3D organotypic cultures

3D organotypic cell cultures showed a multilayer structure.

GFB-16 and HGEK-16 cells were grown to confluence in monolayer cultures and then used to construct 3D organotypic cultures. Figure 4A shows a representative section of this model stained with haematoxylin. HGEK-16 cells formed a multilayered regenerated epithelium with no obvious keratinizing superficial layer on the surface, which is similar to the non-keratinized oral epithelia such as junctional epithelium *in vivo*. The HGEK-16 cells formed multilayers of epithelia and GFB-16 cells fibroblasts were evenly distributed in the collagen gel matrix. Like in monolayer cultures (fig. 4C), HGEK-16 epithelium in 3D organotypic cultures revealed strong CK14 staining (fig. 4B).

The gene expressions levels of cytokeratins and collagens in 3D organotypic cultures were closer to the levels in healthy gingiva than in 2D monolayer cultures.

One of the key differences between *in vitro* and *in vivo* epithelia is that they express epithelial markers differently [Locke et al., 2008]. We expected the CK expression levels in organotypic cultures are closer to the levels found *in vivo* in human tissue than in monolayer culture. Therefore, CK expression in organotypic cultures, healthy gingival tissues and monolayer cultures of HGEK-16 were compared using qRT-PCR. In addition, mRNA expression levels of Col I and Col II were compared between organotypic cultures, clinical samples and GFB-16 cells grown as monolayers.

qRT-PCR analysis showed that the expression levels of CKs in HGEK-16 organotypic cultures are closer to those in gingival tissues than to monolayer cell cultures (fig. 5A, 5B). Especially for CK19, 3D models expression level is 135.91-folds higher than the immortalized cell, which has no significant differences to the level in gingival tissues models (2.50-folds, $p > 0.05$). The levels of CK10, CK13 and CK16 in gingival tissues were significantly higher (465.21-folds, $p < 0.00001$; 1658.49-folds, $p < 0.00001$; 32.07-folds, $p < 0.00001$ for CK10, CK13 and CK16,

respectively) than the levels in the 3D models. However, these values are significantly less than the difference between gingival tissues (2674.31-folds, $p < 0.00001$; 1,785,091.51-folds, $p < 0.00001$; 287.61-folds, $p < 0.00001$ for CK10, CK13 and CK16, respectively) with HGEK-16 grown as monolayers.

The expressions of CK14 and CK18 in 3D models showed no significant differences with monolayer parallel cultures (fig.5 B). Both 3D models and HGEK-16 monolayers expressed significantly lower levels of CK14 compared with gingival tissues (0.027-folds, $P < 0.00001$; 0.032-folds, $P < 0.00001$ for HGEK-16s and 3D models compared with gingival tissue, respectively); while for CK18, HGEK-16 monolayer cells, 3D cultures, and gingival tissues showed no significant difference on expression. Expression for Col I and Col II were found to be significant higher in GFB-16 monolayer (14.49-folds, $P < 0.0001$ and 8.29-folds, $P < 0.00001$ for Col I and Col II, respectively) than the levels in gingival tissue (fig.5 C). However, expression of both collagen genes showed no significant differences between gingival tissues and the 3D model.

Discussion:

To decipher the pathologies of periodontal diseases, it is important to establish *in vitro* models that mimic the mechanisms taking place within gingival tissues. In the present study, we established gingival epithelial and fibroblastic cell lines from human gingiva. Both epithelial and fibroblastic cell lines demonstrated a stable infinite growth with epithelium-like morphology and spindle fibroblastic-shape morphology respectively. Additionally, 3D multilayered organotypic culture was formed by use of embedded fibroblasts in a collagen gel.

It has been long known that connective tissues underlying epithelia not only support the growth but also regulate differentiation of the epithelia [Karring et al., 1975]. Therefore, such organotypic cultures should consist of both epithelium and connective tissues. For the purpose of generating such a model, we developed and characterized an immortalized human gingival epithelial cell line (HGEK-16) and gingival fibroblast cell line (GFB-16) through the expression of HPV16 E6/E7 oncogenes. Stable expression levels of E6 and E7 throughout many passages confirmed successful immortalization. In our study, no morphology difference was observed

between primary cultures and immortalized cell lines even after 50 and 35 passages for HGEK-16 and GFB-16, respectively. Earlier studies have reported immortalized human gingival fibroblasts by gene transfection of human telomerase reverse transcriptase (hTERT), [Kamata et al., 2004; Illeperuma et al., 2012], although other studies have shown that co-expression of hTERT with other immortalizing genes i.e. HPV16 E6/E7 was required for efficiency of immortalization of oral epithelial cells [Kamata et al., 2004].

The HGEK-16 cells had characteristic ‘cobblestone’ appearance of oral epithelial cells, while GFB-16 cells exhibited spindle-shape morphology as their primary counterparts. The qRT-PCR analysis analyses showed that, like primary gingival fibroblasts, GFB-16 cells express highly abundant levels of collagen type I. Regarding the expression of collagen type II in the present experimental system, while its expression is not a typical feature of the gingival mucosa, there is evidence that under certain conditions gingival fibroblasts can express this and have potential to differentiate to chondrocyte-like cells [Yeh et al., 2013].

During periodontal infection, the junctional epithelium is exposed to a biofilm, and this tissue is therefore the subject of intense study into this disease [Bosshardt and Lang, 2005]. Several earlier studies have reported that gingival epithelial cells can form a multilayered epithelium when co-cultured with fibroblastic cells within 3D matrices allowing more direct comparisons to *in vivo* models [Dongari-Bagtzoglou and Kashleva, 2006; Roesch-Ely et al., 2006]. Indeed, when the transformed HGEK cells were co-cultured in a collagen matrix along with HGF, their characteristics were qualitatively and quantitatively closer to those in human gingival tissues than to monolayer cultures. One of the drawbacks of utilizing immortalized cell lines in organotypic cultures is that these cells may show carcinoma-like invasion patterns into the underlying connective tissues especially in the presence of fibroblast collagen matrixes [Costea et al., 2006]. Fortunately, in our model, HGEK-16 showed no invasive trend into the fibroblast/collagen matrix, when both cell lines were used to concomitantly construct an organotypic tissue.

Within the gingiva, the expression patterns of various CKs have been used as molecular indicators for different oral gingival epithelium regions. Previous studies

showed varying expression levels between keratinized region, the non-keratinized oral junctional epithelium and sulcular epithelium [Bragulla and Homberger, 2009; Hsieh et al., 2010]. Therefore, CK10, CK13, CK14, CK 16, CK18 and CK19 were chosen as cell specific markers [Locke et al., 2008]. CK19 is considered as a marker for early differentiation stages of stratified epithelia [Groger et al., 2008], and is mostly found in simple epithelia, such as the junctional epithelium [Mackenzie and Gao, 1993]. Therefore, increased expression of CK19 in the present 3D organotypic tissue model is a common feature of junctional epithelium. CK10 and CK16 are known to be largely expressed in cornifying stratified epithelia [Coulombe and Omary, 2002], as well as in proliferating epithelia [Freedberg et al., 2001]. Thus, healthy gingival tissue samples, which contain mainly keratinized epithelia, expressed very high levels of these keratins. In contrast, the expression of these two CKs was significantly lower in the present organotypic tissue compare to patient. As these are considered as essential for terminal differentiation of keratinized epithelia, low expression of CK10 and CK16 in our model confirms that the formed multilayered epithelia is rather non-keratinized.

In conclusion, the immortalized cell lines generally inherited the characteristics of their parental primary tissue cells. When cultured in 3D collagen matrix, these immortalized cell lines resembled their *in vivo* counterpart more closely than the corresponding monolayer cultures. These results indicate that our new cell lines have potential to be useful tools for studying the physiology and pathobiology of gingival tissue *in vitro*. Moreover, this model could present a practical model for studies of biofilm-gingival tissue interactions *in vitro* [Belibasakis et al., 2013; Belibasakis and Thurnheer, 2013]. The development of this model is not necessarily restricted to the gingiva, but could potentially be expanded with adequate modifications for applications on the respiratory or gastrointestinal tract mucosa, or the skin.

Acknowledgements

The authors would like to thank Dr. Andrew F Irvine for English corrections.

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Tables:

Table 1. List of primers used to check expressions of cell specific markers at the mRNA level.

Name	Sequence: (5' to 3')	Size	Tm (°C)
CK10 Forward primer	CTGACTGAAGAGCTGGCCTA	20	62.3
CK10 Reverse primer	TTCCACATTACATCACCAG	20	62.1
CK13 Forward primer	ATGCTGCTGGACATCAAGAC	20	63.0
CK13 Reverse primer	TGGTAACAGAGGTGCTACGG	20	62.7
CK14 Forward primer	TGCGATCCAGAGGAGAACTG	20	65.6
CK14 Reverse primer	CAGGAGATGATTGGCAGCGT	20	67.6
CK16 Forward primer	GCCAATCCTATTCTTCCCGC	20	66.6
CK16 Reverse primer	GGGGAGATAGCTGGGAAGT	20	64.8
CK18 Forward primer	ATCTTGGTGATGCCTTGGAC	20	63.9
CK18 Reverse primer	CCTGCTTCTGCTGGCTTAAT	20	63.3
CK19 Forward primer	GACATGCGAAGCCAATATGA	20	63.6
CK19 Reverse primer	TCAGTAACCTCGGACCTGCT	20	63.8

GAPDH Forward primer	CAGCCTCCCGCTTCGCTCTC	20	72.8
GAPDH Reverse primer	CCAGCGCCCAATACGACCA	20	74.2
HPV-16 E6 Forward Primer	TGTTTCAGGACCCACAGGA	19	64.4
HPV-16 E6 Reverse Primer	TTGTTTGACAGCTCTGTGCAT	20	64.7
HPV-16 E7 Forward Primer	CTGTTATGAGCAATTAAATGACAGC	25	62.5
HPV-16 E7 Reverse Primer	CCAGCTGGACCATCTATTCA	21	63.9
Col I Forward primer	AAGATGGACTCAACGGTCTC	20	60.7
Col I Reverse primer	CAGGAAGCTGAAGTCGAAAC	20	61.6
Col II Forward primer	ACATCACCTACCACTGCAAG	20	60.2
Col II Reverse primer	TACGTGAACCTGCTATTGCC	20	62.6

Supplement Table 1. The mRNA Expressions of cell markers (average $\pm$ SD) normalized with a housekeeping gene GAPDH.

	HGEK	HGEK-16	GFB	GFB-16	3D model	Gingival tissue
CK10	1.38E-02 $\pm$ 7.26E-04	3.05E-02 $\pm$ 1.89E-02	-	-	1.75E-01 $\pm$ 8.51E-03	8.15E+01 $\pm$ 3.59E+01
CK13	2.17E-03 $\pm$ 1.31E-03	6.03E-04 $\pm$ 9.61E-04	-	-	6.49E-01 $\pm$ 4.75E-02	1.08E+03 $\pm$ 5.07E+02
CK14	2.50E+03 $\pm$ 5.33E+02	3.36E+02 $\pm$ 1.39E+02	-	-	3.98E+02 $\pm$ 2.59E+01	1.22E+04 $\pm$ 4.39E+03
CK16	7.07E+01 $\pm$ 2.15E+00	1.26E+01 $\pm$ 8.97E+00	-	-	1.13E+02 $\pm$ 1.48E+00	3.62E+02 $\pm$ 1.09E+03
CK18	4.45E+01 $\pm$ 1.55E+01	2.81E+01 $\pm$ 7.12E+00	-	-	2.99E+01 $\pm$ 3.65E+00	2.50E+01 $\pm$ 5.70E+00
CK19	2.91E+00 $\pm$ 4.25E-01	7.26E-02 $\pm$ 6.99E-02	-	-	9.87E+00 $\pm$ 1.48E+00	2.47E+01 $\pm$ 2.36E+01
Col I	-	-	2.98E+02 $\pm$ 7.19E+00	1.52E+02 $\pm$ 8.55E+01	2.16E+01 $\pm$ 1.38E+00	1.05E+01 $\pm$ 3.90E+00
Col II	-	-	4.40E+01 $\pm$ 8.89E+00	2.43E+01 $\pm$ 1.13E+01	2.12E+00 $\pm$ 4.01E-1	2.69E+00 $\pm$ 1.24E+00

Figure Legends

Fig. 1. Cell morphologies and growth kinetics. Phase-contrast microphotography for primary human gingival fibroblast (GFB) (A), immortalized gingival fibroblast (GFB-16) (B), primary human gingival epithelial keratinocyte (HGEK) (C) and immortalized human gingival epithelial keratinocyte (HGEK-16) (D), at the passage 2,13,2,16, respectively. Growth kinetics of HGEK-16, GFB-16, HGEK and GFB (E), cells were grown in culture and doubling times were assessed using a haematocytometer. Data were presented till 74 days until GFB cells became senescent. Each dot on the curve represents for the day that subculture the cell lines. The passages were started to present at passage 2, 6, 8, 7 for HGEK, GFB, HGEK-16,

and GFB-16, respectively.

Fig. 2. Expressions of the HPV-16 E6/E7 in immortalized cell lines from different passages. The mRNA expression levels of HPV-16 E6 (A) and E7 (B) were normalized with a housekeeping gene GAPDH. The open cubic box shows the expression levels from HGEK-16 cell line, close round box shows the expression levels from GFB-16.

Fig. 3. Expressions of differentiation markers in primary and immortalized cells from different passages. (A) The values of CK10, CK13, CK14, CK16, CK18 and CK19 (A) represent mean values of 3 or 18 passages respectively for primary epithelial cells (HGEK) or immortalized epithelial cells (HGEK-16) $\pm$ SD as described in materials and methods. The expressions of Collagen I (Col I) and collagen II (Col II) (B) were compared between primary (GFB) or immortalized cells (GFB-16). These values represent mean values of 4 or 10 passages respectively for GFB or GFB-16 $\pm$ SD (Table S1) as described in materials and methods. For a better understanding of data, results were presented on a logarithmic scale. For each marker, mRNA levels were normalized with a housekeeping gene GAPDH. Asterisks correspond to P- value of primary cells versus immortalized cells. *P < 0.05; **P < 0.001; ***P < 0.0001, ****P < 0.00001

Fig. 4. Cell organizations within the 3Dmodel after 14 d in airlifting medium. Light microscopy image of haematoxylin stained 3D tissue (A) show the structures of epithelial and fibroblast cells. Cryostat sections of 3D tissue (B) and monolayer culture (C) using cytokeratin 14 [Yin et al.] and counterstaining with DAPI (blue) distinguished the HGEK-16 cells.

Fig. 5. Expressions of cell markers between immortalised cells, 3D models and healthy gingival tissue samples. The values of CK10, CK13, CK14, CK16, CK18 and CK19 (A, B) represent mean values of 18 passage respectively for immortalized epithelial cells (HGEK-16) or 3 individual samples for 3D models and clinical healthy gingival tissue samples (Gingival tissues) $\pm$ SD as described in materials and methods. The expressions of Collagen I (Col I) and collagen II (Col II) were also shown between these conditions. These values represent mean values of 10 passages

for immortalized cells (GFB-16) or 3 individual samples for 3D models and healthy samples $\pm$ SD (Table S1) as described in materials and methods. For a better understanding of data, results were presented on a logarithmic scale. For each marker, mRNA levels were normalized with a housekeeping gene GAPDH. Asterisks correspond to P-value of primary cells versus immortalized cells. *P < 0.05; **P < 0.001; ***P < 0.0001, ****P < 0.00001.

Abbreviations used in this paper

CK	cytokeratin
Col	collagen
3D	three dimensions
DKSFM	defined keratinocyte serum free medium
DMEM	Dulbecco's modified Eagle medium
DMEM/F-12	Dulbecco's modified Eagle medium: nutrient mixture F-12
FCS	foetal calf serum
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GFB	primary Gingival fibroblast
GFB-16	immortalized gingival fibroblast cell lines
HGEK	primary human gingival epithelium keratinocyte
HGEK-16	immortalized human gingival epithelial cell lines
HPV-16 E6/E7	E6 and E7 oncoproteins of human papillomavirus
hTERT	human telomerase reverse transcriptase
SD	standard deviation
qRT-PCR	quantitative real time polymerase chain reaction

Fig. 1.

A

B

C

D

E

Fig. 2.

Fig. 3.

Fig. 4.
A

B

C

Fig. 5