In vitro reconstruction of human junctional and sulcular epithelium

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Abstract

BACKGROUND: The aim of this study was to develop and characterize standardized *in vitro* three-dimensional organotypic models of human junctional epithelium (JE) and sulcular epithelium (SE).

METHODS: Organotypic models were constructed by growing human normal gingival keratinocytes on top of collagen matrices populated with gingival fibroblasts (GF) or periodontal ligament fibroblasts (PLF). The tissues obtained were harvested at different time points and assessed for epithelial morphology, proliferation (Ki67) and differentiation profile (a panel of cytokeratins - CK, transglutaminase and filaggrin).

RESULTS: The epithelial component in 3 and 5 days organotypics showed limited differentiation and expressed Ki-67, CK 8, 13, 16, 19, and transglutaminase in a fashion closest to control JE samples. PLF supported better than GF higher expression of CK19 and suprabasal proliferation, although statistically significant from GF only at day 5. The rate of proliferating cells in basal layers decreased significantly in 7 and 9 days cultures as well as the percentage of CK19-expressing cells. Day 7 organotypics presented a higher number of cell layers, proliferating cells in suprabasal layers and the CK expression pattern similar to SE.

CONCLUSION: Both time in culture and type of fibroblast had impact on the epithelial phenotype. We suggest that 3 and 5 days cultures with PLF are suitable models for JE, 7 days cultures with PLF or GF as models for SE, while 9 days cultures with GF as models of GE. Such standard, reproducible models can be further used to study periodontal bacteria–host tissue interactions *in vitro*.

Introduction

Anatomically, gingival epithelium consists of oral gingival epithelium (GE), sulcular

epithelium (SE) and junctional epithelium (JE) (1). The JE mediates the attachment of gingiva

to the teeth. JE is multilayered epithelium with permeable structure, high turn orver (the

highest among oral tissues) and limited differentiation, lacking keratinization. The SE makes

the transition between JE and GE and lines the gingival sulcus. The distinctive structure and

function of these epithelia are also reflected in the expression of keratinocyte differentiation

markers such as cytokeratins (CK), filaggrin and transglutaminase (2-4). CK are major

structural proteins expressed by epithelial cells according with the stage of differentiation and

the origin of the epithelium (5-7). Transglutaminase (TG) is an enzyme that plays an

important role in the late differentiation of the mature keratinocyte (8), being expressed in oral

tissues in spinous layers both in orthokeratinized (such as the hard palate) and non-keratinized

(buccal mucosa) epithelia (9). Filaggrin is also a late marker of keratinization, present *in vivo*

in orthokeratinized and parakeratinized areas of human oral epithelium (10, 11).

The native GE was reported to express the cytokeratins 5 and 14 and occasionally CK19 in

the basal layers. Suprabasally were found CK 1 / 10 (characteristic for keratinized squamous

stratified epithelia), CK 6 / 16 (markers of hyperproliferation) as well as transglutaminase

(TG-I) and filaggrin (4, 10). JE has a unique phenotype, expressing cytokeratins specific for

simple epithelia, such as CK 7, 8, 18 and 19 and for basal layers (CK 5 and 14), but also for

non-keratinizing stratified epithelia, CK 13 and 16 (2, 12-14). While expression of CK 8 / 18

in JE may vary in frequency and intensity (2, 4), the presence of CK 19 is regarded as a

consistent marker for JE (13, 14). The SE is a non-keratinizing stratified squamous tissue

lacking stratum granulosum, found in GE (15). SE displays higher number of cell layers and

better differentiated than JE, but expresses CK 19, the marker of JE, to a lesser degree. The proliferation rate in SE is higher than in GE (16).

The SE and JE are continuously challenged by bacterial biofilm from subgingival plaque, thus they represent crucial sites with respect to initiation and development of periodontal diseases. Bacteria can invade through epithelial layers, elaborate bacterial byproducts and induce a local immune response which can lead to breakdown of periodontal tissues. Hence, *in vitro* studies investigating host-bacteria interactions mimicking periodontal conditions have more relevance to be performed by using culture models resembling JE or SE (17). *In vivo*, the phenotype of the epithelium and implicitly the CK expression is influenced by the interactions with the sub-epithelial mesenchyme and eventual pathological changes (such as inflammation, dysplasia or malignant transformation of epithelia) (18-20). Similarly, when oral tissues are reconstructed *in vitro*, as multilayered three-dimensional (3D) organotypic culture models, the CK expression of the epithelial component is strongly influenced by the type of underlying matrix and the origin of fibroblasts contained in it (5, 21, 22).

The aim of this study was thus to develop and characterize standardized models of junctional and sulcular human epithelium that can be used to study periodontal bacteria –host tissue interactions. For this purpose, the morphology, cell proliferation and CK profile of OT models reconstructed with primary human gingival keratinocytes on top of fibroblast populated collagen biomatrices were assessed at different stages of growth. The impact of two different types of oral fibroblasts (periodontal versus gingival) on epithelial differentiation and phenotype was evaluated. We have investigated which combination of type of fibroblasts and growth stage may result in a model that resembles best the junctional or sulcular epithelium. Such model may represent a functional tool for studying periodontal bacteria – host tissue interactions *in vitro*.

Materials and methods

Materials

Dulbecco's Modified Eagle's Medium (DMEM) was acquired from Sigma (St Louis, MO).
Serum-free keratinocyte medium (KSFM), human recombinant epidermal growth factor
(EGF), bovine pituitary extract (BPE), fetal bovine serum (FBS), L-glutamine, penicillin,
streptomycin, and amphotericin B were acquired from GibcoBRL (Grand Island, NY). Cell
culture flasks and plates were from Nunc (Napervile, IL) and center-well organ culture dishes
were from BD Biosciences (San Jose, CA). The Envision+ system used in
immunohistochemistry was from DAKO (Glostrup, Denmark). Antibodies used in this study
are presented in Table 1. Normal human gingival mucosa and specimens served as positive
controls for protein detection. Specimens incubated with antibody diluent only (DAKO,
Glostrup, Denmark) or isotype-matched antibody (DAKO) instead of primary antibody were
used as a negative control.

Cell isolation and culture

Clinically healthy adult volunteers (n=12) undergoing surgical removal of wisdom teeth were
informed about the purpose of study and gave their written consent regarding sample
collection. The study was approved by the Regional Committee for Medical Ethics in
Research. A total of 12 samples of gingival mucosa showing no sign of clinical inflammation
at collection time and seven wisdom teeth were used to generate primary keratinocytes and
fibroblasts. Samples of native normal tissues of JE (n = 6), SE (n = 6) and GE (n = 7) served
as controls for *in vivo* expression patterns of the investigated proteins. Prostate tissue samples
(n=3) were used as controls for simple CKs.

Gingival epithelial cells (GECs) were isolated as previously described through a combination of enzymatic digestion and mechanical separation of cells and cultured in serum-free media (KSFM) supplemented with 1 ng/ml EGF, 25 µg/ml BPE, 20 µg/ml L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B (23). We have previously published the protocol for the isolation and characterization of gingival fibroblasts (GF) and periodontal ligament fibroblasts (PLF) based on alkaline phosphatase (ALP) expression as marker of differentiation (24). Briefly, fibroblast cells were cultured in DMEM supplemented with 10% FBS, 20 µg/ml L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin. The cells used were GECs in their 1st to 3rd passage and GFs or PLFs in their 2nd to 5th passage. All cultures including the organotypic models were kept in a humidified atmosphere at 37°C and supplemented with 5% CO_2.

Construction and harvesting of OT tissue models

In our laboratory, the construction of an OT model of oral mucosa that reaches its maturity at day 9 of development after adding the keratinocytes on top of fibroblast-populated biomatrices has been well established and previously described (25). The multilayered epithelium was elaborated using GECs grown on top of collagen matrices populated with oral fibroblasts. In this study 250,000 fibroblast cells, either GF or PLF (in some cases matched from the same donor), were used for each collagen matrix. A number of 500,000 GECs in 1 ml KSFM were seeded on top of each collagen matrix the following day, considered day 1. The further handling of OTs was carried out as previously described, the tissues being placed on grids and lifted on the air-liquid interface. The harvesting of the OTs was done at different time intervals of their development: at day 3, 5, 7 and 9. Each tissue model was cut in two equal pieces, one half was snap frozen and kept at -80 °C and the other half was formalin-fixed and paraffin-embedded. Six different experiments with GF/ PLF were carried out.

Tissue preparation, staining and analysis

Sections of 5 μm thickness from formalin fixed OT tissues were cut and stained with

haematoxylin–eosin (HE) or used for immunohistochemistry (IHC). IHC staining was carried

out as routinely in our lab and previously described. Information on the primary antibodies

used in the stud is detailed in Table 1. All primary antibodies were applied for 60 min. TE-

buffer (10 mm Tris, 0.1 mm EDTA, pH 9) was used for all markers except for TG (where

citrate buffer pH 6 was used) and 60 min incubation. As positive controls were used tissue

samples of breast epithelium and prostate for markers CK 8 and 19, buccal mucosa for CK 13

and palate epithelium for CK 10, CK 16, transglutaminase and filaggrin.

The HE stained tissues were evaluated under a light microscope (Leica DMLM, GmbH,

Munster, Germany). Epithelial cell layers were counted and evaluated morphologically for

epithelial growth, degree of keratinization and differentiation pattern and compared with

control tissues. Exfoliated cells were not included in the counting of cell layers and the tissue

ends (margins) were also excluded (26, 27). Each tissue was examined with a 40 x objective

and images of three to six fields separated by 334 μm distance were taken with a camera

connected to a computer. By use of Olympus Soft-DP 5.0 software package (Soft Imaging

System GmbH, Münster, Germany) histomorphometric and immunohistochemical analyses

were performed on each image, which had a horizontal width of 334 μm at this magnification.

For quantification, the Ki-67 and CK19-expressing cells and also the negative cells were

counted manually at 400-fold magnification. The Ki-67 immunopositive cells were counted in

all layers in sections for OTs harvested at day 3 and starting from day 5 up they were counted

separately in basal/parabasal and suprabasal regions of the epithelial component. The

delimitation of layers in basal and suprabasal regions was made according to morphology of

the cells in the respective layers (higher ratio nucleus/cytoplasm, vertical or angular

orientation of cell axis on the basal membrane). Cell proliferation was calculated as

percentage of Ki-67 positive cells in the basal layers, but also as total number of Ki-67 expressing nuclei per 334 μm length. The number of proliferating nuclei in suprabasal layers was also registered. The CK 19 - expressing cells were calculated as the percentage of positive cells within all cell layers from day 3 to day 9.

The *in vitro* constructed models were compared at different development stages or related to the type of fibroblasts used in the matrix and also with the control tissues.

Statistical analysis and data presentation

Each individual experiment was run in duplicate or triplicate. The mean of CK 19 or Ki-67 expressing cells was calculated per experiment; and paired *t-test* was performed to compare the GF versus PLF tissues within the same experiment at each stage of differentiation. Data were analyzed for other parameters using analysis of variance (ANOVA) with Bonferroni multiple comparisons by means of the SPSS version 13 statistical software package (SPSS Inc, Chicago, Illinois). P-values less than 0.05 were considered significant.

Results

1. Histomorphometric evaluation of the epithelial compartment in reconstructed tissues

At day 3 of development the epithelium was comprised of 1-4 cell layers (37.73 ± 10.87 µm

thickness, mean and standard deviation) without any sign of differentiation in all OTs. At day

5, the epithelial component displayed 3-5 cell layers (49.79 ± 11.50 µm) and very little

differentiation, as only one superficial cell layer had a more flatten morphology and could be

considered as suprabasal. For both day 3 and day 5 OTs there was no noticeable difference

between OTs constructed using GF or PLF, as assessed by histological analysis of HE stained

tissues (Fig. 1).

At day 7 the OT models had an epithelial component of 9-13 layers (130.93 ± 29.28 µm),

increasing to 11-16 layers at day 9 (190.83 ± 16.78 µm). In all 7 and 9 days OT models the

differentiation was visible and the presence of the spinous layers could be noticed. No

significant difference in the thickness could be observed between OTs constructed with GF or

PLF at days 7 and 9 either. Variations from batch to batch could be observed regarding size of

the cells, total number of layers or degree of differentiation. However, some of the tissues

constructed with GF showed a well differentiated superficial cell layer compared with those

constructed with PLF at day 9, but such differences were not found consistently in all batches.

2. Distribution and quantification of proliferating cells (Ki-67)

In vivo, the cell proliferation rate is an important feature that differentiates JE, having the

highest cell turnover among all types of periodontal and oral tissues. Therefore we have

quantified the Ki-67 expressing cells in the reconstructed OT models for the purpose of

comparison. The distribution and number of Ki-67 positive cells varied in culture with the

time allowed for development and differentiation. Day 3 and 5 OT cultures showed

proliferating cells distributed in all cell layers, similarly with the situation encountered in JE

in vivo. In these OTs we found significantly higher proliferation rate ($p < 0.001$), in cultures

both with GF and PLF, compared to those harvested at later time points (day 7 and 9) (Fig

2A). The type of fibroblasts was not found to influence significantly ($p = 0.38$) the

proliferation rate within the basal and parabasal layers of organotypic models. Interestingly,

significant more Ki-67 positive cells were detected at day 5 in suprabasal layers ($p = 0.018$,

paired *t*-test) in tissues with PLF (2.34 ± 0.39, mean and standard error) than in those with GF

(1.28 ± 0.43). At day 7 fewer Ki-67 expressing nuclei were counted in suprabasal layers,

(1.03 ± 0.51 in GF OTs and 1.13 ± 0.59 in those with PLF, respectively; Fig. 2 B). At day 9,

no Ki-67 expressing cells were present in suprabasal layers.

3. Expression pattern of differentiation markers in reconstructed and control tissues

A summary of expression of the differentiation markers, both in reconstructed tissues and

controls, are presented in Table 2. The CK 13, 16 and 19 were consistently found expressed in

the epithelium of all OT cultures, constructed either with GF or with PLF. In OTs, the marker

for stratified squamous non-keratinized epithelia CK 13 was present throughout all epithelial

layers of day 3 and 5 culture (nearly 100% of cells stained positive). At days 7 and 9, the

expression of CK 13 became polarized, as all cells of suprabasal layers stained strongly for

this marker, while cells in basal layers were positive in a variable proportion from a few

scattered cells to about 50% positive. Slight variations were observed from batch to batch or

even within the same tissue model. The CK 13 staining in JE control tissue was positive in all

layers, in a proportion slightly varying between samples, from 85-100%. The SE control

tissues expressed CK 13 only in suprabasal layers and the staining was gradually lost towards

GE, where this marker was nearly absent.

At day 3, the OTs expressed CK 16 in the cells of the upper layers and sparsely in the basal cell layer, where only a few positive cells were found, matching with the expression of this marker determined in control tissues. In JE, CK 16 was found positive only in half of the samples where was expressed by circa 30 % of cells. OT tissues constructed both with GF or PLF from day 5 to day 9 stained positive for CK 16 only in the suprabasal layers. SE control tissues displayed CK 16 immunopositive cells in the spinous layer and sparsely in basal cells and the staining faded or disappeared towards JE. In GE control tissues we have found CK 16-positive cells in parabasal and suprabasal cells, much stronger stained in the spinous layer.

CK 19 was expressed in all OT reconstructed tissues, in all cell layers, stronger at 3 and 5 days of culture and slightly fading at days 7 and 9, especially in OTs constructed with GF. The percentage of CK 19 positive cells decreased gradually with the time allowed for maturation of the tissue cultures. In GF tissues, from 70.3 ± 2.83 % (mean and standard error) CK19-positive cells determined at day 3 and 67.91 ± 2.03 % at day 5, the percentage decreased further to 53.17 ± 2.65 % at day 7 and 31.24 ± 4.97 % at day 9.Time was found statistically significant by ANOVA test for repeated measurements ($p = 0.003$) as having the main effect for expression of this marker in OT culture. The OTs constructed with PLF expressed CK 19 in a higher percentage than those with GF, as $76.95\% \pm 0.87\%$ of the cells at day 3 stained positively and 72.81 ± 2.72 % at day 5, respectively. The proportion of CK 19 expressing cells decreased further, reaching 56.29 ± 2.04 % at day 7 and 35.45 ± 5.44 % at day 9 in PLF organotypics. Although the expression of CK 19 was maintained higher in PLF than in GF organotypics throughout all development stages, when these groups were compared by use of paired t-test, statistical significance was found only at day 5 ($p = 0.047$). *In vivo*, CK 19 was expressed intensely in all samples in all layers of JE, whilst in control SE the expression changed from intensely positive cells towards apical margin, until almost

disappearing towards the coronal region. In GE there were very few CK 19 positive cells, scattered in the basal layers in rete ridges (Fig. 3).

The marker for simple epithelia CK 8 was weakly positive in few of our OT cultures constructed with both GF and PLF, only at day 3 and not in cultures at day 5 or older. The control tissues did not stain positive for CK 8 either, but few single cells in the basal layers of rete ridges in GE were observed, as for CK 19. The positive control (prostate tissue) showed intense staining for CK 8.

In OT cultures grown to day 3 and 5, the marker for stratified keratinized epithelia CK 10 was absent. In samples harvested at day 7, CK10 positive cells were found sparsely in spinous layers, in circa 20% of the samples, in OT tissues constructed with both GF and PLF. The expression of CK 10 OTs was much stronger at day 9, in spinous and superficial layers, in cultures with both types of fibroblasts. This pattern was observed also in control tissues: absence of CK 10 in JE, very rare positive cells in SE (more towards gingival margin) and strong expression in spinous and superficial layers of GE.

Transglutaminase stabilizes the epithelium by cross-linking constituent proteins, thus it has been used as a marker for epidermal cell maturation. It was expressed in all layers of day 3 and 5 OT cultures, constructed with GF or PLF, whilst in days 7 and 9 it was present in suprabasal layers and seemed stronger in OT with GF. In control JE transglutaminase was found positive in the upper part of the tissue. In SE the staining faded towards apical margin but it was stronger in spinous layers towards gingival margin. Transglutaminase was expressed in spinous layers of GE control tissues.

The other marker of late keratinisation, filaggrin, was not found positive in any of OT cultures. This marker lacked as well from JE and SE control samples, but was found positive in upper layers of GE control tissues.

Discussion

In the present study, aiming to develop and characterize *in vitro* models for periodontal tissue, we found that depending on the time allowed for the model to mature, we can develop models that resemble junctional, sulcular or gingival epithelium by using gingival keratinocytes and periodontal and gingival fibroblasts.

Based on the analyses of tissue morphology and differentiation, correlated with the expression pattern of different markers and the decline of proliferating cells in the basal layers, the OT cultures could be grouped in 'early' (days 3 and 5) and 'late' (day 7 and 9) culture models. The 'early' OT cultures had low number of epithelial layers, none or very little differentiation (one suprabasal layer at day 5) and high number of proliferating cells distributed in all cell layers. The CKs (CK 19, 13, 16 and 8) and transglutaminase normally expressed by native JE (2, 4, 12, 14) were also determined in our 'early' OT models having a distribution pattern similar with the one encountered *in vivo*. The markers CK 10 and filaggrin normally lacking from JE (4, 10, 13, 21) were also lacking from day 3 and 5 OT models. Based on these findings, we suggest that the OT tissues grown for 3 or 5 days could serve as *in vitro* JE models. Particularly, the OTs grown with PLF showed a better preservation of the JE phenotype, reflected by higher percentage of CK 19 positive cells and proliferating cells in the suprabasal cell layers (Fig 4). We propose that day 7 OT cultures could be taken in consideration as a model for SE, due to a higher number of cell layers, limited differentiation, presence of proliferating cells in suprabasal layers and the expression pattern of different CKs similar to the SE. The OT model containing GF matured to day 9 may be used as culture model for GE, as we have already indicated in a previous study (28).

Both the time in culture and type of fibroblasts influenced the tissue morphology, proliferation and differentiation, but only time in culture had a significant impact on expression of CK 19 in our OT models. Our results are in agreement with those of Locke *et*

al. who reported that the type of fibroblasts used in the connective tissue equivalent influenced the CK expression in the epithelial component, but did not induce a complete change of the phenotype (21). However, they reported a markedly less growth of the epithelial component and weaker expression of CK 10 in the OT cultures engineered with PLF, compared to those with GF, having more cell layers and stronger CK 10 expression. In our work we have observed higher differentiation in mature (day 9) OTs constructed with GF than in those with PLF; but the variations in epithelial thickness were not found significant at any time point (data not shown). These differences might be explained by different culture conditions (*e.g.* time allowed for maturation, different culture medium and different number of fibroblasts) used in these two studies.

We have shown here that CK 19 was positive in all OT tissues up till day 9, even if these were constructed with GF. This differentiation marker is distinctive for JE but is sparsely expressed also in GE and it appears to be increased in periodontitis (18, 20, 29, 30). It has been shown that, when cultured *in vitro*, cells isolated from GE were also found to express CK19, similarly with those isolated from JE (31). Gain of CK 19 in the gingival epithelial cells grown in culture both in colonies (monolayers) and in multilayered OT models was reported also by other studies (21, 32). These studies did also point out to a slight change in the phenotype of gingival epithelial cells cultured *in vitro*, expressing markers of differentiation such as CK 1, 10 and 13 less intensely than *in vivo* and at the same time 'gain' or increase in intensity of the expression of cytokeratins characteristic for simple epithelia, CK 8 / 18, 19 and for high proliferating tissues (CK 6 / 16). We also report here a stronger staining for CK 16 and the presence of CK 8 only in day 3 reconstructed tissues, compared to *in vivo* samples, where these markers were found scarce or absent. In conclusion, we agree with the above mentioned studies that expression of differentiation markers in reconstructed culture models is close to the *in vivo* pattern, but not identical.

However, in the present study we show a steady decline in time in the number of CK19 positive cells in OT cultures and determined that time in culture had a significant impact on expression of this marker. This finding indicates once more that 3D organotypic models are more appropriate experimental models and closer to the *in vivo* situation than 2D culture models. The development of such serial models is justified by the continuous and increasing interest of studying interactions between oral bacteria, including putative periodontal pathogens, and human oral tissues (28, 33-37).

In our study, a higher expression of CK 19 in the epithelial compartment was at all time points better supported by PLF than GF, although the type of the fibroblasts used in the connective tissue equivalent became statistically significant just at one time point, at day 5. Of note is that at the same development point (day 5) the presence of PLF in the matrix influenced significantly the distribution pattern of the proliferation cells in the epithelium, PLF supporting suprabasal proliferation.

In conclusion, this study identifies certain culture conditions (time points and type of fibroblasts) that can be reproducibly used in order to develop standardized (serum free) models of human junctional or sulcular epithelium. Based on the characterization of the tissues we made, we suggest the use of the model with PLF between days 3 and 5 as a JE model for bacterial-host cell interactions. As for SE model, we consider appropriate the OT cultures constructed with either PLF or GF grown to day 7; and those with GF at/after day 9 as suitable for a gingival model. Such models may constitute a very useful working tool in investigating the mechanisms involved in periodontium breakdown in periodontal disease, especially in host tissue – bacterial interaction studies.

Acknowledgements

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Table 1. Immunohistochemistry: information on secondary antibodies used.

Antibody	Type	Clone/ number	Titration	Source
CK 8	mouse monoclonal	-	1 : 5	CRUK
CK 10	IgG-1	-	1 : 50	DAKO A/S (Golstrup, Denmark)
CK 13	IgG-1	KS-1A3	1 : 50	Novocastra (Newcastle upon Tyne, UK)
CK 16	mouse monoclonal	-	1 : 5	CRUK
CK 19	mouse monoclonal	-	1 : 5	CRUK
Ki-67	IgG-1	MIB-1	1 : 25	DAKO A/S, Golstrup, Denmark
Filaggrin	IgG-1	15CID	1 : 50	Monosan (Uden, The Netherlands)
TG	rabbit polyclonal	pab0061	1 : 250	Covalab UK Ltd (Cambridge, UK)

CRUK = Cancer Research United Kingdom (Abs were a kind gift from Prof. I.C. Mackenzie, Institute for Cell and Molecular Science, London, UK)

Table 2. Results of immunostaining for a panel of different markers in control tissues and organotypic models. The presence (+) or absence (-) of immunopositive cells, or their presence in only some of the samples (+/-) are marked in the table without indicating the intensity of staining or any quantification.

| Marker | In organotypics* | | | | Control JE | Control SE | | Control GE |
	d3	d5	d7	d9		AM	GM	
CK 8	+	-	-	-	-	-	-	-
CK 10	-	-	+/-	+	-	-	+	+
CK 13	+	+	+	+	+	+	-	-
CK 16	+	+	+	+	+/-	+/-	-	+
CK 19	+	+	+	+	+	+	+	+
Filaggrin	-	-	-	-	-	-	-	+
TG	+	+	+	+	+	+	+	+

* The description of the immunostaining refers to both GF and PLF - constructed organotypics if not otherwise mentioned. AM = apical margin, GM = gingival margin.

Figure legends

Figure 1. Haematoxilin – eosin stained tissues. **A.** Organotypic models constructed with gingival epithelial cells grown on top of collagen matrices populated with GF (gingival fibroblasts) or PLF (periodontal ligament fibroblasts) harvested at different stages of growth. **B.** Native tissues used as controls. Size bar: 200 μm.

Figure 2. Quantification and distribution of proliferating cells (Ki-67 positive) in organotypic models. **A.** Percentage of Ki-67 positive cells in basal and parabasal layers; **B**. Mean (n = 4 independent experiments) of Ki-67 positive cells per 334 μm length in basal (light gray bars) and suprabasal (dark gray bars) layers. Statistical significance: ** * $p < 0.001$; * $p < 0.05$. GF = gingival fibroblasts; PLF = periodontal ligament fibroblasts.

Figure 3. CK 19 staining in organotypics and in control tissues. Size bar: 200 μm.

Figure 4. IHC showing similarities in protein expression found between a representative organotypic culture, PLF – day 5, and control JE. Size bar: 200 μm.
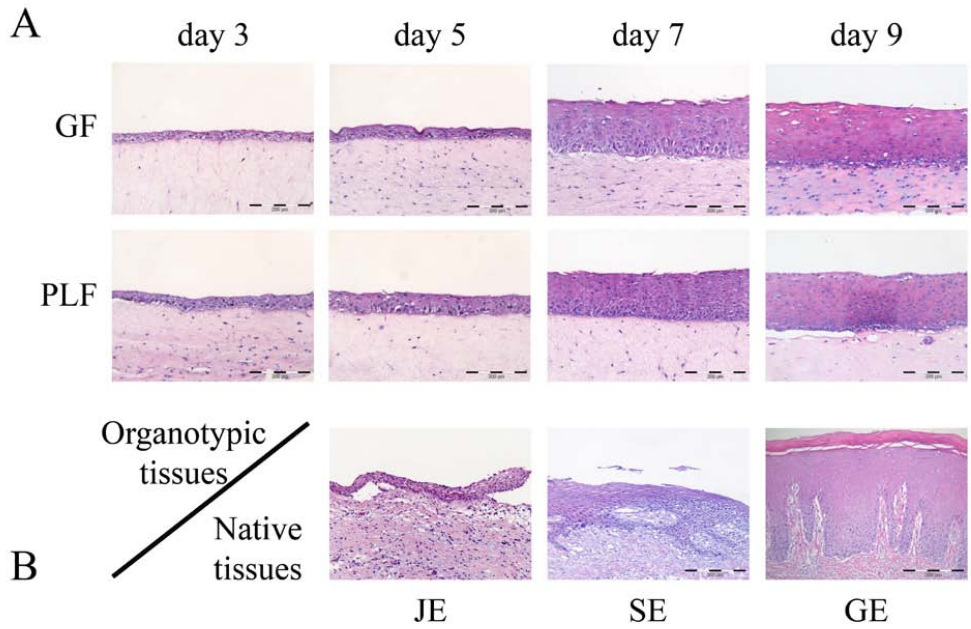
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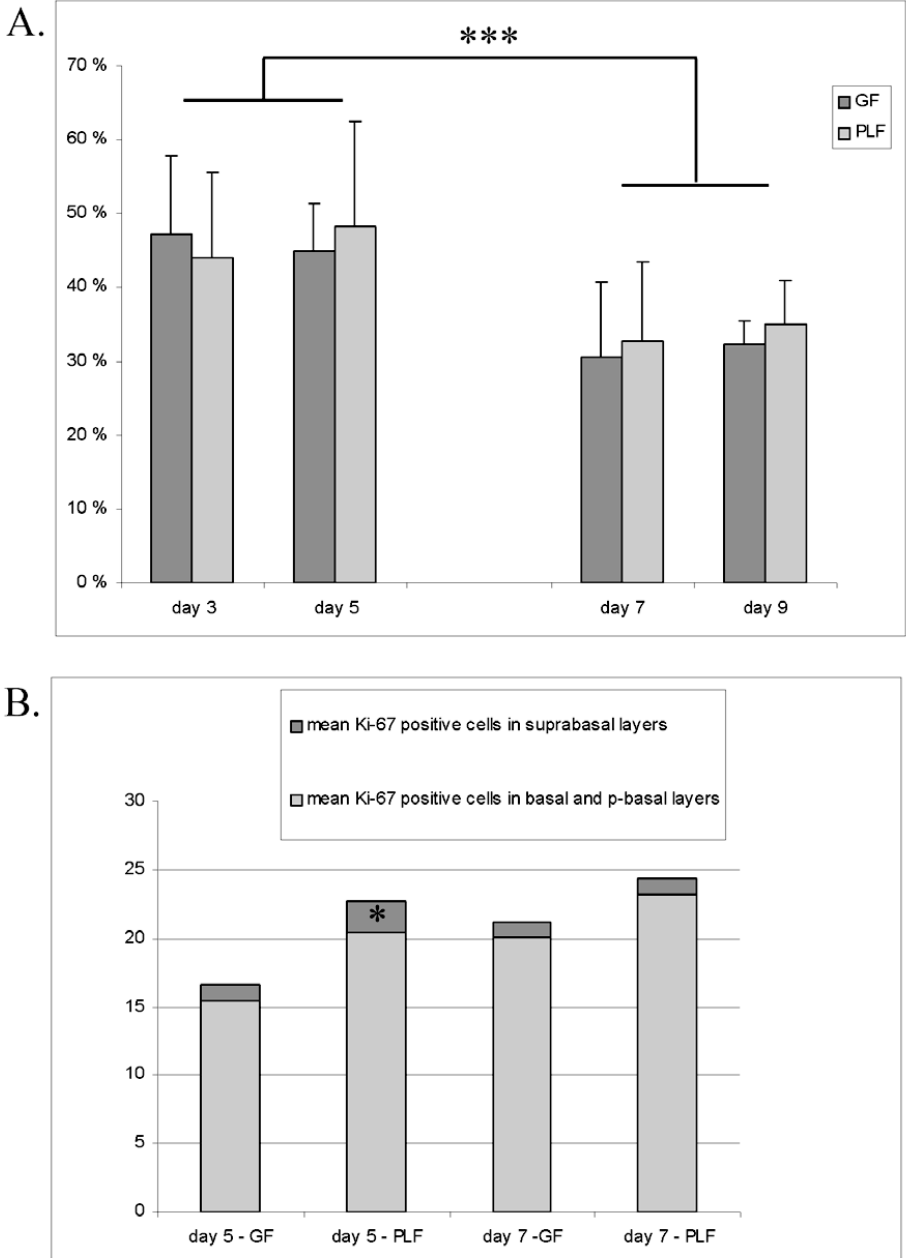
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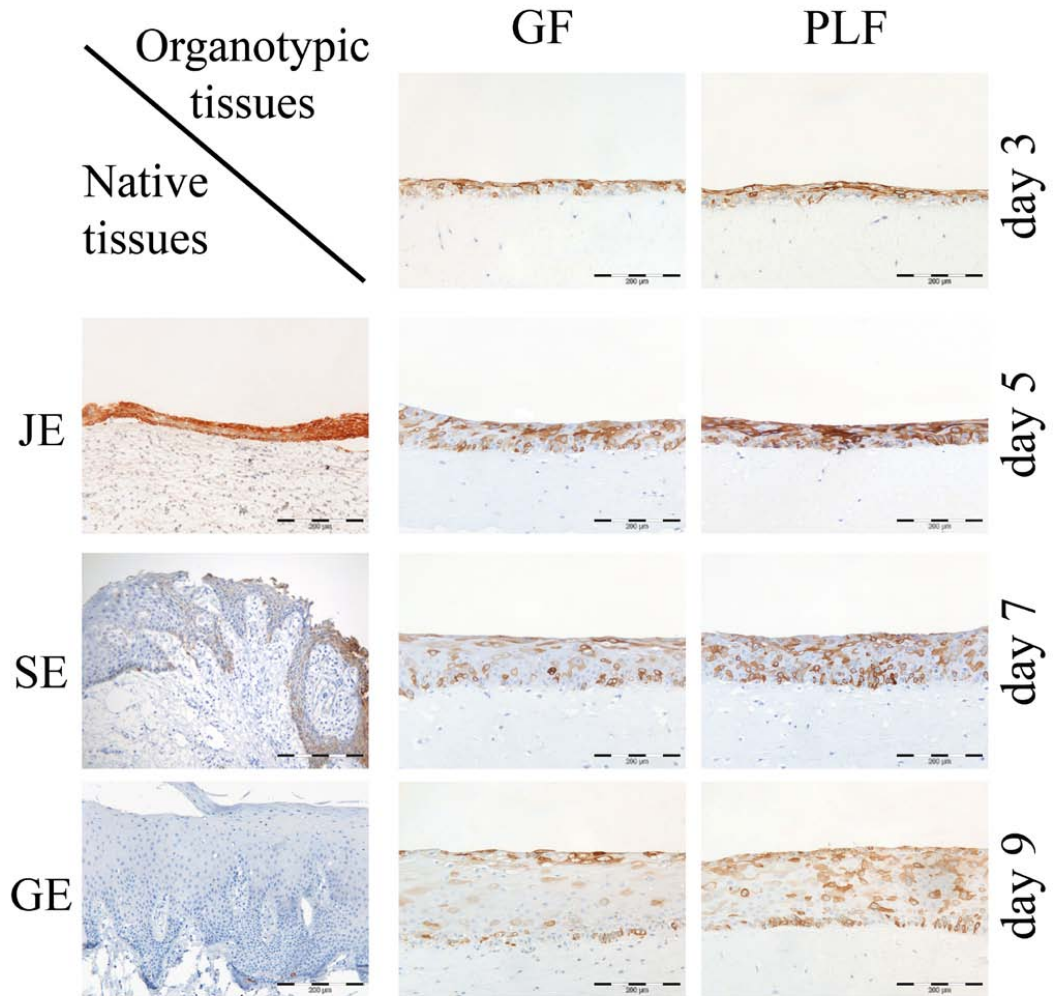
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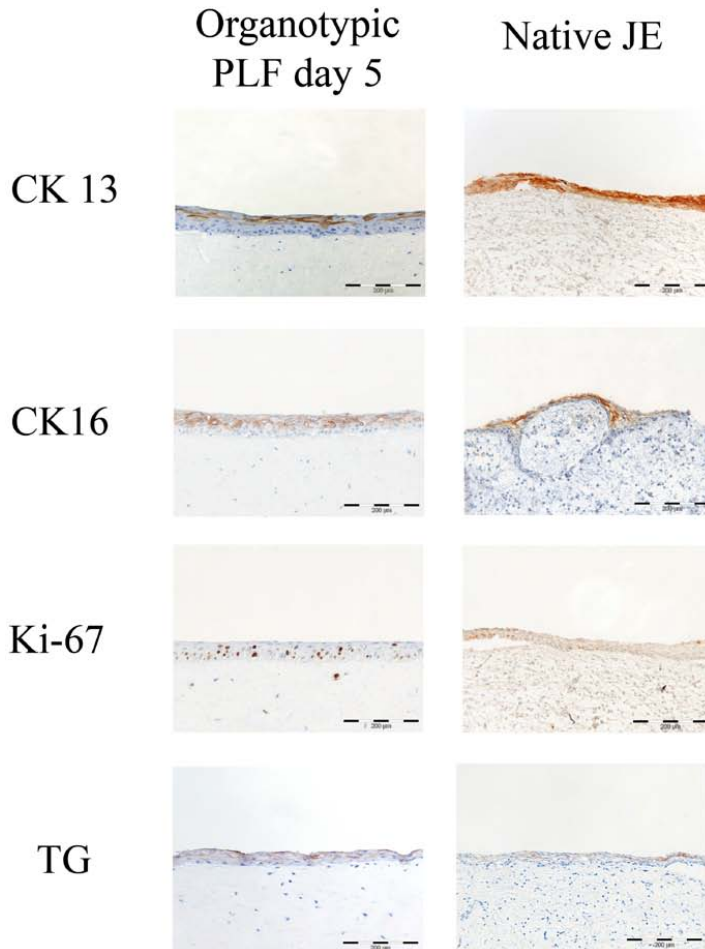
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