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In vitro and *in vivo* cytokeratin patterns of expression in bioengineered human periodontal mucosa

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Background and Objective: Development of human oral mucosa substitutes by tissue engineering may provide new therapeutic tools for the management of periodontal diseases. In this study we evaluated a fibrin–agarose human oral mucosa substitute both *in vitro* and *in vivo*.

Material and Methods: In vitro bioengineered oral mucosa substitutes were developed from irrelevant biopsy samples of human oral gingiva. *In vivo* evaluation of the constructed tissues was performed by implantation into athymic nude mice. The expression of several epithelial markers was assessed by microarray analysis and immunohistochemistry.

Results: Bioengineered oral mucosa samples kept *in vitro* developed a multilayered epithelium that expressed several cytokeratins, including some markers of simple epithelia (cytokeratins 7, 8 and 18), along with markers of stratified epithelia (cytokeratins 5 and 13) and of cell proliferation (proliferating cell nuclear antigen). Bioengineered tissues grafted *in vivo* onto nude mice exhibited very good biointegration with the host, showing a cytokeratin expression pattern that was very similar to that of normal native oral mucosa controls. Histological analysis of the artificial tissues demonstrated that oral mucosa substitutes evaluated *in vivo* were structurally mature, showing some typical structures of human native oral mucosa such as rete ridges and chorial papillae, along with numerous blood vessels at the fibrin–agarose stromal substitute. These structures were absent in samples evaluated *in vitro*.

Conclusion: The results indicate that this model of human oral mucosa, constructed using fibrin–agarose scaffolds, shows similarities to native oral mucosa controls and imply that bioengineered oral mucosa substitutes could eventually be used clinically.

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The oral mucosa is commonly affected by a number of clinical disorders involving the oral cavity. Periodontal disease is one of the pathologies with the highest incidence and prevalence, and it is estimated that 86% of adults have at least moderate periodontal disease (1–3). Other conditions that frequently affect the oral mucosa are oral cancer, leukoplakia, infections and traumatisms.

The oral mucosa consists of two different layers of tissue that are structurally and embryologically

distinct: the superficial epithelium; and the deeper chorion or lamina propria. The epithelium mainly consists of a cell population of keratinocytes which express specific cytokeratins that are responsible for many of the functions of the epithelium. Histologically, the stratified epithelium can be keratinized, parakeratinized or nonkeratinized, depending on the location and functional requirements of this tissue. Both epithelium and chorion are connected by a basal lamina that is synthesized under epithelial–mesenchymal interactions (4,5). This cytoarchitecture of epithelium and chorion allows the avascular epithelium to obtain nutrients and oxygen from the supporting stroma (6).

Cytokeratins are intermediate filament proteins encoded by a large multigene family and expressed by different types of epithelia. These proteins can be found in cells as different combinations of acidic type I and basic type II polypeptides that associate according to molecular weight and isoelectric point (7). As intermediate filaments, the role of cytokeratins in epithelial cells is associated with the cytoskeleton and with cell–cell junctions. For these reasons, cytokeratins are necessary for the maintenance of cell shape, integrity and morphology, as well as for the epithelial cohesion of the normal oral mucosa epithelium (8). As a consequence of their functional role in each type of epithelium, the expression of different groups of cytokeratins appears to be highly specific to each type of epithelium (9). Moreover, the expression pattern of the normal oral mucosa can be altered by several diseases. Therefore, evaluation of the expression of cytokeratins can be used as a diagnostic tool (10,11).

Treatment of the different periodontal pathologies affecting oral mucosa often requires surgical reconstruction or even substitution of the damaged tissues, and oral surgeons are often confronted with a shortage of oral mucosa to replace the excised tissues. The periodontal treatments that have been reported thus far consist of a variety of surgical procedures that can involve the use of organic or synthetic membranes, flaps and implant techniques. However, not all patients can be successfully treated using these techniques. In addition, the management of many periodontal patients may require more than one surgical procedure, which is often associated with high morbidity at the donor and recipient sites (12,13). For these reasons, new sources of normal oral mucosa are necessary.

Tissue engineering is a novel scientific discipline that combines the principles of engineering and biological sciences (14). The main focus of tissue engineering is the development of artificial biological substitutes for all types of human organs and tissues. Thus far, several types of artificial substitutes of the human oral mucosa have been developed in the laboratory through tissue engineering. Most of these oral substitutes use artificial stromas based on type I collagen (15–17), gelatins (18), amniotic membrane (19), acellular dermis (20), fibrin (21) and several synthetic scaffolds (22,23). Recently, we developed a novel stromal scaffold, based on a mixture of agarose and human fibrin, which was efficiently used for the construction of artificial oral mucosa in the laboratory by tissue engineering (12,13). Evaluation of cytokeratin expression in the different oral mucosa substitutes reported by several authors indicated that the cytokeratin-expression pattern in artificial oral mucosa is not always identical to that of normal control oral mucosa (5,11,12,20,24–33).

In the present study we used a novel model of fibrin–agarose oral mucosa to evaluate the cytokeratin-expression pattern in artificial oral mucosa and to determine whether or not this pattern is similar to that of normal control human oral mucosa, with the aim of using this model for clinical therapeutic applications.

Materials and methods

Establishment of primary cultures of oral fibroblasts and keratinocytes

Fifteen small biopsies (average size $2 \times 2 \times 2$ mm) of normal human oral mucosa were obtained from healthy donors at the School of Dental Sciences of the University of Granada. All biopsies corresponded to free gingiva close to the free gingival margin (protective periodontium). Immediately after extraction, all tissues were kept at 4°C in Dulbecco's modified Eagle's minimal essential medium (Sigma-Aldrich, St Louis, MO, USA) supplemented with antibiotics and antimycotics (100 U/mL of penicillin G, 100 mg/mL of streptomycin and 0.25 mg/mL of amphotericin B; Sigma-Aldrich) and processed within 24 h. All patients gave their consent to participate in the study. The work was approved by the local research committee.

Oral mucosa biopsies were washed twice in phosphate-buffered saline and incubated overnight at 4°C in dispase II (5 mg/mL in phosphate-buffered saline; Gibco BRL, Karlsrube, Germany) to detach the epithelium enzymatically from the connective tissue. Subsequently, the detached epithelium was mechanically fragmented into small pieces and each explant piece was cultured in culture flasks using a 3:1 mixture of Dulbecco's modified Eagle's minimal essential medium and Ham's F12 culture medium supplemented with 10% fetal bovine serum, 1% antibiotics, 24 μg/mL of adenine, 0.4 mg/mL of hydrocortisone, 5 mg/mL of insulin, 10 ng/mL of epidermal growth factor, 1.3 ng/mL of triiodothyronine and 8 ng/mL of cholera toxin (all from Sigma-Aldrich). No feeder cells were used in this experiment.

To obtain primary cultures of human oral mucosa fibroblasts, the de-epithelized chorion was digested in a mixture of Dulbecco's modified Eagle's minimal essential medium and 2 mg/mL of *Clostridium histolyticum* collagenase I (Gibco BRL). Detached fibroblasts were collected by centrifugation and expanded in culture flasks containing Dulbecco's modified Eagle's minimal essential medium supplemented with antibiotics (100 U/mL of penicillin G, 100 mg/mL of streptomycin and 0.25 mg/mL of amphotericin B) and 10% fetal bovine serum.

In all cases, cells were incubated at 37°C in 5% carbon dioxide under standard culture conditions. The medium was changed every 3 d, and

subculture of the cells was carried out using a trypsin (0.5 g/L)/EDTA (0.2 g/L) solution at 37°C for 10 min. Keratinocytes and fibroblasts used for tissue engineering of the tissue models always corresponded to the first three cell subcultures.

The following samples were analyzed.

(i) Control samples (controls). As controls, we used normal human native oral mucosa samples corresponding to the trigonus retromolare of healthy donors. These samples were used for histological analysis (hematoxylin and eosin staining) and for immunohistochemistry and microarray studies.

(ii) Bioengineered oral mucosa cultured *in vitro* (*in vitro* samples). These samples corresponded to artificial human oral mucosa generated in the laboratory by tissue engineering. These samples were used for histological analysis (hematoxylin and eosin staining) and for immunohistochemistry and microarray studies.

(iii) Bioengineered oral mucosa implanted *in vivo* in athymic mice (*in vivo* samples). These samples corresponded to artificial human oral mucosa generated by tissue engineering and implanted in athymic nude mice for 4 wk. *In vivo* samples were used for histological analysis (hematoxylin and eosin staining) and immunohistochemistry.

All samples were processed and analyzed using exactly the same protocols.

Generation of human oral mucosa substitutes by tissue engineering (*in vitro* samples)

Biological orthotypical substitutes of the human oral mucosa were developed in the laboratory using Transwell® porous inserts (Costar, Corning Inc., Corning, NY, USA), as described previously (12,13). Briefly, a stromal substitute was first generated by using a mixture of human fibrin obtained from frozen plasma (kindly provided by Dr Fernández-Montoya, Human Tissue Bank of Granada, Spain) and 0.1% agarose. An average of 250 000 cultured fibroblasts was added to 25 mL of the mixture immediately before inducing the polymerization of the artificial stroma on the porous inserts. Once the stroma solidified, cultured keratinocytes were seeded on top of the artificial stroma, and the oral mucosa constructs were cultured for 7–14 d submerged in culture medium. Finally, some of the samples were submitted to an air–liquid culture technique for 8 d to induce the proper differentiation of the multilayered epithelium (17).

In this work, we analyzed mature bioengineered oral mucosa substitutes using a multilayered epithelium submitted to submerged culture conditions for 10 d and to the air–liquid culture technique for 8 additional days (18 d in total).

In vivo evaluation of bioengineered oral mucosa substitutes

To evaluate the *in vivo* behavior of the oral mucosa substitutes generated by tissue engineering, we implanted the artificial tissues in a total of nine 6-wk-old Fox $1^{nu/nu}$ immunodeficient athymic mice (Harlan Laboratories, Indianapolis, IN, USA).

All animals were anesthetized by intraperitoneal injection of a mixture of acepromazine [Calmo-Neosan® (Pfizer, New York, NY, USA); 0.001 mg per g of weight of the animal] and ketamine [Imalgene 1000® (Merial Laboratories, New York, NY, USA); 0.15 mg per g of weight of the animal] after the subcutaneous administration of atropine. All procedures were performed in a biological safety cabinet, and animals were housed in filter-topped cages in a laminar flow cage isolator.

In order to determine the *in vivo* behavior of the artificial tissues, the oral mucosa substitutes were grafted onto the backs of the animals, in direct contact with air. Briefly, we first created a full-thickness skin wound (including the panniculus carnosus) of approximately 1 cm^2 above the shoulder of the mouse, in the interscapular region of the animal. Then, the artificial human oral mucosa substitutes developed by tissue engineering were implanted onto the wound bed of the host mice using 6/0 polydioxanone sutures. All grafts corresponded to mature oral mucosa substitutes (18-d samples). The mice were killed by the administration of a lethal dose of anesthetics [intraperitoneal injection of acepromazine (0.01 mg per g of weight of the animal) and ketamine (1.5 mg per g of weight of the animal)] 4 wk after implantation of the bioengineered oral mucosa, and all grafted tissues were harvested for histological analysis.

Histology and immunohistochemistry

For histological analysis by light microscopy, controls, and in *in vitro* and *in vivo* samples, were fixed in 4% formaldehyde, dehydrated and embedded in paraffin. Four-micrometer-thick sections were stained with hematoxylin and eosin.

In order to determine the protein expression of several cytokeratins, proliferating cell nuclear antigen, and nuclear mitotic apparatus protein in controls, and in *in vitro* and *in vivo* samples, standard immunohistochemical procedures were carried out on formaldehyde-fixed, paraffin-embedded tissue sections. Briefly, paraffin was removed from the tissue sections using xylene, and endogenous peroxidase was quenched in 3% H_2O_2. Then, we used 0.01 M citrate buffer (pH 6.0), at 98°C for 5 min, for antigen retrieval. Incubation with the primary antibodies (all were of mouse origin) was performed for 2 h at 25°C.

Primary antibodies and dilutions used in this work are shown in Table 1. Secondary biotin-conjugated anti-mouse immunoglobulin was used at a 1: 500 dilution, and a horseradish peroxidase-conjugated streptavidin solution was applied for 40 min. Color was developed using a commercial 3-3′ diaminobenzidine kit (Vector Laboratories, Burlingame, CA, USA) and samples were then counterstained in Mayer's haematoxylin and mounted on coverslips for optical evaluation.

Genome-wide gene-expression analysis using oligonucleotide microarrays

Total RNA corresponding to controls and *in vitro* samples was extracted using the Qiagen RNeasy System (Qiagen, Mississauga, ON, Canada), according to the manufacturers' rec-

Table 1. Primary antibodies used in this work

Primary antibodies	Dilution	Source	Reference
Anti-AE1/AE3	Prediluted	Master Diagnostica, Granada, Spain	001000QD
Anti-NuMA	1:20	Calbiochem (Merck KGaA), Darmstadt, Germany	NA08
Anti-pancytokeratin	Prediluted	Master Diagnostica, Granada, Spain	001607QD
Anti-PCNA	1:1000	Sigma-Aldrich, St Louis, MO, USA	P 8825
Anti-CK4	1:1000	Sigma-Aldrich, St Louis, MO, USA	C 5176
Anti-CK7	Prediluted	Master Diagnostica, Granada, Spain	001004QD
Anti-CK8	Prediluted	Master diagnostica, Granada, Spain	005095QD
Anti-CK10	Prediluted	Master diagnostica, Granada, Spain	000150QD
Anti-CK13	1:400	Sigma-Aldrich, St Louis, MO, USA	C 0791
Anti-CK19	Prediluted	Master Diagnostica, Granada, Spain	002163QD

For each case, the dilution used for the immunohistochemical analyses is shown, along with the manufacturer (source) and the reference of each antibody.
CK, cytokeratin; NuMA, nuclear mitotic apparatus protein; PCNA, proliferating cell nuclear antigen.

ommendations. The RNA concentration was determined by the absorbance value at 260 nm, and quality was verified using a Bioanalyzer (Agilent, Santa Clara, CA, USA). Total cDNA was synthesized using a T7-polyT primer and reverse transcriptase (Superscript II; Life Technologies, Inc., Carlsbad, CA, USA) before *in vitro* transcription with biotinylated UTP and CTP (Enzo Diagnostics, Farmingdale, NY, USA). Labeled nucleic acid target was hybridized (45°C for 16 h) to Affymetrix Human Genome U133 plus 2.0 oligonucleotide arrays (Affymetrix, Santa Clara, CA, USA). After automated washing and staining, absolute values of expression were calculated and normalized from the scanned array using the Affymetrix Microarray Suite (Affymetrix).

To select genes encoding cytokeratins that were significantly up-regulated or down-regulated in bioengineered *in vitro* samples in comparison with controls, we used a two-step approach. In the first step the average expression was calculated for native controls and for bioengineered *in vitro* samples, and the fold-change relative expression of controls vs. *in vitro*-generated oral mucosa was obtained. At this step, all cytokeratin genes with a relative fold-change of at least two (i.e. genes whose average expression was at least twice as high than in the controls or in the *in vitro* samples) were selected. In the second step a rank statistical test was used to identify those genes whose absolute expression value was higher in all samples corresponding to one of the comparison groups (controls or *in vitro* samples) and lower in all samples of the other group (no exceptions were allowed). Only genes with a p-value of < 0.01 were selected. All genes fulfilling both criteria were selected as being significantly up-regulated or down-regulated in bioengineered oral mucosa substitutes.

Results

In vitro development of artificial oral mucosa substitutes by tissue engineering

In this work, we were able to generate orthotypical oral mucosa substitutes using previously described cell-culture and tissue-engineering methods and techniques (12,13). Initally, we established primary cultures of human oral keratinocytes and fibroblasts from adult stem cells isolated from small oral mucosa tissue biopsies. In culture, cells showed early attachment to the surface of the culture flasks and a rapid growth rate, reaching cell confluence after 7 d (fibroblasts) and 28 d (keratinocytes) in culture.

Subsequently, construction of artificial oral mucosa substitutes was successfully carried out using porous culture inserts. By using these techniques, we were able to develop a stromal substitute composed of human fibrin and 0.1% agarose with human fibroblasts immersed within, which sustained the proper attachment and growth of human keratinocytes seeded on top. Histological analysis of samples cultured *in vitro* revealed the presence of a fully stratified epithelium that developed on top of the stromal substitute (Fig. 1A).

In vivo evaluation of the oral mucosa grafts on athymic nude mice

In vivo evaluation of our human oral mucosa substitutes showed that the grafted tissues integrated properly into the wound bed of the recipient mice. The surgical procedure was tolerated by all animals, and no intra-operative or postoperative mortality was observed.

Histological analysis of artificial oral mucosa implanted in direct contact with air on the back of the athymic mice showed the presence of a well-formed, integrated, artificial stroma, with a stratified epithelium on top consisting of basal, suprabasal and spinous layers with signs of keratinization in the most apical cell layer. No histological differences were found among samples corresponding to different time periods (1, 2, 3 and 4 wk). Interestingly, the epithelial–stromal junction of the samples grafted on the back of the mice was very similar to the epithelial–stromal junction of human native oral mucosa samples. In both cases, the stroma emitted numerous prolongations that penetrated into the epithelium (chorial papillae) and interdigitated with the prolongations of the epithelium (rete ridges) (Fig. 1B). Specifically, these structures were not found in the *in vitro*-developed samples.

Regarding the stromal substitute, our analysis showed that the grafts

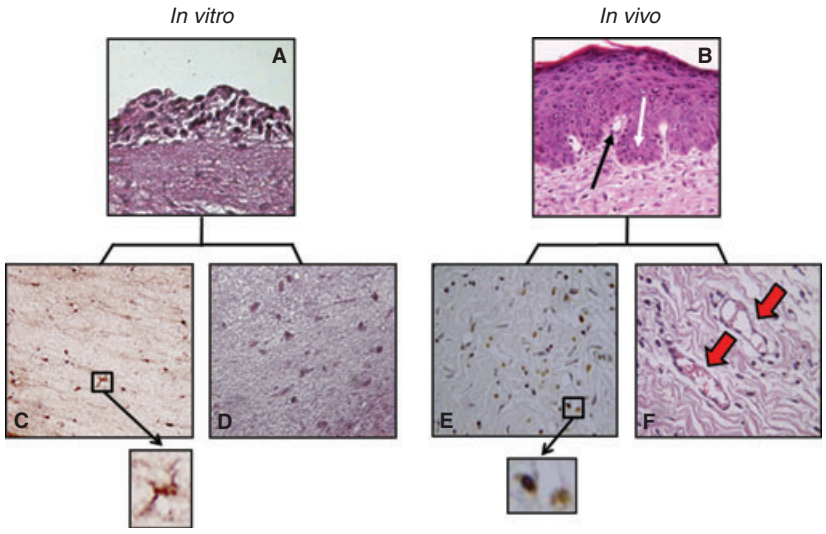

Fig. 1. In vitro and *in vivo* histological analysis of human oral mucosa substitutes developed by tissue engineering. (A) Hematoxylin and eosin staining of samples developed *in vitro* reveals the development of a fully stratified epithelium on these samples. (B) Tissues evaluated *in vivo* showed several chorial papillae (black arrow) and rete ridges (white arrow) (hematoxylin and eosin staining). (C) Nuclear mitotic apparatus protein immunohistochemistry for *in vitro*-developed oral mucosa substitutes demonstrated that all stromal cells were of human origin, whereas around 50% of the cells in the stroma were of human origin for *in vivo* samples (E). (D) The stromal substitute kept *in vitro* did not show any neovascularization, but some blood vessels (red arrows) can clearly be seen after the samples were grafted *in vivo* (F).

implanted in contact with air developed a fully organized stroma, whose cell population was integrated by a mixture of implanted human fibroblasts and host mesenchymal cells that migrated and colonized the grafted stroma. According to the immunostaining results of the human-specific nuclear mitotic apparatus protein, stroma of the *in vivo* samples consisted of 35% human cells and 65% host mice cells (Fig. 1E).

Finally, the proper integration of the grafts was confirmed by the presence of numerous blood vessels in the artificial stroma by light microscopy (Fig. 1F).

In vivo and *in vitro* cytokeratins and proliferating cell nuclear antigen expression in native and bioengineered oral mucosa samples

As shown in Table 2 and Figs 2 and 3, our analysis of the expression of cytokeratins in controls and oral mucosa substitutes allowed us to determine the specific pattern of cytokeratin expression displayed by each type of sample.

Immunohistochemistry analyses for two complexes of several cytokeratins (pancytokeratin and AE1/AE3) revealed that all samples analyzed in this study expressed several types of cytokeratins. Thus, the expression of pancytokeratin (a complex of cytokeratins 5, 6, 8, 18, 10 and 1) was strongly positive in the suprabasal layers of native, oral mucosa control samples. In contrast, the pancytokeratin signal was homogeneously positive in all epithelial layers of oral mucosa substitutes generated *in vitro*, whereas the analysis of samples implanted *in vivo* showed strong expression of pancytokeratin in the suprabasal epithelial layers, similarly to control samples. Evaluation of the expression of AE1/AE3 (a complex of cytokeratins 1, 2, 3, 4, 5, 6, 7, 10, 14, 15 and 16) showed that normal controls displayed positive cytokeratin expression in all epithelial layers, whereas *in vitro*developed samples showed a highly positive expression that was restricted to the suprabasal layers of the bioengineered epithelium, with negative expression in the basal layers. Regarding the oral mucosa constructs implanted on nude mice, AE1/AE3 expression was present in all layers of the epithelium, although the most apical layers showed stronger expression than the rest of the epithelium.

On the other hand, immunostaining for simple epithelium markers (cytokeratins 7, 8 and 18) was negative in the normal oral mucosa control. However, multilayered epithelium of the *in vitro*-developed samples showed positive expression of cytokeratins 7, 8 and 18 in all layers of the epithelium. Finally, similarly to normal controls, samples implanted on athymic mice were negative for cytokeratins 7, 8 and 18.

Stratification markers of nonkeratinized and parakeratinized epithelia (cytokeratins 4 and 13) showed strong

Table 2. Cytokeratin expression, as determined by immunohistochemistry, in the three groups of samples analyzed in this work

	Pancytokeratin	AE1/AE3	CK7	CK8	CK18	CK19	CK4	CK13	CK5	CK10
Control	+ (S)	+ (A)	–	–	–	–	+ (S)	+ (S)	+ (B)	+ (S)
In vitro	+ (A)	+ (S)	+ (A)	+ (A)	+ (A)	+ (A)	+ (A)	+ (S)	+ (A)	–
In vivo	+ (S)	+ (A)	–	–	–	–	+ (S)	+ (S)	+ (B)	+ (S)

Control: normal human native oral mucosa. *In vitro*: *in vitro*-developed oral mucosa substitutes generated by tissue engineering. *In vivo*: bioengineered oral mucosa samples implanted *in vivo* in nude mice. Pancytokeratin is a complex of cytokeratins 5, 6, 8, 18, 10 and 1, whereas AE1/AE3 is a complex of cytokeratins 1, 2, 3, 4, 5, 6, 7, 10, 14, 15 and 16.
(A), cytokeratin expression was found in all epithelial layers; (B), cytokeratin expression in the basal layers of the epithelium; (S), cytokeratin expression was restricted to suprabasal layers; –, no protein expression.

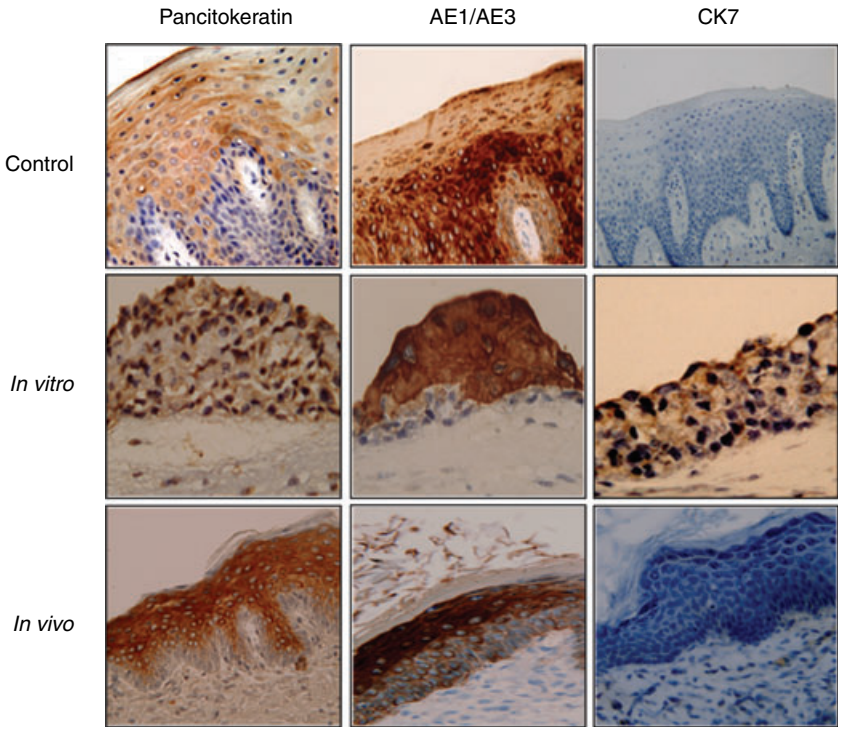

Fig. 2. Illustrative microphotographs corresponding to immunohistochemical analyses carried out for the three different types of samples analyzed in this work for the epithelial markers pancytokeratin, AE1/AE3 and cytokeratin 7 (CK7). Control: normal human native oral mucosa controls. *In vitro*: *in vitro*-developed oral mucosa substitutes generated by tissue engineering. *In vivo*: bioengineered oral mucosa samples implanted *in vivo* onto nude mice.

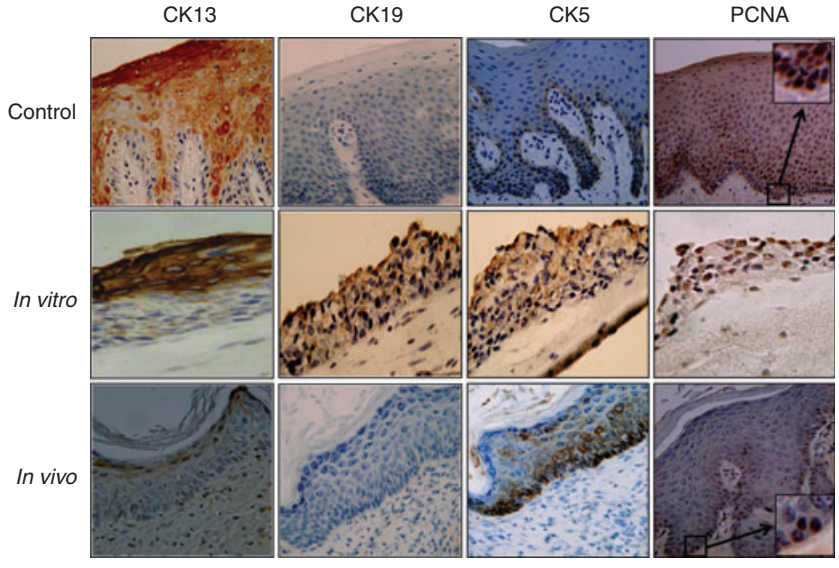

Fig. 3. Illustrative microphotographs of immunohistochemical analyses carried out on the three different types of samples analyzed in this work for the proteins cytokeratin 13 (CK13), cytokeratin 19 (CK19), cytokeratin 5 (CK5) and proliferating cell nuclear antigen (PCNA). Control: normal human native oral mucosa controls. *In vitro*: *in vitro*-developed oral mucosa substitutes generated by tissue engineering. *In vivo*: bioengineered oral mucosa samples implanted *in vivo* onto nude mice.

suprabasal expression in the control oral mucosa, with basal layers of the epithelium being negative. Similarly, *in vitro*-developed oral mucosa also showed expression of both cytokeratin 4 and cytokeratin 13, although the expression of cytokeratin 4 was homogeneous in all layers of the epithelium. Interestingly, when the oral mucosa substitutes were implanted on athymic nude mice, the expression of both cytokeratin 4 and cytokeratin 13 was restricted to suprabasal layers.

Immunohistochemistry for cytokeratin 19 revealed that normal native controls did not express this cytokeratin, whereas *in vitro* oral mucosa substitutes were positive for this cytokeratin marker. However, cytokeratin 19 expression was negative in samples evaluated *in vivo*.

One of the typical markers of keratinized, stratified epithelia is cytokeratin 10. According to our results, both control oral mucosa and samples grafted on nude mice showed suprabasal expression of cytokeratin 10 in the most superficial cell layers, whilst *in vitro*-developed samples did not express this cytokeratin.

Finally, we determined the presence of proliferating basal cells in the epithelium of the oral mucosa samples by immunohistochemisty for proliferating cell nuclear antigen and cytokeratin 5. In this context, normal human native oral mucosa showed strong expression of proliferating cell nuclear antigen protein, especially in basal layers of the stratified epithelium. In contrast, bioengineered tissues kept in culture tended to show homogeneous expression of proliferating cell nuclear antigen in all layers of the artificial epithelium. Interestingly, when the oral mucosa substitutes were implanted in laboratory animals, the distribution of the cells expressing proliferating cell nuclear antigen protein was comparable to that of native normal controls, with a high expression in the basal epithelial layers. However, cytokeratin 5 (a basal cell marker of stratified epithelia) was homogenously expressed by the basal cell layer of the controls, whereas *in vitro* expression of cytokeratin 5 was strong in suprabasal layers of mature epithelium, with several cell

layers being negative for cytokeratin 5 expression in the basal layer. Finally *in vivo* expression of cytokeratin 5 was very similar to control normal oral mucosa, with a strong positive signal in the basal layer.

All the results of the immunohistochemical analyses were confirmed in triplicate.

Microarray gene expression of native and bioengineered oral mucosa samples

RNA expression analysis of oral mucosa samples revealed that the pattern of expression of some cytokeratins could be different for native and *in vitro*-generated samples. In short, *in vitro* bioengineered oral mucosa showed significant overexpression of the gene encoding cytokeratin 7 and significant downregulation of the genes encoding cytokeratins 1, 2A, 2B, 3, 4, 5, 6A, 6B, 10, 13, 14, 15, 16 and 24. Differences were not significant for the genes encoding cytokeratins 1B, 6IRS, 6L, 8, 9, 12, 17, 18, 19, 20, 23, 24, 25A and 25C. As expected, expression of the housekeeping genes glyceraldehyde-3-phosphate dehydrogenase and betaactin (ACTB), along with the ribosomal protein L3 (RPL3), was similar for controls and *in vitro* samples (Table 3).

Discussion

Advances in tissue engineering provide alternative autologous approaches to traditional treatments of pathologies affecting the oral cavity. The use of human bioengineered oral tissue substitutes could be very useful in the management of large reconstructions involving the oral cavity (25,26,34,35), including several diseases and conditions that are currently treated by using connective-tissue grafts, inert grafts or heterologous tissues. In relation to this, the fibrin–agarose artificial oral mucosa constructs could be of potential utility for the clinical treatment of several periodontal disorders, especially in the case of severe periodontal disease with bone and mucosal loss, peri-implant tissue destruction, pocket reduction procedures, exposed tooth roots and other pathologies in which tissue regeneration is necessary. Other potential clinical applications of the bioengineered human oral mucosa include pulp capping/dentin regeneration, treatment of malignant neoplasms of the head and neck, and regeneration for bone grafting of large osseous defects in dental and craniofacial reconstruction (36). Moreover, artificial oral mucosa substitutes have several potential preclinical applications because these tissues could be used as *in vitro* models for investigating the physiology and development of the human oral mucosa and in experimental pharmacology or toxicology tests, thus preventing the need for animal research (27).

The *in vivo* evaluation of artificial oral mucosa by implantation in nude mice is a realistic biological model that has been extensively used by several researchers in order to study the *in vivo* behavior of bioengineered human oral tissues (25,28,29,37). In this regard, the results of this work revealed that the profile of cytokeratin expression was different when the model of artificial oral mucosa was evaluated *in vitro* and then implanted *in vivo* in nude mice. In the first place, all samples analyzed here showed high expression of cytokeratins included in the complexes pancytokeratin and AE1/AE3, suggesting that the expression of several cytokeratins is crucial for the development and functionality of normal human oral keratinocytes at both *in vitro* and *in vivo* levels. Then, *in vitro*-developed samples with a multilayered epithelium showed high expression of several cytokeratin markers of simple epithelium (cytokeratins 7, 8 and 18), which were totally absent in native oral mucosa controls. The most likely explanation for this is that bioengineered multilayered samples are developing new features of stratified epithelia, whereas the typical cytokeratins that were over-expressed during the first stages of epithelial maturation (cytokeratins 7, 8 and 18; markers of monolayered epithelia) could still be present in the cells. Therefore, the expression of these cytokeratins might indicate an intermediate differentiation stage of the *in vitro* multilayered samples. Interestingly, the oral junctional epithelium is considered to be one of the most undifferentiated tissues in the oral cavity, and several studies have demonstrated that this tissue co-expresses some cytokeratins typically found in simple epithelia (cytokeratins 8 and 18) and in stratified epithelia (cytokeratins 5 and 13) (30,31). These findings could point to the possibility that *in vitro*-developed oral mucosa substitutes could be functionally similar to undifferentiated oral junctional epithelia, at least at this stage of development. For that reason, these artificial oral mucosa tissues could eventually be used clinically for the treatment of periodontal disease with extensive soft tissue loss. Similarly, cytokeratin 19 was overexpressed by *in vitro* bioengineered oral substitutes at the protein level, but not in normal native controls. According to some reports (32,33), cytokeratin 19 is expressed during human embryogenesis and could fulfill the same role in the epithelia as cytokeratins 8 and 18. This result would once again support the idea of an early stage of differentiation by *in vitro* samples. However, the expression of proliferating cell nuclear antigen and cytokeratin 5 reveals that most epithelial cells belonging to artificial oral mucosa retain expression signatures that are typical of basal epithelial layers, suggesting that oral mucosa substitutes are highly proliferative and retain basal cell characteristics.

When the artificial tissues were implanted *in vivo*, differences in the expression pattern of some cytokeratins were detected in comparison with *in vitro*-developed samples. On the one hand, the expression of cytokeratins 4 and 13, and of pancytokeratin and AE1/AE3, was similar for *in vitro*-developed samples and oral mucosa substitutes grafted onto nude mice (*in vivo* samples), suggesting that some key cytokeratins are expressed independently of the environmental conditions. On the other hand, and in contrast to *in vitro*-developed oral mucosa substitutes, samples implanted *in vivo* showed high expression of cytokeratins 1 and 10, two well-known markers of epithelial keratinization

Table 3. Microarray expression of several CK and control genes in normal human native controls and bioengineered oral mucosa samples kept *in vitro*

Probe set ID	Mean controls	Mean constructs	Fold change	Rank test	Samples showing higher expression	Gene name	Gene symbol
205900_at	7996.33	15.87	503.97	$p < 0.01$	CONTROLS	Cytokeratin 1	KRT1
237120_at	11.93	23.80	0.50	N.S.	–	Cytokeratin 1B	KRT1B
207908_at	155.30	4.97	31.27	$p < 0.01$	CONTROLS	Cytokeratin 2A	KRT2A
207878_at	9512.77	12.23	777.61	$p < 0.01$	CONTROLS	Cytokeratin 2B	KRT2B
217325_at	154.93	4.43	34.95	$p < 0.01$	CONTROLS	Cytokeratin 3	KRT3
213240_s_at	7268.27	240.33	30.24	$p < 0.01$	CONTROLS	Cytokeratin 4	KRT4
201820_at	10924.93	3073.83	3.55	$p < 0.01$	CONTROLS	Cytokeratin 5	KRT5
214580_x_at / 209125_at	12825.47	3619.57	3.54	$p < 0.01$	CONTROLS	Cytokeratin 6A	KRT6A
213680_at	12302.12	3587.22	3.43	$p < 0.01$	CONTROLS	Cytokeratin 6B	KRT6B
231461_at	5.97	3.17	1.88	N.S.	–	Cytokeratin 6 irs	KRT6IRS
1569909_at	18.07	22.27	0.81	N.S.	–	Cytokeratin 6L	KRT6L
1558393_at/ 1558394_s_at/ 209016_s_at/ 214031_s_at	27.99	461.98	0.06	$p < 0.01$	BIOENGINEERED	Cytokeratin 7	KRT7
209008_x_at/ 214399_s_at/ 216821_at	130.26	69.53	1.87	N.S.	-	Cytokeratin 8	KRT8
208188_at	36.43	26.23	1.39	N.S.	-	Cytokeratin 9	KRT9
207023_x_at/ 210633_x_at/ 213287_s_at	7105.51	992.69	7.16	$p < 0.01$	CONTROLS	Cytokeratin 10	KRT10
207811_at	4.10	1.37	3.00	N.S.	-	Cytokeratin 12	KRT12
207935_s_at	14316.13	1107.97	12.92	$p < 0.01$	CONTROLS	Cytokeratin 13	KRT13
209351_at	13332.67	5950.00	2.24	$p < 0.01$	CONTROLS	Cytokeratin 14	KRT14
204734_at	3801.43	1379.70	2.76	$p < 0.01$	CONTROLS	Cytokeratin 15	KRT15
209800_at	11024.63	2750.70	4.01	$p < 0.01$	CONTROLS	Cytokeratin 16	KRT16
205157_s_at/ 212236_x_at/ 228491_at	3805.78	3372.19	1.13	N.S.	–	Cytokeratin 17	KRT17
201596_x_at	631.67	491.23	1.29	N.S.	–	Cytokeratin 18	KRT18
201650_at	1857.37	1913.20	0.97	N.S.	–	Cytokeratin 19	KRT19
213953_at	9.80	14.80	0.66	N.S.	–	Cytokeratin 20	KRT20
218963_s_at	364.33	1345.23	0.27	N.S.	–	Cytokeratin 23	KRT23
220267_at	981.43	11.83	82.94	$p < 0.01$	CONTROLS	Cytokeratin 24	KRT24
237905_at	19.90	14.83	1.34	N.S.	–	Cytokeratin 25A	KRT25A
240388_at	10.47	17.53	0.60	$p < 0.01$	–	Cytokeratin 25C	KRT25C
212581_x_at	8359.25	8409.20	0.99	N.S.	–	Glyceraldehyde-3-phosphate dehydrogenase	GAPDH
213867_x_at	8336.60	7973.45	1.04	N.S.	–	Beta actin	ACTB
201217_x_at	10441.95	10369.80	1.01	N.S.	–	Ribosomal protein L3	RPL3

Probe Set ID: affymetrix unique identification of each probe set. Mean controls: average expression for all normal human native oral mucosa samples analyzed in this work. Mean constructs: average expression for all bioengineered oral mucosa samples kept *in vitro*. Fold-change: mean controls/mean constructs. Rank test: p value for the test of ranks carried out in this study. Samples showing higher expression: type of sample that shows significantly higher expression for that specific probe set (controls or bioengineered oral mucosa); (–) indicates that none of the samples showed significantly higher expression of that probe set.

(5,38). Immunohistochemical expression of cytokeratin 10 by *in vivo* samples was comparable to that of normal native oral mucosa, which implies that these artificial samples might be able to reproduce the role of native epithelium as a tight protective barrier that allows the epithelium to respond rapidly to a range of stimuli (31). Finally, the tissues evaluated *in vivo*, as is the case of native controls, did not express the low differentiation markers that were found in *in vitro* samples, including cytokeratins 7, 8, 18 and 19.

With regard to the fibrin–agarose stroma substitute, our results revealed that this type of biomaterial is able to integrate into the host tissues, and that no adverse reactions or inflammatory responses were initiated as a consequence. Interestingly, human nuclear mitotic apparatus protein immunostaining of *in vivo* samples evaluated in nude mice demonstrated that a high number of mouse host cells were able to migrate and invade the artificial stroma along with the implanted human

fibroblasts. All of these factors suggest a complete integration of human oral fibroblasts and mice stromal cells into the fibrin–agarose artificial stroma.

Furthermore, the cytokeratin expression profile that we found in the artificial tissue constructs correlated very well with the structure of the different samples. In fact, oral mucosa substitutes evaluated *in vivo* showed specific histological specialization structures at the epithelial–stromal junction (rete ridges and chorial papillae), which play an important role in maintaining the oxygen and nutrient supply to the epithelium *in vivo* (28). The epithelia of both normal native controls and *in vivo* samples are probably exposed to a relative degree of hypoxia as a result of the avascular nature of the epithelium. For that reason, both samples would develop these specialized structures that allow the epithelium to interdigitate with the stroma and facilitate epithelial nutrition and oxygenation. By contrast, in *in vitro* samples submitted to submerged culture, epithelial cells would be able to obtain high amounts of oxygen and nutrients directly from the culture medium that surrounds the epithelium. Thus, these cells would not be exposed to differentiation induction, and typical structures of the chorion and the epithelium (rete ridges and chorial papillae) would not develop.

In summary, in this work we performed several *in vitro* and *in vivo* quality-control analyses (including cytokeratin expression, angiogenesis, cell proliferation and tissue–graft integration analysis) on human artificial oral mucosa generated by tissue engineering. In this way, we were able to establish the relationship between cell differentiation of the epithelial layers and the behavior of the artificial oral mucosa implanted on athymic nude mice. These results enabled us to provide a better understanding of the cytokeratin profiles that characterize the different stages of development and maturation of the epithelium of the oral mucosa substitutes developed in the laboratory by tissue engineering and to establish the usefulness of the fibrin–agarose model both *in vitro* and *in vivo*.

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