RESEARCH ARTICLE

Time-course study of histological and genetic patterns of differentiation in human engineered oral mucosa

M. Alaminos[1,2]*, I. Garzón[1], M. C. Sánchez-Quevedo[1], G. Moreu[3], M. González-Andrades[1], A. Fernández-Montoya[1,4] and A. Campos[1]

[1]*Department of Histology, University of Granada, Spain*
[2]*Fundación FIBAO and University Hospital San Cecilio, Granada, Spain*
[3]*Department of Stomatology, University of Granada, Spain*
[4]*Transfusion Center and Tissue Bank of Granada (CRTS), Spain*

Abstract

The lack of sufficient oral mucosa available for intra-oral grafting is a major surgical problem, and new sources of oral tissues for clinical use are needed. In this regard, some models of engineered oral mucosa have been reported to date, but little is known about the structural and genetic mechanisms that occur during the process of development and maturation of these tissue substitutes. We have carried out a time-course study of the genes and morphological patterns of cell and tissue differentiation that develop in oral mucosa constructs after 3, 7, 11 and 21 days of development. Our electron microscopy and microarray analyses demonstrated that the oral mucosa constructs generated by tissue engineering undergo a progressive process of cell differentiation with the sequential formation and maturation of several layers of epithelium (with expression of stratifin, sciellin, involucrin, trichohyalin and kallikrein 7), intercellular junctions (with expression of plakophilin, desmocollin, desmoglein and cadherins), cytokeratins, a basement membrane (laminins, collagen IV) and the extracellular matrix (biglycan, matrix metalloproteinases). In conclusion, although the level and type of keratinization developed *in vitro* could be different, the oral mucosa substitutes were very similar to the native tissues. Copyright © 2007 John Wiley & Sons, Ltd.

1. Introduction

The lack of sufficient oral mucosa available for intra-oral grafting has been dealt with so far by using split-thickness skin or oral mucosa grafts harvested from donor sites (Izumi *et al.*, 2000). These procedures, however, often require more than one surgical procedure and are associated with morbidity at the donor and the recipient sites.

Construction of artificial organs by tissue engineering (TE) is one of the research fields that has experienced major progress during recent years (Atala, 2000). By using TE techniques, different researchers have developed efficient substitutes of different organs and tissues for therapeutic use, including, among others, human skin (Meana *et al.*, 1998; Llames *et al.*, 2004), cornea (Nishida, 2003; Reichl *et al.*, 2004; Alaminos *et al.*, 2006), bone (Mohammadi *et al.*, 2007) and blood vessels (Pascual *et al.*, 2004). Regarding the oral mucosa, several groups have developed different models of artificial tissues that could eventually be used as organotypic substitutes of the human oral mucosa for reconstruction of oral and maxillofacial tissues (Lauer and Schimming, 2001;

Correspondence to: M. Alaminos, Department of Histology, University of Granada, Avenida de Madrid 11, E-18012 Granada, Spain. E-mail: malaminos@histolii.ugr.es

Schultze-Mosgau et al., 2004; Sanchez-Quevedo et al., 2007). Clinical uses for a tissue-engineered oral mucosa would mainly include intra-oral defects, such as repair of acquired or congenital oral mucosal defects, but also, and strikingly, extra-oral defects, e.g. reconstruction of the cornea (Nakamura et al., 2006), eyelids, conjunctiva, oesophagus, trachea, bladder, urethra or vagina (Feinberg et al., 2005). Other potential uses of artificial oral mucosa constructs are in vitro models to study the biology and pathology of mucosa, and use of the mucosa as a vehicle for delivery and expression of transduced genes (Feinberg et al., 2005). Most oral mucosa substitutes use three-dimensional (3D) co-cultures of the main cell types of the oral mucosa, embedded in different biomaterials. In this regard, our group has recently developed a stromal substitute made of a mixture of fibrin and agarose that demonstrated good biomechanical and structural properties when used for TE purposes (Alaminos et al., 2006; Sanchez-Quevedo et al., 2007).

In designing a TE oral mucosa, it is critical that the constructed tissues have the innate functions seen with natural oral mucosa (especially, acting as a protective covering for the underlying chorion), and that these structures be interactive with their environment, that is, they communicate with the surrounding cells via signalling mechanisms. For these reasons, it is necessary that the oral mucosa constructs emulate the anatomy and the structure of the native organ, and that the level of differentiation is similar in the constructs and in the damaged tissues to be replaced (Feinberg et al., 2005). Hence, evaluation of the degree of cell and tissue differentiation of the constructs developed by TE is mandatory before the artificial tissues can be used clinically. Therefore, it will be necessary to verify that the developed tissues reproduce the structural patterns of differentiation and gene expression that are linked to keratinocyte maturation in native normal oral mucosa, where up to five different patterns of maturation have been described (Moreu et al., 1993; Sanchez-Quevedo et al., 1994). These patterns, initially reported by Dourov (1984) and Kullaa-Mikkonen (1986, 1987) in the gingival epithelium of normal donors, are strongly related to the degree of differentiation of the keratinocytes (Southgate et al., 1987; Moreu et al., 1993; Sanchez-Quevedo et al., 1994). Thus, keratinocytes with surface microvilli (pattern type I) correspond to cells with the lowest level of differentiation, whereas cells with pits (pattern type V) are at the last stages of keratinocytic differentiation (Moreu et al., 1993; Sanchez-Quevedo et al., 1994). Patterns type II, III and IV are usually found in cells with intermediate degrees of differentiation. Many of these patterns are associated with the cell–cell adhesions that exist in the cells and, for example, the deepest layers of the oral mucosa epithelium display a high number of desmosomes and are covered exclusively by microvilli (Hodgkins et al., 1978).

In this study, we have correlated the morphostructural patterns of differentiation, as determined by electron microscopy (EM), and the gene expression profiles in a model of bioengineered human oral mucosa using fibrin–agarose scaffolds.

2. Materials and methods

2.1. Generation of primary cultures of oral mucosa fibroblasts and keratinocytes

Twenty-five small biopsies corresponding to normal human oral mucosa were obtained from healthy donors undergoing minor oral surgery under local anaesthesia. All tissues were washed and transported in Dulbecco's Modified Eayle's Medium (DMEM) medium with antibiotics and antimycotics (500 U/ml penicillin G, 500 mg/ml streptomycin, and 1.25 mg/ml amphotericin B) and processed in the following 24 h. This work was approved by the institutional research committee.

To obtain primary cultures of human oral fibroblasts, all biopsies were washed in phosphate buffered saline (PBS) and incubated overnight at 37 °C in a solution of 2 mg/ml *Clostridium histolyticum* collagenase I (Gibco BRL Life Technologies, Karlsruhe, Germany) in DMEM. Detached fibroblasts were collected by centrifugation and expanded in culture flasks containing DMEM medium supplemented with antibiotics (100 U/ml penicillin G, 100 mg/ml streptomycin, and 0.25 mg/ml amphotericin B) and 10% fetal bovine serum (FBS). To establish primary cultures of oral keratinocytes, undigested oral epithelium was washed in PBS, cut into small explant pieces and co-cultured with a layer of mitomycin C-treated (10 μg/ml) 3T3 feeder cells ($8–10 \times 10^3$ cell/cm^2; Rheinwald and Green, 1975). Keratinocytes culture medium was a 3 : 1 mixture of DMEM and Ham's F12 supplemented with 10% fetal calf serum, 1% antibiotics, 24 μg/ml adenine, 0.4 mg/ml hydrocortisone, 5 mg/ml insulin, 10 ng/ml epidermal growth factor, 1.3 ng/ml triiodothyronine and 8 ng/ml cholera toxin.

All cells were incubated at 37 °C in 5% carbon dioxide under standard culture conditions. The medium was changed every 3 days and subcultivation of the cultured cells was carried out using a trypsin 0.5 g/l–EDTA 0.2 g/l solution at 37 °C for 10 min. All cells used for experimentation were at passages 1–4 (Alaminos et al., 2007).

2.2. Construction of human oral mucosa substitutes by TE

Development of human oral mucosa constructs in the laboratory was carried out using the following previously published methods (Sanchez-Quevedo et al., 2006). Briefly, a stromal substitute made of human fibrin and 0.1% agarose, with fibroblasts immersed within, was developed using Transwell culture inserts with 0.4 μm porous membrane (Costar, Corning Inc., Corning, NY, USA). Twenty-four hours after the stromal matrix substitute had solidified, human oral keratinocytes were

seeded on top of the constructed stroma (approximately 1 000 000 keratinocytes/25 ml construct), and cultured for 21 days submerged in keratinocyte culture medium. Specimens corresponding to oral mucosa substituted were analysed at different times after keratinocyte seeding (3, 7, 11 and 21 days). In this study, a total of 15 human oral mucosa constructs were analysed.

2.3. Microscopic evaluation of the human oral mucosa substitutes

Samples for scanning EM (SEM) were fixed in 2.5% glutaraldehyde and postfixed in 1% osmium tetroxide for 90 min. After fixation, the samples were dehydrated in increasing concentrations of acetone (30%, 50%, 70%, 95% and 100%), critical point-dried, mounted on aluminium stubs, sputter-coated with gold according to routine procedures (Sanchez-Quevedo *et al.*, 1994) and examined in a Quanta 200 scanning electron microscope (FEI, Eindhoven, The Netherlands), using a high vacuum mode. For transmission EM (TEM), samples were fixed, postfixed and dehydrated as described above for SEM, embedded in Spurr's resin and cut into ultrathin sections using an ultramicrotrome. Then, the sections were stained with aqueous uranyl acetate and lead citrate and examined with a EM902 transmission electron microscope (Carl Zeiss Meditec Inc., Oberkochen, Germany).

2.4. Genome-wide gene expression analysis using oligonucleotide microarrays

Total RNA corresponding to oral mucosa constructs was extracted using the Qiagen RNeasy System (Qiagen, Mississauga, Ontario, Canada), according to the manufacturers' recommendations. RNA concentration was determined by absorbency at 260 nm, and quality was verified by using a Bioanalyser (Agilent). Total cDNA was synthesized with 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). Labelled nucleic acid target was hybridized (45 °C for 16 h) to Affymetrix Human Genome U133 plus 2.0 oligonucleotide arrays. After automated washing and staining, absolute values of expression were calculated and Normalized from the scanned array, using Affymetrix Microarray Suite.

For the analysis of the microarray data, average expression corresponding to each group of comparison was calculated for each probe set. The mean expression ratio (fold-change) was then calculated by dividing the mean expression of one group of comparison by that of the other group (Alaminos *et al.*, 2003), and genes with a minimum fold-change of 10 were selected. Gene Ontology analysis of the selected genes was performed using BiNGO (http://www.psb.ugent.be/cbd/papers/BiNGO/), a plug-in for the program Cytoscape (Maere *et al.*, 2005). The set of selected genes was tested for enrichment of any

GO category relating to 'Biological Process', as compared to all annotated genes represented on the array. Scores were evaluated based on the hypergeometric distribution and Bonferroni correction for multiple testing. Thus, the *p* value reflects the likelihood that one observes such an enrichment or higher by chance alone (Boyer *et al.*, 2006).

To identify genes whose expression was statistically significantly associated with specific groups of samples, we used the significance analysis for microarrays (SAM) function of the program TIGR MeV (MultiExperiment Viewer 3.1; Institute for Genomic Research, Rockville, MD, USA; Saeed *et al.*, 2003). We used a δ value that allowed us a false discovery rate of <1 (i.e. < one gene is falsely named). The program is available at http://www.tigr.org/software/tm4. TIGR MeV was also used for hierarchical cluster analysis of the samples by standardizing each expression level of each gene and each sample to mean = 0 and variance = 1.

3. Results

3.1. EM evaluation of the oral mucosa constructs

Construction of oral mucosa substitutes by TE was efficiently carried out by using the methods and techniques descibed above.

TEM analysis of the initial oral mucosa constructs (3 days after keratinocyte seeding) revealed the presence of a single layer of epithelial cells with a smooth surface and small microvilli covering the stromal substitute (Figure 1A). SEM evaluation of the constructs showed that the epithelial cells covered most of the surface of the oral mucosa, with some areas of the stromal substitute exposed and uncovered by keratinocytes (Figure 1B). However, analysis of samples corresponding to day 7 of epithelial maturation revealed that all the surface of the constructs was covered by keratinocytes and that these cells showed more developed microvilli (pattern type I of keratinocytic differentiation) than samples at day 3 (Figure 1C). In addition, TEM analysis of day 7 samples showed a rudimentary basement membrane developing in the interface between the epithelium and the stromal substitute (Figure 1D). Also, TEM analysis of oral mucosa samples at day 11 of epithelial maturation demonstrated the presence of an epithelium with several layers of cells on top of the constructs (Figure 2A). By SEM, the surface of some of the cells showed straight parallel rows of microplicae resembling a pattern II of keratinocytic differentiation, whereas other cells showed smooth surfaces and microvilli (pattern I) (Figure 2B). At this stage of maturation, cell interdigitations and rudimentary desmosomal cell junctions were present (Figure 2A). Finally, samples at day 21 of maturation displayed the highest number of epithelial cell layers (Figure 3A). The surface of these constructs was covered by keratinocytes with pattern II of differentiation, although some curved
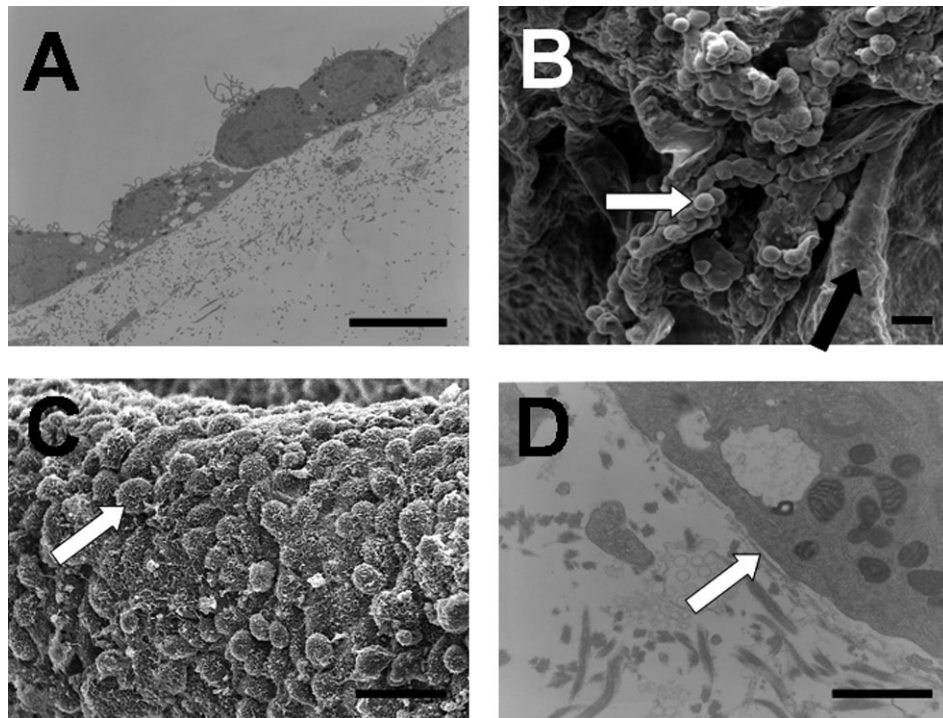
Figure 1. EM analysis of the 3 and 7 day oral mucosa substitutes. (A) TEM evaluation of the 3 day constructs reveals the presence of a cell monolayer on top of the oral mucosa substitute. (B) SEM analysis of the oral mucosa substitutes at 3 days of development shows that some areas of the sample are covered by keratinocytes (white arrow), whereas the stromal substitute is exposed in other areas (dark arrow). (C) Samples of 7 days of development display surface microvilli (pattern type I of keratinocytic differentiation) by SEM (arrow). (D) Development of a rudimentary basement membrane in the interface between the epithelium and the stromal substitute in 7 day oral mucosa substitutes (arrow). Bars = 10 μm (A, B), 20 μm (C) and 100 nm (D)
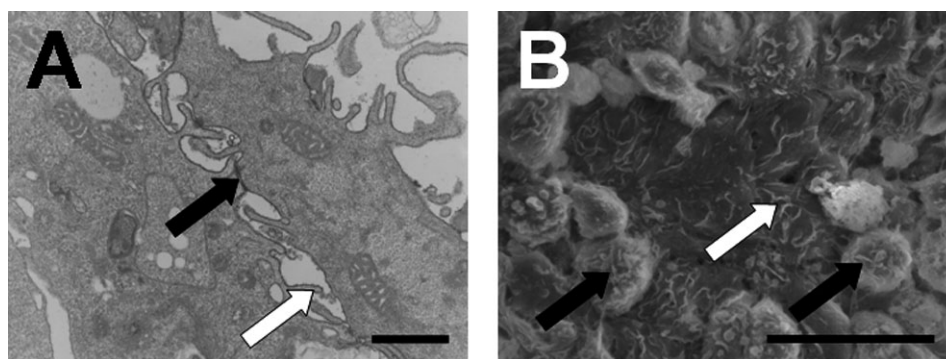

Figure 2. EM analysis of the 11 day oral mucosa substitutes. (A) Epithelial cells on top of the oral mucosa construct are joined by cell interdigitations (white arrow) and rudimentary desmosomes (black arrow). (B) SEM evaluation of the samples uncovers the presence of keratinocytes with microvilli on the surface (pattern I, black arrow) and straight parallel rows of microplicae (pattern II, white arrow). Bars = 1 μm (A), 20 μm (B).

rows of microplicae resembling pattern III began to appear (Figure 3B). In addition, TEM evaluation of the artificial tissues showed that the cell junctions are mostly supported by well-developed desmosomes (Figure 3C).

Furthermore, TEM evaluation of the stromal substitutes of human fibrin and 0.1% agarose demonstrated that oral mucosa fibroblasts immersed in these scaffolds became elongated and spread out in the stromal lattice after 1–3 days of culture (Figure 3D). No contraction of the fibrin–agarose gels was observed in any case. Most of the fibroblasts in the stromal substitute displayed a biosynthetic phenotype, with several nucleoli and a well-developed RER (Figure 3D).

3.2. Genome-wide analysis of gene expression in human oral mucosa constructs

To carry out a comprehensive gene expression analysis in oral mucosa substitutes, we used oligonucleotide arrays with 54 675 probe sets. In the first place, the expression analysis revealed that 1085 genes were at least 10-fold upregulated in the oral mucosa substitutes, with

a stratified epithelium on top in comparison to monolayer oral mucosa (Supplementary Table 1), whereas 627 genes were downregulated in the stratified samples. The analysis of all these genes using the programs Cytoscape and BiNGO demonstrated that most of the 1085 genes that were overexpressed in stratified oral mucosa constructs belonged to specific pathways and gene functions. As shown in Figures 4 and 5, there was a significant enrichment in genes with a role in epidermis development, keratinocyte differentiation, extracellular matrix, cell adhesion and cell junctions, suggesting a higher level of cell differentiation in the stratified oral
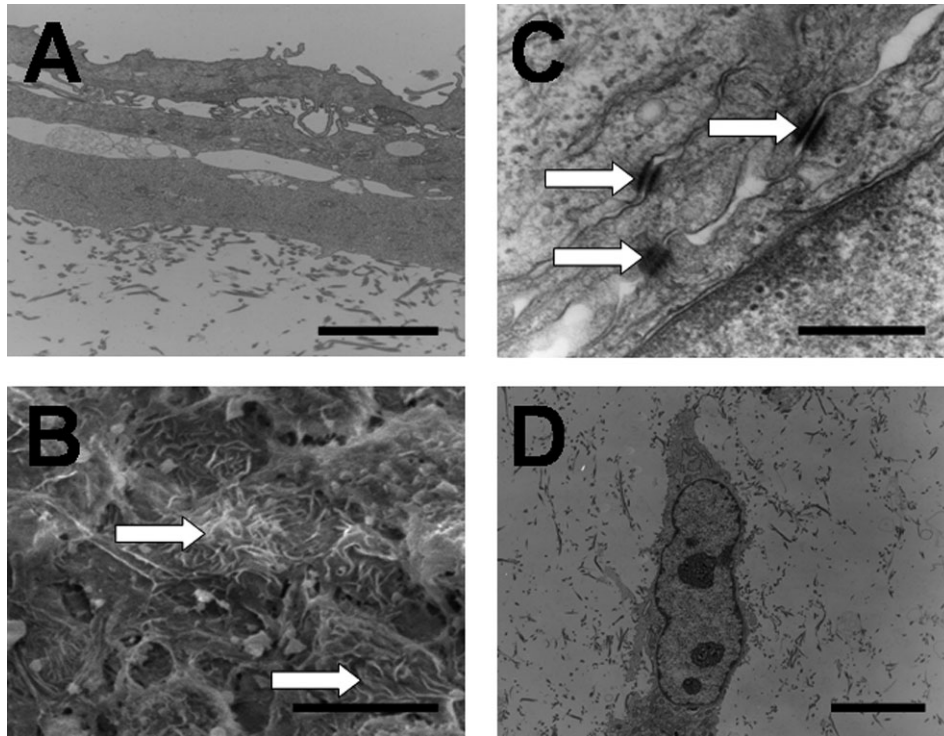

Figure 3. TEM and SEM analysis of oral mucosa substitutes at 21 days of culture. (A) The constructed epithelium shows several layers of keratinocytes on the fibrin–agarose stromal substitute. (B) The surface of the epithelium consists of cells with curve rows of microplicae on the surface (pattern III; arrows). (C) TEM evaluation of the oral mucosa substitutes shows numerous well-developed desmosomes joining the epithelial keratinocytes (arrows). (D) Human oral mucosa fibroblast immersed in the fibrin–agarose scaffold after 21 days of maturation. Bars = 3 μm (A), 10 μm (B, D) and 100 μm (C)
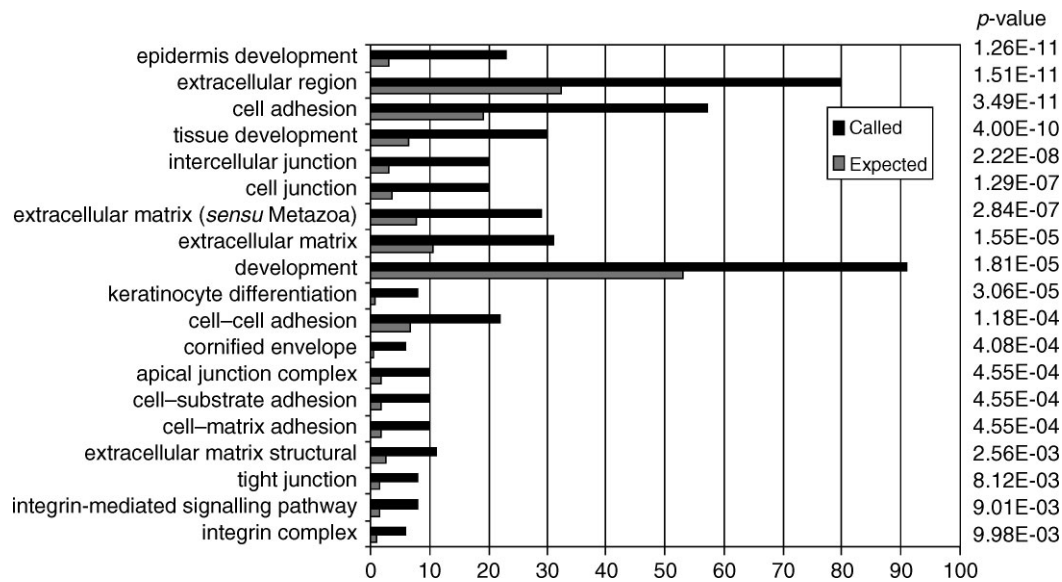

Figure 4. Gene Ontology analysis of genes overexpressed in oral mucosa constructs with a stratified epithelium on top. Black bars represent the observed percentage of overexpressed genes in a particular GO category. Grey bars represent the percentage expected on the basis of all GO-annotated genes on the oligonucleotide array. The significance (*p* value) of this enrichment is based on a hypergeometric distribution

Figure 5. Significant results from the GO analysis using BiNGO are shown within their hierarchical tree of the GO classification system. Each node represents a single GO category. Starting from the most general category, genes are classified stepwise into smaller and more specific categories, which are shown as daughter branches. Branches that were not enriched in our analysis are not shown. The number of genes in each GO category is represented by the node size and the statistical enrichment (*p* value from a hypergeometric distribution test) of these genes is represented as pseudocolour (red, highly enriched; orange, significantly enriched; yellow, potentially enriched; white, non-enriched)
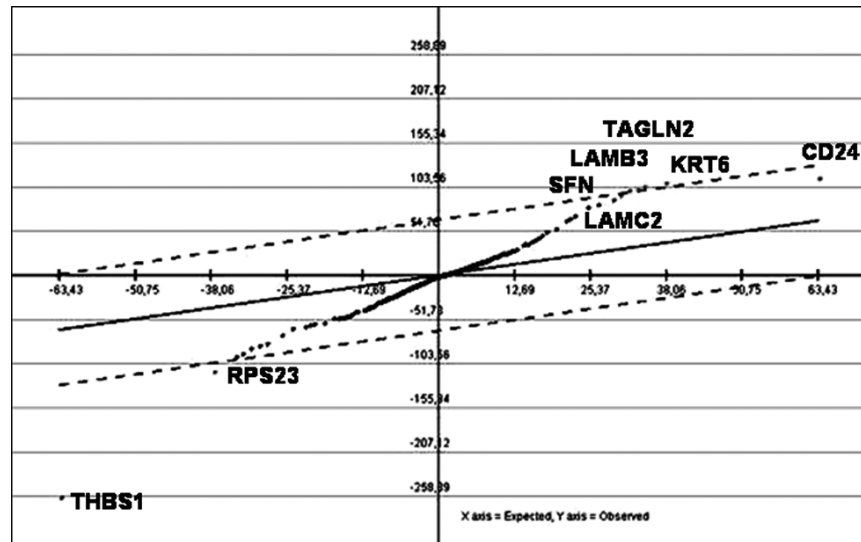
Figure 6. Significance analysis for microarrays (SAM) for the comparison of constructed oral mucosa samples with monolayer epithelium vs. samples with stratificated epithelium and for all genes in the array. The six genes showing positive significant association with samples with stratified epithelium are shown at the right side of the panel, while the two genes overexpressed in samples with monolayer epithelium are shown at the left

substitutes. No specific pathways could be detected among the 627 downregulated genes.

On the other hand, when the oral mucosa substitutes were compared using SAM (Figure 6), we found that six genes were upregulated in mature, stratified constructs, whilst two genes were upregulated in immature oral mucosa with a single layer of epithelial cells on top. Interestingly, the six genes whose expression was higher in stratified samples have been implicated in relevant roles related to the synthesis of the basement lamina (laminins β3 and γ2), epithelial stratification (statifin), regulation of the cell differentiation (CD24), development (transgelin 2) and ectoderm development (keratin 6A), suggesting a higher level of maturation of these samples. In contrast, the two genes that were found upregulated in the immature oral mucosa construct were related to ribosomal synthesis (ribosomal protein S23) and angiogenesis (thrombospondin I).

4. Discussion

The final goal of TE is to develop in the laboratory tissue substitutes that are suitable for clinical use. For that reason, all constructed tissues must be previously analysed and evaluated to ensure that their structure is similar to that of the native, normal tissues to be substituted, and that the functions normally carried out by the native tissues can now be reproduced at both the *in vitro* and *in vivo* levels by the tissue substitutes. In this milieu, previous results recently published by our group revealed that human oral mucosa cells isolated and cultured according to the methods described here showed microanalytical ionic profiles that were compatible with excellent levels of cell viability (Sanchez-Quevedo *et al.*, 2007). In addition, immunohistochemical analyses of

the oral mucosa substitutes generated in the laboratory suggested that our model of fibrin–agarose scaffold was very appropriate for TE of oral mucosa (Sanchez-Quevedo *et al.*, 2007) and cornea (Alaminos *et al.*, 2006).

Although some models of artificial oral mucosa has been reported to date (Lauer and Schimming *et al.*, 2001; Schultze-Mosgau *et al.*, 2004; Sanchez-Quevedo *et al.*, 2007), little is known about the structural and genetic mechanisms that occur during the process of development and maturation of these engineered tissues. In the present work, we have carried out a time-course study of the morphological patterns of cell and tissue differentiation that arise at different stages of this process, including, among others, epithelial stratification and the development of cell junctions and the basement lamina. At the same time, we performed a genome-wide comprehensive gene expression analysis of the oral mucosa substitutes at different stages of development, using high-density oligonucleotide microarrays (Uno and Ueda, 2007). This way, we could establish a relationship between the morphological patterns of differentiation found in the tissues and the expression of genes with a role in the development of those patterns. This information allowed us to contribute to a better understanding of the processes involved in the development and maturation of the artificial oral mucosa tissues generated in the laboratory by TE.

EM analysis of the oral mucosa substitutes revealed the presence of an epithelium on top of the substitutes, and that this epithelium underwent a progressive time-dependent process of histodifferentiation. This process involved the stratification of the epithelial cells after 11 and 21 days of maturation, along with the progressive formation and maturation of desmosomes and cell–cell interdigitations. At the same time, our genetic analysis demonstrated that several

genes with a role in epidermis development and keratinocyte differentiation were upregulated in oral mucosa constructs at the final stages of maturation, including sciellin, involucrin, trichohyalin and kallikrein 7. First, sciellin is a precursor protein that is normally expressed by terminally differentiated keratinocytes of human keratinizing tissues (Kvedar *et al.* 1992), and its presence can be associated with mature keratinocytes in our multilayered oral mucosa substitutes. Second, involucrin is expressed by several layers of the stratified epithelium of native human oral mucosa (Barrett *et al.* 2005) and it has previously been detected in human oral mucosa substitutes developed by TE using collagen scaffolds (Imaizumi *et al.* 2004). Different researchers have previously reported that involucrin is crosslinked into the cornified envelope of oral mucosa keratinocytes during terminal differentiation and, thus, can be used as a marker of epithelial differentiation (Presland and Dale, 2000). Similarly, epidermal and oral keratinocytes express the differentiation marker trichohyalin, which associates with the keratin cytoskeleton during terminal differentiation (Presland and Dale, 2000). Finally, kallikrein 7 (stratum corneum chymotryptic enzyme) is thought to be involved in the physiological process of epithelial desquamation through the proteolysis of intercellular adhesion molecules, such as desmoglein (Ekholm *et al.*, 2000). The overexpression of all these differentiation markers in the mature oral mucosa substitutes suggests that these susbstitutes display genetic similarities with native normal oral mucosa, and could probably exert the same physiological functions *in vivo*. In contrast, immature oral mucosa constructs with a monolayer epithelium on top tended to express a number of genes related to cell proliferation (ribosomal and DNA synthesis proteins, cell membrane components, etc.), rather than genes with a role in mature epithelia. Furthermore, the presence of a stratified epithelium in the oral mucosa constructs coincided with the expression of the gene encoding for stratifin, which has been associated with the stratification of different kinds of epithelia, including the oral mucosa epithelium (Katz and Taichman, 1999).

All these features correlated very well with the progressive development of different types of keratinocytic differentiation patterns in our oral mucosa constructs. Hence, SEM analysis revealed that the keratinocytes of the oral mucosa substitutes initially presented microvilli on their surface (pattern type I), suggesting that the degree of differentiation of these cells was very low (Moreu *et al.*, 1993; Sanchez-Quevedo *et al.*, 1994). However, samples corresponding to 11 and 21 days of maturation tended to show other patterns of keratinocyte differentiation that indicated that these cells were more differentiated. The development of pattern types II and III correlated very well with the expression of genes with a role in epithelial differentiation, discussed above. However, the absence of epithelial cells with pattern types IV and V in the oral mucosa substitutes suggests that the constructs do not reach *in vitro* the degree of maturation found in normal native oral mucosa *in vivo*.

In order to function correctly, stratified epithelia such as the oral mucosa have to maintain tight cell–cell adhesions in the living cells and retain the dead, keratinized squames as a protective sheath prior to being sloughed (Presland and Jurevic, 2002). Intercellular adherens junctions, including desmosomes, are cell adhesion complexes that link epithelial cells to each other and attach intermediate filaments to the cell surface (Presland and Jurevic, 2002). Desmosomes consist of two principal groups of proteins: the desmosomal cadherins (desmogleins and desmocollins), and the cytoplasmic proteins of the desmosomes (which link the desmosomal cadherins to the cytoplasmic keratin filaments; Green and Jones, 1996). In this study, we demonstrated the formation of different types of cell junctions in the oral mucosa substitutes, especially in the mature ones. In these samples, the identification of intercellular junctions by TEM overlapped with the expression of a great number of genes with a role in the synthesis of proteins involved in cell–cell junctions. In short, the presence of well-developed desmosomes in the constructs corresponding to 21 days of maturation was simultaneous with the expression of genes encoding for plakophilin 1, desmocollins 2 and 3, desmoglein 3, plakoglobin, corneodesmosin and cadherins (E-cadherin, CDH3, CDH26, protocadherin β4, protocadherin γA3), suggesting that the cells were actively synthesizing and forming this essential kind of intercellular unions. In addition, the expression of the gene *CD24*, with a role in regulation of the cell differentiation (Jevsek *et al.*, 2006), has recently been implicated in epithelial hemidesmosomes formation (Li *et al.*, 2007). The overexpression of different integrins (α6, β2, β3, β4 and β6 and integrin β-like 1) could be in relationship with the adhesion of the basal layer of keratinocytes and with hemidesmosomes formation. Furthermore, and although our EM analysis did not reveal the presence or other types of cell–cell junctions, the overexpression of genes related to tight junctions (claudin 1, cingulin, tight junction protein 3, CXADR, INADL, AMOTL1) and gap junctions (connexins 30 and 31), suggests that these kinds of intercellular unions might be developing in our constructs. All these results imply that the epithelium of the artificial tissues generated by TE could form a tight barrier that would mimic the *in vivo* properties of the native oral mucosa.

On the other hand, cytokeratins are a family of cytoplasmic proteins that are the predominant cytoskeletal proteins in all epithelia. These filaments function as stress-bearing structures within epithelial cells and are critical for the maintenance of cell shape and viability (Presland and Jurevic, 2002). For those reasons, the expression of the genes encoding for these proteins is strongly necessary for the keratinocytes to exert their functions as a protective barrier of the structures in the oral cavity. In our case, the microarray analysis showed that the oral substitutes at the 21st day of maturation strongly expressed cytokeratins 1, 4, 5, 6, 14, 16, 17 and 23. Expression

of the keratin pair K5–K14 is typical of most stratified epithelia (Presland and Jurevic, 2002), whereas keratin 4 is expressed by stratified, non-keratinized epithelia (Montenegro *et al.*, 1998). However, keratin 1 is expressed by keratinized epithelia like the human epidermis (Lessin *et al.*, 1988). Other cytokeratins, such as keratins 17 and 23, are expressed upon induction in different types of cells. In this regard, Kim *et al.* (2006) showed that keratin 17 is rapidly induced in wounded stratified epithelia and that it regulates cell growth through binding to the adaptor protein stratifin. All these findings suggest that the epithelial cells of the oral mucosa substitutes are synthesizing *in vitro* a high number of cytokeratins of different type, and that these cytokeratins might differ from those expressed by the native normal oral mucosa *in vivo*. Further studies are in need to show which cytokeratins are expressed by the oral mucosa substitutes *in vivo*.

Moreover, ultrastructural analysis of the tissues generated by TE revealed the presence of a basement lamina in the interface between the epithelium and the stromal substitute. The basement lamina is the anchoring complex joining the epithelium and the subjacent connective tissue of skin, oral mucosa and other organs and structures, and is essential for a proper attachment of the epithelium to the lamina propria. Structurally, the basement lamina consists of a lamina densa and a lamina lucida, which can be clearly identified using TEM techniques. The lamina densa layer is mainly formed by type IV collagen, which is organized in a net-like fashion (Timpl *et al.*, 1981). The non-collagenous constituents of the basement lamina mainly comprise laminin, entactin and proteoglycans (Kallioinen *et al.*, 1984). In our study, we detected that the formation of a rudimentary basement lamina in the oral mucosa substitutes was simultaneous with the expression of genes encoding for collagen IV, laminin $\gamma 2$, laminin $\alpha 3$ and other proteins, such as dystonin and hemicentin 1. These findings suggest that a basement lamina is developing between the oral mucosa epithelium and the artificial fibrin–agarose connective tissue with fibroblasts immersed within.

Regarding the stromal substitute of the oral mucosa generated by TE, different kinds of collagens were found overexpressed in the artificial tissues, including collagens I, III, IV, V and XI, along with other genes encoding for proteins of the extracellular matrix [biglycan (Schaefer *et al.*, 2005), matrix metallopeptidase 16 and 28, ADAM metallopeptidase, fibroblast growth factor 1, 18, fibroblast growth factor binding protein 1, etc.]. The expression of these genes suggests that the stromal substitute of fibrin–agarose is likely being progressively modified by the fibroblasts immersed within to generate a structure chemically similar to the native, normal oral mucosa. Noticeably, some genes with a role in angiogenesis [thrombospondin I (de Fraipont *et al.*, 2000; Thakar *et al.*, 2005) in the immature samples and JAG1 (Li *et al.*, 2006) and CEACAM1 (Ergun *et al.*, 2000) in mature oral mucosa constructs] were also overexpressed in the oral mucosa substitutes, implying that the artificial tissues could be

trying to attract blood vessels from peripheral tissues, even when the tissues are kept *in vitro*.

Altogether, our results suggest that the artificial oral mucosa substitutes generated in the laboratory by TE display numerous structural and genetic similarities with human native oral mucosa in both the epithelium and the stroma. Our EM and microarray analyses demonstrate that the tissues undergo a process of cell differentiation with the progressive formation and maturation of several layers of epithelium, intercellular junctions, basement membrane and extracellular matrix. As a result of this process of cell and tissue development and maturation, and although the level and type of keratinization could be different, the oral mucosa substitutes appear to be very similar to the native tissues. Although our findings appear to satisfy the criteria for utilization of the oral constructs in the oral cavity, *in vivo* studies are still needed to uncover the potential utility of these fibrin–agarose oral mucosa constructs for clinical purposes.

Supplementary material

Supplementary electronic material for this paper is available in Wiley InterScience at: http://www.mrw. interscience.wiley.com/suppmat/1932-6254/suppmat/

Acknowledgements

References

Alaminos M, Mora J, Cheung NK, *et al.* 2003; Genome-wide analysis of gene expression associated with MYCN in human neuroblastoma. *Cancer Res* **63**: 4538–4546.

Alaminos M, Sanchez-Quevedo MC, Munoz-Avila JI, *et al.* 2007; Evaluation of the viability of cultured corneal endothelial cells by quantitative electron probe X-ray microanalysis. *J Cell Physiol* **211**: 692–698.

Alaminos M, Sanchez-Quevedo MC, Muñoz-Avila JI, *et al.* 2006; Construction of a complete rabbit cornea substitute using a fibrin–agarose scaffold. *Invest Ophthalmol Vis Sci* **47**: 3311–3317.

Atala A. 2000; Tissue engineering of artificial organs. *J Endourol* **14**: 49–57.

Barrett AW, Morgan M, Nwaeze G, Kramer G, Berkovitz BK. 2005; The differentiation profile of the epithelium of the human lip. *Arch Oral Biol* **50**: 431–438.

Boyer LA, Plath K, Zeitlinger J, *et al.* 2006; Polycomb complexes repress developmental regulators in murine embryonic stem cells. *Nature* **441**: 349–353.

de Fraipont F, El Atifi M, Gicquel C, *et al.* 2000; Expression of the angiogenesis markers vascular endothelial growth factor -A, thrombospondin-1, and platelet-derived endothelial cell growth factor in human sporadic adrenocortical tumors: correlation with genotypic alterations. *J Clin Endocr Metab* **85**: 4734–4741.

Dourov N. 1984; Scanning electron microscopy contribution in oral pathology. *Scan Electron Microsc* **1**: 243–248.

Ekholm IE, Brattsand M, Egelrud T. 2000; Stratum corneum tryptic enzyme in normal epidermis: a missing link in the desquamation process? *J Invest Dermatol* **114**: 56–63.

Ergun S, Kilic N, Ziegeler G, et al. 2000; CEA-related cell adhesion molecule 1: a potent angiogenic factor and a major effector of vascular endothelial growth factor. *Mol Cell* **5**: 311–320.

Feinberg SE, Aghaloo TL, Cunningham LL Jr. 2005; Role of tissue engineering in oral and maxillofacial reconstruction: findings of the 2005 AAOMS Research Summit. *J Oral Maxillofac Surg* **63**: 1418–1425.

Green KJ, Jones JC. 1996; Desmosomes and hemidesmosomes: structure and function of molecular components. *FASEB J* **10**: 871–881.

Hodgkins JFW, Watkins R, Walker DM. 1978; Correlated scanning and transmission electron microscopy of cell surfaces at various levels in human gingival epithelium. *Arch Oral Biol* **23**: 355–357.

Imaizumi F, Asahina I, Moriyama T, Ishii M, Omura K. 2004; Cultured mucosal cell sheet with a double layer of keratinocytes and fibroblasts on a collagen membrane. *Tissue Eng* **10**: 657–664.

Izumi K, Terashi H, Marcelo CL, Feinberg SE. 2000; Development and characterization of a tissue-engineered human oral mucosa equivalent produced in a serum-free culture system. *J Dent Res* **79**: 798–805.

Jevsek M, Jaworski A, Polo-Parada L, et al. 2006; CD24 is expressed by myofiber synaptic nuclei and regulates synaptic transmission. *Proc Natl Acad Sci USA* **103**: 6374–6379.

Kallioinen M, Autio-Harmainen H, Dammert K, Risteli J, Risteli L. 1984; Basement membrane laminin and type IV collagen in various benign and malignant adnexal tumors of the skin: an immunohistochemical study. *J Invest Dermatol* **83**: 276–280.

Katz AB, Taichman LB. 1999; A partial catalog of proteins secreted by epidermal keratinocytes in culture. *J Invest Dermatol* **112**: 818–821.

Kim S, Wong P, Coulombe PA. 2006; A keratin cytoskeletal protein regulates protein synthesis and epithelial cell growth. *Nature* **441**: 362–365.

Kullaa-Mikkonen A. 1986; Scanning electron microscopic study of surface of human oral mucosa. *Scand J Dent Res* **94**: 50–56.

Kullaa-Mikkonen A. 1987; Scanning electron microscopy in oral mucosal research: a review. *Scanning Microsc* **1**: 1145–1155.

Kvedar JC, Manabe M, Phillips SB, Ross BS, Baden HP. 1992; Characterization of sciellin, a precursor to the cornified envelope of human keratinocytes. *Differentiation* **49**: 195–204.

Lauer G, Schimming R. 2001; Tissue-engineered mucosa graft for reconstruction of the intra-oral lining after freeing of the tongue: a clinical and immunohistologic study. *J Oral Maxillofac Surg* **59**: 169–175.

Lessin SR, Huebner K, Isobe M, Croce CM, Steinert PM. 1988; Chromosomal mapping of human keratin genes: evidence of non-linkage. *J Invest Dermatol* **91**: 572–578.

Li H, Yu B, Zhang Y, et al. 2006; Jagged1 protein enhances the differentiation of mesenchymal stem cells into cardiomyocytes. *Biochem Biophys Res Commun* **341**: 320–325.

Li Y, Lin X, Kilani RT, Jones JC, Ghahary A. 2007; 14–3–3 sigma isoform interacts with the cytoplasmic domain of the transmembrane BP180 in keratinocytes. *J Cell Physiol* [Epub ahead of print].

Llames SG, Del Rio M, Larcher F, et al. 2004; Human plasma as a dermal scaffold for the generation of a completely autologous bioengineered skin, *Transplantation* **77**: 350–355.

Maere S, Heymans K, Kuiper M. 2005; BiNGO: a Cytoscape plug-in to assess overrepresentation of gene ontology categories in biological networks. *Bioinformatics* **21**: 3448–3449.

Meana A, Iglesias J, Del Rio M, et al. 1998; Large surface of cultured human epithelium obtained on a dermal matrix based on live fibroblast-containing fibrin gels. *Burns* **24**: 621–630.

Mohammadi Y, Soleimani M, Fallahi-Sichani M, et al. 2007; Nanofibrous poly(ε-caprolactone)/poly(vinyl alcohol)/chitosan hybrid scaffolds for bone tissue engineering using mesenchymal stem cells. *Int J Artif Organs* **30**: 204–211.

Montenegro MA, Ibarra GC, Rojas M. 1998; Cytokeratin expression in human and mouse gingival epithelia. *Rev Chil Anat* **16**: 211–217.

Moreu G, Sanchez-Quevedo MC, Lopez-Escamez JA, et al. 1993; Cell surface patterns in normal human oral gingival epithelium. A quantitative scanning electron microscopy approach. *Histol Histopathol* **8**: 47–50.

Nakamura T, Ang LP, Rigby H, et al. 2006; The use of autologous serum in the development of corneal and oral epithelial equivalents in patients with Stevens–Johnson syndrome. *Invest Ophthalmol Vis Sci* **47**: 909–916.

Nishida K. 2003; Tissue engineering of the cornea. *Cornea* **22**: S28–34.

Pascual G, Rodriguez M, Corrales C, et al. 2004; New approach to improving endothelial preservation in cryopreserved arterial substitutes. *Cryobiology* **48**: 62–71.

Presland RB, Dale BA. 2000; Epithelial structural proteins of the skin and oral cavity: function in health and disease. *Crit Rev Oral Biol Med* **11**: 383–408.

Presland RB, Jurevic RJ. 2002; Making sense of the epithelial barrier: what molecular biology and genetics tell us about the functions of oral mucosal and epidermal tissues. *J Dent Educ* **66**: 564–574.

Reichl S, Bednarz J, Muller-Goymann CC. 2004; Human corneal equivalent as cell culture model for *in vitro* drug permeation studies. *Br J Ophthalmol* **88**: 560–565.

Rheinwald JG, Green H. 1975; Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. *Cell* **6**: 331–343.

Saeed AI, Sharov V, White J, et al. 2003; TM4: a free, open-source system for microarray data management and analysis. *Biotechniques* **34**: 374–378.

Sanchez-Quevedo MC, Alaminos M, Capitan LM, et al. 2007; Histological and histochemical evaluation of human oral mucosa constructs developed by tissue engineering. *Histol Histopathol* **22**: 631–640.

Sanchez-Quevedo MC, Moreu G, Campos A, Garcia JM, Gonzalez-Jaranay M. 1994; Regional differences in cell surface patterns in normal human sulcular epithelium. *Histol Histopathol* **9**: 149–153.

Schaefer L, Babelova A, Kiss E, et al. 2005; The matrix component biglycan is proinflammatory and signals through Toll-like receptors 4 and 2 in macrophages. *J Clin Invest* **115**: 2223–2233.

Schultze-Mosgau S, Lee BK, Ries J, Amann K, Wiltfang J. 2004; *In vitro* cultured autologous pre-confluent oral keratinocytes for experimental prefabrication of oral mucosa. *Int J Oral Maxillofac Surg* **33**: 476–485.

Southgate J, Williams HK, Trejdosiewicz LK, Hodges GM. 1987; Primary culture of human oral epithelial cells. Growth requirements and expression of differentiated characteristics. *Lab Invest* **56**: 211–223.

Thakar CV, Zahedi K, Revelo MP, et al. 2005; Identification of thrombospondin 1 (TSP-1) as a novel mediator of cell injury in kidney ischemia. *J Clin Invest* **115**: 3451–3459.

Timpl R, Wiedemann H, van Delden V, Furthmayr H, Kühn K. 1981; A network model for the organization of type IV collagen molecules in basement membranes. *Eur J Biochem* **120**: 203–211.

Uno K, Ueda HR. 2007; Microarrays: quality control and hybridization protocol. *Methods Mol Biol* **362**: 225–243.