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Ex vivo and *in vivo* modulatory effects of umbilical cord Wharton's jelly stem cells on human oral mucosa stroma substitutes

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Summary. Novel oral mucosa substitutes have been developed in the laboratory using human umbilical cord Wharton's jelly stem cells -HWJSC- as an alternative cell source. In the present work, we have generated human oral mucosa substitutes with oral mucosa keratinocytes and HWJSC to determine the influence of these cell sources on stromal differentiation. First, acellular and cellular stroma substitutes and bilayered oral mucosa substitutes with an epithelial layer consisting of oral mucosa keratinocytes -OM samples- or HWJSC -hOM- were generated. Then, tissues were analyzed by light and electron microscopy, histochemistry and immunohistochemistry to quantify all major extracellular matrix components after 1, 2 and 3 weeks of *ex vivo* development, and OM and hOM were also analyzed after *in vivo* grafting. The results showed that bioengineered oral mucosa stromas displayed an adequate fibrillar mesh. Synthesis of abundant collagen fibers was detected in OM and hOM after 3 weeks, and *in vivo* grafting resulted in an increased collagen synthesis. No elastic or reticular fibers were found. Glycoprotein synthesis was found at the epithelial-stromal layer when samples were grafted *in vivo*. Finally, proteoglycans, decorin, versican and aggrecan were strongly dependent on the *in vivo* environment and the presence of a well-structured epithelium on top. The use of HWJSC was associated to an increased synthesis of versican. These results confirm the usefulness of fibrin-agarose biomaterials for the generation of an efficient human oral mucosa stroma substitute and the importance of the *in vivo* environment and the epithelial-mesenchymal interaction for the adequate differentiation of the bioengineered stroma.

Key words: Human Wharton's jelly stem cells, Bioengineered oral mucosa, Extracellular matrix, Epithelial-mesenchymal transition, Tissue engineering

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Introduction

Oral mucosa is a bilayered structure playing a crucial role as a defensive barrier of the oral cavity (Ramos-e-Silva and Jacques, 2012). The numerous conditions that may affect this structure and the lack of alternative sources or oral mucosa for clinical treatment make necessary the search of oral mucosa substitutes. Advances in the field of tissue engineering allowed the efficient generation of several models of bioengineered human oral mucosa based on oral mucosa cells and biomaterials (Garzon et al., 2009b; Liu et al., 2010; Carriel et al., 2012) that could have potential clinical usefulness. However, the structure and biochemical composition of many of these previously published models does not accurately reproduce the properties of the human native oral mucosa. Therefore, an efficient highly biomimetic oral mucosa substitute with potential clinical application is still in need.

One of the main problems associated to the generation of human oral mucosa substitutes by tissue engineering is the limited proliferation potential and

differentiation capability of cultured adult stem cells derived from the oral mucosa (Garzon et al., 2013). In this regard, human umbilical cord Wharton's jelly stem cells (HWJSC) have high proliferation and differentiation capabilities and they can be harvested by non-invasive procedures with very few ethical concerns (Mechiche Alami et al., 2014). For these reasons, these cells have been widely used in tissue engineering for the generation of different mesodermic cell lineages such as osteoblasts (Than and Gurtner, 2014), chondrocytes (Chen et al., 2013) or cardiomyocytes (Corrao et al., 2013). In this context, our research group recently described the use of HWJSC as an alternative cell source for the generation of an artificial oral mucosa epithelium (Garzon et al., 2013). Although the differentiation capability of these cells to different mesodermic cell lineages had been previously proved (Witkowska-Zimny and Wrobel, 2011), this was one of the first reports demonstrating the epithelial differentiation potential of HWJSC. However, the role of these stem cells in the development of a well-structured bioengineered oral mucosa stroma and their inductive capability on the stromal cells immersed within the biomaterial are not well understood. Generation of a highly biomimetic stroma is a major requirement for the future clinical translation of these oral mucosa substitutes.

In the present work, we carried out a histological, histochemical and immunohistochemical analysis of human oral mucosa substitutes developed by tissue engineering using two different sources of epithelial cells -oral mucosa keratinocytes and HWJSC- to determine the influence of these cell sources on the differentiation levels of the bioengineered oral mucosa stroma.

Materials and methods

Human tissue samples and cell cultures

Small oral mucosa (average size 2x2x2mm) biopsies corresponding to free gingiva close to the free gingival margin (protective periodontum) were obtained from adult healthy donors treated at the School of Dental Sciences of the University of Granada for minor surgical procedures using local anesthesia of normal human oral mucosa. Samples were carefully washed in phosphate buffered saline (PBS) and incubated overnight at 4°C in 2 mg/ml dispase II (Gibco BRL Life Technologies, Karlsruhe, Germany) to enzymatically detach the epithelium from the connective tissue. Subsequently, detached epithelium was cultured in culture flasks using the explant technique with epithelial culture medium. This medium consists of a 3:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 culture medium 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 (all of them from Sigma-Aldrich, St Louis, MO, USA). To obtain primary cultures of human oral mucosa fibroblasts, deepithelized oral mucosa connective tissue was digested in 2 mg/ml Clostridium histolyticum collagenase I (Gibco BRL Life Technologies). Detached fibroblasts were collected by centrifugation (1.000 rpm 10 minutes) and expanded in culture flasks containing DMEM 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.

To isolate human Wharton's Jelly stem cells (HWJSC), human umbilical cords were obtained and processed following protocols previously described by our research group (Garzon et al., 2012). In brief, umbilical cord samples were rinsed in PBS, and fragments of Wharton's Jelly were digested in a mixture of type I collagenase (Gibco BRL Life Technologies) and 0.5 g/l trypsin and 0.2 g/l EDTA (Gibco BRL Life Technologies). Isolated cells were maintained in AmnioMAX culture medium (Gibco BRL Life Technologies).

All patients gave their consent to participate in the study, and this work was approved by the local research committee.

Development of bioengineered oral mucosa samples by tissue engineering

In this study, four types of bioengineered tissues were developed by tissue engineering: 1) acellular fibrin-agarose scaffolds; 2) oral mucosa stroma substitutes consisting of human oral mucosa fibroblasts immersed in fibrin-agarose scaffolds; 3) bilayered oral mucosa substitutes consisting of human oral mucosa fibroblasts immersed in fibrin-agarose scaffolds and an ortotypical epithelium consisting of human oral mucosa keratinocytes. These ortotypical oral mucosa substitutes were called OM; 4) bilayered oral mucosa substitutes consisting of human oral mucosa fibroblasts immersed in fibrin-agarose scaffolds and a heterotypical epithelium consisting of HWJSC. These heterotypical oral mucosa substitutes were called hOM.

To generate a fibrin-agarose scaffold, we used a mixture of fibrin -obtained from frozen human plasma- and 0.1% type VII agarose solved in PBS, tranexamic acid and calcium chloride following previously published protocols (Garzon et al., 2013). In samples containing cells, 250,000 human fibroblasts were added to 5 ml of the scaffold mixture before inducing polymerization and the mixture was aliquoted in Transwell porous inserts (Corning Inc., Corning, NY). In the case of the OM and hOM, oral mucosa keratinocytes (OM) or HWJSC (hOM) were subcultured on top of the stromal substitutes. For a time-course study, all constructs were maintained in culture for 1, 2 and 3 weeks, using air-liquid culture technique for the second and third weeks.

In vivo evaluation of bioengineered OM and hOM substitutes

To evaluate the *in vivo* behavior of the oral mucosa substitutes generated in this work, we implanted the OM and hOM artificial tissues in a total of nine 6-week-old Fox 1nu/nu immunodeficient nude mice (Harlan, Indianapolis, IN). All animals were deeply anesthetized using a mixture of acepromazine (Calmo-Neosan® 0.001 mg per g of weight of the animal) and ketamine (Imalgene 1000® 0.15 mg per g of weight of the animal) after 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 OM and hOM artificial tissues, the oral mucosa substitutes were grafted at the back of the animals following previously published protocols (Garzon et al., 2009a).

Mice were euthanized 30 days after the implantation of the bioengineered oral mucosa, and all grafted tissues were prepared for histological analysis. All experiments were conducted in accordance with animal care protocols of the ethics and animal research committees at the University of Granada, Spain.

Histological, histochemical and immunohistochemical analysis of the stroma extracellular matrix (ECM)

Control human oral mucosa and bioengineered tissues (acellular scaffolds, cellular stroma substitutes, OM and hOM kept in culture for 1, 2 and 3 weeks and *in vivo* implanted OM and hOM) were fixed in 10% buffered formalin and embedded in paraffin, and 5 μm-thick sections were obtained. Deparaffinized tissue sections were stained with hematoxylin and eosin (HE) and histologically analyzed using a light microscope (Nikon Eclipse 90i). For transmission electron microscopy (TEM), samples were fixed, postfixed in 1% osmium tetroxide for 90 minutes and then embedded in spurr's resine and cut into ultrathin sections. For analysis the sections were stained with aqueous uranyl acetate and lead citrate and observed with a transmission electron microscope (EM902; Carl Zeiss Meditec, Inc., Oberkochen, Germany).

To identify fibrillar collagen, samples were stained by picrosirius histochemical methods using picrosirius working solution (Sirius red F3B) for 30 min and counterstaining with Harris's Hematoxylin for 5 min. To evaluate reticular fibers, tissue sections were stained with the Gomori's reticulin metal reduction histochemical method using 1% potassium permanganate, 2% sodium metabisulphite solution and sensibilization with 2% iron alum. Then, samples were incubated in ammoniacal silver and in 20% formaldehyde. Differentiation was carried out with 2% gold chloride and 2% thiosulphate. For elastic fibers, the orcein histochemical method was used. Briefly, samples were incubated in orcein solution for 30 min at 37° and differentiated in acid-alcohol for 15 seconds. To determine tissue glycoproteins, the periodic acid-Schiff histochemical method (PAS) was used. First, 0.5% periodic acid solution was used for 5 min, followed by incubation in Schiff reagent for 15 min and counterstaining with Harris's hematoxylin for 1 min. For proteoglycans detection, samples were incubated in Alcian blue histochemical solution for 30 min and counterstained with nuclear fast red solution for 1 min.

Immunohistochemical analysis of decorin, versican and aggrecan was carried out on formaldehyde-fixed, paraffin-embedded tissue sections using standard immunohistochemical procedures. 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 was performed for 2 h at room temperature. Subsequently, secondary biotin-conjugated anti-mouse antibody was used at 1:500 dilution, and a horseradish peroxidase-conjugated streptavidin solution was applied for 40 min. Color was developed with a commercial 3,3'-diaminobenzidine kit (Vector Laboratories, Burlingame, CA) and samples were then counterstained in Mayer's hematoxylin and mounted on coverslips for optical evaluation.

To quantify the intensity of the histochemistry or immunohistochemistry signal, all procedures were performed at the same time using an automatized protocol to ensure comparability. Then, histological images were taken using a light microscope (Nikon Eclipse 90i) with a DXM1200C digital camera and the intensity of the staining of each specific ECM component was quantified by using ImageJ software as previously reported (Oliveira et al., 2013). All images were taken and analyzed using exactly the same conditions (exposition time, white balance, background, etc.) with the Nikon NIS-Elements software and calibration was performed by using a Nikon MBM11100 stage micrometer. For each image corresponding to each experimental group, 10 small areas were randomly selected at the area of interest and the staining intensity was automatically calculated by the program, and subtracted to the background blank signal. Finally, the signal was referred to the control native oral mucosa (considered as 100%). Comparisons among all groups of samples were carried out using the Kruskal-Wallis test, whereas pair-wise comparisons between two specific groups of samples were done by using the Mann-Whitney test.

Results

Histological analysis using light microscopy and TEM

Histological analysis of the samples generated in this work revealed the presence of a bioengineered oral mucosa stroma consisting of a fibrillar mesh with fibroblasts immersed within in all cellular samples (Fig.

1). In the case of the *ex vivo* OM and hOM substitutes, the number of epithelial cell layers increased with the time in culture, although the typical rete ridges and chorial papillae were not identifiable during the 3 weeks of development in culture. When samples were grafted *in vivo*, numerous blood vessels and a dense mesh of ECM fibers were found in both OM and hOM, with the epithelium showing clear signs of terminal differentiation, including a keratinized layer on top.

Analysis of samples using transmission electron microscopy (Fig. 2) showed that stromal fibroblasts were scattered throughout a dense mesh of properly oriented collagen fibers in control oral mucosa samples. In contrast, the fibrin-agarose biomaterial was configured by numerous randomly oriented fibers. Cells immersed in the stroma of the *ex vivo* OM and hOM samples were surrounded by a mesh of fibers without any defined orientation. However, the analysis of samples grafted *in vivo* revealed that the stromal cells of both OM and hOM were immersed in an ECM rich in properly oriented collagen fibers. Interestingly, the fibrin-agarose lattice was completely substituted by collagen in all *in vivo* implanted tissues.

Analysis of fibrillar ECM components

First, picrosirius histochemical staining (Figs. 3, 4) demonstrated the existence of statistically significant global differences among all study groups ($p<0.001$ for the Kruskal-Wallis test). Specifically, collagen fibers were very abundant in control oral mucosa and *in vivo* implanted OM and hOM, with non-significant differences in collagen content among these three samples. In contrast, the content of collagen was significantly lower in all *ex vivo* samples ($p<0.05$). Among the *ex vivo* samples, the highest amount of collagen corresponded to tissues cultured for 3 weeks, especially those corresponding to hOM, which had significantly higher picrosirius staining intensity than OM ($p<0.05$). Interestingly, OM and hOM kept *ex vivo* for 3 weeks showed significantly higher signal than biomaterials containing stromal cells without an epithelial layer on top ($p<0.05$).

In the second place, the analysis of reticular fibers as determined by Gomori's technique and elastic fibers as determined by orcein staining method showed a complete absence of these types of fibers in control, *ex*

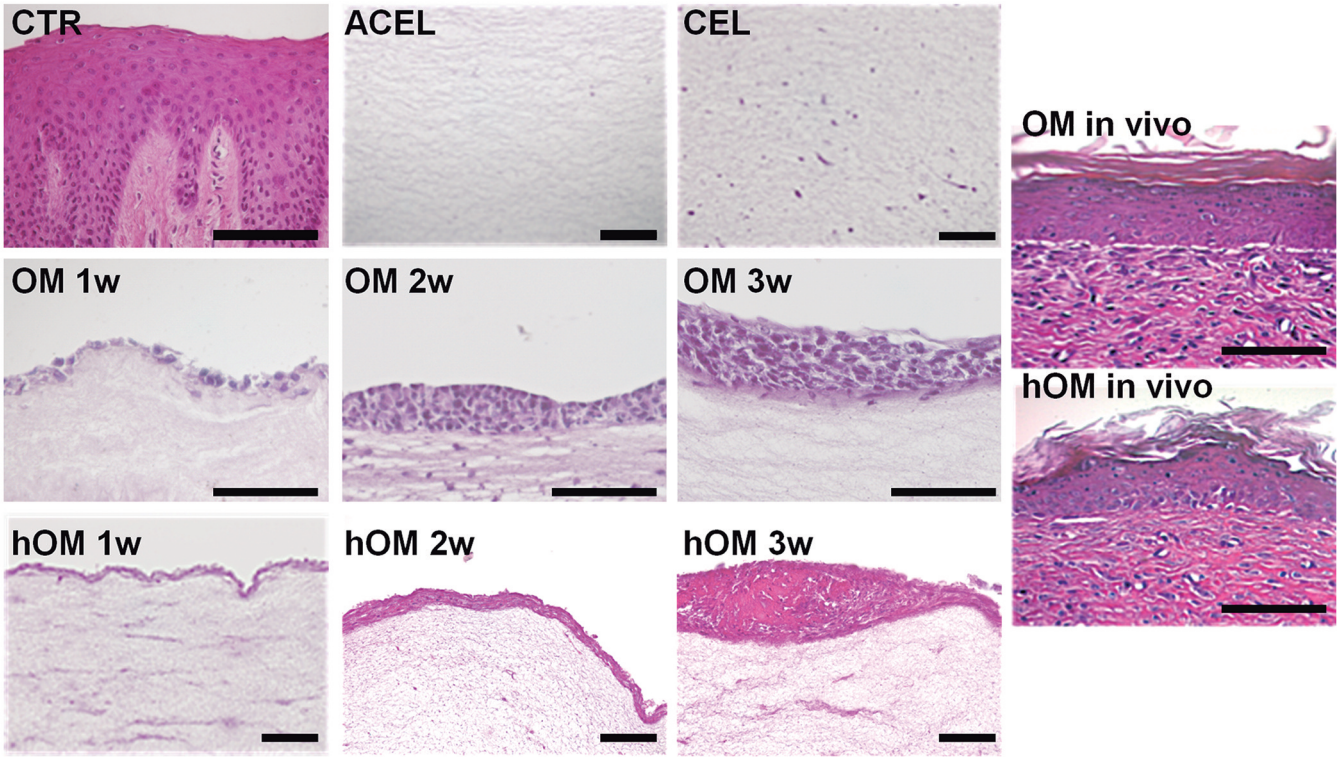

Fig. 1. Analysis of controls and bioengineered tissues using hematoxylin and eosin staining. CTR: control native oral mucosa; ACEL: acellular fibrin-agarose scaffolds; CEL: oral mucosa stroma substitutes consisting of human oral mucosa fibroblasts immersed in fibrin-agarose scaffolds; OM: bilayered oral mucosa substitutes consisting of human oral mucosa fibroblasts immersed in fibrin-agarose scaffolds and an ortotypical epithelium consisting of human oral mucosa keratinocytes; hOM: bilayered oral mucosa substitutes consisting of human oral mucosa fibroblasts immersed in fibrin-agarose scaffolds and a heterotypical epithelium consisting in HWJSC; 1w: samples kept *ex vivo* for 1 week; 2w: samples kept *ex vivo* for 2 weeks; 3w: samples kept *ex vivo* for 3 weeks; *in vivo*: samples grafted in nude mice for 30 days. Scale bars: 100 μm.

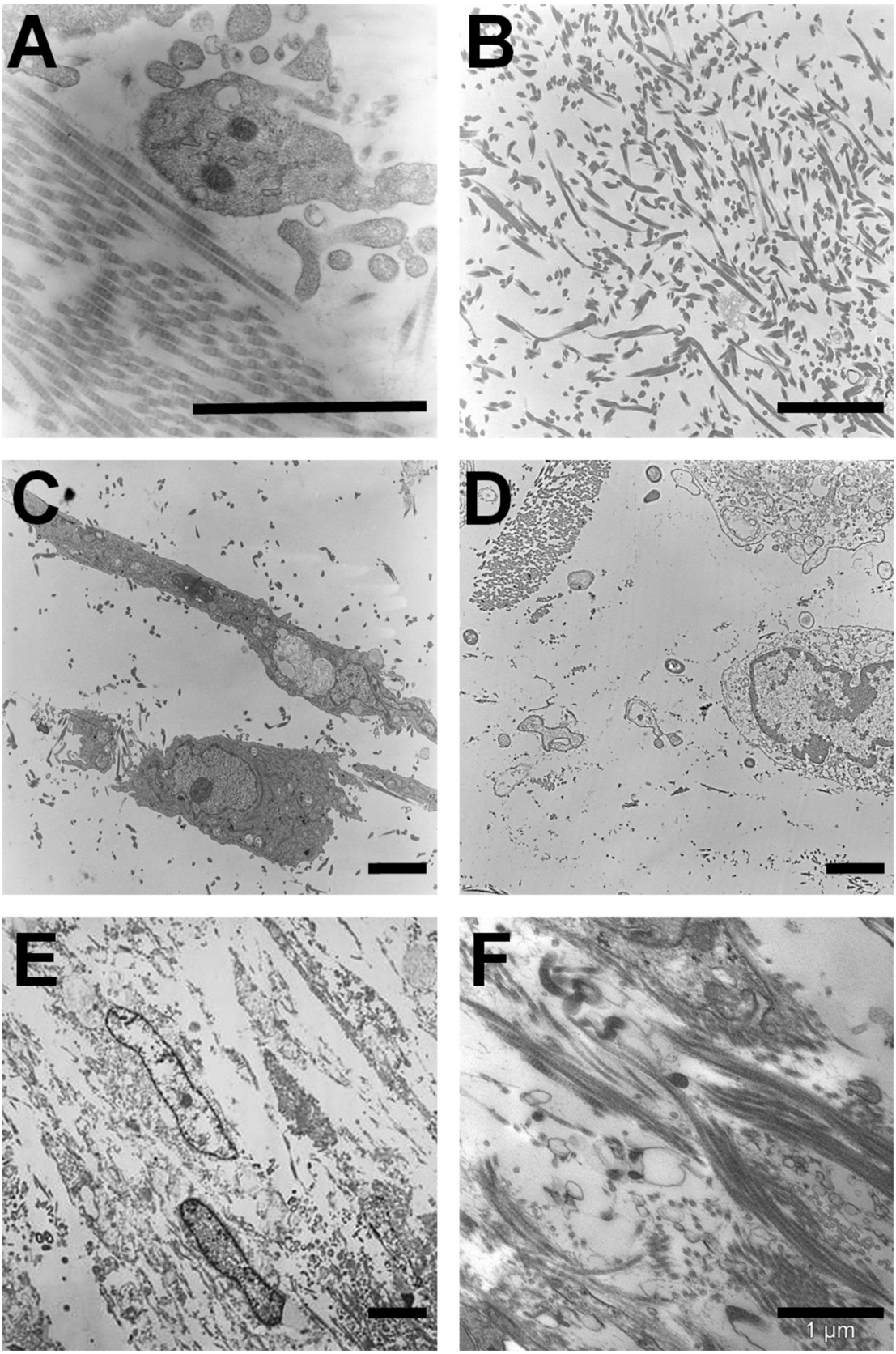

Fig. 2. Transmission electron microscopy analysis of controls and bioengineered tissues. **A.** Control native human oral mucosa. **B.** Acellular fibrin-agarose scaffolds. **C.** Stroma of the bilayered oral mucosa substitutes consisting of human oral mucosa fibroblasts immersed in fibrin-agarose scaffolds and an ortotypical epithelium consisting of human oral mucosa keratinocytes (OM) corresponding to 3 weeks of *ex vivo* development. **D.** Stroma of OM samples grafted *in vivo* for 30 days. **E.** Stroma of the bilayered oral mucosa substitutes consisting of human oral mucosa fibroblasts immersed in fibrin-agarose scaffolds and a heterotypical epithelium consisting of HWJSC (hOM) corresponding to 3 weeks of *ex vivo* development; D: stroma of hOM samples grafted *in vivo* for 30 days. Scale bars: 1 *µ*m.

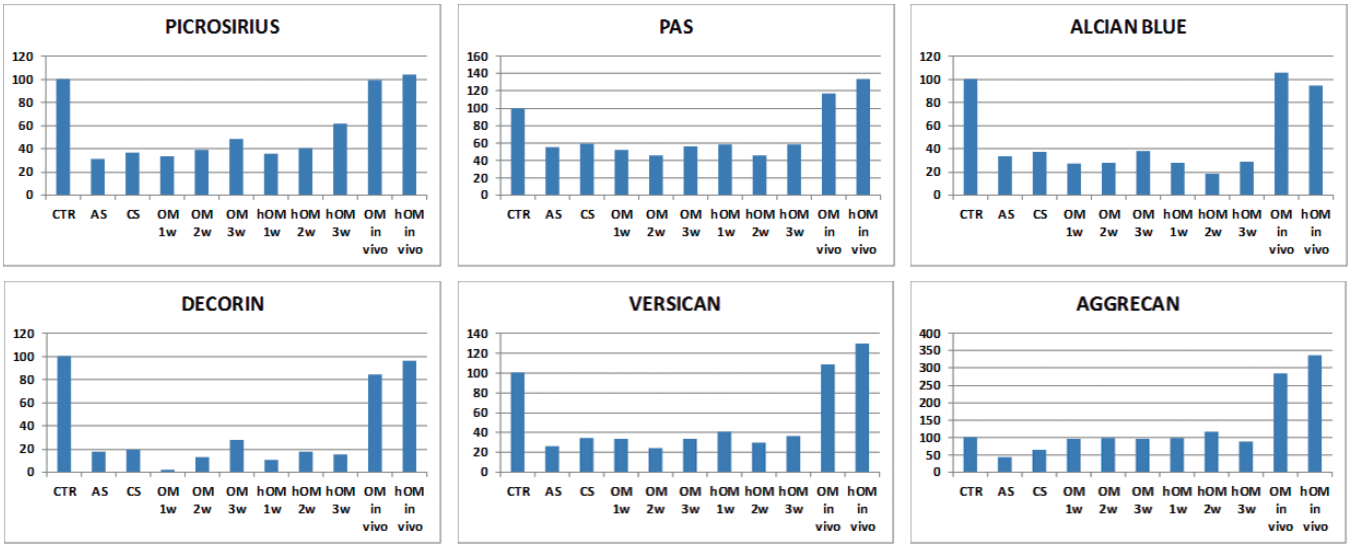

Fig. 3. Quantification of extracellular matrix components in controls and bioengineered tissues as determined by picrosirius, PAS, alcian blue, decorin, versican and aggrecan. The content of each extracellular matrix component is shown as percentage using control oral mucosa as a reference (100%) and correspond to average values for each tissue type. CTR: control native oral mucosa; AS: acellular fibrin-agarose scaffolds; CS: cellular oral mucosa stroma substitutes consisting of human oral mucosa fibroblasts immersed in fibrin-agarose scaffolds; OM: bilayered oral mucosa substitutes consisting of human oral mucosa fibroblasts immersed in fibrin-agarose scaffolds and an ortotypical epithelium consisting of human oral mucosa keratinocytes; hOM: bilayered oral mucosa substitutes consisting of human oral mucosa fibroblasts immersed in fibrin-agarose scaffolds and a heterotypical epithelium consisting of HWJSC; 1w: samples kept *ex vivo* for 1 week; 2w: samples kept *ex vivo* for 2 weeks; 3w: samples kept *ex vivo* for 3 weeks; *in vivo*: samples grafted in nude mice for 30 days.

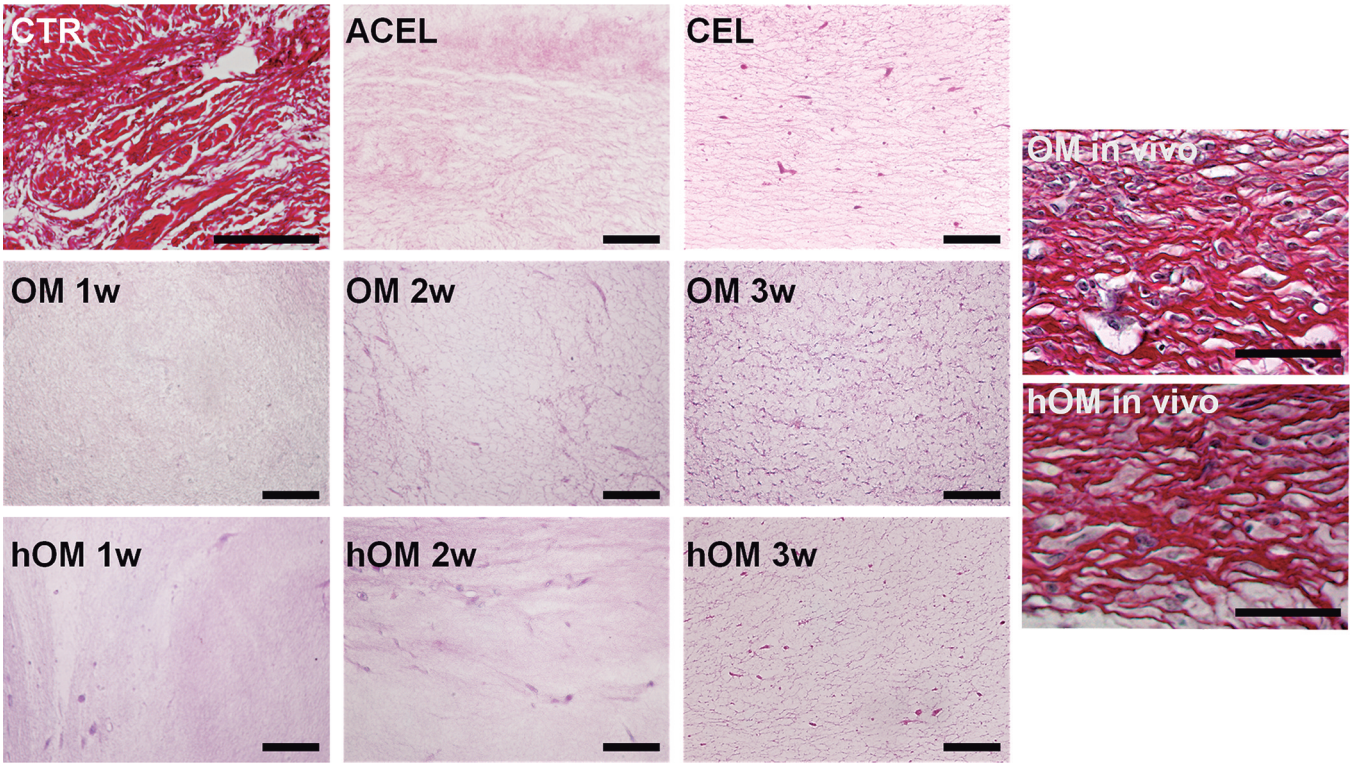

Fig. 4. Histochemical analysis of controls and bioengineered tissues using picrosirius staining. CTR: control native oral mucosa; ACEL: acellular fibrin-agarose scaffolds; CEL: oral mucosa stroma substitutes consisting of human oral mucosa fibroblasts immersed in fibrin-agarose scaffolds; OM: bilayered oral mucosa substitutes consisting of human oral mucosa fibroblasts immersed in fibrin-agarose scaffolds and an ortotypical epithelium consisting of human oral mucosa keratinocytes; hOM: bilayered oral mucosa substitutes consisting of human oral mucosa fibroblasts immersed in fibrin-agarose scaffolds and a heterotypical epithelium consisting of HWJSC; 1w: samples kept *ex vivo* for 1 week; 2w: samples kept *ex vivo* for 2 weeks; 3w: samples kept *ex vivo* for 3 weeks; *in vivo*: samples grafted in nude mice for 30 days. Scale bars: 100 μm.

vivo and *in vivo* all samples (data not shown).

Analysis of non-fibrillar ECM components

The first non-fibrillar components that we analyzed in control and bioengineered samples were the ECM glycoproteins at the epithelial-stromal interphase. In this regard, our results showed statistically significant global differences among all study groups ($p<0.001$ for the Kruskal-Wallis test). PAS staining intensity (Fig. 5) was significantly higher in control oral mucosa and *in vivo* grafted OM and hOM as compared to the rest of samples ($p<0.05$). The low staining intensity found in all *ex vivo* samples suggests that these samples were not able to synthetize a well-developed epithelial-stromal basement membrane during the 3 weeks of culture.

The second non-fibrillar components analyzed here were the ECM proteoglycans. The global analysis of proteoglycans content using alcian blue histochemistry (Fig. 6) confirmed the existence of significant global differences among all study groups ($p<0.001$ for the Kruskal-Wallis test). In short, our analysis demonstrated the presence of these ECM components in control oral mucosa and *in vivo* implanted samples, which were significantly more enriched in these molecules than the rest of samples ($p<0.05$). Differences among these three tissues were non-significant ($p>0.05$), although *in vivo* samples tended to show the highest PAS signal. *ex vivo* developed samples had very few amounts of proteoglycans, with no differences among samples ($p>0.05$).

When specific proteoglycans were analyzed by immunohistochemistry, we found significant differences among all the study groups for decorin, versican and aggrecan ($p<0.001$ for the Kruskal-Wallis test). On the one hand, decorin (Fig. 7) and versican (Fig. 8) confirmed that the highest contents of these two elements corresponded to control human oral mucosa and *in vivo* implanted bioengineered OM and hOM, which had statistically more signal than the rest of samples. The results found for both *in vivo* OM and hOM were similar to those of control native oral mucosa for decorin. However, hOM was significantly more enriched in versican than OM ($p<0.05$). Samples kept *ex*

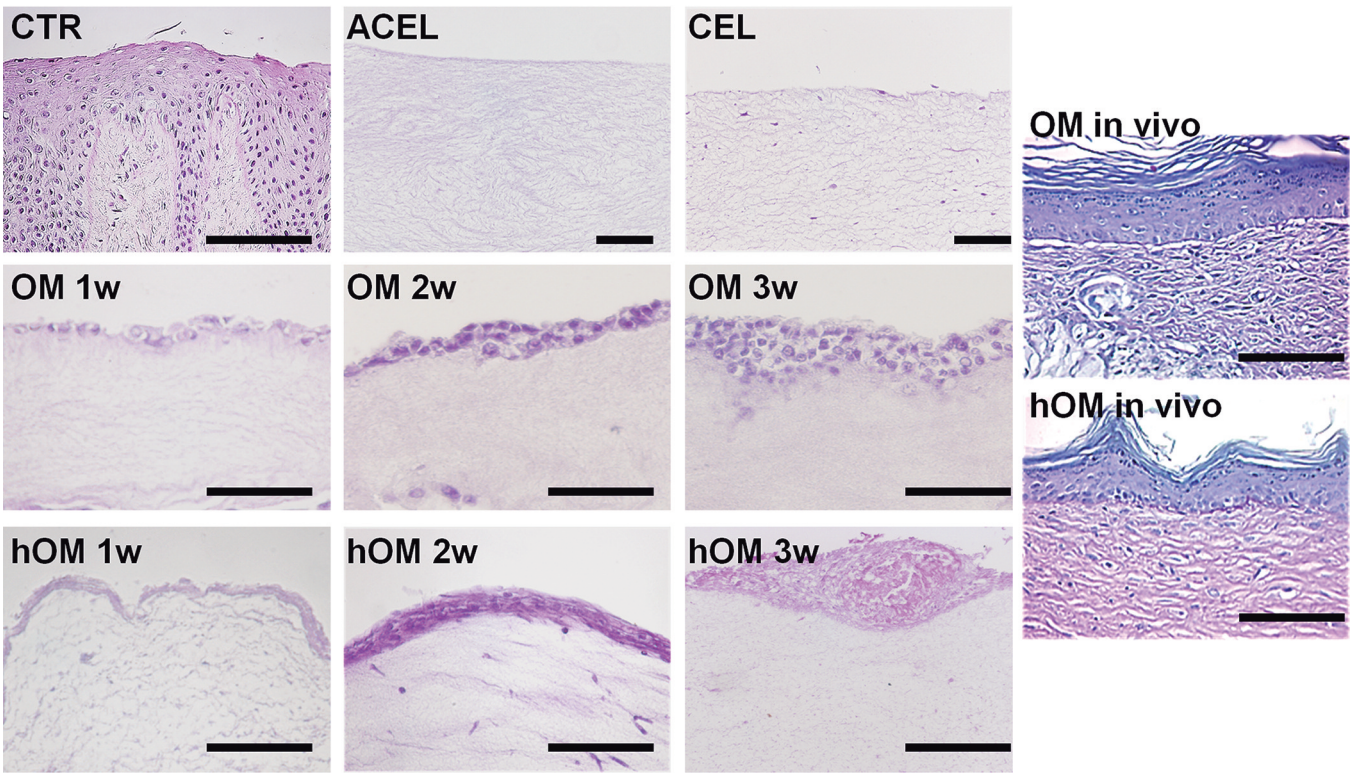

Fig. 5. Histochemical analysis of controls and bioengineered tissues using PAS staining. CTR: control native oral mucosa; ACEL: acellular fibrin-agarose scaffolds; CEL: oral mucosa stroma substitutes consisting of human oral mucosa fibroblasts immersed in fibrin-agarose scaffolds; OM: bilayered oral mucosa substitutes consisting of human oral mucosa fibroblasts immersed in fibrin-agarose scaffolds and an ortotypical epithelium consisting of human oral mucosa keratinocytes; hOM: bilayered oral mucosa substitutes consisting of human oral mucosa fibroblasts immersed in fibrin-agarose scaffolds and a heterotypical epithelium consisting in HWJSC; 1w: samples kept *ex vivo* for 1 week; 2w: samples kept *ex vivo* for 2 weeks; 3w: samples kept *ex vivo* for 3 weeks; *in vivo*: samples grafted in nude mice for 30 days. Scale bars: 100 μm.

vivo showed weak immunostaining signal for decorin and versican, although bioengineered OM kept *ex vivo* for 3 weeks showed a significantly higher content of decorin than the rest of the *ex vivo* samples, including the hOM kept *ex vivo*. On the other hand, aggrecan (Fig. 9) was very abundant in *in vivo* grafted OM and hOM, especially in hOM ($p<0.05$ as compared to *in vivo* OM) and showed weak expression in control native oral mucosa and in samples kept *ex vivo*, which were statistically similar to controls and lower than *in vivo* samples ($p<0.05$).

Discussion

Generation of a bioengineered substitute of the human oral mucosa represents important progress in the field of tissue engineering. In the present work, we have constructed a bioengineered oral mucosa substitute based on natural biomaterials and HWJSC as an alternative source of epithelial cells. Fibrin-agarose biomaterials had been previously used in combination with stem cells for the generation of several artificial tissue models (Cardona Jde et al., 2011; Carriel et al., 2012, 2015), including the human oral mucosa (Garzon et al., 2009a,b), due to its excellent biocompatibility and good biomechanical properties (Scionti et al., 2014). However, the capability of these biomaterials to reproduce the structure and composition of the human native oral mucosa stroma had not been studied to date. Development of an adequate biomimetic stroma substitute is a major requirement for bilayered tissues, since this stroma is responsible for epithelial nutrition and development and the main structural support of the artificial tissue, allowing epithelial-mesenchymal transition (EMT) (Moustakas and Heldin, 2014). In fact, the stroma ECM plays very important roles in cell physiology, cell-cell communication, cell division and differentiation, allowing cell nutrition and oxygen interchange (Badylak, 2007). For all these reasons, characterization of biomaterials used for the development of bilayered tissues such as the oral mucosa is a very important part of the quality control of bioengineered oral mucosa substitutes.

In this regard, the oral mucosa substitutes that we generated in the present work were shown to be adequate bioengineered tissue models since our bioengineered tissues were manufactured using strict cell culture process for autologous human cells. In addition,

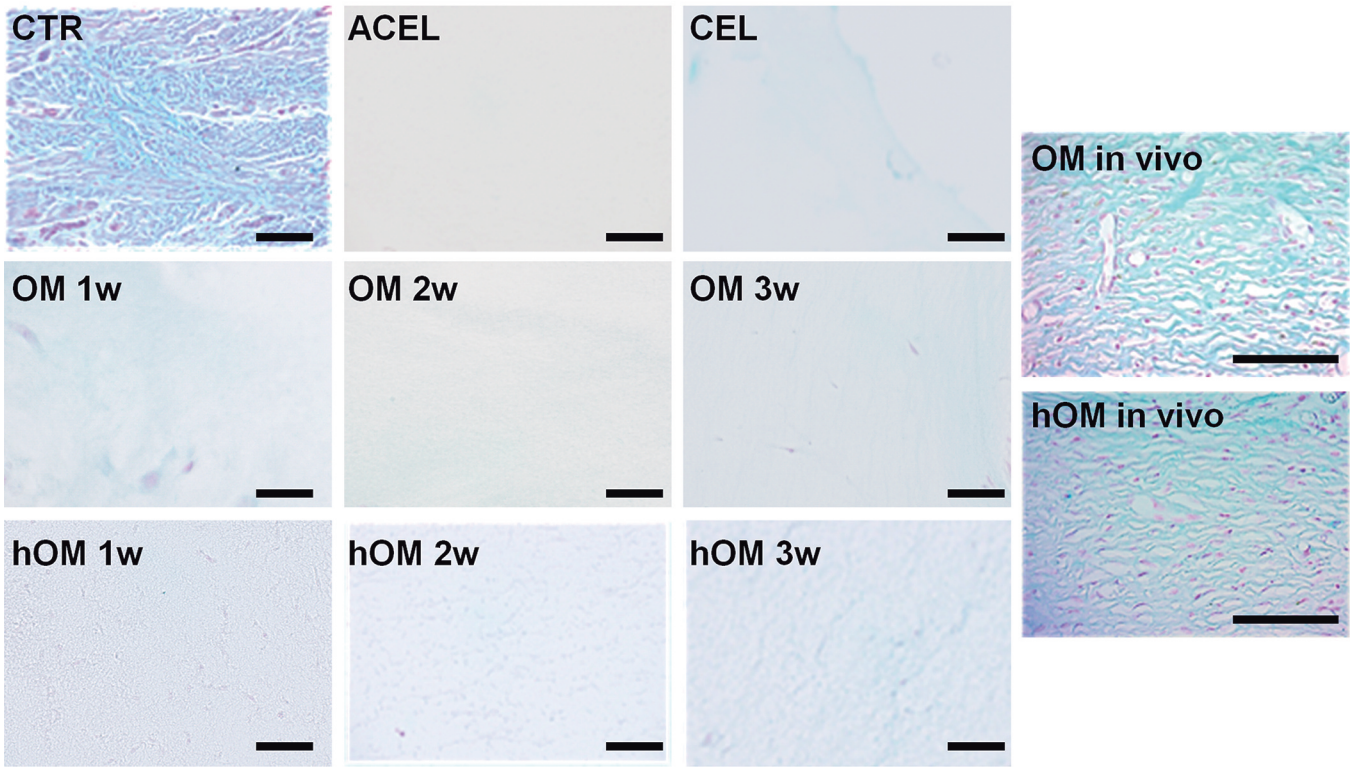

Fig. 6. Histochemical analysis of controls and bioengineered tissues using alcian blue staining. CTR: control native oral mucosa; ACEL: acellular fibrin-agarose scaffolds; CEL: oral mucosa stroma substitutes consisting of human oral mucosa fibroblasts immersed in fibrin-agarose scaffolds; OM: bilayered oral mucosa substitutes consisting of human oral mucosa fibroblasts immersed in fibrin-agarose scaffolds and an ortotypical epithelium consisting of human oral mucosa keratinocytes; hOM: bilayered oral mucosa substitutes consisting of human oral mucosa fibroblasts immersed in fibrin-agarose scaffolds and a heterotypical epithelium consisting of HWJSC; 1w: samples kept *ex vivo* for 1 week; 2w: samples kept *ex vivo* for 2 weeks; 3w: samples kept *ex vivo* for 3 weeks; *in vivo*: samples grafted in nude mice for 30 days. Scale bars: 100 μm.

one of the great advantages of the OM and hOM substitutes developed in this work is that these tissues were generated without using cadaveric substrates or feeder layers as other approaches suggested (Izumi et al., 2000, 2004). This could favor translation into patient care as it has been suggested in the past decade (Izumi et al., 2000, 2004). From the histological point of view, the analysis confirmed the accuracy of the oral mucosa models generated in this work and revealed that the stromal cells immersed in the fibrin-agarose biomaterial were able to spread and grow in this scaffold. However, fibers were randomly dispersed in samples kept *ex vivo* in both the OM and the hOM substitutes. As previously suggested (Carriel et al., 2012), grafting these tissues in athymic mice resulted in high degrees of maturation and differentiation of these stromal substitutes, with the formation of blood vessels and properly aligned collagen fibers, suggesting that these stromal substitutes may be fully functional and display adequate biomechanical properties once implanted *in vivo*. In fact, our analysis of fibrillar collagen content as determined by picrosirius staining showed that the amount of collagen fibers was similar in control oral mucosa and OM and hOM grafted *in vivo*, suggesting that the *in vivo* environment was able to induce the stroma cells to synthetize properly oriented collagen fibers in interaction with the epithelial cells (OM) or the HWJSC used as epithelial substitute (hOM). Although collagen formation was very low *ex vivo*, our results showed that samples kept *ex vivo* for 3 weeks had some collagen content in the stroma. Interestingly, the presence of an epithelium (OM) or an epithelial substitute (hOM) was necessary for collagen formation, thus demonstrating the important role of the epithelial layer and the EMT on stromal physiology. The fact that hOM kept *ex vivo* for 3 weeks had more collagen content than OM confirms the potential usefulness of umbilical cord derived stem cells as alternative cell sources in oral mucosa tissue engineering and suggest that these cells could efficiently cross-talk with stromal cells in a similar or even better way than oral mucosa keratinocytes. Development of a well-structured mesh of fibrillar collagen is very important for cell differentiation and migration not only at the stromal layer, but also at the epithelial tissue layer (Halper and Kjaer, 2014). On the other hand, all bioengineered oral mucosa substitutes showed a complete lack of reticular and elastic fiber synthesis, which was comparable to native oral mucosa controls. Taken together, all these results suggest that

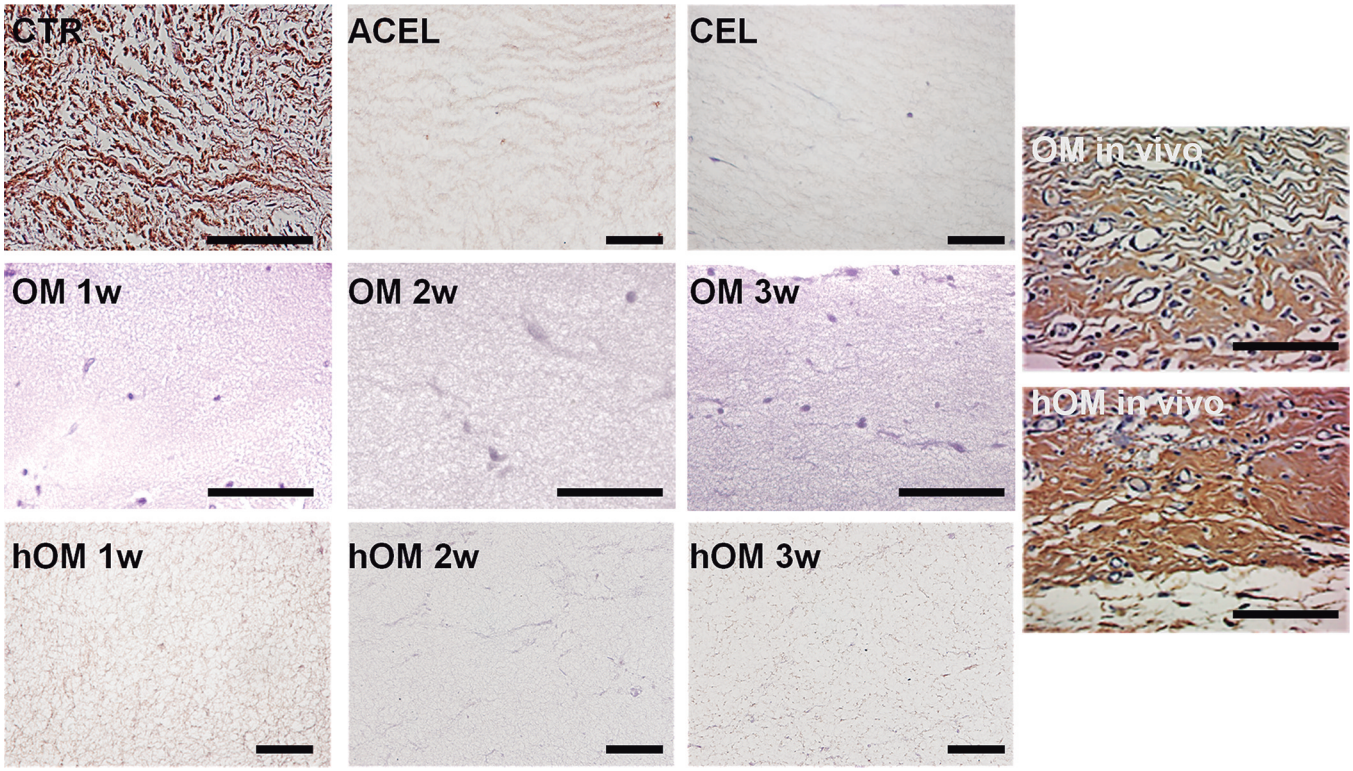

Fig. 7. Immunohistochemical detection of decorin in controls and bioengineered tissues. CTR: control native oral mucosa; ACEL: acellular fibrin-agarose scaffolds; CEL: oral mucosa stroma substitutes consisting of human oral mucosa fibroblasts immersed in fibrin-agarose scaffolds; OM: bilayered oral mucosa substitutes consisting of human oral mucosa fibroblasts immersed in fibrin-agarose scaffolds and an ortotypical epithelium consisting of human oral mucosa keratinocytes; hOM: bilayered oral mucosa substitutes consisting of human oral mucosa fibroblasts immersed in fibrin-agarose scaffolds and a heterotypical epithelium consisting of HWJSC; 1w: samples kept *ex vivo* for 1 week; 2w: samples kept *ex vivo* for 2 weeks; 3w: samples kept *ex vivo* for 3 weeks; *in vivo*: samples grafted in nude mice for 30 days. Scale bars: 100 μm.

both the oral mucosa keratinocytes and the HWJSC may work as inductive agents for stroma cell differentiation in OM and hOM models and open the door to a future clinical translation of both tissue models.

Along with the fibers, one of the most important components of the stroma ECM are the non-fibrillar components, which are essential for the maintenance of the spatial structure and hydration level of tissues (Glim et al., 2014; Halper and Kjaer, 2014). In the first place, our analysis using PAS staining showed that the amount of glycoproteins synthetized by samples kept *ex vivo* was very low and did not differ from acellular biomaterials. This suggests that the EMT was not able to efficiently induce epithelial and stromal cells to synthetize this type of molecule at this stage. In contrast, control native oral mucosa and *in vivo* grafted bioartificial oral mucosa substitutes showed positive PAS staining, revealing that the *in vivo* environment was indeed able to activate the synthesis of glycoproteins in the epithelial-stromal interphase. These results reveal that the formation of a basement membrane is a very complex process requiring not only the presence of a well-structured epithelial and stromal layer, but also an *in vivo* environment. Interestingly, our results showed a non-significant trend to an increased concentration of glycoproteins in the epithelial-stromal interphase of hOM. This suggests that the use of HWJSC may enhance the epithelial-stromal crosstalk process during tissue development.

In the second place, we quantified the presence of proteoglycans in bioengineered tissues. Proteoglycans appear to be essential for several tissue functions, including regulation of protease activity, cellular response to growth factors, cell-cell and cell-matrix interaction and collagen fibrillogenesis (LeBaron et al., 1988; Aviezer et al., 1994). Interestingly, our results showed a good correlation pattern between picrosirius and alcian blue staining, suggesting that the highest contents of fibrillar collagen coincide with the highest proteoglycan levels. Remarkably, the results found for proteoglycan content as determined by alcian blue staining resembled the profile found for glycoproteins, with high expression in control native oral mucosa and OM and hOM grafted *in vivo* and very low or absent signal in all *ex vivo* samples. These results could confirm the idea that the *in vivo* environment was important for an adequate generation of non-fibrillar ECM components. However, the presence of an epithelial layer on top of the stromal substitute does not seem to

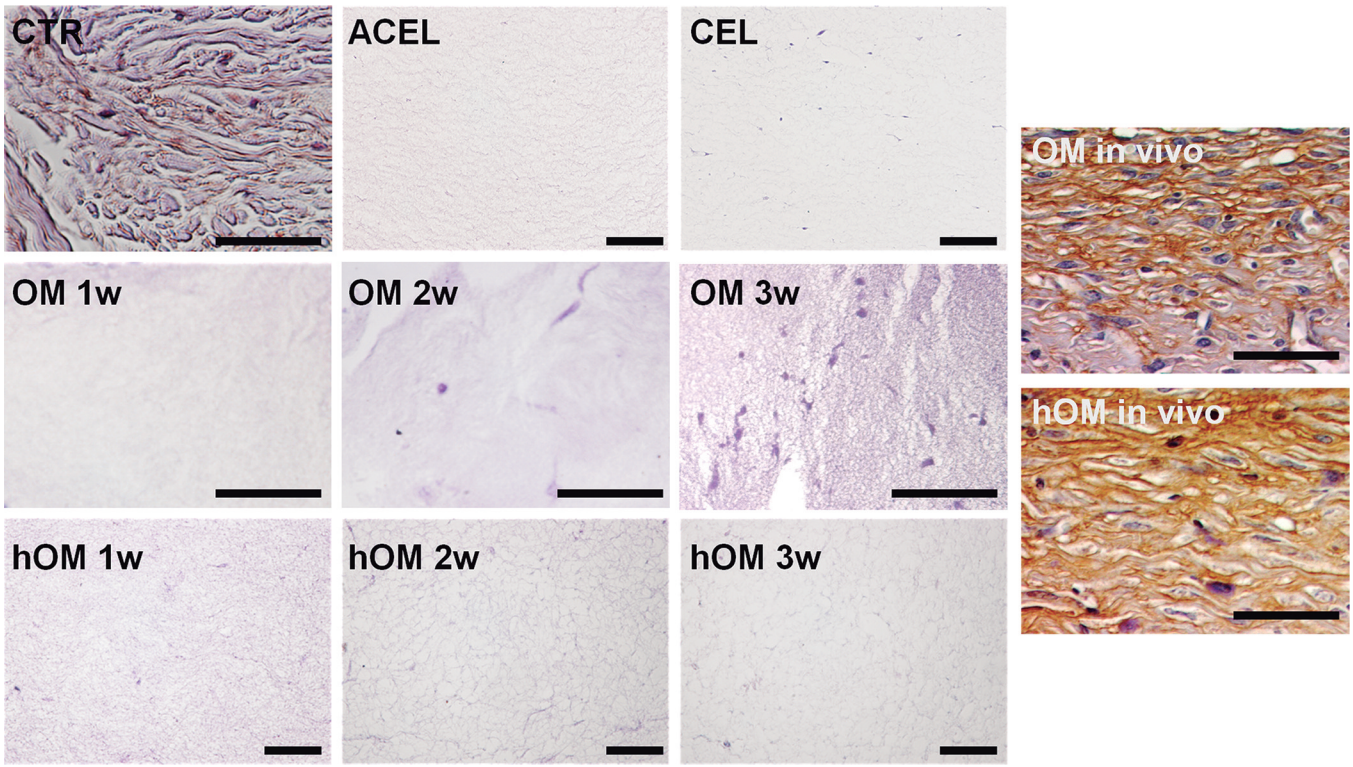

Fig. 8. Immunohistochemical detection of versican in controls and bioengineered tissues. CTR: control native oral mucosa; ACEL: acellular fibrin-agarose scaffolds; CEL: oral mucosa stroma substitutes consisting of human oral mucosa fibroblasts immersed in fibrin-agarose scaffolds; OM: bilayered oral mucosa substitutes consisting of human oral mucosa fibroblasts immersed in fibrin-agarose scaffolds and an ortotypical epithelium consisting of human oral mucosa keratinocytes; hOM: bilayered oral mucosa substitutes consisting of human oral mucosa fibroblasts immersed in fibrin-agarose scaffolds and a heterotypical epithelium consisting of HWJSC; 1w: samples kept *ex vivo* for 1 week; 2w: samples kept *ex vivo* for 2 weeks; 3w: samples kept *ex vivo* for 3 weeks; *in vivo*: samples grafted in nude mice for 30 days. Scale bars: 100 μm.

play a very important role at these stages of development *ex vivo*. Future studies should analyze these ECM components in bioengineered tissues kept *ex vivo* for longer periods of time.

Once the presence of proteoglycans was analyzed globally, we quantified the synthesis of the most important specific proteoglycans of the human ECM. Decorin is a small proteoglycan that joins to collagen fibrils and contributes to determine the final shape of type I collagen fibers (Islam et al., 2013), and versican plays an important role in modulating cell adhesion, migration and proliferation (Yang and Yee, 2014). Therefore, the role of these molecules is crucial for an adequate generation of an efficient oral mucosa stroma substitute by tissue engineering. Quantification of both proteoglycans using immunohistochemistry demonstrated that the expression profile of both molecules was very similar, and it was in agreement with the results obtained by alcian blue staining. This confirms the relevance of the *in vivo* conditions for the synthesis of both ECM components, and the absence of expression of decorin and versican *ex vivo*. Interestingly, the use of an epithelial-like cell layer in hOM resulted in an increased synthesis of versican, which is a potentially useful finding from a translational standpoint. Finally, aggrecan is a highly hydrophilic core proteoglycan that is very abundant in cartilage and water-rich connective tissues (Bernhard and Panitch, 2012).The fact that our results showed a high concentration of this proteoglycan in samples grafted *in vivo* as compared to control oral mucosa could indicate that the water content of tissues grafted on nude mice is abundant during the first days of tissue development *in vivo*. The fact that hOM showed significantly higher aggrecan contents than OM could be explained by the fact that Wharton's jelly is a water-rich mucous connective tissue, and it is likely that HWJSC play a role in the physiology of this tissue.

In summary, our results confirm the usefulness of fibrin-agarose biomaterials for the generation of an efficient human oral mucosa stroma substitute and the importance of the *in vivo* environment. The epithelial-mesenchymal interaction was very important for the adequate differentiation of the bioengineered stroma, and the use of HWJSC was able to induce stromal cell differentiation in a similar way to native oral mucosa keratinocytes shortening the time of fabrication of artificial oral mucosa, one of the future goals as proposed by Izumi (Izumi et al., 2004). Future

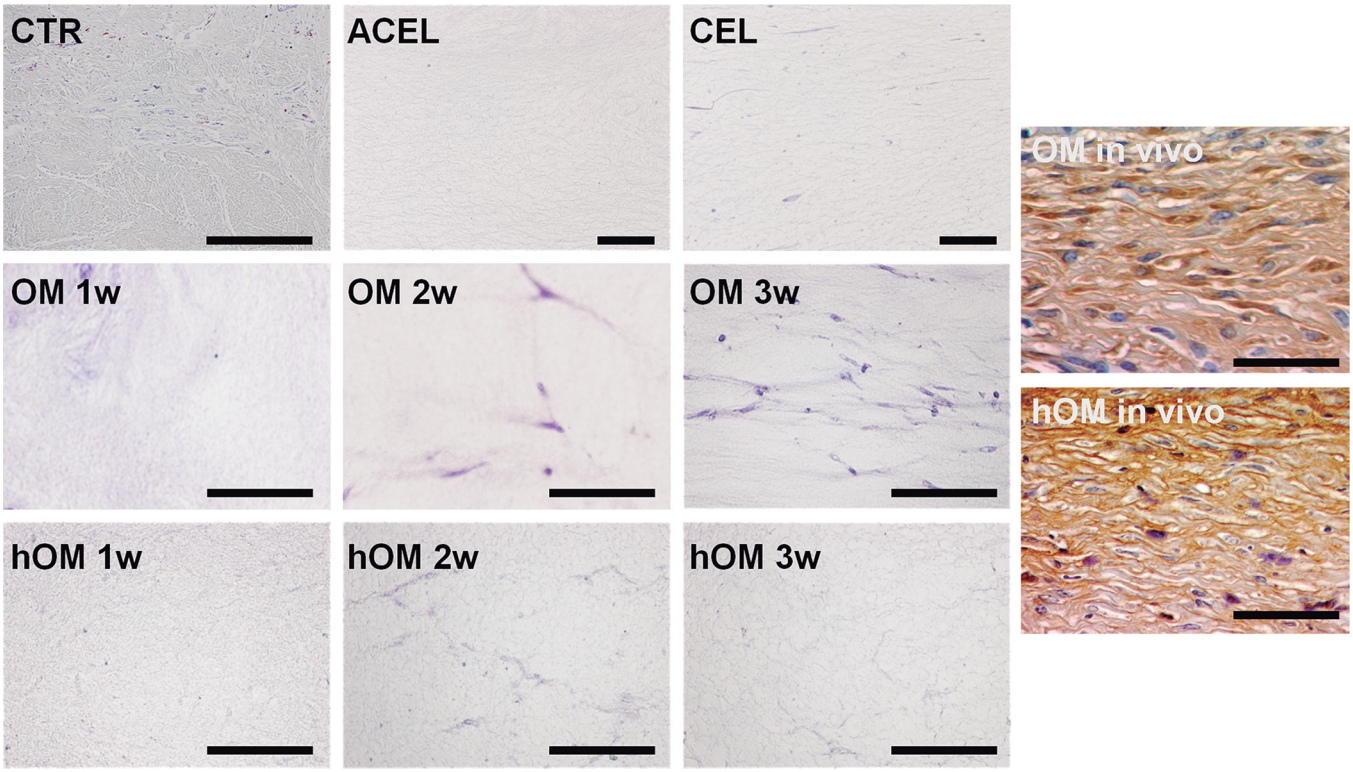

Fig. 9. Immunohistochemical detection of aggrecan in controls and bioengineered tissues. CTR: control native oral mucosa; ACEL: acellular fibrin-agarose scaffolds; CEL: oral mucosa stroma substitutes consisting of human oral mucosa fibroblasts immersed in fibrin-agarose scaffolds; OM: bilayered oral mucosa substitutes consisting of human oral mucosa fibroblasts immersed in fibrin-agarose scaffolds and an ortotypical epithelium consisting of human oral mucosa keratinocytes; hOM: bilayered oral mucosa substitutes consisting of human oral mucosa fibroblasts immersed in fibrin-agarose scaffolds and a heterotypical epithelium consisting of HWJSC; 1w: samples kept *ex vivo* for 1 week; 2w: samples kept *ex vivo* for 2 weeks; 3w: samples kept *ex vivo* for 3 weeks; *in vivo*: samples grafted in nude mice for 30 days. Scale bars: 100 μm.

investigations should be focused on the development of artificial oral mucosa using good manufacture practices in order to demonstrate the efficiency of this bioengineered oral mucosa model.

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